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Metabolism of Unsaturated Monogalactosyldiacylglycerol Molecular Species in *Arabidopsis thaliana* Reveals Different Sites and Substrates for Linolenic Acid Synthesis

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ABSTRACT

Synthesis of unsaturated monogalactosyldiacylglycerol (MGDG) was examined in a mutant of *Arabidopsis thaliana* (L.) Heynh. containing reduced levels of hexadecatrienoic (16:3) and linolenic (18:3) acids in leaf lipids. Molecular species composition and labeling kinetics following the incorporation of exogenous [^{14}C]fatty acids suggest that at least two pathways and multiple substrates are involved in desaturation of linoleic acid (18:2) to 18:3 for production of unsaturated galactolipids. A reduction in 18:3/16:3 MGDG and an increase in 18:2/16:2 MGDG, together with labeling kinetics of these molecular species following the incorporation of exogenous [^{14}C]12:0 fatty acids, suggests that a chloroplastic pathway for production of 18:3 at the *sn*-1 position of MGDG utilizes 18:2/16:2 MGDG as a substrate. This chloroplastic (prokaryotic) pathway is deficient in the mutant. When exogenous [^{14}C]18:1 was supplied, a eukaryotic (cytoplasmic) pathway involving the desaturation of 18:2 to 18:3 on phosphatidylcholine serves as the source of 18:3 for the *sn*-2 position of MGDG. This eucaryotic pathway predominates in the mutant.

The biosynthesis of polyunsaturated chloroplast GL[1] remains a contentious issue in plant lipid biochemistry. In particular, different substrates and cellular compartments appear to be involved in the sequential conversion of saturated FA formed *de novo* in the chloroplast (15).

MGDG of '16:3-plants' is enriched in both 18:3 and 16:3 FA. The major MGDG molecular species are 18:3/16:3 and 18:3/18:3, and it is contended that these are formed via two different biosynthetic pathways (14, 16, 17). The 18:3/16:3 MGDG is thought to arise from DG precursors originating in the chloroplast (the 'prokaryotic' pathway), and the 18:3/18:3 MGDG from a cytoplasmic ('eukaryotic') pathway in which PC provides precursor DG. By this scheme, prokaryotic MGDG is first synthesized as the 18:1/16:0 species within the chloroplast, and this is subsequently desaturated at both positions *in situ* to form 18:3/16:3 MGDG. Conversely, desaturation of the C_{18} FA of eukaryotic MGDG is believed to occur largely outside the chloroplast, such that DG precursors formed from PC already contain 18:2 and possibly 18:3. After galactosylation, the MGDG is desaturated to form the 18:3/18:3 species (16, 20, 21). This eukaryotic pathway predominates in '18:3-plants' (16).

Browse *et al.* (2) have recently isolated a mutant of *Arabidopsis thaliana* (L.) ('JB1') which is deficient in 16:3 and 18:3 FA compared to the wild-type plant. We have selected the JB1 mutant as a potentially useful system in which to examine the participation of different pathways in the formation of unsaturated GL. Our initial approach was to examine the labeling kinetics of unsaturated FA in individual MGDG molecular species following the incorporation of exogenously supplied [^{14}C]18:1 and [^{14}C]12:0 substrates. The data presented here are strongly supportive of more than one pathway being operative in MGDG synthesis, with a nonchloroplastic pathway contributing an increased amount of unsaturated MGDG in the mutant compared to the wild-type *Arabidopsis*. Evidence for different sites and substrates of 18:3 synthesis is presented.

MATERIALS AND METHODS

Plant Material and Growth Conditions. Seeds of the wild type and mutant line JB1 *Arabidopsis thaliana* (L.) Heynh. were sown in a mixture of peat:vermiculite (1:1) irrigated with the mineral solution defined by Somerville and Ogren (19). They were incubated at 4°C for 48 h, and then grown in an 18 h photoperiod (200 $\mu E \cdot m^{-2} \cdot s^{-1}$) at 26°C with a 17°C dark period. *Arabidopsis* seeds were a kind gift of Chris Somerville (MSU-DOE Plant Research Laboratory, Michigan State University).

