Fluxes through the prokaryotic and eukaryotic pathways of lipid synthesis in the '16:3' plant *Arabidopsis thaliana*

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1. The kinetics of [1-¹⁴C]acetate incorporation in *Arabidopsis thaliana* L. (Heyn) showed almost equal labelling of phosphatidylcholine (PC) and diacylgalactosylglycerol (DGG) at early times and the transfer of radioactivity from PC to DGG and diacyldigalactosylglycerol (DDG) at longer times. These kinetics demonstrated the parallel operation of the prokaryotic and eukaryotic pathways of lipid synthesis [Roughan & Slack (1982) *Annu. Rev. Plant Physiol.* 33, 97–132] in this tissue. 2. At 2 h after the application of [1-¹⁴C]acetate, more than 85% of the radioactivity at the *sn*-2 position of each chloroplast lipid was in 16-carbon fatty acids. However, after 60 h, molecular species containing labelled C₁₈ fatty acids at position *sn*-2 and presumably derived from microsomal PC made a large contribution (20–70%) to each chloroplast lipid except phosphatidylglycerol. These findings are consistent with the contention that the chain length of the fatty acid at the *sn*-2 position of glycerol is an accurate predictor of whether a particular lipid molecule has been synthesized by the prokaryotic or eukaryotic pathway. 3. At 30 min after the start of [1-¹⁴C]acetate labelling, only 12.3% of the radioactivity in PC was in saturated fatty acids, but the proportion increased steadily to 24.3% after 142 h. It is suggested that steps involved in the conversion of PC to chloroplast lipids on the eukaryotic pathway discriminate against palmitate-containing species. The step involved does not appear to be transfer of PC to the chloroplast because extrachloroplastic and chloroplast membranes purified from *Arabidopsis* mesophyll protoplasts each contained PC with a fatty acid composition similar to that of the same lipid from leaves. 4. Positional analysis of unlabelled lipids, together with the information summarized above, is used to construct a quantitative scheme of the fluxes through the prokaryotic and eukaryotic pathways during lipid synthesis in *Arabidopsis*. This scheme shows that 38% of the fatty acids synthesized *de novo* in the chloroplast enter the prokaryotic pathway in the chloroplast envelope. Of the 62% which are exported as acyl-CoA species to enter the eukaryotic pathway, 56% (34% of the total) are returned to complete synthesis of the chloroplast's complement of glycerolipids.

INTRODUCTION

A considerable body of data is now seen to support the concept that there are two discrete pathways in plant cells for biosynthesis of lipids and the associated production of polyunsaturated fatty acids. This data comes from [¹⁴C]acetate, ¹⁴CO₂, [³H]glycerol and [¹⁴C]oleate labelling of leaves and algal cells *in vivo* (Nicholls, 1968; Appleby *et al.*, 1971; Slack & Roughan, 1975; Siebertz & Heinz, 1977; Slack *et al.*, 1977; Ohnishi & Yamada, 1980; Gardiner *et al.*, 1982; Williams & Khan, 1982), from labelling experiments with isolated chloroplasts and microsomal fractions *in vitro* (Roughan *et al.*, 1980; Dubacq *et al.*, 1983; Gardiner *et al.*, 1984) and from enzymological studies (Joyard & Douce, 1977; Douce & Joyard, 1979; Block *et al.*, 1983; Frentzen *et al.*, 1983, 1984). The basis of the two-pathway hypothesis (Roughan *et al.*, 1980; Roughan & Slack, 1982) is that fatty acids synthesized *de novo* in the chloroplast (Ohlrogge *et al.*, 1979) may either be used directly for production of chloroplast lipids via the prokaryotic pathway (Roughan *et al.*, 1980; Sparace & Mudd, 1982; Heinz & Roughan, 1983) or be exported as CoA esters

(Block *et al.*, 1983) to enter the eukaryotic pathway at extrachloroplastic sites, particularly in the endoplasmic reticulum (Slack & Roughan, 1975; Slack *et al.*, 1976; Simpson & Williams, 1979; Dubacq *et al.*, 1983). The diacylglycerol moiety of PC synthesized by the eukaryotic pathway is returned to the chloroplast, probably by the action of a phospholipid-transfer protein (Ohnishi & Yamada, 1982; Dubacq *et al.*, 1984), where it contributes to the production of thylakoid lipids (Slack & Roughan, 1975; Williams *et al.*, 1976; Slack *et al.*, 1977).

