Biosynthesis of stearate-rich triacylglycerol in developing embryos and microsomal membranes from immature seeds of *Garcinia indica* Chois.

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Analysis of fatty acid composition and storage lipid content of *Garcinia indica* Chois. during seed development showed high stearate content at the early stages, with progressive increase to 60% of the total fatty acids during development. When ^{14}C-acetate was used as a precursor, it was preferentially incorporated into stearate that, in turn, was esterified to triacylglycerol. Kinetics of incorporation of radioactive stearate into diacylglycerol and triacylglycerol was about two fold higher than that of palmitate during various stages of seed development. Pulse-chase experiments with ^{14}C-acetate provided evidence that phosphatidylcholine is involved in donating stearate and oleate for triacylglycerol biosynthesis. When assays were performed for acyltransferase activities in the microsomal membrane fraction with palmitoyl-, stearoyl-, and oleoyl-CoAs, glycerol-3-phosphate acyltransferase and diacylglycerol acyltransferase showed preference for stearoyl-CoA, whereas lysophosphatidic acid acyltransferase had a preference for oleoyl-CoA. These results indicate that stearic acid preferring triacylglycerol biosynthetic machinery exists in the *G. indica* seeds. These data demonstrate that *G. indica* seeds are a desirable source of acyltransferases for engineering a high stearic acid phenotype in temperate oilseeds.

A few plant species are known to accumulate triacylglycerols (TAGs) that are rich in stearic acid[1]. A major source of stearic acid is obtained from the hydrogenation of C18-unsaturated fatty acids[2]. A limited number of tree species, viz. *Butyrospermum paradoxum* (shea), *Garcinia indica* (kokum), *Mangifera indica* (mango), *Shorea robusta* (sal), *Shorea stenoptera* (borneo tallow) and *Vateria indica* (dhupa) have been reported to accumulate more than 30% stearate in their seed oil[3]. Oils from these plants are solid or semi-solid at ambient temperature. All these plants, except mango, are uncultivated and their oils have wide use as substitutes for cocoa butter and in confections[4]. The mechanism of stearate biosynthesis is well understood. However, the accumulation of stearate in TAG and its absence in membrane lipids are not clear. A prerequisite to such studies would be the analysis of the biosynthetic pathways in plant species accumulating high levels of stearic acid in TAG.

Medium-chain fatty acid biosynthesis in trees has been investigated earlier[5-7]. Recently, the stearoyl-ACP thioesterase of *Garcinia mangostana* has been cloned and protein engineering of the thioesterase has been done[8,9]. However, there is no detailed study of the developmental changes in lipid metabolism in trees producing long-chain fatty acids. We have chosen Kokum (*Garcinia indica* Chois.), an evergreen tree species which grows along the west coast of India and has been reported to accumulate very high levels (56%) of stearic acid in its seed TAG[10].

In the present study, we have examined the changes in storage lipid accumulation and in fatty acid composition of TAG during seed maturation. Labelling experiments were performed with [1-^{14}C]acetate, [1-^{14}C]palmitate, [1-^{14}C]stearate or [1-^{14}C]oleate using immature seeds, and the incorporation of various radiolabels into lipids was investigated. In addition, we examined the TAG biosynthetic enzyme activities in microsomal membranes from developing seeds of *G. indica* using labelled acyl-CoAs. This is a study on lipid metabolism in a high stearic acid-accumulating seed.

Materials and methods

Plant materials and biochemicals

G. indica Chois. trees growing in the mountainous forests of Karnataka, western coast of India were the source for the developing fruits. The fruits were harvested from several trees during March and May for three years and fresh tissue was used for labelling experiments. The seeds were excised out and frozen in liquid nitrogen before storing at − 80°C. Lipid analyses were done using the frozen seeds. [1-^{14}C]acetate (60 mCi mmol^{-1}) was obtained from the Board of Radiation and Isotope Technology, Department of Atomic Energy, Mumbai, India. [1-^{14}C]palmitate (55 mCi mmol^{-1}), [1-^{14}C]palmitoyl-CoA (56.5 mCi mmol^{-1}), [1-^{14}C]stearate (55 mCi mmol^{-1}), [1-^{14}C]oleate (51 mCi mmol^{-1}) and [1-^{14}C]oleoyl-CoA (54.5 mCi mmol^{-1}) were obtained from Perkin Elmer Biosystems. [1-^{14}C]stearoyl-CoA (20 mCi mmol^{-1}) was synthesized as described earlier[11].

