Import of Lyso-Phosphatidylcholine into Chloroplasts Likely at the Origin of Eukaryotic Plastidial Lipids[1]

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Plastids rely on the import of extraplastidial precursor for the synthesis of their own lipids. This key phenomenon in the formation of plastidial phosphatidylcholine (PC) and of the most abundant lipids on earth, namely galactolipids, is poorly understood. Various suggestions have been made on the nature of the precursor molecule(s) transferred to plastids, but despite general agreement that PC or a close metabolite plays a central role, there is no clear-cut answer to this question because of a lack of conclusive experimental data. We therefore designed experiments to discriminate between a transfer of PC, 1-acylglycero phosphorylcholine (lyso-PC), or glycerophosphorylcholine. After pulse-chase experiments with glycerol and acetate, plastids of leek (*Allium porrum* L.) seedlings were purified. The labels of the glycerol moiety and the *sn*-1- and *sn*-2-bound fatty acids of plastidial lipids were determined and compared with those associated with the extraplastidial PC. After import, plastid lipids contained the glycerol moiety and the fatty acids esterified to the *sn*-1 position originating from the extraplastidial PC; no import of *sn*-2-bound fatty acid was detected. These results rule out a transfer of PC or glycerophosphorylcholine, and are totally explained by an import of lyso-PC molecules used subsequently as precursor for the synthesis of eukaryotic plastid lipids.

Galactolipids are the major lipids of photosynthetic tissues, and therefore are the most abundant lipids on earth (Gounaris and Barber, 1983). Their biosynthesis in higher plants involves two different pathways that coexist or not depending on the plant involved (for review, see Mongrand et al., 1998).

The prokaryotic pathway leads to galactolipid synthesis by using only the plastidial enzyme machinery, and differs greatly in this respect from the eukaryotic pathway, which requires close cooperation between the endoplasmic reticulum (ER) and chloroplasts (for review, see Browse and Somerville, 1991). Fatty acids synthesized in the plastid stroma are exported to ER and acylated to glycerophosphate to form phosphatidic acid, which is further converted to phospholipids and particularly to phosphatidylcholine (PC). Some lipids are then transferred (under an unknown form) to chloroplasts, where they account for the presence of PC in these organelles and contribute to the synthesis of the plastidial glycolipids monogalactosyl diacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulfoquinovasyl diacylglycerol. Therefore, the import of lipids from ER membranes to plastids is a major phenomenon of the plant lipid metabolism since it contributes approximately 50% of the total galactolipid formation when the prokaryotic and eukaryotic pathways are operative, and 100% in the fairly common case (for review, see Mongrand et al., 1998) when no prokaryotic synthesis of galactolipids occurs.

That a lipid import is required for plastid lipid synthesis is no longer a matter of debate, but the nature of the lipid link between the ER and the chloroplasts remains unknown. On one hand, plastids contain PC (in the outer leaflet of the envelope outer membrane [Dorne et al., 1985]), but are devoid of CDP-choline diacylglycerol choline-phosphotransferase activity (Joyard and Douce, 1976). One the other hand, several in vivo experiments have evidenced a PC/galactolipid precursor/product relationship in plants (e.g. Roughan, 1970; Slack et al., 1977; Ohnishi and Yamada, 1980; Browse et al., 1986; Williams and Khan, 1996; Mongrand et al., 1997). Therefore, there is general agreement that PC, or a close metabolite, has to be imported from ER membranes to chloroplasts, and that after lipid import, plastidial PC is used as a substrate for eukaryotic galactolipid synthesis (for reviews, see Roughan and Slack, 1982; Somerville and Browse, 1991; Maréchal et al., 1997. It follows that three reasonable hypotheses may be proposed for the chemical form of the lipid link between endomembranes and plastids: (a) glycerophosphorylcholine, but so far no data have established or even suggested a role of this molecule in the plastidial PC synthesis or in the galactolipid accumulation; (b) PC, which for years was thought to be the link between the ER and the plastids. This assumption received some experimental support but now needs to be re-investigated (Mongrand et al., 1997; for reviews, see Kader, 1996; Moreau et al., 1998); or (c) 1-acylglycero phosphorylcholine (lyso-PC), a transfer of which was only recently hypothesized and was immediately supported by several lines of evidence in vitro (Bessoule et al., 1995) and in vivo (Mongrand et al., 1997).

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Nevertheless, no conclusive data have until now been obtained.

We decided to investigate the nature of the lipid link between the ER and the chloroplasts. The rationale of the in vivo experiments described in this paper was to label the glycerol moiety and both fatty acids of the lipids located in the donor (extraplastidial) compartment, to purify at various chase times the acceptor compartment (plastids), and to pay special attention to the label associated with the fatty acids in the *sn*-1 and *sn*-2 positions of the glycerol backbones, as well as with the glycerol moieties, of plastidial PC and galactolipids. For the first time to our knowledge, the labels associated with lipids in the donor compartment have been determined. We describe the nature of the lipid transferred in vivo from extraplastidial membranes to plastids.

