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Galactolipid export from envelope to thylakoid membranes in intact chloroplasts. I. Characterization and involvement in thylakoid lipid asymmetry

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The galactolipid transfer from inner envelope to thylakoid membranes has been studied in intact spinach chloroplasts. Plastids, isolated from mature leaves, were dark-incubated in the presence of UDP-[^{14}C]galactose. After various synthesis periods at 5 or 25°C, intact plastids were reisolated and osmotically lysed. Thylakoid membranes were then prepared by a special procedure which removed ≥ 99% of the envelope amount initially present. Under these conditions, purified thylakoids were found to contain radiolabelled MGDG and DGDG, indicating that galactolipids were exported from the inner envelope. The amounts exported were proportional to the amounts synthesized. About 55% of the MGDG and 25% of the DGDG synthesized in plastids were transferred to thylakoids, irrespectively of incubation time or temperature. The MGDG / DGDG radioactivity ratio was 7 in intact plastids and 18 in thylakoids, suggesting a preferential export of MGDG. Purified thylakoid membranes were then submitted to a lipolytic treatment designed to discriminate between the MGDG and DGDG pools belonging to the outer (stroma-facing) or to the inner monolayer. The radioactivity present in the lyso-products (corresponding to the outer pools) and in the residual parent lipids (corresponding to the inner pools) was measured. The labelled MGDG showed a transmembrane outside : inside distribution (mol%) of 50 : 50, which differed from the native (mass) MGDG asymmetry of 64 : 36. In contrast, the label and mass asymmetries of DGDG gave the same value of 15 : 85. These label distributions were affected neither by incubation time (from 5 to 90 min) nor by temperature (from 5 to 25°C). We discuss the possibilities that transient fusions between the stroma-facing monolayers of the inner envelope and of the thylakoid membrane, and / or galactolipid transfer protein(s), together with lipid translocating activities in thylakoids, may account for the galactolipid export observed in mature spinach chloroplasts.

Introduction

The two envelope membranes greatly contribute to the synthesis and renewal of the main acyl lipids of the chloroplast, the galactolipids. Whatever the prokaryotic or eukaryotic origin of its diacylglycerol backbone, MGDG is formed by a galactosyl transfer between UDP-galactose and diacylgiycerol, catalyzed by the UDP-galactose : diacylglycerol galactosyltransferase (MGDG synthase) [1]. After being disputed for a long time, the final step of DGDG synthesis has been recently assigned to the galactolipid : galactolipid galactosyltransferase [2]. Whereas the pathways leading to the synthesis of galactolipids and to their subsequent desaturation have been extensively studied (see reviews in Refs. 3–6), the problem of the transfer of galactolipids from their site of synthesis (the envelope) to the thylakoid network has received little interest. Yet thylakoids account for more than 90% of the total galactolipid content of chloroplasts. Being essentially unable to synthesize galactolipids [1,7], thylakoids are therefore dependent on a massive import of these lipids from the envelope. Although earlier reports indica

Abbreviations: DGDG, digalactosyldiacylglycerol; HMBA, *p*-hydroxymercuribenzoic acid; MGDG, monogalactosyldiacylglycerol; Mops, 4-morpholinepropanesulfonic acid; Tricine, *N*-[2-hydroxyl-1,1-bis(hydroxymethyl)ethyl]glycine.

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that this galactolipid transport effectively occurred and was rapid [8–10]. little information is presently available on the detailed aspects of this process.

We decided to better characterize the unidirectional galactolipid transfer between the inner envelope membrane and thylakoid membranes. To this aim, intact chloroplasts were first incubated in the presence of UDP-[^{14}C]galactose. Then, the quantitative aspects of this lipid flow were determined under conditions which allowed a complete removal of envelope material from thylakoid membranes. Finally, the molecular mechanism(s) by which the transfer is achieved was investigated by applying a controlled lipolytic treatment to the purified thylakoids. Such a treatment allows to distinguish between lipid molecules that belong to the outer or to the inner leaflet of the thylakoid bilayer [11–15]. Thus we expected this approach to enable us identifying the mechanism(s) by which galactolipids synthesized at the envelope level are eventually recovered at the thylakoid level.

Rationale

If galactolipid molecules are to be exported from the inner envelope membrane to thylakoid membranes, they can do so by anyone of four basic mechanisms.

(a) The inner envelope membrane buds off and releases vesicles which move through the stroma and fuse with the thylakoid membrane.

(b) A lipid transfer protein extracts a galactolipid molecule from the stroma-facing monolayer of the inner envelope membrane, carries it through the stroma and eventually inserts it into the stroma-facing monolayer of the thylakoid membrane.

(c) A partial fusion is triggered between the stroma-facing monolayers of both inner envelope membrane and thylakoid membrane, allowing galactolipid molecules to diffuse laterally within these monolayers, while the inverted micelle at the fusion locus [16] prevents the contents of the two other monolayers to be mixed.

(d) A complete fusion occurs between inner envelope membrane and thylakoid membrane, connecting their stroma-facing monolayers together and their lumen-facing monolayers together, thus allowing an unimpeded lateral diffusion of galactolipid molecules (and of other compounds?) within the whole bilayers.

If the galactolipid molecules to be exported are radioactively labelled (e.g. in their polar headgroup), then the presence of radioactive galactolipids in either monolayer of the thylakoid membrane will depend not only on what transfer mechanism (a, b, c or d) is operative, but also on the topography of galactolipid synthesis in the inner envelope membrane and on the possible occurrence of galactolipid translocation activities across the thylakoid bilayer. Valuable information can be provided by comparing the transmembrane distribution of the labelled galactolipids with the known, native (mass) distribution of galactolipids [11–15] in both the outer (stroma-facing) and the inner (lumen-facing) monolayer of thylakoid membranes. This dual sidedness analysis (radioactivity and mass) can be achieved by treating thylakoids with the lipase from *Rhizopus arrhizus* under non-lytic conditions, so as (i) to hydrolyze all the galactolipid molecules (including the radioactive ones) located in the outer monolayer to lysogalactolipids (and free fatty acids) and (ii) to prevent the remaining galactolipids leaving the inner monolayer (via outward transmembrane movement) by keeping the temperature around 0°C. At this stage, the amount of radioactivity associated with lyso-MGDG (or lyso-DGDG) will therefore represent the amount of exported MGDG (or DGDG) that is located in the outer monolayer of thylakoid membranes at the end of the incubation period of intact plastids with the radioactive precursor. The distribution of the radioactivity between the two monolayers of the thylakoids and its possible change under the influence of time and of various physico-chemical factors can then be studied.

