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Effect of Growth Temperature on the Biosynthesis of Chloroplastic Galactosyldiacylglycerol Molecular Species in *Brassica napus* Leaves[1]

Geoff Johnson and John P. Williams*

Department of Botany and the Centre for Plant Biotechnology, University of Toronto, Toronto, Ontario, Canada M5S 1A1

ABSTRACT

***Brassica napus* leaves developed at low temperature display rapid *in situ* desaturation of monogalactosyldiacylglycerol (MGDG) fatty acids leading to the production of hexadecatrienoic/linolenic acid. This was shown by radioactivity-tracer experiments to occur via a sequence of desaturations proceeding from the initially synthesized palmitic/oleic acid molecular species to palmitic/linoleic acid, palmitoleic/linoleic acid, hexadecadienoic/linoleic acid, hexadecadienoic/linolenic acid, and finally to hexadecatrienoic/linolenic acid. The results suggest that there is increased activity in all five desaturation steps in leaves developed at low temperatures. Labeling data also indicate that there is another pool of MGDG which is more slowly desaturated before galactosylation to digalactosyldiacylglycerol (DGDG). Our data further suggest that relative rates of galactosylation of chloroplastic and cytosolic MGDG molecular species may regulate the final amounts of chloroplastic and cytosolic MGDG and DGDG in the leaf. We have proposed a model for chloroplastic biosynthesis and desaturation of galactosyldiacylglycerols in the leaves of *Brassica napus*, a 16:3 plant.**

We have recently reported (27) the effects of development at low temperature (5 and 10°C) on the level and biosynthesis of unsaturated fatty acids in the polar diacylglycerols of *Brassica napus* leaves (27). *B. napus* is a 16:3[2]-plant possessing 16:3 in MGDG and, to a lesser extent, in DGDG. It has been shown in many 16:3-plants that MGDG is synthesized by major contributions of both chloroplastic and cytosolic pathways, in contrast to 18:3-plants in which the cytosolic pathway is predominant (4, 10, 11, 18, 23). In many organisms, especially plants, development at low temperatures results in increased levels of unsaturated fatty acids in membrane glycerolipids (17, 19, 20). In *B. napus* we have also found that development at low temperature (5–10°C) results in a marked enhancement of the chloroplastic pathway of MGDG synthesis relative to the cytosolic pathway. However, there is a relative reduction in the contribution of the chloroplastic pathway to DGDG biosynthesis at low developmental temperatures. When leaves are fed $^{14}CO_2$ and incubated at 20°C, the fatty acids of MGDG undergo more rapid desaturation if plants are grown at low rather than at high temperatures, consistent with the characteristically increased levels of unsaturation observed at low growth temperatures. A second, slower phase of desaturation accounts for a further gradual increase in the level of unsaturation to that found in the leaf (23). We have shown that the rapid phase of desaturation is dependent primarily on low temperature development rather than on low temperature per se. A series of experiments with leaves developed at low temperature and incubated at different temperatures also demonstrated that desaturation responds in a manner consistent with normal enzyme kinetics. We thus proposed that this rapid desaturation was not directly related to physical factors associated with low temperatures, such as increased O_2 solubility (9), or changes in membrane configuration (13, 14) (for example, decreased membrane fluidity), but rather that modulation of desaturase activity occurs as a developmental response to growth temperature.

We report here on the sequence of reactions in this rapid phase desaturation of chloroplastic MGDG in *B. napus*. We show the effect of growth temperature on the rates of these reactions and also propose a model for galactolipid biosynthesis.

MATERIALS AND METHODS

Brassica napus var Tower plants were developed from seed to the primary leaf stage in growth chambers at 20°C with a 16 h photoperiod and approximate light intensity of 200 μE/m²/s. After 1 week, plants were transferred to growth chambers maintained isothermally at 5, 10, 20, or 30°C, with the same light regime. Secondary leaves with diameters 3 to 4 cm were harvested and allowed 30 min to equilibrate in a growth chamber maintained at 20°C where all $^{14}CO_2$ pulse-chase experiments were conducted. One mCi of $^{14}CO_2$ was generated from [^{14}C]sodium carbonate and offered to the excised leaves in a plexiglass box at atmospheric pressure. After 5 min feeding periods, residual $^{14}CO_2$ was removed, and the leaves were incubated in $^{12}CO_2$ air. Incubation periods were timed from the completion of the feeding period. At various incubation times, lipids were extracted from leaf material in 2:1

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[2] Abbreviations: 16:3, hexadecatrienoic acid; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; 18:3, linolenic acid; 16:0, palmitic acid; 16:1, palmitoleic acid; 16:2, hexadecadienoic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; ACP, acyl carrier protein.

