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## Phospholipases D $\zeta$ 1 and D $\zeta$ 2 have distinct roles in growth and antioxidant systems in *Arabidopsis thaliana* responding to salt stress

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1 Phospholipases D $\zeta$ 1 and D $\zeta$ 2 have distinct roles in growth and antioxidant systems in *Arabidopsis*  
2 *thaliana* responding to salt stress

3  
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26 Short running title

27 Roles of phospholipases D $\zeta$  during salt stress in *Arabidopsis*

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## **Main conclusion**

Phospholipases D $\zeta$  play different roles in Arabidopsis salt tolerance affecting the regulation of ion transport and antioxidant responses.

## **Abstract**

Lipid signalling mediated by phospholipase D (PLD) plays essential roles in plant growth including stress and hormonal responses. Here we show that *PLD $\zeta$ 1* and *PLD $\zeta$ 2* have distinct effects on Arabidopsis responses to salinity. A transcriptome analysis of a double *pld $\zeta$ 1pld $\zeta$ 2* mutant revealed a cluster of genes involved in abiotic and biotic stresses, such as the high salt stress responsive genes *DDF1* and *RD29A*. Another cluster of genes with a common expression pattern included ROS detoxification genes involved in electron transport and biotic and abiotic stress responses. Total SOD activity was induced early in the shoots and roots of all *pld $\zeta$*  mutants exposed to mild or severe salinity with the highest SOD activity measured in *pld $\zeta$ 2* at 14 days. Lipid peroxidation in shoots and roots was higher in the *pld $\zeta$ 1* mutant upon salt treatment and *pld $\zeta$ 1* accumulated H<sub>2</sub>O<sub>2</sub> earlier than other genotypes in response to salt. Salinity caused less deleterious effects on K<sup>+</sup> accumulation in shoots and roots of the *pld $\zeta$ 2* mutant than of wild type, causing only a slight variation in Na<sup>+</sup>/K<sup>+</sup> ratio. Relative growth rates of wild-type plants, *pld $\zeta$ 1*, *pld $\zeta$ 2* and *pld $\zeta$ 1pld $\zeta$ 2* mutants were similar in control conditions but strongly affected by salt in WT and *pld $\zeta$ 1*. The efficiency of photosystem II, estimated by measuring the ratio of chlorophyll fluorescence (Fv/Fm ratio), was strongly decreased in *pld $\zeta$ 1* under salt stress. In conclusion, *PLD $\zeta$ 2* plays a key role in determining Arabidopsis sensitivity to salt stress allowing ion transport and antioxidant responses to be finely regulated.

**Keywords:** Ion relations; Phospholipase D; PLD $\zeta$ ; Reactive oxygen species; Salt stress; Transcriptome.

68  
69 **Introduction**  
70  
71 Salinity is a major environmental constraint on the growth, productivity and diversity of plants. The  
72 effect of salt stress on plants depends on the salt concentration, the duration of exposure and the  
73 plant genotype (Ellouzi et al. 2011). The presence of salt in the environment induces water deficit in  
74 plants because the external water potential is lowered, while ion toxicity and nutritional alterations  
75 disturb ion transport systems (Munns and Tester 2008; Julkowska and Testerink 2015). Salt stress  
76 also causes membrane damage, alters levels of growth regulators, inhibits some enzymes, and  
77 disrupts photosynthesis, and may thus lead to plant death.

78 One of the known plant responses to salt stress is ROS production (for review see Miller et al. 2010;  
79 Ben Rejeb et al. 2014). Plant cells need to regulate ROS production as excess ROS is potentially  
80 harmful to nucleic acids, proteins and lipids, and may therefore lead to cell injury and death (Gill  
81 and Tuteja 2010). ROS produced through NADPH oxidase activity was shown to be mediated by  
82 phospholipid signalling (Zhang et al. 2009). The second messenger phosphatidic acid (PA) is a  
83 phospholipid which targets specific proteins to bring about cellular and physiological changes that  
84 allow plants to adapt to abiotic stresses (for review see Hong et al. 2010; Hou et al. 2016). PA is  
85 formed when phospholipase D (PLD) hydrolyses structural phospholipids at the terminal  
86 phosphoesteric bond with release of the hydrophilic head group. In plants, phospholipase D (PLD)  
87 is predominant among the phospholipase families (for review see Li et al. 2009; McLoughlin and  
88 Testerink, 2013). PLD activity increases rapidly in response to various environmental stresses such  
89 as cold, drought, and salinity (Vergnolle et al. 2005; Bargmann et al. 2009; Hong et al. 2010).  
90 Proline accumulation, another common physiological response to stress, was shown to be  
91 negatively regulated by PLD activity in *Arabidopsis thaliana* (Thiery et al. 2004).

92 In *A. thaliana*, the PLD family includes 12 members that are classified into six types, PLD $\alpha$  (3  
93 isoforms), - $\beta$  (2 isoforms), - $\gamma$  (3 isoforms), - $\delta$ , - $\epsilon$ , and - $\zeta$  (2 isoforms), according to their sequences  
94 and enzymatic properties (Bargmann and Munnik 2006). Biochemical studies have revealed that the  
95 phospholipid-hydrolysing activities of PLD are either calcium-dependent through a C2 domain (C2-  
96 PLD) or **calcium-independent having** pleckstrin homology (PH) and phox homology (PX) domains  
97 (PXPH-PLD) (Meijer and Munnik 2003). Several PLD isoforms have been functionally  
98 characterized. The two PLD $\zeta$ s (*PLD $\zeta$ 1* and *PLD $\zeta$ 2*) are structurally different from other PLDs (for  
99 review see Li et al. 2009). PLD $\zeta$ 1 and PLD $\zeta$ 2 do not have C2 domains and do not require Ca<sup>2+</sup> for  
100 enzymatic activity (Qin and Wang 2002), but they do have PH and PX domains (Qin and Wang  
101 2002). *PLD $\zeta$ 1* gene expression is five-fold greater in roots than in leaves and the gene function is  
102 required for root hair morphogenesis (Li et al. 2006). The *PLD $\zeta$ 2* gene is mainly expressed in roots

103 (Li et al. 2006). *PLDζ2* gene expression is triggered by exogenous auxin (Li and Xue 2007) and  
104 phosphate (Li et al. 2006). *PLDζ1* and *PLDζ2* were shown to be involved in the process by which  
105 root architecture adapts to the lack of phosphate (Li et al. 2006). *PLDζ2* is also involved in vesicle  
106 trafficking and auxin transport (Li and Xue 2007). The *pldζ2* mutant has a reduced halotropic  
107 response, i.e. the capacity to change the direction of root growth to avoid salt, due to impaired  
108 vesicle trafficking (Galvan-Ampudia et al. 2013).

109 In this study, the roles of *PLDζ1* and *PLDζ2* were investigated in Arabidopsis responding to salt  
110 stress. The global transcriptome of the *pldζ1pldζ2* double mutant treated with salt was analysed to  
111 identify candidate genes regulated by these PLDs. Changes in growth, ion balance, ROS content,  
112 antioxidants, and lipid peroxidation were compared in wild type, *pldζ1*, *pldζ2*, and *pldζ1pldζ2* after  
113 short and long periods of salt treatment. *pldζ1* and *pldζ2* mutants responded differently to salt stress  
114 suggesting they have distinct physiological roles in Arabidopsis.

115

116

## 117 **Materials and methods**

### 118 Plant materials and growth conditions

119 *Arabidopsis thaliana* (L.) Heynh ecotype Columbia-0 (Col-0) was used as the wild type in this  
120 study and was obtained from Nottingham Arabidopsis Stock Centre, Loughborough, United  
121 Kingdom. Homozygous T-DNA insertion mutant lines *pldζ1* (SALK\_083090) and *pldζ2A*  
122 (SALK\_094369) were obtained from the Salk Institute, La Jolla, USA (Alonso and Stepanova  
123 2003) and have been described by Li et al. (2006). Double homozygous mutant *pldζ1pldζ2* plants  
124 were isolated from the F2 progeny of crosses between *pldζ1* and *pldζ2*. The presence and  
125 homozygosity of the T-DNA alleles were checked by triplex PCR in all experimental lines using a  
126 primer specific to the left border of the T-DNA and two gene-specific primers flanking the insertion  
127 site (Suppl. Fig. S1 and Suppl. Table S1).

128 Surface sterilized seeds of wild-type and mutant lines were sown onto grids placed on Petri dishes  
129 containing 0.5 × Murashige and Skoog (MS; Murashige and Skoog 1962) solid medium (0.8 %  
130 agar) according to Parre et al. (2007). Seeds were placed in a cold room at 4 °C for 1 day to break  
131 dormancy and then transferred to a growth chamber at 22 °C with continuous light (90 μmol  
132 photons m<sup>-2</sup> s<sup>-1</sup>). Twelve-day-old *Arabidopsis* seedlings were exposed to 200 mM NaCl for up to 24  
133 h.

134 For physiological experiments, wild type, *pldζ1*, *pldζ2* and *pldζ1pldζ2* seeds were germinated in  
135 plastic pots (70 mL) filled with inert sand and watered daily with distilled water for one week.  
136 Seedlings of each genotype were then irrigated with Hewitt nutrient solution (Hewitt 1966) for 3  
137 weeks. The experiments were performed in a glasshouse under controlled conditions, photoperiod  
138 of 16h/8h (day/night), at 20-25 °C and 65-75 % relative humidity. Four-week-old plants were  
139 treated with 75 mM or 150 mM NaCl and collected after 3 h, 24 h and 72 h for short exposure to  
140 salt stress and after 7 and 14 days for long exposure to salinity. Non-stressed plants grown without  
141 added salt were collected and used as controls.

142

### 143 Transcriptome analysis of the *pldζ1pldζ2* double mutant

144 Analysis of the *pldζ1pldζ2* transcriptome was conducted at the Unité de Recherche en Génomique  
145 Végétale (Evry, France) using the Complete Arabidopsis Transcriptome MicroArray (CATMA)  
146 containing 24576 gene-specific tags from Arabidopsis (Crowe et al. 2003). Six biological replicates  
147 and 14 dye swaps (technical replicates) were made. Twelve-day-old seedlings treated for 3 h with  
148 either 200 mM NaCl or 400 mM mannitol for stress conditions or with 0.5 × MS for control  
149 conditions were rapidly washed with water and immediately frozen in liquid nitrogen and stored at

150 –80 °C. Total RNA was isolated from seedlings by the guanidinium thiocyanate-CsCl purification  
151 method (Sambrook et al. 1989). Labelling of cRNAs with Cy3-dUTP or Cy5-dUTP (Perkin-Elmer-  
152 NEN Life Science Products, Courtaboeuf, France), hybridization to slides, and scanning were  
153 performed as described in Lurin et al. (2004). Methods and data were deposited in CATMA  
154 database RS06-01\_PLD ([http://urgv.evry.inra.fr/cgi-bin/projects/CATdb/consult\\_expce.pl?](http://urgv.evry.inra.fr/cgi-bin/projects/CATdb/consult_expce.pl?experiment_id=113)  
155 [experiment\\_id=113](http://urgv.evry.inra.fr/cgi-bin/projects/CATdb/consult_expce.pl?experiment_id=113)) and GEO (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE9459>).  
156 Statistical analysis of transcriptome data was done as in Planchais et al. (2014).

157  
158 Reverse transcription PCR and quantitative PCR analysis of gene expression  
159 Total RNA was extracted from 100 mg of homogenized tissue from different genotypes using the  
160 RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. RNA  
161 was quantified by measuring the absorbance at 260 nm using a Nanovue spectrophotometer  
162 (Nanodrop Spectrophotometer ND 1000, Wilmington, DE, USA). RNA was incubated with DNase  
163 I (Sigma-Aldrich, Saint-Louis, MO, USA) to eliminate genomic DNA. For reverse transcription,  
164 first-strand cDNA was synthesized from 1.5 µg of total RNA using the RevertAid reverse  
165 transcriptase kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). Complementary DNAs were  
166 amplified using DreamTaq Green polymerase (Thermo Fisher Scientific Inc.) and gene-specific  
167 primers (Supplementary Table S1). The *APT1* gene (At1g27450) was used as a positive control for  
168 quantifying relative amounts of cDNA. Amplified PCR fragments were separated on 2 % (w/v)  
169 agarose gels and visualized by staining with ethidium bromide and analysing the image with  
170 GelDoc (Bio-Rad, Hercules, USA). For real-time PCR, 5 µL of diluted cDNA was used with 10 µL  
171 of SYBR<sup>®</sup>GreenqPCR Master Mix (Thermo Fisher Scientific Inc.) and gene-specific primers in an  
172 Eppendorf Master cycler<sup>®</sup> (Eppendorf France SAS, Montesson, France). Critical thresholds (Ct)  
173 were determined by using the Eppendorf Master cycler<sup>®</sup> realplex software and ratios were  
174 calculated by using the method described by Pfaffl (2001). For each gene, a standard curve made  
175 with dilutions of cDNA pools was used to calculate reaction efficiencies, and the levels of gene  
176 transcript were normalized and expressed relative to the amounts observed under control conditions  
177 with *APT1* (At1g27450) as housekeeping gene. All semi-quantitative RT-PCR and real-time PCR  
178 experiments were carried out with three biological replicates.

