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Biosynthesis of Digalactosyldiacylglycerol in Plastids from 16:3 and 18:3 Plants[1]

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ABSTRACT

Intact chloroplasts isolated from leaves of eight species of 16:3 and 18:3 plants and chromoplasts isolated from *Narcissus pseudonarcissus* L. flowers synthesize galactose-labeled mono-, di-, and trigalactosyldiacylglycerol (MGDG, DGDG, and TGDG) when incubated with UDP-[6-^{3}H]galactose. In all plastids, galactolipid synthesis, and especially synthesis of DGDG and TGDG, is reduced by treatment of the organelles with the nonpenetrating protease thermolysin. Envelope membranes isolated from thermolysin-treated chloroplasts of *Spinacia oleracea* L. (16:3 plant) and *Pisum sativum* L. (18:3 plant) or membranes isolated from thermolysin-treated chromoplasts are strongly reduced in galactolipid:galactolipid galactosyltransferase activity, but not with regard to UDP-Gal:diacylglycerol galactosyltransferase. For the intact plastids, this indicates that thermolysin treatment specifically blocks DGDG (and TGDG) synthesis, whereas MGDG synthesis is not affected. Neither in chloroplast nor in chromoplast membranes is DGDG synthesis stimulated by UDP-Gal. DGDG synthesis in *S. oleracea* chloroplasts is not stimulated by nucleoside 5′-diphospho digalactosides. Therefore, galactolipid:galactolipid galactosyltransferase is so far the only detectable enzyme synthesizing DGDG. These results conclusively suggest that the latter enzyme is located in the outer envelope membrane of different types of plastids and has a general function in DGDG synthesis, both in 16:3 and 18:3 plants.

The 16:3 plants differ from 18:3 plants by the occurrence of *cis*- 7,10,13-hexadecatrienoic acid in MGDG[4] of the chloroplasts (7, 16, 17, 24). This type of MGDG is usually called prokaryotic, due to the presence of a C_{16} fatty acid at the *sn*-2 position of the glycerol backbone. According to the plastid-reticular theory, acyl lipids with such a prokaryotic structure are formed within the chloroplast. On the other hand, acyl lipids containing C_{18} fatty acids at *sn*-2 are believed to be derived from diacylglycerol moieties synthesized in the endoplasmic reticulum. These lipids consequently are called eukaryotic (9, 14, 17, 30). In 16:3 plants, the two galactolipids MGDG and DGDG are present in both the prokaryotic and eukaryotic configuration (3, 16, 20), and thus must be derived partially from plastid and partially from reticular diacylglycerol residues. The galactolipids of 18:3 plants, however, are found only in the eukaryotic configuration.

In the membranes of chloroplasts and other plastids, MGDG and DGDG are formed by stepwise galactosylation of diacylglycerol (7, 21, 25, 29). It has been suggested that pro- and eukaryotic galactolipid molecules might be synthesized in plastids by different sets of enzymes, the prokaryotic galactolipids being formed in the inner and eukaryotic lipids in the outer envelope membrane (2, 3, 17, 18). A different localization of UDP-Gal:diacylglycerol galactosyltransferase (MGDG synthase) in the outer envelope membrane of pea (a 18:3 plant) chloroplasts (4), but in the inner envelope membrane of spinach (16:3 plant) chloroplasts (2, 12), was considered evidence for such a hypothesis. Moreover, two distinct enzymatic activities have been proposed for the biosynthesis of DGDG. In 1961, Ferrari and Benson (8) suggested a pathway of DGDG synthesis in which MGDG is galactosylated by UDP-Gal. Using extracts from pea leaves, indeed, Siebertz and Heinz (26) succeeded in measuring DGDG formation from the two precursors UDP-Gal and MGDG. Later, Van Besouw and Wintermans (27) reported for spinach another enzymatic activity, which produces DGDG and diacylglycerol by dismutation of two MGDG molecules. This enzyme, galactolipid:galactolipid galactosyltransferase, subsequently could be localized in the outer envelope membrane of spinach chloroplasts (15). In agreement with a cytosol-facing location, the enzyme activity is lost by pretreatment of chloroplasts with the nonpenetrating (5) protease thermolysin (6). Recently, Heemskerk *et al.* (11, 12) have presented evidence that in spinach chloroplasts DGDG synthesis can be assigned completely to this galactosyltransferase. So far, indirect measurements have revealed that this enzyme is also present in pea chloroplasts (4) and in other types of plastids (1).

To extend the study on the enzymatic mechanism of DGDG synthesis to plants other than spinach, we investigated galactolipid synthesis in chloroplasts isolated from a number of 16:3 and 18:3 plant species including pea. In all these chloroplasts, galactolipid:galactolipid galactosyltransferase proves to be active and is sensitive to thermolysin, whereas no other enzymatic activities of DGDG synthesis could be detected. This suggests that only one enzyme is responsible

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[4] Abbreviations: MGDG, DGDG, and TGDG, mono-, di-. and trigalactosyldiacylglycerol.

for biosynthesis of DGDG in 16:3 and 18:3 plants. Accordingly, the previously suggested DGDG synthesis from UDP-Gal and MGDG in pea (26) is now explained as a concerted action of two well-defined galactosyltransferases.

MATERIALS AND METHODS

Chemicals

Radioactive sodium [1-^{14}C]acetate, UDP-D-[U-^{14}C]Gal, and UDP-D-[6-^{3}H]Gal were supplied by Amersham International (Amersham, U.K.). Radiolabeled [*galactose*-6-^{3}H] MGDG (40 MBq/mmol) was purified from spinach envelope membranes after incubation with UDP-[6-^{3}H]Gal (12). Unlabeled MGDG was isolated from spinach chloroplasts (12). Thermolysin from *Bacillus thermoproteolyticus* (EC 3.4.24.4) was obtained from Sigma (St. Louis, MO). All other reagents were of analytical grade.