$CHCl_3$/MeOH according to Williams and Merrilees (28). Lipids were separated into classes by TLC using the method of Khan and Williams (15). MGDG and DGDG were isolated from the chromatoplates and extracted successively with 2:1 $CHCl_3$/MeOH, 1:1 $CHCl_3$/MeOH, and MeOH. The extracts were dried under N_2 and made to volume in 1:1 $CHCl_3$/MeOH. MGDG and DGDG were separated into component molecular species according to the degree of acyl unsaturation by argentation TLC (22). Resolution of the individual molecular species in bands containing 6, 5, 4, 3, 2, 1, and 0 double bonds was established by careful analysis of quantities and radioactivities in derivatized fatty acids. Fatty acid methyl esters were prepared from each band using 0.5 M sodium methoxide (24). Samples were then analyzed by GLC using a DB-225 megabore capillary column (J & W Scientific), with column conditions as previously described (27). Quantities were determined by flame ionization detection, and radioactivities were determined by splitting the gas flow for subsequent analysis by scintillation spectrophotometry (21). Individual lipid molecular species were identified in bands by their two acyl components that matched in both quantity and radioactivity. Molar quantities of molecular species were calculated by dividing the total of the matching pair by two, and radioactivities by summing the radioactivities of the matching pair. Radioactivities were also determined in the galactosylglycerol fractions of the molecular species bands. The polar fractions were passed through Dowex-50 (H^+ form) to remove silver ions, dried under air, and made to volume for scintillation spectrophotometry.

RESULTS

Effect of Growth Temperature on MGDG and DGDG Molecular Species Composition

Table I contains the molecular species composition of MGDG and DGDG at various growth temperatures (the data for MGDG are a summary of the data presented previously [27]). The presence of increased proportions of 16:3/18:3 MGDG compared to 18:3/18:3 at lower growth temperatures indicates that the chloroplastic pathway of 16/18 MGDG synthesis is stimulated by development at low temperatures. The total level of unsaturation in MGDG is enhanced as growth temperature is lowered, since the decreased levels of 18:3/18:3 are more than compensated for by increases in 16:3/18:3 levels. In DGDG the trend is reversed showing an increased level of cytosolic DGDG at low temperatures. This results in an increase in overall levels of unsaturation due to the higher level of 18:3/18:3 (and 16:3/18:3) compared to 16:0/18:3 and 16:0/18:2. The shift in the relative quantities of chloroplastic and cytosolic molecular species in response to growth temperature is thus opposite for the two galactolipids.

Table I. *Major Molecular Species of MGDG and DGDG of B. napus Leaves Grown at Different Temperatures*

Values are averages of three separate experiments (for MGDG) or two experiments (for DGDG). Standard errors are in parentheses for MGDG.

Lipid	Molecular Species	Growth Temperature (°C)			
		5	10	20	30
		mol %			
MGDG	18:3/18:3	9 (1)	12 (4)	16 (1)	20 (6)
	16:3/18:3	85 (2)	80 (5)	72 (3)	60 (4)
	18:3/18:2	1 (0)	1 (1)	1 (2)	3 (1)
	16:3/18:2	1 (0)	1 (0)	2 (0)	2 (0)
	16:2/18:3	1 (0)	2 (0)	3 (1)	4 (1)
	16:0/18:3	1 (0)	1 (0)	1 (1)	2 (1)
	16:0/18:2	1 (0)	1 (0)	1 (0)	2 (0)
DGDG	18:3/18:3	57	59	47	42
	16:3/18:3	14	11	6	3
	18:3/18:2	1	3	2	4
	16:0/18:3	16	15	24	26
	16:0/18:2	5	4	7	8

Labelling of Fatty Acids in Molecular Species of MGDG

The molecular species (18/18C) derived from the cytosolic pathway contain very little or no radioactivity after 30 min incubation (results not shown). These data are not included in Figures 1 and 2 which, therefore, represent only molecular species formed by the chloroplastic pathway (16/18C).

