Going in the right direction

*Cellular mechanisms underlying root halotropism*

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Chapter 5

PLDζ1 and PLDζ2 are involved in distinct cellular processes during salt stress responses

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Abstract
The increase in soil salinity worldwide is a driver to investigate the cellular mechanisms behind salt tolerance, with the aim to develop new approaches to increase crop resilience. Recently, phospholipase D ζ proteins have been implicated in the response of plant roots to salt stress. Although involvement of both PLDζ1 and PLDζ2 isoforms has been found, their exact roles remain elusive, nor it is clear whether they exhibit redundant functions during salt stress. Here, we investigated salt responses of a pldζ2- and pldζ1/pldζ2- double mutant and compared the results with recent data of pldζ1 (Chapter 4). The pldζ1/pldζ2 mutant was found to largely exhibit the same phenotype as pldζ1 in PIN2 polarity and inhibition of root hair length by salt. On the other hand, the double mutant phenocopied the pldζ2 single mutant with respect to the number of PIN2 containing vesicles in response to salt stress and during early halotropism. No additive phenotypes were observed for the double mutant. Increased root hair length and less effect of salt on root hair length were found in all PLDζ mutants. These effects were largest in the pldζ2 line. These results indicate that PLDζ1 and PLDζ2 are involved in different cellular processes in the salt stress response.

Introduction
While biotic stress is a challenge for most organisms, either mobile or sessile, abiotic stress is a most prominent problem in sessile organisms. Where mobile organisms can simply move away from many harmful factors in their environment, sessile organisms need to cope with the stress or have less and weaker offspring. Amongst all abiotic stresses, high soil salinity can have a devastating effect on crop yield. With an increase in soil salinity worldwide, it has become important to develop crops that are more tolerant to salt, for which fundamental knowledge is required on the processes that occur in plants responding to salt.

The mechanisms behind plant salt-stress tolerance are being addressed using multiple approaches. At the cellular level, vital processes that need to be elucidated include salt sensing (Deinlein et al., 2014; Shabala et al., 2015), Na+ accumulation and transport (Hanin et al., 2016), and regulation of the cell cycle (Martinez-de la Cruz et al., 2015). Regarding plant hormones, roles for auxin (Olatunji et al., 2017; Korver et al., 2018), abscisic acid (Sah et al., 2016), gibberellin (Colebrook et al., 2014), ethylene (Zhang et al., 2016) and jasmonate (Kazan, 2015) have emerged. At the whole-plant level, modulations of the root system, which is normally in contact with the salt, have widely been studied as
a factor in salt tolerance (Koevoets et al., 2016). There, a major role for auxin in plasticity of root system architecture (RSA) in response to stress, as well as in tropisms (Harmer and Brooks, 2018), has become apparent. For most tropism responses, alteration of the auxin flow through the root is required (Li and Xue, 2007; Taniguchi et al., 2010; Galvan-Ampudia et al., 2013). Until recently, the major focus of auxin flow-altering processes has been on polar auxin transport (PAT). However, increasingly more evidence has been found showing active short distance auxin transport, passive auxin transport and auxin conjugation and local biosynthesis (Korver et al., 2018). Nonetheless, the regulation of auxin flow through the internalization of auxin carriers is a major factor during stress. The mechanism of this internalization is still under debate. Both clathrin-mediated endocytosis (CME) (Galvan-Ampudia et al., 2013) and clathrin independent endocytosis (Baral et al., 2015) pathways have been proposed to internalize PIN2 upon salt stress. Nonetheless, the knowledge on plasma membrane (PM)-protein cycling in plants is scarce compared to animals (Murphy et al., 2005). Galvan-Ampudia et al. (2013) suggested that PLDζ2 is required for salt-induced CME and thus the internalization of PIN2. Treatment with an inhibitor known to obstruct mammalian PLDs, FIP1 (5-Fluoro-2-indolyl des-chlorohalopemide) (Su et al., 2009), impeded clathrin localization to the membrane and PIN2 localization (Galvan-Ampudia et al., 2013). Moreover, salt stress has been found to activate other PLD activity (Munnik et al., 2000; Bargmann et al., 2009).