MATERIALS AND METHODS

Materials

High-performance thin-layer chromatography (HP-TLC) plates were Silicagel 60 F254 (Merck, Rahway, NJ). Autoradiography was performed using hyperfilm MP (Amersham, Buckinghamshire, UK). Na-[1-^{14}C]acetate (2 GBq/mmol) was obtained from Commissariat á l'Énergie Atomique (Saclay, France). Na-[^{3}H]acetate (93.2 GBq/mmol), [2–3-^{3}H]glycerol (7.4 GBq/mmol), [^{14}C(U)]glycerol (5.47 GBq/mmol), and [1-^{14}C]oleoyl-coenzyme A (CoA) (2 GBq/mmol) were obtained from DuPont-NEN (Les Ulis, France). Lipases and all other reagents were from Sigma Chemical (St. Louis).

Plant Materials and Pulse/Chase Labeling of Leek Seedlings

Leek (*Allium porrum* var Furor) seeds stored overnight at 4°C were washed five times with distilled water and grown for 15 d at room temperature on a previously described growth medium: 5% (w/v) agar in Heller's solution (see Moreau et al., 1988). Routinely, 6 g of seedlings were gently uprooted, and Na-[1-^{14}C]acetate (8.9 MBq), [2–3-^{3}H]glycerol (7.4 MBq), or both Na-[^{3}H]acetate (55.5 MBq) and [^{14}C(U)]glycerol (455 kBq) were supplied for 2 h. Seedlings were then rinsed eight times with deionized water, and the chase was carried out by adding 1 mL of 0.46 M Na-acetate, pH 5.5, 1 mL of 16 mM glycerol, or both 1 mL of 0.46 M Na-acetate, pH 5.5, and 1 mL of 8 mM glycerol. Seedlings were then replanted at 30°C in low-melting agar (1.5% [w/v] in Heller's solution). At various times, 1 g of seedlings was sampled and cut into small pieces. After that, 0.1 g was used to extract total lipids and 0.9 g to isolate chloroplasts.

Extraction of Total Lipids

Green tissues were weighed (approximately 0.1 g) and ground in a glass-glass tissue grinder with 6 mL of chloroform:methanol:formic acid (10:10:1, v/v). The homogenate was transferred to a screw-capped centrifuge tube and stored overnight at −20°C. The extraction procedure was continued by adding 2.2 mL of chloroform:methanol:water (5:5:1, v/v). The organic phase was washed with 6 mL of 0.2 M H$_3$PO$_4$ and 1 M KCl. Lipids were recovered in the organic phase, dried, and redissolved in 1 mL of chloroform:methanol (2:1, v/v). An aliquot of the lipid extract was evaporated in a scintillation vial, and radioactivity was determined by liquid scintillation counting.

Isolation of Chloroplasts, in Vitro Labeling, and Extraction of Chloroplastic Lipids

All operations were carried out at 4°C. Leek seedlings were weighed and then sliced into small pieces using fine scissors in a homogenization buffer 50 mM Tris, pH 7.5, 0.33 M sorbitol, 5 mM EDTA, and 0.1% (w/v) bovine serum albumin (BSA), as described by Joy and Mills (1987). The homogenate was then strained through two layers of Miracloth (Calbiochem-Novabiochem, San Diego) and centrifuged for 5 min at 3,000*g*. The pellet was suspended in the homogenization buffer and loaded onto a discontinuous Percoll gradient consisting of 5 mL of 80% (v/v) and 10 mL of 40% (v/v) Percoll. After centrifugation at 5,000*g* for 10 min, intact chloroplasts were collected at the 40% to 80% interface, diluted with 20 mL of the homogenization buffer and further centrifuged for 10 min at 3,000*g*. The pellet was resuspended in 1 mL of 50 mM Tris, pH 7.5, and 0.33 M sorbitol (buffer A). The protein content was determined according to the method of Bradford (1976) using BSA as a standard.

To determine the labeling of chloroplastic lipids in vitro, purified chloroplasts (700 μg) were incubated with 2.65 nmol of [1-^{14}C]oleoyl-CoA in 700 μL of buffer A at room temperature for 1 h. After incubation, chloroplasts were spun down for 10 min at 3,000*g* and the pellet was resuspended in 500 μL of buffer A. Aliquots of 100 μg of chloroplasts were incubated with various amounts of unlabeled oleoyl-CoA (0–10 mmol) for 1.5 h. After incubation, lipids was extracted and lipase digestion of chloroplastic PC was carried out as described below.

