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RE-EXAMINING THE INITIAL STEPS OF MEMBRANE AND STORAGE LIPID ASSEMBLY IN PEA LEAVES AND SOYBEAN EMBRYOS:

THE DOMINANT FLUX OF NEWLY SYNTHESIZED FATTY ACID INCORPORATION INTO EXTRA-PLASTIDIC GLYCEROLIPIDS IS THROUGH PHOSPHATIDYLCHOLINE ACYL EDITING

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ABSTRACT

RE-EXAMINING THE INITIAL STEPS OF MEMBRANE AND STORAGE LIPID ASSEMBLY IN PEA LEAVES AND SOYBEAN EMBRYOS: THE DOMINANT FLUX OF NEWLY SYNTHESIZED FATTY ACID INCORPORATION INTO EXTRA-PLASTIDIC GLYCEROLIPIDS IS THROUGH PHOSPHATIDYLCHOLINE ACYL EDITING

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In expanding pea leaves and developing soybean embryos over 95% of fatty acids (FA) synthesized in the plastid are exported for assembly of eukaryotic membrane and storage glycerolipids. It is often assumed that the major products of plastid FA synthesis (18:1 and 16:0) are first acylated to glycerol-3-phosphate (G3P) producing 16:0/18:1 and 18:1/18:1 initial molecular species of phosphatidic acid (PA) which are then converted to phosphatidylcholine (PC), the major eukaryotic phospholipid and site of acyl desaturation. However, by rapid labeling of pea leaf and soybean embryo lipids with [^{14}C]acetate and [^{14}C]glycerol we demonstrate that acyl editing is an integral component of eukaryotic glycerolipid synthesis. First, no precursor-product relationship between diacylglycerol (DAG) and PC [^{14}C]acyl chains was observed at very early time points. Second, analysis of PC molecular species and the positional localization of nascent FA within PC at these early time points showed that the majority of newly synthesized acyl groups (> 90% of the [^{14}C]18:1 and [^{14}C]saturated acyl groups in pea, and >60% in soybeans) were incorporated into PC alongside a

previously synthesized unlabeled acyl group (18:2, 18:3 or 16:0). The nascent FA occupied 62% of the *sn*-2 position in pea leaves and 86% in soybean embryos. Third, [^{14}C]glycerol labeling produced PC molecular species highly enriched with 18:2, 18:3 and saturated acyl chains and not 18:1, the major product of plastid fatty acid synthesis. And fourth, the kinetics of [^{14}C]glycerol and the positional analysis of newly synthesized FA incorporation into triacylglycerol (TAG) of soybean embryos indicated that TAG is synthesized from a DAG molecule generated by removal of the PC phosphocholine head group and not *de novo* synthesized DAG. In conclusion, we propose that most newly synthesized and plastid exported acyl groups are not immediately utilized for PA synthesis, but instead are incorporated directly into PC through an acyl editing mechanism that operates at both *sn*-1 and *sn*-2 positions. The acyl groups removed by acyl-editing are largely used for the net synthesis of PC and other glycerolipids through (G3P) acylation. Very little if any TAG is produced by three consecutive acylations as in the traditional Kennedy pathway. These results provide a more quantitative basis to demonstrate *in vivo* that FA flux in/out of PC by acyl editing, and the inter-converstion of DAG with PC are dominate fluxes in the assembly of eukaryotic membrane and storage glycerolipids in plants.

This dissertation is dedicated to my wife Cassandra. Her loving support, strong encouragement, sacrifice of moving to Michigan, and ability to make me fat and happy has made the perils of graduate school relatively easy to bear.

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1 INTRODUCTION

Fatty acid (FA) based lipids are essential to life and perform a wide variety of functions. Living cells depend upon a hydrophobic bilayer of amphipathic membrane lipids to separate the cell contents from the aqueous environment. Eukaryotic cells accomplish complex metabolism by compartmentalization of reactions within membrane bound organelles. Membrane lipids can be utilized as substrates for metabolic signaling compounds [1-5]. Multi-cellular plants contain fatty acid derived physical barriers against water, solutes, pathogens and solar UV radiation in the form cutin, suberin, and wax [6-11]. Triacylglycerols (TAG) contain three FA bound to a glycerol backbone and are the major form of reduced carbon used for energy storage in plants and animals. In addition to the biological importance of FA containing lipids within an individual organism, many animals rely on plant TAG as a major source of nutrition.

The human population utilizes plant TAG for food, fuel and industrial applications. In 2006, world harvest of plant oil was over 127 million tons [12]. Vegetable oil produced from soybeans represents the largest source of oil for nutritional purposes at approximately 35 million tons produced in 2006 [13]. On average the FA associated with common sources of vegetable oil are composed of 16 and 18 carbon saturated FA (palmitic (16:0) and stearic acid (18:0), respectively) and 18 carbon FA containing 1-3 double bonds (oleic (18:1), linoleic (18:2) and linolenic acid (18:3), respectively). These common FA are useful for

cooking oils and high energy food sources but do not contain the most healthy, oxidatively stable or most useful functional groups required in many highly nutritional foods, fuels or industrial applications, respectively. Nutritionally important unusual FA are the very long chain polyunsaturated fatty acids (PUFA) such as docosahexaenoic acid (DHA) produced in the microalgae *Crypthecodinium cohnii* [14] and usually consumed through fish. An example of industrially important unusual FA is the hydroxyl containing ricinoleic acid produced by the castor plant *Ricinus communis* [15, 16] which can be used in lubricants and as a feedstock to polymers such as nylon.

Most plants cultivated for high yields of vegetable oil (e.g. soybeans and rapeseed) were domesticated based on their value as food/feed and therefore do not contain many of the industrially important unusual FA. Plant species that do accumulate high levels of unusual FA (e.g. castor or tung) typically have agronomical traits that make harvesting large yields difficult and expensive. There is great interest in engineering unusual FA into some of the seasonal high yielding crop species. Additionally, the rising human population and dwindling petroleum reserves increases demand for renewable sources of chemical feed stocks and fuel. Plant oils can be used as direct replacements for diesel fuel and the wide variety of unusual FA could be the base for many industrial applications [12, 13]. Even though there have been many attempts to engineer the FA composition or the quantity of TAG in oilseeds, most cases have been met with only limited success [17-27]. Most of the enzymes responsible for production of the common, and many of the unusual, FA have been identified. However, not all

the factors that regulate FA synthesis or TAG accumulation are known and questions remain about the sequence of reactions in the incorporation of FA into membrane and storage lipids. A greater knowledge of the pathway of membrane lipid and TAG synthesis will allow us to design better strategies for engineering oilseeds in the future. This thesis will address the sequence of reactions that are involved in incorporation of FA into extra-plastidic membrane and storage lipids.

1.1 Background overview

Assembly of most membrane or storage glycerolipids involves four basic steps: 1) FA synthesis; 2) esterification of FA to a glycerol based backbone; 3) addition of the *sn*-3 headgroup or FA; 4) desaturation of acyl chains and/or transfer of modified acyl groups to other lipids. Although this process always begins with fatty acid synthesis, the order of the remaining three steps may vary. In plants these activities may be separated within a particular membrane system or duplicated in different compartments, and thus may involve multiple pools of substrates and trafficking between compartments. Additionally, some activities (in particular desaturation) may be more active with a specific glycerolipid substrate and thus require deacylation of the lipid after desaturation and further utilization of the modified FA to make other lipids. An overview of the current state of knowledge on plant membrane and storage glycerolipid synthesis is presented as a background to the research in this dissertation and to set the stage for the evidence that is presented in later chapters for changes in the current view of FA flux through central plant lipid metabolism.

1.2 Fatty acid synthesis and desaturation

In plants the major site of *de novo* fatty acid synthesis (FAS) for production of membrane and storage lipids is the plastid. This is significantly different from animals and fungi where FAS is primarily cytosolic. The non-cytosolic nature of plant FAS was first determined by subcellular-localization of acyl carrier protein (ACP), an essential component of FAS, to the chloroplasts of spinach [28]. Mitochondria can also synthesize fatty acids, however it appears that mitochondrial FAS is mostly for producing octanoate (8 C) for lipoic acid synthesis [29]. Additionally, stable isotope labeling of *Brassica napus* embryos demonstrated only a plastidic labeling signature in total lipids [30], indicating that any mitochondrial contribution to bulk lipids would be very minor compared to the plastidic FAS. It is possible that mitochondrial FAS may play a more dominant role in lipid synthesis in specific cell types such as epidermal cells involved in wax and cutin synthesis where modified acyl chains are predominant [31, 32]. Since this work deals with membrane and storage lipids from leaves, total seedings and developing embryos, FAS will refer to the major plastidic activity.

The first committed step in FAS is the production of malonyl-CoA by acetyl-CoA carboxylase (ACCase) [33] in the plastid. The three carbon carboxylic acid of malonyl-CoA is transferred to ACP and all further reactions take place with the growing acyl chain on ACP. *De novo* synthesis of a fully reduced 16 or 18 carbon fatty acid takes place by the sequential condensation of the malonyl-

ACP with a growing fatty acyl-ACP. Release of CO_2 drives each condensation reaction, elongating the acyl chain two carbons at a time. The four steps to make the initial four carbon fully reduced acyl-ACP are [34]: First, condensation of the initial malonyl-ACP wtih acetyl-CoA to produce the four carbon 3-ketobutyryl-ACP by 3-ketoacyl-ACP synthase III (KAS III) releasing CO_2. Second, NADPH-dependent reduction by 3-ketoacyl-ACP reductase produces 3-hydroxybutyryl-ACP. Third, removal of water by 3-hydroxyacyl-ACP dehydratase produces a *trans*-Δ^2-butenoyl-ACP. Finally, reduction of the double bond by 2,3-*trans*-enoyl-ACP reductase utilizing NADPH or NADH and producing the saturated four carbon chain, butyryl-ACP. Additional condensations with malonyl-ACP are catalyzed by different enzymes than the original condensation with acetyl-CoA. During elongation from butyryl-ACP (4C) to palmitoyl-ACP (16C) condensation is provided by 3-ketoacyl-ACP synthase I (KAS I). Palmitoyl-ACP can be condensed once more with malonyl-ACP by 3-ketoacyl-ACP synthase II (KAS II) and reduced to produce the 18C saturated stearoyl-ACP. In this manner saturated acyl chains are elongated two carbons at a time and after each condensation the reduction, dehydration and further reduction of the keto-, hydroxyl-, and enoyl- ACPs are catalyzed by the enzymes listed above. 16:0- and 18:0-ACP are the major products of FAS however, the major newly synthesized acyl chain that is incorporated into glycerolipids is the mono-unsaturated oleic acid (18:1). Oleoyl-ACP is produced by the soluble stroma localized stearoyl-ACP desaturase by inserting a double bond at the Δ 9 position [35]. Thus *de novo* FAS and initial desaturation within the plastid of pea leaves,

for example, produces 18:1 (~75%), 16:0 (~20%), and 18:0 (~5%) acyl chains for further lipid metabolism.

Plant membrane and storage glycerolipids mostly contain acyl chains other than the initial products of plastid FAS. Leaf membrane lipid acyl groups typically contain PUFA of either 16- or 18- carbons in length; most commonly 18:2, 18:3 and 16:3. Production of PUFA is accomplished after removal of the *de novo* synthesized acyl groups from ACP and incorporation into membrane lipids within the ER or plastid envelop membranes [36, 37]. Utilization of membrane lipid acyl groups for desaturation in plants differs from that of animals, yeast and fungi where the desaturases act on acyl Coenzyme A (CoA) thioesters as substrates [38, 39]. Additionally, longer chain fatty acids (>18C) can accumulate in some minor membrane lipids such as phosphatidylserine (PS) or storage TAG. Extra-plastidial elongation of acyl groups beyond 18 carbons may utilize glycerolipids as a primer to donate the acyl substrate [40]. The donated acyl chain is elongated two carbons at a time with the steps analogous to plastid FAS except that the intermediates of elongation are bound to CoA instead of ACP [41].

1.2.1 Acetyl-CoA carboxylase: Major FAS regulatory point

Production of malonyl-CoA by acetyl-CoA carboxylase (ACCase) is the first committed step in *de novo* FAS [33] and regulation of ACCase activity ultimately determines the flux through all of FAS [42]. All plants except the grass family Gramineae contain two forms of ACCase, a homomeric and heteromeric

form [43]. The homomeric form produces the cytosolic malonyl-CoA pool, while the heteromeric form is plastid localized and produces the malonyl-CoA pool for *de novo* FAS. A second homomeric form that is plastid localized has been found in *Brassica napus*, however its proportional contribution to FAS is unknown [44]. Since in most plants *de novo* FAS utilizes the products of the plastid localized heteromeric ACCase, the discussion will be limited to this isoform and will be referred to as just ACCase.

There are four subunits that make up ACCase which catalyzes two half reactions. In the first half reaction the biotin carboxylase (BC) catalyzes the ATP and HCO_3^- dependent carboxylation of a biotin prosthetic group attached to the biotin carboxyl carrier protein (BCCP). The activated carboxy-biotin is transferred to the active site of the carboxyltransferase (CT) made up of α-CT and β-CT subunits. CT completes the second half reaction by carboxylation of acetyl-CoA producing malonyl-CoA [45]. The β-CT subunit is encoded by the plastid genome [46] and requires RNA editing for functionality [47], while the other three subunits are all encoded by the nuclear genome and each contains a cleavable plastid targeting peptide [48-50]. All four subunits are coordinately expressed and assembled together in the plastid [51, 52]. It is believed that expression of the β-CT subunit by the plastid coordinates the expression of the other three subunits [53]. The ACCase complex is mostly stroma localized but may be partially associated to the inner envelope membrane in rapidly expanding leaves [54].

ACCase activity can be regulated developmentally or enzymatically. All four subunits of ACCase are developmentally regulated with the highest

expression in the organs with the greatest demand for FA synthesis, for example expanding leaves and developing oil seeds [52, 55]. Increased demand for long chain FA induces expression of all subunits, as demonstrated in *Brassica napus* seeds expressing a lauroyl-acyl carrier protein thioesterase which terminated FAS at 12 carbons starving the cell of long chain FA [56]. The enzymatic activity of ACCase can be activated by the substrate acetyl-CoA [57], and downstream products of FAS such as long chain acyl-CoAs can cause feedback inhibition [58]. Light is a strong regulator of FAS with over five times more FAS in the light than the dark [59]. Some of light's effect on FAS may be due to increases in ACCase enzymatic activity without a change in gene expression [46, 57]. Light causes the pH and Mg^{2+} concentration of the stroma to change toward the enzymatic optima of ACCase [60]. A disulfide bond between the α-CT and β-CT is reduced in the light which increases activity of ACCase. It is believed that the disulfide reduction is mediated through a thioredoxin linked pathway [61-63]. The β-CT subunit of ACCase is also reversibly phosphorylated in the light which has been shown to increase enzymatic activity [64].

Since malonyl-CoA production commits carbon to FAS, this step has been the subject of multiple attempts to increase FAS and thus TAG in oilseeds. Over expression of individual nuclear encoded ACCase subunits has not increased ACCase activity or FAS [65, 66]. Increased expression of the plastid operon that includes β-CT increased leaf longevity and doubled seed yield in tobacco [53]. However, it is unknown if these effects are due to an increase in ACCase alone or if the other gene products in the operon contributed to this phenotype.

Targeting the Arabidopsis homomeric ACCase to the plastid of *Brassica napus* seeds increased *in vitro* ACCase activity, and caused a 5% increase in seed TAG [67]. The strong control that ACCase has on the flux of carbon through FAS indicates the importance of further understanding its regulation in relation to lipid metabolism.

1.3 Esterification of fatty acids to glycerol-*3*-phosphate: *de novo* glycerolipid synthesis.

Glycerolipids (membrane and storage) contain a glycerol moiety as a central backbone in which fatty acyl groups and/or polar headgroups are attached. Polar lipids that make up membranes contain two acyl groups esterified to the *sn*-1 and *sn*-2 positions of glycerol reserving the *sn*-3 position for a polar headgroup. TAG contains a third acyl group esterified to the *sn*-3 position. *De novo* glycerolipid synthesis starts with two sequential acylations of glycerol-3-phosphate (G3P). The first acylation by G3P acyltransferase (GPAT) produces 1-acyl-2-lyso-glycerol-3-phosphate, commonly known as lyso-phosphatidic acid (LPA). The second acylation by lyso-phosphatidic acid acyltransferase (LPAAT) produces 1,2-diacylglycerol-3-phosphate, referred to as phosphatidic acid (PA). These two acylation steps were first elucidated from microsomes of guinea pig liver, with the energy of the reaction provided by activated acyl-CoA [68]. Later it was shown by Eugene Kennedy in rat liver extracts that the diacylglycerol (DAG) backbone produced from *de novo* glycerolipid synthesis and dephosphorylation of PA could be used to make the common membrane lipids phosphatidylcholine

(PC) and phosphatidylethanolamine (PE) through enzymatic reaction with nucleotide activated headgroups, CDP-choline and CDP-ethanolamine, respectively [69]. Kennedy also demonstrated in chicken liver microsomes that DAG could be acylated with acyl-CoA to produce TAG [70]. The fundamental work by Eugene Kennedy in understanding aspects of glycerolipid synthesis has led to the acylations of G3P en route to TAG synthesis in plants to be commonly referred to as the Kennedy pathway.

1.3.1 The two pathways for *de novo* glycerolipid synthesis in plants

Plants contain two pathways for *de novo* glycerolipid synthesis (Figure 1-1) involving the GPAT and LPAAT activities producing the common intermediate PA. 1) The the ER localized "eukaryotic pathway" and, 2) the plastid stroma and envelope localized "prokaryotic pathway". The prokaryotic pathway was named because the plastid LPAAT specifically esterifies a 16 carbon FA to the *sn*-2 position of the glycerol backbone similar to that of cyanobacteria. In contrast, the eukaryotic pathway acyltransferases localize 16 carbon FA, if present at all, to the *sn*-1 position similar to animals [71].

The prokaryotic pathway utilizes the activated acyl-ACPs from FAS and stearoyl-ACP desaturase, to make the initial PA molecular species 18:1/16:0 (molecular species refer the combination of FA esterified within a particular lipid). Prokaryotic PA is used to synthesize phosphatidylglycerol (PG) in all plants and a portion of the plastidic glycolipids in some plants [34]. Plastid localized

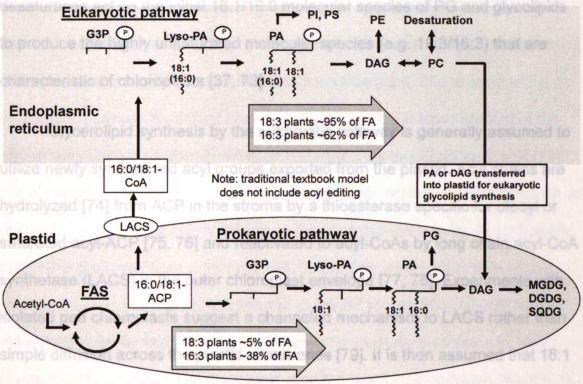

Figure 1-1 Traditional textbook two pathway model of glycerolipid synthesis

The two pathway model of leaf glycerolipid synthesis as described in the text. The model shows general localizationof prokaryotic pathway to the membranes of the plastid and eukaryotic pathway to the endoplasmic reticulum membranes. Estimations of the amount of fatty acids that flow through each pathway are based on the fact that 18:3 plants only make PG by the prokaryotic pathway [71], and the 16:3 plant Arabidopsis leaf measurements [72]. Abbreviations: glycerol-3-phosphate, G3P; lyso-phosphatidic acid, lyso-PA; phosphatidic acid, PA, diacylglycerol, DAG; phosphatidylcholine, PC; phosphatidylinositol, PI; phosphatidylserine, PS, phosphatidylethanolamine, PE; phosphatidylglycerol, PG; monogalactosyldiacylglycerol, MGDG; digalactosyldiacylglycerol, DGDG; suphoquinovosyldiacylglycerol, SQDG; fatty acid, FA; coenzyme A, CoA; acyl carrier protein, ACP; fatty acid synthesis, FAS.

desaturases act on the initial 18:1/16:0 molecular species of PG and glycolipids to produce the highly unsaturated molecular species (e.g. 18:3/16:3) that are characteristic of chloroplasts [37, 73].

Glycerolipid synthesis by the eukaryotic pathway is generally assumed to utilize newly synthesized acyl groups exported from the plastid. Acyl chains are hydrolyzed [74] from ACP in the stroma by a thioesterase specific for oleoyl or saturated acyl-ACP [75, 76] and reactivated to acyl-CoAs by long chain acyl-CoA synthetase (LACS) in the outer chloroplast envelope [77, 78]. Experiments with isolated pea chloroplasts suggest a channeled mechanism to LACS rather than simple diffusion across the plastid membranes [79]. It is then assumed that 18:1 and saturated acyl-CoAs are available within the cyctosol for the ER localized eukaryotic pathway acyltransferases. Utilization of nascent exported 18:1 and 16:0 by the eukaryotic pathway predicts the initial PA molecular species to be 18:1/18:1 and 16:0/18:1. PA is rapidly converted to PC which is the major extraplastidial phospholipid in multi-cellular plants [80] and main site of acyl desaturation for production of all other molecular species [36, 37]. The diacylglycerol backbone of PE, phosphatidylserine (PS), phosphatidylinositol (PI) and storage TAG are also produced by the eukaryotic pathway. Additionally, the eukaryotic pathway can produce the "DAG moiety" for plastid glycolipid synthesis. New insights into the trafficking events by which the DAG moiety is transported into the plastid have been achieved recently [80, 81], and will be briefly discussed below.

The relative amounts of the eukaryotic or prokaryotic pathway that are used to incorporate the acyl chain products of FAS into glycerolipids differs in different types of plants and tissues within the same plant. The amount of prokaryotic pathway in all plants has been related to the relative amount of PA phosphatase activity in the plastid to produce DAG for glycolipid synthesis [73]. "18:3" plants, so called because the leaf galactolipids contain primarily 18:3 FA at the *sn*-2 position, contain very little or no PA phosphatase activity. In 18:3 plants only PG is produced from prokaryotic PA. Therefore, greater than 95% of the FAs produced in the plastid are exported to the ER for acylation to G3P by the eukaryotic pathway and all of the DAG moiety for plastidic glycolipids in 18:3 plants must be imported into the plastid from the eukaryotic pathway. "16:3" plants, so called because the leaf galactolipids contain 16:3 FA at the *sn*-2 position can dephosphorylate PA in the plastid to provide a prokaryotic pathway DAG moiety for glycolipid synthesis. The relative proportion of FAS products utilized by the prokaryotic or eukaryotic pathways varies among different 16:3 plants [82]. In the case of the 16:3 plant *Arabidopsis*, the flux of acyl chains into leaf glycerolipids is approximately 62% eukaryotic and 38% prokaryotic [72], with about half of the galactolipid DAG moieties contributed by the eukaryotic pathway. The eukaryotic pathway is the major pathway of glycerolipid synthesis in non-leaf and oilseed tissue [83], and it is the dominant pathway among angiosperms, of which 88% are 18:3 plants [82].

The two pathway scheme for *de novo* glycerolipid synthesis was first elucidated by radiotracer studies of plant tissue and isolated organelles [71, 84]

and then confirmed genetically through analysis of pathway mutants [34, 85]. Critical experiments in the elucidation of the two pathways involved pulse-chase labeling of FAS with [^{14}C]acetate or $^{14}CO_2$, and [^{3}H]glycerol labeling of the backbone moiety of glycerolipids in leaf tissue of 18:3 and 16:3 plants [86, 87]. Over a period of hours it was evident in the 18:3 plant maize, that newly synthesized FA first accumulated in PC; then during the chase, the labeled FA and glycerol together were lost from PC and accumulated in MGDG. Thus the DAG moiety of eukaryotic synthesized PC was incorporated into a plastid lipid. Rapid synthesis of labeled PC and transfer to MGDG was also evident in spinach, a 16:3 plant, but to a lesser degree due to a portion of the MGDG synthesized by the prokaryotic pathway. Furthermore, isolated chloroplasts from spinach could produce galactolipids from *in situ* synthesized DAG, however PC was only produced from chloroplast-synthesized acyl-CoAs when microsomes were present [84], indicating the physical separation of the two G3P acylation pathways. The *act1* mutant in the 16:3 plant *Arabidopsis thaliana* provided further evidence of the two independent pathways by blocking prokaryotic glycolipid synthesis by a severely reduced plastidial GPAT activity. This forced newly synthesized FA to enter the eukaryotic pathway for acylation to G3P. In the *act1* mutant the eukaryotic pathway provided all of the DAG for glycolipid synthesis, essentially turning a 16:3 plant into a 18:3 plant [88].

1.3.2 Transfer of eukaryotic synthesized DAG to the plastid for glycolipid synthesis

Glycolipids are synthesized from DAG in the plastid envelope. However, in 16:3 and 18:3 plants more than half and all, respectively, of the DAG moiety is produced by the extraplastidial eukaryotic pathway (Figure 1-1). This separation in glycerolipid synthesis activities has led to multiple hypotheses on the mechanism of lipid transfer between the ER and plastid. Pulse-chase labeling in maize demonstrated that the glycerol backbone and acyl chains of PC were a precursor to plastid MGDG synthesis. Careful analysis of radiolabeled glycerol and acyl chains in MGDG molecular species suggested that 18:2/18:2-PC was the donor molecule for the DAG moiety incorporated into MGDG [86]. DAG was hypothesized to be the transfer molecule when a cytosolic phospholipase D and a hypothesized PA phosphatase from cytosolic extracts were added to isolated pea chloroplasts inducing DAG accumulation in the chloroplast [89]. PA (possibly derived from PC) was suggested as the transport molecule when a component of an inner envelope ABC transporter, that could bind PA *in vitro*, was shown to disrupt plastid import of eukaryotic DAG when mutated [90]. Furthermore, such mutants accumulate PA with a eukaryotic fatty acid composition.

An alternative hypothesis to the transfer of a complete DAG moiety originating from PC is the transfer of lyso-PC. *In vitro* experiments suggest that lyso-PC can spontaneously partition through an aqueous environment from isolated microsomes to isolated chloroplasts [91]. Additionally pulse chase labeling of leek seedlings suggested that only the *sn*-1 acyl-chain from PC was transferred to MGDG and DGDG [92, 93]. However the extremely long time points of these experiments (24 hr increments) make it difficult to interpret the

labeling data in relation to the demonstrated rapid transfer of PC to MDGD in other species [72, 86-88]. The transfer lipid may not have to traverse the hydrophilic cytosol at all, but may travel through an ER plastid connection site. Putative connection sites termed plastid associated membranes (PLAMs) containing ER enzymatic activities have been found associated with isolated chloroplasts [94]. Strong physical connections between the ER and chloroplasts have been demonstrated *in vitro* utilizing optical tweezers [95]. Connection sites may allow direct transfer of lipid molecules between the ER and plastid membrane systems as has been demonstrated for ER-mitochondria connection sites in rat liver and yeast [96, 97]. The connection site hypothesis, as opposed to the lyso-PC hypothesis, could also give an explanation of how glycolipids are exported from the plastid under times of phosphate stress [80, 98], and may also be involved in the export of newly synthesized FA to the eukaryotic pathway acyltransferases.

1.4 Long chain acyl-CoA pools for building glycerolipids

Eukaryotic pathway acyl transferases utilize Coenzyme A (CoA) activated acyl groups to drive the esterification of FA to a glycerol backbone. The composition of the FA esterified to CoA (e.g. 16:0, 18:3, etc) and acyltransferase selectivity can affect the molecular species of lipids produced. Acyl-CoAs do not freely diffuse across membranes and transport may be an active process as in mammalian mitochondria [99]. Production of acyl-CoAs from FFA by LACS in

different subcellular compartments may generate multiple pools of acyl groups with different compositions within the cell for different acyltransferase reactions. LACS activity has been shown to take place in the ER [100], chloroplasts [77, 78], mitochondria [101], peroxisomes [102], and lipid bodies [103]. In Arabidopsis LACS is encoded by a family of nine genes [104] of which two are involved in activation of acyl groups for beta-oxidation in peroxisomes [105], at least two located at the plastid envelope are candidates for involvement in export of acyl groups from the plastid to the eukaryotic pathway [106], and one has been shown to be very important for cuticle development [107]. The widespread localization of LACS throughout the cell demonstrates that FFA are generated throughout the cell, almost certainly by lipase activities, and the reutilization of FA by acyltransferases requires the activation to acyl-CoAs. The multiple sites of acyl-CoA synthesis may also be a mechanism for controlling the acyl composition of multiple subpools of acyl-CoA for production of specific lipid molecular species, such as highly saturated or polyunsaturated TAG species.

There is evidence that acyl-CoAs involved in lipid metabolism are not freely soluble or present in membrane bound pools but are bound to acyl-CoA binding proteins (ACBP) that deliver the activated acyl groups to acyltransferases. ACBP have been found in all four eukaryotic kingdoms and some bacteria [108]. ACBP are expressed in all plant organs [109]. High levels of ACBP accumulate in oilseeds during oil deposition [110], suggesting their role in TAG synthesis. Arabidopsis contains two ACBPs that are predicted as cytosolic [111], two ACBPs have been localized to the plasma membrane [112, 113], and

one is targeted to the outside of the cell [114]. Much like the LACS proteins, the wide distribution of ACBP suggests multiple roles in lipid metabolism. RNAi knockdown of a membrane associated ACBP in Arabidopsis increased the 18:2/18:3 ratio of phospholipids [109]. Since acyl chains are desaturated while bound to PC the knockdown of ACBP probably did not affect the desaturation of 18:2. Reduced ACBP levels may affect the delivery of recycled acyl-CoA to acyltransferases after removal of desaturated FA from PC and reactivation to acyl-CoA.

1.5 Synthesis of eukaryotic membrane lipid classes

Leaf glycerolipids are primarily made up of polar membrane lipids. Membrane lipid synthesis starts with PA from *de novo* glycerolipid synthesis (Figure 1-1) and follows one of two routes for acquiring the polar headgroup. (1) Nucleotide-activated head group dependent *sn*-3 esterification of DAG produced from PA by a phosphatase. Cytidine 5'-diphoshphate (CDP) activated choline or ethanolamine is used for PC and PE synthesis and uridine 5'-diphosphate (UDP) activated sugars are utilized in monogalactosyldiacylglycerol (MGDG) and sulfolipid (SL) synthesis [34]. A second galactose can be added to MGDG by reaction of UDP-galactose with the 6-hydoxyl of the MGDG headgroup to produce digalactosyldiacylgycerol (DGDG) [115]. (2) PA is reacted with cytidine 5'-triphosphate to make CDP-DAG. The activated CDP-DAG is then reacted with serine or myo-inositol to produce PS or PI, respectively. PG is synthesized by the reaction of CDP-DAG with G3P, producing first phosphatidylglycerol-phosphate,

and then PG by a phosphatase reaction [34]. Subcellular localization of the enzymes responsible for headgroup addition defines where each lipid is produced. PC, PE, PS, and PI are produced from DAG and PA in the ER [34] while MGDG, DGDG, SL and most of PG are produced within the plastid envelope membranes [73, 81].

1.6 TAG synthesis

The traditional view of TAG synthesis (aka Kennedy Pathway) starts with two consecutive acylations of G3P by the eukaryotic pathway producing PA (Figure 1-2). PA dephosphorylation to form DAG and addition of a third acyl group to the *sn*-3 position of the glycerol backbone by a diacylglycerol acyltransferase (DGAT), produces TAG and completes the Kennedy Pathway [116]. Since TAG is not a substrate for FA desaturation, the acyl-CoA pool that feeds TAG synthesis acyltransferases must contain all the acyl groups found in TAG. Alternatively, the DAG moiety for TAG synthesis can be produced from PC by the reverse reaction of CDP-choline:diacylglycerol cholinephosphotransferase (CPT) [16, 117, 118]. As with the Kennedy pathway final TAG synthesis can be through an acyl-CoA dependent DGAT. Additionally, an acyl-CoA independent mechanism can directly transfer an acyl group from the *sn*-2 position of PC to

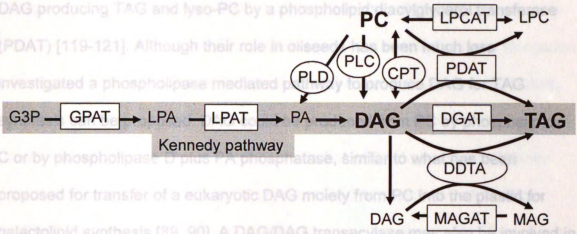

Figure 1-2 Possible enzymatic reactions leading to TAG synthesis in oilseeds

Reactions that may be involved in TAG synthesis as mentioned in the text. Substrates are as text, enzymes are enclosed in boxes. Acyl-CoA pools and how they are generated are not shown. Enzymes utilizing acyl-CoA as an additional substrate are rectangles all other enzymes are in ovals. The traditional Kennedy pathway is highlighted in gray. TAG, DAG and PC are bold because of their central importance in TAG synthesis. Substrates: G3P, glycerol-3-phosphate; LPA, lyso-phosphatidic acid; PA, phosphatidic acid; DAG, diacylglycerol; PC, phophatidylcholine; LPC, lyso-phosphatidylcholine; MAG, monoacylglycerol; TAG, triacylglycerol. Enzymes: GPAT, G3P acyltransferase; LPAT, LPA acyltransferase; LPCAT, LPC acyltransferase; PDAT, phospholipid:diacylglycerol acyltransferase; DGAT, diacylglycerol acyltransferase; MAGAT, monoacylglycerol acyltransferase; DDTA, DAG/DAG trans-acylase; PLD, phospholipase D: PLC, phospholipase C; CPT, diacylglycerol:CDP-choline cholinephosphotransferase.

DAG producing TAG and lyso-PC by a phospholipid:diacylglycerol transferase (PDAT) [119-121]. Although their role in oilseeds has been much less investigated a phospholipase mediated pathway to produce DAG for TAG synthesis can be proposed. DAG could be produced from PC by phospholipase C or by phospholipase D plus PA phosphatase, similar to what has been proposed for transfer of a eukaryotic DAG moiety from PC into the plastid for galactolipid synthesis [89, 90]. A DAG/DAG transacylase may also be involved in TAG synthesis [122] and could transfer FA from the *sn*-2 position of one DAG to the *sn*-3 of another, producing TAG and a *sn*-1-acyl-monoacylglycerol (MAG). Re-acylation of MAG by a monoacylglycerol-acyltransferase (MAGAT) would generate a new DAG for TAG synthesis. Despite the multiple pathways that could be involved in TAG synthesis as indicated by their *in vitro* enzymatic activities, only the DGAT reaction has been conclusively shown to be involved in TAG synthesis *in vivo* [123, 124]. Further knowledge of the actual enzymes and pathways that lead to TAG accumulation will allow better strategies for engineering of TAG quantity and/or FA composition.

1.6.1 Acyl transfer between lipids is required for a majority of TAG synthesis

Synthesis of polyunsaturated TAG molecular species requires significant transfer of acyl chains between lipid species. Phosphatidylcholine is the major site of FA desaturation for membrane lipids and TAG [116], and thus the modified acyl groups need to be removed for incorporation into TAG (at least for

incorporation into the sn-3 position). Additionally, production of many very long chain (>18C) polyunsaturated FA requires desaturation on PC prior to elongation and incorporation into TAG [125]. Desaturated FA may be removed by lipases, reactivated to acyl-CoA and thus be utilized by the Kennedy pathway acyl transferases to acylate the *sn*-1, -2, or -3 position of TAG glycerol backbone. Alternatively, the reverse CPT mechanism of DAG formation is one possible method for incorporation of desaturated acyl chains into the *sn*-1/*sn*-2 positions TAG [16, 117, 118]. The relative amount of Kennedy pathway or reverse CPT used to produce TAG in oilseeds is unknown. It has been suggested from labeling of oilseed species that accumulate PUFA in TAG, that reverse CPT may be a mechanism for channeling desaturated FA from PC into TAG [126, 127]. Further utilization of a PDAT that specifically transfers PUFA from *sn*-2 PC into the *sn*-3 position of TAG [120] may complete a mechanism to specifically produce tri-PUFA molecular species of TAG.

Many plants accumulate unusual FA in seed oil and these unusual FA are mostly excluded from membrane lipids even though most acyl chain modification takes place on the membrane lipid bound acyl chain [128]. An example is ricinolelic acid (12-hydroxyoctadec-9-enoate) which is produced in castor beans (*Ricinus communis*) on PC [129, 130] and accumulates up to 90% of FA in seed oil but is mostly excluded from bulk PC. The exact mechanisms that exclude these unusual FA from membrane lipids and segregate them into TAGs are unknown but identification of unusual FA specific lipases [131] suggest that they may be selectively removed from PC and channeled into TAG through the

Kennedy pathway [17, 116]. In transgenic plants that produce unusual FA in a non native plant species the unusual FA tend accumulate in PC in addition to TAG suggesting that the mechanisms for selective removal of the unusual FA from membrane lipids and incorporation into TAG are not available in the transgenic plant [128]. The final step in TAG production may be provided by a DGAT that is specific for the unusual FA, as demonstrated by the DGAT2 enzymes of castor and tung that appear to preferentially insert ricinoleic and eleostearic acid, respectively into the *sn*-3 position of DAG [132, 133].

It is well documented that in oilseeds it is very important for the plant to be able to transfer unusual modified FA from the site of synthesis in PC to the TAG molecule where the modified FA accumulates. The transfer mechanisms and enzymes could be the same or very similar to those for common desaturated FA. Much of the oilseed data suggests that these deacylation-reacylation enzymatic activities are specific for channeling unusual modified FA into TAG. However, a large number of genes in the *Arabidopsis* genome are annotated as acyltransferases and phospholipases with unknown metabolic function [134], therefore it is possible that similar deacylation-reacylation reactions with common FA may have a more central role in lipid metabolism.

1.7 Suggestive evidence for acyl editing in plants

Maintenance and fine tuning of the fatty acid composition of phospholipid membranes appears to be essential in all organisms. Acyl editing also termed

"remodeling" or "retailoring" is one mechanism to achieve this. Acyl editing involves the deacylation and reacylation of glycerolipids but does not by itself result in net lipid synthesis. Acyl editing was first recognized as a significant pathway of lipid metabolism in animal tissues [135]. In mammalian brain tissue in which phospholipid metabolism consumes 20% of cellular ATP [136], a quarter of that is used for the deacylation-reacylation of phospholipids [137]. Evidence for acyl editing has also been found in prokaryotes and yeast [138, 139].

In plants, acyl editing has not been previously studied as an integral part of central lipid metabolism but there is ample suggestive evidence that it is involved. The acyl editing reaction for phospholipids such as PC can take place through two general mechanisms. The first mechanism involves deacylation by exchange of acyl groups from PC directly onto CoA producing acyl-CoA and lyso-PC (CoA:PC acyl exchange). Deacylation by CoA:PC acyl exchange has been demonstrated in developing safflower cotyledon microsomes, and was attributed to the reverse reaction of lyso-phosphatidylcholine acyltransferase (LPCAT) [140]. LPCAT activity has been extensively reported in isolated plant microsomes [141-144] and chloroplasts [91, 94, 145]. When reported, the specificity of LPCAT activity is mostly at the *sn*-2 position of PC. The identification of LPCAT activities in plant organelles presents the possibility for CoA:PC acyl exchange as an acyl-editing mechanism but does not indicate how prevalent the reverse LPCAT activity is *in vivo*. The second possible mechanism for acyl editing involves deacylation by hydrolytic cleavage of PC by a phospholipase generating a FFA and a Lyso-PC. To complete the acyl editing

cycle the FA must be activated to acyl-CoA by LACS and PC is regenerated by LPCAT activity. It is possible that both acyl chains could be removed by lipases generating a glycerophosphocholine that could be reacylated to PC. However glycerophosphocholine dependent acyl transferases have not been reported in plants. Many plant phospholipases have been identified that could be involved in an acyl editing cycle, of those the majority are PLA_2, which remove acyl chains from the *sn*-2 position [146]. Many of these PLA_2s are specific for unusual FA produced in oilseeds [131]. Removal of acyl chains from the *sn*-1 position has been less prevalent in the plant literature but there are a few examples [147, 148]. Just as for the CoA:PC acyl exchange mechanism, evidence of lipase enzymatic activity that could be involved in acyl editing is not evidence of an actual acyl editing function *in vivo*.

More substantial evidence that acyl editing is involved in central lipid metabolism has come from multiple isotopic labeling studies. Isolated chloroplasts from pea plants could incorporate newly synthesized [^{14}C]acetate labeled FA into PC through a channeled pool of acyl-CoA [79]. Since chloroplasts do not contain the eukaryotic pathway for *de novo* PC synthesis this result suggests an acyl editing mechanism that can incorporate newly synthesized FA into previously synthesized PC in the outer chloroplast membrane or an attached PLAM region. Additionally, oxygen-18 labeling of the carbonyl oxygen of newly synthesized FA demonstrated that the entire pool of PC acyl chains in spinach leaves is under a substantial flux of hydrolytic deacylation and reacylation [74]. This hydrolytic turnover of PC acyl chains occurs at a rate similar to the overall

rate of FAS, and therefore may play a major role in phospholipid metabolism. Additional insightful evidence on acyl editing came from careful comparison of prokaryotic MGDG and eukaryotic PC molecular species after labeling leaf disks of the 16:3 plant *Brassica napus* with [^{14}C]carbon dioxide. MGDG contained the predicted prokaryotic pathway initial molecular species of dual labeled 18:1/16:0. The eukaryotic pathway predicts the major initial PC molecular species to be dual labeled 18:1/18:1. However carbon dioxide pulse-chase labeling produced PC with a large amount of scrambling of labeled FA with unlabeled FA. The authors concluded that the acyl chains of the PC pool are under constant exchange directly after *de novo* PC synthesis and during their prolonged 48 hr chase period [149]. These three lines of evidence suggest that the plant PC pool is under a deacylation-reacylation flux, so that the acyl groups esterified to G3P by the eukaryotic pathway and incorporated into PC are not a permanent fixture of PC. However, how acyl editing is tied in with central membrane and storage lipid synthesis is unknown.

1.8 The *in vivo* labeling approach

Modern biological science benefits from various approaches to study the metabolic pathways of life, each with its own strengths and weaknesses. The pathways of plant lipid metabolism were originally elucidated by two approaches: 1) Discovery of enzymatic activities through *in vitro* assays of plant extracts, 2) step by step pathway development through *in vivo* labeling. Through these

approaches the two pathway hypothesis for *de novo* glycerolipid synthesis was discovered over 25 years ago and it is still valid today [71]. Since that time the advent of genetic and molecular biology approaches has unleashed very powerful tools for discovery of individual genes/enzymes involved in metabolic pathways through mutational analysis. Additionally, the ability to clone and overexpress genes has greatly increased the ability to study the effects of changing one enzyme on metabolism *in vivo* and to purify enzymes for *in vitro* assays. However, the redundancy of genes and metabolic pathways, the lethality of certain gene knockouts, and the ability for plant metabolism to adapt to some changes, can mean that key points of metabolism may not be revealed by genetic or molecular biology techniques alone. Additionally, *in vitro* assays identify what reactions are present but usually cannot determine their relative contributions to metabolic fluxes *in vivo*. Recently, metabolic profiling techniques have allowed us to study differences in metabolism due to different growth, environmental or genetic changes. However, analysis of bulk pools of metabolites may not reveal subcellular compartmentation, metabolic cycles or rapid conversions of one metabolite to another.

