RESEARCH ARTICLE

Metabolically Distinct Pools of Phosphatidylcholine Are Involved in Trafficking of Fatty Acids out of and into the Chloroplast for Membrane Production

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Short title: Role of LPCAT in leaf acyl trafficking

One sentence summary: Chloroplast lysophosphatidylcholine acyltransferases are involved in PC acyl editing with newly synthesized fatty acids, and not in lysophosphatidylcholine transport for galactolipid synthesis.

Footnotes:
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ABSTRACT

The eukaryotic pathway of galactolipid synthesis involves fatty acid synthesis in the chloroplast, followed by assembly of phosphatidylcholine (PC) in the endoplasmic reticulum (ER), and then turnover of PC to provide a substrate for chloroplast galactolipid synthesis. However, the mechanisms and classes of lipids transported between the chloroplast and the ER are unclear. PC, PC-derived diacylglycerol, phosphatidic acid, and lyso-phosphatidylcholine (LPC) have all been implicated in ER-to-chloroplast lipid transfer. LPC transport requires lysophosphatidylcholine acyltransferase (LPCAT) activity at the chloroplast to form PC prior to conversion to galactolipids. However, LPCAT has also been implicated in the opposite chloroplast-to-ER trafficking of newly synthesized fatty acids through PC acyl editing. To understand the role of LPC and LPCAT in acyl trafficking we produced and analyzed the *Arabidopsis thaliana act1 lpcat1 lpcat2* triple mutant. *LPCAT1* and *LPCAT2* encode the major lysophospholipid acyltransferase activity of the chloroplast, and it is predominantly for incorporation of nascent fatty acids exported form the chloroplast into PC by acyl editing. *In vivo* acyl flux analysis revealed eukaryotic galactolipid synthesis is not impaired in *act1 lpcat1 lpcat2* and utilizes a PC pool distinct from that of PC acyl editing. We present a model for the eukaryotic pathway with metabolically distinct pools

of PC, suggesting an underlying spatial organization of PC metabolism as part of the ER–chloroplast metabolic interactions.

INTRODUCTION

Membranes that encompass, subdivide, and provide scaffolds for protein localization are essential to all living cells. In plant leaf tissue, the thylakoid membranes within the chloroplast are composed predominantly from the galactolipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) and are the essential structures that hold the photosynthetic apparatuses. The metabolic pathways of photosynthetic membrane production have been studied for over 40 years through biochemical, genetic, and molecular biology approaches and have been extensively reviewed across the decades (Roughan and Slack, 1982; Browse and Somerville, 1991; Ohlrogge and Browse, 1995; Moreau et al., 1998; Kelly and Dormann, 2004; Benning, 2008, 2009; Shimojima et al., 2009; Li-Beisson et al., 2013; Boudière et al., 2014; Hurlock et al., 2014; Block and Jouhet, 2015; Bastien et al., 2016; Botella et al., 2017; Li-Beisson et al., 2017; LaBrant et al., 2018). This research has led to a complicated metabolic model that requires the trafficking of lipid substrates from the chloroplast, to the endoplasmic reticulum (ER), and then back into the chloroplast to produce galactolipids (Fig. 1). Most of the biosynthetic enzymes and associated genes of fatty acid synthesis, glycerolipid assembly, and fatty acid desaturation crucial to produce galactolipids have been identified. However, a major area of uncertainty surrounds the process of lipid transfer between the chloroplast and the ER, and even the lipid species that is transferred from the ER back into the plastid is still unclear (LaBrant et al., 2018).

Figure 1 displays the current state of the two-pathway model of leaf MGDG synthesis (for more detailed comprehensive recent reviews please see (Li-Beisson et al., 2013; Hurlock et al., 2014; Botella et al., 2017; Li-Beisson et al., 2017; LaBrant et al., 2018)). Fatty acids are synthesized up to 18-carbon saturated or monounsaturated fatty acids while esterified to acyl carrier protein (ACP) within the chloroplast stroma. In some plants these acyl-ACPs can be utilized by the acyl selective glycerol-3-phosphate acyltransferase (GPAT) and lysophosphatidic acid acyltransferase (LPAT) of the

78 "prokaryotic pathway" (*ACT1/ATS1* and *ATS2*, respectively (Kunst et al., 1988; Nishida
79 et al., 1993; Yu et al., 2004)) to produce a molecular species of phosphatidic acid (PA)
80 containing oleate (18:1, number carbons: number double bonds) at the *sn*-1 position,
81 and palmitate (16:0) at the *sn*-2 position. Dephosphorylation produces the diacylglycerol
82 (DAG) substrate of MGDG synthase (MGD1) (Jarvis et al., 2000). Subsequent
83 desaturation of the 18:1/16:0 molecular species to 18:3/16:3 produces the abundant
84 polyunsaturated molecular species of MGDG characteristic of chloroplast membranes.
85 Plants that produce up to half of the MGDG through the prokaryotic pathway are
86 known as 16:3 plants in reference to the 16:3 that accumulates at *sn*-2 of MGDG. Only
87 about 12% of Angiosperms (including *Arabidopsis thaliana*) are 16:3 plants (Mongrand
88 et al., 1998). In contrast to 16:3 plants, 18:3 plants accumulate 18:3 at the *sn*-2 position
89 of MGDG through glycerolipid assembly by the ER-localized "eukaryotic pathway"
90 where *sn*-2 acyltransferases are selective for 18-carbon unsaturated fatty acids. In all
91 plants, the eukaryotic pathway GPAT and LPAT (*AtGPAT9* and *AtLPAT2*, respectively
92 (Kim et al., 2005; Shockey et al., 2016; Singer et al., 2016)) utilize cytosolic acyl-CoA to
93 produce PA, and DAG in a parallel pathway to that in chloroplast (Fig. 1). The *de novo*
94 synthesized DAG is utilized to produce ER phospholipids such as phosphatidylcholine
95 (PC) and phosphatidylethanolamine (PE). PC is also the extra-plastidic site for
96 desaturation of 18:1, to 18:2 and 18:3 through the FAD2 and FAD3 enzymes,
97 respectively (Arondel et al., 1992; Okuley et al., 1994). Eukaryotic MGDG is synthesized
98 from a polyunsaturated DAG backbone derived from PC (Slack et al., 1977). However,
99 the mechanism and location of PC turnover, and the lipid class transferred from the ER
100 to the chloroplast are still unclear (Fig. 1, blue dashed lines). A key aspect of the
101 eukaryotic pathway is that extensive trafficking of acyl groups between the chloroplast
102 and the ER is required.
103 Fatty acid export from the chloroplast begins with hydrolysis of acyl-ACPs by
104 fatty acid thioesterases (Fig. 1) (Bates et al., 2013). The subsequent free fatty acids
105 (FFA) are transported across the chloroplast inner envelope membrane (IEM) by FAX1
106 (Li et al., 2015), and likely other members of the FAX family. The mechanism of FFA
107 transfer across the chloroplast outer envelope membrane (OEM) is not clear but may
108 involve vectoral diffusion driven by activation of FFA to acyl-CoA by long chain acyl-

coenzyme A synthetases (LACS) on the cytoplasmic side of the OEM and/or the ER (Schnurr et al., 2002; Koo et al., 2004; Zhao et al., 2010; Jessen et al., 2015), which prevents FFA diffusion back into the plastid. Original models of the eukaryotic pathway assumed acyl-CoA-containing newly synthesized fatty acids were utilized by the ER-localized GPAT and LPAT to produce initial molecular species of glycerolipids containing 16:0 or 18:1 at *sn*-1, and 18:1 at *sn*-2 prior to further desaturation on PC (e.g. (Ohlrogge and Browse, 1995)). However, metabolic tracing experiments in various plant tissues have demonstrated that the majority of newly synthesized fatty acids exported from the plastid are initially rapidly incorporated into PC by a process known as acyl editing, which is essentially an acyl-CoA:PC fatty acid exchange cycle (Williams et al., 2000; Bates et al., 2007; Bates et al., 2009; Bates et al., 2012; Tjellström et al., 2012; Yang et al., 2017). This exchange between PC and the acyl-CoA pool produces a mixture of nascent fatty acids exported from the plastid with previously synthesized fatty acids derived from PC, some of which may have been desaturated to 18:2, or 18:3. This mixed acyl-CoA pool is thus the substrate for the eukaryotic GPAT and LPAT reactions for *de novo* glycerolipid assembly. In particular, short time point (≤ 1 min) [^{14}C]acetate labeling of fatty acid synthesis in pea (*Pisum sativum*) leaves and Arabidopsis cells was crucial to demonstrating that nascent fatty acids are predominantly incorporated into the *sn*-2 position of PC by a lysophosphatidylcholine acyltransferase (LPCAT)-type reaction faster than incorporation into *de novo* DAG of the eukaryotic pathway (Bates et al., 2007; Tjellström et al., 2012). These results suggest that the lysophosphatidylcholine (LPC) pool and LPCAT enzymes involved in acyl editing may be part of the acyl trafficking of nascent fatty acids from the chloroplast to the ER (Fig. 1).

The lipids DAG, PA, PC, and LPC have all been suggested as the species transported from the ER to the chloroplast in the eukaryotic pathway (Fig. 1). DAG was first suggested to be transferred from the ER to the plastid after metabolic labeling experiments indicated that both the glycerol and fatty acids of PC were incorporated into MGDG together (Slack et al., 1977), and analysis of Arabidopsis PA hydrolase mutants (*pah1* and *pah2*) under phosphate starvation supported this conclusion (Nakamura et al., 2009). However, combining the *pah1* and *pah2* mutants with the *act1* mutation which eliminates the prokaryotic pathway did not appear to affect eukaryotic galactolipid

synthesis under phosphate replete conditions (Fan et al., 2014). Recent work characterizing the Arabidopsis *tgd1-5* mutants has indicated a transporter system involved in transferring the lipid substrate for MGDG synthesis from the chloroplast OEM to the IEM where MGDG synthesis occurs (Xu et al., 2003; Xu et al., 2005; Awai et al., 2006; Lu et al., 2007; Wang et al., 2012b; Wang et al., 2013; Fan et al., 2015). The mutants have impaired eukaryotic MGDG production and accumulate unusual trigalactosyldiacylglycerols (TGDG) as a phenotype. The TGD2 and TGD4 components of this transporter system bind to PA, and isolated chloroplasts from the *tdg1* mutant effectively convert exogenous DAG into MGDG but exogenous PA conversion to MGDG is partially reduced. Therefore, PA has been proposed as the molecule transported from the ER to the plastid (Xu et al., 2005; Lu and Benning, 2009; Wang et al., 2012b; Wang et al., 2013). However, it is also been suggested that the role of PA is to destabilize membranes to reduce the energy barrier to transport of a different lipid species (LaBrant et al., 2018). A recent mathematical modeling approach suggested DAG was a better substrate to transport than PA, but limited PA transport was also required to activate MGDG synthesis (Maréchal and Bastien, 2014). Both DAG and PA can be produced by lipases in the ER, and thus could be transferred from the ER to the chloroplast and then into the IEM for MGDG synthesis. A different approach would be to first move PC from the ER to the chloroplast, and then derive PA or DAG from PC for transport to the IEM by the mechanisms discussed above.

PC is highly abundant in the outer leaflet of the OEM but is not present in other chloroplast membranes (Dorne et al., 1985), while the ER lipid PE is absent from chloroplasts. PC can be selectively transferred over PE from liposomes to isolated chloroplasts, a process which is dependent on the proteins within the chloroplast OEM (Yin et al., 2015), suggesting PC could be directly transferred from the ER, possibly through ER-chloroplast membrane contact sites (Andersson and Dörmann, 2008; Mueller-Schuessele and Michaud, 2018). Recent characterization of the flippase ALA10 (Botella et al., 2016) suggests it could be involved in enriching PC in ER membrane contact sites prior to transfer to the plastid (Botella et al., 2017). Rather than trafficking of a whole membrane lipid, LPC is a more water-soluble derivative of PC that could more easily traverse an aqueous space between the two compartments. The abundant

LPCAT activities associated with the exterior of the chloroplast would then regenerate PC at the OEM (Bessoule et al., 1995; Tjellström et al., 2012). The transfer of LPC from the ER to plastid was supported by long time point (2–100 h) pulse-chase metabolic labeling studies in leek seedlings, which demonstrated a loss of fatty acids from *sn*-1/*sn*-2 PC and the subsequent accumulation in mostly *sn*-1 MGDG (Mongrand et al., 1997; Mongrand et al., 2000). The authors concluded that it was labeled *sn*-1-acyl-LPC that was transferred to the chloroplast and then reacylated with unlabeled fatty acids by LPCAT at the OEM during the chase in route to MGDG production. These results suggest that LPC and chloroplast LPCAT activity may be part of the ER-to-chloroplast lipid transfer reactions of the leaf eukaryotic pathway.

The discussion of previous research above indicates that LPC and LPCAT activity may have roles in both trafficking of fatty acids from the chloroplast to the ER, and from the ER to the chloroplast. Arabidopsis has four enzymes with demonstrated *in vitro* LPCAT activity: AtLPCAT1 and AtLPCAT2 (Stahl et al., 2008; Wang et al., 2012a; Lager et al., 2013; Wang et al., 2014), and the lysophosphatidylethanolmine acyltransferases AtLPEAT1 and AtLPEAT2 (Stalberg et al., 2009; Jasieniecka-Gazarkiewicz et al., 2016). AtLPCAT1 and AtLPCAT2 have a strong preference for 18-carbon unsaturated acyl-CoAs over 16:0-CoA, and thus could produce the *sn*-1/2 18-carbon molecular species of PC (and subsequent MGDG) characteristic of the eukaryotic pathway (Lager et al., 2013). Short timepoint metabolic tracing of lipid metabolism in developing seeds of the LPCAT1 LPCAT2 double mutant (*lpcat1 lpcat2*) indicated the initial incorporation of nascent fatty acids into PC through *sn*-2 acyl editing was abolished (Bates et al., 2012). Instead the acyl groups are rerouted and initially esterified to G3P through the GPAT and LPAT reactions of the eukaryotic pathway prior to *de novo* PC synthesis. This result suggests that the Arabidopsis LPCAT1 and LPCAT2 enzymes are involved in the flux of acyl groups from the plastid to the ER in developing seeds, and that LPEAT1 and LPEAT2 cannot compensate for the loss of LPCATs in the acyl-editing cycle. However, these previous results are not directly applicable to leaves for two reasons: (1) In developing seeds LPCAT1 and LPCAT2 are expressed at 2-3 fold higher levels than LPEAT1 and LPEAT2 (Supplemental Fig. 1) which may explain the lack of compensatory acyl-editing activity by LPEAT1 and

LPEAT2 in the *lpcat1 lpcat2* mutant seeds. In leaves LPEAT1 and LPEAT2 are expressed at similar or even higher levels than LPCAT1 and LPCAT2 (Supplemental Fig. 1). Therefore, it is possible that LPCAT1/LPCAT2 and LPEAT1/LPEAT2 may both contribute the acyl-editing LPCAT activity in leaves. (2) Quantitative acyl flux between the plastid and the ER is distinctly different between developing leaves and developing oilseed tissues. In leaves of 18:3 plants >60% of all fatty acids exported to the ER are reincorporated into the plastid for chloroplast membrane production. However, in oilseeds the major flux of acyl groups is for ER-localized triacylglycerol (TAG) synthesis such that >95% of acyl groups accumulate in extra-plastidial oil bodies and membranes, with very little flux back into the plastid (Li-Beisson et al., 2013). Thus, previous acyl flux studies on developing seeds of *lpcat1 lpcat2* are not appropriate for measuring the role of LPCATs in the flux of acyl groups from the ER to the plastid for galactolipid production in leaves.

