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Probing the limits of very long chain polyunsaturated fatty acid accumulation in transgenic *Brassica napus*

by

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Dedication

To my mother - my best friend and first teacher, a woman of uncommon strength and hard-earned wisdom, who never faltered in her support, but gave me the courage to find my own way.

Abstract

Transgenic oilseeds capable of producing high levels of very long chain polyunsaturated fatty acids (VLCPUFA) represent a promising alternative to marine oils as a source of nutritionally important fatty acids such as arachidonic acid (ARA, $20:4^{\Delta5,8,11,14}$) and eicosapentaenoic acid (EPA, $20:5^{\Delta5,8,11,14,17}$). Previous studies demonstrated that in transgenic oilseeds, elongation of fatty acids from C18 to C20 is a major bottleneck in VLCPUFA accumulation, likely due to limited availability of polyunsaturated C18 acyl-CoA substrates for elongation. The objective of the current study was to determine the underlying biochemical basis for the absence of C18-PUFA in the acyl-CoA pool of transgenic *Brassica napus* engineered to produce ARA and EPA. A comparison of acyl-CoA dependent acyltransferase activities in developing seeds suggested that the high activity of lysophosphatidylcholine acyltransferase (LPCAT) throughout seed development may support the rapid channeling of acyl-CoAs into phosphatidylcholine (PC). However, both ^{14}C-acetate and fatty acid feeding experiments with zygotic embryos of *B. napus* showed that fatty acids are incorporated into triacylglycerol (TAG), with no substantial short- or long-term accumulation in phosphatidylcholine (PC). Additionally, it was demonstrated application of exogenous γ-linolenic acid (GLA, $18:3^{\Delta6,9,12}$) to cultured zygotic embryos increases its availability in the acyl-CoA pool and results in a significant increase in dihomo-γ-linolenic acid (DGLA, $20:3^{\Delta,8,11,14}$). Attempts to manipulate the availability of cytosolic malonyl-CoA through citrate, malonate or acetate supplementation did not result in a further increase in GLA elongation, suggesting

malonyl-CoA availability does not limit overall elongation efficiency in transgenic *B. napus*. Finally, preliminary experiments investigating the fate of acyl groups at the *sn*-2 position of PC revealed considerable movement of acyl groups from PC to phosphatidic acid (PA), suggesting the presence of a highly active phospholipase D. The contribution of this enzyme to acyl group trafficking in the context of VLCPUFA accumulation remains to be determined.

In summary, this study effectively ruled out the hypothesis that acyl-CoA availability for elongation is limited by competition and rapid removal by other acyl-CoA dependent activities, and supports the hypothesis that newly desaturated PUFA are channeled into storage lipids through acyl-CoA independent routes, thereby limiting their availability for elongation.

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Table of Contents

List of Tables

List of Figures or Illustrations

List of symbols, nomenclature, and abbreviations

ACBP: acyl-CoA binding protein

ACCase: acetyl-CoA carboxylase

ACL: ATP:citrate lyase

ACP: acyl carrier protein

ACS: acyl-CoA synthetase

ALA: α-linolenic acid ($18:3^{\Delta 9, 12, 15}$)

ARA: arachidonic acid ($20:4^{\Delta 5,8,11,14}$)

ATP: adenosine triphosphate

BSA: bovine serum albumin

CPT: CDP-choline:diacylglycerol cholinephosphotransferase

DAF: days after flowering

DAG: *sn*-1,2-diacylglycerol

DGAT: diacylglycerol acyltransferase

DGLA: dihomo-γ-linolenic acid ($20:3^{\Delta 8,11,14}$)

DHA: docosahexaenoic acid ($22:6^{\Delta 4,7,10,13,16,19}$)

DTT: dithiothreitol

EDTA: ethylenediaminetetraacetic acid

EPA: eicosapentaenoic acid ($20:5^{\Delta 5,8,11,14,17}$)

ETA: eicosatetraenoic acid ($20:4^{\Delta 8,11,14,17}$)

FA: fatty acid

FAMEs: fatty acid methyl esters

FAS: fatty acid synthase

FFA: free fatty acid

G3P: *sn*-glycerol-3-phosphate

GC/MS: gas chromatography-mass spectrometry

GLA: γ-linolenic acid ($18:3^{\Delta 6,9,12}$)

GPAT: glycerol-3-phosphate acyltransferase

HPLC: high performance liquid chromatography

LA: linoleic acid ($18:2^{\Delta 9,12}$)

LPA: lysophosphatidic acid

LPAAT: lysophosphatidic acid acyltransferase

LPC: lysophosphatidylcholine

LPCAT: lysophosphatidylcholine acyltransferase

LPEAT:lysophosphatidylethanolamine acyltransferase

LPLAT: lysophospholipid acyltransferase

MBOAT: membrane-bound *O*-acyltransferase

MUFA: monounsaturated fatty acid

NADH: nicotinamide adenine dinucleotide

NADPH: nicotinamide adenine dinucleotide phosphate

NT: no treatment

OA: oleic acid ($18:1^{\Delta 9}$)

PA: phosphatidic acid

PAP: phosphatidic acid phosphatase

PC: phosphatidylcholine

PCR: polymerase chain reaction

PDAT: phospholipid:diacylglycerol acyltransferase

PDCT: phospholipid:diacylglycerol cholinephosphotransferase

PEG: polyethylene glycol

PIP_2: phosphatidylinositol 4,5-bisphosphate

PKS: polyketide synthase

PLA_2: phospholipase A_2

PLC: phospholipase C

PLD: phospholipase D

PUFA: polyunsaturated fatty acid

SDA: stearidonic acid ($18{:}4^{\Delta 6,9,12,15}$)

SDS: sodium dodecyl sulfate

SFA: saturated fatty acid

TAG: triacylglycerol

TLC: thin layer chromatography

VLCPUFA: very long chain polyunsaturated fatty acid

1. Introduction

In recent years, there has been increasing awareness of the importance of very long chain polyunsaturated fatty acids (VLCPUFA), such as eicosapentaenoic acid (EPA, $20:5^{\Delta5,8,11,14,17}$) and docosahexaenoic acid (DHA, $22:6^{\Delta4,7,10,13,16,19}$) in the human diet (Griffiths and Morse 2006). These fatty acids are produced naturally by a variety of marine microalgae and are typically obtained in the diet through the consumption of fatty fish. While humans possess the biosynthetic pathways to convert the more abundant dietary precursors, linoleic acid (LA, $18:2^{\Delta9,12}$) and α-linolenic acid (ALA, $18:3^{\Delta9,12,15}$) into VLCPUFA, the efficiency of this process is often limited (Plourde and Cunnane 2007). Moreover, the ratio of omega-6 to omega-3 fatty acids in the Western diet has been estimated to be about 17:1, far in excess of the 4:1 ratio considered to be optimal for human health (Simopoulos 2002). Thus, there has been considerable interest in the development of alternative dietary sources of VLCPUFA and omega-3 fatty acids in particular.

Genetically engineering oilseed crops to produce VLCPUFA could potentially address this dietary imbalance by replacing or supplementing high dietary intakes of omega-6 PUFA with omega-3 VLCPUFA-enriched oils. In addition, development of a plant-based source of VLCPUFA will address growing concerns over the environmental sustainability of harvesting these oils from marine sources. There have been numerous studies demonstrating production of VLCPUFA and their intermediates in transgenic plants, but the levels achieved, particularly for DHA, have often fallen short of commercially viable levels. So

far, production of ARA and EPA has been somewhat more successful, and these studies have revealed some key bottlenecks in the synthesis of VLCPUFA in transgenic plants.

Starting from LA and ALA, the production of ARA and EPA in transgenic plants requires the introduction of at least three genes encoding, most commonly, a Δ6-desaturase, Δ6-elongase, and a Δ5-desaturase (Abbadi et al. 2004). Previous experiments using this minimal set of genes have demonstrated relatively high accumulations of Δ6-desaturation products, but subsequent Δ6-elongation was much less efficient (Abbadi et al. 2004; Wu et al. 2005) Detailed analysis of these transgenic plants suggested a bottleneck in the channeling of newly desaturated acyl groups between phosphatidylcholine (PC), where desaturation occurs, and the acyl-CoA pool, the site of elongation (Abbadi et al. 2004).

Two hypotheses were proposed to explain this so-called "substrate dichotomy" between PC and the acyl-CoA pool (Abbadi et al. 2004). The first possibility, hereafter referred to as the "rapid removal hypothesis", is that newly desaturated Δ6-C18 PUFA are efficiently released from PC into the acyl-CoA pool, but are immediately incorporated into glycerolipids by acyl-CoA-dependent acyltransferases before elongation can take place. The second possibility (hereafter referred to as the "inefficient release hypothesis") is that newly desaturated Δ6-C18 PUFA are not efficiently released from PC, and are instead channeled into storage lipids through various acyl-CoA independent mechanisms

(Dahlqvist et al. 2000; Lu et al. 2009; Stahl et al. 2004), thus effectively limiting their availability for elongation.

The objective of the current study is to determine whether limited acyl-CoA availability for elongation in transgenic *Brassica napus* is the result of "rapid removal" or "inefficient release," and to explore the underlying biochemical basis for the substrate dichotomy problem in VLCPUFA accumulation in *B. napus*. The "rapid removal hypothesis" will be tested through a combination of *in vitro* acyl-CoA dependent acyltransferase assays and fatty acid feeding experiments in zygotic embryos of *B. napus*. In a complementary approach, fatty acid feeding experiments and acyl-CoA analysis will be used to determine whether acyl-CoA availability is the only limitation on elongation efficiency, and whether fatty acid supplementation is sufficient to overcome the substrate dichotomy bottleneck in zygotic embryo cultures. The "inefficient release hypothesis" will be tested through analysis of the fate of acyl groups at the *sn*-2 position of radiolabeled PC incubated with lyophilized microsomes of *B. napus.* The overall goal of this work is to develop strategies for overcoming substrate dichotomy in the future through biotechnological manipulation of the underlying acyl-trafficking pathways.

2. Literature Review

Very long chain polyunsaturated fatty acids and human health

Very long chain polyunsaturated fatty acids (VLCPUFA), such as arachidonic acid (ARA, $20:4^{\Delta5,8,11,14}$), eicosapentaenoic acid (EPA, $20:5^{\Delta5,8,11,14,17}$) and docosahexaenoic acid (DHA, $22:6^{\Delta4,7,10,13,16,19}$), are essential for human health and nutrition. In addition to being important structural components of membrane phospholipids, these fatty acids are biosynthetic precursors to a large group of eicosanoids, including prostaglandins, thromboxanes, and leukotrienes, which have diverse physiological roles including regulation of inflammatory responses, blood clotting, and vasoconstriction/dilation (Riediger et al. 2009). VLCPUFA and their dietary precursors are commonly classified as omega-3 (ω3 or n-3) or omega-6 (ω6 or n-6) fatty acids, according to the number of carbons between the methyl end of the fatty acid and the first point of unsaturation (Griffiths and Morse 2006). In humans, both omega-6 and omega-3 fatty acids are metabolized by the same series of enzymes, but yield products with different and often opposing, physiological roles. Arachidonic acid, for example, is considered to have pro-inflammatory activities, while EPA, an omega-3 fatty acid, has been shown to have anti-inflammatory effects (Riediger et al. 2009). Unlike many lower eukaryotes, humans lack the ability to interconvert between the omega-6 and omega-3 pathways (Ruxton et al. 2007), thus, the relative dietary intake of omega-6 and omega-3 fatty acids is considered to have a major influence on the downstream physiological outcomes.

While both omega-6 and omega-3 PUFA are important for optimal health, the ideal ratio of omega-6 to omega-3 fatty acids is estimated to be 4:1 or lower (Simopoulos 2002). Currently, it is estimated that the ratio consumed in the Western diet is in the range of about 17:1, owing to a combination of low fish consumption and high intake of omega-6 fatty acids (primarily linoleic acid) from plant oils such as sunflower and soybean. This imbalance has been implicated in a wide range of chronic health problems, including increased risk of cardiovascular disease, type-2 diabetes, depression and various other inflammatory diseases (Ruxton et al. 2007; Simopoulos 2008).

In humans, ARA and EPA can be produced from the essential fatty acids, linoleic acid (LA, $18:2^{\Delta 9,12}$) and α-linolenic acid (ALA, $18:3^{\Delta 9,12,15}$), respectively, through a series of desaturation and elongation steps (Figure 2.1). The efficiency of this process, however, differs among individuals and is influenced by a number of factors including age, physical activity, smoking, and various health conditions. In particular, it has been shown that the initial Δ6-desaturation step is rate-limiting (Griffiths and Morse 2006). Interestingly, γ-linolenic acid (GLA, $18:3^{\Delta 6,9,12}$), and its immediate elongation product, dihomo-γ-linolenic acid (DGLA, $20:3^{\Delta 8,11,14}$) are increasingly recognized as exerting anti-inflammatory effects (Kapoor and Huang 2006), while their precursor, LA, and product, ARA, are pro-inflammatory. The pro-inflammatory effects of LA may be a result of its ability to outcompete ALA at the Δ6-desaturation step, which would effectively suppress the omega-3 pathway and its downstream anti-inflammatory effects.

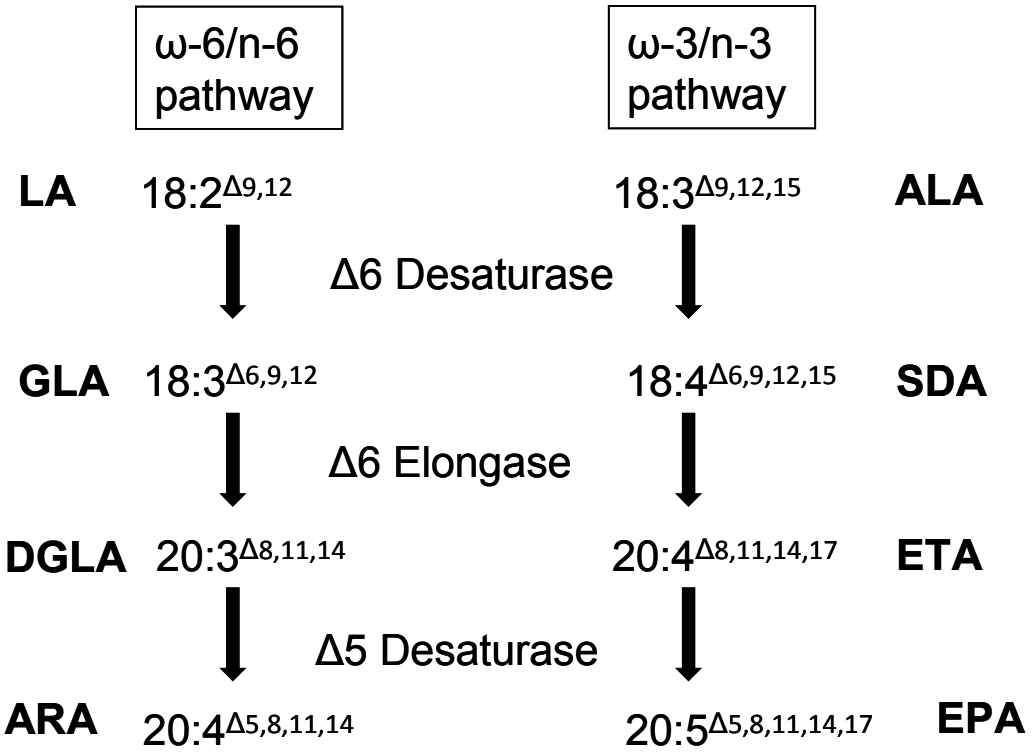

Figure 2.1. VLCPUFA biosynthesis in humans. Pathways for the synthesis of arachidonic acid (ARA) and eicosapentaenoic acid (EPA) from the essential dietary fatty acids, linoleic acid (LA) and α-linolenic acid (ALA) in humans.

Despite having the ability to synthesize EPA from dietary precursors, only a small proportion of EPA is further converted to DHA (Plourde and Cunnane 2007), therefore, dietary consumption of both EPA and DHA is recommended. These fatty acids can be obtained in the diet from oily fish, which accumulate VLCPUFA through the consumption of marine microorganisms. In recent years, there has also been substantial investment in developing omega-3 fortified food products and supplements to help address the high n-6/n-3 ratio of Western diets.

Increasing demand for VLCPUFA, along with growing concerns over the sustainability of wild fish stocks as a supply of VLCPUFA for both humans and aquaculture (Miller et al. 2008), has highlighted a need for alternative commercial sources of dietary VLCPUFA. Modified seed oils are an attractive option for several reasons, including their widespread incorporation in the diet, existing production and processing infrastructure, and their relatively low cost of production compared to emerging technologies, such as microbial fermentation (Raghukumar 2008). Despite more than a decade of ongoing research in this area and substantial progress toward the production of VLCPUFA in transgenic plants, several challenges still remain to achieve commercially viable levels of VLCPUFA, particularly DHA, in oilseed crops. While the basic pathways for VLCPUFA synthesis are known and many of the genes have been cloned from various sources and introduced into plants, the results to date have revealed several "bottlenecks" which will likely be overcome through advances in our understanding of plant lipid metabolism.

2.2 Seed oil biosynthesis

Seed oils are composed primarily of triacylglycerol (TAG), which serves as an energy store to support germination and early seedling development. Conceptually, seed oil biosynthesis can be considered in three parts, fatty acid biosynthesis, formation of the cytosolic acyl-CoA pool, and TAG assembly (Figure 2.2).

Fatty acid biosynthesis occurs in the plastid, beginning with the formation of malonyl-CoA from acetyl-CoA, catalyzed by acetyl-CoA carboxylase (Sasaki and Nagano 2004). The first two carbons in the nascent acyl chain are derived from acetyl-CoA, while each additional two-carbon unit is derived from malonyl-CoA in a repeated cycle of reactions catalyzed by the fatty acid synthase complex (FAS). In most oilseeds, plastidial fatty acid synthesis terminates once the acyl chain reaches 16 to 18 carbons in length, and the first double bond may be introduced by an acyl carrier protein (ACP)-desaturase before the nascent fatty acid is released from ACP via the action of an acyl-ACP thioesterase (Harwood 2005). The resulting free fatty acids are then activated to acyl-CoAs by an acyl-CoA synthetase (ACS) on the outer envelope of the plastid, prior to being released into the cytosol (Schnurr et al. 2002).

