Rapid Kinetic Labeling of Arabidopsis Cell Suspension Cultures: Implications for Models of Lipid Export from Plastids[1][W][OA]

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Cell cultures allow rapid kinetic labeling experiments that can provide information on precursor-product relationships and intermediate pools. T-87 suspension cells are increasingly used in Arabidopsis (*Arabidopsis thaliana*) research, but there are no reports describing their lipid composition or biosynthesis. To facilitate application of T-87 cells for analysis of glycerolipid metabolism, including tests of gene functions, we determined composition and accumulation of lipids of light- and dark-grown cultures. Fatty acid synthesis in T-87 cells was 7- to 8-fold higher than in leaves. Similar to other plant tissues, phosphatidylcholine (PC) and phosphatidylethanolamine were major phospholipids, but galactolipid levels were 3- to 4-fold lower than Arabidopsis leaves. Triacylglycerol represented 10% of total acyl chains, a greater percentage than in most nonseed tissues. The initial steps in T-87 cell lipid assembly were evaluated by pulse labeling cultures with [^{14}C]acetate and [^{14}C]glycerol. [^{14}C]acetate was very rapidly incorporated into PC, preferentially at *sn-2* and without an apparent precursor-product relationship to diacylglycerol (DAG). By contrast, [^{14}C]glycerol most rapidly labeled DAG. These results indicate that acyl editing of PC is the major pathway for initial incorporation of fatty acids into glycerolipids of cells derived from a 16:3 plant. A very short lag time (5.4 s) for [^{14}C]acetate labeling of PC implied channeled incorporation of acyl chains without mixing with the bulk acyl-CoA pool. Subcellular fractionation of pea (*Pisum sativum*) leaf protoplasts indicated that 30% of lysophosphatidylcholine acyltransferase activity colocalized with chloroplasts. Together, these data support a model in which PC participates in trafficking of newly synthesized acyl chains from plastids to the endoplasmic reticulum.

Cell suspension cultures are commonly used and are important model systems for the study of many aspects of plant cell biology, biochemistry, and molecular biology (Razdan, 2003). In contrast with leaf or many other plant tissues that have diverse cell types growing at different rates, the cell population of suspension cultures is much less complex. In liquid medium, many plant cell lines grow rapidly and are readily transformable at high efficiency with *Agrobacterium tumefaciens* (An, 1985; Nagata et al., 2004; Ogawa et al., 2008). This eases the generation and selection of large numbers of independent transgenic lines compared to whole-plant transformation. Although tobacco (*Nicotiana tabacum*) Bright Yellow 2 (BY2) cells have been the most widely used cell culture system, the abundant information and molecular and genetic tools available for Arabidopsis (*Arabidopsis thaliana*) have increased interest in T-87 cells as a model for molecular and biochemical investigations (e.g. Alonso et al., 2010). In addition, a high-throughput procedure for the testing of large numbers of transgenes in a 96-well format has been described (Ogawa et al., 2008).

Arabidopsis T-87 cells originate from seedlings of Arabidopsis (Axelos et al., 1992). To date, studies of their lipid composition or metabolism have not been reported. To evaluate the utility of T-87 cells as a model for lipid synthesis, we analyzed their lipid composition and conducted a set of experiments using [^{14}C]acetate and [^{14}C]glycerol to investigate initial steps in glycerolipid synthesis. The ability to conduct rapid-pulse labeling of T-87 cells provides the ability to track precursor-product relationships and to derive information on precursor pools involved in the incorporation of newly synthesized fatty acids (FAs) into membrane lipids.

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In plants, the incorporation of the newly synthesized acyl chains into glycerolipids occurs by two independent pathways: the prokaryotic pathway inside plastids and the eukaryotic pathway outside the plastid (Frentzen et al., 1983; Heinz and Roughan, 1983). In the eukaryotic pathway, acyl-acyl carrier protein (acyl-ACP) products of FA synthesis are hydrolyzed by plastid acyl-ACP thioesterase reactions and exported to an outer envelope-bound acyl-CoA synthetase. De novo assembly of glycerolipids occurs largely at the endoplasmic reticulum (ER) by two acylations of glycerol-3-P to form phosphatidic acid (PA; Kornberg and Pricer, 1953; Somerville et al., 2000). Phosphatidylcholine (PC), the major phospholipid of plant cells, is synthesized via PA phosphatase action on PA to produce diacylglycerol (DAG) followed by transfer of phosphocholine from DAG-CDP-choline to *sn*-3 of DAG to form PC (Kornberg and Pricer, 1953; Kennedy and Weiss, 1956). The proportion of flux between the prokaryote and eukaryote pathways is not conserved across different plant species or tissues. In 16:3 species, including Arabidopsis, up to 40% of flux in leaves occurs via the prokaryotic pathway (Browse et al., 1986), while 18:3 species, such as pea (*Pisum sativum*) and soybean (*Glycine max*) export >90% of FAs to supply the eukaryotic pathway (Ohlrogge and Browse, 1995; Somerville et al., 2000).

Recently, kinetic labeling experiments of pea leaves and soybean seeds with [^{14}C]acetate has indicated that most FAs synthesized by plastids are initially incorporated into PC via acyl editing of preformed PC, independently of the Kornberg-Pricer pathway. By contrast, [^{14}C]glycerol labeling indicated that most acylation of glycerol-3-P to form PA and DAG uses preexisting rather than newly synthesized acyl groups exported from plastids (Bates et al., 2007, 2009). Acyl editing has also been characterized by the lack of a precursor-product relationship between DAG and PC, by regiochemical analysis of [^{14}C]labeled acyl chains esterified to *sn*-1 and *sn*-2 and by molecular species analysis (Bates et al., 2007).

Investigations of acyl editing by Bates et al. (2007, 2009) were conducted with the 18:3 plants pea and soybean. Similar kinetic analysis of initial FA and glycerolipid labeling has not yet been extended to 16:3 species such as Arabidopsis, although CO_2 labeling of *Brassica napus* leaves (Williams et al., 2000) is consistent with acyl editing. In this study, analysis of initial [^{14}C]acetate and [^{14}C]glycerol labeling of lipids provided evidence for acyl editing in a 16:3 species. In addition, rapid-pulse [^{14}C]labeling provided information on the precursor pools involved in the initial incorporation of [^{14}C]acyl chains into glycerolipids. These data, together with the subcellular distribution of PC and of lysophosphatidylcholine acyltransferase (LPCAT), support a revised model of lipid export from plastids in which PC serves as a carrier of acyl chains from plastids to ER.