[^{14}C]Fatty Acid Incorporation. Leaves (with petioles attached) were removed from plants after 24 d growth. [1-^{14}C]Oleic acid (18:1) (52.6 mCi/mmol; New England Nuclear[2] or [1-^{14}C]lauric acid (12:0) (26 mCi/mmol; Amersham) in 100 μl ethanol was dispersed in 20 ml of mineral solution (19) in 9 cm Petri dishes, and detached leaves (1.5–2.0 g fresh weight) were incubated in this under continuous light (200 $\mu E \cdot m^{-2} \cdot s^{-1}$) for 1 h at 26°C. They were then washed twice in nonradioactive mineral solution in order to remove exogenous radioactivity, and further incubated in nonradioactive mineral solution for specified periods of time under continuous light at 26°C.

Lipid Analysis. Lipids were extracted using the procedure of Bligh and Dyer (1). Separation into neutral lipid, GL, and

[1] Abbreviations: GL, galactolipid(s); FA, fatty acid(s); PL, phospholipid(s) DG, diglyceride(s); TG, triglyceride(s); MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; PC, phosphatidylcholine; C_{16} fatty acids, fatty acids of 16 carbon atoms; C_{18} fatty acids, fatty acids of 18 carbon atoms. In the shorthand numbering system used for identifying fatty acids, the figure preceding the colon indicates the number of carbon atoms in the fatty acid while that following the colon represents the number of double bonds present. Pairs of numbers representing the fatty acids, when separated by a slash, represent the components in the *sn*-1 and *sn*-2 positions, respectively, of the molecular species.

[2] Mention of companies or commercial products does not imply recommendation or endorsement by the United States Department of Agriculture over others not mentioned.

phospholipid (PL) classes was achieved using silica Sep-Pak cartridges (Waters Assoc., Milford, MA). The individual classes were successively eluted with chloroform, acetone, and methanol, respectively. Individual PL and GL components were resolved by TLC on silica gel H using chloroform-acetic acid-methanol-water (75:25:5:2.2, v/v/v/v) as developing solvent, and neutral lipid classes were separated in petroleum ether-ethyl ether-acetic acid (70:30:1, v/v/v). After evaporation of TLC solvents under N_2, the lipid bands were detected by briefly exposing the plate to iodine vapor. To determine radioactivity of the resolved components, the iodine vapor was allowed to dissipate, and then the lipid bands were transferred to scintillation vials and counted using a Packard Tri-Carb (model 2425) liquid scintillation counter. Aliquots of the total lipid fractions were also assayed. MGDG and DGDG separated by TLC were quantified based on sugar content according to Roughan and Batt (13). FA methyl esters were prepared directly with boron trifluoride/methanol (Sigma Chemical Co.) (9). GLC analysis was performed on a Hewlett Packard (model 5880A) gas chromatograph using the procedure of Lynch and Thompson (8).

Molecular Species Analysis. MGDG and DGDG separated by TLC were scraped off the plate and eluted from the silica gel using chloroform-methanol-water (3:5:1, v/v/v). The eluates were adjusted to give proportions of chloroform-methanol-0.1 M KCl (2:3.5:4, v/v/v). The lower phase was collected and dried under a stream of N_2. Samples of MGDG and DGDG (0.2–0.4 μmol) were separated into constituent molecular species using a Waters Assoc. HPLC system (model 6000A) equipped with a model U6K universal injector, and a 25 cm × 4.6 mm Rainin Microsorb (5 μm) reverse-phase column. The mobile phase consisted of methanol-water (96:4, v/v) (18). A flow rate of 1 ml/min was maintained. For direct quantitation, the molecular species were detected by a Tracor 945 Flame Ionization LC Detector (Tracor Instruments, Austin, TX) operated at a block temperature of 160°C (18).

The radioactivity of the resolved molecular species was determined after peaks first eluted through a Waters Assoc. (model 450) Variable Wavelength Detector (operated at 205 nm) and then eluted through a Flo-One Beta (model IC) HPLC Radioactive Flow Detector (Radiomatic Instruments and Chemical Co., Inc., Tampa, FL) with Flo-Scint III (Radiomatic Instruments and Chemical Co., Inc.) as scintillator mixed with the HPLC eluate by means of a pump operating at 2 ml/min. Counting efficiency for [^{14}C] was 80%.

