Biochem. J. (1989) 259, 775–783 (Printed in Great Britain)



# Comparison of glycerolipid biosynthesis in non-green plastids from sycamore (*Acer pseudoplatanus*) cells and cauliflower (*Brassica oleracea*) buds

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The availability of methods to fractionate non-green plastids and to prepare their limiting envelope membranes [Alban, Joyard & Douce (1988) Plant Physiol. **88**, 709–717] allowed a detailed analysis of the biosynthesis of lysophosphatidic acid, phosphatidic acid, diacylglycerol and monogalactosyldiacylglycerol (MGDG) in two different types of non-green starch-containing plastids: plastids isolated from cauliflower buds and amyloplasts isolated from sycamore cells. An enzyme [acyl-ACP (acyl carrier protein):*sn*-glycerol 3-phosphate acyltransferase) recovered in the soluble fraction of non-green plastids transfers oleic acid from oleoyl-ACP to the *sn*-1 position of *sn*-glycerol 3-phosphate to form lysophosphatidic acid. Then a membrane-bound enzyme (acyl-ACP:monoacyl-*sn*-glycerol 3-phosphate acyltransferase), localized in the envelope membrane, catalyses the acylation of the available *sn*-2 position of 1-oleoyl-*sn*-glycerol 3-phosphate by palmitic acid from palmitoyl-ACP. Therefore both the soluble phase and the envelope membranes are necessary for acylation of *sn*-glycerol 3-phosphate. The major difference between cauliflower (*Brassica oleracea*) and sycamore (*Acer pseudoplatanus*) membranes is the very low level of phosphatidate phosphatase activity in sycamore envelope membrane. Therefore, very little diacylglycerol is available for MGDG synthesis in sycamore, compared with cauliflower. These findings are consistent with the similarities and differences described in lipid metabolism of mature chloroplasts from '$C_{18:3}$' and '$C_{16:3}$' plants (those with MGDG containing $C_{18:3}$ and $C_{16:3}$ fatty acids). Sycamore contains only $C_{18}$ fatty acids in MGDG, and the envelope membranes from sycamore amyloplasts have a low phosphatidate phosphatase activity and therefore the enzymes of the Kornberg–Pricer pathway have a low efficiency of incorporation of *sn*-glycerol 3-phosphate into MGDG. By contrast, cauliflower contains MGDG with $C_{16:3}$ fatty acid, and the incorporation of *sn*-glycerol 3-phosphate into MGDG by the enzymes associated with envelope membranes is not limited by the phosphatidate phosphatase. These results demonstrate that: (1) non-green plastids employ the same biosynthetic pathway as that previously established for chloroplasts (the formation of glycerolipids is a general property of all plastids, chloroplasts as well as non-green plastids), (2) the envelope membranes are the major structure responsible for the biosynthesis of phosphatidic acid, diacylglycerol and MGDG, and (3) the enzymes of the envelope Kornberg–Pricer pathway have the same properties in non-green starch-containing plastids as in mature chloroplasts from $C_{16:3}$ and $C_{18:3}$ plants.

## INTRODUCTION

Plastids are able to incorporate *sn*-glycerol 3-phosphate into lysophosphatidic acid (LPA), phosphatidic acid (PA), diacylglycerol (DG) and MGDG (after addition of UDP-galactose) as shown by Douce & Guillot-Salomon (1970). However, most of these data concern chloroplasts (for reviews, see Heinz, 1977; Douce & Joyard, 1980; Roughan & Slack, 1982; Joyard & Douce, 1987); the enzymes of the Kornberg–Pricer pathway have been localized within the chloroplast (Bertrams & Heinz, 1976; Joyard & Douce, 1977; Joyard, 1979; Bertrams & Heinz, 1980; Block *et al.*, 1983; Frentzen *et al.*, 1983; Andrews *et al.*, 1985); the selectivities and specificities of the acyltransferases have been clearly determined (Joyard, 1979; Bertrams & Heinz, 1981; Frentzen *et al.*, 1983), and finally, similarities and differences have been described in lipid metabolism of chloroplasts isolated from $C_{18:3}$ and $C_{16:3}$ plants (Roughan & Slack, 1982; Heinz & Roughan, 1983; Frentzen *et al.*, 1983; Browse *et al.*, 1986). In contrast, very little information is available on non-green plastids; studies have been done using maize (*Zea mays*) etioplasts (Douce & Guillot-Salomon, 1970), chromoplasts (Liedvogel *et al.*, 1978; Liedvogel & Kleinig, 1979), potato (*Solanum tuberosum*) tuber amyloplasts (Fishwick & Wright, 1980) and proplastids from cauliflower buds (Journet & Douce, 1985). The purpose of the present study was to determine whether non-green plastids behave like chloroplasts as far as glycerolipid biosynthesis is concerned.

Abbreviations used: MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SL, sulpholipid; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PA, phosphatidic acid; LPA, lysophosphatidic acid; MG, monoacylglycerol; DG, diacylglycerol; UDP-Gal, UDP-galactose; PMSF, phenylmethanesulphonyl fluoride; ACP, acyl carrier protein; $C_{18:3}$ and $C_{16:3}$ plants, plants with MGDG containing $C_{18:3}$ and $C_{16:3}$ fatty acids.

## MATERIALS AND METHODS

### Chemicals

[1-$^{14}$C]Palmitoyl-ACP (58 Ci/mol), [9,10-$^{3}$H]-palmitoyl-ACP (55 Ci/mol) and [1-$^{14}$C]oleoyl-ACP (56 Ci/mol) were synthesized by using ACP from *Escherichia coli* (kindly supplied by Dr. J. B. Ohlrogge, Michigan State University, East Lansing, U.S.A.). [1-$^{14}$C]-Palmitoyl-CoA (26 Ci/mol), *sn*-[$^{14}$C]glycerol 3-phosphate (170 Ci/mol) and UDP-[$^{14}$C]galactose (333 Ci/mol) were purchased from New England Nuclear.