Therefore, to understand the roles of LPC and LPCAT activity in acyl flux to and from the ER and chloroplast we crossed the Arabidopsis *lpcat1 lpcat2* double mutant (Bates et al., 2012) with the *act1* mutant (Kunst et al., 1988). *ACT1* (also called *ATS1* (Nishida et al., 1993)) encodes the chloroplast GPAT. The *act1* mutant eliminates prokaryotic pathway MGDG synthesis and enhances acyl flux through the eukaryotic pathway, similar to 18:3 plants. Our analysis of lipid accumulation, chloroplast-associated LPCAT activity, and *in vivo* acyl fluxes through both short timepoint metabolic tracing and long timepoint pulse-chase experiments further clarify the role of LPCATs within leaf PC acyl editing, and distinguish a separate metabolically-active pool of PC involved in providing the substrate for chloroplast lipid synthesis.

RESULTS

Production and characterization of the *act1 lpcat1 lpcat2* triple mutant

In Arabidopsis leaves both the eukaryotic pathway and prokaryotic pathway contribute approximately equally to MGDG production (Browse et al., 1986). To better understand the roles of LPCAT1 and LPCAT2 specifically in eukaryotic pathway

galactolipid production we crossed the *lpcat1 lpcat2* double mutant (Bates et al., 2012) with the *act1* mutant, which essentially eliminates prokaryotic pathway galactolipid production (Kunst et al., 1988). The *act1* allele is partially leaky, and residual GPAT activity remaining in the chloroplast is utilized for phosphatidylglycerol (PG) production (Xu et al., 2006). Previous crosses of *act1* with *tgd1-1* (a component of the transporter complex that imports the eukaryotic pathway lipid substrate into the chloroplast) was embryo lethal (Xu et al., 2005). We reasoned if the transport of LPC to chloroplasts and its subsequent conversion to PC by LPCAT at the chloroplast OEM was a key part of the eukaryotic pathway, then the *act1 lpcat1 lpcat2* triple mutant may also demonstrate developmental or vegetative growth defects. Crossed F1 seeds were grown to maturity, and seed was collected and re-sown. The segregating F2 plants were screened for homozygosity of the *lpcat1 lpcat2* double T-DNA mutation by PCR, and for homozygosity of the *act1* mutation by the lack of 16:3 in leaf lipids by gas chromatography (Kunst et al., 1988). During the initial screening no growth phenotypes were observed. Homozygous *act1 lpcat1 lpcat2* triple mutants were subsequently grown side-by-side with parental lines and wild-type Col-0. Across vegetative growth, the size of the triple mutants was within the plant-to-plant variation range of the parental lines (Supplemental Fig. 2). Therefore, the *act1 lpcat1 lpcat2* triple mutation has minimal effects on plant vegetative growth.

To determine if the *act1 lpcat1 lpcat2* triple mutant had defects in leaf lipid production we measured the relative accumulation of leaf membrane lipids at three developmental stages (2, 3, and 4 weeks after germination) in four lines of Arabidopsis: wild-type Col-0, *act1*, *lpcat1 lpcat2* double mutant, and the *act1 lpcat1 lpcat2* triple mutant (Fig. 2). In general the relative accumulation of leaf membrane lipids in the *lpcat1 lpcat2* mutant was similar to Col-0; however as previously characterized, the *act1* mutant has a significant change from Col-0 with less MGDG and PG, and a corresponding increase in PC and DGDG (Fig. 2A-C) (Kunst et al., 1988). Therefore, to understand the effect of the *lpcat1 lpcat2* mutation when acyl flux through the eukaryotic pathway is enhanced in the *act1* background, our main comparison is between *act1* and the *act1 lpcat1 lpcat2* triple mutant. At two and three weeks there were no significant changes in membrane lipid abundance between the two lines (Fig. 2A-B). At four weeks

(Fig. 2C), only PG demonstrated a significant increase from 2.2% ± 1.0% in the *act1* line to 10.1 ± 3.1% in the *act1 lpcat1 lpcat2* line (P-value = 0.0013).

Even though the amount of PC or PE did not significantly change between *act1* and *act1 lpcat1 lpcat2*, there were significant changes to their fatty acid compositions, especially at later stages in development (Fig. 3). There was no change in either PC or PE at 2 weeks after germination (Fig. 3A, 3D). At 3 weeks PC 18:1 content decreased from 23.1% ± 0.3% in the *act1* line to 18.5% ± 0.05% in the *act1 lpcat1 lpcat2* line (Fig. 3B). At 4 weeks PC 18:1 decreased from 29.7% ± 1.1% in *act1* to 20.8% ± 0.6% in *act1 lpcat1 lpcat2*, which was mostly compensated for by significant increases in 18:2 (from 37.8% ± 0.9% to 42.4% ± 0.4%) and 18:3 (from 15.6% ± 0.3% to 17.8% ± 0.5%). The only significant change in PE between *act1* and *act1 lpcat1 lpcat2* was at 4 weeks (Fig. 3F), where 16:0 decreased (from 27.0% ± 0.3% to 25.1% ± 0.3%), 18:2 decreased (from 43.1% ± 0.7% to 40.7% ± 0.5%), and 18:3 increased (from 13.6% ± 0.4% to 16.6% ± 0.7%). Supplemental Figures 3, 4 and 5 report the fatty acid composition for all other lipids measured in Fig. 2 at 2, 3, and 4 weeks respectively. The major galactolipid products of the eukaryotic pathway (MGDG and DGDG) did not have any significant changes in fatty acid composition between *act1* and *act1 lpcat1 lpcat2*. The change in abundance of PG measured at 4 weeks (Fig. 2C), only had a limited effect on its fatty acid composition with a significant increase in 18:3 in the in *act1 lpcat1 lpcat2* line (from 30.7% ± 0.3% to 32.3% ± 0.1%).

Together, the limited effect of the *act1 lpcat1 lpcat2* triple mutation compared to the *act1* mutation alone on plant growth and lipid accumulation across leaf development suggests that LPCAT1 and LPCAT2 are not essential for eukaryotic pathway lipid metabolism when the prokaryotic pathway is limiting. However, other lysophospholipid acyltransferases (such as LPEAT1 and LPEAT2) have demonstrated LPCAT activity *in vitro* (Stalberg et al., 2009; Jasieniecka-Gazarkiewicz et al., 2016), and are expressed at similar levels to *LPCAT1 LPCAT2* in leaves (Supplemental Fig. 1). Thus, it is possible that other lysophospholipid acyltransferases may compensate for the loss of LPCAT activity in the *act1 lpcat1 lpcat2* background. *In vitro* LPCAT activity has also been associated with multiple subcellular membrane fractions (Bessoule et al., 1995; Tjellström et al., 2012; Wang et al., 2014). To measure if the chloroplast-associated

LPCAT activity that has been hypothesized to be involved in the eukaryotic pathway of galactolipid synthesis (Mongrand et al., 1997; Moreau et al., 1998; Mongrand et al., 2000) is actually reduced in the *lpcat1 lpcat2* mutants, we performed LPCAT assays on chloroplasts isolated from each plant line (Fig. 4). The controls Col-0 and *act1* did not exhibit a significant difference in the LPCAT activity that produced [^{14}C]PC from the addition of LPC and [^{14}C]oleoyl-CoA to the isolated chloroplasts. However, [^{14}C]PC production was reduced ~85% in *act1 lpcat1 lpcat2* from wild-type levels (Fig. 4).

There was also no significant difference in [^{14}C]PC synthesis between *lpcat1 lpcat2* and *act1 lpcat1 lpcat2* lines. It is not clear if the residual [^{14}C]PC synthesis within the *lpcat1 lpcat2* backgrounds is chloroplast-localized LPCAT activity, or if it is due to the activity of other lysophospholipid acyltransferases from partial contamination of the isolated chloroplasts with other cellular membrane fractions (Larsson et al., 2007; Stalberg et al., 2009; Bulat and Garrett, 2011; Jasieniecka-Gazarkiewicz et al., 2016). Nevertheless, the major chloroplast-associated LPCAT activity in the *act1 lpcat1 lpcat2* triple mutant was mostly eliminated (Fig. 4), and it had little to no effect on growth or leaf galactolipid accumulation (Fig. 2, Supplemental Fig. 2), suggesting LPCAT activity is not an essential part of eukaryotic pathway galactolipid synthesis. However, the mass accumulation of MGDG and DGDG does not indicate the metabolic pathway of synthesis. To better understand how the loss of the major chloroplast LPCAT activity affects acyl flux out of the chloroplast and through the eukaryotic pathway into galactolipids of the *act1 lpcat1 lpcat2* triple mutant, we moved on to an *in vivo* metabolic labeling approach during the stage of rapid leaf growth (3-week-old plants).

Rapid *in vivo* metabolic labeling to characterize the effect of *lpcat1 lpcat2* on the entry of nascent fatty acids into the eukaryotic pathway through acyl editing

Newly synthesized fatty acids produced in the stroma of the plastid are exported as free fatty acids and esterified to co-enzyme A in the ER for use by the various acyltransferases of the eukaryotic pathway (Li-Beisson et al., 2013). Rapid metabolic labeling experiments in leaves, seeds, and plant suspension cells have demonstrated that nascent fatty acids exported from the plastid are predominantly directly

incorporated into PC through an LPCAT-type reaction within the acyl-editing cycle, rather than first esterified to glycerol-3-phosphate through *de novo* glycerolipid biosynthesis (Bates et al., 2007; Bates et al., 2009; Bates et al., 2012; Tjellström et al., 2012; Yang et al., 2017). This rapid incorporation into PC may take place on the chloroplast surface where significant LPCAT activity resides and ER-plastid connection sites are present (Andersson et al., 2007; Tjellström et al., 2012; Botella et al., 2017). We demonstrated that the *lpcat1 lpcat2* knockout eliminates most of the chloroplast-associated LPCAT activity (Fig. 4). To determine if the knockout of *lpcat1 lpcat2* also affects the initial incorporation of nascent fatty acids into the eukaryotic pathway through PC acyl editing, we followed the continual incorporation of [^{14}C]acetate into fatty acid synthesis and lipid assembly (Allen et al., 2015) in developing leaves of *act1* and *act1 lpcat1 lpcat2* over a short time course from 5 to 60 min (Fig. 5). [^{14}C]acetate incorporation into total lipids was linear and had the same rate of accumulation in each line (Fig. 5A). However, on average *act1* had more total DPM/µg chlorophyll than *act1 lpcat1 lpcat2*. Higher accumulation of label but with the same rate may be due to a difference in [^{14}C]acetate concentration in the incubation medium for each line, or possibly a small difference in total chlorophyll content utilized for normalization. Accumulation of ^{14}C labeled fatty acids into different glycerolipids was also linear, indicating continuous biosynthesis of each lipid over the time course in each line (Fig. 5B-C). Together these results indicate similar rates of total fatty acid biosynthesis in each line, and no indication of lipid degradation during the time course.

The relative accumulation of labeled lipids in *act1* and *act1 lpcat1 lpcat2* from Figure 5 is shown in Figure 6. At 60 min the relative labeling between lipids was similar in both lines; however, the initial incorporation of nascent fatty acids into PC of the *act1 lpcat1 lpcat2* line was delayed compared to *act1* (Fig. 6A). The decrease in PC was mostly compensated for by significant increases in DAG and PE (Fig.6B-C). There was no significant difference measured between the lines for labeling of PA, PG, and PI/PS (Fig. 6B-C), and notably MGDG (Fig. 6D).

To gain a better understanding of the mechanisms of newly synthesized fatty acid incorporation into the eukaryotic pathway, we characterized the positional distribution of the ^{14}C-labeled fatty acids in PC and DAG across the labeling time course

for both *act1* and *act1 lpcat1 lpcat2* (Fig. 7). In *act1* DAG contained similar amounts of nascent fatty acids at both stereochemical positions with a slight preference for *sn*-1 (55-60%) over *sn*-2 (40-45%) across the time course (Fig. 7A). In the PC of *act1* most of the nascent fatty acids accumulated at the *sn*-2 position (~75%) at all time points (Fig. 7C). In *act1 lpcat1 lpcat2*, DAG stereochemical labeling was similar to that of DAG in *act1* with a slight preference for the *sn*-1 position (Fig. 7B). However, PC of *act1 lpcat1 lpcat2* was distinctly different than *act1* PC, with the stereochemical labeling demonstrating a preference for *sn*-1 labeling (~60%) across the time course, similar to DAG from both lines. The rapid incorporation of nascent fatty acids predominantly in *sn*-2 of PC of *act1* (Fig. 5B, Fig. 7C) is characteristic of newly synthesized fatty acids first entering eukaryotic glycerolipids through PC acyl editing. However, in *act1 lpcat1 lpcat2* the initial delay of PC labeling and increase of DAG labeling (Fig. 5C, 6A-B), combined with the similar stereochemical labeling of PC and DAG across the time course (Fig. 7B, 7D) is consistent with an elimination of nascent fatty acid entry into eukaryotic glycerolipids through PC acyl editing and a reorientation of acyl flux to first move through DAG then into PC. These results indicate that LPCAT1 and LPCAT2 are involved in the direct incorporation of nascent fatty acids into PC through acyl editing as the fatty acids exit the chloroplast, and that other lysophospholipid acyltransferases do not compensate for the loss of LPCAT1 and LPCAT2 activity in the leaf acyl editing cycle.

