Last step in the cooperative pathway for galactolipid synthesis in spinach leaves: formation of monogalactosyldiacylglycerol with C18 polyunsaturated acyl groups at both carbon atoms of the glycerol

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In this paper, the formation of monogalactosyldiacylglycerol (MGDG) with C18 polyunsaturated acyl groups at both positions of the glycerol from phosphatidylcholine (PC) of liposomes was demonstrated by a three-step procedure: spinach chloroplasts were first incubated with liposomes of PC-containing polyunsaturated fatty acids at both positions of the glycerol in the presence of phospholipid transfer protein. Chloroplasts were then incubated with phospholipase C in order to form 1,2-diacylglycerol (1,2-DAG) from PC integrated in the chloroplast envelope. Finally, washed chloroplasts were incubated with UDP-gal and MGDG was formed by galactosylation of 1,2-DAG.

Phosphatidylcholine; Diacylglycerol; Galactolipid synthesis; Chloroplast; (Spinach)

1. INTRODUCTION

It now appears clear that two pathways operate

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Abbreviations: C18/C16, C18 at C1, C16 at C2 molecular species of glycerolipids; C18/C18, C18 at C1, C18 at C2 molecular species of glycerolipids; CDP-choline, cytidine diphosphocholine; DGDG, digalactosyldiacylglycerol; FFA, free fatty acids; GLC, gas-liquid chromatography; HPLC, high-performance liquid chromatography; DAG, diacylglycerol; MGDG, monogalactosyldiacylglycerol; PC, phosphatidylcholine; PL, polar lipids; PLTP, phospholipid transfer protein; TLC, thin-layer chromatography; UDP-gal, uridine diphosphogalactose

at the same time for galactolipid biosynthesis in leaves of higher plants [1–3]. An intra-plastidial pathway produces the C18/C16 galactolipids: this is a minor pathway in the leaves of species devoid of hexadecatrienoic acid where most of the galactolipids are C18/C18 species, but in plants containing hexadecatrienoic acid, such as spinach, tomato and tobacco [4], it can be significant because about 20% and more of galactolipids are C18/C16 species. This pathway, often called the 'procaryotic pathway' by analogy with blue-green algae where the same acyl specificity is found [5], implies the existence of 'plastidial desaturases' leading to the formation of di- and trienoic acids. The intra-chloroplastic localization of these desaturases remains unknown. Recent work [6] has shown that 'genetic' mutation in *Arabidopsis thaliana* could affect this intraplastidial pathway.

In leaves of all higher plants, the major pathway

is the 'cooperative' one which is responsible for the formation of C18/C18 species of galactolipids [7]. Studies carried out in vivo and in vitro concur for the following scheme: oleic acid formed by the chloroplast is exported and integrated into PC at the 'microsomal' level [8]. Here, C18:1/C18:1 PC can be converted into the C18:2/C18:2 species by microsomal desaturases [9–12]. The localization of the last desaturase giving C18:3 lipid species remains to be determined; evidence has been presented by Browse and Slack [13] and Dubacq et al. [3] that this activity is linked to the endoplasmic reticulum. Nevertheless, other experiments also strongly suggest a chloroplastic localization for this desaturase [14,15]. However, one of the most important steps in the cooperative pathway concerns the return of PC from the microsomes to the chloroplast and the possible function of this PC as a C18/C18 diacylglycerol donor for subsequent galactosylation. This return was demonstrated to be catalyzed by a PLTP [16] isolated from spinach leaves [17]. The conversion of PC so integrated in the chloroplast envelope into MGDG has been demonstrated by Ohnishi and Yamada [18] and Dubacq et al. [19]. However, in every case, the conversion remained weak (only a few percent of the chloroplastic PC). On the other hand, in a complex mixture including chloroplasts, microsomes, PTLP and radioactive acetate, Dubacq's group has observed the formation of C18/C18 species of MGDG in the chloroplasts. Recently Heemskerk [20] has demonstrated a good conversion of PC into MGDG by isolated envelopes sonicated in the presence of PC liposomes and phospholipase C.

We have now succeeded in converting PC into C18/C18 MGDG and DGDG by temporally separating the three steps of the process. First, PC was targetted to the chloroplast envelope by mixing intact chloroplasts with PC liposomes in the presence of PLTP; then the chloroplasts were pelleted and incubated with phospholipase C and finally, the chloroplasts were repelleted and incubated with UDPgal.