Chemical Synthesis of Different Nucleoside 5′-Diphospho Galactosides

6-*O*-(α-D-galactopyranosyl)-α-D-galactopyranosyl phosphate (m.p. 98–100°C under decomposition) was obtained by extensive hydrogenation over Pd/C of dibenzyl[2,3,4-tri-*O*-benzyl-6-*O*-(2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranosyl)-α-D-galactopyranosyl] phosphate and purification by HPLC (RP 8 column, elution with water) and by passing through an Amberlite IR 120 (H^+ form) column. The benzylated compound ($[\alpha]_D^{22}$ = +55.3, c = 2 in chloroform) was synthesized by reaction of dibenzyl(2,3,4-tri-*O*-benzyl-α-D-galactopyranosyl)phosphate and *O*-(2,3,4,6-tetra-*O*-benzyl-β-D-galactopyranosyl) trichloroacetimidate (28) in presence of trimethylsilyl triflate in dry diethylether at −15°C. The above phosphate ($[\alpha]_D^{22}$ = +50.9, c = 1 in chloroform) was synthesized by reaction of *O*-(6-*O*-acetyl-2,3,4-tri-*O*-benzyl-β-D-galactopyranosyl) trichloroacetimidate (28) with dibenzyl phosphate, isomerization by addition of *p*-toluenesulfonic acid and followed by hydrolysis of the acetyl group using potassium carbonate and methanol.

Bis-triethylammonium [6-*O*(α-D-galactopyranosyl)-α-D-galactopyranosyl] uridine-5′-pyrophosphate (UDP-Gal-Gal): a solution of bis-cyclohexylammonium [6-*O*-(α-D-galactopyranosyl)-α-D-galactopyranosyl] phosphate (49 mg, 77 μmol) was converted into the corresponding bis(tri-*n*-octylammonium) phosphate (19). The latter compound and UMP-morpholidate (71 mg, 102 μmol) were both dried by repeated evaporation with dry pyridine. A round-bottomed flask with molecular sieves (0.4 nm) was dried by heating with a Bunsen burner *in vacuo*. Then, the phosphate and the morpholidate (each in 4 mL dry pyridine) were added under nitrogen. The reaction was monitored by HPLC and after standing for 4 d the mixture was evaporated to dryness. Dissolution in 0.05 M triethylammonium hydrogencarbonate (pH 7.4 with acetic acid), removing of tri-*n*-octylamine by extraction with diethylether, and separation from by-products by preparative HPLC (19) yielded UDP-Gal-Gal as an amorphous solid (25 mg, 33%). Calculated for $C_{33}H_{64}N_4O_{22}P_2 \cdot 3H_2O$ C = 40.25, H = 7.16, N = 5.69; found C = 40.11, H = 6.98, N = 5.79. ^{1}H-NMR (250 MHz, D_2O): δ/ppm = 1.10 (t, J = 7.3 Hz, 18 H, 6 NCH_2CH_3), 3.04 (q, J = 7.3 Hz, 12 H, 6 NCH_2CH_3), 3.50–4.08 (m, 12 H), 4.11–4.35 (m, 5 H, H-2′, H-3′, H-4′, H-5$_A$′, H-5$_B$′), 4.78 (d, $J_{1''',2'''}$ = 3 Hz, 1 H, H-1‴), 5.45 (dd, $J_{1'',P}$ = 7 Hz, $J_{1'',2''}$ = 3 Hz, 1 H, H-1″), 5.78–5.81 (m, 2 H, H-5, H-1′), 7.78 (d, $J_{5,6}$ = 7.8 Hz, 1 H, H-6).

Bis-triethylammonium [6-*O*-(α-D-galactopyranosyl)-α-D-galactopyranosyl] cytidine-5′-pyrophosphate (CDP-Gal-Gal) was synthesized similarly as UDP-Gal-Gal, except that CMP-morpholidate (71 mg, 102 μmol, 1.2 eq.) was used. Preparative HPLC yielded an amorphous solid (14 mg, 17%). ^{1}H-NMR (250 MHz, D_2O): δ/ppm = 1.10, 3.04 and 4.78 as for UDP-Gal-Gal, 3.45–3.97 (m, 12 H), 4.00–4.20 (m, 5 H, H-2′, H-3′, H-4′, H-5$_A$′, H-5$_B$′), 5.45 (m, 1 H, H-1″), 5.80 (d, $J_{1',2'}$ = 4.8 Hz, 1 H, H-1′), 5.96 (br. s, 1 H, H-5), 7.80 (d, $J_{5,6}$ = 8.8 Hz, 1 H, H-6).

Bis-triethylammonium [6-*O*-(α-D-galactopyranosyl)-α-D-galactopyranosyl] adenosine-5′-pyrophosphate (ADP-Gal-Gal) was synthesized similarly as UDP-Gal-Gal, except that AMP-morpholidate (67.5 mg, 113 μmol, 1.2 eq.) was used. Preparative HPLC yielded an amorphous solid (16 mg, 17%). ^{1}H-NMR (250 MHz, D_2O): δ/ppm = 1.10 and 3.04 as for UDP-Gal-Gal, 3.45–3.98 (m, 12 H), 4.00–4.25 (m, 2 H, H-5$_A$′, H-5$_B$′), 4.26–4.41 (m, 3 H, H-2′, H-3′, H-4′), 4.85 (d, $J_{1''',2'''}$ = 3 Hz, 1 H, H-1‴), 5.45 (m, 1 H, H-1″), 5.95 (d, $J_{1',2'}$ = 5.6 Hz, 1 H, H-1′), 8.06 (s, 1 H, H-2), 8.37 (s, 1 H, H-8).

Plants

Spinacia oleracea L. cv Subito (spinach) was grown hydroponically (18). *Avena sativa* L., *Pisum sativum* L. cv Progress No. 9 (pea), *Sinapis alba* subsp. *alba* L. cv Arda Gelbsenf, *Vicia faba* L. and *Zea mays* L. cv Zuckermais were cultivated in a mixture of vermiculite and perlite in the greenhouse. *Nicotiana sylvestris* Spegazz. and *Tropaeolum majus* L. var altum were grown in soil in the greenhouse. Flower-buds from *Narcissus pseudonarcissus* L. subsp. *major* were collected in the botanical garden. Flowers were then allowed to develop at room temperature for 24 h.

Plastids

Intact chloroplasts were purified from young, growing leaves of various plant species by Percoll-gradient centrifugation (11). Table I gives the composition of homogenizing media used for isolation of chloroplasts from various plant species. Purified chloroplasts were finally suspended in a buffer containing 0.33 to 0.37 M sorbitol (as indicated in Table I) and 25 mM Tricine (pH 7.6 with KOH). Spinach chloroplasts for acetate-labeling experiments were isolated by using a rapid purification procedure (19).