Figure 1 illustrates the changing levels of radioactivity in the fatty acid moiety of MGDG molecular species from plants grown at 5°C after feeding and incubation at 20°C. These data can be used as an indication of rates of desaturation and from this the major pathway and sequence of desaturation in the biosynthesis of 16:3/18:3 can be determined. Molecular species are grouped according to the total number of unsaturated bonds in the MGDG molecule. Thus, the insertion of one double bond would move the molecular species into the next grouping while the location of that double bond would determine which molecular species was formed. For example, the initial labeling is found in 16:0/18:1 MGDG; desaturation of 16:0 to 16:1 would lead to 16:1/18:1, whereas the desaturation of 18:1 to 18:2 would yield 16:0/18:2. In this case the results indicate a predominance of radioactivity in 16:0/18:2 leading to the conclusion that the desaturation of 18:1 occurs more readily than 16:0. On the other hand, the second desaturation reaction appears to favor the formation of 16:1/18:2, suggesting that desaturation of 16:0 is more common than desaturation of 18:2 at this level. The third desaturation step appears to result in the conversion of 16:1 to 16:2 yielding predominantly 16:2/18:2 MGDG. The fourth desaturation results in levels of 16:2/18:3 approximately double those of 16:3/18:2 suggesting a somewhat higher rate of desaturation of 18:2 than 16:2 in the 16:2/18:2 molecular species.

Leaves developed at 5°C (and 10°C, not shown) desaturate MGDG-linked fatty acids extensively during the 5 min $^{14}CO_2$ feeding period (Fig. 1). This is indicated by significant labeling in the molecular species of MGDG containing 4 to 6 double bonds at 0 min (immediately following the feeding period). In contrast, little or no labeling of these species is seen at 0 min for leaves grown at 20 or 30°C (results not shown). Leaves developed at 5°C (or 10°C, not shown) desaturate rapidly over the entire chase period of 30 min as indicated by continued increases in the radioactivity of fatty acids in 16:2/18:2, 16:2/18:3, 16:3/18:2, and 16:3/18:3 molecular species. In compar-

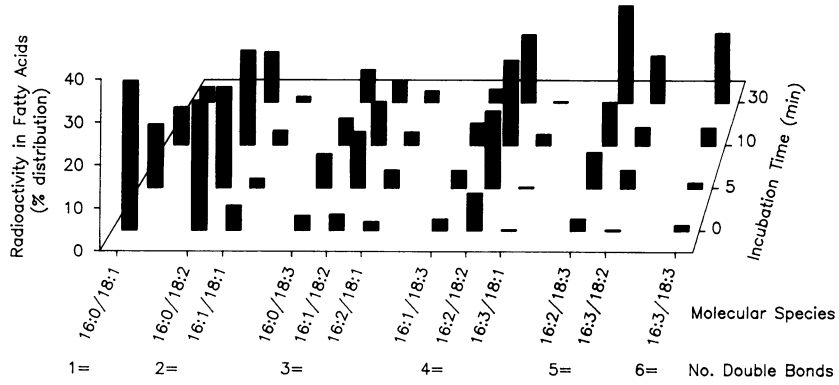

Figure 1. Distributions of radioactivity in the fatty acids of MGDG molecular species after $^{14}CO_2$ labeling and $^{12}CO_2$ chase (at 20°C) for *B. napus* leaves grown at 5°C. Total radioactivities in dpm × 10^{-4} g fresh weight −1 at 0, 5, 10, and 30 min were 3.2, 12.1, 16.3, and 12.1, respectively.

