Suppression of Phospholipase D\textsubscript{c} Confers Increased Aluminum Resistance in \textit{Arabidopsis thaliana}

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Abstract

Aluminum (Al) toxicity is the major stress in acidic soil that comprises about 50% of the world’s arable land. The complex molecular mechanisms of Al toxicity have yet to be fully determined. As a barrier to Al entrance, plant cell membranes play essential roles in plant interaction with Al, and lipid composition and membrane integrity change significantly under Al stress. Here, we show that phospholipase D\textsubscript{c} (PLD\textsubscript{c}) are induced by Al stress and contribute to Al-induced membrane lipid alterations. RNAi suppression of PLD\textsubscript{c} resulted in a decrease in both PLD\textsubscript{c}1 and PLD\textsubscript{c}2 expression and an increase in Al resistance. Genetic disruption of PLD\textsubscript{c}1 also led to an increased tolerance to Al while knockout of PLD\textsubscript{c}2 did not. Both RNAi-suppressed and \textit{pld}\textsubscript{c}1\textsuperscript{-}\textsuperscript{1} mutants displayed better root growth than wild-type under Al stress conditions, and PLD\textsubscript{c}1-deficient plants had less accumulation of callose, less oxidative damage, and less lipid peroxidation compared to wild-type plants. Most phospholipids and glycolipids were altered in response to Al treatment of wild-type plants, whereas fewer changes in lipids occurred in response to Al stress in PLD\textsubscript{c} mutant lines. Our results suggest that PLD\textsubscript{c}s play a role in membrane lipid modulation under Al stress and that high activities of PLD\textsubscript{c}s negatively modulate plant tolerance to Al.

Introduction

Aluminum (Al) toxicity is the major stress in acidic soil mainly because it increases plant susceptibility to other stresses such as nutrient deficiencies and mineral toxicities [1,2]. Acid-soil regions comprise more than 50% of the world’s arable land, and Al toxicity causes large losses in agricultural production [1,2]. Multiple mechanisms have been implicated in plant response and resistance to Al stress [1–3]. Al exposure rapidly inhibits root elongation and subsequently alters root morphology. Some of Al’s effects include alteration of cell wall properties, disruption of membrane integrity, alteration in lipid composition, perturbation of Ca\textsuperscript{2+} homeostasis, dysfunction of mitochondria, denaturation of cellular proteins, damage to DNA, and blockage of cell-cycle progression [1,4–8]. Given the wide range of cellular effects, it is thought that multiple factors, both constitutively expressed and induced, are involved in plant response to Al stress and contribute to Al resistance [2]. The primary line of defense is likely secretion of Al-chelating organic acids including malate or citrate into the rhizosphere by transporters from the Al-activated malate transporter (ALMT) and multidrug and toxin extrusion (MATE) families [9–12]. A large number of other genes are likely involved in the response to Al stress and only a few have been characterized [8].

While the Al stress alters the cell wall, the plasma membrane is the barrier for Al entry into cells and probably the first site of Al damage to the cell. Al stress causes a significant decrease in levels of glycolipids and phospholipids and a loss of membrane integrity [7,13,14]. The total amount of lipids in maize roots and shoots decreases under Al stress, although several phospholipids increase, including phosphatidylethanolamine (PC), phosphatidylglycerol (PG), and monogalactosyldiacylglycerol (MGDG) [15]. Under Al stress, the activities of phosphatidylinositol 4,5-bisphosphate (PIP\textsubscript{2})-specific phospholipase C (PLC) and inositol 1,4,5 trisphosphate (IP\textsubscript{3}) levels decrease whereas those of phosphatidylinositol 4-kinase, phosphatidylglycerol phosphate 5-kinase, and diacylglycerol kinase increase, suggesting alterations in levels of signaling phospholipids [16,17]. In addition, Al stress enhanced the peroxidation of membrane lipids and inactivated membrane proteins because reactive oxygen species accumulated in response to Al stress [6,18–20]. One possible mechanism for Al-induced lipid changes may be that Al binds to negatively-charged lipids, such as PIP\textsubscript{2}, PG, and PF, this binding may alter the ability of proteins to interact with lipids, altering membrane enzyme activities and initiating membrane lipid turnover, or causing changes in membrane permeability and cellular processes [16]. These changes in turn
could alter membrane composition and properties, as well as the activity of membrane-integrated transporters [21–23].

One potential approach for altering plant responses to Al is changing the membrane lipid composition. Elevation of (9Z)-unsaturated long-chain base levels in plant sphingolipids, by constitutively expressing 3E/3Z-desaturase, reversed Al induced root inhibition [7]. Overexpression of a wheat phosphatidylserine synthase gene (TaPSS1) increased PS and PC content in transgenic Arabidopsis and yeast, while deletion of the PSS gene decreased Al resistance in yeast [24]. Al was shown to inhibit the PLC and phospholipase D (PLD) pathways and reduce the formation of phosphatidic acid (PA) in plant tissue cultures [25–28]. However, the effect of PLDs on plant tolerance to Al and the identities of the specific PLDs involved remain unknown.

