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Biosynthesis and Desaturation of Prokaryotic Galactolipids in Leaves and Isolated Chloroplasts from Spinach[1]

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ABSTRACT

Mono- and digalactosyldiacylglycerol (MGDG and DGDG) were isolated from the leaves of sixteen 16:3 plants. In all of these plant species, the *sn*-2 position of MGDG was more enriched in C_{16} fatty acids than *sn*-2 of DGDG. The molar ratios of prokaryotic MGDG to prokaryotic DGDG ranged from 4 to 10. This suggests that 16:3 plants synthesize more prokaryotic MGDG than prokaryotic DGDG. In the 16:3 plant *Spinacia oleracea* L. (spinach), the formation of prokaryotic galactolipids was studied both *in vivo* and *in vitro*. In intact spinach leaves as well as in chloroplasts isolated from these leaves, radioactivity from [1-^{14}C]acetate accumulated 10 times faster in MGDG than in DGDG. After 2 hours of incorporation, most labeled galactolipids from leaves and all labeled galactolipids from isolated chloroplasts were in the prokaryotic configuration. Both *in vivo* and *in vitro*, the desaturation of labeled palmitate and oleate to trienoic fatty acids was higher in MGDG than in DGDG. In leaves, palmitate at the *sn*-2 position was desaturated in MGDG but not in DGDG. In isolated chloroplasts, palmitate at *sn*-2 similarly was desaturated only in MGDG, but palmitate and oleate at the *sn*-1 position were desaturated in MGDG as well as in DGDG. Apparently, palmitate desaturase reacts with *sn*-1 palmitate in either galactolipid, but does not react with the *sn*-2 fatty acid of DGDG. These results demonstrate that isolated spinach chloroplasts can synthesize and desaturate prokaryotic MGDG and DGDG. The finally accumulating molecular species, MGDG(18:3/16:3) and DGDG(18:3/16:0), are made by the chloroplasts in proportions similar to those found in leaves.

The identification of two complementary pathways of acyl lipid synthesis in leaves of 16:3 plants (23) makes it possible to establish the biosynthetic origin of acyl lipids from the pairing of their fatty acids. The so-called prokaryotic pathway in leaf chloroplasts forms acyl lipids with a C_{16} fatty acid at the *sn*-2 position of the diacylglycerol backbone (7). The alternative, eukaryotic pathway in the endoplasmic reticulum synthesizes acyl lipids with only C_{18} fatty acids at the *sn*-2 position. Consequently, galactolipids and other acyl lipids can be divided into pro- and eukaryotic molecular species according to the presence of a C_{16} or C_{18} fatty acid at *sn*-2, respectively (13, 19, 23).

In 16:3 plants such as *Spinacia oleracea* L. (spinach) (6, 14) and *Arabidopsis thaliana* (L.) Heynh. (4, 21), MGDG[3] and DGDG are found in both the pro- and the eukaryotic configuration. The predominant prokaryotic molecular species are MGDG(18:3/16:3) and DGDG(18:3/16:0), which typically differ in their degree of unsaturation. Eukaryotic MGDG and DGDG are mainly found with the $C_{18:3}/C_{18:3}$ combination, except for some DGDG(16:0/18:3) and some minor molecular species carrying $C_{18:2}$. The other group of higher plants, named 18:3 plants, lack galactolipids with a prokaryotic configuration, but have the same eukaryotic molecular species of MGDG and DGDG.

Biosynthesis of prokaryotic MGDG and DGDG has been measured *in vivo* by the labeling of leaves from 16:3 plants with [^{14}C]acetate (4, 26, 29). In *de novo*-made MGDG, both C_{16} and C_{18} fatty acids were labeled and both were gradually desaturated to trienoic fatty acids. Desaturation of newly made DGDG, however, was limited to its labeled C_{18} fatty acids, suggesting a different action of the desaturases on MGDG and DGDG. Strong evidence that desaturation of MGDG fatty acids, indeed, proceeds as a lipid-linked process has been provided by Sato *et al.* (25) for the blue-green alga *Anabaena variabilis*.

Following the initial study of galactolipid desaturation *in vitro* by Roughan *et al.* (22), it was confirmed recently that isolated spinach chloroplasts convert *de novo*-made MGDG(18:1/16:0) to ultimately MGDG(18:3/16:3) (1, 15). In the present paper, we extend this work and report for the first time the synthesis of prokaryotic DGDG(18:1/16:0) and its subsequent desaturation in isolated chloroplasts. The rates observed were low, but are consistent with the small proportions of prokaryotic DGDG that were found in the leaves of sixteen 16:3 plants. A scheme is proposed for the regulation and interdependence of galactolipid synthesis in 16:3 plants, which tries to explain the typical differences in content and fatty acid composition between the major molecular species of MGDG and DGDG.

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[3] Abbreviations: MGDG and DGDG, mono- and digalactosyldiacylglycerol; molecular species of diacyl lipids are represented by a short-hand notation: *e.g.*, MGDG(18:1/16:0) designates monogalactosyl *sn*-1-oleoyl-2-palmitoyl-glycerol.

MATERIALS AND METHODS

Chemicals

Radioactive sodium [1-^{14}C]acetate was supplied by Amersham (U.K.). Thermolysin from *Bacillus thermoproteolyticus* (EC 3.4.24.4) and lipase from *Rhizopus arrhizus* (EC 3.1.1.3) were bought from Sigma (St. Louis, MO). All other reagents were of analytical grade.

Plants

Spinacia oleracea L. (spinach) cv Subito was grown hydroponically, as described by Andrews and Heinz (1). Other plant species, listed in Table I and selected according to published data (16), were collected in the botanical garden in the spring.

Chloroplasts

Only young, growing spinach leaves were harvested and used for the purification of highly desaturating intact chloroplasts by Percoll-gradient centrifugation (1). The isolated chloroplasts were suspended in 0.3 M sorbitol with 40 mM Tricine (pH 7.9 with KOH), and kept on ice until use.

