Running head: Synthesis and plastid export of T-87 lipids

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Article title: **Rapid kinetic labeling of Arabidopsis cell suspension cultures: Implications for models of lipid export from plastids**

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ABSTRACT

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 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 3-4 fold lower than Arabidopsis leaves. Triacylglycerol represented 10% of total acyl chains, a greater percentage than in most non-seed tissues. The initial steps in T-87 cell lipid assembly were evaluated by pulse labeling cultures with [14C]acetate and [14C]glycerol. [14C]acetate was very rapidly incorporated into PC, preferentially at sn-2, and without an apparent precursor-product relationship to diacylglycerol (DAG). In contrast, [14C]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 [14C]acetate labeling of PC implied ‘channeled’ incorporation of acyl chains without mixing with the bulk acyl-CoA pool. Subcellular fractionation of pea-leaf protoplasts indicated that 30% of lysophosphatidylcholine acyltransferase activity co-localized with chloroplasts. Together these data support a model in which chloroplast PC participates in trafficking of newly synthesized acyl chains from plastids to the endoplasmic reticulum.
INTRODUCTION

Cell suspension cultures are commonly used and are important model systems for study of many aspects of plant cell biology, biochemistry and molecular biology (Razdan, 2003). In contrast to 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., 2004b; Ogawa et al., 2008). This eases the generation and selection of large numbers of independent transgenic lines compared to whole-plant transformation. Although tobacco BY2 cells have been the most widely used cell culture system, the abundant information and molecular and genetic tools available for Arabidopsis 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 \[^{14}C\]\text{acetate} and \[^{14}C\]\text{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 into membrane lipids.

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-ACP products of fatty acid synthesis are hydrolyzed by plastid acyl-ACP thioesterase reactions and exported to an outer envelope-bound acyl-CoA (acyl-CoA) synthetase. De novo assembly of glycerolipids occurs largely at the endoplasmic reticulum (ER) by two acylations of glycerol-3-phosphate (G3P) to form phosphatidic acid (PA) (Kornberg and Pricer, 1953; Somerville et al., 2000). 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-Pricer, 1953; Kennedy and Weise 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 and soybean export over 90% of fatty acids to supply the eukaryotic pathway (Ohlrogge and Browse 1995; Somerville et al., 2000).

Recently, kinetic labeling experiments of pea leaves and soybean seeds with $[^{14}\text{C}]$acetate has indicated that most fatty acids synthesized by plastids are initially incorporated into phosphatidylcholine (PC) via ‘acyl-editing’ of preformed PC, independently of the Kornberg-Pricer pathway. In contrast, $[^{14}\text{C}]$glycerol labeling indicated most acylation of G3P to form PA and diacylglycerol (DAG) utilizes pre-existing 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 regio-chemical analysis of $[^{14}\text{C}]$-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 fatty acid and glycerolipid labeling has not yet been extended to 16:3 species such as Arabidopsis, although CO$_2$ labeling of $B$. napus leaves (Williams et al., 2000) is consistent with acyl editing. In this study, analysis of initial $[^{14}\text{C}]$acetate and $[^{14}\text{C}]$glycerol labeling of lipids provided evidence for acyl editing in a 16:3 species. In addition, rapid pulse $[^{14}\text{C}]$-labeling provided information on the precursor pools involved in the initial incorporation of $[^{14}\text{C}]$-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.,
As indicated in Fig. 1, cultures grown with 50 µmol m\(^{-2}\) s\(^{-1}\) light or grown in the dark accumulated similar biomass, fatty acid levels and protein throughout a 7-day cultivation period. Between day 3 and 5, T-87 cell culture dry weights increased 2.7-fold 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 fatty acid synthesis of light-grown T-87 cultures during the 3 to 5 day rapid growth period (calculated from Fig. 1 A and B) was 18 nmol C \(\cdot\) h\(^{-1}\) mg\(^{-1}\) fresh weight (expressed on basis of atoms of carbon) (Fig. 1A and B). For comparison, expanding leaves of 3-week old Arabidopsis plants have a rate of fatty acid synthesis of \(~2.3\) nmol C \(\cdot\) h\(^{-1}\) mg\(^{-1}\) fresh weight (Bao et al., 2000; Bonaventure et al., 2004). Thus, the net rate of fatty acid 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 fatty acid content of T-87 cells was 98-118 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 fatty acids were linoleic (18:2), linolenic (18:3) and palmitic acid (16:0) (Table I). 16:3 represented 1.0-1.5 mol% compared to 13.8 mol% for leaves (Miguel and Browse 1986). The low 16:3 fatty acid 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) (Stand et al., 1999). Low levels of the largely thylakoid associated 16:3 fatty acid 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), phosphatidylserine (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 (Miguel and Browse, 1992; Welti et al., 2002; Li-Bession 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 SQDG
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 (~1/2-1/3 of typical light levels for plants grown in soil) and the high sucrose level in the growth media (90 mM). Sucrose is known to suppress expression of genes associated with photosynthesis (Goldschmitt et al., 1992). Lipid compositions were similar in light and dark, and more closely resembled non-photosynthetic Arabidopsis tissues than leaves. Overall, T-87 cells displayed no major anomalies 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 which changes 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 ESI-MS/MS (Fig. 3A). Molecular species of PC, PE, PI and PA and DGDG were similar to those observed in leaves and roots (Li et al., 2006). 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 fatty acids (>20 carbon) which are characteristic of PS and known to induce membrane curvature (Israelachivlli et al., 1980) were more abundant in T-87 cells compared to leaf tissue (Nerlich et al., 2007; Li et al., 2006). This may relate to the more spherical shape of suspension culture cells compared to leaf tissue.