Positional Distribution of FA in GL and PC. GL molecular species were resolved by HPLC and detected at 205 nm as described above. They were individually collected, dried under N_2, and resuspended in 1 ml of diethyl ether. Fatty acids were hydrolyzed from the *sn*-1 position of the GL by incubating with lipase from *Rhizopus arrhizus* (Boehringer, Mannheim) for 1 h at 37°C (1 ml resuspended lipid combined with 0.25 ml of 10 mM sodium phosphate buffer (pH 7.4) containing 0.5 mM $CaCl_2$, and 35 μl of lipase). The resultant FA and 2-acyl GL were separated by TLC using petroleum ether-diethyl ether-acetic acid (70:30:1, v/v/v). Lipids were eluted from the silica gel and FA identified by GLC following preparation of methyl esters with boron trifluoride/methanol.

PC separated by TLC, and eluted from the silica gel, was dried under N_2 and hydrolyzed with phospholipase A_2 from *Crotalus adamanteus* venom (Sigma Chemical Co.) using the method of Ramesha and Thompson (12). The lipid was resuspended in 1 ml of diethyl ether and combined with 50 μl of 0.1 M sodium borate (pH 7.6), and 0.2 mg of phospholipase A_2 in 50 μl of 0.1 M Tris-HCl (pH 7.6), containing 0.4 mM $CaCl_2$. The preparation was incubated for 1 h at 37°C, and the resultant FA and lysophosphatidylcholine separated by TLC as described above.

Analysis of [^{14}C]FA. The radioactivity in different FA of MGDG and PC eluted from TLC plates, and of FA hydrolyzed from the *sn*-1 or *sn*-2 positions of the lipids as described above, was determined after resolution of *p*-nitrophenacyl derivatives of the FA by HPLC (11). The intact lipids were refluxed for 1 h in methanol-3 N NaOH (9:1, v/v) and after acidification with HCl, the resultant FAs were extracted with petroleum ether, and then dried under N_2. The *p*-nitrophenacyl derivatives, prepared according to Halgunset *et al.* (5), were separated by HPLC using a 15 cm × 4.5 mm Rainin Microsorb (5 μm) reverse-phase column. The mobile phase, delivered at a flow rate of 1 ml/min, consisted of methanol-acetonitrile-water (82:9:9, v/v/v) (7). The derivatives were detected at 254 nm, and radioactivity was analyzed by means of elution through a Flo-One/Beta HPLC Radioactive Flow Detector as described above.

RESULTS

Composition of MGDG and DGDG from Wild type and JB1 Mutant. The FA compositions of MGDG and DGDG from the wild type and JB1 mutant are shown in Table I. Relative amounts of 18:3 and 16:3 in MGDG were reduced in the mutant, and levels of 18:2 and 16:2 were significantly higher. DGDG from JB1 was also relatively low in 18:3, and elevated levels of both 18:1 and 18:2 were found.

The major MGDG molecular species in wild-type *Arabidopsis* was 18:3/16:3 (Table II). This component was reduced in the mutant, and 18:3/16:2 and 18:2/16:2 MGDG were increased, suggesting that these may be immediate precursors of 18:3/16:3 MGDG. On a μmol basis, the ratio of total 18/16C species to 18/18C species was reduced from 19.4 in wild-type MGDG to 2.9 in the mutant, suggesting that the eukaryotic (nonchloroplastic) synthetic pathway contributed an increased amount of MGDG precursors in the mutant. Despite the overall reduction in the amount of MGDG 18:3, there was an increase in the amount of 18:3/8:3 MGDG (μmol/g dry weight) in JB1 *Arabidopsis.*

Changes in DGDG molecular species were also found. The amount of 18:3/18:3 DGDG was reduced in the mutant and 18:3/16:0 was increased (Table III). 18:1/18:2 DGDG, a minor component in the wild type, was the major DGDG molecular species of JB1. The total amount of DGDG (μmol/g dry weight) was reduced by almost 50% in the mutant, and there was an approximate 30% increase in the total amount of MGDG.

Incorporation of [^{14}C]18:1. The incorporation and desaturation of exogenous [^{14}C]18:1 was compared in the wild type and JB1 mutant. Leaves (with petioles attached) were removed from plants and incubated with [^{14}C]18:1 for 1 h at 26°C. They were then washed (removing unincorporated [^{14}C]18:1) and further incubated in nonradioactive medium at 26°C.

