# DEFINING THE SUBSTRATE SPECIFICITY OF AN UNUSUAL ACYLTRANSFERASE: A STEP TOWARDS THE PRODUCTION OF AN ADVANCED BIOFUEL

by

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B.S., Panjab University, Chd, India, 2003
M.S., Kansas State University, 2010

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

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Graduate Group of Biochemistry and Molecular Biophysics

KANSAS STATE UNIVERSITY
Manhattan, Kansas

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# Abstract

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*All may not be mentioned but none is forgotten.*

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# Dedication

*Dedicated to my parents, Late Sh. Madan Lal and Smt. Jai Wanti, my brother, Upinder , my sister, Ranjana, my lovely wife Nisha and my son Laveen*

# Chapter 1 - Overview

## 1.1 Plant oils as renewable feedstocks

Plant oils are rapidly emerging as an alternative to conventional fossil fuel based sources of energy and industrial feedstocks. This is mainly due to the environmental concerns associated with increasing amounts of carbon dioxide and other harmful gases like CO, $NO_x$, and $SO_2$ originating from the use of high amounts of fossil fuels. This has led to the need to develop domestic and renewable sources of feedstocks for fuel and industrial chemicals. The products derived form plant oils offer advantages like being carbon neutral, biodegradable and non-toxic due to their bio-based nature [1]. Vegetable oils are made up of triacylglycerols, which consist of three long chain fatty acids attached to a glycerol backbone. About 79% of total oil production in the world is derived from palm, soybean, rapeseed and sunflower [2]. The production of plant oils has steadily doubled from 88 million metric tons in year 2001 to 176 million metric tons in year 2015 (www.fas.usda.gov). The majority of plant oil produced is used as food, animal feed and for production of industrial chemicals in a ratio of 74:6:20 [3]. The majority of vegetable oil used in industry is for the production of biodiesel while a small part is used as industrial feedstock to produce fatty acid and derivatives, in cosmetics, textiles and leather industry, drying oils, surfactants and petroleum additives [2]. Biodiesel is produced by the transmethylation of vegetable oils, which converts triacylglycerols into fatty acid methyl esters and glycerol as a byproduct [4].

## 1.2 Fatty acid composition affects the properties of vegetable oil

Vegetable oils used in food and feed applications mainly contain five major fatty acids namely palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3). These fatty acids are made up of linear hydrocarbon chains with one or more double bonds (Fig. 1.1). The fatty acid composition of vegetable oil determines its physical and chemical properties, which in turn decides its end use. For example, oils rich in saturated fatty acids are used in preparation of margarines and spreads while oils rich in monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) are useful for cooking and salad oils respectively due to their better nutritional and health properties.

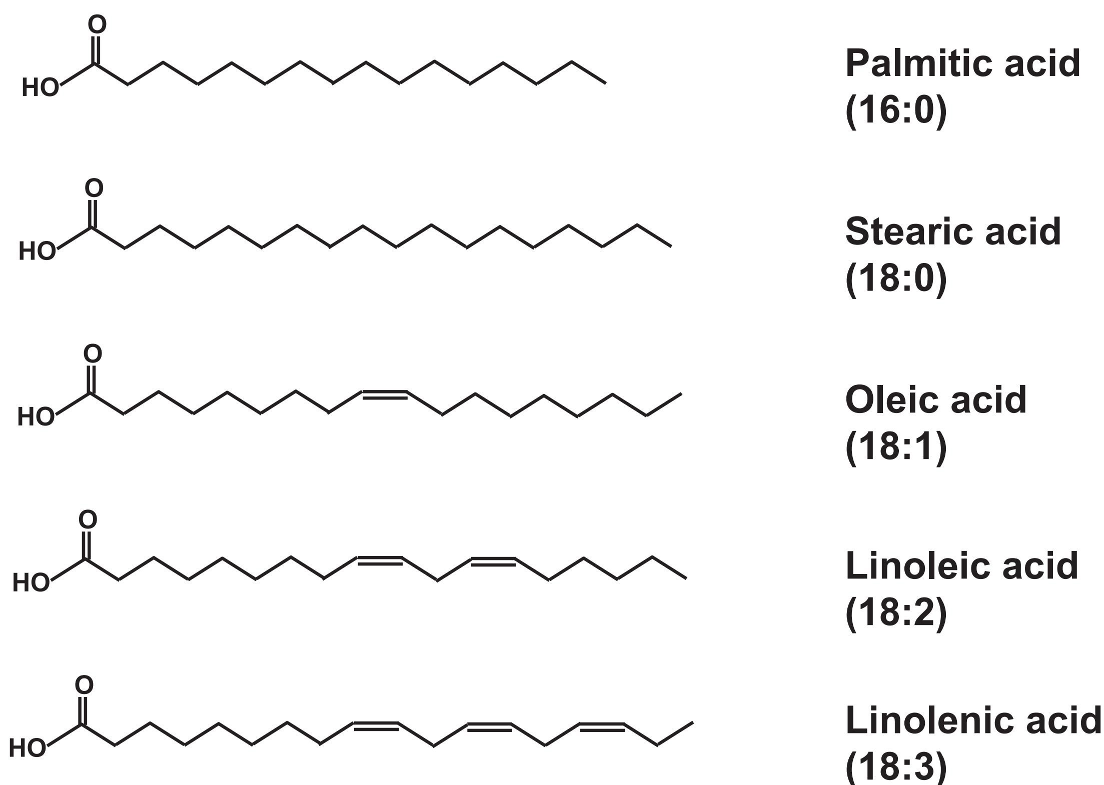
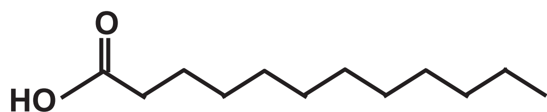
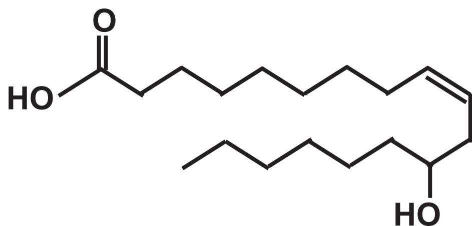



Figure 1.1 **Structures of common fatty acids present in vegetable oils.** Fatty acids are represented as X:Y where X indicates the number of carbon atoms and Y represents the number of double bonds.

However, certain plant oils in nature contain high amounts of fatty acids which are considered unusual because of their unknown role in plant physiology. Some of these fatty acids include ricinoleic acid with a hydroxyl group (found in castor oil), vernolic acid with an epoxy group (*Vernonia galmensis or* iron weed) and the medium chain fatty acid lauric acid (found in coconut and palm kernel oil) (Fig. 1.2). These unusual fatty acids provide very distinct chemical properties to the oil, which are useful for certain applications. For example, lauric acid present in the coconut oil is an excellent surfactant and is used extensively in the synthesis of detergents and soaps as sodium laurate. Similarly, castor oil containing 90 % ricinoleic acid is widely used in manufacturing of lubricants, drying oils, dyes, cold resistant plastics and pharmaceuticals due to its stability at high temperature and resistance to oxidation [5].



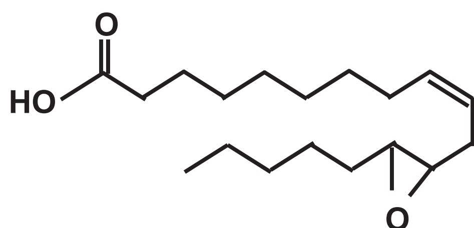




Figure 1.2 **Structures of unusual fatty acids present in vegetable oils.**

## 1.3 Acetyl-TAGs and their potential uses

3-acetyl-1,2-diacylglycerols (acetyl-TAGs) are found abundantly in the seed of certain plant families such as *Celasteraceae* and *Lardizabalaceae* [6,7]. They were also found to be present in low quantities in buffalo milk fat [8]. More recently they were also discovered in deer

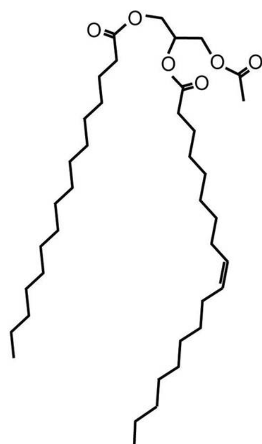


Figure 1.3 **Structure of an acetyl-TAG molecule.** The acetyl-TAGs contain an acetate group at the *sn*-3 position of glycerol backbone instead of a long chain fatty acid.

antlers [9] and insects [10]. They possess different chemical and physical properties compared to usual long chain triacylglycerols (lcTAGs) due to presence of short acetyl group instead of a long chain fatty acid at the *sn*-3 position of the glycerol backbone (Fig 1.3).

### 1.3.1 Biofuel application of acetyl-TAGs

The direct use of vegetable oil as biofuel for diesel engines suffers from problems such as high kinematic viscosity and poor cold temperature properties [11]. This necessitates either modification of engines to preheat the oil or blending them with diesel oil to reduce their viscosity. The other method to reduce the viscosity of vegetable oils is to convert them to biodiesel by chemical transmethylation. All these processes add to the cost and hamper the direct use of vegetable oils as biofuel. Acetyl-TAGs have reduced kinematic viscosity and improved cold temperature properties over traditional vegetable oils that mainly consist of lcTAGs [12,13]. For these reasons, acetyl-TAGs are considered to be useful molecules for development as direct use biofuel. The viscosity of acetyl-TAGs fall in the range of diesel # 4 (5-24 $mm/s^2$), which is mainly used in engines requiring constant power such as low and medium speed engines. Any further reduction in the viscosity of acetyl-TAGs will broaden its scope for use in other types of diesel engines.

### 1.3.2 Industrial application of acetyl-TAGs

In addition to their use as potential biofuel, the acetyl-TAGs can also be used for other industrial applications. For example, acetyl-TAGs are a constituent of ACETEM, which is synonym for the mixture of all the possible acetic acid esters of mono-, and diglycerides of fatty acids. These mixtures are used in food industry as emulsifiers and food surface coating agents besides their other industrial applications such as lubricants, plasticizers and anti-dusting agents [14]. ACETEM are produced by chemical reactions of glycerols with fatty acids and acetic acid and generate a mixture of acetic acid esters of mono and diglycerides. The degree of acetylation of ACETEM decides their physical properties, which in turn decides end use [14]. Acetyl-TAGs produced in the plants can be used in conjunction with chemically produced ACETEM to further enhance their functionalities.

#### 1.3.2.1 Acetyl-TAGs as plasticizers

Plasticizers used to make polyvinyl chloride (PVC) products are generally phthalate-based in nature. The safety concerns associated with phthalate-based plasticizers use has led to

their legislative bans in products such as toys and cosmetics in European Union and Japan. In United States, various manufacturers have also voluntarily eliminated the use of this class of plasticizers. High cost are associated with manufacture of phthalates required in large amounts for PVC products [15]. Plant oil derived products can offer a cheaper and environmental friendly functional alternative to phthalates based plasticizers.

Castor and lesquerella seed oils from that contain hydroxyl fatty acids (HFAs) can be polymerized to form thermosetting polyurethanes upon reaction with diisocyanates. 3-acetyl-1,2-diricinolein rich seed oil will provide a feedstock with monomers containing two rather than three reactive hydroxyls. The polymerization of such compounds will result in more linear rather than cross-linked polyurethanes with high thermoplastic properties. Indeed acetylated versions of castor oil is used to make biodegradable plasticizers such as SOFT-N-SAFE$^{TM}$ . However, along with many other reasons in addition to presence of toxin ricin in the castor seed has hampered its suitability as an agronomic crop. Additionally, chemical processing is required for synthesis of biodegradable plasticizers like SOFT-N-SAFE$^{TM}$. The expression of *Ea*DAcT in combination with the synthesis of HFA will result in to an alternate, greener method of plasticizer production in transgenic plants.

#### 1.3.2.2 Acetyl-TAGs as lubricants

The use of vegetable oils as lubricants offers many advantages over petroleum-based products [16]. However, their reduced oxidative stability and poor low temperature properties hinders their use as lubricants. Modification of the fatty acid composition of the oils was tried to address these issues but improvement in one property often results in exacerbation of the other. For example, an increase in the saturation levels of the oil to improve its oxidative stability leads production of oils with higher pour and cloud points [4]. The lower freezing point of acetyl-TAGs compared to lcTAGs makes them suitable for overcoming this problem. The incorporation of saturated medium chain fatty acids in acetyl-TAGs could offer improved oxidative stability without negatively impacting cold temperature performance.

## 1.4 TAG Biosynthesis in Plants

An overview of the entire TAG biosynthetic pathway, from fatty synthesis to acylation of diacylglycerol, is presented in Chapter 4. Here I will discuss the enzymes involved in the final step of TAG and acetyl-TAG synthesis.

### 1.4.1 DGATs and PDAT

Diacylglycerol acyltransferases (DGATs) are the enzymes involved in the last step of triacylglycerol (TAG) biosynthesis and produce TAGs by transferring an acyl group from an acyl-CoA molecule to the *sn*-3 position of a diacylglycerol molecule [17]. DGATs are further classified in to DGAT1 and DGAT2 based on differences in their amino acid sequence.

The Arabidopsis DGAT1 enzyme was first discovered in three different studies by sequence homology studies to mammalian DGAT enzymes [18-20]. Since then, a large number of DGAT1 have been cloned from a variety of oil seed plants such as olive [21], canola [22], castor bean [23], soybean [24], tung tree [25], *Vernonia galamensis* [26], sunflower [27], *Xanthoceras sorbifolia* [28] and ornamental plants such as *Euonymus alatus* [29], *Tropaeolum majus* [30] and *Echium* [31].

The second class of DGAT in plants was first discovered in Arabidopsis by sequence homology studies to the fungus *Mortierella ramanniana* DGAT2 and was found to show very low sequence similarity to the DGAT1 [32]. Later on additional DGAT2 enzymes were isolated from castor bean [33], tung tree [25], *V. galamensis* [26] and olive [34].

Phospholipid : diacylglycerol acyltransferase (PDAT) like DGATs acts at the last step of TAG biosynthesis to acylate DAG at *sn*-3 position to form TAG. However, unlike DGAT, PDAT transfers the acyl group from the *sn*-2 position of a phospholipid molecule instead of an acyl-CoA. PDAT activity was first discovered in yeast and plants sunflower, castor bean and *Crepis palaestina* [35] but the encoding gene was first identified from Arabidopsis [36]. Later on three PDAT orthologs were identified in castor bean [37,38] and six orthologs were identified in flax [39].

### 1.4.2 Biochemical features and physiological roles of DGATs and PDAT

All the DGAT1 proteins generally contain 9-10 transmembrane domains with a highly hydrophobic N-terminal domain [40]. In contrast, DGAT2 enzymes in plants and other organisms are much shorter in length with only one or two transmembrane domains [17]. Tung

tree DGAT1 and DGAT2 were found to localize to different subdomains of ER although they have similar ER retrieval motifs [25]. DGAT1 activity was also reported in Arabidopsis leaf chloroplasts during senescence [41].

Similar to DGAT1 and DGAT2, PDAT1 is also predicted to be a membrane bound enzyme and is expressed in root, stem and leaves ([36]. The knockdown studies of DGAT1 and PDAT1 have shown their overlapping function in Arabidopsis seed oil synthesis [42]. However, PDAT1 can enhance fatty acid and TAG biosynthesis in leaves, but not in seeds, suggesting a more independent role of PDAT in leaves [43].

Detailed investigations on the substrate specificity of DGAT and PDATs are difficult to perform due to their membrane bound nature and difficulties associated with enzyme purification and substrate solubilization. Hence, assays are generally conducted using microsomal extracts from plants or from yeast expressing the enzymes. The acyl-CoA donor specificities of DGATs from major oil seed crops indicated that they are more specific for long chain acyl-CoAs [40]. DAG specificity studies were mostly done with exogenously added dipalmitin and diolein, hence little information is available on broad range of DAG specificity of these enzymes. Studies conducted using endogenous DAG in microsomal extract were performed for PDAT and DGAT. Castor DGAT2 has high specificity for DAGs containing ricinoleic acid [44] while *V. galamensis* DGAT2 resulted in higher levels of vernolic acids in petunia leaves and soybean seed compared to Vernonia DGAT1 [26]. The PDAT ortholog from castor RcoPDAT1A is specific for ricinoleic acid [37]and 4 functional PDATs out of total 6 PDATs from flax specifically transfer linolenic acid to TAGs [39]. Evidence from the substrate specificity studies, overexpression studies and studies on expression levels of DGAT1, DGAT2 and PDAT in the developing seeds of various plant species suggest that DGAT1 seems to be the major enzyme responsible for the TAG biosynthesis while DGAT2 and PDAT might be responsible for selective accumulation of TAGs with unusual fatty acids [37-39,45].

### 1.4.3 *Ea*DAcT and its biochemical features

The gene responsible for the synthesis of acetyl-TAGs was cloned from *Euonymus alatus* (Burning Bush) by a comparative transcriptomics approach [12]. The enzyme product of the gene was named *Euonymus alatus* diacylglycerol acetyltransferase (*Ea*DAcT). It is a member of the membrane-bound O-acyltransferease (MBOAT) family of enzymes which contains other important enzymes such as DGATs, wax synthases and sterol acyltransferases. MBOATs are

classified as enzymes with several membrane embedded regions and transfer organic acids, usually fatty acids, onto the hydroxyl groups of different kinds of substrates including lipids, proteins and polysaccharides. For example, they can acylate sterols, diacylglycerols and fatty alcohols (lipids), Wingless and ghrelin (proteins) and alginate and lipotechoic acid (polysaccharides) [46]. The family is further subdivided in to three subgroups with subgroup 1 containing enzymes involved in neutral lipid biosynthesis while subgroups 2 and 3 include enzymes involved in protein acylation and phospholipid remodeling respectively [47]. A histidine residue buried in between hydrophobic residues and an asparagine residue present in the hydrophilic region are very well conserved between all the family members and are believed to be active site residues [46,47]. Like other MBOATs, *Ea*DAcT is predicted to have several transmembrane domains based on the presence of several stretches of hydrophobic residues in its amino acid sequence. It synthesizes acetyl-TAGs by acetyl-CoA dependent acylation of DAG at the *sn*-3 position [12].

The substrate specificity studies of microsomal *Ea*DAcT conducted in yeast revealed that *Ea*DAcT could use acetyl-CoA but not oleyl-CoA as an acyl donor [12]. Information regarding the specificity of *Ea*DAcT for acyl-CoAs falling between chain length range of 2-18 carbons was not known. It was also shown that *Ea*DAcT could acetylate yeast endogenous DAGs containing 16 and 18 carbon long fatty acids and an exogenously added smaller chain DAG, 1,2-dihexanoin. However, there was no information available for activity of *Ea*DAcT towards other short and medium chain fatty acid containing DAGs.

It was also known from sequence alignment studies that *Ea*DAcT is more closely related to the Jojoba wax synthase and Arabidopsis sterol acyltransferases than to DGAT1 although it acylates same DAG substrate as DGAT1 [12,13]. Wax synthases and sterol acyltransferases acylate fatty alcohols and sterols respectively to produce their alkyl esters. Also, studies conducted on a number of *Euonymus* species showed the presence of alkyl acetates in their seeds [48] indicating that *Ea*DAcT might also have wax synthase activity.

In Chapter 2, a method to study substrate specificity of *Ea*DAcT by using rather inexpensive unlabeled acyl-CoA substrates and rapid quantification of assay product by using electrospray ionization mass spectrometry (ESI-MS) is described. In Chapter 3, the results from the substrate specificity studies of *Ea*DacT for various chain length acyl-CoA donor substrates and DAG/fatty alcohols acceptor substrates are presented. Chapter 4 is a detailed review on

advantages of Camelina as an oil seed crop with examples of its use for producing various industrially useful molecules and acetyl-TAGs. The research done in this study to further improve the fuel properties of acetyl-TAGs in Camelina, is presented in Chapter 5. At the end, overall conclusions from all these studies and future work are discussed.

# Chapter 2 - Rapid quantification of low-viscosity acetyl-triacylglycerols using electrospray ionization mass spectrometry

## 2.1 Introduction

3-acetyl-1,2-diacyl-*sn*-glycerol (acetyl-TAG) are unusual triacylglycerols (TAGs) with an acetate group at the *sn*-3 position instead of the typical long chain fatty acid. In nature, these molecules are abundantly present in the seeds of plant families in the Celastraceae, Balsaminaceae, Lardizabalaceae, Ranunculaceae and Rosaceae [6,7]. Small quantities of acetyl-TAG have also been discovered in animals and insects [9,10].

Acetyl-TAGs possess very different chemical and physical properties compared to regular TAGs. For example, they possess a lower viscosity and improved cold temperature properties [12,13]. These altered characteristics make acetyl-TAGs useful for different applications, including as an improved low-viscosity straight vegetable oil biofuel. The identification of the *Ea*DAcT acetyltransferase responsible for the synthesis of acetyl-TAG resulted in the generation of transgenic seeds capable of producing high levels of these useful molecules, suggesting a route to the agricultural production of acetyl-TAG [12,13]. To aid in efforts to increase acetyl-TAG levels, a rapid and accurate method to quantify these valuable storage lipids would be very useful. Currently, the quantification of acetyl-TAG from seed oil is based on their separation from other components of a total lipid extract using thin layer chromatography (TLC). The separated TAG fractions are then transmethylated and the subsequent fatty acid methyl esters quantified using gas chromatography. These methods are laborious and time consuming. They also do not provide any information on the types of TAG molecular species present.

In contrast, electrospray ionization mass spectrometry (ESI-MS) based techniques have become increasingly popular for the quantification of lipids, including TAG. The very different molecular masses of acetyl-TAG and regular TAG allow ESI-MS to easily distinguish these two types of TAG species without the need for chromatographic separation. Previously, we have used MS1 scans to successfully quantify acetyl-TAG [5], but found these methods could easily be confounded by the presence of other lipid species, which led to increased ion suppression and background noise. One way to overcome this problem is to quantify TAGs by performing scans for the neutral loss of a fatty acid. Such methods have been used to study relative quantification

of intact DAG and TAG species in mammalian cells [49] and for the quantitative profiling of Arabidopsis seed oil TAGs [50]. These particular methods suffer from the drawback that the neutral loss of each fatty acid needs to be scanned and accounted for in different molecular species. Thus, these methods require multiple scans and therefore tend to be time and computation intensive. However, because acetyl-TAG molecular species can all be detected by the neutral loss of the common *sn*-3 acetate group, these unusual TAG molecules can be quantified with a single scan.

Here, we extend previous neutral loss based methods by developing a method designed to specifically quantify acetyl-TAG. Because acetate represents the shortest fatty acid it was not clear whether the particular methods previously used for regular TAGs would still be valid. We also examined the effects of other fatty acids at the *sn*-3 position, as well as the position of the acetate group on the glycerol backbone. This allowed us to develop a rapid ESI-MS based method for the quantification of acetyl-TAGs. The method avoids the high signal background of MS1 based methods by only detecting acetate containing molecules. Also, because there is only a single scan for the neutral loss of acetate, the data analysis is relatively quick and straightforward. The method was validated by quantifying acetyl-TAG in different biologically relevant lipid samples. The increased sensitivity also allowed the quantification of acetyl-TAGs produced by small scale in vitro enzyme assays. This method therefore provides a rapid way to quantify acetyl-TAGs and other short acyl group containing TAGs.

## 2.2 Material and Methods

### 2.2.1 Synthesis of structured TAG standards

The TAG standards used in this study were synthesized from their respective phosphatidylcholine (PC) orthologs (Avanti polar lipids, Alabaster, AL) using a two-step method. In the first step, PC containing the desired *sn*-1/2 acyl composition was converted to 1,2-DAG using phospholipase C. Acetyl-TAG were subsequently synthesized by treating 1,2-DAG with acetic anhydride/pyridine (3:2) overnight at room temperature [51]. Other TAGs with different chain length *sn*-3 fatty acids were synthesized from 1,2-DAG using the appropriate acyl-chloride (Nu-Check Prep, Waterville, MN) [52]. All TAGs were purified from reaction mixtures using preparative TLC after which they were quantified by GC-FID and their purity confirmed using ESI-MS.

### 2.2.2 Culture condition and lipid preparation

The *Saccharomyces cerevisiae* quadruple knockout strain H1246 was kindly provided by Dr. Sten Stymne (Swedish Agricultural University) and transformed with the native *Ea*DAcT gene in the expression vector pYES-DEST52 [12]. *Ea*DAcT protein expression was induced by growing transformed yeast in selective minimal medium in the presence of galactose for 48 hours with a starting $O.D_{600}$ of 0.02. 30 ml samples were collected at 12h intervals and pelleted by centrifugation at 4000 rpm for 15 minutes. Cell pellets were washed with water to remove residual media and frozen at -20 ˚C until extraction. Lipids were extracted using a chloroform-methanol extraction method [12], resuspended in 500 µl of toluene and stored at -20 ˚C until further analysis.

### 2.2.3 Quantification of acetyl-TAG by gas chromatography

Total lipid extracts were separated on Silica gel 60 TLC plates (Merck, Kenilworth, NJ) using a hexane/diethyl-ether/acetic acid (70:30:1) solvent system and visualized by very brief exposure to iodine vapor. 5 µg triheptadecanoin (Nu-Check Prep, Waterville, MN) was added to the acetyl-TAG bands. Lipids were recovered by scraping the silica, extracting with 5 ml chloroform, drying under nitrogen and dissolving in 500 µl hexane. Acetyl-TAG were converted to their fatty acid methyl esters (FAMEs) using base-catalyzed transmethylation [53]. 50 µg of butylated hydroxytoluene was added to each sample before transmethylation to prevent oxidation. FAMEs were quantified using an Agilent gas chromatograph equipped with a HP-88 (0.25 mm x 100 m) column, a split/splitless injector and flame ionization detector. The carrier gas was helium with a flow rate of 16.4 ml $min^{-1}$. The oven temperature was maintained at 150 ˚C for 1.0 min and then ramped to 175 ˚C at 10 ˚C $min^{-1}$, kept there for 10 minutes, then ramped to 210 ˚C at 5 ˚C $min^{-1}$ and kept there for 4 minutes. FAMEs were identified by comparing their retention times with those in a standard mix.

### 2.2.4 Mass spectrometry analysis of TAG

The samples for mass spectrometry were prepared by dissolving the lipid extracts in 300 µl chloroform to obtain the desired final concentration (125 nM and 500 nM for TAG standard mixes and 1-2 µg/ml of acetyl-TAG purified from *Euonymous* oil or yeast lipid extract) and 700 µl of methanol: 300mM ammonium acetate, 100:5.26 (v/v). 150 nM (final concentration) 15:15:X TAG (where X=2, 4, 6 or 12) was spiked into samples as a normalization standard to

account for instrument variation. Samples were introduced by continuous infusion to the ESI-MS source on a triple quadrupole mass spectrometer (API4000, Applied Biosystem, Foster City, CA, USA). Samples were infused at 30 µl $min^{-1}$ with an autosampler (LC mini PAL, CTC Analytics, AG Zwingen, Switzerland) fitted with an appropriate loop for the acquisition time. TAG were detected and quantified by a series of neutral loss scans targeting the loss of specific fatty acids as neutral ammoniated fragments: NL77.10 (C2:0 for acetyl-TAG); NL105.1 (C4:0 for butyryl-TAG); NL133.2 (C6:0 for hexanoyl-TAG); NL217.3 (C12:0 for dodecanoyl-TAG). The scan speed was 100 µ$sec^{-1}$. The collision energy, with nitrogen in the collision cell, was +20 V; declustering potential was +100 V; entrance potential was +10 V and exit potential was +14 V. One hundred twenty continuum scans were averaged in multiple channel analyzer mode. For all analyses the collision gas pressure was set on 'low', and the mass analyzers were adjusted to a resolution of 0.7 unit full width at half height. The source temperature (heated nebulizer) was 100 ˚C; the interface heater was on; +5.5 kV was applied to the electrospray capillary; the curtain gas was set at 20 (arbitrary units); and the two ion source gases were set at 45 (arbitrary units).

### 2.2.5 Data processing and acetyl-TAG quantification

For acetyl-TAG analyses, the background of each spectrum was subtracted, data were smoothed, and peak areas were integrated using Applied Biosystems Analyst software. Peaks corresponding to the target lipids in these spectra were identified, deconvoluted for M+2 isotopic overlap and corrected for isotopic variation using an inhouse script. Because ionization efficiency is dependent on the total number of carbons and double bonds in the TAG molecule [54], adjustment factors were obtained by measuring the signal from different concentrations of TAG standard mixes. For example, acetyl-TAG standard mixes contained the following molecular species in equimolar quantities (125 nM or 500 nM each species): 3-acetyl-1,2-dipalmitoyl-*sn*-glycerol, 3-acetyl-1,2-distearoyl-*sn*-glycerol, 3-acetyl-1,2-dioleoyl-*sn*-glycerol, 3-acetyl-1,2-dilinoleoyl-*sn*-glycerol,and 3-acetyl-1,2-arachidoyl-*sn*-glycerol, with 3-acetyl-1,2-dipentadecoyl-*sn*-glycerol spiked at 150 nM. The relationship between signal intensity and concentration (referred to as "slope") was calculated for each TAG standard. Multiple linear regression was then used to determine the relationship between the slope and the number of carbons and double bonds. This relationship was then used to correct the signal for a particular TAG molecular species based on number of carbons and double bonds present in that molecule.

### 2.2.6 In vitro acetyltransferase assays

Microsomes were extracted from *S. cerevisae* strain H1246 expressing *Ea*DAcT and acetyltransferase assays performed as previously described [5]. [1-$^{14}$C] acetyl-CoA (Perkin Elmer Life Sciences, Waltham, MA) and [1,2-$^{13}$C] acetyl-CoA (Sigma-Aldrich, St. Louis, MO) were used for radiolabelled based and ESI-MS based assays, respectively. For the radiolabelled-based assay, acetyl-TAG were quantified by separating the lipid extracts from the assay using TLC, scraping the bands and quantifying radioactivity using scintillation counting.

## 2.3 Results and Discussion

### 2.3.1 Effect of number of carbons and double bonds on neutral loss signal intensity

The signal intensity for different TAG molecular species is correlated to aliphatic chain length and unsaturation index [54]. We were curious to see whether similar effects occurred for different acetyl-TAG molecular species, particularly when undergoing the neutral loss of acetate. We also wanted to study the effects of other different short fatty acids on neutral loss signal intensity. Combinations of structured TAGs sharing a common *sn*-3 acyl group but containing varying fatty acids at their *sn*-1 and *sn*-2 were synthesized (Table 2.1). Equimolar mixtures were

Table 2.1 **TAG with specific *sn*-3 acyl group synthesized during the study**

| Type of TAG | TAG molecular species* | | | | | |
|---|---|---|---|---|---|---|
| **Acetyl-TAG** | 15:0/15:0/2:0 | 16:0/16:0/2:0 | 18:0/18:0/2:0 | 18:1/18:1/2:0 | 18:2/18:2/2:0 | 20:0/20:0/2:0 |
| **Butyryl-TAG** | 15:0/15:0/4:0 | 16:0/16:0/4:0 | 18:0/18:0/4:0 | 18:1/18:1/4:0 | 18:2/18:2/4:0 | 20:0/20:0/4:0 |
| **Hexanoyl-TAG** | 15:0/15:0/6:0 | 16:0/16:0/6:0 | 18:0/18:0/6:0 | 18:1/18:1/6:0 | 18:2/18:2/6:0 | 20:0/20:0/6:0 |
| **Dodecanoyl-TAG** | 15:0/15:0/12:0 | 16:0/16:0/12:0 | 18:0/18:0/12:0 | 18:1/18:1/12:0 | 18:2/18:2/12:0 | 20:0/20:0/12:0 |

* The *sn* position of fatty acid is in ascending order from left to right. Fatty acid is represented as X:Y where X is the number of carbon atoms and Y represents number of double bonds.

