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Published in:
Plant Journal

DOI:
10.1046/j.1365-313X.2001.01023.x

Link to publication

Citation for published version (APA):
Characterization of five tomato phospholipase D cDNAs: rapid and specific expression of LePLDβ1 on elicitation with xylanase

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Summary
Phospholipase D (PLD, EC 3.1.4.4.) has been implicated in a variety of plant processes, including signalling. In Arabidopsis thaliana a PLD gene family has been described and individual members classified into α-, β- and γ-classes. Here we describe a second PLD gene family in tomato (Lycopersicon esculentum) that includes three α- and two β-classes. Different expression patterns in plant organs were observed for each PLD. In testing a variety of stress treatments on tomato cell suspensions, PLDβ1 mRNA was found to rapidly and specifically accumulate in response to the fungal elicitor xylanase. The greatest increase was found 2 h after treatment with 100 μg ml⁻¹ xylanase (ninefold). In vivo PLD activity increased nearly threefold over a 1.5 h period of treatment. When the elicitor was injected into tomato leaves, PLDβ1 mRNA accumulation peaked at 2 h (threefold increase), before decreasing to background levels within 72 h. Mutant, non-active xylanase was as effective as the active enzyme in eliciting a response, suggesting that xylanase itself, and not the products resulting from its activity, functioned as an elicitor. When chitotetraose was used as elicitor, no PLDβ1 mRNA accumulation was observed, thus it is not a general response to elicitation. Together these data show that PLD genes are differentially regulated, reflecting potential differences in cellular function. The possibility that PLDβ1 is a signalling enzyme is discussed.

Keywords: phospholipase D, xylanase elicitor, signal transduction, tomato, phosphatidic acid, plant stress.

Introduction
Phospholipase D (PLD, EC 3.1.4.4) catalyses the hydrolysis of structural phospholipids to generate phosphatidic acid (PA) and a free head group. It is prevalent in plants where enhanced activity is associated with the response to: pathogens (Van der Luit et al., 2000; Young et al., 1996); wounding (Lee et al., 1997; Ryu and Wang, 1996, Ryu and Wang, 1998); water stress (Frank et al., 2000; Munnik et al., 2000); nod factor (Den Hartog et al., 2001); oxidative stress (Van Himbergen et al., unpublished data, SILS, University of Amsterdam); ethylene (Fan et al., 1997; Lee et al., 1998); and ABA (Fan et al., 1997; Frank et al., 2000; Jacob et al., 1999; Ritchie and Gilroy, 1998; Ritchie and Gilroy, 2000; Ryu and Wang, 1995). The fact that PLD activity is also stimulated by G-protein activators (De Vrije and Munnik, 1997; Frank et al., 2000; Munnik et al., 1995; Munnik et al., 1998b; Ritchie and Gilroy, 2000; Van Himbergen et al., 1999) indicates that it can transduce extracellular information into intracellular signals and responses. PA is thought to be the active signal, and a number of recent reports bear witness to its biological activity in plants (Den Hartog et al., 2001; Farmer and Choi, 1999; Jacobs et al., 1999; Munnik et al., 1995; Ritchie and Gilroy, 1998; Munnik, 2001). More traditionally, PLD was identified as a catabolic enzyme whose activity was associated with senescence, germination and ripening (Dyer et al., 1994; Munnik et al., 1998a). This clearly illustrates that PLD exists as different enzymes, fulfilling different roles in plants.

From Arabidopsis, four different PLD cDNAs have been cloned. They have been allocated into α, β and γ classes (the latter with two members), based on their amino-acid...
compositions and biochemical properties (Pappan and Wang, 1999; Wang, 1999). Several PLD genes from other plant species have also been cloned, but they all belong to the \( \alpha \) class (Wang, 2000).

Previously we have studied the role of PLD in plant signalling by using a biochemical approach based on \( { }^{32} \text{P} \)-phospholipid labelling and an assay to measure PLD activity \( \text{in vivo} \) (Munnik et al., 1995). To further increase our understanding, a molecular approach has been initiated. Here we describe the cloning and characterization of five tomato cDNAs, and provide evidence for their tissue-specific expression and differential regulation.

