Enhancing the production of acetyl-triacylglycerols through metabolic engineering of the oilseed crop *Camelina sativa*

by

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B.S., Kansas State University, 2016

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

2018

Approved by:

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Abstract

Many *Euonymus* species express an acetyltransferase enzyme in their seeds which catalyzes the transfer of an acetyl group from acetyl-CoA to the *sn*-3 position of diacylglycerol (DAG) producing unusual acetyl-1,2-diacyl-*sn*-glycerols (acetyl-TAG). The presence of the *sn*-3 acetate group gives acetyl-TAG with unique physical properties over regular triacylglycerol (TAG) found in vegetable oils. The useful characteristics of acetyl-TAG oil offer advantages for its use as emulsifiers, lubricants, and 'drop-in' biofuels. One enzyme, *Euonymus alatus* diacylglycerol acetyltransferase (*Ea*DAcT), responsible for acetyl-TAG synthesis in nature was previously isolated from the seeds of *Euonymus alatus* (burning bush) and expressed in the oilseed crop *Camelina sativa.* Expression of *Ea*DAcT successfully led to production of high levels of acetyl-TAG in camelina seeds. To further increase acetyl-TAG accumulation in transgenic camelina seeds, multiple strategies were examined in this study. Expression of a new acetyltransferase enzyme (*Ef*DAcT) isolated from the seeds of *Euonymus fortunei*, which was previously shown to possess higher *in vitro* activity and *in vivo* acetyl-TAG levels compared to *Ea*DAcT, increased acetyl-TAG accumulation by 20 mol%. Suppression of the endogenous competing enzyme DGAT1 further enhanced acetyl-TAG accumulation to 90 mol% in selected transgenic line. Studying the regulation of *Ef*DAcT transcript, protein, and acetyl-TAG levels during seed development further provided new insights on the factors limiting acetyl-TAG accumulation.

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Acknowledgements

I'm extremely grateful to my advisor Dr. Timothy Durrett for providing me with this opportunity, his constant support, encouragement, and patience. I could not have asked for a better mentor.

I would like to express my deepest appreciation to my committee members Dr. Kathrin Schrick and Dr. Lawrence Davis for sharing their knowledge during our weekly lab meetings, which I've learned a lot from, and for their helpful feedback, advise and experimental suggestions to further improve my work.

I would like to thank our past lab member, Kasia Kornacki for starting this project, generating a lot of the preliminary data, and for helping me learn some of the techniques I've used throughout my study.

I want to also thank my current colleagues Jose Aznar Moreno, Karan Aulakh, and Nicholas Neumann for their useful comments, feedback, and discussion on my work and answering lots of my questions.

I truly appreciate the help of our undergraduate students Cole Wilson and Mack Mclntosh in maintaining plants and seed harvesting.

Most of all I want to thank all my family members for their unconditional love and support at all times.

Thank you all,

I could not have done this without you.

Dedication

Dedicated to

my beloved parents

and wonderful siblings

Chapter 1 - Literature Review

1.1 Overview of fatty acids and triacylglycerols synthesis

1.1.1 Plant triacylglycerols

Plant oils mainly consist of triacylglycerols (TAGs), which serve as major lipid storage and energy reserve in plants (Ohlrogge and Browse, 1995). TAGs stored in mature seeds support seed germination and early seedling establishment (Graham, 2008). In vegetative tissues, TAGs have been shown to be involved in remodeling of membrane lipids, cell division, cotyledons expansion, and stomatal opening (Yang and Benning, 2018). TAGs are composed of three fatty acyl groups and one glycerol backbone. A glycerol molecule consists of three carbons each attached to a hydroxyl (HO–) group. The carbons are indicated as *sn*-1, *sn*-2, and *sn*-3.

1.1.2 Fatty acids

Fatty acids found in nature, in plants and animals, are generally composed of even numbers of carbon atoms. Each fatty acid is comprised of an aliphatic hydrocarbon chain with a carboxyl group (−COOH) at one end and a methyl group at the other (Figure 1.1). Some of the major fatty acids that make up TAGs include palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3) (Figure 1.1) (Ohlrogge and Browse, 1995). Fatty acids can be recognized by the shorthand notation CN:D, where N refer to the number of carbon atoms and D refer to the number of double bonds in the chain. The location of double bonds (c) in reference to the carboxyl group or the methyl group is indicated by the nomenclature Δ^c or ω-c (n-c), respectively (Davidson and Cantrill, 1985; Nichols *et al.*, 1986). Based on the presence of double bonds in their backbone, fatty acids are characterized as saturated or unsaturated. Saturated fatty acids contain single bonds between carbon molecules while unsaturated fatty acids contain

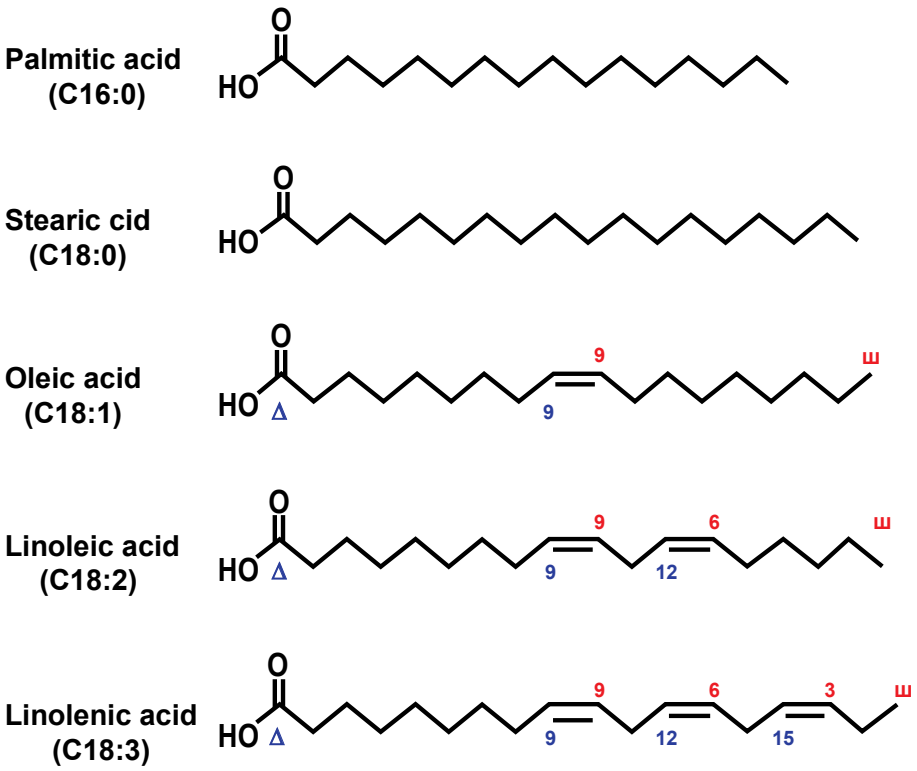

Figure 1.1 Structures of major fatty acids found in vegetable oil.

Fatty acids are designated by CN:D, where N denotes the number of carbon atoms and D indicate the number of double bonds. ω (red) represent the location of double bonds in reference to the methyl group; Δ (blue) represent the location of double bonds in reference to the carboxyl group.

one (monounsaturated) or more (polyunsaturated, PUFA) cis double bonds in their backbone (Rustan and Drevon, 2005). Fatty acids with 20 or more carbons are referred to as very long chain fatty acid (VLCFA). The variance in presence, number and position of the double bonds, along with the length of the hydrocarbon chains in different fatty acids determine the properties of lipids they make up (Dyer *et al.*, 2008). For instance, animal fats are rich in saturated fatty acids which have high melting point and thus make the fat solid at room temperature. In contrast, plant oils

which largely consist of unsaturated fatty acids that have lower melting point, are liquid at room temperature (Berg *et al.*, 2002).

1.1.3 Fatty acid synthesis and acyl editing

Oil synthesis in plants starts with the *de novo* synthesis of fatty acids in the plastid. First, the enzyme acetyl-CoA carboxylase (ACCase) catalyzes the formation of malonyl-CoA from acetyl-CoA and bicarbonate ion (Figure 1.2). This formation of malonyl-CoA is the first committed step of fatty acid synthesis (Ohlrogge and Browse, 1995). The malonyl group of malonyl-CoA is then transferred from coenzyme A to the acyl carrier protein (ACP) by malonyl CoA-acyl carrier protein transacylases (MCAT or FabD) (Figure 1.2) (Li-Beisson *et al.*, 2010). Malonyl-ACP enter a cycle of 4 reactions where condensation, reduction and dehydration occur, leading to the elongation of the fatty acid chain connected to ACP by two carbons. 16:0-ACP is formed after six more repeated cycles which can be further elongated to 18:0-ACP through one more repeated cycle by ketoacyl-ACP synthase II (KASII). 16:0-ACP is hydrolyzed by the acyl-ACP thioesterase B (FATB) to remove the ACP, and exported out of the plastid (Salas and Ohlrogge, 2002). Similar to 16:0-ACP, 18:0-ACP is either hydrolyzed by FATA/B and exported or desaturated to 18:1-ACP by stearoyl-ACP desaturase (SAD) (Figure 1.2). ACP is removed from 18:1-ACP by FATA (Bates *et al.*, 2013).

When ACP is removed, the free fatty acids can be exported out of the plastid with the help of a plastidial fatty acid exporter (FAX1) (Li *et al.*, 2015). The free fatty acids are converted to their CoA forms (i.e. 16:0-CoA, 18:0-CoA, and 18:1-CoA) in the cytosol forming an acyl-CoA pool (Figure 1.2). 18:1-CoA are further modified by two mechanisms. First, it can be elongated into VLCFA-CoA (20:1, 22:1) via the fatty acid elongase complex (Millar and Kunst, 1997). Alternatively, it can be incorporated through a process known as “acyl editing” into

phosphatidylcholine (PC) in the endoplasmic reticulum (ER) for desaturation into PUFA (18:2, and 18:3) (Bates and Browse, 2012). Acyl editing does not lead to the net synthesis of PC, but it is rather a deacylation-reacylation cycle that exchanges acyl groups between PC and CoA (Li-Beisson *et al.*, 2010). Briefly, 18:1-CoA enters the PC pool through its esterification to the *sn*-1 or *sn*-2 position of lyso-PC by acyl-CoA:lyso-phosphatidylcholine acyltransferase (LPCAT) forming 18:1-PC. 18:1-PC is then desaturated into 18:2-PC and 18:3-PC via FATTY ACID DESATURASES 2 and 3 (FAD2 and FAD3), respectively (Okuley *et al.*, 1994; Lou *et al.*, 2014). Modified acyl groups can re-enter the acyl-CoA pool to be utilized for the *de novo* synthesis of diacylglycerol (DAG) (Figure 1.2). Reacylation of lyso-PC with a different 18:1-CoA from the acyl-CoA pool completes the acyl editing cycle (Bates and Browse, 2012).

1.1.4 Triacylglycerol biosynthesis

Fatty acyl-CoAs enter the ER from the acyl-CoA pool to be utilized for TAG synthesis (Figure 1.2). Fatty acid chains are transferred from the CoA group to the *sn*-1 position of glycerol-3-phosphate (G3P) by glycerol 3-phosphate acyltransferase (GPAT) and the *sn*-2 position of lysophosphatidic acid (LPA) by lysophosphatidic acid acyltransferase (LPAAT) to form phosphatidic acid (PA). Phosphatidic acid phosphatase (PAP) then catalyzes the dephosphorylation of PA to yield the *de novo* DAG (Ohlrogge and Browse, 1995). This *de novo* synthesis of DAG and subsequently TAG is commonly known as the Kennedy pathway (Bates and Browse, 2012).

In plants, TAGs can also be synthesized through a PC-derived mechanism. The pathway in which the majority of TAG is synthesized, either from *de novo* DAG or PC-derived DAG, varies depending on the plant species (Bates and Browse, 2012). Multiple pathways appear to interconvert PC and DAG, including the transfer of phosphocholine to DAG by CDP-

choline:diacylglycerol cholinephosphotransferase (CPT) or the action of phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT) which transfers the phosphocholine headgroup between PC and DAG (Li-Beisson *et al.*, 2010). PC-derived DAG can also be synthesized through phospholipase C (PLC) or the combined activities of phospholipase D (PLD) and PAP (Figure 1.2).

Finally, a third fatty acid chain from the acyl-CoA pool or PC becomes esterified to the *sn*-3 position of *de novo* DAG or PC-derived DAG by diacylglycerol acyltransferase-1 (DGAT1) or phospholipid:diacylglycerol acyltransferase 1 (PDAT1), respectively, forming TAG (Bates *et al.*, 2013).

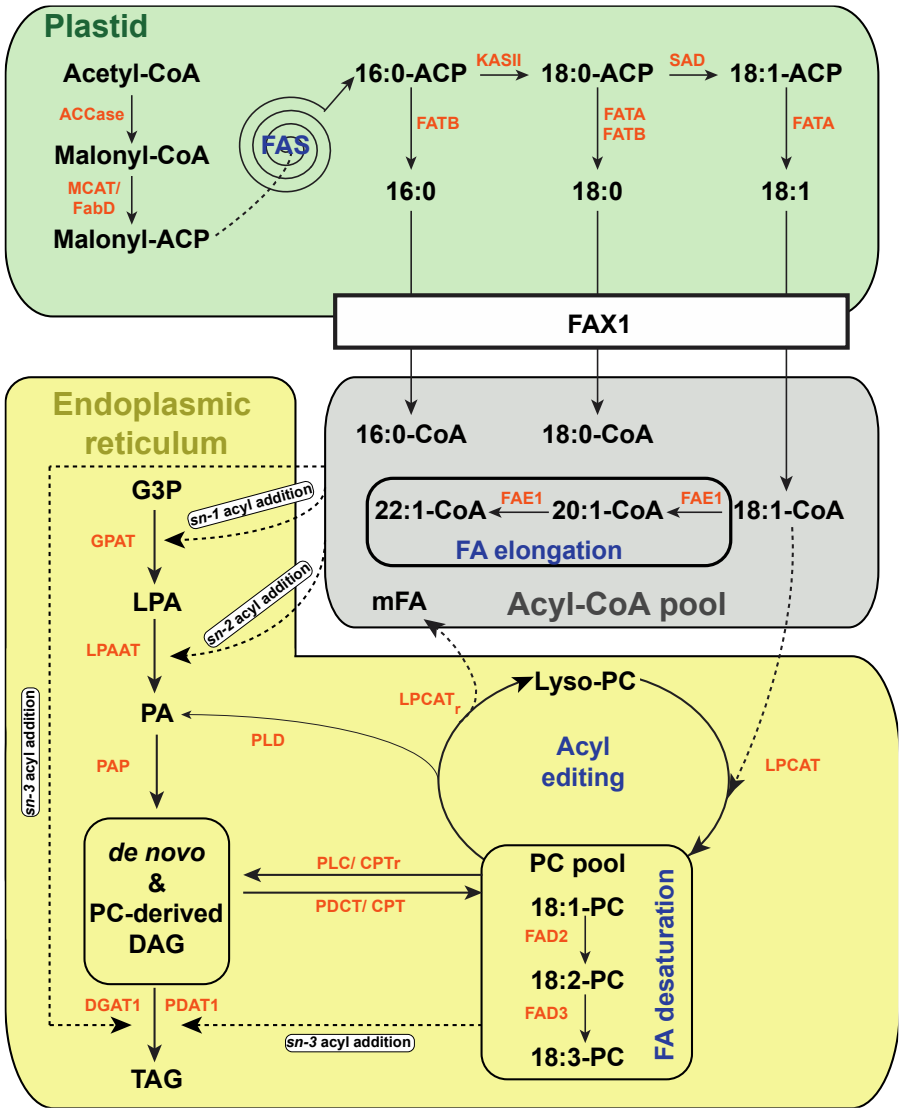

Figure 1.2 Overview of fatty acid and TAG biosynthesis.

Compound abbreviations: ACP, acyl carrier protein; G3P, glycerol-3-phosphate; LPA, lyso-phosphatidic acid; PA, phosphatidic acid; DAG, diacylglycerol; PC, phosphatidylcholine; Lyso-PC, lysophosphatidylcholine; mFA, modified fatty acids; TAG, triacylglycerol. Enzyme (orange) abbreviations: ACCase, acetyl-CoA carboxylase; MCAT, malonyl-CoA-acyl carrier protein transacylase; FAS, fatty acid synthase; FATB, acyl-ACP thioesterase B; FATA, acyl-ACP thioesterase A; KASII, ketoacyl-ACP synthase II; SAD, stearoyl-ACP desaturase; FAX1; fatty acid exporter 1; FAE1; fatty acid elongase 1; LPCAT, acyl-CoA:lyso-phosphatidylcholine acyltransferase; FAD, fatty acid desaturase; PDCT, phosphatidyl-choline:diacylglycerol cholinephosphotransferase; PLC, phospholipase C; PLD, phospholipase D; CPT, CDP-choline:diacylglycerol cholinephosphotransferase; GPAT, glycerol 3-phosphate acyltransferase; LPAAT, lysophosphatidic acid acyltransferase; PAP, phosphatidic acid phosphatase; DGAT1, diacylglycerol acyltransferase 1; PDAT1; phospholipid:diacylglycerol acyltransferase 1.

1.2 The natural occurrence of unusual fatty acids and lipids

Within the plant kingdom there are multiple species that produce high levels of unusual fatty acids or lipid structures in their oil. These structures include differences in fatty acid chain length, the number and position of double bonds, or the addition of functional groups (Ohlrogge, 1994; Voelker and Kinney, 2001; Jaworski and Cahoon, 2003). The unusual structure of these fatty acids and lipids confers them with useful physical properties that enhances their use in food, fuel, or industrial feedstocks (Dyer *et al.*, 2008; Durrett *et al.*, 2008; Lu *et al.*, 2011). Homologues of fatty acid desaturase enzymes that introduce double bonds on fatty acids incorporated into PC are responsible for the variation and synthesis of the unique structure of fatty acids and lipids in majority of the plants (Bates and Browse, 2011; Snapp and Lu, 2013). Below is a brief description of plants that synthesize some of the known unusual fatty acids and lipids found in nature:

1.2.1 Hydroxy fatty acids

Fatty acids containing hydroxy groups (HFA) are synthesized in the seeds of multiple plants in nature. Most commonly known is the synthesis of ricinoleic acid (18:1-OH; 12-hydroxy-

9-cis-octadecenoic acid), an 18-carbon fatty acid with one double bond (between C9 and C10) and a -OH group (at C12), in castor bean (*Ricinus communis*) seeds. Castor oil comprises about 90% ricinoleic acid. It is synthesized by a homolog of the FAD2 enzyme, *Ricinus communis* Δ^{12} fatty acid hydroxylase (RcFAH12), that catalyzes the introduction of a hydroxyl group instead of a double bond at the Δ^{12} position of 18:1-PC (Bafor *et al.*, 1991; van de Loo *et al.*, 1995). In addition to ricinoleic acid in castor, lesquerolic acid (20:1OH, 14-hydroxy-11-eicosenoic acid) constitutes to up to 85% of FA profile in *Physaria lindheimeri and* 60% in *Physaria fendleri* (Hayes and Kleiman, 1996; Jenderek *et al.*, 2009; Chen *et al.*, 2011). Moreover, densipolic acid (18:2-OH, 12-hydroxy-9,15-octadecadienoic acid) was identified in *Paysonia perforata, Paysonia stonensis, Paysonia densipila, Paysonia lyrata,* and *Paysonia lescurii* plants. Auricolic acid (C20:2-OH,14-hydroxy-11,17- eicosenoic acid) was found in in seeds of *Paysonia auriculata* and *Physaria densiflora* (Jenderek *et al.*, 2009). Recently two new 24-carbon HFAs containing two -OH group (at C7, and C18) were discovered in seeds of the Chinese violet cress (*Orychophragmus violaceus*). The new HFAs were named Nebraskanic acid (C24:1-OH,7,18-hydroxy-15- lignoceric acid) and Wuhanic acid (C24:2-OH,7,18-hydroxy-15,21- lignoceric acid) (Li *et al.*, 2018).

1.2.2 Medium chain fatty acids

A number of plants in nature accumulate medium-chain fatty acids (MCFAs) with chain lengths of 8–14 carbons in their seed oils. One of the most valuable MCFA found in nature is lauric acid (C12:0). Lauric acid can be synthesized in coconut (50%), palm, *Umbellularia californica* (California bay tree) (70%), *Cuphea calophylla* (85%), *Laurus nobilis* (58%) and many other plants (Litchfield *et al.*, 1967; Graham, 1989; Pollard *et al.*, 1991). Beside Lauric acid, multiple MCFAs such as myristic (14:0) acid, capric acid (10:0) and caprylic acid (8:0) can be found in the oil of *Cuphea salvadorensis*, *Cuphea koehneana*, *Cuphea pulcherrina*, *Cuphea hookeriana*,

coconut, and the California bay tree (Litchfield *et al.*, 1967; Dehesh *et al.*, 1996; Thelen and Ohlrogge, 2002). MCFAs are believed to be synthesized through their release by specialized FatB acyl-ACP thioesterases in the plastid before entering additional fatty acid synthesis cycles for further elongation (Pollard *et al.*, 1991; Voelker *et al.*, 1992).