2. MATERIALS AND METHODS

2.1. Plant materials

Spinach (*Spinacia oleracea* var. Monstrueux de Viroflay) was grown at 22°C over a 9 h photoperiod. 1-month-old spinach was used to obtain chloroplasts.

Vigna sinensis L. (var. M 53) was grown for 3 days in continuous light at 27°C.

2.2. Chloroplast preparation and incubation

Intact chloroplasts were isolated using the Percoll gradient technique of Mills and Joy [21] starting with about 100 g leaves. The chloroplast pellet was resuspended in 1 ml of the incubation medium used by Roughan et al. [22] to a chlorophyll concentration of 4.0 mg/ml. Under standard conditions, chloroplasts (100–125 μg chlorophyll) were incubated in the medium described by Roughan et al. [22] with or without PLTP (100–120 μg according to the experiments), with [^{14}C]PC liposomes (100 nmol) and UDP-gal (2.5 μmol); the final volume was adjusted to 500 μl and the incubations were performed for 30 min at 25°C in an illuminated Warburg apparatus shaken at 100 strokes/min. In experiments where phospholipase C or cytoplasmic supernatant was used, incubations were performed in three steps: (i) Chloroplasts were incubated for 20 min with [^{14}C]PC liposomes with or without PLTP. (ii) After 2 min centrifugation at 2500 × g chloroplast pellets were resuspended and incubated for 20 min with or without concentrated cytoplasmic supernatant or phospholipase C (0.2–0.6 units phospholipase C from *Bacillus cereus*, grade II from Boehringer). (iii) After centrifugation, in order to remove phospholipase C, the chloroplast pellets were resuspended and incubated again for 20 min with UDP-gal.

After these three sequential incubations, analyses were performed of either all the incubation media, or the chloroplast pellet and the supernatant separated by centrifugation at 2000 × g for 5 min or intact chloroplasts purified by centrifugation through a Percoll gradient according to Mills and Joy [21].

Chlorophyll was estimated by the method of Arnon [23].

PLTP was prepared according to Kader et al. [24] and its activity first tested in the system described by these authors [24].

Liposomes were prepared by mixing radioactive PC isolated from *Vigna* leaves (100 nmol with an average specific activity of 38 MBq/mmol) with 430 nmol soya PC. After evaporation of the sol-

vent, 2.5 ml of the incubation medium were added and the suspension sonicated for 30 min at 15°C under a nitrogen stream.

Concentrated protoplasmic supernatant was prepared by concentrating through dialysis the supernatant obtained after $100000 \times g$ centrifugation for 60 min of a spinach leaf homogenate in the same medium as for chloroplast preparations.

2.3. Lipid analysis

Lipids were extracted according to Bligh and Dyer [25]. Radioactivity in total lipid extracts was measured by scintillation counting of an aliquot corrected for quenching. Separation of lipid classes was performed on silica gel plates using either petroleum ether-diethyl ether-acetic acid (70:30:0.4, by vol.) [26] for analysis of neutral lipids or chloroform-acetone-methanol-acetic acid-water (50:20:10:10:5, by vol.) [27] for analysis of polar lipids.

Radioactive lipids were detected by autoradiography of the plate; then each lipid was scraped off the plate and counted in Permafluor 1. For analysis of radioactivity in the acyl chains, either the total lipid extract or the lipids isolated from the plate were trans-methylated according to Carreau and Dubacq [28]. Methyl esters were analysed by capillary GLC and separated by TLC on silver nitrate-impregnated plates in the solvent proposed by Mangold [26]. Radioactive fatty acids were detected by autoradiography of the plate and counted by liquid scintillation. The analysis of the labelling distribution in fatty acids at each position of the glycerol molecules in PC, DAG and MGDG was performed with the lipase from *Rhizopus arrhizus*, according to Fischer et al. [29]. The resulting products were analysed by TLC ac-

Table 1

Distribution of radioactivity in fatty acids of PC of liposomes, PC transported into the chloroplast and 1,2-DAG

	16:0	18:0	18:1	18:2	18:3
PC-liposomes	18.5	tr.	5	47.0	29.5
PC-chloroplast	17.5	tr.	tr.	52.0	30.0
DAG-chloroplast	25.0	tr.	tr.	46.5	28.5

Results are expressed as the percentage of total radioactivity in fatty acids. tr., trace

cording to Lepage [27]; lysogalactolipids or lysophospholipids and free fatty acids were scraped off the plate and methylated. The distribution of radioactivity was studied by TLC on silver nitrate-impregnated silica gel plates as above.

