Dissecting Arabidopsis phospholipid signaling using reverse genetics

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Arabidopsis PLD\(\alpha_1\) and PLD\(\delta\) are redundantly required for full RPM1- and RPS2-mediated resistance

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Abstract

The *Arabidopsis thaliana* RPM1 resistance (R) protein confers strong resistance against *Pseudomonas syringae* expressing the avirulence (Avr) protein AvrRpm1. Upon AvrRpm1 recognition, the phospholipid phosphatidic acid (PA) is formed. PA can be formed by the action of phospholipase D (PLD). Here we show that Arabidopsis PLDα1 and PLDδ are redundantly required for full RPM1-mediated and RPS2-mediated resistance. Evidence is presented that PLDδ contributes to AvrRpm1-induced PA formation *in vivo*. These data indicate that PA is a positive regulator of Arabidopsis disease resistance.
Introduction

Plants are under constant threat from pathogens. In order to defend themselves, they have evolved sophisticated mechanisms to resist pathogen attack. Broadly, this resistance can be separated into two layers [1]. The first layer of defense consists of cell surface receptors that specifically recognize conserved parts of the pathogen (Pathogen Associated Molecular Patterns, PAMPs), for instance the bacterial flagellum [2-4]. Upon recognition, receptors trigger downstream signaling events that lead to changes in gene expression [5, 6] and ultimately to effective resistance against the pathogen [7], also known as PAMP-triggered immunity (PTI) [1]. However, certain pathogenic bacteria have evolved a protein secretion system that injects proteins directly into the plant cell where they can interfere with the plant’s defense response, resulting in effector-triggered susceptibility (ETS) [1, 8-10]. Successful suppression of PTI causes the pathogen to be virulent. Plants in turn, have evolved a second layer of defense that guards against interference by these pathogen-derived effectors [11]. This layer of defense consists of intracellular R (for Resistance) proteins, which monitor the integrity of the plant’s cellular machinery that might be the target of ETS. In this case, the effector that triggers ETS is called an Avr (avirulence) protein. When an R protein perceives the Avr-induced modification of a plant protein, this results in a very strong and rapid defense response that halts the pathogen, termed effector triggered immunity (ETI) [1]. ETI is often accompanied by localized cell death, also called the hypersensitive response (HR).

The Arabidopsis thaliana – Pseudomonas syringae interaction has become a model system to study R protein-mediated resistance. Several Arabidopsis R genes and Pseudomonas Avr genes have been cloned. Of these, Arabidopsis RPM1 (Resistance against Pseudomonas syringae, pathovar maculicola) has received most attention. RPM1 encodes an NBS-LRR (Nucleotide Binding Site Leucine Rich Repeat)
protein [12]. RPM1 indirectly detects the presence of the Pseudomonas effector, AvrRpm1 [13]. Upon delivery into the plant cell, AvrRpm1 mediates phosphorylation of Arabidopsis RIN4 by an unknown mechanism which in turn is perceived by RPM1, resulting in HR and resistance [14]. In addition, RIN4 is guarded by RPS2, an R protein that detects the perturbation of its guardee by AvrRpt2 [15]. AvrRpt2 probably has additional targets, as it still promotes virulence on rps2 rin4 plants [16].

Several forward genetic screens for loss of R gene-mediated resistance have been performed and have led to the isolation of numerous mutant r alleles [17, 18] and a few loci that function in regulating R protein stability [19-21]. Other proteins have been found to function in both ETI and PTI, such as NDR1 (Nonrace-specific Disease Resistance) [22]. NDR1 is required for resistance against virulent Pseudomonas as well as for the isogenic avirulent strain expressing AvrRpt2 [20]. Apparently, NDR1 is a point of convergence between PTI and ETI. To date, the mechanism by which R proteins activate ETI has remained elusive. It is known that R protein activation results in a multitude of responses, including the elevation of cytosolic calcium [23], formation of reactive oxygen species (ROS) [24] and formation of the phospholipid phosphatidic acid (PA) [25, 26].

