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*When the going gets tough
And the stomach acids flow
When the cold wind of conformity
Is nipping at your nose
When some trendy new atrocity
Has brought you to your knees
Come with us we'll sail the Seas of Cheese*

~Primus

Voor mijn ouders

The role of phospholipase D in plant stress responses

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
prof.mr. P.F. van der Heijden
ten overstaan van een door het college voor promoties ingestelde
commissie, in het openbaar te verdedigen in de Aula der Universiteit
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Bastiaan Otto Robert Bargmann

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Outline of this thesis

This thesis compiles results obtained during Ph.D. studies into the role of phospholipase D in plant stress responses performed in the plant physiology lab at the University of Amsterdam. It contains six chapters: an introduction, four research manuscripts and a general discussion.

Chapter 1 is the introduction to this thesis and has also been published as a review of recent literature entitled The role of phospholipase D in plant stress responses (*Current Opinion in Plant Biology*, October 2006). It cites pertinent literature published before May 2006, and consequently includes chapter 4 (published in *The Plant Journal*, February 2006).

Chapter 2, entitled Multiple PLDs required for salt tolerance in plants, is based on earlier work conducted in dr. Munnik's lab, exploring PLD activity and PA signaling in response to salinity stress. Results show that α - and δ -class PLDs are activated by salt treatment and that knock-out of AtPLD α 1 and AtPLD δ in *Arabidopsis* leads to reduced salt tolerance.

Chapter 3 re-examines PLD involvement in the wounding response and is entitled Analysis of AtPLD α 1 and AtPLD δ function in the *Arabidopsis* wounding response: Locating the site of activity. This research was initiated subsequent to findings presented in chapter 2 concerning PLD activity upon cell-lysis and critically scrutinizes the location of PLD activity during the wounding response, demonstrating that this activity takes place in ruptured cells.

Chapter 4, entitled LePLD β 1 activation and relocalization in suspension-cultured tomato cells treated with xylanase, also builds on previous results obtained in the Munnik lab concerning PLD activation in response to treatment with a fungal elicitor derived from the green mold *Trichoderma viride*. Findings from silencing- and GFP-studies indicate that LePLD β 1 is activated and relocalizes from the cytosol to punctuate structures therein in a tomato cell-suspension culture treated with xylanase.

Chapter 5 moves the study of LePLD β 1 from cell-suspension cultures to entire plants and is entitled Investigating the role of LePLD β 1 in plant-pathogen interactions. Results show that *LePLD β 1* expression is induced *in planta* by xylanase and *Cladosporium fulvum* avirulence proteins and that silencing *LePLD β 1* does not affect elicitor-induced PLD activity or resistance and susceptibility in the interaction with *Fusarium oxysporum*.

Chapter 6 is the General discussion. Here, selected findings presented in this thesis are put in perspective and thoughts about PLD research are discussed.

Chapter 1

Introduction

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Summary

Phospholipase D (PLD) has been implicated in multiple plant stress responses. Its gene transcription and activity increase upon exposure to various stresses and manipulation of PLD protein levels leads to altered stress tolerance. The plant PLD family is relatively large, heterogeneous and different PLD isoforms have been shown to be involved in separate stress responses. PLD and its product phosphatidic acid exert their effects by functioning in signal transduction cascades and by influencing the biophysical state of lipid membranes. In this review, recent advances in the research of PLD involvement in plant stress responses are discussed and put in perspective.

Abbreviations: PLD, phospholipase D; PA, phosphatidic acid; PX, phox homology; PH, pleckstrin homology; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PIP₂, phosphatidylinositolbisphosphate; TLC, thin layer chromatography; NAE, *N*-acylethanolamine; DAG, diacylglycerol; ABA, abscisic acid; ROS, reactive oxygen species; JA, jasmonic acid

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1. Introduction

In nature, plants are exposed to widely varying circumstances. Conditions, such as temperature, water and nutrient availability, herbivore attack and disease pressure, vary from location to location, season to season and day to day. Because of the sessile nature of plants, the only way to thrive/survive is to adapt to changing surroundings quickly and efficiently. Higher plants are profoundly plastic and react to different stresses with specific responses in growth, development and metabolism. Fundamental understanding of these responses has great value, especially for agricultural sciences, as it could aid in the development of stress tolerant crop varieties.

Signal transduction lies at the heart of plant stress responses. Many signaling pathways are activated (often in concert) in response to various stresses in plants, including Ca^{2+} -, protein kinase-, protein phosphatase- and lipid-signaling cascades. It has been proposed that specificity is achieved by the combination and differential timing of the activation of different signaling pathways. The study of lipid signaling is a relatively young, up-and-coming field of research. Several low-abundant lipid species that are rapidly and transiently generated in response to various stresses have been proposed to function as second messengers (Meijer and Munnik, 2003). One of these is phosphatidic acid (PA). This phospholipid constitutes a minor portion of membrane lipids under control conditions and levels significantly increase upon exposure to numerous stresses (Munnik, 2001). Distinct PA generators are activated in stress responses; both phospholipase D (PLD) and the concerted action of phospholipase C and diacylglycerol kinase have been shown to contribute to PA production during stress responses.

This review focuses on the role of PLD in plant stress responses. The plant PLD family, PLD protein-structure and PLD enzymatic activity are canvassed and putative PLD functions are discussed. Furthermore, its involvement in plant responses to a number of prevalent stresses is scrutinized.

2. The phospholipase D enzyme

2.1 The PLD family

In comparison to mammals, which possess two PLD genes, or yeast, containing only one, the PLD family in plants is multitudinous and varied. The genome of the fully sequenced model plant thale cress (*Arabidopsis thaliana*) contains twelve PLD family members and this diversity has been found in assorted higher plant species (Elias *et al.*, 2002). PLD enzymes in eukaryotes are characterized by two highly conserved C-terminal catalytic domains and an N-terminal lipid-binding region (Figure 1). The two catalytic HxKxxxxD (HKD) motifs have been shown to interact and to be essential for lipase activity of rat PLD1 (Xie *et al.*, 2000).

Figure 1. Schematic representation of two PLD sub-families

Plant and mammalian PLD isoforms contain two C-terminal catalytic domains (HKD) and an N-terminal lipid-binding region. Most plant PLD classes harbor an N-terminal calcium-dependent lipid-binding domain (C2), whereas mammalian and plant ζ -class PLDs possess N-terminal phox homology (PX) and pleckstrin homology (PH) lipid-binding domains.

The plant PLD family can be divided into two sub-families based on the N-terminal lipid-binding domains (Figure 1). In *Arabidopsis*, two of the twelve PLDs contain a phox homology (PX) domain and a pleckstrin homology (PH) domain, whereas the remaining ten contain a C2 domain. PX and PH domains have been demonstrated to mediate protein membrane-targeting and are closely linked to polyphosphoinositide signaling (van Leeuwen *et al.*, 2004). C2 domains also mediate localization of soluble proteins to membranes by binding lipids in a calcium-dependent manner (Kopka *et al.*, 1998). Importantly, PX, PH and C2 domains have been implicated in protein-protein interactions as well. The plant PLD family can be subdivided further into six classes, based on sequence homology (Figure 2) and *in vitro* enzymatic activity (Table 1; section 2.2). The *Arabidopsis* genome contains 3 α -, 2 β -, 3 γ -, 1 δ -, 1 ϵ - and 2 ζ -class PLD isoforms; the latter class contains the PX and PH domains and shares more homology with yeast and mammalian PLDs than with other plant PLD classes (Figure 2).

2.2 Enzymatic activity

PLD catalyzes the hydrolysis of structural phospholipids, producing PA and a free headgroup. In plants, PLD has been shown to use phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) as substrates (Pappan *et al.*, 1998). The transphosphatidylation of the lipid moiety from the head group to a water molecule is catalyzed in a two-step reaction (Stuckey and Dixon, 1999). The headgroup is removed during the first step and a covalent phosphatidyl-PLD intermediate is formed between the phosphorus and the histidine in one of the two HKD motifs. Subsequently, this linkage is hydrolysed, releasing the enzyme and yielding PA in the second step.

Figure 2. PLD protein homology

The twelve *Arabidopsis* PLD protein sequences, a human (*HsPLD1*) and yeast (*Saccharomyces cerevisiae*, *ScPLD*) PLD protein sequence (included to illustrate the similarity between PLDs from yeast and animals and ζ -class plant PLDs) were aligned with ClustalX software (Gonnet 250) and are presented in a radial phylogenetic tree created using the TreeViewPPC program.

Aside from their sequences, different PLD classes can be discriminated on the basis of the prerequisites for *in vitro* activity of the purified (recombinant) protein (Table 1). Similar to the mammalian PLDs, the ζ -class PLD activity is calcium-independent and requires the presence of phosphatidylinositolbisphosphate (PIP_2) in the lipid substrate preparation and a neutral pH (Qin and Wang, 2002). The relatively closely interrelated β - and γ -class PLDs require calcium concentrations in the sub μM range, a neutral pH and PIP_2 for *in vitro* activity (Pappan *et al.*, 1997a; Qin *et al.*, 1997). δ -class PLD activity is stimulated by the addition of oleate, is PIP_2 -independent and can be detected in high μM - to low mM calcium concentrations at a neutral pH (Wang and Wang, 2001). Lastly, the α -class PLDs are active at an acidic pH, require mM calcium concentrations and are independent of PIP_2 (Pappan *et al.*, 1997b). Analysis of *in vitro* ϵ -class PLD activity has not been published to date. It must be noted that these conditions are not strictly defined and that there is some overlap. For instance, the *AtPLD* α 1 high calcium requirements can be abated by inclusion of polyphosphoinositides in the substrate preparation and further acidification of the assay buffer (Pappan *et al.*, 1999). Addition of PIP_2 to the substrate preparation in an *AtPLD* δ activity assay alleviates the need for oleic acid (Wang and Wang, 2001). Furthermore, the stimulation of *AtPLD* δ by oleic acid was found to be a surface dilution effect; as Triton X-100 was also demonstrated to stimulate its activity (Qin *et al.*, 2002). In conclusion, the *in vitro* activities likely

have little bearing on the *in vivo* activities, yet, the results obtained from *in vitro* analysis of protein extracts do have value and can give indications as to which PLD class is active where and when.

PLD activity *in vivo* is tightly regulated, as unbridled membrane-hydrolysis would be detrimental to cellular- and organel-integrity. Besides Ca^{2+} and PIP_2 , PLD activity in plants has also been shown to be regulated *in vitro* by α -subunits of heterotrimeric G-proteins (Lein and Saalbach, 2001; Zhao and Wang, 2004). This is in stark contrast to mammals, where PLD is regulated by heterotrimeric G-protein β/γ -subunits (Preininger *et al.*, 2006) and monomeric small G-proteins (Hiroyama and Exton, 2005). In addition, plant PLD activity has been reported to be regulated by actin *in vitro* in line with mammalian and bacterial PLD activity (Kusner *et al.*, 2003). Lastly, plant PLD activity has also been proposed to be regulated by phosphorylation in a study of *in vitro* BoPLD γ activity in wild cabbage (*Brassica oleracea*; Novotna *et al.*, 2003).

Table 1. Prerequisites for *in vitro* activity of the different plant PLD classes

class	PIP ₂ -dependence	pH	[Ca ²⁺]	tested isoform	reference
α	-	acidic	mM	AtPLD α 1	Pappan <i>et al.</i> , 1997b
β	+	neutral	sub μ M	AtPLD β 1	Pappan <i>et al.</i> , 1997a
γ	+	neutral	sub μ M	AtPLD γ 1	Qin <i>et al.</i> , 1997
δ^*	-	.	μ M-mM	AtPLD δ	Wang and Wang, 2001
ϵ	.	.	.	.	.
ζ	+	neutral	0	AtPLD ζ 1	Qin and Wang, 2002

* δ -class PLD activity distinguishes itself by being stimulated by oleic acid (and Triton X-100; Qin *et al.*, 2002)

A period (.) indicates that these conditions have not been thoroughly tested for this particular class

PIP₂, phosphatidylinositolbisphosphate

2.3 Techniques

Basal and, more interestingly, treatment-induced PLD activity can be examined both *in vitro* and *in vivo*. As outlined above, distinct PLD activities can be measured *in vitro* by varying the conditions of the assay. In this way, differences in PLD activities relative to protein content can be studied in separate tissues, subcellular fractions and in response to diverse stimuli. Differences found in such a way could reflect several biochemical processes *in planta*. Firstly, changes in *in vitro* activity could be caused by changes in PLD protein levels, either by altered translation and degradation, in the case of total protein extracts, or by translocation of PLD, in the case of subcellular protein fractions. Secondly, altered *in vitro* activity could be indicative of post-translational modification and regulation of PLD enzymes. Analysis of these changes in time and concomitant analysis of PLD protein levels can aid in distinguishing between these different possibilities.

Analysis of *in vivo* PLD activity necessitates the extraction of lipids from the biological sample. Care must be taken in order to ensure that lipolytic and lipid-modifying enzymatic activity is halted during this process, so that the analyzed lipid profile mirrors the *in vivo* composition (Roughan *et al.*, 1978). Analysis of the extracted phospholipids can give information about the generation of the PLD product PA and consumption of the different PLD substrates. Separation and quantification of the extracted phospholipids can be achieved in several ways; each

with its own advantages and disadvantages. Phospholipids can be separated by thin layer chromatography (TLC), visualized by iodine vapor and quantified by phosphorous content (Ryu and Wang, 1996) or fatty acid content (Wang *et al.*, 2000; Arisz *et al.*, 2000). Alternatively, visualization and quantification can be achieved by radiolabeling biological material with inorganic phosphate ($^{32}\text{P}_i$) and subsequent analysis of the TLC by autoradiography and phosphoimaging (Munnik *et al.*, 1995). Lastly, separation and analysis can be performed by the use of tandem mass-spectrometry (Welti *et al.*, 2002; Arisz *et al.*, 2003).

Identification of *in vivo* PLD activity can be accomplished by different means. PLD activity can be visualized separately from other PA producing reactions by making use of the enzyme's unique ability to use primary alcohols instead of water in its transphosphatidylation reaction (Munnik *et al.*, 1995; Arisz *et al.*, 2003). In this way, the production of phosphatidylalcohol can be taken as a measure of PLD activity. Reduced PA production in PLD knock-down or knock-out lines can give clear indications of the activation of specific PLD isoforms in response to certain cues and genetically modified plant lines have been used successfully in numerous studies of PLD involvement in stress responses (see section 4).

Different inhibitors have been used in PLD research, *e.g.* primary alcohols (Lee *et al.*, 2001; Romanov *et al.*, 2002), lyso-PE (Ryu *et al.*, 1997) and *N*-acylethanolamine (NAE; Motes *et al.*, 2005). However, *in vivo* inhibition of PLD activity has never been convincingly presented. Moreover, these inhibitors likely lack specificity and will have pleiotropic effects, thus, such studies should be interpreted with caution.

An interesting development in the field of lipid studies is the use of recombinant lipid-binding domains fused to fluorescent marker proteins. With this technique, lipid-binding domains specific for particular lipid species can be employed to visualize the generation and intracellular location of lipids produced in response to a certain stimulus. Such analyses have already been performed, for example in the study of $\text{PI}(4,5)\text{P}_2$ and diacylglycerol (DAG) responses (Schultz *et al.*, 2005; reviewed in Varnai and Balla, 2006). The identification of lipid-binding domains specific for PA makes it possible to use this technique in the study of *in vivo* PA production in future (Testerink and Munnik, 2005).

3. Phospholipase D function

3.1 Membrane degradation

An obvious putative function for PLD, or any lipase, is to breakdown membrane lipids and thereby degrade the membrane. If left unchecked, PLD activity can consume the bulk of membrane phospholipids (PC, PE and PG) and generate copious amounts of PA. In fact, the massive lipid breakdown noted in homogenized carrot tissue led to the discovery of PLD activity (Hanahan and Chaikoff, 1947). High PA content induces loss of the membrane bilayer phase, due to the conical shape of this phospholipid as compared to the cylindrical shape of structural phospholipids, and incites the formation of hexagonal phase lipid particles (Kooijman *et al.*, 2003). This process would cause loss of cell membrane integrity

and thus a loss of viability. The generated PA could be broken down further by phosphatases, acylhydrolases and lipoxygenases into soluble molecules, which could subsequently be absorbed and recycled. PLD in plants has been proposed function in this way in processes such as senescence (Fan *et al.*, 1997) and wounding (discussed in section 4.2).

3.2 Vesicular transport

PLD has been shown to be involved in vesicular trafficking events in several independent studies. It has been proposed to function both in the signaling that regulates vesicular transport processes and in the biophysical membrane reorganization during vesicle fission and fusion. In mammalian systems, PLD activity has been reported to be regulated by the small G-protein ARF, an important regulator of vesicular transport (Hiroyama and Exton, 2005; Kahn *et al.*, 2005). Furthermore, PLD has been demonstrated to function as a GTPase-activating-protein for dynamin, which plays an essential role in receptor-mediated endocytosis (Lee *et al.*, 2006). Additionally, PA influences membrane curvature because of its conical shape (Kooijman *et al.*, 2003; Kooijman *et al.*, 2005) and has been shown to affect the insertion dynamin into membrane bilayers (Burger *et al.*, 2000). In plants, PLD has been proposed to play a role in vesicular trafficking during polar cell-expansion in root hairs (Ohashi *et al.*, 2003) and in pollen tubes (Potocky *et al.*, 2003).

3.3 Membrane tethering

PLD has also been proposed to function as a tether between a protein and membranes (Munnik and Musgrave, 2001). This hypothesis makes use of the two-step transphosphatidyl reaction, and states that the phosphatidyl-PLD intermediate could serve as a transient membrane anchor. The hypothesis was based on the finding that a PLD was found to bind microtubules and the plasma membrane in tobacco cell-suspension cultures (Gardiner *et al.*, 2001). Separate groups have presented experimental data that support this hypothesis. The microtubule cytoskeleton organization is disrupted by addition of *n*-butanol in tobacco cell-suspensions (Dhonukshe *et al.*, 2003) and Arabidopsis seedling roots (Gardiner *et al.*, 2003) as well as by the addition of NAE in Arabidopsis seedling roots (Blancaflor *et al.*, 2003; Motes *et al.*, 2005). However, although it has been suggested that AtPLD δ is the corresponding microtubule-binding PLD in Arabidopsis, no lucid evidence to support this statement, such as a study of AtPLD δ -GFP localization, has been presented so far. Moreover, Arabidopsis *pld δ* knock-out and knock-down mutants do not display any growth or developmental phenotype (Li *et al.*, 2004; Katagiri *et al.*, 2001), which one would expect if this is the isoform that is involved in microtubule cytoskeletal organization.

3.4 Signal transduction

The PA produced by PLD has been proposed to be a membranous second messenger molecule. Several modes of action for PA in signal transduction can be imagined, based on recruitment and regulation of target-protein enzymatic activity. Recruitment of soluble proteins to particular membranes can have numerous effects:

(i) localization of a protein to the site where it is active, (ii) localization of a protein to a site where it is modified, (iii) sequestration of a protein from the site where it is active. Alternatively, PA could influence enzyme activity of proteins already residing in the membrane or of recruited proteins or protein complexes. A plethora of PA-binding proteins has been found in different organisms and many have been proposed to function in signaling cascades according to the above mentioned modes (see Testerink and Munnik, 2005). Signaling PA is believed to be principally produced either indirectly by the concerted action of a phospholipase C and diacylglycerol kinase or directly by a PLD (Munnik, 2001). Specificity of PA signals generated by different enzymatic routes or different PLD isoforms has been proposed to be achieved by the cellular- and intracellular location where the PA is produced and the fatty acid composition of the PA precursor (Testerink and Munnik, 2005; Wang *et al.*, 2006).

4. Phospholipase D involvement in plant stress responses

4.1 Absciscic acid, cold-, drought- and salinity-stress

Absciscic acid (ABA) is an important phytohormone that plays a crucial role in several plant stress responses including cold-, drought- and salinity-stress (Verslues and Zhu, 2005; Mahajan and Tuteja, 2005). It effects seed dormancy, induces leaf senescence and promotes stomatal closure upon water-deficiency. PLD has been shown to be involved in ABA responses in seed dormancy in barley (*Hordeum vulgare*; Ritchie and Gilroy, 1998; Ritchie and Gilroy, 2000), senescence in Arabidopsis (Fan *et al.*, 1997) and stomatal regulation in broad bean (*Vicia faba*; Jacob *et al.*, 1999), tobacco (Austin-Brown and Chapman, 2002) and Arabidopsis (Sang *et al.*, 2001a; Zhang *et al.*, 2004; Mishra *et al.*, 2006). *AtPLD α 1*-silenced Arabidopsis plants were reported to have an impaired ability to be rescued by ABA treatment upon drought treatment, excessive waterloss in detached leaves and reduced ABA-induction of stomatal closure (Sang *et al.*, 2001a). *AtPLD α 1* was proposed to mediate ABA signaling by sequestering the negative regulator of ABA signaling, ABI1, at the plasma membrane (see section 3.4; Zhang *et al.*, 2004) and by regulating ABA-induced G-protein signaling (Mishra *et al.*, 2006). Furthermore, PLD has been linked to reactive oxygen species (ROS), which are known to be involved in ABA, cold-, drought- and salinity-stress responses (Laloi *et al.*, 2004; Mahajan and Tuteja, 2005). In Arabidopsis, *AtPLD α 1* and *AtPLD δ* have been implicated in both the production of and responses to ROS (Sang *et al.*, 2001b; Zhang *et al.*, 2003; reviewed in Zhang *et al.*, 2005). *AtPLD δ* expression was shown to be induced by ABA, cold, dehydration and excessive salt in Arabidopsis plants and *AtPLD δ* -silencing indicated that this isoform was, at least in part, responsible for the dehydration-induced PLD activity (Katagiri *et al.*, 2001). Cold-induced freezing tolerance is reportedly impaired in *Atpld δ* T-DNA knock-out Arabidopsis plants and enhanced in *AtPLD δ* -overexpressing plants, which also display decreased and increased freezing-induced PA production, respectively (Li *et al.*, 2004). As a whole, it seems that PLDs (especially α - and δ -class) are involved in multiple aspects of both the overlapping and distinct signaling networks activated by cold-,

drought- and salinity-stress. Significantly, these stresses also bring about major membrane-rearrangement and -remodeling events caused by changes in membrane fluidity and osmotic balance and PLDs might well play important roles in such events.

4.2 Wounding

Wounding entails the loss of cell membrane integrity and can occur due to various factors, *i.e.* herbivory, freezing, severe dehydration, pathogen attack and wind bruising. PA levels have been reported to increase at the wound site and in surrounding (intact) tissues in mechanically wounded castor bean leaves (*Ricinus communis*; Ryu and Wang, 1996) and systemically in soy bean seedlings (*Glycine max*; Lee *et al.*, 2001). Mechanical-wounding- and freezing-induced PA production were demonstrated to be significantly reduced in *AtPLD α 1*-silenced Arabidopsis leaves, denoting this isoform's involvement (Wang *et al.*, 2000; Welti *et al.*, 2002). Wang *et al.* (2000) also showed that *AtPLD α 1*-silencing led to diminished wounding-induced jasmonic acid (JA) production and gene expression. *AtPLD α 1* was proposed to function in the wounding response by producing PA as a precursor for JA synthesis (Wang *et al.*, 2000). Lee *et al.* (2001) suggested that PLD activity lies upstream of wounding-induced MAPK signaling, as evidenced by a correlation of PA production and MAPK activation, the inhibition of MAPK activation by *n*-butanol and MAPK activation by application of exogenous PA in a cell-suspension. Overall, it is clear that PLDs are involved in the wounding response but which PLD-class or -isoform is active where needs to be investigated in more detail in order to get a better understanding of PLD function in this response.