Thermolysin treatment of chloroplasts was as described before (12). Briefly, chloroplasts (0.5 mg Chl/mL) were stirred with thermolysin (1 mg/mL) and $CaCl_2$ (1 mM) at 4°C for 1 h, and the incubation was continued in the presence of 10 mM EDTA for another 10 min. Centrifugation through isotonic 40% (v/v) Percoll then yielded the remaining intact chloroplasts.

Radioactive Labeling of Spinach Leaves and Chloroplasts

[1-^{14}C]Acetate (370 kBq, 2.0 GBq/mmol) was spread with a needle over the surface of a young, detached spinach leaf (3 × 2 cm), which was placed in a moistened glass tube. The leaf was illuminated at 25°C for 2 h, and the lipids were extracted as described below. The *in vivo* labeling experiments were carried out parallel to those with isolated chloroplasts, using the same batch of leaves.

Purified intact spinach chloroplasts (500 μg Chl) were incubated at 25°C with [1-^{14}C]acetate (370 kBq, 2.0 GBq/mmol) in 2.5 mL buffer (pH 7.0), composed of 0.3 M sorbitol, 25 mM Mes, 20 mM Tricine-KOH, 10 mM $KHCO_3$, 5 mM $MgCl_2$, 0.5 mM *sn*-glycerol-3-P, 0.5 mM KH_2PO_4, and 0.5 mM UDP-Gal. Incubation tubes were shaken under illumination during the first 20 min, and then shaken in the dark for another 100 min. These conditions allowed *de novo* synthesis of fatty acids, but simultaneously promoted formation of fatty acid-labeled MGDG and DGDG (10). The long incubation time was necessary to collect sufficient radioactivity in DGDG. Immediately after the incubation, intact chloroplasts were re-

Table I. *Pro- and Eukaryotic Galactolipids in the Leaves from Various 16:3 Plants*

The molar proportions of total MGDG and DGDG ($MGDG_{Tot}/DGDG_{Tot}$) were determined from quantification of the leaf galactolipids by anthrone assays, and the fatty acids of leaf MGDG and DGDG were analyzed as described in "Materials and Methods." The percentage of total C_{16} fatty acids at *sn*-2 in either galactolipid (Σmol% C_{16} at *sn*-2) gave the fraction of prokaryotic MGDG and DGDG (mol% $MGDG_{Pro}$ and mol% $DGDG_{Pro}$). The molar ratios of pro- and eukaryotic galactolipids ($MGDG_{Pro}/DGDG_{Pro}$ and $MGDG_{Eu}/DGDG_{Eu}$) were calculated from these data.

Plant Species	$MGDG_{Tot}/DGDG_{Tot}$	$MGDG_{Pro}$	$DGDG_{Pro}$	$MGDG_{Pro}/DGDG_{Pro}$	$MDGD_{Eu}/DGDG_{Eu}$
	mol/mol	*mol%*		*mol/mol*	
Apiaceae					
Aegopodium podagraria L.	1.56	75.9	20.8	5.69	0.47
Anthriscus sylvestris (L.) Hoffm.	1.66	88.7	26.6	5.54	0.26
Conium maculatum L.	1.68	87.1	22.4	6.53	0.28
Heracleum montegazzianum Somm. et Lev.	1.90	84.7	44.6	3.61	0.52
Brassicaceae					
Alliaria petiolata (Bieb.) Cavara et Grande	1.65	66.7	22.2	4.96	0.71
Arabidopsis thaliana (L.) Heynh.	1.62	60.8	22.6	4.36	0.82
Brassica napus L.	2.08	82.5	24.6	6.98	1.43
Campanulaceae					
Campanula persicifolia L.	1.41	74.0	17.7	5.89	0.45
Chenopodiaceae					
Spinacia oleracea L.	1.87	37.9	17.1	4.14	1.40
Geraniaceae					
Geranium robertianum L.	1.51	46.5	7.1	9.89	0.87
Ranunculaceae					
Aquilegia atrata Koch	1.41	62.1	23.2	3.78	0.70
Ranunculus acris L.	1.65	85.4	21.5	6.55	0.31
Rubiaceae					
Galium aparine L.	1.10	53.2	12.9	4.54	0.59
Solanaceae					
Nicotiana tabacum L.	1.37	40.3	15.5	3.56	0.97
Solanum tuberosum L.	1.89	56.6	12.0	8.91	0.93
Tropaeolaceae					
Tropaeolum majus L.	1.89	69.2	13.4	9.76	0.67

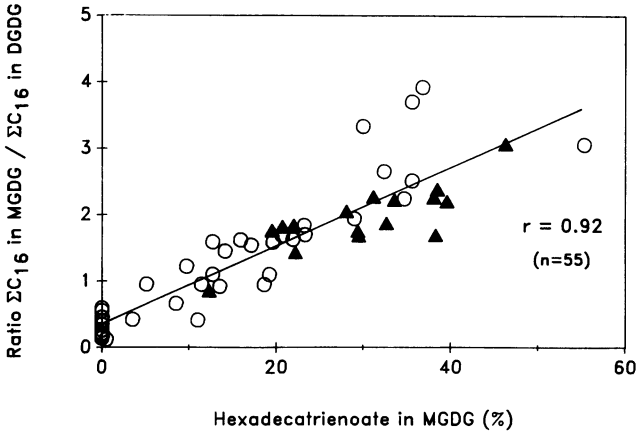

Figure 1. Distribution of C_{16} fatty acids within the galactolipids from leaves of both 18:3 and 16:3 plants. The ratio of C_{16} fatty acids in MGDG to C_{16} fatty acids in DGDG is graphed as a function of the hexadecatrienoate content in MGDG for 55 plant species. Data represented by open circles (○) were calculated from published fatty acids patterns for MGDG and DGDG (3, 6, 12, 16, 17, 28, 29). Closed triangles (▲) were obtained by analysis of the plants listed in Table I. "Typical" 18:3 plants represent the very left part of the graph and "typical" 16:3 plants, the right part of the graph.

purified by Percoll-gradient centrifugation and the lipids were extracted (10).