To extract chloroplastic lipids, chloroplasts were placed in 2 mL of chloroform:methanol (2:1, v/v). The volume of the aqueous phase was completed with water to 0.5 mL. After vortexing, the organic phase was isolated and the aqueous phase was re-extracted with 2 mL of chloroform. The lipid extract was evaporated to dryness and redissolved in 1 mL of chloroform:methanol (2:1). An aliquot of the lipid extract was evaporated in a scintillation vial, and radioactivity was determined by liquid scintillation counting.

Analysis of Labeled Lipids and Lipase Digestion

Individual polar lipids were purified from the extracts by monodimensional HP-TLC using the solvent system described by Vitiello and Zanetta (1978). Neutral lipids were separated by the solvent system described by Juguelin et al. (1986). Lipids were then located by spraying the plates with a solution of 0.1% (w/v) primuline in 80% (v/v) acetone, followed by visualization under UV light. After autoradiography, the silica gel zones corresponding to individual lipids were scraped off, and the radioactivity

associated with the lipids was determined by liquid scintillation counting.

The radioactivity associated with fatty acids esterified to sn-1 and sn-2 positions of total PC and chloroplastic PC was determined by lipase digestion. Under the conditions used, the phospholipase A₂ specificity was determined as described in Mongrand et al. (1997). After HP-TLC, lipid spots were scraped off and sonicated (15 min) in 200 μL of 50 mM Tris-HCl, pH 8.9, and 5 mM CaCl₂. Reactions were started by the addition of 0.2 unit of phospholipase A₂. Incubations were performed for 15 min at 37°C.

After incubation, 2 mL of chloroform:methanol (2:1) was added to stop reactions and to start lipid extraction. The organic phase was washed with 1 mL of 0.2 M H₃PO₄ and 1 M KCl. The aqueous phase was re-extracted by 2 mL of chloroform. Both of the organic phases were combined, evaporated, and lipids were redissolved in a minimal volume of chloroform:methanol (2:1). Lipids were resolved by HP-TLC as described above. After autoradiography, the silica gel zones corresponding to lysolipids and free fatty acids were scraped from the plates and the radioactivity was determined by liquid scintillation counting. The radioactivity associated with fatty acids esterified to the sn-1 and sn-2 positions of galactolipids was determined by *Rhizopus arrhizus* lipase digestion as described in Mongrand et al. (1997). For each pulse-chase experiment, both of the phospholipase A₂ and *R. arrhizus* lipase digestions were carried out at least three times per chase time.

Calculation of the Label Associated with Extraplastidial and Chloroplastic PC in Total Lipid Extract

At each pulse/chase time, the label associated with chloroplastic PC in the chloroplastic lipid extract was determined and expressed as a percentage of the radioactivity incorporated into galactolipids (MGDG plus DGDG). Since the galactolipids are located exclusively in the plastids (Douce and Joyard, 1979), this percentage was multiplied by the label associated with galactolipids in the total lipid extract to obtain the label associated with chloroplastic PC in the total lipid extract. The radioactivity in the extraplastidial PC was calculated by subtracting these values from the label of total PC.

RESULTS

The study was carried out with leek seedlings, which were previously shown to be 18:3 plants (Mongrand et al., 1997, 1998), i.e. a plant in which no prokaryotic synthesis of galactolipids occurs (for reviews, see Somerville and Browse, 1991; Roughan and Slack, 1982). It follows that in all the experiments reported in this paper, both galactolipids and plastidial PC entirely originate from an extraplastidial pool of PC.

In Vivo Labeling of Extraplastidial PC

The in vivo labeling of the acyl chains and of the glycerol moiety of extraplastidial PC in 15-d-old leek seedlings was studied by pulse/chase experiments using acetate and/or glycerol as labeled substrates. After a 2-h pulse with labeled glycerol, around 36% of total lipid label was associated with extraplastidial PC (Fig. 1a). Comparison with the radioactivity associated with total PC (38% of the total lipid label after a 2-h pulse [Mongrand et al., 1997]) showed that immediately after the pulse, labeled PC was mainly located in the extrachloroplastic compartment. During the chase, the label associated with the glycerol moiety of extraplastidial PC decreased from 36% ± 2.4% to 21% ± 1.2% of total labeled lipids.

Using labeled acetate instead of labeled glycerol, basically identical results were obtained: around 30% of the total label was associated with the extraplastidial PC after the pulse, and its radioactivity decreased during the chase from 29.1% ± 4.3% to 11.2% ± 2.3% of total radioactivity (Fig. 1b). This decrease affected the label of the fatty acids esterified to the sn-1 and sn-2 position of the glycerol backbone (Fig. 2). However, it can be noted that after a 2-hour pulse and at the various chase times, the label of sn-2-bound fatty acids of extraplastidial PC was always higher than that of sn-1 bound fatty acids (Fig. 2). From five separate experiments, we observed a reproducible decrease in the label of sn-2-bound fatty acids of extraplastidial PC from 17.0% ± 3.7% (pulse) to 6.9% ± 1.6% (96-h chase) and a label decrease from 12.1% ± 1.8% to 4.4% ± 1.6% in the case of sn-1-bound fatty acids.