4.3 Pathogen interactions

The plant's response to pathogen attack is highly dependent on its ability to recognize the attack in time. Pathogenic elicitors are perceived by the plant and trigger defense responses leading to disease-resistance in the case of an incompatible interaction. In the case of a compatible interaction, the plant lacks the ability to recognize the elicitor(s) and is unable to activate its defense responses in time, leading to disease-susceptibility. In Arabidopsis, α -, β - and γ -class PLD transcripts have been demonstrated to be increased after infiltration with both virulent and avirulent strains of *Pseudomonas syringae* pv. *tomato* (de Torres-Zabala *et al.*, 2002). PLD activity in transgenic Arabidopsis plants inducibly expressing the *P. syringae* avirulence proteins Rpm1 and Rpt2 has been shown to increase in response to induction of elicitor expression (Andersson *et al.*, 2006). Yamaguchi *et al.* (2005) reported increased PLD activity in a rice (*Oryza sativa*) cell-suspension culture treated with an *N*-acetylchitoooligosaccharide elicitor and implied its involvement in ROS production by making use of exogenous application of PA and *n*-butanol as a PLD inhibitor. Increased PLD activity has also been found in tomato (*Lycopersicon esculentum*) cell-suspension cultures treated with the fungal elicitor xylanase (van der Luit *et al.*, 2000) and *LePLD β 1* was shown to be transcriptionally up-regulated both in tomato cell-suspensions and in leaves (Laxalt *et al.*, 2001). A study of *LePLD β 1*-silenced cell-suspensions indicated that this isoform is responsible for the increased elicitor-induced PLD activity and implicated its involvement in ROS

production, polyphenol oxidase activity and cell-wall remodeling (Bargmann *et al.*, 2006). Furthermore, LePLD β 1-GFP was demonstrated to relocate from the cytosol to punctuate structures therein in response to xylanase treatment (Bargmann *et al.*, 2006). Together, these reports indicate that PLD is involved in the plant's response to pathogenic elicitation, although its importance in disease resistance or susceptibility has yet to be demonstrated.

4.4 Phosphate starvation

The phosphorus in phospholipids accounts for a significant share of the phosphate content of plant cells. Upon phosphate starvation this reserve is accessed by phospholipid breakdown; the consequent decline of phospholipids is compensated for by increased galactolipid synthesis (Andersson *et al.*, 2005). Two independent reports have recently demonstrated the involvement of ζ -class PLDs in the response to phosphate starvation (Li *et al.*, 2006; Cruz-Ramírez *et al.*, 2006). The latter study showed that *pld ζ 2* single knock-out mutants have a root growth phenotype when transferred to low phosphate (from 1 mM to 1 μ M P_i) containing agar plates; while the former study found a phenotype (500>25 μ M) only when both ζ -class PLDs were absent in a double knock-out mutant. Li *et al.* (2006) suggested that PA generated by AtPLD ζ s promotes root elongation via activation of an AtPDK1-regulated protein kinase cascade, which has previously been linked to root growth (Anthony *et al.*, 2004). Cruz-Ramírez *et al.* (2006) proposed that PA generated by AtPLD ζ 2 upon phosphate starvation is dephosphorylated by a PA phosphatase, releasing the phosphorus, and that the DAG produced in this way is used for galactolipid synthesis. Analysis of lipid phosphate phosphatase mutants (*e.g.* Katagiri *et al.*, 2005) under phosphate-limited growth conditions could strengthen the latter hypothesis.

5. Perspectives

Over recent years extensive evidence has been accumulated of PLD involvement in a wide variety of plant stress responses. Different roles have been suggested for PLD in these responses, both in stress-induced signaling events and in direct stress responses. It has become clear that members of the large PLD family in plants have individual functions, although some overlap also appears to exist. The α - and δ -class PLDs are the most abundantly expressed isoforms in Arabidopsis plants (Li *et al.*, 2006), have both been linked to water-deficit stresses and are both activated by freezing-induced injury. In contrast, β - and γ -class PLDs have low basal expression levels but are rapidly induced by wounding and pathogenic elicitation. Lastly, ζ -class PLDs have low expression levels and seem to be involved in root architecture and phosphate homeostasis.

Analysis of PLD-silenced lines and T-DNA insertion mutants makes it possible to assess the function of distinct PLD isoforms to various stresses. Although silencing doesn't guarantee complete knock-out or specificity, it is preferred over inhibitor studies, which relatively give the least degree of certainty. Generation of multiple-*pld* knock-out lines could intensify the phenotypes already

described for single knock-out mutants and expose new roles for PLD in future. A better understanding of the functions of the different PLD isoforms will also be aided by intracellular localization studies of the enzymes themselves and of the PA produced in response to particular stresses. Lastly, identification and further examination of the upstream regulatory components involved in PLD activation during plant stress responses is essential to unraveling the roles of this intriguing enzyme family.

6. References

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Chapter 2

Multiple PLDs required for salt tolerance in plants

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Summary

High salinity stress has received much attention because it severely affects crop production worldwide. Analysis and comprehension of the plant's response to excessive salt will aid in the development of salt tolerant crop varieties. Signal transduction lies at the basis of the response to high salinity stress and numerous signaling pathways have been implicated in this process. Here, we provide further evidence for the involvement of phospholipase D (PLD) – mediated lipid signaling. A tomato (*Lycopersicon esculentum*) α -class PLD gene, *LePLD α 1*, is transcriptionally upregulated in cell-suspension cultures treated with salt. We show that the ubiquitous LePLD α 1 protein is active in salt-stressed tomato cells by making use of *LePLD α 1*-silenced cell-suspension cultures. Analysis of the phospholipid composition in *LePLD α 1*-silenced cell-suspensions and tomato plants indicates that there is no effect of LePLD α 1 knock-down on basal phospholipid homeostasis. Interestingly, massive PLD activity is triggered upon loss of cell membrane integrity, invoked by extracellular acidic pH and high Ca^{2+} concentrations. LePLD α 1 is one of the PLDs active under these conditions and is shown to use phosphatidylcholine, phosphatidylethanolamine and phosphatidylglycerol as substrates for the production of phosphatidic acid. In Arabidopsis, both AtPLD α 1 and AtPLD δ are activated in response to salt stress and, although *LePLD α 1*-silenced tomato seedlings displayed no phenotype, *pld α 1* and *pld δ* single- and double-knock-out mutants exhibited enhanced sensitivity to salinity stress when grown on high salt containing agar plates. Implications of these findings are discussed.

Abbreviations: PLD, phospholipase D; PC, phosphatidylcholine; PA, phosphatidic acid; PIP₂, phosphatidylinositolbisphosphate; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; Pbut, phosphatidylbutanol; PIP, phosphatidylinositolmonophosphate; PI, phosphatidylinositol; FDA, fluorescein diacetate; LPA, lysophosphatidic acid; PLC, phospholipase C; DGK, diacylglycerol kinase

Introduction

Plant growth and crop production are severely affected by high salinity stress. Excessive salt has a dually adverse effect on plant cells; it induces hyperosmotic shock and causes ionic imbalance (Flowers, 2004; Mahajan and Tuteja, 2005). The cytoplasm of plant cells is normally hypertonic compared to its surroundings, fostering turgor pressure and thereby plant rigidity. Hyperosmotic extracellular conditions lead to a loss of turgor that necessitates a response from the plant in order to survive. Plants respond by accumulating intracellular osmolites, consequently reacquiring their hypertonic state and regaining/maintaining rigidity (Yancey *et al.*, 1982; Fricke, 2004). Salinity stress is also detrimental because it disturbs ion homeostasis. High cytosolic sodium concentrations are toxic and plants respond to this condition by removing the sodium from the cytosol, making use of antiporter and coporter ion channel activity across the plasma- and vacuolar membranes. This ion flux causes a loss of homeostasis that has to be actively restored (Kinraide, 1998; Zhu, 2003; Yamaguchi and Blumwald, 2005). The activation of the high salinity response comes at a cost and plants that have activated this machinery will have less energy to put into, for instance, fruit production.

Salt tolerance depends on complex signaling networks, allowing plants to respond rapidly and efficiently to high salinity stress (Zhu, 2001). Signal transduction in response to salt stress has become an intensively studied subject because it is believed that a better understanding of this process will lead to the discovery of ways to generate salt tolerant crops that do not have a fitness penalty (Kasuga *et al.*, 1999; Flowers, 2004; Jakab *et al.*, 2005; Yamaguchi and Blumwald, 2005). Numerous signal transduction pathways have been demonstrated to be activated in response to high salinity stress (Munnik and Meijer, 2001; Zhu, 2001; Xiong *et al.*, 2002). Our interest focuses especially on lipid signaling events and in particular on phospholipase D (PLD) activity upon salt stress.

PLD catalyses the hydrolysis of structural phospholipids such as phosphatidylcholine (PC), producing phosphatidic acid (PA) and a free head-group. Roles have been suggested for PLD in numerous processes including vesicular transport, membrane degradation and intracellular signaling. PLD has been reported to be involved in signaling events occurring in response to a multitude of stimuli, *e.g.* freezing, wounding, plant-pathogen interactions, dehydration and salt stress (reviewed in Wang, 2005). PA is believed to act as a second messenger in signaling events; it is generated rapidly and transiently during various stress responses (Munnik, 2001). PA has been proposed to function in signaling cascades by recruiting target proteins to particular membranes and/or influencing their activity (Testerink *et al.*, 2004; Testerink and Munnik, 2005).

Twelve PLD genes are present in the genome of the model plant thale cress (*Arabidopsis thaliana*), whereas just two PLD genes are found in animals. The plant PLD family can be partitioned into six classes, the α -, β -, γ -, δ -, ϵ - and ζ -PLDs, depending on the protein sequence homology and biochemical properties (Qin and Wang, 2002; Wang, 2005). Laxalt *et al.* (2001) cloned five members of the PLD family from tomato and examined their expression in different organs of the plant. These authors went on to investigate changes in *PLD* expression in a tomato cell-

suspension culture treated with various stimuli. For instance, *LePLD β 1* expression was found to be induced by the fungal elicitor xylanase. This finding was investigated further by targeting *LePLD β 1* for RNAi-mediated gene silencing in tomato cell-suspension cultures and subsequent analysis of the effects of *LePLD β 1* knock-down on the response to xylanase (Bargmann *et al.*, 2006). Because *LePLD α 1* expression is increased upon treatment of tomato cell-suspension cultures with salt (Laxalt *et al.*, 2001), we decided to explore this finding in detail in tomato cell-suspensions and plants as well as Arabidopsis plants.

An RNAi construct was used to target *LePLD α 1* for gene silencing in tomato cell-suspension cultures and tomato plants. The effect of silencing was studied in respect to phospholipid composition, *in vitro* PLD activity and *in vivo* PLD activation. High salt-induced PLD activity was examined in *LePLD α 1*-silenced tomato cell-suspension cultures and Arabidopsis *AtPLD α 1* and *AtPLD δ* T-DNA-insertion mutants. The effect of PLD-silencing and knock-out on salt tolerance was investigated by using a plate-root-growth assay.

Results

Silencing LePLD α 1 in a tomato cell-suspension culture

Plant cell-suspension cultures are a useful model system for biochemical research because they can be treated with various conditions in parallel, are homogeneous and can be genetically modified relatively easily and fast. The tomato cell-suspension culture Msk8 has been used in several studies to investigate plant responses to both biotic stress (Felix *et al.*, 1991; Felix *et al.*, 1999) and abiotic stress (Felix *et al.*, 2000). Our lab has also used this cell-suspension culture in the study of lipid signaling and especially the function of PLD therein (van der Luit *et al.*, 1999; Munnik *et al.*, 2000; Laxalt *et al.*, 2001; Bargmann *et al.*, 2006). Here, an RNAi construct was used to knock-down *LePLD α 1* (see Methods). 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 were silenced on the mRNA transcript level (Figure 1a). This finding was confirmed on the protein level by protein blot analysis, showing that the *LePLD α 1* protein was absent in the lines lacking *LePLD α 1* transcript (Figure 1b). These results indicate that we have successfully knocked-down *LePLD α 1* in tomato cell-suspension cultures.

PLD activity can be measured *in vitro* using a fluorescent lipid substrate. Furthermore, several types of *in vitro* plant-PLD activities are distinguishable, depending on their pH, calcium and phosphatidylinositolbispophate (PIP₂) requirements (Wang, 2002). PLD α activity can be distinguished from other classes by performing the assay at an acidic pH (pH 6.5) with high calcium concentrations (10 mM CaCl₂) and omitting PIP₂ in the PC lipid substrate preparation (Pappan *et al.*, 1997). Under these conditions, the PLD activity in silenced cell-suspension cultures was greatly reduced (Figure 1c). Compared to control and non-silenced transgenic cultures, the silenced lines displayed an approximate 90% reduction in α -

class PLD activity. This result indicates that LePLD α 1 is responsible for the bulk of the *in vitro* α -class PLD activity found in a tomato cell-suspension culture.

Phospholipid composition can be monitored *in vivo* by labeling a biological sample with radioactive inorganic phosphate ($^{32}\text{P}_i$) and subsequently extracting, separating and analyzing radiolabeled lipids. Phospholipid species, such as phosphatidylglycerol (PG) or phosphatidylethanolamine (PE), are then quantified and their relative ratio's examined. Moreover, the fatty acid composition of the different phospholipids was scrutinized by transmethylation and subsequent gas chromatographic analysis. No difference in phospholipid composition was discernible when lipids were extracted from labeled empty vector control and *LePLD α 1*-silenced cell-suspension cultures, separated by two-dimensional thin layer chromatography and analyzed (Figure 1d and e). Analysis of the fatty acid composition of the major phospholipid species (data not shown) and of total lipids also showed no difference between control and *LePLD α 1*-silenced cell-suspension cultures (Figure 1f). The data demonstrates that, although the LePLD α 1 protein and most of the *in vitro* α -class PLD activity was lacking in *LePLD α 1*-silenced cell-suspension cultures, there was no effect on phospholipid homeostasis.

Figure 1. Analysis of *LePLD α 1*-silenced tomato cell-suspension cultures

(a) RNA was extracted from 5 independently transformed Msk8 cultures carrying an *LePLD α 1*-silencing construct and one carrying an empty vector (C). RNA was separated by gel electrophoresis, blotted and hybridized with ^{32}P -labeled *LePLD α 1* and *GAPDH* (loading control) probes. (b) Proteins were extracted from the Msk8 lines, separated by SDS-PAGE and blotted or stained with Coomassie Brilliant Blue as a loading control. *LePLD α 1* was detected using a polyclonal antibody directed toward the final 12 amino acids of *LePLD α 1*. (c) Proteins extracts were assayed for *in vitro* α -class PLD activity alongside the extraction buffer alone and commercially available cabbage PLD. The transphosphatidylation of BODIPY-phosphatidylcholine (PC) to BODIPY-phosphatidic acid (PA) and BODIPY-phosphatidylbutanol (Pbut) was visualized by separation of lipids on a TLC plate (n=2). (d-f) Phospholipid- and fatty acid analysis was performed on wildtype (wt) and transgenic Msk8 cell-suspension cultures (carrying an *LePLD α 1*-silencing construct or an empty vector [control]). Lipids were extracted from $^{32}\text{P}_i$ -labeled cell-suspensions and separated by 2-dimensional TLC, the chromatogram was run using an alkaline solvent system in the first dimension and an acid solvent system in the second dimension. The origin of the chromatogram, phosphatidylinositolbisphosphate (PIP₂), phosphatidylinositolmonophosphate (PIP), phosphatidylinositol (PI), PC, phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and PA are indicated. A control line (d) and a representative *LePLD α 1*-silenced line (e) are depicted. (f) Fatty acids from total lipid-extracts were hydrolysed and methyl-esterified for GC-analysis. The 16:0, 18:0, 18:1, 18:2 and 18:3 fatty acid methyl esters were quantified.

LePLD α 1 activity upon loss of cell membrane integrity in tomato cell-suspensions

The α -class PLDs require an acidic pH and mM calcium concentrations in order to be active *in vitro* (Pappan *et al.*, 1997). These conditions are in strong contrast to those found in the cytosol, where the pH is near-neutral (pH $\sim$ 7.2; Gao *et al.*, 2004) and calcium concentrations are in the low- to sub- μ M range ($<10^{-5}$ M; Knight *et al.*, 1997). Rather, these conditions resemble the plant apoplast which is acidic (pH $\sim$ 6.3; Gao *et al.*, 2004) and contains relatively high amounts of free calcium ($>10^{-3}$ M; Björkman and Cleland, 1991; Cabañero *et al.*, 2006).

It has been noted before that there is increased PLD activity upon disruption of compartmentalization (Roughan *et al.*, 1978; Wang, 2005), yet it has not been investigated which PLDs or PLD-classes are activated. Going by the correlation between *in vitro* requirements and *in planta* conditions, it seemed likely that α -class PLDs could become active when apoplastic conditions are encountered. Plant cell-suspension cultures are generally grown in media that are similar to conditions found in the apoplast. In this case, Msk8 tomato cell-suspensions were used that were cultured in Murashige and Skoog medium with a pH of 5.7 and which contains a final calcium concentration of 2 mM (see Methods). In order to achieve loss of cell membrane integrity, cells were snapfrozen in liquid nitrogen and subsequently thawed, this treatment led to a 100% loss of cell membrane integrity as judged by vitality staining with fluorescein diacetate (FDA; data not shown). When $^{32}\text{P}_i$ -labeled cells (100 μ l aliquots) were treated in this way and their phospholipid composition followed through time, a remarkable PA production could be observed (Figure 2). After samples had been removed from the liquid nitrogen, PA levels increased from minor basal levels up to 40% of total radiolabeled phospholipids within 10 minutes, reaching an extraordinary 70% after 160 minutes. Concomitantly, the levels of the structural phospholipids PC, PE and PG decreased dramatically, while levels of phosphatidylinositol (PI) stayed relatively stable. An increase in lysophosphatidic acid (LPA) from negligible levels up to 6% could be seen following the PA increase (Figure 2). These data suggest that the structural phospholipids PC, PE and PG (and not PI) are rapidly and massively converted to PA upon loss of cell membrane integrity.

The PA and LPA increase in *LePLD α 1*-silenced cell-suspension cultures was significantly reduced when compared to control lines (Figure 2b). PA levels were consistently lagging behind the control lines, reaching just 10% after 10 minutes (compared to 40% in the control cells) and reaching merely 60% after 160 minutes. Concurrently, the decrease in PC, PE and PG was also slower in the *LePLD α 1*-silenced cell-suspension cultures. The decrease in phosphatidylinositol-monophosphate (PIP) and PIP₂, both of which completely disappeared within 10 minutes, and the slight decrease in PI seen after 160 minutes were identical in control and silenced cell-suspension cultures (Figure 2b). This result indicates that the PA production observed upon loss of cell membrane integrity is in part derived from *LePLD α 1* activity and that this PLD isoform can use PC, PE and PG as substrates.

Figure 2. PLD activity upon loss of cell membrane integrity

³²P_i-labeled control (black bars) or *LePLDα1*-silenced (hatched bars) cell-suspension cultures were left untreated or snapfrozen-and-thawed for 5 to 160 minutes. Lipids were extracted, separated by alkaline TLC (a) and analyzed by phosphoimaging (b). Phospholipids were quantified as percentage of total radiolabeled lipids and are presented in a histogram $\pm$ SD (n=3).

In order to investigate whether it was indeed the extracellular pH and calcium concentrations that induced the PLD activity after loss of cell membrane integrity, cells were lysed in the presence of Tris-HCl (pH 7.5) and/or EGTA to buffer protons and Ca²⁺, respectively. Buffering the pH of the growth medium to 7.5 with 50 mM Tris-HCl and chelating free [Ca²⁺] with 10 mM EGTA both individually decreased lysis-induced PA production and almost completely blocked this activity when applied in combination (Figure 3). These results show that PLD activity upon loss of cell membrane integrity in a tomato cell-suspension culture is dependent on the low extracellular pH and high calcium concentrations.

Figure 3. pH and calcium dependency of PLD activity upon loss of cell membrane integrity

^{32}P -labeled cell-suspensions were left untreated or snapfrozen-and-thawed for 10 minutes in the absence or presence of 50 mM Tris-HCl pH7.5 and/or 10 mM EGTA. (a) Lipids were extracted, separated by ethyl acetate TLC and analyzed by phosphoimaging. (b) PA was quantified as percentage of total radiolabeled lipids and is presented in a histogram $\pm$ SD (n=3).

LePLD α 1 activity increases upon high salinity stress in a tomato cell-suspension

PLD activity is increased in response to a wide variety of stimuli (Wang, 2002). The acquisition of *LePLD α 1*-silenced cell-suspension cultures allowed us to investigate which stimuli activated this PLD isoform. It has been shown previously that salt-treatment of tomato cell-suspension cultures induces *LePLD α 1* gene expression (Laxalt *et al.*, 2001) and PLD activation (Munnik *et al.*, 2000). As shown in Figure 4a, treatment with 125 mM NaCl induced a clear increase in *LePLD α 1* expression. *LePLD α 1* transcript was not detectable in the silenced lines, either with or without salt treatment. However, we observed a truncated *LePLD α 1* mRNA transcript in line 5 (Figure 4a).

PA increases in response to salt stress have been reported to be produced by both the PLD and phospholipase C/diacylglycerol kinase pathways (PLC/DGK; Munnik *et al.*, 2000). Addition of primary alcohols allows for the distinction of PLD activity from other PA producing reactions, making use of this enzyme's ability to substitute primary alcohols for water in its transphosphatidyl reaction (Munnik *et al.*, 1995). Treatment of cell-suspension cultures with NaCl in the presence of *n*-butanol led to a PA and phosphatidylbutanol (Pbut) accumulation that was dose-dependent (Figure 4b-e). A 15-minute treatment with 500 mM NaCl led to a significant PA accumulation while treatment with 1 and 1.5 M NaCl induced a significant increase in both PA and Pbut. A reduced induction of PA formation could be seen in the *LePLD α 1*-silenced lines upon treatment with 1 and 1.5 M NaCl; the difference in Pbut increase between control and silenced cell-suspension cultures was evident only upon treatment with 1.5 M NaCl. These results show that *LePLD α 1* activity is induced in response to high salinity stress in a tomato cell-suspension culture.

Reduced PLD activity in *LePLD α 1*-silenced lines could only be detected after severe salt treatments of 1 M NaCl or more. The fact that PLD is also active upon loss of cell membrane integrity (Figures 2 and 3) prompted us to investigate whether PLD activity after these high salt treatment might be due to a cell-lysis

effect. Cell-suspension cultures treated with high salt concentrations in the presence of Tris-HCl and EGTA displayed a PLD activation that was identical to that of unbuffered samples (Figure 4b-e). This was true for both control and *LePLD α 1*-silenced cell-suspension cultures. Correspondingly, vitality staining of salt-treated cell-suspension cultures with FDA demonstrated that cells had not lost plasma-membrane integrity (data not shown). These results indicate that *LePLD α 1* activity in a tomato cell-suspension culture treated with high salt concentrations is not due to loss of cell membrane integrity.

Figure 4. Increased *LePLD α 1* expression and activity in tomato cell-suspension cultures treated with salt

(a) RNA from cell-suspension cultures that had been treated with 125 mM NaCl for 5 hours was extracted and analyzed for *LePLD α 1* expression by RNA blot analysis with a ^{32} P-labeled *LePLD α 1* probe, an 18S rRNA probe was used as a loading control. ^{32} P-labeled control (b, d and e solid bars) or *LePLD α 1*-silenced (c, d and e hatched bars) cell-suspensions were left untreated, snapfrozen-and-thawed or treated with an equal volume of cell-free medium supplemented with increasing NaCl concentrations for 15 minutes, either without buffer (black bars) or buffered with 50 mM Tris-HCl pH 7.5 and 10 mM EGTA (gray bars). Lipids were extracted, separated by alkaline TLC and analyzed by phosphoimaging. PA (d) and Pbut (e) were quantified as percentage of total radiolabeled lipids and are presented in a histogram (salt treatment n=2, min and max values indicated; freeze/thaw n=1).

Silencing LePLD α 1 in tomato plants

Previously, Laxalt *et al.* (2001) reported *LePLD α 1* transcript in all tested tomato plant organs. Protein blot analysis of LePLD α 1 protein levels confirmed this finding (Figure 5a), LePLD α 1 could be detected in roots, stems, petioles, leaves, flowers and fruit. Transgenic plant lines were generated carrying the same RNAi construct that was used to knock-down LePLD α 1 in the Msk8 cell-suspension cultures. Several independently transformed lines were obtained and inbred to obtain homozygous lines carrying the silencing construct as well as their isogenic wildtype sibling lines. Three lines were selected in which knock-down of LePLD α 1 was verified by protein blot analysis of proteins extracted from seedlings, probed with the LePLD α 1-specific antibody (Figure 5b). Compared to wildtype (GCR161) tomato seedlings, the *LePLD α 1*-silenced plant lines had reduced LePLD α 1 protein levels, however, a protein band could still be detected in the different silenced lines. Lines 3-1 and 13-3 displayed a relatively clear reduction of LePLD α 1 protein level whereas line 4-10 showed only a slight decrease (Figure 5b). *LePLD α 1*-silenced tomato plants developed normally, displaying no obvious growth phenotype.

Phospholipid analysis was performed on *LePLD α 1*-silenced plants by labeling leaf discs with $^{32}\text{P}_i$, followed by lipid extraction and separation by TLC (Figure 5c). Like the cell-suspension cultures, LePLD α 1 knock-down plant lines exhibited no difference in basal phospholipid composition. However, unlike the cell-suspension cultures, no consistent reduction in cell-lysis induced PLD activity could be observed in *LePLD α 1*-silenced plant lines compared to their isogenic wildtype lines when leaf discs were snapfrozen and thawed (Figure 5c).

