Biosynthesis of digalactosyl diglyceride in *Vicia faba* leaves

J. P. Williams, M. Khan, and S. Leung

Department of Botany, University of Toronto, Toronto, Ontario, Canada

Abstract Developing and mature leaf tissue from *Vicia faba* plants were pulse-fed $^{14}CO_2$. The lipids were extracted at intervals after exposure to light, and the galactolipids monogalactosyl diglyceride (MGDG) and digalactosyl diglyceride (DGDG) were separated. After methylation and methanolysis, gas–liquid chromatography was used to separate the two galactose units of DGDG and the galactose of MGDG. The specific activities of the galactoses and the changes over the time period of the experiment were determined. The results support the view that DGDG is formed by galactosylation of MGDG. This does not take place by a rapid two-enzyme system reaction but more slowly in two phases: galactosylation of a pool of newly formed MGDG and a more random galactosylation of MGDG. There is no evidence of a high turnover of galactose in these lipids.

Supplementary key words galactolipids · chloroplasts · methyl galactosides · gas–liquid chromatography

Monogalactosyl diglyceride (MGDG) and digalactosyl diglyceride (DGDG) are the two major lipid constituents of chloroplast lamellae, and many attempts have been made to determine their functions and biosynthetic pathways (see recent reviews, Refs. 1–3). Most attention has been directed toward determining fatty acid synthesis with both in vivo and in vitro studies, and a few attempts have been made to assess the biosynthesis of the galactose moieties (2, 4). Studies with isolated chloroplast preparations and enzyme suspensions (5–14) have led to the generally accepted idea that MGDG is formed from a diglyceride by galactosylation utilizing UDP-galactose. DGDG is then formed from MGDG by a similar galactosylation step but involving a different enzyme system. No definitive evidence has been produced from in vivo studies to confirm this pathway, nor have attempts been made to determine the nature (other than the fatty acid composition) of the pool of MGDG from which DGDG is formed.

We report here the separation and analysis of the two galactose units of digalactosyl diglyceride after pulse-feeding leaf samples with $^{14}CO_2$. The kinetics of labeling of the two galactoses have been used to confirm, with in vivo studies, the biosynthetic pathway of digalactolipid and to speculate on the nature of the monogalactosyl diglyceride precursor pool.

MATERIALS AND METHODS

Broad beans (*Vicia faba* L. 'Giant Windsor') were grown in growth chambers at 21°C in the dark or under artificial illumination of 1100 foot candles (11.84×10^3 lx).

Developing leaves were obtained by growing plants in complete darkness for 3 wk then exposing them to continuous light for 48 hr. Mature leaves were obtained from plants grown under 16-hr day–8-hr night regimes for 3–4 wk. Developing leaves were harvested and deribbed; leaf discs were taken from mature leaves. The lamina and discs were weighed, spread on damp filter paper in petri dishes, and placed in a bell-jar feeding chamber that was illuminated with high-intensity fluorescent light. The leaves (10–20 g) were allowed to assimilate 600 μCi of $^{14}CO_2$ (5.8 mCi/mmole), generated from $Na_2{}^{14}CO_3$, for 7–10 min. After feeding, the leaves were placed in a growth chamber under continuous illumination and sampled at different time intervals.

Lipids were extracted from 2-g samples by the method of Williams and Merrilees (15). The lipids were separated by thin-layer chromatography on silica gel G (Merck) impregnated with 5% $(NH_4)_2SO_4$ (w/w), using the solvent acetone–benzene–water 91:30:8 (16). The monogalactosyl and digalactosyl diglycerides, located by spraying the plates with 0.05% dichlorofluorescein in methanol, were scraped off and eluted with chloroform–methanol 1:1. The R_f of MGDG was 0.78 and that of DGDG was 0.33. The identity and purity of these lipids was confirmed by quantitative GLC analyses of their fatty acid

Abbreviations: MGDG, monogalactosyl diglyceride; DGDG, digalactosyl diglyceride; Chl, chlorophyll; MG, monogalactosyl diglyceride galactose; DG I and DG II, inner and outer galactose units of digalactosyl diglyceride, respectively; UDP-galactose, uridine diphosphate galactose; GLC, gas–liquid chromatography; TMS, trimethylsilyl.