During the 1 h labeling period with 5 μCi of [^{14}C]18:1 about

Table I. *FA Composition of MGDG and DGDG from Wild Type and JB1 Mutant*

Values represent 4 analyses.

FA	MGDG		DGDG	
	WT[a]	JB1[b]	WT	JB1
	% of total sample weight ± SE			
14:0	0.8 ± 0.2	—[c]	—	—
16:0	2.0 ± 0.4	5.3 ± 0.4	16.0 ± 1.6	17.6 ± 0.9
16:2	1.5 ± 0.2	20.2 ± 1.0	0.8 ± 0.2	1.0 ± 0.2
16:3	41.0 ± 1.9	10.2 ± 0.8	—	—
18:0	—	4.2 ± 1.1	—	—
18:1	1.9 ± 0.5	7.4 ± 0.3	1.2 ± 0.2	25.5 ± 2.6
18:2	1.6 ± 0.2	21.3 ± 1.5	4.1 ± 0.6	21.7 ± 1.9
18:3	51.2 ± 2.1	31.4 ± 1.1	77.9 ± 3.9	34.2 ± 1.0

[a] Wild type. [b] JB1 mutant. [c] Not detected.

Table II. *Molecular Species Composition of MGDG from Wild Type and JB1 Mutant*

MGDG molecular species were quantified by integration of peaks detected by flame ionization detector-liquid chromatography. Values are expressed as mean percent of total sample weight and as μmol per g dry wt ± SE of 4 analyses.

Component[c] Molecular Species	MGDG			
	WT[a]		JB1[b]	
	% total sample wt	*μmol/g dry wt*	*% total sample wt*	*μmol/g dry wt*
18:3/16:3	84.0 ± 1.1	169.6 ± 5.8	20.6 ± 0.9	61.0 ± 4.9
18:3/16:2	1.0 ± 0.2	1.9 ± 0.3	23.2 ± 1.4	68.9 ± 6.2
18:3/18:3	3.2 ± 0.3	6.3 ± 0.9	6.3 ± 0.6	18.8 ± 1.7
18:2/16:2	0.5 ± 0.1	0.9 ± 0.2	18.2 ± 0.7	52.8 ± 5.0
18:2/18:3	1.6 ± 0.2	2.9 ± 0.4	8.1 ± 0.3	24.1 ± 3.2
18:2/18:2	1.3 ± 0.2	2.8 ± 0.5	4.2 ± 0.3	11.2 ± 1.4
18:3/16:0	4.2 ± 0.7	8.2 ± 1.0	1.9 ± 0.2	5.6 ± 1.0
18:1/18:1	0.3 ± 0.1	0.5 ± 0.2	7.2 ± 0.4	21.4 ± 2.7
18:0/18:1	0.5 ± 0.1	0.9 ± 0.2	—[d]	—
18:2/16:0	3.0 ± 0.5	6.0 ± 0.6	9.6 ± 0.5	31.7 ± 3.5
18:1/16:0	0.4 ± 0.1	0.7 ± 0.2	0.7 ± 0.1	1.9 ± 0.2

[a] Wild type. [b] JB1 mutant. [c] Pairs of numbers representing fatty acids and separated by a slash represent the components in the *sn*-1 and *sn*-2 positions, respectively, of the molecular species. The positional distribution of the fatty acids was determined by hydrolyzing the eluted molecular species with lipase from *R. arrhizus*. [d] Not detected.

Table III. *Molecular Species Composition of DGDG from Wild Type and JB1 Mutant*

Analyses were performed as described for MGDG.

Component[c] Molecular Species	WT[a]		JB1[b]	
	% total DGDG	*μmol/g dry wt*	*% total DGDG*	*μmol/g dry wt*
18:3/16:2	1.3 ± 0.3	0.7 ± 0.2	0.5 ± 0.1	0.1 ± 0.05
18:3/18:3	65.4 ± 1.9	37.9 ± 4.1	19.0 ± 0.6	5.9 ± 1.1
18:2/18:3	—[d]	—	1.3 ± 0.2	0.3 ± 0.1
18:1/18:3	2.4 ± 0.2	1.3 ± 0.4	1.9 ± 0.3	0.4 ± 0.1
18:1/18:2	1.0 ± 0.2	0.7 ± 0.2	42.4 ± 1.8	12.9 ± 2.1
18:3/16:0	19.3 ± 1.6	10.9 ± 1.8	32.5 ± 1.9	9.9 ± 1.9
18:2/16:0	10.6 ± 0.5	5.6 ± 1.0	2.4 ± 0.4	0.7 ± 0.2

[a] Wild type. [b] JB1 mutant. [c] The positional distribution of fatty acids was determined by hydrolyzing with lipase from *R. arrhizus*. [d] Not detected.