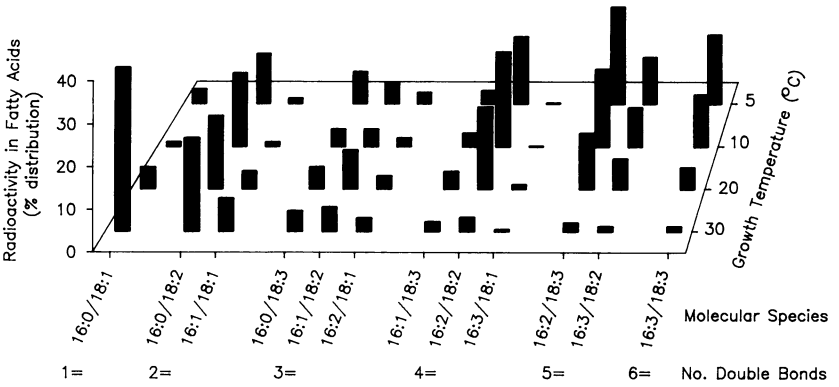

Figure 2. Distributions of radioactivity in the fatty acids of MGDG molecular species after $^{14}CO_2$ labeling and 30 min $^{12}CO_2$ chase (at 20°C) for *B. napus* leaves grown at different temperatures. Total radioactivities in dpm × 10^{-4} g fresh weight^{-1} for leaves grown at 5, 10, 20, and 30°C were 12.1, 12.0, 24.0, and 26.1, respectively.

ison, leaves developed at 20 and 30°C exhibit progressively slower labelling of the highly unsaturated species (Fig. 2).

The results shown illustrate that the production of 16:3/18:3 is the net result of rapid desaturations in a definite pathway, which begins with the 16:0/18:1 precursor. We have no evidence at this time to suggest different pathways of desaturation at different growth temperatures as the data in Figure 2 for each growth temperature appear to follow the same pattern within each group of molecular species.

Labeling of Galactosyl-Glycerol Moieties of MGDG

We have previously determined that the distribution of radioactivity in galactosyl-glycerol fractions of chloroplastic MGDG molecular species immediately after short $^{14}CO_2$ feeding periods indicates attachment of galactose to predominantly 16:0/18:1 diacylglycerol precursors (26). The appearance of radioactivity in the polar fractions associated with progressively more unsaturated species with time indicates continued *in situ* desaturation of the initial 16:0/18:1 precursor. Figure 3 shows that, from 0 to 30 min, leaves developed at low temperatures show greater rates of redistribution of label toward the more unsaturated 16/18C species 16:0/18:3, 16:2/18:2, 16:2(3)/18:3(2), and 16:3/18:3. This confirms that there is a more rapid *in situ* desaturation of chloroplastic MGDG fatty acids in leaves grown at low temperatures.

Radioactivity in the polar moieties of MGDG derived from the cytosolic pathway is immediately found (at 0 min) in the 18:3/18:3 and 18:3/18:2 species. This shows that the diacylglycerol precursors of 18/18C MGDG in *B. napus*, like other 16:3 plants (26), are highly unsaturated. Figure 3 shows that plants grown at 5 and 10°C have initially more labeling in the 18:3/18:3 fraction relative to 18:3/18:2. This may be an effect of growth temperature on the levels of unsaturation in a cytosolic precursor (perhaps phosphatidylcholine).

After 30 min, leaves developed at 5 and 10°C show decreased proportions of radioactivity in the 18:3/18:3 fraction of MGDG relative to the unsaturated 16/18C fractions (Fig. 3). This may reflect enhanced galactosylation of the 18:3/18:3 species of MGDG to 18:3/18:3 DGDG at lower growth temperatures. This interpretation is consistent with the elevated levels of 18:3/18:3 DGDG (and reduced levels in MGDG) found under these conditions. Leaves grown at 20 and 30°C exhibit less turnover (and proportionately less labeling) of the 18:3/18:3 molecular species of MGDG.