In *Arabidopsis*, nine ACS genes have been identified, all of which were shown to have relatively similar substrate specificities, effectively utilizing palmitic, palmitoleic, oleic and linoleic acids, but having different tissue-specific expression patterns (Shockey et al. 2002). Similarly, *B. napus* has at least six ACS

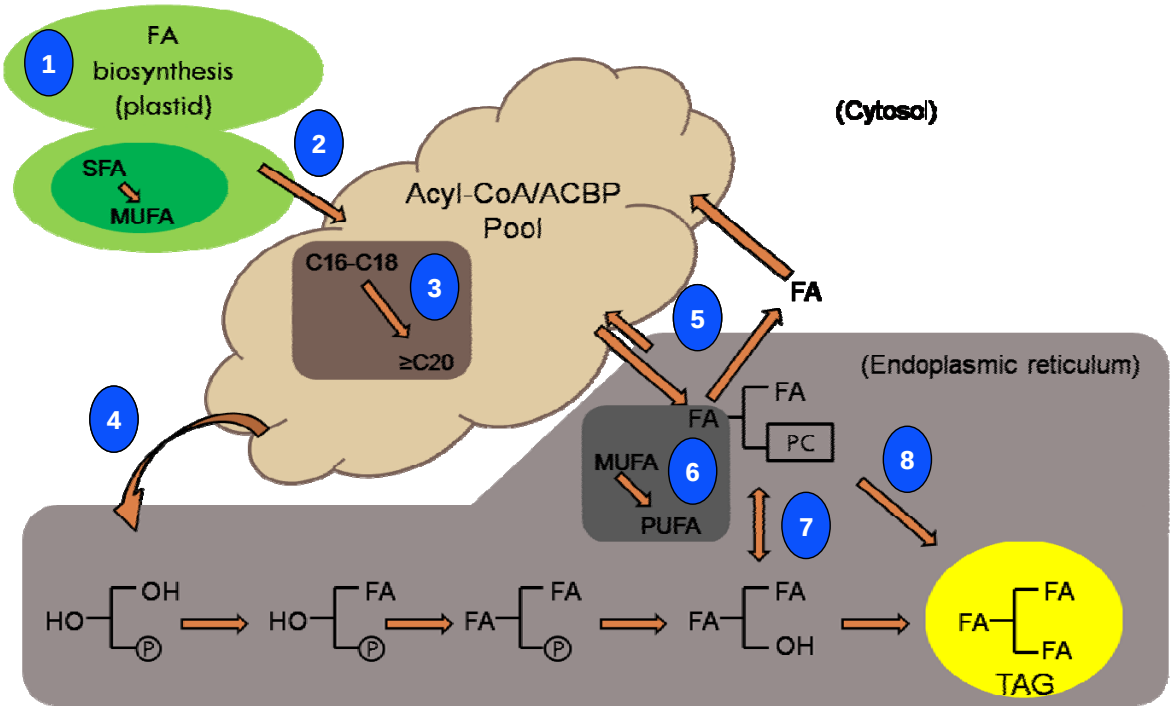

Figure 2.2. Overview of seed oil biosynthesis in developing oilseeds. Fatty acid biosynthesis **(1)** occurs in the plastid, resulting in the formation of saturated (SFA) or monounsaturated (MUFA) fatty acids up to 18 carbons in length. Newly formed fatty acids are released from the plastid and are activated to acyl-CoAs by acyl-CoA synthetase **(2)**. Acyl-CoA binding proteins (ACBPs) facilitate the transport and protection of acyl-CoAs in the acyl-CoA pool. Acyl-CoAs can act as substrates for further elongation **(3)**, acyl-CoA dependent triacylglycerol (TAG) assembly **(4)**, or can be involved in acyl-exchange reactions with membrane phospholipids, primarily phosphatidylcholine (PC) **(5)**. Fatty acid desaturation **(6)** occurs at the *sn*-2 position of PC. Acyl groups on PC can be incorporated into TAG via an acyl-CoA independent route involving exchange of backbones between PC and DAG **(7)** or through acyl transfer **(8)**.

genes (Pongdontri and Hills 2001). Membrane preparations from developing seeds of *B. napus* were able to most effectively use substrates 16-18 carbons in length with double bonds in the Δ9-11 positions (Ichihara et al. 1997). *B. napus* ACS6, which is strongly expressed in developing embryos, also showed similar substrate specificity (Pongdontri and Hills 2001). Interestingly, petroselinic acid, a Δ6 isomer of 18:1, was used considerably less effectively than oleic acid ($18:1^{\Delta 9}$); however, there did not appear to be any difference in specificity between the Δ6 and Δ9 isomers of 18:3 (Pongdontri and Hills 2001).

Most of the cytosolic acyl-CoA pool exists in a bound form with acyl-CoA binding proteins (ACBPs), which facilitate the transport of acyl-CoAs and may play a role in mediating the availability of acyl-CoA substrates for glycerolipid assembly (Burton et al. 2005). Overall, the formation of the cytosolic acyl-CoA pool is the net result of two processes: 1) *de novo* fatty acid biosynthesis which contributes saturated and monounsaturated moieties, and 2) acyl-editing pathways, which result in the exchange of acyl-groups between acyl-CoA and phospholipids, providing a mechanism for channeling substrates toward desaturation and the return of polyunsaturated acyl groups to the acyl-CoA pool (Bates et al. 2007).

This acyl exchange can be facilitated by at least two different mechanisms. One involves lysophosphatidylcholine acyltransferase (LPCAT), which catalyzes the reversible acyl-CoA dependent acylation of lysophosphatidylcholine (LPC) to form phosphatidylcholine (PC)(Stymne and Stobart 1984a; Stymne and Stobart 1984b). Although the forward reaction of LPCAT, toward the formation of PC, is

heavily favored, its reversibility has been demonstrated *in vitro* using an excess of free CoA to shift the equilibrium toward the formation of acyl-CoA (Stymne and Stobart 1984a). Bovine serum albumin, which can bind acyl-CoA, was also required for the reverse reaction to occur *in vitro* (Stymne and Stobart 1984a), and it has since been suggested that ACBP may serve a similar role *in vivo* in facilitating the reversibility of LPCAT (Yurchenko et al. 2009).

A second possible mechanism for acyl-editing involves the hydrolysis of an acyl-group from the *sn*-2 position of PC catalyzed by phospholipase A_2 (PLA_2), which can then be reactivated by an acyl-CoA synthetase, in a process known as the Lands Cycle (Das et al. 2001). There are several PLA_2 isoforms in plants, including a small soluble, calcium-dependent PLA_2 homologous to animal secretory PLA_2, and a membrane-bound calcium-independent form which is homologous to animal intracellular PLA_2 (Lee et al. 2005). The substrate preferences of the individual isoforms from plants are only beginning to be systematically studied, but they are generally believed to play a role in membrane maintenance by removing unusual acyl groups from membrane phospholipids (Stahl et al. 1995).

While both of these acyl-editing mechanisms have been demonstrated *in vitro*, and it has been suggested that acyl-editing constitutes a major flux *in vivo* (Bates et al. 2007), it remains unclear to what extent each enzyme participates in acyl-editing *in vivo*, and what effect they ultimately exert over the fatty acid composition of TAG. At the time these mechanisms were first being described, it was believed that TAG assembly occurred entirely via an acyl-CoA dependent

pathway, thus, it was assumed that TAG composition was essentially a direct reflection of the acyl-CoA pool composition. Several recent developments have forced a re-examination of these assumptions.

The classical "Kennedy pathway" for glycerolipid assembly (Figure 2.3A) involves the sequential acyl-CoA dependent acylation of *sn*-glycerol-3-phosphate (G3P), catalyzed by *sn*-glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidic acid acyltransferase (LPAAT), and diacylglycerol acyltransferase (DGAT), respectively (Weselake 2005). The phosphatidic acid (PA) synthesized by LPAAT is dephosphorylated by a phosphatidic acid phosphatase (PAP) to form DAG, which is the substrate for the final acylation catalyzed by DGAT. PA may also be generated by the activity of phospholipase D (PLD), which catalyzes the hydrolysis of the choline group on PC. PLD has been shown to be active in developing seeds, but it not clear how much this activity contributes to seed oil formation, since both PA and DAG are also important signaling molecules in plants (Li et al. 2009).

DAG is also a substrate for *de novo* PC synthesis, which is catalyzed by the forward reaction of CDP-choline:diacylglycerol cholinephosphotransferase (CPT) (Vogel and Browse 1996). While it was previously believed that CPT could catalyze both the forward and reverse reactions, a new enzyme has recently been described which catalyzes the interconversion of DAG and PC (Lu et al. 2009). This enzyme, phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT) catalyzes a headgroup exchange that does not appear to be dependent on CDP-choline (Lu et al. 2009). This exchange of glycerol backbones between PC

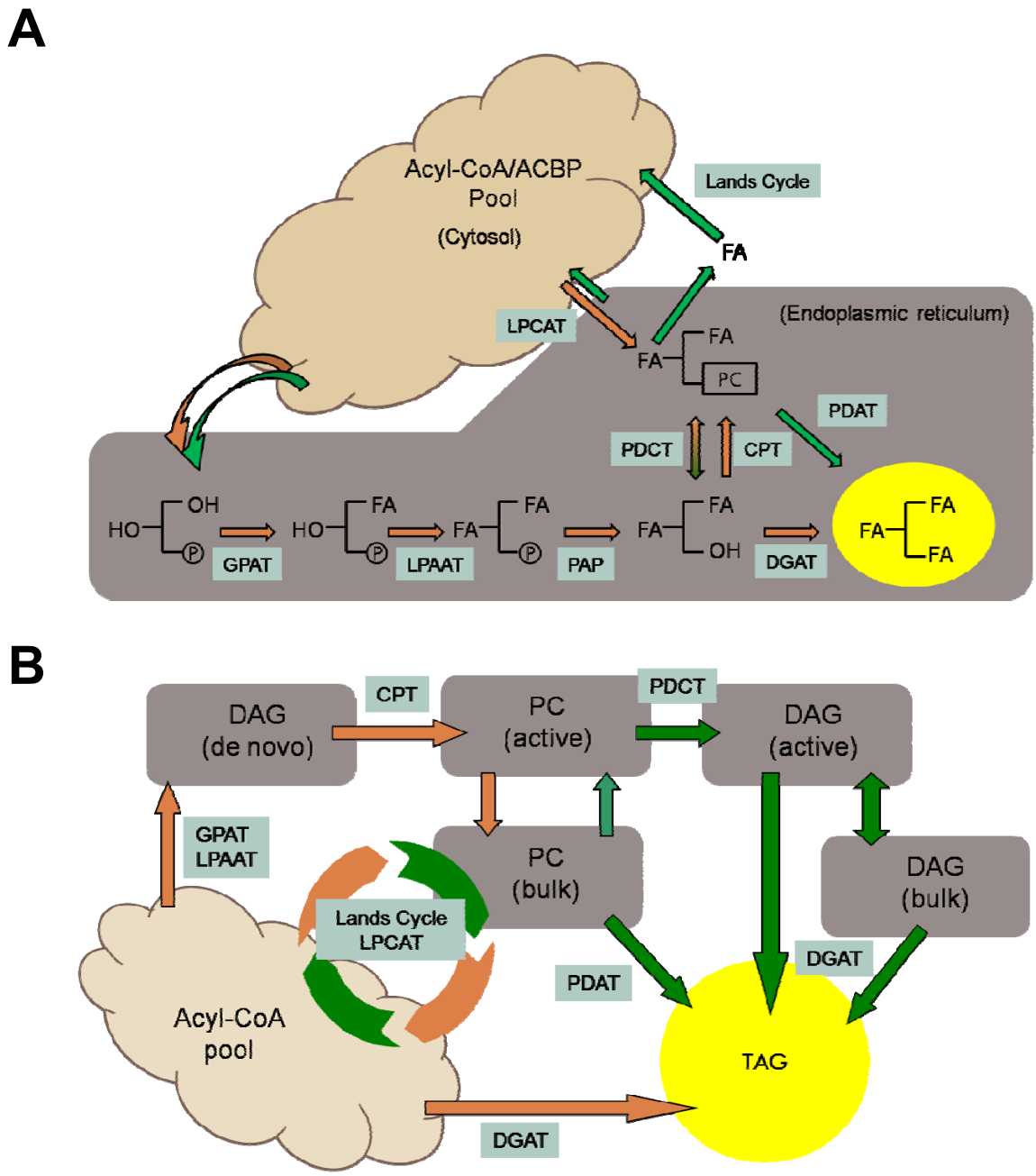

Figure 2.3. Classical and emerging models of seed oil biosynthesis. **A)** The classical understanding of seed oil biosynthesis involves sequential acyl-CoA dependent acylations of *sn*-glycerol-3-phosphate (G3P) catalyzed by GPAT, LPAAT, and DGAT, respectively. Desaturation occurs at the *sn*-2 position of PC, and PUFA can subsequently enter TAG via acyl-CoA dependent or acyl-CoA independent routes. **B)** The emerging view of seed oil biosynthesis based on the work of Bates et al (2009), involves multiple, kinetically distinct pools of PC and DAG. Most newly synthesized acyl groups immediately enter PC and participate in "acyl-editing", while most of the DAG to support TAG synthesis is PC derived. The sequential acylation of G3P accounts for only a small fraction of the TAG produced. Green arrows represent movement of PC-derived acyl groups; orange arrows represent movement of newly formed acyl groups derived from plastidial fatty acid synthesis. See List of Abbreviations for enzyme abbreviations.

and DAG represents another possible mechanism for the entry of PUFA into TAG.

Another acyl-CoA independent mechanism for incorporating PUFA into TAG involves phospholipid:diacylglycerol acyltransferase (PDAT), which catalyzes the direct transfer of an acyl group from the *sn*-2 position of PC to the *sn*-3 position of DAG to form TAG (Dahlqvist et al. 2000; Stahl et al. 2004). It has been suggested that PDAT plays a role in membrane maintenance by sequestering PUFA or other unusual fatty acids in storage lipids (Dahlqvist et al. 2000) but the relative contribution of PDAT to overall rates of TAG synthesis remains in question. Although PDAT appears to be a major contributor to TAG synthesis in yeast, knocking out PDAT in *Arabidopsis* had no effect on oil content or composition (Mhaske et al. 2005). The discovery of an acyl-CoA independent mechanism for acylating PUFA at the *sn*-3 position of TAG, however, opens up the possibility that there is no absolute requirement for PUFA to be released into the acyl-CoA pool to be incorporated into TAG, as was previously believed.

Recently it has been proposed that two kinetically distinct pools of PC and three kinetically distinct pools DAG exist in developing soybean embryos ((Bates et al. 2009), Figure 2.3B). In contrast to the generally accepted Kennedy pathway involving sequential acylations of a glycerol backbone, this new model suggests that only a small proportion of nascent acyl groups are involved in the sequential acylation reactions of the Kennedy Pathway. Instead, nearly 60% of the newly formed acyl groups are directed toward PC in a manner not resulting in *de novo* formation of PC, implying that the forward and reverse reactions of LPCAT play

an important role in "acyl-editing" of bulk PC (Bates et al. 2007; Bates et al. 2009). A smaller proportion of nascent acyl groups (~17%) are involved in the sequential acylation of G3P leading to DAG, but rather than immediately becoming a substrate for DGAT, this newly formed DAG is first cycled through PC, where the acyl groups at the *sn*-2 position can become substrates for desaturation or participate in acyl-exchange reactions such as the reverse reaction of LPCAT. This *de novo* synthesis of PC would occur through the activity of CPT. The newly identified PDCT enzyme, catalyzing the interconversion of PC and DAG, may facilitate the return of PUFA-enriched backbones to DAG, where they subsequently become acyl acceptors for DGAT and/or PDAT (Lu et al. 2009). Together, these acyl-editing and backbone exchange mechanisms facilitate the entry of PUFA into TAG, while the relatively small contribution of the linear Kennedy Pathway (~10% of nascent acyl groups) would facilitate the incorporation of mostly SFA into the *sn*-3 position of TAG (Bates et al. 2009).

Given these developments, it appears that acyl group trafficking via PC plays a much more important role than previously suggested, which may account for some of the challenges encountered in manipulating fatty acid composition through biotechnological approaches.

2.3 VLCPUFA biosynthetic pathways and their expression in transgenic plants

Very long chain polyunsaturated acids are generally defined as fatty acids equal or greater than 20 carbons in length with two or more double bonds. In

most oilseeds, oleic acid (18:1$^{\Delta 9}$) is the major product of *de novo* fatty acid biosynthesis, and can be further desaturated to LA (18:2$^{\Delta 9,12}$) or ALA (18:3$^{\Delta 9,12,15}$) or alternatively, elongated to eicosenoic acid (20:1$^{\Delta 11}$) or erucic acid (22:1$^{\Delta 13}$). A limited number of higher plants, such as borage and evening primrose, are capable of synthesizing γ-linolenic acid (GLA, 18:3$^{\Delta 6,9,12}$), a Δ6 desaturated C18 PUFA (Griffiths et al. 1988a; Shimizu and Nakano 2003). There has been considerable interest in the production of GLA and its omega-3 counterpart, stearidonic acid (SDA, 18:4$^{\Delta 6,9,12,15}$) in transgenic oilseeds, since consumption of Δ6 desaturated C18 PUFA would bypass the rate-limiting Δ6-desaturase in humans. Compared to VLCPUFA production, engineering oilseeds to produce GLA and SDA has been relatively straightforward, minimally requiring the insertion of a single Δ6 desaturase gene (Hong et al. 2002). Insertion of the Δ6-desaturase yielded up to 40% GLA and about 8% SDA in transgenic *Brassica juncea* (Hong et al. 2002). The low accumulation of SDA was probably a reflection of the lower proportion of endogenous ALA in *B. juncea* compared to LA. Similar levels of GLA were obtained in transgenic *B. napus* co-expressing the Δ6-desaturase and a Δ12-desaturase (Liu et al. 2001). Substantially higher levels of SDA were achieved in transgenic soybean co-expressing the Δ6-desaturase and a Δ15-desaturase, which increased the proportion of ALA available for subsequent Δ6-desaturation (Eckert et al. 2006).

Further elongation and desaturation of GLA and SDA to form VLCPUFA in transgenic plants has been a more substantial challenge, requiring the insertion of

several genes derived from microorganisms which naturally accumulate VLCPUFA.

In these organisms, VLCPUFA biosynthesis can occur through two different aerobic desaturation and elongation pathways (Leonard et al. 2004; Uttaro 2006); Figure 2.4). The Δ6-desaturase pathway (Figure 2.4A) is analogous to the one described in humans, whereby GLA and SDA are formed from LA and ALA by a Δ6-desaturase, and subsequently elongated by a Δ6-elongase to form DGLA ($20:3^{\Delta 8,11,14}$) or eicosatetraenoic acid (ETA, $20:4^{\Delta 8,11,14,17}$). DGLA and ETA are further desaturated by a Δ5-desaturase to form ARA or EPA.

In an alternative pathway (Figure 2.4B), LA and ALA can be elongated first by a Δ9-elongase, and then desaturated twice by Δ8- and Δ5-desaturases. In lower eukaryotes, such as the marine □lpine□ *Thraustachytrium*, it is believed that DHA synthesis occurs through further elongation of EPA followed by a final Δ4-desaturation (Qiu 2003). This is in contrast to the mechanism proposed in mammalian systems, which involves partial peroxisomal β-oxidation of 24:6 to form 22:6 (Sprecher et al. 1995).