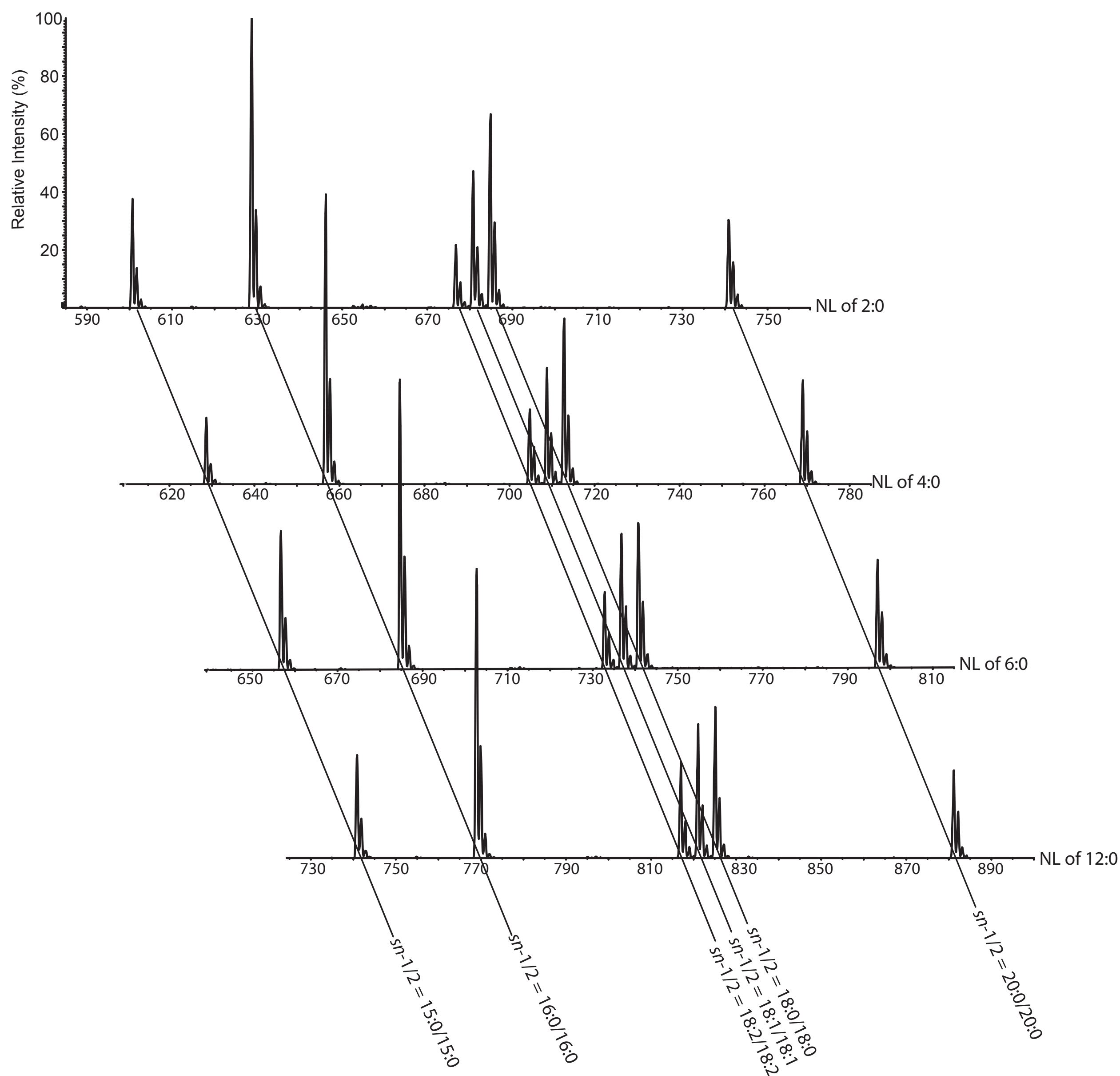



Figure 2.1 **The signal intensity for neutral loss of the *sn*-3 acyl group is dependent on the *sn*-1/2 acyl composition.** ESI-MS spectra from the neutral loss scans of four different TAG standard equimolar mixes (500 nM final concentration for each component). TAGs were detected by the neutral loss of the ammoniated *sn*-3 acyl groups common to their respective mixes. Peaks correspond to the *m*/*z* values of the $[M+NH_4]^+$ adduct. Diagonal lines indicate common *sn*-1/2 moieties. *sn*-3 specific acetate, butyrate, hexanoate and dodecanoate esters of 1,2-dipentadecanoyl-*sn*-glycerol were added as internal standards (150 nM final concentration) to their respective mixes.

analyzed using an ESI-MS neutral loss scan for the common *sn*-3 acyl group (Fig. 2.1). The signal for each molecular species was normalized to that of an 3-acyl-1,2-dipentadecoyl-*sn*-glycerol standard that was spiked to the same level in all the mixtures. The relationship between

signal intensity and concentration (referred to as slope) was calculated for each TAG species. Plotting this slope against the number of carbons at the *sn*-1 and *sn*-2 positions revealed a negative correlation, with larger fatty acids leading to a decrease in NL signal response (Fig. 2.2a). This trend remained consistent for the loss of different chain length *sn*-3 acyl groups varying in number of carbons from 2 to 12. Such an effect might be the result of better protection for the *sn*-3 group from loss during collision by the increase in the bulkiness of groups at the other two positions. A similar effect was observed for the neutral loss of palmitoyl and stearoyl ammonium adducts from TAG [50]. Increasing the total degree of unsaturation from 0 to 4 double bonds at the *sn*-1 and *sn*-2 positions also decreased the NL signal intensity (Fig. 2.2b). It was hypothesized that ammoniation at the double bonds decreases the dissociation of TAG during collision induced dissociation (CID) and produces abundant $MH^+$ ions rather than $DAG^+$ moieties [52]. Therefore more double bonds will result in less $DAG^+$ fragments and less NL signal. Overall, the NL signal intensity for the loss of *sn*-3 acyl group decreased with increases in the total number of carbons and the total degree of unsaturation for a TAG. This trend remains constant for all series of TAG containing *sn*-3 acyl groups with varying short and medium chain lengths (2 to 12 carbons).

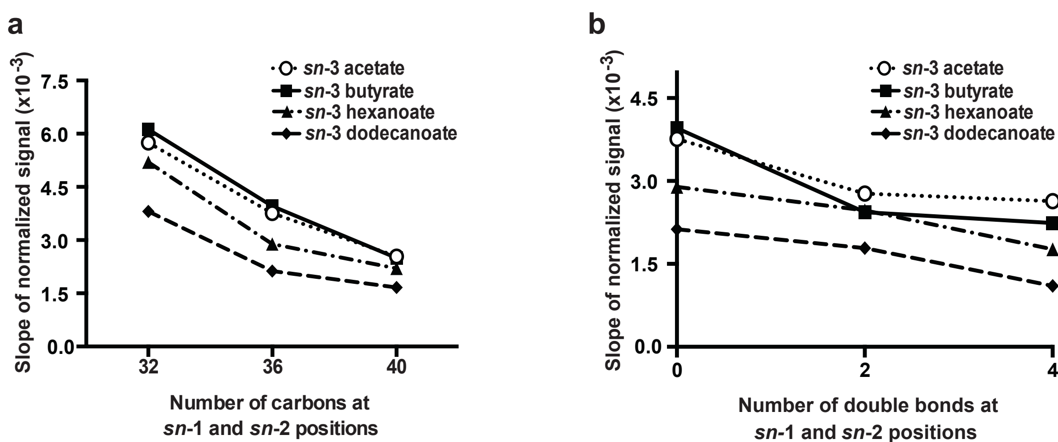



Figure 2.2 **The *sn*-1 and *sn*-2 acyl composition of TAG affects the signal intensity generated by the neutral loss of short chain fatty acids.** The relationship between concentration and the normalized signal from the neutral loss of the common *sn*-3 acyl group shared by a set of TAG molecules differing in their *sn*-1/2 acyl composition was plotted against the total number of carbons (a) and double bonds (b) contained in those *sn*-1 and *sn*-2 positions. The neutral loss signal was normalized to a 3-acyl-1,2-dipentadecanoyl-*sn*-glycerol standard that possessed the same *sn*-3 acyl group as the other TAG molecules in the set.

For TAG molecular species containing same kind of acyl groups at *sn*-1 and *sn*-2 positions, the NL of the *sn*-3 acyl groups tended to result in lower signal response as the size of the neutral loss fragment increased (Fig. 2.3). The only exception was the loss of butyrate for which a higher signal than acetate was recorded under the constant neutral loss scan parameters. This trend was influenced by the instrument settings, especially collision energy and changes in this parameter particularly affected this trend (data not shown). Given our interest in acetyl-TAGs, the instrument settings were optimized for the neutral loss of acetate and not for other acyl groups.

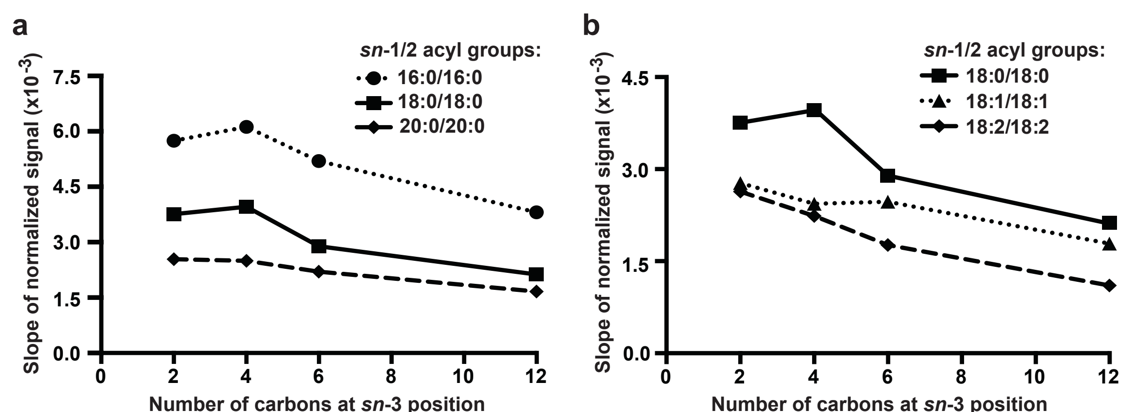



Figure 2.3 **The size of the *sn*-3 acyl group of TAG is negatively correlated with the neutral loss signal intensity**. The relationship between concentration and the normalized signal generated from the neutral loss of the common *sn*-3 acyl group shared by a set of TAG molecules was plotted against the total number of carbons at the *sn*-3 position. The neutral loss signal was normalized to a 3-acyl-1,2-dipentadecanoyl-*sn*-glycerol standard that possessed the same *sn*-3 acyl group as the other TAG molecules in the set. For clarity, data from sets of TAG molecules containing the same *sn*-1/2 acyl groups are separated according to varying numbers of acyl carbons (a) or double bonds (b).

### 2.3.2 Effect of position of acetate groups

It has previously been reported that the loss of the fatty acid from the *sn*-2 center position is unfavorable in CID of TAG [55,56] We were therefore interested to determine whether the position of a much smaller acetate group in acetyl-TAG also affects its neutral loss signal. Samples of 3-acetyl-1,2-dipentadecoyl-*sn*-glycerol or 2-acetyl-1,3-dipentadecoyl-*sn*-glycerol were spiked with an equal quantity of 3-acetyl-1,2-dipalmitoyl-*sn*-glycerol and subjected to a NL scan for the loss of acetate. At two different concentrations, the signal for the loss of acetate from

*sn*-3 position was found to be about three times higher than the signal for loss from *sn*-2 position (Fig. 2.4). This effect is probably due to the greater ease with which the peripheral *sn*-3 group

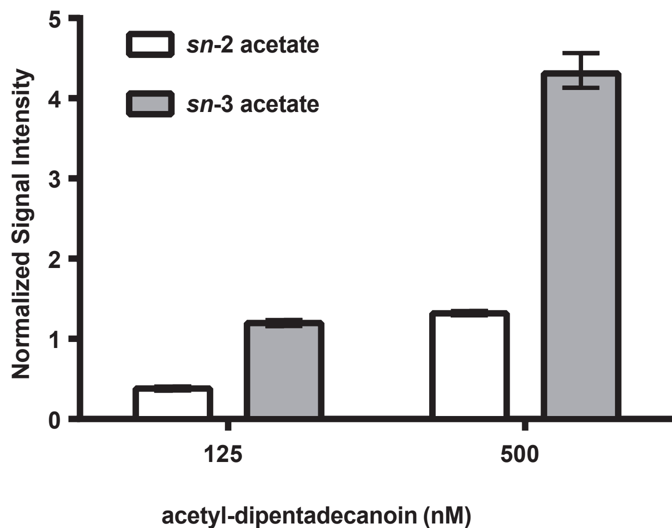



Figure 2.4 **The position of the acetate group affects its neutral loss signal intensity**. The signal intensity for the neutral loss of the acetate group from 3-acetyl-1,2-dipentadecanoyl-*sn*-glycerol or from 2-acetyl-1,3-dipentadecanoyl-*sn*-glycerol was normalized to that from an equimolar amount of 3-acetyl-1,2-dipalmitoyl-*sn*-glycerol present in both the samples.

can be dissociated from parent ion compared to the internal *sn*-2 acetate group which is shielded by bulkier acyl groups. Consequently higher fragmentation and therefore higher signal intensity of *sn*-3 acetate containing acetyl-TAG occurs than *sn*-2 acetate containing acetyl-TAG. The substrate specificity of the plant enzymes means that naturally produced acetyl-TAGs possess an *sn*-3 acetate group [6,12]. However, this result suggests that care should be taken when quantifying acetyl-TAG regioisomers using this neutral loss scan procedure.

### 2.3.3 ESI-MS neutral loss scan based quantification of purified acetyl-TAG

The predictable relationship between the number of carbons and double bonds at the *sn*-1 and *sn*-2 positions in a specific acetyl-TAG molecular species and the signal intensity from the neutral loss of the *sn*-3 acetate group suggested we could use this method to quantify more complex mixtures of acetyl-TAGs. To determine the accuracy of the new quantification method it was tested on acetyl-TAG purified from *Euonymus alatus* seed oil. An 1mg/ml acetyl-TAG stock was analyzed using the newly developed ESI-MS method and compared to the results obtained using standard GC-FID approach. The total acetyl-TAG content was calculated as sum of the different acetyl-TAG molecular species present in *Euonymus* oil (Fig. 2.5). The 0.97 ±

0.01mg/ml value obtained by GC-FID did not differ significantly from the 0.93 ± 0.02mg/ml value obtained using our ESI-MS analysis (Student's *t*-test, $P = 0.106$).

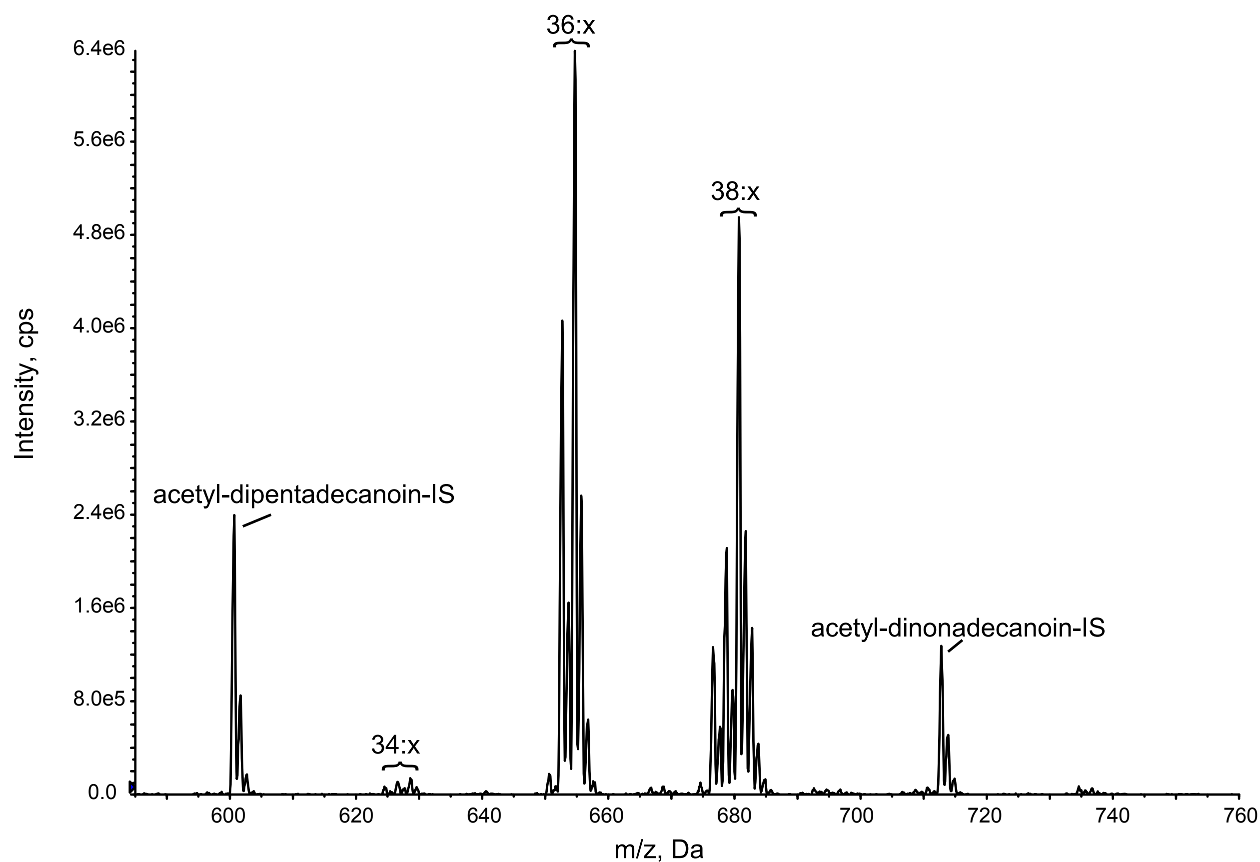



Figure 2.5 **Positive ESI mass spectrum generated by scanning for the neutral loss of ammonium acetate from acetyl-TAGs purified from *Euonymus alatus* seed oil.** Peaks correspond to the *m/z* values of the $[M+NH_4]^+$ adduct. The number of acyl carbons in each series of TAG molecules is indicated; for clarity the number of double bonds (x) is not defined. 3-acetyl-1,2-dipentadecanoyl-*sn*-glycerol and 3-acetyl-1,2-dinonadecanoyl-*sn*-glycerol were added as internal standards

### 2.3.4 Quantification of acetyl-TAG in yeast lipid extracts

To further test this ESI-MS based quantification method in a relevant biological setting, we quantified the production of acetyl-TAG in a TAG deficient yeast strain transformed with *Ea*DAcT. The subsequent acetyl-TAG producing yeast possessed a typical sigmoidal shaped growth curve. The exponential phase started after a lag phase of 12 h and lasted for 24 h after which the cells entered stationary phase. Lipids were extracted from cells collected at different time intervals spread across all three growth phases. The acetyl-TAG content was quantified

using both our new ESI-MS based method or transmethylation followed by GC-FID. Acetyl-TAG accumulation reached its peak by 24 h and declined sharply once the yeast entered the

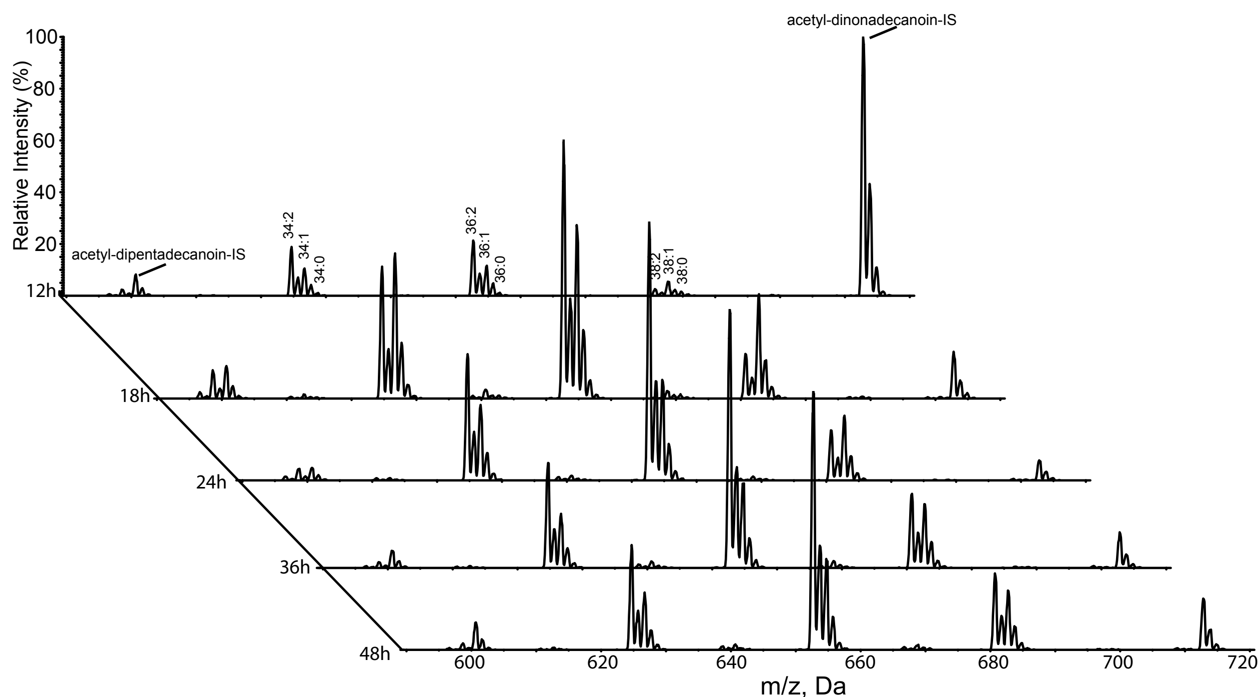



Figure 2.6 **The acetyl-TAG molecular species composition of yeast expressing *Ea*DAcT does not change appreciably in different growth stages.** Positive ESI mass spectra from the neutral loss scan of ammonium acetate of lipids extracted from yeast expressing EaDAcT cultured for different lengths of time. Peaks correspond to the *m/z* values of the $[M+NH_4]^+$ adduct of the intact acetyl-TAG molecule. The number of acyl carbons in each series of TAG molecules is indicated; for clarity, the number of double bonds (x) is not defined. 3-acetyl-1,2-dipentadecanoyl-*sn*-glycerol and 3-acetyl-1,2-dinonadecanoyl-*sn*-glycerol were added as internal standards.

stationary phase (Fig. 2.7). The acetyl-TAG molecular species were dominated by molecules containing two double-bonds consistent with the highly monounsaturated nature of the fatty acids found in yeast [57,58]. The acetyl-TAG molecular species composition changed slightly over the course of the different stages of culture, with a slight increase in the molecular species containing two double bonds at the cost of decrease in species containing one double bond (Fig. 2.6). Importantly, there were no significant differences between the amounts of acetyl-TAG quantified from total lipid extracts using ESI-MS or using GC-FID quantification (Fig. 2.7). Therefore the ESI-MS based method can be used to accurately and rapidly quantify acetyl-TAGs from biological samples.

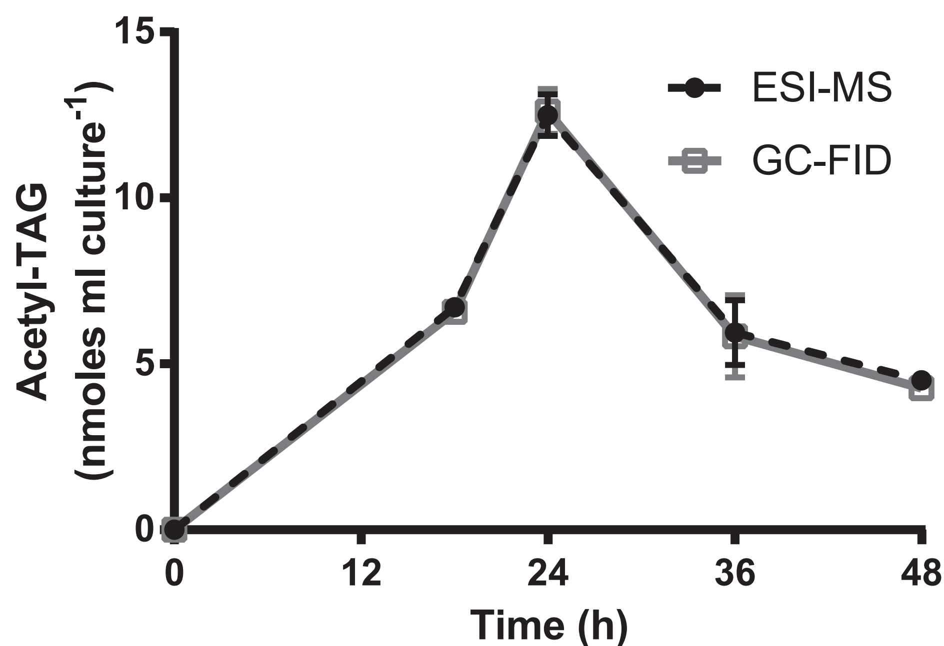



Figure 2.7 **The ESI-MS based method provides accurate quantification of acetyl-TAG produced in yeast.** Total lipids were extracted from *S. cerevisiae* H1246 expressing *Ea*DAcT at different time points and acetyl-TAG content was determined using GC-FID or ESI-MS. Data represents the mean ± SD of three independent experiments. There was no significant difference between values obtained from GC-FID and ESI-MS (Student's *t*-test, 18h: $P = 0.30$; 24h: $P = 0.81$; 36h: $P = 0.89$; 48h: $P = 0.17$.

### 2.3.5 Comparison of ESI-MS analysis based DGAT assay with the radiolabeled substrate based assay

Having demonstrated the ability to quickly and accurately quantify acetyl-TAGs, we wanted to determine whether the method was sensitive enough to accurately quantify the acetyl-TAG products of an in vitro reaction. In the past we have used radiolabeled methods to study some of the in vitro biochemical properties of the *Ea*DAcT acetyltransferase enzyme responsible for the synthesis of acetyl-TAGs [12]. However, an ESI-MS based method would significantly reduce the time required for the assay compared to a radioactivity based quantification. To demonstrate that the newly developed ESI-MS based method can give results comparable to the traditional radioactive based methods, we performed the two assays from the same batch of microsomes at varied concentration of acetyl-CoA, either unlabeled or [$^{14}$C] labeled. Unexpectedly, the microsomes obtained from yeast expressing *Ea*DAcT possessed high levels of acetyl-TAGs, even when no acetyl-TAGs were added (Fig. 2.8). Presumably, the relatively more polar acetyl-TAGs are able to be incorporated into membranes after synthesis via *Ea*DAcT. The in vitro

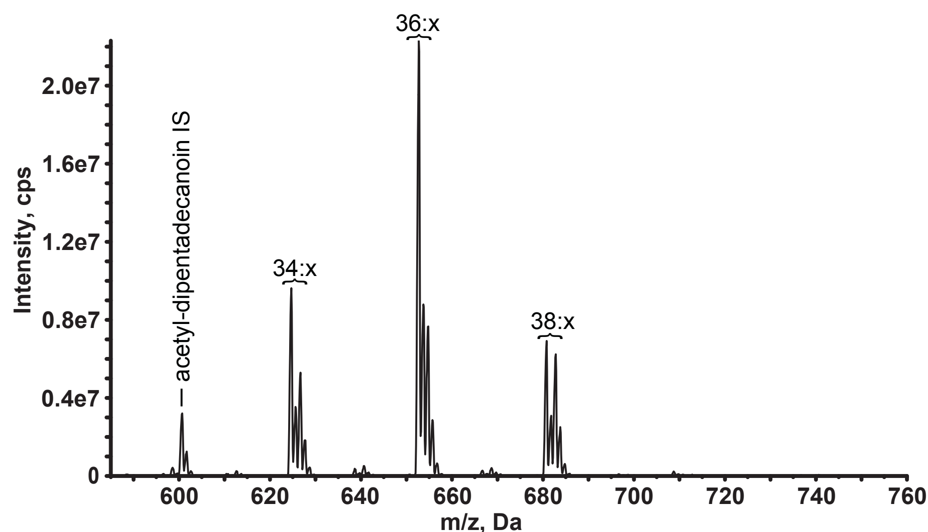



Figure 2.8 **Microsomes isolated from yeast expressing *Ea*DAcT contain acetyl-TAG**. Positive ESI mass spectrum from scanning for the neutral loss of ammonium acetate from lipids extracted from microsomes of yeast expressing *Ea*DAcT. Peaks correspond to the *m*/*z* values of the $[M+NH_4]^+$ adduct of the intact acetyl-TAG molecule. The number of acyl carbons in each series of TAG molecules is indicated; for clarity, the number of double bonds (x) is not defined. 3-acetyl-1,2-dipentadecanoyl-*sn*-glycerol was added as internal standard.

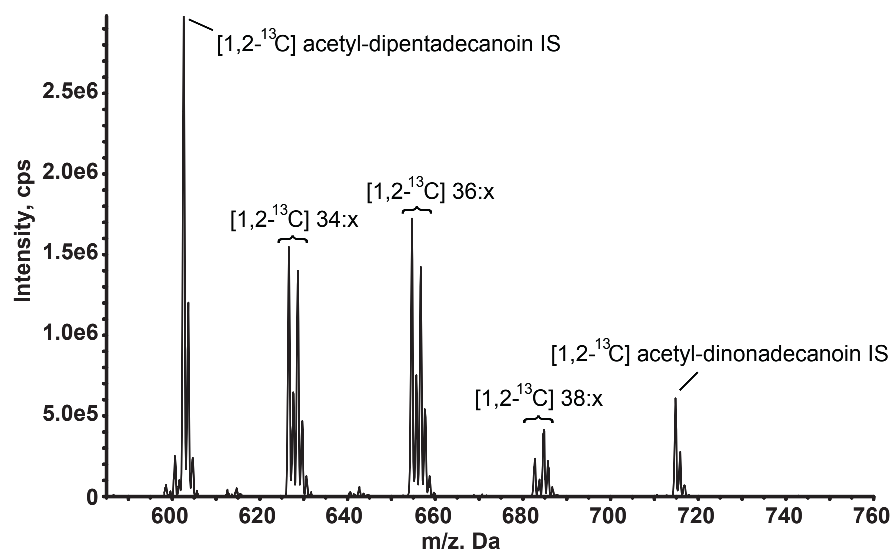



Figure 2.9 **Microsomes containing *Ea*DAcT incubated with [1,2-$^{13}$C]acetyl-CoA produce acetyl-TAG with 2 mass units heavier than endogenous acetyl-TAGs**. ESI mass spectrum from the neutral loss of [$^{13}$C] ammonium acetate from total lipids extracted from an in vitro *Ea*DAcT activity assay using [1,2-$^{13}$C] acetyl-CoA as a substrate. Peaks correspond to *m*/*z* values of the $[M+NH_4]^+$ adduct of the intact acetyl-TAG molecule. The number of acyl carbons in each series of TAG molecules is indicated; for clarity, the number of double bonds (x) is not defined. [1,2-$^{13}$C] 3-acetyl-1,2-dipentadecanoyl-*sn*-glycerol and [1,2-$^{13}$C] 3-acetyl-1,2-dinonadecanoyl-*sn*-glycerol were added as internal standards.

reaction did not produce enough additional acetyl-TAGs that could be correctly estimated above the endogenous background. More importantly however, these results demonstrate that the ESI-MS assay is a rapid and accurate substitute for the more time consuming radiolabeled based assay for quantification of in vitro produced acetyl-TAGs that could be correctly estimated above the endogenous background in the ESI-MS based assay (data not shown). To overcome this,

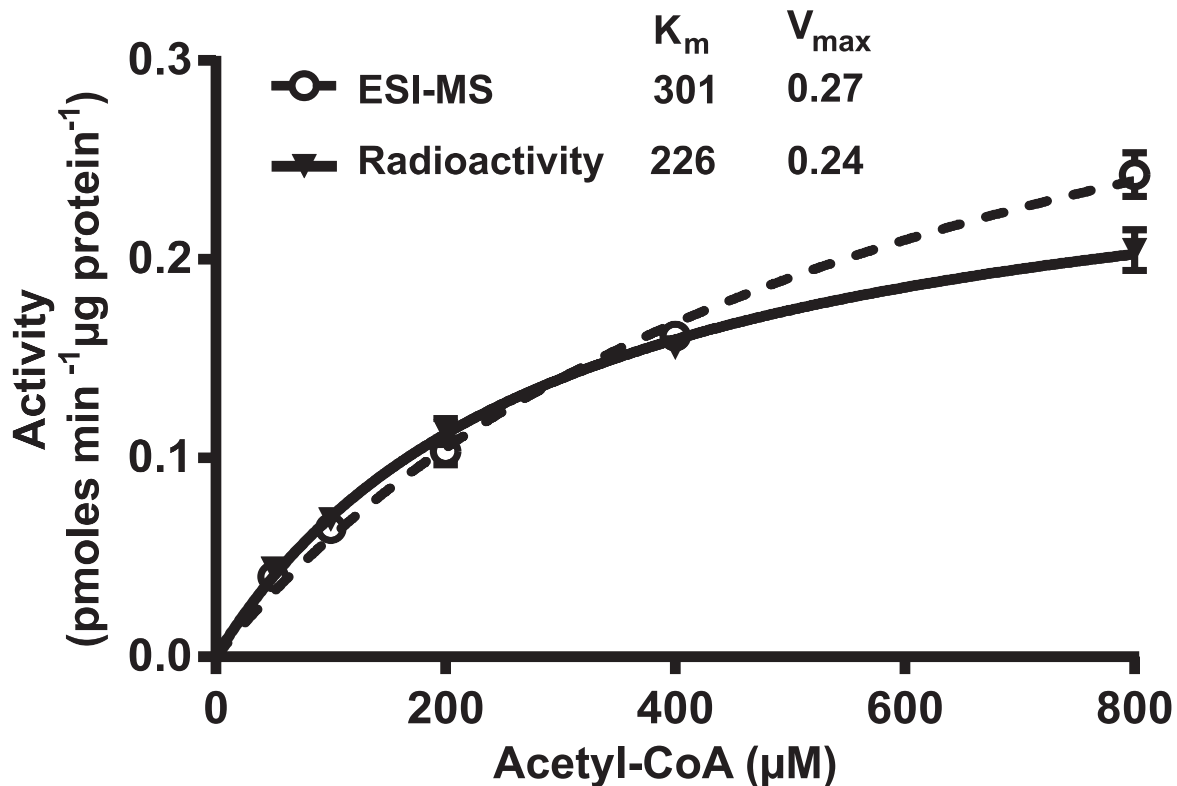



Figure 2.10 **The ESI-MS based method is as effective in quantifying in vitro enzyme activity**. Acetyltransferase enzyme assays were performed from the same batch of microsomes under similar conditions using [1-$^{14}$C] acetyl-CoA for the radioactive assay and [1,2-$^{13}$C] acetyl-CoA for the ESI-MS analysis based assay. Activity values are expressed as the mean ± SD (n=3) of three assays. Curves were fitted using Graphpad Prism (Michaelis-Menten non-linear regression).