PLD catalyzes the hydrolysis of phospholipids to generate PA and a free head group [29,30]. PLD is a major family of lipid-hydrolyzing enzymes in plants and 12 PLDs have been identified in Arabidopsis, including 3 PLDγs that are highly homologous. PLDs are activated under various stress conditions and specific PLD family members have been shown to play roles in plant responses to different stresses [31–32]. However, the biological functions of PLDγs are not well understood. PLDγ1 and PLDγ2 are PIPγ-dependent and require micromolar Ca2+ for activity [33,34]. PLDγ1 and PLDγ2 are 90% identical in amino acid sequences, but they differ in the effect of PIPγ2 and Triton X-100 on their activities [34]. Here, we investigated the effect of PLD mutation on Arabidopsis Al sensitivity, and the results indicate that PLDγ negatively affect plant resistance to Al stress.

### Results

**PLDγ expression in response to Al stress and production of PLDγ-deficient mutants**

To obtain clues about the physiological functions of different PLDs, we examined the expression patterns of PLDs in detached leaves treated with various hormones and abiotic stresses for 3 and 20 h. The level of PLDγ was induced by treatment with AlCl3, cadmium (Cd), H2O2, salicylic acid (SA), and methyl salicylate (MeSA) at 3 h of treatment. The induction of PLDγ by Al-was transient and PLDγ expression fell close to the basal level by 20 h (Fig. 1A). At 20 h, the induction of PLDγmRNA also occurred in response to NaCl, mannitol, CdCl2, MeSA, SA, and abscisic acid (ABA) (Fig. 1A). In contrast, the basal level of PLDγ expression was high and some increase in its expression occurred at 3 h after all treatments, but no induction of mRNA was detectable with 20-h treatments (Fig. 1A). To determine whether both of the two individual PLDγs were induced by Al stress, Arabidopsis seedlings were treated with 100 μM AlCl3 (pH 4.0) and sampled at various time points to examine PLDγ expression with PLDγ1 cDNA and the PLDγ2 5’-UTR as probes (Fig. 1B) [34]. Both PLDγ1 and PLDγ2 were induced transiently by Al stress; the induction peaked earlier for PLDγ1 than PLDγ2, and both fell close to the basal level at 20 h. A survey of EST-based gene expression in Al-stressed rye also identified PLDγ as responsive genes [35].

To characterize the biological functions of the PLDγs, we generated PLDγ mRNA transgenic plants (Fig. 2). Two selected lines, PLDγ2RNAi1 and PLDγ2RNAi2, had decreased levels of PLDγ transcripts (Fig. 2A). Quantification of the RNA blotting data indicated that the PLDγ mRNA levels in PLDγ2RNAi-1 and PLDγ2RNAi-2 plants were reduced by approximately 70–85% and 90%, respectively, compared with wild-type controls (Fig. 2B).

Two T-DNA insertion mutants were isolated from Salk T-DNA insertion lines (Fig. 2C, D). Sequencing indicates that pldγ1-1 contains a T-DNA insert at the 6th exon of PLDγ1, 1912 bp from the start codon (Fig. 2C). Mutant pldγ2-1 has a T-DNA insert at the 6th intron of PLDγ2, 1635 bp from the start codon (Fig. 2D). Because PLDγs share more than 90% identity at the DNA level, distinguishing their transcripts in these mutants was difficult. However, PLDγ2 has a longer 5’-UTR than PLDγ1 [34] so we used the PLDγ1 full-length cDNA and the long 5’-UTR of PLDγ2 cDNA as probes to detect their transcripts in pldγ1-1 and pldγ2-1 mutants. Under strict hybridization conditions, pldγ2-1 plants did not exhibit the higher band, whereas pldγ1-1 did not exhibit the lower band (Fig. 2E). The result indicates that PLDγ1 and PLDγ2 transcripts are absent or decreased substantially in pldγ1-1 and pldγ2-1 mutants, respectively.

**Altered responses of PLDγ mutants to Al stress**

Root elongation of PLDγ mutants and wild-types was measured at different pHs (Fig. 3) and different concentrations of AlCl3 under acidic pH conditions (Fig. 4). The seedling root growth of both ecotypes, Col-0 and WS, was retarded compared with controls, when the plants were transferred to plates containing 100 μM AlCl3 (Fig. 3). The retardation by Al was greater at pH 4.0 than at pH 5.6 (Fig. 3). Low pH (around 4.0) is required.
for Al toxicity, because Al$^{3+}$ is not chelated and precipitated at low pH. Roots of PLDγ RNAi and pldy1-1 seedlings at 50 and 100 μM Al lengthened significantly more than wild-type controls (P<0.01), whereas pldy2-1 did not (Fig. 4A). In addition, PLDγ RNAi mutants increased in the number and length of hairy roots under Al stress conditions (data not shown). The effect of PLDγ mutants on root growth under Al stress was dependent on Al concentration; the root length was similar between PLDγ mutants and wild-types in media without Al, at Al levels higher than 400 μM and media where the pH was raised to pH 5.6, where Al is not toxic (Fig. 3 and 4A).

Callose deposits on cell walls are another commonly observed phenomenon in plants in response to Al stress and have been used as a marker of Al stress intensity and damage [19,36,37]. By staining with aniline blue, callose production in Al-treated roots was visualized. The roots of wild-type plants of both ecotypes show yellowish fluorescent signals compared to the wild-type controls, whereas the staining intensity of pldy1-1 was close to that of its wild-type control (Fig. 4B).