Results

Cloning and sequence analysis of the PLD cDNAs

PCR amplification of tomato cDNA using degenerated PLD primers produced a 530 bp fragment with a predicted amino-acid sequence that was up to 93% identical to a PLD from \textit{Nicotiana tabacum}. Using this fragment as a probe, a tomato cDNA library was screened. After the secondary screening, those with the strongest signals were selected and excised \( \text{in vivo} \). Eventually, five different cDNAs were cloned, sequenced and named \( \text{LePLD}_\alpha \text{1}, \text{LePLD}_\alpha \text{2}, \text{LePLD}_\alpha \text{3}, \text{LePLD}_\beta \text{1} \) and \( \text{LePLD}_\beta \text{2} \) (for \textit{Lycopersicon esculentum} phospholipase D). Southern blot analysis using specific probes (see Experimental procedures) indicated that these genes are present as single copy in the tomato genome (data not shown).

\( \text{LePLD}_\alpha \text{1}, \text{LePLD}_\alpha \text{2} \) and \( \text{LePLD}_\alpha \text{3} \) showed high identity and conserved amino-acid characteristics with \textit{Arabidopsis} class \( \alpha \) PLD (74, 69 and 61%, respectively), while \( \text{LePLD}_\beta \text{1} \) and \( \text{LePLD}_\beta \text{2} \) closely resembled the \( \beta \) class (60 and 67% identity, respectively). Figure 1 shows the amino-acid alignment of five tomato and two \textit{Arabidopsis} PLDs. Identical amino acids are boxed and equivalent amino acids shaded grey.

(a) \( \text{Ca}^{2+} \)-binding domain (C2) at the N-terminus of PLD; arrows indicate the eight \( \beta \)-sheet motifs and the dots the four potential \( \text{Ca}^{2+} \)-amino-acid ligands of the domain.

(b) Putative PIP\(_2\) binding domain; \# indicates the basic amino acids within the domain; asterisks indicate the HKD/phosphatidylyltranserferase motif (catalytic motif). \( \text{LePLD}_\alpha \text{1}, \alpha2, \alpha3, \beta1 \) and \( \beta2 \) have the following respective GenBank accession numbers: AY013252; AY013253; AY013254; AY013255; AY013256. The \textit{Arabidopsis} PLD\(_\alpha \) and \( \beta \) accession numbers are U36381 and U84568, respectively.
These residues are present in the complete C2 domains of PLDβ1 and γ (Qin et al., 1997; Zheng et al., 2000; indicated by dots in Figure 1a). Also in LePLDβ1 and β2, the three putative Ca²⁺-binding amino acids are conserved, whereas PLDαs lacks at least two of them (acidic amino acids have been substituted by basic or uncharged ones; Figure 1a, dots). Arabidopsis PLDβ1 and PLDγ require PIP₂ for activity whereas PLDα does not (Pappan et al., 1997a; Qin et al., 1997). A motif rich in basic amino acids [(K/R)(x)xxxKx(K/R)(K/R)] may bind PIP₂ (Qin et al., 1997). In LePLDβ1 and β2, three of four basic residues are conserved, but one has been replaced by an uncharged polar amino acid (Figure 1b, I and V, respectively, in the second PIP₂-binding domain indicated by #). Both LePLDβ1 and β2 lack the putative myristoylation site present in the γ-like PLDs (Qin et al., 1997; Wang, 1999). All LePLDs contain the two catalytic motifs HxKxxxxD (Figure 1b shows the second motif, indicated by asterisks) that are highly conserved in PLDs (Ponting and Parker, 1996), except for LePLDα3. In vitro expression studies are in progress to test whether that protein has PLD activity.

The PLD gene family is differentially expressed in plant tissues

Organ-specific expression of the tomato PLDs was examined under normal growth conditions. Roots, stems, old leaves (leaf number 13), young leaves (leaf number 4), apical leaves, young flowers, mature flower parts (petals, sepals, pistil, stamen and fertilized ovary) and fruits (green immature (I); green mature (III); orange (III); red (IV)) were harvested from 9-week-old tomato plants. Total RNA was extracted and subjected to RNA gel-blot analysis. Figure 2 shows that when gene-specific PLD probes were hybridized sequentially with the RNA blot, single transcripts of approximately 2.9–3.4 kb were labelled in each hybridization. Figure 2(a) shows the result for the PLDαs. PLDα2 and α3 transcripts were highly expressed in the flower parts, particularly the petals and stamens. In fruit, PLDα3 appeared to be transiently accumulated during early ripening, whereas PLDα2 was accumulated throughout fruit development and maturation. In contrast, a much lower level of PLDα1 was detected in stems and fruit, and a still lower level in roots and young leaves. Figure 2(b) shows that PLDβ1 mRNA was not prevalent. When the PLDβ1 mRNA signals were normalized to those of 18S rRNA, the values for roots and young leaves were higher than for other tissues. PLDβ2 transcripts could not be detected in any of the organs (data not shown). Together, these data show that each PLD has a typical expression pattern.