Some organisms, such as *Schizochytrium* and *Shawanella*, are also capable of synthesizing VLCPUFA through a process catalyzed by polyketide synthase (PKS), which does not require molecular oxygen for desaturation (Metz et al. 2001; Napier 2002). In these systems, fatty acid elongation occurs through an iterative two-carbon chain lengthening process similar to fatty acid synthesis in plants. Unlike plant fatty acid synthase (FAS), which produces saturated fatty acids through a repeated four step process of condensation, reduction,

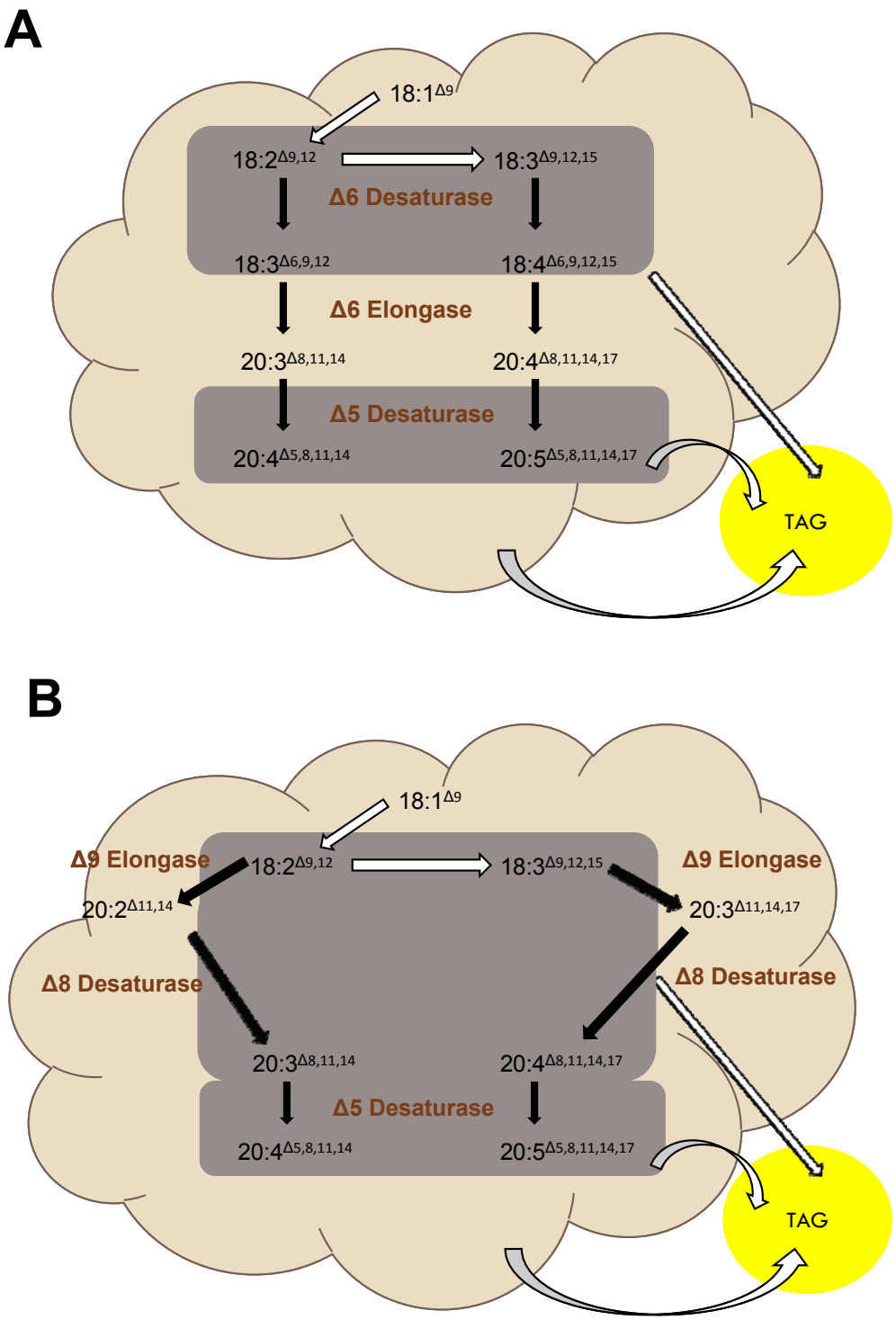

Figure 2.4. Aerobic VLCPUFA biosynthetic pathways. **A)** The Δ6-desaturation pathway. **B)** The Δ9-elongation pathway. Dark shading represents steps occurring in the ER; light shading represents steps occurring in the cytosolic acyl-CoA pool. White arrows represent reactions that occur through endogenous pathways; dark arrows indicate steps that must be introduced through genetic engineering.

dehydration, and reduction, PKS can concurrently introduce double bonds or hydroxyl groups through incomplete cycles omitting dehydration or reduction steps (Qiu 2003). PKS has received relatively little attention as a means of producing VLCPUFA in transgenic plants, but a patent for this technology has been issued to Martek Biosciences, in which it appears that low levels of DHA were obtained in *Arabidopsis* using a plastid-targeted PKS construct (Metz et al. 2007).

Considerably more work has focused on the aerobic desaturation and elongation pathways. A number of desaturase and elongase enzymes have been cloned from various organisms, and several strategies have been used to introduce these pathways into plants. The Δ8-desaturation pathway was first introduced into *Arabidopsis* in 2004, resulting in the accumulation of nearly 7% ARA and 3% EPA in leaves (Qi et al. 2004).

Later the same year, a second group reported the seed-specific expression of the Δ6-desaturation pathway in tobacco and flax, using the minimal set of genes required for EPA biosynthesis (Abbadi et al. 2004). This strategy resulted in nearly 30% C18 Δ6-desaturated PUFA (GLA and SDA), but only about 4% C20 PUFA, suggesting a bottleneck in the Δ6-elongation step. Further analysis indicated that although the recombinant elongase was active *in vitro*, the C18 substrates were not present *in vivo* at sufficient levels in the acyl-CoA pool to support efficient elongation. The authors suggest several possible explanations for this, including a so-called "substrate dichotomy" resulting from the inefficient release of Δ6-desaturated PUFA from PC, where they are formed, into the acyl-

CoA pool, where elongation occurs. It was previously demonstrated using yeast expressing these enzymes that the Δ6-desaturase and Δ6-elongase utilize different acyl carriers as substrates (Domergue et al. 2003). Nevertheless, other groups have since reported increased levels of C20 PUFA in transgenic plants using expression cassettes containing more than the minimal set of three required genes. The addition of a Δ12-desaturase to increase LA, and a second Δ6-elongase to improve elongation efficiency, resulted in up to 25% ARA in transgenic *Brassica juncea* (Wu et al. 2005). The further addition of an omega-3 desaturase to convert ARA to EPA resulted in EPA levels of up to 11%. Finally, a nine-gene construct was used to obtain up to 15% EPA and low levels of DHA; substrate dichotomy was again believed to contribute to inefficient elongation of EPA, resulting in very low yields of DHA (Wu et al. 2005).

In another study (Kajikawa et al. 2008), a liverwort (*Marchantia polymorpha*) Δ6-desaturase that could utilize both glycerolipid and acyl-CoA substrates was used in conjunction with a Δ6-elongase and Δ5-desaturase from the same organism, to achieve high levels (up to 19.5%) of C20 PUFA in soybean seeds. In this case, however, DGLA and ARA were the most abundant C20 PUFA, with EPA representing less than 0.05% of the total fatty acid composition. This suggested that the Δ5-desaturase was not very effective in soybean, although overexpression of the same genes in *M. polymorpha* resulted in a doubling of EPA accumulation, from 5.9% to up to 12.1% (Kajikawa et al. 2008). Similarly, when the minimal set of genes from *Mortierella □lpine* (a filamentous fungus currently used for commercial production of ARA) were introduced into soybean,

ARA represented only about 0.5% of the fatty acid composition in soybean seeds (Chen et al. 2006). Such results underscore the effect of the host and construct on VLCPUFA accumulation. Zero-erucic acid *Brassica carinata,* for example, was shown to be capable of supporting higher levels of EPA production (up to 20%) than zero-erucic acid *Brassica juncea* (5%), when transformed with the same constructs (Cheng et al. 2010). This may be largely explained by the fact that zero-erucic *B. carinata* has much a higher endogenous C18 PUFA content than *B. juncea* or *B. napus*, thus increasing the availability of precursors for VLCPUFA synthesis. Interestingly, it appears that while elimination of erucic acid synthesis in *B. napus* is compensated for by an increase in oleic acid, in zero-erucic acid *B. carinata*, the resulting oleic acid is very efficiently converted to LA and ALA, which increase from 16% to 44% and 16% to 21% respectively in the zero-erucic acid line (Cheng et al. 2010). This pool of C18 PUFA is almost entirely depleted upon the addition of genes for VLCPUFA synthesis. These results suggest that *B. carinata* may have a very efficient acyl-exchange mechanism which could shed further insight on overcoming the substrate dichotomy problems experienced in other hosts.

The use of acyl-CoA dependent desaturase genes has also been explored as a means of overcoming substrate dichotomy in transgenic plants, albeit with limited success. Using Δ6- and Δ5-acyl-CoA desaturases, Hoffman et al (2008), demonstrated that most of the Δ6-C18 PUFA formed in transgenic *Arabidopsis* could be efficiently elongated, but the overall levels of VLCPUFA obtained were less than 1%, considerably lower than what has been achieved previously using

phospholipid desaturases (Abbadi et al. 2004; Qi et al. 2004). A similar reduction in total VLCPUFA was observed in *Arabidopsis* expressing a dual-purpose Δ5/Δ6-acyl-CoA desaturase (Robert et al. 2005). The Δ6-desaturation step itself appears to be limiting in these plants, probably due to limited availability of endogenous acyl-CoA substrates. While the acyl-CoA desaturase was demonstrated to have high activity in yeast (Hoffmann et al. 2008), the uptake of exogenous fatty acids in yeast is directly coupled to acyl-CoA synthesis (Domergue et al. 2003), resulting in high levels of ALA-CoA substrate for the recombinant enzyme. This situation is quite different in plants, since the Δ12 and Δ15 desaturation occurs on PC, and the resulting C18 PUFA must be released into the acyl-CoA pool to be available for desaturation by the acyl-CoA desaturase. Thus, it appears that the substrate dichotomy "bottleneck" has, in this case, only been shifted upstream. While it is widely assumed that endogenous LA and ALA are efficiently released from PC into the acyl-CoA pool, there is little current experimental data quantifying the rate or extent of this exchange which takes into account the possible contribution of PDAT in channeling PUFA directly to TAG. The recent work of Bates et al, (2007; 2009) has provided some key insight into overall patterns of acyl-group trafficking, but the level of labeled PUFA detected in their experiments was relatively low due to the short incubation times used for the flux analysis.

Analyses of the acyl-CoA pool composition of several oilseeds have revealed that the composition of the acyl-CoA pool is not always directly reflected in TAG, often having a higher proportion of saturated fatty acids (i.e. 16:0) and relatively

low levels of PUFA (Abbadi et al. 2004; Larson et al. 2002; Larson and Graham 2001). Such differences, however, could also reflect the substrate preferences of the endogenous acyltransferases which tap into the acyl-CoA pool (Snyder et al. 2009). It is clear from analysis of the positional distribution of PUFA on the glycerol backbone of several seed oils that at least a portion of endogenous PUFA is available in the acyl-CoA pool for acylation at the *sn*-1 position of TAG (Abbadi et al. 2004; Brokerhoff and Yurkowski 1966; Christie et al. 1991; Griffiths et al. 1988a). Interestingly, in both borage and transgenic flax accumulating GLA, virtually none of the GLA was detected at the *sn*-1 position of TAG (Abbadi et al. 2004; Griffiths et al. 1988a). Griffiths et al, (1988a) proposed that this may be due to a GLA-selective DGAT activity preferentially incorporating GLA at the *sn*-3 position in borage. The absence of GLA-CoA in subsequent experiments with transgenic flax (Abbadi et al. 2004) may instead suggest that the specificity of the acyl-editing mechanisms limits the release of GLA into the acyl-CoA pool. Such ambiguities have yet to be resolved by systematic analysis of acyltransferase substrate preferences.

The existence of multiple routes for PUFA incorporation into TAG, coupled with the importance of PUFA in maintaining membrane fluidity, also leaves open the possibility that there may be regulatory factors which play a role in mediating acyl-group trafficking. Fatty acid desaturation can be regulated through signaling cascades triggered by changes in the physical state of the membrane or through feedback inhibition in the presence of PUFA (Aguilar and de Mendoza 2006). It is possible that introduction of foreign desaturases could either trigger or override

endogenous regulatory controls, which could in turn influence which acyl-trafficking pathways predominate. There has been a considerable amount of work focused on studying the regulation of fatty acid desaturation in response to temperature and other stresses (Upchurch 2008), but so far, very little attention has been focused on disruptions brought about through the expression of foreign genes.

In summary, although substrate dichotomy is widely recognized as a limitation on the accumulation of VLCPUFA in transgenic plants, the various mechanisms underlying substrate dichotomy are not well understood. A major goal of the present study is to determine the biochemical basis for substrate dichotomy in transgenic *B. napus,* as a first step in developing strategies for overcoming this bottleneck in VLCPUFA accumulation.

3. Materials and Methods

3.1 Source of chemicals

1-[^{14}C]-γ-linolenic acid was obtained from American Radiolabeled Chemicals (St. Louis, MO). 1-[^{14}C]-α-linolenic acid was obtained from Perkin-Elmer (Waltham, MA). All other radiochemicals were obtained from GE Healthcare (Baie d'Urfe, QC). Phospholipids and diacylglycerol were obtained from Avanti Polar Lipids (Alabaster, AL). Fatty acids and fatty acid methyl esters were obtained from NuChek Prep (Elysian, MN). Chromatography grade solvents were obtained from Fisher Scientific (Whitby, ON). Most other biochemicals and reagents were obtained from Sigma-Aldrich (Oakville, ON).

3.2 Plant material and growth conditions

Seeds from *Brassica napus* L. cv Westar transformed with constructs carrying genes required for VLCPUFA biosynthesis were provided by Dr. Joerg Bauer (BASF Plant Science Company GmbH, Limburgerhof, Germany). The transgenic lines carried constructs required for arachidonic acid (LJB78) or eicosapentaeonic acid (LJB672) biosynthesis, as described in Table 3.1. Both constructs carry genes encoding the minimal set of enzymes required for ARA or EPA synthesis (i.e. Δ6 desaturase, Δ6 elongase and Δ5 desaturase), with the Δ6 desaturases differing in their required substrate (acyl-CoAs in LJB672 vs. phospholipids in LJB78). Δ12- (LJB78 & LJB672) and Δ15 desaturases (LJB672) were included to enhance the accumulation of endogenous LA and ALA precursors, respectively, and in LJB672, an ω-3 desaturase was also

Table 3.1. Summary of constructs and transgenic lines used in this study.

Construct	Genes (Promoters)	Promoter strength	Line #	Generation T-DNA copy #
LJB78	Δ12-Desaturase from *Calendula officinalis* (napin)	strong	28	At least T3 1 copy
	Δ6-Desaturase from *Pythium □lpine□ar* (napin)	strong	67	At least T4 1 copy
	Δ6-Elongase from *Physcomitrella patens* (napin)	strong		
	Δ5-Desaturase from *Thraustochytrium spp.* (napin)	strong		
LJB672	Δ12-Desaturase from *Calendula officinalis* (LuPXR)	weak	11	At least T2 (segregating) 1 copy
	Δ6-Desaturase (acyl-CoA) from *Ostreococcus tauri* (USP)	moderate	18	At least T2 (segregating) Copy number unknown
	Δ6-Elongase from *Physcomitrella patens* (1800bp SBP)	strong	20	At least T2 (segregating) 1 copy
	Δ6-Elongase from *Thalassiosira pseudonana* (GLP)	weak		
	Δ5-Desaturase from *Thraustochytrium spp.,*(Conlinin)	strong		
	ω3-Desaturase from *Phytophtora infestans* (napin)	strong		
	Δ15-Desaturase from *Perilla frutescence* (AtTIP)	very weak		

included to facilitate the conversion of ω-6 intermediates to the ω-3 pathway leading to EPA.

Frozen developing seeds (25-29 days after flowering (DAF) or 36-40 DAF) from each construct were provided by BASF, and in the case of segregating lines, only material from plants confirmed to be transgenic was pooled. Untransformed *B. napus* L. cv Westar grown under the same conditions was similarly harvested, frozen, and provided as a control.

For experiments requiring the use of freshly harvested, unfrozen material, mature seeds from both constructs were provided for propagation. Seeds were sown in 6" square pots containing SunGro Sunshine LA4 Aggregate Plus growing mix (SunGro Horticulture, Vancouver, BC) and were maintained in a growth chamber set at 16°C with a 16h photoperiod (~160 μmol/m^2/s) for approximately 6 weeks, then staked and transferred to the greenhouse. Plants were covered with pollination bags upon onset of flowering. Developing seeds were harvested between 22-25 days after flowering (DAF) and transferred to embryo culture unless otherwise indicated.

3.3 Genomic DNA extraction and PCR analysis

Because lines carrying the LJB672 construct were segregating, the presence of the construct was confirmed by PCR. Young leaf tissue (~0.5g) was collected and flash frozen in a microcentrifuge tube containing a 3mm glass bead. The tissue was subsequently ground to a fine powder using a Biospec Mini Bead Beater (Biospec, Bartlesville, OK), and thoroughly resuspended in 500μL Shorty Buffer (0.2M Tris-HCl, pH 9.0, 0.4M LiCl, 2.5mM EDTA, 1% SDS). The

resulting homogenate was centrifuged in an Eppendorf 5415R microcentrifuge (Mississauga, ON) at 12,000rpm for 5 min at 4°C. Three hundred fifty microliters of the supernatant were transferred to a fresh tube containing an equal volume of cold isopropanol and mixed by inversion, followed by centrifugation at 12,000 rpm for 10min at 4°C. The resulting pellets were washed with 500µL cold 70% ethanol and centrifuged at 12,000 rpm for 5 min at 4°C. The pellets were dried at room temperature for 30-45 min, then resuspended in 100 µL of TE buffer (10mM Tris, pH 8.0, 1mM EDTA) and stored at -20°C until further analysis.

For PCR analysis, 5µL of template DNA was added to a master mix containing 2.5µL 10X PCR buffer, 0.75µL 50mM $MgCl_2$, 1µL each of the forward and reverse primers (20µM), 2µL of dNTPs (2.5mM each), 0.25µL Taq Polymerase and 12.5µL water. dNTPs, Taq polymerase and buffers were obtained from Invitrogen (Mississauga, ON). Primers were synthesized by Integrated DNA Technologies (Coralville, IA) and designed to amplify a 601bp portion of the *O. tauri* Δ6-desaturase (Forward primer: 5'-ATG TGT GTT GAG ACC GAG AAC-3', Reverse primer: 5'-TTC TCT TAT CCC ACC AGA TG-3'). The PCR reaction conditions were 94°C (5 min), then 30 cycles of 94°C (0.5min), 54°C(0.5min), 72°C (1min), followed by extension at 72°C (5min) and 4°C hold.

The band of interest was detected by gel electrophoresis on a 1% Agarose gel containing EtBr, run at 115V for 70min (for large gels), or 100V for 50min (for small gels).

3.4 Enzyme assays

3.4.1 Protein preparation & quantification

Homogenates of developing seeds (25-29 DAF and 36-40 DAF) were prepared by grinding approximately 2g of frozen developing seeds in 4 volumes of grinding buffer (0.5M Sucrose, 0.2M Hepes-NaOH, pH 7.4). Aliquots of homogenate were immediately flash frozen and stored at -80°C until further use. For assays requiring the use of lyophilized microsomes, microsomes were prepared from developing seeds (25-29 DAF) or cell suspension cultures of *B. napus* cv Jet Neuf (Shi et al. 2008) based on the protocol employed by Stahl et al (2004) for assaying PDAT. The tissue was homogenized in 0.1M potassium phosphate buffer (pH 7.2) with 0.1% bovine serum albumin and 0.33M sucrose (6 volumes), filtered through miracloth and centrifuged at 18,000 x *g* at 4°C for 20min. The resulting supernatant was filtered through miracloth into ultracentrifuge tubes and centrifuged at 105,000 x *g* at 4°C for 90min. The pellet was resuspended in 1/10 volume of 0.1M phosphate buffer (pH 7.2), divided into aliquots, flash frozen and stored at -80°C until further use. Protein was quantified in homogenates and microsomes using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Mississauga, ON), which is based on the method of Bradford (1976). All assays were performed in triplicate unless otherwise indicated.

3.4.2 sn-glycerol-3-phosphate acyltransferase

sn-glycerol-3-phosphate acyltransferase (GPAT) was assayed in developing seed homogenates using ^{14}C glycerol-3-phosphate (G3P) as the acyl acceptor and oleoyl-CoA as the acyl donor. The reaction was initiated by the addition of 10µL of homogenate to a reaction mixture (final volume 50 µL) containing (final

concentrations) 40mM Hepes-NaOH (pH 7.0), 5mM EDTA, 1mM DTT, 2.5mg/mL BSA, 15µM 18:1-CoA 100µM ^{14}C G3P (15nCi/nmol). The reaction was allowed to proceed for 10min at 30°C, and was quenched with 2mL of 2:1 (v/v) methanol:chloroform. Phase separation was induced by the addition of 1mL 1M KCl in 0.2M phosphoric acid, followed by vigorous mixing and centrifugation. The organic phase was extracted to a fresh tube and the aqueous phase was re-extracted with an additional 2mL of 2:1 methanol/chloroform. The combined organic phase was evaporated under nitrogen and resuspended in 100µL of chloroform. Seventy microliters were applied to a precoated silica gel G preparative TLC plate (Machery-Nagel, Bethlehem, PA), and developed in 100:40:12:4 (v/v) chloroform:methanol:acetic acid:5% aqueous sodium bisulfite. Radioactive products were visualized using a Typhoon multi-purpose imager (GE Healthcare) in storage phosphor mode. Radioactive spots corresponding to lysophosphatidic acid and its downstream products were scraped into liquid scintillation vials and quantified in a Beckman-Coulter 6500 Liquid Scintillation Counter.