Table IV. *Incorporation of Radioactivity into Lipids of Wild Type and JB1 Arabidopsis Leaves after Labeling with [¹⁴C]18:1*

Analyses were performed immediately after incubating detached leaves with [^{14}C]18:1, or after 3 or 5 h further incubation in nonradioactive medium.

Lipid Class	Time (h) After Labeling					
	WT[a]			JB1[b]		
	0	3	5	0	3	5
	% of total lipid radioactivity					
Polar lipid	58.7	72.0	77.1	69.3	82.6	85.6
DG	3.8	2.7	1.9	4.5	3.2	2.9
FA	32.9	22.2	19.0	22.9	10.6	8.0
TG	4.6	3.1	2.0	3.3	3.6	3.5

[a] Wild type. [b] JB1 mutant.

65% of the isotope was incorporated by the wild-type leaves, and 52% by the mutant. There was a rapid incorporation of radioactivity into polar lipids and radioactivity in free FA decreased (Table IV). After 1 h labeling, about 80% of the total polar lipid counts were found in PC of both leaf types. During subsequent incubation in nonradioactive medium, the radioactivity in wild-type PC slowly continued to increase, while counts in MGDG increased at a faster rate (Table V). A different pattern was seen in JB1 *Arabidopsis*. PC radioactivity declined between 3 and 5 h from labeling, and counts in MGDG, initially rising more slowly than in the wild type, increased at a faster rate during this time. DGDG was only slowly labeled, particularly in the mutant.

Table V. *Incorporation of Radioactivity into PC, MGDG, and DGDG after Labeling Arabidopsis Leaves with [¹⁴C]18:1*

Lipid Class	Time (h) After Labeling					
	WT[a]			JB1[b]		
	0	3	5	0	3	5
	dpm of lipid					
PC	269,100	300,000	310,300	285,800	292,000	210,100
MGDG	5,000	16,800	32,700	3,100	7,500	28,900
DGDG	1,700	3,700	5,600	900	2,500	2,900

[a] Wild type. [b] JB1 mutant.

PC is a proposed site of 18:2 formation in plant tissues (15), and evidence suggests that it acts as a source of unsaturated DG precursors for the synthesis of 18/18C MGDG (16, 20, 21). Leaves of wild-type *Arabidopsis* rapidly incorporated [^{14}C]18:1 into PC (Table VI). Then as the level of [^{14}C]18:1 in PC subsequently declined, [^{14}C]18:2 in PC increased (Table VI). This same pattern was also observed in JB1 PC, but levels of [^{14}C]18:2 were initially found to be higher and [^{14}C]18:1 declined more rapidly. Low levels of [^{14}C]18:3 also accumulated in PC of both leaf types, but by 5 h from labeling the level in JB1 was

Table VI. *Incorporation of Radioactivity into PC C_{18} FA after Labeling with [14C]18:1*

FA	Time (h) After Labeling					
	WT[a]			JB1[b]		
	0	3	5	0	3	5
	dpm of FA					
18:1	223,900	205,500	171,000	220,100	161,400	83,800
18:2	43,800	91,200	133,400	64,000	125,500	113,500
18:3	1,300	3,300	5,900	1,700	5,100	12,800

[a] Wild type. [b] JB1 mutant.