Constitutive and differential expression of tomato PLDs during stress treatments

Tomato cell suspensions were subjected to a variety of treatments previously shown to stimulate PLD activity or to induce PLD expression in other plant systems (see Introduction). Osmotic stress was imposed by adding NaCl or sorbitol (hyperosmotic stress), or by replacing half the medium with water (hypo-osmotic stress). Fungal elicitors, xylanase and chitotetraose (CH₄), were used, as well as H₂O₂ to generate oxidative stress. Hormone treatments were performed by adding abscisic acid (ABA), the ethylene precursor ACC, or CEPA that chemically liberates ethylene (see Experimental procedures). Physical stresses such as heat, cold and drought were also imposed. Cells were harvested after 1 h and RNA blot analysis was performed (Figure 3a). The PLDβ1 hybridization gave some background, probably because it co-migrates with the ribosomal 28S rRNA. The blots were scanned using a phosphoimager, and all hybridization signals were normalized to the levels of 18S rRNA. Figure 3(b) shows the values for PLDβ1 and PLDα1 mRNA accumulation. The PLDβ1 transcript accumulated on treatment with xylanase (more than fivefold) and NaCl (more than twofold) compared to control cells harvested at the beginning or end of...
the experiment (Figure 3b, c₀ and cₙ). Treating seedlings for 3 h with 200 mM NaCl did not enhance PLDβ₁ mRNA accumulation (data not shown), whereas xylanase did (see below). Interestingly, chitotetraose did not elicit the same response as xylanase, suggesting it is not a general elicitor response. The other LePLDs were either not, or hardly, affected by these treatments (Figure 3a). The only other treatment that appeared to have an effect was with ABA, where both PLDα₁ and β₂ levels increased. However, the strong hybridization was over the whole electrophoretic region and the pattern could not be repeated in two subsequent experiments. PLDα₁ and α₂ have a more constitutive expression pattern compared with the rest. Their basal levels are also higher: the transcripts in non-stressed cells (c₀ or cₙ) were detected in autoradiograms exposed for 1 week, while the others required at least 2 weeks. The gapdh (glyceraldehyde-3-phosphate dehydrogenase) mRNA levels did not change during these stress treatments, and the level of 18S rRNA was used as a loading control.

Figure 3. PLD expression patterns in tomato cells subjected to different stress.
(a) RNA blot analysis. Cell suspensions (Msk8) were treated with: NaCl (250 mM); sorbitol (400 mM); water (hypo-osmotic stress); xylanase (100 µg ml⁻¹); chitotetraose (CH₄) (25 ng ml⁻¹); ABA (10 µM); drought (half the medium was removed); cold (4°C); heat (37°C); H₂O₂ (100 µM); CEPA (100 µM); or ACC (10 µM). c₀ and cₙ refer to non-treated cells at the 'start' and 'end' of the experiment, respectively. After 1 h cells were harvested and the RNA isolated. Total RNA was subjected to electrophoresis (10 µg per lane) in formamide gels, transferred to a nylon membrane and hybridized sequentially with PLD, gapdh and 18S rDNA probes.
(b) Relative amounts of PLDβ₁ and PLDα₁ mRNA after 1 h treatment. Signal intensities from the phosphoimages were quantified, normalized against 18S rRNA, and the relative intensities plotted, taking the mRNA level at the start of the experiment as 100% (c₀).

PLDβ₁ mRNA levels and PLD activity in xylanase-treated tomato cells
The pronounced and specific PLDβ₁ mRNA accumulation during xylanase treatment was studied further by performing time- and dose-response experiments. To obtain a cleaner signal on the RNA blots, mRNA was isolated from total RNA and hybridized with PLDβ₁ probe (Figure 4a). Figure 4(b) shows the levels of PLDβ₁ mRNA normalized to the levels of 18S rRNA from at least two experiments, where the Northern blots were performed with total RNA. Expression levels increased within 30 min and increased dramatically within 1–2 h (four- to 10-fold).

