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LIPID SYNTHESIS IN GERMINATING SAFFLOWER SEEDS AND PROTOPLASTS*

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Key Word Index—*Carthamus tinctorius*; Compositae; safflower; lipid biosynthesis; fatty acid composition; galactolipids; phospholipids.

Abstract—Galactolipids and phospholipids rapidly accumulated in a whole seed between 2 and 4 days after germination. However, the rate of incorporation of [^{14}C]acetate into galactolipids was very low. The predominant fatty acid of galactolipids was linolenic acid, while those of phospholipids were linoleic and palmitic acids. Fatty acids of monogalactosyldiacylglycerol in germinating safflower seeds were randomly distributed between the 1- and 2-positions of the glycerol molecule and the distribution in digalactosyldiacylglycerol was slightly non-random, while fatty acids of galactolipids in mature safflower leaves were non-randomly distributed. Triacylglycerol was synthesized in the cotyledon tissue of the germinating seeds simultaneously with its rapid degradation. In addition, lipid biosynthesis in protoplasts is described.

INTRODUCTION

In recent years, biosynthesis of fatty acids [1,2], phospholipids [3,4] and galactolipids [4–10] in plants has been investigated in detail. Lipid metabolism in germinating seeds has been studied by many workers [11–15]. However, we believe that lipid metabolism in developing and germinating seeds should be investigated more quantitatively and systematically. Lipid synthesis by cell homogenates or subcellular fractions of plant tissues often differs from that by intact tissues which are not suitable for quantitative analysis. Plant protoplasts can be obtained more easily [16] than cell suspension cultures, which have been used to study fatty acid biosynthesis or biological membranes [17–20]. We have previously investigated lipid biosynthesis in developing seeds of safflower, which is one of the most important economic oil plants [21]. The purpose of the work reported here has been to obtain basic data on lipid synthesis in germinating seeds and protoplasts of safflower.

RESULTS AND DISCUSSION

Changes in lipid content and fatty acid composition during germination

The dry wt (without seed coat) of seeds slightly changed during germination (ungerminated seed, 16.5 mg/seed; 2 days after germination, 17.3; 4 days, 18.3; 6 days, 15.7; 8 days, 15.5), while the fr. wt markedly increased (17.5 mg/seed, 30.1, 76.1, 127.5 and 213.9, respectively). The triacylglycerol (TG) content remained unchanged during the initial 2 days of germination (*ca* 13 μmol/seed); thereafter it decreased rapidly and linearly to 1 μmol/seed or less until 6 days after germination (Fig. 1). Galactolipids were very minor components in the ungerminated seeds and the 2-day-old seedlings, and their accumulation was observed 3 days after germination (Fig. 1). Monogalactosyldiacylglycerol (MGDG) increased more rapidly than digalactosyldiacylglycerol (DGDG), until the content reached a constant value 6 days after germination. The DGDG content continued to increase as far as it was examined, and approached the MGDG content. After the rapid accumulation of galactolipids, linolenic acid became predominant in these lipids (Fig. 2). The total phospholipid content did not change significantly for the first 2 days, and then increased most rapidly to a maximum (4 days after germination when cotyledons became green); thereafter it decreased slowly to 8 days after germination. A similar decrease in phospholipids was observed by Zimmerman *et al.* [11] in flax seed that was germinated in the dark. Phosphatidylglycerol (PG) began to be synthesized 2.5 days after germination, and it continued to increase until 8 days after germination, in contrast to phosphatidylcholine (PC) and phosphatidylethanolamine (PE). The phosphatidylinositol (PI) content did not change during germination. Linoleic acid was the major fatty acid in PC, PE and PI during the whole period of germination, but it was not dominant in these phospholipids from mature leaves (Fig. 2). This difference in linoleic acid content, a rapid increase of the absolute amount of this fatty acid in phospholipids, and its lowest specific radioactivity in incorporation of [U-^{14}C]acetate (Table 4), suggest that most of the linoleic acid in the phospholipids accumulating during germination was not synthesized *de novo*, but was derived from storage lipids. Hexadecenoic acid, one of the major fatty acids of PG in the mature leaves (Fig. 2), was not detected during germination. It was observed in many plant species that DGDG was richer in saturated fatty acids than MGDG [22]. The two galactolipids of safflower also exhibited a difference in the fatty acid composition (Fig. 2).