1.2.3 Wax esters

Seed lipids and fatty acids are stored in the form of TAGs in almost all known plants species. One exception is the desert shrub jojoba (*Simmondsia chinensis*), where the major seed storage reserves are wax esters instead of regular TAGs. Wax esters are esters of monounsaturated long chain FAs (mostly C20, C22, and C24) and fatty alcohols (Ohlrogge *et al.*, 1978; Pollard *et al.*, 1979). Wax esters biosynthesis in jojoba first involves the elongation of 18:1-CoA from the acyl-CoA pool to monounsaturated long chain FAs-CoA by the fatty acid elongase complex(Voelker and Kinney, 2001). Long-chain acyl-CoAs are then reduced to primary alcohol by fatty acid reductase (FAR) (Wu *et al.*, 1981). Newly synthesized fatty alcohols are esterified to another long-chain acyl-CoA by a fatty acyl-CoA: fatty alcohol acyltransferase known as wax synthase (WS) to form the wax esters (Metz *et al.*, 2000; Lardizabal *et al.*, 2000).

1.3 *Camelina sativa*: an excellent oilseed crop for engineered oil production

Most of the plants that produce unusual fatty acids and lipids with useful industrial properties in their seed oils, are not suitable as crops. Therefore, metabolic engineering of oilseed crops is necessary to produce industrially relevant levels of these high-value unusual fatty acids and oils. Common oilseed crops such as palm (*Elaeis guineensis*), soybean (*Glycine max*), rapeseed (*Brassica napus*), sunflower (*Helianthus annuus*), and peanuts (*Arachis hypogaea*) can be used but their use in edible food for human oil consumption limit their advantages as ideal

platforms for metabolic engineering of industrial oil (Carlsson *et al.*, 2014). On the other hand, camelina [*Camelina sativa* (L.) Crantz] known as false flax, gold-of-pleasure, or wild flax, provides an ideal model for such manipulation due to its appealing agronomic traits, low human consumption, and ease for genetic engineering (Iskandarov *et al.*, 2014; Napier *et al.*, 2014; Bansal and Durrett, 2016a). Camelina is an oilseed crop in the Brassicaceae (mustard) family and is closely related to the model species *Arabidopsis thaliana* (Kagale *et al.*, 2014).

1.3.1 Seed properties

In general, camelina seed size ranges from 1.9 mm^2 to 3 mm^2 while its seed weight ranges from 1 mg-1.5 mg (Berti *et al.*, 2011; Rodríguez-Rodríguez *et al.*, 2013). Compared to different Brassicaceae crops, camelina has smaller seed size and weight (Gugel and Falk, 2006). Camelina seed is comprised of 30%-40% oil, 27% to 32% storage proteins, soluble sugar, starch, crude fiber, minerals and glucosinolates (Budin *et al.*, 1995; Gugel and Falk, 2006; Zubr, 2010). Camelina has an oil content that is higher than soybean and lower than rapeseed and sunflower (Moser, 2010). Its oil is unique in that it contains over 50% of PUFAs which distinguish it from other traditional oilseed crops. It accumulates about 35 to 40 % of omega-3 alpha-linolenic acid (ALA) and 15 to 20% of omega-6 linoleic acid (LA). Camelina oil also contains high levels of monounsaturated fatty acids, oleic acid (12-15%) and eicosenoic acid (14-16%) (Vollmann *et al.*, 2007; Rodríguez-Rodríguez *et al.*, 2013; Pollard *et al.*, 2015). In addition, it has high levels of tocopherols (vitamin E) and antioxidants.

1.3.2 Useful agronomic characteristics

Camelina is widely known for its useful agronomic characteristics. It is a self-pollinating crop with very low outcrossing rate (0.09–0.28%) (Walsh *et al.*, 2012) and a fast-growing plant with a life cycle of three to four months for full plant growth and seed maturation. Due to its short

growing season, camelina can be used in rotation with several other crops such as corn, soybean, sunflower, and wheat (Gesch and Archer, 2013; Gesch *et al.*, 2014; Chen *et al.*, 2015; Berti *et al.*, 2016). Camelina produces high seed yields, ranging from 1500 to ~3000 kg ha^{-1}, depending on the climate, environment, genotypes, and soil nutrients (Zubr, 2003; Vollmann *et al.*, 2007; Berti *et al.*, 2011, 2016; Masella *et al.*, 2014; Obour *et al.*, 2017). Under favorable conditions, seed yields of over 3000 kg ha^{-1} have been reported (Vollmann and Eynck, 2015). Camelina is well adapted to growing on marginal soils and to a wide range of weather conditions. It has been described as a low-input crop, requiring less fertilization, water, and pesticide than other crops. Although camelina yields are comparable to those of representative *Brassica* species (*B. rapa*, *B. juncea*, and *B. napus*) (Gugel and Falk, 2006), yields tend to be lower than other species under optimum precipitation conditions. However, under identical drought conditions, camelina yields surpass that of many oilseed crops including canola, flax, and soybean (Zubr, 1997; Gugel and Falk, 2006; Blackshaw *et al.*, 2011). Camelina is also resistant to many pests and pathogens that affect other oilseed crops yield and growth. Studies have shown that in contrast to canola, camelina is tolerant to many insect pests like flea beetles, root maggots, and diamondback moths (Soroka *et al.*, 2015). Moreover, it confers resistance toward diseases such as Alternaria, black spot and blackleg. Some of the genotypes are also tolerant to downy mildew, sclerotinia stem rot, and brown girdling root rot (Séguin-Swartz *et al.*, 2009; Berti *et al.*, 2016).

1.3.3 Metabolic engineering strategies

Metabolic engineering is performed to improve or introduce the production of specific compounds in an organism through the manipulation of one or multiple genes or enzymatic pathways (DellaPenna, 2001; Pouvreau *et al.*, 2018). Through synthetic biology and metabolic engineering approaches, our understanding of unusual fatty acids and lipids metabolism has

expanded and their production in agronomically favorable crops has become possible (Pouvreau *et al.*, 2018). Since it was shown that camelina is easily amenable to modification through Agrobacterium transformation (Lu and Kang, 2008), in addition to its attractive agronomic traits, it has since emerged as an ideal platform for testing novel metabolic engineering strategies and production of novel traits. As described below, many metabolic engineering strategies such as gene cloning, gene overexpression, gene silencing by RNAi or elimination by CRISPR/Cas9, and gene stacking have been successfully performed in camelina for accumulation of unusual fatty acids and lipids.

HFAs have been synthesized in camelina for industrial application purposes. They are used in the production of chemical feedstock such as polyesters and lubricants. Seed specific expression of RcFAH12 under the control of the phaseolin promoter led to the production of 15% HFA consisting of 18:1-OH and 18:2-OH (Lu and Kang, 2008). HFA levels increased to 22% by the co-expression of RcFAH12 and *Physaria fendleri* 3-ketoacyl-CoA synthase 18 (*Pf*KCS18) (Snapp *et al.*, 2014). PfKCS18 is a fatty acid condensing enzyme that specifically elongates the hydroxylated C18 to C20-HFAs. Co-expression of *Rc*FAH12 and *Pf*KCS18 resulted in the accumulation of 18:1-OH, 18:2-OH, 20:1-OH, and 20:2-OH in camelina seeds (Snapp *et al.*, 2014).

Camelina has also been engineered to produce MCFA. Oil accumulating MCFAs can be used in the production of biodiesel and jet fuel. Overexpression of *Cuphea pulcherrima* FATB2 (*Cp*FatB2) accumulated 24% of C14:0, *Umbellularia californica* FatB1 (*Uc*FatB1) accumulated 18% of C12:0, and *Cuphea hookeriana* FatB2 (*Ch*FatB2) accumulated 10% of 10:0 in camelina seeds (Kim *et al.*, 2015). Expression of *Cp*FatB3 resulted in the accumulation of C8:0, C10:0, C12:0, and C14:0, while *Cp*FatB4 expression accumulated 8% of C14:0 and resulted in a 5-fold

increase of C16:0. Co-expression of the different FatB genes and coconut LPAT *(Cn*LPAT*)* resulted in a further increase of MCFA levels without significant impact on total seed fatty acid content (Kim *et al.*, 2015). In a later study, MCFA levels were increased by the combined expression of *Uc*FatB1 and the RNAi-mediated suppression of camelina KASII gene (Hu *et al.*, 2017).

Wax esters constitute about 76% of the renewable bio-based lubricant spermaceti oil (Ruiz-Lopez *et al.*, 2016). To synthesize wax esters in camelina seeds, jojoba (*Simmondsia chinensis*) wax ester synthase (*Sc*WS) and *Marinobacter aquaeolei* fatty acyl-CoA reductase (*Ma*FAR) were overexpressed. Seed specific expression of *Ma*FAR/*Sc*WS resulted in accumulation of 15– 21% of wax esters in camelina seeds (Iven *et al.*, 2016). Combination of camelina FAD2-RNAi suppression with overexpression of *Lunaria annua* FAE1(*La*FAE1) along with *Sc*FAR and *Sc*WS further increased wax ester accumulation levels (Zhu *et al.*, 2016).

1.4 Acetyl-triacylglycerol

1.4.1 Natural occurrence

Another example of unusual lipid structure found in nature are acetyl-triacylglycerols (acetyl-TAGs). Acetyl-TAGs contain an acetate group at the *sn*-3 position instead of a long chain fatty acid as with regular TAGs (here referred to as lcTAG) found in most vegetable oils (Figure 1.3A). The seeds of many plants in the family of Celastraceae, Lardizabalaceae, Ranunculaceae, Rosaceae, and Balsaminaceae synthesize these unusually structured TAGs at varying levels (Kleiman *et al.*, 1967; Durrett *et al.*, 2010; Sidorov *et al.*, 2014). *Euonymus* species which belong to the family of Celastraceae, use acetyl-TAG as the major lipid storage component. Acetyl-TAG constitutes up to 98% of the oil composition in their seeds.

1.4.2 Properties and applications

The presence of the *sn*-3 acetate group in acetyl-TAGs confers on them favorable physical properties compared to lcTAG. For instance, acetyl-TAG possess a 34%-39% reduction in the kinematic viscosity levels which fall within the range of diesel # 4 (5-24 mm^2 s^{-1}) used in low and medium speed engines (Durrett *et al.*, 2010; Liu *et al.*, 2015a). Additionally, acetyl-TAG oil was found to crystalize at a much lower temperature compared to lcTAG (Liu *et al.*, 2015a, 2015b). The high kinematic viscosity and melting point of regular vegetable oil prevent their use as direct biofuel for diesel engines and require the extra steps of heating and transmethylation (Durrett *et al.*, 2008). Acetyl-TAG oil however bypasses such drawbacks due to its reduced viscosity and superior cold temperature characteristics facilitating its use as a biodiesel 'drop-in'. Acetyl-TAG oil can also be used in producing biodegradable lubricants, plasticizers, and food emulsifiers, analogous to its structurally similar commercial product ACETEM (acetylated mono- and diglycerides) is currently utilized (Gaupp and Adams, 2004; Liu *et al.*, 2015b).

1.4.3 Isolation of acetyl-TAG synthesis enzymes

Acetyl-TAGs are synthesized by the modification of the last rate limiting step of TAG biosynthesis, where an acetyl-CoA is esterified to the *sn*-3 position of DAG instead of acyl-CoA by a diacylglycerol acetyltransferase (DAcT) enzyme (Figure 1.3B). The first enzyme synthesizing acetyl-TAG was discovered from the seeds of *Euonymus alatus* (burning bush), by a comparative transcriptome profiling approach using pyrosequencing technology (Durrett *et al.*, 2010). *Euonymus alatus* endosperm and embryos synthesize high levels of acetyl-TAG, whereas almost only lcTAG is synthesized in aril. Comparing expression levels of genes annotated as acyltransferases from the *Euonymus alatus* endosperm and embryos with that from the aril

identified one enzyme named *Euonymus alatus* diacylglycerol acetyltransferase (*Ea*DAcT) as the enzyme responsible for acetyl-TAG synthesis (Durrett *et al.*, 2010).

*Ea*DAcT belongs to a membrane bound O-acyltransferase (MBOAT) protein family. MBOAT proteins catalyzes O-acylation reactions adding long chain acyl-CoAs as acyl donors to various lipid and protein acceptors (Hofmann, 2000). *Ea*DAcT was however shown to possess an extreme substrate specificity toward the transfer of acetyl-CoA and less efficiently acyl-CoA donors shorter than eight carbons to the *sn*-3 position of DAGs containing PUFA at the *sn*-1 and *sn*-2 positions (Bansal and Durrett, 2016b).

Additional DAcT enzymes capable of synthesizing acetyl-TAG were isolated from different plants belonging to the Celastraceae family (Sidorov *et al.*, 2014; Tran *et al.*, 2017b). Enzymes isolated from *Celastrus scandens*, *Euonymus bungeanus*, *Euonymus atropurpureus*, *Euonymus fortunei*, and *Euonymus kiautschovicus* were expressed in yeast to successfully produce acetyl-TAG. Incubation of yeast microsomes with [^{14}C] acetyl-CoA confirmed that each possesses acetyltransferase activity *in vitro* (Tran *et al.*, 2017b). The enzyme isolated from *Euonymus fortunei*, exhibited the highest acetyl-TAG production levels and acetyltransferase activity when compared to the others.

1.4.4 Expression of *Ea*DAcT in *Camelina sativa* seeds

*Ea*DAcT was isolated form *Euonymus alatus* seeds and expressed in yeast and Arabidopsis, to confirm its ability to synthesize acetyl-TAG. *Ea*DAcT expression successfully produced high levels of acetyl-TAG (Durrett *et al.*, 2010; Liu *et al.*, 2015a; Tran *et al.*, 2017a). To produce relevantly high levels of acetyl-TAG oil, *Ea*DAcT was expressed in camelina under the control of a seed specific promoter. The expression of *Ea*DAcT in camelina produced about 64 mol% of

acetyl-TAG in the best transgenic line with a total average of 58 mol % of acetyl-TAGs (Liu *et al.*, 2015a).

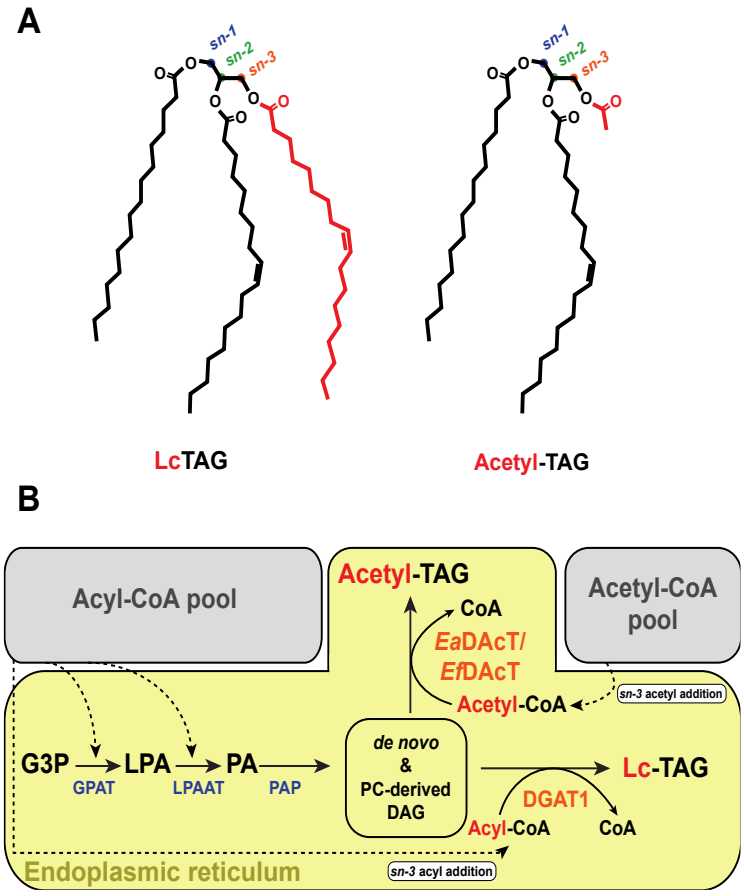

Figure 1.3 Acetyl-TAG structure and synthesis pathway.

A. Structure of triacylglycerol (lcTAG) and acetyl-triacylglycerol (acetyl-TAG) molecule. Acetyl-TAG contains an acetate group (red) at the *sn-3* position of the glycerol backbone instead of a long chain acyl group (red) present in lcTAG. B. A simplified pathway for the synthesis of acetyl-TAG. G3P, glycerol-3-phosphate; LPA, lysophosphatidic acid; PA, phosphatidic acid; DAG, diacylglycerol; LcTAG, triacylglycerol; Acetyl-TAG, acetyl-triacylglycerol; GPAT, glycerol 3-phosphate acyltransferase; LPAAT, lysophosphatidic acid acyltransferase; PAP, phosphatidic acid phosphatase; DGAT1, diacylglycerol acyltransferase 1; DAcT, diacylglycerol acetyltransferase.

1.4.5 Incorporation of new fatty acids into acetyl-TAG

Acetyl-TAG oil properties can be enhanced by incorporation of different high value fatty acids at the *sn*-1 and *sn*-2 position of acetyl-TAG. The majority of camelina fatty acids are PUFAs, which reflect the fatty acid profile of acetyl-TAG produced in transgenic camelina seeds. Polyunsaturated fatty acids were found to be more susceptible to oxidation than monounsaturated fatty acids (Ramos *et al.*, 2009). To increase the oxidative stability of acetyl-TAG oil, *Ea*DAcT was expressed in high-oleic camelina lines generated by the RNAi-mediated suppression of *Camelina sativa* FAD2 and FAE1 genes. 70 mol% of high-oleic acetyl-TAG were successfully accumulated in camelina seed with an oxidative stability index (OSI) of 2.6 (Liu *et al.*, 2015b). Incorporation of high value fatty acids into the *sn*-1 and -2 positions of acetyl-TAG was further extended to introduce MCFAs by the co-expression of *Ea*DAcT or *Ea*DAcT+*DGAT1-RNAi* in *Cp*FatB2+*Ch*FatB2 and *Uc*FatB1+*Cn*LPAAT background (Bansal *et al.*, 2018).

1.4.6 Increasing acetyl-TAG levels

Acetyl-TAG accumulated to up to 64 mol% with expression of *Ea*DAcT alone in camelina seeds. The remaining 36 mol% represented lcTAG levels. To further increase acetyl-TAG production, Liu *et al.*, (2015a) combined *Ea*DAcT expression with RNAi-mediated suppression of the camelina endogenous enzyme DGAT1. DGAT1 catalyzes the transfer of an acyl group from acyl-CoA molecule to the *sn*-3 position of DAG producing lcTAG in camelina thus competing with *Ea*DAcT for the DAG substrate (Figure 1.3B). Suppression of DGAT1 activity is expected to provide higher levels of available DAG substrates to be utilized by *Ea*DAcT. Expression of *Ea*DAcT with *DGAT1-RNAi* in camelina seeds increased acetyl-TAG accumulation by ~20% reaching up to 85 mol% in the best transgenic lines (Liu *et al.*, 2015a). In addition to elimination

or suppression of endogenous competing enzymes, many possibilities remain to be investigated to further maximize acetyl-TAG accumulation levels in camelina seeds.

In this study, multiple strategies to enhance acetyl-TAG accumulation levels in *Camelina sativa* seeds are examined. In Chapter 2, the high activity enzyme *Ef*DAcT is expressed in camelina seeds and its acetyl-TAG production levels are compared with that of *Ea*DAcT. To further enhance acetyl-TAG accumulation, *Ef*DAcT expression is combined with DGAT1 suppression. Acetyl-TAG levels are then quantified over multiple generations to test for phenotypic stability. The effect of producing high levels of acetyl-TAG on seed size, weight, fatty acid content, TAG levels, and germination rate are also studied. Further, *Ef*DAcT transcript and protein expression along with acetyl-TAG accumulation are examined in the developing seeds and the correlation between the final acetyl-TAG levels in mature seeds and enzyme levels is investigated. In Chapter 3, the effect of increasing acetyl-CoA substrate in camelina on the accumulation levels of acetyl-TAG is studied through overexpression of ATP-citrate lyase enzyme in lines expressing *Ef*DAcT alone, or *Ef*DAcT+ *DGAT1-RNAi*.