Figure 4. Xylanase induces PLDβ₁ expression in a dose- and time-dependent manner.
(a) Tomato cell suspensions were treated with 100 µg ml⁻¹ xylanase for up to 2 h or with 10, 50 and 100 µg ml⁻¹ xylanase for 1 h. Poly(A)+ RNA was isolated and subjected to electrophoresis (1.5 µg per lane) in formamide gels and hybridized with PLDβ₁ probe.
(b) PLDβ₁ mRNA levels relative to those in unstressed cells. Signal intensities from the phosphoimages of Northern blots of total RNA (10 µg per lane) hybridized with PLDβ₁ and 18S rRNA probes were quantified. PLDβ₁ mRNA signals were normalized against 18S rRNA and the relative intensities plotted, taking the mRNA level at the start of each experiment as 100% (c₀). The data shown represent means ± SD from at least two independent experiments.
Lipids were extracted, separated by EtAc TLC (b), and the radioactivity from xylanases was used: those from relative to the cell-free medium.

Suspension-cultured tomato cells were labelled with $^{32}$Pi for 3 h and then treated for 1.5 h with xylanase or cell-free medium in the presence of 0.8% 1-propanol. Lipids were extracted and separated using a TLC system that clearly separates the presence of 0.8% 1-propanol. The formation of phosphatidyl propanol (PPro) from all naturally occurring tomato phospholipids (Munnik et al., 1995). Lipids were extracted and separated using a TLC system that clearly separates PPro from all naturally occurring tomato phospholipids (Munnik et al., 1998b). Figure 5(a) shows the PPro spots. Their radioactivity levels were quantified by phosphoimaging and are shown in Figure 5(b). All xylanase species activated PLD, resulting in higher PPro levels. Xylanase from $T. viride$ was the most effective. The increased in PLD activity was threefold, in contrast to an increase of just 1.25-fold when PLD activity was measured only 15 min after adding xylanase (van der Luit et al., 2000). The non-enzymatic form from $T. resei$ was slightly less effective than the wild type, but this is in agreement with earlier reports (Enkerly et al., 1999; Furman-Matarasso et al., 1999). Thus xylanase itself is clearly recognized by tomato cells as an elicitor.

**Figure 5.** Elicitor-stimulated PLD activity in tomato cells. Suspension-cultured tomato cells were labelled with $^{32}$P, for 3 h and then treated for 1.5 h with xylanase or cell-free medium in the presence of 0.8% (v/v) 1-propanol. (a) Lipids were extracted, separated by EtAc TLC (b), and the radioactivity in phosphatidylpropanol (PPro) quantified by phosphoimaging. Different xylanases were used: those from $T. viride$ (Tv) and the wild type $T. resei$ (Tr) as well as a non-catalytic form from $T. resei$ (TrM). Data represent the averages of three independent experiments ± SD and are expressed relative to the cell-free medium.

The response was detected within 1 h using as little as 10 µg mL$^{-1}$ xylanase.

As PLDβ1 mRNA was strongly accumulated after 1 h of xylanase treatment, we tested whether this was correlated with an increase in PLD activity. Plant cells can perceive xylanase directly by a receptor for this protein (Hanania and Avni, 1997) or indirectly via plant cell-wall fragments generated by its enzymatic activity (Bucheli et al., 1990). In order to find out whether the enzymatic activity of xylanase was required, cells were treated with two forms of *Trichoderma resei* xylanase: a mutated form (TrM) that does not have any xylanase activity but can still function as an elicitor (Enkerly et al., 1999); and the wild-type form (Tr) that has both. *Trichoderma viride* xylanase (Tv), the form that was used throughout this study, was used as a control. To measure in vivo PLD activity, we used its ability to transfer the phosphatidyl group of its substrate to 1-propanol. The formation of phosphatidyl propanol (PPro) then provides a relative measure of PLD activity (van der Luit et al., 2000). The non-enzymatic form from $T. resei$ was slightly less effective than the wild type, but this is in agreement with earlier reports (Enkerly et al., 1999; Furman-Matarasso et al., 1999). Thus xylanase itself is clearly recognized by tomato cells as an elicitor.

