A re-examination *in vivo* of the phosphatidylcholine–galactolipid metabolic relationship during plant lipid biosynthesis

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It remains unclear how and in what form the lipids synthesized in plant endoplasmic reticulum are exported to chloroplasts and used as precursors for the biosynthesis of plastid galactolipids, which are the most abundant lipids on Earth. Neither the mechanism of transfer nor the nature of the lipids imported into plastids has been elucidated. To characterize events occurring *in vivo*, the labelling of lipids from 15-day-old leek seedlings (*Allium porrum*, var. *furor*) was studied using pulse–chase experiments. During the chase, a substantial decline in the radioactivity incorporated into phosphatidylcholine (and not in other phospholipids) was accompanied by an increase in the label found in galactolipids. The positional distribution of labelled fatty acids in phosphatidylcholine and galactolipids was further studied as a function of the chase time; whereas phosphatidylcholine was preferentially labelled at the sn-2 position, the increase in radioactivity in galactolipids mainly concerned the sn-1 position. These results strongly suggest that the diacylglycerol moiety of phosphatidylcholine might not be integrated as a whole in the galactolipid.

**INTRODUCTION**

Plant leaf lipids typically contain high levels of trienoic fatty acids, with α-linolenic acid (18:3; C₁₈,₃) or a combination of linolenic and hexadecatrienoic acids (16:3; C₁₆,₃). Leaf lipids are synthesized by two distinct pathways: the ‘eukaryotic’ pathway takes place in the endoplasmic reticulum (ER), whereas the ‘prokaryotic’ pathway is confined to plastids (see [1] for review). Because of the specificity of plant acyltransferases [2,3], it is likely that leaf lipids containing 16:3 fatty acids are exclusively synthesized via the prokaryotic pathway.

In ‘18:3’ plants, both pathways contribute to the synthesis of galactolipids, which are the most abundant chloroplast lipids. Therefore, in these plants, 16:3 fatty acids are found to be present in galactolipids and it has been reported that 16:3 account for around 20% of the fatty acids esterified to mono-galactosyldiacylglycerol (MGD) in several 16:3 plants [4].

In ‘16:3’ plants, phosphatidylglycerol (PG) is the only major product of the prokaryotic pathway. Other lipids are entirely synthesized by the eukaryotic pathway and therefore do not contain 16:3 fatty acids. In these plants, chloroplast lipids (except PG) are synthesized from phosphatidylcholine (PC) molecules first formed in ER membranes (see [1] for a review).

The precursor–product relationship between PC and galactolipids was first described by Slack et al. [5]. After pulse-labelling of maize leaf lipids with [3H]glycerol and [14C]acetate, they observed a decrease in the label found in PC, and a concomitant increase in the radioactivity associated with MGD during the chase. In these experiments, the ratio of the radioactivities incorporated from [3H]glycerol and [14C]acetate into each lipid did not change significantly. Since PC is exclusively synthesized in the extraplasmoidal compartment, it was hypothesized that PC was transferred, as a whole, from the ER to plastids by a protein-mediated process (see [6] for review). This hypothesis took into account the presence of PC in the plastid envelope (although this membrane is unable to synthesize this lipid from diacylglycerol and CDP-choline [7,8]) and the existence of lipid-transfer proteins able to catalyse the transfer of phospholipids between membranes *in vitro* (see [6] for review). Besides this hypothesis, which has recently been revisited [6,9–11], other possibilities are now under investigation [12].

The aim of the present study is to characterize events involved *in vivo* in the precursor–product relationship between PC and galactolipids. We therefore labelled plant lipids with [1-13C]acetate (and [2,3-3H]glycerol) and then carried out a chase for over 100 h during which the label variations in phospholipids and galactolipids were studied. Special attention was paid to the evolution of the label of the acyl moieties esterified to positions sn-1 and sn-2 of the glycerol backbone of PC and galactolipids during the chase.