In Vivo Import of Labeled Molecules into Plastids as a Function of Time

After the pulse with radioactive glycerol, 2.6% ± 0.72% of the total label was found in chloroplastic PC (Fig. 3a). During the first 24 h following the pulse, the radioactivity incorporated into chloroplastic PC increased from 2.6% ± 0.72% to 6.0% ± 1.41%, and then reached a plateau. These
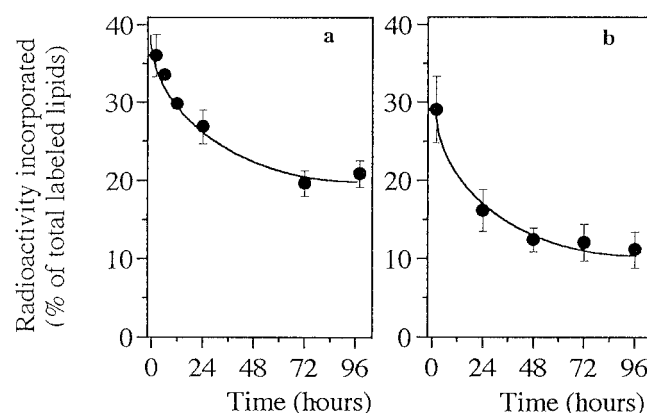
Figure 1. a and b, Variations in label associated with extraplastidial PC following in vivo glycerol and/or Na-acetate pulse/chase experiments. Seedlings were supplied at 0 time with labeled glycerol (a) and/or labeled Na-acetate (b). After the 2-h pulse, seedlings were chased with unlabeled glycerol and/or unlabeled Na-acetate, respectively. At different chase times, chloroplastic and total lipids were extracted and analyzed as described in "Materials and Methods." Results are expressed as percentages of the radioactivity incorporated into total lipids. The error bars represent ±SD of seven separate experiments using glycerol and five separate experiments using acetate.
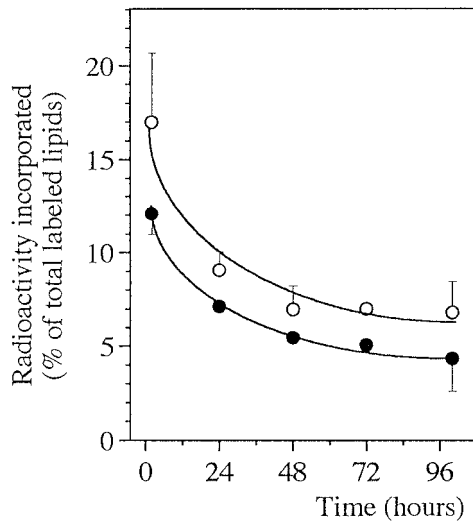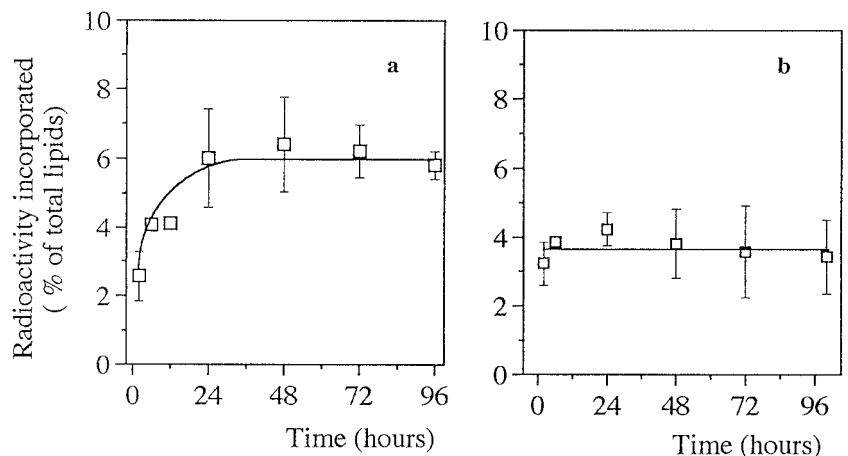
Figure 2. Variations in label associated with fatty acids esterified to *sn*-1 and *sn*-2 positions of extraplastidial PC. Seedlings were supplied at 0 time with labeled Na-acetate. After a 2-h pulse, seedlings were chased with unlabeled Na-acetate. At different chase times, chloroplastic and total lipids were extracted and analyzed as described in "Materials and Methods." Specific lipase treatment of total PC and extraplastidial PC was carried out with phospholipase A$_2$. ●, Label of fatty acids esterified to the *sn*-1 position of extraplastidial PC; ○, label of fatty acids esterified to the *sn*-2 position of extraplastidial PC. Results are expressed as percentages of the total label incorporated into chloroplastic PC. The error bars represent ±SD of five separate experiments.

results indicated an import of glycerol-labeled precursor into chloroplasts during the chase. As shown below, the plateau observed after a 24-h chase is correlated to the synthesis of galactolipids from plastidial PC, and reflects the steady state between import and metabolism.