Analyses

Lipids were extracted from leaves of the 16:3 plants listed in Table I. Subsequently, the galactolipids were separated by TLC for quantification by anthrone and for positional analysis of their fatty acids, as described elsewhere (9). Fatty acids were separated as *p*-bromophenacyl esters by HPLC. Detection of esters was at 254 nm to give molar proportions (9).

Radiolabeled spinach leaves were homogenized in methanol:2-propanol (1:1, v/v) with an Ika-Werk (Staufen, F.R.G.) blender, and lipids were extracted by the addition of chloroform and water. The aqueous phase was reextracted twice with chloroform. The labeled leaf lipids were separated by TLC with chloroform:methanol:water (65:25:4, v/v). Recovered MGDG and DGDG were subjected to a second TLC separation with acetone:benzene:water (91:30:8, v/v), to remove contaminating phospholipids. Losses of label in MGDG and DGDG during this procedure were 3 to 5%, as was checked with [^{14}C]galactose-labeled (11) galactolipids.

Lipids from radiolabeled chloroplasts were extracted with chloroform and methanol, and were purified by TLC in chloroform:methanol:water (65:25:4, v/v) (10). Fatty acid-labeled galactolipids were transmethylated and the resulting methyl esters were separated by radio-HPLC (1). The amount of radioactivity injected was >250 Bq. Positional analysis of labeled fatty acids in the galactolipids was carried out as with unlabeled lipids, using the lipase from *Rh. arrhizus* (9, 26).

RESULTS

Proportions of Prokaryotic Galactolipids in Leaves of 16:3 and 18:3 Plants

The literature provides ample data of the fatty acid composition of MGDG and DGDG in leaves of higher plants. A comparison of 55 of such fatty acid patterns reveals a linear correlation between the percentage of hexadecatrienoate in MGDG from a particular plant species and the ratio of C_{16} fatty acids in MGDG to C_{16} fatty acids in DGDG (Fig. 1). According to this relationship, 16:3 plants have higher levels of C_{16} fatty acids in MGDG than in DGDG, whereas 18:3 plants (at the origin of the graph in Fig. 1) accumulate less C_{16} fatty acids in MGDG than in DGDG. Apparently, in leaves from most plants the C_{16} fatty acids are distributed rather asymmetrically among the two galactolipids. Because the concentration of hexadecatrienoate, being esterified at the *sn*-2 position of MGDG (14), may be considered as a measure for the prokaryotic fraction of MGDG, the above correlation suggests that 16:3 plants contain more prokaryotic MGDG than prokaryotic DGDG.

To verify this hypothesis, we quantified the pro- and eukaryotic galactolipids in leaves from sixteen 16:3 plants. Proportions of prokaryotic galactolipids were calculated from the quantities of total MGDG and DGDG and from the percentage of C_{16} fatty acids found at *sn*-2 in either galactolipid (Table I). All plant species showed a higher percentage of prokaryotic MGDG than of prokaryotic DGDG, and in all plants the molar proportion of prokaryotic MGDG was 4 to 10 times higher than that of prokaryotic DGDG. Analogous quantification of the eukaryotic galactolipids revealed that in 16:3 plants eukaryotic DGDG was relatively abundant, since the molar ratio of eukaryotic MGDG to eukaryotic DGDG was close to unity or even below one (Table I). These values are in good agreement with published data on the molecular species composition of galactolipids in *A. thaliana* (4, 21) and *Brassica napus* L. (30).

Closer examination of the data indicated that the (molar) ratio of prokaryotic MGDG to prokaryotic DGDG was not a function of the percentage of hexadecatrienoate in MGDG (Fig. 2). Thus, the relative preponderance of prokaryotic

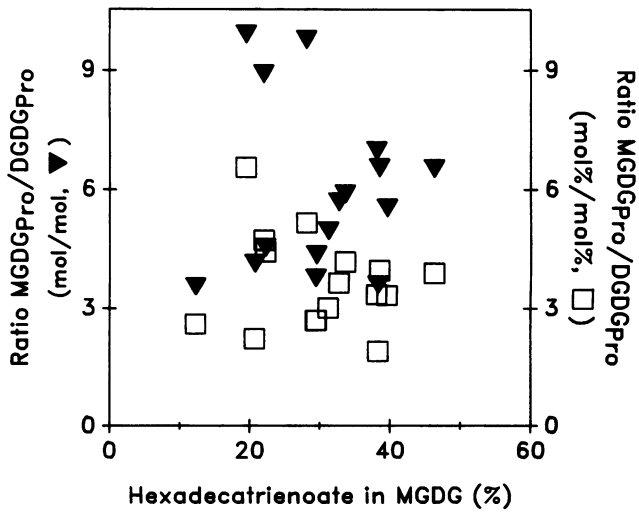

Figure 2. Predominance of prokaryotic MGDG in 16:3 plants. The ratio of prokaryotic MGDG to prokaryotic DGDG is plotted as a function of the percentage of hexadecatrienoate in MGDG. The molar ratio of $MGDG_{Pro}/DGDG_{Pro}$ (▼) is given as well as the percentage ratio (□). Data were obtained from the plants listed in Table I.

Table II. *De Novo Synthesis of Galactolipids in Intact Leaves and Isolated Chloroplasts from Spinach*

Spinach leaves were incubated with [^{14}C]acetate (370 kBq) for 2 h. Chloroplasts (500 μg Chl) purified from the same batch of leaves were incubated for 2 h with [^{14}C]acetate (370 kBq), *sn*-glycerol-3-P, UDP-Gal and various cofactors, under conditions allowing *de novo* fatty acid and galactolipid synthesis. Lipids were extracted, and MGDG and DGDG were purified and transmethylated for analysis of the labeled fatty acids by radio-HPLC. Detailed conditions are given in "Materials and Methods." The incorporation of radioactivity into leaves and chloroplasts is listed as mean values ± SE of five (leaves) or six (chloroplasts) experiments.