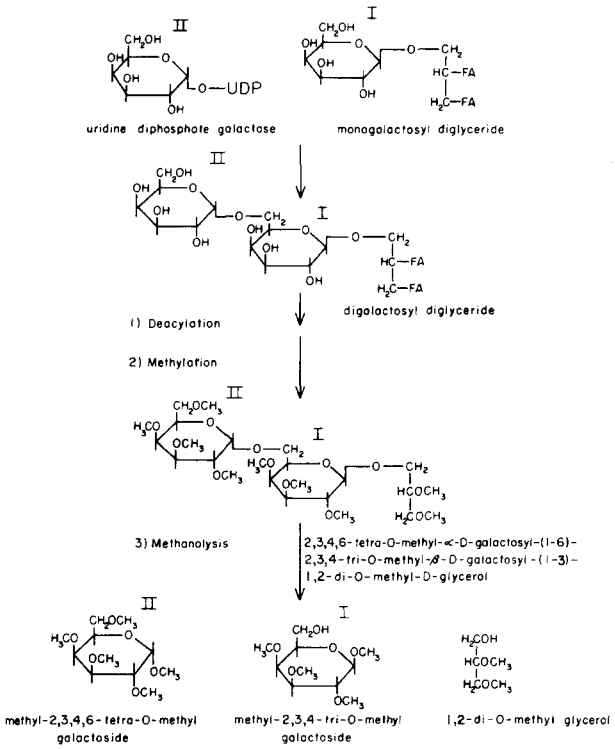

Fig. 1. The generally accepted pathway of the biosynthesis of digalactosyl diglyceride from monogalactosyl diglyceride and UDP-galactose and an outline of the method used to separate the two resulting galactose units. Details of the deacylation, methylation, and methanolysis are in Materials and Methods.

methyl esters and the trimethylsilyl derivatives of the galactose and glycerol moieties.

After drying, the lipids were redissolved in 0.5 ml of dry methanol, and an equal volume of 0.5 N sodium methoxide was added. Deacylation was completed within 1 hr at room temperature, after which the solution was neutralized with ethyl formate and the fatty acid methyl esters were extracted with hexane. Any trace of intact lipid was removed by a further extraction with chloroform.

The resulting galactosyl glycerols were methylated using a combination of the methods of Hakomori (17) and Purdie and Irvine (18). The galactosyl glycerols were dried and redissolved in 0.4 ml of dimethyl sulfoxide. An equal amount of 2.5 M methylsulfinyl anion in dimethyl sulfoxide (prepared according to Conrad [19]) was added after flushing with nitrogen, and the reaction mixture was left for 2 hr at room temperature. After cooling in ice, excess methyl iodide (250 μl) was added dropwise. After 30 min, water was added and the methylated galactosyl glycerols were extracted with chloroform. The combined chloroform extracts were washed once with water. The methylated galactosyl glycerols were further treated by the method of Purdie and Irvine (18) to ensure complete methylation. The dried methylated galactosyl glycerols were redissolved in 250 μl of methyl iodide, 200 mg of silver oxide was added, and the suspension was mixed for 48 hr at room temperature. The methylated products were removed from the silver oxide by several washings with chloroform.

The combined washings were dried and the methylated galactosyl glycerols were methanolyzed with dry methanolic HCl (1.5 N) at 80°C for 4 hr. After neutralization with silver carbonate, the products were removed by washing the residue with chloroform–methanol 1:1. The combined washings were dried and the methylated galactosides were analyzed by GLC before and after TMS derivatization (20). Methyl pentadecanoate (0.20 μmole) was added to each sample as an internal standard prior to injection into the column.

The following GLC conditions were used: packing, 3% JXR on Gas-Chrom Q; column, 6 ft × 4 mm, ID; temperature programming, 100°C for 4 min, increasing at 5°C/min to 200°C; flame ionization detector; carrier gas, nitrogen; Packard model 7401 gas chromatograph. The effluent was divided by a stream splitter in the ratio of 4.8 to the collector and 1 to the flame detector.

The galactosides were collected and their radioactive content was determined by scintillation counting according to the method of Watson and Williams (21). The radioactivity was determined in 15 ml of toluene containing 2,5-diphenyloxazole (PPO) (6 g/l) and *p*-bis-[2-(5-phenyloxazolyl)]-benzene (POPOP) (300 g/l) in a Packard liquid scintillation spectrometer (model 3375) with a counting efficiency of 90%.

