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# Reassessing the role of phospholipase D in the *Arabidopsis* wounding response

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## ABSTRACT

**Plants respond to wounding by means of a multitude of reactions, with the purpose of stifling herbivore assault. Phospholipase D (PLD) has previously been implicated in the wounding response. *Arabidopsis* (*Arabidopsis thaliana*) AtPLDα1 has been proposed to be activated in intact cells, and the phosphatidic acid (PA) it produces to serve as a precursor for jasmonic acid (JA) synthesis and to be required for wounding-induced gene expression. Independently, PLD activity has been reported to have a bearing on wounding-induced MAPK activation. However, which PLD isoforms are activated, where this activity takes place (in the wounded or non-wounded cells) and what exactly the consequences are is a question that has not been comprehensively addressed. Here, we show that PLD activity during the wounding response is restricted to the ruptured cells using $^{32}P_i$-labelled phospholipid analyses of *Arabidopsis pld* knock-out mutants and *PLD*-silenced tomato cell-suspension cultures. *pldα1* knock-out lines have reduced wounding-induced PA production, and the remainder is completely eliminated in a *pldα1/δ* double knock-out line. Surprisingly, wounding-induced protein kinase activation, *AtLOX2* gene expression and JA biosynthesis were not affected in these knock-out lines. Moreover, larvae of the Cabbage White butterfly (*Pieris rapae*) grew equally well on wild-type and the *pld* knock-out mutants.**

*Key-words*: jasmonic acid; phosphatidic acid; PLD.

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*Abbreviations*: JA, jasmonic acid; LPA, lysophophatidic acid; MBP, myelin basic protein; PA, phosphatidic acid; PBut, phosphatidylbutanol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIP, phosphatidylinositolmonophosphate; $PIP_2$, phosphatidylinositolbisphosphate; PLD, phospholipase D.

## INTRODUCTION

Insect herbivory is a consequential stress in higher plants that leads to a loss of nutrients and photosynthetic capacity and, as a result, reduced seed production. Plants respond to the wounding and counter-attack with direct and indirect defensive strategies (Wasternack *et al.* 2006). For example, proteins that interfere with the digestion of plant material in the insect gut are synthesized (Green & Ryan 1972; Orozco-Cárdenas, Narváez-Vásquez & Ryan 2001) and compounds that are toxic or repellant with respect to the herbivore also accumulate in local and systemic parts of the wounded plant (Baldwin 1998; Leitner, Boland & Mithöfer 2005). Additionally, wounded plants emit volatile compounds that attract natural enemies of herbivores (Sabelis, Janssen & Kant 2001; Ament *et al.* 2004). In such a way, plants can fend off an ongoing attack and prepare themselves for further assault.

Wounding-induced signalling molecules, such as JA and systemin, are produced upon wounding and elicit the above-mentioned responses throughout the plant. The plant's wounding response can be partitioned into three territories: (1) the ruptured cells; (2) the local-responding tissue; and (3) the systemic-responding tissue. The ruptured cells emit a non-cell-autonomous, primary signal that is perceived by intact cells in the local-responding tissue. Upon perception of the wounding signal, intact cells in the surrounding tissue respond with changes in gene expression, protein phosphorylation and metabolite production, leading to an induction of defensive strategies and amplification of the primary wounding signal by the production of

secondary signals. Systemic plant tissues perceive the wounding signals and react with a wounding response (Schilmiller & Howe 2005).

PA production by PLD reportedly occurs in all three territories (Ryu & Wang 1996; Lee *et al.* 1997; Lee, Hirt & Lee 2001) and has been proposed to play important roles in the wounding response (Wang *et al.* 2000; Lee *et al.* 2001). PLD catalyses the hydrolysis of structural phospholipids, such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE), producing PA and a free headgroup. In the *Arabidopsis* (*Arabidopsis thaliana*) genome, there are 12 PLD genes, whereas animals only contain two PLDs and yeast even only one. The plant PLD family can be divided into six classes, $\alpha$, $\beta$, $\gamma$, $\delta$, $\varepsilon$ and $\zeta$, based on the enzymes' sequence homology and biochemical properties (Wang 2005). Functions have been suggested for PLD in various processes, including membrane degradation, vesicular transport and intracellular signalling (Wang 2005; Bargmann & Munnik 2006).

The activity of different PLD classes can be separated *in vitro* by varying the buffer in which the assay is performed and the lipid environment in which the substrate is presented (Qin & Wang 2002). Four kinds of *in vitro* PLD activity have been distinguished in this way, depending on their pH, [$Ca^{2+}$], oleate and phosphatidylinositolbisphosphate ($PIP_2$) requirements. The $\alpha$-class PLDs require an acidic pH and millimolar calcium concentrations but do not require inclusion of $PIP_2$ in their lipid substrate preparation. In contrast, $\beta$-/$\gamma$-class PLDs require neutral pH, micromolar calcium concentrations and $PIP_2$. The $\delta$-class PLDs are active at high micromolar to low millimolar calcium concentrations and are stimulated by the inclusion of oleic acid (or TX-100) in the substrate preparation (Qin, Wang & Wang 2002). Lastly, the $\zeta$-class PLDs require a neutral pH and $PIP_2$ but do not require calcium (Qin & Wang 2002).

Although PLD activity has been implicated in the wounding response (Ryu & Wang 1996; Lee *et al.* 1997, 2001; Wang *et al.* 2000), it is not evident which isoforms are involved nor is it apparent where it takes place, that is, in ruptured or intact cells, locally or systemically. Earlier, Wang *et al.* (2000) demonstrated that *Arabidopsis* plants expressing an antisense *AtPLDα1* construct exhibited reduced PA production in wounded leaves, signifying that this isoform is responsible for a part of the PLD activity. The authors proposed that this activity takes place in the intact, responding cells, although this question was never experimentally addressed (Wang *et al.* 2000). Which isoform accounts for the observed residual PLD activity also remains unclear. Similarly, it remains to be shown which PLD isoform is responsible for the systemic PLD activity reported by Lee *et al.* (1997, 2001).

PLD has been proposed to play several roles in the wounding response. Antisense *AtPLDα1* plant lines have been reported to have reduced wounding-induced JA production and impeded wounding-induced gene expression (Wang *et al.* 2000). These authors put forward a model in which AtPLDα1-derived PA is a precursor for JA biosynthesis. In addition, Lee *et al.* (2001) used PLD activity inhibition in soybean (*Glycine max*) seedlings and a cell-suspension wounding model and suggested that PLD activity lies upstream of MAPK signalling.

The aim of this study is to scrutinize the location of wound-activated PLD activity and to assess its role in the downstream wounding response. To this end, two plant systems were employed: *Arabidopsis pld* knock-out mutants and *PLD*-silenced tomato (*Solanum lycopersicon*) cell-suspension cultures. These model systems were used to examine *in vivo* PLD activity, protein kinase activity, gene expression, JA biosynthesis and herbivore performance.

## METHODS

### Plant material

*Arabidopsis thaliana* var. Col-0 T-DNA insertion lines were obtained from the SALK Institute. *pldα1* (SALK_067533) and *pldδ* (SALK_023247) were crossed to obtain the double mutant. The following primers were used to verify genomic insertions:

SALK_067533F 5′-GACGATGAATACATTATCATTGG-3′
SALK_067533R 5′-GTCCAAAGGTACATAACAAC-3′
SALK_023247F 5′-TGTACTCGGTGCTTCGGGAAA-3′
SALK_023247R 5′-TCGAGAAACAATGGTGCGACA-3′
SALK_LeftBorderA 5′-TGGTTCACGTAGTGGGCCATCG-3′

SALK_LeftBorderA was used in combination with SALK_067533R and SALK_023247F. Col-5 (accession number N1644; Nottingham Arabidopsis Stock Centre, University of Nottingham, UK) and *coi1*-16 were obtained from Maarten Koornneef and John Turner, respectively. For routine plant growth, seeds were sown on soil and vernalized at 4 °C for 2 d. For analysis of systemic PA formation, seeds were sown on rockwool cubes. Ordinarily, plants were grown in a growth chamber under a 12 h light/12 h dark regime, with a 23 °C/18 °C cycle and 70% humidity. For the herbivore performance assay, plants were grown under a 9 h light/15 h dark regime. Mas7 (Peninsula Laboratories, Belmont, CA, USA) stock solution of 100 mM was made in water and stored in aliquots at –20 °C.

