Plant phosphatidic acid metabolism in response to environmental stress
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Citation for published version (APA):

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The salt stress-induced lyso-phosphatidic acid response in *Chlamydomonas* is generated through phospholipase-A$_2$ hydrolysis of diacylglycerol kinase-generated phosphatidic acid.
Abstract

The unicellular green alga \textit{Chlamydomonas} has frequently been used as a eukaryotic model system of intracellular phospholipid signalling in response to environmental stress. Previous results showed a rapid salt-induced increase in the putative second messenger, phosphatidic acid (PA), which was suggested to be generated via activation of phospholipase D (PLD) and phospholipase C (PLC) in combination with diacylglycerol kinase (DGK) (Chapter 4; [1, 2]). Also, a rise in lysophosphatidic acid (LPA) was witnessed, which has previously been attributed to the activation of a phospholipase A$_2$, based on pharmacological evidence [3]. So far, it is unknown where this LPA originated from. This question is complicated even more by the fact that both LPA and PA are precursors for the biosynthesis of structural phospholipids and galactolipids. In this study, we have used \textit{in vivo} $^{32}$P-radiolabelling and fatty acid molecular species analysis to answer the question of LPA’s metabolic origin in the salt stress response. Evidence is provided that LPA is formed from a distinct pool of PA, which is characterized by a high \textit{$\alpha$}-linolenic acid (\textit{$\alpha$}-LA; 18:3n3) content. This molecular species is most likely derived from PLC hydrolysis of the polyphosphoinositides, and subsequent phosphorylation of diacylglycerol into PA by DGK. Differential $^{32}$P-radiolabelling assays indicated a leading role for DGK in providing the PLA$_2$ substrate, and argued against the PA pools from PLD or \textit{de novo} synthesis via ER- or plastid-localized routes.
Introduction

The unicellular green alga *Chlamydomonas* has proven to be useful in the study of phospholipid signalling in response to osmotic and salt stress [4, 5]. Recently, particular interest has been focussing on the accumulation of PA as a lipid second messenger in plant and animal systems [6]. In *Chlamydomonas moewusi*, PA is a minor lipid, comprising only 0.67 mole% of total phospholipids ([7], Chapter 3). However, in response to 150 mM NaCl, a 3.6-fold increase is triggered within 5 minutes ([1], Chapter 4). In a recent study, also LPA was shown to accumulate in *C. moewusi* under conditions of salt and non-ionic hyperosmotic stress [3]. The response was dose-dependent within the range of 150 to 400 mM NaCl, reaching a maximum at 300 mM. The present study was devised to elucidate the molecular composition of this lipid and to identify the metabolic pathways underlying its accumulation. This question is complicated by the multiplicity of pathways that can generate LPA.

The primary function of LPA and PA is in the ER and plastidial membranes as intermediates in *de novo* glycerolipid biosynthesis (Fig. 1; Chapter 1). This pathway starts with two consecutive acylations of glycerol3phosphate (Gro3P) to generate first LPA and then PA by the activities of GroP acyltransferase (GPAT) and LPA acyltransferase (LPAAT), respectively. Subsequently, for the synthesis of phosphatidylinositol (PI) or phosphatidylglycerol (PG), PA is converted to cytidine monophosphate-PA (CMP-PA), which reacts with *myo*-inositol or GroP. Alternatively, for the synthesis of phosphatidylethanolamine (PtdEtn) or monogalactosyl diacylglycerol (MGDG), PA is dephosphorylated by PA phosphatase (PAP) to DAG, which acquires its headgroup in a reaction with cytidine diphosphate-ethanolamine (CDP-Etn) or uridine diphosphate-galactose (UDP-Gal), respectively.

Although under steady-state conditions, PA formation by LPAAT may be prevalent, under environmental stress conditions additional pathways are activated which generate a transient accumulation of PA, believed to function as a lipid second messenger. These pathways include phospholipase D (PLD), hydrolyzing PE (or PC) to generate PA, and DAG kinase (DGK) which produces PA by phosphorylation of DAG. Under stimulatory conditions, this DAG can be provided by PLC-mediated hydrolysis of polyphosphoinositides (PPIs), i.e. phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP$_2$) [6, 8, 9].

