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(Galacto)lipid export from envelope to thylakoid membranes in intact chloroplasts. II. A general process with a key role for the envelope in the establishment of lipid asymmetry in thylakoid membranes ☆

A. Rawyler, M. Meylan-Bettex, P.A. Siegenthaler *

Laboratoire de Physiologie Végétale, Université de Neuchâtel, 13, Rue Emile-Argand, CH-2000 Neuchâtel 7, Switzerland

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Abstract

The transfer in organelle of newly synthesized lipid molecules from inner envelope to thylakoid membranes, as well as their subsequent transbilayer distribution in these membranes, have been studied in intact chloroplasts isolated from young and mature spinach, young pea and mature lettuce leaves, using a recently developed methodology (Rawyler, A., Meylan, M. and Siegenthaler, P.A. (1992) Biochim. Biophys. Acta 1104, 331–341). Three radiolabelled precursors were used. UDP-[^{14}C]galactose allowed to follow the fate of mono- and digalactosyldiacylglycerol (MGDG and DGDG) made from polyunsaturated, preexisting diacylglycerol (DAG), whereas [^{14}C]acetate and [^{14}C]glycerol 3-phosphate were used to follow the fate of MGDG and phosphatidylglycerol (PG), respectively, after de novo synthesis. MGDG, DGDG and PG molecules assembled at the envelope level were found to be exportable to thylakoids in amounts strictly proportional to the amounts synthesized, provided that the necessary substrates were not limiting. Lipid export was class-selective; under our conditions, as much as 50–80% of the MGDG, 87% of the PG and 20–30% of the DGDG synthesized were exported to thylakoids. However, within the MGDG class labelled from [^{14}C]acetate, there was hardly any selectivity in the export of its various molecular species. For MGDG, the proportionality coefficient, which reflects the efficiency of the export process, was higher in chloroplasts from young than from mature leaves, and higher in spinach than in pea and lettuce. Temperature affected the efficiency of galactolipid export in a class-dependent way. MGDG synthesis and export had similar Q_{10} values of about 4 in young and 3 in mature spinach leaves, while the Q_{10} of DGDG export was higher than that of its synthesis. In most cases, the transmembrane distribution of labelled lipids in thylakoids was found to match closely the corresponding distribution of mass, regardless of plant age and species and of incubation time and temperature. In some cases however, small but significant differences occurred between the label and the mass transbilayer distributions of MGDG (labelled molecules more inwardly oriented), DGDG and PG (more outwardly oriented). We propose a general model in which the thylakoid lipid asymmetry is primarily preestablished in the chloroplast envelope by the topography of its lipid-synthesizing enzymes, together with the occurrence of relatively fast lateral diffusion and translocation rates of the newly synthesized lipids. Transient fusions between inner envelope and thylakoid membranes would allow lipid export by lateral diffusion and build the observed lipid asymmetry in the latter.

Keywords: Chloroplast; Thylakoid; Lipid synthesis; Mono(di)galactosyldiacylglycerol; Lipid asymmetry

1. Introduction

In chloroplasts of higher plants, the thylakoid membrane network develops an extended surface area which maximizes its absorbance and, consequently, its overall efficiency for photon capture and energy transduction. This is reflected, at the molecular level, by the fact that at least 90% of the chloroplast acyl lipids belong to the thylakoids whereas the remaining 10% are shared between the two envelope membranes [1]. However, thylakoids are deprived of lipid synthesis ability [2,3] and must therefore rely on the chloroplast envelope for their supply of membrane lipids. Lipid export from envelope to thylakoid membranes is thus implicitly assumed, but to date the mechanisms by which this process is achieved remain largely unknown.

☆ The authors are delighted to dedicate this paper to Prof. Hartmut K. Lichtenthaler on the occasion of his 60th anniversary.

Abbreviations: DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; Mops, 4-morpholinopropanesulfonic acid; PG, phosphatidylglycerol; Tricine, *N*-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine.

* Corresponding author. Fax: +41 38 232271.

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Experimental evidence suggesting that galactolipid molecules were exported from the envelope to the thylakoid membranes was first provided by Williams et al. [4] and later confirmed [5,6]. However, apart its occurrence and its rapidity, no information is available on the detailed aspects of this export process. More recently, this problem has been tackled by two groups using different approaches. Morré et al. have used a cell-free system to show that MGDG can be transferred from isolated envelope vesicles to immobilized thylakoid membranes in an ATP- and temperature-dependent manner [7]. In our laboratory, we have worked under more physiological conditions and studied the galactolipid export in organelle using intact spinach chloroplasts supplied with UDP-[^{14}C]galactose [8]. Under these conditions, galactolipids are rapidly and continuously transferred from the inner envelope to the thylakoid membranes, MGDG being preferentially exported with respect to DGDG. This process does not require any intervention of electrochemical gradients. From the transmembrane distribution of labelled galactolipids in thylakoids, we concluded that the export process was most probably due to transient semi-fusion mechanisms between stroma-facing monolayers of inner envelope and of thylakoid membranes [8].

However, our work [8] dealt essentially with methodological aspects, among which the obtention of highly pure, envelope-free thylakoid membranes was a main concern. In particular, we devised a procedure by which thylakoids can be prepared from chloroplasts while removing $\geq 99\%$ of the envelope amount initially present. In addition, UDP-[^{14}C]galactose was the only precursor used, which means that the export process studied in [8] was primarily that of polyunsaturated (hexaene) molecular species of MGDG and DGDG [9]. It would be of interest to know if other lipids synthesized by the plastid (e.g., mono- or oligoene species of MGDG, PG, etc) are also exported to thylakoids via the same mechanism and what is the nature of this mechanism. One may also wonder whether the lipid export process is common to different plants (e.g., those having the so-called 16:3- or 18:3-metabolism) and whether it depends on the developmental stage of the plant (e.g., in chloroplasts from young, vigorously growing seedlings or from mature, fully expanded leaves). The present article is aimed at providing answers to these questions.

2. Material and methods

2.1. Chemicals

UDP-[U-^{14}C]galactose (11.1 GBq/mmol), [1-^{14}C]acetate (1.95 GBq/mmol), L-[U-^{14}C]glycerol 3-phosphate (5.55 GBq/mmol) were purchased from Amersham. The lipase (EC 3.1.1.3) from *Rhizopus arrhizus* was from Boehringer. The phospholipase A_2 (EC 3.1.1.4) from *Vipera russelli*, UDP-galactose and UDP were from Sigma. All other chemicals and solvents were 'purissimum' Fluka products. Bovine serum albumin was defatted as reported in [10].

2.2. Material

Spinach (*Spinacia oleracea*, var. Nobel) and pea (*Pisum sativum*, var. Primavil or Douce Provence) seeds, soaked 10 min in 0.05% sodium hypochlorite and thoroughly washed, were sowed on Vermiculite moistened with a commercial nutrient solution and grown for 10 days (pea) or 21 days (spinach) at 20°C. Seedlings were harvested in the early morning for chloroplast isolation.

Mature spinach and lettuce (*Lactuca sativa*), 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 before being used for chloroplast isolation.

2.3. Methods

Intact chloroplasts were prepared as in [11] and repurified after labelling as in [12]. Incubation with UDP-[^{14}C]galactose, preparation of purified, envelope-free thylakoid membranes, lipolytic treatment of these thylakoids by the lipase from *R. arrhizus*, one-phase lipid extraction as well as lipid separation and analysis were carried out exactly as described [8]. All experiments dealing with galactolipids were done using the procedures given in [8], regardless of the precursor used.