* Part 2 in the series "Lipid Metabolism in Safflower". For Part 1 see ref. [21].

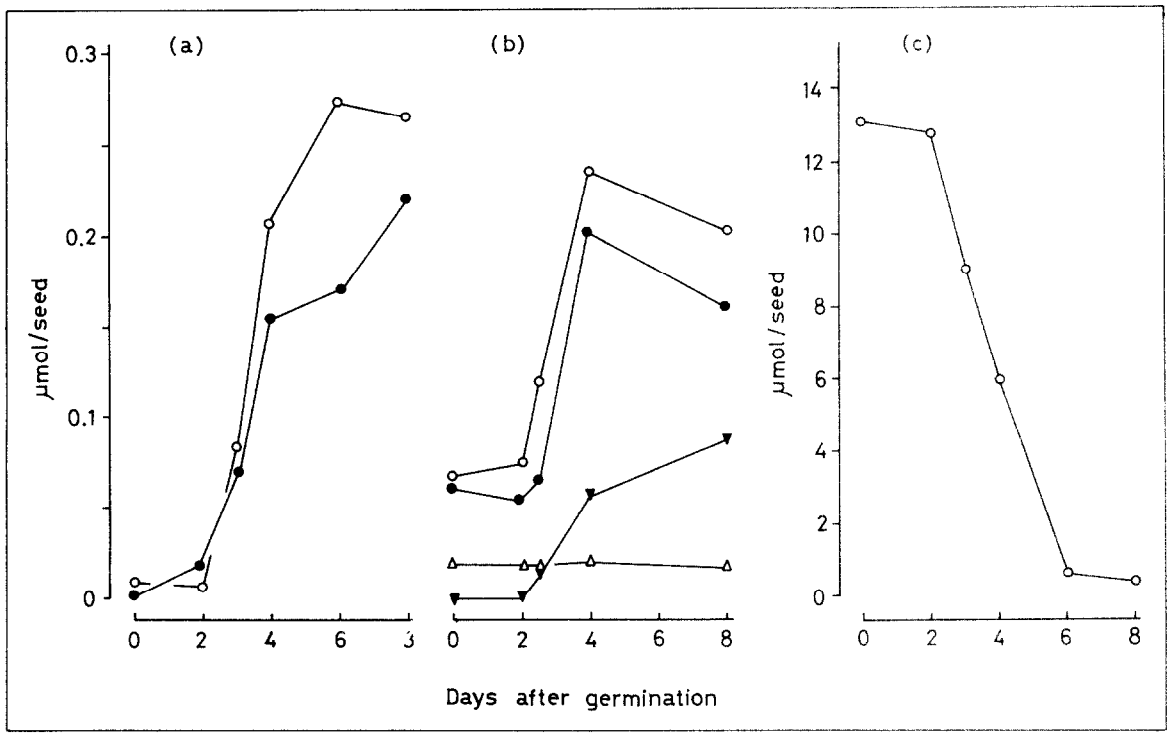

Fig. 1. Changes in the lipid class contents of safflower seeds during germination. (a) Galactolipids: ○——○, MGDG; ●——●, DGDG. (b) Phospholipids: ○——○, PC; ●——●, PE; △——△, PI; ▼——▼, PG. (c) TG.