Evidence for the formation of PA in response to Avr perception comes from tobacco cell suspensions that express the tomato resistance protein Cf4 [25]. Elicitation of these cells with the Cladosporium fulvum avirulence protein Avr4 resulted in the rapid accumulation of PA. PA production was observed in tomato cell suspensions after treatment with the avirulence protein xylanase as well [27]. Part of this PA response originated from the activity of phospholipase D (PLD). Silencing of LePLDβ1 resulted in decreased xylanase-induced PA formation [28]. Recently, PA has been shown to accumulate to high levels in whole Arabidopsis...
leaves after conditional expression of AvrRpm1 [26]. Biochemical evidence supports a role for PLD in this process.

The Arabidopsis genome contains 12 PLD genes. All PLDs have two C-terminal catalytic domains and are subdivided based on sequence homology, their N-terminal lipid-binding domains and their in vitro requirements for activity [29, 30]. PLDγ1 and PLDγ2 contain N-terminal phox homology (PX) and pleckstrin homology (PH) domains. The remaining 10 PLD genes contain an N-terminal C2 domain. C2 domains are able to bind lipids in the presence of calcium. These 10 PLDs are further subdivided based on their in vitro dependency on phosphatidyl inositolbisphosphate (PIP2), oleate, Ca2+ and pH [30, 31]. The predominant PLD activities in Arabidopsis are encoded by PLDα1 [32] and PLDδ [33]. These PLD isoforms are likely candidates to function in (part of) the AvrRpm1-triggered PA formation.

To study the role of PLDα1 and PLDδ in R gene mediated resistance, we took a reverse genetics approach T-DNA mutants were assayed for their response to Pseudomonas syringae, pathovar tomato, expressing avrRpm1 (Pto avrRpm1) and we found that PLDα1 and PLDδ are required for full AvrRpm1-mediated HR. Analysis of the double mutant demonstrated that PLDα1 and PLDδ are redundantly required for various aspects of the response to avirulent Pseudomonas, including restriction of its growth. Furthermore, we show that AvrRpm1-induced PA formation was reduced in a pldδ background.
Results

Isolation of pld1 and pld3 loss of function mutants

In order to establish the importance of PLD activity in the response to AvrRpm1 recognition, we aimed at testing pld mutants for phenotypes when challenged with Pto avrRpm1. Because PLDα1 and PLDδ are responsible for the largest part of the PLD activity in Arabidopsis, we focussed on mutants with T-DNA insertions in these genes. We obtained two T-DNA insertion lines for both PLDα1 and PLDδ from the SALK collection [34]. Those in pld1-1 and pld1-2 are located in the second and third exon of PLDα1 respectively. The T-DNA insertions in pldδ-1 and pldδ-2 are both located in the first intron of PLDδ (Fig. 1a). To confirm that these insertions cause a loss of function, we determined the expression of PLDα1 by western blot analysis and the expression of PLDδ by Q-PCR. As shown in Fig. 1b, the PLDα1-specific antibody detected a protein of the expected size (90 kDa) in wild-type plants but this band was lacking for pld1-1 and pld1-2. Similarly, PLDδ transcripts extending downstream of the insertion in pldδ-1 were not detected by Q-PCR (Fig. 1c). Therefore, pldδ-1 is very unlikely to produce a functional PLDδ protein.

Loss of PLDα1 or PLDδ expression leads to reduced RPM1-mediated HR

Infiltration with a high dose of Pto avrRpm1 results in macroscopically visible HR within 6 hours. We quantified AvrRpm1-induced HR in pldα1 and pldδ mutants by classifying individual leaves for the surface area that showed cell death (Fig. 2). Compared to wild-type, pldα1 and pldδ mutant alleles showed a reduced HR (p < 0.1), while HR was completely absent in the rpm1 mutant. Since independent
Fig. 1. Characterization of pldα1 and pldδ insertion lines
(a) Gene structure of PLDα1 and PLDδ. Filled boxes represent exons, lines represent introns, open boxes represent untranslated regions, grey boxes represent regions that are absent in some splice variants and triangles represent T-DNA insertions. Primers used for Q-PCR are indicated by arrows. Drawing is approximately to scale. (b) PLDα1 expression in pldα1 alleles. Total protein was extracted from indicated genotypes, blotted and probed with a PLDα1-specific antibody (top) or stained with coomassie (bottom). (c) PLDδ expression in pldδ-3 as determined by Q-PCR. PLDδ expression in wild-type was arbitrarily set to 1.

