MODIFICATION OF SEED FATTY ACID COMPOSITION BY CRISPR/CAS9 TARGETING THE FATTY ACID ELONGASE1 IN CAMELINA SATIVA

by

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DEDICATION

I would like to dedicate my thesis to my mother and father who always support me to achieve my goals.

TABLE OF CONTENTS

TABLE OF CONTENTS CONTINUED

LIST OF TABLES

LIST OF FIGURES

Figure Page

LIST OF FIGURES CONTINUED

Figure Page

NOMENCLATURE

ACCase:	acetyl-CoA carboxylase
ACP:	acyl carrier protein
CoA:	coenzyme A
CPT:	CDP-choline:DAG cholinephosphotransferase
DAG:	diacylglycerol
DGAT:	acyl-CoA:DAG acyltransferase
DHAP:	dihydroxyacetone phosphate
ER:	endoplasmic reticulum
FAE1:	fatty acid elongase1
FAS:	fatty acid synthase
FatA:	fatty acyl thioesterase A
FatB:	fatty acyl thioesterase B
FAX1:	fatty acid exporter1
G3P:	*sn*-glycerol-3-phosphate
G3PDH:	*sn*-glycerol-3-phosphate dehydrogenase
GlyceroPC:	glycerophosphocholine
GPAT:	glycerol-3-phosphate acyltransferase
GPCAT:	glycerophosphocholine acyltransferase
KASII:	β-ketoacyl-ACP synthase II
LACS:	long-chain acyl-coenzyme A synthetase
LPA:	lysophosphatidic acid

NOMENCLATURE CONTINUED

LPAAT:	lysophosphatidic acid acyltransferase
$LPCAT^F$:	forward action of lysophosphatidylcholine acyltransferase
$LPCAT^R$:	reverse action of lysophosphatidylcholine acyltransferase
LPCT:	lysophosphatidylcholine transacylase
LysoPC:	lysophosphatidylcholine
PA:	phosphatidic acid
PAP:	phosphatidic acid phosphatase
PC:	phosphatidylcholine
PCho:	phosphocholine
PDAT:	phospholipid:diacylglycerol acyltransferase
PDCT:	phosphatidylcholine:diacylglycerol cholinephosphotransferase
PLA_2:	phospholipase A_2
PLC:	phospholipase C
PLD:	phospholipase D
PUFA:	polyunsaturated fatty acid
SAD:	stearoyl-ACP desaturase
TAG:	triacylglycerol
VLCFA:	very long chain fatty acid

ABSTRACT

The low-input oilseed crop Camelina (*Camelina sativa* (L.) Crantz) is known for its high omega-3 (18:3) content, short growth season, and facile gene transformation. Camelina mostly contains unsaturated fatty acids, however its fatty acid composition needs optimization depending on the end uses, for example reduction of unsaturated fatty acid to use as biodiesels, or enhancing omega-3 fatty acid content to use as nutritional supplements. Very long chain fatty acid (VLCFAs, C20-C24), are undesirable for human consumption, and their accumulation in seed oil also needs to be diminished. VLCFAs are produced by the catalytic action of fatty acid elongase1 (FAE1), and Camelina contains three alleles of *FAE1* genes (*FAE1-A*, *FAE1-B*, and *FAE1-C*) due to its allohexaploid nature. Recently, VLCFAs in camelina were decreased along with polyunsaturated fatty acids (PUFAs) using the RNA interference (RNAi) technology. A low VLCFA line was also isolated from ethyl methanesulfonate (EMS) induced mutants. Sequencing results indicated that *FAE1-B* gene was mutated and resulted in 60% reduction in VLCFAs, but other two *FAE1* copies were presumably still active in the mutant. To address this multiple-allele-knockout-at-once problem, here I investigated the effect of knocking out three alleles of *FAE1* genes using CRISPR technology with egg cell-specific Cas9 expression. Due to the germline mutation, homozygous *FAE1* knockout mutants were successfully created in a single generation. VLCFA accumulation was significantly decreased from 22% of total fatty acids in wild type to less than 2% in transgenic plants, and the C18 unsaturated fatty acids were improved since 18:1 substrates were diverted to desaturation pathway, rather than elongation. Analysis of the fatty acid composition of four transgenic generations indicated that the mutations that cause low VLCFA genotype were heritable. There was no significant difference observed in seed weight, plant height, total oil content, and seed germination in Cas9-induced mutants compared to the wild type. This study showed that polyploid Camelina can be modified rapidly and effectively through CRISPR/Cas9 to achieve desired fatty acid composition.