Labeling of Lipids	Leaves	Chloroplasts
	kBq/leaf	*kBq/mg Chl*
Total acyl lipids	21.6[a] ± 5.17	71 ± 8.5
MGDG	6.0 ± 3.00	26 ± 5.6
DGDG	0.54 ± 0.11	2.5 ± 0.69
	kBq/kBq	
Ratio MGDG/DGDG	11.0 ± 1.31	12.1 ± 1.50

[a] $n = 3$.

MGDG over DGDG seems not to be determined by the degree to which the prokaryotic pathway contributes to galactolipid synthesis. In DGDG the percentage of C_{16} fatty acids, consisting mostly of palmitate esterified at *sn*-1 or *sn*-2, was relatively constant for all 16:3 and for 18:3 plants (data not shown; see ref. 14). Consequently, Figure 1 depicts the increase in prokaryotic MGDG in plants with increasing levels of hexadecatrienoate, whereas the proportion of C_{16} fatty acids in DGDG (mainly eukaryotic, but also some prokaryotic) remains roughly constant.

Formation and Desaturation of Prokaryotic Galactolipids in Detached Spinach Leaves

The 16:3 plant spinach was used to follow the synthesis of prokaryotic galactolipids both *in vivo* and *in vitro*. In agreement with previous findings (26, 27), the treatment of detached leaves with [^{14}C]acetate resulted in a high MGDG labeling, but in low DGDG labeling. After 2 h of incubation, 30% of the radioactivity recovered from total acyl lipids was found in MGDG, but only 3% in DGDG (Table II). The *de novo* synthesized C_{18} fatty acids in both galactolipids were highly desaturated: 87% of the labeled oleate in MGDG and 61% in DGDG was converted to polyunsaturated products (Fig. 3). In contrast, the labeled C_{16} fatty acid palmitate were

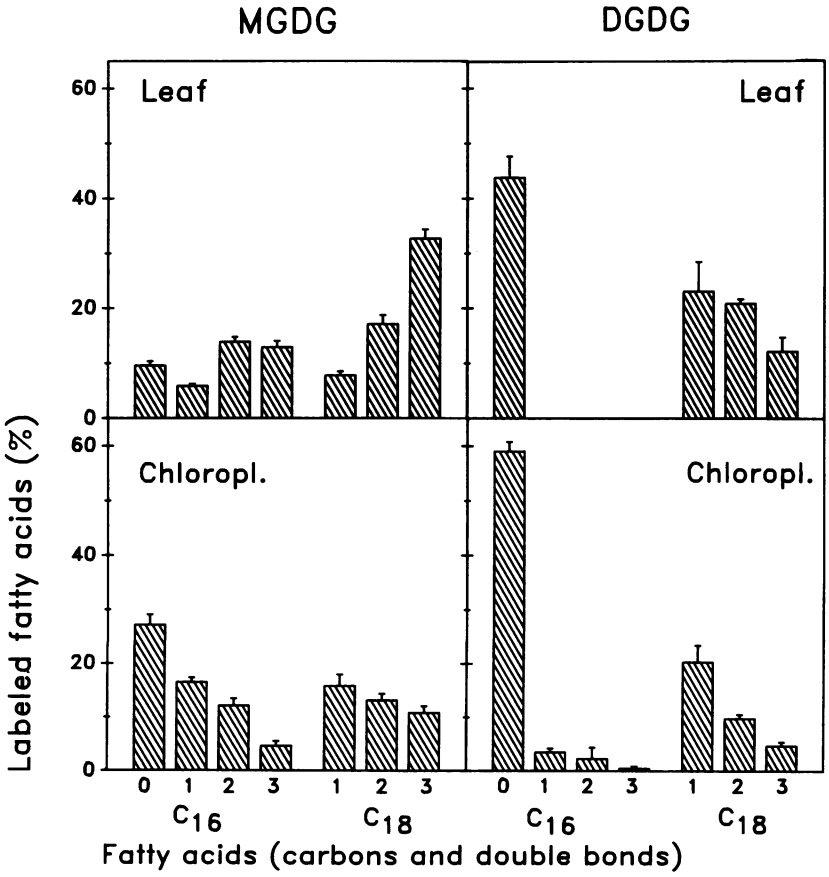

Figure 3. Desaturation of *de novo*-made galactolipids in leaves and isolated chloroplasts from spinach. Leaves were incubated with [^{14}C]acetate and labeled galactolipids were analyzed as described in Table II. The C_{16} fatty acids represent 42.3 (MGDG) and 43.8% (DGDG) of the labeled fatty acids. Intact chloroplasts were incubated with [^{14}C]acetate and cofactors, and labeled galactolipids were analyzed as described in Table II. The C_{16} fatty acids represent 60.4 (MGDG) and 65.3% (DGDG) of the labeled fatty acids. Data are mean values ± SE of five (leaves) or six (chloroplasts) independent experiments.

only desaturated in MGDG, but not in DGDG. Positional analysis of the fatty acid labeling indicated that the newly synthesized MGDG and DGDG were mainly, but not completely, in the prokaryotic configuration, because low quantities of C_{18} fatty acids were found at the *sn*-2 position, apart from the expected high levels of C_{16} fatty acids. The desaturation of palmitate at *sn*-2 was high in MGDG, but absent in DGDG (Fig. 4). The corresponding *sn*-1 position of both leaf galactolipids contained almost exclusively labeled polyunsaturated C_{18} fatty acids (data not shown).