In many families of higher plants, PG is the only major product of the prokaryotic pathway, and the remaining chloroplast lipids are synthesized entirely by the eukaryotic pathway (Roughan & Slack, 1982, 1984). However, in the members of some (but not all) primitive angiosperm families, both pathways contribute to the synthesis of DGG and to a lesser extent SL and DDG (Siebertz & Heinz, 1977; Williams & Khan, 1982). Because DGG formed via the prokaryotic pathway contains a high level of C_{16:3} fatty acid at the *sn*-2 position (Siebertz & Heinz, 1977; Roughan *et al.*, 1979), these species may be distinguished as '16:3 plants' in contrast with the other angiosperms ('18:3 plants') whose DGG

Abbreviations used: ACP, acyl carrier protein; DDG, diacyldigalactosylglycerol; DGG, diacylgalactosylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; SL, sulpholipid (diacylsulphoquinovosylglycerol).

contains predominantly α -linolenate (Jamieson & Reid, 1971).

In order to place these general concepts on a quantitative basis, we have used positional analyses of individual lipids to measure the fluxes through the prokaryotic and eukaryotic pathways during lipid synthesis by the 16:3 plant *Arabidopsis thaliana*. First, however, we present evidence to show that such analyses are a valid way of estimating fluxes through the two pathways.

MATERIALS AND METHODS

Materials

Arabidopsis thaliana L. (Heyn) (race Columbia) plants were grown under continuous illumination ($200 \mu\text{E} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$) at 22°C as described by Somerville & Ogren (1982). Sodium [$1\text{-}^{14}\text{C}$]acetate (2.15 GBq/mmol) was obtained from Amersham International, Amersham, Bucks., U.K., and *Rhizopus* lipase (product no. L-4384; 431 000 units/mg of protein) from Sigma, St. Louis, MO, U.S.A. Sodium methoxide was prepared as described previously (Slack *et al.*, 1976).

Labelling of plants

Sodium [$1\text{-}^{14}\text{C}$]acetate was diluted to 1.9 MBq/ml with water without the addition of ethanol or surfactants. Each pot of plants (175 cm^2 soil surface area) was sprayed with 1 ml of the dilute solution with a perfume atomizer. At various times after application of the label samples, weighing about 1 g fresh wt. after removal of roots and stems, were harvested and killed by immersion in liquid N_2 .

Lipid extraction

Each sample from the labelling experiments was ground under liquid N_2 in a pre-cooled mortar and pestle, transferred to a screw-capped centrifuge tube with 12 ml of chloroform/methanol/formic acid ($10:10:1$, by vol.) and stored overnight at -20°C . This procedure precluded the formation of phosphatidylmethanol (Roughan *et al.*, 1978). After centrifuging, the supernatant was decanted and the tissue pellet re-extracted with 4.4 ml of chloroform/methanol/water ($5:5:1$, by vol.). The combined extracts were washed with 6 ml of $0.2 \text{ M-H}_3\text{PO}_4/1 \text{ M-KCl}$ (Hajra, 1974) and the lipids recovered in the chloroform phase were dried under N_2 and taken up in 0.5 ml of chloroform.

Unlabelled *Arabidopsis* plants were also killed in liquid N_2 and samples ($4\text{--}6 \text{ g}$ fresh wt.) extracted by the method described above but using appropriately larger volumes. The lipid extracts were dried under vacuum and taken up in 2 ml of chloroform.

