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Development of High Value oil traits using the model oilseed crop *Camelina sativa*

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By

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Development of High Value oil traits using the model oilseed crop *Camelina sativa*

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Plant oils are an important source of food, fuel, and feed in our society today. The oil found in the seeds of plants is composed almost entirely of triacylglycerol (TAG) molecules, which consist of three fatty acids esterified to a glycerol backbone. As crude oil supplies decline, vegetable oils are gaining traction as a renewable substitute to petroleum-based materials in fuels, lubricants, and specialty oleochemicals. However, as it currently stands vegetable oils do not possess the properties necessary to fill the void of a petroleum free world.

To address this problem, plant biotechnologists have done extensive work on genetic engineering the fatty acid biosynthetic pathway to produce designer oils that are specialized for nutritional or industrial use. However, the bottleneck seems to be the uptake of these specialized oils into TAG. To further investigate this problem, I have chosen to study fatty acid biosynthetic genes from the species *Thunbergia laurifolia* which naturally produces 90% petroselinic acid (18:1Δ6). This species was chosen because of the unusually high accumulation of one single fatty acid, therefore the hypothesis is that the enzymes involved in the pathway to produce this novel fatty acid are highly specific. In this study I successfully engineered camelina to produce ≥25% 18:1Δ6 using a specialized Δ6 desaturase, fatty acid thioesterase A, and lysophosphatidic acyltransferase from *T. laurifolia*

Acknowledgments

I want to first and foremost thank my parents for supporting me through my academic journey. They constantly pushed me to achieve my goals and helped me out along way. I also want to thank all of my close friends and family that were there for me throughout this entire process.

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ABBREVIATIONS

DGAT	Diacylglycerol acyltransferase
LPAT	Lysophosphatidic acid transferase
GPAT	Glycerol-3-Phosphate acyltransferase
PUFAs	Polyunsaturated Fatty Acids
TAG	Triacylglycerol
DAG	Diacylglycerol
MAG	Monoacylglycerol
ACP	Acyl Carrier Protein
ER	Endoplasmic reticulum
FAS	Fatty acid synthase
FatA	Fatty acyl thioesterase A
FatB	Fatty acyl thioesterase B
FAD2	Fatty Acid Desaturase
FAX1	Fatty Acid Exporter 1
PUFAs	Polyunsaturated Fatty Acids
FAMEs	Fatty Acid Methyl Esters
KAS	3-ketoacyl-ACP synthase
VLCFA	Very long chain fatty acids
MCFA	Medium chain fatty acids

Fatty Acid Nomenclature

x:y	x is the number of carbon atoms in the fatty acid chain and y is the number of double bonds in the fatty acid chain.
Δ^x	indicated the position of the double bond at the x^{th} carbon relative to the carboxyl end of the fatty acid

1 Introduction

1.1 Fatty Acids in Plants

Plant oils are an irreplicable part of our society today due to their use in foods, animal feeds, and industry. The ultimate use of these oils is determined by their fatty acid composition. Fatty acids are categorized depending on their saturation levels which include saturated and unsaturated, chains lengths such as short, medium, long, and very long chains. The five most common types of fatty acids in plants are palmitic (16:0), stearic (18:0), oleic (18:1Δ9), linoleic (18:2Δ9,12), and α-linolenic (18:3Δ9,12,15). Fatty acids can also contain several unusual compounds. An unusual fatty acid is a given fatty acid that is found in only a select few plants. Unusual fatty acids can contain functional groups such as hydroxyl residues and epoxide rings such as in ricinoleic acid (18:1Δ9, 12-OH) and vernolic acid (18:1Δ9, 12-epoxy) [2] (Figure 1). There are a number of food and nonfood applications of plant oils in that they are mostly consumed as cooking and salad oils, while industrial applications can consist of biofuels, lubricants, surfactants, drying oils, plasticizers, and ink production [3].

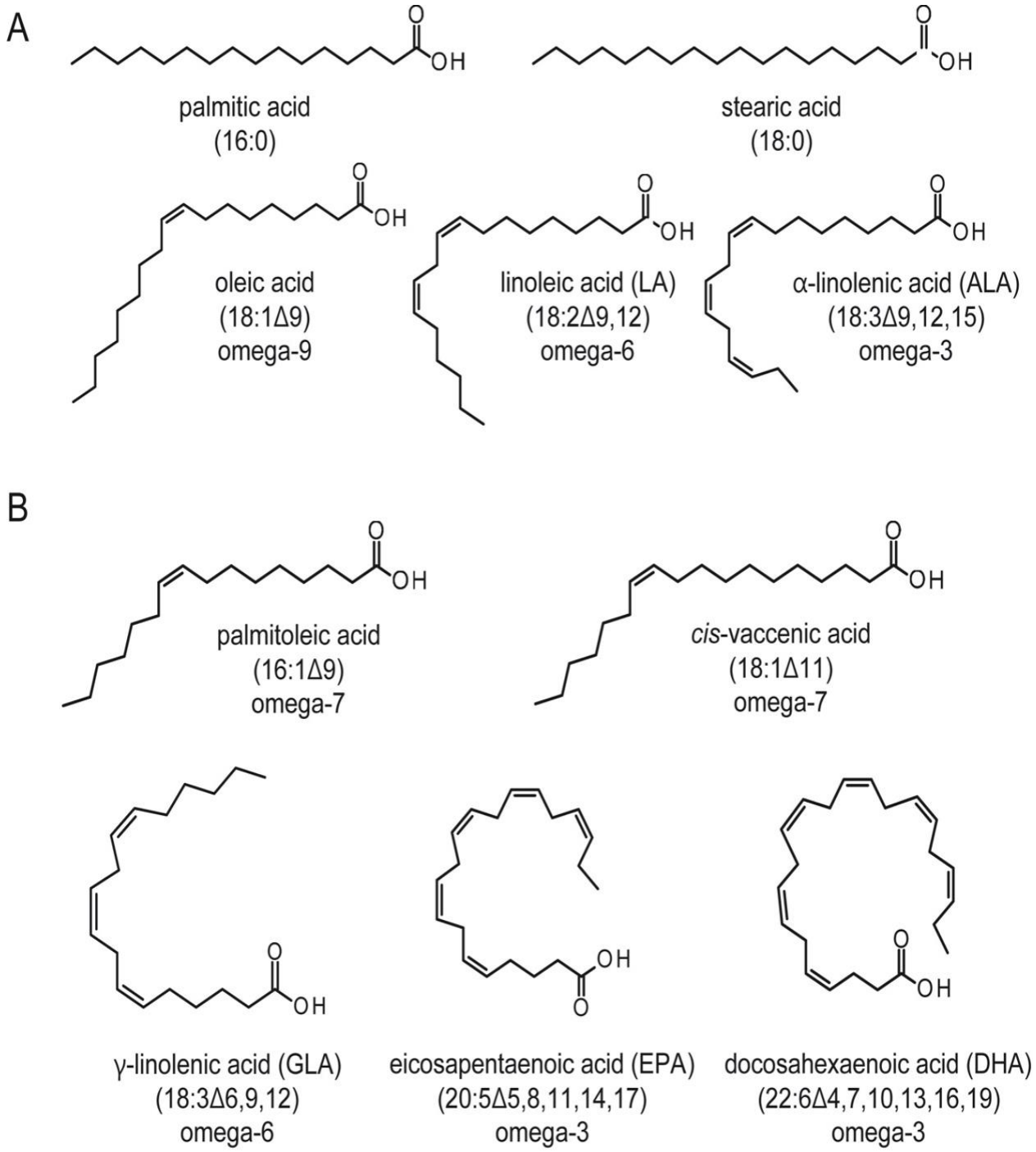

Figure 1 (A) Chemical structures of some common fatty acids (B) and examples of some health-promoting fatty acids that are targets for biotechnology [2]

Vegetable oils principally consist of triglycerides or triacylglycerols (TAG), which are composed of three fatty acids esterified to a glycerol backbone. These oils are commercially obtained from seeds of plants such as soybeans and canola and the oil can be extracted from the mesocarps that surround seeds of plants such as olive and palm [4]. Usually, the oils are extracted from these sources via hexane or they can be extracted by pressing.

The world consumption of vegetable oils is currently at 200 million metric tons annually and is steadily increasing, mostly due to the increasing demand for the production of fried and processed foods as well as salad oils [5]. The top oil producing crops in 2020 were palm oil (75.45 Million Metric Tons (Mt)), soybean (59.48 Mt), rapeseed (27.64 Mt), sunflower (19.02 Mt), palm kernel oil (8.51 Mt), peanut oil (6.17Mt), cottonseed (4.89 Mt), coconut (3.67 Mt), and olive (3.1 Mt) [6]. The major oilseed producing countries are Indonesia (49.43 Mt), China (28.86 Mt), Malaysia (22.28 Mt), European Union (18.53 Mt), United States (13.04 Mt), Brazil (10.08), Argentina (9.11 Mt), and other remaining countries (58.24 Mt) [6].

In the United States, soybean oil is the dominant oilseed crop, accounting for about 90% of total oil production [6]. Soybean oil is a rich source of essential fatty acids linoleic and the omega-3 fatty acid, α-linolenic. However, because of the low melting point of these fatty acids possess, they often need to be hydrogenated to be solid at room temperature to produce margarines. Hydrogenation is a chemical process that transforms liquid vegetable oil to a solid state by heating and pressurizing the oil in the presence of

hydrogen and a catalyst (typically powdered nickel compound). This process generates *trans* fatty acids which have been shown to be linked to coronary heart disease [7]. As a result, there has been considerable interest in engineering oilseed crops can produce a healthy, *trans*-fat-free alternative. In addition to the health benefit, polyunsaturation of fatty acids leads to increased oxidation rate of the oils and promotes rancidity of fried and baked foods using these such oils [8].

Although global plant oil production is heavily directed towards food use, a significant proportion is also used in the oleochemical industry. Medium chain fatty acids, such as laurate (12:0) that are mainly produced from palm kernel oil and coconut oil, are used in the production of surfactants such as soaps, detergents, and related personal care products [9]. Other more specialized oils such as castor oil which contains the hydroxy fatty acid ricinoleic acid (12-hydroxy 18:1Δ9) are used in the production of certain nylons [10]. Tung oil (*Vernicia Fordii*) contains high levels of the conjugated fatty acid α-eleostearic acid (18:3Δ9,11,13), which is highly valued for its furniture protection due to its unique drying properties [11]. However, due to the limited agronomic properties of some of the plant species that these fatty acids are isolated from, large-scale production is not always economically feasible. This presents the opportunity for genetically engineering a more robust and agronomically versatile oilseed crop for the production of these specialized, and higher value oils.

1.2 Fatty Acid Metabolism

Lipids serve many functions in plants. As the major component of biological membranes, they form a hydrophobic barrier that is critical to life. Membranes not only separate cells from their surroundings, they also serve as separators for the contents of organelles, such as the chloroplast and mitochondria, from the cytoplasm. This cellular compartmentalization depends on polar lipids forming a bilayer that prevents free diffusion of hydrophilic molecules between different organelles in the cell and prevents diffusion in and out of the cell. The membranes of chloroplasts mainly contain galactolipids, while the membranes external to plastids are composed of mixtures of phospholipids. Although a gram of leaf tissue may contain as much as $1m^2$ of membrane, lipids make up a relatively small portion of the total mass of plant tissue.

Fatty acid biosynthesis in plants takes place in the plastids, organelles in plants that are widely thought to have originated from a photosynthetic bacterial symbiont [12]. The first step in the fatty acid biosynthesis pathway is the conversion of acetyl-CoA to malonyl-CoA via acetyl-CoA carboxylase (ACCase). Next, the malonyl-CoA is transferred to ACP (acyl carrier protein). This reaction is carried out by the actions of malonyl-CoA:ACP transacylase. The biosynthesis of all fatty acids involves the central cofactor of ACP. This is a small protein about 80 amino acids long and contains a phosphopantetheine prosthetic group covalently linked to a serine residue near the middle of the peptide chain. This phosphopantetheine group, which is also found in coenzyme

A, contains a terminal sulfhydryl. The thioester linkage forms between a fatty acid and this sulfur is a high-energy bond with a free energy of hydrolysis similar to that of ATP.

From here the assembly of the fatty acid begins when a carbon-carbon bond forms between the C-1 of the acetate group and the C-2 of the malonyl group on the ACP, this reaction releases a CO2 molecule. This addition of a 2 carbon chain results in the formation of acetoacetyl-ACP (Figure 2). A three reaction sequence of reduction, dehydration, and reduction again finally produce a fully reduced acyl-ACP. The condensation reaction to form a new carbon-carbon bond is catalyzed by 3-ketoacyl-ACP synthase (KAS). All plants found to date contain three KAS enzymes (I, II, III) and the difference between them is the substrate specificity. KASI is most active with C4-C14 acyl-ACP substrates, KASII prefers C10-C16, and finally, KASIII has a strong preference for acetyl-CoA rather than acyl-ACP. These enzymes work in sequence KASIII initiates fatty acid biosynthesis by using acetyl-CoA as a primer. KASI then extends the acyl chain to C12-C16 and finally KASIII completes the synthesis to C18. These FAS reactions increase the chain length from C2 to C18 by two carbon atoms at a time.

FAS or fatty acid synthase refers to all enzyme activities in fatty acid biosynthesis, excluding ACCase. In nature the reactions carried out by FAS are essentially the same, however, two different types have been found. Type I FAS is what is normally found in animals and yeast, this is a single multifunctional enzyme complex characterized by large (250kDa) subunit size. Type II FAS is found in most plants and bacteria, this is different from Type I in that each enzyme resides on an individual protein and these proteins can

be readily separated from the other reactions of FAS. Type I functions much more like large protein complex while type II functions as more of a metabolic pathway of ordered reactions.