Salt tolerance in plants can be assayed by measuring seedling root-growth on agar plates with or without added NaCl. One-week-old tomato seedlings that had been grown on standard agar plates were transferred to new plates containing 0, 125 or 250 mM NaCl and rotated 180°. Primary root-growth after 24 hours was greatly reduced on plates with 125 mM NaCl compared to control plates and was completely abolished when seedlings were transferred to plates containing 250 mM NaCl (Figure 5d). *LePLD α 1*-silenced plant lines had the same magnitude of growth reduction on plates containing 125 mM NaCl as their isogenic wildtype control lines and also exhibited complete growth inhibition on 250 mM NaCl. These results show that, although it was able to reduce LePLD α 1 protein levels, the introduction of a *LePLD α 1*-silencing construct did not induce changes in basal phospholipid composition and did not affect cell-lysis induced PLD activity nor did it lead to altered salt tolerance in tomato seedlings.

Figure 5. Silencing *LePLDα1* in tomato plants and the effect on salt tolerance.

(a) Proteins were extracted from roots (R), stems (S), petioles (P), leaves (L), flowers (FI) and fruit (Fr) harvested from mature tomato plants. Proteins were separated by SDS-PAGE and blotted or stained with Coomassie Brilliant Blue as a loading control. *LePLDα1* was detected using an antibody directed towards the final 12 amino acids of *LePLDα1*. A precision protein marker (M) was used to gauge the size of the detected band. (b) Protein blot analysis of *LePLDα1* protein levels was performed on proteins extracted from roots and stems of one-week-old wildtype (GCR161; wt) and *LePLDα1*-silenced (lines 3-1, 4-10 and 13-3) tomato seedlings. (c) Leaf discs were excised from fully expanded leaves from wildtype (line 13-2, black bars) and *LePLDα1*-silenced (line 13-3, hatched bars) plant lines and labeled overnight with $^{32}\text{P}_i$. Leaf discs were either left untreated (-) or snapfrozen and thawed for 15 minutes (f/t). Lipids were extracted, separated by alkaline TLC and analyzed by phosphoimaging, PA was quantified as percentage of total phospholipids and is presented in a histogram $\pm$ SD (n=3). (d) Tomato seeds from wildtype (line 3-10) and *LePLDα1*-silenced (line 3-1) plant lines were sown on agar plates and grown vertically in a growth chamber. After 1 week, seedlings were transferred to fresh plates supplemented with 0, 125 or 250 mM NaCl, rotated 180° and placed back in the growth chamber. Plates were scanned after 24 hours. A representative silenced line is shown (line 3).

Salt tolerance in Arabidopsis requires both AtPLDα1 and AtPLDδ

Whereas gene-silencing can be incomplete and inconsistent, T-DNA insertional mutagenesis gives a higher degree of confidence that the gene-of-interest is no longer functional. *AtPLDα1* and *AtPLDδ* are the two predominant PLD isoforms present in *Arabidopsis* and both have been linked to plant responses to water stress (Sang *et al.*, 2001; Katagiri *et al.*, 2001). T-DNA insertion lines were obtained for both genes (SALK_067533 and SALK_023247) and were inbred to generate homozygous knock-out lines. Subsequently, a *pldα1/pldδ* double mutant was generated. Knock-out was verified, in the case of *AtPLDα1*, by protein blot analysis (Figure 6a) and, in the case of *AtPLDδ*, by RT-PCR (Figure 6b). Both the individual and double mutants developed normally, exhibiting no obvious phenotype when grown under standard greenhouse- and growth chamber-conditions.

When leaf discs from wildtype (Col-0), *pldα1*, *pldδ* and *pldα1/pldδ* Arabidopsis plants were labeled and lipids were extracted and analyzed, no difference in basal composition levels could be detected (Figure 6c). Treatment of leaf discs with high salt concentrations caused up to a five-fold increase in PA levels within 15 minutes in wildtype plants. However, when the *pld* mutants were treated with high salt concentrations, they consistently displayed a lower PA accumulation. Salt-induced PA levels were always lower in *pldα1* mutants when compared to wildtype, lower still in *pldδ* mutants, and lowest of all in *pldα1/pldδ* double mutants, reaching only 17% of total labeled phospholipids after treatment with 1.5 M NaCl, where wildtype plants PA levels reached up to 28% (Figure 6d). The salt-induced PIP₂ increase was not significantly different in the *pld* knock-out mutants (data not shown).

Figure 6. Salt-induced PLD activation in Arabidopsis T-DNA insertion lines

T-DNA insertion was verified for *pldα1* and *pldδ* single and double knock-out mutants, in the case of *AtPLDα1* by protein blot analysis of leaf protein extracts (a), and in the case *AtPLDδ* by RT-PCR amplification of *AtPLDδ* and *AtTUA4* cDNA (b). Leaf discs were excised from fully expanded leaves from control and *pld* knock-out plant lines and labeled overnight with ³²P. Leaf discs were treated with increasing NaCl concentrations for 15 minutes. Lipids were extracted, separated by alkaline TLC (c) and analyzed by phosphoimaging (d). PA was quantified as percentage of total radiolabeled lipids and is presented in a histogram ±SD (n=3).

Root-growth of *pld* knock-out mutants and wildtype Arabidopsis seedlings that had been grown vertically on agar plates and transferred to fresh plates was identical; growing approximately 4 mm per day during the first four days after transfer (Figure 7). When seedlings were transferred to plates supplemented with 75 mM NaCl, growth in wildtype and *pldα1* mutant seedlings was unaffected but the *pldδ* and *pldα1/pldδ* mutants grew marginally slower (Figure 7). Transfer to plates containing 150 mM NaCl caused a clear reduction in root growth in wildtype seedlings to approximately 1.5 mm per day. In *pld* knock-out lines, this growth inhibition was even greater: *pldα1* mutants grew approximately 1 mm per day and *pldδ* mutants about 0.5 mm per day, whereas *pldα1/pldδ* double mutants hardly grew at all (Figure 7). These results show that AtPLDα1 and AtPLDδ are required for the salt-induced PA production and salt-tolerance in Arabidopsis.

Figure 7. Reduced salt tolerance in Arabidopsis *pld* mutants

Seeds from wildtype (Col-0, black circles), *pldα1* (open squares), *pldδ* (open triangles) or *pldα1/pldδ* double (open diamonds) knock-out mutant lines were sown on agar plates and grown vertically in a growth chamber. After 3 days seedlings were transferred to fresh plates supplemented with 0, 75 or 150 mM NaCl. Plates were scanned after 8 days (a) and primary root growth was followed and averaged $\pm$ SE during 4 days after transfer (b; $n=12-16$; a representative experiment is presented).

Discussion

Silencing LePLDα1 in tomato cell-suspension cultures and plants

The PLD family in plants consists of numerous classes, which often contain several isoforms (Elias *et al.*, 2002), and the PA generated by PLDs in response to a multitude of stimuli has been proposed to play an important role in stress signal transduction (Testerink and Munnik, 2005). Which PLD class and, more specifically, which PLD isoform is activated in response a certain cue is a question that needs to be answered in order to better understand PLD function. Genetic modification makes it possible to remove specific PLD isoforms from the plant transcriptome, either by silencing or by T-DNA-insertional knock-out. Anti-sense and RNAi silencing of PLDs has been used in studies done in different species to examine the role of PLD in plant processes, *e.g.* development (Ohashi *et al.*, 2002), senescence (Ryu *et al.*, 1995), the defense response (Bargmann *et al.*, 2006) and waterloss (Katagiri *et al.*, 2001; Sang *et al.*, 2001). Arabidopsis *pld* T-DNA insertion mutants have also been employed to investigate PLD function (Zhang *et al.*, 2003; Li *et al.*, 2006).

The introduction of an RNAi silencing-construct in a tomato cell-suspension culture led to an essentially complete loss of *LePLDα1* transcript and

LePLD α 1 protein in three independent transformants (Figure 1a and b). Consequently, the *in vitro* α -class PLD activity in silenced lines was greatly reduced, reaching at most 10% of control cell-suspension cultures (Figure 1c). The remaining activity could be due to incomplete silencing or due to the presence of another (α -class) PLD in the Msk8 proteome. The latter explanation seems more likely because, as stated above, the silencing is virtually complete and because another α -class PLD gene, *LePLD α 2*, is known to also be expressed in Msk8 cells (Laxalt *et al.*, 2001). This finding suggests that LePLD α 1 is responsible for most of the α -class PLD activity found in an Msk8 tomato cell-suspension culture. Nonetheless, the lack of functional LePLD α 1 did not induce any changes in basal phospholipid composition or the fatty acid composition of total lipids (Figure 1d-f). These results suggest that LePLD α 1 does not function in the maintenance of basal phospholipid homeostasis, or, if it does, the loss of LePLD α 1 can be compensated for, perhaps by another PLD.

Silencing *LePLD α 1* in tomato plants did not lead to a complete lack of detectable LePLD α 1 protein. Rather, three independent plant lines that were selected on the basis of their transcriptional silencing showed residual LePLD α 1 levels when examined by protein blot analysis (Figure 5b). The level of knock-down was also found to be inconsistent; comparing LePLD α 1 protein levels in samples from silenced- and wildtype lines, taken from various organs from plants of varying age, the knock-down was not always as apparent, and sometimes even indiscernible (data not shown). Furthermore, in contrast to silenced cell-suspension cultures, no difference in PLD activity after loss of cell membrane integrity in leaf discs could be observed (Figure 5c). This distinction can be explained by the incomplete knock-down seen on the protein level, however, the possibility that another PLD isoform in plants is activated under these conditions cannot be excluded. In summary, the lack of a lipid phenotype in *LePLD α 1*-silenced tomato plants can be rationalized both by incomplete silencing and partial redundancy.

PLD activation upon loss of cell membrane integrity

The noncytosolic conditions required for *in vitro* α -class PLD activity have been a riddle for a long time (Roughan and Slack, 1976; Heller, 1978; Pappan and Wang, 1999). Of course *in vitro* activity does not necessarily reflect *in vivo* activity; there might be factors *in planta* which alleviate the need for an acidic pH and mM calcium concentrations that are missing in the *in vitro* activity assay. There are, for instance, reports of AtPLD α 1 interacting with a heterotrimeric G-protein α -subunit (Zhao and Wang, 2004) and regulation of PLD by phosphorylation (Novotna *et al.*, 2003). Alternatively, there might be (transient) micro-environments within the cytosol that do meet α -class activity requirements, particularly near membranes and in the vicinity of Ca²⁺ and proton channels (Berridge *et al.*, 2003). Conversely, α -class PLD activity does not necessarily have to be restricted to the cytosolic or intracellular milieu. An α -class PLD has been found in the cell wall of rice leaves in an immunochemical electron-microscopy study done with a peptide-specific antibody (McGee *et al.*, 2003). Lastly, α -class PLDs could function in the biochemical events following loss of cell membrane integrity, when they come in

contact with apoplastic conditions. It has been noted previously that there is strong PLD activity upon “wounding, tissue handling, or uncontrolled lipid extraction” (Wang, 2005), and it was proposed that this “deregulated” activity might be due to disruption of cellular compartments.

By making use of *LePLD α 1*-silenced tomato cell-suspension cultures, we can now demonstrate that this α -class PLD is indeed active in apoplastic conditions. Cell-suspensions cultures lacking *LePLD α 1* showed a significant delay in PA accumulation after cell membrane integrity had been lost due to a snapfreeze-and-thaw treatment (Figure 2). We were subsequently able to show that it is the acidic pH and Ca^{2+} accessibility that determine this activity by removing the protons and Ca^{2+} ions from the growth medium using Tris/HCl and EGTA (Figure 3). In addition, we can stipulate that *LePLD α 1* uses PC, PE and PG as substrates after loss of cell membrane integrity, as can be concluded from their delayed hydrolysis in an *LePLD α 1*-silenced cell-suspension culture (Figure 2). There is, however, residual PLD activity in the silenced lines (Figures 1, 2 and 4), suggesting that there is another PLD isoform that is also active. *LePLD α 2* seems a good candidate as it too is expressed in Msk8 cell-suspensions (Laxalt *et al.*, 2001). Nevertheless, we cannot exclude other, still undiscovered α -class PLD isoforms or even other PLD classes. In conclusion, we have established that there is a massive PLD-mediated hydrolysis of bulk structural lipids when cell membrane integrity is lost, that α -class PLDs are, at least in part, responsible for this hydrolysis and that the extracellular conditions govern this activity. This finding calls for the re-examination of earlier reports of α -class PLD involvement in stresses that could lead to disruption of cell compartments, such as wounding (Wang *et al.*, 2000) and freezing (Welti *et al.*, 2002).

Activation of multiple PLDs in the high salinity stress response

PLD-derived PA has previously been shown to transiently accumulate in response to high salinity stress. Munnik *et al.* (2000) described the salt-induced PLD activation in *Chlamydomonas moewusii* and plant cell-suspension cultures from alfalfa and tomato. PLD expression has also reported in response to high salinity stress. In Arabidopsis, *AtPLD δ* (and not *AtPLD α 1*) expression was rapidly and strongly increased when plants were treated with NaCl (Katagiri *et al.*, 2001). In tomato cell-suspension cultures, *LePLD α 1* expression was shown to increase more than two-fold upon salt exposure (Laxalt *et al.*, 2001). This finding was used as a starting point for further research into the role of PLD in salinity stress.

LePLD α 1-silenced cell-suspension cultures exhibited a compromised salt-induced PA and Pbut increase, illustrating that this particular PLD isoform is activated under these circumstances (Figure 4b-d). The remaining increase in PA seen in *LePLD α 1*-silenced lines can be explained in three ways: incomplete silencing, activation of a second PLD or generation of PA by another route. The combination of PLC and DGK activities also produces PA and is known to be activated in response to different stresses in plants, including high salinity stress (Munnik and Meijer, 2001; Testerink and Munnik, 2005). This route accounts for part of the remaining PA response seen in the *LePLD α 1*-silenced cell-suspension

cultures, but the fact that residual Pbut formation can also be detected in the silenced lines indicates that there is also still PLD activity present (Figure 4b-e). Laxalt *et al.*, (2001) reported that *LePLD α 2* expression was also increased in tomato cell-suspension cultures treated with salt and this isoform might well explain the residual PLD activity. Nevertheless, even though *LePLD α 1* protein levels were indiscernible by protein blot analysis, some activity due to incomplete silencing cannot be excluded.

Arabidopsis AtPLD α 1 knock-out lines displayed a PA increase in response to high salinity stress in leaf discs that was only slightly lower than that of wildtype (Figure 6c and d), again indicating that alternate routes of PA synthesis must be active. Correspondingly, it can be concluded that *AtPLD δ* activity is also increased upon high salt treatment because leaf discs taken from *AtPLD δ* T-DNA insertion lines exhibited a reduced PA response as well, while a *pld α 1/pld δ* double mutant line showed even less of a PA increase (Figure 6c and d). Yet, there was still a significant PA increase in the double mutant, which can again be accounted for by the activity of PLC/DGK and another, unknown PLD, as residual Pbut formation could be detected (data not shown). To our knowledge, this is the first report of *in planta* PA production in response to high salinity stress; similar results have been obtained with intact seedlings (data not shown). Altogether, it has been established that in *Arabidopsis* PLD activity is increased upon high salt stress and evidence has been presented that indicates that both α - and δ -class PLDs are responsible for this increase.

PLD function in the high salinity stress response

Now that it has been established that PLDs are activated in response to high salinity stress, an obvious question arises: what function do PLD and PA fulfill in the plant's response to high salt? That they are important is attested by the fact that *Arabidopsis pld* mutants have a growth defect when placed on high salt containing media (Figure 7).

PLD and its product PA have been implicated in intracellular signaling by many independent studies (Wang 2005; Testerink and Munnik, 2005). The PA formed in response to high salt treatment has also been proposed to function in signaling that leads to plant adaptation to this stress. PA is known to bind and affect the activity of signaling protein phosphatases and protein kinases (Testerink and Munnik, 2005). In relation to high salinity stress, PA has been found to bind Snf-related protein kinases (Testerink *et al.*, 2004) which are activated in response to high salt (Munnik *et al.*, 1999; Hrabak *et al.*, 2003; Boudsocq *et al.*, 2004). Recently, Zhang *et al.* (2006) suggested that PLD-derived PA regulates the activity of vacuolar H⁺-ATPases upon high salt treatment, which help maintain the proton gradient that drives Na⁺/H⁺ antiporter activity. Lastly, *AtPLD α 1* has been shown to be intricately involved in the response to the salt-inducible hormone abscisic acid (Zhang *et al.*, 2004; Mishra *et al.* 2006). Together, these findings argue a strong case for PLD function in high-salinity-stress signaling. Yet, it must also be taken into account that hyperosmotic stress induces significant membrane remodeling and rearrangements and that PLD activity could be physically involved in such membrane alterations (Gigon *et al.*, 2004).

Methods

Plant material

Tomato plants (*Lycopersicon esculentum* cv. GCR161) were grown on soil under 13.5h light/ 10.5h dark regime in the greenhouse. *Arabidopsis thaliana* var. Col-0 T-DNA insertion lines were obtained from the SALK Institute (Alonso *et al.*, 2003). Homozygous lines were generated for *AtPLD α 1* (SALK_067533) and *AtPLD δ* (SALK_023247), which were consequently crossed and inbred to obtain lines that were homozygous for both insertions. The following primers were used to verify genomic insertions by PCR:

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, in compliance with the orientation of the T-DNA insertion. For routine plant growth, seeds were sown on soil and vernalized at 4° C for 2 days. Plants were grown in a growth chamber under a 12h light/12h dark regime at 21° C and 70% humidity.

Cell-suspension cultures

Suspension-cultured cells (*Lycopersicon esculentum* cv. Mill. line Msk8; Felix *et al.*, 1991) were grown at 24°C in the dark, shaking at 125 r.p.m. in MS medium supplemented with 3% (w/v) sucrose, 5.4 μ M NAA, 1 μ 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 days after weekly subculturing.

Cloning of the silencing construct

For the silencing vector, an inverted repeat (RNAi) construct specific for *LePLD α 1* was generated targeting the gene's 3' UTR. PCR amplification of the *LePLD α 1* cDNA, cloned previously by Laxalt *et al.* (2001), was performed with the following oligonucleotides: 1_5'-CGGGATCCCCATCGATCAGTCAATTAAGCATCTC-3'(reverse) with a *Bam*HI and a *Cl*al restriction site, 2_5'-CCGGAATTCCCCCGACACCAAGG-3' (forward) with an *Eco*RI restriction site and 3_5'-CCGGAATTCATCCAGAAAGTGAGG-3' (forward) with an *Eco*RI restriction site. The PCR products resulting from primer combinations 1-2 and 1-3 were ligated in a 1-2/3-1 orientation into the pGreen1K binary vector which was modified to contain the 35S-Tnos cassette from pMON999.

Tomato plant and cell-suspension transformation

All constructs were transferred to *Agrobacterium tumefaciens* strain EHA105 carrying the pJIC.SaRep plasmid. Tomato plants were transformed as specified by van Roekel *et al.* (1993). Cell-suspension culture transfection was achieved as described by Bargmann *et al.* (2006).

in vitro PLD α activity assay

PLD α -like activity was assayed by using a combined protocol of Pappan *et al.* (1997) and Ella *et al.* (1994). Briefly, 10 μ g of total protein extract was incubated with 250 μ 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 CaCl₂ and 1% (v/v) n-butanol, for 1 hour at 30 °C. Cabbage PLD (1 U; type V, Sigma-Aldrich, Steinheim, Germany) was used as a positive control. Lipids were extracted as described before (van der Luit *et al.*, 2000) and separated on silica 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). BODIPY-lipids were visualized under UV light and quantified by fluorimaging (Molecular Dynamics, Sunnyvale CA, USA).

in vivo phospholipid analysis

Cell-suspension cultures were labeled by incubation of 100 μ l aliquots with 100 μ Ci carrier-free PO₄³⁻ in growth medium. Treatments were performed in the presence of 0.5% (v/v) n-butanol when indicated and treatments were stopped and lipids extracted as described before (van der Luit *et al.*, 2000). Leaf discs (Ø 5 mm) were labeled by floating them overnight on 100 μ l 10 mM MES buffer (2-[N-Morpholino]ethane sulfonic acid) pH 5.7 (KOH) supplemented with 100 μ Ci carrier-free PO₄³⁻ in a 2 ml microcentrifuge tube (Frank *et al.*, 2000). Treatments were stopped by addition of perchloric acid to a final concentration of 5% (w/v). Leaf discs were then transferred to a new tube containing 375 μ l CHCl₃/MeOH/HCl (50 : 100 :

1 [v/v]) where lipids were extracted during vigorous shaking for 10 minutes. A two-phase system was induced by addition of 375 μ l CHCl_3 and 200 μ l 0.9% (w/v) NaCl. The remainder of the extraction was performed as described before (van der Luit *et al.*, 1999).

For quantitative analysis, lipids were separated on silica 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% $\text{NH}_4\text{OH}/\text{H}_2\text{O}$ (90 : 70 : 4 : 16 [v/v]; Munnik *et al.*, 1994) when indicated. For qualitative analysis, lipids were separated in 2 dimensions; using CHCl_3 /MeOH/25% $\text{NH}_4\text{OH}/\text{H}_2\text{O}$ (90 : 70 : 4 : 16 [v/v]) in the first dimension and CHCl_3 /Me₂Co/MeOH/HAc/ H_2O (80 : 30 : 28 : 26 : 15 [v/v]) in the second dimension. Phospholipids were visualized by autoradiography and quantified by phosphoimaging (Molecular Dynamics, Sunnyvale CA, USA).

The fatty acid composition of total lipids was determined by transmethylation of the extracted lipids and subsequent gas chromatographic analysis of the fatty acid methyl esters as described by Arisz *et al.* (2000).

RNA blot analysis

Total RNA from tomato cell-suspension cultures was isolated using the Trizol-LS reagent method (Gibco, Gaithersburg, MD, USA). 10 μ g RNA was separated by denaturing 1.4% (w/v) formaldehyde-agarose gel electrophoresis, transferred onto Hybond-XL nylon membranes (Amersham Pharmacia, Buckinghamshire, England), and hybridized with ^{32}P -labeled probes in modified Church solution at 65°C. Membranes were washed 3 times for 15 minutes with wash buffer (1xSSC, 0.1% [w/v] SDS) and the probe signal was visualized by autoradiography.

Protein blot analysis

Protein extraction buffer (9.5 M Urea, 0.1 M Tris-HCl pH 6.8, 2% [w/v] SDS and 2% [v/v] $\square$ ME) was added to an equal volume of ground leaf tissue, vortexed and centrifuged in an eppendorf centrifuge for 10 min at 1000 g. 4x Sample buffer (8% [w/v] SDS, 40% [v/v] glycerol, 20% [v/v] $\square$ ME, 240 mM Tris-HCl pH 6.8 and 0.08% [w/v] Bromophenol Blue) was added to the supernatant and samples were loaded onto a 10% SDS-PAGE gel, blotted on nitrocellulose and incubated o/n in 10 ml PBST (0.8 % [w/v] NaCl, 0.02% [w/v] KCl, 0.144 % [w/v] Na_2HPO_4 , 0.02 % [w/v] KH_2PO_4 and 0.05% [v/v] Tween-20) with 5% (w/v) powdered milk and affinity-purified polyclonal anti-LePLD α 1 antibody (rabbit; Eurogentech, Liege, Belgium). Antibodies were generated using the final 12 amino acids of the LePLD α 1 protein: N-TKSDYLPNLTT-C. The blot was washed three times with PBST, incubated for 1h in 10 ml PBST with 5% (w/v) powdered milk and horseradish peroxidase-coupled goat anti-rabbit IGG antibody (Pierce, Rockford, IL, USA) and washed three more times in PBST. The peroxidase activity was detected by enhanced chemiluminescence (Amersham, Buckinghamshire, UK). A duplicate gel was stained with Coomassie Brilliant Blue (0.25% [w/v] CBB, 30% [v/v] methanol and 10% [v/v] acetic acid) as a loading control.

RT-PCR analysis

cDNA was synthesized as described in Ament *et al.* (2004). cDNA from wildtype (Col-0) and homozygous *AtPLD α 1*, *AtPLD δ* and *AtPLD α 1/ δ* -double T-DNA insertion lines was used as a template for the amplification of *AtPLD δ* (with primers: *AtPLD δ RT-F* 5'-CGAGACCTTCCCAGATGTTG-3' and *dT₁₈*) and the α -tubulin encoding *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 -labeled *AtPLD δ* and *AtTUA4* probes. The signal was visualized by phosphoimaging (Molecular Dynamics, Sunnyvale CA, USA).