Preparation of chloroplast and extrachloroplastic membrane fractions

Leaf slices (1 mm) were used as the starting material for protoplast preparation. Production, purification and rupture of protoplasts were carried out as described by Somerville *et al.* (1981). Chloroplasts were pelleted by centrifugation for 4 min at 800 g in a swing-out rotor and then the essentially colourless supernatant was centrifuged for 60 min at $105\,000 \text{ g}$ to pellet the extrachloroplastic membranes. Each pellet was resuspended in a small

volume of buffer and extracted by the method of Bligh & Dyer (1959).

Separation of lipids

Small quantities of individual lipids were purified from the extracts either by a one-dimensional t.l.c. method on $(\text{NH}_4)_2\text{SO}_4$ -impregnated plates (Khan & Williams, 1977) or by two-dimensional t.l.c. as described by Roughan *et al.* (1978). Larger quantities of unlabelled lipids for lipase analysis were prepared by DEAE-cellulose column chromatography (Roughan, 1985) followed by preparative t.l.c. on silica-gel G plates using chloroform/methanol/acetic acid/water ($85:15:10:3$, by vol.) as solvent. Lipids were located by light staining with I_2 vapour.

Labelling experiments

The lipids from one chromatographic separation were transferred to scintillation vials and counted for radioactivity in 0.5% *p*-terphenyl in xylene/Triton X-100/water ($6:3:1$, by vol.). Lipids from a second separation were transferred to screw-capped tubes and transmethylated with sodium methoxide (Slack *et al.*, 1976). The resulting fatty acid methyl esters, after addition of a carrier mixture, were separated by argentation t.l.c., and then counted for radioactivity as described above. A final separation provided lipids that were extracted from the silica gel and treated with lipase as detailed below. The fatty acid methyl esters derived from the intact lipids, the lyso-lipids and the released fatty acids were hydrogenated (Appelqvist, 1972) and then separated on the basis of chain length by reverse-phase t.l.c. (Heinz & Roughan, 1983). The bands containing $\text{C}_{16:0}$ and $\text{C}_{18:0}$ methyl esters were located by autoradiography and then counted for radioactivity as described above.

Analysis of unlabelled lipids

Individual lipids separated by t.l.c. were transmethylated with sodium methoxide after addition of a known amount of $\text{C}_{17:0}$ methyl ester. The resulting methyl esters were then quantified by g.l.c. using an internal-standard method. Separation of $\text{C}_{16:3}$ from $\text{C}_{18:0}$, and $\text{C}_{16:2}$ from $\text{C}_{17:0}$, was achieved by using a 4 m EGS column as described by Browse *et al.* (1986).

Lipase analyses, including t.l.c. purification of the lyso-derivatives and fatty acids, were carried out by the method of Siebertz & Heinz (1977), except that $50 \text{ mM-H}_3\text{BO}_3$ was added to the buffer used for lipase digestion to minimize intramolecular acyl transfer on the lyso-lipids produced. Fatty acid methyl esters were formed from the untreated lipids and the lyso-derivatives using sodium methoxide and from the fatty acids using methanolic HCl . The fatty acid composition of each compound was then determined by g.l.c. analysis.

RESULTS AND DISCUSSION

Labelling kinetics *in vivo*

Arabidopsis plants were labelled with sodium [$1\text{-}^{14}\text{C}$]acetate 19 days after sowing. Preliminary experiments showed that incorporation of radioactivity into lipids was rapid in the first $40\text{--}60 \text{ min}$ after application of the label, but then slowed so that very little further incorporation occurred beyond 90 min after application. In the experiment reported here, the total radioactivity in polar

Fig. 1. Redistribution of radioactivity among the polar lipids of *Arabidopsis* leaves following [1- 14 C]acetate labelling

Plants were sprayed with sodium [1- 14 C]acetate at zero time, and lipids from samples of leaves were separated by t.l.c. as described in the Materials and methods section. (a): $\square$, DGG; $\circ$, PC; $\bullet$, DDG; $\triangle$, SL. (b): $\bullet$, PG; $\circ$, PE; $\triangle$, PI.

lipids reached a maximum (3.3 kBq/g fresh wt.) in the sample taken 90 min after application of [1- 14 C]acetate and declined during the remainder of the experiment to 1.6 kBq/g fresh wt. at 142 h. Growth of the plants during the experiment (from 85 to 150 mg fresh wt. per plant) accounts for much of this decline, and there was probably also some turnover of lipids.