Material and Methods

Chemicals

UDP-[U-^{14}C]galactose (11.1 GBq/mmol) was purchased from Amersham. The lipase (EC 3.1.1.3) from *Rhizopus arrhizus* was from Boehringer. Tricine, Mops, UDP, UDP-galactose and (HMBA) were from Sigma. Percoll was a Pharmacia product. All other chemicals and solvents were 'purissimum' products from Fluka. Bovine serum albumin was defatted as described [17].

Media

The following media (A to F) were used in these experiments. Medium A: 330 mM sorbitol/5 mM $MgCl_2$/50 mM Mops-KOH (pH 7.2). Medium B: 40% Percoll/330 mM sorbitol/0.5 mM UDP/50 μM HMBA/50 mM Mops-KOH (pH 7.8). Medium C: 2 mM EDTA-Na_2/0.5 mM UDP/50 μM HMBA/10 mM Tricine-KOH (pH 7.8). Medium D: 5% Percoll/300 mM sorbitol/0.5 mM UDP/50 μM HMBA/10 mM Tricine-KOH (pH 7.8). Medium E: 300 mM sorbitol/0.5 mM UDP/50 μM HMBA/10 mM Tricine-KOH (pH 7.8). Medium F: 200 mM sorbitol/50 mM KCl/5 mM $MgCl_2$/25 mM Tricine-NaOH (pH 8) and containing defatted bovine serum albumin (100 mg/ml).

Material

Mature spinach leaves, obtained from the local market, were stored in the dark at 4°C for 1–3 days to reduce starch content. Washed leaves were illuminated

for about 30 min on melting ice before being used for chloroplast isolation.

Preparation of chloroplasts

Intact chloroplasts were prepared from spinach leaves by the method of Mourioux and Douce [18]. On a routine basis, these chloroplasts presented a 90% intactness as determined by the ferricyanide assay [19].

Incubation with UDP-[^{14}C]galactose

Fresh intact chloroplasts, suspended in medium A at 2 mg chlorophyll/ml, were equilibrated 5 min at the desired temperature (5 or 25°C). Galactolipid synthesis was started by adding, to 1.5 ml of the chloroplast suspension, UDP-[U-^{14}C]galactose (440 MBq/mmol) to a final concentration of 67 μM and the mixture was then incubated at 5 or 25°C in darkness for time periods ranging from 5 to 90 min. The incubation was terminated by rapid cooling of the mixture on melting ice followed by immediate processing. All subsequent operations were carried out at 0–2°C.

Repurification of labelled chloroplasts

The chilled chloroplast suspension was rapidly layered on 10 ml of medium B and the intact chloroplasts remaining in the preparation were reisolated according to Mills and Joy [20]. The presence of both UDP and HMBA ensured an essentially complete inhibition of UDP-galactose : diacylglycerol galactosyltransferase and of galactolipid : galactolipid galactosyltransferase activities [21] in this step (and in the next ones).

Preparation of purified thylakoid membranes

The pellet of repurified, labelled intact chloroplasts was adjusted to a final volume of 2 ml with medium E. Aliquots were sampled for chlorophyll (2 × 20 μl) and lipid radioactivity (2 × 20 μl) determinations. The remaining suspension was diluted with 30 ml of medium C to osmotically disrupt the chloroplasts. After 2 min, isotonic conditions were restored by adding 3.6 ml 3 M sorbitol and the suspension was centrifuged for 5 min at 16500 × g (Sorvall swing-out HB-4 rotor). The pellets were suspended in 18 ml of medium E, then 8 ml of medium D were injected below this dilute suspension and the tubes were centrifuged for 15 min at 20000 × g (HB-4 rotor, slow acceleration). The pellets were washed once more in 30 ml of medium E and spun for 5 min at 16500 × g (HB-4 rotor). The resulting pellets consisted of purified thylakoids from which ≥ 99% of the initial envelope membranes were removed (see Results). On a chlorophyll basis, the yield of thylakoids varied from 80 to 90% with this method.

Lipolytic treatment of purified thylakoids

The purified labelled thylakoids were pooled and resuspended in medium F so as to obtain a total volume of 3 ml. Aliquots were sampled for chlorophyll (2 × 40 μl) and lipid radioactivity (2 × 40 μl) determinations. The chlorophyll concentration was usually between 0.4 and 0.6 mg/ml. The thylakoid suspension was placed in a brown glass vial, immersed in melting ice, and continuously stirred. After sampling a 0.3 ml control aliquot (at time zero) for lipid extraction, the lipase was added (≈ 200 units/mg chlorophyll) and the incubation was carried out at 0°C for 25 min. Aliquots (0.3 ml) were sampled at regular time intervals for lipid extraction.

Lipid extraction

The lyso-compounds generated by the lipase are much more polar than their parent lipids, so that the risk of loosing at least part of these lyso-compounds in the aqueous phase of conventional two-phase extraction systems cannot be neglected. To avoid such pitfalls, we have used a one-phase extraction procedure [22] based on the use of isopropanol and hexane (2:3, v/v). The 20-μl and 40-μl samples were extracted into 4 ml of this solvent mixture, to which was added a thylakoidal lipid extract (equivalent to 150 μg chlorophyll) as carrier. The 0.3-ml aliquots were extracted into 10 ml of the solvent mixture. The monophasic extracts were vigorously vortexed, incubated at room temperature for 15 min with occasional shaking, vortexed again, and finally centrifuged for 5 min at 3000 × g. The protein pellet, firmly adhering to the bottom of the tube, was totally depigmented. The supernatants were quantitatively transferred to new tubes, dried under nitrogen and the lipids taken up in about 400 μl chloroform/methanol (8:2, v/v).