The molar response factors for methyl-2,3,4,6-tetra-*O*-methyl galactoside and methyl-2,3,4-tri-*O*-methyl galactoside and its TMS derivative were determined using 2,3,4,6-tetramethyl glucose obtained from K & K Laboratories, Plainview, N.Y. The molar response factors of the galactosides were assumed to be identical with those of the glucosides.

The quantities of the galactosyl diglycerides were estimated by GLC from their fatty acid methyl esters. The methyl esters were chromatographed in 4 ft × 4 mm ID columns with 10% EGSS-X on Gas-Chrom P at 180°C (isothermal). The quantity of each fatty acid was calculated, using methyl pentadecanoate as internal standard, and the total was divided by two to give the total quantity of lipid.

Chlorophyll was determined according to the method of Arnon (22).

RESULTS

The generally accepted pathway of conversion of MGDG to DGDG and the method of separating the two

galactose units used in this study are outlined in **Fig. 1.** Galactosylation of MGDG results in the formation of DGDG, which, therefore, contains two galactose units of differing origin. The galactose unit attached to the glycerol or the inner galactose (DG I) would be of MGDG origin; the outer galactose (DG II) would be of UDP-galactose origin.

Separation and purification of the galactolipids followed by deacylation and methylation result in the production of methylated galactosyl glycerols. Methanolysis of the digalactosyl glycerol yields two methylated galactosides differing by one methyl group, each galactoside representing a galactose unit in the original molecule. Methyl-2,3,4,6-tetra-*O*-methyl galactoside is derived from the outer galactose unit (DG II); methyl-2,3,4-tri-*O*-methyl galactoside is from the inner galactose unit (DG I). Methanolysis of methylated monogalactosyl glycerol results in the production of methyl-2,3,4,6-tetra-*O*-methyl galactoside. The two methylated galactosides were separated by GLC. Improved separation is obtained, on nonpolar packings, by trimethylsilylation of the free hydroxyl group of methyl-2,3,4-tri-*O*-methyl galactoside.

The methyl tetra-*O*-methyl galactoside was identified by comparison with standard galactose after methylation. Two isomers were separated with retention temperatures of 136°C and 138°C, respectively. The presence or absence of free hydroxyl groups was confirmed by trimethylsilylation. Only the compound identified as methyl tri-*O*-methyl galactoside (retention temperatures for two isomers: 141°C and 142°C) reacted with the TMS reagent. The TMS derivative of methyl tri-*O*-methyl galactoside gave only one peak with a retention temperature of 154°C. Similar results were obtained with prepared methyl glucoside standards that have retention temperatures slightly lower than their corresponding galactosides.

Complete methylation of the galactosyl glycerols using this technique has been confirmed by GLC of the products.[1] Only one product was obtained from each galactosyl glycerol with a molar response similar to the calculated expected value. On methanolysis, in both studies, the methylated monogalactosyl glycerol yielded only methyl tetra-*O*-methyl galactoside, with no traces of other galactosides, indicating complete methylation. Similarly, the methylated digalactosyl glycerol yielded only two galactosides, methyl tetra- and methyl tri-*O*-methyl galactosides. Approximately equal quantities of the two galactosides were recovered after GLC, giving an average ratio in the 21 samples used in these experiments of 0.92:1, methyl tetra-*O*-methyl galactoside to methyl tri-*O*-methyl galactoside.

[1] Williams, J. P., G. R. Watson, M. Khan, S. Leung, A. Kuksis, O. Stachnyk, and J. J. Myher. Submitted for publication.

Using nine radioactive samples of each lipid as standards, the recovery of ^{14}C as methyl galactosides was estimated after methylation and methanolysis of the galactosyl glycerols and GLC of the galactosides. Despite the many steps involved in these techniques and the loss of the activity in the glycerol moiety, which was not recovered in this estimate, the average recovery was estimated to be 64% and 72% for mono- and digalactosyl glycerol, respectively. Since the loss of activity was not due to incomplete methylation, it had no effect on the specific activity determinations, which were made by comparing the radioactivity collected with the quantity determined for each galactoside after GLC.

Two types of leaf tissue were examined in this study, expanding or developing leaves and mature leaves. The developing leaves were taken from plants that had been etiolated and then exposed to 48 hr of light. At this stage the prolamellar bodies of the etioplasts have been considerably reduced or have disappeared (23), and the thylakoids of the chloroplasts are developing rapidly. In contrast, the chloroplasts of the mature leaves do not appear to be growing or expanding.