Following the recently discovered role of PLDζ1 in early halotropism- and gravitropism responses, in root system architecture under salt stress, and PIN2 recycling during salt stress (Chapter 4), we here functionally addressed the role of PLDζ2 in salt stress. Using fluorescent PLDζ fusions of both PLDζ1 and PLDζ2, we studied their subcellular localization and found differences in root cap cells where they located to the plasma membrane and tonoplast, respectively, under control conditions. Salt stress induced a shift in the membrane/cytosol ratio for both PLDζs, however, no role for PLDζ2 in either early halo- or gravitropism was observed. Also, we found an apolar PIN2 distribution in the pldζ1/ pldζ2 mutant but not in the pldζ2 mutant under control conditions. Knockouts of PLDζ1 and PLDζ2 do not lead to major changes in performance or RSA during salt stress, indicating a minor role for both PLDζs in these processes. However, interestingly, all mutants had longer root hairs in control conditions and lower reduction of root hair length during salt stress. These results indicate that PLDζ1 and not PLDζ2 regulates PIN2 sub-cellular localization, however, both PLDζs are involved in root hair length.
Results

PLDζ1 and PLDζ2 show different subcellular localization in LRC and similar localization in epidermal cells

To study the subcellular localization of PLDζ1 and PLDζ2, FP-fusions were constructed and imaged with and without salt stress. In lateral root cap (LRC) cells, plasma membrane (PM) localization was observed for PLDζ1, visible by co-localization with the styryl dye, FM4-64, whereas PLDζ2 was localized to the tonoplast. Both PLDζ1 and PLDζ2 were also found to localize to intracellular compartments (Figures 1a-b). Surprisingly, no differences in subcellular localization were observed in epidermal root cells (Figure 1a), where both fusion proteins were located at the PM or at intracellular compartments, and in the cytosol.

When seedlings were exposed to 120 mM NaCl for 1h, the contrasting localization of PM and tonoplast from respectively PLDζ1 and PLDζ2 in LRC cells did not change, although a signal-intensity shift from membrane to cytosol was observed for both (Figure 1c). In epidermal cells, their subcellular localization did not change either and again a shift from PM to cytosol was found (Figure 1c). Interestingly, the total PLDζ-YFP signal decreased in both lines in root epidermal cells upon salt stress (figure 1d). Based on their location, these findings suggest distinct roles for PLDζ1 and PLDζ2 in LRC cells and a potentially redundant role in epidermal cells.

Upon salt treatment, less intracellular structures containing PIN2-GFP are observed in pldζ2- and pldζ1/pldζ2 roots

To further investigate the cellular roles of PLDζ1 and PLDζ2 during salt stress, we examined the subcellular localization of the auxin efflux carrier, PIN2 using a functional FP fusion (Xu and Scheres, 2005), in both pldζ2 single- and pldζ1/pldζ2 double-KO mutants. Loss of PLDζ1 affected PIN2 polarity in control conditions and led to a PIN2- and AUX1-polarity shift upon salt stress (Chapter 4). However, such change in PIN2 polarity was not reported for salt-stressed pldζ2 (Galvan-Ampudia et al., 2013). Given the opposite PLDζ1 and PLDζ2 phenotypes, we were interested in the PIN2 polarity during salt stress in the pldζ1/pldζ2-double KO mutant.

First, we confirmed the pldζ2 phenotype, with no differences in PIN2 polarity during control conditions and no differences in PIN2-polarity shift at the different sides of the membranes compared to wildtype during salt stress (figure 2a-d). However, in contrast to what we found before, no decrease in PIN2-GFP signal intensity in intracellular PIN2-GFP in pldζ2 was found (Galvan-
Ampudia et al., 2013), which is probably due to the length of the salt treatment. Here, we focused on short-term changes (30 min) while earlier work was conducted after longer salt treatments (60 min). The \textit{pldζ1/pldζ2}-double mutant, similar to the \textit{pldζ1}-single mutant (Chapter 4), showed a decrease in PIN2-GFP signal intensity at the apical side of the PM and an increase at the lateral side of the PM in control conditions (Figure 2a and c). During salt stress, PIN2-GFP signal decreased in the apical and lateral sides of the PM similar to wildtype, however, a more apolar distribution is still observed (Figure 2b). After 30 min of salt stress, the \textit{pldζ1/pldζ2} line has the same PIN2 distribution compared to 5 min of salt treatment and no longer differed from the wildtype roots (Figure 2d). These results show that the \textit{pldζ1/pldζ2} line phenocopies the \textit{pldζ1} line, and that PLDζ1, but not PLDζ2, is involved in PIN2's polarity.

PIN2 internalization was proposed to be altered in the \textit{pldζ2} mutant (Galvan-Ampudia et al., 2013). Therefore, the number of vesicles in the cytosol were quantified under control- and salt stress conditions (figure 3a-b). Under control conditions, no significant differences were found between the amount of vesicles per cell, possibly due to high variation. However, during salt stress, both \textit{pldζ2} and \textit{pldζ1/pldζ2} lines showed a significantly lower amount of vesicles in the cytosol, which is in agreement with earlier results (Galvan-Ampudia et al., 2013). Thus, \textit{pldζ1/pldζ2} behaves similar to the \textit{pldζ1} with respect to PIN2 polarity, but is more like \textit{pldζ2} in the amount of PIN2-containing vesicles during salt stress. These results suggest distinct, non-overlapping functions for PLDζ1 and PLDζ2 in the cellular response to salt stress.

