Remodeling of triacylglycerol synthesis in emerging oilseed crops: genetic engineering for high-value seed oil

by

Linah M. Alkotami

B.S., Kansas State University, 2016
M.S., Kansas State University, 2018

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submitted in partial fulfillment of the requirements for the degree

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Department of Biochemistry and Molecular Biophysics
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Abstract

The utilization of vegetable oils, which predominantly consist of triacylglycerols (TAG), for diverse industrial purposes is hindered by many challenges due to their unfavorable physical properties. To overcome this constraint, metabolic engineering studies have focused on modifying plant seeds to produce oil with improved physical properties in non-food crops. However, altering oil composition to generate novel TAG molecules has proven to be challenging due to pathway bottlenecks and their potential impact on plant growth and seed viability. One unique class of TAG, known as acetyl-1,2-diacyl-*sn*-glycerols (acetyl-TAG), naturally constitutes more than 90% of the seed oil in *Euonymus* species. Acetyl-TAG is characterized by a distinct structure in which an *sn*-3 acetate replaces the long acyl group found in regular TAG. Consequently, acetyl-TAG oil exhibits lower viscosity, making it advantageous for applications as an emulsifiers, lubricants, or "drop-in" biofuel. Previous attempts to produce acetyl-TAG in oilseed crops such as soybean (*Glycine max*), *Camelina sativa*, and pennycress (*Thlaspi arvense*) have only achieved an average of 45-80% acetyl-TAG. The primary objective of this study is to engineer acetyl-TAG production in oilseed crops to match the highest naturally occurring levels in *Euonymus* seeds. Furthermore, the study aims to investigate the impact of acetyl-TAG on plant growth, seed properties, seed lipids, and global gene expression. The non-food crops camelina and pennycress were chosen due to their ease of transformation using an Agrobacterium-mediated floral-dip method. To achieve the desired outcome, multiple strategies were employed. Initially, an acetyltransferase enzyme (EfDAcT) from *Euonymus fortunei* seeds, known for its higher activity compared to the previously used *Euonymus alatus* enzyme (EaDAcT), was introduced. Subsequently, the endogenous TAG-synthesizing enzyme DGAT1, which competes with EfDAcT for the diacylglycerol (DAG) substrate, was inhibited. Lastly, *EfDAcT* was expressed in FATTY ACID ELONGASE1 (FAE1)

mutant lines to enhance acetyl-CoA availability for acetyl-TAG synthesis while eliminating very long-chain fatty acids (VLCFA), which are unfavorable substrates for EfDAcT. Using these strategies, transgenic plants capable of producing 98% acetyl-TAG in pennycress seeds and 93% acetyl-TAG in camelina seeds were generated. Compared to wild-type seeds, transgenic seeds exhibited higher accumulation of TAG and polar lipids, while maintaining comparable or slightly lower levels of total fatty acid content. Seeds that produced high levels of acetyl-TAG showed a slight delay in germination with no significant impact on plant growth. Global transcript levels in camelina transgenic seeds indicated changes in gene expression, including those involved in flavonoid biosynthesis and hormone response. Overall, this study demonstrates the feasibility of achieving nearly complete alteration of the type of oil synthesized by a plant and offer valuable insights into the genetic and metabolic mechanisms underlying lipid biosynthesis.

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Approved by:

Major Professor
Timothy Durrett

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Dedication

I dedicate this dissertation to my parents for their unconditional love and support.

List of Abbreviations

ABA - Abscisic acid
ACCase - Plastid acetyl-CoA carboxylase
Acetyl-TAG - Acetyl-triacylglycerol
ACL - ATP-citrate lyase
ACP - Acyl carrier protein
CRISPR - Clustered regularly interspaced short palindromic repeats
Cas9 - CRISPR-associated protein 9
DAG - Diacylglycerol
DAcTs - Diacylglycerol-acetyl-CoA acetyltransferases
DGAT1 - Diacylglycerol acyltransferase 1
DGDG - Digalactosyldiacylglycerol
DOG1 - Delay of germination 1
DAF - Days after flowering
EaDAcT - *Euonymus alatus* diacylglycerol acetyltransferase
EfDAcT - *Euonymus fortunei* diacylglycerol acetyltransferase
ER - Endoplasmic reticulum
ESI-MS - Electrospray ionization mass spectrometry
FAD - Fatty acid desaturase
FAE1 - Fatty acid elongase 1
FAMEs - Fatty acid methyl esters
FAs - Fatty acids
FDR - False Discovery Rate
FFA - Free Fatty Acids
FLS - Flavonol synthase
GA - Gibberellic acid
GO - Gene ontology
GPAT - Glycerol-3-phosphate acyltransferase
HA - Hemagglutinin tag
KAS - Ketoacyl-ACP synthase
KEGG - Kyoto Encyclopedia of Genes and Genomes
LD - Lipid droplets
lc-TAG - Long chain triacylglycerol
LPAT - Acyl-CoA:lysophosphatidic acid acyltransferase
LPC - Lysophosphatidylcholine
LPCAT - Lyso-PC acyltransferase
LPE - Lysophosphatidylethanolamine
MALDI-MS - Matrix-assisted laser desorption/ionization mass spectrometry
MBOAT - Membrane-bound O-acyltransferase
MCFA - Medium-chain fatty acids
MGDG - Monogalactosyldiacylglycerol
MALDI-MS - Matrix-assisted laser desorption/ionization mass spectrometry
MUFAs - Monounsaturated FAs
PCA - Principal component analysis
PDCT - Phosphatidylcholine:diacylglycerol cholinephosphotransferase

PDAT - Phospholipid-diacylglycerol acyltransferase
PE - Phosphatidylethanolamine
PG - Phosphatidylglycerol
PI - Phosphatidylinositol
PLS-DA - Partial Least-Squares Discriminant
PP2C - Protein phosphatase 2C
PUFAs - Polyunsaturated FAs
PS - Phosphatidylserine
ROD1 - Reduced oleate desaturase 1
RNAi - RNA interference
RNA-Seq - RNA sequencing
RT-qPCR - Reverse transcription-quantitative polymerase chain reaction
SDP1 - Sugar-dependent1
TAG - Triacylglycerols
TCA - Tricarboxylic acid
TT - Transparent testa
VLCFA - Very long-chain fatty acids
WRI1 - Wrinkled1
WT - Wild-type

Chapter 1 – Literature Review

As of 2023, the global demand for vegetable oil continues to rise. Global vegetable oil consumption increased from 197 million metric tons in the 2018/2019 marketing year to 213 million metric tons in 2022/2023 (United States Department of Agriculture; USDA). This growth can be attributed to the expanding use of vegetable oil, in various industries beyond food, including cosmetics, pharmaceuticals, chemical feedstocks, and transportation fuels like biodiesel and sustainable aviation fuel (SAF) (Dyer *et al*., 2008; Lu *et al*., 2011). Over 15% of global vegetable oil is used for biodiesel production due to the urgent need to mitigate climate change by transitioning away from fossil fuels. To aid with this, researchers have been aiming to improve oil yields and quality for biodiesel production. However, a fundamental understanding of the structures, pathways, and regulation of vegetable oil biosynthesis and degradation is first necessary. This knowledge can pave the way for using natural or engineered oilseed crops to meet the increasing demand for vegetable oil sustainably.

1.1 TRIACYLGLYCEROLS SIGNIFICANCE

Vegetable oils consist of triacylglycerols (TAG) and minor quantities of other lipids, including phospholipids, diacylglycerols (DAG), monoacylglycerols (MAG), sterols, and trace amounts of free fatty acids (FFA). TAG represent the major components of common vegetable oils, typically accounting for more than 90% of their composition (Bates *et al*., 2013). TAGs are non-membrane storage lipids mainly produced in the seeds and fruit of plants and in a very small amounts in vegetative tissues such as leaves, roots, and stems (Xu & Shanklin, 2016). The oil content in various plant species can vary significantly, ranging from as little as 1% to as much as 70% of the total dry weight of the seed (Ohlrogge & Browse, 1995).

During seed development, TAGs are assembled in the endoplasmic reticulum (ER) in seed tissues such as the embryo and/or endosperm (Chapman & Ohlrogge, 2012). TAGs accumulate between the leaflets of the ER membrane, leading to the formation of lipid droplets (LD) or oil bodies as independent organelles in the cytosol. They are stored in the core of LDs surrounded by a monolayer of phospholipids and LD-associated proteins (Martin & Parton, 2006). TAGs have been associated with many processes including lipid homeostasis, cellular energy balance, flower and pollen development, seedling establishment and post-germination organ development (Yang & Benning, 2018). They serve as a carbon and energy storage compounds which are hydrolyzed by the action of TAG lipases upon seed germination and in times of energy and carbon deficiency (Eastmond, 2006). The biosynthesis and degradation of TAGs involve complex enzymatic reactions and regulatory mechanisms which can be manipulated to enhance TAG production or modify TAG composition. This holds significant promise for producing high-value oils, such as those rich in omega-3 fatty acids or oils with enhanced functional properties, addressing diverse needs in both industrial applications and nutritional contexts.

1.2 TAG AND FATTY ACID STRUCTURE

The structure of TAG is characterized by a glycerol backbone in which all three hydroxyl groups are esterified to fatty acid chains (Figure 1.1a). Fatty acids (FAs) consist of a hydrocarbon chain with a carboxylic acid functional group on one end. A simplified nomenclature for FAs involves specifying the number of carbons in the chain followed by the number of double bonds, separated by a colon (Figure 1.1a and b). For example, 18:3 indicates a fatty acid with 18 carbon atoms and three double bonds. Additionally, the positions of double bonds can be further denoted as omega-c (ω-c or n-c), indicating their distance (c) relative to the methyl end, or delta-c (Δ-c), indicating

their distance relative to the carboxyl end (Figure 1.1c). FAs can be classified as saturated FAs (SFA, with no double bonds), monounsaturated FAs (MUFAs, with one double bond) or polyunsaturated FAs (PUFAs, with two or more double bonds) based on the presence or absence of double bonds. Additionally, FAs are distinguished by their carbon chain length, ranging from short-chain fatty acids (SCFAs, with 6 carbons or less), medium-chain fatty acids (MCFA, with 8–14 carbons), long-chain fatty acids (LCFA, with 16 to 18 carbons) or very long-chain fatty acids (VLCFA, with 20 carbons or more).

The composition of fatty acids determines the nutritional value, physicochemical properties, and industrial uses of oil (Avato & Tava, 2022; Dyer *et al*., 2008; Ohlrogge, 1994). Major FAs, found in membrane lipids and storage lipids of plants, typically consist of an even number of carbons within their aliphatic chains and cis double bonds. These FAs typically range from 16 to 18 carbons in length and contain 1 to 3 double bonds. They include palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1, Δ9), linoleic acid (18:2, Δ9,12), and α-linolenic acid (18:3, Δ9,12,15; Figure 1.1c). These major FAs are usually optimized for consumption in the context of food and feed applications. However, in nature, a diverse array of unusual FAs exists, accumulating in non-membrane lipids such as seed storage lipids of various native plant species (Badami & Patil, 1980b; Ohlrogge *et al*., 2018; Smith, 1971). These unusual FAs can make up as much as 90% of the total FAs present in the oils of native species. They include atypical fatty acid chain lengths (from C8 to C14, and from C20 to C22), double bonds in unconventional positions, or contain functional groups such as acetyl, hydroxyl, epoxy, and cyclopropane (Figure 1.2) (Avato & Tava, 2022; Cahoon & Li-Beisson, 2020; Dyer *et al*., 2008). Due to their distinct physical and chemical properties, oils containing unusual FAs hold immense promise as raw materials for industrial purposes. These oils have the potential to serve as excellent feedstocks for various industries,

including the production of lubricants, paints, cosmetics, and biofuels (Avato & Tava, 2022; Durrett *et al*., 2008; Dyer *et al*., 2008).

1.3 TAG METABOLISM

TAG metabolism involves multiples steps that occur in separate subcellular compartments. The major steps include the synthesis of FAs in the plastids, modification of FAs in the ER, and assembly of FAs onto the glycerol backbone of TAG in the ER. The mechanisms of fatty acid synthesis, TAG metabolism, and regulation have been extensively reviewed (Bates, 2022; Chapman & Ohlrogge, 2012; Li-Beisson *et al*., 2013; Ohlrogge & Browse, 1995; Yang *et al*., 2022). The review provided here is based on the model system Arabidopsis (*Arabidopsis thaliana*). An overview of the pathways discussed relating to TAG synthesis is depicted in Figure 1.3.

1.3.1 Acetyl-CoA substrate

De novo FA synthesis requires acetyl-CoA, ATP, and NAD(P)H as substrates. Acetyl-CoA is the immediate carbon precursor for FA synthesis in the plastid, and it also plays a central role in numerous metabolic pathways linking anabolism and catabolism, such as the biosynthesis of flavonoids and isoprenoids, metabolism of certain amino acids, mitochondrial respiration, and various acetylation reactions (Oliver *et al*., 2009; Pietrocola *et al*., 2015). Several enzymes have been proposed to produce acetyl-CoA in different subcellular locations including the plastids (for *de novo* FA biosynthesis), cytosol (for isoprenoids, flavonoids and malonate derivatives biosynthesis, and FA elongation), mitochondria (for the TCA cycle), and peroxisomes (the product of β-oxidation of fatty acids) due to its impermeability through membranes (Fatland *et al*., 2002; Pietrocola *et al*., 2015; Schwender & Ohlrogge, 2002). Acetyl-CoA synthetase (ACS), responsible

for activating acetate into its CoA-ester, was initially identified as the primary acetyl-CoA-generating enzyme in plants (Smirnov, 1960). However, subsequent studies have proposed that ACS does not play a major role in providing acetyl-CoA for FA biosynthesis, instead most of the carbon directed toward *de novo* FA synthesis is utilized by the plastidial pyruvate dehydrogenase complex (PDHC) (Oliver *et al*., 2009; Reid *et al*., 1977). PDHC is a large multienzyme structure that catalyzes the oxidative decarboxylation of pyruvate, yielding acetyl-CoA, NADH, and CO2 (Johnston *et al*., 1997). Pyruvate is mainly generated from photosynthetically assimilated hexose or triose carbons precursors (Rawsthorne, 2002; Sagun *et al*., 2023). It can be produced within the plastid through plastidial glycolysis, or from cytosolic glycolysis products and malate imported into the plastid by special transporters (Dennis & Miernyk, 1982; Denyer & Smith, 1988; Kang & Rawsthorne, 1994; White *et al*., 2000). In the cytosol, acetyl-CoA is generated by the heteromeric ATP-citrate lyase (ACL) composed of ACLA and ACLB subunits. ACL catalyzes the ATP-dependent reaction of citrate, exported from the tricarboxylic acid (TCA) cycle (Lonien & Schwender, 2009), and CoA to form acetyl-CoA and oxaloacetic acid (Fatland *et al*., 2002; Fatland *et al*., 2005). Although the direct contribution of ACL to plastidial *de novo* fatty acid synthesis remains controversial (Fatland *et al*., 2000; Rangasamy & Ratledge, 2000), it generates cytosolic acetyl-CoA for fatty acid elongation (Baud *et al*., 2003; Cassagne *et al*., 1994).

1.3.2 ***De novo*** **FA synthesis**

1.3.2.1 Plastidial acetyl-CoA carboxylase (ACCase)

De novo fatty acid synthesis in plants is a ubiquitous process found in all plant cells and predominantly results in the production of 18:1, with smaller amounts of 16:0 and 18:0 FAs within the plastid stroma (Ohlrogge & Browse, 1995). ATP-dependent acetyl-CoA carboxylase (ACCase)

is responsible for catalyzing the first committed step of fatty acid biosynthesis by converting acetyl-CoA and bicarbonate into malonyl-CoA (Alberts & Vagelos, 1968; Sasaki & Nagano, 2004). In dicots, plastid ACCase is a heteromeric complex that is composed of four subunits, biotin carboxylase (BC), biotin carboxyl carrier protein (BCCP), and α- and β carboxyltransferase (α-CT and β-CT) (Konishi & Sasaki, 1994; Salie & Thelen, 2016; Sasaki *et al*., 1993). HtACCase serves as a key regulatory point in controlling the flux of carbon into de novo fatty acid biosynthesis. Manipulating htACCase activity has been explored as a strategy to enhance seed oil production. Several negative regulators of htACCase have been identified, including increased 18:1 concentration (Andre *et al*., 2012; Yu *et al*., 2021), alterations in chloroplast stromal pH due to light exposure (Ye *et al*., 2020), and a family of BCCP homologs proteins called BADCs (biotin attachment domain containing) that bind to BC but lack the biotinylation site necessary for carboxylation activity (Salie *et al*., 2016). Downregulating BADCs in Arabidopsis has resulted in elevated htACCase activity and significantly increased seed oil content, with levels reaching up to 32.6% (Keereetaweep *et al*., 2018). Overexpressing individual htACCase subunits, however, showed varying effects. In upland cotton, overexpression of individual htACCase subunits including β-CT enhanced seed oil accumulation by 17-22% (Cui *et al*., 2017) while overexpressing β-CT and BC in tobacco led to enhanced ACCase activity but had no significant impact on seed oil content (Madoka *et al*., 2002; Shintani *et al*., 1997). In Arabidopsis developing seeds, α-CT was found to be 3- to 10-fold less abundant than β-CT (Wilson & Thelen, 2018). Consequently, overexpression of pea (*Pisum sativum*) α-*CT* in both Arabidopsis and camelina (*Camelina sativa*) led to higher htACCase activity during early seed development and increased seed oil content up to 14% (M. Wang *et al*., 2022).

1.3.2.2 FA synthase complex (FAS) reactions and FA export

The subsequent step in fatty acid synthesis involves the conversion of malonyl-CoA, generated by htACCase, into malonyl-acyl carrier protein (malonyl-ACP) through the action of a malonyl-CoA:ACP malonyltransferase (MCAT/MCAMT; Figure 1.3) (Jung *et al*., 2019; Lessire & Stumpe, 1983). Malonyl-ACP then partakes in a condensation reaction with acetyl-CoA as an initial substrate, which is facilitated by β-ketoacyl synthase III (KAS III) (Clough *et al*., 1992; Jaworski *et al*., 1989), resulting in the formation of 3-ketobutyl-ACP. This condensation reaction marks the initiation of a cycle comprising four enzymatic reactions constituting the fatty acid synthase complex (FAS) (Brown *et al*., 2006). The process continues with the reduction of 3-ketobutyl-ACP to 3-hydroxylacyl-ACP, followed by dehydration into enoyl-ACP (Mou *et al*., 2000), and culminating in a second reduction step to yield the elongated 4:0-ACP. The resulting newly synthesized acyl-ACP chain, now a 4:0-ACP, re-enters the FAS cycle repeatedly with malonyl-ACP. With each cycle, malonyl-ACP contributes two carbon units, resulting in the gradual elongation of the acyl chain. This continues over six additional cycles to generate 16:0-ACP or over seven cycles to produce 18:0-ACP. The condensation reactions from 4:0-ACP until the production of 16:0-ACP are enzymatically catalyzed by KAS I, while the condensation of 16:0-ACP with malonyl-ACP to produce 18:0-ACP is facilitated by KAS II (Figure 1.3) (Shimakata & Stumpf, 1982).

The majority of 18:0-ACP is desaturated by the stromal Δ9 stearoyl-ACP desaturase (SAD) (Kachroo *et al*., 2007; Lightner *et al*., 1994) to 18:1-ACP. In seeds, about 38% of 16:0-ACP and 18:1-ACP are then used to generate glycerolipids in the plastid (prokaryotic pathway), the rest is hydrolyzed along with 18:0-ACP to free fatty acids by acyl-ACP thioesterases A and B for export and generation of glycerolipids in the ER (FATA and FATB; Figure 1.3) (Jones *et al*., 1995; Mayer

& Shanklin, 2007). FATA enzymes tend to exhibit a preference for 18:1-ACP as their substrate, while FATB enzymes have been observed to favor 16:0-ACP and 18:0-ACP substrates (Bonaventure *et al*., 2003; Salas & Ohlrogge, 2002). In plants that produce MCFA like palm kernel (*Elaeis guineensis* Jacq.), coconut (*Cocos nucifers* L.), and Cuphea, release from the FAS machinery occur earlier to produce acyl-ACPs containing 8 to 14 carbons (Graham, 1989). The hydrolysis of these medium chain acyl-ACPs (8 to 14 carbons) to free fatty acids (FFAs) is facilitated by specialized acyl-ACP thioesterases with specific affinity for MCFA (Dehesh *et al*., 1998; Pollard *et al*., 1991). FFAs generated at the inner plastid envelope membrane (IE) are transported by FATTY ACID EXPORT (FAX1, 2, and 4) proteins (Li *et al*., 2015; Li *et al*., 2020). Subsequently, FFAs are activated to CoA esters by a long-chain acyl-CoA synthetase (LACS9, 1, and 4) (Jessen *et al*., 2015; Schnurr *et al*., 2002; Shockey *et al*., 2002; Zhao *et al*., 2010) in the plastid outer envelope (OE) and exported from the plastid to the cytosol forming an acyl-CoA pool. ATP-binding cassette transporter 9 (ABCA9), localized at the ER membrane, further transport acyl-CoAs into the ER for glycerolipid assembly (Figure 1.3) (Kim *et al*., 2013; Li *et al*., 2016).

1.3.3 FA modification

1.3.3.1 FA elongation

Within the cytosol, 18:1-CoA can undergo elongation in seeds to produce VLCFA, such as eicosenoic acid (20:1, Δ11) and erucic acid (22:1, Δ13), through the fatty acid elongase (FAE) complex (Figure 1.3) (Cassagne *et al*., 1994; Kunst *et al*., 1992; Pollard & Stumpf, 1980). The FAE complex is a membrane-bound multienzyme complex that functions similarly to the plastidial FAS, in which each cycle of fatty acid elongation adds two carbons to the acyl chain and involves

four reactions: condensation, reduction, dehydration, and a second reduction (Bessoule *et al*., 1989; Cassagne *et al*., 1994; Haslam & Kunst, 2013). However, the initial substrate of the FAE complex is a fatty acyl-CoA, 18:1-CoA, instead of acetyl-CoA (Agrawal & Stumpf, 1985). Malonyl-CoA, synthesized by the cytosolic homomeric ACCase complex, contributes two carbon units per cycle to the FAE complex. The condensation reaction in the FAE complex is facilitated by FATTY ACID ELONGASE1 (FAE1), which encodes the rate-limiting enzyme for seed VLCFA production, 3-ketoacyl-CoA synthase (KCS) (James, 1990; James *et al*., 1995; Lemieux *et al*., 1990; Millar & Kunst, 1997). FAE1 is a seed-specific enzyme that plays a critical role in determining the length of VLCFA in various plant species, leading to variations in their abundance and distribution, even within the same plant family. For example, in the Brassicaceae family, the content of erucic acid (22:1) can vary significantly, with species like rapeseed (*Brassica napus*), *Crambe abyssinica*, and pennycress (*Thlaspi arvense*) having erucic acid content ranging from 40 to 60% in their seed oil, while Arabidopsis contains less than 2.5% erucic acid (Claver *et al*., 2020; Sun *et al*., 2013; P. Wang *et al*., 2022). Mutation of *FAE1* in various oilseeds, including Arabidopsis, rapeseed, camelina, and pennycress, leads to a significant reduction in the accumulation of VLCFA to less than 2% of the total FA content (James, 1990; Liu *et al*., 2022; McGinn *et al*., 2019; Ozseyhan *et al*., 2018).

1.3.3.2 Acyl-editing

Upon being transported into the ER, almost 60% of the newly synthesized 18:1-CoA are integrated into the membrane lipid phosphatidylcholine (PC), as confirmed by several *in vivo* isotopic tracing studies (Bates, 2022; Bates *et al*., 2009; Bates *et al*., 2012; Bates *et al*., 2007; Karki *et al*., 2019; Zhou *et al*., 2020). This integration is facilitated by acyl editing and the Lands cycle or PC-

diacylglycerol (DAG) exchange (Figure 1.3) (Bates *et al*., 2012). Acyl editing comprises a cycle of PC:acyl-CoA exchange without net PC synthesis or degradation and involves remodeling of 18:1 through desaturation to PUFA or other modifications such as hydroxylation. Acyl-CoA:lysoPC acyltransferase 1 and 2 (LPCAT1/2) transfers 18:1 from 18:1-CoA to the *sn*-2 position of lyso-PC (LPC), resulting in the formation of 18:1-PC. A preference for incorporating 18:1-CoA into the *sn*-2 position of PC has been reported, although the specific ratio of *sn*-1 to *sn*-2 acyl editing varies among species and tissues (Bates, 2022; Bates *et al*., 2009; Bates *et al*., 2012; Bates *et al*., 2007; Karki *et al*., 2019; Lager *et al*., 2013; Zhou *et al*., 2020). Modification of 18:1-PC is catalyzed by fatty acid desaturases. In Arabidopsis, fatty acid desaturase 2 (FAD2) converts 18:1-PC to 18:2-PC, followed by FAD3-mediated desaturation to 18:3-PC (Arondel *et al*., 1992; Okuley *et al*., 1994). Alternatively, in certain species like castor (*Ricinus communis*), which produce the hydroxy fatty acid (HFA) ricinoleic acid (18:1OH) in its seeds, a fatty acid hydroxylase 12 (RcFAH12) enzyme homolog to Arabidopsis FAD2, hydroxylates 18:1 at the *sn*-2-PC position, yielding 18:1OH-PC (van de Loo *et al*., 1995). After synthesis on PC, these modified fatty acids (mFAs) can be released from PC to enter the acyl-CoA pool through two mechanisms, direct transfer from PC to CoA facilitated by the reverse activity of LPCAT (Lager *et al*., 2013; Stymne & Stobart, 1984), or through a process involving phospholipase A2 (PLA2), known as the Lands cycle (Lands, 1965), which hydrolyzes mFAs from PC, generating FFAs, followed by the reactivation of FFAs to acyl-CoAs via ATP-dependent acyl-CoA synthetases (Shockey *et al*., 2003; Shockey *et al*., 2002). 18:1-PC that enter acyl editing and modification can also be produced from de novo DAG by either CDP-choline:DAG cholinephosphotransferase (CPT), which results in a net production of PC from DAG (Slack *et al*., 1983; Slack *et al*., 1985), or PC:DAG cholinephosphotransferase (PDCT), encoded by the *REDUCED OLEATE*

DESATURASE 1 (*ROD1*) gene in Arabidopsis. PDCT catalyzes the interconversion between PC and DAG generating new molecular species of PC and DAG, and as a result, there is no net accumulation of PC (Lu *et al*., 2009). The simultaneous mutation of *rod1* and *lpcat1/lpcat2* in Arabidopsis seeds led to a notable reduction of around 66% in the PUFA content of seed TAG. This observation suggested that PC-DAG interconversion facilitated by PDCT and acyl editing collectively contribute to two-thirds of the PUFA flux from PC to TAG in Arabidopsis seeds (Bates *et al*., 2012).

1.3.4 Incorporation of FA into DAG

Diacylglycerol (DAG) serves as the immediate substrate used for TAG production in the ER and is synthesized either *de novo* through the Kennedy pathway, or it can be derived from PC (Figure 1.3) (Bates, 2016; Li-Beisson *et al*., 2013; Ohlrogge & Browse, 1995). PC-derived DAG can exhibit distinct molecular compositions compared to de novo DAG, since PC serves as the site for acyl editing and FA modification in the ER (Bates, 2022; Bates *et al*., 2009).

1.3.4.1 *De novo* DAG (Kennedy pathway)

The Kennedy pathway utilizes the acyl-CoA pool to sequentially acylate glycerol-3-phosphate (G3P) (Kennedy, 1961; Weiss *et al*., 1960). G3P is mainly synthesized from dihydroxyacetone phosphate (DHAP), derived from glycolysis, by glycerol-3-phosphate dehydrogenase1 (GPDH) (Finlayson & Dennis, 1980; Gee *et al*., 1988; Shen *et al*., 2006). However, glycerol kinases have also been implicated in the production of G3P from glycerol in the cytosol (Hippmann & Heinz, 1976; Sadava & Moore, 1987). In Arabidopsis, acyl-CoA:glycerol-3-phosphate acyltransferase 9 (GPAT9) initiates the process of acylation in the Kennedy pathway by transferring an acyl group

from acyl-CoA to the *sn*-1 position of G3P, generating lysophosphatidic acid (LPA) (Shockey *et al*., 2016). Acyl-CoA:lysophosphatidic acid acyltransferase (LPAT) follows by forming phosphatidic acid (PA) through the transfer of an acyl group from acyl-CoA to the *sn*-2 position of LPA (Kim & Huang, 2004; Kim *et al*., 2005). Finally, a phosphatidic acid phosphatase enzyme (PAP; *PAH1* and *PAH2 in Arabidopsis*) removes the phosphate group at the *sn*-3 position to produce de novo DAG (Eastmond *et al*., 2010; Nakamura *et al*., 2009). *De novo* DAG can be utilized for the synthesis of *de novo* TAG directly or to produce ER membrane lipids phosphatidylethanolamine (PE) and PC. PC is produced by either CPT or PDCT for acyl-editing (Lu *et al*., 2009; Slack *et al*., 1983; Slack *et al*., 1985; Wang *et al*., 2012).

1.3.4.2 PC-derived DAG

Isotopic tracing of glycerol incorporation into glycerolipids have indicated that over 80% of seed TAG in Arabidopsis, camelina and soybean (*Glycine max*), originates from PC-derived DAG (Bates, 2022; Bates & Browse, 2011; Bates *et al*., 2009; Yang *et al*., 2017). There are several mechanisms that can contribute to the production of PC-derived DAG for TAG biosynthesis (Bates, 2022). PC-derived DAG can be produced by the reverse action of CPT as demonstrated by labelling experiment in linseed and safflower seeds (Slack *et al*., 1983; Slack *et al*., 1985), or PDCT, which involves the exchange of the phosphocholine headgroup between PC and DAG (Lu *et al*., 2009). Additionally, PC-derived DAG can be produced through alternative mechanisms involving phospholipase C (PLC), which cleaves PC directly into DAG (Cai *et al*., 2020; Hong *et al*., 2016; Wang, 2001), and a sequential process involving phospholipase D (PLD) to generate PA, followed by PAP activity to convert PA into DAG (Lee *et al*., 2011; Li *et al*., 2009; Yang *et al*., 2017).

1.3.5 TAG synthesis

The final step of TAG synthesis involves the transfer of a third acyl group to the *sn*-3 position of DAG. This acylation reaction is catalyzed by an acyl-CoA-dependent acylation of DAG through acyl-CoA:diacylglycerol acyltransferases (DGATs) or, alternatively, through an acyl-CoA independent mechanism mediated by phospholipid:diacylglycerol acyltransferase (PDAT) (Figure 1.3) (Zhang *et al*., 2009). Studies using isotopic tracing of acyl or glycerol flux through the lipid metabolic network in developing Arabidopsis and soybean seeds have revealed the existence of at least three distinct pools of DAG involved in this process. These pools include de novo DAG and an initially produced PC-derived DAG, both of which are small and rapidly turned over. The DAG initially derived from PC was suggested to partition into LD and is maintained in equilibrium with PC through AtPDCT, creating a third larger, more slowly turned over DAG pool (Bates, 2022; Bates *et al*., 2009; Regmi *et al*., 2020). Emerging evidence suggests that the composition of TAG and the regulation of their biosynthesis from various DAG pools are influenced by substrate channeling toward specific enzymes and the spatial separation of different acyltransferases within the ER, forming distinct metabolons. This regulation is partly controlled by the relative tissue expression, substrate selectivity, and enzymatic activities of these enzymes (Bates, 2022; Regmi *et al*., 2020; Zhang & Fernie, 2021).

1.3.5.1 DGAT

DGAT enzymes, which transfer acyl groups from acyl-CoA to the *sn*-3 position of DAG to form TAG, are expressed ubiquitously in various organisms, including bacteria, algae, plants, and mammals (Chen *et al*., 2022). In plants, three classes of DGATs have been identified: membrane-

bound DGAT1 and DGAT2, as well as a soluble cytosolic DGAT3 class (Cao *et al*., 2013; Lardizabal *et al*., 2001; Routaboul *et al*., 1999; S. Saha *et al*., 2006; Shockey *et al*., 2006; Zou *et al*., 1999). DGAT1 and DGAT2 are structurally distinct integral membrane proteins with different localizations within the ER membrane, tissue expression patterns, and substrate preferences (Bates, 2022; Li, Yu, & Hildebrand, 2010; Regmi *et al*., 2020; Shockey *et al*., 2006; Zhou *et al*., 2013). In Arabidopsis, DGAT1 is primarily expressed in developing seeds and pollen, with low expression in vegetative tissues. In contrast, DGAT2 is highly expressed in vegetative tissues but has low expression in late seed development stages (Schmid *et al*., 2005; Zhou *et al*., 2013). In most plants, DGAT1 is the major enzyme responsible for seed TAG synthesis, while DGAT2 only significantly contributes when overexpressed (Lardizabal *et al*., 2008; Regmi *et al*., 2020; Zhou *et al*., 2013). Mutations in Arabidopsis DGAT1 reduce seed oil content by 20% and alter FA composition, including an increase in 18:3 FA, predominantly at the *sn*-3 position of TAG, and a decrease in 20:1 and 18:1 FAs (Katavic *et al*., 1995; Routaboul *et al*., 1999; Zhang *et al*., 2009). Similar results were observed in in camelina seeds suppressing DGAT1 (Alkotami *et al*., 2021). In contrast, mutations of DGAT2 in Arabidopsis did not significantly affect seed oil accumulation in wild-type or *dgat1-1* mutant background, suggesting a minor role for DGAT2 in TAG synthesis (Zhang *et al*., 2009). Unlike Arabidopsis, in some plant species such as castor, tung tree (*Vernicia fordii*), and *Vernonia galamensis*, the DGAT2 enzyme plays a more significant role in synthesizing TAG in seeds. In these species, DGAT2 is primarily responsible for accumulating TAG containing unusual FA like hydroxy FAs, conjugated FAs, and epoxy FAs (Kroon *et al*., 2006; Li, Yu, *et al*., 2010; Shockey *et al*., 2006).

In addition to other enzymes involved in PC and TAG assembly, DGAT1 and DGAT2 distribution and interactions within the ER subdomains are not uniform. For example, both tung

tree DGAT1 and DGAT2 were found to localize to distinct and separate subdomains within the ER (Shockey *et al*., 2006). Additionally, yeast two-hybrid analysis revealed that Arabidopsis DGAT1 (AtDGAT1) interacts with AtPDCT and AtLPCAT2 but not with AtDGAT2 or AtGPAT9 (Regmi *et al*., 2020; Shockey *et al*., 2016). Similarly, both castor DGAT2 (RcDGAT2) and soybean DGAT2 (GmDGAT2) interact with AtPDCT and AtLPCAT2 but not AtDGAT1 (Regmi *et al*., 2020). This direct physical interactions between enzymes of the Kennedy pathway and the acyl editing cycle, indicated a significant crosstalk and potential metabolic channeling between these two pathways (Regmi *et al*., 2020; Shockey *et al*., 2016; Wang *et al*., 2012). Isotopic tracing experiments using the Arabidopsis *dgat1-1* mutant complemented with overexpression of AtDGAT1, RcDGAT2, or GmDGAT2, revealed that AtDGAT1 utilizes the initially produced small pool of PC-derived DAG not accessible to AtPDAT1 or any of the overexpressed DGAT2s, possibly through substrate channeling (Regmi *et al*., 2020). In contrast, AtPDAT1 and each of the overexpressed DGAT2s utilize the larger, slowly turned over PC-derived DAG pool. This is further supported by localization experiments indicating that DGAT2 can also associate with LDs (Aymé *et al*., 2014; Kwiatkowska *et al*., 2012). These findings suggested that AtDGAT1 and various plant DGAT2s operate in separate TAG-producing metabolons or interactomes, each utilizing distinct pools of PC-derived DAG for TAG synthesis (Bates, 2022; Regmi *et al*., 2020).

Unlike DGAT1 and DGAT2, DGAT3 is a soluble enzyme first identified in peanut (S. Saha *et al*., 2006). DGAT3 genes have since been identified in various higher plants, including Arabidopsis, tung tree, peanut, oil palm (*Elaeis guineensis),* maize, upland cotton (*Gossypium hirsutum*), camelina, and soybean (Cao *et al*., 2013; Gao *et al*., 2021; Hernández *et al*., 2012; Rosli *et al*., 2018; Saikat Saha *et al*., 2006; Xue *et al*., 2022; Yan *et al*., 2018; Zhao *et al*., 2021). DGAT3 contains a [2Fe-2S] domain in its C-terminal region and features an N-terminal chloroplast transit

peptide, suggesting potential localization in either the cytosol or chloroplast (Aymé *et al*., 2018; Hernández *et al*., 2012). Arabidopsis DGAT3 (AtDGAT3) is expressed throughout plant development, particularly higher during late seed maturation, and surpasses AtDGAT1 and AtDGAT2 expression levels in germinating seeds and young seedlings (Schmid *et al*., 2005). Although its exact role remains unclear, AtDGAT3 may participate in recycling of FA into TAG in young seedlings, especially when FA breakdown is inhibited (Hernández *et al*., 2012). Additionally, research in *Chlamydomonas reinhardtii* suggests that DGAT3 enzymes may play a role in accumulating TAG in the chloroplast in response to limited light conditions (Carro *et al*., 2022). Arabidopsis DGAT3 mutants have not been characterized but transient expression of AtDGAT3 in *tobacco* leaves resulted in a significant increase in TAG content, yet in contrast to AtDGAT2, AtDGAT3 accumulated less TAG than DGAT1 (Hernández *et al*., 2012). In camelina, knockout of *CsDGAT3 resulted in* 19% lower fatty acid content in seeds and decrease in 18:3 and 20:1 compared to the wild-type (Lee *et al*., 2022). Characterization of DGAT3 from various other plant species, such as camelina, cotton and soybean, has revealed their potential for enhancing seed oil content and improving FA composition in seeds when overexpressed (Gao *et al*., 2021; Xue *et al*., 2022; Zhao *et al*., 2021), making them promising targets for genetic oilseed improvement alongside DGAT1 and DGAT2.

1.3.5.2 PDAT

PDAT catalyzes the transfer of an acyl group from the *sn*-2 position of PC to the *sn*-3 position of *sn*-1,2-DAG, resulting in the formation of TAG and LPC. PDAT is a *homolog* of the human enzyme LECITHIN:CHOLESTEROL ACYL-TRANSFERASE (LCAT), and is present in diverse organisms, including yeast, fungi, microalgae, and plants (Dahlqvist *et al*., 2000; Stahl *et al*., 2004;

Stahl *et al*., 2008; Yoon *et al*., 2012). Arabidopsis possesses two PDAT enzymes, AtPDAT1 and AtPDAT2. These enzymes are characterized by the presence of a C-terminal ER retrieval motif, a brief cytoplasmic tail at the N-terminus, and a region spanning the ER membrane (Stahl *et al*., 2004). Compared to AtDGAT1, the expression levels of AtPDAT1 and AtPDAT2 are relatively lower. AtPDAT1 exhibits ubiquitous expression throughout the plant, with the highest expression observed in flowers and pollen. In contrast, AtPDAT2 is primarily expressed during late seed development (Schmid *et al*., 2005). Research in Arabidopsis has shown that a knockout mutation of AtPDAT1, does not significantly affect seed oil accumulation (Mhaske *et al*., 2005). Similarly, overexpression of AtPDAT1 in wild-type Arabidopsis plants did not lead to an increase in oil content or changes in fatty acid composition (Stahl *et al*., 2004). However, when RNA interference (RNAi) was used to downregulate either AtPDAT1 in the *dgat1* background or AtDGAT1 in the *pdat1* background, a significant reduction of oil content in Arabidopsis seeds, ranging from 70% to 80%, was observed. Attempts to generate a *pdat1/dgat1* double mutant were unsuccessful due to pollen lethality (Zhang *et al*., 2009). In contrast, *pdat2*/*dgat1* double mutants were able to survive, with no reduction in TAG content greater than what was observed in mutants lacking *dgat1* only, indicating a minor role for PDAT2 in TAG accumulation (Zhang *et al*., 2009). These data suggest that while the individual knockout or overexpression of AtPDAT1 might not have a significant effect on oil accumulation, its role becomes more apparent acting as a compensatory mechanism when AtDGAT1 is absent. PDAT1 has also been implicated in Arabidopsis plant stress responses. Plants with reduced PDAT1 are more sensitive to cold exposure, while plants overexpressing PDAT1 have increased tolerance to both heat and cold stress (Demski *et al*., 2020; Mueller *et al*., 2017).

In vitro assays of AtPDAT1 have demonstrated its ability to utilize various phospholipids as acyl donors and to accept acyl groups ranging from C10 to C22, with its activity rate notably influenced by the acyl composition, showing the highest activities for acyl groups containing multiple double bonds, epoxy groups, or hydroxy groups (Stahl *et al*., 2004). Furthermore, yeast two-hybrid assays and isotopic tracing of glycerol flux have revealed that AtPDAT1 interacts with AtDGAT1, as well as both GmDGAT2 and RcDGAT2, and, similar to GmDGAT2 and RcDGAT2, it utilizes the larger, more slowly turned over PC-derived DAG pool (Regmi *et al*., 2020). PDAT1 homologs from different species can exhibit varying substrate specificities and activity levels, which can impact the fatty acid composition of their seed oils (Marmon *et al*., 2017). For instance, plants with high levels of PUFA like camelina and flax (*Linum usitatissimum*) have been shown to possess high PDAT activity since PC is the ER-localized site for PUFA synthesis (Marmon *et al*., 2017; Pan *et al*., 2013). When camelina PDAT1 expression was altered, seed oil content remained largely unchanged, but overexpression led to an increase in 18:2 fatty acids and a decrease in 18:3, while down-regulation resulted in higher levels of 18:1 and 20:1 and lower levels of 18:2 (Marmon *et al*., 2017). Additionally, in transgenic Arabidopsis seeds expressing RcFAH12, which produces HFA, coexpression of castor PDAT1 (RcPDAT1A), but not AtPDAT1, led to an increase in seed oil content and the percentage of HFAs in the seed oil. This indicates that castor PDAT1 has evolved to be more efficient at incorporating HFAs into TAG compared to AtPDAT1 (van Erp *et al*., 2011).

1.4 METABOLIC ENGINEERING OF OILSEED CROPS FOR THE PRODUCTION OF UNUSUAL TAGs

1.4.1 The purpose and potential of metabolic engineering

The metabolic engineering of unusual fatty acids in oilseeds plays an important role in agricultural and industrial biotechnology. Unusual fatty acids, such as MCFA, hydroxylated, epoxy, or fish-oil-type fatty acids eicosapentaenoic acid (EPA; 20:5Δ5,8,11,14,17) and docosahexaenoic acid (DHA; 22:6Δ4,7,10,16,19), have unique properties that render them valuable for various applications, ranging from enhanced nutritional profiles in food and feed to the production of biofuels and industrial chemicals (Avato & Tava, 2022; Cahoon *et al*., 2010; Damude & Kinney, 2008; Durrett *et al*., 2008; Dyer *et al*., 2008; Ohlrogge *et al*., 2018). These fatty acids are often not naturally produced by traditional oilseed crops or produced at low levels. Many native plants that accumulate unusual fatty acids do not possess the necessary agronomic traits required for large-scale production, as they often suffer from drawbacks such as incompatibility with various environmental conditions, low seed yields, the potential for toxic by-products, non-uniform maturation of seeds, and a lack of automated seed harvesting technology (Horn *et al*., 2016; Jaworski & Cahoon, 2003; Severino *et al*., 2012). Due to these limitations, researchers have turned to alternative oilseed crops which can be genetically engineered to produce higher quantities of unusual FAs while offering favorable agronomic properties (Bansal & Durrett, 2016a; Blackshaw *et al*., 2011; McGinn *et al*., 2019; Msanne *et al*., 2020; Napier *et al*., 2019; Yuan & Li, 2020). These alternative crops are more amenable to metabolic engineering efforts and can be better tailored to meet specific industrial or nutritional demands for unusual fatty acids production.

1.4.2 Plant platforms for metabolic engineering

1.4.2.1 *Camelina sativa*

Camelina sativa, commonly known as false flax or simply camelina, is a dicotyledonous, self-pollinating member of the mustard family (Brassicaceae). The cultivation of camelina has a rich history dating back to approximately 500-600 BCE, notably in regions such as France and Germany. Presently, it is primarily grown in Europe and North America, with significant cultivation in Canada and the Upper Midwest of the U.S. including states like Minnesota, North Dakota, South Dakota, and Montana (Bouby, 1998; Robinson, 1987; Zubr, 1997). It has gained recognition as an ideal platform for metabolic engineering due to its advantageous agronomic attributes (Bansal & Durrett, 2016a; Iskandarov *et al*., 2014; Malik *et al*., 2018; Putnam *et al*., 1993). Camelina outperforms some Brassicaceae oilseed crops under stress conditions due to its adaptability to diverse environmental conditions combined with its minimal requirements, including low inputs of fertilizer, water, and pesticide inputs (Gugel & Falk, 2006; Putnam *et al*., 1993; Zubr, 1997). It has a short growth cycle of approximately 85-100 days with rapid seed maturation and flowering making it suitable for rotational or relay cropping, maximizing land use during agricultural off-season (Gesch *et al*., 2014; Neupane *et al*., 2022). Camelina seed characteristics, such as single seed weight (0.7mg-1.5 mg), and nutritional content, including protein (25%-28%) and oil (30%-45%), vary based on environmental conditions and cultivar types (Neupane *et al*., 2022; Obour *et al*., 2017). Generally, its oil yield is similar to *Brassica juncea* and *Brassica rapa*, but twice the oil yield of soybeans (Blackshaw *et al*., 2011). Camelina oil is characterized by its high content of PUFA, particularly linoleic acid (18:2) and linolenic acid (18:3), constituting about 45% to 60% of the oil, along with slightly lower levels of MUFA oleic acid (18:0) and eicosenoic acid (20:1), which make up approximately 30% of its composition

(Moser, 2010; Obour *et al*., 2017; Putnam *et al*., 1993). Camelina oil, containing erucic acid and glucosinolate, is unsuitable for food consumption. However, it serves as an excellent feedstock for biodiesel production, mitigating ethical concerns associated with utilizing food crops for industrial purposes (Malik *et al*., 2018). Camelina-derived biodiesel have demonstrated the potential to significantly reduce greenhouse gas emissions by ~75% compared to standard petroleum and have been successfully used in road and military aviation fuels production (Moser, 2010; Patil *et al*., 2009; Shonnard *et al*., 2010; Stamenković *et al*., 2023). Camelina is closely related to Arabidopsis and has an allohexaploid genome which is sequenced and publicly available (Hutcheon *et al*., 2010; Kagale *et al*., 2014; Kang *et al*., 2011). In addition to its current non-food status, favorable agronomic traits, and availability of genetic information, biotechnologically, camelina is a prime candidate due to its ease of transformation using standard floral-dip methods, making it amenable to genetic manipulation (Lu & Kang, 2008). These features enabled the utilization of biotechnological approaches, such as the insertion of recombinant genetic elements, suppression or elimination of endogenous genes using RNA interference (RNAi) or Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR-associated protein 9 (CRISPR-Cas9), for the rapid improvement of specific traits (Gao, 2021). For instance, to eliminate VLCFAs in camelina, homozygous *fae1* mutants were generated by simultaneously targeting all three FAE1 *homoeologous* genes through CRISPR-Cas9 (Ozseyhan *et al*., 2018). All three homoeologs were effectively altered at the single guide RNA (sgRNA) target sites, mainly through deletions near the protospacer-adjacent motif (PAM) sites, leading to translational frameshifts and subsequent inactivation of the FAE1 enzyme. This resulted in a significant reduction in VLCFAs, less than 2% of the total fatty acids compared to over 22% in wild type (Ozseyhan *et al*., 2018). Additional

studies that use biotechnological tools to alter camelina oil have recently been reviewed (Cai *et al*., 2022; Malik *et al*., 2018; Napier & Betancor, 2023; Sagun *et al*., 2023).

1.4.2.2 *Thlaspi arvense* L. (Field Pennycress)

Thlaspi arvense L. (Field Pennycress; hereafter referred to as pennycress) is a spring or winter annual plant of the Brassicaceae family. While initially native to Eurasia, pennycress has become fully naturalized in the continental United States (US) (Best & McIntyre, 1975; McGinn *et al*., 2019). Its domestication has recently gained attention due to its potential as a model plant platform for genetic modification and as an oilseed cash cover crop grown throughout the U.S. Midwest Corn Belt, including states such as Illinois, Michigan, Minnesota, and Missouri (McGinn *et al*., 2019). Pennycress possesses a valuable combination of traits, such as short life cycle with early maturity, cold tolerance, overwintering, and minimal agricultural inputs such as pesticides, fertilizer, and water (Moser *et al*., 2009; Phippen & Phippen, 2012). Spring pennycress germinates in early spring and does not need exposure to cold temperatures to initiate flowering. In contrast, winter pennycress typically germinates during the fall, often after corn or soybean harvest in late September or early October (Fan *et al*., 2013). These winter varieties can endure freezing conditions, remaining green throughout the winter, and eventually flower in early spring. They produce seeds ready for harvest from mid-May to June, well before soybeans or corn are planted (Isbell, 2008; Sedbrook *et al*., 2014). This unique growth pattern allows pennycress to seamlessly integrate into the corn-soybean crop rotation without requiring additional land development (McGinn *et al*., 2019). As a cover crop, pennycress delivers crucial ecosystem benefits including reduced soil erosion, decreased nutrient leaching, effective suppression of spring weeds, the creation of habitats for insects, and an early-season food source for pollinators (Eberle *et al*., 2015;

Groeneveld & Klein, 2015; Isbell, 2008; Johnson *et al*., 2015; McGinn *et al*., 2019). Pennycress seeds are similar in size to camelina seeds, weighing between 0.6 to 1.3 mg each. They are rich in oil, containing approximately 24% to 39% oil content and around 19% protein on a dry weight basis (Hojilla-Evangelista *et al*., 2013; Moser *et al*., 2009; Sedbrook *et al*., 2014). Compared to other oilseed crops, pennycress has a relatively high oil yield. It produces an average of 1500 kg of seeds per hectare, which translates to 600 to 1200 liters of oil per hectare, surpassing the oil yields of soybean and camelina, which produce approximately 450 liters and 420 to 640 liters per hectare, respectively (Moser *et al*., 2009). This oil is primarily composed of erucic acid (22:1; 28-39%), eicosenoic acid (20:1; 8-10%), oleic acid (18:1; 8-17%), linoleic acid (18:2; 19-22%), and linolenic acid (18:3; 8-15%) (Moser *et al*., 2009; Sedbrook *et al*., 2014). The high content of erucic acid in pennycress seeds holds significant value for industries such as lubricants, plastics, cosmetics, and jet fuel. Previous research has shown that fuels derived from pennycress possesses excellent physical properties and could be classified as advanced biofuels, offering over 50% reductions in greenhouse gas emissions compared to petroleum-based counterparts (Fan *et al*., 2013; Moser, 2012).

Pennycress has a diploid genome with a size of approximately 539 Mb, organized into 7 chromosomes ($2n = 14$). This diploid nature distinguishes it from camelina and makes it a more manageable and amenable candidate for genetic modification and breeding. A draft genome sequence using the winter annual line MN106 have been released in earlier studies (Dorn *et al*., 2013; Dorn *et al*., 2015). More recently, the entire genome and transcriptome has been sequenced in multiple pennycress accessions, as well as in the inbred line Spring 32-10, characterized by minimal seed dormancy and the ability to flower without cold treatment (Dorn *et al*., 2018; García Navarrete *et al*., 2022; McGinn *et al*., 2019; Nunn *et al*., 2022). Pennycress is closely related to

several agriculturally significant plants, including rapeseed (*Brassica rapa* and *Brassica napus* varieties), canola, camelina, and the model plant Arabidopsis, with approximately 86% of the genes in pennycress having homologous counterparts in the Arabidopsis genome (McGinn *et al*., 2019; Warwick *et al*., 2010). In addition to its diploid genome, the availability of genomic and transcriptomic sequences, and genetic similarities with Arabidopsis, pennycress can be easily transformed using the Agrobacterium-mediated floral dip method with a vacuum infiltration step (McGinn *et al*., 2019). These attributes enabled scientists to rapidly develop molecular tools for the domestication of pennycress, transforming it into a profitable oilseed cover crop and supporting breeding efforts. Key traits targeted for domestication included reducing seed dormancy to enhance germination, minimizing pod shattering to reduce seed losses before harvest, decreasing seed glucosinolate and seed coat fiber content to enhance meal nutritional value and taste, and lowering seed oil erucic acid content to create an edible oil akin to canola (Chopra *et al*., 2020; McGinn *et al*., 2019; Phippen *et al*., 2022; Sedbrook *et al*., 2014; Vaughan *et al*., 2007). For instance, a CRISPR-Cas9-based approach was successfully employed to introduce 4bp-deletion and single insertion mutations into the pennycress *FAE1* gene, resulting in the elimination of erucic acid in the seed oil (McGinn *et al*., 2019). Additional biotechnological studies aimed at modifying pennycress have been reviewed in recent publications (Park & Kim, 2022; Sagun *et al*., 2023).

1.4.3 Factors affecting plant metabolic engineering studies

1.4.3.1 Transgenic source

In plant metabolic engineering, the choice of transgene source—the organism or species from which the foreign gene is isolated— significantly influences the transgene functionality within a plant host. For instance, a comparison of DGAT1 genes from seven different plants revealed that

when expressed in yeast cultures, DGAT1 genes from Jatropha, sesame, sunflower and Vernonia led to more than ten-fold increase of TAG content when compared to DGAT1 genes from Arabidopsis, castor bean, and soybean (Hatanaka *et al*., 2022). Likewise, in both coconut endosperm and California bay seeds, MCFA lauric acid (12:0) is the dominant fatty acid and it is produced by acyl-ACP thioesterase (FatB), comprising around 46% to 52% in coconut and approximately 70% in California bay (Jing *et al*., 2011; Pollard *et al*., 1991; Voelker *et al*., 1992; Yuan *et al*., 2017). Heterologous expression of California bay FatB in transgenic Arabidopsis seeds led to the successful accumulation of 12:0 and 14:0 MCFA with a higher accumulation of 12:0 compared to 14:0 mirroring the trend observed in California bay seeds (Voelker *et al*., 1992). However, when coconut FatB was expressed in Arabidopsis, the resulting transgenic seeds contained only 1.6% of 12:0 and 8.6% of 14:0 (Yuan *et al*., 2017), suggesting that California bay FatB is a more suitable transgene to produce 12:0 in transgenic Arabidopsis seeds. Another example involves the production of the omega-6 fatty acid, γ−linolenic acid (GLA; 18:3, Δ6,9,12), using Δ6-desaturase isolated from borage seed (Sayanova *et al*., 1997), and the fungus *Pythium irregulare (Hong et al., 2002)*. Expression of borage Δ6-desaturase in *Brassica juncea* led to the accumulation of approximately 10% GLA in transgenic seeds (Qiu *et al*., 2002), whereas expression of *Pythium irregulare* Δ6 desaturase, significantly increased GLA levels, reaching up to 40% of total seed fatty acids (Hong *et al*., 2002).