Lipid separation and analysis

Each lipid extract was quantitatively spotted on silicagel plates using an automatic sample applicator (Linomat IV, Camag). Plates were developed in acetone/toluene/water/acetic acid (100:25:9:1, by vol.). After a brief drying, plates were lightly sprayed with 0.01% primuline and viewed under UV light. Zones containing MGDG, DGDG, their corresponding lyso-compounds and the oligogalactolipids (tri- and tetragalactosyldiacylglycerol) were scraped into scintillation tubes, dispersed in 4 ml of scintillation liquid (Optiphase 'HiSafe II', Pharmacia) and the radioactivity was determined in a Kontron Betamatic II counter. Alternatively, the amounts of MGDG, DGDG and of their corresponding lyso-derivatives were determined by measuring the galactose content of these lipids [23]. The data were then normalized to the sums (MGDG + lyso-MGDG) and (DGDG + lyso-DGDG) and ex-

pressed as plots of percentage of initial MGDG (or DGDG) content versus hydrolysis time.

Preparation of ^{14}C-labelled envelopes

Purified envelopes were prepared from intact spinach chloroplasts essentially as described by Nguyen and Siegenthaler [24]. Labelling of envelope membranes was carried out in 2 ml of a medium containing 300 mM sorbitol/10 mM $MgCl_2$/10 mM Tricine-KOH (pH 7.8)/70.1 mM UDP-[U-^{14}C]galactose (864 MBq/mmol) and envelopes (1 mg protein/ml), and incubated at 25°C for 90 min. Membranes were then washed twice in 30 ml of medium E (without inhibitors) by pelleting the material at 90000 × *g* for 60 min. Labelled envelopes (about 24.7 kBq of lipid radioactivity per mg protein) were stored in 20 mM Mops-KOH (pH 7.6)/50% glycerol at −20°C.

Other techniques

Established methods were used to determine chlorophyll [25], protein [26] and phospholipid phosphorus [27].

Results

Essential for our study was the availability of thylakoids from which any envelope material was efficiently removed. We required our purification procedure to fulfill two independent criteria. First, purified thylakoid membranes should be free of any galactolipid synthesizing activities (such as UDP-galactose : diacylglycerol galactosyltransferase and galactolipid : galactolipid galactosyltransferase) which are considered to be exclusive envelope markers [1,3]. Second, if ^{14}C-labelled envelope membranes are added to non-labelled, osmotically disrupted chloroplasts, then the label should be completely removable from thylakoids. This second criterion is more demanding than the first one, which would be partly invalidated if thylakoids were to display any endogenous galactolipid synthesis activity – a possibility that cannot be ruled out a priori [8]. Preliminary attempts to purify thylakoid membranes by simple differential centrifugation indicated that 5 to 6 washing steps were required to remove ≥ 99% of the envelope material initially present. We noticed however that the envelope material was washed away more efficiently at low than at high ionic strength and that the presence of divalent cations adversely affected the separation of envelopes from thylakoids.

Separate experiments with isolated, fluorescamine-tagged envelopes showed that low density Percoll cushions were able to retain (or to float) envelopes while still allowing a good pelleting of thylakoids. The progress of thylakoid membrane purification after repeated washings on 5 (or 10)% Percoll cushions was determined according to the abovementioned criteria

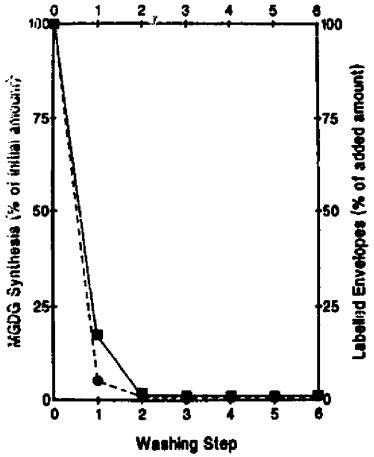

Fig. 1. Progress of the purification of thylakoid membranes, assessed by the residual amount of MGDG synthase activity (criterion 1; ■) and by the residual amount of prelabelled envelope membranes added to non-labelled, disrupted chloroplasts (criterion 2; ●). When required (criterion 2), UDP-[U-^{14}C]galactose-labelled envelope membranes (200–300 μg protein, corresponding to 1.6–2.4 kBq of MGDG and 1.3–2.0 kBq of DGDG radioactivity) were added to osmotically disrupted chloroplasts (3.75 mg chlorophyll). After having restored isotonic conditions, an aliquot was taken for direct lipid extraction of thylakoid membranes or for galactolipid synthesis (step 0). The remaining thylakoid membranes were spun down (step 1) and further purified by repeated centrifugations through 5 (or 10)% Percoll cushions (steps 2–6) (see Material and Methods). At each step, representative aliquots were taken for direct lipid extraction of thylakoid membranes or for galactolipid synthesis. Galactolipid synthesis was carried out in 1 ml medium A containing chloroplast membranes (about 150 μg chlorophyll) and 0.1 mM UDP-[U-^{14}C]galactose (90.9 MBq/mmol) at 25°C during 30 min. Lipids were extracted [28] and separated by TLC on silicagel plates developed in $CHCl_3/CH_3OH/25\%\ NH_3/H_2O$ (65:35:3:2, v/v). The MGDG and DGDG radioactivity was normalized to chlorophyll or to phosphatidylglycerol content, both methods giving similar results. The MGDG synthase activity in step 0 was between 55 and 65 nmol MGDG/mg chlorophyll per h (= 100%).