3.4.3 Lysophosphatidic acid acyltransferase

Lysophosphatidic acid acyltransferase (LPAAT) was assayed using developing seed homogenates in the presence of ^{14}C oleoyl-CoA and unlabeled lysophosphatidic acid, essentially according to the method described by Sorensen et al (2005), except that the reaction mixture contained 11µM oleoyl-CoA (radiospecific activity 5.7nCi/nmol), and the reaction was allowed to proceed for 10min at 30°C. Lipids were extracted twice with 1mL of 1:1 (v/v)

chloroform/methanol, using 0.5mL 1M KCl in 0.2M phosphoric acid to induce a phase separation. The lipid extracts were separated by TLC as described above, using 85:15:10:4 (v/v) chloroform/methanol/acetic acid/water as the mobile phase. Radioactive spots corresponding to phosphatidic acid were scraped and quantified as described above.

3.4.4 Diacylglycerol acyltransferase

Diacylglycerol acyltransferase (DGAT) was assayed using developing seed homogenates, essentially as described by Weselake et al (2008) using ^{14}C-oleoyl-CoA (5.7nCi/nmol) as the acyl donor. Lipids were extracted with 2mL 2:1 (v/v) chloroform/methanol in the presence of 1mL 1M KCl in 0.2M phosphoric acid, followed by a second extraction with 2mL chloroform. TLC plates were run as described above using 80:20 (v/v) hexane/ether as the mobile phase. Spots corresponding to TAG were quantified as above.

3.4.5 Lysophosphatidylcholine acyltransferase

Lysophosphatidylcholine acyltransferase (LPCAT) was assayed in developing seed homogenates according to the method of Furukawa-Stoffer et al (2003), with the reaction volume scaled down to 70μL and a final ^{14}C-oleoyl-CoA concentration of 13μM (5.7nCi/nmol). Lipids were extracted as per Furukawa-Stoffer et al. (2003), with the volumes scaled down by one half. TLC plates were run as described above, using 33:45:5 (v/v) chloroform/methanol/ammonium hydroxide, or 25:25:25:10:9 (v/v) methyl acetate/n-propanol/chloroform/methanol/0.25% aqueous KCl (Vitiello and Zanetta 1978), which was found to give better resolution.

For LPCAT specificity assays, ^{14}C-ALA-CoA and ^{14}C-GLA-CoA were enzymatically synthesized from their respective free fatty acids according to the method of Taylor et al. (1990). The synthesized acyl-CoAs were initially purified by washing the aqueous reaction mixture several times with diethyl ether to remove free fatty acids, and subsequently further purified by solid phase extraction as described by Taylor et al. (1990).

3.4.6 Phospholipid:diacylglycerol acyltransferase

Phospholipid:diacylglycerol acyltransferase (PDAT) assays were based on the protocol of (Dahlqvist et al. 2000). Aliquots (20µL) of microsomal fractions of developing seeds and cell suspension cultures were prepared as described above and lyophilized. 1-palmitoyl, 2-[^{14}C]-linoleoyl-PC (5nmol; 53nCi/nmol) was added to the lyophilized microsome in benzene and the benzene was immediately evaporated under nitrogen. The microsomes were reconstituted in 100µL 50mM potassium phosphate (pH 7.2), thoroughly mixed, and incubated at 30°C for 60min. Assays were quenched by the addition of 2:1 (v/v) chloroform/methanol and extracted as described above for DGAT. TLC was performed as described and developed half-way using 60:30:3:1 (v/v) chloroform/methanol/acetic acid/water, then dried and fully developed in 80:20 (v/v) hexane/ether. Radioactive spots were visualized as described above and scraped for counting.

3.5 Zygotic embryo culture

Developing siliques were collected between 22-25 DAF and the seeds were immediately removed and rinsed at least three times with sterile water. Zygotic

embryos (cotyledonary stage) were dissected out of the seed coat under sterile conditions, rinsed in sterile water, and transferred to 24-well plates containing 0.5mL embryo culture media (Table 3.2). Plates were incubated at room temperature (~24°C) under low light conditions (~37μmol/m^2/s), with no shaking. Embryos were routinely incubated overnight in culture media prior to feeding experiments.

3.6.Fatty acid feeding in embryo culture

3.6.1 Unlabeled fatty acids

Fatty acids for feeding experiments were prepared fresh in 5% (w/v) Pluronic F127, sonicated for 10min, and filter sterilized prior to use. Feeding experiments were typically performed in a 24-well plate format with 600μL total volume per well, consisting of 100μL 30mM fatty acid, 500μL of embryo culture media and ~2-5 embryos/well. An equal volume of 5% Pluronic F127 was fed as a negative control. For experiments where additional treatments were applied (i.e. co-factors for elongation, enzyme inhibitors, as described in Results), the treatment was applied in 50μL of an appropriate solvent, and the solvent was also used alone as a negative control. Cultures were maintained as described above for the duration of the experiment. At the end of the experiment, the media was removed and the embryos washed at least five times with sterile water to ensure that both the fatty acid and any residual media were removed. Embryos were processed immediately or frozen until further use. Embryos destined for acyl-CoA extraction were stored at -80°C, while those destined for fatty acid analysis

Table 3.2. Media composition for zygotic embryo culture of *B. napus*.

	Final concentration (mM)		Final concentration mg/L
KNO_3	19	EDTA	14.9
NH_4NO_3	10	$FeSO_4*7H_2O$	11.1
$CaCl_2$	5.99	H_3BO_4	12.4
$MgSO_4$	1.5	$MnSO_4*H_2O$	33.6
KCl	4.69	$ZnSO_4*7H_2O$	21
KH_2P0_4	1.25	KI	1.66
Sucrose	60	$Na_2MoO_4*2H_2O$	0.5
Glucose	40	$CuSO_4*5H_2O$	0.05
		$CoCl_2*6H_2O$	0.05
	Final concentration (g/L)	nicotinic acid	5
PEG-4000	220	pyridoxine HCl	0.5
		Thiamine HCl	0.5
		Folic Acid	0.5
		Biotin	0.05
		Inositol	100

were typically stored in solvent (i.e. chloroform) at -20°C until extractions or methylations could be performed.

3.6.2 ^{14}C precursor feeding experiments

For feeding radiolabeled precursors, several modifications to the feeding procedure were required. Embryos were precultured overnight as described above, but were washed thoroughly with PEG-free media and weighed prior to feeding, so that the incorporation of radiolabel could be corrected on a per mg fresh weight basis. PEG-free media (identical to the composition in Table 3.2 without the PEG-4000) was required because the addition of solvent to quench the assay caused precipitation of the PEG and interfered with subsequent lipid extractions. For a similar reason, fatty acids were prepared in 1% Pluronic F127, which did not interfere with the lipid extractions.

For fatty acid feeding assays, embryos were placed in disposable glass tubes containing 90µL PEG-free media and the feeding was initiated by the addition of 10µL fatty acid (4mM stock, 1.1mCi/mmol radiospecific activity). At designated time intervals, the assay was quenched with 2mL 2:1 chloroform/methanol. Alternatively, chloroform alone was used if lipid extractions could not be immediately performed. This was to avoid the possible formation of phospholipid artefacts reported with prolonged exposure of plant tissues to methanolic solvents (Roughan et al. 1978). In that case, an appropriate volume of methanol was added immediately prior to extraction, and extractions were performed as previously described for DGAT assays above. TLC was

routinely performed by a partial ascension in 60:30:3:1 (v/v) chloroform/methanol/acetic acid/water followed by a full ascension in 80:20 (v/v) hexane/ether. Plates were scanned and scraped as previously described.

Radiolabeled acetate feeding was performed using a similar procedure, using 10µL of 1mM ^{14}C acetate (57mCi/mmol radiospecific activity) in place of the fatty acid.

3.7 Preparation and GC/MS analysis of fatty acid methyl esters (FAMEs)

Methanolic HCl was prepared by the gradual addition of 20mL acetyl chloride to 100mL cold methanol. Fatty acid methyl esters (FAMEs) were routinely prepared by direct methylation of developing embryos by adding 1mL of methanolic HCl to the embryos and incubating for 1h at 80°C. The methylation was stopped by the addition of 1 mL 0.9% aqueous NaCl, and the FAMEs were extracted twice with 2mL hexane. The resulting extract was dried down under nitrogen and resuspended in iso-octane for gas chromatography/mass spectrometry (GC/MS) analysis. The GC/MS system and method parameters are summarized in Table 3.3. FAMEs were routinely analyzed in split injection mode; splitless mode was used for trace samples. Peak identification was determined on the basis of retention time relative to external standards (Nu-Chek Prep GLC Standards 96 and 421A with GLA and SDA methyl ester added) and spectral matching using the NIST05 Mass Spectral Database.

Table 3.3. Chromatographic conditions for GC/MS analysis of FAMEs.

Chromatographic System	
Chromatographic System	Agilent 6890N w/7863B autosampler
Inlet	Split/splitless
Detector	Agilent 5975B Inert XL MSD
Column	DB-23, 30m x 0.24mm i.d. x 0.25µm
Carrier gas	Helium, constant flow mode, 40cm/sec
Inlet Temperature	290°C
Injection Volume	1µL
MS Transfer Temperature	250°C
MS Parameters	Scan (30-350amu), threshold 300 MS Quad 150°C MS Source (EI mode) 230°C
Experimental Conditions, GC/MS	**Split Injection Method**
Split ratio	10:1
Oven Temperature	165°C, hold 4min, 10°C/min to 180°C, hold 5 min, 10°C/min to 230°C hold 5 min
MS Parameters	Solvent delay 2min
Experimental Conditions, GC/MS	**Splitless Injection Method**
Oven Temperature	90°C, 10°C /min to 180°C hold 5min, 5°C/min to 230°C
MS Parameters	solvent delay 4min

Analysis of the acyl-CoA pool composition in developing embryos

Acyl-CoA analysis was performed based on the procedure described by Larson and Graham (2001), which relies on the detection of fluorescent acyl *etheno* CoA derivatives. Developing embryos were harvested from fatty acid feeding experiments as described previously, placed in a microcentrifuge tube with a 3mm glass bead and flash frozen in liquid nitrogen. The tissue was homogenized in a Biospec Mini Bead Beater (Biospec, Bartlesville, OK) for 1 min, then 200µL of freshly prepared extraction buffer (4mL 1:1 (v/v) isopropanol/potassium phosphate buffer, pH 7.2, 50µL glacial acetic acid, 80µL 50mg/mL BSA) were added and the mixture was homogenized for an additional 30s. Four hundred microliters of petroleum ether saturated with 1:1 (v/v) isopropanol/water were added, and then the mixture was vortexed thoroughly and centrifuged briefly (~45s) to induce a phase separation. The upper phase was removed and the lower phase was washed again several times 400µL of the saturated petroleum ether, until no green color was observed in the upper phase (~3-4 washes). Five microliters of saturated ammonium sulphate was added to the lower phase, followed by 600µL of 2:1 (v/v) methanol/chloroform. The mixture was vortexed and incubated on ice for 20min, and then centrifuged at 21,130 x *g* for 2 min. The supernatant was transferred to a 2mL glass autosampler vial and evaporated to dryness under vacuum (Savant AES200 SpeedVac). The resulting residue was resuspended in 100µL 0.5M chloroacetylaldehyde (in 0.15M trisodium citrate/citric acid, pH 4.0, 0.5% SDS) and derivatized at 85°C for 20min. The cooled solution was transferred to a Costar Spin-X (0.45µm nylon filter) spin

column and centrifuged at 21,130 x *g* for 1min. The filtrate was transferred into 250µL glass autosampler vial insert and analyzed by high performance liquid chromatography (HPLC) within two days. HPLC method parameters are provided in Table 3.4 and Figure 3.1.

Table 3.4. Chromatographic conditions for analysis of acyl-CoAs.

Chromatographic System Parameters	
Chromatographic System	Agilent 1200 HPLC with quaternary pump, autosampler, thermostatted column compartment, diode array detector (DAD) and fluorescence detector (FLD)
Column	LUNA-18(2) (150mm x 2mm x 5µm)
Mobile Phase	A: 1% acetic acid B: 90% acetonitrile, 1% acetic acid C: 0.25% triethylamine, 0.1% tetrahydrofuran D: 90% acetonitrile
Flow rate	0.75mL/min from 0-10.1min, 0.5mL/min from 10.1-67min; 0.75mL/min from 67.1-75min
Mobile Phase Gradient	See Figure 3.1
Injection volume	20µL
Column Temperature	40°C
FLD signals	320nm Excitation, 420nm Emission

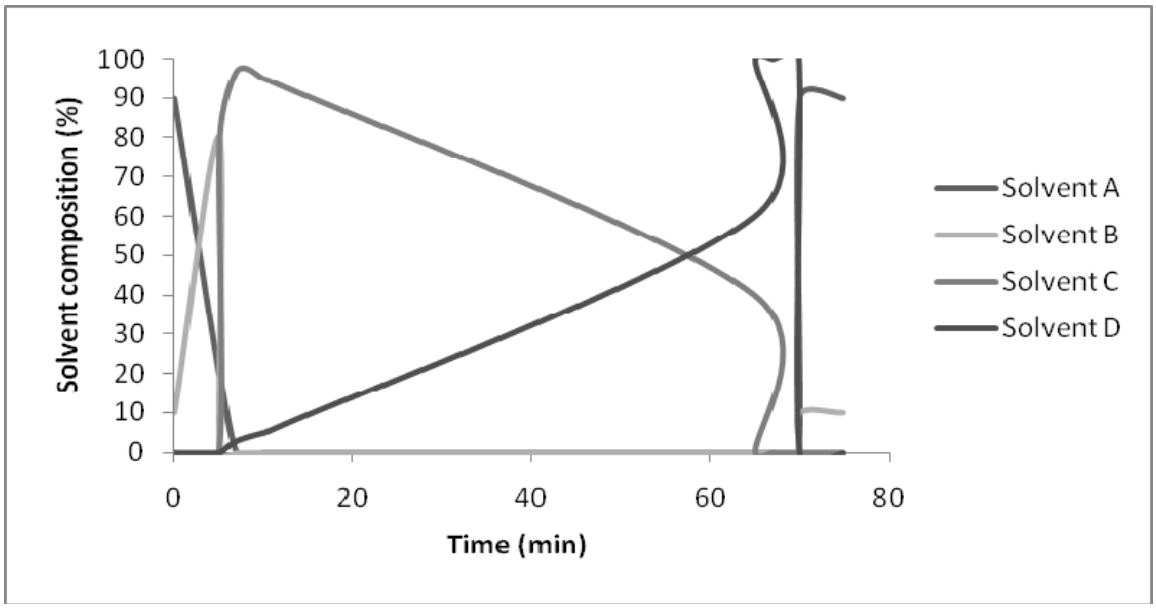

Figure 3.1. HPLC mobile phase gradient composition for analysis of acyl-CoAs. Solvent A: 1% (v/v) acetic acid, Solvent B: 90% (v/v) acetonitrile, 1% (v/v) acetic acid, Solvent C: 0.25% (v/v) triethylamine, 0.1% (v/v) tetrahydrofuran, Solvent D: 90% (v/v) acetonitrile. (Balance of all solvents is HPLC grade water).

5. Results

4.1 Investigating the role of acyl-CoA dependent acyltransferases in the "rapid removal" of acyl-CoAs

The near absence of Δ6-desaturated C18 PUFA in the acyl-CoA pool of flax and tobacco plants transformed with the minimal set of genes for VLCPUFA synthesis (Abbadi et al. 2004) suggested two possibilities; the newly desaturated PUFA were either not effectively released into the acyl-CoA pool or else they were rapidly being removed from the acyl-CoA pool by acyltransferases before elongation could occur. We initially investigated the "rapid removal hypothesis" by comparing the activities of acyl-CoA dependent acyltransferases in developing seeds. The activities of GPAT, LPAAT, DGAT, and LPCAT were assayed in homogenates of developing seeds at two developmental stages (25-29 DAF and 36-40 DAF), using 18:1-CoA as the acyl-donor (Figure 4.1). While there was some variation in the absolute activities for individual lines, particularly at 25-29 DAF, the overall trends were similar for all lines. At both developmental stages, LPCAT and LPAAT had the highest specific activities, while DGAT and GPAT activities were much lower. LPCAT activity remained relatively high at 36-40 DAF while other activities dropped off considerably. In *B. napus* cv Westar, it was previously shown that DGAT activity peaks during the active phase of oil accumulation (~27 DAF), and falls rapidly thereafter (Weselake et al. 1993). The relative activities observed at 25-29 DAF were consistent with previous studies (Furukawa-Stoffer et al. 2003; Sorensen et al. 2005; Weselake et al. 1993). The low activities of GPAT and DGAT throughout seed development made these

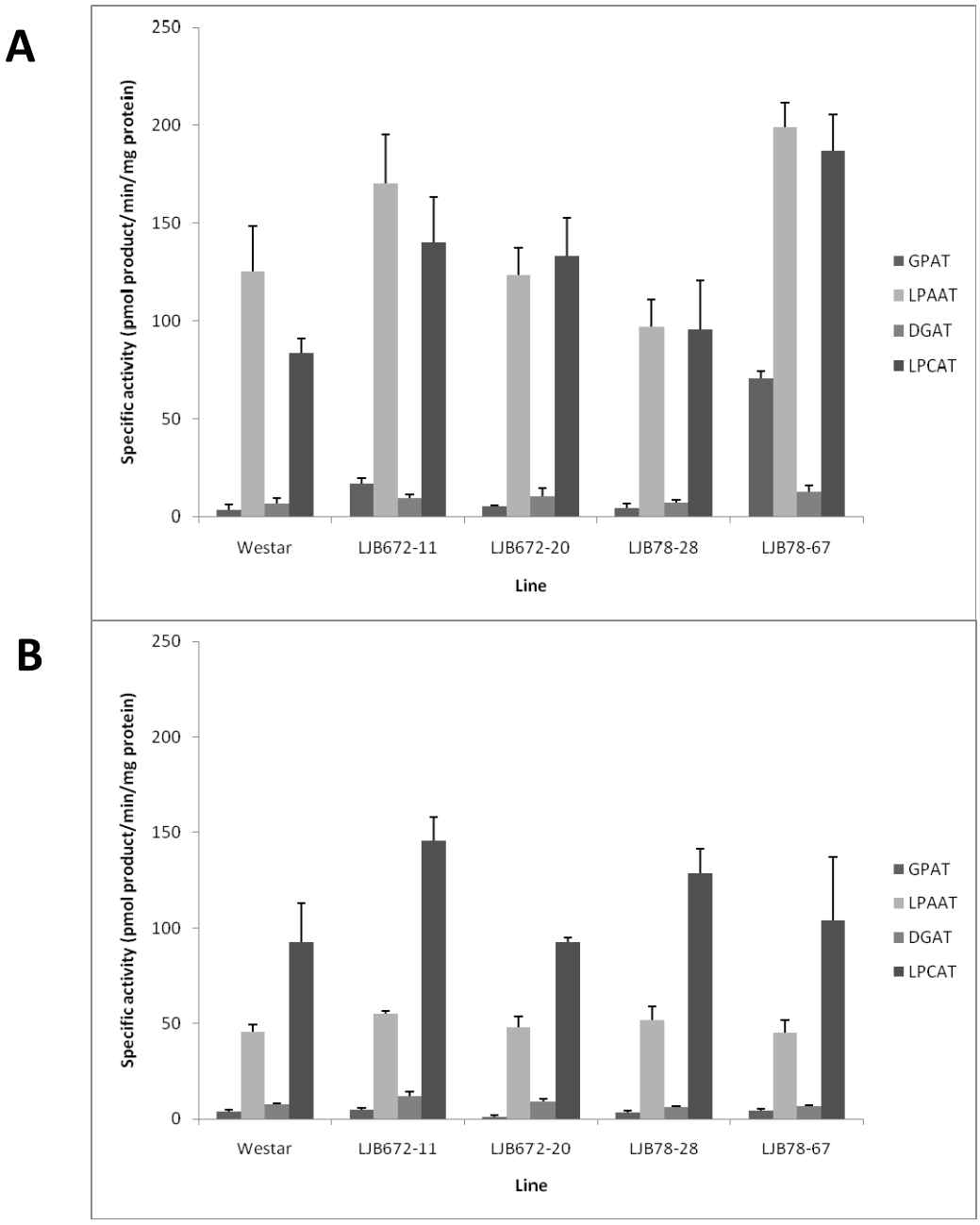

Figure 4.1. Comparison of acyl-CoA dependent acyltransferase activities in developing seeds of *B. napus*. Assays were performed homogenates from developing seeds harvested at two developmental stages: 25-29 DAF (**A**) and 36-40 DAF (**B**) and ^{14}C-18:1-CoA as the acyl donor. Two transgenic lines from each construct (LJB672 and LJB78) were analyzed, with untransformed Westar as a control. Error bars represent standard deviation of triplicate assays.

enzymes unlikely candidates for the rapid removal of acyl-CoAs, while the high activity of LPCAT even beyond the active phase of oil accumulation suggested a possible role for LPCAT in acyl-editing worth further investigation.