Chapter 2 - A high activity acetyltransferase from *Euonymus fortunei* enhances acetyl-triacylglycerols accumulation in transgenic camelina seeds

A manuscript to be submitted to the *Plant Journal*

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2.1 SUMMARY

Acetyl-triacylglycerols (acetyl-TAG) contain an acetate group in the *sn*-3 position instead of the long chain fatty acid present in regular triacylglycerols (TAG). The acetate group confers acetyl-TAG with unique physical properties such as reduced viscosity and lower freezing point providing advantages for its use as emulsifiers, lubricants, and 'drop-in' biofuels. The enzyme responsible for acetyl-TAG synthesis was previously isolated from the seeds of *Euonymus alatus* (burning bush). Expression of *Euonymus alatus* diacylglycerol acetyltransferase (*Ea*DAcT) in the oilseed crop *Camelina sativa* successfully produced acetyl-TAG in its seeds. Later work isolated a new acetyltransferase enzyme (*Ef*DAcT) from the seeds of *Euonymus fortunei*, which possessed higher *in vitro* activity compared to *Ea*DAcT. In this study, the seed specific expression of *Ef*DAcT in camelina resulted in 20 mol% increase in acetyl-TAG levels as compared to *Ea*DAcT. Coupling *Ef*DAcT expression with RNA-induced suppression of an endogenous competing enzyme DGAT1 further enhanced acetyl-TAG accumulation to 90 mol% in the best transgenic line. Accumulation of high levels of acetyl-TAG was stable over multiple generations with minimal effect on size, dry weight, fatty acid content, and germination of seeds. Analysis of *Ef*DAcT mRNA transcript and protein during seed development revealed a correlation between their levels and a limited window

of *Ef*DAcT protein expression. However in mature seeds, acetyl-TAG levels did not correlate with *Ef*DAcT protein levels suggesting that other unidentified factors limit acetyl-TAG accumulation.

2.2 INTRODUCTION

The seeds of many *Euonymus* species synthesize 3-acetyl-1,2-diacyl-*sn*-glycerols (acetyl-TAGs) as their main storage lipid (Kleiman *et al.*, 1967; Durrett *et al.*, 2010; Sidorov *et al.*, 2014). The *sn*-3 acetate group in these unusually structured TAGs confers favorable physical properties compared to the regular triacylglycerols (TAG; here referred to as lcTAG to indicate the presence of the *sn*-3 long chain fatty acid) found in most vegetable oils. For example, acetyl-TAG possess reduced viscosity, superior cold temperature characteristics, and a low caloric content. Oil with such properties can be used in a wide variety of applications, including as emulsifiers, food coating agents, biodegradable lubricants and 'drop-in' diesel replacements (Durrett *et al.*, 2010; Liu *et al.*, 2015a, 2015b).

Production of industrially relevant quantities of acetyl-TAG oil cannot be achieved from the non-agronomic *Euonymus* species and will therefore require metabolic engineering of existing oilseed crops. *Camelina sativa* (also known as false flax), is a member of the Brassicaceae (mustard) family and is closely related to the model plant Arabidopsis. Camelina possesses many favorable agronomic characteristics such as a short growing season, minimal use of water and fertilizer, and resistance to cold and drought. It is easily engineered using *Agrobacterium*-mediated transformation and has proven a useful platform for the synthesis of unusual lipids through biotechnology approaches (Haslam *et al.*, 2016; Bansal and Durrett, 2016a). A comparative transcriptome profiling approach identified *Euonymus alatus* diacylglycerol acetyltransferase (*Ea*DAcT) as the enzyme responsible for acetyl-TAG synthesis in the seeds of the burning bush

shrub (Durrett *et al.*, 2010). *Ea*DAcT belongs to the membrane bound O-acyltransferase (MBOAT) protein family, members of which add long fatty acyl chains to various lipid and protein acceptors (Hofmann, 2000). In contrast to other MBOATs which utilize long chain acyl-CoAs as acyl donors, *Ea*DAcT efficiently uses acetyl-CoA to acylate the *sn*-3 position of sn-1,2-diacylglycerol (DAGs) to form acetyl-TAG (Durrett *et al.*, 2010; Bansal and Durrett, 2016b). With regard to acyl acceptor specificity, substrate selectivity experiments demonstrated that *Ea*DAcT preferentially acetylates DAG containing more polyunsaturated fatty acids (Bansal and Durrett, 2016b).

Isolation of *Ea*DAcT and its subsequent expression in yeast, Arabidopsis, and camelina successfully produced high levels of acetyl-TAG (Durrett *et al.*, 2010; Liu *et al.*, 2015a; Tran *et al.*, 2017a). Acetyl-TAG levels in camelina were further increased by the suppression of endogenous diacylglycerol acyltransferase 1 (DGAT1) activity responsible for the synthesis of lcTAG (Liu *et al.*, 2015a). *Ea*DAcT has also been co-expressed with enzymes responsible for the production of medium-chain fatty acids (MCFAs) (Bansal *et al.*, 2018) and high oleic fatty acid content (Liu *et al.*, 2015b) in other studies to incorporate useful fatty acid chains in acetyl-TAG to broaden the oil applications.

We recently identified and characterized additional diacylglycerol acetyltransferase enzymes from different plants that synthesize acetyl-TAG (Tran *et al.*, 2017b). These enzymes are all similar to *Ea*DAcT and can synthesize [^{14}C] acetyl-TAG in yeast microsomes when incubated with [^{14}C] acetyl-CoA. One such enzyme, *Ef*DAcT, isolated from *Euonymus fortunei*, possessed elevated in vitro activity compared to *Ea*DAcT. Further, when expressed in yeast, *EfDAcT* produced almost 7-fold higher levels of acetyl-TAG in comparison to *EaDAcT*, consistent with the higher *in vitro* activity.

Here we demonstrate that *EfDAcT* expression in camelina seeds results in the production of higher acetyl-TAG levels compared to *EaDAcT*. We combine *EfDAcT* expression with DGAT1 suppression to further enhance acetyl-TAG accumulation. Our results show that acetyl-TAG successfully accumulates to levels beyond those reported with any modified oil content in an engineered oilseed crop, with only minor impacts on key seed properties. We quantified *EfDAcT* mRNA transcript and protein expression and acetyl-TAG accumulation during seed development to examine the relationship between enzyme levels and the final acetyl-TAG levels in mature seeds. The results provide new insights on factors limiting acetyl-TAG accumulation.

2.3 RESULTS

2.3.1 Seed expression of *Ef*DAcT results in higher levels of acetyl-TAG

Previously we showed that *Ef*DAcT possesses higher *in vitro* acetyltransferase activity compared to six other diacylglycerol acetyltransferase enzymes including *Ea*DAcT (Tran *et al.*, 2017b). To test whether *Ef*DAcT would produce higher levels of acetyl-TAG than *Ea*DAcT in seeds, we expressed hemaglutinin (HA)-epitope tagged versions of both genes under the control of the soybean glycinin promoter in wild-type camelina. The addition of the HA epitope to the N-terminus of EaDAcT does not affect the activity of the enzyme when expressed in yeast (Tran *et al.*, 2017b). Transgenic T_2 seed from lines with single transgene insertions were identified based on the presence of DsRed fluorescence and their acetyl-TAG levels were quantified. Expression of *Ef*DAcT resulted in a mean of 72 mol% acetyl-TAG in T_2 seed, which is significantly greater than the mean of 52 mol% acetyl-TAG observed for *Ea*DAcT lines (Figure 2.1A). Furthermore, the highest levels of acetyl-TAG achieved with the expression of *Ef*DAcT was 81 mol%, which is greater than the highest *Ea*DAcT line that accumulated 65 mol% acetyl-TAG.

Suppression of DGAT1 in transgenic camelina seeds expressing *Ea*DAcT resulted in higher acetyl-TAG levels compared to *Ea*DAcT alone (Liu *et al.*, 2015a). We therefore expressed both *EaDAcT* and *EfDAcT* in combination with *DGAT1-RNAi*. In agreement with our previous work (Liu *et al.*, 2015a), *EaDAcT+DGAT1-RNAi* expression resulted in a mean acetyl-TAG accumulation of 68 mol%, higher than that observed for *EaDAcT* alone (Figure 2.1A). However, expression of *Ef*DAcT+*DGAT1-RNAi* increased acetyl-TAG accumulation to levels beyond those achieved for either *Ea*DAcT+*DGAT1-RNAi* or *Ef*DAcT alone. Lines expressing EfDAcT+*DGAT1-RNAi* possessed a mean of 80 mol% acetyl-TAG, 12 mol% higher than *Ea*DAcT+*DGAT1-RNAi* and 8 mol% higher than *Ef*DAcT expression alone (Figure 2.1A). Further, the highest producing transgenic *EfDAcT+DGAT1-RNAi* line accumulated up to 87 mol% of acetyl-TAG, higher than levels observed for *EaDAcT+DGAT1-RNAi*.

The fatty acid composition of acetyl-TAG in T_2 seed expressing *Ef*DAcT+*DGAT1-RNAi* was similar to that of *Ea*DAcT+*DGAT1-RNAi* (Liu *et al.*, 2015a), with lower levels of very long chain fatty acids compared to lcTAG from the same seeds, along with an increase in the polyunsaturated fatty acids 18:2 and 18:3 (Figure 2.1B).

2.3.2 Acetyl-TAG levels in T_2 seed are predictive of levels in subsequent generations

As the construction of homozygous lines is time consuming and requires the generation of many plants, we were interested to know whether acetyl-TAG levels in seeds from hemizygous T_2 plants were predictive of the acetyl-TAG levels in the derived homozygous T_3 plants. Based on T_2 seed accumulation levels, a range of the highest, medium, and lowest acetyl-TAG producing lines expressing *Ea*DAcT, *Ef*DAcT, and *Ef*DAcT+*DGAT1-RNAi* were selected for propagation to T_3 seed. Homozygous T_3 seeds were identified based on segregation of DsRed fluorescence and acetyl-TAG levels quantified. The acetyl-TAG levels of homozygous T_3 seed were typically

similar to those of the original T_2 seed for all individual transgenic lines (Figure 2.2A). Thus, analysis of T_2 seed can be successfully predict acetyl-TAG levels in subsequent generations and therefore used to identify high acetyl-TAG producing lines. The similarity in acetyl-TAG levels between homozygous T_3 seed and parental T_2 seed was surprising given the two-fold transgene

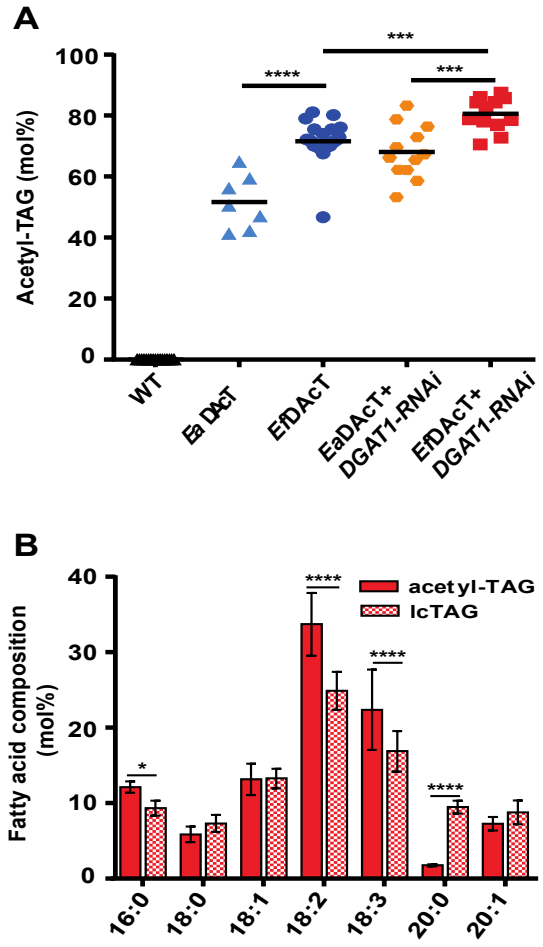

Figure 2.1 Expression of *Ef*DAcT results in higher levels of acetyl-TAG in transgenic camelina seeds.

A. Scatter plot of acetyl-TAG content of T_2 seed from independent transgenic lines expressing E*a*DAcT or *Ef*DAcT alone or in combination with the RNAi knockdown of DGAT1. Horizontal lines represent the mean acetyl-TAG content for each group. WT, Wild-type. B. Mean fatty acid composition of acetyl-TAG and lcTAG fractions of T_2 seed from 12 independent transgenic lines expressing *Ef*DAcT+*DGAT1-RNAi*. Error bars represent SD. Asterisks indicate significant difference (*, $P \leq 0.05$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$; Mann–Whitney U-test).

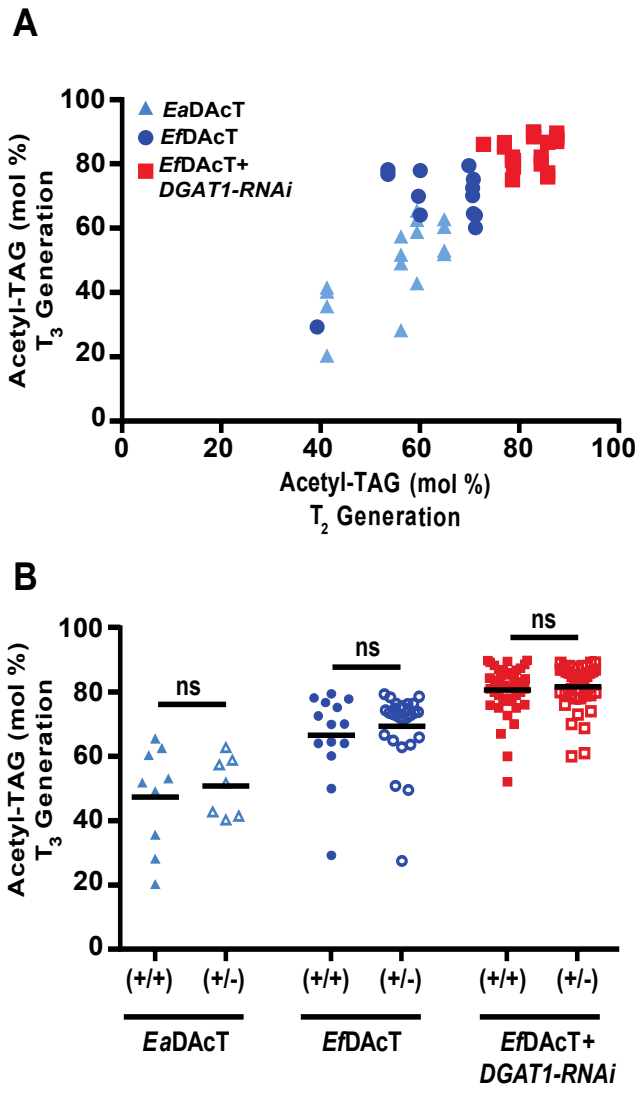

Figure 2.2 The acetyl-TAG accumulation in T_2 seed correlates with accumulation levels in T_3 seed.

A. Correlation of acetyl-TAG levels in T_2 seed and in the subsequently generated homozygous T_3 seed of transgenic lines expressing *Ea*DAcT, *Ef*DacT, or *Ef*DAcT+*DGAT1-RNAi*. Each data point represents the acetyl-TAG content of T_3 seed of individual plants plotted against the acetyl-TAG content of T_2 seed from independent transgenic lines. B. Scatter plot of acetyl-TAG content of T_3 seed from independent homozygous (+/+) and hemizygous (+/-) transgenic plants expressing E*a*DAcT, *Ef*DAcT, or *Ef*DAcT+*DGAT1-RNAi*. ns, not significant; Mann–Whitney U-test.

dosage level difference. However, quantification of the acetyl-TAG levels in the hemizygous T_3 siblings grown alongside the homozygous plants, revealed no significant difference in acetyl-TAG levels compared to the homozygous plants expressing two copies of EaDAcT, EfDAcT or EfDAcT+DGAT1-RNAi (Figure 2.2B). These results further confirm that one copy of the transgene is sufficient to produce high acetyl-TAG levels.

To investigate the stability of acetyl-TAG levels, four independent lines expressing *Ef*DAcT or *EfDAcT+DGAT1-RNAi* were propagated to produce T_4 seed. Acetyl-TAG levels in transgenic lines expressing either *EfDAcT* or *Ef*DAcT+*DGAT1-RNAi* remained relatively consistent in T_2, T_3, and T_4 seed (Figure S2.1) confirming the stability of the phenotype over multiple generations. For instance, line J2, the highest producing *E*fDAcT+*DGAT1-RNAi* transgenic line with 87 mol% of acetyl-TAG in T_2 seed, accumulated 89 mol% in T_3 seed and a mean of 90 mol% in T_4 seed.

2.3.3 Production of high of acetyl-TAG levels has a minor impact on seed properties

We quantified different seed properties in wild-type and four independent T_3 homozygous lines expressing *Ef*DAcT+*DGAT1-RNAi* that produce the highest levels of acetyl-TAG yet reported in transgenic camelina seeds, to determine whether the accumulation of these high levels of acetyl-TAG impacts seed properties. The transgenic lines showed increases of 1% to 14% in seed weight and 6% to 20% in seed size compared to wild-type seed (Figure 2.3A and 2.3B). Similar to observations in our previous study (Liu *et al.*, 2015a), all of the transgenic lines accumulated higher levels of total TAG (Figure 2.3C). However, some of the transgenic lines possessed a slightly lower seed fatty acid content compared to the wild-type (Figure 2.3D).

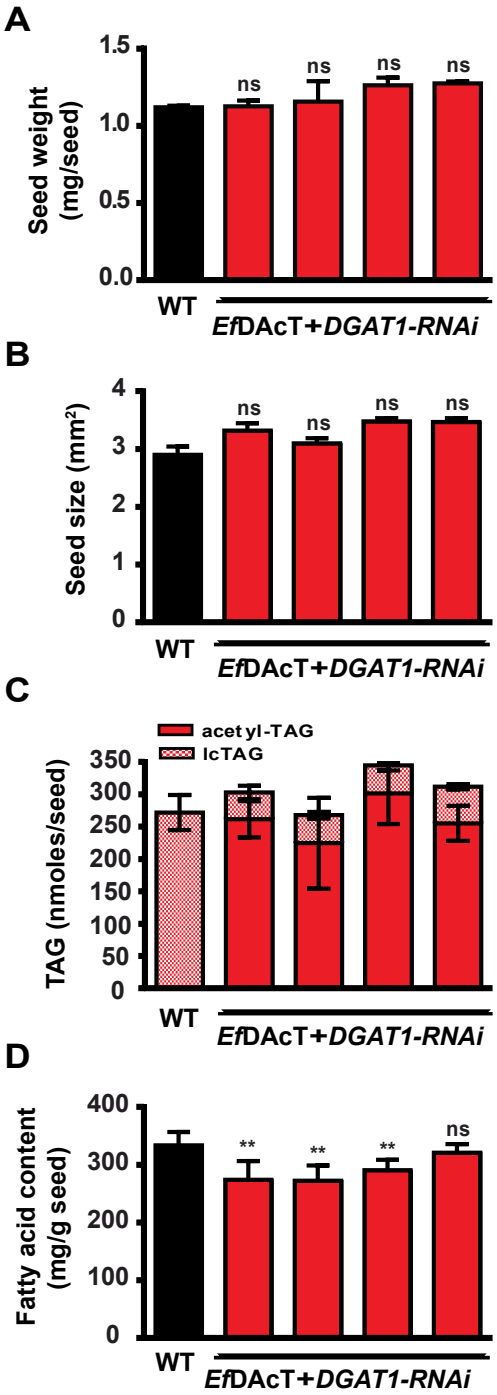

Figure 2.3 Accumulation of high levels of acetyl-TAG has little or no impact on seed properties.

Seed weight (A), seed size (B) seed TAG (C) and fatty acid content (D) of mature seed harvested from wild type (WT) and four independent T_3 homozygous transgenic lines expressing *Ef*DAcT+*DGAT1-RNAi*. Values represent the mean ± SD of three biological replicates. Asterisks indicate significant difference compared to the wild type control (**, $P \leq 0.01$; ns, not significant; Mann–Whitney U-test).

In addition, we examined the germination of T_4 seed from homozygous lines expressing *EfDAcT* or *EfDAcT +DGAT1-RNAi* that accumulate high levels of acetyl-TAG. Transgenic lines expressing either of the transgenes showed a significant delay in seed germination compared to the wild-type. For example, two days after imbibition, radicals had emerged from 100% of the wild-type seed but from only ~40% and ~20% of *Ef*DAcT and *Ef*DAcT+*DGAT1-RNAi* seed, respectively. However, by day 4 almost all of the transgenic seeds had germinated (Figure 2.4A). Similarly, cotyledon emergence in all *Ef*DAcT and *Ef*DAcT+*DGAT1-RNAi* seeds were delayed by one and two days, respectively (Figure 2.4B). Eventually, all transgenic seeds with an emerged radicle exhibited cotyledons and were capable of developing into mature plants. Together, these results indicate that the accumulation of high levels of acetyl-TAG has minor impact on key traits of camelina seeds.