Salt stress in *C. moewusi* triggers the formation of PA via both PLD- and PLC/DGK-mediated pathways [1, 2], and the accumulation of LPA was considered to be produced from PA through a PLA$_2$ activity, based on PLA$_2$ inhibitors [3]. However, the picture is more complicated since LPA and PA are also product and precursor in the *de novo* glycerolipid biosynthesis pathway (Fig. 1). This raises the possibility that LPA is generated as precursor of PA, rather than being its hydrolytic product. Here we present evidence, using fatty acid analysis and differential $^{32}$P-radiolabelling, that the accumulated LPA is mainly derived from a subpool of PA that is generated via DGK, characterized by an enrichment in 18:3.
(α-linolenic acid, α-LA). A model illustrating how the metabolic pools of PA and LPA are related is presented.

Material and Methods

Cell cultures, treatment and lipid extraction

*Chlamydomonas moewusii* strain UTEX 10 (mating type minus) from the Culture Collection of Algae, University of Texas (Austin) was autotrophically grown as described before [10]. Petri dishes containing cultured cells of ~18 d old were flooded with with 20 ml HMCK (10 mM HEPES, 1 mM MgCl₂, 1 mM CaCl₂, 1 mM KCl; pH 7.4) and after 16 hrs, suspensions of swimming gametes were harvested [7].

For fatty acid analyses, treatments were conducted in separation funnels containing 60 ml cells (density 2.0 * 10⁷ cells/ml) to which 20 ml buffered NaCl solution (or only buffer) was added. Reactions were stopped by the addition of perchloric acid to a final conc. of 5% (w/v) and lipids extracted essentially by a previously described method with adjustment for the larger extraction volume (Chapter 4; [1]).

From the extracts, PA and LPA were purified by column-adsorption chromatography on a 2 g-silica column (Sep-pak plus). The lipid extract was dissolved in hexane and applied to the column. Elution solvents were (1) hexane-Et₂O (99:1, 18 ml), (2) hexane-Et₂O (4:1, 15 ml), (3) CHCl₃ (10 ml), (4) Me₂CO-CHCl₃ (2:1, 25 ml), (5) Me₂CO-MeOH (29:1, 10 ml), (6) Me₂CO-MeOH (19:1, 30 ml), (7) Me₂CO-MeOH (2:1, 25 ml), (8) CHCl₃-MeOH-H₂O (1:2:0.8, 19 ml). The last eluate was collected in 10 ml tubes in 4 portions of 4.75 ml. To extract lipids from these aqueous eluates, 3.75 ml CHCl₃ and 1 ml 0.9% (w/v) NaCl was added, tubes were vigorously shaken and centrifuged to separate 2 phases of which the lower one, containing LPA and PA, was dried down in a gyrovap at 50°C. The concentrated extracts were further purified by TLC using silica gel 60 plates (Merck) in a solvent of CHCl₃-MeOH-NH₄OH-H₂O (90:70:4:16, by vol.). ³²P-lipid markers served to calculated the recovery af each lipid after purification and for their localization on the TLC plate.

For ³²P-labelling experiments, 100 ml aliquots of cell suspension were labelled with 50 µCi carrier-free ³²Pᵢ (Fig. 5), or 50 µCi ³²Pᵢ in a 1 mM K-Pi buffer at pH 7.4 (Fig. 6). Treatments and lipid extraction were performed as described previously (Chapter 4; [1]), separation of the extracted lipids was by TLC using the solvent specified above.

Fatty acid derivatization and analysis

Lipid spots were scraped off the TLC plates directly into the transmethylation reagent. Known concentrations of heneicosanoic (21:0) acid methyl esters served as internal standard. The concentrated FAME extract was analyzed in a GC (Varian Chrompack, Bergen op Zoom, NL) equipped with a 50 m WCOT fused silica column and FID with
carrier gas N₂ at 30 ml min⁻¹. Operating conditions were 180° isothermal or temp. programmed 180° to 220° at 0.5° min⁻¹ with injector and detector temps. at 250° and 270°, respectively.

