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### Going in the right direction

*Cellular mechanisms underlying root halotropism*

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# Chapter 5

## PLD $\zeta$ 1 and PLD $\zeta$ 2 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  $\zeta$  proteins have been implicated in the response of plant roots to salt stress. Although involvement of both PLD $\zeta$ 1 and PLD $\zeta$ 2 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 *pld $\zeta$ 2*- and *pld $\zeta$ 1/pld $\zeta$ 2*- double mutant and compared the results with recent data of *pld $\zeta$ 1* (Chapter 4). The *pld $\zeta$ 1/pld $\zeta$ 2* mutant was found to largely exhibit the same phenotype as *pld $\zeta$ 1* in PIN2 polarity and inhibition of root hair length by salt. On the other hand, the double mutant phenocopied the *pld $\zeta$ 2* 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 PLD $\zeta$  mutants. These effects were largest in the *pld $\zeta$ 2* line. These results indicate that PLD $\zeta$ 1 and PLD $\zeta$ 2 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<sup>+</sup> 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 PLD $\zeta$ 2 is required for salt-induced CME and thus the internalization of PIN2. Treatment with an inhibitor known to obstruct mammalian PLDs, FIPI (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 PLD $\zeta$ 1 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 PLD $\zeta$ 2 in salt stress. Using fluorescent PLD $\zeta$  fusions of both PLD $\zeta$ 1 and PLD $\zeta$ 2, 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 PLD $\zeta$ s, however, no role for PLD $\zeta$ 2 in either early halo- or gravitropism was observed. Also, we found an apolar PIN2 distribution in the *pld $\zeta$ 1/pld $\zeta$ 2* mutant but not in the *pld $\zeta$ 2* mutant under control conditions. Knockouts of PLD $\zeta$ 1 and PLD $\zeta$ 2 do not lead to major changes in performance or RSA during salt stress, indicating a minor role for both PLD $\zeta$ s 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 PLD $\zeta$ 1 and not PLD $\zeta$ 2 regulates PIN2 sub-cellular localization, however, both PLD $\zeta$ s are involved in root hair length.

## Results

### **PLD $\zeta$ 1 and PLD $\zeta$ 2 show different subcellular localization in LRC and similar localization in epidermal cells**

To study the subcellular localization of PLD $\zeta$ 1 and PLD $\zeta$ 2, FP-fusions were constructed and imaged with and without salt stress. In lateral root cap (LRC) cells, plasma membrane (PM) localization was observed for PLD $\zeta$ 1, visible by co-localization with the styryl dye, FM4-64, whereas PLD $\zeta$ 2 was localized to the tonoplast. Both PLD $\zeta$ 1 and PLD $\zeta$ 2 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 PLD $\zeta$ 1 and PLD $\zeta$ 2 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 PLD $\zeta$ -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 PLD $\zeta$ 1 and PLD $\zeta$ 2 in LRC cells and a potentially redundant role in epidermal cells.

### **Upon salt treatment, less intracellular structures containing PIN2-GFP are observed in *pld $\zeta$ 2*- and *pld $\zeta$ 1/pld $\zeta$ 2* roots**

To further investigate the cellular roles of PLD $\zeta$ 1 and PLD $\zeta$ 2 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 *pld $\zeta$ 2* single- and *pld $\zeta$ 1/pld $\zeta$ 2* double-KO mutants. Loss of PLD $\zeta$ 1 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 *pld $\zeta$ 2* (Galvan-Ampudia et al., 2013). Given the opposite PLD $\zeta$ 1 and PLD $\zeta$ 2 phenotypes, we were interested in the PIN2 polarity during salt stress in the *pld $\zeta$ 1/pld $\zeta$ 2*-double KO mutant.