**Effect of PLDγ mutations on Al-induced organic acid secretion and Al content**

Al stress also induces plasma membrane transporters to secrete organic acids that can chelate Al, and the release of citric acid and malic acid to root environments is closely associated with Al-tolerance in plants [9–12]. After treatment of roots of wild-type and PLDγ mutants with 50 μM AlCl$_3$ for 5 h, the secretion of citric acid and malic acid into the media was measured. The roots of pldy1-1 excreted significantly higher levels of citrate and malate into the media (Fig. 5A). Al accumulation in Al-treated pldy1 mutants and wild-type was measured in Al-treated roots by inductively coupled plasma mass spectrometry (ICP-MS). We did not detect significant differences in root Al accumulation between pldy1-1, pldy2-1, and Col-0 roots treated with 50 μM Al for 5 h (Fig. 5B).

**Altered oxidative stress in PLDγ mutants in response to Al stress**

Al stress induces oxidative stress in plant roots, and increases in oxidative stress are regarded as one of the causes of root damage by Al [6,18,20,37]. The reactive oxygen species (ROS)-detecting agent, 6-carboxy-2,7-dihlorodihydrofluorescein diacetate (2,7-DCFDA), was used to examine whether there is any alteration in ROS production in PLDγ mutants compared to wild-type plants under Al stress. Roots of seedlings grown in 50 and 100 μM AlCl$_3$ displayed strong 2,7-DCFDA fluorescence (Fig. 6A). The signal was much stronger in roots than in other tissues (data not shown), which is consistent with the observation that the Al-induced formation of ROS occurs first in Arabidopsis roots [6,18,20]. RNAi1, RNAi2, and pldy1-1 exhibited much weaker fluorescent staining than wild-type, and the lower signal intensity occurred in both root cells and throughout the whole root apex in seedlings grown in 100 μM AlCl$_3$. The fluorescent staining of pldy2-1 was stronger than the other mutants but slightly weaker than wild-type Col-0 (Fig. 6A). Glutathione S-
transferase (GST) is up-regulated in oxidative stress caused by many adverse environments [38]. AtGST1 expression increased upon Al stress; AtGST1 expression in both pldy-1-1 and RNAi mutants was higher than their respective wild type controls and pldy-2-1 (Fig. 6B and C).

To examine the potentially altered balances in oxidative stress and antioxidant systems, the ascorbate-glutathione antioxidant cycle system was examined by assaying the activities of ascorbate peroxidase (APX) and glutathione reductase (GR) in PLD mutants and wild-type plants that were exposed to Al for 5 h. RNAi

Figure 3. Root growth of PLD<sup>c</sup> mutants and wild-type Arabidopsis under Al stress. (A–D) PLD<sup>c</sup> RNAi mutants and wild-type WS in 1/8 MS medium containing 100 μM AlCl<sub>3</sub> (A and B) or in 1/8 MS medium without AlCl<sub>3</sub> (C and D) at two different pHs. (E–H) Col-0, pldy-1-1, pldy-2-1 mutants on 1/8 MS plates containing 100 μM AlCl<sub>3</sub> (E and F) and 1/8 MS medium without AlCl<sub>3</sub> (G and H). Photos are representatives from at least three independent experiments (7 day-old seedlings post treatment).

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mutants and pld1-1 plants had higher APX and GR enzymatic activities than wild-types, while activities of the two enzymes in pld2-1 were comparable to those in wild-type plants (Fig. 6D and E). In addition, PLDγ RNAi and PLDγ mutants had significantly lower levels of lipid peroxidation, as assayed by the thiobarbituric acid reaction (TBARS), whereas the lipid peroxidation value of pld2-1 was similar to that of wild-type (Fig. 6F). Of all the genotypes, PLDγ/RNAi2 had the highest APX and GR activities and lowest lipid peroxidation value (Fig. 6).

Membrane lipid changes upon Al stress

To examine lipid changes occurring as a result of Al stress and PLDγ-deficiencies, seedlings of PLD mutants and corresponding wild-types were transferred to 100 µM AlCl3 for two days. Roots and shoots (including stems and rosette leaves) under the Al-stressed and normal conditions were collected separately for phospholipid and glycolipid analysis by an ESI-MS/MS method described previously [43]. Compared with untreated roots, the levels of several phospholipids, PC, PE, PI, and PS, were increased in roots of both WS and Col-0 seedlings under Al treatment (Fig. 7C). Changes in response to Al treatment of plasticid lipids, DGDG, MGDG, and PG, were minor (Fig. 7A and C), but a significant drop in MGDG levels was observed in the roots of Col-0 plant under Al stress (Fig. 7C). In shoots of wild-type plants, the most consistent change due to Al treatment, occurring in both ecotypes, were decreases in the levels of DGDG and lysoPC (Fig. 7B and D).

Without Al stress, PA was lower in pld1-1 and pld2-1 roots and RNAi lines leaves (Fig. 7A and B), suggesting that PLDγ1 and PLDγ2 play a role in the basal accumulation of PA. With Al stress, the level of PA tended to increase in Col-0 and the mutants pld1-1 and pld2-1, and the percentage of increase was greater in the mutants, particularly in pld2-1. The results suggest that these PLDs are not responsible for Al-induced PA increase. This observation is consistent with an earlier finding that Al inhibits phosphoinositide-dependent PLD activity in vitro [27].