CHAPTER ONE

INTRODUCTION

Fatty Acid Composition in Plant Oils

Plant oils are an irreplaceable part of human life since its direct usage as foods or indirect usage as industrial products affect our daily life. The end uses of plant oils are determined by their fatty acid composition. Fatty acids are categorized depending on their saturation levels such as saturated or unsaturated, chain lengths such as short, medium, long, and very long chains, and presence of unusual compounds such as hydroxyl, epoxy or conjugated groups (Gunstone, 1998; Jaworski & Cahoon, 2003). Plant oils mostly contain five types of fatty acids: palmitic (16:0, carbon number: double bond number), stearic (18:0), oleic (18:1), linoleic (18:2), and α-linolenic (18:3) acids. There are a plethora of food and non-food applications of plant oils in that they are mostly consumed as cooking and salad oils, while industrial applications consist of biofuels, lubricants, surfactants, drying oils, plasticizers, and ink production (Berti et al., 2016; Dyer et al., 2008).

Common vegetable oils on the market are palm (69.33 Million Metric Tons (Mt)), soybean (56.20 Mt), rapeseed (canola) (28.45Mt), sunflower seed (17.71 Mt), palm kernel (8.09 Mt), peanut (5.94 Mt), cottonseed (5.02 Mt), coconut (3.44 Mt), and olive oils (2.70 Mt) (USDA/FAS, December 2017). Major vegetable oil producing countries are Indonesia (43.91 Mt), China (28.58 Mt), Malaysia (22.93 Mt), European Union (22.93 Mt), United States (11.65 Mt), Argentina (10.22 Mt), Brazil (8.97 Mt), and others (52.10 Mt) (USDA/FAS, December 2017). Even though those plant oils are leading products in the

market, they contain very less amount of health-promoting fatty acids such omega-3 (Fig. 1).

Omega-3 is an umbrella term that tells the first double bond location from the methyl end of a fatty acid chain. Plant oils containing high 18:3 (ALA) are considered as high omega-3 plants, while there are very long-chain omega-3 fatty acids that stored in fish oils such as 20:5 (EPA) and 22:6 (DHA). These fatty acids are essential, meaning humans cannot produce by themselves and they need to consume them in regular basis. In addition, ALA conversion to EPA and DHA is low, thus omega-3 fatty acids from fish and plants should be consumed individually (Abedi & Sahari, 2014). For example, American Heart Association (AHA) recommends fish consumption at least twice a week to prevent and reduce heart diseases. Major omega-3 producing plants are flax, chia, camelina, and walnut (Abedi & Sahari, 2014; Lane et al., 2014; Zubr, 1997). Flaxseed oil has the highest omega-3 amount in commercial vegetable oils.

A healthy diet requires a balance between omega-3 and omega-6 containing fatty acids. A new study showed that a correlation between high omega-6 consumption and risk of obesity (Simopoulos, 2016). In Western diet, omega-6 containing plant oils such as soybean oil are excessively consumed, so that the ratio of omega-6/omega-3 becomes as high as 10-30/1 (Abedi & Sahari, 2014). In contrast, Indians and Japanese have much higher omega-3 consumption such as 1/30-70 and 1/2-4, respectively (Abedi & Sahari, 2014).

Very long chain fatty acids (VLCFAs) are also undesired types of fatty acids for human consumption. Although erucic acid (22:1) has been used as a slip agent in

polyethylene, its consumption is not recommended. For example, rapeseed oil is not suitable for human consumption, and traditional breeding of rapeseed in 1970s helped reduce the erucic acid content from >40% to <5%, and then, low erucic acid rapeseed (LEAR) was designated as canola (Lin et al., 2013).