The kinetics of labelling of individual polar lipids (Figs. 1a and 1b) during the early part of the experiment are similar to those reported for other 16:3 plants (Slack & Roughan, 1975; Siebertz & Heinz, 1977; Williams & Khan, 1982). DGG and PC contain nearly equal amounts of radioactivity in the sample at 0.5 h, and most of the other polar lipids (i.e. PE, PG, PI, SL) contain activity approximately in proportion to their relative masses in the tissue (Table 3 below). However, DDG, which represents 15% of the polar-lipid fatty-acid mass, contains only 2% of the total polar-lipid radioactivity at the shortest time.

Transfer of 14 C label from PC to DGG and DDG has been observed in 16:3 plants (Slack & Roughan, 1975), but the process is relatively slow, so that little change in the level of label in PC may be observed even after a 10 h chase (Williams & Khan, 1982). By continuing to sample *Arabidopsis* plants for over 100 h (Fig. 1), it was possible to show the fall in PC label and the increases in DGG and DDG that accompany the operation of the eukaryotic pathway. Furthermore, the detailed kinetics shown in Fig. 1 allow an estimate to be made of the contributions of the prokaryotic and eukaryotic pathways to the

synthesis of DGG and DDG. Assuming that radioactivity in these compounds at early times has entered via the prokaryotic pathway whereas radioactivity from the eukaryotic pathway has entered after longer times, then it may be calculated that a minor, but significant, proportion of the label in DGG (perhaps 20–25%) was derived from the eukaryotic pathway. In contrast, the eukaryotic pathway appeared to contribute over 80% of the label found in DDG by the end of the experiment. In a similar way it may be calculated that approx. 60% of the 14 C label entering PC at early times was metabolized further during the course of the experiment.

Positional distribution of labelled fatty acids

The starting point of the prokaryotic pathway is the synthesis of PA in the chloroplast envelope by the sequential action of acyl-ACP, glycerol-3-phosphate acyltransferase and acyl-ACP:lysophosphatidic acid acyltransferase. These enzymes have been shown to produce almost exclusively 1- $C_{18:1}$,2- $C_{16:0}$ -PA (Frentzen *et al.*, 1983), and this is in agreement with the finding that isolated chloroplasts (in which only the prokaryotic pathway can operate) synthesize PA, diacylglycerol, DGG and PG, which contain only C_{16} fatty acids at *sn*-2 (Roughan *et al.*, 1980; Heinz & Roughan, 1983). However, the microsomal acyltransferase isoenzymes, which are responsible for PA synthesis by the eukaryotic pathway, give rise to lipids that contain a C_{18} fatty acid at *sn*-2 and either a C_{18} or C_{16} fatty acid at position 1 (Frentzen *et al.*, 1984). The importance of these points is illustrated by an experiment in which the positional distribution of labelled fatty acids in individual lipids was measured on samples taken 2 h and 60 h after [1- 14 C]-acetate labelling (Table 1). After 2 h, each of the chloroplast lipids contains approximately equal label in C_{16} and C_{18} fatty acids, but the label at position *sn*-2 of these lipids is dominated by C_{16} fatty acids. The major extrachloroplastic lipids, PC and PE, contain less label in C_{16} fatty acids and, at position *sn*-2 of these lipids, C_{18} fatty acids represent more than 90% of the radioactivity. After 60 h the proportion of radioactivity in C_{16} fatty acids at *sn*-2 of DGG, DDG and SL had fallen significantly. In the case of SL, a substantial decrease in position-*sn*-2 16-carbon label occurred, even though the overall C_{16}/C_{18} labelling ratio remained close to 1.