2.4. Labelling of PC

For labelling PC young *V. sinensis* L. seedlings were used because the very high rate of growth allowed a good labelling of the lipids. Radioactive PC for liposomes was prepared by placing [1-^{14}C]acetate (1.9 GBq/mmol, from CEA, France) droplets on developing *V. sinensis* leaves for 3 days. Then leaves were fixed in boiling water, lipids extracted and PC prepared by HPLC according to Trémolières [30]. The specific activity of PC was determined by counting radioactivity and measuring the mass of the fatty acids by capillary GLC. However, as our PC preparation contains several molecular species and moreover each fatty acid does not have the same specific activity, the value used for expression of the results only represents an average specific activity of 38 MBq/mmol. Nevertheless, we have verified that the distribution of radioactivity amongst the fatty acids was the same in PC liposomes, in the PC transferred to the chloroplast and in diacylglycerols obtained after phospholipase C treatment of the chloroplast (table 1). If most of the radioactivity is found in palmitate, linoleate and linolenate, we can assume that all the main PC species were transported and converted into diacylglycerols at the same rate in our system. Consequently, the results have been expressed either in mol PC transported into the chloroplast, or in mol DAG obtained by lipase digestion, or in mol galactolipids formed by galactosylation of the DAG.

3. RESULTS

3.1. Transfer of PC catalysed by PLTP

Table 2 shows the effect of addition of PLTP on the transfer of PC from liposomes to the chloroplast as measured by incorporation of radioactive PC into the crude chloroplast pellet recovered at the end of the incubation. There was a 2–2.5-fold increase in radioactive PC incorporated into the chloroplast pellet when PLTP was added. In contrast, this incorporation decreased in

Table 2

Transfer and metabolisation of PC from PC liposomes to chloroplasts in the presence of PLTP and UDP-gal

		None	+ PLTP	+ PLTP + UDP-gal	+ 2 PLTP + UDP-gal
		(nmol/mg chlorophyll) (%)			
Pellet	PC	158.5 (26.60)	337.4 (66.70)	256.5 (58.20)	299.2 (68.90)
	DAG	5.1 (0.80)	11.8 (2.30)	6.4 (1.45)	7.5 (1.70)
	MGDG	0.38 (0.06)	0.28 (0.05)	0.57 (0.12)	0.57 (0.13)
Supernatant	PC	428.6 (72.00)	151.0 (29.80)	172.7 (39.20)	118.0 (27.20)
	DAG	2.4 (0.40)	5.5 (1.09)	4.6 (1.04)	8.3 (1.90)
	MGDG	0.34 (0.06)	0.31 (0.06)	0.40 (0.09)	0.46 (0.10)
Total		595	506	441	434

Labelled PC liposomes (100 nmol) were incubated for 30 min with spinach chloroplasts (100–125 μg chlorophyll) with or without PLTP (200–400 μg protein) or UDP-gal (2.5 μmol) in a total volume of 500 μl; the results are expressed as nmol/mg chlorophyll, the percentage being given in parentheses

about the same proportion in the supernatant. Addition of PLTP or UDP-gal decreased the total radioactivity found in the incubation medium suggesting an increase in degradation of PC. On the other hand, addition of UDP-gal slightly decreased the transport of PC by PLTP as about 10% less radioactivity was found in the PC of the pellet when UDP-gal was added. Nevertheless, addition of more PLTP slightly increased the transfer of PC towards the chloroplast; the radioactivity of the MGDG was always very low although it was marginally higher when UDP-gal was added. While little radioactivity (fig.1) occurred in 1,2-DAG under our conditions, one might suspect that some cofactors necessary for the conversion of PC into DAG were lacking.