The variation in the label of the chloroplastic PC acyl chains was also studied as a function of time by supplying leek seedlings with labeled acetate. In contrast with the results observed using glycerol, the total radioactivity associated with the acyl chains of chloroplastic PC remained almost constant during the pulse/chase, and represented approximately 3.5% of the total lipid label (Fig. 3b). There-
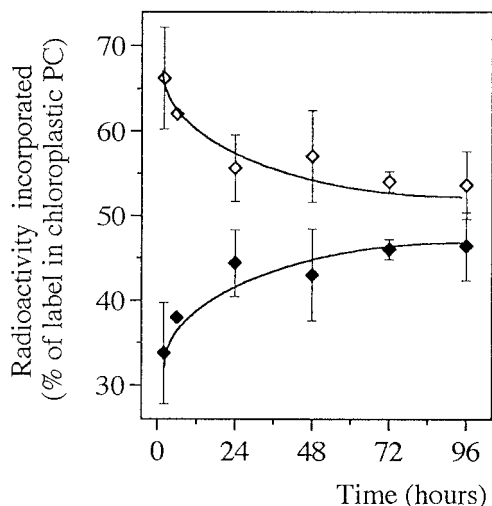
fore, unexpectedly, no import of labeled fatty acids into chloroplastic lipids seemed to occur during the chase. This apparent lack of label import into plastids resulted from the superimposition of two phenomena that were clearly evidenced when the label of the fatty acids esterified to the *sn*-1 and to the *sn*-2 positions of chloroplastic PC was studied (Fig. 4). After the pulse, the label of the fatty acids esterified to the *sn*-1 position was repeatedly 2 times lower than that of the *sn*-2-bound fatty acids: 33% ± 6% and 66% ± 6% of the plastidial PC label were associated to the *sn*-1 and *sn*-2 positions, respectively (i.e. approximately 1.2% and 2.4% of the total lipid label, respectively). During the chase, an increase in the label associated with the *sn*-1 position of chloroplastic PC was observed (from 33% ± 6% after the pulse to 46% ± 5% of the plastidial PC label after 96 h of chase), whereas the fatty acid radioactivity in the *sn*-2 position decreased from 66% ± 6% after the pulse to 54% ± 5% after 96 h of the chase. These two phenomena were prominent during the first 24 h following the pulse. These results indicated an import of labeled fatty acids esterified to the *sn*-1 position of plastidial PC during the chase, whereas no import of labeled fatty acids esterified to the *sn*-2 position seemed to occur.

After demonstrating an in vivo differential labeling of *sn*-1- and *sn*-2-bound fatty acids of chloroplastic PC, we analyzed the kinetics of the fatty acid labeling of MGDG and DGDG. Results (Fig. 5) showed that during the chase, the increase in the fatty acid label due to an import of labeled molecules from the extraplastidial compartment did not affect the two acylable positions of the glycerol backbone to the same extent. Whereas the fatty acid label bound to the *sn*-2 position of the galactolipids remained almost constant during the chase, the radioactivity associated with fatty acids esterified to the *sn*-1 position increased from 2.7% ± 0.8% after the pulse to 9.1% ± 1.1% after a 96-h chase in MGDG, and from 1.7% ± 1.2% to 4.8% ± 2% in DGDG.

During the chase, the glycerol labeling of galactolipids increased (Fig. 5), as did the acetate label associated with fatty acids esterified to the *sn*-1 position of galactolipids. When the molecular species of lipids were analyzed, it appeared that while palmitic acid accounted for 25% of the