Fatty acid distribution and molecular classes of galactolipids

Fatty acids of MGDG in the 4-day-old and 8-day-old seedlings were randomly distributed between the 1- and 2-positions of the glycerol molecule, while the fatty acid distribution of DGDG was slightly non-random (Table 1). These distribution patterns differed from those of galactolipids in the mature leaves, in which the distribution was distinctly non-random. Fatty acids of phospholipids were non-randomly distributed at every stage of growth (K. Ichihara and M. Noda, unpublished data). The random distribution of fatty acids in galactolipids of the seedlings may be due to a lack of substrate-specificity of enzymes that participate in galactolipid synthesis. It is interesting that linolenic acid of DGDG from the seedlings was preferentially esterified at the 1-position in spite of its high degree of unsaturation (Table 1). In *Chlorella* and *Anabaena*, linolenic acid of MGDG was preferentially located at the 1-position [23]. The general rule of fatty acid distribution appears not to be applied to linolenic acid.

Although the separation of neutral lipid classes into fractions of differing numbers of double bonds is easily achieved by argentation chromatography [24], the equivalent resolution of more polar lipids is generally difficult. Arvidson [25] reported the fractionation of animal lecithins into molecular classes by argentation TLC. Harwood [26] has successfully separated intact phosphatidylcholine and phosphatidylethanolamine of soya bean into fractions, and the fractionation of sulpholipid has been achieved by Heinz *et al.* [27]. Other authors [28, 29] reported fractionation of these lipids after removal or modification of the more polar groups. Nichols *et al.* [30] demonstrated that good separation of MGDG was obtained by TLC on a $AgNO_3$–Si gel plate without modification. Heinz *et al.* [7] reported the fractionation of DGDG. In the present work, DGDG could be separated directly into several fractions according to degree of unsaturation by TLC on $AgNO_3$–Si gel H using a solvent mixture saturated with $AgNO_3$ (Table 2). The structures of the individual molecular species of MGDG and DGDG were not determined completely in the present work. Although it is now under investigation, we can cite one of the results. The DGDG class with five double bonds, which was obtained from the 4-day-old seedlings, was composed of 55.6% 1-linoleoyl-2-linolenoyl and 44.4% 1-linolenoyl-2-linoleoyl species (K. Ichihara and M. Noda, unpublished data). This suggests that the fatty acid distribution is considerably randomized at the level of molecular species.

Incorporation of [U-^{14}C]acetate into lipid classes and individual fatty acids by cotyledons

When oil-rich seeds germinate, there is a rapid disappearance of TG. Zimmerman *et al.* [11] emphasized that TG might be catabolized in one tissue of flax seedlings and resynthesized in another tissue simultaneously. While soya bean cotyledons incorporated little [U-^{14}C]acetate into TG, its axis could do so readily [15]. Safflower cotyledons rapidly catabolized and synthesized TG simultaneously (Table 3). Lipase activity rises sharply in the early stages of seed germination [31, 32]. On the other hand, it is known that lipase catalyses not only hydrolysis of acylglycerols but also synthesis *in vitro* [33]. However, it is not clear whether or not lipase participates in TG synthesis in plant seedlings.