In vivo labeling approaches are still the only way to study the kinetics of metabolites moving through pathways *in vivo* and the relative contributions of parallel or convergent pathways. Recently the *in vivo* labeling approaches have been reinvigorated with the use of stable isotope labeling in conjunction with mass spectrometry, nuclear magnetic resonance (NMR) and sophisticated software for analysis. Stable isotope labeling over prolonged periods and

analysis of the composition of the label in metabolic end products (e.g. membrane and storage lipids, starch and cell walls, and proteins) allows a steady-state determination of the relative fluxes through different known metabolic pathways. Steady-state labeling that does not fit known metabolic pathways can suggest areas of uncertainty in the pathway that need to be revised [150]. The sensitivity of mass spectrometry and NMR often limit stable isotope labeling to prolonged periods and analysis of steady-state metabolism. *In vivo* labeling with radioisotopes is usually the most sensitive method for detecting small amounts of labeled compounds as they move through metabolic pathways [151]. Rapid dynamic labeling with radioisotopes, as opposed to long term steady-state labeling, involves introducing a labeled compound to the plant or tissue and rapidly stopping metabolism to determine how the labeled compound has been metabolized. Multiple labelings of different time periods allows us to generate kinetic curves for accumulation of label overtime in different metabolic products. Metabolic pathway precursor-product relationships can then be determined by the relative time it takes for each metabolite to accumulate label linearly, which corresponds with the complete labeling of all precursor pools.

The research in this dissertation utilizes *in vivo* dynamic radiolabeling to investigate if acyl editing is involved in central lipid metabolism. The textbook eukaryotic pathway hypothesizes that newly synthesized FA are exported from the plastid, activated to acyl-CoA, and esterifed to G3P producing initial PA molecular species of 18:1/18:1 and 16:0/18:1. The PA is then converted to PC for further desaturation of the nascent FA. Additionally, the initial PA may be used by

the Kennedy pathway to produce TAG in oilseed tissue. However, evidence that nascent FA are channeled directly into PC in isolated chloroplast [79] through a possible acyl editing mechanism suggest that there may be alternative mechanisms at work for incorporation of newly synthesized FA into membrane lipids. Additionally, some unusual FA produced in the plastid that are not further modified on PC are transiently incorporated into PC prior to accumulation in TAG [152, 153], suggesting there may also be an alternative to direct plastid export of acyl groups into the Kennedy pathway for TAG synthesis. The goals of this dissertation have been to test the glycerolipid synthesis mechanisms *in vivo*. The next two chapters utilize *in vivo* kinetic labeling techniques to investigate the initial events by which newly synthesized FA and the glycerol backbone are incorporated into eukaryotic membrane and storage lipids. The results in pea leaves and in developing soybean embryos demonstrate that the initial flux of FA into glycerolipids occurs through PC acyl editing and that this pathway should be considered a major part of eukaryotic glycerolipid synthesis.

2 INCORPORATION OF NEWLY-SYNTHESIZED FATTY ACIDS INTO CYTOSOLIC GLYCEROLIPIDS IN PEA LEAVES OCCURS VIA ACYL EDITING

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2.1 Abstract

In expanding pea leaves over 95% of fatty acids (FA) synthesized in the plastid are exported for assembly of eukaryotic glycerolipids. It is often assumed that the major products of plastid FA synthesis (18:1 and 16:0) are first incorporated into 16:0/18:1 and 18:1/18:1 molecular species of phosphatidic acid (PA) which are then converted to phosphatidylcholine (PC), the major eukaryotic phospholipid and site of acyl desaturation. However, by labeling lipids of pea leaves with [^{14}C]acetate, [^{14}C]glycerol and [^{14}C]carbon dioxide we demonstrate that acyl editing is an integral component of eukaryotic glycerolipid synthesis. First, no precursor-product relationship between PA and PC [^{14}C]acyl chains was observed at very early time points. Second, analysis of PC molecular species at these early time points showed that >90% of newly synthesized [^{14}C]18:1 and [^{14}C]16:0 acyl groups were incorporated into PC alongside a previously synthesized unlabeled acyl group (18:2, 18:3 or 16:0). And third, [^{14}C]glycerol labeling produced PC molecular species highly enriched with 18:2, 18:3 and 16:0 FA, and not 18:1, the major product of plastid fatty acid synthesis. In conclusion, we propose that most newly synthesized acyl groups are not immediately utilized for PA synthesis, but instead are incorporated directly into PC through an acyl editing mechanism that operates at both *sn*-1 and *sn*-2 positions. Additionally, the acyl groups removed by acyl-editing are largely used for the net synthesis of PC through glycerol-3-phosphate acylation.

2.2 Introduction

In plants acyl carrier protein (ACP)-dependent *de novo* fatty acid synthesis (FAS) is restricted to organelles [28]. Essentially all acyl chains for membrane and storage lipid synthesis are produced in the plastid [30, 34]. The initial products of FAS, 16- and 18-carbon fatty acids (FAs) esterified to ACP, are incorporated into glycerolipids by one of two routes: (i) acyl-ACP is used directly by acyltransferases of the "prokaryotic" pathway within the plastid, or (ii) the acyl-ACP thioester bond is hydrolyzed during acyl export from the plastid prior to FA reactivation and incorporation into glycerolipids by acyltransferases of the "eukaryotic" pathway outside the plastid. This two pathway mechanism for glycerolipid synthesis was first elucidated by radio-tracer studies [71], and was confirmed genetically by analysis of *Arabidopsis* mutants [34, 154]. The proportions of nascent FA (i.e. immediate products of FAS) incorporated into the eukaryotic and prokaryotic pathways vary widely among plants and different tissues within the same plant. The eukaryotic pathway predominates in non-photosynthetic tissues of all higher plants. In 18:3 plants, so called because they accumulate predominantly 18:3 and not 16:3 in their leaf galactolipids, 95% of the FA produced in the plastid is exported for assembly into glycerolipids by the eukaryotic pathway. Only phosphatidyl-glycerol (PG) is synthesized directly in the plastid from acyl-ACPs. This is in contrast to leaves of 16:3 plants which can also synthesize glycolipids by the prokaryotic pathway. Consequently, only about 60% of FA produced in the chloroplast by 16:3 plants is exported to the eukaryotic

pathway [72]. 18:3 plants, which include pea, make up about 88% of angiosperm species [82].

It is generally assumed that the major exported products of chloroplast FAS, namely 18:1 and 16:0 FA, are transferred to the outer envelope of the plastid where they are activated to acyl-CoAs by a long chain acyl-CoA synthetase [77, 78]. The eukaryotic pathway for *de novo* PC synthesis then utilizes this pool of newly synthesized acyl-CoAs for sequential *sn*-1 and *sn*-2 acylations of glycerol-3-phosphate to produce 18:1/18:1 and 16:0/18:1 molecular species of phosphatidic acid (PA). PA is rapidly converted to phosphatidylcholine (PC) by the action of PA phosphatase and CDP-choline:1,2-diacyl-*sn*-glycerol cholinephospho-transferase [34]. Desaturation of 18:1 to 18:2 and then 18:3 on PC produces the abundant polyunsaturated molecular species of PC [36, 37]. However, several lines of evidence suggest that this model may be inadequate and needs to take account of acyl editing. We define acyl editing, often also termed "remodeling" or "re-tailoring," as any process that exchanges acyl groups between polar lipids but which does not by itself result in the net synthesis of the polar lipids. Acyl editing has long been considered an important facet of phospholipid metabolism [135]. Acyl editing relevant to this work can occur through two mechanisms. In plants acyl editing via a CoA:PC acyl exchange mechanism was demonstrated in microsomes isolated from developing seeds and was attributed to a reverse reaction of lyso-phosphatidylcholine acyl-transferase (LPCAT) [140]. LPCAT activity has also been described in isolated chloroplasts [91, 94, 145] as well as microsomes [141-144]. Thus LPCAT allows

for a mechanism for acyl editing, although the *in vitro* results do not indicate how prevalent the reaction might be *in vivo*. In this context, isolated pea chloroplasts incubated with [^{14}C]acetate immediately label PC with newly synthesized FA through a channeled pool of acyl-CoA [79]. As chloroplasts cannot synthesize PC *de novo*, this indicated a functional mechanism for PC synthesis with nascent FA through an acyl editing mechanism. A second mechanism for acyl editing involves hydrolysis of the phospholipid, such as PC to lyso-PC or even to glycerolphosphorylcholine (GPC), activation of the released free fatty acid and its re-utilization for phospholipid synthesis from lyso-PC or GPC. Based on ^{18}O labeling there is some indication that acyl-chains esterified to bulk cellular PC are under a constant flux of acyl editing that proceeds wholly or in part through a hydrolytic deacylation- reacylation cycle [74].

The most direct line of evidence of acyl editing in plants comes from a careful analysis of the molecular species of monogalactosyl-diacylglycerol and PC after labeling leaf disks of the 16:3 plant *Brassica napus* with carbon dioxide [149]. [^{14}C]Carbon dioxide labeling produced initial acyl-labeled species as expected for prokaryotic monogalactosyldiacylglycerol, namely dual labeled 16:0/18:1. By contrast for PC a high degree of scrambling between labeled and unlabeled acyl chains was noted. The authors concluded that there was continuous exchange of acyl groups between all molecular species of PC immediately after labeling and during the prolonged pulse-chase period of 48 hours. In this paper we augment and extend these important observations and conclusions in several ways:

(1) We perform rapid kinetic studies to more carefully address PC labeling and that of its precursors, PA and 1,2-diacyl-*sn*-glycerol (DAG). We address whether the initial incorporation of nascent fatty acids occurs via acyl editing, or if there is a rapid incorporation by *de novo* PC synthesis via glycerol-3-P (G3P) and DAG, which is followed by rapid acyl editing of PC.

(2) We perform both total molecular species and stereochemical analyses on acyl-labeled PC using *in vivo* experiments with expanding pea leaves and seedlings. Pea is an 18:3 plant, so this complements the analysis done previously with the 16:3 plant *B. napus* [149].

(3) We track molecular species of PC labeled in the glycerol backbone. When combined with the analysis of acyl labeling, this allows us to propose that *sn*-1 acyl editing is as important a component as *sn*-2 acyl editing.

(4) As a control, we perform rapid labeling experiments *in planta* using carbon dioxide. The results parallel those obtained with excised tissue assays, indicating that there are no wound responses that compromise the metabolic conclusions obtained from excised tissue experiments.

From these studies we analyze possible models by which newly synthesized FA are incorporated into eukaryotic lipids. The data presented in this paper and from other studies do not allow us to unambiguously define one particular model, but do narrow down the possible routes in which nascent FA are incorporated into eukaryotic glycerolipids and suggest future directions to re-examine this important yet poorly understood area of plant lipid metabolism.

2.3 Experimental procedures

Plant Materials—All experiments were performed with Garden pea (Pisum *sativum* L. cv Little marvel) grown in soil/perlite/vermiculite (1:1:1) mixture at 22-25 °C under a day/night 8/16-h photoperiod of white light at 185-210 μmol m^{-2} s^{-1}. Leaves, or in some cases, whole seedlings were harvested 8 days after sowing.

Radiochemicals—[1-^{14}C]Acetic acid, sodium salt (specific activity 50 mCi/mmol), [^{14}C(U)]glycerol (specific activity 150 mCi/mmol), and [^{14}C]sodium bicarbonate (specific activity 50 mCi/mmol) were from American Radiolabeled Chemicals, Inc. (St. Louis, MO).

Leaf [^{14}C]Acetate or [^{14}C]Glycerol Labeling—Leaf labeling experiments used ~ 0.3 g fresh weight of pea leaf strips per assay, incubated in the light (180-220 μmol m^{-2} s^{-1}) at 22-24 °C with reciprocal shaking in 5 ml media containing 20 mM MES pH 5.5, 0.1x MS salts, and 0.01% Tween-20. Cut leaves were placed in media without radioisotope and pre-incubated in ambient light for 5 min. Labeling was started by the addition of radioactive substrate and strong illumination. The reaction was quenched by transfer of leaves into isopropanol at 80 °C for 10 min. For labeling of acyl groups 250 μCi of [1-^{14}C]acetic acid (1.0 mM) was used per replicate. For [^{14}C]glycerol labeling of PC molecular species each incubation contained 47 μM [^{14}C(U)]glycerol (39 μCi). For [^{14}C]glycerol time course the labeling media contained 25 μM [^{14}C(U)]glycerol (18 μCi) and the media was reused for consecutive 1, 3, 6 and 9 min time points. *In vivo* labeling with excised plant tissue can produce considerable variance between samples due to

differences in development and in uptake of substrate. To minimize such variance each data point for total incorporation into lipids was normalized against the trend line for all time points to allow improved kinetic plots (Figures 2-1 and 2-3).

Seedling [^{14}C]Carbon Dioxide Labeling— Assays were conducted with 8-day potted pea seedlings in a closed 9.5 L glass desiccator under 250 μmol m^{-2} s^{-1}white light. [^{14}C]CO_2 was released by injection of 1 ml H_2SO_4 through the desiccator lid into a vial containing aqueous [^{14}C]$NaHCO_3$. The head space of the vial was quickly flushed with 30 ml air and the desiccator sealed for the desired time. Labeling was stopped by removal of the shoots at the base of the first leaves and quenching in 80 °C isopropanol. The assay used ~ 700 μCi substrate and ~ 40 seedlings. The seedlings from each labeling were split into two replicate samples (~ 20 seedlings each) for analysis. Although each labeling was nominally for 5 min, about 5 min was required to remove all the seedlings and quench, so the assay is reported as of 5-10 minutes duration.

General Methods—Lipids were extracted from hot isopropanol quenched tissue with hexane/isopropanol [155] after homogenization with a mortar and pestle. Chlorophyll was determined spectrophotometrically at 652 nm in 20% aqueous acetone [156]. Radioactivity in the total lipid samples, eluted lipids or organic and aqueous phases recovered from transmethylation was quantified by liquid scintillation counting (Beckman Instrument Inc., Fullerton, CA), while radioactivity on TLC plates was visualized and quantified by electronic radiography (Packard Instrument Co., Meriden, CT). $AgNO_3$-TLC plates were

prepared by impregnating Partisil® K6 silica gel 60 Å TLC plates (Whatman, Maidstone, UK) with 10% $AgNO_3$ in acetonitrile (w/v), drying in air and activating at 110 °C for 5 min. Fatty acid methyl esters (FAMEs) were quantified by gas chromatography (GC) using a flame ionization detection and a DB-23 capillary column (30 m length x 0.25 mm inner diameter, 0.25 μ film thickness; J&W).

Lipid Class Analysis—For kinetic analyses polar lipids were separated on a K6 silica TLC plates impregnated with 0.15 M ammonium sulfate and heat activated (110 °C for 3 hr) prior to lipid loading. Plates were developed in acetone/toluene/water (91:30:8, v/v/v) [157]. DAG was analyzed by first acetylating an aliquot of the total lipids in acetic anhydride/pyridine, (3:2, v/v) and then separating the 1,2-diacyl-3-acetyl-glycerols by silica TLC developed in hexane/diethyl ether/acetic acid (50:50:1, v/v/v). PA was isolated by a first preparative silica TLC separation using a K6 plate developed in chloroform/methanol/water (65:25:4, v/v/v), and further purified on a second K6 plate developed in chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, v/v/v/v/v). For preparative TLC all solvents contained 0.01% butylated hydroxyl-toluene antioxidant. Lipids were eluted from TLC silica with chloroform/methanol/water (5:5:1, v/v/v). Chloroform and 0.88% aq. KCl were added to give chloroform/methanol/water ratios of 2:1:1, (v/v/v), resulting in a phase separation. The aqueous phase was back extracted with chloroform and lipids were recovered from the combined chloroform phases. For [^{14}C]glycerol and [^{14}C]carbon dioxide labeled lipids the proportion of label in the acyl groups versus the backbone/head group was determined by transmethylation [158] and

scintillation counting of the separated organic and aqueous phases. Aqueous phase radioactivity from PC was determined to be in the glycerol backbone by silica TLC developed in methanol/water/28% NH_4OH/3M NaCl (50:26.6:17.9:3.4, v/v/v/v), as sample radioactivity co-migrated with glycerol and glycerol-3-phosphate standards, but not choline.

Radiolabeled Acyl Group Composition—FAMEs were prepared from purified individual lipids or total lipids by base-catalyzed transmethylation [158]. Recovered FAMEs were separated based on the number of double bonds by $AgNO_3$-TLC, the plates being developed to ¾ height with hexane/diethyl ether (1/1, v/v), then fully with hexane/diethyl ether (9:1, v/v).

PC Molecular Species Determination—PC was separated from other lipids by silica TLC (K6 plates developed with chloroform/methanol/acetic acid (75:25:8, v/v/v)). DAG was produced from the purified PC by phospholipase C (*B. cereus*, Sigma) digestion [159]. The DAG was acetylated as described above and the 1,2-diacyl-3-acetyl-glycerols separated into molecular species based on the number of double bonds by argentation-TLC [159], using a triple development (½ then ¾ development in chloroform/methanol (96:4, v/v), then fully in chloroform/methanol (99:1, v/v)). The proportion of radioactivity in each band was determined by electronic autoradiography, then each band was eluted, the recovered lipids transmethylated, and the [^{14}C]FAME analyzed by $AgNO_3$-TLC, as described above. To determine endogenous acyl groups from isolated PC molecular species, triheptadecanoin was added as an internal standard to each fraction during elution and prior to transmethylation and GC analysis of

FAME. When necessary 1,2-diacyl-3-acetyl-glycerol fractions recovered from $AgNO_3$ TLC plates were further purified by reverse phase TLC on Partisil® KC18 silica gel 60 Å plates (Whatman, Maidstone, UK) developed with methanol/acetone/water (75:25:2, v/v/v).

PC Acyl Group Stereochemistry—PC was isolated as described above and digested with phospholipase A_2 (*Crotalus atrox*, Sigma) [159]. Briefly, PC was dissolved in 1 ml diethyl ether and 0.5 unit PLA_2 in 0.1 ml of 50 mM Tris-HCl pH 8.7, 5 mM $CaCl_2$. The reaction was mixed vigorously for 5 min then the ether was evaporated under N_2. To extract lipids 3.8 ml chloroform/methanol (2/1, v/v) and 1 ml 0.15 M acetic acid were added, the mixture vortexed, the chloroform phase collected and the aqueous phase back extracted with 2.5 ml chloroform. Reaction products were separated on silica TLC plates developed with chloroform/methanol/acetic acid/water (50:30:8/4, v/v/v/v). Radioactivity in the free fatty acid and lyso-PC fractions was quantified by electronic autoradiography, then each product eluted and transmethylated by heating at 50 °C in 5% sulfuric acid in methanol for 30 min. Labeled and unlabeled FAME compositions were determined as described above.

Lipid Molecular Species by Mass Spectrometry—Pea leaf samples were analyzed by ESI-MS/MS by the Kansas Lipidomics Research Center. Extraction of lipids was conducted by their standard *Arabidopsis* leaf protocol (Web site: http://www.k-state.edu/lipid/lipidomics/leaf-extraction.html). The data set from this analysis is available as a supplement in Appendix A, Figure 5-1: S1A-S1M.

Net Rate of Fatty Acid Deposition—The net rate of fatty acid synthesis by pea leaves was determined by harvesting 10 leaves from pea seedlings at the start and end of the light cycle for three days. After immediate weighing to determine fresh weight, FAME and chlorophyll contents were measured as described above. Triheptadecanoin was added during the lipid extraction as an internal standard, providing methyl heptadecanoate after transmethylation for FAME analysis by GC.

2.4 Results

Kinetics of Glycerolipid Labeling from [^{14}C]Acetate— Relationships between precursor and product pools in metabolic pathways are revealed by kinetic labeling experiments. The linear accumulation of a product, once reached, coincides with steady state labeling of all precursor pools. In this context [^{14}C]acetate is ideal for the study of acyl lipid metabolism as it is rapidly taken up by excised leaf tissue and utilized for FAS, while the lipids are labeled in their acyl groups, with minimal head group labeling [79]. The kinetics of [^{14}C]acetate labeling of glycerolipids from rapidly expanding excised pea leaves over a period of 9 minutes is shown in Figure 2-1. PC was the major radiolabeled glycerolipid with over 65% of the label at all time points. Furthermore, steady state labeling was established very rapidly, with no detectable lag (Figure 2-1A) and thus suggested that PC was a very early product of nascent FA incorporation into eukaryotic glycerolipids. A second independent 9 min time course gave similar

results (data not shown). Phosphatidylethanolamine (PE) was labeled in a similar manner (Figure 2-1C), with no obvious lag phase, but PC labeling was 15-fold greater than PE labeling despite endogenous PC levels only being twice that of PE (Appendix A, Figure 5-1:S1A).

Both the PA and the DAG pools were still filling after the onset of PC steady state labeling. The label in PA reached a maximum by 3 min whereas DAG labeling increased throughout the time course (Figure 2-1B). Thus, although PA and DAG are intermediates in the *de novo* synthesis of PC via acylation of glycerol-3-P, these [^{14}C]acyl labeled intermediates do not show a precursor-product relationship relative to PC labeling, and thus represent different glycerolipid pools. Of course, we cannot rule out that very small labeled PA and DAG pools might contribute to PC labeling in a precursor-product relationship, since experimentally, quenching in these kinetic assays limits the temporal resolution of the method. A statistical analysis suggested about 0.5 min variance (95% CI) in rate extrapolations to zero time. In conclusion, we were not able to detect a [^{14}C]acyl labeled glycerolipid precursor pool to the labeling of PC.

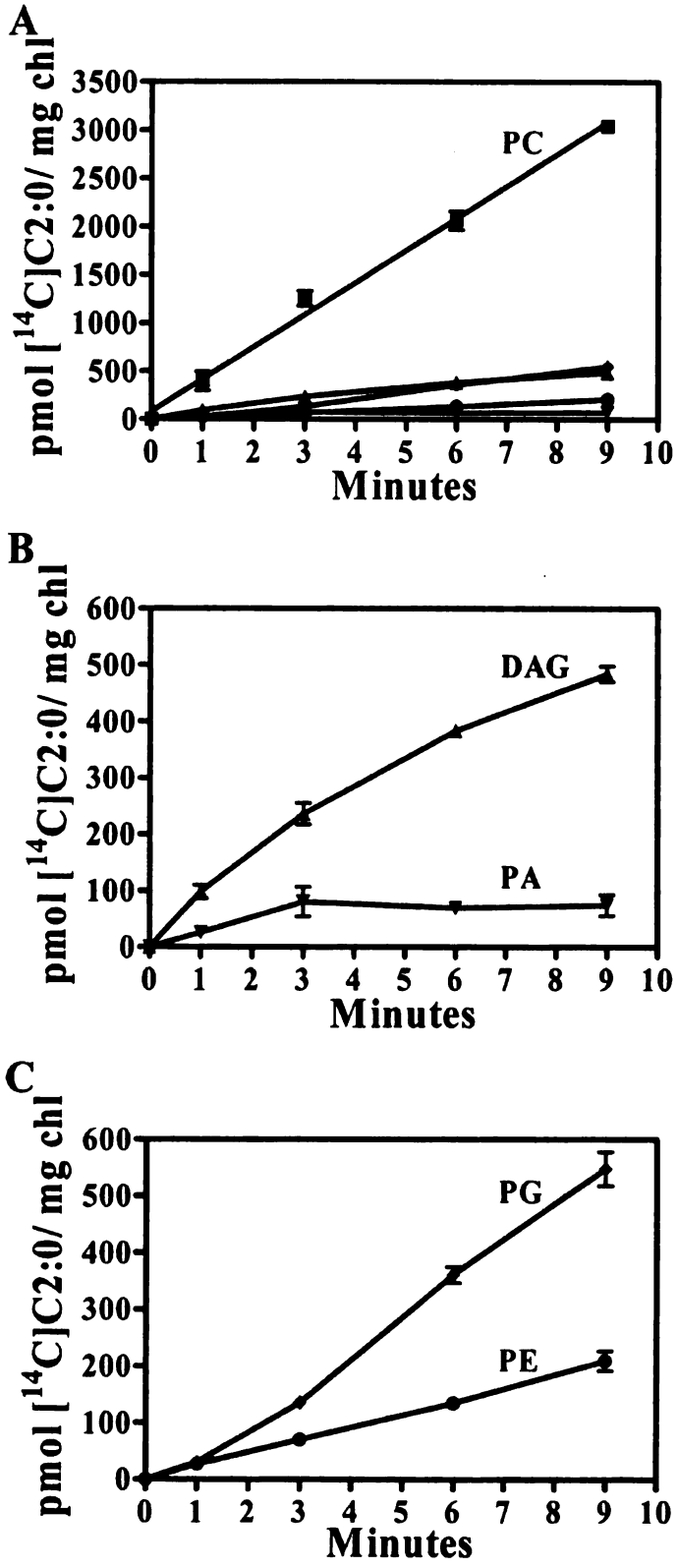

Figure 2-1 Time course for [^{14}C]acetate incorporation into the acyl groups of glycerolipid classes by excised pea leaves.

A-C: Results expressed as mean pmoles [^{14}C]acetate incorporation per mg chlorophyll (pmol [^{14}C]C2:0/ mg chl) for each lipid class ± S.E. (triplicate determinations). Glycerolipid classes are PC, squares; DAG, triangles; PA, inverted triangles; PG, diamonds; PE, circles.

Acyl Compositions from [^{14}C]Acetate Labeling of Lipids—Figure 2-2 shows the composition of labeled fatty acids from the [^{14}C]acetate time course, for total lipids, PC, PA, DAG, PG and PE. Oleate was the major product in total lipids (Figure 2-2A), with saturates, mainly palmitate, decreasing from 32% at 1 min to about 24% for the remainder of the assay period. The reason for this decline is unknown. There was no detectable desaturation of 18:1 at the earliest time point but desaturation to 18:2 was detectable from 3 min onwards. PC had less saturates (11% falling to 8%) whereas PE had more saturates (54% falling to 38%). By the end of the time course desaturation produced 7% linoleate in PC (Figure 2-2B). In contrast to PC and PE, PA (Figure 2-2C) contained high levels of saturates (>60%) throughout the time course and had an acyl composition with the closest match to PG. PA is also an intermediate of the prokaryotic glycerolipid synthesis pathway, and so the labeled PA pool is likely the precursor for prokaryotic PG labeling. In this context, close inspection of Figure 2-1B and 2-1C shows a kinetic precursor-product relationship between PA (label reaching a maximum at 3 min) and PG (lag phase ending at approximately 3 min), confirming this conclusion. DAG contained an intermediate level of saturates compared to PC and PE. The origin of the labeled DAG is not certain. It is probably largely eukaryotic in origin, and may arise in part from the reverse action of CDP-choline:DAG cholinephospho-transferase or phospholipase C on labeled PC, or from acyl groups edited from PC re-entering the eukaryotic *de novo* glycerolipid synthesis pathway. Any of these explanations is supported by

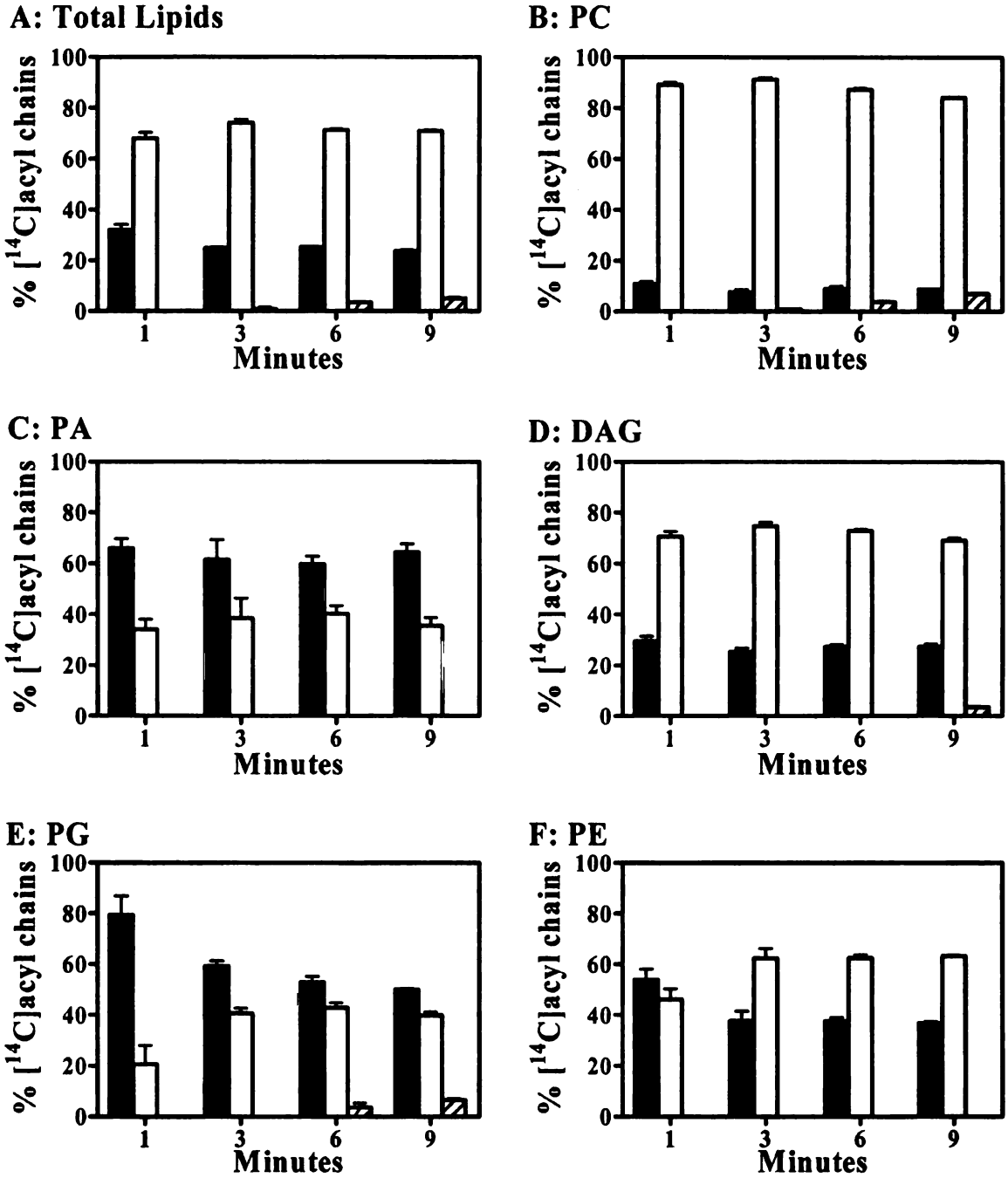

Figure 2-2 [^{14}C]Acyl composition of [^{14}C]acetate labeled lipids
Total lipids and isolated lipid classes from the time course assays run with excised pea leaves, as shown in Fig. 1, were analyzed for their labeled acyl group distribution. Results expressed as mean ± S.E. (triplicate determinations). Each bar represents the % of total lipid radioactivity present in saturates (16:0>18:0) (black bars), monoenes (18:1) (white bars), and dienes (18:2) (hatched bars). PG also contained 4% trienes (18:3) at 9 min. **A**, Total lipid FAMEs; **B**, PC; **C**, PA; **D**, DAG; **E**, PG; **F**, PE.

the appearance of labeled linoleate in DAG by 9 min (Figure 2-2D). Whatever its origin, the difference in labeled acyl group composition of PA and DAG compared to PC supports the conclusion from the kinetic data that they do not represent precursors of initial PC acyl labeling.

The Kinetics of Glycerolipid Labeling from [^{14}C]Glycerol—[^{14}C]Glycerol is rapidly taken up by pea leaves and incorporated into the glycerol backbone of glycerolipids. In addition, acyl groups also become labeled because glycerol-3-phosphate equilibrates with glycolytic precursors, leading to plastid acetyl-CoA production [86]. To separately analyze the label from the backbone/head-group and the acyl-chains, isolated lipid classes were transmethylated. Analysis of the aqueous fraction from PC by TLC indicated that the radioactivity was contained in the glycerol backbone and not the choline headgroup, as noted before [86]. In marked contrast to acetate labeling, [^{14}C]glycerol incorporation into lipids exhibited a lag (Figure 2-3A). At the earliest time points, radioactivity in DAG was approximately equal to PC. We assume that the labeled PA includes a large contribution from the plastid component. However, chloroplast lipid assembly in 18:3-plants does not require PA conversion to DAG, so we also assume the labeled DAG is largely associated with extra-plastidial membranes.

Two lines of argument support the notion that the biosynthesis of PC from labeled acetate (Figure 2-1) and from labeled glycerol (Figure 2-3) report different metabolic processes. First, for *de novo* PC synthesis the relative

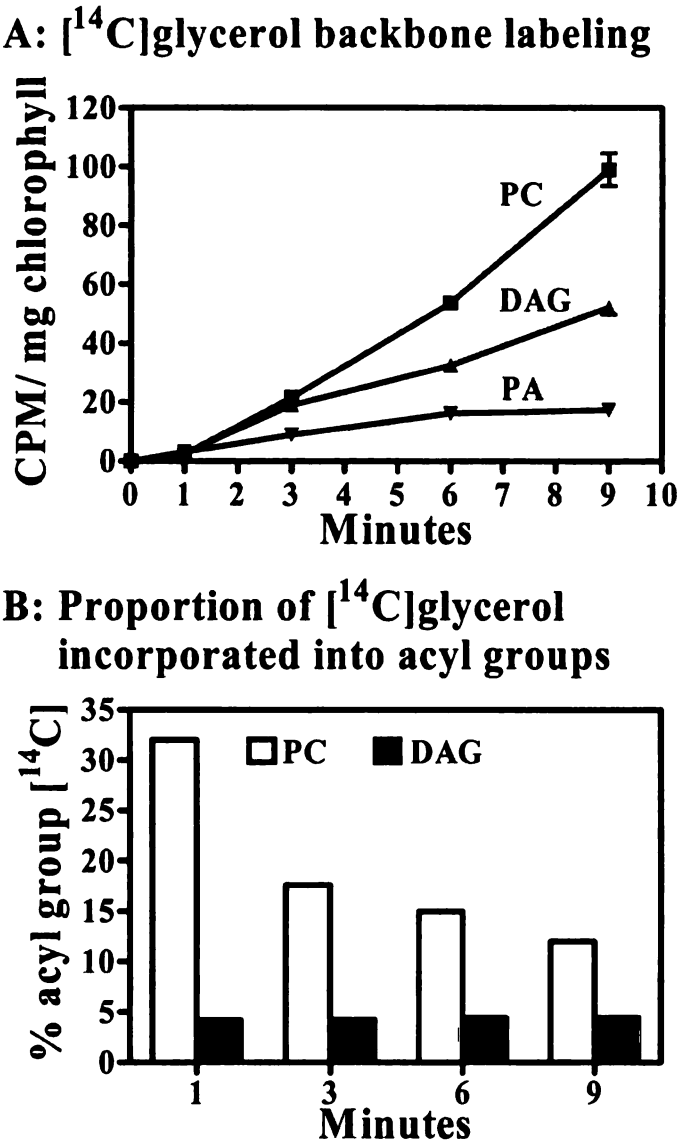

Figure 2-3 Time course for incorporation of label from [^{14}C]glycerol into the backbone and acyl groups of glycerolipids by excised pea leaves.

A: The amount of radioactivity in the glycerol backbone of each lipid. Results expressed as mean ± S.E. (triplicate determinations): PC, squares; DAG, triangles; PA, inverted triangles. **B**: The % of total lipid [^{14}C]glycerol labeling that is in the acyl chains. The three samples of PC and DAG (Figure 3A replicates) were each combined for the glycerol to acyl group determination. PC, white bars; DAG, black bars.

movement of label from PA to DAG to PC should be same for both glycerol and acyl group labeling strategies. However, at the earliest time points, it is clear that acetate acyl chain labeling produces PC >> DAG (Figure 2-1A) whereas glycerol backbone labeling produces DAG ≈ PC (Figure 2-3A), suggesting separate metabolic processes. Second, when we analyze the different kinetics of acyl chain and glycerol labeling of PC and DAG from [^{14}C]glycerol labeling (Figure 2-3B), the amount of label in the acyl chains of DAG remained fairly constant (4-5%) while the acyl label in PC fell from 32% at 1 min to 12% at 9 min. The difference is explained by postulating two different pathways for PC synthesis. About 10% of the label from exogenous glycerol is rapidly utilized for *de novo* FAS and labeled acyl groups move rapidly to PC, and then to DAG, as described for Figure 2-1. The remainder of the labeled glycerol moves through PA to DAG, which has a half life for pool filling of about 3-4 minutes, and then to PC. Under these conditions the fraction of PC which is acyl labeled is initially high but declines steadily, while this simple model predicts that the acyl labeled fraction of DAG remains low and fairly constant. This is what is observed. Together the glycerol backbone and acyl chain labeling from [^{14}C]glycerol and the [^{14}C]acetate acyl group labeling provide evidence for a separate (acyl-editing) pathway for newly synthesized FA to rapidly enter PC without *de novo* PC synthesis by the eukaryotic pathway.

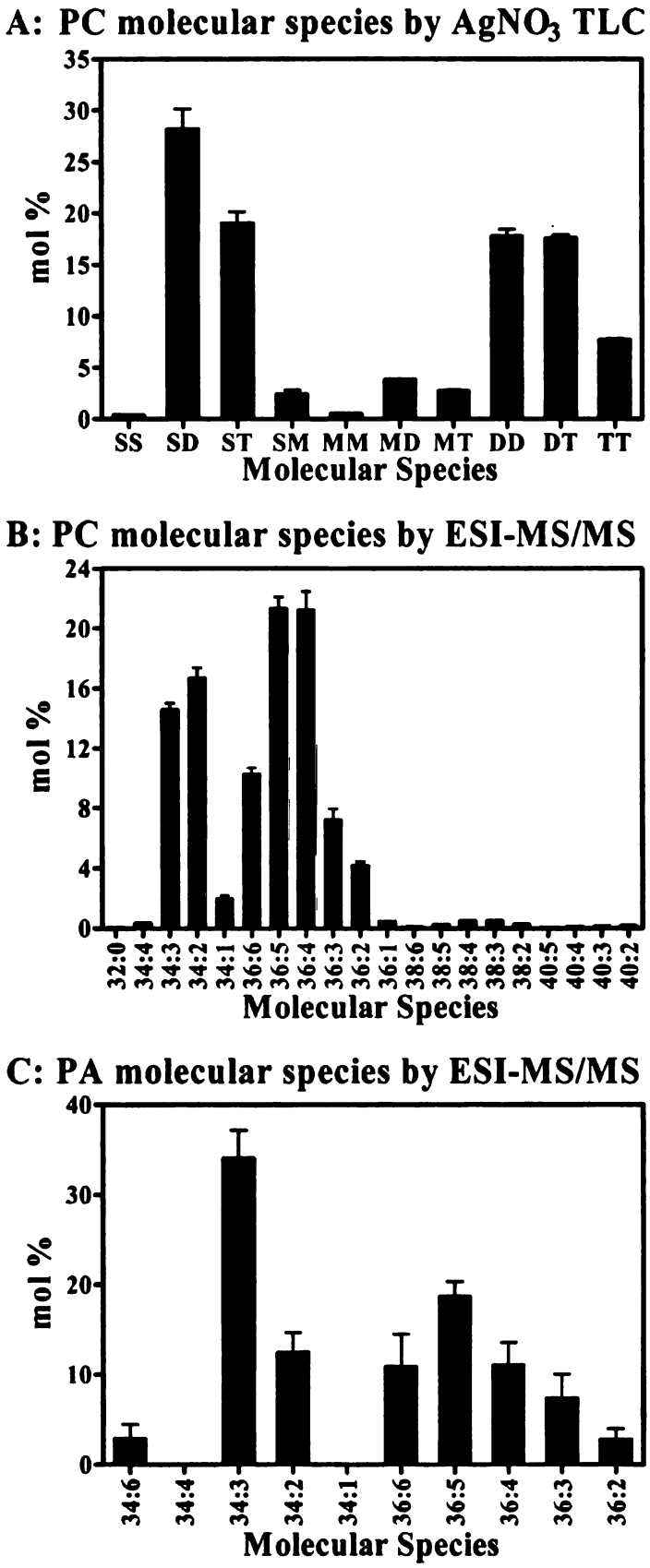

Figure 2-4. Molecular species analyses of endogenous PC and PA from pea leaves.

Data is presented as mole percentage of each molecular species within that lipid class (100%). **A**: PC molecular species as determined by $AgNO_3$ TLC/GC (three determinations). Molecular species are reported as the combination of two acyl groups: S, saturates (16:0>18:0); M, monoenes (18:1); D, dienes (18:2); and T, trienes (18:3). **B-C**: molecular species as determined by ESI-MS/MS (five separate measurements). Molecular species reported as (# total carbons: # total double bonds). **B**: PC. **C**:PA. All error bars are standard error.

Glycerolipid Molecular Species in Expanding Pea Leaves—Molecular species of PC from expanding pea leaves were analyzed by GC of FAMEs following $AgNO_3$-TLC separation of the corresponding 1,2-diacyl-3-acetyl-glycerols. The data are shown in Figure 2-4A, and are very similar to a published analysis of PC molecular species from pea leaf microsomes as determined by reverse-phase HPLC [143]. In addition pea leaf PC molecular species from expanding leaves were determined by ESI-MS/MS (Figure 2-4B). The major fatty acids of pea leaf PC are 18:2 (44%), 18:3 (31%), 16:0 (18%), 18:0 (4.2%) and 18:1 (2.6%). The major molecular species that we observe are the expected combinations of these fatty acids, given that saturates are confined to the sn-1 position. Oleic (18:1) and palmitic (16:0) are the major products of chloroplast FAS (Figure 2-2A). We note that if these nascent FA were incorporated via de novo PC synthesis without any contribution from acyl editing then 18:1/18:1-PC would be the most abundant initial species formed. However this species represents less than 1% of the total PC (MM, Figure 2-4A). Likewise, the PA molecular species contain only trace amounts of 16:0/18:1 (C34:1) and 18:1/18:1 (C36:2) based on ESI-MS/MS (Figure 2-4C). The majority of the C36:2 PA (and PC) species will be 18:0/18:2. Total PA contributes 0.1-0.4 mole % to total pea leaf membrane lipids. The PA profile is quite similar to that for PC, but it does contain significantly more 16:0/18:3 (34:3). PA may include biosynthetic pools, pools derived from lipid signaling via phospholipase D [4] and DAG kinase, and the putative PA pool for delivery of eukaryotic molecular species to the plastid for

galactolipids synthesis [90]. PA also contains a small amount of 34:6, which is presumed to be 18:3/16:3, and may therefore indicate a plastid contribution.