These data are consistent, therefore, with the parallel synthesis of DGG, DDG and SL by both the prokaryotic and eukaryotic pathways. The prokaryotic pathway leads to the rapid labelling of C_{18}/C_{16} species of these lipids, and these predominate at early times. Species with 14 C-labelled C_{18} fatty acids at position *sn*-2 appear later, when sufficient time has elapsed for radioactivity to pass through the extrachloroplastic PC pool of the eukaryotic pathway. The data in Table 1 also indicate that DGG, DDG and SL receive very different proportions of C_{18}/C_{18} and C_{16}/C_{18} species via the eukaryotic pathway. Thus SL must receive mainly C_{16}/C_{18} species, so that the percentage of labelled C_{16} fatty acids in the intact lipid decreases only from 55.1 to 48.2%, whereas that at position *sn*-2 shows a decrease from 84.8 to 34.8%. On the other hand, DGG receives almost no labelled C_{16}/C_{18} species from the eukaryotic pathway, since at 60 h C_{16} radioactivity at the *sn*-2 position still accounts for all of the C_{16} label in the intact lipid. In DDG the situation is intermediate between these two extremes.

In contrast with these results for DGG, DDG and SL,

Table 1. Positional distribution of ^{14}C -labelled C_{16} and C_{18} fatty acids in *Arabidopsis* lipids

Lipids from *Arabidopsis* plants sampled 2 h and 60 h after labelling with $[1-^{14}\text{C}]$ acetate were treated with *Rhizopus* lipase. Fatty acid methyl esters derived from untreated lipids and from the lyso-derivatives were hydrogenated and then separated according to chain length by reverse-phase t.l.c. The lyso-lipids contain fatty acids from the *sn*-2 position of the parent molecule.

Sampling time (h)	Lipid	Proportion of radioactivity in each chain length (% of the sum)			
		Untreated lipid		Lyso-derivative	
		C_{16}	C_{18}	C_{16}	C_{18}
2	DGG	46.3	53.7	95.8	4.2
	DDG	44.0	56.0	85.8	14.2
	SL	55.1	44.9	84.8	15.2
	PG	55.4	44.6	94.2	5.8
	PC	13.0	87.0	0.8	99.2
	PE	28.3	71.7	9.2	90.8
60	DGG	39.3	60.7	81.0	19.0
	DDG	24.3	75.7	30.6	69.4
	SL	48.2	51.8	34.8	65.2
	PG	58.0	42.0	91.6	8.4
	PC	21.4	78.6	3.0	97.0
	PE	28.2	71.8	5.4	94.6

the distribution of radioactivity between C_{16} and C_{18} chains in PG did not change between 2 and 60 h, either in the intact lipid or in the lyso-derivative, which represents the *sn*-2 position of the molecule. It appears that very little PG is synthesized via the eukaryotic pathway.

The major phospholipids of the eukaryotic pathway, PC and PE, also show essentially no change in the distribution of radioactivity between C_{16} and C_{18} fatty acids at position *sn*-2 of the molecule. However, for PC the proportion of label in C_{16} fatty acids in the intact lipid increases from 13.0% at 2 h to 21.4% at 60 h (Table 1). This result was shown more clearly when argentation t.l.c. was used to separate fatty acid methyl esters prepared from the PC of plants sampled at different times after $[1-^{14}\text{C}]$ acetate labelling (Fig. 2). The data suggest that the further metabolism of PC to DGG, DDG and SL by the eukaryotic pathway discriminates in favour of $\text{C}_{18}/\text{C}_{16}$ molecular species so that the remaining pool of PC becomes progressively enriched in $\text{C}_{16}/\text{C}_{18}$ molecular species.