In vitro PLA₂ assay of phospholipids

TLC-separated lipids were recovered from the silica as described by Arisz et al. (2000) and dissolved by sonication in 1 ml ethylether/MeOH (98:2, by vol). Five units of PLA₂ (bee venom PLA₂, Sigma) was added in 100 μl 100 mM Tris HCl buffer (pH 8.9) containing 9.1 mM CaCl₂. After 3 hrs incubation at 25 °C with frequent shaking, reactions were stopped by adding 20 μl 0.5 M EDTA. Lipid products were extracted after evaporation of the ether phase by three consecutive extractions of the aqueous phase. Pooled extracts were evaporated, and the precipitate was resolved into CHCl₃ for TLC purification and analysis. For the purpose of accurate quantitation of LPA in fatty acid analyses, a C17-LPA standard was prepared from the corresponding, commercially available, di-C17-PA (Sigma), by in vitro PLA₂ digestion.

Results

When Chlamydomonas cells were metabolically labelled with ³²P-P₁ and subsequently treated with 300 mM NaCl for 5 min, several changes in the ³²P-phospholipid pattern can be observed (Fig. 2) as reported earlier. Thus, typical ³²P-increases were found in PA [2], LPA [3], diacylglycerol pyrophosphate (DGPP, [2, 11]), PIP [12], PI(3,5)P₂ [13] and PI(4,5)P₂ [5, 13]. The increase in ³²P-PA has been suggested to result from a PLD pathway, in which the structural phospholipid PE is hydrolyzed, and via the combined activities of the

![Diagram of lipid synthesis](image.png)

Figure 1. PA is formed by successive acylations of Gro3P and LPA for de novo synthesis of glycerolipids, but it can also be formed through the stress-induced activities of PLC/DGK and PLD. For the synthesis of PI and PG(P), PA is converted to cytidine monophosphate-phosphatidic acid (CMP-PA) by CMP-PA synthase (CDS), and for the synthesis of PE and MGDG, it is dephosphorylated by PAP. Note that the PA/DAG substrates in the synthesis of PE and MGDG are in actual fact different pools localized at the ER and plastidial envelope membrane, respectively. During salt stress, Chlamydomonas cells accumulate the alternative PA metabolites DGPP and LPA. The latter is suggested to originate from PA-hydrolyzing PLA₂ activity.
PLC/DGK pathway [1, 2]. PLC hydrolyzes PI4P and PI(4,5)P$_2$ into inositolpolyphosphates (InsPPs) and DAG, and the latter can be converted to PA via DGK (Fig. 1). DGPP is the phosphorylated product of PA, which may represent an attenuation of PA as a signalling molecule, but it could also be a phospholipid signal itself [11, 14].

Like PA, the study of LPA, is complicated by the multiplicity of pathways that synthesize it (Fig. 1). LPA is formed in the pathway of de novo PA synthesis, but it can also result from acyl hydrolysis of PA by a PLA$_2$ activity. The first possibility implies that LPA is precursor to PA, whereas the latter implies that LPA is the product of LPA. To study these product-precursor relationships, fatty acid analysis have been performed, as phospholipid classes can have characteristic ‘fatty acid fingerprints’ that are inherited by their metabolites.

As shown in Fig. 3, salt stress induced major increases in 16:0 and 18:1n9 in both LPA and PA pools. However, the LPA generated in response to salt contained $\alpha$-linolenic acid ($\alpha$-LA, 18:3n3; Fig. 3b, d; peak 8). Since this species was hardly detectable under steady-state conditions, and increased in response to salt stress to become one of its major constituents (Fig. 3b, d), these results strongly suggest activation of a novel metabolic pathway, rather than an upregulation of de novo synthesis. Consequently, this possibility was further investigated.

Interestingly, $\alpha$-LA was not particularly abundant in the pool of PA, in both conditions (Fig. 2a, 2c). To explain this difference, it was hypothesized that the $\alpha$-LA-enriched LPA was perhaps derived from a preceding, short-lived part of the PA increase, with a similar $\alpha$-LA enrichment in its fatty acid composition. To test this idea, we treated cells for 30 s and 5 min with 300 mM NaCl and quantitatively analyzed the fatty acid compositions of LPA and PA (Fig. 4).