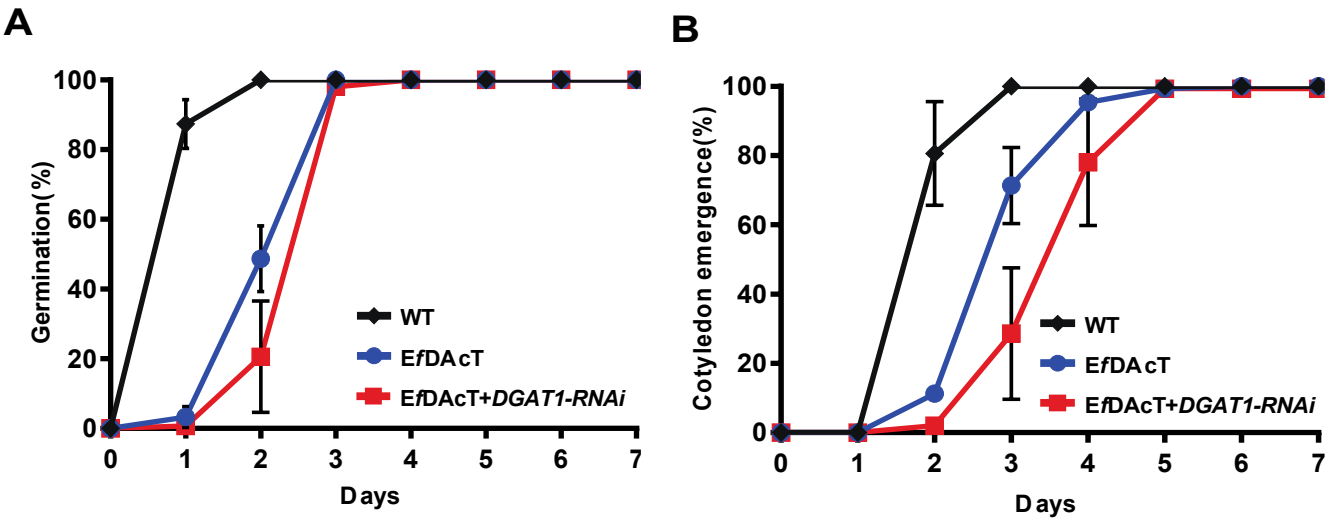

Figure 2.4 Seeds with high acetyl-TAG content germinate later than wild-type seed.

Seed germination (A) and cotyledon emergence (B**)** of wild-type (WT) and transgenic homozygous high acetyl-TAG producing lines expressing *Ef*DAcT or *Ef*DAcT+*DGAT1-RNAi*. Seeds were considered germinated based on radicle emergence. Values shown represent the mean ± SD of three biological replicates.

2.3.4 Accumulation of acetyl-TAG and lcTAG during seed development

We collected developing seeds from T_3 plants homozygous for either *EfDAcT* or *EfDAcT + DGAT1-RNAi* and quantified their TAG content, in order to better understand the accumulation of acetyl-TAG in the highest producing transgenic lines. In transgenic seed expressing *EfDAcT*, acetyl-TAG levels increased from 10 to 30 days after flowering (DAF) before gradually decreasing during late seed development and in mature seeds (Figure 2.5). In *EfDAcT + DGAT1-RNAi* transgenic seed, acetyl-TAG accumulation followed the same trend, increasing rapidly until a peak at 30 DAF, and then dropping slightly at 40 DAF and in mature seed (Figure 2.5). Developing *EfDAcT + DGAT1-RNAi* seed showed lower accumulation of lcTAG compared to *EfDAcT* alone, particularly from 20 DAF onwards (Figure 2.5). These results were consistent for multiple independent transgenic lines: in three additional high acetyl-TAG producing lines homozygous for *EfDAcT + DGAT1-RNAi*, acetyl-TAG levels increased during early seed development with accumulation reaching a peak at 30 DAF, followed by decreasing levels during late seed development (Figure S2.2).

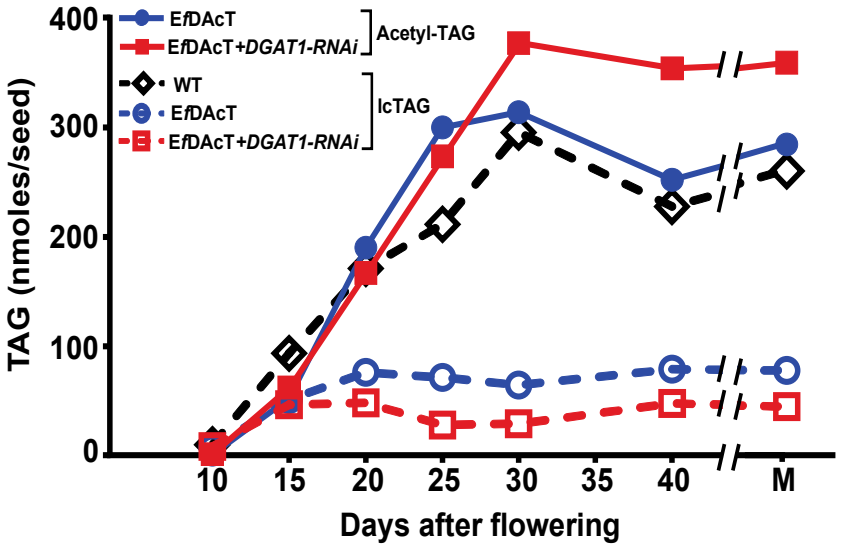

Figure 2.5 Acetyl-TAG and lcTAG content in developing wild-type and transgenic camelina seeds.

Average acetyl-TAG (solid lines) and lcTAG (dashed lines) levels in the developing seed of wild-type (WT) and transgenic T_4 homozygous high acetyl-TAG producing lines expressing *Ef*DAcT or *Ef*DAcT+*DGAT1-RNAi*. Values shown represent the mean of three biological replicates. Error bars were omitted for clarity. M, mature harvested seed

2.3.5 Transcript accumulation reflects *Ef*DAcT-HA protein content in developing transgenic seeds

In addition to studying the accumulation of acetyl-TAG during seed development we also quantified the levels of *Ef*DAcT-HA transcript and protein in the same seeds. In both seeds expressing *Ef*DAcT and *Ef*DAcT+*DGAT1-RNAi*, *Ef*DAcT transcript levels continued to increase during early seed development reaching a peak of transcript expression at 20 DAF before slowly dropping in late seed development (Figure 2.6A). Immunoblot analysis on the same seeds to detect the HA epitope fused to EfDAcT showed that the protein was only present at high levels for a relatively short window during seed development (Figure 2.6B). Accumulation of *EfDAcT* is first evident at 15 DAF in both *EfDAcT* and *EfDAcT+DGAT1-RNAi* transgenic lines, with protein levels reaching a peak at 20 DAF in *EfDAcT + DGAT1-RNAi* transgenic seed and then dropping until reaching a barely detectable signal at 40 DAF (Figure 2.6B). In seed expressing *Ef*DAcT alone, protein levels are more consistent between 15 to 25 DAF, but as in the *EfDAcT+DGAT1-RNAi* seed, they decrease at 30 DAF and are barely detectable at 40 DAF. *Ef*DAcT protein accumulation therefore is consistent with the transcript expression pattern in developing seeds, suggesting that *Ef*DAcT protein might be controlled at the transcription level. Moreover, *Ef*DAcT-HA transcript and protein data coincides well with the pattern of acetyl-TAG accumulation (Figure 2.5), where the decrease in *Ef*DAcT-HA transcript and protein signal at 30 and 40 DAF were accompanied

with a drop in acetyl-TAG levels, while the highest acetyl-TAG levels at 30 DAF followed the peak production of *Ef*DAcT-HA transcript and protein at 20 DAF.

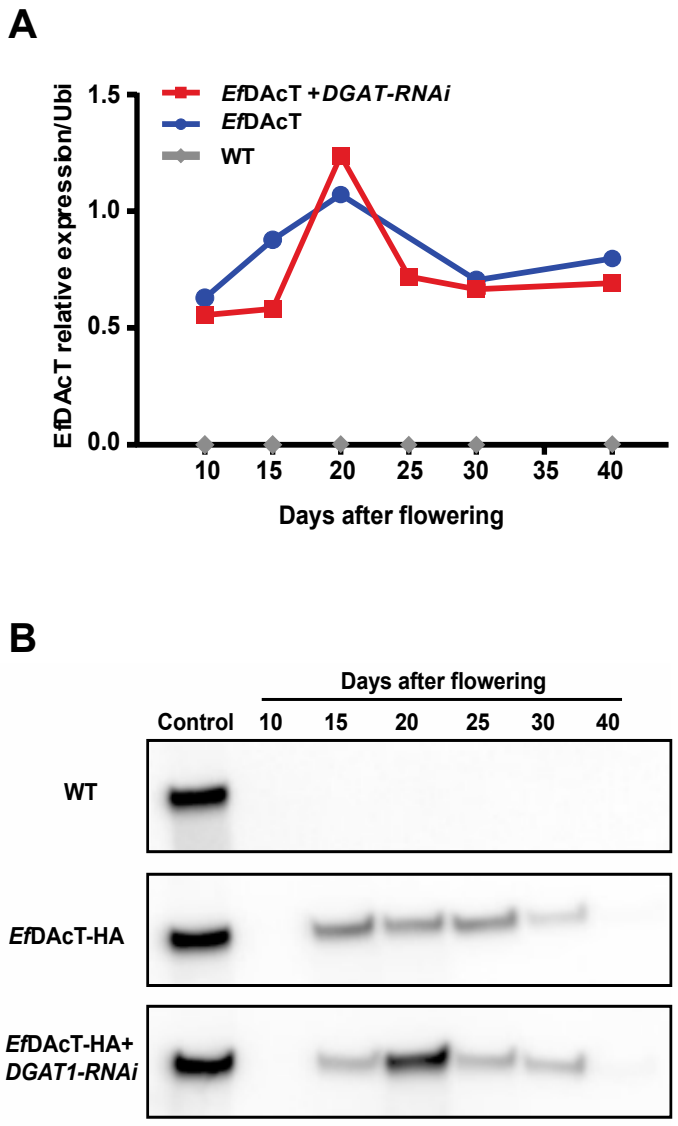

Figure 2.6 Transcript levels reflect EfDAcT protein content during seed development.

RT-qPCR analysis of *Ef*DAcT mRNA gene expression (A) and western blot of protein content (B) in the developing seeds of wild-type (WT) and transgenic T_4 homozygous high acetyl-TAG producing lines expressing *Ef*DAcT or *Ef*DAcT+*DGAT1-RNAi*. EfDAcT protein levels were detected by immunoblotting for the HA epitope tag. Yeast microsomes expressing *Ef*DAcT-HA were used as a positive control. *Ef*DAcT relative expression levels were normalized to the reference gene ubiquitin.

2.3.6 EfDAcT protein levels are not predictive of acetyl-TAG accumulation

Since acetyl-TAG production correlated well with *Ef*DAcT-HA protein level during seed development (Figure 2.5), we tested whether *Ef*DAcT-HA protein levels were predictive of the final acetyl-TAG levels in mature seeds. We selected *Ef*DAcT and *Ef*DAcT + *DGAT1-RNAi* transgenic lines that synthesized different levels of acetyl-TAG and quantified *Ef*DAcT protein levels in developing seed at 20 DAF as well as the eventual acetyl-TAG levels in mature seed for each plant. In lines expressing EfDAcT alone and that accumulate lower amounts of acetyl-TAG, there is an approximately linear relationship between EfDAcT protein levels and acetyl-TAG accumulation. However, above ~75 mol%, this linear relationship disappears (Figure 2.7A). In *EfDAcT+DGAT1-RNAi* lines, all of which accumulate levels of acetyl-TAG greater than 70 mol%, there is no apparent correlation between EfDAcT protein levels at 20 DAF and eventual acetyl-TAG accumulation. While plants with the highest levels of EfDAcT accumulation tended to produce the highest levels of acetyl-TAG, a few plants with much lower protein levels (<0.5 normalized units) also synthesized acetyl-TAG levels of ~90 mol%, similar to those with EfDAcT levels of ~1.0 normalized units, or higher (Figure 2.7B).

2.4 DISCUSSION

The results presented here show that seed-specific expression of *Ef*DAcT led to 20 mol% increase of acetyl-TAG levels compared to *Ea*DAcT (Figure 2.1A). The high activity of *Ef*DAcT in yeast (Tran *et al.*, 2017b) successfully translated into camelina, increasing acetyl-TAG accumulation in transgenic lines. Conserved amino acids residues found in all DAcT enzymes have been studied and multiple residues such as S253, H257 and D258 were found crucial for the acetyltransferase activity (Tran *et al.*, 2017b). However, the role of different amino acid residues

of DAcT enzymes with various acetyltransferase activity levels has not been conclusively explored to date. *Ef*DAcT

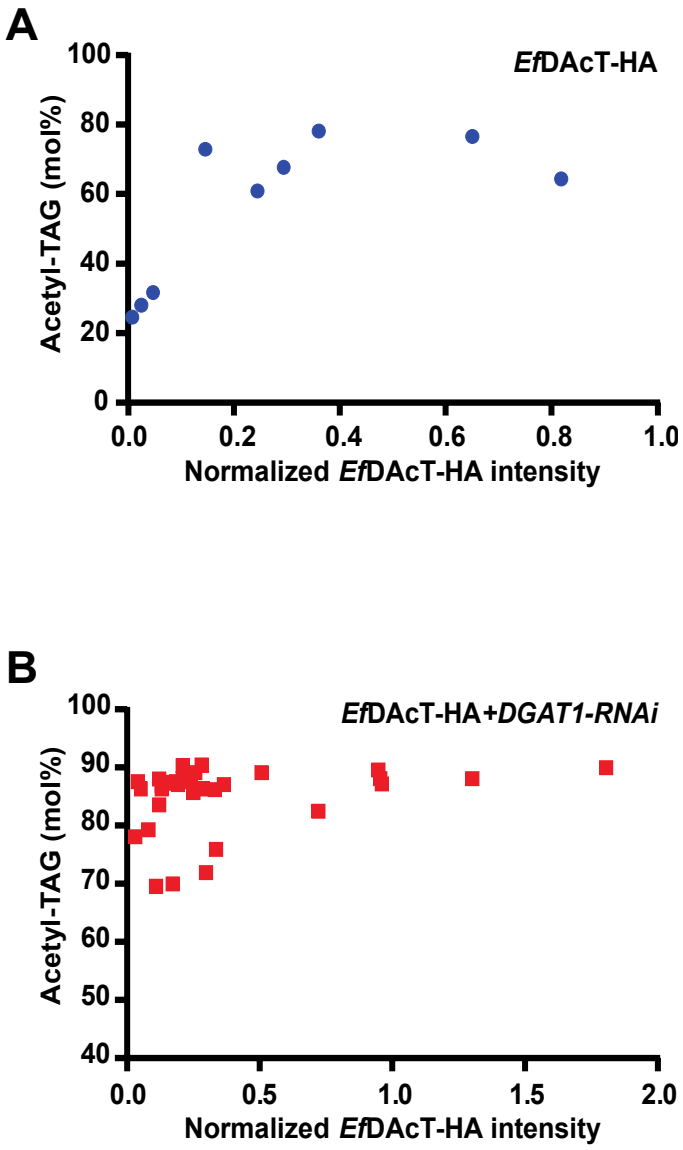

Figure 2.7 Acetyl-TAG accumulation does not correlate with *Ef*DAcT-HA protein levels.

Final acetyl-TAG content in mature seeds expressing *Ef*DAcT (A) or *Ef*DAcT+*DGAT1-RNAi* (B) plotted against the EfDAcT-HA protein levels in seeds from the same plant collected 20 DAF. *Ef*DAcT-HA levels were normalized to yeast microsomes expressing *Ef*DAcT-HA as a positive control.

and *Ea*DAcT share 94% protein sequence identity with only 22 amino acid differences. The position, charge, or size of some of these amino acid residues might contribute to variation in the protein stability and enzyme activity, but the mechanism behind those differences remains unknown. In a previous study, site-directed mutagenesis (SDM) of a putative functional motif in DGAT1 enzyme where a serine residue was substituted by alanine, resulted in an enhanced activity and 20%–50% increase in oil accumulation (Xu *et al.*, 2008). This strategy can be utilized to further understand the mechanisms governing the efficiency of acetyltransferase activity in diverse DAcT enzymes.

Expression of *Ef*DAcT combined with DGAT1 suppression led to the highest accumulation of modified lipids in an oilseed crop. DGAT1 and phospholipid:diacylglycerol acyltransferase 1 (PDAT1) are known to catalyze the last rate limiting step of TAG biosynthesis (Zhang *et al.*, 2009). However, earlier work demonstrated that DGAT1 suppression increased acetyl-TAG levels whereas combining PDAT1 suppression with DGAT1 suppression did not in an increase (Liu *et al.*, 2015a; Bansal *et al.*, 2018). Similar results were observed in an attempt to increase hydroxy fatty acid (HFA) levels in Arabidopsis, where suppression of PDAT1 in a *dgat*1 mutant background expressing DGAT2 and PDAT1 enzymes from castor failed to increase HFA levels. Further, seeds from many of these lines were wrinkled and germinated poorly (van Erp *et al.*, 2015). Both studies suggest that suppression of both DGAT1 and PDAT1 negatively affect seed development and cannot be complemented through the expression of alternative enzymes introduced transgenically (Liu *et al.*, 2015a; van Erp *et al.*, 2015). Therefore, only DGAT1 suppression was implemented in the current work to enhance acetyl-TAG levels.

In the highest producing transgenic line expressing *Ef*DAcT+ *DGAT1-RNAi*, 87 mol% of total TAGs in T_2 seed were acetyl-TAG (Figure 2.1A). T_4 seed from the same line contained an

average of 90 mol% acetyl-TAG, the highest levels of modified TAG structures thus far produced in a transgenic seed. Previous efforts to alter camelina seed oil have typically achieved lower levels of the desired lipid product. For example, levels of 8%–30% of medium chain fatty acids (MCFA) (Kim *et al.*, 2015; Hu *et al.*, 2017) or 22% of HFA (Snapp *et al.*, 2014) have been achieved in transgenic camelina seed. The very high levels of acetyl-TAG achieved are likely obtained due to *Ef*DAcT modifying the final step of TAG biosynthesis. In contrast, in the other studies, the incorporation of unusual fatty acids into TAG, not only requires expression of enzymes necessary for the synthesis, but also the coordinated expression of specialized acyltransferases to prevent bottlenecks in fatty acid trafficking.

Acetyl-TAG accumulating in camelina seeds expressing *Ef*DAcT + *DGAT1-RNAi* possessed a significant increase in the PUFA and decrease in the VLCFA compared to the lcTAG in the same seeds (Figure 2.1B), consistent with our previous observations with *Ea*DAcT expression in camelina seeds (Liu *et al.*, 2015a). The decrease in VLCFA in acetyl-TAG is due to the presence of an acetate group in the *sn*-3 position, where VLCFA are preferentially incorporated in the lcTAG produced in other Brassicaceae species (Takagi and Ando, 1991; Taylor *et al.*, 1995). In vitro selectivity assays demonstrated that *Ea*DAcT preferentially acetylate DAGs with high levels of unsaturation compared to saturated DAGs (Bansal and Durrett, 2016b). The higher levels of PUFA in acetyl-TAG synthesized with *Ef*DAcT imply that like *Ea*DAcT, *Ef*DAcT possesses a similar preference for PUFA DAG.

The stability of high acetyl-TAG levels in the best transgenic lines over multiple generations (Figure S2.1) suggests that gene silencing does not occur in these lines, thus rendering them suitable for crossing to transgenic lines containing additional useful traits to further expand their utility. Importantly, and consistent with the stability of the phenotype over multiple

generations, very high levels of acetyl-TAG were achieved with minor impact on key seed properties. Since acetyl-TAG utilizes two acyl chains instead of three acyl chains with lcTAG, acetyl-TAG producing lines accumulate more overall TAG molecules compared to wild-type plants (Figure 2.3C) (Liu *et al.*, 2015a). This increase in TAG content likely explains the higher seed weight and size for most transgenic lines (Figure 2.3A and B). However, total fatty acid content was slightly reduced in most of the transgenic lines (Figure 2.3D). The minor reduction in fatty acid could be caused by bottlenecks in the acetyl-TAG synthesis at these high levels of accumulation. For example, limitations in acetyl-CoA availability could prevent the complete incorporation of all available fatty acids into acetyl-TAG.

Successful seed germination requires mobilization of stored lipid reserves. Previously, acetyl-TAG and lcTAG were shown to metabolize at similar rates in germinating seeds (Liu *et al.*, 2015a). Nonetheless, in this study a two day delay in germination of transgenic lines accumulating high levels of acetyl-TAG was observed compared to the wild-type, with *Ef*DAcT+*DGAT1-RNAi* expressing seeds showing a larger effect (Figure 2.4). Endogenous camelina lipases responsible for the mobilization of lcTAG in germinating seed might function less efficiently with the unusually structured acetyl-TAG compared to lcTAG, explaining the delay. In addition, Arabidopsis mutant *dgat1* seeds also germinate later than wild-type seed (Lu and Hills, 2002), possibly explaining why the *DGAT1-RNAi* lines experience a further delay in germination.

For this work, *Ef*DAcT was highly expressed under the control of the soybean seed-specific glycinin promoter. Previous studies in plants indicate that when RNA levels are abundant, transcript expression generally reflects protein content levels (Sun *et al.*, 2001; Cao *et al.*, 2013; Ponnala *et al.*, 2014). Analysis of *Ef*DAcT mRNA and protein levels in the developing seeds of the transgenic lines revealed a well correlated pattern of *EfDAcT* transcript with protein. Here,

*Ef*DAcT protein was present only in a limited window of expression between 15 and 30 DAF and was barely detectable in later stages of seed development (Figure 2.6). A similar expression pattern was observed for genes involved in TAG and fatty acid synthesis in Arabidopsis, castor bean, and camelina (Ruuska *et al.*, 2002; Chen *et al.*, 2007; Abdullah *et al.*, 2016). *Ef*DAcT protein and transcript showed maximum expression levels at 20 DAF, the stage at which acetyl-TAG levels are most rapidly increasing (Figure 2.5). Acetyl-TAG levels reach a peak of accumulation at 30 DAF, coincident with the reduction of *Ef*DAcT transcript and protein content (Figure 2.6).