Based on this information, the substrate specificity of LPCAT was examined more closely using the forward reaction leading to PC. Developing seed homogenates (25-29 DAF) of *B. napus* cv Westar were used to compare the activity of LPCAT using 18:1-CoA, α-18:3-CoA or γ-18:3-CoA as acyl donors. A-18:3-CoA was used as effectively as 18:1-CoA, while it appears that γ-18:3-CoA was less effectively utilized (Figure 4.2).

Since the high activity of LPCAT throughout seed development suggested a possible role in acyl-editing, the effect of inhibiting LPCAT on the fatty acid composition of developing embryos was examined. Several inhibitors of LPCAT have been reported previously in microbial and mammalian systems. Sesamin was previously shown to inhibit LPCAT in *Mortierella □lpine,* a natural producer of ARA, but did not appear to affect LPCAT activity in sunflower microsomes (Chatrattanakunchai et al. 2000). Zinc was shown to inhibit *Saccharomyces cerevisiae* LPCAT *in vitro* through interaction with a high-affinity zinc binding site (Richard and McMaster 1998). A human membrane-bound *O*-acyltransferase (MBOAT) with LPCAT activity exhibited sensitivity to thimerosal (Gijon et al. 2008), an organo-mercury compound commonly used in biochemical studies as a sulfhydryl reagent (Elferink 1999). In homogenates of a microspore-derived cell suspension culture of *B. napus* cv Jet Neuf, however, none of these compounds

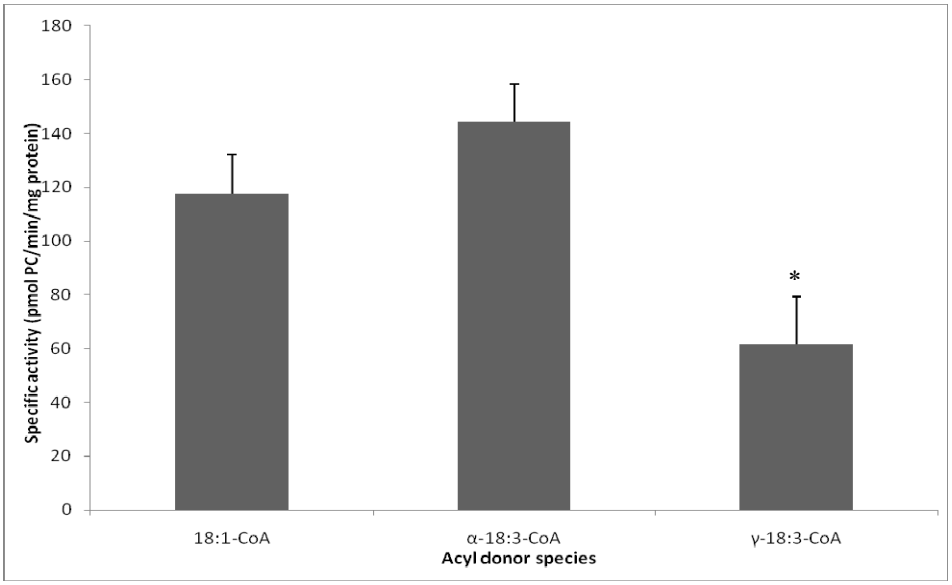

Figure 4.2. Specificity of LPCAT toward different acyl-CoA substrates. Assays were performed using homogenates of developing Westar seeds (25-29 DAF). Error bars represent the standard deviation of triplicate assays. An asterisk (*) denotes a statistically significant difference compared to 18:1-CoA (t-test, $p<0.05$).

had an inhibitory effect on LPCAT activity *in vitro* (Figure 4.3). Ethanol, which was used as a solvent for sesamin, appeared to have a mild stimulatory effect. Inhibition was initially tested using the concentration of inhibitor previously demonstrated to be effective in other systems, but no effect was observed in *B. napus* even at considerably higher concentrations (data not shown). Furthermore, no effect on VLCPUFA composition was observed when zygotic embryos of *B. napus* were cultured in the presence of these compounds (data not shown).

The high LPCAT activity observed in *in vitro* assays suggested that this enzyme might support a rapid flux of acyl-CoAs toward PC, consistent with the observations of Bates et al (2007; 2009). Since *in vitro* acyltransferase assays are not necessarily directly representative of the *in vivo* situation, we further investigated the partitioning of acyl groups between PC and TAG by incubating zygotic embryos for up to an hour in the presence of ^{14}C-labeled oleic acid (18:1), linoleic acid (18:2), or GLA (γ-18:3). Both transgenic and non-transgenic lines exhibited similar trends in the incorporation of all three fatty acids (Figure 4.4). Of the three fatty acids, it appeared that ^{14}C-labeled 18:1 accumulated to a lesser extent in TAG than the 18:2 and GLA, but this is likely due to dilution of the label by the large endogenous pool of 18:1. Overall, the amount of ^{14}C-labeled fatty acid accumulating in PC was small compared to TAG, and remained relatively constant over the course of the incubation, while TAG increased throughout. The same trend was observed during a longer four-day labeling period (Figure 4.5),

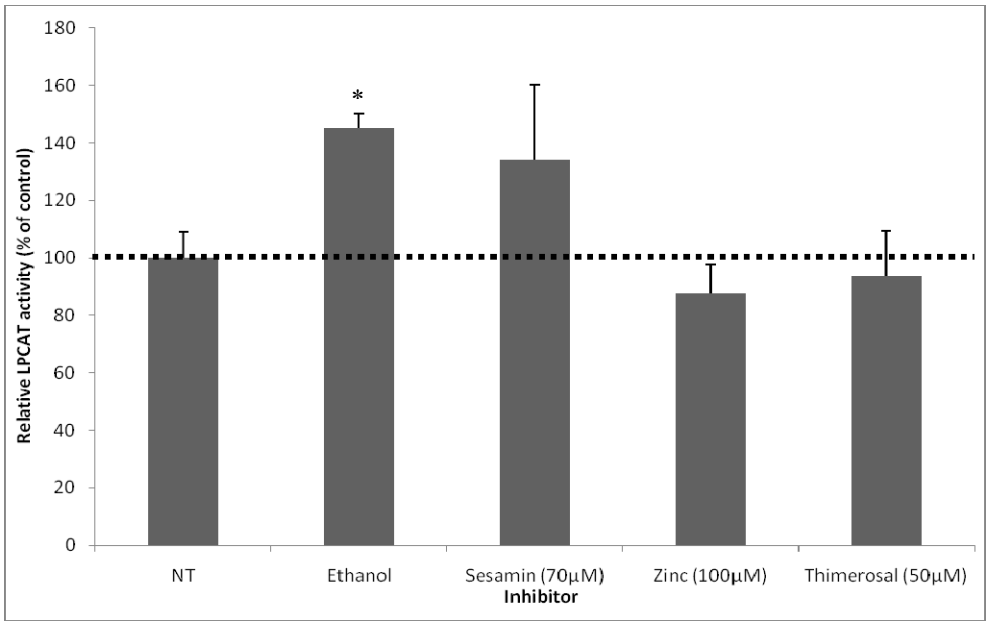

Figure 4.3. Effect of inhibitors on LPCAT activity *in vitro*. Effect of sesamin (Chatrattanakunchai et al. 2000), zinc (Richard and McMaster 1998), or thimerosal (Gijon et al. 2008) on LPCAT activity in homogenate of microspore-derived cell suspension cultures of *B. napus* L. cv Jet Neuf. The concentration shown represents the final concentration of the potential inhibitor in the reaction mixture. The reaction mixtures were pre-incubated at 30°C for 15 minutes in the presence of the inhibitor prior to initiating the reaction by the addition of ^{14}C-18:1-CoA. Bars represent the relative specific activity compared to a control pre-incubated with an equal volume of water (NT) or ethanol (in the case of sesamin). Error bars represent the standard deviation of triplicate assays. An asterisk (*) denotes a statistically significant difference compared to NT (t-test, $p<0.05$).

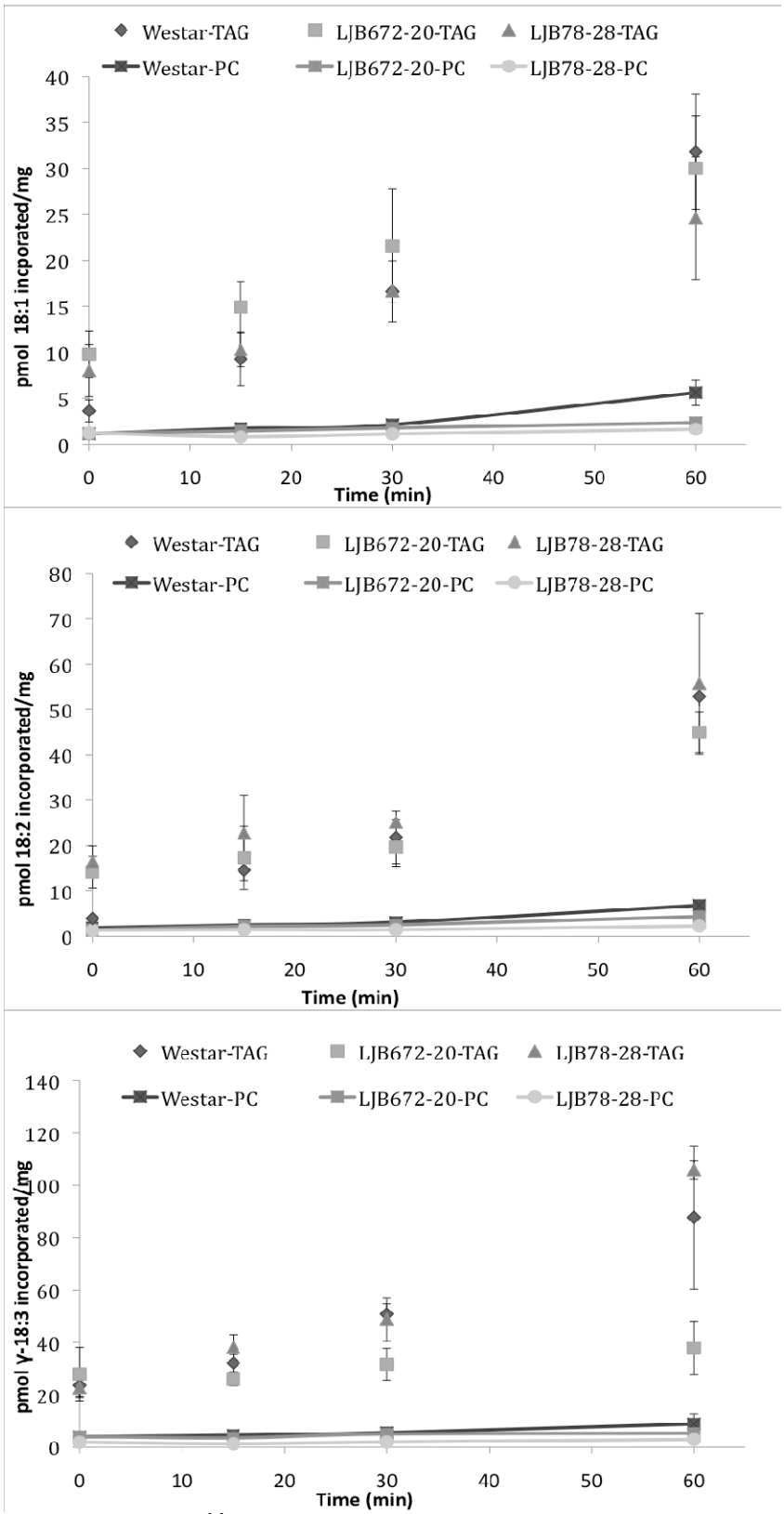

Figure 4.4. Incorporation of ^{14}C-fatty acids into PC and TAG. Incorporation of ^{14}C-labeled **A)** oleic acid (18:1), **B)** linoleic acid (18:2), **C)** Gamma linolenic acid (γ-18:3) fatty acid into PC and TAG in zygotic embryos of *B. napus.* Each FA (radiospecific activity ~1.1mCi/mmol) was supplied at a final concentration of 400µM in a total assay volume of 100µL. One transgenic line per construct was analyzed, with Westar as an untransformed control. Error bars represent the standard deviation of triplicate assays.

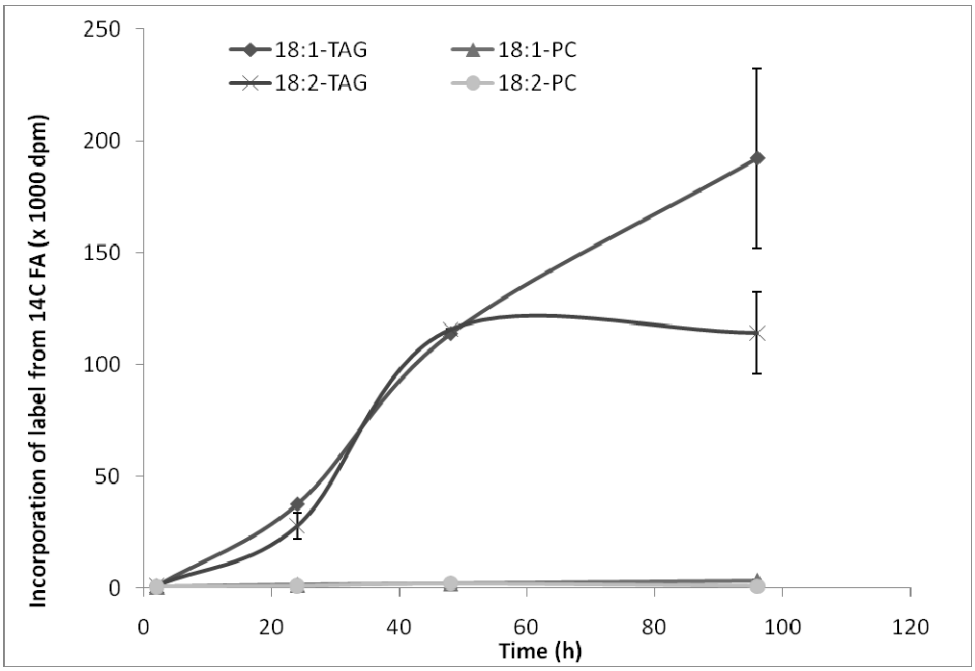

Figure 4.5. Incorporation of ^{14}C fatty acids into TAG and PC in zygotic embryos of *B. napus* (Westar) over 96h. Embryos were maintained in embryo culture media (see Table 3.2) supplemented with 100µL of 4mM ^{14}C-18:1 or ^{14}C-18:2 FA (radiospecific activity ~1.1mCi/mmol). Error bars indicate standard deviation of duplicate assays.

where the label in TAG increased and the label in PC remained at relatively low levels over the four days. This trend did not agree with the high LPCAT activities observed *in vitro* or the recent labeling experiments of Bates et al, (2007; 2009), where the majority of newly formed fatty acids rapidly entered PC. While both exogenous and newly formed endogenous fatty acids must enter the acyl-CoA pool prior to participating in acylation reactions, it is possible that the excess exogenous fatty acids had an influence on acyltransferase activities that may not be representative of the *in vivo* situation. Therefore, a similar experiment was performed using ^{14}C-labeled acetate in place of the fatty acids, and the incorporation of label from nascent fatty acids was monitored. Over a 30 minute time course, the labeling pattern from acetate was similar to that observed with fatty acids, confirming the earlier observations (Figure 4.6). There was also a large amount of label occurring in phosphatidic acid (PA). Diacylglycerol (DAG) was also heavily labeled (Figure 4.7A), but this was difficult to quantify reliably due to the presence of labeled pigments which ran with DAG. This was also observed with the fatty acid feeding experiments (Figure 4.7B).

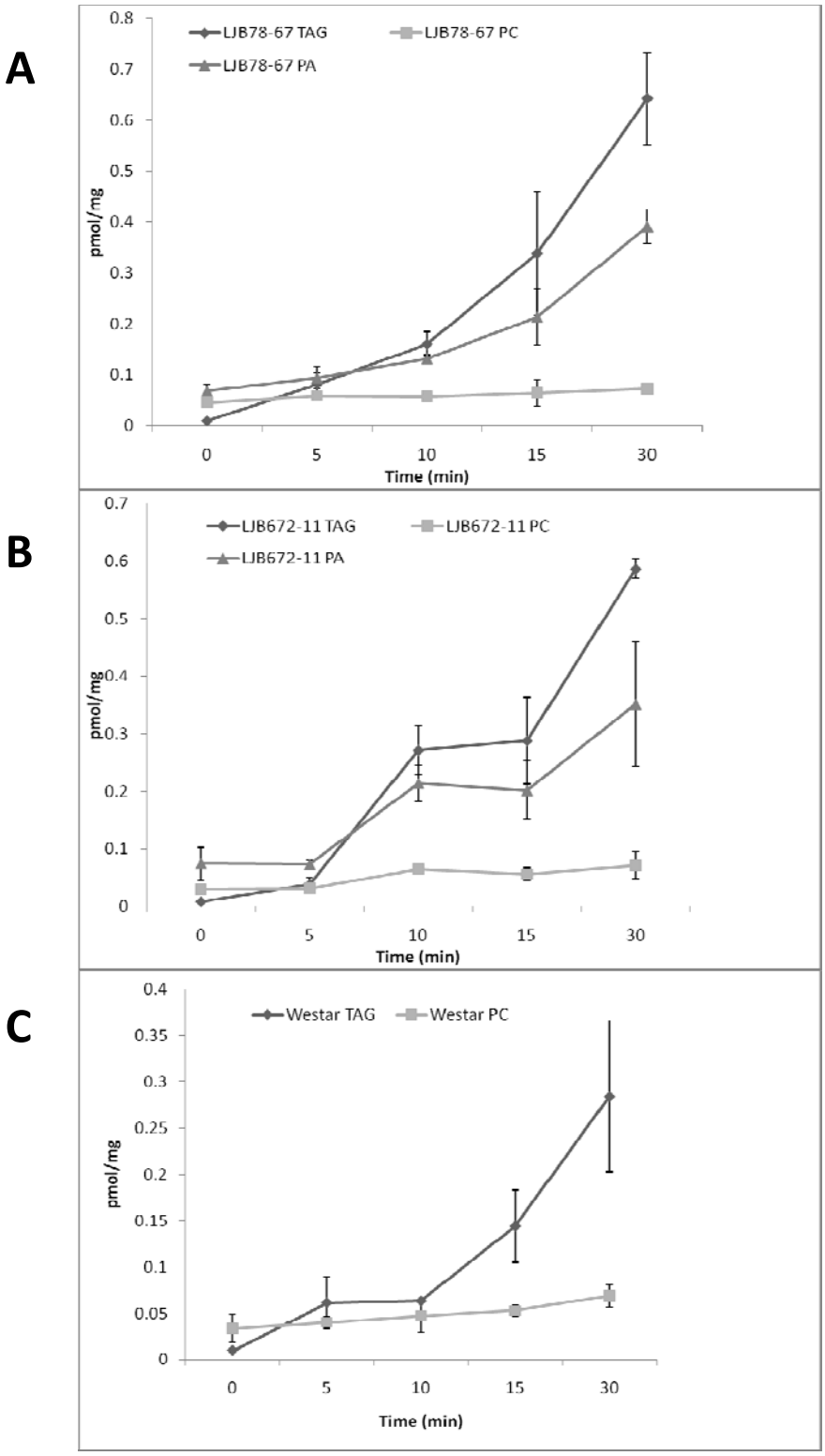

Figure 4.6. Incorporation of ^{14}C-acetate into lipids of zygotic embryos of *B. napus.* **A)** LJB78-67, **B)** LJB672-11, **C)** Westar. Note: PA was observed in Westar but was not quantified due to poor chromatographic resolution. ^{14}C-acetate was supplied at a final concentration of 100µM in an assay volume of 100µL (radiospecific activity: 57mCi/mmol). Error bars indicate standard deviation of triplicate assays.