**EXPERIMENTAL**

**Materials**

TLC plates were high-performance TLC (HPTLC) silica-gel 60 plates (Merck 60 F254). Autoradiography was performed using hyperfilm MP (multipurpose) (Amersham). Sodium [1-13C]acetate (53.9 Ci/mol) and [2,3-3H]glycerol (200 Ci/mol) were obtained from CEA (Saclay, France) and Dupont–NEN (Les Ullis, France) respectively. Lipases and all other reagents were from Sigma Chemical Co. (St Louis, MO, U.S.A.).

**Plant materials and pulse–chase labelling of leek seedlings**

Leek (*Allium porrum*, var. *furor*) seeds, stored overnight at 4 °C, were sterilized with sodium hypochlorite in the presence of 0.5% Triton X-100 for 2 min and then washed with distilled water. They were then grown for 2 weeks at room temperature in small vials (approx. 8–10 seeds per vial) on a previously described growth medium [13].

Sodium [1-13C]acetate (200 µCi) or [2,3-3H]glycerol (200 µCi) was supplied for 2 h. Seedlings were then rinsed eight times with deionized water. The chase was carried out by adding 1 ml of

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*Abbreviations used:* DGD, digalactosyl diacylglycerol; ER, endoplasmic reticulum; HPTLC, high-performance TLC; lyso-PC, 1-acylglycerol-phosphocholine (lysophosphatidylcholine); MGD, monogalactosyldiacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol.

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0.46 M sodium acetate, pH 5.5, or 16 mM glycerol, for 2 h. Seedlings were then rinsed eight times with deionized water.

Analysis of labelled lipids
At various times, green tissues (approx. 0.1 g) were harvested, weighed and ground in a glass–glass tissue grinder with 6 ml of chloroform/methanol/formic acid (10:10:1, by vol.). The homogenate was transferred to a screw-capped centrifuge tube and stored overnight at −20 °C. The extraction procedure was continued by adding 2.2 ml of chloroform/methanol/water (5:5:1, by vol.). The organic phase was washed with 3 ml of 0.2 M H3PO4/1 M KCl [14,15]. Lipids were recovered in the chloroform phase, dried and redissolved in 1 ml of chloroform/methanol (2:1, v/v). An aliquot of the lipid extract was evaporated in a scintillation vial, and radioactivity was determined by liquid-scintillation counting.

Individual lipids were purified from the extracts by monodimensional TLC using the solvent system described by Vitiello and Zanetta [16]. Lipids were then located by spraying the plates with a solution of 0.001 % primuline in 80 % acetone, followed by visualization under UV light. After autoradiography, the silica gel zones corresponding to individual lipids were scraped from the plates and the radioactivity associated with the lipids was determined by liquid-scintillation counting.

Lipase digestion
The radioactivity associated with fatty acids at the sn-1 and sn-2 positions of PC, MGD and digalactosyl diacylglycerol (DGD) was determined by lipase digestion. After TLC, lipid spots were scraped off the plates and sonicated (15 min) in 100 μl of 10 mM Tris/HCl at pH 7.5. Reactions were started by the addition of 0.2 units of phospholipase A2 and 8000 units of Rhizopus arrhizus delemar lipase to PC and galactolipids respectively. Incubations were carried out for 15 min (MGD, PC) or 30 min (DGD) at 37 °C.

After incubation, 2 ml of chloroform/methanol (2:1) were added in order to stop reactions and to start lipid extraction. The organic phase was re-extracted in chloroform and the resulting organic phases were combined, dried and redissolved in a minimal volume of chloroform/methanol (2:1). Lipids were resolved by TLC as described above. After autoradiography, the silica gel zones corresponding to lyso lipids and non-esterified fatty acids were scraped from the plates and the radioactivity was determined by liquid-scintillation counting. At each time and for each lipid, three lipase digestions were carried out.

The specificity of phospholipase A2 was checked by hydrolysing 1-palmitoyl-2-[3H]Clinoeyl-PC and di[14C]oleoyl-PC. After hydrolysis of 1-palmitoyl-2-[3H]Clinoeyl-PC, 97 % of the radioactivity was associated with non-esterified fatty acids and 3 % with 1-acyl glycerophosphorylcholine (lyso-PC). When di[14C]oleoyl-PC was treated with phospholipase A2, 48 % was found in lyso-PC and 52 % in non-esterified fatty acids.