3.2. Conversion of PC into DAG

In order to increase the formation of DAG we first added concentrated cytoplasmic supernatant to the incubation medium. As seen in table 3 the increase in formation of DAG was minor under these conditions and no increase in radioactivity of MGDG was measured. However, if phospholipase C was added to chloroplasts re-isolated after incubation with PLTP, a dramatic increase in formation of radioactive 1,2-DAG was observed, from 0.2 nmol/mg chlorophyll to 136.9 nmol/mg chlorophyll for the highest concentration of phospholipase C. After washing off phospholipase C by centrifugation, if the chloroplasts were re-incubated for 20 min with UDP-gal significant incorporation of radioactivity into MGDG was observed, from 0.58 to 20.4 nmol/mg chlorophyll. As seen in table 3 and fig.1, slight radioactivity was recovered in DGDG (up to 6.3 nmol/mg chlorophyll). Indeed, it was at the lowest phospholipase C concentration that the highest
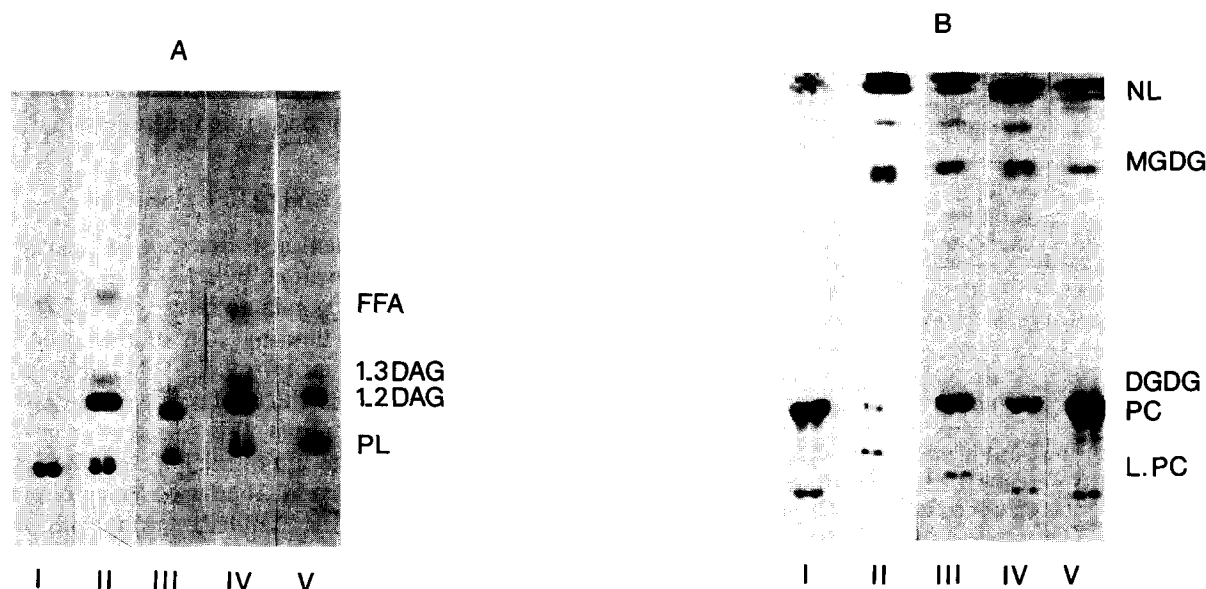
Fig.1. Autoradiography of the total lipids after incubation of chloroplasts with [^{14}C]PC liposomes. Lanes: I, crude chloroplast pellet after incubation without phospholipase C (0.2 U); II, crude chloroplast pellet after incubation with phospholipase C (0.2 U); III, total incubation medium after incubation with phospholipase C (0.2 U); IV, purified chloroplast pellet from lane III; V, supernatant from lane III. Incubations were performed in 3 steps as described in tables 3 and 4. (A) Neutral lipids analysed according to Mangold [26]. (B) Polar lipids analysed according to Lepage [27].

radioactivity was found in MGDG, indicating a probable effect of excessive lipase on chloroplast membrane organization.

After the three successive incubations (the first with PLTP, the second with phospholipase C and the last with UDP-gal), when intact chloroplasts were purified through Percoll, MGDG was found to contain 15% of the total lipid radioactivity in the intact chloroplasts while it represented only 1.5% of that in the supernatant at the top of the gradient. Slight radioactivity was always found in FFA even without phospholipase C treatment (fig.1) but this was too low to account for the formation of MGDG.

Table 3

Effect of phospholipase C addition on PC metabolisation by chloroplasts

	With super-natant	+ PhL C (0.2 U) + UDP-gal + PLTP	+ PhL C (0.4 U) + UDP-gal + PLTP	+ PhL C (0.6 U) + UDP-gal + PLTP
FFA	1.0	12.5	10.9	16.8
DAG	0.9	95.9	102.5	136.9
MGDG	0.58	20.4	12.2	11.3
DGDG	–	4.7	4.2	6.3
PC	82.0	6.67	7.1	4.2

Chloroplasts (100–125 μg chlorophyll) were first incubated for 20 min with PC liposomes and PLTP as in table 2. After pelleting they were incubated for 20 min with phospholipase C (0.2–0.6 U). After repelleting they were incubated for 20 min with UDP-gal (2.5 μmol). Results expressed as nmol/mg chlorophyll