Pulse-chase metabolic labeling to characterize the effect of *lpcat1 lpcat2* on the PC–MGDG precursor–product relationship of the eukaryotic pathway

To measure acyl flux through the longer-term precursor–product relationships within leaf lipid metabolism, we performed a pulse-chase metabolic tracing experiment. Three-week-old rosettes from both the *act1* and *act1 lpcat1 lpcat2* lines were pulsed with [^{14}C]acetate for 15 min, the radioisotope was washed off, and then the samples were chased without radiolabel for an additional 51 h to measure the redistribution of fatty acids as eukaryotic pathway intermediates turn over with time (Fig. 8). Similar to the short-timepoint continuous [^{14}C]acetate labeling experiment (Fig. 5, 6), at the end of

388 the pulse most of the newly synthesized radioactive fatty acids were in PC in both the
389 *act1* (Fig. 8A) and *act1 lpcat1 lpcat2* (Fig. 8B) lines. During the chase the radioactivity in
390 PC rapidly declined, and the fatty acids were redistributed predominantly into MGDG,
391 followed by PE, and then TAG. All other measured lipids (PG, PA, DAG, PI/PS, DGDG)
392 contained only minor amounts of radioactivity over the chase period (Fig. 8A-B). When
393 the relative labeling of individual lipids was compared between the plant lines, there was
394 no statistical difference in the labeling pattern for the major labeled lipids PC, MGDG,
395 and PE. PC levels differed only at the 51 h time point where there was more labeled PC
396 in *act1 lpcat1 lpcat2* than in *act1* (Fig. 8C-E). In addition, there was no difference
397 between the lines over the time course for labeling of DAG (Fig. 8C) and PA (Fig. 8E),
398 which are intermediates of glycerolipid synthesis. This result suggests that the
399 quantitative turnover of PC to provide the substrate for MGDG synthesis within the
400 eukaryotic pathway is not affected by the *lpcat1 lpcat2* mutations.
401 Interestingly, TAG labeling was significantly different between the two plant lines
402 (Fig. 8F). TAG accumulation is very minor in leaf tissue under normal circumstances
403 (typically < 1% of total lipid mass), but a dynamic small pool of TAG that is constantly
404 synthesized and degraded can be measured through radiolabeling (Fan et al., 2014;
405 Tjellström et al., 2015). Both lines demonstrated the same trend, with the continual
406 increase of labeled fatty acid in TAG until the 22 h timepoint, and then a decrease
407 through the remaining time course. However, starting at the 4 h timepoint the *act1* line
408 had a significantly higher proportion of labeled fatty acids in TAG than the *act1 lpcat1*
409 *lpcat2* line did (Fig. 8F).
410 To gain a better understanding of the role of the LPCAT1 LPCAT2 enzymes in
411 leaf eukaryotic pathway metabolism, we analyzed the changes in both the labeled fatty
412 acid composition (Fig. 9), and their stereochemical location (Fig. 10) within DAG, PC,
413 and MGDG across the [^{14}C]acetate pulse-chase time course (Fig. 8). DAG, PC, and
414 MGDG each had a unique profile of ^{14}C-fatty acid accumulation. However, when the
415 composition of labeled fatty acids within each lipid was compared between the *act1* and
416 *act1 lpcat1 lpcat2* lines there was no significant differences between the lines (Fig. 9).
417 There was limited change in the composition of DAG over the 51 h chase period (Fig.
418 9A-B), consistent with its role as an intermediate of lipid metabolism that is not a

substrate of fatty acid desaturases. However, PC and MGDG had larger changes in composition as both lipid classes are substrates for desaturases, and the acyl groups that are initially incorporated into PC eventually accumulate in MGDG (Fig. 8). In PC the major change in composition was a decrease in monoenoic fatty acids, with a concomitant increase in both dienoic and trienoic fatty acids as a proportion of the labeled fatty acids remaining in PC (Fig. 9C-D). The major change in MGDG was a large increase in trienoic fatty acids (Fig. 9F). Considering the precursor–product relationship of acyl transfer between PC and MGDG (Fig. 8), these results are consistent with the loss of nascent ^{14}C-18:1 initially incorporated into PC (through both desaturation and acyl transfer) and its subsequent accumulation as ^{14}C-18:3 in MGDG. These results are consistent with the current understanding of the eukaryotic pathway (Fig. 1). Together with Figure 8, this result suggests the *lpcat1 lpcat2* mutations have little to no effect on the flux of total acyl groups (Fig. 8) or select fatty acids (Fig. 9) from PC to MGDG within the eukaryotic pathway.

Despite the similarities of acyl accumulation and composition over the pulse-chase between the *act1* and *act1 lpcat1 lpcat2* lines, the stereochemical location of the labeled acyl groups revealed significant differences between the lines (Fig. 10). During the pulse-chase experiment labeled DAG can represent *de novo* DAG at early time points. At later time points labeled DAG can represent *de novo* DAG synthesized with fatty acids removed from other lipids, and DAG derived from membrane lipid or TAG turnover. The ^{14}C-fatty acid stereochemistry in DAG throughout the pulse-chase time course indicated more labeled fatty acids at the *sn*-1 position than the *sn*-2 position (Fig. 10A), similar to the short timepoint continuous [^{14}C]acetate labeling experiment (Fig. 7A-B). There was no significant difference between *act1* or *act1 lpcat1 lpcat2* at any time point, indicating the loss of LPCAT1 LPCAT2 does not affect initial or prolonged DAG metabolism. The *act1* PC stereochemistry was similar to the continuous labeling experiment (Fig. 7C) throughout the pulse-chase with more ^{14}C-fatty acids at *sn*-2 than *sn*-1 (Fig. 10B), consistent with the incorporation of nascent fatty acids into *sn*-2 PC by LPCAT-mediated acyl editing. The *act1 lpcat1 lpcat2* PC stereochemistry was initially similar to the short timepoint continuous labeling experiment (Fig. 7D) with more label at *sn*-1 than *sn*-2, consistent with the loss of rapid incorporation of nascent fatty acids into

PC through acyl editing. However, after 4 hours of chase there was a switch in stereochemistry, with more labeled fatty acids in the *sn*-2 position rather than the *sn*-1 (Fig. 10B), more similar to PC from the *act1* mutant. At all timepoints the labeled fatty acid stereochemistry in PC was significantly different between the *act1* and *act1 lpcat1 lpcat2* lines. For MGDG, the early timepoints indicated similar stereochemical localization of the ^{14}C-fatty acids in both lines, at later time points in the chase more *sn*-1 labeled fatty acids accumulated in both lines (Fig. 10C). It is important to point out that MGDG accumulated as the major labeled lipid by the end of the chase period (Fig. 8A-B), and that the stereochemistry of labeled acyl groups was similar to that of DAG throughout the time course (Fig. 10A) and that of initial PC of the *act1 lpcat1 lpcat2* line (Fig. 7D, 10B), but not similar to the labeled PC which remains near the end of the time course in both lines (Fig. 10B).

DISCUSSION

Biochemical, genetic, and molecular biology research on plant membrane lipid assembly over the past 40 years has indicated a complicated metabolic network of reactions (Fig. 1) that requires the trafficking of intermediates between multiple subcellular compartments to produce the diverse molecular species of lipids crucial to cellular function. While many of the acyltransferases and desaturases involved in lipid assembly have been identified the pathways of acyl trafficking, the identity of lipid intermediates, and the trafficking proteins involved in the eukaryotic pathway have remained more elusive. Major advances over the past 15 years include the identification of a free fatty acid transporter for export of nascent fatty acids from the plastid (Li et al., 2015), the determination that nascent fatty acids exported from the chloroplast in leaves are predominantly first incorporated into PC by an LPCAT-type reaction of acyl editing rather than the initial attachment to G3P through *de novo* glycerolipid assembly (Bates et al., 2007), and the characterization of a protein complex involved in the transport of the eukaryotic pathway assembled lipid intermediate into the plastid for galactolipid production (Xu et al., 2005). However, the exact lipid species transported from the ER to the chloroplast has remained unclear; DAG, PA, PC and LPC have all been suggested.

Eukaryotic pathway-derived lipids are characterized by 18-carbon fatty acids at the *sn*-2 position, while the *sn*-2 acyltransferases of the prokaryotic pathway in the plastid utilize 16-carbon fatty acids. If LPC is transported to the outer membrane of the plastid it must be acylated to PC by an LPCAT activity prior to turnover to DAG or PA to produce the correct molecular species of the eukaryotic pathway. Since LPCAT activity has been implicated in both the trafficking of fatty acids from the chloroplast to the ER in seeds (Bates et al., 2012) and from the ER to the chloroplast in leaves (Mongrand et al., 1997; Moreau et al., 1998; Mongrand et al., 2000), we sought to gain a better understanding of the roles of LPCAT1 and LPCAT2 in leaf acyl trafficking by analyzing the lipid accumulation and acyl fluxes within the *act1 lpcat1 lpcat2* mutant background.

LPCAT1 and _LPCAT2_ encode chloroplast-localized LPCATs that are involved in the direct incorporation of newly synthesized fatty acids into PC through acyl editing

The rapid incorporation of nascent fatty acids into predominantly the *sn*-2 position of PC through acyl editing, rather than through *de novo* glycerolipid synthesis, was originally characterized in developing pea leaves, an 18:3 plant (Bates et al., 2007). In this study the *act1* mutation (Kunst et al., 1988) was utilized to essentially convert Arabidopsis into an 18:3 plant. Here we demonstrate that in *act1* leaves nascent fatty acids are also predominantly incorporated initially into the *sn*-2 position of PC, consistent with a highly active PC acyl-editing cycle in Arabidopsis leaves (Fig. 5B, 6A, 7C). When LPCAT1 and LPCAT2 were additionally mutated in the *act1 lpcat1 lpcat2* line, the chloroplast-associated LPCAT activity was reduced at least 85% (Fig. 4), the initial incorporation of nascent fatty acids into PC was reduced concomitantly with an increase into DAG (Fig. 6A-B), and the stereochemistry of incorporation into PC was completely switched to favor *sn*-1 in a proportion similar to the rapidly synthesized *de novo* DAG (Fig. 7B,D). These results suggest that without chloroplast-associated LPCAT1 and LPCAT2 activity the newly synthesized fatty acids are rerouted to enter PC through the GPAT and LPAT reactions of *de novo* glycerolipid synthesis rather than LPCAT-based acyl editing in leaves (Fig. 11). It is not clear if the residual ~15% of wild-

type LPCAT activity measured in the *act1 lpcat1 lpcat2* isolated chloroplasts is due to other chloroplast-associated lysophospholipid acyltransferases (LPLAT) (Larsson et al., 2007; Stalberg et al., 2009; Bulat and Garrett, 2011; Jasieniecka-Gazarkiewicz et al., 2016), or due to partial contamination of the isolated chloroplasts with other cellular membrane fractions containing LPLATs (Larsson et al., 2007; Tjellström et al., 2012). However, the complete switch in the *in vivo*-labeled PC stereochemistry suggests that any other putative chloroplast-associated LPLATs do not compensate for the lack of LPCAT1 and LPCAT2 in the direct flux of nascent fatty acids into PC through acyl editing.

Roles of PC acyl editing in leaves

Here we demonstrate a role for LPCAT1 and LPCAT2 in the direct incorporation of newly synthesized fatty acids into PC as they exit the chloroplast in leaves and show that this role is dispensable in the *lpcat1 lpcat2* background (Fig. 11A-B). However, the role of LPCAT1 and LPCAT2 in leaves likely extends beyond trafficking of nascent fatty acids to PC. PC is the site of ER-localized fatty acid desaturation (Li-Beisson et al., 2013). Previously, LPCAT1 and LPCAT2 were demonstrated to be involved in acyl flux through PC to provide polyunsaturated fatty acids (PUFA) for seed triacylglycerol biosynthesis (Bates et al., 2012; Wang et al., 2012a). Acyl flux through PC for PUFA production is likely also a key role for acyl editing in leaves. Recent work has indicated that the amount of PUFA that accumulate in ER lipids is related to both desaturase activity and the rate of acyl flux through PC. When acyl flux slows down, more PUFA accumulate due to enhanced residence time on PC for desaturation (Maatta et al., 2012; Meï et al., 2015; Botella et al., 2016). While young leaves are expanding, acyl flux through PC is high for membrane lipid production, and little change in PC fatty acid composition was observed in the *act1 lpcat1 lpcat2* mutant (Fig. 3A). However, as leaves matured, more 18:2 and 18:3 and less 18:1 accumulated in PC as compared to *act1* (Fig. 3B,C), and a similar change was observed in PE (Fig. 3F). This result suggests that LPCAT1- and LPCAT2-based acyl editing has homeostatic roles likely involving distribution of PUFA to other lipids across the leaf life cycle. In the *lpcat1*

543 *lpcat2* mutant background, the plant may compensate for the loss of acyl editing by
544 increasing other mechanisms of acyl flux through PC as indicated in seeds (Bates et al.,
545 2012; Wang et al., 2012a), or providing PUFA from chloroplast sources. Recently, a PG
546 lipase was implicated in the export of PUFA from the chloroplast for seed oil
547 biosynthesis (Wang et al., 2017; Aulakh and Durrett, 2019). The only lipid with a change
548 in abundance in the *act1 lpcat1 lpcat2* line was PG (Fig. 2C). It is possible that the loss
549 of LPCAT1- and LPCAT2-based acyl editing has activated this or other mechanisms of
550 chloroplast-to-ER trafficking of PUFA. However, the *in vivo* metabolic labeling
551 experiments (Fig. 6, 8) did not measure a significant difference in PG labeling,
552 suggesting acyl flux through PG may be a minor contribution to ER PUFA content.
553 The only lipid that had significant differences in [^{14}C]fatty acid accumulation
554 between lines across the pulse-chase time course was TAG (Fig. 8F). The *act1 lpcat1*
555 *lpcat2* line accumulated less labeled TAG than did the *act1* line. TAG does not
556 accumulate to high mass levels in leaves, but a small metabolically-active pool that is
557 constantly synthesized and turned over is believed to act as a free fatty acid buffer
558 during times of high rates of fatty acid synthesis or stress (Xu and Shanklin, 2016).
559 Recently, phospholipid:diacylglycerol acyltransferase (PDAT) was demonstrated to be a
560 key part of TAG production in Arabidopsis leaves (Fan et al., 2013a; Fan et al., 2013b;
561 Fan et al., 2014). PDAT transfers a fatty acid from the *sn*-2 position of PC to DAG,
562 producing TAG and LPC. LPCAT works in tandem with PDAT to regenerate PC from
563 the co-produced LPC (Xu et al., 2012). Together, PDAT and LPCAT could lead to
564 channeling of nascent fatty acids exported from the plastid into PC and then TAG during
565 high rates of fatty acid synthesis. The reduced TAG labeling in the *act1 lpcat1 lpcat2*
566 mutant is likely due to inefficient PDAT activity without an LPCAT to regenerate the PC
567 substrate. Together, these results suggest a variety of possible roles for LPCAT1- and
568 LPCAT2-based acyl editing in leaves.
569
570 **MGDG production from PC is independent of LPCAT1 and LPCAT2**
571
572 LPCAT activity has been implicated in the acylation of LPC transported from the
573 ER to the chloroplast as part of eukaryotic pathway trafficking of substrates for MGDG

574 synthesis. This previous conclusion originally came from *in vitro* experiments
575 demonstrating the transfer of LPC from isolated microsomes to isolated chloroplasts
576 from leek seedlings, and its acylation to PC by the chloroplast-associated LPCAT
577 activity (Bessoule et al., 1995). Further *in vivo* metabolic labeling pulse-chase
578 experiments in leek seedlings demonstrated that PC containing predominantly *sn*-2
579 labeled fatty acids gave rise to MGDG labeled mostly at *sn*-1 (Mongrand et al., 1997;
580 Mongrand et al., 2000). The conclusion was that only the *sn*-1 fatty acid was transferred
581 to the chloroplast and, combined with the previous *in vitro* experiments, suggested that
582 LPC was the molecule transferred from the ER to the chloroplast.