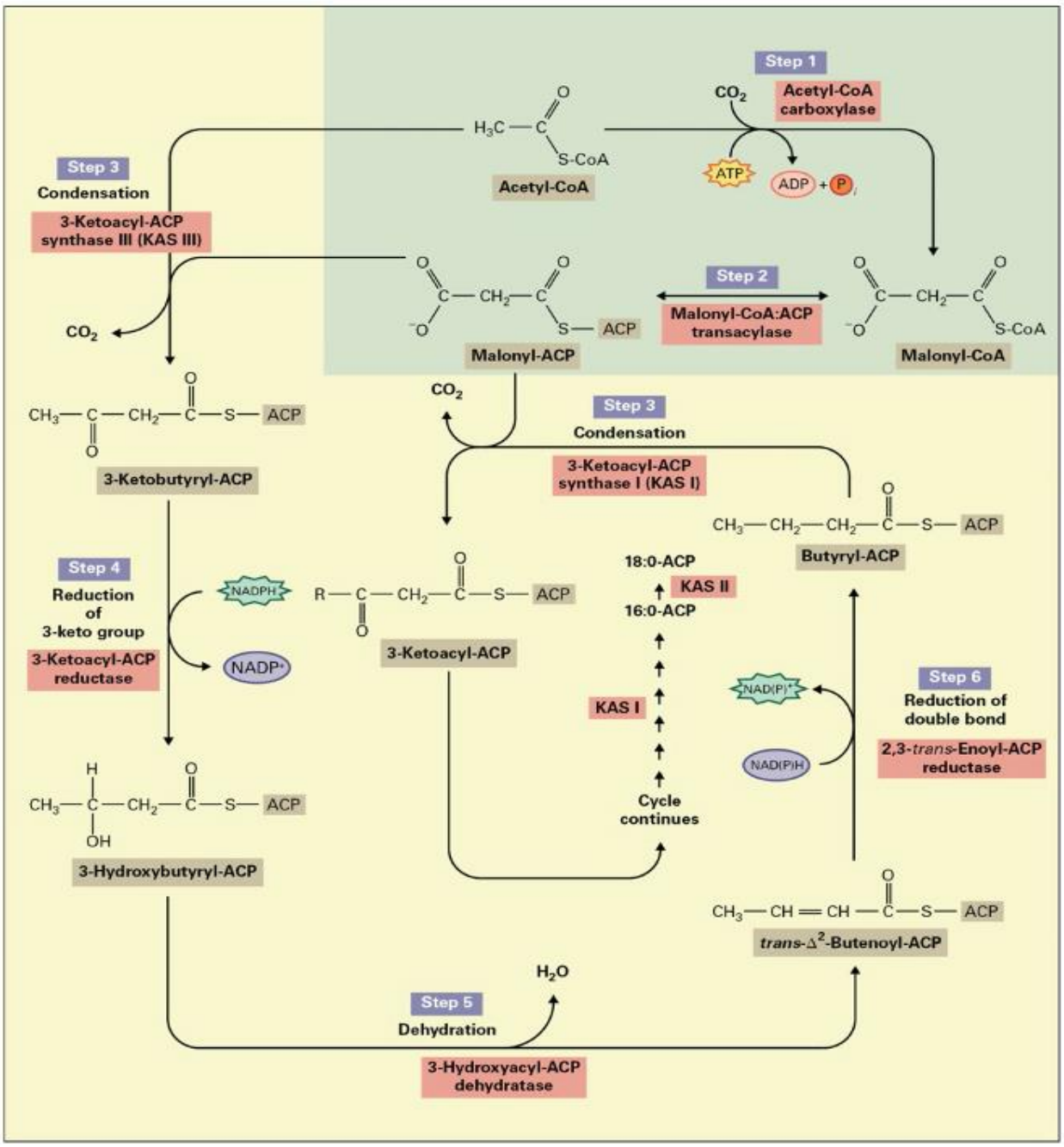

Figure 2 Overview of fatty acid synthesis. Fatty acids grow by addition of two carbon units. The reactions highlighted in yellow show how malonyl-CoA enters the cycle: those that are highlighted in orange represent the cyclic reactions. Synthesis of a C16 fatty acid requires that the cycle be repeated seven times. During the first turn of the cycle, the condensation reaction (step 3) is catalyzed by ketoacyl-ACP synthase (KAS) III. For the next six turns of the cycle, the condensation reaction is catalyzed by isoform I of KAS. Finally, KAS II is used during the conversion of 16:0 to 18:0 [1]

The product obtained to this point is either 16:0-ACP or 18:0-ACP.

To terminate the FAS cycle and be exported for further acyl editing/elongation, fatty acids are cleaved off of ACP. The thioester bond between the fatty acids and the ACP is hydrolyzed by a fatty acid thioesterase enzyme of which there are two gene classes in higher plants, FatA and FatB [13]. The main difference between the two types of thioesterases is their substrate specificities. FatA is primarily responsible for the hydrolysis of unsaturated acyl substrates (18:1-ACP) whereas FatB preferentially acts on saturated acyl-ACPs with acyl chain length varying from 8 to 18 carbons. Due to the diversity of substrates accepted by the FatB, it has been further classified into three subclasses. Subclass I FatB prefer 14-16 carbon acyl-ACPs, subclass II prefer a broader range of 8-16, and finally, subclass III acts predominantly on 8-carbon-acyl-ACP chains [14].

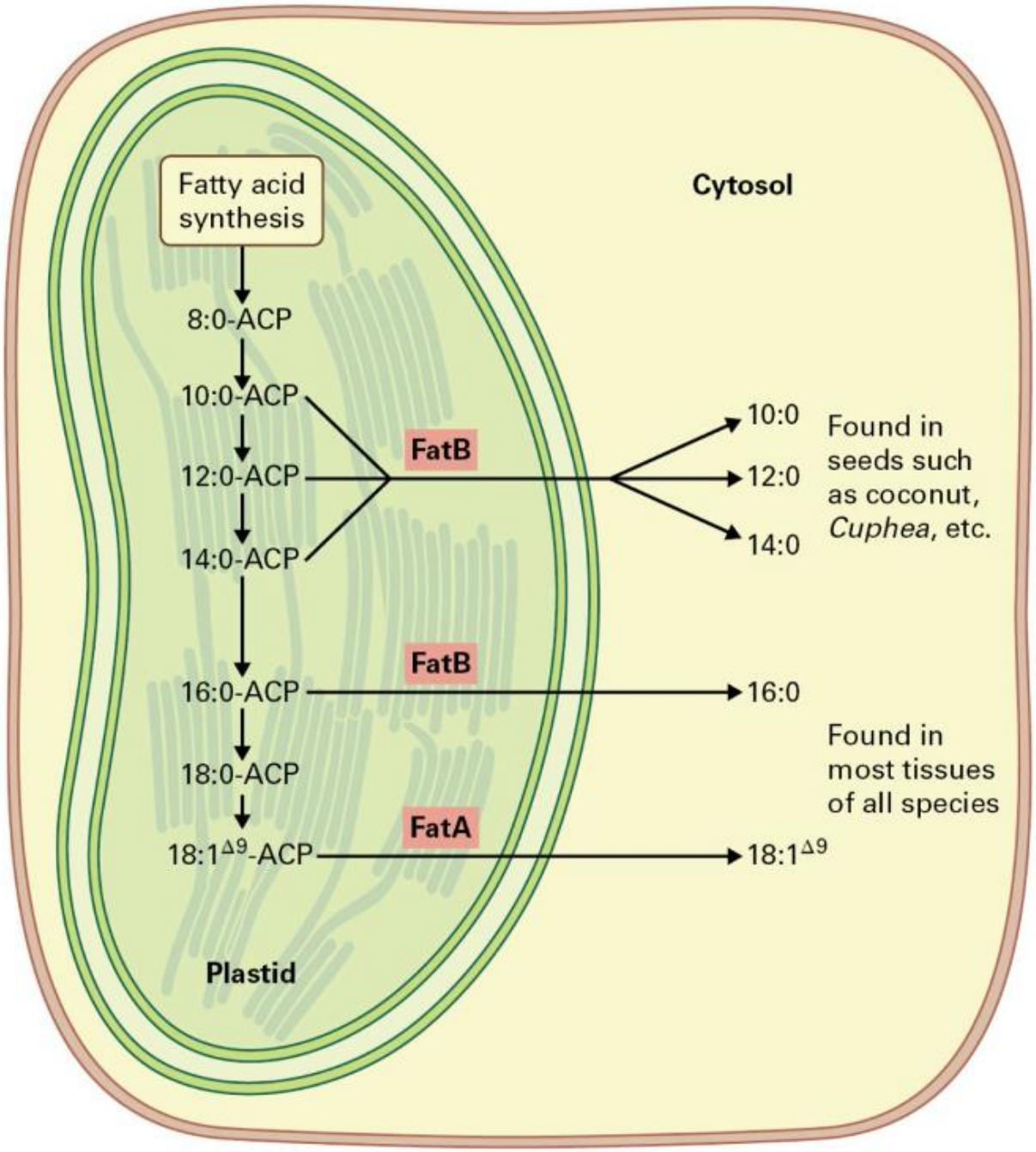

Figure 3 Principal Types of acyl-ACP thioesterases in plants. The FatA class of thioesterase is most active with 18:1Δ9 and the FatB class is most active with saturated acyl-ACPs. The FatA 18:1Δ9 thioesterase and the FatB 16:0-ACP thioesterase are found in all plant tissues. Some FatB thioesterases, especially those most active on acyl-ACP acyl groups shorter than C16, are species specific [1].

If membranes only contained saturated and trans-unsaturated fatty acids, the hydrophobic lipid tails would form a semi-crystalline gel which would impair the permeability barrier which would interfere with membrane mobility. *Cis*-double bonds introduce kinks in the fatty acid chain which lowers the melting point and makes them more fluid, allowing easier transport through membranes. For example, the desaturation of stearic acid (18:0) to oleic (18:1Δ9) decreases the melting temp from 69℃ to 13.4℃.

The first desaturation reaction is carried out by stearoyl-ACP desaturase (SAD). This enzyme adds a double bond to the Δ9 position of 18:0-ACP producing 18:1-ACP. The fatty acid biosynthetic reactions in the plasmid produce mostly 16:0, 18:0, and 18:1Δ9 fatty acids, however, in some plants produce shorter carbon chains such as palm kernel and coconut [15].

Upon cleavage from the ACP, the fatty acids are exported from the plastid to the cytosol via fatty acid exporter (FAX1) [16]. Once in the cytosol, the free fatty acids (FFAs) are then made into temporary compounds with coenzyme A (CoA). This acyl-CoA pool is the main donor of fatty acids for TAG synthesis and membrane lipids. The fatty acids can now either be elongated from long chain fatty acid (18:1) to very long chain fatty acids (VLCFAs: 20:1 and 22:1) [17] or be further desaturated to 18:2 via fatty acid desaturase 2 (*FAD2*).

Pathways producing membrane lipids such as phosphatidylcholine (PC) and storage lipids such as TAG use the same substrates in endoplasmic reticulum (ER), membrane lipid synthesis has priority due to the importance of cellular functioning and survival over storing lipids for germination and reproduction. PC is the site for further desaturation

from 18:1 to 18:2 via fatty acid desaturase 2 (FAD2) [18]. Fatty acids can be further desaturated via fatty acid desaturase 3 (FAD3) which produces 18:3 from the 18:2 substrate [19]. In Arabidopsis, both of these enzymes are located in the ER.

The backbone molecule from which TAG is formed is glycerol-3-phosphate (G3P) which is produced from dihydroxyacetone phosphate (DHAP) via glycerol-3-phosphate dehydrogenase (G3PDH) in the cytosol. Fatty acids are acylated onto the *sn*-3 positions of the G3P molecule in a stepwise reaction known as the Kennedy Pathway [20]. First *sn*-1 and *sn*-2 positions are incorporated by glycerol-3-phosphate acyltransferase (GPAT) to form lysophosphatidic acid (LPA) and lysophosphatidic acid acyltransferase (LPAT) to form phosphatidic acid (PA) respectively. The second step is the removal of the phosphate group on the *sn-3* from PA by phosphaditic acid phosphatase (PAP) to form diacylglycerol (DAG). The final step is the addition of a fatty acid at the *sn*-3 position of DAG to form TAG by the diacylglycerol transferase enzyme (DGAT). [21, 22].

There is an alternative pathway available for TAG biosynthesis where *de novo* DAG is incorporated into membrane lipid phosphatidylcholine (PC) where it is can then be further modified (desaturation, hydroxylation, etc.) [23]. Once incorporated into PC, a new pool is produced which is termed “PC-Derived DAG” this pool can be used as a substrate for TAG biosynthesis as well. The first step in becoming a membrane lipid is for the had group at the *sn*-3 position of *de novo* DAG to change from hydroxyl to a phosphocholine group by CDP-choline:DAG choline phosphotransferase (CPT) [24, 25]. Once these membrane lipids are formed, the fatty acid modification begins on the *sn*-2 position via DGAT, there is also another enzyme that facilitates the same reaction of

adding a fatty acid onto the *sn-2* position, this enzyme is called phospholipid:diacylglycerol acyltransferase (PDAT) [26]. The difference between the two enzymes is the substrate specificity, PDAT prefers the PC pool as the substrate while DGAT prefers to use the acyl-CoA pool. Multiple studies have been done to further examine these two enzymes. Double mutants of both *pdat1* and *dgat1* in Arabidopsis were unable to be obtained, as expected because TAG synthesis is essential for plant cellular function [27]. *pdat1* mutants did not change the fatty acid profile and did not alter total oil content in Arabidopsis, suggesting that when *pdat1* is not functioning properly *dgat1* can facilitate all needed reactions [28]. On the other hand, a *dgat1* mutant line of Arabidopsis showed a 30% reduction in total oil content [22, 27] and significant upregulation of *pdat1* [29]. It is also worth noting that *pdat1* and *dgat1* expression changes depending on the plant species. For example, the expression of *dgat1* in sunflower is 5 times higher than *pdat1*, while in safflower (*Carthamus tinctorium*) *pdat1* is expressed 5 times more than *dgat1* [15].

The synthesis of unusual fatty acids has always been of interest for biotechnologists due to their potential economic value. An unusual fatty acid is any fatty acid that contains fewer than 16 or more than 18 carbons, variant double bond positions (*cis/trans* orientation), acetylenic or triple bonds, or carbon side-chain modifications [30]. These unusual fatty acids often make up the majority of plant seed oils such as in *Thunbergia laurifolia* (≥90%). Despite this large investment in photosynthetic carbon, the biological significance of these unusual fatty acids is still largely a mystery. There is speculation that some of these unique fatty acids can confer resistance to pathogenic or predatory

attack, which then promoted selective advantage during the evolutionary process [31]. Some fatty acids also possess anti-inflammatory properties such as borage *(Borago officinalis)* and evening primrose *(Oenothera biennis)*. Both of these plants produce high amounts of the unusual fatty acid ɣ-linolenic acid (18:3Δ6,9,12) because the pathway for the production of this fatty acid is of potentially high economic value, the elucidation of the specialized genes involved in the biosynthetic pathway has been a target for scientists since the 1980s [32]. Efforts to date have been successful in engineering model oilseed crops to produce unusual fatty acids, however, obtaining production levels that are like those of the host plant has been difficult. This difficulty has often been attributed to the downstream enzymes in seeds of host plants to effectively link the unusual fatty acids to the glycerol backbone [33]. This thesis is an examination of specialized acyltransferase proteins and their role in the storage of unusual fatty acids.

