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Phospholipases Dα and δ are involved in local and systemic wound responses of cotton (*G. hirsutum*)

Angeliki Bourtsala[a], Theodora Farmaki[b], Dia Galanopoulou[a,*]

[a] National and Kapodistrian University of Athens, Department of Chemistry, Panepistimiopolis, 15771 Athens, Greece
[b] Institute of Applied Biosciences, Centre for Research and Technology, 57001 Thessaloniki, Greece

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ABSTRACT

Phospholipases D (PLDs) catabolize structural phospholipids to produce phosphatidic acid (PtdOH), a lipid playing central role in signalling pathways in animal, yeast and plant cells. In animal cells two PLD genes have been studied while in model plant *Arabidopsis* twelve genes exist, classified in six classes (α-ζ). This underlines the role of these enzymes in plant responses to environmental stresses. However, information concerning the PLD involvement in the widely cultivated and economically important cotton plant responses is very limited. The aim of this report was to study the activity of conventional cotton PLD and its participation in plant responses to mechanical wounding, which resembles both biotic and abiotic stresses. PLDα activity was identified and further characterized by transphosphatidylation reaction. Upon wounding, cotton leaf responses consist of an acute *in vitro* increase of PLDα activity in both wounded and systemic tissue. However, determination of the *in vivo* PtdOH levels under the same wounding conditions revealed a rapid PtdOH formation only in wounded leaves and a late response of a PtdOH increase in both tissues. Expression analysis of PLDα and PLDδ isoforms showed mRNA accumulation of both isoforms in the wounded tissue, but only PLDδ exerts a high and sustainable expression in systemic leaves, indicating that this isoform is mainly responsible for the systemic wound-induced PtdOH production. Therefore, our data suggest that PLDα and PLDδ isoforms are involved in different steps in cotton wound signalling.

1. Introduction

Phospholipases D (PLDs, EC 3.1.4.4) are hydrolases present in all bacteria, plant and animal cells studied so far. These enzymes catabolize structural phospholipids to produce phosphatidic acid (PtdOH), which can act as a second messenger in the cell [1–3]. There is, however, an interesting evolutionary notion: twelve PLD genes exist in *A. thaliana* (and a similar or greater diversity exists in many plants) when there is only one in *S. cerevisiae* and two genes (*PLD1* and *PLD2*) in animal cells [4–7]. This underlines the important regulatory role of PLD superfamily in plants and supports the idea of a possible participation in the membrane reorganization events as it has been already proposed for model membranes [8]. Such a PLD function could be attributed to its fusogenic product, PtdOH, as in the case of tobacco pollen tube growth [9].

PLDs contain two HKD catalytic motifs which form a cone-shaped active site, well-adapted for the recognition of their substrate [10], and they are active in lipid-water interfaces/lipid dispersions in water. In the presence of short-chain primary alcohols, PLDs exchange the polar headgroup of membrane lipids with the alcohol group and this transphosphatidylation reaction produces lipids not normally existing in membranes, which cannot be further metabolized [11]. Accordingly, this reaction is used for PLD activity identification.

Arabidopsis PLDs are classified into 6 classes (α-ζ) according to their requirements for optimal activity *in vitro* and their sequence similarity. They all possess the HKD catalytic motifs; however, PH and PX domains for phosphoinositide binding exist only in PLDζ (which shares more homology to yeast and mammalian isoforms), while PLDα, β, γ, δ and ε contain the calcium- and lipid-binding C2 domain instead [3]. The most widespread PLD isoforms in plants are PLDα and δ and there are multiple reports suggesting their involvement in signalling pathways connected to plant responses and adaptation to various abiotic or biotic stresses: salinity [12], drought [13,14], cold stress [15], mechanical wounding [16–18] or pathogen elicitors [19] induce specific PLD isoforms.

Cotton (*Gossypium hirsutum*) is a widely cultivated and economic-

Abbreviations: HKD, catalytic motif; PLD, catalytic motif containing Histidine-Lysine-Aspartic acid residues; HRM, High Resolution Melting; PLD, phospholipase D; PtdCho, phosphatidylcholine; PtdEth, phosphatidylethanolamine; PtdEtOH, phosphatidylethanol; PtdOH, phosphatidic acid.

* Corresponding author.
E-mail addresses: abourtsala@chem.uoa.gr (A. Bourtsala), mfarmaki@certh.auth.gr (T. Farmaki), galanopoulou@chem.uoa.gr (D. Galanopoulou).

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ally important plant. However, information on the mechanisms participating in cotton responses to different forms of environmental stress is rather limited. Recently, two PLDα genes were identified in the genome of *G. hirsutum* [15]. Their ORFs encode a polypeptide of 807 amino acids with a predicted molecular mass of 91.6 kDa sharing 81–82% homology with PLDα1 and PLDα2 from *A. thaliana*. *Gh*PLDα1 and *Gh*PLDα2 apparently participate in the response to environmental stress, since their expression was found to be acutely upregulated by cold stress, in a light-regulated manner. Importantly, upregulation was suppressed when plants were acclimated before applying the cold treatment [15]. In addition, two very recent illuminating reports on *G. arboreum* and *G. hirsutum* PLD genes, respectively, have been published [20,21]. In the latter, 40 genes were identified in the allotetraploid *G. hirsutum* genome and 20 in its diploid progenitor *G. raimondii*. These PLD genes, together with 19 previously identified from *G. arboreum*, were examined in detail. Quantitative Real-Time PCR documented that all *Gh*PLD genes were expressed and each had a unique spatial and developmental expression pattern suggesting involvement in cotton growth and development [21].