RESULTS AND DISCUSSION

Growth and Lipid Composition of T-87 Cells in Light and Dark

The lipid composition of T-87 cells was determined to provide baseline data for future lipid research and to evaluate the extent to which Arabidopsis cell culture lipids resemble tissues growing in planta. T-87 cells can grow with or without the provision of light (Alonso et al., 2010). As indicated in Figure 1, cultures grown with 50 μmol m^{-2} s^{-1} light or grown in the dark accumulated similar biomass, FA levels, and protein throughout a 7-d cultivation period. Between days 3 and 5, T-87 cell culture dry weights increased 2.7- and 3.1-fold for light and dark conditions (Fig. 1A), which corresponds to a biomass doubling rate of 35 and 31 h, respectively. The net rate of FA synthesis of light-grown T-87 cultures during the 3- to 5-d rapid growth period (calculated from Fig. 1, A and B) was 18 nmol C h^{-1} mg^{-1} fresh weight (FW; expressed on the basis of atoms of carbon; Fig. 1, A and B). For comparison, expanding leaves of 3-week-old Arabidopsis plants have a rate of FA synthesis of approximately 2.3 nmol C h^{-1} mg^{-1} FW (Bao et al., 2000; Bonaventure et al., 2004). Thus, the net rate of FA synthesis in T-87 cells is 7- to 8-fold higher compared to young plants with rapidly expanding leaves and 50-fold higher compared to leaves in the dark (Browse et al., 1981).

At day 5, the total FA content of T-87 cells was 98 to 118 μg mg^{-1} dry weight, which is 1.5- to 2-fold higher than levels observed in Arabidopsis leaves (40 μg FA mg^{-1} dry weight; e.g. Yang and Ohlrogge, 2009; Li-Beisson et al., 2010). The most abundant FAs were linoleic (18:2), linolenic (18:3), and palmitic acid (16:0; Table I). 16:3 represented 1.0 to 1.5 mol % compared to

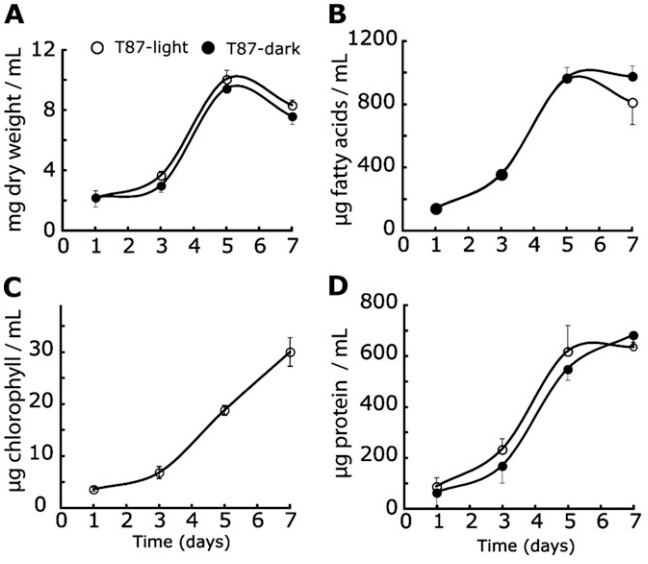

Figure 1. Growth of T-87 cells with light (white symbols) or without light (black symbols). Accumulation of biomass (A), FAs (B), chlorophyll (C), and protein (D). Data are means ± SD of three replicates.

Table I. *FA composition of total lipids extracted from light- or dark-grown T-87 cultures*

Means ± SD from three replicates. 18:1 represents the sum of 18:1Δ9 and 18:1Δ11. Data for Arabidopsis leaf and tobacco BY2 cells are presented for comparison.

FA	T-87 (Light)	T-87 (Dark)	Arabidopsis Leaf[a]	BY2
16:0	22.4 ± 0.48	20.8 ± 0.21	15.0	23.4 ± 0.06
16:1	2.2 ± 0.11	3.1 ± 0.58	3.8	Trace
16:3	1.8 ± 0.04	1.0 ± 0.02	13.8	Trace
18:0	2.8 ± 0.05	1.3 ± 0.00	1.0	4.3 ± 0.01
18:1	9.8 ± 0.14	14.9 ± 0.59	3.5	12.3 ± 0.13
18:2	22.7 ± 0.20	29.5 ± 0.65	15.7	47.5 ± 0.01
18:3	37.0 ± 0.46	27.8 ± 0.65	46.0	9.6 ± 0.01
Other	1.3 ± 0.04	1.6 ± 0.30	1.7	2.9 ± 0.04

[a]Values from Miquel and Browse (1992).

13.8 mol % for leaves (Miguel and Browse, 1986). The low 16:3 FA level is similar to 15-d-old Arabidopsis roots (1.5 mol%; Beaudoin et al., 2009). One clear difference between T-87 cells grown in light or dark was the accumulation of chlorophyll. While dark-grown cultures were yellow, light-grown T-87 cultures were pale green and contained about 0.2 mg chlorophyll per gram dry weight or 2% of the levels observed in leaf tissue (10 mg/g dry weight; Strand et al., 1999). Low levels of the largely thylakoid-associated 16:3 FA in T-87 light compared to leaf tissue are consistent with the low chlorophyll content.

The major phospholipids of light- and dark-grown T-87 cells were PC and phosphatidylethanolamine (PE) at 45 and 12.6 mol %, respectively (Fig. 2). Other phospholipids, including phosphatidylinositol (PI), phosphatidylglycerol (PG), phosphatidyl-Ser (PS), and PA, were minor components at 6.6, 4.1, 4.1, and 1.6 mol %, respectively. Lysophospholipids (lysoPC, lysoPE, and lysoPG) represented <0.5 mol %. The relative proportions of phospholipids in T-87 cell cultures (PC > PE > PI, PS > PA) were similar to values reported for other Arabidopsis tissues (Miquel and Browse, 1992; Welti et al., 2002; Li-Beisson et al., 2010). Monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) were 12.2% and 4.2% (mol), respectively, and together represented 16.4% of all glycerolipids (excluding sulfoquinovosyl diacylglycerols and cardiolipin, which were not analyzed; Fig. 2). This level of galactolipids in T-87 cells was 3.4-fold lower in comparison to Arabidopsis leaves, but the 3:1 proportion between MGDG and DGDG was similar to leaf (Miquel and Browse, 1992). Lipids primarily localized in the thylakoids (MGDG, DGDG, and PG) were slightly more abundant in light than dark-grown T-87 cultures, indicating some influence of light on lipid composition. The lower levels of 16:3, MGDG, DGDG, and PG compared to leaves likely reflect both the lower light of 50 μmol m^{-2} s^{-1} (approximately one-half to one-third of typical light levels for plants grown in soil) and the high Suc level in the growth media (90 mM). Suc is known to suppress expression of genes associated with photosynthesis (Goldschmidt and Huber, 1992). Lipid compositions were similar in light and dark and more closely resembled nonphotosynthetic Arabidopsis tissues than leaves. Overall, T-87 cells displayed no major anomalies in lipid composition related to their growth in suspension cultures.