### Cell-suspension cultures

Suspension-cultured cells (*Solanum lycopersicon* Mill.; line Msk8; Felix *et al.* 1991) were grown at 24 °C in the dark shaking at 125 r.p.m. in Murashige and Skoog medium supplemented with 3% (w/v) sucrose, 5.4 $\mu$M 1-naphthaleneacetic acid, 1 $\mu$M 6-benzyladenine and vitamins (pH was adjusted to 5.7 with 1 M KOH) as described by Felix *et al.* (1991) and used 4–6 d after weekly subculturing.

For the *LePLDα1*-RNAi construct, an inverted repeat specific for *LePLDa1* was generated targeting the gene's 3′ UTR. PCR amplification of *LePLDa1* cDNA was performed with the oligonucleotides: 1_5′-CGGGATCCCCATCGATCAGTCAATTAAAGCATCTC-3′ (reverse) with

*Bam*HI and *Cla*I restriction sites, 2_5′-CCGGAATTCCC CCGACACCAAGG-3′ (forward) with an *Eco*RI restriction site and 3_5′-CCGGAATTCCATCCAGAAAGTG AGG-3′ (forward) with an *Eco*RI restriction site. PCR products resulting from primer combinations 1-2 and 1-3 were ligated in a 1-2/3-1 orientation into pGreen1K, which was modified to contain the 35S-Tnos cassette from pMON999. Cell-suspension culture transformation was achieved as described by Bargmann *et al.* (2006).

## *In vivo* phospholipid analysis

Suspension-cultured cells (100 $\mu$L aliquots in 2 mL Eppendorf tubes, Eppendorf, Germany) were labelled in growth medium supplemented with 10 $\mu$Ci $^{32}PO_4^{3-}$ (carrier free) for 3 h. Wounding was induced by freezing cells in liquid nitrogen and thawing. When indicated, incubations were performed in the presence of 0.5% (v/v) *n*-butanol. Treatments were stopped by adding 5% perchloric acid (final concentration), and lipids were extracted as described before (van der Luit *et al.* 2000). Leaf discs (5 mm Ø) were labelled by incubation with 10 $\mu$Ci carrier-free $^{32}PO_4^{3-}$ on 100 $\mu$L 10 mM MES buffer [2-(N-Morpholino)ethane sulfonic acid] pH 5.7 (KOH) in a 2 mL microcentrifuge tube (Frank *et al.* 2000). Two-week-old *Arabidopsis* seedlings, grown on rockwool cubes, were labelled by pipetting 100 $\mu$L water containing 100 $\mu$Ci carrier-free $^{32}PO_4^{3-}$ onto the rockwool and leaving them overnight under fluorescent light in a fume hood. Wounding was induced by freezing/thawing or a hemostat, as indicated. Treatments were stopped by incubation with 5% (v/v) perchloric acid. Plant material was transferred to a new tube containing 375 $\mu$L $CHCl_3$/MeOH/HCl [50:100:1 (v/v)] shaken vigorously for 10 min. A two-phase system was induced by the addition of 375 $\mu$L $CHCl_3$ and 200 $\mu$L 0.9% (w/v) NaCl. The remainder of the extraction was performed as described before (van der Luit *et al.* 2000). Lipids were separated on thin-layer chromatography (TLC) plates using the organic upper phase of an ethyl acetate mixture, ethyl acetate/*iso*-octane/formic acid/water [12:2:3:10 (v/v); Munnik *et al.* 1998], or using an alkaline solvent system, $CHCl_3$/MeOH/25% $NH_4OH$/$H_2O$ [90:70:4:16 (v/v); Munnik, Irvine & Musgrave 1994], when indicated. Radio-labelled phospholipids were quantified by phosphoimaging (Molecular Dynamics, Sunnyvale, CA, USA).

## RNA and protein blot analysis

RNA blot analysis was performed as described by Bargmann *et al.* (2006). Protein extraction buffer [9.5 M urea, 0.1 M Tris-HCl pH 6.8, 2% (w/v) sodium dodecyl sulphate (SDS) and 2% (v/v) $\beta$-mecraptoethanol] was added to an equal volume of ground leaf tissue, vortexed and centrifuged in an Eppendorf centrifuge for 10 min at 1000 *g*. Samples were separated by 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) gel electrophoresis, blotted on nitrocellulose and incubated overnight with polyclonal anti-LePLD$\alpha$1 antibody (rabbit; Eurogentech, Liege, Belgium). Antibodies were generated using the final 12 amino acids of LePLD$\alpha$1: N-TKSDYLPPNLTT-C. Peroxidase activity of horseradish peroxidase-coupled goat anti-rabbit immunoglobulin G antibody (Pierce, Rockford, IL, USA) was detected by enhanced chemiluminescence (Amersham, Buckinghamshire, UK). A duplicate gel was stained with Coomassie Brilliant Blue as a loading control.

## *In vitro* PLD$\alpha$ activity assay

PLD$\alpha$ activity was assayed by using a combined protocol of Pappan, Zheng & Wang (1997) and Ella *et al.* (1994). Briefly, 10 $\mu$g of total protein extract was incubated with 250 $\mu$M BODIPY-PC as a substrate in a buffer containing 50 mM MES pH 6.5, 80 mM NaCl, 0.5 mM SDS and 10 mM $CaCl_2$ and 1% (v/v) *n*-butanol, for 1 h at 30 °C. Cabbage PLD (1 U; type V; Sigma-Aldrich, Steinheim, Germany) was used as a positive control. Lipids were extracted as described earlier and separated by ethyl acetate. TLC BODIPY-lipids were visualized by fluoroimaging.

## In-gel kinase assay

Proteins were extracted from ground plant and cell-suspension material using 1 vol of extraction buffer [50 mM Tris-HCl pH 7.5, 5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM ethylene glycol tetraacetic acid (EGTA), 2 mM dithiothreitol (DTT), 25 mM soduimfluoride (NaF), 1 mM $Na_3VO_4$, 50 mM $\beta$-glycerophosphate, 1× complete protease inhibitor cocktail] and a 15 min 10 000 *g* centrifugation. Samples were assayed for protein content and 10 $\mu$g protein was loaded onto a 10% SDS–PAGE gel containing 4 $\mu$g mL$^{-1}$ myelin basic protein (MBP) (Upstate, Lake Placid, NY, USA). The gel was washed three times for 30 min with wash buffer [25 mM Tris-HCl pH 7.5, 500 $\mu$M DTT, 100 $\mu$M $Na_3VO_4$, 5 mM NaF, 500 $\mu$g mL$^{-1}$ bovine serum albumin, 0.1% (v/v) Triton X-100] and renatured overnight in renaturation buffer (25 mM Tris-HCl pH 7.5, 1 mM DTT, 100 $\mu$M $Na_3VO_4$, 5 mM NaF). The gel was washed three times for 30 min in reaction buffer (25 mM Tris-HCl pH 7.5, 1 mM DTT, 100 $\mu$M $Na_3VO_4$) and incubated in reaction buffer supplemented with 25 $\mu$M cold dATP and 50 $\mu$Ci $^{32}$P-labelled $\gamma$-ATP for 1 h. The reaction was stopped, and the gel was washed six times for 30 min with stop buffer [1% (w/v) $Na_2H_2P_2O_7$, 5% (v/v) trichloric acid]. The gel was dried, and the signal was visualized by phosphoimaging.

## RT-PCR analysis

cDNA was synthesized as described in Ament *et al.* (2004) and used as a template for amplification of *AtPLD$\delta$* (with primers: AtPLD$\delta$RT-F 5′-CGAGACCTTCCCAGATGT TG-3′ and $dT_{18}$) and *AtTUA4* (with primers: AtTUA4RT-F 5′-CCAGCCACCAACAGTTGTTC-3′ and AtTUA4RT-Rev 5′-CACAAGACGAGATTATAGAGA-3′). PCR products were separated by gel electrophoresis, blotted onto nitrocellulose and hybridized with $^{32}$P-labelled

*AtPLDδ* and *AtTUA4* probes. The signal was visualized by phosphoimaging.

### Oxylipin analysis

Oxylipins were extracted, derivatized and analysed as previously described by Stumpe *et al.* (2005). Pentafluorobenzyl esters were analysed by gas chromatography/mass spectrometry using the following ions and retention times for quantification: *m*/*z* 215 ($D_6$-JA; $R_f$ = 14.11, 14.46 min), 209 (JA; $R_f$ = 14.15, 14.51 min), 237 (OPC-4; $R_f$ = 16.76, 16.98 min), 265 (OPC-6; $R_f$ = 18.84, 19.08 min), 293 (OPC-8; $R_f$ = 20.72, 20.95 min), 296 ($D_5$-oPDA; $R_f$ = 20.8, 21.18, 21.52 min), 291 (oPDA; $R_f$ = 20.84, 21.22, 21.56 min) and 263 (dinor-oPDA; $R_f$ = 18.94, 19.39, 19.75 min).