(Fig. 1). A simple differential centrifugation (step 1) removed 95% of the added envelope material and more than 80% of the MGDG synthase activity initially present in disrupted chloroplasts. Such results were routinely obtained provided that isotonic conditions (≈ 300 mM sorbitol) were restored within 1 to 2 min after osmotic disruption of chloroplasts and that the chlorophyll concentration did not exceed 0.1 mg/ml. The residual amount of contaminating envelopes could be further reduced to very low levels (≤ 1%) by pelleting thylakoid membranes through 5 (or 10)% Percoll cushions (Fig. 1, steps 2–6). A single Percoll step (step 2) was sufficient to eliminate most of the residual contamination. Contrarily to a recent claim [7], our purified thylakoid preparations did contain phosphatidylcholine. Indeed, the phosphatidylcholine to phos-

phatidylglycerol molar ratio decreased from 0.68 ± 0.04 ($n = 3$) in broken chloroplasts (step 0) to 0.28 ± 0.02 ($n = 3$) in purified thylakoid membranes (steps ≥ 2).

In a next step, the export – in its broadest meaning – of galactolipid molecules from inner envelope membrane to thylakoid membranes was investigated by measuring how much and how fast radioactivity appeared in purified thylakoids after feeding intact chloroplasts with UDP-[^{14}C]galactose. Fig. 2A shows that the amount of labelled MGDG found in purified thylakoids depended linearly on the amount of labelled MGDG synthesized in intact chloroplasts. About 55% of the MGDG molecules synthesized at the inner envelope level were thus recovered in thylakoids. At 25°C, this linearity was obeyed up to about 40 min of synthesis. Longer incubation times (up to 90 min) resulted in a levelling off of MGDG synthesis without however affecting MGDG export (not shown). The transfer of lipids from inner envelope membrane to thylakoids is a rapid phenomenon: after 5 min of synthesis at 5°C, labelled galactolipids were easily detectable in purified thylakoids. As shown in Fig. 2B, the export and synthesis rates of MGDG were also linearly related, at least up to 40 min of incubation.

The synthesis rate of MGDG increased by a factor of 7 between 5 and 25°C (Table I). Interestingly, the export rate of MGDG was raised in the same proportion, suggesting that a close link exists between export and synthesis mechanisms. DGDG was not only synthesized at a much slower rate than did MGDG, but its export also was lower: 24% only of the newly made DGDG molecules were eventually recovered in thylakoid membranes (Table I).

When thylakoids were treated with the lipase from *Rhizopus arrhizus* under non-lytic conditions, only those galactolipids belonging to the outer monolayer were hydrolyzed. By measuring either the mass (galactose assay) or the radioactivity content of both galactolipids and of their respective lyso-derivatives during the course of the lipolytic treatment, the hydrolysis patterns shown in Figs. 3 and 4 were obtained. Fig. 3 indicates that in terms of mass, the MGDG content of thylakoids rapidly decreased to an equilibrium value at about 35% whereas the amount of lyso-MGDG reached a plateau at 65%. However, the amount of radioactivity associated with MGDG was fully stabilized already when 50% of the initial label content of MGDG had been converted into its lyso-derivative. On the other hand, closely overlapping curves were obtained when both mass and label were measured in DGDG and its

TABLE I

Synthesis of galactolipids in intact chloroplasts and their export from inner envelope membrane to thylakoids at two different incubation temperatures

The 'total S' corresponds to the amount of radioactivity incorporated in chloroplast galactolipids (MGDG + DGDG + tri- + tetragalactosyldiacylglycerol). The 'total E' corresponds to the amount of radioactivity incorporated in thylakoid galactolipids.

	5°C	25°C	
	MGDG ($n = 3$)	MGDG ($n = 8$)	DGDG ($n = 3$)
Units: nmol precursor/mg chlorophyll per h			
Synthesis (S) rate	5.3 ± 0.4	37.1 ± 9.7	1.4 ± 0.4
Export (E) rate	2.5 ± 0.5	22.0 ± 5.3	0.4 ± 0.1
Units: percent			
Synthesis (% of total S)	74.8 ± 3.5	79.4 ± 5.8	11.7 ± 1.1
Export (% of total E)	82.8 ± 5.2	93.2 ± 3.1	5.3 ± 2.5
Export (% of S)	47.7 ± 6.1	56.1 ± 5.3	23.8 ± 13.6

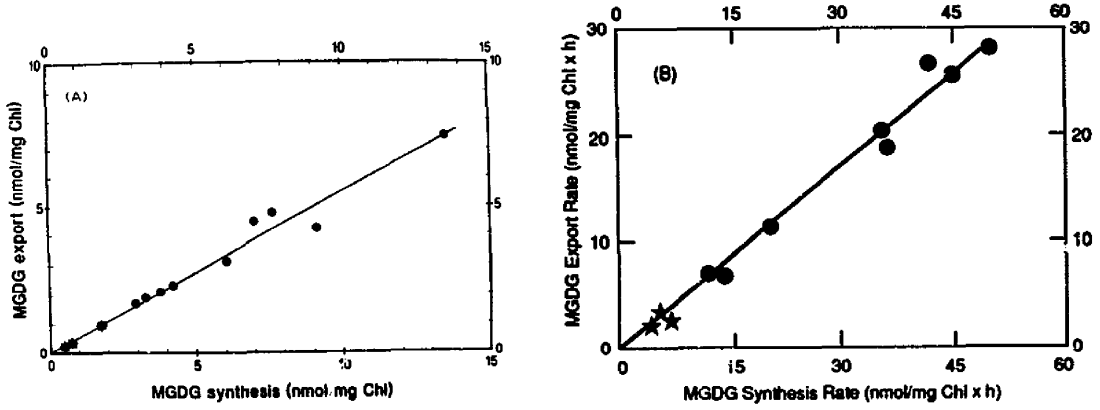

Fig. 2. (A) The dependence of MGDG export from inner envelope membrane to thylakoids on MGDG synthesis in intact chloroplasts. Each data point corresponds to a single experiment carried out for different synthesis time periods (ranging from 5 to 40 min) at 5°C (•) or at 25°C (●). (B) The corresponding relation between the export rate and the synthesis rate of MGDG. Chl = chlorophyll.