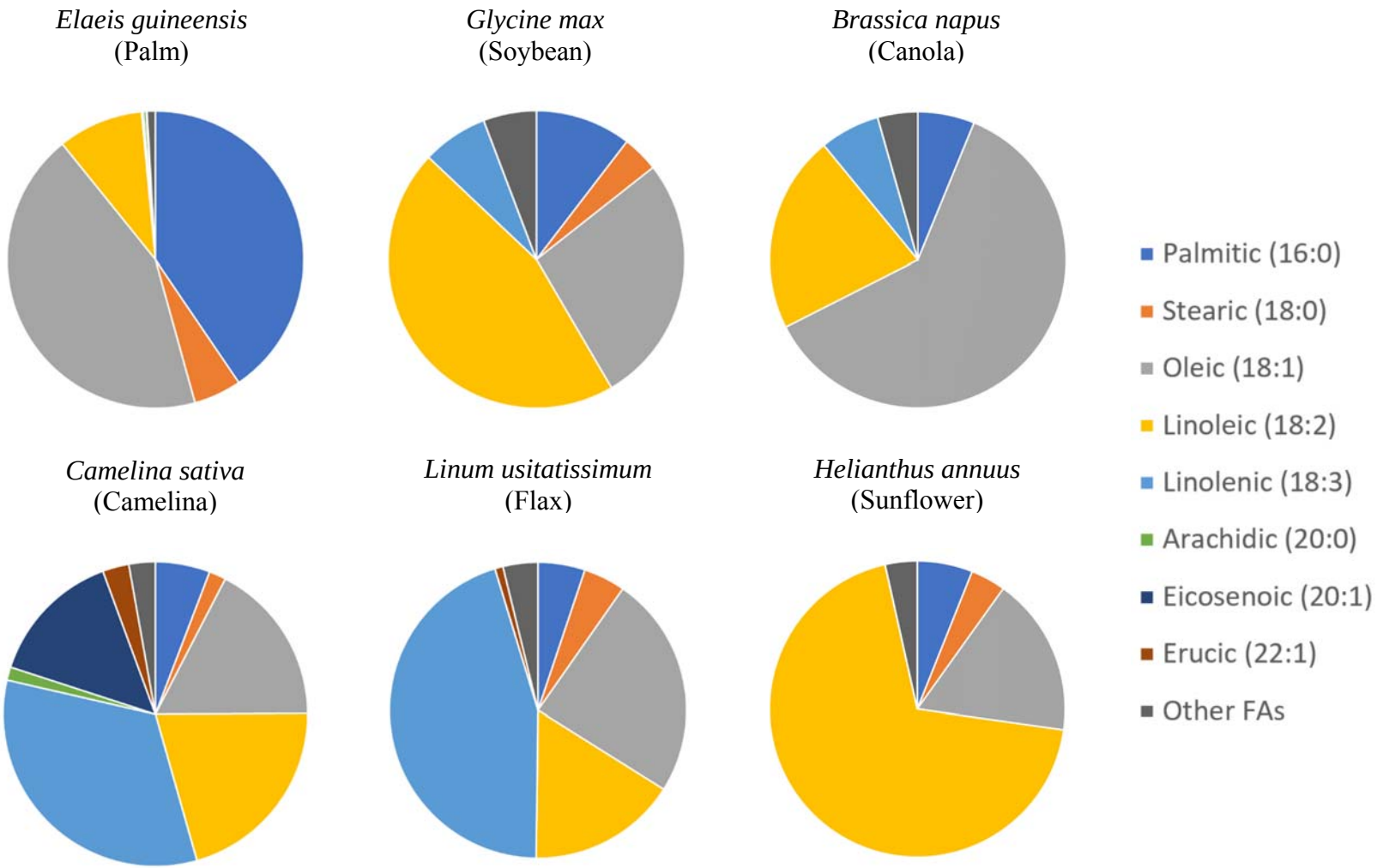

Figure 1. Fatty Acid Profiles of Some Common Plant Oils (D.H. Putnam et al., 1993; Montoya et al., 2014).

Understanding Fatty Acid Metabolism in Plants

The primary form of storage lipids for most plants is triacylglycerol (TAG), except jojoba that accumulates fatty acids in the form of wax esters. Esterification of three fatty acids to the glycerol backbone produces TAG. Fatty acid composition determines the quality and end uses plant oils. For instance, high amount of polyunsaturated fatty acids is much preferable than saturated fatty acids for human consumption. On the other hand, monounsaturated fatty acids meet the criteria for industrial application, but polyunsaturated fatty acids are oxidized.