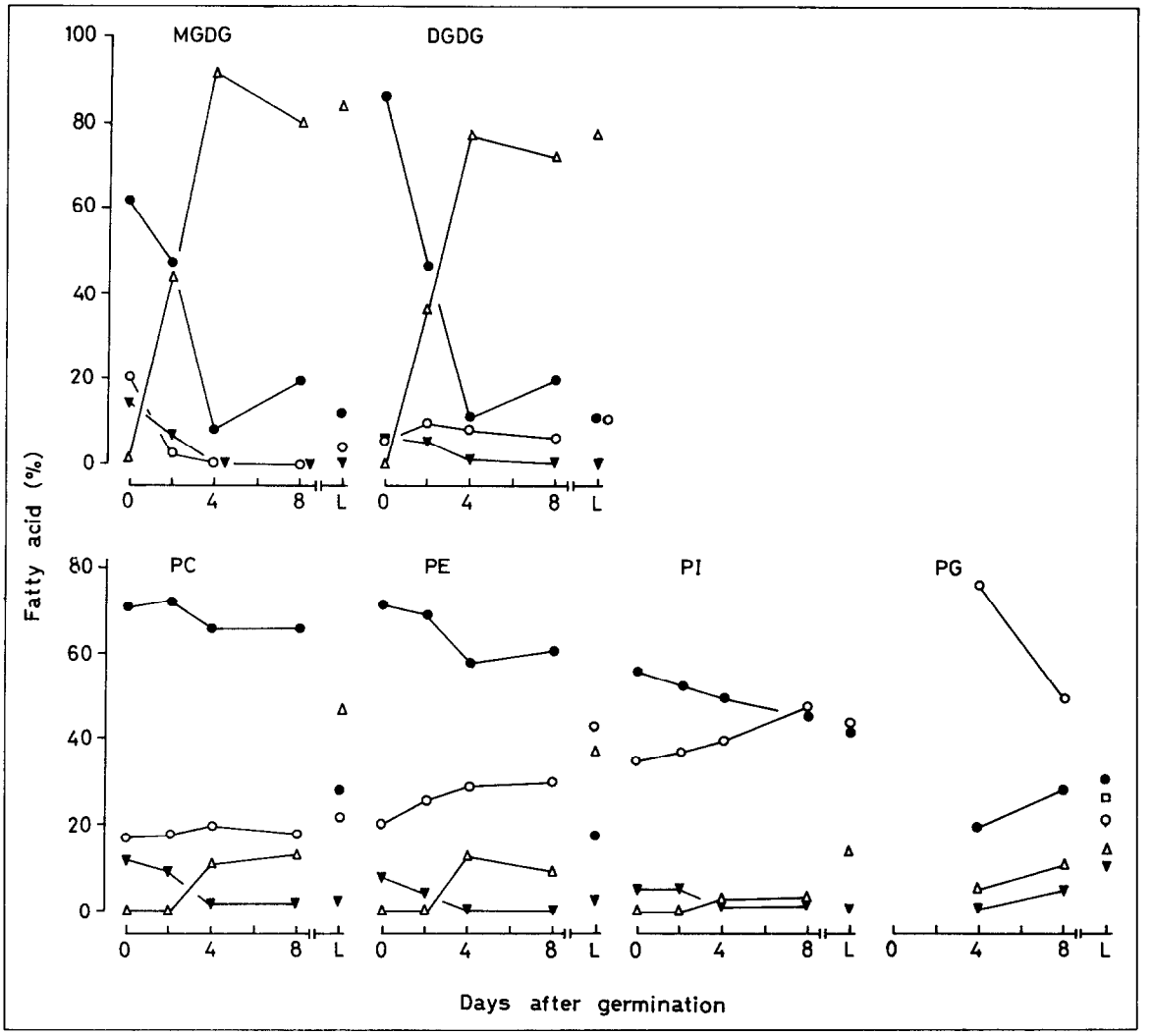

Fig. 2. Changes in the fatty acid compositions of galactolipid and phospholipid classes during germination. ○——○, Palmitic acid; ▼——▼, oleic acid; ●——●, linoleic acid; △——△, linolenic acid. L, Leaves of 210-day-old safflower. Stearic acid occurred in most of these fractions but was less than 3% of the total, except for the PG fraction from the 8-day-old seedlings, in which the stearic acid content was 7.6%. Hexadecenoic acid (□) was detected only in the PG fraction from the mature leaves and it was esterified at the 2-position of the glycerol molecule.