[1,2-$^{13}$C] labeled acetyl-CoA was used for the ESI-MS based assay. This enabled us to specifically quantify the [$^{13}$C] acetyl-TAG produced during the assay over endogenous background of normal acetyl-TAG by scanning for the neutral loss of the heavier [$^{13}$C]-labeled acetate group (Fig. 2.9). The two assays yielded similar enzyme activity curves (Fig. 2.10) resulting in similar apparent $K_m$ and $V_{max}$ values. The $K_m$ value is high given that the reported concentration of acetyl-CoA in plants and yeast are in range of 5 to 30 µM [59,60]. One

explanation is that microsomes are relatively crude enzyme preparations and therefore contain other enzymes, many of which could also utilize acetyl-CoA. Indeed, problems about thioester hydrolases present in microsomes have long been noted [61]. Therefore, the interference present from other acetyl-CoA utilizing proteins in microsomes might have caused this shift in $K_m$ value. More importantly however, these results demonstrate that the ESI-MS assay is a rapid and accurate substitute for the more time consuming radiolabeled based assay for quantification of in vitro produced acetyl-TAG.

## 2.4 Conclusions

We developed an ESI-MS based method for the absolute quantification of TAGs containing acetate and other short acyl groups. In order to achieve this, we determined the effect of the acyl group composition on the neutral loss signal, something which has never been performed for short fatty acids. The method compared well to traditional methods of quantification and was sensitive enough to quantify the products of a small scale in vitro enzyme reaction. Because this new method involves the direct infusion of total lipids, it eliminates the need for the laborious process of acetyl-TAG fractionation from other lipid components before quantification. Further, it provides information about the amount of different molecular species. ESI-MS based quantification therefore represents a useful tool with which to understand the production of acetyl-TAGs in transgenic plants, as well as to better study the substrate specificity of unusual acetyltransferases such as *Ea*DAcT.

# Chapter 3 - Defining the substrate specificity of *Ea*DAcT: a key to altering acetyl-TAG fatty acid composition

## 3.1 Introduction

The enzyme *Euonymus alatus* diacylglycerol acetyltransferase (*Ea*DAcT) produces acetyl triacylglycerols (acetyl-TAGs) by transferring an acetyl group from acetyl-CoA to the *sn*-3 position of diacylglycerol (DAG) acyl acceptor substrates [12]. *Ea*DAcT belongs to the membrane-bound O-acyltransferase (MBOAT) family of enzymes, which catalyze the acyl-CoA dependent acylation of hydroxyl groups present on small hydrophobic substrates such as cholesterol and diacylglycerols, as well as small peptides and larger protein substrates [46,47]. *Ea*DAcT is unusual in that it uses the shortest possible acyl-CoA and not longer chain CoAs. EaDAcT acylates a common DAG substrate as other important MBOAT enzyme diacylglycerol acyltransferase 1 (DGAT1) to synthesize triacylglycerol (TAG). Surprisingly, phylogenetic studies of *Ea*DAcT and other MBOATs revealed its unusually close relationship with wax synthases and sterol acyltransferases instead of DGAT1 [12].

*Ea*DAcT can also produce alkyl acetates by esterifying the acetate group onto a fatty alcohol. *Ea*DAcT was shown to produce medium chain alkyl acetates, which act as insect pheromone-like compounds [62]. These small and medium chain alkyl esters can also be useful as direct use biodiesel [63] and in various industrial applications such as flavors, fragrances, solvents, cosmetics and surface coating agents [64].

Acetyl-TAGs possess different properties than usual long chain triacylglycerols (lcTAGs) due to the presence of the shortest possible acyl group at the *sn*-3 position. The different chemical properties of acetyl-TAGs compared to lcTAGs, such as reduced kinematic viscosity and lower freezing temperatures makes them suitable candidate for a reduced viscosity straight vegetable oil biofuel [13,65]. The physical and chemical properties of acetyl-TAGs and alkyl acetates is dependent on their molecular species composition, which in turn is dependent on two factors: first, the availability of different acyl donors and acceptor substrates to the enzyme and second, the substrate specificity of *Ea*DAcT to utilize those substrates. Therefore, the knowledge of the substrate specificity of *Ea*DAcT will be helpful in achieving a desired acetyl-TAG/alkyl

acetate molecular species composition profile in an oil seed crop. In this study yeast was chosen as a model system to study the enzyme characteristics of *Ea*DAcT because of problems obtaining fresh *E. alatus* seeds year around to extract the enzyme. The other advantage was the availability of a yeast strain deficient in TAG production, which meant a smaller number of DAG utilizing side reactions and low background for the ESI-MS analysis of longer acyl-CoA reactions. Therefore, *Ea*DAcT was overexpressed in yeast and the microsomal fraction was used for conducting substrate specificity assays.

Here we describe the acyl donor and acceptor substrate specificity of *Ea*DAcT. We found that in addition to acetyl-CoA, *Ea*DAcT could also use other short chain length acyl-CoAs as acyl donors, though it had high preference for acetyl-CoA. It was also found that *Ea*DAcT can effectively esterify acetyl groups to its acyl acceptor substrates *sn*-1,2-DAGs and fatty alcohols with highly variable chain lengths to make acetyl-TAGs and alkyl acetates respectively. Moreover, *Ea*DAcT showed high preference for DAGs with greater unsaturation levels and fatty alcohols with medium chain lengths. Overall, our results suggest that *Ea*DAcT is a valuable enzyme for producing a variety of desired acetyl-TAGs and alkyl acetates.

## 3.2 Materials and Methods

### 3.2.1 DGAT assay for *Ea*DAcT

Microsomes were extracted from *S. cerevisae* strain H1246 expressing *Ea*DAcT and DGAT assays were performed as previously described [12]. Radiolabeled and ESI-MS based methods of detection were same as described in Chapter 2.

#### 3.2.1.1 Optimization of DGAT assay conditions

Optimization studies were performed using radiolabeled acetyl-CoA. For the protein concentration optimization, total microsomal protein concentration varying from 20 µg to 100 µg per assay was used. Similarly, for incubation time optimization, 80 µg total microsomal protein was used per assay and incubated for different time periods varying from 5 minutes to 45 minutes. An [1-$^{14}$C] acetyl-CoA (Perkin Elmer Life Sciences, Waltham, MA) concentration of 125 µM and incubation temperature of 30 $^{o}$C was used for both the optimization studies.

#### 3.2.1.2 Acyl-CoA specificity assays

Unlabeled acetyl-CoA, butyryl-CoA, hexanoyl-CoA, octanoyl-CoA, decanoyl-CoA, dodecanoyl-CoA, tetradecanoyl-CoA, hexadecanoyl-CoA and octadecenoyl-CoA (Sigma-Aldrich, St. Louis, MO) were used for ESI-MS based assays. A 10 mM stock in sodium acetate buffer (pH 5.5) was prepared for each unlabeled acyl-CoA and stored as aliquots at -80 $^{o}$C. For acyl-CoA chain length specificity assays, a final concentration of 250 µM unlabeled acyl-CoAs varying in acyl chain length from 2 to18 was achieved by appropriate dilution in final reaction mixture (100 µl final volume). After the assay, 3-acyl-1,2-dipentadecanoin internal standard was added before lipid extraction to achieve a final concentration of 250 nM in 600 µl of chloroform. TAGs formed during the reaction were quantified using ESI-MS neutral loss scan of the *sn*-3 acyl group. The operating conditions of mass spectrometer and data processing were same to as described in Chapter 2. The processed signal obtained for different TAG molecular was quantified by normalization to the internal standard.

For detailed kinetic studies on acetyl-CoA, butyryl-CoA and hexanoyl-CoA, assays were conducted with different concentrations of acyl-CoAs varying from 50 µM to 1500 µM with the same batch of microsomes. [1,2-$^{13}$C] acetyl-CoA was used instead of unlabeled acetyl-CoA to remove the background signal as explained in Chapter 2. 3-acyl-1,2-dinonadecanoin was added as an extraction standard and 3-acyl-1,2-dipentadecanoin was added as a technical standard to achieve final concentrations of 100 nM and 125 nM in the 600 µl of chloroform. TAG molecular species for all the three acyl-CoA were quantified using the ESI-MS neutral loss scan analysis of *sn*-3 acyl-group after correction using slope calculated from the TAG standard curves as described in detail in Chapter 2.

#### 3.2.1.3 DAG specificity assay

Yeast microsomes containing *Ea*DAcT were incubated with three different concentrations (200 µM, 400 µM and 800 µM) of [1,2-$^{13}$C] acetyl-CoA and acetyl-TAGs species formed from the endogenous DAGs were quantified using ESI-MS. The endogenous *sn*-1,2-DAGs from the same batch of yeast microsomes were isolated by separation of total lipid extract of microsomes on Silica gel 60 TLC plates (Merck, Kenilworth, NJ) impregnated with borate, using a chloroform/acetone (80:10) solvent system and visualized by staining with 2,7-dichlorofluorescein. These DAGs were then chemically acetylated using acetic anhydride and

pyridine [66] to determine their concentrations using the ESI-MS based quantification method for acetyl-TAGs (Chapter 2).

For testing *Ea*DAcT activity towards specific DAGs, microsomes were supplemented with 1,2-dilauroyl-*sn*-glycerol, 1,2-dimyristoyl-*sn*-glycerol and 1,2-distearoyl-*sn*-glycerol. 20 mM stocks of 1,2-dilauroyl-*sn*-glycerol and 1,2-dimyristoyl-*sn*-glycerol (Cayman Chemical Company, Ann Arbor, MI) were prepared in ethanol while the stock (20 mM) of 1,2-distearoyl-*sn*-glycerol was prepared in toluene. The reaction was started by adding 2 µl of DAG stock in the final assay volume of 100 µl (final concentration = 400 µM). Microsomes without the exogenous addition of DAGs were used as controls.

For determining in vivo DAG specificity, yeast expressing *Ea*DAcT was grown in synthetic medium with galactose (2 % w/v) for 24 hours. Total lipids were extracted from 50 ml of culture medium using a chloroform-methanol extraction [12]. *sn*-1,2-DAGs and acetyl-TAGs were separated by TLC using the chloroform/acetone (80:10) solvent system. The bands were visualized by staining with 2,7-dichlorofluorescein. 3-acetyl-1,2-dinonadecanoin (100 pmoles) was added at each spot and bands were scraped to extract the acetyl-TAGs and *sn*-1,2-DAGs. DAGs were then chemically acetylated as described above to analyze them as acetyl-TAGs using the developed ESI-MS neutral loss scan based method.

### 3.2.2 Fatty alcohol acetyltransferase assay of *Ea*DAcT

Alcohol acetyltransferase assays were conducted using radiolabeled [1-$^{14}$C] acetyl-CoA substrate. Oleyl alcohol was used for all the optimization studies for which a 1.0 mM stock was made in toluene and stored at -20 $^{o}$C. DMSO was used as a solubilizing agent for fatty alcohols. The desired volume of fatty alcohol stock was added at the bottom of the tube and toluene was evaporated under nitrogen. DMSO (15 µl) was added over the fatty alcohol and tube was gently vortexed to dissolve the alcohol. Microsomes (20 µl = 80 µg total protein) were added and the tube was incubated on ice for 5 minutes. DGAT reaction buffer (60 µl) (pH 7.4) was added to the tube to make a total reaction volume of 100 µl and the components were gently mixed. The reaction was started by adding 5 µl of [1-$^{14}$C] acetyl-CoA (final concentration = 125 µM). Lipids were extracted using hexane-isopropanol extraction and separated on Silica gel 60 TLC plates using a hexane/diethyl-ether/acetic acid (70:30:1) solvent system with oleyl acetate as a reference standard. The alkyl acetate product was estimated by counting radioactivity incorporated in to band corresponding to oleyl acetate standard.

Fatty alcohols varying in chain length from 8 to 22 were purchased from Nu-Chek Prep Inc. (Waterville, MN). Stocks (1.0 mM) were made in toluene and kept at -20 $^{o}$C. A final concentration of 125 µM in 100 µl assay mixture was used for in vitro chain length specificity assay of *Ea*DAcT.

### 3.2.2.1 Optimization of alcohol acetyltransferase assay conditions

Surfact-Amps detergent sampler (Fisher Scientific, Pittsburgh, PA) consisting of a number of ionic (sodium deoxycholate), non-ionic (NP-40, octyl beta-thioglycoside, octyl beta-glycoside, Brij-35 and Brij-58) detergents was used to enhance solubility of fatty alcohols in alcohol acetyltransferase assay. Stocks were made in water by dilution of original 10 % (w/v) stock and used at required concentration in the assay in place of DMSO. Assay was conducted as mentioned above.

### 3.2.2.2 In vivo assay for alcohol acetyltransferase activity

The plasmid pPT534 harbouring *Apis mellifera* fatty acid reductase 1(*Am*FAR1) in yeast expression vector pESC-URA ([67] was kindly provided by Dr Xiao Qiu (University of Saskatchewan). Hemagglutinin (HA) tagged *Ea*DAcT open reading frame (ORF) was amplified from pYES2/CT-*Ea*DAcT-HA using the primers 5'-ATGCGGCCGCGATGGATGCTCATCAAGAGATCAAG-3' and 5'-GGAAGATCTCACAAATCCCATGTAGGA-3' and digested with NotI and BglII. The amplified fragments were cloned into corresponding sites of yeast expression vector pESC-URA and pPT534 to obtain the pESC-URA-*Ea*DAcT-HA single expression and pESC-URA-*Am*FAR1-*Ea*DAcT-HA coexpression vectors respectively. The *Saccharomyces cerevisiae* quadruple knockout strain H1246 was transformed with empty vector pESC-URA, pPT534, pESC-URA-*Ea*DAcT-HA and pESC-URA-*Am*FAR1-*Ea*DAcT-HA. The transformants were grown in synthetic medium-ura-trp with 2% (w/v) galactose at 30 $^{o}$C under shaking at 225 rpm for 72 hours to obtain an $O.D_{600}$ of approximately 3.2-3.4. The yeast cells were harvested from 50 ml culture and lypholized to calculate their dry weight. Lipids were extracted using a chloroform-methanol extraction method [12]and resuspended in 1.0 ml of toluene.

### 3.2.2.3 Analysis of fatty alcohol and alkyl acetates

For fatty alcohol analysis, 300 µl of the total lipid extract was used. Heptadecanol (100 nmoles) was added as an internal standard and lipids were transmethylated in the presence of

5.0% (v/v) methanolic $H_2SO_4$ at 90 °C for 1.5 hour. NaCl (1.5 ml) was added to stop the reaction and total FAMEs and fatty alcohols were extracted twice using 2 ml of hexane. The organic phase was evaporated under nitrogen. For TMS derivatization of fatty alcohols, 100 µl of BSTFA and 100 µl of pyridine were added to tubes. Tubes were sealed with screw caps under nitrogen and heated at 110 °C for 10 minutes. Solvent was evaporated under $N_2$ and samples were suspended in 200 µl of hexane to be analyzed using GC-MS.

For alkyl acetate analysis, 80 µl of total lipid extract was separated into different lipid components using Silica gel 60 TLC plates with a hexane/diethyl-ether/acetic acid (70:30:1) solvent system and oleyl acetate as a standard. Pentadecyl acetate (8.5 nmoles) was added to the bands corresponding to oleyl acetate. Bands were scraped and lipids were extracted using hexane and analyzed using GC-MS.

FAMEs, fatty alcohols and alkyl acetates were quantified using an Agilent GC-MS system equipped with a DB-5ms (0.25 mm x 60 m) column, a split/splitless injector and a single quad mass spectrometer. The injector was operated in splitless mode with an injection volume of 2.0 µl. The carrier gas was helium with a total flow rate of 9.0 ml $min^{-1}$. The oven temperature was maintained at 60 ˚C for 2.0 min and then ramped to 200 ˚C at 50 ˚C $min^{-1}$, kept there for 1.0 minute, then ramped to 280 ˚C at 10 ˚C $min^{-1}$ and kept there for 3 minutes. The mass quad was maintained at 150 °C and source temperature was 230 °C. The detector was set to detect fragment ions with a mass range between 50-650. FAMEs, fatty alcohols and alkyl acetates were identified by matching their fragmentation patterns against reported spectras present in NIST database. The quantification was performed by normalization to internal standard and reported as µg per mg dry weight of yeast.

## 3.3 Results

### 3.3.1 Optimization of yeast microsomal system for *Ea*DAcT activity assay

Before studying substrate specificity, the optimum conditions for diacylglycerol acetyltransferase (DAcT) activity of microsomal *Ea*DAcT were determined. The optimum value for incubation temperature (30 °C) and pH (7.5) were known from previous studies and used for further assay optimization. The reaction was found to be linear with increasing total microsomal protein concentration between 20 to 80 µg (Fig. 3.1a). Hence, to keep the reaction in a linear range for kinetic assays and still get maximum product formation within the detection range

of the instrument, a concentration of 80 µg total protein/per assay was selected for further assays. With a total microsomal protein concentration of 80 µg, the acetyl-TAG product formation increased sharply for first 5 minutes after which acetyl-TAGs accumulated slowly and reached steady state in 20 minutes (Fig. 3.1b).

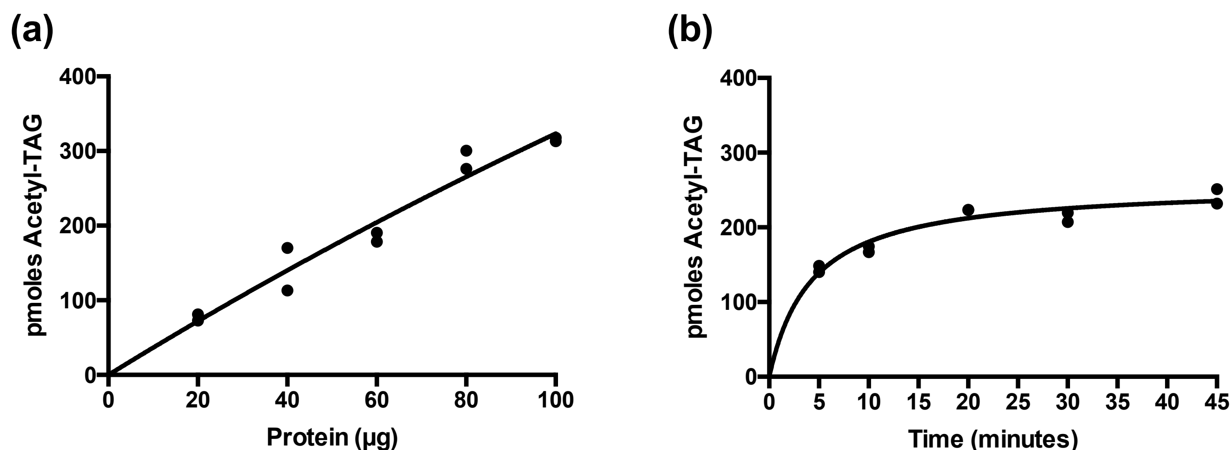



Figure 3.1 **Optimization of in vitro DAcT assay conditions for microsomal *Ea*DAcT.** In vitro assays were conducted using microsomal fractions from yeast expressing *Ea*DAcT and incubated with [1- $^{14}$C] acetyl-CoA (125 µM) at 30 $^{o}$C. [1- $^{14}$C] acetyl-TAG product was determined by quantifying the radioactivity in acetyl-TAG band. A. Effect of the amount of total microsomal protein on acetyl-TAG formed in 25 minutes of incubation. B. Effect of different incubation times on the amounts of acetyl-TAG formed using 80 µg total microsomal protein.

The calculation of initial reaction velocity for each substrate concentration is recommended for doing the Michaelis-Menten analysis mainly to avoid the effects of reverse reaction for conversion of enzyme substrate (ES) complex to enzyme and substrate. The possibility of this occurring can be neglected due to the following reasons: 1) a large substrate to product ratio at all times due to low product accumulation and 2) low thermodynamic feasibility of reconstitution of acetyl-CoA from acetate and free CoA. Therefore, to maximize product accumulation, an incubation time of 25 minutes was selected to do the analysis under the steady state conditions.

### 3.3.2 *Ea*DAcT has high preference for acetyl-CoA as acyl donor

The acyl-CoA preference of *Ea*DAcT was first tested for different chain length acyl-CoAs varying in carbon chain length from 2 to18 carbon atoms using an ESI-MS based assay. Only acyl-CoA with acyl chain length up to 6 carbons showed detectable product levels (Fig. 3.2).

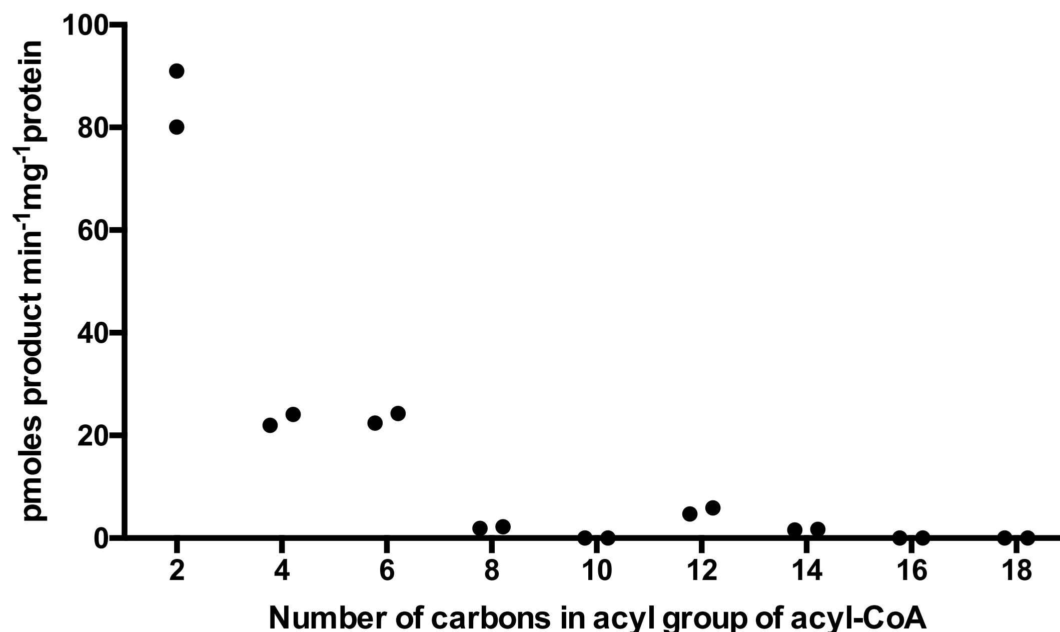



Figure 3.2 ***Ea*DAcT has negligible activity for higher chain length acyl-CoA**. In vitro assays for microsomal *Ea*DAcT were performed with equal amounts of unlabeled acyl-CoA of different acyl chain lengths. Reaction products were quantified using ESI-MS based analysis for the neutral loss scan of *sn*-3 acyl group. TAG reaction products were calculated by normalizing to the neutral loss signal of a 3-acyl-1,2-dipentadecanoyl-*sn*-glycerol standard that possessed the same *sn*-3 acyl group as the other TAG molecules.

The activity of *Ea*DAcT for acetyl-CoA was approximately 4 times higher compared to other short chain acyl-CoAs such as butyryl-CoA and hexanoyl-CoA. *Ea*DAcT used butyryl-CoA and hexanoyl-CoA with equal efficiency. However, *Ea*DAcT showed negligible activity for acyl-CoA with acyl chain longer than 8 carbon atoms. These results are consistent with the previous results where no *Ea*DAcT activity was observed for oleyl-CoA [12]. This result clearly distinguishes *Ea*DAcT from plant DGAT1 enzymes that have high preference for long chain acyl-CoAs and negligible activity for short chain acyl-CoAs [40].

Further detailed kinetic studies were conducted using acetyl-CoA, butyryl-CoA and hexanoyl-CoA. The microsomal *Ea*DAcT followed Michaelis -Menten kinetics for all the three acyl-CoAs (Fig. 3.3). The $K_m$ value was found be surprisingly high for the acetyl-CoA (590.4 µM) followed by hexanoyl-CoA (347.2 µM) and butyryl-CoA (75.11 µM) (Table 3.1). One possible reason for this might be lower substrate availability to enzyme either due to rapid degradation of acetyl-CoA by thioesterases [61] and/or competition from other acetyl-CoA

Table 3.1 **Enzyme kinetic parameters for different acyl-CoA substrates for microsomal *Ea*DAcT**

| Acyl-CoA | $K_m$ (µM) | $V_{max}$ (pmoles $min^{-1}$) | $V_{max}/K_m$ ( ml $min^{-1}$) |
|---|---|---|---|
| Acetyl-CoA | 590.4 | 30.8 | 0.52 |
| Butyryl-CoA | 75.1 | 6.4 | 0.85 |
| Hexanoyl-CoA | 347.2 | 5.7 | 0.16 |

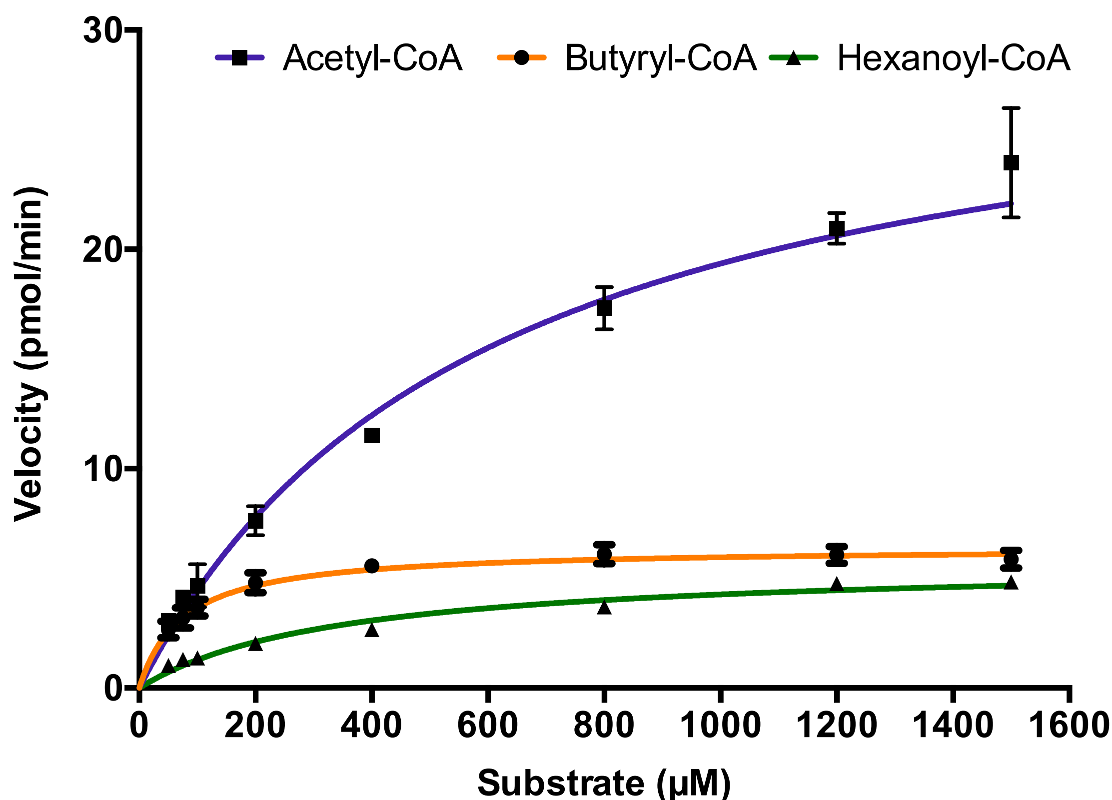



Figure 3.3 ***Ea*DAcT has a high preference for acetyl-CoA**. Enzyme activity curves for *Ea*DAcT exposed to different acyl-CoA donor substrates. Assays were performed from the same batch of microsomes under similar conditions using [1,2-$^{13}C_2$] acetyl-CoA, butryl-CoA or hexanoyl-CoA. Reaction products were quantified using ESI-MS by normalization to a 3-acetyl-1,2-pentadecanoin internal standard. Curves were fitted using Michaelis-Menten non-linear regression fit using Graph-pad Prism software. Values are expressed as mean ± SD (n=3).

utilizing reactions occurring simultaneously in the microsomes. The increasing incorporation of acetyl-CoA into non-specific products was noted in the radioactive assays with increasing acetyl-CoA concentrations (data not shown). Thus, higher overall acetyl-CoA concentrations in the assay mixture would be needed to reach actual $V_{max}$ and $V_{max}/2$, resulting in higher apparent $K_m$. $K_{cat}$ value could not be calculated due to inability to measure the actual enzyme concentration. As the same amount of total protein was used for all the assays, instead of calculating specificity

constant $K_{cat}/K_m$, the $V_{max}/K_m$ value was used to determine the specificity. As a consequence of the higher $K_m$ value of acetyl-CoA, $V_{max}/K_m$ of acetyl-CoA was little higher than that for butyryl-CoA (Table 3.1). However, both the overall $V_{max}$ values and average velocity ($V_{av}$) values for any given substrate concentration were substantially higher for acetyl-CoA than for butyryl-CoA and hexanoyl-CoA (Table 3.1 and Fig. 3.3). This demonstrates that *Ea*DAcT preferentially utilizes acetyl-CoA to esterify an acetyl group on to the *sn*-3 position of DAG compared to other higher chain length acyl-CoAs.

### 3.3.3 DAG acyl acceptor substrate specificity of *Ea*DAcT

Information about *Ea*DAcT's preference for DAG is important in order to synthesize acetyl-TAG with desired fatty acid composition at the *sn*-1/2 positions. The two important parameters that are crucial to chemical and physical properties of acetyl-TAG are chain length and saturation indices of constituent fatty acids. Previous studies have shown that *Ea*DAcT could esterify a DAG containing short chain fatty acids [12]. Hence, to further determine the preference of *Ea*DAcT for DAG with different chain length fatty acid and saturation indices, in vivo and in vitro studies were conducted in yeast as the model system.

#### 3.3.3.1 *Ea*DAcT has high preference for unsaturated fatty acid containing DAGs in vitro

Detailed in vitro kinetic studies to determine different chain length DAG specificity for any membrane bound enzyme are difficult to perform due to the variable solubility of hydrophobic substrates containing different chain length fatty acids in the aqueous buffer solution. Because of this, the result of any specificity assay using different chain length DAGs will be dependent on both the actual enzyme specificity and accessibility of DAG substrates to the enzyme. We attempted three approaches to achieve equal accessibility of DAG substrates in a microsome based assay system. The first was to remove all the endogenous DAG from the microsomes and supply exogenous DAG to the enzyme by solubilizing them into microsomes. To achieve this, microsomes were washed with acetone to remove DAGs. Though this strategy successfully removed most of DAG, the enzyme lost its activity (data not shown). The second strategy was to purify *Ea*DAcT by solubilization of microsomes and reconstitution into liposomes. However, the solubilization of *Ea*DAcT using different detergents and its reconstitution in to lecithin liposomes also resulted in to loss of enzyme activity. The third strategy was to express the enzyme in a cell free translation system in presence of liposomes and then provide equivalent amount of DAGs in liposomes. Here, a commercially available

translation system containing all the components of protein synthesizing machinery of wheat germ was used. This system does not contain any membranes for insertion of membrane proteins and hence was supplemented with lecithin liposomes to incorporate the membrane protein. In this system, the protein was expressed very well but the activity assay did not result in any product formation, probably due to improper folding of enzyme during reconstitution in to liposomes.

Therefore, to determine DAG preference of *Ea*DAcT, we performed selectivity assays instead of specificity assays. These assays were performed by providing different DAGs at equivalent concentrations all in a single assay instead of using one substrate at a time. The enzyme preference of different DAGs can then be determined by comparing their relative incorporation in to acetyl-TAG product.

DAG molecular species containing monounsaturated 16 and 18 carbon fatty acids were found to be the dominant species in yeast microsomes (Fig 3.4a). In vitro selectivity assays were performed for these DAG species using different concentrations of [1,2-$^{13}$C] labeled acetyl-CoA. The concentration of acetyl-TAG species formed during the reaction was very low (100-200 pmoles) compared to corresponding DAG species initially present in microsomes (µmoles) (Fig 3.4 a and b). The low amount of acetyl-TAG synthesis in the assay might be due to factors such as utilization of acetyl-CoA in side reactions of other proteins in microsomes, partial degradation of added acetyl-CoA and low accessibility of microsomal DAGs to the enzyme. Although the initial amounts of DAG molecular species with same number of carbons with two double bonds was lower than DAGs with one double bond, incorporation of the former into acetyl-TAGs was significantly higher than the latter for all the different concentrations of acetyl-CoA (Fig 3.4 a and b). These results indicate that *Ea*DAcT preferentially acetylates DAG species with higher unsaturation levels than those with lower saturation levels. The DAG species containing only saturated fatty acids were not incorporated at all in acetyl-TAGs (Fig. 3.4b). This might be be due to their very low abundances in microsomes (Fig 3.4a), due to low preference of *Ea*DAcT for these DAGs or a combination of both effects.