**Accumulation of PLDβ1 mRNA in tomato leaves treated with xylanase**

To find out whether xylanase also elicits the accumulation of PLDβ1 mRNA in plants, fully expanded leaves were injected with *T. viride* xylanase. As shown in Figure 6(a), xylanase treatment induced a defence response, illustrated by the formation of necrotic regions after 72 h (Figure 6a), which was absent when leaves were injected with water. Figure 6(b) shows the PLDβ1 mRNA levels in leaflets injected with either water (c, control) or xylanase (x, 2.5 µg mL$^{-1}$) for 2, 4 and 72 h. The blot was scanned using a phosphoimager and all hybridization signals were normalized to the levels of 18S rRNA (Figure 6c). An increase in PLDβ1 was apparent after 2 h xylanase treatment (more than threefold compared to water), but thereafter declined over a long period, approaching the background level at 72 h (Figure 6b, c). When this blot was hybridized with PLDa1, no differences were found between control and xylanase treatments (Figure 6b, c). The injection itself slightly stimulated the transient accumulation of PLDβ1 and PLDa1 transcripts in both control and treated plants. As the same effect was found for gapdh transcript levels (data not shown), this probably reflects a slight increase in leaf metabolism during the first hours of treatment. As the extracellular pathogenesis-related protein PR-1 plays a role in plant defence (Alexander et al., 1992), we used its expression as a positive control during these experiments. Figure 6(b) shows that PR-1 mRNA accumulated much later than PLDβ1 mRNA.

The mutant xylanase (TrM) was also effective in eliciting PLDβ1 mRNA accumulation (Figure 6d, left panel), although slightly less when compared with *T. viride* (Figure 6d, right panel).

**Discussion**

We have described five tomato phospholipase D cDNAs. They are representatives of a gene family similar to the one reported for *Arabidopsis* (Wang, 1999; Wang, 2000). As more than one gene has also been reported for rice (Morioka et al., 1997), *Craterostigma plantagineum* (Frank
et al., 2000) and cabbage (Pannenberg et al., 1998), we can assume that all higher plants possess multigene PLD families. Despite the family relationship, there is no evidence for gene redundancy or for different PLDs playing similar roles, because gene replacement or silencing and over-expression studies in yeast, animal and plant cells have shown that the remaining family members cannot compensate for the altered expression of their kin (Colley et al., 1997; Fan et al., 1997). This indicates that different PLDs play different roles in cell physiology.

The diversity in plant PLDs was made clear by Wang’s group (Kansas State University), who separated them into three classes based on their genetic and biochemical properties (reviewed by Pappan and Wang, 1999). The five tomato genes described here fall into the α and β classes, based solely on sequence data. The presence of two PLDβs is noteworthy, because only one other in Arabidopsis has so far been described. With the exception of these three PLDβs and two Arabidopsis PLDγ cDNAs (Pappan et al., 1997a; Qin et al., 1997), all PLDs reported in databases (about 25) belong to the α class, probably reflecting the general abundance of PLDα mRNAs. Whereas Arabidopsis has only one α, tomato has three, with clear differences in tissue expression.

One of the reasons for the occurrence of PLD gene families is that PLD is involved in very different cell functions, ranging from large-scale phospholipid catabolism to the localized production of PA as second messenger (reviewed by Munnik et al., 1998a; Munnik, 2001). Therefore the question is whether the tomato PLDs described here have a metabolic or signalling function. One can expect PLDs involved in signalling to be low abundance proteins and, if they are activated by Ca2+, they must respond to in vivo cytosolic concentrations. This favours the PLDβs as signalling enzymes because they are less prevalent than PLDαs (Fan et al., 1999), and one of the

Figure 6. Xylanase-activated PLDβ1 expression and necrosis in tomato leaves.
(a) Photograph of tomato leaves 72 h after injecting water or xylanase.
(b) RNA blot analysis. Total RNA from water- and xylanase- (20 µL of 2.5 µg µL−1) injected leaflets (2, 4 and 72 h) was subjected to electrophoresis (10 µg per lane) in formamide gels and hybridized sequentially with PLDβ1, PLDα1, PR-1 and 18S rDNA probes.
(c) Changes in PLDβ1 and PLDα1 mRNA levels relative to control experiments. Signal intensities were quantified as described and normalized against the 18S rRNA signal. Relative intensities were plotted against water control.
(d) Xylanase catalytic activity is not required to elicit PLDβ1 expression in tomato leaves. Tomato leaves were injected with inactive xylanase (20 µL of 5 µg µL−1) from T. resei (TrM) or active xylanase from T. viride (Tv, 2.5 µg µL−1). After 2, 4 and 6 h, RNA was extracted and analysed as described before. C2 and C6 refer to water-injected leaves after 2 and 6 h (left panel); in the right panel C is the same after 4 h. As a loading control, EtBr-stained 28S rRNA is shown.
documented PLDβ1 properties is activation by 0.5–20 μM Ca^{2+} (Pappan et al., 1997b; Qin et al., 1997), concentrations that arise locally during signalling (Trewavas, 1999). In contrast, Wang’s group noted that PLDs were activated by non-cytosolic millimolar concentrations of Ca^{2+} or low, non-cytosolic pHs (Zheng et al., 2000).

