Formation of Acylmonogalactosyldiglyceride and Degradation of Polar Lipids in Leaf Homogenates of Tobacco

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Acylmonogalactosyldiglyceride (acyl-MGDG) was isolated from leaf homogenates of tobacco, and its structure was determined to be 1,2-di-*O*-acyl-3-*O*-(6-*O*-acyl- β -D-galactopyranosyl)-sn-glycerol by decomposition analyses, PMR, CMR and GC-MS. This compound had an identical structure with that found in disrupted spinach leaves, except for a significant difference in fatty acid composition. This compound was detected in disrupted leaves of tobacco but was not found in normal green, senescent and cured leaves. Monogalactosyldiglyceride and digalactosyldiglyceride decreased abruptly during incubation of leaf homogenates, which was accompanied by the rapid formation of acyl-MGDG.

During senescence¹⁾ or curing,^{2,3)} the lipid components of tobacco leaves are drastically broken down, and some of the degradation products have been known to contribute to the aroma and taste of tobacco.⁴⁾ Since post-harvest treatment of tobacco leaves includes laborious and energy wasting processes, methods for a direct use of the homogenates of the harvested leaves as tobacco material has been sought in many countries.^{5,6)} Successive incubation of the homogenates has been recognized to be a very important process for producing aroma and taste. Similar to conventional curing, homogenization and incubation of green tobacco leaves also served to degrade the lipid components. One purpose of this report is to show the mode of degradation of the lipid components during incubation of the homogenates of green tobacco leaves. In

the course of the above study, it was found that a lipid compound, which has never been detected in either green or cured leaves, increased to a significant amount during the incubation process.

Heinz⁷⁾ detected a lipid compound which was formed during the homogenization of spinach leaves, and identified it to be a species of acyl-MGDG. From the similarity of its chromatographic behavior, we suspected that the lipid compound, which only appeared during incubation of tobacco homogenates, was a similar compound to the acyl-MGDG detected in homogenized spinach leaves. This paper deals with the isolation, identification and mode of formation of the compound produced in homogenized tobacco leaves.

MATERIALS AND METHODS

Abbreviations: acyl-MGDG, acylmonogalactosyldiglyceride; MGDG, monogalactosyldiglyceride; DGDG, digalactosyldiglyceride; SQDG, sulfoquinovosyldiglyceride; PG, phosphatidylglycerol; PC, phosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; TLC, thin layer chromatography; HPLC, high performance liquid chromatography; GLC, gas liquid chromatography.

Materials and detection of unknown lipid A. Tobacco plants (*Nicotiana Tabacum* L. cv. Bright Yellow) were grown in a green house. Normal green leaves of the middle stalk position and senescent and flue-cured leaves of the lower stalk position were frozen in liquid nitrogen and lyophilized. The normal green leaves were homogenized for 3 min with a Waring Blender, kept for 3 hr at 40°C and lyophilized. The lipids were extracted from these dried

samples by the procedure of Folch *et al.*⁸⁾ and were chromatographed on Silicagel G (Merck) TLC with a solvent system of chloroform-methanol-acetone-ammonia (50:5:50:0.5, v/v). Only in the homogenized leaves was an unknown lipid A (U-A) detected. This was less polar than MGDG and gave a Rhodamine 6G and anthrone positive single spot (*R_f* 0.5) under the above conditions.

Isolation of U-A. Two kg of tobacco green leaves were homogenized with 3 liters of water. The homogenates were kept for 3 hr at 40°C and then lyophilized. The dried sample was extracted twice with 6 liters of chloroform-methanol (2:1, v/v) and washed twice with 5% NaCl. After mixing and washing the extracts, the chloroform layer was evaporated *in vacuo* at 35°C. Separation of the crude lipids (22 g) was performed with silicic acid (500 g, Wakogel C-200) column chromatography by the method of Ohmori and Yamada⁹⁾ with a slight modification. β -Carotene and chlorophyll were successively eluted with chloroform and 5% acetone in chloroform (v/v). The U-A fraction was eluted with 3 liters of 20% acetone in chloroform (v/v). The eluate was immediately dried *in vacuo* at 35°C, yielding 3.6 g of yellowish syrup. This was redissolved in chloroform and the solution was further chromatographed on a silicagel column (100 g, Wakogel C-300) with 10% acetone in chloroform (v/v). The anthrone positive fractions were concentrated and further purified with HPLC (3 ml/min flow of chloroform, Shodex GPC H202 column, RI detector). The final purification was performed by preparative TLC on Silicagel G with a solvent system of chloroform-methanol-acetone-ammonia (50:5:50:0.5, v/v). In this way 320 mg of pure substance was recovered. MGDG and DGDG were purified from nonhomogenized leaves of tobacco by silicic acid chromatography with a solvent series of chloroform-acetone, and by preparative TLC on Silicagel G with chloroform-methanol-water (65:25:4, v/v). Hydrogenation was performed over Pd/C in ethyl acetate.