### Plants

Cauliflower (*Brassica oleracea* L.) buds were purchased from local markets. Sycamore (*Acer pseudoplatanus* L.) cells were grown as a suspension in a liquid medium as described by Bligny (1977).

### Preparation of cauliflower bud plastids

The top 1 cm of cauliflower florets from 15 kg of total inflorescence (corresponding to about 4–5 kg of tissue) were disrupted with a 1-gallon Waring Blendor at low speed for 3 s in chilled extraction medium (1 litre/kg of tissue) containing 0.3 M-mannitol, 20 mM-tetrasodium pyrophosphate, 4 mM-cysteine, 1 mM-EDTA, 0.1% (w/v) defatted bovine serum albumin, pH 7.6 (adjusted with HCl). The plant material was homogenized in three successive batches. All the operations were carried out at 0–4 °C. A crude plastid pellet was obtained as described by Journet & Douce (1985). This crude preparation was then purified twice by isopycnic centrifugation in Percoll gradients (Journet & Douce, 1985). Integrity and purity of the plastids was measured as described by Journet & Douce (1985). Starting from 4–5 kg of material, the yield of highly purified intact plastids was 50–80 mg of proteins.

### Preparation of sycamore cell plastids

Sycamore plastids were prepared from protoplasts. A sample (150–200 g) of sycamore cells were harvested during exponential growth (3–5 days after the beginning of the culture). The evening before the experiment the cells were transfered to a sucrose-free medium in order to decrease the amount of starch within amyloplasts [about 15 h of starvation are necessary, as found by Journet *et al.* (1986)]. This step was essential to improve the yield of intact plastids at the end of the preparation. The cells were washed twice in culture medium and 0.5 M-mannitol. Protoplasts were then prepared essentially by the method previously described by Journet *et al.* (1986). The protoplasts pellet was then washed once and resuspended in 150 ml of the following medium: 0.5 M-mannitol/20 mM-Mops/NaOH (pH 7.5)/2 mM-EDTA/bovine serum albumin (1 g/l)/0.4 mM-spermidine/7 mM-$\beta$-mercaptoethanol/1 mM-phenylmethylsulphonyl fluoride (PMSF)/10 $\mu$M-leupeptine/1 mM-benzamidine hydrochloride/5 mM-6-aminohexanoic acid/1% (w/v) insoluble polyvinylpyrrolidone (K c.a. 25; Serva). Rupture of the protoplasts was achieved by two successive filtrations on a 20 $\mu$m-mesh-size nylon blutex. A crude plastid preparation was then obtained by fractionation of the whole homogenate by differential centrifugations, essentially as described by Journet *et al.* (1986). Aliquots (2 ml) of the crude plastid pellet were then layered on top of 8 ml of 50% (v/v) Percoll cushions containing 0.5 M-mannitol/10 mM-Mops/NaOH (pH 7.5)/2 mM-EDTA/10 $\mu$M-leupeptin/1 mM-PMSF/1 mM-benzamidine hydrochloride/5 mM-6-aminohexanoic acid. After 10 min centrifugation at 1500 *g* (Sorvall, HB 4), purified plastid pellets were recovered. The plastids obtained were almost devoid of contamination by extraplastidial membranes (mitochondria) and cytosol, as judged by the essential absence of marker enzymes for these compartments. From 150–200 g of sycamore cells, the yield of purified intact plastid was about 1–1.5 mg of protein.

### Preparation of envelope membranes from cauliflower bud plastids or sycamore cell amyloplasts

Intact cauliflower plastids (0.5 ml, 50–80 mg of protein) were frozen (for 30 min at −80 °C), then thawed to room temperature, treated with 3 ml of hypo-osmotic swelling medium (10 mM-Tricine/NaOH(pH 7.8)/4 mM-$MgCl_2$) and homogenized smoothly with a Potter–Elvehjem apparatus with a loose-fitting pestle (Alban *et al.*, 1988). The broken plastid suspension (3.5 ml, 50–80 mg of protein) was layered on top of a continuous or a discontinuous sucrose gradient as described by Alban *et al.* (1988). The membrane fraction corresponding to the envelope membranes was recovered by centrifugation as described by Alban *et al.* (1988). By using this procedure, the yield of purified envelope membranes was 1–1.5 mg of protein (i.e. about 2% of the plastid proteins), starting from 4–5 kg of cauliflower buds.

The preparation of envelope membranes from sycamore cells was by a similar procedure. The differences from the method described above are the following: (*a*) a simple osmotic shock was sufficient for disruption of the envelope membranes; (*b*) the swelling medium as well as the different layers of the sucrose gradient were devoid of $MgCl_2$; (*c*) proteinase inhibitors were added in all the medium used and at all the steps of the purification (in absence of these inhibitors, the envelope polypeptide pattern was totally altered). With sycamore amyloplasts, only a starch pellet, devoid of green membranes, was obtained at the bottom of the tube. From 150–200 g of isolated sycamore cells, the yield of purified envelope membranes was only 0.2–0.3 mg of protein (i.e. about 20–30% of the amyloplast proteins).

### Measurements of enzymic activities in plastid subfractions

We measured the distribution of enzymes in 100 $\mu$l aliquots of the different fractions obtained.

Glucose-6-phosphate isomerase (phosphoglucose isomerase) was assayed as described by Simcox *et al.* (1977).

Determination of MGDG synthase (or UDP-galactose:diacylglycerol galactosyltransferase) was done by monitoring the incorporation of galactose from UDP-[$^{14}$C]galactose into MGDG, as described by Douce (1974).

Acyl-CoA (ACP):*sn*-glycerol-3-phosphate acyltransferase and acyl-CoA (ACP):monoacyl-*sn*-glycerol-3-phosphate acyltransferase were measured as described by Joyard & Douce (1977). In most cases, we used acyl-CoA rather than acyl-ACP, the physiological acyl donor (see below). The use of acyl-ACP was restricted to studies of the specificity and the selectivity of the enzyme.