Labeling of Fatty Acids in DGDG Molecular Species

The individual fatty acids of four chloroplastic DGDG molecular species become sufficiently labeled for accurate determination only after 30 min incubation. Figure 4 indicates that in leaves developed at 5 and 10°C, proportionately more radioactivity is found in 16:0/18:2 compared to 16:0/18:1, whereas in leaves grown at 20 and 30°C, the reverse is true. These distributions of radioactivity do not reflect quantitative distributions of these DGDG molecular species in the leaf (Table I). Since 16:0/18:3 is the major chloroplast species at all growth temperatures, the distribution of radioactivity after 30 min appears to suggest that subsequent *in situ* desaturation of 16:0/18:1(2) to 16:0/18:3 occurs in DGDG. Our

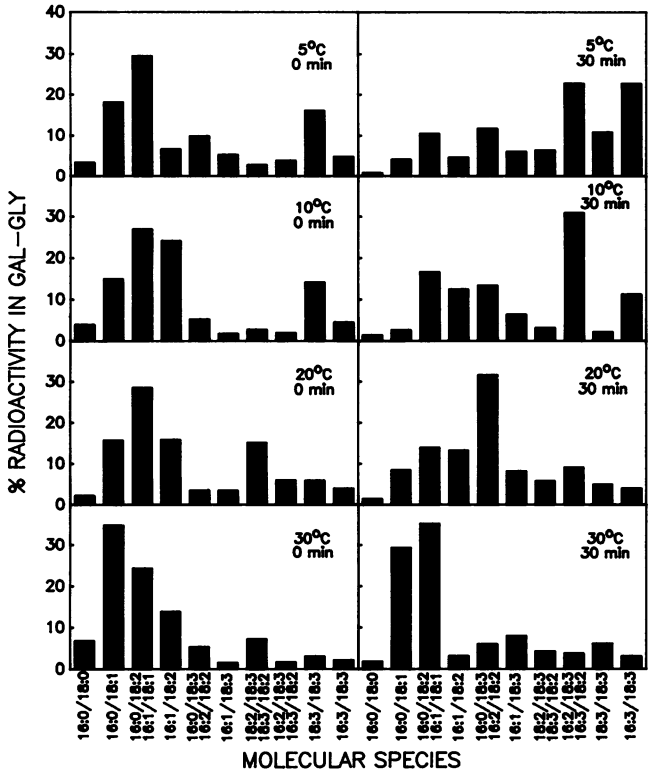

Figure 3. Distributions of radioactivity in the galactosyl-glycerol moieties of MGDG molecular species after $^{14}CO_2$ labeling and $^{12}CO_2$ chase (at 20 C) for *B. napus* leaves grown at different temperatures. Total radioactivities in dpm × 10^{-4} g fresh weight^{-1} at 0 and 30 min were 6.2 and 14.1 (5°C), 7.9 and 14.8 (10°C), 6.8 and 22.9 (20°C), and 5.8 and 19.4 (30°C). Bands separated by argentation TLC are ordered from saturated to unsaturated, left to right. The molecular species indicated are the major molecular species in each band.

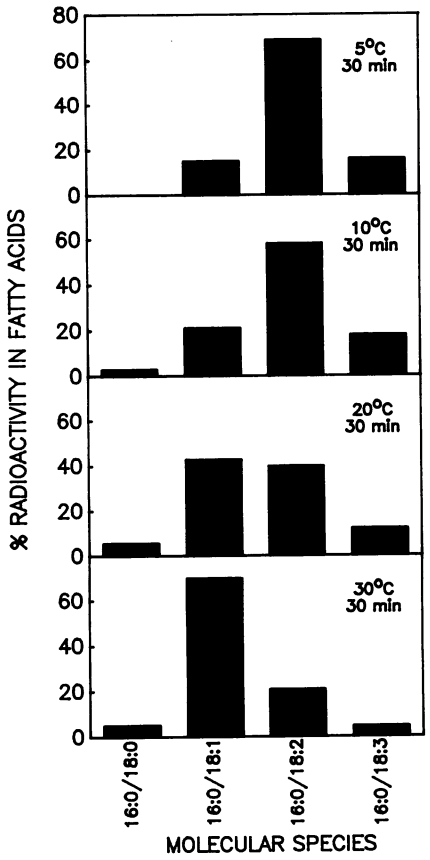

Figure 4. Distributions of radioactivity in the fatty acids of DGDG molecular species after $^{14}CO_2$ labeling and 30 min $^{12}CO_2$ chase (at 20°C) of *B. napus* leaves developed at different temperatures. Total radioactivities in dpm × 10^{-4} g fresh weight^{-1} were 1.0, 1.7, 2.9, and 1.3 for leaves developed at 5, 10, 20, and 30°C, respectively.

previous data, however, with incubation periods of up to 24 h, indicate that *in situ* desaturations do not occur in DGDG (23). The distribution of radioactivity in DGDG more likely reflects the distribution of radioactivity in the corresponding precursor MGDG species.