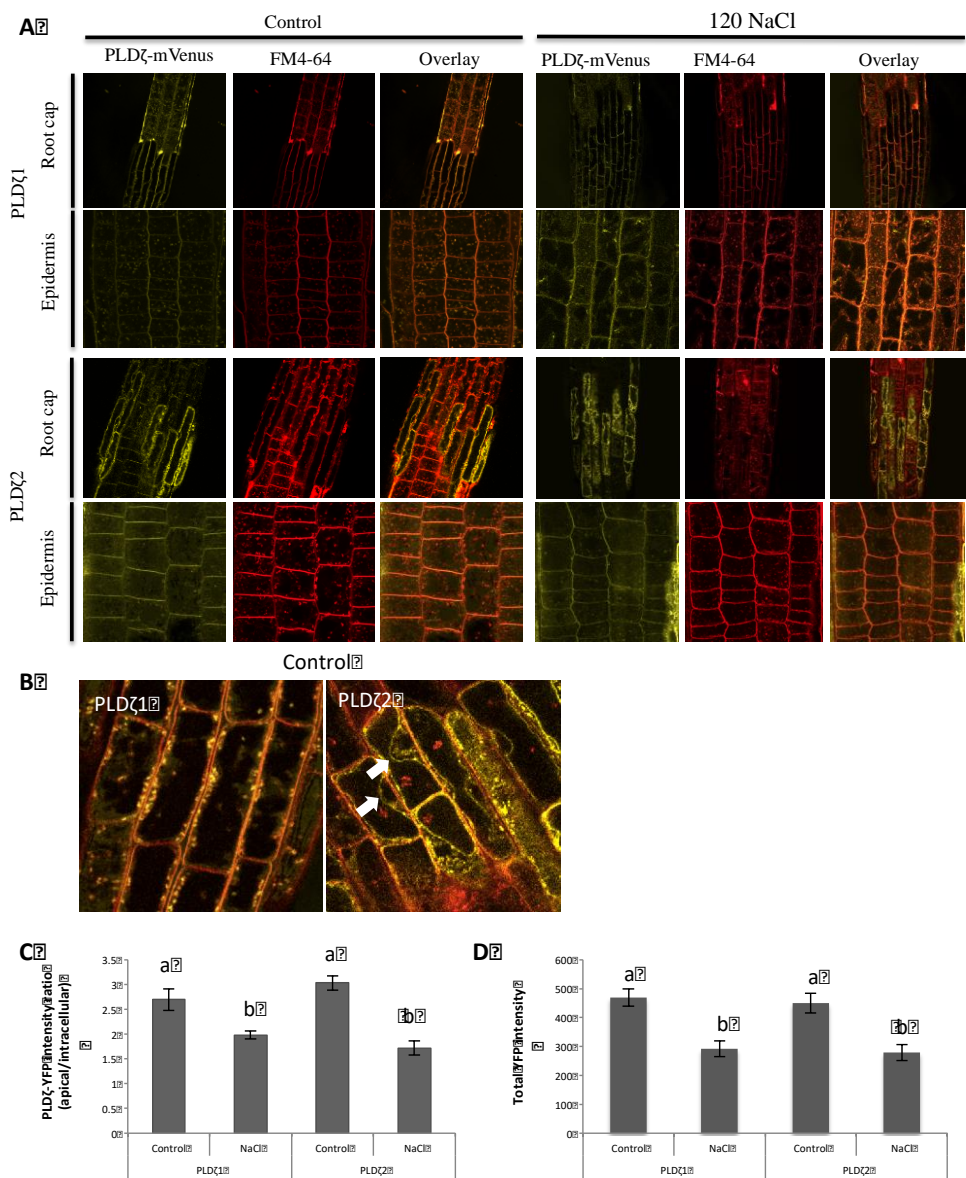
First, we confirmed the *pld $\zeta$ 2* phenotype, with no differences in PIN2 polarity during control conditions and no differences in PIN2-polarity shift at the different sides of the membranes compared to wildtype during salt stress (figure 2a-d). However, in contrast to what we found before, no decrease in PIN2-GFP signal intensity in intracellular PIN2-GFP in *pld $\zeta$ 2* 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 *pldζ1/pldζ2*-double mutant, similar to the *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 *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 *pldζ1/pldζ2* line phenocopies the *pldζ1* line, and that PLDζ1, but not PLDζ2, is involved in PIN2's polarity.

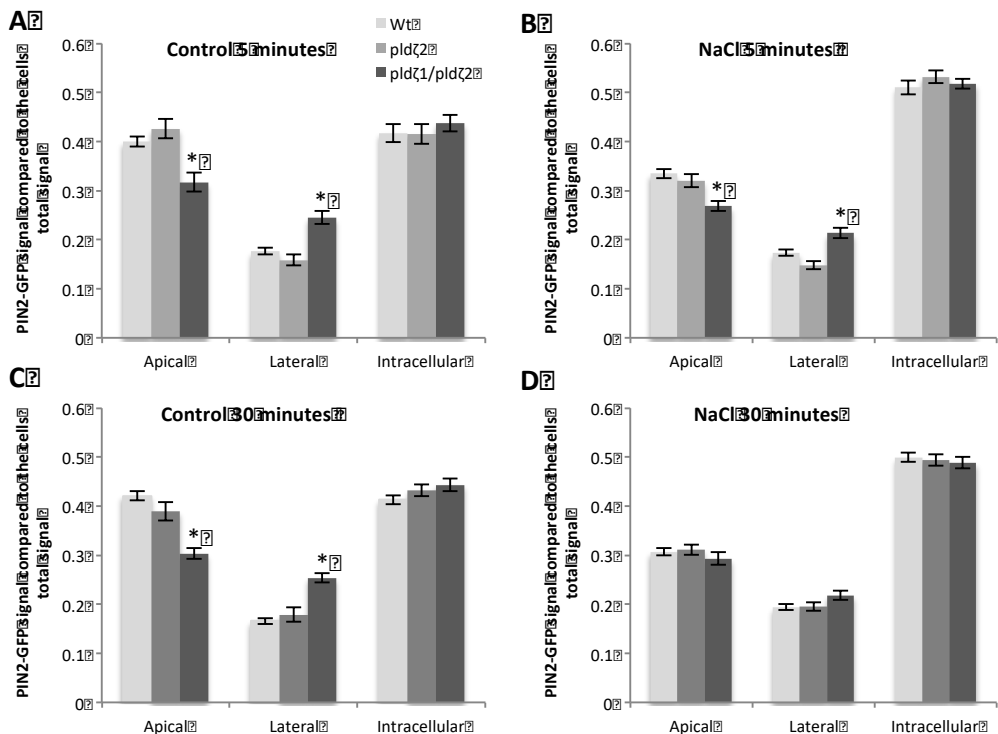
PIN2 internalization was proposed to be altered in the *pldζ2* mutant (Galvan-Ampudia et al., 2013). Therefore, the number of vesicles in the cytosol were quantified under control- and salt stress conditions (figure 3a-b). Under control conditions, no significant differences were found between the amount of vesicles per cell, possibly due to high variation. However, during salt stress, both *pldζ2*- and *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, *pldζ1/pldζ2* behaves similar to the *pldζ1* with respect to PIN2 polarity, but is more like *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.