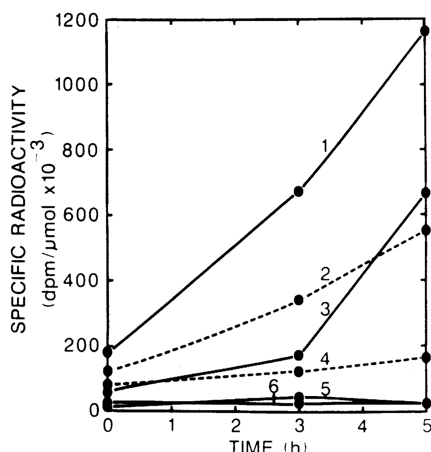

FIG. 1. Rate of labeling of wild-type MGDG molecular species following incubation with [14C]18:1. Isolated leaves were labeled for 1 h with [14C]18:1, washed, and further incubated in nonradioactive medium for 0 to 5 h. Radioactivity of 18:2/18:3 (1), 18:2/16:0 (2), 18:3/18:3 (3), 18:1/16:0 (4), 18:2/18:2 (5), and 18:1/18:1 (6) MGDG is recorded as dpm/μmol.

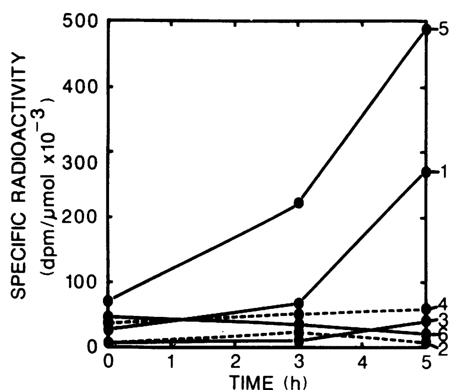

FIG. 2. Rate of labeling of mutant JB1 MGDG molecular species following incubation with [14C]18:1. Details of the labeling protocol are as given in Figure 1. Radioactivity of 18:2/18:3 (1), 18:2/16:0 (2), 18:3/18:3 (3), 18:1/16:0 (4), 18:2/18:2 (5), and 18:1/18:1 (6) MGDG is recorded as dpm/μmol.

twice that in wild-type PC. Ninety to 95% of the [14C]18:3 was found in the *sn*-2 position of PC of both leaf types (data not shown).

We found a rapid and selective labeling of 18/18C MGDG molecular species. In the wild type, radioactivity accumulated primarily in 18:2/18:3, and more slowly in 18:3/18:3 MGDG (Fig. 1). By hydrolysis with *Rhizopus* lipase, it was found that 18:3/18:3 was labeled only in the *sn*-2 position. In JB1, 18:2/18:2 MGDG was the most rapidly labeled species, and increases

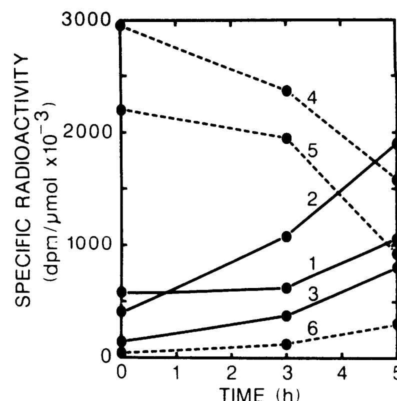

FIG. 3. Rate of labeling of wild-type MGDG molecular species following incubation with [14C]12:0. The same labeling conditions employed with [14C]18:1 were used. Radioactivity of 18:2/18:2 (1), 18:2/18:3 (2), 18:3/18:3 (3), 18:2/16:2 (4), 18:3/16:2 (5), and 18:3/16:3 (6) MGDG is expressed as dpm/μmol.

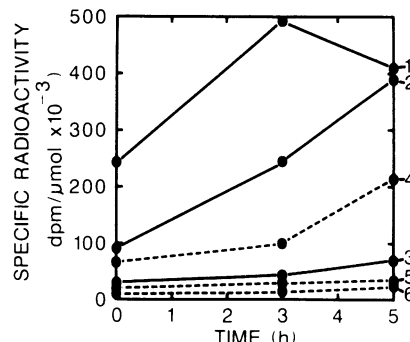

FIG. 4. Rate of labeling of mutant JB1 MGDG molecular species following incubation with [14C]12:0. Radioactivity of 18:2/18:2 (1), 18:2/18:3 (2), 18:3/18:3 (3), 18:2/16:2 (4), 18:3/16:2 (5), and 18:3/16:3 (6) MGDG is expressed as dpm/μmol.

in radioactivity of 18:2/18:3 and 18:3/18:3 were relatively delayed, especially during the first 3 h after labeling (Fig. 2). [14C]18:3 was again found only in the *sn*-2 position of 18:3/18:3 MGDG.