The distribution and composition of pairs of *O*-acyl chains on polar lipids constitute the molecular species within a lipid class. This arrangement of acyl chains influences the physical properties of membranes that change in response to osmotic and temperature stress (Lynch and Thompson, 1984). To further compare T-87 lipids to Arabidopsis plants, the molecular species of phospholipids and galactolipids were characterized by electrospray ionization-tandem mass spectrometry (Fig. 3A). Molecular species of PC, PE, PI, PA, and DGDG were similar to those observed in leaves and roots (Li et al., 2006a). However, compared to data for leaves (Miquel and Browse 1992), and consistent with the lower 16:3 levels, there was a lower abundance of 16:x/18:x (34:0–34:6) molecular species of MGDG compared to 18:x/18:x (36:1–36:6). In addition, molecular species with longer chain FAs (>20 carbon) that are characteristic of PS and known to induce membrane curvature (Israelachvili et al., 1980) were more abundant in T-87 cells compared to leaf tissue (Li et al., 2006a; Nerlich et al., 2007). This may relate to the more spherical shape of suspension culture cells compared to leaf tissue.

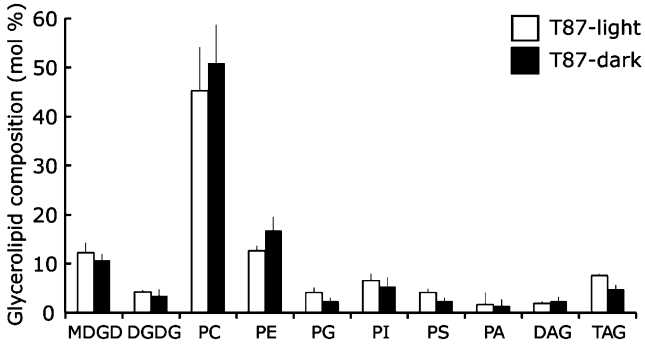

Figure 2. Glycerolipid composition of T-87 cells grown in light (white bars) or in dark (black bars). Data are means ± SD (n = 5).

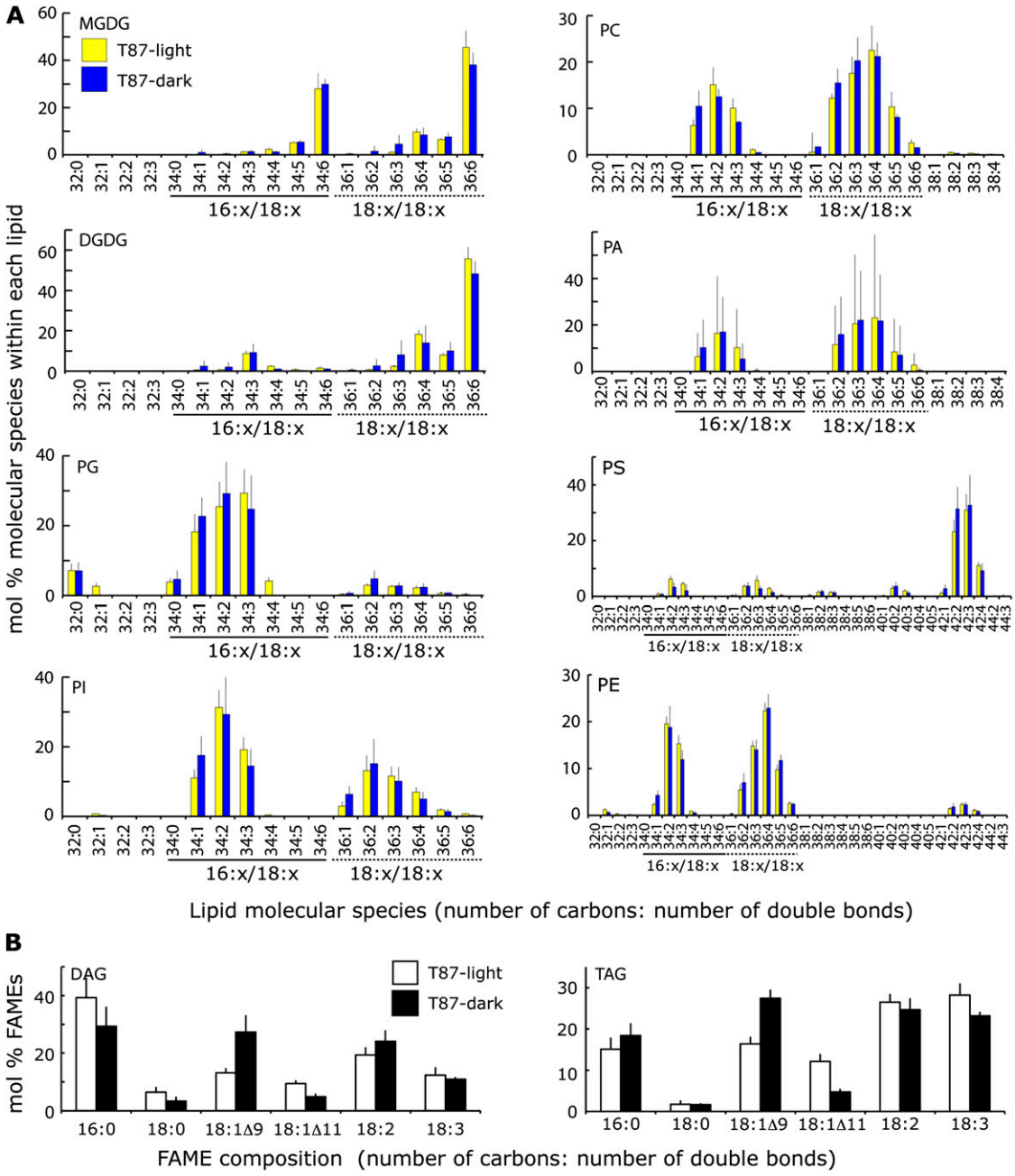

Figure 3. Glycerolipid composition of T-87 cells grown in light or dark. Molecular species of polar glycerolipids (A) and FA composition of neutral glycerolipids (B). Bar height represents means ± SD of five biological replicates.

T-87 Cells Accumulate Triacylglycerol

Triacylglycerol (TAG) is a major component of seeds and pollen but is usually found at very low levels in vegetative tissues. For example, TAG is <1% of lipids of leaves during growth from seedlings until senescence of Arabidopsis, *Brachypodium*, and switchgrass (*Panicum virgatum*; Yang and Ohlrogge, 2009). By contrast, T-87 cells accumulated TAG as a major neutral lipid, in addition to the polar lipids described above. TAG represented 10.8 mol % of total FAs (7.6 mol % of glycerolipids) in light-grown T-87 cells (Fig. 2). Linoleic and linolenic acids were the predominant FAs (>50% of total) of TAG for T-87 cells grown in either light or dark (Fig. 3B). The TAG FA composition was distinct from Arabidopsis seeds, which include ~26% FAs with chain length >C18 (largely 20:1; Li et al., 2006b). By contrast, the FA compositions in TAG derived from T-87 cells was very low in eicosenoic acid (20:1) or other >C18 FAs. In agreement with the content based on FA levels, after [^{14}C]acetate labeling (0–60 min), TAG accounted for 11% of total

[^{14}C]label recovered in lipids at 60 min (Supplemental Fig. S1). The occurrence of TAG in T-87 cells and the convenient transformation with *Agrobacterium* suggests that T-87 cells could be a useful model system for evaluating TAG metabolism in nonseed tissues and to evaluate gene candidates involved in TAG accumulation.