We have also monitored the time course of LPA, PA, DG and MGDG formation in mixtures of envelope

membranes and stroma. The experiments were done essentially as described by Joyard & Douce (1977).

After these various incubations, lipids from the reaction mixtures were extracted with chloroform/methanol (1:1, v/v) by the method of Hajra (1974). They were chromatographed on silica-gel 60 t.l.c. plates (Merck), with chloroform/methanol/water (65:25:4, by vol.) as solvent. Glycerolipids were located under u.v. light (360 nm) after spraying the plates with anilinonaphthalene sulphonate (0.2%, w/v, in methanol). Individual lipids were identified by their reactions with specific spray reagents and by comparing their values with those of reference standards.

### Determination of the specificities and selectivities of acyltransferases

The methods used were based on those described by Frentzen *et al.* (1983).

For the acyl-CoA(ACP):*sn*-glycerol-3-phosphate acyltransferase, stroma from cauliflower plastids were incubated at 25 °C in 0.25 M-Mops/NaOH, pH 7.1, containing 1 mM-*sn*-glycerol 3-phosphate, 15 $\mu$g of bovine serum albumin, 2 $\mu$M-acyl-ACP (as indicated, $^3$H- and/or $^{14}$C-labelled palmitoyl- and/or oleoyl-ACP) and stroma corresponding to 50 $\mu$g of protein, in a total volume of 40 $\mu$l. After 1 h incubation, the pH of the incubation mixture was lowered to 6.0 by the addition of HCl, in order to dephosphorylate the LPA synthesized into MG (as described by Joyard & Douce, 1977). After 30 min of incubation at pH 6.0, the lipids were extracted by the procedure of Hajra (1974). 1-Acyl- and 2-acyl-*sn*-glycerol were separated on silica-gel t.l.c. plates impregnated with boric acid [4% (w/v) in 0.01% (w/v) $Na_2CO_3$] in the following solvent: chloroform/acetone (17:5, v/v). The radioactive spots were located by autoradiography. The position of 1-acyl- and 2-acyl-*sn*-glycerol was determined by comparison of the $R_F$ values of the radioactive spots with those of standards.

Studies with acyl-CoA(ACP):MG 3-phosphate acyltransferase were made at 25 °C in the following incubation mixture: 0.25 M-Mops/NaOH, pH 7.4, 30 $\mu$g of bovine serum alumin, 25 $\mu$M-LPA (i.e.1-oleoyl-*sn*-glycerol 3-phosphate, synthesized from chloroplast stroma as described by Joyard & Douce, 1977), 0.8 $\mu$M-[$^3$H]palmitoyl-ACP, 0.4–1.6 $\mu$M-[$^{14}$C]palmitoyl-CoA, as indicated, and cauliflower envelope membranes corresponding to 25 $\mu$g of protein, in a total volume of 40 $\mu$l. After 2 min incubation the reaction was stopped by the addition of chloroform/methanol (1:1, v/v) and the lipids were extracted by the method of Hajra (1974) and analysed as described above.

### Analyses of fatty acids in plastid galactolipids

Envelope lipids were extracted as described above. Glycerolipids were separated by two-dimensional t.l.c., and the fatty acids from MGDG and DGDG were analysed by gas chromatography as described by Douce & Joyard (1980).

### Other assays

Proteins were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. The radioactivity was determined using a scintillation counter (Intertechnique) after addition of 10 ml of ACS medium (Amersham) to the samples to be analysed.

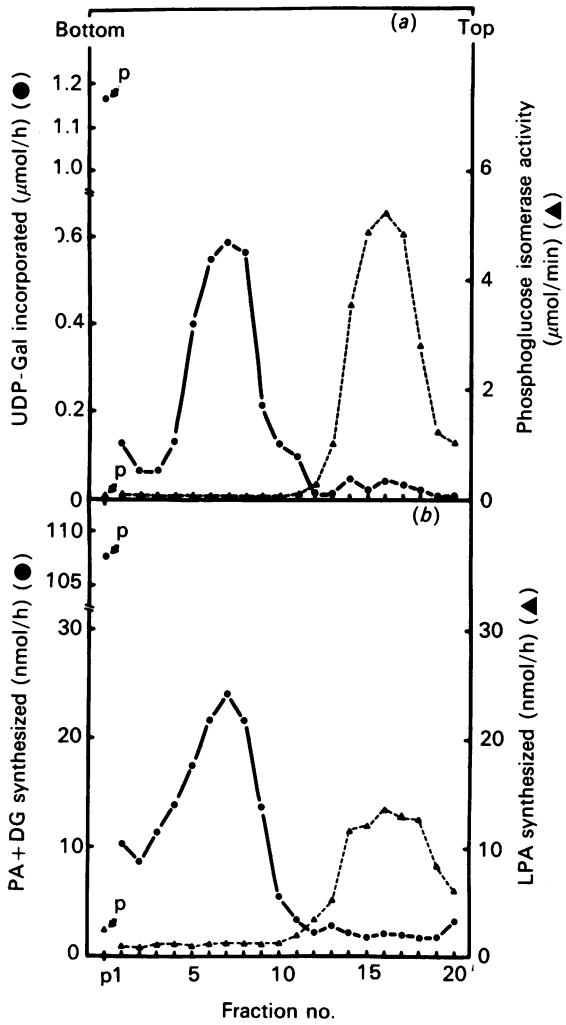


**Fig. 1. Distribution of marker enzymes (*a*) and acyltransferases (*b*) along the linear sucrose gradient used for fractionation of cauliflower bud plastids**

Experimental details for the rupture of plastids, sucrose-gradient centrifugation and enzyme assays are given in the Materials and methods section. We used cauliflower plastids corresponding to 60 mg of protein for this fractionation. About 20 fractions (650 $\mu$l each) were collected and assayed for enzyme activities. Glucose-6-phosphate isomerase (phosphoglucose isomerase activity) was used as a marker for stroma. MGDG synthase (UDP-galactose incorporated) was used as a marker for envelope membranes. The total recovery of the proteins and of the activities in the different fractions analysed was between 95 and 99% of that loaded on top of the gradient. Abbreviation: p, pellet.