1.4.3.2 Promoter choice

The choice of promoter can also significantly impact the success of metabolic engineering efforts. Promoters are sequences of DNA located upstream of the transcription start site (TSS) of a gene (Conaway & Conaway, 1993). These sequences contain binding sites for transcription initiation

and regularity elements that determine when, where, and how strong a specific gene is expressed within cells (Franco-Zorrilla *et al*., 2014; Hernandez-Garcia & Finer, 2014). Factors such as developmental stage, tissue specificity, and optimal expression levels must all be considered before heterologous expression of genes. A promoter active only during seed development may be ideal for altering fatty acid metabolism in seeds, as it ensures the engineered genes are expressed at the right time and place to influence the desired metabolic pathways, preventing unwanted accumulation and off-target effects on other plant tissues (Millar *et al*., 1998).

For instance, transgenic Arabidopsis plants overexpressing Arabidopsis FAE1 using the cauliflower mosaic virus (CaMV 35S) constitutive promoter accumulated VLCFA in non-seed tissues resulting in altered plant morphology which included failure of flowering shoots to elongate, a modified spatial pattern of siliques, an altered floral phenotype, and a large accumulation of anthocyanins (Millar *et al*., 1998). Conversely, no morphological changes were reported in transgenic Arabidopsis plants overexpressing Arabidopsis FAE1 using the seed-specific phaseolin promoter (Ma *et al*., 2021). Furthermore, to achieve desired end-product levels in seeds during multi-gene engineering, careful selection of seed-specific promoters with optimal expression should be prioritized, while avoiding the use of multiple copies of the same promoter to prevent DNA instability and transgene silencing (Shockey *et al*., 2015). For example, varying levels of the conjugated fatty acid α-eleostearic acid (EA) were observed in transgenic Arabidopsis seeds expressing tung tree bifunctional fatty acid conjugase/desaturase driven by different seed-specific promoters including *β-conglycinin* α′-subunit, phaseolin, Arabidopsis *2S-3*, napin, and Arabidopsis *FAE1*. The highest amount of EA was obtained using the β-conglycinin promoter while the lowest was obtained using the FAE1 promoter (Shockey *et al*., 2015), emphasizing that the amount of product obtained is affected by promoter choice.

1.4.3.3 Suitable background

In addition to favorable agronomic properties of the plant host and polyploidy, a background suitable to produce high amount of engineered product is necessary. If such a background is not available, it can be created through various techniques, including increasing the availability of transgene-preferred substrates, eliminating endogenous competitive enzymes, and introducing multiple genes encoding downstream enzymes with a high affinity for the desired product. These modifications can be achieved using advanced genetic tools including T-DNA insertions and mutagenesis, CRISPR-Cas9, and RNAi.

1.4.3.3.1 Substrate availability and elimination of competitive enzymes

Substrate availability can influence the activity of transgenes in metabolic engineering studies (Bates, 2016; Bates & Browse, 2011). When pennycress FAE1, responsible for producing 22:1 VLCFA in pennycress seeds, was introduced into wild-type Arabidopsis seeds, a significant increase in 22:1 content was observed. However, when expressed in an Arabidopsis mutant lacking VLCFAs, including 20:1, pennycress FAE1 did not lead to significant accumulation of VLCFA, indicating that the enzyme's efficiency was reduced in the absence of its 20:1 substrate (Claver *et al*., 2020). To increase the availability of substrate molecules required for specific product biosynthesis, overexpression of enzymes involved in substrate synthesis or elimination of competitive enzymes utilizing the same substrates can be employed. CL37 is a transgenic Arabidopsis line expressing castor RcFAH12, involved in HFA biosynthesis, under the seed-specific phaseolin promoter in an Arabidopsis *fae1* mutant background (Lu *et al*., 2006). **Overexpression of** the transcription factor WRINKLED1 (WRI1), which positively regulates the

expression of genes involved in fatty acid synthesis, in transgenic Arabidopsis CL37 seeds, increased HFA accumulation by 8%- 31% (Adhikari *et al*., 2016). Alternatively, when the *fae1* and *fad2* genes, which utilize the same 18:1 substrate as RcFAH12, were mutated, the accumulation of HFA increased from 9.6 mol% in transgenic Arabidopsis lines expressing RcFAH12 alone to 19.2 mol% in transgenic lines expressing RcFAH12 in a *fae1/fad2* mutant background (Smith *et al*., 2003).

1.4.3.3.2 Expression of downstream enzymes

In some cases of plant metabolic engineering studies, the requirement for the expression of downstream enzymes with preference and selectivity for the engineered compounds is necessary to ensure their successful incorporation into a final product (Bates *et al*., 2014; Lunn *et al*., 2018; Lunn *et al*., 2022). For instance, Arabidopsis CL37 lines expressing RcFAH12, accumulate only ~17 mol% of HFA, lower than the 90% naturally produced in castor seed, and experience a 35-45% reduction in seed oil content compared to wild-type seeds (van Erp *et al*., 2011). This low amount of HFA and drop in oil content was attributed to the inefficient conversion of HFA-PC to HFA-DAG and its subsequent transfer into TAG due to Arabidopsis endogenous enzymes preferentially utilizing non-HFA substrates, causing accumulation of HFA and subsequent feedback inhibition of FA synthesis (Bates & Browse, 2011; Bates *et al*., 2014; Lunn *et al*., 2022). To overcome this challenge, a multi-gene approach was adopted, where a combination of enzymes involved in TAG biosynthesis from castor, specifically RcPDAT, RcGPAT9, and RcLPAT, were introduced into Arabidopsis CL37 transgenic lines. This strategy significantly increased HFA accumulation to 35 mol% while also increasing oil content to levels equivalent to those found in wild-type plants (Lunn *et al*., 2019). In camelina plants, a similar approach was employed, where

five essential castor genes involved in HFA biosynthesis, including RcFAH12, RcPDAT1-2, RcPDCT, and RcLPCAT, were co-expressed. This led to a notable increase in HFA content, reaching 25 mol% in seed oil (M.-E. Park *et al*., 2022), compared to 15 mol% obtained by expression of RcFAH12 alone (Snapp *et al*., 2014). This level was further increased to over 31% by knocking out the Arabidopsis *FAE1* gene in these plants (M.-E. Park *et al*., 2022).

1.4.3.4 Effect on seed properties and plant growth

Plant metabolic engineering can impact not only the desired metabolic pathways but also plant physiology and growth. Alterations in metabolic flux may redirect resources away from essential processes or cause feedback inhibition, potentially affecting seed properties such as seed vigor, yield, and overall plant fitness (Lunn *et al*., 2018). Accumulation of conjugated fatty acids (Cahoon *et al*., 2006), epoxy fatty acids (Li *et al*., 2012), and HFA (Adhikari *et al*., 2016; van Erp *et al*., 2011), in transgenic Arabidopsis caused reduction in seed oil content, seedling establishment, and/or seed germination (Lunn *et al*., 2018). When seeds begin to germinate, their metabolism relies on stored FAs derived from TAG through β-oxidation. This process is essential for sustaining early growth and hypocotyl elongation in seedlings after radicle emergence (Chapman & Ohlrogge, 2012; Lunn *et al*., 2018). In a study characterizing the Arabidopsis HFA synthesizing line CL37 (Lunn *et al*., 2018), it was found that although this line achieved parental levels of radicle emergence, it exhibited significant reductions in cotyledon opening (2.3-fold) and hypocotyl elongation (5.6-fold) compared to its *fae1* parent. Moreover, fully opened cotyledons of CL37 displayed chlorosis and a 1.6-fold reduction in chlorophyll content. However, the introduction of castor acyltransferases with preference for HFA, RcDGAT2 or RcPDAT1, along with RcFAH12, resulted in notable improvements. These enhancements included a 1.9- and 1.5-

fold increase in cotyledon opening and a 3.4-fold and 2.8-fold increase in hypocotyl elongation, respectively, when compared to CL37. Although neither of these lines achieved cotyledons and hypocotyls equivalent to those of the *fae1* parent, seedlings with fully opened cotyledons in the RcDGAT2 or RcPDAT1 co-expression lines exhibited healthy, green growth with no reduction in chlorophyll content. Additionally, seedlings that produced fully opened cotyledons almost uniformly developed two true leaves in all lines (Lunn *et al*., 2018). These findings indicated that CL37 experiences significant restrictions in its reproductive development compared to its non-HFA-expressing *fae1* parent. However, these defects were partially alleviated in lines co-expressing RcDGAT2 or RcPDAT1 due to increased flux of HFAs from PC into TAG and enhanced lipid mobilization (Lunn *et al*., 2018).

1.5 ACETYLTRIACYLGLYCEROLS (ACETYL-TAG)

1.5.1 Discovery of Acetyl-TAG in Nature

3-acetyl-1,2-diacyl-*sn*-glycerol (acetyl-TAG) are unusual TAG that have an acetate group esterified at the *sn*-3 position of the glycerol backbone and long-chain acyl groups esterified at the *sn*-1 and *sn*-2 positions (Figure 1.4). In literature, they are also referred to as 1,2-diacyl-3-acetins (monoacetotriglycerides) (Kleiman *et al*., 1967), acetodiacylglycerols (Parodi, 1975), monoacetyldiglycerides (MADG) (Limb *et al*., 1999), 1,2-diacyl-3-acetyl-*sn*-glycerols (acTAG) (Milcamps *et al*., 2005), and acDAG (Sidorov *et al*., 2013). Acetyl-TAG were first reported in the seeds of *Impatiens roylei* from the Balsaminaceae plant family (Kaufmann & Keller, 1948). Subsequent studies showed that they are produced naturally in the seeds of many species including those belonging to the Celestraceae family (spindle-tree family) as well as some members of the Ericales, Lardizabalaceae, Ranunculales and Rosaceae families (Bagby & Smith, 1967; Kleiman

et al., 1967). In *Euonymus* species (Celestraceae), acetyl-TAG is predominately produced in the seeds, although trace amounts have been observed in the fruit arils as well. The abundance of acetyl-TAG varies among species, with levels ranging from 13% to 98% in seeds (Kleiman *et al*., 1967; Sidorov *et al*., 2014). *Euonymus alatus*, commonly known as the Burning Bush due its bright red leaves color in fall, is a member of the *Euonymus* L. genus in the Celastraceae family and is commonly used in Chinese folk medicine. Its natural habitat includes eastern Russia, Japan, China, and Korea, but it has been cultivated as an ornamental plant in both Europe and North America. The aril of *Euonymus alatus* fruit was found to mainly consist of regular TAG, constituting approximately 92% of the total lipid content, while acetyl-TAG only accounted for about 0.5% (Durrett *et al*., 2010). Conversely, in the whole seed, acetyl-TAG was the dominant form, comprising up to 92% of the TAG, with regular TAG making up around 3% (Durrett *et al*., 2010). Further analysis revealed that in the endosperm, acetyl-TAG represented about 94.5% of the total lipid content, while regular TAG was present at a much lower level of about 1.6%. In the embryo, acetyl-TAG accounted for approximately 65.6% of the total lipid, while regular TAG constituted about 26% (Durrett *et al*., 2010).

The occurrence of acetyl-TAG has also been reported in other organisms. Acetyl-ATGs were detected in milk fat lipids and bovine udders (Limb *et al*., 1999; Parodi, 1975), deer antlers (Pham *et al*., 2019; Yang *et al*., 2004), and the larvae of the goldenrod gall fly (*Eurosta solidagini*) (Marshall *et al*., 2014). Acetyl-TAG is believed to play a role in freeze tolerance in the goldenrod gall fly, accumulating before overwintering and decreasing in the spring (Marshall *et al*., 2014). Despite this finding, the specific physiological and functional role of acetyl-TAG in plant seeds and other animal organisms remains to be fully explored.

1.5.2 Properties and Applications of Acetyl-TAG

The presence of an acetyl group instead of a longer fatty acyl group gives acetyl-TAG altered physical and chemical properties that make them of particular interest in various industrial applications. Acetyl-TAG purified from *Euonymus alatus* seed oil possessed 39% reduction in kinematic viscosity compared to regular TAG in soybean oil, meeting the ASTM specifications for Diesel Grade No. 4-D, commonly used in low- and medium-speed engines (Durrett *et al*., 2010). Unlike conventional vegetable oils, which have high viscosity and require transesterification to produce biodiesel, acetyl-TAG oil allows the possibility of direct combustion without the need for further processing (Durrett *et al*., 2008). In addition, acetyl-TAG extracted from camelina seed oil, and from the fat body of the goldenrod gall fly, was reported to have lower melting temperatures (Liu, Tjellström, *et al*., 2015; Marshall *et al*., 2014). Due to the similarity of acetyl-TAG to current synthetic acetylated mono- and diacylglycerols (ACETEM), acetyl-TAG-rich oils offers potential benefits in areas where low viscosity and melting point is desirable, such as in the formulation of biodegradable lubricants and plasticizers, as well as food-grade coatings and emulsifiers (Gaupp & Adams, 2004; Liu, Tjellström, *et al*., 2015).

Moreover, acetyl-TAG is also considered an emerging functional lipid in the dietary and health sectors. Acetyl-TAG oil has reduced-calorific values (Durrett *et al*., 2010), and is a natural equivalent to commercial low-calorie fats such as SALATRIM (Smith *et al*., 1994), and active ingredient in functional food like EC-18 (Kim *et al*., 2009; Yang *et al*., 2004). SALATRIM products, which consist of a combination of long-chain saturated fatty acids (such as stearic) and short-chain fatty acids (acetic, propionic, and/or butyric) (Smith *et al*., 1994), have demonstrated efficacy in reducing hunger and increasing fullness in clinical trials when compared to typical fats (Sørensen *et al*., 2008). EC-18 is a synthetic acetyl-TAG with a 1-palmitoyl-2-linoleoyl-3-

acetylglycerol structure in which its natural analogue (PLAG) has been previously isolated from the antlers of sika deer (Kim *et al*., 2009; Yang *et al*., 2004). It has been shown to play a role in the treatment of cancers (Kim *et al*., 2009), sepsis (Hong *et al*., 2010), asthma (Shin *et al*., 2014), inflammations (Shin *et al*., 2016), and arthritis (Kim *et al*., 2017; J. S. Park *et al*., 2022). Introducing acetyl-TAG to edible oilseed crops could therefore offer a desirable dietary supplement for human consumption with beneficial therapeutic properties.

1.5.3 Discovery and Isolation of Acetyltransferases

Similar to regular TAG, in which DGAT1 acylates the *sn*-3 hydroxyl group of DAG, acetyl-TAG are produced through the acetylation of the *sn*-3 hydroxyl group of DAG by the action of diacylglycerol:acetyl-CoA acetyltransferases (DAcTs; Figure 1.5). Initially, a gene showing high sequence similarity to *DGAT1* gene family, was identified and isolated from the developing seeds of *Euonymus alatus* (Milcamps *et al*., 2005). Yeast microsomes transformed with the *Euonymus DGAT1* gene (EaDGAT1) exhibited a 40-fold higher acetyltransferase activity in comparison to yeast transformed with either the empty vector or the Arabidopsis DGAT1. Nevertheless, overexpression of EaDGAT1 in yeast led to a 5-fold increase in regular TAG and only very minimal accumulation of acetyl-TAG (0.26% of regular TAG) (Milcamps *et al*., 2005). A subsequent comparative transcriptomic analysis was conducted on the developing endosperm and aril tissues of *Euonymus alatus* fruit using pyrosequencing technology with the same aim of isolating the enzyme responsible for acetyl-TAG synthesis (Durrett *et al*., 2010). The study revealed an uncharacterized ER-localized membrane-bound O-acyltransferase (MBOAT) family member, that was abundant in the endosperm but absent from the aril, matching the accumulation pattern of acetyl-TAG in these tissues. Unlike the previously isolated EaDGAT1, this MBOAT,

named *Euonymus alatus* diacylglycerol acetyltransferase (EaDAcT), exhibited acyltransferase activity but lacked long-chain acyltransferase activity. When introduced into yeast, EaDAcT facilitated the accumulation of acetyl-TAG, while regular TAG were not produced (Durrett *et al*., 2010). This finding marked the initial isolation of an enzyme specifically involved in acetyl-TAG synthesis. Interestingly, despite both DGAT1 and EaDAcT acylating DAG, EaDAcT showed a more distant relationship to DGAT1 and a closer association with *Simmondsia chinensis* (jojoba) wax synthase (ScWS) and Arabidopsis sterol acyltransferase (AtASAT1).

Following the discovery of EaDAcT, several other acetyltransferases with similar sequences have been isolated from various *Euonymus* species. These enzymes include EtDAcT, EbDAcT, EkDAcT and EfDAcT isolated from *E. atropurpureus*, *E. bungeanus*, *E. kiautschovicus* and *E. fortunei*, respectively (Tran *et al*., 2017), as well as EeDAcT from *E. europaeus (Mihálik et al., 2020)*. Like EaDAcT, these newly discovered DAcT enzymes did not produce regular TAG when expressed in yeast, and all displayed acetyltransferase activity *in vitro* (Gajdoš *et al*., 2020; Tran *et al*., 2017). However, their acetyltransferase activity varied, and this difference was reflected in their capacity to synthesize acetyl-TAG (Tran *et al*., 2017). For example, yeast expressing EfDAcT demonstrated the highest acetyltransferase activity *in vitro* and accumulated the highest amount of acetyl-TAG *in vivo*, which was 6.7 times higher than the amount achieved with EaDAcT expression (Tran *et al*., 2017). EfDAcT shares 94% sequence identity with EaDAcT with a 22 amino acids difference (Tran *et al*., 2017). Despite the high sequence similarity of EfDAcT and EaDAcT, the specific factors responsible for their divergent acetyltransferase activities remain currently unknown. These differences in activity could be attributed to subtle variations in structure and active site residues, substrate specificity, regulatory mechanisms, or other factors influencing the enzymatic function of these homologous enzymes in their respective

species. Further research is needed to elucidate the exact reasons for the observed differences in acetyltransferase activity between EfDAcT and EaDAcT.

1.5.4 Substrate Specificity of Acetyltransferases

Insights into the substrate specificity of acetyltransferases were obtained through *in vitro* substrate specificity assays of isolated enzymes, and by analyzing the fatty acid composition and positional-species composition of seed oils across multiple species belonging to the *Euonymus* L. genus within the Celastraceae family (Bansal & Durrett, 2016b; Sidorov *et al*., 2016). *In vitro* assays of EaDAcT revealed that despite its substantial sequence similarity and identity to AtASAT1 and ScWS, it has a distinct preference for acetyl-CoA as the acyl donor substrate (Bansal & Durrett, 2016b). EaDAcT displayed an ability to also utilize acyl-CoAs with chain lengths shorter than eight carbons, although with decreased efficiency. However, it exhibited minimal to no activity with acyl-CoAs exceeding eight carbons. Additionally, the enzyme is capable of acetylating DAG molecules with a range of acyl chain lengths, particularly favoring DAGs with unsaturated fatty acids (FAs) over saturated FAs. Notably, DAG species containing only saturated fatty acids longer than 14 carbons at *sn*-1 and *sn*-2 positions were not incorporated at all in acetyl-TAG. Beyond its primary activity, EaDAcT also showed that it can acetylate aliphatic chain fatty alcohols in vitro to produce alkyl acetates (Bansal & Durrett, 2016b). A comprehensive analysis spanning 14 species within the *Euonymus* L. genus unveiled distinct trends of Acetyl-TAG composition. Species falling under the *Euonymus* section (*E. bungeanus, E. europaeus, E. hamiltonianus, E. phellomanus, E. semiexsertus, and E. sieboldianus*) displayed the highest levels of acetyl-TAG positional species with one or two oleic acids (Sidorov *et al*., 2016). Furthermore, the seed compositions across almost all *Euonymus* species featured acetyl-TAG enriched in both oleic and

linoleic fatty acids predominantly esterified at the *sn*-2 position of the glycerol backbone. Conversely, saturated fatty acid residues were absent from the *sn*-2 position of acetyl-TAG (Sidorov *et al*., 2016).

Additionally, it has been established that the acetylation activity of the studied acetyltransferase, such as EaDAcT, is exclusively specific to the *sn*-3 position of the glycerol backbone (Bansal & Durrett, 2016b; Kleiman *et al*., 1967). Nevertheless, it is plausible to speculate that other types of acyltransferases in nature might possess the ability to utilize acetyl-CoA as a substrate at different positions on the glycerol backbone. A case in point involves the neutral lipids of *Polygala virgata* and *Rumex paulsenianus* (Polygalaceae family) seeds. Research have showed that around 74% of the total TAG in *Polygala virgata* seeds is of an *sn*-2-acetyl-TAG structure (Smith *et al*., 1977; Smith *et al*., 2018). Similarly small amount of *sn*-2-acetyl-TAG were detected in *Rumex paulsenianus* seeds (Gusakova *et al*., 1990). However, the specific enzymes accountable for *sn*-2-acetyltransferase activity in these instances remain to be identified and characterized.

1.5.5 Production of Acetyl-TAG in Transgenic Plants

In recent years, some progress has been made in the production of acetyl-TAG in transgenic plants through genetic engineering. This progress began with the model plant *Arabidopsis thaliana*, where successful production of acetyl-TAG was demonstrated in seeds through the expression of the acetyltransferase EaDAcT. In these transgenic Arabidopsis seeds, the accumulation of acetyl-TAG reached up to 40 mol%, while regular TAG comprised 60 mol% in homozygous T3 transgenic lines. Some changes in the fatty acid composition of seed lipids were recorded, particularly a rise in linoleic acid (18:2) (Durrett *et al*., 2010). Subsequent efforts extended to oilseed crops such as camelina and soybean. Expression of EaDAcT in soybean seeds led to the

accumulation of acetyl-TAG up to 70 mol%, while in camelina, acetyl-TAG levels ranged from 47 to 64 mol% across independent lines (Liu, Rice, *et al*., 2015). In comparison to WT and regular TAG fraction of transgenic camelina seeds, acetyl-TAG displayed an approximate twofold decrease in VLCFA content, coupled with an increase in the presence of polyunsaturated fatty acids (PUFA) such as 18:2 and 18:3 (Liu, Rice, *et al*., 2015). Since the *sn*-3 position of acetyl-TAG is occupied with acetate, these results align with the known pattern within the Brassicaceae plant family, where VLCFA tend to predominantly occupy the *sn*-1 and *sn*-3 positions of TAG molecules (Takagi & Ando, 1991; Taylor *et al*., 1995). The feasibility of scaling up acetyl-TAG production was explored by cultivating the highest acetyl-TAG/TAG ratio transgenic camelina lines in field conditions. Notably, field cultivation of the transgenic camelina lines resulted in acetyl-TAG levels consistent with growth-chamber-grown plants, with an average composition of 64.6 mol% in some cases. Acetyl-TAG accumulation did not appear to significantly impact key seeds traits or the overall morphology of camelina plants (Liu, Rice, *et al*., 2015). Pennycress seeds were also successfully engineered to produce acetyl-TAG by transformation of EaDAcT. Similar to camelina, pennycress transgenic seeds exhibited lower levels of VLCFA such as C22:1 in acetyl-TAG compared to regular TAG (McGinn *et al*., 2019). Furthermore, the gene responsible for acetyl-TAG synthesis from *E. europaeus* (EeDAcT) was introduced into tobacco plants. The successful overexpression of the EeDAcT gene in these transgenic tobacco plants facilitated the accumulation of acetyl-TAG within immature seeds (Mihálik *et al*., 2020).

Additionally, the synthesis of acetyl-TAG can be combined with the production of other unusual FAs by leveraging the unique enzymatic capabilities present in multiple species. This allows the production of acetyl-TAG containing unusual FAs at the *sn*-1 and *sn*-2 positions. For instance, a high-oleic camelina line, developed through the RNAi-mediated suppression of FAD2

and FAE1 (Nguyen *et al*., 2013), was genetically engineered to express EaDAcT, thereby facilitating the production of acetyl-TAG oils enriched with high levels of oleic acid (Liu, Tjellström, *et al*., 2015). Field trials of high-oleic camelina lines expressing EaDAcT, demonstrated an accumulation of approximately 70 mol% acetyl-TAG. The molecular species of acetyl-TAG synthesized in high-oleic camelina predominantly consisted of acetyl-dioleoyl-glycerol. As with previous results in wild-type background, the introduction of EaDAcT into field-grown high-oleic transgenic camelina had minimal effects on oil content, seed weight, seed yield, and harvest index (Liu, Tjellström, *et al*., 2015). Another example involves transgenic camelina lines that were initially engineered to synthesize medium-chain fatty acids (MCFAs; C6–C14) through the expression of MCFA-specific thioesterases and acyltransferases (*Cuphea palustris* CpFatB2, *Cuphea hookeriana* ChFATB2, *Umbellularia californica* UcFatB1, and *Cocos nucifera* CnLPAT). These lines were subsequently transformed with EaDAcT to produce acetyl-TAG containing MCFAs (Bansal *et al*., 2018). The integration of EaDAcT led to acetyl-TAG levels of approximately 50 mol% in the T3 seed of the most promising transgenic lines within the UcFatB1+CnLPAT background, and approximately 34 mol% within the CpFatB2+ChFatB2 background. Nevertheless, it's important to highlight that acetyl-TAG in these instances demonstrated reduced concentrations of MCFAs compared to the standard TAG produced within the corresponding lines. For instance, in UcFatB1+CnLPAT lines, 12:0 levels in acetyl-TAG were, on average, half of those in TAG. Similarly, within the CpFatB2+ChFatB2 lines, 10:0 levels in acetyl-TAG were around four times lower than those in TAG (Bansal *et al*., 2018). Pennycress was also engineered to produce acetyl-TAG rich in MCFAs. Lines expressing EaDAcT were crossed with lines producing elevated levels of MCFAs, resulting in plants with the capacity to synthesize acetyl-TAG containing MCFAs (Esfahanian *et al*., 2021). Co-expressing either

UcFatB1 + CnLPAT or CpFatB2 + ChFatB2 alongside EaDAcT accumulated approximately 45-55 mol% of acetyl-TAG in the seeds of the most promising transgenic lines, mirroring the levels achieved with EaDAcT expression alone in wild-type lines. In lines where CpFatB2 + ChFatB2 were expressed, acetyl-TAG displayed reduced levels of MCFAs shorter than 14:0 in comparison to TAG. On the contrary, lines expressing UcFatB1 + CnLPAT alongside EaDAcT demonstrated a tendency towards higher levels of 8:0 and 12:0 in acetyl-TAG (Esfahanian *et al*., 2021). These findings resemble the results observed in camelina acetyl-TAG MCFA lines.

1.5.6 Increasing Acetyl-TAG Levels in Transgenic Plants

As previously discussed, expression of isolated acetyltransferases alone such as EaDAcT in oilseed crops resulted in a maximum of 70 mol% of acetyl-TAG production as described in soybean and high-oleic camelina seeds (Liu, Rice, *et al*., 2015; Liu, Tjellström, *et al*., 2015). To further increase acetyl-TAG production in transgenic plants, studies have investigated how EaDAcT interacts with key TAG biosynthetic enzymes. By examining the interplay between EaDAcT and DGAT1 in Arabidopsis, it was revealed that in an Arabidopsis *dgat1* mutant background, acetyl-TAG levels increased significantly to approximately 60–65 mol% of total TAG in contrast to the 45 mol% observed in the wild-type background (Liu, Rice, *et al*., 2015). Given the intricate genome complexities of camelina, which harbors up to three homoeologs of each enzyme expressed in seeds, researchers designed an RNAi hairpin loop to target DGAT1. By simultaneously suppressing all three camelina DGAT1 homoeologs and co-expressing EaDAcT, acetyl-TAG accumulation of around 85 mol% was achieved in the best lines (Liu, Rice, *et al*., 2015). This success extended to high-oleic camelina lines, where co-expression of EaDAcT with DGAT1 RNAi led to similar results (Liu, Tjellström, *et al*., 2015). Acetyl-TAG increase in *dgat1*

mutant/suppressed lines highlighted the shared utilization of a DAG pool by both EaDAcT and DGAT1, despite their relatively low sequence identity and predicted differences in membrane topology. Interestingly, suppression of PDAT1, another enzyme involved in TAG synthesis, did not significantly influence acetyl-TAG levels in both high-oleic and wild-type camelina backgrounds (Liu, Rice, *et al*., 2015; Liu, Tjellström, *et al*., 2015). In the MCFA backgrounds, co-expression of EaDAcT with DGAT1 RNAi led to approximately 77 mol% acetyl-TAG accumulation in UcFatB1+CnLPAT lines and ~42 mol% in CpFatB2 + ChFatB2 lines (Bansal *et al*., 2018). However, simultaneous suppression of both DGAT1 and PDAT1 did not yield significant gains, echoing earlier observations in the wild-type background. One plausible explanation for this trend is that the downregulation of both DGAT1 and PDAT1, due to their roles in seed development as demonstrated in Arabidopsis (Zhang *et al*., 2009), likely affect the embryo development process in camelina. Despite these efforts to enhance acetyl-TAG production, the levels achieved remained lower than the natural amounts of acetyl-TAG observed in *Euonymus* seeds (Kleiman *et al*., 1967).

1.6 RESEARCH OVERVIEW

The primary aim of the work in this dissertation is to build upon previous attempts to optimize and increase acetyl-TAG production in transgenic oilseed crops. This work involved multiple metabolic engineering strategies, with the goal of surpassing 90 mol% acetyl-TAG content – akin to the levels naturally occurring in *Euonymus* seeds (Figure 1.5b). Chapter 2 of this dissertation describes the alternative expression of *EfDAcT* from *Euonymus fortunei* in transgenic camelina seeds instead of *EaDAcT*. This choice is attributed to EfDAcT higher acetyltransferase activity. Additionally, this section explores the synergistic approach of combining *EfDAcT* expression with

the suppression of the endogenous TAG-synthesizing enzyme *DGAT1*. Chapter 3 focuses on characterizing the top-performing acetyl-TAG producing line in transgenic camelina through RNA-seq and lipidomic analyses. The goal is to identify comprehensive changes in gene expression and lipid composition resulting from *EfDAcT* expression and acetyl-TAG accumulation. Chapter 4 explores the impact of altering the fatty acid composition of camelina and pennycress lines, which are engineered to express *EfDAcT*, on the accumulation of acetyl-TAG. Specifically, this involves eliminating VLCFA and enhancing acetyl-CoA availability via *fae1* mutation. Chapter 5 serves as the concluding chapter, summarizing the significant findings and conclusions drawn from each preceding chapter while also suggesting potential avenues for future research.

FIGURES

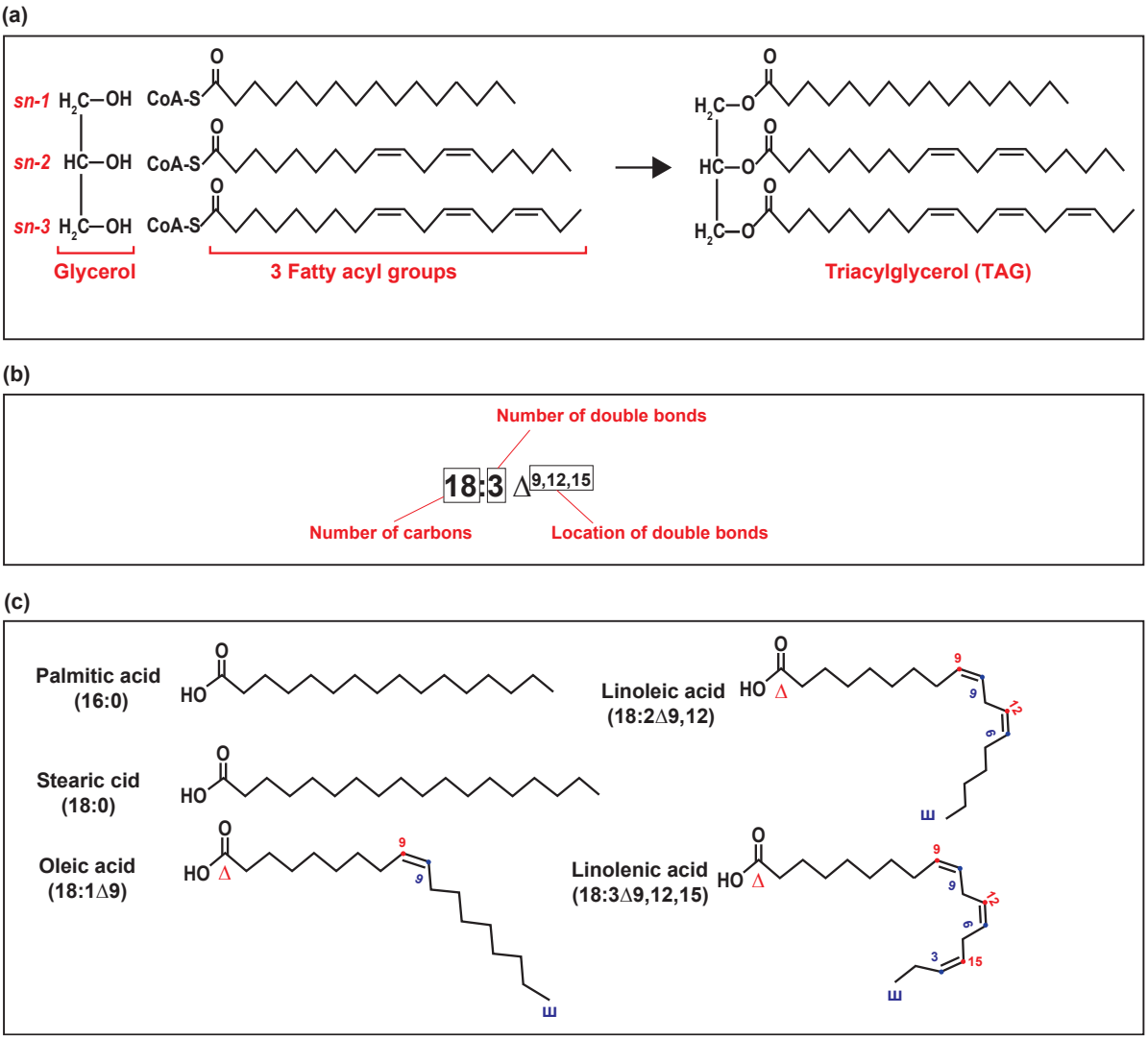


Figure 1.1. Structures of triacylglycerol and common fatty acids.

(a) General structure of a triacylglycerol (TAG) molecule. *sn* represents a stereospecific numbering (*sn*) system to denote the position of carbon atoms/fatty acids on the glycerol backbone. (b) Example of a fatty acid nomenclature. (b) Structures of major fatty acids found in oilseeds. Δ (red) represent the location of double bonds from the carboxyl group; ω (blue) represent the location of double bonds from the methyl group.

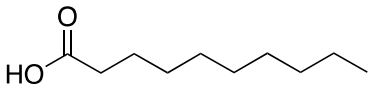
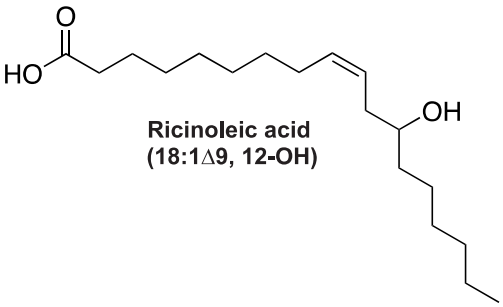
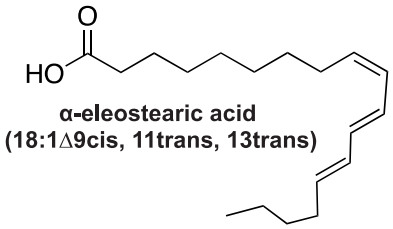
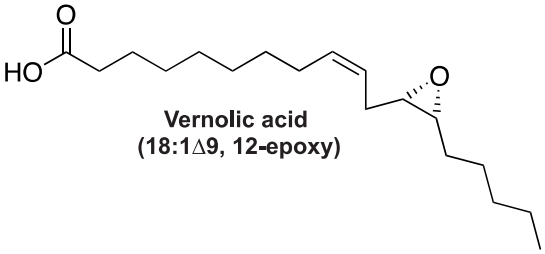
Unusual Fatty acids	Structure	Example of natural occurrence
Medium chain fatty acid	 Capric acid (10:0)	*Cuphea viscosissima* (Clammy waxweed)
Hydroxy fatty acid	 Ricinoleic acid (18:1△9, 12-OH)	*Ricinus communis* (Castor bean)
Conjugated fatty acid	 α-eleostearic acid (18:1△9cis, 11trans, 13trans)	*Vernicia fordii* (Tung tree)
Epoxy fatty acid	 Vernolic acid (18:1△9, 12-epoxy)	*Vernonia galamensis* (Ironweed)

Figure 1.2. Structures and natural sources of unusual fatty acids in plants.

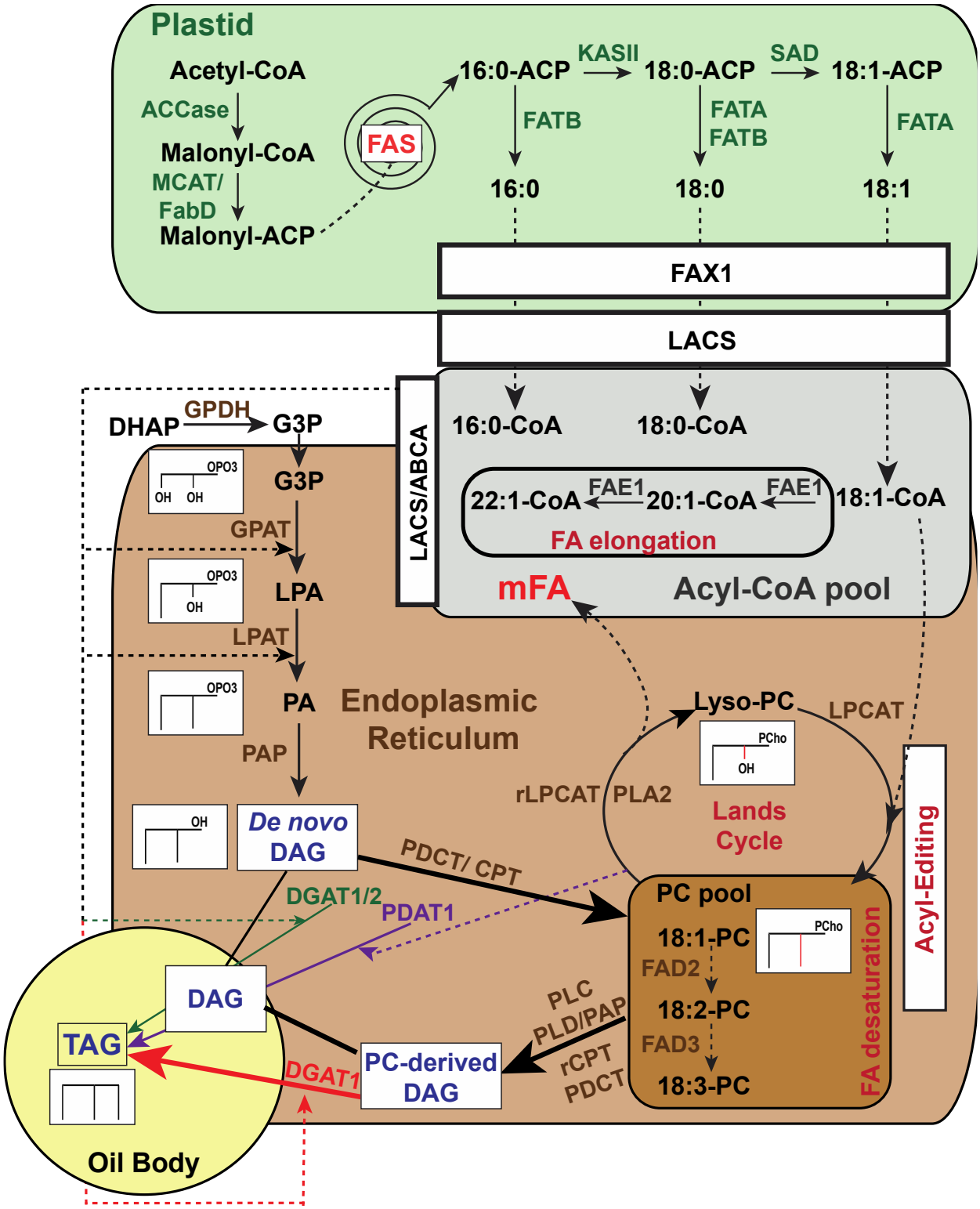


Figure 1.3. Overview of fatty acid and TAG biosynthesis

Dashed arrows represent fatty acid transport/addition. Abbreviations: ACP, acyl carrier protein; DHAP, dihydroxyacetone phosphate; G3P, glycerol-3-phosphate; LPA, lyso-phosphatidic acid; PA, phosphatidic acid; DAG, diacylglycerol; PC, phosphatidylcholine; Lyso-PC, lysophosphatidylcholine; TAG, triacylglycerol; mFA, modified fatty acids. 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; LACS , long-chain acyl-CoA synthetase; ABCA, ATP-binding cassette transporter; 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; LPAT, lysophosphatidic acid acyltransferase; PAP, phosphatidic acid phosphatase; DGAT, diacylglycerol acyltransferase; PDAT1; phospholipid:diacylglycerol acyltransferase 1. r, reverse activity.

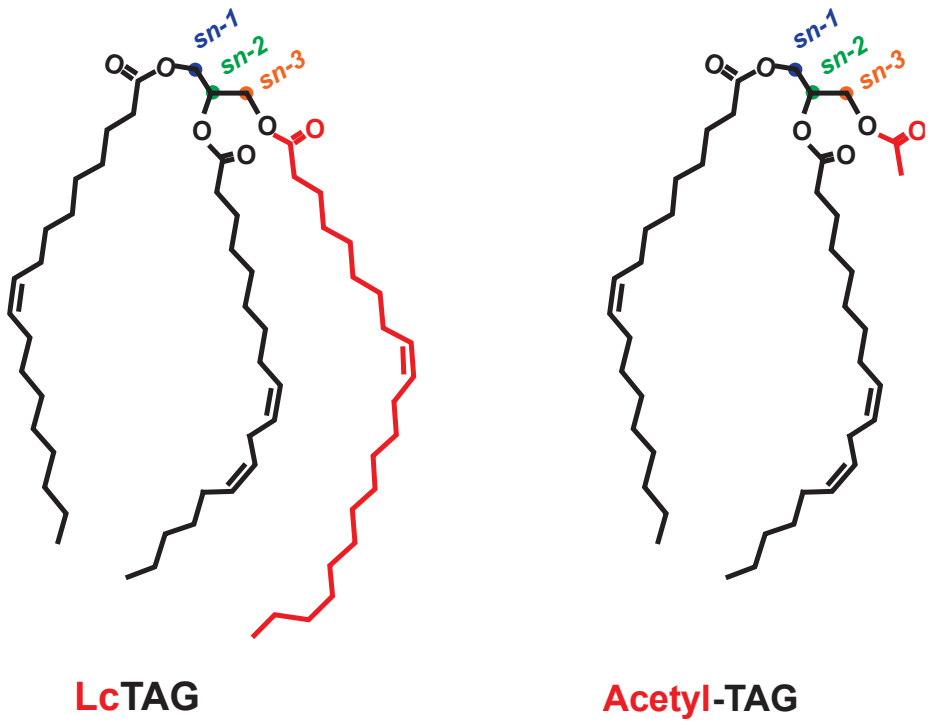


Figure 1.4. Acetyl-TAG structure.

Structure of a 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 the long chain acyl group (red) present in lcTAG.

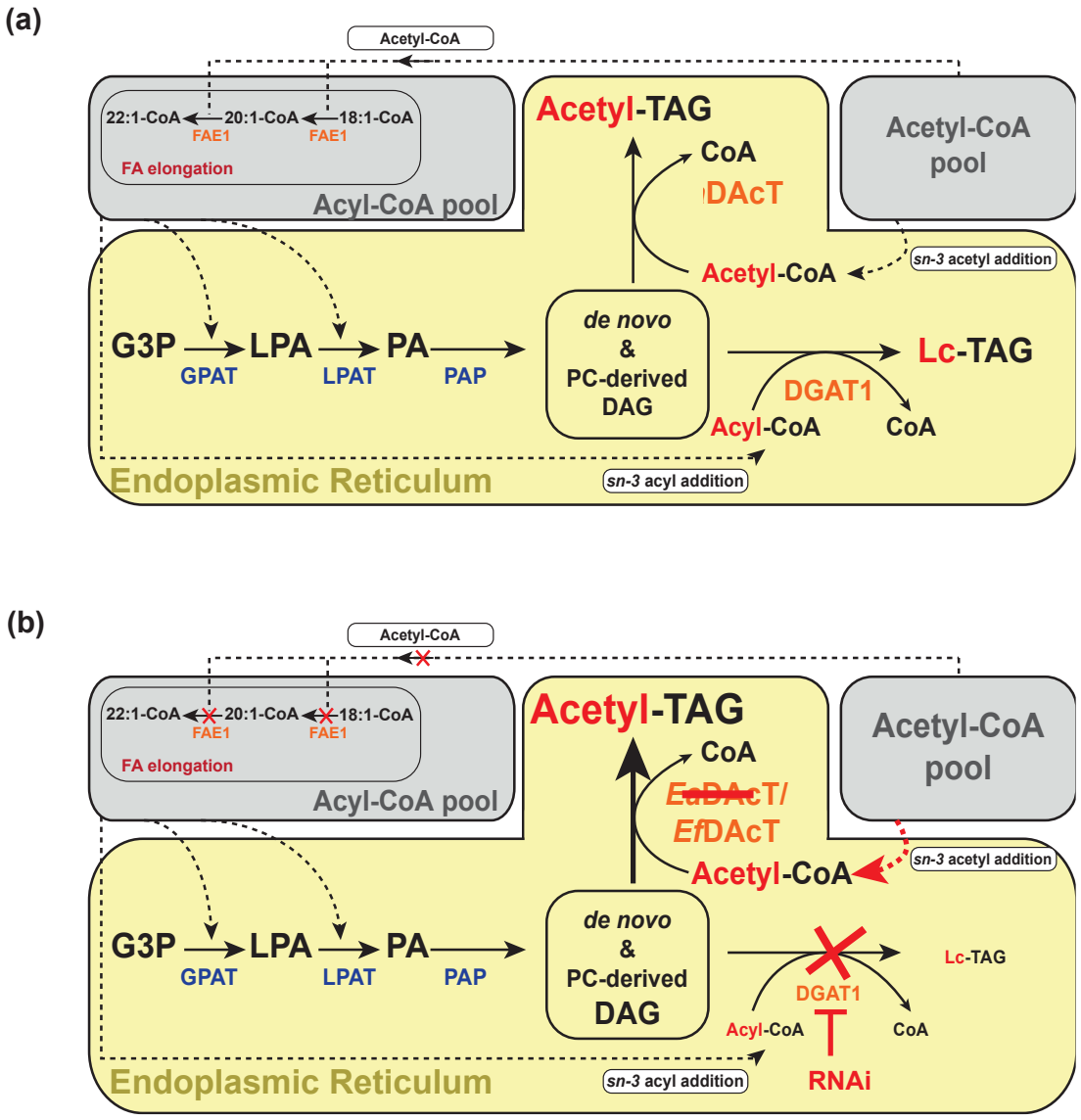


Figure 1.5. A simplified pathway of acetyl-TAG biosynthesis.

(a)Overview of the last step in acetyl-TAG synthesis. Acetyl-TAGs are synthesized by the action of DAcT, which transfer an acetate group from acetyl-CoA to the sn-3 position of DAG. In contrast, lc-TAGs are produced by DGAT1, which transfers an acyl group to the sn-3 position of DAG. (b) Summary of the approaches employed in this study to increase acetyl-TAG accumulation. These approaches involve introducing a high-activity acetyltransferase (EfDAcT), utilizing RNAi to inhibit DGAT1 and elevate DAG levels, and *fae1* mutation to raise acetyl-CoA levels. Abbreviations: G3P, glycerol-3- phosphate; LPA, lysophosphatidic acid; PA, phosphatidic acid; DAG, diacylglycerol; LcTAG, triacylglycerol; Acetyl-TAG, acetyl-triacylglycerol. FAE1, fatty acid elongase 1; GPAT, glycerol 3-phosphate acyltransferase; LPAT, lysophosphatidic acid acyltransferase; PAP, phosphatidic acid phosphatase; DGAT1, diacylglycerol acyltransferase 1; DAcT, diacylglycerol acetyltransferase; EfDAcT, *Euonymus fortunei* diacylglycerol acetyltransferase.

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Chapter 2 - Expression of a high-activity diacylglycerol acetyltransferase results in enhanced synthesis of acetyl-TAG in camelina seed oil

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ABSTRACT

Acetyl-triacylglycerols (acetyl-TAG) contain an acetate group in the *sn*-3 position instead of the long-chain fatty acid present in regular triacylglycerol (TAG). The acetate group confers unique physical properties such as reduced viscosity and a lower freezing point to acetyl-TAG, providing advantages for use as emulsifiers, lubricants, and 'drop-in' biofuels. Previously, the synthesis of acetyl-TAG in the seeds of the oilseed crop camelina (*Camelina sativa*) was achieved through the heterologous expression of the diacylglycerol acetyltransferase gene *EaDAcT*, isolated from *Euonymus alatus* seeds that naturally accumulate high levels of acetyl-TAG. Subsequent work identified a similar acetyltransferase, EfDAcT, in the seeds of *Euonymus fortunei*, that possesses higher *in vitro* activity compared to EaDAcT. In this study, the seed-specific expression of *EfDAcT* in camelina led to a 20 mol% increase in acetyl-TAG levels over that of *EaDAcT*. Coupling *EfDAcT* expression with suppression of the endogenous competing enzyme DGAT1 further enhanced acetyl-TAG accumulation, up to 90 mol% in the best transgenic lines. Accumulation of

high levels of acetyl-TAG was stable over multiple generations, with minimal effect on seed size, weight, and fatty acid content. Slight delays in germination were noted in transgenic seeds compared to the wild type. EfDAcT transcript and protein levels were correlated during seed development with a limited window of EfDAcT protein accumulation. In high acetyl-TAG producing lines, EfDAcT protein expression in developing seeds did not reflect the eventual acetyl-TAG levels in mature seeds, suggesting that other factors limit acetyl-TAG accumulation.

INTRODUCTION

The wide diversity of structures present in seed storage lipids throughout the plant kingdom imparts different properties to these seed oils, making them useful for specific applications. For example, the seeds of many *Euonymus* species synthesize 3-acetyl-1,2-diacyl-*sn*-glycerols (acetyl-TAG) as their main storage lipid (Kleiman *et al*., 1967; Sidorov *et al*., 2014; Tran, Shelton, *et al*., 2017). The *sn*-3 acetate group in these unusually structured triacylglycerols (TAG) confers favorable physical properties compared to the typical TAG (here referred to as lcTAG to indicate the presence of the *sn*-3 long-chain fatty acid) found in most vegetable oils. Acetyl-TAG exhibit reduced viscosity, possess superior cold-temperature characteristics, and have 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, Rice, *et al*., 2015; Liu, Tjellström, *et al*., 2015).

Poor agronomic properties or restricted areas for cultivation greatly limit the production of industrially relevant quantities of modified oils, including acetyl-TAG. The goal, therefore, of many metabolic engineering efforts is to develop transgenic oil seeds capable of synthesizing the unusual lipids produced by other plant species. A comparative transcriptome profiling approach

identified *Euonymus alatus* diacylglycerol acetyltransferase (EaDAcT) as the enzyme responsible for acetyl-TAG synthesis in the seeds of the burning bush shrub (Durrett *et al*., 2010). Expression of *EaDAcT* in yeast and different plant species successfully led to the production of high levels of acetyl-TAG (Durrett *et al*., 2010; Liu, Rice, *et al*., 2015; McGinn *et al*., 2019; Tran, Breuer *et al*., 2017). For example, expression of *EaDAcT* in the oilseed crop camelina (*Camelina sativa*) resulted in acetyl-TAG levels between 47 mol% and 64 mol% in transgenic seeds. Acetyl-TAG content in Arabidopsis (*Arabidopsis thaliana*) and camelina were further increased by the elimination or suppression of the endogenous diacylglycerol acyltransferase 1 (DGAT1) activity responsible for the synthesis of lcTAG. RNA interference (RNAi)-mediated suppression of the three camelina *DGAT1* homoeologs resulted in acetyl-TAG content as high as 85 mol% in the seeds of the best transgenic line (Liu, Rice, *et al*., 2015). Importantly, the synthesis of such high levels of exogenous lipids did not appear to affect the physiology and function of the transgenic seed, with no obvious effects on germination and only minor reductions in seed oil content. The subsequent field growth of high-producing acetyl-TAG lines enabled the production of large quantities of acetyl-TAG, allowing functional analysis of acetyl-TAG properties, including viscosity and freezing point. While the synthesis of 85 mol% in camelina represents one of the highest levels of synthesis of novel oil compositions in seeds, this level is still lower than that in the seeds of *E. alatus* and other species that naturally produce acetyl-TAG (Durrett *et al*., 2010; Sidorov *et al*., 2014). Furthermore, the development of oilseeds producing pure acetyl-TAG would eliminate the need for post-harvest purification of these useful lipid molecules, greatly reducing production costs.

EaDAcT 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, EaDAcT

efficiently uses acetyl-CoA to acylate the *sn*-3 position of *sn*-1,2-diacylglycerol (DAG) to form acetyl-TAG (Bansal and Durrett, 2016; Durrett *et al*., 2010). With regard to acyl acceptor specificity, substrate selectivity experiments demonstrated that EaDAcT preferentially acetylates DAG containing more polyunsaturated fatty acids (PUFA; Bansal and Durrett, 2016). We recently identified and characterized additional DAcT enzymes from different plants that synthesize acetyl-TAG. All these enzymes possess a high level of primary sequence identity with EaDAcT and can synthesize [^{14}C] acetyl-TAG in yeast microsomes when incubated with [^{14}C] acetyl-CoA. One such enzyme, EfDAcT, isolated from *Euonymus fortunei*, possessed higher *in vitro* activity compared to EaDAcT. Furthermore, yeast expressing *EfDAcT* produced almost sevenfold more acetyl-TAG than yeast expressing *EaDAcT*, consistent with its higher *in vitro* activity (Tran, Shelton, *et al*., 2017).

Here, we demonstrate that *EfDAcT* expression in camelina seeds results in the production of higher acetyl-TAG levels compared to *EaDAcT*. We combined *EfDAcT* expression with DGAT1 suppression to further enhance acetyl-TAG accumulation, resulting in 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 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, thereby providing additional insights about factors limiting acetyl-TAG accumulation.

RESULTS

Seed expression of *EfDAcT* results in higher levels of acetyl-TAG

Previously we demonstrated that EfDAcT possesses higher *in vitro* acetyltransferase activity compared to six other DAcT enzymes, including EaDAcT (Tran, Shelton *et al*., 2017). To test whether EfDAcT would produce higher levels of acetyl-TAG than EaDAcT in seeds, we used the strong seed-specific soybean (*Glycine max*) glycinin promoter to express hemagglutinin (HA) epitope-tagged versions of both genes in wild-type camelina. Transgenic T_2 seed from lines with single transgene insertions were identified based on the segregation of DsRed fluorescence and their acetyl-TAG levels were quantified. Expression of *EfDAcT* resulted in a mean of 72 mol% acetyl-TAG in T_2 seed, significantly greater than the mean of 52 mol% acetyl-TAG observed for *EaDAcT* lines (Figure 2.1a). Furthermore, the highest level of acetyl-TAG achieved with the expression of *EfDAcT* was 81 mol%, higher than that in the best *EaDAcT* line, which accumulated 65 mol% acetyl-TAG. Acetyl-TAG from seeds expressing *EfDAcT* contained relatively high levels of 18:2, on average 32 mol%, more than in lcTAG from the same seeds or from wild-type seeds (Figure 2.1b). Analysis of fatty acid profiles revealed that lcTAG produced in *EfDAcT* seeds contained higher levels of the very long-chain fatty acids (VLCFA) 20:0 and 20:1 compared to the acetyl-TAG fraction. Interestingly, the levels of 20:0 in lcTAG from the transgenic seeds was threefold higher, about 5 mol%, than the amounts typically found in wild-type seeds.