Chromoplasts were isolated from the coronae (paracorollae) of *N. pseudonarcissus* flowers according to Liedvogel *et al.* (22), except that the grinding medium was composed of 0.45 M sucrose, 25 mM Tricine (pH 7.6 with KOH), 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 0.2% (w/v) PVP. Sucrose-gradient centrifugation yielded a Chl-free, orange-colored chromoplast fraction between 15 and 40% (w/v) sucrose. The chromoplasts were suspended in a medium containing 0.45 M sucrose, 25 mM Tricine (pH 7.6 with KOH), and 5 mM 2-

Table I. *Media Used for Isolation of Intact Chloroplasts*

Chloroplasts were isolated from various plant species using homogenization media of indicated composition. All media were buffered with 25 mM Tricine (pH 7.6 with KOH). Yields of intact chloroplasts (in μg Chl/g leaf) are given as obtained in a typical experiment. Abbreviations: merc., 2-mercaptoethanol; X^{2+} indicates 1 mM $MgCl_2$, 1 mM $MnCl_2$ plus 2 mM EDTA.

Plant species	Sorbitol	X^{2+}	BSA	PVP	Ascorbate	Merc.	Yield
	M		%		*mM*		*μg Chl/g*
P. sativum	0.33	−	0.1	0	0	0	82
S. oleracea	0.33	−	0.1	0	0	0	143
N. sylvestris	0.33	+	0.1	0.1	0	5	23
T. majus	0.33	+	0.1	0.1	0	5	12
V. faba	0.33	+	0.1	0.1	0	5	75
S. alba	0.35	+	0.1	0	0	5	110
A. sativa	0.37	+	0.2	0	2	0	28
Z. mays	0.37	+	0.2	0	2	0	60

mercaptoethanol. Microscopic examination showed yellow-orange organelles, part of which enclosed pigmented crystals.

Thermolysin treatment of purified chloroplasts and chromoplasts was carried out according to Heemskerk *et al.* (15), except that the incubation medium contained sorbitol as given in Table I. Chloroplasts (0.5 mg Chl/mL) were incubated under stirring with thermolysin (1 mg/mL) in presence of 1 mM $CaCl_2$ at 4°C for 1 h. Control chloroplasts were incubated similarly, except that incubations contained additional 10 mM EDTA. After treatment, intact chloroplasts were repurified by Percoll-centrifugation (15). Thermolysin treatment of chromoplasts (0.5 mg carotenoid/mL) was similar, but using the sucrose-containing suspension medium followed by repurification on sucrose gradient (22).

Plastid Membranes

Total envelope membranes from spinach and pea were isolated from the intact chloroplasts as described by Heemskerk *et al.* (12). Chromoplast membranes from *N. pseudonarcissus* were prepared by disrupting the organelles with a tight-fitting Potter homogenizer in a medium containing 25 mM Tricine and 5 mM 2-mercaptoethanol (pH 7.6 with KOH). Total chromoplast membranes were then collected by centrifugation at 100,000g_{max} for 2 h (4°C).

Incubations of Plastids

Total galactolipid synthesis in intact chloroplasts (50 μg Chl) was measured (11) by incubation with 0.12 mM UDP-[6-^{3}H]Gal (1.28 GBq/mmol) in 200 μL medium (final volume), containing 0.33 to 0.37 M sorbitol (as indicated in Table I), 30 mM Tricine (pH 7.2 with KOH), and 10 mM $MgCl_2$ for 10 to 30 min at 30°C. Total galactolipid synthesis in chromoplasts was measured similarly, except that the incubation medium contained 0.45 M sucrose instead of sorbitol.

Spinach chloroplasts (1.25 mg Chl) were labeled with [1-^{14}C]acetate (370 kBq, 2.0 GBq/mmol) by incubation in 1.0 mL buffer composed of 0.33 M sorbitol, 20 mM Tricine (pH 8.0 with KOH), 10 mM $KHCO_3$, 0.5 mM *sn*-glycerol-3-P and 0.3 mM KH_2PO_4 (final concentrations) with shaking and illumination at 30°C. After 20 min of incubation, the chloroplasts were pelleted, washed, and resuspended in a buffer containing 330 mM sorbitol and 25 mM Tricine (pH 7.2 with KOH). Generally, three separate experiments were carried out, each in three parallels.

Enzymatic Assays with Membranes

Galactosyltransferases in pea, spinach and *N. pseudonarcissus* membrane fractions were assayed as described elsewhere (12). Briefly, for measuring UDP-Gal:diacylglycerol galactosyltransferase (EC 2.4.1.46), membranes were sonicated with dispersed dioleoyl-PC and preincubated with phospholipase C from *Bacillus cereus* (EC 3.1.4.3) (0.3 units/incubation) until all PC was converted into dioleoylglycerol (15 min at 35°C). Then sodium desoxycholate was added for solubilization and incubations were started by addition of UDP-Gal in a final volume of 220 μL, containing 27 to 35 μg membrane protein, 406 nmol desoxycholate, 116 nmol dioleoylglycerol, 50 mM Tricine (pH 7.2 with KOH), and 0.2 mM UDP-[^{3}H]Gal (10 min). Galactolipid:galactolipid galactosyltransferase (EC 2.4.1.184) activity was measured after sonication of the membranes with [^{3}H]MGDG/desoxycholate (1:2, mol/mol) micelles. Incubations of 250 μL contained 54 nmol desoxycholate, 26.6 nmol [^{3}H]MGDG, 18 to 24 μg protein of chloroplast envelope membranes, or 22 to 27 μg protein of chromoplast membranes and 50 mM Tricine (pH 7.2 with KOH). Incubations were started by addition of 10 mM $MgCl_2$ and stopped after 10 min at 35°C.

Lipids

Lipid extraction, lipid separation by TLC, and analysis of labeled and unlabeled lipids was as described elsewhere (11). Lipids extracted from leaves were separated by TLC in acetone:benzene:water (91:30:8, v/v). Recovered leaf DGDG was repurified by TLC using the same solvent, to remove contaminating phosphatidylethanolamine.