Formation and Desaturation of Prokaryotic Galactolipids in Spinach Chloroplasts

To follow the synthesis of prokaryotic galactolipids *in vitro*, intact spinach chloroplasts were isolated and incubated with [^{14}C]acetate, *sn*-glycerol-3-P, and UDP-Gal (22). The labeling rate of DGDG was low compared to that of MGDG (Fig. 5), despite the attempt to stimulate DGDG formation by the presence of Mg^{2+} and a neutral pH value (10). After 2 h of incubation, the ratio of label in MGDG to that in DGDG was 12.1 ± 1.50 (Table II).

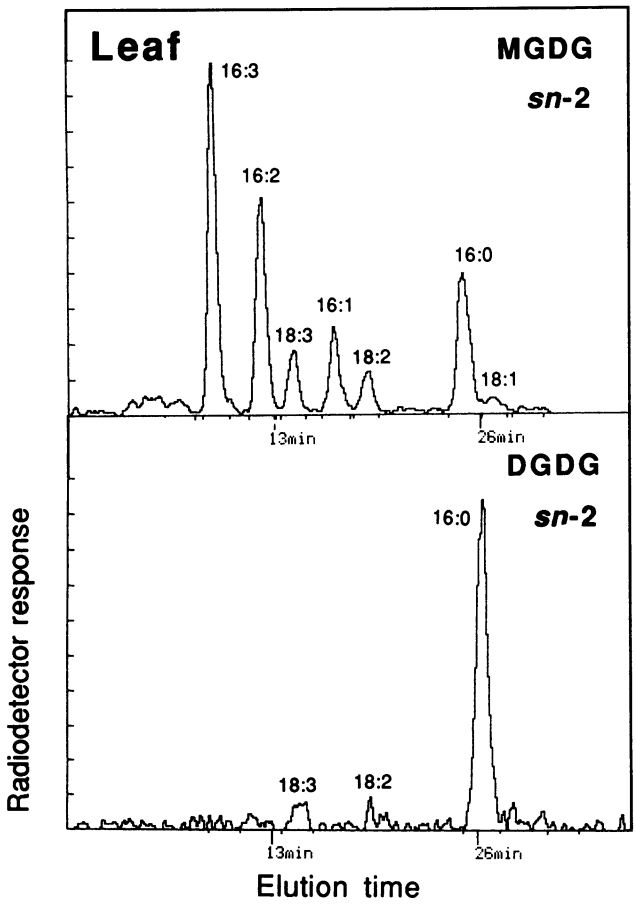

Figure 4. Desaturation of *sn*-2 fatty acids from MGDG and DGDG in spinach leaves. A detached leaf was incubated with [^{14}C]acetate for 2 h, the isolated galactolipids were treated with *Rh. arrhizus* lipase, and the fatty acids at *sn*-1 and *sn*-2 were analyzed by radio-HPLC. Radiochromatograms are shown of the *sn*-2 methylated fatty acids of MGDG and DGDG. The peaks in the chromatogram of DGDG are somewhat retarded due to changes in chromatographic conditions.

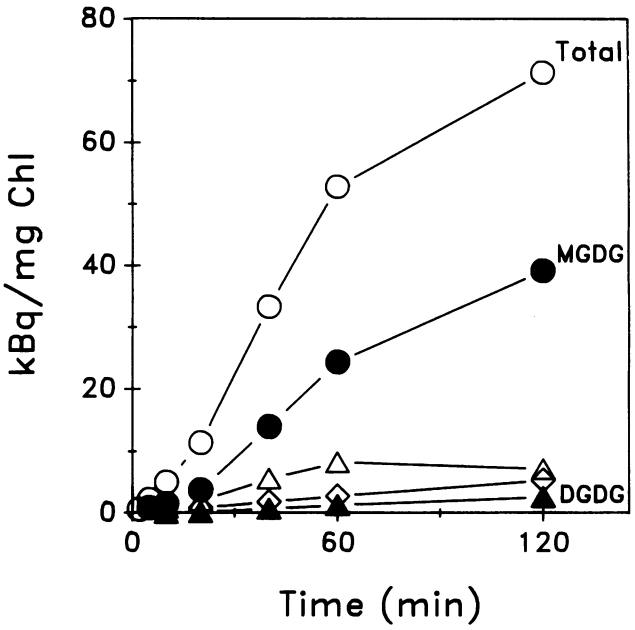

Figure 5. Time course of [^{14}C]acetate incorporation into the acyl lipids of spinach chloroplasts. Isolated chloroplasts (1.2 mg Chl) were incubated with [^{14}C]acetate (370 kBq), *sn*-glycerol-3-P, UDP-Gal, $MgCl_2$, and cofactors at pH 7.0 in a total volume of 1.2 mL (see "Materials and Methods"). The incubation mixture was illuminated only during the first 20 min of incubation. Aliquots of 50 μL were taken at the indicated times and analyzed. Data are mean values of duplicate incubations for the incorporation of label into total acyl lipids (○), MGDG (●), DGDG (▲), diacylglycerol (△), and sulfolipid + phosphatidylglycerol (◇).

Kinetic studies have shown that isolated spinach chloroplasts are able to desaturate MGDG(18:1/16:0), formed *de novo* from [^{14}C]acetate (1, 15, 22). Similarly, in the present experiments labeled palmitate and oleate from MGDG were desaturated to hexadecatrienote and linolenate, respectively. The poor labeling of DGDG permitted its analysis only after longer times of incubation. After 2 h, palmitate and oleate in DGDG were desaturated to the extent of 9 and 43%, compared to 55 and 61% desaturation in MGDG, respectively (Fig. 3). Desaturation of the fatty acids in sulfolipid (18) was still lower than that in DGDG (data not shown).