The aim of this study was to obtain a global view of *G. hirsutum* PLDs and their possible participation in plant responses to environmental stresses. For this, we have identified, for the first time in *G. hirsutum*, PLDα activity and further investigated the involvement of PLDα activity in local and systemic responses to mechanical wounding. This stress resembles wounding from wind or hail or wounding by herbivores, insect feeding on leaves or exposure to pathogens, stresses known to deteriorate the membrane and elicit responses throughout the plant. PLDα activity from cotton plants or leaves was partially purified and assayed *in vitro* and further characterized by a transphosphatidylation reaction. Our data show that, upon wounding, responses in cotton plants involve an acute PLDα activity increase together with an increased local PtdOH formation. Analysis of putative PLD substrates suggests that PtdOH production may result from different endogenous substrates. Expression analysis of PLDα and PLDδ isoforms showed mRNA accumulation of both isoforms in the wounded tissue. However, only PLDδ exerts a high and sustainable expression in systemic leaves, indicating that this isoform is mainly responsible for the systemic wound-induced PtdOH production.

2. Materials and methods

2.1. Plant material and stress treatment

Gossypium hirsutum var. ACALA SJ2 was used for this study. Cotton plants were grown in a growth chamber with 60–70% humidity, for 6 weeks (6-w-old plants), at 23 °C, under a photoperiod of 16 h light/8 h dark. Illumination of 110 μE m^{-2} s^{-1} PAR was supplied by cool-white fluorescent tungsten tubes (Osram, Germany). Mechanical wounding was performed by cutting each leaf (local tissue) four times with a pair of scissors across the mid-vein. Leaves of non-wounded plants served as control. Following wounding, leaves were detached at specific time points as indicated. For the systemic study, the neighboring non-wounded (systemic) leaves were also detached at the same time points. All samples were snap-frozen in liquid nitrogen immediately after harvesting and used for protein or RNA isolation or immersed in hot isopropanol for lipid extraction.

2.2. PLD activity isolation

Collected tissue from 6-w-old cotton plants (aerial part of plant or detached leaves) was ground in liquid nitrogen with a mortar and pestle into fine powder which was dispersed in 3.5 volumes of extraction buffer containing 50 mM Tris (pH 7.5), 10 mM KCl, 2 mM DTT (dithiothreitol), 1 mM EDTA and 0.5 mM PMSF (phenylmethanesulfonyl fluoride). The mixture was filtered through two layers of cheesecloth and centrifuged at 1500 *g* for 15 min to remove particles plus nuclei. The supernatant was heated at 55 °C for 5 min, immediately frozen on ice and centrifuged at 10,000 *g* for 5 min. The new supernatant was centrifuged at 100,000 *g* for 45 min. The 10,000 *g* pellet fraction (intact organelles and debris) as well as the 100,000 *g* pellet (microsomal) fraction were resuspended in extraction buffer for the PLD activity assay. Total protein assay was performed according to the Lowry method [22].

For wounding experiments, PLD activity was isolated from leaves. In this case, the only modification on the above-described protocol was that 7 volumes of extraction buffer were added to the powdered sample.

2.3. PLDα activity assay and transphosphatidylation reaction

Endogenous PLDα activity was assayed, using PtdCho dispersions as substrate, based on the formation of PtdOH as previously described [23]. Briefly, 10–15 μg protein were incubated at 30 °C for 30 min in an assay mixture containing 100 mM HEPES (pH 6.5), 50 mM $CaCl_2$, 0.5 mM SDS (sodium dodecyl sulfate) and 2 mM PtdCho (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine) as substrate, in a total volume of 200 μl. Reaction was started by the addition of protein preparation. Identification of the activity was performed by transphosphatidylation reaction in the presence of ethanol. In this case, PLDα activity was assayed based on the formation of both PtdOH and phosphatidylethanol (PtdEtOH). For the transphosphatidylation reaction, absolute ethanol was added to the assay mixture at a final concentration of 1%, v/v.

The reaction was stopped by adding 750 μl of chloroform/methanol (1:2), followed by 200 μl of chloroform and 200 μl of 2 M KCl. After vortexing, the two phases were separated by centrifugation at 500 *g* for 5 min and the chloroform phase, containing the lipids, was collected. For hydrolysis and transphosphatidylation activity assays, based on PtdOH and PtdEtOH determination, respectively, lipids were separated by TLC on oxalate-impregnated heat-activated silica gel H plates [24] using chloroform/methanol/ammonium hydroxide (65:35:5, v/v/v) as solvent system. TLC plates were prepared by mixing silica gel H with 2.1% potassium oxalate at a ratio of 1:2.6, w/v. After chromatogram development, bands corresponding to authentic lipid standards (PtdOH, Rf=0.10, PtdCho, Rf=0.48, PtdEtOH, Rf=0.82) were identified by exposure to iodine vapor. PtdOH (and PtdEtOH in the case of transphosphatidylation reaction) band was scrapped off the plate and eluted from the silica gel by a modification of the Bligh and Dyer method [25]. For the modification, acidification of the aqueous phase was achieved by adding 1.1 N HCl (final concentration). Phosphorus content of the extracts was quantified by phosphorus determination according to Bartlett [26], as modified by Marinetti [27]. Briefly, extracts were dried under a stream of nitrogen and lipids were digested with perchloric acid 70% at 180 °C for 1 h. After cooling, ammonium molybdate and aminonaftholsulfonic acid reagent were added. Samples were heated for 10 min in boiling water and, after 20 min, color was quantified at 820 nm.