Storage lipids are important resources for germination, and some plants may store carbohydrates. The food reserve of cereals such as wheat, barley, oat, and rye is starch, while the oilseeds such as palm, soybean, rapeseed, and sunflower seed accumulates oils in their seeds (Kermode, 2011). Moreover, these storage products are placed in two different compartments: endosperm or cotyledon. Cereals keep their storage products in their endosperm; however, soybean, peanut, pea, bean, *Arabidopsis thaliana* and *Camelina sativa* keep storage products in their cotyledons (Kermode, 2011). Since Camelina is an oilseed crop, oil accumulation in seeds will be further discussed.

Fatty acid production in plants is essential for two reasons: first, membrane lipids such as phospholipids are vital for cellular functioning, and second, storage oils are important for survival and reproduction for plants. Oil biosynthesis in plants is a complex metabolic process that requires coordination between plastid and endoplasmic reticulum (ER). Basically, fatty acid synthesis consists of three steps: first, the production of glycerol backbone in the cytosol, second, the formation and, if necessary, the modification of fatty

acids in both plastic and ER, and, third, the esterification of fatty acids onto glycerol backbones in ER. Fatty acid mechanism can be summarized as pushing newly produced fatty acids from plastids into cytosol, pulling modified fatty acids from membrane lipids such as phosphatidylcholine (PC), rechanneling fatty acids for TAG accumulation, and protecting TAG products from β-oxidation (Bates, 2016; Li-Beisson et al., 2016).

Generation of fatty acids takes place in the plastid (Fig. 3). The first step towards fatty acid nascence is the production of malonyl-CoA from acetyl-CoA by acetyl-CoA carboxylase (ACCase). Fatty acid chains are further elongated by a series of condensation, reduction, and dehydration reactions. These fatty acid synthase (FAS) reactions increase the length of fatty acids from 2:0 to 16:0 with 2 carbons at a time. Then, 16:0 fatty acids are elongated to 18:0 by β-ketoacyl-ACP synthase II (KASII), and stearoyl-ACP desaturase (SAD) adds a double bond into 18:0 to form 18:1. The fatty acid reactions in the plastid produce mostly 18:1, and relatively low 18:0, and 16:0 fatty acids, but some plants such as coconut or palm kernel can produce shorter fatty acids (Bates, 2016). Fatty acids, also known as acyls, are attached to the acyl-acyl carrier protein (ACP) during the elongation/desaturation process in the plastid (Ohlrogge et al., 1979). To be exported from plastid, fatty acids must be removed from ACP and released into the cytosol as free fatty acids (FFAs). The thioester bond between fatty acids and ACP is terminated by thioesterases, FatA that preferentially hydrolyzes 18:1 and FatB that hydrolyzes saturated fatty acids, 18:0 and 16:0 (Dormann et al., 1995; Salas & Ohlrogge, 2002). It is worth noting that both thioesterases are able to remove both saturated and unsaturated fatty acids from ACP; however, the efficient removal depends on substrate specificity. Once fatty

acids become free fatty acids, they are exported to cytosol via plastidial fatty acid exporter (FAX1) (Li et al., 2015) and then, free fatty acids made temporary compounds with coenzyme A (CoA) in the cytosol. Acyl-CoA pool is the main donor of fatty acids for membrane lipids and TAG synthesis. Elongation of long chain fatty acid (18:1) into very long-chain fatty acids (VLCFAs: 20:1 and 22:1) take place in the acyl-CoA pool via fatty acid elongase 1 (FAE1) (Kunst et al., 1992) (Fig. 2).