Analysis of Molecular Species of PC from [^{14}C]Acetate Labeling—To examine the initial molecular species of PC produced from incorporation of nascent FA, pea leaves were labeled with [^{14}C]acetate for 5 min. This time was a compromise between allowing enough labeling of PC for the analysis, yet keeping the amount of labeled 18:2 to a minimum (Figure 2-2B). Each labeled molecular species, analyzed as the 1,2-diacyl-3-acetyl-glycerol derivative, was also analyzed for labeled fatty acid composition. Figures 2-5A and 2-5B show the distribution of PC molecular species by mass and [^{14}C]acyl group, respectively. If acyl chains enter eukaryotic phospholipid synthesis via glycerol-3-phosphate acylation without acyl editing the predicted initial molecular species of PC would be 16:0/18:1 and 18:1/18:1 (S/M and M/M, respectively). Each will be dual labeled with newly synthesized FA, and if the saturates are 10% of the total label (Figure 2-2B) then the S/M to M/M labeling ratio will be approximately 1:4. This does not occur. Also, the S/M molecular species was only labeled with oleate; the corresponding palmitate was unlabeled. The MM molecular species which would be predicted to contain 80% of the initial label actually represented only 6.5% of all the labeled molecular species. It is unclear if this species contains one or both acyl groups labeled. The data in Figure 2-5B indicate that all other labeled PC molecular species contain one labeled acyl group (18:1 or 16:0/18:0) together with an unlabeled acyl group (18:2, 18:3, or 16:0/18:0). Thus > 90% of

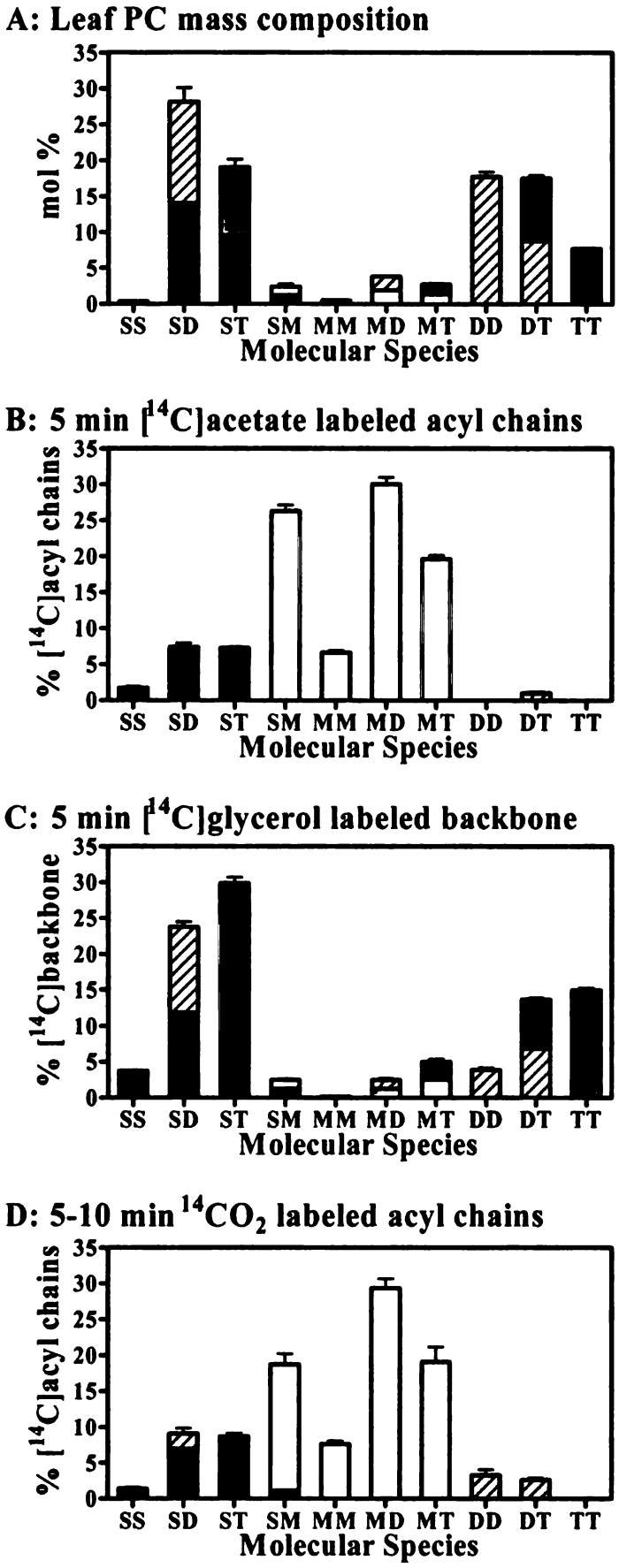

Figure 2-5 Analysis of endogenous PC molecular species and those labeled after short time incubations of excised pea leaves with [^{14}C]acetate or [^{14}C]glycerol or of seedlings with [^{14}C]CO_2.

A-D: Molecular species are represented as pairs of FA, with bar heights represent % of species among the total. Bar shading represent % of FA per species (mass or ^{14}C): S, saturates (16:0>18:0), black bars; M, monoenes (18:1), white bars; D, dienes (18:2), hatched bars; and T, trienes (18:3), gray bars. **A:** Endogenous PC molecular species mass composition from Figure 2-4A. **B:** 5 min [^{14}C]acetate labeled PC molecular species. Results expressed as mean ± S.E. (triplicate determinations). **C:** 5 min [^{14}C]glycerol labeled PC molecular species, label was in the glycerol moiety only (label in acyl chains has been subtracted). Results expressed as mean ± S.E. (duplicate determinations). **D:** 5-10 min [^{14}C]CO_2 acyl chain labeled PC molecular species. Results expressed as mean ± S.E. (quadruplicate determinations).

the molecular species of [^{14}C]PC contain one newly synthesized (labeled) FA in the same molecule as a previously synthesized (unlabeled) FA. This finding strongly suggests acyl editing as a mechanism of PC synthesis with nascent FA.

Analysis of Molecular Species of PC Synthesized de novo from [^{14}C]Glycerol—Because acyl editing only exchanges acyl groups it does not result in net synthesis of phospholipid. However, in order for a leaf to grow net (i.e. *de novo*) PC synthesis is required. To determine the PC molecular species and hence ascertain which acyl chains are involved in *de novo* eukaryotic glycerolipid synthesis, pea leaves were incubated with [^{14}C]glycerol. The PC glycerol backbone will be labeled regardless of whether the acyl chains are nascent FA or from acyl-editing. Figure 2-5C shows the distribution of label among PC molecular species after 5 min [^{14}C]glycerol labeling. The data are corrected to give only the glycerol backbone labeling by subtracting the small amount of acyl labeling (Figure 2-3B) that occurs from [^{14}C]glycerol. The PC molecular species distribution obtained with glycerol backbone labeling (Figure 2-5C) is quite different from that for acetate acyl labeling (Figure 2-5B), but closely resembles the endogenous mass distribution (Figure 2-5A), with the exception of a significant reduction in 18:2-containing molecular species and an increase in 18:3-containing species. The major initial PC molecular species predicted from incorporation of only nascent FAs, 18:1/18:1, was less than 0.2% of labeled species. The total 18:1 content of the glycerol-labeled PC is 5%, some of which may derive from nascent FA. We know that at this time point the ratio of unsaturates to saturates in PC from nascent FA is about 10:1 (Figure 2-2B) and

that there are negligible polyunsaturates. Thus we infer that at the most there is a 5.5% contribution of newly synthesized FA to *de novo* PC biosynthesis, or, conversely, that the bulk (≥ 95%) of the acyl groups in PC molecular species labeled via glycerol backbone come from acyl editing. Figure 2-5D, which describes PC molecular species analysis from [^{14}C]carbon dioxide labeling, will be described in a later section.

Stereochemistry of PC acylation—Molecular species of PC can be defined by acyl composition and/or by stereochemistry. To determine the stereospecific incorporation of newly synthesized FA, PC isolated from 5 min incubations of pea leaves with [^{14}C]acetate was digested with phospholipase A_2 and the reaction products analyzed (Figure 2-6). 62% of the label was incorporated at the *sn*-2 position and 38% at the *sn*-1 position. Saturates were predominately found in the *sn*-1 position, indicating that the PLA_2 digestion was *sn*-2-specific. Although *de novo* PC synthesis requires equimolar acylations at the *sn*-1 and *sn*-2 position we cannot assume that this will be the case for the 16:0 and 18:1 labeled FA if they are mixing with an endogenous acyl-CoA pool containing 18:2 and 18:3, since we do not know the acyl transferase specificity at each position. Previous workers have reported either a similar *sn*-1 to *sn*-2 distribution of acyl label, for PC from 30 minute acetate labeling of spinach leaves [84], or, using a 2 hour

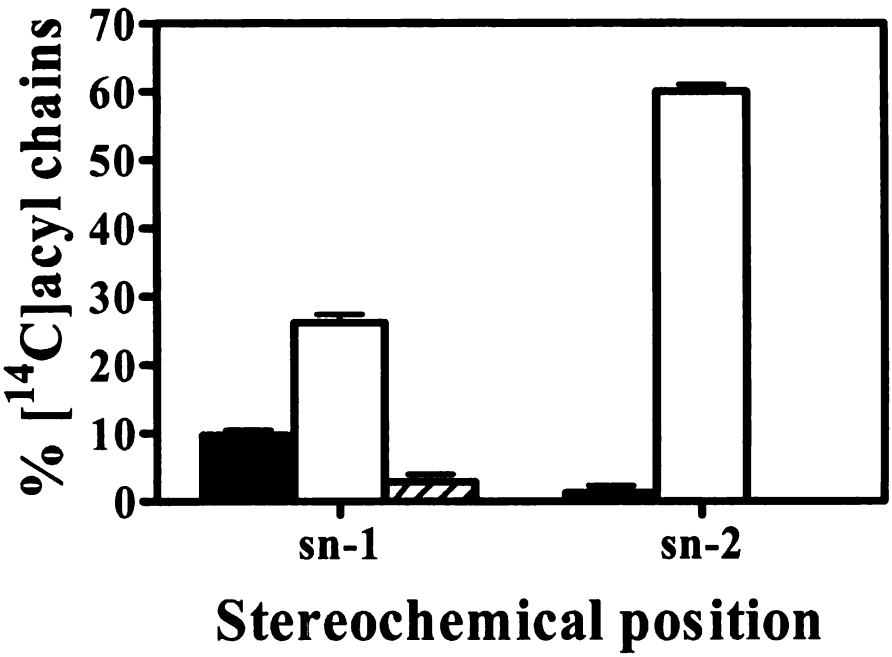

Figure 2-6 Stereochemical distribution of [^{14}C]acyl chains in PC.
Pea leaves were labeled for 5 min with [^{14}C]acetate. FAMEs generated from the fatty acid and lyso-PC products of phospholipase A_2 digestion of isolated PC were separated based on the number of double bonds: saturates (16:0>18:0), black bars; monoenes (18:1), white bars; dienes (18:2), hatched bars. Results expressed as mean ± S.E. (triplicate determinations).

acetate labeling of leek leaves, a 2:1 to 3:2 enrichment in the *sn*-2 position [92, 93]. The unequal stereochemical labeling complements the conclusion that nascent FAs are incorporated into PC along side previously synthesized FA.

Unlabeled FA Composition of Endogenous PC, LPC, and Various Labeled PCs—From Figure 2-5B we have estimated the composition of the unlabeled FAs that are associated with the nascent, [^{14}C]acetate-labeled FAs. The calculated values are shown in Figure 2-7, along with the acyl composition of endogenous PC (calculated from Figure 2-4A) and of endogenous lyso-PC (obtained by ESI-MS/MS, Appendix A Figure 5-1:S1K). To a first approximation all three profiles are similar. The unlabeled, paired acyl groups from [^{14}C]acetate-labeled PC have a slightly reduced 18:2 and increased saturates compared to endogenous PC. The unlabeled fatty acids must have had an inverted stereochemical distribution to that of the labeled FA measured in Figure 2-6; that is, 62% *sn*-1 and 38% *sn*-2. The fact that the FA profile of lyso-PC was similar to that of total PC allows us to propose that the endogenous lyso-PC pool is produced by approximately equal *sn*-1 and *sn*-2 deacylations, whether catalyzed by hydrolysis or by PC acyl exchange with CoA. Thus the appropriate lyso-PC pool is available for both *sn*-1 or *sn*-2 acylations with newly synthesized FA. The unequal stereochemical acylation with nascent FA found in PC (Figure 2-6) therefore may arise from *sn*-2 acyl migration in lyso-PC to the more thermodynamically stable *sn*-1 position, or possibly a higher reacylation of the

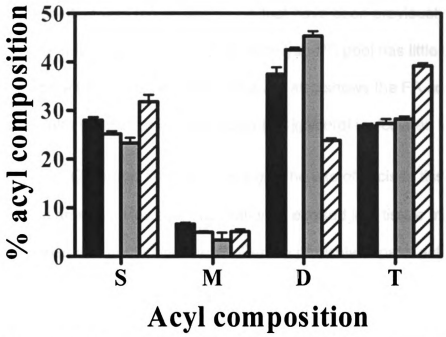

Figure 2-7 Comparison of unlabeled fatty acid compositions deduced from [^{14}C]acetate and [^{14}C]glycerol labeled PC molecular species to endogenous pea leaf PC and lyso-PC compositions.

Black bars: [^{12}C]FA in the same molecule as [^{14}C]FA labeled molecular species, as calculated from figure 5B. White bars: endogenous PC FA mass composition, as calculated from Figure 4A. Gray bars: endogenous pea leaf lyso-PC FA mass composition, as calculated from ESI-MS/MS (supplemental Figure S1K, 5 determinations). Hatched bars: FA composition calculated from [^{14}C]glycerol labeled PC molecular species, as calculated from Fig. 5D. All error bars are standard error. S, saturates (16:0>18:0); M, monoenes (18:1); D, dienes (18:2); T, trienes (18:3).

sn-1 position with the unlabeled FA that have been previously removed by acyl editing. It is also possible that the bulk lyso-PC pool has little to do with the incorporation of nascent FA. Figure 2-7 also shows the FA composition of molecular species of PC labeled by [^{14}C]glycerol (calculated from Figure 2-5C).

[^{14}C]Carbon Dioxide Labeling—The use of excised tissue facilitates rapid kinetic analysis. However, incubation of excised leaf tissue might cause a wound response that could alter metabolism. In particular, lipase activities may be rapidly and transiently induced that could alter phospholipid metabolism. To control for this possibility whole pea seedlings were labeled for 5-10 minutes with [^{14}C]CO_2. Label is rapidly incorporated into both the acyl-chains and glycerol backbone of lipids. Figure 2-8 compares the distribution of acyl group labeling among lipid classes for [^{14}C]CO_2 labeling of whole seedlings and [^{14}C]acetate labeling of cut leaves at approximately equivalent assay times. Both labeling experiments indicated that PC is the major recipient of newly synthesized FA and that the relative acyl group labeling among other lipid classes is similar. Additionally, molecular species of PC were analyzed for acyl-chain labeling from whole seedlings with [^{14}C]CO_2 (Figure 2-5D). This distribution should be compared with that from [^{14}C]acetate labeling of cut leaves (Figure 2-5B). The slightly longer time of assay (approximately 10 minutes including seedling removal and quenching) for [^{14}C]carbon dioxide compared to [^{14}C]acetate

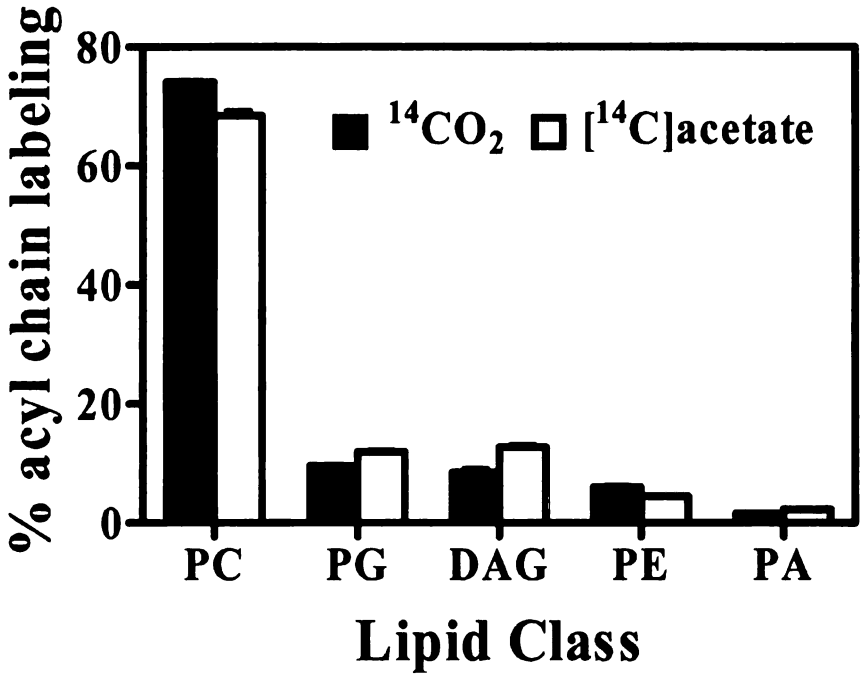

Figure 2-8 Distribution of lipid classes, labeled in their acyl chains, after incubation of pea leaves with [^{14}C]CO_2 or [^{14}C]acetate.

Black bars: ~5-10 min [^{14}C]CO_2 labeling of intact pea seedlings, with results expressed as mean ± S.E. (quadruplicate determinations). White bars: 6 min [^{14}C]acetate labeling of excised pea leaves (Data from Fig. 2-1, results expressed as mean ± S.E. (triplicate determinations)).

labeling allowed slightly greater desaturation of 18:1 to 18:2. However, it is clear that the pattern of nascent FA acyl labeling from [^{14}C]CO_2 matches that of [^{14}C]acetate. We conclude that the relative proportions of individual lipids and initial PC molecular species labeled in incubations of excised leaves was not due to a change in lipid metabolism caused by a wound response but is indicative of the *in planta* lipid metabolism.

2.5 Discussion

Largely through analyses of the kinetics, molecular species and stereochemistry of the immediately labeled products of eukaryotic glycerolipid synthesis we demonstrate that, in rapidly expanding pea leaves, (1) there was no detectable precursor-product relationship for PA and DAG in the incorporation of nascent FA into PC, (2) nascent FA were incorporated into PC more rapidly than the *de novo* synthesis of PC from glycerol, suggesting two systems for the incorporation of acyl groups into PC, (3) greater than 90% of nascent FA were incorporated into PC molecules in combination with an endogenous FA, (4) greater than 95% of *de novo* synthesized PC was esterified by recycled acyl groups, (5) acyl editing can take place at both the *sn*-1 and *sn*-2 positions, and (6) that pea leaves contain an endogenous lyso-PC pool that could be involved in acyl editing. Experiments with carbon dioxide labeling of whole seedlings demonstrated that the metabolism seen in excised leaves is not due to a wound response.

These results lead us to conclude that the major pathway for the incorporation of newly synthesized FA into eukaryotic glycerolipids is largely through acyl editing of PC and that *de novo* synthesis of eukaryotic glycerolipids primarily utilizes acyl chains recycled from acyl editing. We will discuss these results in terms of the possible mechanisms and flux models (Figure 2-9) that can be proposed to drive acyl editing.

18:1/18:1 and 16:0/18:1 Are Not Initial Molecular Species of Eukaryotic Glycerolipid Synthesis—The classical scheme for eukaryotic phospholipid synthesis in leaves [34, 37], as summarized in Figure 2-9, Model 1, predicts the biosynthesis of dual-labeled 16:0/18:1 and 18:1/18:1 PA, DAG and then PC species sequentially. However, our kinetic analysis failed to detect precursor-product relationships between PA, DAG and PC pools for acyl group labeling (Figures 2-1 and 2-2). Furthermore, we have not been able to observe the expected, dual-labeled 16:0/18:1 and 18:1/18:1 PC species. Based on estimates of FAS fluxes and endogenous PA and PC molecular species pool sizes (see supplement Note 1, Appendix A), labeled 16:0/18:1 and 18:1/18:1 molecular species of PC should have been easily detected by our analysis if Model 1 was correct. Additionally, glycerol labeling of *de novo* synthesized PC revealed mostly esterification with endogenous saturates and polyunsaturates, and not newly synthesized saturates and oleate. Taken together these three lines of evidence strongly suggest that 16:0/18:1 and 18:1/18:1 molecular species are not the first products of nascent FA incorporation into eukaryotic glycerolipids and that Model 1 is incorrect.

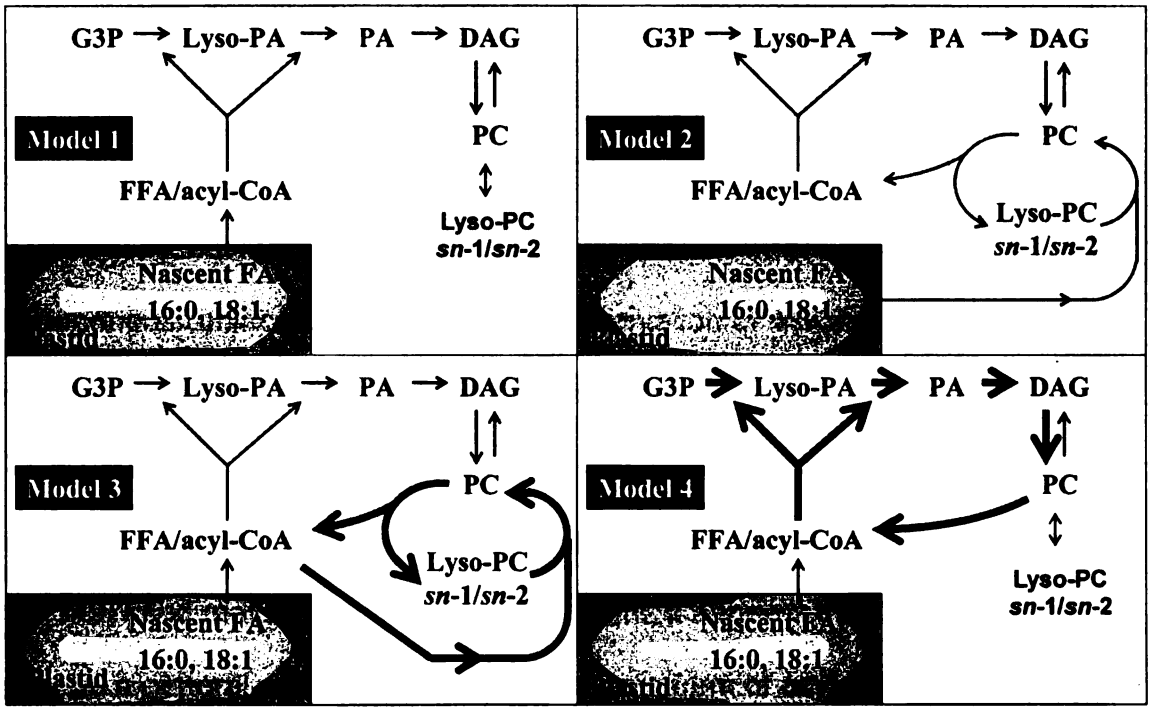

Figure 2-9 Four possible models describing pathways and fluxes for the incorporation of nascent FA, exported from the chloroplast immediately after *de novo* FAS, into PC.

For simplicity, the models are set up not to depend on enzyme specificity, and show only PC. The mechanisms for removal of fatty acids from PC and provision of acyl-CoA are not defined, while the width of the arrows gives an indication of relative flux. The grey box represents the chloroplast; G3P, glycerol-3-phosphate; FFA, free fatty acid. Model 1 shows a description lacking any acyl editing. Models 2 and 3 are considered the best representations for the acyl editing that accompanies in the incorporation of nascent FA into PC, but a definitive choice of the most correct model is not yet possible.

Most Newly Synthesized FAs Enter PC by sn-1 and sn-2 Acyl Editing, while de novo PC Synthesis Primarily Uses Recycled Acyl Groups—We have shown that the majority of newly synthesized (labeled) FAs in pea leaves are immediately incorporated into PC molecular species where > 90% are paired with endogenous (unlabeled) FAs. A similar observation of >60% incorporation of nascent FA paired with endogenous FA is also reported by Williams et al. [149] for *Brassica napus* leaves 1hr after a pulse of carbon dioxide labeling. Because we have used rapidly expanding leaves from an 18:3 plant, whereas Williams et al. [149] used mature leaves from a 16:3 plant, it appears that the observation may be a general one. The result implies some form of acyl editing. Either the nascent FA are used directly in an acyl editing process (Figure 2-9, Model 2), or acyl groups are released from endogenous lipids and mix with the nascent FA prior to completing the acyl editing cycle and/or acylation of glycerol-3-P for *de novo* PC synthesis (Figure 2-9, Models 3 and 4). Models 2-4 will be discussed in more detail later. Whatever the acyl editing mechanism, net (i.e. *de novo*) phospholipid synthesis from glycerol-3-P is required for the leaf to grow. Glycerol labeling showed that *de novo* PC synthesis utilized mainly endogenous palmitate (+ stearate), linoleate and linolenate and not newly synthesized FA. Thus the FA used for *de novo* PC synthesis must be largely (≥ 95%) recycled from acyl editing, and, almost certainly, largely from PC. The fact that saturated FAs are major acyl groups released by acyl editing suggests that *sn*-1 hydrolysis or CoA:PC *sn*-1 acyl exchange contributes in a major way to this process. This is confirmed by the results from the acetate labeling experiments shown in Figure

2-5B and Figure 2-6, namely that > 90% of the molecular species contain only one labeled acyl group and 40% of the labeled acyl groups are at the *sn*-1 position. For Models 2 and 3, this implies that a *sn*-1 acyl editing process for incorporation of nascent FA into PC represents 30-40% of the net flux. This conclusion from our *in vivo* labeling represents a substantial biochemical activity largely overlooked or underrepresented by previous *in vitro* analyses of PC acyl metabolism [94, 140] and *in vivo* labeling studies [149].

Proposed Models for Acyl Editing—Questions then arise as to the pathways, fluxes and mechanisms for the metabolism of PC synthesis utilizing nascent FA from the chloroplast. The puzzle is complex because of the large number of unknowns, and our current data do not completely resolve the models proposed. However, it is instructive to review possible models to highlight the unknowns requiring resolution. Three models are considered which might accommodate a flux of x moles of nascent FA from the chloroplast, producing 0.5x moles of net PC synthesis (Figure 2-9, Models 2-4). For simplicity, the models were initially set up not to depend on enzyme specificity, and show only PC. The mechanisms for removal of fatty acids from PC and provision of acyl-CoA are not defined. They may be via phospholipase action with activation of the released free fatty acid by an acyl-CoA synthetase, or via direct CoA:PC acyl exchange.

Model 2 describes a situation whereby nascent FA are channeled to lyso-PC. Their incorporation allows just one labeled FA per re-synthesized PC molecule, as observed experimentally. The endogenous FA released from PC is

concomitantly used in the *de novo* synthesis of PC. The endogenous FA may enter a general acyl-CoA pool, and the model does not rule out other sites of phospholipid acyl editing also supplying this pool. Model 2 implies acyl transfer reactions for nascent FA incorporation and *de novo* PC synthesis may be spatially distinct metabolic processes. The model cannot accommodate the catabolism of PC to GPC, because the acylation of GPC by the nascent, labeled FA will lead to two labeled acyl groups per PC molecule. As the endogenous, unlabeled acyl distribution in PC molecular species from acetate labeling closely matches the endogenous PC acyl distribution (Figure 2-7), the lyso-PC and the FA pools produced by PC editing must have the similar acyl profiles. This is confirmed by the endogenous LPC analysis. The fact that both lyso-PC and unlabeled FA in FA/FA*-PC have compositions identical to the bulk PC does not absolutely require *sn*-1 and *sn*-2 deacylations to be on an equimolar basis. However, the phospholipase/acyl exchange activities (and any lyso-PC isomerization) will control the sn-1 and sn-2 distributions observed in acetate-labeled acyl groups. Thus Model 2 can accommodate the excess sn-2 acylation noted in acetate labeling. Model 2 requires an explanation for the reduction in 18:2 FA content for glycerol-labeled PC, by, for example, proposing a preference for the transfer of 18:2-containing acylglycerol moieties to the chloroplast for galactolipid synthesis [86].

Model 3 describes a situation whereby the nascent FAs are directed to a general acyl-CoA pool, along with FAs from PC acyl editing. Thus the pairing of labeled and unlabeled acyl groups in PC is caused by a high flux of acyl editing,

which greatly dilutes out nascent FA in the common acyl-CoA pool. In this model it is possible to propose that PC editing stops at lyso-PC or instead goes through two deacylation steps to produce GPC. However, because GPC-dependent acyltransferases have not been reported in plants, we will focus on the lyso-PC example, as shown in Figure 2-9 Model 3. An analysis of the molecular species of PC labeled from acetate showed that ≥ 90% had single acyl group labeling. Without any substrate specificity constraints, this requires that > 90% of the flux of acyl groups in the acyl-CoA pool originate from unlabeled fatty acids (Appendix A, Figure 5-2). Consistent with this observation, analysis of the molecular species of PC with glycerol backbone labeling required that ≥ 95% of the flux for *de novo* PC synthesis come from acyl editing. With this model the *sn*-1 and *sn*-2 distributions for nascent FA do not have to fit a 1:1 ratio. Also, it is easy to explain the deficit of 18:2 acyl groups in the *de novo* PC labeling with glycerol. We can simply assert that there is some acyl-CoA selectivity in the *de novo* synthesis at either *sn*-1 or *sn*-2 acylation. A moderate change in the *de novo* synthesis pathway acyl composition will have little effect on the endogenous acyl group composition measured by acetate labeling. Of course, the simplicity of this explanation does not prove it.

In Models 2 and 3 the acyl editing cycle (phospholipid deacylation and reacylation) is distinct from *de novo* phospholipid synthesis. It is, however, possible to have *de novo* phsopholipid synthesis be part of the acyl editing cycle (Figure 2-9, Model 4). This requires PC to be completely degraded to glycerol or glycerol-3-P, and choline or choline-P. In this model there is only one pathway for

the synthesis of PC, and if the PC turnover rate is high relative to nascent FA production, much of the labeling data is consistent with this model. However, a comparison of labeling kinetics for DAG and PC using either acetate or glycerol substrates strongly suggested that we were dealing with two distinct metabolic processes for PC synthesis. This is inconsistent with Model 4, and on this basis, we rule it out as a dominant pathway of leaf cytosolic glycerolipid synthesis.

In this Discussion we have arrived at the conclusion that one of a pair of simple models (Figure 2-9, Models 2 and 3), offers the best description of cytosolic glycerolipid synthesis in pea leaves. The models present quantitatively very different magnitudes for the fluxes involved in acyl editing, and give different views on how such acyl editing integrates with the incorporation into PC of nascent FA exported from the chloroplast. Currently, we cannot categorically rule out either Model 2 or Model 3. Furthermore, it is always possible to build more complexity into models, including substrate specificity and the possibility of some hybrid combination. Future oxygen-18 and acyl-CoA pool labeling studies may yield pertinent information on the mechanism of acyl editing: lipolytic or through CoA:PC acyl exchange. Enzymology with isolated chloroplasts and microsomes may yield useful information on transacylation and *de novo* acyl transferase mechanisms and selectivity. Such studies must take into account the very substantial *sn*-1 component identified for acyl editing. Furthermore, endogenous PC is 2-fold higher than PE, but acyl labeling of PC from acetate (and also from glycerol) is 15-fold greater. This is despite the total leaf lyso-PE pool being approximately equivalent to lyso-PC (Appendix A Figure 5-1:S1B). Thus, there is

7.5 or 15 fold preference for incorporation into PC via acyl editing that will have to be taken into account in defining any mechanism. A complete understanding may require the study of metabolism in KO mutants. However, with so many genes annotated as lipases, acyl hydrolases and transacylases [134], most with unknown functions, this may not be a facile approach either to this tricky question. Defining the mechanisms of the incorporation of nascent fatty acids into glycerolipids will help our understanding of lipid turnover and be relevant to research areas with more practical applications such as the integration of seed triacylglycerol synthesis with phospholipid turnover and the effects of biotic and abiotic stresses on phospholipid metabolism.

2.6 Footnotes

This work was supported by the US Department of Energy grant DE-FG02-87ER13729. ESI-MS/MS analysis was provided by the Kansas Lipidomics Research Center.

Abbreviations: ACP, acyl carrier protein; DAG, diacylglycerol; FA, fatty acid; FAME, fatty acid methyl ester; FAS, fatty acid synthesis; GC, gas chromatography; GPC, glycerophosphorylcholine; LPCAT, lyso-phosphatidylcholine acyltransferase; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol. Fatty acids are described by the convention "carbon number:number of double bonds." For example, oleic acid is represented as 18:1. The molecular species of

phospholipids are described by FA/FA. When known, the *sn*-1 position is represented first, but often the stereochemistry is uncertain. For example, both 1-oleoyl-2-linoleoyl-*sn*-phosphatidylcholine and 1-linoleoyl-2-oleoyl-*sn*-phosphatidylcholine are described as 18:1/18:2-PC.

3 THE DOMINANT INITIAL FLUX OF FATTY ACIDS INTO SOYBEAN OIL IS THROUGH PHOSPHATIDYLCHOLINE ACYL EDITING

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The results of this chapter will be submitted for publication in plant physiology.

The ESI-MS/MS analysis of endogenous soybean lipid compositions was provided by Tim Durrett. The data is presented in Appendix B Tables 6-2, 6-3, 6-4. The ESI-MS/MS data was reformatted for discussion of soybean lipid metabolism and comparison to labeled lipid data in Table 3-1 and Figures 3-1, 3-6, and 3-8.

3.1 Abstract

The reactions leading to triacylglycerols (TAG) synthesis in oilseeds have been well characterized. However, quantitative analyses of acyl group and glycerol backbone fluxes that comprise extraplastidic phospholipid and TAG synthesis, including acyl editing and phosphatidylcholine-diacylglycerol (PC-DAG) inter-conversion, are lacking. To investigate these fluxes we rapidly labeled soybean (*Glycine max*) embryos with [^{14}C]acetate and [^{14}C]glycerol. Cultured intact embryos that mimic *in planta* growth were used to minimize any changes in metabolism due to wounding. The initial kinetics of newly synthesized acyl chain and glycerol backbone incorporation into PC, DAG and TAG were analyzed along with their initial labeled molecular species and regiochemical compositions. Over 60% of the newly synthesized fatty acids enter glycerolipids through PC acyl editing, largely at the *sn*-2 position. This flux, mostly of oleate, was about 4-fold greater than the flux of nascent [^{14}C]fatty acids into *sn*-1 and *sn*-2 positions of DAG through *de novo* glycerolipid synthesis. The total flux for PC acyl editing was estimated to be 2 to 7 times the flux of nascent fatty acids entering the eukaryotic pathway. Thus recycled acyl groups (16:0, 18:1, 18:2 and 18:3) provide most of the acyl chains for *de novo* glycerolipid synthesis. Our results also suggest that the DAG utilized for TAG synthesis is mostly derived from PC, whereas *de novo* synthesized DAG is mostly used for PC synthesis. These results provide a quantitative demonstration that acyl flux in/out of PC by acyl editing is the dominate flux in soybean embryos during TAG synthesis.

3.2 Introduction

In plants essentially all acyl chains for membrane and storage lipid synthesis are produced in the plastid by acyl carrier protein (ACP) dependent *de novo* fatty acid synthesis (FAS) [28, 34, 160]. However, in oilseed tissue these acyl groups are utilized almost completely (> 95%) by the extra-plastid pathways of *de novo* glycerolipid synthesis, collectively termed the "eukaryotic pathway" [34]. The plastid acyl-ACPs are hydrolyzed and the free fatty acid end products (usually 18:1>>16:0>18:0) are transported across the plastid envelope [74]. Once activated to acyl-CoAs [77, 78], these acyl groups are available within the endoplasmic reticulum (ER) for incorporation by the acyltransferases of the eukaryotic pathway. The acyl-CoAs may be used for the sequential *sn*-1 and *sn*-2 acylations of G3P to produce phosphatidic acid (PA) [34]. PA is then converted to *sn*-1,2-diacylglycerol (DAG) by the action of PA phosphatase. DAG represents an important branch point between neutral and membrane lipid biosynthesis. DAG may be acylated to produce TAG, or converted to phosphatidylcholine (PC) by CDP-choline:1,2-diacyl-*sn*-glycerol cholinephosphotransferase (CPT), with an analogous reaction to form phosphatidylethanolamine (PE) [34]. However, in both leaves [161] and in oilseeds the flux of nascent fatty acids into PC dominates over the synthesis of PE, PS and other phospholipids of the eukaryotic pathway. Desaturation of 18:1 to 18:2 and then 18:3 on PC produces the abundant polyunsaturated molecular species of PC [36, 37].

Recently we investigated the initial steps in eukaryotic membrane lipid synthesis in expanding pea (*Pisum sativum*) leaves through *in vivo* labeling of the acyl groups and backbones of glycerolipids [162]. The results were inconsistent with a pathway in which newly synthesized fatty acids are directly esterified to G3P during *de novo* glycerolipid synthesis. Instead almost all newly synthesized fatty acids were esterified to a *sn*-1 or *sn*-2 lyso-PC through an "acyl editing" process resulting in molecular species of PC with one newly synthesized and one previously synthesized FA. In contrast, *de novo* glycerolipid synthesis in pea leaves primarily utilized recycled acyl groups released from PC during acyl editing. Acyl editing, often also termed "remodeling" or "re-tailoring," is defined as any process that exchanges acyl groups between polar lipids but which does not by itself result in the net synthesis of the polar lipids. Acyl editing may proceed by at least two mechanisms. The first mechanism involves CoA:PC acyl exchange producing lyso-PC and acyl-CoA, which was demonstrated in microsomes isolated from developing seeds and was attributed to a reverse reaction of lyso-phosphatidylcholine acyltransferase (LPCAT) [140]. There are several reports of high LPCAT activity in seeds [130, 140, 142, 144, 163]. A second mechanism for acyl editing involves hydrolysis of PC to lyso-PC, activation of the released free fatty acid and its re-utilization for phospholipid synthesis from lyso-PC by LPCAT.

Plants exhibit both acyltransferase and transacylase mechanisms for the acylation of DAG to TAG. Three classes of diacylglycerol:acyl-CoA *sn*-3 acyltransferase have been identified, namely the ER membrane bound DGAT1

[164] and DGAT2 [165, 166] classes, and third a soluble cytosolic DGAT class [167]. In addition, the transacylase PDAT (phospholipid:diacylglycerol acyltransferase) allows the direct transfer of acyl groups from the *sn*-2 position of PC to DAG, producing TAG and lyso-PC products [119]. A DAG:DAG transacylase activity has also been described [122]. In addition to this diversity of TAG synthesis mechanisms and biosynthetic genes, oil-accumulating tissues may utilize different strategies to enrich for polyunsaturated fatty acids (PUFA) in TAG. PC is the major site of fatty acid desaturation and related reactions, while evidence for a direct role for DAG or TAG as a desaturase substrate is lacking. The conversion of PC to DAG (for TAG synthesis) may enrich for PUFAs in TAG. This conversion may be accomplished by a phospholipase or the reverse action of CTP-choline:diayclglycerol cholinephosphotransferase [117, 118]. Secondly, PUFA may be enriched in TAG by mechanisms of PC acyl editing. One such general mechanism has already been described above, as PC acyl editing may enrich the PUFA acyl-CoA pool by either a transacylase or lipase/LPCAT mechanism [162]. A second possible mechanism arises from the action of PDAT in TAG synthesis. The LPC produced by PDAT would be re-acylated by the action of LPCAT.

From the above outline it is clear that there are a number of alternative metabolic routes for TAG synthesis. These include the recycling of intermediates of membrane lipid biosynthesis. The alternatives may vary with tissue, species and development. Different metabolic labeling experiments have been used to probe the sequence of reactions for TAG synthesis *in vivo* from a variety of

oilseed species. Utilization of a PC-derived DAG moiety has been proposed as the major pathway for TAG synthesis in excised linseed and soybean cotyledons [127]. Glycerol backbone labeling of developing safflower cotyledons suggested that the DAG pool feeding TAG synthesis was in equilibrium with PC, however a similar experiment with avocado mesocarp suggested that PC was not involved in TAG synthesis [168]. This led the authors to propose that PC backbone turnover is used primarily in high PUFA containing TAG synthesis. Phosphocholine head group labeling in linseed cotyledons was also consistent with the concept that DAG is in equilibrium with PC, allowing the production of a highly unsaturated DAG pool prior to incorporation into TAG [117]. The authors suggested that the reversible CPT reaction is most likely responsible for this PC-DAG inter-conversion. Labeling of safflower cotyledon slices with 16:0 and 18:1 free fatty acids allowed an alternative mechanism for PUFA enrichment in TAG to be proposed. An *sn*-2 specific LPCAT could direct 18:1 towards desaturation on PC and release PUFA to the acyl-CoA pool for the Kennedy pathway [169]. Although there is now significant *in vivo* evidence for PC acyl editing in the eukaryotic pathway in plant leaves [162], the *in vivo* evidence for such a cycle in oilseeds is still minimal.

We took several steps to extend and improve on previous studies of oilseed metabolism to determine the pathway of FA incorporation into membrane and storage lipids in developing soybeans. To avoid potential artifacts associated with wounding due to excision of tissues we utilized cultured soybean (*Glycine max*) embryos which closely mimic *in planta* growth and oil accumulation [170].

To provide an analysis of the initial events in eukaryotic glycerolipid synthesis we performed kinetic labeling studies with time points shorter than previous studies. Labeling was conducted with both [^{14}C]acetate and [^{14}C]glycerol and the labeled compositions, regiochemistry, and molecular species of labeled PC, DAG and TAG, along with endogenous molecular species compositions were determined. Together these approaches have allowed us to develop a more quantitative flux model of glycerolipid synthesis *in vivo* that demonstrates that acyl fluxes in and out of PC by acyl editing is a dominate flux in soybean during TAG synthesis. A better understanding of the pathways of TAG biosynthesis in developing soybeans may aid future efforts to engineer soybean oil with improved or novel properties.

3.3 Results

Embryo culture and endogenous lipid compositions

Accurate interpretation of metabolic labeling studies requires that during labeling the tissue functions as it would *in vivo*. In this context the use of cultured embryos (as opposed to embryo or seed slices in simple buffered media) minimizes lipase activation and other perturbations of lipid metabolism due to wounding. Thus we have utilized a soybean embryo culture system that allows dissected zygotic embryos to develop as *in planta* for over two weeks [170]. Radioactive precursors are incubated at low concentrations (acetate and glycerol at 0.5-1 mM) relative to the culture media, which contains sucrose (140 mM),

glucose (70 mM) and amino acids (48 mM) at levels that mimic the apoplastic concentration surrounding the embryo in planta. This minimizes changes in metabolism due to alternative carbon source utilization during the labeling.

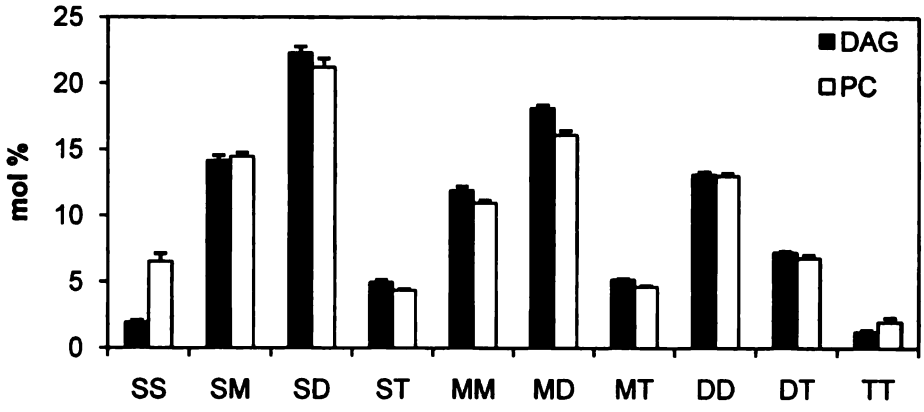
Relationships between precursor and product pools in metabolic pathways are revealed by kinetic labeling experiments. The linear accumulation of a product, once reached, coincides with steady state labeling of all precursor pools. In this context the duration of labeling needs to be short enough so that precursor pools have not filled before time point sampling begins. Soybean embryo DAG and PC had very similar FA compositions (Table 3-1) and molecular species profiles (Figure 3-1A). FA are desaturated on PC, but it is DAG that is the precursor for TAG. The combined PC/DAG pool is expected to provide precursor DAG for TAG synthesis. TAG FA composition and molecular species distribution are shown in Table 3-1 and Figure 3-1B, respectively. Developing soybean embryos of ~15-20 mg dry weight were pre-cultured for ~3 days before addition of radioactive substrates, to allow metabolism to equilibrate past any transient response due to the initiation of embryo culture. Cultured embryos accumulate ~6 mg total biomass per day [170] therefore each embryo doubles in biomass during the pre-culture. If the ratio of DAG:PC:TAG (Table 3-1) stays the same during pre-culture the initial pool sizes during labeling would be 208:542:9260 nmoles lipid embryo^{-1} respectively (~ 29280 nmoles total FA embryo^{-1}). Developing soybeans produce ~2.7 nmoles FA min^{-1} embryo^{-1} [170]. As about 93% of newly synthesized FA are used to produce TAG, of which 1/3 are utilized

Table 3-1 Endogenous lipid pool sizes and fatty acid composition

nmol lipid embryo^{-1}	DAG	±	PC	±	TAG	±
	20.8	0.63	54.2	2.8	926	17.9
% mol FA						
16:0	14.42	0.43	17.14	0.42	14.93	0.25
18:0	8.21	0.38	9.40	0.31	4.55	0.04
18:1	30.60	0.34	28.56	0.24	26.35	0.2
18:2	36.89	0.33	35.04	0.49	41.18	0.2
18:3	9.88	0.27	9.87	0.34	12.33	0.12
20:0					0.66	0.03

DAG, PC and TAG amounts as measured by ESI-MS/MS of 13-14 mg dry weight developing embryos, before pre-culture. The fatty acid composition as calculated from the molecular species presented in Figure 3-1. Fatty acid values are represented as percent mol among each lipid class.