## RESULTS AND DISCUSSION

### Localization of the enzymes of the Kornberg–Pricer pathway in cauliflower plastids and in sycamore amyloplasts

We analysed the distribution of acyl-CoA(ACP):*sn*-glycerol-3-phosphate acyltransferase, acyl-CoA (ACP):MG 3-phosphate acyltransferase, MGDG synthase (a marker enzyme for envelope membranes) and phosphoglucose isomerase (a marker enzyme for the stroma), along the sucrose gradient used for the fraction-

ation of cauliflower plastids (Fig. 1). As expected, phosphoglucose isomerase was found exclusively in the top part of the gradient, whereas MGDG synthase was mostly present in the yellow band corresponding to the plastid envelope (Alban *et al.*, 1988). The two acyltransferases were clearly localized in two different fractions: the first acyltransferase, involved in LPA synthesis, and the second acyltransferase, involved in the synthesis of PA, were respectively found in the soluble fraction and the envelope membrane fraction. Some activity for the second acyltransferase and for MGDG synthase was recovered in the green pellet (see below).

The localization of acyltransferases was also carried out in sycamore amyloplasts. Fig. 2 demonstrates that, again, the first acyltransferase was found in the stroma phase, whereas synthesis of PA was possible only after addition of envelope membranes.

These results are identical with those found in chloroplasts (Joyard & Douce, 1977), but contrast with those described for potato tuber amyloplasts by Fishwick & Wright (1980), where the first acyltransferase was found to be bound to the amyloplast envelope membranes. Indeed, Fig. 3 clearly demonstrates that envelope membranes from sycamore amyloplasts and from cauliflower bud plastids are unable to incorporate directly *sn*-[$^{14}$C]glycerol 3-phosphate into glycerolipids; addition of a soluble fraction containing the first acyltransferase is absolutely necessary for further synthesis of PA by envelope membranes. In fact, Fishwick & Wright (1980) used amyloplasts from potato tubers, which are extremely difficult to obtain pure and intact, owing to the huge starch grains they contain. We believe that this could explain the contradictory results obtained.

Together, these results demonstrate that starch-containing plastids behave exactly like chloroplasts with respect to the recovery of the first acyltransferase in the soluble phase of plastids. It is likely that this enzyme should work in close association with the envelope inner membrane, since LPA synthesized is directly transferred into the membrane during synthesis (Joyard & Douce, 1977). The velocity of the incorporation of *sn*-[$^{14}$C]glycerol 3-phosphate into PA is lower (especially in sycamore amyloplasts) than that found in chloroplasts: 5–10, 30–60 and 80–150 nmol of *sn*-glycerol 3-phosphate were incorporated/h per mg of envelope protein respectively for sycamore, cauliflower and spinach (*Spinacia oleracea*) envelope membranes. This could reflect the various requirements for glycerolipid biosynthesis in the different plastids analysed; sycamore amyloplasts are almost restricted to their limiting envelope membranes, whereas in chloroplasts, thylakoids represent the bulk of membranes and thus more lipids are necessary for membrane biogenesis.

Some acyltransferase and MGDG synthase activities were also recovered in the green pellet from cauliflower bud plastids (see Fig. 1). The presence of green membranes (containing very low amounts of chlorophyll, i.e. 0.5 $\mu$g/mg of protein) is attributable to the few internal membranes of cauliflower plastids (Journet & Douce, 1985). The presence of MGDG synthase in this pellet is due to a contamination by envelope membranes; after two careful washings of the green pellet, MGDG synthase activity in the pellet is less than 5% (on a protein basis) of the activity in envelope membranes (Fig. 4*a*). We have verified that the substrate (DG) was not limiting in this experiment. Several other envelope enzymes (acyl-CoA synthetase and thioesterase) are also removed by washing of the pellet (Alban *et al.*, 1988). In contrast, several washings are so efficient at removing acyl-CoA (ACP):MG 3-phosphate acyltransferase; more than 25% (on a protein basis) of the initial activity remains in the pellet after two successive washings (Fig. 4*b*). Thus it is possible that some membrane-bound acyltransferase is present in the internal membranes of cauliflower plastids. In fact, these internal membranes are clearly connected to the envelope inner membrane (Journet & Douce, 1985). In addition, electrophoretic analyses of the different membrane fractions suggest that some polypeptides are present in both membrane systems (Alban *et al.*, 1988). Thus it is possible that the membrane-bound acyltransferase is present in both envelope and internal membranes from cauliflower plastids. The situation is different from that found in mature chloroplasts: the membrane-bound acyltransferase is concentrated in the envelope membranes and no connection between thylakoids and the envelope inner membrane is visible [see Joyard & Douce (1987) for a discussion of this problem].