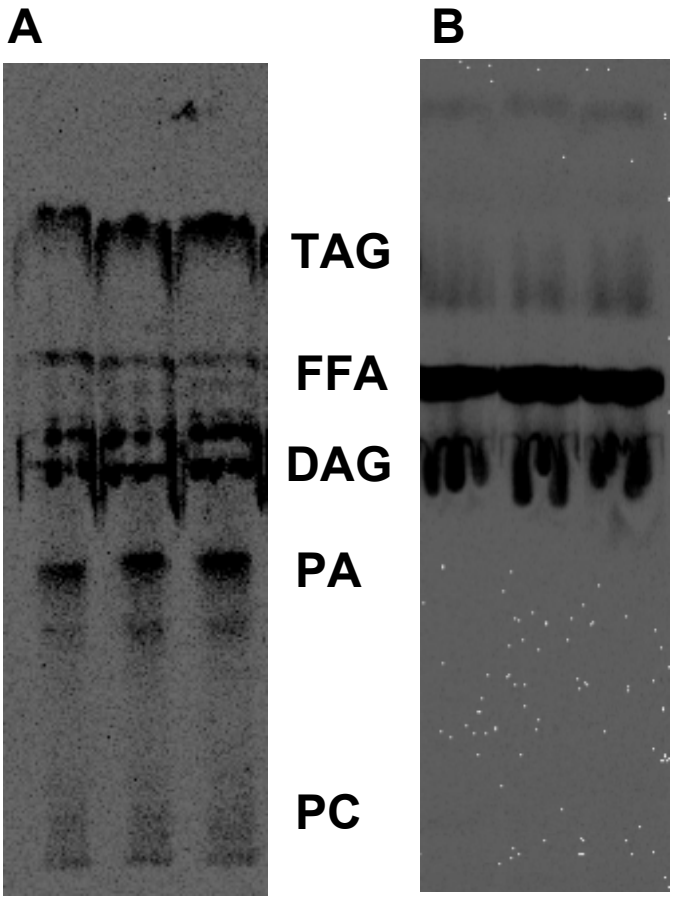

Figure 4.7. Thin layer chromatography of labeled lipids from A) ^{14}C acetate and B) ^{14}C fatty acid labeling experiments. Three replicate lanes are shown for each panel. Note that in ^{14}C fatty acid feeding experiments, the radiospecific activity was approximately 50x less than for ^{14}C -acetate, thus band intensities are not directly comparable.

4.2 Investigating the elongation and persistence of exogenous precursors in the acyl-CoA pool of zygotic Brassica napus embryos

Having established that zygotic embryos of *B. napus* are capable of utilizing exogenous fatty acids in TAG synthesis, we performed longer-term feeding experiments using unlabeled fatty acids, to determine whether these acyl groups are available for elongation and whether elongation proceeds efficiently when substrates are available. Embryos were cultured for four days in the presence of various fatty acid intermediates in the VLCPUFA pathway, including the endogenous PUFA precursors, LA and ALA (Figure 4.8). In transgenic embryos representing both constructs (LJB78 and LJB672), application of exogenous GLA consistently resulted in an increase in its immediate elongation product, DGLA (Figure 4.8C). This increase, however, was small relative to the total incorporation of the exogenous fatty acid, and downstream products were not substantially increased. Other fatty acids did not have a pronounced effect on downstream products. Partial elongation of exogenous GLA to DGLA suggested that at least some of the GLA was available in the acyl-CoA pool. This was verified by analyzing the acyl-CoA pool of embryos after four days of culture in the presence of exogenous GLA, which showed a clear increase in 18:3 in GLA treated embryos relative to the control (Figure 4.9). It should be noted that the chromatographic method does not resolve isomers of 18:3; the small peak in untreated embryos corresponds to endogenous ALA, or in the case of transgenic embryos, the sum of endogenous ALA and background levels of GLA accumulating as a result of the transgene expression.

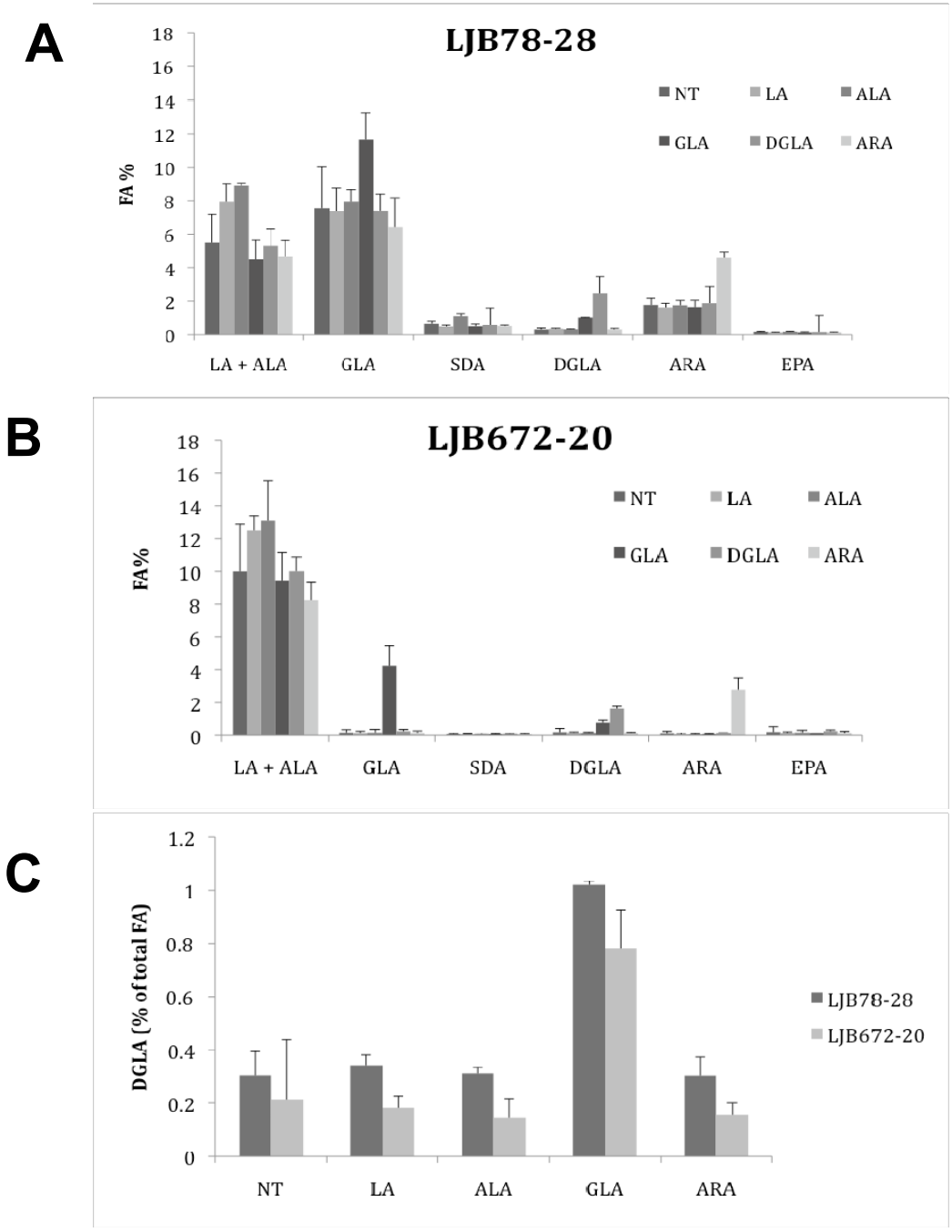

Figure 4.8. Effect of exogenous PUFA precursors on elongation. Changes in the PUFA composition of zygotic embryos of *B. napus* cultured in the presence of exogenous fatty acids for 96h. **A)** PUFA composition of zygotic embryos of Construct LJB78, Line 28 cultured in the presence of 5mM LA, ALA, GLA, DGLA, ARA, or 5% (w/v) Pluronic F127 (NT). **B)** PUFA composition of zygotic embryos of Construct LJB672, Line 20 cultured in the presence of 5mM LA, ALA, GLA, DGLA, ARA, or 5% (w/v) Pluronic F127 (NT). **C)** Proportion of DGLA (% of total FA) in zygotic embryos of LJB78-28 (dark bars) and LJB672-20 (light bars) in response to treatment with various exogenous FA. Error bars represent standard deviation of at least triplicate assays. Treatment with exogenous GLA resulted in a significant increase in DGLA, compared to treatment with Pluronic F127 alone (t-test, $p<0.05$).

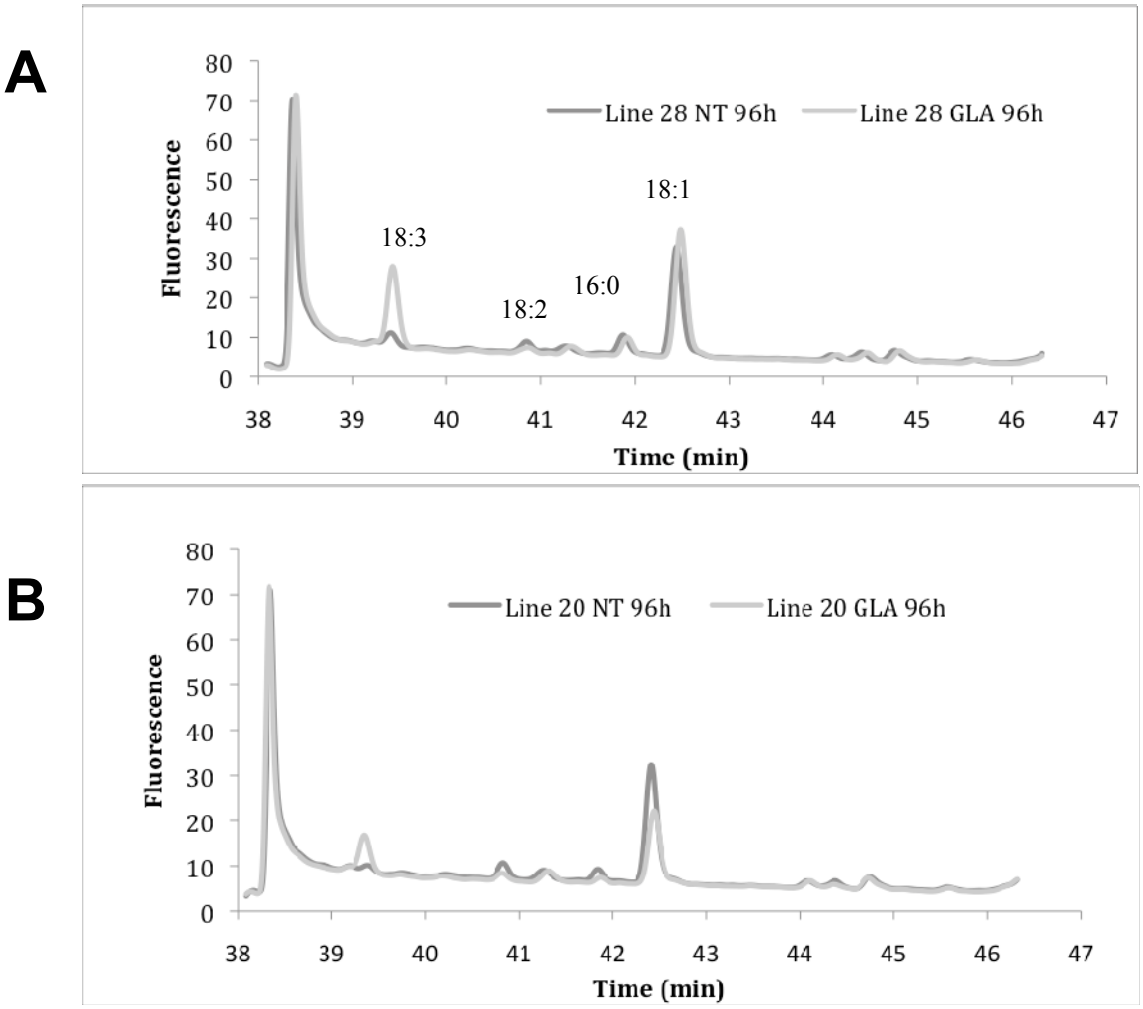

Figure 4.9. Acyl-CoA pool composition of *B. napus* embryos fed exogenous fatty acids. HPLC chromatograms illustrate the acyl-CoA composition of zygotic embryos of **A)** LJB78-28 and **B)** LJB672-20, following incubation with exogenous GLA (5mM final concentration) or Pluronic F127 (NT) for 96h. Similar results (not shown) were observed for Westar.

In a second experiment, the persistence of GLA in the acyl-CoA pool was explored by culturing embryos in the presence of exogenous GLA for four days, then thoroughly washing the embryos to remove the exogenous fatty acid and culturing them for a further three days. The acyl-CoA composition was monitored during this post-treatment period to determine how quickly GLA-CoA is depleted from the acyl-CoA pool in the absence of an exogenous supply. In both transgenic and untransformed Westar embryos, exogenous GLA accumulated in the acyl-CoA pool during the 96 hour feeding period, and was slowly depleted upon removal of the exogenous source of GLA (Figure 4.10). Downstream elongation products were not detected in the acyl-CoA pool, but this may have been a consequence of the limit of detection for the method. As before, analysis of the total fatty acid composition showed an increase in DGLA after GLA supplementation (Figure 4.11).

Despite relatively high levels of GLA-CoA accumulating in the acyl-CoA pool, it appears that only a small fraction of this substrate was utilized for Δ6-elongation. Since GLA-CoA is not rapidly removed from the acyl-CoA pool, this raises the question of whether other co-factors for elongation may be limiting the efficiency of the Δ6-elongase. Cytosolic elongation requires, in addition to an acyl-CoA substrate, a source of cytosolic malonyl-CoA and reducing power. As with fatty acid biosynthesis, malonyl-CoA is produced from acetyl-CoA via the activity of an acetyl-CoA carboxylase. Metabolic flux studies in high-erucic acid *B. napus* embryos have demonstrated that most of the cytosolic acetyl-CoA pool is derived from citrate exported from the mitochondria, and this export of citrate

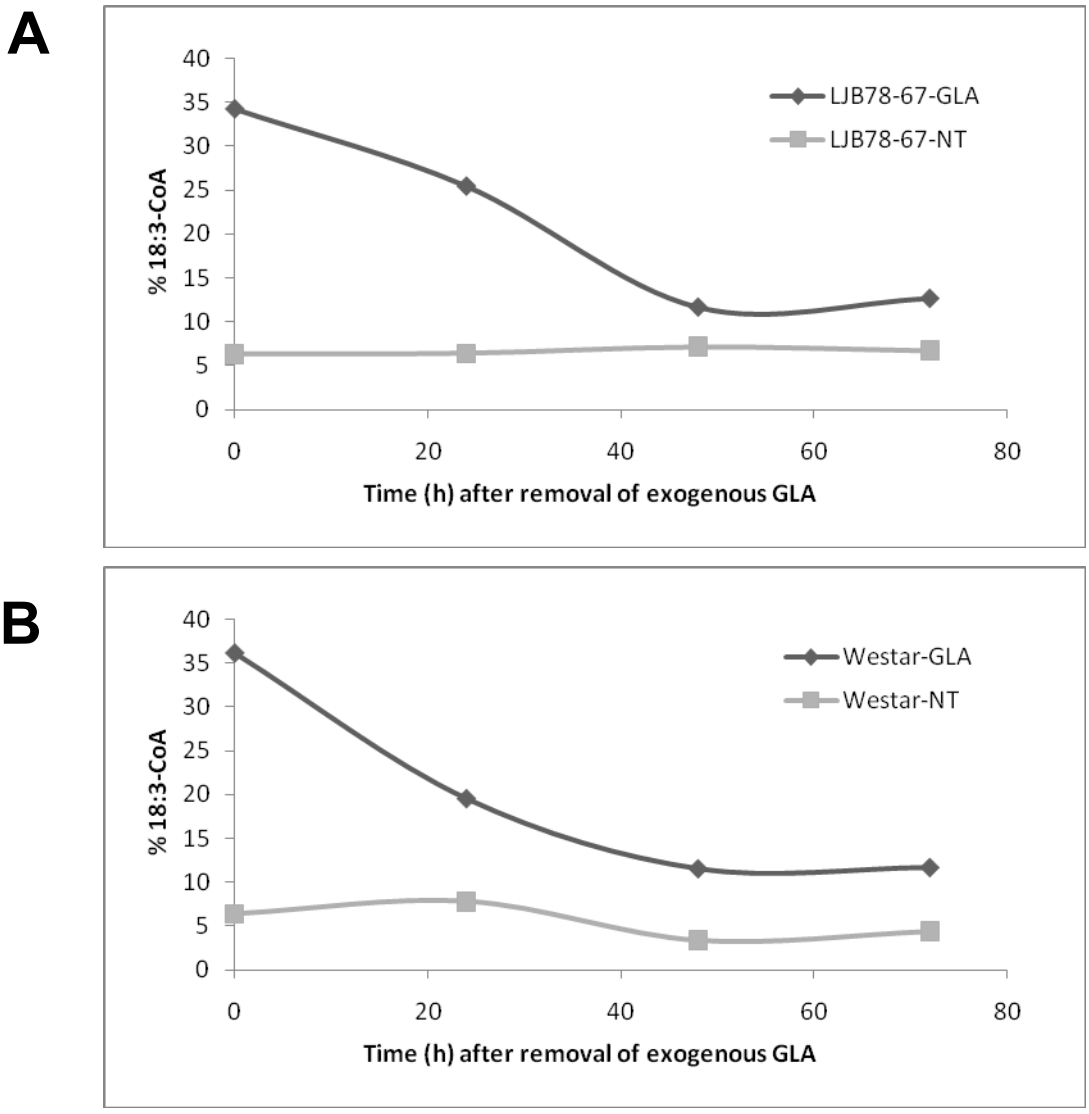

Figure 4.10. Persistence of exogenous FA in the acyl-CoA pool. Zygotic embryos of *B. napus* **A)** LJB78-67 or **B)** Westar were cultured for four days in the presence of 5mM GLA (GLA) or Pluronic F127 (NT), then washed thoroughly and incubated without exogenous fatty acid for 72h, and the persistence of GLA-CoA in the acyl-CoA pool was monitored by HPLC. FA feeding was performed in duplicate, with one replicate per line used for acyl-CoA analysis and one replicate used for GC/MS analysis of fatty acid composition (Figure 4.11).