We further checked that under the conditions of the study the Rhizopus lipase specifically hydrolysed fatty acids esterified to the sn-1 position of galactolipids [17]. Taking advantage of the fact that 85–100 % of C16 fatty acids esterified to galactolipids from 18:3 plants were found to be associated with the sn-1 position [17,18], we checked the specificity of the Rhizopus lipase by determining the distribution of palmitate in non-esterified fatty acids and lyso-galactolipids obtained upon treatment of leek MGD and DGD with this enzyme. Under our conditions, 90 % of C16 fatty acids were present in the non-esterified fatty acid fraction and 10 % in lyso-galactolipids.

Nevertheless, 2-acylglycerogalactolipid obtained by lipase digestion could tend to racemize, thus giving rise to complete hydrolysis of some galactolipids. To determine the extent of this hydrolysis, labelled [3H]glycerol MGD and [3H]glycerol DGD were biosynthesized by incubating seedlings with [3H]glycerol, and further purified by HPTLC. The effect of Rhizopus lipase (1 μl) was checked on MGD (five assays) and DGD (five assays). In control experiments (MGD, five assays; DGD, five assays), lipase was replaced by 1 μl of water. After incubation, lipids were extracted, separated by HPTLC and their label was determined.

The lipase hydrolysed 54.2 ± 4.8 % of [3H]MGD and 63 ± 4.8 % of [3H]DGD (no hydrolysis of galactolipids was observed in the control experiments). After incubation, the sum of the label found in lyso-galactolipids and non-hydrolysed galactolipids extracted from assays containing lipase (3018 ± 292 d.p.m. and 1328 ± 185 d.p.m. for MGD and DGD respectively) did not significantly differ from the label found in the lipid extracted from assays carried out in the absence of Rhizopus lipase (3259 ± 312 and 1265 ± 245 d.p.m. respectively). Hence, no significant amount of lyso product was further hydrolysed during the experiments. These results confirm previous data obtained by Fisher et al. [17], who showed that after a 20 min incubation, fewer than 2.5 % of fatty acids associated with the sn-2 position of galactolipids were hydrolysed by Rhizopus lipase.

Analysis of fatty acids from leek seedlings
Whole lipid extracts were obtained, as described above in the Analysis of labelled lipids section, from unlabelled seedlings. Fatty acid methyl esters were obtained as described by Miquel and Browse [15] and analysed by gas chromatography on a 15 m × 0.53 mm Carbowax column (Altech, Deerfield, IL, U.S.A.). The gas chromatograph was programmed for an initial temperature of 160 °C for 1 min, followed by a 20 °C/min ramp to 190 °C and a secondary ramp of 5 °C/min to 210 °C. This temperature was maintained for 1 min.

RESULTS
The leek is an ‘18:3’ plant
To study the precursor–product relationship between PC and galactolipids in plant cells, we chose to avoid any prokaryotic synthesis of galactolipids during our experiments; i.e. we chose to study lipid metabolism using an 18:3 plant. Because our previous in vitro experiments concerning the transfer of lipids between ER and plastids were carried out using subfractions of A. porrum cells [12], we first checked whether leek, like most monocotyledons, is a 18:3 plant.

Table 1 gives the fatty acid composition of the lipids extracted.

Table 1 Fatty acid composition of total lipids, PC and galactolipids from leek seedlings

<table>
<thead>
<tr>
<th>Fatty acid (mol%)</th>
<th>Total lipids</th>
<th>PC</th>
<th>MGD</th>
<th>DGD</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>22.0</td>
<td>22.3</td>
<td>3.0</td>
<td>13.3</td>
</tr>
<tr>
<td>16:1</td>
<td>1.0</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>16:2</td>
<td>nd</td>
<td>nd</td>
<td>1.8</td>
<td>2.1</td>
</tr>
<tr>
<td>16:3</td>
<td>nd</td>
<td>1.8</td>
<td>0.9</td>
<td>2.1</td>
</tr>
<tr>
<td>18:0</td>
<td>3.6</td>
<td>nd</td>
<td>nd</td>
<td>2.0</td>
</tr>
<tr>
<td>18:1</td>
<td>3.7</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
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<tr>
<td>nd</td>
<td>72.2</td>
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Abbreviation: nd, not detected.
Lipid metabolism was further studied by supplying [1-14C]acetate and [2,3-3H]glycerol. As expected, in the case of PC and galactolipids convincingly show that the backbone of PC, MGD and DGD. Interestingly, these results are similar to those obtained by Kunst et al. [22] using an Arabidopsis thaliana mutant (act 1) deficient in glyceral-3-phosphate activity. Hence, the results shown in Figure 1(a) indicate, to our knowledge for the first time, that the kinetics of lipid metabolism in a 18:3 plant closely resembles those of a 16:3 plant defective in the synthesis of prokaryotic lipids.