Figure 3. Variations in label associated with chloroplastic PC following in vivo glycerol and/or Na-acetate pulse/chase experiments. Seedlings were supplied at 0 time with labeled glycerol (a) and/or labeled Na-acetate (b). After a 2-h pulse, seedlings were chased with unlabeled glycerol and/or unlabeled Na-acetate, respectively. At different chase times, chloroplastic and total lipids were extracted and analyzed as described in "Material and Methods." Results are expressed as percentages of radioactivity incorporated into total lipids. The error bars represent ±SD of seven separate experiments using glycerol and five using acetate.
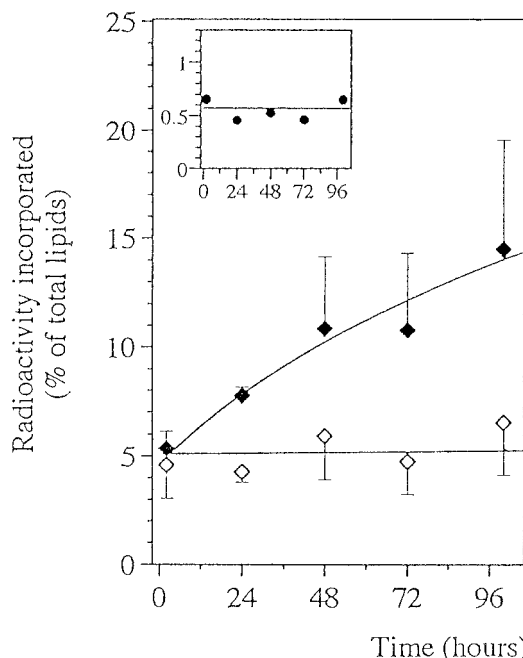
Figure 4. Variations in label associated with fatty acids esterified to the sn-1 and sn-2 positions of chloroplastic PC. Seedlings were supplied at 0 time with labeled Na-acetate. After the 2-h pulse, seedlings were then chased with unlabeled Na-acetate. At different chase times, chloroplastic and total lipids were extracted and analyzed as described in "Materials and Methods." Specific lipase treatment was carried out with phospholipase A₂. ●, Label of fatty acids esterified to the sn-1 position of chloroplastic PC; ○, label of fatty acids esterified to the sn-2 position of chloroplastic PC. Results are expressed as percentages of the total label incorporated into chloroplastic PC, and the error bars represent ±SD of five separate experiments.

labeled fatty acids associated with PC after the pulse, only labeled 18:2 and 18:3 fatty acids were esterified to MGDG after the chase. This result is in agreement with the fatty composition of MGDG, which does not contain palmitic acid (for review, see Browse and Somerville, 1991). In contrast to MGDG, DGDG usually contains 16:0 fatty acids and, in good agreement, labeled palmitic acid was found to be esterified to DGDG after a 96-h chase.

We also determined the total imported fatty acid label associated with either the sn-1 or the sn-2 position of the glycerol backbones of plastidial PC, MGDG, and DGDG.

Figure 6. Variations in label associated with fatty acids esterified to sn-1 and sn-2 positions of eukaryotic chloroplastic lipids. ◆, Total label associated with fatty acids esterified to the sn-1 position of eukaryotic chloroplastic lipids; ◇, total label associated with fatty acids esterified to the sn-2 position of eukaryotic chloroplastic lipids (i.e. MGDG, DGDG, and chloroplastic PC). Results are expressed as percentages of the total label, and the error bars represent ±SD of four separate experiments. Inset, Variations in the ratio of the radioactivities associated with the sn-1 fatty acids and with the glycerol of total eukaryotic chloroplastic lipids.

The results (Fig. 6) showed that no import of labeled fatty acid associated with the sn-2 position occurred during the chase and that the acetate labeling of eukaryotic lipids in plastids resulted from an import of radioactivity exclusively associated with the sn-1-bound fatty acids. This import matched the label decrease associated with the sn-1-bound fatty acids of PC in the donor compartment (shown
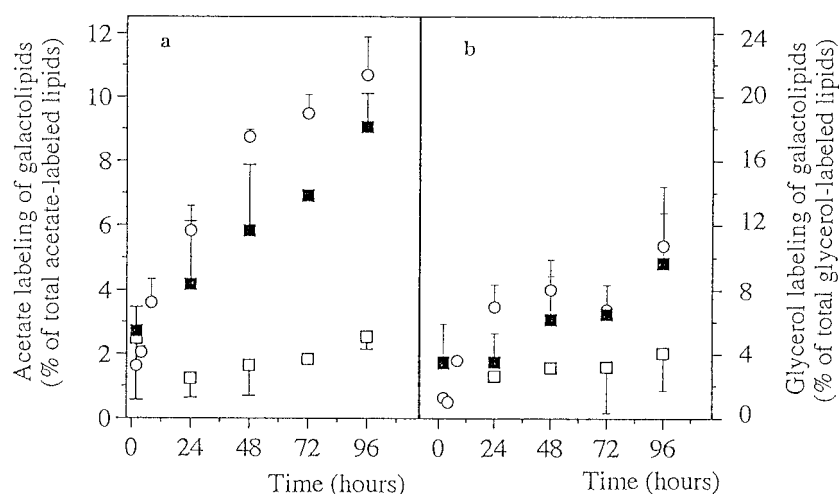
Figure 5. Distribution of label associated with glycerol and with sn-1- and sn-2-bound fatty acids of MGDG and DGDG during chase. The acetate label incorporated into the fatty acids esterified to the sn-1 (■) and sn-2 (□) positions of MGDG (Fig. 5a) and DGDG (Fig. 5b) was determined using R. arrhizus lipase (see "Materials and Methods"). Results are expressed as percentages of the acetate label incorporated into total lipids. ○, Glycerol label of MGDG (a) and DGDG (b) expressed as percentages of the glycerol label incorporated into total lipids. The error bars represent ±SD of four separate experiments.

in Fig. 2). Moreover, the ratio of the radioactivities associated with the *sn*-1 fatty acids and with the glycerol of the chloroplastic lipids remained constant during the chase (Fig. 6, inset).