179  
180 Measurements of plant biomass, maximum efficiency of PSII photochemistry, and leaf water status  
181 Fresh weight (FW) and dry weight (DW) of rosettes and roots were measured at each experimental  
182 time point to evaluate leaf and root growth. The accumulation of biomass was estimated by  
183 determining the relative growth rate (RGR), which is a measure of biomass production relative to

184 treatment duration and/or initial plant size. RGR was calculated as  $\Delta \ln(DW)/\Delta t$ , where DW is the dry  
185 weight,  $\ln$  is the natural logarithm and  $\Delta$  represents the difference between final and initial values for a given  
186 time interval  $\Delta t$  (Hunt, 1990). The maximum efficiency of PSII photochemistry ( $F_v/F_m$ ) was  
187 monitored throughout the experiment using a portable mini-PAM fluorometer (ADC BioScientific  
188 LC pro System Serial, Hoddesdon, UK).

189 The relative leaf water content (RWC) was calculated as  $100 \times (FW - DW) / (TW - DW)$ , where TW was  
190 the turgid weight of leaves saturated for 24 h in deionized water at 4 °C in darkness and DW was  
191 obtained after oven-drying the leaves at 60 °C for 72 h according to Ellouzi et al. (2013).

192

#### 193 Measurement of ion content

194 Desiccated leaf and root tissues were ground to a fine powder and then broken down with  
195 concentrated 0.5 % HNO<sub>3</sub> according to Deal (1954). Na<sup>+</sup> and K<sup>+</sup> contents were determined using  
196 flame emission photometry (Corning, Tewksbury, MA, USA). In emission photometry the soluble  
197 mineral component is injected into an air-propane flame. Following thermal excitation a  
198 characteristic spectrum of lines is emitted that are selected by monochromator filters. Behind each  
199 filter a photoreceptor cell detects the intensity of the emitted light which is proportional to the  
200 amount of the emitting element contained in the vaporized solution.

201

#### 202 Histochemical detection of hydrogen peroxide

203 The localization of hydrogen peroxide was determined according to Ben Rejeb et al. (2015). Fresh  
204 leaf and root samples were collected from each mutant and infiltrated with a solution containing 0.5  
205 mg ml<sup>-1</sup> diaminobenzidine (DAB) and 10 mM Mes buffer pH 5.8. Samples were then kept at room  
206 temperature until the brown residue generated by H<sub>2</sub>O<sub>2</sub>-DAB polymerization developed. Samples  
207 were incubated for 60 min in boiling ethanol before being photographed.

208

#### 209 Measurement of hydrogen peroxide concentration

210 Fresh leaf and root samples were ground in 0.2 % trichloroacetic acid (TCA) in a mortar and pestle  
211 chilled on ice. The homogenate was centrifuged at 15000 g for 20 min at 4 °C. The supernatant was  
212 mixed with 10 mM sodium phosphate buffer pH 7 and 1 M KI. The H<sub>2</sub>O<sub>2</sub> content was determined  
213 by comparing the absorbance of the sample at 390 nm with a standard calibration curve, expressing  
214 values in  $\mu\text{mol H}_2\text{O}_2 \text{ g}^{-1} \text{ DW}$ .

215

#### 216 Membrane lipid peroxidation assays



217 Levels of lipid peroxidation were assessed by measuring the amount of malondialdehyde (MDA) in  
218 tissue. Fresh leaf and root samples were homogenized in 10 % TCA. The homogenate was  
219 centrifuged at 15000 g for 20 min at 4 °C. The supernatant was collected and mixed with 0.5 %  
220 thiobarbituric acid in 20 % TCA. Samples were heated at 95 °C for 25 min in a water bath, and then  
221 cooled on ice. The samples were centrifuged at 10000 g for 10 min and the absorbance of solutions  
222 at 532 and 600 nm was recorded. The MDA level was calculated using the extinction coefficient for  
223 MDA [ $\epsilon = 155 \mu\text{M cm}^{-1}$ ] expressed in nmol MDA g<sup>-1</sup> DW.

224

#### 225 Protein extraction and antioxidant enzyme assays

226 Leaf and root samples of each genotype were homogenized in a chilled mortar containing 10 %  
227 (w/w) poly-vinyl-polypyrrolidone in 50 mM potassium phosphate buffer pH 7, 0.1 mM  
228 ethylenediaminetetraacetic acid (EDTA) and 1 mM dithiothreitol. Homogenates were then  
229 centrifuged at 10000 g for 15 min at 4 °C. The supernatants were collected and stored at -20 °C for  
230 protein and enzyme assays. The soluble protein content of leaves and roots was estimated according  
231 to Bradford (1976) with bovine serum albumin as the standard. Superoxide dismutase (SOD,  
232 EC.1.15.1.1) activity was assayed based on the inhibition of nitro blue tetrazolium (NBT) reduction  
233 (Beyer and Fridovich 1987). The assay was conducted in 50 mM potassium phosphate pH 7.8  
234 containing 50  $\mu\text{L}$  of enzyme extract, 2.25 mM NBT, 13 mM methionine, 2 mM riboflavin and 1  
235 mM EDTA. The reaction was started by illuminating the sample for 15 min and stopped by  
236 switching the light off. The amount of blue formazan formed was determined by measuring the  
237 absorbance at 560 nm. One unit of SOD activity (U) was defined as the amount of enzyme that  
238 caused 50 % inhibition of NBT reduction.

239

#### 240 Statistical analysis

241 Differences between measurements and between genotypes at different times were evaluated by  
242 analysis of variance (one-way ANOVA) using SPSS (Chicago, IL, USA). Differences were  
243 considered as statistically significant when  $P \leq 0.05$ .

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## 251 Results

252 Analysis of the *pldζ1pldζ2* double mutant transcriptome

253 If Arabidopsis PLD $\zeta$  act in lipid signalling in response to salt stress then a *pldζ1pldζ2* double  
254 mutant, lacking both PLD $\zeta$  and PLD $\zeta$ 2, would be expected to differ from WT in the way it responds  
255 to salt stress. We first investigated gene expression in WT after 3 h exposure to salt or mannitol.  
256 This was to test whether the ionic stress component of the response could be distinguished from the  
257 osmotic stress component. A large common set of genes was induced (560 genes) or repressed (221  
258 genes) by the two individual treatments, which was attributed to the similar effects of osmotic stress  
259 on the plants (Supplementary Fig. S2). However a smaller subset of genes was specifically induced  
260 (170 genes) or repressed (82 genes) only by salt. For example, the known salt-stress responsive  
261 gene *DDF1* was specifically upregulated in response to NaCl in WT but was not affected by  
262 hyperosmotic stress caused by mannitol. Global transcriptome analysis of the *pldζ1pldζ2* double  
263 mutant also revealed common sets of genes that were triggered or repressed by both NaCl and  
264 mannitol, but also differential expression of subsets of genes in a salt-specific and mannitol-specific  
265 manner (data not shown). In further experiments we were thus able to distinguish between ionic and  
266 osmotic stress effects, focusing on the former. As some genes were differentially and specifically  
267 regulated by NaCl, the perception and transduction of the ionic component of the salt stress signal  
268 must have occurred within the 3 h of the stress treatment.

269 We then compared differences in the NaCl stress transcriptomes of *pldζ1pldζ2* and WT with the  
270 WT NaCl stress response. In WT genes were either induced (642 genes) or repressed (241 genes)  
271 after 3 h salt stress (Fig. 1). Twenty of the salt-induced genes in WT were found to be expressed at  
272 lower levels in *pldζ1pldζ2* after 3 h salt treatment than in WT in the same conditions (intersection in  
273 Fig. 1a). This cluster of genes induced in WT, but less strongly induced in *pldζ1pldζ2* was defined  
274 as cluster 1 (Fig. 1 and Suppl. Fig. S3). Analysis of gene ontology using BAR Classification  
275 SuperViewer (<http://bar.utoronto.ca>) showed an over-representation of genes involved in abiotic  
276 and biotic stresses in cluster 1 (Suppl. Fig. S4). It was noted that the salt-stress responsive genes  
277 *DDF1* and *RD29A* (Magome et al. 2008) were present in cluster 1. We investigated the expression  
278 of these two genes in WT, the *pldζ1* and *pldζ2* single mutants and the *pldζ1pldζ2* double mutant  
279 using RT-PCR and quantitative PCR (Fig. 2a, c). The RT-PCR analysis confirmed that *DDF1*  
280 expression was induced by salinity in WT plants (Fig. 2a). *DDF1* gene expression was induced in  
281 *pldζ1pldζ2* but not as strongly (4.9-fold) as in WT (6-fold) confirming the transcriptome data (Fig.  
282 2c). In comparison, *DDF1* expression was induced 9.7-fold in *pldζ2* and 7.8-fold in *pldζ1* (Fig. 2a,  
283 c). Similarly, *RD29A* expression was strongly induced by salt treatment in WT (Fig. 2a, c) but less

284 so in *pldζ1pldζ2* as expected from the transcriptomic data. Of the four genotypes tested *pldζ2* again  
285 showed the highest level of salt-induction with a 10.4-fold increase in *RD29A* expression.

286 Another cluster of genes, cluster 2, was identified by comparing the differences in the *pldζ1pldζ2*  
287 and WT transcriptomes between control and salt stress conditions. In this cluster 23 genes were  
288 deregulated in the double mutant in both conditions, 8 upregulated and 15 downregulated (Fig. 1 b,  
289 Suppl. Fig. S5). Cluster 2 gene functions mainly related to electron transport and biotic and abiotic  
290 stresses (Suppl. Fig. S4). The ROS detoxification genes *SOD1* (At1g08830) and *CCS1* (*Copper*  
291 *Chaperone for SOD1*, At1g12520) were overexpressed in *pldζ1pldζ2* compared to WT after salt  
292 treatment and in control conditions (Suppl. Fig. S5).

293

294 ROS detoxification was impaired in *pldζ* mutants

295 We verified whether *SOD1* expression was altered in *pldζ* single and double mutants by RT-PCR,  
296 and compared it to expression of two other ROS detoxifying genes, ascorbate peroxidase 1 (*APX1*)  
297 and catalase (*CAT2*), which are commonly used as oxidative burst markers (Fig. 2b). *APX1*  
298 expression was slightly higher in *pldζ2* than in WT and *pldζ1*, but was lower in *pldζ1pldζ2*. *SOD1*  
299 gene expression was upregulated by salt treatment in both *pldζ* single mutants but not in WT.  
300 Expression of *CAT2* was highest in *pldζ2* compared to the other genotypes even in control  
301 conditions (Fig. 2).

302 To test whether the lack of both or either PLD $\zeta$  affected the ROS detoxification activity of SOD,  
303 SOD activity was monitored for 14 days in the shoots and roots of WT and *pldζ* mutants exposed to  
304 moderate (75 mM NaCl) or severe salinity (150 mM NaCl) (Fig. 3). In leaves treated with 75 mM  
305 NaCl SOD activity increased transiently in the first 72 h up to 316 % in WT, 169 % in *pldζ1* and  
306 108 % in *pldζ1pldζ2*, declining less rapidly then remaining constant until the end of the time course.  
307 In roots, the kinetics of SOD activity were markedly different in *pldζ1pldζ2* in comparison to other  
308 genotypes as the sharp transient increase occurred within the first three days of mild salt treatment  
309 (Fig. 3). Under the same salt conditions, no significant variations were detected in stressed roots of  
310 WT and *pldζ1* mutants. By contrast SOD activity in both the shoots and roots of *pldζ2* treated with  
311 75 mM NaCl increased linearly to reach a plateau that remained higher than levels in the other  
312 genotypes at the end of the 14-day experiment. Upon severe NaCl stress, SOD activity in leaves of  
313 *pldζ2* and *pldζ1pldζ2* mutants showed a distinct profile with a transient maximum activity at 3 h,  
314 remaining higher than that of WT and *pldζ1* mutants until the end of the salt treatment (Fig. 3). No  
315 substantial changes in SOD activity were observed in shoots of WT and *pldζ1* mutants during stress  
316 from 150 mM NaCl. SOD activities increased distinctly in the roots of all genotypes except *pldζ1*  
317 under severe salt stress. Although there was a transient maximal peak in SOD activity in WT during

318 the first 72 h of exposure to 150 mM NaCl, the highest SOD activities were measured in *pldζ2* and  
319 *pldζ1pldζ2* mutants after 14 days.

