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

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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.

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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

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