A: DAG and PC molecular species



B: TAG molecular species

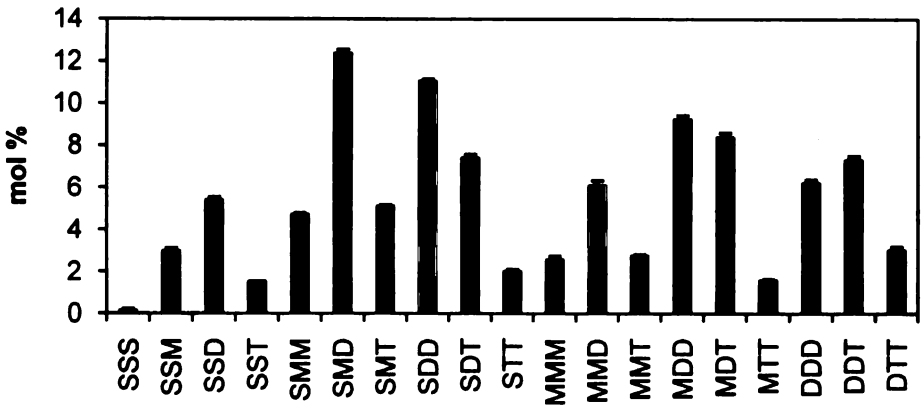


Figure 3-1 Endogenous molecular species compositions of DAG, PC and TAG.

A-B, Endogenous glycerolipid molecular species from developing soybean embryos of 13-14 mg dry weight per embryo as determined by ESI-MS/MS. Molecular species are represented as two or three FA, with bar heights represent % of species among the total (random stereochemistry). For comparison to molecular species separated by argentation TLC the molecular species are grouped based on the individual FA number of double bonds: Total saturates (16:0, 18:0, 20:0, etc), S; monoenes (18:1), M; dienes (18:2), D; trienes (18:3), T. Actual molecular species measurements of the non grouped lipid molecular species for TAG, DAG, and PC are in Appendix B Tables 6-1, 6-2, 6-3, respectively. A, DAG (black bars) and PC (white bars) were analyzed as their *sn*-1,2-diacyl-3-acetyl-glycerol derivatives. B, TAG.

for *sn*-3 acylation, then ~1.7 nmoles FA min^{-1} $embryo^{-1}$ flux through the DAG moiety precursor pool into TAG. Therefore turnover time of the combined DAG/PC pool (1500 nmoles FA) would be 882 min (14.7 hrs) if all the DAG and PC takes part in acyl metabolism. Thus our assays of 2-30 min will be effective measures of initial rates and products but will not directly measure the long term precursor-product relationships of FA flux through the DAG moiety into TAG. However, these longer term relationships may be inferred through full analysis of the molecular species of products observed and consideration of the end point compositions.

[^{14}C]Acetate labeled fatty acid products

[^{14}C]acetate is an ideal substrate for the study of acyl lipid metabolism because it rapidly enters plant tissue and is highly specific for incorporation into newly synthesized FA . Transmethylation of total lipid extracts resulted in >96% of the radioactivity recovered in the hexane soluble fraction. TLC analysis of total lipids at 10 min of labeling indicated <9% of the radioactivity migrated with free sterols or waxes. Thus, >87% of [^{14}C]acetate incorporated into soybean lipids is in the acyl moiety of glycerolipids (examples of TLC separation of labeled lipid classes are in Appendix B Figure 6-3). The rate of [^{14}C]acetate incorporation into soybean lipid acyl fraction was 3.2 µmol [^{14}C]acetate $embryo^{-1}$ day^{-1} (data taken from experiment shown in Figure 3-2), and is approximately 1/10 of total embryo fatty acid synthesis rate expressed on a C2 unit incorporation basis (35 µmol acetyl-CoA $embryo^{-1}$ day^{-1} [170]. Therefore, assuming an equal distribution and utilization of acetate within the embryo tissue, statistically most of the fatty acids

synthesized during the labeling period contained a single [^{14}C]C2 unit. [^{14}C]Oleate (18:1) and [^{14}C]saturated FA (16:0, 18:0) (Appendix B Figure 6-1) are the immediate products of fatty acid synthesis. At 30 min of labeling less than 3% of the labeled FA were desaturated to [^{14}C]linoleate (18:2); therefore short time point labeling represents the initial acyl transferase reactions of newly synthesized FA, prior to further metabolism. Total labeled saturates increased from 23% of total FA at 2 min to 42% by 30 min of labeling. Most of the increase was due to an increase in stearic acid (18:0) labeling. Chemical α-oxidation was employed to determine the position of the label along the [^{14}C]18:0 acyl chain (Appendix B Figure 6-2). Label was evenly distributed along the chain. This result suggested that the 18:0 was a direct product of fatty acid synthesis and not labeled by a high specific activity cytosolic elongation as reported for other very long chain FA [171, 172]. The reason for 18:0>16:0 production when endogenous composition is 16:0>18:0 is unknown. The 18:0>16:0 labeling at early time points in soybean embryos is not expected to affect the total saturates acyl group fluxes as 16:0 and 18:0 behave in a similar fashion in acyl transfer reactions, and have the similar positional distributions in the major lipid classes.

Kinetics of [^{14}C]acetate labeling indicate independent rates of lipid class acylation

To determine the initial rates and precursor-product relationships for the incorporation of newly synthesized FA into soybean glycerolipids we followed

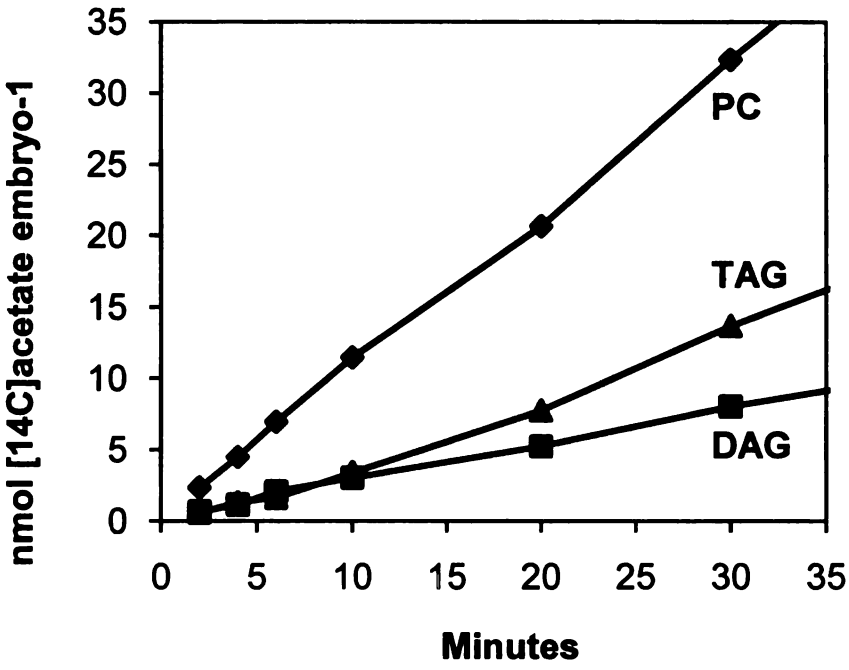


Figure 3-2 Time course for [^{14}C]acetate incorporation into the acyl groups of the major labeled soybean embryo lipids.

Results are based on the total radioactivity in each lipid class isolated by TLC and measured by liquid scintillation counting. Results expressed as nmol of [^{14}C]acetate incorporated per embryo for each lipid. Glycerolipid classes are PC, diamonds; TAG, triangles; DAG, squares. Time points in minutes are, 2, 4, 6, 10, 20, 30 and 120. The graph shows the first 35 min to focus on initial kinetics.

[^{14}C]acetate labeling into the major labeled soybean embryo lipids (Figure 3-2). At all time points PC, DAG and TAG represent ~85% of labeled glycerolipids. PC labeling was linear and gave the highest initial rate (1.15 nmol [^{14}C]acetate embryo^{-1} min^{-1}). DAG labeling was also linear over the 30 minute period and gave a lower initial rate (0.36 nmol [^{14}C]acetate embryo^{-1} min^{-1}). The initial rate of TAG labeling was the lowest of the three major species (0.26 nmol [^{14}C]acetate embryo^{-1} min^{-1}), however TAG labeling accelerated and accumulated more total radioactivity than DAG over time. Other membrane lipids contained very little of the newly synthesized FA. Phosphatidylethanolamine (PE) contained less than 8% of the radioactivity observed in PC even though PE mass abundance is approximately half that of PC. Phosphatidic acid (PA) is an intermediate of *de novo* glycerolipid synthesis, but PA labeling was barely detectable (less than 2% of that for PC). The high rate of fatty acid incorporation into PC is similar to acyl editing described in pea leaves where PC is the first incorporation product of chloroplast exported nascent FA [162].

The rate of incorporation of nascent FA into PC is independent of the incorporation of nascent FA into DAG or TAG indicating independent acylation reactions. As it is reasonable to assume that the specific radioactivity of the fatty acids for each process is identical, the relative initial acylation rates are about 10:3:2 respectively. The accelerating rate for TAG synthesis over the 30 minute period may represent the combination of three possible effects: (i) increasing contribution from very long-chain saturates, (ii) an accelerated initial rate, possibly due to higher levels of stearate, and (iii) the movement of labeled

diacylglycerol moieties from DAG and PC pools to TAG becomes measurable, although longer term assays will be required to detect concomitant reductions in DAG and PC labeling. In the next five sections we describe the acetate labeled PC, DAG and TAG products in more detail and summarize the product structures and kinetic results.

[^{14}C]Acetate labeled FA compositions differ among glycerolipid classes

Fatty acid methyl esters (FAME) of PC, DAG and TAG were separated by argentation TLC to determine [^{14}C]FA composition based on the number of double bonds (Figure 3-3). At 2 min of labeling [^{14}C]18:1 made up >95% of the labeled FA in PC and total labeled saturates slowly increased to approximately 25% at 30 min. By contrast total labeled saturates were relatively constant in DAG and TAG over the first 30 min of labeling. The average amount of saturate radioactivity between 4 and 30 min of labeling is 29% in DAG and 75% in TAG. The lower amount of labeled saturates at 2 min most likely represented acyl group pool filling. By 120 min radioactivity is detected in polyunsaturated fatty acids (PUFA) in all lipids, and the relative proportions of saturates have dropped as the newly synthesized FA are slowly equilibrated through metabolism to the endogenous FA compositions. The very different initial [^{14}C]FA compositions among PC, DAG and TAG reflects different metabolic processes for incorporation of nascent FA into glycerolipids, which is consistent with their independent acyl labeling kinetics.

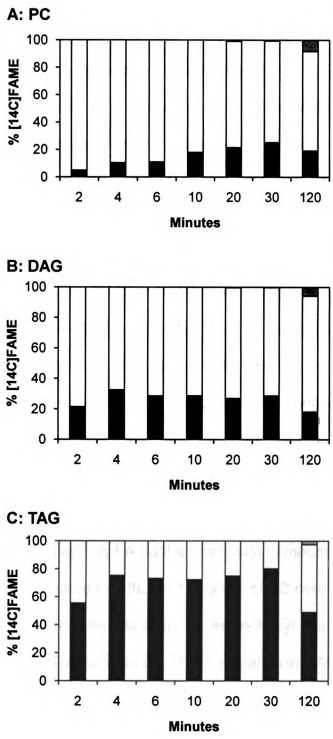


Figure 3-3 Fatty acid composition of [^{14}C]acetate labeled lipids.

A-C. Lipid classes from the time course assays were transmethylated and the FAME separated based on the number of double bonds by $AgNO_3$-TLC, and quantified by electronic autoradiography. Each bar shows the distribution of radioactivity among different acyl groups at each time point. Bar shading: total saturates, S, black; monoenes (18:1), M, white; dienes (18:2), D, gray. A, PC; B, DAG; C, TAG.

Differential positional acylation of [^{14}C]acetate labeled FA among lipid classes

The regiochemistry of labeled FA distributions in PC, DAG and TAG was determined by enzymatic digestion (Figure 3-4). At 2 min 86% of the newly synthesized FA in PC was esterified at the *sn*-2 position (Figure 3-4A). Over 10 min of labeling, the label at *sn*-1 increased from 14% to 26%, which was mostly due to an increase in saturates. The unequal distribution of nascent FA incorporation into PC is reminiscent of our previous results in pea leaves at 5 minutes, in which acyl editing produced PC labeled at *sn*-1 and *sn*-2 positions of 38 % and 62%, respectively [162]. However, acyl editing in soybean embryos appears to be more *sn*-2 specific than in pea leaves. The increase in PC *sn*-1 saturate labeling may reflect a slow conversion of *de novo* synthesized DAG into PC. An alternative explanation is that the high [^{14}C]*sn*-2 composition of early labeled PC may reflect a large amount of *sn*-1 re-acylation with unlabeled recycled saturated FA. As nascent [^{14}C]stearate replaces unlabeled palmitate in the acyl editing FA pool an increasing amount of labeled saturates are incorporated into the *sn*-1 position of PC over time. In this scenario *sn*-1 acyl editing may be just as active as *sn*-2 acyl editing.

The position of [^{14}C]FA acylation in DAG differs significantly from PC, in that at 2 min of labeling there is more *sn*-1 labeled FA than *sn*-2 (Figure3-4B). The relative *sn*-1 to *sn*-2 positional labeling equilibrated in DAG by 20 min. Two possible causes for this equilibration are: (i) equilibration of newly synthesized FA into a reacycled acyl group pool feeding *de novo* glycerolipid synthesis; or

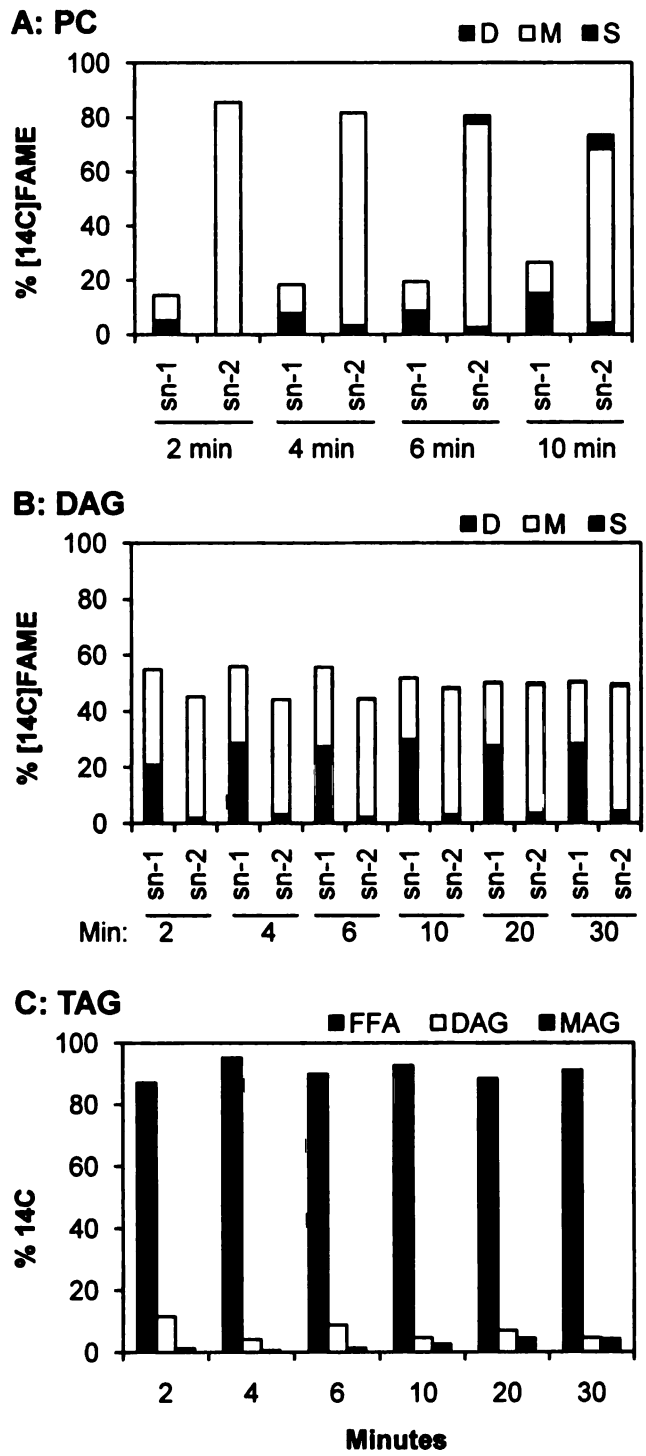


Figure 3-4 Positional analysis of [^{14}C]acetate labeled acyl groups in different lipids.

Major labeled lipids from the [^{14}C]acetate labeling time course were subject to lipase positional analysis. Bar heights represent percent of lipid label in that position. A-B. *Bar shading* represents the labeled FA as a percent of the whole lipid: total saturates, S, black; monoenes, M, (18:1), white; dienes, D, (18:2), gray. A, PC PLA_2 digestion. FAMEs generated from lipase products lyso-PC (*sn*-1) and FFA (*sn*-2) were further analyzed by $AgNO_3$-TLC. B, Acetylated DAG pancreatic 1,3 TAG lipase digestion. FAMEs generated from lipase products, FFA (*sn*-1) and 1-O-2-acyl-3-acetyl glycerol (*sn*-2) were further analyzed by $AgNO_3$-TLC. C. TAG pancreatic 1,3 TAG lipase digestion. Bar shading: FFA (*sn*-1 or *sn*-3), black; DAG (*sn*-1,2 DAG or *sn*-2,3 DAG), white; MAG (*sn*-2), gray.

(ii) highly *sn*-2 labeled PC conversion to DAG to balance out the higher *sn*-1 *de novo* synthesized DAG. Since PC labeling at 20 min is over 4 times that of DAG (Figure 3-2), the proportion of labeled PC moving back to DAG must be minor otherwise the DAG stereochemistry would reflect PC stereochemistry.

The results of the positional analysis of nascent [^{14}C]FA incorporation into TAG differ from both that of PC and DAG. Lipase digestion of TAG indicates that almost the entire label at early time points was *sn*-1 or *sn*-3 (lipase product: free fatty acid, Figure 3-4C). Labeled FA can be incorporated into only the *sn*-3 position of TAG by acylation of an unlabeled DAG; however any *sn*-1 labeling must come from *de novo* synthesis of DAG. Since the DAG regiochemistry is approximately equal at *sn*-1 and *sn*-2 positions, the amount of label at *sn*-1 of TAG is predicted to be at most equal to the amount of label at *sn*-2. However, the end product of the lipase digestion, MAG, which contains the sn-2 label, is less than 2% of the total end products (Figure 3-4). If any of the highly *sn*-2 labeled PC has been converted to DAG and then TAG, this would further reduce the amount of label at *sn*-1. Therefore we conclude that initial incorporation of nascent FA into TAG is almost completely localized to the *sn*-3 position.

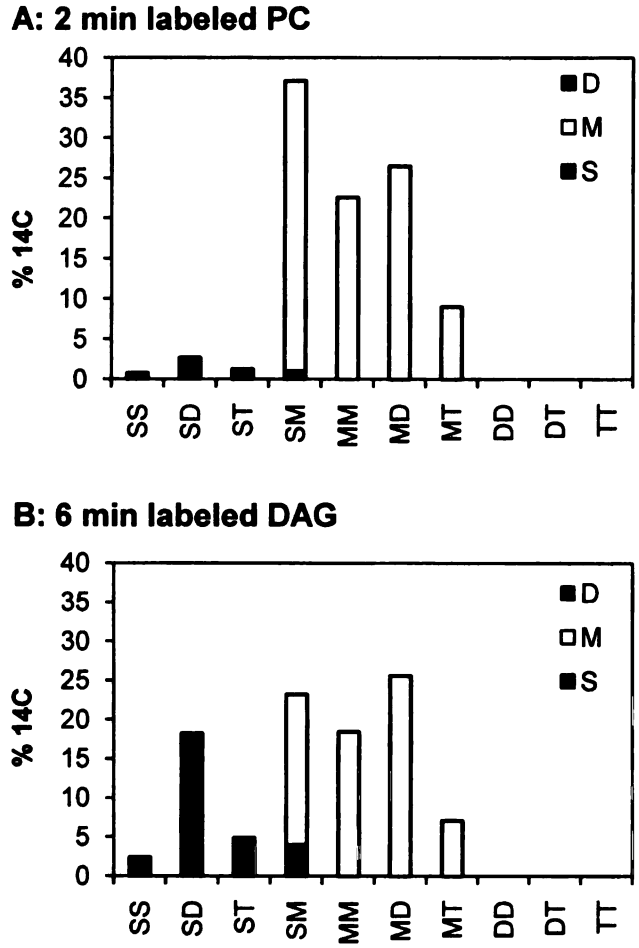


Figure 3-5 Molecular species compositions of [^{14}C]acetate labeled PC and DAG.

A-B, molecular species are represented as pairs of FA, with bar heights represent % of species among the total labeled lipid. Bar shading represent % of [^{14}C]FA among species. In species with only one color it means that only the colored FA is labeled the other FA within the molecular species is unlabeled. *Bar shading:* S, total saturates, black bars; M, monoenes (18:1), white bars; D, dienes (18:2), gray bars. Molecular species of labeled lipids were determined for the earliest time points that had enough radioactivity for the analysis. Examples of TLC systems used in molecular species analysis are in Appendix B Figure 6-3. A, 2 min labeled PC. B, 6 min labeled DAG. Additional time point labeled molecular species compositions for PC and DAG are in Appendix B Figures 6-4 and 6-5, respectively.

[^{14}C]acetate labeled PC and DAG molecular species contain one newly synthesized and one previously synthesized FA

The unequal [^{14}C]FA positional labeling of DAG and PC suggest at least some nascent, labeled FA are incorporated into DAG and PC next to an unlabeled FA. To further investigate this facet of product structure the molecular species of [^{14}C]acetate labeled PC and DAG were analyzed (Figure 3-5). Examples of the TLC systems used in molecular species analysis are given in Appendix B Figure 6-3, while the shorthand nomenclature used to describe DAG, PC and TAG molecular species is contained in the legend to Figure 3-1. At 2 min the labeled molecular species SM made up 37% of total radioactivity in PC, with almost all the label as [^{14}C]M (Figure 3-5A). Thus 35% of the total PC must be [^{12}C]S[^{14}C]M, while only up to 2% of total PC could be the dual labeled species [^{14}C]S[^{14}C]M. The majority of other species (40%) consisted of unlabeled PUFA alongside a nascent labeled S or M. Assigning the labeled MM molecular species of PC (22%) precisely as single or dual labeled is more problematic. Given that labeled oleate was highly incorporated at the sn-2 position in total PC the possibility that MM was largely the dual labeled species is highly unlikely. Furthermore, at least 76% of nascent FA were incorporated into PC alongside unlabeled S, D or T in proportions relative to their endogenous composition and the position of acylation. Endogenous PC contained 30% M (Table 1), which was almost as much as S and T together. Therefore it is highly likely that nascent

[^{14}C]S and [^{14}C]M were mostly incorporated next to endogenous unlabeled M in the labeled SM and MM molecular species, respectively.

[^{14}C]Fatty acid positional analysis of DAG indicated that up to 90% of molecular species could be produced solely from matching *sn*-1 and *sn*-2 acylations with nascent [^{14}C]S and [^{14}C]M (Figure 3-4B). However, molecular species of [^{14}C]acetate labeled DAG at 6 min (3- 5B) indicated that 56% of initial labeled molecular species contained a nascent labeled FA next to an unlabeled PUFA. In the labeled DAG molecular species SM, the unequal S and M labeling indicated that at least an additional 15% of total DAG is [^{12}C]S[^{14}C]M. Therefore molecular species analysis of DAG indicates that at least 71% of DAG contains a newly synthesized FA incorporated in the same molecule as previously synthesized FA. As with the PC, this analysis cannot reveal how much of the remaining SM and MM are dual labeled but it is probably much less than 29%. We conclude that initial [^{14}C]acetate labeled PC and DAG are comprised predominantly of molecular species that contain one labeled newly synthesized FA and one unlabeled previously synthesized FA.

[^{14}C]Acetate labeled TAG molecular species indicates bulk DAG was used for *sn*-3 acylation with nascent [^{14}C]FA

Six minutes is the earliest time point where enough radioactivity has accumulated in TAG to conduct a full molecular species analysis. At this time the TAG molecular species distribution labeled with nascent FA does not match the

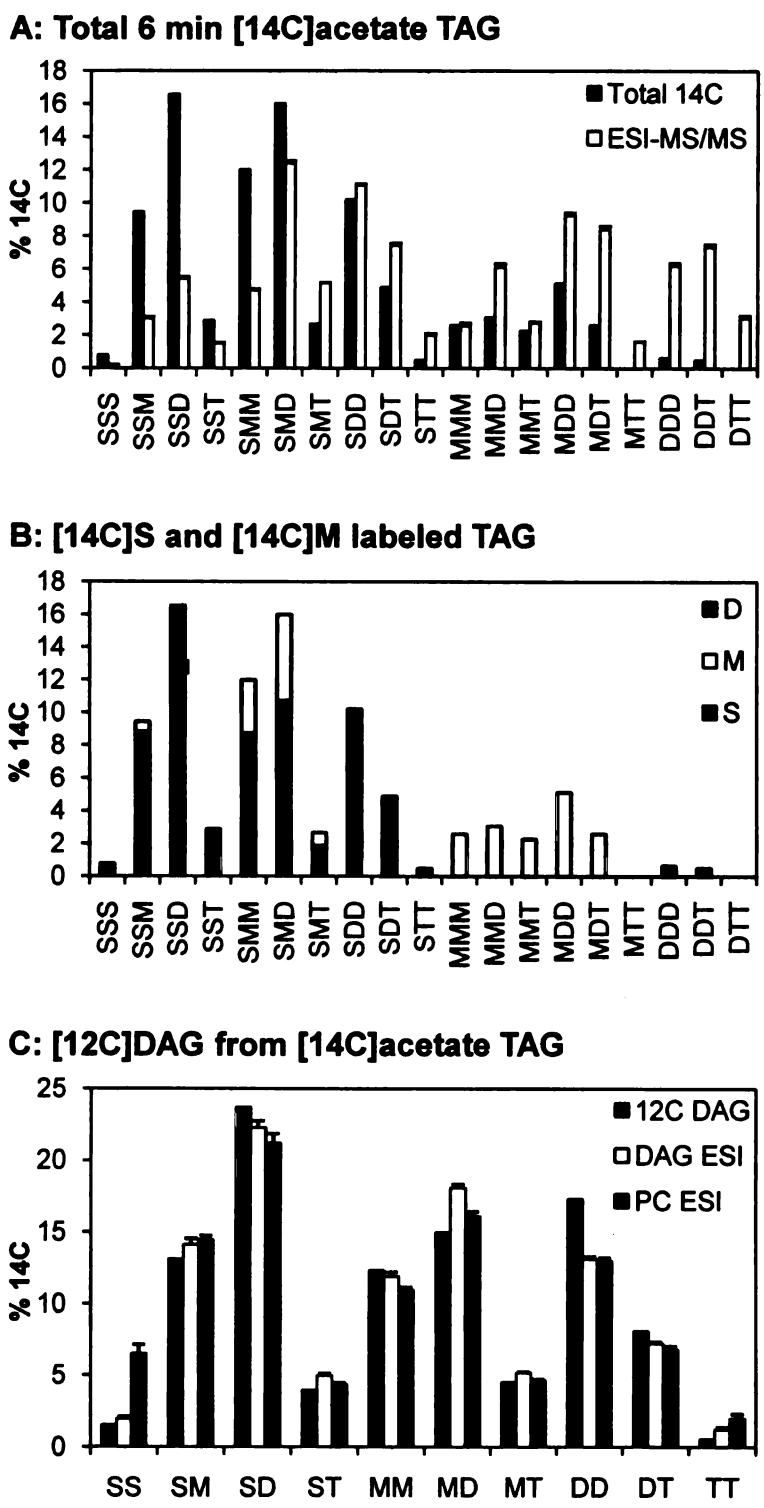


Figure 3-6 Six min [^{14}C]acetate labeled TAG molecular species.

A, Total 6 min [^{14}C]acetate labeled molecular species (Black bars), compared to endogenous molecular species (White bars).B, 6 min [^{14}C]acetate labeled TAG molecular species with [^{14}C]FA labeling. Bar shading represents the labeled FA within each molecular species. Saturates, S, black; monoenes (18:1), M, white; dienes (18:2), D, gray; trienes, T. C, the calculated unlabeled DAG molecular species associated with labeled TAG in panel B, assuming one *sn*-3 labeled FA per molecular species, black bars. Endogenous molecular species from Figure 3-1, DAG (white bars), PC (gray bars).

endogenous molecular species distribution (Figure 3-6A) but, as expected, is enriched in species containing S and M and is deficient in species containing two or three polyunsaturated fatty acids. The positional analysis described above indicated that nascent FA incorporation into TAG was primarily in the *sn*-3 position. Therefore the [^{14}C]acetate-labeled TAG molecular species in Figure 3-6A each contain one labeled S or M and two unlabeled FA. The relative proportions of [^{14}C]S and [^{14}C]M labeled TAG are shown in Figure 3-6B. The calculated unlabeled [^{12}C]DAG molecular species composition associated with the *sn*-3 [^{14}C]FA labeled TAG was very similar to the endogenous bulk DAG and PC molecular species compositions (Figure 3-6C). Therefore it appears that DAG generated from the bulk PC pool is utilized for TAG synthesis with newly synthesized FA. Furthermore, inspection of Figure 3-6B reveals that there is little DAG selectivity when considering either [^{14}C]S or [^{14}C]M individually for esterification at the *sn*-3 position of DAG. However, this does not rule out selectivity of DAG molecular species for esterification of 18:2 or 18:3 to the *sn*-3 position.

Conclusions from [^{14}C]acetate labeling

The [^{14}C]acetate labeling kinetics, and the different labeled FA composition, positional acylation and molecular species of DAG, PC and TAG reveal that newly synthesized DAG is not a direct immediate precursor for nascent FA incorporation into PC or TAG. Three different acyltransferase pathways (*de novo sn*-1 and *sn*-2 acylations to produce DAG, PC acyl editing,

and *sn*-3 acyl transfer to produce TAG) act independently of each other for the incorporation of nascent FA into glycerolipids. This clarifies ambiguities in previous seed labeling studies about the overlap of these pathways, at least for developing soybean embryos. The rapid labeling of PC largely at the *sn*-2 position suggests an acyl editing mechanism in which FA is largely removed from the *sn*-2 position of PC to generate *sn*-1-acyl-2-lyso-PC, which is then re-esterified with nascent FA. The GPAT and LPAT of *de novo* glycerolipid synthesis utilize nascent acyl groups mixed with unlabeled acyl groups to produce the equal regiochemical labeling and mixed molecular species labeling of [^{14}C]acetate labeled DAG. Presumably some of the unlabeled acyl groups released from PC during acyl editing are recycled for *de novo* glycerolipid synthesis. The labeled acyl composition, positional, and molecular species analyses of TAG at early time points demonstrate clearly that nascent FA are esterified to the *sn*-3 position of DAG by an acyltransferase system that is selective towards saturates but not to DAG molecular species. Furthermore, the DAG is likely derived from the bulk PC pool. Direct incorporation of nascent FA by PC acyl editing, *de novo* DAG synthesis and TAG synthesis from preformed DAG together account for about 85% of all newly synthesized FA incorporated into cytosolic glycerolipids, while the ratio of initial rates of incorporation (approximately 10:3:2, respectively), demonstrates that the major flux is a rapid acyl editing mechanism with PC, similar to our previous results in pea leaves [162].

[^{14}C]glycerol labeling of lipid backbones and acyl chains indicate a common backbone pathway and separate acylation pathways among lipid classes

Lipids produced by *de novo* glycerolipid synthesis can be tracked with [^{14}C]glycerol, which is rapidly taken up by developing soybeans and incorporated into the backbone of glycerolipids through G3P acylation. The polar head-group of PC is typically not labeled from glycerol in short duration incubations (lasting a few hours or less) [86, 162]. However, in addition to backbone labeling, acyl groups are also rapidly labeled because G3P can provide precursors for plastidic acetyl-CoA synthesis. The glycerol backbone and acyl moiety labeling can be measured separately after lipid transmethylation. For cultured soybean embryos the labeled nascent FA composition produced by [^{14}C]glycerol was very similar to that from [^{14}C]acetate (data not shown) indicating that *de novo* fatty acid synthesis products were not dependent on the substrate.

The kinetics of [^{14}C]glycerol backbone labeling of PC, DAG and TAG (Figure 3-7A) was distinctly different from acyl labeling of glycerolipids by [^{14}C]acetate (Figure 3-2). At 2 min of [^{14}C]glycerol labeling the backbone incorporation into DAG was already linear and was approximately four times the initial rate for PC and more than eight times that of TAG. However, PC [^{14}C]backbone labeling rapidly accelerated such that by 30 min PC contained more label than DAG and by 120 min it was approaching the ~2:1 molar ratio of endogenous PC:DAG. Thus the DAG and PC labeling showed the expected

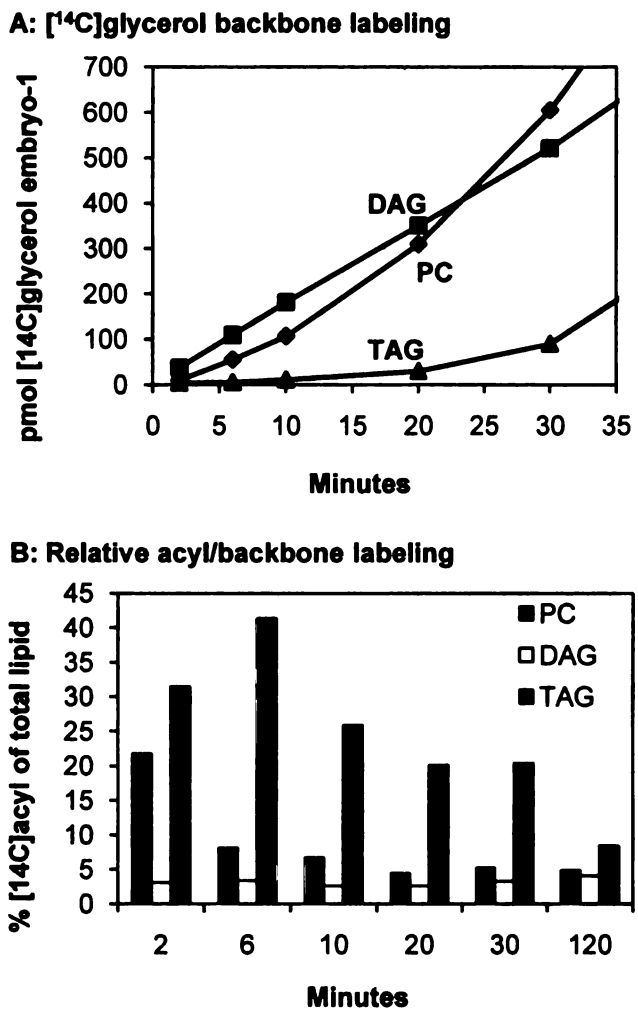


Figure 3-7 Time course for the incorporation of [^{14}C]glycerol into the backbone and acyl groups of the major labeled soybean embryo lipids.

[^{14}C]glycerol labeled lipids were isolated by TLC and transmethylated to separate the glycerol moiety from the acyl groups, each fraction quantified by liquid scintillation counting. A, time course of only glycerol backbone moiety labeling. Results expressed as pmol [^{14}C]glycerol incorporated per embryo over the time course (2, 6, 10, 20, 30 120 min). The graph shows the first 35 min to focus on initial kinetics. B, % of total lipid [^{14}C]glycerol labeling that is in the acyl chains over the time course. Bar shading: PC, black bars; DAG, white bars; TAG, gray bars.

precursor-product relationship kinetics. Incorporation of [^{14}C]glycerol into the backbone of TAG lagged significantly behind that of DAG and PC. TAG labeling was still accelerating after 120 min but had not reached the level of labeling observed for PC or DAG even though endogenous levels of TAG accumulate to ~20 times that of PC. The extreme lag of TAG (as compared to PC) behind DAG labeling suggests that the bulk of TAG synthesis (> 98%) is not related to rapidly labeled *de novo* synthesis of DAG. TAG labeling is more consistent with a precursor-product relationship with PC. The backbone labeling kinetics in developing soybeans are consistent with a major TAG synthesis pathway of G3P to DAG, to PC, to DAG, and finally to TAG and complement the molecular species analysis of [^{14}C]acetate labeled TAG, that had an unlabeled DAG moiety very similar to bulk DAG and PC. The Discussion elaborates on this hypothesis and includes evidence from mathematical modeling of the labeling data through different possible pathways.

Approximately 5-10% of [^{14}C]glycerol is rapidly incorporated into acyl chains. Steady-state [^{14}C]glycerol labeling of acyl and backbone precursor pools coincides with a constant acyl to backbone labeling ratio. The dual acyl/backbone labeling from [^{14}C]glycerol confirms the conclusions made from separate [^{14}C]acetate acyl and [^{14}C]glycerol backbone labeling experiments. Figure 3-7B shows the percent of acyl labeling from [^{14}C]glycerol acyl/backbone labeled lipids. At 2 min PC contains 22% of the label in the acyl chains, a value which drops to a steady-state level of ~5% by 20 min. This is consistent with rapid incorporation of nascent FA into PC through acyl editing while the backbone label lags through

the *de novo* glycerolipid synthesis via the DAG pool. The constant ~5% acyl labeling of DAG over the time course represents nascent FA and backbone entering glycerolipids together by *de novo* G3P acylation. Initially TAG contains the largest proportion of radiolabel in acyl chains and it takes over 120 min to equilibrate to the level of PC and DAG. This result supports the hypothesis that nascent FA can be esterified at the *sn*-3 position to a DAG pool that is not immediately produced from *de novo* synthesized DAG and that TAG steady-state labeling is consistent with backbone lagging through the large PC/DAG bulk pool.

Analysis of acyl chains utilized by *de novo* glycerolipid synthesis through molecular species separation of [^{14}C]glycerol backbone labeled lipid classes

Separation of the backbone-labeled lipid molecular species at early time points allows for analysis of the acyl groups used for acylation of G3P. The initial molecular species of backbone-labeled DAG and PC are very similar (Figure 3-8 A-B, respectively), indicating *de novo* synthesized DAG is converted to PC without molecular species selectivity. The slightly higher SD and lower MM levels in labeled DAG compared to labeled PC might indicate a differential utilization of DAG molecular species by a low flux pathway, such as for PE or PI synthesis. The newly synthesized backbone labeled molecular species composition (Figure 3-8A, B) of DAG and PC contain relatively less polyunsaturated molecular species than the endogenous PC and DAG molecular species,

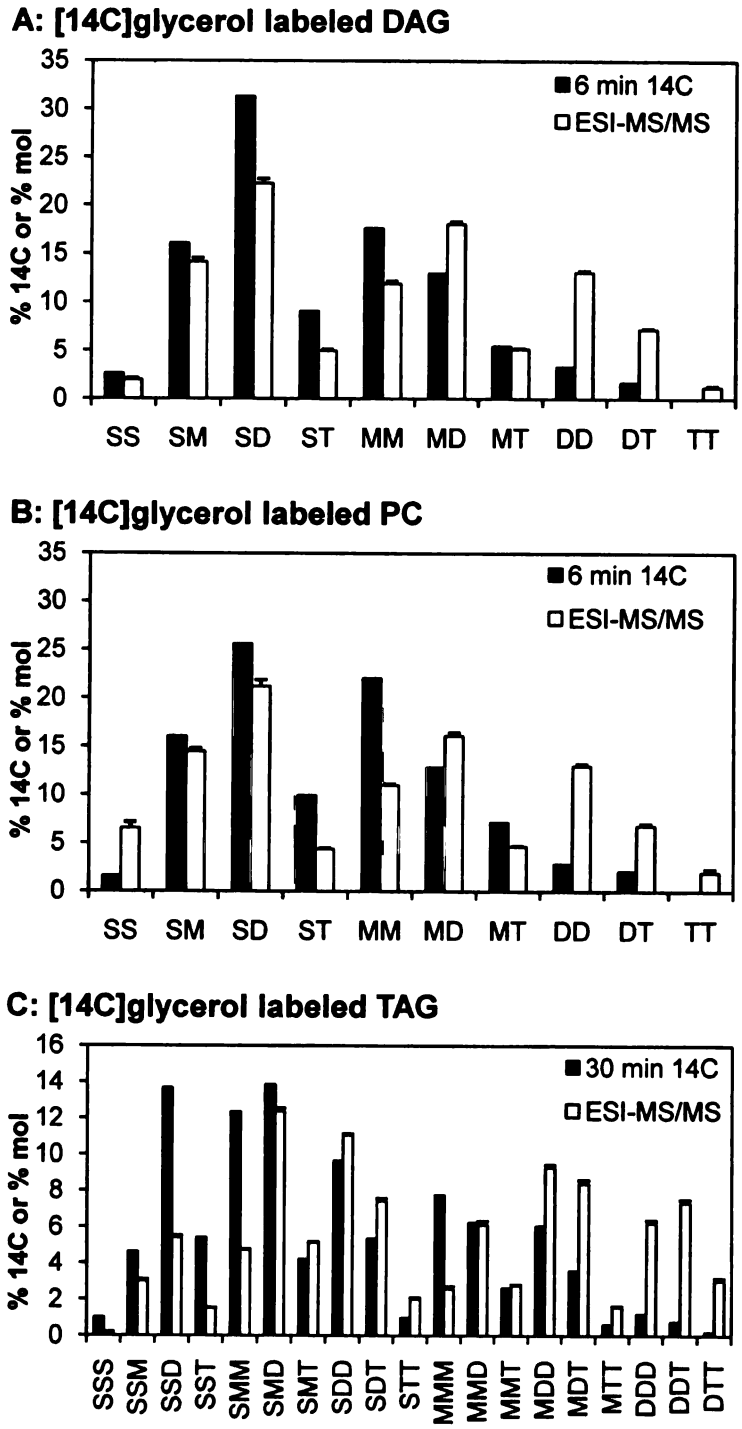


Figure 3-8 Molecular species composition of [^{14}C]glycerol backbone labeled PC, DAG and TAG.