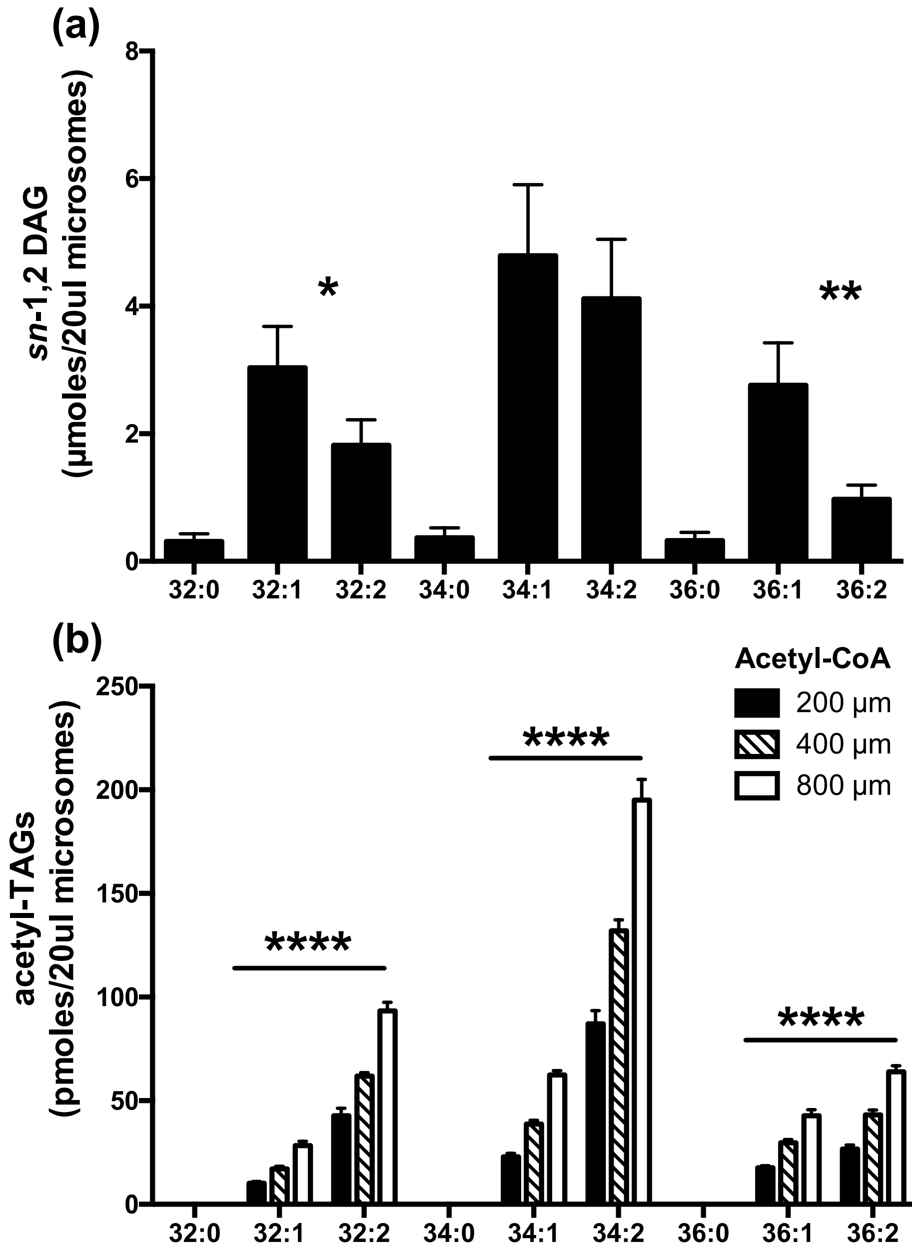



Figure 3.4 ***Ea*DAcT has high preference for unsaturated fatty acid containing DAGs in vitro**. Graphs representing the amount of different molecular species of (a) *sn*-1,2-DAG initially present in microsomes and (b) acetyl-TAGs formed during the reaction in vitro. The acetyl-TAGs and *sn*-1,2-DAGs were analyzed from same batch of microsomes from yeast expressing *Ea*DAcT grown for 24 h in synthetic medium with galactose. Asterisks represent significant difference between levels of either DAG species or acetyl-TAG molecular species (* $p<0.05$; unpaired *t*-test). Values are expressed as mean ± SD (n=3 for DAGs and n=6 for acetyl-TAG).

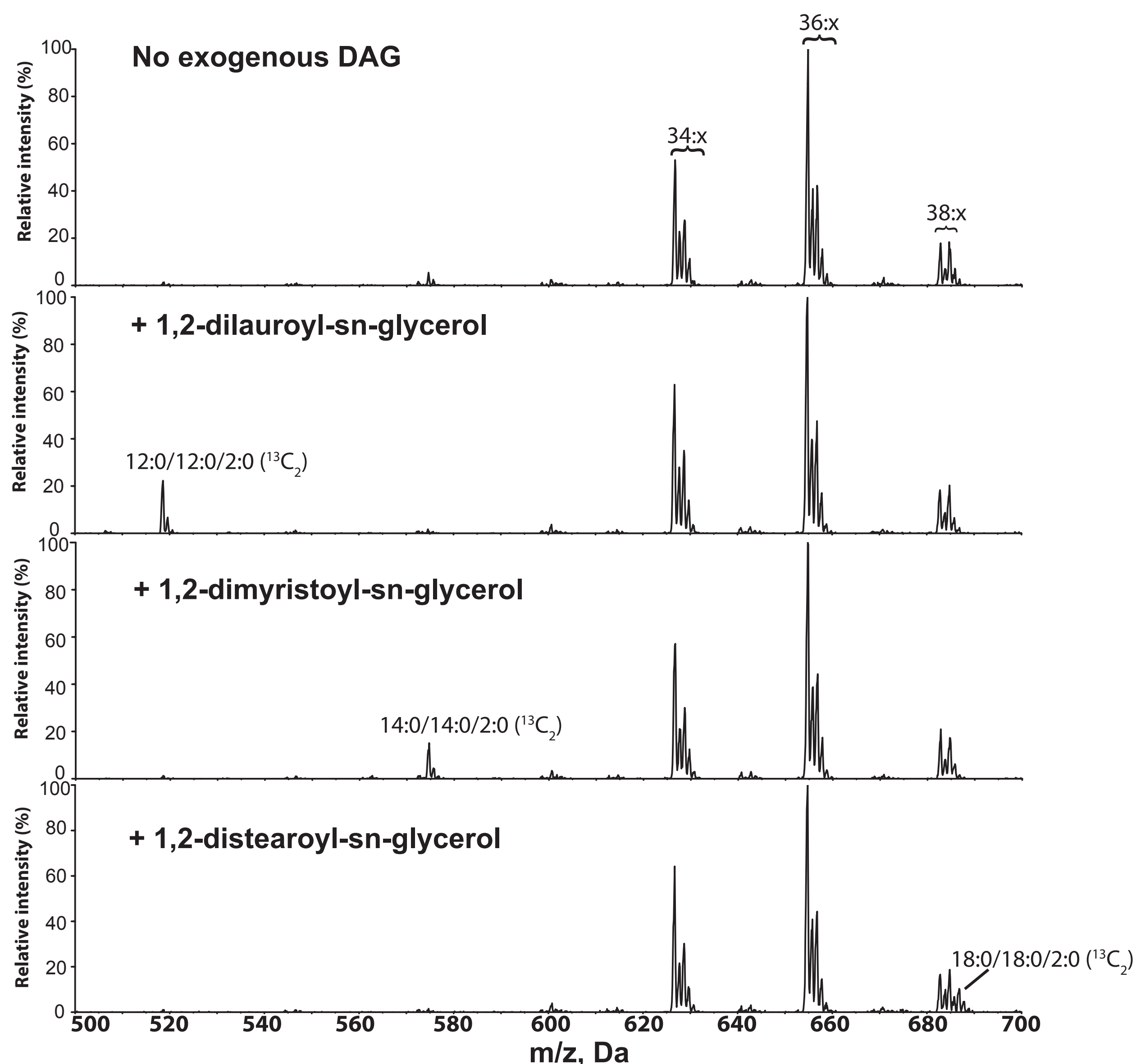



Figure 3.5 ***Ea*DAcT can acetylate *sn*-1,2-DAGs containing different chain length fatty acids.** Positive ESI mass spectra obtained from the neutral loss of [1,2-$^{13}C_2$] ammonium acetate from total lipids extracted from an in vitro *Ea*DAcT activity assay using [1,2-$^{13}$C] acetyl-CoA incubated with or without any exogenous *sn*-1,2-DAGs as substrates. Peaks correspond to *m*/*z* values of the $[M+NH_4]^+$ adduct of the intact acetyl-TAG molecule. The number of acyl carbons in each series of TAG molecules is indicated; for clarity, the number of double bonds (x) is not defined.

### 3.3.3.2 *Ea*DAcT can acetylate *sn*-1,2-DAGs with medium chain length fatty acids in vitro

One of goal of my work is to synthesize low molecular weight acetyl-TAGs by incorporating medium chain length fatty acids (MCFA) to lower their viscosity further (see chapter 5). However, no previous information regarding *Ea*DAcT preference for DAG

containing MCFAs was available. Hence *Ea*DAcT activity for DAGs containing MCFA was tested in yeast microsomes containing EaDAcT.

Very low amounts of DAG species containing the MCFAs lauric acid (12:0) and myristic acid (14:0) were also noted in the microsomes probably due to low amounts of 12:0 and 14:0 fatty acids in the yeast [58,68]. To increase the concentration of DAGs containing MCFA, 1,2-dilauroyl-sn-glycerol and 1,2-dimyristoyl-sn-glycerol DAGs were added to the microsomes, but levels equivalent to endogenous DAGs could not be achieved (data not shown). Hence, selectivity assays comparing DAGs containing MCFAs with endogenous DAGs were not possible. Therefore, qualitative in vitro assays using microsomes were done to demonstrate activity of *Ea*DAcT towards *sn*-1,2-DAGs containing medium chain fatty acids. The ESI-MS spectra showed peaks corresponding to the presence of 3-acetyl-1,2- dilauroyl-*sn*-glycerol and 3-acetyl-1,2- dimyristoyl-*sn*-glycerol when microsomal *Ea*DAcT was incubated with exogenous DAGs containing lauric acid and myristic acid respectively (Fig. 3.5). This indicates that *Ea*DAcT could esterify *sn*-1,2-DAGs containing lauric acid, myristic and stearic acid. These results suggest that *Ea*DAcT should be able to synthesize MCFA containing acetyl-TAGs in oil seed crops.

#### 3.3.3.3 *Ea*DAcT can acetylate *sn*-1,2-DAGs containing an *sn*-2 acetyl group

To test the effect of the presence of another acetate group in a DAG on *Ea*DAcT activity, we tested a DAG with an acetate group at *sn*-2 position. The results showed that even a DAG with an acetate group at *sn*-2 position could be acetylated by *Ea*DAcT at *sn*-3 position (Fig. 3.6).

The results from these in vitro studies along with results from previous study [12] proved that *Ea*DAcT could potentially acetylate any *sn*-1,2-DAGs containing saturated and unsaturated fatty acids with chain length varying from 2-18. However, *Ea*DAcT did not show any activity towards *sn*-1 MAGs, *sn*-2 MAGs or *sn*-1,3-DAGs (data not shown). These results suggest that *Ea*DAcT is highly specific for esterifying acetyl group to only the *sn*-3 positions of an *sn*-1,2-DAG acceptor substrates.

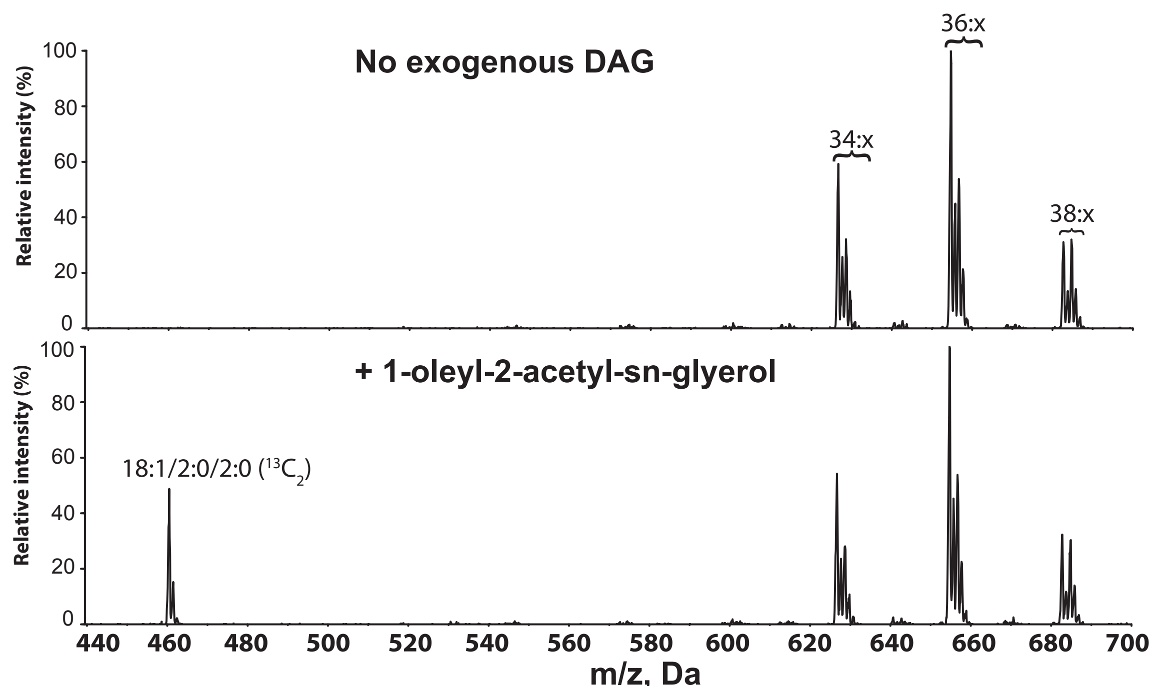



Figure 3.6 ***Ea*****DAcT can acetylate *sn*-1,2-DAGs containing an *sn*-2 acetyl group.** Positive ESI mass spectra obtained from the neutral loss of [1,2-$^{13}C_2$] ammonium acetate from total lipids extracted from an in vitro *Ea*DAcT activity assay using [1,2-$^{13}$C] acetyl-CoA incubated with or without any exogenous 1-oleyl-2-acetyl-sn-glycerol as substrates. Peaks correspond to *m*/*z* values of the $[M+NH_4]^+$ adduct of the intact acetyl-TAG molecule. The number of acyl carbons in each series of TAG molecules is indicated; for clarity, the number of double bonds (x) is not defined.

### 3.3.3.4 *Ea*DAcT can acetylate *sn*-1,2-DAGs with MCFAs in yeast

The ability of of *Ea*DAcT to acetylate DAGs with different chain lengths in vivo was tested in the quadruple knockout yeast strain H1246 that lacks endogenous TAG production [69]. The total lipid fraction of this yeast strain overexpressing *Ea*DAcT was analyzed using ESI-MS neutral loss scan to detect acetyl-TAGs and residual DAGs. The DAGs extracted from the yeast were chemically acetylated to convert them to corresponding acetyl-TAG, which can be easily quantified using our established ESI-MS neutral loss scan based method.

The results showed that 16 and 18 carbon fatty acid containing acetyl-TAGs molecular species were the most abundant molecular species in yeast. Acetyl-TAG species with MCFAs (12 and 14) were also present in lipid extract, but in very small amounts (Fig. 3.7a and c).

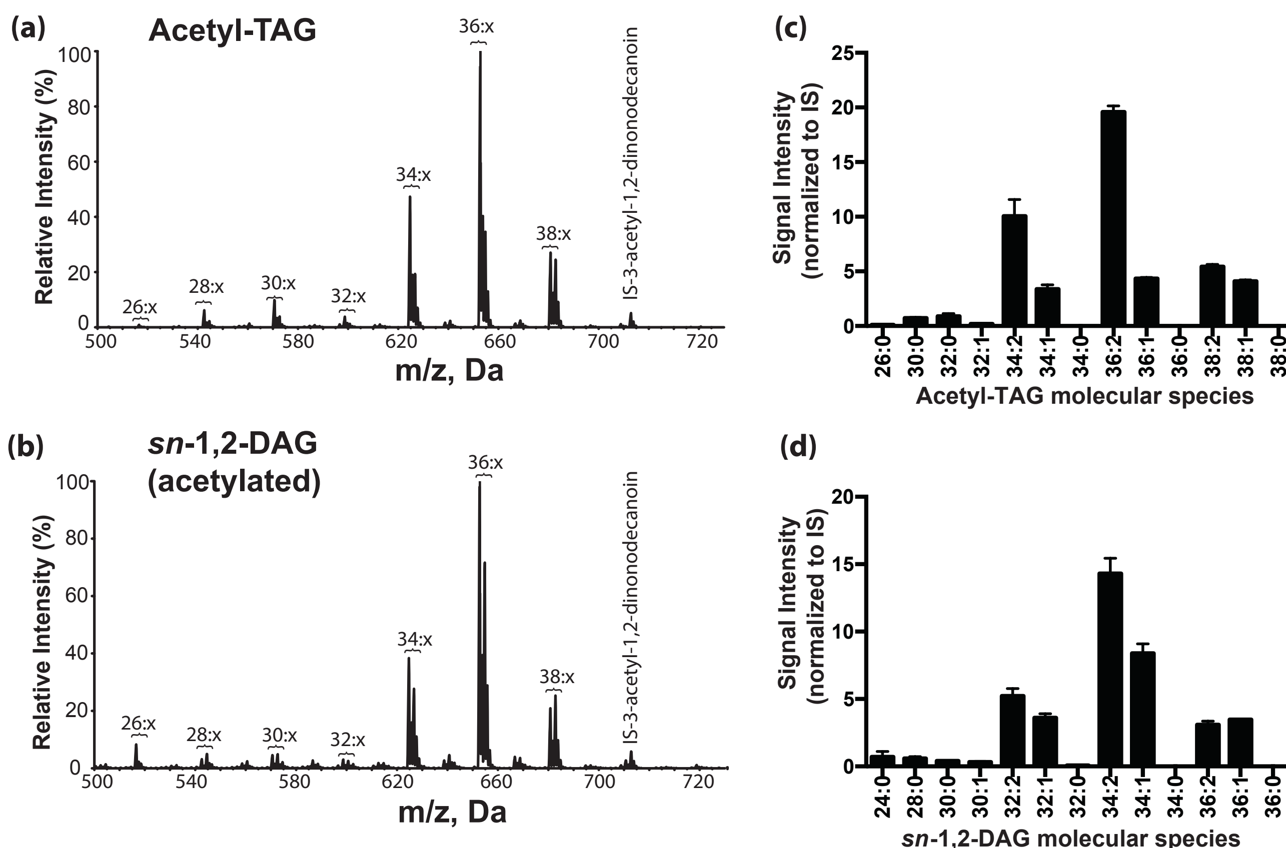



Figure 3.7 **The abundance of an acetyl-TAG molecular species and its corresponding *sn*-1,2-DAG in vivo**. Positive ESI mass spectra generated by scanning for the neutral loss scan of ammonium acetate from the acetyl-TAG (a) or chemically acetylated *sn*-1,2-DAG fraction (b) from yeast expressing *Ea*DAcT. Peaks correspond the to *m*/*z* values of the $[M+NH_4]^+$ adduct of the intact acetyl-TAG or chemically acetylated *sn*-1,2-DAG molecule. The number of acyl carbons in each series of TAG molecules is indicated; for clarity, the number of double bonds (x) is not defined. Quantification of different acetyl-TAG molecular species (c) and chemically acetylated *sn*-1,2-DAG molecular species (d) was done by normalizing their signal to 3-acetyl-1,2-dinonadecanoyl-*sn*-glycerol internal standard signal spiked to same amounts in both fractions. DAG and acetyl-TAGs molecular species are represented as X:Y, where X represents the total number of carbons and Y represents the number of double bonds. Values are expressed as mean ± SD (n=3).

A similar trend was observed for the amounts of long and medium chain fatty acid containing DAGs. The relatively low amounts of MCFA-containing acetyl-TAGs and DAGs might be due to the relatively low amount of C12:0 and C14:0 fatty acids compared to long chain fatty acids in yeast [58,68]. However, these results indicate that *Ea*DAcT can acetylate a variety of *sn*-1,2-DAGs containing medium and long chain fatty acids in vivo. These results are consistent with

the in vitro results and again emphasize that *Ea*DAcT could be used for producing low molecular weight acetyl-TAGs in plants.

#### 3.3.3.5 *Ea*DAcT preferentially acetylates DAGs with high saturation indices in vivo

The estimation of DAG selectivity of *Ea*DacT based on the amounts of corresponding acetyl-TAG species in this in vivo system would not be accurate due to large variability in amounts of *sn*-1,2-DAGs available to *Ea*DAcT (Fig 3.7). Hence, a normalized value of acetyl-TAG molecular species to the corresponding residual *sn*-1,2-DAGs species was calculated to determine relative selectivity of *Ea*DAcT for endogenous DAGs. A higher acetyl-TAG/*sn*-1,2-DAG ratio for a particular DAG species would be directly correlated to greater conversion of that DAG to its corresponding acetyl-TAG species and hence is a better substrate for *Ea*DAcT compared to other DAGs. Results of analysis of acetyl-TAG/*sn*-1,2-DAG ratio for abundant acetyl-TAG molecular species showed higher activity of *Ea*DAcT towards DAGs with higher

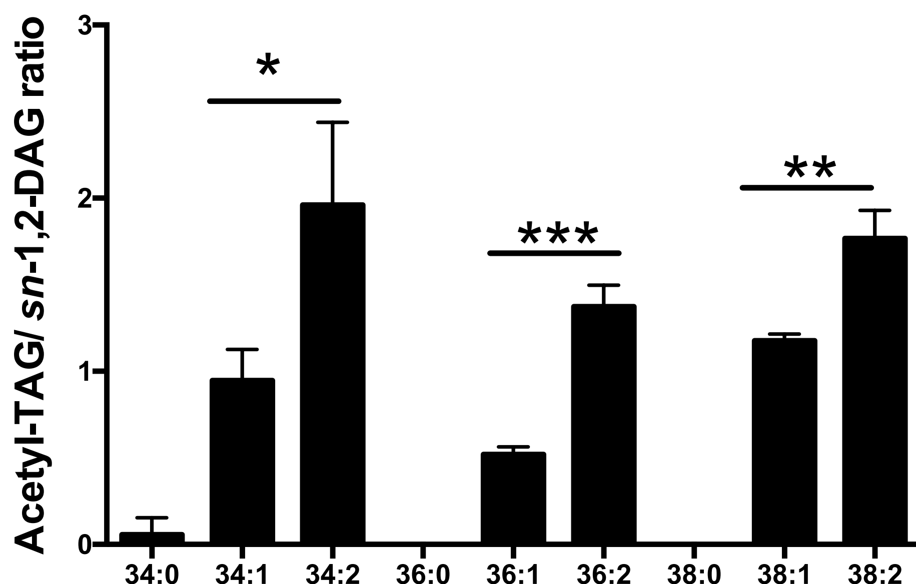



Figure 3.8 ***Ea*DAcT preferentially acetylates DAGs with higher unsaturation indices in vivo**. A plot of ratios of amounts of acetyl-TAGs to corresponding *sn*-1,2-DAG molecular species relative to internal standard 3-acetyl-1,2-dinonadecanoyl-*sn*-glycerol. The acetyl-TAGs and *sn*-1,2-DAGs were analyzed from same amount of total lipids extracts of yeast expressing *Ea*DAcT grown for 24 h in synthetic medium with galactose. Acetyl-TAGs molecular species are represented as X:Y, where first letter represents total number of carbons and second letter represents number of double bonds. Asterisks represent significant difference (*$p<0.05$; unpaired *t*-test). Values are expressed as mean ± SD (n=3).

unsaturation levels (Fig. 3.8). These results are consistent with the in vitro results, which also showed high preference of *Ea*DAcT for DAGs with high unsaturation levels (Fig. 3.4). Low abundance DAGs containing lauric, myristic, palmitic and stearic acid are not included in this analysis because of high possibility of error in their measurement.

Together, these studies showed that *Ea*DAcT can acetylate a wide variety of *sn*-1,2-DAGs with differences in chain lengths and saturation indices under in vivo and in vitro conditions. Although, the actual specificity of *Ea*DAcT for the different DAG species could not be determined due to differential solubility and availability of these DAGs, a relative estimate of DAG acceptor preference was gained from acetyl-TAG/DAG ratio obtained from in vivo and in vitro assays. Based on these results, it appears that preference of *Ea*DAcT for DAG increases with an increase in the unsaturation index of the substrate.

### 3.3.4 Fatty alcohol acyl acceptor substrate specificity of *Ea*DAcT

Based on sequence alignment, the *Ea*DAcT protein shows high similarity and identity to the Jojoba wax synthase and the Arabidopsis sterol acyltransferase [12]. Hence, the activity of *Ea*DAcT towards various alcohols and sterols was tested under in vitro and in vivo conditions. In earlier in vitro assays, we did not see formation of any [1-$^{14}$C] sterol acetate from endogenous sterols in microsomes. To eliminate the possibility that no sterols are present in microsomes, we also tested sterol acyltransferase activity with exogenous addition of cholesterol. However, no cholesterol acetate product was observed, further confirming that *Ea*DAcT does not possess sterol acetyltransferase activity.

#### 3.3.4.1 *Ea*DAcT can acetylate a variety of aliphatic chain fatty alcohols in vitro

Before studying the substrate specificity, the assay conditions for alcohol acetyltransferase using yeast microsomes containing *Ea*DAcT were optimized. Unlike DAGs, fatty alcohols are not present endogenously in the yeast microsomes and needed to be supplied exogenously. The addition of DMSO was found to be necessary to provide the activity due to insoluble nature of fatty alcohols in an aqueous buffer system (Fig. 3.9a). A concentration of 10-15 % (v/v) in the assay mixture was optimal for activity. The alkyl acetate product was found to

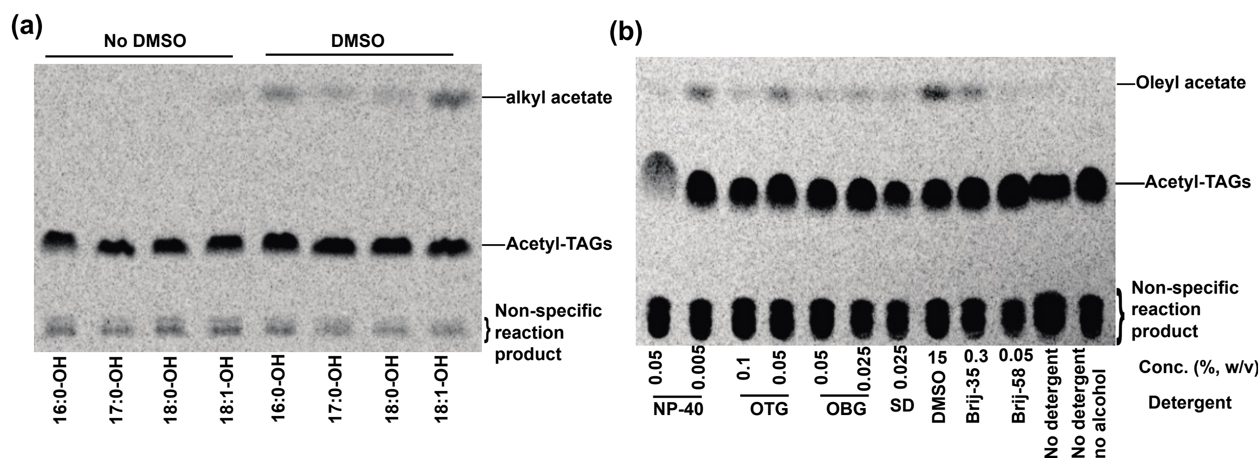



Figure 3.9 **Optimization of in vitro wax synthase assay conditions for microsomal *Ea*DAcT**. Autoradiograms of TLC separation of total lipids extract microsomal *Ea*DAcT incubated with different fatty alcohols and [1-$^{14}$C] acetyl-CoA (a) Effect of addition of DMSO on alkyl acetate synthesis (b) Effect of different detergents on the activity of *Ea*DAcT for oleyl acetate synthesis. In two numeral code nomenclature of fatty alcohols first and second numeral represent number of carbons and number of double bonds respectively. NP-40, nonyl phenoxypolyethoxylethanol-40; OTG, octyl beta-thio-glucoside; OBG, octyl-beta-glucoside; SD, sodium deoxycholate.

be much lower than the acetyl-TAG product. This might either be due to lower reactivity of *Ea*DAcT towards alcohols than towards DAGs or low solubility and accessibility of alcohol substrate to microsomal *Ea*DAcT. Hence, a number of detergents at concentrations which did not result in loss of enzyme activity, were tested to increase the alkyl acetate product formation. The product accumulation was maximum when DMSO was used, followed by addition of non-ionic detergents in the order brij-35 (0.3%) > NP-40 (0.005%) > OTG (0.05%) > OBG (0.025%) (Fig. 3.9b). Addition of the ionic detergent sodium deoxycholate was also not effective in producing alkyl acetate product. DMSO was still found to provide the best product formation and was used for later studies. *Ea*DAcT produced alkyl acetate product for a pH range between 7.0-8.5 with maximum oleyl acetate production at a pH 7.5 (Fig. 3.10a). The oleyl acetate accumulated sharply with an increase in the oleyl alcohol substrate concentration from 25 μM to 125 μM. A gradual decrease was observed when the oleyl alcohol concentration was increased from 125 μM to 1000 μM probably due to detergent effects of the hydrophobic fatty alcohol substrate (Fig. 3.10b). Hence, a pH of 7.5 and an oleyl alcohol concentration of 125 μM were selected for further assays. Once the assay conditions were optimized, specificity assays for fatty alcohol acceptors were done for alcohols with carbon chain lengths varying from 8 to 22. No product

was detected for either very short chain fatty alcohols 8:0-OH and 10:0-OH or very long chain 18:0-OH and 22:0-OH.

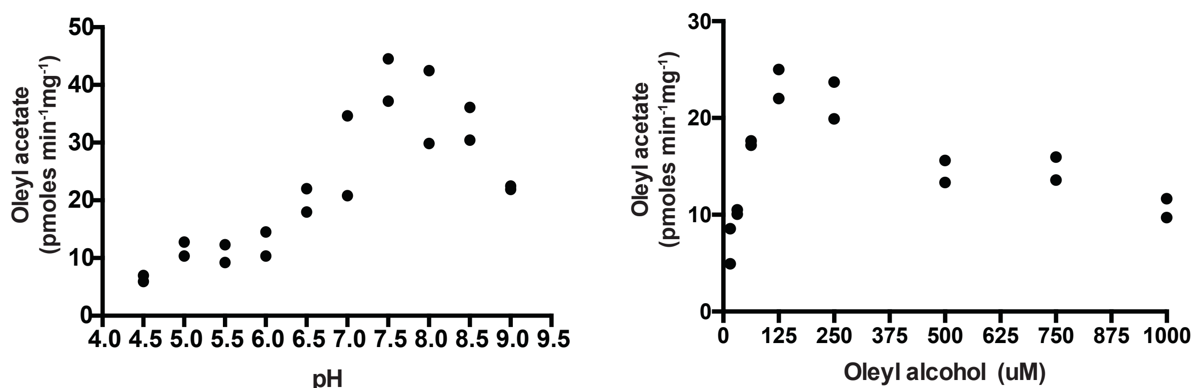



Figure 3.10 **Optimization of in vitro wax synthase assay conditions for microsomal *Ea*DAcT.** Plots of *Ea*DacT activity at different pH and oleyl alcohol concentrations. (a) Effect of assay pH on oleyl acetate synthesis. (b) Effect of different oleyl alcohol concentrations on oleyl acetate synthesis by *Ea*DAcT.

Higher amount of alkyl acetates were produced from 12:0-OH and 14:0-OH with only a very small amount for longer chain fatty alcohols 15:0-OH, 16:0-OH and 17:0-OH (Fig. 3.11). These results suggest that *Ea*DAcT has higher specificity towards fatty alcohols with medium chain length as compared to either short or long chain fatty alcohols. Aromatic alcohols such as cinnamyl alcohol, benzyl alcohol and 3-phenyl-1-propanol were also tested as substrates but no products were detected (data not shown).

Further characterization of the alcohol acetyltransferase activity of *Ea*DAcT along with characterization of another native yeast acetyltransferase was done in collaboration with a partner group in Sweden. In addition to producing low amounts of alkyl acetates, in vitro alcohol acetyltransferase enzyme assay using yeast microsomes also resulted in the formation of comparatively large amounts of acetyl-TAGs due to presence of endogenous DAGs (Fig. 3.9 a and b). This complicated the interpretation of alcohol specificity results due to competition between two activities of *Ea*DAcT for the same acyl-CoA donor substrate. Ideally, a microsomal assay system devoid of endogenous DAGs would provide better measure of specificity of *Ea*DAcT for alcohols by avoiding any competing DAcT reactions. Washing of microsomes with cold (-80 $^{o}$C) acetone removes neutral lipids from microsomes without greatly affecting the enzyme activity [70]. Our partner group in Sweden tested a large variety of saturated and unsaturated fatty alcohols varying in chain length and number and position of double bonds in a

better in vitro system using acetone washed microsomes. Additionally, feeding assays were performed by adding different fatty alcohols in the yeast growing medium. In their study, similar trends to ours were observed for different chain length saturated alcohols. Also, it was found that *Ea*DAcT showed higher activity towards unsaturated fatty alcohols than their corresponding saturated counterparts [71].