### Herbivore performance assay

The assay was performed as described earlier (de Vos *et al.* 2006). Briefly, 6-week-old *Arabidopsis* plants were transferred to modified magenta boxes. One *P. rapae* caterpillar in larval stage L2 was placed on each plant and allowed to feed for 96 h. Caterpillar weight was determined at $t = 0$ and at $t = 96$ h, and the relative weight increase of multiple larvae during this period was averaged.

## RESULTS

### AtPLDα1 is activated after wounding

As shown in Fig. 1a, mechanical wounding of leaf discs induced a rapid and transient increase of PA. Interestingly, wounded leaf discs from *pldα1* knock-out lines produced significantly less PA (Fig. 1a; two-tailed paired *t*-test, $P < 0.05$ at 10 and 20 min). The kinetics of the transient PA response in wild-type and *pldα1* knock-out lines were similar. These results correlate well with the earlier study of antisense *AtPLDα1* plants (Wang *et al.* 2000), demonstrating that AtPLDα1 is activated upon wounding and that there is likely another PLD activated under these circumstances. Our data also shows that an overnight incubation of the leaf discs left them responsive despite earlier wounding by excision from the plant.

### AtPLDα1 is activated in ruptured cells

It is unclear whether the wounding-induced *AtPLDα1* activity originates from the ruptured cells or the intact cells. The correlation between the *in vitro* α-class PLD enzymatic requirements and the conditions found in the plant apoplast and vacuole, namely an acidic pH (pH ~6.3; Gao *et al.* 2004) and millimolar calcium concentrations (>$10^{-3}$ M; Björkman

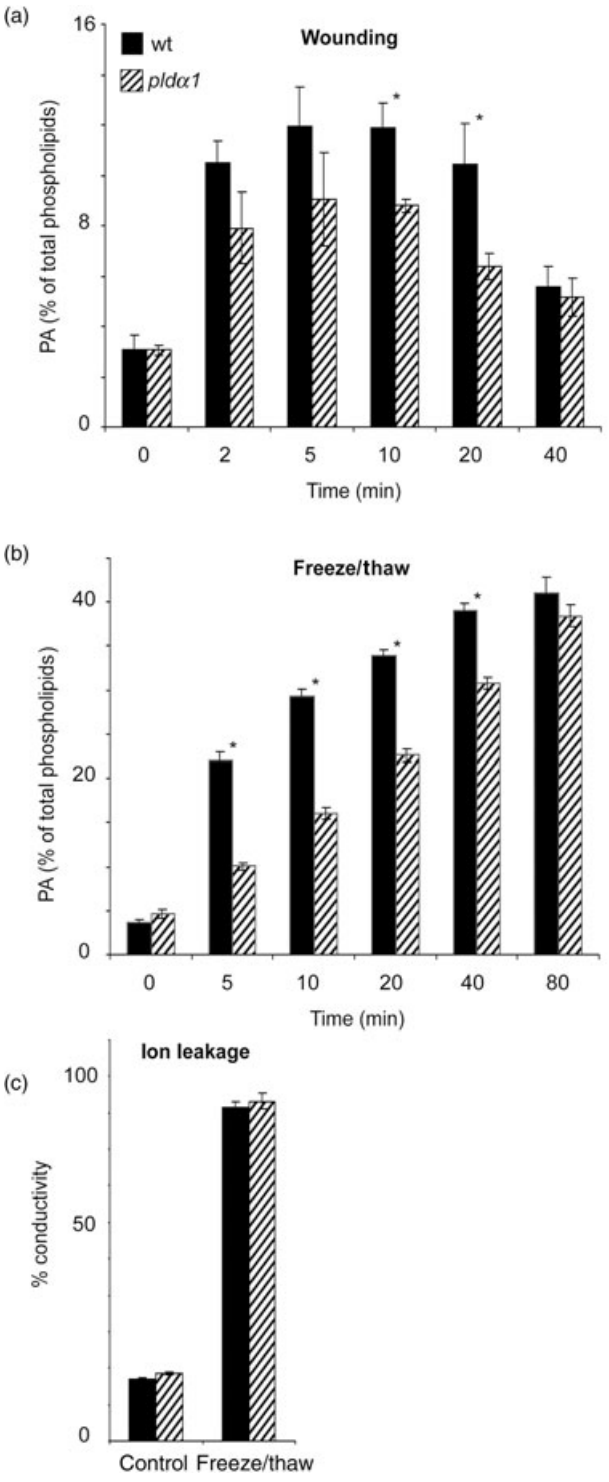


**Figure 1.** AtPLDα1 activity after wounding and loss of cell membrane integrity. Leaf discs from wild-type (wt; Col-0) and *pldα1* knock-out (SALK_067533) lines were labelled overnight, floating on buffer containing $^{32}P_i$. Discs were mechanically wounded with a hemostat (a) or snap frozen and thawed (b). Lipids were extracted at the indicated time points, separated by thin-layer chromatography (TLC) and analysed by phosphoimaging. PA was quantified as percentage of total radio-labelled lipids and is presented in a histogram ± SD ($n = 3$). (c) Ion leakage before and after snap freezing and thawing was quantified as conductivity of the labelling buffer relative to boiled samples and is presented in a histogram ± SD ($n = 3$). Asterisks indicate a significant difference between mutant and wt (two-tailed paired *t*-test, $P < 0.05$).

& Cleland 1991; Cabañero *et al.* 2006) indicate that AtPLD$\alpha$1 could become active upon disruption of cellular compartmentalization. To address this question, analyses of solely dead or living cells would be required.

The response in dead cells can be assessed by rupturing every cell in the leaf disc. This can be achieved by snap freezing the leaf discs in liquid nitrogen and subsequent thawing; the formation of ice crystals causes mechanical damage, disrupting cellular compartmentalization. Analysis of leaf discs that had been snap frozen and thawed revealed dramatically increased PA levels (Fig. 1b). The increase in PA in the *pld$\alpha$1* mutant was consistently lower than in wild type (Fig. 1b). Analysis of electrolyte leakage showed that cells had been ruptured equally in both the wild-type and mutant leaf discs (Fig. 1c). These data indicate that AtPLD$\alpha$1 becomes active upon loss of cell membrane integrity and that at least part of the increase in PA measured in wounded leaf discs is caused by AtPLD$\alpha$1 activity in the ruptured cells.

Having established that AtPLD$\alpha$1 is active in ruptured cells, our next efforts were directed towards assessing PLD activity in the remaining territories in the wounding response, namely, the local and systemic tissues. To assess AtPLD$\alpha$1 activity in the intact tissue of a wounded leaf disc, half of a leaf disc was wounded and the wounded and uninjured halves were dissected and analysed separately.

As shown in Fig. 2, PA levels were not increased in unwounded halves of wounded leaf discs, whereas wounded halves showed an increase that was the same as in non-dissected wounded leaf discs. Treatment in the presence of *n*-butanol, allowing exclusive visualization of PLD activity via PLD-catalysed transphosphatidylation (Munnik *et al.* 1995), showed a PBut accumulation that mirrored that of PA (Fig. 2b). These results suggest that the increase in PA in wounded *Arabidopsis* leaf discs is not produced in intact tissue but only in the ruptured cells.

## PLD is activated locally

PLD has also been implicated in the systemic wounding response. Lee *et al.* (1997, 2001) reported increased systemic PA levels in seedlings within minutes after wounding. Although Wang *et al.* (2000) investigated local and systemic JA levels and gene expression in wounded *Arabidopsis* plants, their report did not present data concerning systemic PA levels. This hiatus prompted us to investigate whether PLD activity increases systemically in *Arabidopsis* and, if so, whether the AtPLD$\alpha$1 isoform is involved. When the first true leaf of seedlings was mechanically wounded and PA levels were followed for 20 min in both the wounded and in the second, systemic, true leaf, PA production in the wounded leaf was rapid and substantial (Fig. 2c). In contrast, no statistically significant increase in PA was detected in the systemic leaf; instead, levels remained basal throughout the 20 min following treatment (two-tailed paired *t*-test, $P < 0.05$). These results indicate that there is no systemic PA response in wounded *Arabidopsis* plants within the first 20 min.