PC is the major substrate for the desaturation of oleate to linoleate in the eukaryotic pathway, and Fig. 2 shows the resulting shift in label from $\text{C}_{18:1}$ to $\text{C}_{18:2}$ (and $\text{C}_{18:3}$) fatty acids during the experiment. During the time (12–50 h) when label was most rapidly being transferred from PC to DGG, the label in PC was predominantly in $\text{C}_{18:2}$ fatty acid, but there were also significant amounts of both $\text{C}_{18:3}$ and $\text{C}_{18:1}$ fatty acids. Selection for molecular species containing particular C_{18} fatty acids cannot be discounted, but the data are consistent with the conclusion that eukaryotic DGG when first synthesized contains a range of unsaturated C_{18} fatty acids (Williams, 1980).

Fatty acid composition of chloroplast and extrachloroplasmic PC pools

Recently evidence has been presented that PC transfer processes (with or without the assistance of a transfer

Fig. 2. Distribution of labelled fatty acids of PC after $[1-^{14}\text{C}]$ acetate labelling of *Arabidopsis* leaves

PC purified from plants labelled during the experiment described in Fig. 1 was treated with sodium methoxide and the resulting fatty acid methyl esters were separated by argentation t.l.c. □, Saturated; ●, monoenoic; △, dienoic; ○, trienoic.

protein) may show selectivity for particular molecular species (Childs *et al.*, 1985). To find out whether transfer of lipid back to the chloroplast envelope was the process that leads to enrichment of PC with labelled $\text{C}_{16:0}$ fatty acid in *Arabidopsis*, we prepared chloroplasts and

Table 2. Fatty acid composition of chloroplast and extra-chloroplastic PC

Chloroplasts and an extrachloroplastic-membrane fraction were prepared from *Arabidopsis* protoplasts as described in the Materials and methods section. Phosphatidylcholine was purified by t.l.c. from the lipids of these fractions and from the lipids of the protoplasts and leaves used in preparing them. After transmethylation the fatty acid composition of the PC was determined by g.l.c.

Fatty acid	Organ... Proportion of total PC (mol%)...	Fatty acid composition (%)			
		Chloro- plasts	Extra- chloroplast membranes	Proto- plasts	Leaves
		58.3	41.7	—	—
C _{16:0}		18.4	21.9	18.1	19.9
C _{18:0}		2.5	2.5	3.1	2.5
C _{18:1}		8.3	8.6	9.5	6.9
C _{18:2}		36.8	35.6	36.6	38.7
C _{18:3}		32.5	31.0	32.1	31.6

extrachloroplastic membranes from *Arabidopsis* mesophyll protoplasts. Only 1% of protoplast chlorophyll was present in the extrachloroplastic membranes, and less than 5% of the total PE (a negative marker for chloroplasts; Mazliak, 1977), was found in the chloroplast fraction, indicating a very low level of cross-contamination. Approx. 60% of the total PC was found in the chloroplasts and 40% in the extrachloroplast membranes (Table 2). However, the fatty acid composition of the PC in both fractions was very similar to that of the original protoplasts and the leaves from which they were derived (Table 2). These data indicate that, if PC transfer is the means by which eukaryotic pathway lipids are returned to the chloroplast, then it is the reactions of diacylglycerol formation and/or synthesis of DGG, DDG and SL that select against C_{18:0}-fatty-acid-containing species. Furthermore, the PC transfer must be freely reversible, since the chloroplast PC does not contain a higher proportion of C_{18:0} fatty acid than the extrachloroplastic pool.

Calculation of fluxes through the two pathways

The results presented above confirm the conclusion, reached from other evidence (Roughan & Slack, 1982, 1984), that the chain length of the fatty acid at position *sn*-2 of a glycerolipid molecule is an accurate predictor of whether the particular molecule has been synthesized on the prokaryotic (C₁₆ fatty acid at *sn*-2) or eukaryotic (C₁₈ fatty acid at *sn*-2) pathway. Positional analysis of labelled fatty acids (Table 1) gives results that vary according to the time that elapses between application of the radiotracer and sampling. On the other hand, a mass analysis of lipids from unlabelled leaves will reflect the net lipid metabolism of those leaves. Table 3 shows the mass and fatty acid composition of the major polar lipids of *Arabidopsis* leaves. The fatty acid composition of the lyso-derivative of each lipid (which represents the *sn*-2 position) obtained after lipase digestion is also shown. The total proportion of C₁₆ fatty acids at position *sn*-2 of each lipid is an estimate of the proportion of that lipid