Radiolabel Incorporation into T-87 Cells to Probe Initial Kinetics of Lipid Biosynthesis

The rapid growth and uniform properties of T-87 cultures are particularly useful for studying precursor-product relationships through rapid pulse radioisotope labeling. Incorporation of label into lipids was determined beginning 15 s after addition of isotope to shaking cell cultures. The initial steps of FA and glycerol incorporation into glycerolipids were investigated by labeling experiments using [^{14}C]acetate and [^{14}C]glycerol. Exogenously supplied [^{14}C]acetate is rapidly taken up, converted to acetyl-CoA, and incorporated into the acyl chains of glycerolipids (Roughan et al., 1976; Bao et al., 2000; Koo et al., 2004). By contrast, [^{14}C]glycerol is primarily incorporated into the glycerol backbone of glycerolipids (Slack et al., 1977). Thus, experiments with [^{14}C]acetate and [^{14}C]glycerol can distinguish the assembly of the glycerol backbone from incorporation of acyl chains into glycerolipids. In addition to establishing general aspects of T-87 lipid biosynthesis, the experiments below were designed to evaluate the extent to which acyl editing reactions participate in FA incorporation into glycerolipids and to investigate pool sizes of intermediates in this process. Initial experiments showed little difference between light- and dark-grown cultures; in the sections below, all experiments reported are on light-grown T-87 cell culture.

[^{14}C]Acetate Labeling

To identify the initial products of T-87 acyl lipid metabolism, [^{14}C]acetate was added to cultures and radiolabeled lipids were analyzed by thin-layer chromatography (TLC) and autoradiography. Incorporation of [^{14}C]acetate into lipids was linear over a 10-min time course. The highest [^{14}C]label (35%–55%) was associated with PC and was 3- to 7-fold higher than DAG from 30 s to 10 min (Fig. 4A). This pattern of rapid [^{14}C]acetate incorporation into PC is similar to results observed by Bates et al. with pea leaves (Bates et al., 2007) and soybean seeds (Bates et al., 2009). Incorporation of [^{14}C]acetate into other glycerolipids is presented in Supplemental Figs. S2 and S3.

We further analyzed the abundance and identity of [^{14}C]labeled acyl chains at *sn*-1 and *sn*-2 positions of PC and DAG labeled for 5 min. The regiospecific distribution of [^{14}C]acyl chains of PC was determined by digestion with phospholipase A_2. Analysis of [^{14}C] label in free FA (FFA) and lysoPC products indicated that 74% of the radiolabel was associated to the *sn*-2

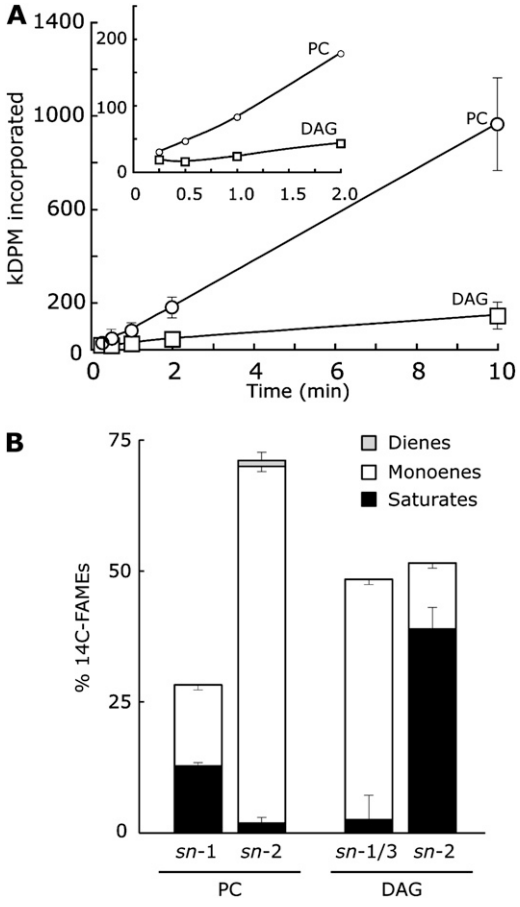

Figure 4. A, Time course of [^{14}C]acetate incorporation into PC (circles) and DAG (squares) of light-grown T-87 cells. Two-milliliter cultures were sampled. Insert graph expands scale of early time points. B, Distribution of radiolabeled acyl chains at *sn*-1 and *sn*-2 of PC and DAG after 5 min [^{14}C]acetate labeling. Black bars, saturated acyl chains; white bars, monoenes (18:1); and gray bars, dienes (18:2).Values in A and B represent means ± SD of three biological replicates.

position (Fig. 4B). Further analysis of the radiolabeled acyl groups by silver TLC revealed that >90% of the [^{14}C]label at the *sn*-2 position was monoenes, whereas <3% was in the form of saturates and dienes. By contrast, at *sn*-1, there was a 45/55 distribution between saturates and monoenes, with trace amounts of dienes (Fig. 4B).

To determine the regiospecificity of acyl chain labeling in DAG, pancreatic lipase was used to selectively remove the acyl groups from the *sn*-1/*sn*-3 position but not the acyl chain at *sn*-2 (Christie, 2003). In contrast with PC, DAG had a much more even distribution of [^{14}C]acyl chains on the glycerol backbone, with 52% and 48% of the radiolabel from [^{14}C]acetate associated with *sn*-2 and *sn*-1, respectively. In addition, the distribution of [^{14}C]saturated and unsaturated FAs in DAG was substantially different from that of PC (Fig. 4B).

DAG labeled in these experiments could originate either from the prokaryotic pathway in plastids or the

extraplastidial eukaryotic pathway. DAG synthesis via the eukaryotic pathway will initially have either a 16:0 or 18:1 FA at *sn*-1 and an 18:1 FA at *sn*-2. DAG synthesized inside the plastid (via the prokaryotic pathway) will initially have 18:1 FA at the *sn*-1 position and 16:0 FA at the *sn*-2 position. The composition of radiolabeled acyl chains of DAG was intermediate between prokaryotic and eukaryotic DAG (Fig. 4B). Based on the amount of saturates at *sn*-2, we estimate that approximately 75% of DAG labeled with [^{14}C] acetate after 5 min of incubation was of prokaryotic origin. This was consistent with findings in spinach (*Spinacia oleracea*) leaves (a plant with 16:3 metabolism), where 74% of [^{14}C]acetate labeled DAG was of prokaryotic origin (Roughan et al., 1980).