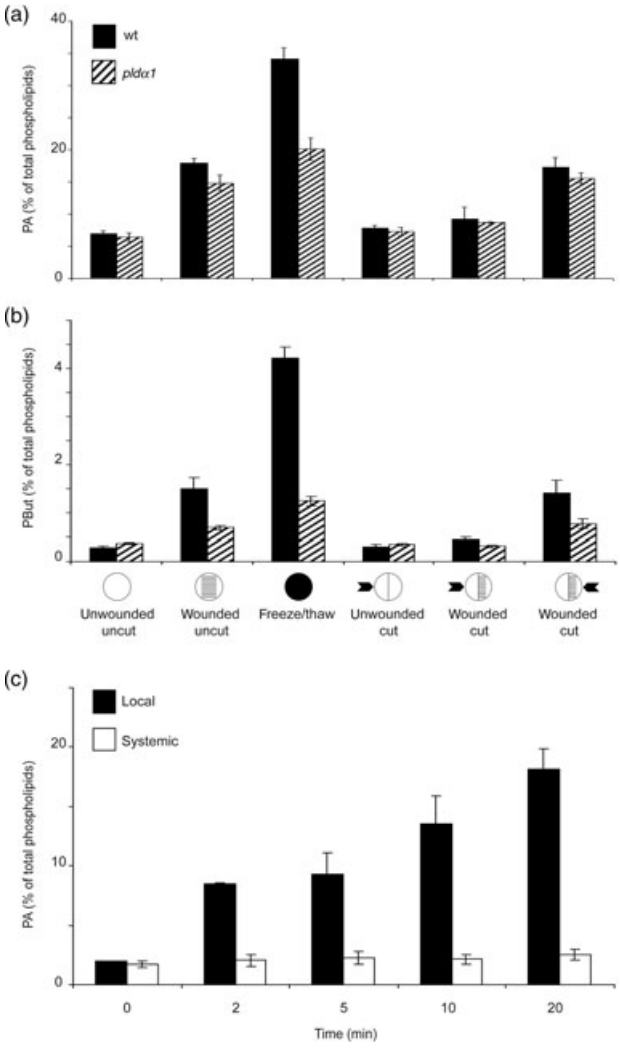


**Figure 2.** Location of wounding-induced PLD activity in *Arabidopsis* leaf discs and plants. Leaf discs from wild-type (wt) and *pld$\alpha$1* knock-out lines were labelled overnight, floating on buffer containing $^{32}P_i$. Leaf discs were left untreated, wounded with a hemostat (25 or 50% of the leaf disc area) or snap frozen and thawed in the presence of 0.5% *n*-butanol. Lipolytic activity was stopped after 15 min by the addition of perchloric acid. Control and 25% wounded leaf discs were dissected in two halves (the analysed half is indicated with an arrow ➡). Lipids were extracted, separated by thin-layer chromatography (TLC) and analysed by phosphoimaging. PA (a) and PBut (b) were quantified as percentage of total radio-labelled lipids and are presented in a histogram ± SD ($n = 3$). (c) The first leaf of 10-day-old $^{32}P_i$-labelled *Arabidopsis* Col-0 plants was wounded using tweezers. The wounded leaf (local) and second (systemic) leaf were harvested at the indicated time points. Lipids were extracted at the indicated time points, separated by TLC and analysed by phosphoimaging. PA was quantified as percentage of total radio-labelled lipids and is presented in a histogram ± SD ($n = 3$).

## PLD$\alpha$1 activity in a cell-suspension wounding model

PLD and PA involvement in the wounding response has been previously studied utilizing a cell-suspension

wounding model (Lee *et al.* 2001). The authors showed that addition of ruptured cells to a soybean cell-suspension activated a wounding-induced MAPK (Lee *et al.* 2001). Moreover, they showed that the addition of PA to the cell-suspension culture could induce MAPK activation. These results were interpreted to suggest that PLD activity in the intact, responding cells was upstream of the observed MAPK activation. However, neither PLD activity nor PA levels were measured in this report. Nonetheless, a cell-suspension wounding model would be well suited for differentially labelling and analysing the PA levels in ruptured and intact cells independently.

To investigate the involvement of tomato PLD$\alpha$1 (LePLD$\alpha$1) in the wounding response, an RNAi construct targeting the gene's 3′ UTR was used to knock down *LePLDα1* in a tomato cell-suspension culture (Msk8). Five independently transformed cell-suspension culture lines were obtained, as well as an empty-vector control line. RNA blot analysis showed that three of the five lines carrying the RNAi construct displayed a negligible mRNA transcript level (Fig. 3a). This finding was confirmed by protein blot analysis (Fig. 3b). An assay for *in vitro* activity demonstrated that the silenced cell-suspension cultures displayed a greatly reduced PLDα activity (Fig. 3c). Together, these results show that we were able to successfully knock down *LePLDα1* in tomato cell-suspension cultures.

Increased PLD activity upon disruption of compartmentalization has been noted before (Roughan, Slack & Holland 1978; Wang 2005), yet it is unknown which PLDs are involved. As mentioned earlier, it seems likely that the $\alpha$-class PLDs becomes active when apoplastic conditions are encountered. In general, cell-suspension cultures are grown in media with conditions that mimic the apoplast. In this case, cells were cultured in Murashige and Skoog medium with a pH of 5.7 and a final $Ca^{2+}$ concentration of 2 mM (see 'Methods'). In order to achieve a loss of cell membrane integrity, cells were snap frozen and thawed. Vitality staining with fluorescein diacetate revealed that this treatment led to a 100% cell death (data not shown). When cells were treated in this way, a remarkable PA production could be observed (Fig. 3d). Concomitantly, the levels of the structural phospholipids PC, PE and phosphatidylglycerol (PG) decreased dramatically, whereas levels of phosphatidylinositol (PI) remained relatively stable. An increase in lysophophatidic acid (LPA) could be seen following the PA increase (Fig. 3d). These data suggest that the structural phospholipids PC, PE and PG (and not PI) are rapidly and massively converted to PA by PLD upon loss of cellular membrane integrity. Phosphatidylinositolmonophosphate (PIP) and $PIP_2$ levels also decreased, possibly because of the action of phospholipase C, which, in combination with diacylglycerol kinase, may also contribute to the increased PA levels (Mosblech *et al.* 2008).

The PA and LPA increases in *LePLDα1*-silenced cell-suspension cultures were significantly reduced when compared with those in control lines (Fig. 3d). Concurrently, the decreases in PC, PE and PG were also slower. These results indicate that the PA production observed upon loss of cell membrane integrity is in part derived from LePLD$\alpha$1 activity, and that this isoform uses PC, PE and PG as substrates, but not PI.

In order to investigate whether it was indeed the extracellular pH and $Ca^{2+}$ concentrations that induced the PLD activity after loss of cell membrane integrity, cells were lysed in the presence of 50 mM Tris-HCl (pH 7.5) and/or 10 mM EGTA to buffer protons and $Ca^{2+}$ ions, respectively. Buffering the growth medium and chelating the free $Ca^{2+}$ ions both decreased the lysis-induced PA production individually, and almost completely blocked this activity when applied in combination (Fig. 4). These results show that PLD activity upon loss of cell membrane integrity in a tomato cell-suspension culture is dependent on the extracellular low pH and high [$Ca^{2+}$].

A wounding model system analogous to the one employed by Lee *et al.* (2001) was set up to examine PLD activity in the responding cells. Cells that had been ruptured (snap frozen and thawed 5 min) were added to a cell-suspension culture in a 1:9 ratio, and the induced protein kinase activity towards MBP was analysed by in-gel kinase assays (Fig. 5a). A band of approximately 48 kDa was detected, which displayed a fast and transient activation in response to the treatment, with a maximum activity at 5 min that declined again after 20 min. Analysis solely of the ruptured cells showed that this MBP kinase did not originate from these cells (Fig. 5a). This protein kinase activity is similar to the 48 kDa MBP kinase found by Stratmann & Ryan (1997) in wounded tomato leaves. Once more, a dramatic increase in PLD activity could be observed in the snap frozen and thawed cells (Fig. 5b), and an *LePLDα1*-silenced line exhibited reduced PLD activity. Interestingly, no increase in PA levels could be detected when intact cells were treated with unlabelled ruptured cells (Fig. 5c). In contrast, cells treated with mas7, a strong elicitor of PLD responses (Munnik *et al.* 1996, 1998; Van Himbergen *et al.* 1999; van der Luit *et al.* 2000), displayed a clear PA response within 20 min (Fig. 5c). These results further indicate that wounding-induced PLD activation is restricted to the ruptured cells and does not occur in the intact, responding cells.