Plate root-growth assay

Seeds were sterilized, placed on MS agar plates and vernalized for 2 days at 4° C. Plates were placed vertically in growth chambers under a 18h light/6h dark regime at 21° C and 70% humidity. Seedlings (3-4 days old in the case of Arabidopsis and 7-10 days old in the case of tomato) were transferred to fresh plates supplemented with NaCl. Plates were rotated and placed back vertically in the growth chamber. Arabidopsis primary root elongation was scored daily during the first four days; pictures were taken after 8 days.

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Chapter 3

Analysis of AtPLD α 1 and AtPLD δ function in the Arabidopsis wounding response: Locating the site of activity

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Summary

The phospholipase D (PLD) AtPLD α 1 has previously been implicated in the wounding response in Arabidopsis (*Arabidopsis thaliana*). It has been proposed that AtPLD α 1 is activated in intact cells in response to an unknown wounding signal and that the produced phosphatidic acid (PA) serves as a precursor for jasmonic acid synthesis. Here, we show that PLD activity during the wounding response originates from the ruptured cells using $^{32}\text{P}_i$ -mediated phospholipid analysis and Arabidopsis *pld* knock-out mutants. *pld* α 1 knock-out lines have reduced wounding-induced PA production and the remaining production is eliminated in a *pld* α 1/ δ double knock-out line. Dissection of wounded leaf material and examination of PLD activity in a tomato (*Lycopersicon esculentum*) cell-suspension wounding-model showed that this activity takes place in the ruptured cells and is not detectable in intact cells. Furthermore, evidence is provided that indicates that extracellular PA derived from the ruptured cells elicits responses in intact cells. Addition of PA to a cell-suspension induced a myelin basic protein (MBP)-kinase activity similar to that induced by ruptured cells, whereas ruptured *LePLD* α 1-silenced cells induced an activation inferior to that induced by control cells. Wounding-induced *in planta* MBP-kinase activation and AtLOX2 expression was, however, not influenced by knock-out of either or both AtPLD α 1 and AtPLD δ and the larvae of the Cabbage White butterfly (*Pieris rapae*) grew equally well on wildtype as on the *pld* knock-out mutants. In conclusion, the role of PLD in the wounding response is discussed and a model is put forward proposing that PA could serve as a non-cell-autonomous wounding signal.

Abbreviations: PLD, phospholipase D; PA, phosphatidic acid; MBP, myelin basic protein; PC, phosphatidylcholine; PIP₂, phosphatidylinositolbisphosphate; JA, jasmonic acid; PIP, phosphatidylinositolmonophosphate; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; Pbut, phosphatidylbutanol

Introduction

Insect herbivory is a consequential stress in higher plants that leads to a loss of nutrients and photosynthetic capacity and therefore reduced seed production. Plants respond to the wounding caused by the feeding behavior and counter the attack with both direct and indirect defensive strategies (Wasternack *et al.*, 2006). Accumulation of proteins that interfere with the digestion of plant material in the insect gut, *i.e.* proteinase inhibitors, occurs upon perception of wounding in plants (Green and Ryan, 1972; Casaretto *et al.*, 2004). Compounds that are toxic or repellant with respect to the herbivore are also synthesized and accumulated in local and systemic parts of the wounded plant (Baldwin, 1998; Leitner *et al.*, 2005). Additionally, wounded plants emit volatile compounds that attract natural enemies of the herbivores, *e.g.* parasitoids (Sabelis *et al.*, 2001; Ament *et al.*, 2004). In such a way, plants can fend off an ongoing attack and prepare themselves for further assault.

The plant response to wounding can be partitioned into three territories: (i) the ruptured cells, (ii) the local responding tissue and (iii) the systemic responding tissue. The ruptured cells emit a non-cell-autonomous, primary signal (or signals) 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 (reviewed in Schilmiller and Howe, 2005).

Phospholipase D (PLD) catalyses the hydrolysis of structural phospholipids such as phosphatidylcholine (PC), producing phosphatidic acid (PA) and a free head-group. There are twelve PLD genes present in the *Arabidopsis* genome (*Arabidopsis thaliana*), whereas just two PLD genes are present in animals and only one in yeast. The plant PLD family can be divided into six classes, the α -, β -, γ -, δ -, ϵ - and ζ -PLDs, depending on the enzymes' sequence homology and biochemical properties (Wang, 2005). 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 and Wang, 2002). Four kinds of *in vitro* PLD activity can be distinguished in this way, depending on their pH-, $[\text{Ca}^{2+}]$ -, oleate- and phosphatidylinositolbisphosphate (PIP_2)-requirements. The α -class PLDs require an acidic pH and mM calcium concentrations but do not require inclusion of PIP_2 in their lipid substrate preparation. In contrast, β/γ -class PLDs require a neutral pH, μM calcium concentrations and PIP_2 . δ -class PLDs are active at high μM and low mM calcium concentrations and are stimulated by inclusion of oleic acid in the substrate preparation. Lastly, the ζ -class PLDs require a neutral pH and PIP_2 but do not require calcium.

Functions have been suggested for PLD in various processes including membrane degradation, vesicular transport and intracellular signaling (reviewed in Meijer and Munnik, 2003; Wang, 2005). There have been reports of PLD involvement in signaling events occurring in response to a multitude of stimuli, *e.g.* cold (Li *et al.*, 2004, Vergnolle *et al.*, 2005), plant-pathogen interactions (Laxalt *et*

al., 2001; Yamaguchi *et al.*, 2005; Bargmann *et al.*, 2006), dehydration (Frank *et al.*, 2000; Katagiri *et al.*, 2001) and salt stress (Munnik *et al.*, 2000). PA is believed to act as a second messenger in these signaling events, it is generated rapidly and transiently during such stress responses (Munnik, 2001). PA has been proposed to function in signaling cascades by recruiting target proteins to particular membranes and/or influencing their activity (Testerink and Munnik, 2005).

PLD activity has also been implicated in the wounding response. PA production has been reported to increase at the wounding site and in the local responding tissue in castor bean leaves (*Ricinus communis*, Ryu and Wang, 1996; Ryu and Wang, 1998) and systemically in wounded seedlings of several plant species (Lee *et al.*, 1997; Lee *et al.*, 2001). However, which PLD isoform(s) is(are) active is not clear nor is it apparent where (*i.e.* in the ruptured or the intact cells) this activity takes place. Wang *et al.* (2000) demonstrated that in *AtPLD α 1*-silenced Arabidopsis plants the PA production in wounded leaves was diminished, indicating that this isoform is active in the ruptured cells and/or the local intact tissue. Furthermore, the silenced plant lines displayed reduced wounding-induced jasmonic acid (JA) production and decreased wounding-induced gene expression. The authors put forward a model in which *AtPLD α 1* is activated in the intact cells in response to an unidentified wounding signal and suggested that the PA produced in this way is used as a precursor for JA biosynthesis. However, the precise location of the *AtPLD α 1* activity in the Arabidopsis wounding response was not examined in this communication.

The correlation between the *in vitro* PLD α requirements and the conditions found in the plant apoplast and vacuole (namely an acidic pH and mM calcium concentrations) suggests that *AtPLD α 1* would be activated upon disruption of cellular compartmentalization. Therefore, in contrast to the proposed model, the reduced PA production in wounded leaves of *AtPLD α 1*-silenced Arabidopsis plants reported previously (Wang, *et al.*, 2000) could be accounted for by *AtPLD α 1* activation in ruptured cells. Thus, the aim of this study is to determine the location of PLD activity in the wounding response. PLD activity was monitored in ruptured cells, local responding tissues and in systemic leaves of Arabidopsis wildtype and *pld* knock-out lines. In addition, a cell-suspension wounding-model was employed to more readily differentiate between ruptured and responding cells. Furthermore, wounding-induced protein-kinase activation and *AtLOX2* gene expression were examined and herbivore performance on the *pld* knock-out lines was assayed.

Results

Increased AtPLD α 1 activity after wounding

Phospholipid metabolism can be monitored *in vivo* by labeling biological material with radioactive inorganic phosphate and subsequent extraction and analysis of radiolabeled lipids. In this case, leaf discs (5 mm Ø) were excised from Arabidopsis leaves and incubated overnight floating on a buffer (pH 5.8) containing $^{32}\text{P}_i$. Mechanical wounding of leaf discs with a hemostat induced a rapid and transient increase of PA levels (Figure 1a and b). PA levels in wildtype lines quadrupled within 5 min, reaching up to 12% of total radiolabeled lipids, and then decreased again between 10 and 40 min down to 6%. Wounded leaf discs from *pld α 1* knock-out lines produced notably less PA, exhibiting at most a threefold increase in PA levels (Figure 1b). The kinetics of the transient PA response were similar in the wildtype and *pld α 1* knock-out lines. These results correlate well with the earlier report of reduced PA formation in wounded *AtPLD α 1*-silenced Arabidopsis leaves (Wang *et al.*, 2000). They also show that an overnight incubation left the leaf discs responsive to wounding despite previous wounding by excision from the plant.

Yet, it is unclear whether the wounding-induced AtPLD α 1 activity originates from the ruptured cells or the intact cells. The analysis of solely dead or living cells is required in order to address this question. A way of analyzing the response in exclusively dead cells is by rupturing every cell in the leaf disc. This can be achieved by snapfreezing 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 snapfrozen-and-thawed revealed dramatically increased PA levels within 5 min, surpassing 20% of total radiolabeled phospholipids (Figure 1c and d). This increase continued for up to 80 min, leveling out at approximately 40% of total radiolabeled lipids. The increase in PA levels in the *pld α 1* mutant was consistently lower than in wildtype leaf tissue, achieving only 10% of total radiolabeled lipids in 5 min, but eventually catching up to wildtype levels after 80 min, reaching 38% (Figure 1d). Analysis of electrolyte leakage showed that cells had been ruptured equally in both the wildtype and mutant leaf discs (Figure 1e). These data indicate that AtPLD α 1 becomes active upon loss of cell membrane integrity and that at least part of the increase in PA levels measured in wounded leaf discs is due to AtPLD α 1 activity in the ruptured cells.

Figure 1. AtPLD α 1 activity after wounding and loss of cell membrane integrity

Leaf discs from wildtype (Col-0) and *pld α 1* knock-out (SALK_067533) lines were labeled overnight floating on buffer containing $^{32}\text{P}_i$. Discs were mechanically wounded with a hemostat (a and b) or snapfrozen-and-thawed (c and d). Lipids were extracted at the indicated timepoints, separated by alkaline TLC (wildtype samples are depicted in a and c) and analyzed by phosphoimaging (b and d). The origin of the chromatogram, phosphatidylisitolbisphosphate (PIP₂), phosphatidylinositolmonophosphate (PIP), phosphatidic acid (PA), phosphatidylinositol (PI), phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) are indicated (a and c). PA was quantified as percentage of total radiolabeled lipids and is presented in a histogram $\pm$ SD (b and d; wt, black bars; *pld α 1*, hatched bars; n=3). (e) Ion-leakage before (control) and after snapfreezing-and-thawing (f/t) was quantified as conductivity of the labeling buffer relative to boiled samples and is presented in a histogram $\pm$ SD (wt, black bars; *pld α 1*, hatched bars; n=3).

Location of wounding-induced PLD activity in *Arabidopsis*

Having established that AtPLD α 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 α 1 activity in the intact tissues of a wounded leaf disc, half of a $^{32}\text{P}_i$ -labeled leaf disc was wounded with a hemostat after which the wounded and uninjured halves were dissected and analyzed separately. The treatment was administered in the presence of *n*-butanol with the purpose of highlighting PLD activity. Addition of primary alcohols allows for the distinction of PLD activity, as opposed to activity of other PA producing reactions, making use of this enzyme's ability to substitute primary alcohols for water in its transphosphatidylation reaction (Munnik *et al.*, 1995). As shown in Figure 2, PA and phosphatidylbutanol (Pbut) 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. Again, the PLD activity in the *pld α 1* mutant line was consistently lower after both wounding and snapfreezing-and-thawing, as can be seen by a reduced PA and Pbut accumulation (Figure 2). These results show that the increase in PA levels in wounded *Arabidopsis* leaf discs is due to PLD activity in the ruptured cells and that this is not a response produced in the intact tissue.

Figure 2. Locating PLD activity in a wounded Arabidopsis leaf disc

Leaf discs from wildtype (Col-0) and *pldα1* knock-out lines were labeled overnight floating on buffer containing $^{32}\text{P}_i$. Leaf discs were left untreated, wounded with a hemostat (25% or 50% of the leaf disc area) or snapfrozen-and-thawed (100%) in the presence 0.5% *n*-butanol. Lipolytic activity was stopped after 15 min by addition of perchloric acid. Control and 25% wounded leaf discs were dissected in two halves (the analyzed half is indicated with an arrow ►). Lipids were extracted, separated by alkaline TLC and analyzed by phosphoimaging. (a). The origin of the TLC, PIP₂, PIP, PA, PI, PC, PE, PG and phosphatidylbutanol (Pbut) are indicated. (b) PA and Pbut were quantified as percentage of total radiolabeled lipids and are presented in a histogram $\pm$ SD (wt, black bars; *pldα1*, hatched bars; $n=3$).

PLD activity has also been suggested to be involved in the systemic wounding response. Lee *et al.*, (2001) reported increased systemic PA levels in tomato seedlings within minutes after wounding. Although Wang *et al.*, (2000) investigated local and systemic JA levels and gene expression in wounded Arabidopsis plants, this report did not present data concerning the systemic PA levels. This hiatus prompted us to investigate whether PLD activity increases systemically in Arabidopsis and, if so, whether the AtPLD α 1 isoform is involved. Plants were grown on rockwool and radioactively labeled by addition of $^{32}\text{P}_i$ to the growth medium one day before treatment. The first true leaf was mechanically wounded and PA levels were followed for 20 min in both the wounded and in the second, unwounded leaf. PA production in the wounded leaf was rapid and substantial (Figure 3), reaching up to 20% of total radiolabeled lipids in 20 min. In contrast, no increase in PA was detected in the systemic leaf, instead levels remained basal throughout the 20 min following treatment (Figure 3). This result indicates that there is no systemic PA response in wounded Arabidopsis plants.

Figure 3. Local and systemic PA production in wounded Arabidopsis plants

The first leaf of 10-day-old $^{32}\text{P}_i$ -labeled Arabidopsis Col-0 plants was wounded using tweezers. The wounded leaf and second (systemic) leaf were harvested after 0, 2, 5, 10 and 20 min. Lipids were extracted, separated by TLC and analyzed by phosphoimaging. PA was quantified as percentage of total radiolabeled lipids and is presented in a histogram $\pm$ SD (n=3).

PLD α 1 activity in a cell-suspension wounding model

PLD and PA involvement in the wounding response has been studied previously utilizing a wounding model that makes use of a cell-suspension culture (Lee *et al.*, 2001; Lee *et al.*, 2003). The authors showed that addition of ruptured cells to a soy bean (*Glycine max*) cell-suspension induced the activation of a wounding-induced MAPK (Lee *et al.*, 2001) and rearrangement of the actin cytoskeleton (Lee *et al.*, 2003). Moreover, these authors showed that addition of PA to the cell-suspension culture could induce MAPK activation and cytoskeletal rearrangements. These results were interpreted to suggest that PLD activity in the intact, responding cells was positioned upstream of the observed wounding responses. However, measurements of PLD activity or PA levels in these cells were not included in these reports.

A cell-suspension wounding model is well suited for differentially prelabeling the ruptured or the intact cells and analyzing changes in the radioactive PA levels in either of the two independently. We have previously used a tomato (Msk8) cell-suspension culture to study lipid signaling in response to biotic (van der Luit *et al.*, 2000; Laxalt *et al.*, 2001; Bargmann *et al.*, 2006) and abiotic stresses (Munnik *et al.*, 2000). Here, Msk8 cell-suspensions were used to set up a wounding model system in order to examine the location of PLD activity in the wounding response. Cells that had been ruptured (snapfrozen-and-thawed 5 min) were added to a cell-suspension culture in a 1:9 ratio and the induced protein-kinase activity towards myelin basic protein (MBP) was analyzed by in-gel-kinase assay (Figure 4a). An approximately 48 kDa band was detected that 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 of solely the ruptured cells showed that this MBP-kinase did not originate from these cells (Figure 4a). This protein-kinase activity is similar to the 48 kDa MBP-kinase found by Stratmann and Ryan (1997) in wounded tomato leaves.

As with the Arabidopsis leaf discs, a dramatic increase in PLD activity could be observed in the snapfrozen-and-thawed cells when they were prelabeled

with $^{32}\text{P}_i$ and their phospholipid composition analyzed (Figure 4b). PA levels rose to almost 50% of total radiolabeled lipids in 15 min and up to 60% in 30 min. Similar to the *Arabidopsis pldal* knock-out line, an *LePLD α 1*-silenced Msk8 cell-suspension culture exhibited reduced PLD activity upon loss of cell membrane integrity, indicating that α -class PLDs are also active in this wounding model. PA levels in the silenced cells were notably lower than in the control culture, reaching only 30% in 15 min (Figure 4b). Likewise, PA levels were monitored in $^{32}\text{P}_i$ -labeled intact cells treated with unlabeled ruptured cells. Interestingly, no increase in PA levels could be detected in these cells (Figure 4c). When cells were treated with mas7, a strong elicitor of PLD responses (van der Luit *et al.*, 2000), a clear PA response was seen within 20 min (Figure 4c), indicating that the cells were responsive. These results further indicate that wounding-induced PLD activation is restricted to the ruptured cells and does not occur in the intact, responding cells.

Figure 4. MBP-kinase and PLD activity in a cell-suspension wounding-model

(a) Msk8 cells were incubated with 10% (v/v) ruptured (snapfrozen-and-thawed 5 min) cells for up to 20 min. Proteins were extracted from samples taken after 0, 2, 5, 10 and 20 min 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) $^{32}\text{P}_i$ -labeled empty vector control (black bars) or *LePLD α 1*-silenced (hatched bars) cell-suspensions were left untreated or snapfrozen-and-thawed for 15 or 30 min. Lipids were extracted, separated by TLC and analyzed by phosphoimaging. PA was quantified as percentage of total radiolabeled lipids and is presented in a histogram $\pm$ SD (n=3). (c) $^{32}\text{P}_i$ -labeled cell-suspensions were left untreated, incubated with 10% (v/v) unlabeled, ruptured cells for up to 20 min or treated with 5 μM mas7 for 20 min. PA was quantified and is presented in a histogram $\pm$ SD (n=3).

Having demonstrated that the PLD activity in this cell-suspension wounding-model is restricted to the ruptured cells, the role of PLD in the wounding response needed to be reassessed. Lee *et al.* (2001 and 2003) used exogenous application of PA to mimic endogenous PA production, and showed that MAPK activation and cytoskeletal rearrangements could be induced similar to those induced by the addition of ruptured cells. This exogenous PA application, however, actually mimics the addition of ruptured cells, since bulk phospholipids in these cells are rapidly hydrolysed to PA (Figure 4b). These results led us to hypothesize that, in fact, the PA produced in the ruptured cells elicits a response in the intact cells. In-gel-kinase assays were undertaken to investigate whether exogenous PA could also elicit MBP-kinase activity in the Msk8 wounding model. As shown in Figure 5a, a rapid and transient activation of a 48 kDa kinase was apparent when cell-

suspensions were treated with PA, but not when treated with PC. This activity peaked at 5 min and declined again after 20 min. Secondly, whereas a clear activation with a peak at 5 min followed by a gradual decline could be seen when ruptured control cells were added, the activity elicited by ruptured *LePLD α 1*-silenced cells was inferior and peaked at 10 min (Figure 5b). These results show that PA on its own can elicit a (wounding) response and that ruptured cells with compromised PA production elicit a diminished response.

Figure 5. MBP-kinase activation induced by exogenous PA

Empty vector control Msk8 cell-suspensions were incubated with 100 μ M PA or PC (a), or with 10% (v/v) ruptured (snapfrozen-and-thawed 5 min) cells from control or *LePLD α 1*-silenced Msk8 cell-suspensions (b). Proteins were extracted from samples taken after 2, 5, 10 and 20 min and assayed for protein-kinase activity towards MBP by in-gel-kinase analysis.

The Arabidopsis pld α 1/ δ double mutant and the wounding response

Although the Arabidopsis *pld α 1* 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 (Figure 1a-d, Figure 2). This indicates that there is likely another PLD isoform active in the ruptured cells. *AtPLD δ* seems a good candidate because, like *AtPLD α 1*, it is active in relatively high Ca^{2+} concentrations (Qin *et al.*, 2002) and *AtPLD δ* is highly expressed (Li *et al.*, 2006). *pld α 1/ δ* double knock-out mutants were generated by crossing homozygous *AtPLD α 1* and *AtPLD δ* T-DNA-insertion lines and inbreeding subsequent generations to obtain lines homozygous for both insertions. Knock-outs were verified by genomic PCR (Figure 6). No obvious growth or developmental phenotype was observed under standard greenhouse- and growth chamber conditions in either of the single mutants or the double mutant.

Figure 6. Verification of Arabidopsis *pld α 1* and *pld δ* knock-outs

Genomic DNA were extracted from wildtype (wt, Col-0), *AtPLD α 1* (SALK_067533), *AtPLD δ* (SALK_023247) and *AtPLD α 1/ δ* -double T-DNA insertion lines and was used to verify the presence of the wildtype *AtPLD α 1* (a) and *AtPLD δ* (b) gene or the SALK_067533 (a) and SALK_023247 (b) T-DNA insertion by PCR. Products were separated by gel electrophoresis alongside a DNA size ladder (L).

Leaf discs from wildtype and *pld* knock-out lines were prelabeled with $^{32}\text{P}_i$ and wounded or snapfrozen-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 (Figure 1a-d, Figure 2), the PA and Pbut production in the *pld α 1* mutant line was consistently lower than in the wildtype line (Figure 7). In contrast, PA and Pbut increases in the *pld δ* knock-out line were closer to wildtype levels (Figure 7). However, in the double mutant, a complete lack of wounding- and rupture-induced PA and Pbut increases was observed; levels remained at or below the levels measured in untreated leaf discs (Figure 7). These results indicate that AtPLD δ is responsible for the residual PLD activity seen upon wounding and loss of membrane integrity in the *pld α 1* knock-out line.

Figure 7. PLD activity after wounding and loss of cell membrane integrity in Arabidopsis *pld* mutants

Leaf discs from wildtype (Col-0) and *pld α 1*, *pld δ* and *pld α 1/ δ* double knock-out lines were prelabeled overnight with $^{32}\text{P}_i$, left untreated, wounded or snapfrozen-and-thawed for 15 min in the presence 0.5% *n*-butanol. Lipids were extracted, separated by TLC and analyzed by phosphoimaging. PA (a) and Pbut (b) were quantified as percentage of total radiolabeled lipids and are presented in a histogram $\pm$ SD ($n=3$).

Rapid and transient MAPK activation in response to wounding has also been found in Arabidopsis (Ichimura *et al.*, 2000). AtMPK4 and AtMPK6 are 47 kDa and 44 kDa wounding-activated MBP-kinases, respectively. MBP-kinases of corresponding sizes were activated in wildtype Arabidopsis leaves in response to mechanical wounding (Figure 8a). Activity was detectable within 2 min and kept on increasing up until the last measured timepoint (20 min). Surprisingly, the MBP-kinase activation seen in the *pld α 1/ δ* double mutant was identical to the activation seen in wounded wildtype leaves (Figure 8a). In order to examine conditions comparable to the assays for wounding-induced PLD activity (Figure 1, 2 and 7), protein extracts from wounded leaf discs were analyzed for MBP-kinase activity. The same activation as in whole leaves could be observed in both wildtype and mutant leaf discs, with the exception that in this case the wounding-induced activity declined again within the assay period (Figure 8b). 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 *pld α 1/ δ* double mutant.

Figure 8. MBP-kinase activation in wounded Arabidopsis *pldα1/δ*-double mutants

Fully expanded leaves from 6-week-old wildtype and *pldα1/δ*-double knock-out lines were wounded with a hemostat (a). Leaf discs that had been excised from fully expanded leaves of wildtype and *pldα1/δ*-double knock-out lines were incubated on labeling buffer overnight and subsequently wounded with a hemostat (b). Proteins were extracted from samples taken after 0, 2, 5, 10 and 20 min and assayed for protein-kinase activity towards MBP by in-gel-kinase analysis.