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Lipid analysis

Lipids were extracted from 10 g of seeds from each stage of development by first grinding the frozen tissue in liquid nitrogen to a fine powder in a mortar and pestle. The powder was extracted with 20 ml of boiling isopropanol. The mixture was then centrifuged briefly, the supernatant removed and the extraction repeated. The pooled isopropanol extracts were brought to dryness on a rotary evaporator. The tissue residue was then re-extracted twice with 38 ml of chloroform–methanol–10% acetic acid (1 : 2 : 0.8, v/v). After centrifugation, the supernatant was added to the isopropanol extract and 20 ml each of chloroform and water was added to the mixture. The biphasic system was mixed and centrifuged. The lower chloroform phase was dried in a rotary evaporator. The lipid residue was dissolved in chloroform–methanol (1 : 1, v/v) and stored at – 20°C. Phospholipids were estimated as described by Barlett[12].

The total lipid extract from each developmental stage was loaded onto a preparative TLC plate (silica gel G, Merck) and developed in petroleum ether (60 to 70°C)–ethyl ether–acetic acid (80 : 20 : 1, v/v) to purify TAG, or developed in chloroform–methanol–water (65 : 25 : 4, v/v) to purify phosphatidylcholine (PC). TAG and PC were scraped-off and eluted from the silica with chloroform–methanol (1 : 1, v/v). The purified TAG and PC from each stage was converted to fatty acid methyl esters (FAMEs) by heating at 75°C for 60 min in 4.5 ml of methanolic–HCl (0.6 N), prepared by diluting 5 ml of concentrated HCl with 90 ml methanol[13,14]. The FAMEs were purified by preparative thin-layer chromatography (TLC) and analysed under gas chromatography–electrospray ionization mass spectrometry (GC–EIMS) conditions using VG AutoSpecM mass spectrometer (Hewlett-Packard, Palo Alto) equipped with HP 5890 series II gas chromatograph fitted with a HP-5 capillary column (30 m × 0.32 mm i.d.). The chromatograph was programmed for an initial temperature of 100°C for 5 min followed by a 10°C min^{-1} ramp to 220°C. The final temperature was maintained for 10 min[14].

Positional analysis of *sn*-2 fatty acid in TAG was carried out using *Rhizopus arrhizus* lipase[15]. The purified TAG samples from various stages of seed development were separately treated with commercially available lipase and monoacylglycerol was purified by preparative silica-TLC. Monoacylglycerol was converted to FAME and analysed by GC-EIMS as described above.

[1-^{14}C]Acetate labelling of G. indica seeds

In the [1-^{14}C]acetate labelling experiments, 500 mg of fresh seeds from various developmental stages were cut into thin slices and soaked in 0.5 ml of 0.1 M Tris-HCl, pH 6.8 containing 10 mg ml^{-1} streptomycin sulphate and

2.5 µCi [1-^{14}C]acetate (84 µM). Seeds at various developmental stages were incubated at 30°C for 4 h and stage-3 seeds were incubated for various time points. After incubation, the tissue was washed in cold buffer and the lipids extracted. The total lipid extract was loaded onto pre-coated silica gel TLC (AL SIL G, Whatman) and neutral lipids were separated using petroleum ether (60 to 70°C)–ethyl ether–acetic acid (80 : 20 : 1, v/v) as the solvent system[16]. Phospholipids were separated using chloroform–methanol–water (65 : 25 : 4, v/v) as the solvent system[15]. Labelled TAG, diacylglycerol (DAG), PC and phosphatidylethanolamine (PE) bands corresponding to the standards were scraped and counted in a liquid scintillation counter. For fatty acid analysis, the labelled TAG or PC was purified from each developmental stage, and converted to FAME. The FAMEs were separated on reverse phase TLC (RP-18, F254s, Merck) with acetic acid–water (90 : 10, v/v) as the solvent system[17]. Following autoradiography, the radioactivity on the TLC plate was quantified by liquid scintillation counting. Each set of experiments was repeated three times.

Pulse-chase labelling was carried out in stage-3 immature seeds. The immature seeds were pulsed with [1-^{14}C]acetate for 15 min. After labelling, the tissues were chased with unlabelled acetate. At different time intervals, total lipids were extracted and analysed in silica-TLC.

Metabolic labelling of seeds with [1-^{14}C]labelled fatty acids

Fresh seeds (500 mg) were cut into thin slices and soaked in 1 ml of 100 mM Tris-HCl (pH 6.8) containing 5 µCi (91 µM) of [1-^{14}C]palmitate or [1-^{14}C]stearate, or 5 µCi (98 µM) of [1-^{14}C]oleate. Fatty acids were converted to their potassium salts by heating in 10 µl of 4.5% KOH at 60°C for 10 min[15]. The labelling was carried out at 30°C for 4 h and stopped by adding 1 ml of boiling acetic acid–isopropanol (1 : 9, v/v) followed by lipid extraction. The chloroform phases were evaporated to dryness under a stream of nitrogen and applied onto silica-TLC plates for resolution of TAG, DAG and phospholipids.