A-C, molecular species are represented as a combination of two or three FA. Total saturates, S; monoenes (18:1), M; dienes (18:2), D; trienes (18:3), T. [^{14}C]glycerol labeled molecular species are backbone labeling only (acyl chain labeling has been subtracted), black bars. Molecular species were determined at the earliest time point with enough radioactivity for analysis (Figure 3-7A). The endogenous mass molecular species compositions are from ESI-MS/MS measurements, white bars. A, 6 min labeled DAG, 10 & 30 min time points are in Appendix B Figure 6-7B. B, 6 min labeled PC, 10 & 30 min time points in Appendix Figure 6-7A. C, 30 min labeled TAG was the minimum labeling time with enough radioactivity for analysis.

respectively. Because acyl group desaturation is believed to occur on PC, but not DAG, *de novo* DAG has to be converted to PC for further desaturation. And as mentioned earlier the endogenous bulk PC and bulk DAG molecular species compositions are very similar (Figure 3-1A). From this deduction and the observation that the bulk DAG pool molecular species profile (Figure 3-1A) is similar to that used for TAG synthesis (Figure 3-6B) and not the *de novo* synthesized DAG composition (Figure 3-8A), we conclude that the immediately *de novo* synthesized DAG can contribute only a relatively small fraction to the total endogenous DAG pool. Instead, the bulk of the endogenous DAG must be generated by another route. That route involves the removal of the phosphocholine head-group from PC to produce DAG, by one of several possible mechanisms such as a reversal of the cholinephosphotransferase reaction or by phospholipase C action.

[^{14}C]Glycerol labeling of the TAG backbone was insufficient for molecular species analysis until 30 min of labeling. However, at 30 min the [^{14}C]glycerol backbone labeled TAG molecular species (Figure 3-8C) showed a correspondence to that of initial [^{14}C]glycerol labeled PC and DAG in that the labeled molecular species composition was relatively less polyunsaturated as compared to the endogenous composition of the corresponding lipid. The calculated FA composition esterified to backbone labeled PC, DAG and TAG was also very similar (Appendix B Figure 6-8). However, the FA composition of TAG labeled with [^{14}C]glycerol in the backbone was enriched in S and M, and depleted

in D and T, when compared to the FA composition of endogenous TAG. Further interpretation of these results is left to the Discussion section.

3.4 Discussion

The goal of this study is to provide a quantitative analysis of acyl fluxes in the developing embryo of an oilseed, from the point where free fatty acids are exported from the plastid to the formation of TAG. It is a timely exercise, given the advances in identification of acyltransferase genes in recent years. The literature describing *in vivo* labeling of developing seeds suggests various metabolic pathways. However, collectively, it suffers from a number of problems. (1) The tissue is excised and immediately incubated, often in the absence of an osmoticum or nutrients. Thus its physiological state relative to the in planta situation is uncertain. (2) It has been particularly difficult to assess flux directions and pools for the DAG-PC conversions. (3) Interpretation based on initial rates of reaction is under-utilized, and there is often a lack of comprehensive product analysis. (4) Quantitative conclusions have rarely been possible. In order to undertake initial rate studies we have used C2/C3 precursors. These are rapidly taken up and utilized, allowing the system to quickly reach a (quasi) steady state labeling condition and hence the opportunity to obtain initial rate data. By contrast labeled hexose substrates face very large dilutions and take too long to reach a steady state. At the acetate concentrations employed a significant

absolute rate of incorporation (about 10%) relative to the endogenous rate of C2 utilization for fatty acid synthesis was measured.

Newly synthesized FA are incorporated into cytosolic glycerolipids through three independent acyltransferase pathways

Through analysis of the kinetics of glycerolipid acyl labeling from acetate, and analysis of the FA composition, position of acylation, and molecular species of these products, we demonstrate that three different acyltransferase systems are responsible for incorporation of newly synthesized FA into cytosolic glycerolipids. ***1) The major flux of nascent FA is through acyl editing of PC.*** The highest rate of FA incorporation was into PC. There was no detectable kinetic lag to the onset of steady-state labeling of PC, indicating that PC is the immediate product of an acyltransferase utilizing nascent FA. Additionally the labeled FA composition and stereochemistry of PC indicated it was not related to the initial labeled DAG, a possible precursor. Molecular species and FA positional analyses of PC demonstrated that at least 80% of newly synthesized FA are esterified in the same molecule as pre-existing FA. Finally, [^{14}C]glycerol acyl and backbone labeling showed that initial labeled acyl groups and backbone are incorporated into PC independently. Together these results reveal that about 57% of newly synthesized FA are directly esterified to the *sn*-1 or *sn*-2 position of PC through acyl-editing mechanisms. Because GPCAT activity has not been described in developing oilseeds, whereas microsomal *sn*-2 LPCAT and acyl exchange activities are high, we ascribe the acyl editing to acylation of lyso-PC

[130, 140, 142, 144, 163]. ***2) Newly synthesized FA mixed with recycled FA to acylate G3P for de novo glycerolipid synthesis.*** Approximately 17% of newly synthesized FAs in glycerolipids were incorporated into DAG, approximately equally distributed between the *sn*-1 and *sn*-2 positions. At least 71% of DAG molecular species contained newly synthesized and pre-existing FA in the same molecule. [^{14}C]glycerol acyl/backbone labeling demonstrated that nascent acyl groups and backbone are incorporated into DAG simultaneously. These results demonstrate that the GPAT and LPAT of the eukaryotic *de novo* glycerolipid synthesis pathway utilize a mixed pool of acyl-CoA containing newly synthesized and recycled FA. The recycled FA most likely originate from PC acyl editing. ***3) Newly synthesized FA were utilized for sn-3 acylation of pre-existing DAG to generate TAG.*** Approximately 11% of newly synthesized FA was directly incorporated into the *sn*-3 position of TAG, with negligible labeling in the *sn*-1 and *sn*-2 positions. Nascent FA incorporation into TAG was kinetically independent of *de novo* DAG synthesis. TAG molecular species analysis indicated that the DAG used had the same composition as the endogenous bulk DAG derived from PC. Therefore the direct incorporation of nascent FA into TAG utilized a pre-existing DAG pool, not *de novo* synthesized DAG. In a later section below we use the relative rates of these three independent acyl transferase pathways to construct a more quantitative model of TAG synthesis in soybeans. This model takes account of PC acyl editing, which has not be previously included in models of oilseed TAG metabolism.

At least two systems of DAG *sn*-3 acylation producing TAG can be identified

The first flux, as mentioned above, involves 11% of the total FA labeling that is directly incorporated into the *sn*-3 position of TAG. Other nascent FA are incorporated into glycerol lipids through acyl editing of PC, and *de novo* DAG synthesis which constitute approximately 57% and 17% of the total fatty acid labeling, respectively. The remaining 15% of acyl label unaccounted in the above three glycerolipid classes is present largely in minor phospholipid species. As TAG constitutes approximately 93% of the endogenous acyl lipid mass, eventually about 31% of the nascent fatty acids must end up in the *sn*-3 position of TAG. Thus about 20% of the nascent fatty acids incorporated into glycerolipids other than TAG must eventually move through into the *sn*-3 position of TAG to add to the 11% that are immediately incorporated. This represents a second, distinctive flux of FA into the *sn*-3 position of TAG.

When compared to endogenous TAG, the molecular species analysis of TAG from [^{14}C]acetate labeling (Figure 3-6C) also strongly suggests two pathways to TAG, of approximately equal contribution. We estimated that TAG labeled with nascent FA at the *sn*-3 position provides approximately 35% of total TAG synthesis and has a total FA composition of 39% S, 29% M, 25% D and 7% T, with the unlabeled DAG coming from the endogenous DAG pool (Figure 3-6B) and *sn*-3 made up of 75% S and 25% M (Figure 3-3C). Since the endogenous TAG composition is 20% S, 27% M, 41% D and 12% T, it is likely that a separate contribution to TAG synthesis is required to provide the remaining 65% of TAG.

This separate TAG synthesis must produce TAG of the composition 10% S, 26% M, 49% D and 15% T to balance out the endogenous composition. It is clear that this second TAG synthesis component is very different, containing reduced saturates and much higher PUFA than the initial [^{14}C]acetate labeled TAG. The DAG moiety presumably comes from the bulk unsaturated DAG/PC pool, with a selectivity for *sn*-3 acylation for PUFA.

The molecular species analysis of [^{14}C]glycerol labeled TAG (Figure 3-8C) is also consistent with two pathways to TAG. We can group the molecular species of TAG into three groups; (1) high specific activity species SSS, SSM, SSD, SST, SMM, MMM (about 16% of TAG endogenous mass); (2) low specific activity species MTT, DDD, DDT, DTT (about 20% of endogenous TAG mass) (and possibly STT, MDD and MDT), and (3) the other species, of intermediate specific activity (about 64% of endogenous TAG mass). Clearly, rapidly labeled (i.e. high specific activity) molecular species are dominated by high levels of S and M, consistent with the nascent fatty acid labeling, while slowly labeled species have high levels of D and T. Time is required for further desaturation of the PC species derived from *de novo* synthesized DAG, to eventually produce DAG species with both positions occupied by PUFA. These can then be utilized by the TAG synthesis system that acylates the *sn*-3 position with PUFA, to produce TAG molecular species with all three positions occupied by PUFA. A PUFA selective DGAT or PDAT may be involved in this system.

A DAG/DAG transacylase [122] has been proposed for the synthesis of TAG. Unsaturated FA would be transferred from the *sn*-2 position of one DAG

molecule to the *sn*-3 position of another, producing TAG and a *sn*-1-acyl-MAG. Re-acylation of MAG by MAGAT would bring about a significant enrichment of label in the sn-2 position of DAG. This is not observed. Therefore this pathway is not a major route of TAG synthesis in developing soybean embryos.

Interconversion of DAG and PC and the supply of DAG for TAG synthesis

It is clear from previous studies on oilseed metabolism that DAG and PC can be inter-converted. This is one way in which PUFA can be enriched for TAG biosynthesis. This inter-conversion is usually postulated to result from a highly reversible CPT [117, 118, 173]. It has been suggested that the PC and DAG pools are in equilibrium. The kinetics of glycerol backbone labeling by soybean embryos shown in Figure 3-7 are similar to those observed for detached linseed cotyledons [127], and provides the strongest evidence that the majority of TAG synthesis is from bulk DAG, via retro-conversion from PC, and not *de novo* synthesized DAG. If TAG and PC synthesis competed for the *de novo* DAG pool, with no opportunity for PC conversion back to DAG (Figure 3-9, Model A), TAG should label up 20x more rapidly than PC, to generate the desired ratio of end products. However, the converse is observed: PC labels up about 10x more rapidly than TAG (Figure 3-7A). The observation that the DAG pool used for *sn*-3 acylation with [^{14}C]acetate labeled nascent acyl groups closely resembles the endogenous DAG and PC molecular species profiles (Figure 3-6B), and not that of the de novo DAG pool (Figure 3-8B), is also consistent with DAG first moving to PC, then back to DAG.

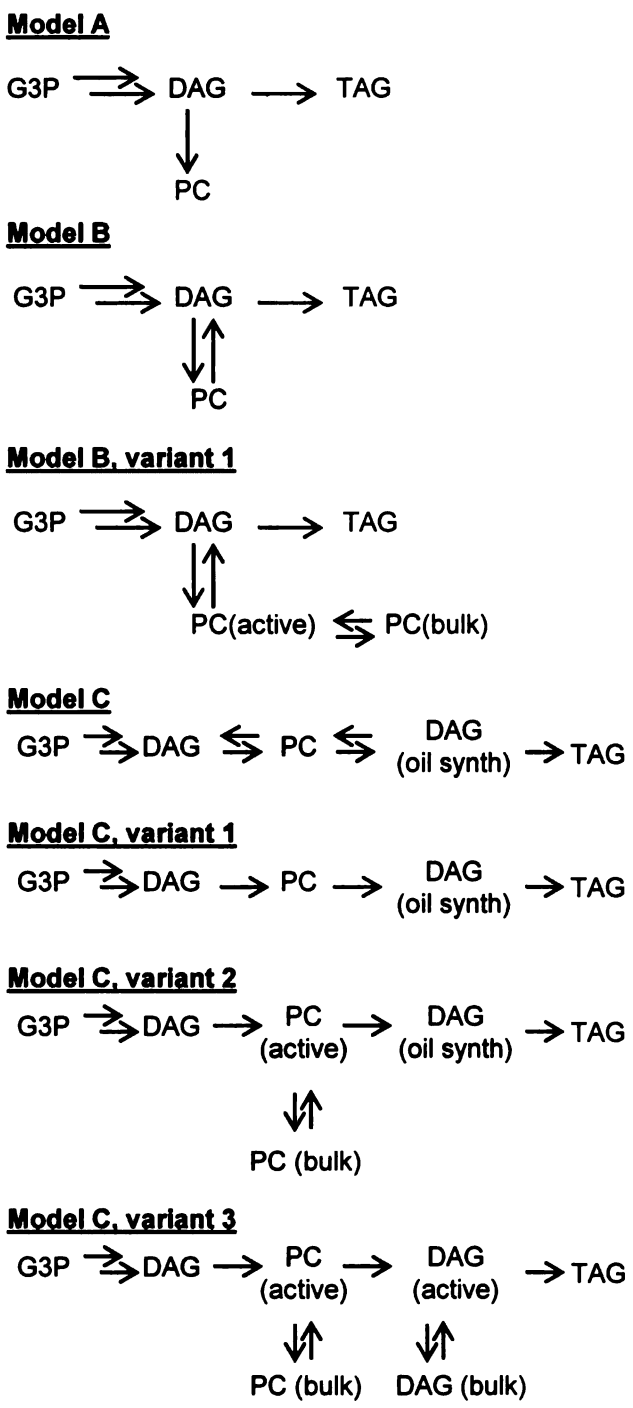


Figure 3-9 Representations of mathematical models for TAG synthesis.

Mathematical models tested against the kinetic labeling data by running simulations. Descriptions of the simulations are in Appendix B. As mentioned in the text the mathematical model that most fits the acetate and glycerol labeling is Model C, variant 3.

The required DAG-PC inter-conversion might be supplied by different mechanisms, each of which may display explicit kinetic consequences. The simplest would be where both PC and DAG are the bulk biosynthetic pools (Figure 3-9, Model B). However, the DAG pool could be split into *de novo* and oil synthesis (oil synth) pools. Such a general mechanism (Figure 3-9, Model C) might be simplified if the reversible reactions were replaced by unidirectional reactions (Figure 3-9, Model c, variant 1). Pool filling simulations for Models B and C, and several variants, were run to see how closely they would conform to the experimental kinetics of glycerol backbone labeling shown in Figure 3-7A. The variants included splitting PC and DAG pools into two pools, which would consist of a small biosynthetically active pool in kinetic equilibrium with a large bulk pool, for example (Figure 3-9 Model B, variant 1). Details of these simulations are provided in the Appendix B. Conditions were set to allow a simulation of glycerol backbone labeling from DAG that produced continuous DAG labeling but where PC labeling crossed over DAG labeling at about 30 minutes as in the experimental results (Figure 3-7A). The backbone labeling models were then tested against the acyl chain labeling results (Figure 3-2) to see if, when PC was labeled through acyl editing, how quickly would the acyl label move from PC to DAG. If the equilibration of [^{14}C]acetate acyl label at the *sn*-1 and *sn*-2 positions of DAG is from PC conversion to DAG, in 30 minutes of assay no more than about 10% of the labeled acyl groups that accumulated in DAG can originate from PC (which is mostly *sn*-2 labeled through acyl editing, Figure 3-4A-B). However, the total label in PC is 3 times that of DAG, so only

about 3% of the total labeled acyl groups in PC originating from acyl editing can move through to DAG in 30 minutes. Simulations of acyl labeling with Model B failed to meet this criterion. In a simulation of Model B, variant 1 with the acyl editing input through PC(bulk), the movement of labeled acyl groups from acyl editing into DAG was still far too fast. By contrast, models of type C were generally much more successful at describing the DAG-PC precursor-product glycerol labeling kinetics and yet having slow movement of acyl editing labeling of PC move through into DAG.

A observation in setting up the model C concerns the possibility of splitting the PC pool into a small, active pool and a large, bulk pool (Figure 3-9, Model C, variant 2). If this is done, then acyl editing must be largely a function of the bulk pool, not the active pool. Although several variants of Model C gave good simulations, one problem was that they predicted movement of glycerol labeling into TAG that was much slower than observed. This could be remedied by splitting the DAG(oil synth) pool into a small active and a large bulk component (Figure 3-9 Model C, variant 3). The de novo DAG pool must be small because it is compositionally distinct from the bulk DAG pool. In addition, in leaves, the endogenous DAG is very small, constituting less than 1% of the PC (Mhaske V. and Pollard M., unpublished data). This DAG presumably encompasses the *de novo* synthesized DAG pool we propose in Model C. In summary, the kinetics of [^{14}C]glycerol labeling suggest that the major flux of glycerol-3-P through *de novo* synthesis into DAG is for PC synthesis, with almost none of the *de novo* synthesized DAG being channeled directly to TAG. The conversion of *de novo*

synthesized DAG to PC is fairly rapid, but residence in the large PC pool is much longer to allow for further desaturation, before conversion back to DAG for TAG synthesis. The *de novo* synthesized DAG and the DAG pool destined for oil biosynthesis are kinetically distinct and not in rapid equilibrium.

A flux model for glycerolipid synthesis in developing soybean embryos

Our analysis of glycerolipid acyl group and glycerol backbone labeling allows us to generate a model of the flux of acyl groups during oil synthesis (Figure 3-10). The individual elements of this model have already been discussed in the three preceding sections. A detailed step-by-step logic of model construction, along with its implicit assumptions and calculations, is presented in Appendix B. The flux model structure is based on the kinetic model C, variant 3 (Figure 3-9) which fit the [^{14}C]acetate and [^{14}C]glycerol labeling data. The flux model tracks 100 moles of fatty acids synthesized in the plastid through lipid metabolism over a small time increment. The fluxes are determined based off of initial rates of nascent FA incorporation into extra-plastidic glycerolipids (Figure 3-2), their composition and molecular species (Figures 3-3, 3-4, 3-5, 3-6), along with determinations of the acyl groups used by *de novo* glycerolipid synthesis (Figure 3-8), and utilizes endogenous lipid compositions (Table 3-1, Figure 3-1) and mass balance to predict fluxes. Assuming steady state metabolism the end point reached in the developing soybean embryo accumulating oil will be 93 moles of acyl groups in TAG, 1.4 moles in DAG, 3.6 moles in PC and 2 moles in

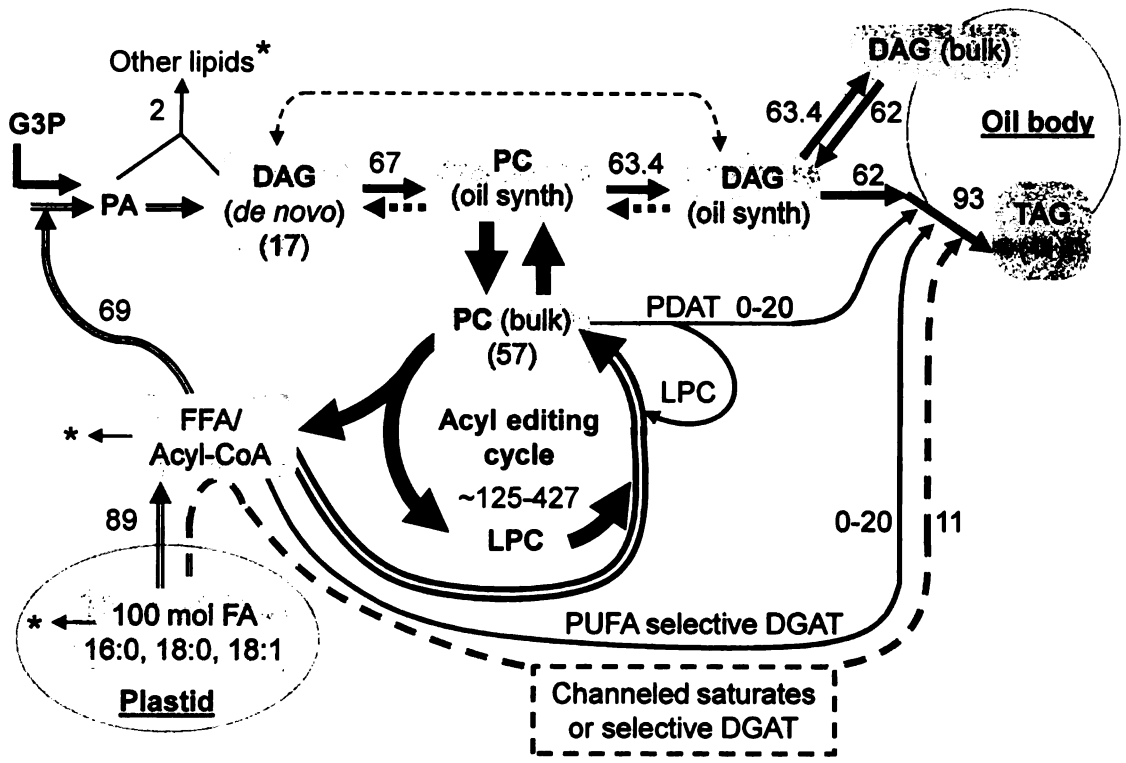


Figure 3-10 Soybean TAG synthesis flux model.

The model assumptions and flux calculations are in Appendix B. The model follows 100 moles of newly synthesized FA in the plastid through the major fluxes of lipid metabolism such that final accumulation of FA are TAG 93 moles, PC 3.6 moles, DAG 1.4 moles, other lipids 2 moles. Other membrane lipids in soybean embryos are mostly made up of PE and PI. The arrows demonstrate the flux reactions with the major metabolite pools highlighted in boxes. All of the arrows represent steady-state fluxes, the black arrows are the route that nascent FA take for incorporation into glycerolipids as measured by [^{14}C]acetate labeling. Dashed arrows represent uncertainty for that reaction. The numbers next to flux arrows represent steady-state fluxes or flux regions as calculated in Appendix B and utilizing mass balance for unknowns. The numbers in parentheses within the substrate pools represent the major initial accumulation of nascent FA in glycerolipids PC, DAG and TAG accumulate ~85% of initial FA. The * represents small fluxes of acyl groups to other lipids which is ≤ 2% of the total steady-state acyl groups. ~15% of nascent FA initially accumulate in lipids other than PC, DAG and TAG.

other lipids (based on the values in Table 3-1). The model shows the fluxes, or expected flux ranges, for both the newly synthesized fatty acids and for the total flux. It should be noted that in terms of an activity (expressed in moles fatty acid accumulated unit time^{-1} unit fresh weight^{-1}) the relative rate of lipid synthesis in soybean is slow (about 5 fold less) compared to other oilseeds such as safflower and rapeseed. Thus the model may or may not be a quantitative or qualitative representation of other oilseeds which accumulate palmitate, stearate, oleate, linoleate and linolenate.

The model has a single PC pool. Although it is possible to consider organelles and specific domains of the endomembrane system giving rise to distinct sub-pools of PC, to date we have no kinetic or metabolic evidence to support such a conclusion. By contrast, our analysis strongly suggests three DAG pools. Pools for the immediate product of de novo synthesis of DAG and for TAG synthesis each contain only a small mole fraction of the total DAG, while the bulk DAG pool may be associated with the oil body fraction. We cannot rule out the possibility that a very small fraction of the *de novo* DAG pool is utilized directly for TAG synthesis by the traditional Kennedy pathway, as indicated by the dotted arrow. Likewise, the reversibility of PC-DAG inter-conversions is not clear: back reactions are indicated as dotted lines. An important aspect of PC metabolism is the acyl editing cycle. This is the largest acyl flux in the cytosol. Our analysis cannot place an exact value on this total flux, but an analysis of saturated fatty acid mass balance suggests a lower limit of over twice the nascent fatty acid incorporation into PC (about 125 moles time increment^{-1}), while

an upper limit may be over seven fold (about 427 moles time increment^{-1}). In considering an acyl editing cycle previously, from the data obtained from pea leaves, we considered the possibility that there was a "channeled" mole of mole exchange of nascent fatty acids with acyl groups released by acyl editing. However, if the acyl editing cycle flux is significantly larger than the incorporation of nascent acyl groups into PC via a PC:lyso-PC acyl editing cycle, then the simplest way to depict the process is via a bulk acyl-CoA pool where acyl groups released by acyl editing and from *de novo* fatty acid synthesis mix, as shown in Figure 3-10.

A surprise from the labeling was that we could make a kinetic distinction for two TAG synthesis systems. TAG synthesis will require 31 nmoles of acylation at the *sn*-3 position. About 11 moles are provided by the immediate incorporation of nascent FA, with a high preference for saturates. Analysis of the molecular species of [^{14}C]FA labeled TAG suggests that this direct acylation utilizes the bulk DAG pool, not the *de novo* synthesized DAG pool. The high *sn*-3 [^{14}C]saturated FA labeling of bulk DAG suggest that either a specific pool of acyl-CoAs high in nascent saturates is delivered to a DGAT enzyme, or that the DGAT has a strong selectively for saturates from the bulk acyl group pool (Figure 3-10). At least two mechanisms, both of which are drawn into Figure 10, may provide the remaining 20 mol FA required for TAG synthesis, including essentially all the tri-PUFA TAG molecular species. A PUFA selective DGAT2 may utilize the bulk acyl pool or a PDAT reaction may transfer *sn*-2 PUFA from PC to DAG, generating TAG. The LPC produced by a PDAT reaction will add an

incremental flux to the PC acyl editing cycle (Figure 3-10). It is noteworthy that the stereochemical analysis of TAG from soybean oil shows that the *sn*-1 and *sn*-3 positions have quite similar acyl compositions. Such observations have lead to speculations that the enzymes responsible for TAG synthesis are quite non-specific. However, at least in soybean, there is the coincidental summation of two routes with distinct specificities to give the overall composition. PC has long been considered the site of FA desaturation. In developing soybeans acyl editing, DAG-PC-DAG interconversion and a specific TAG synthesis system all contribute to the biosynthesis of TAG containing PUFA at higher levels than found in PC.

Linking the Model to Questions of Biochemistry and Cell Biology

Acyl Editing: The LPCAT reactions which are hypothesized to produce the acyl editing measured in pea leaves [162] and soybean embryos was first suggested as a mechanism to allow enrichment of PUFA for TAG synthesis in oilseeds. Subsequently, an *in vitro* mechanism, acyl exchange, a reversal of the LPCAT reaction, was proposed as a mechanism for acyl editing. It was demonstrated qualitatively by feeding free fatty acids to seeds [169]. In this study acyl editing is demonstrated as a major flux for endogenously synthesized fatty acids. Our previous identification of acyl editing in pea leaves and now in developing soybeans suggests that PC acyl editing may be a ubiquitous part of plant lipid metabolism. TAG synthesis in the cell must accommodate acyl editing, and indeed TAG composition may be significantly modified by its action. However,

there are likely to be more fundamental reasons for acyl editing mechanisms. First, it may be important in membrane dynamics and homeostasis. Control of the low level of lyso-PC may be critically important due to its detergent effects on membranes. Second, during periods of high fatty acid synthesis it ensures that levels of saturated-oleoyl and dioleoyl molecular species of membrane phospholipids (which may affect membrane fluidity, especially in the cold [174]) are kept low. Third, a high cycling rate allows for rapid metabolic changes in times of stress. Thus there is an advantage of adaptability for membrane biogenesis. Fourth, cycles in biology add robustness to metabolic networks. Fifth, PC is appears to be the acyl flux bank of the plant cell, rather than other phospholipids such as PE or PI, which have more important and specific functions. The idea that a PC:lyso-PC cycle is the principle acyl acceptor system in the cytosol of the plant cell is reinforced by the metabolic analysis we performed on the *fatB* mutant, where additional acyl flux was directed into PC and PC acyl cycling increased [161]. And finally, acyl editing may allow the removal of oxidized acyl groups in membrane lipids.

In seed, as in leaf [162], PC editing is an order of magnitude greater than PE editing. Also, seed acyl editing of PC at the *sn*-2 position is particularly dominant over that at the *sn*-1 position. This coincides with in vitro measurements of acyl exchange in developing seeds, where over 90% of the measured exchange is at the *sn*-2 position [143]. Assays of lyso-PL acyltransferase activity in microsomes developing soybean cotyledons with exogenous acceptor show a higher Vmax for LPEAT than LPCAT, but with

endogenous lyso-lipid acceptors the LPCAT activity was significantly higher [175]. Thus further experiments are required to reveal whether lyso-PC generation is by way of a phospholipase A and/or a transacylase (eg. acyl exchange by a reversal of LPCAT) mechanism.

DAG-PC Inter-Conversion and Localization of DAG pools: In leaf tissue DAG is a very minor glycerolipid. From this observation alone it may be inferred that CPT operates in the direction DAG → PC, with very little reverse reaction. As DAG is a component of lipid signaling cascade [3], cellular control of DAG is likely to be tight. Thus our finding that there are at least two, and probably three, kinetically distinct DAG pools is hardly surprising. CPT would certainly be associated with the *de novo* DAG synthesis pool. Whether the reverse CPT reaction would also produce the DAG pool required for TAG synthesis is questionable. If so, it may be another gene product or post-translationally modified enzyme. In order for CPT to provide a flux running in the reverse direction from that associated with *de novo* glycerolipid synthesis would require a CPT with a locally different equilibrium position and /or a DAG removal mechanism such a phase partitioning to another membrane domain. It is also possible that a phospholipase C like activity provides the function of generating a bulk DAG pool for TAG synthesis from bulk PC. Thus there is no requirement for a rapidly reversible DAG-PC inter-conversion.

The identification of distinct kinetic DAG pools raises the question of localization. In a comparative study of CPT and DGAT in microsomes from

several different seeds [16] found little DAG selectivity for either enzyme. They concluded that the exclusion of unusual fatty acids from membrane lipids was not achieved on the basis of CPT or DGAT specificities and postulated distinctly separate pools of DAG as an explanation. The concept of different endomembrane domains is well known. However, the location of the *de novo* DAG pool is unclear. This DAG pool may feed into a small, discrete PC pool, or into the bulk PC pool. If the former is the case, then TAG synthesis may occur from the bulk pool (which may itself have a more complex structure than we can observe here). However, if the latter is the case, then TAG synthesis will occur from a sub-domain. Or both DAG pools could each represent separate ER sub-domains, connected by the bulk PC pool. However, the bulk DAG pool is most likely associated with the oil bodies. It is a reasonable hypothesis that DAG is also phase partitioned along with TAG into the oil bodies during their biogenesis.

TAG Synthesis: Despite the identification of three classes of genes that encode for enzymes of TAG synthesis in plants, and the observation that DGAT1 and DGAT2 proteins from tung seeds, when tagged, localize to different ER domains in tobacco and onion [133], the identification of kinetically distinct TAG synthesis routes with a preference for saturated or polyunsaturated FA was unexpected. It raises questions about acyltransferase genes, enzyme specificity and acyl-CoA channeling.

Considering the synthesis of TAG highly enriched with saturates at the *sn*-3 position, this is presumably a DGAT activity, and not PDAT, as it utilizes

nascent fatty acids immediately. Furthermore, PDAT would transfer unsaturates from the *sn*-2 position of PC. However, DGAT1 does not seem strong candidates for this activity. The *dgat1* mutant of Arabidopsis has a slight increase in total saturates in TAG, with the fraction in the sn-3 position decreasing only slightly [176]. DGAT2 has been implicated in the production of TAG containing high amounts of novel unsaturated fatty acids, and on this basis also does not seem to be a good candidate. Perhaps another DGAT is responsible? This DGAT may have a very strong selectivity for saturates if it utilizes the mixed acyl-CoA pool. A caveat concerns acyl group channeling via ACBP. The numerous acyl-CoA binding proteins (ACBP) in plants [108-111, 113] may be involved in shuttling acyl groups to different fates. Selective transfer of newly synthesized saturated acyl groups to DGAT via a specific ACBP may have produced the observed kinetics.

Considering the synthesis of TAG highly enriched with PUFA at the sn-3 position, including tri-PUFA TAG species, DGAT and PDAT mechanisms may be invoked. Both are shown in Figure 3-10. TAG synthesis via various transacylases of the PDAT family would transfer a *sn*-2 PUFA from PC to *sn*-3 DAG producing TAG and lyso-PC [119, 120, 177]. Thus PDAT would leave a footprint indistinguishable from *sn*-2 acyl editing. However, it cannot be the major producer of lyso-PC for acyl editing because it would not allow recycling of FA to feed *de novo* glycerolipid synthesis. For a putative DGAT to be involved, it would either have to have a strong preference for PUFA acyl-CoAs, or such molecules would have to be preferentially delivered to the site of the enzyme(s). It is

noteworthy that highly *sn*-2 labeled PC from acyl editing is not immediately available for conversion to DAG and then to TAG, otherwise TAG would contain more *sn*-2 label at early time points. A simple explanation for this is that the bulk PC pool that fills from acyl editing, creating a kinetic delay in its utilization.

3.5 Conclusion

Through *in vivo* labeling experiments we have shown that an acyl editing cycle and generation of multiple sub pools of DAG are important for the synthesis of TAG in developing soybeans. Similar research in the leaves of peas suggests that acyl editing may be a ubiquitous part of plant acyl lipid metabolism. The individual enzymes/genes that are involved in these processes are unknown and thus represent an important area for further research on plant oil synthesis. As there are many unknown genes in Arabidopsis annotated as acyl transferases [134], further analysis of the acyl editing mechanism to determine if lipases or transacylases are involved may be very useful for final identification of the enzymes involved. Ultimate identification of acyl editing enzymes may require analysis of mutants. The model of newly synthesized acyl group fluxes may be useful in the interpretation of changes in lipid quantities or FA compositions identified during mutant screens or in targeted knockouts. It is also possible that not all the gene products/routes in the metabolism scheme have been identified. In this context, the use of quantitative metabolic analysis taking into account acyl editing with mutant lines may provide further clues to new genes with overlapping roles in the processes. The fact that soybean oil is a major worldwide source of

vegetable oil, with genetically engineered oil compositions in commercial production and under development, adds a biotechnology dimension to this study. Our identification of the major flux reactions of acyl groups from synthesis in the plastid to accumulation in TAG may allow a more directed approach toward identifying enzymes that might be useful in the engineering of TAG. The enzymatic reactions involved in acyl editing may also be important for transferring unusual FA from their site of synthesis on *sn*-2 PC to the three backbone locations of TAG. The synthesis of TAG and phospholipids must be intricately coordinated as both products require the synthesis of DAG. Production of different lipids from DAG may be controlled by utilizing multiple DAG pools in different locations. Identification of the sites and enzymes in each location may allow more efficient engineering of novel lipid metabolizing enzymes to their site of action in oilseed crop plants. Thus, a better understanding of the pathways of TAG biosynthesis including acyl edting and DAG production in developing soybeans may aid future efforts to engineer soybean oil with improved or novel properties.

3.6 Materials and methods

Plant material-Developing soybean (*Glycine max)* embryos were harvested from plants grown in the greenhouse at ~24° C, supplemented with lights to maintain a 15 hr day. Embryos were harvested and cultured in media containing the carbohydrates, amino acids, inorganic salts and light conditions required for embryo growth to mimic *in planta* development as demonstrated

previously [178]. Harvested embryos (~21 mg dry weight/embryo for [^{14}C]acetate labeling and ~15 mg dry weight/embryo for [^{14}C]glycerol labeling) were equilibrated in culture conditions for 2.5 days before starting labeling experiments.

Radiochemicals—[1-^{14}C]Acetic acid, sodium salt (specific activity 50 mCi/mmol), and [^{14}C(U)]glycerol (specific activity 150 mCi/mmol) were from American Radiolabeled Chemicals, Inc. (St. Louis, MO).

[^{14}C]acetate and [^{14}C]glycerol labeling- Pre-cultured embryos were moved to a single beaker containing fresh culture media plus radioactive substrate [^{14}C]acetate (1 mM) or [^{14}C]glycerol (0.5 mM) to start the labeling reaction. The media volume was just enough to cover all the embryos and was gently shaken in a water bath at 27° C under 30-40 µmol m^{-2} s^{-1} of white light. At each time point the labeling reaction was quenched by transfering three embryos (four during [^{14}C]glycerol labeling) to 6 ml of 85° C isopropanol for 10 min.

General methods- Lipids were extracted from hot isopropanol quenched tissue with hexane/isopropanol [155] after homogenization with a mortar and pestle. Radioactivity in the total lipid samples, eluted lipids or organic and aqueous phases recovered from transmethylation was quantified by liquid scintillation counting (Beckman Instrument Inc., Fullerton, CA), while radioactivity on TLC plates was visualized and quantified by electronic radiography (Packard Instrument Co., Meriden, CT). $AgNO_3$-TLC plates were prepared by impregnating Partisil® K6 silica gel 60 Å TLC plates (Whatman, Maidstone, UK) with 10% $AgNO_3$ in acetonitrile (w/v), drying in air and activating at 110 °C for 5 min.

Unlabeled fatty acid methyl esters (FAMEs) were quantified by gas chromatography (GC) using a flame ionization detection and a DB-23 capillary column (30 m length x 0.25 mm inner diameter, 0.25 μ film thickness; J&W). For preparative TLC all solvents contained 0.01% butylated hydroxyl-toluene antioxidant.

Polar lipid class analysis- polar lipid separation by TLC, recovery of polar lipids from TLC plates, PC molecular species separation, and PC FA positional localization were done as reported previously [162].

Neutral lipid class analysis-DAG and TAG were isolated by first acetylating an aliquot of the total lipids in acetic anhydride/pyridine, (3:2, v/v) and then separating the 1,2-diacyl-3-acetyl-glycerols (acetylated DAG) from TAG by silica TLC developed in hexane/diethyl ether/acetic acid (70:30:1, v/v/v). Individual lipids were eluted from TLC silica by chloroform/methanol (4:1, v/v). Methanol and 0.88% aq. KCl were added to give chloroform/methanol/water ratios of 2:1:1, (v/v/v), resulting in a phase separation. The aqueous phase was back extracted with chloroform and lipids were recovered from the combined chloroform phases.

Radiolabeled acyl group analysis-FAME were prepared from total lipid extracts by heating to 80° C in 5% H_2SO_4 in methanol for 60 min. The organic soluble FAME were separated from the aqueous soluble compounds by the addition of water and hexane. FAME were prepared from purified individual lipids by base-catalyzed transmethylation [158]. For [^{14}C]glycerol labeled lipids the proportion of label in the acyl groups versus the backbone/head group was determined by transmethylation [158] and scintillation counting of the separated

organic and aqueous phases. Aqueous phase radioactivity from PC was determined to be in the glycerol backbone and not the choline moiety previously [162]. [^{14}C]FAME compositions were determined by separation based on the number of double bonds by $AgNO_3$-TLC, the plates being developed to ¾ height with hexane/diethyl ether (1:1, v/v), then fully with hexane/diethyl ether (9:1, v/v). Saturated FAME were further separated by removal from $AgNO_3$-TLC with $CHCl_3$/MeOH (2:1, v/v) and separated on KC18 reversed phase silica gel 60 Å TLC plates (Whatman, Maidstone, UK) by development in acetonitrile/methanol/water (65/35/0.5, v/v/v).

Molecular species separation of radiolabeled DAG and TAG- Acetylated DAG isolated from TLC was separated into molecular species based on the number of double bonds by $AgNO_3$-TLC [159], using a triple development (½ then ¾ development in chloroform/methanol (96:4, v/v), then fully in chloroform/methanol (99:1, v/v)). When necessary the saturate/diene and monoene/monoene molecular species were eluted from the silica [159] together and further separated on 15% $AgNO_3$-TLC plates at -20° C with the same triple development as above. TAG molecular species were separated by $AgNO_3$-TLC in two batches both at -20° C. The more saturated species (0-4 double bonds) were separated by triple development (first 60% then 75% development in chloroform/methanol (96:4, v/v) then full in chloroform/methanol (98:2, v/v)). The more unsaturated species (5-9 double bonds) were separated by triple development (60%, 75%, then full development in chloroform/methanol 94:6, v/v). When necessary the saturate/monoene/monoene and

saturate/saturate/diene molecular species were eluted together and separated by $AgNO_3$ TLC at -20° C by triple development in chloroform/methanol (99:1, v/v). The proportion of radioactivity in each molecular species band for DAG and TAG was determined by electronic autoradiography, then each band was eluted, the recovered lipids transmethylated, and the [^{14}C]FAME analyzed by $AgNO_3$-TLC, as described above.

Regiochemistry of radiolabeled DAG and TAG-Collected acetylated DAG and TAG were digested with porcine pancreatic lipase (sigma) [159]. The digestion products were separated on silica TLC developed with hexane/diethyl ether/acetic acid (70:30:2, v/v/v). The amount of radioactivity in MAG, DAG, free fatty acids, and TAG was quantified by electrionic autoradiography, then each band was eluted, the recovered lipids transmethylated, and the [^{14}C]FAME analyzed by $AgNO_3$-TLC, as described above.

Endogenous molecular species compositions- Total lipids from four soybean embryos (approx 13-14 mg dry weight), cultured under identical conditions to those used for labeling, were extracted according to Hara and Radin (1978). TAG concentrations were measured directly using electrospray ionization mass-spectrometry (ESI-MS). DAG and PC were converted to their *sn*-1,2-diacyl-3-acetyl-glycerol (ac-TAG) derivatives by digestion with pancreatic lipase C (for PC) followed by acetylation prior to ESI-MS analysis.
ESI-MS in positive ion mode was performed by direct infusion with a Shimadzu (Columbia, MD) SIL-5000 autosampler into a Waters (Milford, MA) Quattro micro mass spectrometer. 10µl of sample in toluene was introduced to the electrospray

source by flow injection analysis conveyed by a 65:32:3 chloroform:methanol:100mM ammonium acetate solution at a flow rate of 0.1 ml/min. The capillary, extractor, and cone voltages were 3.2 kV, 2.0 V, and 40 V respectively. The source and desolvation temperatures were 110 and 350 °C, respectively. The desolvation gas flow rate was 400 l/hr. Mass spectra were collected for 2 min; the *m/z* range scanned in the MS measurements was from 500 to 1000 (1 sec/scan) and in the MS^2 measurements from 20 to the mass of the parent ion. Collision-induced dissociation used argon as the collision gas (2×10^{-3} mbar) with the collision energy set at 22 eV. Mass spectra data was acquired with MassLynx 4.0 software; $[TAG\text{-}NH_4]^+$ ion peaks were smoothed and integrated using QuanLynx software.

In order to correct for the effect of the number of acyl chain carbons and double bonds on the signal strength [179], TAG standards with varying acyl chain length and number of double bonds were analyzed at different concentrations. After correcting for natural isotope abundance effects, the ion peak intensities for each TAG standard were normalized to the internal standard (10μM triheptadecanoin). The normalized peak intensity was plotted against TAG concentration. The slope of this standard curve was determined for each TAG standard. Multiple linear regression was then used to create a correction function relating the slope of the standard curve to the number of acyl chain carbons and double bonds.

To determine the concentration of soybean TAG or ac-TAG molecular species, the ion peak intensities were deisotoped and corrected for natural

isotope abundance and then normalized to the 10μM triheptadecanoin internal standard. The correction function for acyl chain carbons and double bonds was then applied to determine the absolute concentration of each TAG species. When necessary, ESI-MS^2 was used to determine the relative abundance of isomers with the same *m/z* value. The moles $embryo^{-1}$ of each molecular species measured for TAG, DAG, PC are in Appendix B Tables 6-1, 6-2, 6-3, respectively.