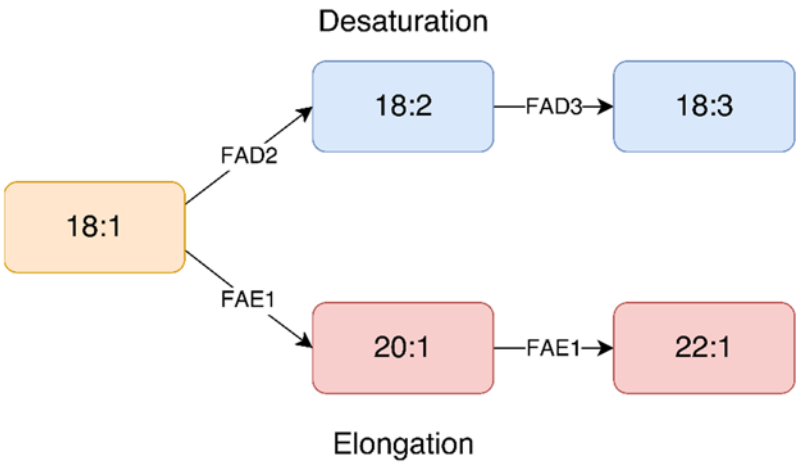

Figure 2. Basic Fatty Acid Elongation and Desaturation Mechanism. The oleic acid substrate (18:1) is either elongated by fatty acid elongase (FAE1) or desaturated by fatty acid desaturases (FAD2 and FAD3).

Even though the pathways producing membrane lipid such as phosphatidylcholine (PC) and storage lipid such as triacylglycerol (TAG) use the same substrates in the endoplasmic reticulum (ER), membrane lipid biogenesis has a priority because cellular functioning and survival are more important than storing lipid for germination and reproduction. PC is the site for desaturation where 18:1 becomes 18:2 via fatty acid desaturase 2 (FAD2) (Okuley et al., 1994), and 18:3 is obtained from 18:2 via FAD3 (Browse et al., 1993; Sperling et al., 1993) (Fig. 2).

The glycerol backbone of plant lipids is made of glycerol-3-phosphate (G3P) that is produced from dihydroxyacetone phosphate (DHAP) via glycerol-3-phosphate dehydrogenase (G3PDH) in the cytosol. Then, fatty acids are acylated stepwise onto three *sn*-positions of G3P. First, *sn*-1 and *sn*-2 positions of G3P are incorporated by glycerol-3-phosphate acyltransferase (GPAT) to form lysophosphatidic acid (LPA) and lysophosphatidic acid acyltransferase (LPAAT) to form phosphatidic acid (PA), respectively. Second, phosphate group at *sn*-3 is removed from PA by phosphatic acid phosphatase (PAP) to form *de novo* diacylglycerol (DAG). Final step is the fatty acid addition at *sn*-3 position of DAG to form TAG by acyl-CoA: diacylglycerol acyltransferase (DGAT) (Hobbs et al., 1999; Routaboul et al., 1999; Zou et al., 1999). This linear fatty acid synthesis is called the Kennedy pathway (Kennedy, 1961). There is an alternative pathway for TAG biosynthesis where *de novo* DAG first becomes membrane lipid phosphatidylcholine (PC) for modification (desaturation, hydroxylation, etc.) (Roughan & Slack, 1982) and then PC produces new DAG pool called “PC-derived DAG” that provides substrate for TAG (Bates & Browse, 2011). To become membrane lipid, *de novo* DAG

changes head group at *sn*-3 position from hydroxyl to phosphocholine group by CDP-choline:DAG cholinephosphotranferase (CPT) (Goode & Dewey, 1999; Slack et al., 1983). Once membrane lipids are settled, fatty acid modification starts on at *sn*-2 position of PC. The rate limiting step of TAG biosynthesis is the addition of fatty acid into *sn*-3 position via DGAT. Another enzyme phospholipid:diacylglycerol acyltransferase (PDAT) can also add fatty acids onto the same position (Dahlqvist et al., 2000). The difference is that *DGAT* uses acyl-CoA pool as substrate; however, *PDAT* uses PC pool as substrate, specifically *PDAT* removes fatty acids at *sn*-2 position on PC for TAG biosynthesis. Mutants in Arabidopsis indicated that *pdat1* mutant did not change total oil content and fatty acid profile (Mhaske et al., 2005; M. Zhang et al., 2009). On the other hand, *dgat1* mutant reduced 30% of total oil content (Katavic et al., 1995; M. Zhang et al., 2009; Zou et al., 1999) and *PDAT1* was upregulated (Xu et al., 2012). Neither *pdat1* mutant nor *dgat1* mutant was lethal and they can compensate one another when either of them is absent (M. Zhang et al., 2009). As expected, *pdat1 dgat1* double mutant cannot be obtained since TAG is essential for storage lipid and pollen (M. Zhang et al., 2009). *PDAT* and *DGAT* expression can change depending on plant species. For example, sunflower expressed 5 times more *DGAT1* comparing to *PDAT1*, however *PDAT1* in safflower is expressed 1.5 times more than *DGAT1* (Bates, 2016).