Positional analysis of the labeled lipids showed that *de novo*-made MGDG and DGDG were completely in the prokaryotic configuration, *i.e.* either galactolipid contained only labeled C_{16} fatty acids at the *sn*-2 position (Fig. 6, A and B). Nevertheless, the degree of desaturation at *sn*-2 was different for each galactolipid. Labeled palmitate in MGDG was converted to the C_{16} mono-, di-, and trienoic fatty acids, but palmitate in DGDG was hardly desaturated. We would like to point out the labeling of unsaturated C_{16} fatty acids, notably of $C_{16:1}$, at the *sn*-1 position of MGDG and DGDG (Fig. 6, A and B). Apparently, when localized at *sn*-1, palmitate can be desaturated in both galactolipids. The resulting $C_{16:1}$ at the *sn*-1 position of MGDG was only poorly desaturated further, unlike $C_{16:1}$ at *sn*-2 (Fig. 6A).

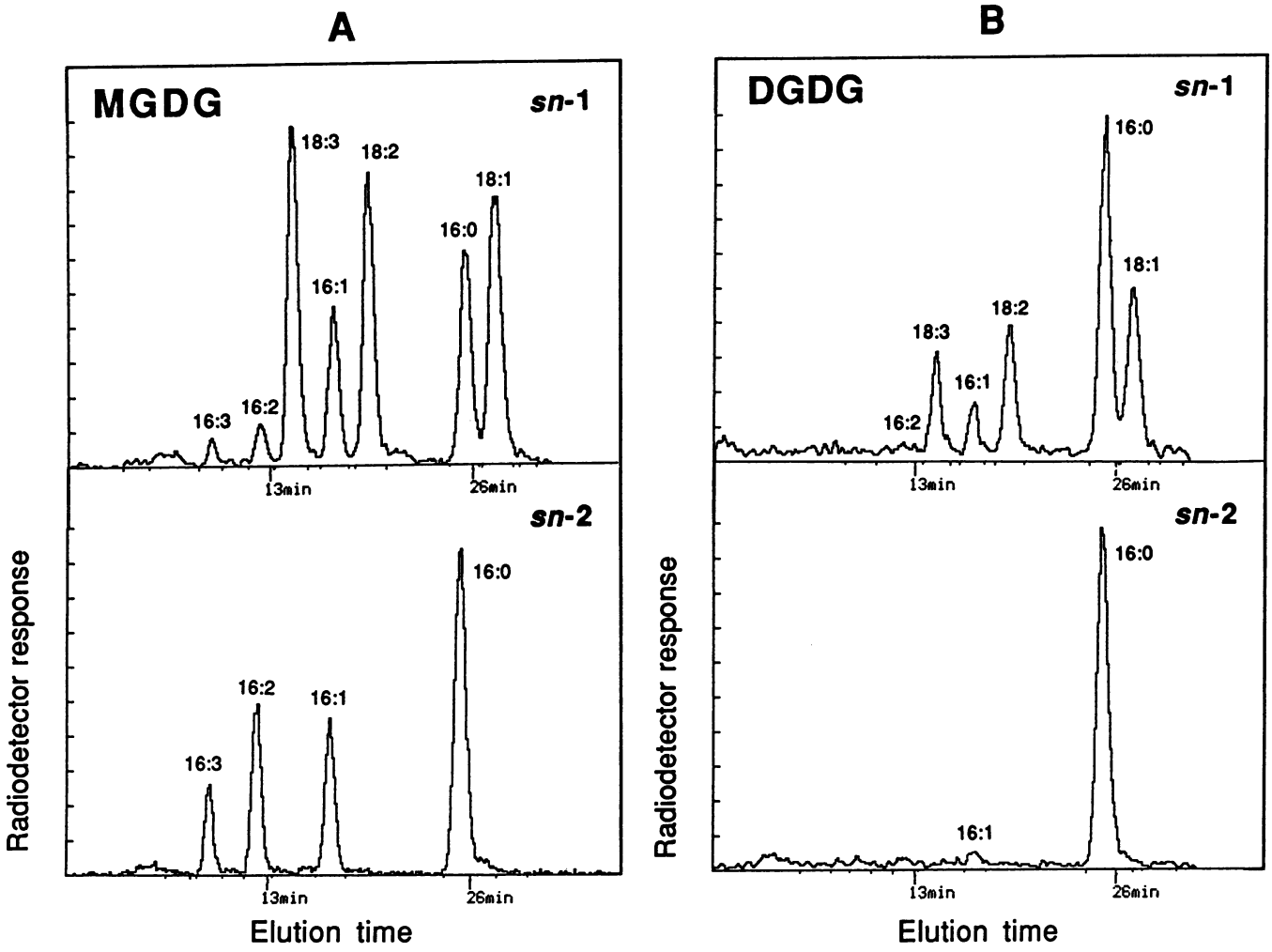

Figure 6. Desaturation of *sn*-1 and *sn*-2 fatty acids from MGDG and DGDG in isolated spinach chloroplasts. Chloroplasts were labeled with [^{14}C]acetate in the presence of UDP-Gal for 2 h (see "Materials and Methods"). Galactolipids were purified and treated with *Rh. arrhizus* lipase for analysis of the labeled fatty acids at the *sn*-1 and *sn*-2 positions. Radiochromatograms of the fatty acid methyl esters from MGDG (A) and DGDG (B) were obtained by radio-HPLC.

Galactolipid:galactolipid galactosyltransferase is known to form DGDG by the transfer of a galactosyl group from one MGDG to another MGDG molecule (10, 12). Its activity in spinach chloroplasts is inhibited by treatment of chloroplasts with the nonpenetrating proteinase thermolysin (10). Accordingly, thermolysin treatment inhibited the labeling of DGDG from [^{14}C]acetate by 65 ± 9% (mean ± SE, $n = 3$). In contrast, the desaturation of neither C_{16} nor C_{18} fatty acids in *de novo* made MGDG was inhibited significantly by thermolysin treatment (data not shown).