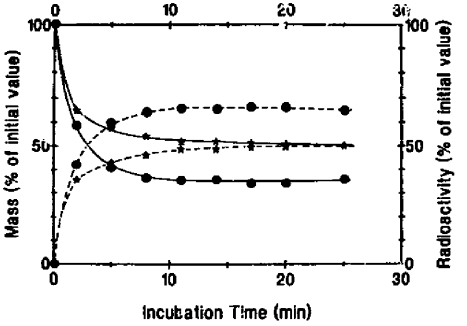

Fig. 3. Decrease in MGDG (———) and parallel increase in lyso-MGDG (- - - -) in thylakoid membranes treated by the lipase of *Rhizopus arrhizus* under non-lytic conditions. The lipid mass (●) and the radioactivity amount (*) associated with each lipid was determined as described in Materials and Methods and plotted as percentage of the initial corresponding value versus incubation time in the presence of the lipase.

lyso-derivative during the lipolytic treatment (Fig. 4). In this case, the plateau was obtained when the conversion extent reached 15%.

The transmembrane distributions of mass and label for both MGDG and DGDG were derived from semi-log plots of the data of Figs. 3 and 4 by extrapolation to zero time of the equilibrium phase, as previously described [11,13–15] and the results are depicted in Table II. The outer monolayer accounted for 64% of the MGDG and 15% of the DGDG content of thylakoids. In contrast with this asymmetry, the newly synthesized MGDG molecules were always integrated into thylakoid membranes in a fully symmetrical way (50% outside : 50% inside), regardless of the temperature at which synthesis and export of galactolipids occurred in intact chloroplasts. This discrepancy between mass and label distributions of MGDG resulted in a marked difference between the specific radioactivity of the

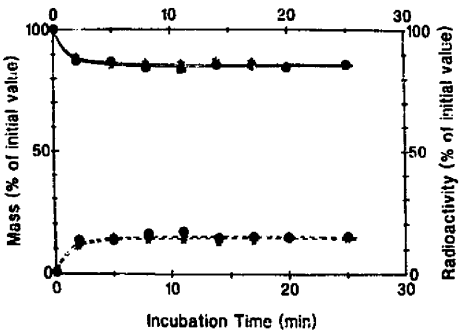

Fig. 4. Decrease in DGDG (———) and parallel increase in lyso-DGDG (- - - -) in thylakoid membranes treated by the lipase of *Rhizopus arrhizus* under non-lytic conditions. The symbols and the experimental conditions are those of Fig. 3.

TABLE II

Mass and label distribution of galactolipids between the outer and inner monolayers of thylakoid membranes

Intact chloroplasts were incubated with UDP-[^{14}C]galactose for various time periods ranging from 5 to 90 min at either 5°C or 25°C. Thylakoid membranes were then purified and submitted to the lipolytic treatment described in Material and Methods. The specific radioactivity of a given galactolipid in the outer (o) or inner (i) monolayer was calculated as: $(L \times l_{(o,i)})/(M \times m_{(o,i)})$ where L = total amount of lipid radioactivity in thylakoid membranes (dpm/mg chlorophyll); M = total amount of lipid in thylakoid membranes (nmol/mg chlorophyll); (l_o, l_i) and (m_o, m_i) = fractions of L, respectively, of M, attributable to the outer or the inner monolayer of thylakoid membranes.

	5°C	25°C	
	MGDG ($n \simeq 3$)	MGDG ($n = 8$)	DGDG ($n = 3$)
Units: mol% outer : mol% inner			
Mass asymmetry	65:35 (±2)	64:36 (±2)	15:85 (±2)
Label asymmetry	50:50 (±2)	49:51 (±1.5)	14:86 (±1)
Specific radioactivity ratio (outer/inner)	0.564 ± 0.045	0.537 ± 0.031	0.963 ± 0.053

outer and inner MGDG pools. On the other hand, there was no difference between the mass and the label distributions of DGDG over the whole range of

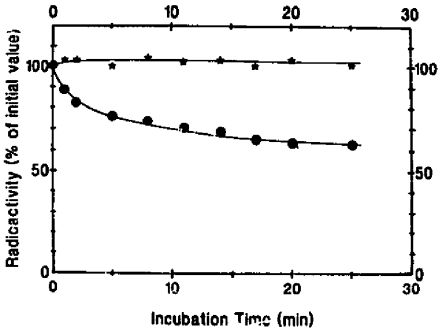

Fig. 5. Hydrolysis kinetics of galactolipids in isolated, UDP-[^{14}C]galactose-prelabelled envelopes treated by the lipase of *Rhizopus arrhizus*. Labelled envelopes (295 μg protein, corresponding to 2.34 kBq of MGDG and 1.90 kBq of DGDG radioactivity) were resuspended in 3 ml of medium F containing also non-labelled thylakoid membranes (0.5 mg chlorophyll/ml) in order to reproduce the conditions under which the lipolytic treatment of labelled thylakoids (see Figs. 3 and 4) was carried out. The mixture was supplemented with 4 mM UDP and 50 μM HMBA so as to block galactosyltransferase activities [21]. In separate experiments, we have ascertained that UDP (tested up to 5 mM) and HMBA (up to 250 μM) did not inhibit the lipase activity. The lipase was then added (100 units/ml) and the suspension was incubated at 0°C. The hydrolysis was followed by measuring the change in the radioactivity content of MGDG (●) and of DGDG (*) as described in Materials and Methods. The results presented are the average of two experiments (±S.D.). Similar results were obtained when thylakoids were omitted from the envelope suspension.