Various Procedures

Chl and protein were quantified as before (11). Carotenoids were quantified in ethanol by determination of optical density

Table II. *Fatty Acid Composition of Galactolipids from Leaves of the Plants used in This Study*

Plant Species	Fatty Acid (% of total)							
	16:0	16:1	16:2	16:3	18:0	18:1	18:2	18:3
S. oleracea								
MGDG	3.3	1.2	0.7	17.1	0.8	5.5	2.2	69.1
DGDG	8.1	0.3	0.2	5.9	0.6	4.1	3.7	76.9
N. sylvestris								
MGDG	3.4	0.8	1.6	14.1	0.7	2.4	4.4	72.5
DGDG	11.4	0.6	0.3	1.4	1.5	3.6	4.2	77.0
S. alba								
MGDG	3.6	1.0	1.9	20.7	0.9	6.0	5.3	60.7
DGDG	12.4	0.7	0.4	2.5	2.5	6.5	7.9	67.1
T. majus								
MGDG	2.8	0.8	1.0	23.3	0	3.7	2.4	66.0
DGDG	12.9	0.5	0.2	2.8	1.4	2.8	3.5	75.5
V. faba								
MGDG	4.2	1.0	0	0	1.3	5.6	7.0	81.0
DGDG	11.4	0	0	0	2.8	3.9	6.9	75.1
P. sativum								
MGDG	2.7	0.5	0	0	1.7	2.3	2.1	90.7
DGDG	9.1	0.3	0	0	2.8	1.9	2.3	83.3
A. sativa								
MGDG	1.3	0.3	0.1	0	0.3	1.3	3.7	93.4
DGDG	10.3	0.5	0	0	0.9	2.9	3.5	81.9
Z. mays								
MGDG	2.7	0.4	0.1	0	0.7	2.0	8.0	86.1
DGDG	12.0	0.3	0	0	2.4	3.1	9.5	72.3

at 442 nm (2430 cm^{-1} of 1% w/v solution). UDP-Gal-Gal was dissolved in water, ADP-Gal-Gal and CDP-Gal-Gal were dissolved in DMSO:water (30:70, v/v). Concentrations of digalactosyl derivatives were determined using molar absorption coefficients of UDP (10,000, 262 nm), ADP (15,400, 259 nm) and CDP (15,400, 259 nm).

RESULTS

Fatty Acid Composition of Galactolipids in Leaves of 16:3 and 18:3 Plants

Eight 16:3 and 18:3 plant species were selected from which it was possible to isolate intact chloroplasts. Table II gives the fatty acid composition of the galactolipids extracted from leaves of these plants. In the 16:3 plants *S. oleracea*, *N. sylvestris*, *S. alba*, and *T. majus*, MGDG contains high levels of hexadecatrienoate, whereas DGDG is rich in palmitate. Hexadecatrienoate in MGDG is present almost exclusively at the *sn*-2 position, whereas palmitate in DGDG of 16:3 plants is distributed roughly equally over *sn*-1 and *sn*-2 (7, 16). Therefore, the data of Table II suggest that in the 16:3 plants MGDG is more enriched than DGDG in percentage of prokaryotic molecular species (with C_{16} fatty acids at *sn*-2). The same is true for the 16:3 plant *Arabidopsis thaliana* (L.) Heynh., where molecular species of MGDG and DGDG have been analyzed extensively (3, 23).

In the 18:3 plants *V. faba*, *P. sativum*, *A. sativa*, and *Z. mays*, DGDG differs from MGDG in a high percentage of palmitate (Table II). Since in 18:3 plants DGDG palmitate is esterified at *sn*-1 (16), Table II indicates that MGDG and DGDG of 18:3 plants are almost completely eukaryotic.

Isolation of Plastids

Intact chloroplasts were isolated from young leaves using different homogenization media, as indicated in Table I. Despite efforts to optimize the isolation procedure, yields of intact chloroplasts vary considerably with the type of plant species (Table I), partially as a consequence of differences in Chl content of leaves, but also due to a different fraction of chloroplasts that was broken upon homogenization. Additionally, chromoplasts were isolated from coronae (paracarollae) of the 18:3 plant *N. pseudonarcissus.*

Effect of Thermolysin Treatment on Galactolipid Synthesis

All isolated intact chloroplasts synthesize MGDG, DGDG, and TGDG from the precursor UDP-[^{3}H]Gal, although the rate of galactose incorporation on a Chl basis is variable between species (Fig. 1). There were no typical differences between 16:3 and 18:3 plants. In all plant species, treatment of chloroplasts with the nonpenetrating protease thermolysin results in a considerable reduction of total galactolipid synthesis, compared to untreated chloroplasts (Fig. 1A). Generally, thermolysin treatment causes a shift in the distribution of labeled galactolipids: percentage of label in MGDG increases at the expense of DGDG and TGDG (Fig. 1B). However, some differences exist between the various plants. Whereas in most types of chloroplasts inhibition of DGDG

