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

Johan W. M. Heemskerk*2, Hermann Schmidt, Ute Hammer, and Ernst Heinz

Institut für Allgemeine Botanik, University of Hamburg, Ohnhorststr. 18, 2000 Hamburg 52, Federal Republic of Germany

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 C16 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 [1-14C]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(18:3/16:0) 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 C16 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 C18 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 C16 or C18 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. (21), MGDG 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 C18:3/C18:3 combination, except for some DGDG(16:0/18:3) and some minor molecular species carrying C18:2. 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 [1-14C]acetate (4, 26, 29). In de novo-made MGDG, both C16 and C18 fatty acids were labeled and both were gradually desaturated to trienoic fatty acids. Desaturation of newly made DGDG, however, was limited to its labeled C18 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.

1 This work was supported by the Deutsche Forschungsgemeinschaft (Federal Republic of Germany). J. H. acknowledges a grant from the Federation of European Biochemical Societies.

2 Present address and corresponding address: Department of Biochemistry, B.M.C., University of Limburg, P.O. 616, 6200 MD Maastricht, The Netherlands.

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 monogalactosyldiacylglycerol sn-1-oleoyl-2-palmitoyl-glycerol.
MATERIALS AND METHODS

Chemicals

Radioactive sodium [1-14C]acetate was supplied by Amer-sham (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 1 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 CaCl2 (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

[1-14C]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 [1-14C]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 KHCO3, 5 mM MgCl2, 0.5 mM sn-glycerol-3-P, 0.5 mM KH2PO4, 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 1. Pro- and Eukaryotic Galactolipids in the Leaves from Various 163 Plants

The molar proportions of total MGDG and DGDG (MGDGmol/DGDGmol) 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 C16 fatty acids at sn-2 in either galactolipid (Σ% mol% C16 at sn-2) gave the fraction of prokaryotic MGDG and DGDG (mol% MGDGmol and mol% DGDGmol). The molar ratios of pro- and eukaryotic galactolipids (MGDGmol/DGDGmol and MGDGmol/DGDGmol) were calculated from these data.

<table>
<thead>
<tr>
<th>Plant Species</th>
<th>MGDGmol/DGDGmol</th>
<th>MGDGmol</th>
<th>DGDGmol</th>
<th>MGDGmol/DGDGmol</th>
<th>MGDGmol/DGDGmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apiaceae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aegopodium podagraria L.</td>
<td>1.56</td>
<td>75.9</td>
<td>20.8</td>
<td>5.69</td>
<td>0.47</td>
</tr>
<tr>
<td>Anthriscus sylvestris (L.) Hoffm.</td>
<td>1.66</td>
<td>88.7</td>
<td>26.6</td>
<td>5.54</td>
<td>0.26</td>
</tr>
<tr>
<td>Conium maculatum L.</td>
<td>1.68</td>
<td>87.1</td>
<td>22.4</td>
<td>6.53</td>
<td>0.28</td>
</tr>
<tr>
<td>Heracleum montegazzianum Somm. et Lev.</td>
<td>1.90</td>
<td>84.7</td>
<td>44.6</td>
<td>3.61</td>
<td>0.52</td>
</tr>
<tr>
<td>Brassicaceae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alliaria petiolaris (Bieb.) Cavara et Grande</td>
<td>1.65</td>
<td>66.7</td>
<td>22.2</td>
<td>4.96</td>
<td>0.71</td>
</tr>
<tr>
<td>Arabidopsis thaliana (L.) Heynh.</td>
<td>1.62</td>
<td>60.8</td>
<td>22.6</td>
<td>4.36</td>
<td>0.82</td>
</tr>
<tr>
<td>Brassica napus L.</td>
<td>2.08</td>
<td>82.5</td>
<td>24.6</td>
<td>6.98</td>
<td>1.43</td>
</tr>
<tr>
<td>Campanulaceae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Campanula persicifolia L.</td>
<td>1.41</td>
<td>74.0</td>
<td>17.7</td>
<td>5.89</td>
<td>0.45</td>
</tr>
<tr>
<td>Chenopodiaceae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spinacia oleracea L.</td>
<td>1.87</td>
<td>37.9</td>
<td>17.1</td>
<td>4.14</td>
<td>1.40</td>
</tr>
<tr>
<td>Geraniaceae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Geranium robertianum L.</td>
<td>1.51</td>
<td>46.5</td>
<td>7.1</td>
<td>9.89</td>
<td>0.87</td>
</tr>
<tr>
<td>Ranunculaceae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aquilegia atrata Koch</td>
<td>1.41</td>
<td>62.1</td>
<td>23.2</td>
<td>3.78</td>
<td>0.70</td>
</tr>
<tr>
<td>Ranunculus acris L.</td>
<td>1.65</td>
<td>85.4</td>
<td>21.5</td>
<td>6.55</td>
<td>0.31</td>
</tr>
<tr>
<td>Rubiaceae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Galium aparine L.</td>
<td>1.10</td>
<td>53.2</td>
<td>12.9</td>
<td>4.54</td>
<td>0.59</td>
</tr>
<tr>
<td>Solanaceae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nicotiana tabacum L.</td>
<td>1.37</td>
<td>40.3</td>
<td>15.5</td>
<td>3.56</td>
<td>0.97</td>
</tr>
<tr>
<td>Solanum tuberosum L.</td>
<td>1.89</td>
<td>56.6</td>
<td>12.0</td>
<td>8.91</td>
<td>0.93</td>
</tr>
<tr>
<td>Tropaeaceae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tropaeolum majus L.</td>
<td>1.89</td>
<td>69.2</td>
<td>13.4</td>
<td>9.76</td>
<td>0.67</td>
</tr>
</tbody>
</table>
Figure 1. Distribution of C18 fatty acids within the galactolipids from leaves of both 18:3 and 16:3 plants. The ratio of C18 fatty acids in MGDG to C18 fatty acids in DGDG is graphed as a function of the hexadecatrienoiate content in MGDG for 55 plant species. Data represented by open circles (C) 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" 16:3 plants represent the very left part of the graph and "typical" 18:3 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 antrone 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 aceton: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 [14C]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 hexadecatrienoiate in MGDG from a particular plant species and the ratio of C16 fatty acids in MGDG to C16 fatty acids in DGDG (Fig. 1). According to this relationship, 16:3 plants have higher levels of C16 fatty acids in MGDG than in DGDG, whereas 18:3 plants (at the origin of the graph in Fig. 1) accumulate less C16 fatty acids in MGDG than in DGDG. Apparently, in leaves from most plants the C16 fatty acids are distributed rather asymmetrically among the two galactolipids. Because the concentration of hexadecatrienoiate, 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 eu-