Already within 30 s of NaCl stimulation, a substantial increase in PA was found, which was characterized by the abundance of palmitic (16:0) and oleic acids (18:1), while $\alpha$-LA (18:3), the hallmark of stimulated LPA, was relatively minor at both time points (Fig. 4a, b). While
this appears to argue against α-LA-enriched LPA resulting from a preceding phase of PA formation, it could be derived from a metabolically separated subpool of PA, containing a relatively high α-LA content. Indeed, the increase in α-LA was found in the same order of magnitude in both lipids, when plotted along the same axis (Fig. 4c).

As salt stress-induced PA in *Chlamydomonas* is of mixed origin, it was hypothesized that LPA formation selectively drew upon one of the PA metabolic pools, explaining the disparate fatty acid compositions of PA and LPA. The LPA molecular species would then reflect the composition of either the PLD substrate, PE, or PLC’s substrate, the PPIs. PE has been shown to contain mainly 18:1n9 (92 mole%) and in the MS spectrum the (18:1)2 species was predominant accounting for > 95% of the total PE ([1], Chapter 4). Although a minor amount of α-LA (2 mole%) was present in PE ([7], Chapter 3), it probably is absent from the PA produced by PLD-mediated PE hydrolysis during salt stress ([1], Chapter 4). While this suggested that PLD-derived PA could not lead to the increase in α-LA-containing LPA, it could still be precursor to the 18:1-containing LPA.

In contrast, PPIs contain α-LA and also 18:1n7, a hallmark-fatty acid of PI and its derivatives (Chapter 3, [7]). So their hydrolysis by PLC and subsequent phosphorylation of DAG could generate these molecular species of PA (Fig. 3a/c, Fig. 4; [1], Chapter 4). If this PA pool would be substrate to a PLA2, it might account for the increase in α-LA- and 18:1n7 species of LPA.
Table 1. Fatty acid compositions (mol%) of PE and PI (parents) from *C. moewusii*, and their lyso-derivatives (daughters) acquired by *in vitro* PLA2–catalyzed digestion

<table>
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<tr>
<th></th>
<th>16:0</th>
<th>18:1n9</th>
<th>18:1n7</th>
<th>18:2n6</th>
<th>18:3n3</th>
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<tr>
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<tr>
<td>PE</td>
<td>5</td>
<td>92</td>
<td>0</td>
<td>1</td>
<td>2</td>
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<tr>
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<td>50</td>
<td>14</td>
<td>20</td>
<td>9</td>
<td>7</td>
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<tr>
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<tr>
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<td>0</td>
<td>0</td>
<td>3</td>
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<tr>
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<td>4</td>
<td>13</td>
<td>64</td>
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Figure 4. Increments in 18:2- and 18:3-containing PA (a) and LPA (b) are registered after 30 s and 5 min of hyperosmotic stress in *Chlamydomonas*. Two fatty acids that are characteristic for PPIs, 18:1n7 and 18:3n3, increase in LPA and PA during treatment (c). Lipids were isolated from suspensions of *Chlamydomonas* gametes after challenge with 300 mM NaCl for the respective times.
PLA₂ hydrolysis of PA from a mixed PLD- and PLC/DGK-derived origin could thus account for the production of the corresponding LPA species, provided that these fatty acids are esterified to the sn-1-OH position of the respective substrate lipids, i.e. PE and PPIs. To test this, a positional study of the fatty acids in PE and PI, which is similar to the PPIs (Chapters 3, 4; [1, 7]), was performed (Table 1). First, the lipids were purified from a total lipid extract and subsequently digested in vitro using PLA₂ from bee venom to generate the corresponding lysophospholipids, which were purified on TLC and analyzed for their fatty acids by GC.

As shown in Table 1, the α-LA and 18:1n7 content was not decreased in LPI compared to its parent PI, suggesting that these fatty acids were predominantly linked to the sn-1 position which is resistant to PLA₂ digestion. These results are in agreement with the suggested pathway in *Chlamydomonas*, involving PLA₂-hydrolysis of PA derived from PPIs.