### ***pldζ1/pldζ2* and *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 *pldζ2*-single and *pldζ1/pldζ2*-double mutants. No differences in the initial halotropic response of both *pldζ2*- and *pldζ1/pldζ2* mutants were observed, although after approximately 11 hrs, *pldζ1/pldζ2* exhibited a smaller angle in growing away from the salt gradient, as compared to wildtype and *pldζ2* (Figure 4a-b). This difference remained during the rest of the experiment (24 hrs). Under control conditions in the long-term halotropism assay, *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



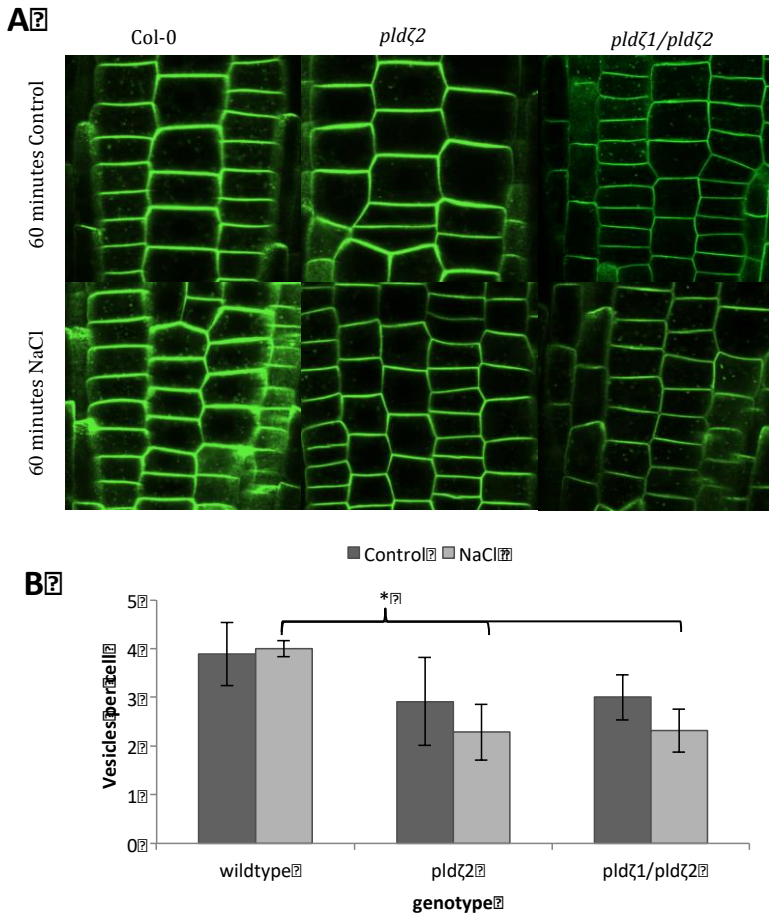
**Figure 1. Subcellular localization of PLD $\zeta$ 1-YFP and PLD $\zeta$ 2-YFP with and without salt stress.** (A) Confocal microscope images of PLD $\zeta$ 1-mVenus and PLD $\zeta$ 2-mVenus lines, stained with FM4-64. Channels shown are PLD $\zeta$ -mVenus (yellow), FM4-64 (red) and an overlay of both channels in root cap and epidermal cells. In root cap cells PLD $\zeta$ 1 localizes to the plasma membrane and intracellular structures. PLD $\zeta$ 2 localizes to the tonoplast in the root cap cells furthest from the root tip. In epidermal tissue PLD $\zeta$ 1 localizes to the plasma membrane and intracellular structures containing FM4-64. PLD $\zeta$ 1 also localizes to large intracellular structures ( $\pm 1 \mu\text{m}$ ) which do not contain FM4-64. Epidermal PLD $\zeta$ 2 localizes to the PM and intracellular structures. (B) Enlarged confocal microscope images of root cap cells in both PLD $\zeta$ 1-mVenus and PLD $\zeta$ 2-mVenus line; arrows indicate tonoplast localization of PLD $\zeta$ 2. (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 PLD $\zeta$ 1-YFP and PLD $\zeta$ 2-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 PLD $\zeta$ 1-YFP and PLD $\zeta$ 2-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 *pldζ1/pldζ2* and no differences in the *pldζ2* 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 *pldζ1/pldζ2* 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 *pldζ1/pldζ2* mutant behaved similar to *pldζ2*.