RNAi-mediated suppression of *DGAT1* in transgenic camelina seeds expressing *EaDAcT* resulted in higher acetyl-TAG levels compared to *EaDAcT* expression alone (Liu, Rice, *et al*., 2015) We therefore expressed both *EaDAcT* and *EfDAcT* in combination with an RNAi hairpin loop targeting *DGAT1*. In agreement with our previous work (Liu, Rice, *et al*., 2015), *EaDAcT+DGAT1-RNAi* expression resulted in a mean acetyl-TAG accumulation of 68 mol%,

higher than that observed for *EaDAcT* expression alone. However, expression of *EfDAcT+DGAT1-RNAi* increased acetyl-TAG accumulation to a mean of 80 mol%, higher than the levels achieved for either *EaDAcT+DGAT1-RNAi* or *EfDAcT* alone (Figure 2.1a). Furthermore, the highest producing transgenic *EfDAcT+DGAT1-RNAi* line accumulated approximately 87 mol% of acetyl-TAG, higher than levels observed for the best *EaDAcT+DGAT1-RNAi* line (approximately 81 mol%). The fatty acid composition of acetyl-TAG in seeds expressing *EfDAcT+DGAT1-RNAi* showed slightly elevated levels of 18:2, but not as high as those observed with only *EfDAcT* expression, such that 18:2 and 18:3 were present in equal amounts (Figure 2.1c). LcTAG from *EfDAcT+DGAT1-RNAi* seeds contained lower levels of 20:1 and increased amounts of 18:3 compared to lcTAG from *EfDAcT* seeds. Levels of 20:0 in lcTAG from these seeds were also higher than in lcTAG in wild-type seeds.

To determine whether some of the changes in fatty acid composition were due to the effect of *DGAT1* suppression, we generated and characterized camelina lines expressing only the *DGAT1-RNAi* cassette. Seeds from multiple independent transgenic lines possessed lower levels of 20:1 and increased amounts of 18:3 (Supplementary Figure B.1a), similar to previous results suppressing the three camelina *DGAT1* homeologues (Marmon *et al*., 2017). Consistent with the suppression of DGAT1, these seeds possessed lower amounts of TAG (Supplementary Figure B.1b). *DGAT1-RNAi* seeds also tended to be larger than wild-type seeds, again comparable to earlier work (Marmon *et al*., 2017), though this effect was less pronounced in some lines and not statistically significant (Supplementary Figure B.1c).

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

As the development of homozygous transgenic lines is time consuming and requires the generation of many plants, we were interested to know whether acetyl-TAG levels in transgenic T_2 seed (a 2:1 mixture of seed hemizygous:homozygous for the transgene) were predictive of the acetyl-TAG levels in subsequent homozygous T_3 seeds. Based on T_2 seed accumulation levels, we selected a range of the highest, medium, and lowest acetyl-TAG producing lines expressing *EaDAcT*, *EfDAcT*, or *EfDAcT+DGAT1-RNAi* for propagation to T_3 seed. Homozygous T_3 seeds were identified based on segregation of DsRed fluorescence and acetyl-TAG levels were quantified. The acetyl-TAG levels of homozygous T_3 seed were similar to those of the original T_2 seed for all individual transgenic lines (Figure 2.2a). To further ensure the stability of acetyl-TAG levels, four independent lines expressing *EfDAcT* or *EfDAcT+DGAT1-RNAi* were propagated for another generation. Acetyl-TAG levels in the resulting homozygous T_4 seed expressing either *EfDAcT* or *EfDAcT+DGAT1-RNA*i were generally consistent with the levels observed in T_2 and T_3 seeds (Supplementary Figure B.2), confirming the stability of the phenotype over multiple generations. For instance, line J2, the highest producing *EfDAcT+DGAT1-RNAi* transgenic line with 87 mol% of acetyl-TAG in T_2 seed, accumulated 89 mol% in T_3 seeds and a mean of 90 mol% in T_4 seeds. Thus, high levels of acetyl-TAG accumulation in seeds remain stable over multiple generations, allowing the analysis of T_2 seeds to confidently identify high acetyl-TAG producing lines.

The similarity in acetyl-TAG levels between homozygous T_3 seeds and parental T_2 seeds was surprising given the transgene dosage difference. Therefore, we also quantified the acetyl-TAG levels in the transgenic seeds derived from the hemizygous T_2 plants grown alongside their homozygous siblings. There were no significant differences in acetyl-TAG levels in transgenic seeds harvested from plants hemizygous or homozygous for *EaDAcT*, *EfDAcT*, or

EfDAcT+DGAT1-RNAi (Figure 2.2b), suggesting that one copy of the transgene is sufficient to produce high acetyl-TAG levels.

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

In some cases, the synthesis of unusual fatty acids has been associated with deleterious effects on seed quality, including reduced oil content and poor germination (Burgal *et al*., 2008; Li *et al*., 2012). We, therefore, quantified different seed properties of *EfDAcT+DGAT1-RNAi* lines that produce the highest levels of acetyl-TAG yet reported in transgenic camelina seeds, to determine whether the accumulation of such high levels of unusual storage lipids impacts seed quality traits. Homozygous T_4 seeds from four independent lines showed increases of 1–14% in weight and 6–20% in size compared to wild-type seeds (Figure 2.3a,b). Similar to our previous observations (Liu, Rice, *et al*., 2015), the transgenic lines tended to accumulate higher levels of total TAG, though these differences were not statistically significant due to large variability among replicates (Figure 2.3c). Some of the acetyl-TAG-producing seeds possessed a slightly lower fatty acid content compared to wild-type seeds (Figure 2.3d), again similar to our previous work with camelina plants expressing *EaDAcT+DGAT1-RNAi* (Liu, Rice, *et al*., 2015).

We also examined the germination of T_4 seed from homozygous lines expressing *EfDAcT* or *EfDAcT+DGAT1-RNAi* that accumulate very high levels of acetyl-TAG. Transgenic seed expressing either of the transgenes showed a significant delay in germination on damp filter paper compared to wild-type seed. For example, 2 days after imbibition, radicals had emerged from 100% of the wild-type seed but from only approximately 50% of *EfDAcT* and *EfDAcT+DGAT1-RNAi* seed. However, by day 4 almost all of the transgenic seeds had germinated (Figure 2.4a). Eventually, all transgenic seeds with an emerged radicle exhibited cotyledons and developed into

mature plants. Similar trends were noted when the seeds were germinated on agar plates (Supplementary Figure B.3a) with transgenic seeds exhibiting delays in radical and cotyledon emergence (Supplementary Figure B.3b,c). Furthermore, slightly reduced seedling weight and considerably lower chlorophyll content were observed in the transgenic seedlings (Supplementary Figure B.3d,e). On soil, the transgenic plants were slightly smaller than wild-type plants (Supplementary Figure B.3f).

Subsequent germination studies on agar plates using freshly harvested seeds revealed no differences between seeds from wild-type or transgenic plants expressing *DGAT1-RNAi*, *EaDAcT*, *EfDAcT*, or *EfDAcT+DGAT1-RNAi* with 100% of seeds in all genotypes germinating after 1 day (Supplementary Figure B.4a). Cotyledons emerged slightly faster from *EfDAcT* and *DGAT1-RNAi* seeds compared to wild-type seeds, whereas this process appeared slower in *EaDAcT* and *EfDAcT+DGAT1* seeds (Supplementary Figure B.4b). When freshly harvested seeds were germinated on soil, transgenic lines expressing different combinations of *EaDAcT*, *EfDAcT*, and *DGAT1-RNAi* appeared no different from wild-type plants (Supplementary Figure B.5).

The levels of acetyl-TAG in the seeds did not appear to greatly affect the delay in germination. For example, seeds from an *EfDAcT* line with 78 mol% acetyl-TAG germinated faster than those with 28 mol% acetyl-TAG; likewise, *EfDAcT+DGAT1-RNAi* seeds with 87 mol% acetyl-TAG germinated faster than seeds with 53 mol% acetyl-TAG (Supplementary Figure B.6). We quantified the lcTAG and acetyl-TAG levels in the germinating seeds to see if the delay in germination was connected to an inability to utilize the acetyl-TAG present in the transgenic seeds. In *EfDAcT* seeds, acetyl-TAG levels decreased at the same rate as lcTAG levels in germinating wild-type seeds. However, mobilization of acetyl-TAG in *EfDAcT* and *EfDAcT+DGAT1-RNAi* seeds was delayed by 1 day compared to that of lcTAG in wild-type seeds (Figure 2.4b). In both

transgenic lines, the relatively small amounts of lcTAG also decreased during germination, though at a slower rate than acetyl-TAG.

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 acetyl-TAG producing 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 seeds, 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 seeds (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 three additional independent transgenic lines where acetyl-TAG levels also increased during early seed development with accumulation reaching a peak at 30 DAF, followed by decreasing levels during late seed development (Supplementary Figure B.7). Acetyl-TAG accumulation, therefore, follows a consistent pattern over development in transgenic seed.

Transcript accumulation reflects EfDAcT protein content in developing transgenic seeds

In addition to studying the accumulation of acetyl-TAG during seed development, we also quantified the transcript and protein levels of EfDAcT in the same seeds. In both seeds expressing *EfDAcT* or *EfDAcT+DGAT1-RNAi*, *EfDAcT* transcript levels increased during early seed development, reaching a peak 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 protein is 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 *EfDAcT* alone, protein levels are more uniform from 15 to 25 DAF, but as in the *EfDAcT+DGAT1-RNAi* seeds, they decrease at 30 DAF and are barely detectable at 40 DAF. EfDAcT protein accumulation is therefore consistent with the transcript expression pattern in developing seeds, indicating that EfDAcT protein accumulation might be controlled at the transcriptional level, though additional work is needed to confirm this observation. Moreover, EfDAcT transcript and protein levels correlate well with the pattern of acetyl-TAG accumulation (Figure 2.5). For example, the decrease in EfDAcT transcript and protein levels at 30 and 40 DAF is accompanied with a reduction in acetyl-TAG levels, while the highest acetyl-TAG levels at 30 DAF follow the peak production of EfDAcT transcript and protein at 20 DAF.

EfDAcT protein levels do not correlate with acetyl-TAG accumulation

Since acetyl-TAG production correlated well with the EfDAcT-HA protein level during seed development (Figure 2.5), we tested whether EfDAcT-HA protein levels were predictive of the final acetyl-TAG levels in mature seeds. We selected *EfDAcT* and *EfDAcT+DGAT1-RNAi* transgenic lines that synthesized different levels of acetyl-TAG and quantified EfDAcT protein levels in developing seeds at 20 DAF as well as the eventual acetyl-TAG levels in mature seeds for each plant. In lines expressing *EfDAcT* alone that accumulate lower amounts of acetyl-TAG,

there is an approximately linear relationship between EfDAcT protein levels and acetyl-TAG accumulation. However, above 80 mol%, this linear relationship disappears (Figure 2.7a). Consistent with this threshold, 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 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 synthesize acetyl-TAG levels of approximately 90 mol%, similar to those with EfDAcT levels of approximately 1.0 normalized units or higher (Figure 2.7b).

We also quantified *DGAT1* expression in developing seeds from *EfDAcT* and *EfDAcT+DGAT1-RNAi* lines that produce different amounts of acetyl-TAG (Figure 2.7c). In general, *EfDAcT+DGAT1-RNAi* lines possessed lower levels of *DGAT1* transcript, demonstrating the efficacy of the *DGAT1-RNAi* cassette, consistent with the lower levels of lcTAG 20:1 in these lines compared to those in *EfDAcT* lines (Figure 2.1b,c). However, no obvious correlation between *DGAT1* expression in the transgenic seeds and eventual acetyl-TAG accumulation was evident.

DISCUSSION

EfDAcT expression results in increased acetyl-TAG levels

Expression of *EfDAcT* in camelina seeds resulted in higher levels of acetyl-TAG accumulation than with the related enzyme EaDAcT (Figure 2.1a), consistent with the higher *in vitro* activity of EfDAcT (Tran, Shelton, *et al*., 2017). Similar to our previous results with EaDAcT, the suppression of the lcTAG-synthesizing enzyme DGAT1 further enhanced acetyl-TAG levels, to as high as approximately 90 mol% in some cases (Figures 1a and S2b). The difference in acetyl-

TAG accumulation between the two acetyltransferases was smaller when combined with *DGAT1* suppression. One possible explanation for these results is that the higher acetyltransferase activity of EfDAcT enables it to better compete with DGAT1 for DAG compared to EaDAcT. This advantage, however, becomes less relevant when *DGAT1* is suppressed. Consistent with this idea, there is a partial initial linear relationship between EfDAcT protein accumulation and acetyl-TAG accumulation in the absence of *DGAT1* suppression (Figure 2.7a), but such a relationship is not evident in *EfDAcT+DGAT1-RNAi* lines (Figure 2.7b).

In addition to DGAT1, phospholipid:diacylglycerol acyltransferase 1 (PDAT1) can also catalyze the final step of lcTAG biosynthesis in developing seeds (Dahlqvist *et al*., 2000). The fatty acid composition of lcTAG in *EfDAcT+DGAT1-RNAi* seeds implies that this residual lcTAG is synthesized by PDAT1. LcTAG from these lines contains more 18:3 and less 20:1 compared to lcTAG in *EfDAcT* seeds (Figure 2.1b,c), similar to changes in the fatty acid composition in Arabidopsis *dgat1* mutant seeds, where PDAT1 is responsible for TAG synthesis (Zhang *et al*., 2009). However, our earlier work demonstrated that while eliminating or suppressing DGAT1 activity enhances acetyl-TAG levels, the combined suppression of both *PDAT1* and *DGAT1* did not result in any further increase (Bansal *et al*., 2018; Liu, Rice, *et al*., 2015). Similar results were observed in Arabidopsis expressing the castor hydroxylase FAH12, where suppression of *PDAT1* in a *dgat1* mutant background expressing the castor DGAT2 and PDAT1 enzymes failed to increase hydroxy-fatty acid (HFA) levels. Furthermore, seeds from many of these lines were wrinkled and germinated poorly (van Erp *et al*., 2015). The observations from these studies suggest that suppression of both *DGAT1* and *PDAT1* negatively affects seed development, which cannot be complemented through the expression of alternative enzymes introduced transgenically. Whether this is due to differences in activity of the introduced enzymes or caused by altered timing

or location of expression, or combinations thereof, remains to be determined. Given the lack of effect of targeting *PDAT1* expression, only *DGAT1* suppression was implemented in the current work to enhance acetyl-TAG levels.

The acetyl-TAG molecules synthesized in camelina seeds expressing *EfDAcT* or *EfDAcT+DGAT1-RNAi* possessed higher levels of PUFA and lower levels of VLCFA compared to the lcTAG produced in the same seeds (Figure 2.1b,c), similar to our previous observations with camelina plants expressing *EaDAcT+DGAT1-RNAi* (Liu, Rice, *et al*., 2015). *In vitro* selectivity assays have demonstrated that EaDAcT preferentially acetylates more unsaturated DAG (Bansal and Durrett, 2016). The higher levels of PUFA in acetyl-TAG synthesized with EfDAcT imply that like EaDAcT, EfDAcT possesses a similar preference for PUFA DAG. The decrease in VLCFA in acetyl-TAG is likely 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). As the noticeably higher levels of 20:0 in lcTAG occur in both *EfDAcT* and *EfDAcT+DGAT1-RNAi* seeds (Figure 2.1b,c), this phenotype is due to the expression of EfDAcT and not the suppression of DGAT1, a conclusion supported by the fatty acid profile of *DGAT1-RNAi* seeds where 20:0 levels are not elevated (Supplementary Figure B.1a). One possible explanation is that EfDAcT discriminates against acetylating DAG molecules containing 20:0, thus enriching this fatty acid in the lcTAG fraction, though more work is needed to explore this idea.

High acetyl-TAG accumulation does not affect seed quality

In the highest acetyl-TAG line expressing *EfDAcT+DGAT1-RNAi*, 87 mol% of total TAGs in T_2 seeds were acetyl-TAG (Figure 2.1a). T_4 seeds from the same line contained an average of

90 mol% acetyl-TAG, the highest levels of modified TAG structures thus far produced in transgenic seeds (Supplementary Figure B.2b). In contrast, previous efforts to alter camelina seed oil content have typically achieved lower levels of the desired lipid product. For example, levels of 8–30% of medium-chain fatty acids (MCFA; Hu *et al*., 2017; Kim *et al*., 2015) or 22% of HFA (Snapp *et al*., 2014) have been achieved in transgenic camelina seeds. These very high levels of acetyl-TAG achieved here are likely due to EfDAcT modifying the final step of TAG biosynthesis. Generally, 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. Such bottlenecks can affect seed viability, reduce germination rates, and lower oil content (Burgal *et al*., 2008; Li *et al*., 2012). However, very high levels of acetyl-TAG were achieved with a minor impact on key seed properties (Figures 3 and 4), consistent with the synthesis of acetyl-TAG occurring at the end of a biosynthetic pathway, thus preventing the accumulation of deleterious lipid intermediates. Since acetyl-TAG utilizes two acyl chains instead of three acyl chains with lcTAG, seeds synthesizing acetyl-TAG tend to accumulate more overall TAG molecules compared to wild-type plants (Figure 2.3c), in agreement with previous work (Liu, Rice, *et al*., 2015). However, this increase in TAG content is unlikely to explain the higher seed weight and size for the transgenic lines (Figure 2.3a,b). We observed that camelina lines where *DGAT1* was suppressed using the same RNAi hairpin used in combination with *EfDAcT* expression also tended to produce slightly larger seeds compared to wild-type plants (Supplementary Figure B.1c). These results are consistent with previous work where suppression of camelina *DGAT1* homeologues using artificial microRNAs also resulted in slightly larger seed mass (Marmon *et al*., 2017). Here, it will be interesting to

quantify other components such as carbohydrates or proteins in seeds where *DGAT1* is suppressed, to determine whether increases in these fractions contribute to the observed larger seed size.

Transgenic seeds accumulating acetyl-TAG germinated later compared to wild-type seeds (Figures 4a and S3), though this phenotype was not observed with freshly harvested seeds (Supplementary Figure B.4). This delay is unlikely to be caused by endogenous camelina lipases functioning less efficiently with the unusually structured acetyl-TAG because the breakdown of acetyl-TAG occurred at roughly the same rate as that of lcTAG in wild-type seed, once it was initiated (Figure 2.4b). However, the activity of the endogenous lipases, most likely orthologs of SUGAR-DEPENDENT1 (SDP1) (Eastmond, 2006), towards acetyl-TAG will need to be quantified *in vitro* to confirm their relative effectiveness against these unusual storage lipids. The delay in germination is also unlikely to be connected to the suppression of *DGAT1*, as there were minimal differences in the timing of the germination of *EfDAcT* and *EfDAcT+DGAT1-RNAi* seeds. Instead, the germination phenotype observed is somehow connected to the accumulation of acetyl-TAG, and also appears to be affected by the age of the seeds (Figures S3 and S4). Similar to our results, delays in the germination of camelina seeds expressing RcFAH12 that accumulate HFA have also been noted; here again though, after the initial delay, the transgenic seeds catch up and eventually all germinate (Snapp *et al*., 2014). Future work is, therefore, needed to better understand how the accumulation of unusual lipids affects the timing of seed germination, particularly when the activity of endogenous enzymes is altered.

EfDAcT expression does not limit very high levels of acetyl-TAG accumulation

Expression levels, and therefore phenotypic effects, are usually positively correlated with gene copy number (Schubert *et al*., 2004). Interestingly in our study average acetyl-TAG accumulation

was similar in hemizygous and homozygous lines where one and two copies of the transgene are present, respectively, at a single locus (Figure 2.2b). The lack of distinction between homozygous and hemizygous genotypes on the basis of trait accumulation levels has also been observed in rapeseed (*Brassica napus*) synthesizing MCFAs and Arabidopsis transformed with a polyketide synthase-like to enable biosynthesis of very long-chain polyunsaturated omega-3 fatty acids (Voelker *et al*., 1996; Walsh *et al*., 2016). Our results suggest that *EfDAcT* expression does not limit the accumulation of acetyl-TAG, and are consistent with the lack of correlation between EfDAcT protein and acetyl-TAG levels when the latter are greater than 70 mol% (Figure 2.7a,b). Thus, in transgenic lines expressing *EfDAcT+DGAT1-RNAi* with high acetyl-TAG levels, other factors likely restrict acetyl-TAG accumulation. For example, acetyl-CoA levels in developing camelina seeds might not be sufficient to support even higher levels of acetyl-TAG synthesis. Another intriguing possibility is that the availability of glycerol-3-phosphate (G3P) backbone caused by the higher overall synthesis of TAG (Figure 2.3c) could limit the synthesis of DAG and subsequently acetyl-TAG. This hypothesis could also explain the lower fatty acid levels in the transgenic seeds (Figure 2.3d), as insufficient amounts of G3P could prevent the complete incorporation of all available fatty acids into acetyl-TAG or lcTAG.

The temporal expression of *EfDAcT* is consistent with acetyl-TAG accumulation

Other factors, such as timing of EfDAcT expression, could also limit acetyl-TAG accumulation. In this study, *EfDAcT* was highly expressed using the seed-specific soybean glycinin promoter. While this promoter has been used to drive the expression of different exogenous biosynthetic genes in camelina seed (Iskandarov *et al*., 2017; Liu, Rice, *et al*., 2015; Nguyen *et al*., 2015), detailed analysis of gene expression and accumulation of resulting products has not been

conducted. Quantification of EfDAcT mRNA and protein levels in transgenic camelina seeds revealed a generally well-correlated pattern of *EfDAcT* transcript with protein during seed development. A similar correlation between mRNA and protein levels across seed development has also been observed for the DGAT2 acyltransferase in developing tung (*Vernicia fordii*) seeds (Cao *et al*., 2013). EfDAcT transcript and protein showed maximum expression levels at 20 DAF (Figure 2.6), the stage when acetyl-TAG levels are most rapidly increasing (Figure 2.5). In the later stages of seed development (30 DAF and onwards), EfDAcT protein accumulation decreases to barely detectable levels by 40 DAF. Consistent with the low levels of enzyme present, acetyl-TAG levels decrease slightly. Extending the expression of EfDAcT to later stages of seed development could, therefore, potentially represent another approach to increase acetyl-TAG levels. Such a strategy has been used successfully in Arabidopsis to enhance oil content (Kanai *et al*., 2016). In addition, the slight reduction in acetyl-TAG levels could be caused by endogenous lipases present in the developing seeds. Suppression of orthologs of the triacylglycerol lipase SDP1 in rapeseed oil and jatropha (*Jatropha curcas*) led to higher seed oil content in the seeds of these species (Kelly *et al*., 2013; Kim *et al*., 2014). A similar approach targeting *SDP1* expression in camelina seeds could, therefore, also be used to eliminate turnover of acetyl-TAG during late seed development.

CONCLUSION

We have demonstrated that the seed-specific expression of *EfDAcT* in camelina, combined with suppression of endogenous TAG synthesis, leads to acetyl-TAG levels as high as 90 mol%, the highest levels of modified lipids yet reported in a transgenic oilseed crop. These high levels of acetyl-TAG were stable over multiple generations and had minimal effect on most seed properties.

However, germination was slightly delayed in lines that accumulate acetyl-TAG. The lack of correlation between EfDAcT protein and acetyl-TAG levels in high-accumulating lines and the lack of distinction between homozygous and hemizygous siblings suggest that expression of *EfDAcT* is not limiting further acetyl-TAG accumulation. Instead, the reductions in fatty acid content in transgenic lines imply that the flux of other substrates involved in acetyl-TAG synthesis such as G3P or acetyl-CoA might not be sufficient for maximal production of acetyl-TAG. Optimizing the temporal and spatial expression of acetyl-TAG synthesis, as well as preventing lipase-mediated turnover, could also further enhance levels of acetyl-TAG.

MATERIALS AND METHODS

Plant material and growth conditions

Transgenic and wild-type *C. sativa* 'Suneson' were grown in growth chambers 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 threads and the tagged pods were 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 multiple days before analysis.

Vector construction and plant transformation

The construction of yeast expression vectors containing *EaDAcT-HA* and *EfDAcT-HA* was described previously (Tran, Shelton, *et al*., 2017). Importantly, the addition of the HA epitope to EaDAcT does not affect the activity of the enzyme when expressed in yeast. The open reading frames of the epitope-tagged acetyltransferases were amplified with the primers EaDAcT-HA-F,

EfDAcT-HA-F, and Ea/fDAcT-HA-R (Table S1) and then cloned into the binary vector pBinGlyRed3, where expression is driven by the soybean glycinin promoter. The vector contains the fluorescent protein DsRed as a visual selectable marker. An expression cassette containing the *DGAT1-RNAi* hairpin sequence driven by the *B. napus* oleosin promoter (Liu, Rice, *et al*., 2015) was then added to combine *EaDAcT* or *EfDAcT* expression with *DGAT1* suppression. This DGAT1-RNAi cassette was also ligated into pBinGlyRed3 by itself to enable suppression of *DGAT1*. 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 *C. sativa* 'Suneson' via a floral dip vacuum infiltration method (Lu and Kang, 2008). Transgenic T_1 seeds 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 χ^2 test for a 3:1 segregation ratio were used for further analysis and propagation.

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 gel TLC plates and separated using a 70:30:1 hexane/diethyl-ether/acetic acid (v/v/v) solvent system. TLC plates were sprayed with 0.2% (w/v) 2,7-dicholorofluorescein in 96% methanol and visualized under UV light. Triheptadecanoin 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 (FAMEs) were quantified using a standard gas chromatography method (Aznar-Moreno and Durrett, 2017). Acetyl-TAG and lcTAG levels were quantified as described previously (Liu, Rice, *et al*., 2015). The total fatty acid contents of mature seeds were determined by quickly homogenizing 20 seeds in 1 ml toluene with a PT2500E polytron (Kinematica AGd) followed by transmethylation and gas chromatography (Aznar-Moreno and Durrett, 2017).

Analysis of seed properties

Seed weight was determined by carefully weighing 100 dry seeds on a Sartorius CP225D analytical balance. Seed size was calculated using ImageJ software to quantify the area of 300 randomly selected seeds per replicate using a previously established method (Herridge *et al*., 2011). For germination assays, seeds from wild-type and transgenic plants grown at the same time were sown on wet filter paper in Petri dishes or surface-sterilized with 70% ethanol for 1 min followed by 50% (v/v) bleach and 0.05% Tween 20 for 10 min and planted on 1% agar plates. Plates were placed in a growth chamber under a 16 h light/8 h dark cycle at a constant temperature of 21°C. Germination and cotyledon emergence were scored daily for 7 days. Seeds were considered germinated based on radicle emergence. A pool of 15 germinated seeds were collected each day, immediately frozen in liquid nitrogen, and stored at −80 °C until further analysis of lipids, dry weight (lyophilization), and chlorophyll content.

Chlorophyll content

Fifteen germinated seeds at each indicated stage were finely ground with liquid nitrogen. 1 ml of pre-cooled 80% (v/v) buffered acetone (2.5 mm sodium phosphate pH 7.8) solution was added to

the ground seeds. Samples were centrifuged at 15 000 ***g*** for 5 min and supernatants were transferred to fresh tubes. Extraction was repeated four to five times. Absorbance of the combined extracts for each sample was measured at wavelengths of 646.6 and 663.6 nm. The total chlorophyll content was calculated by the formula given in Porra *et al*. (1989). The leaf chlorophyll content of plants grown on soil was measured using a SPAD-502 Chlorophyll Meter (Konica-Minolta).

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 15 min at 4°C. The final supernatants were transferred to fresh tubes and stored at −80°C until further use. Protein concentrations were determined with a Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). Protein samples were incubated with loading buffer (125 mm Tris-HCl pH 6.8, 8 m urea, 10% SDS, 0.004% [w/v] bromophenol blue, 10% β-mercaptoethanol) for 45 min at 37°C and then resolved on a 10% acrylamide gel (GenScript, Piscataway, NJ, USA) using SDS-PAGE. Yeast microsomes (1 µg protein) expressing EfDAcT-HA (Tran, Shelton, *et al*., 2017) 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 (Supplementary Figure B.8). The membrane was then 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, and 0.1% [v/v] Tween 20) for 1 h. The membrane

was rinsed with PBS five times for 5 min each and then incubated with mouse anti-HA primary antibody (1:5000 dilution in PBS; clone 2-2.2.2.14; Thermo Scientific) at 4°C overnight. The membrane was then rinsed with PBS five times for 5 min each and incubated with goat anti-mouse secondary antibody (1:5000 dilution in PBS; Thermo Scientific) for 1 h at room temperature. Antibodies were detected using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) and protein bands were visualized using an Azure c600 Chemiluminescent Western Blot Imaging System. EfDAcT-HA protein abundance was quantified and normalized to the protein abundance of the yeast positive control using densitometry analysis with ImageJ software (Abràmoff *et al*., 2004).

RNA extraction

Total RNA was isolated from developing seeds using a previously established method (Chang *et al*., 1993) with the following modifications: 60 µl of 10% SDS and 1 ml of pre-warmed CTAB extraction buffer (2% cetyltrimethylammonium bromide, 25 mm EDTA, 100 mm Tris-HCl pH 8.0, 2.0 m NaCl, 0.5 g L^{-1} spermidine, 2% polyvinylpyrrolidone K 30, 50 µg ml^{-1} proteinase K, and 2% β-mercaptoethanol) were added to the ground tissue. Samples were incubated at 65°C for 10 min with vigorous shaking and centrifuged at 13 000 ***g*** for 10 min at 4°C. An equal volume of 25:24:1 phenol:chloroform:isoamyl alcohol (v/v/v) was added to the supernatant and centrifuged at 13 000 ***g*** for 10 min at 4°C. This phenol:chloroform:isoamyl alcohol extraction step was performed 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 20 000 ***g*** for 1 h 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.

Reverse transcription quantitative PCR (RT-qPCR) analysis

For each sample, 4 μg of total RNA was digested with RNase-free DNaseI (Promega, Madison, WI, USA) to remove any genomic DNA contamination. RNA concentrations were quantified with a NanoDrop 2000 spectrophotometer (Thermo Scientific). One microgram of RNA was used for cDNA synthesis using qScript™ cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA). Quantitative PCR was performed on a CFX96 real-time PCR system (Bio-Rad) using PerfeCTa® SYBR® Green SuperMix (Quanta Bioscience) according to the manufacturer's instructions. *EfDAcT* and *DGAT1* gene expression levels were normalized to the camelina *Ubiquitin* and *GAPDH* genes using the $2^{-\Delta Ct}$ method. Primers used to quantify expression of these genes are listed in Supplementary Table B.1.

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AUTHOR CONTRIBUTIONS

TPD and LA designed the research; TNTT, SC, and CK constructed transformation vectors and generated transgenic plants synthesizing acetyl-TAG; CK and LA characterized acetyl-TAG producing lines; CW and GM generated and characterized *dgat1*-RNAi transgenic plants; LA, CK, and TPD wrote the manuscript with input from all authors.

CONFLICT OF INTEREST

CK, TNTT, and TPD have filed a patent application covering the use of EfDAcT to synthesize acetyl-TAG.

FIGURES

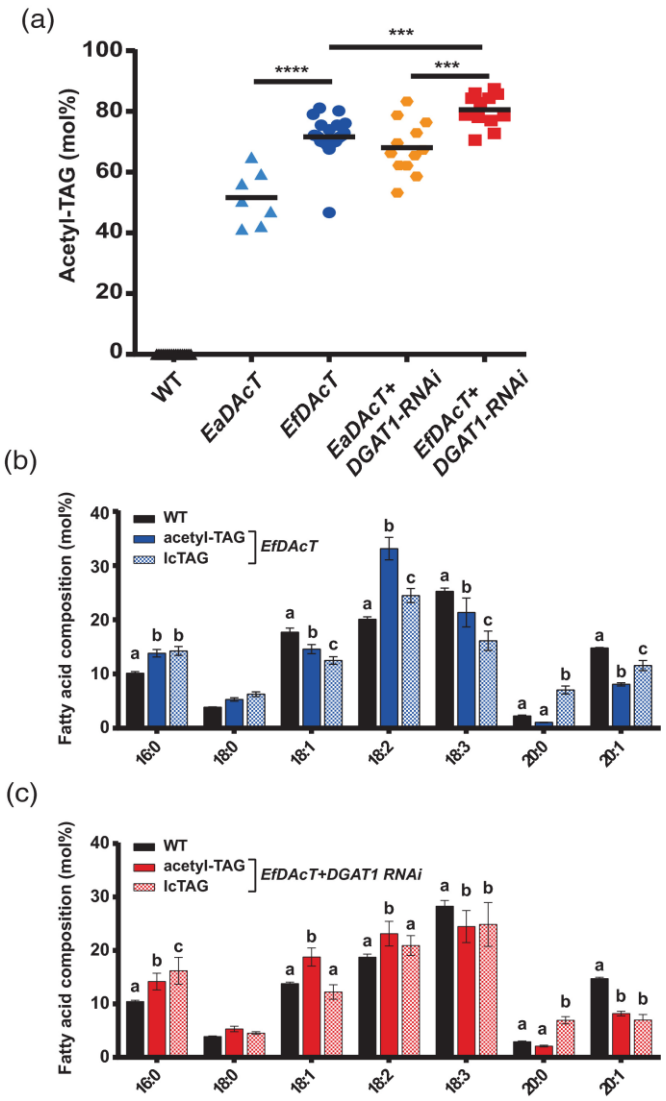


Figure 2.1. Expression of *EfDAcT* results in higher levels of acetyl-TAG in transgenic camelina seeds.

(a) Scatter plot of acetyl-TAG content of T_2 seed from independent transgenic lines expressing *EaDAcT* or *EfDAcT* alone or in combination with the RNAi-mediated suppression of *DGAT1*. Horizontal lines represent the mean acetyl-TAG content for each group. Asterisks indicate significant differences (**$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$; Mann–Whitney U-test). (b, c) Mean fatty acid composition of acetyl-TAG and lcTAG fractions of T_3 seed from independent transgenic lines expressing *EfDAcT* (b) or *EfDAcT+DGAT1-RNAi* (c). Error bars represent standard deviation. Different letters indicate significant differences by two-way anova with Dunnett's multiple comparison test. WT, wild type.

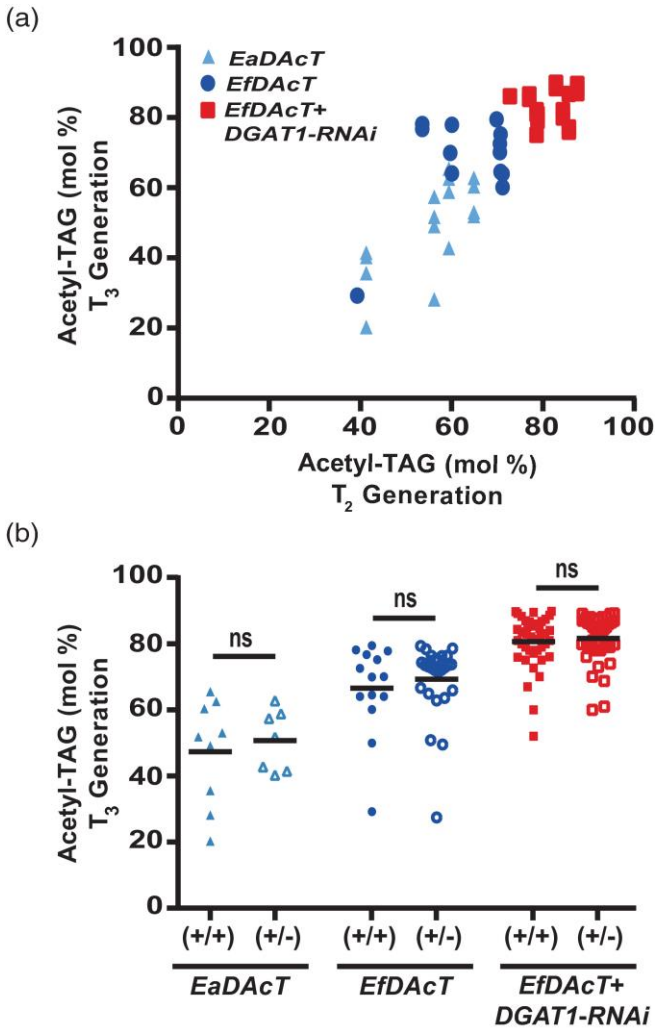


Figure 2.2. Acetyl-TAG accumulation in transgenic T_2 seed correlates with accumulation levels in T_3 seed.

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

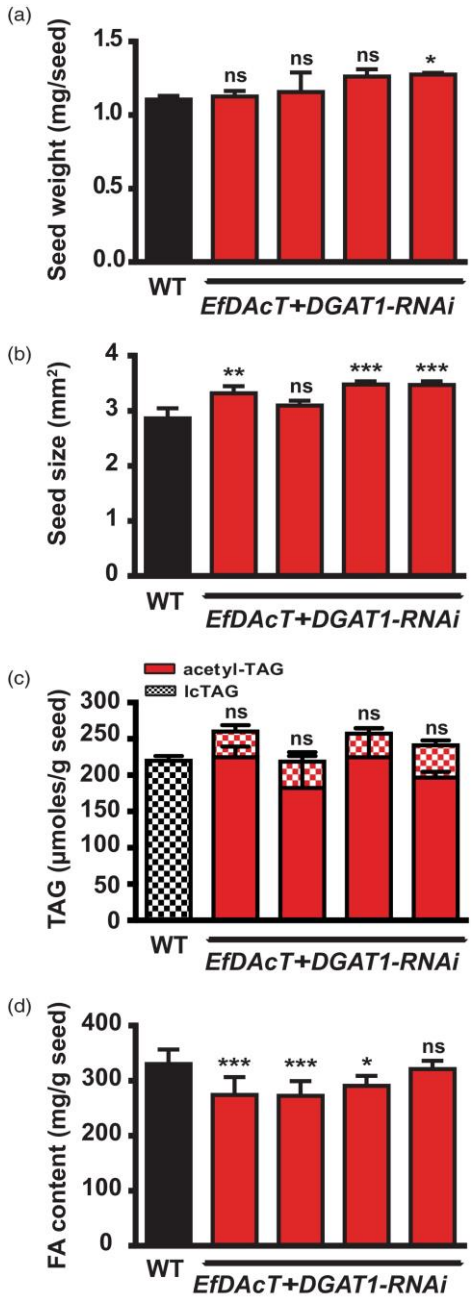


Figure 2.3. Accumulation of high levels of acetyl-TAG has minimal impact on seed properties.

Seed weight (a), seed size (b), seed TAG content (c), and seed fatty acid content (d) of mature seed harvested from wild type (WT) and four independent T_3 homozygous high acetyl-TAG producing lines expressing *EfDAcT+DGAT1-RNAi*. Values represent the mean ± standard deviation of three biological replicates. Asterisks indicate significant differences compared to wild type (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ns, not significant; one-way anova with Dunnett's multiple comparison test).

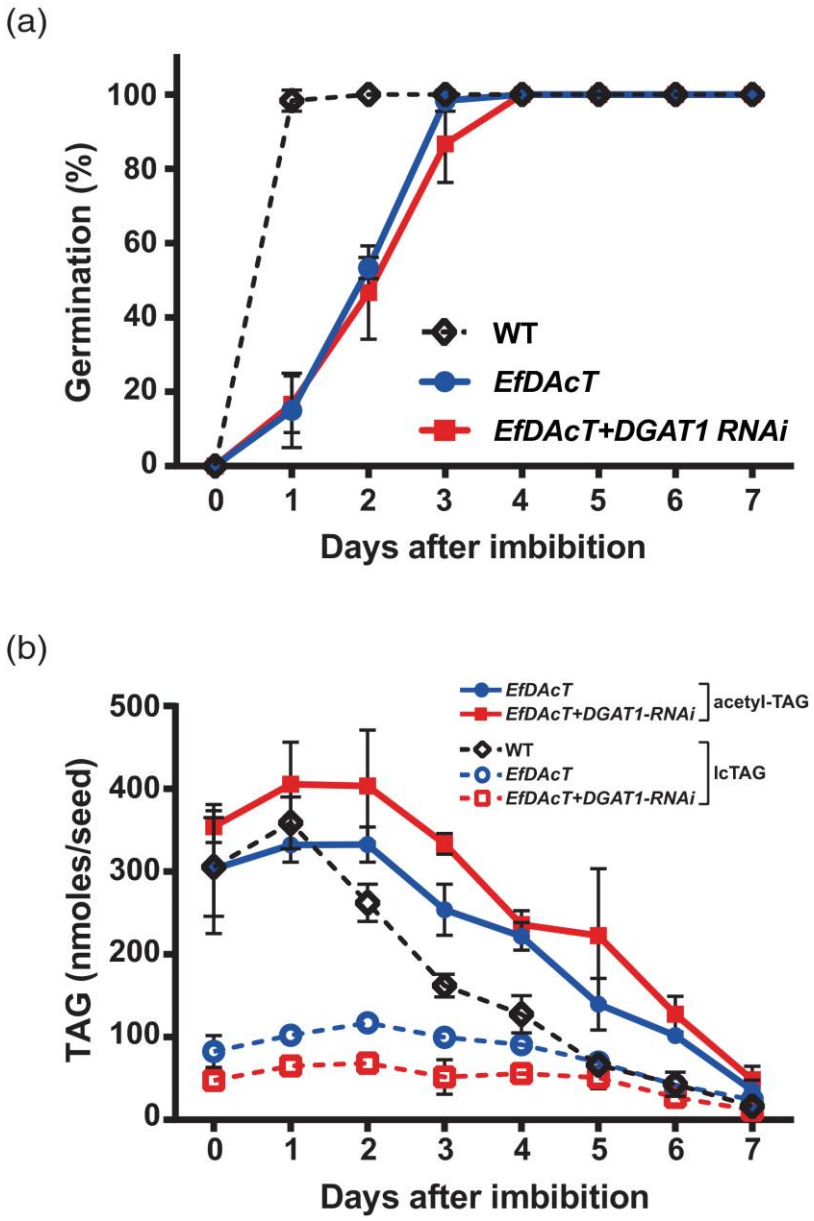


Figure 2.4. Germination of seed with high acetyl-TAG levels is slightly delayed.

Germination rates (a) and TAG content (b) of seeds from wild type (WT) and transgenic homozygous high acetyl-TAG producing lines expressing *EfDAcT* or *EfDAcT+DGAT1-RNAi*. Values shown represent the mean ± standard deviation of three biological replicates and are representative of two independent experiments.

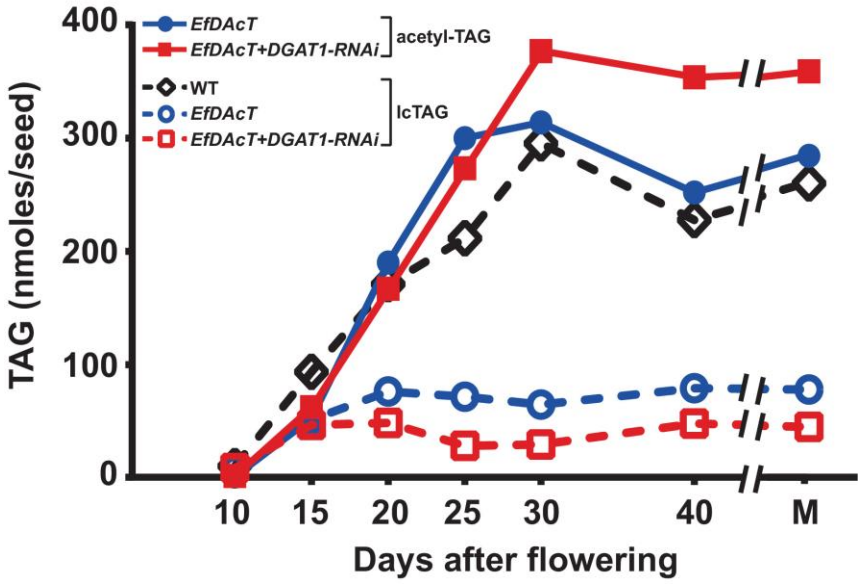


Figure 2.5. Acetyl-TAG and lcTAG content in developing wild-type and transgenic camelina seeds.

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 *EfDAcT* or *EfDAcT+DGAT1-RNAi*. Values represent the mean of three biological replicates and are representative of two independent experiments. Error bars were omitted for clarity. M, mature harvested seed.

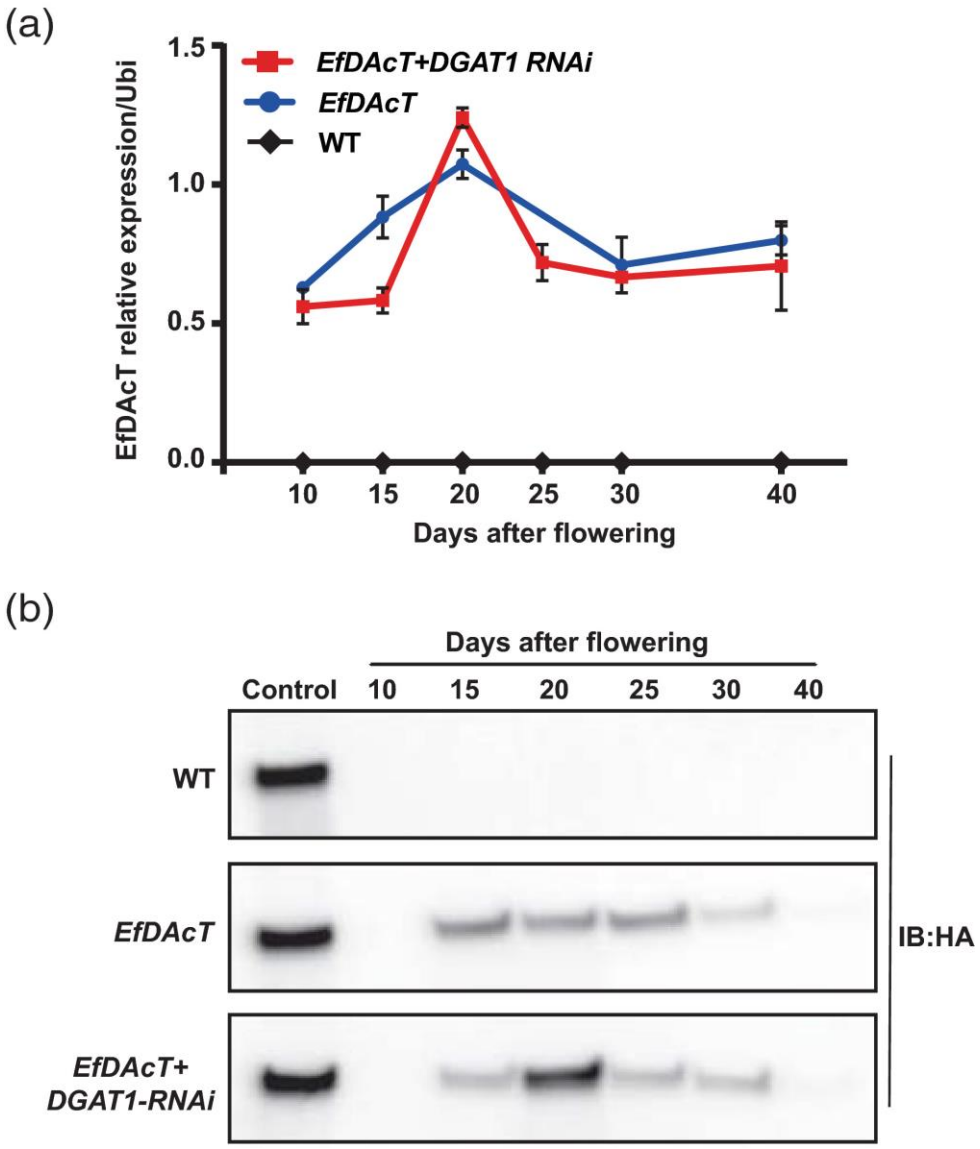


Figure 2.6. *EfDAcT* transcript levels reflect EfDAcT protein content during seed development.

EfDAcT mRNA levels (a) and protein content (b) in the developing seeds of wild type (WT) and homozygous T_4 high acetyl-TAG producing lines expressing *EfDAcT* or *EfDAcT+DGAT1-RNAi*. *EfDAcT* transcript levels were quantified using reverse transcription quantitative PCR (RT-qPCR) and normalized to the camelina *Ubiquitin* reference gene. Similar results were obtained when normalized to *GAPDH* expression. EfDAcT protein was detected by immunoblotting for the C-terminal HA epitope. Yeast microsomes expressing *EfDAcT-HA* were used as a positive control.

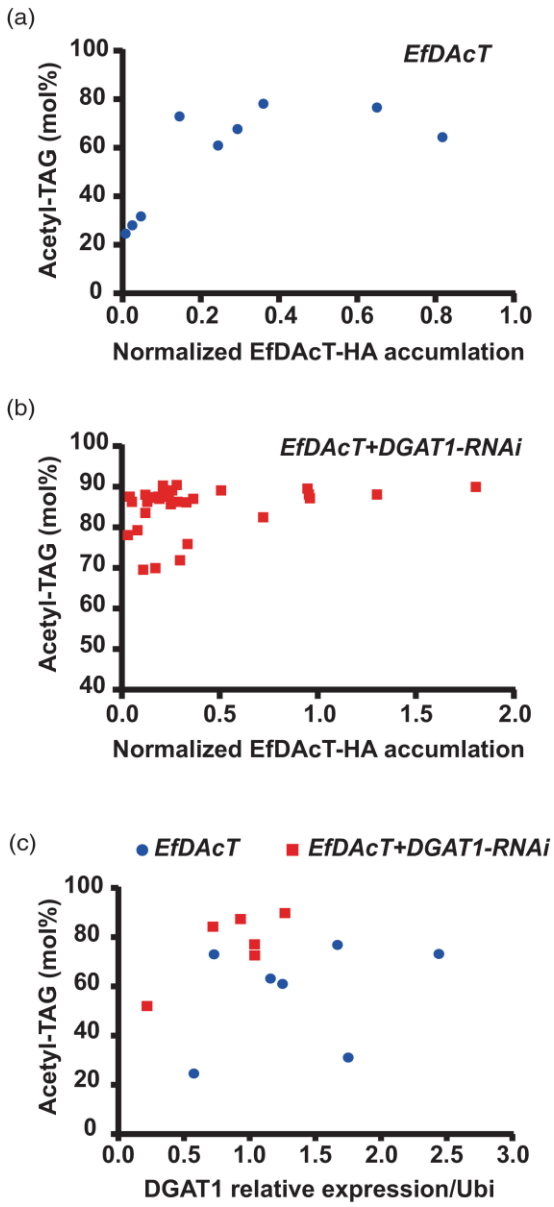


Figure 2.7. Acetyl-TAG accumulation does not correlate with EfDAcT-HA protein levels nor the amount of *DGAT1* suppression.

Final acetyl-TAG content in mature seeds expressing *EfDAcT* (a) or *EfDAcT+DGAT1-RNAi* (b) plotted against the EfDAcT-HA protein levels in seeds from the same plant collected at 20 DAF. Developing EfDAcT-HA levels were normalized to those of yeast microsomes expressing *EfDAcT-HA* included in every immunoblot. (c) Final acetyl-TAG content in mature seeds expressing *EfDAcT* or *EfDAcT+DGAT1-RNAi* plotted against the relative amount of *DGAT1* transcript in seeds from the same plant collected at 20 DAF. *DGAT1* transcript levels were normalized to the *Ubiquitin* reference gene and expressed relative to these normalized levels in wild-type plants (set at 1.0).

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Chapter 3 - Integrated lipidomic and transcriptomic analysis of transgenic camelina acetyl-TAG producing seeds

ABSTRACT

Many *Euonymus* species produce an unusual structure of triacylglycerols (TAG) referred to as acetyl-TAG. Acetyl-TAG contains an acetate group at the *sn*-3 position instead of a long chain fatty acid present in regular TAG of common vegetable oil. This structural difference provides acetyl-TAG with desirable physical properties, such as reduced viscosity, facilitating its potential use in a wide range of industrial applications. We have previously reported that heterologous expression of *Euonymus fortunei* diacylglycerol:acetyl-CoA acetyltransferase (EfDAcT), along with the RNAi-mediated suppression of the endogenous TAG competing enzyme DGATl, results in high levels of acetyl-TAG (> 85%) in *Camelina sativa* seeds. Transgenic seeds exhibited larger seed weight, a slight reduction in total fatty acid content, and a two-day delay in germination. Here, we performed comparative transcriptomic and targeted lipidomic analysis to further investigate the consequence of acetyl-TAG accumulation in developing camelina seeds. Transgenic seeds displayed reduced accumulation of regular TAG and elevated levels of diacylglycerol (DAG), phosphatidylcholine (PC), and phosphatidylglycerol (PG). Enrichment analysis of differentially expressed genes revealed changes in processes associated with flavonoid biosynthesis, response to hormones, as well as lipid metabolic processes. This combined approach revealed a distinct lipidomic and transcriptomic profile of the transgenic lines and identified potential targets for further engineering of transgenic lines capable of producing high levels of acetyl-TAG while preserving seed properties.

INTRODUCTION

Vegetable oils are a fundamental component of the human diet and play a crucial role in nutrition. They are also significant contributors to chemical feedstocks and renewable biofuels, serving a wide range of industrial applications (Durrett *et al*., 2008; Dyer *et al*., 2008). Vegetable oils are composed of triacylglycerol (TAG), molecules with three fatty acids esterified to a glycerol backbone. While most oilseeds produce and store TAGs with a set of common fatty acids (16 to 18 carbons with 0 to 3 *cis* double bonds), many plant species diverge from this norm by synthesizing and accumulating unique TAGs with unusual fatty acids structures. The unusual fatty acids include atypical fatty acid chain lengths, non-standard double bond positions, and functional groups such as acetylenic, cyclopropane, epoxy, or hydroxy (Badami & Patil, 1980a; Cahoon & Li-Beisson, 2020; Ohlrogge *et al*., 2018). These unconventional TAGs diversify the properties and quality of oils, enhancing their nutritional value and suitability for industrial applications (Avato & Tava, 2022; Dyer *et al*., 2008). Despite the potential of some plants to produce unusual TAGs, their poor agronomic traits limit their ability for large-scale oil production. To overcome this limitation, researchers have turned to easily transformable emerging oilseed crops like *Camelina sativa* to engineer the production of these unconventional TAGs (Bansal & Durrett, 2016a; Yuan & Li, 2020). However, engineering crops presents its own set of challenges. One primary issue is that the engineered crops often struggle to reach the levels of unusual TAGs naturally produced in native species (Cai *et al*., 2022). Furthermore, the accumulation of these unique TAGs can have adverse effects on seed properties and can hinder overall plant growth (Bates *et al*., 2014; Lunn *et al*., 2018). To address these bottlenecks and meet the increasing demand for high-quality oils and renewable biofuels, it is essential to gain a deep understanding of the intricate molecular mechanisms governing oil biosynthesis and regulation in plants.