583 The *act1* mutation eliminates the prokaryotic pathway of MGDG synthesis. When
584 this mutant was crossed with the *tgd1-1* mutant (a part of the OEM to IEM transporter
585 that provides substrate for chloroplast lipid synthesis) no viable double mutants were
586 recovered (Xu et al., 2005), indicating that disruptions of the eukaryotic pathway in the
587 *act1* background are lethal. However, we demonstrate that the *act1 lpcat1 lpcat2* triple
588 mutation causes at least an 85% reduction in chloroplast LPCAT activity (Fig. 4), little to
589 no growth alteration (Supplemental Fig. 2), and no effect on the accumulation of
590 galactolipids (Fig. 2). Therefore, we conclude that LPCAT1 and LPCAT2, and LPC
591 trafficking are not a key part of eukaryotic pathway galactolipid synthesis. However, we
592 cannot rule out that the residual ~15% of wild-type LPLAT activity associated with
593 isolated *act1 lpcat1 lpcat2* chloroplasts may represent a minimal flow of LPC
594 transported from the ER for other purposes, such as incorporation of PC into the outer
595 leaflet of the chloroplast OEM. If this minimal flow of LPC occurs, the formation of PC
596 must be through a LPLAT other than LPCAT1 and LPCAT2.

597 To gain a better understanding of the mechanisms involved in the PC–MGDG
598 precursor–product relationship we performed a long term [^{14}C]acetate pulse-chase
599 experiment. The *act1* mutant and *act1 lpcat1 lpcat2* triple mutant showed little difference
600 in quantitative turnover of initially-labeled PC or in the subsequent incorporation of the
601 labeled fatty acids into MGDG (Fig. 8). In addition, the labeled fatty acid composition of
602 DAG, PC, and MGDG was the same between the two lines across the pulse-chase time
603 course (Fig. 9). These results further suggest that the *lpcat1 lpcat2* mutation does not
604 affect the ER-to-chloroplast trafficking of the eukaryotic pathway. When the

605 stereochemistry of labeled fatty acids in PC and MGDG of *act1* were analyzed, we
606 found a similar result to that of the leek seedlings (Mongrand et al., 2000) where PC
607 was mostly *sn*-2 labeled and the labeled MGDG that accumulated from turnover of PC
608 was mostly *sn*-1 labeled (Fig. 10). From the *act1* labeling data alone (in an essentially
609 18:3 plant, similar to leek), the transfer of LPC would make sense. However, the
610 stereochemical analysis of DAG, PC, and MGDG of the *act1 lpcat1 lpcat2* line revealed
611 a different underlying mechanism.

612 In both the short continuously labeling time course and at the end of the pulse
613 (time 0), the stereochemistry of DAG and PC from *act1 lpcat1 lpcat2* were very similar,
614 with more nascent labeled fatty acids at *sn*-1 than *sn*-2 (Fig. 7, 10). Therefore, the lack
615 of LPCAT1- and LPCAT2-based acyl editing leads to nascent fatty acid incorporation
616 into PC though eukaryotic *de novo* glycerolipid assembly, which dictates the
617 stereochemical distribution of fatty acids in DAG and PC. The labeled fatty acid
618 stereochemical distribution that accumulates in MGDG over time in both lines (Fig. 10C)
619 is also very similar to the *de novo* synthesized DAG and PC (Fig. 10A-B). Therefore, we
620 conclude that the DAG backbone utilized to synthesize eukaryotic MGDG is derived
621 from a PC pool produced from *de novo* eukaryotic glycerolipid assembly and is distinct
622 from the pool of PC undergoing LPCAT1- and LPCAT2-based acyl editing.

623 Figure 11 incorporates the [^{14}C]acetate pulse-chase data onto new models of
624 eukaryotic pathway acyl flux that demonstrate the metabolically distinct pools of PC
625 involved in acyl editing and eukaryotic pathway MGDG production. When LPCAT1 and
626 LPCAT2 are present in *act1* the labeling of PC is dominated by the rapid *sn*-2 acyl
627 editing (Fig. 11A). Acyl editing is a constant exchange of acyl groups in PC with the
628 acyl-CoA pool, and it allows the PUFA produced on PC to be utilized by the GPAT and
629 LPAT reactions of the eukaryotic pathway (Bates et al., 2007; Bates et al., 2009; Bates
630 et al., 2012; Bates, 2016). Therefore, during the pulse-chase the labeled acyl groups
631 can leave PC by acyl editing and are assembled into DAG with more *sn*-1 labeling than
632 *sn*-2, which is then used for PC synthesis. If this *de novo* synthesized pool of PC is
633 rapidly turned over to produce the substrate for MGDG synthesis, MGDG will have the
634 same *sn*-1-labeled stereochemistry, and it would not have much effect on the
635 stereochemistry of "total labeled PC", which is dominated by the separate highly-labeled

636 acyl-editing PC pool. It is only when LPCAT activity is removed in the *act1 lpcat1 lpcat2*
637 triple mutant that the flux through *de novo* PC to MGDG can be measured separately
638 from the acyl-edited PC pool (Fig. 10), which reveals a clear PC–MGDG precursor–
639 product relationship (Fig. 11B-C). Therefore, the glycerol backbone and both fatty acids
640 (derived mostly from PC acyl editing) that are assembled onto *de novo* PC are
641 ultimately the "DAG backbone" utilized for chloroplast MGDG synthesis. This model is
642 also supported by recent characterization of an unusual Δ6 desaturated fatty acid
643 produced transgenically in Arabidopsis leaves at only the *sn*-2 position of PC. However,
644 the Δ6D fatty acid was redistributed approximately equally to the *sn*-1 and *sn*-2
645 positions of MGDG (Hurlock et al., 2018). Removal of the Δ6D fatty acid from PC by
646 acyl editing (Fig. 11A) and its subsequent incorporation into both positions of *de novo*
647 DAG by GPAT/LPAT activities of the eukaryotic pathway prior to MGDG synthesis is
648 consistent with our new model of acyl flux.
649
650 **Changes to eukaryotic pathway acyl flux within the *act1 lpcat1 lpcat2* background**
651
652 In wild-type and *act1* leaves, the PC acyl-editing cycle may occur by at least
653 three mechanisms (Bates, 2016): (1) both the forward and reverse reactions of LPCAT
654 (Lager et al., 2013; Jasieniecka-Gazarkiewicz et al., 2016); (2) a phospholipase A_2
655 (PLA_2) hydrolysis of PC to LPC and a free fatty acid (FFA), FFA activation to acyl-CoA
656 by LACS, and LPC conversion to PC by the forward LPCAT reaction using a different
657 acyl-CoA (also known as the Lands Cycle (Lands, 1965)); (3) either mechanism 1 or 2
658 plus a LPC:LPC transacylase (LPCT) (Lager et al., 2015) and a glycerophosphocholine
659 acyltransferase (GPCAT) (Lager et al., 2015; Gląb et al., 2016). LPCT transfers an acyl
660 group from one LPC to another producing PC and glycerophosphocholine, which is then
661 converted back to PC by the combined action of GPCAT and LPCAT. In relation to the
662 multiple possible enzymatic mechanisms for acyl editing, two important details from the
663 [^{14}C]acetate pulse-chase experiment must be pointed out. (1) In both *act1* and *act1*
664 *lpcat1 lpcat2*, DAG has approximately 30% ^{14}C-PUFA at the end of the pulse (time 0
665 chase), indicating newly synthesized 18:1 is rapidly incorporated into PC for
666 desaturation, and then incorporated into DAG. (2) The *act1 lpcat1 lpcat2* PC

667 stereochemical labeling within the pulse-chase experiment switches from more *sn*-1
668 label at time 0 to more *sn*-2 label at the end of the time course. Based on the
669 mechanisms of acyl editing, multiple possible scenarios could explain both the *act1*
670 *lpcat1 lpcat2* DAG PUFA content and the stereochemistry switch in PC (Fig. 11B-C).
671 First, in model Fig. 11B the *lpcat1 lpcat2* mutation eliminates acyl chain removal
672 from PC by acyl editing (e.g. eliminating acyl-editing mechanism 1). Therefore, the
673 PUFA-labeled DAG represents PC-derived DAG after desaturation. In model 11B, the
674 switch in labeled PC stereochemistry may be through selective molecular species
675 trafficking. Not all PC that is synthesized *de novo* is turned over for chloroplast lipid
676 synthesis. Some PC has a structural role within various cellular endomembrane
677 systems. It is possible that the turnover of mostly *sn*-1 labeled molecular species for
678 chloroplast lipid synthesis has left behind a majority of molecular species that contain
679 *sn*-2 labeled fatty acids.
680 Second, the *lpcat1 lpcat2* mutation eliminates the LPCAT portion of a Lands
681 Cycle, but not the continual generation of LPC by PLA_2. In model Fig. 11C red and blue
682 arrows only, PLA_1- or PLAB-based turnover of LPC generated by the PLA_2 would
683 completely remove the fatty acids from PC leading to complete PC turnover.
684 Considering PC is also undergoing desaturation (Fig. 9), the [^{14}C]18:1 originally
685 incorporated into PC will be converted to [^{14}C]18:2 and [^{14}C]18:3 over time. When these
686 fatty acids are removed and then reutilized for *de novo* glycerolipid synthesis it will
687 produce *de novo* DAG containing PUFA, and the labeled fatty acid stereochemistry in
688 PC will then be determined by the acyl selectivity of GPAT/LPAT. This will lead to
689 different labeled stereochemical molecular species of PC produced from the ^{14}C-PUFA
690 and newly synthesized ^{12}C-18:1 during the chase. In support this hypothesis, increased
691 expression of various lipases with as-yet uncharacterized functions were measured in
692 developing seeds of the *lpcat1 lpcat2* mutant, suggesting the possibility of a modified
693 method to remove PUFA from PC in the *lpcat1 lpcat2* background (Wang et al., 2012a).
694 Third (model Fig. 11C, all arrows), PLA_2 activity in *act1 lpcat1 lpcat2* would
695 produce LPC, which could be converted back to PC by LPCT (as in acyl-editing
696 mechanism 3). This type of reaction would transfer an *sn*-1 acyl group from one LPC to
697 a second LPC and thus could move a labeled fatty acid from the *sn*-1 to *sn*-2 position in

PC. The GPC also produced can be reacylated to LPC by GPCAT, and thus could produce a cycle of *sn*-1/*sn*-2 acyl switching within the *lpcat1 lpcat2* background. From the current experiments it is not clear which of these three possibilities may be occurring, but it is likely that a pool of PC that remains in the ER (model 1, Fig. 11B) may be undergoing acyl turnover (models 2/3, Fig. 11C), which leads to a different stereochemistry of the labeled fatty acids in PC over time (Fig. 10B).

Current model for leaf glycerolipid synthesis and trafficking in wild-type Arabidopsis leaves

Figure 12 is a modification of Fig. 1 based on our current results and displays the current areas of uncertainty in the eukaryotic pathway of leaf glycerolipid synthesis. The model no longer indicates that LPC can be transferred from the ER to the chloroplast for galactolipid production. It also has three metabolically distinct pools of PC. PC(1) is involved in LPCAT1- and LPCAT2-based acyl editing and may be located at the chloroplast surface or a ER-chloroplast membrane contact site. A membrane contact site might make the most sense because it would allow PC containing newly synthesized 18:1 to diffuse through the ER to the FAD2 and FAD3 enzymes for desaturation. PC(2) is the pool that is produced by *de novo* PC synthesis within the eukaryotic pathway; it is a substrate for desaturases and provides PC that migrates through the ER to other locations. PC(3) is the pool that is turned over to produce the substrate for MGDG synthesis, and it may have been further desaturated by FAD2 and FAD3 than PC(2). In addition, the exact PC(3)-derived intermediate (DAG or PA) transported to the IEM by the TGD1-5 complex is still unclear. The location of PC(3) turnover is also unclear; it could be the ER, the chloroplast surface, or a ER-chloroplast membrane contact site. Recent characterization of the ALA10 flippase mutant suggest the likely involvement of a membrane contact site for ER-to-chloroplast trafficking (Botella et al., 2016). Considering the multiple possible roles for membrane contact sites in acyl trafficking, it may be plausible that there are ER-chloroplast contact sites with specific functions in acyl export from the chloroplast involving LPCAT1- and LPCAT2-

based acyl editing and separate contact sites with specific function in eukaryotic lipid import into the chloroplast for galactolipid production.

The findings presented here strongly enhance our understanding of eukaryotic pathway of membrane lipid production in leaves by demonstrating that (1) *LPCAT1* and *LPCAT2* encode the major chloroplast-associated LPCAT activities; (2) the major role of LPCAT1 and LPCAT2 is for direct incorporation of newly synthesized fatty acids into PC through acyl editing as the fatty acids are transported out of the chloroplast; (3) LPCAT1 and LPCAT2 activity is not involved in the transfer of LPC from the ER to the chloroplast within eukaryotic pathway galactolipid production; (4) the PC–MGDG precursor–product relationship of acyl flux involves removal of acyl chains from PC by acyl editing prior to *de novo* PC synthesis and the subsequent turnover of PC for MGDG production; and (5) PC acyl editing and PC turnover for MGDG production involve metabolically distinct pools of PC. This last result suggests that an underlying spatial organization of distinct PC metabolism may be a key part of the efficient acyl trafficking through the eukaryotic pathway. While there is still uncertainty regarding which PC-derived lipid is trafficked from the ER to the chloroplast, the Arabidopsis *act1 lpcat1 lpcat2* line characterized here may be particularly useful for future studies because it allows for metabolic tracing of the PC–MGDG precursor–product relationship without the complications of acyl flux around the PC acyl-editing cycle.

