MANIPULATING OIL SEED BIOCHEMISTRY TO ENHANCE THE PRODUCTION OF ACETYL-TAGS

by

CATHERINE KORNACKI

B.S., Webster University, 2013

A THESIS

Submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Biochemistry and Molecular Biophysics Graduate Group

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2016

Approved by:

Major Professor
Timothy P. Durrett, PhD

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Abstract

Using vegetable oils directly as an alternative biofuel presents several problems as such oils typically possess poor fuel qualities including high viscosity, low volatility, and poor cold temperature properties. The ornamental shrub *Euonymus alatus* produces unusual acetyl-1,2-diacyl-*sn*-glycerols (acetyl-TAGs) that have an acetyl group in the *sn*-3 position instead of a long chain fatty acid. The presence of this *sn*-3 acetyl-group give acetyl-TAGs properties desirable for biofuels, such as reduced viscosity, compared to the normal long chain triacyglycerols found in most vegetable oils. Acetyl-TAGs are synthesized by the *Euonymus alatus* diacylglycerol acetyltransferase (*Ea*DAcT) and *Euonymus fortunei* diacylglycerol acetyltransferase (*Ef*DAcT) enzymes. Both enzymes catalyze the transfer of an acetyl group from acetyl-CoA to diacylglycerol (DAG) to produce acetyl-TAGs. Previous work demonstrated that expression of *Ea*DAcT combined with the suppression of a diacylglycerol aceyltransferase (DGAT1) in *Camelina sativa* led to seeds with 85 mol % acetyl-TAGs. Increasing acetyl-TAG levels further was explored using two strategies. Over expression of citrate lyase to increase the pool of acetyl-CoA to be used as a substrate for the acetyltransferase enzymes failed to increased levels of acetyl-TAGs. A second approach involved expressing *Ef*DAcT in *Camelina sativa*. *Ef*DAcT has demonstrated higher activity *in vitro* and *in vivo* and its expression in yeast leads to approximately 50 % higher levels of acetyl-TAGs compared to *Ea*DAcT. The expression of *Ef*DAcT coupled with the suppression of DGAT1 in *Camelina sativa* resulted in 90 mol % acetyl-TAGs in the transgenic seeds. Levels of *Ef*DAcT protein analyzed in developing transgenic *Camelina sativa* seeds across a 40 day time period were highest at 15 and 20 days after flowering. Following these time points acetyl-TAG

accumulation increased rapidly, coinciding with the higher enzyme expression levels. The optimization of additional promoters to ensure expression of *Ef*DAcT in the last half of seed development could represent another way to further increase acetyl-TAGs in the future.

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Acknowledgements

First and foremost I would like to thank my major advisor Dr. Timothy P Durrett, Assistant Professor, Department of Biochemistry and Molecular Biophysics for his unwavering support and guidance. I am indebted and forever grateful for his patience, inspiring mentorship, and unyielding positivity.

I would like to thank my advisory committee Dr. Kathrin Schrick and Dr. Michael Zolkiewski for their wisdom, helpful instruction, and providing recommendations throughout the course of my research and studies. I am truly humbled and tremendously appreciative of their guidance and advice.

My family is my source of strength and refuge without whom I would not be able complete my work. Their ceaseless encouragement and support have sustained and motivated me in accomplishing my goals and aspirations.

To my amazing colleges whose company I enjoy, Sunil, Tam, Karan, Jose, Garrison, and James, thank you for your friendship.

Thank you all.

Catherine Kornacki

Dedication

Dedicated to my wonderful husband Vipin.

My love, my joy, you are my hiding place.

Chapter 1 - Introduction and literature review

1.1 Vegetable oils as biofuels

In today's modern society there is a growing need and desire for alternative energy and renewable fuel sources that reduce environmental impact. The release of harmful gases and toxic waste products from the burning of fossil fuels has prompted a demand for alternate fuels that do not contribute any additional pollution. In comparison, vegetable oils are non-toxic, biodegradable, and carbon neutral as the plants that produce the oil will use and therefore displace carbon dioxide while growing. Additionally, concerns about the availability and cost of fossil fuels has created a demand for research and procurement of chemical products and fuel acquired from renewable resources.

Historically, vegetable oils were commonly used as a source to create light and heat until fossil fuels and electricity became the dominant means of providing energy to large populations. The ability for vegetable oils to be used in a wide range of industrial and nutritional feedstocks has reignited an interest to expand upon their functionality. Vegetable oil fatty acids and their derivatives are used for textiles, lubricants, soaps, detergents, and cosmetics [1]. For the manufacturing of biodiesel, the triacylglycerol molecules are fragmented into the glycerol backbone and fatty acid methyl esters.

1.2 Fatty acids structure affects their chemical and physical properties

Plant and vegetable oils are commonly made of triacylglycerols with a structure consisting of three long chain fatty acids attached to a glycerol backbone (Figure 1.1). Fatty acids typically consist of a chain of hydrocarbons with zero, one, or more double bonds. The five most common fatty acids in commercial plant oils are palmitic acid (16:0), stearic acid (18:0) oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3)

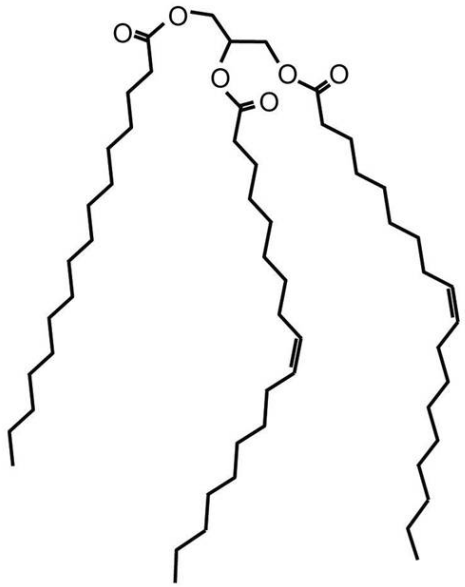

Figure 1.1 **Structure of a long chain TAG molecule.** A triacylglycerol (lcTAG) possesses 3 long chain fatty acids esterified to a glycerol backbone.

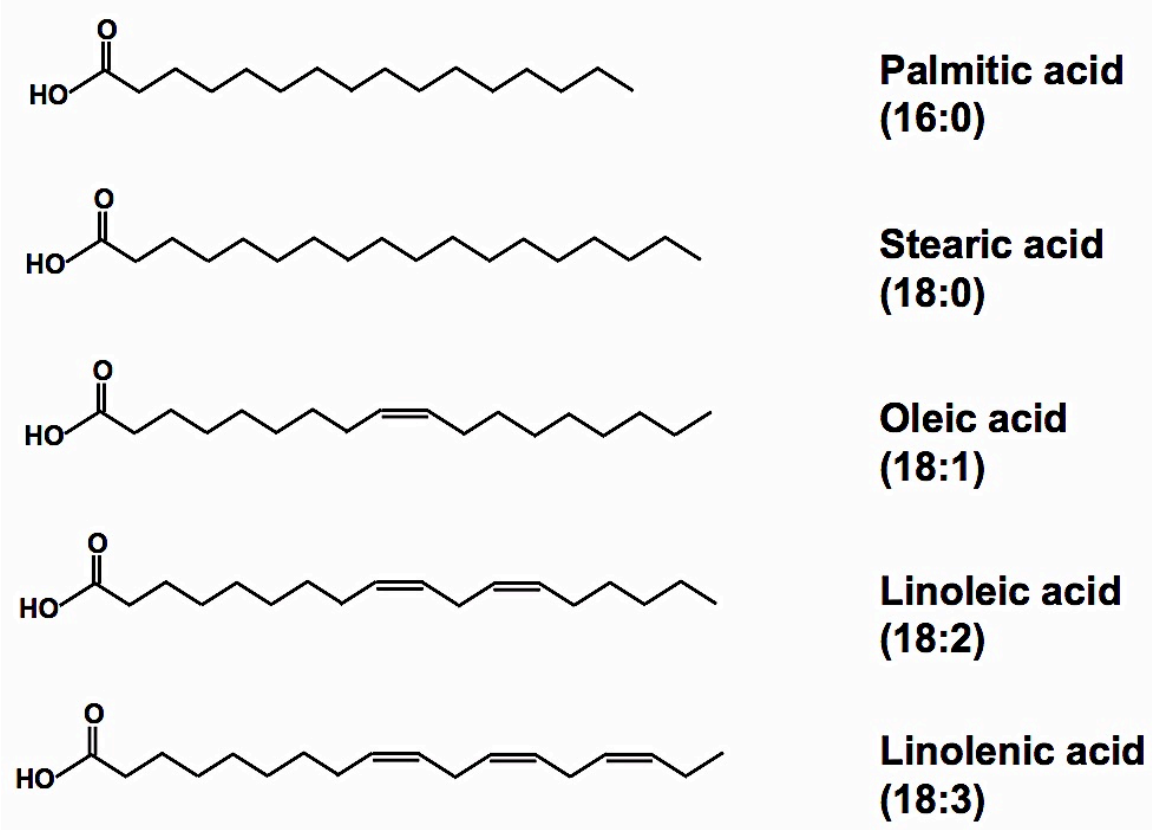

Figure 1.2 **Common fatty acids and their structure.** Fatty acids are denoted as X:Y where X represents the number of carbon atoms and Y represents the number of double bonds.

(Figure 1.2). The structure and composition of a fatty acid determines its physical and chemical properties, which affects how that particular fatty acid will be used. In a nutritional capacity, unsaturated fatty acids are liquid at room temperature and commonly found in vegetable oils used for cooking, including olive and canola oil. Saturated fatty acids, such as palmitic and stearic acids, are solid at room temperature and normally found in spreads such as butter and margarine. Industrially, the production of many soaps and detergents are derived from lauric acid (12:0) [1].

1.3 Lipid synthesis in plants

Triacylglycerol (lcTAG) structure is characterized by three long chain fatty acids esterified to the *sn*-1, *sn*-2, and *sn*-3 positions of a glycerol backbone (Figure 1.1). In most plants, triacylglycerols predominantly serve as a source of energy and are commonly stored within the plants seeds as oil. As such, they also help support the growth and development of seedlings during the early stages of germination. The *de novo* synthesis of fatty acids occurs within the plant's plastids where the first committed step is catalyzed by the enzyme acetyl-CoA carboxylase in a two-step reaction to convert acetyl-CoA into malonyl-CoA. It then enters the type II fatty acid synthase complex where through a series of four sequential reactions, two carbon units are added to the growing fatty acid that is attached to an acyl carrier protein (ACP). The fatty acid is removed from the ACP by acyl-ACP thioesterases and then transported to the cytosol and endoplasmic reticulum for further modification.

Synthesized fatty acids exit the plastid and are esterified onto Coenzyme A (CoA). The fatty acyl-CoAs are then attached to the *sn*-1 and *sn*-2 positions of glycerol 3-phosphate (G3P) through reactions within the endoplasmic reticulum. The initial step in

TAG synthesis is catalyzed by glycerol-3-phosphate acyltransferase (GPAT) using an acyl-CoA to acylate glycerol-3-phosphate (G3P) into lysophosphatic acid (LPA). Lysophosphatic acid is then acylated again by lysophosphatidic acid acyltransferaese enzyme (LPAAT) to form phosphatidic acid (PA). PA phosphatases dephosphorylate PA to form *de novo* DAGs [2]. The diacylglycerol acetyltransferase enzymes (DGAT) catalyze the final step of TAG synthesis by adding a long chain fatty acid onto the *sn*-3 position of the glycerol backbone. In comparison, phospholipid: diacylglycerol acetyltransferase (PDAT) catalyzes the transfer of an acyl group from the *sn*-2 position of phospholipids to form TAGs [3].

1.4 Synthesis of triacylglycerol is performed by several enzymes

In plants and animals, the DGAT enzymes use acyl-CoA as a substrate for the transfer of an acyl group onto the *sn*-3 position of the DAG glycerol backbone. DGAT1 and DGAT2 enzymes are responsible for the majority of TAG synthesis through the acylation of DAG. PDATs use either a phosphotidylcoline (PC) or phosphotidylethanolamine (PE) as substrates to transfer an acyl group to produce TAGs.

1.4.1 DGAT1

The DGAT1 gene was first identified through sequence comparison to the acyl CoA:cholesterol acyltransferase (ACAT) enzyme, a known acetyltransferase that uses fatty acyl-CoA substrates and cholesterol to synthesize cholesterol esters. The DGAT1 gene was first cloned in mice and expressed in insect cells where the [^{14}C] oleoyl-CoA substrate was exposed to a variety of possible acceptors to examine if the radio labeled substrate would be incorporated into triacylglycerols. It was determined that DAG was the acceptor molecule. Radiolabeled triacylglycerol levels significantly increased within

the cell membrane consequently revealing DGAT1 activity [4].

The mouse DGAT1 gene was used to find and clone an *Arabidopsis thaliana* homologue that was expressed in insect cells [5]. A mutation in the gene encoding the DGAT1 enzyme affected its activity in Arabidopsis and led to altered fatty acid composition and decreased lipid content [6, 7]. Another study reported similar results and reasoned that there must be an additional enzyme contributing to TAG synthesis since considerable levels of TAG were still produced in Arabidopsis seeds [8].