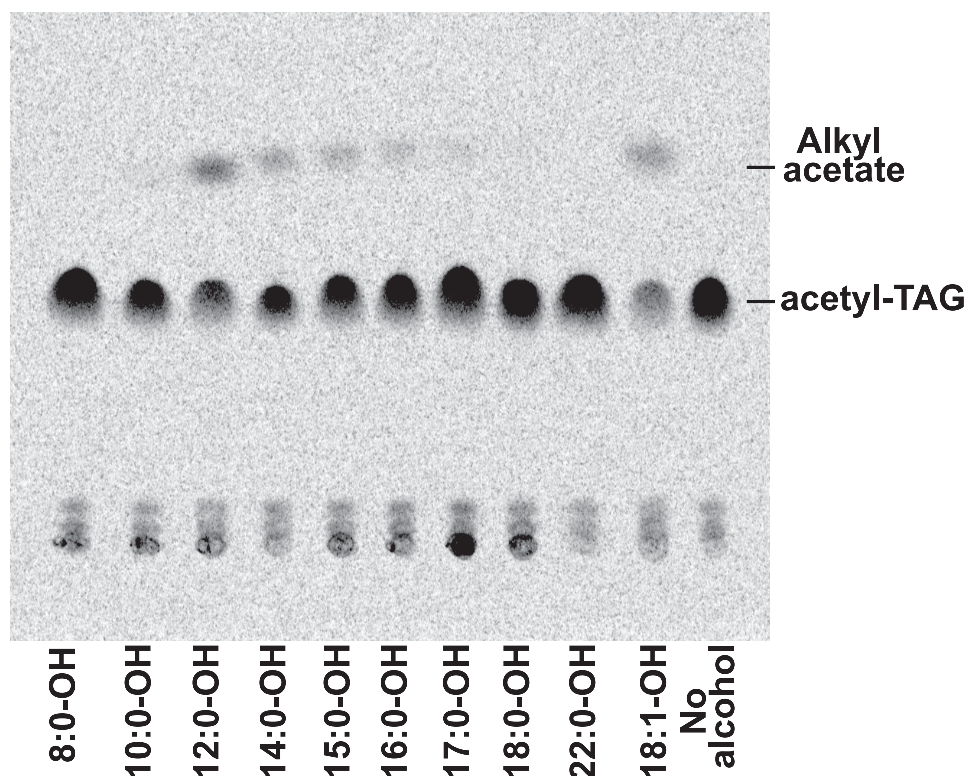



Figure 3.11 ***Ea*DacT has high specificity for medium chain length alcohols compared to short and long chain alcohols in vitro**. Autoradiogram of total lipids extracted from in vitro assay of microsomal *Ea*DAcT incubated with equal amounts of different chain length alcohols and [1-$^{14}$C] acetyl-CoA.

**3.3.4.2 *Ea*DAcT can acetylate fatty alcohol produced in yeast to form alkyl acetates in vivo**

The in vivo alcohol acetyltransferase activity of *Ea*DAcT was also examined by heterologous coexpression of *Ea*DAcT and honeybee fatty alcohol reductase (*Am*FAR1) genes to produce the fatty alcohols in vivo. *Am*FAR1 was previously reported to produce fatty alcohols in the yeast background H1246 hence was used for this study [67]. The results showed that the yeast expressing *Am*FAR1 produced hexadecanol (16:0-OH) and octadecanol (18:0-OH) (Fig.

3.12a). The amount of 18:0-OH was almost 9 fold greater than levels of 16:0-OH (Fig. 3.12b) which was in agreement with the results reported before in this yeast strain expressing *Am*FAR1 [67]. Two extra peaks were observed for the yeast coexpressing *Ea*DAcT and *Am*FAR1

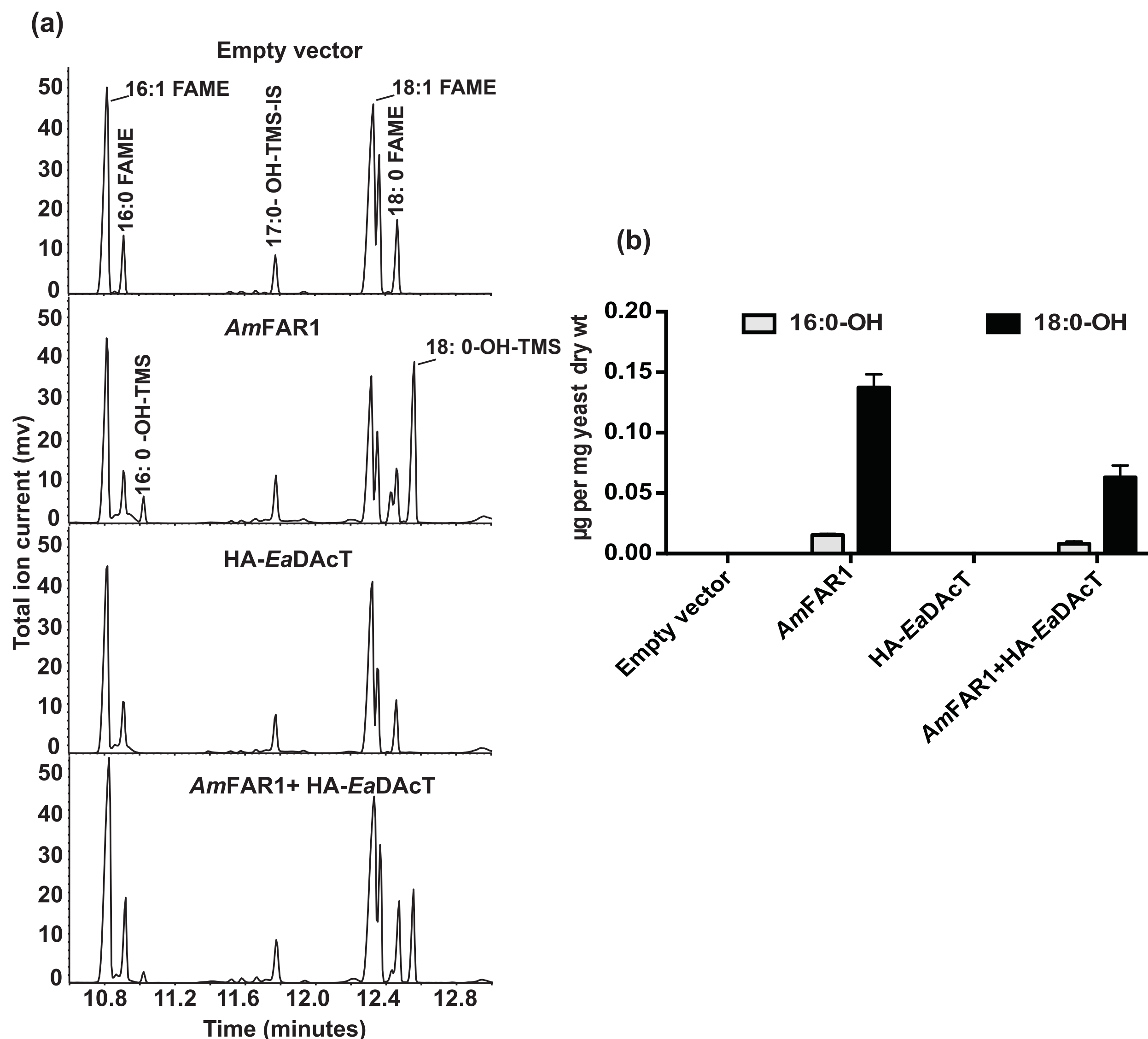



Figure 3.12 **Yeast expressing *Am*FAR1 produce fatty alcohols in vivo.** Total lipids were extracted from *S. cerevisiae* H1246 expressing empty vector, *Apis mellifera* fatty acid reductase (*Am*FAR1), *Ea*DAcT or *Ea*DAcT+*Am*FAR1 genes and separated by TLC. (a) GC-MS chromatograms of fatty alcohol fraction after TMS derivatization. (b) Quantification of fatty alcohols using GC-MS. Values are expressed as mean ± SD (n=3).

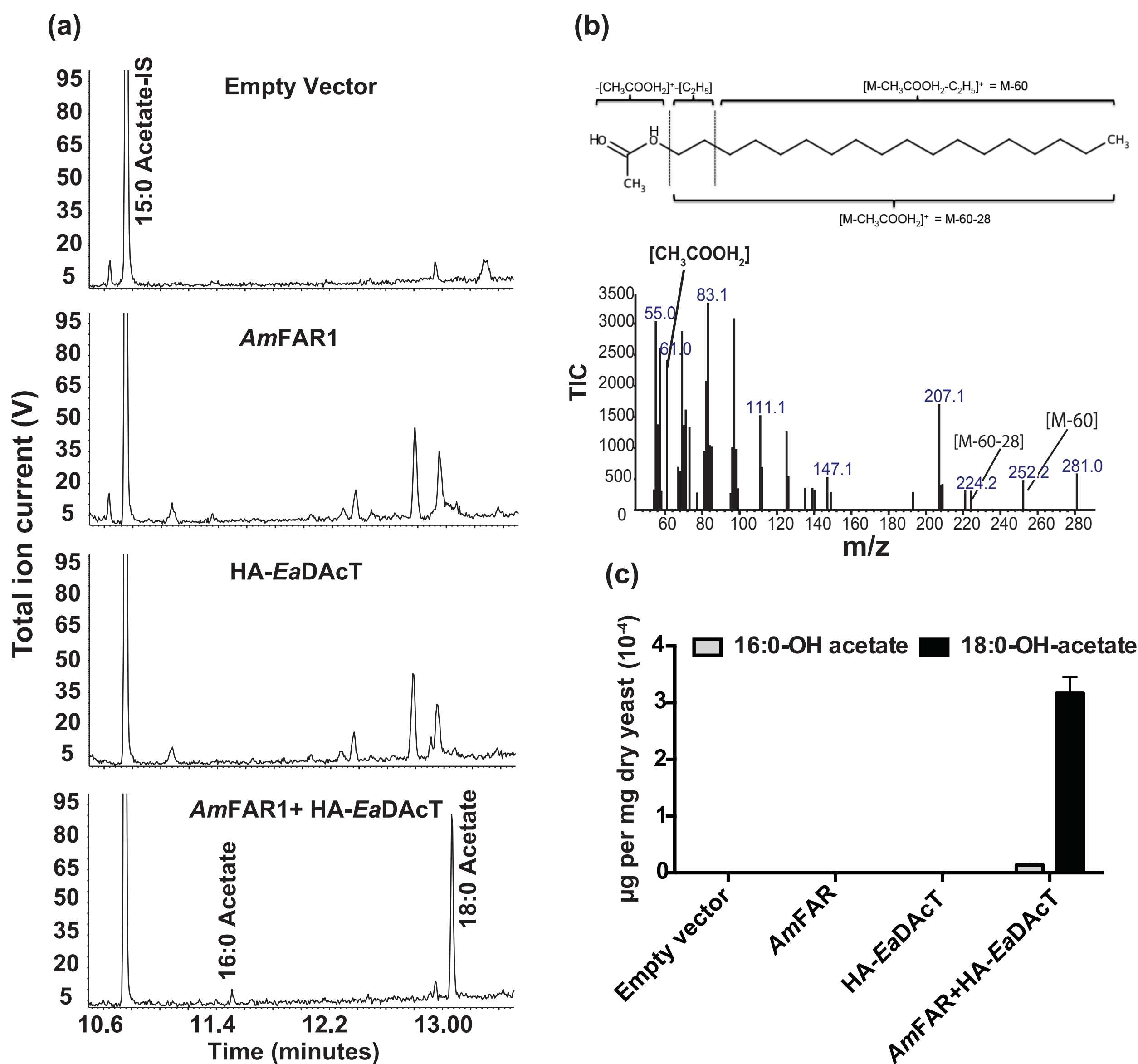



Figure 3.13 ***Ea*DAcT can acetylate fatty alcohols in vivo**. Total lipids were extracted from *S. cerevisiae* H1246 expressing empty vector, *Apis mellifera* fatty acid reductase (*Am*FAR1), *Ea*DAcT or *Ea*DAcT+*Am*FAR1 genes and separated by TLC. (a). GC-MS chromatograms of alkyl acetate fraction. (b). Mass spectrum of stearyl acetate (M=312.2) labeled with acharacteristic fragment ions such as acetate $[CH_3COOH_2]^+$, loss of an acetyl group from parent ion [M-60] and loss of an acetyl and an ethylene group [M-60-28]. (c). Quantification of alkyl acetates using GC-MS. Values are expressed as mean ± SD (n=3).

compared to empty vector, *Am*FAR1 and *Ea*DAcT alone controls (Fig. 3.13a). The mass spectra of bigger peak matched the characteristic fragmentation pattern of stearyl acetate confirming the production of alkyl acetate by *Ea*DacT in vivo (Fig. 3.13b). However, the amounts of alcohol acetates produced were significantly lower than the alcohols produced (Fig 3.13c). This might

indicate the low preference of *Ea*DAcT for these long chain saturated alcohols, again in agreement with the in vitro assay results.

## 3.4 Discussion

*Ea*DAcT is an unusual MBOAT in that it is the only known member of the enzyme family which uses the shortest possible acyl-CoA donor, namely acetyl-CoA. Previous work showed that it could not utilize oleyl-CoA as substrate, but no information was available regarding its ability to use acyl-CoAs with chain lengths falling in between the range of 2 to18 carbons. Another unusual aspect of this enzyme is its rather close phylogenetic relationship with the jojoba wax synthase and Arabidopsis sterol acyltransferase rather than with the triacylglycerol producing diacylglycerol acyltransferase (DGAT1) enzymes, given that *Ea*DAcT produces unusual TAG molecules called acetyl-TAGs. Hence, the presented work was done to better define the substrate specificity of *Ea*DAcT to further understand its unusual activity, which will be useful in its future use for various applications.

This study showed that though *Ea*DAcT can utilize other acyl donors with short acyl chains, it had a high preference for acetyl-CoA (Fig. 3.3). Unexpectedly, the $K_m$ values obtained were substantially higher (Table 3.1) than the endogenous acetyl-CoA concentrations in plant and yeast. Interference from other enzymes and proteins in the microsomal system competing for externally added acetyl-CoA substrate might be the reasons for the high apparent $K_m$. A purified enzyme system is therefore required for the assay to determine the actual $K_m$. But the more valuable result was that *Ea*DAcT was able to produce large amounts of acetyl-TAGs by esterifying acetate from acetyl-CoA on to DAG molecules (Fig. 3.3). This was a very useful result especially for a future strategy where very high acetyl-TAGs production will need to be achieved in oil seed crops by overexpression of *Ea*DAcT and providing it with increased flux of acetyl-CoA using other metabolic engineering strategies. Also, *Ea*DAcT showed no activity with acyl-CoA molecules with acyl groups containing more than 6 carbons. (Fig. 3.2). This means that in any oil seed crop designed to produce acetyl-TAGs by *Ea*DAcT expression, there will be no competing side reactions of *Ea*DAcT with abundantly available long chain acyl-CoA. This increases the probability of achieving future goals of very high acetyl-TAGs production in engineered oil seed crops. A goal of my study is to produce low molecular weight acetyl-TAGs by incorporating MCFAs in them. For that, *Ea*DAcT will be expressed in Camelina lines

producing high amounts of MCFAs. Results from this study suggest that *Ea*DAcT will not able be to use medium chain acyl-CoAs present in those lines on to DAGs. Hence the synthesis of undesirable MCFA containing TAGs by *Ea*DAcT as a side reaction will be completely avoided.

Similarly, the knowledge of DAG acceptor specificity was important for producing oil seed lines with high amounts of short and medium chain fatty acids containing acetyl-TAG in final part of project. The initial plan was to conduct the DAG acceptor specificity study for different chain length DAG substrates by providing equivalent amounts of short, medium and long chain DAGs. Yeast microsomes were found to have endogenous *sn*-1,2-DAGs which were useful for conducting acyl-CoA assay without the need to add any exogenous DAGs. However, it was difficult to outcompete these endogenous DAGs due to differential solubility of added DAGs. A few other possible strategies to control for the availability of DAG substrate were tried in this study but proved to be unsuccessful. Hence, preference of *Ea*DAcT the for DAGs abundantly available endogenously in microsomes and yeast cells was determined using in vitro and in vivo assays respectively. The levels of each endogenous *sn*-1,2-DAGs species initially present in the microsomes were determined and compared to the acetyl-TAGs formed during the assay for different acetyl-CoA concentrations. From the data it was inferred that *Ea*DAcT preferentially acetylated DAGs with two double bonds instead of DAGs with one double bond even though the latter was present at higher concentrations (Fig. 3.4). Under in vivo conditions, the ratio of amount of acetyl-TAG species produced to the residual amount of corresponding DAG species was calculated to get a better measure of specificity (Fig. 3.8). This ratio provided a normalized value of acetyl-TAG and accounted for initial variability in amounts of different DAG species. The results from in vivo studies matched with the in vitro results, which suggested that *Ea*DAcT prefers DAGs with high unsaturation indices. Results from both of these studies suggested that *Ea*DAcT preference for a DAG increases with an increase in its unsaturation index. However, *Ea*DAcT specificity towards DAGs containing MCFAs, could not be determined due to their very low abundances as endogenous DAGs in yeast microsomes and low solubility as exogenous DAGs. Hence qualitative in vitro assays were performed to determine whether *Ea*DAcT could acetylate the DAGs containing MCFAs. Indeed, it was found that *Ea*DAcT could acetylate DAGs containing MCFAs to produce acetyl-TAGs (Fig 3.5). However, very low amount of acetyl-TAGs produced might indicate either low specificity of *Ea*DAcT for these DAGs or low accessibility of these DAGs to enzyme. With the knowledge that *Ea*DAcT

can produce acetyl-TAGs containing MCFA and the urgency to start plant transformations early to obtain homozygous lines, we went ahead and expressed *Ea*DAcT in Camelina lines producing DAG containing MCFAs to produce acetyl-TAG with lower molecular mass.

Given the phylogenetic similarity of *Ea*DAcT to jojoba wax synthase, the fatty alcohol esterification activity of *Ea*DAcT was tested. The results from this study (Fig 3.11) and a collaborative study [62] concluded that *Ea*DAcT has high preference for medium chain saturated and unsaturated alcohols. This activity was later successfully tested by our collaborators through transient expression in tobacco plant to produce variety of saturated and unsaturated alcohol acetates, which are major components of insect pheromones [62]. Yeast expressing *Ea*DAcT fed with different chain length fatty alcohols also showed higher specificity for the medium chain length fatty alcohols compared to long chain fatty alcohols [71]. Interestingly, small but significant amounts of saturated and unsaturated alkyl acetates were observed in the seed and arils of various *Euonymous* species [48]. Given this additional activity of *Ea*DAcT, it is tempting to speculate that orthologs of this enzyme might also be involved in the in vivo production of these alkyl acetates in these acetyl-TAG producing species.

## 3.5 Conclusions

Overall, it can be concluded that *Ea*DAcT can use a variety of acyl donor and acceptor substrares but it has high preference for acetyl-CoA and DAGs. For DAGs it prefers more unsaturated molecules. However, it can acetylate DAGs containing MCFA to produce acetyl-TAGs. This enzyme has already been expressed successfully in the model plant Arabidopsis and the oil seed crop Camelina and soybean for modification of physical properties of their seed oil [13]. The results from this study provided important information regarding the enzyme's ability to use DAGs containing medium chain fatty acids to produce reduced molecular mass acetyl-TAGs. As discussed in the next chapter, this feature will be utilized to produce MCFA acetyl-TAGs in Camelina in order to produce a low viscosity straight vegetable oil biofuel.

# Chapter 4 - *Camelina sativa:* an ideal platform for the metabolic engineering and field production of industrial lipids

## 4.1 Introduction

Dwindling cheap fossil fuel reserves and concerns about climate change have increased the need to obtain fuel and chemical products from renewable sources. While plant oils have long been used as sources of fuel and as feedstocks for industrial chemistry, commercially grown oil seed crops only synthesize a low diversity of fatty acids, limiting the chemical functionality of the oil. However, within the plant kingdom a great diversity of lipids has been found with different structures and fatty acids, many of which confer chemically useful functions [72]. The isolation of enzymes from different plants and an increased understanding of metabolic fluxes in developing seeds have allowed the production of high levels of unusual lipids in transgenic seeds. Until recently, much of this work was pioneered in the model plant *Arabidopsis thaliana*, which is easily transformed and possesses a great many mutants affecting different aspects of lipid biosynthesis [73,74]. Not only have these mutants provided insights into the synthesis of fatty acids and subsequent flux into storage lipids, but they have also provided a useful genetic background for the synthesis of unusual fatty acids. However, the small seed yield and lack of large scale field growth options have limited the ability to test the functionality of any transgenic oil produced in Arabidopsis. Instead, insights obtained from work with this model species typically have had to be transferred to oil seed crops. Here we describe the use of the emerging oil seed crop *Camelina sativa* as an appealing alternative for the production of modified lipids in transgenic plants. We will discuss why Camelina is particularly attractive as an industrial seed crop, both from an agronomic perspective as well as from a seed metabolic engineering system. To illustrate this potential of Camelina, we will describe examples of the metabolic engineering of unusual lipids in this oil seed crop. In particular, we focus on the synthesis of very high levels of acetyl-TAGs in Camelina, the highest accumulation of unusual lipids achieved so far in transgenic seeds. Further, the field growth of these high acetyl-TAG lines allowed the production of large quantities of these unusual lipids, permitting meaningful property testing.

## 4.2 *Camelina sativa*: an underdeveloped oil seed crop

*Camelina sativa*, also known as false flax or gold of pleasure, is a member of the Brassicaceae family. Despite appearing to have been used as an oil seed crop since the Iron Age, Camelina's use waned during the Middle Ages [75]. However, its various positive agronomic and environmental features, along with the development of tools for genetic manipulation, have contributed to an increased interest of scientists and farmers in Camelina for use as an industrial oilseed crop.

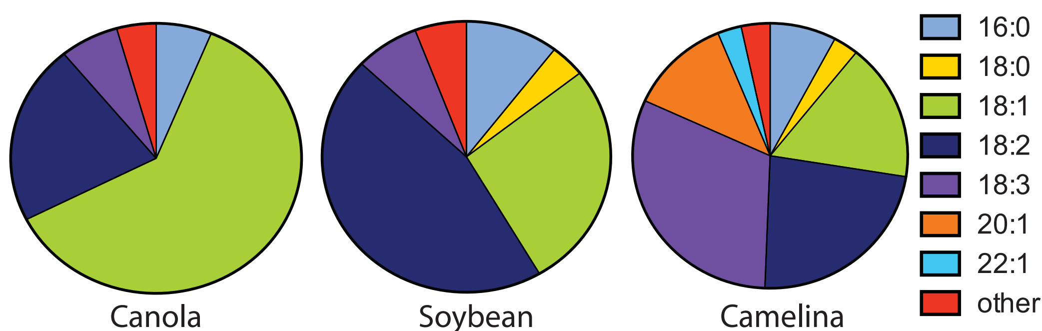



Figure 4.1 **Fatty acid composition of seed oil from canola, soybean and Camelina**. Values are from Putnam et al. (1993).

### 4.2.1 Fatty acid profile of Camelina

Camelina oil contains high proportions of the polyunsaturated fatty acids (PUFAs) linoleate (18:2)[1] and linolenate (18:3) compared to soybean and canola oil (Fig. 4.1). This high degree of unsaturation renders Camelina oil highly prone to oxidation making it less suitable for certain applications. For example, biodiesel derived from Camelina possesses a lower oil stability index (OSI) compared to biodiesel from other feedstocks [76]. Below we describe successful mutagenic and biotechnology approaches to decrease the PUFA content of Camelina oil to make it more oxidatively stable.

### 4.2.2 Agronomic advantages of Camelina

Camelina has a number of excellent agronomic properties which make it valuable as an oilseed crop. For example, Camelina has a relatively short growing season (85 to 100 days), and possesses winter and spring variaties, facilitating rotation with other crops [77]. Further, the oil

[1] Fatty acids are represented as X:Y where X indicates the number of carbon atoms and Y represents the number of double bonds. In some situations, the position of double bonds from the carboxyl group is designated by delta (Δ). Therefore linoleate can be represented as 18:2 or 18:2Δ9,12.

yield of Camelina (variable from 400 to 850 kg $ha^{-1}$, depending on the study) is typically comparable with that of *Brassica juncea* and *Brassica rapa* and higher than that of soybean [77,78]. While the oil yield is somewhat lower than that of canola, studies have shown that the cost of production of seed oil from Camelina can be less than half that from rapeseed due to comparatively low input requirements [79]. For example, Camelina requires only low amounts of fertilizer, with optimum nitrogen and phosphorus requirements of about 80-100 kg $ha^{-1}$ and 50 kg $ha^{-1}$, respectively [80,81]. Increasing the nitrogen content above optimum was found to increase seed yield depending on soil potential and precipitation [82,83]. Camelina is also capable of tolerating conditions of water stress and is therefore less dependent on irrigation. For example, in one drought stress study, Camelina possessed a higher seed yield (1383 kg $h^{-1}$) than *B. juncea* (933 kg $ha^{-1}$) and *Brassica carinata* (711 kg $ha^{-1}$) [84]. Similarly, a two-year study in western Canada showed high drought and flea beetle resistance of Camelina over commercially grown crops such as *B. rapa* and canola [85]. The evapotranspiration of Camelina was found to be 332 to 371mm, much lower than that of vegetable and grain crops (600-655mm), offering one explanation for the increased tolerance of low water stress [86]. Camelina also appears to be more resistant to diseases like black spot (*Alternaria brassicae*) and blackleg (*Leptosphaeria maculans*) which are important pathogens of rapeseed and canola [87-89]. Additionally, some Camelina genotypes are resistant to other common Brassicaceae diseases like sclerotinia stem rot, brown girdling rot and downy mildew [90]. Such germplasm could therefore be used to breed for resistance in other backgrounds.

### 4.2.3 Environmental benefits of Camelina as an industrial oilseed crop.

Ideally, crops grown for industrial feedstocks or biofuel purposes should not displace other crops grown for food. The agronomic features of Camelina mean that it is well suited for this role. For example, its low water and fertilizer requirements mean it can be cultivated on marginal lands. However, recent work has shown that other alternative biofuel crops, such as *Jatropha curcas*, require some irrigation for optimal growth and need to be supplemented with fertilizers to produce seed oil if grown on marginal lands [91]. These results suggest that even for crops supposedly suitable for growth on marginal lands, yield under unfavorable conditions without some sort of agricultural input will be low.

Therefore, instead of trying to grow Camelina on marginal lands, a more effective strategy would be to take advantage of its short generation time and cultivate it on currently used

cropland as part of multi crop rotation system. For example, Camelina can be grown as a rotation crop during the fallow years with wheat and dryland cereals, like oats and barley, without affecting the yield of these crops [92]. The same study predicted that the roughly 5-7 million acres of land available through such a fallow rotation would provide a Camelina oil yield of 750 to 1000 million gallons per year. Yield returns of Camelina- soybean and Camelina-sunflower were 82% and 72% respectively of their monocropped counterparts however the net economic returns for Camelina-soybean double crop were higher than soybean alone [93]. In a subsequent study, winter Camelina grown as double crop and relay crop with soybean showed similar net economic returns compared to the mono-cropped full season soybean [94]. Further, life cycle assessments of biodiesel produced from camelina grown under different conditions, showed reduced energy, non-renewable energy inputs and overall green house gas emissions compared to diesel fuel as well as biodiesel from other oilseed crops [95,96], making Camelina a more environmental friendly substitute than other biofuel crops.

### 4.2.4 Manipulating lipid metabolism in Camelina

In addition to its useful agronomic traits, the recent development of tools and resources for Camelina have facilitated its emergence as an ideal platform for the metabolic engineering of oil composition and subsequent growth in the field. Importantly, Camelina can be rapidly and easily transformed using agrobacterium-mediated floral-dip methods [97,98]. The development of these methods makes Camelina transformation much simpler and quicker compared to the tissue-culture based approaches required for the transformation of other oilseed crops. Transgenic seeds are easily identified using a variety of selectable markers, including seed fluorescence and resistance to specific herbicides or antibiotics [98]. The existence of multiple selectable markers is useful for stacking different transgenic traits. Additionally, knowledge of the seed transcriptome [99] and the recent sequencing of the genome [100] allow current and future work in Arabidopsis to be easily applied to Camelina. For example, analysis of genomic sequence revealed that over 90% of the Arabidopsis genes involved in lipid metabolism were also present in the Camelina genome [73,100]. Similar results were also obtained through analysis of the seed transcriptome, which suggested that approximately 80% the expressed lipid related genes were 80% or more identical compared to their Arabidopsis orthologs [99].

#### 4.2.4.1 Overcoming the hexaploid genome of Camelina

One potential drawback to engineering metabolic fluxes in Camelina, is its remarkably undifferentiated hexaploid genome [100]. Therefore, enzymatic activity could be encoded by up to three similar gene homeologues making modifying endogenous metabolic fluxes more difficult. Indeed, earlier work has demonstrated that all three copies of *CsFAD2* and *CsFAE1* are expressed in developing seeds [101,102]. Further, all three *CsFAD2* genes encode functional desaturase enzymes based on their ability to synthesize linoleic acid when expressed in yeast [102].

Consequently, RNA interference (RNAi) has been used to facilitate post-transcriptional gene silencing of targeted genes. The high similarity of the three Camelina sub-genomes [99,100] means that all three homeologues can be targeted with a single RNAi construct. Thus RNAi against *CsFAD2* was successful in increasing oleic acid levels to around 50% compared to about 15% in wild type seeds [99,103]. The simultaneous silencing of both *CsFAD2* and *CsFAE1* further increased the levels of oleic acid to 70% in the best lines [99]. As we describe below, other RNAi based strategies have been successfully used to target specific endogenous Camelina activities that compete with the production of a desired lipid.

While RNAi-based approaches have the advantage of targeting all homeologues encoding a particular enzyme activity, in some cases mutagenesis of the genes might be more desirable. For example, the phenotype in a mutant background would be more genetically stable than that achieved through RNAi. Further, some farmers and markets might be more receptive to the adoption of a non-transgenic biofuel crop, such as high-oleic Camelina. Given the hexaploid genome of Camelina, in most cases three separate mutagenic events will be required for complete elimination of enzymatic activity. For example, mutation of the *CsFAD2-2* gene increased oleic acid content from 17% to 27%, lower than the 38-51% range observed with RNAi of *CsFAD2* [102]. This is not surprising given that more than 80% of Camelina genes potentially involved in different aspects of acyl-lipid metabolism were present in three homeologous copies [100]. However, the fact that even a small increase was observed suggests, at least for the case of *CsFAD2*, that the three genes contribute additively to activity. Further, the presence of phenotype, albeit small, suggests that high throughput screening for mutants with altered fatty acid composition is a viable approach towards obtaining mutations in different homeologues.

The emergence of a number of different genome editing technologies offers new strategies with which to generate mutants in a target gene. The CRISPR/Cas9/sgRNA-mediated targeted gene modification method has been shown to be effective in inducing targeted gene mutations in Arabidopsis and other plant systems [104]. This method relies on expression of the nuclease domain containing Cas9 protein and a single-stranded guide RNA (sgRNA) that confers DNA target specificity. Binary vectors expressing both Cas9 and a sgRNA have been developed and successfully transformed into Arabidopsis using a standard floral dip method [105]. Importantly, the mutations can be stably inherited and the process appears specific as other genes are not mutated [106]. However, if desired, careful selection of the sgRNA allows for the selective targeting of multiple genes containing identical stretches of sequence [107,108]. Thus, given the high degree of similarity between all three Camelina sub-genomes, in most cases it should be possible to simultaneously mutate all three homeologues of a specific gene.

With the demonstrated effectiveness of RNAi and the promise of CRISP/Cas9 genome editing, altering endogenous lipid biosynthetic fluxes can now be achieved seemingly at will. Thus mutants analogous to those in Arabidopsis should be easily generated, further facilitating the use of Camelina as an ideal oil seed metabolic engineering platform. Before discussing specific examples of Camelina metabolic engineering, a brief overview of fatty acid and TAG biosynthesis is presented.

## 4.3 TAG Biosynthesis in Plants

TAGs are the major seed storage lipids of most plants and are used to provide energy and carbon during germination. The overall pathway for the synthesis of TAG in the seeds of higher plants is complex (Fig. 4.2); for a more detailed description of this important process, readers are encouraged to refer to recent and more in-depth reviews [73,109,110].

### 4.3.1 Fatty acid synthesis

Briefly, fatty acids are synthesized in the plastids by a type II fatty acid synthase complex similar to that found in prokaryotes [111]. A repeated series of condensation, reduction and dehydration reactions add two carbon units to the elongating fatty acid chain, which is connected to an acyl carrier protein (ACP). Typically, fatty acids up to 16 or 18 carbons in length are synthesized, though shorter molecules are possible in other species. Introduction of a double bond through the action of a Δ9-desaturase can also occur in the plastid. Acyl-ACP thioesterases

are responsible for the removal of the growing fatty acid from ACP. Of the two types of thioesterases, FatA enzymes preferentially remove oleate (18:1) whereas FatB thioesterases tend to remove shorter, saturated fatty acids. The fatty acids synthesized in the plastid are exported to the cytosol and converted to CoA forms, which can be used as acyl donor substrates by acyltransferases.