[^{14}C]Glycerol Labeling

In contrast with [^{14}C]acetate labeling of PC, [^{14}C] glycerol was most rapidly incorporated into DAG over the 10-min time course, while PC labeling showed a distinct lag. At the earliest time points (15 and 30 s), label from [^{14}C]glycerol in PC was undetectable, and at 1 min, label in DAG was 200-fold greater than PC (Fig. 5). Unlike [^{14}C]acetate labeling, these results are consistent with the precursor-product relationship between DAG and PC for de novo PC biosynthesis (Kennedy and Weiss, 1956).

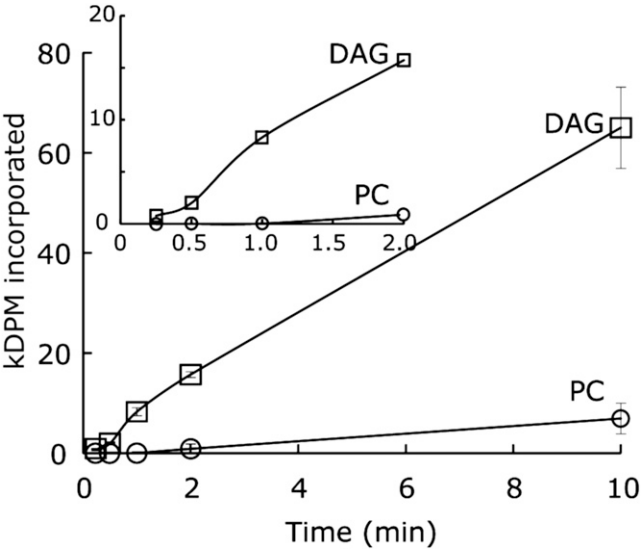

Figure 5. Time course of [^{14}C]glycerol incorporation into PC (circles) and DAG (squares) in light-grown T87 cells. Two-milliliter cultures were sampled. Inset graph expands scale of early time points. Values represent means ± SD of three biological replicates. Note: These values have not been corrected for the small amount of glycerol that enters acyl chains via glycerol metabolism to acetyl-CoA (Slack et al., 1977). Our analysis indicated 1.5% to 4% of the radioactivity from glycerol labeling was located in the acyl chains. Similar values have been reported for [^{14}C]glycerol labeling of DAG in soybean embryos (Bates et al., 2009).

Together, the acetate and glycerol experiments clearly demonstrate that initial incorporation of acyl chains into PC and incorporation of glycerol into the glycerolipid backbone occur through different pathways. Furthermore, acyl editing is a major pathway for incorporation of newly synthesized FAs into glycerolipids of T-87 cells, consistent with the acyl editing pathway described by Bates et al. (2007, 2009). This conclusion is supported by three lines of evidence: (1) the rapid incorporation of [^{14}C]acyl chains into PC relative to DAG (Fig. 4A); (2) the preferential incorporation of [^{14}C]acetate label into *sn*-2 of PC compared to *sn*-1. This pattern differed from DAG where radiolabeled acyl chains were almost evenly distributed between *sn*-1 and *sn*-2 (Fig. 4B). And (3) the major initial product of [^{14}C]glycerol labeling was DAG, whereas PC labeling lagged at least 1 min (Fig. 5).

Are Newly Synthesized Acyl Chains Channeled into PC at the Plastid Envelope? Consideration of Lag Times and Precursor Pool Sizes

The initial kinetics and lag time for radiolabel incorporation into products can provide information on the pool sizes of intermediates of a metabolic pathway. Linear kinetics of incorporation of label into product will not occur until the intermediate pools of a pathway are saturated with radioactivity (Segel, 1976). A large endogenous pool will result in a longer lag time prior to linear incorporation of radiolabel compared to a smaller endogenous pool.

Rapid and linear incorporation of [^{14}C]acetate into PC was previously observed in studies on pea leaves and soybean embryos (Bates et al., 2007, 2009). A lag could not be detected in [^{14}C]acetate incorporation into PC by pea leaves, but the earliest time sampled was 30 s and the variance was estimated at 30 s.

We took advantage of the ability to rapidly manipulate T-87 cell cultures to determine the length of time for linear [^{14}C]acetate incorporation into PC. This lag time was determined from analysis of seven independent biological replicates. By extrapolation of time-course labeling data to the *x* axis (time), an average lag of 5.4 ± 4.4 s (Fig. 6A) was determined.

How does the observed 5.4 s lag compare with the lag predicted for saturation of intermediate pools? The precursor pools involved in [^{14}C]acetate incorporation into glycerolipids include acetyl-CoA, acyl-ACP, FFA, and acyl-CoA. Literature values for chloroplast acetyl-CoA, acyl-ACP, and FFA are approximately 10, 5, and 1 pmol C mg FW^{-1} (Post-Beittenmiller et al., 1992; Soll and Roughan, 1982; Koo et al., 2004). Acyl-CoA levels in seedlings have been reported at 80 pmol C mg FW^{-1} (Larson and Graham, 2001). Although the subcellular distribution of long-chain acyl-CoA is not known, we estimate a cytosolic acyl-CoA pool size of approximately 82% of total cellular acyl-CoA or 66 pmol C mg FW^{-1} (see Supplemental Text S1 for calculation). The sum of acetyl-CoA, acyl-ACP, FFA, and acyl-CoA precursor pools is 82 pmol C mg FW^{-1}.

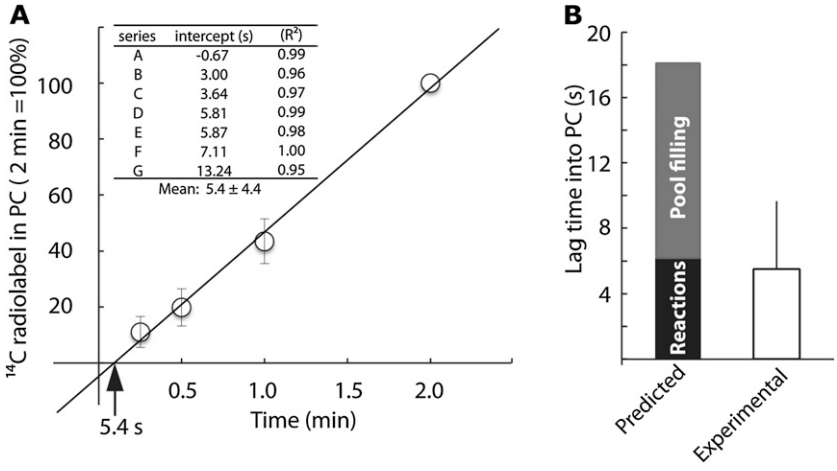

Figure 6. Determination of lag time for incorporation of [^{14}C]acetate into PC. A, Data from seven biological replicates were used to calculate the time-axis intercept. To allow comparisons between independent experiments, [^{14}C]acetate incorporations at 2 min were normalized to 100%. Averages ± SD ($n = 7$) are plotted for each time point. Arrow indicates time-axis intercept, and the inset table represents individual time-axis intercept and R^2 values for each of the seven time courses. B, Predicted time required for reactions from [^{14}C]acetate to PC (black bar) and for pool filling (gray bar). There are uncertainties in the predicted lag time values because of variation in data reported for pool sizes and turnover numbers. Based on literature values, we estimate an error range of ±20% and ±7% for predicted values shown. White bar presents means ± SD for time-axis intercept data from A.