## The *Arabidopsis pldα1/δ* double mutant and the wounding response

Although the *Arabidopsis pldα1* knock-out line exhibited a reduced PA response to wounding and loss of membrane integrity, there was still a considerable production of PA in this line (Figs 1a–d & 2). This indicates that there is likely another PLD isoform active in the ruptured cells. AtPLD$\delta$ seemed a good candidate because, like AtPLD$\alpha$1, it is active in relatively high $Ca^{2+}$ concentrations (Qin *et al.* 2002) and *AtPLDδ* is relatively highly expressed (Li *et al.* 2006). *pldα1/δ* double knock-out mutants were generated and verified by genomic PCR, protein blot analysis and RT-PCR (Fig. 6). No obvious growth or developmental phenotype was observed under standard greenhouse and growth chamber conditions in either single or double mutants.

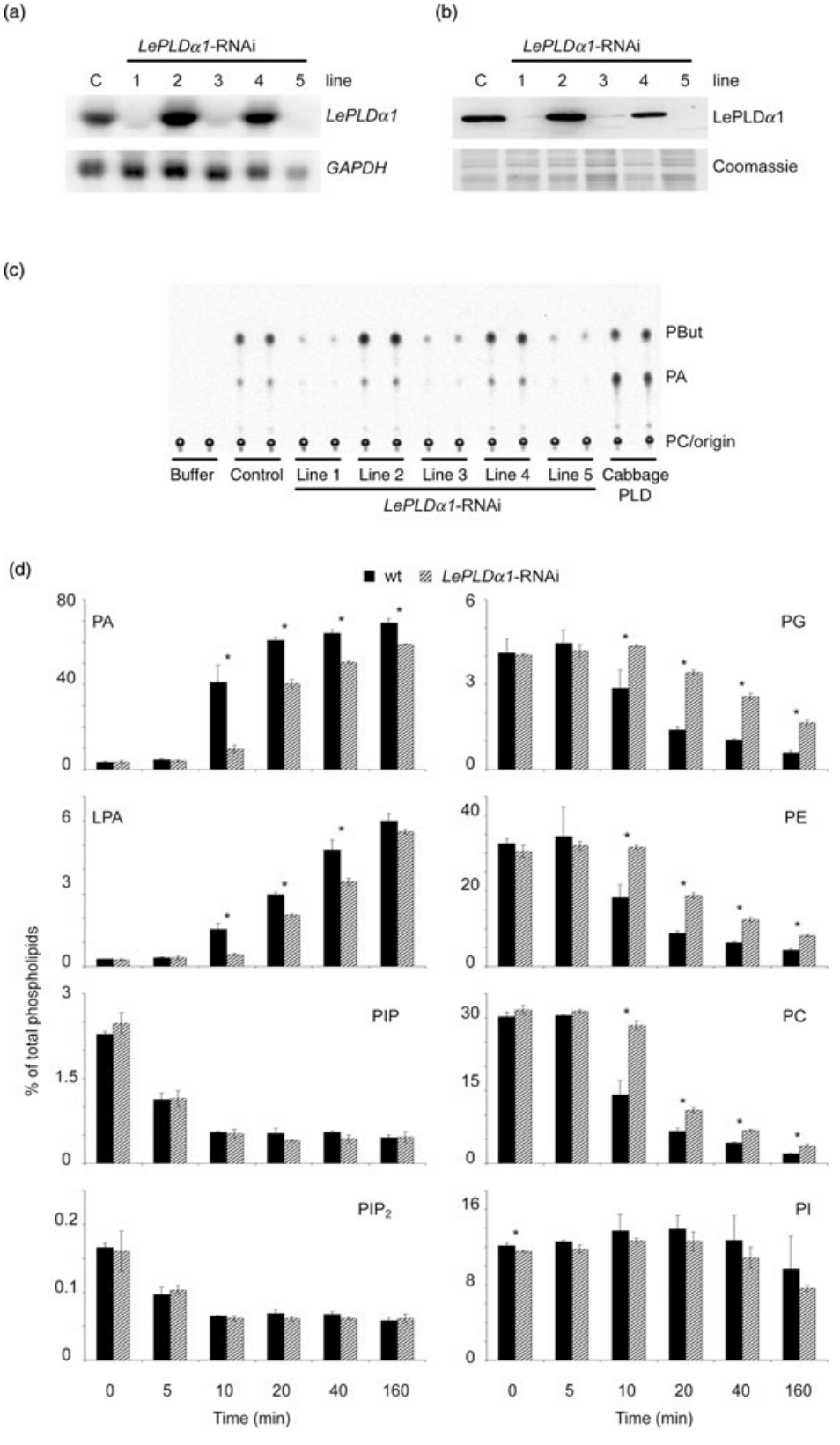


**Figure 3.** Analysis of *LePLDα1*-silenced tomato cell-suspension cultures. (a) RNA was extracted from five independently transformed Msk8 cultures carrying an *LePLDα1*-silencing construct and one carrying an empty vector as control (C). RNA was separated by gel electrophoresis, blotted and hybridized with $^{32}$P-labelled *LePLDα1* and *GAPDH* (loading control) probes. (b) Proteins were extracted from the Msk8 lines, separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and blotted or stained with Coomasssie Brilliant Blue as a loading control. LePLDα1 was detected using a polyclonal antibody directed towards the final 12 amino acids of LePLDα1. (c) Protein extracts were assayed for *in vitro* α-class PLD activity alongside the extraction buffer alone and commercially available cabbage PLD. The transphosphatidylation of BODIPY-PC to BODIPY-PA and BODIPY-PBut was visualized by the separation of lipids on a thin-layer chromatography (TLC) plate ($n = 2$). (d) $^{32}P_i$-labelled control or *LePLDα1*-silenced (line 1) cell-suspension cultures were left untreated or snap frozen and thawed for 5–160 min. Lipids were extracted, separated by TLC and analysed by phosphoimaging. Phospholipids were quantified as percentage of total radio-labelled lipids and are presented in a histogram ± SD ($n = 3$). Asterisks indicate a significant difference between mutant and wild type (wt) (two-tailed paired *t*-test, $P < 0.05$).

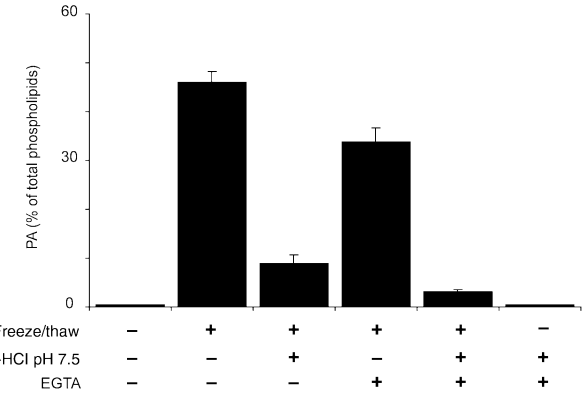


**Figure 4.** pH and calcium dependency of PLD activity upon loss of cell membrane integrity. $^{32}P_i$-labelled cell suspensions were left untreated or snap frozen and thawed for 10 min in the absence or presence of 50 mM Tris-HCl pH 7.5 and 10 mM EGTA. Lipids were then extracted, separated by thin-layer chromatography (TLC) and analysed by phosphoimaging. PA was quantified as percentage of total radio-labelled lipids and is presented in a histogram ± SD ($n = 3$).

Leaf discs from wild-type and *pld* knock-out lines were wounded or snap frozen and thawed in the presence of *n*-butanol in order to assess the effect of individual and combined *pld* knock-outs on the induction of PA and PBut production. In agreement with earlier results (Figs 1 and 2), the PA and PBut production in the *pldα1* mutant line was consistently lower than in the wild-type line (Fig. 7). In contrast, PA and PBut increases in the *pldδ* knock-out line were closer to wild-type levels (Fig. 7). However, in the double mutant, no increase of wounding- and cell lysis-induced PA and PBut was observed (Fig. 7). When followed for longer periods of time, up to 80 min, the double mutant still did not display a notable increase in PA production when snap frozen and thawed (Supporting Information Fig. S1). These results indicate that AtPLDδ is responsible for the residual PLD activity seen upon wounding and loss of membrane integrity in the *pldα1* knock-out line and suggest that AtPLDα1 can compensate for the loss of AtPLDδ in the *pldδ* mutant. Lastly, these results indicate that these two PLD isoforms together account for all the discernible activity seen in response to these treatments.