Microsomal membrane preparation

Microsomal membranes were prepared as described previously[18]. Frozen, developing *G. indica* cotyledons of stage 3 (10 g) were ground in a pre-chilled mortar and pestle for 5 min with two parts (w/v) of 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 2 µM leupeptin, 0.1 mM phenylmethylsulphonyl fluoride, 1 mM β-mercaptoethanol, 10 mM KCl, 1 mM MgCl$_2$ and 0.25 M sucrose. The crude homogenate was passed through two layers of cheesecloth and the filtrate was centrifuged at 18,000 g for 15 min. The resulting supernatant was further centrifuged at 150,000 g for

90 min to obtain the microsomal membrane fraction. The pellet was washed with grinding medium and resuspended in a small volume of homogenizing medium without sucrose, with the aid of a glass homogenizer. All operations were carried out at 4°C. Microsomal membranes were either used immediately or frozen as small aliquots in liquid nitrogen and stored at − 80°C. Protein concentrations were determined by the Coomassie dye-binding method[19] using bovine serum albumin as the standard.

Enzyme assay

Microsomal membranes were assayed for G3P acyltransferase, LPA acyltransferase and DAG acyltransferase activities by incubation with different [^{14}C]acyl-CoAs as described earlier[20]. The assay mixture consisted of 50 mM Tris-HCl, pH 8.0, 20 μM G3P or LPA or DAG, 60 to 74 μg of microsomal membrane proteins and 20 μM [1-^{14}C]palmitoyl-CoA or [1-^{14}C]stearoyl-CoA or [1-^{14}C] oleoyl-CoA in a total volume of 100 μl. The assay was carried out at 30°C for the indicated time period. Reactions were stopped by extracting lipids, as described by Bligh and Dyer[21]. Lipids were scraped from silica-TLC plates and radioactivity measured in a liquid scintillation counter. Boiled membranes were used as a control and control values were subtracted from the values measured in the presence of active microsomal membranes.

Results

Developmental profile of G. indica seeds

G. indica trees begin flowering in mid-December and continue until mid-February. Fruits start appearing from mid-January and are fully ripe by May. The fleshy rind of the fruit is juicy and there are 3 to 8 seeds per fruit. The fruits are light green until the last stage of development, then turn red after ripening and become dark brown at maturation. Mature seeds accumulate solid edible oil. Accumulation of TAG in the seed was analysed at seven different stages of development. The developmental stages selected for detailed analyses were based on the morphological characteristics of the fruits (weight and diameter) rather than the days after flowering, because time of flowering varied considerably in a single tree and among trees. The mean weight and diameter of fruit at each developmental stage were as follows: stage 1, 2.5 g, 1.6 cm; stage 2, 6.2 g, 2.2 cm; stage 3, 9.3 g, 2.6 cm; stage 4, 13.8 g, 2.9 cm; stage 5, 21.7 g, 3.4 cm; stage 6, 27 g, 4 cm; stage 7, 34.5 g, 4.4 cm. Moisture content of the seeds was determined by weighing seeds from freshly excised fruit and weighing the same seeds after complete lyophilization. Moisture content in seeds decreased from 73% in stage 1 to 43% in stage 7 (data not shown). The weight of

the fruit and the seed increased until stage 7, after which ripening started.

Lipid content of the seeds at each stage of development was quantified. The amount of lipid increased from 6.5% of wet weight in stage 1 to 20% (195 mg g^{-1}) of wet weight in stage 7. Neutral lipid content varied from 68% in stage 1 to 89% in stage 7. Phospholipid content was 20% of the total lipids at stage 1 and decreased to 3% of total lipids at stage 7 (Table 1).

Fatty acid composition of seed TAG

FAMEs of TAG from each developmental stage were prepared and analysed by gas chromatography–mass spectrometry (GC–MS). The analysis in kokum seeds revealed that stearic acid and oleic acid were the major fatty acids in TAG followed by palmitic acid (Table 2). It was noted that oleic acid content decreased from 47 to 34% and similarly, palmitic acid also decreased from 10 to 4% during seed maturation. In contrast, stearic acid increased from 42% in stage 1 to 60% in stage 7. Minor fatty acids such as linoleic acid were not detected initially and started appearing from stage 4 (0.2%), whereas arachidic acid (0.2%) remained constant throughout the development (Table 2). Positional distribution analyses of fatty acids in TAG indicated that sn-2 position was esterified with oleic acid.

Fatty acid composition of seed PC

PC from seed at each developmental stage was converted to FAME and analysed by GC–MS. It was observed that oleic acid was the major fatty acid and its content increased gradually from 48% in stage 1 to 55% of total fatty acid content of PC in mature seed. Stearic acid content of PC decreased from 46% in stage 1 to 40% in stage 7. Palmitic acid levels in PC were close to 5% at all stages of development. Linoleic acid levels decreased significantly from 1.2% in the early stages to 0.3% at seed maturity.