A property of signalling enzymes in general is that treatments that activate them often rapidly enhance expression of their genes. The response could be a positive feedback mechanism to prime the cell for further stimulation (Hirt, 1999; Yamamoto and Matsui, 1998). In our experiments, PLDα expression levels did not respond markedly to treatments that can induce PLD signalling; however PLDβ1 expression did increase, particularly when tomato cells were challenged with xylanase. The response was rapid (within 60 min), dramatic (up to ninefold increase in mRNA after 2 h treatment; Figure 4) and transient (Figure 6). Xylanase is a proteinaceous elicitor that evokes a number of defence responses in tomato cells, ranging from the oxidative burst to ethylene biosynthesis and hypersensitive cell death (Felix et al., 1991, Felix et al., 1993, Felix et al., 1999). As we have shown here with intact plants, the reaction became visible as necrosis after a few days (Figure 6a). Such responses are activated by elicitor-induced signalling, which can involve a number of different routes (e.g. MAP kinases; Meskiene and Hirt, 2000), but in particular, xylanase treatment of tomato cells evokes lipid signalling that leads to PA formation within minutes (Van der Luit et al., 2000). Much of this lipid signal originated from phospholipase C and diacylglycerol kinase activities, but some was produced by PLD activation. When measured after 15 min xylanase treatment, the PLD activity was 1.25-fold compared with the control. However, as reported here, when PLD activity was measured after PLDβ1 expression had increased (1.5 h), the xylanase-stimulated activity was threefold. The accompanying increase in PLDβ1 expression therefore suggests that this gene could be responsible for xylanase-induced PLD signalling. Interestingly, xylanase itself must be the elicitor, as claimed recently (Enkerly et al., 1999; Furman-Matarasso et al., 1999), because non-catalytic xylanase was able to stimulate both PLD activity and the expression of PLDβ1. A xylanase receptor in the plasma membrane presumably binds this agonist before relaying the signal to effector enzymes such as PLD.

Although xylanase induced the expression of PLDβ1, chitotetraose did not. At first sight this is surprising, because chitinases are another elicitor that induces similar responses in tomato cells (Felix et al., 1993, Felix et al., 1999; Van der Luit et al., 2000). Nonetheless, differences are known: for example both cause alkalinization of the cell medium, but the kinetics for xylanase are slower. Also, xylanase activates the transcription of phenyl-ammonia-lyase and ACC oxidase, whereas chitotetraose does not. Significantly, Van der Luit et al. (2000) emphasized that xylanase activated PLD, whereas chitotetraose did not. Thus the elicitor-induced expression of PLDβ1 is specifically correlated with elicitor-induced PLD signalling. Experiments with other elicitors or pathogens are now needed to test this correlation further.

The importance of PLD as a signalling enzyme lies in the production of PA as a potential second messenger in plant cells (Munnik, 2001). Evidence for such a role is based on hormone and stress treatments stimulating PLD activity and PA production. In some cases, when PA was added to non-treated cells it activated the same responses (Jacob et al., 1999; Munnik et al., 1995; Ritchie and Gilroy, 1998). If PA is a second messenger, it must activate downstream components. Some are now being identified in plants, for example, PA has recently been shown to activate a MAP kinase signalling in soybean cells (Lee et al., 2001) and in vitro, it activates a calcium-dependent protein kinase from carrot cells (Farmer and Choi, 1999). In animal cells, PA is known to increase cytosolic Ca^{2+} concentrations and to stimulate the activities of several signalling enzymes, including isoforms of PLC, PIP 5-OH kinase, PLD, Raf kinase and cAMP phosphodiesterase (Munnik et al., 1998a; T.M., unpublished results). Finally, PA has been shown to stimulate a kinase that mediates the functional reconstitution of the NADPH oxidase complex in neutrophils (McPhtail et al., 1999), and homologues of a similar complex have been identified in tomato cells (Keller et al., 1998; Xing et al., 1997). We therefore speculate that the PA generated in xylanase-treated cells triggers the oxidative burst as part of a defence response that eventually leads to hypersensitive cell death. Together, these data emphasize that PLD and its product PA must be considered as important new signalling components in eukaryotes.