Labeling of Total Fatty Acid Fractions of MGDG and DGDG

Table II shows the ratio of total fatty acid labeling in MGDG *versus* DGDG at different growth temperatures. These results appear to indicate an effect of growth temperature on galactosyltransferase activity in the chloroplastic pathway. The data suggest that the rate of galactosyltransferase is stimulated at lower growth temperatures.

Table II. *Ratio of Radioactivity in the Total Fatty Acid Fractions of MGDG versus DGDG in Leaves Grown at Various Temperatures*

Leaves were fed $^{14}CO_2$ and incubated in $^{12}Co_2$ at 20°C as described in "Materials and Methods." The values are averages of two experiments (5 and 10 min) or three experiments (0 and 30 min). Radioactivities (cpm) measured in DGDG fatty acids were in the ranges: 330–1,773 (5°C), 686–3,542 (10°C), 540–3,028 (20°C), and 343–2,962 (30°C) cpm. Radioactivities (cpm) in MGDG fatty acids were in the ranges: 2,085–14,161 (5°C), 7,429–29,307 (10°C), 8,977–33,247 (20°C), and 11,142–64,317 (30°C) cpm.

Incubation Time	Growth Temperature (°C)			
	5	10	20	30
min	*MGDG/DGDG dpm fatty acid*			
0	9.1	15.2	20.0	36.2
5	9.5	10.8	24.9	32.6
10	8.5	10.8	9.6	27.2
30	8.5	8.2	11.5	22.7

DISCUSSION

We have shown that leaves developed at low temperatures have the potential to desaturate newly synthesized MGDG fatty acids more rapidly when fed and incubated at 20°C than plants grown at high temperatures (27). This phenomenon appears as an apparently distinct phase resulting in the rapid desaturation of 16:0 to 16:3 and 18:1 to 18:3. A slower phase of desaturation was evident in previous experiments with *Brassica napus*, where radioactivity in 16:3 and 18:3 steadily accumulated over 24 h (23). It is only after a 24 h chase period that the labeling distribution in MGDG (and DGDG) fatty acids approach their actual quantitative distributions in the leaf.

The rapid phase of desaturation in MGDG occurs mainly within 30 min of feeding. We have terminated our incubations at 30 min since it is at this point that chloroplastic DGDG becomes strongly (and differentially) labeled at the various growth temperatures (Fig. 4; Table II). We are also confident of little or no interference from radioactivity derived from the cytosolic pathway at this point in the incubation.

The rapid phase is characterized by labeling in a definite series of molecular species: 16:0/18:1, 16:0/18:2, 16:1/18:2, 16:2/18:2, 16:2/18:3, and 16:3/18:3. This implies that five desaturase enzymes act in series, each with a degree of molecular species specificity. The activities of these desaturase enzymes are somehow modulated by growth temperature and are not directly influenced by physical factors associated with low temperature (27). This pathway of desaturation is shown diagrammatically in Figure 5 where the extent of each desaturase reaction is indicated by the thickness of the connecting lines between molecular species. Each of the molecular species in this pathway become labeled at different rates (Fig. 2). Andrews and Heinz (1) recently showed that progressive desaturations of MGDG-linked fatty acids to 16:3 and 18:3 in isolated spinach chloroplasts also occurred with differential rates. These authors found that after pulse labeling with ^{14}C-acetate, the distribution of radioactivity in MGDG fatty acids was identical in both envelope and thylakoid fractions at all times. Thus, it was not certain whether complete desaturation occurred in both membranes or whether there was transfer of molecular species before desaturation was complete.