AtLOX2, a lipoxygenase involved in JA synthesis, is transcriptionally upregulated in response to wounding in Arabidopsis (Bell and Mullet, 1993). In an *AtPLDα1*-silenced Arabidopsis line, the wounding-induced expression of *AtLOX2* was reported to be adversely affected (Wang *et al.*, 2000). These authors showed that, whereas there was a distinct induction of expression in wildtype plants within 1 hour, there was hardly any increase in *AtLOX2* mRNA transcript levels even up to 4 hours after wounding in *AtPLDα1*-silenced lines. We therefore decided to follow the wounding-induced *AtLOX2* expression in our *pld* knock-out lines (Figure 9). In contrast to expectations, there was no evident difference in the induction of *AtLOX2* expression between wildtype and mutant lines. This finding shows that, like MAPK activation, wounding-induced *AtLOX2* expression is not affected in the single *pldα1* and *pldδ* or the double *pldα1/δ* knock-out mutants.

Figure 9. Wounding-induced *AtLOX2* expression in Arabidopsis *pld* mutants

Fully expanded leaves from 6-week-old wildtype (wt, Col-0) and *pldα1*, *pldδ* and *pldα1/δ*-double knock-out lines were wounded with a hemostat. After 0, 0.5, 1, 2 and 4 hours RNA was extracted and *AtLOX2* expression analyzed by RNA blot. Ribosomal RNA (rRNA) is presented as a loading control.

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 and Howe, 2005). Consequently, mutants with impaired basal defenses or an impaired wounding response are more nutritious to herbivores (Li *et al.*, 2003). The fact that reduced wounding-induced JA levels have been reported in *AtPLDα1*-silenced Arabidopsis plants (Wang *et al.*, 2000) prompted us to investigate 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 wildtype and mutant Arabidopsis plants over a period of 4 days, was measured and averaged (Figure 10). A glabrous Arabidopsis variety (Col-5) and the JA-insensitive *coi1-16* mutant (Ellis and Turner, 2002) were assayed as positive controls along with the wildtype (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 to performance on the wildtype plants, the weight gain achieved on *pldα1*, *pldδ* or *pldα1/δ* double knock-outs did not differ significantly from that on wildtype (Figure 10). These data indicate that the wounding response in the *pld* mutants is not affected in such a way that it influences herbivore performance.

Figure 10. Herbivore performance on Arabidopsis *pld* mutants

Pieris rapae larvae (stage L2) were allowed to feed on wildtype (Col-0), glabrous (Col-5), JA-insensitive (*coi1-16*), or *pldα1*, *pldδ* and *pldα1/δ* double knock-out lines for 96 hours. The caterpillars were weighed prior to placement and after 96 hours, the fold increase in weight is presented in a histogram $\pm$ SE (n=19-28). Asterisks indicate statistically significant differences compared to Col-0 (Fisher's LSD-test; $p \leq 0,05$).

Discussion

PLD activity in the Arabidopsis wounding response

The abundance of divergent PLD isoforms in higher plants, as compared to yeast and animals, implies that the different plant PLDs have distinct functions. Knock-out of specific PLD isoforms is a useful tool for gaining new insight into the function of the different family members. Here, we have demonstrated that AtPLD α 1 and AtPLD δ become active in wounded Arabidopsis plants and have resolved the location (*i.e.* the ruptured cells rather than the intact cells) of this activity using $^{32}\text{P}_i$ -mediated phospholipid analysis of *PLD* T-DNA insertion lines and a cell-suspension wounding model.

The fact that massive PLD activation can be observed in leaf discs and cell-suspension cultures after cellular compartmentalization has been lost by snapfreezing-and-thawing (Figures 1, 2, 4 and 7) indicates that at least part of the PA increase seen in mechanically damaged leaf material (Figures 1, 2, 3 and 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. Purified, recombinant proteins of both of these PLDs have been analyzed *in vitro* (Pappan and Wang, 1999; Qin *et al.*, 2002). These studies indicated that they are active at mM Ca^{2+} concentrations and, in the case of AtPLD α 1, at acidic pH. Taken together, these data suggest that AtPLD α 1 and AtPLD δ become active autonomously upon encountering apoplastic conditions, which closely resemble the conditions required for *in vitro* activity. This conclusion implies that the PLD activity reported to occur in response to other stresses that could cause loss of cellular compartmentalization, such as freezing, H_2O_2 and severe dehydration (Frank *et al.*, 2000; Katagiri *et al.*, 2001; Welti *et al.*, 2002; Zhang *et al.*, 2003; Li *et al.*, 2004), might also take place upon rupture of cells.

Dissection of wounded Arabidopsis leaf discs (Figure 2) 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 (Figure 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 when the primary leaf of an untouched Arabidopsis plant was mechanically wounded (Figure 3). This indicates that PLD is not activated systemically in a wounded Arabidopsis plant. These results contradict earlier reports of wounding-induced PLD activation in intact tissues (Ryu and Wang, 1996; Lee *et al.*, 1997; Ryu and Wang, 1998; Lee *et al.*, 2001). This disparity can be explained by differences in lipid extraction methods, lipid analysis, plant species, examined tissues or circumstances of plant growth. Our lab has developed sensitive phospholipid-analysis techniques that have been used to detect PA signaling events in numerous plant systems (Frank *et al.*, 2000; Munnik *et al.*, 2000; den Hartog *et al.*, 2001; den Hartog *et al.*, 2003; Bargmann *et al.*, 2006). Although it cannot be fully excluded that there is some undetectable PLD activity, we deduce that PLD activity in the Arabidopsis wounding response is restricted to ruptured cells.

PLD activity in the wounding response has been proposed to be positioned upstream of MAPK activation (Lee *et al.*, 2001) as well as JA production and *AtLOX2* gene expression (Wang *et al.*, 2000). The fact that a wounding-induced MBP-kinase response can be measured in intact cells that do not display a detectable PA response (Figure 2, 4 and 8b) 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 (Figure 8). Lee *et al.* (2001) based their hypothesis on results showing activation of MAPK by the application of exogenous PA and inhibition of wounding-induced MAPK activation by *n*-butanol. We now show that adding exogenous PA actually mimics the addition of ruptured cells. Furthermore, the wounding-induced *AtLOX2* expression in- and herbivore performance on the single- and double *pld* knock-out lines was not affected (Figures

9 and 10). This discrepancy with the expectations derived from earlier reports could perhaps be due to the fact that these authors used a silencing strategy whereas we have used T-DNA-insertion mutants. Alternatively, variations in growth conditions and/or treatments may have differed materially.

PA as a non-cell-autonomous wounding signal

One of the central questions in the research into the plant wounding response pertains to the identity of the non-cell-autonomous (primary) wounding signals generated at the wound-site that elicit a response in the intact tissues (Schilmiller and Howe, 2005). Numerous candidate signals have been volunteered. Systemin (Ryan, 2000), pectic polysaccharides (Stratmann and Ryan, 1997), reactive oxygen species (Huang *et al.*, 2004), electrical pulses (Wildon *et al.*, 1992) and hydraulic pressure (Malone and Alarcon, 1995) have all been proposed as wounding signals. On the whole, it seems there is an assortment of prospective local signals that are able to elicit a response from intact cells. Yet, for most, the antithesis has not been clearly demonstrated, *i.e.* that the truancy of a proposed primary signal, for instance by genetic modification, leads to an absence of the wounding response. This can be rationalized by the existence of various parallel local wounding signal transduction pathways.

If PLD activity in the intact, responding cells is not involved in the Arabidopsis wounding-response, could PLD activity in the ruptured cells somehow play a role in the process? Application of exogenous PA to a tomato cell-suspension induces an MBP-kinase activation similar to that elicited by the addition of ruptured cells (Figure 5). This result resembles the activation of a wound-induced MAPK by the addition of PA in a soy bean cell-suspension culture (Lee *et al.*, 2001). In fact, the application of exogenous PA has been shown to induce various responses in plants in a number of different studies. Lee *et al.*, (2003) demonstrated that PA also induces actin cytoskeleton rearrangements in a soy bean cell-suspension culture. Huang *et al.* (2006) show that exogenous PA induces changes in the actin cytoskeleton in an Arabidopsis cell-suspension culture. The production of reactive oxygen species has been reported to be induced by PA in a tobacco cell-suspension culture (de Jong *et al.*, 2004), a rice cell-suspension culture (Yamaguchi *et al.*, 2004) and in Arabidopsis leaves (Sang *et al.*, 2001), while Park *et al.* (2004) showed that PA causes cell-death in detached Arabidopsis leaves. We have found that ruptured *LePLD α 1*-silenced cells, which exhibit reduced PA production upon loss of membrane integrity (Figure 4b), elicit a weakened MBP-kinase activation when compared to control cells (Figure 5b). Together, these findings argue for PA as a non-cell-autonomous primary wounding signal. Nonetheless, the fact that the Arabidopsis *pld α 1/* δ double knock-out still exhibits wounding-induced MBP-kinase activation and *AtLOX2* expression (Figure 8 and 9) and does not behave differently in a herbivore performance assay (Figure 10) suggests that there are alternative/additional wounding signals *in planta*.

An intriguing question is how PA reaches the intact, responding cells and also how PA elicits a subsequent response in these cells. Treatment of tobacco cell-suspension cultures and pollen tubes with fluorescently labeled PA has demonstrated that plants cells can incorporate this phospholipid when applied

exogenously (Dhonukshe *et al.* 2003; Potocky *et al.*, 2003). Once in the membrane, exogenous PA could function as a second messenger in signaling cascades just as it has been proposed to do when produced intracellularly (reviewed in Testerink and Munnik, 2005; Wang, 2006). Yet, how the PA, which is presumably in lipid vesicles or particles, crosses the plant cell wall is unclear. Extracellular lipid transfer proteins could well be involved, they have been shown to mediate JA perception in tobacco (Buhot *et al.*, 2004) and have been implicated in systemic signaling in response to pathogenic elicitation (Maldonado *et al.*, 2002). Further research is required to ascertain which factors, if any, are needed for the cellular incorporation of exogenous PA.

Methods

Plant material

Arabidopsis thaliana var. Col-0 T-DNA insertion lines were obtained from the SALK Institute (Alonso *et al.*, 2003). Homozygous lines were generated for *AtPLD α* (SALK_067533) and *AtPLD δ* (SALK_023247), which were consequently crossed and inbred to obtain lines that were homozygous for both insertions. The following primers were used to verify genomic insertions by PCR:

SALK_067533F 5'-GACGATGAATACATTATCATTTGG-3'

SALK_067533R 5'-GTCCAAAGGTACATAACAAC-3'

SALK_023247F 5'-TGTAACCGTGCTTCGGGAAA-3'

SALK_023247R 5'-TCGAGAAACAATGGTGCGACA-3'

SALK_LeftBorderA 5'-TGGTTCACGTAGTGGGCCATCG-3'

SALK_LeftBorderA was used in combination with SALK_067533R and SALK_023247F, in compliance with the orientation of the T-DNA insertion. The Col-5 (accession number N1644, Nottingham *Arabidopsis* Stock Centre) variety and the *coil-16* were obtained from the labs of Maarten Koornneef and John Turner, respectively. For routine plant growth seeds were sown on soil and vernalized at 4° C for 2 days. For the analysis of systemic PA formation seeds were sown on rockwool cubes and vernalized at 4° C for 2 days. Ordinarily, plants were grown in a growth chamber under a 12h light/12h dark regime with a 23°C/18°C cycle and 70% humidity. For the herbivore performance assay plants were grown under short day conditions, *i.e.* 9h light/15h dark regime.

Cell-suspension cultures

Suspension-cultured cells (*Lycopersicon esculentum* Mill.; line Msk8; Felix *et al.*, 1991) were grown at 24°C in the dark at 125 rpm in MS medium supplemented with 3% (w/v) sucrose, 5.4 μ M NAA, 1 μ 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 days after weekly subculturing.

For the silencing vector, an inverted repeat (RNAi) construct specific for *LePLD α* was generated targeting the gene's 3' UTR. PCR amplification of the *LePLD α* cDNA, cloned previously by Laxalt *et al.* (2001), was performed with the following oligonucleotides: 1_5'-CGGGATCCCCATCGATCAGTCAATTAAAGCATCTC-3'(reverse) with a *Bam*HI and a *Clal* restriction site, 2_5'-CCGGAATTCCCCGACACCAAGG-3' (forward) with an *Eco*RI restriction site and 3_5'-CCGGAATTCCATCCAGAAAGTGAGG-3' (forward) with an *Eco*RI restriction site. The PCR products resulting from primer combinations 1-2 and 1-3 were ligated in a 1-2/3-1 orientation into the pGreen1K binary vector which was modified to contain the 35S-Tnos cassette from pMON999. Cell-suspension culture transfection was achieved as described by Bargmann *et al.* (2006).

RNA blot analysis

Total RNA from ground leaf tissue was isolated using an RNeasy plant mini kit (QIAGEN, The Netherlands). 10 μ g RNA was separated by denaturing 1.4% formaldehyde-agarose gel electrophoresis and transferred onto Hybond-XL nylon membranes (Amersham Pharmacia, Buckinghamshire, England). The blot was hybridized with a ³²P-labeled *AtLOX2* probe in modified Church solution at 65°C.

Membranes were washed 3 times for 15 min with wash buffer (1xSSC, 0.1% (w/v) SDS) and probe signal was visualized by autoradiography.

in vivo phospholipid analysis

Cell-suspension cultures were labeled by incubation with 100 μCi carrier-free $^{32}\text{PO}_4^{3-}$ in growth medium. Treatments were performed in the presence of 0.5% (v/v) n-butanol when indicated and treatments were stopped and lipids extracted as described before (van der Luit *et al.*, 2000). Leaf discs (5 mm $\varnothing$) were labeled by incubation with 100 μCi carrier-free PO_4^{3-} on 100 μl 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 labeled by pipetting 100 μl water containing 100 μCi carrier-free PO_4^{3-} onto the rockwool and leaving them overnight under fluorescent light in a fume hood. Treatments were stopped by incubation with 5% (w/v) perchloric acid. The plant material was then transferred to a new tube containing 375 μl $\text{CHCl}_3/\text{MeOH}/\text{HCl}$ (50 : 100 : 1 [v/v]) where lipids were extracted during vigorous shaking for 10 min. A two-phase system was induced by addition of 375 μl CHCl_3 and 200 μl 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 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: $\text{CHCl}_3/\text{MeOH}/25\% \text{NH}_4\text{OH}/\text{H}_2\text{O}$ (90 : 70 : 4 : 16 [v/v]; Munnik *et al.*, 1994) when indicated. Radiolabeled phospholipids were visualized by autoradiography and quantified by phosphoimaging (Molecular Dynamics, Sunnyvale CA, USA).

In-gel-kinase assay

Proteins were extracted from ground plant- and cell-suspension material using 1 volume of extraction buffer (50 mM Tris/HCl pH 7.5, 5 mM EDTA, 5 mM EGTA, 2mM DTT, 25 mM NaF, 1 mM Na_3VO_4 , 50 mM β -glycerophosphate, 1x complete protease inhibitor cocktail) and a 15 minute 10,000g centrifugation. Samples were assayed for protein content according to Bradford (1976) and 10 μg protein was loaded onto a 10% SDS-PAGE gel containing 4 $\mu\text{g}/\text{ml}$ Myelin Basic Protein (Upstate, Lake Placid NY, USA). The gel was washed 3 times for 30 min with wash buffer (25 mM Tris/HCl pH 7.5, 500 μM DTT, 100 μM Na_3VO_4 , 5 mM NaF, 500 $\mu\text{g}/\text{ml}$ BSA, 0.1% [v/v] Triton X-100) and renatured overnight in renaturation buffer (25 mM Tris/HCl pH 7.5, 1 mM DTT, 100 μM Na_3VO_4 , 5 mM NaF). The gel was washed 3 more times for 30 min in reaction buffer (25 mM Tris/HCl pH7.5, 1 mM DTT, 100 μM Na_3VO_4) and then incubated in reaction buffer supplemented with 25 μM cold dATP and 50 μCi ^{32}P -labeled γ -ATP for 1 hour. The reaction was stopped and the gel was washed 6 times for 30 min with stop buffer (1% [w/v] $\text{Na}_2\text{H}_2\text{P}_2\text{O}_7$, 5% [v/v] trichloric acid). The gel was dried and the signal was visualized by autoradiography and quantified by phosphoimaging (Molecular Dynamics, Sunnyvale CA, USA).

Herbivore performance assay

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

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Chapter 4

LePLD β 1 activation and relocation in suspension-cultured tomato cells treated with xylanase

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Summary

Phospholipase D (PLD) has been implicated in various cellular processes including membrane degradation, vesicular trafficking and signal transduction. Previously, we described a PLD gene family in tomato (*Lycopersicon esculentum*) and showed that gene expression of one of them, *LePLD β 1*, was induced by treatment with the fungal elicitor xylanase. To further investigate the function of this PLD, a gene-specific RNAi construct was used to knock down *LePLD β 1* transcript levels in suspension-cultured tomato cells. Silenced cells exhibited a strong decrease in xylanase-induced PLD activity and responded to xylanase treatment with a disproportionate oxidative burst. Furthermore, *LePLD β 1*-silenced cell-suspension cultures were found to have increased polyphenol oxidase activity, secreted less of the β -D-xylosidase LeXYL2 and secreted and expressed more of the xyloglucan-specific endoglucanase inhibitor protein XEGIP. Using an *LePLD β 1*-GFP fusion protein for confocal laser scanning microscopy-mediated localization studies, untreated cells displayed a cytosolic localization whereas treatment with xylanase induced relocalization to punctuate structures within the cytosol. Possible functions for PLD β in the plant-pathogen interaction are discussed.

Abbreviations: PLD, phospholipase D; PLC, phospholipase C; DGK, diacylglycerol kinase; PA, phosphatidic acid; PIP₂, phosphatidylinositolbisphosphate; PR protein, pathogenesis related protein; ROS, reactive oxygen species; PPO, polyphenol oxidase

Introduction

A plant's susceptibility or resistance to a certain pathogen often depends on its ability to recognize pathogen attack. Once the pathogen has been perceived, the plant can respond with a plethora of defensive strategies (Nimchuck *et al.*, 2003). For example, plants can accumulate compounds and proteins that are toxic to the attacker, limit their nutritional value with respect to the pathogen or fortify their cell-walls (Dixon, 2001; Howe, 2005). Together, such adaptations constitute the plant defense response.

Phospholipid signaling has recently been implicated in plant responses to various environmental cues, both biotic and abiotic (Meijer and Munnik, 2003; Wang, 2004). Especially phosphatidic acid (PA) mediated signal transduction has been shown to play an important role in numerous processes (Munnik, 2001; Testerink and Munnik, 2005). The lipid second messenger PA can be generated by two distinct enzymatic routes, either directly by phospholipase D (PLD) or via the combined action of phospholipase C (PLC) and diacylglycerol kinase (DGK).

PLD catalyses the hydrolysis of structural phospholipids producing PA and a free head-group (Wang, 2000). Functions for PLD have been suggested in membrane degradation (Ryu and Wang, 1995), vesicular trafficking (Roth *et al.*, 1999), membrane tethering (Gardiner *et al.*, 2001; Dhonukshe *et al.*, 2003) and intracellular signaling (Munnik, 2001; Wang, 2002; Laxalt and Munnik, 2002). Plants possess a vast variety of PLDs, whereas mammals have just two PLDs and only one has been found in yeast (Elias *et al.*, 2002, Qin and Wang, 2002, Meijer and Munnik, 2003).

The *Arabidopsis* genome contains 12 PLDs that can be subdivided into several classes depending on their sequence similarity, substrate specificity and enzymatic requirements. *Arabidopsis* has two calcium-independent PLDs, AtPLD ζ 1 and AtPLD ζ 2, that are similar to mammalian and yeast PLDs with regard to their N-terminal lipid binding domains, namely a Pleckstrin homology domain and a PHOX homology domain (Qin and Wang, 2002). The remaining ten PLDs in the *Arabidopsis* genome harbor an N-terminal C2 domain and are subdivided into the α , β , γ , δ and ϵ PLD classes (Wang, 2000; Elias *et al.*, 2002). Whereas α -class PLDs require millimolars of calcium and low pH for *in vitro* activity, the β - and γ -class PLDs require phosphatidylinositol 4,5-bisphosphate (PIP₂) and physiological calcium concentrations (low micromolar range) and pH (neutral) for *in vitro* activity (Wang, 2000). The δ -class PLD distinguishes itself in that its *in vitro* activity is stimulated by oleic acid (Wang and Wang, 2001). The *in vitro* activity of PLD ϵ has not been studied to date.

Evidence of PA signaling as a part of the plant defense response has been observed in several independent studies (Laxalt and Munnik, 2002). An increase in expression of various *Arabidopsis* PLDs was demonstrated in response to both virulent and avirulent strains of *Pseudomonas syringae* (Zabela *et al.*, 2002). The same is true for several PLDs in rice treated with *Xanthomonas oryzae* (Young *et al.*, 1996). In addition, a change in PLD membrane distribution upon microbial infiltration was observed (Young *et al.*, 1996). Furthermore, PLC/DGK-generated PA was shown to be crucial for the oxidative burst response following the Avr4/Cf-

4 interaction in transgenic tobacco cell-suspensions (de Jong *et al.*, 2004). Recently, PLD activation in response to the elicitor chitosan has been ascertained in rice cell-suspension cultures and this activity was also suggested to be involved in the oxidative burst response (Yamaguchi *et al.*, 2003, 2004, 2005).

Earlier, our laboratory demonstrated PLC and PLD activation in response to pathogenic elicitation in tomato cell-suspension cultures (Msk8; *Lycopersicon esculentum* Mill.) treated with xylanase (van der Luit *et al.*, 2000). Subsequently, we showed that expression of one PLD, *LePLD β 1*, is specifically up-regulated after xylanase treatment (Laxalt *et al.*, 2001). Xylanase (endo-1,4- β -xylanase), from the green mold *Trichoderma viride*, is a family 11 glucoside hydrolase that is characterized by its ability to break down various xylans to produce short-chain xylo-oligosaccharides. It is a potent elicitor of plant defense responses in tomato (Enkerli *et al.*, 1999; Furman-Matarasso *et al.*, 1999). Recently, two likely xylanase receptors were cloned from tomato, establishing the relationship between this elicitor and the plant defense response as a ligand-receptor interaction (Ron and Avni, 2004).

In this report we investigate the role of *LePLD β 1* in the Msk8 response to xylanase elicitation. A silencing strategy was employed to resolve whether diminished *LePLD β 1* expression influences the xylanase-induced PLD activity and the phenotypic consequences of silencing are explored. A GFP-tagging approach was used to examine the influence of xylanase on the intracellular *LePLD β 1* localization.

Results

Rapid LePLD β 1 expression and LePLD β 1 activation in response to xylanase treatment

In untreated cell-suspension cultures, the *LePLD β 1* transcript was hardly detectable by Northern blot analysis. Basal expression levels could only be visualized after a long exposure (Figure 1a) but levels rapidly increased when cells were treated with 100 μ g/ml xylanase (Figure 1b). Xylanase exposure led to a readily detectable *LePLD β 1* transcript level within three hours and this expression was still present after 24 hours, albeit to a lesser extent. The induction of *LePLD β 1* expression preceded the increase in Pathogenesis Related protein 1 (*PR1*) transcript level, which was detected only after 24 hours of xylanase treatment (Figure 1b).

In order to study *LePLD β 1* function in more detail, *LePLD β 1* expression was knocked down in Msk8 cell-suspension cultures using a gene-specific RNAi construct (see Experimental Procedures). Several silenced lines were obtained that had diminished basal *LePLD β 1* transcript levels compared to empty-vector control lines (Figure 1a). Upon xylanase treatment, the effect of the silencing construct on *LePLD β 1* expression was more obvious; a representative silenced line displaying a $\pm$ 90% reduction in transcript level is shown in Figure 1b.

Figure 1. A gene-specific RNAi construct reduces *LePLDβ1* transcript levels in Msk8 cells.

Total RNA was isolated, separated by electrophoresis and gel blots were hybridized with ^{32}P -labeled *LePLDβ1* and *PR1* probes, the signal was visualized by autoradiography. Ethidium bromide staining of ribosomal RNA (rRNA) is presented as a loading control. The basal expression levels in three independent empty-vector- and three independent *LePLDβ1*-silenced lines are shown (a). Empty-vector control cultures and *LePLDβ1*-silenced cultures were incubated for 3 or 24 hours with or without 100 $\mu\text{g/ml}$ xylanase. A representative silenced line is shown (b).