mutant alleles of both PLDα1 and PLDδ exhibited a reduced HR, both genes seem to be involved in this defense response.
Leaves were classified according to the percentage of leave surface that showed HR symptoms. The insertion lines were compared pairwise to wild-type using \( \chi^2 \) analysis. Statistical significant differences are indicated by * (\( p < 0.1 \)). This experiment was independently repeated with consistent results.

**PLD\( \alpha \)1 and PLD\( \delta \) have partially overlapping functions in RPM1- and RPS2-mediated responses**

To investigate the effect of combined loss of PLD\( \alpha \)1 and PLD\( \delta \) on HR, a double mutant was constructed by crossing \( \text{pld} \alpha 1 \) and \( \text{pld} \delta 1 \). These parental lines will be referred to as \( \text{pld} \alpha 1 \) and \( \text{pld} \delta 1 \) from here onwards. The \( \text{pld} \alpha 1 \text{pld} \delta 1 \) double mutant developed normally. As was observed with both single mutants, the \( \text{pld} \alpha 1 \text{pld} \delta 1 \) double mutant showed a reduced HR, 6 hours after infiltration with Pto avrRpm1 (Fig. 3a). The HR of the double mutant appeared to be slightly more reduced compared to the single mutants but this was not statistically significant. Possibly, the severity and speed of cell-death development (visible symptoms within 6 hours) prevented us from detecting subtle differences between genotypes. The HR in response to Pto avrRpt2 is delayed, compared to the RPM1-mediated HR (24 hrs compared to 6 hrs) and this allowed us to score for more intermediate levels of HR. As a control, the ndr1 mutant was used which does not show an RPS2-mediated HR [35] (Fig. 3b). Comparison to wild-type revealed a statistical significant reduction
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of RPS2-mediated HR, only in the \textit{pld\alpha{1} pld\delta} double mutant, suggesting a redundant role for \textit{PLD\alpha{1}} and \textit{PLD\delta} here.

A quantitative marker for HR is electrolyte leakage [14]. Infiltration of a high dose of \textit{Pto avrRpm1} resulted in electrolyte leakage within 4 hrs, whereas only background leakage was observed in \textit{rpm1} (Fig. 4a). The kinetics of the response measured were in agreement with those published by others [14, 15]. The \textit{pld\alpha{1} pld\delta} double mutant showed a statistical significant reduction in RPM1-mediated electrolyte leakage after 7 hrs and onwards. The response to \textit{Pto avrRpt2}, which exhibits different kinetics (electrolyte leakage after 5 hours), was reduced in \textit{pld\alpha{1} pld\delta} but the effect was not as severe as in \textit{ndr1} (Fig. 4b).
Fig. 4. Electrolyte leakage after infection with *Pto avrRpm1* or *Pto avrRpt2* 
(a) AvrRpm1-mediated electrolyte leakage. Error bars represent standard errors. Four to six leaf discs per replicate were used. The three genotypes were different at the last three time points according to Tukey (*α* = 0.05). Similar results were obtained in four out of five replicate experiments. (b) AvrRpt2-mediated electrolyte leakage. Error bars represent standard errors. Four to five leaf discs per replicate were used. Statistically significant differences between Col-0 and *pld*Δ1 *pld*Δ3 were observed at the last two time points (Tukey, *α* = 0.05)
PLD\(\alpha 1\) and PLD\(\delta\) are redundantly required for full RPM1- and RPS2-mediated resistance