Table 3. Mass and fatty acid compositions of *Arabidopsis* leaf lipids and their lyso-derivatives

Polar lipids were separated by two-dimensional t.l.c. or a combination of DEAE-cellulose column chromatography and t.l.c. The mass and fatty acid compositions of the lipids and their lyso-derivatives, resulting from digestion with *Rhizopus* lipase, were determined by g.l.c. analysis as outlined in the Materials and methods section. Abbreviation used: tr, trace.

Lipid (Lyso-derivative)	Mass of fatty acids		Fatty acid composition (mol%)						Total C ₁₆ fatty acids at <i>sn</i> -2 (mol%)
	(mol/g fresh wt.)	(mol/1000 mol)	C _{16:0}	Other C ₁₆	C _{18:0}	C _{18:1}	C _{18:2}	C _{18:3}	
DGG	3.56	390	1.0	35.6	tr	tr	2.4	60.8	70.2
DDG	1.39	152	tr	70.2	tr	tr	1.2	28.4	
PC	1.99	218	12.0	2.6	tr	1.2	3.7	79.6	11.9
PE	0.96	100	9.1	2.8	tr	tr	4.1	83.4	
PG	0.74	81	21.5	tr	1.8	4.9	34.8	36.5	1.0
SL	0.26	28	1.0	tr	tr	4.9	46.9	46.6	
PI	0.27	30	29.0	tr	1.8	3.5	37.4	27.7	
			tr	tr	tr	1.7	59.5	37.8	tr
			32.3	20.0	tr	4.9	10.6	31.5	83.5
			42.6	40.7	tr	0.7	8.0	8.0	
			43.3	—	1.2	2.1	4.7	48.7	
			63.4	—	tr	2.0	2.8	31.7	63.4
			47.5	—	3.2	1.7	23.6	23.5	
			15.9	—	2.6	2.7	40.7	35.5

Fig. 3. A flow diagram showing the fluxes (mol/1000 mol) of fatty acids during lipid synthesis by *Arabidopsis* leaves

Further abbreviations used: DAG, diacylglycerol; NFA, non-esterified fatty acid.

that is synthesized via the prokaryotic pathway, with the remainder being formed via the eukaryotic pathway.

Fig. 3 shows the fluxes of fatty acids through the prokaryotic and eukaryotic pathways during lipid synthesis by *Arabidopsis* leaves. Fluxes of other components may be calculated from the structure of the various lipid molecules. Additional information on the fatty acid composition at intermediate points is given in Table 4. The following sequence of calculations was used in the analysis.

1. An original input of 1000 mol of fatty acids synthesized in the chloroplast as acyl-ACP species has a composition calculated from Table 3, with the assumption that C_{16:0} lipid gives rise to unsaturated C₁₆ fatty acids and that polyunsaturated C_{18:1} fatty acids are derived from C_{18:1} lipid.
 2. The tissue complement of each lipid was divided between the prokaryotic and eukaryotic pathways on the basis of the content of C₁₆ fatty acids at the *sn*-2 position (Table 3).
 3. Prokaryotic lipids with the exception of PG were assumed to be 1-C₁₈, 2-C₁₆ molecular species. The molecular-species composition of these lipids produced by the eukaryotic pathway was then calculated by difference.
 4. Since chloroplast PG is known to contain di-C₁₆ molecular species (Murata, 1983), the eukaryotic PG was assumed to comprise 1-C_{16:0}, 2-C₁₈ species synthesized in the endoplasmic reticulum and mitochondria (Moore, 1974) and the fatty acid composition of the prokaryotic PG, synthesized in the chloroplast envelope (Sparace & Mudd, 1982) was calculated by difference.
 5. The distribution of PC between the chloroplast and the extrachloroplast membranes shown in Table 2 applies to the mesophyll cells from which the protoplasts were derived. Other tissues of the leaf, such as the cuticle and the vascular strands, will have a much higher proportion of their total PC in extraplastid membranes. Our preliminary estimates suggest that nearly 30% of the leaf PC is in achlorophyllous cells. On this basis 40% of the total leaf PC was assigned to the chloroplast compartment. The PC of spinach (*Spinacia oleracea*) chloroplasts has been shown to be located almost exclusively in the outer monolayer of the outer envelope membrane (Dorne *et al.*, 1984).
 6. The data in Table 3 suggest that 16% of *Arabidopsis* PI may be synthesized by the prokaryotic pathway. However, because of the small amount of lyso-PI available for g.l.c. analysis, the possibility of PI contamination with SL during t.l.c., and, in the absence of supporting enzymological or radiotracer data, only PI synthesis in the extrachloroplast compartment is shown in Fig. 3.
 7. The fatty acid compositions and fluxes at intermediate points in the two pathways (Table 4) were calculated from summing of the values downstream.
- The values shown for the metabolism of PC to DDG