320  
321 *pldζ* mutants differentially accumulate ROS in response to salt stress  
322 The presence of H<sub>2</sub>O<sub>2</sub> is an indicator of oxidative stress. The differential accumulation of H<sub>2</sub>O<sub>2</sub> in  
323 leaves and roots over the duration of the stress was evaluated in the four genotypes using DAB,  
324 which stains tissues brown in the presence of H<sub>2</sub>O<sub>2</sub> (Fig. 4). Leaves and roots grown in the absence  
325 of salt did not stain brown with DAB. After 3 h salt treatment, whole leaves and roots of WT and  
326 *pldζ1pldζ2* and *pldζ1* were stained brown with DAB (Fig. 4). Although the assay is qualitative, the  
327 intensity of the staining consistently increased over the duration of the experiment. Roots and  
328 shoots of *pldζ2* mutant were the most faintly stained, indicating these tissues had the lowest  
329 amounts of H<sub>2</sub>O<sub>2</sub>.

330 The differences observed in DAB staining in *pldζ* mutants led us to quantify H<sub>2</sub>O<sub>2</sub> content. H<sub>2</sub>O<sub>2</sub>  
331 measurements corresponded with the DAB staining. In the absence of salt stress, H<sub>2</sub>O<sub>2</sub> did not  
332 accumulate above basal levels in any of the four genotypes. Under mild salt stress, H<sub>2</sub>O<sub>2</sub> content  
333 increased steadily in shoots and roots in all four genotypes, accumulating faster during the first 24 h  
334 (Fig. 5). H<sub>2</sub>O<sub>2</sub> accumulated at a similar rate in WT and *pldζ1*, but *pldζ2* and *pldζ1pldζ2* accumulated  
335 less than half of the amount measured in WT (Fig. 5). Under severe salt stress, *pldζ2* leaves and  
336 roots had the lowest levels of H<sub>2</sub>O<sub>2</sub> compared to the other *pldζ* genotypes.

337 Oxidative stress can lead to damage of structural macromolecules including lipids. Lipid  
338 peroxidation in leaves and roots was estimated by quantifying malondialdehyde (MDA), which  
339 forms when ROS attack lipids. The accumulation of MDA greatly increased in both leaves and  
340 roots growing under salt stress (Fig. 6). However the genotypes accumulated MDA differently. The  
341 highest amounts of MDA were found in leaves and roots of salt-stressed *pldζ1*, while *pldζ2*  
342 accumulated the least MDA in leaves and roots in response to both moderate and severe salt stresses  
343 (Fig. 6).

344  
345 Ion distribution was altered in *pldζ* mutants responding to salt stress  
346 The distribution of ions between leaves and roots of WT and the *pldζ* mutants exposed to 14 days of  
347 either moderate or severe salinity is shown in Table 1. Changes in Na<sup>+</sup> and Cl<sup>-</sup> content followed the  
348 same profile over the 14 days in all genotypes and under both salt treatments, increasing linearly  
349 (data not shown) and reaching high levels by the end of the experiment. These final levels were  
350 higher in roots than in leaves (Table 1). There were some differences between genotypes. *pldζ1*  
351 mutants stressed by 150 mM NaCl had the most Na<sup>+</sup> in leaves (7.3 mmol g<sup>-1</sup> DW) and roots (8.4

352 mmol g<sup>-1</sup> DW) (Table 1). This increase was concomitant with the highest Cl<sup>-</sup> accumulation, which  
353 was greater in roots (5.8 mmol g<sup>-1</sup>DW) than in leaves (3.9 mmol g<sup>-1</sup> DW) (Table 1). However, Na<sup>+</sup>  
354 content increased more than Cl<sup>-</sup> content within leaves and roots upon salt stress. When grown in  
355 150 mM NaCl, *pldζ2* mutants contained the least Na<sup>+</sup> (3 mmol g<sup>-1</sup> DW in leaves and 3.9 mmol g<sup>-1</sup>  
356 DW in roots) (Table 1) and the least Cl<sup>-</sup> (2.1 mmol g<sup>-1</sup> DW in leaves and 3.1 mmol g<sup>-1</sup> DW in roots).  
357 Na<sup>+</sup> accumulation concomitant with K<sup>+</sup> loss was higher in roots than in leaves in all genotypes  
358 under salt stress (Table 1). Salt-stressed *pldζ1* mutants showed the most drastic decrease in K<sup>+</sup>  
359 content with 6-fold and 22-fold decreases in leaves and roots, respectively, when treated with 150  
360 mM NaCl (Table 1). By contrast, only a slight variation in Na<sup>+</sup>/K<sup>+</sup> ratio was observed in *pldζ2*  
361 mutant. Salinity effects on K<sup>+</sup> accumulation were less severe in shoots (2-fold decrease) and roots  
362 (7-fold decrease) in *pldζ2* than in other genotypes.

363  
364 Relative growth rate, relative water content and efficiency of chlorophyll fluorescence in *pldζ*  
365 mutants during salt stress

366 Relative growth rate (RGR) of WT, *pldζ1*, *pldζ2* and *pldζ1pldζ2* had decreased after 14 days of  
367 exposure to NaCl (Fig. 7). RGRs of *pldζ1pldζ2* and *pldζ2* mutants were less affected by salt than  
368 those of *pldζ1* and WT, which were severely inhibited by even mild salt stress. By measuring the  
369 change in relative water content (RWC) over time, we found that salt stress induced dehydration in  
370 leaves of all genotypes (Fig. 8). *pldζ1* mutants became the most dehydrated with RWC decreases of  
371 50 % in 75 mM NaCl and 66 % in 150 mM NaCl compared to the same genotype at the same  
372 developmental stage in control conditions (Fig. 8). The *pldζ1pldζ2* leaves only lost 40 % of RWC  
373 compared to plants grown in control conditions.

374 The *Fv/Fm* ratio is a convenient measure of photosynthesis efficiency at the photosystem II level  
375 (PSII). The *Fv/Fm* ratios for all genotypes were constant in control conditions but decreased under  
376 salt stress (Fig. 8). After 14 days of salt treatment, the *Fv/Fm* ratios of *pldζ1* plants had decreased  
377 by as much as 64 % in 150 mM NaCl. By contrast *pldζ1pldζ2* plants maintained higher *Fv/Fm* ratio  
378 values, which were 1.16 and 1.32-fold higher than those measured in WT, respectively under mild  
379 and severe stress, suggesting PSII functions better under stress in the absence of PLDζ (Fig. 8).

380

381

## 382 Discussion

383 Recent studies have provided valuable insights into the molecular and cellular mechanisms by  
384 which plants respond to and tolerate salinity stress (Deinlein et al. 2014; Julkowska and Testerink  
385 2015; Slama et al. 2015). Although *Arabidopsis thaliana* is considered to be a glycophyte, it has

386 been widely used as a genetic model to investigate salt signalling mechanisms. Salt treatment  
387 significantly inhibits growth of *A. thaliana* with intracellular Na<sup>+</sup> concentration increasing at the  
388 expense of K<sup>+</sup> (Ghars et al. 2008; Ellouzi et al. 2011). The replacement of K<sup>+</sup> by Na<sup>+</sup> affects the  
389 cell's Na<sup>+</sup>-sensitive enzymes, including components of the photosynthetic machinery, that  
390 determine plant growth and yield. Tolerance to salt stress depends on complex signalling networks  
391 enabling plants to respond rapidly and efficiently to this constraint (Zhu 2002). Many signal  
392 transduction pathways have been shown to be stimulated in response to high salinity (Bragmann et  
393 al. 2009; Julkowska and Testerink 2015). Lipid mediators are key components in the signalling  
394 network of plant stress adaptation, including adaptation to salinity (Julkowska and Testerink 2015).  
395 As an enzyme responsible for producing PA, we focused on the roles of the two *A. thaliana* PLD $\zeta$   
396 in salt tolerance.

397 To identify changes in gene expression affected by PLD $\zeta$  activity, the transcriptome of a double  
398 *pld $\zeta$ 1pld $\zeta$ 2* mutant was compared to the WT transcriptome. In gene cluster 1, the stress-responsive  
399 genes *DDF1* and *RD29A* (Thiery et al. 2004; Magome et al. 2004; Magome et al. 2008) were  
400 strongly induced by severe salt stress (200 mM NaCl) within 3 h in the WT but were less strongly  
401 induced in *pld $\zeta$ 1pld $\zeta$ 2* under the same conditions. Transgenic Arabidopsis lines overexpressing  
402 *DDF1* were shown to have increased tolerance to high salt stress (170 mM NaCl) by repressing  
403 plant growth through the induction of genes coding gibberellin (GA) deactivating enzymes  
404 (Magome et al. 2004; Magome et al. 2008). We found that the *pld $\zeta$ 2* single mutant showed higher  
405 expression of *DDF1* and *RD29A* under salt stress compared with the WT and other *pld $\zeta$  mutants*  
406 (Fig. 2). The expression of auxin-responsive genes such as *IAA5*, *IAA19*, and *GH3-3* was previously  
407 found to be reduced in *pld $\zeta$ 2* mutants of Arabidopsis in response to external IAA (Li and Xue  
408 2007). Therefore, *PLD $\zeta$ 2* may be specifically involved in the regulation of gene expression in stress  
409 responses and hormonal signalling for growth. Controlled growth reduction may be an effective  
410 strategy to save energy and minimize ROS accumulation while facing the deleterious impact of salt  
411 stress (Rangani et al. 2016).

412 Transcriptomic analysis revealed a second gene cluster that included ROS detoxification genes such  
413 as *SOD1* and *CCS1*, which were overexpressed in *pld $\zeta$ 1pld $\zeta$ 2* mutants (Suppl. Fig. S5). It is known  
414 that ROS act as second messengers in intracellular signalling cascades to trigger plant tolerance to  
415 various abiotic and biotic stresses (Ben Rejeb et al. 2014; Mittler, 2016). When *Cakile maritima*, a  
416 salt-tolerant plant, is treated with salt, there is a transient and rapid increase in SOD activity and  
417 H<sub>2</sub>O<sub>2</sub> content within 4 h. The SOD activity measured in *C. maritima* was eight-fold higher than was  
418 found in Arabidopsis (Ellouzi et al. 2013). In the present study, the highest *SOD1*, *CAT2* and *APX*  
419 gene expression was observed in *pld $\zeta$ 2* mutant under salt stress (Fig. 2). High expression of *SOD*

420 under salt stress corresponded with high SOD activity and low H<sub>2</sub>O<sub>2</sub> levels in leaves and roots of  
421 *pldζ2* mutants, indicating that *PLDζ2* participates in the regulation of ROS generation upon salt  
422 stress in Arabidopsis. Several reports show that different PLD family enzymes function in concert  
423 with ROS to mediate tolerance responses to various abiotic stresses (Hong et al. 2010, Singh et al.  
424 2012). For example, mutation in *PLDα1* leads to PA deficiency, reduced plasma membrane  
425 NADPH oxidase activity, and less ROS in stomata guard cells in response to abscisic acid (Zhang et  
426 al. 2009). As a consequence, *plda1* mutants can not fully close stomata leading to increased water  
427 loss (Zhang et al. 2009). We found that *pldζ1* mutants accumulated H<sub>2</sub>O<sub>2</sub> faster in leaves and roots  
428 than *pldζ2* mutants do in response to salt stress. Moreover, *pldζ1* plants displayed the lowest SOD  
429 activity which, associated with the highest levels of H<sub>2</sub>O<sub>2</sub>, implies that *pldζ1* is more sensitive to  
430 salt stress than WT and the other *pldζ* mutants. Interestingly other *pld* mutants such as *plda1*, *plda3*  
431 and *pldδ* have also been shown to be hypersensitive to salt treatment (Hong et al. 2008; Bargmann  
432 et al. 2009).

433 Salinity had several striking effects on physiological indexes with distinctions becoming clear  
434 between WT and *pldζ1* versus *pldζ2* and *pldζ1pldζ2*. The *pldζ1* mutant showed the highest Na<sup>+</sup>  
435 content in leaves and roots compared with the other genotypes upon salt stress, leading to a drastic  
436 decrease in K<sup>+</sup> content in roots and shoots (Table 1). The loss of K<sup>+</sup> could be explained by  
437 downregulation of the expression of genes involved in K<sup>+</sup> transport like the HAK5 transporter gene.  
438 HAK5 is expressed under the control of *DDF2* gene, a transcription factor which is homologous to  
439 *DDF1* (Hong et al. 2013). The massive K efflux could also be mediated by the opening of outward-  
440 rectifying depolarisation-activated (GORK) channels, an outward K<sup>+</sup> channel (for review see  
441 Anshütz et al., 2014). Phospholipase Dζ could be involved in the regulation of ion channels by  
442 modulating their lipid environment. For example in yeast cells, many ion channels have been shown  
443 to be located in plasma membrane microdomains called lipid rafts with channel activities dependent  
444 on the lipid raft composition (for review see Mollinedo 2012).