Expression levels, and therefore phenotypic effects, are usually positively correlated with gene copy number (Schubert *et al.*, 2004). Interestingly in our study, the mean acetyl-TAG accumulation was similar in homozygous lines where two copies of the transgene are present and in hemizygous lines where one copy is present at a single locus (Figure 2.2B). The lack of distinction between homozygous and hemizygous state on trait accumulation levels was previously observed in rapeseed synthesizing MCFA (Voelker *et al.*, 1996), and Arabidopsis transformed with a polyketide synthase-like to enable biosynthesis of very long chain polyunsaturated (VLC-PUFA) omega-3 fatty acids (Walsh *et al.*, 2016). These results are in agreement with what was observed when we compared acetyl-TAG level in mature seeds with *Ef*DAcT protein content in the developing seeds of the same line. The data indicate that *Ef*DAcT protein levels in the developing seeds of transgenic lines producing more than 70 mol% of acetyl-TAG, did not correlate with acetyl-TAG levels in its mature seeds (Figure 2.7). The absence of correlation between *Ef*DAcT protein expression and the final acetyl-TAG levels may explain why both homozygous and hemizygous transgenic lines accumulate similar levels of acetyl-TAG. Thus, in the high producing transgenic lines, *Ef*DAcT protein levels reach a threshold beyond which, higher

levels of protein do not result in more acetyl-TAGs, suggesting that other factors such as the availability of glycerol backbone or acetyl-CoA may restrict acetyl-TAG accumulation.

2.5 CONCLUSION

We have demonstrated that seed specific expression of *Ef*DAcT in camelina results in up to 90 mol% of acetyl-TAG accumulation. The higher acetyltransferase activity of *Ef*DAcT increased acetyl-TAG to the highest levels of modified lipids reported to date in a transgenic oilseed crop. High levels of acetyl-TAG were confirmed to be stable over at least three generations and had a minimal effect on seed properties. Acetyl-TAGs are associated with reduced viscosity, high caloric content, and superior cold temperature properties (Durrett *et al.*, 2010; Liu *et al.*, 2015a). Increasing their levels to maximum accumulation in the seed will further enhance camelina oil physical properties and render it more suitable to a wide variety of industrial applications. The lack of correlation between *Ef*DAcT protein and acetyl-TAG levels, and the lack of distinction between homozygous and hemizygous lines, suggest that expression of *Ef*DAcT is not limiting further acetyl-TAG accumulation. Instead, the reductions in fatty acid content in transgenic lines, implies that the flux of other substrates involved in acetyl-TAG synthesis such as acetyl-CoA might not be sufficient for maximal production of acetyl-TAG.

2.6 MATERIALS AND METHODS

2.6.1 Plant material and growth conditions

Transgenic and wild-type *Camelina sativa* 'Suneson' were grown in a growth chamber under a 16 h light/8 h dark cycle, at a constant temperature of 21°C. To obtain seeds at different stages of development, emerging flowers were tagged using colored thread and the tagged pods

harvested at the indicated intervals. Collected seeds were immediately frozen in liquid nitrogen and stored at −80°C until further analysis. Harvested mature seeds were dried in a vacuum desiccator for several days before analysis.

2.6.2 Vector construction and generation of transgenic plants

The construction of yeast expression vectors containing *Ea*DAcT-HA and E*f*DAcT-HA has been described previously (Tran *et al.*, 2017b). The open reading frames of the epitope tagged acetyltransferases were amplified with the primers *Ea*DAcT-HA-F, *Ef*DAcT-HA-F and *Ea/f*DAcT-HA-R (Table S2.1) then cloned into the binary vector pBinGlyRed3 under control of the soybean glycinin promoter (Nguyen *et al.*, 2013). The vector contains the fluorescent protein DsRed as a visual selectable marker. Expression cassettes containing the *DGAT1-RNAi* hairpin sequence driven by the *Brassica napus* oleosin promoter (Liu *et al.*, 2015a) were then added. The fidelity of the different cloning steps was verified by sequencing all introduced DNA. The transformation constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 and transformed into wild-type *Camelina sativa* 'Suneson' via a floral dip vacuum infiltration method (Lu and Kang, 2008). Transgenic T_1 seed were identified by visual screening for DsRed fluorescence using a red lens filter and a green LED light. Transgenic lines segregating for a single transgenic event were identified by counting the ratio of red to non-red fluorescent seeds in 100 T_2 seeds. Lines that passed the chi-square test for a 3:1 segregation ratio were used for further analysis and propagation.

2.6.3 Lipid analysis

Lipids were extracted from mature and developing seeds using a hexane–isopropanol extraction method (Li *et al.*, 2006). Tripentadecanoin (NuChek Prep, New Elysian, MN) was added as an internal standard during lipid extraction. Lipid extracts were loaded on K6 Silica TLC

plates and separated using a 70:30:1 hexane/ diethy-ether/ acetic acid (v/v/v) solvent system. TLC plates were sprayed with 0.2% (w/v) 2,7-dichlorofluorescein in 96% methanol and visualized under UV light. Triheptadecanoin (NuChek Prep, New Elysian, MN) was added as an internal standard to the acetyl-TAG and lcTAG bands, which were then scraped and transmethylated directly using an acid-catalyzed method. The resulting fatty acid methyl esters (FAME) were quantified using a standard gas chromatography method (Aznar-Moreno and Durrett, 2017). Acetyl-TAG and lcTAG levels were quantified as described previously (Liu *et al.*, 2015a). The total fatty acid contents of mature seeds were determined by quickly homogenizing 20 seeds in 1 ml toluene with a PT2500E polytron (Kinemtica AGd) followed by transmethylation and gas chromatography analysis (Li *et al.*, 2006).

2.6.4 Analysis of seed properties

Seed weight was determined by carefully weighing 100 dry seeds per replicate on a Sartorius CP225D analytical balance. Seed size was calculated using ImageJ software following a previously established method (Herridge *et al.*, 2011) based on 300 randomly selected seeds per replicate. For seed germination, seeds from wild-type and transgenic plants grown at the same time were planted on petri dishes containing wet filter paper. Plates were placed in a growth chamber under a 16 h light/8 h dark cycle, and a constant temperature of 21°C. Seeds were considered as germinating based on the emergence of the radicle.

2.6.5 Protein extraction and immunoblotting

Fifteen developing seeds were finely ground with liquid nitrogen, added to 400 µl of ice-cold extraction buffer (100 mM Tris-HCL pH 8.0, 10% (v/v) glycerol, 1 mM DTT, and 1 mM PMSF) and mixed vigorously. Samples were centrifuged at 1000 g for 5 min at 4°C after which the supernatants were removed and centrifuged again at 10,000 g for 15min at 4°C. The final

supernatants were transferred to new tubes and stored in -80°C until further use. Protein concentrations were determined with a Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Protein samples were incubated with loading buffer (125 mM Tris-HCl pH6.8, 8M urea, 10% SDS, 0.004% (w/v) bromophenol blue, 10% B-mercaptoethanol) for 45min at 37°C and then resolved on 10% SDS-PAGE (GenScript, Piscataway, NJ). Yeast microsomes (1µg protein) expressing E*f*DAcT-HA (Tran *et al.*, 2017b) were added to all gels as a positive control and to normalize protein accumulation between different blots. The resolved proteins were transferred onto a nitrocellulose membrane, which was then stained with Ponceau S for visualization of total protein (Figure S2.3). Membrane was blocked with 5% (w/v) non-fat milk powder in PBST buffer (0.8% NaCl, 0.02% KCl, 0.024% KH_2PO_4, 0.144% Na_2HPO_4, 0.1% (v/v) Tween 20) for 1h. The membrane was rinsed with PBS 5 times for 5 min each and then incubated with mouse anti-HA primary antibody (1:5,000 dilution in PBS; clone 2-2.2.2.14; Thermo Scientific) at 4°C overnight. The membrane was then rinsed with PBS 5 times for 5 min each, and then incubated with goat anti-mouse secondary antibody (1:5,000 dilution in PBS; Thermo Scientific) for 1 hour at RT. Antibodies were detected using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, IL) and protein bands were visualized using an Azure c600 Chemiluminescent Western Blot Imaging System. E*f*DAcT-HA protein abundance was quantified and normalized to the protein abundance of the yeast positive control using densitometry analysis with ImageJ software.

2.6.6 RNA extraction

Total RNA was isolated from developing seeds using a previously established method (Chang *et al.*, 1993) with the following modifications: 60µl of 10%SDS and 1ml of pre-warmed CTAB extraction buffer (2% cetyltrimethylammonium bromide, 25mM EDTA, 100 mM Tris-HCl

pH8.0, 2.0M NaCl, 0.5 g/L spermidine, 2% polyvinylpyrrolidone K 30, 50µg/ml proteinase K, and 2% B-mercaptoethanol) were added to the ground tissue. Samples were incubated at 65°C for 10min with vigorous shaking and centrifuged at 13,000g for 10min at 4°C. An equal volume of 25:24: 1 phenol:chloroform: isoamyl alcohol was added to the supernatant and centrifuged at 13,000g for 10min at 4°C. This phenol:chloroform: isoamyl alcohol extraction step was repeated twice. The aqueous phase containing RNA was mixed with LiCl to a final concentration of 2.5 M and incubated at -20°C overnight. RNA was precipitated by centrifuging at 20,000g for 1h at 4°C. The RNA pellet was washed with 80% ethanol twice, resuspended in RNase free DEPC-treated water and stored at -80°C until further use.

2.6.7 Quantitative reverse transcription PCR (RT-qPCR) analysis

For each sample, 4µg of total RNA were digested with RNase-free DNaseI (Promega, Madison, WI) to remove any genomic DNA contamination. RNA concentrations were quantified with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Rockford, IL). 1µg of RNA was used for cDNA synthesis using qScript™ cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD). Quantitative PCR was performed on a CFX96 (Bio-Rad) using PerfeCTa® SYBR® Green SuperMix (Quanta Bioscience, Gaithersburg, MD) according to manufacturer's instructions. E*f*DAcT gene relative expression normalized to camelina ubiquitin and GAPDH gene was calculated using the comparative $2^{-\Delta Ct}$ method (Winer *et al.*, 1999).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

2.7 SUPPLEMENTARY DATA

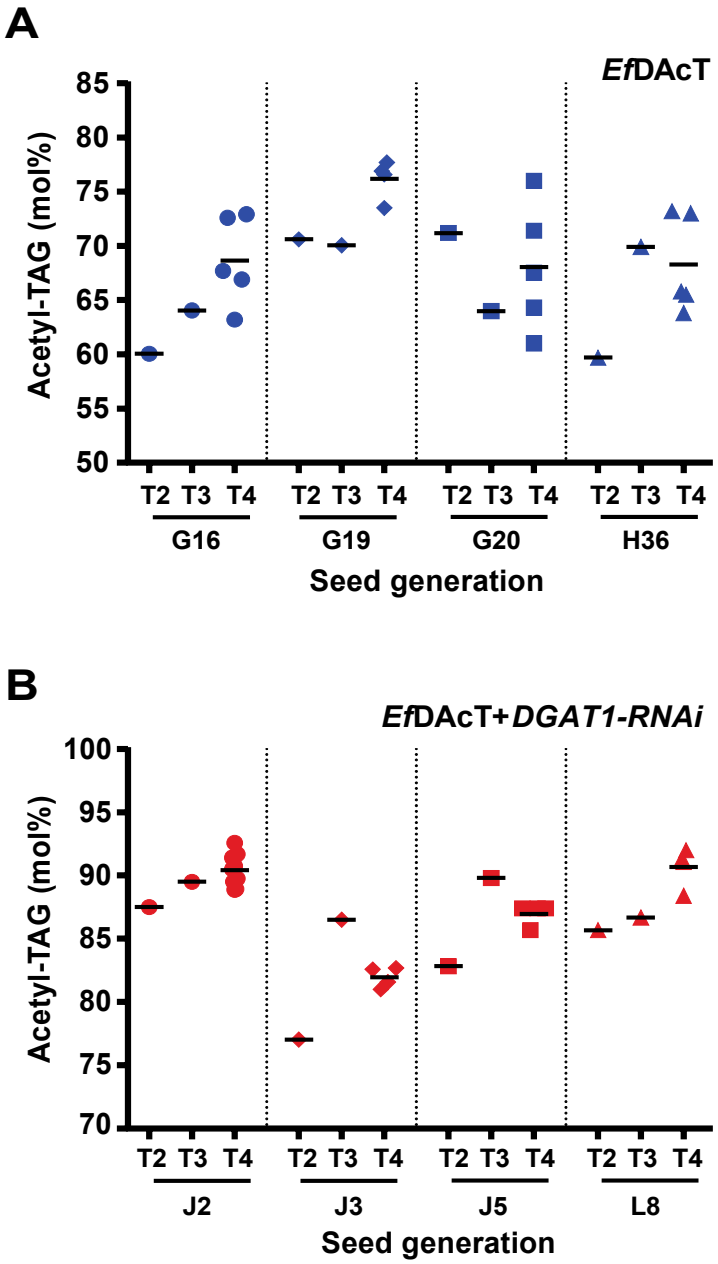

Figure S 2.1 Acetyl-TAG levels in transgenic camelina seeds are stable across multiple generations.

Acetyl-TAG content in mol% of T_2, T_3, and T_4 seed generation from transgenic lines expressing *Ef*DacT (A), or *Ef*DAcT+*DGAT1-RNAi* (B). Each data point represents the acetyl-TAG content of seeds from an individual plant. Horizontal lines represent the average levels of acetyl-TAG.

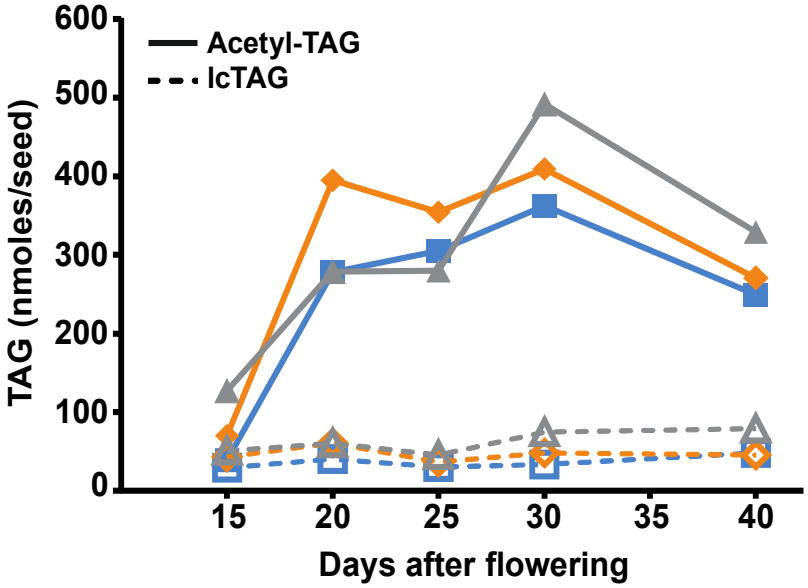

Figure S 2.2 Acetyl-TAG accumulation follows the same pattern in independent transgenic lines.

Acetyl-TAG (solid lines) and lcTAG (dashed lines) levels in the developing seed of three independent transgenic T_4 homozygous high acetyl-TAG producing lines expressing *Ef*DAcT+*DGAT1-RNAi*.

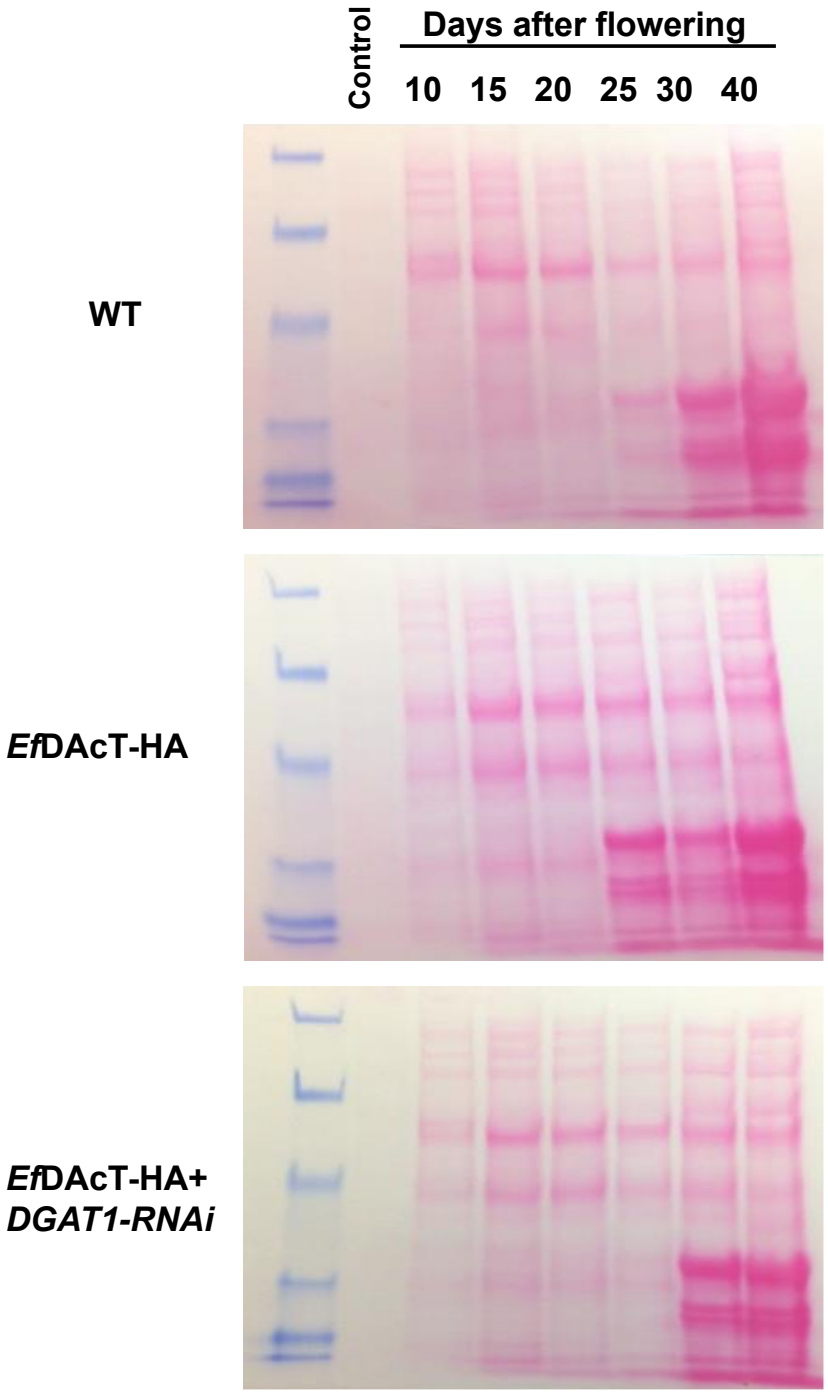

Figure S 2.3 Total protein in developing seeds of wild-type and transgenic camelina lines.

Ponceau S staining of total proteins in the developing seeds of wild type (WT) and transgenic T_4 homozygous high acetyl-TAG producing lines expressing *Ef*DAcT or *Ef*DAcT+*DGAT1-RNAi*. Control is yeast microsomes expressing *Ef*DAcT-HA protein.

Table S 2.1 Primers used for vector construction and RT-qPCR amplification.

Primer	Sequence
*Ea*DAcT-HA-F	5’-ACCCAATTGATGATGGATGCTCATCAA-3’
*Ef*DAcT-HA-F	5’- ACCCAATTGATGATGGATGTTCATCAA -3’
*Ea/f*DAcT-HA-R	5’-AGAGCTAGCTTAAGCGTAATCTGGAA-3’
*Ef*DAcT-RT-F	5’- CGGCTTCTTCCTTGCTTGGC -3’
*Ef*DAcT-RT-R	5’- GGATTTCGCTTGACTGTGATG GG -3’
Ubiquitin-RT-F	5’- GATGAGCTTGTTGGAATGCAGGGTCCAG-3’
Ubiquitin-RT-R	5’- GCCATGACACGTGATACAGTATAACCCGTC-3’
GAPDH-RT-F	5’- GATGGAAAGATCACAGGAGATGTTGAGGC-3’
GAPDH-RT-R	5’- GCTATCTCGTTTGCAATCTTTGCGCCC-3’

Chapter 3 - Expression of citrate lyase increases acetyl-TAG levels in transgenic camelina seeds

3.1 INTRODUCTION

Euonymus fortunei diacylglycerol acetyltransferase (*Ef*DAcT) transfers an acetate group from acetyl-CoA to the *sn*-3 position of *sn*-1,2-diacylglycerol (DAG) producing acetyl-TAG. Compared to regular TAGs (lcTAGs), which contain a long chain fatty acid at the *sn*-3 position, acetyl-TAGs have unique physical and chemical properties that make them suitable to a wide range of industrial applications (Durrett *et al.*, 2010; Liu *et al.*, 2015a, 2015b). *Ef*DAcT possesses the highest acetyltransferase activity in yeast when compared to six other DAcT enzymes (Tran *et al.*, 2017b), making it the best enzyme variant for producing acetyl-TAG. To produce high levels of acetyl-TAG-containing oil, *Ef*DAcT was expressed in *Camelina sativa* seeds in which an average of 72 mol% was achieved. The average acetyl-TAG accumulation was further increased to 80 mol% by the suppression of the endogenous camelina lcTAG synthesizing enzyme, diacylglycerol acyltransferase 1 (DGAT1) (Chapter 2). Analysis of *Ef*DAcT protein levels suggested that expression of the enzyme was no longer limiting acetyl-TAG accumulation. Instead, at these high levels of acetyl-TAG synthesis, the supply of other factors required for the synthesis for acetyl-TAG, such as acetyl-CoA, might be limiting.