1.4.2 DGAT2

The existence of the DGAT2 enzyme was put forth through studies on DGAT1. The removal of DGAT1 activity reduced TAG synthesis yet did not completely eliminate it. One explanation for the decrease but not the entire elimination of TAGs was that there must be another enzyme with a similar function to complete the last step in TAG synthesis [8]. The presence of the DGAT2 enzyme later was confirmed when its sequence was first identified in the fungus *Mortierella ramanniana*. It was also shown that DGAT2 does not possess high sequence similarity to DGAT1 [10]. Through comparisons in tung, Arabidopsis, and rice it was found that both enzymes share only 50% amino acid sequence similarity with DGAT1 having ten transmembrane domains and DGAT2 having only two [12]. Also, both enzymes are located in separate regions of the endoplasmic reticulum [12].

Despite catalyzing the same reaction, DGAT1 and DGAT2 do not have identical functions in vivo. A study found that mice with a suppressed DGAT1 enzyme were resistant to diet induced obesity and remained slim compared to the fat wild type mice [9]. It was thought that an additional enzyme with a similar function must be present

since TAG synthesis was not entirely eliminated. Another study found that mice would die a few hours after birth with a DGAT2 knockout indicating its importance in early development [11]. In plants, while DGAT1 may have a more dominant role in TAG synthesis, DGAT2 is thought to play a role in the incorporation of unusual fatty acids into TAGs [12, 13]. Studies examining the evolutionary relationship between the two enzymes found that DGAT1 and DGAT2 genes were ubiquitously found in major eukaryotic groups. Additional evolutionary and phylogenic analyses determined that they have evolved separately and show structural and molecular divergence. However, they also demonstrated functional convergence. This further emphasizes their similar yet non-redundant functions [14].

1.4.3 PDAT1

Phospholipid: diacylglycerol acyltransferase (PDAT) enzyme activity also catalyzes the last step of TAG synthesis. However, instead of using acyl-CoA, PDATs use a phosphotidylcoline (PC) or phosphotidylethanolamine (PE) molecules as a substrate. An acyl group is transferred from the *sn*-2 position of the phospholipids onto the *sn*-3 position of DAG to produce normal long chain TAGs. The activity of PDAT was first characterized in microsomes of yeast, castor bean, sunflower, and *Crepis palaestina* where a wide range of [^{14} C] labeled phospholipids were incubated with DAG to quantify the accumulation of TAG [15]. Like the DGAT enzymes, PDAT1 is also a membrane bound enzyme and synthesizes TAG molecules [16].

To better understand the role of PDAT1 in plant lipid metabolism, one study examined the effects of its overexpression in Arabidopsis and determined that TAG levels and fatty acid synthesis increased in the leaves, not the seeds [17]. The knockdown

of PDAT1 alone in Arabidopsis did not demonstrate a significant change in fatty acid composition or TAG accumulation in its seeds [18]. However, the combined suppression of DGAT1 and PDAT1 resulted in compromised seed embryo development and a 70% - 80% decrease in oil content in Arabidopsis seeds, a notable increase from the suppression of DGAT1 alone [16]. This revealed the overlapping yet not redundant function of the PDAT1 enzyme with a more independent role in Arabidopsis leaves.

Acetyl-TAGs as biofuels

The *Euonymus alatus* (Burning Bush) plant produces unusual acetyl-1,2-diacyl-*sn*-glycerols (acetyl-TAGs) in its seeds. Acetyl-TAGs are a subclass of lipids that have an acetyl group in the *sn*-3 position instead of a long chain fatty acid (Figure 1.3). The presence of this *sn*-3 acetyl group confers several advantageous physical and chemical properties to acetyl-TAGs making them suitable for biofuels.

1.5.1 Acetyl-TAGs possess lowered viscosity

The absence of a long chain fatty acid in the *sn*-3 position of the glycerol backbone gives acetyl-TAGs reduced viscosity. (Figure 1.3). Viscosity is a significant component of biofuels and lubricants, which determines their unique functionality and consequent use in industrial applications. The viscosity of vegetable oils tends to be unfavorably high at approximately 30mm^2s^{-1} [19, 20] and prevents their direct use in diesel engines. Therefore, to reduce viscosity, the oil must first be heated before use. It may also undergo chemical transmethylation or be mixed with other diesel oil to reduce their viscosity. In contrast, acetyl-TAGs may be used to bypass these limitations as they demonstrate a 39% reduction in kinematic viscosity compared to normal TAGs [21]. The viscosity of acetyl-TAGs (20 mm^2s^{-1}) meets the viscosity specifications for diesel #4

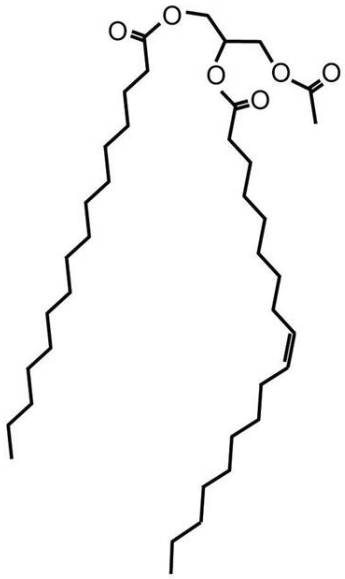

Figure 1.3 **Acetyl-TAG molecule structure.** Instead of a long chain fatty acid, acetyl-TAGs possess an acetate group at the *sn*-3 position of the glycerol backbone.

(5 - 24 $mm^2 s^{-1}$ at 40 °C) which is used in engines working at a constant low to medium speed such as heavy generators and ships [22].

1.5.2 Improved cold temperature properties of acetyl-TAGs

Another important aspect of using vegetable oils for various industrial applications is their response to cold temperatures. At low temperatures, vegetable oils tend to crystallize and solidify into gels clogging fuel lines and engine pipes. Acetyl-TAGs may be used to overcome these obstacles. Differential scanning calorimetry used to assess the thermal properties of acetyl-TAGs compared to normal TAGs determined that high oleic acetyl-TAGs crystallized at -64 °C compared to TAGs at -34 °C [22]. As acetyl-TAGs possess improved cold temperature properties it is feasible that they will contribute to higher quality biofuels and lubricants.

1.6 Synthesis of acetyl-TAGs

1.6.1 *Ea*DAcT enzyme synthesizes acetyl-TAGs

The synthesis of acetyl-TAGs is catalyzed by the *Euonymus alatus* diacylglycerol acetyltransferase (*Ea*DAcT) enzyme [21]. The structure of *Ea*DAcT is unknown as it is membrane bound within the endoplasmic reticulum. *Ea*DAcT is part of the membrane bound O acyltransferase (MBOAT) family that is distinguished by their acyl-CoA dependent reactions that catalyze the transfer of an acyl group onto a wide variety of substrates [23]. At the end of the triacylglycerol synthesis pathway, instead of using an acyl-CoA, *Ea*DAcT transfers an acetyl group from acetyl-CoA onto the *sn*-3 position of a DAG molecule to produce acetyl-TAGs [21].

1.6.2 *Ef*DAcT enzyme possesses higher activity

The *Euonymus fortunei* diacylglycerol acetyltransferase (*Ef*DAcT) enzyme is an ortholog of *Ea*DAcT that is native to the *Euonymus fortunei* (Winter Creeper) plant and catalyzes the formation of acetyl-TAGs in its seeds. Both *Ea*DAcT and *Ef*DAcT are unusual within the MBOAT family in that they both use acetyl-CoA, the smallest molecule available as an acyl-CoA donor. Both enzymes have exactly 636 amino acids and 96.5% sequence similarity. Interestingly, *Ef*DAcT has higher activity in vitro and in vivo (Figure 1.4).

Previous work was performed on a group of DAcT enzyme orthologs to quantify their production of acetyl-TAGs. Yeast microsomes containing the enzymes were incubated with [^{14}C] labeled acetyl-CoA. The lipids were extracted and quantified demonstrating that *Ef*DAcT produced the highest levels of acetyl-TAGs (Figure 1.4a). In another experiment, a group of DAcT enzyme orthologs were expressed separately in

yeast. Using mass spectrometry, the amount of acetyl-TAGs were measured demonstrating again that *Ef*DAcT possesses the highest activity (Figure 1.4b).

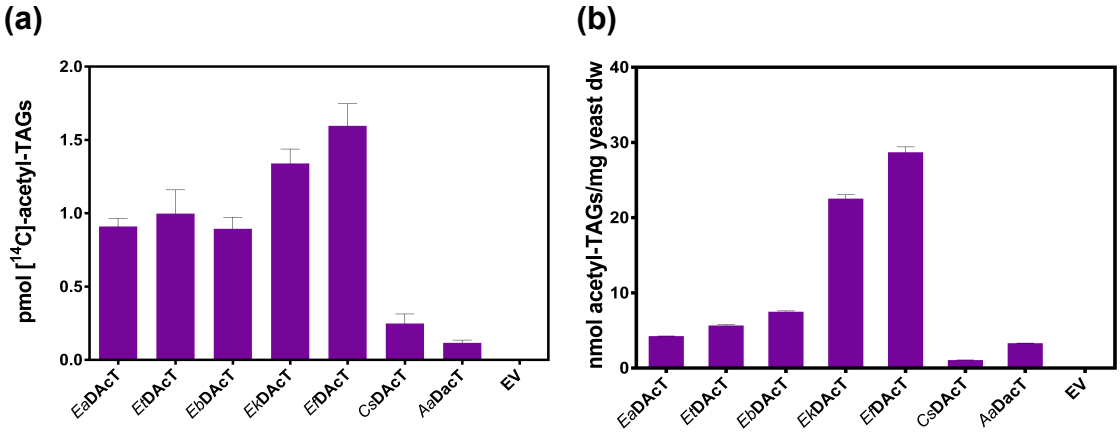

Figure 1.4 ***Ef*DAcT demonstrates higher activity in vitro and in vivo.** Enzymatic activity and acetyl-TAG accumulation was quantified in several DAcT enzyme orthologs. (a) Lipids extracted from yeast microsomes transformed with the enzymes and incubated with ^{14}C acetyl-CoA. (b) Quantification of acetyl-TAGs from yeast cultures expressing different acetyltransferase enzymes. Figured modified from Tran et al. (2017) Plant J. 92: 82-94. © 2017 The Authors and John Wiley & Sons, Ltd.[1]

1.6.3 Acetyl-TAG synthesis in Arabidopsis

The expression of *Ea*DAcT was first pioneered through work with Arabidopsis. Under the control of a seed specific promoter, the expression of *Ea*DAcT led to the production of 45 mol% of acetyl-TAGs within the seeds [21]. Since DGAT1 is the dominant enzyme in producing normal long chain triacylglycerols, it was reasoned that when suppressed, *Ea*DAcT would be able to use more of the DAG substrate to produce higher levels of acetyl-TAGs. The accumulation of acetyl-TAGs increased to 60 - 65 mol % in plant lines expressing *Ea*DAcT in a *dgat1* mutant background [22]. However, there are limitations in working with Arabidopsis when attempting to maximize the amount of

[1] Tran et al (2017) was submitted and published while this thesis was placed under embargo. The addition of the copyright information therefore represents a modification of the original text.

total oil production. This includes small seed size and inability to be grown as a field crop eliminating the possibility of mass production of seed oil. These restrictions can be overcome through work with *Camelina sativa*. Its valuable agronomic qualities demonstrate incredible promise as an industrial oil seed crop for the production of acetyl-TAGs.

1.7 *Camelina sativa*: An emerging oil seed platform

1.7.1 Agronomic qualities of *Camelina sativa*

Camelina sativa is a member of the Brassicaceae (mustard) family and commonly known as false flax or gold of pleasure. It is native to South Eastern Europe and South Western Asia [24]. It is an ancient crop that was originally cultivated in Europe during the Iron Age as vegetable oil fuel source. Although the use of *Camelina sativa* declined throughout the Middle Ages, [24] within the past few decades it has gained considerable attention as more research has rediscovered its many useful agronomic attributes. Also, its potential to be used as a biofuel feedstock has made *Camelina sativa* an attractive emerging oilseed platform. It may be simply transformed using an agrobacterium floral dip vacuum infiltration method [25]. Some of its beneficial attributes include its moderately short growth cycle requiring approximately 85 - 100 days to reach maturity and ability to be grown as both a spring and winter crop [26]. As such, *Camelina sativa* has been shown to be compatible with cover crops used in the fall and early spring to prevent soil erosion producing increased crop yields [27]. Its ability to grow well on poor quality land without the requirement of supplemental fertilizer or nutrients has given *Camelina sativa* a reputation for being a low input oil seed crop. Its many advantageous qualities make it an ideal platform for the production of acetyl-TAGs.

1.7.2 *Camelina sativa* and acetyl-TAGs

Since acetyl-TAGs possess valuable characteristics in demand for a variety of industrial and nutritional applications, their mass production would be useful in an oil seed crop. Much of the research regarding lipid metabolism and production of acetyl-TAGs in Arabidopsis has translated well into *Camelina sativa* [22]. Approximately 90% of the genes in Arabidopsis that are responsible for the regulation of lipid metabolism are also found in *Camelina sativa* [28]. The expression of *Ea*DAcT in *Camelina sativa* resulted in an average of 55 mol% acetyl-TAGs accumulation within the seeds with the highest accumulating lines yielding 64 mol % acetyl-TAGs. Also the expression of *Ea*DAcT in combination with the suppression of the DGAT1 led to 85 mol % acetyl-TAGs in the best plant lines [22].