assay, *pldζ1/pldζ2* was found to grow at a significant larger positive angle than wildtype after 11hrs. For the *pldζ2* mutant, no difference with wildtype was found. These results confirm involvement of PLDζ1 in gravitropism, while showing no role for PLDζ2. In addition to that, data also indicated that after ~11 hrs, the skewing phenotype of *pldζ1/pldζ2* 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 *pldζ1/pldζ2* mutant was found to have a similar phenotype to the *pldζ1* mutant during the first hrs of gravitropism.



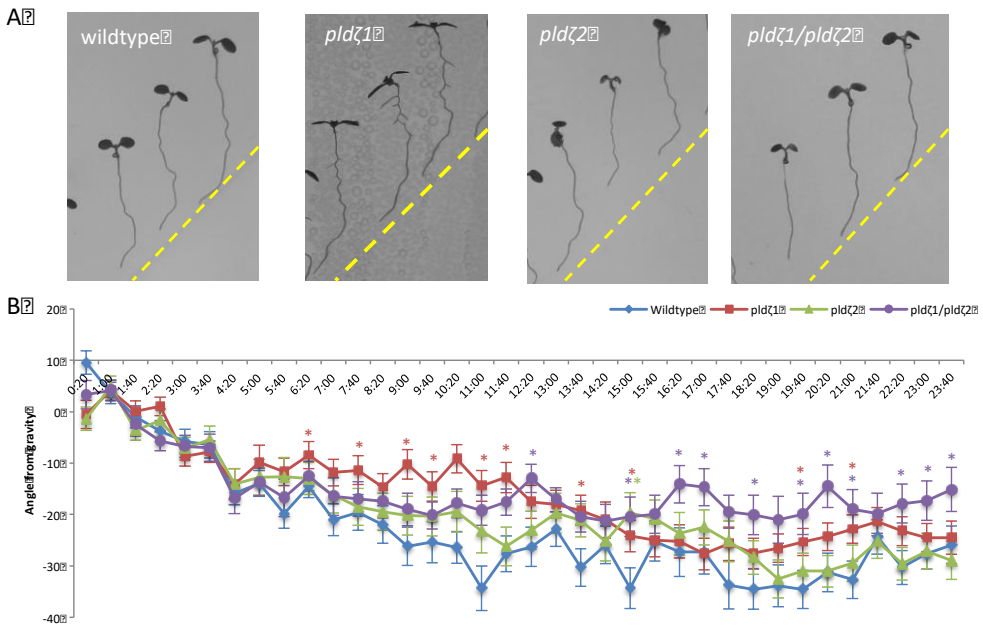
**Figure 3. Less intracellular structures containing PIN2-GFP are observed upon NaCl treatment in *pldζ2* and *pldζ1/pldζ2* *Arabidopsis thaliana* mutants.** Confocal images and quantification of wildtype, *pldζ2* and *pldζ1/pldζ2* lines expressing PIN2-GFP. (A) Confocal images showing PIN2-GFP localization in wildtype, *pldζ2* and *pldζ1/pldζ2* lines. In control conditions no clear differences were observed between wildtype, *pldζ2* and *pldζ1/pldζ2*. Upon salt stress, no change in intracellular structures containing PIN2-GFP were observed in wildtype while in the different *pldζ* mutants there seemed to be a decrease of intracellular structures containing PIN2-GFP. (B) Quantification of the amount of intracellular structures containing PIN2-GFP in the different genotypes in either control or a one hour 120 mM NaCl treatment. Asterisks show significant differences between the lines in one treatment ( $p < 0.05$ , univariate ANOVA followed by Tukey's post hoc test in SPSS 24).