PLDα activity was assayed using either unlabelled or radioactive PtdCho as substrate. For substrate preparation, 0.4 μmol of carefully dried under a stream of nitrogen PtdCho (Sigma-Aldrich, Germany) was dispersed in water to a final concentration of 20 mM by 20 min-bath sonication at room temperature. In case of using radioactive PtdCho, 22 nCi of PtdCho, L-a-dipalmitoyl [2-palmitoyl-9,10-^{3}H(N)] (ARC, USA) was mixed with 0.4 μmol of unlabelled PtdCho and then dispersed as described above. In the case of radioactive substrate, PtdOH band was scrapped off as described and counted using a liquid scintillation counter, after addition of 0.5 ml of methanol and toluene-based scintillation cocktail.

2.4. Lipid extraction

For lipid determination, a modification of *A. thaliana* extraction protocol was followed [28]. Briefly, plant tissue (leaves) was immersed

in preheated (75 °C) isopropanol containing 0.01%, w/v BHT (butylated hydroxytoluene) for 15 min immediately after sampling in order to eliminate any endogenous lipolytic activity and, also, avoid undesirable transphospatidylation [29]. Chloroform and water were subsequently added to the isopropanol at a final ratio of 1:2:0.4 (chloroform/isopropanol/water, v/v/v) followed by agitation at room temperature for 1 h. Lipids were re-extracted 5 times with 4 ml of chloroform/methanol (2:1), containing 0.01% BHT, for 30 min. The combined extracts were washed twice with 1 ml of 1 M KCl and 2 ml of water. The solvent of the extracts was then dried under a stream of nitrogen. Lipids were dissolved in 0.5 ml of chloroform/methanol (2:1) and stored at −20 °C until use. Data were normalized with dry weight of the leaves. For each extraction, one leaf was used (20–30 mg of dry weight).

2.5. Lipid analysis

Separation of lipids was performed by TLC of lipid extracts on oxalate-impregnated heat-activated silica gel H plates, prepared as described above, using chloroform/methanol/ammonium hydroxide (65:35:5) as solvent system. An amount of 15–35 μg of total lipid phosphorus was applied on the plate for each separation. Lipids corresponding to PtdOH, PtdCho and phosphatidylethanolamine (PtdEth, Rf=0.68), were identified by exposure to iodine vapor (Suppl. Fig. 1), scrapped off the plate and eluted from the silica gel by the modification of Bligh and Dyer method described above. Phospholipid content was subsequently quantified by lipid phosphorus determination, as described in Section 2.3.

2.6. RNA isolation and cDNA preparation

Total RNA was isolated from wounded or systemic leaves at specific time points using a plant RNA isolation kit (NucleoSpin RNA plant, Macherey-Nagel, USA) according to the manufacturer's protocol. cDNA was synthesized using the Superscript Reverse Transcriptase (Invitrogen, USA) and random hexamers.

2.7. Quantitative Real-Time PCR and HRM

Real-Time PCR was performed as previously described [15]. An RG6000 (Corbett Research Pvt. Ltd., Sydney, Australia) Real-Time PCR system was used. PCR was performed using the Platinum Quantitative PCR SuperMix-UDG, SYTO9 (Invitrogen, USA). Relative gene expression was performed using ubiquitin as control and C_Ts from triplicate reactions were recorded.

The following primers were used for PLDα [15]:

*Gh*PLDα1-1, 5′→3′ ACTCACTCTCACTTCTGCCTTG;
*Gh*PLDα1-2, 5′→3′ GCTCACTTTCACTGCTGCTTTG;
*Gh*PLDα2-1, 5′→3′ TTATCTTATGTTAATATCTCTCTGGC;
*Gh*PLDα2-2, 5′→3′ TTATCTTATGTTAATATCTCTCTGTC.

Reverse for all of them: 5′→3′ GCCAATGCCAATAGTCTCCTGAACATTTG. The following primers were used for PLDδ: 5′CAGGTTGTTGGAACCATGTTTACAC3′ as forward and 5′CAGGAAAAGTA;GGATTATGAAAATCATCC3′ as reverse primer.

The program used is described in reference 15. Identification of the quantified products was performed by High Resolution Melting (HRM) as well as by sequencing.