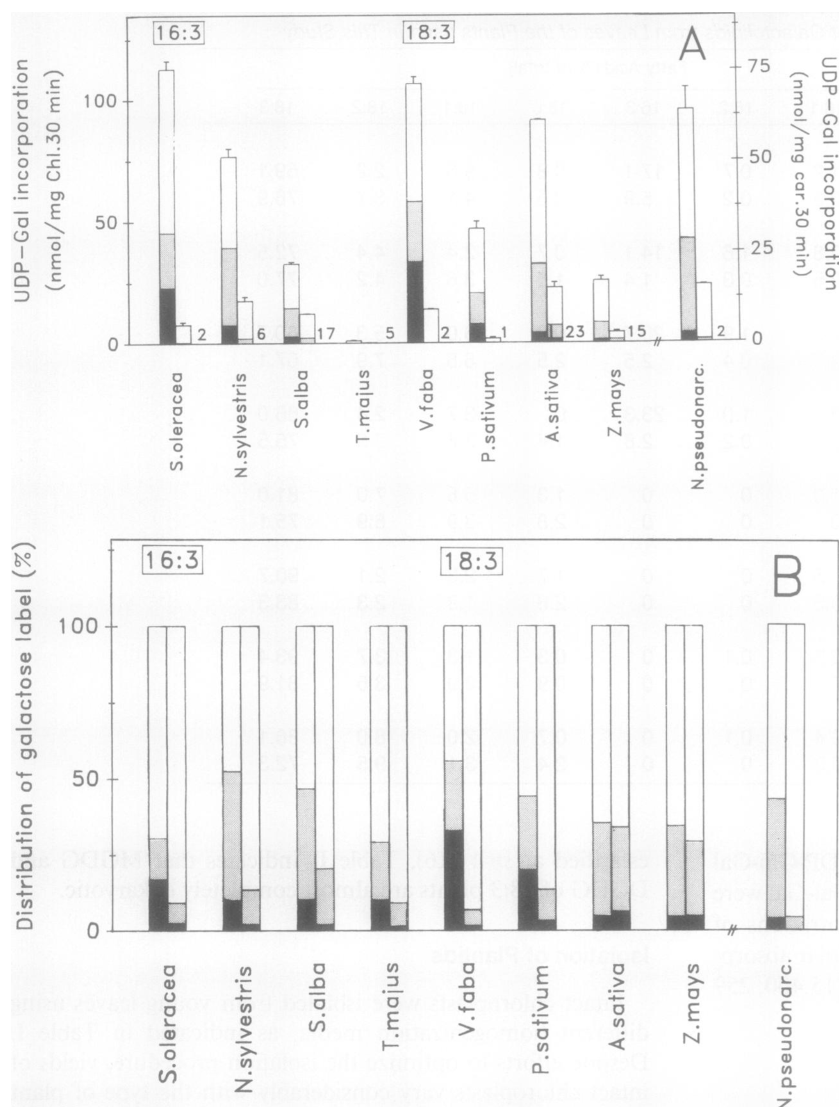

Figure 1. Galactolipid synthesis by untreated and thermolysin-treated chloroplasts and chromoplasts. Intact chloroplasts from 16:3 and 18:3 plants and chromoplasts from *N. pseudonarcissus* were either untreated (left bars) or treated with thermolysin (right bars) and then incubated with 0.12 mM UDP-[^{3}H]Gal and 10 mM MgCl, for 30 min. A, Formation of labeled MGDG (white area), DGDG (dotted), and TGDG (black). Numbers indicate synthesis of DGDG + TGDG in thermolysin-treated plastids, relative to that in untreated plastids (set as 100%). B, Percentage distribution of label in different galactolipids: MGDG (white areas), DGDG (dotted), and TGDG (black). Data are mean values ± SD (n = 3) of representative experiment.

and TGDG synthesis by thermolysin was almost complete (≥94%), inhibition was less severe (77–85%) in chloroplasts from *S. alba*, *A. sativa*, and *Z. mays* (Fig. 1A).

Similar to chloroplasts, chromoplasts isolated from *N. pseudonarcissus* are able to synthesize galactolipids (21) and form labeled MGDG, DGDG, and TGDG from UDP-[^{3}H]Gal (Fig. 1). Synthesis of DGDG and TGDG is inhibited almost completely after treatment of the chromoplasts with thermolysin (Fig. 1).

It has been shown previously with spinach chloroplasts that UDP-Gal-derived synthesis of MGDG, DGDG, and TGDG is the result of two cooperatively acting enzymes: UDP-Gal:diacylglycerol galactosyltransferase, which forms MGDG from UDP-Gal plus diacylglycerol, and galactolipid:galactolipid galactosyltransferase, producing DGDG and TGDG by galactosyl transfer from MGDG to another galactolipid molecule with concomitant formation of diacylglycerol. The released diacylglycerol then can be reused for MGDG synthesis by the first enzyme (11). Thus, all galactose label from UDP-[^{3}H]Gal is first incorporated into MGDG and labeled MGDG then is converted partially to DGDG and TGDG. Consequently, the ratio of UDP-Gal:diacylglycerol galactosyltransferase activity to galactolipid:galactolipid galactosyltransferase activity can be roughly estimated as the ratio of label in (MGDG + DGDG + TGDG)/(DGDG + TGDG). Under the assumption that such a mechanism of galactolipid formation is valid for plant species other than spinach, calculation of this ratio shows that in all types of plastids thermolysin treatment has a differentially inhibitory effect on galactolipid:galactolipid galactosyltransferase activity, although the effect of thermolysin is rather small in chloroplasts from *S. alba*, *A. sativa*, and *Z. mays* (Table III).

Thermolysin treatment is known to inhibit quite specifically the activity of spinach galactolipid:galactolipid galactosyltransferase (DGDG and TGDG synthesis), but not of spinach UDP-Gal:diacylglycerol galactosyltransferase (6, 13, 15). For an accurate measurement of these two galactosyltransferases, it is necessary to work with isolated membrane

Table III. *Ratio of Galactosyltransferase Activities in Untreated and Thermolysin-Treated Plastids*

Chloroplasts and chromoplasts were treated with thermolysin or untreated, as described in "Materials and Methods." Plastids were incubated with 0.12 mM UDP-[^{3}H]Gal and 10 mM $MgCl_2$ for 10 or 30 min, and galactolipid synthesis was measured. Numbers represent ratio of UDP-Gal:diacylglycerol galactosyltransferase activity to galactolipid:galactolipid galactosyltransferase activity, which was calculated as ratio of label in (MGDG + DGDG + TGDG)/(DGDG + TGDG). Mean values are given of three independent experiments.

	Untreated		Thermolysin-Treated	
	10 min	30 min	10 min	30 min
	ratio of label			
Chloroplasts				
S. oleracea	4.5	3.3	26.1	11.4
N. sylvestris	4.3	3.2	14.0	7.0
S. alba	2.5	2.0	6.6	4.4
T. majus	6.2	3.4		10.7
V. faba	2.7	1.8	20.2	15.0
P. sativum	3.4	2.5	10.7	7.9
A. sativa	4.1	2.3	5.5	2.9
Z. mays	6.0	2.9	6.7	3.3
Chromoplasts				
N. pseudonarcissus	2.5	1.9	19.5	11.2

fractions, in which either enzyme can be assayed separately (12). Table IV demonstrates that, after thermolysin treatment of the organelles, chromoplast membranes from *N. pseudonarcissus* and pea chloroplast envelope membranes are strongly reduced in galactolipid:galactolipid galactosyltransferase activity, but not in MGDG synthase activity. Thus, the reduced UDP-Gal incorporation in intact plastids of *N. pseudonarcissus*, pea, and spinach after thermolysin treatment (Fig. 1A) seems to be caused completely by inactivation of galactolipid:galactolipid galactosyltransferase, the capacity of MGDG synthesis remaining unchanged.

Taken together, these results indicate that galactolipid synthesis in the various types of plastids proceeds similarly as described for spinach chloroplasts (11): treatment of plastids with thermolysin causes inactivation of galactolipid:galactolipid galactosyltransferase, which results in a deficient supply of diacylglycerol and, as a consequence, in reduced incorporation of UDP-Gal into galactolipids. The incorporated galactose in thermolysin-treated chloroplasts accumulates into MGDG, since its conversion into DGDG and TGDG is blocked. However, chloroplasts from *S. alba*, *A. sativa*, and *Z. mays* seem to behave differently (see below).