The acquisition of *LePLDβ1*-silenced lines allowed us to test whether this PLD is responsible for the xylanase-induced PLD activity in Msk8 cell-suspensions reported previously (van der Luit *et al.*, 2000; Laxalt *et al.*, 2001). *In vivo* changes in phospholipid composition can be monitored by pre-labeling cells with radioactive phosphate. After treatment, lipids can be extracted, separated by thin layer chromatography and quantified by phosphoimaging. PLD activity can be visualized using this enzyme's unique transphosphatidyltransferase activity, which entails that in the presence of primary alcohols, PLDs can transfer the lipid moiety of its substrate to the alcohol producing a phosphatidylalcohol (Munnik *et al.*, 1995; Munnik, 2001). In this case, cells were treated with xylanase in the presence of 1-butanol. PLD activity in Msk8 cells incubated with 100 $\mu\text{g/ml}$ or 200 $\mu\text{g/ml}$ xylanase increased 2- and 2.5-fold, respectively, within 1 hour as measured by the production of phosphatidylbutanol in xylanase treated cultures compared to mock treated cultures (Fig. 2a and 2b). However, in *LePLDβ1*-silenced cell-suspension cultures this increase in activity was markedly reduced, reaching at most a 1.5-fold increase (Fig. 2a and 2b). These results indicate that both *LePLDβ1* expression and *LePLDβ1* activity are increased upon xylanase treatment.

Figure 2. Xylanase-induced PLD activation is strongly reduced in *LePLDβ1*-silenced cells.

³²P_i-labeled empty-vector control and *LePLDβ1*-silenced cell-suspension cultures were incubated for 1 hour with 0, 100 or 200 µg/ml xylanase in the presence of 0.5% (v/v) 1-butanol. Lipids were extracted and separated by TLC, radioactivity was visualized by autoradiography (a). The origin, phosphatidic acid (PA) and phosphatidylbutanol (Pbut) are indicated. Two independent experiments were quantified by phosphoimaging. The fold increase in Pbut formation in xylanase treated- compared to mock treated cultures is presented in a histogram with standard deviations (b). Empty-vector control samples are represented by black bars and *LePLDβ1*-silenced samples by hatched bars. A representative silenced line is shown.

Enhanced ROS response in LePLDβ1-silenced cell-suspension cultures

The plant defense response consists of a wide variety of reactions, *e.g.* accumulation of phytoalexins, PR (pathogenesis related) proteins, proteinase inhibitors and glucanase inhibitors; lignification and callose deposition. One of the earliest measurable responses to elicitor perception is the production of ROS. We therefore decided to study this phenomenon in Msk8 cell-suspension cultures exposed to xylanase and examine in particular whether silencing *LePLDβ1* affected the response.

Hydrogenperoxide is a typical ROS produced by plant cells in response to pathogenic elicitation. An assay adapted from Felix *et al.* (1999) was used to study H₂O₂ production by making use of the oxidation of 5-amino-salicylic acid (ASA) by H₂O₂ peroxidases, which leads to the formation of a dark stain. Control cells and *LePLDβ1*-silenced cells were spread as flat lawns in Petri dishes and test solutions containing different amounts of xylanase were applied locally. In the presence of ASA, a dark stain was visible after 24 hours around the areas where cells had responded with H₂O₂ production; 50 ng of xylanase barely induced macroscopic staining while 5 µg induced massive staining around the area of application (Fig. 3). The *LePLDβ1*-silenced cell-suspension cultures responded to the same range of elicitor concentrations, however, the staining was more intense and typically gave higher backgrounds (Fig. 3).

Figure 3. ASA-mediated hydrogenperoxide staining is more intense in *LePLDβ1*-silenced lines.

Aliquots of empty-vector control cell-suspension cultures and *LePLDβ1*-silenced cell-suspension cultures with or without 10 mM 5-amino-salicylic acid were placed in Petri dishes. Most of the medium was removed leaving a lawn of cells; 1 μ l of cell free medium (negative control) or 1 μ l cell free medium containing 5, 50, 500 or 5000 ng xylanase was applied onto the lawn as indicated. Petri dishes were analyzed optically by flat-bed scanner after 24 hours. A representative silenced line is shown.

These results prompted us to look at the H_2O_2 production in more detail, focussing on the immediate ROS response following elicitation, termed the oxidative burst. H_2O_2 production was monitored by measuring the decrease in fluorescence of the H_2O_2 -sensitive probe pyranin in a microtiter-plate assay. This reporter loses its fluorescence at 512 nm when oxidized by H_2O_2 peroxidases (Apostol *et al.*, 1989; de Jong *et al.*, 2004). The oxidative burst response during the first 30 minutes following elicitation with 50 ng/ml, 500 ng/ml and 5 μ g/ml xylanase was examined. Control Msk8 cells clearly responded to 500 ng/ml and 5 μ g/ml xylanase within the assay period, displaying a biphasic response to 5 μ g/ml xylanase, with an initial strong burst between 5 and 10 minutes, followed by a secondary weaker burst after 15 minutes (Fig. 4a). As with the ASA assay (Fig. 3), the *LePLDβ1*-silenced cell-suspension cultures responded to the same range of elicitor concentrations, however, the biphasic response seen in control cell-suspension cultures was not discernable in silenced lines. Instead, the initial strong burst continued throughout the assay period (Fig. 4b). Together, these results show that silencing *LePLDβ1* affects the ROS response to xylanase in tomato cells.

Figure 4. *LePLDβ1*-silenced cells respond to xylanase with a disproportionate oxidative burst. H₂O₂ mediated decrease in pyranin fluorescence was followed for 30 minutes in 1 minute intervals after elicitation with 0 ng/ml (◆), 50 ng/ml (■), 500 ng/ml (▲) and 5 µg/ml (●) xylanase. An empty-vector control cell-suspension culture (a) and an *LePLDβ1*-silenced cell-suspension culture (b) were assayed in triplicate and averaged. Fluorescence is presented relative to initial fluorescence. A representative silenced line is shown.

Elevated polyphenol oxidase activity in LePLDβ1-silenced cell-suspensions

Polyphenol oxidases (PPOs) oxidize phenols to form quinones: highly reactive molecules that can covalently link encountered nucleophiles resulting in the formation of melanin-like black or brown condensation polymers. PPO enzymes are present in the thylakoid lumen and their phenolic substrates in the vacuole. Upon loss of cell integrity, enzyme and substrate converge and quinones are produced (Koussevitsky *et al.*, 2004). PPO has been linked to several plant-pathogen interactions. Its activity and gene expression is wounding- and pathogen-inducible and over-expression of PPO in tomato has been shown to increase resistance to the bacterial pathogen *Pseudomonas syringae* (Thipyapong and Steffens, 1997; Howe and Ryan, 1999; Li and Steffens, 2002).

When shaking in the incubator, there was no visible difference between the control and *LePLDβ1*-silenced cell-suspensions. We noticed that the culture media of the silenced lines developed an orange/brown coloration when the old culture flasks were left standing on the lab-bench after subculturing. To further investigate this color change, 3 ml of one-week-old control and *LePLDβ1*-silenced cell-suspension cultures was transferred to test-tubes and photographed after 0, 24 and 48 hours. Whereas control cell-suspension cultures showed no or hardly any coloration, even after 48 hours, the silenced lines displayed a strong coloration of the medium within 24 hours and the formation of a dark precipitate within 48 hours (Fig. 5a). This coloration was derived from the plant cells, as cell-free medium from one-week-old cultures displayed no color change (data not shown). FDA staining of cell-suspensions that had been left standing showed that both the control- and silenced cell-suspensions had started to lose cell integrity within 24 hours and that, in both cases, all cells had perished within 48 hours (data not shown). Coloration of the medium could be abated by co-incubation with 10 mM of the anti-oxidant ascorbic acid (data not shown). The discharged compound was most likely an oxidated phenolic. We tried to identify it by mass spectrometry but, unfortunately,

definitive identification of the compound(s) was not possible due to its resistance to fragmentation (data not shown).

These observations prompted us to examine whether there was more PPO activity in the silenced lines. Assaying for PPO activity in protein extracts of untreated cell cultures and cultures that had been left standing for 24 hours showed that silenced lines indeed had more PPO activity (Fig. 5b). Purple coloration caused by oxidation of *p*-phenylenediamine, indicative of PPO activity, was more intense in protein extracts of silenced lines than in control culture protein extracts, irrespective of whether cell-suspensions had been left standing (Fig. 5b). These results indicate that knocking down *LePLDβ1* expression affects Msk8 cell-suspension cultures' phenolic metabolism.

Figure 5. *LePLDβ1*-silenced cells have increased polyphenol oxidase activity.

3 ml aliquots of week-old empty-vector control and *LePLDβ1*-silenced cell-suspension cultures were transferred to 12 ml tubes and left standing at room temperature. Pictures were taken after 0, 24 and 48 hours (a). Cellular protein extracts from control and silenced cultures that had been left standing for 0 or 24 hours were assayed for polyphenol oxidase activity by monitoring the color change from clear to purple due to oxidation of *p*-phenylenediamine (b). A representative silenced line is shown.

Anomalous protein secretion in culture medium of LePLDβ1-silenced cell-suspensions

The plant cell-wall is a sturdy encasing that keeps the cell from bursting, protects it against dehydration and also serves as a physical barrier to invasive attackers. It is composed of polysaccharides (cellulose, hemicelluloses and pectins), proteins and aromatic compounds such as lignins (Goujon *et al.*, 2003). During development, the cell-wall undergoes several modifications to allow for cell-expansion and differentiation. In order to execute these changes, plant cells secrete cell-wall-modifying enzymes that can hydrolyse and/or reattach cell-wall components. Plant pathogens also secrete cell-wall-modifying enzymes; in their case, to breach the

plant's protective barrier or to make oligo- and mono-saccharides accessible for nutritional value (Igawa *et al.*, 2004).

The altered phenolic composition of the silenced cell-suspension culture media led us to investigate the protein composition of the media. Accordingly, medium proteins were precipitated, separated by SDS-PAGE and stained with Coomassie. Two protein bands were observed, with an apparent mass of 51 and 67 kDa (Fig. 6a), that clearly differed in abundance when the medium of silenced lines was compared to that of control cell-suspensions. Both bands were excised, digested with trypsin and analyzed by Mass Spectrometry. Strikingly, the 67 kDa band that was less abundant in *LePLDβ1*-silenced cell-suspension culture medium could be identified by the MSMS of 6 peptides as LeXYL2, a β-D-xylosidase (total MASCOT score 249; accession number AB041812; Itai *et al.*, 2003). Plant β-D-xylosidases have been suggested to be involved in cell-wall remodeling (Goujon *et al.*, 2003). Surprisingly, the 51 kDa band that was more abundant in silenced cell-suspension culture medium was identified by the MSMS of 4 peptides as the xyloglucan-specific endoglucanase inhibitor protein XEGIP (total MASCOT score 113; accession number AY155579; Qin *et al.*, 2003). This protein has been shown to form a complex with the xyloglucan-specific endoglucanase from *Aspergillus aculeatus*, inhibiting its glucanase activity. For both identified proteins the theoretical mass corresponds well with the observed mass on SDS-PAGE.

XEGIP expression in control- and silenced lines was subsequently analyzed (Fig. 6b). Whereas untreated control cell-suspension cultures exhibited no detectable *XEGIP* transcript, elicitation with 100 µg/ml xylanase induced clear expression within 3 hours. In contrast, untreated *LePLDβ1*-silenced cell-suspension cultures already exhibited *XEGIP* expression and this transcript level was increased considerably beyond control cell-suspension culture levels after xylanase treatment (Fig. 6b). These results suggest that diminished *LePLDβ1* expression leads to an altered expression- and protein secretion-pattern in tomato cell-suspension cultures, especially of proteins involved in xyloglucan hydrolysis and/or the plant defense response.

Figure 6. *LePLDβ1*-silenced cells have an altered secretion and expression pattern.

Culture medium proteins from one-week-old cell-suspension cultures were precipitated and separated by SDS-PAGE (a). Indicated bands (<) were excised and analyzed by Mass Spectrometry. *XEGIP* expression was analyzed by Northern blot in control and *LePLDβ1*-silenced cell-suspension cultures after 0 and 3 hours of incubation with 100 µg/ml xylanase (b). rRNA is shown as a loading control. A representative silenced line is shown.

Relocalization of LePLD β 1-GFP after xylanase treatment

Localization studies concerning PLD β s have, up to now, focussed on PLD activity distribution. These studies have implied that PIP $_2$ -dependent, low micromolar [Ca $^{2+}$] requiring PLD activity is present both in the cytosol and on various membranes (Pappan *et al.*, 1997). However, such activity assays do not distinguish between PLDs of the β - and γ -class, let alone between members of the same class.

Polyclonal antibodies directed against LePLD β 1 were generated but, unfortunately, immuno-localization studies failed due to a lack of specificity of the antibodies (data not shown). We therefore employed a GFP-tagging strategy in combination with confocal laser scanning microscopy to examine the intracellular distribution of LePLD β 1 in Msk8 cells upon xylanase exposure. In untreated cells, the LePLD β 1-GFP signal was cytosolic (Fig. 7b). Intriguingly, after xylanase treatment the LePLD β 1-GFP signal relocalized to punctuate structures in the cytosol (Fig. 7d). These punctuate structures were 1 to 2 μ m in diameter, mobile and mainly localized close to the plasma membrane (Supplemental Fig. 1a and 1b). In contrast, the LePLD α 1-GFP signal in Msk8 cells did not relocalize in response to xylanase but remained cytosolic (Fig. 7a and 7c). These results suggest that specifically LePLD β 1 localization in Msk8 cells is altered in response to xylanase.

Figure 7. Xylanase treatment induces relocalization of LePLD β 1-GFP.

Msk8 cell-suspension cultures carrying an *LePLD α 1-GFP* (a and c) or an *LePLD β 1-GFP* (b and d) expression vector were analyzed by confocal laser scanning microscopy. Cells were left untreated (a and b) or incubated with 100 μ g/ml xylanase for 3 hours (c and d). The white bar represents 20 μ m.

Discussion

LePLD β 1 activation in response to xylanase

The plant PLD family is a diverse group that can be subdivided into several classes: the α , β , γ , δ , ϵ and ζ classes (Wang, 2000; Elias *et al.*, 2002). The PLD ζ s resemble mammalian and yeast PLDs, possessing an N-terminal PHOX homology and Pleckstrin homology domain, while the remaining classes all have C2 domains in their N-terminal region. The fact that this multitude of PLDs can be found in all higher plants examined so far suggest that they have specific functions and are not

redundant. The occurrence of so many PLDs in plants makes it difficult to determine which one is activated in response to a certain stimulus.

Here, we have shown that we are able to silence *LePLD β 1* expression in Msk8 cell-suspension cultures using an RNAi construct (Fig. 1a and 1b). Basal *LePLD β 1* transcript levels were absent in cultures expressing a *LePLD β 1*-silencing construct (Fig. 1a). Xylanase treatment of cell-suspensions led to a prolific induction of *LePLD β 1* expression that was markedly reduced in silenced lines, although some induction ($\pm 10\%$) was still detectable (Fig. 1b). Xylanase concentrations as low as 10 $\mu\text{g/ml}$ have been shown to induce *LePLD β 1* expression and PLD activation in Msk8 cells (Laxalt *et al.*, 2001; van der Luit *et al.* 2000). In this case, higher concentrations were used in the expression and activity assays (100 and 200 $\mu\text{g/ml}$; Figures 1 and 2) in order to make the difference between control- and silenced lines more distinct. In this context, it is important to note that our xylanase concentrations are not necessarily comparable to those used by others. For example, we have used a commercially available xylanase (Fluka; Laxalt *et al.*, 2001; van der Luit *et al.*, 2000; see Experimental Procedures), while others have used a xylanase fraction that they purified themselves (Bailey *et al.*, 1992; Enkerli *et al.*, 1999; Furman-Matarasso *et al.*, 1999; Ron and Avni, 2004).

Xylanase also induces an increase in PLD activity, as can be measured by the increased phosphatidylbutanol formation in xylanase treated cell-suspensions compared to mock-treated cell-suspensions. *LePLD β 1*-silenced cell-suspension cultures reached only a third of the increase in PLD activity seen in control cultures (Fig. 2a and 2b), indicating that *LePLD β 1* is responsible for the xylanase-induced PLD activity. The remaining increase in activity observed in silenced lines can be explained by incomplete gene-silencing or by the activity of another PLD. The fact that we can still detect residual expression in the silenced lines argues for the former explanation although the latter cannot be excluded.

To our knowledge, no localization studies have been done to date with respect to PLDs of the β class specifically. Assays for PIP2-dependent, low micromolar $[\text{Ca}^{2+}]$ requiring PLD activity in protein isolates have demonstrated that this PLD activity can be found both in cytosolic- and membrane-fractions (Pappan *et al.*, 1997). However, these enzymatic requirements are also present in PLDs of the γ -class (Pappan *et al.*, 1998; Fan *et al.*, 1999). Consequently, going by these studies, it is unclear whether *PLD β* is present at all in untreated plants, and, if so, whether it is soluble or on the membrane. Fan *et al.* (1999) tried to detect Arabidopsis *PLD β* in different tissues using antibodies but were unable to show the presence of this isoform, although they could detect *PLD β* mRNA. Our expression studies show that *LePLD β 1* transcript is detectable in untreated Msk8 cell-suspension cultures (Fig. 1a).

Expression of an *LePLD β 1*-GFP fusion protein confers a cytosolic fluorescent signal in untreated cell-suspension cultures (Fig. 7b). Upon treatment with xylanase, the *LePLD β 1*-GFP signal relocalized to punctuate structures in the cytosol, whereas *LePLD α 1*-GFP remained diffuse throughout the cytosol (Fig. 7c and 7d). This relocalization is another strong indication that *LePLD β 1* is activated by xylanase treatment.

LePLD β 1 involvement in the plant defense response

LePLD β 1-silenced cell-suspension cultures responded differently to xylanase than did control cultures. The oxidative burst, as measured by the pyranin assay, was much more intense in silenced lines (Fig. 4). It might seem that *LePLD β 1* is a negative regulator of the oxidative burst response to xylanase. Yet, this is difficult to rhyme with the fact that the PA produced by PLC is a positive regulator of the oxidative burst (de Jong *et al.*, 2004). This interpretation is also in conflict with the findings of Yamaguchi *et al.* (2005), who suggest that the PLD activity observed after chitosan treatment of rice cell-suspension cultures positively regulates ROS production, defense-related gene-expression and phytoalexin accumulation.

However, untreated *LePLD β 1*-silenced cell-suspension cultures were already aberrant. They displayed higher background staining in the ASA assay (Fig. 3), and showed pyranin oxidation in the absence of xylanase (Fig. 4b). Furthermore, silenced lines had higher PPO activity (Fig. 5b), secreted less LeXYL2 (Fig. 6a) and expressed and secreted more XEGIP (Fig. 6). Increased PPO activity and secretion of proteins that inhibit pathogenic cell-wall-degrading enzymes are clear indicators of the plant defense response (York *et al.* 2004; Thipyapong *et al.*, 2004). A decreased secretion of endogenous cell-wall-modifying enzymes has to our knowledge not been reported as a response to pathogenic elicitation but it is comprehensible that a plant would suspend developmental cell-wall-modification while it deals with a pathogen. Hence, the lack of basal PLD β 1 levels already has an effect on plant defense. It appears that *LePLD β 1*-silenced cell-suspension cultures are responding without elicitation; or that they are primed to respond and that the slightest elicitation sets them off. However, it seems that only a subset of defensive strategies are primed, as *PR1* expression is not affected by the silencing (Fig. 1b).

LePLD β 1 function in the plant defense response still remains undefined. The silencing strategy employed here has shed some light but there is still much to be discovered. *LePLD β 1*-GFP in Msk8 cells relocalized to what appear to be vesicles after xylanase treatment (Fig. 7d and Supplemental Fig. 1). One could speculate that these are endocytic vesicles; this fits with the fact that the recently cloned tomato xylanase receptors have an endocytosis signal (Ron and Avni, 2004). Alternatively, these punctuate structures could be secretory vesicles delivering proteins or other compounds to the cell surface in response to elicitation. *Arabidopsis* PLD β 1 has been reported to bind actin *in vitro* (Kusner *et al.*, 2003), maybe PLD β 1 can form a tether between vesicular membranes and the actin cytoskeleton, as has been proposed for the plasma membrane and cortical microtubules (Dhonukshe *et al.*, 2003). Co-localization studies and studies with cytoskeleton-affecting drugs could yield enlightening results in the future.

Methods

Cell-suspension cultures

Suspension-cultured cells (*Lycopersicon esculentum* Mill.; line Msk8; Felix *et al.*, 1991) were grown at 24°C in the dark at 125 r.p.m. in MS medium supplemented with 3% (w/v) sucrose, 5.4 μ M NAA, 1 μ 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 days after weekly subculturing.

Construction of silencing and GFP-tagging plasmids

For the silencing plasmid, an inverted repeat (RNAi) construct specific for *LePLD β 1* was generated targeting the gene's 3' UTR. PCR amplification of the *LePLD β 1* cDNA, cloned previously by Laxalt *et al.* (2001), was performed with the following oligonucleotides: 1 5'-CGGGATCCCCATCGATGATCACTTCACTCATAAAGAC-3' (reverse) with a *Bam*HI and a *Clal* restriction site, 2 5'-CCGGAATTCGTTCTCTGGAAGGCACCAGAGATACT-3' (forward) with an *Eco*RI restriction site and 3 5'-CCGGAATTCGGCCAGAGGGTAATCCGACCAG-3' (forward) with an *Eco*RI restriction site. The PCR products resulting from primer combinations 1-2 and 1-3 were ligated in a 1-2/3-1 orientation into the pGreen1K binary vector which was modified to contain the 35S-Tnos cassette from pMON999 (Verdonk *et al.*, 2005).

GFP-fusion constructs were generated by cloning *LePLD α 1* and *LePLD β 1* into pEZR(K)-LN. The pEZR(K)-LN plasmid was created by ligating the expression cassette from pEZR-LN (David Ehrhardt, Stanford University. More information is available at <http://deepgreen.stanford.edu/>) into pCambia 2300. The following oligonucleotides were used to add restriction sites to previously cloned cDNAs: *LePLD α 1* 5'-CCGTCGACATGGCTCAGATTCAGTTCATGG-3' (forward) with a *Sall* restriction site and 5'-GGGGATCCGTAGTGAGGTTGGGAGGAAGGTAG-3' (reverse) with a *Bam*HI restriction site; *LePLD β 1* 5'-GCGAATTCTAATGGCTCATTCTCTTATTC-3' (forward) with an *Eco*RI restriction site and 5'-GGAGATCTATGGTGAGATTTTCTTGAACACCAGTGA-3' (reverse) with a *Bgl*II restriction site.

Cell-suspension transformation

All constructs were transferred to *Agrobacterium tumefaciens* strain EHA105 carrying the pJIC.SaRep plasmid. Transfection was achieved by co-cultivating 8 ml of 4-day-old Msk8 cells for 3 days at 23°C in a Petri dish with 200 μ l of an overnight *A. tumefaciens* culture carrying the appropriate vector, in the presence of 0.2 mM acetosyringon. The cells were then plated on filters in Petri dishes with supplemented MS/agar containing 250 μ g/ml carbenicilin and 40 μ g/ml kanamycin. Every 3 days, cells were transferred to a new Petri dish containing carbenicilin and increasing amounts of kanamycin (twice on 40 μ g/ml, twice on 100 μ g/ml and twice on 200 μ g/ml). After 3 weeks, developing calli were transferred to individual Petri dishes with 250 μ g/ml carbenicilin and 200 μ g/ml kanamycin. Pieces of independently transformed calli were transferred to liquid MS medium containing the same antibiotic concentrations and cultured as described above.

in vivo PLD measurements

To assay PLD activity in living cells, the production of phosphatidylbutanol was measured (Munnik *et al.*, 1995). In brief, cells were pre-labeled with ^{32} P_i for 3 hours and subsequently treated with cell-free medium with or without xylanase (*Trichoderma viride*, Fluka BioChemika, Buchs, Switzerland) in the presence of 0.5% (v/v) 1-butanol. Incubations were stopped and lipids extracted as described before (van der Luit *et al.*, 2000). The ^{32} P-labeled phosphatidylbutanol was separated from the rest of the phospholipids on heat-activated TLC plates using the organic upper phase of an ethyl acetate mixture: ethyl acetate/iso-octane/formic acid/water (12 : 2 : 3 : 10; Munnik *et al.*, 1998). Lipids were visualized by autoradiography and quantified by phosphoimaging (Molecular Dynamics, Sunnyvale CA, USA).