### 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



**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

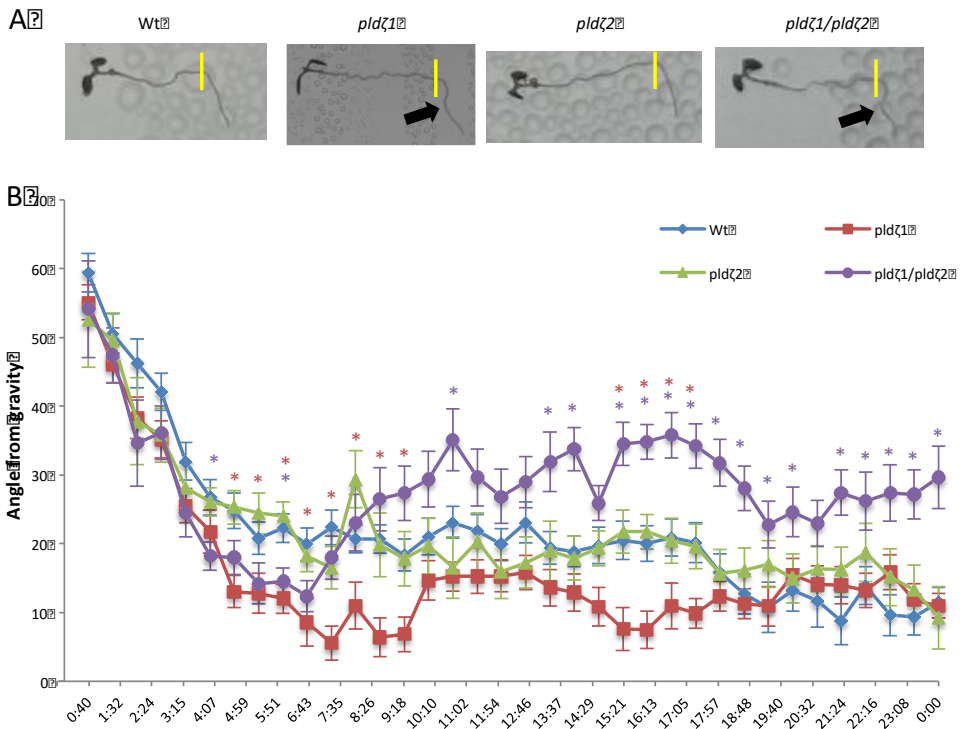
Plant phospholipase D (PLD) proteins have been of interest to different fields of research. Their biochemical action leads to production of PA, with subsequent effects on bio-membranes, including vesicular trafficking (Li and Xue, 2007) and plant signalling (Testerink and Munnik, 2005). Genetic evidence 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 PLD $\zeta$ 2 in tropic responses has been described; *pld $\zeta$ 2* 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 PLD $\zeta$ 1 and PLD $\zeta$ 2 has not received much attention, except for the observation that during phosphate starvation, *pld $\zeta$ 1/pld $\zeta$ 2* double KO plants have shorter main roots and longer lateral roots (Li et al., 2006).

Here, we report differences in the subcellular localization of PLD $\zeta$ 1-mVenus and PLD $\zeta$ 2-mVenus in lateral root cap cells, suggesting unrelated roles for PLD $\zeta$ 1 and PLD $\zeta$ 2 in cellular processes. The *pld $\zeta$ 1/pld $\zeta$ 2*-double mutant phenocopied *pld $\zeta$ 1* in PIN2 polarity, early gravitropic response and root hair length, while it phenocopied *pld $\zeta$ 2* in the amount of PIN2-containing vesicles during salt stress and halotropism. Furthermore, increased elongation of root hairs was observed in all PLD $\zeta$  mutants, while root hair length was less affected by salt for all mutants.

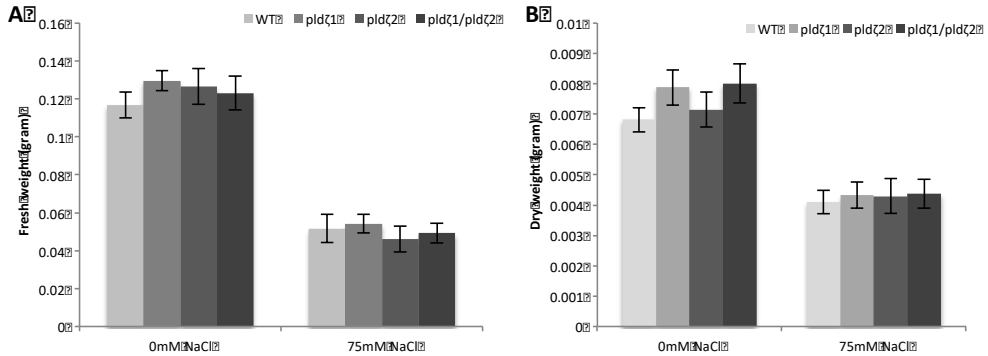
### PLD $\zeta$ 1 and PLD $\zeta$ 2 differ in root cap sub-cellular localization