2.4. Incubations with [1-^{14}C]acetate and L-[U-^{14}C]glycerol 3-phosphate

Fresh intact chloroplasts (equivalent to 3 mg chlorophyll) were added to a medium consisting of 330 mM sorbitol/50 mM Mops-NaOH (pH 7.9)/10 mM $NaHCO_3$/2 mM EDTA-Na_2/2 mM $MgCl_2$/1 mM $MnCl_2$/0.4 mM KH_2PO_4, supplemented with 2.5 mM glycerol 3-phosphate, 0.1 mM UDP-galactose and 0.18 mM sodium [1-^{14}C]acetate (718 Mbq/mmol). This suspension (total volume = 10 ml) was incubated as a thin (0.25 cm) layer under white light (60 W/m^2) for 20–60 min at 25°C. Alternatively, fresh intact chloroplasts (equivalent to 3 mg chlorophyll) were added to a medium consisting of 330 mM sorbitol/50 mM Tricine-KOH (pH 7.9)/10 mM $KHCO_3$/10 mM $MgCl_2$/2 mM $MnCl_2$/0.2 mM KH_2PO_4/0.2 mM sodium acetate, supplemented with 2 mM CTP and 0.33 mM L-[U-^{14}C]glycerol 3-phosphate (18.4 MBq/mmol). This suspension (total volume = 15 ml) was incubated as a thin (0.35 cm) layer under white light (60 W/m^2) for 60–90 min at 25°C. In both cases, intact labelled chloroplasts were then repurified as in [12].

2.5. Phospholipase A_2 treatment of purified thylakoids

Thylakoid membranes were suspended in a medium consisting of 200 mM sorbitol/50 mM KCl/25 mM Mops-KOH (pH 7.0)/5 mM $MgCl_2$/5 mM $CaCl_2$, supplemented with defatted bovine serum albumin (25 mg/ml), in a total volume of 3 ml and at a chlorophyll concentration of 0.5–0.6 mg/ml. After sampling aliquots for chlorophyll (2×40 μl) and radioactivity (2×40 μl) determinations, phospholipase A_2 was added (0.7 units/mg chlorophyll) and the incubation was carried out at 15°C for 50 min under gentle, continuous stirring. Aliquots (0.3 ml) were taken at regular time intervals for a two-phase lipid extraction.

2.6. Two-phase lipid extraction

The thylakoid membrane aliquot (0.3 ml) was added to a mixture of 6.5 ml chloroform/methanol (53:37, v/v) and of 0.1 ml 250 mM EGTA, followed by strong vortexing. The two-phase separation was obtained by adding 3.2 ml 0.5 M KCl, followed by vigorous shaking and centrifugation ($3000 \times g$, 5 min).

2.7. Lipid separation and analysis

The resulting lower phase was dried under N_2 flow and the lipids, taken up in chloroform/methanol (8:2, v/v), were applied quantitatively on silicagel plates using an automatic sample applicator (Linomat IV, Camag). Plates were developed in chloroform/methanol/25% aqueous NH_3/water (65:35:3:2, by volume), dried in air, lightly sprayed with 0.01% primuline and viewed under UV light. Zones of interest (PG, MGDG, and chlorophylls) were scraped into glass tubes and analyzed for lipid phosphorus [13], lipid galactose [14] and pheophytin [15] content. Silicagel zones containing radioactive PG were scraped into scintillation vials, dispersed in 4 ml of scintillation liquid (Optiphase HiSafe II, Pharmacia) and the radioactivity determined in a Kontron Betamatic II counter. Lipid phosphorus and radioactivity amounts in PG were finally normalized with respect to MGDG and to the pigment contents. These normalized data were then semilogarithmically plotted against time to obtain the mass and label transmembrane distributions of PG in thylakoid membranes, as described elsewhere [8,10,16].

2.8. Other techniques

The quantitative determination of chlorophylls and of proteins was done according to the methods of Bruinsma [17] and Markwell et al. [18], respectively. Additional details are given in the legends to figures and tables.

3. Results

For the sake of simplicity, the methodological aspects of our approach of the lipid export process will not be

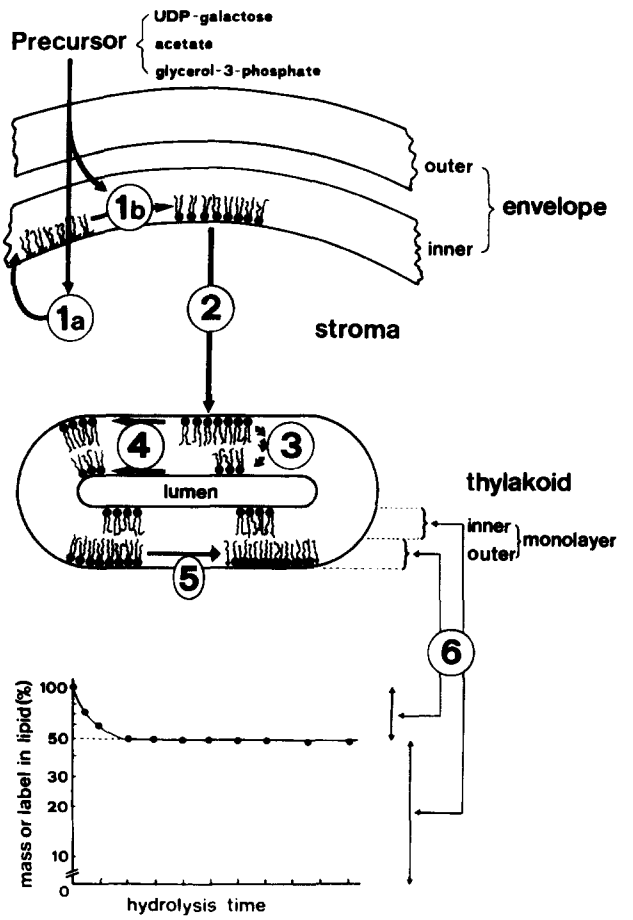

Fig. 1. Strategy used to study the lipid export in intact chloroplasts. In the first four 'in organelle' steps (1–4), intact chloroplasts are provided (step 1) with a suitable radioactive precursor together with all necessary cofactors and allowed to synthesize membrane lipids [9] either from the de novo pathway (step 1a: labelled acetate or glycerol 3-phosphate) or by galactosylation of preexisting lipids (step 1b: labelled UDP-galactose). Concomitantly, some of the newly made lipids are exported (step 2) from their site of synthesis (the inner envelope membrane) to their final destination (the thylakoids). When present in the thylakoid membrane, these lipids may undergo transbilayer movement (step 3) and thereby may be redistributed between the outer (stroma-facing) and inner (lumen-facing) monolayers. Finally, lipids may also diffuse laterally (step 4). Since a method is available to prepare pure, envelope-free thylakoid membranes from intact chloroplasts [8], then a comparative determination of the amount of lipid radioactivity in the intact plastids and in the corresponding thylakoid membranes will give direct information on the extent, rate and selectivity of the lipid export process. In addition, purified thylakoid membranes can be treated with lipolytic enzymes [8,10]; for instance, both the lipase from R. arrhizus and the phospholipase A_2 will generate a free fatty acid (single-tailed arrow) and the corresponding lyso-lipid (single-tailed circle) from galactolipids and phosphatidylglycerol (double-tailed circles), respectively (step 5). Then, a comparative sidedness analysis of the lipid mass and label distribution in both monolayers of the thylakoid membranes can be performed (step 6). This should help identifying which export mechanisms – involving stromal intermediates (vesicles, lipid transfer protein) or not (semi- or complete fusion) – are at work [8].