PLDγ mutants show fewer significant changes in all major phospholipids and glycolipids than wild-types under Al stress (Fig. 6B and D). All mutants and wild-type controls showed a decrease in MGDG/DGDG ratio after Al treatment in roots (Fig. 8). On the contrary, the MGDG/DGDG ratio in shoots was increased in all wild-types and mutants under Al stress. The results suggest that lipids in roots and shoots change differently in response to Al stress.

Discussion

The study shows that the suppression of PLDγ renders Arabidopsis seedlings more tolerant to Al stress. Analysis of the single gene knockouts suggests that PLDγ1 is the gene responsible for most of the observed Al tolerance in the PLDγ RNAi lines. This is not surprising given that PLDγ1 is expressed in the roots where most of the Al toxicity is observed while PLDγ2 predominantly expressed in the inflorescence [44]. This suggests that Al stress is similar to other abiotic and biotic stress conditions, such as drought, hyposmotic stress, and phosphorous starvation where PLDs have been implicated in mediating dynamic changes of membrane phospholipids and glycolipids [31,32,40].

The increased Al tolerance in PLDγ RNAi and pld1-1 plants is accompanied by the decreased levels of ROS, lipid peroxidation, and callose deposition in roots, and by the increases in the expression of protective GST and activities of redox enzymes APX

![Figure 4. Root length and callose accumulation of PLDγ mutants and wild-type roots. (A) Quantification of root length of PLDγ mutants and wild-type seedlings at various concentrations of AlCl₃ (pH 4.0). Four day-old seedlings were transferred to 1/8 MS containing indicated levels of AlCl₃. Roots were measured 5 days after transfer. The values of RNAi1 and RNAi2 were significantly different (P<0.05) from those of WS and the values for pld1-1 were significantly different than the values for Col-0 at 50 and 100 µM AlCl₃. Data represent the mean ± SD (N=50). (B) Callose staining in roots. Seven day-old seedlings were treated with 100 µM AlCl₃ in 1/8 MS solution (pH 4.0) for 5 h and roots were stained for callose with ammine blue as described in “Materials and Methods”. Images are representatives of three experiments and more than 25 roots. Control was Col-0 roots without Al treatment; all genotypes had no staining without the Al treatment. doi:10.1371/journal.pone.0028086.g004](image)

![Figure 5. Al content in roots and organic acid released by roots of PLDγ mutants and wild-type. Wild-type, pld1-1, and pld2-1 mutant seedlings were treated in 1/8 MS medium (pH 4.0) containing 50 µM AlCl₃ for 5 hrs. Media were used for organic acid analysis with GC-MS (A) and roots were washed for ICP-MS analysis of Al content (A). Data are from three samples and presented with means ± S.D. * indicates p<0.05, significant difference in Student’s t test. doi:10.1371/journal.pone.0028086.g005](image)
and GR. ROS generation and oxidative stress are known to be increased by Al treatment of Arabidopsis [4,6,18,20,37] and responses such as increasing GST expression and activating redox enzymes are used to protect membrane lipids from peroxidation [4,6,18,20,38]. Al stress increases ROS production in all genotypes, but Al-resistant PLD\textsubscript{\textgamma} mutants accumulate less ROS than wild-type roots, suggesting that the ROS level is associated with cell damage. ROS is a double-edged sword: at low levels and earlier stages of stress, ROS may trigger signaling and defense responses such as increasing callose production and rigidification of the cell wall [6,18–20,27,37,38]. However, a large increase in ROS can result in and/or be indicative of cell damage. Studies have suggested that mitochondrial dysfunction is an Al toxicity mechanism due to increased mitochondria membrane permeability, excess ROS generation, and redox signals [4,6,20,38,41]. Subcellular localization results indicate that PLD\textgamma\textsubscript{1} is primarily associated with membrane fractions, including mitochondrial and plasma membranes and nucleus [42]. Since the expression of PLD\textgamma\textsubscript{s} is induced by H\textsubscript{2}O\textsubscript{2} and Al stresses, and PLD\textgamma\textsubscript{1} can also be phosphorylated, which might regulate PLD\textgamma\textsubscript{1} activity in lipid metabolism [43], it would be possible that PLD\textgamma\textsubscript{s} are involved in signal transduction and mitochondrial or plasma membrane lipid hydrolysis under Al stress. Thus, PLD\textgamma\textsubscript{s} might be required for certain Al-activated stress responses such as ROS generation, redox status change, and lipid peroxidation. PLD\textgamma\textsubscript{1}-deficient mutants thus have smaller changes in lipid compositions or oxidative stress damage under Al stress since the suppression of PLD\textgamma\textsubscript{s} may partially block ROS production and attenuate Al-induced damage. Furthermore, PLD\textgamma\textsubscript{1} RNAi and pld\textgamma\textsubscript{1-1} mutants displayed higher ROS-scavenging enzyme activity. APX and GR are responsible for regeneration of two important antioxidants, ascorbate and glutathione [41]. The higher activities of these enzymes in PLD\textgamma\textsubscript{1} RNAi and pld\textgamma\textsubscript{1-1} mutants are consistent with the decreased lipid peroxidation and membrane damage.