The time required to saturate 82 pmol of intermediate pools can be calculated from the equation: lag time = ln2/k, where k = rate of FA synthesis/substrate pool (Segel, 1976). Based on the rate of FA synthesis of 4.8 pmol C s^{-1} mg^{-1} FW (calculated per second from Figure 1B) a lag of 11.9 s is predicted to saturate the above intermediate pools and achieve linear ^{14}C-label incorporation into PC.

A second major factor influencing the lag in linear [^{14}C]labeling of PC will be time required for enzyme catalysis. Beginning with acetyl-CoA synthetase, there are 39 reactions catalyzed by 13 enzymes that are required for biosynthesis of oleic acid (the major plastid FA product) and its incorporation into PC by acyl editing. The catalytic constant (Kcat) or turnover number of each enzyme determines the time required for the reaction. Available values for turnover numbers for the reactions are summarized in Supplemental Table S1. Since the reactions occur sequentially, the times are additive. The plant stearoyl-ACP desaturase has a very low turnover number of 0.5 s^{-1} (Whittle and Shanklin, 2001; Rogge and Fox, 2002) and therefore will contribute 2 s to the lag. Based on Kcat data, the eight ketoacyl-ACP synthase reactions required to synthesize a C18 FA contribute 2.3 s to the lag. Long-chain acyl-CoA synthetase (LACS) would contribute 0.8 s. Kcat values for three enzymes are not available. The other pathway enzymes have higher turnover numbers (11–265 s^{-1}) and together would contribute 0.9 s. A conservative estimate for the combined lag times introduced by the enzymatic reactions between [^{14}C]acetate and PC is 6.2 s.

The total lag time predicted from the enzyme turnover time (6.2 s) plus the precursor pools (11.9 s) is 18.1 s (Fig. 6B). The observed 5.4 ± 4.4 s (Fig. 6) lag time is statistically different ($P < 0.05$) compared to the predicted lag time of 18.1 s (Fig. 6B). Therefore, it is clear that [^{14}C]label moves into PC much more quickly than expected based on the intermediate pools. The most straightforward interpretation for this observation is that intermediates in the pathway undergo substrate channeling and therefore do not mix with the bulk pools. In particular, because the acyl-CoA pool would introduce the largest lag (9.5 s), we infer that the bulk cytosolic acyl-CoA pool is not an intermediate. Instead, we propose a model in which acyl-CoA synthesized at the envelope by LACS does not mix with the bulk cytosolic acyl-CoA pool before incorporation into PC. The corollary hypothesis is that, after activation of newly synthesized FA to CoA at the plastid envelope, substrate channeling directly delivers acyl groups to the PC acyl editing cycle.

Substrate channeling has previously been proposed for chloroplast FA synthesis (Roughan and Ohlrogge, 1996; Roughan, 1997) and for activation by LACS of the FFA released by acyl-ACP thioesterase after FA synthesis (Koo et al., 2004). Although there are uncertainties (see below), the results above imply that substrate channeling occurs not only for FA and acyl-CoA synthesis but extends to the incorporation of newly synthesized acyl chains into PC. Below we present additional data related to this hypothesis.

Substantial LysoPC Acyl Transferase Activity Is Associated with Chloroplasts

Although in vivo data are lacking, based on in vitro assays, LPCAT is a strong candidate for the enzyme activity that is responsible for PC acyl editing (Bates et al., 2007; Ståhl et al., 2008). LPCAT transfers acyl

chains from acyl-CoA onto lysoPC and also catalyzes the reverse reaction (Stymne and Stobart, 1984). The forward and reverse reactions together constitute one possible acyl-exchange mechanism between acyl-CoA and PC.

LPCAT activity is generally considered to act at the ER, although its subcellular localization has not been well established. Addition of [^{14}C]acyl-CoA to isolated pea, spinach, or leek (*Allium porrum*) chloroplasts results in [^{14}C]PC as the major labeled glycerolipid (Bertrams et al., 1981; Bessoule et al., 1995; Kjellberg et al., 2000). LPCAT activity has also been directly assayed in chloroplast envelopes isolated from pea leaves (Kjellberg et al., 2000). This activity was unaffected by thermolysin treatment, suggesting it resides in the inner envelope or the inner face of the outer envelope. Although ER contamination of the chloroplasts was not assessed, these results indicate there is a plastid envelope-associated LPCAT activity, which potentially is involved in acyl editing. Kjellberg et al. (2000) did not assay other subcellular fractions and thus did not determine whether the envelope-associated LPCAT is a minor or a more substantial proportion of total cellular LPCAT.

To further investigate a possible role for acyl editing at the chloroplast envelope, we determined the subcellular distribution of LPCAT activity. Because isolation of intact organelles with high yield from T-87 cells is problematic, we fractionated lysed pea leaf protoplasts by ultracentrifugation on a linear Suc gradient (Fig. 7A). We observed a clear correspondence between peaks of LPCAT activity and chlorophyll distribution. Approximately 30% of all LPCAT activity was associated with chloroplasts (Fig. 7B, shaded region). LPCAT activity was also observed in fractions enriched in ER and in plasma membranes as determined by marker enzyme activity (Fig. 7C). LPCAT activity recovered at the top of the gradient (presumably light microsomes and soluble proteins) was recovered in the pellet after centrifugation at 100,000g_{max}, indicating that this LPCAT activity is also membrane bound. Approximately 24% of the cytochrome c reductase ER marker was associated with chloroplast fractions and may represent plastid-associated membranes (PLAMs; Andersson et al., 2007) or other ER-plastid associations (Kaneko and Keegstra, 1996; Hanson and Köhler, 2001).