Desaturation of 18:1 takes place at the *sn*-2 position of phosphatidylcholine (PC) (Lu et al., 2009). *FAD2* and *FAD3* enzymes can add double bonds to produce 18:2 and 18:3, respectively. Some enzymes such as phospholipase A_2 (PLA_2), reverse action of acyl-CoA: lysophosphatidylcholine acyltransferase ($LPCAT^R$), *PDAT* can hydrolyze fatty acid

at *sn*-2 position of PC. Disassemble of fatty acid at *sn*-2 of PC generates lysoPC (LPC), having only fatty acid at *sn*-1 position. Removal of fatty acid at *sn*-1 of LPC by lysophosphatidylcholine transacylase (LPCT) produces glyceroPC (GPC), having no fatty acid attached to glycerol backbone (Lager et al., 2015). Re-acylation of GPC via acyl-CoA: glycerophosphocholine acyltransferase (GPCAT) can produce LPC, indicating *sn*-1 position of PC can also be controlled along with GPAT in Kennedy pathway (Lager et al., 2015). Moreover, the forward action of LPCAT ($LPCAT^F$) can re-acylate LPC to form PC (Bates et al., 2012). Since PDAT contributes TAG biosynthesis by removing *sn*-2 fatty acid of PC, this reaction also provides LPC substrate for $LPCAT^F$ (Dahlqvist et al., 2000).

Even though the acyl editing function of PLA_2 and $LPCAT^R$ enzymes in Lands cycle is the similar, PLA_2 reaction is an energy intensive process since removed fatty acids from the *sn*-2 positions are in the free fatty acids (FFAs) form and production of acyl-CoAs from FFAs consumes 2 ATPs by the action of long-chain acyl coenzyme A synthetase (LACS) (Shockey et al., 2003). However, $LPCAT^R$ directly transfers fatty acids in the acyl-CoA form, so that it is more efficient than PLA_2. By the actions of PLA_2 and $LPCAT^R$, acyl-CoA pool is enriched with modified fatty acids. The *lpcat1 lpcat2* double mutant in Arabidopsis showed that expression of *PLA* was upregulated and LPC accumulation was increased due to lack of *LPCAT*, indicating the overlapping function of *PLA* and *LPCAT* (Bates et al., 2012; L. P. Wang et al., 2012). Another example demonstrates the contribution of LPCAT activities towards desaturation. Mutant yeast cells with no endogenous TAG synthesis could produce polyunsaturated fatty acids (PUFAs) after flax *FAD2*, *FAD3*, and *DGAT1*-

1 were overexpressed. PUFA content was increased with overexpression of flax *LPCAT1* along with other enzymes mentioned above (Pan et al., 2015).

PC-derived DAG pool exists due to the flux of *de novo* DAG pool through PC pool and accepted as the main source of DAG for TAG biosynthesis (Lu et al., 2009). For instance, in soybeans, [^{14}C] glycerol metabolic labeling studies demonstrated that the flux was towards PC-derived DAG rather than linear Kennedy pathway (Bates et al., 2009). Seeds of the reduced oleate desaturase 1 (*rod1*) mutant in Arabidopsis accumulate 40% less PC-modified fatty acids in TAG in the (Lu et al., 2009). *ROD1* gene encodes an enzyme, PC:DAG cholinephosphotransferase (PDCT), that exchanges head group at *sn*-3 position among *de novo* DAG, PC, and PC-derived DAG (Lu et al., 2009). Another example is that castor fatty acid hydroxylase 12 (RcFAH12) can help produce 17% hydroxy fatty acids (HFAs) in Arabidopsis (Lu & Kang, 2008), and co-expression of *RcFAH12* and *RcPDCT* improves the HFA level up to 22% (Hu et al., 2012); therefore, *PDCT* help channel PC-modified FAs into TAG. On the other hand, as expected, Arabidopsis *pdct lpcat1 lpcat2* triple mutant cannot efficiently produce PUFAs (66% reduction) in TAG since the crosstalk among DAG, PC, and LPC pools are interrupted (Bates et al., 2012).