METHODS

Plant materials

Arabidopsis thaliana lines used in this study include: wild-type Columbia-0 (Col-0), *act1* mutant (ACT1, At1G32200; Kunst et al., 1988), *lpcat1 lpcat2* double mutant (LPCAT1, AT1G12640; LPCAT2, AT1G63050; Bates et al., 2012), and the *act1 lpcat1 lpcat2* triple mutant generated here.

Plant germination and growth

Seeds were sterilized in aqueous 10% bleach, 27% ethanol, and 0.1% SDS, rinsed with water 5 times and applied to germination plates (1x MS salts, 0.05% MES

759 free acid, 1% sucrose, and 0.8% Agar, pH 5.7) in a 0.1% agar solution. The plates were
760 incubated at 4 °C for 3 days, then placed in a growth chamber under ~150 µmol
761 photons m^{-2} s^{-1} white light using 14 h/10 h day/night cycle at 23 °C constant temperature
762 until all lines germinated and produced two true leaves (approximately 7-10 days). The
763 seedlings were then transferred to soil and placed back into the growth chambers. All
764 plants were watered 3 times a week with on watering consisting of Peter's NPK 20-20-
765 20 (0.957g/l) fertilizer solution. During the crossing and harvest of seeds, the plants
766 were grown at the same growth condition but with constant light.
767
768 **Production of *act1 lpcat1 lpcat2* triple mutant**
769 The *act1* and *lpcat1 lpcat2* were crossed via cross-pollination by hand. The
770 screening of *act1* was done by identifying absence of hexadecatrienoic acid (16:3) in
771 whole leaf FAME by gas chromatography. The screening of *lpcat1 lpcat2* was done by
772 PCR of leaf tissue with previously described primers (Bates et al., 2012; Xu et al.,
773 2012). With the Phire Plant Driect PCR Mastermix (Thermo Scientific) as per the
774 manufacturer's instructions.
775
776 **Production of FAME and gas chromatography**
777 Plant tissue and collected lipid samples were converted to fatty acid methyl
778 esters (FAME) with an internal 17:0 TAG standard by heating to 85 °C for 1.5 h in 5%
779 sulfuric acid in methanol. After forcing a phase separation by adding hexane and 0.88%
780 potassium chloride, the hexane phase containing the FAME was analyzed by gas
781 chromatography with flame ionization detection on a Shimadzu GC-2010 with a
782 RESTEK Rtx®-65 column (30m, 0.25mm ID, df = 0.25 µm), with method run parameters
783 of 190 °C prior for 2 min, and then the temperature increased to 270 °C at 10 °C/min
784 and held at 270 °C for 2 min. The detector was set at 275 °C.
785
786 **Lipid extraction**
787 The lipid extraction is based off of Hara and Radin (1978). Plant tissues were
788 quenched in 80-85 °C isopropanol with 0.01% butylated hydroxytoluene for 10 min. The
789 tissue was homogenized with polytron and moved to new glass tubes. The polytron was

790 washed with isopropanol and hexane to recover all remaining sample and combined
791 with the ground tissue to a final proportion of hexane/isopropanol/water of 6/4/0.2
792 (v/v/v). The polytron was washed further between samples to avoid cross-
793 contamination. Lipids were collected into the hexane phase by adding half of the sample
794 volume of 6.6% sodium sulfate. The aqueous phase was back extracted using
795 hexane/isopropanol (7/2) and combined with the previous hexane extract. The
796 combined organic sample was dried down under nitrogen and resuspended in known
797 volume of toluene. For radiolabeled samples, the lipids were resuspended in
798 chloroform/methanol (2:1) and subjected to a second phase separation by the addition
799 of 0.88% potassium chloride to remove any excess radiolabel. The organic phase was
800 collected, dried down in nitrogen, and resuspended in known volume of toluene.
801 Chlorophyll was measured as in Arnon (1949). Each replicate was a separate extraction
802 of enough leaf material from many plants to make approximately 0.3 g fresh weight.
803
804 **Chloroplast isolation and LPCAT assays**
805 Arabidopsis lines were grown on soil in a growth chamber set to 12/12-h
806 day/night cycle, 25°C, and 150 µmol photons m^{-2} s^{-1} light. 25 grams of leaf tissue was
807 harvested at 36 days (Col-0, *lpcat1 lpcat2*) and 44 days (*act1*, *act1 lpcat1 lpcat2*) after
808 16 hours of dark treatment. Chloroplast isolation was as described by (Kubis et al.,
809 2008), with a modified concentration of 0.33 M sorbitol (instead of 0.3 M) for the
810 isolation and resuspension buffers. The chlorophyll content of isolated chloroplasts was
811 measured as in Arnon (1949) with a Thermo Scientific Genesys 50 UV-Vis
812 spectrophotometer.
813 LPCAT assays were performed on chloroplasts equivalent to 150 µg chlorophyll,
814 in 0.3 mL in a 1.5-mL tube containing 1 mM soy LPC, and 13.6 µM [^{14}C]oleoyl-CoA 55
815 mCi/mmol (American Radiolabeled Chemicals, Inc.), at 30 °C, with 300 rpm mixing for
816 30 minutes on an Eppendorf Thermomixer. The reaction was stopped by adding 1.2 mL
817 $CHCl_3$/MeOH/Formic Acid (2/1/.1, v/v/v) and vigorous vertexing. The assay mixture was
818 transferred to 8 mL glass tubes and the assay vessel was washed once with a second
819 aliquot of $CHCl_3$/MeOH/formic acid, then combined with the previous extract. Addition
820 of 0.3 mL KCl to the mixture and centrifugation at 2000 g produced phase separation,

and the lower organic phase was removed to a new 8 mL glass tube. The aqueous phase was washed with 1 mL of $CHCl_3$ and combined with previous organic phase. The $CHCl_3$ extract was evaporated under a stream of N_2 and resuspended in 100 µL $CHCl_3$. Two aliquots of 5 µL were dissolved in 5 mL Eco-Scint liquid scintillation cocktail (National Diagnostics) and radioactivity measured with a Packard 2200CA Liquid Scintillation Counter to quantify the radioactivity in the whole extract. The remaining extract was loaded onto Millipore-Sigma Silica gel 60 TLC plates in 1 cm bands with non-radioactive lipid standards in adjacent lanes. The TLC plate was developed in $CHCl_3$/MeOH/acetic acid/acetone/water (35/25/4/14/2, v/v/v/v/v). After development, the TLC plate was air dried and stained with iodine vapor for visualization of lipid mass bands, and the standards were marked with a radioactive dot. The TLC plate was placed against phosphor imaging screen for 24 hours and developed by a GE Typhoon FLA 7000 phosphor imager. Identification of radioactive lipids from the assays was based on comigration with lipid standards. Relative quantification of all radioactive bands was by ImageQuant software version 7.0.

***In vivo* [^{14}C]acetate metabolic labeling**

For both continuous and pulse-chase labeling 3-week-old plant tissue was floated on incubation medium consisting of 20 mM MES, 0.1X MS salts, and 0.01% Tween 20 at pH 5.5, in a shaking water bath at 23 °C under ~150 µmol photons m^{-2} s^{-1} white light.

For the continuous labeling, leaves were harvested into the incubation media and placed in the shaking water bath to equilibrate temperature. To start the time course the medium was removed and replaced with incubation medium containing 0.255 mM [1-^{14}C]acetate sodium salt 55 mCi/mmol (American Radiolabeled Chemicals Inc.) at 12.75 µCi/ml. The leaves were incubated for different timepoints (5, 10, 15, and 60 minutes) after which they were removed from the medium and quenched in isopropanol with 0.01% BHT at 85 °C prior to lipid extraction. Fifteen leaves were used per time point replicate. The labeled medium was reused between different timepoints of same replicate/plant line. Each timepoint was harvested/radiolabeled separately in replicates of three per plant line.

For the pulse-chase labeling, whole rosettes were harvested by removing the roots, and immediately placed into the incubation medium. Once rosettes for all timepoints for each replicate/plant line were collected, the incubation medium was removed and replaced with medium containing 0.153 mM [^{14}C]acetate at 7.65 µCi/ml. After 15 mins of pulse, the radiolabeled medium was removed, and the tissues were washed 5 times using incubation medium. The 0-timepoint was immediately collected and the remaining rosettes were incubated in incubation medium for 1, 4, 22, 28, and 51 hours. The collected tissues were quenched in isopropanol with 0.01% BHT at 85 °C prior to lipid extraction. Three to four whole rosettes were used per time point replicate. Three replicate pulse-chase time courses were carried out per line, and the radiolabeled medium for the pulse was reused for the three replicates within each line.

Analysis of radioactivity of extracts in disintegrations per minute (DPM) by liquid scintillation counting in EcoScint Original scintillation fluid (National Diagnostics) was on a Beckman Coulter LS 6500 liquid scintillation counter. Relative radioactivity of lipids separated on TLC plates was measured using phosphor imaging on a GE Typhoon FLA7000, and ImageQuant analysis software.

Glycerolipid and FAME separations by TLC

Polar lipids were separated using thin layer chromatography (TLC) plates (20 x 20 cm Analtech Silica gel HL 250 µM thickness) pre-treated with 0.15 M ammonium sulfate and baked at 120 °C for 3 hours. Less than 250 µg lipid was loaded per cm and separated in toluene/acetone/water (30/91/7, v/v/v). Neutral lipids were loaded directly onto the untreated EMD Millipore silica gel 60 20 x 20 cm TLC plates and separated in hexane/ether/acetic acid (70/30/1, v/v/v). FAME were loaded onto EMD Millipore plates treated in 7.5% silver nitrate in acetonitrile and baked at 100 °C for 5 minutes prior to use. The FAME were first separated to 75% of plate height in hexane/ether (1/1, v/v), then fully developed in hexane/ether (9/1, v/v).

Stereochemical Analysis of ^{14}C labeled lipids

Lipids were separated by TLC, stained with 0.005% primulin in acetone/water (4/1, v/v) and visualized under UV light. Polar lipids were eluted from silica gel with

chloroform/methanol/water (5/5/1, v/v/v), and the chloroform phase was collected after phase partitioning with 0.88% KCl. Neutral lipids were eluted from silica gel with chloroform/methanol (9/1, v/v). All lipids were dried under nitrogen prior to being suspended in diethyl ether for lipase digestion.

Stereochemical analysis of DAG and MGDG was done by enzymatic digest with lipase from *Rhizomucor miehei* (Sigma). Buffer consisting of 50 mM boric acid and 5 mM calcium chloride at 7.8 pH and the lipase were added at 4:1 ratio for diacylglycerol (DAG) and 39:1 ratio for MGDG. The reaction was carried out for 15 minutes for both DAG and MGDG with a goal of 50% to 60% and 20% to 30% digestion respectively. The reaction was stopped by adding chloroform/methanol (1:1), the chloroform phase was collected for TLC. The digested lipids were separated using hexane/diethyl ether/acetic acid (35/70/1.5, v/v/v) for DAG and acetone/toluene/water (91/30/7.5, v/v/v) for MGDG on non-treated silica TLC plates.

Stereochemical analysis of PC was done with Phospholipase A_2 from bee venom (*Apis mellifera*) (Sigma). Buffer containing 50 mM Tris-HCl and 5 mM calcium chloride at 8.7 pH and PLA_2 were added such that the enzyme was about 0.25 units. The reaction was carried out for 5 minutes and the reaction mixture was dried down under nitrogen. The digested lipids were extracted by adding chloroform/methanol/0.15 M acetic acid (38/19/15, v/v/v) and collecting the organic phase. The lipids extracted were separated using chloroform/methanol/acetic acid/water (50/30/8/4, v/v/v/v) on silica TLC plates.

Data analysis

All calculations from raw data were done in Microsoft Excel. Graphing and statistical analysis done with GraphPad Prism version 7.04.

Accession Numbers

LPCAT1 (At1g12640), *LPCAT2* (At1g63050), *ACT1/ATS1* (At1g32200)

Supplemental Data

Supplemental Figure 1. Relative gene expression of LPCAT1, LPCAT2, LPEAT1, LPEAT2 in leaves and seeds of wild-type Arabidopsis.

Supplemental Figure 2. Pictures of growth of Col-0, *act1, lpcat1 lpcat2,* and *act1 lpcat1 lpcat2.*

Supplemental Figure 3. Lipid fatty acid composition at 2 weeks.

Supplemental Figure 4. Lipid fatty acid composition at 3 weeks.

Supplemental Figure 5. Lipid fatty acid composition at 4 weeks.

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Author Contributions

NK, BSJ, and PDB designed the research; performed research; analyzed data; and wrote the paper.

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Figure legends

Figure 1. Model of wild-type Arabidopsis acyl trafficking within leaf glycerolipid synthesis

The model focuses on the trafficking of acyl groups between the chloroplast and the ER for MGDG synthesis. The model is centered around the ER "PC pool", which is involved in *de novo* PC synthesis, desaturation, acyl editing, and turnover to produce the substrate for MGDG production. Chloroplast OEM PC produced from transfer of LPC or PC may also be a substrate for MGDG synthesis. Key enzymes/transporters are in yellow, uncertain reactions are in blue and have dashed lines. Abbreviations are as in text.

Figure 2. Membrane lipid composition of leaves across development from wild-type and mutant lines. The relative abundance of leaf membrane lipids was determined at three developmental stages: two weeks (A), three weeks (B), and four weeks (C) after germination. The data represent the average and standard error of 2-4 biological replicates. Significant (P-value < 0.05, Two-way ANOVA with multiple

comparisons) differences within individual lipid abundances between *act1* and *act1 lpcat1 lpcat2* are indicated by asterisks above the bars.

Figure 3. Fatty acid composition of PC and PE across leaf development from wild-type and mutant lines. The fatty acid composition of PC (A-C) and PE (D-F) were determined at three developmental stages: two weeks (A, D), three weeks (B, E), and four weeks (C, F) after germination. The data represent the average and standard error of 2-4 biological replicates. Significant (p-value < 0.05, Two-way ANOVA with multiple comparisons) differences within individual lipid abundances between *act1* and *act1 lpcat1 lpcat2* are indicated by asterisks above the bars.

Figure 4. LPCAT activity in isolated chloroplasts. Isolated chloroplasts from Col-0, *act1*, *lpcat1 lpcat2*, and *act1 lpcat1 lpcat2* were incubated with 1 mM soy LPC, and 13.6 µM [^{14}C]oleoyl-CoA for 30 min at 30 °C and radioactivity incorporated into PC measured. The data represent the average and SEM of three independent assays from chloroplasts isolated from each line. Significant (p-value < 0.05, students t-test) differences from the Col-0 control are indicated by asterisks above the bars.