3. Results

3.1. G. hirsutum *PLDα activity is localized in the microsomal fraction*

PLDαs are the conventional plant PLDs. In the present study, we first characterize PLDα activity in cotton. For this, the aerial part of 6-w-old plants was collected, snap-frozen in liquid nitrogen and homogenized. The powdered samples were mixed with extraction buffer and fractionated by differential centrifugation. The resulting fractions were subsequently assayed for PLDα activity (assay conditions: pH 6.5, 50 mM Ca^{2+} concentration [23]) using PtdCho dispersions as substrate. PLDα specific activity of different fractions was determined based on the PtdOH formation, as described in Materials and methods section. Specific activities are presented in Fig. 1.

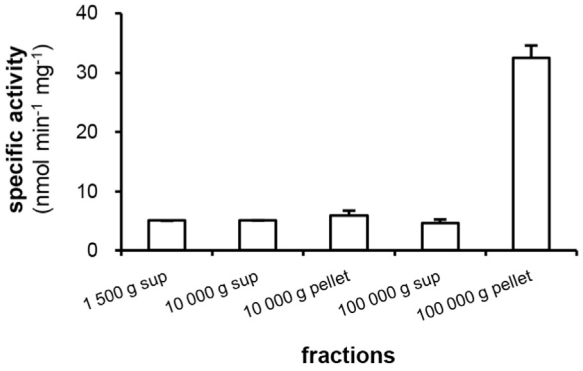

Fig. 1. *Purification of G. hirsutum PLDα activity*. PLDα activity was isolated from the aerial part of 6-w-old cotton plants and fractionated by centrifugation as described in Materials and methods. In the resulting fractions, activity was assayed using unlabelled PtdCho as substrate. For the estimation of the PLDα specific activity, the PtdOH formed was quantified by phosphorus determination after TLC separation. Results are means ± S.D. of three experiments.

As shown in Fig. 1, the microsomal fraction (100,000 *g* pellet) was remarkably (6.4-fold) enriched in PLDα activity compared to the starting homogenate (1500 *g* supernatant). Accordingly, 100,000 *g* pellet fraction was chosen for the rest of the activity experiments. The 100,000 *g* pellet fraction was also used for transphosphatidylation reaction in the presence of 1% ethanol [30] in order to exclude the possibility that the reaction product, PtdOH, is derived from the combination of other enzyme activities present in the microsomal fraction. Quantification of PtdOH and PtdEtOH, after TLC separation and phosphorus determination, resulted in the specific activities presented in Table 1. As expected, the hydrolytic activity was inhibited by ethanol, confirming that the PLDα present in the microsomal fraction was responsible for the production of PtdOH detected *in vitro*. The rates of the competing reactions exhibit a ratio of 2.7 (transphosphatidylation to hydrolysis, Table 1), suggesting a strong *in vitro* inhibition of PLDα hydrolytic activity by the primary alcohol used. This 70% inhibition is comparable to the inhibition observed for PLDs from other plant species in the presence of a primary alcohol [31].

To investigate the involvement of PLDα activity in cotton leaves upon wounding and further analyze local and systemic responses, basal enzyme activity of leaves was first determined. Quantification of leaf PLDα activity was carried out after detachment of leaves and fractionation of their homogenate by centrifugation using [^{3}H]PtdCho as substrate, as described in Materials and methods. The enzyme assay

Table 1
Effect of ethanol on the hydrolytic activity of PLDα. Microsomal fraction (100,000 *g* pellet) from 6-w-old cotton plants was obtained as described in Fig. 1. PLDα activity was assayed in the presence or absence of 1% ethanol in the assay mixture. For the estimation of hydrolysis and transphosphatidylation specific activities, PtdOH and PtdEtOH were quantified by phosphorus determination after TLC separation. Results are means ± S.D. of three experiments.

Ethanol concentration	Specific activity (nmol min^{-1} mg^{-1})	
	hydrolysis	transphosphatidylation
0%	31.99 ± 1.99	–
1%	13.98 ± 1.90	37.43 ± 1.08

in the leaf fractions revealed a PLDα activity distribution similar to that of the aerial part of plant (Fig. 1) with the microsomal fraction showing enrichment compared to the starting homogenate. Interestingly, the microsomal fraction from leaves showed a 5 times lower activity when compared to the corresponding aerial part of plant fraction. This is not unusual, as previous studies revealed the same for a variety of plants and seeds [32]. In the case of seeds, the highest activity after germination was found in the roots and the cotyledons, suggesting that PLD activity could be related to the development of plants [32].

3.2. Wounding of cotton leaves results in both local and systemic increase of PLDα activity

In 6-w-old cotton plants, wounding was applied across the mid-vein of leaves as described in Materials and methods. The neighboring non-wounded leaves served for the systemic response analysis. At the indicated time points, wounded and systemic leaves were collected, snap-frozen in liquid nitrogen and fractionated and the microsomal fractions were subsequently tested *in vitro* for PLDα activity. The assay of PLDα activity was performed using radioactive PtdCho dispersions. The percentage increase in specific activity of the samples tested compared to control (time=0 min, 7.56 ± 0.57 nmol min^{-1} mg^{-1}) are shown in Fig. 2.