TABLE III

Effect of a contamination of thylakoid membranes by a residual 1% or 5% of the original envelope amount on the values of the transmembrane radioactivity ratios for MGDG and DGDG

Fraction or parameter	Radioactivity (dpm)		Asymmetry	
	MGDG	DGDG	MGDG	DGDG
Intact chloroplasts [a]	355 292	45 151	–	–
Thylakoids [a,b]	196 103	14 342	–	–
Removed envelopes [a,c]	159 189	30 809	–	–
Measured label ratio [d]	–	–	49:51	14:86
1% contamination level ('removed envelopes' represent 99%):				
1% envelopes	1608	311	–	–
Thylakoids (corrected)	194 495	14 031	–	–
Corrected label ratio [e]	–	–	49.1:50.9	14.3:85.7
5% contamination level ('removed envelopes' represent 95%):				
5% envelopes	8378	1622	–	–
Thylakoids (corrected)	187 725	12 720	–	–
Corrected label ratio [e]	–	–	49.6:50.4	15.8:84.2

[a] Radioactivity data are taken from a typical experiment and are expressed on the basis of 1 mg chlorophyll.

[b] Including 1% or 5% residual contaminating envelopes.

[c] The radioactivity of the 'Removed envelopes' is obtained by subtracting the 'Thylakoids' radioactivity value from the 'Intact chloroplasts' value.

[d] Taken from Table II; each ratio is given as (mol% outside): (mol% inside).

[e] The corrected label ratio was calculated using the fact that 35% of the MGDG and 0% of the DGDG content of contaminating envelopes was degraded (see text and Fig. 5). *Example of calculation*: for 1% contamination level, the removed envelopes (99%) contain 159189 dpm in MGDG. Accordingly, 1% contaminating envelopes corresponds to 159189/99 = 1608 dpm in MGDG. Therefore, the radioactivity associated with the thylakoidal MGDG corresponds to 196 103–1608 = 194 495 dpm. Thus, the relative MGDG amount R_0 (mol%) assignable to the outer thylakoid monolayer is given from: $(R_0 \times 194\,495) = (49 \times 196\,103) - (35 \times 1608)$, and one finds $R_0 = 49.1$ mol%.

incubation conditions tested, so that the specific radioactivity ratio of outer to inner DGDG remained close to unity (Table II).

Control experiments were also carried out to assess the possible influence of contaminating (labelled) envelopes – even in low amounts – on the value of the transmembrane ratios for MGDG and DGDG in thylakoids. To this end, we have investigated the hydrolytic behaviour of galactolipids in isolated envelopes treated by the lipase of *Rhizopus arrhizus* under conditions that matched exactly those used for the sidedness studies in labelled thylakoids. Fig. 5 shows that in isolated envelopes, the lipase-catalyzed degradation of MGDG effectively occurred, although at a slower rate than in thylakoids (compare with Fig. 3). After 25 min of incubation at 0°C, only 35% of the initial MGDG radioactivity of isolated envelopes was converted into lyso-MGDG radioactivity whereas DGDG was not hydrolyzed under these conditions (Fig. 5). Under our conditions, it can be calculated that the specific radioactivity of galactolipids is 10-times higher in envelopes than in thylakoids. A small amount of contaminating envelopes in the thylakoid fraction could thus be expected to biase the transmembrane distribution of labelled galactolipids in thylakoid membranes, particularly if the hydrolysis extents of galactolipids in envelopes and in purified thylakoids differ markedly (compare Figs. 3 and 4 with Fig. 5). The data of Fig. 5 were then used to determine whether the contamination of thylakoid membranes by a residual 1% or 5% of the original envelope amount would affect the value of the transmembrane radioactivity ratios for MGDG and DGDG reported in Table II. The result of such calculations is shown in Table III. It can be seen that the presence of contaminating envelope material in the thylakoid fraction has no influence on the label asymmetry ratio of both MGDG and DGDG, provided that the contamination level does not exceed 5%. At higher levels (e.g. $\geq 10\%$), the corrected DGDG label ratio begins to deviate significantly from the measured one, whereas the MGDG label ratio remains essentially unchanged (not shown). We routinely worked at a contaminating level of 1% or lower (Fig. 1), which not only prevented such biased estimations to be made (Table III), but also conferred a high degree of reliability to our results.

Discussion

In this article, we have addressed the question of the galactolipid export between the envelope and the thylakoid network of intact chloroplasts. Here, we are mainly concerned with the description of our experimental approach of the problem and with its validation. We have therefore deliberately set aside the more physiological aspects of this problem (using plants of various ages and species as well as several radiolabelled precursors) which will be dealt with in a forthcoming paper of this series.

The general strategy was (a) to provide intact plastids with UDP-[^{14}C]galactose and to allow them to synthesize their own galactolipids (mostly hexaene molecular species) through the galactosyl transferase activities of the envelope [29]; (b) to allow the newly made galactolipids to be transferred in organello from their site of synthesis (the envelope) to their final destination (the thylakoid membrane); and (c) to allow the newly made galactolipid molecules, now inserted into the thylakoid membrane, to be redistributed (or not) between its outer and inner monolayers.

Purification of thylakoid membranes

In this context, it was of the utmost importance to prepare very pure thylakoid membranes, so that any label found in thylakoids could be safely attributed to the galactolipid export mechanism(s), but not to a

trivial contamination of thylakoids by some residual envelope material. The results presented in Fig. 1 indicate that this goal has been reached. Indeed, 99% of both the endogenous envelope amount (according to criterion 1) and of the added envelope material (according to criterion 2) were removed from thylakoids already after step 2 (Fig. 1). The constancy of the thylakoidal MGDG/DGDG label ratio during the removal of added prelabelled envelopes (results not shown) suggests that under our experimental conditions, no particular portion of either outer or inner envelope membrane remains preferentially associated with thylakoid membranes. In addition, the very low amount of radioactivity left in purified thylakoids indicates that spontaneous exchange of (labelled) galactolipid molecules, if it exists, does not occur to a sizable extent between envelope vesicles and thylakoid membranes. However, we cannot exclude the possibility (see below) that such exchange may occur with higher efficiency during galactolipid transfer within the intact plastid.