Table 4

Distribution of radioactivity into lipids of total incubation medium, purified chloroplast pellets and supernatant

	Total in-cubation medium	Percoll gradient	
		Pellet	Supernatant
FFA	5.3	4.0	4.8
DAG	80.7	62.0	68.8
MGDG	5.04	14.5	1.5
DGDG	tr.	tr.	tr.
PC	9.0	19.8	24.2

At the end of the third incubation as described in table 3, chloroplasts were purified by centrifugation through a Percoll layer (as described in section 2) and radioactive lipids analysed in the pellet and supernatant. Results are expressed as percent

3.3. Fatty acid distribution between C1 and C2 of the glycerol in PC and MGDG

In order to demonstrate that MGDG is really directly formed by galactosylation of the 1,2-DAG resulting from the phospholipase C digestion it is necessary to establish whether the acyl distribution between C1 and C2 in the glycerol is the same in both PC and MGDG. To determine this distribution, PC from liposomes or PC isolated from chloroplasts and MGDG were treated with lipase A1. As seen in table 5 the same acyl group distribution was found in PC from liposomes or from chloroplasts and in MGDG: at C1 both C16

Table 5

Distribution of acyl groups between C1 and C2 in PC ⎡ liposomes, PC of the chloroplasts and MGDG

Lipids	C16:0	C18:1	C18:2	C18:3
Liposomes (1)	37	3	36	24
PC (2)		7	58	35
Chloroplasts (1)	35		39	27
PC (2)			66	34
MGDG (1)	50		31	19
(2)			62	38

Results are expressed as percentage of the radioactivity recovered in the main fatty acids for C1 and C2 in the glycerol

and C18 acyl groups were found while at C2 only C18 acyl groups occurred, demonstrating that MGDG with C18 polyunsaturated acyl groups at the two positions can be formed in the system.

4. CONCLUSIONS AND DISCUSSION

Here, it has been demonstrated that PC brought to the chloroplast envelope by PLTP can be used for the synthesis of galactolipids of C18/C18 species. The crucial limiting step in this process seems to be the formation of 1,2-DAG from the PC integrated into the chloroplast envelope. With isolated chloroplasts alone, this formation was so low that only a few percent of the PC could be converted into 1,2-DAG and then into galactolipids when UDP-gal was added. Addition of phospholipase C dramatically increased the formation of 1,2-DAG and consequently the formation of galactolipids by galactosylation of these molecules. Analysis of the fatty acid distribution in each position of the glycerol clearly demonstrates that PC is mainly used as 'diacylglycerol donor' for the formation of galactolipids since the same distribution is found on C1 and C2 both in PC and in MGDG. A PC with the C18 acyl groups at both the carbons of the glycerol but with the C16 acyl groups only at the C1 is typically extra-chloroplastic [31]; by using such a PC we have synthesized a galactolipid with C18 polyunsaturated fatty acids at both the position C1 and C2 of the glycerol. This can be considered as a typically 'eucaryotic' galactolipid synthesized in vivo by a 'cooperative pathway' implicating the extrachloroplastic compartment [7].

We cannot assume from this experiment that whole chloroplasts are necessary for the set of reactions demonstrated here. On the contrary the fact that radioactive MGDG was also found in the supernatant and the previous work of Heemskerk [20] on sonicated chloroplast envelopes indicate that broken chloroplasts or sonicated envelopes can work. Nevertheless, the fact that the highest percentage of radioactive MGDG was found in a chloroplast pellet obtained by centrifugation through a Percoll gradient at the end of the three incubations can, at least, indicate that MGDG with a eucaryotic acyl distribution can be incorporated into the chloroplast in this way. Furthermore, the fact that radioactive MGDG was obtained by

galactosylation from chloroplasts re-isolated after phospholipase C treatment indicates that 1,2-DAG of the chloroplast envelope (and not the 1,2-DAG released in the supernatant) was involved.

The physiological significance of this experiment has yet to be established, bearing in mind the addition of phospholipase C. It could be supposed that in vivo a cytoplasmic phospholipase or more likely a CDP-choline transferase working reversibly is active during chloroplast formation but at a sufficiently low rate not to destroy the chloroplast envelope.

Current work is aimed at decreasing the concentration of phospholipase C to the lowest level giving sufficient 1,2-DAG and on the other hand to examine which enzyme could be physiologically involved in the formation of 1,2-DAG.

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