One particular class of unusual TAGs is acetyl-TAGs, which naturally occur in the seeds of many *Euonymus* species and is characterized by the presence of an acetate group at the *sn*-3 position of the glycerol backbone (Kleiman *et al*., 1967; Sidorov *et al*., 2014). This distinctive feature provides acetyl-TAGs with improved physical properties, such as reduced viscosity, making them highly attractive for various industrial applications (Durrett *et al*., 2010; Liu, Rice, *et al*., 2015; Liu, Tjellström, *et al*., 2015). Previously, we have successfully produced significant levels of acetyl-TAGs in transgenic camelina seeds (Alkotami *et al*., 2021) through the heterologous expression of *EfDAcT*, a diacylglycerol:acetyl-CoA acetyltransferase (DAcT) enzyme responsible for acetyl-TAG synthesis in the seeds of *Euonymus fortunei* (Tran *et al*., 2017), and the use of RNA interference (RNAi) to suppress the endogenous TAG competing enzyme, diacylglycerol acyltransferase 1 (DGAT1) (Liu, Rice, *et al*., 2015). Expression of *EfDAcT+DGAT1-RNAi* in camelina seeds resulted in an average of 80 mol% acetyl-TAG and exceeded 85 mol% in the best transgenic lines (Alkotami *et al*., 2021). However, transgenic seeds exhibited notable changes in seed properties, including an increase in seed size, a reduction in total fatty acid content, and a delay in germination by two days (Alkotami *et al*., 2021).

To decipher the impact of acetyl-TAG accumulation in camelina seeds, we employed a comprehensive strategy involving transcriptomic and targeted lipidomic analyses of *EfDAcT+DGAT1-RNAi* transgenic seeds across four developmental stages. Our main objectives were to gain insights into the molecular processes affected by the synthesis and storage of acetyl-TAGs in late seed development and to identify potential targets to further refine the genetic engineering of these seeds.

RESULTS

Reduction of total FA content in *EfDAcT+DGAT1 RNAi* developing seeds

In order to examine how the accumulation of acetyl-TAG affects lipid composition and gene expression during late seed development, we collected developing seeds at 20, 30, 40, and 50 days after flowering (DAF) from both wild-type (WT) camelina plants and high acetyl-TAG producing plants expressing *EfDAcT+DGAT1 RNAi* with a *DsRED* fluorescence marker (Discosoma sp. Coral; Matz *et al*., 1999) for transgene detection. We first examined the total fatty acid (FA) content and composition in these developing seeds to track their changes throughout different stages of seed development. Total FA content increased from 20 DAF to 30 DAF in both WT and the transgenic *EfDAcT+DGAT1 RNAi* seeds, with a 51% increase in the WT and a 26% increase in the transgenic seeds (Figure 3.1a). However, after 30 DAF, the transgenic seeds showed a decrease in FA content in late seed development (40 and 50 DAF). In comparison to the WT, the FA content of the transgenic lines was lower at 30, 40, and 50 DAF (Figure 3.1a). While the composition of different FAs in both WT and transgenic seeds remained relatively stable across different developmental stages, the transgenic seeds possessed a different FA composition profile when compared to the WT seeds (Figure 3.1b). These changes were consistent across all stages of seed development and included a significant decrease in the levels of 20:1 and 18:3 FAs and an increase in 18:2 and 18:1 FAs (Figure 3.1b).

Altered accumulation of TAG, DAG, PC, and PG in transgenic seeds

To further investigate the changes in the accumulation of different lipids classes during seed development, targeted lipidomic profiling of WT and transgenic camelina seeds at 20, 30, 40 and 50 DAF was performed. A total of 13 glycerolipid categories were analyzed. Out of these, 12

classes, containing a total of 426 different molecular species, passed the limit of detection (Table 3.1). Principal component analysis (PCA) (Jolliffe, 2002) applied to the lipidomic data demonstrated a distinct separation between the WT and *EfDAcT+DGAT1 RNAi* genotypes (Figure 3.2a). Within each genotype, the biological replicates of each developmental stage grouped together, emphasizing a unique lipid profile associated with *EfDAcT+DGAT1 RNAi* seeds compared to the WT. Correlation analysis of total lipids in each class revealed that the accumulation of acetyl-TAG in the transgenic lines exhibited a strong positive correlation with DAG, PC, and PG, but a negative correlation with TAG (Figure 3.2b).

The total TAG levels, encompassing both regular TAG and acetyl-TAG, increased from 20 to 30 DAF, followed by a decline in later stages of seed development, mirroring the total FA content accumulation pattern. Despite this decrease, total TAG levels in the transgenic lines consistently exceeded those of WT at all developmental stages (Figure 3.3). In general, transgenic lines showed higher accumulation of glycerolipids and phospholipids, with the exception of PE, and slightly lower levels of galactolipids (Figure 3.3). Total DAG and PG levels were significantly elevated in transgenic lines throughout seed development (Figure 3.3). Total PC and LPC levels increased at 30, 40, and 50 DAF, while PA, PI, and PS levels showed an increase at 50 DAF. Notably, the transgenic lines also exhibited lower levels of MGDG and DGDG at 40 and 50 DAF.

Upregulation of less-unsaturated DAG, PC, and PG species in transgenic seeds

An analysis of fold change in the accumulation of lipid molecular species revealed that a significant portion, 356 (or 83.6%), exhibited differences in at least one developmental stage when comparing the transgenic lines to the WT (Table 3.1). Beside acetyl-TAG which accumulate in the transgenic lines only, DAG, TAG, PG, and PC exhibited notable differences, with roughly 97%,

93%, 92%, and 84% of their molecular species differentially accumulating in the transgenic seeds, respectively (Table 3.1). Among the 356 differentially accumulated (DA) lipid species, 237 (or 66.6%) were consistently altered in all developmental stages (Figure S1). As expected, majority of these shared species belonged to the lipid classes of acetyl-TAG, TAG, and DAG (Supplementary Figure C.1).

To gain a deeper understanding of what specific changes were associated with the top DA lipid classes (TAG, DAG, PC, LPC, and PG, Table 3.1), we examined the composition of their respective molecular species (Supplementary Figure C2-4). The variations between the transgenic lines and WT at different developmental stages were diverse, with the most prominent differences observed at 40 and 50 DAF. TAG molecular species with 54 and 56 carbons and 3-7 double bonds, showed the greatest reductions in the transgenic seeds (Supplementary Figure C.2). Among the DAG species, those containing 38 and 40 carbons (DAG(38:2), DAG(40:3), and DAG(40:4); Supplementary Figure C.3) demonstrated the highest difference, with DAG species containing 22:1 FA and 18:2 FA contributing significantly to the observed increase (Supplementary Figure C.3). the most significant difference was observed in the accumulation of PC molecules containing 34 and 38 carbons, with only 2-3 double bonds, as well as LPC molecules featuring 16:0 FAs, and PG molecules with 34 and 36 carbons and only 2-3 double bonds (Supplementary Figure C.4 and C.5).

Transgenic seeds display increased transcriptome profile variability in late stages of seed development

To investigate the dynamic changes in the camelina transcriptome caused by acetyl-TAG accumulation during late seed development, high-throughput RNA sequencing (RNA-Seq)

analysis was performed on developing seeds of both the transgenic lines and WT at 20, 30, 40, and 50 DAF. Over 30 million reads for each sample were generated by the RNA-Seq analysis, and more than 96% of the sequenced reads were successfully mapped against the annotated camelina reference genome (Kagale *et al*., 2014). PCA analysis of the transformed raw read counts indicated that the transgenic lines and WT clustered more closely based on the developmental stage rather than their genotype (Figure 3.4a). Notably, the 40 and 50 DAF developmental stages clustered together, suggesting a higher similarity in their transcriptome profiles compared to 20 and 30 DAF (Figure 3.4a and b). Partial Least-Squares Discriminant (PLS-DA) analysis, a "supervised" version of PCA that achieves dimensionality reduction with full awareness of class labels (Barker & Rayens, 2003; Ruiz-Perez *et al*., 2020), revealed that the most significant separation between the transgenic lines and WT occurred at 40 and 50 DAF (Figure 3.4b).

Differential expression analysis of the raw read counts (DESeq2; FDR $\leq$ 0.05, log2foldchange $\geq$ 1 and $\leq$ -1) (Love *et al*., 2014) revealed a combined total of 6248 differentially expressed genes (DEG) between the transgenic lines and the WT for all stages (Figure 3.4c). Given the hexaploid nature of camelina genome, the list of DEGs included genes that displayed differential expression in one, two, or three homoeologs. The 40 DAF stage exhibited the highest number of DEGs. Among these, 519 upregulated genes and 410 downregulated genes were also shared with 50 DAF (Supplementary Figure C.6). Interestingly, out of all the DEGs, less than 1% were shared between all stages with 14 exhibiting upregulation, and 9 exhibiting downregulation (Supplementary Figure C.6). The list of commonly upregulated genes included *EfDAcT* and *DsRED* (Supplementary Figure C.6 and Supplementary Figure C.7b), which confirms their expression in the transgenic lines. It also encompassed genes associated with embryo development, such as *MATERNAL EFFECT EMBRYO ARREST 8 (MEE8)*, and *LATE EMBRYOGENESIS*

ABUNDANT 38 (LEA38), as well as genes linked to hormone response, including *AGAMOUS-LIKE 15* and *LIPOXYGENASE 2* (*LOX2*; Supplementary Figure C.6a). Conversely, the list of commonly downregulated genes comprised all three homoeologs of *DGAT1* (Supplementary Figure C.6 and Supplementary Figure C.7a), validating the efficiency of RNAi suppression. Additionally, the list included genes related to abscisic acid (ABA) response (UDP-Glycosyltransferase Superfamily Protein) and phosphate ion homeostasis (*NITRATE-INDUCIBLE 1* and *GLYCEROL-3-PHOSPHATE PERMEASE 1*; Supplementary Figure C.6b).

Expression pattern of genes related to FA biosynthesis and lipid metabolism pathways

Given the changes observed in FA profile and lipid accumulation in our transgenic lines, we examined the expression patterns of DEGs associated with FA and lipid metabolism pathways. More than 45 genes were differentially expressed (Figure 3.5), including *DGAT1*. In addition to *DGAT1*, only 14 genes exhibited differential expression in more than one homeolog and were predominantly upregulated at 40 and/or 50 (Supplementary Figure C.8-11). Among these 14 genes, key regulatory proteins were identified, including GLB1, which binds to the biotin carboxylase carrier protein (BCCP) subunits of plastid heteromeric acetyl-coenzyme A carboxylase (HtACCase) and negatively regulates its expression under 2-oxoglutarate limitation (Feria Bourrellier *et al*., 2010) (Figure 3.5; Supplementary Figure C.8a). Conversely, ABA insensitive 4 (ABI4), involved in ABA signal transduction and reported to activate DGAT1 expression in Arabidopsis seedlings under nitrogen deficiency (Yang *et al*., 2011), showed downregulation at 40 and 50 DAF (Figure 3.5; Supplementary Figure C.8b). Other notable upregulated genes included *ACYL CARRIER PROTEIN (ACP) DESATURASE 5 (AAD5)*, a member of the stearoyl-ACP desaturase family (SAD) responsible for producing oleic acid (18:1;

Figure 3.5; Supplementary Figure C.9a); *FATTY ACID DESATURASE 3 (FAD3),* involved in synthesizing linoleic acid (18:2) from linolenic acid (18:3; Figure 3.5; Supplementary Figure C.9b); *PI SYNTHASE (PIS),* contributing to PI synthesis from PA in the endoplasmic reticulum (ER; Figure 3.5; Supplementary Figure C.10a); *DAG KINASE 3 (DKG3),* producing PA by phosphorylating DAG (Figure 3.5; Supplementary Figure C.10b); and two members of the phosphoethanolamine N-methyltransferase (PEAMT) family (*NMT1* and *NMT3*) catalyzing the last two methylation steps of PE for PC synthesis in the ER (Figure 3.5; Supplementary Figure C.10c). *REDUCED OLEATE DESATURATION 1 (ROD1),* functioning as PC:DAG cholinephosphotransferase in the ER, exhibited upregulation at 40 DAF in one homeolog and downregulation at 50 DAF in another (Figure 3.5; Supplementary Figure C.10e). Additionally, *SUGAR-DEPENDENT 1-LIKE (SDP1L),* associated with the initial step of TAG degradation, displayed upregulation at 20 DAF, while *PLASTID LIPASE1 (PLIP1)*, involved in the hydrolysis of polyunsaturated FA (PUFA) from plastid PG, and *CALEOSIN1 (CLO1),* encoding a caleosin protein found within seed lipid droplets, were upregulated at 40 and 50 DAF (Figure 3.5; Supplementary Figure C.11). The remaining DEGs similarly showed upregulation at 40 and/or 50 DAF, but exclusively in one homeolog (Figure 3.5; Supplementary Figure C.12).

Functional and pathway enrichment analysis of *EfDAcT+DGAT1 RNAi* developing seed

To understand the biological implication of the DEGs, processes significantly affected in the transgenic lines compared to the WT in each developmental stage were identified by Gene ontology (GO) enrichment analysis. GO enrichment analysis revealed 105 upregulated and 50 downregulated processes at 20 DAF, 36 upregulated and 52 downregulated processes at 30 DAF, 116 upregulated and 55 downregulated processes at 40 DAF, and 98 upregulated and 65

downregulated processes at 50 DAF. Upregulated GO biological processes shared between at least 3 developmental stages included Glucan metabolic process, Response to hormones, and Response to Karrikin (Figure 3.6a). Downregulated GO biological processes shared between at least 3 developmental stages included Response to lipid, TAG metabolic process, Phosphate ion homeostasis, Regulation of seed germination, and Regulation of seedling development (Figure 3.6b).

40 DAF and 50 DAF showed a significant overlap in their upregulated processes. These included processes associated with Sulfate reduction and assimilation, Cellular response to sulfur starvation, Flavonoid biosynthesis, and PC metabolism (Figure 3.6a). Similarly, there was considerable overlap in their downregulated processes. These processes included Cytochrome complex assembly, Photosynthesis, and Root development (Figure 3.6b). To identify and map the key pathways affected in the transgenic lines, functional pathway analysis was performed on a complied list of all DEGs across all developmental stages. This analysis was carried out using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. 19 KEGG pathways were enriched, out of which only five have an FDR (adjusted *p*-value or *q*-value) ≤ 0.05, which included, Metabolic pathways, Biosynthesis of secondary metabolites, Amino sugar and nucleotide sugar metabolism, Starch and sucrose metabolism, and Flavonoid biosynthesis (Figure 3.6c).

Identification of target genes within significant pathways of *EfDAcT+DGAT1 RNAi* developing seed

To further identify target genes associated with the changes observed in the transgenic lines, we examined the list of DEGs related to the significant pathways identified from KEGG analysis (Figure 7c; Flavonoid biosynthesis, Amino sugar and nucleotide sugar metabolism, and Starch and

sucrose metabolism). Many genes encoding key enzymes in flavonoid biosynthesis exhibited upregulation in more than one homeolog at 40 and 50 DAF (Figure 3.7). These included 4 members of the TRANSPARENT TESTA (TT) family (*TT4, TT5, TT6,* and *TT7*), two flavonol synthases (*FLS1* and *FLS3*), a chalcone isomerase like family protein (*CHIL*), and three MYB transcription factors (*MYB11, MYB12,* and *MYB111*) known for their redundant regulatory role in flavonoid biosynthesis. *TT4*, catalyzing the rate-limiting step of flavonoid biosynthesis and FLSl, catalyzing the conversion of dihydroflavonols to flavonoid were consistently upregulated in all homoeologs at 40 and 50 DAF (Figure 3.7).

Within the amino sugar and nucleotide sugar metabolism pathway, four genes showed distinct expression patterns across multiple homoeologs (Supplementary Figure C.13a). These included genes encoding two chitinase family proteins (*CT* and *CT2*) and a UDP-D-glucuronate 4-epimerase (*GEA6*), involved in chitinase and pectin biosynthesis in the cell wall, respectively. *CT* was downregulated at 30 and 40 DAF, *CT2* was upregulated at 20 DAF, and *GEA6* was upregulated at 40 and 50 DAF. The fourth DEG encodes a putative glucose-6-phosphate acetyltransferase (*GNA1*) contributing to UDP-acetylglucosamine biosynthesis, and displayed upregulation at 50 DAF.

Similarly, in the starch and sucrose metabolism pathway, 11 DEGs were identified (Figure S14b). Within this set, three genes involved in trehalose biosynthesis, with *TREHALOSE-PHOSPHATASE SYNTHASE 8 (TSP8)* and *TSP9* downregulated at 40 and 50 DAF, and *TREHALOSE-PHOSPHATE PHOSPHATASE G (TPPG)* upregulated at 40 and 50 DAF. Further, *ONE GLYCOSYL HYDROLASE (GH17)* and three *β-GLUCOSIDASES (BGLU3, BGLU16,* and *BGLU25)* engaged in cell wall polysaccharide metabolism demonstrated varied expression profiles. *GH17* experienced upregulation at 40 and 50 DAF, *BGLU3* was downregulated at 30

DAF, *BGLU16* displayed upregulation at 40 DAF in one homeolog and downregulation at 50 DAF in the other, and BGLU25 exhibited upregulation at 20 DAF. Moreover, three genes dedicated to sucrose biosynthesis—*SUCROSE SYNTHASE 6 (SUC6), SUCROSE PHOSPHATE SYNTHASE 2 (SPS2)*, and *SPS4*— displayed upregulation in later seed developmental stages. Additionally, *ADP-GLUCOSE PYROPHOSPHORYLASE 3 (APL3),* a key gene governing the rate-limiting step in starch biosynthesis, exhibited significant upregulation at 40 and 50 DAF.

Expression of *EfDAcT+DGAT1 RNAi* alters the expression of genes associated with hormone response

In addition to lower FA content, transgenic seeds expressing *EfDAcT+DGAT1 RNAi* exhibited a slight increase in seed weight and a delay in seed germination (Alkotami *et al*., 2021). We examined the list of DEGs involved in hormone response (Figure 3.8), processes that were either upregulated or downregulated in at least 3 developmental stages in the transgenic seeds (Figure 3.6), as the phenotypic changes observed in the transgenic seeds might be associated with changes in hormone levels. Notably, genes involved in the degradation/inactivation of cytokinin, abscisic acid (ABA), and jasmonic acid (JA) were downregulated, while those involved in gibberellic acid (GA) inactivation were upregulated (Figure 3.8). Cytokinin and brassinosteroid-regulated genes associated with cell division were upregulated, reflecting potential changes in growth regulation. Additionally, genes influenced by auxin, crucial for cell enlargement and plant growth, displayed varied expression patterns, with a majority being upregulated.

GA signaling promotes stem growth and seed germination. In the transgenic seeds, the expression of *SLEEPY1 (SLY1),* a gene responsible for facilitating the degradation of GA signaling negative regulators known as DELLA proteins, was decreased (Figure 3.8). Conversely genes

induced by ABA, a key regulator of seed dormancy, displayed contrasting patterns of gene expression. In transgenic seeds, essential components governing ABA signaling exhibited upregulation. This includes multiple genes encoding the ABA receptor pyrabactin resistance-like (*PYL*), sucrose non-fermenting-1-related protein kinase 2 (*SnRK2*), and delay of germination 1 (*DOG1*; Figure 3.8). *PYL* and *SnRK2* activation trigger downstream targets promoting dormancy, while *DOG1* inhibits the activity of protein phosphatase 2C (*PP2C*), an inhibitor of *SnRK2* kinases.

DISCUSSION

Despite the successful accumulation of acetyl-TAG in transgenic camelina seeds, their levels fall short of those naturally occurring in species like *Euonymus* (Alkotami *et al*., 2021; Kleiman *et al.*, 1967). Transgenic seeds also exhibit notable changes in seed properties, including reduction in fatty acid content, increase in seed weight, and delay in seed germination (Alkotami *et al*., 2021). Many studies have utilized omics analysis to gain insights into the oil biosynthesis mechanisms in oilseed crops, aiming to enhance oil levels or optimize the production of designer oils (Abdullah *et al*., 2018; Arias *et al*., 2023; Horn *et al*., 2016; Li *et al*., 2023). Similarly, here we adopted a comprehensive approach, integrating lipidomic and transcriptomic analysis of transgenic camelina seeds accumulating more than 85 mol% acetyl-TAG over seed development to understand the impact of acetyl-TAG accumulation on seeds biology. By employing this approach, we observed changes in lipid composition, alterations in gene expression, and modulation of multiple metabolic pathways. Furthermore, this approach facilitated the identification of candidate genes that can be utilized to address the observed limitations in transgenic seeds.

Decrease of FA content in transgenic seeds accompanies upregulation of flavonoid and starch biosynthesis

The observed decrease in FA content in transgenic seeds during seed development (Figure 3.1a) aligns with earlier findings in dry seeds (Alkotami *et al*., 2021). Transcriptomic analysis of transgenic seeds revealed upregulation in genes related to starch and flavonoid biosynthesis in later stages of seed development (Figures 3.7, 3.8; Supplementary Figure C.13). This suggests a redirection of carbon flux from oil synthesis towards starch and flavonoid production, which correlates with the observed increase in seed weight and decrease in total FA levels in transgenic seeds (Alkotami *et al*., 2021). A comparative analysis of flavonoids and starch levels between transgenic lines and the WT would validate this shift. Additionally, targeting genes involved in starch and flavonoid biosynthesis presents an opportunity to enhance total FA content, as demonstrated in a previous study where the loss of TT4 led to increased FA biosynthesis in Arabidopsis seeds (Xuan *et al*., 2018).

The acetyl-TAG pathway, producing TAG molecules with only two FAs, might delay the incorporation of FAs into glycerol backbones in transgenic lines compared to the WT, potentially inhibiting FA synthesis. This hypothesis aligns with Bates *et al*. (2014), in which inefficient utilization of unusual FAs was found to induce posttranslational inhibition of genes involved in *de novo* FA synthesis. The proposed posttranslational inhibition could also explain the lack of downregulation in genes involved in FA synthesis in the transgenic lines (Figure 3.5). Further studies, including flux analysis, are warranted to understand FA metabolism in these transgenic seeds.

Elevated levels of DAG and PC in transgenic seeds suggest a bottleneck in their utilization to produce acetyl-TAG

The targeted lipidomic analysis revealed an impact on various lipid classes in transgenic seeds (Figure 3.3). Acetyl-TAG accumulation exhibited a negative correlation with TAG levels, highlighting the competition between acetyl-TAG and regular TAG synthesis pathways (Figure 3.2b). Conversely, acetyl-TAG accumulation showed a positive correlation with DAG, PC, and PG (Figure 3.2b). The increase in PC levels in the transgenic lines was further associated with the upregulation of NMT1 and NMT3 phosphoethanolamine N-methyltransferases, essential for PC synthesis (Figure 3.5; Supplementary Figure C.10c). The role of NMT1 and NMT3 was previously demonstrated in Arabidopsis, where the *nmt1 nmt3* double mutant showed significantly reduced PC levels and exhibited altered lipid profiles (Chen *et al*., 2018). The increase in DAG and PC levels, although surprising, given the increase in total TAG (acetyl-TAG+TAG) and decrease in FA content of transgenic seeds (Figure 3.1a; Figure 3.3), suggests that glycerol backbone availability is not a limiting factor for acetyl-TAG production. Instead, a bottleneck exists in the utilization of DAG and PC for acetyl-TAG synthesis, which could be influenced by fatty acid composition or acetyl-CoA levels.

The decrease in specific TAG species, particularly those containing with 54 and 56 carbons and 3-7 double bonds, coupled with the accumulation of DAGs containing VLCFA, suggests a preference for less unsaturated acetyl-TAGs lacking VLCFA (Supplementary Figure C.2 and C.3). This observation is consistent with the decrease in 20:1 VLCFA and increase in 18:2 FA in developing seeds (Figure 3.1b) and dry seeds (Alkotami *et al*., 2021). Furthermore, it aligns with EaDAcT preference for acetylating DAGs with unsaturated FAs over saturated FAs and VLCFA, as demonstrated in previous research (Bansal & Durrett, 2016b). These results present an

opportunity to optimize acetyl-TAG production by eliminating VLCFA and increasing acetyl-CoA levels in camelina seeds.

FATTY ACID ELONGASE1 (FAE1), is a major component of the fatty acid elongase complex and encodes the rate-limiting enzyme for seed VLCFA production. Mutation of *FAE1* in camelina resulted in the accumulation of less than 2% of VLCFA in mutant seeds compared to 22% in WT seeds (Ozseyhan *et al*., 2018). Given that FAE1 utilizes acetyl-CoA as a substrate, *fae1* mutant lines could possibly contain higher levels of acetyl-CoA. Another strategy to increase acetyl-CoA levels is to overexpress ATP-citrate lyase (ACL) which catalyzes the ATP-dependent reaction of citrate and CoA to form acetyl-CoA (Fatland *et al*., 2002; Fatland *et al*., 2005). Previous research has demonstrated the effectiveness of this approach, with overexpression of rat liver ACL resulting in a fourfold increase in total ACL activity and a corresponding 16% increase in fatty acid content in tobacco leaves (Rangasamy & Ratledge, 2000).

Upregulation of genes involved in seed dormancy and ABA signaling correlate with delayed germination

Changes in hormone response-related genes provide insights into the observed phenotypic changes in transgenic seeds (Figure 3.8). For example, at 40 and 50 DAF, all three homoeologs of camelina cytokinin oxidase/dehydrogenase 5 (CKX5) were downregulated. CKX, a key negative regulator of cytokinin accumulation, when downregulated, has been reported to significantly boost cytokinin levels in transgenic plants, consequently increasing seed yield (Ashikari *et al*., 2005; Yeh *et al*., 2015; Zeng *et al*., 2022). Conversely, two homoeologs of camelina SLEEPY1 (SLY1) were downregulated at 40 and 50 DAF. SLY1 interacts with and inhibits DELLA genes, known negative regulators of GA signaling and seed germination. *Sly1* mutants display increased sensitivity to

ABA-induced inhibition of seed germination and increased dormancy in seeds (Steber *et al*., 1998; Strader *et al*., 2004). Additionally, transgenic seeds demonstrated upregulation in DELAY OF GERMINATION 1 (DOG1) and in the pyrabactin resistance 1 (PYR1)/PYR1-like (PYL)/regulatory component of ABA receptor (RCAR) proteins. DOG1 and PYR/PYL inhibit ABA signaling negative regulators, clade A type 2C protein phosphatases (PP2Cs). Upregulation of DOG1 and PYR/PYL has been reported to induce hyperdormancy in seeds (Mosquna *et al*., 2011; Nishimura *et al*., 2018). Upregulation of ABA signaling components and GA inactivation genes, in addition to downregulation of ABA degradation genes correlate with delayed germination. These genes serve as promising targets for further investigation and potential manipulation to address delayed germination in transgenic seeds.

CONCLUSION

The complex molecular processes affected by acetyl-TAG synthesis in transgenic camelina seeds were examined in this study. The alterations in lipid composition, specific lipid molecular species, transcriptome profile, and enriched pathways offer important information on acetyl-TAG regulation paving the way for tailored improvements in these transgenic seeds. Furthermore, the identification of target genes within specific pathways creates opportunities for subsequent research to manipulate these pathways and obtain high acetyl-TAG with improved overall seed traits. The findings of this study contribute to our understanding of the broader implications of lipid engineering on seed biology and establish a foundation for the targeted advancement of producing high-quality oils with enhanced industrial applications.

MATERIALS AND METHODS

Plant material and growth conditions

Transgenic camelina 'Suneson' seeds expressing *EfDAcT+DGAT1-RNAi* were previously generated (Alkotami *et al*., 2021). Transgenic and wild-type camelina plants were grown in growth chambers with a 16-hour light and 8-hour dark cycle, using a soil mixture composed of Berger BM6 All-Purpose soil mix, vermiculite, and perlite in a 4:2:1 ratio. The light intensity was maintained at 250 μmol m^{-2} s^{-1}, and the temperature was controlled at 21°C. To collect seeds at various developmental stages, newly emerging flowers were marked using colored threads, and the siliques marked in this manner were collected at 20, 30, 40 and 50 days after flowering (DAF). The harvested siliques were promptly frozen in liquid nitrogen and stored at −80°C until further analysis. Before lipid and RNA extraction, 20 developing seeds pooled from 4 plants were crushed in liquid nitrogen to fine ground tissue.

Lipid analysis

Lipids were extracted from ground tissues of 20 developing seeds using a single-extraction method (Shiva *et al*., 2018) as follows: ground tissues were placed in an 8-mL glass vial containing 1 mL of hot isopropanol with 0.01% BHT, maintaining a temperature of 85°C for 15 minutes. After cooling to room temperature, 4 mL of extraction solvent (comprising chloroform-methanol-water in a 40:55:5, v/v/v ratio) were added. The samples were shaken on an orbital shaker at 100 rpm for 24 hrs. The resulting lipid extracts were transferred to a new vial and stored at -20°C until further analysis. For targeted lipidomic analysis, lipid extracts were prepared for mass spectrometry as outlined in Shiva *et al*. (2013). Prepared samples were analyzed on a triple quadrupole mass spectrometer with an electrospray ionization source (API 4000 QTRAP, Applied

Biosystems, Foster City, CA), as described previously (Lee *et al*., 2011). Fatty acid content and composition was determined by first drying 300 μL of lipid extracts using a stream of nitrogen. Subsequently, 500 μL of toluene was added to the dried lipid extracts, along with 30 nmoles of tri17:0-TAG (NuChek Prep, New Elysian, MN) serving as an internal standard. Fatty acid methyl esters (FAME) were generated as described previously (Li *et al*., 2006). FAME samples were analyzed on a DB23 column, using a Shimadzu GC-2010 plus gas chromatograph with a flame ionization detector (FID) following a standard gas chromatography method (Aznar Moreno & Durrett, 2017).

RNA extraction and RNA-seq analysis

RNA was isolated from ground tissues of 20 developing seeds using the RNAqueous Total RNA Isolation Kit (Ambion, Austin, TX, USA) followed by a subsequent lithium chloride precipitation step according to the manufacturer's instructions. Genomic DNA contamination was removed by treating the isolated RNA with the TURBO DNA-free Kit (Invitrogen, Carlsbad, CA, USA). RNA quality was assessed with the Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA), confirming RNA Integrity Number (RIN) of ≥8 for all samples. Extracted RNA samples were stored at -80°C for later use. RNA-seq analysis was performed by the Genome Sequencing Facility at the University of Kansas Medical Center. The analysis included mRNA library preparation, mRNA library quality checks, indexing, and sequencing (30 million, 101 paired-end reads) on the Illumina NovaSeq6000 platform.

RNA-seq data filtering and analysis

Raw RNA sequencing reads were checked for quality control and trimmed using fastp (S. Chen *et al*., 2018). Clean reads were mapped to the camelina reference genome with Ensembl gene annotations using the STAR Aligner (Dobin *et al*., 2013) with default parameters in addition to "outSAMtype BAM SortedByCoordinate". An average mapping of 96% for all samples was achieved. The option "quantMode GeneCounts" within STAR was used to generate raw count data. Clean raw reads were also mapped to the camelina reference transcriptome with Ensembl gene annotations using Salmon (Patro *et al*., 2017) to calculate transcripts per million (TPM).

Raw read counts obtained from STAR were processed using the Integrated Differential Expression and Pathway (iDEP) tool (Ge *et al*., 2018) for differential expression analysis (DE) and Gene Ontology (GO) enrichment analysis (http://bioinformatics.sdstate.edu/idep/). For read count filtering, data was normalized by the count per million (CPM) function in edgeR (Robinson *et al*., 2010). Only genes with a minimum of one CPM in at least 12.5% of the samples were retained. Differential expression analysis was performed on the filtered read counts using DESeq2 (Love *et al*., 2014). The *P* values were adjusted in DESeq2 for multiple testing using the Benjamini–Hochberg method. Genes with a False Discovery Rate (FDR; or adjusted *P*-value) ≤ 0.05 and fold change ≥ 2 were considered as differentially expressed genes (DEGs). A list of all camelina DEGs was converted to Arabidopsis homologs and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed on the list using the ShinyGo tool (http://bioinformatics.sdstate.edu/go/) (Ge *et al*., 2020).

Plot generation and statistical analysis

Principal component analysis (PCA), correlation heatmap student t-test and differential lipids analysis were performed using MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/) (Pang *et al*., 2021). The *P* values for differential lipids analysis were adjusted for multiple testing using the Benjamini–Hochberg method. Lipids with an FDR (adjusted *P*-value) ≤ 0.05 and fold change ≥ 2 were considered as differentially accumulated (DA). Venn plots were generated using jvenn (https://jvenn.toulouse.inrae.fr/app/index.html) (Bardou *et al*., 2014).

FIGURES

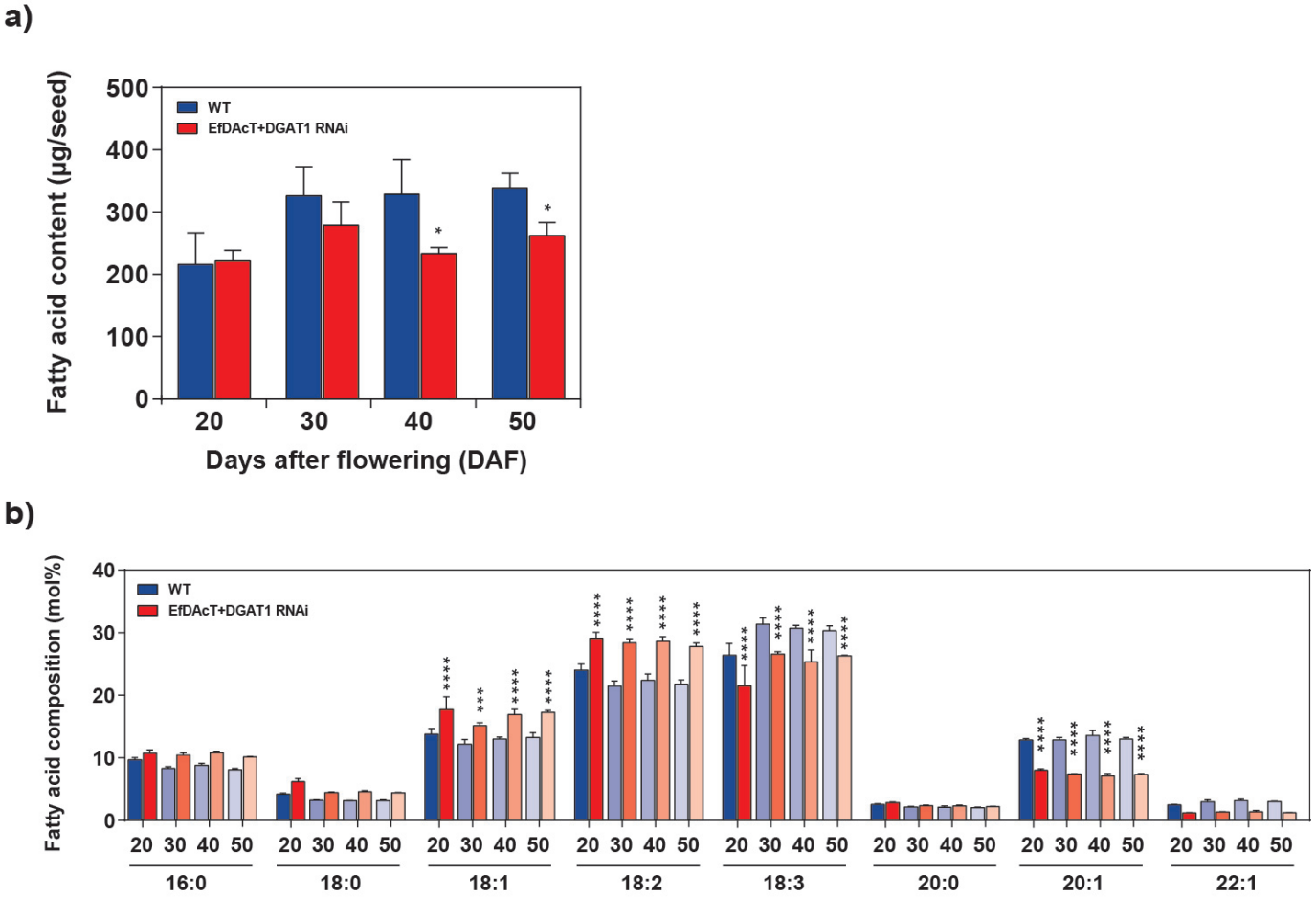


Figure 3.1. Fatty acid content and composition of *EfDAcT+DGAT1 RNAi* developing seeds.

(a) Seed fatty acid content and **(b)** fatty acid composition of camelina wild-type (WT) and homozygous transgenic seeds expressing *EfDAcT+DGAT1 RNAi*. Data represent mean ± standard error, n ≥ 3 biological reps. Asterisks indicate statistical difference from WT seeds (Student t-test; * $p \leq 0.05$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

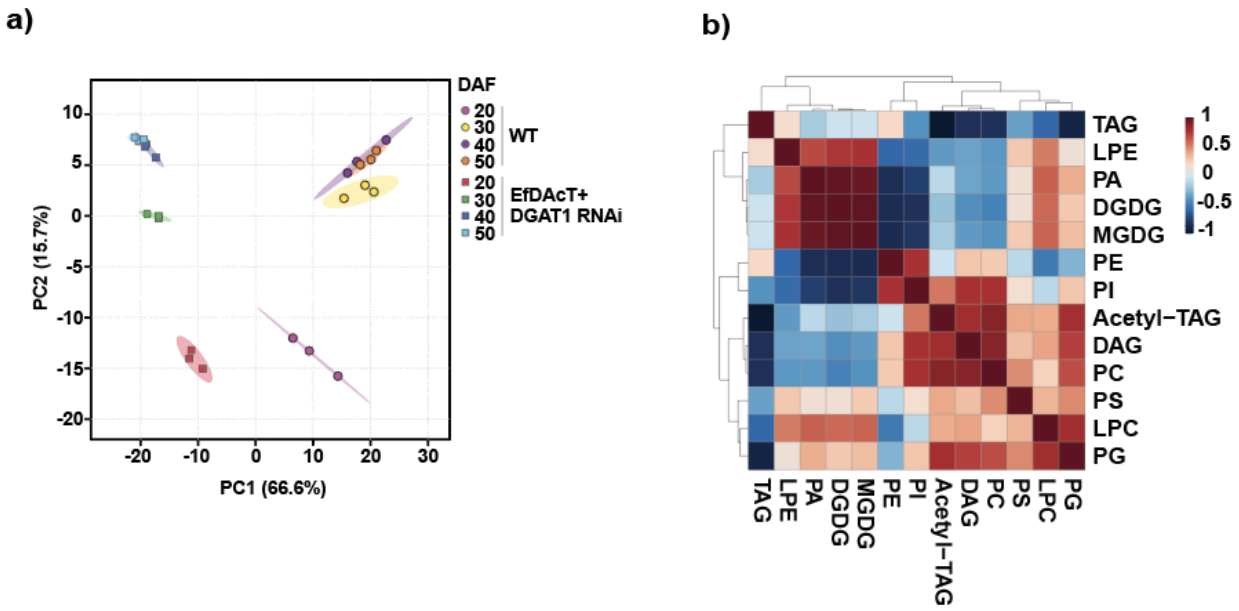


Figure 3.2. PCA plot and correlation heatmap of different lipid classes detected in transgenic camelina developing seeds.

(a) Principal component analysis (PCA) of all detected lipids in camelina wild-type (WT) and homozygous transgenic seeds expressing *EfDAcT+DGAT1 RNAi.* **(b)** Correlation heatmap (Pearson) of different lipid classes detected in camelina WT and homozygous transgenic seeds expressing *EfDAcT+DGAT1 RNAi.* Abbreviations: DAF, Days after flowering. TAG, triacylglycerol; Acetyl-TAG, acetyl-triacylglycerol; DAG, diacylglycerol; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; PC, phosphatidylcholine; LPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine; PA, phosphatidic acid; PI, phosphatidylinositol; PS, phosphatidylserine; PG, phosphatidylglycerol.

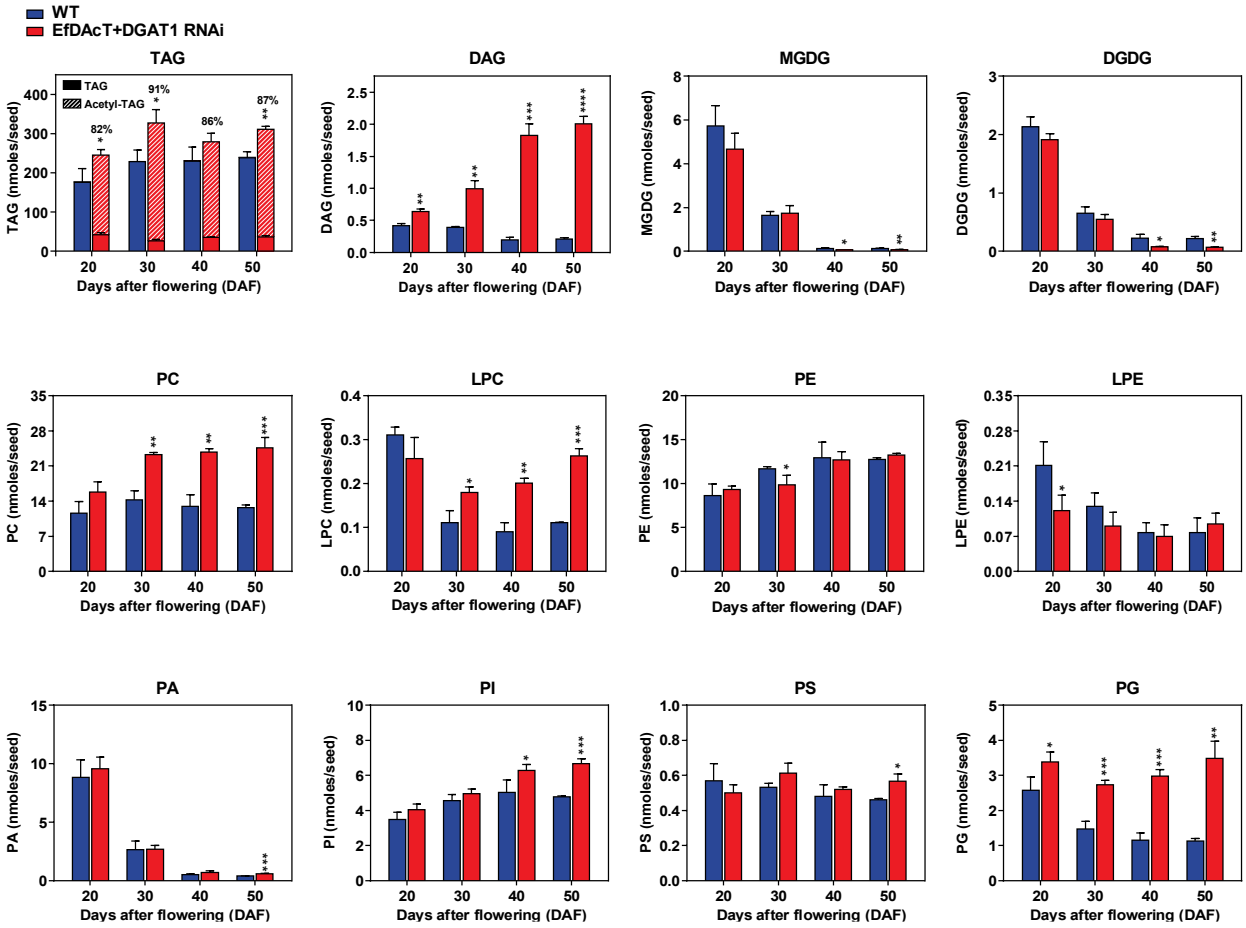


Figure 3.3. Total contents of different lipid classes across seed development.

Accumulation of total detected lipids in each class in camelina wild-type (WT) and homozygous transgenic seeds expressing *EfDAcT+DGAT1 RNAi*. The numerical value above each bar in the TAG plot indicate the percentage of acetyl-TAG in regard to total TAG (TAG + Acetyl-TAG). Data represent mean ± standard error, n= 3 biological reps. Asterisks indicate statistical difference from WT seeds (Student t-test; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$). Abbreviations: TAG, triacylglycerol; Acetyl-TAG, acetyl-triacylglycerol; DAG, diacylglycerol; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; PC, phosphatidylcholine; LPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine; PA, phosphatidic acid; PI, phosphatidylinositol; PS, phosphatidylserine; PG, phosphatidylglycerol.

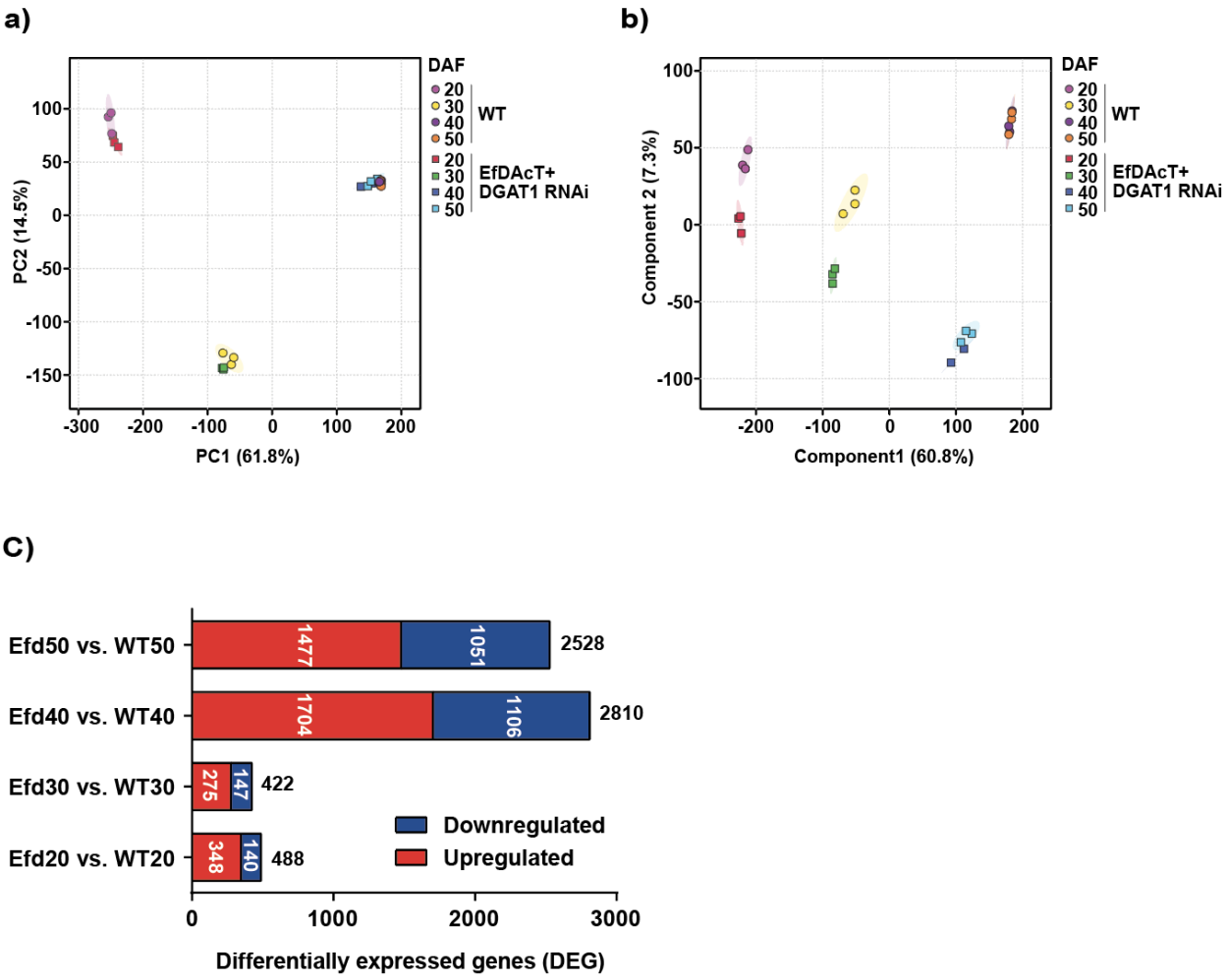


Figure 3.4. Statistical analysis of DEGs in transgenic seeds across seed development.

(a) Unsupervised (Principal component analysis plot; PCA) and **(b)** supervised (Partial Least-Squares Discriminant; PLS-DA) multivariate analysis of differentially expressed genes (DEGs) in camelina wild-type (WT) seeds and homozygous transgenic seeds expressing *EfDAcT+DGAT1 RNAi*. **(c)** Number of DEGs in transgenic seeds relative to the WT at each developmental stage. False Discovery Rate (FDR) ≤ 0.05 and fold change ≥ 2. The numerical values following Efd and WT indicate the respective seed development stage. The numerical value on top of each bar represents the total number of DEGs. DAF, Days after flowering; Efd, *EfDAcT+DGAT1 RNAi*.

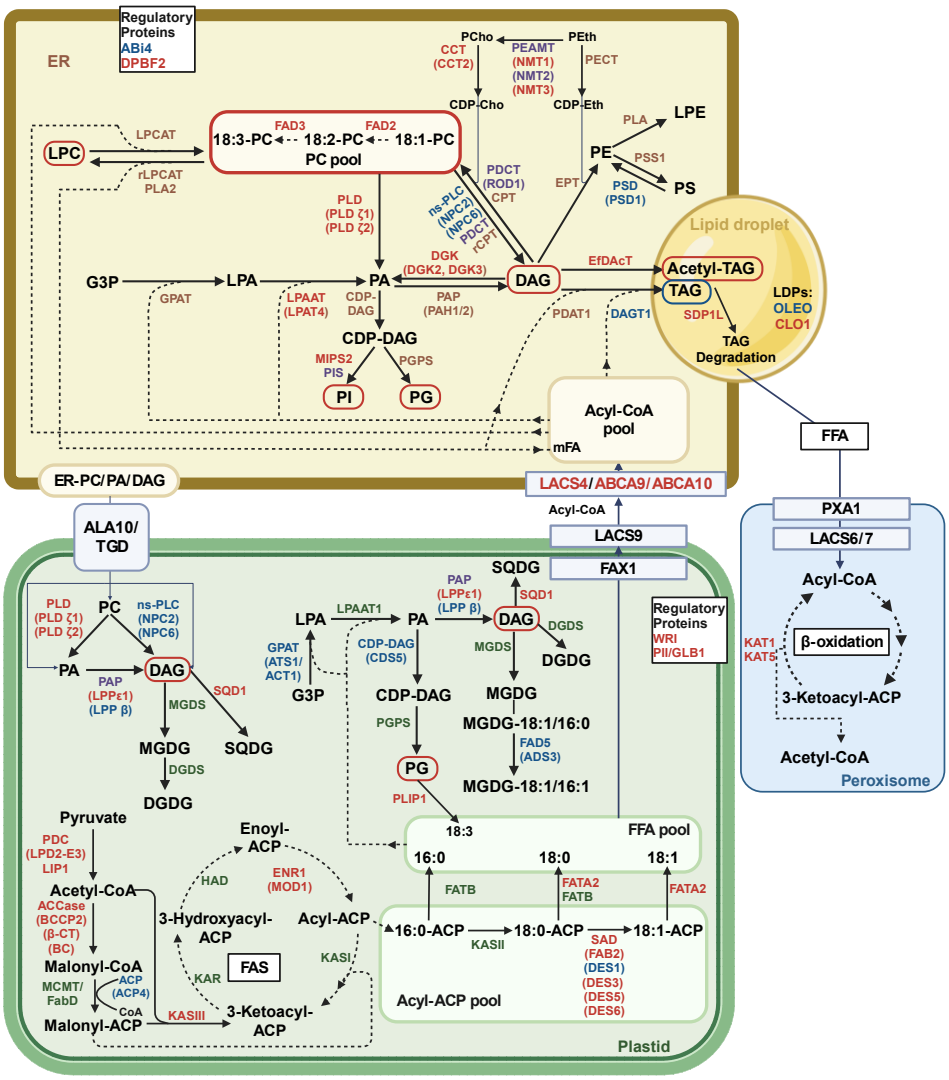


Figure 3.5. Fatty acids and lipid metabolism of *EfDAcT+DGAT1 RNAi* developing seeds.

Red-colored genes signify upregulated differentially expressed genes (DEGs) at any stage of seed development in the transgenic seeds, while blue-colored genes denote downregulated DEGs. Purple-colored genes indicate instances of both upregulation and downregulation in different camelina homoeologs. Genes in brackets represent different members/isoforms of the preceding label. Red boxes represent upregulated lipid classes. Differential expression of transcript and lipids is based on a False Discovery Rate (FDR) ≤ 0.05 and fold change ≥ 2. Dashed lines indicate the synthesis, addition, or degradation of fatty acids (FAs). Abbreviations: **G3P**, glycerol-3-phosphate; **LPA**, lysophosphatidic acid; **PA**, phosphatidic acid; **CDP,** cytidine diphosphate; **DAG**, diacylglycerol; **PG**, phosphatidylglycerol; **PI**, phosphatidylinositol; **PC**, phosphatidylcholine;

LPC, lysophosphatidylcholine; **Cho**, choline; **PCho**, phosphocholine; **Etn**, ethanolamine; **PEth**, phosphoethanolamine; **PE**, phosphatidylethanolamine; **LPE**, lysophosphatidylethanolamine; **PS**, phosphatidylserine; **MGDG**, monogalactosyldiacylglycerol; **DGDG**, digalactosyldiacylglycerol; **SQDG**, sulfoquinovosyl diacylglycerol; **TAG**, triacylglycerol; **Acetyl-TAG**, acetyl-TAG. **ER**, endoplasmic reticulum; **FFA**, free fatty acid; **mFA**, modified fatty acid; **ACP**, acyl carrier protein; **ALA10**, aminophospholipid atpase 10; **TGD**, trigalactosyldiacylglycerol; **FAX1**, fatty acid export 1; **LACS**, long chain acyl-CoA synthetase; **ABCA**, ATP-binding cassette (ABC) transporter subfamily A; **PXA1**, peroxisomal ABC transporter 1; **WRI1**, WRINKLED 1; **ABI4**, ABA insensitive 4; **DPBF2**, DC3 promoter-binding factor 2; **PDC**, pyruvate dehydrogenase complex; **LPD2-E3**, dihydrolipoamide dehydrogenase2-E3 component; **LIP1**, Lipoate Synthase 1; **ACCase**, plastid acetyl-CoA carboxylase; **BCCP**, biotin carboxyl carrier protein; **β-CT**, β-carboxyltransferase; **BC**, biotin carboxylase; **MCMT**, malonyl-CoA: ACP malonyltransferase; **FAS**, fatty acid synthase complex**; ENR1,** ENOYL-ACP REDUCTASE 1; **KAS**, ketoacyl-ACP synthase; ; **KAR**, ketoacyl-ACP reductase; **HAD**, hydroxyacyl-ACP dehydrase; **SAD**, stearoyl-ACP desaturase; **FAB**, fatty acid biosynthesis; **AAD**, Acyl-ACP desaturase; **FAT**, acyl-ACP *thioesterase;* ***GPAT****;* glycerol-3-phosphate acyltransferase; ***ACT1****;* acyltransferase 1; **LPAAT1**, LPA acyltransferase 1; **PAP**, PA phosphatase; **LPP**, lipid phosphate phosphatase; **MGDS**, MGDG synthase; **DGDS**, DGDG synthase; **SQD1**, SQDG synthase 1; **CDS5**, CDP-DAG synthase 5; **PGPS**, PG phosphate synthase; **PLIP1**, plastid lipase 1; **PLD**, phospholipase D; **ns-PLC/NPC**, non-specific phospholipase C; **MIPS2**, myo-inositol-1-phosphate synthase 2; **PIS**, phosphatidylinositol synthase; **PAH**, PA phosphohydrolase; **DKG**, DAG kinase; **PDCT**, PC:DAG cholinephosphotransferase; ***ROD1****, reduced oleate desaturase 1*; **CPT**, CDP-choline:DAG cholinephosphotransferase; **r**, reverse; **FAD**, fatty acid desaturase; **LPCAT**, *lyso*-PC acyltransferase; **PLA**, phospholipase A; **PEAMT**, phosphoethanolamine (PEA) N-methyltransferase (NMT); **CCT**, CTP:phosphorylcholine cytidylyltransferase; **PECT**, CTP:phosphorylethanolamine cytidylyltransferase; **EPT**, DAG:ethanolaminephosphotransferase; **PSD**, PS decarboxylase; **PSS**, PS synthase; **EfDAcT***, Euonymus fortunei* diacylglycerol acetyltransferase; **DGAT1**, diacylglycerol acyltransferase 1; **PDAT1**, phospholipid: diacylglycerol acyltransferase 1; **OLEO**, oleosin **; CLO1**, caleosin 1; **SDP1L**, sugar-dependent 1-like; **KAT**, 3-keto-acyl-CoA thiolase.