\textit{pldζ1/pldζ2} and \textit{pldζ2} have normal short-term halotropic response

To assess whether the observed cellular phenotypes translate to an effect on the halotropic response, short- and long-term halotropism assays were performed on \textit{pldζ2}-single and \textit{pldζ1/pldζ2}-double mutants. No differences in the initial halotropic response of both \textit{pldζ2}- and \textit{pldζ1/pldζ2} mutants were observed, although after approximately 11 hrs, \textit{pldζ1/pldζ2} exhibited a smaller angle in growing away from the salt gradient, as compared to wildtype and \textit{pldζ2} (Figure 4a-b). This difference remained during the rest of the experiment (24 hrs). Under control conditions in the long-term halotropism assay, \textit{pldζ1/pldζ2} was found to skew more (Supplemental figure S1a), which may explain the smaller angle by which it grew away from the salt gradient. During long term-salt exposure, no significant differences between mutants and wildtype were found
Figure 1. Subcellular localization of PLDζ1-YFP and PLDζ2-YFP with and without salt stress. (A) Confocal microscope images of PLDζ1-mVenus and PLDζ2-mVenus lines, stained with FM4-64. Channels shown are PLDζ-mVenus (yellow), FM4-64 (red) and an overlay of both channels in root cap and epidermal cells. In root cap cells PLDζ1 localizes to the plasma membrane and intracellular structures. PLDζ2 localizes to the tonoplast in the root cap cells furthest from the root tip. In epidermal tissue PLDζ1 localizes to the plasma membrane and intracellular structures containing FM4-64. PLDζ1 also localizes to large intracellular structures (± 1 μm) which do not contain FM4-64. Epidermal PLDζ2 localizes to the PM and intracellular structures. (B) Enlarged confocal microscope images of root cap cells in both PLDζ1-mVenus and PLDζ2-mVenus line; arrows indicate tonoplast localization of PLDζ2. (C) Quantification of the ratio of YFP intensity between the apical and the intracellular YFP signal in root epidermal cells in control- or salt (120 mM NaCl) treatment for both PLDζ1-YFP and PLDζ2-YFP. Letters show significance groups. (D) Quantification of the total YFP intensity in root epidermal cells during control and salt (120 mM NaCl) treatment for both PLDζ1-YFP and PLDζ2-YFP. Letters show significance groups. (p<0.05, in a univariate ANOVA followed by Tukey’s post hoc test in SPSS 24).
Figure 2: PIN2-GFP intensities in root epidermal cells show an apolar distribution in \textit{pld\{1\}/pld\{2\}} and no differences in the \textit{pld\{2\}} line. The PIN2-GFP intensity was measured for the apical and lateral sides of the epidermal root cells next to the intracellular signal during control conditions after 5 (A) and 30 min (C) and for salt stress conditions (120 mM NaCl) for 5 (B) and 30 min (D). Only \textit{pld\{1\}/pld\{2\}} was found to significantly differ from wildtype, the PIN has a more apolar distribution than wild-type cells during control conditions. Asterisks depict significant differences with the wildtype (p<0.05, in an univariate ANOVA followed by Tukey’s post hoc test in SPSS 24).

(Supplemental figure S1c-d). Again, the \textit{pld\{1\}/pld\{2\}} mutant behaved similar to \textit{pld\{2\}}.

assay, \textit{pld\{1\}/pld\{2\}} was found to grow at a significant larger positive angle than wildtype after 11hrs. For the \textit{pld\{2\}} mutant, no difference with wildtype was found. These results confirm involvement of PLD\{1\} in gravitropism, while showing no role for PLD\{2\}. In addition to that, data also indicated that after \textasciitilde 11 hrs, the skewing phenotype of \textit{pld\{1\}/pld\{2\}} becomes visible in the halotropic response as well as during gravitropic challenging of the root. This might indicate a time span for processes that suppress the direction of ‘normal’ root growth. Strikingly, in contrast to halotropism, the \textit{pld\{1\}/pld\{2\}} mutant was found to have a similar phenotype to the \textit{pld\{1\}} mutant during the first hrs of gravitropism.
 Shoot growth during salt stress is not influenced by PLDζ1 and PLDζ2

pldζ2 and pldζ1/pldζ2 were found to exhibit very mild RSA phenotypes in both control- and salt stress conditions (Supplemental figure S2). To test whether the mild RSA phenotype and the changes that occur in root growth upon salt stress have an effect on the performance of plants grown on soil, we measured the fresh- and dry weight of the shoot of wildtype, pldζ1, pldζ2 and pldζ1/pldζ2 under control and mild salt conditions. As shown in Figure 6, mild salt stress (75 mM) reduced shoot growth by ~60%, but we found no significant difference among genotypes.
**pldζ1-, pldζ2- and pldζ1/pldζ2 mutants exhibit elongated root hairs**