Our results clearly showed that: (a) the eukaryotic lipids imported into plastids were labeled by a concomitant import of glycerol and of *sn*-1-bound fatty acids occurring at the same rate, and (b) the *sn*-2 position of the eukaryotic plastid lipids synthesized during the chase was esterified by unlabeled fatty acid and not by labeled fatty acids originating from the *sn*-2 position of extraplastidial PC. Indeed, the decrease in the radioactivity associated with the *sn*-2-bound fatty acids of extraplastidial PC during the chase (Fig. 2) was accompanied by an increase of the same order in the radioactivity associated with free fatty acids (from 35.2% ± 2.2% to 43.7% ± 4.8%, see Fig. 7). Therefore, when expressed as percentage of the total radioactivity incorporated into lipids, the decrease in the label of fatty acids esterified to the *sn*-1 and *sn*-2 positions of extraplastidial PC matched the increase in the label of plastidial lipids and of free fatty acids, respectively, which is in agreement with the fact that the total amount of radioactivity did not vary greatly during the chase (see also Mongrand et al., 1997). In addition, 70% of the fatty acids esterified to the *sn*-1 position of PC during the pulse were transferred to plastids during the chase (47% in MGDG and 23% in DGDG), while 30% remained associated with the extraplastidial PC. These results are in good agreement with those obtained with Arabidopsis leaves: 342 molecules were transferred to plastids, while 131 molecules of PC remained in the extraplastidial compartment (Browse et al., 1986). Using the same approach (mass analysis of lipids from unlabeled plants), we obtained similar proportions in leek seedlings: 532 molecules were transferred (310 in
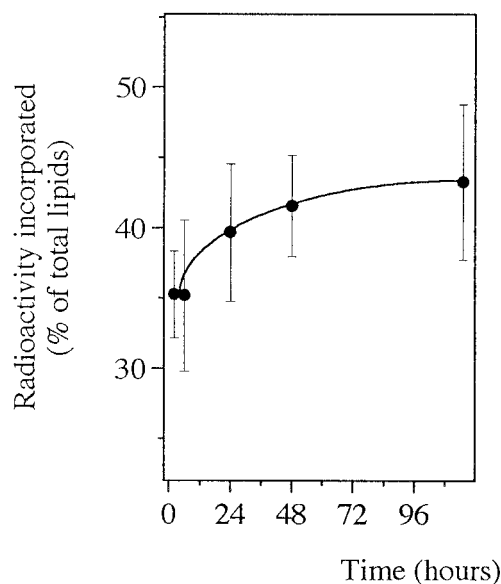
Figure 7. Variations in radioactivity associated with free fatty acids during pulse/chase experiments. Results are expressed as percentages of the acetate label incorporated into total lipids, and the error bars represent ±SD of four separate experiments.

Table I. *In vitro chloroplastic acyltransferase assays*

Intact chloroplasts (700 μg) were incubated with 2.6 nmol (3.7 μM) of [1-^{14}C]oleoyl-CoA (2 GBq/mmol) for 1 h at room temperature under illumination. After centrifugation at 3,000g for 10 min to eliminate unreacted labeled oleoyl-CoA, chloroplasts (100 μg) were incubated with various amounts of unlabeled oleoyl-CoA for 1.5 h under the same conditions. Lipids were then extracted and the radioactivity associated with fatty acids esterified to the *sn*-1 and *sn*-2 positions of chloroplastic PC was measured. Results represent the mean values ± SD of four separate experiments. Similar results were obtained with osmotically broken chloroplasts.

OleoylCoA	Label Incorporation into Chloroplastic PC	Positional Analysis	
μM	dpm/100 μg of chloroplasts	% sn-1	% sn-2
0	667 ± 154	13.2 ± 3.4	86.8 ± 3.4
5	599 ± 178	12.0 ± 2.8	88.0 ± 2.8
10	604 ± 126	10.0 ± 2.4	90.0 ± 2.4
20	668 ± 130	12.5 ± 3.7	87.5 ± 3.7
30	712 ± 314	10.5 ± 2.6	89.5 ± 2.6
50	606 ± 220	12.2 ± 3.3	87.8 ± 3.3
100	794 ± 96	9.5 ± 2.5	90.5 ± 2.5

MGDG and 167 in DGDG), while 192 molecules of PC remained in the extraplastidial compartment (Mongrand, 1998).