PLD\textgamma\textsubscript{s} could affect membrane lipid metabolism under Al stress by affecting lipid degradation and/or by lipid remodeling, such as replacement of phospholipids with glycolipids under phosphorous starvation [40,44]. Degradation is primarily detrimental and leads to fatty acid release and oxidation. The treatment of plants with Al and other metals causes changes in the composition and levels of phospholipids and glycolipids [4,5,15,28,45–48]. Lipid composition affects the membrane integrity, fluidity, and biological functions of proteins intrinsic to and associated with membranes. The biological significance of the lipid changes is not well understood and enzymes catalyzing the membrane lipid changes in response to metal stresses are not defined. Our results suggest that suppression of PLD\textgamma\textsubscript{s} decreased Al-induced lipid changes and lipid peroxidation. An increase in lipid peroxidation after Al treatments was associated with a decrease in polyunsaturated fatty acids in sorghum, and the decrease on polyunsaturated fatty acids was more severe in an Al-sensitive sorghum cultivar than in an Al-tolerant one [4,5,15]. A similar association between decreased lipid peroxidation and enhanced Al tolerance was also observed in sorghum and rice cultivars, as well as transgenic Arabidopsis plants [18,19,49,50]. The results suggest that the suppression of PLD\textgamma\textsubscript{s} decreases lipid hydrolysis and partially blocks Al-induced membrane damage.

The lipid compositional results indicate that plant roots and shoots respond differently to Al stress. MGDG/DGDG ratio was decreased in roots, but increased in shoots in mutants and wild types after Al treatment. Earlier studies indicated that Al increased MGDG/DGDG ratio in membranes from maize roots [15] and from roots of the Al-tolerant wheat cultivar [5]. Thus, our results in roots are different from those reported in maize and wheat. It is not clear whether it is due to a difference between monocots and dicots. In addition, PLD\textgamma RNAi mutants had a lower root MGDG/DGDG ratio than WS control under normal growth conditions. In pld\textgamma\textsubscript{1-1}, pld\textgamma\textsubscript{2-1}, and Col-0 seedlings, MGDG/DGDG ratio in roots all decreased in response to Al stress, and pld\textgamma\textsubscript{2-1} had a larger decrease than did pld\textgamma\textsubscript{1-1} and Col-0. Thus, the present data showing a drop in MGDG/DGDG ratio in all genotypes and no consistent trend for difference in amount of change between wild-types and mutants, do not support a positive association of increased Al tolerance with increased MGDG/DGDG ratio in roots. In contrast, our data suggest that Al-resistant lines are likely to maintain a relatively constant MGDG/DGDG ratio in leaves.

Characterization of various Arabidopsis Al-sensitive mutants suggests that tolerance of Arabidopsis to Al toxicity involves more than one mechanism, and the mechanism may be unique to specific plant species [51]. Secretion of organic acids, such as citric acid and malic acid [12,52], increase in the rhizosphere pH [53], relocation of Al to less sensitive tissues [3], and improved integrity and function of membranes [7] are all potential mechanisms for Al tolerance. An Al tolerance phenotype may result from one or more mechanisms. The present results indicate that increased changes in major membrane glycerolipids are positively associated with Al sensitivity and that PLD\textgamma\textsubscript{s} are involved in Al-induced cell damage. The lipid changes in response to Al are distinctively different in roots and leaves. PLD\textgamma\textsubscript{s} are involved in the Al resistance by affecting Al-evoked lipid hydrolysis that results in alterations in oxidative stress. The generation of double and triple mutants of PLD\textgamma\textsubscript{s}, which has been difficult because the genes are linked in tandem [54], will aid further elucidation of the role of PLDs in plant response to Al toxicity.

**Materials and Methods**

**Plant Materials and Mutant Isolation**

*Arabidopsis thaliana* PLD\textgamma\textsubscript{1} (At4g11850) and PLD\textgamma\textsubscript{2} (At4g1830) were cloned as previously described [33,34]. T-DNA insertion knockouts for PLD\textgamma\textsubscript{1} and PLD\textgamma\textsubscript{2} were isolated from Salk lines SALK\_113873 (PLD\textgamma\textsubscript{1}) and SALK\_014510 (PLD\textgamma\textsubscript{2}) obtained from
ABRC (Columbia OH), by using gene specific primers, 5'-TCATATGGTGAGGTTTTCTTGTAG-3' for PLDc1 or 5'-ATGTCAATGGGAGGAGGG-3' (for PLDc2) with a T-DNA left border primer. These two homozygous mutants show a cosegregation between their PLDc deletions with kanamycin resistance, suggesting that these mutants have a single T-DNA insertion.