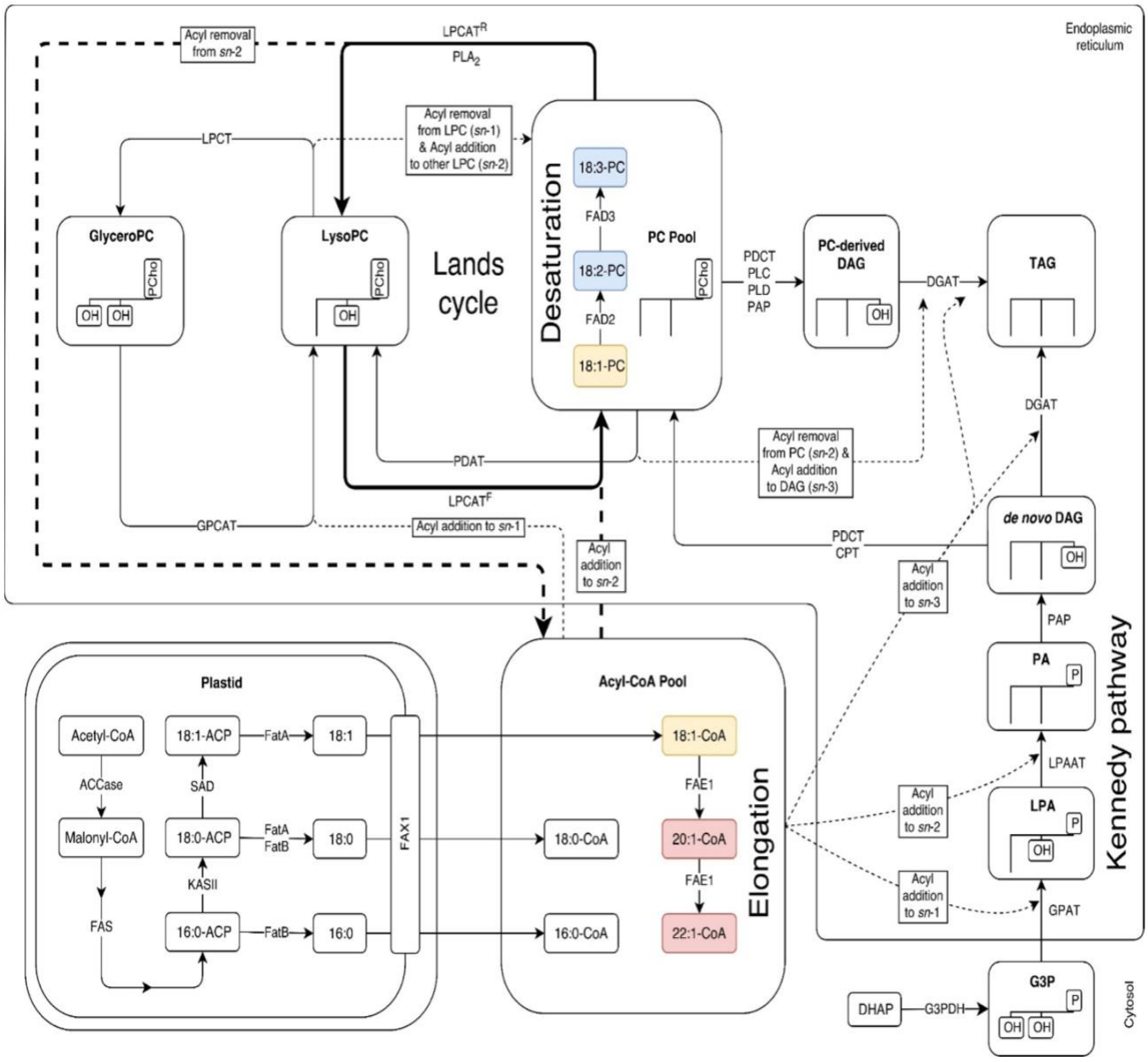

Figure 4 Overview of metabolic pathway for triacylglycerol synthesis [1]

1.3 Petroselinic Acid

Petroselinic acid (18:1Δ6) was first identified in the seed oil of parsley (*Petroselinium crispum*), an Apiaceae species. This discovery was made by Vongerichten and Köhler (1909) and was also later detected in the seed oil of English ivy (*Hedera helix*) a species in the Araliaceae species by Palazzo and Tamburello (1914). It has since been identified in many plant families such as Apiaceae, Araliaceae, and Garryaceae where reports have shown that it can constitute up to 85% seed oil [34]. Recently, we have discovered that 18:1Δ6 is present in the seeds of *T. laurifolia* at levels above 90%. This finding creates an incredible opportunity to further study the genes responsible for such a high accumulation of this novel fatty acid.

Petroselinic acid is a double bond positional isomer of oleic acid (18:1Δ9), the unique position of the double bond gives an increased melting temperature to 18:1Δ6 (30°C) compared to oleic acid (14°C). In addition to the increased melting temp, 18:1Δ6 also has a higher oxidative stability when compared to oleic acid [35]. Oxidative stability of oils is very important in terms of cooking and nutrition. The process of fatty acid oxidation affects not only the shelf life but can also affect the taste and smell of these oils. The universally accepted test for the measurement of oxidative stability is the Rancimat Test. This is a test that uses a constant airflow and gradually increasing temperatures to artificially speed up and mimic natural aging processes, therefore determining oxidative stability of various compounds.

Elucidation of the biosynthetic pathway of 18:1Δ6 was started off by the discovery of a plastid located, Δ4-acyl ACP desaturase found in *Coriandrum sativum* [36]. It was

determined that this desaturase required an acyl-ACP as a substrate, at the time oleic acid was the only known fatty acid to be desaturated by an acyl-ACP desaturase. The cDNA from the desaturase was used to express the protein in tobacco leaf, the result was the accumulation of 18:1Δ6 [37]. This was the first known instance of the production of 18:1Δ6 in transgenic plants. The pathway for production in coriander is detailed in Figure 6.

In coriander, 16:0-ACP is desaturated at the Δ4 position and then is elongated to produce 16:1Δ4-ACP. We believe that there is a more direct and more efficient pathway to get to 18:1Δ6 by directly desaturating 18:0-ACP using a specialized Δ6 desaturase from *T. laurifolia.*

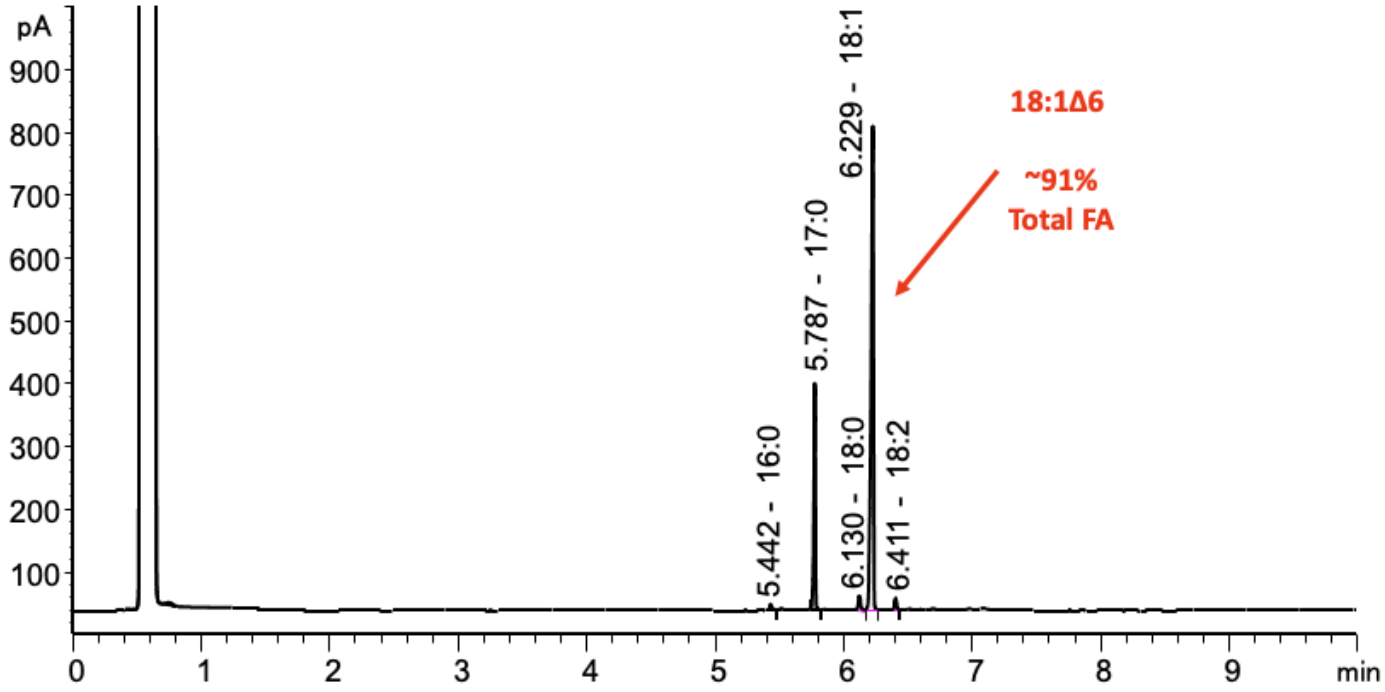

Figure 5 Chromatogram showing fatty acid profile of T. Laurifolia seeds along with the 17:0 standard, there is an abnormally large peak where 18:1 should be. However, because oleic acid and petroselinic acid are positional isomers of each other, we cannot separate them on a standard GC column. Figure provided by Dr. Edgar Cahoon.

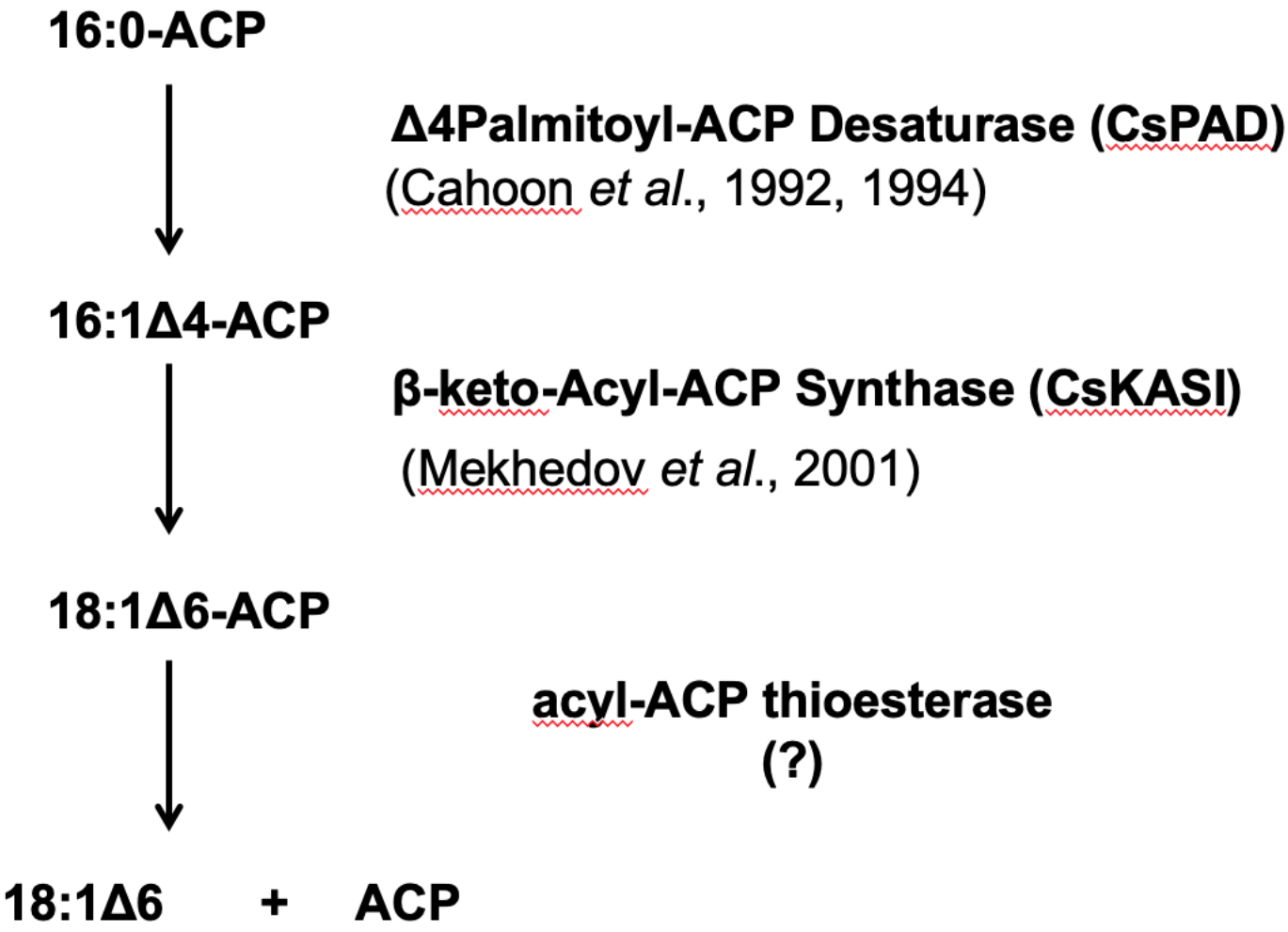

Figure 6 Biosynthetic pathway for production of petroselinic acid in *Coriandrum sativum*

1.4 Advantageous agronomic Traits of Camelina

Camelina sativa (L.) Crantz or camelina commonly known as false flax or gold of pleasure is a fall or spring planted oilseed crop [38]. Seeds and capsules of *Camelina sativa* ssp. *C. lincola* have been found in excavations from the bronze age in Scandinavia and Western Europe [39]. Evidence suggests that the cultivation of camelina began in early France around 500 BC [40]. The first documented planting of camelina in North America was in the Red River in 1863 Valley which forms the border between Minnesota and North Dakota [41]. During this time, it is reported that camelina was used as a fuel oil for lighting, meal for livestock, and soap making.

The name camelina was derived from the Greek word chamai, dwarf and linon, flax [42]. Camelina is a close genetic relative of the model plant *Arabidopsis thaliana* (or Arabidopsis), both are members of the Brassicaceae. Camelina has also been sequenced [43] and was revealed to have an undifferentiated allohexaploid genome with a comparatively large number of genes and a low percentage of repetitive DNA. Camelina is a dicotyledonous, monocarpic, self-pollinating oilseed crop that is very hardy and able to grow in various environmental conditions [39, 42].

Recently camelina has garnered much interest by biotechnologists as a viable industrial bio-platform crop. Although the oil yield is usually less than canola, studies have shown that the economic cost of camelina oil is less than half that of rapeseed due to the low input requirements of camelina [44]. The oil content of camelina seeds is mostly dominated by PUFAs linoleic (18:2) and linolenic (18:3) acid which totals around 50% of

the fatty acid profile depending on the cultivar and weather conditions in which it was grown [45].

Perhaps one of the biggest reasons for the resurgence of the cultivation of camelina is the need for sustainable and renewable bio-sources of fossil fuel alternatives. It has already been proven by the US Air Force and the US Navy that a blend of standard jet fuel (kerosene) and camelina based jet fuel could be used as fuel to fly an A-10 Thunderbolt, F-22 Raptor, and F/A-18 Super Hornet fighter jets. They also found that this blend of jet fuel produced 75% fewer emissions than standard petroleum [46].

Camelina has proven to be a very hardy crop, its adaptability to many soil types and weather conditions distinguishes it from other crops. Camelina requires low inputs such as fertilizer, water, and pesticides which makes it an ideal crop for areas where rainfall is insufficient, fertility of the soil is low, and weather is harsh [39]. The lifecycle of camelina is between 85-100 days [47] this would allow it to be used as a short seasoned crop that could be used for rotational or relay purposes instead of keeping the ground empty for a fallow year. The seed yield of camelina is in the range of 677-1306 kg/ha, while the seed oil yield is about 234-445 kg/ha [48].