No detectable radioactivity accumulated in 18:3/16:3, 18:3/16:2, or 18:2/16:2 MGDG of either leaf type at any time after administering [14C]18:1. Even by 8 h, these species remained unlabeled (data not shown). In both leaf types, radioactivity was found in 18:1/16:0 and 18:2/16:0 MGDG, but not in 18:3/16:0. The specific activity of 18:2/16:0 increased much more slowly in the mutant.

Incorporation of [14C]12:0. To examine further MGDG synthesis and FA desaturation, leaves were labeled with exogenous [14C]12:0. This is considered to give rise to C_{16} and C_{18} FA only via enzymatic elongation pathways operating in the chloroplast.

There was no radioactivity present as [14C]12:0 in MGDG (or DGDG) following labeling but radioactive C_{16} and C_{18} FA were found in the component molecular species indicating rapid elongation of [14C]12:0. In contrast to the findings with [14C]18:1, unsaturated C_{18} FA formed from [14C]12:0 appeared in 18:3/16:3, 18:3/16:2, and 18:2/16:2 MGDG as well as in 18/18C molecular species.

The most rapidly labeled molecular species of wild-type MGDG were 18:3/16:2 and 18:2/16:2. Their radioactivity subsequently declined and activity in the more unsaturated species 18:3/16:3 increased (Fig. 3). A strikingly different pattern was seen in JB1 (Fig. 4). Each of these 18/16C species was labeled much more slowly, and radioactivity accumulated in 18:2/16:2

MGDG, particularly between 3 and 5 h from labeling. Analyses of total MGDG [^{14}C]FA showed increased levels of both [^{14}C] 16:2 and [^{14}C]18:2 during this time and relatively low levels of [^{14}C]16:3 and [^{14}C]18:3 compared to the wild-type MGDG (data not shown). Coincidentally, radioactivity accumulated in the JB1 18:2/16:2 species.

A greater proportion of the total MGDG radioactivity was always in the 18:2/18:2 and 18:2/18:3 molecular species in JB1 (Figs. 3 and 4). Analysis of radioactivity at the *sn*-1 and *sn*-2 positions of 18:3/18:3 MGDG by *Rhizopus* lipase revealed a further specific difference between the wild type and the mutant. [^{14}C]18:3 was esterified to both positions of the molecule in contrast to the exclusive location at the *sn*-2 position after desaturation of exogenous [^{14}C]18:1. The specific activity of [^{14}C] 18:3 at *sn*-1 of the wild-type lipid remained between 2 and 3 times higher than *sn*-2 [^{14}C]18:3. There was a much slower labeling of JB1 18:3/18:3 and, at all times analyzed, [^{14}C]18:3 at *sn*-2 had the highest specific radioactivity (data not shown).

DISCUSSION

We have selected the *Arabidopsis* 'JB1' mutant as a unique system in which to examine the formation of GL polyunsaturated FA in higher plants. The FA compositions of MGDG and DGDG (Table I) confirmed that desaturase enzymes acting on both C_{16} and C_{18} FA are affected by the mutation(s), as identified by Browse *et al.* (2). Comparison of molecular species compositions allowed assessment of the relative contributions of the proposed eukaryotic and prokaryotic pathways for synthesis of MGDG. We found a selective reduction in the levels of certain unsaturated 18/16C MGDG molecular species compared to the wild type, while more of the available 18:3 was paired with a second C_{18} FA. By direct mass determinations (FID-HPLC), we found increases in the amounts of 18:2/18:3 and 18:3/18:3 MGDG in JB1 *Arabidopsis*, despite the overall reduction in 18:3. This provided evidence for different pathways being operative in MGDG synthesis in *Arabidopsis* as previously proposed for 16:3 plants (14, 16, 17). Since eukaryotic (18/18C) MGDG molecular species contributed increased amounts of the total MGDG in JB1, the mutation(s) may be specifically affecting the desaturation of FA in the prokaryotic pathway of MGDG synthesis, which is proposed to occur within the chloroplast.