When [2,3-3H]glycerol was instead of [1-14C]acetate, similar results were observed: a substantial decline in the radioactivity in PC (from 39 ± 2 % to 22 ± 1 %) was accompanied by an increase (from 4.5 ± 0.3 % to 30 ± 4 %) in the label in galactolipids (Figure 1b). Under the conditions in Figure 1(b), less than 1 % of the label incorporated into lipids was associated with the fatty acids and more than 99 % was associated with the polar head. The results in Figure 1(b) therefore show that the glycerol moiety of PC was incorporated into galactolipids. These results are hence consistent with the precursor–product relationship between PC and ‘eukaryotic’ galactolipids proposed by Slack et al. [5].

In addition, and as described for maize leaves [5], the percentages of radioactivity associated with PC after the pulse were of the same order of magnitude when [1-14C]acetate and [2,3-3H]glycerol were used; and in the same way, whatever the chase time, the percentage of remaining labelled PC did not depend on the labelled substrate used. Because the decrease in PC (expressed as the percentage of radioactivity incorporated into polar lipids) was only compensated for by an increase in galactolipids, this increase did not depend on the labelled substrate used. In other words, irrespective of the chase-time, the ratio of the radioactivities incorporated into PC from [1-14C]acetate and [2,3-3H]glycerol was approximately unity (when the label was expressed as the percentage of radioactivity incorporated), and this ratio was necessarily similar for galactolipids. Therefore, while confirming the PC–galactolipid relationship, these results gave no clue as to the mechanism(s) involved in this metabolism.

A kinetic study in vivo of lipid metabolism in an 18:3 plant

Lipid metabolism was further studied by supplying [1-14C]acetate to 15-day-old A. porrum seedlings for 2 h and by carrying out a chase for 100 h. At various times, lipids from a batch of seedlings were extracted. The total incorporation of the labelled substrate could vary from one batch to another, but by expressing results as the percentage of radioactivity incorporated into lipids (as in many other studies; see for example [21–24]), no significant variation in the results was observed from one experiment to another, irrespective of batches having incorporated the higher or lower amounts of radioactivity. Results are given Figure 1(a).

The labelling kinetics of lipids from leek seedlings showed that the label was primarily contained in PC and that, in contrast to what is observed using 16:3 plants [21], the radioactivity associated with galactolipids after the pulse reached only 9 ± 0.7 % of the label incorporated into polar lipids. As in Arabidopsis [21], label incorporation into phospholipids other than PC remained almost constant during the chase [6 ± 1 %, in phosphatidylinositol (PI) and 30 ± 2 %, in phosphatidylethanolamine (PE) + PG]. The only important changes occurring during the chase were: (1) a substantial decline in the radioactivity incorporated into PC (from 46 ± 0.2 % immediately after the pulse to 25 ± 0.1 % after 100 h); and (2) an increase in the label found in galactolipids (from 6.5 ± 0.5 % to 19 ± 1 %, and from 2.5 ± 0.2 % to 7.5 ± 1 % in MGD and DGD respectively). From phosphatidylcholine to eukaryotic galactolipids: a study in vivo

Figure 1 Redistribution of radioactivity among the polar lipids of leek seedlings following [1-14C]acetate and [2,3-3H]glycerol labelling