Table 1
Distribution of radioactive label in some lipid classes of intact chloroplasts isolated from various plant species and of thylakoid membranes purified therefrom

Precursor	Lipid class	Label incorporation (% of total) [a] in:	
Plant		chloroplasts	thylakoids
UDP-[^{14}C]galactose:			
Mature spinach	MGDG [b]	79.4 ± 5.8 (8)	93.2 ± 3.1 (8)
	DGDG [b]	11.7 ± 1.1 (3)	5.3 ± 2.5 (3)
Young spinach	MGDG [b]	83.7 ± 4.6 (4)	93.1 ± 2.6 (4)
	DGDG [b]	7.8 ± 0.6 (3)	3.6 ± 0.4 (3)
Young pea	MGDG [b]	66.5 ± 5.2 (7)	86.9 ± 5.3 (7)
	DGDG [b]	18.7 ± 4.7 (7)	9.2 ± 3.3 (7)
Mature lettuce	MGDG [b]	52.5 ± 8.4 (5)	83.8 ± 9.3 (3)
	DGDG [b]	36.6 ± 3.5 (3)	19.4 (1)
[^{14}C]Glycerol 3-phosphate:			
Mature spinach	PG [c]	74.3 ± 6.9 (2)	91.2 ± 0.7 (2)

Incubation of intact chloroplasts was carried out at 25°C and lasted 5–60 min in the presence of UDP-[^{14}C]galactose (+darkness) and 60–90 min in the presence of [^{14}C]glycerol 3-phosphate (+light). Values given are averages ± S.D., and the number of experiments is mentioned in parentheses.
[a] % of total label incorporation in each fraction.
[b] Remaining label was in tri- and tetragalactosyldiacylglycerol.
[c] Most of the remaining label was found in diacylglycerol and in phosphatidic acid.

discussed here, because they have been extensively covered in [8]. However, a condensed description of the strategy is outlined in Fig. 1 for the reader's convenience.

We shall first present results dealing with lipid synthesis and export, that is, steps 1a, 1b and 2 in Fig. 1. Radioactivity was readily incorporated into lipids of intact chloroplasts and of thylakoids upon incubation of the former with appropriate labelled precursors (Tables 1 and 2). For instance, in the presence of UDP-[^{14}C]galactose (Table 1), the label was found primarily in MGDG and, to a lesser extent, in DGDG. The label distribution between these two lipid classes depended on the plant species. In spinach chloroplasts, MGDG synthesis was largely favoured over that of DGDG (with a MGDG/DGDG label ratio of 7–11) whereas it was much less so in pea (ratio of 3.5) and especially in lettuce (ratio of 1.4) chloroplasts. Both labelled galactolipids were also found in thylakoid membranes, where the preferential accumulation of MGDG – relatively to DGDG – was clearly evident (the MGDG/DGDG label ratio was 18–26 for spinach, 9.4 for pea and 4.3 for lettuce thylakoids). With [^{14}C]glycerol 3-phosphate as precursor (Table 1), PG accounted for about 74% and 91% of the label incorporated in chloroplasts and thylakoids, respectively. Finally, when the de novo lipid synthesis was carried out with spinach chloroplasts illuminated in the presence of [^{14}C]acetate, all those lipid classes which were synthesized by the chloroplast (including diacylglycerol, free fatty acids, MGDG, DGDG and phosphatidic acid) were also found in thylakoids, and in comparable proportions (Table 2). Here again, and regardless of the developmental stage of the spinach leaf, the accumulation of MGDG in thylakoid membranes was favoured over that of DGDG. Indeed, the MGDG/DGDG label ratio was 3.6–4.5 in chloroplasts but rose up to 6.5–9.6 in thylakoids of young and mature leaves, respectively (Table 2). However, a closer look at these data

Table 2
Distribution of radioactive label in some lipid classes of intact chloroplasts isolated from mature or young spinach leaves and of thylakoid membranes purified therefrom

Precursor	Lipid class	Label incorporation (% of total) [a] in	
Plant		chloroplasts	thylakoids
[^{14}C]Acetate:			
Mature spinach	diacylglycerol + free fatty acids	59.3	70.5
	MGDG	33.0	26.8
	DGDG	7.3	2.8
	phosphatidic acid	0.4	tr
Young spinach	diacylglycerol + free fatty acids	55.6	60.7
	MGDG	33.1	33.3
	DGDG	9.2	5.1
	phosphatidic acid	1.0	0.8

The incubation of intact chloroplasts was carried out at 25°C in the light and lasted 60 min in the presence of [^{14}C]acetate, UDP-galactose and glycerol 3-phosphate. The indicated values are those of a single experiment.
[a] % of total label incorporation in each fraction. tr, traces.

reveals that the marked increase in the thylakoidal MGDG/DGDG label ratios was rather due to a strong restriction of the DGDG export towards thylakoids, as shown by the relative proportion of DGDG in thylakoids being at least half of that in whole chloroplasts (Tables 1 and 2), whereas MGDG and PG were only slightly enriched (Table 1) or not at all (MGDG in Table 2).

The quantitative relation between lipid synthesis (measured as the amount of radiolabel accumulated in chloroplast lipid classes) and lipid export (determined as the amount of radiolabel present in the corresponding lipid classes of thylakoid membranes) has been studied in various plants and with different lipid precursors. Fig. 2 shows two sets of data obtained after incubation of chloroplasts isolated from mature spinach leaves with either [^{14}C]acetate or UDP-[^{14}C]galactose. In both cases, the amount of labelled MGDG found in thylakoids depended linearly on the amount of labelled MGDG synthesized in the intact plastid. Both data sets were found to extrapolate almost exactly through the origin of the graph and a regression analysis indicated that their slope was similar (see also Table 3), irrespectively of the precursor used. This linear dependence of MGDG export upon MGDG synthesis was maintained at least up to 40 min with UDP-[^{14}C]galactose and to 60 min of incubation with [^{14}C]acetate. Similar results were obtained with all plants and all precursors so far tested, as reported in Table 3. In addition, Table 3 shows that the value of the coefficient *m*, which represents the proportion of chloroplast lipid synthesis used for export toward thylakoids, was influenced by several parameters. Thus, for MGDG, young spinach leaves had a higher value of *m* than mature spinach leaves, both at 5 and 25°C. The same trend could be observed when comparing young pea leaves with mature lettuce leaves. This was not the case, however, for DGDG, for which the value of *m*

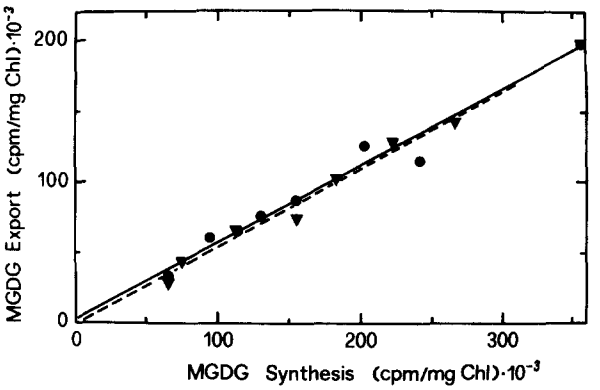

Fig. 2. The dependence of MGDG export from the inner envelope membrane to thylakoids on MGDG synthesis in intact chloroplasts isolated from mature spinach leaves. Chloroplasts were incubated for various time periods (ranging from 5 to 60 min) at 25°C in the presence of UDP-[^{14}C]galactose (+darkness) (●) or of [^{14}C]acetate (+light) (▼). Chloroplasts and thylakoids (purified therefrom) were extracted, lipids were separated and MGDG radioactivity was determined and expressed on a chlorophyll basis [8]. The linear regression analysis of these data is presented in Table 3. Chl, chlorophyll.