Table 1. Content and composition of lipids at different developmental stages in seeds of G. indica

Stage	Total lipid	Neutral lipid	Phospholipid
		(mg g fresh tissue^{-1})	
1	65.1 ± 5.1	44.0 ± 3.9	13.0 ± 1.0
2	76.1 ± 6.4	52.4 ± 5.4	12.4 ± 0.9
3	100.1 ± 9.2	78.2 ± 4.8	11.2 ± 0.8
4	112.7 ± 8.5	97.7 ± 7.6	10.2 ± 0.7
5	125.3 ± 12.7	110.3 ± 8.9	8.1 ± 0.7
6	147.6 ± 10.9	131.2 ± 10.1	6.0 ± 0.4
7	195.5 ± 5.2	174.6 ± 13.6	6.3 ± 0.4

Amount of various lipids was determined as described in the text. Values are mean ± SD of four determinations.

Labelling of G. indica seed using [1-^{14}C]acetate

In an attempt to understand TAG biosynthesis, [1-^{14}C] acetate incorporation into various lipids during seed development was carried out. Stage 3 was active in synthesizing various lipids and hence this stage was used for standardizing the conditions for [1-^{14}C]acetate incorporation at different time intervals. The rate of incorporation into total lipids was maximum at 0.5 h (0.96 nmol h^{-1} g seed^{-1}) and decreased nearly 7-fold at 12 h. The amount of radioactivity in TAG accounted for nearly 30% of the total lipids at 30 min and remained constant with increase in time, while DAG and PC accounted for 18 and 26%, respectively, at 30 min and increased thereafter. Label in free fatty acids decreased from 16% at 30 min to 8% at 12 h (Figure 1).

Incorporation of [1-^{14}C]acetate into TAG and PC reached a maximum at stage 3 and decreased gradually during maturation. At stage 3, 66% of the radiolabel was associated with TAG, 19% with PC and 15% with DAG and PE (Figure 2).

The pattern of incorporation of ^{14}C-acetate into various fatty acids esterified to TAG and PC was analysed by reverse-phase TLC. This revealed that most radioactivity was associated with the stearoyl moiety on TAG, which reached a maximum at stage 3 and declined thereafter. Incorporation of label into palmitate + oleate of TAG was lower than stearate at all stages of development. In the case of PC, stearate had lower radioactivity compared to oleate + palmitate and maximum labelling was observed at stage 3 (Table 3).

Incorporation of [1-^{14}C]fatty acids into G. indica seed lipids

To get an insight into the synthesis of stearate-rich TAG in G. indica, fresh seeds from each developmental stage were labelled with potassium salts of [1-^{14}C]palmitate, [1-^{14}C]stearate or [1-^{14}C]oleate (Figure 3). After incubation for 4 h at 30°C, the amount of radioactivity in various lipids was estimated. Incorporation of [^{14}C]palmitate into TAG did not show any stage-specific variation, whereas oleate incorporation into TAG increased at stage 6. Labelling experiment (Figure 3) and endogenous fatty acid composition of TAG (Table 2) showed that C18-fatty acids accumulated in TAG during seed development. The incorporation of stearate and palmitate into DAG was the same at the initial stages of development. At stage 7, incorporation of palmitate to DAG reduced while the incorporation of stearate increased significantly. PC was labelled to a greater extent compared to other labelled products. The label in PC gradually increased during seed development.

Table 2. Fatty acid composition of triacylglycerols at various stages of seed maturation in G. indica

Stage	Fatty acid composition (% wt)				
	C16 : 0	C18 : 0	C18 : 1	C18 : 2	C20 : 0
1	10.4 ± 0.2	41.7 ± 0.5	46.8 ± 0.6	n.d.	0.2 ± 0.01
2	6.5 ± 0.2	45.4 ± 2.1	47.5 ± 0.4	n.d.	0.2 ± 0.02
3	6.8 ± 0.5	45.8 ± 2.9	46.8 ± 1.1	n.d.	0.2 ± 0.01
4	5.7 ± 0.5	50.2 ± 2.3	43.5 ± 1.4	0.2 ± 0.01	0.2 ± 0.03
5	5.4 ± 0.5	56.6 ± 1.4	36.7 ± 2.6	0.9 ± 0.01	0.2 ± 0.01
6	4.0 ± 1.0	59.4 ± 2.3	35.0 ± 1.4	1.3 ± 0.1	0.2 ± 0.01
7	4.1 ± 1.0	59.6 ± 3.7	34.2 ± 2.4	1.8 ± 0.1	0.2 ± 0.01