Rapid and transient MAPK activation in response to wounding has also been found in *Arabidopsis* (Ichimura *et al.* 2000). AtMPK4 and AtMPK6 are 47 and 44 kDa wounding-activated MBP kinases, respectively. MBP kinases of corresponding sizes were activated in *Arabidopsis* leaves in response to mechanical wounding (Fig. 8a). Surprisingly, the MBP kinase activation seen in the *pldα1/δ* double mutant was identical to the activation seen in wounded wild-type leaves (Fig. 8a). In order to examine conditions comparable with the assays for wounding-induced PLD activity (Figs 1, 2 & 7), protein extracts from wounded leaf discs were analysed for MBP kinase activity. Again, the same activation as in whole leaves could be observed in both wild-type and mutant leaf discs, with the

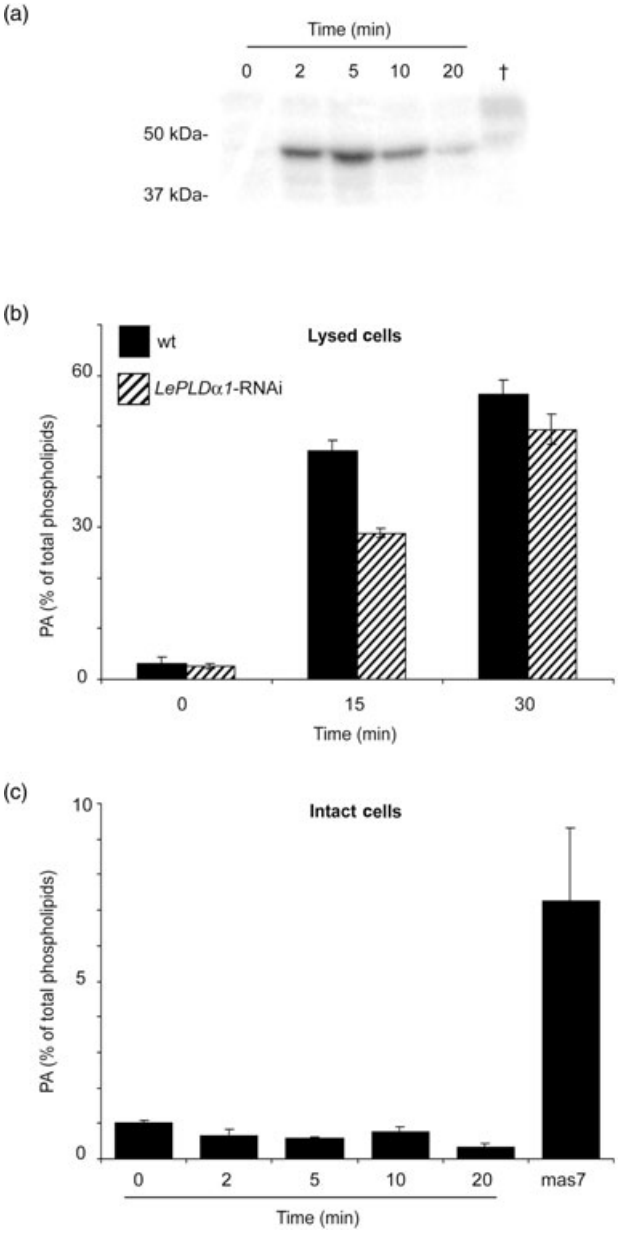


**Figure 5.** Myelin basic protein (MBP) kinase and PLD activity in a cell-suspension wounding model. (a) Msk8 cells were incubated with 10% (v/v) ruptured (snap frozen and thawed for 5 min) cells for up to 20 min. Proteins were extracted from samples taken at the indicated time points and from ruptured cells incubated on their own for 10 min (†). Samples were assayed for protein kinase activity towards MBP by in-gel kinase analysis. (b) $^{32}P_i$-labelled empty vector control or *LePLDα1*-silenced cell suspensions were left untreated or snap frozen and thawed for 15 or 30 min. (c) $^{32}P_i$-labelled cell suspensions were left untreated, incubated with 10% (v/v) unlabelled, ruptured cells for up to 20 min or treated with 5 μM mas7 for 20 min. Lipids were extracted, separated by thin-layer chromatography (TLC) and analysed by phosphoimaging. PA was quantified as percentage of total radio-labelled lipids and is presented in a histogram ± SD ($n = 3$). wt, wild type.

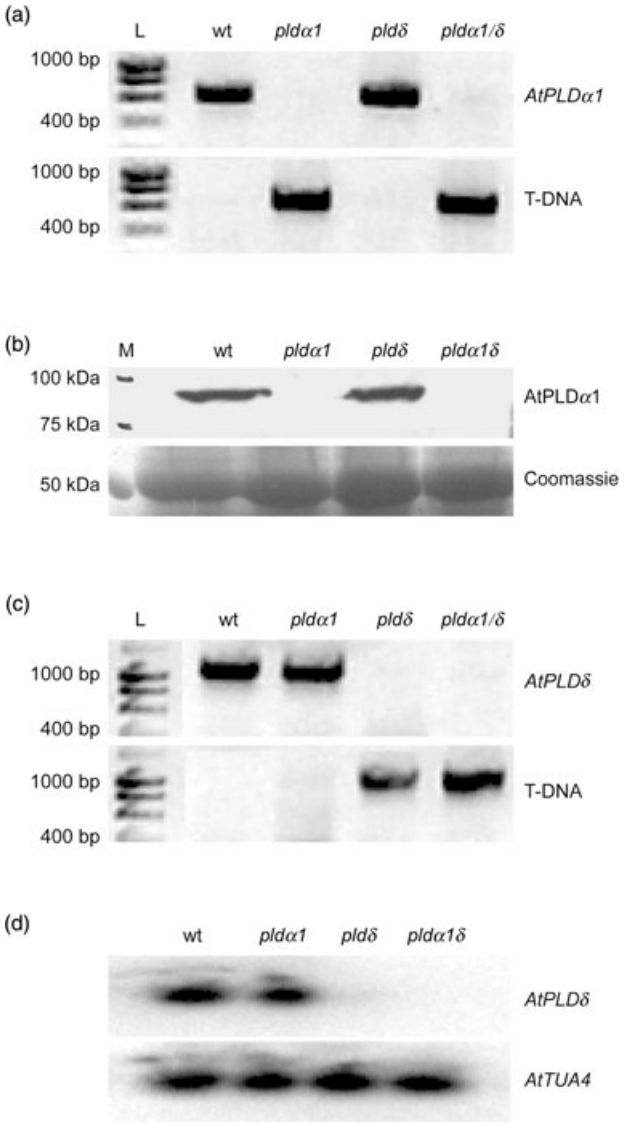


**Figure 6.** Verification of *Arabidopsis pldα1* and *pldδ* knock-outs. Genomic DNA was extracted from wild type (wt; Col-0), and T-DNA insertion lines and was used to verify the presence of the wt *AtPLDα1* gene and SALK_067355 T-DNA insertion (a) and the *AtPLDδ* gene and SALK_023247 T-DNA insertion (c) by PCR. Products were separated by gel electrophoresis alongside a DNA size ladder (L). Knock-out of *AtPLDα1* was confirmed by protein blot analysis of leaf protein extracts; M, marker (b), and knock-out of *AtPLDδ* was confirmed by RT-PCR with *AtTUA4* as a loading control (d).

exception that in this case the wounding-induced activity declined again within the assay period (Fig. 8a). These results show that, although the wounding-induced PA increase is completely abolished in this mutant, the wounding-induced MAPK activation is not affected in the *pldα1/δ* double mutant.

*AtLOX2*, encoding a lipoxygenase involved in JA synthesis, is transcriptionally up-regulated in response to wounding in *Arabidopsis* (Bell & Mullet 1993). In an *Arabidopsis* line expressing the antisense *AtPLDα1*, the wounding-induced expression of *AtLOX2* was reported to be adversely affected (Wang *et al.* 2000). We therefore decided to follow the wounding-induced *AtLOX2* expression in the *pld* knock-out lines (Fig. 8b). In contrast to expectations, there was no evident difference in the induction of *AtLOX2* expression between wild-type and mutant lines. This finding shows that wounding-induced *AtLOX2* expression is not affected in *pldα1* nor in the *pldδ* knock-out mutants or *pldα1/δ* double knock-out mutant.