3.7 Footnotes

Abbreviations: ACP, acyl carrier protein; CPT, CDP-choline:1,2-diacyl-*sn*-glycerol cholinephosphotransferase ; DAG, *sn*-1,2-diacylglycerol; DGAT, diacylglycerol:acyl-CoA *sn*-3 acyltransferase; ER, endoplasmic reticulum; FA, fatty acid; G3P, glycerol-3-phosphate; LPCAT, *lyso*-phosphatidylcholine:acyl-CoA acyltransferase; PC, phosphatidylcholine; PDAT, (phospholipid:diacylglycerol acyltransferase); PE, phosphatidylethanolamine; PUFA, polyunsaturated fatty acid; TAG, triacylglycerol.

4 CONCLUSION

The two pathway hypothesis of eukaryotic and prokaryotic glycerolipid assembly (Figure 1-1) was first proposed over 25 years ago based on radiotracer studies of plant tissue and isolated organelles [71, 84]. The age of genetics and molecular biology further confirmed this seminal work [85]. Since that time identification of PC acyl exchange reactions [140], hydrolytic turnover of PC acyl groups [74], channeling of nascent FA from the plastid into PC [79], and the mixing of nascent FA with previously synthesized FA during PC synthesis [149] have suggested that the steps of the eukaryotic pathway may be further refined. A re-examination of plant glycerolipid synthesis requires that new investigations circumvent the limitations of previous studies. Therefore, very rapid (as short as 1 min) *in vivo* kinetic labeling studies were conducted with [^{14}C]acetate and [^{14}C]glycerol. Controls with [^{14}C]CO_2 and media containing additional carbon sources mimicking *in planta* conditions were utilized to limit changes in metabolism due to wounding or utilization of exogenously applied metabolic tracer compounds, respectively. Additionally, analysis included not only precursor-product relationships between labeled lipid classes, but also analysis of the regiochemistry and molecular species of lipids from the initial incorporation of labeled nascent FA and glycerol backbones into eukaryotic glycerolipids.

4.1 Acyl editing conclusions

Expanding pea leaves and developing soybean embryos were utilized for *in vivo* of labeling of the initial events of eukaryotic membrane and storage lipid synthesis. To ensure the *in vivo* relevance of the metabolic labeling data from exogenously applied [^{14}C]acetate or [^{14}C]glycerol to excised pea leaves, intact planted pea seedlings growing in pots were also labeled with $^{14}CO_2$. The results with the $^{14}CO_2$ precursor confirmed the conclusions based on exogenously applied [^{14}C]acetate and [^{14}C]glycerol metabolic tracers. Likewise, developing soybean embryos were pre-cultured for three days in media that allows excised embryos to mimic *in planta* growth. Trace amounts of [^{14}C]acetate or [^{14}C]glycerol were added to the culture media for labeling experiments which allowed exogenous tracers to be applied without wounding the embryos or changing the carbon sources used for heterotrophic growth and represent methodological improvements over most previous studies that utilized seed slices and a single radiolabeled carbon source. These controls allow us to be more confident that the labeling results reflect the reactions of central lipid metabolism that occur *in vivo.*

Kinetic analysis of rapid [^{14}C]acetate and [^{14}C]glycerol incorporation into lipids allowed precursor-product relationships to be established for newly synthesized FA and glycerol backbone incorporation into eukaryotic glycerolipids, respectively. Stereochemical analysis of [^{14}C]acetate labeled FA in different lipids indicated that different mechanisms are employed for incorporation of nascent FA into PC, DAG and TAG. Molecular species separation of labeled lipids allowed determination of: (1) pre-existing FA that occur in the same molecule as

the [^{14}C]acetate labeled newly synthesized FA; (2) the FA esterifed to [^{14}C]glycerol during *de novo* glycerolipid synthesis. Finally, endogenous compositions of different lipid molecular species were determined utilizing ESI-MS/MS techniques which demonstrated differences between the steady state molecular species and those of *de novo* synthesized lipids. Together these analyses of *de novo* synthesized glycerolipids and incorporation of newly synthesized FA into glycerolipids were used to develop a revised model of eukaryotic membrane and storage lipid synthesis in plant leaves and seeds.

The pathway of plant eukaryotic membrane lipid synthesis shown in most textbooks and reviews [34, 85] predicts that newly synthesized acyl groups exported from the plastid are first esterified to G3P producing initial PA molecular species of 18:1/18:1 and 16:0/18:1. PA is then converted to PC the major site of acyl desaturation, and that desaturated PC may be in equilibrium with DAG to produce other desaturated membrane or storage lipids (Figures 1-1 and 2-9 model 1). However, rapid labeling of the initial steps of eukaryotic lipid synthesis in pea leaves indicated that this simple model is incorrect and the major flux of newly synthesized FA into eukaryotic glycerolipids is not by G3P acylation but through acyl editing of PC, producing initial molecular species of PC containing one newly synthesized FA and one previously synthesized FA. Acyl editing involves a cycle of deacylation and reacylation of PC that by it self does not lead to net glycerolipid synthesis and produces an acyl group and lyso-PC as intermediates. Two models of acyl editing were possible in pea leaves (Figure 2-9, model 2 and 3). (1) Plastid FA export is tied to acyl editing in a mole for mole

fashion, with newly synthesized FA directly esterified to the lyso-PC intermediate and the released acyl groups utilized for *de novo* glycerolipid synthesis (Figure 2-9 model 2). (2) The acyl editing cycle is at a much higher rate than nascent FA synthesis such that a mixed acyl group pool is mostly FA recycled from acyl editing, and nascent FA in the mixed pool are rapidly incorporated into PC through the high flux of acyl editing (Figure 2-9 model 3). Labeling of initial glycerolipid synthesis in developing soybean embryos also indicated acyl editing as the major pathway for incorporation of newly synthesized FA into extra-plastidial glycerolipids. However, only the high acyl editing flux pathway is consistent with the soybean labeling results (Figure 3-10). Therefore, incorporation of nascent FA into glycerolipids through a high flux of acyl editing is probably the more accurate model in pea leaves and other plants.

Research in this thesis demonstrates that incorporation of newly synthesized FA into extra-plastidial glycerolipids occurs through acyl editing in leaves and developing embryos from peas and soybeans, respectively. Pea and soybean are both "18:3" plants [82] and are both in the legume family *Fabaceae* of the order *Fabales*. Similar PC molecular species labeling results have also been observed in the leaves of the "16:3" plant *Brassica napus* [149] (order *Capparales*, family *Brassicaceae*) suggesting that acyl editing may be a ubiquitous part of plant leaf lipid metabolism. Additionally, lysophosphatidylcholine acyltransferase enzymatic activity has been found in a wide variety of plant leaf [91, 94, 143, 145] and oilseed tissues [130, 140, 142,

144, 163] further suggesting that acyl editing may be a central part of plant lipid metabolism.

In soybean embryos the labeling experiments and endogenous PC, DAG and TAG molecular species compositions suggested that TAG was produced primarily from a PC derived DAG moiety generated by the removal of the phosphocholine headgroup (Figure 3-10). A traditional Kennedy pathway involving three consecutive backbone acylations was either very minor or nonexistent. The utilization of the PC DAG moiety for TAG synthesis has been suggested by *in vitro* assays of safflower [118] and sunflower [173] that demonstrated the reversibility of CDP-choline:diacylglycerol cholinephosphotransferase. *In vivo* pulse-chase backbone labeling demonstrated the utilization of PC backbone for TAG synthesis in linseed [117, 127] and safflower [168]. In each of these studies it is assumed that the DAG pool is in equilibrium with the DAG moiety of PC and that this equilibrated DAG pool is utilized for TAG synthesis. However, in the soybean embryo labeling reported in chapter three the positional localization of nascent [^{14}C]FA incorporation into PC (>86% *sn*-2), DAG (equal *sn*-1 and *sn*-2) and TAG (>98% *sn*-3) suggested that newly synthesized DAG and acyl edited PC are not in rapid equilibrium and neither is rapidly used for TAG synthesis.

Safflower (*Carthamus tinctorius* L.) oil contains over 75% PUFA and avocado (*Persea americana*) oil less than 15%. The major initial product of [^{14}C]glycerol labeling in safflower cotyledons was PC yet it was TAG in avocado mesocarp [168]. Thus, it has been suggested that utilization of the PC-DAG

moiety for TAG synthesis may only take place in oilseeds that utilize PC for FA modification and accumulate large amount of desaturated or unusual FA in TAG. However, the unusual FA petroselinic acid (18:1*cis*Δ6) produced in coriander and carrot endosperm [152] and the unusual FA 16:1-Δ6 produced in *Thunbergia alata* [153] are both synthesized in the plastid, yet still flux through PC prior to accumulation into TAG. If the PC-DAG moiety containing the unusual FA is used for TAG synthesis it would imply that FA modification on PC is not required for conversion of PC to TAG. Additionally, in *Brassica napus* which contains 30% PUFA in seed TAG, four hour labeling of developing embryos with acetate and glycerol led the authors to suggest the Kennedy pathway is the major route to TAG synthesis [180, 181]. It is clear that multiple pathways may be involved in TAG synthesis in different plant species and that utilization of the PC-DAG moiety for TAG is a mechanism that may or may not be used by various oilseed plants. However, the variability in the techniques of labeling studies in different plants (variability of oil tissue and developmental stage, wounding effects of excised tissue, different labeling time periods, FA or backbone labeling only, labeling with or without stereochemical and/or molecular species analyses) makes determinations of the sequence of reactions involved in the TAG synthesis pathway difficult to deduce from most previous studies of oil biosynthesis. The soybean inital glycerolipid synthesis studies presented here demonstrate that very short time point labeling kinetics and analyses of both the acyl and backbone components of TAG synthesis through regiochemistry and molecular species analyses are essential to generate an accurate model of TAG

synthesis. The soybean TAG synthesis model indicates that the PC-DAG moiety utilized by TAG synthesis is not the same as the DAG produced by *de novo* glycerolipid synthesis and therefore a direct Kennedy pathway may not be required in oilseeds (Figure 3-10). Whether a direct Kennedy pathway is a more major TAG biosynthesis route in other oilseeds will require additional in depth investigations of TAG synthesis in different plant species.

4.2 Additional significance of acyl editing and PC derived TAG synthesis

The process of acyl editing described in this thesis has possible significance for understanding additional areas of basic plant lipid biology. The mechanism of fatty acid export from the plastid for utilization by the eukaryotic pathway acyltransferases has yet to be elucidated. Experiments with isolated pea chloroplasts indicate that newly synthesized FA are channeled into PC, possibly on the outer chloroplast envelope [79]. Isolated chloroplasts cannot synthesize PC *de novo*; therefore acyl editing is very likely involved in incorporation of newly synthesized FA into PC at the plastid envelope. Lateral diffusion of PC through the PLAM [94, 95] to the cellular endomembrane system would allow a mechanism of plastid FA export that would not require acyl-CoA to pass through the cytosol.

Acyl editing may also be involved in ensuring that newly synthesized glycerolipids do not disturb the integrity of cellular membranes. PUFA containing lipids are required for proper membrane physiology and function, especially at low temperatures [174, 182]. Desaturation of 18:1 to 18:2 and 18:3 lagged

significantly behind the rate of 18:1 production in pea and soybean (Figures 2-2 and 3-3, respectively). However, acyl editing produced initial molecular species of PC with mixtures of previously synthesized PUFA with newly synthesized saturated or monounsaturated FA (Figures 2-5 and 3-5). The mixed FA molecular species may benefit the plant by not allowing accumulation of relatively saturated lipid molecular species produced from only newly synthesized FA (e.g. 16:0/18:1, 18:1/18:1). Without acyl editing, plant tissue that is rapidly producing lipids (e.g. expanding leaves or developing oilseeds) may accumulate relatively saturated molecular species in biosynthetic regions of the ER membrane that could affect the membrane physical properties and function.

The supply of FA has been shown to be one of the limiting factors of TAG accumulation in oilseeds [183]. High levels of externally supplied FA produces a feedback control on fatty acid synthesis through modulation of plastidic ACCase activity [58]. In soybean embryos most newly synthesized FA are initially incorporated into glycerolipids through acyl editing (Figure 3-10). Acyl editing enzymes may be able to regulate or sense free FA levels which could be involved in the signaling to modify ACCase activity. In plant species that accumulate unusual FA in TAG the amount of unusual FA in membrane lipids is typically very minor. However, in transgenic plants producing unusual FA the membrane lipids can sometimes accumulate more unusual FA than TAG [128]. An acyl editing scheme that selectively removes modified FA from PC for TAG synthesis and incorporates newly synthesized FA into PC to be further modified may be involved in plant species that naturally produce unusual FA. Likewise, in

transgenic plants that express the biosynthetic enzymes for unusual FA but do not express unusual FA selective acyl editing enzymes, the unusual FA may not be efficiently removed from the site of synthesis on PC. A similar mechanism selective for PUFA may be involved in plant species that accumulate large amounts of PUFA in TAG.

The kinetic modeling of [^{14}C]acetate and [^{14}C]glycerol labeling of soybeans (Figures 3-9 and 3-10) suggested at least three DAG sub-pools that are kinetically distinct. The individual DAG sub-pools may suggest separate sub-cellular localizations that are related to separate functions. Soybean TAG synthesis primarily utilized the bulk DAG produced from bulk PC. The PC headgroup removal to generate the bulk DAG may be localized to an ER region associated with a developing oil body for TAG synthesis. A possible function of utilizing bulk PC in a separate location for TAG synthesis may be to ensure the use of desaturated PC (and thus DAG) and not *de novo* synthesized DAG/PC or acyl edited PC which would contain at least one acyl group that was not desaturated or otherwise modified. Utilization of PC that diffuses laterally through the membrane from the site of desaturation to the proximity of the oil body may be a mechanism to separate the steady-state desaturated bulk PC/DAG for polyunsaturated TAG synthesis without the need for a Kennedy pathway that utilizes a high PUFA acyl-CoA pool or enzymes that are selective for PUFA.

4.3 Future research on acyl editing and TAG synthesis pathways

The next logical step in the investigation of lipid synthesis through acyl editing is to identify the enzymes/genes responsible for the deacylation and reacylation of both the *sn*-1 and *sn*-2 position of PC. The acyl editing model presented (Figure 3-10) utilizes a *sn*-1/*sn*-2 lyso-PC intermediate. Therefore an LPCAT enzyme is probably involved in the lyso-PC reacylation and possibly the PC deacylation by the reverse action of LPCAT [140]. *Arabidopsis thaliana* contains 11 gene candidates for lyso-phospholipid acyltransferases [134], and recently two Arabidopsis genes have been expressed in yeast and the activities demonstrated to be broad specificity lyso-phospholipid acyltransferases with the highest activity for lyso-PC [184]. Alternatively phospholipases may be involved in the deacylation of PC of which Arabidopsis has at least one PLA_1 and six PLA_2 gene candidates [134]. Assuming the knockout of acyl editing enzymes are not lethal, the redundancy in Arabidopsis suggests that a forward genetic screen is unlikely to yield which gene products are involved in acyl editing. A reverse genetic approach may be more suitable; however redundancy may also be a problem and may require testing many combinations of gene knockouts. Additionally, the labeling experiments and analysis methods presented in this thesis to investigate acyl editing are laborious and time consuming and thus are not suitable for screening of many different plants. A possible preliminary labeling experiment to analyze reverse genetic candidates would be to rapidly pulse label fatty acid synthesis with [^{14}C]acetate and measure the ratio of [^{14}C]DAG to [^{14}C]PC. If acyl editing is reduced in the mutants the initial labeling ratio should

increase. Further analysis will be required to determine if a change in DAG and PC labeling is actually due to a change in acyl editing.

A possible non metabolic labeling based analysis method to analyze reverse genetic acyl editing mutant candidates would be to use mass spectrometry to quantify lipid molecular species. If acyl editing is knocked out or significantly reduced by a mutation then more nascent FA might enter *de novo* glycerolipid synthesis and thus initially produce larger amounts of 18:1/18:1 & 16:0/18:1molecular species in PA, DAG and PC. Additionally, levels of lipid molecular species that have nascent FA/previously synthesized FA composition (e.g. 18:1/18:3) produced from acyl editing might be reduced. However, desaturation of the initially produced molecular species would presumably not be affected and any change in molecular species abundances may be small and transient. Therefore to have the highest probability of success, tissue that is rapidly producing lipids such as young expanding leaves should be utilized.

Since there are many possible gene candidates for acyl editing enzymes further analysis of the mechanism of acyl editing to determine whether lipases or transacylases are involved could narrow down the number of possibilities. In this regard incorporation of oxygen-18 into the carbonyl oxygen of newly synthesized FA through [^{18}O]acetate or $H_2{}^{18}O$ labeling and measuring ^{18}O content with mass spectrometry would allow for measurements of FA hydrolysis. Each time a FA is hydrolyzed by a lipase and re-esterified there is 50% exchange of the carbonyl oxygen with the cellular H_2O [74]. If acyl editing proceeds by way of a lipase then there would be different amounts of ^{18}O in the acyl-CoA pool and PC. However, if

acyl groups are removed from PC by a transacylation reaction such as reverse LPCAT then ^{18}O content of the acyl-CoA pool and PC should be the same. Similar experiments utilizing [^{18}O]phosphate may be able to determine if PC headgroup removal to produced DAG for TAG synthesis is by way of a phospholipase D and PA phosphatase (as suggested for eukaryotic galactolipid DAG production [89, 90]) or reverse action of cholinephosphotransferase [16, 117, 118].

Genetic approaches to identify the enzymes of acyl editing would be most successful in the model species *Arabidopsis thaliana*, which has a fully sequenced genome and many genetic/molecular biology tools available. However, acyl editing was demonstrated in pea and soybean and not Arabidopsis. Thus identification of the acyl editing enzymes from pea and soybean by enzyme purification may be a logical approach. In this regard LPCAT activity has been measured in microsomes of many different leaf [91, 94, 143, 145] and oilseed tissue [130, 140, 142, 144, 163], which suggest that at least partial purification of enzymatic activity is possible. Preliminary experiments suggest that an acyl editing activity is associated with isolated pea leaf chloroplasts (Appendix C). Interestingly in five minutes of [^{14}C]acetate labeling of pea chloroplasts only nascent [^{14}C]18:1 was incorporated into PC and was located ~93% at the *sn*-2 position of PC. Similarly most *in vitro* LPCAT activities measured in microsomes were also mostly *sn*-2 specific. Therefore the substantial *sn*-1 acyl editing activity identified by *in vivo* labeling in pea leaves (Figure 2-6) may be a soluble activity that is lost by isolation of chloroplasts or

microsomes alone. If an enzyme is able to be purified the amino acid sequence may be identified by Edman degradation or mass spectrometry based sequencing. Once the protein/gene sequence is known genetic and molecular biology techniques could be utilized to confirm its function by analysis of acyl editing in knockout/knockdown mutants and over expressers.

The models of pea leaf and soybean acyl editing presented describe an acyl editing cycle involving nascent saturated and monounsaturated FA incorporation into the *sn*-1 and *sn*-2 position of PC. Some evidence in chapter three suggests that within the acyl editing cycle and glycerolipid synthesis there could be different mechanisms involved in saturate and 18:1 incorporation into glycerolipids which should be studied further. Soybean saturate labeling increased with time in proportion to 18:1 in PC, while saturate labeling was constant and in very different proportions to 18:1 in DAG and TAG (Figure 3-7B). Additionally, the primarily *sn*-2 and 18:1 specific acyl editing in pea chloroplasts (Appendix C) suggest that separate enzymatic activities and possibly locations are involved with incorporation of nascent 18:1 into PC as compared to nascent saturates in pea leaves. The high *sn*-3 saturate labeling of nascent FA incorporation into TAG of developing soybeans may suggest that there is a specific channeling of saturated FA to a DGAT. Release of newly synthesized saturated FA and 18:1 from ACP at the termination of fatty acid synthesis is carried out by two separate thioesterases, FATB and FATA respectively. The Arabidopsis *fatb* mutant which reduces the amount of saturated FA exported from the plastid [161, 185, 186] and increases total 18:1 plastid export and

breakdown may be useful for analysis of acyl editing mechanisms. If there is a saturate specific acyl editing enzyme that is required for nascent saturated FA incorporation into glycerolipids then a mutant should have a change in lipid composition similar to *fatb*, and the double mutant lipid composition should be similar to the single mutants. If the excess 18:1 exported from the plastid in the *fatb* mutant is incorporated into glycerolipids through acyl editing then the rate of nascent FA incorporation into PC should increase. If acyl editing is required for turnover and breakdown of the excess 18:1 in the *fatb* mutant, then β-oxidation in an acyl editing and *fatb* double mutant might be reduced as compared to the *fatb* mutant alone. Additionally, a *fatb* and 18:1 acyl editing mutant may accumulate excess 18:1 containing PC molecular species as compared to wild type.

Soybean embryo fatty acid synthesis produced more newly synthesized [^{14}C]acetate labeled 18:0 than 16:0. However, the endogenous FA mass composition contains more 16:0 than 18:0. The newly synthesized [^{14}C]18:0 that is initially more abundant than newly synthesized [^{14}C]16:0 in soybean glycerolipids may be a transient pool of 18:0 that is used for synthesis of other products such as sphingolipids, cutin, or wax. Alternatively, 18:0 may be desaturated while bound to PC or within the recycled acyl-CoA pool. The plastidic stearoyl-ACP desaturase is considered the only known 18:0 desaturase in plants [34]. Identification of an extra-plastidic stearate desaturase would be novel to plant lipid metabolism. Further [^{14}C]acetate soybean labeling with much longer time points and/or pulse-chase [^{14}C]acetate labeling experiments may be

able to reveal the down steam products of the initially high 18:0 production for additional investigation.

The identification of multiple sub-pools and DAG that are not in equilibrium suggests that the enzymes producing each sub-pool are not in the same cellular location. Even though we could not distinguish multiple pools of PC kinetically, acyl edting may be at a separate location than *de novo* PC synthesis. Cell biology localization techniques such as green fluorescent protein (GFP) fusions of the enzymes for *de novo* glycerolipid synthesis, acyl editing, and TAG synthesis could be employed to confirm the separate localization hypothesis. An interesting facet of localization studies would be whether different isoforms of enzymes localize to the same sub-cellular location. For instance if plastid associated acyl editing is only *sn*-2 specific then a *sn*-2 LPCAT may be localized to the PLAM region, but an *sn*-1 LPCAT that utilizes saturates may be soluble or localized to an acyl-editing domain of the ER. A hypothesis as to why many transgenic plants expressing FA modification enzymes do not accumulate the same quantities of unusual FA in TAG as do the native species is that unknown factors channel the unusual FA from site of synthesis to incorporation into TAG. The GFP fusions used for localization studies could also be used for co-immunoprecipitation studies to identify proteins interacting with the PC acyl editing enzymes or the TAG synthesis DGATs and PDATs. Identification of the enzymes required for incorporation of modified FA into TAG and the ability to express them in the correct location to be efficiently utilized will greatly increase

our ability to generate transgenic crops that accumulate unusual FA of interest in TAG.

This dissertation has demonstrated that FA incorporation into membrane lipids and TAG involves large fluxes of acyl editing in and out of PC in pea leaves and developing soybeans. The relation of acyl editing to previous studies suggest that acyl editing may be involved in many different plant species and further research may imply that it is a central part of plant glycerolipid metabolism. The acyl editing models presented update the simple *de novo* glycerolipid synthesis model found in most lipid biosynthesis reviews and textbooks. Further research of the mechanisms of acyl editing and the enzymes/genes involved will deepen the understanding of plant lipid synthesis and possibly increase our ability to modify the incorporation of FA into plant lipids to suit our needs for food, fuel and industrial applications.

5 APPENDIX A: CHAPTER 2 SUPPLEMENT

NOTE 1

Discussion: 18:1/18:1 and 16:0/18:1 Are Not Initial Molecular Species of Eukaryotic Glycerolipids

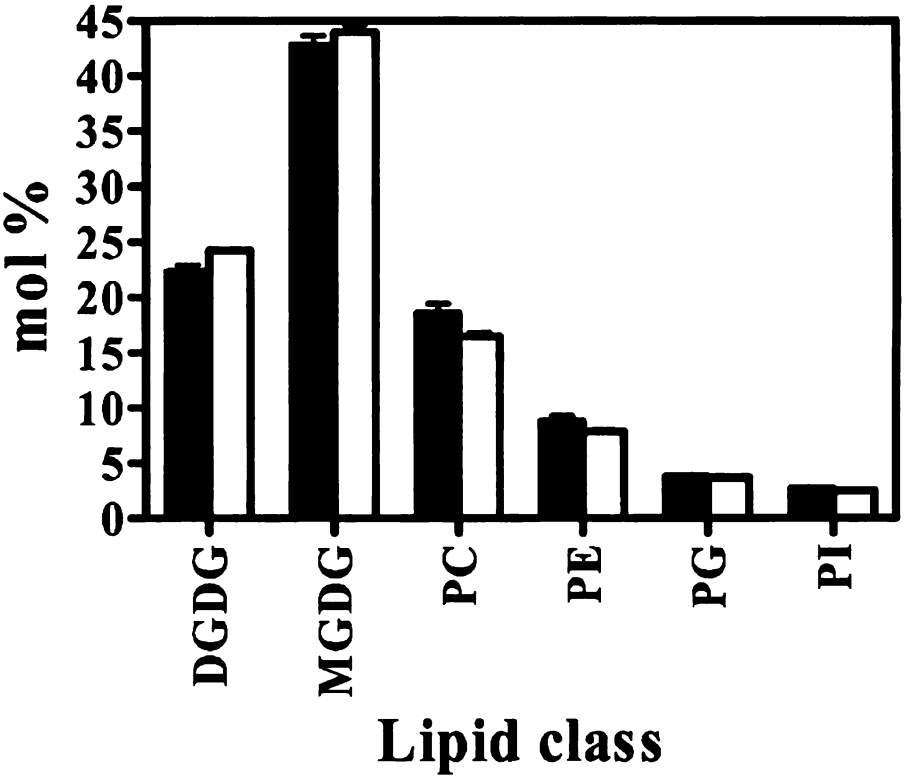
Rapidly expanding pea leaves at day 8 and day 9 produce about 2.2 and 0.6 µmol FA/hr/g fresh wt. respectively in the light, and contain 20-25 µmol FA/g fresh wt. Although endogenous 16:0/18:1 and 18:1/18:1 molecular species of PA and PC can arise by non-biosynthetic mechanisms, if we treat them as essentially all biosynthetic, produced by *de novo* synthesis from glycerol-3-P, and estimating their PA and PC levels at 0.002 and 0.2 mole % respectively, the residence time for any labeled FA passing through these molecular species pools is calculated as 0.7-3 sec and 1.1-5 minutes respectively. Thus, a pool of 16:0/18:1 and 18:1/18:1 molecular species of PC should have been easily detected by our analysis.

SUPPLEMENTAL FIGURES

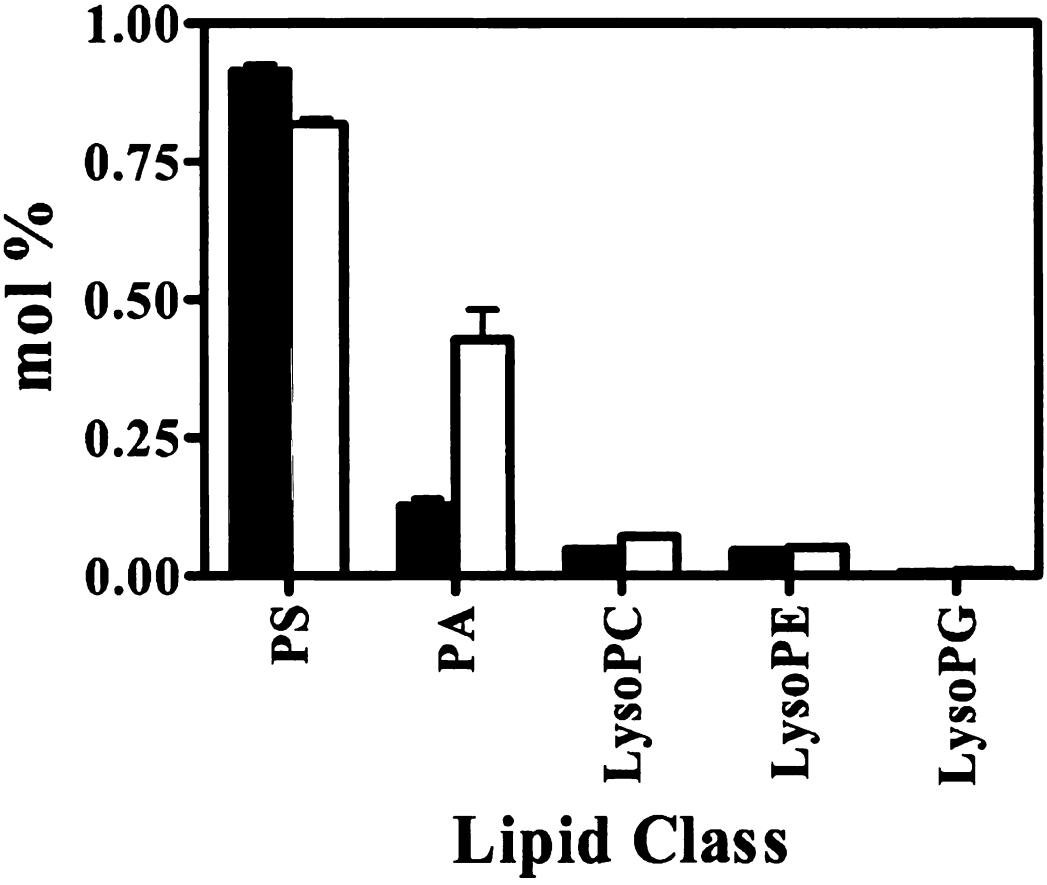
Figure 5-1 Supplemental Figures S1A-S1M: Lipidomics of pea leaves and acetate labeled pea leaves.

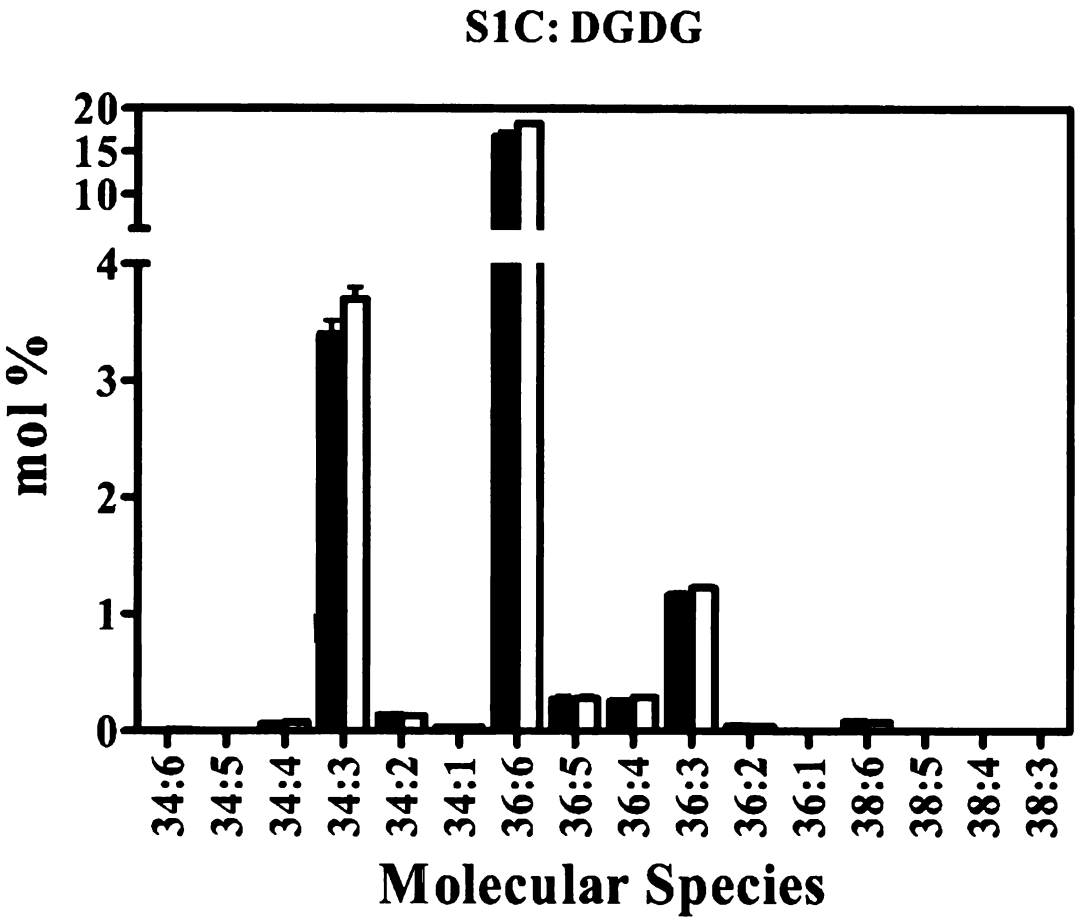
Pea leaf lipid samples were analyzed by ESI-MS/MS by the Kansas Lipidomics Research Center. Extraction of lipids was conducted by their standard *Arabidopsis* leaf protocol (Web site: http://www.k-state.edu/lipid/lipidomics/leaf-extraction.html). Data is represented as mol percent of total lipids measured. Error bars are standard error, $n = 5$. Black bars: 8 day old pea leaves cut and quenched immediately. White bars: 8 day old pea leaves cut into strips and labeled with 1 mM cold (^{12}C) acetate for 5 min and quenched immediately. Mock labeling conditions were the same as for [^{14}C]acetate labeling. **A-B:** The sum of total molecular species for each lipid class measured. **C-M:** Individual molecular species for each lipid class measured. **A:** Digalactosyldiacylglycerol (DGDG), Monogalactosyldiacylglycerol (MGDG), PC, PE, PG, phosphatidylinositol (PI). **B:** phosphatidylserine (PS), PA, Lyso-PC, Lyso-PE, Lyso-PG. **C:** DGDG. **D:** MGDG. **E:** PC. **F:** PE. **G:** PG. **H:** PI. **I:** PS. **J:** PA. **K:** Lyso-PC. **L:** Lyso-PE. **M:** Lyso-PG.

S1A: Total molecular species by lipid class



S1B: Total molecular species by lipid class




S1C: DGDG
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15
10
4
3
2
1
0
mol %
34:6
34:5
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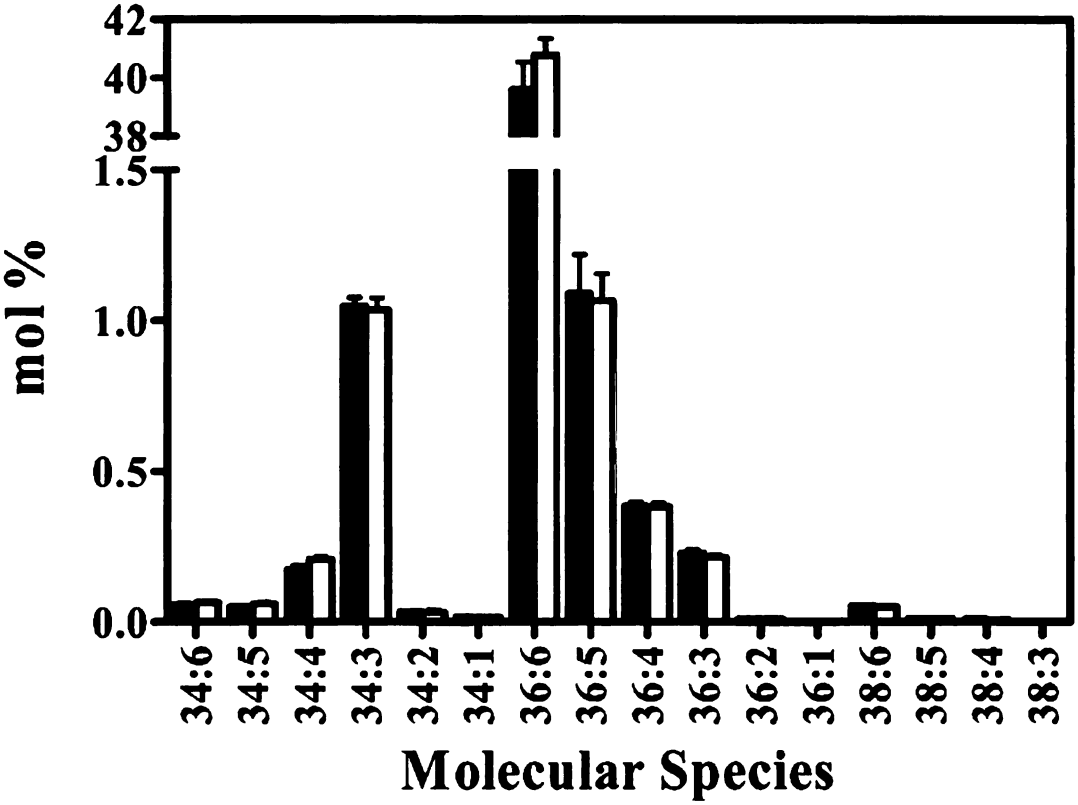
34:3
34:2
34:1
36:6
36:5
36:4
36:3
36:2
36:1
38:6
38:5
38:4
38:3
Molecular Species

S1D: MGDG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40
38
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1.0
0.5
0.0
mol %
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34:1
36:6
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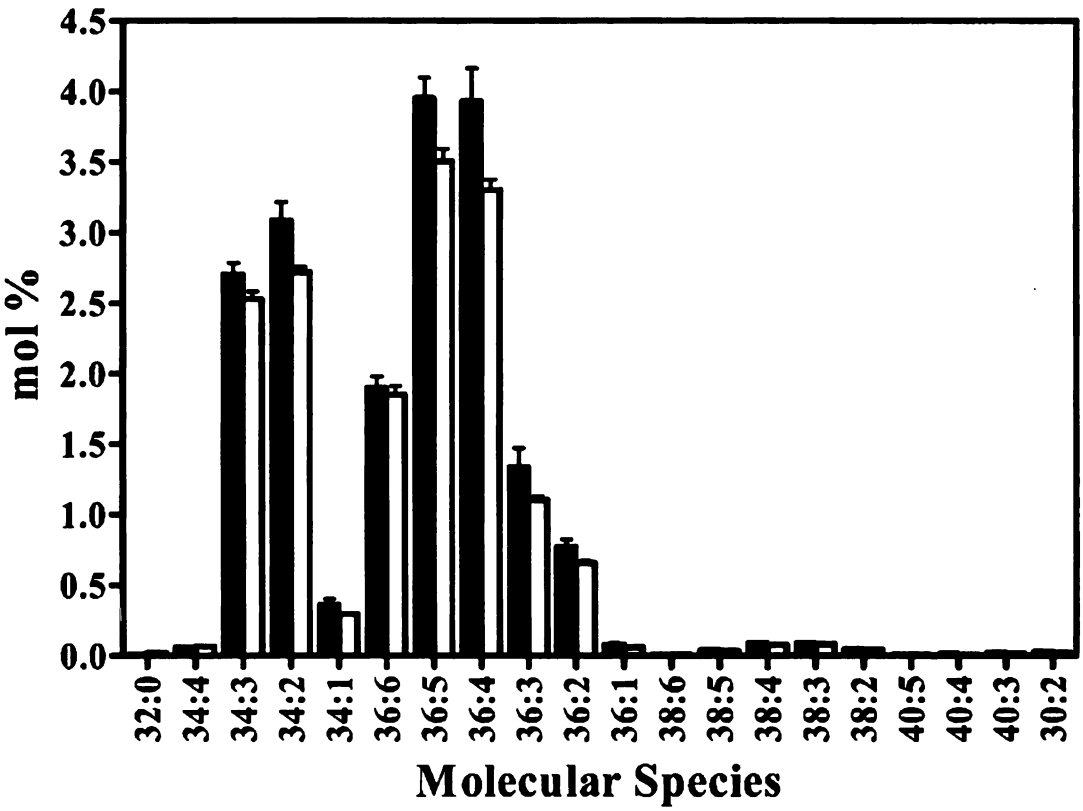
36:4
36:3
36:2
36:1
38:6
38:5
38:4
38:3
Molecular Species

S1E: PC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34:3
34:2
34:1
36:6
36:5
36:4
36:3
36:2
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38:6
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38:4
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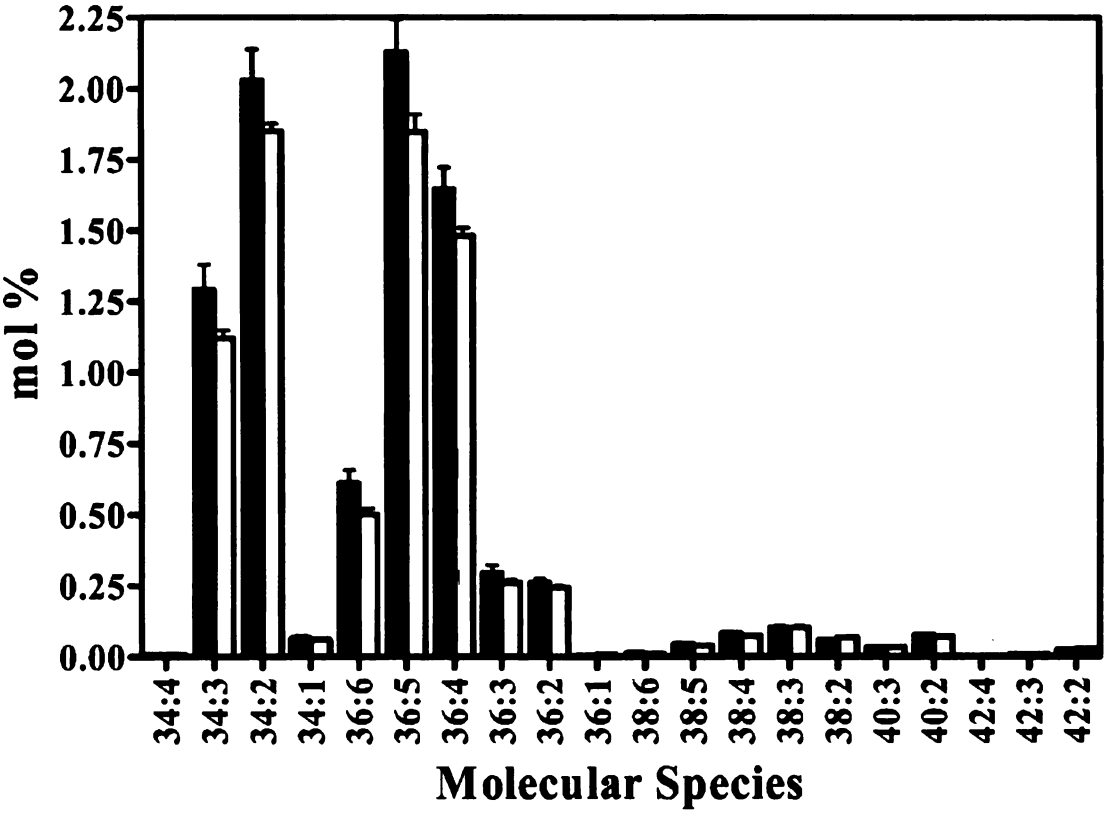
40:5
40:4
40:3
30:2
Molecular Species