1.7.3 Effects on *Camelina sativa* phenotype

Since the pathways of lipid metabolism are altered, there is concern if transgenic plants and seeds are negatively affected in their growth and development. It is reported that the transformation and production of acetyl-TAGs in *Camelina sativa* does not dramatically affect plant characteristics. Acetyl-TAGs are normally metabolized by seed TAG lipases and are able to be used as an energy source [22]. There is no observable change in the height and morphology between the wild type and transgenic plants. The average seed weights of wild type, *Ea*DAcT, and *Ea*DAcT coupled with DGAT1 suppression are similar to each with no major differences. The overall oil content of the seeds expressing *Ea*DAcT and the suppression of DGAT1 was reduced 7.5 - 11% compared to the wild type controls and the seeds expressing *Ea*DAcT alone [22]. The accumulation of high levels of acetyl-TAGs might be an acceptable trade off with the

reduction in overall oil production. Such queries may be addressed through further research into metabolic flux, breeding, genetic manipulation, and biotechnological approaches to compensate for the loss of total seed oil content.

1.8 Conclusions

Understanding TAG synthesis lays the foundation to our understanding of acetyl-TAG production and the engineering of metabolic fluxes within lipid pathways. Here we also use this approach in an effort to maximize acetyl-TAG accumulation while employing several new strategies. In Chapter 2, we study the production of acetyl-TAGs with the expression of *Ef*DAcT alone and in combination with DGAT1 suppression through lipid analysis in transgenic seed. We also track acetyl-TAG levels and enzyme protein accumulation in developing seeds to understand the relationship between enzyme expression and the generation of enzyme product. In Chapter 3, citrate lyase is over expressed in *Camelina sativa* in an attempt increase levels of acetyl-CoA substrate used by the acetyltransferase enzymes. This is also done in combination with the suppression of DGAT1.

Chapter 2 – Enhancing acetyl-TAG production with an improved acetyltransferase

2.1 Introduction

The unusual triacylglycerol 3-acetyl-1,2-diacyl-*sn*-glycerol (acetyl-TAG) possesses an acetate group at the *sn*-3 position of the glycerol backbone instead of a normal long chain fatty acid (Figure 1.3). The presence of this acetate group gives acetyl-TAGs advantageous properties desirable for various biofuel and industrial applications. They have superior cold temperature properties and low viscosity making acetyl-TAGs preferable as a vegetable oil biofuel [21, 22]. The synthesis of acetyl-TAGS are catalyzed by diacylglycerol acetyltransferase (DAcT) enzymes from various species, including *Euonymus alatus* and *Euonymus fortunei*. The enzyme *Ef*DAcT has demonstrated higher activity in vitro and in vivo through experiments where the production of acetyl-TAGs was quantified for different DAcT enzyme orthologs (Figure 1.3). Here we validate that the expression of *Ef*DAcT in *Camelina sativa* yields higher levels of acetyl-TAGs in T_2 and T_3 seed compared to the expression of *Ea*DAcT. The diacylglycerol acyltransferase (DGAT1) enzyme competes with both enzymes for their diacylglycerol (DAG) substrate to produce normal lcTAGs. We show that the combination of *Ef*DAcT expression and DGAT1 suppression leads to significantly higher levels of acetyl-TAG accumulation in T_2 seed compared to the expression of the *Ea*DAcT. Since the expression of *Ef*DAcT alone and *Ef*DAcT coupled with the suppression of DGAT1 consistently generated high amounts of acetyl-TAGs, we tracked their production in developing T_4 seeds over time alongside wild type controls. The pattern of enzyme expression levels within the developing seeds reveals their relationship with the synthesis of acetyl-TAGs.

2.2 Materials and Methods

2.2.1 Construction of binary vectors, generation of transgenic *Camelina sativa*, and selection of transgenic seed.

Constructs were assembled in the T-DNA binary vector pBinGlyRed which contains the fluorescent protein visual marker *Ds*Red as a selectable marker and the glycinin promoter for the seed specific expression of *Ea*DAcT-HA and *Ef*DAcT-HA alone or in combination with DGAT1-RNAi. An oleosin promoter was used for the suppression of the DGAT1-RNAi hairpin. Constructs were then transformed into the *Agrobacterium tumefaciens* strain GV3101 and subsequently transformed into flowering wild type *Camelina sativa* using the *Agrobacterium* mediated floral dip vacuum infiltration method [25]. Harvested T_1 seeds were inspected for the *Ds*Red visual marker under a green LED light and red filter lens. T_1 seeds expressing a red fluorescence were planted while seeds without the visual marker were removed. 100 T_2 transformant seeds from each T_1 plant were selected at random and screened for a 3:1 ratio of fluorescent vs. non-fluorescent seeds. Plant lines passing the chi-squared test were analyzed for their oil content. Seeds without the visual marker were not analyzed. Selected T_2 seeds were propagated to the T_3 generation, where again 100 seeds were randomly selected, examined for fluorescence, and analyzed for their lipid content.

2.2.2 Lipid analysis

Between 15 mg and 20 mg of dried seeds were heated at 85 °C in 2.0 ml of isopropanol and ground in glass tubes using a 7.0 mm Polytron probe (PT2500E, Kinematica AG, Switzerland). Afterwards 2.5 ml of 6.6 % w/v K2SO4, 3.0 ml hexane, and 50 µg of tripentadecanonin (NuChek Prep, Waterville, MN) internal standard were added to the seeds. The tubes were vortexed, centrifuged, and the organic phase was

removed and placed in another tube. This was repeated again with 2 ml hexane/ isopropanol (7:2). The combined organic phases were evaporated under nitrogen gas, dissolved in chloroform, and stored in -20 °C until used for further work.

To separate the acetyl-TAGs and lcTAGs, 50 µl of the lipid extract dissolved in chloroform was added to Silica gel 60 TLC plates and placed in a hexane/diethyl-ether/glacial acetic acid (70:30:1) solvent system. When dried, the plate was sprayed with 2,7-dichlorofluorescein and placed under a UV light for visualization. Then, 30 µg of heptadecanoin (NuChek Prep, Waterville, MN) was added to each TAG spot, which were scraped off into a separate tube and dissolved in 500 µl toluene.

The TAGs were subjected to acid based transmethylation where 1 ml mixture of methanol/sulphuric acid/0.2 % butylated hydroxytoluene (0.95:0.05:0.025) was added to each tube and heated at 90 – 95 °C for 30 - 45 minutes. When cooled to room temperature 1.5 ml of 0.9 % KCl and 2.0 ml hexane was added and vortexed. Tubes were centrifuged with the organic phase separated and added into another tube. This was repeated again with 2.0 ml hexane.

After evaporation with nitrogen gas the lipid was dissolved in 500 µl hexane. All samples were analyzed using an Agilent gas chromatography machine equipped with a 30 meter DB-23 column with a flame ionization detector. The oven temperature was maintained at 200 ˚C for 2.0 minutes and ramped to 240 ˚C at 10˚C min^{-1} and kept there. FAMEs were identified by analyzing their retention times and normalized to the internal standards, tripentadecanonin and triheptadecanoin.

2.2.3 Developing T_4 seeds across time and enzyme expression

Selected plant lines that accumulated the highest levels of acetyl-TAGs were

propagated to the T_4 generation. Plant lines expressing *Ef*DAcT alone and *Ef*DAcT coupled with the suppression of DGAT1 were grown along with wild type controls. Flowering buds were tied off with differed colored string 5 days apart from each other to track the production of TAGs over seed development. After 40 days, labeled seeds from each time point were frozen in liquid nitrogen and stored in -80 °C. Then, 20 seeds were subjected to lipid analysis, as previously described, to observe the accumulation of acetyl-TAGs and lcTAGs over time.

2.2.3.1 Seed microsome preparation

For each time point, 40 seeds were collected and pooled from wild type plants or transgenic lines expressing, *Ef*DAcT, and *Ef*DAcT with DGAT1-RNAi. Samples and extracts were kept on ice throughout preparation. Seeds were placed in 4.0 ml extraction buffer (100mM Tris-HCl pH 8.0, 10% glycerol (v/v), 1 mM DTT, 250 mM PMSF inhibitor cocktail) ground with a 7.0 mm Polytron probe, and centrifuged at 4,000 rpm for 30 minutes at 4 °C. The supernatant was separated and centrifuged again at 100,000 g for 1 hour at 4 °C. The microsome pellet was dissolved in 300 µl extraction buffer (100 mM Tris-HCl ph 8.0, 10% glycerol (v/v)) and stored in -80 °C until further use.

2.2.3.2 Western blotting and protein quantification

Dissolved microsomes from each time point and 2X sample buffer (12.5 mM Tris-chloride, 8M Urea, 10% SDS, 100 µl of 4% bromophenol blue, 10 mM DTT) were mixed together in a 1:1 ratio. 50 µl of the mixture was pipetted into 10% sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and subjected to electrophoresis at 110 volts for 1 hour 45 minutes. Protein from the gels was transferred onto a nitrocellulose membrane through electroblotting at 60 volts for 1 hour. The

membrane was washed with PBS (0.8% NaCl, 0.02% KCl, 0.024% KH2PO4, 0.144% Na2HPO4 dissolved in dH2O), incubated in 5 % blocking buffer (5% skim milk powder dissolved in PBS) for 1 hour, washed again, and incubated overnight with 1:2000 primary antibody mouse anti-HA. The membrane was washed again, incubated with 1:5000 secondary antibody goat anti-mouse for one hour, washed again and incubated for 4 minutes with Femto substrate (Fisher Scientific PI-34085) mixed in a 1:1 ratio. The membrane was immediately put in an Azure Biosystems imager machine for chemiluminescent signal detection of the secondary antibody. Protein bands were normalized and calculated against the control of 2.5 µg/µl yeast microsomes of *Ef*DAcT.

2.3 Results and Discussion

2.3.1 Expression of *Ef*DAcT in *Camelina sativa* leads to increased levels of acetyl-TAGs

Previously, the overexpression of *Ea*DAcT alone in wild type *Camelina sativa* lead to an accumulation of 47 to 64 mol % acetyl-TAGs depending on the transgenic line [22]. Our results are consistent with this data where the transformation of *Camelina sativa* with *Ea*DAcT-HA yielded 64 mol % for the best plant line with an average of 53 mol % acetyl-TAGs for 7 independent plant linee in T_2 seed (Figure 2. 1). Like *Ea*DAcT, *Ef*DAcT catalyzes the same reaction to produce acetyl-TAGs. Since it demonstrates higher activity in vitro and in vivo (Figure 1.3), we hypothesized that it would increase the production of acetyl-TAGs when expressed in plant seeds. Compared to *Ea*DAcT, the expression of *Ef*DAcT-HA led to a significant increase in the accumulation of acetyl-TAGs, where the best plant line yielded 81 mol % acetyl-TAGs with an average of 71 mol % for 23 independent plant lines in T_2 seed (Figure 2.1).

In transgenic T_2 seed, the fatty acid composition of *Ea*DAcT-HA and

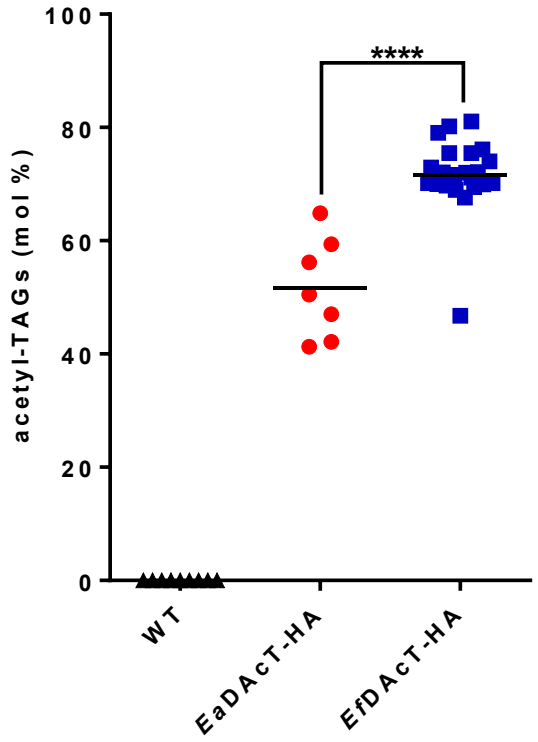

Figure 2.1 ***Ef*DAcT expression leads to increased acetyl-TAG accumulation in T_2 seed.** Scatter plot of the distribution of acetyl-TAGs, in T_2 seed from 7 independent lines expressing *Ea*DAcT and 23 independent lines expressing *Ef*DAcT. Seeds without the visual *Ds*Red transformation marker were excluded from analysis. Horizontal lines represent the average value from each group. Asterisks indicate significant difference (Mann-Whitney U-test, ****, $P < 0.0001$).

*Ef*DAcT-HA are similar to each other (Figure 2.2a and 2.2b). For both enzymes, acetyl-TAGs are enriched at the 18:1, 18:2, and 18:3 fatty acids compared to lcTAGs, consistent with a previous study [22]. However, for *Ef*DAcT-HA, the levels of acetyl-TAG enrichment are higher in these fatty acids than *Ea*DAcT-HA.

Since T_2 generation seeds are segregating for the transgene, we were curious to know if the acetyl-TAG levels in these seeds were predictive of levels in T_3 seeds homozygous for the insertion. Therefore we selected a range of the highest, medium, and lowest acetyl-TAG producing T_2 seed lines for propagation to the T_3 generation.

Homozygous T_3 seed were analyzed for their acetyl-TAG content and compared to their T_2 generation counterparts.