n.d., Not detected; TAG from each developmental stage was converted to FAME and analysed on GC–MS.
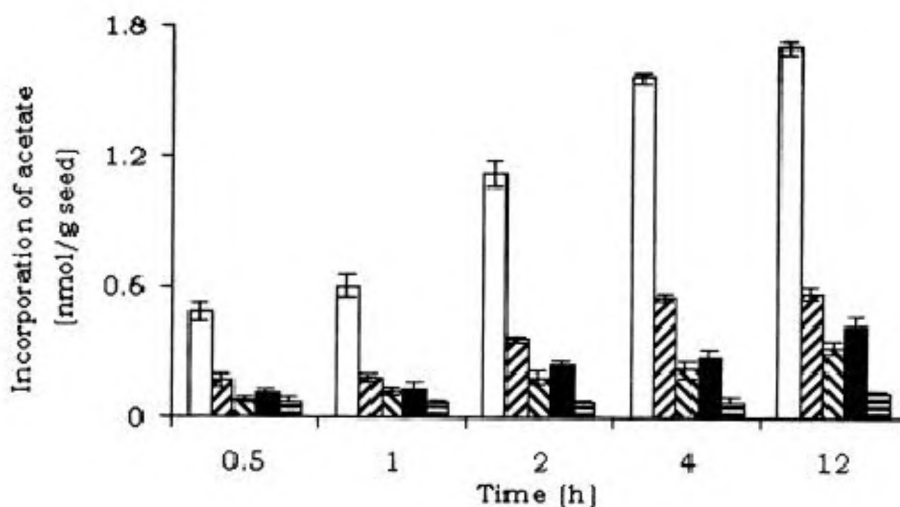Values are mean per cent ± SD of two determinations.

Figure 1. Time-course of [1-^{14}]acetate incorporation into lipids by G. indica seed. Developing seeds of stage 3 (500 mg) were incubated in the presence of ^{14}C-acetate. At the indicated time, the amount of radioactivity incorporated into total lipids (□), TAG (▨), DAG (◨), PC (■) and fatty acids (▤) was determined. Values are expressed as mean ± SD of three determinations.

Pulse-chase labelling of *G. indica* seed lipids with [1-^{14}C]acetate

In vivo labelling of acyl chains of various lipids in stage-3 immature seeds was studied by pulse-chase experiments. After 15 min of pulse with labelled acetate, the chase was carried out for 4 h. Amount of label recovered in the total lipid was relatively stable during the chase period. PC was the most heavily labelled lipid (45%) at the end of pulse and subsequently, declined steadily during the chase period. The amount of label that was lost from PC was found in TAG (Figure 4 *a*). Other phospholipids contained a low level of radiolabel throughout the chase period and these lipids were not characterized. The distribution of label in stearate (Figure 4 *b*) and oleate (Figure 4 *c*) in PC, DAG and TAG was analysed during the chase period of the experiments. Among the acyl groups, stearate was labelled higher compared to oleate. During the chase, radioactivity in the stearoyl and oleoyl moieties of PC declined, and concurrent increases of radiolabel in DAG and TAG were observed (Figure 4 *b* and *c*). These results suggest that PC is involved in donating both stearate and oleate to neutral lipids in kokum seed.

TAG biosynthetic enzyme activities in *G. indica* seed microsomal membranes

G3P acyltransferase, LPA acyltransferase and DAG acyltransferase activities in the microsomal membranes from stage-3 immature seeds were measured in the presence of exogenously added acyl acceptor such as G3P, LPA or DAG. It was observed that all the acyltransferases involved in TAG biosynthesis were very active towards stearoyl-CoA and less active towards oleoyl-CoA, and that the reaction was linear with time up to 30 min (data not shown). The acyl donor specificity for acyltransferases was stu-

died by providing palmitoyl-, stearoyl- and oleoyl-CoAs separately as the substrate (Figure 5). Oleoyl-CoA was preferentially used by LPA acyltransferase. G3P acyltransferase and DAG acyltransferase preferred stearoyl-CoA over oleoyl-CoA. These results suggest that the G3P acyltransferase and DAG acyltransferase in developing *G. indica* seeds prefer long-chain saturated fatty acids, in particular, stearate.

Discussion

Stearic acid is a low-abundance fatty acid in all plant cell membranes[1]. It has been shown in *fab2* mutant of *Arabidopsis thaliana* that increased levels of stearate in leaves retard plant growth and development[22]. Leaves of *G. indica* do not contain significant amount of stearate (data not shown), but the seeds of *G. indica* accumulate a large quantity of stearate (60%), and most of it is esterified to TAG. Developing seeds of *G. indica* have the ability to incorporate various exogenously supplied precursors into lipids.