remained unchanged between spinach chloroplasts from young or mature leaves. The value of *m* varied also according to the plant species. With UDP-[^{14}C]galactose as a precursor for MGDG, *m* was significantly greater in young spinach than in young pea, and in mature spinach than in mature lettuce. Moreover, Table 3 shows that temperature diversely affected the value of *m*; indeed, for MGDG, *m* increased only by a factor of 1.2 between 5 and 25°C, both in young and in mature spinach leaves, whereas it almost doubled for DGDG in the same temperature range in young spinach seedlings. Finally, Table 3 shows that in spinach chloroplasts, close to 90% of the newly made PG molecules were exported towards thylakoids.

Table 3
Relationship between the extent of lipid synthesis (*S*) (cpm/mg chlorophyll) from various ^{14}C-labelled precursors in intact chloroplasts and the extent of intraplastidial lipid export (*E*) (cpm/mg chlorophyll) from inner envelope to thylakoid membranes in various plant species, given by the slope *m*, the constant term *h* (cpm/mg chlorophyll) and the correlation coefficient *r* of the linear regression equation $E_T = m * S_T + h$, computed from a number *n* of experiments.

Plant species	Labelled precursor	*T* (°C)	Lipid class	*m*	*h* [a] ×10^{-3}	*r*	*n*
Mature spinach	acetate	25	MGDG	0.551	−1.93	0.991	7
	UDP-gal	5	MGDG	0.435	0.12	0.999	2
	UDP-gal	25	MGDG	0.534	3.25	0.986	9
	UDP-gal	25	DGDG	0.317	−0.02	0.999	2
Young spinach	UDP-gal	5	MGDG	0.674	0.51	0.981	5
	UDP-gal	25	MGDG	0.819	−3.49	0.989	4
	UDP-gal	5	DGDG	0.167	0.17	0.988	5
	UDP-gal	25	DGDG	0.320	0.36	0.995	4
Mature lettuce	UDP-gal	25	MGDG	0.477	−1.36	0.995	3
Young pea	UDP-gal	25	MGDG	0.588	−1.35	0.989	4
	UDP-gal	25	DGDG	0.193	−0.60	0.952	4
Spinach	G-3-*P*	25	PG	0.871	−0.18	0.991	3

T, temperature; UDP-gal, UDP-[^{14}C]galactose; G-3-*P*, [^{14}C]glycerol 3-phosphate.
[a] These values of *h* are actually small with respect to the range of S_T and E_T values, and in each case, they were very close to the origin of the plot (see also Fig. 2).

Table 4
Q_{10} values of UDP-[^{14}C]galactose-dependent galactolipid synthesis and export in chloroplasts isolated from young and mature spinach leaves

Spinach leaves	T	Synthesis				Export			
		MGDG		DGDG		MGDG		DGDG	
		rate	Q_{10}	rate	Q_{10}	rate	Q_{10}	rate	Q_{10}
	25	1.014		0.251		0.796		0.080	
Young			3.9		3.8		3.9		4.5
	5	0.067		0.017		0.051		0.004	
	25	0.692		0.024		0.366		0.008	
Mature			2.8		–		2.9		–
	5	0.088		n.d.		0.042		n.d.	

Rates of synthesis and export at 5 and 25°C were determined from 5 min incubation periods in the presence of the precursor and expressed as nmol precursor incorporated/(mg chlorophyll) per min. The Q_{10} values were then calculated (assuming Q_{10} constancy over the 5–25°C range) as (rate at 25°C/rate at 5°C)$^{1/2}$. T, temperature (°C).
n.d., not determined.

The role of the temperature was also assessed by determining the Q_{10} values of galactolipid synthesis and export rates in spinach chloroplasts from young and mature leaves, using UDP-[^{14}C]galactose as precursor. Table 4 indicates that in young spinach leaves, the synthesis and the export of MGDG as well as the synthesis of DGDG had very similar Q_{10} values whereas the Q_{10} of DGDG export was significantly higher. In addition, the corresponding Q_{10} values for MGDG were markedly lower in mature leaves than in young leaves.

It is well known that the MGDG molecules synthesized de novo from labelled acetate are primarily monoene species which then undergo further intrachloroplastic desaturation steps [9,19], giving thus rise to a number of different molecular species [20]. We took advantage of this fact and allowed intact chloroplasts to incorporate [^{14}C]acetate into MGDG, which was then exported to thylakoids. Both plastidial and thylakoidal MGDG classes were isolated and further fractionated by argentation thin-layer chromatography. Under our conditions, they were shown (Table 5) to consist of four molecular species ranging from monoene (major species) to tetraene (minor one). These four MGDG species had similar relative proportions in chloroplasts and in thylakoids. Accordingly, they were all exported essentially to the same extent.

We report now some results on the transmembrane distribution adopted by the newly synthesized lipid molecules after their insertion into thylakoid membranes, that is, steps 3–4 in Fig. 1. Lipolytic treatments of thylakoids were carried out (steps 5–6 in Fig. 1) with the lipase from *R. arrhizus* or with *Vipera russelli* phospholipase A_2 to determine the transbilayer distribution of galactolipids (MGDG and DGDG), respectively of PG, both in terms of mass and of radioactivity incorporated, after incubation of intact chloroplasts in the presence of three different ^{14}C-labelled precursors. Fig. 3 summarizes these data. Concerning the transmembrane distribution of mass, the outer thylakoid monolayer was enriched in MGDG (57–65 mol%) and in PG (72 mol%) whereas the inner monolayer was markedly enriched in DGDG (85–90 mol%) (Fig. 3, black rectangles). This was observed for each plant species, in full agreement with earlier studies [8,10,16,23]. However, the transmembrane distribution of label showed two different trends. In the first one, it matched exactly the corresponding mass distribution. This was the case for DGDG in mature spinach, for MGDG in young spinach, for DGDG in young pea and for both galactolipids in mature lettuce (Fig. 3B, D, G, H and I). In the second trend, the distribution of label differed significantly from the corresponding mass distribution, and in a way that depended on the lipid class considered. For instance, in mature spinach and in young pea, the newly synthesized MGDG molecules (from either UDP-

Table 5
Comparison between the MGDG molecular species synthesized de novo from [^{14}C]acetate in intact chloroplasts and those found in thylakoid membranes isolated from these chloroplasts

MGDG	Chloroplasts		Thylakoids		
	IOD	relative IOD (% of total)	IOD	relative IOD (% of total)	export (%)
18:1/16:0	24.90	49.9	10.51	45.9	42.2
18:2/16:0	10.63	21.3	4.88	21.3	45.9
18:3/16:0	11.43	22.9	6.01	26.2	52.6
18:3/16:1	2.96	5.9	1.50	6.5	50.5
Total	49.92	100	22.90	100	46.0 [a]