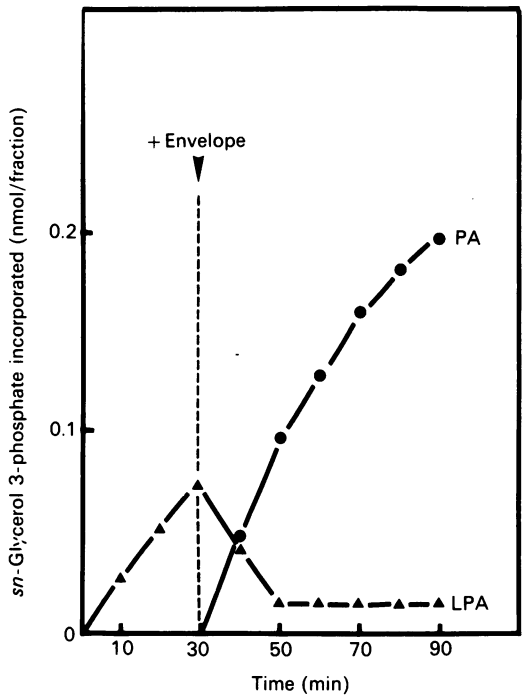


**Fig. 2. Biosynthesis of LPA and PA by stroma and envelope membranes from sycamore-cell amyloplasts**

The soluble enzymes from amyloplasts (equivalent to 410 $\mu$g of protein) were first incubated in presence of *sn*-[$^{14}$C]glycerol 3-phosphate (1 mM, 3.3 mCi/mmol) and oleoyl-CoA (100 $\mu$M). After 30 min incubation (arrow), envelope membranes from sycamore amyloplasts (equivalent to 300 $\mu$g of protein) were added, together with palmitoyl-CoA (100 $\mu$M). The volume of each fraction was 100 $\mu$l. The experimental conditions for preparation of stroma and envelope membranes from sycamore amyloplasts and for assay of the enzymes are described in the Materials and methods section. Note that LPA is synthesized in the stroma fraction, whereas PA is formed only when envelope membranes are present.

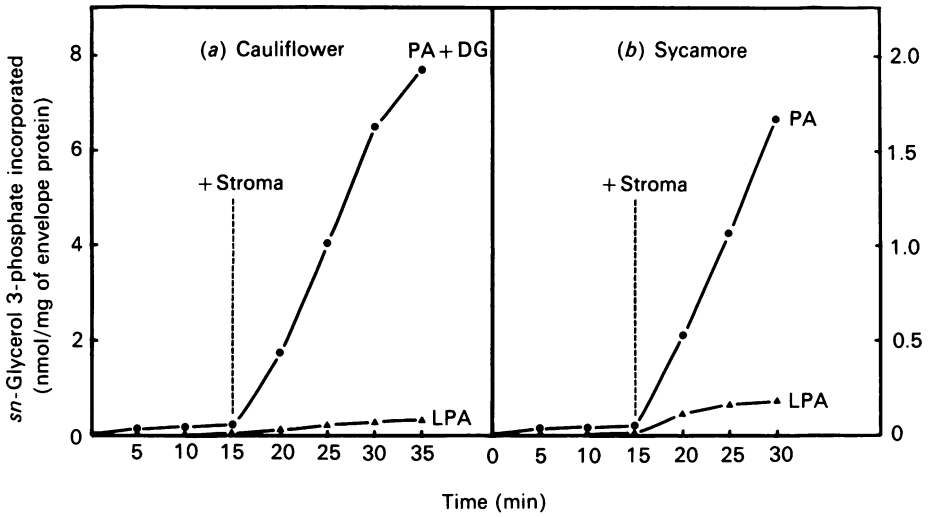


**Fig. 3. Biosynthesis of LPA and PA by envelope membranes and stroma from cauliflower plastids (*a*) and from sycamore amyloplasts (*b*)**

The experimental conditions are similar to those of Fig. 2, except that envelope membranes were first incubated in absence of any soluble enzymes, which were added after 15 min incubation (broken line). Note that the envelope membranes are unable to form LPA in the absence of the stroma fraction. When both envelope membranes and stroma are present, LPA and PA are formed. The amounts of protein used were: (*a*) 400 $\mu$g and 1 mg of protein respectively for envelope and stroma from cauliflower plastids (total volume 800 $\mu$l; volume of each fraction 100 $\mu$l); (*b*) 200 $\mu$g and 450 $\mu$g of protein respectively for envelope and stroma from sycamore amyloplasts (total volume 700 $\mu$l; volume of each fraction 100 $\mu$l).

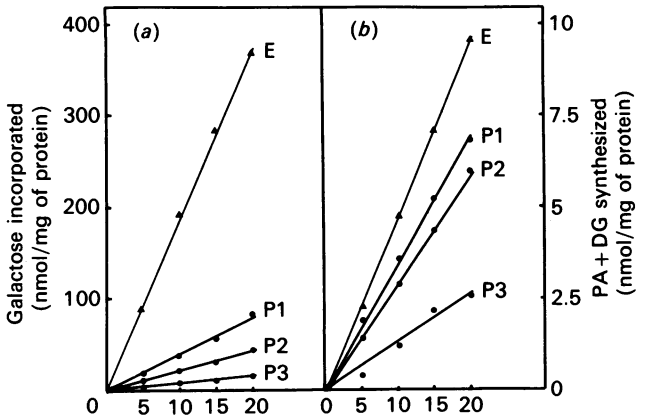


**Fig. 4. Time-course of incorporation of galactose from UDP-[$^{14}$C]galactose and *sn*-[$^{14}$C]glycerol 3-phosphate into envelope (E) and pellet (P) from cauliflower plastids**

The experimental conditions for preparation and for assays of enzymic activities are described in the Materials and methods section. P1 corresponds to the crude membrane pellet, whereas P2 and P3 correspond to the membranes obtained after one and two washings respectively. The washing of the membrane fractions was done as described by Alban *et al.* (1988). The amounts of protein used were: (*a*) 225 $\mu$g of protein from envelope and pellet (total volume 900 $\mu$l; volume of each fraction 200 $\mu$l); (*b*) 250 $\mu$g of protein for envelope and pellet, and 3 mg of protein of stroma, which is necessary for LPA biosynthesis (total volume 500 $\mu$l; volume of each fraction 100 $\mu$l).