S1F: PE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mol %
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34:2
34:1
36:6
36:5
36:4
36:3
36:2
36:1
38:6
38:5
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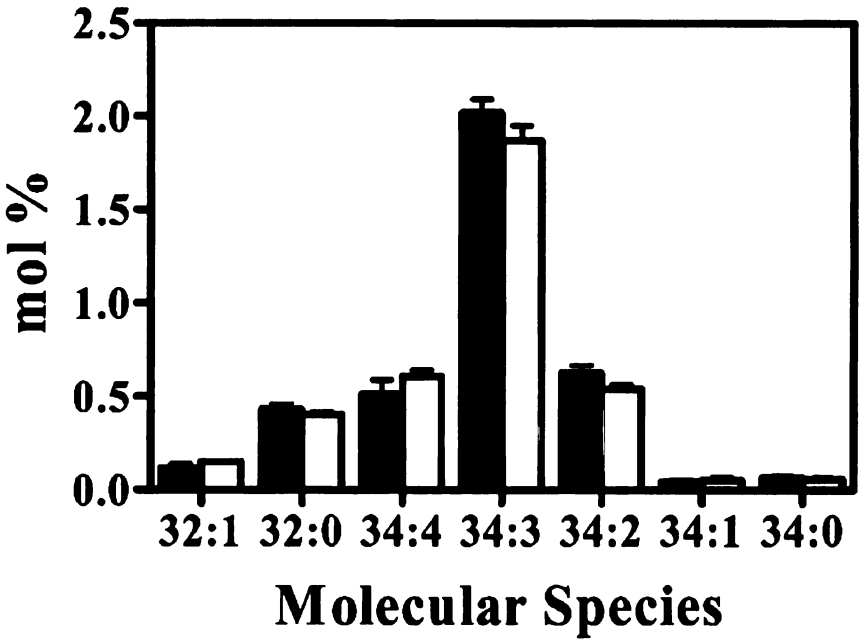
38:3
38:2
40:3
40:2
42:4
42:3
42:2
Molecular Species

S1G: PG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mol %
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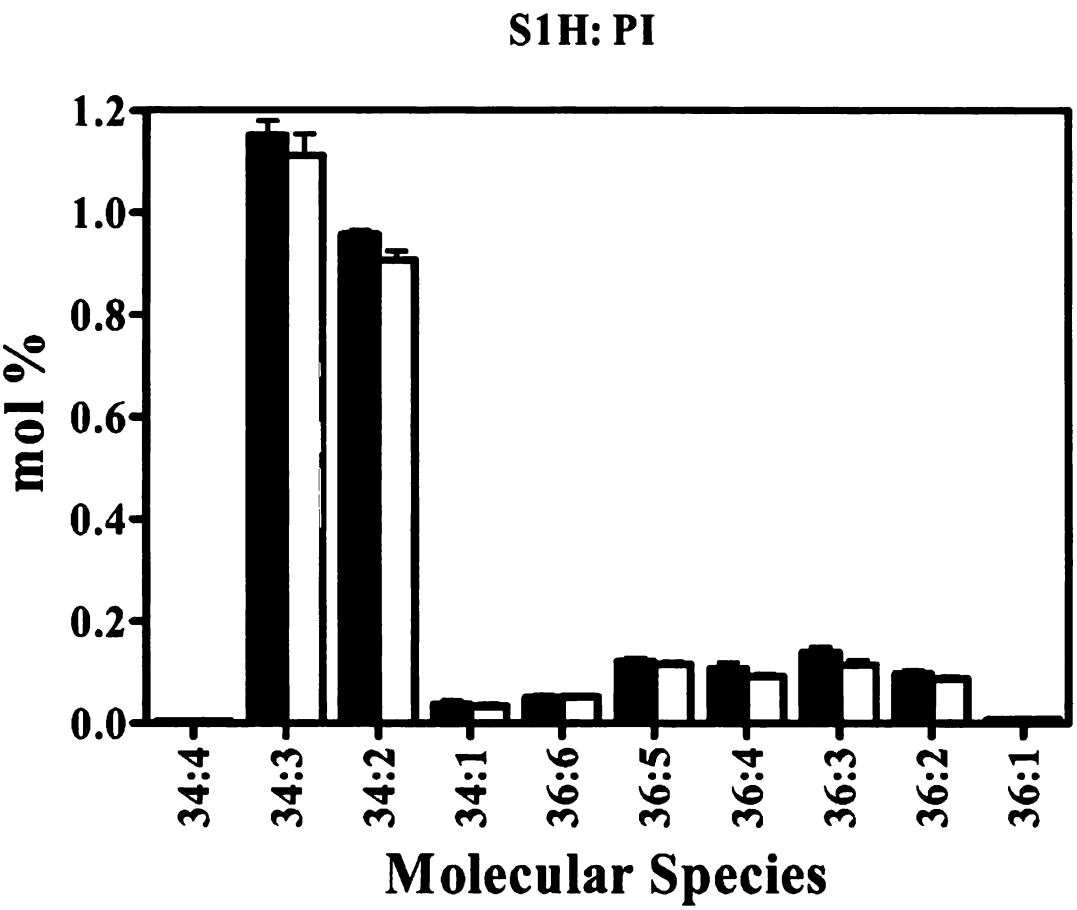
Molecular Species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1.0
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0.6
0.4
0.2
0.0
mol %
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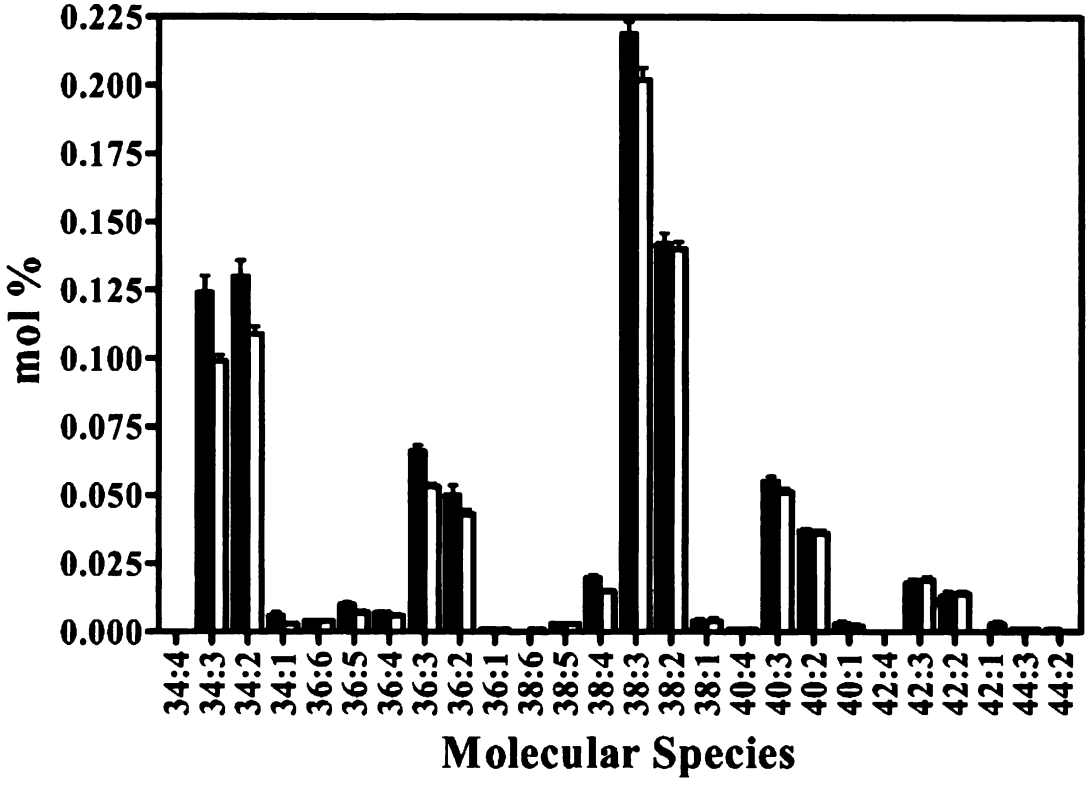
34:1
36:6
36:5
36:4
36:3
36:2
36:1
Molecular Species

S1I: PS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0.050
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0.000
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36:3
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38:4
38:3
38:2
38:1
40:4
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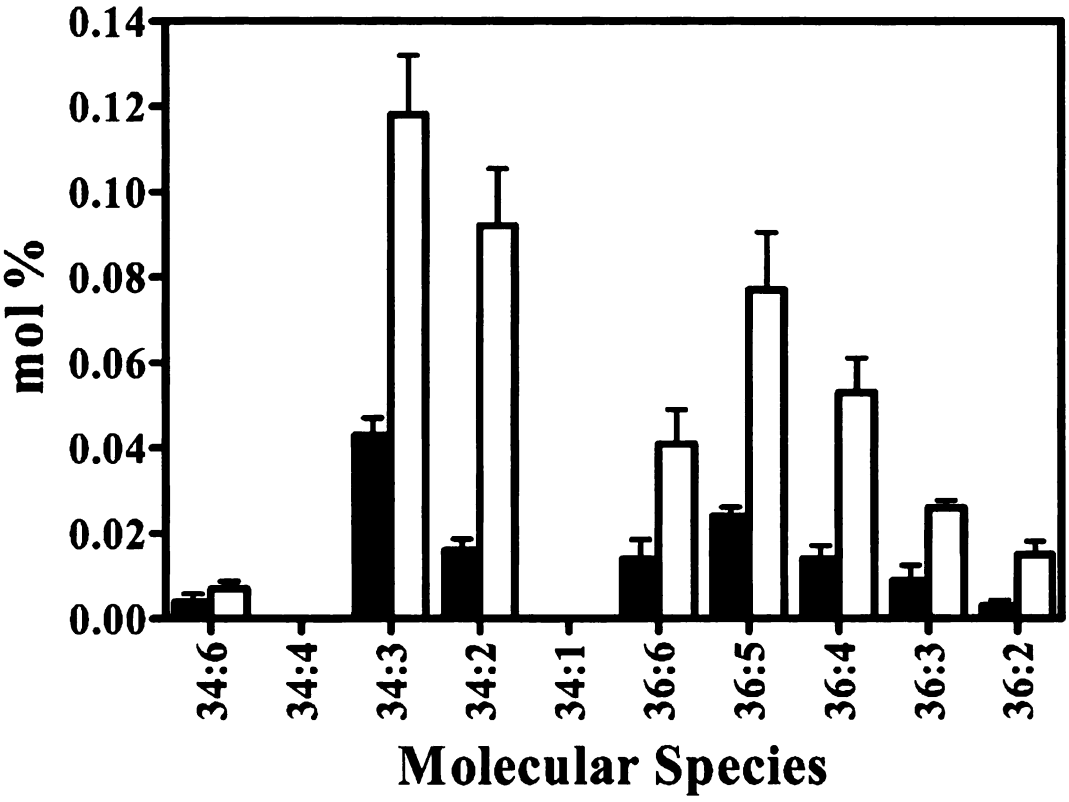
40:2
40:1
42:4
42:3
42:2
42:1
44:3
44:2
Molecular Species

S1J: PA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0.08
0.06
0.04
0.02
0.00
mol %
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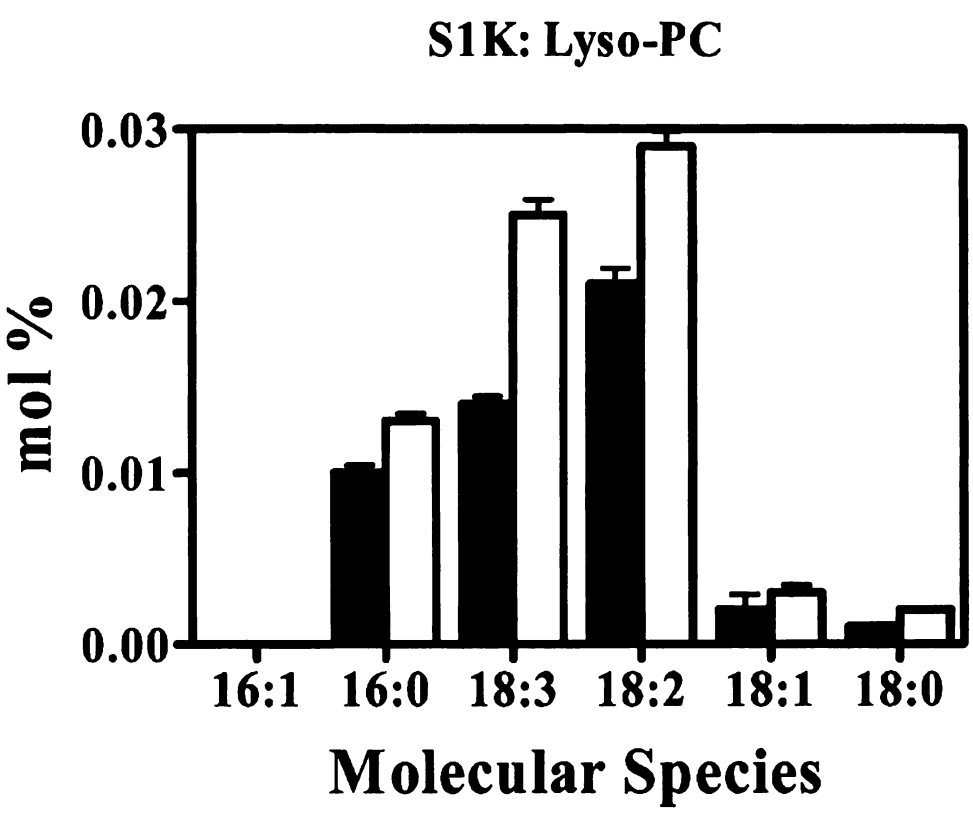
36:6
36:5
36:4
36:3
36:2
Molecular Species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0.02
0.01
0.00
mol %
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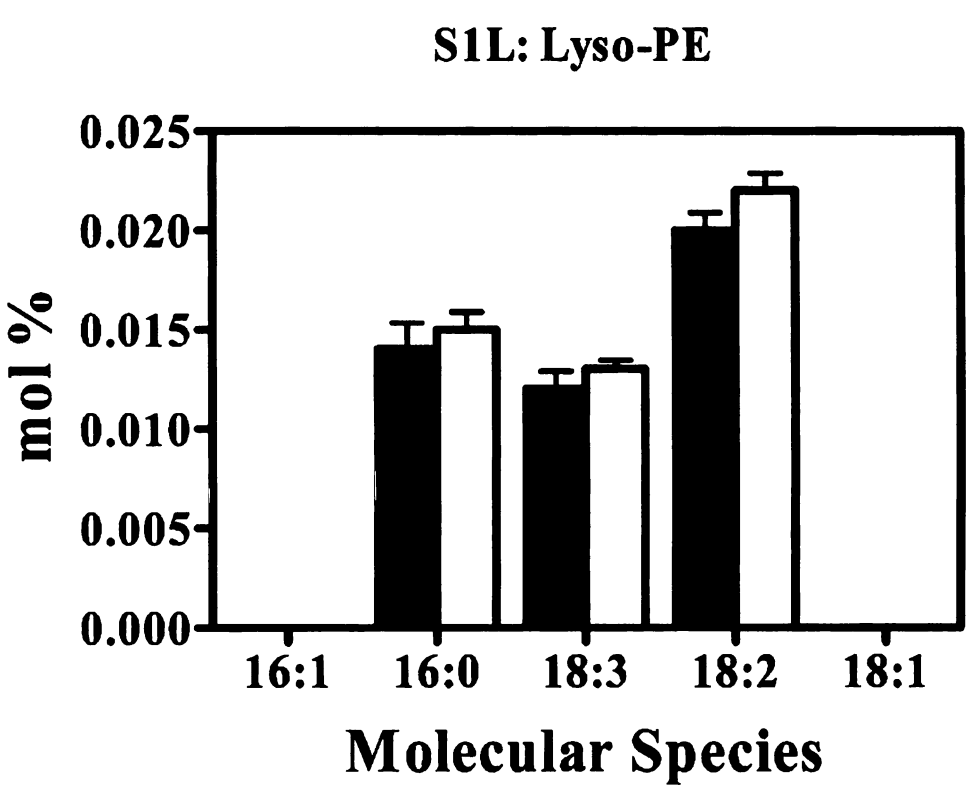
16:0
18:3
18:2
18:1
18:0
Molecular Species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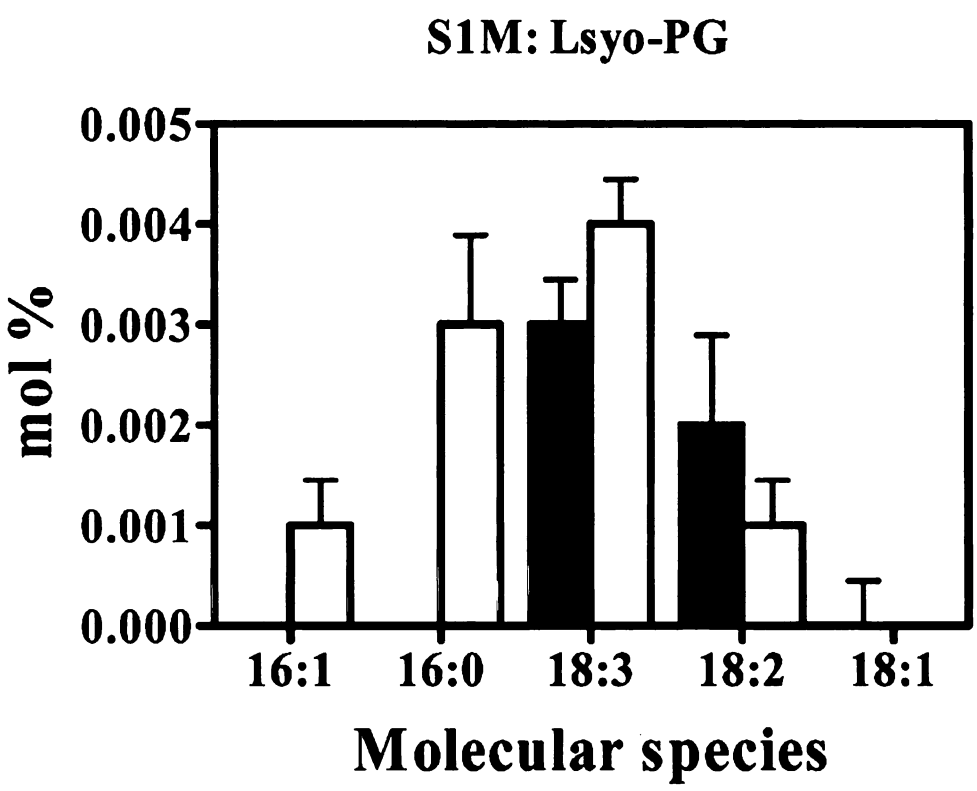
0.015
0.010
0.005
0.000
mol %
16:1
16:0
18:3
18:2
18:1
Molecular Species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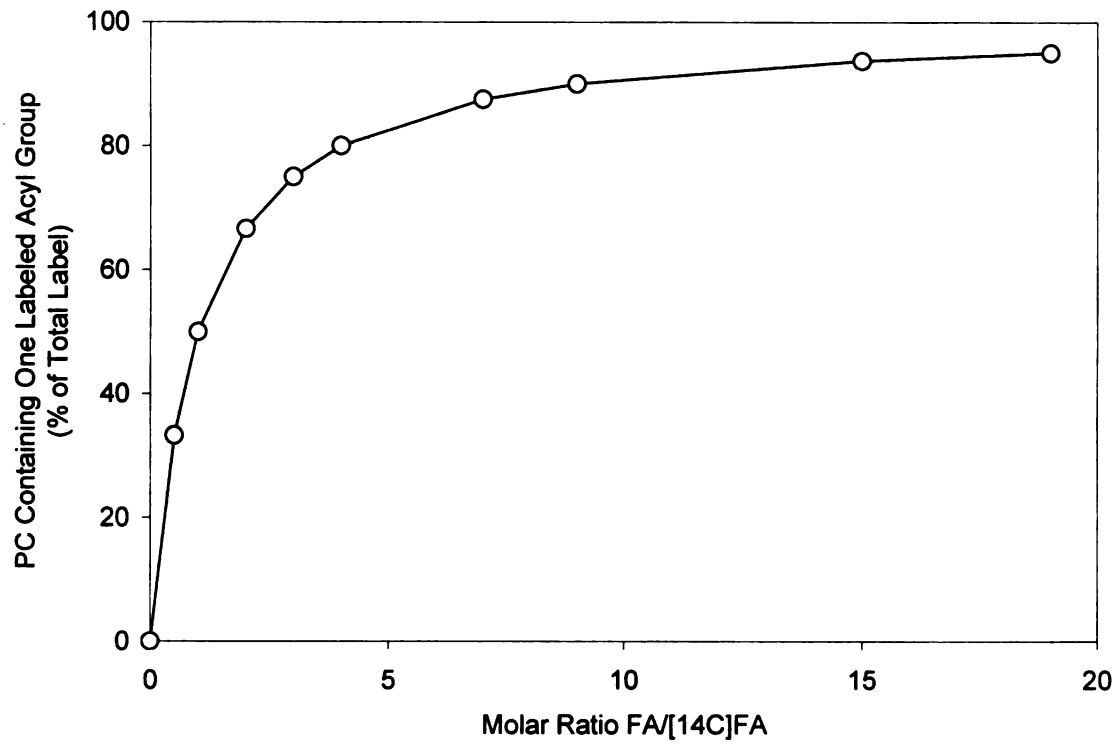
0.004
0.003
0.002
0.001
0.000
mol %
16:1
16:0
18:3
18:2
18:1
Molecular species

Figure 5-2 Estimates of single and dual acyl-labeled PC molecular species for Model 3 (Figure 2-9).

Calculation of PC molecular species containing one labeled acyl group (PC[FA*FA]) or two labeled acyl groups (PC[FA*FA*]), based on x moles of labeled fatty acid (FA*) and y moles of unlabeled fatty acid (FA) distributed randomly between sn-1 and sn-2 positions. The distribution is calculated as PC[FA*FA]/(PC[FA*FA*] + PC[FA*FA]). The small amount of labeled saturates largely at the sn-1 position (Figure 2-6) will not greatly skew these results.



6 APPENDIX B: CHAPTER 3 SUPPLEMENT

6.1 Supplement Figures for Chapter 3 Results

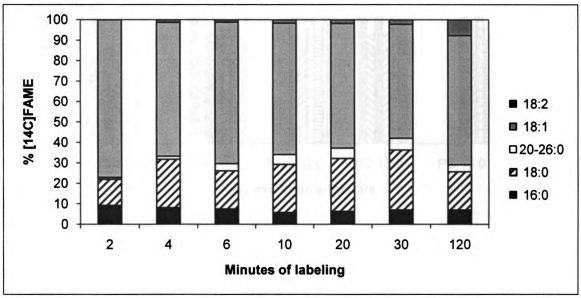


Figure 6-1 [^{14}C]Fatty acid composition of total [^{14}C]acetate labeled soybean lipids.

Total lipid FAMEs from each [^{14}C]acetate labeling time point were produced by acid catalyzed transmethylation. FAME were separated based on the number of double bonds by $AgNO_3$-TLC and based on chain length/unsaturation by RP-TLC. In $AgNO_3$-TLC the saturated FAME co-migrated and in RP-TLC 16:0/18:1 co-migrated. FAME bands that co-migrated with standards were quantified by electronic autoradiography. The individual FA compositions were calculated based on their relative quantities in each TLC system. Color labels: Black, 16:0; hatched, 18:0; white, the sum of 20:0, 22:0, 24:0, 26:0; light gray, 18:1; dark gray, 18:2.

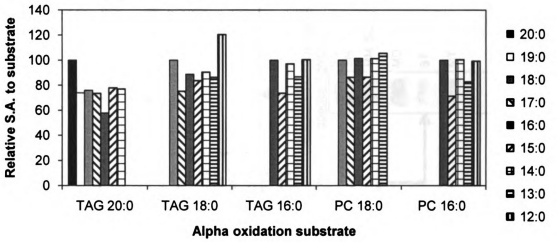


Figure 6-2 Alpha oxidation of [^{14}C]acetate labeled saturated FA from TAG and PC.

Individual saturated FA acids from TAG and PC were subject to chemical alpha oxidation. Each reaction is a mixture of FA from 20, 30, 120 & 360 min acetate labeling. The reaction produced a series of FA each shortened by 1C from the carboxyl end. Results are expressed as relative specific activity to that of the full length reaction substrate. The X-axis groups each individual substrate reaction products with decreasing chain length from left to right. Individual FA products bar shading is in the key.

Method: Individual saturated FAME from TAG and PC were collected by first $AgNO_3$ then reversed phase TLC (as in methods) and saponified to FA. 100 ug of non labeled carrier FA was added to each reaction with the respective FA chain length. The reaction was started by adding 10 mg of finely ground $KMnO_4$ and 0.5 ml acetone to each dried FA sample and heated to 60° C. After six hours remaining $KMnO_4$ was reduced in the cooled sample by adding 1 ml of 0.5M $FeSO_4$ in 0.5 N H_2SO_4. FA were extracted with hexane and FAME generated by reaction in 5% sulfuric acid in MeOH at 50° C for 30 min. FAME extracted by hexane were split into two aliquots, mass quantified by GC-FID, and radioactivity quantified by reversed phase TLC and electronic autoradiography.

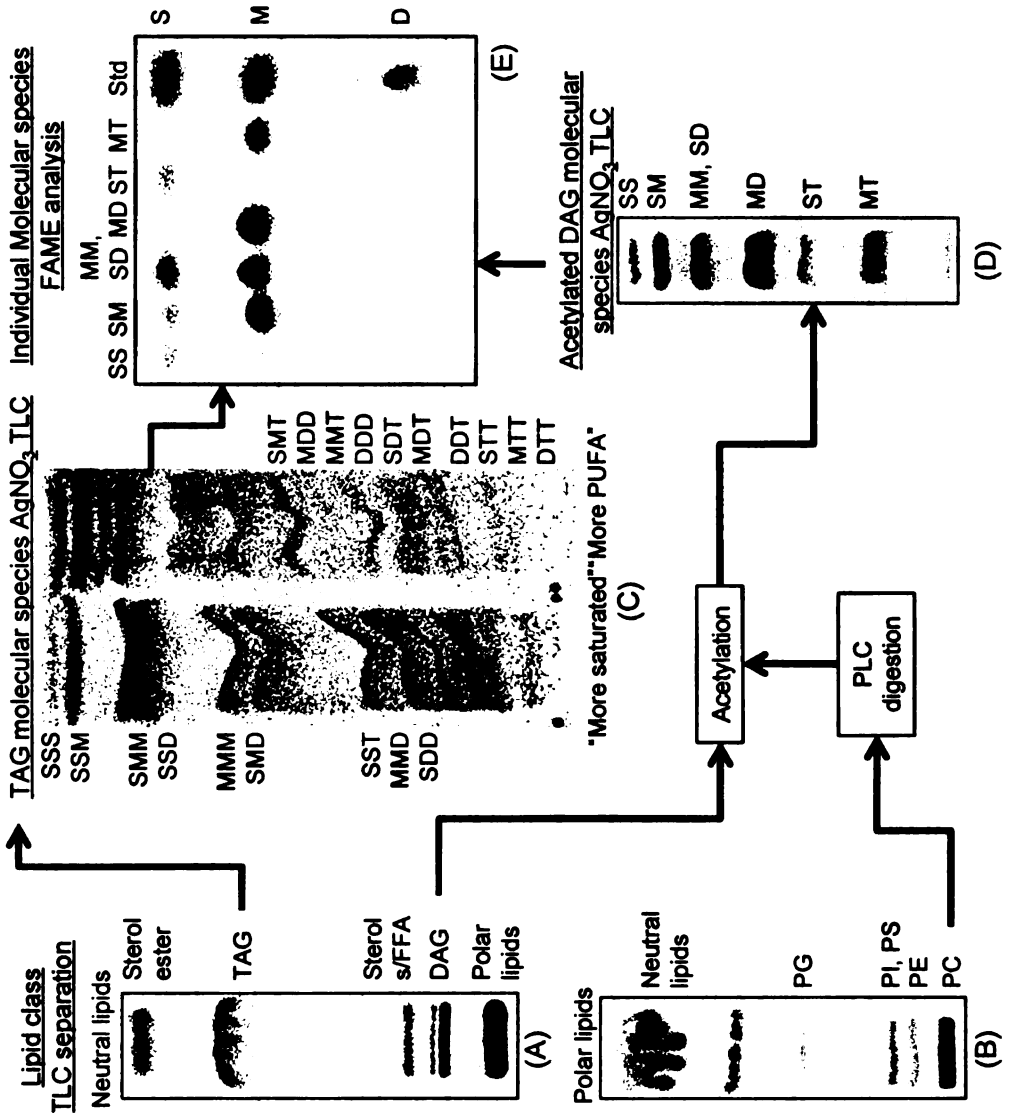


Figure 6-3 TLC image examples and flow chart for analysis of radiolabeled glycerolipids.

A-E. Examples of the major TLC systems used during analysis of radiolabeled glycerolipid classes, molecular species, and FA composition. Molecular species are represented as a combination of two or three FA, total saturates, S; monoenes (18:1), M; dienes (18:2), D; trienes (18:3), T. **A,** Neutral lipid class TLC, pictured 6 min [^{14}C]acetate. **B,** Polar lipid class TLC, pictured 6 min [^{14}C]acetate. **C,** TAG molecular species argentation TLC, pictured 30 min [^{14}C]glycerol. **D,** Molecular species separation of PC and DAG as 1,2-diacyl-3-acetyl-glycerols, pictured 6 min [^{14}C]acetate PC. **D,** FAME argentation TLC, pictured FAMEs from different 6 min [^{14}C]acetate PC molecular species.

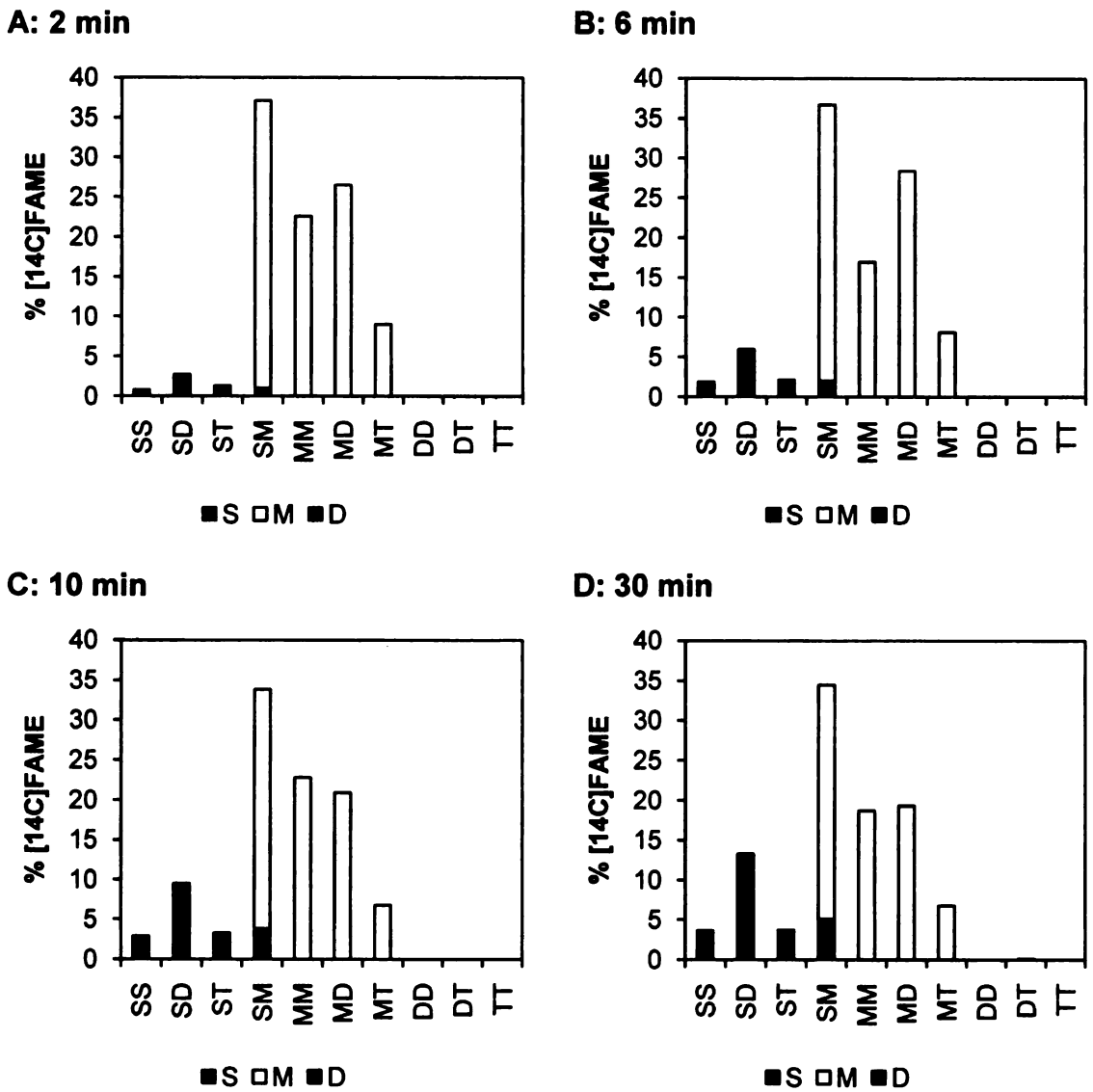
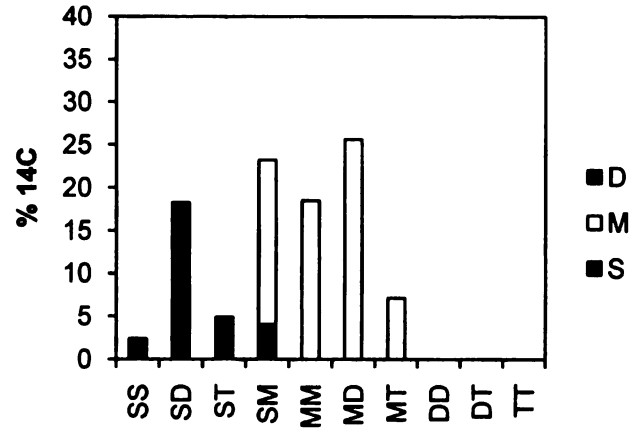


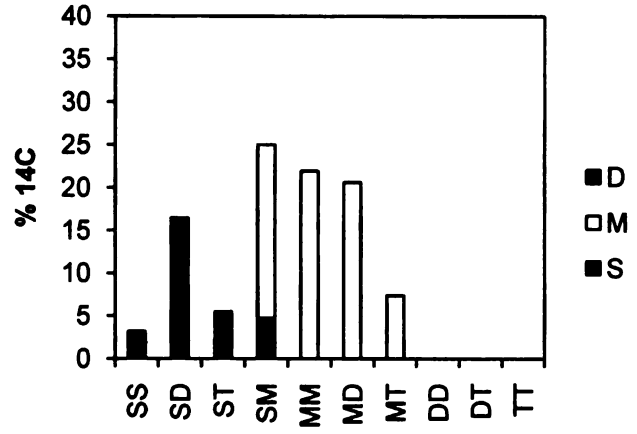
Figure 6-4 [^{14}C]acetate labeled PC molecular species time course.

A-D, molecular species are represented as pairs of FA, with bar heights represent % of species among the total. Bar shading represent % of [^{14}C]FA among species. In species with only one color it means that only that FA is labeled the other FA within the molecular species is unlabeled. *Bar shading:* S, total saturates, black bars; M, monoenes (18:1), white bars; D, dienes (18:2), gray bars. **A-D** Time of [^{14}C]acetate labeling: **A**, 2 min; **B**, 6 min; **C**, 10 min; **D**, 30 min.

A: 6 min



B: 10 min



C: 30 min

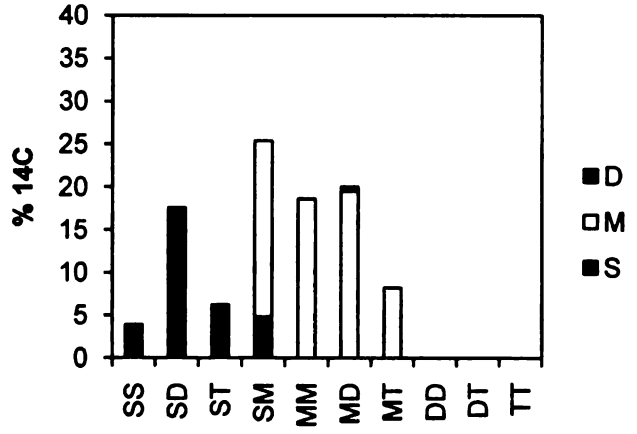
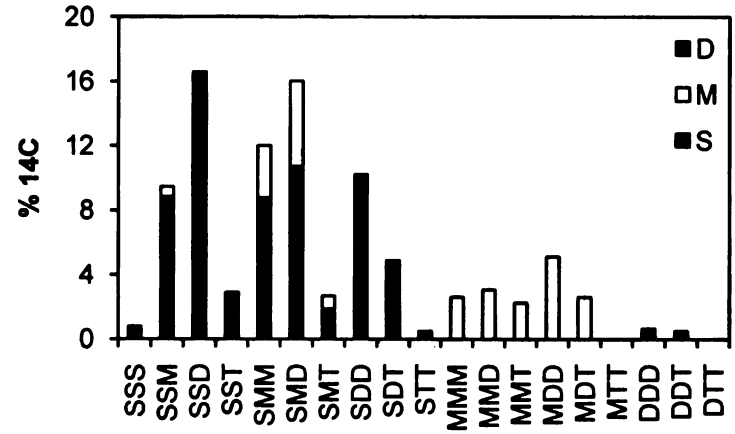


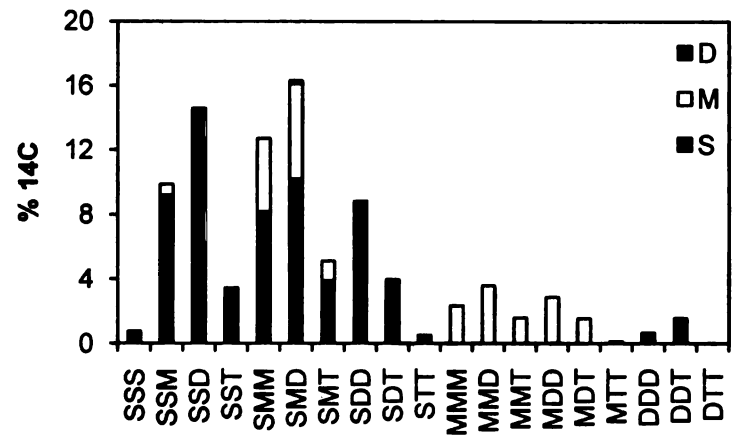
Figure 6-5 [^{14}C]acetate labeled DAG molecular species time course.

A-C, molecular species are represented as pairs of FA, with bar heights represent % of species among the total. Bar shading represent % of [^{14}C]FA among species. In species with only one color it means that only that FA is labeled the other FA in the molecular species is unlabeled. *Bar shading:* S, total saturates, black bars; M, monoenes (18:1), white bars; D, dienes (18:2), gray bars. **A-C,** Time of [^{14}C]acetate labeling: **A**, 6 min; **B**, 10 min; **C**, 30 min.

A: 6 min



B: 10 min



C: 30 min

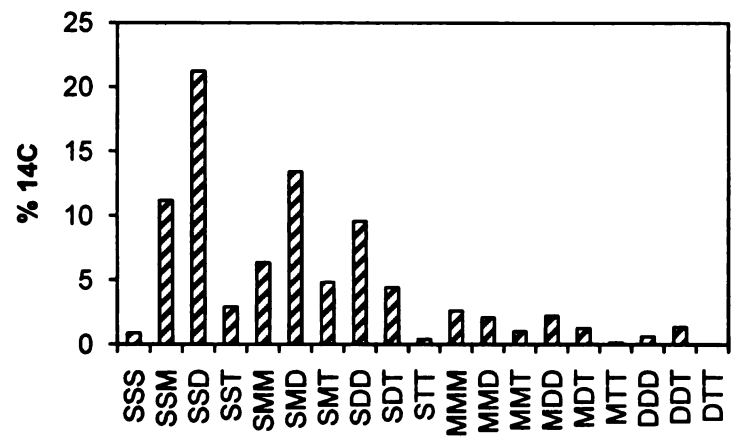


Figure 6-6 [^{14}C]acetate labeled TAG molecular species time course.

A-C, molecular species are represented as three of FA, with bar heights represent % of species among the total. **A-B,** bar shading represent % of [^{14}C]FA among species. In species with only one color it means that only that FA is labeled the other FA in the molecular species is unlabeled. *Bar shading:* S, total saturates, black bars; M, monoenes (18:1), white bars; D, dienes (18:2), gray bars. **A** 6 min labeling; **B,** 10 min labeling. **C,** Only the total [^{14}C] label per molecular species is reported.

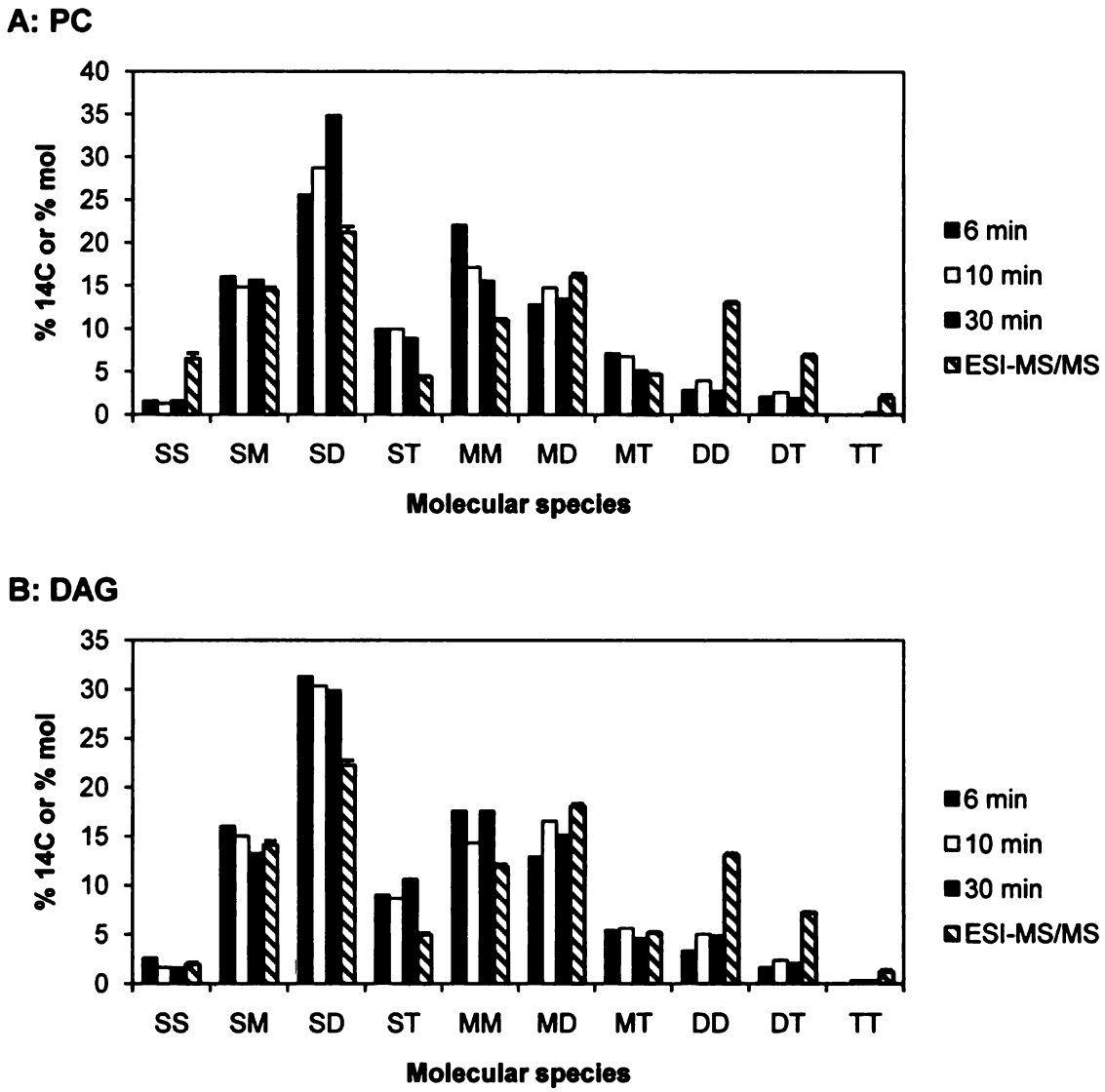


Figure 6-7 [^{14}C]glycerol labeled molecular species time course of PC and DAG.