In this particular experiment, spinach chloroplasts from mature leaves were incubated with [^{14}C]acetate for 60 min at 25°C (see Materials and methods); thylakoids were then isolated and purified as in [8]. Lipids were extracted from both chloroplasts and thylakoids and MGDG, quantitatively isolated as a class by thin-layer chromatography, was rechromatographed on silicagel 60 plates (predeveloped with 5% $AgNO_3$ in methanol, air-dried and stored in darkness) using a mixture of chloroform/methanol/water (65:35:5, by volume) to separate the molecular species. After autoradiography of the plate, the film (Kodak X-OMAT) was scanned (transmission mode) and the digitalized image thus obtained was processed using the BioImage System (Millipore). The data output (expressed as IOD, integrated optical density for each band, and corresponding to a radioactivity amount) was normalized with respect to the chlorophyll content of the initial lipid extract, thus allowing the amounts of each MGDG molecular species in both chloroplasts and thylakoids to be compared. The amount of MGDG exported to the thylakoid membranes could then be calculated: Export = 100 × (IOD in thylakoids)/(IOD in chloroplasts). Tentative identification of MGDG molecular species was done according to published data [20–22].
[a] The export of MGDG class, determined by conventional liquid scintillation counting, was 46.5% in this particular experiment. This underlines the good agreement between the direct radioactivity measurements and the densitometric analysis of autoradiograms.

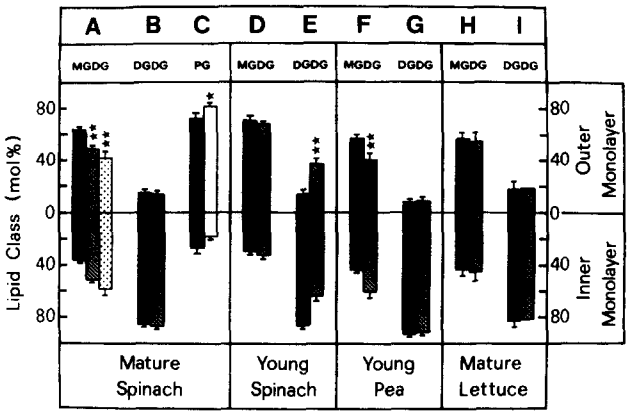

Fig. 3. Comparison between the mass (■) transbilayer distribution of MGDG, DGDG and PG in thylakoid membranes of different plant species and the label transbilayer distribution of the same lipid classes after incubation of intact chloroplasts at 25°C with UDP-[^{14}C]galactose (hatched), [^{14}C]acetate (stippled) or [^{14}C]glycerol 3-phosphate (□) as precursor. The data were submitted to paired analysis of variance, comparing label distribution with mass distribution for each lipid class. Where indicated by one or two stars (*, **), the difference was either significative ($P < 5\%$) or highly significative ($P < 1\%$), respectively. For each lettered case, the number of experiments (given in parentheses from left to right) was (5;11;7) for A, (5;3) for B, (4;3) for C, (4;6) for D, (4;3) for E, (6;7) for F, (6;6) for G, (2;3) for H and (2;1) for I.

[^{14}C]galactose or [^{14}C]acetate) were more inwardly distributed (by 15–20 mol%) than the bulk of MGDG (Fig. 3A and F). On the other hand, the newly synthesized DGDG molecules in young spinach were more outwardly oriented (by about 25 mol%) than the bulk of DGDG (Fig. 3E); likewise, the labelled PG molecules in mature spinach were more outwardly distributed (by about 10 mol%) than the bulk of PG (Fig. 3C).

Since at least four labelled MGDG molecular species were found in purified thylakoids after incubation of intact chloroplasts with [^{14}C]acetate (see Table 5), it was of interest to know whether these various MGDG species adopted a similar transbilayer distribution or not. To this end, we submitted thylakoid membranes (previously purified from [^{14}C]acetate-labelled chloroplasts) to a lipolytic treatment [8]. Both MGDG and its corresponding lyso-derivative were then quantitatively isolated as lipid classes and resolved by argentation thin-layer chromatography into molecular species, the radioactivity of which was quantitatively determined (Fig. 4). Since there was little difference between the hydrolysis curves of monoene and diene MGDG species, as well as between those of triene and tetraene species, we pooled them into two groups (mono- + diene; tri- + tetraene) of MGDG species and compared them to that of the globally labelled MGDG class. Fig. 4 clearly indicates that the enzymatic degradation of MGDG in thylakoid membranes was a biphasic process. The first, rapid phase was essentially independent of the unsaturation degree of MGDG species, and resulted in a general 30% decrease in MGDG content within two minutes of lipase treatment. The second phase corresponded to the establishment of an almost horizontal plateau, the level of which being now characteristic of the MGDG molecular species considered. After semilog transformation of the data (see legend to Fig. 4), extrapolation of each plateau to zero time yielded values (open symbols) which represent that percentage of a given MGDG molecular species attributable to the inner thylakoid monolayer. From these values, the outside/inside distribution (in mol%) can be derived. Thus the mono- and diene MGDG species, which together accounted for 72% of the total MGDG label, showed a ratio of 27:73. On the other hand, the tri- and tetraene MGDG species, which together accounted for 28% of the total MGDG label, adopted a 65:35 distribution. From these data, one can calculate the theoretical percentage of total labelled MGDG attributable to the inner thylakoid monolayer: $(0.72 \times 73) + (0.28 \times 35) = 62.4\%$. This value is in good agreement with the experi-

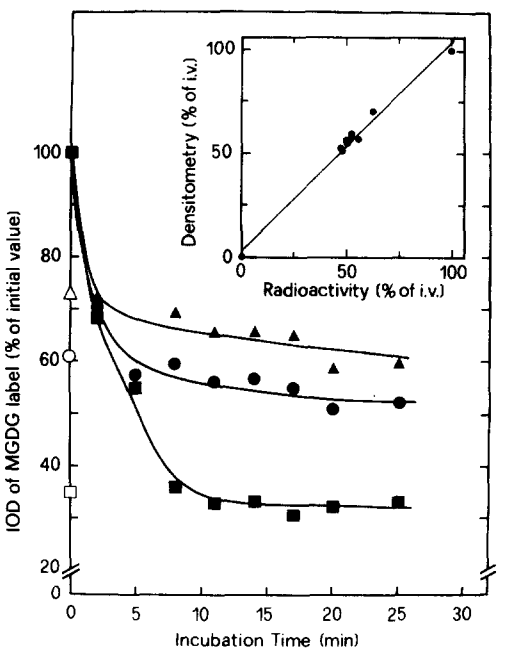

Fig. 4. Time-course of MGDG (class and molecular species) hydrolysis in thylakoid membranes treated by the lipase from *R. arrhizus*. Spinach chloroplasts from mature leaves were incubated with [^{14}C]acetate for 60 min at 25°C. After lipid extraction and separation, the silicagel plate was autoradiographed and the film scanned and processed with the BioImage System (Millipore). Alternatively, MGDG spots were scraped off for scintillation counting. As shown by the inset, there was a very good agreement (slope = 1.009; $r = 0.993$) between radioactivity directly measured by scintillation counting and that estimated from the densitometric analysis of the autoradiogram. The integrated optical density (IOD) of each MGDG molecular species was determined, together with that of the 1-lyso derivative. Data were normalized with respect to the sum [(MGDG) + 2 × (lyso-MGDG)] and expressed as % of initial value (i.v.). ●, total MGDG class; ▲, 18:1/16:0 + 18:2/16:0 molecular species; ■, 18:3/16:0 + 18:3/16:1 molecular species. Open symbols on the ordinate axis correspond to the extrapolation to zero time of the hydrolysis curves, plotted semilogarithmically against time. By linear regression analysis of the second (linear) phase of these plots, one thus finds 73% for mono- + diene MGDG (△), 35% for tri- + tetraene MGDG (□) and 61% for the total labelled MGDG (○).

mental value of 61% (see open circle in Fig. 4) determined in a parallel experiment by scintillation counting, and falls within the general range of values represented in Fig. 3A (lower dotted bar).