The proportion of [U-^{14}C]acetate incorporated into galactolipids was only 3–5% of the total (Table 3). As shown in Fig. 1, these lipids were accumulated rapidly in the seedlings. This apparent conflict may be explained, in part, by assuming the presence of a relatively large pool of precursor(s) of galactolipids. When pumpkin leaves were exposed to $^{14}CO_2$ or [^{14}C]acetate, the gross and specific radioactivities of galactolipids were lower than those of phospholipids [34]. This observation can be explained by a difference between the turnover rates of these lipids, because pumpkin leaves originally contain galactolipids. At the initial period of incubation after $^{14}CO_2$ feeding of broad bean, the radioactivity in galactolipids was very low, and then it increased slowly over 72 hr [35]. Immature maize lamina rapidly incorporated $^{14}CO_2$ and [^{14}C]acetate into galactolipids [36]. Thus, the rate of galactolipid synthesis differs with plant species, leaf age, substrates, etc. These variations are probably due to differences in the size of a pool of precursor(s) of galactolipids and their turnover rates in the plant tissues examined. The labelling pattern of phospholipids in germinating safflower seeds somewhat differed from those observed in germinating soya bean [37, 38]. PC had the highest radioactivity of the phospholipid classes, and PI had the lowest activity (Table 3). The ^{14}C distribution reflects the accumulation rates of the individual phospholipid classes, which are shown in Fig. 1.

When cotyledons were not treated in boiling water prior to lipid extraction, an unknown phospholipid was detected. This phospholipid became the major one when fresh seedlings were soaked in MeOH prior to homogenizing. The IR spectrum of the phospholipid lacked characteristic absorption bands of *N*-acyl PE. The ratio of P/glycerol/ester bond was 1:1:2.4. The R_f value of the unknown lipid on Si gel G TLC did not agree with that of phosphatidic acid ($CHCl_3$–

Table 1. Positional distribution of fatty acids in galactolipids

Source of galactolipids	Galacto-lipid class	Position	Fatty acid (%)				
			16:0	18:0	18:1	18:2	18:3
Seedling (4-day-old)	MGDG	1	0.0	0.0	0.0	7.3	92.7
		2	0.0	0.0	0.0	6.5	93.5
		Total	0.0	0.0	0.0	6.9	93.1
	DGDG	1	4.5	3.6	1.2	8.2	82.5
		2	9.9	1.4	0.0	10.4	78.3
		Total	7.2	2.5	0.6	9.3	80.4
Seedling (8-day-old)	MGDG	1	0.0	0.0	0.0	16.5	83.5
		2	0.0	0.0	0.0	16.5	83.5
		Total	0.0	0.0	0.0	16.5	83.5
	DGDG	1	5.8	1.5	0.0	14.6	78.2
		2	5.2	1.5	0.0	19.8	73.6
		Total	5.5	1.5	0.0	17.1	75.9
Leaf*	MGDG	1	6.1	0.0	0.0	11.4	82.4
		2	0.7	0.0	0.0	8.4	91.0
		Total	3.4	0.0	0.0	9.9	86.7
	DGDG	1	14.9	0.0	0.0	7.6	77.5
		2	4.5	0.0	0.0	11.2	84.3
		Total	9.7	0.0	0.0	9.4	80.9

* From 210-day-old safflower plants.

Table 2. Molecular classes of galactolipids

Age of seedling	Galactolipid class	Molecular class (%) (Number of double bond)				
		0–2	3	4	5	6
4-Day-old	MGDG	0.0	0.0	5.1	11.7	83.2
	DGDG	6.8	19.6		5.8	67.8
8-Day-old	MGDG	0.0	0.0	13.1	20.2	66.7
	DGDG	13.7	20.9		13.8	51.6

MeOH–Me_2CO–25% NH_3, 70:30:4:1.4). On the basis of these data, this phospholipid is probably phosphatidylmethanol (PM) formed by phospholipase D during extraction [39]. This artefact occurred in the experiments on incorporation of the labelled substrate because cotyledons and protoplasts were not treated in boiling water (Tables 3 and 5).

Mature safflower seeds contain a large amount of linoleic acid, but linolenic acid was not detected. The latter appear as the seeds germinate [40]. At the two different stages of germination, the specific radioactivity of linolenic acid was much higher than that of linoleic acid (Table 4). Cotyledons 5 days after germination primarily incorporated [U-^{14}C] acetate into saturated fatty acids, which were the predominant acids of PG in the seedlings and important components of the other phospholipids and neutral lipids.