PLD $\zeta$ 1-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, PLD $\zeta$ 2 localization was observed near the apex. Transient expression of PLD $\zeta$ 2-GFP showed localization of PLD $\zeta$ 2 to the tonoplast of guard cells and epidermal cells in leaves (Yamaryo et al., 2008). During phosphate starvation PLD $\zeta$ 2 remained at the tonoplast but its distribution was found to be uneven (Yamaryo et al., 2008). Here, we used stable arabidopsis transformants, expressing PLD $\zeta$ 1-YFP and PLD $\zeta$ 2-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, PLD $\zeta$ 1-YFP was found to localize to endosomal structures in both root

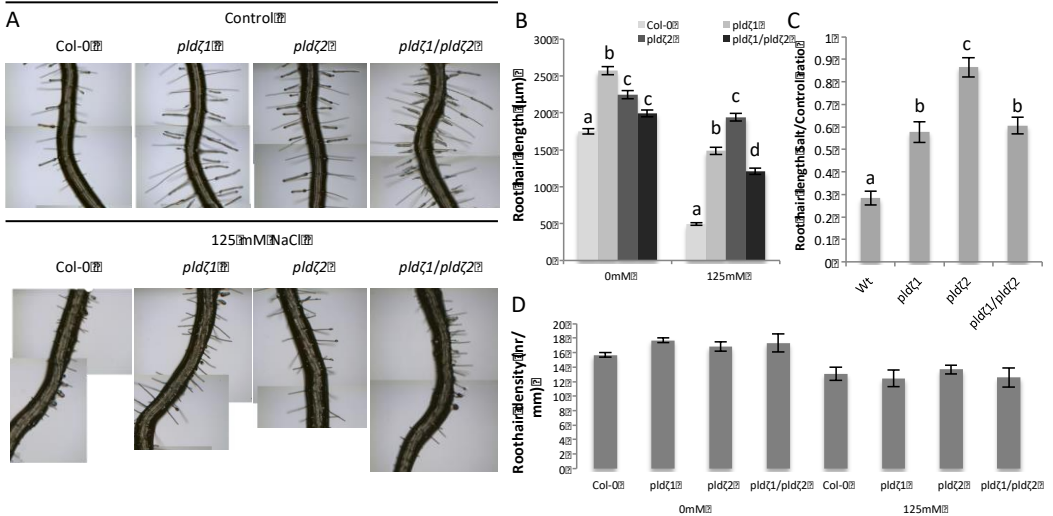
epidermal- and lateral root cap cells. A weak signal was also observed at the PM. PLD $\zeta$ -mVenus was found to localize to the tonoplast and to endosomal structures in the root cap, while in root epidermal cells, PLD $\zeta$ -mVenus was found at the PM, and not the tonoplast. Furthermore, a strong signal was observed in the cytosol and endosomal structures. It must be noted that the PLD $\zeta$ -mVenus fusion protein is expressed in the *pld $\zeta$ 1/pld $\zeta$ 2*-double mutant, putatively influencing PLD $\zeta$ -mVenus localization. Nonetheless, our data suggests a distinct, so far unknown, role for PLD $\zeta$ 2 in lateral root



**Figure 5: The *pld $\zeta$ 1/pld $\zeta$ 2* line has an exaggerated gravitropic response while *pld $\zeta$ 2* 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 *pld $\zeta$ 1/pld $\zeta$ 2* or *pld $\zeta$ 1* and wildtype (p < 0.05, univariate ANOVA followed by Tukey's post hoc test in SPSS24; purple asterisks = *pld $\zeta$ 1/pld $\zeta$ 2*, red asterisks = *pld $\zeta$ 1*). Shown are combined results from three biological replicates (n=36 for wildtype n=36 for *pld $\zeta$ 1*, n=23 for *pld $\zeta$ 2*, and n=23 for *pld $\zeta$ 1/pld $\zeta$ 2*).



**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).



**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 PLD $\zeta$ 2 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 PLD $\zeta$ 's during abiotic stress has so far received little attention. Only the aforementioned uneven distribution on the tonoplast during phosphate starvation of PLD $\zeta$ 2 was reported (Yamaryo et al., 2008). Since we are interested in the role of PLD $\zeta$ 1 and PLD $\zeta$ 2 during salt stress, we monitored the changes in PLD $\zeta$  localization after salt treatment. We discovered a shift in the PM/intracellular ratio after 1 h of salt stress for both PLD $\zeta$ 1 and PLD $\zeta$ 2, 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 PLD $\zeta$ s are involved in different aspects of the PIN2 cycling pathway**