The prevalence of PLDαs in plant cells suggests a metabolic rather than a signalling role. Their dependence on millimolar Ca^{2+} concentrations or low pH for activity (Qin et al., 1997) suggests a catabolic role in plant vacuoles or during cell lysis, and is definitely not conducive to a role in signalling. The fact that a PLDα-antisense plant exhibited delayed senescence in response to hormone treatment (although it did not delay senescence in the control; Fan et al., 1997) is in line with this thinking, and suggests that they could be involved in large-scale lipid degradation associated with cell death. They could also be involved in lipid mobilization during seed formation and germination (Pappan and Wang, 1999). Lipid degradation and mobilization were the traditional roles ascribed to PLD and supported by the early work on PLD expression showing that levels were low in dry seed but increased during seed germination (Dyer et al., 1994; Ryu and Wang, 1996; Ueki et al., 1995). Although much of this work was performed before the discovery of PLD classes, it presumably depicts the expression of the PLDαs. We therefore
suggest that the high expression levels of tomato PLDcs in stamens and petals reflects this traditional role. For example, they could be involved in tapetum disintegration and petal wilting. It has also been reported that the phospholipid content of tomato fruits declines during ripening (Güçlü et al., 1989), therefore the high expression levels of two PLDcs in fruit could mean that they are involved in this process.

In contrast to our own results, where we did not observe stress-induced LePLD mRNA accumulation, two treatments have been shown to activate the expression of PLDcs in other systems. When rice leaves recognized the pathogen Xanthomonas oryzae, PLD expression increased (Young et al., 1996). This increase was on a time scale of days rather than minutes, and the enzyme appeared to have a role in membrane degradation leading to permeabilization, indicating a catabolic rather than a signalling role. Secondly, when the resurrection plant Craterostigma plantagineum was dehydrated, expression of the CpPLD-2 gene markedly increased (Frank et al., 1995). This increase was on a time scale of days rather than minutes, and the enzyme appeared to have a role in membrane degradation leading to permeabilization, indicating a catabolic rather than a signalling role.

In vivo PLD measurements

To assay PLD activity in living cells, the production of phosphatidylpropanol (PPro) was measured (Munnik et al., 1995). In brief, cells were pre-labelled with $^{32}$P, for 3 h and subsequently treated with cell-free medium or xylanase in the presence of 0.8% (v/v) 1-propanol for 1.5 h. Incubations were stopped and the lipids extracted as described before (Van der Luit et al., 2000). The $^{32}$P-labelled PPro was separated from the rest of the phospholipids on a heat-activated TLC plate using the organic upper phase of an ethyl acetate mixture: acetate/octane/formic acid/water (12 : 2 : 3 : 10; Munnik et al., 1995b). Lipids were visualized by autoradiography and quantified by phosphoimaging (Molecular Dynamics, Sunnyvale, CA, USA).

Screening tomato cDNA library – isolation of PLD cDNA clones

Based on the conserved amino-acid regions of all aligned PLDs, two degenerate oligonucleotides (oligo) were synthesized: oligo 1: 5’-(AC)G(ACGT)G(A)CC(AGCT)TGGCA(CT)GA(CT)-3’ and oligo 2: 5’-(AGCT)CC(CT)TC(A)GCTGGCCACAT(A)GCTGG-3’. With these oligonucleotides a PLD fragment was amplified using tomato cDNA made from total RNA from roots and stems using SUPERSCRIPT II for RT-PCR ( Gibco BRL, Breda, the Netherlands) and an oligo d’T primer as a template. The PCR program consisted of 30 cycles of 45 sec at 95°C, 45 sec at 53°C and 1 min at 72°C. The 530 bp product was cloned into pGEM-T easy (Promega, Leiden, the Netherlands) and transformed into Escherichia coli strain XL1-Blue. The PLD sequence was confirmed by the Thermo sequenase fluorescent-labelled primer cycle sequencing kit (Amersham Pharmacia Biotech, Roosendaal, the Netherlands).