The lipid content of individual seeds of camelina ranges roughly from 35-45% which almost double that of soybean (18-22%) [45]. The fatty acid profile of camelina as previously eluded to is as follows: palmitic (16:0, 5.3-6.8%), stearic (18:0, 2.5-2.7%), oleic (18:1, 12.6-18.6%), linoleic (18:2, 14.3-19.6%), α-linolenic (18:3, 32.6-38.4%), arachidic (20:0, 1.2-1.5%), eicosenoic (20:1, 12.4-16.8%), eicosadienoic (20:2, 1.3-19%),

eicosatrienoic (20:3, .8-1.7%), behenic (22:0, .2-.3%), erucic (22:1, 2.3-2.9%) and others (1.2-3.7%) [45].

Polyunsaturated fatty acids (PUFAs) make >50% of the total oil in camelina seeds, which poses quite a high risk for oxidation of the oils due to the low oxidative stability PUFAs. Oxidation of oils is the undesirable set of reactions by which an oxygen atom steals an electron from the double bond. The result of this reaction can shift a double bond from the *cis* to *trans* double bond isomerization.

Various diseases and pests that cause a decrease in yield in other oilseed crops have shown to have less effect on camelina. Camelina, for example, has significant resistance to diseases such as *Alternaria* black spot and blackleg, and some cultivars are resistant to sclerotinia stem rot, brown girdling root rot, and downy mildew [49]. However, there are a few diseases to which camelina is susceptible to such as clubroot, white rust, and aster yellows disease [49]. Pests that normally target canola on Canadian prairies such as flea beetles, root maggots, diamondback moths, bertha armyworms, leafhoppers, grasshoppers, cutworms, and lygus bugs all have shown to have little effect on camelina [50].

As mentioned earlier the genome of camelina was sequenced [43] and was revealed to have an allohexaploid genome. This had been previously speculated after confirmation that camelina contained three copies of *FAD2* and *FAE1* [51]. Camelina has three sub-genomes with two genomes containing seven chromosomes each which are very similar, the third sub genome contains six chromosomes which are slightly different [52]. Since the size of the camelina genome (785 Mb) [43] is roughly six times larger

than the Arabidopsis genome (135 Mb) [53] it is hypothesized that a whole genome triplication event could have occurred. In addition to the sequencing of the genome, transcriptional analysis of different tissues was taken at different stages of the camelina lifecycle. This data can be found on *Camelina sativa* electronic Fluorescent Pictograph (eFP) (http://bar.utoronto.ca/efp_camelina/cgi-bin/efpWeb.cgi).

1.5 Industrial Uses

Vegetable oils represent a substantial chemical reserve of free energy. This is because fatty acids are much more reduced organic molecules than carbohydrates. As stated before, plant oils consist of triglycerides which are three fatty acids esterified to a glycerol backbone. The physical and chemical traits of the TAG molecule are dependent on the three fatty acids that compose them. Fatty acids themselves are carboxylic acids of highly reduced hydrocarbon chains. As stated before, the fatty acid profile of plants is mostly dominated by a select few fatty acids being 16-18 carbons long and containing double bonds on the Δ9, 12, and 15 positions.

Camelina has a high oil content with unique properties that could be beneficial in both industry and nutrition. Based on studies done in by the USDA lab in Illinois, camelina oil yield can reach 106 to 907 L ha-1, which is significantly greater than soybean [45]. The total oil content is about 60% polyunsaturated fatty acids mainly linolenic acid (18:2) and α-linolenic acid (18:3). Because of the high content of omega-3 α-linolenic camelina oil has been promoted for both human and animal nutrition.

However, undesirable longer chain fatty acids such as erucic acid (22:1) poses a problem because of the adverse health. Erucic acid is actually classified as a natural toxin due to the detrimental effects on heart muscle functions [54]. However, fatty acids longer than 20C have recently been all but eliminated from camelina using CRISPR-Cas9 and targeting the FAE1 gene in camelina which is responsible for the elongation of carbons longer than 18C [55].

After oil is extracted from the seeds of camelina the meal can be used as feed for livestock. The meal is very nutritional with high levels of omega-3 fatty acids (>35%), vitamin E, crude protein (>45%), and fiber (11%) [56]. Again the problem with the feed is the high proportion of erucic acid which has been shown to create fat deposits in heart muscles and myocardial lesions in experimental animals. However with the recent advancements using CRISPR to knockout the genes that produce these fatty acids more research will need to be done on the nutritional properties of the meal.

Perhaps one of the more promising industrial uses that could come out of camelina are the bio based products like polymers, varnishes, paints, cosmetics and dermatological products. The high proportion of unsaturated fatty acids in camelina means that it can be easily epoxidized and used in many industrial applications such as lubricants, resins, coatings, and adhesives. A group in the Biomaterials Department of Kansas State University, Manhattan, KS showed that epoxidized camelina oil has potential in the biopolymer industry for making pressure-sensitive adhesives, resins, and coatings [57]. They were able to optimized the epoxidation process for camelina oil using formic acid and hydrogen peroxide.

2 Objectives

The goal of this study was to engineer the fatty acid biosynthetic pathway of camelina to increase the production of the monounsaturated fatty acid 18:1Δ6, while simultaneously decreasing undesirable VLCFA. To do this we cloned various biosynthetic genes from *T. laurifolia,* a plant that produces extremely high amounts of 18:1Δ6 (+91%). The specific genes to be cloned include a Δ6 desaturase, thioesterase A, and we also tested out various acyltransferase genes (LPAT/DGAT). Camelina was chosen as the model crop for this study because it possesses some special features such as high seed oil and yield, high low input requirements, short growing season, easy transformation, drought and cold tolerance, and a similar protein content to soybeans. Camelina also possesses some VLCFA which may not be as desirable such as in the case of rapeseed [58]. It has been shown through various studies that monounsaturated fatty acids (MUFAs) have a positive impact on cardiovascular health compared to saturated fatty acids [59] [60]. Therefore, increasing MUFAs and decreasing saturated fatty acids and VLCFAs is of both economic and nutritional importance to both the producer and consumer of these plant oils.

We are currently in the midst of a genetic engineering revolution. Advances such as CRISPR/Cas9 [61], *Agrobacterium* transformation [62], and RNA interference [63] are revolutionizing the way scientist are able to knockout unwanted gene function, test out novel function of new genes, or knockdown genes for lower expression. Previous technologies did not have the flexibility of cutting specificity (meganucleases) or target

specificity (zinc finger nuclease) and delivery and expression in plants were difficult due to such large construct size (e.g., Transcription activator-like effector nuclease).

It is our hypothesis that the acyltransferase genes involved in the Kennedy pathway and TAG synthesis are highly specific for their fatty acid substrate and these enzymes can create a bottleneck when trying to engineer plants to produce these novel fatty acids. Due to the high specificity of these enzymes we not only need to add them to the pathway, but we also need to make sure the substrates are readily available to be used. For this, we have chosen to add the Δ6 desaturase from *T. laurifolia* and also the FatA thioesterase from the same species. It is our hypothesis that adding these enzymes, along with the acyltransferases involved in the Kennedy pathway will significantly increase the production of 18:1Δ6.

3 Materials and Methods

3.1 Plant Materials and Growth Conditions

Camelina (*camelina sativa)* cv. Suneson was grown in 3.5x3.5 inch plastic pots containing farfad germinatino mix media (Hummert International, Saint Louis, MO, USA). Under greenhouse conditions with 14-h day length (24-26°C) and 8-h dark (18-20°C) with natural and supplemental lighting at 400-500 μmoles/m^2/s. Transgenic camelina lines were generated via GV3101 *Agrobacterium* strain according to Lu and Kang (2008). After transformation seeds, T_1 seeds were planted in flats, and the first true leaves were allowed to emerge. A 0.01% Basta solution was sprayed onto the surface of the leaves at 3-4 day intervals. After 2 weeks of growth in the flats, the Basta resistant young plants were transferred to 3.5x3.5 inch pots.

3.2 Vector Construction

gDNA was isolated from *T. laurifolia* and used as a template for PCR using the following primers:

Oligo Name	*Oligo Sequence (5' to 3')*
TL-DGAT1F	TCAGGAATTTAGAGATTTTGCG
TL-DGAT1R	TGTTTGGAGGCATTGTTTCA
TL-DGAT2F	TTGAGAGAGTTCATCTTTCCCTTT
TL-DGAT2R	CTACAAAGAAATCAAGGGACCG
TL-LPAT1F	GAGTATCGTCAGCAAAATGGGA
TL-LPAT1R	AAGAAAGCCTGGCAAAGAAA
TL-LPAT2F	TATCTTGGATTCATTGCGGC
TL-LPAT2R	CTTAGACGAAGCCATACCCAAA

Table 1 List of primers used to obtain acyltransferase genes from *T. laurifolia* gDNA

The parameters for the PCR were as follows: initial denaturation at 95ºC, 32 cycles of denaturing at 95ºC for 30 seconds, annealing at 56ºC for 30 seconds, extension at 72ºC for 2 min 30 seconds, and final extension at 72ºC for 4 min. Since DNA consists of a negatively charged sugar-phosphate backbone, gel electrophoresis (125 volts) was used to separate the bands. PCR products were ran on a 1% agarose gel with ethidium bromide and seen under a UV light, PCR products of the correct size were cut from the gel and purified using ZymocleanTM Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA). Upon purification, these fragments were ligated into PCR-Blunt II TOPO vector using Zero BluntTM TopoTM PCR cloning kit (Thermo Fisher Scientific, Waltham, MA, USA). Once ligated, the plasmid was transformed into competent *E.Coli* DH5α. Transformation protocol was initiated with thawing 50 μL in an ice bucket. Once thawed to a liquid 2 μL of the new plasmid construct was added and gently mixed by tapping with the index finger. The mixture was then left to sit on ice for 20 min and then heat shocked at 42ºC for 35 seconds. Later, 1mL of LB medium was added and the entire mixture was placed in the 37ºC shaker for 1 hour to allow the cells to recover. While the cells recovered a Kanamycin LB agar plate was taken from the refrigerator and placed in a 37ºC incubator to warm it. One hour later the tube was taken from the 37ºC shaker and the full 1mL of recovered cells was plated onto the now warmed LB + Kanamycin plate and the cells were allowed to incubate overnight. The next day, single colonies were picked off the plate and place in liquid LB + Kanamycin media, and allowed to grow for 12-24 hours. Cloudy LB medium was purified by using IBI Scientific High-Speed plasmid Mini Kit (MIDSCI, St. Louis, MO, USA). The new PCR Blunt II vector was

digested for 1 hour at 37ºC with *EcoRI* enzyme (Thermo Fisher Scientific, Waltham, MA, USA) in order to verify that the gene had in fact been inserted into the vector. Singe DNA consists of a negatively charged sugar phosphate-backbone, gel electrophoresis was used to separate the bands of the digest. If bands were the correct size on the gel, the entire plasmid would be sent to be sequenced at Eurofins Genomics (Eurofins, Louisville, KY, USA).

Once genes were verified to be the correct sequence, the PCR-Blunt II TOPO vector was used as the template for another PCR reaction. The purpose of this reaction was to add *notI* cut sites to the ends of the genes so that the gene could be inserted into the BetaConHygHGGT vector, which harbors the seed specific BetaConGlycinin promoter. The parameters for the PCR were as follows: initial denaturation at 95ºC, 32 cycles of denaturing at 95ºC for 30 seconds, annealing varied from 57 - 63 ºC, extension at 72ºC for 2 min 30 seconds, and final extension at 72ºC for 4 min. Once PCR was completed the *notI* enzyme was added directly to the pcr product mix and was digested for 1hr at 37ºC. The BetaConHygHGGT vector was also simultaneously digested at 37 ºC. Once digested both reactions were run on a 1% agarose gel with ethidium bromide. The sizes of the digested PCR fragments are as follows: DGAT1 1622bp, DGAT2 1595bp, LPAT1 1121bp, LPAT2 1166bp, and digested BetaConHygHGGT vector was 7167bp.

Oligo Name	*Oligo Sequence (5' to 3')*
TL-DGAT1-NotIF	ATTTAAGATATACTGCGGCCGCAAAATGGCGATCCTGG
TL-DGAT1-NotIR	TTTTAGTTCATACTGCGGCCGCCTATGGGGCGCT
TL-DGAT2-NotIF	AGATATACTGCGGCCGCAAAATGGCGATCCTGG
TL-DGAT2-NotIR	GTTCATACTGCGGCCGCCTATGTAGCGCT
TL-LPAT1-NotIF	CCGCGGCCGCGAATTCATGGAATTATCTTTGCCCTTTATCGA
TL-LPAT1-NotIR	CTGCGGCCGCCTCGAGCTAGCAATAGCGAATGAGTTCAT
TL-LPAT2-NotIF	AGATATACTGCGGCCGCAAAATGGCGATTGCA
TL-LPAT2-NotIR	GTTCATACTGCGGCCGCTCAGTTTATCTTCTCTGC

Table 2 List of primers used for adding NotI restriction enzyme site to ends of each of the acyltransferase genes

The purpose of this was to insert our gene of interest in between the Beta-Conglycinin promoter and phaseolin terminator. The Beta-Conglycinin promoter has shown to be a very strong seed specific promoter and is often used in agricultural biotechnology studies [64]. Once ligated into BetaConHygHGGT vector, the entire cassette was digested out via *AscI* and ligated into the *AscI* site of pBinGlyBAR1+cFAE1 RNAi [65]. This same method was repeated 4 times for each of the acyltransferase genes (LPAT1, LPAT2, DGAT1, DGAT2)

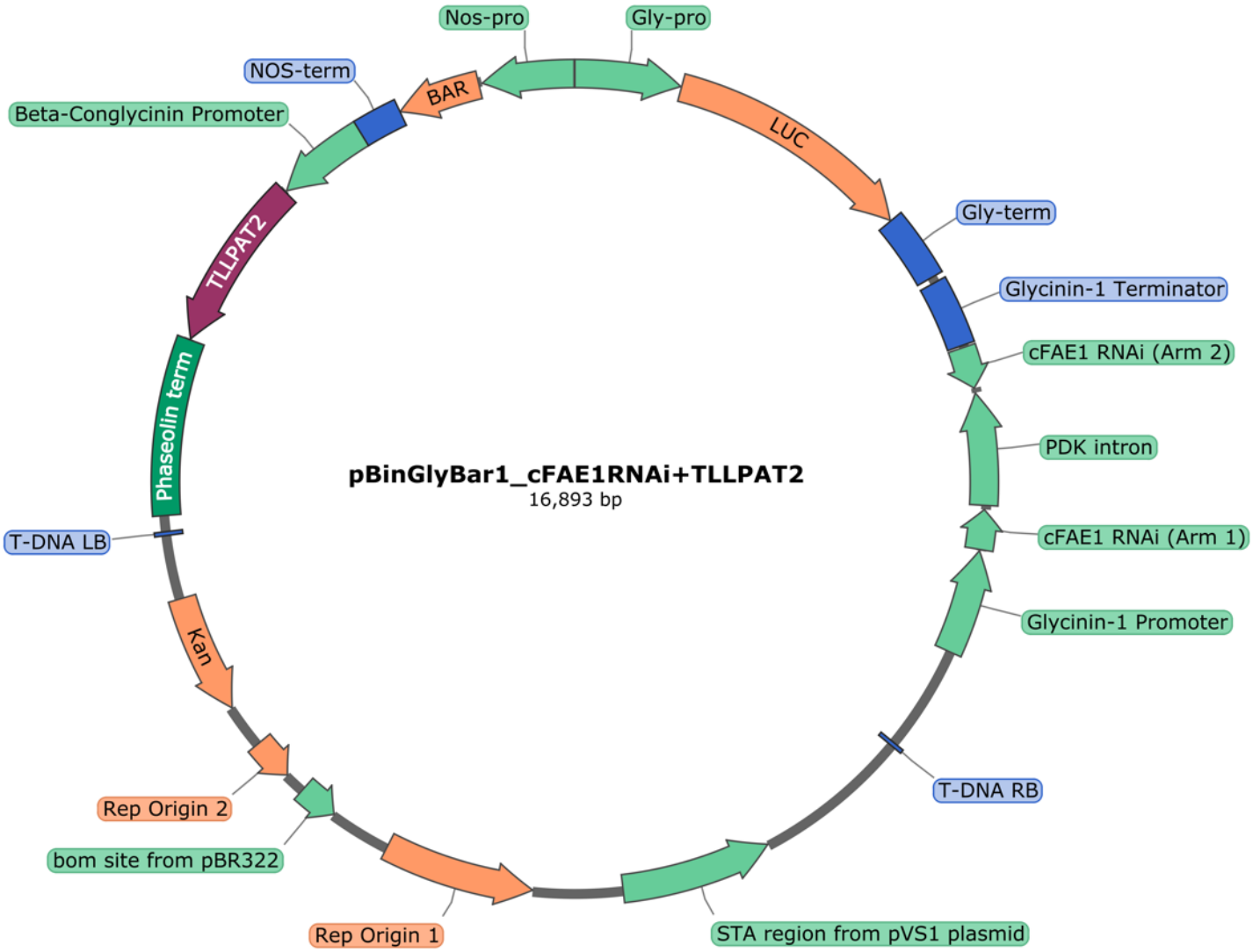

Figure 7 Example of construct used in this study. This vector map is showing the TL-LPAT2 acyltransferase gene that has been inserted between the phaseolin seed specific promoter/terminator cassette. The backbone of this construct also possesses the camelina *cFAE1* RNAi hairpin.