Labeling kinetics of FA in individual MGDG molecular species following the incorporation of [^{14}C]18:1 and [^{14}C]12:0 strongly support different pathways of C_{18} FA desaturation. Unsaturated 18/18C MGDG is thought to originate from DG precursors derived from PC via the eukaryotic pathway (20, 21). The exogenously incorporated [^{14}C]18:1 clearly entered the pathway leading to the synthesis of these species of MGDG. A decline in PC radioactivity between 3 and 5 h from labeling the JB1 mutant was coincidental with a faster rate of MGDG labeling (Table V), and this was due mainly to increases in radioactive 18:2/18:3 and 18:3/18:3 species (Fig. 2). Levels of [^{14}C]18:2 and [^{14}C]18:3 increased more rapidly in JB1 PC than in the wild type, implying either an increase in desaturase activity or reduced PC turnover. The specific activities of PC and of the 18/18C MGDG are supporting of the former explanation.

Since the 18:3/16:3, 18:2/16:3, and 18:2/16:2 species remained unlabeled, they appear to be metabolically isolated from this synthetic pathway. Prokaryotic MGDG is normally thought to be assembled exclusively from ACP-FA synthesized within the chloroplast (14–17). We did, however, find [^{14}C]-labeled 18:1/16:0 and 18:2/16:0 of initially very high specific radioactivity. The positioning of the [^{14}C]18:1 and [^{14}C]18:2 at *sn*-1 suggested their assembly within the chloroplast from imported [^{14}C] 18:1. It was recently reported that FA of eukaryotic-type lipids can be transported into the prokaryotic path in the unicellular alga *Dunaliella salina* (10, 11) and we may be seeing this interchange here.

We are particularly concerned with the site of 18:3 formation. The exclusive location of [^{14}C]18:3 at the *sn*-2 position of the 18:3/18:3 MGDG molecular species following [^{14}C]18:1 labeling suggests a remarkable metabolic segregation of the two FAs. They either have very different rates of turnover and/or arise from different precursors. Positioning of [^{14}C]-C_{18} FA at *sn*-2 suggested an original eukaryotic assembly. Further, the labeling patterns supported 18:2/18:2 MGDG being a direct precursor of the 18:3/18:3 species. This would mean 18:2 desaturases acting on the *sn*-1 position of MGDG while the 18:3 at *sn*-2 may be formed on a PC substrate by a nonchloroplast desaturase(s).

[^{14}C]FA formed by elongation of [^{14}C]12:0 clearly entered both paths of MGDG synthesis. The elongation and desaturation to form [^{14}C]16:0 and [^{14}C]18:1 are assumed to occur solely by chloroplast enzymes, which are known to utilize FA as large as 12:0 as substrates (6). These would then be used either in MGDG assembly within the chloroplast, or exported for synthesis of eukaryotic lipids (14–17). Incorporation of [^{14}C]12:0 therefore allowed us to follow the utilization of newly synthesized [^{14}C] 16:0 and [^{14}C]18:1 in the different pathways of GL synthesis.

In the wild type, the labeling kinetics of unsaturated 18/16C MGDG suggested that progressive desaturation of 18:2/16:2 MGDG produced the 18:3/16:2 species. This was only very slowly labeled in JB1, and the accumulation of [^{14}C]-labeled 18:2/16:2 suggested that these desaturations were inhibited. Relatively more of the total MGDG radioactivity was found in 18/18C species, and particularly in 18:2/18:2 and 18:2/18:3. This was supportive of an increased amount of MGDG being formed by the eukaryotic pathway, and of the mutation(s) primarily affecting chloroplast desaturases.

Further work with this system should also clarify the biosynthetic pathway of DGDG, which may be formed from precursor MGDG molecules (21). The levels of 18:1 in DGDG of both the wild type and JB1 mutant were higher than other reported values (2, 3), which may be a reflection of different growth conditions. The amount of DGDG (μmol/g dry weight) was decreased in the mutant, and the incorporation of radioactivity into this lipid was reduced after labeling with [^{14}C]18:1 or [^{14}C]12:0 compared to the wild type. The changes in DGDG molecular species did not follow those found in MGDG. Since the amount of 18:3/18:3 DGDG was lower in JB1 and 18:1/18:2 DGDG was increased, desaturation of C_{18} FA may occur on DGDG as well as MGDG.

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