Finally, it must be noted that all transmembrane distribution ratios of labelled lipids determined so far were constant (within experimental error) over the whole range of incubation times (5 to 90 min) in the presence of a given labelled precursor. They were thus independent of the amount of lipid synthesized and exported, at least within the abovementioned range, and no influence of age or of species could be detected. Moreover, we could not observe any noticeable change in these ratios even in those cases where label and mass transmembrane distributions were significantly different (e.g., Fig. 3A and F). Unlike export, the asymmetric distribution of label in thylakoid lipids depends therefore neither on the developmental nor on the metabolic status of the plant.

4. Discussion

The results presented in this article confirm and extend those reported previously [8]. Altogether, they suggest the following picture, which will be discussed below. The (in vitro) intrachloroplastic export of acyl lipids from their initial biosynthesis site (the inner envelope membrane) to their final destination site (the thylakoid membrane) is a very general process which occurs in various higher plants and involves most of the lipid classes of the inner chloroplast envelope. Lipid export is a continuous and relatively fast process, with a marked temperature dependence. It is also characterized by an efficiency and a class (but not molecular species) selectivity which depend at least in part on the developmental and metabolic status of the plant. In the last step of the export process, those lipid molecules now located in thylakoid membranes adopt a transmembrane distribution that is either definitive or (particularly when desaturation is still in progress) liable to further adjustments.

The use of different lipid precursors has thus revealed that not only galactolipids but also PG molecules are exported towards thylakoids after being assembled at the envelope level (Tables 1–3). Although we did not carry out similar experiments with sulfoquinovosyldiacylglycerol (which is also synthesized by the chloroplast envelope [24,25]), it is however sensible to suggest that lipid export is extendable, qualitatively speaking, to all lipid classes present in the inner chloroplast envelope. An additional peculiarity of the lipid transfer process is its class selectivity, which expresses the fact that certain lipids (including MGDG and PG) are systematically exported towards thylakoids in clear preference to others (e.g., DGDG) (Tables 1 and 2). Our data indicate that this class selectivity can be accounted for more by a restricted export of DGDG rather than by an enhanced export of MGDG and PG. This suggests that the topography of lipid-synthesizing enzymes at the envelope level is involved in this selectivity. Indeed, DGDG is formed by a dismutation of two MGDG molecules catalyzed by the galactolipid:galactolipid galactosyltransferase [20,26], which is localized in the outer envelope membrane [27]. The synthesis and the export of DGDG require thus the following sequence of events: (i) new MGDG molecules, made in the inner envelope membrane [28], diffuse to the outer envelope membrane, probably through the multiple contact sites established between both membranes [29,30]; (ii) the MGDG molecules now dismute to diacylglycerol and DGDG molecules, a part of which (iii) may leave then the outer membrane and diffuse back to the inner membrane via the contact sites. DGDG's availability for export is however limited by the obligatory passage of DGDG in the outer envelope membrane and its subsequent local dilution in it and by expectedly slow rates of transbilayer movement through both envelope membranes, resulting in the low values of m for DGDG in Table 3 and consistent with the particularly high Q_{10} value for DGDG export (Table 4). In contrast, lipids such as MGDG and PG are assembled exclusively in the inner envelope membrane [28,31] and it is therefore conceivable that their export towards thylakoids remains unimpeded.

In [8], we envisaged the lipid export from envelope to thylakoid membranes as the result of four possible mechanisms, namely (a) the inner envelope membrane buds off and releases vesicles which move through the stroma space and fuse with thylakoids; (b) a lipid transfer protein extracts a lipid molecule from the inner envelope membrane, carries it through the stroma and inserts it into the thylakoid membrane; (c) partial or (d) complete fusions are triggered as transient events between inner envelope and thylakoid membranes without occurrence of stromal intermediates. The mechanism(s) responsible for the intrachloroplastic lipid export should account at least for: (i) the linear dependence of lipid export on lipid synthesis (Fig. 2; Table 3); (ii) the comparable export efficiency of all molecular species within a given lipid class (e.g., MGDG) (Tables 3 and 5) but the different export efficiencies between lipid classes (Tables 1–3); (iii) the almost immediate establishment of a stable transmembrane distribution (see text in Results) for each lipid subclass in thylakoids after labelling with a given precursor (Fig. 3); (iv) the marked differences between the transmembrane distributions of newly synthesized MGDG subclasses (Fig. 3) and molecular species, particularly mono- + diene, tri- + tetraene and native (mostly hexaene) species (Fig. 4). Mechanisms (b) and (c), which transfer lipid molecules only between the stroma-facing monolayers of inner envelope and thylakoid membranes, cannot account for these features, unless one makes the additional postulate – for which there is no experimental evidence to date – of the occurrence of fast transbilayer movements in thylakoid membranes. On the other hand, mechanisms (a) and (d) involve complete fusion events during which both mono-

layers of each membrane merge. Accordingly, they are the simplest mechanisms able to satisfy requirements (i) to (iv).

Our results do not allow to discriminate unequivocally between transient fusion and vesicular mechanisms. Both could coexist, at least at an early developmental stage [32]. This view is supported by the data of Moreau et al. [33] which indicate that in a cell-free system from rat liver, the lipid trafficking and sorting between the endoplasmic reticulum and the Golgi apparatus exhibited two kinetically distinct components, an ATP-stimulated vesicular transfer and an ATP-independent non-vesicular transfer. However, we showed that the efficiency of the intrachloroplastic MGDG export was identical in the light and in the dark (Fig. 2) and therefore essentially independent of ATP supply, which suggests a non-vesicular mechanism. Moreover, the Q_{10} values determined here (Table 4) are higher (by 1–2 units) than those observed for the cell-free ATP-dependent MGDG transfer between isolated envelope vesicles and immobilized thylakoids [7] in the same temperature range; this suggests that the intraplastidial lipid transfer is achieved either with an improved efficiency and/or by a mechanism other than that alluded to in [7]. We would therefore consider that the intrachloroplastic lipid export is primarily achieved by a mechanism involving transient complete fusions between inner envelope and thylakoid membranes, although a vesicular transfer cannot be excluded. This conclusion differs somewhat from that proposed earlier [8] which was based on a set of data limited to the use of UDP-[^{14}C]galactose as sole precursor.