3.3 Camelina Transformation

Camelina transformation has a well-established and robust protocol that was first developed by the Lu lab [62]. Camelina was planted roughly one month before transformation, the ideal time to transform camelina at an early flowering stage when many large buds are present [66]. The camelina used for this project had previously been engineered to contain both the *T .laurifolia* Δ6 desaturase and also the *T. laurifolia* fatty acid thioesterase A (FatA). This specific line had been grown to homozygosity and the selection marker used for that project was dsRed. The transformation efficiency is normally very low, it has been reported to be around 1% [62]. Once plants were ready for transformation 500 mL of *Agrobacterium* containing the pBinGlyBAR1+cFAE1 vector with each acyltransferase gene was grown in liquid LB with rifampicin (50mg/mL) and kanamycin (100mg/mL) in a 28 ºC rotating shaker, the bacteria were allowed to grow overnight. The following morning the OD_{600} value of the culture was measured via the spectrophotometer, if the OD value was 0.8-1.5 then the transformation process could commence. Once grown to an acceptable level the *Agrobacterium* solution was divided into 200mL centrifuge tubes and spun down in a Beckman Coulter Avanti J-26 XPI Centrifuge at 4000 RPM for 15 min (Beckman Coulter, West Sacramento, California, USA). Pink *Agrobacterium* residues were observed for each tube and the supernatant was discarded. The *Agrobacterium* pellet was resuspended in an infiltration solution. The volume of infiltration solution used was always double the volume used to grow *Agrobacterium* (for example in this experiment, we grew 500 mL of agro so we make 1L of infiltration solution) the infiltration solution

contained 2.2g MS Media, 50g sucrose, 200 µl Silwett L77 surfactant, and 1L Q-pod Milli Q ultrapure water (Millipore Corporation, Billerica, Massachusetts, USA). Camelina plants were placed in a techni-dome vacuum desiccator chamber (Bel-Art – SP Scienceware, Wayne, NJ, USA) a container containing the resuspended *Agrobacterium* in infiltration solution was placed in the middle of the container to submerge all inflorescences during transformation. Plants were held inside the chamber with sustained vacuum pressure for five min. After this, the pressure valve was opened slowly as to not damage the plant tissue. After the transformation treatment, the plants were laid down horizontally in flats and covered with a black sheet and allowed to sit overnight. The next day the plants were placed back in the same greenhouse, and carefully watered without wetting the flower buds. The same transformation protocol was repeated 7-10 days later to increase transformation efficiency.

3.4 Screening and Confirmation of Transgenic Plants

Camelina plants were allowed to complete their lifecycle and when seed capsules turned a light brown color they were harvested. The harvesting of the *Agrobacterium* treated plants was done with a 1.7 mm sieve. Since the selection marker used in this project was basta resistance, the entire tray of transformed camelina was harvested and then immediately spread onto flats so that the plants could germinate and be sprayed with basta for selection. Basta resistant two week old seedlings that showed resistance to .01% basta were transplanted into 3.5” x 3.5” pots and allowed to complete their lifecycle.

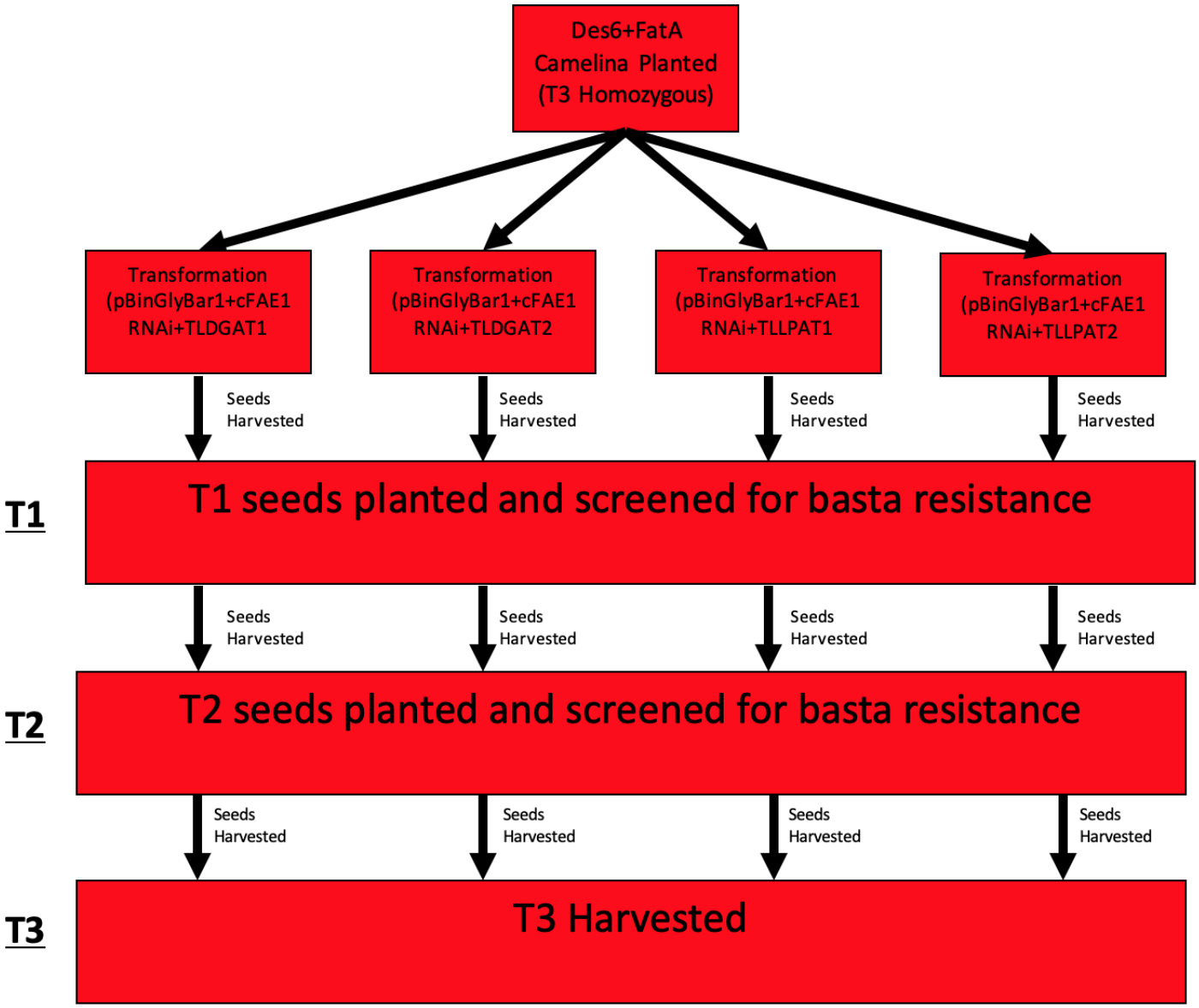

Figure 8 Experimental Design Overview

3.5 Fatty Acid analysis

For fatty acid analysis, 5 camelina seeds were placed in a ThermoFisher 8 mL test tube. Because the target fatty acid of this project is 18:1Δ6 which is an isomer of the very abundant oleic acid (18:1Δ9) we needed to figure out a way to separate these two. Our first approach to this was to change the column to a 60 m length x 0.25 mm inner diameter HP-88 column (Agilent, Lexington, MA, USA). In order to separate the fatty acids from the glycerol backbone, a transesterification reaction is needed. Normally, fatty acid methyl esters (FAMEs) are preferred for gas chromatography-flame ionization detection (GC-FID) analysis. However, since the fatty acid we are trying to quantify is so similar to oleic we needed to create isopropyl esters to allow for better separation between the 18 carbon isomers.

To prepare the seeds for analysis, 1 mL of isopropanol with 1.5% H_2SO_4 was added to the test tube and seeds were crushed. The tube was then tightly capped and placed on a heat block set to 98 ºC for 1 hour. After 1 hour had passed test tubes were taken off the heat block and allowed to cool. Once cool, 1 mL of H_2O and 1 mL of heptane was added to the tube. The tube was then shaken and spun down to separate, once separated the top layer was taken off and this was run through the GC-FID (7890A GC System Agilent Technologies).

In an effort to further verify the position of the double bond, the fatty acid isopropyl esters were derivatized by reaction with dimethyl disulfide in the presence of iodine as previously described [67]. In this reaction, the dimethyl disulfide reacts directly

with the double bond. As a result, the mass spectra obtained from these derivatives contain, in addition to the molecular ion, at least three abundant ions diagnostic for the position of the double bond (Figure 9). These ions include those of fragments corresponding to the portions of the molecule on either side of the double bond and an ion formed by the loss of methanol.

To quantify the amount of specific fatty acids at the *sn-2* position, TAG was column purified (Superclean™ LC-Si SPE 3 mL Tube, Sigma Aldrich, St. Louis, MO). Once pure TAG was obtained samples were dried down under nitrogen and then redissolved in 1 mL of ethyl ether. To this test tube, 800 µl of 5mM borate buffer pH 7.8 was added and then 200 µl of lipase from *Rhizomucor miehei* was added. The entire reaction was placed in the 37 ºC incubator and shaken at 350 rpm. Every 15 min the tube was taken out and given a good shake by hand. After 1 h the tube was removed, 1.5 mL of 2:1 (v/v) methanol chloroform was added, followed by 0.5 mL chloroform. The tube was shaken well and spun down for 5 min at setting 4 on the clinical centrifuge. The upper phase was discarded, the lower phase was recovered and a few drops of ethanol was added. The solution was dried under nitrogen and resuspended in ~100 µl of 6:1 chloroform:methanol. Now the digested TAG solution needed to be ran on a 5 x 20 cm silica TLC plate in order to visualize the separation of the MAG (monoacylglycerol), DAG (diacylglycerol), and undigested TAG. The solvent system used was 20:80 (v/v) heptane:ethyl ether. The plate was ran about ½ to 2/3 of its length. Once separated, the MAG and DAG could be scraped off the plate, transesterified, and analyzed on the GC as previously described.

4 Results

4.1 Genetic Engineering, Confirmation, and Analysis

DGAT and LPAT enzymes are responsible for the addition of fatty acids onto the glycerol backbone. Recently, it has become increasingly clear that these enzymes are highly specific for a substrate that are produced by their host species. For example, coconut (*Cocos nucifera*) mainly produces short chain saturated fatty acids like lauric acid (12:0, 49% of total F.A). When the LPAT enzyme from coconut was expressed in camelina, the transgenic seeds showed inclusion of lauric acid at the *sn*-2 position of TAG [68], which naturally does not occur in WT camelina. This is also an interesting result because saturated fatty acids are not normally found at the *sn*-2 position of the TAG molecule, leading to the hypothesis that the LPAT enzyme is extremely substrate specific.

Previously our lab has done work on putting the *T. laurifolia* FatA and the Δ6 desaturase into camelina. The result of this work is was ~9% 18:1Δ6 production in camelina. To further build on this work we decided that the next thing that needed to be done is to try to knock down the FAE1 activity in camelina to increase carbon flux to the medium chain fatty acids such as petroselinic, while simultaneously knocking down undesirable VLCFAs. To do this we chose the vector pBinGlyBar1+cFAE1 RNAi [65] to be the building point for further experiments.