The acetate group transferred by *Ef*DAcT to synthesize acetyl-TAG likely originates from a cytosolic acetyl-CoA pool. In the cytosol, ATP-citrate lyase (ACLY) catalyzes the ATP-dependent reaction of citrate, produced in the mitochondria by citrate synthase, and CoA to form acetyl-CoA and oxaloacetate (Ratledge *et al.*, 1997; Schwender and Ohlrogge, 2002). In animals,

ACLY is a homomeric enzyme (Elshourbagy *et al.*, 1990), while a heteromeric structure consisting of ACLA and ACLB subunits was reported in Arabidopsis plants (Fatland *et al.*, 2002).

Due to its role in acetyl-CoA formation, it was hypothesized that overexpression of ACLY would lead to higher acetyl-TAG accumulation levels when co-expressed with *Ef*DAcT. In this study, the *Rattus norvegicus* ATP: citrate lyase (*Rn*ACLY) homotetramer enzyme (Elshourbagy *et al.*, 1990; Rangasamy and Ratledge, 2000) was overexpressed in camelina seeds with either *Ef*DAcT or *Ef*DAcT+*DGAT1-RNAi*. A mammalian rather than a plant enzyme was used as the homomeric structure of the *Rn*ACLY requires the expression of only one gene. Previous work has shown that expression of *Rn*ACLY in chloroplasts results in increased fatty acid synthesis due to increased supply of acetyl-CoA in tobacco (Rangasamy and Ratledge, 2000). Acetyl-TAG levels were examined in the segregating T_2 seed and the derived T_3 seed from homozygous plants. To investigate the stability of acetyl-TAG accumulation, the highest producing *Rn*ACLY+*Ef*DAcT+*DGAT1-RNAi* transgenic line was further propagated to T_4 seed and acetyl-TAG levels were analyzed.

3.2 RESULTS AND DISCUSSION

3.2.1 Co-expression of citrate lyase and *Ef*DAcT does not increase acetyl-TAG accumulation

Cytosolic acetyl-CoA is utilized by *Ef*DAcT to acetylate DAG molecules in the last step of acetyl-TAG biosynthesis. At high levels of acetyl-TAG accumulation, *Ef*DAcT expression no longer limits formation of acetyl-TAG (Chapter 2). It was hypothesized that overexpressing *Rn*ACLY would increase the acetyl-CoA pool available for *Ef*DAcT and, thus generate more acetyl-TAG. *Rn*ACLY was overexpressed in wild type camelina seeds along with *Ef*DAcT-HA under the control of the soybean glycinin seed-specific promoter (Liu *et al.*, 2015a) (Figure 3.1).

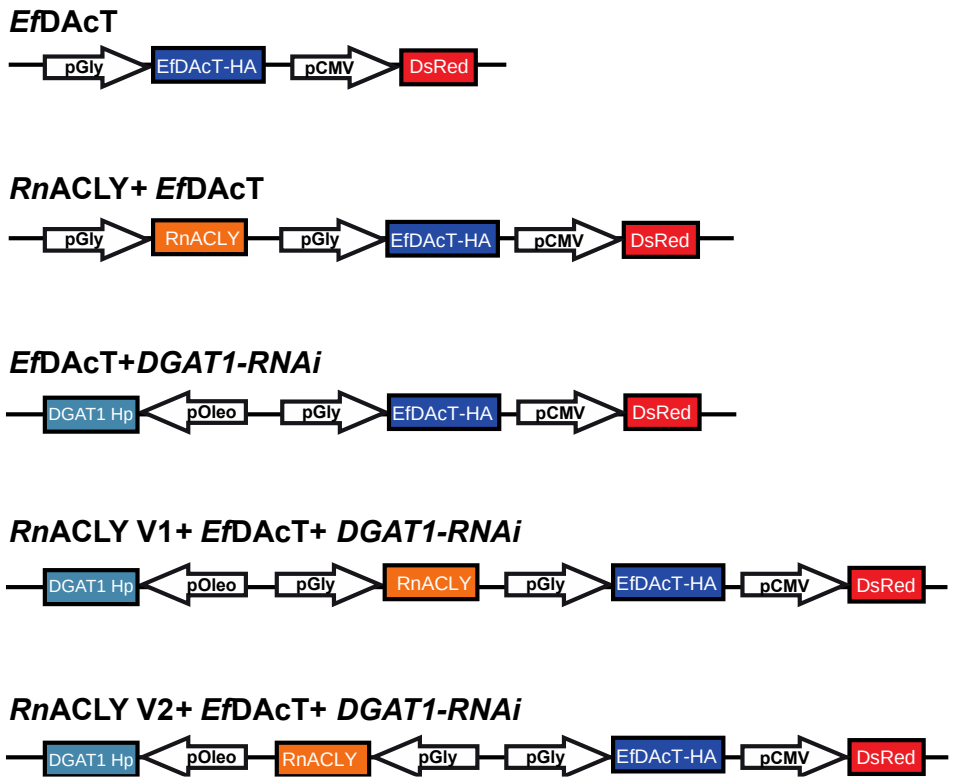

Figure 3.1 Constructs used to express *Ef*DAcT, *Rn*ACLY+ *Ef*DAcT, or in combination with *DGAT1-RNAi*.

Representation of the constructs used in this study. pGly, soybean glycinin promoter; pCMV, Cauliflower Mosaic virus 35S promoter; DsRed, Discosoma sp. fluorescent protein; pOleo, *Brassica napus* oleosin promoter; *Ef*DAcT, *Euonymus fortunei* diacylglycerol acetyltransferase; *Rn*ACLY, *Rattus norvegicus* ATP-dependent citrate lyase; DGAT1, diacylglycerol acyltransferase-1; Hp, hairpin.

Transgenic camelina lines with single transgene insertions were selected based on a 3:1 DsRed fluorescence segregation ratio in T_2 seed. Quantification of acetyl-TAG levels in the transgenic T_2 seed from these lines revealed that overexpression of *Rn*ACLY did not significantly increase acetyl-TAG accumulation in *Ef*DAcT expressing lines. *Rn*ACLY+ *Ef*DAcT overexpressing lines accumulated a mean of 74 mol% of acetyl-TAG, a 2 mol% increase from that with *Ef*DAcT expression alone (72 mol%) with the highest producing transgenic line accumulating up to 80

mol% in T_2 seed (Figure 3.2A). Cytosolic acetyl-CoA plays role in the elongation of C18 and subsequent formation of very long chain fatty acid (VLCFA) C20 and C22 by fatty acid elongase1 (FAE1) (Pollard and Stumpf, 1980; Fatland *et al.*, 2002; Hutcheon *et al.*, 2010). However, RNAi-mediated suppression of *FAE1* in camelina seeds, which should increase acetyl-CoA levels, failed to increase acetyl-TAG accumulation when *Ea*DAcT was expressed (Liu *et al.*, 2015b). Similarly, the minor increase in acetyl-TAG levels, even when more acetyl-CoA is available for *Ef*DAcT utilization, suggests the presence of other limiting factors to acetyl-TAG accumulation such as the availability of the glycerol backbone/DAG substrate.

In agreement to previous results (Liu *et al.*, 2015b) (Figure 2.1B), the fatty acid composition of acetyl-TAG with *Rn*ACLY overexpression showed an increase in the polyunsaturated fatty acids (PUFA) compared to lcTAG confirming the substrate specificity of *Ef*DAcT (Bansal and Durrett, 2016b). An increase in very long chain fatty acid (VLCFA) is observed in lcTAG (Figure 3.3A) consistent with the presence of acetate group at the *sn*-3 position of acetyl-TAG (Liu *et al.*, 2015a).

3.2.2 Overexpression of citrate lyase combined with *Ef*DAcT expression and DGAT1 suppression slightly increases acetyl-TAG levels

The endogenous camelina TAG biosynthetic enzyme, DGAT1 competes with *Ef*DAcT for DAG substrate to synthesize lcTAG. RNAi-mediated suppression of DGAT1 coupled with *Ef*DAcT expression increases acetyl-TAG accumulation to a mean of 80 mol% in T_2 transgenic seed, reaching up to 87 mol% in the highest producing transgenic line (Figure 2.1A). Since higher levels of acetyl-TAG were not achieved with overexpression of *Rn*ACLY and *Ef*DAcT alone, combining DGAT1 suppression might elevate acetyl-TAG accumulation. Two constructs that

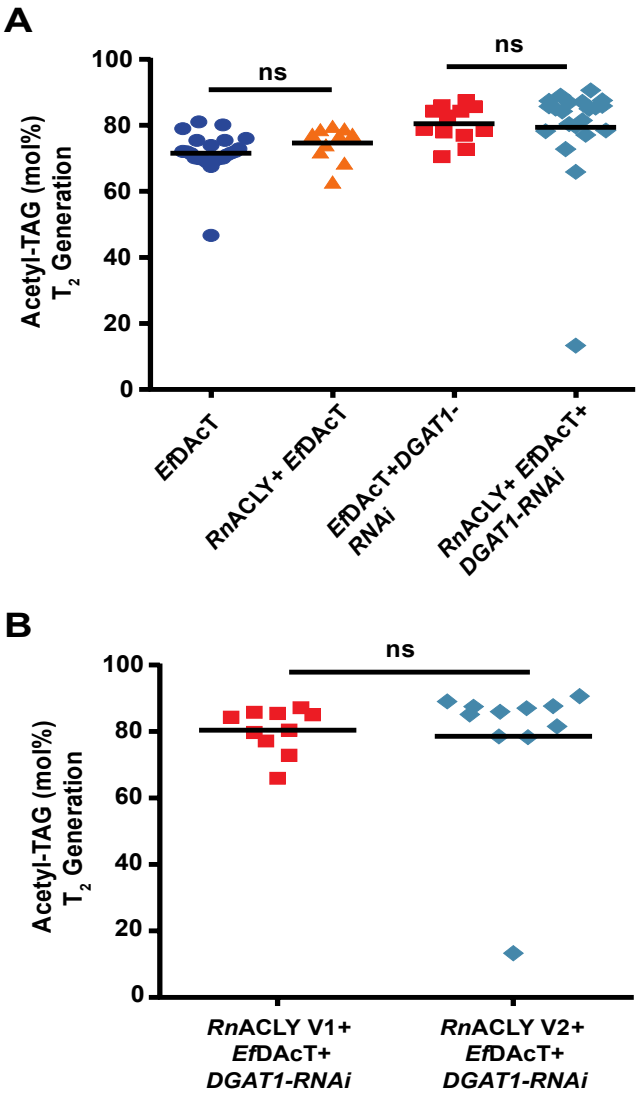

Figure 3.2 Acetyl-TAG levels of T_2 seed transgenic lines overexpressing *Rn*ACLY in combination with *Ef*DAcT or *Ef*DAcT +*DGAT1-RNAi*.

A. Scatter plot of acetyl-TAG content of T_2 seed from independent transgenic lines expressing E*f*DAcT alone, *Ef*DAcT with *Rn*ACLY expression, or in combination with the RNAi knockdown of DGAT1. Horizontal lines represent the mean acetyl-TAG content for each group. C. Scatter plot of acetyl-TAG levels in T_2 seed transgenic lines expressing E*f*DAcT+ *DGAT1-RNAi* with two different orientations of *Rn*ACLY in the binary vector. ns, not significant (Mann–Whitney U-test).

differ in the orientation of *Rn*ACLY driven by the glycinin promoter were used in this study (Figure 3.1). Both constructs expressed an RNAi hairpin loop that targets DGAT1 expression under the control of the oleosin promoter (Liu *et al.*, 2015a), and *Ef*DAcT-HA driven by the glycinin promoter (Figure 3.1). The two constructs were expressed in camelina and accumulated similar levels of acetyl-TAG in T_2 seeds when compared to each other (Figure 3.2B), suggesting that the orientation of the *Rn*ACLY expression cassette is not an important factor. Compared to *Ef*DAcT+*DGAT1-RNAi*, overexpression of *Rn*ACLY together with *Ef*DAcT expression and RNAi of DGAT1 led to a mean of 83 mol% of acetyl-TAG (Figure 3.2A). The highest producing transgenic line expressing *Rn*ACLY+*Ef*DAcT+*DGAT1-RNAi* accumulated 90 mol% of acetyl-TAG in T_2 seed. Cytosolic acetyl-CoA is a key precursor for different pathways that synthesize multiple metabolites in plants such as flavonoids, amino acids, lipids, elongated fatty acids, sugars, mevalonate-derived isoprenoids, cuticular waxes and sterols (Fatland *et al.*, 2002, 2005). The small increase in acetyl-TAG levels with *Rn*ACLY overexpression even with DGAT1 suppression suggests that acetyl-CoA levels might be preferably utilized by such pathways instead of *Ef*DAcT, although further work is needed to confirm this idea.

Analysis of the fatty acid profile in lines expressing *Rn*ACLY+*Ef*DAcT+*DGAT1-RNAi* showed the expected increase in PUFA 18:2 and 18:3 in acetyl-TAGs and VLCFA in lcTAGs (Figure 3.3B), consistent with previous results.

3.2.3 Homozygous expression of *Rn*ACLY+*Ef*DAcT+*DGAT1-RNAi* significantly increases acetyl-TAG accumulation in T_3 seed generation

In T_2 seed, the plants are hemizygous for the transgenic cassette. To analyze whether *Rn*ACLY overexpression would increase acetyl-TAG to significant levels in seeds from homozygous plants, a high acetyl-TAG producing line expressing *Ef*DAcT+*DGAT1-RNAi* or

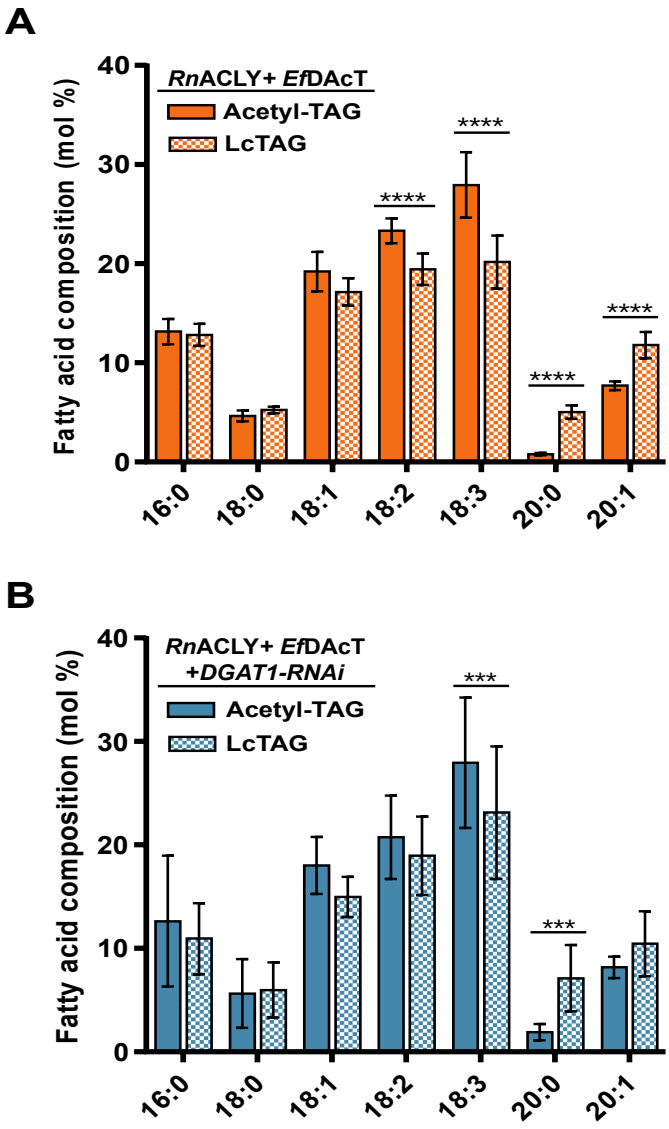

Figure 3.3 Fatty acid composition of T_2 seed transgenic lines overexpressing *Rn*ACLY in combination with *Ef*DAcT or *Ef*DAcT +*DGAT1-RNAi*.

Mean fatty acid composition of acetyl-TAG and lcTAG fractions of T_2 seed from multiple independent transgenic lines overexpressing *Rn*ACLY with *Ef*DAcT alone (A) or *Ef*DAcT+*DGAT1-RNAi* (B). Error bars represent SD. Asterisks indicate significant difference (***, $P \leq 0.001$; ****, $P \leq 0.0001$; Mann–Whitney U-test).

*Rn*ACLY+*Ef*DAcT+*DGAT1-RNAi* was propagated to produce T_3 seed. Homozygous T_3 seed (identified based on DsRed fluorescence) expressing *Rn*ACLY+*Ef*DAcT+*DGAT1-RNAi*

significantly increased the mean acetyl-TAG levels to 90 mol%, reaching up to 92 mol% in some plants (Figure 3.4A). These results suggest that for *Rn*ACLY expression, gene dosage is important. This is in contrast to what was observed with *Ef*DAcT expression (Chapter 2). To further investigate whether transgene dosage is important, acetyl-TAG levels in T_3 hemizygous seed were also analyzed. A slight increase in acetyl-TAG accumulation was observed with homozygous *Rn*ACLY+*Ef*DAcT+*DGAT1-RNAi* T_3 seed lines compared to the hemizygous lines (Figure 3.4B). In earlier work, acetyl-TAG accumulated to similar levels in *Ef*DAcT+*DGAT1-RNAi* implying that one copy of the transgene is sufficient (Chapter 2, Figure 2.2B). However, our data suggests that this might not be the case with *Rn*ACLY overexpression where a small increase of acetyl-TAG levels in homozygous lines is shown. To further confirm this observation, additional independent T_3 homozygous and hemizygous lines expressing either *Ef*DAcT+*DGAT1-RNAi* or *Rn*ACLY+*Ef*DAcT+*DGAT1-RNAi* need to be analyzed. To examine the stability of acetyl-TAG accumulation with *Rn*ACLY overexpression, a homozygous *Rn*ACLY+*Ef*DAcT+*DGAT1-RNAi* T_3 high acetyl-TAG producing line was further propagated to the next generation. In agreement with previous work (Chapter 2, Figure 2.S1B) acetyl-TAG levels remained stable in T_4 seed generation with a mean of 91 mol% acetyl-TAG (Figure 3.4C).

3.4 CONCLUSION

Earlier work revealed that high acetyl-TAG levels in transgenic camelina seeds can be achieved by the expression *Ef*DAcT, a high activity acetyltransferase, in combination with the RNAi-mediated suppression of DGAT1 (Chapter 2). It was hypothesized that overexpression of *Rn*ACLY to increase the acetyl-CoA pool for *Ef*DAcT acetylation of DAGs, can further enhance acetyl-TAG accumulation. The data presented here, shows that *Rn*ACLY overexpression along

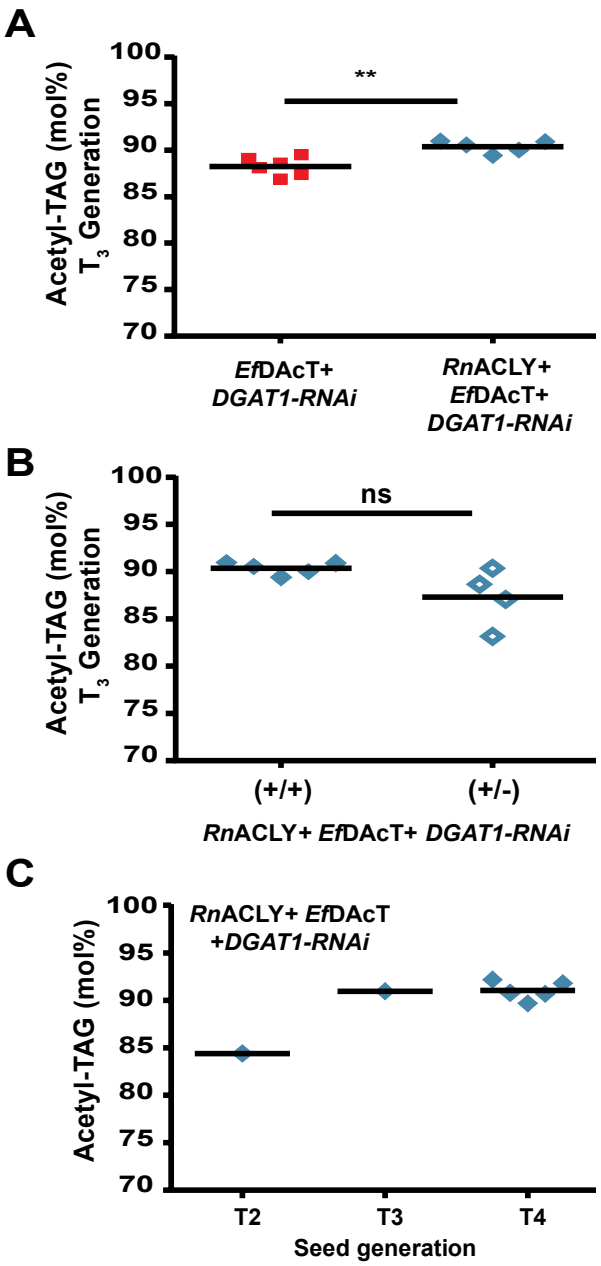

Figure 3.4 Acetyl-TAG levels of T_3 and T_4 transgenic lines overexpressing *Rn*ACLY in combination with *Ef*DAcT +*DGAT1-RNAi*.