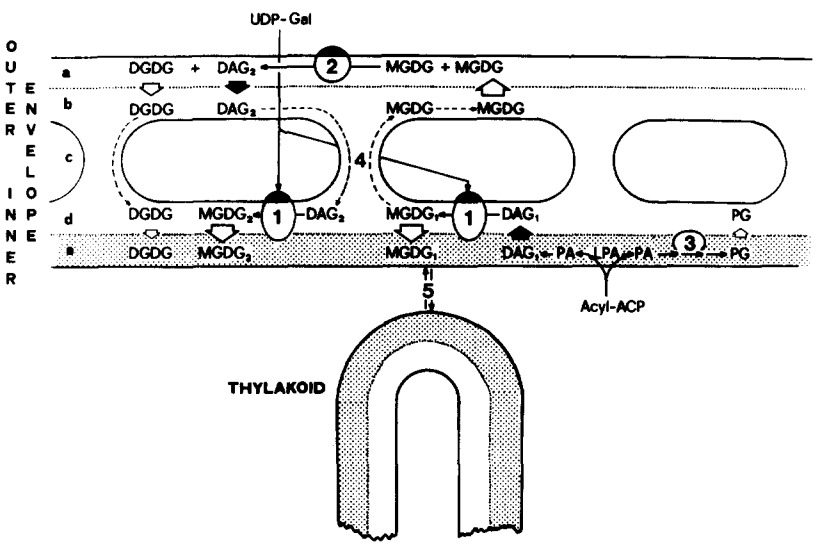

Fig. 5. A model for the origin of lipid asymmetry in thylakoid membranes and for the fundamental role played in this process by chloroplast envelope membranes. From top to bottom: outer envelope membrane with its two monolayers (a, b), separated from the inner envelope membrane with its two monolayers (d, e) by the intermembrane space (c). Because of the cytoplasmic origin of UDP-galactose [38] and of the impermeability of the inner envelope membrane to this compound, we propose that galactosylation of the diacylglycerol DAG_1 (synthesized de novo through the Kornberg-Pricer pathway) by the UDP-galactose:diacylglycerol galactosyltransferase (1) occurs at the cytoplasmic side of the inner envelope membrane to yield $MGDG_1$. This $MGDG_1$ may then undergo inwardly oriented transbilayer movement at a relatively high, non-limiting rate (large open arrow), possibly in a quasi-one step transfer [39]; it may also diffuse laterally untill it reaches a contact site (4) which enables $MGDG_1$ to enter [40] and equilibrate [39] in the outer envelope membrane. From the known localization [27] of the galactolipid:galactolipid galactosyltransferase (2), dismutation of MGDG molecules is then assumed to occur at the cytoplasmic side of the outer envelope membrane to yield DGDG [26] and DAG_2. After synthesis, DGDG must first be translocated inwardly, then diffuse across contact sites (4) to reach the cytoplasmic side of the inner membrane, where it accumulates because of a slow rate (small open arrow) of transbilayer movement to the stroma side of this membrane. As usual for most DAG [41,42], DAG_2 is assumed to be translocated very rapidly (large black arrows) across the outer envelope bilayer and to diffuse via contact sites (4) towards the cytoplasmic side of the inner envelope membrane where it is eventually galactosylated by (1) to $MGDG_2$ and inwardly translocated as described for $MGDG_1$. Desaturases, which have been claimed to reside in a hydrophobic region of the bilayer [19], are not represented in our model, but in order to account for their NAD(P)H and ferredoxin requirements [36], they are expected to function in the inner envelope membrane [37]. Lastly, the assembly of PA (phosphatidic acid) from LPA (lyso-phosphatidic acid) requires acyl residues of stromal origin and accordingly takes place at the stroma side of the inner envelope membrane [43–45]. Since the other cofactors required for PG synthesis (glycerol 3-phosphate and CTP) must obviously be available in the stroma [46,47], the subsequent steps (3) leading to the formation of PG [48,49] are assumed to be also localized at the stroma side of the inner envelope membrane, where PG accumulates preferentially because of a slow rate (small open arrow) of outwards transbilayer movement. The overall result is the synthesis-dependent establishment of a lipid asymmetry in the inner envelope membrane, with the stroma side being primarily enriched in PG and (to an extent which depends on its desaturation level) in MGDG, and the cytoplasmic side enriched in DGDG. By analogy with MGDG synthesis, one may even suggest that SQDG synthesis [25] occurs at the cytoplasmic side of the inner envelope membrane, giving thus rise to a marked enrichment of this monolayer in SQDG, provided that its translocation rate is slow. Lipid export from the inner envelope membrane to thylakoids (5) would then be achieved by transient fusion events [8,32,50] connecting both stroma-facing monolayers (dotted zones) together and both complementary monolayers (lumenal for thylakoids and cytoplasmic for inner envelope membrane) together. Possible mechanisms for such fusion events have been described [51].

It is however worth mentioning that evidence has been recently presented for a rapid assembly of functional thylakoid membranes in close association with the chloroplast envelope in greening *Chlamydomonas* cells [34].

Three other facts deserve special consideration. Firstly, stable transbilayer distributions of label (Fig. 3) have been obtained independently of the temperature and time for which intact chloroplasts were incubated in the presence of the labelled precursor (see text Results). Secondly, whenever significative differences between mass and label transmembrane distributions do occur (Fig. 3A, C, E and F), they seem to depend on the lipid class involved. Thus, all newly inserted MGDG molecules are more inwardly oriented than the bulk of MGDG (Fig. 3A and F) whereas the opposite is true for DGDG (Fig. 3E) and PG (Fig. 3C). Thirdly, within a single lipid class (and this concerns at least MGDG made by the de novo pathway), the transbilayer distribution of label clearly depends on the unsaturation degree of the various molecular species. Indeed, only those MGDG species in which at least three double bonds have been introduced were able to adopt the definitive MGDG asymmetry (Fig. 4). The eventuality that the difference between the hydrolysis curves of the various MGDG molecular species might simply be due to substrate specificity of the lipase from *R. arrhizus* can be dismissed on the basis of (i) the almost identical initial hydrolysis rates of these species (Fig. 4) and (ii) the reported lack of such specificity for this enzyme [35]. We suggest that the establishment of the definitive thylakoidal asymmetry of the de novo synthesized MGDG is also linked to the progress of desaturation of this molecule in the inner envelope membrane [36]. This implies the occurrence of an endless diffusional shuttle of MGDG molecules between both membranes.

We then propose to add a new competence to the already impressive list [30,37] of biochemical functions carried out by the chloroplast envelope of higher plants, namely the biogenesis of lipid asymmetry in thylakoid membranes. The topography of the various lipid-synthesizing and -desaturating enzymes in envelope membranes, the occurrence of contact sites and the relative rates of lateral diffusion and of transbilayer movement of the various lipid molecules would play key roles in establishing the proper transmembrane distribution of each lipid in the inner envelope membrane. Transient fusions with thylakoids (between stroma-facing sides and lumen-facing sides) would then allow the newly synthesized lipid molecules to diffuse laterally within each monolayer into thylakoid membranes, where further minor adjustments in lipid asymmetry (if required) can still be made (via transbilayer movements). This model is further described and discussed in Fig. 5. It is attractive because most of its features are already well documented. In addition, it requires a minimum number of sensible assumptions on the relative rates of transbilayer movement of the various lipids involved. Indeed, it should be recalled that bilayer-forming lipids (e.g., DGDG and PG) are much more reluctant than nonbilayer-lipids (e.g., diacylglycerol, polyunsaturated MGDG) to undergo transbilayer movement [52,53]. Finally, the model allows a single fusion mechanism to account for both lipid export to and gross lipid asymmetry in thylakoid membranes, while not excluding later and minor adjustments leading to the ultimate transbilayer distribution of acyl lipids in these membranes. A straightforward prediction of this model is that PG, although present in the outer envelope membrane as a whole [28], will hardly be found at its cytoplasmic side, and indeed this has been shown to be the case [54]. Clearly, a detailed investigation of the lipid sidedness in isolated chloroplast envelope membranes should provide useful information on the validity of this model.