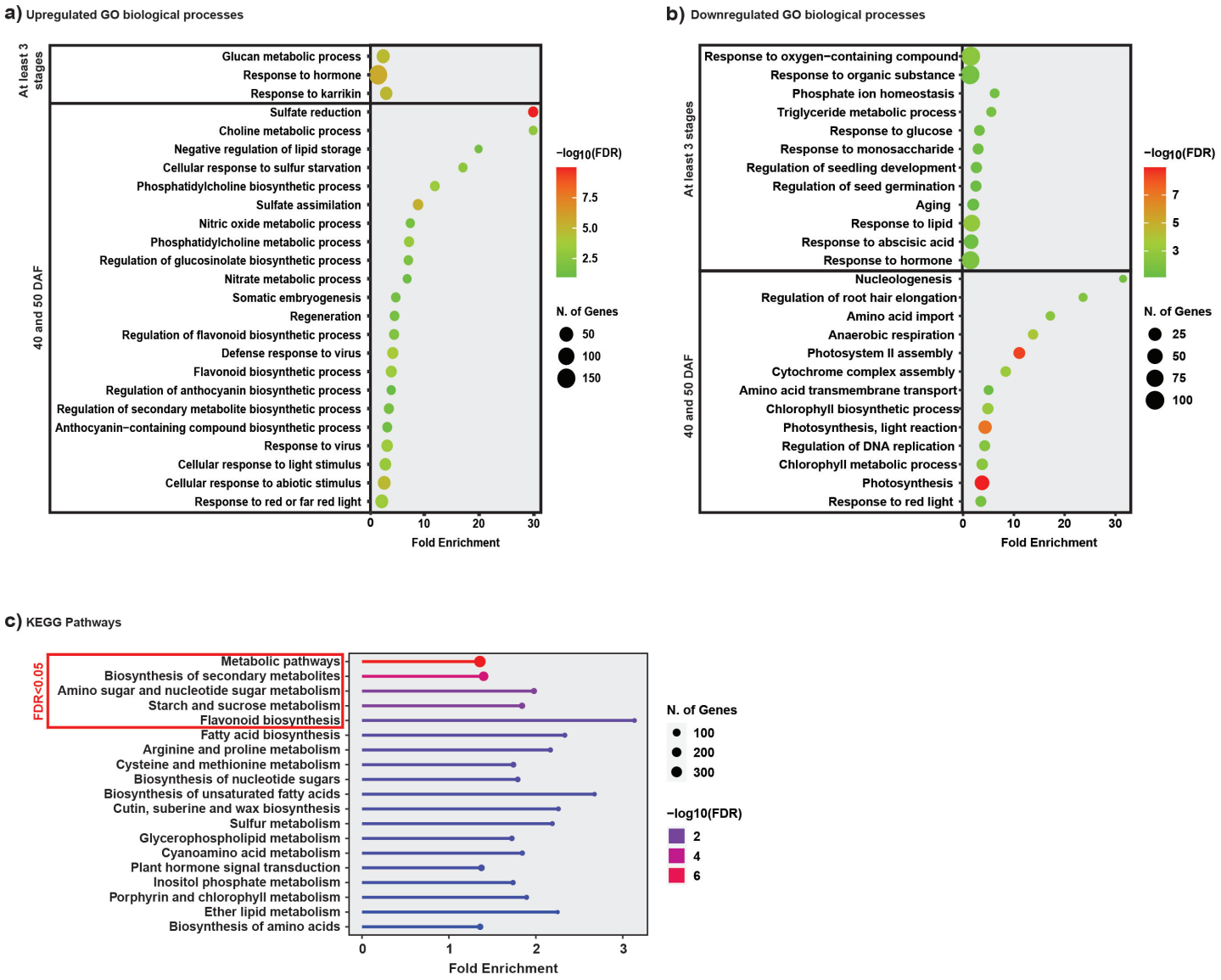


Figure 3.6. GO and KEGG pathway enrichment analysis of DEGs.

Gene Ontology (GO) enrichment analysis of differentially expressed genes (DEGs). Only shared significant biological processes **(a)** upregulated or **(b)** downregulated in transgenic seeds compared to the wild-type (WT) at least during three developmental stages or at 40 and 50 DAF are shown. **(c)** *Kyoto Encyclopedia of Genes and Genomes (*KEGG) pathway analysis of all DEGs. Circle size corresponds to the gene number, and different colors indicate the range of False Discovery Rate (FDR) values. DAF, Days after flowering.

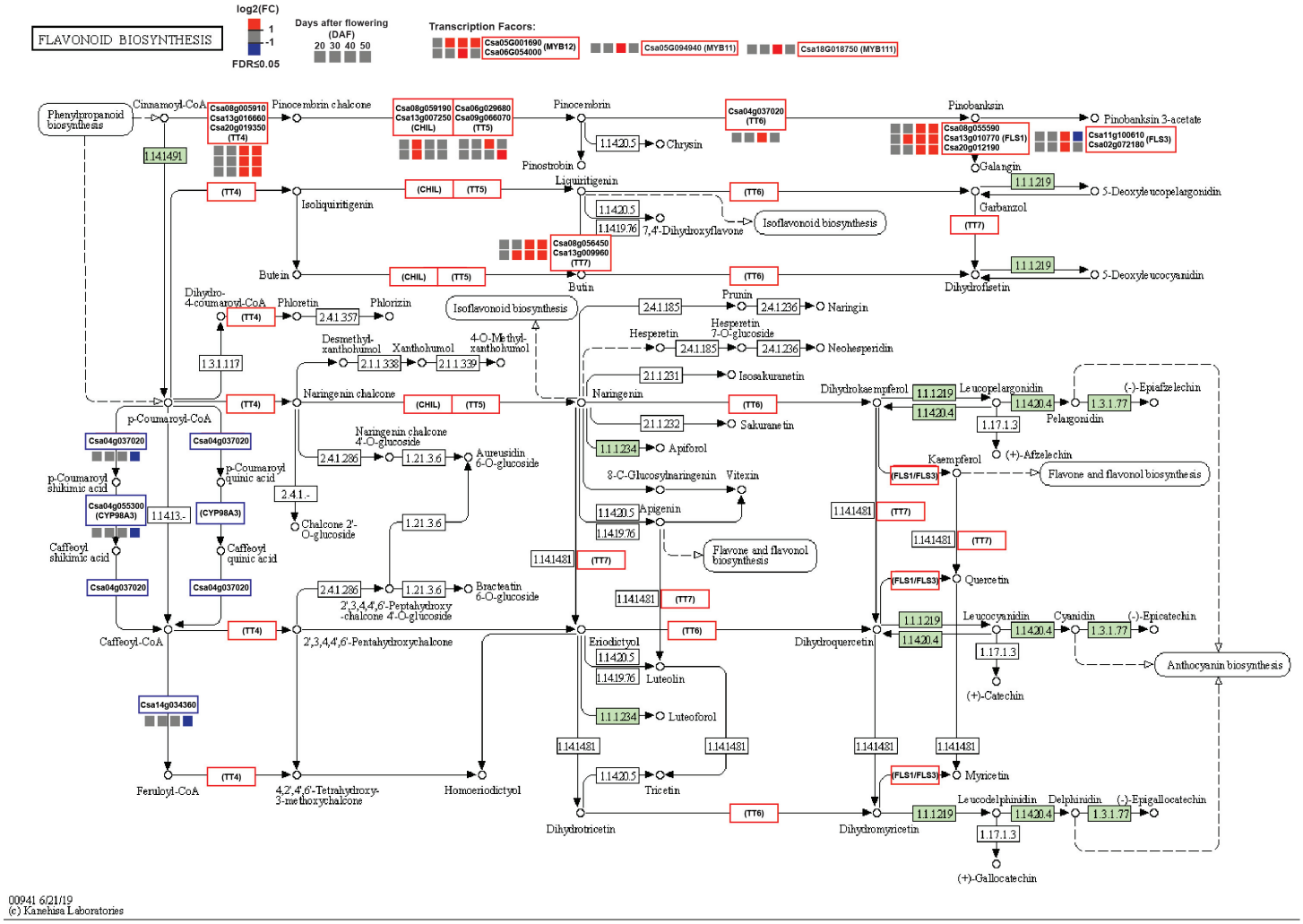


Figure 3.7. DEGs involved in flavonoid biosynthesis.

KEGG map of the flavonoid biosynthesis pathway. Differentially expressed genes (DEGs) upregulated in homozygous transgenic camelina seeds expressing *EfDAcT+DGAT1 RNAi* compared to the wild-type (WT), with an FDR ≤ 0.05 and fold change ≥ 2, are highlighted in red, while downregulated genes are highlighted in blue. Boxes represent developmental stages at 20, 30, 40, and 50 days after flowering (DAF) from left to right.

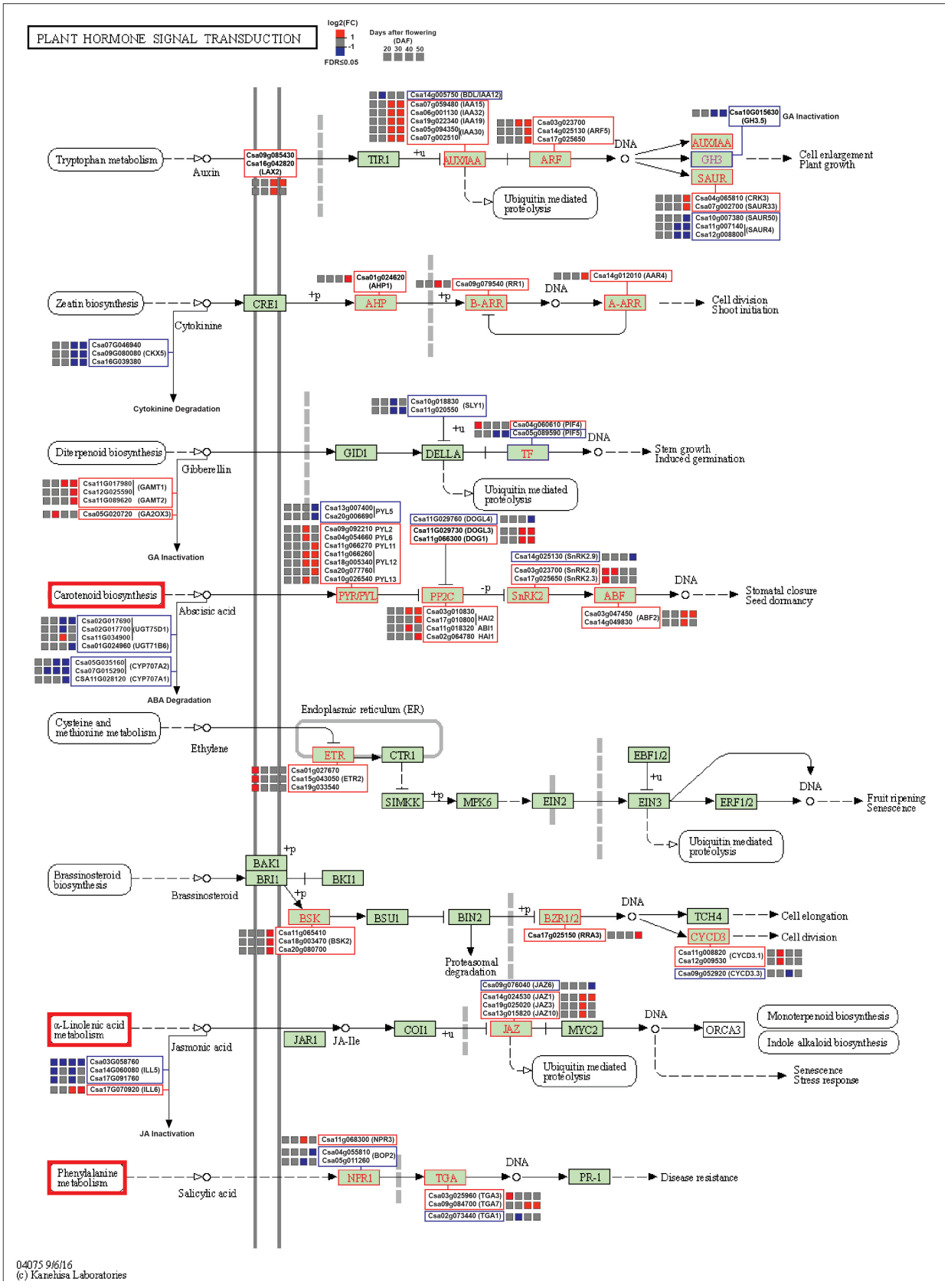

PLANT HORMONE SIGNAL TRANSDUCTION
log2(FC)
1
-1
FDR≤0.05
Days after flowering (DAF)
20 30 40 50
Tryptophan metabolism
Auxin
Csa09g085430
Csa16g042820 (LAX2)
TIR1
+u
AUX/IAA
ARF
DNA
AUXIAA
GH3
SAUR
Cell enlargement
Plant growth
Ubiquitin mediated proteolysis
Csa14g005750 (BDL/IAA12)
Csa07g059480 (IAA15)
Csa06g001130 (IAA32)
Csa19g022340 (IAA19)
Csa05g094350 (IAA30)
Csa07g002510
Csa03g023700
Csa14g025130 (ARF5)
Csa17g025650
Csa10G015630 (GH3.5)
GA Inactivation
Csa04g065810 (CRK3)
Csa07g002700 (SAUR33)
Csa10g007380 (SAUR50)
Csa11g007140
Csa12g008800 (SAUR4)
Zeatin biosynthesis
Cytokinine
CRE1
+p
AHP
B-ARR
A-ARR
Csa01g024620 (AHP1)
Csa09g079540 (RR1)
Csa14g012010 (AAR4)
Cell division
Shoot initiation
Csa07G046940
Csa09G080080 (CKX5)
Csa16G039380
Cytokinine Degradation
Diterpenoid biosynthesis
Gibberellin
GID1
DELLA
TF
Stem growth
Induced germination
Csa10g018830
Csa11g020550 (SLY1)
Csa04g060610 (PIF4)
Csa05g089590 (PIF5)
Csa11G017980 (GAMT1)
Csa12G025590
Csa11G089620 (GAMT2)
Csa05G020720 (GA2OX3)
GA Inactivation
Ubiquitin mediated proteolysis
Csa13g007400
Csa20g006690 PYL5
Csa09g092210 PYL2
Csa04g054660 PYL6
Csa11g066270 PYL11
Csa11g066260
Csa18g005340 PYL12
Csa20g077760
Csa10g026540 PYL13
Csa11G029760 (DOGL4)
Csa11G029730 (DOGL3)
Csa11g066300 (DOG1)
Carotenoid biosynthesis
Abscisic acid
PYR/PYL
PP2C
-p
SnRK2
ABF
Stomatal closure
Seed dormancy
Csa14g025130 (SnRK2.9)
Csa03g023700 (SnRK2.8)
Csa17g025650 (SnRK2.3)
Csa03g047450
Csa14g049830 (ABF2)
Csa02G017690
Csa02G017700 (UGT75D1)
Csa11G034900
Csa01G024960 (UGT71B6)
Csa05G035160 (CYP707A2)
Csa07G015290
CSA11G028120 (CYP707A1)
ABA Degradation
Csa03g010830
Csa17g010800 HAI2
Csa11g018320 ABI1
Csa02g064780 HAI1
Cysteine and methionine metabolism
Ethylene
Endoplasmic reticulum (ER)
ETR
CTR1
Csa01g027670
Csa15g043050 (ETR2)
Csa19g033540
SIMKK
+p
MPK6
EIN2
EBF1/2
+u
EIN3
ERF1/2
DNA
Fruit ripening
Senescence
Ubiquitin mediated proteolysis
Brassinosteroid biosynthesis
Brassinosteroid
BAK1
BRI1
BKI1
+p
BSK
BSU1
BIN2
BZR1/2
Csa17g025150 (RRA3)
TCH4
CYCD3
Cell elongation
Cell division
Csa11g065410
Csa18g003470 (BSK2)
Csa20g080700
Proteasomal degradation
Csa11g008820 (CYCD3.1)
Csa12g009530
Csa09g052920 (CYCD3.3)
α-Linolenic acid metabolism
Jasmonic acid
JAR1
JA-Ile
COI1
JAZ
MYC2
ORCA3
Monoterpenoid biosynthesis
Indole alkaloid biosynthesis
Senescence
Stress response
Csa09g076040 (JAZ6)
Csa14g024530 (JAZ1)
Csa19g025020 (JAZ3)
Csa13g015820 (JAZ10)
Csa03G058760
Csa14G060080 (ILL5)
Csa17G091760
Csa17G070920 (ILL6)
JA Inactivation
Phenylalanine metabolism
Salicylic acid
Csa11g068300 (NPR3)
Csa04g055810 (BOP2)
Csa05g011260
NPR1
TGA
PR-1
Disease resistance
Csa03g025960 (TGA3)
Csa09g084700 (TGA7)
Csa02g073440 (TGA1)
04075 9/6/16
(c) Kanehisa Laboratories

Figure 3.8. DEGs involved in plant hormone signal transduction.

KEGG pathway map of plant hormone signal transduction. Differentially expressed genes (DEGs) upregulated in homozygous transgenic camelina seeds expressing *EfDAcT+DGAT1 RNAi* compared to the wild-type (WT), with an FDR ≤ 0.05 and fold change ≥ 2, are highlighted in red, while downregulated genes are highlighted in blue. Boxes represent developmental stages at 20, 30, 40, and 50 days after flowering (DAF) from left to right.

TABLES

Table 3.1. List of measured and detected lipid classes in wild-type (WT) and transgenic seeds

Lipid class analyzed	Abbreviation	# of molecular species that exceeded the limit of detection	# of lipids that are DA in at least one stage	% of lipids that are DA in at least one stage
Triacylglycerol	TAG	210	195	92.9%
Acetyl-triacylglycerol	Acetyl-TAG	63	63	100%
Diacylglycerol	DAG	34	33	97.1%
Monogalactosyldiacylglycerol	MGDG	9	6	66.7%
Digalactosyldiacylglycerol	DGDG	11	5	45.5%
Phosphatidic acid	PA	10	2	20%
Phosphatidylcholine	PC	19	16	84.2%
Phosphatidylethanolamine	PE	22	9	40.9%
Phosphatidylglycerol	PG	12	11	91.7%
Phosphatidylinositol	PI	14	6	42.9%
Phosphatidylserine	PS	15	7	46.7%
Lyso-phosphatidylethanolamine	LPE	3	0	-
Lyso-phosphatidylcholine	LPC	4	3	75%
Lyso-Phosphatidylglycerol	LPG	0	-	-
Total	**-**	**426**	**356**	**83.6%**

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Chapter 4 - Remodeling of TAG composition to generate novel oil in transgenic oilseeds

In the process of submission

Linah Alkotami[1], Dexter J. White[1], Kathleen M. Schuler[2], Maliheh Esfahanian[3], Brice A. Jarvis[3], Andrew E. Paulson[4], Somnath Koley[5], Chaofu Lu[6], Doug K. Allen[5,7], Young-Jin Lee[4], John C. Sedbrook[3], Timothy P. Durrett[1]

[1] Department of Biochemistry and Molecular Biophysics, Kansas State University, Manhattan, KS
[2] Division of Biology, Kansas State University, Manhattan, KS
[3] School of Biological Sciences, Illinois State University, Normal, IL
[4] Department of Chemistry, Iowa State University, Ames, IA
[5] Donald Danforth Plant Science Center, St. Louis, MO
[6] Department of Plant Sciences and Plant Pathology, Montana State University, Bozeman, MT
[7] United States Department of Agriculture, Agricultural Research Service, St. Louis, MO

ABSTRACT

Acetyl-TAG are distinct triacylglycerols (TAG) in which an *sn*-3 acetate replaces the conventional long acyl group, yielding superior industrial oils with improved viscosity and freezing points. In *Euonymus* seeds, acetyl-TAG are synthesized by diacylglycerol acetyltransferases (DAcTs) utilizing diacylglycerol (DAG) and acetyl-CoA as substrates. Although heterologous DAcT expression has resulted in the production of acetyl-TAG in various transgenic oilseed crops, acetyl-TAG levels have remained below those naturally produced in *Euonymus* seed oil. Here, we report the generation of camelina and pennycress transgenic lines accumulating native-like levels at 93 mol% and 98 mol% of acetyl-TAG, respectively. These levels were achieved by expression of *EfDAcT*, a high-performance enzyme from *E. fortunei*, and suppression of the endogenous TAG-

synthesizing enzyme DGAT1 in lines with mutations in *FATTY ACID ELONGASE1* (*FAE1*). Characterization of the ultra-high-acetyl-TAG pennycress seeds revealed that residual TAG localized primarily to the embryonic axis. The accumulation of high acetyl-TAG levels displayed no major detrimental effect on seed properties or plant growth despite a lower germination rate. Our results demonstrate the feasibility of metabolically engineering designer oils by modifying the genetic background of oilseed crops through substrate manipulation and elimination of competing pathways.

INTRODUCTION

Euonymus species, belonging to the Celastraceae family, naturally accumulate more than 90% of 3-acetyl-1,2-diacylglycerol (acetyl-TAG) in their seeds as the major form of carbon storage (Kleiman *et al*., 1967; Sidorov *et al*., 2014). The *sn*-3 acetate group imparts altered physical and chemical properties to the resulting oil, such as reduced-calorific values, lower kinematic viscosity and freezing temperatures, expanding its applicability for various industries including food, chemical feedstock, and biofuels (Durrett *et al*., 2010; Liu, Rice, *et al*., 2015; Liu, Tjellström, *et al*., 2015). This intriguing composition has been attributed to the unique activity of *Euonymus* diacylglycerol acetyltransferases (DAcTs), that utilize diacylglycerol (DAG) and acetyl-CoA as substrates to synthesize acetyl-TAG (Durrett *et al*., 2010; Tran *et al*., 2017).

While native species like *Euonymus* often possess poor agronomic traits that prevent them from being cultivated as crops, their unique enzymatic machinery responsible for synthesizing these unusual TAGs can be harnessed to engineer oilseed crops with tailored oil profiles (Cai *et al*., 2022; Haslam *et al*., 2016). Camelina (*Camelina sativa*) and pennycress (*Thlapsi arvense* L.) have emerged as promising candidates for genetic engineering efforts due to their short life cycle,

adaptability to diverse environments, and well-established transformation protocols facilitating efficient genetic modifications (Lu & Kang, 2008; Malik *et al*., 2018; McGinn *et al*., 2019). Pennycress additionally hold an advantage due to its diploid genome, which simplifies genetic manipulation and minimizes gene redundancy that is common in polyploid species like camelina (Dorn *et al*., 2015; Kagale *et al*., 2014).

Previously, multiple DAcT enzymes with varying acetyltransferase activities have been isolated from different *Euonymus* species seeds (Durrett *et al*., 2010; Tran *et al*., 2017). Heterologous expression of the DAcT enzyme isolated from *Euonymus alatus* (EaDAcT), successfully produced acetyl-TAG in oilseeds including soybean, camelina, and pennycress, albeit achieving only 55-70 mol% in the best transgenic seeds (Liu, Rice, *et al*., 2015; Liu, Tjellström, *et al*., 2015; McGinn *et al*., 2019). Suppression of *DIACYLGLYCEROL ACYLTRANSFERASE1* (*DGAT1*) by RNA interference (RNAi), which catalyzes the final step of endogenous TAG synthesis, further enhanced acetyl-TAG production (Alkotami *et al*., 2021; Liu, Rice, *et al*., 2015; Liu, Tjellström, *et al*., 2015). *DGAT1* suppression coupled with the expression of *Euonymus fortunei* DAcT (EfDAcT), possessing higher *in vitro* activity relative to EaDAcT (Tran *et al*., 2017), resulted in 80 mol% acetyl-TAG, with some camelina lines exceeding 87 mol% (Alkotami *et al*., 2021). Despite being the highest level ever achieved in transgenic oilseed crops, acetyl-TAG levels remained below those naturally produced in many *Euonymus* seeds, reaching up to 98%. Our previous successes suggest that it may be possible to further optimize the metabolic pathways and boost the accumulation of acetyl-TAG to their natural levels.

In oilseeds, particularly those of the Brassicaceae, acetyl-CoA is utilized by the fatty acid elongase complex (FAE) to elongate oleic acid (18:1) to very-long-chain fatty acids (VLCFAs), such as eicosenoic acid (20:1) and erucic acid (James *et al*., 1995; Kunst *et al*., 1992). This process,

involving acetyl-CoA, implies potential competition in transgenic seeds producing acetyl-TAG between the *FATTY ACID ELONGASE 1* (*FAE1*) gene—encoding the key enzyme in the FAE complex—and DAcT for acetyl-CoA. CRISPR-Cas9 mutagenesis of *FAE1*, was shown to deplete VLCFAs levels in camelina and pennycress seeds (McGinn *et al*., 2019; Ozseyhan *et al*., 2018).

We therefore aimed to characterize existing camelina and pennycress *fae1* mutant lines and leverage their potential use to improve acetyl-TAG production. By fine-tuning the fatty acid composition and availability of DAG substrate in the plant genetic background used to produce acetyl-TAG, we document near quantitative remodeling of the entire endogenous TAG profile.

RESULTS

Pennycress *fae1* seeds possess elevated levels of acetyl-CoA

We hypothesized that seeds with *fae1* mutations would contain higher levels of acetyl-CoA accessible to EfDAcT, and ultimately lead to a greater accumulation of acetyl-TAG. To assess our hypothesis, we characterized *fae1* mutant lines of camelina *(Ozseyhan et al., 2018)* and pennycress (McGinn *et al*., 2019), two emerging oilseed crops that produce varying amount of VLCFA. Fatty acid composition, seed weight and fatty acid content were analyzed in wild type and *fae1* mutants. Camelina wild-type seeds accumulated over 20 mol% of VLCFA, with 14 mol% of the VLCFA content attributed to 20:1 (Figure 4.1a). In contrast, pennycress wild-type seeds accumulated over 55 mol% of VLCFA, with 22:1 representing approximately 43 mol% of the total VLCFA content (Figure 4.1a). Mutant *fae1* seeds of both camelina and pennycress contained small amount of VLCFA (<2%) and increased levels of 18:1, 18:2 and 18:3 (Figure 4.1a). Interestingly, the proportions of these fatty acids differed between the two species. In camelina, the major fatty acid is 18:3, mirroring the wild-type background, whereas pennycress *fae1* mutants primarily

accumulated 18:1 as their major fatty acid (Figure 4.1a). The average weight and fatty acid content of camelina *fae1* seed was slightly decreased relative to wild-type in camelina but not pennycress seeds (Figures 4.1b and 1c). Acetyl-CoA levels in developing camelina *fae1* seeds were not significantly different from that of the wild-type seeds (Figure 4.1c). However, an increase of 57% of acetyl-CoA levels was observed in developing pennycress *fae1* seeds (Figure 4.1d) compared to the wild-type control. These findings may reflect the differences in the contribution of FAE1 in these two species, and relatively lower amounts of VLCFA in wild-type seeds of camelina compared to pennycress.

Camelina *fae1* mutation slightly increases acetyl-TAG accumulation

To test the impact of the *fae1* mutation on acetyl-TAG accumulation in camelina, *EfDAcT* was expressed in *fae1* mutant seeds, either alone or in combination with *DGAT1 RNAi*. On average, acetyl-TAG content in *fae1* T2 seeds segregating for *EfDAcT* was 75 mol%, higher than the 71 mol% previously observed in transgenic seeds with a wild-type background. Likewise, expression of *EfDAcT+DGAT1 RNAi* resulted in 87 mol% acetyl-TAG in *fae1* seeds, compared to 80 mol% in wild-type seeds (Figure 4.2a) (Alkotami *et al*., 2021).

The two highest acetyl-TAG producing lines from each genotype were then grown together to directly compare acetyl-TAG accumulation. *EfDAcT* homozygous lines accumulated 88 mol% and 81 mol% of acetyl-TAG in *fae1* and wild-type seeds, respectively (Figure 4.2b). *EfDAcT+DGAT1 RNAi* homozygous *fae1* lines accumulated on average 90 mol% of acetyl-TAG, with 93 mol% in the best line. These levels were comparable to the 89 mol% of acetyl-TAG and a high of 92 mol% observed in the homozygous *EfDAcT+DGAT1 RNAi* lines in wild-type background (Figure 4.2b). TAG fatty acid composition of *EfDAcT* and *EfDAcT+DGAT1 RNAi*

transgenic lines had higher levels of 18:2 and lower levels of 18:3 and 20:1 compared to wild-type seeds (Supplementary Figure D.1a). Similar to previous results (Alkotami *et al*., 2021), VLCFAs 20:0 and 22:1 were mainly present in the endogenous TAG fraction of the transgenic lines (Supplementary Figure D.1a). Transgenic seeds in the *fae1* background contained higher levels of 18:2 and lower levels of 18:3 (Supplementary Figure D.1b).

To better compare the impact of mutating *fae1* on acetyl-TAG production without the confounding effect of different transgenic loci possessing varying levels of expression stemming from random insertions during plant transformation, we crossed stable high acetyl-TAG producing lines expressing either *EfDAcT* or *EfDAcT+DGAT1 RNAi* in a wild-type background with the *fae1* mutant line. As camelina possesses three *FAE1* homoeologs that contribute to VLCFA production (Hutcheon *et al*., 2010; Ozseyhan *et al*., 2018), identification of transgenic lines in a segregating backcross population where all three *FAE1* homoeologs are homozygous mutant would be particularly onerous. Instead, we measured acetyl-TAG and 20:1 levels in individual transgenic seeds derived from segregating F2 seed populations. Transgenic *EfDAcT* F2 seed possessed a low negative correlation between acetyl-TAG accumulation and 20:1 levels (Figure 4.2c) with the lowest 20:1 level (4.4 mol%) corresponding to the highest level of acetyl-TAG (84 mol%). In contrast, transgenic seeds from the *fae1* x *EfDAcT+DGAT1 RNAi* F2 cross, showed no correlation between acetyl-TAG and 20:1 levels (Figure 4.2d).

Transgenic *fae1* pennycress seeds accumulate up to 98 mol% of acetyl-TAG

Given the observed elevation of acetyl-CoA levels upon near elimination of VLCFA in *fae1* pennycress seeds (Figure 4.1e), we explored whether this modification would result in higher accumulation of acetyl-TAG. Stable transgenic pennycress lines in both wild-type and *fae1*

backgrounds expressing *EfDAcT* with and without *DGAT1 RNAi*, were generated (Supplementary Figure D.2). For each genotype generated, the three independent lines exhibiting the highest acetyl-TAG levels were selected for further propagation (Supplementary Figure D.2b). In accordance with our hypothesis, *fae1* mutation significantly increased acetyl-TAG level accumulating up to 98 mol% in the best *EfDAcT+DGAT1 RNAi* transgenic line (Figure 4.3a), mirroring or exceeding the naturally occurring levels in many *Euonymus* seeds (Durrett *et al*., 2010; Kleiman *et al*., 1967). On an average basis, expression of *EfDAcT* in *fae1* seeds resulted in 95 mol% of acetyl-TAG while *EfDAcT+DGAT1 RNAi* expression accumulated 96 mol% (Figure 4.3a). In contrast, wild-type lines expressing either *EfDAcT* alone or *EfDAcT+DGAT1 RNAi* resulted in only 48 mol% of acetyl-TAG and 79 mol% of acetyl-TAG, respectively (Figure 4.3a).

The ultra-high levels of acetyl-TAG in the *fae1* background were further confirmed by electrospray ionization mass spectrometry (ESI-MS) analysis (Figure 4.3b, Supplementary Figure D.3). Trace amounts of TAG in the *fae1* background, less than 4% and 1% of total signal intensity, were observed in *EfDAcT* and *EfDAcT+DGAT1 RNAi* seeds, respectively. The majority of the residual TAG molecular species in *EfDAcT+DGAT1 RNAi* seeds possessed 54 carbons (Figure 4.3b), while in *EfDAcT*, seed TAG molecular species were similar to those found in non-transgenic *fae1* seed, being dominated by molecular species with 54 carbons, but also possessing molecular species containing VLCFA (Supplementary Figure D.3). Wild-type seeds and transgenic seeds expressing *EfDAcT* and *EfDAcT+DGAT1 RNAi* in the wild-type background possessed a more complicated molecular species profile for both acetyl-TAG and TAG, reflective of the ability to synthesize high levels of VLCFA (Supplementary Figure D.3). ESI-MS results were confirmed by fatty acid analysis of total TAG that indicated significantly lower accumulation of 22:1 and higher accumulation of 18:1 in transgenic seeds in the wild-type background (Supplementary Figure

D.4a). This alteration in the fatty acid composition of the transgenic lines was mainly associated with changes observed in the acetyl-TAG fraction, consistent with the camelina results shown here and previous work in pennycress (Supplementary Figure D.1a) (McGinn *et al*., 2019). In *fae1* seeds, expression of *EfDAcT* or *EfDAcT+DGAT1 RNAi* resulted in a reduction of 18:3 and an increase in 18:1 compared to the *fae1* control (Supplementary Figure D.4b).

To further confirm the effect of the *fae1* mutation on acetyl-TAG synthesis, we crossed high acetyl-TAG producing lines expressing *EfDAcT+DGAT1 RNAi* in both *fae1* mutant or wild-type backgrounds with non-transgenic plants of the other background. For each cross, the segregating F2 population was genotyped to identify transgenic plants either in a wild-type background or those homozygous for *fae1*. F3 seeds harvested from these plants were screened for DsRed fluorescence to identify lines homozygous for the transgene. The cross between *fae1* and *EfDAcT+DGAT1 RNAi* (wild-type) resulted in 82 mol% acetyl-TAG in seeds homozygous for *fae1* (Figure 4.3c), elevated relative to the 71 mol% observed in wild-type siblings (Figure 4.3c) and lower than the 96 mol% obtained by stable transformation (Figure 4.3a). Likewise, the cross between wild-type and *EfDAcT+DGAT1 RNAi* (*fae1)* yielded acetyl-TAG levels of 74 mol% in wild-type segregants, lower than the 96 mol% of acetyl-TAG in siblings homozygous for *fae1* (Figure 4.3c), and slightly lower than the 79 mol% of acetyl-TAG obtained by stable transformation (Figure 4.3a).

DGAT1 expression decreases in transgenic pennycress lines expressing EfDAcT only

To investigate whether the expression of *EfDAcT* in the wild-type or *fae1* mutant background influences genes encoding acyltransferases involved in seed TAG synthesis, we quantified the relative expression of *DGAT1* and *phospholipid diacylglycerol acyltransferase 1* (*PDAT1*) in the

highest acetyl-TAG producing pennycress lines. We also measured the levels of *EfDAcT* and *FAE1* to confirm their presence or depletion. The relative expression of *DGAT1* was reduced in the *DGAT1-RNAi* lines as anticipated. Interestingly, in lines expressing only *EfDAcT*, *DGAT1* levels were slightly decreased in the wild-type background and significantly reduced in the *fae1* background (Figure 4.4a). The *fae1* mutation alone in the non-transgenic control did not impact *DGAT1* expression, suggesting that only the combination of *fae1* mutation and *EfDAcT* expression had a measurable effect on *DGAT1* expression. In contrast to *DGAT1*, the relative expression of *PDAT1* was not affected by *EfDAcT* expression or *fae1* mutation (Figure 4.4b). Analysis of *fae1* mutants showed greatly reduced *FAE1* expression but low levels of transcript were still detected (Figure 4.4c). As expected, *EfDAcT* was only expressed in the transgenic lines (Figure 4.4d). However, the levels of *EfDAcT* expression varied among the transgenic lines, with no apparent correlation between *EfDAcT* expression and acetyl-TAG levels, similar to prior results (Alkotami *et al*., 2021).

Residual TAG in pennycress transgenic seeds localizes to the embryonic axis

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) imaging was performed on mature pennycress seeds to determine the localization of the acetyl-TAG and residual TAG in the high acetyl-TAG producing lines (Figure 4.5). Consistent with the ESI-MS results (Figure 4.3b, Supplementary Figure D.3), low levels of TAG were detected in transgenic *fae1* seeds. With *EfDAcT* expression, 54:X TAG was present in both the cotyledons and the embryonic axis, whereas the less abundant 52:X TAG was only present in the embryonic axis. In *fae1* seeds expressing *EfDAcT+DGAT1 RNAi*, where endogenous TAG levels were lower (Figure 4.3), both 54:X and 52:X TAG were mostly localized to the embryonic axis.

Acetyl-TAG were present in both the embryonic axis and the cotyledons, though specific molecular species were more abundant in one tissue relative to the other. For example, in the *fae1* background, 38:X acetyl-TAG was lower in the embryonic axis compared to the cotyledons; in contrast, 36:X acetyl-TAG was slightly more abundant in the embryonic axis (Figure 4.5). The relative accumulation of these acetyl-TAG molecular species directly corresponds to the levels of their DAG precursor molecules, with 36:X DAG being lower in the embryonic axis compared to the cotyledons, whereas 34:X DAG is more abundant in the embryonic axis (Supplementary Figure D.5). Interestingly, TAGs in non-transgenic *fae1* plants showed a similar relation to DAG. 54:X TAG was higher in the cotyledons relative to the embryonic axis, and 52:X TAG was more abundant in the embryonic axis. Thus, results imply that acetyl-TAG and TAG are made from the same common DAG pool.

Acetyl-TAG production in transgenic *fae1* lines has minimal impact on seed properties and plant growth

We quantified seed TAG levels, seed fatty acid content, seed weight, and seed size to examine the effect of high acetyl-TAG production on transgenic camelina and pennycress seed properties. In general, all transgenic lines in camelina and pennycress exhibited slight increases in TAG content, seed weight, and seed size compared to the wild-type and *fae1* control lines, while the fatty acid content was slightly reduced (Supplementary Figure D.6 and D.7). For instance, pennycress lines expressing *EfDAcT+DGAT1 RNAi*, which were the most productive in terms of acetyl-TAG synthesis in wild-type and *fae1* background, demonstrated increases up to 14.7% and 52% in total TAG accumulation, respectively (Supplementary Figure D.7a). However, the fatty acid content of

these lines exhibited up to 16% decrease in wild-type seeds and up to 4.6% decrease in *fae1* seeds (Supplementary Figure D.7b).

Seeds from pennycress transgenic lines along with wild-type and *fae1* control lines were further plated on 1% agar plates to monitor germination rates and subsequently transferred to soil to record their growth phenotype. Germination started on the second day after plating for all lines. All germinated seeds advanced to the cotyledon stage (Supplementary Figure D.8); however, the transgenic lines, particularly those expressing *EfDAcT+DGAT1 RNAi*, exhibited slower germination rates compared to the wild-type and *fae1* control lines (Figure 4.6a; Supplementary Figure D.8). By the ninth day, *EfDAcT+DGAT1 RNAi* transgenic lines had an average germination rate of 86%-92% in the wild-type background compared to 98%-100% in the wild-type control, and 74%-88% in *fae1* background compared to 95%-98% in the *fae1* control (Figure 4.6a). The time to flowering and plant height were measured for all genotypes (Figure 4.6b; Supplementary Figure D.9). Similar to the wild-type, flowering time for the *EfDAcT* and *EfDAcT+DGAT1 RNAi* plants in the wild-type background was between 30-37 days after planting (Figure 4.6b). On average, *fae1* plants took two days longer to flower compared to the wild type plants. In terms of plant height at various growth stages, the transgenic lines generally exhibited heights similar to the wild-type or *fae1* control plants, with the exception of *EfDAcT fae1* plants, which were shorter (Figure 4.6c). For example, at 51 days after planting, the *fae1* control plants and *EfDAcT+DGAT1 RNAi* plants had an average height of 62-64 cm, while *EfDAcT* plants had an average height of 56 cm (Figure 4.6c). Although *EfDAcT fae1* plants showed slightly slower growth, this variation in the average flowering time and height was not consistent in other independent lines grown at different times.

DISCUSSION

To enhance acetyl-TAG production in transgenic oilseed crops, we optimized endogenous metabolic pathways by eliminating the synthesis of VLCFAs and simultaneously suppressing the TAG-synthesizing enzyme DGAT1. The most successful transgenic lines accumulated 93 mol% acetyl-TAG in camelina seeds and 98 mol% acetyl-TAG in pennycress seeds (Figure 4.2b, Figure 4.3a), closely resembling levels present in *Euonymus* seeds (Bagby & Smith, 1967; Kleiman *et al*., 1967) and exceeding our previous reports in transgenic oilseed crops (Alkotami *et al*., 2021; Liu, Rice, *et al*., 2015; Liu, Tjellström, *et al*., 2015).

FAE1, a key component of the fatty acid elongase complex, plays an essential role in elongating 18:1 fatty acid using acetyl-CoA as a substrate, leading to the production of VLCFA (Millar & Kunst, 1997). Our rationale for blocking VLCFA synthesis through fae*1* mutation was to elevate acetyl-CoA levels – a key substrate for EfDAcT-mediated acetyl-TAG synthesis (Bansal & Durrett, 2016). Consistent with previous studies (McGinn *et al*., 2019; Ozseyhan *et al*., 2018), CRISPR-induced *fae1* mutants of camelina and pennycress showed negligible amounts of VLCFA in seeds. Examination of acetyl-CoA levels revealed that pennycress *fae1* seeds accumulated 57% more acetyl-CoA compared to wild-type seeds (Figure 4.1e). Wild-type pennycress inherently contains more than 40 mol% of VLCFAs (McGinn *et al*., 2019) (Figure 4.1a), which is notably higher than the approximately 20 mol% found in camelina (Figure 4.1a). The much greater reliance on fatty acid elongation in pennycress, compared to camelina, in development of wild-type fatty acid composition is a potential explanation for the observed elevated acetyl-CoA levels in pennycress *fae1* mutants but not in camelina in the absence of elongation (Figure 4.1c).

The higher levels of acetyl-CoA in the *fae1* background in pennycress versus camelina is one likely explanation for why larger increases in acetyl-TAG accumulation were observed in pennycress. However, other non-exclusive mechanisms could also explain the particularly large increases in acetyl-TAG accumulation in the *fae1* pennycress mutant. For example, the removal of VLCFAs might prevent the efficient functioning of pennycress DGAT1 to synthesize TAG. In Brassicaceae species, VLCFA are located at the *sn*-1 and *sn*-3 positions of TAG molecules (Takagi & Ando, 1991; Taylor *et al*., 1995), with the majority of VLCFA at the *sn*-3 being products of DGAT1 activity (Katavic *et al*., 1995; Woodfield *et al*., 2018). This suggests that the increase of acetyl-TAG in pennycress *fae1* mutants might be caused by EfDAcT outcompeting DGAT1 due to unavailability of the VLCFA substrates utilized by DGAT1.

Alternatively, we observed that *DGAT1* expression was reduced when *EfDAcT* was expressed (Figure 4.4a), with much greater suppression in the *fae1* background. This reduced expression of *DGAT1* presumably also enables synthesis of acetyl-TAG via EfDAcT to outcompete that of endogenous TAG via DGAT1. While the mechanism mediating this decrease in *DGAT1* expression is unclear, it is tempting to speculate that *DGAT1* gene expression is somehow regulated by DGAT1 activity itself, possibly because of reduced activity due to lack of VLCFA substrate.

When DGAT1 is not present, the primary pathway for TAG production involves PDAT1 transferring polyunsaturated fatty acids (PUFAs) from the *sn*-2 position of phosphatidylcholine (PC) to the *sn*-3 position of DAG (Stahl *et al*., 2004; Zhang *et al*., 2009). In the suppressed *DGAT1* transgenic lines expressing *EfDAcT*, acetyl-TAG levels in camelina wild-type and *fae1* mutant background were not significantly different (Figure 4.2b). This suggests that camelina PDAT1

does not preferably utilize DAG containing VLCFA, as elimination of VLCFA does not seem to influence PDAT1 ability to produce TAG in the *DGAT1-RNAi* transgenic lines (Figures 4.2b and 2d). This is further supported by the reduced levels of VLCFA in *DGAT1-RNAi* lines (Supplementary Figure D.1a) (Alkotami *et al*., 2021; Marmon *et al*., 2017) and the fact that *fae1* mutant lines expressing *EfDAcT* alone possess similar levels of acetyl-TAG compared to wild-type lines expressing *EfDAcT+DGAT1 RNAi* (Figure 4.2b). In pennycress, however, PDAT1 might preferably utilize DAG containing VLCFA as observed by the lower levels of acetyl-TAG in transgenic wild-type background compared to *fae1* mutant background in *DGAT1-RNAi* lines (Figure 4.3a and 3b). With near elimination of VLCFAs in *DGAT1-RNAi* lines, pennycress PDAT1 activity is limited due to reduced substrate availability, allowing EfDAcT to produce very high levels of acetyl-TAG. This can explain the similar acetyl-TAG levels achieved in *fae1* mutant background expressing *EfDAcT* with or without *DGAT1-RNAi* (Figure 4.3a), as well as the lower accumulation of 18:2 and 18:3 in the *fae1* mutant transgenic lines (Supplementary Figure D.4b). Future research can focus on lipid flux analysis in high acetyl-TAG-producing lines, as well as conducting enzyme activity and substrate selectivity tests on pennycress DGAT1 and PDAT1. By employing flux analysis, we can gain insights into substrate allocation and rate of substrate channeling towards EfDAcT or pennycress DGAT1 and PDAT1. Furthermore, enzyme activity and substrate selectivity assays for pennycress DGAT1 and PDAT1 can help determine their preference for VLCFA and whether their activity decreases with depletion of their preferred substrates.

Given the substantial acetyl-TAG accumulation and variations in fatty acid composition, we explored whether acetyl-TAG and residual TAG produced in the transgenic lines show

differential seed tissue localization compared to TAG of the control backgrounds. All transgenic lines accumulated acetyl-TAG in the cotyledons and the embryonic axis (Figure 4.5), indicating that EfDAcT is able to acetylate DAG molecular species in both tissues. The spatial distribution pattern of PC, DAG, acetyl-TAG, and TAG containing VLCFAs in the cotyledons relative to the embryonic axis observed in our pennycress wild-type control and transgenic seeds (Figure 4.5; Supplementary Figure D.5) resembled the results observed in earlier studies in camelina, rapeseed, and pennycress seeds (Horn *et al*., 2013; Jarvis *et al*., 2021; Lu *et al*., 2020). FAE1 expression has been demonstrated to be predominantly concentrated in the cotyledons as opposed to the embryonic axis (Lu *et al*., 2020; Rossak *et al*., 2001), which may underlie the preferential distribution of PC, DAG, TAGs containing VLCFAs to the cotyledonary tissues. In the *fae1* mutant background, residual TAG (~2-4 mol%) in the transgenic lines were almost exclusively enriched in the embryonic axis (Figure 4.5). Similar results were observed in high and low erucic acid rapeseed lines where significantly less abundant TAG molecular species were localized to the embryonic axis (Lu *et al*., 2020). In our transgenic lines, this observation suggests that DGAT1-RNAi and EfDAcT might more effectively target or compete with endogenous TAG-synthesizing enzymes in the cotyledonary tissues compared to the embryonic axis, which raises the possibility that employing tissue-specific promoters to express *EfDAcT* and to suppress endogenous TAG-acyltransferases within the embryonic axis could offer another strategy to eliminate the remaining residual TAG.

The accumulation of ~98 mol% of acetyl-TAG in transgenic seeds has no major impact on plant growth as no significant morphological differences were observed between pennycress transgenic plants and the wild-type or *fae1* control plants (Supplementary Figure D.9; Figures 4.6b

and 6c). Engineering unusual lipids in oilseeds has typically affected seed characteristics and decreased oil levels (Esfahanian *et al*., 2021; Lunn *et al*., 2018; Snapp *et al*., 2014). Previously we have reported that in the wild-type background, accumulation of acetyl-TAG in transgenic camelina seeds resulted in a slight increase in seed TAG, seed weight and seed size and a decrease in total fatty acid content (Alkotami *et al*., 2021; Liu, Rice, *et al*., 2015; Liu, Tjellström, *et al*., 2015). In this study, similar results were observed in transgenic camelina and pennycress *fae1* lines (Supplementary Figure D.6; Supplementary Figure D.7). The higher levels of overall seed TAG are probably caused by the absence of an acyl chain at the *sn*-3 position of acetyl-TAG. This absence might promote an increased availability of free fatty acids for further integration into additional TAG glycerol backbones (Alkotami *et al*., 2021; Liu, Rice, *et al*., 2015; Liu, Tjellström, *et al*., 2015). Though, an increased availability of fatty acids, if not rapidly incorporated into TAG molecules, can triggers feedback inhibition of de novo fatty acid synthesis (Andre *et al*., 2012; Bates, 2016; Bates *et al*., 2014). This phenomenon could possibly account for the slight reduction in the overall fatty acid content observed in the transgenic lines (Supplementary Figure D.6b; Supplementary Figure D.7b). Pennycress transgenic seeds exhibited reduced germination rates when compared to both the wild-type and *fae1* control seeds with *EfDAcT+DGAT1 RNAi fae1* lines displaying a reduction ranging from 7% to 24% compared to the *fae1* control seeds (Figure 4.6a). In our previous study, we demonstrated that transgenic camelina seeds accumulating acetyl-TAG in a wild-type background also exhibited delayed germination, though this effect was not present in freshly harvested seeds (Alkotami *et al*., 2021), indicating that the germination delay associated with acetyl-TAG accumulation varies with seed age. The cause of reduced germination in the transgenic seeds accumulating acetyl-TAG remains unclear as germination is a highly complex trait regulated by numerous processes (Koornneef *et al*., 2002). Germination initiation

relies on the imbibition phase involving water uptake and influenced by hormone balance, seed coat permeability and seed dormancy (Koornneef *et al*., 2002). Future experiments are needed to examine whether acetyl-TAG accumulation impact any of these factors altering germination rates.

In summary, we have successfully generated transgenic lines that mimic the natural production levels of acetyl-TAG found in native species, providing economically viable sources of low-viscosity oil for potential applications across various industries. These lines can serve as invaluable tools for lipid labeling and multi-omics studies, offering insights into the impact of unusual TAG accumulation on the reorganization of lipid metabolism and other biological processes. Our results underscore the ability of replicating native-like levels of unusual TAG in transgenic oilseed crops through the coordinated expression of the most efficient biosynthetic genes, ensuring the presence of suitable substrates by manipulating the fatty acid composition of the host plant, and eliminating competitive metabolic pathways.

MATERIALS AND METHODS

Plant material and growth conditions

The following ecotypes were used in this study: Camelina 'Suneson' and pennycress "Spring-32" (McGinn *et al*., 2019). CRISPR-Cas9-induced *fae1* mutant camelina and pennycress seeds were previously generated (McGinn *et al*., 2019; Ozseyhan *et al*., 2018). Plants were grown on soil (4:2:1 mixture of Berger BM6 All-Purpose soil mix:vermiculite:perlite) in growth chambers under a 16 h light/8 h dark cycle at a light intensity of 250 μmol m^{-2} s^{-1}, and a temperature of 21°C. To obtain developing seeds for metabolite and RT-qPCR analysis, emerging flowers were tagged

using colored threads. Tagged pods were harvested at 20 DAF (days after flowering), immediately frozen in liquid nitrogen and stored at −80°C until further analysis.

Generation of transgenic plants

Previously generated pBinGlyRed3 plasmids containing *EfDAcT*-HA and *EfDAcT*-HA + *DGAT1-RNAi* cassette (Alkotami *et al*., 2021; Tran *et al*., 2017) were introduced into *Agrobacterium tumefaciens* strain GV3101 and transformed into camelina and pennycress wild-type and *fae1* mutants plants via a floral dip vacuum infiltration method (Lu & Kang, 2008; McGinn *et al*., 2019). Transgenic seeds were identified by visual screening for DsRed in mature seeds for camelina and in seedlings for pennycress due to pennycress dark seed coat as described previously (Alkotami *et al*., 2021; McGinn *et al*., 2019).

Generation of crosses and genotyping of transgenic *fae1* mutant lines

Crosses were generated by transferring pollen from a mature flower of the male donor (transgenic plants expressing *EfDAcT*-HA or *EfDAcT*-HA + *DGAT1-RNAi*) onto the stigma of the emasculated (via removal of siliques and all immature anthers from unopened flower buds) maternal plants (wild-type or *fae1* mutant plants). Successful F1 crosses were identified by visual screening for DsRed and propagated to the F2 generation. DsRed positive segregating F2 camelina seeds were directly used for lipid analysis. DsRed positive F2 pennycress seeds were propagated to the F3 generation to identify plants homozygous for *EfDAcT* (DsRed screening) and *fae1* mutation (genotyping). Genotyping for *fae1* was performed using derived cleaved amplified polymorphic sequence analysis (dCAPS) (Neff *et al*., 1998) as illustrated in Supplementary Figure D.10.

Genomic DNA was extracted from leaves as previously described (Edwards *et al*., 1991). Genotyping primers are listed in Supplementary Table C.1.

Lipid analysis

Total lipids were extracted from mature seeds using a hexane–isopropanol based method (Li *et al*., 2006). 100 μg of tri15:0-TAG (NuChek Prep, New Elysian, MN) was added as an internal standard during lipid extraction. For quantification of acetyl-TAG/TAG content and FA composition, total lipid extracts were loaded onto thin layer chromatography silica gel 60 plates for separation with a 70:30:1 hexane:diethyl ether:acetic acid (v/v/v) solvent system. TAG and acetyl-TAG bands were scraped and subjected to acid-catalyzed transesterification following the Li *et al*. (2006) method. Fatty acid methyl esters (FAMEs) extracts were analyzed on a DB23 column, using a Shimadzu GC-2010 plus gas chromatograph with a flame ionization detector (FID) (Aznar Moreno & Durrett, 2017). Acetyl-TAG and regular TAG levels were calculated as described previously (Liu, Rice, *et al*., 2015). For ESI-MS analysis, neutral lipids were collected by passage of total lipid extracts over a small silica column with 99:1 (v/v) chloroform: methanol. Neutral lipids samples were prepared for MS following Bansal and Durrett (2016) and analyzed on an electrospray ionization triple quadrupole mass spectrometer (Sciex QTRAP 6500+, Sciex, Framingham, MA, USA). Total fatty acids content of mature seeds was determined by quickly homogenizing 20 seeds in 1 ml toluene with a PT2500E polytron (Kinematica AGd) followed by transesterification and gas chromatography as stated above.