To confirm and further investigate the origin of LPA, a differential labelling technique was applied [8, 15]. This strategy takes advantage of the extremely fast 32P-radiolabelling of the cellular ATP pool when cells are only briefly incubated with 32P-Pi. As a consequence, lipids, which are direct products of ATP-dependent phosphorylation, such as DGK-generated PA, are quickly labelled. whereas PA that is derived from PLD activity is slowly labelled because of the relatively slow labelling kinetics of PE, which takes hours-days [8, 15, 16]. Thus, *Chlamydomonas* cells were labelled with 32Pi for only 1 min and subsequently treated with 300 mM NaCl for different periods of time, ranging from 1-20 min, or mock-treated with buffer alone. Lipids were then extracted, separated by TLC, and quantitatively analyzed by phosphoimaging (Fig. 5). Analysis of 32P-PA and 32P-LPA revealed rapid increases, both peaking after 10 min of treatment (Fig. 5a). The increase in PA was larger, and LPA seemed to lag slightly behind, although this was less marked than previously found [3]. Under non-stimulated conditions, the levels of 32P-PA and 32P-LPA remained low.

The labelling kinetics of PIP₂ and PIP (Fig. 5b and c) were more complex. The transient peaks of 32P-PIP₂ and 32P-PIP in unstimulated conditions is characteristic of their extremely rapid turnover and small pool size, which has been scrutinized in an earlier study [15]. The pulse/chase pattern was also seen under salt stress, but with increased 32P-PIP₂ and 32P-PIP levels, suggesting increased PI- and PIP-kinase activities. During the first 2 min of salt treatment, 32P-PIP levels were lower than the controls (Fig. 5c). Potentially, this could reflect the activation of a PIP-kinase to replenish the PIP₂ that is consumed by PLC. Alternatively, PIP itself could be a PLC substrate.

Figure 5d shows the characteristic slow labelling pattern of PE, which is slightly enhanced under salt-stress conditions. Nonetheless, the rapid and transient labelling of LPA and PA in this experiment is clearly more in agreement with their origin in the PPIs rather than from PLD-mediated hydrolysis of PE.

To examine this further, we labelled cells for extended time periods with 32P in the presence of unlabelled carrier phosphate to deliberately impede the rapid label incorporation into
the DGK product. Under these conditions, $^{32}$P-lipid levels will, over time, reflect actual quantities rather than turnover rates. Thus, radioactivity in the structural phospholipids such as PE, keeps on increasing for days, while the quantitatively minor PPIs will reduce, reflecting their actual levels (Chapter 3, [7]). The opposite labelling trends of PE and PPI must be reflected in their products PA PE and PA PPI, which provides a means to distinguish them and to determine which pathway is activated during salt stress, a PLD- or PLC-mediated pathway, respectively. Thus, PA derived from PLD activity will reflect the steadily increasing labelling of $^{32}$P-PE, and PA generated by the PLC/DGK pathway will show a decreasing labelling.

Under non-stimulated conditions, the amount of $^{32}$P-label in PE increased massively with increasing labelling times (Fig. 6a). Also LPA and PA showed increased labelling with time, probably reflecting the extensive equilibration of the precursors in de novo synthesis. Stimulation of cells with 300 mM NaCl for 5 min (Fig. 6b) induced increased $^{32}$P-PA levels at all labelling times, and in $^{32}$P-LPA until 1 day of labelling (compare Fig. 6a and 6b). However, the $^{32}$P-LPA response was lower at longer labelling times. Strikingly, after 2 days of labelling, salt stress did not induce an increase in $^{32}$P-LPA anymore, instead it declined.