**T-87 cells accumulate TAG**

TAG is a major component of seeds and pollen, but is usually found at very low levels in vegetative tissues. For example, TAG is less than 1% of lipids of leaves during growth from seedlings until senescence of Arabidopsis, *Brachypodium* and Switchgrass (Yang and Ohlrogge, 2009). In 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 fatty acids (7.6 mol% of
glycerolipids) in light-grown T-87 cells (Fig. 2). Linoleic and linolenic acids were the predominant fatty acids (>50% of total) of TAG for T-87 cells grown in either light or dark (Fig 3B). The TAG fatty acid composition was distinct from Arabidopsis seeds which include ~26% fatty acids with chain length >C18 (largely 20:1), (Li et al., 2006). In contrast, the fatty acid compositions in TAG derived from T-87 cells was very low in eicosenoic acid (20:1) or other >C18 fatty acids. In agreement with the content based on fatty acid levels, after [14C]acetate labeling (0-60 min) TAG accounted for 11% of total [14C]-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 non-seed 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 fatty acid and glycerol incorporation into glycerolipids were investigated by labeling experiments using [14C]acetate and [14C]glycerol. Exogenously supplied [14C]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). In contrast, [14C]glycerol is primarily incorporated into the glycerol backbone of glycerolipids (Slack et al., 1977). Thus, experiments with [14C]acetate and [14C]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 fatty acid incorporation into glycerolipids and to investigate pools 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.

**[14C]acetate labeling**

To identify the initial products of T-87 acyl lipid metabolism, [14C]acetate was added to
cultures and radiolabeled lipids were analyzed by 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 3-7 fold higher than DAG from 30 s to 10 min (Fig. 4 A). 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 FFA and lysoPC products indicated that 74% of the radiolabel was associated to the sn-2 position (Fig. 4B). Further analysis of the radiolabeled acyl groups by silver TLC revealed that over 90% of the $[^{14}C]$-label at the sn-2 position was monoens whereas less than 3 % was in the form of saturates and dienes. In contrast, at sn-1 there was a 45/55 distribution between saturates and monoens, 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 to 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 fatty acids 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 extra-plastidial 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 leaves (a plant with 16:3 metabolism), where 74% of $[^{14}C]$acetate labeled DAG was of prokaryotic origin (Roughan et al., 1980).
[\textsuperscript{14}C]glycerol labeling

In contrast to [\textsuperscript{14}C]acetate labeling of PC, [\textsuperscript{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 [\textsuperscript{14}C]glycerol in PC was undetectable and at one minute label in DAG was 200 fold greater than PC (Fig. 5). Unlike [\textsuperscript{14}C]acetate labeling, these results are consistent with the precursor-product relationship between DAG and PC for de novo PC biosynthesis (Kennedy and Weise, 1956).

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 fatty acids 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 [\textsuperscript{14}C]-acyl chains into PC relative to DAG (Fig. 4A); 2) the preferential incorporation of [\textsuperscript{14}C]acetate label into sn-2 of PC compared to sn-1. This pattern differed from DAG where radiolabelled acyl chains were almost evenly distributed between sn-1 and sn-2 (Fig. 4B); 3) the major initial product of [\textsuperscript{14}C]glycerol labeling was DAG whereas PC labeling lagged at least one min (Fig. 5).

Are newly synthesized acyl chains channeled into PC at the plastid envelope? Consideration of lag times and precursor pools 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 also 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 [\textsuperscript{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 [\textsuperscript{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 $[^{14}\text{C}]$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 $5.4 \pm 4.4$ s (Fig. 6A) was determined.