The quantity of chlorophyll and galactolipids in the leaf samples over the time periods of the experiments are listed in **Tables 1** and **2.** The developing leaves are clearly synthesizing both chlorophyll and galactolipids, whereas little or no net synthesis occurs in the mature leaves. There appears to be little or no obvious degeneration of the chloroplast in the tissues even after 48–72 hr, judging by the chlorophyll and lipid contents.

The specific radioactivity of the galactose units of monogalactosyl and digalactosyl diglycerides are also listed in Tables 1 and 2. The first samples from both types of tissue were taken only 7–10 min after the introduction of $^{14}CO_2$ into the feeding chamber. By this time, the galactose unit of monogalactosyl diglyceride (MG) and the outer galactose unit of digalactosyl diglyceride (DG II) had high specific activities. In contrast, the inner galactose unit (DG I) had a relatively low specific activity.

The higher specific activity of DG II than DG I is consistent with the view that digalactosyl diglyeride is formed by the addition of newly synthesized radioactive galactose to existing, mainly nonradioactive, monogalactosyl diglyceride. If this were not the case, both galactose units would be expected to have similar specific activities. There was no indication of rapid turnover of the galactose units over the time periods used in these experiments. There seems little chance, therefore, that the high specific activity of DG II (or MG) resulted from a high turnover rate.

In the developing leaf tissue, the specific activity of DG I increased during the 24–48-hr period after pulse-feeding. This increase in activity presumably occurred by conversion of newly formed radioactive MGDG to DGDG. The

TABLE 1. Galactolipid and chlorophyll content of developing leaves and specific activities of the galactose units after $^{14}CO_2$ pulse-feeding

Time	Quantity[a]			Specific Activity[b]			
	Chl[c]	MGDG	DGDG	MG[c]	DG I	DG II	Ratio DG II/DG I
hr	*μmoles/g fresh wt*			*dpm/μmole* × 10^{-5}			
0[d]	1.44	3.93	2.70	1.74	0.22	1.04	4.7
0.25	2.02	3.80	2.77	2.98	0.23	1.21	5.3
0.5	2.59	3.94	2.72	4.80	0.30	1.40	4.7
1	2.13	4.12	2.70	4.40	0.46	1.33	2.9
2	2.28	4.12	2.75	3.90	0.46	1.04	2.3
4	2.25	4.42	2.77	2.86	0.51	1.11	2.2
6	2.19	4.40	2.78	2.30	0.68	1.24	1.8
8	2.34	5.05	3.25	2.20	0.76	1.13	1.5
16	2.61	5.42	3.44	2.03	0.85	1.42	1.6
24	2.68	5.61	3.18	1.59	1.04	1.12	1.1
48	2.77	5.55	4.13	1.47	1.04	1.04	1.0
72	3.22	6.07	4.49	0.93	0.83	0.71	0.85

[a] Determined from fatty acid methyl esters.
[b] Calculated from galactoside samples that contained between 581 and 49,651 cpm/vial.
[c] Chl, chlorophyll; MG, monogalactosyl diglyceride galactose; DG I and DG II, digalactosyl diglyceride galactoses (Fig. 1).
[d] Sample taken immediately after 7–10-min feeding period.

specific activity of MG bears this out, reaching a peak at 0.5–1 hr then dropping as the specific activity of DG I increases. Part of the drop in specific activities in the later times is, however, due to an increase in the total amount of galactolipids in the tissue and not to a loss of radioactivity.

In the mature leaf tissue, the specific activity of DG I also increased over a period of 24 hr, supporting the findings in the developing leaf tissue. The MG was rapidly labeled but the specific activity did not decrease as found in the developing leaves. There are probably two reasons for this. First, the lipid content of the leaves remained constant and there was, therefore, less dilution of the radioactivity with newly synthesized lipid. Second, there is in this tissue a considerable amount of recycling of respired $^{14}CO_2$ by photosynthesis,[2] resulting in a slow but continuous increase in activity in both galactolipids. The loss in activity of MGDG by galactosylation to DGDG is probably balanced by this process.