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**Figure 5.** $^{32}$P-phospholipid changes in *Chlamydomonas* cells prelabelled with $^{32}$P for 1 min and subsequently treated for different periods of time with buffer alone (mock treatment, open symbols) or buffer supplemented with 300 mM NaCl (closed symbols). Total lipids were extracted and separated by TLC, and subsequently radioactivity in the phospholipid spots was determined by phosphoimaging.
Figure 6. Changes in $^{32}$P-PA (white bars), $^{32}$P-LPA (grey bars) and $^{32}$P-PE (black bars) from *Chlamydomonas* cells, prelabelled with a solution of $^{32}$P$_{i}$ + unlabelled P$_{i}$ for different periods of time (X-axis), and subsequently treated for 5 min with buffer (a and c) or buffer supplemented with 300 mM NaCl (b and d). Radioactivity, which was measured in phospholipid spots by phosphoimaging, is expressed in arbitrary units (AU, a and b); alternatively, radioactivity levels were expressed as fraction of the total labelled phospholipids (c and d).

(Fig. 6a, 6b). Clearly, the declining trend in NaCl-induced $^{32}$P-LPA was the opposite of the increase in $^{32}$P-PE, again suggesting that PLD activity could not be responsible for the bulk of $^{32}$P-LPA accumulation. Moreover, non-stimulated $^{32}$P-LPA levels, presumably reflecting *de novo* LPA synthesis, kept on increasing in radioactivity over the entire sampling period (Fig. 6a), which again is in contrast with the decline in the salt-stimulated $^{32}$P-LPA response. This result argues against a major contribution of *de novo* synthesis to the $^{32}$P-LPA response and is in agreement with a leading role for DGK in providing PA as substrate to PLA$_{2}$.

The opposite $^{32}$P-labeling trends of LPA and PE are even clearer when the data are compared as percentage of the total $^{32}$P-lipid (Fig. 6c, d).

**Discussion**

In recent years, the rapid accumulation of PA has been shown to be an early hallmark of plant stress responses. Being the product of the stress-inducible activities of PLD and
DGK, but also the precursor in glycerolipid biosynthesis, the study of PA is complex. In Chlamydomonas cells, salt stress activates both PLD and DGK, generating a PA increment of mixed origin. A recent study showed that 32P-LPA also accumulated, which was proposed to be due to PLA2 activity on PA [3]. The present work used a combination of fatty acid analysis and differential radiolabelling techniques to provide evidence for a pathway connecting PLC/DGK signalling to the LPA response. A model is presented that maps out different metabolic PA and LPA pools and their precursors, and shows where the NaCl-induced LPA response fits in (Fig. 7).

LPA accumulates under salt stress due to PLA2 activity

The enzymes GPAT and LPAAT are responsible for de novo synthesis of LPA and PA, respectively (Fig. 1). This route prevails at basal conditions, but its activity might be induced under stress conditions, similar to observations of de novo synthesized DAG in hypo-osmotically stressed Dunaliella salina [17]. However, different lines of evidence indicate that the salt-induced LPA response in Chlamydomonas is not a consequence of stimulated GPAT activity. First, the α-LA-rich LPA under salt stress is clearly different.
from *de novo* synthesized LPA molecular species (Fig. 3b, 3d, 7). Second, under basal conditions, $^{32}$P-radiolabelling of LPA occurs slowly, reaching equilibrium after days, whereas salt-stimulated LPA showed a contrary labelling trend (Fig. 6). As a product of enhanced GPAT activity, LPA would display the slow equilibration kinetics of its precursors, which is not the case.

Similarly we have presented several lines of evidence to suggest that the LPA increase is due to a PA-specific PLA$_2$ activity. The increase in LPA concurs with the rise in PA, but the latter sets in a bit earlier ([3]; Fig. 5a). Moreover, LPA’s rapid labelling kinetics (Fig. 5) and peculiar fatty acid composition (Fig. 3, 4) are consistent with its origin from a specific subpool of PA. In addition, no other lysophospholipids were found. These results are also in agreement with our previous study, showing that pharmacological inhibition of PLA$_2$ abrogated the LPA response [3]. This treatment simultaneously increased the $^{32}$P-level of PA and its metabolite DGPP, suggesting that PA kinase (PAK) and PLA$_2$ compete for the same PA pool (Fig. 1).