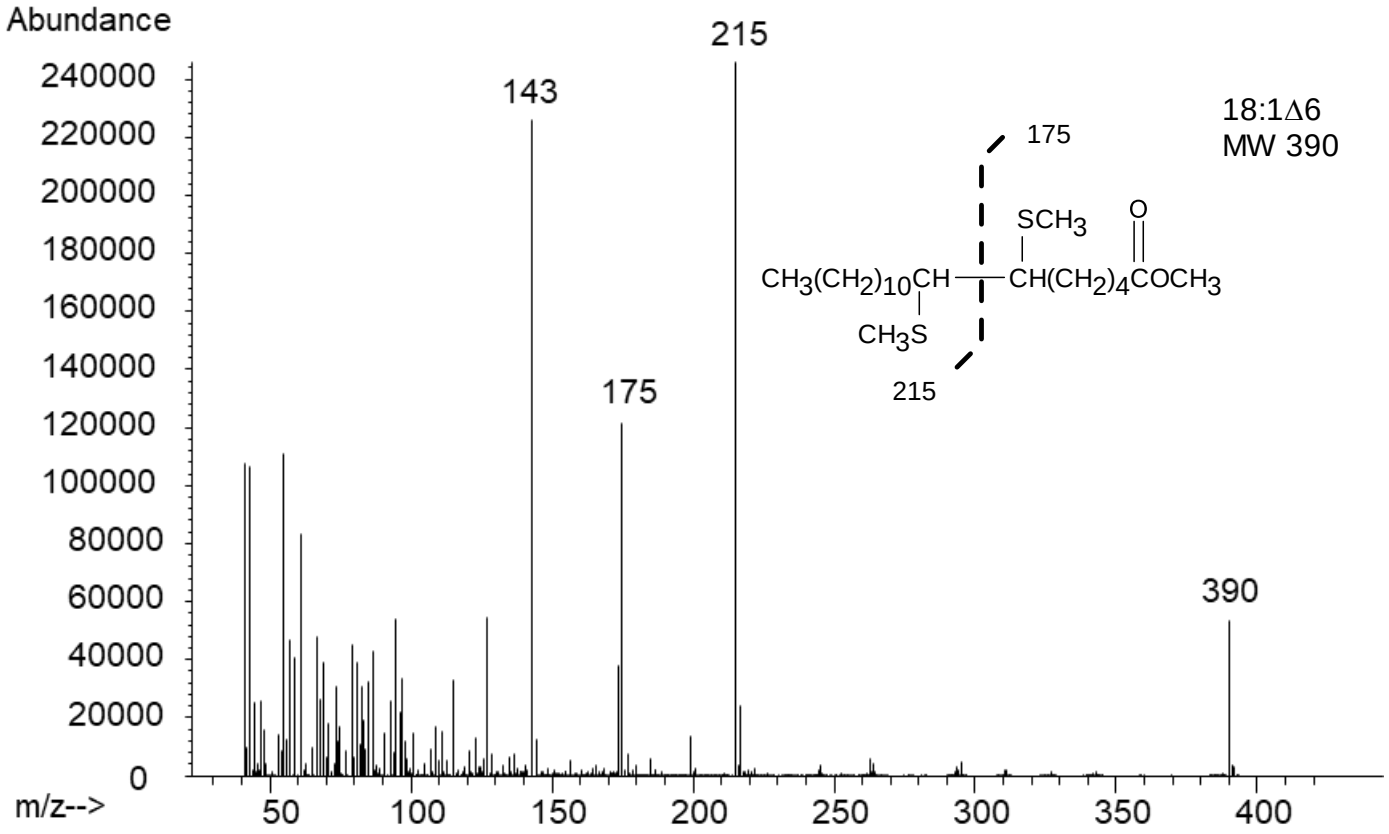

Figure 9 Mass spectra of dimethyl disulfide derivatized fatty acid isopropyl esters obtain from the seeds of *T. laurifolia*

Fatty acid elongase 1 from camelina (*cFAE1*) catalyzes the rate limiting step in VLCFA production by extending the length of fatty acids from 18 up to 22 carbons long. Malonyl-CoA and two carbon molecules are used to elongate oleic acid (18:1Δ9) to eicosenoic acid (20:1Δ11) by the action of FAE1, this reaction is carried out in the acyl-CoA pool. Further extension by FAE1 can also occur, this reaction results in the product of euricic acid (22:1Δ13).

When the *cFAE1* RNAi knockdown vector was introduced into camelina we saw a decrease in VLCFA of ≥20 carbons. In WT camelina the levels of 20:1Δ11 are between 11-12% whereas with the *cFAE1* RNAi construct they drop down to around 4% (Figure 10). This confirms to us that our FAE1 knockdown is in fact working and we have knocked down FAE1 below the levels at which it normally operates. The transformed plants with decreased VLCFA appeared healthy and showed no signs of a fitness disadvantage compared to WT camelina.

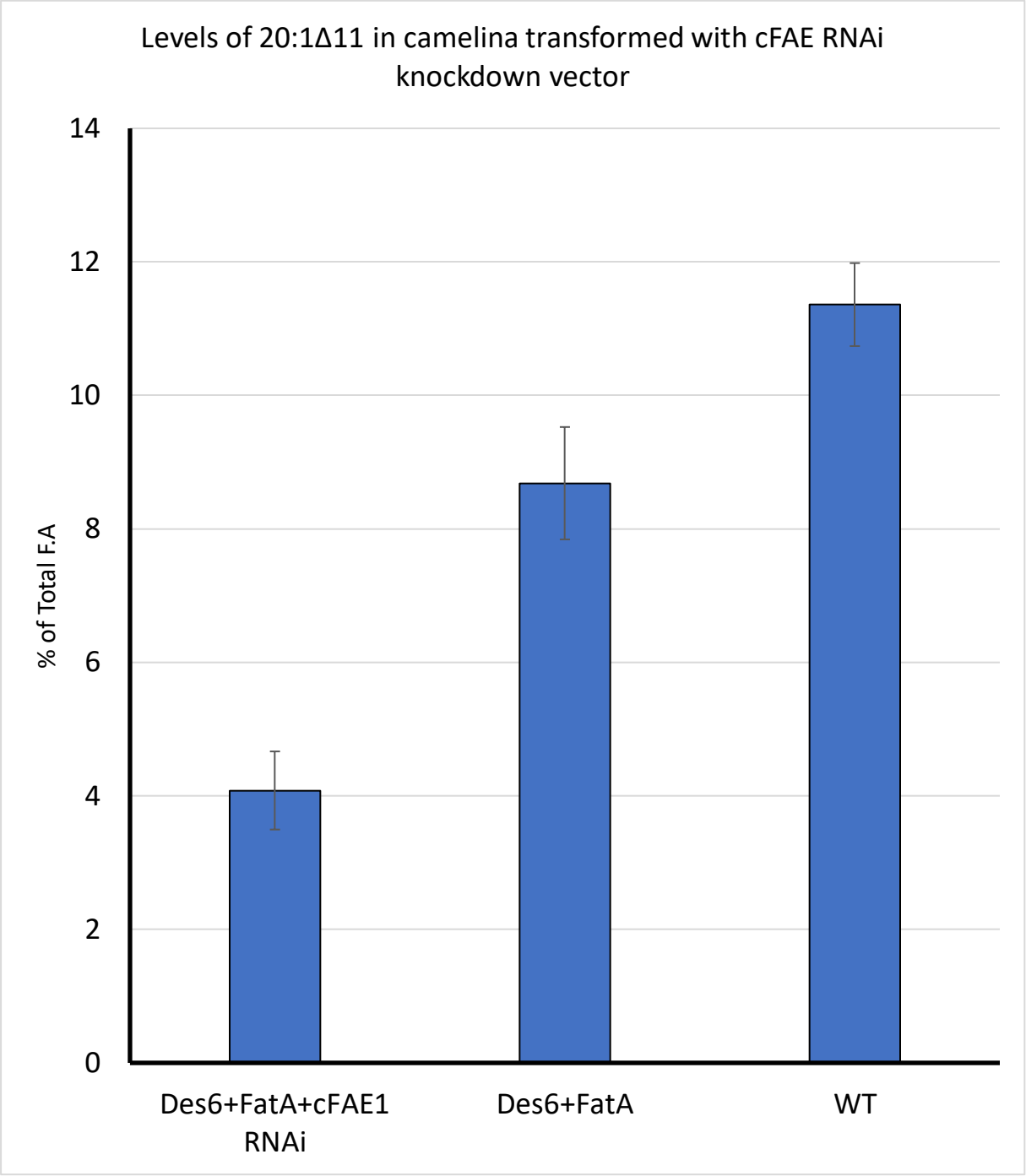

Figure 10 Seeds from T3 plants expressing the *cFAE1* RNAi hairpin were subjected to fatty acid isopropyl ester analysis and relative content of 20:1Δ11 is expressed as the percentage of total fatty acids. Single seed samples were analyzed.

	16:0	18:0	18:1Δ6	18:1Δ9	18:2	18:3	20:0	20:1Δ8	20:1Δ11	22:1Δ10	18:1Δ6 derived F.As
FAE1(9-1)A	7.73%	5.44%	9.51%	3.41%	7.93%	27.53%	4.974%	4.97%	4.70%	1.55%	16.03%
FAE1(9-1)B	8.07%	6.62%	11.79%	4.05%	7.72%	26.39%	6.165%	6.17%	4.29%	1.39%	19.35%
FAE1(9-1)C	9.91%	6.97%	10.51%	2.64%	8.74%	27.08%	5.864%	5.86%	4.01%	1.08%	17.46%
FAE1(9-1)D	10.40%	7.31%	10.51%	2.77%	9.17%	28.42%	6.155%	6.16%	4.21%	1.14%	17.80%
FAE1(9-1)E	7.87%	6.74%	10.77%	3.64%	8.37%	25.72%	5.898%	5.90%	4.52%	1.40%	18.07%
FAE1(5-5)A	7.98%	5.35%	11.10%	3.09%	7.27%	28.75%	4.557%	4.56%	3.49%	1.63%	17.29%
FAE1(5-5)B	8.57%	6.35%	10.55%	4.58%	8.38%	29.38%	4.39%	4.39%	3.66%	1.09%	16.03%
FAE1(5-5)C	7.92%	5.19%	10.95%	3.64%	6.77%	34.04%	3.965%	3.97%	3.04%	1.30%	16.22%
FAE1(5-5)D	8.12%	5.62%	11.59%	3.45%	6.83%	32.80%	4.489%	4.49%	3.39%	1.13%	17.21%
FAE1(5-5)E	7.47%	5.29%	9.39%	4.95%	7.94%	34.85%	3.739%	3.74%	5.23%	0.90%	14.04%
FAE1(3-3)A	6.51%	4.64%	10.25%	2.72%	7.61%	32.50%	4.373%	4.37%	4.03%	1.67%	16.29%
FAE1(3-3)B	7.53%	6.48%	8.42%	4.37%	8.58%	29.33%	5.155%	5.15%	4.51%	1.29%	14.86%
FAE1(3-3)C	6.28%	5.39%	9.32%	3.70%	8.94%	31.31%	5.425%	5.42%	4.47%	1.64%	16.39%
FAE1(3-3)D	6.20%	4.66%	8.89%	3.33%	9.16%	32.67%	4.556%	4.56%	3.46%	1.59%	15.03%
FAE1(3-3)E	6.58%	4.52%	8.99%	4.01%	9.25%	31.18%	3.412%	3.41%	4.20%	1.44%	13.84%
TLDes6+FatA	9.94%	6.00%	9.05%	6.23%	9.36%	27.55%	5.637%	5.84%	8.684%	1.37%	16.25%
WT	8.34%	3.55%	0.00%	13.98%	14.18%	35.03%	1.802%	0.00%	11.36%	0.00%	0.00%

Table 3 Single seed analysis of camelina seeds expressing *cFAE1* RNAi knockdown the seeds were subject to fatty acid isopropyl analysis and the relative content of each fatty acid is expressed as a percentage of total lipids derived from the seed

In order to further test this hypothesis that specialized acyltransferase genes are needed to facilitate storage of 18:1Δ6 into TAG, we constructed vectors harboring both DGAT and LPAT genes obtained from *T. laurifolia*. Transcriptome analysis was done on *T. laurifolia* by previous members of the Cahoon Lab to identify said genes. This data revealed two DGAT genes and two LPAT genes from *T. laurifolia* which are the genes tested in this study. The *T. laurifolia* biosynthetic genes were cloned into the pBinGlyBAR1+cFAE1 RNAi vector and were transformed as previously described [62]. The camelina used for transformation already had been engineered with a Δ6 desaturase and fatty acid thioesterase (FatA) from *T. laurifolia* these plants were homozygous. Selection of positive transformants from the T_1 generation was carried out by using 0.01% basta solution, plants showing Basta resistance and dsRed fluorescence were grown up to T_2, and seeds were analyzed for their fatty acid content. We transformed a total of five different plasmids into the camelina (Table 2).

Plasmid Name	*# of T2 Lines obtained after basta selection*
pBinGlyBar1+cFAE1 RNAi	14
pBinGlyBar1+cFAE1 RNAi+TLDGAT1	3
pBinGlyBar1+cFAE1 RNAi+TLDGAT2	26
pBinGlyBar1+cFAE1 RNAi+TLLPAT1	19
pBinGlyBar1+cFAE1 RNAi+TLLPAT2	11

Table 4 Vectors constructed in this study and transgenic lines obtained after *Agrobacterium* transformation and basta resistance selection

Seeds were harvested from all surviving T_2 lines that showed basta resistance. To evaluate the function of the fatty acid biosynthetic genes that were inserted red seeds from each of the lines were analyzed by gas chromatography. It is expected that the background Δ6 desaturase and FatA are linked to the dsRed reporter gene. The first transformations we did were all built using a vector harboring camelina FAE1 RNAi hairpin. As stated before this vector was assembled as previously described [65]. The results from the T_2 generation are listed below in Table 3. As stated before, the data in Table 3 represent 5 red seeds that were selected from each line for analysis. The lines listed in Table 3 were all replanted for the next generation. The level of 18:1Δ6 produced in each line is also shown in Figure 11. In terms of 18:1Δ6 produced, the tops lines only produced about 13% petroselinic. However, the standard deviation between the lines was quite high at 2.5%. It should be noted however that at this point in the experiment not all lines were homozygous.

	16:0	18:0	18:1Δ6	18:1Δ9	18:2	18:3	20:0	20:1Δ8	20:1Δ11	22:1Δ10
cFAE1RNAi3	8.8%	8.2%	11.4%	5.0%	11.0%	25.0%	7.4%	5.9%	4.3%	1.6%
cFAE1RNAi5	9.9%	9.8%	6.8%	8.6%	18.2%	21.4%	7.3%	3.5%	7.1%	0.8%
cFAE1RNAi9	9.1%	8.8%	9.8%	6.7%	12.8%	24.9%	6.9%	4.9%	6.0%	1.2%
cFAE1RNAi+LPAT1(5)	9.8%	5.9%	13.1%	2.7%	9.0%	31.3%	3.7%	6.7%	6.2%	1.7%
cFAE1RNAi+LPAT1(7)	9.9%	8.2%	7.5%	9.3%	13.0%	33.4%	2.2%	3.2%	6.6%	0.7%
cFAE1RNAi+LPAT1(9)	12.6%	10.9%	6.0%	9.2%	12.3%	28.5%	8.0%	1.2%	6.1%	0.2%
cFAE1RNAi+LPAT1(11)	8.7%	6.1%	13.0%	4.0%	7.6%	32.6%	3.6%	6.6%	5.6%	1.8%
cFAE1RNAi+LPAT2(2)	9.0%	8.6%	11.8%	5.6%	11.0%	23.7%	7.7%	6.2%	4.5%	1.8%
cFAE1RNAi+LPAT2(3)	11.6%	8.5%	6.4%	8.1%	21.4%	25.9%	3.4%	1.9%	6.3%	1.8%
cFAE1RNAi+LPAT2(7)	9.7%	7.8%	12.6%	5.2%	9.0%	27.4%	5.6%	6.3%	4.0%	1.8%
cFAE1RNAi+LPAT2(10)	9.1%	9.0%	8.9%	7.5%	15.5%	24.4%	6.4%	3.9%	5.7%	0.9%
cFAE1RNAi+DGAT1(1)	10.7%	7.4%	13.0%	13.0%	13.0%	35.3%	1.3%	2.6%	7.7%	0.3%
cFAE1RNAi+DGAT1(2)	17.5%	12.8%	11.6%	11.6%	14.4%	31.8%	1.2%	2.9%	0.2%	0.5%
cFAE1RNAi+DGAT1(3)	10.6%	7.6%	12.6%	4.6%	9.4%	31.1%	3.5%	4.9%	4.2%	2.0%
cFAE1RNAi+DGAT2(6)	8.6%	5.6%	11.7%	4.2%	7.2%	33.9%	3.6%	6.5%	3.9%	1.8%
cFAE1RNAi+DGAT2(15)	8.5%	5.5%	12.1%	3.1%	6.5%	34.2%	3.3%	6.5%	5.0%	1.5%
cFAE1RNAi+DGAT2(16)	11.1%	8.6%	13.3%	6.0%	9.6%	35.1%	1.4%	3.3%	3.7%	0.7%
cFAE1RNAi+DGAT2(20)	8.6%	5.9%	11.8%	3.1%	8.3%	31.3%	5.1%	7.5%	4.2%	2.3%
WT	8.1%	4.0%	0.0%	13.6%	14.7%	35.0%	1.3%	0.0%	11.4%	0.0%
Des6+FatA	9.3%	6.8%	8.8%	6.1%	8.6%	23.4%	5.6%	4.3%	7.8%	1.0%