### 4.3.2 Incorporation of fatty acids into TAGs

Newly synthesized fatty acids are rapidly incorporated into phosphatidylcholine (PC) [51,112]. There they can be further modified by additional desaturation or the incorporation of other functional groups. An acyl editing pathway cycles fatty acids between PC and the acyl-CoA pool without the net synthesis of PC. Recent work has suggested that the movement of fatty acids both into and out of the PC pool is mediated by the forward and reverse reactions of lysophosphatidylcholine acyltransferases (LPCAT) [112-114]. Incorporation of fatty acids into TAG occurs via the Kennedy Pathway [115], which starts with sequential acylation of glycerol-3-phosphate by glycerol-3-phosphate acyltransferases (GPATs) and lysophosphatidic acid acyltransferases (LPAATs) using acyl-CoA to produce phosphatidic acid (PA). PA is then dephosphorylated by PA phosphatases to create *de novo* diacylglycerol (DAG). DAG can then be used as a substrate in two different acyltransferase reactions that synthesize TAG. Diacylglycerol acyltransferases (DGAT) transfer the acyl group from acyl-CoAs to the *sn*-3 position of DAG to produce TAG [116]. Alternatively, phospholipid:diacylglycerol acyltransferases (PDAT) transfer the *sn*-2 acyl group from phospholipids to DAG to form TAG [36,117]. In Arabidopsis, DGAT1 is responsible for the synthesis of the majority of TAG [42], but in other species, such as flax, PDAT activity also plays an important role [39].

In addition to the *de novo* DAG pool formed via the Kennedy Pathway, labeling experiments have suggested the existence of a second, PC-derived DAG pool [51,112]. In Arabidopsis, phosphatidylcholine: diacylglycerol cholinephosphotransferase (PDCT), which transfers the phosphocholine headgroup from PC to DAG, appears to be responsible for the bulk of the interconversion between PC and DAG [118]. However, other mechanisms to convert PC to DAG, such as the action of a phospholipase C or the reverse reaction of CDP-choline:diacylglycerol cholinephosphotransferase (CPT), are also possible [119]. This PC-derived DAG pool can then converted to TAG via DGAT or PDAT activity.

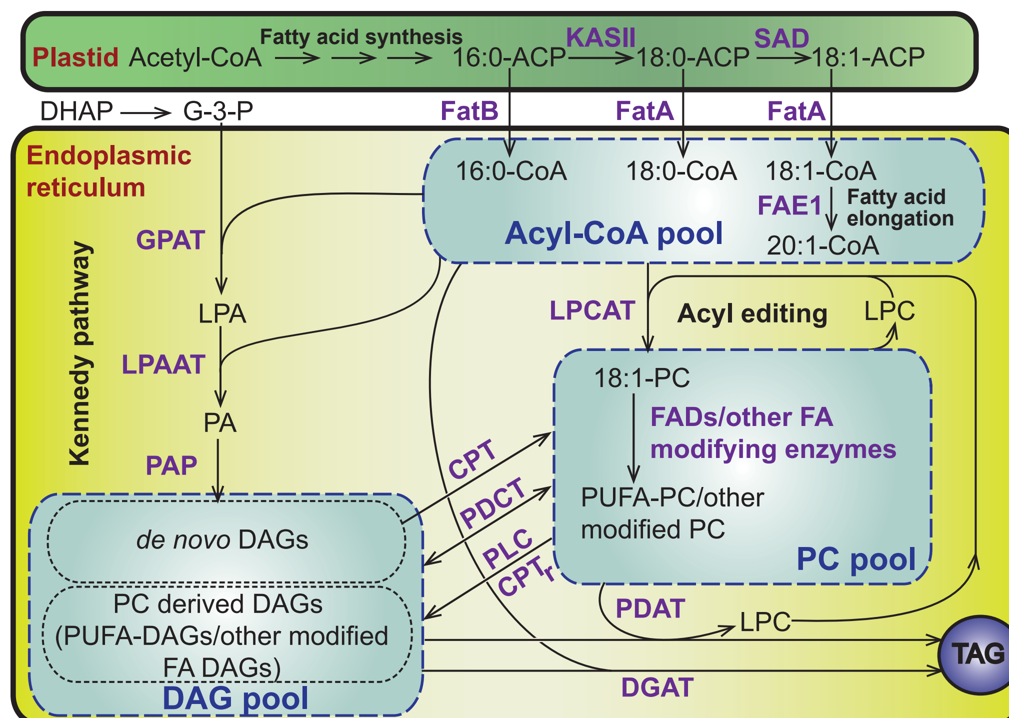



Figure 4.2 **Overview of triacylglycerol biosynthesis in seeds.** Dashed borders are used to indicate different metabolite pools within the endoplasmic reticulum.

Compound abbreviations: ACP, acyl carrier protein; CoA, coenzyme A; DAG, diacylglycerol; DHAP, dihydroxyacetone phosphate; G-3-P, glycerol-3-phosphate; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; PA, phosphatidic acid; PC, phosphotidylcholine; PUFA, polyunsaturated fatty acid; TAG, triacylglycerol

Enzyme abbreviations: CPT, CDP-choline:diacylglycerol cholinephosphotransferase; $CPT_r$, reverse activity of CPT; DGAT, diacylglycerol acyltransferase; FAD, fatty acid desaturase; Fat, fatty acid thioesterase; GPAT, glycerol-3-phosphate acyltransferase; KAS, ketoacyl-ACP synthase; LPAAT, lysophosphatidic acid acyltransferase; LPCAT, lysophosphatidylcholine acyltransferase; PAP, phosphatidic acid phosphatase; PDAT, phosphatidylcholine: diacylglycerol acyltransferase; PDCT, phosphatidylcholine: diacylglycerol cholinephosphotransferase; PLC, phospholipase C; SAD, stearoyl desaturase.

### 4.3.3 Production of TAG containing unusual fatty acids in Arabidopsis

A large number of plants also contain unusual fatty acid in TAGs [72]. These FA are typically derived from the further modification of fatty acids on PC molecules. Interestingly, many of the enzymes that catalyze these modifications like hydroxylation or epoxylation are variants of the membrane-bound desaturases responsible for the synthesis of polyunsaturated fatty acids [120]. One of the most highly studied unusual fatty acids is ricinoleic acid, a hydroxylated fatty acid which accounts for up to 90% of the fatty acids found in castor oil [72].

Ricinoleic acid is synthesized from oleic acid esterified to the *sn*-2 position of PC by the castor oleate Δ-12 hydroxylase (*Rc*FAH12) [121,122]. However, expression of *Rc*FAH12 in Arabidopsis led to seed oil containing relatively low levels (around 17%) of hydroxy fatty acids [123]. Similar low results were also noted in efforts to engineer the synthesis of other unusual fatty acids in Arabidopsis [124]. These low levels of modified fatty acids in the seeds of transgenic plants appear to be caused by their inefficient removal from the site of their synthesis on PC and subsequent incorporation into TAG [38,112,125]. Indeed, the coexpression of castor derived enzymes with higher specificity for hydroxy fatty acid containing substrates increased the accumulation of these unusual fatty acids in the transgenic seed oil. For example, expression of a castor DGAT2 ortholog (*Rc*DGAT2) along with *Rc*FAH12 increased hydroxy fatty acid levels from 17% to 28% [44]. Likewise, co-expression of a castor PDAT led to similar sized increases [38]. Stacking these two acyltransferases with *Rc*FAH12 only led to a small, but significant, further increase over either acyltransferase alone [38]. Finally, the addition of PDCT cloned from castor increased hydroxy fatty acids from 10% with only *Cs*FAH12 to ~20% [126]. An increased understanding of the flux of fatty acids from the site of synthesis to incorporation will lead to even higher increases of industrially useful fatty acids in the future.

## 4.4 Synthesis of unusual lipids in Camelina

Much of what we have learned from engineering oil seed metabolism in Arabidopsis has translated remarkably well to Camelina, not only with regard to hydroxy fatty acids but also with the production of omega-7 fatty acids and acetyl-TAGs.

### 4.4.1 Production of high levels of hydroxy fatty acids in Camelina

Transformation of Camelina with *Rc*FAH12 under control of the seed specific phaseolin promoter yield lines capable of accumulating just over 6% ricinoleic acid along with smaller quantities of other hydroxy fatty acids [98]. Combining the expression of *Rc*FAH12 with a fatty acid condensing enzyme from *Physaria fendleri* (*Lf*KCS), responsible for the elongation of hydroxy fatty acids, increased the accumulation of the elongated hydroxy fatty acids such as lesquerelic acid to 8% [103]. Interestingly, these seeds also accumulated slightly higher levels of ricinoleic acid leading to an overall increase in total hydroxy fatty acids from an average of 14% with *Rc*FAH alone to 19% with the addition of *Lf*KCS. The increase was attributed to a more efficient removal of the elongated hydroxy fatty acids from PC, thus helping overcome the

bottleneck in this process. Presumably, coexpression of castor acyltransferases in Camelina will lead to similar increases to hydroxyl fatty acids, similar to what was observed in Arabidopsis.

### 4.4.2 Production of omega-7 unsaturated fatty acids

Omega-7 unsaturated fatty acids such as palmitoleic acid (16:1Δ9) and vaccenic acid (18:1Δ11) possess a number of physical properties that make them useful for industrial and biofuel applications. For example, it is predicted that shorter, monounsaturated fatty acids such as palmitoleic acid contain the optimal balance of chain length and double bonds to allow improved biodiesel cold-temperature properties and reduced $NO_x$ emissions while maintaining ignition quality and oxidative stability [4]. Omega-7 fatty acids can also be used as a source of valuable chemical precursors for industrial applications. In particular, the location of the single double bond in these fatty acids allows them to be used for the metathesis based production of 1-octene, a monomer in the synthesis of polyethylene.

Accumulation of high levels of omega-7 fatty acids was achieved in transgenic Arabidopsis seeds through the expression of a Δ9-18:0-ACP desaturase engineered with enhanced specificity towards 16:0-ACP and an extraplastidial Δ9-16:0-CoA desaturase. Additionally, the activity of KASII which also uses 16:0-ACP as a substrate was suppressed and the elongation of 16:1Δ9 was prevented by using an *fae1* mutant background. Through this expression of exogenous genes combined with the elimination of competing pathways, omega-7 fatty acid levels as high as 67 mol % were achieved, essentially the same as the levels achieved in seeds that naturally accumulate these unusual fatty acids [127].

A similar strategy was subsequently employed in Camelina. However, instead of using mutant backgrounds, RNAi was used to suppress expression of the endogenous *KASII* and *FAE1* genes. This suppression, combined with the expression of the same substrate-optimized Δ9-18:0-ACP desaturase as before and a Δ9-16:0-CoA desaturase from *C. elegans*, led to total omega-7 fatty acid levels of 44 mol % [128]. Retransformation of this line with another copy of the Δ9-18:0-ACP desaturase and RNAi against FatB expression resulted in omega-7 fatty acids levels of 66%, one of the highest levels of unusual fatty acids synthesized in a transgenic oil seed [128]. Initial data from greenhouse-grown plants suggest that this high accumulation of omega-7 fatty acids did not affect the oil content, seed weight or germination efficiency [127]; future work will confirm whether these results translate under field conditions.

### 4.4.3 Production of high levels of acetyl-TAGs in Camelina

The seeds of *Euonymus alatus* (Burning Bush) and a few other plant species synthesize 3-acetyl-1,2-diacyl-*sn*-glycerols (acetyl-TAGs), unusual TAGs with an *sn*-3 acetate group [6,7]. Acetyl-TAGs possess different physical properties compared to regular seed oils, making them potentially useful for a number of different applications. For example, acetyl-TAGs possess a kinematic viscosity that is approximately 40% lower than that of typical TAGs [12]. As the high viscosity of vegetable oils prevents their direct use in standard diesel engines [129], one potential application for acetyl-TAGs is as an improved low-viscosity straight vegetable oil (SVO) biofuel. Indeed, acetyl-TAGs possess a viscosity in the range of Diesel #4, a heavier grade diesel used in the constant speed engines found in locomotives, ships and heavy generators. Further, acetyl-TAGs also possess improved cold temperature properties compared to regular TAGs [13,65], an important consideration, especially in colder climates. In addition to being used as a fuel, these properties of acetyl-TAGs lend themselves to other useful applications such as biodegradable lubricants, transformer oils and other products.

The gene encoding the diacylglycerol acetyltransferase (DAcT) activity required for the synthesis of acetyl-TAGs in *Euonymus alatus* (Burning Bush) was cloned using a comparative transcriptomic approach [12]. Expression of *Ea*DAcT in wild-type Arabidopsis seeds resulted in the accumulation of up to 45 mol % of acetyl-TAGs in the transgenic seed oil. Similar results were obtained in Camelina where lines transformed with *Ea*DAcT accumulating on average 55 mol % acetyl-TAGs [13]. Because *Ea*DAcT uses the same DAG substrate as the endogenous DGAT1 and PDAT enzymes responsible for the synthesis of regular triacylglycerols, the elimination of this competing pathway represents one approach to increasing acetyl-TAG levels. Indeed, expression of *Ea*DAcT in the Arabidopsis *dgat1* background led to acetyl-TAG levels of 60 – 65 mol % in the highest accumulating lines. Further, this result could be replicated in Camelina: *Ea*DAcT expression combined with the RNAi-mediated suppression of the three Camelina DGAT1 homeologues led to transgenic lines capable of synthesizing up to 85 mol % acetyl-TAGs [13]. Further analysis of the high acetyl-TAG lines revealed that the trait was stable through multiple generations. The average seed mass was not altered relative to wild-type controls and seed oil content was reduced only slightly. Importantly, germination rates of the seeds accumulating high levels of acetyl-TAGs were not greatly different from wild-type seed. These results stand in contrast to the synthesis of other unusual lipids in transgenic seeds where

major reductions in seed size, oil content and germination were noted [26,38,125,130]. Typically these problems are associated with bottlenecks in the flux of unusual fatty acids from the PC pool where they are synthesized [131]. The synthesis of acetyl-TAGs avoids such constraints because *Ea*DAcT functions at the end of a biosynthetic pathway and uses the readily available acetyl-CoA as a direct substrate. Further, because it appears that germinating seedlings can metabolize acetyl-TAGs as well as the regular TAGs stored in a seed [13], the complete replacement of regular TAG storage reserved with these unusually structured molecules should not pose a problem during the germination process.

#### 4.4.3.1 Enhancing the functionality of acetyl-TAGs

The fatty acid composition of the acetyl-TAGs produced in different transgenic plant species is reflective of DAG molecular species found in those plants. By altering the composition of the DAG pool it should therefore be possible to incorporate different fatty acids at the *sn*-1/2 positions of acetyl-TAGs and further modulate the properties of these unusual lipids.

This scenario has already been successfully demonstrated with the production of high-oleic acetyl-TAGs [65]. High oleic Camelina lines generated using RNAi against *CsFAD2* and *CsFAE1* [99] were transformed with *Ea*DAcT and RNAi against DGAT1 and PDAT. The resultant lines produced up to 70 mol % acetyl-TAGs. Further, 3-acetyl-1,2-dioleoyl-*sn*-glycerol was the most abundant acetyl-TAG molecular species, comprising 47% of all acetyl-TAG molecular species [65]. The high proportion of oleic acid in the *sn*-1 and *sn*-2 positions of the acetyl-TAGs changed the physical properties of these molecules. For example, high-oleic acetyl-TAGs possessed a high oxidative stability index [132] reflective of the replacement of polyunsaturated fatty acids with the more stable mono-unsaturated oleic acid.

The ability to incorporate unusual fatty acids into acetyl-TAGs therefore allows a combinatorial approach in order to achieve a wider range of molecules with desired properties and increased value. For example, because viscosity appears to primarily depend on molecular weight [133], it is tempting to speculate that the incorporation of medium chain fatty acids at *sn*-1/2 will lead to further reductions in the viscosity of acetyl-TAGs. *In vitro* experiments suggest that *Ea*DAcT is capable of acetylating diacylglycerol molecules with fatty acids as short as caproic acid [12] suggesting that the synthesis of acetyl-TAGs with a wide variety of fatty acids at *sn*-1/2 is not excluded from a substrate specificity point of view.

### 4.4.4 Functional testing of modified oil from transgenic Camelina

The larger seeds of Camelina compared to Arabidopsis offer an obvious advantage in terms of producing larger quantities of seed oil for physical and chemical property testing. Thus, analysis of Camelina oil using differential scanning calorimetry (DSC) containing high levels of omega-7 fatty acids revealed alterations in thermal properties, such as a lower crystallization onset temperature, compared to seed oil from wild-type plants [128]. Similar analyses revealed that acetyl-TAGs possess improved cold-temperature properties, with crystallization of the α-polymorphic form occurring 18 ˚C lower than typical triacylglycerols [13]. Further, the ability to grown Camelina in the field allows for the generation of large quantities of modified oil seeds, permitting analyses that require larger amounts of material. For example, the standard tests for quantifying the cloud point or the pour point of a fuel each require 40ml of sample. Other, more elaborate analyses such as cetane number determination consume up to 1L of the test fuel. Thus, the field growth of high acetyl-TAG Camelina lines allowed the generation of large quantities of modified oil, allowing the demonstration that transgenically produced acetyl-TAGs also possess a 40% reduction in kinematic viscosity compared to regular seed oil [13]. Interestingly, high-oleic acetyl-TAGs possessed a higher viscosity than acetyl-TAGs synthesized in a wild-type Camelina background, probably due to the reduced levels of polyunsaturated fatty acid levels in the high-oleic acetyl-TAGs [65]. Such physical property information is particularly valuable as it suggests that additional factors beyond molecular weight are important for viscosity. Importantly, insights such as this would not have been achievable without the ability to specifically engineer a designed oil trait in Camelina and then obtain large quantities of the resulting oil.

## 4.5 Future Directions

While Camelina already possesses many positive agronomic traits, it has not been the subject of much breeding, suggesting that further improvements in these characteristics are still possible. For example, increasing yield and seed oil content will enhance the competitiveness of Camelina compared to other oil seed crops. Improving tolerance to heat and other abiotic stresses will increase options for growth, both geographically and with regard to seasonal timing. A number of studies surveying different Camelina genotypes have demonstrated significant genetic

variation in a number of important traits [134-137], providing a basis for future breeding programs. However, the undifferentiated allohexaploid nature of the Camelina genome with multiple homeologues expressed will mean a complex mode of inheritance for various phenotypes, complicate breeding efforts. In this regard, the application of next generation sequencing technologies to develop dense marker maps and rapidly genotype individuals should allow the implementation of genomic selection models to speed up the breeding process [138].

## 4.6 Conclusion

Based on its ease of transformation and knowledge of its lipid biosynthetic pathways, Arabidopsis has long been used as the preferred model system with which to engineer the production of modified lipids. In addition to the examples that we detail, the production of many other unusual lipids, such as wax esters and epoxy-fatty acids, has been demonstrated in Arabidopsis [139,140]. However, the development of an effective transformation method has allowed transgenic Camelina lines to be developed almost as easily as with Arabidopsis. Further, by allowing the manipulation of endogenous pathways, RNAi-mediated suppression and the potential of genome-editing techniques has effectively compensated for the lack of Camelina lipid biosynthetic mutants. The ease therefore of manipulating lipid composition, combined with the ability to grow Camelina in the field, make it an ideal platform to develop industrial lipids in transgenic oil seed crops. While this review has focused on the production of industrial lipids, it is worthwhile noting that Camelina has recently also been used for the production of nutritionally valuable omega-3 fatty acids [141,142]. This work involved the expression of multiple desaturase and elongase genes to successfully reconstitute eicosapentaenoic acid (20:5) or docosahexaenoic acid (22:6) biosynthetic pathways. Likewise, Camelina has also been used as a platform for the synthesis of the polymer poly-3-hydroxybutyrate (PHB) [143]. It is therefore tempting to speculate that much future seed metabolic engineering will be performed in Camelina rather than Arabidopsis, allowing for more rapid introduction of plants capable of producing industrial seed oils into the field.

# Chapter 5 - Metabolic engineering of the oil seed crop *Camelina sativa* to generate acetyl glycerides containing medium chain fatty acids

## 5.1 Introduction

Seed oil from some species in the *Celastraceae* and *Lardizabalaceae* plant families contain high amounts of 3-acetyl-1,2-diacyl-*sn*-glycerol (acetyl-TAG) [6,7,144]. The presence of an acetyl group instead of a fatty acyl group at the *sn*-3 position of acetyl-TAGs provides them with different physical and chemical properties compared to conventional triacylglycerol (refered to as lcTAGs). For example acetyl-TAGs possess low calorific values, reduced viscosity and lower freezing temperatures than lcTAGs, which makes them suitable for applications such as a low viscosity biofuel [12,13]. In addition, synthetic acetyl glyceride mixtures are also used in various other industrial aplications such as emulsifiers, food coating agents and plasticizers [14].

The enzyme responsible for the production of acetyl-TAGs was identified from *Euonymus alatus* (Burning Bush) and named *Euonymus alatus* diacylglycerol acetyltransferase (*Ea*DAcT). It belongs to the membrane-bound O-acyltransferase (MBOAT) family and catalyzes the acetyl-CoA dependent acetylation of *sn*-1,2-DAGs at the *sn*-3 position [12]. DGAT1 and DGAT2 are two enzymes which produce lcTAGs by the acyl-CoA dependent acylation of DAGs [40]. While DGAT1 is also a member of MBOAT family [46], DGAT2 belongs to a different enzyme class due to low sequence similarity with DGAT1. Phospholipid : diacylglycerol acyltransferase (PDAT) also acylates DAG by transfering an acyl group from the *sn*-2 position of phosphotidylcholine (PC) or phosphotidylethanolamine (PE) to produce lcTAG [35]. The exact role of DGATs and PDAT in plant is still unknown but certain evidence suggest that DGAT1 is the major enzyme reponsible for TAG biosynthesis while DGAT2 and PDAT are more active in plants which accumulate unusual fatty acids [45]. Surprisingly, *Ea*DAcT was found to be more closely related to wax synthase and sterol acyltransferases [12,13] instead of DGAT1 with which it shares a common DAG substrate. *Ea*DAcT was successfully overexpressed in seeds of Arabidopsis, Camelina and Soybean without drastically affecting any

seed traits [13,65] and with high production levels of acetyl-TAGs (85 mol %) in Camelina indicating that *Ea*DAcT is a suitable candidate for producing acetyl-TAGs in seed oil crops.

The term straight vegetable oil (SVO) biofuel refers to the direct use of vegetable oil without any processing as a fuel in diesel engines. SVO offers various advantages over diesel oil such as being biodegrable, non-toxic and carbon neutral with low $SO_2$ and $NO_x$ emissions due to their plant origin [145]. However, SVO biofuels suffer from problems such as high viscosity and poor cold temperature properties [11]. The high kinematic viscosity (30-40 $mm/s^2$ at $40^oC$) of vegetable oils leads to their poor flow, atomization and vaporization causing injector nozzle deposits, incomplete combustion and high particulate emissions [146]. Either the use of modified engines which involve preheating of the oil or blending with diesel is required to avoid these difficulties [147,148]. Another option is to convert the oil to biodiesel (fatty acid methyl esters) by transmethylation [4]. However, these methods comes with the disadavntages such as the high cost involved with the modification of engines and environmental hazards associated with the disposal of crude glycerol generated during biodiesel production.

The viscosity of acetyl-TAGs falls in the range of diesel #4 (5-24 $mm/s^2$ at 40 $^oC$) suggesting they can be used as SVO biofuels for low and medium speed engines [12,13]. The fact that acetyl-TAGs can be produced in high quantities in the emerging oil seed crop Camelina [149] make them as ideal candidate for research involving further reduction in viscosity and improvement of other fuel properties. The kinematic viscosity of a triacylglycerides (TAGs) increases linearly with increasing molecular mass of constituent fatty acids [150,151]. Further, the reduced viscosity of acetyl-TAGs with lower average molecular mass compared to lcTAGs [12,13] is additional evidence that molecular mass is an important factor which determines the viscosity of TAGs. Hence, decreasing the average molecular mass of acetyl-TAGs is predicted to lead to further reduction in viscosity.

Medium chain length fatty acids (MCFAs) having carbon chain lengths between C8 to C14 are produced in high quantities in the seed oil of plants such as Cuphea, palm kernel and coconut [2,152]. They are produced by the action of specific thioesterases which cleave the growing acyl-ACP to release shorter fatty acids [153,154]. MCFAs were successfully produced and incorporated in the seed oil of Camelina by transgenic coexpression of MCFA specific thioesterases and acyltransferases [155]. For example, coexpression of California Bay thioesterase (*Uc*FatB1) and coconut lysophophatidyl acyltransferase (*Cn*LPAAT) genes, specific

for the production and eventual transfer of lauric acid to lysophosphatidic acid respectively, in Camelina resulted in nearly 28% lauric acid (12:0) production. Similarly, expression of Cuphea thioesterases *Ch*FatB2 and *Cp*FatB2 alone or in combination resulted in 10% of capric acid and 25% of lauric and myristic acid in Camelina, respectively.

This study was conducted with the aim of further reducing the viscosity of acetyl-TAGs further by incorporation of medium chain fatty acids (MCFAs). Overexpression of *Ea*DAcT combined with DGAT1 and PDAT downregulation in oil seed crop Camelina lines producing high levels of MCFAs was successful in achieving high amounts of acetyl-TAG levels. However, incorporation of MCFAs in to acetyl-TAGs was inefficient in these lines. The accumulation of high acetyl-TAGs did not affect germination but caused a small reduction in seed oil content. The kinematic viscocity of acetyl-TAGs containing small amounts of MCFAs was also found to be increased compared to acetyl-TAGs produced in wild type Camelina. The possible reasons for low incorporation of MCFAs in acetyl-TAGs and increase in their viscosity are discussed.

## 5.2 Materials and Methods

### 5.2.1 Materials

The agrobacterium strain GV3101 was kindly provided by Dr Kathrin Schrick (Kansas State University). Heptadecanoin and pentadecanoin were purchased from Nu Chek Prep Inc. (Waterville, MN).

### 5.2.2 Plant transformation vectors and generation of transgenic plants

Binary expression vectors of pBlb series (pBinGlyRed2 backbone) were kindly provided by Dr John Ohlrogge (Michigan State University). Plasmids expressing *Ea*DAcT alone or also with DGAT RNAi and/or PDAT RNAi [13,65] were modified by insertion of basta herbicide resistance gene (*BAR*) from *Streptomyces hygroscopicus* [156]. The *BAR* gene along with NOS promoter was amplified from the plasmid pBinGlyBar1 [99] using the primer sequences 5’-GCAGAGCTCGATCTCGGTGACGGGCAGGACCGGA-3’ and 5’-AGCGATCGGCACGCTGCCGCAAGCACTCAGGGC-3’ digested with Sac1 and Pvu1. The amplified fragments were cloned in to corresponding sites in the pBlb series of vectors. The orientation of transgenes and promoters used for *Ea*DAcT expression and for RNAi constructs are summarized schematically in Fig. 5.1.

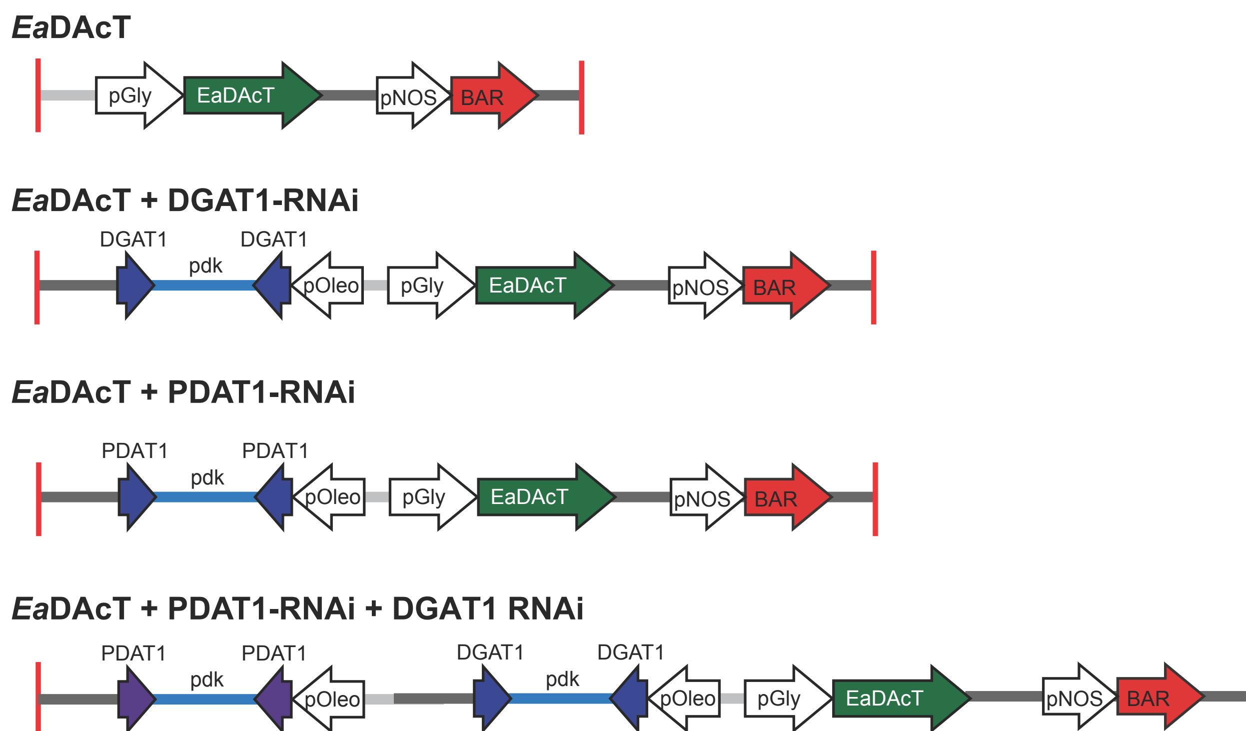



Figure 5.1 **Constructs used to express *Ea*DAcT and suppress Camelina acyltransferases**. Shown are the regions flanked by T-DNA borders (vertical red lines). Promoter regions are indicated by white arrows, terminator sequences are represented by dark gray lines. DGAT1, portion of Camelina DGAT1 gene; BAR, *Streptomyces hygroscopicus* protein phosphinothricin acetyltransferase; *Ea*DAcT, native *Ea*DAcT coding sequence; pCMV, Cauliflower Mosaic virus 35S promoter; PDAT1, portion of Camelina PDAT1 gene; pdk, pdk intron from pHANNIBAL; soybean glycinin promoter; pOleo, *Brassica napus* oleosin promoter.

Transgenic Camelina lines producing medium chain fatty acids [155] were kindly provided by Dr. Edgar Cahoon (University of Nebraska, Lincoln). Each of the four lines, *Ch*FatB2, *Ch*FatB2 + *Cp*FatB2, *Uc*FatB1 + *Cn*LPAAT, and *Cp*FatB2 + *Uc*FatB1 was transformed with all four different vectors using Agrobacterium mediated floral dip vacuum infiltration [98]. Positive transformants were selected based on Basta herbicide resistance in $T_1$ seedlings. All the $T_1$ seeds were grown to the 4 leaf stage after which they were sprayed with 0.01 % (w/v) Basta herbicide solution (Bayer crop science, Raleigh, NC) four times at intervals of 2 days between each spray. The survivors were selected and further propagated to obtain $T_2$ seeds. 16 $T_2$ seeds from survivors were planted and screened for Basta resistance. The $T_3$ plants from the lines segregating with 3:1 (survivors: non survivors) ratio and passing the chi-square test were further propagated to collect $T_3$ seeds. 16 seeds from each surviving plant of a $T_3$ line were further screened. The plant with all the seeds surviving the Basta spray was designated as a

homozygous plant. All the homozygous lines obtained from each independent transformation event were further tested for acetyl-TAG content.

### 5.2.2 Lipid analysis

Approximately 30 mg of dried seeds were ground in 2.0 ml of hot isopropanol using a 7.0 mm Polytron probe (Kinematica Inc. Bohemia, NY). Tripentadecanoin (100 µg ) was added as internal standard. To the ground seeds, 3.0 ml of hexane and 2.5 ml of 6.6 % w/v $K_2SO_4$ were added. Tubes were vortexed and organic phase was separated by centrifugation and collected in a separate tube. Lipids were extracted one more time with 2 ml hexane/ isopropanol (7:2). The organic phases were combined and evaporated under nitrogen. The lipids were resuspended in 1.0 ml of toluene and stored at -20 $^oC$ till further analysis.

Lipid extract (100 µl) was loaded on Silica gel 60 TLC plates and developed using a hexane/diethyl-ether/acetic acid (70:30:1) solvent system to separate acetyl-TAGs and lcTAGs. The TAGs were stained with 2,7- dichlorofluorescein and visualized under UV light. Heptadecanoin (100 µg) was added to each band. The bands were scraped, resuspended in 1.0 ml of hexane and directly transmethylated using a base catalyzed mechanism [53]. FAMEs were analysed by gas chromatography using a DB-23 capillary column with a flame ionization detector [73]. The area of FAMEs peaks were corrected for FID response, normalized to the internal standard and summed to quantify total FAMEs as moles. The moles of acetyl-TAGs and lcTAGs were calculated by dividing the number of moles of FAMEs by 2 and 3 respectively.

For the ESI-MS analysis, samples were prepared by dissolving total lipid extract from transgenic seeds in 300 µL of chloroform and 700 µL of methanol/ammonium acetate (100:5.26) was added. The samples were then directly infused in to a triple quadrupole mass spectrometer (API4000, Applied Biosystems, Foster City, CA) operated under preset condition for MS1 positive scan mode to detect all the acetyl-TAGs and lcTAGs. Samples were also scanned for the neutral loss of ammonium acetate (77.10) in a MS/MS mode under the conditions described in chapter 2 to specifically detect acetyl-TAG molecular species.