As shown in Fig. 2, a rapid increase in PLDα activity in both wounded and systemic leaves was observed upon wounding. The local PLDα activity induction, however, occurred in two bursts, at 5 and 30 min, respectively, returning to basal levels in 60 min. In contrast, the PLDα activity increase of systemic leaves was transient, reaching basal levels in 30 min. These results suggest an immediate local activation of pre-existing PLDα activity. The wounding signal is likely transduced to the neighboring leaves triggering a systemic response involving the observed systemic PLDα activation. The simultaneous presence of signal in damaged and systemic leaves is in good agreement with previous studies in plants, where wounding stimulation of PLD was not limited to the site of wounding but it also occurred at distal sites [33]. In this report, the authors suggest that PLD activation is attributed to decompartmentalization in the case of local response and translocation of cytosolic PLD to microsomal membranes in the case of distal response. Although the data presented in Fig. 2 do not rule out translocation, data of Fig. 1 support localization of cotton PLDα mainly in membranes.

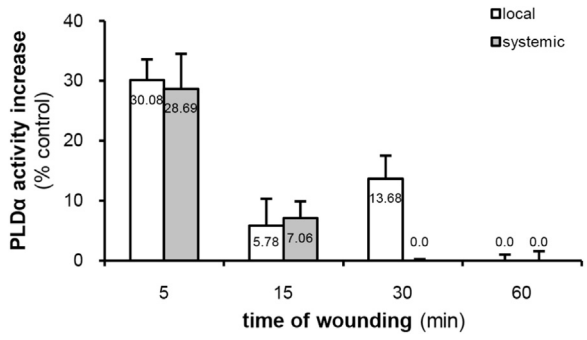

Fig. 2. *Effect of mechanical wounding on PLDα activity of wounded and systemic leaves.* Wounded (local response) or neighboring non-wounded (systemic response) cotton leaves subjected to mechanical wounding for the indicated time points were homogenized and fractionated as described in Materials and methods. The resulting microsomal fractions were assayed for PLDα activity using [^{3}H]PtdCho as substrate. For the estimation of PLDα specific activity, the [^{3}H]PtdOH formed was quantified by scintillation counting after TLC separation. Results are shown as percentage increases of the PLDα specific activities over control and are means ± S.D. of three experiments. Percentage changes in activity are significantly different ($P < 0.01$).

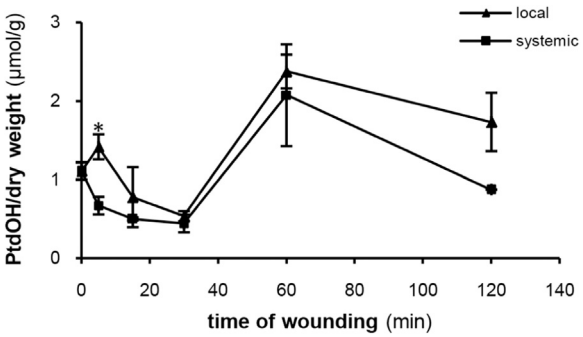

Fig. 3. *Effect of wounding on PtdOH levels of cotton leaves.* Wounded (local response) or neighboring non-wounded (systemic response) cotton leaves from plants subjected to mechanical wounding for the indicated time points were detached and immediately immersed in hot isopropanol. Lipid extraction was then performed as described in Materials and methods, followed by TLC separation and phosphorus determination of the isolated lipids. PtdOH levels of wounded and systemic leaves are shown as µmol PtdOH/g dry weight of tissue (leaves). Results are means ± S.D. of four samples from two independent experiments (*$P < 0.01$ compared to control).

3.3. Phospholipid determination reveals different PtdOH production pattern during local and systemic responses and differential consumption of PLD endogenous substrates

Since there is clear evidence that cotton PLDα is activated during stress ([15]; Fig. 2), the endogenous formation of the enzyme product, PtdOH, was determined at different time points after wounding, during local and systemic responses. In parallel, we determined the endogenous levels of putative PLD substrates, PtdCho and PtdEth. For these experiments, the total lipid extraction protocol described in Materials and methods was followed. The lipid extracts were subjected to TLC separation and the phosphorus content of the isolated lipids was determined. The levels of PtdOH in cotton leaves, given as µmol/g of dry weight, are presented in Fig. 3.

As shown in Fig. 3 and in accordance to PLDα activity results, an increase in PtdOH levels occurred in wounded leaves immediately after wounding (almost 30% in 5 min). This confirms the immediate local activation of PLDα and suggests an acute signalling role for PtdOH when cotton plants are mechanically wounded. There is also a second, delayed increase of PtdOH in 60 min, possibly connected to the second burst in PLDα activation shown in Fig. 2, although in 60 min constitutive PtdOH synthesis is likely to have altered the levels of PtdOH. The systemic PtdOH production was lower compared to the locally observed in all time points tested (Fig. 3). More importantly, in this case, the upregulation of PtdOH levels occurs only in 60 min, when there was no systemic PLDα activity detected *in vitro*. This 2-fold systemic upregulation could be attributed to the expression of at least one different PLD isoform and will be discussed in the following sections. In both cases, analysis at 3.5 h showed that the 2h-PtdOH (almost control) levels were not further changed (data not shown).

Surprisingly, concerning the main PLD substrates in plants, there was a different pattern of endogenous PtdCho and PtdEth consumption with time. The calculated phospholipid ratio changes upon wounding, compared to control, are presented in Fig. 4B and C; basal levels of endogenous lipids are presented in 4A.