Not only is substantial LPCAT activity associated with chloroplasts, but PC, its substrate (reverse reaction), is also the major phospholipid of the chloroplast envelope (Dorne et al., 1985). In fact, it can be estimated that 40% of total cellular PC of leaves is localized in the outer envelope (Supplemental Text S2). PC is also a major phospholipid of oilseed plastids (Miernyk, 1985). Therefore, after FAs are exported from the plastid and esterified to CoA by LACS, the acyl-CoA would encounter both abundant PC substrate and LPCAT activity. The colocalization of the LACS and LPCAT enzymes and the substrates for acyl editing at the same site as FA export supports a hypothesis that acyl groups are channeled into PC at the chloroplast envelope without mixing with the bulk acyl-CoA pool. As noted above,

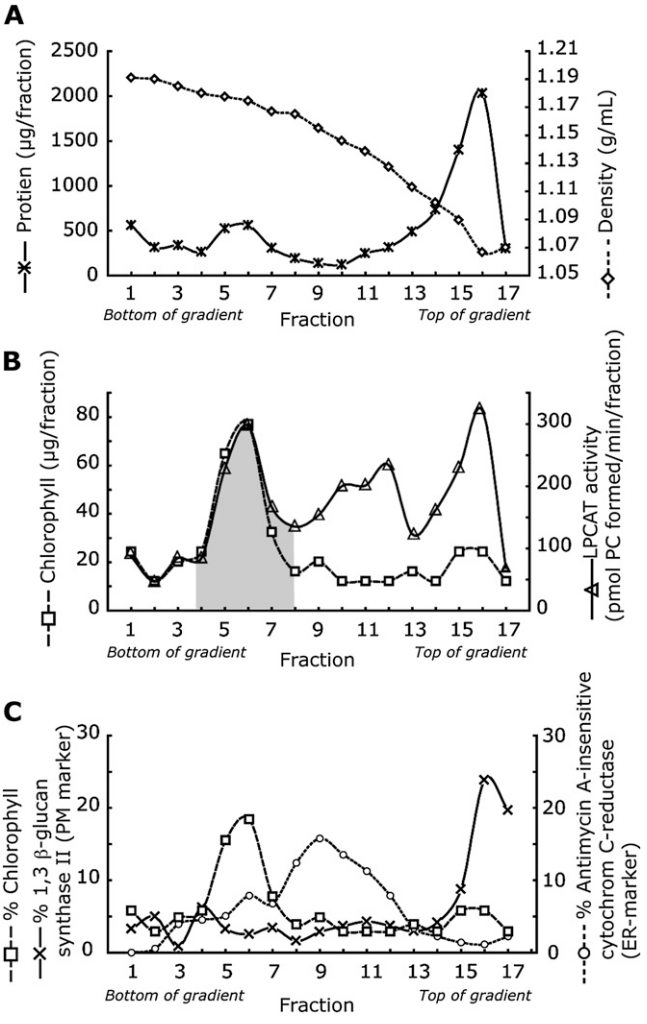

Figure 7. Subcellular fractionation of pea leaf protoplasts. Fractions from Suc gradient were assayed for: A, distribution of protein content and density; B, LPCAT activity (18:1-CoA/18:1-lysoPC) and chlorophyll content; and C, antimycin A-insensitive cytochrome c reductase (ER marker) and 1,3 β-glucan synthase II (plasma membrane marker). Relative percentage of abundance of chlorophyll is displayed for reference; 100% cytochrome c reductase = 63 nmol min^{-1} mg protein^{-1}, and 100% glucan synthase II = 57 nmol min^{-1} mg protein^{-1}. Assays were performed on duplicate biological samples, and representative values are displayed.

this scenario is also supported by the very short lag time for [^{14}C]acetate incorporation into PC (Fig. 6).

CONCLUSION/PERSPECTIVE

In this study, we provided data on the lipid composition and initial reactions of glycerolipid biosynthesis of T-87 cell cultures. Together with high-throughput transformation methods, these data should be useful as a baseline for design and analysis of additional experiments, for example, the testing of functions of lipid biosynthesis or regulatory genes. In addition, labeling data indicated that a major flux of newly

synthesized FA into glycerolipids occurs via acyl editing. Thus, this pathway is widespread, occurring in 16:3 and 18:3 plants.

The very rapid labeling of PC acyl chains (Figs. 4A and 6), together with the subcellular distribution of LPCAT (Fig. 7B) and of PC, provides insight into models of plant lipid trafficking. The two-pathway model of plant lipid metabolism (Roughan and Slack, 1984) describes major trafficking of acyl chains from the plastid to the ER and the return of acyl chains from ER to plastid. In this model, acyl chains first synthesized in the plastid are exported to the ER for incorporation into glycerolipids and for further desaturation. After desaturation, the return of acyl chains from the ER to plastids is most often considered to involve PC as a carrier (Somerville et al., 2000; Benning, 2008). By contrast, the trafficking of newly synthesized acyl chains from the plastid to the ER has generally been assumed to occur through an acyl-CoA pool in the cytosol, followed by their incorporation into ER glycerolipids. However, in vivo evidence for acyl-CoA as a carrier of acyl chains from plastid to ER is lacking. From the data presented in this study, instead of acyl-CoA movement through an acyl-CoA pool in the cytosol, we propose that newly synthesized acyl chains enter PC via substrate channeling at the plastid envelope and that PC (rather than acyl-CoA) may then serve as a carrier of acyl chains from plastids to the ER. Thus, PC may be central to acyl fluxes that occur in both directions between plastids and the ER. This hypothesis is supported by (1) association of 30% of pea leaf LPCAT activity with chloroplasts; (2) localization of 40% of leaf PC in the chloroplast envelope; (3) very rapid incorporation of acyl groups into PC by acyl editing with a lag time less than predicted if flux is through the bulk acyl-CoA pool; and (4) very rapid CoA dependent incorporation of [^{14}C]acetate into polar lipids by isolated chloroplasts (Koo et al., 2004).

Several aspects of this alternative scenario of acyl export from plastids are uncertain. Direct in vivo evidence that acyl editing occurs via the LPCAT enzyme is lacking. In addition, it is uncertain if PC acylation occurs via LPCAT that is an integral component of the chloroplast envelope or LPCAT associated with plastid-associated membranes (Andersson et al., 2007) or with other ER to chloroplast contact sites or associations (Xu et al., 2008). Finally, uncertainties in the subcellular distribution of acyl-CoA pools (and extrapolation to T87 cells) contribute to the provisional nature of this model. Nevertheless, consideration of PC as a carrier for acyl-chains from the plastid to the ER, as well as for the reverse traffic, may be useful in building a more complete understanding of acyl lipid metabolism in plants.

MATERIAL AND METHODS

Plant Material

Arabidopsis (*Arabidopsis thaliana*) T-87 cells were grown either in light (40–50 μmol m^{-2} s^{-1}) or in the dark at 120 rpm in media as described by Alonso et al. (2010). Cell cultures were maintained by a 1:9 (culture:fresh media) split every 7 d. Pea (*Pisum sativum*) cv Little marvel was cultivated in soil: vermiculite:perlite mixture (1:1:1) under 180 to 200 μmol m^{-2} s^{-1}white light with a 16/8-h (day/night) photoperiod at 22°C to 25°C.