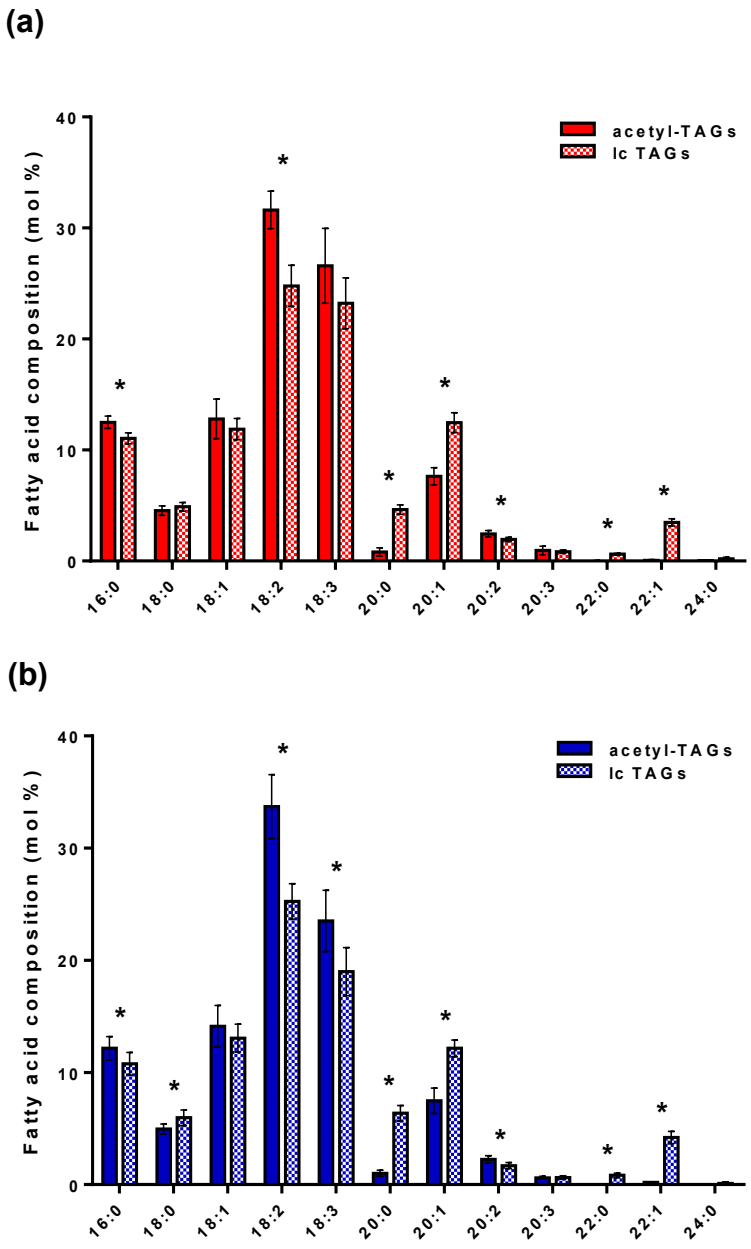

Figure 2.2 **Fatty acid composition of T_2 seed expressing *Ea*DAcT or *Ef*DAcT.** (a) Average fatty acid composition of T_2 seed expressing *Ea*DAcT in 7 independent plant lines. (b) Average fatty acid composition of T_3 seed expressing *Ef*DAcT in 21 independent plant lines. Seeds without the visual *Ds*Red transformation marker were excluded from analysis. Asterisks indicate significant difference (Students t-test, *, $P < 0.05$)

2.3.2 *Ea*DAcT and *Ef*DAcT have stable levels of acetyl-TAGs in the T_3 generation

When comparing transgenic lines for *Ea*DAcT-HA there was a slight, non-significant decrease in the average amount of acetyl-TAGs from 53 mol % to 47 mol % for 9 independent homozygous lines (Figure 2.3a). A similar result was observed for *Ef*DAcT-HA where the average level of acetyl-TAG accumulation was reduced from 71 mol % in the T_2 generation to 67 mol % for 21 independent lines in the T_3 generation (Figure 2.3b). This difference was also statistically non-significant.

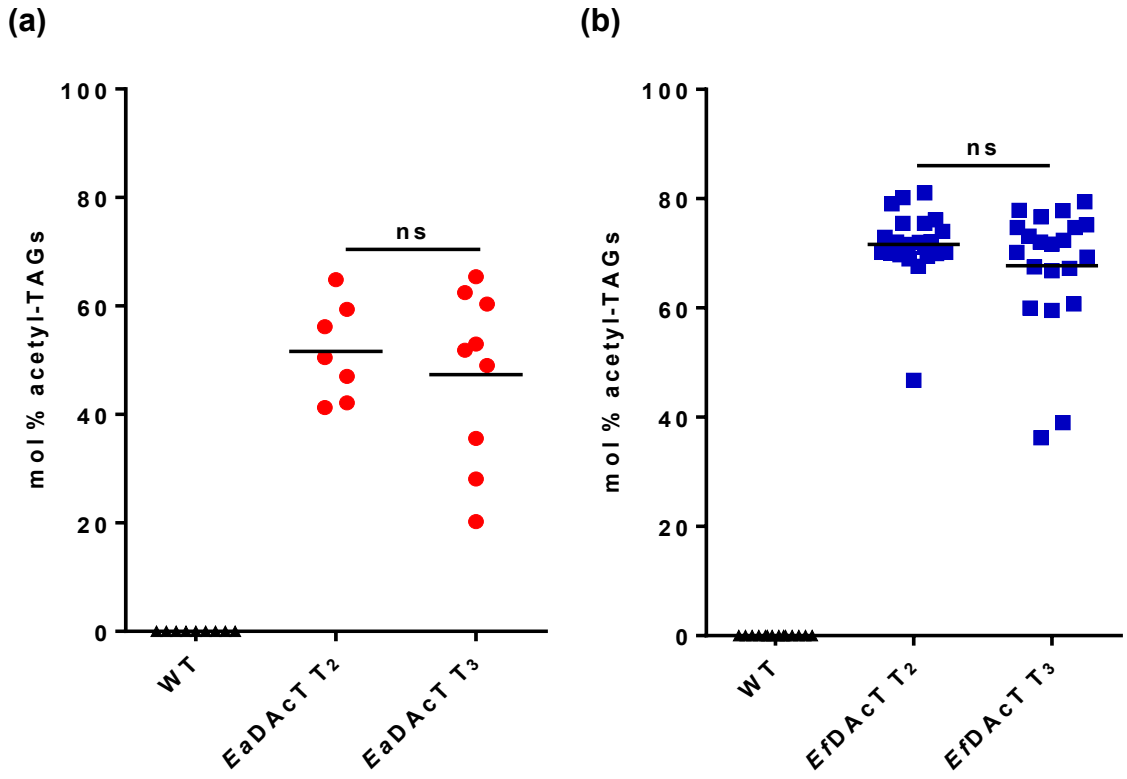

Figure 2.3 **Acetyl-TAG accumulation remains stable into the T_3 generation in transgenic *Camelina sativa* expressing *Ea*DAcT or *Ef*DAcT.** (a) Scatter plot of the distribution of acetyl-TAGs in T_2 seed from 7 independent lines and T_3 seed from 9 independent homozygous lines expressing *Ea*DAcT (b) Scatter plot of the distribution of acetyl-TAGs in T_2 seed from 23 independent lines and T_3 seed from 21 independent homozygous lines expressing *Ef*DAcT. Seeds without the visual *Ds*Red transformation marker were excluded from analysis. Horizontal lines represent the average value from each group. (Mann-Whitney U-test, ns indicates no significance.

Since we observed a small drop in the average accumulation of acetyl-TAGs, we tested T_3 plant lines that were hemizygous and compared them to their T_3 homozygous counterparts to see if this observation was consistent. For *Ea*DAcT-HA, 7 independent T_3 hemizygous lines yielded an average of 50 mol % acetyl-TAGs (Figure 2.4). For *Ef*DAcT-HA, 23 independent T_3 hemizygous lines yielded an average of 71 mol % acetyl-TAGs (Figure 2.4). Although the average acetyl-TAG content of T_3 hemizygous seeds is slightly higher than homozygous seeds, there was no statistical difference between them for both *Ea*DAcT-HA and *Ef*DAcT-HA. Some of the T_3 hemizygous plant lines demonstrated lower acetyl-TAG accumulation compared to their homozygous counterparts. For instance, in *Ea*DAcT-HA, out of a total of 16 plants, only 2

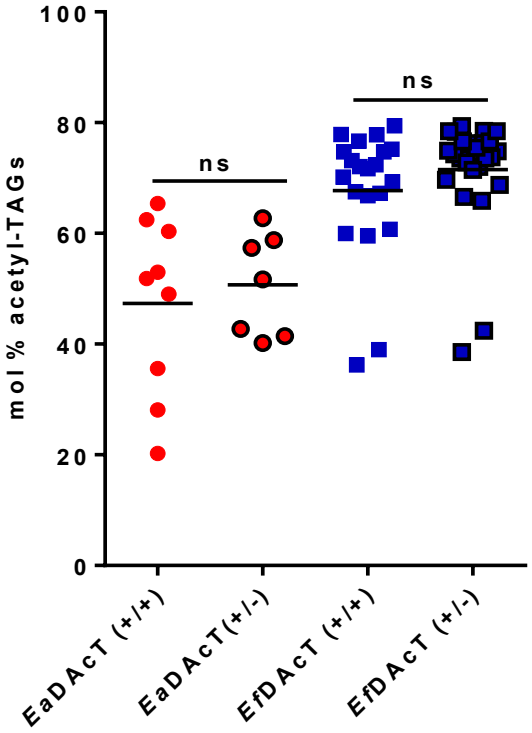

Figure 2.4 **The average levels of acetyl-TAGs are similar between T_3 homozygous and hemizygous seed.** Scatter plot of the distribution of acetyl-TAGs in T_3 hemizygous and homozygous seed expressing either *Ea*DAcT or *Ef*DAcT. Horizontal lines represent the average value from each group. (Kruskal-Wallis test with Dunn multiple comparisons, ns indicates no significance).

homozygous seed lines displayed higher levels of acetyl-TAGs. For, *Ef*DAcT-HA, out of 46 plants, only 1 homozygous seed line accumulated more acetyl-TAGs compared to their hemizygous counterparts. The variances between the T_3 homozygous and hemizygous plant lines are small and within the tolerated margin of error. Therefore, there is no significant difference between the two populations for either enzyme. Overall, acetyl-TAG levels remain stable with minor fluctuations between the T_2 and T_3 generations for both homozygous and hemizygous seed.

As a whole there is a positive correlation between the T_2 and T_3 generations for both *Ea*DAcT and *Ef*DAcT with a total combined R^2 value of 0.7694 (Figure 2.5). This observation is important because analysis and selection for transgenic seeds at the T_2 generation is reflective of acetyl-TAG levels in seeds at the T_3 generation. This data suggests that we may analyze T_2 seeds and select high acetyl-TAG accumulating lines to

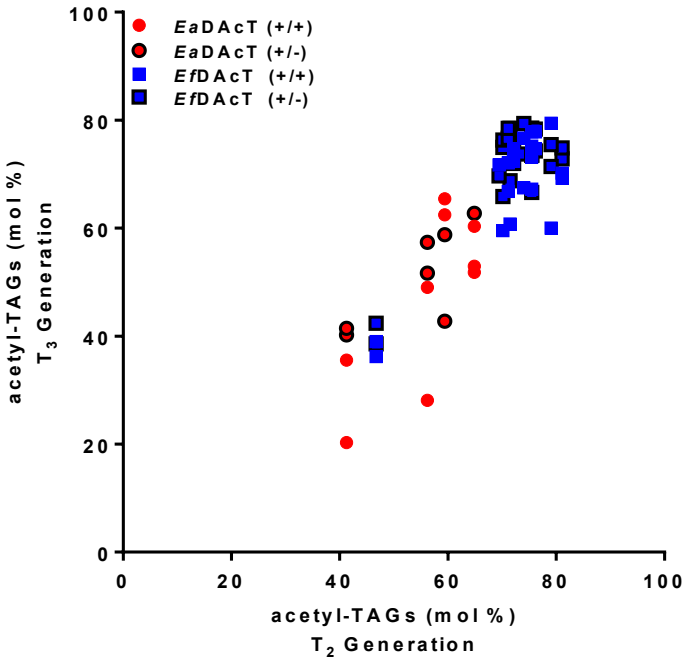

Figure 2.5 **Positive correlation between the T_2 and T_3 generation for both *Ea*DAcT and *Ef*DAcT.** Linear regression comparing the T_2 generation and T_3 seed hemizygous and homozygous expressing *Ea*DAcT or *Ef*DAcT. $R^2 = 0.7694$.

grow into the next generation knowing that the amount of acetyl-TAGs will remain relatively stable. The fatty acid compositions for *Ea*DAcT in both of the T_2 and T_3 generations are similar to each other (Figures 2.3a & 2.6a). For *Ef*DacT, the