At the first time point examined locally (5 min), a decrease of PtdEth is observed compared to the total phospholipids, while a decrease of PtdCho occurs from 30 to 120 min (Fig. 4B). These results suggest a possible shift in cotton PLD substrate preference in a time-dependent manner after wounding. This may also indicate the expression of multiple PLD isoforms. The apparent enhanced presence of PtdEth in 30 min can be attributed to the constitutive lipid metabolism in cotton plants [34]. The "consumption pattern" is essentially the same in systemic leaves (Fig. 4C).

A

phospholipid	% total
PtdCho	54.05 ± 1.14
PtdEth	40.33 ± 0.45
PtdOH	5.63 ± 0.70

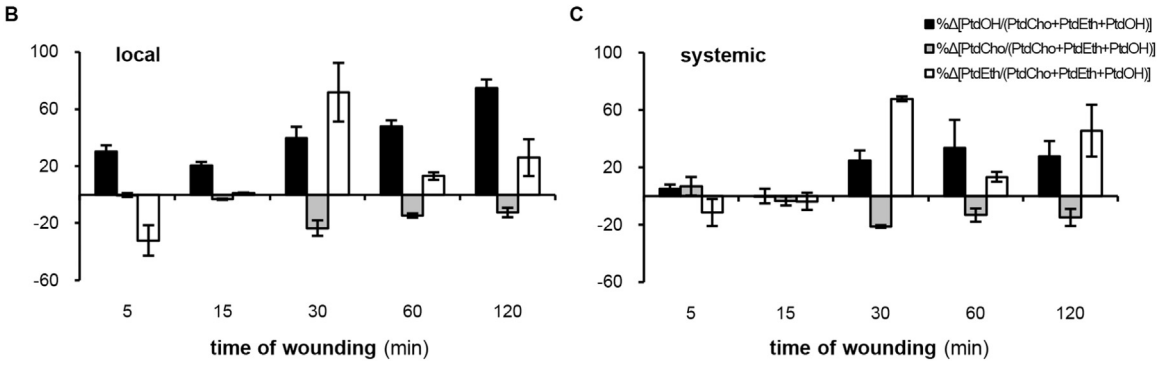

Fig. 4. *Effects of wounding on phospholipid content of cotton leaves.* Wounded (local response) or neighboring non-wounded (systemic response) cotton leaves from plants subjected to mechanical wounding for the indicated time points were detached and immediately immersed in hot isopropanol. The lipid extraction was performed as described in Materials and methods, followed by TLC separation and phosphorus determination of the isolated lipids. (A) Basal levels of major phospholipids (PtdCho and PtdEth) and PtdOH in cotton leaves are presented as percentage of total (PtdCho+PtdEth+PtdOH) lipid content (B) PtdOH, PtdCho and PtdEth levels of wounded leaves are shown as percentage changes of the corresponding basal ratio. (C) PtdOH, PtdCho and PtdEth levels of systemic leaves are shown as percentage changes of the corresponding basal ratio. (A, B and C), results are means ± S.D. of four samples from two independent experiments.

3.4. Wounding differentially upregulates PLDα and PLDδ expression during local and systemic responses

According to a recent paper of Tang et al. [21], *G. hirsutum* possesses several PLDα and PLDδ gene that are expressed, each having a unique expression pattern. Certain PLDα genes have been previously studied in detail during cold stress [15].

To examine whether a different stress (wounding of cotton leaves) affects the expression of these PLDα and of PLDδ as well, quantitative PCR analysis was performed using cDNAs obtained from wounded and systemic leaves. The expression levels of the PLDα and PLDδ isoforms were determined at several time points after wounding in order to assess the involvement of expression of these isoforms in both local and systemic responses. As *G. hirsutum* is an allotetraploid plant, derived from its diploid progenitors *G. raimondii* and *G. arboreum*, primers designed for 2 *GrPLDα* and 2 *GaPLDα*, respectively, were used for PLDα expression analysis [15]. Primers that identify two PLDδ isoforms were also used (see Materials and methods). For these experiments, ubiquitin served as a control gene and identification of the quantified products was performed by HRM.

As shown in Fig. 5A–E, increase in the expression of all PLD isoforms examined was observed. All 4 PLDα mRNAs were accumulated locally 30 min after wounding (3- to 5-fold increase) and this increase persisted up to 3.5 h. No changes, however, were observed in *G. hirsutum* PLDα expression in systemic leaves. On the contrary, strong PLDδ mRNA accumulation was observed during both local and systemic responses after 30 min (Fig. 5E, 8- and 6.7-fold, respectively). It is of further interest that, in the case of systemic response, PLDδ mRNA levels were further increased reaching 15-fold change compared to control. This high systemic expression of PLDδ is in accordance with our conclusion concerning PtdOH increase in systemic cotton leaves, which was attributed to a PLD isoform other than PLDα and will be discussed in the next section.

In an attempt to find whether *G. hirsutum* possesses also PLDδ activity, leaves from non-wounded plants were assayed under conditions suitable for PLDδ (pH 7.0, 100 μM Ca^{2+} concentration, 0.6 mM oleate [35]). In these preliminary experiments, PLDδ specific activity of the 100,000 *g* pellet fraction (0.20 ± 0.03 nmol min^{-1} mg^{-1}) was much lower than PLDα activity and this is in agreement with the low basal RNA levels of PLDδ observed in cotton leaves.