## Specificities and selectivities of the acyltransferases from cauliflower bud plastids

The soluble acyltransferase from cauliflower plastids, when incubated in presence of palmitoyl- and/or oleoyl-ACP, led almost exclusively to the formation of 1-acyl-*sn*-glycerol 3-phosphate, as shown in Table 1. This is just like what has been shown for the chloroplast acyltransferase by Joyard (1979) and Bertrams & Heinz (1981), using acyl-CoA, and by Frentzen *et al.* (1983), who used acyl-ACP. The same positional distribution was obtained with either a short or a long incubation time. However, the rates of acylation were much higher with oleoyl-ACP than with palmitoyl-ACP (Table 1); this is somewhat different from the chloroplast situation, where similar rates were obtained (Frentzen *et al.*, 1983). Finally, the specificity for oleic acid was confirmed when mixtures of palmitoyl- and oleoyl-ACP were offered (Table 1). Therefore the characteristic property of the soluble acyltransferase, namely to direct oleic acid from oleoyl-ACP to the *sn*-1 position of *sn*-glycerol 3-phosphate, can be extended from chloroplasts to non-green plastids.

Acyl-CoA(ACP):MG 3-phosphate acyltransferase, i.e. the membrane-bound acyltransferase, converts 1-oleoyl-*sn*-glycerol 3-phosphate into PA; therefore, the only position available for further esterification is the *sn*-2 position. Thus the membrane-bound acyltransferase cannot display any positional specificity. When a mixture of palmitoyl-ACP and oleoyl-ACP was offered, almost all (more than 95%) of the fatty acids incorporated into PA was palmitic acid. This is in complete agreement with data obtained with chloroplast envelopes using acyl-CoA (Joyard, 1979) or acyl-ACP (Frentzen *et al.*, 1983) thioesters. To investigate whether the membrane-bound acyltransferase displays a specificity towards acyl-ACP instead of acyl-CoA, envelope membranes from cauliflower plastids were incubated in presence of LPA and mixtures of palmitoyl-ACP and palmitoyl-CoA. Fig. 5 demonstrates that, even with excess acyl-CoA thioesters, the major part of the palmitic acid esterifying the *sn*-2 position of PA was derived from acyl-ACP and not from acyl-CoA.

**Table 1. Specificities and selectivities of acyl-ACP:*sn*-glycerol 3-phosphate acyltransferase from cauliflower plastids**

The soluble enzymes from the stroma were prepared from cauliflower plastids and assayed as described in the Materials and methods section. The amount of protein used for each assay was 50 $\mu$g. The incubation mixtures contained the following radioactive substrates: 2 $\mu$M-[$^{14}$C]palmitoyl('16:0')-ACP or [$^{14}$C]oleoyl('18:1')-ACP, or 1 $\mu$M-[$^{3}$H]palmitoyl-ACP + 1 $\mu$M-[$^{14}$C]oleoyl-ACP (total vol. 40 $\mu$l). After 1 h incubation, the pH of the incubation mixture was lowered to 6.0 by addition of HCl in order to permit the enzymic dephosphorylation of LPA into MG. MGs esterified at the *sn*-1 or *sn*-2 position were analysed as described in the Materials and methods section.

| Product formed... | 1-Acyl-*sn*-glycerol | | 2-Acyl-*sn*-glycerol | |
|---|---|---|---|---|
| Substrate(s) | (pmol/mg of protein) | (%) | (pmol/ml of protein) | (%) |
| [$^{14}$C]16:0-ACP | 80 | 91.6 | 7.3 | 8.4 |
| [$^{14}$C]18:1-ACP | 1022 | 99.6 | 4.1 | 0.4 |
| [$^{3}$H]16:0-ACP + [$^{14}$C]18:1-ACP | 761 | 98.5 | 11.6 | 1.5 |
| Percentage of total radioactivity in 1-acyl-*sn*-glycerol | | | | |
| From $C_{16:0}$ fatty acid... | | 22 | | |
| From $C_{18:1}$ fatty acid... | | 78 | | |

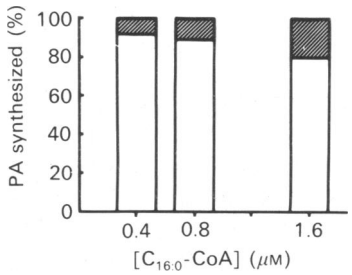


**Fig. 5. Preference for ACP-thioesters and CoA-thioesters by acyl-ACP (CoA):monoacylglycerol-3-phosphate acyltransferase from cauliflower plastid envelope membranes**

Envelope membranes (corresponding to 25 $\mu$g of protein) were incubated for 2 min in presence of 25 $\mu$M-LPA, 0.8 $\mu$M-[$^{3}$H]palmitoyl-ACP and various amounts of [$^{14}$C]-palmitoyl-CoA (total volume 40 $\mu$l). The experimental conditions for incubation and analyses are described in the Materials and methods section. ▨, from $C_{16:0}$-CoA; □, from $C_{16:0}$-ACP.

In conclusion, these experiments demonstrate that the specificities and selectivities for acylation of *sn*-glycerol 3-phosphate are identical in chloroplasts and non-green plastids. The first step is acylation of *sn*-glycerol 3-phosphate at the *sn*-1 position using oleic acid from oleoyl-ACP; 1-oleoylglycerol 3-phosphate is then esterified at the available *sn*-2 position, using palmitic acid from palmitoyl-ACP. The resulting PA has a typical prokaryotic structure. This PA can then be used for diacylglycerol and MGDG biosynthesis.