Up to this point, responses after infiltration of relatively high doses of avirulent bacteria have been described. Although informative, such high doses may not reflect the natural situation in which bacteria enter the leaf in small numbers. Therefore, we set out to test the ability of the \(pld\) mutants to restrict the growth of low doses of avirulent Pseudomonas. The \(pld\alpha 1\) \(pld\delta\) double mutant allowed approximately 10-fold more bacterial growth than wild-type, demonstrating that PLD\(\alpha 1\) and PLD\(\delta\) are redundantly required for full RPM1-mediated resistance (Fig. 5a). The average bacterial titer in the single mutants was marginally higher than in wild-type but this difference was not statistically significant. Bacterial titer measured after 3 days was 10,000-fold higher in \(rpm1\) compared to wild-type, demonstrating that RPM1-mediated resistance is sufficient to restrict the growth of \(Pto\ avrRpm1\) under our experimental conditions. To substantiate these data, time-course experiments after challenge with \(Pto\ avrRpm1\) (Fig. 5b) and \(Pto\ avrRpt2\) were performed (Fig. 5c). The results show that \(pld\alpha 1\) \(pld\delta\) allowed approximately 10-fold more growth of both \(Pto\ avrRpm1\) and \(Pto\ avrRpt2\) compared to wild-type and this difference was sustained over 4 days.

AvrRpm1 induced PA formation is reduced in a \(pld\delta\) background

Conditional expression of AvrRpm1 resulted in RPM1-mediated PA formation [26]. In order to establish the contribution of PLD\(\alpha 1\) and PLD\(\delta\) to this response, transgenic Arabidopsis harbouring \(avrRpm1\) under control of a dexamethasone-inducible promoter (\(DEX::avrRpm1\)) was crossed with the \(pld\alpha 1\) \(pld\delta\) double mutant. Although various mutant combinations that were genetically homozygous for the \(DEX::avrRpm1\) transgene were isolated, these lines did not respond to dexamethasone. Western blot analysis revealed that these lines fail to express
significant amounts of AvrRpm1 after dexamethasone induction. Recently, it was published that transgenes under control of the 35S promoter are silenced in various
AvrRpm1 expression was induced by treatment with 20 μM dexamethasone for 2 hours. Phospholipids were then extracted, separated by TLC and PA was quantified as a percentage of total labeled phospholipids. Error bars represent standard errors.

T-DNA tagged backgrounds, including SALK lines [36, 37]. To circumvent this problem, we obtained another pldδ allele (pldδ-3) from the Wisconsin collection that does not contain a 35S promoter [38]. pldδ-3 was reported to be a null allele [39]. Accordingly, pldδ-3 was crossed with DEX::avrRpm1, selfed and lines homozygous for DEX::avrRpm1 and PLDδ or pldδ were selected in the F3. As these plants were in a mixed Col/WS background, multiple independent F3 families were selected for analysis. Dexamethasone treatment resulted in a ~ 4 fold increase in PA levels in two PLDδ lines after 2 hrs (Fig. 6). This induction was ~ 40% lower in three lines homozygous for the pldδ-3 allele. These results strongly suggest that part of the AvrRpm1-induced PA formation is derived from PLDδ.
A reverse genetics approach was undertaken to establish the contribution of \textit{PLD\alpha1} and \textit{PLD\delta} to R-mediated HR and resistance. Both \textit{pld\alpha1} and \textit{pld\delta} alleles displayed a slightly reduced HR in response to AvrRpm1. As we observed similar phenotypes for independent alleles, it is likely that these were caused by mutations in the \textit{PLD} genes. A \textit{pld\alpha1 pld\delta} double mutant was also affected in AvrRpt2-induced HR. This phenotype was accompanied by reduced electrolyte leakage. Both \textit{Pto avrRpm1} and \textit{Pto avrRpt2} proliferated to higher levels in \textit{pld\alpha1 pld\delta}. In contrast, the single mutants did not allow more growth than wild-type, indicating that \textit{PLD\alpha1} and \textit{PLD\delta} are redundantly required for full R-mediated resistance.

Conditional expression of AvrRpm1 in a \textit{pld\delta} background demonstrated that \textit{PLD\delta} contributes to AvrRpm1-induced PA formation. The reduced PA formation correlates with reduced resistance phenotypes that we observed for mutants containing a \textit{pld\delta} allele. Thus, these data are consistent with the hypothesis that reduced PLD-mediated PA formation causes the observed phenotypes. However, we cannot exclude that loss of \textit{PLD\alpha1} and \textit{PLD\delta} caused a loss of resistance via another mechanism than reduced PA formation.