Analysis of endogenous fatty acids in TAG at different developmental stages (Table 2) reveals that stearic acid is the major fatty acid and its concentration increases during maturation (42 to 60%). When acetate is the radio-tracer, most of the synthesized stearate is esterified into TAG (Table 3). [^{14}C]Acetate incorporation by kokum seeds is about 2.5 to 3.5-fold higher into TAG than into PC (Figure 1). Hawkins and Kridl[9] have studied a related tree species and have identified an acyl-ACP thioesterase that is involved in stearate accumulation. Recently, acyl-CoA-independent PC : DAG acyltransferase has been reported to synthesize TAG in certain oilseeds[23]. It is therefore possible that PC may be involved in donating both stearic and oleic acids to TAG in kokum seed (Figure 4).
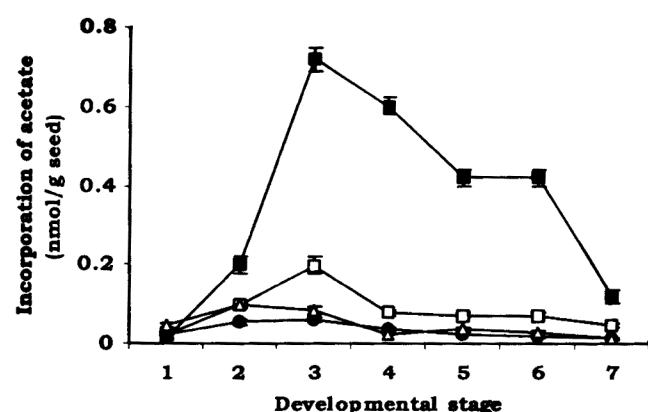

Figure 2. Incorporation of [1-^{14}C]acetate into lipids by developing seeds of *G. indica*. Seed slices (500 mg) from each stage were incubated for 4 h with ^{14}C-acetate and the radioactivity in TAG (■), DAG (●), PC (□) and PE (△) quantified. Data are expressed as mean ± SD of three sets of determinations.

Table 3. Incorporation of [1-^{14}C]acetate into various fatty acids of triacylglycerol (TAG) and phosphatidylcholine (PC)

	Acetate incorporation into			
	TAG		PC	
	C18 : 0	C16 : 0 + C18 : 1	C18 : 0	C16 : 0 + C18 : 1
Stage	(nmol g fresh tissue^{-1})			
1	0.09 ± 0.004	0.04 ± 0.003	0.06 ± 0.003	0.08 ± 0.004
2	0.23 ± 0.02	0.11 ± 0.002	0.15 ± 0.01	0.21 ± 0.01
3	0.40 ± 0.03	0.28 ± 0.02	0.32 ± 0.02	0.45 ± 0.04
4	0.33 ± 0.04	0.24 ± 0.02	0.28 ± 0.02	0.40 ± 0.03
5	0.14 ± 0.009	0.07 ± 0.004	0.09 ± 0.007	0.18 ± 0.015
6	0.11 ± 0.008	0.06 ± 0.004	0.07 ± 0.004	0.13 ± 0.008
7	0.06 ± 0.004	0.02 ± 0.001	0.03 ± 0.001	0.07 ± 0.004

Incorporation of [^{14}C]acetate into lipids was carried out for 4 h and the labelled TAG and PC from seed at various developmental stages were purified and converted to FAME. Radiolabelled FAMEs were resolved on reverse phase TLC. Values are mean ± SD of three observations.

Incorporation of various [14C]fatty acids into *G. indica* seed lipids revealed a higher level of incorporation into DAG and a lower level into TAG and phospholipids (Figure 3). A similar observation was made in cocoa seeds[24]. When developing safflower and sunflower cotyledons were incubated with radiolabelled fatty acid, a substantial amount was esterified to DAG[25]. Accumulation of DAG could be due to shortage of acyl-chain supply (acyl-CoA) or DAG acyltransferase might have a higher K_m for acyl-CoA than other acyltransferases[26]. In addition, DAG acyltransferase may exert significant flux control over TAG-accumulation[24].