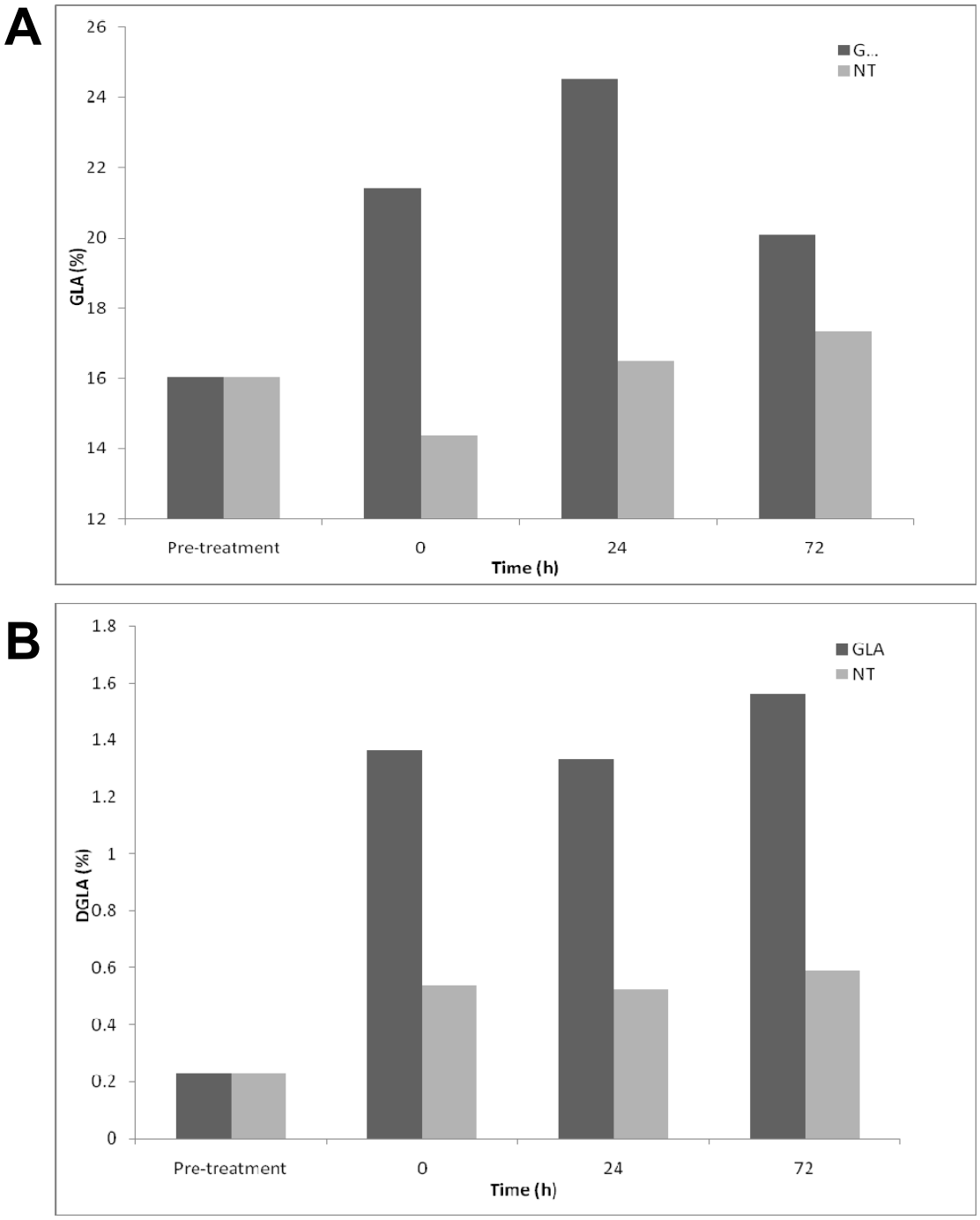

Figure 4.11. Changes in GLA and DGLA content of total lipids in acyl-CoA persistence experiment. Zygotic embryos of LJB78-67 were cultured in the presence of exogenous GLA (GLA, dark barks) or Pluronic F127 (NT, light bars) for 96h, then washed thoroughly and incubated for a further 72h without exogenous FA. Changes in the **A)** GLA and **B)** DGLA content of total lipids were monitored by GC/MS. Pre-treatment refers to the composition prior to any treatment; 0h corresponds to the composition at the end of the 96h feeding period after which the supply of exogenous FA was removed. One replicate per line was analyzed for FA composition; a second replicate was used for acyl-CoA analysis (Figure 4.10).

represents the largest flux of carbon leaving the mitochondria (Schwender et al. 2006). In high-erucic *B. napus,* erucic acid ($22:1^{\Delta 13}$) accounts for up to 60% of the total fatty acid composition, thus, a large pool of cytosolic acetyl-CoA must be directed toward fatty acid elongation. In low-erucic acid *B. napus,* including the cultivar Westar used in this study, erucic acid accounts for, by definition, less than 2% of the total fatty acid composition. Since there is no endogenous elongase activity requiring a source of acetyl-CoA, it seemed plausible that the formation of cytosolic acetyl-CoA might be down-regulated or otherwise redirected to support other metabolic processes.

To test whether co-factor availability could limit Δ6-elongation efficiency, embryos were cultured in the presence of GLA and supplemented with malonate, citrate, or acetate, at a range of concentrations. After four days of incubation, no substantial increases in total C20 fatty acid content were evident at any of the concentrations tested (Figure 4.12).

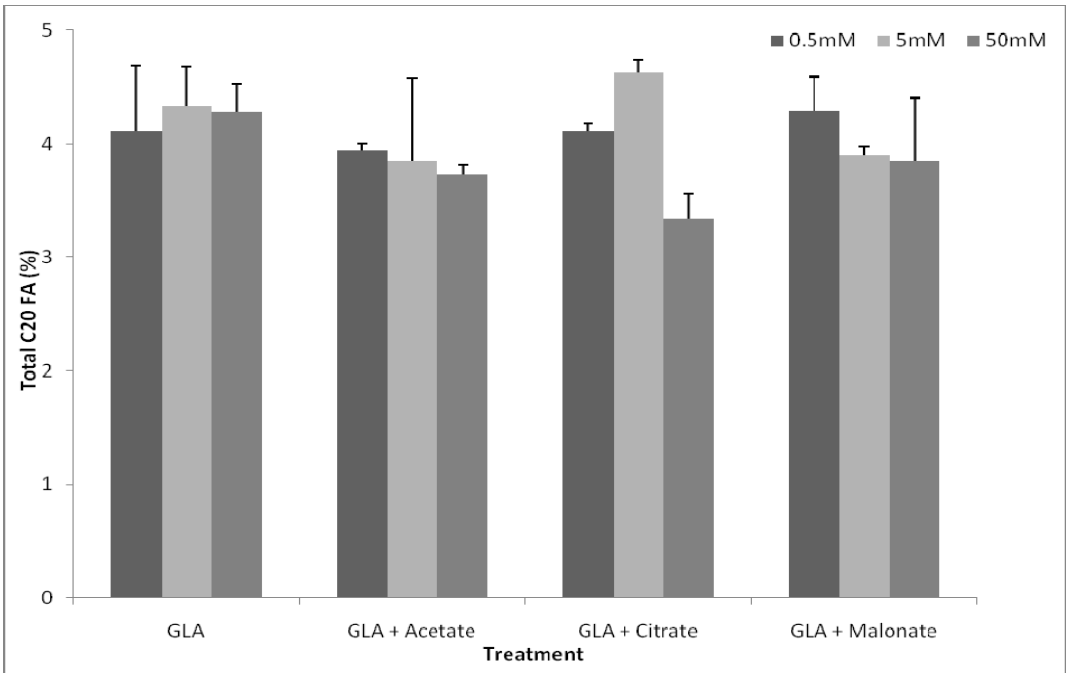

Figure 4.12. The effect of various malonyl-CoA precursors on fatty acid elongation. Zygotic embryos of *B. napus* line LJB672-18 were cultured for four days in the presence of 5mM GLA alone, or 5mM GLA with acetate, citrate, or malonate at the concentrations indicated. FA composition of total lipids was then determined by GC/MS and the total content of C20 fatty acids (products of cytosolic elongation) was compared. Error bars represent the range of duplicate assays.

4.3 Investigating the fate of acyl groups at the sn-2 position of phosphatidylcholine (PC)

Based on the results above, we could effectively rule out the possibility that acyl groups are rapidly removed from the acyl-CoA pool and thus unavailable for elongation. After rejecting the "rapid removal hypothesis", we explored the alternative possibility that newly desaturated acyl groups are not efficiently released into the acyl-CoA pool and instead enter TAG through an acyl-CoA independent route. We investigated the fate of acyl groups at the *sn*-2 position of PC by incubating lyophilized microsomes with phosphatidylcholine containing radiolabeled 18:2 at the *sn*-2 position. This assay was based on the protocol published for assaying PDAT (Dahlqvist et al. 2000; Stahl et al. 2004). Although there was some formation of radiolabeled TAG (Figure 4.13), there was also considerable label in DAG and PA. The formation of DAG was observed previously in PDAT assays (Dahlqvist et al. 2000), but the formation of PA was unexpected. While PC/DAG interconversion has been described in the context of seed oil biosynthesis (Bates et al. 2009; Stobart and Stymne 1985; Vogel and Browse 1996), no such activity has been described in this context for the conversion of PC to PA. This conversion can be catalyzed by a phospholipase D (PLD), which is present in developing oilseeds (Novotna et al. 2000; Ryu et al. 1996; Xu et al. 1997), but has mostly been described as an enzyme involved in signaling and stress responses (Li et al. 2009). Since our initial assays were performed with microsomes from a microspore-derived cell suspension culture of *B. napus*, we considered the possibility that PA formation was unique to the

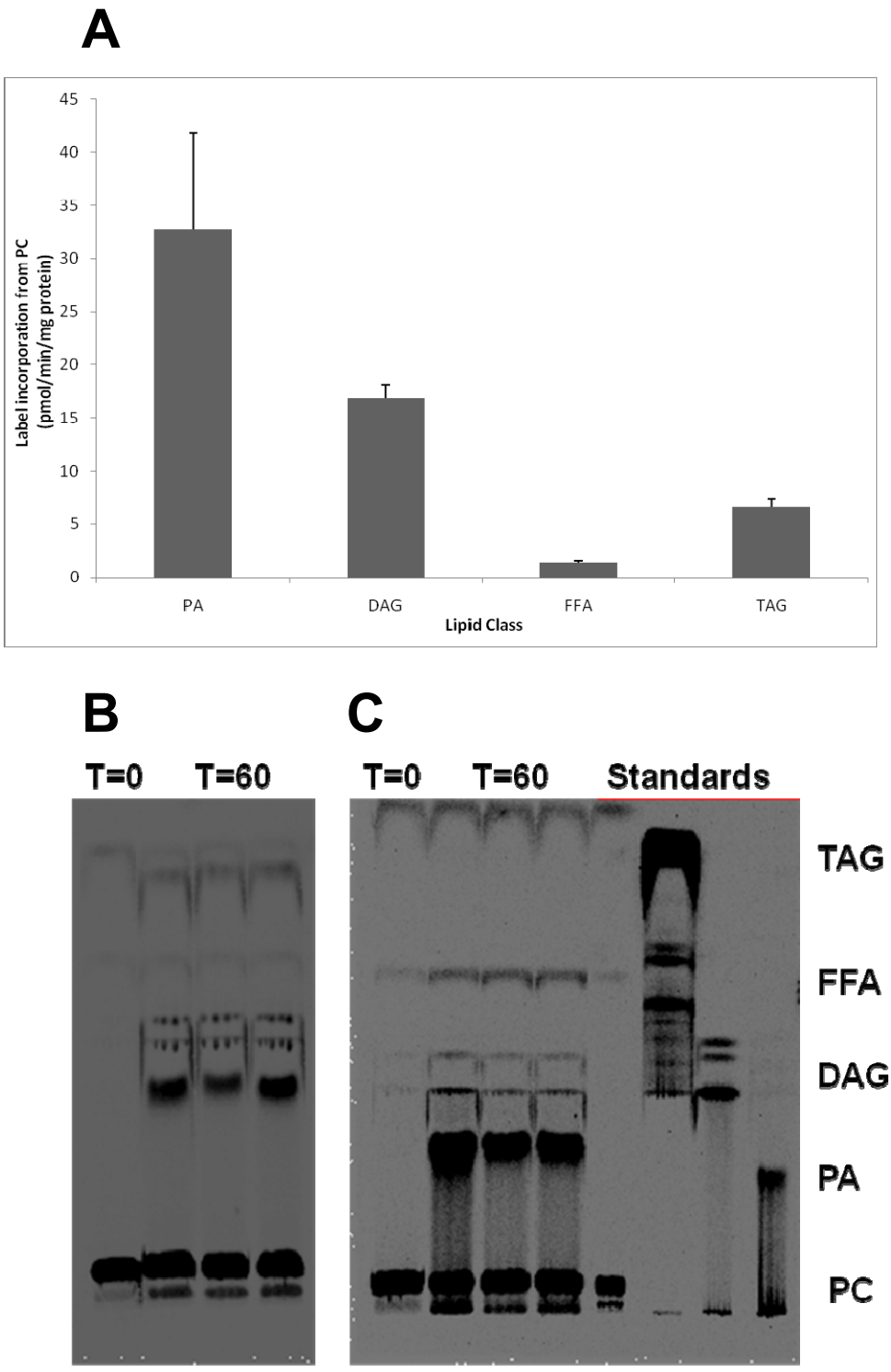

Figure 4.13. Incorporation of label from ^{14}C-PC in lyophilized microsomes of *B. napus.* A) Incorporation of label into different lipid classes in microsomes of microspore derived cell suspension cultures of *B. napus.* Error bars represent standard deviation from triplicate assays. **B)** Thin layer chromatography of lipids from microsomes of cell suspension cultures and **C)** microsomes of developing seeds (Westar) incubated with ^{14}C-PC. T=0 lane represents quenched (negative control), T=60 lanes represent triplicate 60min assays.

culture and repeated the assay using microsomes from developing seeds, obtaining similar results (Figure 4.13C).

5. Discussion

The overall goal of this study was to determine the biochemical basis for the inefficient elongation of Δ6-desaturated C18-PUFA to longer chain VLCPUFA such as ARA and EPA. It was previously proposed that the availability of Δ6-desaturated C18-PUFA in the acyl-CoA pool was a major limitation on subsequent elongation efficiency (Abbadi et al. 2004). This study expands on these findings by further exploring two possible explanations for the limited availability of Δ6-desaturated C18-PUFA in the acyl-CoA pool, as well as by demonstrating that this "bottleneck" in VLCPUFA biosynthesis can be partially overcome when the levels of Δ6-desaturated C18-PUFA in the acyl-CoA pool are increased.

One hypothesis explaining the absence of PUFA in the acyl-CoA pool is that they are rapidly removed through the activity of various acyltransferases and thus are unavailable for elongation. A comparison of acyl-CoA dependent acyltransferase activities at two stages during seed development showed that LPCAT and LPAAT had high specific activities compared to GPAT and DGAT. This was entirely consistent with previous findings which demonstrated that DGAT has the lowest specific activity of all the *sn*-glycerol-3-phosphate pathway enzymes (Perry et al. 1999). LPAAT activity was also previously shown to be high compared to DGAT in both *B. napus* and flax (Perry et al. 1999; Sorensen et al. 2005). LPCAT was shown to be highly active in microspore-derived cell suspension cultures of *B. napus* (Furukawa-Stoffer et al. 2003), and was inferred

based on the rapid incorporation of acyl-CoAs into PC in microsomes from developing sunflower (Stymne and Stobart 1984b). In this study, LPCAT activity in all lines remained at substantially higher levels late in seed development, while GPAT, LPAAT, and DGAT activity was reduced. This observation is consistent with a role for LPCAT in "acyl-editing" of membrane phospholipids, which might be expected to continue even after storage lipid biosynthesis tapers off. The acyl-editing activity of LPCAT would involve both the forward and reverse reactions (Yurchenko et al. 2009).

We also examined the substrate specificity of LPCAT, in order to determine whether it possessed high activity toward Δ6-desaturated C18-PUFA. Based on our preliminary experiments, 18:1-CoA and ALA-CoA were utilized at similar rates, while the activity toward GLA-CoA was significantly reduced. There is little data available concerning the substrate specificity of other plant LPCATs, but recent data on two *Arabidopsis* LPCAT isoforms showed higher specificity toward 18:1-CoA than 18:2-CoA (Shen et al. 2008). Interestingly, the *Arabidopsis* LPCATs (particularly AtLPCAT2) also appeared to exhibit high specificity toward 16:0-CoA and 18:0-CoA. This is surprising considering that saturated moieties are typically discriminated against in the *sn*-2 position of both phospholipids and triacylglycerol (Brokerhoff and Yurkowski 1966; Christie et al. 1991), and *B. napus* LPCAT was previously shown to be less active toward saturated acyl-CoAs, particularly at higher substrate concentrations (Furukawa-Stoffer et al. 2003).

It should be noted, however, that substrate specificity data alone may not be entirely indicative of the *in vivo* situation where different acyl-CoA species co-exist in a mixed and dynamic pool, and where their availability to certain enzymes may be mediated in part by acyl-CoA binding proteins (ACBPs) (Burton et al. 2005; Faergeman et al. 2007). Recently, it was demonstrated that ACBP enhances the exchange of acyl groups between PC and the acyl-CoA pool, and that *B. napus* ACBP binds 18:1-CoA more effectively than ALA-CoA (Yurchenko et al. 2009). Thus, in drawing conclusions about acyltransferase substrate preferences, it is important to consider not only the substrate specificity, but also the substrate selectivity (the tendency for an enzyme to "choose" a particular substrate from a mixed pool), as well as the influence of ACBP binding specificity.

Several inhibitors of LPCAT were tested in *in vitro* assays and in an embryo culture system to determine the effect of reduced LPCAT activity on fatty acid composition, and trafficking of PUFAs in particular. No inhibition of LPCAT activity was detected in *in vitro* assays, and no major changes were observed in the fatty acid composition of zygotic embryos cultured in the presence of these inhibitors. This was not entirely surprising in the case of sesamin, which was previously shown to inhibit *Mortierella* LPCAT but not LPCAT from developing sunflower seeds or guinea pig liver (Chatrattanakunchai et al. 2000). Zinc was shown to inhibit yeast LPCAT *in vitro*, and it was suggested that this may occur through a high-affinity zinc binding site (Richard and McMaster, 1998). At that time, no LPCAT gene or cDNA had been cloned,

so the presence and position of this binding site was never confirmed. Based on sequences currently available, the *Saccharomyces cerevisiae* LPCAT (LCA1; (Chen et al. 2007)) (Genbank accession: BAF93897) polypeptide is considerably longer (619 residues) than the 462-465 residue *Arabidposis* LPCATs (At1g12640 & At1g63050; (Shen et al. 2008)). It is possible that the zinc binding site occurs in a region not conserved between plant and yeast LPCAT. Similarly, while human MBOAT5 displayed strong sensitivity to thimerosal, a related protein, MBOAT2, was only marginally inhibited (Gijon et al. 2008), suggesting that sensitivity to thimerosal is also not universal among LPCATs.