Incorporation of [U-^{14}C] *acetate into lipids by protoplasts*

It was relatively difficult to isolate protoplasts from 3-day-old seedlings, while a large amount of protoplasts was obtained more easily from cotyledons 5 days after germination. The activity of lipid synthesis from [U-^{14}C] acetate differed much between the two protoplast preparations from 3-day-old and 5-day-old seedlings (Table 5). The high activity of protoplasts from 5-day-old seedlings suggests that the protoplasts suffered no mechanical damage. The striking difference between lipid synthesis of the cotyledonous tissue and protoplasts is the lower proportion of ^{14}C-labelled polyenoic fatty acids in the latter; only saturated and monoenoic acids were labelled in protoplasts. Stearns *et al.* [14] reported that trienoic fatty acids did not become significantly labelled in soya been suspension cultures until 4 hr of incubation and the radioactivity of these acids continued to rise from

Table 3. Incorporation of [U-^{14}C]acetate into lipids by cotyledons of germinating safflower seeds

Age of cotyledon	Total incorporation (nmol/seed)	Distribution of ^{14}C among lipid classes (%)												
		SE	TG	FA	ST	DG	MGDG	DGDG	SG	PM	PG	PE	PI	PC	Others
3-Day-old	88.1	4.8	10.3	4.3	1.1	14.3	2.9	0.4	0.8	—	← 55.6 →				5.5
5-Day-old	39.2	11.4	13.5	13.2	2.6	9.0	3.4	1.2	1.8	4.5	7.1	11.1	2.6	15.1	3.5

The sterol ester (SE) and TG fractions may be slightly contaminated with polyacetylenic hydrocarbons and a polyacetylenic epoxide, respectively. See the text and ref. [40] regarding polyacetylenes. ST, Free sterol; SG, sterol glycoside.

Table 4. Incorporation of [U-^{14}C]acetate into fatty acid classes by cotyledons of germinating safflower seeds

Age of cotyledon	Distribution of ^{14}C among fatty acid classes (%)			
	Saturated	Monoenoic	Dienoic	Trienoic
3-Day-old	30.2 (3.4)	29.8 (3.3)	33.0 (0.4)	7.1 (5.1)
5-Day-old	65.8 (6.0)	9.7 (0.9)	14.3 (0.2)	10.3 (1.0)

Figures in parentheses represent the values of specific radioactivity and are given in % of total radioactivity/% of total wt.

4 to 85 hr. Safflower protoplasts may also require a much longer incubation time for polyenoic acid synthesis. This problem should be solved by long-term incubation of protoplasts under sterile conditions. The absence of linolenic acid might depress galactolipid synthesis.

Safflower seedlings contain polyacetylenes, which are absent in the mature seeds [40, 41] and derived from oleic acid [42]. A polyacetylenic epoxide, 1,2-epoxy-3,11-tridecadiene-5,7,9-triyne, was the most abundant polyacetylene in the seedlings and was found in chloroplasts [40]. It is noteworthy that protoplasts synthesize the polyacetylene that is more unsaturated than polyenoic fatty acids (Table 5).

At the initial stage of incubation with [U-^{14}C]acetate, PC, free fatty acid (FA) and diacylglycerol (DG) were mainly labelled. It is notable that the label of TG increased most rapidly with incubation time in spite of the occurrence of strong lipase activity. This observation seems to suggest that DG acyltransferase and lipase are located in different cellular compartments. Since germinating seeds have strong phospholipase D activity [43], it is possible that phospholipids labelled initially were converted into TG via phosphatidic acid and DG.