As mentioned above, the label associated with the fatty acids esterified to the *sn*-2 position of plastidial lipids remained constant and did not decrease during the chase (see Fig. 6), strongly suggesting that no acyl exchange occurred in the *sn*-2 position of the plastidial lipids. This point was also examined in vitro. After incubation of purified chloroplasts with [^{14}C]oleoyl-CoA (first incubation), plastids were spun down to eliminate unreacted labeled oleoyl-CoA and incubated in the presence or absence of unlabeled oleoyl-CoA (second incubation). Under these conditions, and as already observed (Bessoule et al., 1995), PC was the only labeled lipid. The activity was very low and the weak radioactivity was almost exclusively associated with the *sn*-2 position of PC (Table I). The addition of unlabeled oleoyl-CoA—whatever the amount used—did not induce a decrease in the specific radioactivity of chloroplastic PC, clearly showing that no acyl exchange occurred in vitro in addition to in vivo. It follows that the weak labeling of plastidial PC during the incubation involved an acylation of a low amount of endogenous lyso-PC rather than an acyl exchange. In addition, the data gathered from these in vitro experiments are in agreement with results from the in vivo pulse chase experiments, since they suggest that no extensive remodeling of lipids occurred after their import into plastids.

DISCUSSION

The aim of this study was to investigate in vivo the lipid trafficking from the extraplastidial compartment (donor compartment) to the chloroplasts (acceptor compartment). Since in this kind of experiment (e.g. Browse et al., 1986), the chase is followed over a period of days, it might be inferred that the transfer is quite a slow mechanism. Nevertheless, not only the constant rate of the transfer (*k*) but

also the amount of molecules involved (A) must be taken into account ($v = kA$). In the present study, we show that, like Arabidopsis leaves (Browse et al., 1986), approximately 70% to 75% of the PC located in ER membranes is involved in the plastid lipid biosynthesis in leek seedlings (see above). Since PC represents 40% to 45% of total lipids located in ER membranes of leek seedlings (Moreau et al., 1998), it appeared that during the course of the experiments described in this paper, 15% to 20% of total ER lipids were transferred to plastids during the first 24 h of chase.

There were three hypotheses concerning the nature of the molecules transferred: (a) glycerophosphorylcholine, (b) PC molecules, or (c) lyso-PC.

The first hypothesis may be ruled out for several reasons. First, chloroplastic membranes are devoid of glycerophosphorylcholine acyltransferase activity (Bessoule et al., 1995), so it seems unlikely that an import of glycerophosphorylcholine in plastids leads to a PC synthesis. Second, since fatty acids synthesized during the chase were unlabeled, and even if some—as yet unobserved—glycerophosphorylcholine acyltransferase activity was present in the plastids, a differential label variation of glycerol and fatty acids of plastid lipids would be expected, and this was not found. Third, even if some fatty acids remaining labeled during the chase unexpectedly acylated glycerophosphorylcholine in the plastids, this would lead to a similar label variation of the fatty acids esterified to the sn-1 and sn-2 positions of eukaryotic plastid lipids, and this was also not observed.

Regarding the transfer of PC, the label of fatty acids esterified to the sn-2 position of extraplastidial PC was always higher than that of the fatty acids esterified to the sn-1 position. It follows that if PC were to be transferred as a whole from the ER to the plastids, the rate of incorporation of labeled fatty acids in the sn-2 position of the imported lipids ($V_2 = k$ [PC$_{ER}sn$-2]) would be higher than that in the sn-1 position ($V_1 = k$ [PC$_{ER}sn$-1]), resulting in a higher import of labeled fatty acids esterified to the sn-2 position than to the sn-1 position. Our data are therefore not compatible with a PC transfer, unless a special pool of extraplastidial PC (as yet never evidenced) specifically and exclusively labeled at the sn-1 position (because $V_2 = 0$, see Fig. 6) was transferred to plastids. Therefore, a transfer of PC appears highly unlikely.

A transfer of lyso-PC has also been proposed (Bessoule et al., 1995). According to this hypothesis, lyso-PC formed from extraplastidial PC reaches the plastids, where it is acylated by a lyso-PC acyl-CoA acyltransferase. In agreement with this proposal, this enzyme has been evidenced in the plastid envelope, and its properties are highly compatible with the formation of plastid lipids (Bessoule et al., 1995). Our data add new in vivo evidence that this pathway is operative. The study of the label variation of extraplastidial and plastidial PC and galactolipids during the chase and the comparison of the labels associated with glycerol and with the acyl moieties of these lipids establish, for the first time to our knowledge, that the glycerol moiety is transferred concomitantly with—and at the same rate

as—fatty acids esterified to the sn-1 position. Furthermore, fatty acids originating from the sn-2 position of extraplastidial PC are not associated with plastid lipids. These results, which strongly suggest a transfer of lyso-PC molecules between ER and plastids, underline the prominent role of the plastidial lyso-PC:acyl-CoA acyltransferase in plastidial lipid synthesis. Purification of this protein is now under way in our laboratory.

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