PLD $\zeta$ 2 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 *pld $\zeta$ 2* mutant during salt stress. Because PLD $\zeta$ 1 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 *pld $\zeta$ 1/pld $\zeta$ 2* mutant could be expected to have the same internalization phenotype as *pld $\zeta$ 2*. Indeed, the *pld $\zeta$ 1/pld $\zeta$ 2* mutant showed the same reduction of PIN2-GFP containing vesicles as *pld $\zeta$ 2*. These findings implicate a role for PLD $\zeta$ 2 in the internalization of PIN2 during salt stress where PLD $\zeta$ 1 might be involved in the proper recycling of PIN2. This theory is supported by the apolar PIN2 distribution that we found in *pld $\zeta$ 1/pld $\zeta$ 2*, which is similar to *pld $\zeta$ 1*. Together, these results provide evidence that PLD $\zeta$ 1 and PLD $\zeta$ 2 affect the PIN2 localization during salt stress via distinct processes. PLD $\zeta$ 1 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 PLD $\zeta$ 1 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 PLD $\zeta$ 2 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, PLD $\zeta$ 2 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 *pld $\zeta$ 1* single mutant (Ohashi et al., 2003) also provide evidence for the role of PLD $\zeta$ 1 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 PLD $\zeta$ 2 does not influence halotropism or gravitropism**

Both PLD $\zeta$ 1 (Chapter 4) and PLD $\zeta$ 2 (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 *pld $\zeta$ 2* single or the *pld $\zeta$ 1/pld $\zeta$ 2*-double mutant. This contrasts to previous results on the halotropic response of *pld $\zeta$ 2* (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. PLD $\zeta$ 1 was reported before to have a weaker halotropic response. This result might be explained by the altered PIN2 polarity caused by loss of PLD $\zeta$ 1. Assuming a shift from PIN2 from the apical to the lateral membrane is needed for normal halotropic response, putatively the loss of PLD $\zeta$ 1 and PLD $\zeta$ 2 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 PLD $\zeta$ 1 regulated PIN2 recycling (Chapter 4). However, in the *pld $\zeta$ 1/pld $\zeta$ 2* double mutant, due to loss of PLD $\zeta$ 2, 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 *pld $\zeta$ 1/pld $\zeta$ 2* double mutant is explained by the skewing phenotype of the mutant.

Remarkably, loss of PLD $\zeta$ 2 did not result in a change in gravitropic growth while loss of PLD $\zeta$ 1 resulted in an exaggerated gravitropic response. Interestingly, the *pld $\zeta$ 1/pld $\zeta$ 2*-double mutant showed an exaggerated gravitropic response similar, although weaker, to *pld $\zeta$ 1*. 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 *pld $\zeta$ 1/pld $\zeta$ 2* 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 PLD $\zeta$ 1 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 PLD $\zeta$ 2, or PLD $\zeta$ 1 and PLD $\zeta$ 2, induces only minor changes in RSA. To rule out an effect of PLD $\zeta$ 1 or PLD $\zeta$ 2 in shoot performance on salt, we measured fresh- and dry weights after 3 weeks of growth on saline soil. Indeed, no differences between the PLD $\zeta$  mutants and wildtype were found.

### **Root hair length is regulated by PLD $\zeta$ 1 and even more by PLD $\zeta$ 2**

In growing root hairs and pollen tubes, a PtdIns(4,5)P<sub>2</sub> (PIP<sub>2</sub>) 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*). PLD $\zeta$ 1 has been reported to be a direct transcriptional target of *GL2* (Ohashi et al., 2003). Induced PLD $\zeta$ 1 expression leads to abnormal root hair patterning whereas inducible suppression of PLD $\zeta$ 1 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<sup>+</sup> in the cytoplasm as fast as 5 min after salt treatment (Halperin and Lynch, 2003). Interestingly, we found longer root hairs in all PLD $\zeta$  (single and double) mutants under control conditions. Moreover, loss of PLD $\zeta$ 2 leads to the lowest root hair length inhibition by salt stress. This is consistent