Inducible-RNAi inhibition of PLDζ1 has been reported to result in stunted root hairs in Arabidopsis (Ohashi et al., 2003). To assess the role of PLDζ1 and PLDζ2 in root hair development and patterning upon salt stress, 4-day old seedlings of wildtype, *pldζ1*, *pldζ2* and *pldζ1/pldζ2* were transferred to half strength MS plates containing 0 or 125 mM NaCl for 5 days of further growth. Both single- and double mutants were found to have longer root hairs during control- and salt conditions (Figures 7a-b). Also, the root hair length of the mutants was less

![Representative images of seedlings from wildtype, *pldζ1*, *pldζ2*, and *pldζ1/pldζ2* mutants during the time lapse experiment on a 200 mM NaCl gradient. Yellow dashed lines show the border between new and old medium.](image)

![Quantification of the root tip angle over 24 hrs, each time point shows the change after 40 min. Asterisks show significant difference with wildtype (p<0.05 in a univariate ANOVA followed by Tukey’s post hoc test in SPSS 24), the color of the asterisks correspond to the different mutants. Shown are combined results from three biological replicates. For wildtype, n=34, for *pldζ1*, n=42, for *pldζ2*, n=35, and for *pldζ1/pldζ2*, n=37.](image)

**Figure 4: Short-term halotropic response of pldζ1, pldζ2 and pldζ1/pldζ2- double mutant.**

(A) Representative images of seedlings from wildtype, *pldζ1*, *pldζ2*, and *pldζ1/pldζ2* mutants during the time lapse experiment on a 200 mM NaCl gradient. Yellow dashed lines show the border between new and old medium. (B) Quantification of the root tip angle over 24 hrs, each time point shows the change after 40 min. Asterisks show significant difference with wildtype (p<0.05 in a univariate ANOVA followed by Tukey’s post hoc test in SPSS 24), the color of the asterisks correspond to the different mutants. Shown are combined results from three biological replicates. For wildtype, n=34, for *pldζ1*, n=42, for *pldζ2*, n=35, and for *pldζ1/pldζ2*, n=37.

affected by salt, with *pldζ2* showing the lowest response to salt and *pldζ1/pldζ2* having a similar phenotype as *pldζ1*. Previous literature on the inducible-*pldζ1* mutant also reported that root hairs were deformations (Ohashi et al., 2003) but
this was not found for stable mutant lines (Li et al., 2006). No differences in root hair density (Figure 7c) or patterning were found either.

**Discussion**

Plant phospholipase D (PLD) proteins have been of interest to different fields of research. Their biochemical action leads to production of PA, with subsequent effects on bio-membranes, including vesicular trafficking (Li and Xue, 2007) and plant signalling (Testerink and Munnik, 2005). Genetic evidence has implicated their physiological impact on plant development (Zhang et al., 2017), biotic interactions (Zhao, 2015) and abiotic stress (Hong et al., 2010; Hong et al., 2016). A role for PLDζ2 in tropic responses has been described; pldζ2 mutant plants were reported to suppress gravitropism and to facilitate hydrotropism (Taniguchi et al., 2010), and were compromised in their halotropic response (Galvan-Ampudia et al., 2013). Nevertheless, putative redundancy of PLDζ1 and PLDζ2 has not received much attention, except for the observation that during phosphate starvation, pldζ1/pldζ2 double KO plants have shorter main roots and longer lateral roots (Li et al., 2006).

Here, we report differences in the subcellular localization of PLDζ1-mVenus and PLDζ2-mVenus in lateral root cap cells, suggesting unrelated roles for PLDζ1 and PLDζ2 in cellular processes. The pldζ1/pldζ2-double mutant phenocopied pldζ1 in PIN2 polarity, early gravitropic response and root hair length, while it phenocopied pldζ2 in the amount of PIN2-containing vesicles during salt stress and halotropism. Furthermore, increased elongation of root hairs was observed in all PLDζ mutants, while root hair length was less affected by salt for all mutants.