Figure 5. Initial incorporation of [^{14}C]acetate-labeled nascent fatty acids into leaf lipids. Continuous [^{14}C]acetate labeling of 3-week-old leaves over a 1 hour time course. (A) Total ^{14}C accumulation in organic extractable lipids, and linear regression. Significant differences (students t-test, p-value <0.05) in lipid labeling between lines at each time point are indicated by asterisks above the data points. (B-C) Incorporation of [^{14}C]acetate into major labeled membrane lipids and DAG in the *act1* and *act1 lpcat1 lpcat2* lines respectively.

Figure 6. Relative accumulation of [^{14}C]acetate-labeled nascent fatty acids into leaf lipids. The relative labeling of individual lipids to the total labeled lipids in each line compared between lines. (A) PC. (B) DAG and PA. (C) PE, PG, and PI/PS. (D) MGDG. All data points are average and SEM from three sets of independently-labeled plants. Significant differences (students t-test, p-value <0.05) in lipid labeling between lines at

each time point are indicated by asterisks above the data points. An α above the data indicates a p-value <0.07.

Figure 7. Stereochemical analysis of [^{14}C]acetate-labeled nascent fatty acids incorporation into DAG and PC. Continuous [^{14}C]acetate-labeled DAG and PC from Figures 5 and 6 were collected and subjected to lipase-based regiochemical analysis of ^{14}C fatty acid locations in the *sn*-1 or *sn*-2 position of the glycerol backbone. (A) *act1* DAG. (B) *act1 lpcat1 lpcat2* DAG. (C) *act1* PC. (D) *act1 lpcat1 lpcat2* PC. All data points are average and SEM from three sets of independently-labeled plants.

Figure 8. Pulse-chase [^{14}C]acetate tracking of leaf lipid precursor–product relationships. A 15 min [^{14}C]acetate pulse of 3-week-old whole rosettes was followed by a chase up to 51 hours. (A-B) Relative labeling of lipids within *act1* (A), or *act1 lpcat1 lpcat2* (B). (C-F) Relative labeling of major labeled individual lipids to the total labeled lipids in each line compared between lines. (C) PC and DAG. (D) MGDG. (E) PE and PA. (F) TAG. All data points are average and SEM from three sets of independently-labeled plants, except for PA which had 1–3 replicates. In (C-F), significant differences (students t-test, p-value <0.05) in lipid labeling between lines at each time point are indicated by asterisks above the data points.

Figure 9. Radiolabeled fatty acid composition of DAG, PC, and MGDG over the [^{14}C]acetate pulse-chase time course. The radiolabeled fatty acids in different lipids from Fig. 8 are represented as total saturated fatty acids (e.g. 16:0, 18:0), monoenoic fatty acids (e.g. 18:1), dienoic (e.g. 18:2), and trienoic (e.g. 18:3). The proportion of each fatty acid within each lipid is compared between plant lines with *act1* as solid lines, and *act1 lpcat1 lpcat2* as dashed lines. (A-B) DAG. (C-D) PC. (E-F) MGDG. All data points are average and SEM from three sets of independently-labeled plants from Fig. 8. Significant differences (students t-test, p-value <0.05) in lipid labeling between lines at each time point are indicated by asterisks above the data points.

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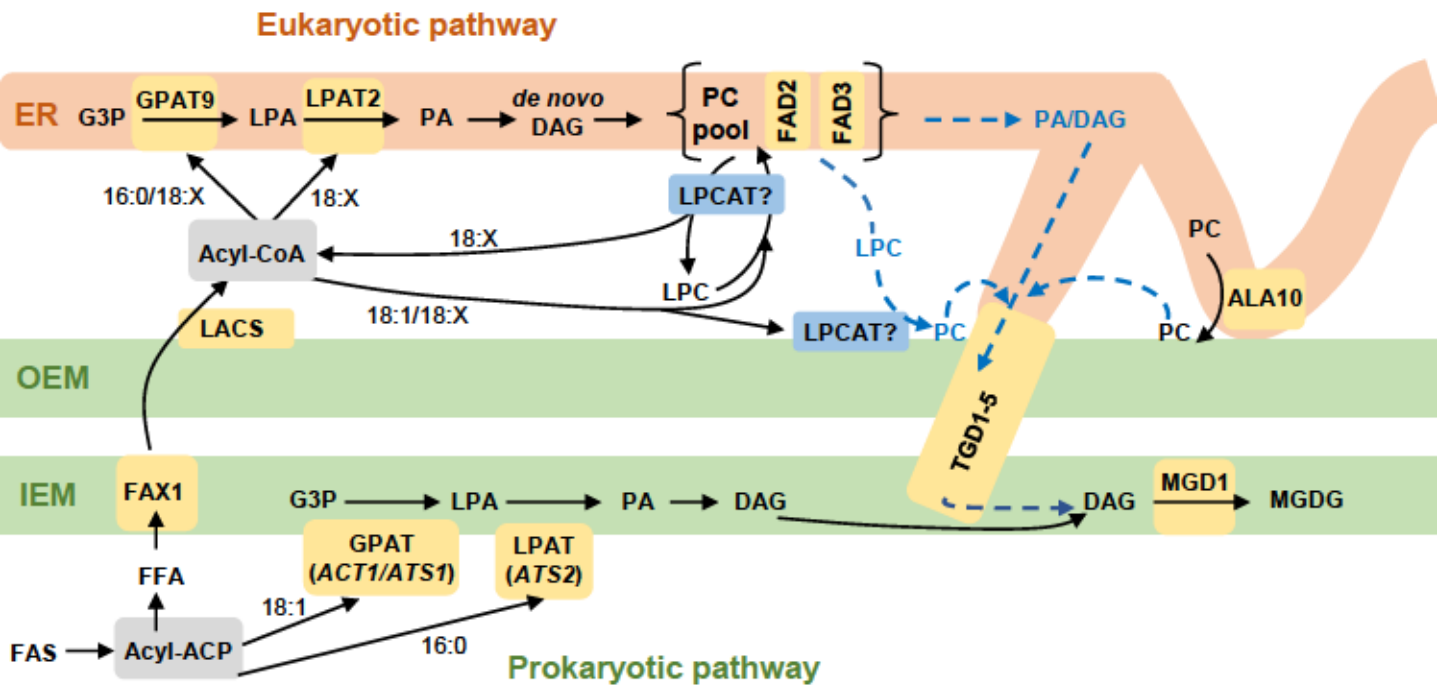
Figure 11: Models of [^{14}C]acetate pulse-chase labeling of MGDG synthesis in *act1* and *act1 lpcat1 lpcat2* leaves.

The models indicate the relative rate of labeled fatty acid flux through the eukaryotic pathway of MGDG synthesis within the pulse-chase experiment. Red solid lines represent initial reactions, blue large dashed lines represent the next set of reactions labeled over time, and the green small dashed lines represent the slowest set of reactions labeled over time within each model. Likewise, for the DAG, PC, and MGDG pools, the major labeled stereochemical position at various time points is indicated by the position noted with an asterisk and color coding the same as the lines. No specific time points are intended, and each model color coding is independent from the others, representing only relative labeling within each model. Abbreviations are as in the text. A, *act1*. B, *act1 lpcat1 lpcat2* with no PC acyl chain removal from residual acyl-editing mechanisms. C, *act1 lpcat1 lpcat2* with compensating acyl-editing reactions that lead to acyl chain removal from PC and incorporation into the acyl-CoA pool, and the switching of PC labeled stereochemistry from *sn*-1 to *sn*-2.

Figure 12. Updated model of wild-type Arabidopsis acyl trafficking within leaf glycerolipid assembly clarifying the role of LPCAT1 and LPCAT2.

The model focuses on the trafficking of acyl groups between the chloroplast and the ER for MGDG synthesis. Here the model separates PC involved in acyl editing "PC(1)" from PC synthesized *de novo* "PC(2)" and PC that provides the substrate for MGDG synthesis "PC(3)". The model also allows that PC acyl editing may take place in the ER

or at the chloroplast surface, which could be a way to move acyl groups into the ER by PC movement through membrane contact sites. The PC(3) pool is derived from *de novo* synthesized PC(2) which may have been further desaturated by FAD2 and FAD3. The substrate for MGDG synthesis may come from turnover of the PC(3) pool in the ER, or turnover of the PC(3) at the chloroplast surface. Key enzymes/transporters are in yellow, uncertain reactions are in blue and have dashed lines. Abbreviations are as in text, rLPCAT is the reverse LPCAT reaction



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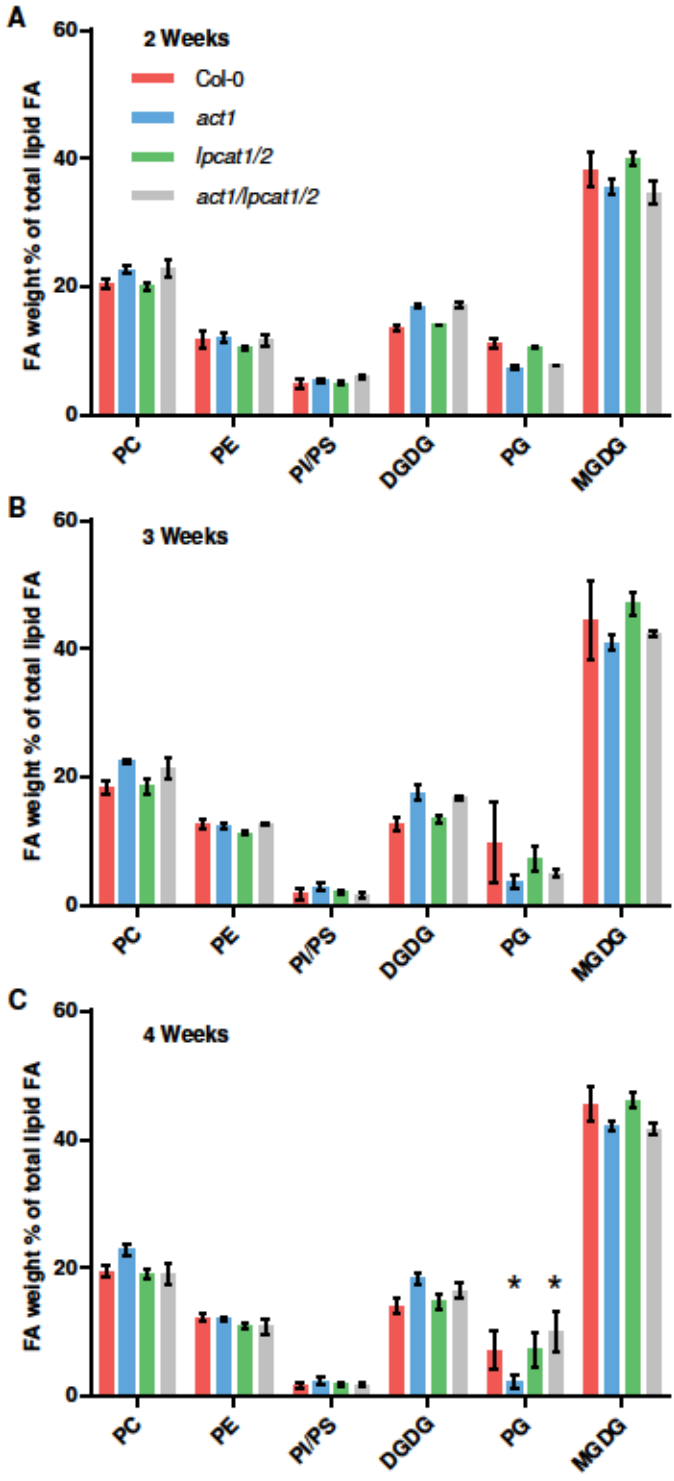

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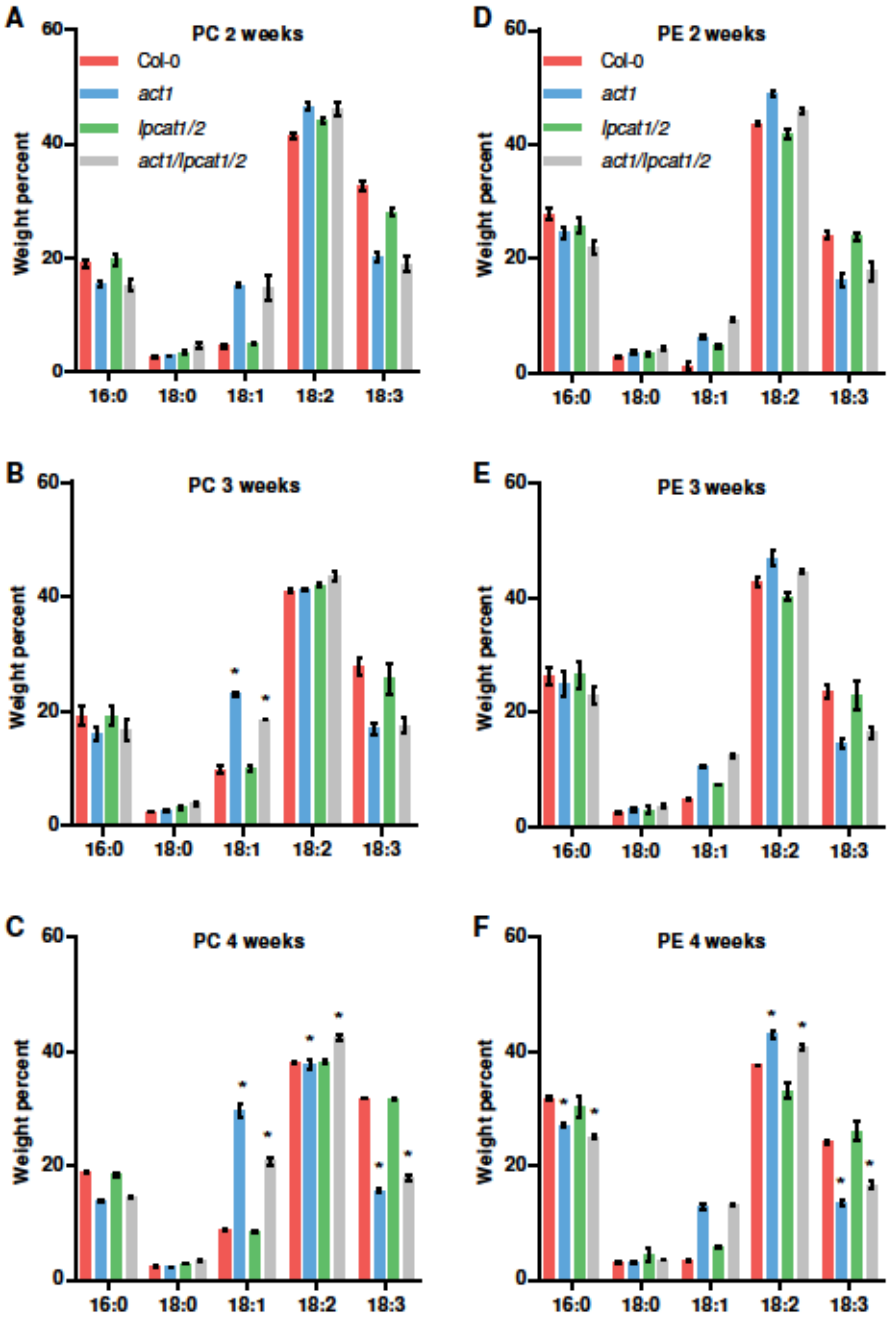