PC-derived DAG can be produced by other enzymes besides PDCT such as phospholipase C (PLC), phospholipase D (PLD), and phosphatidic acid phosphatase (PAP). PLC cleaves phosphocholine headgroup of PC in one step reaction (Nakamura et al., 2005); however, PLD first cleaves choline group of PC, then PAP removes phosphate group of PC so that they generate PC-derived DAG (Chen et al., 2011; Lee et al., 2011).

Finally, the rate limiting enzymes DGAT and PDAT insert third fatty acids on DAG to form TAG. The newly produced TAGs are enclosed in oil bodies covered with monolayer membranes and proteins called oleosins to prevent them from β-oxidation (Goepfert & Poirier, 2007).

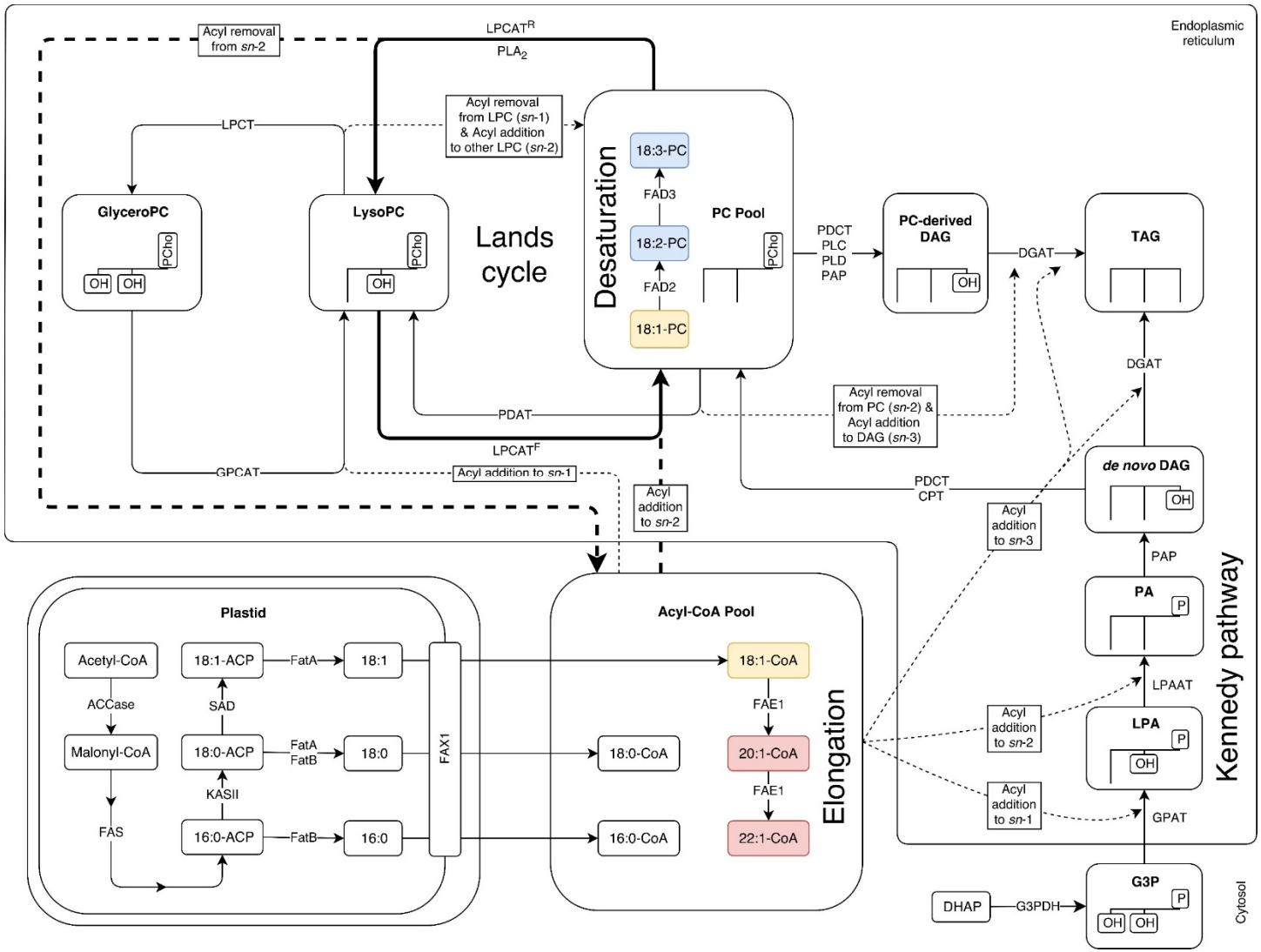

Figure 3. Metabolic Pathway for Triacylglycerol Synthesis.

Once Forgotten Precious: *Camelina sativa*

Camelina [*Camelina sativa* (L.) Crantz], known as false flax, wild flax, and German sesame, is a re-emerging oilseed crop that has great potentials due to its adaptability on marginal lands, low input requirements, high omega-3 content, short growth season and facile transformation (Berti et al., 2016). The name of Camelina was derived from Greek *chamai*, dwarf and *linon*, flax (Plessers et al., 1962); Romans called it 'gold of pleasure' since its oil is good for massage. Camelina is closely related to *Arabidopsis thaliana*, a member of mustard (Brassicaceae) family. Camelina is a dicotyledonous, monocarpic, self-pollinating oilseed crop that grows on various environmental conditions; however, heavy clay soil limits its growth (Plessers et al., 1962; Zubr, 1997). Although there is a plethora of end uses of camelina oil such as omega-3 supplements, biodiesels, jet fuels, adhesives, resins (Berti et al., 2016), camelina meal, as well, is a valuable commodity for animal feed such as cows, chickens, and salmons due to its high protein content (Zubr, 2003).