**PLDc RNAi Construct**

The sense cDNA exon (362 bp) and the following intron (283 bp) were amplified by PCR with forward primer GIR51: 5'-CCGCTCGAGTGGTAATGAGTGTGTAGGAGTTC-3' (XhoI underlined) and reverse primer GIR31: 5'-CCGGAATTCTTGCTACAACAAAACAAAAGCTT-3' (EcoRI underlined). Antisense cDNA (362 bp) was amplified with forward primer GIR52: 5'-CCGGAATTCTTTTGAATCCCAGAAGACTC-3' (EcoRI underlined) and reverse primer GIR32: 5'-CTAGCTAGTCTAATGGGAGGAGGG-3' (XbaI underlined). The two PCR products were digested with EcoRI and then ligated into a fragment of 1007 bp containing two inverted cDNA repeats separated by an intron. This fragment was further digested with XhoI and XbaI restriction enzymes, and the resulting fragment was purified and then ligated into pKYLX71-35S2 binary vector at XhoI and XbaI sites. The resulting RNAi construct was confirmed by sequencing and used to transform *Agrobacterium tumefaciens* strain GV301 and then Arabidopsis plants, using the floral dip method. F1 to F3 progeny were screened on kanamycin plates and using PCR. Two homozygous lines, PLDcRNAi1 and PLDcRNAi2, were obtained with dramatically decreased PLDc transcripts as indicated by northern blotting.

**Root Elongation Assay**

All seeds from PLDc1 and PLDc2 T-DNA knockouts, PLDc RNAi mutants, or their wild-type controls (WS for RNAi mutants and Columbia ecotype Col-0 for T-DNA knockouts) were collected at the same stage for the root elongation test. Seeds were surface-sterilized and germinated on 1/8 Murashige and Skoog (MS) plates containing 1% sucrose and 0.8% agar. Four to seven day-old seedlings were transferred to 1/8 MS containing 50, 75, 100, 150, 200, 250, and 300 μM of AlCl3, 1% sucrose, and 0.8% phytagar (pH 5.6 or pH 4.0, pH was adjusted with 5 mM MES). Plates were vertically cultivated under cold fluorescent light with a 16 h/8 h light period at 23°C. The newly elongated roots were measured.

**RNA Blotting**

Total RNA was isolated from roots or leaves of Arabidopsis plants with a cetyltrimethylammonium bromide extraction method [55]. Equal amounts of total RNA (10 μg) were separated by 1% formaldehyde agarose denaturing gel electrophoresis and stained with ethidium bromide for visualization. RNA was transferred to nylon membranes and probed with radioactively labeled probes. The blots were autoradiographed and analyzed by image analysis.

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**Figure 7. Lipid changes as affected by Al stress and PLDc mutations.** Seven day-old PLDc mutant and wild type seedlings were treated with (+Al) or without 100 μM AlCl3 (−Al) in 1/8 MS solution (pH 4.0) for 2 days. Roots and shoots (rosette leaves and stems) were harvested separately for lipid extraction as described in “Material and Methods”. Data represent means ± SD (N = 5). (A) and (B) Lipid profiles of PLDc RNAi mutants and wild-type (WS) seedlings in roots and shoots, respectively. (C) Lipid profiles of PLDc1-KO and PLDc2-KO and wild-type (Col-0) seedlings in roots and shoots, respectively. (Label ‘a’ above the bar indicates that the value of the Al-treated is significantly different from that of non-treated control at P<0.05. Label ‘b’ above the bar indicates that the mutant values are significantly different from wild type at P<0.05. Label ‘c’ above the bar indicates that the mutant values are significantly different from wild type and non-Al treatment control at P<0.05. doi:10.1371/journal.pone.0028086.g007
transferred to nylon membranes. The entire coding regions of PLDγ1 and PLDγ2 5′-UTR, or full-length AtGST1 cDNA were used as hybridization probes. The DNA probes were labeled with [α-32P] dCTP by random priming. The hybridization, washing, and visualization were performed as described previously [35].

Microscopic Observations

Cell death caused by Al treatments was monitored by DAPI staining. After treatment of 7 day-old seedlings with 100 μM Al3+ in 1/8 MS medium for 5–8 h, roots from different plants were fixed with ethanol and stained with DAPI for 15 min. ROS generation in roots was observed by using 2,7-DCFDA as a detecting agent. Seedlings were treated as above for 5 h and then were put into a fresh PBS buffer; 5 μg/ml of 2,7-DCFDA were added to stain the ROS. Roots were imaged under a fluorescence microscope (Nikon Eclipse 800, Japan) with excitation at 488 nm and emission at 530 nm. Callose production in Al-treated roots was visualized by staining with aniline blue as described by [3]. The root region of 7 day-old seedlings was exposed to 1/8 MS medium containing 100 μM Al for 5 h, fixed with formaldehyde under vacuum, and then stained with 0.1% (w/v) aniline blue in 0.1 M K2HPO4 (pH 9.0). Callose was imaged with a fluorescence microscope (Nikon Eclipse 800, Japan) with excitation at 356 nm and emission at 515 nm.