Influence of UDP-Gal on DGDG Synthesis

To study the effect of UDP-Gal on DGDG synthesis, plastid membranes were sonicated with [*galactose*-^{3}H]MGDG and formation of labeled DGDG was measured. Table IV shows that in chloroplast envelope membranes of pea and in chromoplast membranes of *N. pseudonarcissus*, like in spinach envelope membranes, MGDG-dependent DGDG synthesis is not stimulated by UDP-Gal. Consequently, this provides no evidence for activity of the hypothetical UDP-Gal:MGDG galactosyltransferase.

It might be possible that a second DGDG forming enzyme causes the incomplete inhibition of DGDG synthesis in thermolysin-treated chloroplasts of *S. alba*, *A. sativa*, and *Z. mays*. However, when chloroplast membranes from these species were incubated with [*galactose*-^{3}H]MGDG, there was no stimulating effect of UDP-Gal on DGDG synthesis (Table

Table IV. *Activities of Galactosyltransferases in Membranes Isolated from Untreated and Thermolysin-Treated Chromoplasts and Chloroplasts*

Membrane fractions were isolated from thermolysin-treated and untreated organelles. Activities of UDP-Gal:diacylglycerol galactosyltransferase (UDGT, MGDG synthase) and galactolipid:galactolipid galactosyltransferase (GGGT, synthesis of DGDG and TGDG) were assayed separately (see "Materials and Methods"). In some incubations, assays for GGGT contained additionally 0.5 mM UDP-Gal, as indicated. Mean values are given of duplicate incubations. (Data for spinach envelope membranes are obtained from refs. 12 and 13.)

	Enzymatic Activity	Untreated	Thermolysin-Treated
		nmol/mg protein·h	
Chromoplast membranes			
N. pseudonarcissus	UDGT	257	284
	GGGT	568	48
	GGGT + UDP-Gal	478	61
Chloroplast envelopes			
P. sativum	UDGT	342	321
	GGGT	2433	142
	GGGT + UDP-Gal	2209	146
Chloroplast envelopes			
S. oleracea	UDGT	77	89
	GGGT	549	24
	GGGT + UDP-Gal	554	

Table V. *Effect of UDP-Gal on DGDG Synthesis in Broken Chloroplasts of Various Plant Species*

Purified chloroplasts were broken, sonicated with [^{3}H]MGDG/desoxycholate (1:2, mol/mol), and incubated in the presence or absence of UDP-Gal for 20 min at 35°C. Incubations of 300 μL (final volume) contained chloroplasts (35–45 μg Chl), 51 nmol desoxycholate, 25 nmol [^{3}H]MGDG, 50 mM Tricine (pH 7.2 with KOH), and 10 mM $MgCl_2$. Conversion of label from MGDG into DGDG is shown. Mean values ± SD (n = 3) are given.

Chloroplasts	UDP-Gal	Synthesis of DGDG
	mM	*nmol/mg Chl·h*
S. alba	0	271 ± 7.1
	0.33	262 ± 5.5
A. sativa	0	194 ± 11.3
	0.33	208 ± 6.6
Z. mays	0	108 ± 4.4
	0.33	105 ± 6.1

V), again excluding UDP-Gal:MGDG galactosyltransferase activity.

Other Possible Pathways of DGDG Synthesis

We finally considered the possibility that nucleoside 5′-diphospho digalactosides might act as precursors for DGDG biosynthesis, resembling the oligosaccharide transfer during glycoprotein synthesis in the endoplasmic reticulum. Three compounds were chemically synthesized: 6-*O*-(α-D-galactopyranosyl)-α-D-galactopyranosyladenosine-5′-pyrophosphate and the corresponding cytidine-5′-pyrophosphate and uridine-5′-pyrophosphate derivates (ADP-Gal-Gal, CDP-Gal-Gal, and UDP-Gal-Gal, respectively). In order to test biological function of these compounds, spinach chloroplasts were prelabeled with [^{14}C]diacylglycerol by incubation with [^{14}C] acetate (10). When these chloroplasts were subsequently incubated with UDP-Gal-Gal, ADP-Gal-Gal, or CDP-Gal-Gal, none of the digalactosides led to formation of labeled DGDG (Table VI). Furthermore, addition of a spinach leaf extract enriched in cytosol did not induce DGDG synthesis with UDP-Gal-Gal, nor did breakage of the prelabeled chloroplasts (data not shown).

DISCUSSION

Galactolipid:Galactolipid Galactosyltransferase in Various Plastids

Our results demonstrate that UDP-Gal:diacylglycerol galactosyltransferase and galactolipid:galactolipid galactosyltransferase are active in chromoplasts and in chloroplasts of 16:3 and 18:3 plants. The presence of galactolipid:galactolipid galactosyltransferase in plastids is concluded from the coinciding production of DGDG and TGDG (Fig. 1), the inhibitory effect of thermolysin on DGDG and TGDG synthesis (Table III) and from the use of a specific assay (Table IV). In all plastids studied, galactolipid:galactolipid galactosyltransferase is sensitive toward the nonpenetrating protease (5, 6) thermolysin, which suggests a uniform location of the transferase at the cytosolic face of the outer envelope membrane.

Various authors have provided additional evidence for galactolipid:galactolipid galactosyltransferase activity in the outer membrane of various types of plastids, although its function in DGDG synthesis was not established. Cline and Keegstra (4) measured high DGDG and TGDG production in outer envelope membrane of pea chloroplasts. Alban *et al.* (1) isolated envelope membranes from nongreen plastids of cauliflower (*Brassica oleracea* L.) buds and from amyloplasts of sycamore (*Acer pseudoplatanus* L.) cells. The authors found accumulation of TGDG and diacylglycerol in the envelope membranes, unless the plastids were treated with thermolysin before isolation of the membranes.

Thermolysin treatment only partially suppressed the synthesis of DGDG and TGDG in chloroplasts of *S. alba*, *A. sativa*, and *Z. mays* (Fig. 1). This suggests incomplete proteolysis of galactolipid:galactolipid galactosyltransferase in these chloroplasts, since there is no evidence for a second DGDG forming activity. At present, it is not clear whether the decreased sensitivy to thermolysin is caused by changes in accessibility and localization of the enzyme or by changes in its chemical structure.