Similarly, wounding-induced JA synthesis has been reported to be negatively affected in antisense *AtPLDα1 Arabidopsis* plants (Wang *et al.* 2000). However, when we measured JA levels and those of its precursors oxophytodienoic acid (oPDA), dinor-oPDA, and the 3-oxo-2-(pent-$2_0$-enyl)-cyclopentane-1-octanoic acids OPC-8, OPC-6 and OPC-4 (Afitlhile *et al.* 2005) in the wounded *pld* mutants, no significant difference could be detected between wild type and the lines missing either one or both AtPLDα1 and AtPLDδ (Fig. 8c; two-tailed paired *t*-test, $P < 0.05$, data not

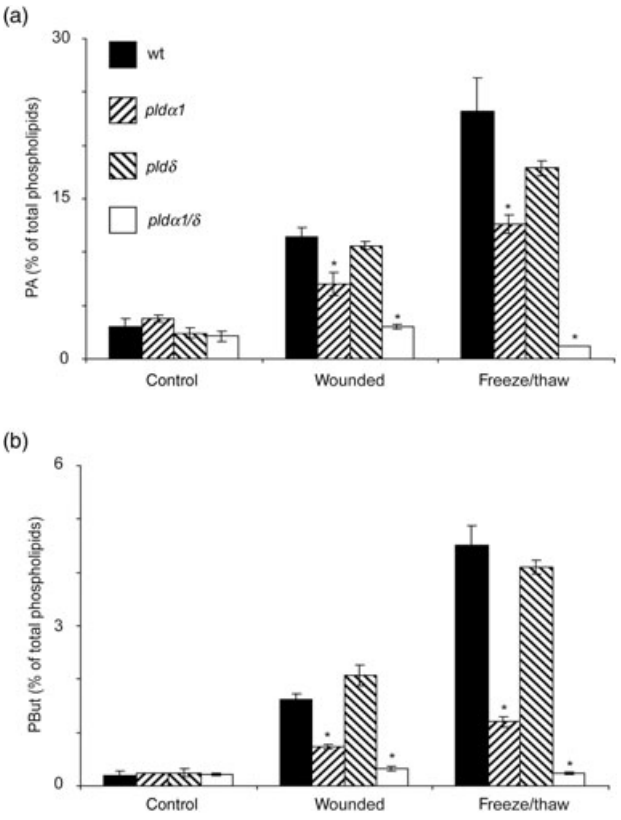


**Figure 7.** PLD activity after wounding and loss of cell membrane integrity in *Arabidopsis pld* mutants. Leaf discs from wild-type (wt), *pldα1*, *pldδ* and *pldα1/δ* knock-out lines were labelled overnight with $^{32}P_i$, left untreated, wounded or snap frozen and thawed for 15 min in the presence 0.5% *n*-butanol. Lipids were extracted, separated by thin-layer chromatography (TLC) and analysed by phosphoimaging. PA (a) and PBut (b) were quantified as percentage of total radio-labelled lipids and are presented in a histogram ± SD ($n = 3$). Asterisks indicate a significant difference between mutant and wild type (two-tailed paired *t*-test, $P < 0.05$).

shown). These results show that even though the wounding-induced PLD activity is completely lacking in the *pldα1/δ* double mutant, JA biosynthesis was not disturbed.

Plants respond to wounding with the production of proteins and chemicals that are toxic to potential future attackers or that affect herbivore appetite or digestion (Schilmiller & Howe 2005). Consequently, mutants with impaired basal defenses or an impaired wounding response are more nutritious to herbivores (Li *et al.* 2003). We investigated whether herbivore performance was affected on the *Arabidopsis* single and double *pld* knock-out lines. The weight gain of Cabbage White butterfly larvae (*Pieris rapae*) while feeding on wild-type and mutant *Arabidopsis* plants over a period of 4 d was measured and averaged (Fig. 8d). A glabrous *Arabidopsis* variety (Col-5) and the JA-insensitive *coi1*-16 mutant (Ellis & Turner 2002) were assayed as positive controls along with the wild type (Col-0) and the single and double *pld* knock-out mutants. Glabrous and JA-insensitive lines have previously been shown to have reduced resistance to *P. rapae* herbivory (Reymond *et al.* 2004). Whereas the caterpillar weight gain was significantly greater on the Col-5 and *coi1*-16 lines as compared with the performance on the wild-type plants, the weight gain achieved on *pldα1*, *pldδ* or *pldα1/δ* double knock-outs did not differ from that on wild type (Fig. 8d). These data indicate that the wounding response in the *pld* mutants is not affected in any such way that it influences herbivore performance.

## DISCUSSION

### Where is PLD activity located upon wounding?

The fact that massive PLD activation can be observed in leaf discs and cell-suspension cultures in which cellular compartmentalization is lost by snap freezing and thawing

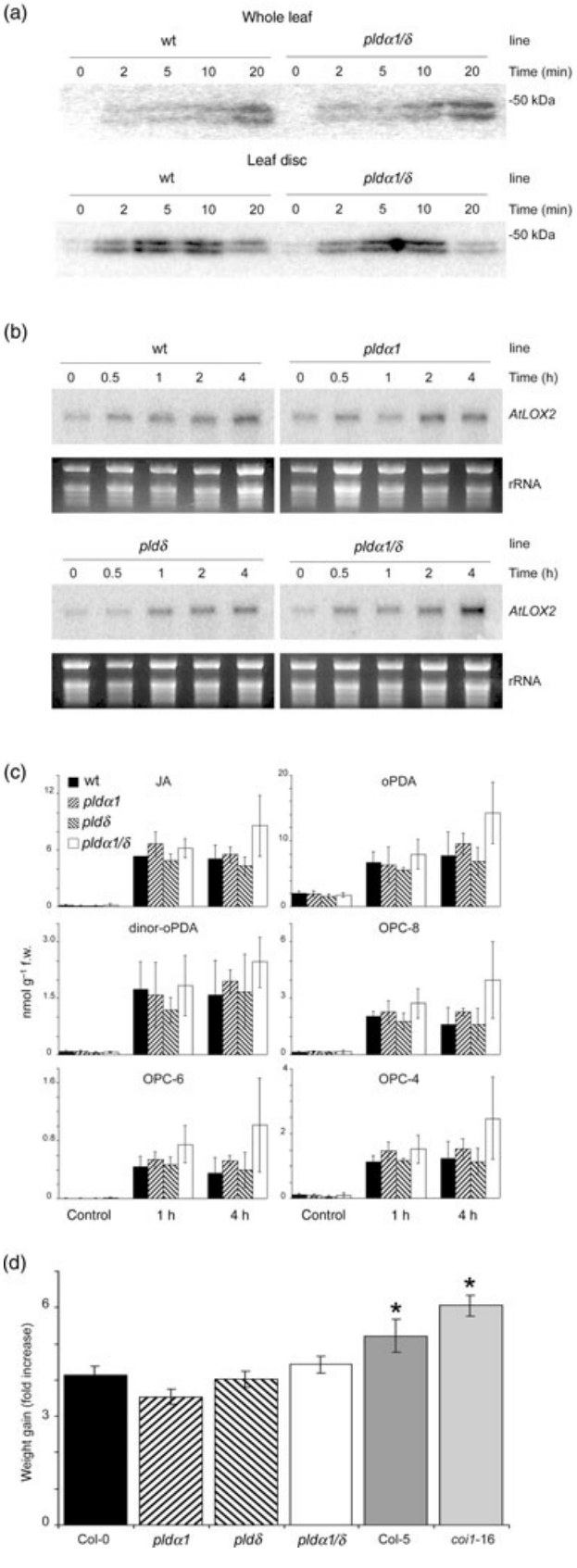


**Figure 8.** Analysis of the wounding response in *Arabidopsis pld* mutants. (a) Fully expanded leaves from 6-week-old plants and leaf discs that had been excised from fully expanded leaves and incubated on labelling buffer overnight were wounded with a hemostat. Proteins were extracted from samples taken at the indicated time points and assayed for protein kinase activity towards MBP by in-gel kinase analysis. (b) Fully expanded leaves from 6-week-old plants were wounded with a hemostat, and RNA was extracted at the indicated time points. *AtLOX2* expression was analysed by RNA blot, ribosomal RNA (rRNA) is presented as a loading control. (c) JA and its precursors oxophytodienoic acid (oPDA), dinor-oPDA, OPC-8, OPC-6 and OPC-4 were measured by gas chromatography/mass spectrometry (GC/MS) analysis of extracts of fully expanded leaves from 6-week-old plants that had been wounded with a hemostat and harvested at the indicated time points. Results [nmol $g^{-1}$ fresh weight (FW)] are presented in a histogram ± SD ($n = 3$). (d) *Pieris rapae* larvae were allowed to feed on wild-type (wt; Col-0), glabrous (Col-5), JA-insensitive (*coi1*-16), or *pldα1*, *pldδ* and *pldα1/δ* double knock-out lines for 96 h. The caterpillars were weighed prior to placement, and after 96 h, the fold increase in weight is presented in a histogram ± SE ($n = 19$–28). Asterisks indicate statistically significant differences from Col-0 (Fisher's LSD test; $P < 0.05$).