Table 4. Flux and chain-length composition of fatty acids at various points in the flow diagram of lipid synthesis by *Arabidopsis* leaves

The chain-length composition of the fatty acids in eukaryotic-pathway lipids were calculated after subtracting the contribution of prokaryotic-pathway lipids as described in the Results and discussion section

Point in the pathway	Flux (mol/1000 mol of fatty acids)	Fatty acid composition (mol%)	
		C ₁₆	C ₁₈
Entering the prokaryotic pathway	385	50.1	49.9
Entering the eukaryotic pathway	615	19.0	81.0
Synthesis of PC (gross)	473	13.8	86.2
Further metabolism of PC	255	7.2	92.8
Synthesis of eukaryotic DDG	110	3.5	96.5
Synthesis of eukaryotic DDG	134	8.5	91.5
Synthesis of eukaryotic SL	10	31.7	68.3

and DDG (Fig. 3 and Table 4) are in good agreement with the estimates made from Fig. 1.

Responsibilities of the two pathways for chloroplast lipid synthesis

Overall, almost equal amounts of the chloroplast lipids are produced by the prokaryotic and eukaryotic pathways in *Arabidopsis*. However, the quantities of individual lipids synthesized by the two routes are very different. All the chloroplast PG is synthesized by the prokaryotic pathway, whereas chloroplast PC is a product of the eukaryotic pathway. The prokaryotic pathway predominates in the synthesis of DGG and SL, but DDG is derived very largely from the eukaryotic pathway.

DDG is thought to be synthesized from DGG, whereas DGG itself and SL are synthesized from diacylglycerol (Douce & Joyard, 1979). Given these relationships, why should DDG be derived mainly from the eukaryotic pathway and its precursor be derived mainly from the prokaryotic pathway? We suggest that chloroplast lipid synthesis from diacylglycerol occurs at two distinct sites: one for the prokaryotic pathway (DAG derived from chloroplast PA) and the other for the eukaryotic pathway (diacylglycerol derived from PC of the endoplasmic reticulum). The two sites may correspond to the inner and outer chloroplast envelopes. The degree of access to each site by the enzymes of DGG, DDG and SL synthesis could then explain why the prokaryotic and eukaryotic pathways produce different proportions of each of these lipids. It is also possible that distinct isoenzymes may exist at the two sites.

The DGG, DDG and SL synthesized via the eukaryotic pathway each shows a fatty acid composition (Table 4) that is distinct from that of PC, their common precursor. Thus synthesis of both DGG and DDG discriminates against molecular species containing $C_{16:0}$ fatty acids, but DDG contains more $C_{16:0}$ fatty acid than does DGG. On the other hand, the SL produced by the eukaryotic pathway is considerably enriched in $C_{16:0}$ fatty acids relative to PC. The molecular-species specificities of these synthetic reactions contribute in determining the overall fatty acid composition of each lipid (Table 3) and may therefore be necessary to produce the precise mixture of lipid species required for proper functioning of the thylakoid membranes.

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