Galactolipid export: quantitative aspects

We can now consider galactolipid export as accounting for about 99% of the label found in purified thylakoids. This lipid transfer presents three main characteristics.

Firstly, and in agreement with earlier reports [8–10], it is a rapid process. Under our conditions, radioactive lipids were detected in thylakoids already after 5 min of synthesis at 5°C (Fig. 2), but this is not the lower limit. For instance, Bertrams et al. [10] could detect labelled galactolipids in thylakoids a few seconds following the addition of UDP-[^{3}H]galactose to intact plastids. However, the purity of the thylakoid fraction was not rigorously assessed by these authors [10] and the contribution of contaminating envelopes was probably not marginal (see also Ref. 9).

Secondly, the transfer is a continuous process and, at a given temperature, its amplitude depends only on the amount of lipids available for transfer, that is, on the relative surface area of the inner envelope membrane. This is shown by the linear relation between MGDG synthesis and export, as well as between their corresponding rates (Fig. 2). Moreover, this straight line extrapolated through the origin of the graph. Thus, as soon and long as galactolipid synthesis proceeds steadily in the intact plastid, a constant percentage of the newly made lipids is exported to the thylakoid network (Table I). This is in contrast with earlier works [10,30] which have reported a time-dependent shift of the lipid radioactivity from the inner envelope in favour of thylakoids. Such a shift would only be expected if the precursor supply to the intact chloroplast was severely limited. Indeed, a limited amount of UDP-galactose would allow MGDG synthesis for a short time only, so that incubations lasting longer than this short synthesis time would merely favour the galactolipid export to thylakoids at the expense of envelopes. Bertrams et al. [10] worked with a ratio of 0.019 nmol UDP-galactose/mg chlorophyll, which is clearly limitating. In the present work, we have used a much higher (about 1800-fold) ratio of 33.5 nmol UDP-galactose/mg chlorophyll, which suppressed the abovementioned limitation for much longer incubation times (up to 40 min at 25°C, see Fig. 2). However, the substrate limitation and the subsequent radioactivity shift from envelopes to thylakoids became evident for prolonged incubations (between 50 and 90 min at 25°C), as indicated by our observation that MGDG synthesis was progressively levelled off whereas MGDG export was not affected (results not shown). We interpret therefore the data of Fig. 2 and Table I as suggesting the occurrence of a continuous flow of galactolipid molecules from envelopes to thylakoids. At the rates reported in Table I, this flow could have completely renewed the average MGDG content of thylakoid membranes (about 1300 nmoles/mg chlorophyll) in 22 days at 5°C and in $2\frac{1}{2}$ days at 25°C. These values can be instructively compared with the 6–12 h half-life of the thylakoidal 32 kDa herbicide-binding polypeptide [31].

Thirdly, the galactolipid transfer process exhibits a remarkable class selectivity. This conclusion is based on the observation that the MGDG/DGDG label ratio, which was 7 in chloroplasts, increased up to 18 in thylakoids, thus indicating a preferential export of the non-bilayer forming MGDG (compare lines 3 and 4 in Table I). Together, MGDG and DGDG accounted for the quasi totality of the lipids exported at 25°C (Table I). This means that oligogalactolipids, although present in the envelope (where they amount to 9% of the total label incorporated in chloroplast lipids; calculated from Table I, line 3), are not exportable. This class selectivity in galactolipid export raises the question of how the relative proportions of these lipids in thylakoid membranes (MGDG, 55 mol%; DGDG, 27 mol%) can be maintained in the long term. One may envisage that a backflow of lipids of the same quantitative importance occurs from thylakoids in the direction of envelopes, but at the present time we have no experimental evidence in support of this hypothesis.

The lipid class selectivity exhibited by the galactolipid export implies that this process cannot be achieved by the formation of vesicles budding off from the inner envelope membrane. Indeed, such vesicles should have the same lipid composition as the inner envelope, except if they derive from specialized regions (highly enriched in MGDG) of this membrane. In the latter case however, it remains to understand how bilayer vesicles could be formed from a lipid mixture containing 90–95 mol% of the non-bilayer forming MGDG [32]. Moreover, vesicles originating from the

inner envelope membrane have been observed essentially in greening plastids or in chloroplasts from young leaves, but hardly in mature plastids from fully developed leaves [33].

Galactolipid sidedness in thylakoid membranes

The use of lipolytic enzymes to determine the transbilayer distribution of lipids in thylakoid membranes as well as the validity of this technique have been already described and discussed [11–15]. Here, we have simply employed this technique as a convenient tool to find out the localization of the newly synthesized galactolipid molecules in thylakoid membranes.

Since the elucidation of the galactolipid export mechanism depends essentially on the determination of the transmembrane distribution of labelled galactolipids in thylakoids (see Rationale), it was desirable to 'freeze' as much as possible the chloroplast system at the end of the incubation period with the labelled precursor. A complete 'freezing' was, however, unrealistic because the purification procedure to be applied to thylakoids lasted about 45 min. We took great care to carry out the entire purification procedure as well as the subsequent lipolytic treatment at 0–2°C to minimize further uncontrollable rearrangments of labelled galactolipids across the thylakoid membrane.

The horizontal plateaus of the hydrolysis curves (Figs. 3 and 4) demonstrate that, in terms of mass, galactolipid degradation stopped completely when 64% of the MGDG and 15% of the DGDG content of thylakoids was removed, that is, when the outer monolayer of thylakoid membranes was depleted of all its galactolipid molecules [11–15]. The flatness of these plateaus also indicates that the residual galactolipids – which are assigned to the inner monolayer – remained in place and therefore did not undergo any outward transbilayer movement under our experimental conditions. The same remarks apply, mutatis mutandis (Table II) to the labelled galactolipids. In addition, the parallel behaviour of the hydrolysis curves for mass and radioactivity (Figs. 3 and 4) suggests that upon insertion into thylakoid membranes, the labelled galactolipid molecules do not form segregated pools of altered accessibility to the lipase, but rather dilute rapidly and homogeneously into the bulk of membrane galactolipids.