4. Discussion

It is now well established that plants use extensively PLD/PtdOH system in order to regulate the transduction of environmental signals and/or reorganize their membranes [1–7]. In general, PLDs play an important role in plant growth and development. The main body of information concerning environmental stresses comes from the model plant *A. thaliana*, although PLD genes have been identified in many plants; this concerns mostly PLDα and PLDδ isoforms [13,14,17]. These two isoforms are the most widely studied in plants. They show different requirements in pH and Ca^{2+} concentration *in vitro* and, in addition, PLDδ is stimulated by oleate [35]. However, and despite the fact that information from economically important cultivated cotton plant responses would be of great value, data on cotton PLDs is rather limited. There are only few reports dealing either with possible enzyme involvement in the regulation of secondary cell wall synthesis in cotton fibers [36,37] or with the involvement of cotton PLD responses and plant adaptation to cold stress [15].

In this study we identify, for the first time, and characterize PLDα activity in cotton (*G. hirsutum*) plants. Mechanical wounding was applied on cotton leaves and changes in the activity of microsomal fractions of wounded and systemic leaves were followed *in vitro* at several time points after wounding. A rapid increase in PLDα activity in both wounded and systemic leaves was observed upon wounding, in agreement with previous reports on plant PLD activation as a rapid (seconds to minutes) response both in damaged plant cells and at distal sites from wounding [33,38]. In the present study, however, local PLDα activity induction occurred in two bursts, at 5 and 30 min, respectively, returning to basal levels in 60 min. In contrast, the PLDα activity increase of systemic leaves was transient, reaching basal levels in 30 min. These results were confirmed by determination of endogenous PtdOH levels at the same time points post-wounding. In agreement with PLDα activity increase, an increase in PtdOH levels occurred in wounded leaves immediately after wounding (almost 30% in 5 min), linking PtdOH formation to PLDα upregulation. There is also a second,