Seedlings were supplied at zero time with [1-14C]acetate (a) or with [2,3-3H]glycerol (b). At different chase times, lipids were extracted and separated by TLC as described in the Experimental section. For each lipid, values are expressed as a percentage of the radioactivity incorporated into polar lipids. The error bars, when present, represent the mean values ± S.D. of three analyses; when absent the S.D. is inferior to 0.5 % and values represent the means of three analyses. ○, PG; □, MGD; ◆, DGD; +, PI; △, PG + PE; □, PA; ×, sulfoquinovosyl diacylglycerol.
radioactivity incorporated into polar lipids) chiefly involved the sn-2 position, the radioactivity of which decreased from 35% to 20% of the polar lipid label, and to a lesser extent the sn-1 position (from 14% to 10.5%). This decrease in the radioactivity of the acyl chain esterified to the sn-2 position of PC was prominent during the first 24 h after the chase. During the pulse, the PC label is likely to result from neosynthesis (labelled fatty acids esterified to the sn-1 position of PC and galactolipids during a pulse–chase experiment. Since all the fatty acids released from the sn-2 position of PC during the chase (and mostly during the first 24 h; see Table 2 and Figure 2a) were not re-incorporated into the polar lipid fraction, the total radioactivity incorporated into polar lipids slightly decreased, as already observed with *Arabidopsis* [21]. In our hands the relative labelling of polar lipids decreased by approx. 10–15% during the chase. Hence, for a given amount of labelled molecules remaining constant during the chase, an increase was detected by expressing results as a percentage of the radioactivity incorporated into polar lipids. This increase should not appear when the radioactivity of total lipids (including polar lipids, non-esterified fatty acids and neutral lipids) is taken into account.

We recalculated the results of the present study as a function of the radioactivity associated with the total lipids (instead of polar lipids). As shown in Figure 3 (open symbols), the sum of the radioactivities esterified to the sn-1 position of galactolipids and showed, unexpectedly, that whereas PC was always more labelled in the sn-2 than in the sn-1 position, the increase in the galactolipid label occurred mainly, if not exclusively, at the sn-1 position.
and PC remained constant during the chase; the increase in the label associated with the sn-1 position of galactolipids was exactly compensated for by the decrease observed in labelling at the sn-1 position of PC. The results obtained by using this mode of calculation are not essentially different from the previous ones, but they show that PC sn-1 labelling may be the sole ‘precursor’ of galactolipid sn-1 labelling.

DISCUSSION

The present study provides the first experimental arguments that the diacylglycerol moiety of PC might not be the substrate of galactolipid-forming enzymes. Whereas the overall analysis of the label distribution does not preclude a direct precursor–product relationship between PC and galactolipids, and as such does not contradict former analyses [5,21], the positional distribution of the label almost rules out this possibility because of the very different distribution of the radioactivities at the sn-1 and sn-2 positions of PC and galactolipids during the chase.

As noted in the Introduction, a transfer of PC molecules between the ER and chloroplasts was first suggested by Slack et al. [5], who showed that during the chase the [3H]glycerol/[14C]fatty acid ratio is the same in PC as in galactolipids and does not vary. These results could be taken as indicative of the incorporation of the diacylglycerol moiety of PC as a whole into galactolipids during the chase. Nevertheless, the expression of the data as a percentage of polar lipid radioactivity could be misleading and does not make it possible to draw clear-cut conclusions about the precursor–product relationship between PC and galactolipids. From the results obtained by Slack et al. [5] and expressed as d.p.m./g of fresh weight of lamina, we calculated that the decrease in labelled glycerol content in PC between 20 and 27 h of chase was compensated for by a similar increase in galactolipids (41 × 10^4 and 50 × 10^4 d.p.m. respectively), but that only half of the labelled fatty acids released from PC were recovered into galactolipids (135 × 10^3 and 68 × 10^3 d.p.m. respectively). Of course, the results given in Table 2 and Figure 2 could tentatively be explained by a mere hydrolysis of the fatty acids esterified to the sn-2 position of PC, followed by a subsequent and specific acylation to the sn-1 position of galactolipids. This hypothesis, however, can be ruled out for at least two reasons:

1. The glycerol backbone of PC was incorporated into galactolipids during the chase (Figure 1b); (2) labelled fatty acids were not exclusively incorporated at the sn-1 position of galactolipids during the pulse: at the beginning of the chase 9 ± 3 % (n = 6) of the total radioactivity of the polar lipids was found in fatty acids esterified to the sn-1 position and 8 ± 4 % (n = 6) to the sn-2 position of the galactolipids.