Measurements of Al accumulation and organic acid secretion

Accumulation of aluminum in roots and secretion of organic acids into medium upon AlCl3 treatment were determined by growing seedlings on 1/2 MS medium plates oriented vertically. After 7 days, the plants were transferred to 1/8 MS liquid medium (pH 4.0) for adaptation for 2 h followed by 50 μM AlCl3 Al-treatment for 5 h. After treatment, the media were collected for analysis of glycerolipids was performed as previously described [4]. GL and PL were quantified using an Agilent 6890 GC coupled to a 5973 MSD. One μL was injected in a split/splitless injector at 280°C at a split ratio of 15:1. Separation was achieved on DB-5 MS column (J&W Scientific, 60 m, 0.25 mm i.d., and 0.25 μm film). Helium was the carrier gas at a constant flow of 1 mL/min. The GC temperature program was 80°C for 2 min then ramped to 315°C at 5°C/min was held at 315°C for 12 min. The transfer line to the mass spectrometer was set to 280°C and the MS source was set to 230°C. Mass spectra were scanned from m/z 50–500 at an acquisition rate of 2 spectra/s. Malic and citric acid were quantified using authentic standard that was methylated and analyzed using the same procedure.

Enzyme Activity and Lipid Peroxidation Assay

Whole roots of Al-treated plants (100 μM Al treatment for 5 h) were frozen in liquid nitrogen. Enzyme extraction, activity assay, and lipid peroxidation determination by measuring the level of thiobarbituric acid reactive substances (TBARS) were conducted as previously described [57]. APX activity was expressed as μmol ascorbate oxidized/mg protein per min; GR activity was expressed as nmol NADPH oxidized/mg protein min, and lipid peroxidation was expressed as nmol TBARS/g fresh weight (FW).

Lipid Extraction and ESI-MS/MS Analysis

Wild-type, PLDγ1 knockouts (pld1-1, pld2-1), and RNAi mutant plants were germinated and grown on 1/8 MS plates for 6–7 days in a growth chamber and then transferred to 1/8 MS medium (pH 4.0) containing 100 μM AlCl3 for 2 days. Roots and shoots (leaves and stems) from the seedlings were collected and immediately immersed in isopropanol (preheated to 75°C) to inactivate lipolytic enzymes. Lipid extraction and ESI-MS/MS analysis of glycerolipids was performed as previously described [39]. Five replicates of each treatment for each wild type or mutant were processed and analyzed.

Statistical Analysis

The t-test for replicates of lipid data was performed. A difference between two groups of data is considered significant when p<0.05 in the Student’s t-test.

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Author Contributions

Conceived and designed the experiments: XW. Performed the experiments: JZ, CW MB IB. Analyzed the data: JZ, CW MB IB. Contributed reagents/materials/analysis tools: RW LWS IB. Wrote the paper: JZ XW.

References

1. Ma JF, Ryan PR, Dellaiaze E (2001) Aluminum tolerance in plants and the complexing role of organic acids. Trends Plant Sci 6: 273–278.

2. Kochian LV, Heckenga OA, Paterson MA (2004) How do crop plants tolerate acid soils? Mechanisms of aluminum tolerance and phosphorus efficiency. Annu Rev Plant Biol 55: 459–493.

3. Larson PR, Degenhardt J, Tai CY, Stenzler LM, Howell SH, Kochian LV (1998) Aluminum-resistant Arabidopsis mutants that exhibit altered patterns of aluminum accumulation and organic acid release from roots. Plant Physiol 117: 9–18.

4. Zhang G, Slaski JJ, Arambaihl DJ, Taylor GJ (1996) Aluminum-induced alteration in lipid composition of microsomal membrane from an aluminum-
25. Li L, Fleming N (1999) Aluminum fluoride inhibits phospholipase D activation in Arabidopsis roots from ATR-activated cell-cycle arrest. Curr Biol 16: 1495–1500.

27. Quintal-Tun F, Munoz-Sanchez JA, Ramos-Diaz A, Escamilla-Becomo A, Gunse B, Poschenrieder C, Barcelo J (1997) Water transport properties of roots of a maize variety. Plant Physiol 116: 10–10.

28. Rounds M, Larsen PB (2006) Aluminum-dependent root-growth inhibition in Arabidopsis results from ATR-activated cell-cycle arrest. Curr Biol 16: 1495–1500.

29. Wang X (2000) Multiple forms of phospholipase D in plants: the gene family, catalytic and regulatory properties, and cellular functions. Prog Lipid Res 39: 566–573.

30. Wang X (2005) Regulatory functions of phospholipase D and phosphatidic acid metabolism from the phosphoinositide pathway in Arabidopsis. J Plant Physiol 160: 1297–1303.

31. Hong Y, Pan X, Wei L, Wang X (2008a) Phospholipase D enzyme is involved in hypersensitive responses in Arabidopsis. Plant Cell 20: 803–816.

32. Hong Y, Zheng S, Wang X (2008) Dual functions of phospholipase D in plant response to drought. Molecular Plant 1: 262–269.

33. Qin W, Pappan K, Wang X (1990) Molecular heterogeneity of phospholipase D (PLD) cloning of PLDδ and regulation of plant PLDζ, β and γ by polyphosphoinositides and calcium. J Biol Chem 272: 20267–20273.

34. Qin C, Li M, Qin W, Bahn SC, Wang C, et al. (2006) Expression and characterization of Arabidopsis phospholipase D7. Biochim Biophys Acta 1761: 1450–1458.

35. Shemesh MAR, Butler E, Huetta AR, Wilson CF, Anderson Q, et al. (2002) Expression sequenced tag-based gene expression analysis under aluminum stress in rice. Plant Physiol 130: 1749–1761.