**PLDζ1 and PLDζ2 differ in root cap sub-cellular localization**

PLDζ1-YFP has previously been reported to reside mostly in vesicles in the plant root cortex cells, and at the bulges of newly forming epidermal root hairs (Ohashi et al., 2003). In growing root hairs, PLDζ2 localization was observed near the apex. Transient expression of PLDζ2-GFP showed localization of PLDζ2 to the tonoplast of guard cells and epidermal cells in leaves (Yamaryo et al., 2008). During phosphate starvation PLDζ2 remained at the tonoplast but its distribution was found to be uneven (Yamaryo et al., 2008). Here, we used stable arabidopsis transformants, expressing PLDζ1-YFP and PLDζ2-mVenus under the control of their own promoter, and focussed on their localization in root epidermal- and lateral root-cap cells. Similar to its localization in cortical root cells, PLDζ1-YFP was found to localize to endosomal structures in both root
epidermal- and lateral root cap cells. A weak signal was also observed at the PM. PLDζ2-mVenus was found to localize to the tonoplast and to endosomal structures in the root cap, while in root epidermal cells, PLDζ2-mVenus was found at the PM, and not the tonoplast. Furthermore, a strong signal was observed in the cytosol and endosomal structures. It must be noted that the PLDζ2-mVenus fusion protein is expressed in the pldζ1/pldζ2-double mutant, putatively influencing PLDζ2-mVenus localization. Nonetheless, our data suggests a distinct, so far unknown, role for PLDζ2 in lateral root

Figure 5: The pldζ1/pldζ2 line has an exaggerated gravitropic response while pldζ2 alone does not differ from wildtype. (A) Representative pictures of seedlings 24 hours after the re-orientation of the agar plate. Yellow lines show the point of the root tip immediately after a 90° re-orientation, the black arrow shows the exaggerated response. (B) Quantification of the differences in the angle of root growth over time. Angles between the position of the root tip were calculated every 40 min. Asterisks show significant differences between pldζ1/pldζ2 or pldζ1 and wildtype (p<0.05, univariate ANOVA followed by Tukey’s post hoc test in SPSS24; purple asterisks = pldζ1/pldζ2, red asterisks = pldζ1). Shown are combined results from three biological replicates (n=36 for wildtype n=36 for pldζ1, n=23 for pldζ2, and n=23 for pldζ1/pldζ2).
Figure 6: No difference in shoot fresh- and dry weight in *pldζ2* or *pldζ1/pldζ2* under mild salt stress. (A) Fresh weight of wildtype, *pldζ1*, *pldζ2* and *pldζ1/pldζ2* after 3 weeks of growth on soil watered with rainwater or rainwater containing 75 mM NaCl. No significant differences were found. (B) Dry weight. No significant differences were found (p<0.05, univariate ANOVA followed by Tukey post hoc in SPSS 24).

![Image of fresh and dry weight graphs]

Figure 7: *pldζ1*, *pldζ2* and *pldζ1/pldζ2* mutants have longer root hairs than wildtype and a lower salt-induced root hair length decrease. (A) Representative images of root hairs on wildtype-, *pldζ1*, *pldζ2* and *pldζ1/pldζ2* roots from the transfer point on control and salt (125mM) treatment plates. (B) Average root hair length for all lines on both control and salt treatment plates. On control plates wildtype root hairs had the shortest length, followed by *pldζ2* and *pldζ1/pldζ2*. The *pldζ1* line had the longest root hairs on control plates. On plates containing 125 mM NaCl, wildtype had the largest reduction in root hair length. *pldζ2* had the longest root hairs on salt followed by *pldζ1* and *pldζ1/pldζ2*. (C) Response of root hair length to salt. Ratio between the root hair length on salt and control has been calculated showing a significant weaker response to salt for all mutant lines with. *pldζ2* shows almost no response. (D) Root hair density of wildtype, *pldζ1*, *pldζ2* and *pldζ1/pldζ2* during control and salt treatment. No significant differences were found in either treatment between the lines. Letters show significance groups (For (B) statistics were done per treatment for (D) no letters are shown due to the lack of differences, p<0.05 using univariate ANOVA followed by Tukey post hoc in SPSS 24).
cap cells. One possibility is that PLDζ2 is involved in auxin transport out of the vacuole where auxin is stored to create local auxin maxima, although the role of short distance-auxin transport to change local auxin concentrations remains elusive (Korver et al., 2018).

Changes in the subcellular localization of PLDζ’s during abiotic stress has so far received little attention. Only the aforementioned uneven distribution on the tonoplast during phosphate starvation of PLDζ2 was reported (Yamaryo et al., 2008). Since we are interested in the role of PLDζ1 and PLDζ2 during salt stress, we monitored the changes in PLDζ localization after salt treatment. We discovered a shift in the PM/intracellular ratio after 1 h of salt stress for both PLDζ1 and PLDζ2, supporting a role for these PLDs in the internalization of membrane proteins upon salt stress. We found this to be in agreement with studies showing auxin carrier and aquaporin internalization upon salt stress (Zhang et al., 2010; Martiniere et al., 2012; Galvan-Ampudia et al., 2013; Korver et al., Chapter 4 this thesis).