We are unable to speculate on the location(s) of desaturases involved in the rapid phase of desaturation, with the exception of the oleoyl desaturase. A clear increase in apparent activity of an oleoyl desaturase is seen for leaves grown at 5 and 10°C compared to leaves grown at 20°C. This effect is seen at the early incubation times (27), and is due to more rapid labeling of 16:0/18:2 in leaves grown at low temperature compared to 20°C (data not shown). In leaves developed at 5°C, considerable desaturation of 16:0/18:1 to 16:0/18:2 has occurred during the 5 min feeding period (Fig. 1). Since envelope-bound UDP:diacylglycerol galactosyltransferase (2, 12) generates the 16:0/18:1 precursor (1, 11, 23, 26) for the oleoyl desaturase, we believe that both enzymes must be located in the envelope in close association with each other.

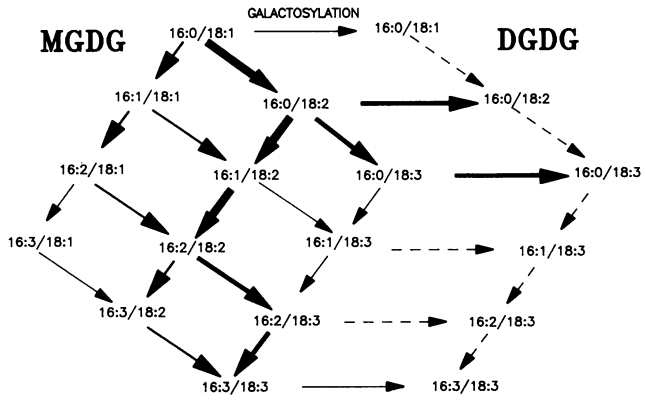

Figure 5. Proposed model for biosynthesis and desaturation of galactosyldiacylglycerols in the chloroplastic pathway.

Leaves developed at 30°C show a more extreme effect of temperature on apparent oleoyl desaturase activity because even after 30 min incubation levels of radioactivity in 16:0/18:1 are high (Fig. 2). Desaturation of 16:0/18:1 to 16:0/18:2 may be thermolabile since we have shown that leaves developed at low temperatures lose the ability to rapidly desaturate beyond 16:0/18:1 after transfer to 30°C (25). In *Escherichia coli* it has been shown that the anaerobic desaturation of palmitoleoyl-ACP to *cis*-vaccenoyl-ACP is regulated by the thermolability of 3-ketoacyl-ACP synthetase II, which is synthesized at all temperatures but only active at low temperatures (3). In *Bacillus*, it has been suggested that aerobic desaturation of palmitate is regulated by a thermolabile desaturase modulator (5).

At low growth temperatures, the galactose-glycerol fraction of 18:3/18:3 MGDG has elevated levels of radioactivity relative to the 18:2/18:3 fraction and to 16/18 fractions immediately after $^{14}CO_2$-feeding (Fig. 3). The fact that these levels drop after 30 min incubation may signify galactosylation of 18:3/18:3 MGDG to 18:3/18:3 DGDG. This is consistent with the quantitative increases of 18:3/18:3 DGDG relative to 18:3/18:3 MGDG observed at lower growth temperatures. Quantitative proportions of the major chloroplastic species of DGDG are also modified in response to low growth temperatures, with 16:0/18:2 and 16:0/18:3 levels decreasing and 16:3/18:3 increasing. The altered proportions of these DGDG chloroplastic species are paralleled by similar trends in the corresponding MGDG molecular species compositions (Table I). The labeling distributions in 16:0/18:1, 16:0/18:2, and 16:0/18:3 DGDG species at 30 min also reflect trends in labeling of the corresponding MGDG species. All these data suggest that the molecular species content of DGDG is dependent on the pool(s) of MGDG from which DGDG is formed and does not involve subsequent desaturation of the DGDG molecule. The relative rates of galactosylation of the various cytosolic and chloroplastic molecular species of MGDG may be one means by which the final amounts of chloroplastic and cytosolic species in both MGDG and DGDG is regulated. Other reactions are also likely to be regulated (6–8, 16).

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