Table 5 Fatty acid profile of top T2 lines

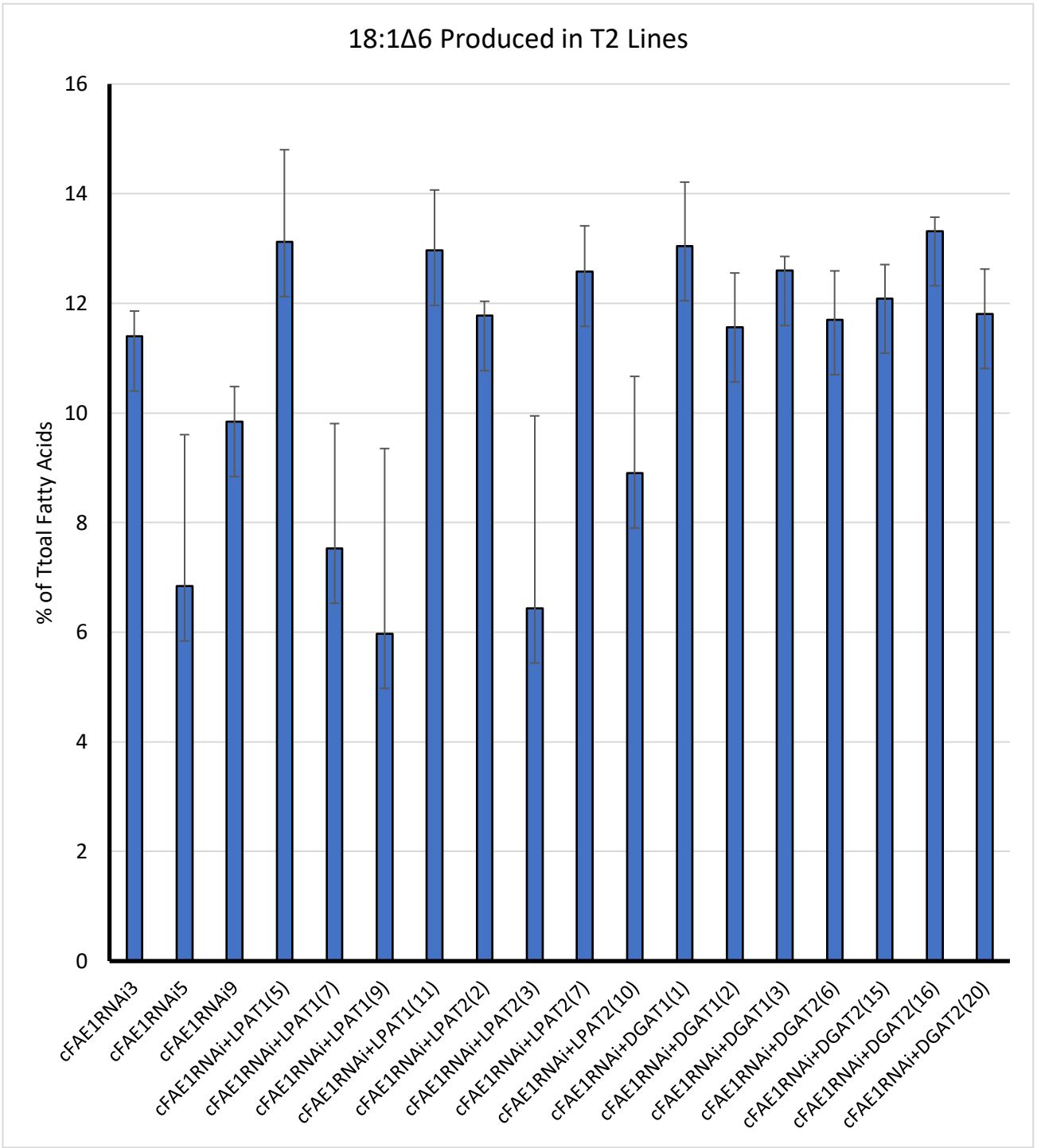

Figure 11 Seeds from T2 plants with the designated transgenes were subjected to fatty acid isopropyl analysis, and the relative content of 18:1Δ6 expressed as the percentage of total fatty acids. Five seeds from each plant were analyzed and expressed as a mean +/- S.E.M (Standard Error of Mean).

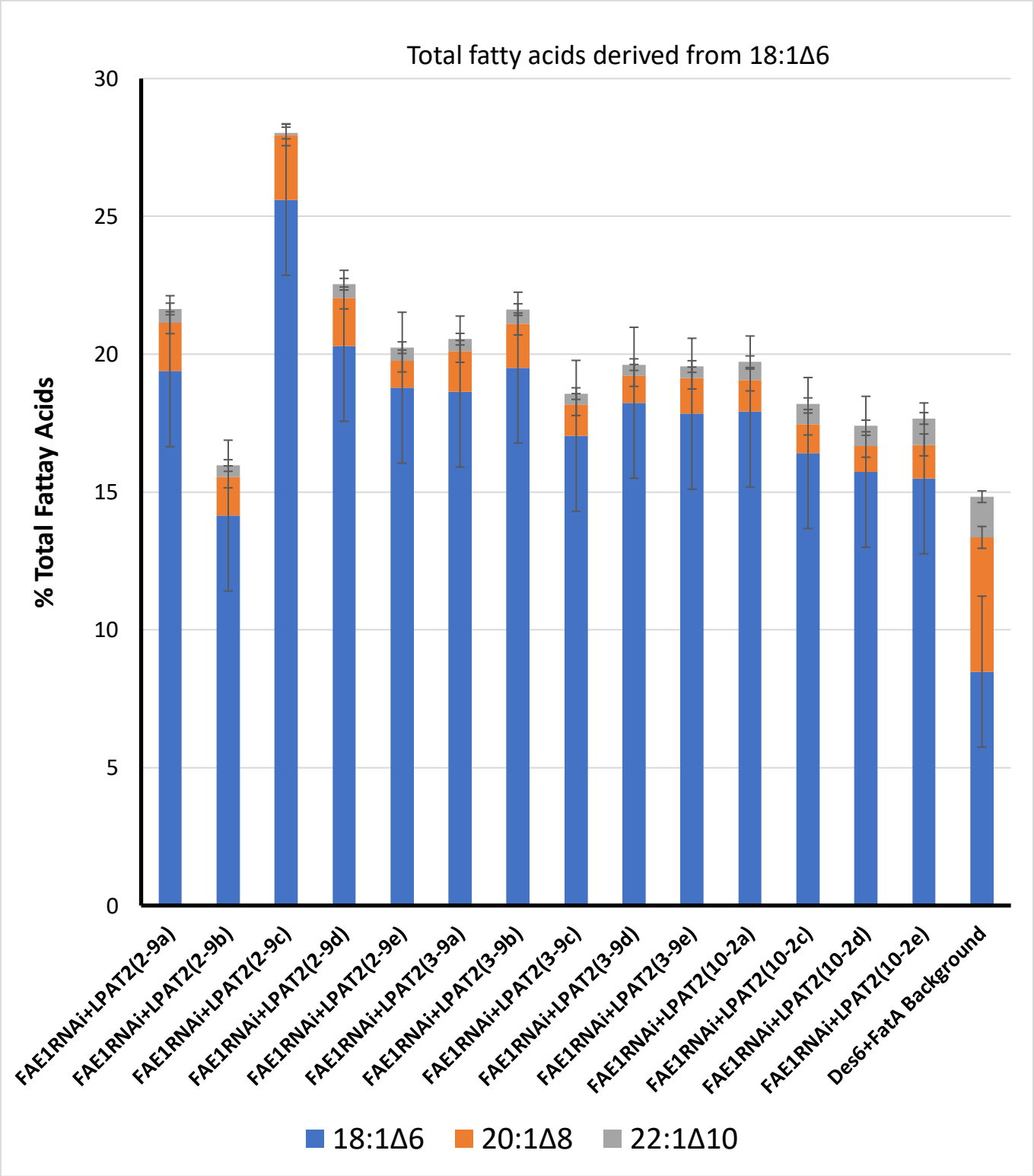

Figure 12 Seeds from best performing T_2 lines expressing the TL-LPAT2 gene. Seeds were subject to fatty acid isopropyl ester analysis and relative content of each fatty acid is expressed as the mole percentage of total fatty acids. five seeds of each plant were analyzed and expressed as mean +/- S.E.M.

For T_3 generation, 18 red seeds were planted from each of the T_2 lines shown in Table 3. The plants were grown in the greenhouse alongside WT control plants. The T_3 generation showed a considerable increase in the amount of petroselinic produced compared to T_2. This could be because for the T_3 generation we did some single seed fatty acid analysis compared to the T_2 generation where we just did bulk five seed analysis to determine which lines were the best. It could also be due to the fact of not all plants in the T_2 generation were homozygous for the inserted gene yet.

The gene that seemed to have the most impact on the production of 18:1Δ6 was the LPAT2, specifically the highest producing lines were (2-9, 3-9, and 10-2) (Figure 12). In all the lines shown in the graph, the levels of 18:1Δ6 were over 17% which is the highest ever reported in a transgenic plant to date. In addition to the increased production of 18:1Δ6, the levels of the 20 carbon long fatty acids were also decreased. The percentage of 20:1Δ11 was decreased from 11.3% in WT and 7.8% in the Des6FatA down to 4.33% on average in the T_3 $LPAT_2$ generation. Brown seeds were also analyzed from each of the lines listed below, however, they showed no FAE1 RNAi activity or LPAT2 activity. Due to the high levels of 18:1Δ6 we felt that single seed analysis was needed to be done on the seeds in order to get a better idea of the fatty acid profile.

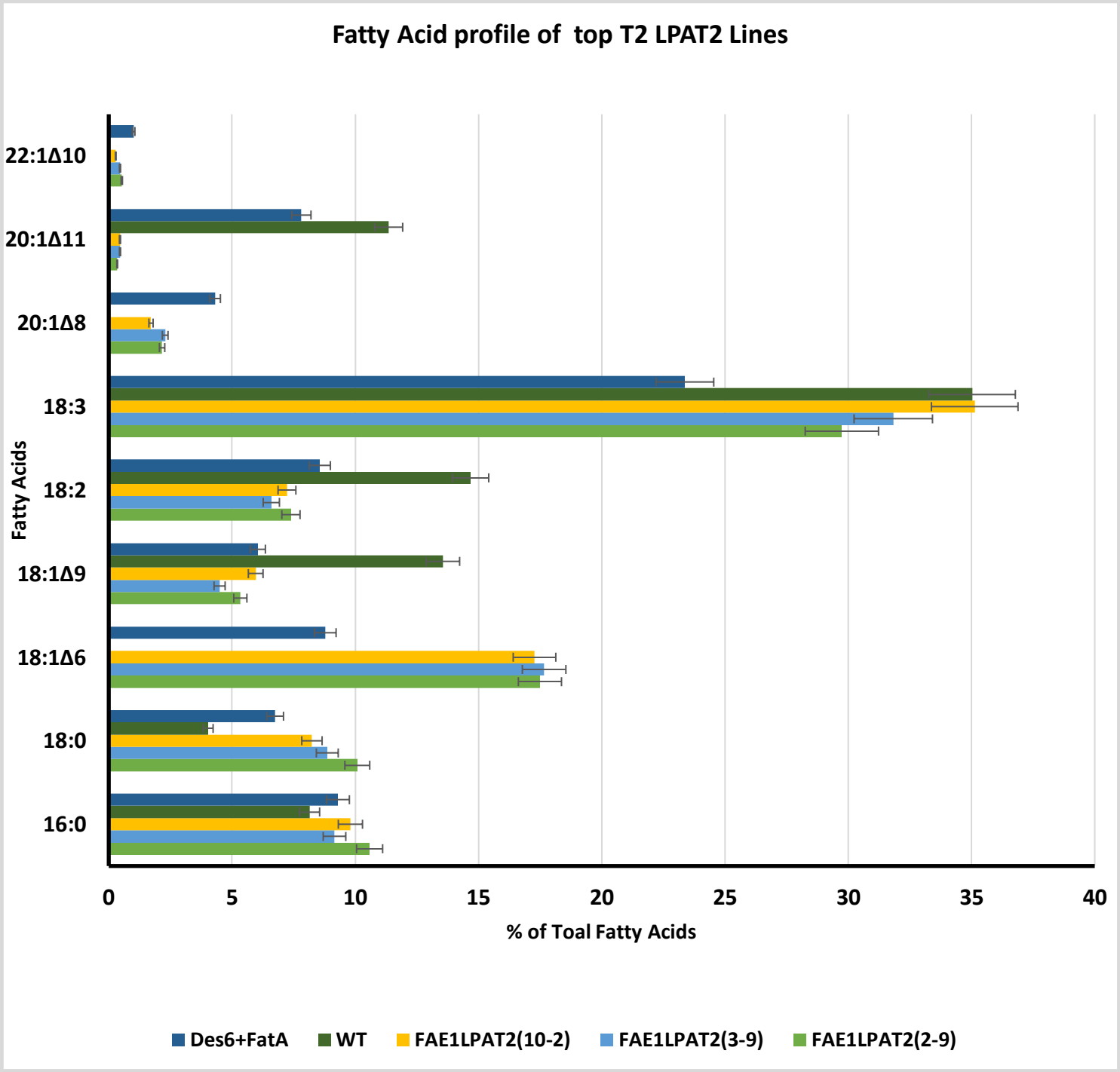

Figure 13 Single seeds from the top performing plants expressing the TL-LPAT2 gene were subject to fatty acid isopropyl ester analysis. The relative content of 18:1Δ6, 20:1Δ8, and 22:1Δ10 is expressed as a percentage of the total fatty acid content of each seed. Five single seeds were selected form each of the 3 lines were analyzed and expressed as mean +/- S.E.M.