445 RGR measurements showed that WT and *pldζ1* have only a limited capacity to withstand the  
446 presence of salt. Sensitivity of WT and *pldζ1* to salt stress was associated with a decline in the leaf  
447 water content, increase in Na<sup>+</sup> and Cl<sup>-</sup> preferentially accumulated in roots, early induction of  
448 oxidative stress (excess H<sub>2</sub>O<sub>2</sub> and MDA), and inhibition of photosynthesis from the toxicity of the  
449 salt ions (low *Fv/Fm* ratio). For most markers *pldζ1* was more sensitive than WT, indicating that  
450 *PLDζ1* is required in the regulation of growth in normal conditions. Interestingly and as described  
451 by Ohashi et al. (2003) and Chen et al. (2013), this PLD isoform is greatly involved in the root  
452 development and growth and the lack of this PLD may lead to enhanced salt stress sensitivity  
453 (Wang, 2005).

454 Conversely, *pldζ2* mutants tolerated salt stress better than WT. This response may be related to its  
455 ability to minimize redistribution of Na<sup>+</sup> and Cl<sup>-</sup> to the roots, resulting in the lowest Na<sup>+</sup>/K<sup>+</sup> ratio  
456 and consequently better retention of K<sup>+</sup> in these organs. *pldζ2* grew less than *pldζ1 pldζ2* double  
457 mutant, which produced more biomass in both roots and leaves. We can consider growth reduction  
458 in *pldζ2* to be a strategy to maintain performance under salinity by first controlling ion homeostasis.  
459 The activation of the Salt Overly Sensitive (SOS) signalling pathway is a key mechanism for Na<sup>+</sup>  
460 exclusion in roots (Zhu, 2000). PLDs were shown to interact strongly with SOS and plasma  
461 membrane transporters under salt stress (Yu et al. 2010). Notably, the activation of PLD in salt  
462 stressed tobacco elevated the PA level which is a direct stimulator of SOS1, a central regulator of  
463 ion homeostasis (Gardiner et al. 2001). Other studies suggested that there is a functional connection  
464 between the activation of *PLDζ2* and SOS3, which is more prominent in the roots and has a crucial  
465 role in Na<sup>+</sup>/K<sup>+</sup> dynamics (Muzi et al. 2016). Much less is known about the cross-talk between PLD  
466 and SOS signalling pathways under salt stress. However, the above findings will guide future  
467 research to elucidate the mechanism of the salt tolerance in *pldζ2*.

468 It was clear from our results that the *pldζ2* mutant was the most tolerant of salt stress as evidenced  
469 by the lowest Na<sup>+</sup>/K<sup>+</sup> ratio. It has long been known that salinity stress triggers a dramatic increase in  
470 ROS accumulation in plant tissues. Interestingly, our results showed that salt-stressed *pldζ2* plants  
471 also displayed the lowest levels of H<sub>2</sub>O<sub>2</sub> and MDA detected in both leaves and roots. In addition,  
472 mild and severe salt stresses triggered higher SOD activity in *pldζ2* than in the other genotypes  
473 which could explain why H<sub>2</sub>O<sub>2</sub> and MDA levels were lower in both leaves and roots. The oxidative  
474 burst indicators SOD, H<sub>2</sub>O<sub>2</sub> and MDA indicate the presence of an efficient antioxidant defence in  
475 *pldζ2* mutant. The lack of *PLDζ2* may therefore allow a better performance of the plants facing  
476 moderate and severe salinity due to a primed antioxidant defence system.

477 In conclusion, our study suggests that in Arabidopsis *PLDζ* genes encode isoforms that have distinct  
478 roles in plant growth particularly under salt stress. The *pldζ2* KO mutant was more salt-tolerant than  
479 the WT as growth, water status, ion homeostasis and antioxidant defence systems were all better  
480 adjusted to withstanding salinity. Possibly *pldζ* are differently regulated in halophytes enabling  
481 them to tolerate salinity. Looking for natural variants or mutations in *PLDζ2* gene might be a way to  
482 improve salt tolerance in glycophytic crops.

483

484

#### 485 **Author contribution statement**

486 AS and CA conceived and designed the research. Data collection, analysis and interpretation were  
487 performed by AO, HE, SP and BF. SP conducted the transcriptomic analysis. DDV was involved in



488 the molecular analysis of *pldζ* mutant and in obtaining the double *pldζ1pldζ2* mutant. PC  
489 contributed to study conception and design. AO, HE, SP wrote the manuscript. AS and CA  
490 supervised manuscript preparation and correction. All authors read and approved the manuscript.

491

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645

646

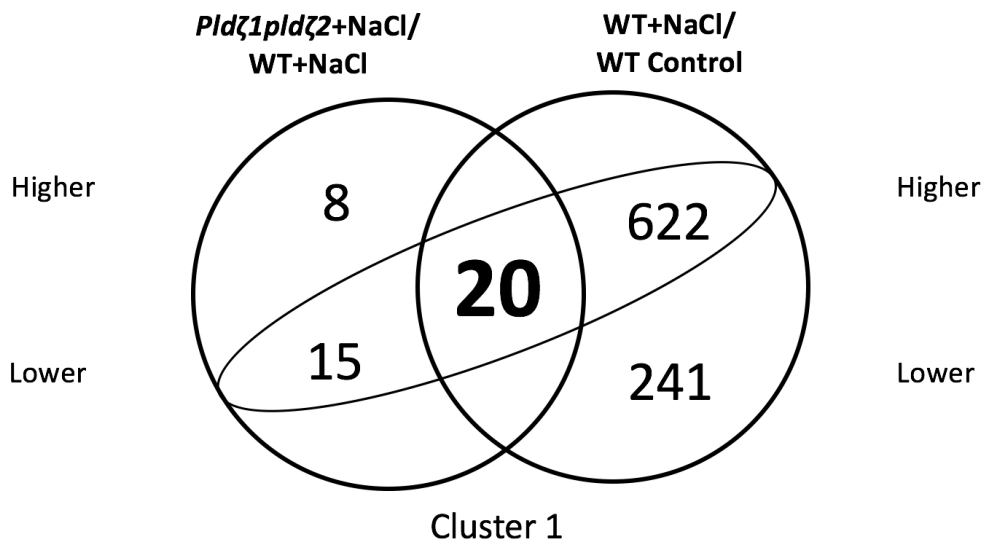
647 Table 1. Ion content (mmol g<sup>-1</sup> DW) of leaves and roots of WT and *pldζ1*, *pldζ2* and *pldζ1pldζ2*  
 648 mutants after 14 days of mild or severe salt stress. Data are means of three replicates ± S.E. Means  
 649 indicated by the same superscript letters are not significantly different at P ≤ 0.05 in one-way  
 650 ANOVA.

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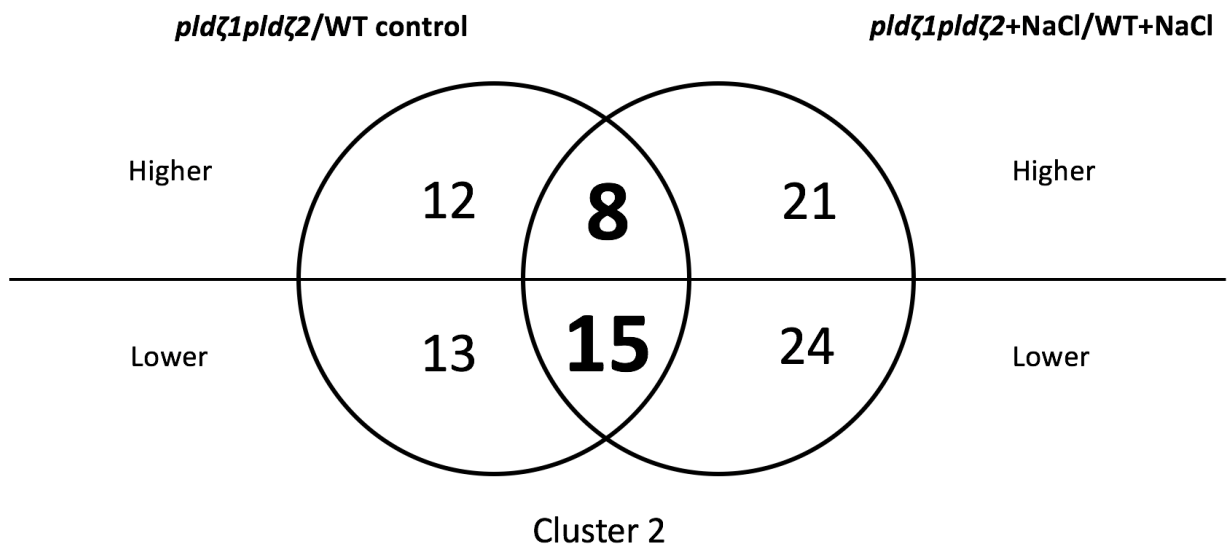
Leaves		Genotype	WT	<i>pldζ1</i>	<i>pldζ2</i>	<i>pldζ1pldζ2</i>
NaCl (mM)	Ion content					
0	Na <sup>+</sup>		0.14 ± 0.01 <sup>a</sup>	0.14 ± 0.00 <sup>ab</sup>	0.14 ± 0.01 <sup>ab</sup>	0.15 ± 0.00 <sup>b</sup>
75	Na <sup>+</sup>		2.36 ± 1.09 <sup>b</sup>	3.30 ± 0.91 <sup>d</sup>	1.33 ± 0.19 <sup>a</sup>	2.89 ± 0.87 <sup>c</sup>
150	Na <sup>+</sup>		4.50 ± 1.06 <sup>c</sup>	7.31 ± 1.06 <sup>d</sup>	3.00 ± 1.10 <sup>a</sup>	3.92 ± 1.08 <sup>b</sup>
0	K <sup>+</sup>		2.79 ± 0.24 <sup>b</sup>	1.86 ± 0.02 <sup>a</sup>	2.32 ± 0.21 <sup>ab</sup>	2.07 ± 0.17 <sup>a</sup>
75	K <sup>+</sup>		1.33 ± 0.15 <sup>b</sup>	0.76 ± 0.03 <sup>a</sup>	1.30 ± 0.27 <sup>b</sup>	0.65 ± 0.01 <sup>a</sup>
150	K <sup>+</sup>		0.77 ± 0.05 <sup>b</sup>	0.31 ± 0.00 <sup>a</sup>	1.12 ± 0.22 <sup>b</sup>	0.28 ± 0.02 <sup>a</sup>
0	Cl <sup>-</sup>		0.03 ± 0.00 <sup>a</sup>	0.03 ± 0.00 <sup>a</sup>	0.09 ± 0.01 <sup>b</sup>	0.02 ± 0.00 <sup>a</sup>
75	Cl <sup>-</sup>		2.37 ± 0.55 <sup>c</sup>	2.14 ± 0.32 <sup>a</sup>	1.66 ± 0.32 <sup>b</sup>	1.70 ± 0.09 <sup>a</sup>
150	Cl <sup>-</sup>		3.08 ± 0.85 <sup>b</sup>	3.88 ± 0.66 <sup>d</sup>	2.09 ± 0.53 <sup>a</sup>	3.23 ± 1.021 <sup>c</sup>
0	Na <sup>+</sup> /K <sup>+</sup>		0.04 ± 0.00 <sup>a</sup>	0.07 ± 0.00 <sup>c</sup>	0.06 ± 0.00 <sup>b</sup>	0.07 ± 0.00 <sup>c</sup>
75	Na <sup>+</sup> /K <sup>+</sup>		1.80 ± 0.06 <sup>b</sup>	4.38 ± 0.19 <sup>c</sup>	1.05 ± 0.11 <sup>a</sup>	4.56 ± 0.30 <sup>c</sup>
150	Na <sup>+</sup> /K <sup>+</sup>		5.88 ± 0.44 <sup>b</sup>	23.88 ± 0.22 <sup>d</sup>	2.88 ± 0.51 <sup>a</sup>	14.30 ± 1.40 <sup>c</sup>
Roots						
0	Na <sup>+</sup>		0.12 ± 0.00 <sup>a</sup>	0.11 ± 0.01 <sup>a</sup>	0.22 ± 0.01 <sup>b</sup>	0.13 ± 0.00 <sup>a</sup>
75	Na <sup>+</sup>		3.73 ± 1.11 <sup>b</sup>	3.72 ± 1.19 <sup>b</sup>	2.91 ± 0.66 <sup>a</sup>	2.89 ± 0.97 <sup>a</sup>
150	Na <sup>+</sup>		6.43 ± 1.31 <sup>b</sup>	8.42 ± 1.16 <sup>c</sup>	3.92 ± 0.91 <sup>a</sup>	5.88 ± 1.08 <sup>b</sup>
0	K <sup>+</sup>		1.14 ± 0.21 <sup>b</sup>	0.65 ± 0.24 <sup>a</sup>	0.98 ± 0.07 <sup>ab</sup>	0.94 ± 0.02 <sup>ab</sup>
75	K <sup>+</sup>		0.40 ± 0.01 <sup>c</sup>	0.10 ± 0.00 <sup>a</sup>	0.52 ± 0.02 <sup>d</sup>	0.28 ± 0.00 <sup>b</sup>
150	K <sup>+</sup>		0.09 ± 0.01 <sup>b</sup>	0.04 ± 0.00 <sup>a</sup>	0.15 ± 0.00 <sup>c</sup>	0.08 ± 0.00 <sup>b</sup>
0	Cl <sup>-</sup>		0.04 ± 0.00 <sup>a</sup>	0.03 ± 0.00 <sup>a</sup>	0.06 ± 0.01 <sup>b</sup>	0.03 ± 0.00 <sup>a</sup>
75	Cl <sup>-</sup>		4.10 ± 0.91 <sup>c</sup>	4.08 ± 0.90 <sup>c</sup>	1.91 ± 0.36 <sup>a</sup>	3.50 ± 0.33 <sup>b</sup>
150	Cl <sup>-</sup>		5.08 ± 1.05 <sup>c</sup>	5.72 ± 0.28 <sup>d</sup>	3.00 ± 0.83 <sup>a</sup>	4.00 ± 0.83 <sup>b</sup>
0	Na <sup>+</sup> /K <sup>+</sup>		0.11 ± 0.00 <sup>a</sup>	0.21 ± 0.06 <sup>ab</sup>	0.23 ± 0.01 <sup>b</sup>	0.13 ± 0.01 <sup>ab</sup>
75	Na <sup>+</sup> /K <sup>+</sup>		9.61 ± 0.52 <sup>b</sup>	35.14 ± 2.75 <sup>c</sup>	5.54 ± 0.13 <sup>a</sup>	10.22 ± 1.05 <sup>b</sup>
150	Na <sup>+</sup> /K <sup>+</sup>		70.67 ± 2.81 <sup>b</sup>	246.95 ± 40.1 <sup>d</sup>	55.41 ± 20.0 <sup>a</sup>	72.91 ± 1.30 <sup>c</sup>