36. Schreiner KA, Hodnett J, Taylor GJ (1994) Aluminum-induced deposition of (1,3)-glucans (calleose) in [Triticum aestivum L. Plant Soil 162: 273–280.

37. Jones DL, Blancfleur EB, Kochian LV, Gilroy S (2006) Spatial coordination of uptake, storage and transport of aluminum in maize. Plant Physiol 142: 750–761.

38. Novoa G, Foyer CH (1994) Regulation of carboxyl and glutathione: keeping active oxygen under control. Annu Rev Plant Biol 55: 49–279.

39. Fan L, Zheng S, Cui D, Wang X (1999) Subcellular distribution and tissue expression of phospholipase Dalpa, Dbeta, and Dgamma in Arabidopsis. Plant Physiol 119: 1371–1378.

40. Novotna Z, Linek J, Hyncik R, Martinez J, Potocky M, et al. (2003) Plant PIP-dependent phospholipase D activity is regulated by phosphorylation. FEBS Lett 530: 50–54.

41. Cruz-Ramirez A, Oropesa-Alurto A, Razo-Hernandez F, Ramirez-Chavez E, Herrera-Estrella L. (2006) Phospholipase D2γ plays an important role in extracellular galactolipid biosynthesis and phospholipase recycling in Arabidopsis roots. Proc Natl Acad Sci USA 103: 6763–6770.

42. Hernandez LE, Cooke DT (1999) Modifications of the root plasma membrane lipid composition of cadmium-tREATED Plant sativum. J Exp Bot 48: 1357–1361.

43. Quartacci MF, Cosi N, Navari-Izzo F (2001) Lipids and NADPH-dependent superoxide production in plasma membrane vesicles from roots of wheat grown under copper deficiency or excess. J Exp Bot 52: 77–84.

44. Quartacci MF, Pinzino C, Sgherri CLM, Dalla Vecchia F, Navari-Izzo F (2000) Growth in excess copper induces changes in the lipid composition and fluidity of PSII-enriched membranes in wheat. Physiol Plant 108: 87–93.

45. Orsini O, Bossanova N, Zunino M, Cherif A, Ghorbal MH (1997) Cadmium- and copper-induced changes in tomato membrane lipids. Photoscience 4: 1334–1345.

46. Peixoto PHP, Cambara J, Sant’Anna R, Mosquim PR, Moreira MA (1999) Aluminum effects on lipid metabolism in the rhizosphere. J Exp Bot 50: 137–143.

47. Orsini O, Bossanova N, Zunino M, Cherif A, Ghorbal MH (1997) Cadmium- and copper-induced changes in tomato membrane lipids. Photoscience 4: 1334–1345.

48. Orsini O, Bossanova N, Zunino M, Cherif A, Ghorbal MH (1997) Cadmium- and copper-induced changes in tomato membrane lipids. Photoscience 4: 1334–1345.

49. Orsini O, Bossanova N, Zunino M, Cherif A, Ghorbal MH (1997) Cadmium- and copper-induced changes in tomato membrane lipids. Photoscience 4: 1334–1345.

50. Orsini O, Bossanova N, Zunino M, Cherif A, Ghorbal MH (1997) Cadmium- and copper-induced changes in tomato membrane lipids. Photoscience 4: 1334–1345.

51. Orsini O, Bossanova N, Zunino M, Cherif A, Ghorbal MH (1997) Cadmium- and copper-induced changes in tomato membrane lipids. Photoscience 4: 1334–1345.

52. Orsini O, Bossanova N, Zunino M, Cherif A, Ghorbal MH (1997) Cadmium- and copper-induced changes in tomato membrane lipids. Photoscience 4: 1334–1345.

53. Orsini O, Bossanova N, Zunino M, Cherif A, Ghorbal MH (1997) Cadmium- and copper-induced changes in tomato membrane lipids. Photoscience 4: 1334–1345.

54. Orsini O, Bossanova N, Zunino M, Cherif A, Ghorbal MH (1997) Cadmium- and copper-induced changes in tomato membrane lipids. Photoscience 4: 1334–1345.

55. Orsini O, Bossanova N, Zunino M, Cherif A, Ghorbal MH (1997) Cadmium- and copper-induced changes in tomato membrane lipids. Photoscience 4: 1334–1345.

56. Orsini O, Bossanova N, Zunino M, Cherif A, Ghorbal MH (1997) Cadmium- and copper-induced changes in tomato membrane lipids. Photoscience 4: 1334–1345.

57. Orsini O, Bossanova N, Zunino M, Cherif A, Ghorbal MH (1997) Cadmium- and copper-induced changes in tomato membrane lipids. Photoscience 4: 1334–1345.

58. Orsini O, Bossanova N, Zunino M, Cherif A, Ghorbal MH (1997) Cadmium- and copper-induced changes in tomato membrane lipids. Photoscience 4: 1334–1345.

59. Orsini O, Bossanova N, Zunino M, Cherif A, Ghorbal MH (1997) Cadmium- and copper-induced changes in tomato membrane lipids. Photoscience 4: 1334–1345.

60. Orsini O, Bossanova N, Zunino M, Cherif A, Ghorbal MH (1997) Cadmium- and copper-induced changes in tomato membrane lipids. Photoscience 4: 1334–1345.