Our attempts to measure PA in a \textit{pld\alpha1} background after conditional expression of AvrRpm1 failed, because the transgenic lines lost their responsiveness to dexamethasone over generations. This reduced responsiveness is probably caused by trans-inactivation of the 35S promoters present in the T-DNA’s used for mutagenesis and for dexamethasone induction [36, 37].

In a \textit{pld\delta} background, AvrRpm1-induced PA formation was reduced but not abolished. As \textit{PLD\alpha1} and \textit{PLD\delta} are redundantly required for RPM1-mediated
resistance, we suspect that PLDα1 is also activated in response to AvrRpm1 recognition. In this scenario, either PLDα1- or PLDδ-mediated PA formation is sufficient for full resistance but loss of both PLDs cannot be compensated for.

How could PLD-mediated PA formation contribute to resistance? PA has been shown bind to several proteins [40]. These PA binding proteins include signaling proteins such as CTR1 [41], SnrK2.10 [40] and PDK1[42]. The latter activates and interacts with the protein kinase OX11/AGC2-1 in a PA-dependent manner [43]. oxil mutants were affected in their resistance against Hp Emco5 [44]. It is possible that PA generation is perceived by PDK1 and is translated in OXI1 activation that mediates appropriate defense responses. Downstream of OX1 are PTI1-2 [45], a protein kinase that shares similarity with tomato Pti kinase [46] and the mitogen associated kinases MPK3 and MPK6 which have been associated in defense responses [5, 47]. Alternatively, PA could bind to heterodimeric capping protein [48] or AGD7 (Arf GAP domain) [49] and influence cytoskeleton dynamics and membrane trafficking which have been proposed to play a role in pathogen resistance [50].

Comparison to rpm1 showed that pldα1 pldδ has residual resistance. It is possible that other PLD isoforms contribute to resistance or that RPM1 activation leads to a divergent response, including PLD activation. PLDα1 and PLDδ were required for responses mediated by both RPM1 and RPS2 (Fig.5b,c). As xylanase induced PLD activity in tomato [27, 28], it is conceivable that PLD activation is a general response to R protein activation.
Materials and methods

Plant material

*pld* mutants were obtained from the SALK collection [34] or the Wisconsin collection [38] (table 1). Homozygous lines were selected by PCR, using gene-specific primers in combination with a T-DNA border primer (table 2). The *pld*1 *pld*Δ double mutant was constructed by crossing *pld*1-1 with *pld*Δ-1 and a line homozygous for both mutations was identified in the F2 by PCR. *rpm1* was *rpm1*-3 in a glabrous background. *ndr1* [35], *Dex::avrRpm1* [26] and *pld*Δ-3 [39] have been described. For conditional expression of AvrRpm1 in *pld*Δ-3 and *PLD*Δ sibling controls, *Dex::avrRpm1* was crossed with *pld*Δ-3. Plants that were at least hemizygous for *Dex::avrRpm1* were identified in the F2 by localized application of dexamethasone and the presence of *pld*Δ-3 was determined by PCR. Plants that were homozygous for *pld*Δ-3 or *PLD*Δ were selfed and F3 families for *Dex::avrRpm1* were identified.

Table 1.

<table>
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<th>reverse</th>
<th>border primer with</th>
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<td>787</td>
<td>788 LBa</td>
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<td>470 LBa</td>
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<td><em>pld</em>Δ-2</td>
<td>SALK_023808</td>
<td>469</td>
<td>470 LBa</td>
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<tr>
<td><em>pld</em>Δ-3 (WS)</td>
<td>PLDdF</td>
<td>PLDdR</td>
<td>JR70</td>
</tr>
</tbody>
</table>

Growth conditions

Plants were grown in a growth chamber at 21°C, 70% humidity under a 11 hours photoperiod. *Pto* expressing *avrRpm1* or *avrRpt2* was grown in liquid King’s B medium containing the appropriate antibiotic. The next day, bacteria were resuspended in 10 mM MgSO4 (or deionized water for electrolyte leakage assays) and diluted for use in bioassays.
Table 2.