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**a**



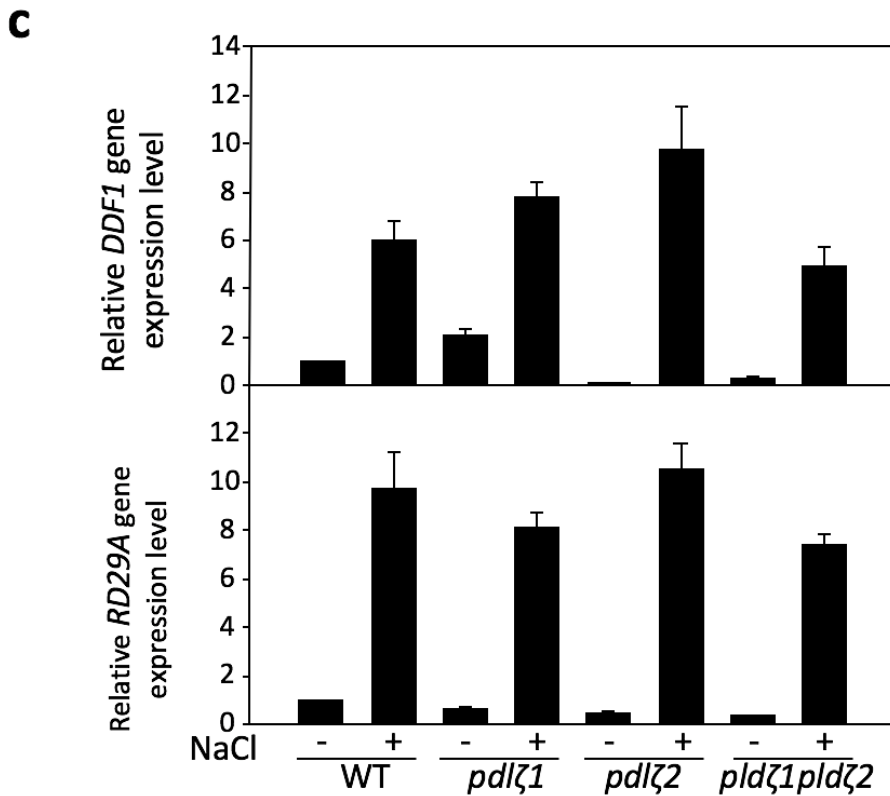
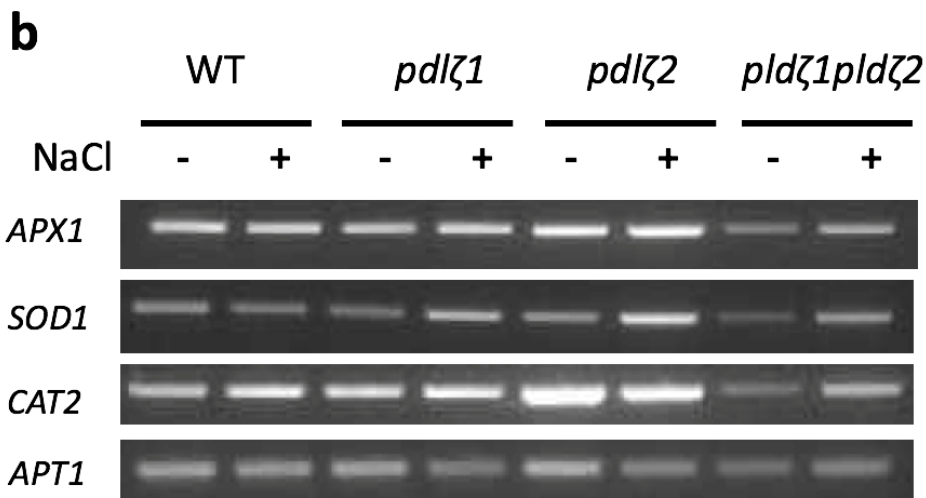
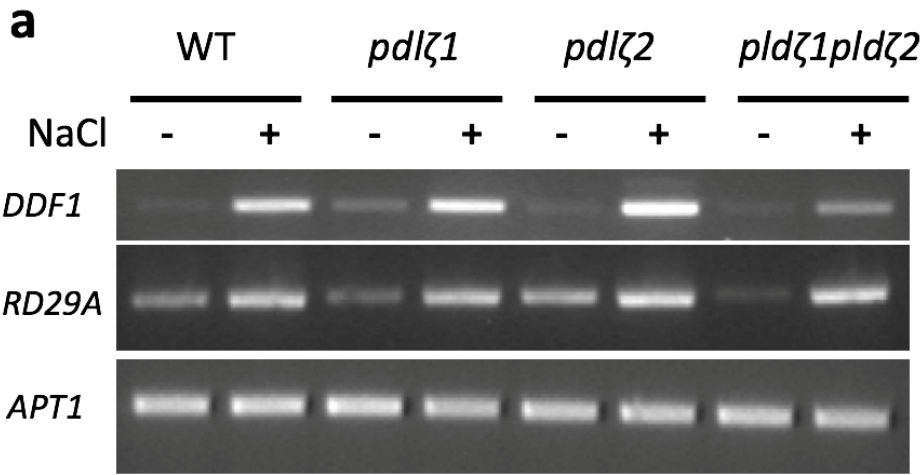
**b**



657  
 658 **Figure 1.** Numbers of differentially expressed genes in WT and *pldζ1pldζ2* transcriptome  
 659 comparisons. Venn diagrams show how gene clusters were defined. **(a)** Cluster 1 (intersection in  
 660 bold) includes genes which are induced by salt stress in WT and are expressed at lower levels in the  
 661 double mutant under salt stress than in WT. **(b)** Cluster 2 (intersection in bold) includes genes  
 662 which are up or down regulated in *pldζ1pldζ2* compared to WT in both control and salt stress  
 663 conditions.

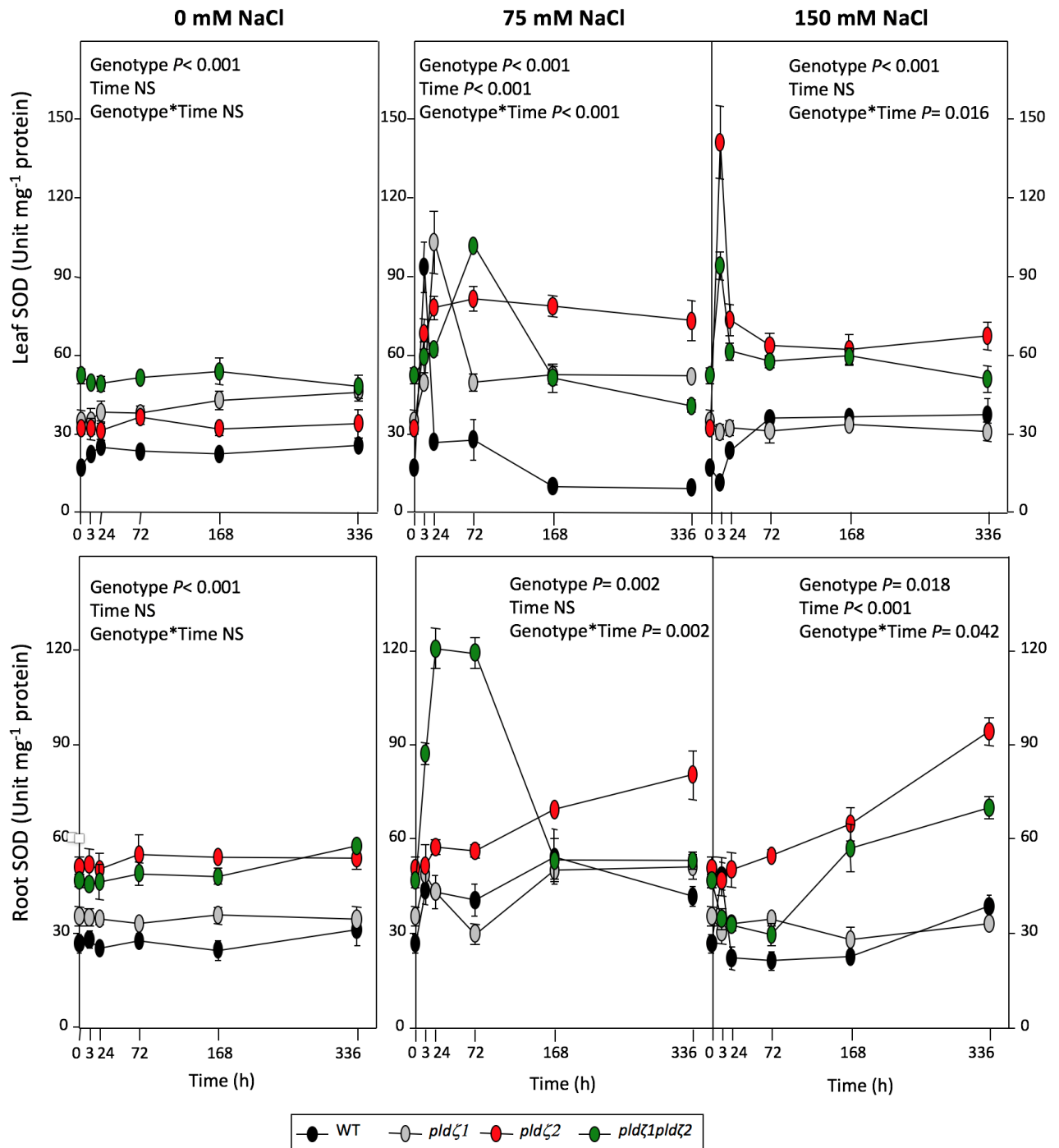
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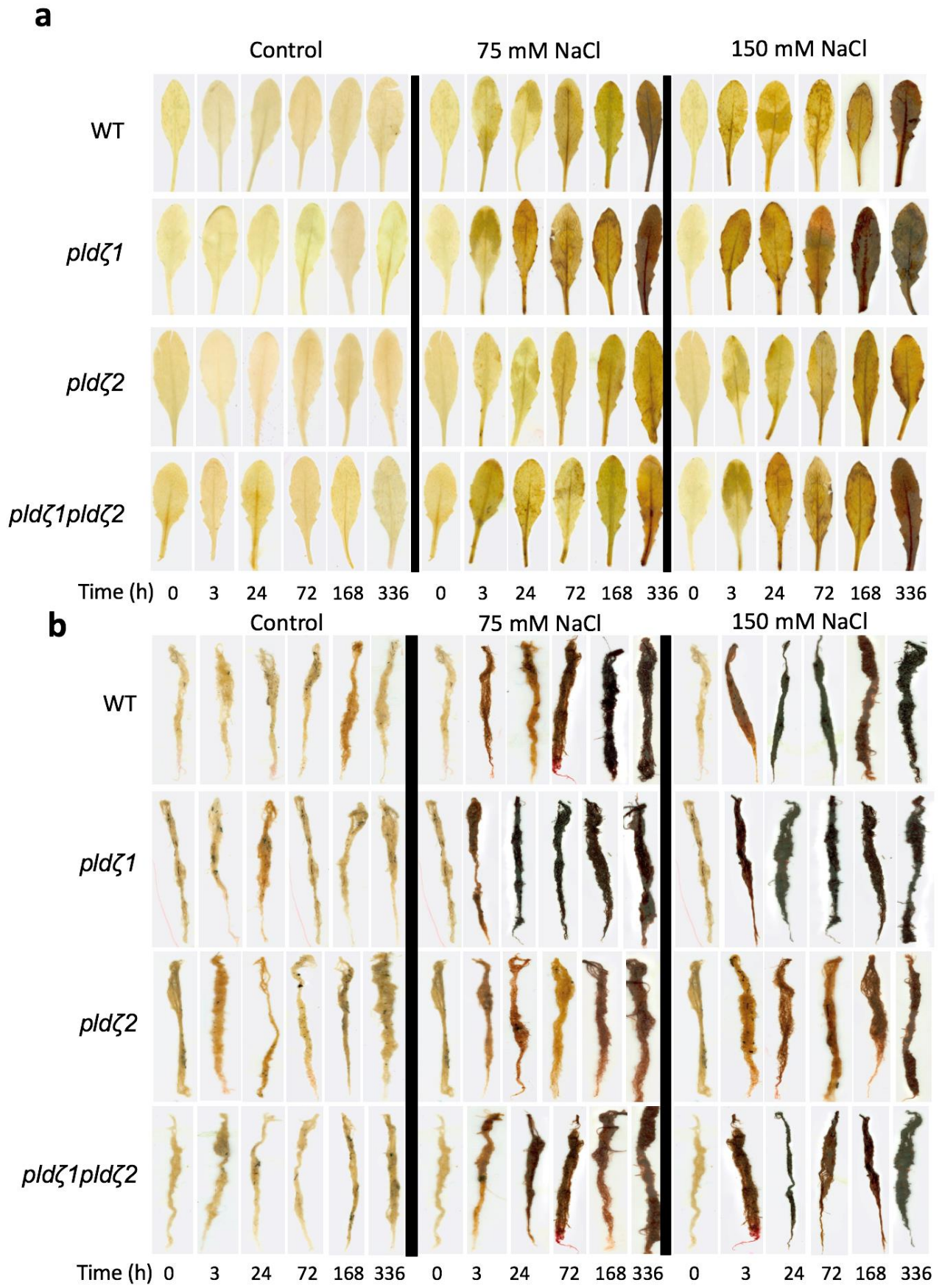
667 **Figure 2.** Gene expression in wild-type (WT), *pldζ1*, *pldζ2* and *pldζ1ζ2* plants treated (+) or not  
668 treated (-) with 200 mM NaCl for 3 h. *DDF1* and *RD29A* (a), and *APX1*, *SOD1* and *CAT2* (b)  
669 expression was estimated by RT-PCR with *APT1* as a loading control. (c) Quantitative RT-PCR of  
670 *DDF1* and *RD29A* expression in WT, *pldζ1*, *pldζ2* and *pldζ1ζ2* seedlings. *DDF1* transcript  
671 abundance was expressed as a ratio of the value for WT in control conditions. *APT1* gene  
672 expression was used as a standard. Mean and standard errors are based on three technical repeats  
673