Analysis of seed properties and plant growth

Seed weight was determined by weighing 100 dry seeds on a Sartorius CP225D analytical balance. Seed size of ~ 200 randomly chosen seeds was calculated utilizing ImageJ software, using a well-established method (Herridge *et al*., 2011). Germination assays were performed as previously described (Alkotami *et al*., 2021). Mature seeds were surface-sterilized and planted on 1% agar plates. Plates were placed in a growth chamber and germination were scored daily for 9 days. Plant growth phenotypes were analyzed in transgenic plants grown alongside wild-type and *fae1* mutants. Flowering time, measured in days after planting, was determined when the first inflorescence appeared at any node in the main stem. Plant height, in centimeters (cm), was determined at 23, 37, and 51 days after planting by measuring the distance from the soil surface to the highest point of the main stem.

Acetyl-CoA quantification

Metabolites were extracted from ~100 mg (fresh weight) camelina seeds as previously described (Ma *et al*., 2017) using 1200 µL cold (-20°C) methanol:chloroform solution (7:3 v/v), 480 µL chilled (4°C) double-distilled water, and internal standards (Pipes, norvaline, and ribitol). The upper polar phase was vacuum dried in a SpeedVac overnight at 8°C, resuspended with 50 µL of 50% methanol with 0.2% formic acid, and passed through a 0.45-µm centrifugal filter (Costar, Corning Inc.). The filtered solution was then transferred to insert glass vials (Agilent, Xpertek) for LC-MS analysis. Samples were analyzed by LC-MS/MS using a Shimadzu HPLC system (UFLC-XR; Shimadzu Corporation, Kyoto, Japan) connected to an electrospray ionization triple-quadrupole MS (Sciex QTRAP 6500). Metabolites were separated on an Imtakt Intrada organic acid column (150 × 2 mm, 3 µm; Kyoto, Japan) and detected in negative ion mode following

previously described methods and MS parameters (Koley *et al*., 2022). For quantification of pool sizes, metabolite concentrations and recoveries were calculated based on calibration curves of the individual standard and the internal standard ribitol, respectively.

MALDI-MSI sample preparation

Desiccated seeds were lightly fixed using slight modifications of a previously published method (Horn *et al*., 2012). Briefly, a 50 mM PIPES buffer, adjusted to a pH of 7.2 with potassium hydroxide, was prepared. Desiccated seeds were placed in a 4 % (w/v) formaldehyde in the PIPES buffer and allowed to sit in the solution for 30 min. Excess fixative was washed with three successive 5-min PIPES buffer washes. The lightly fixed seeds were then embedded in 10% w/v gelatin, flash-frozen using liquid nitrogen, and equilibrated to -20°C in a freezer for at least 8 h. The embedded seeds were sectioned to a 20 µm thickness at -20 °C using a cryo-microtome (Leica CM1850, Leica Microsystems; Buffalo Grove, IL, USA), collected on Cryo-Jane tape (Leica Biosystems; Wetzlar, Germany), and then mounted onto glass slides. After sectioning, slides were stored at -80°C until sample preparation. Samples were placed on a -80°C prechilled aluminum block and lyophilized under vacuum for 1.25 hours. After drying, tissue integrity was checked under a microscope, and optical images were obtained using a BX53 microscope (Olympus; Shinjuku City, Tokyo, Japan). 6.5 mM potassium acetate in methanol was applied using a TM sprayer (HTX Technologies; Chapel Hill, NC, USA). A flow rate of 0.03 mL/min was used for a total of eight passes with 3 mm spacing in a crisscross pattern at a velocity of 1200 mm/min with a nitrogen gas pressure of 10 psi and nozzle temperature of 30 °C. 2,5-dihydroxyacetophenone (DHAP) in 90:10 acetonitrile:water (20 mg/mL) was sprayed onto the sample surface as a matrix using the TM sprayer (flow rate of 0.10 mL/min, sixteen passes, 3 mm spacing, crisscross pattern,

a velocity of 1200 mm/min, nitrogen gas pressure of 10 psi, and nozzle temperature of 30 °C). Gold was then sputtered on top of the sample for 20 s at 40 mA using a Cressington 108 auto Sputter Coater (Ted Pella; Redding, CA, USA).

MALDI-MSI data acquisition

Mass spectral data were acquired using a high-resolution mass spectrometer (Q Exactive HF Orbitrap, Thermo Scientific; San Jose, CA, USA), equipped with a Spectroglyph MALDI source (Kennewick, WA, USA). All experiments were performed using a mass resolution of 120,000 at *m/z* 200, maximum injection time of 250 ms, *m/z* range of 500–1200, and a raster step of 20 μm. Data were analyzed using Xcalibur (Thermo Scientific, V4.2.47), Image Insight (Spectroglyph), and MSiReader (North Carolina State University; Raleigh, NC, USA) software. All mass spectrometry images were generated with a ±10 ppm mass tolerance due to mass drift across datasets.

RT-qPCR analysis

Total RNA was isolated from 15 pooled pennycress developing seeds using the RNAqueous Total RNA Isolation Kit (Ambion, Austin, TX, USA) followed by lithium chloride precipitation according to the manufacturer's instructions. Total RNA was digested with TURBO DNA-*free* Kit (Invitrogen, Carlsbad, CA, USA) to remove any genomic DNA contamination. RNA concentrations were quantified with a NanoDrop 2000 spectrophotometer (Thermo Scientific; San Jose, CA, USA). 1 μg of total RNA was used for cDNA synthesis using iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA). Quantitative PCR was performed on a CFX96 real-time PCR system (Bio-Rad) using iTAQ Universal SYBR Green Supermix (Bio-Rad) following the

manufacturer's guidelines. mRNA levels were normalized to the geometric mean of the reference genes *UBC21* and *TIP41* using the $2^{-\Delta Ct}$ method (Livak & Schmittgen, 2001). Primers used for RT-qPCR are listed in Supplementary Table C.1.

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AUTHOR CONTRIBUTIONS

TPD, JCS and LA designed the experiments; LA, DJW, and KMS generated transgenic camelina plants, characterized acetyl-TAG producing lines and performed data analysis; ME and BAJ generated transgenic pennycress plants; AEP performed MALDI-MS seed imaging; SK performed metabolite (acetyl-CoA) analysis; CL provided *fae1* camelina mutant seeds; LA, and TPD wrote the manuscript with input from all authors.

FIGURES

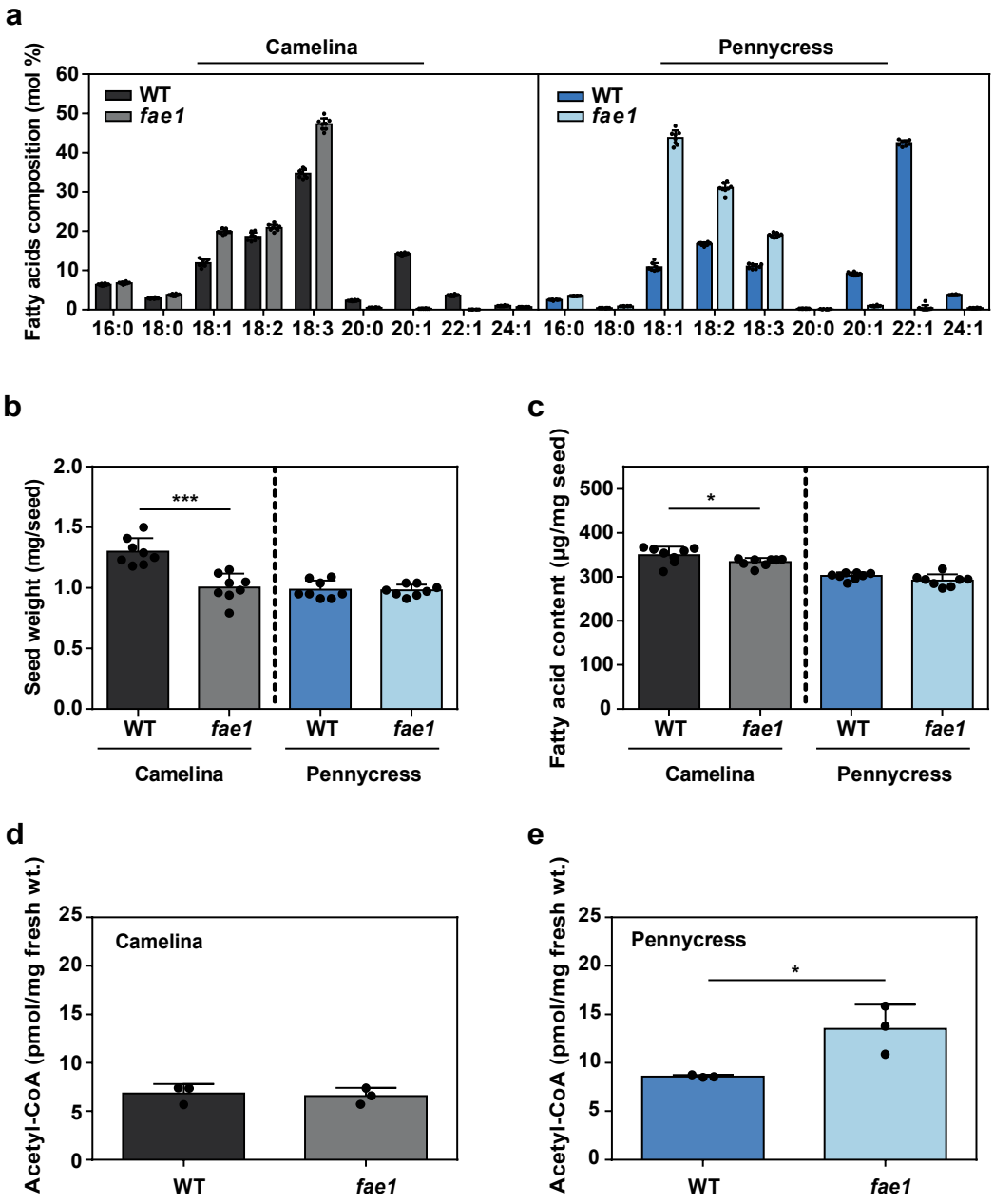


Figure 4.1. Pennycress *fae1* mutant seed possess increased levels of acetyl-CoA.

Seed fatty acid composition **(a)**, seed weight **(b)**, seed fatty acid content **(c)** and seed acetyl-CoA levels **(d-e)** of camelina and pennycress wild-type and *fae1* mutant seed. Data represent mean ± standard error, n= ≥ 3 biological reps. Asterisks indicate statistical difference from wild-type seeds (Student t-test; * $p \leq 0.05$, *** $p \leq 0.001$).

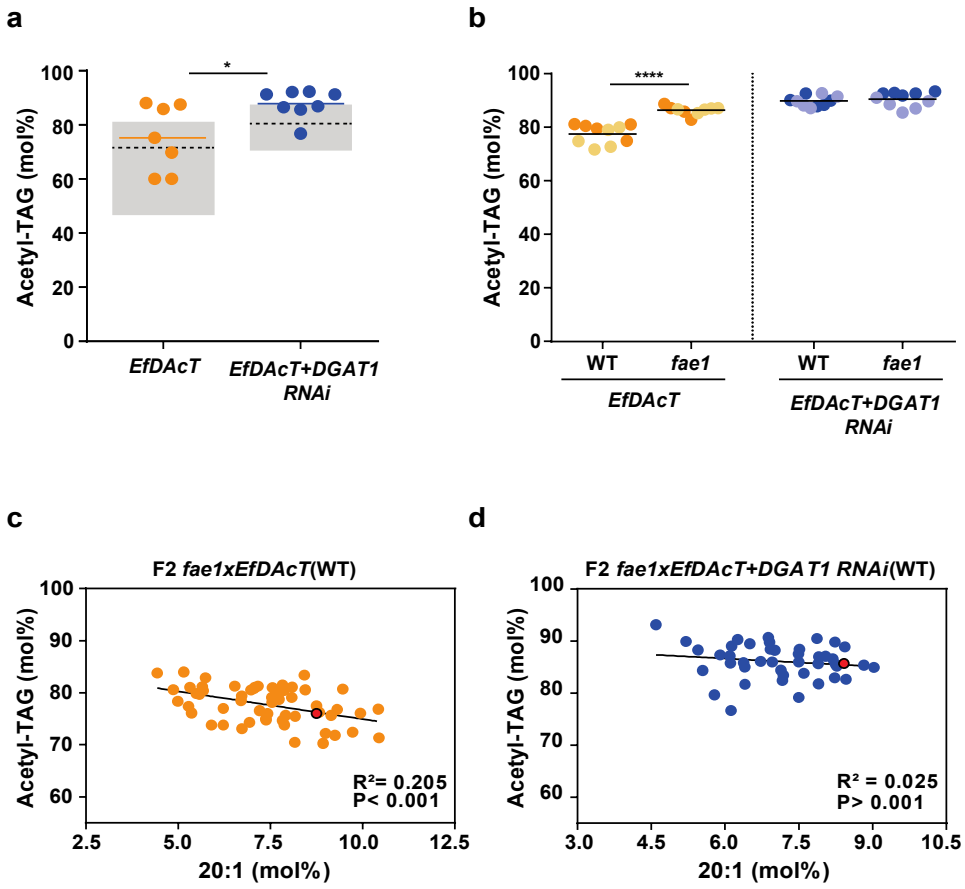


Figure 4.2. Mutation of *fae1* slightly affect acetyl-TAG accumulation in transgenic camelina lines.

(a) Scatter plot of acetyl-TAG content in segregating T2 seeds of transgenic *fae1* lines expressing either *EfDAcT* or *EfDAcT+DGAT1 RNAi*. Gray boxes represent acetyl-TAG values in wild-type seeds, with the top and bottom edges indicating the maximum and minimum values, respectively (Alkotami *et al.* 2021). Horizontal lines represent mean values, n ≥ 3 independent transgenic lines. **(b)** Scatter plot of acetyl-TAG content in homozygous wild-type and *fae1* transgenic lines expressing *EfDAcT* or *EfDAcT+DGAT1 RNAi*. Each independent line is represented by a distinct color. Horizontal lines represent mean values, n ≥ 3 biological replicates. Asterisks indicate statistical difference from wild-type seeds (One-way ANOVA followed by Sidak's multiple comparisons test; * $p \leq 0.05$, **** $p \leq 0.0001$). **(c-d)** Linear regression analysis of acetyl-TAG accumulation and 20:1 VLCFA levels in segregating F_2 seed from crosses between *fae1* and transgenic wild-type seeds expressing *EfDAcT* **(c)** or *EfDAcT+DGAT1 RNAi* **(d)**. The red data point indicates 20:1 and acetyl-TAG accumulation in the original transgenic parent segregating for *EfDAcT* **(c)** or *EfDAcT+DGAT1 RNAi* **(d)**. All other data points represent 20:1 and acetyl-TAG content in segregating F2 seed.

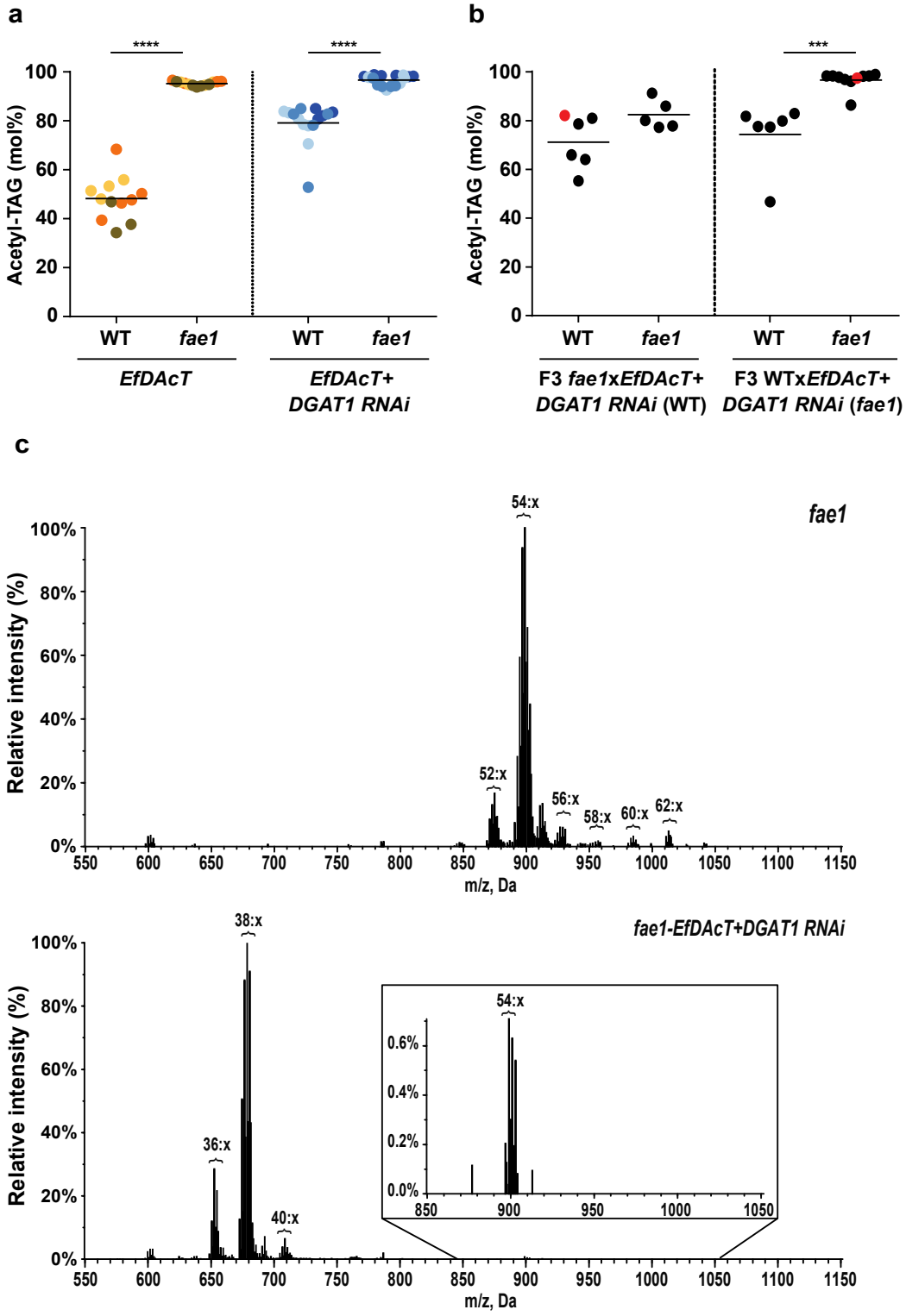


Figure 4.3. ***fae1*** **mutation enhances acetyl-TAG accumulation in transgenic pennycress seeds.**

(a) Scatter plot of acetyl-TAG content in homozygous transgenic wild-type and *fae1* lines expressing *EfDAcT* or *EfDAcT+DGAT1 RNAi*. Each independent line is represented by a distinct color. Horizontal lines represent mean values, n= three independent transgenic lines with ≥ 3 biological reps. Asterisks indicate statistical difference from wild-type or *fae1* seeds (One-way ANOVA followed by Sidak's multiple comparisons test; *** $p \leq 0.001$, **** $p \leq 0.0001$). **(b)** Scatter plot of acetyl-TAG levels obtained from homozygous F3 seed crosses between *fae1* or wild-type seeds and transgenic seeds expressing *EfDAcT+DGAT1 RNAi*. Red data points represent acetyl-TAG levels in the transgenic parent used in each cross. **(c)** Positive ESI-MS spectra of neutral lipids from *fae1* mutant control and homozygous pennycress seeds expressing *EfDAcT+DGAT1 RNAi* in *fae1* background. Signal peaks represent *m*/*z* values of the $[M + NH_4]^+$ adduct. The number of acyl carbons in each series of acetyl-TAG and TAG molecular species is indicated; the number of double bonds (x) is not delineated.

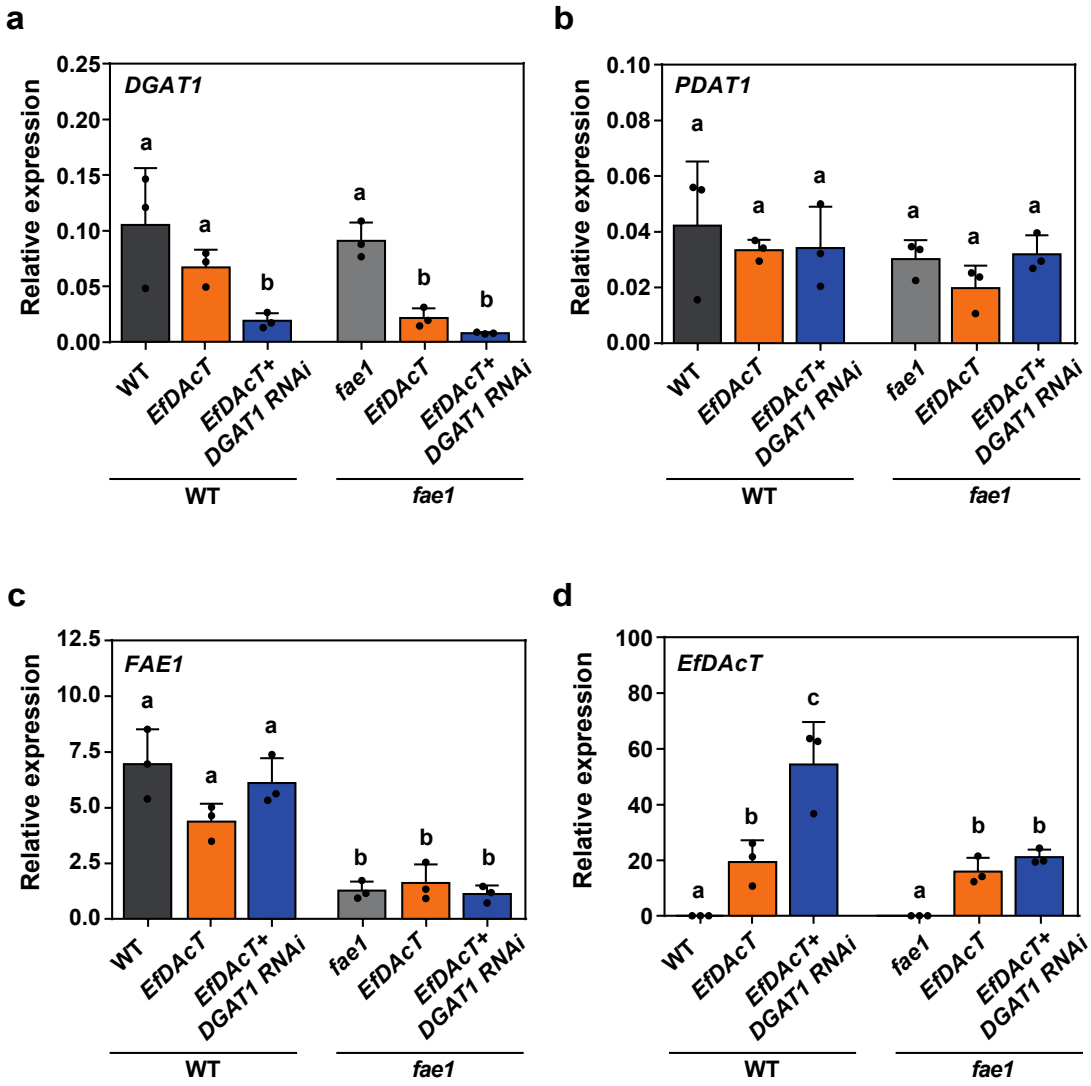


Figure 4.4. Assessment of gene expression levels of TAG-related genes in transgenic pennycress lines.

Quantitative real-time PCR analysis of *DGAT1* (**a**), *FAE1* (**b**), *EfDAcT* (**c**), and *PDAT1* (**d**) in transgenic wild-type and *fae1* developing seeds 20 days after flowering expressing *EfDAcT* or *EfDAcT+DGAT1 RNAi*. Gene expression levels were normalized to the geometric mean of the reference genes *TIP4* and *UBC*. Data represent mean ± standard error, n= 3 biological reps. Different letters indicate statistical difference between genotypes (One-way ANOVA followed by Tukey's multiple comparisons test; $p \leq 0.05$).

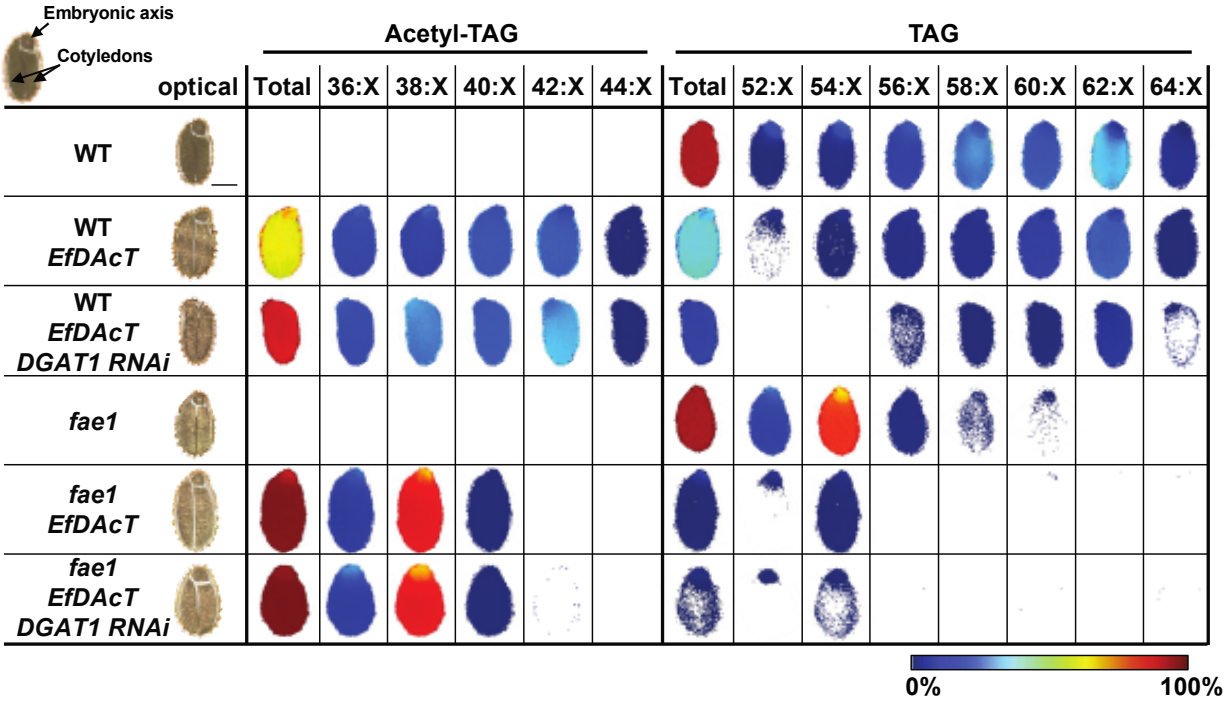


Figure 4.5. MALDI-MS imaging of TAG and acetyl-TAG in pennycress mature seeds.

MALDI-MS images of TAG potassium adducts $[M+K]^+$ in mature pennycress seeds from wild-type, *fae1* mutant, and homozygous pennycress transgenic lines expressing *EfDAcT* or *EfDAcT+DGAT1 RNAi.* Images shown are summed ion images of acetyl-TAG and TAG molecular species with the same number of acyl carbons regardless of the number of double bonds (x). Normalized ion intensities are shown using a jet color scale, with red representing 100% of the total TAG signal. Interference signals are removed with more than 2ppm mass error after internal calibration. The scale bar represents 500 μm. Optical images are included for reference.

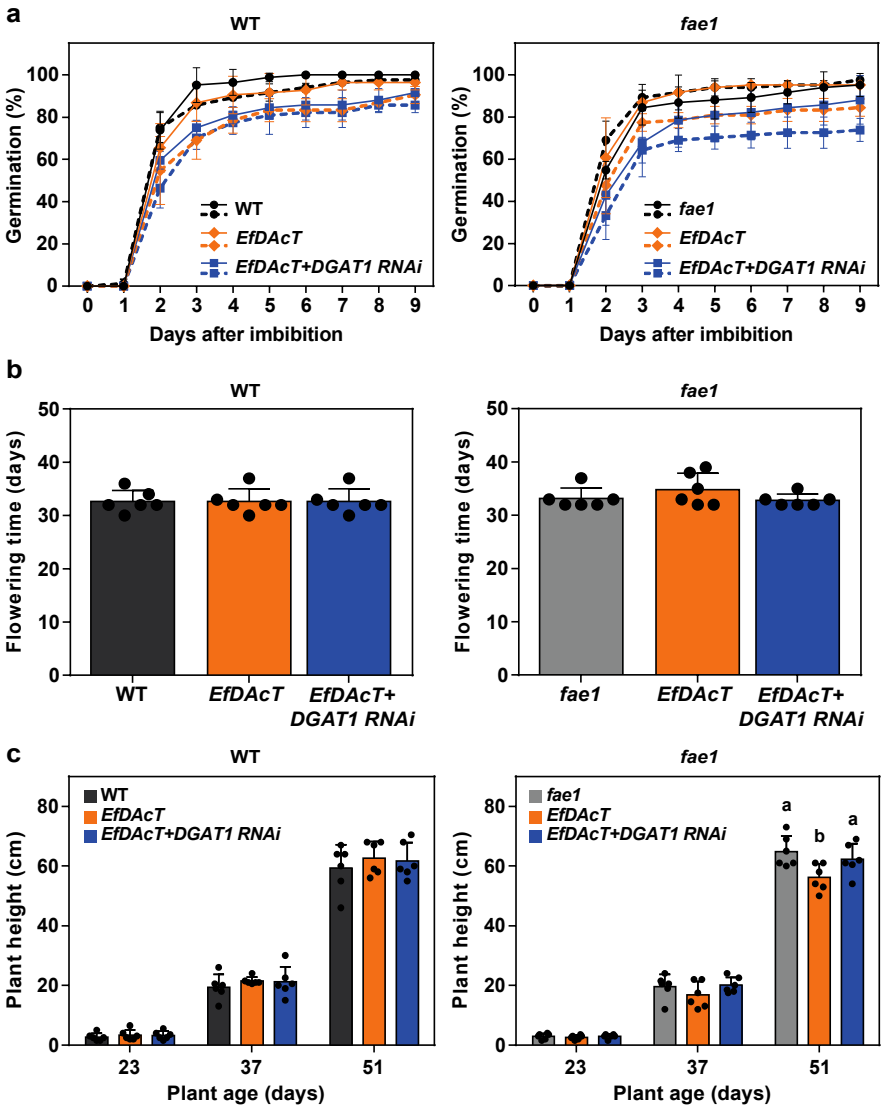


Figure 4.6. Seed germination and plant traits of pennycress high acetyl-TAG producing lines. **(a)** Germination rates of seed from homozygous transgenic lines expressing *EfDAcT* or *EfDAcT+DGAT1 RNAi* in both wild-type and *fae1* backgrounds. Solid and dashed lines indicate different independent lines. Representative images of germination plates are shown in Supplementary Figure D.8. Data represent mean ± standard error, n= 3 biological reps. **(b)** Time in days until first flower opening. **(c)** Plant height measurements at 21, 37, and 51 days after planting. Data represent mean ± standard error, n= two independent transgenic lines with 3 biological reps. Different letters indicate statistical difference between genotypes (One-way ANOVA followed by Tukey's multiple comparisons test; $p \leq 0.05$).

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Chapter 5 – Summary and future directions

SUMMARY

The primary focus of this dissertation was to optimize the acetyl-TAG biosynthesis pathway in transgenic oilseeds, specifically camelina and pennycress, to produce native-like levels which can reach more than 90 mol% of acetyl-TAG in some *Euonymus* species. To achieve this goal, multiple strategies were utilized (Figure 1.5b) including:

1) **Strategy: Expression of *EfDAcT* to efficiently produce acetyl-TAG (Chapter 2). Rationale:** *EfDAcT* is an acetyltransferase isolated from *Euonymus fortunei* seeds, to efficiently produce acetyl-TAG in camelina seeds. Previous work revealed that *EfDAcT* exhibited superior acetyltransferase activity *in vitro* and *in vivo* compared to multiple acetyltransferases, including the first identified acetyl-TAG synthesizing enzyme, *EaDAcT*, from *Euonymus alatus* seeds (Durrett *et al*., 2010; Tran *et al*., 2017). **Results:** *EfDAcT* expression accumulated 72 mol% of acetyl-TAG in camelina seeds, a 20 mol% increase compared to *EaDAcT* expression.
2) **Strategy: *DGAT1* Knockdown to suppress the production of endogenous TAG (chapter 2). Rationale:** *DGAT1* catalyzes the acylation of DAG with acyl-CoA, resulting in the formation of endogenous TAG in oilseeds. In transgenic seeds expressing *EfDAcT*, *DGAT1* and *EfDAcT* compete for the DAG precursor to produce either TAG or acetyl-TAG, respectively. **Results:** Combining *EfDAcT* with RNAi suppression of *DGAT1* increased average acetyl-TAG accumulation to 80 mol% with the highest lines reaching up to 90 mol% in homozygous seeds.

3) **Strategy: Expression of *EfDAcT+DGAT1 RNAi* in a *fae1* mutant background (Chapter 4). Rationale:** FAE1, is a key component of the fatty acid elongase complex and participates in elongation of 18:1 fatty acid to VLCFA using acetyl-CoA as a substrate (Millar & Kunst, 1997). In transgenic seeds, acetyl-CoA is also utilized by EfDAcT as a substrate for acetyl-TAG synthesis. Additionally, *EfDAcT* transgenic seeds in chapter 2 and 3 showed reduction in VLCFA accumulation compared to the wild-type suggesting that EfDAcT does not prefer to utilize DAG substrates containing VLCFA. **Results:** *fae1* mutation resulted in an increase in acetyl-CoA levels in pennycress but not in camelina. Expression of ***EfDAcT+DGAT1 RNAi* in *fae1* mutant background resulted in an average of 90 mol% of acetyl-TAG in homozygous camelina seeds and 96 mol% in homozygous pennycress seeds.**

Overall, the final chapter (chapter 4) demonstrate that by combining these strategies, acetyl-TAG levels in the best transgenic lines reached 98 mol% in pennycress seeds and 93 mol% in camelina seeds (Figures 4.2b, and 4.3a). This marks the first instance where transgenic oilseed crops were successfully engineered to achieve or exceed the naturally occurring levels of unique oils found in native species.

FUTURE DIRECTIONS

The successful generation of native-like acetyl-TAG levels in transgenic oilseeds opens up avenues for future research and evaluation of these lines. Characterization of the high acetyl-TAG producing lines in camelina and pennycress in chapter 2, 3 and 4 revealed changes in seed properties which included slight increase in seed weight, reduced total fatty acid content, and reduced germination rates. Changes in seed storage reserves including oil content, protein and

carbohydrate can affect seed weight (Duan *et al*., 2023). Chapter 3 transcriptomic analysis indicated the upregulation of genes linked to starch and sucrose metabolism, as well as amino sugar and nucleotide sugar metabolism. While this information suggests potential contributors to the observed increase in seed weight, quantification of carbohydrate and protein levels in the transgenic seeds will be necessary to confirm whether either or both contribute to the increase in seed weight.

The decrease in total fatty acid content in transgenic lines might be due to feedback inhibition of fatty acid biosynthesis. Although no downregulation was observed in genes related to fatty acid biosynthesis in the transgenic lines based on the transcriptomic analysis in Chapter 3, some might be post-transcriptionally or post-translationally regulated (Hajduch *et al*., 2010). Genes involved in fatty acid biosynthesis such as WRINKLED1 (*WRI1*) can be genetically modified to increase total oil content in the transgenic lines (Adhikari *et al*., 2016). For example, overexpression of Arabidopsis *WRI1* in camelina seeds resulted in a 14% increase in oil content (An & Suh, 2015). An additional approach to increase total fatty acid content, is to eliminate negative regulators of fatty acid synthesis or to eliminate non-essential competing pathways. Malonyl-CoA serves as a substrate for both fatty acid and flavonoid biosynthesis, which indicates a potential interplay between these pathways. The transcriptomic analysis in Chapter 3 identified an upregulation in flavonoid biosynthesis in transgenic seeds. Previous study in Arabidopsis have demonstrated that the loss of TRANSPARENT TESTA 4 (TT4), which catalyzes the initial step of flavonoid biosynthesis, reduces flavonoid biosynthesis and increases seed fatty acid content (Xuan *et al*., 2018). *TT4* therefore could serve as a target for elimination in transgenic seeds to increase total fatty acid content.

The reduced and delayed germination observed in acetyl-TAG lines, indicates a potential link between altered lipid composition and seed germination. In Chapter 3, transgenic seeds displayed upregulation of dormancy-related genes, including the key regulator Delay of Germination 1 (DOG1). DOG1 serves as a master regulator of primary dormancy, collaborating with ABA to delay germination (Carrillo-Barral *et al*., 2020; Huo *et al*., 2016; Nishimura *et al*., 2018). Targeting DOG1 emerges as a potential strategy to restore germination in the transgenic seeds.

To gain a deeper understanding of how lipid pathways are affected and rerouted by acetyl-TAG accumulation and genetic modification of enzymes involved in oil biosynthesis in different plants, comparative characterization of the high acetyl-TAG lines through acyl flux analysis in camelina and pennycress seeds might be beneficial. Expression of *EfDAcT+DGAT1 RNAi* in a *fae1* mutant background to produce acetyl-TAG in camelina and pennycress yielded varying levels of acetyl-TAG accumulation, as discussed in Chapter 4. The observed differences in accumulation between the two plants, particularly considering the higher content of VLCFA in pennycress compared to camelina, raise the possibility that the elimination of VLCFA might influence the activity of endogenous TAG-synthesizing enzymes, specifically DGAT1 and PDAT1, in pennycress. This alteration could allow EfDAcT to better outcompete these enzymes in pennycress but not in camelina. Future studies could confirm this hypothesis by investigating the substrate specificity of DGAT1 and PDAT1 in both pennycress and camelina. To further enhance acetyl-TAG levels in camelina, the generation of knockout lines in *DGAT1* and *PDAT1*, followed by crossing with *fae1* mutant lines to express *EfDAcT*, could be explored.

Lastly, previous research has indicated variations in trait measurements when comparing laboratory or greenhouse-grown plants to those cultivated in the field (Aryal, 2018; Liu, Tjellström, *et al*., 2015; Usher *et al*., 2017). Therefore, field evaluations of high acetyl-TAG lines

will be essential to examine whether their performance concerning acetyl-TAG levels, seed properties, and overall plant fitness remains stable and similar to laboratory settings. Field trials will also allow for the evaluation of stress response against common biotic and abiotic stresses.

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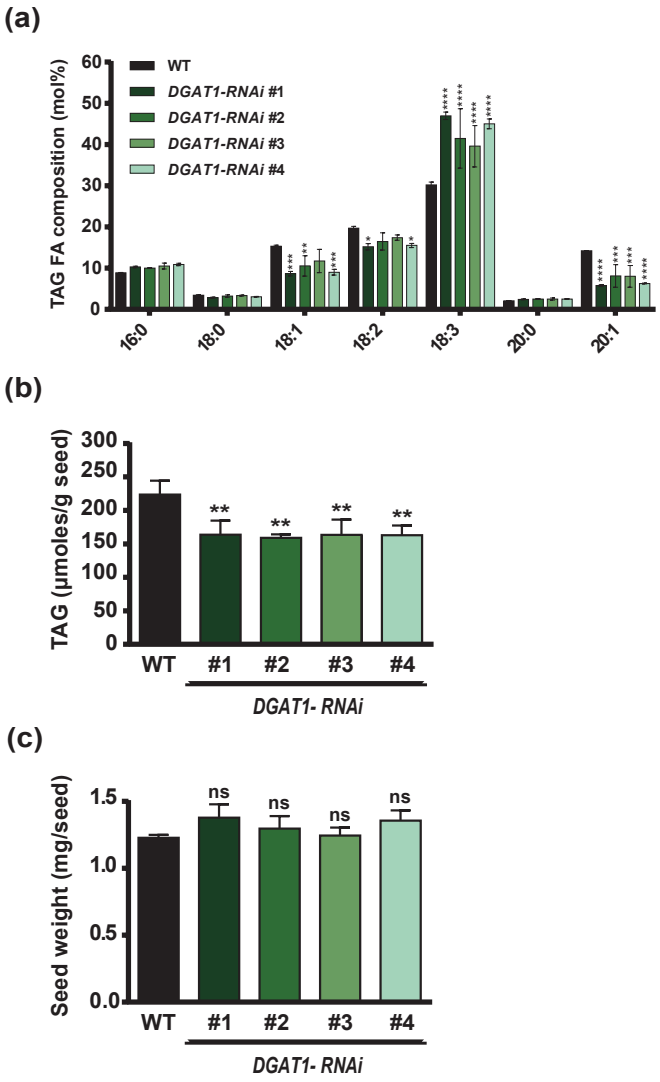
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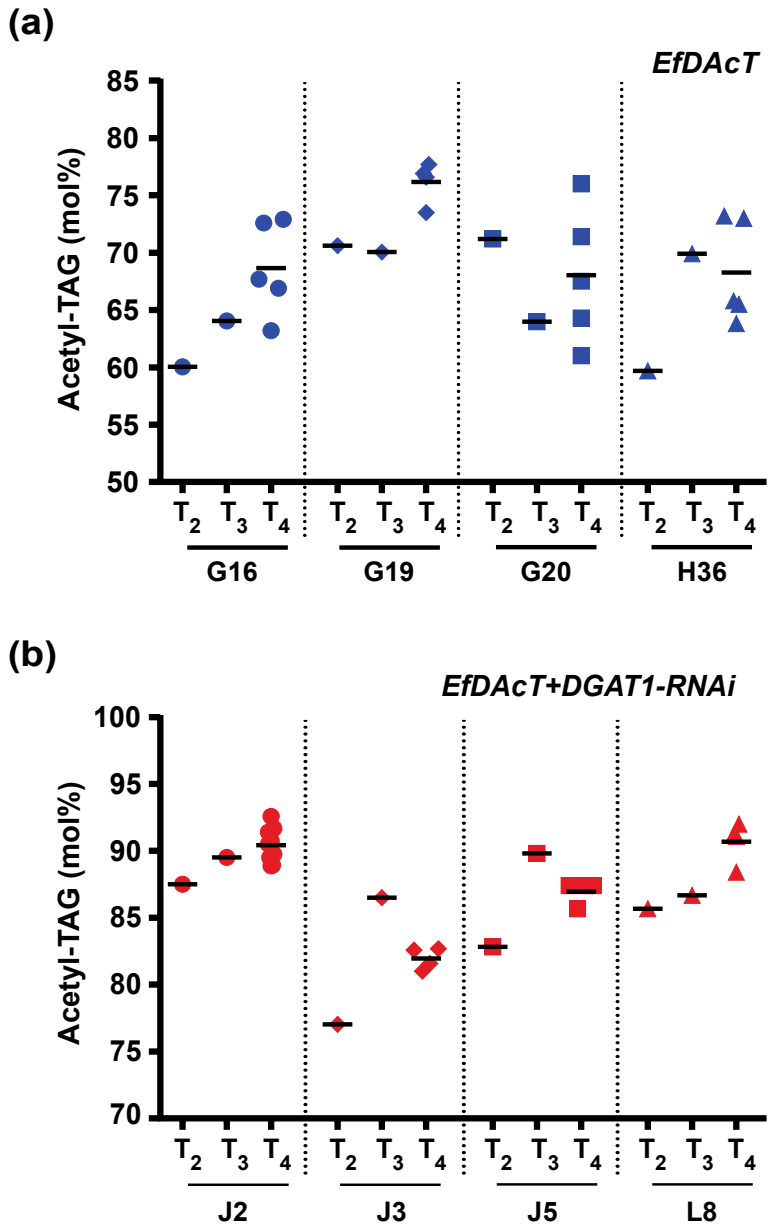
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Institution name	Kansas State University
Expected presentation date	Dec 2023
Order reference number	032221
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Appendix B - Chapter 2 Supplementary Materials



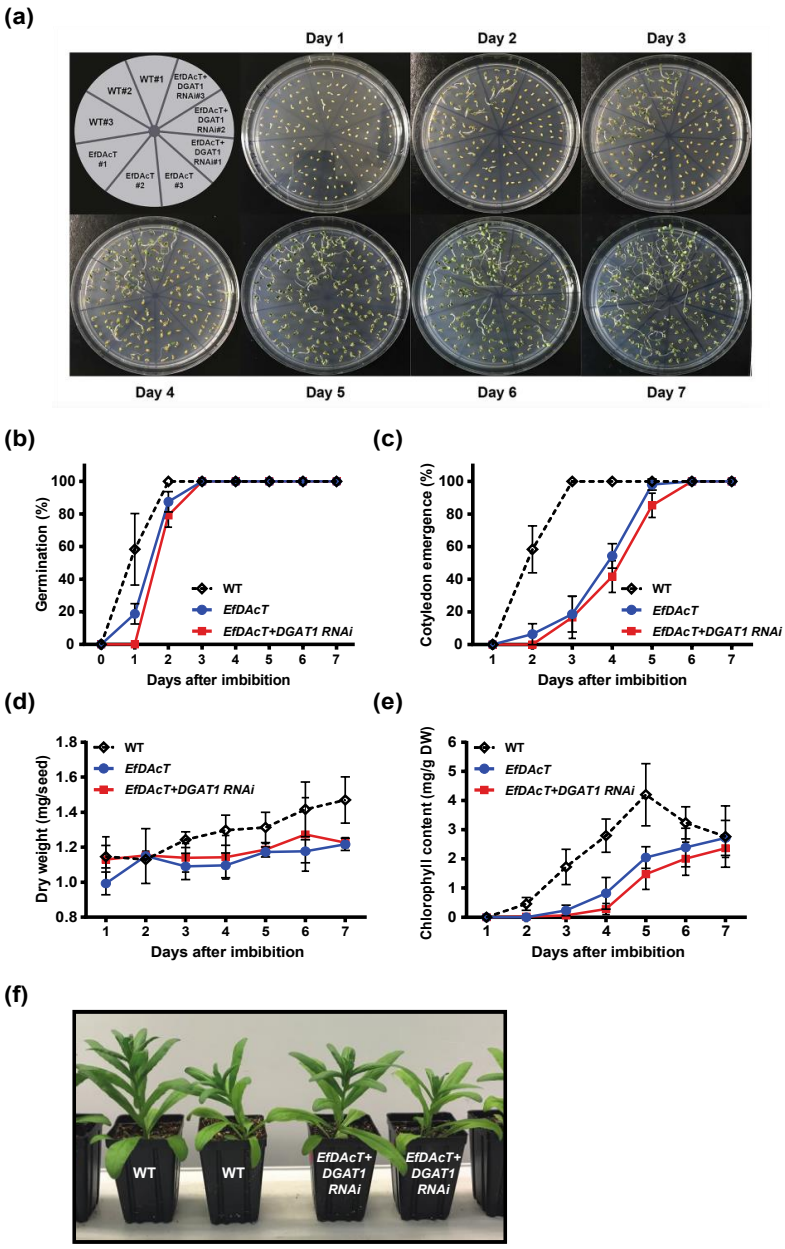
Supplementary Figure B.1. Suppression of DGAT1 in camelina alters seed fatty acid composition.

Fatty acid composition (A), seed TAG (B), and seed weight (C) of mature seed harvested from wildtype (WT) and four independent T3 homozygous lines expressing DGAT1-RNAi. Values represent the mean + SD of three biological replicates. Asterisks indicate significant difference compared to the wild type control (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$; ns, not significant; one-way ANOVA with Dunnett's multiple comparison test).



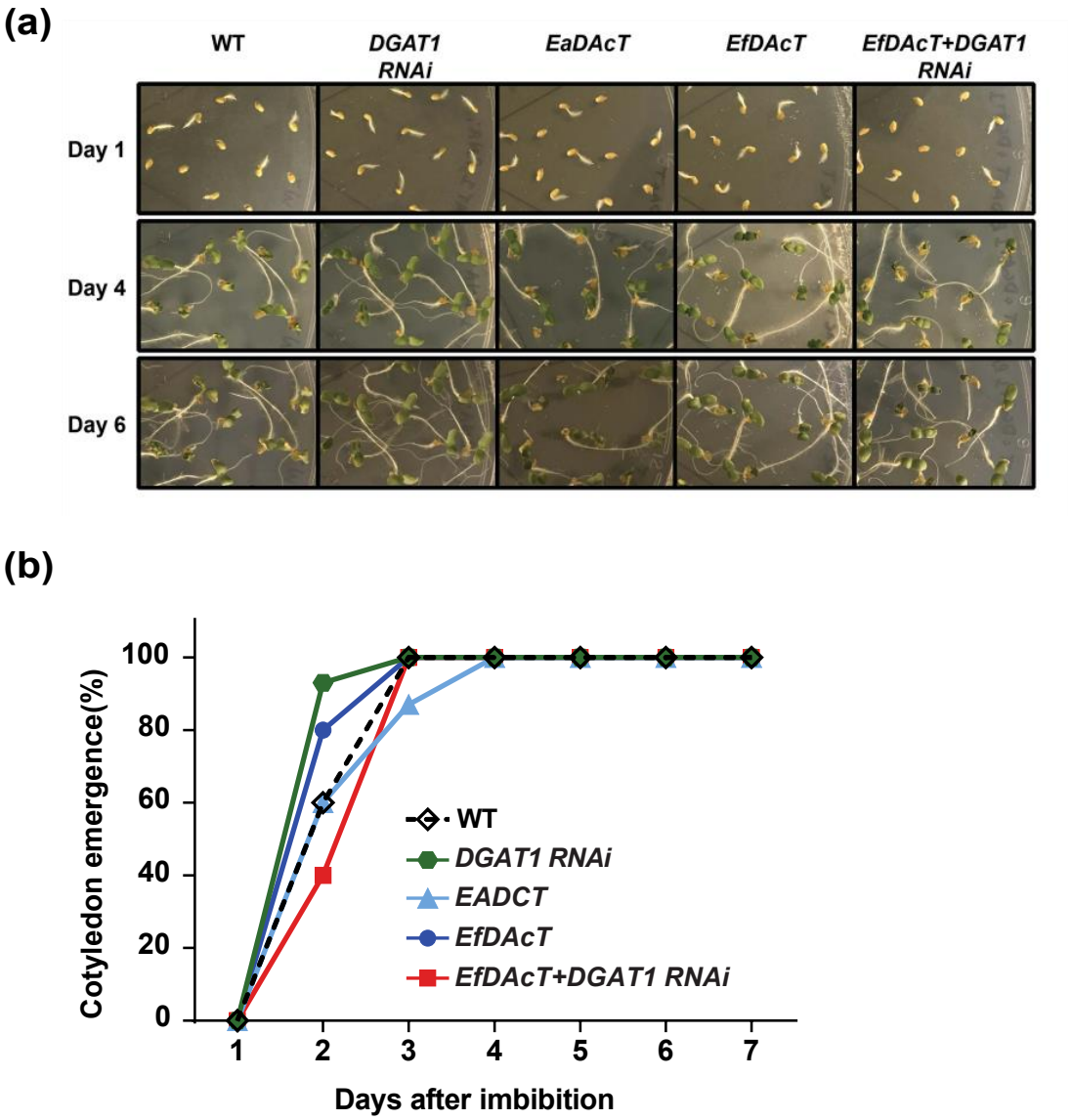
Supplementary Figure B.2. Acetyl-TAG levels in transgenic seeds are stable across multiple generations.

Acetyl-TAG content of transgenic seed collected from T2, homozygous T3 and homozygous T4 plants expressing *EfDAcT* (A) or *EfDAcT+DGAT1-RNAi* (B). Each data point represents acetyl-TAG content of seeds from an individual plant. Horizontal lines represent the mean acetyl-TAG levels for each group of plants.



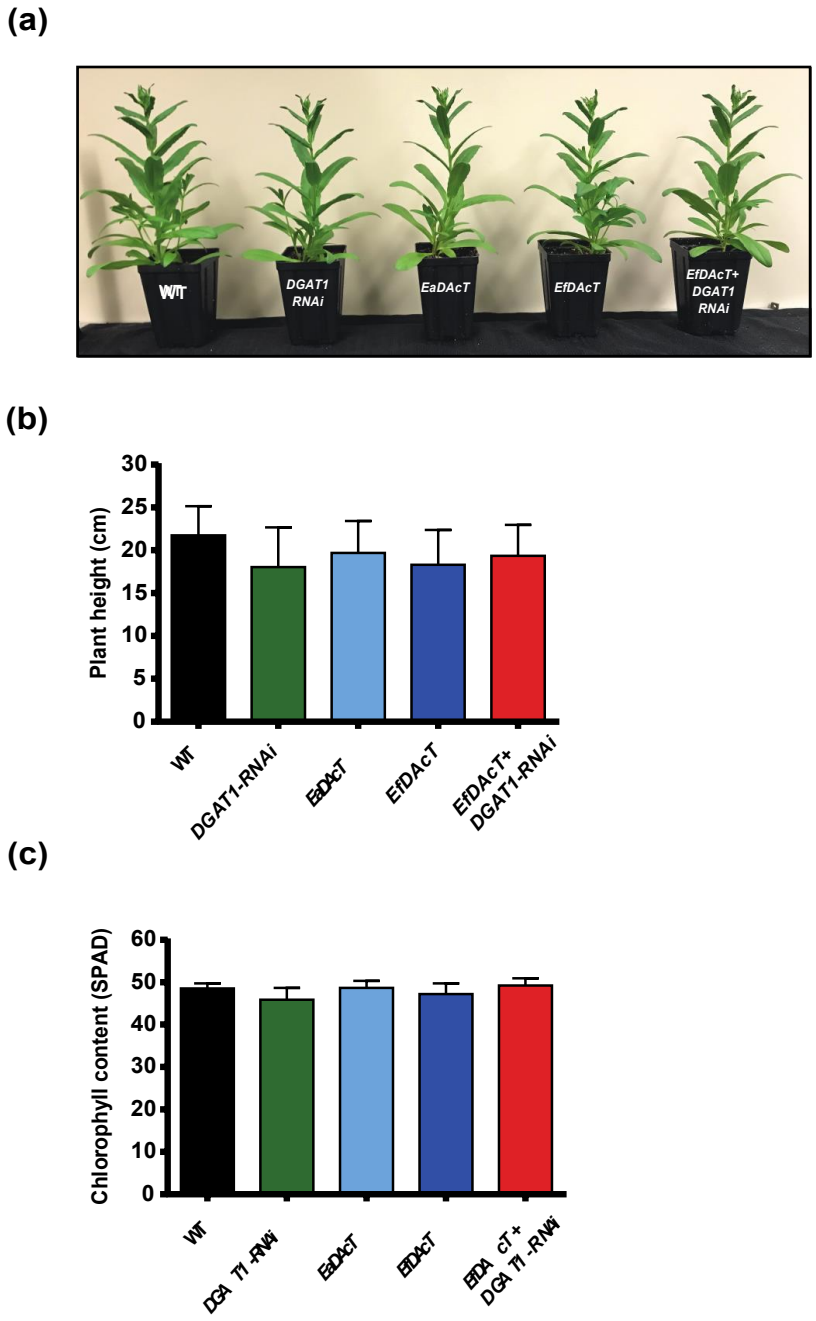
Supplementary Figure B.3. Germination of seed containing acetyl-TAG is delayed.