### Diacylglycerol and MGDG formation in isolated envelope membranes

Using envelope membranes from sycamore amyloplasts and from cauliflower bud plastids, we have monitored the incorporation of *sn*-glycerol 3-phosphate into LPA, PA, DG and MGDG. Fig. 6 demonstrates that different patterns were obtained. In both cases, PA formation is linear with time for almost all of the experiment. However, before addition of UDP-Gal, the major difference between cauliflower and sycamore envelope membranes is the apparent lack of DG formation in sycamore. In contrast, DG formation almost parallels PA synthesis in cauliflower envelope membranes (Fig. 6). Since the chloroplast envelope PA phosphatase had a rather alkaline optimum pH (Joyard & Douce, 1979), we measured the effect of pH on DG formation in cauliflower and sycamore envelopes. Fig. 7 demonstrates that the low level of DG formation in sycamore envelope membranes is not due to a different pH optimum; only 10–25% of PA was converted into DG in sycamore envelope membranes, whereas in cauliflower the level of DG synthesized was almost the same as that of PA. In fact, this difference was expected, because sycamore, in contrast with cauliflower, has MGDG molecules almost devoid of $C_{16}$ fatty acids; as shown in Table 2, sycamore contains only MGDG with $C_{18}$ fatty acids, i.e. the so-called 'eukaryotic' structure for glycerolipids (see Heinz, 1977), whereas cauliflower MGDG contains both $C_{16:3}$ and $C_{18:3}$. This distinction between '$C_{16:3}$' and '$C_{18:3}$' plants is strongly documented (see, for instance, Heinz, 1977; Siebertz *et al.*, 1979; Harwood, 1980; Joyard & Douce, 1987) and is due to major differences in the biosynthesis of plastid glycerolipids (Heinz & Roughan, 1983). Sycamore amyloplasts, as '$C_{18:3}$' chloroplasts [such as pea (*Pisum sativum*)], have a very low PA phosphatase activity (Heinz & Roughan, 1983; Frentzen *et al.*, 1983; Gardiner & Roughan, 1983). In contrast, cauliflower plastids, like $C_{16:3}$ chloroplasts (such as those of spinach), present a very active PA phosphatase. Gardiner *et al.* (1984) and Kosmac & Feierabend (1985) have suggested that the low level of DG formed in these plants was due to a non-enzymic breakdown. To analyse this we have monitored the incorporation of *sn*-glycerol 3-phosphate into DG using envelope membranes from sycamore amyloplasts. After 1 h incubation, the mixture was boiled and the formation of PA and DG was monitored. Fig. 8 demonstrates that boiling immediately stops PA and DG formation, despite the high level of PA already formed. This result suggests that diacylglycerol formation in envelope membranes from sycamore cells

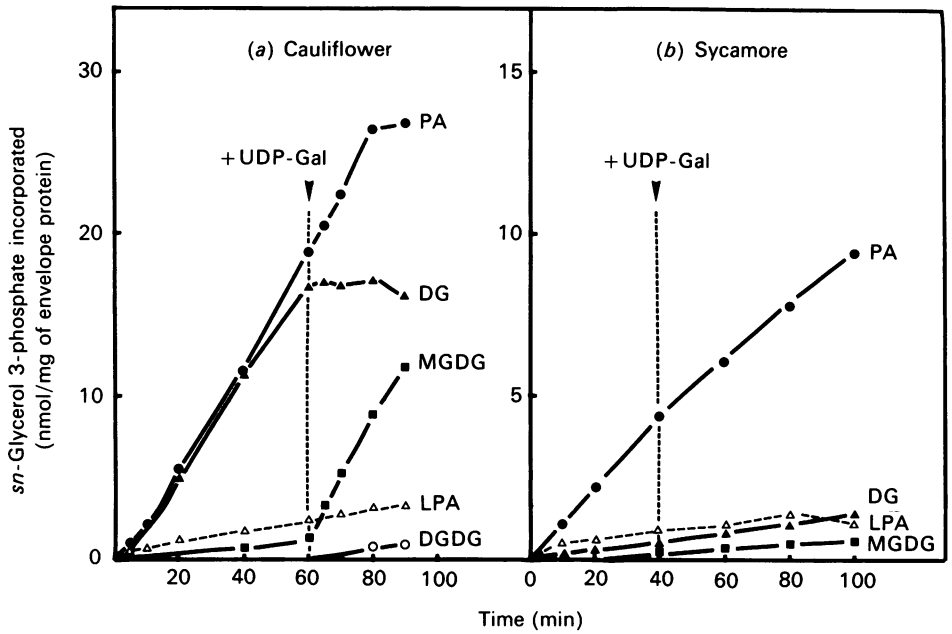


**Fig. 6. Effect of UDP-galactose on the incorporation of *sn*-[$^{14}$C]-glycerol 3-phosphate into envelope lipids from cauliflower plastids (*a*) and sycamore amyloplasts (*b*)**

The experimental conditions for the preparation of the fractions, incubation and analyses of the labelled compounds are described in the Materials and methods section. Note that the major difference between cauliflower and sycamore is the very low level of DG (and therefore of MGDG) formed in sycamore. The amounts of protein used were: (*a*) 1 mg and 500 $\mu$g of protein respectively for stroma and envelope membranes from cauliflower plastids (total volume 1 ml; volume of each fraction 100 $\mu$l); (*b*) 410 $\mu$g and 300 $\mu$g of protein respectively for stroma and envelope membranes from sycamore amyloplasts (total volume 700 $\mu$l; volume of each fraction 100 $\mu$l).