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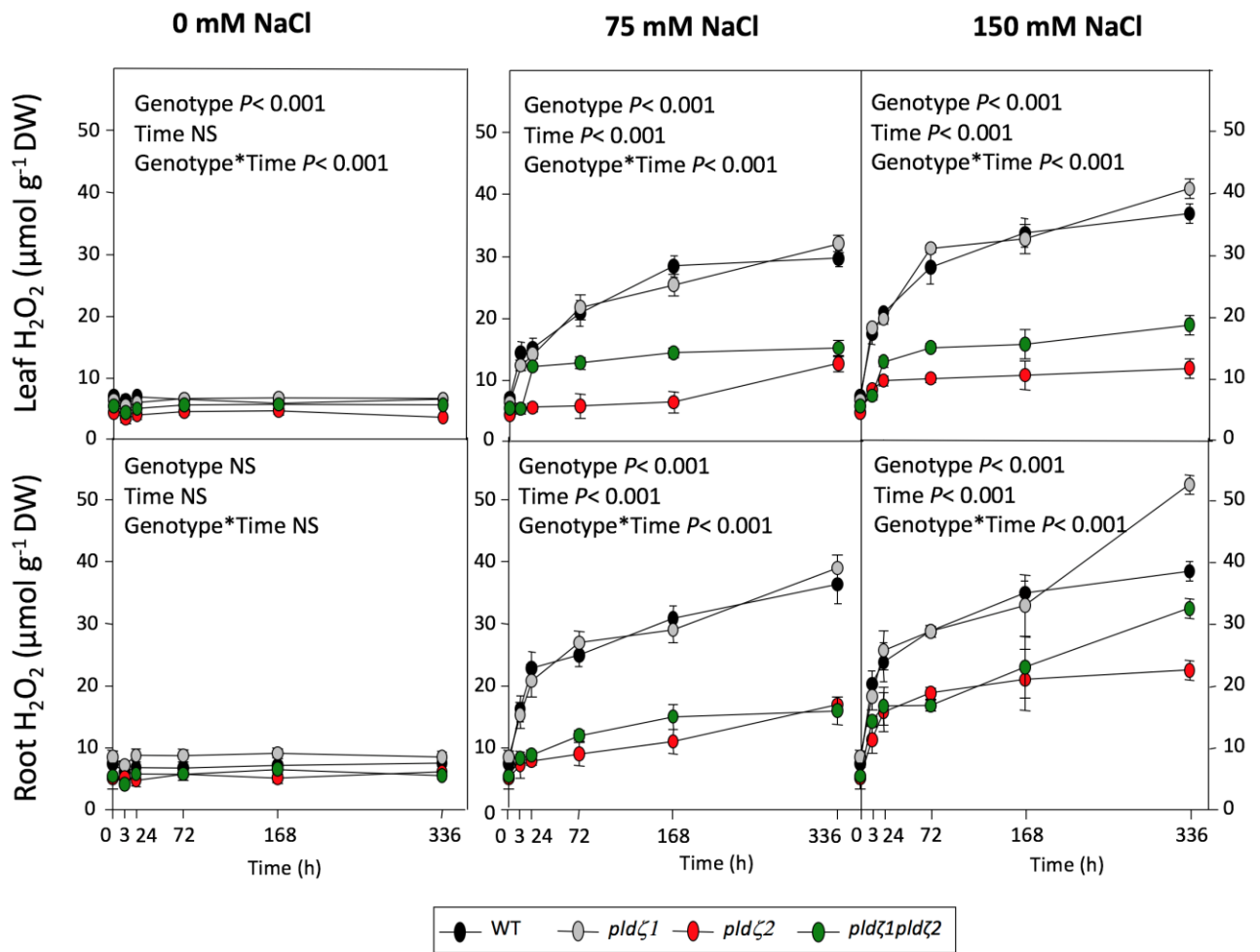
676 **Figure 3.** Changes in superoxide dismutase (SOD) activity in leaves and roots of wild type and  
 677 *pldζ1*, *pldζ2* and *pldζ1pldζ2* mutants of *A. thaliana* plants grown for 14 days under moderate (75  
 678 mM NaCl) or severe salinity (150 mM NaCl). Data are means ± SE of 3 replicates. Results of  
 679 statistical analysis are given in each panel (ANOVA,  $P \leq 0.05$ ). NS, not significant.

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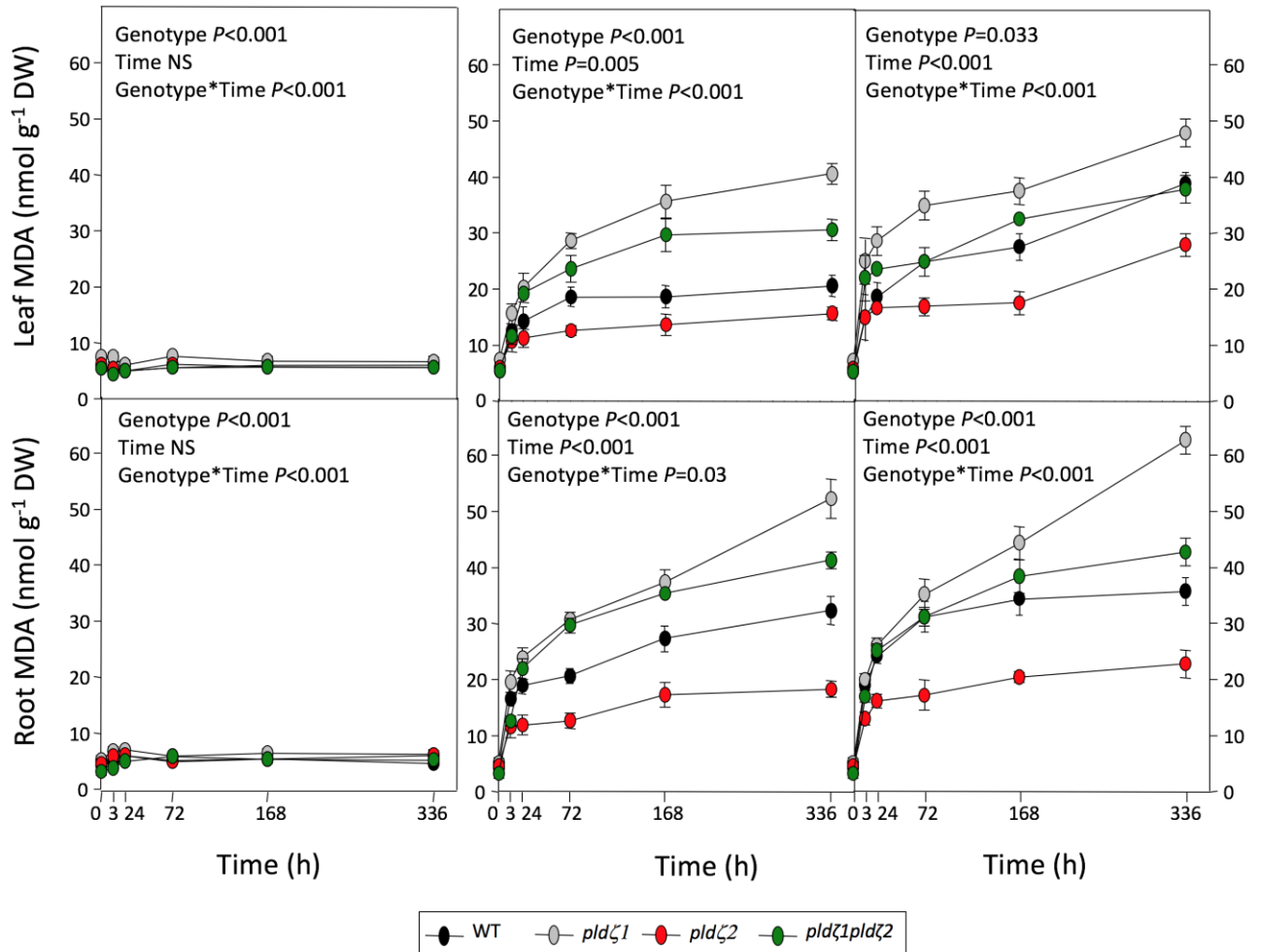


682 **Figure 4** Histochemical detection of hydrogen peroxide in leaves (a) and roots (b) of wild type and  
683 *pldζ1*, *pldζ2* and *pldζ1pldζ2* mutants of *A. thaliana* plants grown for 14 days under moderate (75  
684 mM NaCl) or severe salinity (150 mM NaCl). Brown residue from diaminobenzidine staining  
685 indicates sites of H<sub>2</sub>O<sub>2</sub> accumulation.