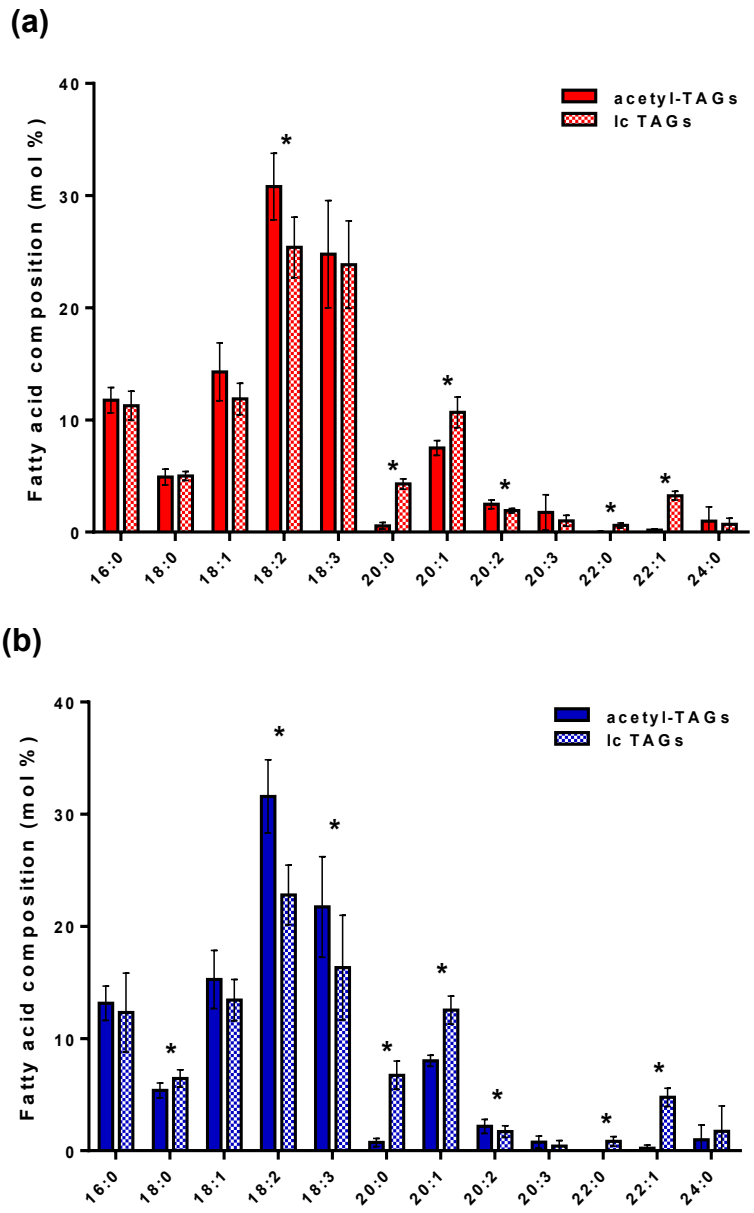

Figure 2.6 **Fatty acid composition of T_3 seed expressing *Ea*DAcT or *Ef*DAcT.** (a) Average Fatty acid composition of T_3 seed expressing *Ea*DAcT in 9 homozygous independent plant lines. (b) Average fatty acid composition of T_3 seed expressing *Ef*DAcT in 23 independent plant lines. Asterisks indicate significant difference Seeds without the visual *Ds*Red transformation marker were excluded from analysis. Asterisks indicate significant difference (Students t-test, *, $P < 0.05$).

fatty acid compositions are also alike (Figures 2.3b & 2.6b). As previously observed in the T_2 generation, acetyl-TAGs are likewise enriched at the 18:1, 18:2, and 18:3 fatty acids compared to lcTAGs for the T_3 generation. Again, in these fatty acids, the levels of acetyl-TAG enrichment in *Ef*DAcT-HA are higher than *Ea*DAcT-HA.

2.3.3 DGAT1 suppression leads to increased levels of acetyl-TAGs

In the last step of triacylglycerol synthesis, a long chain fatty acid is added to the *sn*-3 position of the glycerol backbone by the enzyme DGAT1. This reaction might compete with *Ea*DAcT or *Ef*DAcT for their common DAG substrate. DNA constructs with RNAi sequences designed to suppress DGAT1 expression were co-expressed with *Ea*DAcT or *Ef*DAcT.

For both *Ea*DAcT and *Ef*DAcT in combination with the suppression of DGAT1, the levels of acetyl-TAGs significantly increased to an average of 63 mol % and 80 mol % respectively for the T_2 generation (Figure 2.7). The highest levels of acetyl-TAG accumulation for both *Ea*DAcT and *Ef*DAcT plus the knockdown of DGAT1 are 83 mol % and 86 mol % respectively (Figure 2.7). This further supports the notion that the *Ef*DAcT enzyme is able to increase acetyl-TAG production in *Camelina sativa* particularly in combination with the knockdown of DGAT1.

The fatty acid composition for *Ea*DAcT and *Ef*DAcT coupled with the suppression of DGAT1 exhibits a similar trend previously demonstrated in both enzymes without the DGAT1 knockdown. Similarly, acetyl-TAGs are enriched at the 18:1, 18:2, and 18:3 fatty acids for both enzymes (Figure 2.8a & 2.8b). For these fatty acids, levels of acetyl-TAG enrichment are higher in *Ef*DAcT+DGAT1 than in *Ea*DAcT+DGAT1.

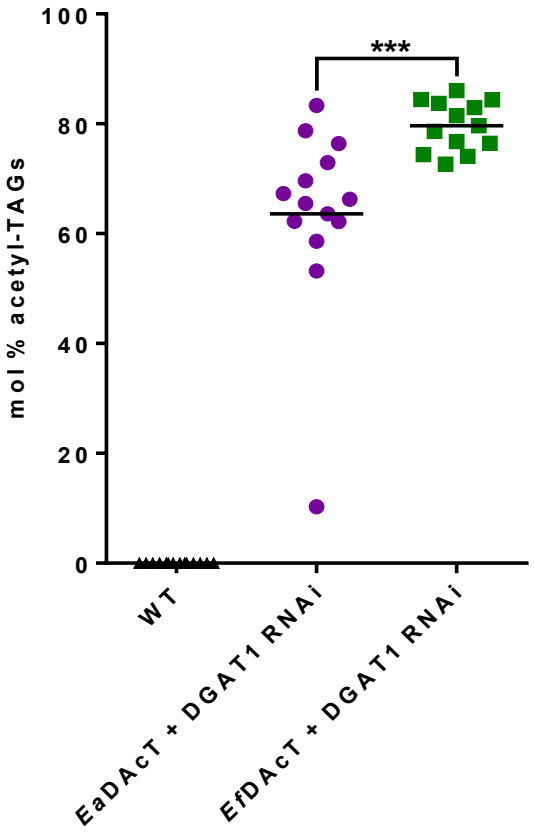

Figure 2.7 **Transgenic *Camelina sativa* leads to increased levels of acetyl-TAGs in T_2 seeds expressing *Ef*DAcT + DGAT1 RNAi.** Scatter plot of acetyl-TAG levels from T_2 transgenic *Camelina sativa* seed expressing *Ea*DAcT + DGAT1 RNAi or *Ef*DAcT + DGAT1 RNAi. Seeds without the visual *Ds*Red transformation marker were excluded from analysis. Asterisks indicate significant difference (Mann-Whitney U-test, ***, $P < 0.001$).

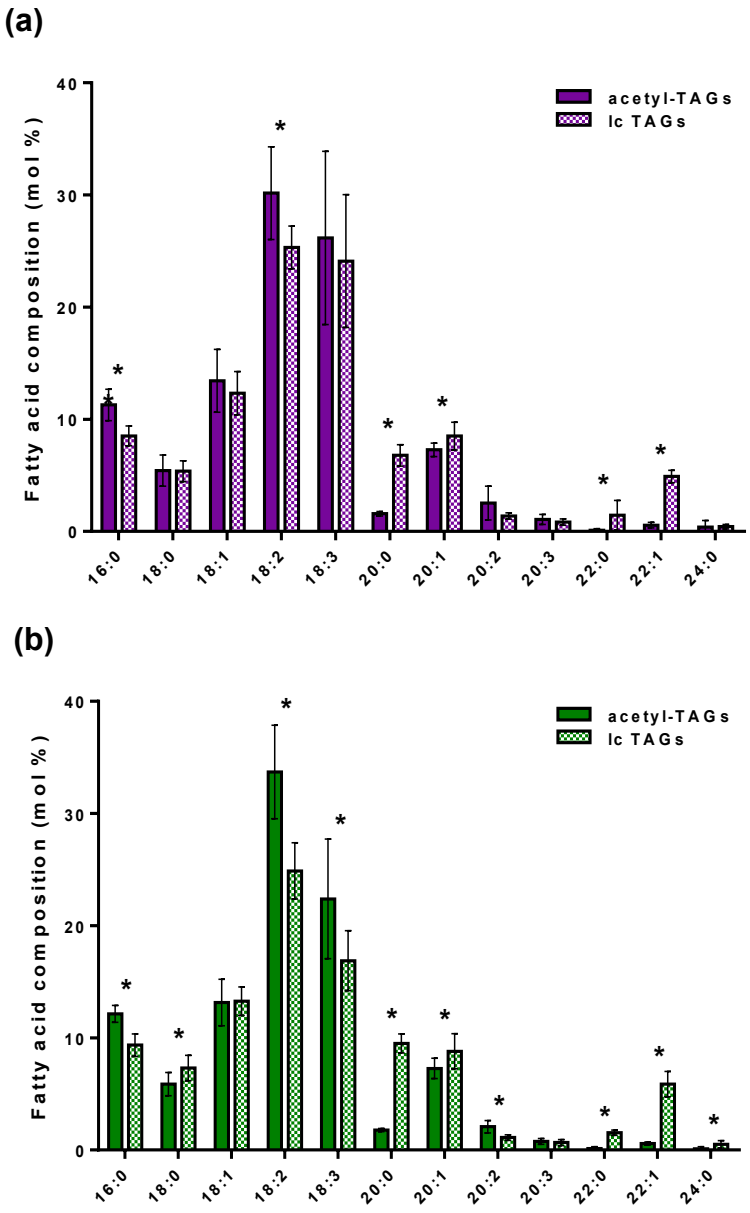

Figure 2.8 **Fatty acid composition of T_2 seed expressing *Ea*DAcT + DGAT1 RNAi or *Ef*DAcT + DGAT1 RNAi.** (b) Average fatty acid composition of T_3 seed expressing *Ea*DAcT + DGAT1 RNAi in 13 independent plant lines. (c) Average fatty acid composition of T_3 seed expressing *Ef*DAcT + DGAT1 RNAi in 12 independent plant lines. Seeds without the visual *Ds*Red transformation marker were excluded from analysis. Asterisks indicate significant difference (Students t-test, *, $P < 0.05$).

2.3.4 Accumulation of TAGs in developing T_4 seeds expressing *Ef*DAcT or *Ef*DAcT + DGAT1 RNAi

In an effort to understand the accumulation of acetyl-TAGs in developing transgenic *Camelina sativa* seeds, high yielding *Ef*DAcT and *Ef*DAcT + DGAT1 RNAi T_3 plants were grown along with wild type plants to produce T_4 seed. Seeds were collected over the course of development and analyzed for their acetyl-TAG and lcTAG content (Figure 2.9a). For wild type plants, the production of lcTAGs follows a sigmoidal curve correlating with previous publications studying the oil content in developing *Camelina sativa* seed [29]. For *Ef*DAcT, acetyl-TAG development increased rapidly during the 15 and 20 day time points reaching a peak at the 25 days after flowering time point while lcTAG production remain relatively constant and low. In comparison, *Ef*DAcT combined with the suppression of DGAT1 demonstrates a slightly different trend. The levels of acetyl-TAGs increase throughout the 15, 20, and 25 day time periods and peak at the 30 day time point before dropping at the 40 day time point. Accumulation of lcTAG remains low with a small dip between the 20 and 25 day time points. Interestingly, this small dip in lcTAG accumulation mirrors a large increase in acetyl-TAG accumulation between the 20 and 25 day time points. Since DGAT1 is suppressed, more of the DAG substrate is free to be used for the synthesis of acetyl-TAGs, not lcTAGs. When the production of acetyl-TAGs quickly increases, generation of lcTAGs decreases. This data shows how rapidly acetyl- TAGs are synthesized in the early stages of development before accumulation stabilizes. Analysis of dry T_4 seed reveals that expression of *Ef*DAcT-HA yields 77 mol % acetyl- TAGs. *Ef*DAcT coupled with DGAT1 suppression contain 90 mol % acetyl-TAGs in its seed (Figure 2.9b).