To explain how sn-1-labelled products were synthesized from sn-1- and sn-2-labelled substrates, the general scheme must then include an additional event between PC and galactolipid, irrespective at this stage of the analysis of the compartmentalization of PC and galactolipids. For example, it could be suggested that the transacylation by unlabelled fatty acids occurring during the chase (see the Results section) produced some PC molecules labelled exclusively in the sn-1 position, and that for unknown reasons, these ‘neo-transacylated’ PC molecules were the only ones which could be transferred (by an unknown mechanism) to the plastids. But one can also imagine that the acylation by unlabelled fatty acids occurred not before, but after, the transfer of molecules to the chloroplasts. We recently proposed that the transfer of molecules between the ER and chloroplasts could involve a partial hydrolysis of PC molecules in the ER, a partition of the resulting lyso-PC between ER membranes, cytosol and the chloroplast envelope, followed by acylation of lyso-PC by a plastidial lyso-PC acyltransferase. We demonstrated the presence of such an enzyme in the envelope of chloroplasts from leek seedlings and we showed that in the presence of ER membranes from A. porrum seedlings containing labelled lyso-PC, chloroplasts were able, in vitro, to synthesize labelled PC [12]. The results of the present study are in good agreement with this hypothesis: after its synthesis, the labelled PC present in ER membranes could be hydrolysed and the resulting lyso-PC molecules could be ‘exported’ to the chloroplast, then acylated by (chiefly) unlabelled fatty acids produced by plastids during the chase. The resulting molecules, labelled mainly in the sn-1 position could then be used subsequently for the synthesis of galactolipids.

The ‘lyso-PC pathway’, as the other hypothesis mentioned above, could also explain why in the act 1 mutant [22] the block in the prokaryotic pathway is compensated for by an increased flux through the eukaryotic one, and why in tomato cell cultures [25], while both 1-O-octadecenoyl and 2-O-octadecenoyl glycerol can be used as substrates for PC synthesis, only PC molecules which are hydrolysable in sn-2 (i.e. PC synthesized from 1-O-octadecenoyl glycerol) are used as precursors for galactolipid synthesis (see [12] for discussion). Moreover, this hypothesis and the results of the present study may explain why in Arabidopsis and in maize the label in C_18 fatty acids esterified to PC increases during the chase from 13% to 21.4% and from 6.1% to 13.3% respectively [5,21]. Since palmitate is almost exclusively esterified to the sn-1 position of PC, this increase could simply reflect an increase in the percentage of the label found at the sn-1 position, due to hydrolysis of labelled fatty acids esterified to the sn-2 position.

In addition, because in plant cells ER membranes desaturate oleic acid esterified to PC [26,27], and because desaturation intimately involves the exchange of C_18 acids at the sn-2 position of PC, fatty acids esterified to the sn-2 position of PC could be desaturated before being hydrolysed. This mechanism could be an efficient way for the endomembranous system to provide, at the same time, linoleic acid to the extraplastidial membranes and lipids for plastid biogenesis. This link between desaturation and lyso-PC synthesis in the ER remains hypothetical; however, it must be pointed out that in an Arabidopsis mutant deficient in the activity of a microsomal oleoyl phosphatidylcholine de-
The 18:1-containing lipids accumulated in the ER could be not efficiently transferred to chloroplasts [15]. Together with the data of other studies described above, the present results suggest that in vivo, as in vitro [12], lyso-PC rather than PC could be transferred from the ER to chloroplasts. Interestingly, it has not been demonstrated that PC present in the chloroplast envelope is the precursor of galactolipids. Hence, lyso-PC could be the common precursor of PC and galactolipids, without any precursor–product relationship between these chloroplast lipids. This important point for the understanding of the lipid metabolism in plant cells will now be investigated in our laboratory.

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