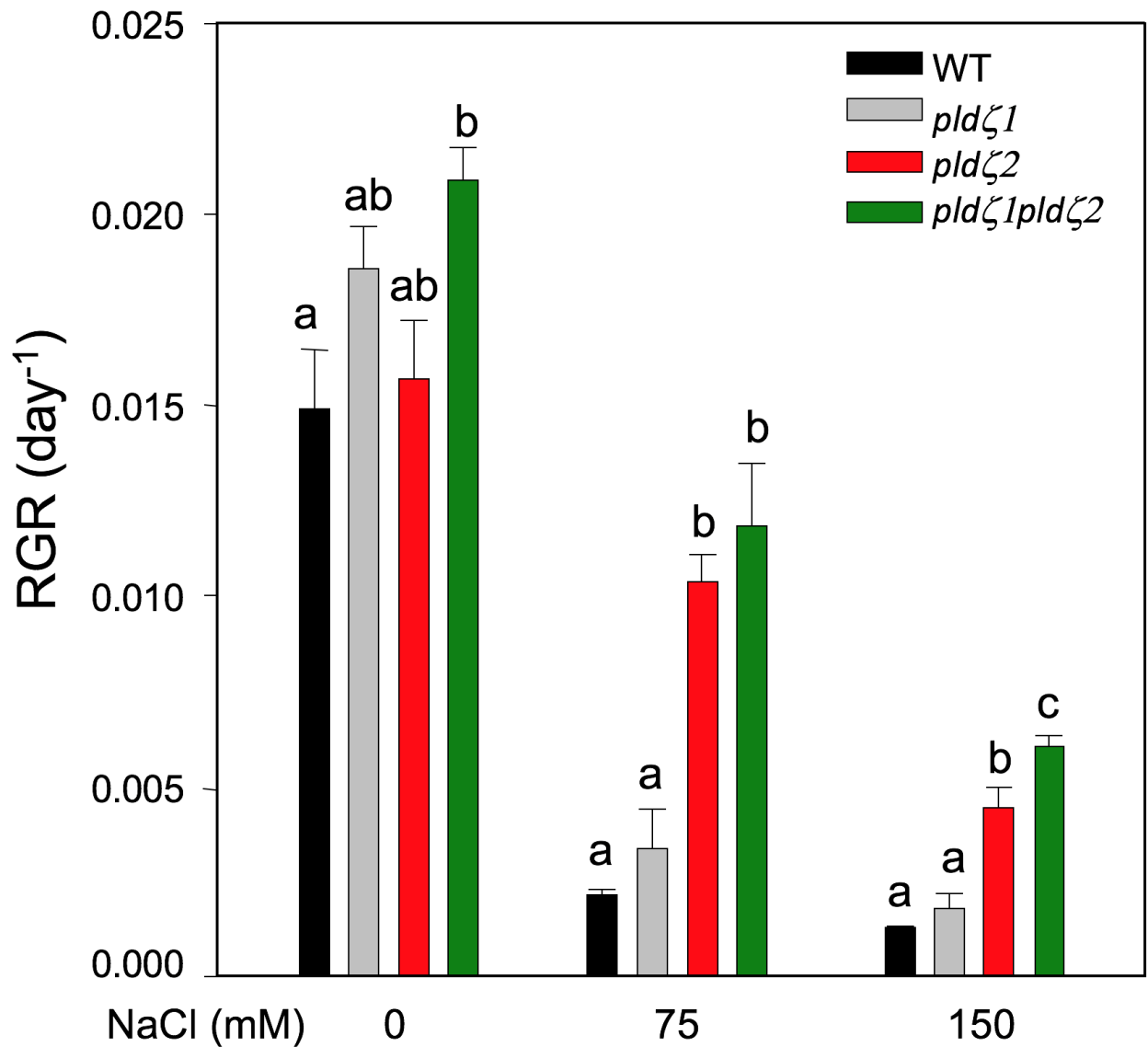
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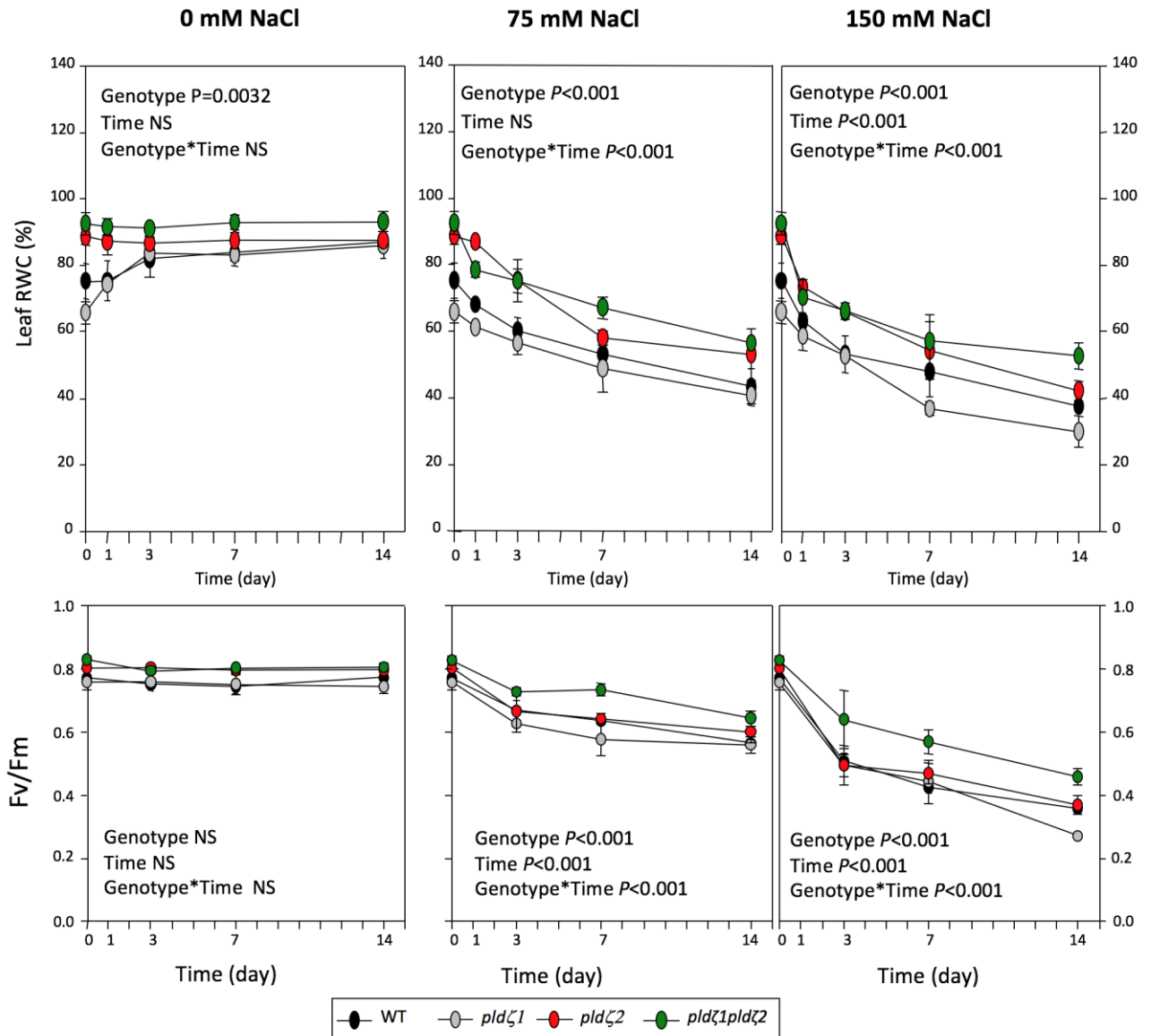
687  
 688 **Figure 5.** Changes in hydrogen peroxide levels in leaves and roots of wild type and *pldζ1*, *pldζ2*  
 689 and *pldζ1pldζ2* mutants of *A. thaliana* plants grown for 14 days with moderate (75 mM NaCl) or  
 690 severe salinity (150 mM NaCl). Data are means ± SE of 3 replicates. Results of statistical analysis  
 691 are given in each panel (ANOVA,  $P \leq 0.05$ ). NS, not significant.  
 692



693  
 694 **Figure 6.** Changes in malondialdehyde (MDA) levels in leaves and roots of wild type and *pldζ1*,  
 695 *pldζ2* and *pldζ1pldζ2* mutants of *A. thaliana* plants grown for 14 days under moderate (75 mM  
 696 NaCl) or severe salinity (150 mM NaCl). Data are means ± SE of 3 replicates. Results of statistical  
 697 analysis are given in each panel (ANOVA,  $P \leq 0.05$ ). NS, not significant.  
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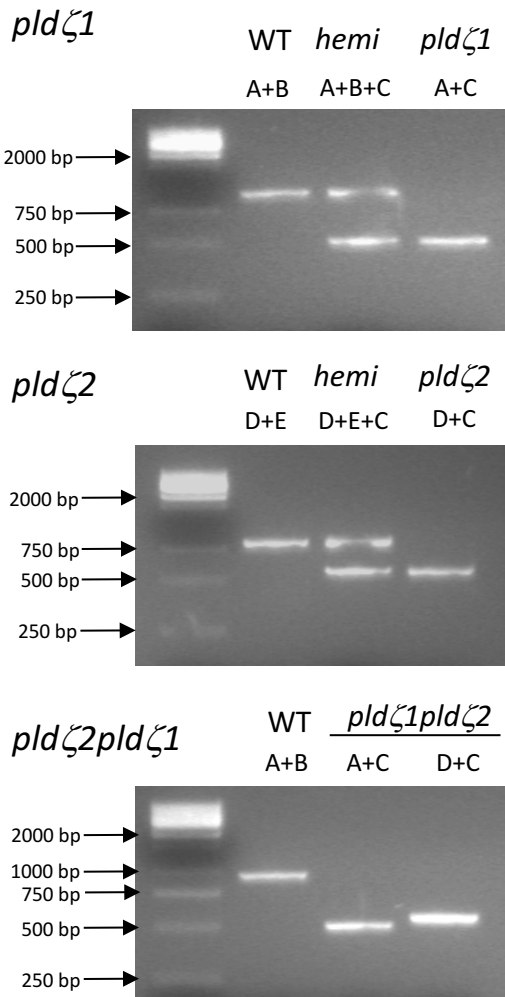
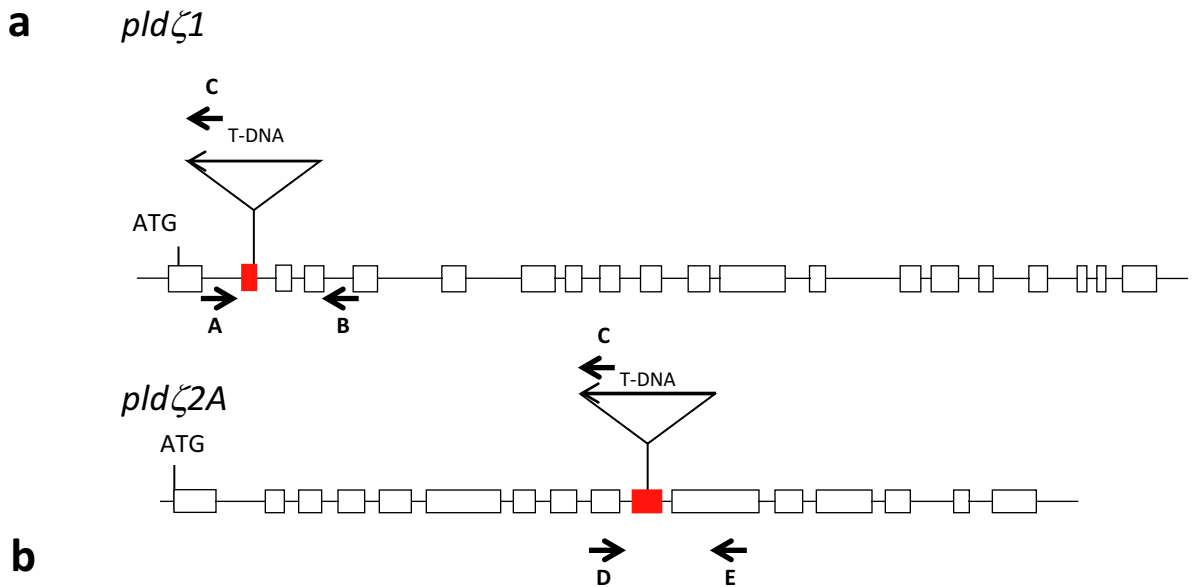