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References

[1] Joyard, J. and Douce, R. (1976) Physiol. Vég. 14, 31–48.
[2] Douce, R. (1974) Science 183, 852–853.
[3] Dorne, A.J., Joyard, J. and Douce, R. (1990) Proc. Natl. Acad. Sci. USA 87, 71–74.
[4] Williams, J.P., Simpson, E.E. and Chapman, D.J. (1979) Plant Physiol. 63, 669–673.
[5] Joyard, J., Douce, R., Siebertz, H.P. and Heinz, E. (1980) Eur. J. Biochem. 108, 171–176.
[6] Bertrams, M., Wrage, K. and Heinz, E. (1981) Z. Naturforsch. 36c, 62–70.
[7] Morré, D.J., Morré, J.T., Morré, S.R., Sundqvist, C. and Sandelius, A.S. (1991) Biochim. Biophys. Acta 1070, 437–445.
[8] Rawyler, A., Meylan, M. and Siegenthaler, P.A. (1992) Biochim. Biophys. Acta 1104, 331–341.
[9] Heemskerk, J.W.M. and Wintermans, J.F.G.M. (1987) Physiol. Plant. 70, 558–568.
[10] Siegenthaler, P.A., Rawyler, A. and Smutny, J. (1989) Biochim. Biophys. Acta 975, 104–111.
[11] Mourioux, G. and Douce, R. (1981) Plant Physiol. 67, 470–473.
[12] Mills, J.R. and Joy, K.W. (1980) Planta 148, 75–83.
[13] Rouser, G., Fleischer, S. and Yamamoto, A. (1970) Lipids 5, 494–496.
[14] Roughan, P.G. and Batt, R.D. (1968) Anal. Biochem. 22, 74–84.
[15] Lichtenthaler, H.K. (1987) Methods Enzymol. 148, 350–382.
[16] Rawyler, A. and Siegenthaler, P.A. (1985) Biochim. Biophys. Acta 815, 287–298.
[17] Bruinsma, J. (1961) Biochim. Biophys. Acta 53, 576–578.
[18] Markwell, M.A.K., Haas, S.M., Bieber, L.L. and Tolbert, N.E. (1978) Anal. Biochem. 87, 206–210.
[19] Browse, J. and Somerville, C. (1991) Annu. Rev. Plant Physiol. Plant Mol. Biol. 42, 467–506.
[20] Heemskerk, J.W.M., Bögemann, G., Helsper, J.P.F.G. and Wintermans, J.F.G.M. (1988) Plant Physiol. 86, 971–977.
[21] Siebertz, H.P., Heinz, E. Joyard, J. and Douce, R. (1980) Eur. J. Biochem. 108, 177–185.
[22] Heinz, E. and Roughan, P.G. (1983) Plant Physiol. 72, 273–279.
[23] Rawyler, A., Unitt, M.D., Giroud, C., Davies, H., Mayor, J.P.,

Harwood, J.L. and Siegenthaler, P.A. (1987) Photosynthesis Res. 11, 3–13.
[24] Heinz, E., Schmidt, H., Hoch, M., Jung, K.H., Binder, H. and Schmidt, R.R. (1989) Eur. J. Biochem. 184, 445–453.
[25] Seifert, U. and Heinz, E. (1992) Bot. Acta 105, 197–205.
[26] Heemskerk, J.W.M., Storz, T., Schmidt, R.R. and Heinz, E. (1990) Plant Physiol. 93, 1286–1294.
[27] Dorne, A.J., Block, M.A., Joyard, J. and Douce, R. (1982) FEBS Lett. 145, 30–34.
[28] Block, M.A., Dorne, A.J., Joyard, J. and Douce, R. (1983) J. Biol. Chem. 258, 13281–13286.
[29] Cremers, F.F.M., Voorhout, W.F., Van der Krift, T.P., Leunissen-Bijvelt, J.J.M. and Verkleij, A.J. (1988) Biochim. Biophys. Acta 933, 334–340.
[30] Douce, R. and Joyard, J. (1990) Annu. Rev. Cell Biol. 6, 173–216.
[31] Andrews, J. and Mudd, J.B. (1985) Plant Physiol. 79, 259–265.
[32] Carde, J.P., Joyard, J. and Douce, R. (1982) Biol. Cell 44, 315–324.
[33] Moreau, P., Rodriguez, M., Cassagne, C., Morré, M.M. and Morré, D.J. (1991) J. Biol. Chem. 266, 4322–4328.
[34] Hoober, J.K., Boyd, C.O. and Paavola, L.G. (1990) Plant Physiol. 96, 1321–1328.
[35] Slotboom, A.J., De Haas, G.H., Bonsen, P.P.M., Burbach-Westerhuis, G.J. and Van Deenen, L.L.M. (1970) Chem. Phys. Lipids 4, 15–29.
[36] Schmidt, H. and Heinz, E. (1990) Proc. Natl. Acad. Sci. USA 87, 9477–9480.
[37] Joyard, J., Block, M.A. and Douce, R. (1991) Eur. J. Biochem. 199, 489–509.
[38] Murphy, D.J. (1982) in Biochemistry and Metabolism of Plant Lipids (Wintermans, J.F.G.M. and Kuiper, P.J.C., eds.), pp. 51–56, Elsevier Biomedical Press, Amsterdam.
[39] Simbeni, R., Paltauf F. and Daum, G. (1990) J. Biol. Chem. 265, 281–285.
[40] Van Venetië, R. and Verkleij, A.J. (1982) Biochim. Biophys. Acta 692, 397–405.
[41] Hamilton, J.A., Bhamidipati, S.P., Kodali, D.R. and Small, D.M. (1991) J. Biol. Chem. 266, 1177–1186.
[42] Allan, D., Thomas, P. and Michell, R.H. (1978) Nature 276, 289–290.
[43] Joyard, J. and Douce, R. (1977) Biochim. Biophys. Acta 486, 273–285.
[44] Block, M.A., Dorne, A.J., Joyard, J. and Douce R. (1983) J. Biol. Chem. 258, 13273–13280.
[45] Andrews, J., Ohlrogge, J.B. and Keegstra, K. (1985) Plant Physiol. 78, 459–465.
[46] Heldt, H.W. (1976) in The Intact Chloroplast (Barber, J., ed.), pp. 215–234, Elsevier/North Holland Biomedical Press, Amsterdam.
[47] Dyer, T.A. (1984) in Chloroplast Biogenesis (Baker, N.R. and Barber, J., eds.), pp. 23–69, Elsevier, Amsterdam.
[48] Mudd, J.B. and DeZacks, R. (1981) Arch. Biochem Biophys. 209, 584–591.
[49] Andrews J. and Mudd, J.B. (1985) Plant Physiol. 79, 259–265.
[50] Heber, U. and Heldt, H.W. (1981) Annu. Rev. Plant Physiol. 32, 139–168.
[51] Siegel, D.P. (1986) Biophys. J. 49, 1171–1183.
[52] Homan, R. and Pownall, H.J. (1988) Biochim. Biophys. Acta 938, 155–166.
[53] Cullis, P.R. and De Kruijff, B. (1979) Biochim. Biophys. Acta 559, 399–420.
[54] Dorne, A.J., Joyard, J., Block, M.A. and Douce, R. (1985) J. Cell Biol. 100, 1690–1697.