A. Scatter plot of acetyl-TAG levels of T_3 seed from independent transgenic lines expressing E*f*DAcT+ *DGAT1-RNAi* or in combination with *Rn*ACLY overexpression. B. Scatter plot of acetyl-TAG levels of T_3 seed from independent homozygous (+/+) and hemizygous (+/-) transgenic lines expressing *Rn*ACLY+*Ef*DAcT+*DGAT1-RNAi*. C. Acetyl-TAG levels of homozygous high acetyl-TAG producing line expressing *Rn*ACLY + *Ef*DAcT+*DGAT1-RNAi* in T_2, T_3, and T_4 seed generations. Horizontal lines represent the mean acetyl-TAG content for each group. Asterisks indicate significant difference (**, $P \leq 0.01$; ns, not significant; Mann–Whitney U-test).

with *Ef*DAcT+*DGAT1-RNAi* accumulated an average of 90 mol% of acetyl-TAG in homozygous T_3 seed generation, the highest average of acetyl-TAG or any unusual lipids yet reported. The high levels of acetyl-TAG in camelina seeds remained relatively stable in the next generation. The impact of *Rn*ACLY overexpression on oil content, and other agronomically important seed properties such as germination remains to be analyzed. In addition, future studies will examine the expression of RnACLY protein and the levels of acetyl-CoA in transgenic seed to determine whether levels of the enzyme or acetyl-CoA are limiting acetyl-TAG accumulation.

3.3 MATERIAL AND METHODS

3.3.1 Plant material and growth conditions

Camelina sativa plants were grown under a 16 h light/8 h dark cycle, and a constant temperature of 21°C inside growth chambers. Harvested mature seeds were dried in a vacuum desiccator for multiple days before analysis.

3.3.2 Vector construction and generation of transgenic plant

The open reading frames *Rn*ACLY were amplified with the following primers: 5'-TGCGGCCGCATGTCAGCCAAGG-3' and 5'- CGCGGCCGCTTACATGCTCATGTGTTC-3' then cloned into the *Not*I site of vector pKMS3 (Nguyen *et al.*, 2013), inserting the gene in between the soybean glycinin promoter and terminator. The pGly::*Rn*ACLY::Glyterm cassette was then inserted into the binary vector E*f*DAcT-HA+*DGAT-RNAi* (Chapter 2). The fidelity of the different cloning steps was verified by sequencing all introduced DNA. The transformation constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 and transformed into wild-type *Camelina sativa* 'Suneson' via a floral dip vacuum infiltration method (Lu and Kang, 2008). Transgenic T_1 seed were identified by visual screening for DsRed fluorescence using a red lens

filter and a green LED light. Transgenic lines segregating for a single transgenic event were identified by counting the ratio of red to non-red fluorescent seeds in 100 T_2 seeds. Lines that passed the chi-square test for a 3:1 segregation ratio were used for further analysis and propagation.

3.3.3 Lipid extraction

Lipids were extracted from mature seeds following a hexane–isopropanol extraction method (Li *et al.*, 2006). Briefly for each sample, the dry weight of 15 seeds was determined before adding 2 ml of isopropanol and heating for 10 min at 85˚C. Samples were quickly homogenized with a PT2500E polytron (Kinemtica AGd). 3 ml of hexane, 50 µg of tripentadecanoin (C15:0) (NuChek Prep, New Elysian, MN) (internal standard), and 2.5 ml of 6.6% (w/v) Na_2SO4 were added. Samples were vortexed and centrifuged for 5 min at 2500 rpm. The upper organic phase was transferred to new pre-weighted labeled glass tube. To the lower aqueous phase, 2 ml of 7:2 Hexane: Isopropanol were added for re-extraction, samples were vortexed and centrifuged for 5 min at 2500 rpm. The new upper organic phase from the re-extraction was transferred and combined with the previous one. Samples were dried under nitrogen gas, and chloroform was added based on the lipids weight to reach a final concentration of 10 µg/µl. Lipids sample were stored at -20˚C until further analysis.

3.3.4 Thin layer chromatography

500 µg of lipid extracts were loaded on K6 Silica TLC plates and separated using a 70:30:1 hexane/ diethyl-ether/ acetic acid (v/v/v) solvent system for 30-45 min. TLC plates were sprayed with 0.2% (w/v) 2,7-dicholorfluorescein in 96% methanol and visualized under UV light to mark the acetyl-TAG and lcTAG bands. 30 µg of triheptadecanoin (C17:0) (NuChek Prep, New Elysian, MN) was spotted as an internal standard to each acetyl-TAG and lcTAG bands on the plate. Each

band was scraped, transferred to a labeled glass tube where 500 µl of toluene was added. Samples were either transmethylated directly or stored at -20˚C until the next day.

3.3.5 Transmethylation and gas chromatography

Samples were transmethylated using a previously established method (Li *et al.*, 2006). To each sample, 1 ml of 5% sulfuric acid in methanol mixture, and 25 ul of 0.2% (w/v) butylated hydroxytoluene were added. Samples were vortexed and heated for 45 min-1h at 90˚C and then cooled to room temperature before adding 1.5 ml of 0.9% (w/v) KCl and 2 ml of hexane. Samples were vortexed and centrifuged for 5 min at 2500 rpm, 25˚C. The upper organic phase was transferred to new labeled glass tubes and dried under nitrogen gas. 300 µl of hexane was added to the dry samples which were analyzed by gas chromatography (Shimadzu GC-2010 plus) equipped with a 30 X 0.25 mm DB-23 column (AgilentTechnologies) with a flame ionization detector. The GC conditions were: flame ionization detector temperature, 260˚C; oven temperature was maintained at 140˚C for 1 min, then increased to 250˚C at 10˚C min^{-1}, temperature was held for 4 min. Fatty acid methyl esters (FAME) peak areas were corrected for FID response and normalized to the internal standards (Li *et al.*, 2006). Acetyl-TAG and lcTAG levels were quantified as described previously (Liu *et al.*, 2015a).

Chapter 4 - Summary and Future Directions

4.1 SUMMARY

Acetyl-TAGs are synthesized in the seeds of plant species in the Celastraceae, Lardizabalaceae, Ranunculaceae, Rosaceae, and Balsaminaceae families. Acetyl-TAGs are unique in that they contain an acetate group at the *sn*-3 position instead of a long fatty acid as with regular TAGs (lcTAGs). The presence of the short acetyl group imparts acetyl-TAG oil with useful physical properties such low viscosity and low freezing point that can be used in variety of industrial applications such as biodegradable lubricants, emulsifiers, food coating agents, plasticizers and biodiesel 'drop-in' replacements (Durrett *et al.*, 2010; Liu *et al.*, 2015a, 2015b). To produce industrially relevant levels of acetyl-TAG in seed oil, the enzyme that synthesizes acetyl-TAG in the seed of *Euonymus alatu*s, a plant that belongs to the Celastraceae family, was previously isolated (Durrett *et al.*, 2010) and expressed in *Camelina sativa* seeds (Liu *et al.*, 2015a, 2015b). Acetyl-TAG was successfully produced at high levels in camelina seeds with *Ea*DAcT expression. These levels were further increased when DGAT1, the endogenous lcTAG synthesizing enzyme was knocked down by RNAi-mediated suppression (Liu *et al.*, 2015a).

The goal of this study was to further maximize acetyl-TAG production in camelina seeds. Multiple approaches were employed to obtain higher acetyl-TAG levels and to understand the bottlenecks acting on acetyl-TAG synthesis.

In Chapter 2, a high activity acetyltransferase enzyme isolated from *Euonymus fortunei* (*Ef*DAcT), which accumulated increased acetyl-TAG levels in yeast as compared to *Ea*DAcT (Tran *et al.*, 2017b), was expressed in camelina under the control of a strong seed specific promoter. *Ef*DAcT expression increased the average acetyl-TAG levels by 20 mol% compared to

*Ea*DAcT expression (52 mol%). Suppression of DGAT1 along with *Ef*DAcT expression enhanced acetyl-TAG accumulation to an average of 80 mol%, higher that that with *Ef*DAcT expression alone (72 mol%) or *Ea*DAcT+*DGAT1-RNAi* (68 mol%).

Multiple agronomically important seed properties were examined to ensure that no negative impact was associated with high acetyl-TAG accumulation. The data showed that acetyl-TAG levels in the highest producing transgenic lines expressing *Ef*DAcT or *Ef*DAcT +*DGAT1-RNAi* remained stable in the T_4 seed generation. Most of the transgenic lines exhibited a 100% seed germination rate with only a two-day delay compared to the wild-type. Minor increases in seed weight and seed size of transgenic lines expressing *Ef*DAcT +*DGAT1-RNAi* were observed which, possibly due to the increase in seed TAG levels. High acetyl-TAG transgenic lines exhibited slightly lower total fatty acids content compared to the wild-type.

In addition, *Ef*DAcT transcript, *Ef*DAcT protein, and acetyl-TAG levels were examined in the developing seeds to better understand the mechanism underlining acetyl-TAG accumulation in transgenic camelina. *Ef*DAcT protein showed a limited window of expression from 15 to 30 DAF, reaching a peak at 20 DAF and a barely detectable signal at 40 DAF. *Ef*DAcT mRNA transcript followed a similar pattern of expression. Acetyl-TAG accumulation, which was consistent across four independent transgenic lines, increased dramatically during seed development, concurrent with the stage that transcript and protein showed maximum expression. At 30 DAF, acetyl-TAG levels peaked and then gradually decreased at 40 DAF and in the mature seeds, correlating with the reduction in *Ef*DAcT mRNA transcript and protein at 30 DAF and in subsequent seed development stages.

The relationship between *Ef*DAcT protein levels and the final acetyl-TAG levels in mature seed was further examined. *Ef*DAcT protein was correlated with acetyl-TAG levels in transgenic

lines accumulating less than 75 mol% of acetyl-TAG. At higher levels of acetyl-TAG, the linear relationship disappears suggesting that *Ef*DAcT protein is not limiting. This data is consistent with the unexpected results showing that homozygous and hemizygous transgenic lines accumulated similar levels of acetyl-TAG. Given these results, it was hypothesized that substrates such as fatty acids, the glycerol backbone, or acetyl-CoA might limit acetyl-TAG accumulation.

In Chapter 3, overexpression of *Rn*ACLY was used to increase acetyl-CoA production and hence acetyl-TAG accumulation. The data shows that overexpression of *Rn*ACLY only slightly increased acetyl-TAG levels in T_2 seed lines expressing *Ef*DAcT or *Ef*DAcT+*DGAT1-RNAi*. However, analysis of acetyl-TAG levels in homozygous T_3 seed lines revealed a significant increase in acetyl-TAG accumulation with an average of 90 mol% in *Rn*ACLY+*Ef*DAcT+*DGAT1-RNAi* compared to *Ef*DAcT+*DGAT1-RNAi* alone. Analysis of acetyl-TAG levels in homozygous and hemizygous transgenic lines expressing *Rn*ACLY+*Ef*DAcT+*DGAT1-RNAi* showed a slight increase in acetyl-TAG accumulation in the homozygous lines. Furthermore, a high acetyl-TAG producing line expressing *Rn*ACLY+*Ef*DAcT+*DGAT1-RNAi* accumulated an average of 91 mol% acetyl-TAG in T_4 seed demonstrating the stability of acetyl-TAG levels over at least three generations.

4.2 FUTURE DIRECTIONS

Collectively, this study shows that increases in average acetyl-TAG levels in transgenic camelina were achieved. The average acetyl-TAG levels started with 68 mol% in *Ea*DAcT+*DGAT1-RNAi* and increased throughout the study by applying multiple synthetic biology approaches to 90 mol% with *Rn*ACLY+*Ef*DAcT+*DGAT1-RNAi* expression in homozygous transgenic line. The average accumulations reported here are the highest levels of

modified TAGs achieved in an engineered oilseed crop to date. Further maximization of acetyl-TAG levels will require better understanding of the pathways governing both acetyl-TAG and lcTAG synthesis and the roles of the enzymes involved in other stages of camelina growth and development. Based on the results generated in this study, future work will examine *Rn*ACLY+*Ef*DAcT+*DGAT1-RNAi* expressing lines in more detail. *Rn*ACLY protein expression, enzyme activity, and acetyl-CoA levels will be studied during seed development and in the final mature seeds through enzyme activity assay and western blotting, to investigate whether the enzyme or acetyl-CoA levels correlate to acetyl-TAG accumulation. If *Rn*ACLY expression reflects that of *Ef*DAcT accumulation with limited window of expression from 15 DAF to 30 DAF, multiple seed specific promoters will be used to extend the expression of the proteins to late seed development. Fatty acid content will be measured to determine whether the reduction in fatty acid levels observed with *Ef*DAcT+*DGAT1-RNAi* expression is compensated for by increasing acetyl-CoA availability. Other strategies such as overexpression of the transcription factor WRINKLED1 (WRI1), which regulates the expression of enzymes involved in fatty acid synthesis pathways, may be used to further increase fatty acids content in the transgenic lines. Seed germination rate, yield, size and weight will also be analyzed to test whether expression of *Rn*ACLY+*Ef*DAcT+*DGAT1-RNAi* is not negatively affecting camelina seed properties.

References

Abdullah, H.M., Akbari, P., Paulose, B., Schnell, D., Qi, W., Park, Y., Pareek, A. and Dhankher, O.P. (2016) Transcriptome profiling of Camelina sativa to identify genes involved in triacylglycerol biosynthesis and accumulation in the developing seeds. *Biotechnology for Biofuels*. **9**, 136.

Aznar-Moreno, J.A. and Durrett, T.P. (2017) Simultaneous Targeting of Multiple Gene Homeologs to Alter Seed Oil Production in Camelina sativa. *Plant and Cell Physiology*. **58**, 1260–1267.

Bafor, M., Smith, M.A., Jonsson, L., Stobart, K. and Stymne, S. (1991) Ricinoleic acid biosynthesis and triacylglycerol assembly in microsomal preparations from developing castor-bean (Ricinus communis) endosperm. *Biochemistry Journal*. **280**, 507–514.

Bansal, S. and Durrett, T.P. (2016a) Camelina sativa: An ideal platform for the metabolic engineering and field production of industrial lipids. *Biochimie*. **120**, 9–16.

Bansal, S. and Durrett, T.P. (2016b) Defining the extreme substrate specificity of Euonymus alatus diacylglycerol acetyltransferase, an unusual membrane-bound O-acyltransferase. *Bioscience Reports*. **36**, e00406.

Bansal, S., Kim, H.J., Na, G., Hamilton, M.E., Cahoon, E.B., Lu, C. and Durrett, T.P. (2018) Towards the synthetic design of camelina oil enriched in tailored acetyl-triacylglycerols with medium-chain fatty acids. *Journal of experimental botany*. **69**, 4395–4402.

Bates, P.D. and Browse, J. (2011) The pathway of triacylglycerol synthesis through phosphatidylcholine in Arabidopsis produces a bottleneck for the accumulation of unusual fatty acids in transgenic seeds. *The Plant Journal*. **68**, 387–399.

Bates, P.D. and Browse, J. (2012) The significance of different diacylgycerol synthesis pathways on plant oil composition and bioengineering. *Frontiers in Plant Science*. **3**.

Bates, P.D., Stymne, S. and Ohlrogge, J. (2013) Biochemical pathways in seed oil synthesis. *Current Opinion in Plant Biology*. **16**, 358–364.

Berg, J., Tymoczko, J. and Stryer, L. (2002) Fatty Acids Are Key Constituents of Lipids. In:

Biochemistry. 5th edition. New York, NY, W H Freeman. p.

Berti, M., Gesch, R., Eynck, C., Anderson, J. and Cermak, S. (2016) Camelina uses, genetics, genomics, production, and management. *Industrial Crops and Products*. **94**, 690–710.

Berti, M., Wilckens, R., Fischer, S., Solis, A. and Johnson, B. (2011) Seeding date influence on camelina seed yield, yield components, and oil content in Chile. *Industrial Crops and Products*. **34**, 1358–1365.

Blackshaw, R., Johnson, E., Gan, Y., May, W., McAndrew, D., Barthet, V., McDonald, T. and Wispinski, D. (2011) Alternative oilseed crops for biodiesel feedstock on the Canadian prairies. *Canadian Journal of Plant Science*. **91**, 889–896.

Budin, J.T., Breene, W.M. and Putnam, D.H. (1995) Some compositional properties of camelina (Camelina sativa L. Crantz) seeds and oils. *Journal of the American Oil Chemists' Society*. **72**, 309–315.

Cao, H., Shockey, J.M., Klasson, K.T., Chapital, D.C., Mason, C.B. and Scheffler, B.E. (2013) Developmental Regulation of Diacylglycerol Acyltransferase Family Gene Expression in Tung Tree Tissues. *PLOS ONE*. **8**, e76946.

Carlsson, A.S., Zhu, L.-H., Andersson, M. and Hofvander, P. (2014) Platform crops amenable to genetic engineering – a requirement for successful production of bio-industrial oils through genetic engineering. *Biocatalysis and Agricultural Biotechnology*. **3**, 58–64.

Chang, S., Puryear, J. and Cairney, J. (1993) A simple and efficient method for isolating RNA from pine trees. *Plant Molecular Biology Reporter*. **11**, 113–116.

Chen, C., Bekkerman, A., Afshar, R.K. and Neill, K. (2015) Intensification of dryland cropping systems for bio-feedstock production: Evaluation of agronomic and economic benefits of Camelina sativa. *Industrial Crops and Products*. **71**, 114–121.

Chen, G.Q., Lin, J.-T. and Lu, C. (2011) Hydroxy fatty acid synthesis and lipid gene expression during seed development in Lesquerella fendleri. *Industrial Crops and Products*. **34**, 1286–1292.

Chen, G.Q., Turner, C., He, X., Nguyen, T., McKeon, T.A. and Laudencia-Chingcuanco, D. (2007)

Expression Profiles of Genes Involved in Fatty Acid and Triacylglycerol Synthesis in Castor Bean (Ricinus communis L.). *Lipids*. **42**, 263–274.

Davidson, B.C. and Cantrill, R.C. (1985) Fatty acid nomenclature. *SAMJ*. **67**, 20.

Dehesh, K., Jones, A., Knutzon, D.S. and Voelker, T.A. (1996) Production of high levels of 8:0 and 10:0 fatty acids in transgenic canola by overexpression of Ch FatB2, a thioesterase cDNA from Cuphea hookeriana. *Plant Journal*. **9**, 167–172.

DellaPenna, D. (2001) Plant metabolic engineering. *Plant Physiology*. **125**, 160–163.

Durrett, T.P., Benning, C. and Ohlrogge, J. (2008) Plant triacylglycerols as feedstocks for the production of biofuels. *Plant Journal*. **54**, 593–607.

Durrett, T.P., McClosky, D.D., Tumaney, A.W., Elzinga, D.A., Ohlrogge, J. and Pollard, M. (2010) A distinct DGAT with sn-3 acetyltransferase activity that synthesizes unusual, reduced-viscosity oils in Euonymus and transgenic seeds. *Proceedings of the National Academy of Sciences of the United States of America*. **107**, 9464–9469.

Dyer, J.M., Stymne, S., Green, A.G. and Carlsson, A.S. (2008) High-value oils from plants. *The Plant Journal*. **54**, 640–655.

Elshourbagy, N.A., Near, J.C., Kmetz, P.J., Sathe, G.M., Southan, C., Strickler, J.E., Gross, M., Young, J.F., Wells, T.N. and Groot, P.H. (1990) Rat ATP citrate-lyase. Molecular cloning and sequence analysis of a full-length cDNA and mRNA abundance as a function of diet, organ, and age. *Journal of Biological Chemistry*. **265**, 1430–1435.

van Erp, H., Shockey, J., Zhang, M., Adhikari, N.D. and Browse, J. (2015) Reducing Isozyme Competition Increases Target Fatty Acid Accumulation in Seed Triacylglycerols of Transgenic Arabidopsis. *Plant Physiology*. **168**, 36–46.