### 5.2.3 Positional analysis of TAGs

The *sn*-2 fatty acid composition of acetyl-TAGs and lcTAGs in $T_4$ seed oil were determined by using the *sn*-2 specific lipase of *Thermomyces lanuginosus* (Sigma- Aldrich, St. Louis, MO). 1.5 mg of TLC purified acetyl-TAGs and lcTAGs was dissolved in 1.0 ml of diethyl ether. Lipase

(5000 units) in 1.0 ml of Tris buffer (50 mM, pH 7.2) was then added. The digestion was performed by incubating the samples at 37 $^{o}$C for 30 minutes under shaking conditions. After the reaction, lipids were extracted twice using 3.0 ml of diethyl ether. The solvent was evaporated under $N_2$ and lipids were resuspended in 100 µl of chloroform. The lipids were separated on a boric acid impregnated silica gel plates using a chloroform/acetone (80:10) solvent system to separate 1-monoacylglycerol (MAG) and 2-MAG. Tripentadecanoin (10 µg) was added to the bands corresponding to 2-MAG. Bands were scraped and lipids were extracted using 2.0 ml of toluene. The solvent was evaporated and lipids were redissolved in 500 µl of hexane and transmethylated using base catalyzed reaction as described earlier. FAMEs were quantified by gas chromatography and represented as mol %.

### 5.2.4 Seed trait analysis

Seeds from $T_3$ homozygous lines showing high acetyl-TAGs/lcTAG ratios were further propagated to the $T_4$ generation. Four seeds were planted from each of wild type, 3 parental lines ($T_6$ seeds) and 5 different transgenic lines ($T_4$ seed) in 4 pots (3.5" x 6" deep). 36 pots were arranged in a random fashion in two flats (10" x 20") and kept at 21 $^{o}$C under 18 hours light and 6 hours dark cycles in a growth chamber. The two highest acetyl-TAGs producing lines were again selected to grow up to $T_5$ generation under similar conditions. The acetyl-TAG contents (mol %) were analyzed as described above. The oil content of the seeds was analyzed gravimetrically. Approximately 100 mg of $T_4$ seeds were dried to a constant weight in a desiccator. Dried seeds were ground using a polytron and lipids were extracted using hexane/isopropanol as described earlier. The lipids were dried to constant weight in a desiccator and weighed in a precision balance (Mettler Toledo XS105, Mettler-Toledo LLC, Columbus, OH) with an accuracy of ± 0.1 mg. The oil content is calculated as percent dry seed weight basis.

Germination testing was performed using $T_4$ seeds on soil and on MS medium. 36 seeds were sterilized using fumigation and planted on ½ strength MS medium without sucrose. Plates were incubated 21 $^{o}$C under 18 hours light and 6 hours dark cycle in an incubator. Similarly, 36 seeds were planted on the soil in pots and incubated in the growth chamber at 21 $^{o}$C under 18 hours light and 6 hours dark cycle. The plates and pots were monitored every day for the emergence of cotyledons.

### 5.2.5 Purification of acetyl-TAGs and lcTAGs and viscosity analysis

To generate large amount of oil for viscosity analysis, $T_5$ plants from the two highest acetyl-TAG yielding lines and $T_7$ plants from corresponding parental lines were grown on a bigger scale, first in the growth chamber up to flowering stage and then in a green-house under conditions mentioned above. For a single round of oil extraction, 25 g seeds were ground and packed in to a cellulose thimble (33 x 118 mm). The thimble was fitted into a soxhlet apparatus and the oil was extracted by continuous reflux of hexane through the seeds. The samples were reduced in volume using a rotavap and dried to a constant weight in 50 ml glass tubes under $N_2$ gas. The oil was stored at 4 $^oC$ until further purification.

The crude oil was fractionated on a 1000 ml silica gel column (diameter 64 mm). For every 20 g of oil, 400 g of silica gel was used. The silica gel was first saturated with hexane. 50 ml fractions were collected using step gradient with increasing diethyl ether in hexane: from 100:0, 95:5, 90:10, 80:20 to 70:30 (v/v). Fractions were tested for purity using TLC and a hexane/diethyl ether/acetic acid (70:30:1) solvent system. The fractions containing either pure acetyl-TAGs or lcTAGs were combined. Samples were reduced in volume using a rotavap and dried to constant weight under $N_2$ to obtain purified acetyl-TAGs and lcTAGs. The purity of the fractions was confirmed using ESI-MS. The kinematic viscosity of purified acetyl-TAGs and lcTAGs was measured using a calibrated Ubbelohde viscometer (Cannon Instruments, State College, PA) according to the ASTM D445 method [157].

## 5.3 Results and Discussion

### 5.3.1 DGAT1 knockdown resulted in high levels of acetyl-TAGs in Camelina

In our yeast studies it was seen that *Ea*DAcT could acetylate medium chain fatty acids containing DAGs to produce acetyl-TAGs. Hence, *Ea*DAcT was expressed in Camelina lines producing high levels of medium chain fatty acids (MCFA) to produce acetyl-TAGs with low molecular weight. These MCFA lines were generated by the seed specific expression of MCFA producing thioesterases (FatBs) and MCFA specific lysophosphatidic acid acyltransferases (LPAATs) and can produce up to 53 mol % MCFAs in their seed lipids [155]. *Ea*DAcT was expressed in four different Camelina genetic backgrounds each producing different types and amounts of MCFAs (Table 5.1).

Table 5.1 **Fatty acid compositions of seed lipids from MCFA producing Camelina background used in this study**

| Genotype | 8:0 | 10:0 | 12:0 | 14:0 | 16:0 | 18:0 | 18:1 | 18:2 | 18:3 | 20:0 | 20:1 | 22:1 | MCFA (C8-C14) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Wild type** | - | - | - | - | 8.9 ± 0.9 | 4.0 ± 0.7 | 10.6 ± 0.7 | 25.1 ± 1.1 | 40.5 ± 2.0 | 1.6 ± 0.0 | 7.5 ± 0.5 | 1.8 ± 0.1 | **0** |
| ***Ch*FatB2** | 0.7 ± 0.1 | 10.3 ± | 0.9 ± 0.0 | 0.7 ± 0.0 | 9.0 ± 0.3 | 4.7 ± 0.3 | 14.8 ± 0.6 | 30.0 ± 0.3 | 19.9 ± 0.8 | 1.9 ± 0.1 | 5.8 ± 0.1 | 1.2 ± 0.0 | **12.6** |
| ***Ch*FatB2 + *Cp*FatB2** | - | 8.7 ± 0.5 | 0.8 ± 0.5 | 9.3 ± 0.0 | 12.4 ± 0.2 | 3.4 ± 0.0 | 21.6 ± 0.9 | 16.2 ± 0.4 | 16.1 ± 0.5 | 2.1 ± 0.0 | 7.4 ± 0.1 | 1.9 ± 0.0 | **18.8** |
| ***Uc*FatB1 + *Cn*LPAAT** | - | - | 28.4 ± 1.5 | 3.8 ± 0.2 | 5.6 ± 0.4 | 3.1 ± 0.6 | 7.4 ± 0.4 | 13.6 ± 0.3 | 30.4 ± 0.7 | 1.2 ± 0.1 | 4.9 ± 0.3 | 1.5 ± 0.1 | **32.2** |
| ***Cp*FatB2 + *Uc*FatB1** | - | - | | 36.9 ± 1.9 | 16.8 ± 0.6 | 3.0 ± 0.3 | 3.1 ± 0.3 | 19.2 ± 0.6 | 16.0 ± 2.0 | 1.9 ± 0.2 | 2.3 ± 0.2 | 0.9 ± 0.1 | **53.7** |

Adapted from Kim et al., 2015

*Ea*DAcT might compete with DGAT1 and PDAT enzymes for the same DAG pool in seeds for acetyl-TAG synthesis [13,65]. Hence, it was hypothesized that knocking down these two enzymes might increase DAG flux to *Ea*DAcT, resulting in higher acetyl-TAG synthesis. Therefore, *Ea*DAcT expression was combined with the RNAi mediated suppression of either DGAT1 or PDAT alone or in combination. Because of the hexaploid nature of the Camelina genome [100], each gene is present as three homeologous copies. Hence, to effectively reduce gene expression, RNAi constructs designed to have common complementary sequence from all the three homoeologues of DGAT1 and PDAT RNAi gene were used to knockdown all the three copies of the DGAT1 and PDAT genes in transgenic Camelina [12].

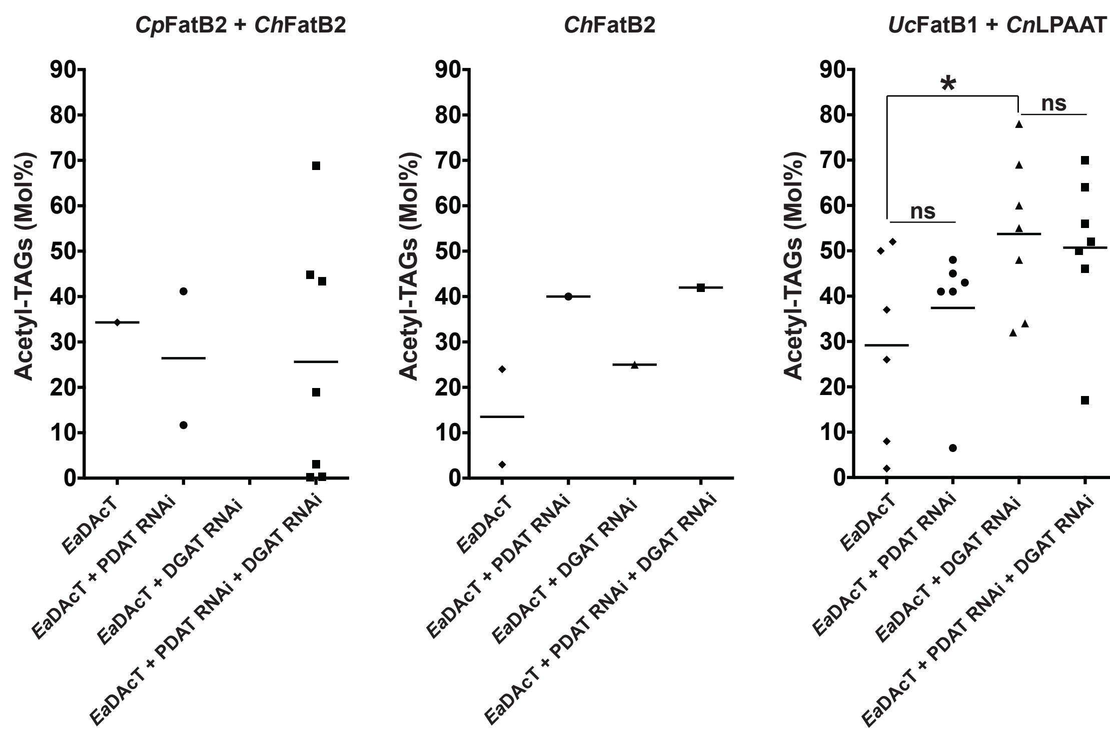



Figure 5.2 ***Ea*****DAcT expression with simultaneous DGAT1 knockdown results in high levels of acetyl-TAGs in Camelina.** Scatter plots of the distribution of acetyl-TAG composition of $T_3$ seeds from independent homozygous Camelina lines expressing medium chain specific fatty acyl thioestersases (FatB), lysophosphatidyl acyltransferases (LPAAT) along with *Ea*DAcT alone or in combination with RNAi constructs targeting Camelina DGAT11 and PDAT homeologues. Horizontal lines represent the mean values for each sample group. Asterisks indicate significant difference ($p<0.05$; t-test). ns, not significant.

The number of independent $T_3$ lines needed to test our hypothesis with statistical significance was obtained for one Camelina line with the genotype *Uc*FatB1 + *Cn*LPAAT. The acetyl-TAG contents of a total of 23 independent $T_3$ homozygous or near homozygous lines were determined. The levels of acetyl-TAG achieved in some of the best $T_3$ lines were in the range of 64-77 mol %. These acetyl-TAG levels are consistent with levels reported (74-86 mol %) for wild type Camelina expressing *Ea*DAcT [13]. The results showed that only DGAT1 knockdown resulted in significant increase in the acetyl-TAG accumulations over *Ea*DAcT expression alone (Fig. 5.2). *Ea*DAcT expression along with PDAT knockdown produced similar acetyl-TAG levels as *Ea*DAcT alone expressing lines while PDAT knockdown combined with DGAT1 knockdown did not result in any further acetyl-TAG increase over DGAT1 knockdown alone. Previous studies in Arabidopsis and Camelina showed similar results [13,65]. The authors

attributed these effects to either detrimental effects of the combined knockdown of PDAT and DGAT1 on embryo and seedling development or inefficient suppression of PDAT expression by RNAi mediated knockdown. Future work will involve expression of *Ea*DAcT in DGAT1 and PDAT complete knockout lines for these MCFA Camelina lines to achieve even higher levels of acetyl-TAGs. The generation of such DGAT1 and PDAT knockout lines can be achieved by emerging precise genome editing technique like CRISPR/Cas9 [158] in the near future.

No transformants expressing *Ea*DAcT in combination with DGAT1 and/or PDAT RNAi constructs were observed for Camelina with *Cp*FatB2 + *Uc*FatB1 genetic background. It might be due the strong lethal effects of DGAT1 and PDAT knockdown on seed and embryo viability even in heterozygous conditions in this particular background. However, two independent $T_3$ lines expressing *Ea*DAcT alone was obtained for this background with acetyl-TAG levels of 3 mol % and 17 mol % (data not shown). Only a small number of independent $T_3$ homozygous lines for *Ea*DAcT and RNAi expression were obtained for two of the other three transgenic MCFA producing Camelina parental lines (Fig. 5.2). The best lines from background *Cp*FatB2+ *Ch*FaTB2 and *Ch*FaTB2 have acetyl-TAG levels of 69 mol % and 42 mol % respectively. Although a higher number of $T_2$ heterozygous lines were obtained for these two parental lines but for most of them we were unable to isolate homozygous $T_3$ lines (data not shown). This might due to enhanced negative effects of DGAT1 and PDAT knockdown on the seed viability in $T_3$ generation due to increased gene dosage effect in these genotypes.

### 5.3.2 *Ea*DacT can produce medium chain acetyl-TAGs in Camelina

$T_3$ seeds from homozygous transgenic lines were tested for the presence of medium chain acetyl-TAGs. The ESI-MS analysis of total lipids from these transgenic lines showed the presence of acetyl-TAG with lower molecular mass peaks compared to wild type Camelina expressing *Ea*DAcT (Fig 5.3a). The lower molecular mass of these peaks suggested the incorporation of MCFAs in to acetyl-TAGs. Further confirmation of presence of MCFAs in these low molecular mass acetyl-TAG species was revealed by daughter ion scans of the parent molecule (Fig. 5.3b).

These results proved that *Ea*DAcT could synthesize acetyl-TAGs containing MCFAs. *Ea*DAcT was able to acetylate DAGs such as 1,2-dilaurin-*sn*-3-glycerol and 1,2-myristoyl-*sn*-3-

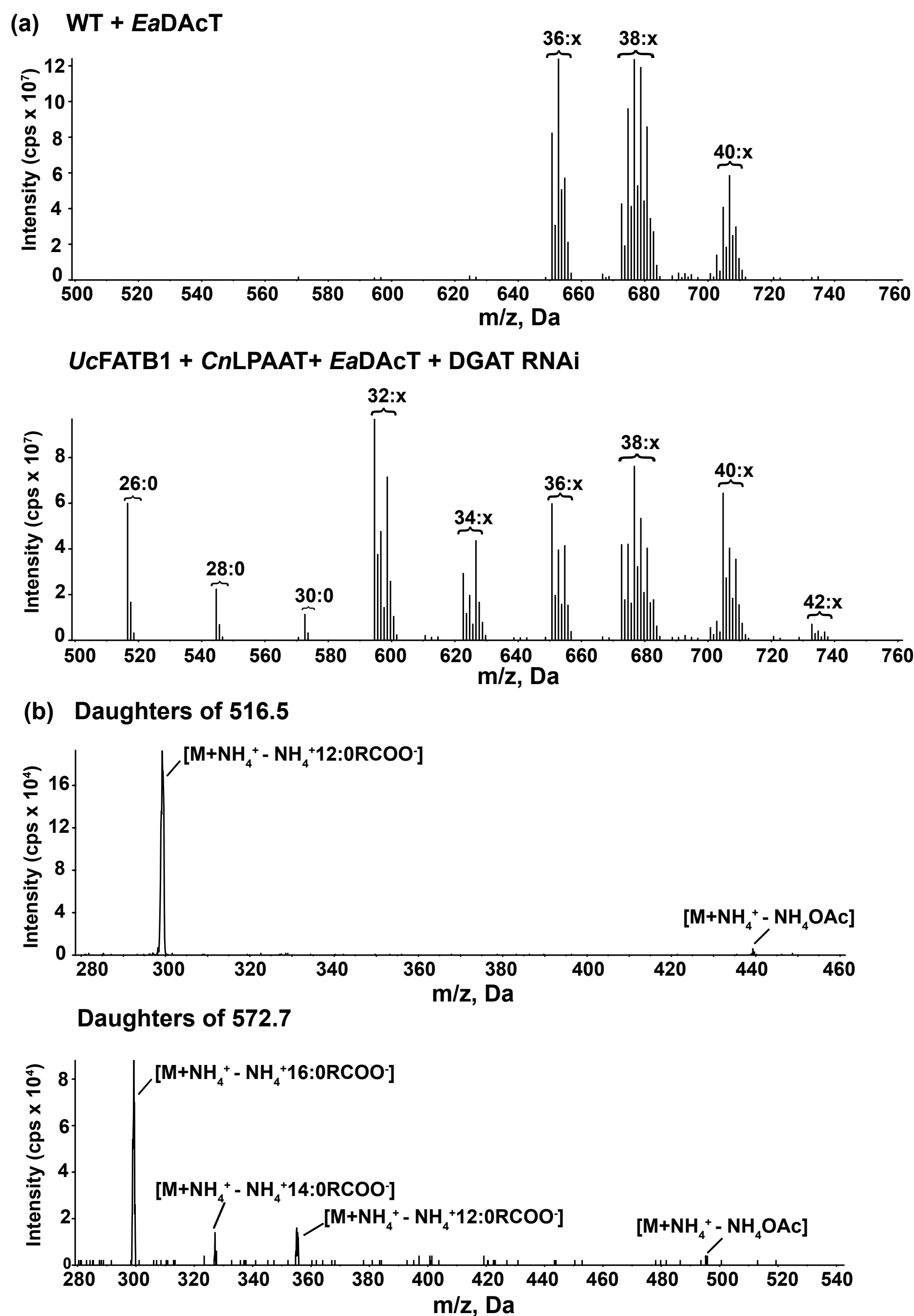



Figure 5.3 ***Ea*DacT can produce medium chain acetyl-TAGs in Camelina.** (a) Positive ESI-MS$^2$ spectra scanning from the neutral loss of ammonium acetate to detect acetyl-TAGs molecular species. Lipids were extracted from wild type plant and MCFA producing Camelina $T_3$ transgenic seeds expressing *Ea*DAcT. Signal peaks possess the m/z value of $[M+NH_4]^+$ adduct. For clarity, only the number of acyl carbons and not the number of double bonds (x) in each series of acetyl-TAG molecular species is indicated. (b) ESI-MS$^2$ daughter scans of medium chain acetyl-TAGs from Camelina seed expressing *Ea*DAcT. Shown are the fragment peaks for acetyl-TAGs with $[M+NH_4]^+$ adducts with masses of 516.5 and 572.7.

glycerol to synthesize acetyl-dilaurin (26:0) and acetyl-dimyristin (30:0) respectively. These results are consistent with our in vitro and in vivo results in yeast (Chapter 3).

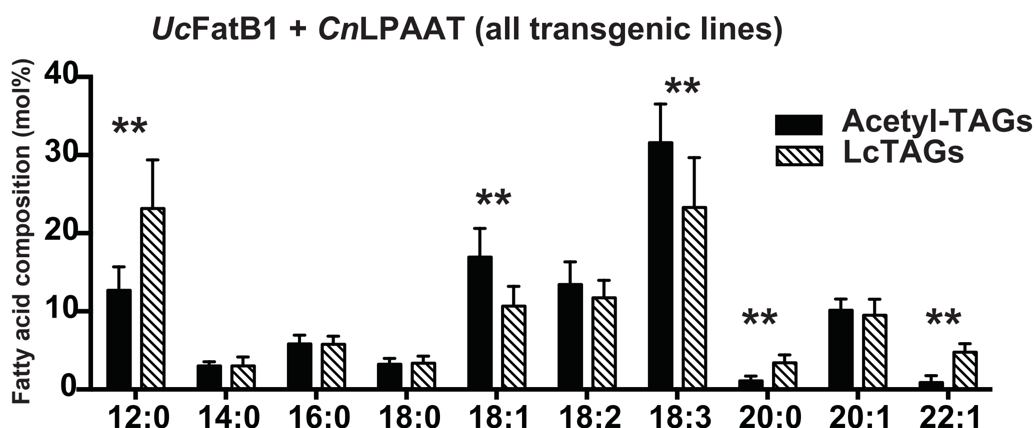



Figure 5.4 **Acetyl-TAGs contain lower levels of MCFA than lcTAGs.** Mean fatty acid composition of acetyl-TAGs and lcTAG fractions from the $T_3$ seed of 23 independent homozygous lines expressing *Ea*DAcT and DGAT RNAi. Error bars represent SD from mean. ** indicates $p < 0.01$; unpaired *t*-test.

### 5.3.3 Acetyl-TAGs contain lower levels of MCFA than lcTAGs

The Camelina line with genotype *Uc*FatB1 + *Cn*LPAAT produces 28 mol % 12:0 as the major MCFAs in the seed lipids [155]. To determine the amount of MCFAs incorporated in to acetyl-TAGs, fatty acid compositional analysis of acetyl-TAGs and lcTAGs from all the *Ea*DAcT expressing transgenic lines obtained from this background line was performed. Acetyl-TAGs were found to have lower concentrations of lauric acid (12.2 ± 3.5 mol %) compared to lcTAGs (23.2 ± 6.2 mol %) (Fig. 5.4).

The unsaturated fatty acids oleic acid and linolenic acid were significantly higher in acetyl-TAGs than lcTAGs. This is consistent with in vitro results where *Ea*DAcT showed high preference for DAGs containing unsaturated fatty acid (Chapter 3). This relative enrichment of unsaturated fatty acid and reduction of very long chain fatty acids (VLCFA) in acetyl-TAGs compared to lcTAGs is consistent with previous results [13]. This effect is proposed to be a consequence of expected enrichment of VLCFA at *sn*-1 and *sn*-3 positions of lcTAGs [159]. A similar trend of medium and long chain fatty acid incorporation in acetyl-TAGs and lcTAGs was observed in lines with other backgrounds.

### 5.3.4 Inefficient incorporation of MCFAs in acetyl-TAGs is due to their low abundances at *sn*-2 position

DAGs are synthesized in the Kennedy pathway by sequential acylation of glycerol-3-phosphate at *sn*-1 position by GPAT and *sn*-2 position by LPAAT [115]. In *Brassica napus,* a close relative of Camelina, endogenous GPATs and LPAATs are known to preferentially acylate unsaturated 16 and 18 carbon-containing acyl groups on *sn*-1 and *sn*-2 positions respectively [160]. In the Camelina line *Uc*FatB1 + *Cn*LPAAT used in this study, medium chain acyl-CoA specific *Cn*LPAAT [161,162] is expected to be in competition with endogenous LPAATs to produce DAG. Due to three copies of endogenous LPAAT compared to one copy of *Cn*LPAAT, a higher endogenous LPAAT activity than *Cn*LPAAT activity is a strong possibility. This lower *Cn*LPAAT activity might further result in to low incorporation of MCFA at *sn*-2 position of DAGs. The low incorporation of MCFA at *sn*-2 positions of DAGs will result in their lower incorporation in acetyl-TAGs and lcTAGs. The effect was expected to affect MCFA incorporation in to acetyl-TAG more than lcTAGs due to the simple reason that DGAT1 can add one more MCFAs in to lcTAGs by acylation at *sn*-3 position whereas *Ea*DAcT will only transfer an acetate group at *sn*-3 position. Because of this reason, incorporation of high levels of MCFAs into acetyl-TAGs is particularly dependent on the efficient incorporation of MCFAs at the *sn*-2 position of DAGs.

Indeed, analysis of stereospecific fatty acid composition revealed relatively low amounts of lauric acid compared to 18 carbon unsaturated fatty acids at the *sn*-2 positions of acetyl-TAGs and lcTAGs (Fig. 5.5) suggesting that endogenous LPAAT activity dominated over *Cn*LPAAT activity. This in turn means that DAG pools in seeds had lower concentration of DAGs containing MCFA compared to DAGs with 18 carbon unsaturated fatty acids. The lower amounts of DAGs containing MCFAs could also be a result of insufficient MCFA available for *Cn*LPAAT.

This trend of DAG composition might be directly related to acetyl-TAG fatty acid composition with low abundance of 12:0 compared to unsaturated fatty acids (Fig 5.3). Incorporation of low amounts of 20:0 and 22:1 in to acetyl-TAGs can be also be explained by their low enrichment at *sn*-2 positions (Fig 5.5). Similarly, higher incorporation of 18:3 fatty acid in to acetyl-TAGs can be directly correlated to its high abundance at the *sn*-2 position of TAGs as reported in Arabiodopsis [159]. One of the Camelina transgenic line *Cp*FatB2 + *Ch*FaTB2

without any *Cn*LPAAT showed even lower amounts of MCFA at *sn*-2 positions which was reflected in very low incorporation of MCFA in the acetyl-TAGs and lcTAGs in that line (data not shown). This further substantiates the fact that *sn*-2 incorporation of MCFA is crucial for

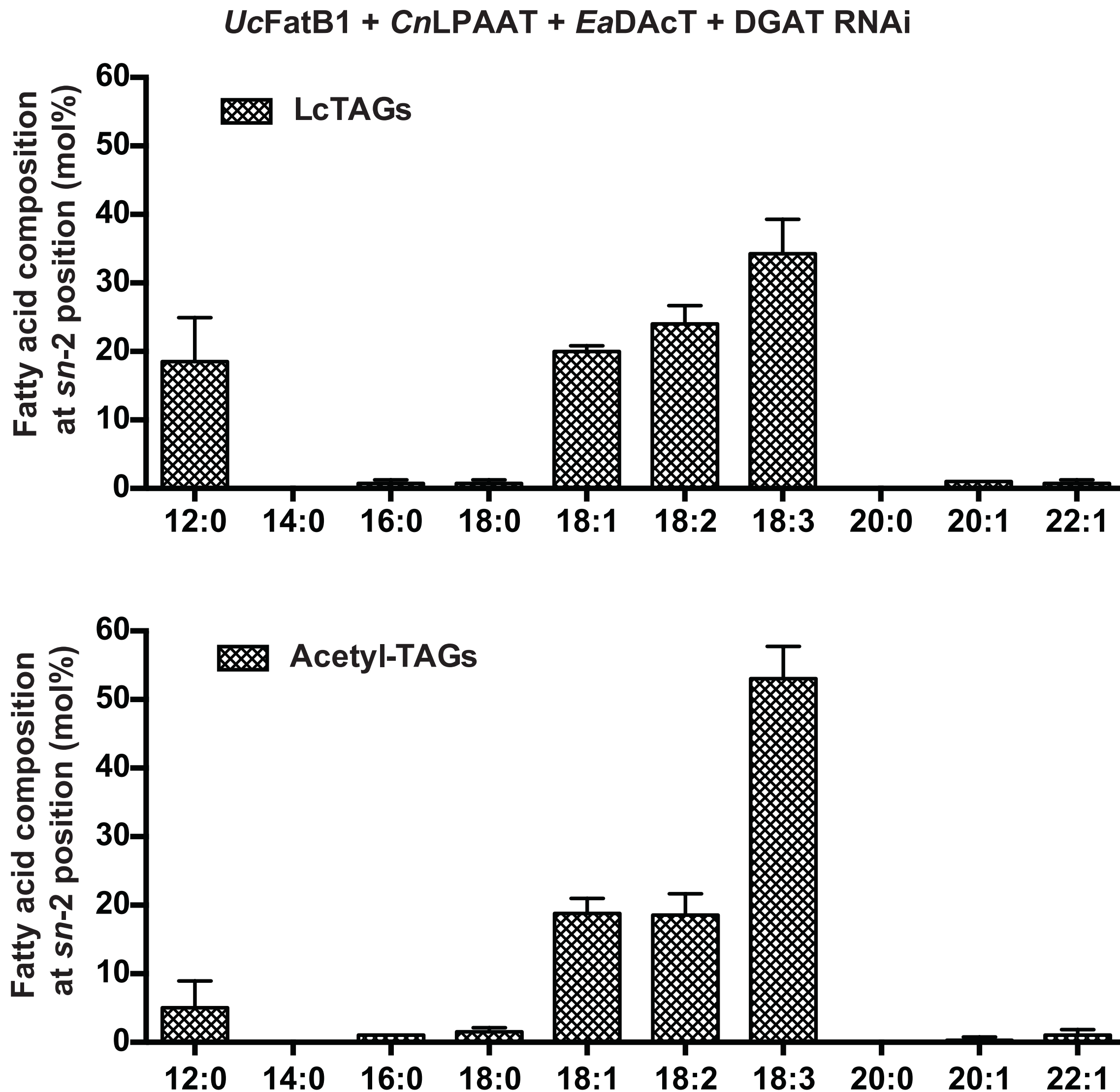



Figure 5.5 **Inefficient incorporation of MCFAs in acetyl-TAGs is partially due to their low abundance at *sn*-2 position**. Mean fatty acid composition at *sn*-2 positions of lcTAGs and acetyl-TAGs from the $T_4$ seed of 4 independent homozygous Camelina *Uc*FatB1 + *Cn*LPAAT lines expressing *Ea*DAcT gene in combination with DGAT1 RNAi. Error bars represent SD from mean (n=4)

their final incorporation in to acetyl-TAGs. This result is also consistent with results from previous study [13]. It will be interesting to see if knockdown of native LPAATs in these

Camelina lines can enhance the MCFA incorporation at *sn*-2 position in DAGs and subsequently in acetyl-TAGs. Another approach could be expression of newer LPAATs from Cuphea [163] along with *Cn*LPAAT to further enhance MCFA incorporation in to DAGs.

### 5.3.5 Analysis of seed traits of acetyl-TAG producing transgenic lines

To determine the effect of high acetyl-TAG accumulation in the MCFA producing parental lines on seed, various performance parameters were tested.

#### 5.3.5.1 High acetyl-TAG production trait is stable

The problem of foreign gene silencing in transgenic crops due to epigenetic effects is very well known [164,165]. As our high yielding Camelina lines are harbouring at least 6

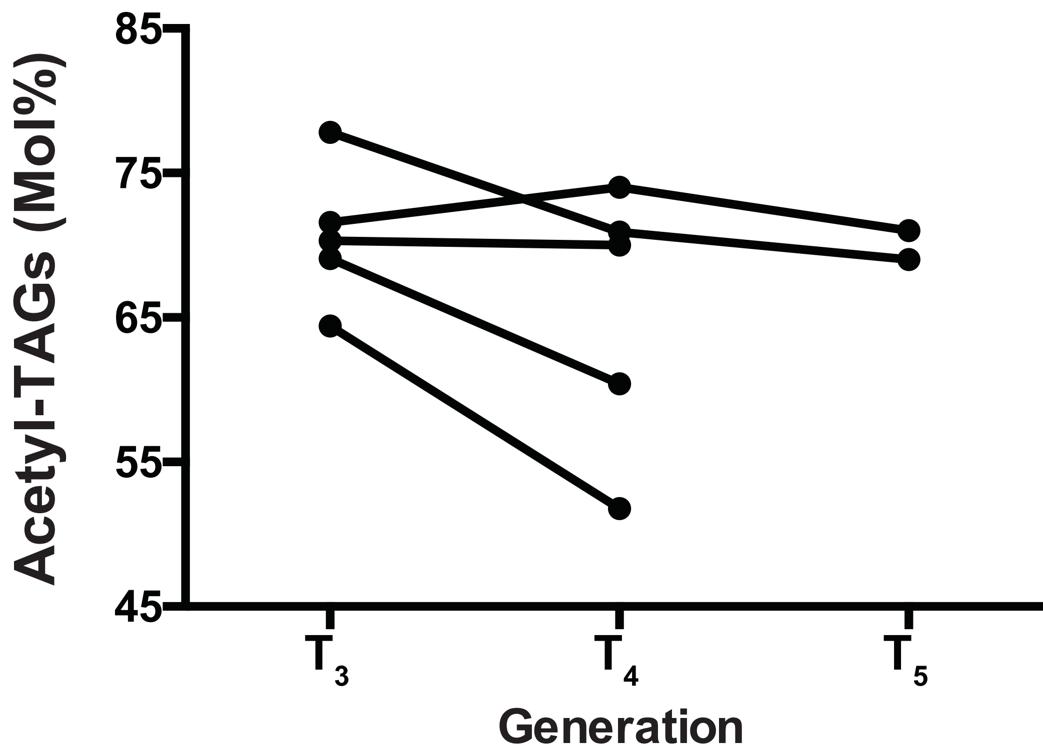



Figure 5.6 **High acetyl-TAG production trait is stable**. Acetyl-TAG content of 5 independent homozygous high yielding medium chain acetyl-TAGs producing Camelina lines across different generations.

different transgenes, either diminished trait or complete loss of transgenic trait over generations is highly plausible. Hence we monitored acetyl-TAG levels in some of the high acetyl-TAG yielding lines up to the $T_5$ generation.