In both tissues, the increase in specific activity of DG I and, therefore, the conversion of MGDG occurred in two phases. The first phase was a fairly rapid increase in activity (although slower than found in MG and DG II) up to 1–2 hr, presumably at the expense of a high specific activity pool of MGDG. Over 25% of the activity accumulated in DG I in 24 hr was taken up in the first hour after feeding. The second phase was a much slower but continuous increase in activity and occurred over a period of at least 24 hr in both tissues. The slower incorporation of activity into DG I in this phase could not have been due solely to the dilution of radioactivity in the precursor MGDG pool. This must have occurred by galactosylation of a larger pool of MGDG with a lower specific activity.

It seems probable, therefore, that DGDG is formed from a pool of newly formed MGDG and also by random galactosylation in the chloroplast. Despite the wide variation in specific activities in the three galactose units in the early times of both tissues, after 24–48 hr they were very similar. The fact that the specific activity of MG does not fall considerably below DG I would suggest that not all of the MGDG molecules were susceptible to galactosylation in this time.

DISCUSSION

Our results clearly support the view that digalactosyl diglyceride is formed by galactosylation of monogalactosyl diglyceride and to our knowledge represent the first evidence of this reaction from in vivo studies.

In addition to this, our results indicate that the galactose units of galactolipids do not turn over rapidly but are fairly stable entities. These results and other data from our laboratory support the results of Trémolières (2) and Ferrari and Benson (4), which indicated more rapid incorporation of ^{14}C into the galactose rather than the fatty acids of galactolipids. The incorporation of activity into the fatty acids of *Vicia faba* leaves continues for several days,[3] in contrast to the rapid incorporation into galactose. This slow incorporation of activity is due to the time taken for the synthesis and the desaturation of the fatty acids. The comparatively more rapid incorporation of ^{14}C

[2] Williams, J. P., and S. Leung. Unpublished results.

[3] Williams, J. P., G. R. Watson, M. Khan, and S. Leung. Submitted for publication.

TABLE 2. Galactolipid and chlorophyll content of mature leaves and specific activities of the galactose units after $^{14}CO_2$ pulse-feeding

Time	Quantity[a]			Specific Activity[b]			
	Chl[c]	MGDG	DGDG	MG[c]	DG I	DG II	Ratio DG II/DG I
hr	*μmoles/g fresh wt*			*dpm/μmole* × 10^{-4}			
0[d]	2.17	4.16	2.33	4.54	0.38	5.64	14.9
0.25	2.21	3.58	2.58	5.57	0.52	5.60	10.8
0.5	2.27	3.57	2.33	5.76	0.78	7.07	9.1
1	2.17	3.35	2.34	4.28	1.16	7.13	6.2
2	2.25	3.90	2.35	5.89	1.22	7.62	6.2
4	2.23	3.60	2.51	6.36	1.75	8.11	4.6
12	2.29	3.66	2.27	6.17	2.66	6.31	2.4
24	2.44	3.37	2.50	7.36	4.35	8.23	1.9
48	2.14	3.35	2.63	5.15	4.21	5.56	1.3

[a] Determined from fatty acid methyl esters.
[b] Calculated from galactoside samples that contained between 406 and 7500 cpm/vial.
[c] Chl, chlorophyll; MG, monogalactosyl diglyceride galactose; DG I and DG II, digalactosyl diglyceride galactoses (Fig. 1).
[d] Sample taken immediately after 7–10-min feeding period.

into galactose is not, therefore, due to the rapid turnover of galactose, as suggested by Ferrari and Benson (4), but to the time taken for the incorporation of ^{14}C into the fatty acids.

A number of workers have shown in isolated chloroplast preparations or extracts, using UDP-[^{14}C]galactose, that the synthetic mechanisms of MGDG and DGDG differ considerably. Eccleshall and Hawke (11) were able to obtain MGDG synthesis but little DGDG synthesis in spinach chloroplasts. Ongun and Mudd (6) found that the enzyme that synthesizes MGDG in spinach chloroplasts appeared to be more tightly bound to the membranes than that responsible for DGDG synthesis. Mudd, van Vliet, and van Deenen (10) found a difference in the two enzyme rates at different pH's and Mudd et al. (14) found a difference in the effect of inhibitors on the same two enzymes from spinach. At least two different enzymes exist, therefore, which not only react differently to pH and inhibitors but are possibly located in different parts of the chloroplast or cell. Our data clearly support this idea indicating that DGDG synthesis does not involve a two-enzyme system converting diglyceride to MGDG to DGDG in rapid succession. Rather, it appears to be a separate process (or processes) involving galactosylation of at least two pools of MGDG, which results in a relatively slow conversion of radioactive MGDG to DGDG.

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