Our investigations on the preferences for acylation with various acyl-CoAs by the microsomal membranes showed that G3P acyltransferase and DAG acyltransferase had a preference for stearoyl-CoA. However, LPA acyltransferase showed higher activity with oleoyl-CoA compared
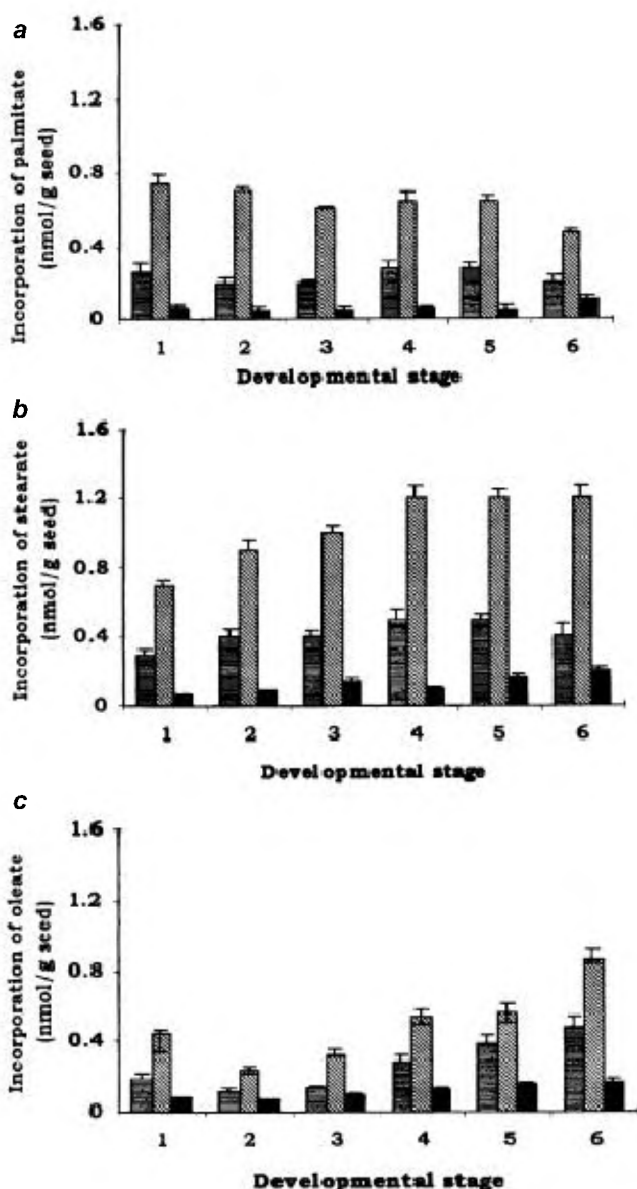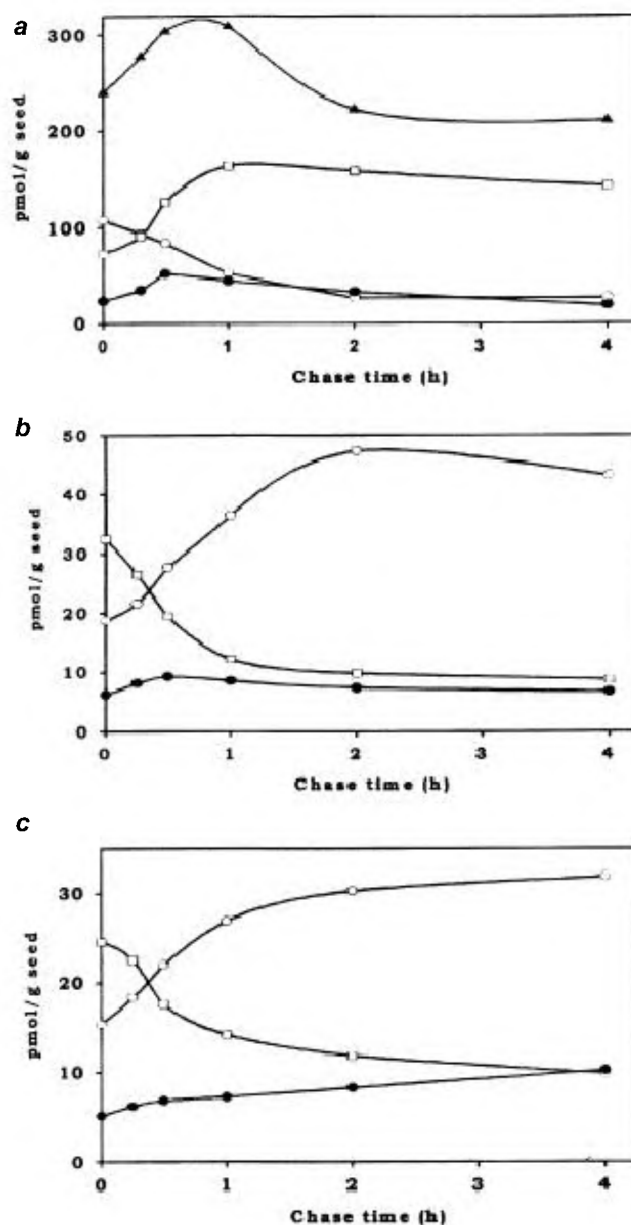
Figure 3. Incorporation of 14C-labelled fatty acids into lipids by developing seeds of *G. indica*. Seeds at various developmental stages (500 mg) were incubated with [1-14C]palmitate *a*, [1-14C]stearate *b*, and [1-14C]oleate *c*, for 4 h, and incorporation of radioactivity into TAG (⊟), DAG (◘) and PC (■) by developing seeds was determined. Values are mean ± SD of three determinations.