Hydrolysis and methanolysis of U-A. U-A was partially hydrolyzed with 0.4% KOH in ethanol at 60°C for 15 min and the hydrolyzate was neutralized with 0.2 N H₂SO₄ and passed through Dowex 50 (H⁺). The effluent was purified by Silicagel G TLC with *n*-butanol-acetic acid-water (5:4:1, v/v) and partially acetylated with acetic anhydride in pyridine at room temperature. The reaction mixture was subjected to GLC. An acid hydrolysis was performed with 2 N HCl at 110°C for 3 hr. The hexane-soluble fraction from the hydrolyzate was methanolized with 5% sulfuric acid in methanol (v/v) at 40°C for 16 hr and the methyl ester solution was subjected to GLC. The water-soluble fraction was condensed *in vacuo*. The condensate was analyzed by Silicagel G TLC with *n*-butanol-acetic acid-water (5:4:1, v/v), and partially trimethylsilylated with hexamethyldisilazane and trimethylchlorosilane in pyridine. The reaction mixture was subjected to GLC.

GLC. The compounds in the alkaline hydrolyzate of U-A were analyzed as acetyl derivatives at 200~250°C on a 3 mm $\times$ 2 m glass column packed with 1% OV 101 on Chromosorb W. The sugar moiety was analyzed as a trimethylsilyl ether derivative at 170°C on the same OV 101 column. The fatty acid methyl esters were chromatographed at 200°C on a 3 mm $\times$ 2 m glass column packed with 5% 1,4-butanediol glycol succinate polyester on Chromosorb W. Peaks in the chart were identified by comparing their retention times with those of the standard specimens and the content was determined by area measurement, employing a Hewlett-Packard integrator with methyl arachidate as an internal standard.

Molar ratio. Galactose in the U-A was determined by the anthrone method.¹⁰⁾ The fatty acid ester was determined by GLC with methyl arachidate as an internal standard. Glycerol was determined by the periodate oxidation-chromotropic acid technique.¹¹⁾

Spectroscopy. The spectra were recorded on the following spectrometers: IR, Jasco IR-G; mass, Hitachi M-80; PMR, JM-PS-100; CMR, JEOL FX 100.

Polar lipid separation and analysis. The lipids were extracted from 1 g of lyophilized leaf homogenates, which had been kept for 0~24 hr at 40°C, with chloroform-methanol (1:2, v/v) according to Allen and Good.¹²⁾ The U-A was separated by one dimensional TLC with a solvent system of chloroform-methanol-acetone-ammonia (50:5:50:0.5, v/v). Other polar lipids were separated by two dimensional TLC, developed with chloroform-methanol-water (65:25:4, v/v) in the first direction and with chloroform-methanol-isopropylamine-ammonia (65:35:0.5:5, v/v) in the second direction.¹²⁾ Each lipid area was scraped off, methanolized and determined as previously described.²⁾

RESULTS

Identification of acyl-MGDG from tobacco leaf homogenates

It has been known that disrupted spinach leaves accumulated acyl-MGDG in accordance with decreasing MGDG.⁷⁾ The physical and chemical properties of the spinach acyl-MGDG were examined by Heinz and his collaborators.^{7,13)} From the similarities of chromatographic behavior, tobacco acyl-MGDG was suspected to have a similar structure to that of spinach. To identify the structure of tobacco acyl-MGDG, the physical and chemical properties were compared with those of spinach acyl-MGDG. Very similar infrared

FIG. 1. Infrared Spectra of MGDG, DGDG and U-A from Tobacco.

spectra were shown between tobacco (Fig. 1) and spinach acyl-MGDG,⁷⁾ showing a strong absorption at 1730 cm^{-1} due to the ester bonds, at 2800 cm^{-1} due to the methyl and methylene groups of fatty acids and at 3500 cm^{-1} due to the OH groups of the sugar moiety. The finger print region of U-A was almost identical with that of spinach acyl-MGDG. These results indicate that U-A has a very similar structure to spinach acyl-MGDG. Acid hydrolysis of U-A gave fatty acids, glycerol and galactose. Deacylation by mild alkaline hydrolysis of U-A gave 3-O- β -D-galactosyl-*sn*-glycerol, which was identified by GLC co-chromatographed with the authentic substance, and GC-MS as the acetate derivative. The molar ratio of glycerol-galactose-fatty acid esters was 1:1:3. These results almost corresponded with those of spinach.⁷⁾ The fatty acid composition of U-A before hydrogenation was (% values in weight): 16:0, 8; 16:3, 8; 18:0, 2; 18:1, 1; 18:2, 5; 18:3, 76 and after hydrogenation, 16:0, 25; 18:0, 75. The percentage of hexadecatrienoic acid (16:3) in U-A was a little lower than that (11%) in MGDG from tobacco.