Seedling establishment on 1% agar plates (A) and the corresponding germination rates (B), cotyledon emergence (C), dry weight (D), and chlorophyll content (E) of seeds from wild-type (WT) and transgenic homozygous high acetyl-TAG producing lines expressing *EfDAcT* or *EfDAcT+DGAT1-RNAi*. Values shown represent the mean ± SD of three biological replicates. (F) Growth phenotype of wild-type (WT) plants and transgenic homozygous acetyl-TAG producing lines expressing *EfDAcT+DGAT1-RNAi* grown on soil for 24 days.



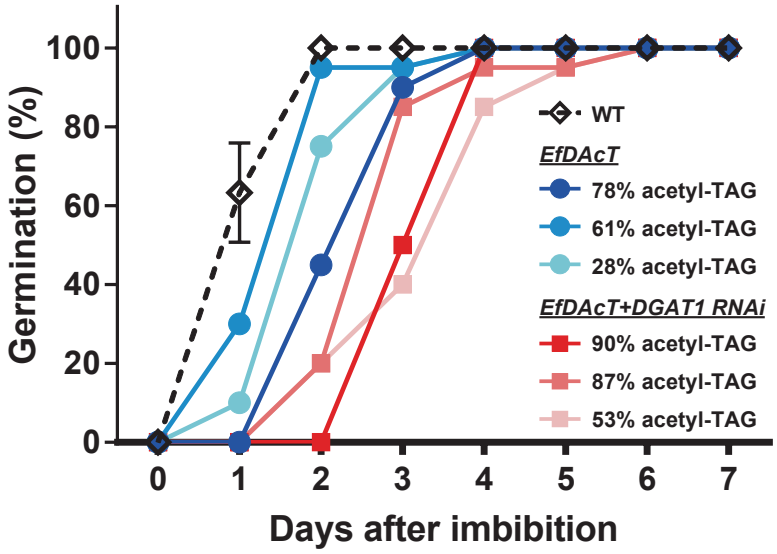
Supplementary Figure B.4. Germination of freshly harvested seed containing acetyl-TAG is not delayed.

Representative images of seeds germinated on 1% agar plates at 1 day after imbibition, and subsequent seedling morphology at days 4 and 6 (A), and the corresponding cotyledon emergence (B), of freshly harvested seeds from wild-type (WT), and homozygous transgenic lines expressing *DGAT1-RNAi*, *EaDAcT*, *EfDAcT* or *EfDAcT+DGAT1-RNAi*.



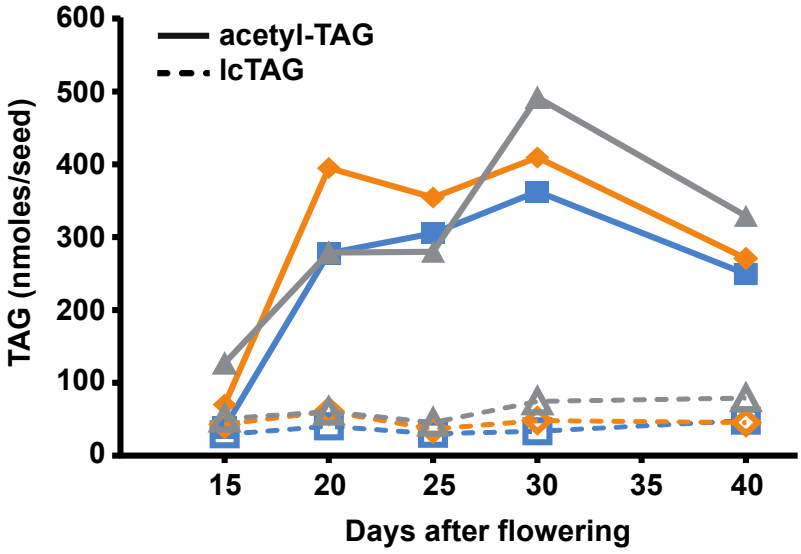
Supplementary Figure B.5. Transgenic plants possess no visual phenotypes compared to wild-type plants.

Growth phenotype (A), plant height (B), and leaf chlorophyll content (SPAD values; C) of wildtype (WT) plants and homozygous transgenic lines expressing *DGAT1 -RNAi*, *EaDAcT*, *EfDAcT* or *EfDAcT+DGAT1-RNAi* grown on soil for 28 days from freshly harvested seeds. Values shown represent the mean + SD of three biological replicates.



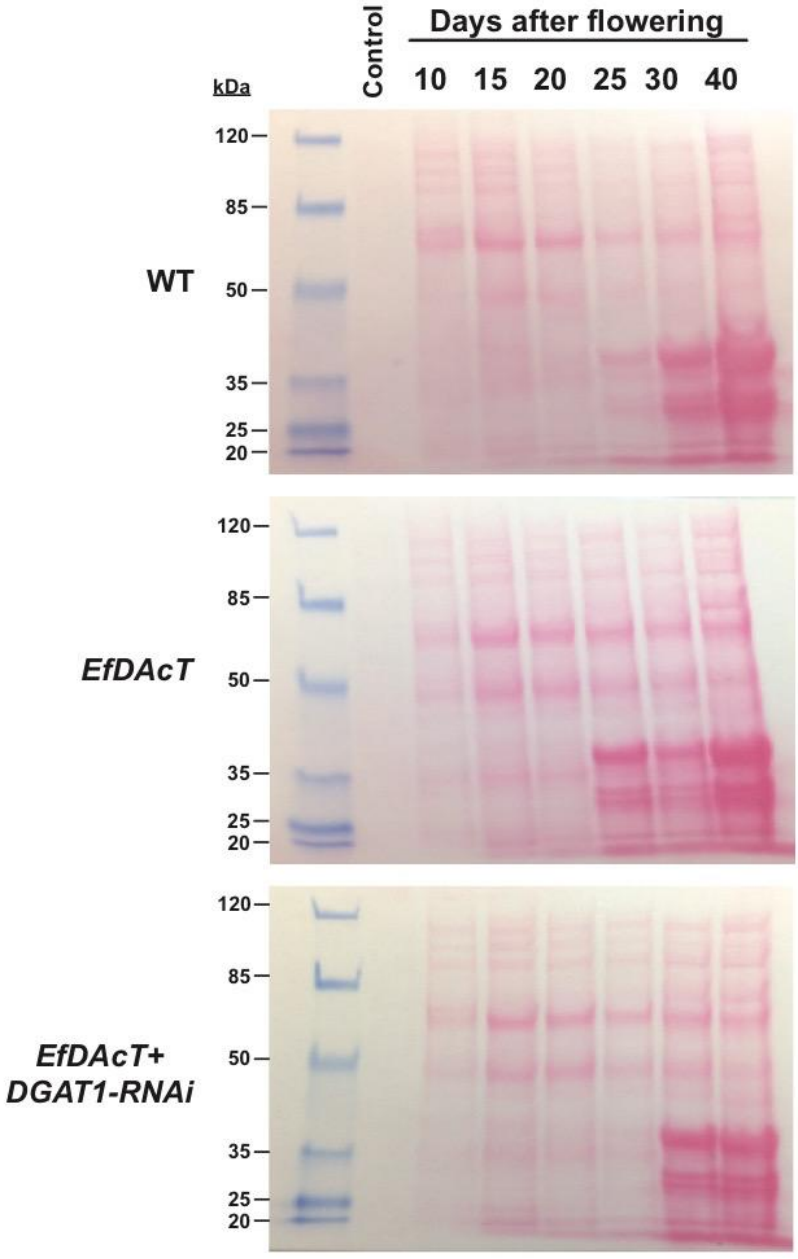
Supplementary Figure B.6. Acetyl-TAG levels do not impact the germination delay.

Germination rates of seeds from wild-type (WT) and a range of transgenic homozygous acetyl-TAG producing lines expressing *EfDAcT* or *EfDAcT+DGAT1-RNAi*.



Supplementary Figure B.7. Acetyl-TAG accumulation follows the same pattern in independent transgenic lines.

Acetyl-TAG (solid line) and lcTAG (dashed line) accumulation in the developing seed of three independent T4 homozygous high acetyl-TAG producing lines expressing *EfDAcT+DGAT1-RNAi*. For each developmental stage, seeds were pooled from multiple plants per transgenic line.



Supplementary Figure B.8. Total protein in developing seeds of wild-type and transgenic camelina lines.

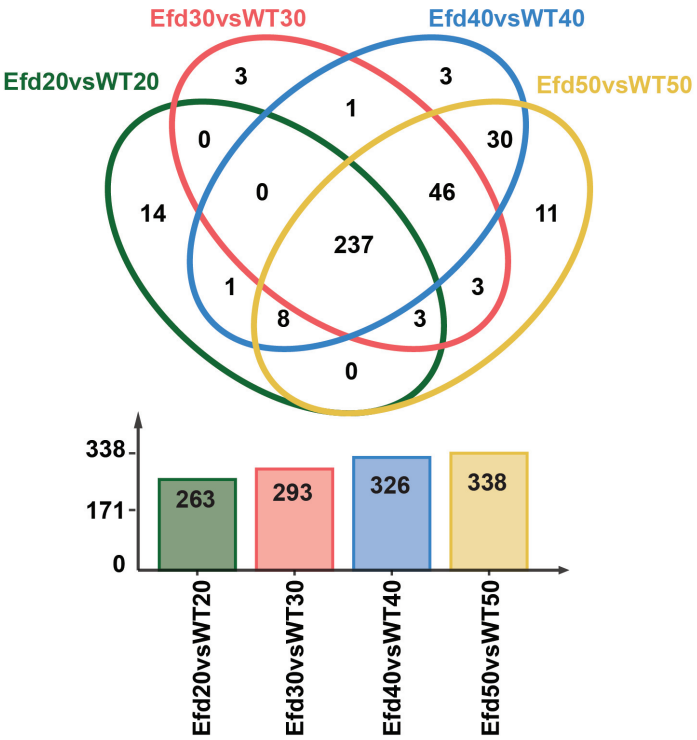
Ponceau S staining of total protein extracts from the developing seeds of wild-type (WT) and homozygous T4 plants expressing *EfDAcT* or *EfDAcT+DGAT1-RNAi* prior to immunoblotting for the HA epitope tag (Figure 6). Yeast microsomes expressing EfDAcT-HA were used as the positive control.

Supplementary Table B.1.Primers used for vector construction and RT-qPCR amplification of different genes in chapter 2

Primer	Sequence
EaDAcT-HA-F	5'-ACCCAATTGATGATGGATGCTCATCAA-3'
EfDAcT-HA-F	5'- ACCCAATTGATGATGGATGTTCATCAA -3'
Ea/fDAcT-HA-R	5'-AGAGCTAGCTTAAGCGTAATCTGGAA-3'
EfDAcT-RT-F	5'- CGGCTTCTTCCTTGCTTGGC -3'
EfDAcT-RT-R	5'- GGATTTCGCTTGACTGTGATG GG -3'
DGAT1-RT-F	5'- GACGTTAGGGATCGGATTGA -3'
DGAT1-RT-R	5'- TCTCCCTAGCTCTCCGATG-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'

Appendix C - Chapter 3 Supplementary Materials

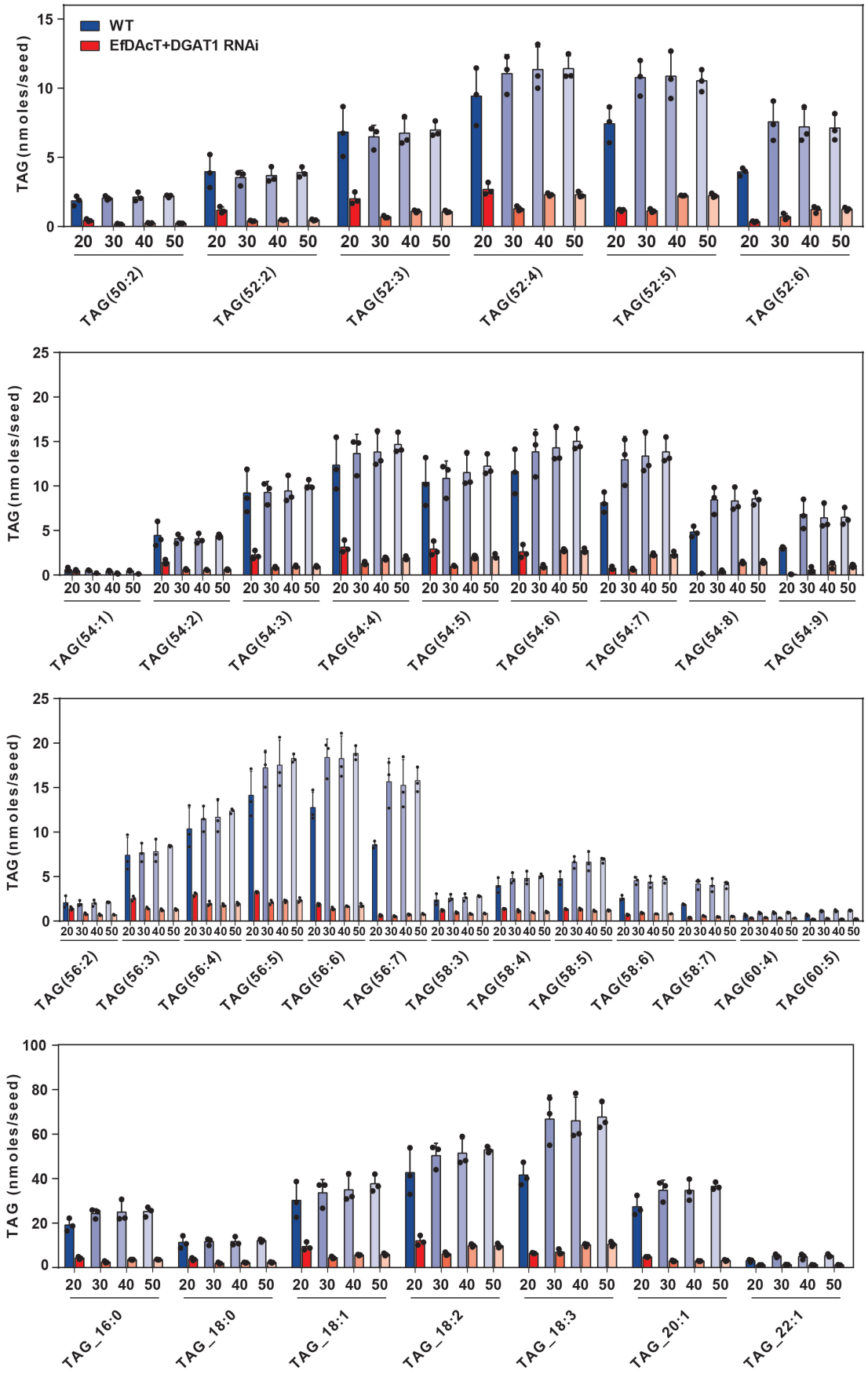
a)



b)

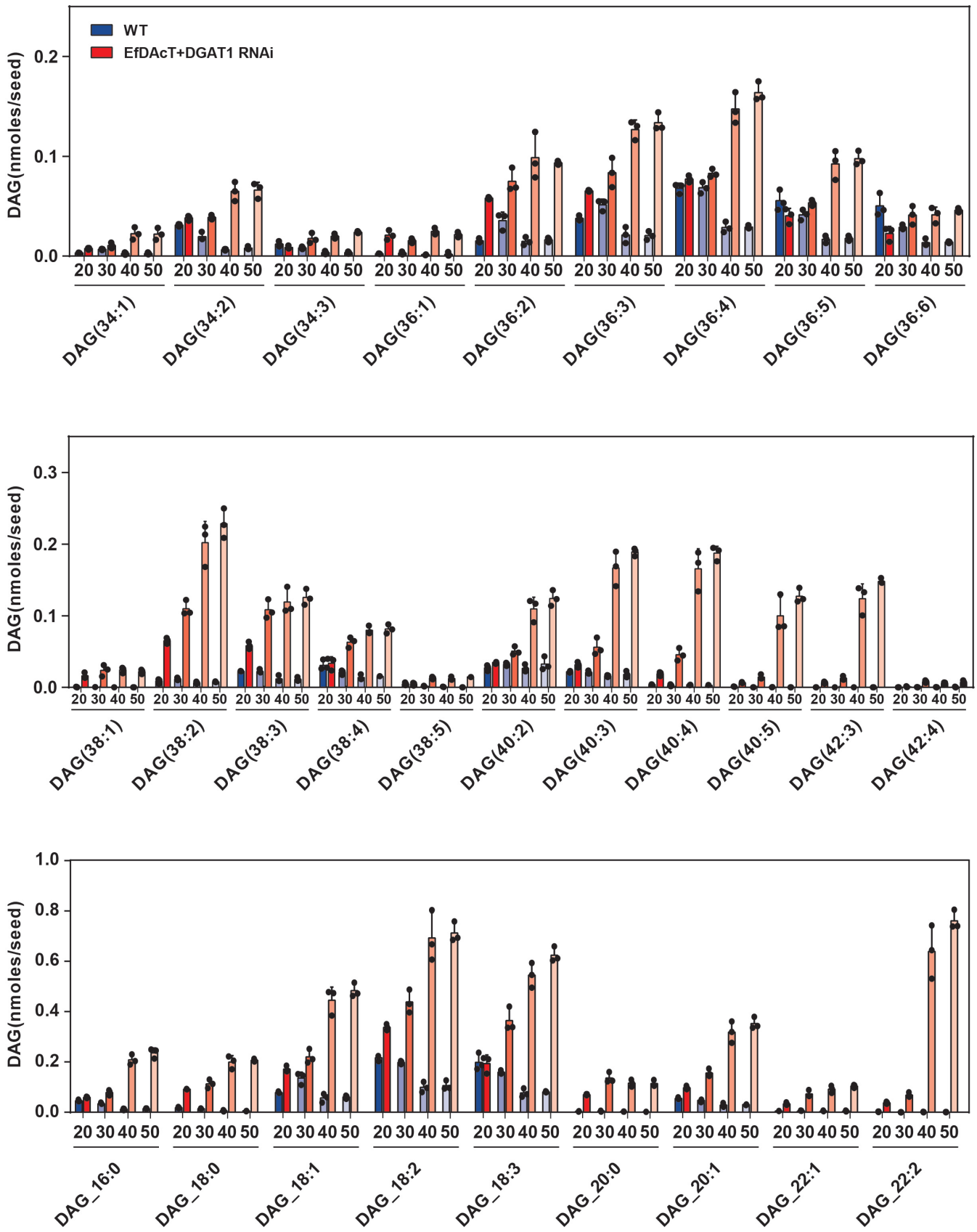
Lipid class	# of DA lipids common in all stages	% of DA lipids common in all stages
TAG	149	71%
Acetyl-TAG	63	100%
DAG	16	47.1%
MGDG	0	-
DGDG	1	9.1%
PA	0	-
PC	5	26.3%
PE	1	4.5%
PG	2	16.7%
PI	0	-
PS	0	-
LPE	0	-
LPC	0	-
LPG	0	-
Total	**237**	**66.6%**

Supplementary Figure C.1. Differentially accumulated lipids identified in transgenic seeds. **(a)** Venn diagram illustrating the overlap of differentially accumulated (DA) lipid molecular species, meeting criteria of FDR ≤ 0.05 and fold change ≥ 2, in homozygous transgenic camelina seeds expressing *EfDAcT+DGAT1 RNAi* compared to the WT. The numerical values following Efd and WT indicate the respective seed development stage. The bar graph depicts the total number of DA lipid molecular species at each developmental stage. **(b)** Table representing the number of DA lipid molecular species in each lipid class that are consistently observed across all developmental stages. WT, wild-type; Efd, *EfDAcT+DGAT1 RNAi*.



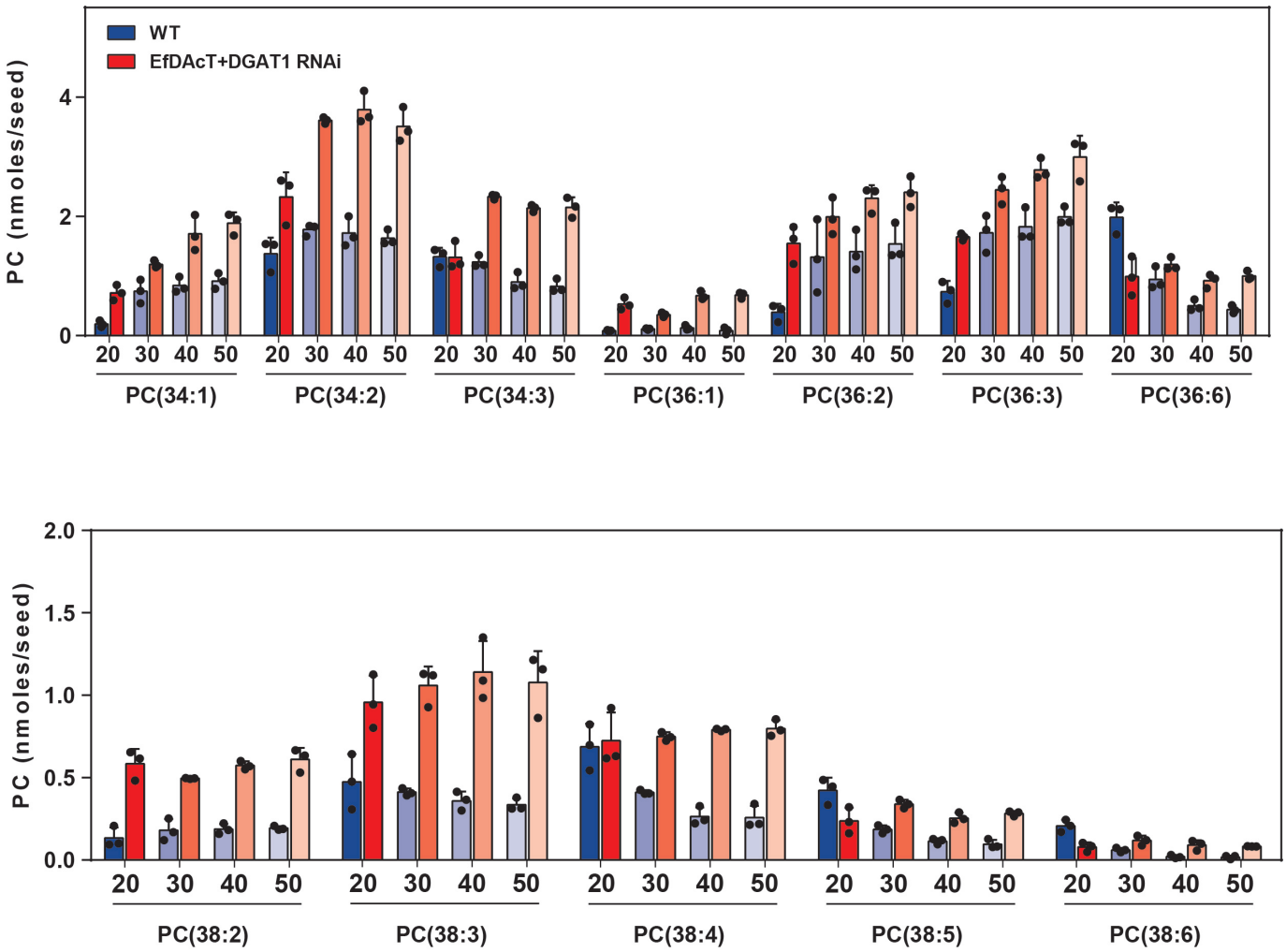
Supplementary Figure C.2. Distribution of TAG molecular species across seed development.

The content of different TAG molecular species in camelina wild-type (WT) seeds and homozygous transgenic seeds expressing *EfDAcT+DGAT1 RNAi*. Each molecular species is annotated as (X:Y), where X represents the total carbon number, and Y indicates the total number of double bonds. The last panel shows the sum of all TAG molecular species containing a specific fatty acid. The developmental stages are denoted by 20, 30, 40, and 50. Data represent mean ± standard error, n=3 biological reps. DAG, triacylglycerol.



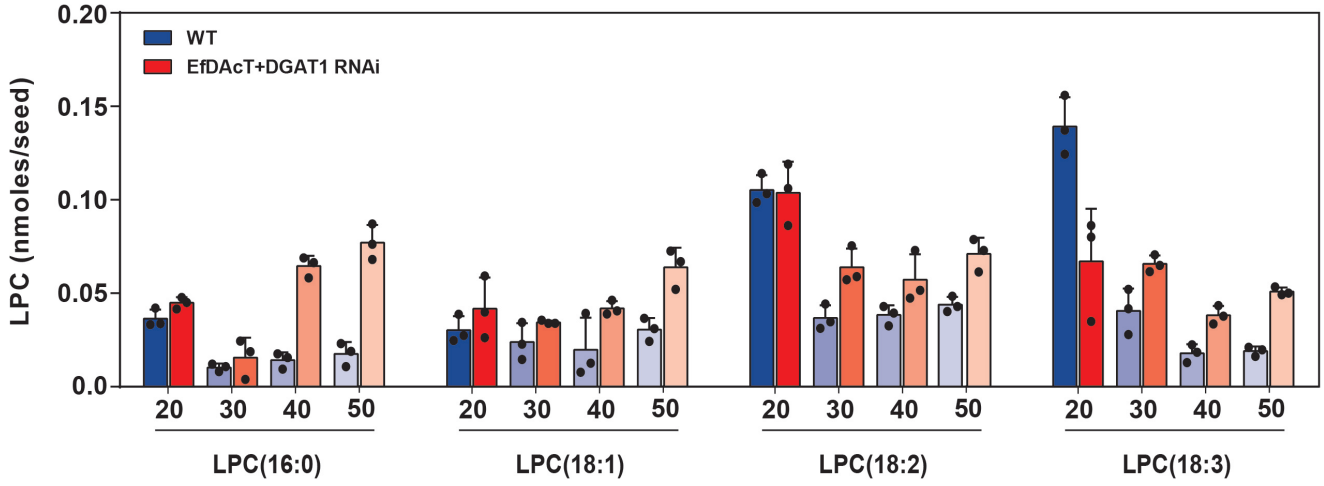
Supplementary Figure C.3. Distribution of DAG molecular species across seed development.

The content of different DAG molecular species in camelina wild-type (WT) seeds and homozygous transgenic seeds expressing *EfDAcT+DGAT1 RNAi*. Each molecular species is annotated as (X:Y), where X represents the total carbon number, and Y indicates the total number of double bonds. The last panel shows the sum of all DAG molecular species containing a specific fatty acid. The developmental stages are denoted by 20, 30, 40, and 50. Data represent mean ± standard error, n=3 biological reps. DAG, diacylglycerol.

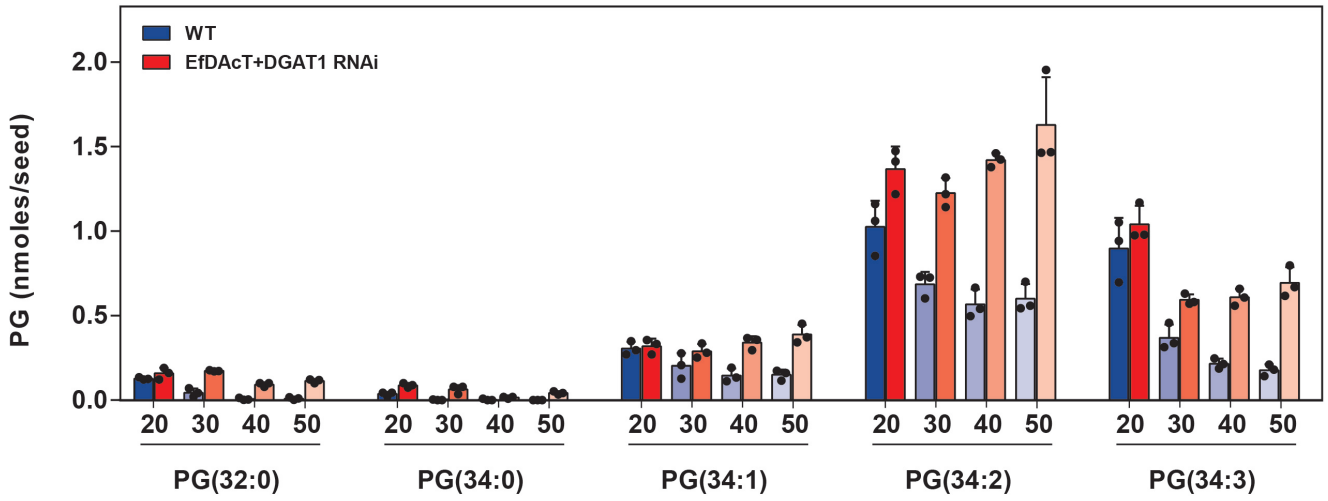


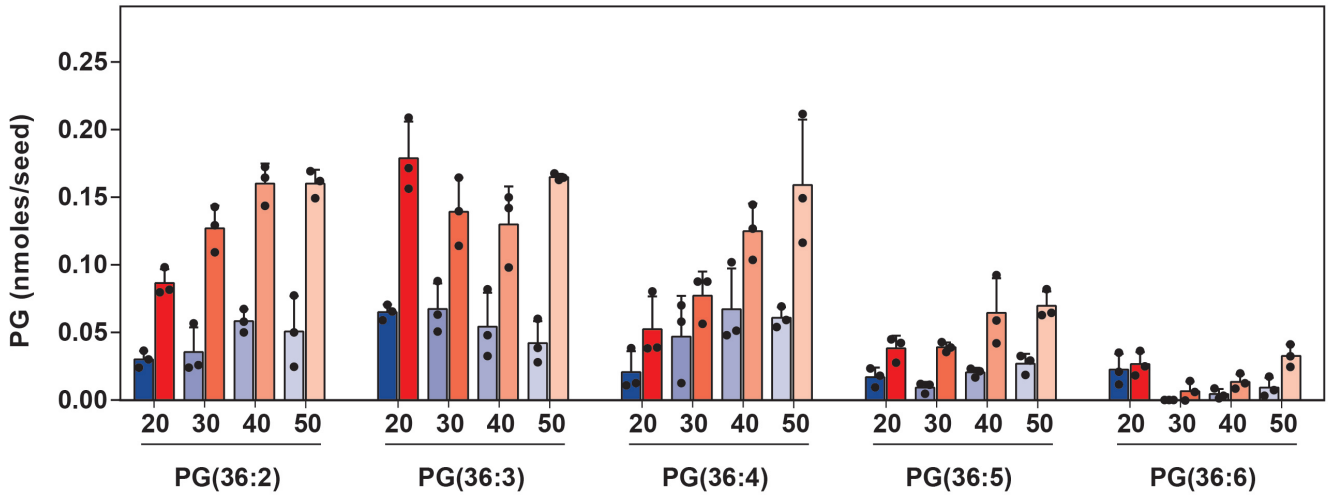
Supplementary Figure C.4. Distribution of PC molecular species across seed development.
The content of different PC molecular species in camelina wild-type (WT) seeds and homozygous transgenic seeds expressing *EfDAcT+DGAT1 RNAi*. Each molecular species is annotated as (X:Y), where X represents the total carbon number, and Y indicates the total number of double bonds. The developmental stages are denoted by 20, 30, 40, and 50. Data represent mean ± standard error, n=3 biological reps. PC, phosphatidylcholine.

a) LPC


WT
EfDAcT+DGAT1 RNAi
LPC (nmoles/seed)
0.20
0.15
0.10
0.05
0.0
20 30 40 50
LPC(16:0)
LPC(18:1)
LPC(18:2)
LPC(18:3)

b) PG

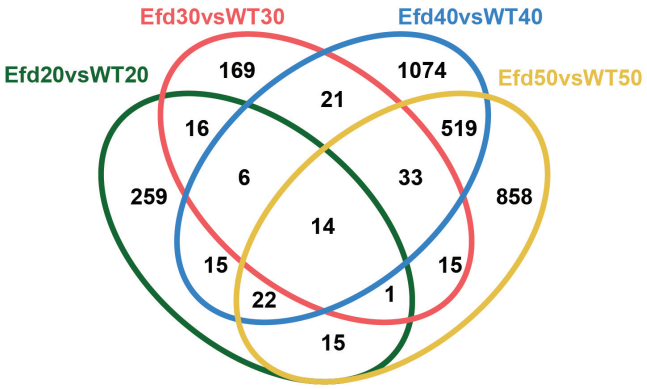

WT
EfDAcT+DGAT1 RNAi
PG (nmoles/seed)
2.0
1.5
1.0
0.5
0.0
20 30 40 50
PG(32:0)
PG(34:0)
PG(34:1)
PG(34:2)
PG(34:3)


PG (nmoles/seed)
0.25
0.20
0.15
0.10
0.05
0.00
20 30 40 50
PG(36:2)
PG(36:3)
PG(36:4)
PG(36:5)
PG(36:6)

Supplementary Figure C.5. Distribution of LPC and PG molecular species across seed development.

The content of different **(a)** LPC and **(b)** PG molecular species in camelina wild-type (WT) seeds and homozygous transgenic seeds expressing *EfDAcT+DGAT1 RNAi*. Each molecular species is annotated as (X:Y), where X represents the total carbon number, and Y indicates the total number of double bonds. The developmental stages are denoted by 20, 30, 40, and 50. Data represent mean ± standard error, n=3 biological reps. LPC, lysophosphatidylcholine; PG, phosphatidylglycerol.

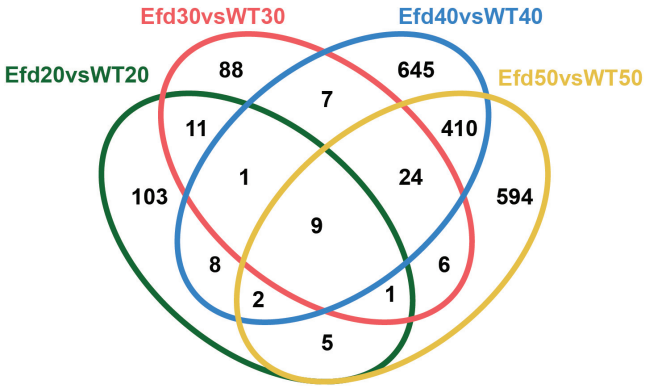
a) Upregulated DEG



List of upregulated DEG common in all stages

Camelina Gene ID	Arabidopsis homologue	Description	Biological Function
-	-	EfDAcT- *Euonymus Fortune* Diacylglycerol Acetyl-Coa Acetyltransferases	Synthesis of acetyl-TAG
-	-	Dsred- *Discosoma* Sp. Red Fluorescent Protein	Fluorescent marker
CSA03G029830	AT1G25310	Maternal Effect Embryo Arrest 8 (MEE8)	Embryo development ending in seed dormancy
CSA11G002140	AT4G39180	Secretion 14 (Sec14)	Phosphatidylcholine biosynthetic process, phospholipid transport
CSA08G052090	AT4G02380	Late Embryogenesis Abundant 38	Cellular response to water deprivation, response to cold, response to oxidative stress
CSA11G070940	AT5G13790	Agamous-Like 15	Embryo development ending in seed dormancy, floral organ abscission, gibberellin catabolic process
CSA00382S230	AT3G45140	Lipoxygenase 2	Response to fungus, response to Jasmonic acid, response to wounding
CSA11G074260 CSA12G072080	AT2G12190 AT5G36220	Cytochrome P450 Superfamily Proteins	Response to abscisic acid, Response to oxidative stress
CSA15G018530	AT1G54030	Endoplasmic Reticulum Morphology 3	Endoplasmic reticulum organization, intracellular protein transport
CSA17G095230	AT1G56130	Impaired in Glycan Perception 2	Protein phosphorylation
CSA12G081270	AT3G48960	Ribosomal Protein El13y	Translation
CSA12G081270	AT3G61111	Ribosomal Protein Es27x	Ribosomal small subunit assembly, translation
CSA09G039770	-	Unannotated	

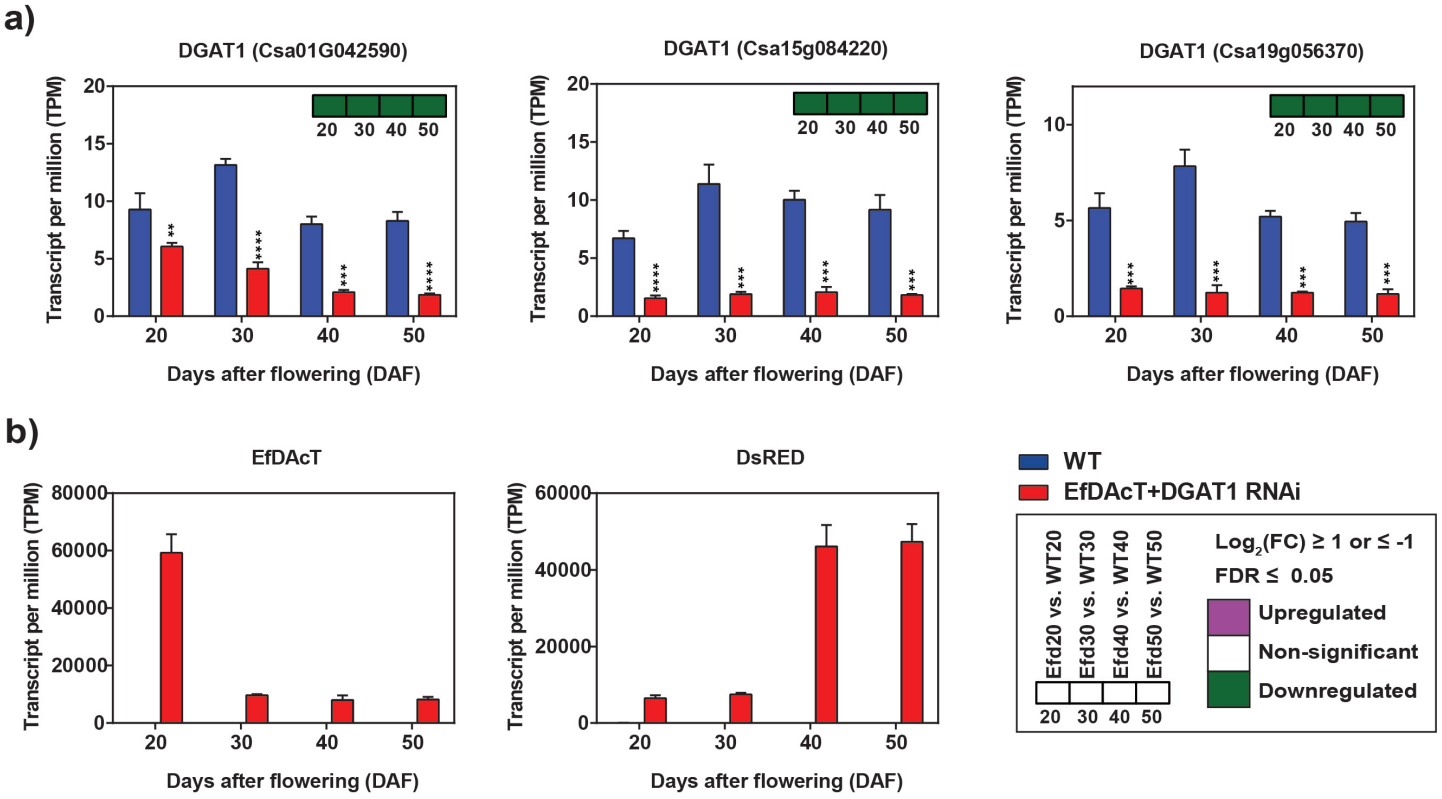
b) Downregulated DEG



List of downregulated DEG common in all stages

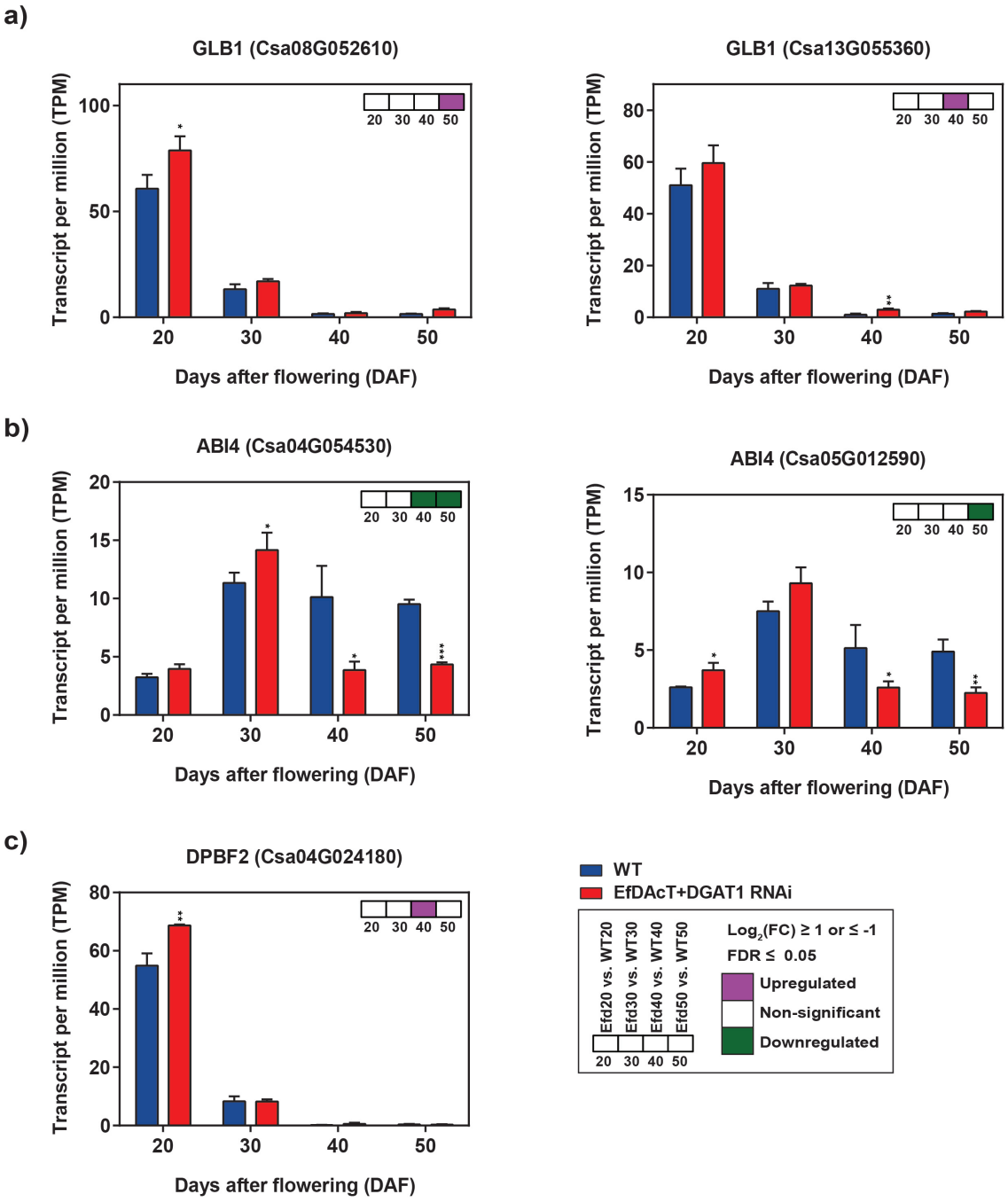
Camelina Gene ID	Arabidopsis homologue	Description	Biological Function
CSA01G042590 CSA15G084220 CSA19G056370	AT2G19450	Diacylglycerol Acyltransferase1 (DGAT1)	TAG synthesis
CSA02G007770 CSA04649S010	AT3G21790	UDP-Glycosyltransferase Superfamily Protein	Response to lipid, response to water deprivation, Response to abscisic acid
CSA17G075410	AT1G47980	Unknown Protein	Desiccation-like protein, Response to abscisic acid
CSA01G042590	AT1G68670	Nitrate Inducible 1	Phosphate ion homeostasis, Response to abscisic acid
CSA04G030290	AT3G47420	Glycerol-3-Phosphate Permease 1	Phosphate ion homeostasis
CSA03G006330	AT1G05000	Atypical Dual-Specificity Phosphatase 1	Dephosphorylation

Supplementary Figure C.6. DEGs identified in in transgenic seeds across seed development. Venn diagram illustrating the overlap of **(a)** upregulated or **(b)** downregulated differentially expressed genes (DEGs), meeting criteria of FDR ≤ 0.05 and fold change ≥ 2, in homozygous transgenic camelina seeds expressing *EfDAcT+DGAT1 RNAi* compared to the WT. The tables present the list of DEGs shared across all developmental stages. Numerical values following Efd and WT indicate the respective seed development stage. WT, wild-type; Efd, *EfDAcT+DGAT1 RNAi*.



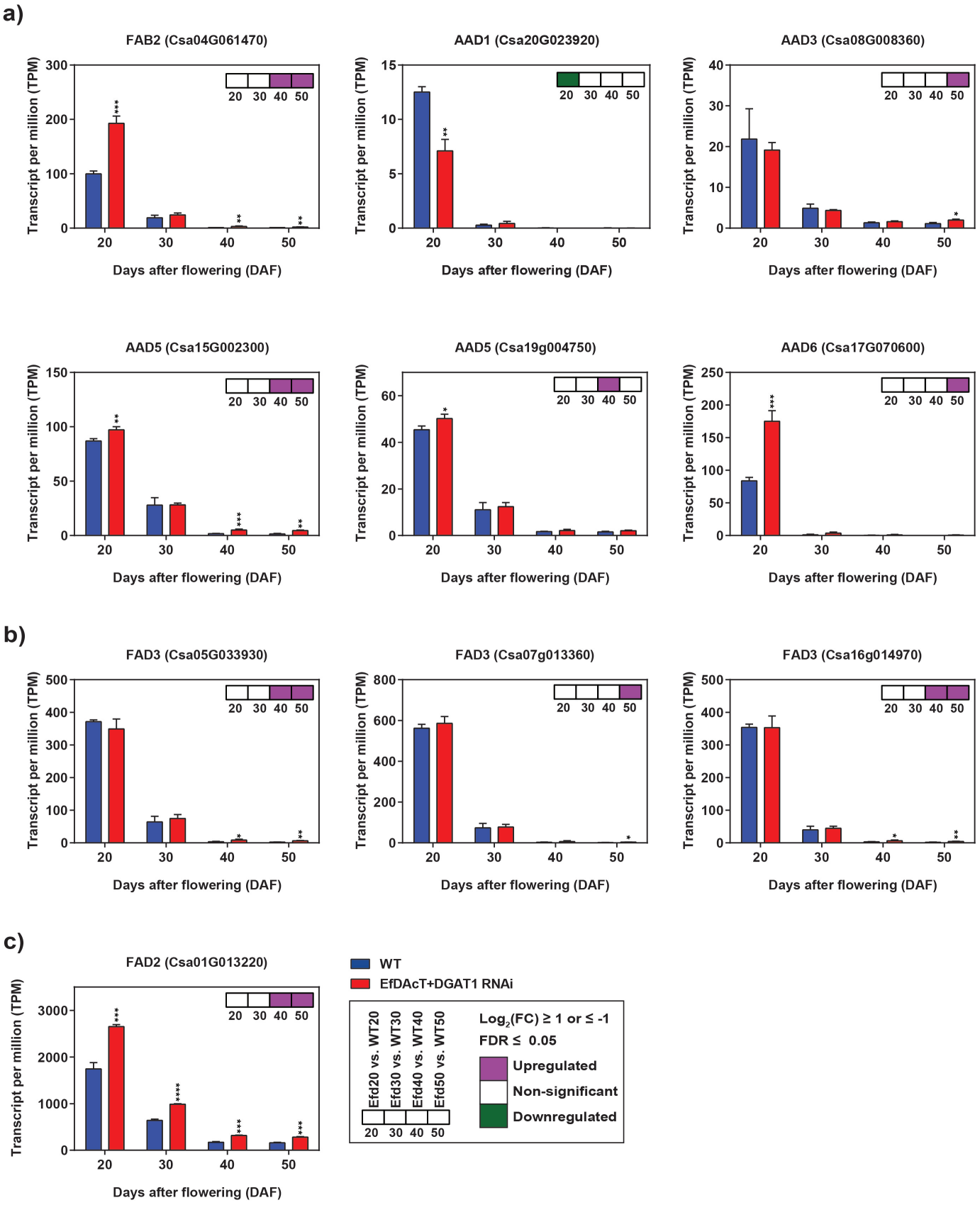
Supplementary Figure C.7. Expression profiles of DGAT1, EfDAcT, and DsRED in WT and transgenic seeds.

Expression profiles of **(a)** *DGAT1*, **(b)** *EfDAcT* and *DsRED* in the developing seeds of camelina wild-type (WT) and homozygous transgenic seeds expressing *EfDAcT+DGAT1 RNAi*. Bars represent the mean TPM (Transcripts Per Million) value of 3 biological replicates ± standard error. Asterisks indicate statistical difference from WT seeds (Student t-test; ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$). Boxes denote upregulation (purple) or downregulation (green) based on an FDR ≤ 0.05 and fold change ≥ 2 at 20, 30, 40, and 50 days after flowering (DAF) from left to right. DGAT1, diacylglycerol acyltransferase 1; EfDAcT, *Euonymus fortunei* diacylglycerol acetyltransferase; *DsRED*, Discosoma red fluorescent protein.



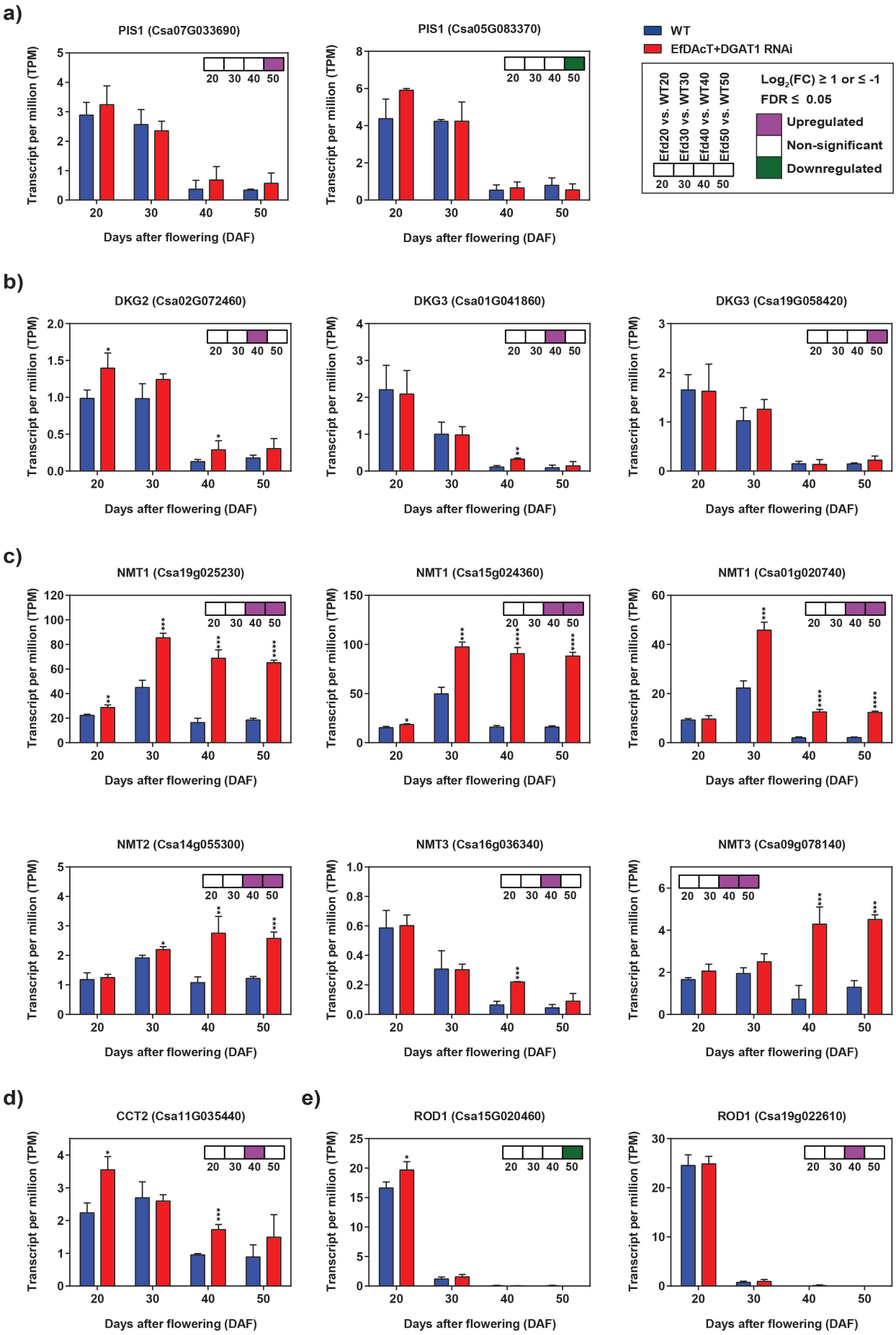
Supplementary Figure C.8. Expression profiles of regulatory genes related to lipid metabolism in WT and transgenic seeds.