The acetyl-TAG content of 3 out of 5 independent high yielding homozygous lines showed small variations from $T_3$ to $T_4$ generation (Fig. 5.6). The small changes in acetyl-TAG levels in most lines between generations might have been caused by effect of variability in the growing conditions and/or differential levels of suppression of DGAT1 and PDAT by their RNAi. However, for the two highest acetyl-TAG yielding lines, acetyl-TAG levels were relatively stable up to the $T_5$ generation. This is important especially in a case where these lines need to be further improved by breeding with lines possessing other important commercial seed traits. The large change in one line may be due to partial gene silencing either due to epigenetic factors or transcriptional gene silencing. Overall, these results indicated that the acetyl-TAG production trait was inherited quite stably across generations in these transgenic lines.

**5.3.5.2 Oil content was reduced slightly in the high acetyl-TAGs yielding lines**

To determine the effect of *Ea*DAcT expression and DGAT1 and PDAT knockdown on total seed oil content in MCFAs producing lines, the oil content of $T_4$ seeds from high yielding lines was analyzed gravimetrically. The value of oil content for wild type seeds was 27.6 % DW which was much lower than that of reported values of ~39% [13,65].

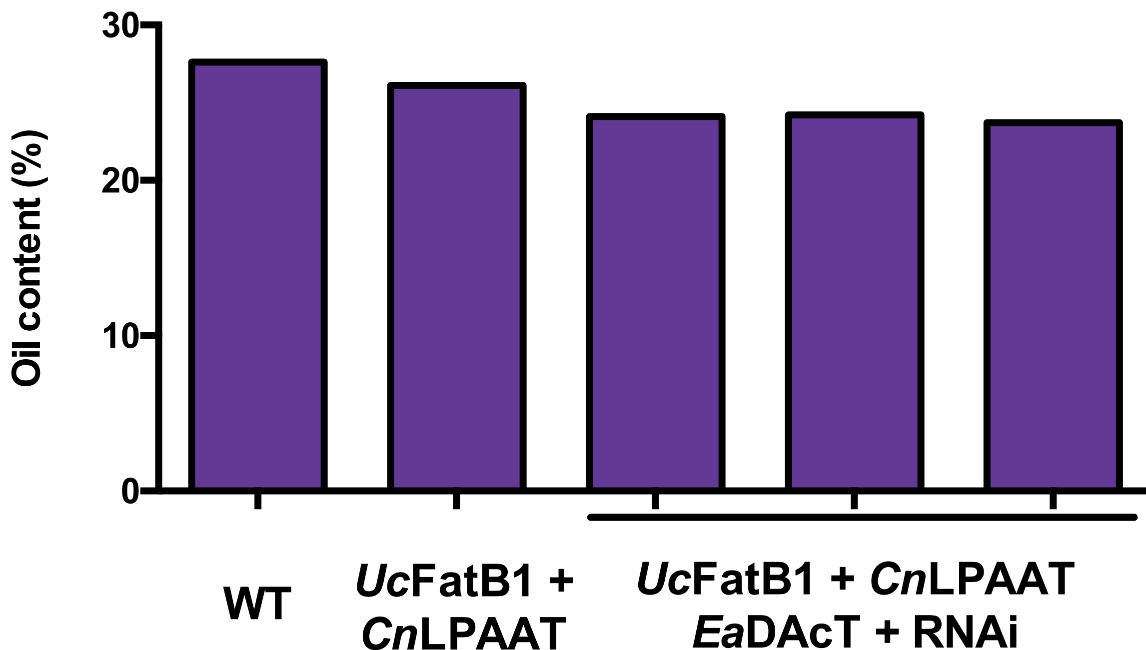



Figure 5.7 **Oil content was reduced slightly in the high acetyl-TAGs yielding lines**. Seed oil content of wild type Camelina, MCFA Camelina line (*Uc*FatB1+*Cn*LPAAT) and three independent homozygous lines ($T_4$ seeds) expressing *Ea*DAcT and DGAT1 RNAi

This might be due to different growing conditions from those studies which were conducted at large scale levels in the fields compared to our studies which were done in growth chambers in pots. However, a small decrease was observed between the high acetyl-TAG yielding lines and the control parental line (Fig. 5.7).

For example the parental line with *Cn*LPAAT and *Uc*FatB1 genes has oil content of 26.1% of DW while the high yielding lines obtained from this parental line showed oil contents in the range between 23.7 to 24.2% with a decrease of 7.1 to 9.0%. Previous studies have also reported similar level of reductions in a DGAT1 mutant line in Arabidopsis [20] and DGAT1 knockdown lines in Camelina [13,65]. Two possible reasons for low oil contents were suggested in the latter Camelina studies. One was that substrates such as acetyl-CoA and glycerol-3-phosphate for the synthesis of acetyl-TAG might be limiting. The other reason might be the relatively late expression of *Ea*DAcT under the glycinin promoter versus the earlier knockdown of DGAT1 that is under the oleosin promoter [65]. These hypotheses might be tested in future in acetyl-CoA overproduction lines or by expression of both *Ea*DAcT and DGAT1 RNAi under the same promoter.

**5.3.5.3 Germination is not affected in high yielding transgenic lines**

TAGs are a major metabolic reserve in seeds and are required for proper germination. To test the effect of presence of high levels of acetyl-TAGs on seed germination, $T_4$ seeds from high acetyl-TAGs yielding lines were tested for germination on soil and MS medium. Germination was defined as complete emergence of cotyledons [166].

Seeds from two parental MCFA lines germinated at a rate of 89% and 97% on soil. All the high acetyl-TAG yielding lines showed germination rates of 89% or above, except for one line with a lower (81%) rate of germination (Table 5.2). Because the other lines expressing the same genes are not affected, the lower germination rate might be due to a deleterious insertion effect in that particular line. A smaller seed size and lower oil content compared to the other transgenic line was also noted for this line (data not shown). Similar trends were also observed when the seeds were germinated on MS medium with an even higher germination percentage for most of lines (Table 5.2). Because TAGs are major storage reserve and need to be mobilized during the germination it can be concluded that all TAGs were mobilized in these lines. Previous studies have also reported efficient breakdown of acetyl-TAGs and lcTAGs during germination in high acetyl-TAG yielding Camelina lines [13].

Table 5.2 **Germination test of seeds from high acetyl-TAG yielding transgenic lines**

| Genotype | Line number | Germination (%) | |
|---|---|---|---|
| | | **On soil** | **On MS media** |
| Wild type | | 86 | 97 |
| *Uc*FatB1 + *Cn*LPAAT ($T_6$) | | 97 | 97 |
| *Uc*FatB1 + *Cn*LPAAT + *Ea*DAcT + DGAT RNAi ($T_4$) | 43 | 97 | 94 |
| | 83 | 81 | 86 |
| | 119 | 92 | 94 |
| | 132 | 100 | 92 |
| *Cp*FatB2 + *Ch*FatB2 ($T_6$) | | 89 | 97 |
| *Cp*FatB2 + *Ch*FatB2 + *Ea*DAcT + DGAT RNAi + PDAT RNAi ($T_4$) | 134 | 89 | 94 |

Seeds were grown in growth chambers under 16 hours light and 8 hours dark cycle. Germination is defined as complete emergence of cotyledons.
**Abbreviation:** *Ch- Cuphea hookeriana; Cn- Coccus nucifera; Cp- Cuphea palustris;* DAcT- Diacylglycerol acetyltransferase; DGAT- diacylglycerol acyltransferase; *Ea- Euonymus alatus;* Fat- fatty acid thioesterase; LPAAT- lysophosphatidic acid acyltransferase; PDAT- Phospholipid:diacylglycerol acyltransferase; *Uc- Umbellularia californica.*

### 5.3.6 Physical properties of transgenic acetyl-TAGs

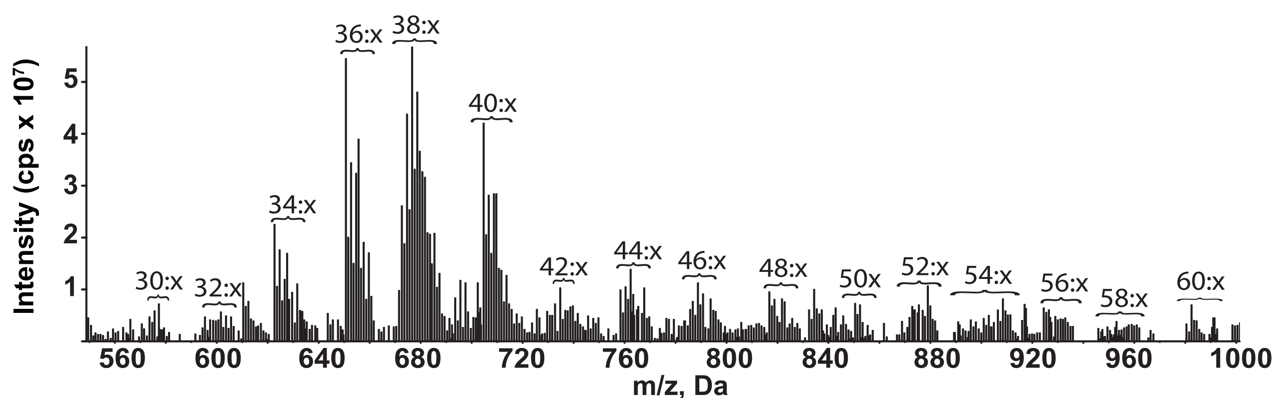
The viscosity of vegetable oils is an important parameter for its use as fuel, biodegradable lubricant, and other industrial applications. The kinematic viscosity of aliphatic hydrocarbons increases with increase in fatty acid chain length [147]. Also, tests conducted on mixtures of pure lcTAGs and short chain length TAGs showed a positive linear relationship between average molecular mass of TAG species and their kinematic viscosities [150,151]. Similarly, we hypothesized that reducing the average molecular weight of acetyl-TAG molecular species might decrease their kinematic viscosity. This was done by the incorporation of MCFAs into acetyl-TAGs, reducing the overall molecular weight of the acetyl-TAGs and thus eventually further lowering their viscosity. To test this idea, $T_4$ seeds from two high medium chain acetyl-

TAG yielding lines from two different MCFA background lines were grown on a scale to yield enough oil (~20 ml) for viscosity testing. The oils from the mature seeds of parental lines and transgenic lines were extracted using organic solvents and further fractionated in to pure acetyl-TAGs and lcTAGs.

### 5.3.6.1 Purification of acetyl-TAGs and lcTAGs

Acetyl-TAGs were purified using silica gel fractionation and a solvent gradient. Because of involvement of a solvent gradient and collection of various small fractions, there was a

**(a) Original oil**




**(b) Purified acetyl-TAGs**

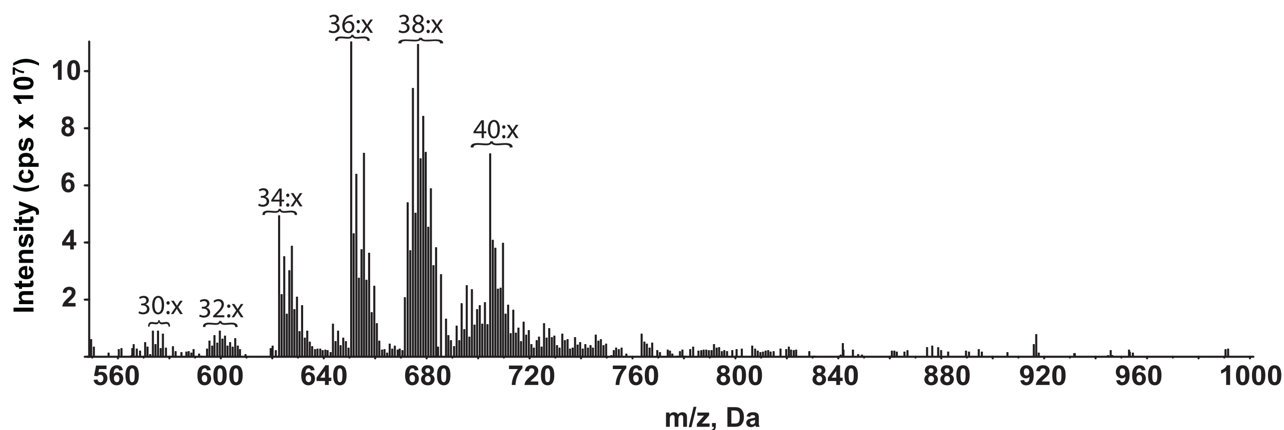



Figure 5.8 **Large scale purification of acetyl-TAGs from crude seed oil of high acetyl-TAG yielding Camelina line.** Representative positive ESI mass spectra of (a) total lipid extract and (b) purified acetyl-TAG fraction of transgenic $T_5$ seeds from one of high yielding MCFAs producing Camelina line expressing *Ea*DAcT. Signal peaks possess the m/z value of $[M+NH^4]^+$ adduct. For clarity, only the number of acyl carbons and not the number of double bonds (x) in each series of TAG molecular species is indicated.

possibility of losing different MCFA containing acetyl- TAGs molecular species. Hence, the acetyl-TAG containing fractions obtained during the fractionation were combined and tested for purity and fatty acid composition. All the acetyl-TAG molecular species present in the original oil were also present in the purified acetyl-TAGs (Fig. 5.8).

The fatty acid composition also matched closely with the original acetyl-TAG composition (Table 5.3). Similar tests were done to confirm the purity of lcTAGs obtained from the seed oil of parental lines.

Table 5.3 **Fatty acid composition of the purified acetyl-TAG and lcTAGs from seed oil of transgenic lines**

| Genotype | Fatty acid composition (Mol %) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Wild type (LcTAGs) | Fatty acid | 8:0 | 10:0 | 14:0 | 16:0 | 18:0 | 18:1 | 18:2 | 18:3 | 20:0 | 20:1 | 22:0 | 22:1 |
| | Original | - | - | - | 7.7 | 3.3 | 12.9 | 21.7 | 32.0 | 2.3 | 13.8 | 0.4 | 2.5 |
| | Purified | - | - | - | 8.0 | 3.3 | 11.7 | 21.9 | 33.3 | 2.2 | 13.0 | 0.4 | 2.5 |
| *Cp*FatB2 + *Ch*FatB2 (LcTAGs) | Fatty acid | 8:0 | 10:0 | 14:0 | 16:0 | 18:0 | 18:1 | 18:2 | 18:3 | 20:0 | 20:1 | 22:0 | 22:1 |
| | Original | 0.7 | 6.7 | 7.0 | 10.9 | 3.7 | 17.2 | 15.2 | 21.8 | 2.3 | 9.5 | 0.5 | 2.3 |
| | Purified | 0.7 | 6.5 | 6.8 | 10.6 | 3.5 | 17.0 | 15.5 | 23.1 | 2.2 | 9.1 | 0.5 | 2.3 |
| *Cp*FatB2 + *Ch*FatB2 + *Ea*DAcT + DGAT RNAi + PDAT RNAi (acetyl-TAGs) | Fatty acid | 8:0 | 10:0 | 14:0 | 16:0 | 18:0 | 18:1 | 18:2 | 18:3 | 20:0 | 20:1 | 22:0 | 22:1 |
| | Original | 0 | 0.1 | 5.9 | 12.3 | 3.9 | 19.6 | 20.2 | 26.6 | 1.7 | 7.6 | 0.2 | 0.8 |
| | Purified | 0 | 0.3 | 5.7 | 12.0 | 3.8 | 19.0 | 20.4 | 27.8 | 1.6 | 7.4 | 0.1 | 0.7 |
| *Uc*FatB + *Cn*LPAAT (LcTAGs) | Fatty acid | 8:0 | 12:0 | 14:0 | 16:0 | 18:0 | 18:1 | 18:2 | 18:3 | 20:0 | 20:1 | 22:0 | 22:1 |
| | Original | - | 30.6 | 4.0 | 5.1 | 2.3 | 7.5 | 11.3 | 29.0 | 1.4 | 6.5 | 0.2 | 2.0 |
| | Purified | - | 26.1 | 3.7 | 5.5 | 2.8 | 9.4 | 12.1 | 26.6 | 1.9 | 9.0 | 0.4 | 2.6 |
| *Uc*FatB + *Cn*LPAAT + *Ea*DAcT + DGAT RNAi (acetyl-TAGs) | Fatty acid | 8:0 | 12:0 | 14:0 | 16:0 | 18:0 | 18:1 | 18:2 | 18:3 | 20:0 | 20:1 | 22:0 | 22:1 |
| | Original | - | 11.4 | 2.9 | 6.0 | 3.3 | 15.1 | 13.8 | 33.7 | 1.9 | 10.3 | 0 | 1.6 |
| | Purified | - | 14.2 | 3.3 | 6.1 | 2.9 | 12.1 | 13.6 | 36.6 | 1.6 | 8.2 | 0 | 1.4 |

**Abbreviation:** *Ch- Cuphea hookeriana; Cn- Coccus nucifera; Cp- Cuphea palustris;* DAcT- Diacylglycerol acetyltransferase; DGAT- diacylglycerol acyltransferase; *Ea- Euonymus alatus;* Fat- fatty acid thioesterase; LPAAT- lysophosphatidic acid acyltransferase; PDAT- Phospholipid:diacylglycerol acyltransferase; *Uc- Umbellularia californica*.

### 5.3.6.2 Decreasing the molecular weight of acetyl-TAGs is not sufficient to reduce their viscosity

The viscosities of purified lcTAGs from wild type Camelina oil and parental MCFA producing lines and acetyl-TAGs obtained from their *Ea*DAcT expressing transgenic lines were measured according to the standard ASTM D445 method. The viscosity value of the purified lcTAGs (32.5 ± 0.1 $mm^2/s$) from Camelina wild type seed oil showed a small increase from the previously reported values of 30.6 $mm^2/s$ [13,167]. This might be due to small

differences in the method of oil extraction and purification. The viscosity of lcTAGs from the parental lines *Cp*FatB2+*Ch*FatB2 was 6.0% more than wild type while lcTAGs from parental lines *Uc*FatB1+*Cn*LPAAT had 9.5% lower viscosity than wild type. This might be due to high levels of long and saturated fatty acids 16:0 and 14:0 in first line compared to second line, which have more lauric acid. The acetyl-TAGs containing MCFAs from two different parental lines showed viscosity values of 25.6 and 22.7 $mm^2/s$ (Table 5.4). As expected acetyl-TAGs from the both the lines showed reduced viscosities compared to lcTAGs from the

Table 5.4 **Kinematic viscosity of purified acetyl-TAGs and lcTAGs**

| Genotype | TAG Type | Kinematic Viscosity ($mm^2/s$) at 40 $^0C$ |
|---|---|---|
| **Wild type** | LcTAGs | 32.5 ± 0.1 |
| ***Cp*FatB2 + *Ch*FatB2** | LcTAGs | 34.5 ± 0.5 |
| ***Cp*FatB2 + *Ch*FatB2 + *Ea*DAcT + DGAT RNAi + PDAT RNAi** | Acetyl-TAGs | 25.6 ± 0.2 |
| ***Uc*FatB1 + *Cn*LPAAT** | LcTAGs | 29.4 ± 0.1 |
| ***Uc*FatB1 + *Cn*LPAAT + *Ea*DAcT + DGAT RNAi** | Acetyl-TAGs | 22.7 ± 0.1 |

Viscosity was determined according to standard method ASTM D445.
**Abbreviations:** *Ch- Cuphea hookeriana; Cn- Cocos nucifera; Cp- Cuphea palustris;* DAcT- Diacylglycerol acetyltransferase; DGAT- diacylglycerol acyltransferase; *Ea- Euonymus alatus;* Fat- fatty acid thioesterase; LPAAT- lysophosphatidic acid acyltransferase; PDAT- Phospholipid:diacylglycerol acyltransferase; *Uc- Umbellularia californica*

parental lines. These reductions were in the range of 22.5% to 25.8%. The previously reported values of acetyl-TAGs of Camelina wild type seed oil is 20.6 $mm^2/s$ [13]. Our expectations were to decrease the viscosity of acetyl-TAGs further by incorporating MCFAs in to acetyl-TAGs. Instead, increase of 26.2% and 11.8 % were observed over the acetyl-TAGs from wild type Camelina.

The increase observed might be due to increased saturation levels of acetyl-TAGs due to incorporation of lauric and palmitic acids in these lines. A previous study from our group has also reported increase in viscosity with high oleic acid containing acetyl-TAGs [65]. In these lines, higher oleic acid levels were achieved at a cost of decreased levels of linoleic and linolenic acids which resulted in increase in overall saturation levels of acetyl-TAGs. Also a positive correlation was observed between viscosities of oils and increasing levels of saturation [168]. The second reason could be low incorporation of MCFA in to acetyl-TAGs, which did not lower their overall molecular weight appreciably. Both of these problems may be overcome by finding desaturases specific for MCFAs to produce monounsaturated MCFAs and LPAATs specific for these MUFA MCFAs with higher activities to acylate them on to *sn*-2 position. It can be concluded that a balance between saturation levels and the molecular weight of oil acetyl-TAGs might might be critical and have to be considered at same time before any future attempts to decrease the viscosity further.

## 5.4 Conclusions

This study successfully achieved high levels of production of acetyl-TAGs in MCFA producing lines of Camelina. Although levels of acetyl-TAGs molecular species containing MCFAs were low, one advantage of producing them in the oil seed crop Camelina is that large amounts of acetyl-TAGs can be obtained from these lines by field growth. From these large amounts of acetyl-TAGs, MCFAs containing acetyl-TAGs, which are expensive to synthesize chemically, can be obtained by further purification. A detailed future study can be planned from these purified acetyl-TAGs containing either long or medium chain fatty acids. Viscosity values can be obtained from mixture of known compositions to build a model relating composition of acetyl-TAGs with viscosity. Based on the prediction of that model, desired combination of acetyl-TAG molecular species can be generated using engineered Camelina backgrounds producing appropriate amounts of those fatty acids.

# Chapter 6 - Conclusions and Future work

Vegetable oils mainly consisting of long chain triacylglycerols (lcTAGs) have the potential to be used as direct use biofuel but they suffer from problems such as high viscosity and poor cold temperature properties [11]. 3-acetyl-1,2-diacyl-*sn*-glycerol (acetyl-TAGs) are different from lcTAGs due to the presence of an acetyl group at the *sn*-3 position instead of a long chain fatty acid. Acetyl-TAGs possess lower viscosity and improved cold temperature properties compared to lcTAGs [12,13]. Further improvement in their properties can be achieved by modifying the fatty acid composition of acyl-chains present at the *sn*-1 and *sn*-2 positions of the molecule. The substrate specificity of *Ea*DAcT for its acyl-CoA donor and acceptor substrates was studied in a yeast model system. This was done with a novel ESI-MS based method for quantification of DGAT assay products, enabling use of non-radiolabeled substrates. Based on the knowledge of substrate specificity, *Ea*DAcT was expressed in Camelina transgenic lines producing abundant medium chain fatty acids (MCFAs) to synthesize acetyl-TAGs with improved fuel properties.

Typically, substrate specificity of acyltransferases is tested by incubating microsomal extract containing desired enzyme with radiolabeled acyl-CoA and DAG and measuring the amount of radioactive product. This microsomal assay for *Ea*DAcT had certain limitations: for example, due to non- specific reactions from other enzymes in microsomes, the desired assay product need to be separated by performing TLC which is time consuming. Also, unavailability and high cost of synthesis of all different chain length radiolabeled acyl-CoAs made the substrate specificity studies highly difficult. Hence, we developed a DGAT assay based on the use of non-radiolabeled substrates and quantification of product by electrospray ionization mass spectrometry (ESI-MS) (Chapter 2). This method was based on the detection of all the TAGs molecular species containing same *sn*-3 acyl group by neutral loss scan of *sn*-3 acyl group in a single scan. As the neutral loss signal is affected by the chain length and saturation indices of the fatty acids [54], a series of TAG standards with a specific *sn*-3 acyl group and fatty acids of different chain length and saturation levels at *sn*-1 and *sn*-2 positions were synthesized. The response factors calculated from these standards were used to correct and quantify the signal for TAGs present in the samples. This accuracy of this method was further tested to quantify purified acetyl-TAGs of *Euonymus alatus* seed oil and acetyl-TAGs present in the lipid extract of

yeast expressing *Ea*DAcT. The values closely matched with the values obtained from GC-FID quantification. This method was next tested by quantification of acetyl-TAG produced in an in vitro enzyme assay. The results obtained from this method were found to be highly comparable to results obtained from the radioactive assay.

*Ea*DAcT is a member of MBOAT family of enzymes and synthesizes acetyl-TAG by the acetyl-CoA dependent acylation of diacylglycerol (DAG) [12]. To test the acyl-CoA specificity of *Ea*DAcT under in vitro conditions, microsomes from the yeast expressing *Ea*DAcT were incubated with a series of different unlabeled acyl-CoAs varying in acyl group chain length from 2 to 18 carbons. The product formed was quantified using ESI-MS neutral loss scans of *sn*-3 acyl group with the method described in Chapter 2. It was found that although *Ea*DAcT can use other small chain acyl-CoAs such as butryl-CoA and hexanoyl-CoA, it has high specificity for acetyl-CoA. Further, *Ea*DAcT has negligible activity for acyl-CoAs with chain length greater than 6 carbons. Detailed studies were conducted with acetyl-CoA, butyryl-CoA and hexanoyl-CoA using a wide range of acyl-CoA concentrations to determine kinetic parameters. The substrate conversion rate for any concentration of acetyl-CoA was higher than that for butyryl-CoA and hexanoyl-CoA, again confirming higher specificity of *Ea*DAcT for acetyl-CoA. However, the higher apparent $K_m$ recorded for acetyl-CoA than butyryl-CoA and hexanoyl-CoA might be caused by side reactions utilizing acetyl-CoA and rapid acetyl-CoA hydrolysis by the enzymes present in the microsomal system. Future work will involve development of a purified enzyme system to more accurately determine the enzyme kinetic parameters using surface dilution kinetics.

The activity of *Ea*DAcT for DAG acceptor substrates with different chain length and saturation indices was tested under in vitro and in vivo conditions using endogenously present DAGs. However, the presence of very low amounts of DAG containing medium chain length fatty acids (MCFAs) in yeast microsomes and their low solubility in aqueous buffer allowed only qualitative tests for these DAGs. However, results from both in vitro and in vivo studies indicated that *Ea*DAcT could acetylate a variety of DAGs with highly variable fatty acid chain lengths but has high preference for DAGs with high unsaturation levels. Substrates such as 1-monoacylglycerols (MAGs), 2-MAGs, and *sn*-1,3-DAGs did not get acetylated by *Ea*DAcT in vitro suggesting that *Ea*DacT specifically acetylates *sn*-1,3-DAGs.

Phylogenetic evidences demonstrated that *Ea*DAcT has high sequence similarity with wax synthases and sterol acyltransferases [12]. Hence, we decided to test the *Ea*DAcT ability to acetylate fatty alcohols and sterols. DMSO as a solubilizing agent for fatty alcohols was found to be essential to observe alcohol acetyltransferase activity under in vitro conditions. Other assay conditions such as type of solubilizing agent, pH and fatty alcohol concentration were also optimized. Fatty alcohols with chain length ranging between 8 to 22 carbons were tested under optimized assay conditions. *Ea*DAcT was found to show higher activity for medium chain fatty alcohols such as 12:0-OH and 14:0-OH. The ability of *Ea*DAcT to acetylate fatty alcohol produced in vivo was also tested in yeast. The yeast expressing *Ea*DAcT and honey bee fatty acid reductase (*Am*FAR1) genes was able to produce alkyl acetates further confirming wax synthase activity of *Ea*DAcT. Similar results were obtained from further studies done in collaboration using endogenous DAG free microsomes in vitro and by fatty alcohol feeding assays in vivo [71]. *Ea*DAcT did not show any activity either for the exogenously added cholesterol or for any other sterols endogenously present in the yeast suggesting that *Ea*DAcT has no sterol acetyltransferase activity.

The viscosity of vegetable oils is an important parameter that governs their use as a direct use biofuel. The viscosity of acetyl-TAGs produced in wild type Camelina (20 $mm^2/s$) falls in the range of diesel #4 (5-24 $mm^2/s$) used in low and medium speed engines [13]. Any further reduction in the viscosity of acetyl-TAGs will increase their usefulness. Previous studies have suggested that molecular mass of constituent TAGs is directly correlated to their viscosity [150,151]. Based on this and the fact that *Ea*DAcT can acetylate DAG containing MCFAs (Chapter 3), lower molecular mass acetyl-TAGs were synthesized in oil seed crop Camelina by incorporation of MCFAs in them to further reduce their viscosity.

Transgenic Camelina lines producing high levels of MCFAs were used to produce acetyl-TAGs. These lines were obtained by the expression of medium chain specific fatty acyl thioesterases (FatBs) and lysophosphatidyl acyltransferases (LPAATs) isolated from plants producing high levels of MCFAs in their TAGs [155]. Four lines producing high levels of capric acid, lauric acid and myristic acid were transformed with *Ea*DAcT. In addition, diacylglycerol acyltransferase 1 (DGAT1) and and phospholipid : diacylglycerol acyltransferase (PDAT) enzymes, which utilize the same DAG substrate as *Ea*DAcT, were also knocked down alone or in combination to minimize the competition and enhance acetyl-TAG production.

Homozygous $T_3$ lines producing high levels of acetyl-TAGs (64 – 77 mol %) were obtained for 2 out of 4 MCFAs producing genetic backgrounds. DGAT1 knockdown was effective in increasing the acetyl-TAG levels over the lines with *Ea*DAcT expression alone. The fatty acid compositions of acetyl-TAGs and lcTAGs from these lines showed poor incorporation of MCFAs in the acetyl-TAGs compared to lcTAGs. *sn*-2 fatty acid compositional analysis of acetyl-TAGs showed low abundance of MCFAs at their *sn*-2 position which in turn indicates poor incorporation of MCFAs at the *sn*-2 position of DAGs. Either the low concentration of DAGs containing MCFAs and/or low specificity of *Ea*DAcT for these DAGs might be reasons for inefficient concentrations of acetyl-TAGs containing MCFAs.

The seed traits of some of the high acetyl-TAG yielding lines were tested. The acetyl-TAG producing trait was consistent upto $T_5$ generation for the two highest acetyl-TAGs yielding lines. There was no effect on seed germination. However, the oil contents of these lines were reduced compared to wild type Camelina.

$T_5$ seeds from two of the highest acetyl-TAG yielding lines and corresponding parental lines ($T_7$ seeds) were produced at a scale to yield enough acetyl-TAGs and lcTAGs for testing viscosity. The oil was extracted with hexane using soxhlet extraction and fractionated into acetyl-TAG and lcTAGs by column purification using silica gel and a step gradient of diethyl ether in hexane. The viscosity of acetyl-TAGs from two lines with different MCFA backgrounds (25.6 and 22.7 $mm^2/s$ at 40 $^oC$) were found to 26.2 % and 11.8 % higher than viscosity of acetyl-TAGs produced in wild type Camelina (20.2 $mm^2/s$). Inefficient lowering of molecular mass due to low incorporation of MCFAs and/or increase in relative amount of saturated fatty acids compared to acetyl-TAGs produced in wild type Camelina might be the reasons for this increase. The other possibility is difference in techniques used for oil extraction, purification and viscosity testing used in this and previous studies. Overall, it was concluded that saturation levels of fatty acids of acetyl-TAGs needs to be considered in addition to lowering the molecular mass.

## Future directions

### Increasing MCFAs incorporation into acetyl-TAGs

One possible explanation for the insufficient decrease in the viscosity of acetyl-TAGs is inefficient lowering of average molecular mass of acetyl-TAGs. It was because of low incorporation of MCFA in to acetyl-TAGs. One of the possible reasons for this was low *sn*-2

incorporation of MCFAs in to the DAGs. This in turn might be the result of either low activity of medium chain specific *Cn*LPAAT or higher activity of Camelina endogenus LPAATs outcompeting *Cn*LPAAT activity or both effects.

Future work will involve generation of Camelina lines where endogenous LPAAT activity will be suppressed to see its effect of MCFA incorporation in to acetyl-TAGs. For this, endogenous LPAATs orthologs of Camelina need to be identified using Camelina transcriptome and genome databases [99,100]. Once known, LPAATs knockout lines can be generated using the CRISPR/Cas9 genome editing techniques [158]. In the event that endogenous LPAATs knockout is lethal for the plant, RNAi based suppression of endogenous LPAATs can be tried instead of complete knockout.

The other option is to express MCFA specific LPAATs with higher activities than *Cn*LPAAT or add more number of MCFA specific LPAATs in *Cn*LPAAT backgrounds. Recently, novel LPAATs specific for capric acid (10:0) and myristic acid (14:0) from Cuphea were discovered and successfully expressed in Camelina [163]. Those lines can be crossed with the high yielding acetyl-TAG producing lines obtained from this study to possible imcorporate more MCFA incorporation in acetyl-TAGs.

**Increasing the unsaturation levels of acetyl-TAGs**

The other possible explanation of increase in viscosity of acetyl-TAGs containing MCFA was increase in the saturation levels due to incorporation of saturated fatty acids such as lauric acid (12:0), myristic acid (14:0) and palmitic acid (16:0). Maintaining high unsaturation levels and reduction of the molecular weight of acetyl-TAGs can be achieved by expressing desaturases, which can produce unsaturated MCFAs.

A fatty acyl-ACP desaturase, which acts on 14:0-ACP to produce 14:1-ACP was cloned from *Pelargonium x hortorum* [169]. This enzyme can be further used as a model to develop desaturases, which can produce unsaturated MCFAs, by protein engineering. Another way to find MCFA specific desaturases is to screen mutant population of this plant or related species. However, the first step will be to test the *Ea*DAcT ability to acylate the DAGs containing unsaturated MCFAs which can be determined by assay techniques developed in this study.

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