(Figs 1–5 & 7), indicates that a large proportion of the PA increase seen in mechanically damaged leaf material (Figs 1, 2 & 7) is derived from ruptured cells. Analysis of the PA response in *pldα1/δ* double knock-out lines shows that virtually all the wounding-induced PLD activity can be ascribed to these two isoforms (Fig. 7). Purified, recombinant proteins of both of these PLDs have been analysed *in vitro* (Pappan & Wang 1999; Qin *et al.* 2002). These studies indicated that they are active at millimolar $Ca^{2+}$ concentrations and, in the case of AtPLDα1, at acidic pH. Taken together, these data suggest that AtPLDα1 and AtPLDδ become active autonomously upon encountering apoplastic conditions, which resemble the conditions that are required *in vitro*.

Dissection of wounded *Arabidopsis* leaf discs (Fig. 2a,b) indicates that the PLD activity seen in this material is restricted to the ruptured cells. This finding is corroborated by the analysis of PLD activity in a tomato cell-suspension wounding model (Fig. 4). In this system, the activity is also massive in ruptured cells and not detectable in the intact cells. Additionally, no significant increase in systemic PA levels could be measured in mechanically wounded *Arabidopsis* plants (Fig. 2c). These results demonstrate that PLD activity upon wounding is restricted to the ruptured cells and not present in the intact, responding cells. These findings contradict earlier reports of wounding-induced PLD activation in intact tissues (Ryu & Wang 1996, 1998; Lee *et al.* 1997, 2001). This disparity can be explained by differences in lipid extraction methods, lipid analysis, plant species, examined tissues or circumstances of plant growth. Our laboratory has developed sensitive phospholipid analysis techniques that have been used to detect PA signalling events in numerous plant systems (Munnik *et al.* 1995, 1996, 2000; Frank *et al.* 2000; den Hartog, Musgrave & Munnik 2001; den Hartog, Verhoef & Munnik 2003; Bargmann *et al.* 2006). Although it cannot be fully excluded that there is some undetectable PLD activity, we deduce that PLD activity in *Arabidopsis* wounding response is restricted to ruptured cells.

## Does PLD activity play a role in the wounding response?

Results in earlier studies of PLD activity in the wounding response, using PLD inhibition and plant lines expressing antisense *AtPLDα1* constructs, have been interpreted in a way that suggests that it is required for wild-type responses to wounding. Our analyses of four separate wounding response assays, however, show that the knock-out of *AtPLDα1* and *AtPLDδ* does not influence the wounding response (Fig. 8), even though the *pldα1/δ* double mutant completely lacks wounding-induced PA accumulation (Fig. 7).

PLD activity in the wounding response has been proposed to be positioned upstream of MAPK activation (Wang *et al.* 2000; Lee *et al.* 2001). The fact that a wounding-induced MBP kinase response can be measured in intact cells that do not display a detectable PA response (Figs 2, 5 & 8a) suggests that PLD activity in these cells is not involved in the protein kinase activation. This conclusion is strengthened by the finding that wounding-induced protein kinase activation is not impaired in the *pldα1/δ* double knock-out mutant (Fig. 8a). Lee *et al.* (2001) based their hypothesis on results showing MAPK activation by application of exogenous PA and inhibition of wounding-induced MAPK activation by *n*-butanol. Application of exogenous PA has been shown to induce numerous responses, including the production of reactive oxygen species (Sang, Cui & Wang 2001; de Jong *et al.* 2004), cytoskeletal rearrangements (Lee, Park & Lee 2003; Huang *et al.* 2006) and cell death (Park *et al.* 2004). However, it does not necessarily mimic intracellular PLD activation. The specificity of PLD inhibition by primary alcohols is also disputable; it has been shown that alcohols induce changes in microtubule organization (Dhonukshe *et al.* 2003; Gardiner *et al.* 2003; Motes *et al.* 2005) that might well indirectly influence MAPK activation.

An *Arabidopsis* line expressing an antisense *AtPLDα1* construct was reported to have perturbed wounding-induced gene expression (e.g. *LOX2*), as well as JA production (Wang *et al.* 2000). These authors suggested that the PA produced by AtPLDα1 upon wounding was a precursor for JA synthesis. In contrast, we found that *AtLOX2* expression and the synthesis of JA and its peroxisomal precursors were not affected in wounded *pldα1*, *pldδ* or *pldα1/δ* knock-out mutants (Fig. 8b,c), indicating that these PLD isoforms are not involved in these wounding responses. This discrepancy is hard to explain; however, it could be accounted for by the fact that the former study made use of a silencing strategy, whereas we used T-DNA insertion lines. Wang *et al.* (2000) used an antisense line created previously (Fan, Zheng & Wang 1997) that expressed 785 bp of *AtPLDα1* (bp 1446–2231 of the *AtPLDα1* cDNA) in reverse orientation. This region shows high similarity to several other PLD isoforms in *Arabidopsis* and could conceivably be affecting their expression, as well as other, secondary silencing effects.

Pertaining to the lack of an effect on JA levels, DONGLE (DGL) and DEFECTIVE IN ANTHER DEHISCENCE (DAD1) are plastidic galactolipases that have been demonstrated to be necessary and sufficient for JA production (Hyun *et al.* 2008). These authors suggested that PLD was only involved in the wounding-induced expression of the genes encoding these lipases and not in the synthesis of JA precursors. A number of studies have indicated that JA originates from the plastidial oxylipin precursors 12-oPDA and dinor-oPDA (Wasternack *et al.* 2006). Certain JA precursors appear to be esterified to plastidial galactolipids, termed Arabidopsides, which are being discussed as another source of JA (Kourtchenko *et al.* 2007). In addition, cross-talk of oxylipins with phospholipids at the signalling levels has previously been proposed (Mosblech *et al.* 2008), in which the phospholipids do not act as biosynthetic precursors of JA.

Lastly, herbivore performance on the *pld* mutants was also not altered compared with that on wild type, as seen by the weight gain of Cabbage White butterfly caterpillars

(Fig. 8d). These larvae did show enhanced performance on glabrous and JA-insensitive plant lines, demonstrating the efficacy of the assay. Taken together, these results show that no function can be ascribed to these two PLD isoforms in the wounding response, even though they are responsible for all the detectable PLD activity upon wounding.

All these results together demonstrate that the role of PLDs in the *Arabidopsis* wounding response needs to be reassessed. It cannot be fully excluded that PLDs have some kind of function in this response, but it can be stated that their role is not of great consequence and that the PLD-produced PA does not seem to be a precursor for JA synthesis as formerly proposed. The fact that PA is produced in massive amounts by ruptured cells at the wound site (Figs 1–5 & 7) and that it has been shown to elicit a number of responses in living cells (Lee *et al.* 2001, 2003; Sang *et al.* 2001; de Jong *et al.* 2004; Park *et al.* 2004; Huang *et al.* 2006) could indicate that this molecule might have some non-cell-autonomous signalling function. However, in the wounding response, this putative cell-to-cell PA signalling is apparently not required for a wild-type reaction as determined by kinase activation, induced *AtLOX2* expression, JA production or herbivore resistance (Fig. 8).

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** PLD activity upon loss of cell membrane integrity in the *pldα1/δ* double knock-out line. Leaf discs from wild-type (Col-0) and *pldα1/δ* double knock-out lines were labelled overnight floating on buffer containing $^{32}P_i$. Discs were snap frozen and thawed. Lipids were extracted at the indicated time points, separated by TLC and analysed by phosphoimaging. PA was quantified as percentage of total radio-labelled lipids and is presented in a histogram ± SD ($n = 3$). Asterisks indicate a significant difference between mutant and wild type (two-tailed paired *t*-test, $P < 0.05$).

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