The PLDζs are involved in different aspects of the PIN2 cycling pathway

PLDζ2 was proposed to be involved in the internalization of PIN2 during halotropism (Galvan-Ampudia et al., 2013). Here, we confirmed the reduced amount of PIN2-GFP containing vesicles in the pldζ2 mutant during salt stress. Because PLDζ1 had been proposed to be involved in the recycling instead of internalizing of PIN2, because of differences in PIN2-polarity shifts during salt stress (Chapter 4), the pldζ1/pldζ2 mutant could be expected to have the same internalization phenotype as pldζ2. Indeed, the pldζ1/pldζ2 mutant showed the same reduction of PIN2-GFP containing vesicles as pldζ2. These findings implicate a role for PLDζ2 in the internalization of PIN2 during salt stress where PLDζ1 might be involved in the proper recycling of PIN2. This theory is supported by the apolar PIN2 distribution that we found in pldζ1/pldζ2, which is similar to pldζ1. Together, these results provide evidence that PLDζ1 and PLDζ2 affect the PIN2 localization during salt stress via distinct processes. PLDζ1 is possibly involved in recruitment of protein kinases and phosphatases to the PM, to regulate the phosphorylation status of PINs, thus maintaining their polarity (Kleine-Vehn et al., 2009; Ganguly et al., 2012; Guo et al., 2015). Another possible role for PLDζ1 could be in the inhibition of the lateral diffusion of PIN2 in the PM, which is required for endured polar detention (Kleine-Vehn et al., 2011). Our suggestion that PLDζ2 is involved in the internalization of PIN2 and not in its polarity is supported by a previous report, that inhibition of PIN2 endocytosis did not affect the auxin mediated-PIN2 dynamics (Jasik et al., 2016).
However, PLDζ2 could putatively regulate halotropism timing by regulating the amount of PIN2 available for cycling.

The discovery of defects in root hair pattern formation in the pldζ1 single mutant (Ohashi et al., 2003) also provide evidence for the role of PLDζ1 in recycling. For appropriate root hair polarity, which is required for straight root hair growth, cellular gradients are required that depend on phosphoinositide metabolism and the correct cytoskeleton arrangement (Yoo et al., 2012). More evidence showing the importance of PIN cycling is seen in a phosphatidylinositol monophosphate 5-kinase 2 (pip5k2) KO mutant. Less vesicle trafficking was observed in this mutant using FM4-64. This led to inhibited cycling of PIN proteins and delayed redistribution of PIN2 during gravitropism (Mei et al., 2012). Moreover, the pip5k2 mutant has reduced lateral root formation, showing the importance of PIN cycling during root developmental processes.

**Loss of PLDζ2 does not influence halotropism or gravitropism**

Both PLDζ1 (Chapter 4) and PLDζ2 (Li and Xue, 2007; Taniguchi et al., 2010; Galvan-Ampudia et al., 2013) have been found to be involved in the root growth direction during tropism responses. Here, unexpectedly, we did not observe any changes in either short-term (less than 12 hrs) or long-term (4 days) halotropic response of either the pldζ2 single or the pldζ1/pldζ2-double mutant. This contrasts to previous results on the halotropic response of pldζ2 (Galvan-Ampudia et al., 2013), where a smaller angle was observed after 24 hrs on a salt gradient. This might be explained by the high biological variation of the current halotropism assay and the small difference that was observed previously. PLDζ1 was reported before to have a weaker halotropic response. This result might be explained by the altered PIN2 polarity caused by loss of PLDζ1. Assuming a shift from PIN2 from the apical to the lateral membrane is needed for normal halotropic response, putatively the loss of PLDζ1 and PLDζ2 might complement each other to restore normal PIN2 polarity during salt stress. The cell already has elevated levels of PIN2 at the lateral membrane through defective PLDζ1 regulated PIN2 recycling (Chapter 4). However, in the pldζ1/pldζ2 double mutant, due to loss of PLDζ2, less apical PIN2 is internalized, resulting in a PIN2 polarity more similar to the wildtype plants during salt stress. Whether this scenario is realistic might be assessed using computational modelling. The smaller angle away from the in-plate gradient after 11h of exposure to a salt gradient of the pldζ1/pldζ2 double mutant is explained by the skewing phenotype of the mutant.
Remarkably, loss of PLDζ2 did not result in a change in gravitropic growth while loss of PLDζ1 resulted in an exaggerated gravitropic response. Interestingly, the pldζ1/pldζ2-double mutant showed an exaggerated gravitropic response similar, although weaker, to pldζ1. This suggests that during gravitropism, proper recycling of auxin carrier proteins plays a more prominent role than internalization. Computational modelling could again be useful to test this hypothesis.

Strikingly, in both short-term halotropism assay and gravitropism assay, skewing to the right was observed after 11 hrs in the pldζ1/pldζ2 double mutant. This indicates a timing mechanism for the abolishment of tropic responses. So far, timing of environmental induced changes in root growth has only been reported for growth rate (Geng et al., 2013), not direction.