RNA blot analysis

Total RNA from tomato cell-suspension cultures was isolated using the Trizol-LS reagent method (Gibco). 10 μ g RNA was separated by denaturing 1.4% formaldehyde-agarose gel electrophoresis, transferred onto Hybond-XL nylon membranes (Amersham Pharmacia, Buckinghamshire, England), and hybridized with ^{32} P-labeled probes in modified Church solution at 65°C. Membranes were washed 3 times for 15 minutes with wash buffer (1xSSC, 0.1% (w/v) SDS) and probe signal was visualized by autoradiography.

Hydrogenperoxide measurements

The ASA assay was performed as described by Felix *et al.* (1999). Briefly, 10 ml 5-day-old Msk8 cell-suspensions with or without 10 mM 5-amino-salicylic acid were placed in 6 cm petri dishes. Most of the medium was removed using a narrow-tipped pipette, leaving a layer of wet cells. Cell free medium in a volume of 1 μ l containing 0 ng/ml, 5 ng/ml, 50 ng/ml, 500 ng/ml or 5 μ g/ml xylanase was applied locally onto the lawn of cells and staining was visualized after 24 hours using a flat-bed scanner.

The pyranin oxidative burst assay was performed as described previously (de Jong *et al.*, 2004). In short, Msk8 cells were washed and resuspended to a 100 mg/ml cell-density in assay buffer (5 mM MES/NaOH, pH 5.7, 175 mM mannitol, 0.5 mM K₂SO₄, 0.5 mM CaCl₂). Cells were dispensed in 250 μ l aliquots in 24-well microtiter plates and allowed to equilibrate overnight in the dark at 20°C. To measure the elicitor-induced oxidative burst, 250 μ l assay buffer, supplemented with the elicitor and the fluorescent probe pyranin (8-Hydroxy-1,3,6-pyrenetri-sulfonic acid; Aldrich Chemical Company, Milwaukee WI, USA) was added to the cells to produce the desired final concentration of elicitor and 10 μ g/ml pyranin. The quenching of pyranin fluorescence due to the production of H₂O₂ was recorded at 1 min intervals using an excitation wavelength of 405 nm and emission wavelength of 512 nm in a fluorescence spectrophotometer (Perkin Elmer).

Polyphenol oxidase assay

Msk8 cells were ground in liquid nitrogen and taken up in extraction buffer (0.1 M Tris-HCl pH 7.0, 0.1 M KCl, 1% (v/v) Triton, 1 mM EDTA pH 8.0 and 5% (w/v) PVPP; Li and Steffens, 2002). This suspension was centrifuged at 4°C for 20 minutes at 10,000 g and the supernatant was transferred to new tubes and assayed for protein content according to Bradford (1976). Protein extracts were spotted onto Hybond-ECL nitrocellulose membrane (Amersham Pharmacia, Buckinghamshire, England) in a 1 μ l volume containing 10 mg/ml protein. The membrane was then placed onto Whatman 3MM paper soaked in PPO staining solution (0.2 M sodium phosphate, 0.1 M citric acid, 15 mM catechol and 0.05% (w/v) *p*-phenylenediamine; Howe and Ryan, 1999) for 1 hour, rinsed with water and imaged with a flat-bed scanner.

Identification of culture medium proteins

Protein from 3 ml of culture medium was precipitated overnight at -20°C with 4 volumes precipitation buffer (0.1 M sodium acetate, 95% (v/v) ethanol). The protein was spun down by centrifugation for 10 minutes at 15,500 g and the pellet was resuspended in 100 μ l loading buffer (8% (w/v) SDS, 40% (v/v) glycerol, 20% (v/v) β ME, 240 mM Tris-HCl pH 6.8 and 0.08% (w/v) Bromophenol Blue). Samples were separated using an 8% SDS-PAGE gel and stained with Brilliant Blue G-colloidal Coomassie (SIGMA). Selected bands were identified by mass spectrometry as described by Testerink *et al.* (2004).

Confocal scanning laser fluorescence microscopy

Microscopic imaging was performed as described previously (Dhonukshe *et al.*, 2003). Briefly, 200 μ l 4 to 6-day-old cell-suspension culture samples were prepared in 8-well NUNC chambers (Nunc, Naperville IL, USA). Images were acquired using confocal laser scanning microscopy based on the Zeiss LSM 510 System with a x40 water-immersion objective (Zeiss, Jena, Germany). Images were captured using LSM 510 image-acquisition software (Zeiss).

Supplementary material

Supplemental Figure 1. Xylanase-induced relocation of LePLD β 1-GFP.

Msk8 cell-suspension cultures carrying an *LePLD β 1-GFP* expression vector were analyzed by confocal laser scanning microscopy. Cells were incubated with 100 μ g/ml xylanase for 3 hours. (a) A time-lapse animation spanning 12 minutes is shown, the width of the image is equal to 75 μ m. (b) A 60 μ m deep 3D-stack of several cells rotating 360° is depicted, the width of the image is equal to 130 μ m. This material is available as part of the online article from <http://www.blackwell-synergy.com>

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Chapter 5

Investigating the role of LePLD β 1 in plant-pathogen interactions

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Summary

Phospholipase D (PLD) has been linked to the plant stress responses including the pathogen defense response. Recently, it was shown that *LePLD β 1* gene expression and LePLD β 1 phospholipase activity are activated in tomato cell-suspension cultures treated with the fungal elicitor xylanase. We have examined LePLD β 1 function in tomato plants using a silencing strategy. Several transgenic plants no longer displayed xylanase-induced *LePLD β 1* gene expression. Nonetheless, silenced lines exhibited no developmental phenotype and the lack of LePLD β 1 did not cause changes in basal lipid metabolism. There was no effect on a xylanase-induced increase in *in vitro* PLD activity or the xylanase-induced hypersensitive response in tomato plant leaves. Furthermore, *LePLD β 1* gene expression was found to be induced during an incompatible tomato-*Cladosporium fulvum* interaction, but *LePLD β 1*-silenced tomato plant lines did not show altered susceptibility or resistance when challenged with the fungal pathogens *C. fulvum* or *Fusarium oxysporum* f. sp. *lycopersici*.

Abbreviations: PLD, phospholipase D; PA, phosphatidic acid; HR, hypersensitive response; PIP₂, phosphatidylinositolbisphosphate; PIP, phosphatidylinositolmonophosphate; PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol

Introduction

Phospholipase D (PLD) hydrolyses structural phospholipids in a transphosphatidyltransfer reaction with water, producing a free head-group, such as ethanolamine, and phosphatidic acid (PA; Wang, 2000). PA is a low-abundant membrane phospholipid that has been shown to function as a lipid second messenger in several plant systems (Munnik, 2001; Testerink and Munnik, 2005; Wang, 2005). In comparison to yeast and animals, which contain one and two PLD genes, respectively, plants possess an expanded family of PLDs that can be divided into numerous different classes, based on their genomic- and protein-sequence similarity and biochemical requirements for *in vitro* phospholipase activity. Twelve PLD genes have been identified in the Arabidopsis genome: three α -, two β -, three γ -, one δ -, one ϵ - and two ζ -PLDs (Wang, 2005). This plethora of PLD isoforms in plants suggests that they have individual functions and that they are activated under different circumstances. Indeed, knock-down or knock-out of specific PLD isoforms has been shown to have distinct effects. *AtPLD α 1*-silenced Arabidopsis plants have an altered response to wounding (Wang *et al.*, 2000), induction of senescence (Fan *et al.*, 1997), water loss (Sang *et al.*, 2001a), superoxide generation (Sang *et al.*, 2001b) and freezing (Welti *et al.*, 2002). Arabidopsis *AtPLD δ* knock-out lines are more sensitive to H₂O₂ (Zhang *et al.*, 2003) and freezing (Li *et al.*, 2004). Silencing *AtPLD ζ 1* in Arabidopsis leads to a misshapen root hair phenotype (Ohashi *et al.*, 2003). Finally, silencing *LePLD β 1* in tomato cell-suspension cultures causes an altered response to the fungal elicitor xylanase (Bargmann *et al.*, 2006).

The elaborate early signaling events downstream of elicitor perception are only just beginning to be unraveled. In response to pathogen-derived stimuli, evidence has been presented for calcium and protein-kinase signaling (Nürnberg and Scheel, 2001; Romeis, 2001; Pedley and Martin, 2006) as well as lipid signaling (van der Luit *et al.*, 2000; Laxalt *et al.*, 2001; Meijer and Munnik, 2003; de Jong *et al.*, 2004; Shah, 2005). Several studies have linked PLD and plant-pathogen interactions. PLD activity was found to increase in rice cell-suspension cultures treated with an *N*-acetylchitoooligosaccharide elicitor, and was proposed to positively regulate the reactive oxygen species production (Yamaguchi *et al.*, 2003; Yamaguchi *et al.*, 2005). Additionally, an increase in PLD expression was found in rice infected with the bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae* (Young *et al.*, 1996). De Torres Zabela *et al.* (2002) showed that numerous PLDs were differentially expressed when Arabidopsis plants were challenged with *Pseudomonas syringae* pv. *tomato*. In tomato, the expression of a specific β -class PLD, *LePLD β 1*, was induced by the fungal elicitor xylanase (Laxalt *et al.*, 2001). Xylanase has also been shown to induce a rapid increase in *in vivo* PLD activity in tomato cell-suspension cultures (van der Luit *et al.*, 2000; Laxalt *et al.*, 2001). It has subsequently been demonstrated that *LePLD β 1* is responsible for this increase, as *LePLD β 1*-silenced cell-suspension cultures exhibited impaired xylanase-induced PLD activity. Furthermore, *LePLD β 1*-silenced cell-suspension cultures had an excessive oxidative burst in response to xylanase, higher polyphenol oxidase activity, altered gene-expression and protein-secretion patterns. Lastly, *LePLD β 1*-

GFP was shown to relocalize to punctuate structures in transgenic tomato cell-suspension cultures treated with xylanase (Bargmann *et al.*, 2006).

The aim of this study is to investigate the link between LePLD β 1 and pathogen-induced defense responses in tomato plants. *LePLD β 1*-silenced tomato plants were examined on the level of phospholipid composition, PLD activity, the xylanase-induced hypersensitive response (HR) and susceptibility or resistance to the leaf mold *Cladosporium fulvum* and the wilting disease *Fusarium oxysporum* f.sp. *lycopersici*.

Results

LePLD β 1 expression is induced by xylanase and silenced by an RNAi construct

In tomato plants *LePLD β 1* transcripts were only found in the roots of mature, soil-grown plants (Laxalt *et al.*, 2001). In agreement, expression in aerial parts of untreated tomato seedlings is hardly detectable by RNA blot analysis (Figure 1). Additionally, Laxalt *et al.* (2001) showed that *LePLD β 1* transcript levels were dramatically increased in leaves treated with the fungal elicitor xylanase. This protein originates from the green mold *Trichoderma viride* and is a secreted glucoside hydrolase. It is known to be a potent elicitor of plant defense responses in tomato (Enkerli *et al.*, 1999; Furman-Matarasso *et al.*, 1999; Ron and Avni, 2004). Correspondingly, when seedlings were cut and placed in water containing 2.5 mg/ml xylanase *LePLD β 1* transcript levels increased to amounts that could readily be detected by RNA blot analysis (Figure 1). Mock treatment of seedlings with just water did not have any effect, indicating that xylanase and not wounding or other experimental factors induced expression (Figure 1).

Transgenic tomato plants bearing a gene-specific RNAi-silencing construct for *LePLD β 1* were generated in order to study the role of LePLD β 1 in plants. This construct was used previously to successfully silence *LePLD β 1* in tomato cell-suspension cultures (Bargmann *et al.*, 2006). Several independent transgenic lines were selected and these were inbred to obtain homozygous lines containing the silencing construct. Their isogenic wildtype sibling lines were obtained simultaneously. All transgenic plant lines developed normally, exhibiting no obvious growth phenotype. The fact that expression levels could be increased to detectable levels by xylanase treatment allowed us to ascertain whether introduction of the silencing construct had affected the *LePLD β 1* mRNA abundance. Transgenic plant lines were obtained that exhibited no detectable transcript levels, either with or without xylanase treatment, while their isogenic wildtype lines showed a clear induction of expression (Figure 1). These results show that *LePLD β 1* expression in tomato seedlings is xylanase-inducible and that *LePLD β 1* transcript levels can be successfully knocked-down in transgenic tomato plants using a gene-specific RNAi-silencing construct.

Figure 1. Silencing xylanase-induced *LePLDβ1* expression in tomato plants.

3 one-week-old *LePLDβ1*-silenced (S) and isogenic wildtype (wt) tomato seedlings were cut at ground level and placed in water with or without 2.5 mg/ml xylanase for 3 hours. RNA was extracted, separated by gel electrophoresis, blotted onto nitrocellulose and hybridized with a ^{32}P -labeled *LePLDβ1* probe. The signal was visualized by autoradiography. Ethidium bromide staining of ribosomal RNA (rRNA) is presented as a loading control. Two independent transformant lines are shown.

Xylanase-induced PLD activity

The acquisition of transgenic plant lines with knocked-down *LePLDβ1* expression gave us the opportunity to test whether lack of this PLD isoform affects phospholipid composition and in particular whether there was an effect on the expected xylanase-induced PLD activity. PA production can be monitored *in vivo* by radio-isotopically labeling phospholipids and extracting, separating and analyzing them after treatment. Cell suspensions can be relatively easily labeled by incubating a small volume of cell-suspension culture with $^{32}\text{P}_i$ in the growth medium. However, radioactively labeling entire tomato plants is not practical. Instead, we opted to label leaf discs by floating them on buffer containing $^{32}\text{P}_i$. Analysis showed that there are no differences in basal phospholipid levels between *LePLDβ1*-silenced and isogenic wildtype plant lines (Figure 2). Surprisingly, no increase in PA levels could be detected when leaf discs were treated with xylanase for up to one hour (Figure 2). These results suggest that silencing *LePLDβ1* does not affect phospholipid homeostasis and that xylanase does not elicit a PA response in tomato leaf discs.

Figure 2. Phospholipid composition of *LePLDβ1*-silenced tomato plant leaf discs

$^{32}\text{P}_i$ -labeled leaf discs from isogenic wildtype (wt) or *LePLDβ1*-silenced (line 3) tomato plants were treated with 100 $\mu\text{g}/\text{ml}$ xylanase for 0, 15, 30 and 60 min or left untreated (c60). Lipids were extracted, separated by alkaline TLC and visualized by phosphorimaging. The origin of the chromatogram, phosphatidylinositolbiphosphate (PIP_2), phosphatidylinositolmonophosphate (PIP), phosphatidic acid (PA), phosphatidylinositol (PI), phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) are indicated.

The failure to detect *in vivo* xylanase-induced PLD activity, as reflected by altered PA levels, prompted us to investigate xylanase-induced *in vitro* PLD activity and to ascertain whether silencing *LePLD $\beta 1$* affected this activity. Since xylanase induced *LePLD $\beta 1$* expression, an increase in *in vitro* β/γ -class activity was anticipated and this increase was expected to be absent or less intense in *LePLD $\beta 1$* -silenced plants. PLD activity can be monitored *in vitro* by making use of a fluorescent lipid substrate. In this case the conversion of fluorescently labeled NBD-phosphatidylcholine (PC) to NBD-PA was measured. The *in vitro* activity of different PLD classes can be separated by varying the buffer in which the assay is performed and lipid environment in which the substrate is presented (Qin and Wang, 2002). Four kinds of *in vitro* PLD activity can be distinguished in this way, depending on their pH-, $[Ca^{2+}]$ -, oleate- and phosphatidylinositolbisphosphate (PIP₂)-requirements. The α -class requires an acidic pH and mM calcium concentrations but does not require PIP₂ in its lipid substrate preparation. β/γ -class PLDs are active at a neutral pH and μ M calcium concentrations and require PIP₂. δ -class PLDs are active at μ M to mM calcium concentrations and are stimulated by inclusion of oleic acid. Lastly, the ζ -class PLDs require a neutral pH and PIP₂ but do not require calcium. Proteins were extracted from leaves and assayed for β/γ -class PLD activity. This activity was significantly increased in leaves that had been treated with xylanase compared to mock-treated leaves (Figure 3), but, surprisingly, this increase was also present in leaves from *LePLD $\beta 1$* -silenced plants and was not notably different from the increase in leaves from isogenic wildtype plants (Figure 3). These results show that xylanase induces an increased β/γ -class PLD activity in tomato leaves and suggests that, although its expression is induced upon treatment with xylanase, *LePLD $\beta 1$* is not a major contributor to this increase.

Figure 3. Xylanase-induced increase in *in vitro* β/γ -class PLD activity

Fully expanded leaflets were cut from isogenic wildtype and *LePLD $\beta 1$* -silenced (line 3) tomato plants and were placed with their petioles in water with or without 2.5 mg/ml xylanase for 3 h in a flow cabinet. Proteins were extracted and assayed for β/γ -class PLD activity (μ M Ca^{2+} , pH 7.0, with addition of PIP₂, see Methods) using fluorescent NBD-PC as a substrate. NBD-PA was quantified as a percentage of total fluorescence and is presented in a histogram (avg. n=2; min and max values are indicated).

LePLD β 1-silenced plants respond to xylanase with a normal HR

Xylanase is an elicitor of the HR in tomato (Ron and Avni, 2004). The HR involves the production of reactive oxygen species and antimicrobial compounds, cell-wall fortification and programmed cell-death, resulting in necrotic lesions (Pontier *et al.*, 1998). *LePLD β 1*-silenced cell-suspension cultures were hypersensitive to xylanase and responded with an excessive production of reactive oxygen species (Bargmann *et al.*, 2006). This finding led us to question whether *LePLD β 1*-silenced plants would display an abnormal HR upon treatment with xylanase.

When tomato leaves were pressure infiltrated with xylanase, lesions became visible within a few days (two-five), appearing as dry, yellow patches (Figure 4). *LePLD β 1*-silenced plants developed lesions just like their isogenic wildtype counterparts. There was no difference in the timing or intensity of the HR in two independent silenced plant lines compared to their isogenic wildtype controls (Figure 4). These results indicate that silencing *LePLD β 1* does not affect the HR in tomato leaves treated with xylanase.

Figure 4. Xylanase-induced hypersensitive response in *LePLD β 1*-silenced tomato plants

Six-week-old *LePLD β 1*-silenced and isogenic wildtype tomato plants were pressure infiltrated with water (left half) or 1 mg/ml xylanase (right half) using a syringe without needle. Photos were taken after 4 days. Two independent lines are shown (lines 3 and 10).

*LePLD β 1 expression in the interactions between tomato and *Cladosporium fulvum**

The tomato response to xylanase has recently been proposed to be mediated by specific recognition of xylanase by membrane spanning receptor-like proteins named LeEIX1 and LeEIX2 (Ron and Avni, 2004). The LeEIX receptors are structurally similar (31% identity) to known tomato disease resistance proteins: the *Cf* family. This family of genes mediates the race-specific recognition of avirulence factors from the leaf mold *Cladosporium fulvum* and their gene-products are required for the induction of defense-related gene-expression, HR and resistance in the respective incompatible interactions (Rivas and Thomas, 2005).

The interaction with the fungus *C. fulvum* is one of the most intensively studied gene-for-gene systems in tomato (Joosten and de Wit, 1999). Several fungal *Avr* genes and their corresponding tomato *R* genes have been isolated and examined. An elegant transgenic system has been set up in which tomato plant lines carrying *R* genes have been crossed with lines lacking the functional *R* gene that have been genetically modified to express fungal *Avr* genes (de Jong *et al.*, 2002). Seedlings expressing both the matching *Cf* and *Avr* genes develop necrotic lesions within days after emergence of the hypocotyls and die within a week after emergence. Seedlings expressing either the *Cf-9/Avr9* and *Cf-4/Avr4* combinations or the separate *R* and *Avr* genes were harvested up to 6 days post emergence and assayed for *LePLDβ1* expression. As shown in Figure 5, a clear induction of *LePLDβ1* expression could be observed in seedlings expressing *Cf-9* and *Avr9* and in seedlings expressing *Cf-4* and *Avr4*, whereas no such induction could be observed in seedlings expressing the *Cf* or *Avr* genes alone. The *LePLDβ1* expression correlated with the expression of *LeHSR203*, a marker of the HR in tomato (Pontier *et al.*, 1998). These results indicate that induction of *LePLDβ1* expression is not limited to xylanase treatment but also takes place in the *Cf-9/Avr9* and *Cf-4/Avr4* interactions.

Figure 5. *LePLDβ1* expression induced by transgenic expression of *Cladosporium fulvum* *Avr* genes in tomato

Tomato seedlings expressing *Cf-9/Avr9*, *Cf-4/Avr4*, *Avr4*, *Avr9*, *Cf-4* or *Cf-9* were harvested between 1 and 6 days post emergence (d.p.e.). RNA was extracted, separated by gel electrophoresis, blotted onto nitrocellulose and hybridized with ³²P-labeled *LePLDβ1* and *LeHSR203* probes. The signal was visualized by autoradiography, ethidium bromide staining of rRNA is presented as a loading control.

C. fulvum and *F. oxysporum* interactions with *LePLDβ1*-silenced tomato plants

Having established that *LePLDβ1* is expressed upon incompatible plant-pathogen interactions, both after recognition of xylanase (Figure 1) and *C. fulvum* *Avr* proteins (Figure 5), we next wanted to test the *LePLDβ1*-silenced lines' interactions with plant pathogens. Regrettably, the incompatible tomato-*C. fulvum* interaction could not be assayed because the silenced lines had been generated in a *Cf-0* background, GCR161. In the compatible interaction, silenced plants exhibited no altered susceptibility to *C. fulvum*, showing the same level of disease symptoms as their isogenic wildtype control lines (Figure 6). This result suggests that *LePLDβ1* is not involved in basal defenses that influence susceptibility in a compatible tomato-*C. fulvum* interaction.

Figure 6. *Cladosporium fulvum* susceptibility in *LePLDβ1*-silenced tomato plants

LePLDβ1-silenced and isogenic wildtype tomato leaves (line 3) were inoculated with *Cladosporium fulvum* spores (race 5) or mock treated. Pictures were taken after 21 days. Four representative leaflets are shown.

Fusarium oxysporum f.sp. *lycopersici* was a more suitable candidate for use in a biocompatibility assay. *F. oxysporum* is a soil-borne pathogen that attacks the roots, colonizes the xylem vessels and consequently spreads throughout the plant, causing fusarium wilt in tomato (Mes *et al.*, 2000). The gene-for-gene interaction between this fungus and tomato has also been intensively studied and both *R* genes in the plant and *Avr* genes in the pathogen have been cloned (Simons *et al.*, 1998). The *LePLDβ1*-silenced transgenic tomato plant lines were generated in the GCR161 background that confers resistance to *F. oxysporum* race 1 isolates but is susceptible to race 2 and 3 isolates (Simons *et al.*, 1998), allowing us to test for both altered susceptibility and resistance. Furthermore, basal *LePLDβ1* expression in tomato plants was found in roots (Laxalt *et al.*, 2001), which correlates with the site of initial infection by this pathogen.

Bioassays were undertaken in order to determine whether silencing *LePLDβ1* expression would affect the resistance or susceptibility of tomato to this fungus. Three isolates of *F. oxysporum* belonging to race 1, 2 and 3 (isolates 004, 007 and 029, respectively) were tested on two independent *LePLDβ1*-silenced plant lines and their isogenic-wildtype controls. Ten-day-old seedlings were either infected with one of the isolates using the root-dip method or mock-treated with water and fresh weight was determined after 3 weeks. C32 (Moneymaker) tomato plants, which are susceptible to *F. oxysporum* race 1, were taken along as a positive control for virulence of the pathogen and showed a clear reduction in plant growth when infected with isolate 004 (Figure 7a). As expected, infection with the

GCR161-incompatible race 1 isolate 004 did not have an effect on plant growth in wildtype lines (Figure 7a). Interestingly, the growth of *LePLD β 1*-silenced plant lines was not different from mock-treated plants (Figure 7a). In contrast to isolate 004, the GCR161-compatible *F. oxysporum* isolates 007 (race 2) and 029 (race 3) caused a strong decrease in plant growth in the GCR161 background, both in the *LePLD β 1*-silenced plant lines and their isogenic-wildtype controls (Figure 7b). Infection with isolate 007 generally had less severe consequences for plant growth than infection with isolate 029. However, there was no notable gain or loss of susceptibility to either isolate in the two tested *LePLD β 1*-silenced plant lines as compared to their isogenic-wildtype controls (Figure 7b). These results show that silencing *LePLD β 1* does not affect resistance or susceptibility in tomato-*F. oxysporum* interactions.