Lack of Evidence for Alternative Pathways of DGDG Synthesis

Siebertz and Heinz (26) have reported DGDG synthesis by a putative UDP-Gal:MGDG galactosyltransferase (8) in homogenized pea leaves. However, such an enzymatic activity could not be measured in the present experiments: UDP-Gal did not stimulate MGDG-dependent DGDG synthesis in membrane fractions from *S. oleracea*, *S. alba*, *P. sativum*, *A. sativa*, and *Z. mays* chloroplasts or from *N. pseudonarcissus* chromoplasts (Table IV and V). Another possible pathway of DGDG synthesis, which was not studied so far, is one using nucleoside 5′-diphospho digalactosides. We were unable to demonstrate involvement of these compounds in DGDG formation (Table VI). Therefore, galactolipid:galactolipid galactosyltransferase is, so far, the only known enzyme forming

Table VI. *Effect of Nucleoside 5′-Diphospho Digalactosides on DGDG Synthesis*

Spinach chloroplasts (1.25 mg Chl) were preincubated with [^{14}C] acetate (see "Materials and Methods") for 20 min and washed. Incorporation of label into chloroplast lipids was 69.1 kBq/mg Chl, from which 54% was present in diacylglycerol. Subsequently, aliquots of the washed chloroplasts (70 μg Chl, assay volume of 250 μL) were incubated in the presence of 330 mM sorbitol, 25 mM Tricine (pH 7.2 with KOH), and 0.5 mM $MgCl_2$ with 0.25 mM of the indicated digalactoside derivatives for 30 min at 30°C. All incubations contained 6% (v/v) DMSO.

Added Nucleotide	Labeled product	
	MGDG	DGDG
	kBq	
None	0.21	0.018
ADP-Gal-Gal	0.26	0.013
CDP-Gal-Gal	0.18	0.012
UDP-Gal-Gal	0.30	0.018
UDP-Gal	1.55	0.036

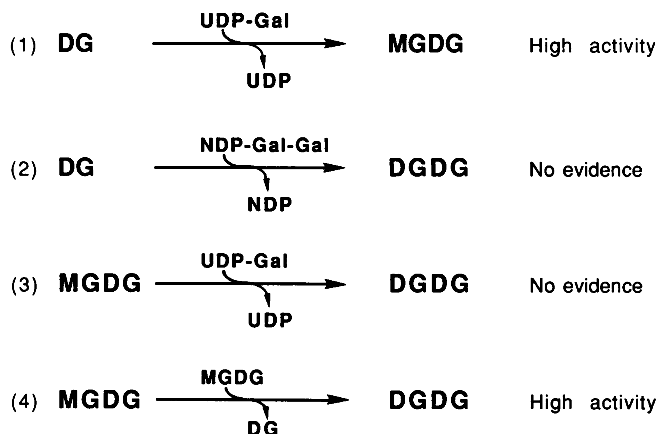

Figure 2. Evidence for enzymatic reactions of galactolipid synthesis in plastids of higher plants. The reactions catalyzed by UDP-Gal:diacylglycerol galactosyltransferase (1) and galactolipid: galactolipid galactosyltransferase (4) are demonstrated experimentally in 16:3 and 18:3 plants. Note that reaction (4) can also proceed in reversed direction (see ref. 13). Abbreviations: NDP indicates ADP, CDP, or UDP.

DGDG, as is summarized in Figure 2. A criticism against the biosynthetic function of this galactosyltransferase is its production of TGDG and other unnatural galactolipids (6, 7). In this context, Heemskerk *et al.* (11) have shown before that formation of unnatural galactolipids is caused by high concentrations of (unchelated) divalent cations in the incubation medium.

In view of the present experiments, the previously reported UDP-[^{14}C]Gal- and MGDG-stimulated synthesis of DGDG in pea leaves (with low activity of 6 nmol/mg protein·h) (26) may be satisfactorily explained by concerted action of UDP-Gal: diacylglycerol galactosyltransferase and galactolipid: galactolipid galactosyltransferase. Under diacylglycerol-limited conditions, the latter transferase may produce diacylglycerol from externally added MGDG, which in turn is converted with UDP-[^{14}C]Gal into galactose-labeled MGDG. Subsequently, this MGDG is galactosylated to labeled DGDG by action of galactolipid:galactolipid galactosyltransferase.

Galactolipid Synthesis in 16:3 and 18:3 Plants

We found no typical differences between 16:3 and 18:3 plants in the synthesis of galactolipids from labeled UDP-Gal: the same enzymatic activities seem to be involved and the effect of thermolysin treatment is quite similar. This is in agreement with data from Heinz and Roughan (18), who reported equal rates of UDP-Gal-derived MGDG synthesis in chloroplasts from 16:3 and 18:3 plants. The same authors have reported that chloroplasts from 16:3 plants efficiently produce MGDG from [^{14}C]acetate, whereas chloroplasts from 18:3 plants are greatly reduced in such MGDG production. Since in the latter case deficient MGDG synthesis is a consequence of deficient formation of prokaryotic diacylglycerol (18), the same plastid galactosyltransferases nevertheless can be active in 16:3 and 18:3 plants, despite the lack of generation of prokaryotic diacylglycerol in the 18:3 plants.

UDP-Gal:diacylglycerol galactosyltransferase has been localized in the inner envelope membrane of spinach chloroplasts (2, 12), but in the outer envelope membrane of pea chloroplasts (4). Our results indicate that in either plant species activity of this enzyme is not affected by thermolysin treatment, in contrast to the DGDG synthase (Table IV). Consequently, the equal effect of thermolysin on galactolipid synthesis in spinach and pea chloroplasts (Fig. 1) suggests a similar regulation of MGDG, DGDG, and TGDG formation in these 16:3 and 18:3 plants, despite the reported difference in localization of MGDG-producing galactosyltransferase.

Both 16:3 and 18:3 plants share the eukaryotic pathway of galactolipid synthesis, as is inferred from the presence of eukaryotic galactolipids in either type of plants (3, 14, 20, 23). The fatty acid composition of galactolipids in the studied 16:3 plants suggest that the proportion of eukaryotic DGDG is higher than that of eukaryotic MGDG (Table II) (2, 23). It is tempting to conclude that it is the peripheral location of galactolipid:galactolipid galactosyltransferase in the outer envelope membrane of plastids, which determines the preferential synthesis of eukaryotic DGDG.