A cDNA library from stems and roots of 11-week-old tomato plants that were infected with Fusarium oxysporum f. sp. lycopersici (race 2) was constructed using a ZAP-cDNA synthesis kit and a ZAP-cDNA Gigapack II gold cloning kit (Stratagene, La Jolla CA, USA). The cDNA library was transformed into E. coli strain XL1-Blue. A total of 1.2 x 10^6 plaques were transferred onto Hybond-N filters (Amersham). The 530 bp fragment was labelled by random priming (Stratagene) with [a$^{32}$P]-dATP and used as a hybridization probe. Filters were hybridized in modified Church’s solution (0.5 M phosphate buffer pH 7.2, 7% SDS, 10 mM EDTA; Church and Gilbert, 1984) overnight at 65°C. The filters were washed twice with 2 x SSC, 0.1% SDS for 15 min, 1 x SSC, 0.1%...
SDS for 30 min and 0.2 × SSC, 0.1% SDS for 15 min at 65°C. The phage DNAs isolated from the cDNA library were excised in vivo with ExAssist helper phage to recover pACT2 according to the instructions of the manufacturers (Stratagene). DNA sequencing was as described above. Analyses of the nucleotide and amino acids sequences were performed with CLUSTAL W multiple sequence alignment software, and the homology and identity were done using BLAST 2.0 at the National Center for Biotechnology Information (NCBI) (Altschul et al., 1997).

Probe selection

In order to isolate PLD-specific probes, a PCR reaction was performed using the PLD-containing pACT2 plasmid as templates, and oligo 1 and a pACT2-specific plasmid oligonucleotide as primers. The PCR products, of approximately 1.5 kb which corresponded to the 3′ region of the PLDs, were purified from agarose gel using the Ultrafree-MC centrifugal filter units (Millipore, Bedford, MA, USA) and labelled as described above. No cross-hybridization was found between the probes (not shown). A glyceraldehyde-3-phosphate dehydrogenase (gapdh) fragment (1 kb) from petunia and a tomato extracellular PR-1 clone P6 (van Kan et al., 1992) fragment (0.7 kb) were also used as probes. A radish 18S rDNA EcoRI fragment of 1.7 kb (Grellet et al., 1989) was used as a loading control probe in Northern blot experiments. The specific radioactivity of all probes was the same.

Northern blot analysis

Total RNA from tomato cells was isolated using the Trizol-LS reagent (Gibco) method. For isolating RNA from the different plant organs, hot phenol was used. Briefly, ground tissue was incubated at 65°C in 1/3 phenol and 2/3 1% SDS, 10 mM EDTA and 0.2 M NaAc pH 5. After phenol extraction, the RNA was precipitated overnight at 4°C in 2 M LiCl. Poly(A)+ RNA was isolated from total RNA using an mRNA purification kit according to the manufacturer’s instructions (Qiagen, Leusden, the Netherlands). For Northern blot analysis, 10 μg total RNA or 1.5 μg poly(A)+ was separated by denaturing 1.4% formaldehyde–agarose gel electrophoresis, transferred onto Hybond-XL (Amersham) nylon membranes, and hybridized with the different probes in modified Church solution at 65°C. The same washing conditions as described above were used. Hybridizing bands were visualized by autoradiography and quantified by phosphoimaging (Molecular Dynamics). Before re-hybridization, bands were stripped by washing the membrane with boiling 0.1% SDS.

Acknowledgements

Financial support for this work was provided by the Fundación Antorchas (A.M.L.), the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET; A.M.L.), the Royal Netherlands Academy of Arts and Sciences (KNAW; T.M.) and the Netherlands Organization for Scientific Research (NWO-PULS 805.48.05; T.M.).

We thank Dr G. Felix for kindly providing the xylanases from T. resei, Dr J. van Kan and Dr F. Grellet for kindly providing the PR-1 and the 18S rRNA probes, respectively. We thank our colleagues for helpful discussions.

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Isolation and characterization of an unusual repeated sequence from the ribosomal intergenic spacer of the crucifer *Sisymbrium irio*. Plant Mol. Biol. 12, 695–706.


Accession numbers: *LePLDα1*, AY013252; *LePLDα2*, AY013253; *LePLDα3*, AY013254; *LePLDβ1*, AY013255; *LePLDβ2*, AY013256.