Figure 7. *Fusarium oxysporum*-resistance and -susceptibility in *LePLD β 1*-silenced tomato plants
 (a) Ten-day-old GCR161, *LePLD β 1*-silenced (S), isogenic wildtype (wt) and race-1-susceptible C32 seedlings were infected with *Fusarium oxysporum* f.sp. *lycopersici* (Fol) race 1 isolate 004 or mock-treated with water. Fresh weight was determined 3 weeks after infection and is presented in a histogram $\pm$ SD (n=7-10). (b) GCR161, *LePLD β 1*-silenced (S) and isogenic wildtype (wt) seedlings were infected with Fol race 2 isolate 007 and race 3 isolate 029 or mock-treated. Fresh weight three weeks after infection is presented $\pm$ SD (n=38-40).

Discussion

Silencing LePLD β 1 in tomato plants

Previously, it was demonstrated that the *LePLD β 1* transcript levels, which are hardly detectable by RNA blot analysis in untreated tomato leaves, are dramatically increased to readily detectable levels after treatment with the endo-1,4- β -xylanase from *Trichoderma viride* (Laxalt *et al.*, 2001). Xylanase-induced increase in transcript levels also occurs in tomato cell-suspension cultures, and the *LePLD β 1* expression can be silenced by the introduction of a gene-specific RNAi construct (Bargmann *et al.*, 2006). The same construct was used to generate transgenic tomato plants with impaired *LePLD β 1* gene-expression. Several independent transformants were obtained and *LePLD β 1*-silenced lines were selected that no longer displayed an induction of *LePLD β 1* gene-expression when seedlings were treated with xylanase (Figure 1).

LePLD β 1-silenced tomato plants did not reveal a developmental phenotype under normal greenhouse conditions. This is perhaps not surprising, as *LePLD β 1* has a very low basal expression level. When Laxalt *et al.* (2001) analyzed the *LePLD β 1* expression in separate organs of 9-week-old, soil-grown tomato plants, only the roots had a detectable transcript level. In contrast, *LePLD β 1*-silenced tomato cell-suspension cultures, which also have hardly detectable basal *LePLD β 1* expression levels, exhibited altered protein-secretion and gene-expression patterns, increased polyphenol oxidase activity and increased hydrogenperoxide production even when cells had not been treated with xylanase (Bargmann *et al.*, 2006). This difference between the effect of *LePLD β 1*-silencing in untreated cell-suspension cultures and plants might be due to the expression of a PLD isoform in plants that is able to compensate for the loss of *LePLD β 1* and that is not expressed to sufficient levels in cell-suspension cultures.

In spite of the fact that xylanase-induced *LePLD β 1* expression was successfully knocked-down in tomato plant leaves, the xylanase-induced HR was not different in *LePLD β 1*-silenced plant lines (Figure 4). *LePLD β 1*-silencing in tomato cell-suspension cultures gave rise to a more severe oxidative burst in response to elicitation with xylanase (Bargmann *et al.*, 2006). In contrast, the HR to xylanase in the *LePLD β 1*-silenced plants' leaves infiltrated with xylanase did not deviate from the response in isogenic wildtype leaves in severity or timing.

Measuring PLD activity in LePLD β 1-silenced plants

In a tomato cell-suspension culture, treatment with xylanase induces a massive PA response, this PA is mostly derived from the PLC/DGK pathway but also in part from PLD activity, as can be determined from the production of phosphatidylalcohol when cells are treated in the presence of a primary alcohol (van der Luit *et al.*, 2000). The response is fast (within 4 min) and transient (basal levels were regained after 100 min). In leaf discs, however, we have not been able to detect a PA response to xylanase (Figure 2). Lipid extraction at multiple timepoints up to 60 min after start-of-treatment failed to give an increase in PA levels. This lack of a PA response might be due to the fact that the leaf discs had been excised the day before and that this wounding desensitized the material. Alternatively, the timing of activation could differ between cell-suspensions and leaves. Lastly, perhaps only a small subset of the cells within leaf tissues is responsive to xylanase, tissue specific expression of the xylanase receptors *LeEIX1* and *LeEIX2* has not been studied to date (Ron and Avni, 2004). PLD responses in leaf discs have been measured previously only in response to drought stress, which would affect all the cells in a leaf disc (Frank *et al.*, 2000).

The activity of different PLD-classes can be separated *in vitro* depending on the biochemical conditions used (Wang, 2002). Basal β/γ -class PLD activity was detectable in tomato leaves (Figure 3). However, the basal β/γ -class PLD activity in *LePLD β 1*-silenced lines was not different from that of the isogenic wildtype control lines. Moreover, the more than two-fold increase in activity induced by treatment with xylanase was also present in the silenced lines (Figure 3). In theory, this finding can be explained by incomplete silencing or by redundancy of *LePLD β 1* in this process. On the transcript level, *LePLD β 1*-silencing after xylanase treatment is

unequivocal (Figure 1), nonetheless, it cannot be excluded that on the protein level this silencing effect is less explicit. Alternatively, LePLD β 1 activity could represent only an insignificant share of the basal and xylanase-induced β/γ -class PLD activity in tomato plants. In Arabidopsis, increased expression of both the β - and γ -class PLDs was observed after challenge with *P. syringae* (de Torres Zabela *et al.*, 2002).

LePLD β 1-silencing and tomato pathogen-resistance and -susceptibility

PLD has been shown to be intimately involved in plant-pathogen relationships by numerous independent studies. Differential PLD gene expression in response to various pathogens and elicitors has been observed (Young *et al.*, 1996; Laxalt *et al.*, 2001; de Torres Zabela *et al.*, 2002) as well as increased PLD activity *in vitro* (Yamaguchi *et al.*, 2003) and *in vivo* (van der Luit *et al.*, 2000; Laxalt *et al.*, 2001; Yamaguchi *et al.*, 2005; Bargmann *et al.*, 2006). Yet, to date, there have been no reports of plants with compromised PLD activity having an altered pathogen-susceptibility or -resistance.

The abundance of PLD genes combined with the labor-intensiveness of genetic modification of tomato plants and plant-pathogen bioassays makes it necessary to carefully select which PLD isoform(s) to choose for further investigation. LePLD β 1 is a good candidate for research into plant-pathogen interactions because it has previously been found to be specifically expressed, activated and to relocalize in response to treatment with an elicitor of plant defense responses, xylanase (Laxalt *et al.*, 2001; Bargmann *et al.*, 2006). Furthermore, *LePLD β 1*-silenced tomato cell-suspension cultures displayed defense responses in the absence of the elicitor and responded more severely to elicitation with enhanced elicitor-induced gene-expression and reactive oxygen species production (Bargmann *et al.*, 2006). Lastly, we now show that *LePLD β 1* expression is also induced in incompatible tomato-*C. fulvum* interactions (Figure 5). These findings suggested that both the basal and induced defenses might be affected in *LePLD β 1*-silenced plants and, therefore, that both pathogen-resistance and -susceptibility could be influenced.

However, *LePLD β 1*-silenced plant lines did not show altered resistance or susceptibility to *C. fulvum* or *F. oxysporum* f. sp. *lycopersici* (Figures 6 and 7). These results suggest that *LePLD β 1*-expression is not of vital importance in these interactions. These findings are in agreement with the fact that *in vitro* β/γ -class PLD activity is not reduced in *LePLD β 1*-silenced plants (Figure 3) and the fact that no difference in xylanase-induced HR development could be detected in silenced lines (Figure 4). Taken together, it seems that another β/γ -class PLD (or PLDs) can compensate for the loss of LePLD β 1 in tomato plants. In tomato, only one other β -class PLD (*LePLD β 2*) and no γ -class PLDs have been found in a screen for PLD genes in a tomato cDNA-library (Laxalt *et al.*, 2001). Analysis of plants with multiple PLDs knocked-down could yield more lucid effects on pathogen resistance and/or susceptibility in the future.

Methods

Plant material, plant transformation and growth conditions

The *LePLD β 1*-silencing construct was inserted into the genome of tomato plants cv. GCR161; plants were grown under standard greenhouse conditions. The *LePLD β 1*-silencing construct was cloned as described by Bargmann *et al.* (2006), and plants were transformed as specified by van Roekel *et al.* (1993). The transgenic *Cf-Avr* tomato plants were generated as described previously (de Jong *et al.*, 2002).

Xylanase treatment

Xylanase (*Trichoderma viride*, Fluka BioChemika, Buchs, Switzerland) was dissolved in water. For RNA blot analysis, seedlings were cut at ground level and placed with their stems in 2 ml microcentrifuge tubes containing xylanase or water alone and left in a horizontal laminar flow cabinet for 3 hours. For the *in vitro* PLD assay mature leaflets were cut from plants, placed with their petioles in water or xylanase solution as described above. Pressure infiltration in leaves was performed on the bottom side of mature leaflets using a 1 ml syringe without needle, as a control leaflets were infiltrated with water.

RNA blot analysis

Total RNA was isolated from ground tomato tissue using the Trizol-LS reagent method (Gibco). 10 μ g RNA was separated by denaturing 1.4% formaldehyde-agarose gel electrophoresis, transferred onto Hybond-XL nylon membranes (Amersham Pharmacia, Buckinghamshire, England), and hybridized with ^{32} P-labeled probes in modified Church solution at 65°C. Membranes were washed 3 times for 15 min with wash buffer (1xSSC, 0.1% (w/v) SDS) and probe signal was visualized by autoradiography.

in vivo PLD activity measurements

Leaf discs (5 mm ϕ) were labeled by incubation with 100 μ Ci carrier-free PO_4^{3-} on 100 μ 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). Treatments were stopped by addition of 50% (w/v) perchloric acid to a final concentration of 5%. Leaf discs were then transferred to a new tube containing 375 μ l $\text{CHCl}_3/\text{MeOH}/\text{HCl}$ (50 : 100 : 1 [v/v]) where lipids were extracted during vigorous shaking for 10 min. A two-phase system was generated by addition of 375 μ l CHCl_3 and 200 μ 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 TLC plates using an alkaline solvent system: $\text{CHCl}_3/\text{MeOH}/25\% \text{NH}_4\text{OH}/\text{H}_2\text{O}$ (90 : 70 : 4 : 16 [v/v]; Munnik *et al.*, 1994). The radioactive signal was visualized by autoradiography and quantified by phosphoimaging (Molecular Dynamics, Sunnyvale CA, USA).

in vitro PLD activity measurements

The *in vitro* β/γ -class PLD activity was measured basically as described previously (Pappan *et al.*, 1997). Total protein (10.000 g supernatant) from mock- or xylanase-treated leaves ground in liquid nitrogen was extracted using a buffer composed of 50 mM Tris-HCl pH 7.0, 10 mM KCl, 1 mM EDTA, 2 mM DTT and 1x complete protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). 10 μ g of protein was incubated with 100 μ l 0.4 mM PC/PE/PIP₂ lipid vesicles for 15 min at 30° C in reaction buffer containing 100 mM MES-KOH pH 7.0, 80 mM KCl, 5 μ M CaCl_2 and 2 mM MgCl_2 . The vesicles were composed of 0.54% NBD-PC (1-Oleoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-sn-Glycero-3-Phosphocholine), 4.86% PC (16:0, 16:0), 87% PE (18:1, 18:1) and 7.6% PI4,5P₂ (porcine-brain), percentages represent mol%; all lipids were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Lipids in organic solvents were mixed in the aforementioned proportions, vacuum dried and taken up in reaction buffer using a sonication bath. After the 15 minute incubation, reactions were stopped using 5% (final concentration; w/v) perchloric acid and lipids were extracted as described before (van der Luit *et al.*, 2000). Extracted lipids were separated on TLC plates using the organic upper phase of an ethyl acetate mixture: ethyl acetate/*iso*-octane/formic acid/water (12 : 2 : 3 : 10; Munnik *et al.*, 1998). NBD-lipids were visualized and quantified by fluoroimaging (Molecular Dynamics, Sunnyvale CA, USA).

Bioassays

LePLD β 1-silenced and isogenic wildtype tomato plants were inoculated with *Cladosporium fulvum* race 5 spores as described earlier (Westerink *et al.*, 2004). Briefly, a suspension of conidia ($\sim 5 \times 10^6$ conidia per

ml) was used to spray the lower leaf surface of five-week-old tomato plants. Pictures were taken after 3 weeks.

Fusarium oxysporum f.sp. *lycopersici* isolates Fol004, Fol007 and Fol029 were used for bioassays on the *LePLD β 1*-silenced and isogenic wildtype tomato plant lines. The assays were performed as described previously (Mes *et al.*, 1999). Briefly, ten-day-old seedlings were inoculated by dipping the roots in a conidial suspension and grown for 3 weeks after which fresh weight of plants cut just above the cotyledons was determined.

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Chapter 6

General discussion

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Plants vs. animals

There is a divergence in the evolution of the PLD family in plants versus animals. Whereas the latter have just one PLD subfamily, *i.e.* the PX-PH PLDs, plants possess an additional subfamily, namely the C2 PLDs. Interestingly, the opposite seems to be true for the phosphoinositide-specific phospholipase C (PLC) family, which consists of five classes in animals and is restricted to one class in plants (Cockcroft, 2006; Meijer and Munnik, 2003; Mueller-Roeber and Pical 2002). Moreover, phosphatidic acid (PA) seems to play a central role in plant PLD and PLC signaling, whereas diacylglycerol (DAG) appears to be the prominent second messenger in animal PLD and PLC signaling events. The well-known downstream target of PLC-generated DAG in animals, protein kinase C, is absent in plants and the DAG produced by PLC in plants is rapidly phosphorylated by DAG-kinases to yield PA. In contrast, PLD derived PA in animals has been reported to be dephosphorylated by lipid phosphate phosphatases to yield DAG (Pyne, *et al.*, 2005). An intriguing exemption is the proposed function of the ζ -class (PX-PH) PLDs in the plant response to phosphate starvation (Cruz-Ramirez *et al.*, 2006). This work suggests that these animal-like PLDs in plants work together with PA phosphatase to generate free phosphate and DAG.

The difference in PA and DAG generation between plants and animals is demonstrated by the preliminary results shown in Figure 1. As iterated in Chapters 2 and 3, PLD activity in plants is massively triggered upon loss of cell membrane integrity, leading to a substantial PA accumulation. However, when HeLa cells are subjected to the same snapfreeze-and-thaw treatment, bulk structural lipids (with the exception of phosphatidylinositol [PI]) are rapidly broken down yet no PA accumulation can be observed. This membrane degradation could be PLD-independent but the fact that PI, which is not a PLD substrate, remains suggests that, like in plants, PLD activity in animals is also triggered upon disruption of compartmentalization. Furthermore, the lack of PA accumulation indicates that it, too, is quickly broken down (to DAG) during this process.

Figure 1. Phospholipid turnover after cell-lysis in plants and animals

HeLa (human) and Msk8 (tomato) cells were labeled overnight with $^{32}\text{P}_i$ and left untreated (control) or snapfrozen and thawed (f/t). Lipids were extracted, separated by alkaline TLC and visualized by phosphoimaging. The origin of the chromatogram, phosphatidylinositolbisphosphate (PIP_2), phosphatidylinositolmonophosphate (PIP), phosphatidic acid (PA), phosphatidylinositol (PI), phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) are indicated. The experiment with HeLa cells was only done once.

Redundancy

The abundance of PLD genes in plants is puzzling. The fact that the sub-families and the different PLD classes are conserved in higher plants (Elias *et al.*, 2002) suggests that they serve certain separate and essential purposes. However, knock-out of a single PLD isoform leaves residual PLD activity and does not lead to severe phenotypes. This point is especially clear in the analysis of PLD in the salinity stress response (Chapter 2). In this study, the knock-out of AtPLD α 1 and AtPLD δ alone led to marginal decreases in salt-induced PLD activity and salt-tolerance whereas the *pld α 1/ δ* double knock-out mutant exhibited unequivocal reduction in activity and tolerance. Thus it appears that there is at least some overlap in AtPLD α 1 and AtPLD δ function during the salinity stress response. The PLD activity triggered upon loss of cell membrane integrity is also a concerted action of these two PLD isoforms in Arabidopsis, as demonstrated in Chapter 3. Nonetheless, knock-down of AtPLD α 1 and AtPLD δ has also been shown to have seemingly opposed effects; silencing *AtPLD α 1* resulted in freezing tolerance (Welti *et al.*, 2002) whereas *pld δ* knock-out mutants exhibit increased freezing sensitivity (Li *et al.*, 2004).

The conserved presence of different PLD isoforms within the same class found in different plant species, e.g. PLD α 1, PLD α 2 and PLD α 3, implies that these class-members also have separate functions. Alternatively, such homologs could serve the same function but be expressed in different tissues or under distinct circumstances. The γ -class PLDs in Arabidopsis are exceptionally homologous and are found in tandem on the same chromosome. Additionally, this class of PLDs appears to be present only in *Brassicaceae* (personal communication Martin Potocky, Department of Plant Physiology, Charles University, Prague, Czech Republic). Yet, most research to date has focused on single PLD knock-down or knock-out; the analysis of multiple-PLD knock-outs, both within one class and spanning several classes, will give more information about overlap and separation of the functions of plant PLDs.

GFP studies

GFP-mediated localization studies can be valuable in dissecting the roles of the different PLD families and isoforms therein. The two PLD isoforms found in animals have different cellular localizations in most of the studied cell types, e.g. perinuclear vesicular compartments and the plasma membrane were stained by HsPLD1b and HsPLD2, respectively (Sarri *et al.*, 2003; Hiroyama and Exton, 2005). In plants, GFP-studies of PLD localization have not been used extensively. AtPLD ζ 1-GFP has been analyzed in Arabidopsis root hairs (Ohashi *et al.*, 2003) and we have presented data concerning the localization of two LePLD-GFP fusions in tomato cell-suspensions (Chapter 4; Bargmann *et al.*, 2006). This latter study showed that, although both LePLD α 1-GFP and LePLD β 1-GFP have a cytosolic localization in control cells, only LePLD β 1-GFP relocalized to punctuate structures within the cytosol upon treatment with the fungal elicitor xylanase. LePLD α 1 is activated by salt treatment (Chapter 2), however, microscopic studies of salt treated cells is challenging due to the hyperosmotic-shock-induced plasmolysis. In the end,

no strong conclusions concerning the localization of LePLD α 1 during high salinity treatments could be drawn from our LePLD α 1-GFP studies.

An important factor in GFP-fusion studies is the functionality of the fusion protein. The best way to prove the functionality is to express the fusion protein using the native promotor and achieve complementation of a knock-out. However, in the case of our tomato cell-suspension studies, a silencing strategy was used, excluding this possibility. We can therefore not say with certainty that the LePLD α 1-GFP and LePLD β 1-GFP fusion proteins were functional. In fact, there are indications that they were not functional and were actually interfering with the activity of the native PLDs, since the activity seen in salt-treated LePLD α 1-GFP cell-suspensions and in xylanase-treated LePLD β 1-GFP cell-suspensions was reduced compared to control lines (data not shown). These were both C-terminal fusions, made with the intent of keeping the N-terminus free as that is the site where the lipid-binding domains reside, which are believed to regulate localization. However, the C-terminal GFP fusion appears to interfere with the PLD activity, thereby creating a dominant negative effect, taking the place of the native PLD enzymes. Nonetheless, these findings indicate that the relocalization of LePLD β 1-GFP after xylanase treatment is representative of the localization of the native LePLD β 1 because it apparently interferes with the native protein's activity.

A future study of PLD δ localization promises interesting results; this PLD isoform is reportedly localized on the plasma-membrane and bound to microtubules (Gardiner *et al.*, 2001; Wang and Wang, 2001). Furthermore, it is known to be activated in response to dehydration (Katagiri *et al.*, 2001), H₂O₂ (Zhang *et al.*, 2003), cold (Li *et al.*, 2004), salinity stress and wounding (Chapters 2 and 3).

Silencing, knock-out and other genetic modification studies

Silencing strategies are useful tools in reverse-genetics studies. The effect of knock-down of a gene-of-interest can give strong indications of its involvement in certain processes. We have successfully employed silencing-constructs for *LePLD α 1* and *LePLD β 1* to demonstrate their involvement in salinity stress, wounding and plant-pathogen interactions. Analysis of PLD responses in *PLD*-silenced cell-suspension cultures established the activation of particular PLD isoforms in response to specific environmental cues. However, silencing strategies do entail some complications that have to be considered. Silencing is often not complete; experimentally measured residual enzymatic activity can therefore not be unambiguously explained but could be attributed to both incomplete silencing and the activity of a separate, redundant, enzyme. Furthermore, silencing is not always gene-specific; especially in species for which the genomic sequence is not yet available, designing a gene-specific silencing construct is often abstruse. We have designed silencing constructs targeting the genes' 3' UTRs, where the least homology can be found, retaining less than 70% identity with the sequences of other cloned tomato *PLDs*. Northern blot analysis of the expression of other *PLDs* in the *LePLD α 1*- and *LePLD β 1*-silenced lines indicated that the silencing construct did not affect their transcript levels (data not shown). Nonetheless, some level of cross-silencing cannot be fully excluded.

T-DNA-insertional mutagenesis can give a higher degree of certainty that the gene-of-interest is completely and specifically knocked-out. Knock-out lines can

subsequently be crossed to produce double knock-outs and so on. Examination of partial complementation of knock-outs by reintroduction of mutated genes can give valuable information about a protein's involvement in certain processes. For example, Mishra *et al.* (2006) used the reintroduction of an *AtPLD α 1* mutant that was impaired in G-protein-binding to study the role of *AtPLD α 1* in ABA signaling. The reintroduction of mislocalized and enzymatically hyper- or inactive PLDs may yield interesting results in the future.

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Samenvatting

Zowel in de natuur als in de tuinbouw staan planten blootgesteld aan steeds variërende condities. Bepaalde veranderingen in omstandigheden dwingen de plant om zich aan te passen, aangezien ze niet kunnen weglopen en beschutting kunnen zoeken. Zulke levensbedreigende veranderingen noemen we stress en de aanpassingen van de plant noemen we de stress respons. Aan de basis van dergelijke aanpassingen ligt het waarnemen van de verandering, gevolgd door het doorgeven van een signaal dat leidt tot aangepast functioneren van de plant.

Het doorgeven van een signaal van buiten naar de interne compartimenten van de cel, ofwel signaaltransductie, wordt vaak bewerkstelligd door de productie van signaalstoffen (second messengers). Dit zijn moleculen die normaal, d.w.z. als er geen stress wordt ervaren, nauwelijks voorkomen maar wiens niveaus onder stress condities tijdelijk toenemen. Deze signaalstoffen beïnvloeden de activiteit van componenten verder in de signaaltransductie keten die uiteindelijk leiden tot de aanpassing. Fosfatidylzuur is zo een signaalstof. Het is een fosfolipide, dat wil zeggen twee vetzuren aan een glycerol keten met een fosfaat groep. Onder normale omstandigheden komt dit molecuul voor in kleine hoeveelheden, die fors kunnen toenemen als reactie op uiteenlopende stress condities. Fosfatidylzuur wordt geproduceerd door, onder andere, phospholipase D (PLD). Dit enzym verwijderd de kopgroep van structurele fosfolipiden, zoals fosfatidylcholine, met als resultaat fosfatidylzuur en een losse kopgroep. Hogere planten bezitten een uitgebreide familie van PLD enzymen en er zijn meerdere aanwijzingen waaruit blijkt dat PLD betrokken is bij fosfatidylzuur productie tijdens stress condities. Echter, welke PLD wanneer wordt geactiveerd en hoe die productie leidt tot de uiteindelijke aanpassingen is een puzzel die nog verre van af is.

Dit proefschrift beschrijft onderzoek naar de rol van PLD in de aanpassingen van planten die plaatsvinden onder verschillende stress condities. Hoofdstuk 1 is een literatuur samenvatting waarin wordt uitgelegd hoe we denken over de mogelijke functies van PLD. In hoofdstuk 2 laten we zien dat specifieke PLD enzymen (nl. AtPLD α 1 en AtPLD δ) worden geactiveerd door zout stress en ook nodig zijn voor adequate aanpassingen die leiden tot zouttolerantie. Hoofdstuk 3 betreft de rol van deze twee PLDs in de respons van planten op verwonding, hier wordt aangetoond dat de PLD activiteit tijdens verwonding zich beperkt tot de verwondde (kapotte) cellen. In hoofdstukken 4 en 5 wordt PLD betrokkenheid bij plant-pathogeen interacties onderzocht, de rol van een specifieke PLD in tomaat, LePLD β 1, wordt bestudeerd aan de hand van celsuspensies en bio-assays met hele planten. Het laatste hoofdstuk, hoofdstuk 6, is een algemene discussie die ingaat op een aantal punten uit de voorgaande hoofdstukken en gedachtes over verder PLD onderzoek openbaart.

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