699  
700 **Figure 7.** Relative growth rate (RGR) in *A. thaliana* single and double *pldζ* mutants grown for 14  
701 days under control conditions or moderate (75 mM NaCl) or severe salinity (150 mM NaCl). Means  
702 indicated by different letters are significantly different ( $P \leq 0.05$ ) as determined by one-way  
703 ANOVA.  
704

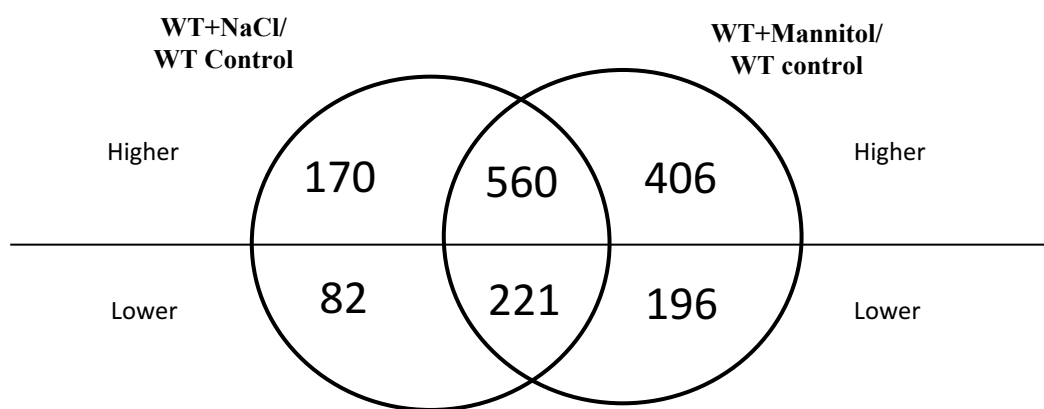


705  
 706 **Figure 8.** Changes in the relative water content (RWC) and in  $F_v/F_m$  ratios of leaves and roots of  
 707 wild type, and *pldζ1*, *pldζ2* and *pldζ1pldζ2* mutants of *A. thaliana* plants grown for 14 days under  
 708 moderate (75 mM NaCl) or severe salinity (150 mM NaCl). Data are means  $\pm$  SE of 3 replicates.  
 709 Results of statistical analysis (ANOVA,  $P \leq 0.05$ ) are given in each panel. NS, not significant  
 710





**Supplementary Figure S1** Map and genotyping of *pldζ1*, *pldζ2* and *pldζ1pldζ2* mutants. (a) Map of T-DNA insertion site in *pldζ1* and *pldζ2* genes. Primers used for PCR are represented by arrows. (b) 1 % agarose gels of PCR products for *PLDζ1* WT allele (A+B), *pldζ1* T-DNA flanking sequence (A+C), or both (A+B+C) in hemizygous plants (top gel). PCR products for WT *PLDζ2* WT allele (D+E), *pldζ2* T-DNA flanking sequence (D+C), or both (D+E+C) in hemizygous plants (middle gel). WT allele amplification (A+B), *pldζ1* T-DNA flanking sequence (A+C) and *pldζ2* T-DNA flanking sequence (D+C) in *pldζ1pldζ2* (bottom gel).



**Supplementary Figure S2** Venn diagram of WT transcriptomes under salt and mannitol stresses in comparison to control growth conditions. Numbers of differentially expressed genes for each comparison are shown.

AGI	Name	WT+NaCl / WT control		pld $\zeta$ 1pld $\zeta$ 2+NaCl / WT+NaCl	
		Log <sub>2</sub> Ratio	Pvalue	Log <sub>2</sub> Ratio	Pvalue
AT1G12610	DDF1 transcription factor	1.96	0.00E+0	-1.89	0.00E+0
AT1G73480	alpha/beta hydrolase family	3.40	0.00E+0	-1.04	0.00E+0
AT5G52310	COR78 (COLD REGULATED 78) (RD29A)	4.04	0.00E+0	-1.03	5.60E-12
AT2G16500	ADC1_ arginine decarboxylase 1	0.89	1.17E-4	-0.98	1.12E-10
AT1G58360	AAP1; amino acid permease 1	1.95	0.00E+0	-0.86	1.77E-7
AT2G38240	oxidoreductase. 2OG-Fe(II) oxygenase	1.23	2.24E-11	-0.81	2.50E-6
AT5G13750	ZIFL1 zinc induced facilitator-like 1	1.81	0.00E+0	-0.78	9.68E-6
AT2G46370	JAR1 (JASMONATE RESISTANT 1)	0.83	1.15E-3	-0.76	2.66E-5
AT4G26080	ABI1 (ABA INSENSITIVE 1) PP2C family	1.48	0.00E+0	-0.75	4.51E-5
AT1G51090	metal ion binding	2.99	0.00E+0	-0.74	1.05E-4
AT1G72770	HAB1; PP2C homologue to ABI1	2.53	0.00E+0	-0.73	1.22E-4
AT3G07700	Protein kinase superfamily protein	0.90	8.80E-5	-0.73	1.57E-4
AT1G01650	SPPL4( SIGNAL PEPTIDE PEPTIDASE-LIKE 4)	0.73	4.31E-2	-0.71	3.41E-4
AT3G48990	AMP-dependent synthetase and ligase	0.75	2.14E-2	-0.71	3.61E-4
AT1G29400	AML5 (ARABIDOPSIS MEI2-LIKE PROTEIN 5)	0.79	5.23E-3	-0.70	5.50E-4
AT5G17760	P-loop nucleoside triphosphate hydrolase	1.43	0.00E+0	-0.70	6.32E-4
AT1G76960	unknown protein	0.85	7.24E-4	-0.67	2.25E-3
AT2G35940	EDA29 (BEL1-like homeodomain 1)	0.80	3.45E-3	-0.65	5.59E-3
AT4G15530	PPDK pyruvate orthophosphate dikinase	0.90	1.04E-4	-0.64	8.57E-3
AT5G55970	RING/U-box superfamily protein	1.25	5.59E-12	-0.61	2.51E-2

**Supplementary Figure S3** List of genes in Cluster 1. Genes are involved in abiotic or biotic stimulus and response to stress. Expression data is given as log<sub>2</sub> ratios.

**Cluster 1 (Figure 1)****Biological process**

	Normed to Freq. In Arabidopsis set ( $\pm$ bootstrap StdDev, p-value)		
	Frequency in Cluster 1	Std dev	P value
response to abiotic or biotic stimulus (Input set freq.: 0.65; 0.13)	4.96	0.795	0.0000009492
other biological processes (Input set freq.: 0.45; 0.12)	3.65	0.874	0.0002548
response to stress (Input set freq.: 0.5; 0.14)	3.45	0.681	0.0001537
developmental processes (Input set freq.: 0.4; 0.13)	3.02	0.91	0.002149
transport (Input set freq.: 0.35; 0.12)	2.82	0.814	0.006256
signal transduction (Input set freq.: 0.15; 0.07)	2.12	1.185	0.115

**Cluster 2 (Figure 4)****Biological process**

	Normed to Freq. In Arabidopsis set ( $\pm$ bootstrap StdDev, p-value)		
	Frequency in Cluster 2	Std dev	P value
electron transport or energy pathways (Input set freq.: 0.28; 0.02)	13.07	4.609	4.157e-06
response to stress (Input set freq.: 0.76; 0.13)	5.47	0.807	1.850e-10
response to abiotic or biotic stimulus (Input set freq.: 0.61; 0.13)	4.62	0.885	2.780e-07
other biological processes (Input set freq.: 0.57; 0.13)	4.28	0.815	2.565e-06
transport (Input set freq.: 0.38; 0.1)	3.57	1.227	7.785e-04
cell organization and biogenesis (Input set freq.: 0.33; 0.11)	2.81	0.94	6.493e-03
developmental processes (Input set freq.: 0.33; 0.12)	2.62	0.781	9.223e-03
signal transduction (Input set freq.: 0.14; 0.05)	2.53	1.084	0.083

[http://bar.utoronto.ca/ntools/cgi-bin/ntools\\_classification\\_superviewer.cgi#annotation\\_list](http://bar.utoronto.ca/ntools/cgi-bin/ntools_classification_superviewer.cgi#annotation_list)

**Supplementary Figure S4** SuperViewer classification for genes listed in clusters 1 and 2.

AGI	Name	<i>pldζ1ζ2</i> / WT control		<i>pldζ1ζ2</i> +NaCl / WT+NaCl	
		Log2 Ratio	Pvalue	Log2 Ratio	Pvalue
AT5G35935	unknown protein_copia-like	1.64	0.00E+0	1.73	0.00E+0
AT1G23390	unknown protein	1.28	0.00E+0	0.78	1.40E-5
AT1G08830	CSD1; copper. zinc superoxide dismutase	1.27	0.00E+0	0.78	1.02E-5
AT1G66100	toxin receptor binding_ thionin. putative	1.23	0.00E+0	0.65	4.41E-3
AT1G12520	CCS1; superoxide dismutase copper chaperone	1.04	5.30E-9	0.81	2.04E-6
AT3G02380	COL2 transcription factor	0.81	3.05E-4	0.64	7.26E-3
AT5G48485	DIR1 lipid transfer protein (LTP)	0.74	3.99E-3	0.60	4.57E-2
AT4G09320	NDPK1 nucleoside diphosphate kinase type 1	0.72	7.93E-3	0.73	1.58E-4
AT3G48990	AMP-dependent synthetase and ligase	-0.84	7.33E-5	-1.06	0.00E+0
AT5G08530	CI5. NADH-ubiquinone oxidoreductase 51 kDa	-0.85	5.72E-5	-0.95	9.52E-10
AT3G23820	GAE6	-0.87	2.15E-5	-1.09	0.00E+0
AT5G05170	CESA3 (CELLULASE SYNTHASE 3)	-0.92	2.63E-6	-0.98	1.12E-10
AT4G32410	CESA1 (CELLULASE SYNTHASE 1)	-0.94	9.08E-7	-1.00	3.92E-11
AT3G46970	ATPHS2	-1.01	3.21E-8	-1.09	0.00E+0
AT1G59870	ABC transporter family protein	-1.08	8.23E-10	-1.04	0.00E+0
AT2G25490	EBF1 (EIN3-BINDING F BOX PROTEIN 1)	-1.15	1.12E-11	-1.31	0.00E+0
AT4G38770	PRP4 (PROLINE-RICH PROTEIN 4)	-1.29	0.00E+0	-0.94	1.15E-9
AT3G60750	transketolase_ transketolase. putative	-1.31	0.00E+0	-1.01	1.12E-11
AT3G33002	ribosomal protein S2p family	-1.33	0.00E+0	-1.11	0.00E+0
AT5G42020	ATP binding_ luminal binding protein 2 (BiP-2)	-1.38	0.00E+0	-1.53	0.00E+0
AT3G09440	HSP70-3	-1.61	0.00E+0	-1.38	0.00E+0
AT4G30650	low temperature and salt responsive protein	-1.82	0.00E+0	-1.11	0.00E+0
AT5G02500	HSC70-1	-1.83	0.00E+0	-1.39	0.00E+0

**Supplementary Figure S5** List of genes in cluster 2. Genes are involved in electron transport, biotic and abiotic stress and stress responses. Expression data are given as log<sub>2</sub> ratios.

**Supplementary Table S1** List of primer sequences used for RT-PCR analysis and genotyping.

For RT-PCR

Gene name	Accession number	5'-3' Sequence
DDF1-F	AT1G12610	GGGACTTATCCCACAGCAGA
DDF1-R	AT1G12610	ATCATTGGATTCCGGCACC
RD29AF	AT5G52310	CAAAACAGAGCACTTACACAGAGAA
RD29AR	AT5G52310	CATAATCTCTACCCGACACACTTTT
APT1F	AT1G27450	GAGACATTTTGCCTGGGATT
APT1R	AT1G27450	CGGGGATTTTAAGTGAACA
APX1F	X59600.1	CTGACATTCCTTCCACCCT
APX1R	X59600.1	CAGACCTATCCTTGTGGCAT
CSD1F	AT1G08830	GGTTTCCATGTCCATGCTCT
CSD1R	AT1G08830	ATTGTGAAGGTGGCAGTTCC
CAT2F	AT4G35090.1	AACTCTGGTGCTCCTGTATGG
CAT2R	AT4G35090.1	CTCCAGTTCTCTTGGATGTG

For genotyping

A	PLDzeta1F	At3g16785	TCAGAATCACTTAAGAGGAGATGGG
B	PLDzeta1R	At3g16785	TTTTCGCATAGTCACTTGCTGT
C	LBb1	T-DNA left border	GCGTGGACCGCTTGCTGCAACT
D	PLDzeta2F	At3g05630	TCTCTGTTTTGGGCGGTACGA
E	PLDzeta2R	At3g05630	AAAATGTTTCAGCGTTCTGGAT