with the negative effect of PLD $\zeta$ 2 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 PLD $\zeta$ 2 and in lesser extent by PLD $\zeta$ 1. Following the reduction of root hair number and length in wildtype roots during salt stress, the elongated roots in PLD $\zeta$  might influence performance on saline soils through Na<sup>+</sup> accumulation in the longer root hair. However, we did not find any differences in performance of the PLD $\zeta$  mutants. So, although PLD $\zeta$ s 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 *pld $\zeta$ 2* mutant is a tDNA insertion line (SALK\_094369). The *pld $\zeta$ 1/pld $\zeta$ 2* is a cross between *pld $\zeta$ 1* (SALK\_083090) and *pld $\zeta$ 2* (SALK\_094369). The *pld $\zeta$ 2/PIN2-YFP* and *pld $\zeta$ 1/pld $\zeta$ 2/PIN2-YFP* were created by crossing the KO mutants with a PIN2-GFP line. The WT/PLD $\zeta$ 1-YFP line was obtained from Takashi Aoyama (Ohashi et al., 2003). *pld $\zeta$ 1/pld $\zeta$ 2/PLD $\zeta$ 2-mVenus* was created by crossing *pld $\zeta$ 1/pld $\zeta$ 2* with PLD $\zeta$ 2-mVenus. Primers used for genotyping: for PLD $\zeta$ 1, forward, tgaagagcatggaattttcg and reverse gtgatcgtctctgtctctcgc and for PLD $\zeta$ 2, forward, cttcatgagccttcagaatgc and reverse, cggcatttacctctgtgtacag. 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  $\mu$ mol/m<sup>2</sup>/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  $\mu$ mol/m<sup>2</sup>/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 0mM, 75mM 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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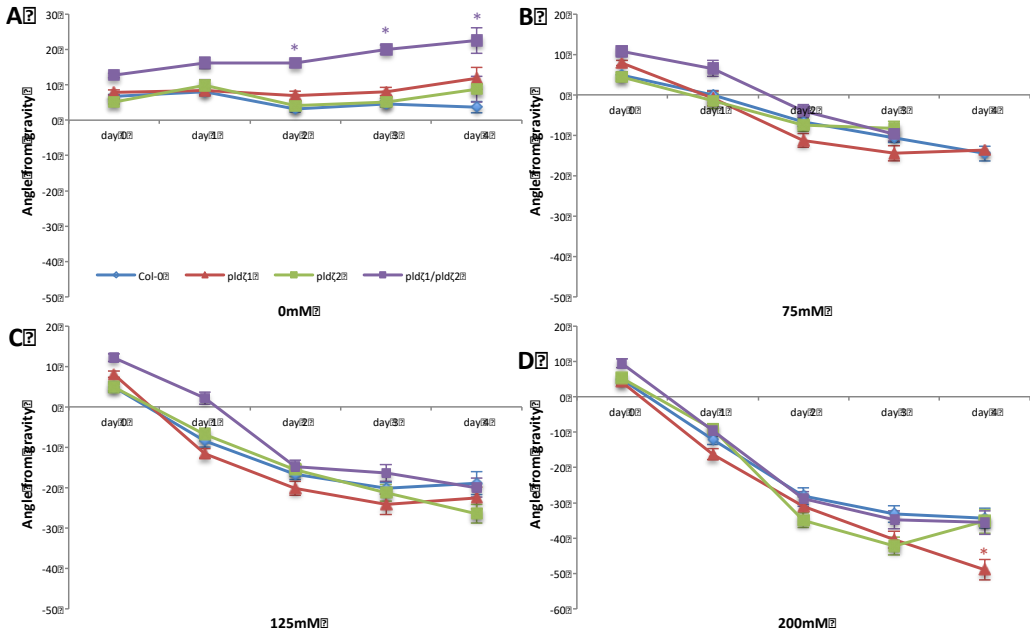
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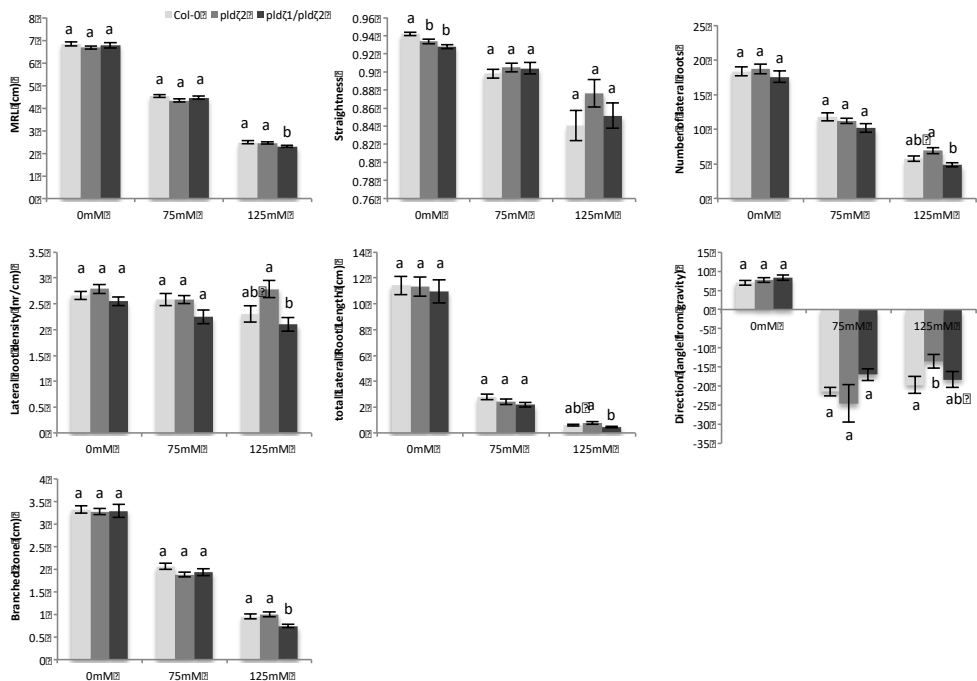
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## Supplemental information



**Supplemental figure S1: Long-term halotropic response in *pld1*, *pld2* and *pld1/pld2*-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.  $n = \sim 70$  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).