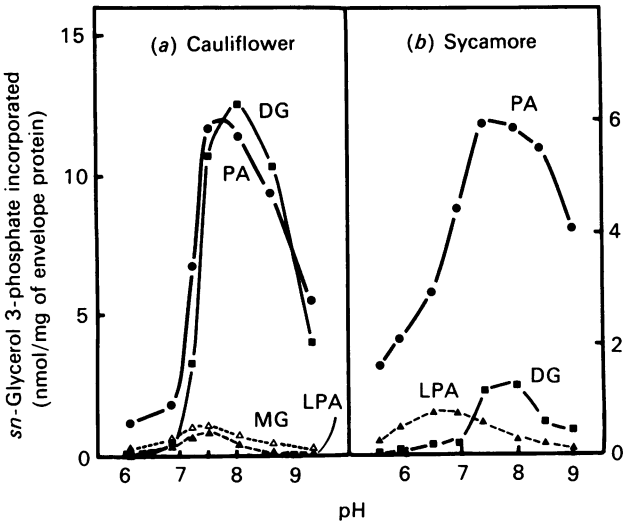


**Fig. 7. Effect of pH on the incorporation of *sn*-[$^{14}$C]glycerol 3-phosphate into envelope lipids from cauliflower plastids (*a*) and sycamore amyloplasts (*b*)**

The experimental conditions for preparation of the fractions, incubation and analyses of the synthesized glycerolipids are described in the Materials and methods section. The different buffers used were: pH 5.5–6.5, 50 mM-Mes; pH 7–7.5, 50 mM-Mops; pH 8–9, 50 mM-Tricine/NaOH. Incubation was for 1 h. The amounts of protein used for each condition were: (*a*) 200 $\mu$g and 100 $\mu$g respectively for stroma and envelope membranes from cauliflower plastids; (*b*) 55 $\mu$g and 25 $\mu$g respectively for stroma and envelope membranes from sycamore amyloplasts. The total volume for each incubation was 100 $\mu$l.

was indeed enzymic. It is not yet known, however, whether this reduced level of activity is due to a lower amount of enzyme or to the presence of regulatory molecules which control the activity of the enzyme.

Finally, the difference in the level of diacylglycerol synthesis in plastid envelope membranes from cauliflower and from sycamore led to different levels of MGDG synthesis after addition of UDP-Gal (Fig. 6). Immediately after addition of UDP-Gal, MGDG is formed at the expense of DG in cauliflower envelopes; MGDG synthesis proceeds at a rate obviously identical with that of DG formation. In envelope membranes from sycamore amyloplasts, MGDG synthesis proceeds at a low rate, most of the radioactivity incorporated into glycerolipids being recovered in PA (Fig. 6).

In conclusion, we have found that amyloplasts from sycamore cells and plastids from cauliflower buds contain the same biosynthetic pathways for LPA, PA, DG and MGDG as mature chloroplasts. In addition, the similarities and differences described in lipid metabolism of mature chloroplasts from $C_{18:3}$ and $C_{16:3}$ plants by Heinz & Roughan (1983) are also valid for non-green plastids. Despite the strong structural differences, amyloplasts from sycamore cells and pea chloroplasts do not behave differently as far as glycerolipid biosynthesis is concerned. The same is true for cauliflower bud plastids and spinach chloroplasts. In fact, one must bear in mind that, despite their major structural differences, a common feature shared by all kinds of plastids is their limiting envelope membranes (Douce *et al.*, 1984). Therefore the observation that glycerolipid biosynthesis in envelope membranes is a general feature of all envelope membranes from any kind of plastids clearly indicates the major

**Table 2. Fatty acid composition of envelope galactolipids from cauliflower plastids and sycamore amyloplasts**

Envelope galactolipids were extracted and analysed as described in the Materials and methods section. The composition shown is from a representative experiment. –, not detected; tr, < 0.5%.

| Lipid | Fatty acid... | Composition (%, w/w) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | $C_{16:0}$ | $C_{16:1}$ | $C_{16:3}$ | $C_{18:0}$ | $C_{18:1}$ | $C_{18:2}$ | $C_{18:3}$ |
| Cauliflower | | | | | | | | |
| MGDG | | 6 | tr | 14 | tr | 0.5 | 2 | 77 |
| DGDG | | 8.5 | tr | 2 | 0.5 | 4.5 | 6 | 78 |
| Sycamore | | | | | | | | |
| MGDG | | 2 | – | – | tr | 7 | 25 | 66 |
| DGDG | | 7 | – | – | 1 | 3.5 | 24.5 | 63 |

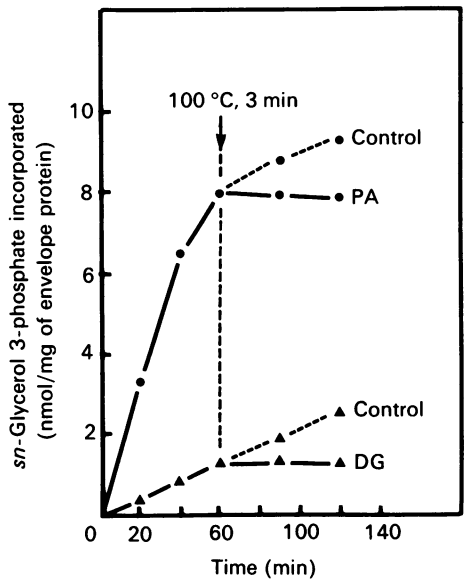


**Fig. 8. Formation of PA and DG in envelope membranes from sycamore amyloplasts**

The experimental conditions for the preparation of the fractions, incubation and analyses of the synthesized glycerolipids are described in the Materials and methods section. After 1 h incubation, the incubation mixture was boiled for 3 min (arrow) to stop the synthesis of envelope glycerolipids. The amounts of protein used for this experiment are 250 $\mu$g and 120 $\mu$g respectively for stroma and envelope membranes (total volume 600 $\mu$l; volume of each fraction 100 $\mu$l).

importance of this membrane system in the cell, at least for plastid biogenesis. These biosynthetic capacities are not restricted to chloroplasts with a well developed thylakoid network, but are also present in plastids whose membrane structures are almost limited to their envelope membranes. The presence of these enzymes probably reflects the flexibility of envelope membranes surrounding the starch grains. For instance, in storage tissues, in roots or in isolated cells such as sycamore, expansion of the amyloplasts envelope keeps pace with the growth of the starch granule inside it, and vice versa (Briarty *et al.*, 1979).

Professor John B. Ohlrogge is gratefully acknowledged for providing us with the acyl-ACP used in some of these experiments.

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Received 14 September 1988/23 November 1988; accepted 28 November 1988