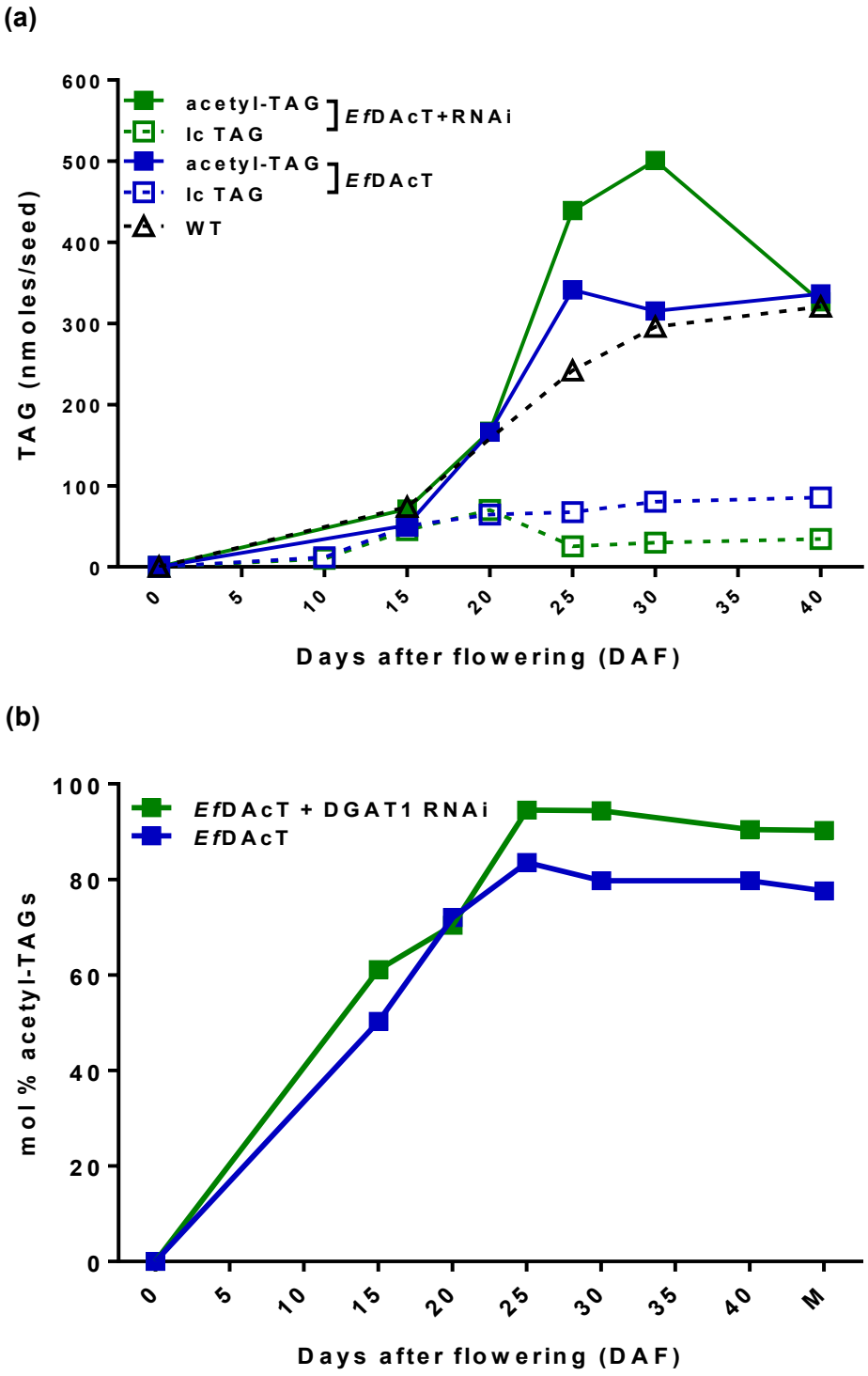

Figure 2.9 **Accumulation of acetyl-TAG and lcTAG in developing transgenic *Camelina sativa* seeds in high yielding *Ef*DAcT and *Ef*DAcT + DGAT1 RNAi.** (a) Acetyl-TAG and lcTAG levels in developing wild type seeds and homozygous T_4 seeds expressing *Ef*DAcT alone or *Ef*DAcT with DGAT1 suppression. (b) Acetyl-TAG composition over time for developing seeds expressing *Ef*DAcT or *Ef*DAcT with DGAT1 suppression. Dry mature seed is denoted by M.

2.3.5 Protein accumulation in T_4 developing seeds

We also analyzed levels of *Ef*DAcT-HA protein in T_4 developing seeds. For both, enzyme accumulation first appears at the 15 day time point reaching a peak at the 20 day time point. At the 25 day time point the signal has diminished and is not detected at the other time points (Figure 2.10). This data correlates well with the pattern of acetyl-TAG accumulation previously demonstrated in Figure 2.5 with the highest levels of enzyme accumulation occurring just prior to the rapid increase in acetyl-TAG accumulation in the seeds. Towards the end of seed development, we see no enzyme expression, which coincides with the decrease and subsequent stabilization of acetyl-TAG levels. Although the expression of *Ef*DAcT with the DGAT1 knockdown produces higher levels of acetyl-TAG accumulation over time, the expression of *Ef*DAcT alone displays a slightly stronger signal on the Western blot. Future work into protein accumulation may help clarify this result.

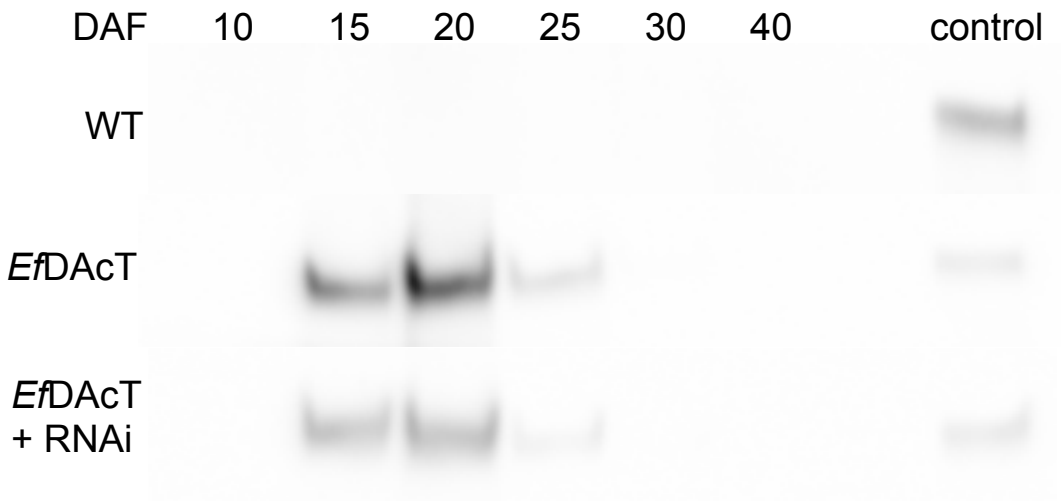

Figure 2.10 **Enzyme accumulation across developing seeds.** Protein extraction from 40 seeds across 10, 15, 20, 25, 30, and 40 day time points were collected from 12 plants expressing wild type, *Ef*DAcT, and *Ef*DAcT + DGAT1 RNAi. Seed microsomes were subjected to Western blotting. Control is 2.5 µg/µl *Ef*DAcT yeast microsomes.

2.4 Conclusions

This research was conducted with the purpose of maximizing acetyl-TAG accumulation in the seeds of *Camelina sativa* through metabolic engineering of its lipid synthesis pathways. This study demonstrates that obtaining high levels of acetyl-TAG is successfully achieved through the expression of *Ef*DAcT and *Ef*DAcT coupled with the suppression of DGAT1. When attempting to maximize the production of acetyl-TAGs, it is preferable to use *Ef*DAcT over *Ea*DAcT, especially with the DGAT1 knockdown. Protein expression levels in developing transgenic seed correspond with acetyl-TAG accumulation. Our data suggests that if *Ef*DAcT accumulation levels are increased, then acetyl-TAG synthesis may increase even further. Western blotting exhibited a slightly stronger signal for the expression of *Ef*DAcT alone in developing seeds despite *Ef*DAcT coupled with DGAT1 suppression producing higher levels of acetyl-TAGs (Figure 2.9). Previously, the expression of *Ef*DAcT+DGAT1 RNAi in dry T_2 seed led to significantly higher levels of acetyl-TAGs than *Ef*DAcT alone (Figures 2.1, 2.7). Although *Ef*DAcT alone exhibits higher protein levels in the Western Blot, it is not sufficient enough to outperform *Ef*DAcT+DGAT1 RNAi acetyl-TAG accumulation even though it demonstrates lower amounts of protein. This suggests that DGAT1 suppression contributes more towards acetyl-TAG production than increased *Ef*DAcT-HA protein levels by itself. Even higher levels of acetyl-TAGs are possible through optimization of the glycinin promoter to obtain high transgene and protein expression. The manipulation of lipid synthesis pathways in *Camelina sativa* with the introduction of an improved enzyme, *Ef*DAcT, presents a novel approach to enhancing the production of acetyl-TAGs.

Chapter 3 - Overexpression of citrate lyase

3.1 Introduction

The *Euonymus alatus* diacylglycerol acetyltransferase (*Ea*DAcT) and *Euonymus fortunei* diacylglycerol acetyltransferase (*Ef*DAcT) enzymes synthesize acetyl-TAGs at the end of a metabolic pathway. Both enzymes use acetyl-coenzyme A (acetyl-CoA) as a substrate and transfer the acetyl group to the *sn*-3 position of the glycerol backbone. We hypothesized that increasing acetyl-CoA levels would also enhance acetyl-TAG accumulation. It is known that the enzyme ATP:citrate lyase catalyzes the cleavage of citrate to produce ADP, oxaloacetate, and acetyl-CoA. Therefore, one way to increase the supply of acetyl-CoA is though the introduction of citrate lyase. Previously, the over expression of *Rattus norvegicus* ATP: citrate lyase (*Rn*ACLY) targeted to plastids in tobacco led to an increase of fatty acids by 16 % and did not significantly alter overall fatty acid composition [30]. We hypothesized that if both *Ea*DAcT and *Ef*DAcT enzymes have an abundant supply of acetyl-CoA substrate, then the production of acetyl-TAGs may increase. We tested this through the expression of *Rn*ACLY in the cytosol. Here we co-express *Rn*ACLY with both acetyltransferase enzymes to see whether increasing acetyl-CoA leads to higher acetyl-TAGs levels. *Ef*DAcT competes with the diacylglycerol acyltransferase (DGAT1) enzyme for their common DAG substrate.

3.2 Materials and methods

3.2.1 Plasmid construction and transformation of *Camelina sativa*

Constructs were assembled in the T-DNA binary vector pBinGlyRed which contains the fluorescent protein *Ds*Red as a selectable marker and the glycinin promoter for the seed specific expression of *Ea*DAcT and *Ef*DAcT in combination with *Rn*ACLY.

An oleosin promoter was used for the suppression of the DGAT1-RNAi hairpin. Constructs were then transformed into the *Agrobacterium tumefaciens* strain GV3101 and subsequently transformed into flowering wild type *Camelina sativa* using the *Agrobacterium* mediated floral dip vacuum infiltration method [25]. Harvested T_1 seeds were inspected for the *Ds*Red visual marker under a green LED light and red filter lens. T_1 seeds expressing a red fluorescence were planted while seeds without the visual marker were removed. 100 T_2 transformant seeds from each T_1 plant were selected at random and screened for a 3:1 ratio of fluorescent vs. non-fluorescent seeds. Seeds without the visual marker were not analyzed. Selected T_2 seeds were propagated to the T_3 generation, where again 100 seeds were randomly selected, examined for fluorescence, and analyzed for their lipid content.

3.2.2 Lipid analysis

Between 15 mg and 20 mg of dried seeds were heated at 85 °C in 2.0 ml of isopropanol and ground in glass tubes using a 7.0 mm Polytron probe (PT2500E, Kinematica AG, Switzerland). Afterwards 2.5 ml of 6.6 % w/v K2SO4, 3.0 ml hexane, and 50 µg of tripentadecanonin (NuChek Prep Waterville, MN) internal standard were added to the seeds. The tubes were vortexed, centrifuged, and the organic phase was removed and placed in another tube. This was repeated again with 2 ml hexane/ isopropanol (7:2). The combined organic phases were evaporated under nitrogen gas, dissolved in chloroform, and stored in -20 °C until used for further work.

To separate the acetyl-TAGs and lcTAGs, 50 µl of the lipid extract dissolved in chloroform was added to Silica gel 60 TLC plates and placed in a hexane/diethyl-ether/glacial acetic acid (70:30:1) solvent system. When dried, the plate was sprayed with

2,7-dichlorofluorescein and placed under a UV light for visualization. Then, 30 µg of heptadecanoin (NuChek Prep Waterville, MN) was added to each TAG spot, which were scraped off into a separate tube and dissolved in 500 µl toluene.

The TAGs were subjected to acid based transmethylation where 1 ml mixture of methanol/sulphuric acid/0.2 % butylated hydroxytoluene (0.95:0.05:0.025) was added to each tube and heated at 90 - 95 ° C for 30 - 45 minutes. When cooled to room temperature 1.5 ml of 0.9 % KCl and 2.0 ml hexane was added and vortexed. Tubes were centrifuged with the organic phase separated and added into another tube. This was repeated again with 2.0 ml hexane.

After evaporation with nitrogen gas the lipid was dissolved in 500 µl hexane. All samples were analyzed using an Agilent gas chromatography machine equipped with a 30 meter DB-23 column with a flame ionization detector. The oven temperature was maintained at 200 ˚C for 2.0 minutes and ramped to 240 ˚C at 10˚C min^{-1} and kept there for 4 minutes. FAMEs were identified by analyzing their retention times, normalized to the internal standards, tripentadecanonin and heptadecanoin, and quantified as nmoles.

3.3 Results and discussion

3.3.1 Citrate lyase over expression does not significantly increase acetyl-TAG accumulation

Previous work showed that the overexpression of citrate lyase in tobacco led to an increase in overall fatty acid synthesis [30]. It was hypothesized that the expression of *Rn*ACLY with *Ea*DAcT or *Ef*DAcT would also increase acetyl-CoA levels. If there is more substrate for the acetyltransferase enzymes to use then there may be an increase in acetyl-TAG production as well. Acetyl-TAG accumulation resulted in an average of 56 mol % for T_2 seed from 5 independent transgenic plant lines expressing

*Ea*DAcT+*Rn*ACLY (Figure 3.1). This is not statistically significant compared to the expression of *Ea*DAcT alone which yielded an average of 51 mol % acetyl-TAGs. Also, the co-expression of *Ef*DAcT with citrate lyase yielded an average of 60 mol % acetyl-TAGs in 7 independent T_2 plant lines (Figure 3.1). Compared *Ef*DAcT's average of 71 mol %, the decrease in the average production of acetyl-TAGs for the co-expression *Ef*DAcT and *Rn*ACLY is statistically significant. Interestingly, the expression of *Ef*DAcT and *Rn*ACLY resulted in plant lines synthesizing a wide range of acetyl-TAG levels from 39 - 76 mol %.