Figure 4. Pulse-chase labelling of *G. indica* seed lipids with [1-14C]acetate. Stage-3 immature seeds were pulsed with [14C]acetate for 15 min; after labelling, the tissues were then chased with unlabelled acetate. At different time intervals, total lipids were extracted and analysed in silica-TLC. *a*, Amount of radioactivity incorporated into total lipids (?), PC (O), DAG (●) and TAG (□) at indicated time points after the label was removed. *b* and *c*, Radiolabelled PC, DAG and TAG from the various chase periods were purified and converted to FAME. The labelled FAMEs were resolved on reverse-phase TLC. The proportion of radioactivity in the stearoyl *b* and oleoyl *c* moieties of PC (□), DAG (●) and TAG (O) was determined. Values are mean of three separate experiments from the same batch of seeds and each performed in duplicate.
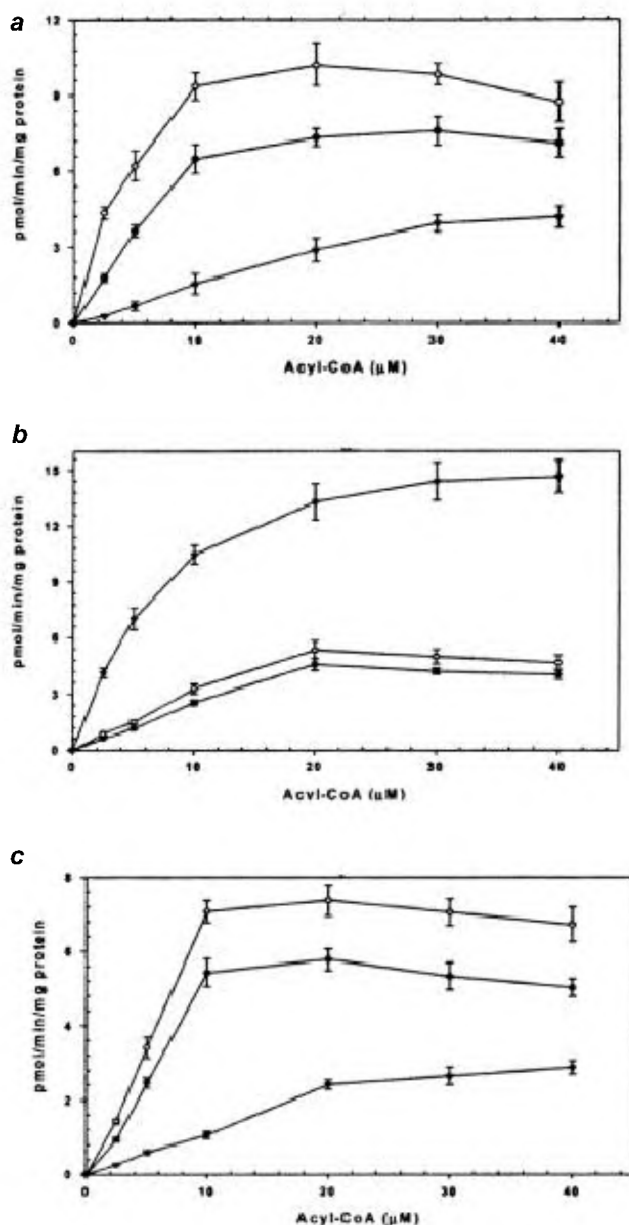
Figure 5. Substrate saturation studies of membrane-bound acyltransferase activities towards various acyl-CoAs. *a*, G3P acyltransferase; *b*, LPA acyltransferase, and *c*, DAG acyltranferase activities were measured as a function of palmitoyl-CoA (●), stearoyl-CoA (O) and oleoyl-CoA (▼) concentrations and 50 μM concentration of appropriate acyl acceptor. Assays were carried out for 20 min at 30°C with ~ 75 μg membrane proteins. Each point is the mean ± SD of three determinations, each performed in triplicate.

to palmitoyl- and stearoyl-CoAs (Figure 5). These results suggest the presence of specific acyltransferases for channelling of C-18 fatty acids into the seeds of *G. indica*. Fatty acid-specific acyltransferases have also been reported in the developing seeds of palm[27] and *Cuphea*[28].

It is evident from the results presented here that developing seeds of *G. indica* mainly synthesize stearate and oleate-containing molecular species of TAG. First, the analysis of endogenous TAG showed that it is composed

mainly of C18-fatty acids. Secondly, when the labelled palmitate was supplied, the cotyledon slices showed lower incorporation into TAG compared to stearate. Thirdly, when the microsomal membranes were incubated with [14C]labelled palmitoyl-, stearoyl- or oleoyl-CoAs, stearoyl-CoA was more active in the formation of LPA and TAG. This could be due to the presence of stearate-preferring G3P acyltransferase and DAG acyltransferase in this system. *G. indica* is an ideal model system to study the biosynthesis of stearate and assembly of stearate-rich TAG in their seed oils.

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