DISCUSSION

Formation and Desaturation of Prokaryotic Galactolipids *in Vivo* and *in Vitro*

Our results indicate that isolated spinach chloroplasts synthesize prokaryotic MGDG and DGDG with high levels of unsaturated fatty acids, when incubated with [^{14}C]acetate, *sn*-glycerol-3-P, and UDP-Gal at a neutral pH value. The [^{14}C] acetate-based galactolipid synthesis in chloroplasts resembled *de novo* galactolipid synthesis in spinach leaves in various aspects. In either case, the labeling rate of MGDG was much higher than that of DGDG (Table II). Both *in vitro* and *in vivo*, the ratio of label in C_{16} to C_{18} fatty acids was quite similar for MGDG and for DGDG (see legend to Fig. 3), which is compatible with a precursor-product relationship between the mono- and digalactolipid. In chloroplasts and in leaves, C_{18} fatty acids at *sn*-1 of MGDG and DGDG were desaturated well, but C_{16} fatty acids at *sn*-2 were desaturated only in MGDG (Figs. 4 and 6). Together, this demonstrates that isolated spinach chloroplasts are capable of synthesizing and desaturating prokaryotic mono- and digalactolipids, yielding finally MGDG(18:3/16:3) and DGDG(18:3/16:0) in similar proportions as are formed in the leaves. Therefore, the apparently poor DGDG labeling in chloroplasts cannot be expected to become much better, in view of the equally poor DGDG labeling in spinach leaves and the small proportion of prokaryotic DGDG that is present in leaves. Table I

suggests that this low content of prokaryotic DGDG is a general property of 16:3 plants. This is in agreement with the generally slow formation of prokaryotic DGDG *in vivo* that has been measured by various authors in a number of 16:3 plants (4, 26, 29).

Selectivity of Galactosyltransferases and Desaturases

In green algae, it has been demonstrated that the fatty acids in both MGDG and DGDG can be desaturated as lipid-linked substrates (5, 8). Our results with spinach are compatible with such lipid-linked desaturation. The typical differences between the desaturation pattern of *de novo* made MGDG and DGDG, and especially the lack of desaturated C_{16} fatty acids at *sn*-2 of DGDG, suggest that prokaryotic DGDG was formed predominantly by the galactosylation of more saturated MGDG molecular species, *i.e.* of only those species containing palmitate at the *sn*-2 position. In isolated envelope membranes, galactolipid:galactolipid galactosyltransferase discriminates only moderately between molecular species of MGDG which vary in the degree of fatty acid unsaturation (11). Thus, it is unlikely that the transferase would utilize only $C_{16:0}$-, and not $C_{16:1}$- or $C_{16:2}$-containing species of MGDG. The observed selective formation of DGDG with saturated *sn*-2 palmitate, therefore, must be regulated by a different mechanism, putatively by spatial restriction of the galactosyltransferase activity to the pool of newly made MGDG(18:1/16:0), after which the $C_{18:1}/C_{16:0}$ molecular species of MGDG and DGDG each are channeled to desaturation and are converted gradually to MGDG(18:3/16:3) and DGDG(18:3/16:0), respectively. Galactosylation of molecular species of MGDG with a higher degree of unsaturation then is thought to be only a minor route of DGDG formation, similar to what has been proposed for green algae (5, 8).

Our experiments with isolated chloroplasts (Figs. 3 and 6) gave some information on the specificities of recently identified plastid desaturases, ω3, ω6, and ω9 (2, 3, 20). The ω6 oleate and ω3 linoleate desaturases were more active with MGDG than with DGDG fatty acids. The activity of the palmitate desaturase (ω9 or Δ^7) was high with *sn*-2-bound palmitate in MGDG, but was negligible with *sn*-2 palmitate in DGDG. Working with spinach chloroplasts, Andrews and Heinz (1) have shown that palmitate at *sn*-2, similarly, was not desaturated in two other chloroplast-synthesized lipids, sulfolipid and phosphatidylglycerol. Together, these results suggest that desaturation of *sn*-2 palmitate may be specific for MGDG.