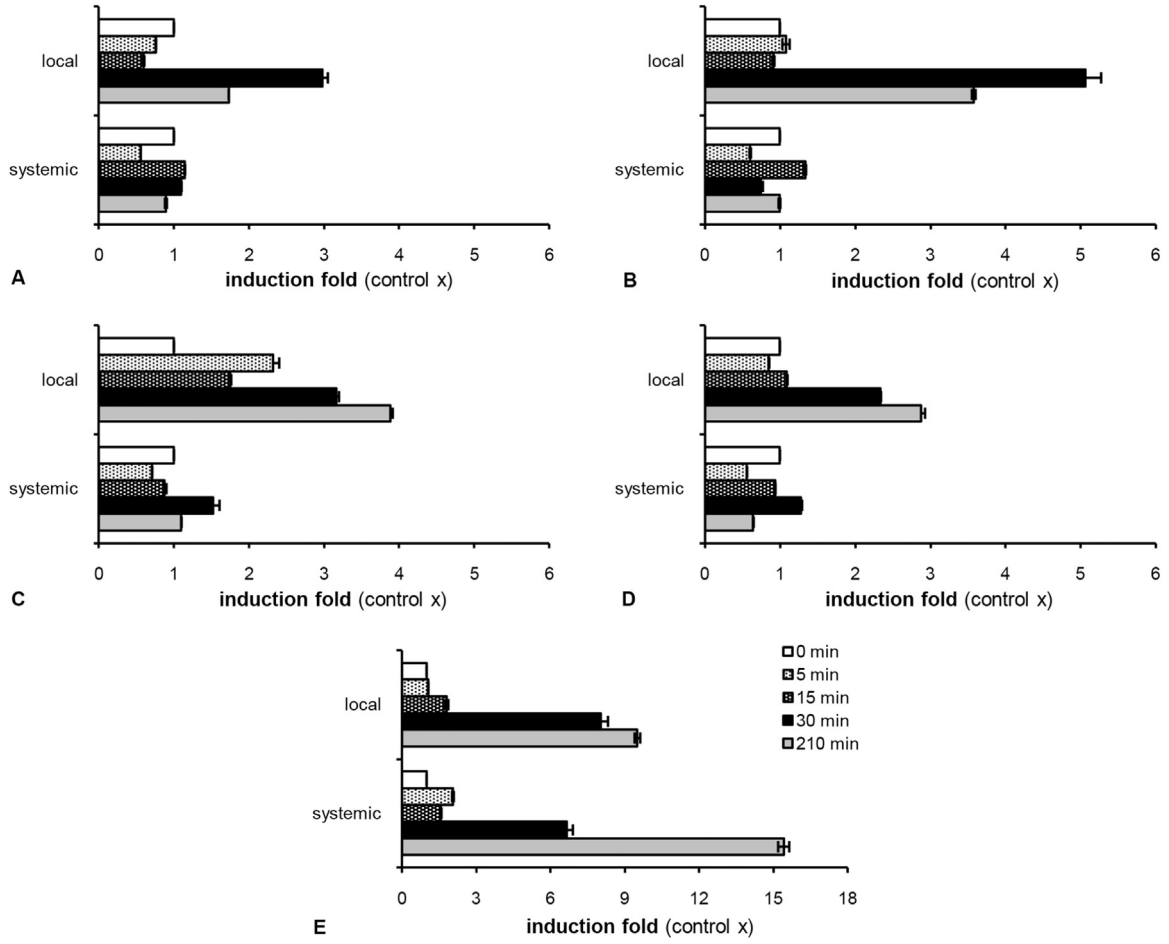

Fig. 5. *Effects of wounding on cotton PLDα and PLDδ expression*. Wounded (local response) or neighboring non-wounded (systemic response) cotton leaves subjected to mechanical wounding for the indicated time points were collected. After leaf RNA extraction and reverse transcription, the expression levels of (A) *Gh*PLDα1-2 (GU569956.1), (B) *Gh*PLDα1-1 (GU569957.1), (C) *Gh*PLDα2-2 (GU569958.1), (D) *Gh*PLDα2-1 (GU569953.1) and (E) *Gh*PLDδ (AY138251.1 and AY138252.1) isoforms were analyzed by Real-Time PCR. (A–E) Results are shown as fold changes of basal values (non-wounded leaves, t=0) and are means ± S.D. of triplicate reactions.

delayed increase of PtdOH in 60 min, possibly connected to the second burst in PLDα activation or to combined activities of PLDα and other PLD isoforms expressed later or, even, to changes in whole phospholipids signalling and/or metabolism. Importantly, the upregulation of PtdOH levels in systemic leaves occurs only in 60 min, when there is no systemic PLDα activity detected. This systemic PtdOH upregulation could be also attributed to different PLD isoforms, with the δ being a prevalent candidate.

Although PLDδ was found to be involved in several stress types in *Arabidopsis*, little is known concerning wounding and the involvement of this isoform [18]. Our results, together with findings from these other stress types applied [14,39–42], show that PLDδ is involved in plant signalling connected to environmental stresses possibly playing a complementary to PLDα role. The involvement of PLDδ in the systemic response of cotton leaves was confirmed by expression experiments. The expression analysis in the wounded tissue showed mRNA accumulation of both isoforms 30 min after wounding. No changes, however, were observed in PLDα expression in systemic leaves where only PLDδ exerts a high and sustainable expression. This suggests that this isoform is mainly responsible for the systemic wound-induced PtdOH production observed. The PLDα activation found *in vitro* in systemic leaves 5 min after wounding is not accompanied by an increase in PtdOH levels possibly because the *in vivo* conditions (pH and Ca^{2+} concentration in this tissue) were not suitable for PtdOH production. Therefore, we suggest that our data strongly support a model where the PtdOH increase in non-wounded leaves (and, possibly, the delayed local PtdOH accumulation) is driven by PLDδ activity. The two isoforms apparently exhibit non-identical patterns of transcriptional regulation and, more importantly, they have different biochemical properties; this suggests a differential involvement in cotton plant stress responses for PLDα and PLDδ isoforms.

According to several reports, PtdCho, used as the *in vitro* PLDα substrate in this study, is one of the main substrates of this activity, although PtdEth and phosphatidylglycerol are equally good substrates for the conventional PLDs. In our study, when we analyzed endogenous substrates at several time points after wounding, we observed a shift in PLD substrate preference from PtdEth (in 5 min, when there was a 30% increase in the PLDα activity) to PtdCho (in 30–120 min). These changes in substrate preference are possibly connected to the differential expression of the two PLD isoforms and suggest that PtdEth could be a better substrate for cotton PLDα. We have recently confirmed this by PLDα activity assays using fluorescent (NBD-PtdCho and -Eth) substrates (Bourtsala et al., unpublished data).

To conclude, in this report we demonstrate the existence of a microsomal PLDα activity in *G. hirsutum*. Mechanical wounding causes a rapid upregulation of pre-existing PLDα activity both in wounded and systemic leaves, although this upregulation is accompanied by an equally rapid PtdOH increase only in the wounded leaves. However, in both wounded and systemic leaves, doubling of PtdOH levels is observed as a late response. Therefore, PLDα and δ are expressed differentially in response to wounding with PLDα displaying the strongest wound induction. However, it is only the PLDδ that exerts a high expression during systemic response to wounding. These data support a model where the local PLDα stimulation/PtdOH increase has

possibly a dual function: it may serve locally for cell membrane reorganization after the non-regulated loss of its integrity and systemically for a PLDδ-involving mechanism that prevent further damage of the plant tissue [43,44]. We believe that studies on stress-responsive enzymes of economically important plants, like cultivated cotton, may present opportunities for improving plant tolerance to environmental stresses and aid in the development of stress-tolerant species. We also propose that *G. hirsutum* is a plant very suitable for better understanding the roles of PLD/PtdOH system during plant defense responses.

Author contribution

T. Farmaki and D. Galanopoulou conceived the study and designed the research. A. Bourtsala and T. Farmaki performed experiments. A. Bourtsala, T. Farmaki and D. Galanopoulou analyzed data and wrote the paper.

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Appendix A. Transparency document

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.12.001.

Appendix B. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.12.001.

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