FIG. 2. PMR Spectrum of Hydrogenated U-A in Pyridine.
TMS=0.

The PMR spectrum of hydrogenated U-A (Fig. 2) showed an identical profile with hydrogenated 6-*O*-acyl- β -D-galactosyl diglyceride in the range from 4.0 to 6.0 ppm, where methine and methylene protons of the sugar and glycerol moieties were assigned.¹³⁾ The overall profiles of the PMR spectra of the acetyl derivatives of hydrogenated tobacco U-A and MGDG were also identical with those of spinach MGDG and acyl-MGDG,¹³⁾ respectively. In each case, 4 acetoxy signals (1.96, 2.03, 2.04 and 2.14 ppm) were shown in MGDG, whereas 3 signals (1.96, 2.04 and 2.14 ppm) were shown in either spinach acyl-MGDG or U-A. These results indicated that acylation occurred in the 6-*O*-position of galactose. The mass spectrum of the acetyl derivatives of hydrogenated U-A contained the same fragment ions as those of the model compound acyl-MGDG (18:0/18:0/18:0 species),¹⁴⁾ showing the presence of ions at m/z 1,118 by the loss of acetic acid and at m/z 896 and m/z 608 due to the galactosyldiglyceride and diglyceride portions of the parent molecules, respectively. But in the case of U-A, the fragment ions derived from the 18:0/18:0/16:0 species were also observed. The ions at m/z 555 and 527 showed the presence of one

stearoyl or palmitoyl group at the terminal of galactose. These results further confirmed that tobacco U-A was acyl-MGDG. The basic structure of 3-*O*- β -D-galactosyl-*sn*-glycerol and the three acylated positions were the same as those of spinach acyl-MGDG.

The CMR spectrum of tobacco acyl-MGDG is shown in Fig. 3. The differences between the acyl-MGDG and MGDG spectra were found in the range around 170 ppm where the carbonyl groups of ester linkages are assigned, *i.e.* acyl-MGDG showed 3 signals (173.14, 173.49, 173.61) and MGDG showed 2 signals (173.38, 173.73). The present signals from tobacco MGDG almost correspond with the signals of MGDG obtained by Johns *et al.*¹⁵⁾ Five signals (127.16, 127.80, 128.21, 130.14, 131.90) could be assigned to double bonds of the linolenate, and resonances of methyls and methylenes of fatty acid were observed between 14~34 ppm. Seven signals (62.72, 62.95, 68.10, 68.69, 70.15, 72.76, 73.44) and a signal at 103.88 ppm, which corresponded to a chemical shift of the C-1 carbon of methyl- β -D-galactopyranoside,¹⁶⁾ could be assigned to the carbons of galactose and glycerol, respectively. In spite of the same basic structure, the fatty acid residues of tobacco

FIG. 3. CMR Spectrum of Acyl-MGDG from Tobacco in CDCl_3 .
TMS=0.

acyl-MGDG showed a different composition from those of spinach acyl-MGDG.⁷⁾ Although the fatty acid composition changed during incubation, the species gap exceeded the extent of the changes. In general, tobacco acyl-MGDG contained more 16:0 and less 16:3 than spinach acyl-MGDG.

Changes in acyl-MGDG and other polar lipids during incubation of leaf homogenates

Changes in the polar lipids and free fatty acid pool in the homogenized leaves are shown in Fig. 4. Acyl-MGDG, which was not found in nonhomogenized leaves, accumulated dramatically during incubation of the leaf homogenates at 40°C, and reached 80% of the

initial level of MGDG in 3 hr. This accumulation was accompanied by a rapid decrease of MGDG and DGDG, which were the most abundant glycolipids in normal leaves before homogenization. The other glycolipid SQDG did not decrease so dramatically as MGDG and DGDG. Phospholipids such as PC, PG and PE, which were minor polar lipids, also decreased considerably. The free fatty acid pool increased at the very early stage of incubation, then remained at a constant level and again increased during the latter stages of incubation. Changes in the fatty acid composition of acyl-MGDG, MGDG and DGDG are shown in Table I. Acyl-MGDG contained hexadecatrienoic acid, which was characteris-

FIG. 4. Changes in the Compositions of Polar Lipids and Free Fatty Acids in the Leaf Homogenates of Tobacco.

(a): ○—○, MGDG; ●—●, DGDG; △—△, SQDG; ▲—▲, PG; ○---○, acyl-MGDG.

(b): ○—○, PI; △—△, PE; ●—●, PC; ▲—▲, free fatty acid pool.

The leaf homogenates of tobacco were incubated at 40°C, and the samples were withdrawn at designated periods during incubation.