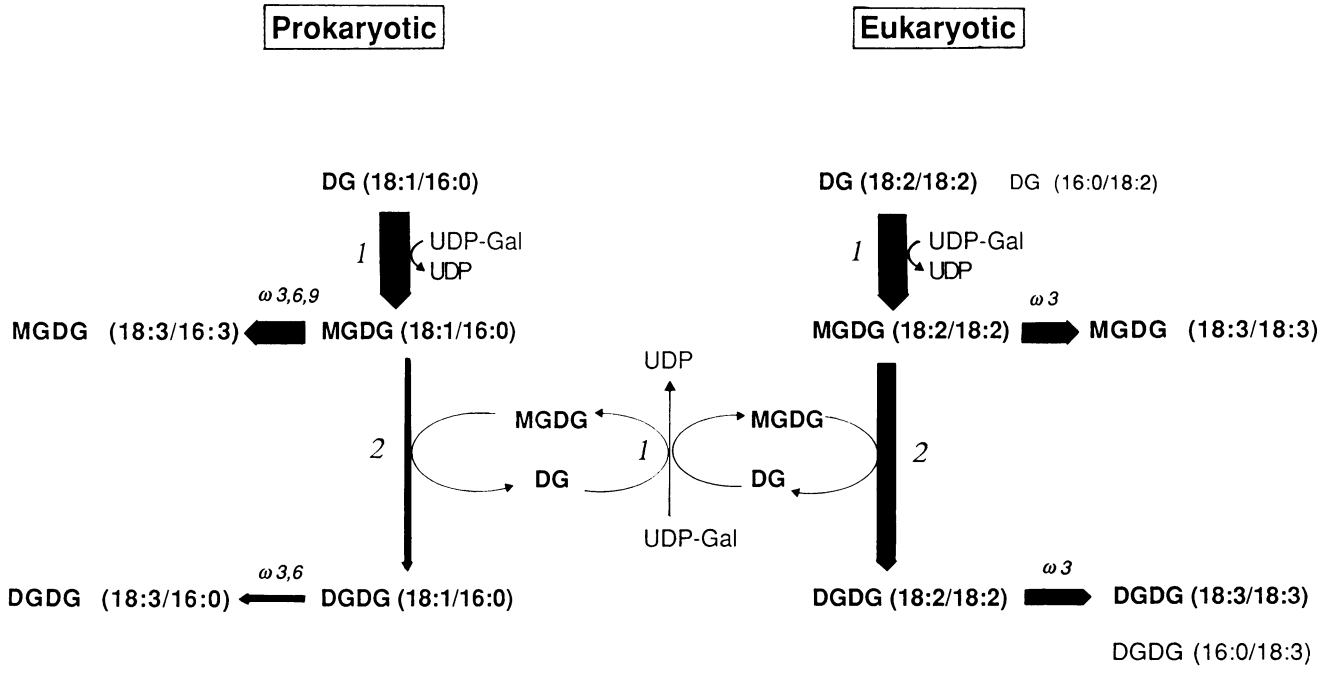

Figure 7. Simplified scheme for the synthesis of pro- and eukaryotic galactolipids in spinach and other 16:3 plants. *De novo*-made diacylglycerol, DG(18:1/16:0), is formed in chloroplasts by the prokaryotic pathway, is galactosylated to MGDG(18:1/16:0) and, at a slow rate, to DGDG(18:1/16:0). Desaturation of MGDG(18:1/16:0) competes with galactosylation and results, finally, in MGDG(18:3/16:3). Desaturation of DGDG(18:1/16:0) results in DGDG(18:3/16:0). The galactosylation of polyunsaturated MGDG species is thought to be a minor pathway and is not indicated. Eukaryotic diacylglycerol, DG(18:2/18:2), transported or released to the chloroplasts by an unknown mechanism, is galactosylated relatively fast to MGDG and to DGDG. Both galactolipids are subsequently desaturated to the $C_{18:3}/C_{18:3}$ molecular species. A similar reaction sequence, starting from DG(16:0/18:2), may lead to the formation of eukaryotic DGDG(16:0/18:3). The corresponding MGDG(16:0/18:3) is not well documented in the literature, and is therefore omitted. During the formation of DGDG by galactolipid:galactolipid galactosyltransferase diacylglycerol is produced, which is reconverted into MGDG. In 18:3 plants the prokaryotic pathway is absent. Note that this scheme includes only one enzyme transferring galactose residues from a water-soluble nucleotide of cytosolic origin. Abbreviations for enzymes: *1*, UDP-Gal:diacylglycerol galactosyltransferase (EC 2.4.1.46); *2*, galactolipid:galactolipid galactosyltransferase (EC 2.4.1.184); ω3,6,9, ω3, ω6, and ω9 desaturase (EC 1.3.?.?).

In addition, we found that palmitate located at the *sn*-1 position of both MGDG and DGDG was desaturated to $C_{16:1}$. This formation of $C_{16:1}$ at *sn*-1 of MGDG and DGDG may be catalyzed by the same $\omega 9$ or Δ^7 desaturase (although the position of the double bond in the *sn*-1 fatty acid has not been determined), since $C_{16:1}$ formation in MGDG was accompanied by that of $C_{16:2}$ and $C_{16:3}$ (Fig. 6). On the other hand, $C_{16:1}$ desaturation at *sn*-1 in MGDG was much lower than that at *sn*-2, which suggests either a preferent reaction of the desaturase with palmitate at *sn*-2 or involvement of a second activity desaturating the palmitate at *sn*-1. Recently, Roughan *et al.* (24) have reported high desaturation *in vivo* of [^{14}C]palmitate and [^{14}C]stearate that were applied to spinach leaves as free fatty acids and were incorporated into the *sn*-1 position of MGDG. The present findings indicate that this type of desaturation reaction can, indeed, be carried out by isolated chloroplasts. If it turns out that the same enzyme can desaturate palmitate and stearate at *sn*-1 as the enzyme desaturating palmitate at *sn*-2 (with identified double bond patterns), then one can conclude that the $C_{16:0}$-desaturase is a $\omega 9$, and not a Δ^7, desaturase.

Regulation of Galactolipid Synthesis in Spinach Leaves

In Figure 7 we present a simplified scheme for the regulation and interdependence of galactolipid synthesis in spinach. It is based on the following observations and premises: (a) Within a cell, the plastid and endoplasmic reticular pathways form diacylglycerol molecules with the pro- and eukaryotic configuration, respectively. These diacylglycerols are the precursors of pro- and eukaryotic galactolipids. (b) The synthesis of prokaryotic MGDG is high compared to that of prokaryotic DGDG. (c) Eukaryotic MGDG and DGDG are synthesized at similar rates. (d) Only two galactosyltransferases are known which synthesize galactolipids (10–12). (e) *In vitro,* either galactosyltransferase discriminates only moderately between various molecular species of MGDG or DGDG, but polyenoic MGDG is galactosylated faster than oligoenoic MGDG (11). (f) There are typical differences in desaturation between *de novo*-made prokaryotic MGDG and DGDG, *i.e.* palmitate at *sn*-2 is desaturated in MGDG but not in DGDG. (g) From (e) and (f), it is likely that synthesis of MGDG is linked to DGDG synthesis and that fatty acids are desaturated in both MGDG and DGDG.

According to this scheme, the preponderance of prokaryotic MGDG relative to prokaryotic DGDG is a consequence of the low galactosylation rate of MGDG(18:1/16:0). The galactosylation of more unsaturated molecular species of MGDG may occur as a minor path (not indicated) and result in the formation of low amounts of DGDG species containing unsaturated C_{16}-fatty acids at *sn*-2. A functional association of galactosyltransferases and desaturases in the plastid (envelope) membranes may ensure rapid channeling of the intermediate products, finally resulting in the polyunsaturated galactolipid species that are accumulated in leaves.

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