A-B, molecular species are represented as a combination of two FA. Total saturates, S; monoenes (18:1), M; dienes (18:2), D; trienes (18:3), T. [^{14}C]glycerol labeled molecular species are backbone label only (acyl chain labeling hase been subtracted). Bar shading represents labeling time: 6 min, black bars; 10 min, white bars; 30 min, gray bars. The endogenous mass molecular species compositions are from ESI-MS/MS measurements, hatched bars. **A,** PC. **B,** DAG.

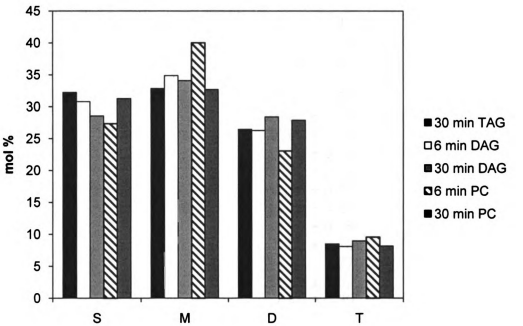


Figure 6-8 Fatty acid composition of [^{14}C]glycerol labeled TAG, DAG and PC molecular species.

The fatty acid composition of acyl groups esterified during *de novo* glycerolipid synthesis with glycerol-3-phosphate acylation. Values are calculated from the [^{14}C]glycerol backbone labeled molecular species in figures 7C (TAG) and S7 (PC and DAG). Fatty acids are represented as number of double bonds: saturates, S; monoenes, M; dienes, D; trienes, T. Bar shading: 30 min TAG, black bars; 6 min DAG, white bars; 30 min DAG, light gray bars; 6 min PC, hatched bars; 30 min PC, dark gray bars.

TAG Molecular Species	nanomoles TAG/embryo
PPI	4.19 ± 0.17
PPL	12.92 ± 0.35
PPO	4.52 ± 0.35
PPS	0.66 ± 0.27
PLL	8.03 ± 0.28
PLI	26.86 ± 1.23
PLL	38.60 ± 1.11
POI	15.78 ± 0.45
POL	37.72 ± 2.13
PSI	2.16 ± 0.12
POO	13.70 ± 0.26
PLI	9.26 ± 0.18
PSO	6.02 ± 0.78
LII	14.10 ± 0.77
LLI	34.12 ± 1.82
OII	7.40 ± 0.39
OLT	39.06 ± 1.75
LLL	28.94 ± 1.29
SII	1.33 ± 0.05
OLL	43.15 ± 1.75
OOI	12.88 ± 0.52
SLI	7.69 ± 0.31
OOL	28.44 ± 2.06
OLL	10.49 ± 0.76
SOI	5.27 ± 0.38
SOL	16.20 ± 0.76
OOO	11.95 ± 0.56
SSI	0.79 ± 0.03
SOO	8.23 ± 0.40
SSL	2.97 ± 0.14
SSO	3.35 ± 0.21
ALL	2.43 ± 0.09
AOI	2.85 ± 0.11
AOL	3.70 ± 0.20

Table 6-1 TAG Molecular Species Composition of Soybean Embryos

Individual TAG molecular species of developing soybean embryos were quantified using ESI-MS and ESI-MS^2. Values represent nanomoles of TAG per embryo (± standard deviation). Descriptions of the various molecular species do not imply any specific stereochemical arrangement of the different acyl chains. Abbreviations for fatty acids: P = palmitate (16:0), S = stearate (18:0), O = oleate (18:1), L = linoleate (18:2), I = linolenate (18:3) and A = arachidonate (20:0)

DAG Molecular Species	picomoles DAG/embryo
PP	19.75 ± 2.94
PI	384.64 ± 22.84
PL	1629.70 ± 146.31
PO	878.23 ± 80.62
PS	80.59 ± 11.76
II	125.62 ± 10.41
LI	751.03 ± 39.99
OI	537.02 ± 30.00
LL	1364.04 ± 76.21
SI	130.86 ± 8.31
OL	1884.44 ± 119.75
SL	692.70 ± 43.88
OO	1239.94 ± 78.56
SO	594.25 ± 46.74
SS	105.53 ± 11.42

Table 6-2 DAG Molecular Species Composition of Soybean Embryos

DAG molecular species of developing soybean embryos were converted to their *sn*-1,2-diacyly-3-acetyl-glycerol derivatives and then quantified using ESI-MS and ESI-MS^2. Values represent picomoles of DAG per embryo (± standard deviation). Descriptions of the various molecular species do not imply any specific stereochemical arrangement of the different acyl chains. Abbreviations for fatty acids: P = palmitate (16:0), S = stearate (18:0), O = oleate (18:1), L = linoleate (18:2), and I = linolenate (18:3).

PC Molecular Species	picomoles PC/embryo
PP	952.25 ± 118.71
PI	832.32 ± 97.16
PL	3827.04 ± 456.43
PO	2141.75 ± 245.10
PS	524.49 ± 76.10
II	523.51 ± 98.42
LI	1836.70 ± 218.43
OI	1244.15 ± 128.81
LL	3483.64 ± 360.67
SI	358.86 ± 37.87
OL	4360.23 ± 460.21
SL	1912.57 ± 186.51
OO	2964.48 ± 289.10
SO	1730.68 ± 165.72
SS	259.02 ± 43.78

Table 6-3 PC Molecular Species Composition of Soybean Embryos

DAG molecular species of developing soybean embryos were converted to their *sn*-1,2-diacyly-3-acetyl-glycerol derivatives and then quantified using ESI-MS and ESI-MS^2. Values represent picomoles of PC per embryo (± standard deviation). Descriptions of the various molecular species do not imply any specific stereochemical arrangement of the different acyl chains. Abbreviations for fatty acids: P = palmitate (16:0), S = stearate (18:0), O = oleate (18:1), L = linoleate (18:2), and I = linolenate (18:3).

6.2 Supplement: Kinetic modeling of pool filling

To choose between alternative models of TAG synthesis which take into account various options for DAG-PC interconversions simulations of kinetic labeling were undertaken (Figure 3-9, and 6-9). The expectation was not that any of the more complex models would exactly match the actual kinetics measured (Figures 3-2, 3-7). Instead the modeling is used to formulate the best descriptions of acyl metabolism.

In such modeling of this *in vivo* data there is a list of implicit assumptions:

(1) The cells are in a uniform physiological state.

(2) Although exogenous "non-physiological" substrates may penetrate differently, may perturb metabolism slightly differently, they effectively report the bulk tissue metabolism.

(3) *In vivo* variance, including that generated by using different experiments used for acetate and glycerol labeling is small.

(4) We assume that there is no futile cycling of acyl groups, that TAG is a static end product, and that different lipid molecular species have similar time constants for pool filling.

(5) Minor acyl fluxes, such as to sphingolipids, plastid lipids, PE etc. are ignored.

The modeling is set up with 1000 moles FA in total PC and 400 moles in total DAG (based off of Table 3-1), which are invariant in size over the hour period the simulation is run. The inputs are either 100 moles/hr of FA into the

system into the DAG(input) pool, or 300 moles via acyl editing into a bulk or small active PC pool. The output is 100 moles/hr of FA into TAG. A simple Excel spreadsheet is used to run the simulation, using 0.01 hour increments, that is, with 1 mole FA input per time increment when modeling for *de novo* DAG input. The models simulated are based off those represented in Figure 3-9. In the case of the simplest model tested, model B (Figure 6-9a), the rate constant for DAG to TAG conversion k_1 will be 0.25 hr^{-1}, to allow a fixed rate of TAG synthesis of 100 moles/hr (400 moles DAG x 0.25 hr^{-1}). The rate constants for the DAG to PC (k_2) and PC to DAG conversions (k_3) can be set at any value, but because the DAG and PC pool sizes must be kept constant, the ratio of k_2:k_3 = 2.5. The values of k_2 and k_3 were then varied to allow a simulation of glycerol backbone labeling from DAG that produced continuous DAG labeling but where PC labeling crossed over DAG labeling at about 30 minutes as in the experimental results (Figure 3-7A). This occurred when the forward and back reactions for the DAG to PC interconversion were 20 times the rate of TAG synthesis (Figure 6-9a). These parameters were then used to test what would be predicted for an acyl editing input (Figure 6-9b). At 30 minutes about 21% of the PC would be converted to DAG. This is inconsistent with experimental observations that show about 3% of the PC have actually moved from PC to DAG. Thus model B fails to predict the slow movement of labeled PC to DAG. If at 0.5 hr 21% PC was converted to DAG that would change the composition and stereochemistry of the DAG product dramatically.

Additional pool filling simulations for Models B and C (including variants) were run to see how closely they would conform to the experimental kinetics of glycerol backbone labeling, of TAG labeling, and of acyl editing. Some of these are shown in Figure 6-9. Variants on model B suffered the same problem as the simplest form of model B (too much back conversion of acyl labeled PC to DAG at early time points). By contrast, variants on model C was generally much more successful at describing the DAG-PC precursor-product glycerol labeling kinetics and yet having slow movement of acyl editing labeling of PC move through into DAG. Model C variants have as a common theme at least two kinetically distinct DAG pools, DAG(in) for the immediate products of de novo DAG synthesis, and DAG(out), which supplies TAG synthesis. The DAG input pool is kept small (2% of PC), in keeping with the observation of very small DAG pools observed in leaf, because the *de novo* DAG pool must be compositionally distinct from the bulk DAG pool, and because glycerol labeled DAG moves through to PC very significantly within 30 minutes (PC pool filling to TAG takes 10 hours, so if 15 min pool filling of DAG occurs, it will be 1/40 of PC). To fit for the most simplified version of Model C ($\rightarrow$ DAG $\rightarrow$ PC $\rightarrow$ DAG $\rightarrow$TAG) gives are rather flattened DAG filling curve (6- 9c) compared to Figure 3-7, although this can be attenuated by adding a direct low flux loop from DAG(in) to DAG(out). Thus Model C, variant 1 seems unlikely.

Splitting the PC pool into a small, active pool and a large, bulk pool Model C, variant 2 (Figure 6-9d) greatly alleviates the problem of slow DAG pool filling

at later times seen in Model C, variant 1. Keeping the active PC pool at 2% of the total PC, and therefore at the same size as DAG(in), requires that the interchange between the active and bulk PC pools be 3 times the rate of TAG synthesis. If this is done, then acyl editing must be largely a function of the bulk pool (Figure 6-9e), not the active pool (Figure 6-9f). In proposing two PC pools, it is not clear whether the desaturases act on PC during its residence in the small, active PC pool, or the bulk PC pool, or both. One problem with simulations of Model C, variants 1 (Figure 6-9c) and 2 (Figure 6-9d), was that they predicted movement of glycerol labeling into TAG that was much slower than observed (Figure 3-7A). This could be remedied by splitting the DAG(out) pool into a small active and a large bulk component (Figure 6-9g, Model C, variant 3). Of course, with multiple TAG synthesis routes, this partitioning of DAG(out) may still be a simplification. More generally, the splitting of the intermediate pools of PC and DAG allows for a large degree of flexibility in modeling TAG synthesis, with many combinations of pool sizes and exchange rates. The exercise of modeling initial kinetics has lead to Model C, variant 3 as the preferred model, giving a reasonable though not an exact simulation. However, many more complex variants are possible and cannot be adequately distinguished. Clearly, additional methods, such as longer term and pulse-chase labeling, will be required to refine the model.

Figure 6-9 Kinetic modeling of pool filling simulations

Graphs of simulations of pool filling, either from *de novo* DAG acyl labeling (it will be the same for glycerol backbone *de novo* labeling), or from acyl editing inputs. The pool filling represents the labeling experiments, where nascent, labeled acyl groups are available from fatty acid synthesis for glycerolipid synthesis.

Figure 6-9a: Pool filling from de novo DAG synthesis using model B.
Figure 6-9b: Pool filling from acyl editing using model B.
Figure 6-9c: Pool filling from de novo DAG synthesis using model C, variant 1.
Figure 6-9d: Pool filling from de novo DAG synthesis using model C, variant 2.
Figure 6-9e: Pool filling from acyl editing via the bulk PC pool using model C, variant 2.
Figure 6-9f: Pool filling from acyl editing via the active PC pool using model C, variant 2.
Figure 6-9g: Pool filling from de novo DAG synthesis using model C, variant 3.

Figure 6-9a: Pool filling from de novo DAG synthesis using model B.

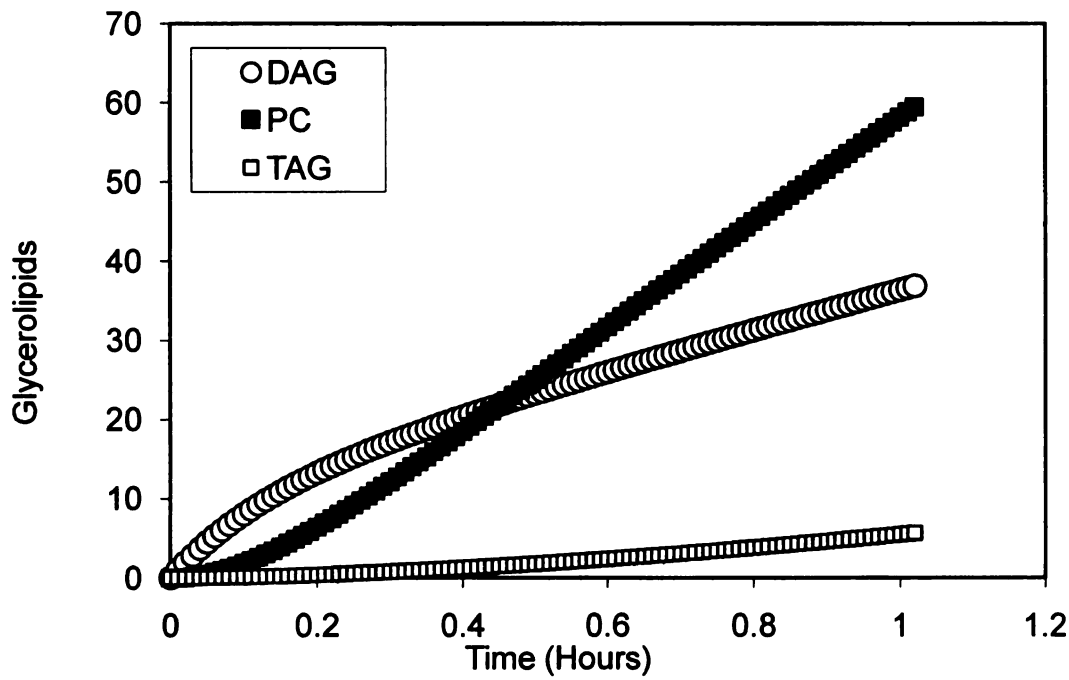


Figure 6-9b: Pool filling from acyl editing using model B

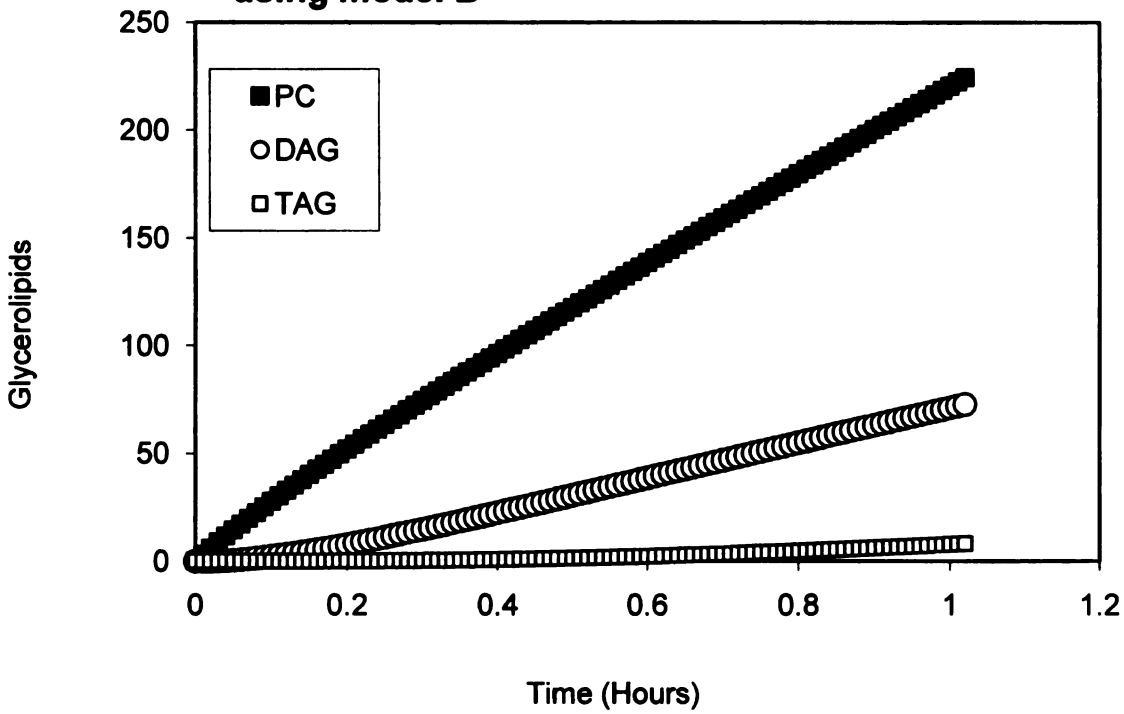


Figure 6-9c: Pool filling from de novo DAG synthesis using model C, varient 1.

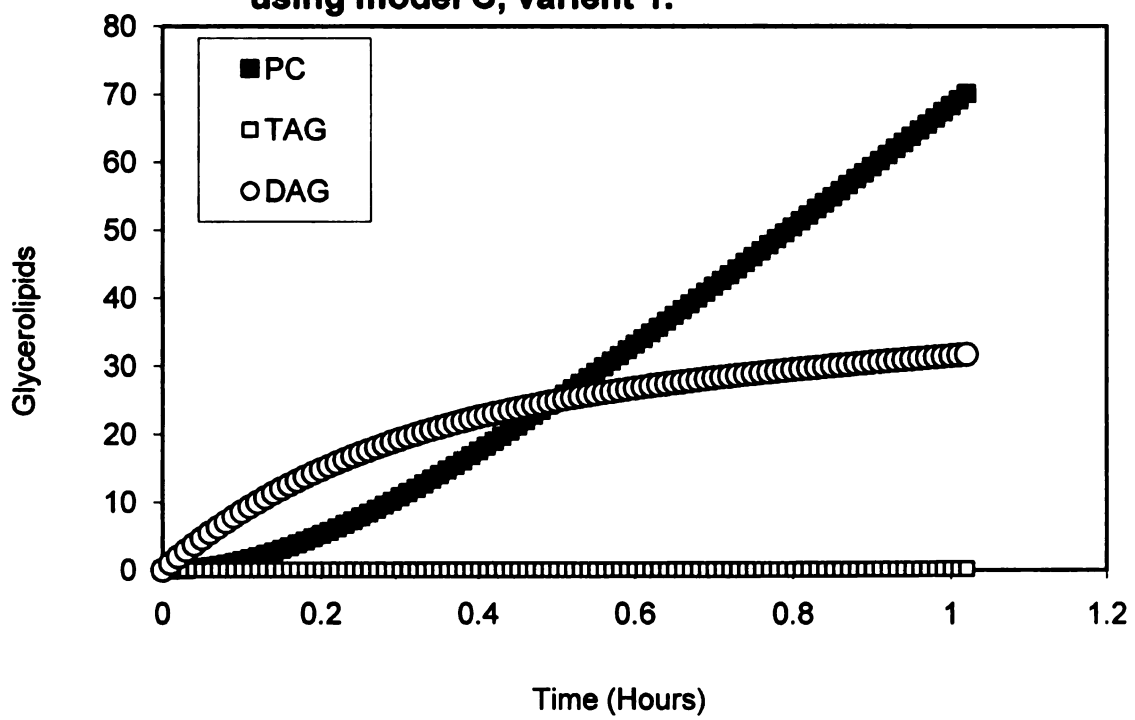


Figure 6-9d: Pool filling from de novo DAG synthesis using model C, variant 2.

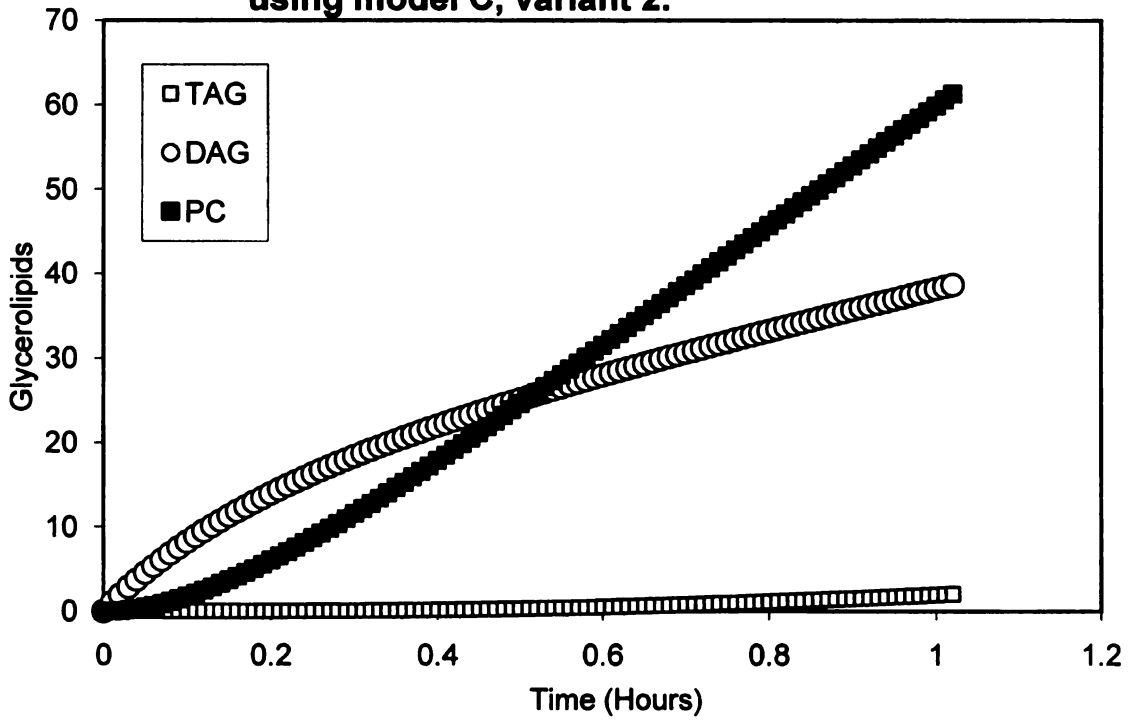


Figure 6-9e: Pool filling from acyl editing via the bulk PC pool using model C, variant 2.

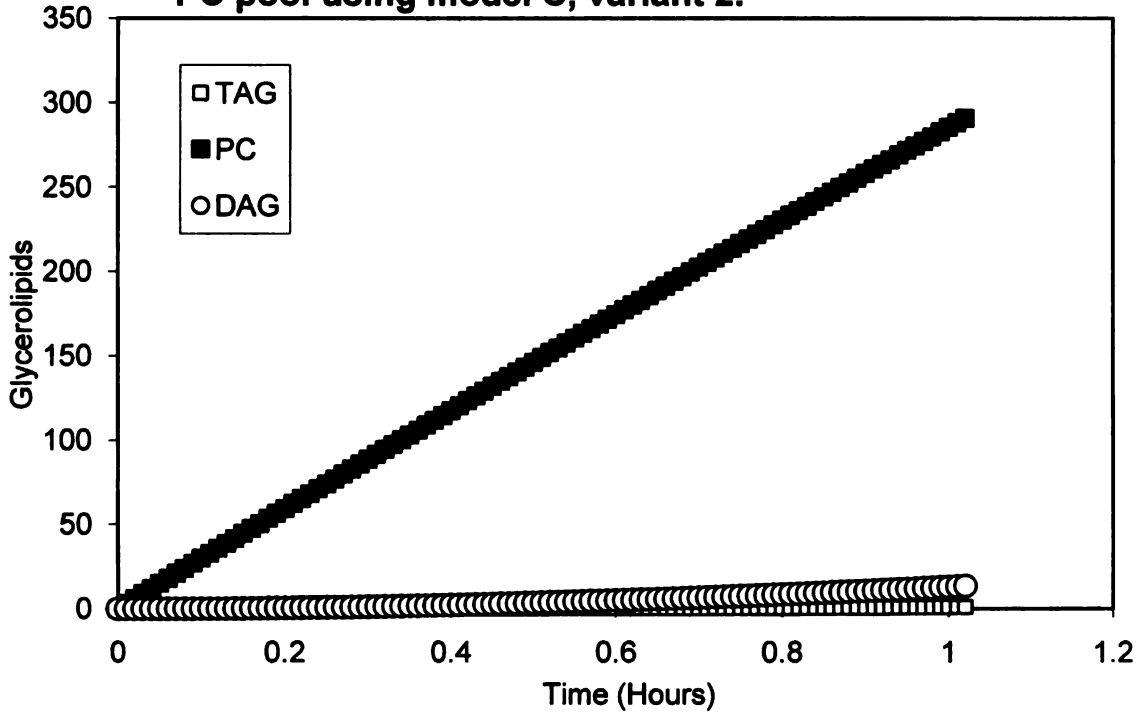


Figure 6-9f: Pool filling from acyl editing via the active PC pool using model C, variant 2.

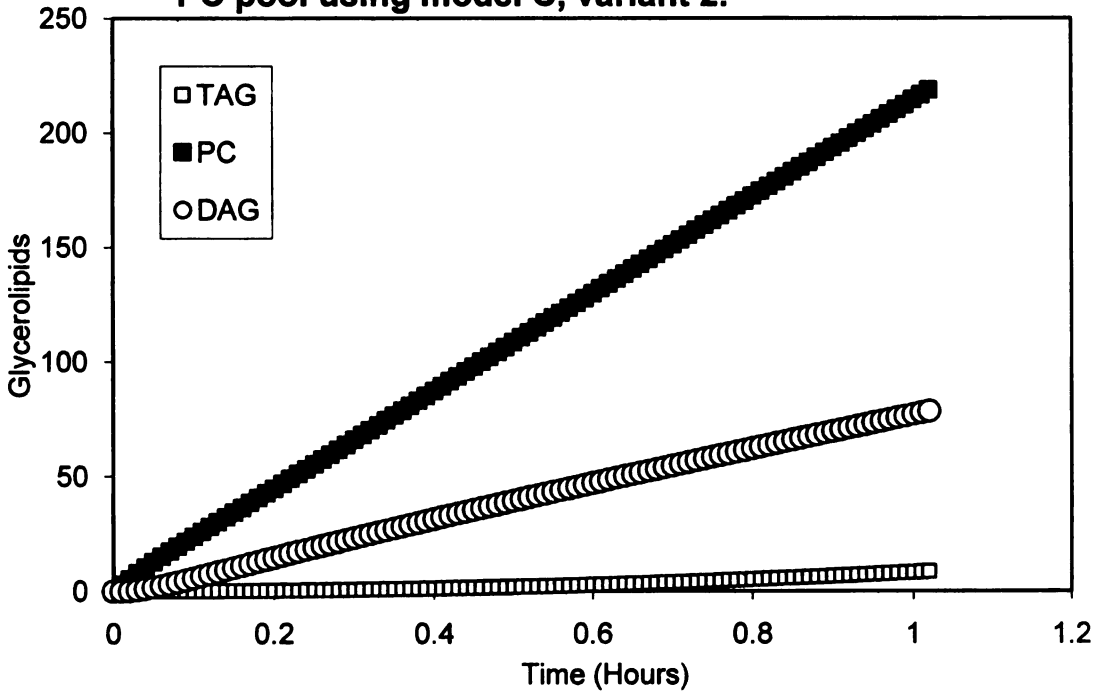
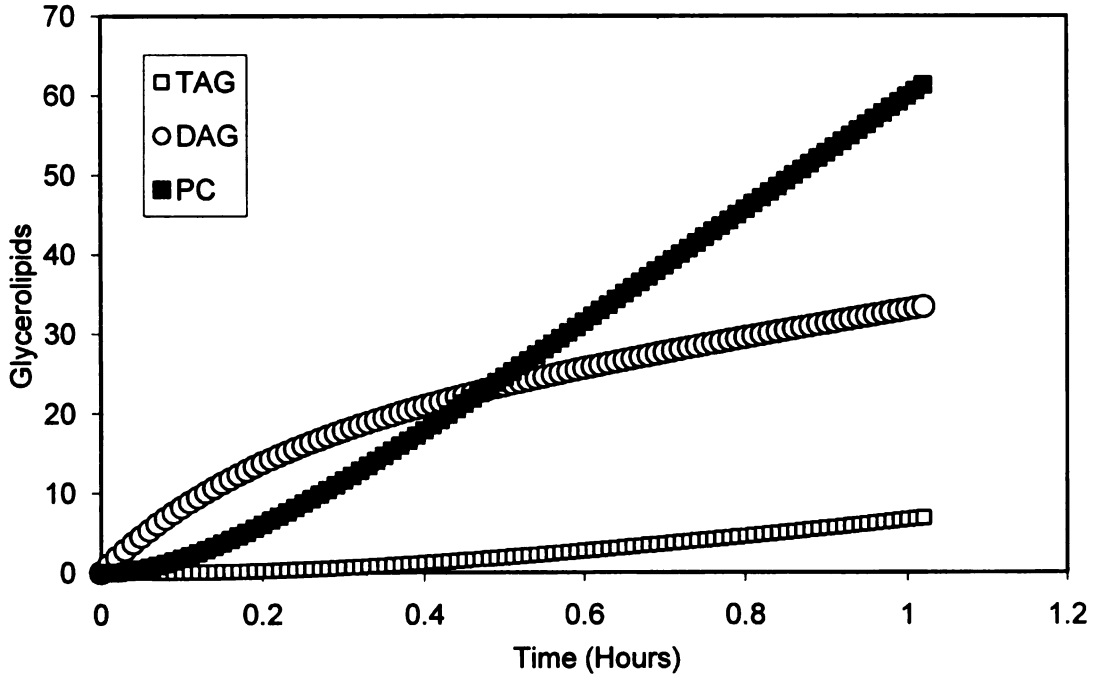


Figure 6-9g: Pool filling from de novo DAG synthesis using model C, variant 3.



6.3 Discussion of soybean TAG synthesis flux model Figure 3-10

Model assumptions

The following assumptions used to generate a model of acyl group fluxes in soybean embryos are based on the endogenous lipid compositions and the [^{14}C]acetate/[^{14}C]glycerol labeling results and conclusions. 1) *De novo* synthesis of DAG and acyl editing of PC are metabolically distinct processes. 2) Nascent FA directly enter PC through *sn*-1/*sn*-2 acyl editing. 3) Nascent FA can mix with recycled FA released from acyl editing for *de novo* glycerolipid synthesis. 4) The major flux of *de novo* synthesized DAG is for PC synthesis. 5) TAG synthesis utilizes the bulk DAG pool (not *de novo* DAG) generated from bulk PC. 6) The bulk DAG generating reaction is in a different location or selective such that *de novo* synthesized PC and acyl edited PC are not rapidly used for TAG synthesis. 7) In a soybean embryo rapidly accumulating oil 90% of FAS is ultimately used for TAG synthesis and most of the PC/DAG produced is turned over for TAG synthesis. 8) Newly synthesized FA are incorporated into PC:DAG:TAG at relative initial rates of approximately 4:1:1, respectively. Thus the initial FAS product incorporation into glycerolipids: 60% PC, 15% DAG, 15% TAG (total 90%).

Table 6-4 TAG synthesis model: nascent FA flux value calculations

	Total [^{14}C]FA	S (% ^{14}C)	M (% ^{14}C)	Nascent S Flux	Nascent M Flux
PC	57	5	95	2.9	54.1
DAG	17	29	71	4.9	12.1
TAG	11	75	25	8.2	2.8
Other	15	29	71	4.4	10.6

[^{14}C]acetate labeling values used to calculate the flux of newly synthesized saturated (S, 16:0, 18:0) and monounsaturated (M, 18:1) FA through the lipid metabolism model towards TAG. Total [^{14}C]FA values are based on the fact that 85% of newly synthesized FA are incorporated into PC, DAG and TAG at a ratio of 10:3:2. The remaining 15% most likely is incorporated into other membrane lipids. Nascent S & M flux values are The model assumes that 93% of FA will ultimately flux into TAG to generate the endogenous lipid composition.

Supplement: step by step logic in flux model construction

Model development assumes 100 moles of FAS and utilizes mass balance along with the initial nascent FA incorporation rates and compositions (Table 6-4). Newly synthesized FA are incorporated into PC:DAG:TAG at relative initial rates of 10:3:2, respectively. Thus of initial FAS product incorporation into glycerolipids: 57% PC, 17% DAG, 11% TAG (total 85%). If 93 moles of FA are ultimately incorporated into TAG (based on Table 1, and that PC represents ~1/2 of soybean membrane lipids) and the remaining 7 moles for membrane lipid synthesis then 31 and 3.5 moles of G3P are required for acyl group esterification, respectively. *De novo* synthesis of 34.5 mol of total glycerolipids would require 69 mol of FA to flux through PA and DAG into the *sn*-1/*sn*-2 positions of membrane lipids and TAG. The remaining 31 moles from FAS will ultimately be esterifed to

the *sn*-3 position of TAG. If 32 moles of nascent [^{14}C]FA are used by *de novo* glycerolipid synthesis (Table 6-4, DAG + other lipids), then an additional 37 moles of recycled [^{12}C]FA are required to balance *de novo* synthesis. Presumably this is provided by the acyl chains released by acyl editing to allow 57 mol of nascent [^{14}C]FA incorporation into PC through Lyso-PC acylation. However, 57 mol of PC acyl editing will not provide enough saturates to sustain *de novo* glycerolipid synthesis. Saturates make up ~29% of acyl groups entering *de novo* glycerolipid synthesis, as estimated from [^{14}C]glycerol labeling (Figure 6-8). Thus total saturates would comprise 20 moles of the 69 moles acylated to G3P. Newly synthesized FA would contribute 9.3 moles of saturates (Table 6-4), requiring 10.7 moles of recycled saturates. However, 57 mol of PC acyl editing with equal *sn*-1/*sn*-2 contribution could only provide up to 7.6 mol of saturated FA (PC 26.5% S x 28.5 mol *sn*-1 FA release). Additionally, nascent FA incorporation into PC was 14% *sn*-1 and 86% *sn*-2. Saturates are predominately at the *sn*-1 position, therefore if the labeling reflects the positional release of FA during acyl editing much less saturates would be released and recycled into *de novo* synthesis. Therefore the flux of acyl editing cannot be one acyl group released from PC for each nascent acyl group incorporated, and must be 1.4 times that required for incorporation of nascent FA into PC with the possibility of being much greater. The excess flux of recycled acyl groups produced during acyl editing may be re-incorporated into PC through the acyl editing cycle (Figure 3-10), with the entire process reducing the saturated concentration of PC and redistributing FA between the *sn*-1/*sn*-2 positions.

It is very unlikely that nascent acyl groups are channeled directly into Lyso-PC that is under a high flux of reacylation with recycled acyl groups, but more likely the nascent acyl groups mix with the recycled acyl groups of the rapid acyl editing cycle (Figure 3-10). Initial PC and DAG contain different [^{14}C]FA incorporation rates and compositions indicating there is either different mixed pools of new and recycled acyl groups feeding *de novo* synthesis and acyl editing or that the acyl editing flux is selective and fast enough that over three times as much nascent FA enter PC than DAG (Figure 3-2) through *de novo* synthesis from the same mixed pool. To determine the relative flux of acyl groups around the acyl editing cycle we consider how many more FA must be removed from the mixed acyl pool by acyl editing compared *de novo* synthesis to produce the different [^{14}C]FA labeling amounts and stereochemistry of PC and DAG. To simplify the compositional and stereochemical differences of acylation we consider the relative flux of [^{14}C]M into the *sn*-2 position of both lipids, assuming initial labeling of PC to be 100% *sn*-2 M and DAG to be 50% *sn*-2 M. In this scenario 54.1 moles nascent [^{14}C]M enter *sn*-2 PC and 11.4 moles enter *de novo* glycerolipid synthesis (*de novo* DAG + other membrane lipids, Table 6-4). Therefore the flux of nascent [^{14}C]M into *sn*-2 PC is a maximal 4.8 times faster than esterification of *sn*-2 G3P, any greater flux would lower the amount of nascent acyl groups entering *de novo* glycerolipid synthesis. However, if there are any enzymatic selectivites of LPAT or acyl editing enzymes there is a possibility that the flux could be lower.

TAG synthesis will require 31 nmoles of *sn*-3 acylation. About 11 moles of this flux is provided by the immediate incorporation of nascent FA, with a high preference for saturates (Table 6-4). Analysis of the molecular species of [^{14}C]FA labeled TAG suggests that this direct acylation utilizes the bulk DAG pool, not the *de novo* synthesized DAG pool, and provides about 35% of the molecular species in endogenous TAG. The other 65% of TAG synthesis would be compensated by utilizing the recycled acyl-CoA pool from acyl edting and also PDAT reactions.

7 APPENDIX C: PC ACYL EDITING IN ISOLATED PEA CHLOROPLASTS

Introduction

Isolated pea chloroplasts have been shown to channel newly synthesized FA into PC, even though chloroplasts cannot synthesized PC *de novo* [79]. To determine if acyl editing is involved in this channeling of newly synthesized FA into PC, isolated pea leaf chloroplasts were labeled with [^{14}C]acetate. The [^{14}C]FA labeled PC was analysed for labeled FA composition, regiochemistry, and molecular species.

Results and Discussion

After 5 min of [^{14}C]acetate labeling of isolated pea chloroplasts over half the radioactivity was in free fatty acids and acyl CoA as reported previously [79]. PC contained the most labeled FA out of all labeled polar lipids and was entirely [^{14}C]18:1. The stereochemical analysis indicated that ~93% of labeled FA were localized to the *sn*-2 position. Separation of labeled molecular species (Figure 7-1) demonstrated that nascent [^{14}C]FA were incorporated next to unlabeled saturates, mono-, di- and tri-unsaturates. Together these results are very similar to the acyl editing measured by *in vivo* labeling of pea leaves, with the exception of a highly *sn*-2 specific FA incorporation.

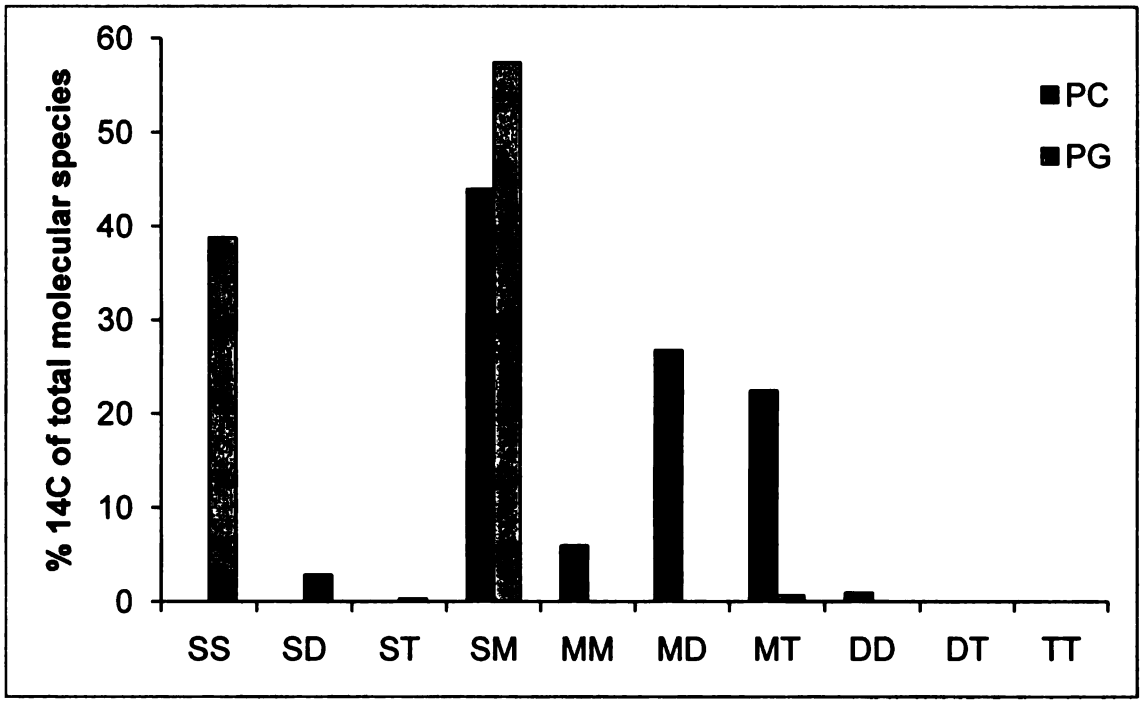


Figure 7-1 [^{14}C]acetate labeled PC and PG molecular species from isolated chloroplasts.

Isolated chloroplasts were labeled with [^{14}C]acetate for five minutes. Molecular species of PC and PG were separated by argentation TLC. Bar heights represent percent of labeled species among each lipid. Molecular species are represented as a pair of FA. S, saturates; M, monounsaturates; D, diunsaturates, triunsaturates. Black bars, PC. Gray bars, PG.

The radiolabeled FA composition and molecular species of the prokaryotic lipid PG were analyzed to ensure that the PC labeling results were from acyl editing and not due to a transfer of the PC phosphocholine headgroup to a prokaryotic pathway synthesized DAG. PG from five minute [^{14}C]acetate labeled pea leaf chloroplast contained approximately equal amounts of saturated and monounsaturated radiolabeled FA. The molecular species labeled were predominantly fully saturated and saturated/monounsatured (Figure 7-1). Together the PG labeling is the expected result of a *de novo* synthesized prokaryotic lipid. The difference in PG and PC labeling confirms that incorporation of newly synthesized FA into PC of isolated chloroplasts is not from prokaryotic pathway produced DAG.

Therefore, incorporation of newly synthesized FA into PC of isolated pea chloroplasts is through acyl editing of the *sn*-2 position. In pea leaves PC *sn*-2 acyl-editing may be located within the outer chloroplast envelope or associated with the chloroplast through the PLAM region. The lack of PC *sn*-1acyl editing in isolated chloroplasts compared to intact leaves may be because this process takes place within a different cellular membrane, involves soluble enzymes, or the activity may be disrupted by chloroplast isolation.

Methods

Pea plants were grown, chloroplasts isolated, labeling conditions and lipid extraction were as done previously [79]. Labeling was started by adding 5 µCi of 50 mCi/mmol [^{14}C]acetate resuspened in labeling media to 0.4 mg chlorophyll of chloroplasts with a final volume of 0.2 ml. Chloroplasts were incubated at room

temperature under ~180 mol of photons/m2/s of white light, with gentle shaking for 5 min. The labeling reaction was stopped by adding 2 ml of chloroform/methanol (1:1, v/v) to start the lipid extraction. Lipid separation, radioactive fatty acid composition, regiochemistry and molecular species were determined as done previously [162].

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