TABLE I. CHANGES IN THE FATTY ACID COMPOSITION OF ACYL-MGDG, MGDG AND DGDG IN THE LEAF HOMOGENATES OF TOBACCO

Lipid	Fatty acid	Percentage of the total fatty acids (w/w)							
		Incubation time (hr)							
		0	0.25	0.5	1.0	2.0	3.0	5.0	24.0
Acyl-MGDG	16:0	—	15	17	11	9	9	11	10
	16:1	—	tr	tr	1	1	1	tr	tr
	16:3	—	5	6	8	7	8	8	8
	18:0	—	tr	3	2	2	2	2	2
	18:1	—	tr	2	2	1	1	1	1
	18:2	—	6	5	5	6	5	6	6
MGDG	18:3	—	73	67	71	74	74	72	73
	16:0	3	3	4	5	6	9	12	28
	16:1	1	tr	tr	tr	tr	tr	tr	tr
	16:3	11	12	11	11	11	9	7	4
	18:0	tr	tr	tr	tr	1	3	3	6
	18:1	tr	tr	tr	tr	tr	3	3	6
DGDG	18:2	4	6	6	6	6	6	6	6
	18:3	81	79	79	78	76	70	69	50
	16:0	15	18	18	19	20	21	20	35
	18:0	3	3	3	3	3	5	4	14
	18:1	2	3	3	2	3	3	2	10
	18:2	5	6	5	6	6	6	6	tr
	18:3	75	70	71	70	68	65	68	41

tic of MGDG, by 5~8% throughout the period. This indicates that MGDG might be a good acceptor of fatty acids and be transformed to acyl-MGDG by transacylation. Comparing the ratio of 16:3 to 16:0 between the MGDG at the initial time and the acyl-MGDG formed after 3 hr incubation, the latter had a lower ratio than the former. The proportion of 18:3 decreased from 81 to 50% in MGDG and 75 to 41% in DGDG, but this decrease was not observed in acyl-MGDG.

DISCUSSION

Acyl-MGDG was isolated from the leaf homogenates of tobacco in the present experiment. Its structure was determined to be 1,2-di-*O*-acyl-3-*O*-(6-*O*-acyl- β -D-galactopyranosyl)-*sn*-glycerol, which was coincident with that isolated from spinach leaf homogenates by Heinz,⁷⁾ and Heinz and Tulloch.¹³⁾ They demonstrated the structure by PMR and detected that only position 6 of the galactose moiety in MGDG was acylated. We ascer-

tained that acyl-MGDG from the leaf homogenates of tobacco constituted only 6-*O*-acyl galactose moiety by comparing the physical data of PMR with that of spinach acyl-MGDG.¹³⁾ However, the fatty acid composition of acyl-MGDG differed significantly between tobacco and spinach. Acyl-MGDG from tobacco had a lower and higher proportion of 16:3 and 16:0, respectively, compared with that from spinach.⁷⁾ In spinach leaves the acyl-MGDG accounted for 20% of the leaf lipids after 3 hr of incubation at 4°C.¹⁷⁾ Contrary to that in tobacco leaves the acyl-MGDG formation was little at 4°C, although it was very actively produced at 40°C. From the results, we suspected that the acylation enzyme activity is not as strong in tobacco as it is in spinach. Acyl-MGDG from tobacco was formed in disrupted leaves and not found in normal green, senescent and cured leaves. This suggests that the acyl-MGDG-forming enzyme may be compartmentalized within healthy cells. Recent results from Heinz *et al.*¹⁸⁾ showed that this enzyme was contained in

chloroplast envelope membranes. In isolated chloroplasts from tobacco leaves, the acyl-MGDG formation was demonstrated by incubating them at 40°C under either the native condition, or under the disrupted condition by ultrasonic treatment (unpublished data). This shows that the activity to change MGDG to acyl-MGDG might be located in the chloroplasts. However, the amount of acyl-MGDG from the chloroplast fraction seemed to be smaller compared with that formed in the leaf homogenates. The reason why the leaf homogenates produced more acyl-MGDG may be that the acylation ability appeared more strong under acidic condition because the leaf homogenates had a lower pH value than the chloroplast fraction.¹⁸⁾ Comparing the fatty acid composition between acyl-MGDG and MGDG, the former contained a lower ratio of hexadecatrienoate and higher ratio of palmitate. DGDG had a rather higher ratio of palmitate than acyl-MGDG and MGDG. The amount of acyl-MGDG reached 80% of that of the initial MGDG after 3 hr of incubation. These suggest that the fatty acid moiety of acyl-MGDG, which increased during incubation of the leaf homogenates, was transferred from the polar lipids other than MGDG, possibly from DGDG. The characterization and localization of the enzyme concerned with the acylation of MGDG should be further studied.

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