The precursor pools involved in $[^{14}\text{C}]$acetate incorporation into glycerolipids include acetyl-CoA, acyl-acyl carrier protein (acyl-ACP), free FA (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 supplement text 1 for calculation). The sum of acetyl-CoA, acyl-ACP, FFA and acyl-CoA precursor pools is 82 pmol C • mg FW$^{-1}$.

Considering the kinetics of pool filling as a first order kinetic process, the time required to saturate 82 pmol of intermediate pools can be calculated from the equation: lag time = $\ln2/k$ where $k$=rate of fatty acid synthesis / substrate pool (Segel, 1976). Based on the rate of fatty acid synthesis of 4.8 pmol C s$^{-1}$ mg$^{-1}$ fresh weight (calculated per s from Fig. 1B) a lag of 11.9 s would occur to saturate the above intermediate pools and achieve linear $^{14}\text{C}$-label incorporation into PC.

A second major factor influencing the lag in linear $[^{14}\text{C}]$ 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 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 supplement Table SI. 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 8 KAS 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 to 265 s$^{-1}$) and together would contribute 0.9 s. A conservative estimate for the combined lag times introduced by the enzymatic reactions between $[^{14}\text{C}]$acetate and PC is 6.2 s The total lag time predicted from the
enzyme turnover time plus the precursor pools is 18.1 s (6.2 s + 11.9 s) (Fig. 6C). The observed 5.4 ± 4.4 s (Figs 6A and B) 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 [14C]-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 conclude 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 fatty acid synthesis (Roughan and Ohlrogge, 1996; Roughan, 1997) and for activation by LACS of the free FA 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 fatty acid 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 [14C]acyl-CoA to isolated pea, spinach or leek chloroplasts results in [14C]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 ultra-centrifugation on a linear sucrose 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 endoplasmic reticulum 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 100 000 g_{\text{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 (PLAM; 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 (Supplement Text 2). PC is also a major phospholipid of oilseed plastids (Miernyk, 1985). Therefore, after fatty acids 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 fatty acid 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, this scenario is also supported by the very small lag time for [14C]acetate incorporation into PC (Fig. 6A and B).

**Conclusions/Perspective**
In this study we have 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 as well as 18:3 plants.

The very rapid labeling of PC acyl chains (Fig. 4A, Fig. 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). In 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; 4) very rapid CoA dependent incorporation of [*14C*]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 PLAM (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 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 in Alonso et al., 2010. Cell cultures were maintained by a 1:9 (culture: fresh media) split every seven days. Pisum sativum cv. Little marvel was cultivated in soil:vermiculite:perlite mixture (1:1:1) under 180-200 µmol m\(^{-2}\) s\(^{-1}\)white light with a 16/8 h (day/night) photoperiod at 22-25°C.

Lipid Extraction and analysis

Cultured cells were harvested by centrifugation (1800g\(_{\text{max}}\)) and washed with distilled water three times. Cells were resuspended in boiling isopropanol, 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 co-migration relative to standards. Total fatty acid, DAG and triacylglycerol (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 GC on a DB23 column (30 m x 0.25 mm x 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 GC-FID 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 Kansas Lipidomics Research Center (www.k-state.edu/lipid/lipidomics; Welti and Wang, 2004).

**Radio-labeling of lipids**

For $[^{14}C]$-labeling studies, five-day 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), 85-95% 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, Meriden) and scintillation counter (LS 6500, Beckman Coulter). In preliminary experiments we observed less reproducible results if cells were not pre-equilibrated or were allowed to settle before addition of radiolabel. Up to 30% of [1-$^{14}$C]acetate was incorporated into non-glycerolipid product, which co-migrated 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$_2$SO$_4$ in methanol and 200 µL toluene at 85°C, 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). Regio-chemistry 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). Regio-chemistry 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 free fatty acids) were separated on TLC plates and each band was transmethylated to FAMEs and separated by silver TLC (10% (w/v) AgNO₃).