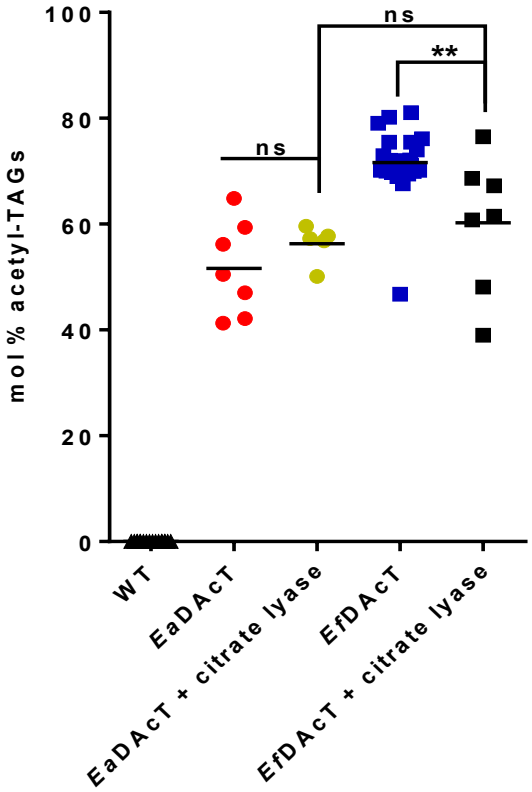

Figure 3.1 **Over expression of citrate lyase does not significantly increase acetyl-TAG production.** Seeds without the visual *Ds*Red transformation marker were excluded from analysis. Scatter plot of the distribution of acetyl-TAGs in T_2 seed with the co-expression of either *Ea*DAcT or *Ef*DAcT with citrate lyase. Horizontal lines represent the average value from each group. ns indicates no significance. Asterisks indicate significant difference (Kruska-Wallis test with Dunn multiple comparisons, **, $P < 0.01$

These results imply that the over expression of citrate lyase by itself is not adequate to significantly increase acetyl-TAG accumulation. The broad variance in acetyl-TAG levels for *Ef*DAcT's co-expression with citrate lyase suggests that there may be a difference in lipid metabolism between each individual *Camelina sativa* plant. It is possible that acetyl-CoA may build up at particular points throughout the lipid synthesis pathway limiting its ability to be used exclusively by *Ea*DAcT or *Ef*DAcT. Or it may be used elsewhere and funneled into other regulatory pathways since it is a commonly used metabolite.

The fatty acid composition for T_2 seed co-expressing *Ea*DAcT and citrate lyase exhibits a similar pattern to previous results. On average, acetyl-TAGs are enriched with 18:1, 18:2, and 18:3 fatty acids Figure (3.2a). The co-expression of *Ef*DAcT and citrate lyase also led enrichment of 18:1 and 18:2 fatty acids in acetyl-TAGs (Figure 3.2b). Compared to the co-expression of *Ea*DAcT and citrate lyase, *Ef*DAcT and citrate lyase demonstrate greater levels of acetyl-TAG enrichment at the 18:2 fatty acid with a smaller deviation from the average.

3.3.2 The co-expression of *Ef*DAcT and citrate lyase coupled with the suppression of DGAT1 leads to high levels of acetyl-TAGs

Since the average levels of acetyl-TAGs remained low with the over expression of citrate lyase, it was thought that the suppression of DGAT1 would assist in increasing its production. Since the *Ef*DAcT enzyme consistently produced the highest levels of acetyl-TAGs, we combined the expression *Ef*DAcT and citrate lyase with DGAT1 suppression. Interestingly, the average accumulation of acetyl-TAGs increased to an average of 84 mol % for 5 independent transgenic plants. The best plant line produced 87 mol % acetyl-TAGs (Figure 3.3). This is a significant difference from the expression of *Ef*DAcT alone

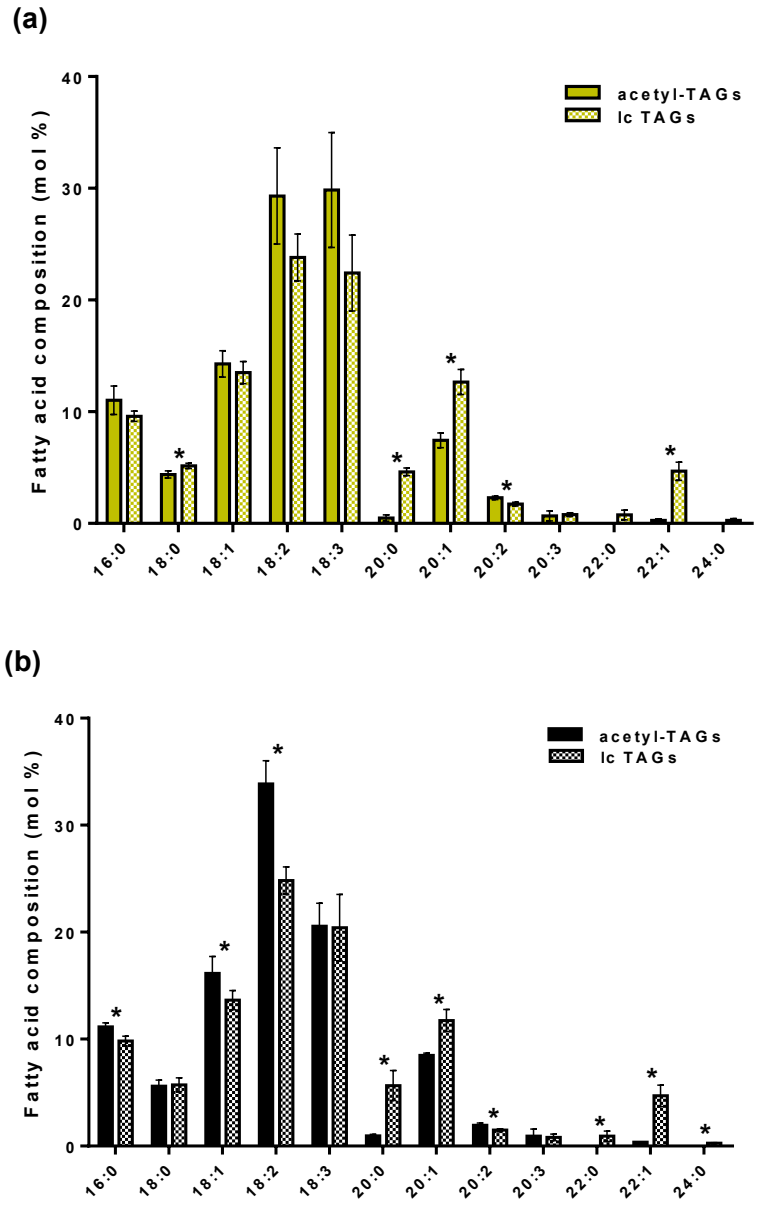

Figure 3.2 **Fatty acid composition of citrate lyase co-expressed with either *Ea*DAcT or *Ef*DAcT.** (b) Average fatty acid composition of T_2 seed from 5 independent plant lines expressing *Ea*DAcT and *Rn*ACLY. (c) Average fatty acid composition of T_2 seed from 7 independent plant lines expressing *Ef*DAcT and *Rn*ACLY. Seeds without the visual *Ds*RED transformation marker were excluded from analysis. Asterisks indicate significant difference (Students t-test, *, $P < 0.05$).

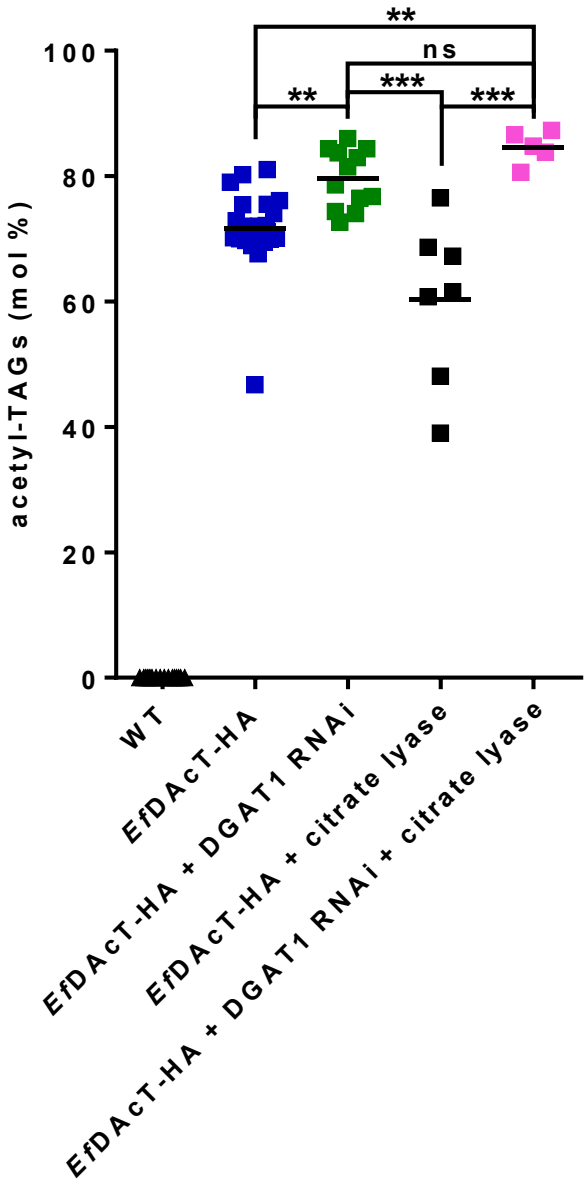

Figure 3.3 **Co-expression of *Ef*DAcT and citrate lyase combined with DGAT1 suppression significantly increases acetyl-TAG production.** Scatter plot of the distribution of acetyl-TAGs in T_2 seed from 5 independent lines expressing *Ef*DAcT and citrate lyase with the suppression of DGAT1. Seeds without the visual *Ds*Red transformation marker were excluded from analysis. Horizontal lines represent the average value from each group. ns indicates no significance. Asterisks indicate significant difference (Kruskal-Wallis test with Dunn multiple comparisons, *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$).

and the co-expression of *Ef*DAcT and citrate lyase. There is no significant difference between *Ef*DAcT+DGAT1 RNAi and *Ef*DAcT+*Rn*ACLY+DGAT1 RNAi. The suppression of DGAT1 may enable other unknown changes within lipid metabolism that allow acetyl-CoA to be used by the acetyltransferase enzymes. This data highlights the complexity of modifying metabolic flux within *Camelina sativa* plants, especially while using citrate lyase.

3.4 Conclusions

Acetyl-TAG accumulation failed to increase with the co-expression of *Rn*ACLY with *Ea*DAcT or *Ef*DAcT. It still remains to be determined if the overexpression of *Rn*ACLY results in increased acetyl-CoA levels. Further, even if there is an oversupply of acetyl-CoA, there is no guarantee that it will only be used by the acetyltransferase enzymes as it is a common metabolite used in many biochemical reactions. However, the co-expression of *Ef*DAcT with *Rn*ACLY and the suppression of DGAT1 significantly increased the production of acetyl-TAGs comparted to *Ef*DAcT+*Rn*ACLY. There is no significant difference between *Ef*DAcT coupled with *Rn*ACLY and the suppression of DGAT1 and *Ef*DAcT+DGAT1-RNAi. These results indicate that the over expression of *Rn*ACLY in combination with acetyltransferase enzymes is not sufficient enough to enhance acetyl-TAG synthesis. There must be further modification, particularly with DGAT1 suppression, of the lipid synthesis pathway to achieve this. Our data suggests that alteration of metabolic pathways in *Camelina sativa* is dynamic and involves intricate modification beyond increasing the supply of acetyl-CoA. This data is particularly interesting and raises many questions as to why a dramatic change in acetyl-TAG levels is observed.

With the over expression of citrate lyase, it is unknown how much acetyl-CoA is generated and how much of it is used by the acetyltransferase enzymes to produce acetyl-TAGs. In future work, developing seeds from *Camelina sativa* expressing *Rn*ACLY may will be analyzed for their acetyl-CoA content. Also, western blotting to quantify levels of *Rn*ACLY expression may provide insight into the accumulation of the enzyme. Since acetyl-CoA is a commonly used metabolite, future research should also focus on metabolic flux in developing seeds to investigate its dynamic role in lipid synthesis and the effects of any potential buildup and limited availability typically associated with the oversupply of substrate.

Chapter 4 - Conclusions and future work

In recent years, interest in utilizing renewable resources has grown through concerns of the damaging effects of climate change the limited availability of fossil fuels. The demand for utilizing renewable resources has created an interest in using vegetable oils and exploiting their beneficial qualities to reduce negative environmental impact. Since vegetable oils may be used various industrial applications, it becomes necessary to improve upon their chemical and physical characteristics to expand upon their functionality. Plant and vegetable oils are typically composed of long chain triacylglycerol (lcTAG) molecules comprised of a glycerol backbone with three long chain fatty acids. The structure of TAGs determines their chemical and physical properties that impacts how they may be used for particular industrial applications. When using vegetable oils directly as a biofuel, several problems emerge that limit its ability to be used in engines subsequently requiring structural modification of TAGs.