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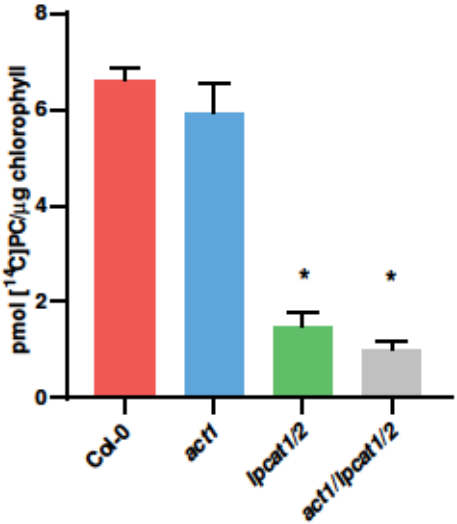

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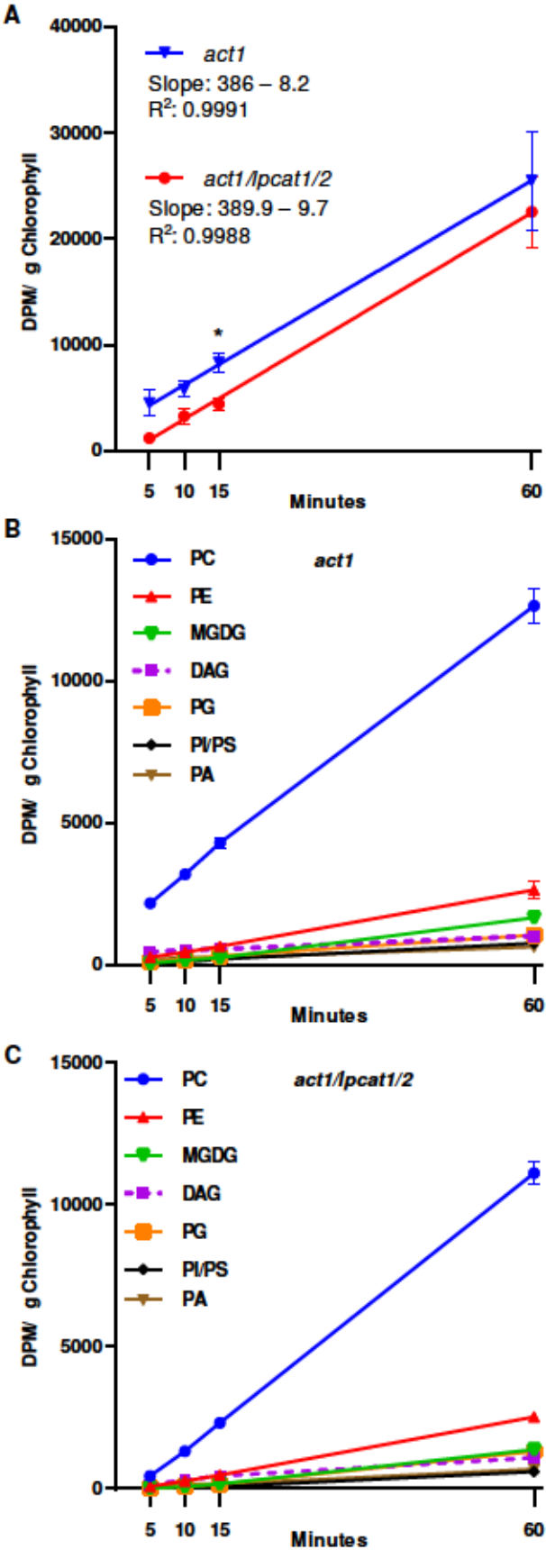

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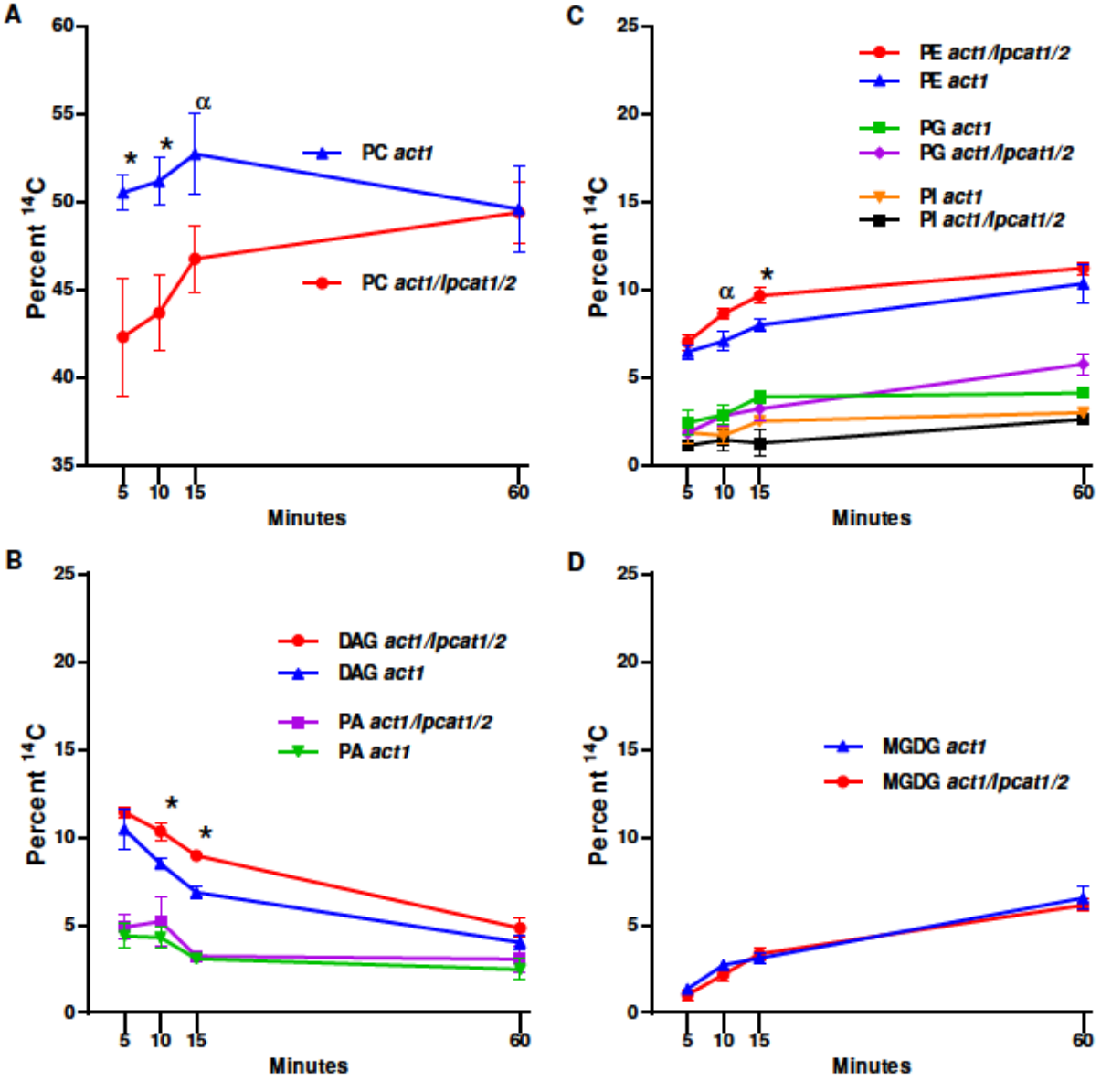

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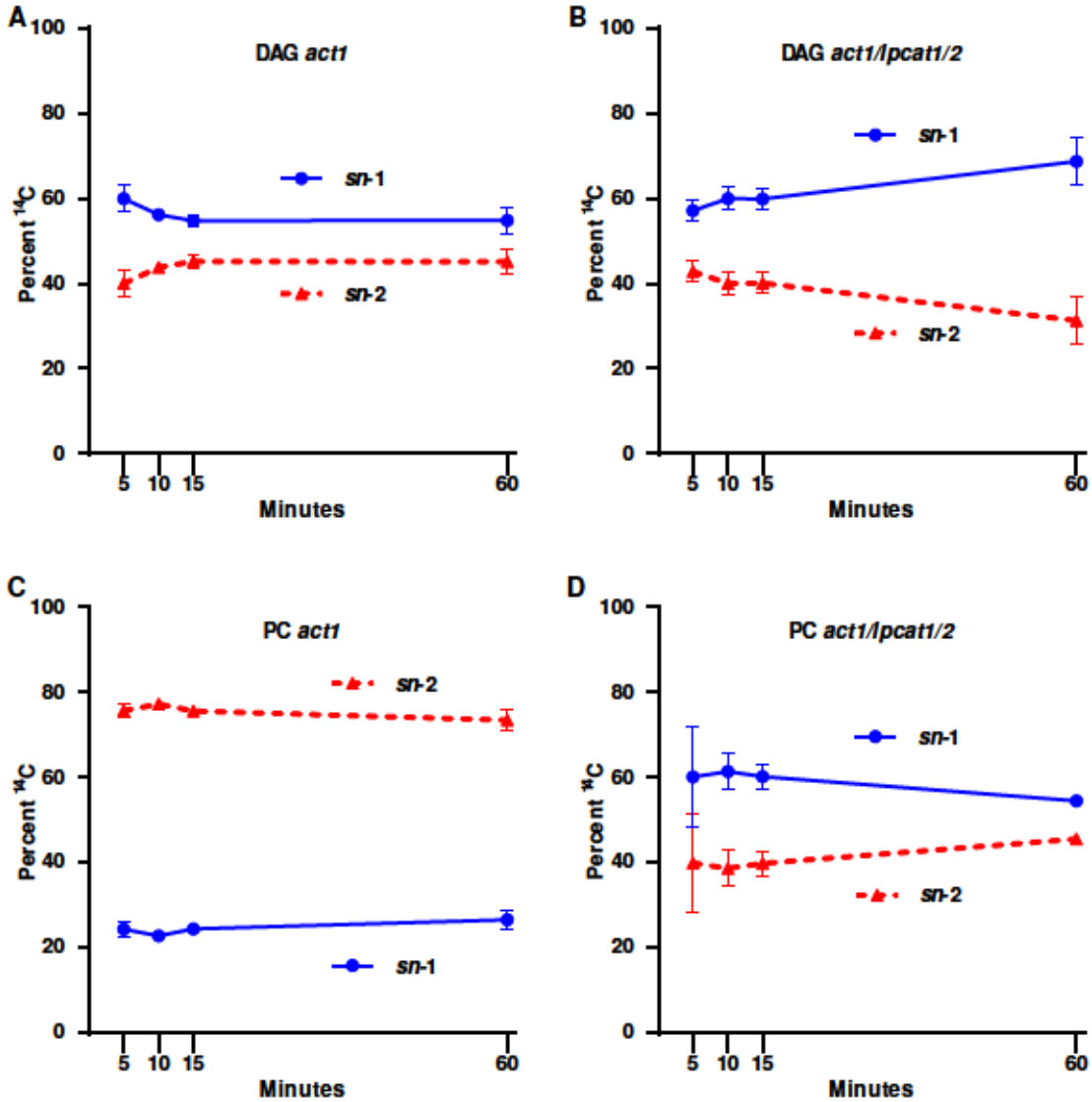

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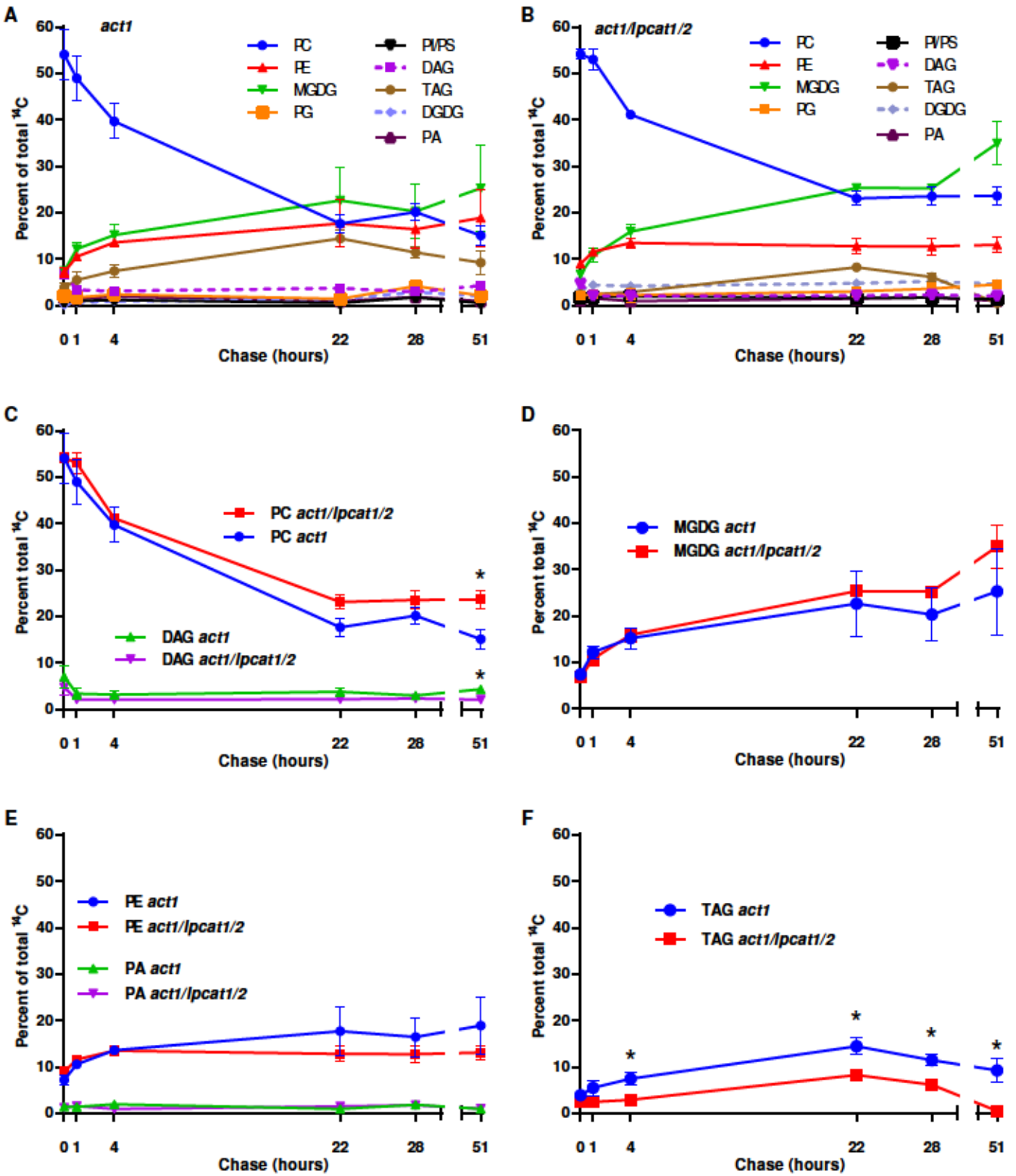