**Minor differences in RSA do not affect shoot growth on saline soil**

Loss of PLDζ1 has been observed to affect average lateral root length but not number of lateral roots (Chapter 4). Different RSA-adaptation strategies have been found for Arabidopsis accessions during salt stress (Julkowska et al., 2014). However, loss of PLDζ2, or PLDζ1 and PLDζ2, induces only minor changes in RSA. To rule out an effect of PLDζ1 or PLDζ2 in shoot performance on salt, we measured fresh- and dry weights after 3 weeks of growth on saline soil. Indeed, no differences between the PLDζ mutants and wildtype were found.

**Root hair length is regulated by PLDζ1 and even more by PLDζ2**

In growing root hairs and pollen tubes, a PtdIns(4,5)P$_2$ (PIP2) gradient is found localized in the tip (van Leeuwen et al., 2007; Munnik and Nielsen, 2011), indicating importance of lipid signalling in root hair protein polarity. For proper root hair patterning, an array of transcription factors (TFs) and other targets are regulated by the homeobox gene GLABRA2 (GL2). PLDζ1 has been reported to be a direct transcriptional target of GL2 (Ohashi et al., 2003). Induced PLDζ1 expression leads to abnormal root hair patterning whereas inducible suppression of PLDζ1 resulted in defective root-hair initiation. Moreover, repression of genes encoding structural cell wall components and genes involved in the differentiation of trichoblasts is reported in response to salt stress (Dinneny et al., 2008). During salt stress, a decrease in root hair length and density has been reported (Wang et al., 2008). Additionally, root hairs were found to accumulate Na$^+$ in the cytoplasm as fast as 5 min after salt treatment (Halperin and Lynch, 2003). Interestingly, we found longer root hairs in all PLDζ (single and double) mutants under control conditions. Moreover, loss of PLDζ2 leads to the lowest root hair length inhibition by salt stress. This is consistent
with the negative effect of PLDζ2 on root hair length during other abiotic stress, e.g. phosphate starvation (Su et al., 2018). These results show negative regulation of root hair length by PLDζ2 and in lesser extent by PLDζ1. Following the reduction of root hair number and length in wildtype roots during salt stress, the elongated roots in PLDζ might influence performance on saline soils through Na⁺ accumulation in the longer root hair. However, we did not find any differences in performance of the PLDζ mutants. So, although PLDζs are involved in root reduction of root hair length during salt stress, the effect of these changes for whole plant performance under salinity stress remains unknown.

Methods

Plant materials and growth conditions
The wildtype (WT) used was Arabidopsis thaliana, ecotype Columbia-0 (Col-0). All mutants are within this background. The pldζ2 mutant is a tDNA insertion line (SALK_094369). The pldζ1/ pldζ2 is a cross between pldζ1 (SALK_083090) and pldζ2 (SALK_094369). The pldζ2/PIN2-YFP and pldζ1/ pldζ2/PIN2-YFP were created by crossing the KO mutants with a PIN2-GFP line. The WT/PLDζ1-YFP line was obtained from Takashi Aoyama (Ohashi et al., 2003). pldζ2/PLDζ2-mVenus was created by crossing pldζ1/ pldζ2 with PLDζ2-mVenus. Primers used for genotyping: for PLDζ1, forward, tgaaagcatggaaattttcg and reverse gtgatcgtctctgtctctcgc and for PLDζ2, forward, cttcagccttcagatgc and reverse, cggcatttacctctggtacag. General growth conditions on agar plates (0.5x MS, supplemented with 0.1% 2(N-morpholino)ethanesulphonic acid (MES) buffer, 0.5% sucrose and 1% agar) were in a climate chamber with long day period (16 hours light at 130 µmol/m²/s) at 22°C and 70% humidity. Seeds were sterilized using 50% bleach and stratified for at least 2 days at 4°C. For soil experiments, seeds sterilized with 50% bleach were stratified in 0.1% agar in the dark for at least 2 days and then placed on sieved sowing ground. Plants were then grown were in a climate chamber with short day period (11 h light at 130 µmol/m²/s) at 22°C and 70% humidity.

Halotropism plate assays and gravitropism plate assays
For the halotropism plate assays (both during time-lapse imaging and long-term halotropism assays), 10 seeds were germinated in a diagonal line on half strength MS plates. When the seedlings were 5 days old, the bottom corner (in diagonal line 0.5 cm below the root tips) of the agar was removed and replaced by control half-strength MS agar without salt or half strength MS agar containing 200 mM NaCl. For the time-lapse experiment the plates were placed in a climate chamber containing the time-lapse set-up. For the latter, all plates were imaged every 20 min by infrared photography. Images were then analysed using ImageJ. For the long-term halotropism assay, a dot was placed
immediately after replacing the agar and every 24 hrs after the start of the treatment. After 4 days of growth, the plates were scanned and the images were analysed using ImageJ. In the gravitropism assay, 12 plants were germinated on half strength MS plates and after 5 days of growth the plates were re-orientated by turning 90° and placed in the climate chamber containing the time-lapse set-up, after which they were imaged and analysed as above.