The protoplast preparation appeared to contain sufficient cofactors because the addition of a mixture of 0.1 mM CoA, 0.1 mM NADH, 0.1 mM NADPH and 2 mM ATP neither affected lipid synthesis nor was it somewhat inhibitory. To examine the effect of photosynthesis on lipid synthesis, protoplasts from 5-day-old seedlings were incubated with [U-^{14}C]acetate under dark and light conditions. Comparison of the data of the two experiments revealed that lipid synthesis in protoplasts was independent of photosynthesis, at least during a short period (1 hr) of incubation. There was no great change in labelling patterns of fatty acids and lipid classes. In addition, lipid synthesis by protoplasts under aerobic and anaerobic conditions was investigated. Under N_2, desaturation of fatty acids was depressed, but the total incorporation of ^{14}C into lipids and the distribution of ^{14}C among lipid classes were unchanged. In these experiments, protoplasts were superior in reproducibility of lipid synthesis to the cotyledon tissue.

EXPERIMENTAL

Seeds of safflower (*Carthamus tinctorius* L.) were washed with H_2O and germinated at 24° ± 1° under illumination (10^3 lx).

Determination of lipids and analysis of fatty acids. Germinating seeds were treated in boiling water for 4 min to inactivate phospholipase D. Lipids were extracted with $CHCl_3$–MeOH (1:1) and fractionated into neutral lipids, glycolipids and phospholipids by CC [21]. The glycolipid fraction was separated into MGDG and DGDG by TLC on Si gel G with $CHCl_3$–MeOH–Me_2CO (20:4:1). Phospholipid classes were isolated from the phospholipid fraction by TLC on Si gel G with $CHCl_3$–MeOH–Me_2CO–25% NH_3 (70:30:4:1.4) or $CHCl_3$–MeOH–H_2O (65:35:4). The purified lipids were determined by the hydroxamate method of ref. [44]. Fatty acid methyl esters were prepared from lipid classes by methanolysis with NaOMe–MeOH and analysed by GLC (15% DEGS).

Analysis of galactolipids. The positional distribution of fatty acids in galactolipids was analysed by the method of ref. [45]. Galactolipids were directly separated into several groups of molecular species by TLC on $AgNO_3$–Si gel H (15:85). The solvent systems were $CHCl_3$–MeOH–Me_2CO–H_2O (40:15:2:2) for MGDG, and $CHCl_3$–MeOH–H_2O–Me_2CO–PrOH (50:36:10.5:5:5) satd with $AgNO_3$ (*ca* 4% wt/vol.) for DGDG. The individual groups were visualized with 2′,7′-dichlorofluorescein, and recovered from the absorbent with $CHCl_3$–MeOH–H_2O (10:15:6). The extracts were washed with H_2O to remove $AgNO_3$. Fatty acids of these fractions were converted to their methyl esters with NaOMe–MeOH. Methyl heptadecanoate was added as an internal standard, and the individual fractions (molecular classes) were determined by GLC of the fatty acid methyl esters.

Incorporation of [U-^{14}C]acetate into lipids by cotyledons. Cotyledons of 3-day-old or 5-day-old safflower seedlings were incubated in 0.1 ml 10 mM Na [U-^{14}C]acetate (2 μCi) at 25° for 3 hr under illumination. Lipids were extracted by the method of ref. [46], and the total radioactivity was measured in 10 ml toluene scintillation fluid containing 20 mg PPO and 0.5 mg POPOP. Total lipids were separated into lipid classes by one-dimensional TLC on Si gel G. The solvent systems used were: (i) hexane–C_6H_6–HOAc (25:75:1), double development (for separation into neutral lipid classes); (ii) $CHCl_3$–Me_2CO–HOAc (25:75:1) to a height of 15 cm for the first development and Me_2CO–HOAc (100:1) to a height of 9 cm for the second development (for separation into galactolipid classes); (iii) Me_2CO–HOAc (100:1) for the first development and $CHCl_3$–MeOH–Me_2CO–H_2O (70:30:4:1) for the second development (for separation into phospholipid classes). A 1% soln of I_2 in EtOH was sprayed on the chromatograms. Bands corresponding to individual lipid classes were scraped off and transferred to counting vials. The scintillation liquid consisted of 0.4% PPO, 0.01% POPOP, 7.5% naphthalene (wt/vol.) and toluene–1,4-dioxane–ethylene glycol monoethyl ether (1:1:1). The incorporation of ^{14}C into individual fatty acid classes was measured by the same procedure as described in the previous paper [21].