Expression profiles of **(a)** *GLB1*, **(b)** *ABI4* and **(c)** *DPBF2* in the developing seeds of camelina wild-type (WT) and homozygous transgenic seeds expressing *EfDAcT+DGAT1 RNAi*. Bars represent the mean TPM (Transcripts Per Million) value of 3 biological replicates ± standard error. Asterisks indicate statistical difference from WT seeds (Student t-test; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$). Boxes denote upregulation (purple) or downregulation (green) based on an FDR ≤ 0.05 and fold change ≥ 2 at 20, 30, 40, and 50 days after flowering (DAF) from left to right. ABI4, ABA insensitive 4*; DPBF2, DC3 promoter-binding factor 2*.


a)
FAB2 (Csa04G061470)
AAD1 (Csa20G023920)
AAD3 (Csa08G008360)
AAD5 (Csa15G002300)
AAD5 (Csa19g004750)
AAD6 (Csa17G070600)
b)
FAD3 (Csa05G033930)
FAD3 (Csa07g013360)
FAD3 (Csa16g014970)
c)
FAD2 (Csa01G013220)
Transcript per million (TPM)
Days after flowering (DAF)
20 30 40 50
WT
EfDAcT+DGAT1 RNAi
Efd20 vs. WT20
Efd30 vs. WT30
Efd40 vs. WT40
Efd50 vs. WT50
Log2(FC) ≥ 1 or ≤ -1
FDR ≤ 0.05
Upregulated
Non-significant
Downregulated

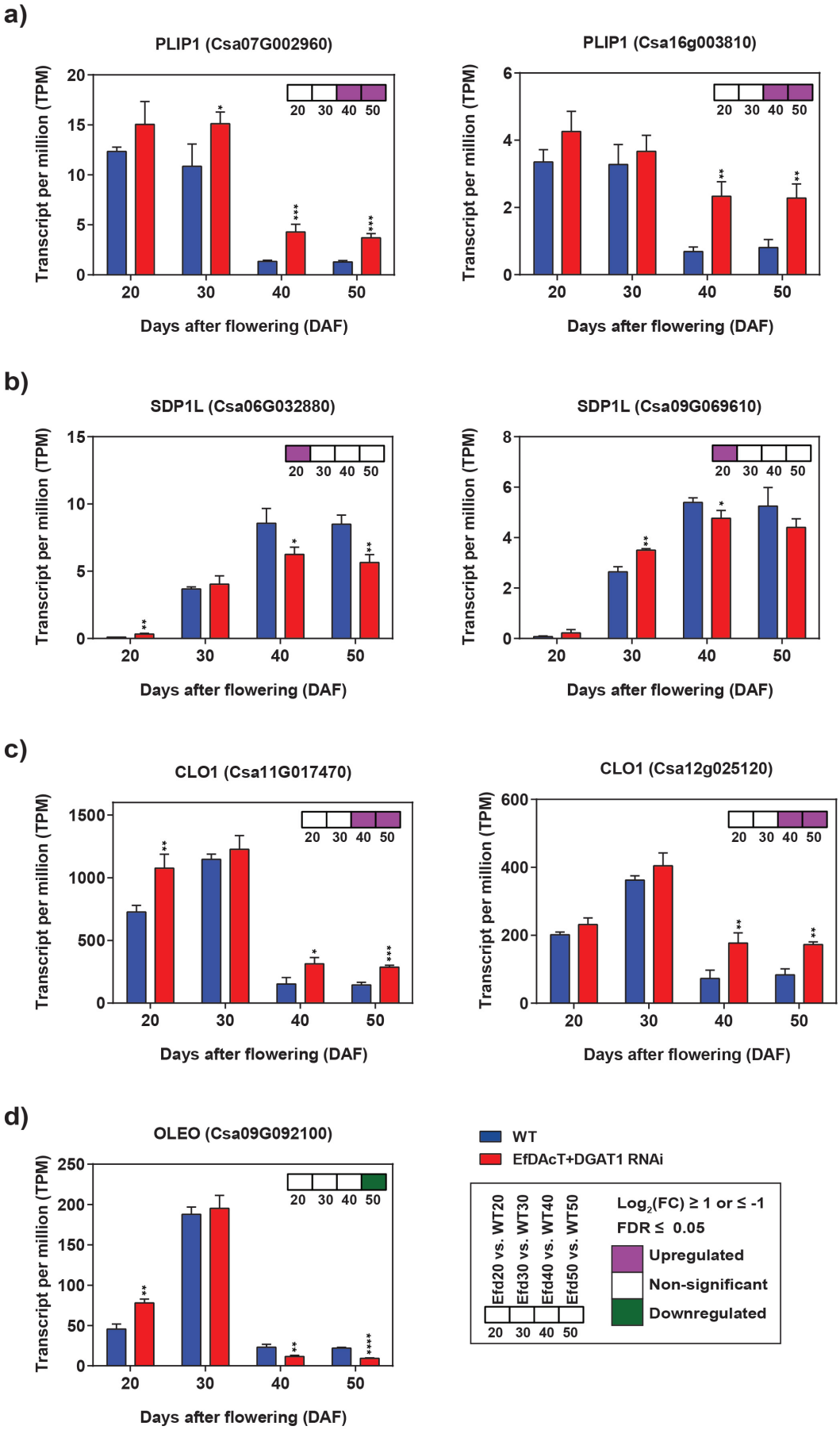
Supplementary Figure C.9. Expression profiles of genes related to fatty acid desaturation in WT and transgenic seeds.

Expression profiles of **(a)** *stearoyl-ACP desaturases* (*FAB2, AAD1,3,5,* and *6)*, **(b)** *FAD3* and **(c)** *FAD2* in the developing seeds of camelina wild-type (WT) and homozygous transgenic seeds expressing *EfDAcT+DGAT1 RNAi*. Bars represent the mean TPM (Transcripts Per Million) value of 3 biological replicates ± standard error. Asterisks indicate statistical difference from WT seeds (Student t-test; ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$). Boxes denote upregulation (purple) or downregulation (green) based on an FDR ≤ 0.05 and fold change ≥ 2 at 20, 30, 40, and 50 days after flowering (DAF) from left to right. FAB, fatty acid biosynthesis; AAD, Acyl-ACP desaturase; FAD, fatty acid desaturase.


a)
PIS1 (Csa07G033690)
PIS1 (Csa05G083370)
WT
EfDAcT+DGAT1 RNAi
Efd20 vs. WT20
Efd30 vs. WT30
Efd40 vs. WT40
Efd50 vs. WT50
Log2(FC) ≥ 1 or ≤ -1
FDR ≤ 0.05
Upregulated
Non-significant
Downregulated
b)
DKG2 (Csa02G072460)
DKG3 (Csa01G041860)
DKG3 (Csa19G058420)
c)
NMT1 (Csa19g025230)
NMT1 (Csa15g024360)
NMT1 (Csa01g020740)
NMT2 (Csa14g055300)
NMT3 (Csa16g036340)
NMT3 (Csa09g078140)
d)
CCT2 (Csa11G035440)
e)
ROD1 (Csa15G020460)
ROD1 (Csa19g022610)
Transcript per million (TPM)
Days after flowering (DAF)
20 30 40 50

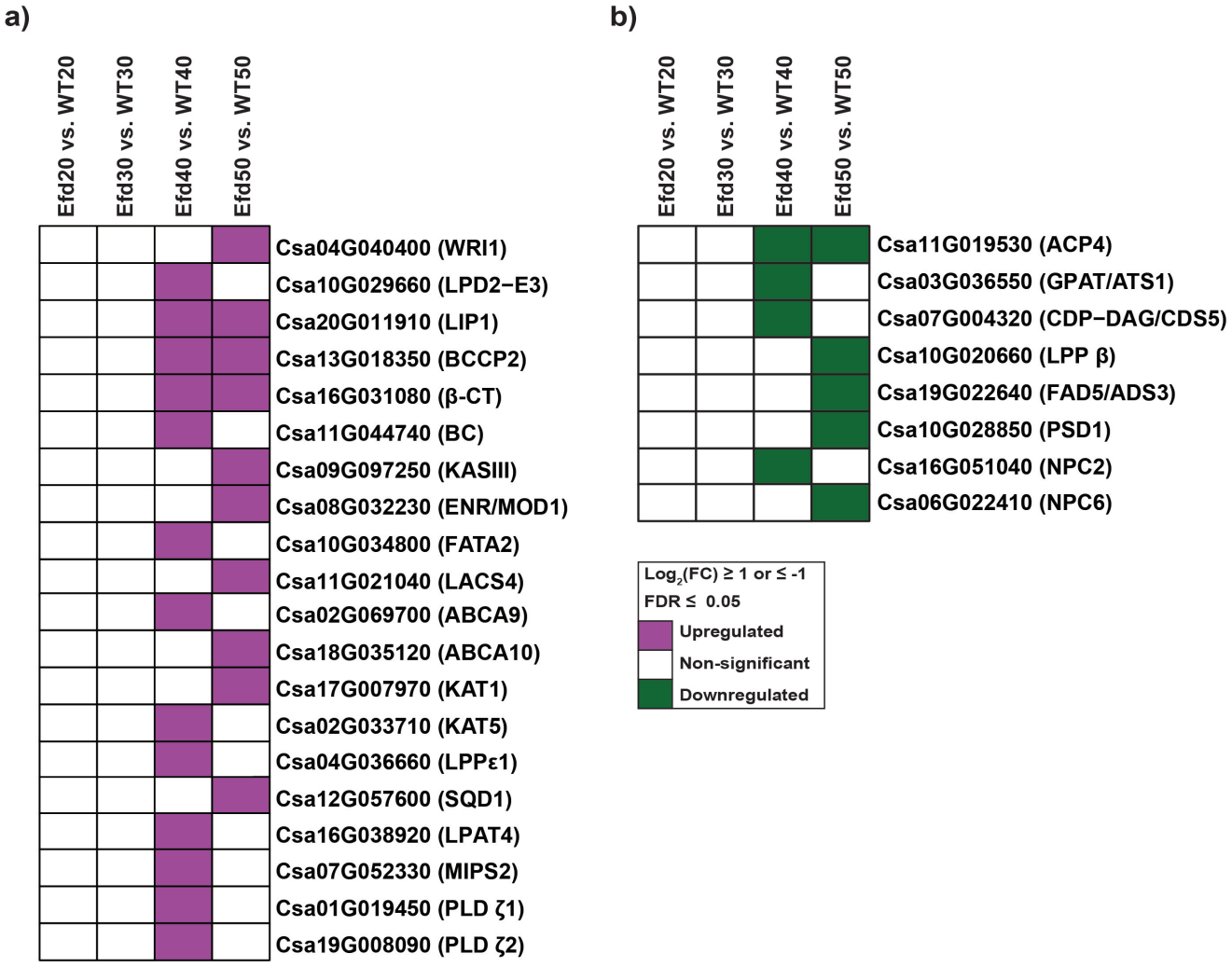
Supplementary Figure C.10. Expression profiles of genes related to phospholipid metabolism in WT and transgenic seeds.

Expression profiles of **(a)** *PIS1*, **(b)** *DKG*, **(c)** *NMT,* **(d)** *CCT2,* and **(e)** *ROD1* in the developing seeds of camelina wild-type (WT) and homozygous transgenic seeds expressing *EfDAcT+DGAT1 RNAi*. Bars represent the mean TPM (Transcripts Per Million) value of 3 biological replicates ± standard error. Asterisks indicate statistical difference from WT seeds (Student t-test; ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$). Boxes denote upregulation (purple) or downregulation (green) based on an FDR ≤ 0.05 and fold change ≥ 2 at 20, 30, 40, and 50 days after flowering (DAF) from left to right. PIS, phosphatidylinositol synthase; DKG, diacylglycerol kinase; NMT, N-methyltransferase; CCT, CTP:phosphorylcholine cytidylyltransferase; *ROD1, reduced oleate desaturase 1*.


a)
PLIP1 (Csa07G002960)
PLIP1 (Csa16g003810)
b)
SDP1L (Csa06G032880)
SDP1L (Csa09G069610)
c)
CLO1 (Csa11G017470)
CLO1 (Csa12g025120)
d)
OLEO (Csa09G092100)
Transcript per million (TPM)
Days after flowering (DAF)
20 30 40 50
WT
EfDAcT+DGAT1 RNAi
Efd20 vs. WT20
Efd30 vs. WT30
Efd40 vs. WT40
Efd50 vs. WT50
Log2(FC) ≥ 1 or ≤ -1
FDR ≤ 0.05
Upregulated
Non-significant
Downregulated

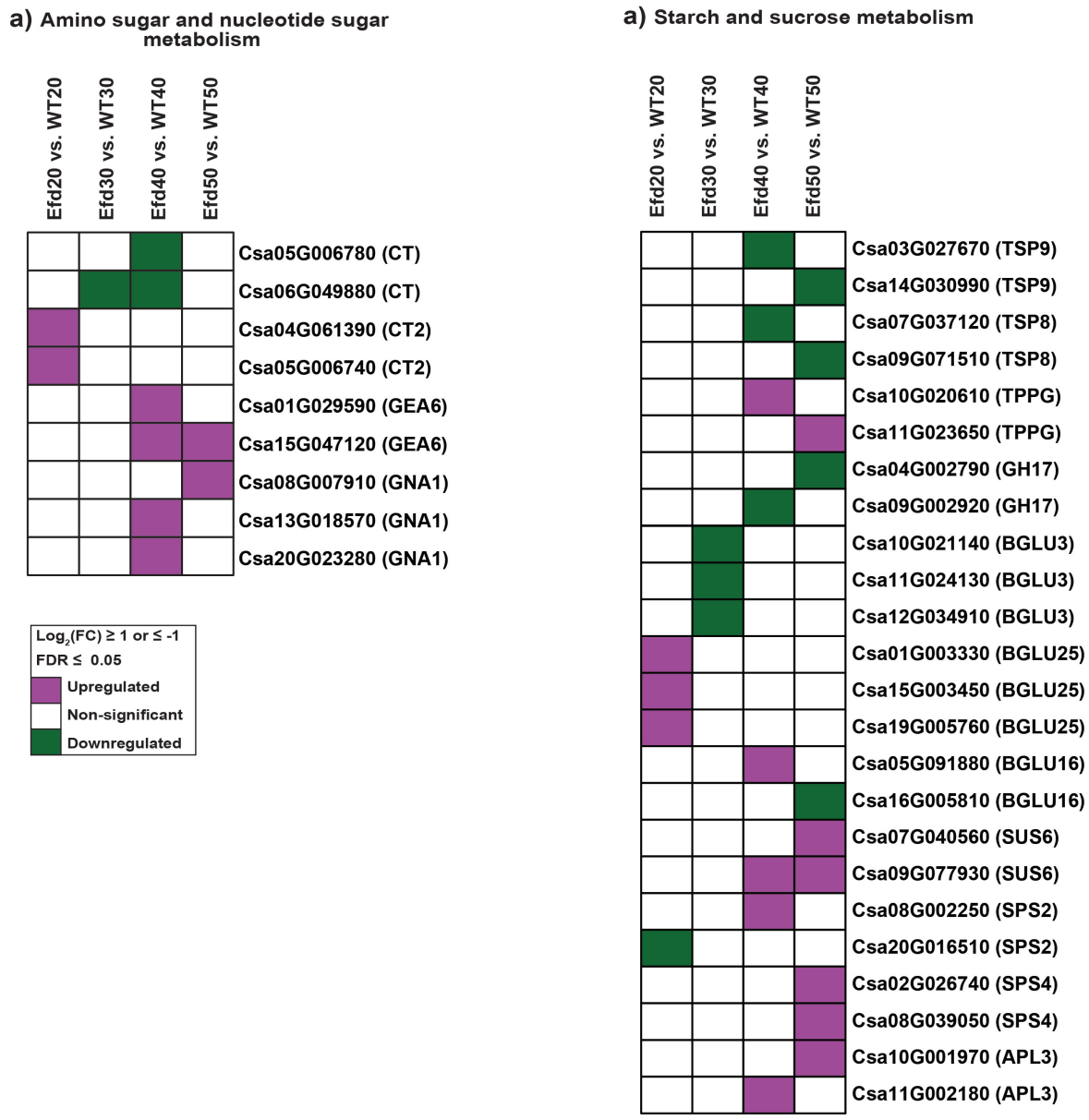
Supplementary Figure C.11. Expression profiles of genes related to lipases and lipid droplet proteins in WT and transgenic seeds.

Expression profiles of **(a)** *SDP1L*, **(b)** *CLO* and **(c)** *OLEO* in the developing seeds of camelina wild-type (WT) and homozygous transgenic seeds expressing *EfDAcT+DGAT1 RNAi*. Bars represent the mean TPM (Transcripts Per Million) value of 3 biological replicates ± standard error. Asterisks indicate statistical difference from WT seeds (Student t-test; ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$). Boxes denote upregulation (purple) or downregulation (green) based on an FDR ≤ 0.05 and fold change ≥ 2 at 20, 30, 40, and 50 days after flowering (DAF) from left to right. **SDP1L**, sugar-dependent 1-like; **OLEO**, oleosin **; CLO1**, caleosin 1.



Supplementary Figure C.12. Differential expression of genes related to fatty acid and lipid metabolism.

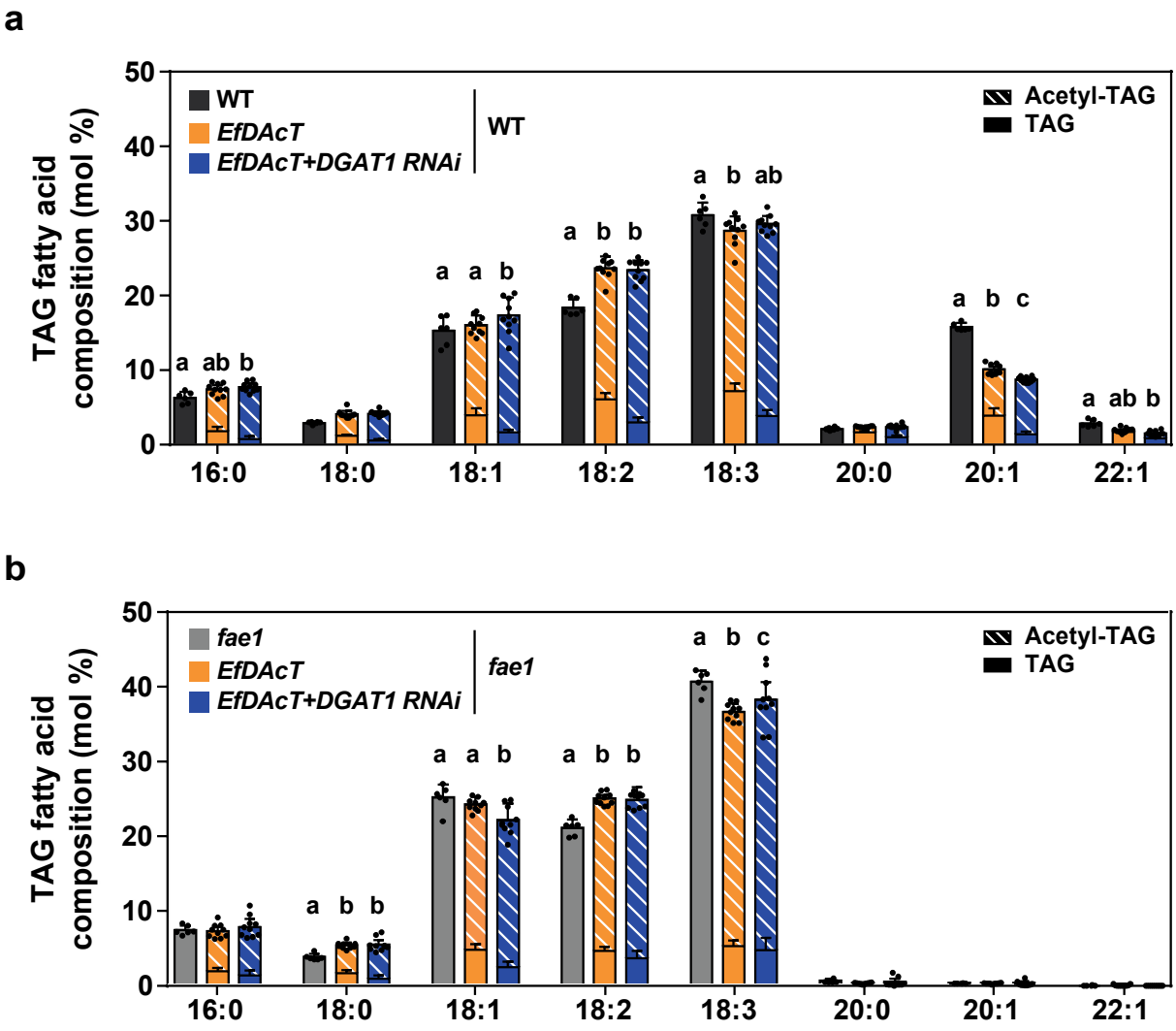
(a) upregulated and **(b)** downregulated differentially expressed genes (DEGs) involved in fatty acid or lipid metabolism in only one camelina homeolog. Boxes denote upregulation (purple) or downregulation (green) based on an FDR ≤ 0.05 and fold change ≥ 2 at 20, 30, 40, and 50 days after flowering (DAF) from left to right. Numerical values following Efd and WT indicate the respective seed development stage. WT, wild-type; Efd, EfDAcT+DGAT1 RNAi; WRI1, wrinkled 1; LPD2-E3, dihydrolipoamide dehydrogenase2-E3 component; LIP1, lipoate synthase 1; BCCP, biotin carboxyl carrier protein; β-CT, β-carboxyltransferase; BC, biotin carboxylase; KAS, ketoacyl-ACP synthase; ENR1, ENOYL-ACP REDUCTASE 1; FATA2, acyl-ACP *thioesterase A2;* LACS, long chain acyl-CoA synthetase; ABCA, ATP-binding cassette (ABC) transporter subfamily A; KAT, 3-keto-acyl-CoA thiolase; LPP, lipid phosphate phosphatase; SQD1, SQDG synthase 1; LPAT4, lysophosphatidic acid acyltransferase 4; MIPS2, myo-inositol-1-phosphate synthase 2; PLD, phospholipase D; ACP4, acyl carrier protein 4; *GPAT;* glycerol-3-phosphate acyltransferase; CDS5, CDP-DAG synthase 5; FAD5, fatty acid desaturase 5; PSD1, phosphatidylserine decarboxylase 1; NPC, non-specific phospholipase C.



Supplementary Figure C.13. Differential expression of genes related to significant pathways identified from KEGG analysis.

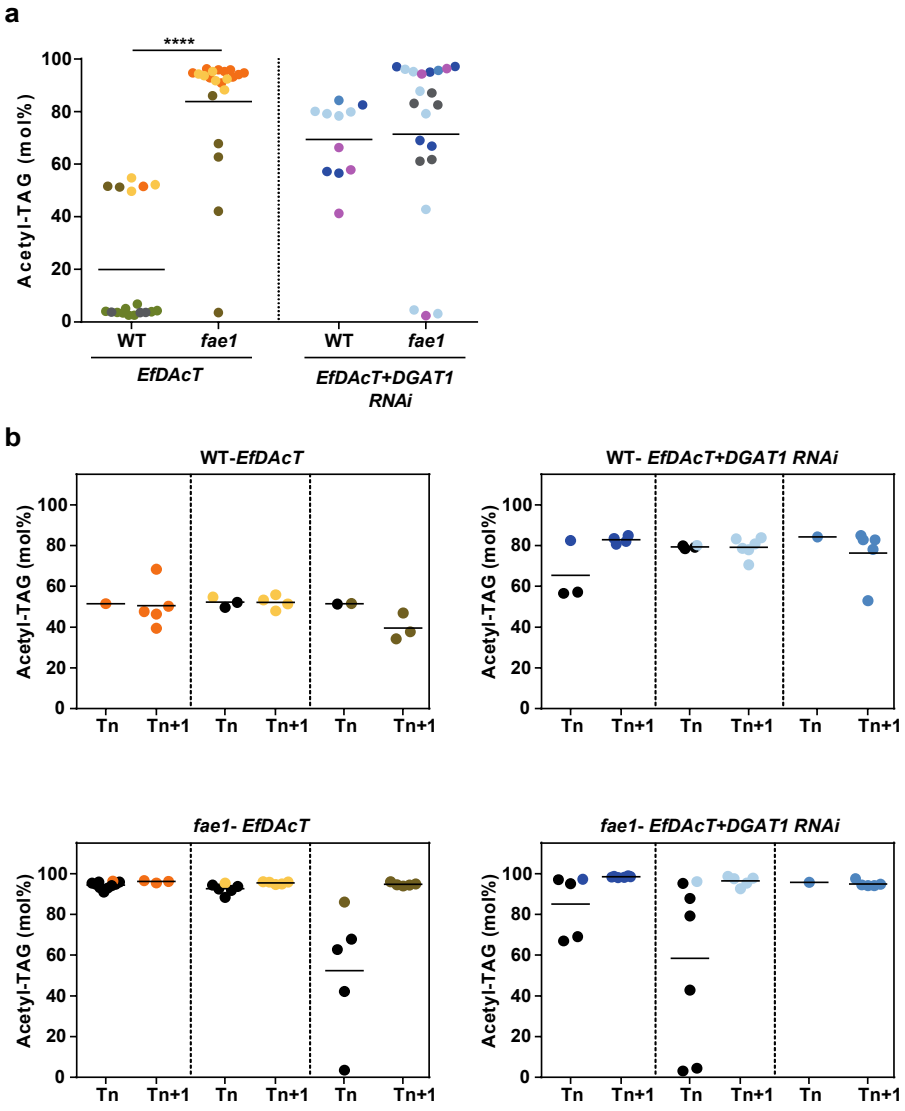
Differentially expressed genes (DEGs) involved in **(a)** amino sugar and nucleotide sugar metabolism, and **(b)** starch and sucrose metabolism in more than one camelina homeolog. Boxes denote upregulation (purple) or downregulation (green) based on an FDR ≤ 0.05 and fold change ≥ 2 at 20, 30, 40, and 50 days after flowering (DAF) from left to right. Numerical values following Efd and WT indicate the respective seed development stage. WT, wild-type; Efd, EfDAcT+DGAT1 RNAi; CT, Chitinase family protein; GEA6, UDP-D-glucuronate 4-epimerase 6; GNA1, glucose-6-phosphate acetyltransferase 1; TSP9, trehalose -6-phosphatase synthase S9; TPPG, trehalose-phosphate phosphatase G; GH17, O-Glycosyl hydrolases family 17 protein; BGLU, beta glucosidase; SUS6, sucrose synthase 6; SPS, sucrose phosphate synthase; APL3, glucose-1-phosphate adenylyltransferase large subunit 3.

Appendix D - Chapter 4 Supplementary Materials



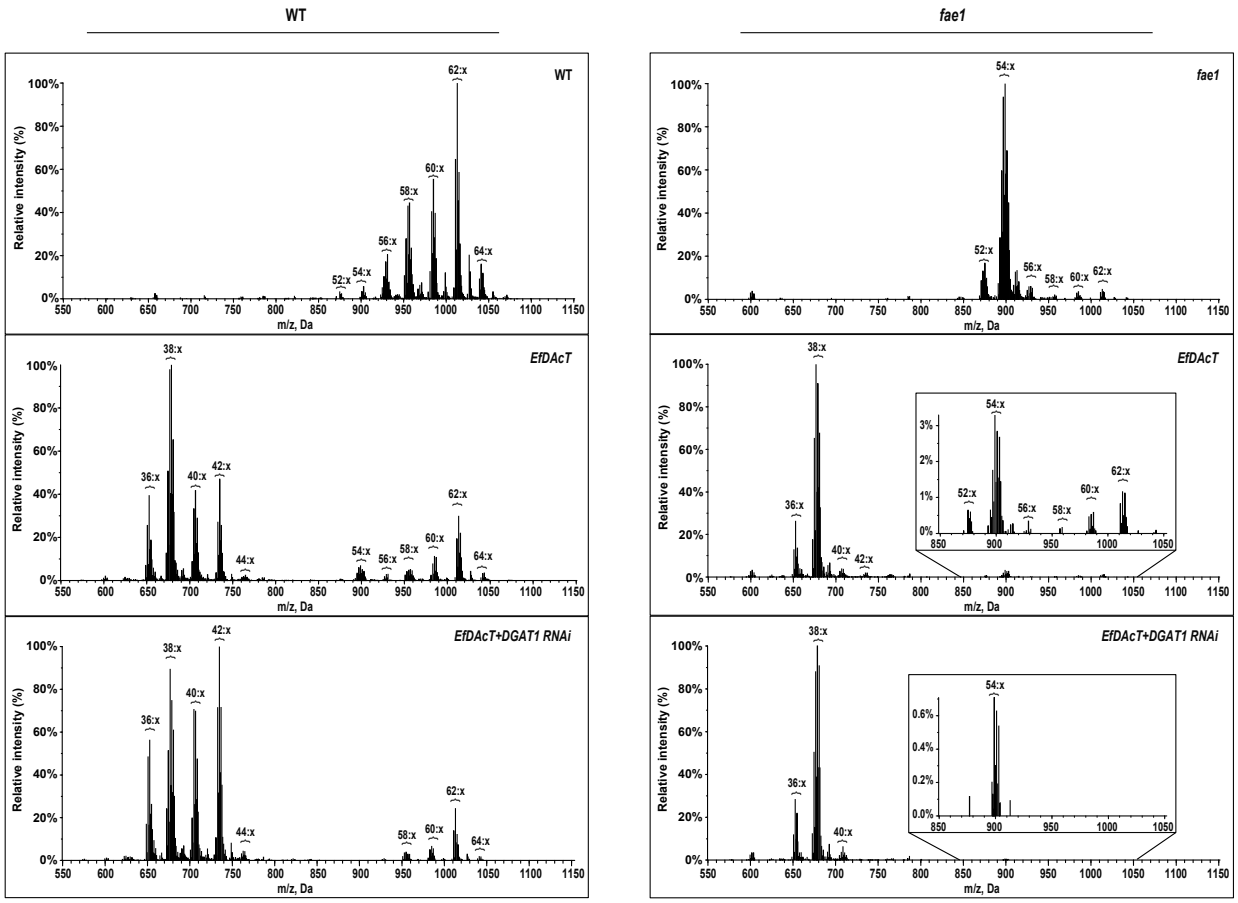
Supplementary Figure D.1. Seed TAG fatty acid composition of transgenic camelina wild-type and *fae1* lines

(a-b) Fatty acid composition of acetyl-TAG and regular TAG fractions of homozygous transgenic lines expressing *EfDAcT* or *EfDAcT+DGAT1-RNAi* in wild-type seeds **(a)** and *fae1* seeds **(b)**. Data represent mean ± standard error of two independent transgenic lines with ≥ 3 biological replicates each. Different letters indicate statistical difference between genotypes (One-way ANOVA followed by Tukey's multiple comparisons test; $p \leq 0.05$).

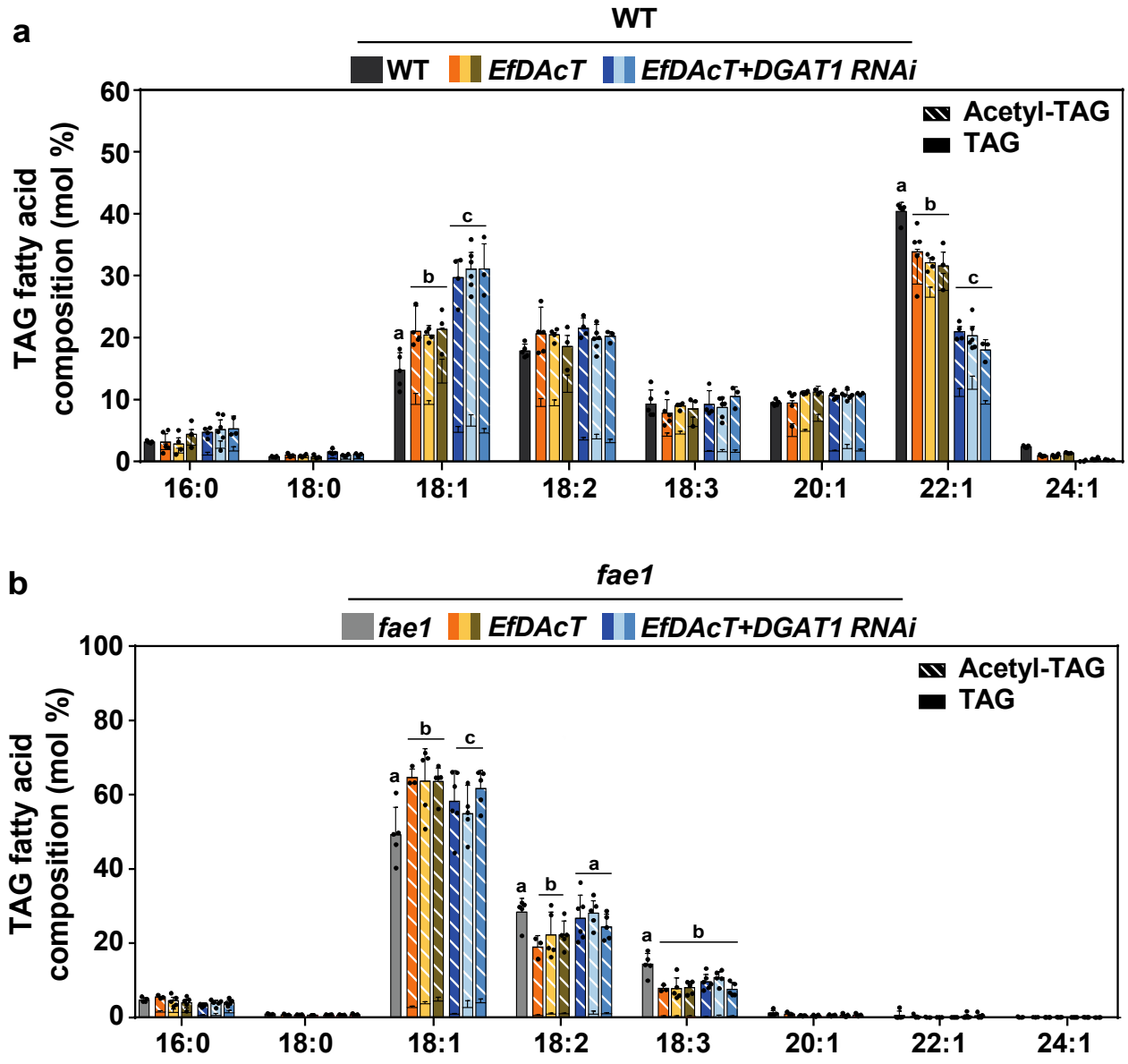


Supplementary Figure D.2. Enhanced acetyl-TAG accumulation in transgenic *fae1* pennycress seeds is stable in different generations.

(a) Scatter plot of acetyl-TAG content in homozygous transgenic wild-type and *fae1* lines expressing *EfDAcT* or *EfDAcT+DGAT1 RNAi*. Some lines were grown on different dates. **(b)** Tracking of acetyl-TAG levels in homozygous transgenic lines expressing *EfDAcT* or *EfDAcT+DGAT1 RNAi* in wild-type or *fae1* seeds. T_{n+1} represents the subsequent seed generation that follows T_n. Each independent line is represented by a distinct color. Black data points represent acetyl-TAG levels in seed from sibling plants in the T_n generation. Horizontal lines represent mean values, n= ≥ 1 biological reps. Asterisk indicate statistical difference from wild-type seeds (One-way ANOVA followed by Sidak's multiple comparisons test; **** $p \leq 0.0001$).

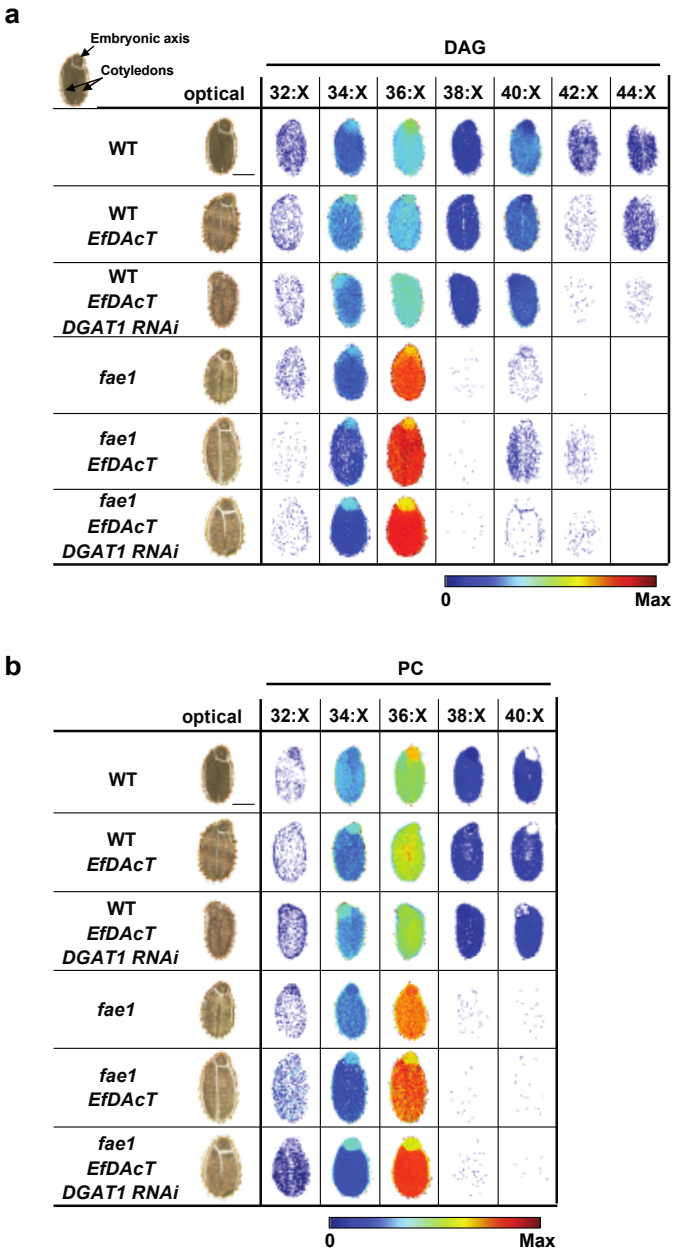


Supplementary Figure D.3. ESI-MS spectra of neutral lipids from pennycress mature seeds. Positive ESI-MS spectra of neutral lipids from wild-type, *fae1* mutant, and homozygous pennycress transgenic lines expressing *EfDAcT* or *EfDAcT+DGAT1 RNAi.* Signal peaks represent *m*/*z* values of the $[M + NH_4]^+$ adduct. The number of acyl carbons in each series of acetyl-TAG and TAG molecular species is indicated; the number of double bonds (x) is not defined for clarity.

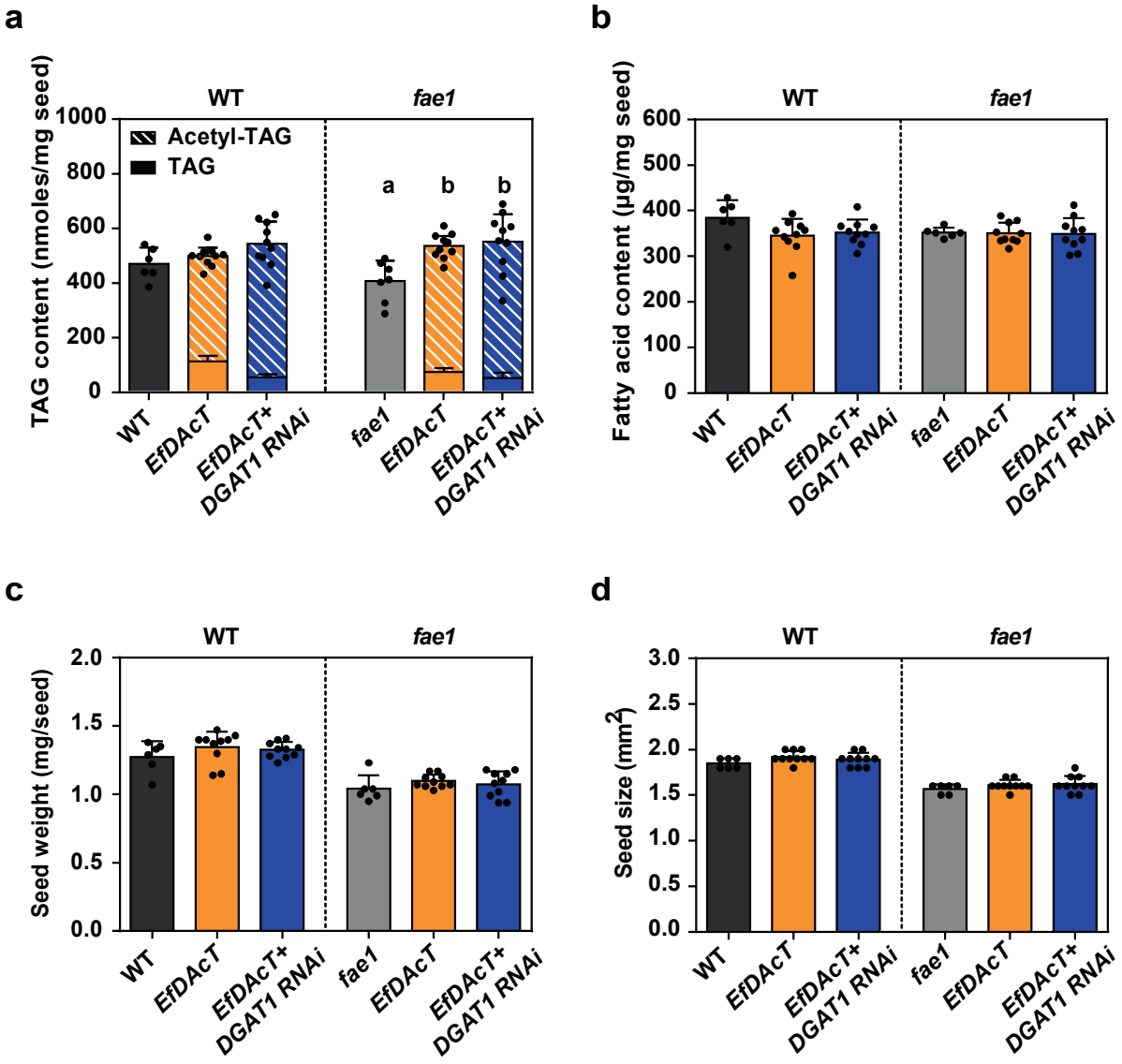


Supplementary Figure D.4. Seed TAG fatty acid composition of transgenic pennycress wild-type and *fae1* lines.

(a-b) Fatty acid composition of acetyl-TAG and regular TAG fractions of homozygous transgenic lines expressing *EfDAcT* or *EfDAcT+DGAT1-RNAi* in wild-type seeds **(a)** and *fae1* seeds **(b)**. Data represent mean ± standard error, n=≥ 3 biological reps. Each independent line is represented by a distinct color. Different letters indicate statistical difference between genotypes (One-way ANOVA followed by Tukey's multiple comparisons test; $p \leq 0.05$).

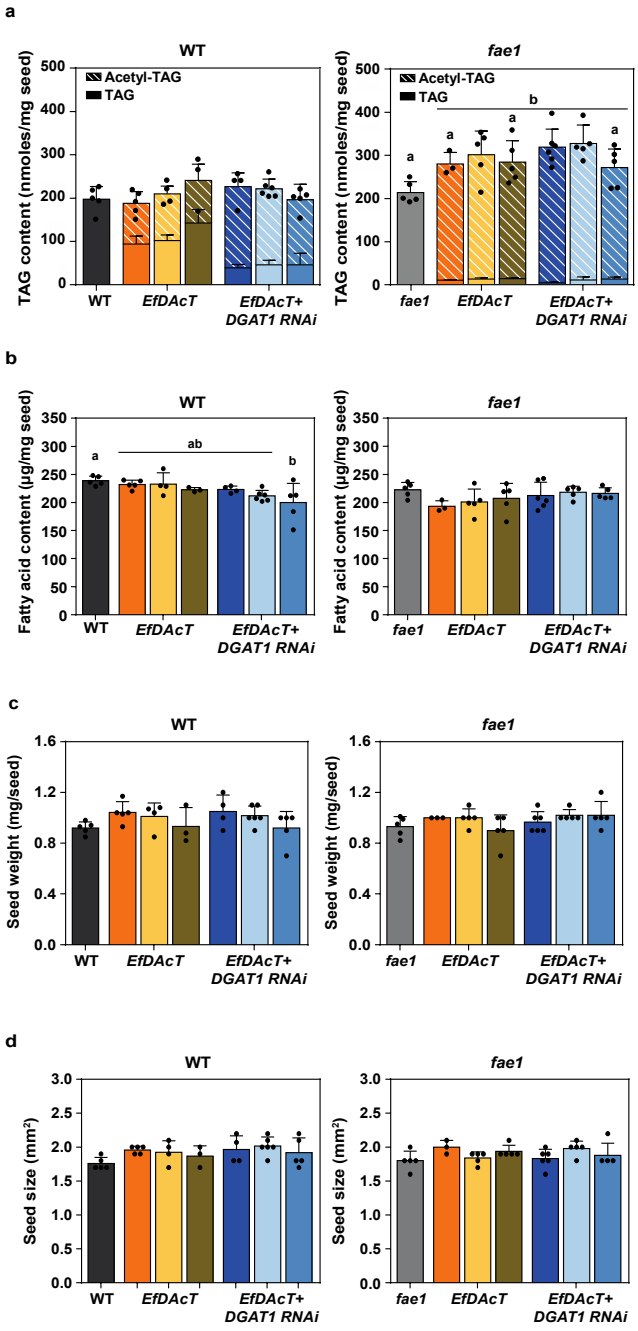


Supplementary Figure D.5. MALDI-MS imaging of DAG and PC in pennycress mature seeds. MALDI-MS images of DAG **(a)** and PC **(b)** potassium adducts $[M+K]^+$ in mature pennycress seeds from wild-type, *fae1* mutant, and homozygous pennycress transgenic lines expressing *EfDAcT* or *EfDAcT+DGAT1 RNAi.* Images shown are summed ion images of DAG **(a)** or PC **(b)** molecular species with the same number of acyl carbons and different number of double bonds (x). Normalized ion intensities are shown using a jet color scale, with red representing 100% of the DAG **(a)** or PC **(b)** signal. The scale bar represents 500 µm. Optical images are included for reference.



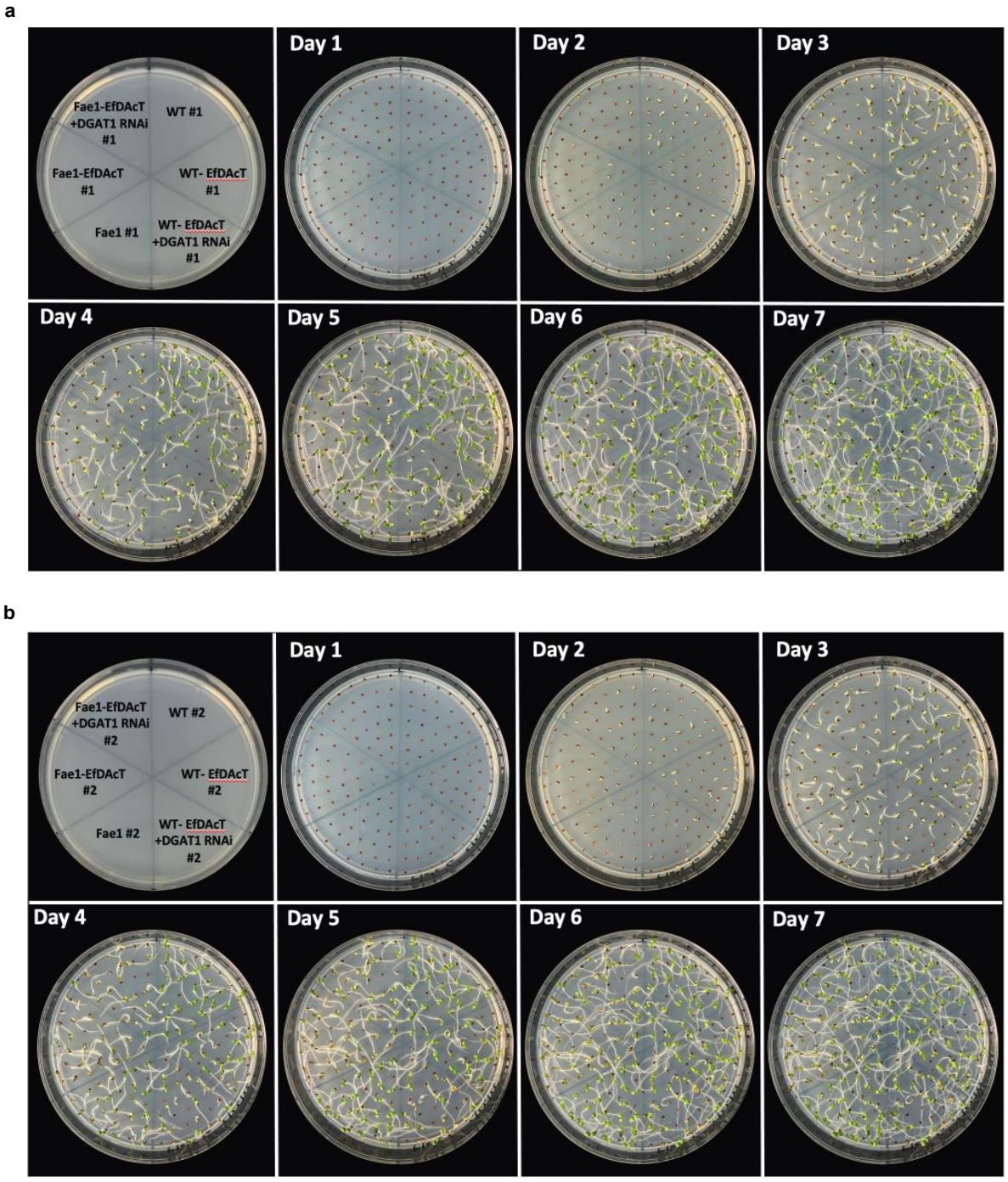
Supplementary Figure D.6. Analysis of seed traits in transgenic camelina wild-type and *fae1* lines.

Acetyl-TAG and regular TAG content **(a)**, seed fatty acid content **(b)**, seed weight **(c)**, and seed size **(d)** of homozygous transgenic wild-type and *fae1* lines expressing *EfDAcT* or *EfDAcT+DGAT1 RNAi*. Data represent mean ± standard error of two independent transgenic lines with ≥ 3 biological replicates each. Different letters indicate statistical difference between genotypes (One-way ANOVA followed by Tukey's multiple comparisons test; $p \leq 0.05$).



Supplementary Figure D.7. Ultrahigh acetyl-TAG levels have minimal effects on transgenic pennycress seed.

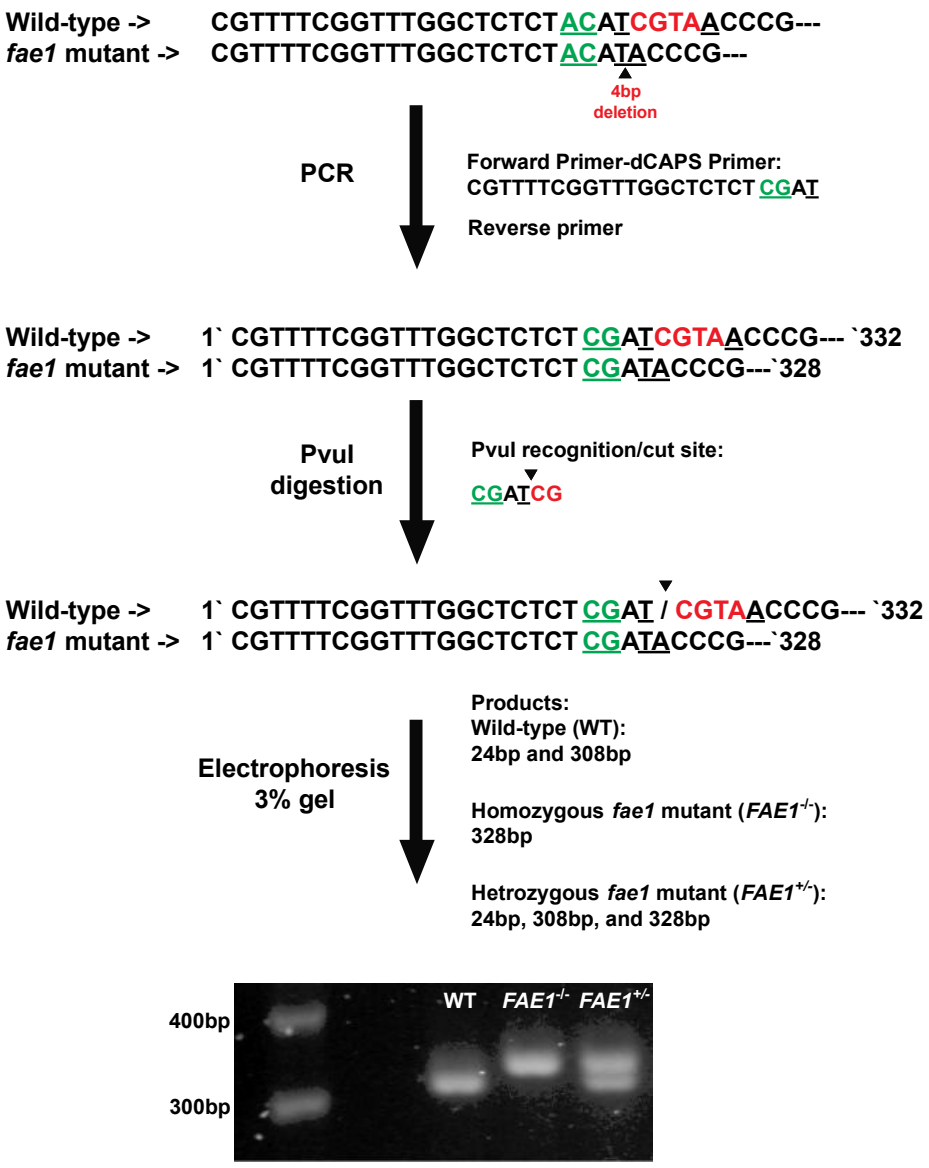
Acetyl-TAG and regular TAG content **(a)**, seed fatty acid content **(b)**, seed weight **(c)**, and seed size **(d)** of selected homozygous transgenic lines expressing *EfDAcT* or *EfDAcT+DGAT1 RNAi* in both wild-type and *fae1* backgrounds. Each independent line is represented by a distinct color. Data represents mean ± standard error, n ≥ 3 biological reps. Different letters indicate statistical difference between genotypes (One-way ANOVA followed by Tukey's multiple comparisons test; $p \leq 0.05$).



Supplementary Figure D.8. Germination phenotype of transgenic pennycress seeds.
Representative images of seed germination assays on 1% agar plates of homozygous transgenic wild-type and *fae1* lines expressing *EfDAcT* or *EfDAcT+DGAT1 RNAi*. The two panels feature images from different independent lines.



Supplementary Figure D.9. Plant growth phenotypes of transgenic pennycress lines.
Representative images of wild-type, *fae1* mutant, and homozygous pennycress transgenic plants expressing *EfDAcT* or *EfDAcT+DGAT1 RNAi* at 21, 37, and 51 days after planting.



Supplementary Figure D.10. Illustration of pennycress *fae1* mutants genotyping using dCAPS. Red letters represent the 4bp deleted nucleotides in pennycress *fae1* mutant sequence. Black underlined letters represent the nucleotides flanking the 4bp deletion site. A dCAPS primer containing two mismatches (green underlined nucleotides) to the pennycress *FAE1* sequence create a PvuI recognition site in wild-type sequence but not in the *fae1* mutant. The resulting PCR products are subsequently digested by PvuI and separated on a 3% agarose gel to assess the presence or absence and zygosity of the mutant *fae1* allele. Illustration was adapted from www.ncbi.nlm.nih.gov/probe/docs/techdcaps/

Supplementary Table D.1. List of pennycress genotyping and RT-qPCR primers

Genotyping		
Primer Name	**Primer Sequence**	**Arabidopsis Homologue**
FAE1-PvuI_dCAPS-F	5'-CGTTTTCGGTTTGGCTCTCTCGAT-3'	AT4G34520
FAE1-R	5'-CGAATAGTTTTTCGAGCGCA'3'	
RT-qPCR		
Primer Name	**Primer Sequence**	**Arabidopsis Homologue**
DGAT1-RT-F	5'-TCACTATGCCGACGGAGAACG-3'	AT2G19450
DGAT1-RT-R	5'-GGGTGCGGAATCGGAAAGAGG-3'	
PDAT1-RT-F	5'-CCTCACGATTCCCCGAAACC-3'	AT5G13640
PDAT1-RT-R	5'-CGCAGACACAACCGATGAACC-3'	
FAE1-RT-F	5'-GGCTCTACGCAATGTCAAGGC-3'	AT4G34520
FAE1-RT-R	5'-GACCGACCGTTTTGGACACG-3'	
TIP41-RT-F	5'-TGAGATTGAGAGAGACGAGAATGC-3'	AT4G34270
TIP41-RT-R	5'-TTGCTTCCCTCCAACAGCTTTCAC-3'	
UBC-RT-F	5'-ACGAGCGAGACTGTTCAAGGAG-3'	AT5G25760
UBC-RT-R	5'-TTGAGGAGGTTGCAGAGGATATGG-3'	