karyotic galactolipids in leaves from sixteen 16:3 plants. Pro-

portions of prokaryotic galactolipids were calculated from the quantities of total MGDG and DGDG and from the percentage of C16 fatty acids found at sn-2 in each 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 eu-karyotic galactolipids revealed that in 16:3 plants eu-karyotic DGDG was relatively abundant, since the molar ratio of eu-karyotic MGDG to eu-karyotic 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 hexadecatrienoiate in MGDG (Fig. 2). Thus, the relative preponderance of prokaryotic...
Spinach leaves were incubated with [14C]acetate (370 kBq) for 2 h. Chloroplasts (500 μg Chl) purified from the same batch of leaves were incubated for 2 h with [14C]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.

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

<table>
<thead>
<tr>
<th>Labeling of Lipids</th>
<th>Leaves</th>
<th>Chloroplasts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>kBq/leaf</td>
<td>kBq/mg Chl</td>
</tr>
<tr>
<td>Total acyl lipids</td>
<td>21.6 ± 5.17</td>
<td>71 ± 8.5</td>
</tr>
<tr>
<td>MGDG</td>
<td>6.0 ± 3.00</td>
<td>26 ± 5.6</td>
</tr>
<tr>
<td>DGDG</td>
<td>0.54 ± 0.11</td>
<td>2.5 ± 0.69</td>
</tr>
<tr>
<td>Ratio MGDG/DGDG</td>
<td>11.0 ± 3.1</td>
<td>12.1 ± 1.5</td>
</tr>
</tbody>
</table>

* 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 [14C]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...
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).

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 hexadecatrienol 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 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 (□).
Figure 6. Desaturation of sn-1 and sn-2 fatty acids from MGDG and DGDG in isolated spinach chloroplasts. Chloroplasts were labeled with [14C]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 [14C]acetate by 65 ± 9% (mean ± se, n = 3). In contrast, the desaturation of neither C16 nor C18 fatty acids in de novo made MGDG was inhibited significantly by thermolysin treatment (data not shown).

DISCUSSION

Formation and Desaturation of Prokaryotic Galactolipids

Our results indicate that isolated spinach chloroplasts synthesize prokaryotic MGDG and DGDG with high levels of unsaturated fatty acids, when incubated with [14C]acetate, sn-glycerol-3-P, and UDP-Gal at a neutral pH value. The [14C]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 C16 to C18 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, C18 fatty acids at sn-1 of MGDG and DGDG were desaturated well, but C16 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 C16 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 C16ω7, and not C16:1- or C16:2-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 C18:1/C16:0 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 desaturation (ω9 or Δ7) 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-synthetized lipids, sulfolipid and phosphatidylglycerol. Together, these results suggest that desaturation of sn-2 palmitate may be specific for MGDG.

### Abbreviations

- MGDG: Methylglyceridegalactoside
- DGDG: Diacylglyceridegalactoside
- UDP-Gal: UDP-Galactose
- DG: Diacylglycerol

**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 C18:3/C18:3 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, ω6, and ω9 desaturase (EC 1.3.1.7).
In addition, we found that palmitate located at the sn-1 position of both MGDG and DGDG was desaturated to C16:1. This formation of C16:1 at sn-1 of MGDG and DGDG may be catalyzed by the same ω9 or δ desaturase (although the position of the double bond in the sn-1 fatty acid has not been determined), since C16:1 formation in MGDG was accompanied by that of C16:2 and C16:3 (Fig. 6). On the other hand, C16:1 desaturation at sn-1 in MGDG was much lower than that at sn-2, which suggests either a preferential 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 [14C]palmitate and [14C]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 C16:0-desaturase is a ω9, and not a δ, 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 C16-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.

ACKNOWLEDGMENT

We thank M. Frenzen for very helpful discussions.

LITERATURE CITED