Galactolipid export: the possible mechanisms

We have already dismissed the possibility that the transfer of galactolipids could be achieved by a vesiculation process, at least in mature chloroplasts (see above). Two other mechanisms, not mutually exclusive, can then be envisaged.

The first mechanism involves the participation of fusion events between the inner envelope membrane and the thylakoid membrane, thereby excluding any water-soluble intermediate in the transfer process. The fusion should be of transient nature and therefore a reversible event. Moreover, the lifetime of the transitory fused structure should be long enough (e.g. of the order of 10^{-3} s) to allow a sizable diffusional flow of (galacto)lipid molecules between membranes via the fusion locus [16], but also short enough to account for the rather scarce evidence for such structures obtained from electron microscopy [33] or inferred from metabolic studies [34]. The similar acyl lipid composition of both inner envelope and thylakoid membranes [35], the low hydration capability of MGDG and DGDG [36], the abundance of the H_{II} phase-forming MGDG [32] together with the high ratio of non-bilayer to bilayer lipids in the outer monolayer of thylakoid membranes [12,13], are expected to facilitate membrane apposition, aggregation, destabilization and fusion [37]. Depending on whether the fusion process connects the inner envelope to the thylakoid membrane by their stroma-facing monolayers only or by establishing a continuum between their whole bilayers, a partial or complete scrambling of lipid molecules should result. However, it is difficult to reconcile a complete fusion mechanism – which postulates an unimpeded lateral diffusion of lipid and protein molecules – with the lipid class selectivity exhibited by the transfer process (Table I) and with the very different polypeptide electrophoretic profiles shown by the inner envelope and the thylakoid membrane [38,39]. Indeed, a complete fusion between the bilayers of both membranes should have yielded similar MGDG/DGDG label ratios for intact plastids (galactolipid synthesis) as well as for purified thylakoids (galactolipid export), which was not the case (Table I). Therefore, a galactolipid transfer mechanism involving transient fusions between the stroma-facing monolayers only of both membranes (e.g. via inverted micellar intermediates, see Ref. 16) is more likely to occur. However, this mechanism implies that the MGDG/DGDG label ratios be the same in the stroma-facing monolayer of the inner envelope membrane and in the whole thylakoid membrane, except if the lateral diffusion rates of these two lipids differ markedly.

The second mechanism involves the participation of soluble stroma protein(s) endowed with galactolipid transfer activity. The lipid class selectivity of the transfer process (Table I) could thus be easily explained by a preferential interaction of this protein with MGDG rather than with DGDG. The plausibility of this mechanism is also supported by the discovery, in spinach chloroplast extracts, of a soluble 30 kDa protein able to transfer (spin-labelled) MGDG molecules in a donor/acceptor vesicle system [40]. Assuming that the putative protein accounts for 0.5% of the soluble protein content of plastids and using an average value of 7.5 mg stroma protein/mg chlorophyll [41], a specific

transfer activity of about 0.6 μmol MGDG (mg protein)$^{-1}$ h^{-1} can be derived from Table I. This value, similar to that determined for a spinach leaf phospholipid transfer protein [42], may thus reflect maintenance and turnover processes occurring within the intact plastid.

At this stage, the two proposed mechanisms can only explain the galactolipid export and its class selectivity (Table I). They cannot, however, account for the three following facts: (a) labelled galactolipids can be found in the inner monolayer of thylakoid membranes as early as their presence can be detected in whole thylakoids; (b) the transmembrane label ratios for MGDG and DGDG are constant over the whole range of incubation conditions tested (from 5 min at 5°C to 90 min at 25°C); (c) the mass and label asymmetry ratios for MGDG are different whereas they are similar for DGDG (Figs. 3 and 4; Table II). This issue can be solved for both mechanisms together if one postulates the occurrence of inwardly directed galactolipid translocating activities in thylakoid membranes. These activities should be triggered as soon as galactolipid molecules penetrate into the outer monolayer and controlled in such a way as to reorient the newly inserted galactolipids according to their respective label asymmetry ratios, independently of the incubation temperature (Table II). Such translocating activities might be aimed at maintaining transmembrane asymmetry, e.g. that of DGDG. In addition, they might be a simple way to relieve the excess of internal stress caused by the entry of H_{II} phase-forming lipids (e.g. MGDG molecules) in the outer monolayer of an already frustrated [37] membrane [12].

Finally, it is worth mentioning that the galactolipid export reported here presents several analogies with the intramitochondrial phospholipid transfer recently described in yeast mitochondria [43]. Indeed, both processes operate in the absence of any electrochemical (proton) gradient and involve a rapid, synthesis-linked lipid transfer between two membranes (possibly achieved via semi-fusion mechanisms and/or specific contact sites) and the occurrence of transmembrane translocating activities.

Concluding remarks

We have shown that in mature spinach chloroplasts, a rapid and continuous transfer of galactolipid occurs from the inner envelope to the thylakoid membranes, MGDG being preferentially exported with respect to DGDG. The transmembrane distribution of the newly inserted galactolipid molecules in thylakoids suggests that the export mechanism consists of transient and partial fusions between stroma-facing monolayers of both membranes (followed by lateral diffusion of galactolipid molecules) and/or of galactolipid transport by soluble protein(s) through the stroma compartment, thus resulting in the insertion of new galactolipid molecules in the outer monolayer of thylakoid membranes. In a second phase, the newly inserted galactolipids are rapidly redistributed across the thylakoid bilayer via translocating mechanisms able to maintain the native (mass) asymmetry of DGDG and, to a lesser extent, that of MGDG.

The present results show that the approach described in the Rationale is valid and reliable. We are currently using this procedure to study how lipid export is achieved in plastids of young, vigorously growing spinach seedlings as well as in plants with eukaryotic lipid metabolism, employing several different labelled precursors. The outcome of these studies will be reported in a near future.

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