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LITERATURE CITED

1. **Alban C, Joyard J, Douce R** (1988) Preparation and characterization of envelope membranes from nongreen plastids. Plant Physiol **88:** 709–717
2. **Block MA, Dorne A-J, Joyard J, Douce R** (1983) Preparation and characterization of membrane fractions enriched in outer and inner envelope membranes from spinach chloroplasts. J Biol Chem **258:** 13273–13286
3. **Browse J, Warwick N, Somerville CR, Slack CR** (1986) Fluxes through the prokaryotic and eukaryotic pathways of lipid synthesis in the '16:3' plant *Arabidopsis thaliana.* Biochem J **235:** 25–31
4. **Cline K, Keegstra K** (1983) Galactosyltransferases involved in galactolipid biosynthesis are located in the outer membrane of pea chloroplast envelopes. Plant Physiol **71:** 366–372
5. **Cline K, Werner-Washburne M, Andrews J, Keegstra K** (1984) Thermolysin is a suitable protease for probing the surface of intact pea chloroplasts. Plant Physiol **75:** 675–678
6. **Dorne A-J, Block MA, Joyard J, Douce R** (1982) The galactolipid:galactolipid galactosyltransferase is located on the outer surface of the outer membrane of the chloroplast envelope. FEBS Lett **145:** 30–34
7. **Douce R, Joyard J** (1980) Plant galactolipids. *In* PK Stumpf, EE Conn, eds, The Biochemistry of Plants. A Comprehensive Treatise, Vol 4. Academic Press, New York, pp 321–362
8. **Ferrari RA, Benson AA** (1961) The path of carbon in photosynthesis of the lipids. Arch Biochem Biophys **93:** 185–192
9. **Frentzen M, Heinz E, McKeon TA, Stumpf PK** (1983) Specificities and selectivities of glycerol 3-phosphate acyltransferase and monoacylglycerol 3-phosphate acyltransferase from pea and spinach chloroplasts. Eur J Biochem **129:** 629–636
10. **Gardiner SE, Roughan PG, Browse J** (1984) Glycerolipid labelling kinetics in isolated intact chloroplasts. Biochem J **224:** 637–643
11. **Heemskerk JWM, Bögemann G, Helsper JPFG, Wintermans**

JFGM (1988) Synthesis of mono- and digalactosyldiacylglycerol in isolated spinach chloroplasts. Plant Physiol **86:** 971–977

12. **Heemskerk JWM, Jacobs FHH, Scheijen MAM, Helsper JPFG, Wintermans JFGM** (1987) Characterization of galactosyltransferases in spinach chloroplast envelopes. Biochim Biophys Acta **918:** 189–203
13. **Heemskerk JWM, Jacobs HH, Wintermans JFGM** (1988) UDPGalactose-independent synthesis of monogalactosyldiacylglycerol. An enzymatic activity of the spinach chloroplast envelope. Biochim Biophys Acta **961:** 38–47
14. **Heemskerk JWM, Wintermans JFGM** (1987) Role of the chloroplast in the leaf acyl-lipid synthesis. Physiol Plant **70:** 558–568
15. **Heemskerk JWM, Wintermans JFGM, Joyard J, Block MA, Dorne A-J, Douce R** (1986) Localization of galactolipid:galactolipid galactosyltransferase and acyltransferase in outer envelope membrane of spinach chloroplasts. Biochim Biophys Acta **877:** 281–289
16. **Heinz E** (1977) Enzymatic reactions in galactolipid biosynthesis *In* M Tevini, HK Lichtenthaler, eds, Lipids and Lipid Polymers in Higher Plants, Springer-Verlag, Berlin, pp 102–120
17. **Heinz E, Roughan PG** (1982) De novo synthesis, desaturation and acquisition of monogalactosyl diacylglycerol by chloroplasts from "16:3"- and "18:3"-plants *In* JFGM Wintermans, PJC Kuiper, eds, Biochemistry and Metabolism of Plant Lipids. Elsevier, Amsterdam, pp 169–182
18. **Heinz E, Roughan PG** (1983) Similarities and differences in lipid metabolism of chloroplasts isolated from 18:3 and 16:3 plants. Plant Physiol **72:** 273–279
19. **Heinz E, Schmidt H, Hoch M, Jung KH, Binder H, Schmidt RR** (1989) Synthesis of different nucleoside 5′-diphospho-sulfoquinovoses and their use for studies on sulfolipid biosynthesis in chloroplasts. Eur J Biochem **184:** 445–453
20. **Kunst L, Browse J, Somerville C** (1988) Altered regulation of lipid biosynthesis in a mutant of *Arabidopsis* deficient in chloroplast glycerol-3-phosphate acyltransferase activity. Proc Natl Acad Sci USA **85:** 4143–4147
21. **Ledvogel B, Kleinig H** (1979) Galactolipid synthesis in chromoplasts in vitro. Planta **144:** 467–471
22. **Liedvogel B, Sitte P, Falk H** (1976) Chromoplasts in the daffodil: fine structure and chemistry. Cytobiologie **12:** 155–174
23. **Norman HA, St John JB** (1986) Metabolism of unsaturated monogalactosyldiacylglycerol molecular species in *Arabidopsis thaliana* reveals different sites and substrates for linolenic synthesis. Plant Physiol **81:** 731–736
24. **Roughan PG, Slack CR** (1982) Cellular organization of glycerolipid metabolism. Annu Rev Plant Physiol **33:** 97–132
25. **Siebertz HP, Heinz E, Joyard J, Douce R** (1980) Labelling *in vivo and in vitro* of molecular species of lipids from chloroplast envelopes and thylakoids. Eur J Biochem **108:** 177–185
26. **Siebertz M, Heinz E** (1977) Galactosylation of different monogalactosyldiacylglycerols by cell-free preparations from pea leaves. Hoppe Seyler's Z Physiol Chem **358:** 27–34
27. **Van Besouw A, Wintermans JFGM** (1978) Galactolipid formation in chloroplast envelopes. I. Evidence for two mechanisms in galactosylation. Biochim Biophys Acta **529:** 44–53
28. **Wegmann B, Schmidt RR** (1987) The application of the trichloroacetimidate method to the synthesis of α-D-gluco and α-D-galactopyranosides. J Carbohydr Chem **6:** 357–375
29. **Williams JP, Watson GR, Khan MU, Leung S** (1975) Galactolipid synthesis in *Vicia faba* leaves. Plant Physiol **55:** 1038–1042
30. **Williams JP, Khan MU, Mitchell K** (1988) The molecular species of diacylglycerol precursors used in monogalactosyldiacylglycerol biosynthesis in the leaves of a number of higher plant species. Plant Cell Physiol **29:** 849–854