<table>
<thead>
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<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>470</td>
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<td>787</td>
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<td>788</td>
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<tr>
<td>UBI10 R</td>
<td>AAA GAG ATA ACA GGA ACG GAA ACA TAG T</td>
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</table>

**Bioassays**

Four weeks after sowing, three to five leaves (the fourth true leaf and younger leaves) were syringe inoculated with either a high dose (OD 0.02-0.05, 1.0 – 2.5 x 10⁷ colony forming units (cfu) per ml) of *Pto* for HR symptoms and electrolyte or a low dose (OD 0.00005, 1.0 x 10⁴ cfu/ml) for bacterial growth assays. HR symptoms were scored 6 hours (Pto avrRpm1) or 24 hours (Pto avrRpt2) after inoculation. Electrolyte leakage was monitored by floating leaf discs on deionized water and measuring the conductivity with a Radiometer Copenhagen CDM80 conductometer equipped with a type CDC114 electrode at room temperature. Bacterial growth was measured by grinding 2 leaf discs per replicate in 10 mM MgSO₄ and plating 10-fold dilutions in duplicate on KB plates containing 25 μg/ml rifampicin. Plates were left at room temperature for three days and colonies were counted.

**Protein extraction and western blot**

Protein extraction buffer (9.5 M Urea, 0.1M Tris-HCl pH 6.8, 2% (w/v) SDS and 2% (v/v) β-mercapto-ethanol) was added to an equal volume of ground leaf tissue, mixed and centrifuged in an appendorf centrifuge for 10 min at maximal speed. Sample buffer was added to the supernatant and samples were loaded on a 10%
SDS-PAGE gel, blotted on nitrocellulose and incubated overnight in PBST with 5% (w/v) powdered milk and the an affinity purified polyclonal peptide specific anti-LePLDα1 antibody which also detects AtPLDα1 (rabbit; Eurogentech, Liege, Belgium). The blot was washed three times in PBST and incubated for 1 hour with an appropriate peroxidase-conjugated secondary antibody. The peroxidase activity was detected by enhanced chemiluminescence (Amersham, Buckinghamshire, UK). Duplicate gels stained with Coomassie Brilliant Blue (0.25% (w/v) CBB, 30% (v/v) methanol and 10% (v/v) acetic acid) of the blot served as loading control.

**RNA extraction and Q-PCR**

Total RNA was extracted as described previously [51]. Gene expression was analyzed by quantitative RT-PCR. Five μg of RNA was digested with Turbo DNase-free (Ambion, Huntingdon, United Kingdom) according to the manufacturer’s instructions. To check for contamination with genomic DNA a PCR was performed on the DNase-treated RNA. DNA-free RNA was converted to cDNA using oligo-dT18 primers, dNTPs, and SuperScript III Reverse Transcriptase (Invitrogen, Breda, The Netherlands) according to the manufacturer’s instructions. Quantitative PCR was performed on the cDNA using SYBR Green Supermix reagent (Invitrogen) in a final volume of 15 μL, following the manufacturer’s protocol, using the Applied Biosystems 75000 real time PCR machine. Gene-specific primers for *PLDδ* were PLDd4363F and PLDd4440R. Primers for the reference gene *UBI10* (At4g05320) were UBI10 F and UBI10 R. C₅ values were normalized to C₅ of UBI10, after which the fold-differences in transcript levels were calculated.

**Dexamethasone induced AvrRpm1 expression and phospholipid analysis**

For the determination of AvrRpm1-induced PA formation, leaf discs from four-week old plants labelled overnight in 100 μl labeling buffer (2.5 mM MES, 1mM KCl pH 5.7 with KOH) in 2ml Eppendorf tubes by the addition of 1 μl ³²P-labeled PO₄. The next day, 100 μl labeling buffer supplemented with 20 μM
dexamethasone and 0.005% (v/v) Silwet was added. The treatment was stopped after 2 hours by addition of 5% (v/v) final concentration of perchloric acid. Lipid extraction and separation was essentially done as described previously [27].

Acknowledgments

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