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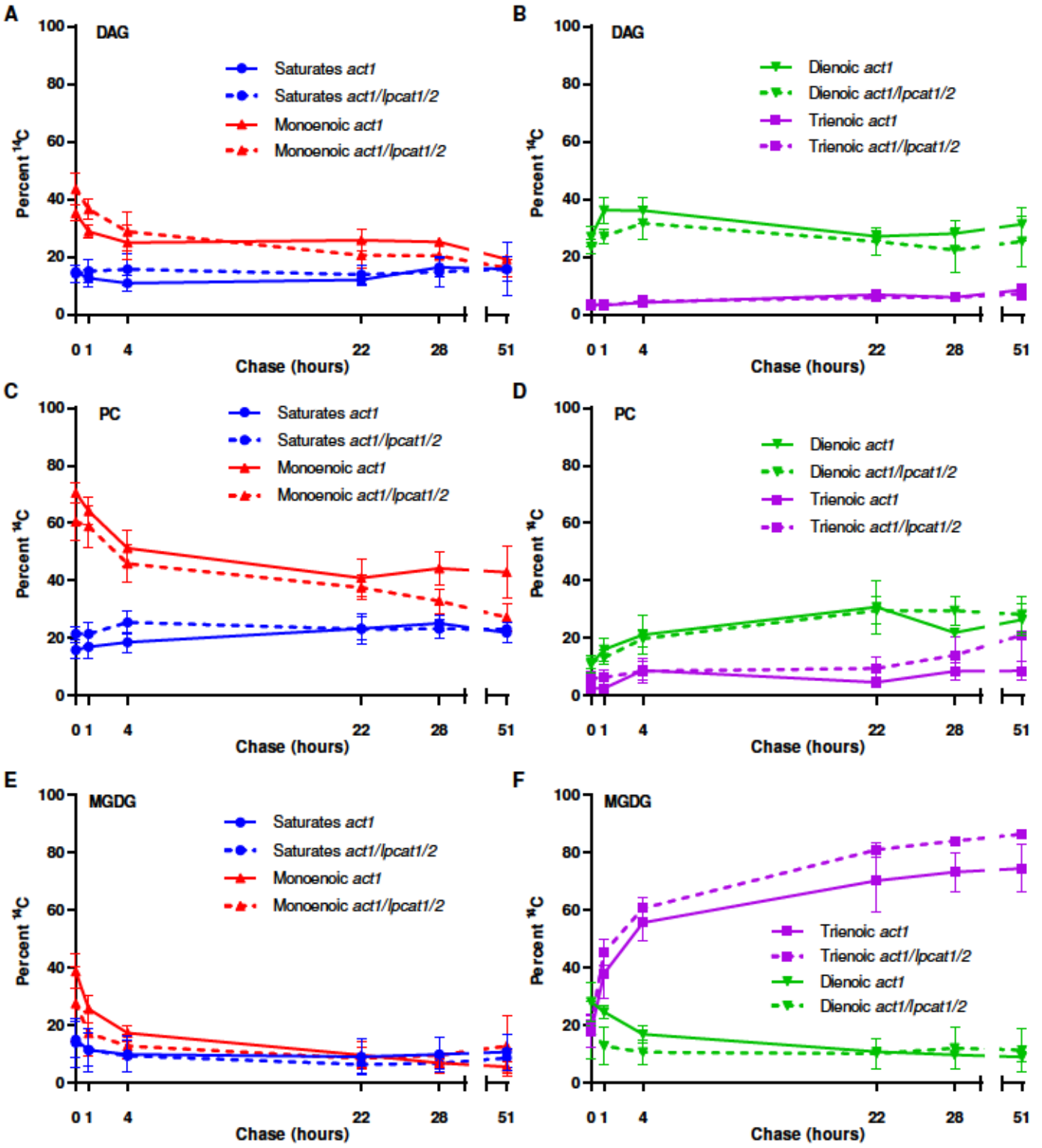

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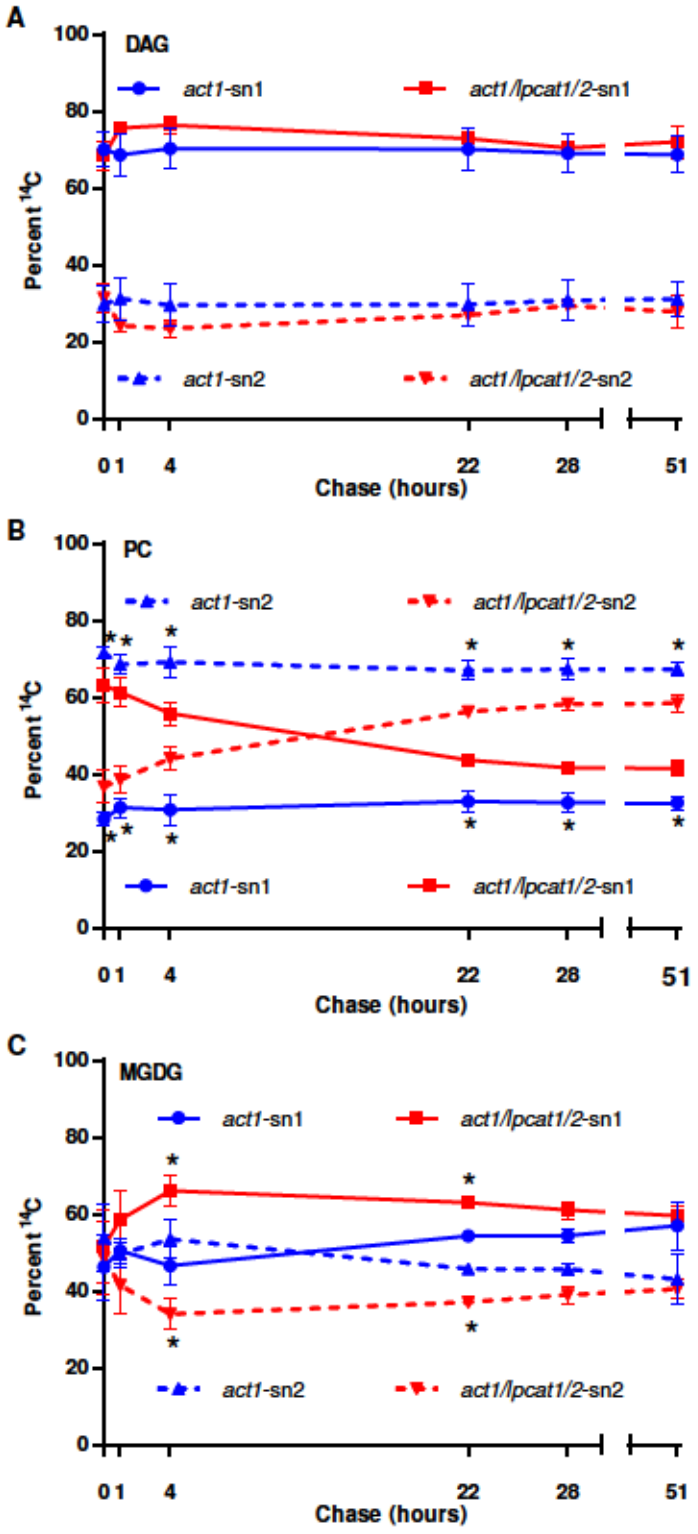

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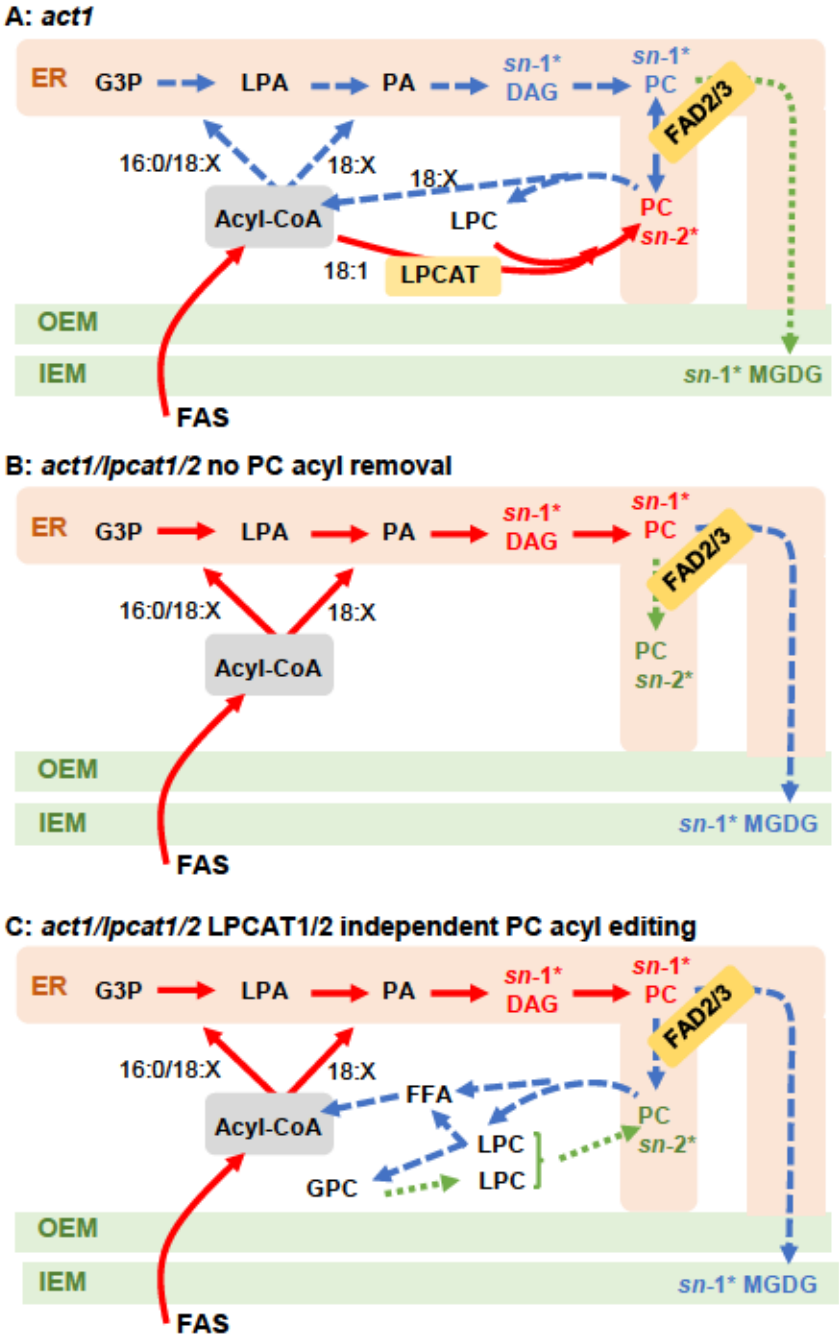

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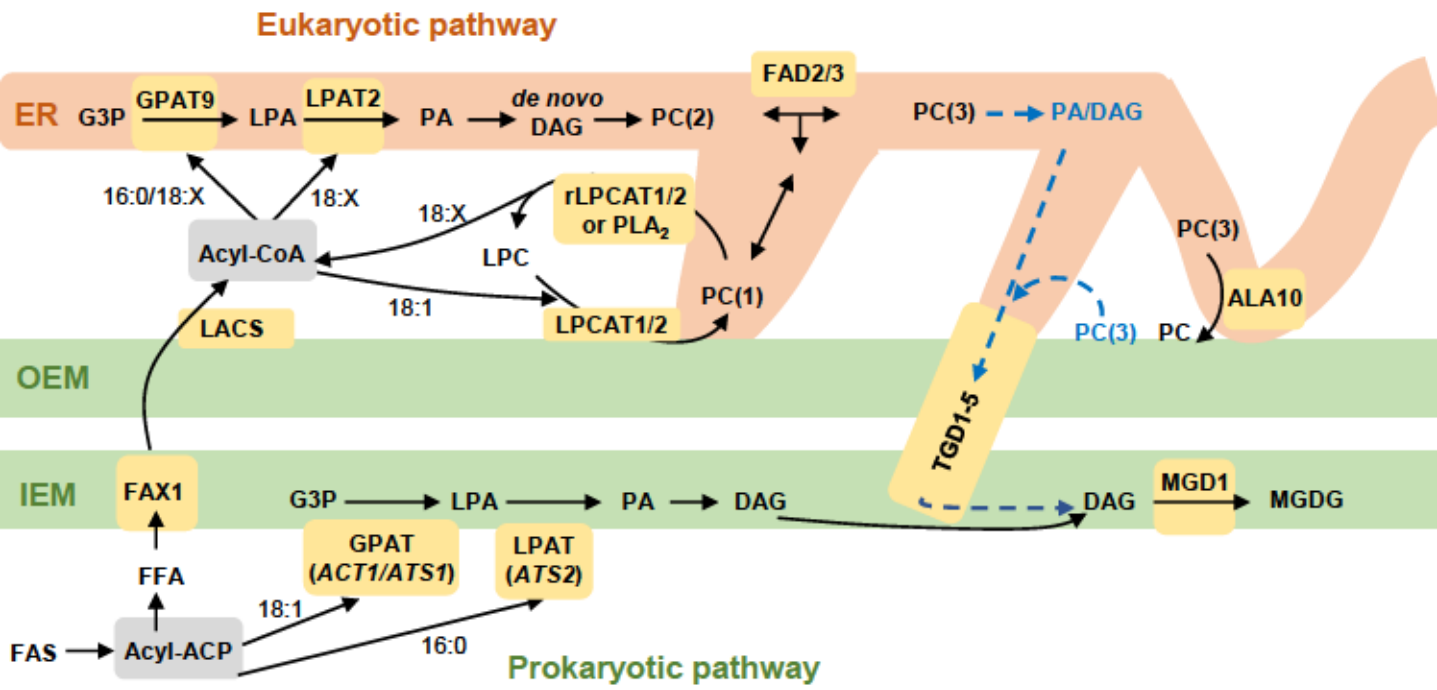

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