**Salt-stress-induced PLA$_2$ hydrolyses a subpool of PA that is generated via the PLC-DGK route**

As summarized in Fig. 1 and 7, several potential pathways could generate PA during salt stress. *De novo* synthesis of PA implicates LPA as precursor, while the PLC/DGK and PLD pathways may provide PA as substrate to PLA$_2$, generating LPA as product. Previous studies have implicated the PLC/DGK and PLD pathways in the salt response in *Chlamydomonas* ([1, 2]; Chapter 4). PLD activity was probed using its transphosphatidylation activity: in the presence of a primary alcolhol, such as n-butanol, the alcohol can serve as acceptor of the phosphatidyl moiety, generating phosphatidylbutanol (PBut). PBut accumulation thus reflects PLD activity, and its fatty acid composition matches that of PLD’s substrate and its normal product, PA. Thus, we previously could infer from the PBut fatty acid composition that salt-induced PLD activity does not produce $\alpha$-LA-containing PA [1], which is in agreement with our present conclusion that PLA$_2$‘s substrate is provided by another pathway (Fig. 7). NaCl-stimulated LPA species contain several fatty acids which are characteristic of PIP$_2$, PIP and PI: $\alpha$-LA, 18:1n7 and 18:2n6 (Fig. 7). Each one is present at the sn-1 position of PI (Table 1), suggesting that sequential PIP and/or PIP$_2$ hydrolysis by PLC and subsequent phosphorylation of DAG could provide the PA substrate to PLA$_2$ (Fig. 7). This was further supported by our $^{32}$P-radiolabelling experiments. Under pulse-labelling conditions, which favour labelling of the products of DGK, salt induced rapid and large $^{32}$P-PA- and $^{32}$P-LPA increments. Conversely, under conditions, which favour label incorporation into PLD’s substrate, $^{32}$P-LPA levels declined, likely following the decreased specific radioactivity of ATP. Interestingly, hyperosmotic stress in *Arabidopsis* seedlings induced the formation of DAG and PA species with a high 18:2n6 and 18:3n3
content, which was speculated to reflect a PLC/DGK pathway, drawing on a PPI pool with increased PUFA levels [18].

The finding that pharmacological inhibition of PLA$_2$ not only decreases salt-induced LPA formation in *Chlamydomonas*, but simultaneously increases the levels of PA and DGPP [3], has interesting implications, as it suggests that PLA$_2$ and PAK compete for the same PA substrate pool. According to the present data, this is the PA pool generated through the PLC/DGK pathway. As DGK and PAK are predicted to be mainly localized at the plasma membrane ([8, 19]; Chapter 2), PLA$_2$ might be active there as well. Like PAK, it might represent a pathway to attenuate PA signalling. Alternatively, LPA has signalling functions itself.

**LPA signalling**

In animal systems, LPA is an intercellular signalling molecule that is ligand for G-protein coupled EDG receptors [20]. However, these receptors are missing from the *Chlamydomonas* and *Arabidopsis* genomes (unpublished), and in a unicellular alga it seems unlikely that LPA is secreted and sensed by other cells.

From *Arabidopsis*, a putative PA-PLA$_1$ gene was cloned, *SRG1*, of which the KO mutant displayed a reduced gravitropic response [21]. In rat testis, a PA-preferring PLA$_2$ activity has been found, which also hydrolyzes lysobisphosphatidic acid [22]. Recently, putative targets for PA have been picked up in a proteomics screen using PA-affinity beads [23]. It will be interesting to test LPA as a competitor in those assays as some of the proteins may be LPA targets.

A novel *Arabidopsis* PLA$_2$, encoded by *SOBER1*, with no homology to any of the plant PLA families, was suggested to suppress the elicitor-induced induction of a hypersensitive response by reducing the accumulation of PA [24]. Based on *in vitro* activity of recombinant SOBER1, its substrate was speculated to be PC rather than PA.