Table 5. Incorporation of [U-^{14}C]acetate into lipid classes by safflower protoplasts

Age*	Incubation time (hr)	Incorporation into total lipids (nmol/mg protein)	Distribution of ^{14}C among lipid classes (%)											
			TG	EP	FA	DG	MG + UL	MGDG + ASG + SG	DGDG	PM	PG + PE	PI	PC	UPL
3-Day-old	0.2	0.10	1.2	1.0	32.7	18.7	12.7	0.5	0.6	0.8	11.0	5.4	13.4	2.0
	0.5	0.17	2.8	1.0	19.3	25.1	6.8	0.6	1.4	0.9	19.4	3.4	17.7	1.6
	1.0	0.21	5.0	2.3	15.8	24.0	8.8	0.8	0.7	1.2	19.7	4.3	16.3	1.1
	3.0	0.26	8.9	4.1	10.4	24.1	9.7	1.3	0.9	1.7	21.0	3.4	13.7	0.8
5-Day-old	0.2	0.66	2.4	0.5	21.0	19.5	7.3	1.4	0.9	1.3	11.5	2.4	27.3	4.5
	0.5	1.84	3.8	0.6	17.3	22.5	7.6	1.3	0.3	0.4	17.3	2.5	24.8	1.6
	1.0	3.77	7.4	0.8	12.4	22.4	10.0	1.0	0.2	0.6	23.4	2.5	18.4	0.9
	3.0	4.89	14.7	1.3	13.1	19.9	8.2	1.6	0.3	0.4	20.6	2.7	16.5	0.7

* Age of seedlings from which protoplasts were isolated.

EP, 1,2-Epoxy-3,11-tridecadiene-5,7,9-triyne; MG, monoaclyglycerol; UL, unidentified lipids; ASG, acylated sterol glycoside; UPL, unidentified polar lipids recovered from the origin of the chromatograms.

Isolation of protoplasts. Protoplasts were prepared from cotyledons of 3-day-old and 5-day-old seedlings which were grown at 24° ± 1° under illumination. Cotyledons were sliced into pieces and incubated in 50 mM K-Pi buffer (pH 6.0) containing 0.7 M sorbitol and 2% Macerozyme R-10 (a polygalacturonase preparation from *Rhizopus* sp., Kinki Yakult MFG. Co., Japan) at 30° for 2.5 hr. The incubation mixture was decanted and the cotyledons were washed twice with 0.7 M sorbitol. The residue was suspended in a medium (pH 5.0) containing 0.7 M sorbitol and 2% Cellulase 'ONOZUKA' R-10 (a cellulase preparation from *Trichoderma viride*, Kinki Yakult MFG. Co., Japan). After digestion at 30° for 2 hr with gentle shaking, the incubation mixture was filtered through a nylon mesh. Protoplasts were collected by low speed centrifugation (200 g, 1 min), washed 3 × with 0.7 M sorbitol, and resuspended in the same soln.

Incorporation of [U-^{14}C] *acetate into lipids by protoplasts.* Protoplasts were incubated in 0.1 M K-Pi buffer (pH 6.0) containing 0.7 M sorbitol, 5 mM $KHCO_3$, 0.5 mM $MnCl_2$ and 90 μM Na [U-^{14}C] acetate (2 μCi). The reaction was carried out at 30° for 0.2–3.0 hr under illumination (1500 lx). The radioactivity of each lipid class was measured by the same procedure described above. The incorporation of ^{14}C into individual fatty acid classes was measured according to the procedure described in the previous paper [21].

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