Fatland, B.L., Ke, J., Anderson, M.D., Mentzen, W.I., Cui, L.W., Allred, C.C., Johnston, J.L., Nikolau, B.J. and Wurtele, E.S. (2002) Molecular characterization of a heteromeric ATP-citrate lyase that generates cytosolic acetyl-coenzyme A in Arabidopsis. *Plant physiology*. **130**, 740–756.

Fatland, B.L., Nikolau, B.J. and Wurtele, E.S. (2005) Reverse Genetic Characterization of Cytosolic

Acetyl-CoA Generation by ATP-Citrate Lyase in Arabidopsis. *The Plant Cell*. **17**, 182 LP-203.

Gaupp, R. and Adams, W. (2004) Acid esters of mono- and diglycerides. In: R J Whitehurst (ed.). *Emulsifiers in Food Technology*. Oxford, UK, Blackwell Publishing. pp. 59–68.

Gesch, R.W. and Archer, D.W. (2013) Double-cropping with winter camelina in the northern Corn Belt to produce fuel and food. *Industrial Crops and Products*. **44**, 718–725.

Gesch, R.W., Archer, D.W. and Berti, M.T. (2014) Dual Cropping Winter Camelina with Soybean in the Northern Corn Belt. *Agronomy Journal*. **106**, 1735–1745.

Graham, I.A. (2008) Seed Storage Oil Mobilization. *Annual Review of Plant Biology*. **59**, 115–142.

Graham, S.A. (1989) Cuphea: A new plant source of medium-chain fatty acids. *Critical Reviews in Food Science and Nutrition*. **28**, 139–173.

Gugel, R.K. and Falk, K.C. (2006) Agronomic and seed quality evaluation of Camelina sativa in western Canada. *Canadian Journal of Plant Science*. **86**, 1047–1058.

Haslam, R.P., Sayanova, O., Kim, H.J., Cahoon, E.B. and Napier, J.A. (2016) Synthetic redesign of plant lipid metabolism. *The Plant Journal*. **87**, 76–86.

Hayes, D.G. and Kleiman, R. (1996) A detailed triglyceride analysis ofLesquerella fendleri oil: Column chromatographic fractionation followed by supercritical fluid chromatography. *Journal of the American Oil Chemists' Society*. **73**, 267–269.

Herridge, R.P., Day, R.C., Baldwin, S. and Macknight, R.C. (2011) Rapid analysis of seed size in Arabidopsis for mutant and QTL discovery. *Plant Methods*. **7**, 1–11.

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

Hu, Z., Wu, Q., Dalal, J., Vasani, N., Lopez, H.O., Sederoff, H.W. and Qu, R. (2017) Accumulation of medium-chain, saturated fatty acyl moieties in seed oils of transgenic Camelina sativa. *PLOS ONE*. **12**, e0172296.

Hutcheon, C., Ditt, R., Beilstein, M., Comai, L., Schroeder, J., Goldstein, E., Shewmaker, C., Nguyen, T., De Rocher, J. and Kiser, J. (2010) Polyploid genome of Camelina sativa revealed by

isolation of fatty acid synthesis genes. *BMC Plant Biology*. **10**, 233.

Iskandarov, U., Kim, H.J. and Cahoon, E.B. (2014) Camelina: An Emerging Oilseed Platform for Advanced Biofuels and Bio-Based Materials. In: Maureen C McCann, Marcos S Buckeridge, & Nicholas C Carpita (eds.). *Plants and BioEnergy*. New York, NY, Springer New York. pp. 131–140.

Iven, T., Hornung, E., Heilmann, M. and Feussner, I. (2016) Synthesis of oleyl oleate wax esters in Arabidopsis thaliana and Camelina sativa seed oil. *Plant Biotechnology Journal*. **14**, 252–259.

Jaworski, J. and Cahoon, E.B. (2003) Industrial oils from transgenic plants. *Current Opinion in Plant Biology*. **6**, 178–184.

Jenderek, M.M., Dierig, D.A. and Isbell, T.A. (2009) Fatty-acid profile of Lesquerella germplasm in the National Plant Germplasm System collection. *Industrial Crops and Products*. **29**, 154–164.

Kagale, S., Koh, C., Nixon, J., Bollina, V., Clarke, W.E., Tuteja, R., Spillane, C., Robinson, S.J., Links, M.G., Clarke, C., Higgins, E.E., Huebert, T., Sharpe, A.G. and Parkin, I.A.P. (2014) The emerging biofuel crop Camelina sativa retains a highly undifferentiated hexaploid genome structure. *Nature Communications*. **5**, 3706.

Kim, H.J., Silva, J.E., Vu, H.S., Mockaitis, K., Nam, J.-W. and Cahoon, E.B. (2015) Toward production of jet fuel functionality in oilseeds: identification of FatB acyl-acyl carrier protein thioesterases and evaluation of combinatorial expression strategies in Camelina seeds. *Journal of Experimental Botany*. **66**, 4251–4265.

Kleiman, R., Miller, R.W., Earle, F.R. and Wolff, I.A. (1967) (S)-1,2-diacyl-3-acetins: Optically active triglycerides from Euonymus verrucosus seed oil. *Lipids*. **2**, 473–478.

Lardizabal, K.D., Metz, J.G., Sakamoto, T., Hutton, W.C., Pollard, M.R. and Lassner, M.W. (2000) Purification of a Jojoba Embryo Wax Synthase, Cloning of its cDNA, and Production of High Levels of Wax in Seeds of Transgenic Arabidopsis. *Plant Physiology*. **122**, 645–656.

Li-Beisson, Y., Shorrosh, B., Beisson, F., Andersson, M.X., … Ohlrogge, J. (2010) Acyl-Lipid Metabolism. *The Arabidopsis Book*. e0133.

Li, N., Gügel, I.L., Giavalisco, P., Zeisler, V., Schreiber, L., Soll, J. and Philippar, K. (2015) FAX1, a

novel membrane protein mediating plastid fatty acid export. *PLoS biology*. **13**, e1002053.

Li, X., Teitgen, A.M., Shirani, A., Ling, J., Busta, L., Cahoon, R.E., Zhang, W., Li, Z., Chapman, K.D., Berman, D., Zhang, C., Minto, R.E. and Cahoon, E.B. (2018) Discontinuous fatty acid elongation yields hydroxylated seed oil with improved function. *Nature Plants*. **4**, 711–720.

Li, Y., Beisson, F., Pollard, M. and Ohlrogge, J. (2006) Oil content of Arabidopsis seeds: the influence of seed anatomy, light and plant-to-plant variation. *Phytochemistry*. **67**, 904–915.

Litchfield, C., Miller, E., Harlow, R.D. and Reiser, R. (1967) The triglyceride composition of 17 seed fats rich in octanoic, decanoic, or lauric acid. *Lipids*. **2**, 345–350.

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

Liu, J., Tjellström, H., McGlew, K., Shaw, V., Rice, A., Simpson, J., Kosma, D., Ma, W., Yang, W., Strawsine, M., Cahoon, E., Durrett, T.P. and Ohlrogge, J. (2015b) Field production, purification and analysis of high-oleic acetyl-triacylglycerols from transgenic Camelina sativa. *Industrial Crops and Products*. **65**, 259–268.

van de Loo, F.J., Broun, P., Turner, S. and Somerville, C. (1995) An oleate 12-hydroxylase from Ricinus communis L. is a fatty acyl desaturase homolog. *Proceedings of the National Academy of Sciences*. **92**, 6743–6747.

Lou, Y., Schwender, J. and Shanklin, J. (2014) FAD2 and FAD3 desaturases form heterodimers that facilitate metabolic channeling in vivo. *The Journal of biological chemistry*. **289**, 17996–18007.

Lu, C. and Hills, M.J. (2002) Arabidopsis mutants deficient in diacylglycerol acyltransferase display increased sensitivity to abscisic acid, sugars, and osmotic stress during germination and seedling development. *Plant physiology*. **129**, 1352–1358.

Lu, C. and Kang, J. (2008) Generation of transgenic plants of a potential oilseed crop Camelina sativa by Agrobacterium-mediated transformation. *Plant Cell Reports*. **27**, 273–278.

Lu, C., Napier, J.A., Clemente, T.E. and Cahoon, E.B. (2011) New frontiers in oilseed biotechnology: meeting the global demand for vegetable oils for food, feed, biofuel, and industrial applications. *Current Opinion in Biotechnology*. **22**, 252–259.

Masella, P., Martinelli, T. and Galasso, I. (2014) Agronomic evaluation and phenotypic plasticity of *Camelina sativa* growing in Lombardia, Italy. *Crop and Pasture Science*. **65**, 453–460.

Metz, J.G., Pollard, M.R., Anderson, L., Hayes, T.R. and Lassner, M.W. (2000) Purification of a jojoba embryo fatty acyl-coenzyme A reductase and expression of its cDNA in high erucic acid rapeseed. *Plant physiology*. **122**, 635–644.

Millar, A.A. and Kunst, L. (1997) Very-long-chain fatty acid biosynthesis is controlled through the expression and specificity of the condensing enzyme. *The Plant Journal*. **12**, 121–131.

Moser, B.R. (2010) Camelina (Camelina sativa L.) oil as a biofuels feedstock: Golden opportunity or false hope? *Lipid Technology*. **22**, 270–273.

Napier, J.A., Haslam, R.P., Beaudoin, F. and Cahoon, E.B. (2014) Understanding and manipulating plant lipid composition: Metabolic engineering leads the way. *Current opinion in plant biology*. **19**, 68–75.

Nguyen, H.T., Silva, J.E., Podicheti, R., Macrander, J., Yang, W., Nazarenus, T.J., Nam, J.-W., Jaworski, J.G., Lu, C., Scheffler, B.E., Mockaitis, K. and Cahoon, E.B. (2013) Camelina seed transcriptome: a tool for meal and oil improvement and translational research. *Plant Biotechnology Journal*. **11**, 759–769.

Nichols, P.D., Guckert, J.B. and White, D.C. (1986) Determination of monounsaturated fatty acid double-bond position and geometry for microbial monocultures and complex consortia by capillary GC-MS of their dimethyl disulphide adducts. *Journal of Microbiological Methods*. **5**, 49–55.

Obour, A.K., Obeng, E., Mohammed, Y., Ciampitti, I., Durrett, T.P., Aznar Moreno, J.A. and Chen, C. (2017) Camelina seed yield and fatty acids as influenced by genotype and environment. *Agronomy Journal*.

Ohlrogge, J. and Browse, J. (1995) Lipid biosynthesis. *Plant Cell*. **7**, 957–970.

Ohlrogge, J.B. (1994) Design of new plant products: engineering of fatty acid metabolism. *Plant physiology*. **104**, 821.

Ohlrogge, J.B., Pollard, M.R. and Stumpf, P.K. (1978) Studies on biosynthesis of waxes by developing jojoba seed tissue. *Lipids*. **13**, 203–210.

Okuley, J., Lightner, J., Feldmann, K., Yadav, N., Lark, E. and Browse, J. (1994) Arabidopsis FAD2 gene encodes the enzyme that is essential for polyunsaturated lipid synthesis. *The Plant Cell*. **6**, 147–158.

Pollard, M., Martin, T.M. and Shachar-Hill, Y. (2015) Lipid analysis of developing Camelina sativa seeds and cultured embryos. *Phytochemistry*. **118**, 23–32.

Pollard, M.R., Anderson, L., Fan, C., Hawkins, D.J. and Davies, H.M. (1991) A Specific Acyl-ACP Thioesterase Implicated in Medium-Chain Fatty-Acid Production in Immature Cotyledons of Umbellularia californica. *Archives of Biochemistry and Biophysics*. **284**, 306–312.

Pollard, M.R., McKeon, T., Gupta, L.M. and Stumpf, P.K. (1979) Studies on biosynthesis of waxes by developing jojoba seed. II. the demonstration of wax biosynthesis by cell-free homogenates. *Lipids*. **14**, 651–662.

Pollard, M.R. and Stumpf, P.K. (1980) Biosynthesis of C(20) and C(22) Fatty Acids by Developing Seeds of Limnanthes alba: CHAIN ELONGATION AND Delta5 DESATURATION. *Plant physiology*. **66**, 649–655.

Ponnala, L., Wang, Y., Sun, Q. and Wijk, K.J. (2014) Correlation of mRNA and protein abundance in the developing maize leaf. *The Plant Journal*. **78**, 424–440.

Pouvreau, B., Vanhercke, T. and Singh, S. (2018) From plant metabolic engineering to plant synthetic biology: The evolution of the design/build/test/learn cycle. *Plant Science*. **273**, 3–12.

Ramos, M.J., Fernández, C.M., Casas, A., Rodríguez, L. and Pérez, Á. (2009) Influence of fatty acid composition of raw materials on biodiesel properties. *Bioresource Technology*. **100**, 261–268.

Rangasamy, D. and Ratledge, C. (2000) Genetic Enhancement of Fatty Acid Synthesis by Targeting Rat Liver ATP:Citrate Lyase into Plastids of Tobacco. *Plant Physiology*. **122**, 1231–1238.

Ratledge, C., Bowater, M.D. V and Taylor, P.N. (1997) Correlation of ATP/citrate lyase activity with lipid accumulation in developing seeds of Brassica napus L. *Lipids*. **32**, 7–12.

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

Ruiz-Lopez, N., Broughton, R., Usher, S., Salas, J.J., Haslam, R.P., Napier, J.A. and Beaudoin, F. (2016) Tailoring the composition of novel wax esters in the seeds of transgenic Camelina sativa through systematic metabolic engineering. *Plant Biotechnology Journal*. **15**, 837–849.

Rustan, A.C. and Drevon, C.A. (2005) Fatty Acids: Structures and Properties. *eLS*.

Ruuska, S.A., Girke, T., Benning, C. and Ohlrogge, J.B. (2002) Contrapuntal networks of gene expression during Arabidopsis seed filling. *Plant Cell*. **14**, 1191–1206.

Salas, J.J. and Ohlrogge, J.B. (2002) Characterization of substrate specificity of plant FatA and FatB acyl-ACP thioesterases. *Archives of Biochemistry and Biophysics*. **403**, 25–34.

Schubert, D., Lechtenberg, B., Forsbach, A., Gils, M., Bahadur, S. and Schmidt, R. (2004) Silencing in Arabidopsis T-DNA Transformants: The Predominant Role of a Gene-Specific RNA Sensing Mechanism versus Position Effects. *The Plant Cell*. **16**, 2561 LP-2572.

Schwender, J. and Ohlrogge, J.B. (2002) Probing in vivo metabolism by stable isotope labeling of storage lipids and proteins in developing Brassica napus embryos. *Plant physiology*. **130**, 347–361.

Séguin-Swartz, G., Eynck, C., Gugel, R.K., Strelkov, S.E., Olivier, C.Y., Li, J.L., Klein-Gebbinck, H., Borhan, H., Caldwell, C.D. and Falk, K.C. (2009) Diseases of Camelina sativa (false flax). *Canadian Journal of Plant Pathology*. **31**, 375–386.

Sidorov, R.A., Zhukov, A. V, Pchelkin, V.P., Vereshchagin, A.G. and Tsydendambaev, V.D. (2014) Content and fatty acid composition of neutral acylglycerols in Euonymus fruits. *Journal of the American Oil Chemists' Society*. **91**, 805–814.

Snapp, A. and Lu, C. (2013) Engineering industrial fatty acids in oilseeds. *Frontiers in Biology*. **8**, 323–332.

Snapp, A.R., Kang, J., Qi, X. and Lu, C. (2014) A fatty acid condensing enzyme from Physaria fendleri increases hydroxy fatty acid accumulation in transgenic oilseeds of Camelina sativa. *Planta*. **240**, 599–610.

Soroka, J., Olivier, C., Grenkow, L. and Séguin-Swartz, G. (2015) Interactions between Camelina sativa (Brassicaceae) and insect pests of canola. *The Canadian Entomologist*. **147**, 193–214.

Sun, W., Bernard, C., Van De Cotte, B., Van Montagu, M. and Verbruggen, N. (2001) At-HSP17.6A, encoding a small heat-shock protein in Arabidopsis, can enhance osmotolerance upon overexpression. *The Plant Journal*. **27**, 407–415.

Takagi, T. and Ando, Y. (1991) Stereospecific analysis of triacyl-sn-glycerols by chiral high-performance liquid chromatography. *Lipids*. **26**, 542–547.

Taylor, D., Giblin, E., Reed, D. and Hogge, L. (1995) Stereospecific analysis and mass spectrometry of triacylglycerols from Arabidopsis thaliana; (L.) Heynh. Columbia seed. *Journal of the American Oil Chemists' Society*. **72**, 305–308.

Thelen, J.J. and Ohlrogge, J.B. (2002) Metabolic engineering of fatty acid biosynthesis in plants. *Metabolic Engineering*. **4**, 12–21.

Tran, T.N.T., Breuer, R.J., Avanasi Narasimhan, R., Parreiras, L.S., Zhang, Y., Sato, T.K. and Durrett, T.P. (2017a) Metabolic engineering of Saccharomyces cerevisiae to produce a reduced viscosity oil from lignocellulose. *Biotechnology for Biofuels*. **10**, 69.

Tran, T.N.T., Shelton, J., Brown, S. and Durrett, T.P. (2017b) Membrane topology and identification of key residues of EaDAcT, a plant MBOAT with unusual substrate specificity. *The Plant Journal*. **92**, 82–94.

Voelker, T. and Kinney, A.J. (2001) Variations in the Biosynthesis of Seed-Storage Lipids. *Annu Rev Plant Physiol Plant Mol Biol*. **52**, 335–361.

Voelker, T.A., Hayes, T.R., Cranmer, A.M., Turner, J.C. and Davies, H.M. (1996) Genetic engineering of a quantitative trait: metabolic and genetic parameters influencing the accumulation of laurate in rapeseed. *The Plant Journal*. **9**, 229–241.

Voelker, T.A., Worrell, A.C., Anderson, L., Bleibaum, J., Fan, C., Hawkins, D.J., Radke, S.E. and Davies, H.M. (1992) Fatty acid biosynthesis redirected to medium chains in transgenic oilseed plants. *Science*. **257**, 72–74.

Vollmann, J. and Eynck, C. (2015) Camelina as a sustainable oilseed crop: Contributions of plant breeding and genetic engineering. *Biotechnology Journal*. **10**, 525–535.

Vollmann, J., Moritz, T., Kargl, C., Baumgartner, S. and Wagentristl, H. (2007) Agronomic evaluation of camelina genotypes selected for seed quality characteristics. *Industrial Crops and Products*. **26**, 270–277.

Walsh, K.D., Puttick, D.M., Hills, M.J., Yang, R.-C., Topinka, K.C. and Hall, L.M. (2012) Short Communication: First report of outcrossing rates in camelina [Camelina sativa (L.) Crantz], a potential platform for bioindustrial oils. *Canadian Journal of Plant Science*. **92**, 681–685.

Walsh, T.A., Bevan, S.A., Gachotte, D.J., Larsen, C.M., … Metz, J.G. (2016) Canola engineered with a microalgal polyketide synthase-like system produces oil enriched in docosahexaenoic acid. *Nature Biotechnology*. **34**, 881–887.

Winer, J., Jung, C.K.S., Shackel, I. and Williams, P.M. (1999) Development and Validation of Real-Time Quantitative Reverse Transcriptase–Polymerase Chain Reaction for Monitoring Gene Expression in Cardiac Myocytesin Vitro. *Analytical Biochemistry*. **270**, 41–49.

Wu, X.-Y., Moreau, R.A. and Stumpf, P.K. (1981) Studies of biosynthesis of waxes by developing jojoba seed: III. Biosynthesis of wax esters from Acyl-CoA and long chain alcohols. *Lipids*. **16**, 897–902.

Xu, J., Francis, T., Mietkiewska, E., Giblin, E.M., Barton, D.L., Zhang, Y., Zhang, M. and Taylor, D.C. (2008) Cloning and characterization of an acyl-CoA-dependent diacylglycerol acyltransferase 1 (DGAT1) gene from Tropaeolum majus, and a study of the functional motifs of the DGAT protein using site-directed mutagenesis to modify enzyme activity and oil content. *Plant Biotechnol J*. **6**, 799–818.

Yang, Y. and Benning, C. (2018) Functions of triacylglycerols during plant development and stress.

Current Opinion in Biotechnology. **49**, 191–198.

Zhang, M., Fan, J., Taylor, D.C. and Ohlrogge, J.B. (2009) DGAT1 and PDAT1 Acyltransferases Have Overlapping Functions in Arabidopsis Triacylglycerol Biosynthesis and Are Essential for Normal Pollen and Seed Development. *Plant Cell*. **21**, 3885–3901.

Zhu, L.-H., Krens, F., Smith, M.A., Li, X., … Cahoon, E.B. (2016) Dedicated Industrial Oilseed Crops as Metabolic Engineering Platforms for Sustainable Industrial Feedstock Production. *Scientific Reports*. **6**, 22181.

Zubr, J. (2010) Carbohydrates, vitamins and minerals of Camelina sativa seed. *Nutrition & Food Science*. **40**, 523–531.

Zubr, J. (1997) Oil-seed crop: Camelina sativa. *Industrial Crops and Products*. **6**, 113–119.

Zubr, J. (2003) Qualitative variation of Camelina sativa seed from different locations. *Industrial Crops and Products*. **17**, 161–169.