**Salt stress-responses in Chlamydomonas and other green algal systems**

An LPA response has also been reported in hyperosmotically stressed *Dunaliella salina* [25]. Interestingly, in this system, also $^{32}$P-lyso-PC increased while $^{32}$P-PA decreased. Such differences may be related to the fact that *D. salina* lives in saline environments and is extremely salt-tolerant. Therefore, phospholipid changes were only found when salt concentrations went up from 1.7 to 3.4 M NaCl, which may induce effects of NaCl toxicity, disturbed ionic balance and/or membrane destabilization. In *D. salina*, such stress leads to a cell volume decrease, plasma membrane infolding and shrinking of nuclear and plastidial membranes [25]. In contrast, hypo-osmotic stress was found to increase the level of PA in *D. salina*, and both PLC/DGK and *de novo* synthesis pathways were implicated in the response [17, 26].
In another unicellular green alga, *Micrasterias denticulata*, salt stress has been shown to induce all kinds of morphological changes, such as cytoplasmic vacuolization, deformation of mitochondria and ultrastructural changes in ER and Golgi [27]. After only 5 min of stress treatment a rapid accumulation of reactive oxygen species (ROS) was registered, and after prolonged salt stress, several symptoms of programmed cell death (PCD) became manifest. Interestingly, PLC and PLD have been implicated in PCD in suspension-cultured tomato and rice cells [28, 29], and phosphoinositide-dependent kinase 1 (PDK1), which mediates responses to ROS [30], has been identified as a target of PA [31].

With respect to osmoregulation, *Chlamydomonas* has a unique mechanism based on the function of its contractile vacuole, which has been implicated in the elimination of water in a low osmotic potential environment [32]; however, its function in hyperosmotic stress has yet to be evaluated.

**Possible functions of free fatty acids and their metabolites**

In *Chlamydomonas*, the major molecular species of PI that have 18:3, are 18:3/16:0 and 18:3/18:3 ([1]; Chapter 4, Fig. 7). Upon PLA$_2$ hydrolysis this would generate free palmitic acid and $\alpha$-LA. Free fatty acids and their metabolites may have functions in the regulation of enzymes, such as PLD$,\delta$, whose activity is enhanced in the presence of oleate [33]. Moreover, $\alpha$-LA can be metabolized to octadecanoids such as oxophytodienoic acid and jasmonic acid, which acts as growth factor and modulator of stress resistance, in particular during wounding and pathogen infection in land plants [34]. Previously, JA accumulation has been directly linked to PLD$\alpha$1-derived PA production during the wounding response [35], however, this could not be reproduced by Bargmann et al. [36]. Alternatively, two chloroplast-localized galactolipases/PLAs, AtDAD1 and AtDGL1, were found to be responsible for the wounding-induced accumulation of JA, whereas PLD$\alpha$1 was required for their transcriptional upregulation [37].

In an evolutionary distant diatom, *Thalassiosira rotula*, wounding has been reported to trigger PLA$_2$ activity and to release C20 polyunsaturated fatty acids within minutes. These fatty acids were further metabolized to the defensive aldehydes 2,4-decadienal and 2,4,7-decatrienal [38]. In *Arabidopsis* leaves, wounding induced the formation of hexanal through a lipid hydrolyzing activity releasing $\alpha$-LA as its precursor [39]. Apart from the role of these aldehydes in toxic defense against grazers, they have been speculated to be chemical signals of unfavourable growth conditions, inducing programmed cell death (PCD) within phytoplankton communities [4].

**Biophysical aspects of PLA$_2$-mediated LPA formation**

Due to an intramolecular hydrogen bond between the headgroup of LPA and the hydroxyl on the glycerol backbone, LPA carries more negative charge than PA [40]. Moreover, at neutral pH, LPA has the shape of an inverted cone, whereas PA has a cone
shape, which is even more pronounced if it contains PUFAs like \( \alpha \)-LA [41]. Hence, the interconversion of PA and LPA by PLA\(_2\) and LPAAT may affect local membrane charge and curvature depending on the membrane environment. Both effects may contribute to the physiological significance of the mammalian LPAATs CtBP/BARS [42] and endophilin 1 [43] in vesicle formation. The response to hyperosmotic stress in *Chlamydomonas* may require membrane surface reduction by endocytosis to adapt to the decreased cell volume.

The question of where the different pools of PA and LPA, characterized in this study (Fig. 7), are located in the cell will be important. Lipid biosensors for PA and LPA may yield valuable information as to where and when, PA and LPA are formed [44].

**Acknowledgement**

We thank Harold Meijer (Wageningen University) for his contribution to the \(^{32}\text{P}\)-labelling experiments.
References


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