Confocal microscopy

The images were acquired using a Nikon Ti inverted microscope in combination with an A1 spectral confocal scanning head. For GFP fusion proteins excitation/emission wavelengths used were 488 nm/505-555 nm. For YFP and mVenus excitation/emission wavelengths were 514 nm/525-555 nm. The analysis of the images was performed using Fiji (http://fiji.sc) software. All measured membranes were corrected for their size so the artificial unit used depicts average transport capacity over the membrane.

Root system architecture assay

pldζ2, pldζ1/pldζ2 and WT plants were germinated on half strength MS plates. Four days after germination the seedlings were transferred to half strength MS plates with either 0 mM, 75 mM and 125 mM of NaCl. Four seedlings were transferred to each plate, resulting in 20 replicas per line per treatment. Plates were placed in the climate chamber following a randomized order. After 6 days the plates were scanned using an Epson Perfection V800 scanner at a resolution of 400 dpi. Root phenotypes were quantified using the SmartRoot (Lobet et al., 2011) plugin for ImageJ. Statistical analysis was performed in R with RStudio using two-way ANOVA with Tukey’s post hoc test for significance.

Salt performance assay

pldζ2, pldζ1/pldζ2 and WT plants were germinated on sowing soil. After one week of growth seedlings were transferred to the treatment trays in a randomized order, with 2 seedlings per pot. A day before transfer of seedlings the dried treatment trays were treated with 4 L of rainwater containing salt for a final concentration of 75 mM NaCl or no salt for the control treatment. Per line, per treatment 20 replicates are used. After one week of growth on the treatment tray the seedlings were thinned to one seedling per pot, when necessary seedlings from the same tray and genotype were transferred to an empty pot. After the initial treatment water, the trays were watered with rainwater once per week. Three weeks after the start of the treatment the shoots the plants were harvested and weighed for freshweight. Plants were then individually stored in paper bags and dried at 70 °C in a stove for 3 weeks and dryweight was measured.

Root hair experiment
pldζ1, pldζ2, pldζ1/pldζ2 and wildtype plants were germinated on half strength MS plates. Four days after germination the seedlings were transferred to half strength MS plates with either 0 mM, or 125 mM NaCl. Four seedlings were transferred to each plate, resulting in 20 replicas per line per treatment. Plates were placed in the climate chamber following a randomized order. After 5 days of growth on the treatment plates the new growth of each root was photographed under an Olympus BH-2 microscope with a Infinity 1 microscope camera. The amount of root hair was counted and the length measured using ImageJ software.

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References


Yoo CM, Quan L, Cannon AE, Wen J, Blanchafior EB (2012) AGD1, a class 1 ARF-GAP, acts in common signaling pathways with phosphoinositide metabolism and the actin cytoskeleton in controlling Arabidopsis root hair polarity. Plant J 69: 1064-1076

Supplemental figure S1: Long-term halotropic response in *pldξ1, pldξ2* and *pldξ1/pldξ2*-double mutants. Quantification of the angle of the main root compared to the direction of gravity over 4 days on either a 0 mM NaCl (A), 75 mM NaCl (B), 125 mM NaCl (C) or 200 mM NaCl gradient. Positive angles correspond with growth towards the salt medium, negative angles correspond with growth away from the salt medium. Asterisks show significant difference with wildtype (p<0.05 in a univariate ANOVA followed by Tukey’s post hoc test in SPSS 24), the color of the asterisks correspond to the different mutants. n= ~70 for all lines and treatments.
Supplemental figure S2: Root system architecture shows mild differences between wildtype, *pldζ2* and *pldζ1/pldζ2* during salt stress. The only difference found in control condition was on 'straightness', where wildtype showed a straighter root than both mutants (B). During mild salt stress (75 mM NaCl) no significant differences in RSA between the lines were found. For more severe salt stress (125 mM NaCl) a difference between wildtype and *pldζ2* was found on root growth direction (F). Differences between wildtype and *pldζ1/pldζ2* were found on main root length (A) and length of branched zone (G). Most differences were observed between *pldζ2* and *pldζ1/pldζ2*. Main root length (A), number of lateral roots (C), Lateral Root density (D), total lateral root length (E) and length of branched zone were found to be significantly different. Letters show significance groups between the different lines in one treatment (p<0.05 using univariate ANOVA followed by Tukey post hoc in SPSS 24).