Acetyl-1,2-diacyl-*sn*-glycerols (acetyl-TAGs) are an unusual triacylglycerol molecule that possess an acetyl group at the *sn*-3 position of the glycerol backbone. The absence of a long chain fatty acid at the *sn*-3 position gives acetyl-TAGs advantageous characteristics, such as a 39 % reduction in viscosity and superior cold temperature properties, making their use preferable over normal lcTAGs [21, 22]. Their improved qualities make them an attractive alternative to be used for higher quality lubricants and biofuels.

The synthesis of acetyl-TAGs is catalyzed by the *Euonymus alatus* diacylglycerol acetyltransferase (*Ea*DAcT) enzyme and yielded an average of 53 mol % acetyl-TAGs when expressed in Camelina seed. Since acetyl-TAGs exhibit valuable properties it

becomes necessary to maximize their production. The *Euonymus fortunei* diacylglycerol acetyltransferase (*Ef*DAcT) ortholog of *Ea*DAcT has demonstrated higher activity in vitro and in vivo and produces higher levels of acetyl-TAGs in transgenic *Camelina sativa* seeds. Compared to *Ea*DAcT, the expression *Ef*DAcT led to an average of 71 mol % acetyl-TAGs in T_2 seed.

When propagated to the next generation, average acetyl-TAG accumulation slightly decreased in T_3 homozygous seed for both acetyltransferase enzymes. The expression of *Ea*DAcT yielded an average of 47 mol % acetyl-TAGs while *Ef*DAcT produced an average of 67 mol % acetyl-TAGs. Since hemizygous seeds in the T_2 generation yielded higher averages of acetyl-TAG content, T_3 hemizygous seed was also analyzed. Both *Ea*DAcT and *Ef*DAcT demonstrated slightly higher averages of 50 mol % and 71 mol % acetyl-TAGs for their T_3 hemizygous seed. It is unclear why hemizygotes display slightly higher levels of acetyl-TAG accumulation.

We also explored other strategies to see if even higher levels of acetyl-TAG accumulation could be obtained. Since the diacylglycerol acetyltransferase (DGAT1) enzyme competes with both *Ea*DAcT and *Ef*DAcT enzymes for their common DAG substrate, we hypothesized that its suppression would enable the other enzymes to synthesize even higher levels of acetyl-TAGs. Our results supported this idea, as the expression of *Ea*DAcT coupled with the suppression of DGAT1 lead to an increased average of 63 mol % acetyl-TAGs in T_2 seed. Similarly *Ef*DAcT combined with the DGAT1 suppression yielded an average 80 mol % acetyl-TAGs in T_2 seed. As the *Ef*DAcT enzyme consistently demonstrates higher acetyl-TAG accumulation, plant lines expressing *Ef*DAcT alone or coupled with the suppression of DGAT1 were propagated to

the next generation. Transgenic T_4 seed expressing *Ef*DAcT alone or combined with the suppression of DGAT1 yielded an average of 77 mol % and 90 mol % acetyl-TAGs respectively. Protein accumulation was analyzed in T_4 developing seed expressing *Ef*DAcT alone or *Ef*DAcT in combination with the knockdown of DGAT1 across a period of 40 days. Through Western blotting it was observed that protein expression is highest during the early stages of seed development, especially at the 15 and 20 day time points. This data implies that when more enzyme is present there is greater levels of acetyl-TAG accumulation.

Another strategy in our effort to increase acetyl-TAGs levels included the over expression of the *Rattus norvegicus* ATP: citrate lyase (*Rn*ACLY) to generate more acetyl-CoA substrate for the acetyltransferase enzymes. However, the co-expression of *Ea*DAcT and *Rn*ACLY resulted in an average of 56 mol % acetyl-TAGs. This is not statistically significant from the expression of *Ea*DAcT alone. Also, the co-expression of *Ef*DAcT and *Rn*ACLY resulted in an average of 60 mol % acetyl-TAGs, a significant decrease compared to the expression of *Ef*DAcT alone and suppression of DGAT1. However, the co-expression of *Ef*DAcT and *Rn*ACLY with the knockdown of DGAT1 resulted in high levels of acetyl-TAGs with an average of 84 mol %.

Future directions

Our research primarily focused on attempting to maximize the production of acetyl-TAGs though various modifications of the lipid synthesis pathway in *Camelina sativa*. The data from the over expression of *Rn*ACLY revealed that metabolic pathways within *Camelina sativa* are complex and requires more understanding in order to be successfully modified for the increased production of acetyl-TAGs. Future work should focus on

quantifying how much protein is present in dry and developing transgenic seeds expressing *Ea*DAcT, *Ef*DAcT and *Rn*ACLY. Since *Ef*DAcT expression levels were only expressed in early seed development, it would be beneficial to optimize the glycinin promoters to ensure high transgene expression. If there is more *Ef*DAcT activity expressed in late seed development, perhaps instead of plateauing more acetyl-TAGs may accumulate in the seeds. Since the overexpression of *Rn*ACLY yielded no significant increase in the average amount of acetyl-TAGs, the quantification of the enzyme's activity would reveal its level of expression. This may be accomplished through activity assays on developing transgenic seeds. Also, quantifying the amount of acetyl-CoA produced through the over expression of *Rn*ACLY would give valuable insights if there are any differences in metabolite accumulation between plants. Enzyme expression levels of *Rn*ACLY may also be investigated through Western blotting. This research would serve to provide greater understanding of the lipid synthesis pathway in *Camelina sativa* and how to maximize acetyl-TAG production.

References

[1] J.M. Dyer, S. Stymne, A.G. Green, A.S. Carlsson, High□value oils from plants, The Plant Journal 54 (2008) 640-655

[2] S.B. Weiss, E.P. Kennedy, The enzymatic synthesis of triglycerides, J.Am.Chem.Soc. 78 (1956) 3550-3550

[3] P.D. Bates, The significance of different diacylgycerol synthesis pathways on plant oil composition and bioengineering, Frontiers in plant science 3 (2012) 147

[4] S. Cases, S.J. Smith, Y.W. Zheng, H.M. Myers, S.R. Lear, E. Sande, S. Novak, C. Collins, C.B. Welch, A.J. Lusis, S.K. Erickson, R.V. Farese Jr, Identification of a gene encoding an acyl CoA:diacylglycerol acyltransferase, a key enzyme in triacylglycerol synthesis, Proc.Natl.Acad.Sci.U.S.A. 95 (1998) 13018-13023

[5] D.H. Hobbs, C. Lu, M.J. Hills, Cloning of a cDNA encoding diacylglycerol acyltransferase from Arabidopsis thaliana and its functional expression, FEBS Lett. 452 (1999) 145-149

[6] V. Katavic, D.W. Reed, D.C. Taylor, E.M. Giblin, D.L. Barton, J. Zou, S.L. Mackenzie, P.S. Covello, L. Kunst, Alteration of seed fatty acid composition by an ethyl methanesulfonate-induced mutation in *Arabidopsis thaliana* affecting diacylglycerol acyltransferase activity, Plant Physiol. 108 (1995) 399-409

[7] J. Zou, Y. Wei, C. Jako, A. Kumar, G. Selvaraj, D.C. Taylor, The Arabidopsis thaliana TAG1 mutant has a mutation in a diacylglycerol acyltransferase gene, The Plant Journal 19 (1999) 645-653

[8] J. Routaboul, C. Benning, N. Bechtold, M. Caboche, L. Lepiniec, The TAG1 locus of Arabidopsis encodes for a diacylglycerol acyltransferase, Plant Physiology and Biochemistry 37 (1999) 831-840

[9] S.J. Smith, S. Cases, D.R. Jensen, H.C. Chen, E. Sande, B. Tow, D.A. Sanan, J. Raber, R.H. Eckel, R.V. Farese, Obesity resistance and multiple mechanisms of triglyceride synthesis in mice lacking Dgat, Nat.Genet. 25 (2000) 87-90

[10] K.D. Lardizabal, J.T. Mai, N.W. Wagner, A. Wyrick, T. Voelker, D.J. Hawkins, DGAT2 is a new diacylglycerol acyltransferase gene family: purification, cloning, and expression in insect cells of two polypeptides from *Mortierella ramanniana* with diacylglycerol acyltransferase activity, J.Biol.Chem. 276 (2001) 38862-38869

[11] S.J. Stone, H.M. Myers, S.M. Watkins, B.E. Brown, K.R. Feingold, P.M. Elias, R.V. Farese Jr, Lipopenia and skin barrier abnormalities in DGAT2-deficient mice, J.Biol.Chem. 279 (2004) 11767-11776

[12] J.M. Shockey, S.K. Gidda, D.C. Chapital, J.C. Kuan, P.K. Dhanoa, J.M. Bland, S.J. Rothstein, R.T. Mullen, J.M. Dyer, Tung tree DGAT1 and DGAT2 have nonredundant functions in triacylglycerol biosynthesis and are localized to different subdomains of the endoplasmic reticulum, Plant Cell 18 (2006) 2294-2313

[13] R. Li, K. Yu, D.F. Hildebrand, DGAT1, DGAT2 and PDAT expression in seeds and other tissues of epoxy and hydroxy fatty acid accumulating plants, Lipids 45 (2010) 145-157

[14] A.C. Turchetto-Zolet, F.S. Maraschin, G.L. de Morais, A. Cagliari, C.M. Andrade, M. Margis-Pinheiro, R. Margis, Evolutionary view of acyl-CoA diacylglycerol

acyltransferase (DGAT), a key enzyme in neutral lipid biosynthesis, BMC evolutionary biology 11 (2011) 1

[15] A. Dahlqvist, U. Stahl, M. Lenman, A. Banas, M. Lee, L. Sandager, H. Ronne, S. Stymne, Phospholipid:diacylglycerol acyltransferase: an enzyme that catalyzes the acyl-CoA-independent formation of triacylglycerol in yeast and plants, Proc.Natl.Acad.Sci.U.S.A. 97 (2000) 6487-6492

[16] M. Zhang, J. Fan, D.C. Taylor, J.B. Ohlrogge, DGAT1 and PDAT1 acyltransferases have overlapping functions in Arabidopsis triacylglycerol biosynthesis and are essential for normal pollen and seed development, Plant Cell 21 (2009) 3885-3901

[17] J. Fan, C. Yan, X. Zhang, C. Xu, Dual role for phospholipid:diacylglycerol acyltransferase: enhancing fatty acid synthesis and diverting fatty acids from membrane lipids to triacylglycerol in Arabidopsis leaves, Plant Cell 25 (2013) 3506-3518

[18] V. Mhaske, K. Beldjilali, J. Ohlrogge, M. Pollard, Isolation and characterization of an Arabidopsis thaliana knockout line for phospholipid: diacylglycerol transacylase gene (At5g13640), Plant Physiology and Biochemistry 43 (2005) 413-417

[19] J. Blin, C. Brunschwig, A. Chapuis, O. Changotade, S. Sidibe, E. Noumi, P. Girard, Characteristics of vegetable oils for use as fuel in stationary diesel engines—Towards specifications for a standard in West Africa, Renewable and Sustainable Energy Reviews 22 (2013) 580-597

[20] H.M. Paulsen, V. Wichmann, U. Schuemann, B. Richter, Use of straight vegetable oil mixtures of rape and camelina as on farm fuels in agriculture, Biomass Bioenergy 35 (2011) 4015-4024

[21] T.P. Durrett, D.D. McClosky, A.W. Tumaney, D.A. Elzinga, J. Ohlrogge, M. Pollard, A distinct DGAT with *sn*-3 acetyltransferase activity that synthesizes unusual, reduced-viscosity oils in *Euonymus* and transgenic seeds, Proc.Natl.Acad.Sci.U.S.A. 107 (2010) 9464-9469

[22] J. Liu, A. Rice, K. McGlew, V. Shaw, H. Park, T. Clemente, M. Pollard, J. Ohlrogge, T.P. Durrett, Metabolic engineering of oilseed crops to produce high levels of novel acetyl glyceride oils with reduced viscosity, freezing point and calorific value, Plant Biotechnology Journal 13 (2015) 858-865

[23] K. Hofmann, A superfamily of membrane-bound O-acyltransferases with implications for wnt signaling, Trends Biochem.Sci. 25 (2000) 111-112

[24] K. Knörzer, Entwicklung und Ausbreitung des Leindotters (Camelina saliva s. 1.), Berichte der Deutschen Botanischen Gesellschaft 91 (1978) 187-195

[25] C. Lu, J. Kang, Generation of transgenic plants of a potential oilseed crop Camelina sativa by Agrobacterium-mediated transformation, Plant Cell Rep. 27 (2008) 273-278

[26] D. Putnam, J. Budin, L. Field, W. Breene, Camelina: a promising low-input oilseed, New crops.Wiley, New York 314 (1993)

[27] R.G. Robinson, Camelina: a useful research crop and a potential oilseed crop (1987) Minnesota Agricultural Experiment Station Bulletin 579-1987.

[28] S. Kagale, C. Koh, J. Nixon, V. Bollina, W.E. Clarke, R. Tuteja, C. Spillane, S.J. Robinson, M.G. Links, C. Clarke, The emerging biofuel crop *Camelina sativa* retains a highly undifferentiated hexaploid genome structure, Nature Communications 5 (2014) 3706

[29] M.F. Rodríguez-Rodríguez, A. Sánchez-García, J.J. Salas, R. Garcés, E. Martínez-Force, Characterization of the morphological changes and fatty acid profile of developing *Camelina sativa* seeds, Industrial Crops and Products 50 (2013) 673-679

[30] D. Rangasamy, C. Ratledge, Genetic enhancement of fatty acid synthesis by targeting rat liver ATP:citrate lyase into plastids of tobacco, Plant Physiol. 122 (2000) 1231 -1238