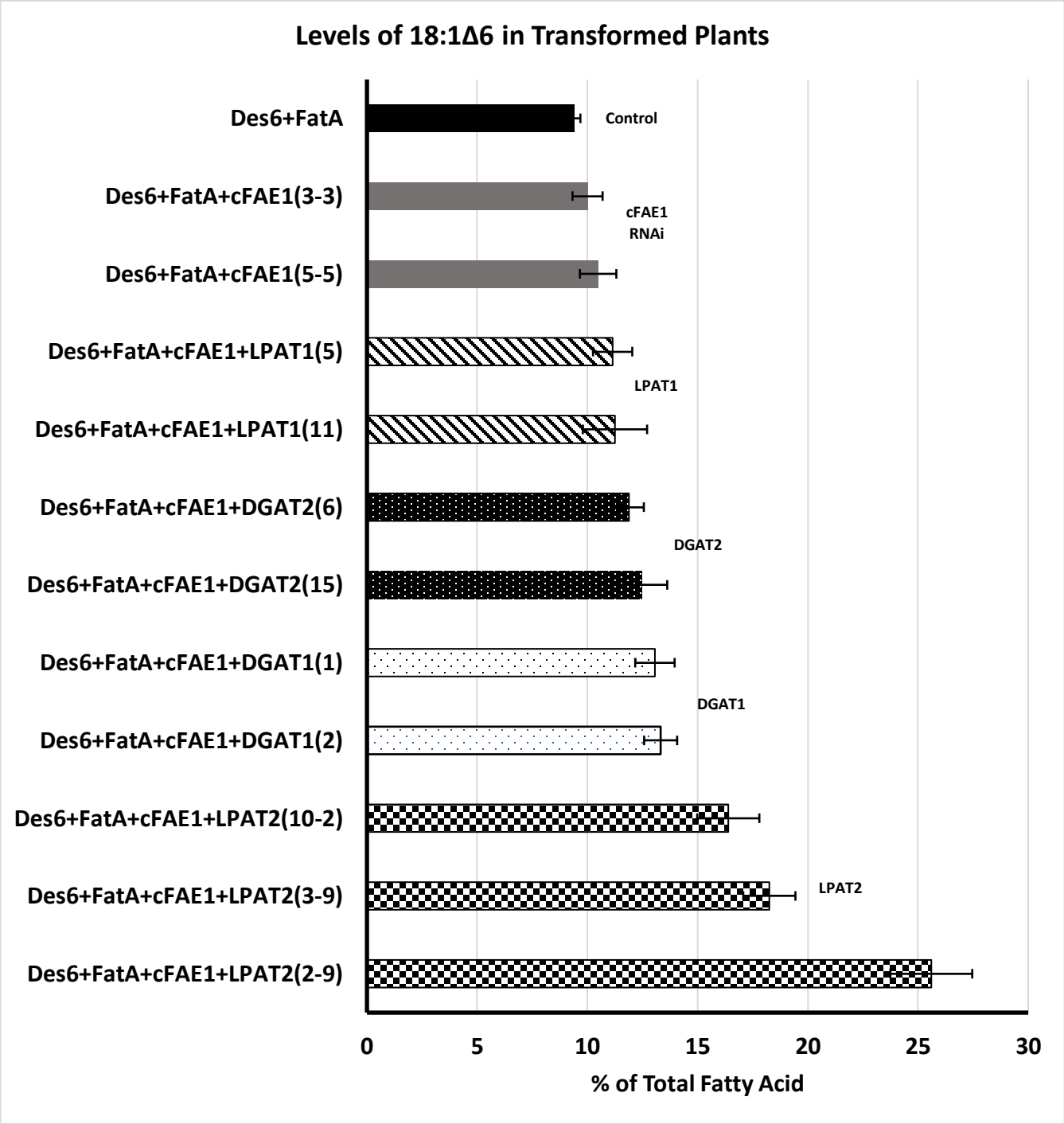

Figure 14 Single seeds were subject to fatty acid isopropyl ester analysis and the relative content of 18:1Δ6 is expressed as a percentage of the total fatty acids obtained from each seed. Varying amounts of single seeds were analyzed from each line and are expressed as mean +/- S.E.M.

In the single seed analysis of the T_2, we were surprised to see that in one of our samples the total fatty acids derived from 18:1Δ6 accounted for 28% of the total fatty acids in the seed (Figure 12). This is almost a two-fold increase compared to the background Des6+FatA construct which only produced 14%.

In order to further confirm the activity of the LPAT2 enzyme TAG was column purified and then digested via *R. miehei* lipase (Sigma Aldrich, St. Louis, MO). This digestion yields MAG, DAG, and TAG for our purposes we are mostly interested in the MAG and DAG that is produced from this reaction because this enzyme favors the two outside (*sn*-1 and *sn*-3) positions of the TAG molecule. Therefore the MAG that is leftover from this reaction should give us an accurate representation of which fatty acids are being esterified to the TAG molecule at the *sn-2* position. Unsurprisingly, when we added in the *T. laurifolia* LPAT2 we saw an increase of 18:1Δ6 at the *sn-2* position from 2.4% in the background to 14% in the plants with the specialized acyltransferase gene (Figure 15).

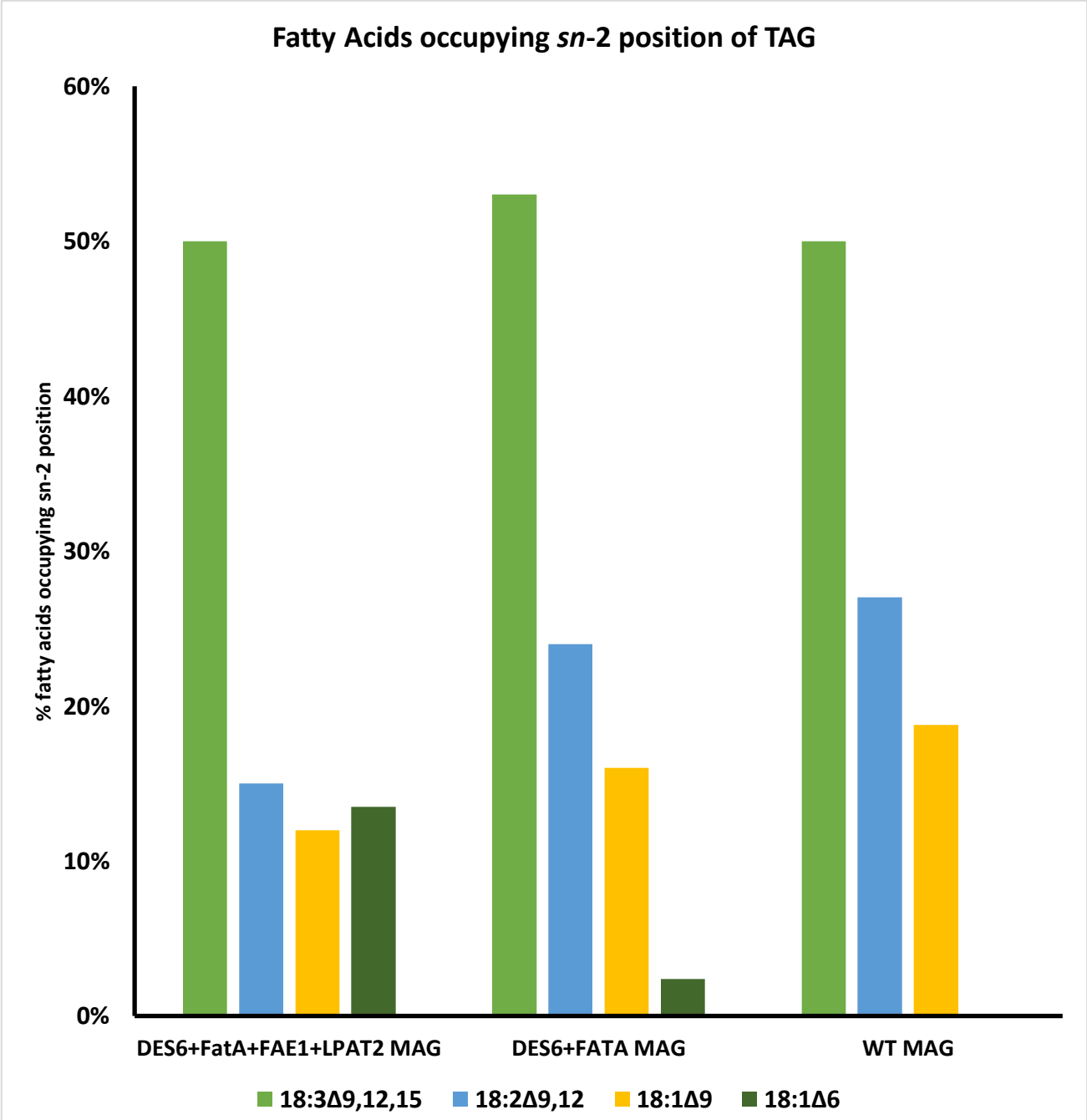

Figure 15 Results of *Rhizomucor miehei* lipase digest. 20 seeds from each line were subject to column chromatography in order to obtain pure TAG. The pure TAG was then digested via R. miehei lipase and the resulting MAG, DAG, and TAG was separated on TLC silica plates. Upon separation the MAG, DAG, and TAG was scraped off the plates and transesterified to create fatty acid isopropyl esters for GC analysis. The relative percentage of 18:1Δ6 is expressed as a percentage of total fatty acids in TAG.

5 Discussion

Camelina is a promising crop for plant biotechnology research due to its low input requirements, ease of transformation, and our knowledge of its genome. In this study, we aimed to study the aspects of the fatty acid biosynthetic pathway that are seen as bottlenecks in the attempt to engineer plants to produce unusual fatty acids. Specifically, I focused on acyltransferase enzymes that facilitate the esterification of acyl-CoA fatty acids onto the glycerol backbone to form TAG. In addition to working with these enzymes, we also engineered a Δ6 desaturase and a specialized fatty acid thioesterase A that we used as the starting point for our experiments.

Vegetable oils are composed almost entirely of triacylglycerol molecules. These storage molecules consist of three fatty acids esterified to a glycerol backbone and they represent a renewable source of raw materials that can be easily and economically extracted from seeds. The fatty acid profile and their distribution in TAGs of plant oils determines the oil quality, physicochemical properties, and uses [69]. Over the past couple of decades, we have gained an increased understanding of plant lipid metabolism and its regulation, along with the characterization of fatty acid biosynthesis and how these fatty acids are then assembled into TAGs [70].

It is estimated that there are nearly 400,000 different plant species in the world. This represents a wealth of genetic information that is yet to be tapped into. With advances in sequencing technology, scientists have been able to mine these genomes for specialized fatty acid biosynthetic genes. Various genetic modification tools have been developed including gene editing and synthetic biology techniques, which allow the rapid

assembly of novel pathways in oilseed crops for commercial production of high levels of designer oils and high value compounds.

Up until the last ten years, most of this work was being done in *Arabidopsis thaliana* due to the ease of transformation, short lifespan, and our knowledge of its genome. However, this plant has poor agronomic traits such as small seed size and its inability to be used for large scale field cultivation. Camelina has emerged as a promising platform for testing out these specialized fatty acid biosynthetic genes because it possesses several valuable agronomic traits. Camelina has a relatively short lifecycle, low water, and fertilizer requirements, and seed oil content that is nearly double that of soybeans [45]. These traits along with the extremely easy and robust transformation method have made camelina an extremely attractive platform for rapid testing of fatty acid biosynthetic genes.

In this study, I concentrated on engineering camelina with the genes required to produce 18:1Δ6. Petroselinic acid is a positional isomer to oleic acid (18:1Δ9), it exhibits some attractive properties such as anti-aging and anti-inflammatory activity [71]. Further, this rare fatty acid displays significant potential for the chemical industry due to the unique position of its double bond. Oxidative cleavage leads to industrially interesting compounds lauric acid and adipic acid, the latter is an important precursor for nylon production with 2.5 billion kg being commercially produced annually. Along with the potential use in industry, 18:1Δ6 has a higher oxidative stability and increase melting temperature (30℃) compared to oleic acid (13.4℃).

Previous work by members of the Cahoon lab has shown that engineering camelina with a Δ6 desaturase and specialized fatty acid thioesterase from *Thunbergia laurifolia* resulted in 9% 18:1Δ6 production in the seeds of camelina. In an attempt increase production and further build out the fatty acid biosynthetic pathway we chose to transform these existing plants with specialized acyltransferase genes in order to increase the adoption of these unique fatty acids onto the TAG storage molecules. We transformed a total of 5 plasmids into the camelina engineered with the Δ6 desaturase and FatA (Table 2). We also decided to build all of our cloning constructs into a *cFAE1* RNAi knockdown vector [65]. The reasoning behind this was to knock down the unwanted VLCFAs that camelina produces while simultaneously increasing carbon flow to MCFA.

In plants, saturated and monounsaturated fatty acids are exported from the plastids to the cytosol following their release from fatty acid synthesis and ACP by either FatB or FatA. Once in the cytosol the fatty acids are linked to CoA where they will serve as substrates the GPAT, LPAT, and DGAT enzymes. Although GPATs and DGATs typically use both saturated and monounsaturated acyl CoA substrates, researchers have shown that LPAT is active with C16 and C18 mono and polyunsaturated acyl CoAs with *cis*-Δ9 unsaturation but much less active with saturated acyl CoAs and C18 and C22 unsaturated acyl CoAs with *trans*- Δ9 unsaturation or with *cis*-unsaturation in positions other than Δ9 [72, 73]. Based on these findings LPATs have often been researched in metabolic pathway engineering experiments because they are highly specific and are

often the limiting factor in getting seed oils with >66% of a single fatty acid onto TAG molecules [74].

Through my research, I was able to engineer camelina to produce ≥25% 18:1Δ6, this is the highest ever produced in a transgenic plant to date (Figure 14). We found that the most efficient enzyme in esterifying 18:1Δ6 onto TAG was the *T. laurifolia* LPAT2. This enzyme adds the specific fatty acid to the *sn*-2 position of the TAG molecule. To confirm that 18:1Δ6 was being added onto TAG at that position, we performed a digest of column purified TAG. The lipase used was isolated from the fungus *R. miehei*, this lipase works by first cutting the fatty acids on the outside *sn*-1 and *sn*-3 which leaves the *sn*-2 position of TAG still attached. When we ran the digested TAG from our best LPAT2 line compared to the controls in our experiment we saw that the fatty acid content of 18:1Δ6 at the *sn*-2 position increased from 2.4% to 13.5%, confirming that the LPAT2 was adding this unique fatty acid onto the TAG molecule.

Future experiments that combined the *T. laurifolia* LPAT2 with the DGAT1 would be interesting because DGAT1 was the next most efficient enzyme that we found (producing roughly 13% 18:1Δ6). Another idea for future experiments would be to either knockdown or knockout some of the endogenous camelina enzymes such as the Δ9 desaturase, it would be interesting to see how much of an effect this would have on the 18:1Δ6 production because we were still seeing a substantial amount of oleic acid being produced. Something we have started trying to do recently is to cross our top producing petroselinic line with a Crispr *FAE1* knockout line that we obtained from collaborators at Montana State University. The reasoning behind this is that we had previously shown

that knocking down the camelina *FAE1* RNAi helped increase 18:1Δ6 production, so if we could get this enzyme completely knocked out it could further increase 18:1Δ6. At the time of writing this thesis the crosses have been grown up to F2 generation and the *FAE1* alleles need to be screened in order to determine which lines are homozygous for the Crispr knockout.

In conclusion, the oilseed crop camelina has great potential in food and non-food applications. It is necessary to improve fatty acid composition in its oil to meet different economic and agronomic requirements. In this study I showed that a specialized lysophosphatidic acid acyltransferase from the species *Thunbergia laurifolia* is needed to produce high levels of the novel fatty acid, 18:1Δ6. The combination of a specialized Δ6 desaturase, fatty acid thioesterase A, lysophosphatidic acyltransferase, and also the knockdown of camelina FAE1 enzyme resulted in the production of greater than 25% 18:1Δ6 in camelina. This is the highest amount of 18:1Δ6 produced in a transgenic oilseed plant to date, and we hope that this research will help guide future biotechnologists in the pursuit of engineering the fatty acid biosynthetic pathway.

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