Lipid Extraction and Analysis

Cultured cells were harvested by centrifugation (1,800g_{max}) and washed with distilled water three times. Cells were resuspended in boiling isopropanol and heated for 10 min, and lipids were extracted according to Hara and Radin (1978). Neutral lipids were separated on K6 TLC plates using hexane: diethyl ether:acetic acid (70:30:1, v/v/v) as the mobile phase. Polar lipids were separated on ammonia-impregnated K6 TLC plates with acetone:toluene: water (91:30:8, v/v/v) as the mobile phase. Lipids were identified by comigration relative to standards. Total FA, DAG, and TAG content was quantified by acid-catalyzed transmethylation to fatty acid methyl esters (FAMEs; Browse et al., 1986) with triheptadecanoic (Tri-17:0-TAG) and tripentadecanoic (Tri-15:0-TAG) internal standards. FAMEs were analyzed by gas chromatography on a DB23 column (30 m × 0.25 mm × 0.2 mm; J&W Scientific) and detected by flame ionization. Levels of glyco- and phospholipids were separated by solid-phase extraction according to Andersson et al. (2005). Purity of glyco- and phospholipids was examined by TLC, and their abundances were quantified as FAMEs by gas chromatography-flame ionization detection as above. The relative proportions of individual molecular species were determined for five biological replicates. Samples were analyzed by electrospray ionization triple quadrupole mass spectrometry with internal standard for each phospholipid and galactolipid class at the KS Lipidomics Research Center (www.k-state.edu/lipid/lipidomics; Welti and Wang, 2004).

Radiolabeling of Lipids

For [^{14}C]labeling studies, 5-d T-87 cell cultures were used. From a 50-mL T-87 cell culture (light), approximately one-fourth the volume was transferred to a 25-mL Erlenmeyer flask and allowed to equilibrate with gentle shaking (120 rpm) for 3 h prior to the addition of 150 μCi [1-^{14}C]acetate (specific activity 52 mCi/mmol) or 16 μCi [^{14}C-(U)]glycerol (specific activity 150 mCi/mmol). Samples (2 mL) were quickly removed at each time point, while shaking was maintained, and transferred to 10 mL of boiling isopropanol to quench metabolism. After hexane-isopropanol extraction (Hara and Radin, 1978), 85% to 95% of supplied [^{14}C]label was recovered in the lipid and aqueous phase. Radiolabeled lipids were separated by TLC as described above. Incorporation of radiolabel into lipids was determined with an Instant Imager electronic autoradiography (Packard) and scintillation counter (LS 6500; Beckman Coulter). In preliminary experiments, we observed less reproducible results if cells were not preequilibrated or were allowed to settle before addition of radiolabel. Up to 30% of [1-^{14}C]acetate was incorporated into nonglycerolipid product, which comigrated with squalene, and sterols.

Analysis of Radiolabeled Acyl Chains

[^{14}C]acyl chains from PC and DAG were isolated by preparative TLC followed by transmethylation to FAMEs using 2 mL of 5% H_2SO_4 in methanol and 200 μL toluene at 85°C for 1 h. FAMEs were extracted with hexane after adding 0.9% NaCl and were separated on 10% (w/v) $AgNO_3$ impregnated TLC as previously described (Bates et al., 2009). Regiochemistry of acyl chains of DAG was performed as previously described (Christie, 2003). Briefly, 1,2-DAG was purified by preparative TLC. After acetylation of DAG, the product TAG was treated with pancreatic lipase to release *sn*-1/*sn*-3 acyl chains. Lipase digestion products were separated on TLC, and the monoacylglycerol band was transmethylated to FAMEs and separated on TLC plates impregnated with 10% (w/v) $AgNO_3$ as described (Bates et al., 2009). Regiochemistry of PC was performed as described by Bates et al. (2007) with PC purification by preparative TLC and subsequent digestion using phospholipase A_2 from *Crotalus atrox*. The digestion products (lysoPC and FFAs) were separated on TLC plates, and each band was transmethylated to FAMEs and separated by silver TLC (10% [w/v] $AgNO_3$).

Protoplast Isolation and Fractionation

The lower epidermis of rapidly expanding pea leaves (7–8 d old) was abraded with a nylon brush and sliced into 1-mm strips. Prior to digestion,

leaf strips were incubated for 1 h in preplasmolysis media (330 mM sorbitol, 1 mM $CaCl_2$, and 10 mM MES, pH 6.0). The leaf strips were transferred to digestion media (2% cellulase, 0.2% mazerozyme, 550 mM sorbitol, 1 mM $CaCl_2$, 0.25% BSA, and 10 mM MES, pH 6.0) and incubated at 30°C, approximately 30 to 40 μmol m^{-2} s^{-1}, with gentle agitation for 3 h. Released protoplasts were separated from the leaf tissue by filtration through 100-μm nylon mesh, and leaf tissue was washed twice with wash buffer (550 mM sorbitol, 1 mM $CaCl_2$, and 10 mM MES, pH 6.0) to collect additional protoplasts. The resulting protoplasts were washed twice by spinning ($70g_{max}$ for 5 min) and further purified using differential centrifugation in combination with stacked 35% and 25% Percoll gradients (3 mL each) with 5 mL of wash buffer on top. After centrifugation ($250g_{max}$ for 10 min), intact protoplasts were collected at the 25% interphase and washed twice by centrifugation ($70g_{max}$ for 5 min) in wash buffer to remove residual Percoll. Protoplasts were ruptured by three passes thorough a 20-μm nylon mesh (Nishimura et al., 1976). The ruptured protoplasts (approximately 1 mL) were separated on a 11-mL 20% to 60% linear Suc gradient (10 mM HEPES, pH 7.0, and 1 mM $CaCl_2$) at $100{,}000g_{max}$ for 4 h. Fractions (700 μL) were collected and frozen in liquid nitrogen and stored at −80°C until further analysis. Chloroplasts were identified by the presence of chlorophyll as described (Arnon, 1949). Protein content was determined using bicinchoninic acid reagent (Smith et al., 1985).

Enzyme Assays

LyosPC acyl transferase activity was assayed at room temperature in a final volume of 30 μL containing 5 μg protein, 23 μM lysoPC (18:1), and 70 μM [^{14}C] 18:1-CoA (0.01 μCi), in 50 mM HEPES, pH 8.0, and 10 mM KCl. Reactions were initiated by adding the enzyme and terminated by the addition of 10 μL acetonitrile:acetic acid (4:1). The entire reaction mixture was loaded on K6 TLC plates and developed with $CHCl_3$:MeOH:HAc:H_20 (85:15:10:3.5). Quantification of radiolabeled products was done by autoradiography as above. Marker enzymes were used to identify membranes in fractions collected from the subcellular fractionation. ER was identified by NADH-dependent, antimycin A-insensitive cytochrome c reductase (Hodges and Leonard, 1974) and plasma membranes by 1,3-β-glucan synthase (Fredrikson and Larsson, 1989).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. [^{14}C]acetate incorporation and [^{14}C]labeled acyl composition into DAG and TAG.

Supplemental Figure S2. [^{14}C]acetate incorporation into major glycerolipids (15 s–10 min).

Supplemental Figure S3. [^{14}C]acetate incorporation into major glycerolipids (5–60 min).

Supplemental Table S1. Turnover number of enzymes involved in fatty acid synthesis and calculation of reaction times for [^{14}C]acetate incorporation into PC.

Supplemental Text S1. Estimation of cytosolic acyl-CoA pool.

Supplemental Text S2. Calculation of cellular distribution of PC.

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