In mammalian systems, it has recently been shown that several enzymes exhibiting LPCAT activity are actually members of a more general family of lysophospholipid acyltransferases (LPLATs), which overlap in their headgroup specificity. For example, mouse MBOAT1 possessed LPCAT and LPEAT (lysophophatidylethanolamine) activities, as well as lower activity toward lysophophatidylserine (Hishikawa et al. 2008). Similarly, two *Arabidopsis* LPEATs also exhibited LPAAT and LPCAT activity (Stalberg et al. 2009). Given the apparent catalytic flexibility of this family of enzymes, a more careful dissection of various LPCAT/LPLAT activities in plants might be best achieved using a combination of knockout mutants and characterization of individual substrate preferences using recombinant systems. Such an approach may shed further insight into which individual enzymes are most directly involved in trafficking of PUFA.

Given the challenges and the limitations of assaying acyltransferase activities *in vitro*, we also performed a series of fatty acid feeding experiments in a zygotic embryo culture system, which would more closely mimic the *in vivo* situation. Exogenous fatty acids were incorporated into the acyl-CoA pool and other lipids, including TAG. Of the various fatty acids applied, the most pronounced effect was an increase in DGLA in response to exogenous GLA. The increase in DGLA, however, was small compared to the increase in GLA available in the acyl-CoA pool for elongation. To determine whether this was due to rapid removal of GLA from the acyl-CoA pool, we monitored the acyl-CoA pool composition over several days after the source of exogenous GLA was removed, and found that GLA-CoA was slowly removed but remained above background levels after three days of incubation. These observations effectively ruled out the rapid removal hypothesis.

Since GLA-CoA availability was, in this case, not a limiting factor for subsequent elongation, we tested whether co-factor availability for elongation might pose a further limitation on elongation efficiency. No increase in total C20 fatty acid content was observed after supplementing the media with various concentrations of citrate, malonate, or acetate, all of which are precursors for the formation of cytosolic malonyl-CoA which supports fatty acid elongation beyond C18. This suggested that malonyl-CoA is probably not limiting fatty acid elongation, at least within the embryo culture system. It is possible that while the embryo culture media may contain adequate carbon to support formation of endogenous malonyl-CoA, the situation may be different *in planta*. It was

previously demonstrated that over-expression of rat liver ATP:citrate lyase (ACL) in tobacco leaf plastids led to a 16% increase in fatty acid content, presumably due to the increased availability of acetyl-CoA feeding into plastidial fatty acid biosynthesis (Rangasamy and Ratledge 2000). Likewise, over-expression of the cytosolic form of acetyl-CoA carboxylase (ACCase) in the plastids of *B. napus* seeds led to a 5% increase in seed oil content (Roesler et al. 1997). The activity of ACL in *B. napus* seeds is closely coordinated with ACCase activity, with both peaking during the active phase of seed oil accumulation (Ratledge et al. 1997). It has been further suggested through antisense suppression studies in *Arabidopsis* that ACL represents a non-redundant source of cytosolic acetyl-CoA; even mild suppression of ACL expression resulted in a number of phenotypic changes, including reduced growth rate, reduction in cuticular wax deposition and reduced oil content (Fatland et al. 2005). While no change in fatty acid composition was observed, the authors suggested this may have been due to poor expression of the antisense construct in the embryo under the 35S CaMV promoter. Considering that even mild disruption of the cytosolic acetyl-CoA supply caused severe phenotypic effects, and that substantial gains in oil content could be derived from plastidial over-expression of ACL or ACCase, it is reasonable to speculate that cytosolic over-expression of these enzymes under an appropriate promoter may result in an increase in fatty acid elongation beyond C18.

Fatty acid elongation also requires a source of reducing power, usually NADPH. It seems unlikely, however, that the supply of reductant represents a significant bottleneck in fatty acid elongation, since it has been calculated that in

B. napus embryos, photosynthetic activity provides more reductant than is required to support oil synthesis (Ruuska et al. 2004).

It should be noted that in the case of the transgenic lines, it is possible that the expression of one or more transgenes (including the elongase) could be suppressed due to the presence of multiple copies of the same promoter. In the case of the LJB78 construct, all four transgenes were placed under the direction of a strong napin promoter. In the case of the LJB672 construct, different promoters were used for each transgene, but because these lines were segregating, it is possible that differences in zygosity could also have an impact on the extent to which the desired phenotype is expressed. It should also be noted that in many cases, we were only able to analyze one transgenic line per construct, which does not permit us to fully determine whether there are differences resulting from positional effects of the construct insertion. Differences in elongation efficiency and acyl-trafficking between constructs and/or between transgenic lines within a construct may be attributable in part to one or more of these factors, which should be confirmed through further study.

To complement the longer-term (4 day) fatty acid feeding experiments with unlabeled fatty acids, we also performed short-term (60 min) incubations using radiolabeled fatty acids. The radiolabeled fatty acids were incorporated into a number of lipid classes, including PC, DAG, and TAG. Similar results were obtained using radiolabeled acetate, where there also appeared to be substantial labeling in PA. In each case, the rate of incorporation and the total label incorporated was higher in TAG than PC, which disagrees with several previous

studies. In developing soybean embryos, labeled acetate was rapidly incorporated into PC, and to a lesser extent, DAG and TAG (Bates et al. 2009; Slack et al. 1978). The rate of lipid biosynthesis in soybean, however, is about 5-fold less than *B. napus* (Bates et al. 2009), so some of this difference could be due to the relatively slow rate of TAG biosynthesis in soybean compared to *B. napus*. In tissue slices of sunflower or safflower cotyledons incubated with labeled oleate, label was initially incorporated into PC and DAG at a higher rate than TAG, but after two hours of incubation, the amount of label in TAG was equal or greater than the amount of label in PC (Griffiths et al. 1988b). In contrast, cotelydon slices from sunflower incubated with palmitate or linoleate produced a more similar labeling pattern over two hours to what we observed in *B. napus* embryos, with most of the label occurring in TAG (Griffiths et al. 1988b).

It should be noted that these previous studies were performed using between 70-500mg of tissue for each replicate, which is considerably higher than the 25-30mg of tissue (~8 embryos) used in our experiments. In our case, it was important to be able to directly compare different transgenic lines at the same stage of development grown at the same time. Growth space limitations, as well as the time required to dissect embryos prior to each experiment, imposed a practical limit on sample size in our experiments, and also limited us in many instances to analyzing only one transgenic line per construct in each experiment. To put this in perspective, (Bates et al. 2009) used three soybean embryos (~20mg each (dwt)) per time point in their acetate labeling experiments. To obtain 25-30mg total (fwt) *B. napus* embryos, per time point, we required ~2.5 times more

embryos. This was a considerable logistical challenge, which was also compounded by the fact that not all the lines flowered synchronously in the greenhouse. Now that it has been established that the lines behave relatively alike in side-by-side comparisons of their overall labeling pattern, it may be reasonable to focus on one line at a time in larger scale experiments, which might also allow a more detailed examination, for example, of acyl-group modifications (i.e. desaturation, elongation) through analysis of the fatty acid composition of the labeled lipids. We attempted some preliminary experiments using silver-nitrate TLC to separate acyl groups according to their degree of unsaturation, but found this method lacked both the sensitivity for our small sample sizes, and the resolution to separate acyl groups with the same degree of unsaturation but different chain lengths. For example, it was not possible to separate ALA and GLA from DGLA, which would have been a major product of interest. The latter issue could likely be addressed with further development, or through an alternative strategy such as radio-gas chromatography, but even with radio-GC, the issue of sensitivity must still be addressed.

Having established that PUFA-CoAs are not rapidly removed from the acyl-CoA pool, and after examining the short-term acyl-group trafficking pattern through labeling studies, which suggested that acyl groups do not remain sequestered in PC, the next approach was to examine the fate of acyl-groups at the *sn*-2 position of PC. Incubation of lyophilized microsomes with PC containing a radiolabeled 18:2 acyl group at the *sn*-2 position resulted in the formation of primarily PA, with various amounts of DAG and TAG formed depending on the

source of the microsomes. This was surprising, since the assays were performed under the conditions previously described for PDAT (Dahlqvist et al. 2000; Stahl et al. 2004), where the major products were TAG and DAG. A key difference between our assays and those of Dahlqvist et al. (2000) and Stahl et al. (2004), was the TLC solvent system used to resolve the reaction products. In the previous PDAT papers, the solvent system was 35:70:1.5 or 30:70:1.5 hexane/diethyl ether/acetic acid (v/v), which would have resolved neutral lipids but likely not individual phospholipid species. Indeed, this is what is shown in the figures in the paper of Stahl et al. (2004). In that paper, the authors note that when polar lipids were resolved, they did not detect any activity in polar lipids except for a minor, unidentified polar compound that did not co-elute with any known phospholipids, but the results of that analysis are not shown (Stahl et al. 2004). In previous experiments in our laboratory focusing on the reverse reaction of LPCAT, a labeled band not corresponding to TAG was routinely observed in the organic fraction after incubating microsomes with labeled PC; this may have been PA, but was never identified because the major products of interest in that case were in the aqueous fraction (Yurchenko, personal communication)

PA can be formed from PC and other phospholipids through the activity of a phospholipase D (PLD). PA itself is believed to mediate a wide range of physiological functions including biotic and abiotic stress responses, early seedling development and senescence (Li et al. 2009). Several forms of PLD have been described in plants; *Arabidopsis* has 12 PLD genes representing six different types (Li et al. 2009). PLD has also been described in developing

oilseeds, including castor bean (Xu et al. 1997), soybean (Ryu et al. 1996), and *B. napus* (Novotna et al. 2000). In developing soybean, PLD expression was highest during the early to middle stages of seed development (Ryu et al. 1996), while in castor bean, PLD expression levels were very high in the most metabolically active tissues, including developing seeds (Xu et al. 1997). In *B. napus*, both phosphatidylinositol 4,5-bisphosphate (PIP_2)-dependent and independent forms of PLD were active during seed development, but the activity peaked at different times; the PIP_2-independent PLD activity was very high around 34 DAF and fell thereafter, while the PIP_2-dependent PLD activity rose sharply late in seed development (~48 DAF) (Novotna et al. 2000). The activity of both PLDs were associated with both microsomal and cytosolic fractions, with most of the PIP_2-independent PLD activity occuring in the microsomal fraction during earlier developmental stages, and PIP_2-dependent PLD activity increasing in the cytosolic fractions late in seed development (Novotna et al. 2000). Our observed PA formation in microsomes from 25-29 DAF developing seeds is most consistent with the timing and localization of the PIP_2-independent PLD activity. Most plant PLDs, however, require micromolar to millimolar concentrations of calcium (Wang 2001). Of the nine *Arabidopsis* PLDs that have been characterized so far, only one, PLDζ1, does not require calcium, and this PLD is PIP_2-dependent (Li et al. 2009). Since no exogenous calcium was provided in our assay, it remains unclear whether the PA formation we observed is due to a PLD activity, and if so, which type.

A relatively small amount of FFA was observed in incubations with labeled PC, presumably arising from the activity of a phospholipase A_2. This was also consistent with previous assays of PDAT (Dahlqvist et al. 2000). This FFA could contribute a minor flux of acyl groups back into the acyl-CoA pool via the Lands Cycle. It should be noted that this probably does not represent the total PLA_2 activity, since, as noted before, no calcium was added to the reaction mixture and there are both calcium-dependent and calcium-independent forms of plant PLA_2 (Wang 2001).

In microsomes from microspore-derived cell suspension cultures, but not microsomes from developing seeds, there was also some movement of label from PC to TAG, which could occur directly through the activity of PDAT, or indirectly by exchange of the backbone with DAG followed by a subsequent acylation. Since there was also movement of label from PC to DAG, either route could explain our results. The movement of label from PC to DAG was also demonstrated in the original papers describing PDAT (Dahlqvist et al. 2000; Stahl et al. 2004), where DAG was frequently the predominant product observed in PDAT assays. For example, in castor bean microsomes, DAG was the major product when oleoyl-PC was provided, but TAG was the major product when ricinoloeyl-PC was provided; in other cases, such as sunflower and hawk's beard (*Crepis palaestina*), only DAG was formed in the presence of oleoyl-PC (Dahlqvist et al. 2000). The relatively minor formation of TAG is also in agreement with the observation an *Arabidopsis* mutant deficient in PDAT activity did not exhibit disruptions in oil content or fatty acid composition (Mhaske et al.

2005). This is in contrast to studies demonstrating that a reduction in DGAT activity results in decreased seed oil content and changes in fatty acid composition (Katavic et al. 1995; Lock et al. 2009).

In *Arabidopsis*, over-expression of PDAT also had little impact on oil content or composition, despite the relatively low levels of PDAT expression in the wild-type (Dahlqvist et al. 2000). The fact that PDAT was more active toward PC containing unusual acyl groups, such as ricinoleoyl-PC, vernoloyl-PC and arachidonyl-PC (Stahl et al. 2004) highlights its possible role as a membrane "proofreading" enzyme, thus, it may not be surprising that in our results and others, PDAT appears to play a minor role in TAG synthesis when only common endogenous substrates such as oleoyl-PC and linoleoyl-PC are present. To date, the characterization of PDAT toward unusual substrates has been strictly *in vitro*; it would be interesting to further explore whether PDAT expression levels change in response to major disruptions in lipid composition, such as from VLCPUFA formation in transgenic plants.

Evidence of DAG formation from PC is not only consistent with previous observations in PDAT assays, but also with the more recent model proposed by Bates et al (2009), which involves multiple, kinetically distinct pools of PC and DAG, and suggests a mechanism for PUFA enrichment in TAG through PC/DAG interconversion. Several enzymes may be responsible for this activity; until recently it was believed to be catalyzed either by a phospholipase C (PLC) (Wang 2001) or via the reverse activity of CDP-choline:diacylglycerol cholinephosphotransferase (CPT) (Vogel and Browse 1996). PLC activity was

previously shown to be high in *B. napus* microsomes around 34 DAF (Novotna et al. 2000). Recently, a new enzyme, phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT) has been identified which catalyzes the interconversion of PC and DAG (Lu et al. 2009). Unlike CPT, which is involved in the *de novo* synthesis of PC from DAG and CDP-choline, PDCT activity increased with the addition of exogenous PC and did not require CDP-choline (Lu et al. 2009). *Arabidopsis reduced oleate desaturation1* (*rod1*) mutants deficient in PDCT activity exhibited a reduction in 18:2 and 18:3 in the seed oil, compensated for by increases in 18:1 and 20:1 (Lu et al. 2009).

It should be noted that while *in vitro* studies using lyophilized membranes are invaluable for the characterization of enzymes utilizing PC as a substrate, we must exercise caution in extrapolating the *in vivo* relevance of these results since the membrane preparation may not accurately represent the actual membrane organization *in vivo*; for example, kinetically distinct pools of substrate may become homogenous, and various subdomains of the ER may become disrupted. Movement of label from exogenous PC in this artificial system may therefore not necessarily mirror the trafficking of acyl groups and backbones between different substrate pools *in vivo*.

6. Conclusions and Future Directions

In summary, the results of this study have demonstrated that PUFA-CoA are not rapidly removed from the acyl-CoA pool, and that GLA-CoA, when present, can be partially elongated to form DGLA. While the efficiency of this elongation was still quite low relative to the amount of substrate available, we were unable to increase the efficiency of elongation through supplementation with compounds involved in the formation of the cytosolic acetyl-CoA pool, suggesting that elongation co-factor availability did not limit elongation efficiency in our embryo culture system. Incubation of zygotic embryos with radiolabeled acetate and various fatty acids resulted in similar trends in the labeling pattern, with TAG formation outpacing PC formation in each case; the fact that label did not continue to increase in PC over an extended incubation suggested that there is an efficient mechanism for channeling acyl groups from PC into TAG.

Our preliminary work investigating the fate of acyl groups at the *sn*-2 position of PC agreed with previous findings that PDAT does not appear to play a major role in the trafficking of common fatty acids such as 18:2 into TAG, but we have not yet ruled out the possibility that PDAT may be more important in the context of channeling VLCPUFA into TAG. We also detected conversion of PC to DAG, and release of labeled FFA from PC, consistent with previous observations. Despite previous reports of phospholipase D activity in developing oilseeds, our observation of PA as a major product has not been reported in microsomes of other species assayed under similar conditions. Further validation of this result is needed, as is a more thorough exploration of the fate of acyl

groups on PC under different assay conditions. A comparison of PDAT activity between the transgenic lines may shed insight into whether PDAT activity changes in response to VLCPUFA accumulation. Use of commercially available 18:2-labeled PC may not be the best substrate for detecting such differences, since it appears PDAT has a preference for unusual acyl groups and has relatively low activity toward common endogenous substrates such as 18:2-PC.

One major issue that has not yet been addressed is what mechanism is responsible for the return of acyl groups from PC to the acyl-CoA pool. The recent work of Bates et al (2007; 2009) suggests that this represents a major flux in lipid metabolism, but this is in contrast to several previous observations of substrate dichotomy between PC and the acyl-CoA pool in relation to the accumulation of unusual fatty acids, including VLCPUFA, in transgenic plants. Having effectively ruled out the rapid removal hypothesis as an explanation for the absence of Δ6-desaturated C18-PUFA in the acyl-CoA pool, the most likely explanation is that PUFA are inefficiently released from PC into the acyl-CoA pool following desaturation. This is also supported by the observations of Hoffman et al (2008), where it appeared that the efficiency of Δ6-desaturation by an acyl-CoA dependent desaturase was limited by the availability of endogenous PUFA-CoA, which must also be released from PC into the acyl-CoA pool.

It is clear that further work needs to be done to better understand the contributions of the reverse LPCAT reaction and the Lands Cycle to acyl-editing in the context of PUFA trafficking, including their regulation *in vivo* and associated substrate specificities. Such studies face a number of technical

challenges, not only surrounding the availability of radiolabeled PC substrates containing unusual acyl groups, but also the involvement of acyl-CoA binding proteins which can mediate these activities both *in vitro* and *in vivo* and have specificities of their own to consider (Yurchenko et al. 2009). It may be insightful to study mechanisms of acyl exchange in systems that more efficiently accumulate VLCPUFA, such as low-erucic acid *B. carinata* (Cheng et al. 2010).

From the perspective of developing strategies for overcoming substrate dichotomy issues in the synthesis of unusual fatty acids in transgenic plants, an "ideal" system might, as others have speculated, involve an entirely acyl-CoA dependent pathway. The challenges encountered by Hoffmann et al (2008) suggest that it may also be necessary to introduce acyl-CoA dependent Δ12 and Δ15 desaturase activities in order to shift the formation of endogenous PUFA precursors to the acyl-CoA pool. Acyl-CoA dependent Δ12 desaturation has been described in insects (Blomquist et al. 1991). More recently, genetic analysis has shown that the Δ12 desaturase from cricket may have evolved from an ancestral Δ9 desaturase similar to the animal stearoyl-CoA desaturases, while a more distantly related Δ12 desaturase from beetle may have evolved independently (Zhou et al. 2008). No acyl-CoA dependent Δ15 desaturase has been described to date, but ongoing studies into the molecular mechanisms underlying the positional specificity of desaturases may suggest strategies for introducing the required specificity through mutagenesis. For example, substitution of five amino acid residues in a Δ6-16:-0-ACP desaturase resulted in an enzyme that functions as a Δ9-18:0-ACP desaturase; while chain length specificity could be shifted from

18:0 to 16:0 by substitution of two residues (Cahoon et al. 1997). The phospholipid-dependent Δ12 desaturase from *Arabidopsis* was shown to acquire hydroxylase activity similar to castor bean through the substitution of just four residues (Broadwater et al. 2002). Such studies suggest that desaturases are amenable to rational modification for altered substrate specificity. Given the increasingly diverse range of desaturase activities now documented from various organisms and the availability of sequence information for desaturases differing in substrate specificity, it may only be a matter of time before it is possible to engineer customized desaturase activities with the required specificity.

7. References

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