**Protoplast isolation and fractionation**

The lower epidermis of rapidly expanding pea leaves (7-8-days old) was abraded with a nylon brush and sliced into 1mm strips. Prior to digestion, leaf-strips were incubated for 1 h in pre-plasmolysis media (330 mM sorbitol, 1 mM CaCl₂, 10 mM MES pH 6.0). The leaf strips were transferred to digestion media (2% cellulase, 0.2% mazerozyme, 550 mM sorbitol, 1mM CaCl₂, 0.25% BSA, 10 mM MES pH 6.0) and incubated at 30° C, ~30-40 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 CaCl₂, 10 mM MES pH 6.0) to collect additional protoplasts. The resulting protoplasts were washed twice by spinning (70 gₘₐₙₐₓ for 5 min) and further purified using differential centrifugation in combination with stacked 35% and 25% Percoll gradients (3 mL each) with 5 mL wash buffer on top. After centrifugation (250 gₘₐₙₐₓ for10 min), intact protoplasts were collected at the 25%-interphase and washed twice by centrifugation (70 gₘₐₙₐₓ 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 (~1 mL) were separated on a 11 mL 20-60% linear sucrose gradient (10 mM HEPES pH 7.0, 1 mM CaCl₂) at 100 000 gₘₐₙₐₓ for 4 hours. 700 µL fractions 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**

LysoPC 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 [¹⁴C] 18:1-CoA (0.01 µCi), in 50 mM HEPES pH8.0, 10mM 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 CHCl₃:MeOH:HAc:H₂O (85:15:10:3.5). Quantification of radiolabeled products was by autoradiography as above. Marker enzymes were used to identify
membranes in fractions collected from the subcellular fractionation. Endoplasmic reticulum 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).

**Supplement data**
The following data are available in the on-line version of this article

**Supplemental Fig. 1** [14C]acetate incorporation and [14C]-labeled acyl composition into DAG and TAG

**Supplemental Fig. 2** [14C]acetate incorporation into major glycerolipids (15 s-10 min)

**Supplemental Fig. 3** [14C]acetate incorporation into major glycerolipids (5-60 min)

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

**Supplemental Text 1** Estimation of cytosolic acyl-CoA pool

**Supplemental Text 2** Calculation of cellular distribution of PC

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FIGURE LEGENDS

Figure 1
Growth of T-87 cells with light (open symbols) or without light (solid symbols). Accumulation of A, biomass B, fatty acids C, chlorophyll and D, protein. Data are Mean ± SD of three replicates.

Figure 2
Glycerolipid composition of T-87 cells grown in light (white bars) or in dark (black bars). Data are mean ± SD (n = 5). Abbreviations: Monogalactosyl-diacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidic acid (PA), diacylglycerol (DAG) and triacylglycerol (TAG).

Figure 3
Glycerolipid composition of T-87 cells grown in light or dark. A, molecular species of polar glycerolipids and B, fatty acid composition of neutral glycerolipids. Bar height represents mean ± SD of five biological replicates.

Figure 4
A, time course of [14C]acetate incorporation into PC (circles) and DAG (squares) of light-grown T-87 cells. 2 mL 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 [14C]acetate labeling. Black bars: saturated acyl chains, white bars: monoenyes (18:1) and grey bars: dienes (18:2). Values in A and B represent mean ± SD of three biological replicates.

Figure 5
Time course of [14C]glycerol incorporation into PC (circles) and DAG (squares) in light-grown T87 cells. 2 mL cultures were sampled. Inset graph expands scale of early time points. Values represent mean ± SD of three biological replicates. Note: These values have not been corrected for the small amount of glycerol labeled that enters acyl chains via glycerol metabolism to acetyl-
CoA (Slack et al., 1977). Our analysis indicated 1.5-4% of the radioactivity from glycerol labeling was located in the acyl chains. Similar values have been reported for [14C]glycerol labeling of DAG in soybean embryos (Bates et al., 2009).

**Figure 6**
Determination of lag time for incorporation of [14C]acetate into PC. A, Data from seven biological replicates were used to calculate the time-axis intercept. To allow comparisons between independent experiments, [14C]acetate incorporations at 2 min were normalized to 100%. Average ± SD (n=7) are plotted for each time point. Arrow indicates time axis intercept, inset table represent individual time axis intercept and R² values for each of the seven time courses. B, Predicted time required for reactions from [14C]acetate PC (black bar) and for pool filling (grey 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 mean ± SD for time-axis intercept data from Fig. 6A.

**Figure 7**
Subcellular fractionation of pea leaf protoplasts. Fractions from sucrose gradient were assayed for distribution of A, 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), 1,3 β-glucan synthase II, (plasma membrane marker). Relative % abundance of chlorophyll is displayed for reference. 100% cytochrome c reductase = 63 nmol min⁻¹ mg protein⁻¹; 100% glucan synthase II = 57 nmol min⁻¹ mg protein⁻¹. Assays were performed on duplicate biological samples: representative values are displayed.
Table I

Fatty acid composition of total lipids extracted from light or dark grown T-87 cultures Mean ± 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.

| Fatty acid | T-87 (light) | T-87 (dark) | Arabidopsis leaf† | 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 |

† values from Miquel and Browse 1992,