Archaeological records indicated that the oldest Camelina seed remnants (4,000 BCE) were discovered at Auvernier, Switzerland in Europe (Zohary et al., 2012). Camelina seeds were also found in France during early Iron Age (Bouby, 1998). Another finding showed that Tollund man, a well-preserved corpse lived in Denmark during Iron Age, consumed *Camelina linicola* seeds based on his stomach content analysis (Zubr, 1997). Dönmez and Belli (2007) found camelina seeds in a storage vessel in Van, Turkey. Camelina cultivation was halted after World War II since the demands changed towards other commercial crops (Zubr, 1997). Nonetheless, the underlying reason why Camelina is grown again in the last decade or so is based on the search for sustainable and renewable

bio-sources of fossil fuel alternatives. It was already proven by US Air Force and US Navy that the blend of standard jet fuel (kerosene) and camelina-based jet fuel could successfully fly A-10 Thunderbolt, F-22 Raptor and F/A-18 Super Hornet fighter jets (Roberts, 2014). It is noteworthy that biodiesel from Camelina oil produces 75% less emission than standard petroleum (Roberts, 2014). Camelina cultivation was successfully achieved across the continental U.S. such as Southwest (Hunsaker et al., 2011), Pacific Northwest (Schillinger et al., 2012), North and Central Plains (Aiken et al., 2015), and Corn Belt regions (Gesch, 2014).

Adaptability to many soil types and weather conditions distinguishes Camelina from other crops. That Camelina requires low inputs such as water, fertilizer, and pesticide makes it an ideal crop for areas where rainfall is insufficient, fertility is low, and weather is harsh (Zubr, 1997). Under identical drought conditions, Camelina yields more than *B. rapa, B. juncea,* and *B. napus* in western Canada (Gugel & Falk, 2006). Camelina grows between 85-100 days (Bansal & Durrett, 2016) so that it is a short-seasoned crop that can used as rotation or relay purposes rather than keeping the ground empty for fallow year. Camelina seed yield is in the range of 677–1306 kg/ha, and oil yield is about 234–445 kg/ha in Central Montana (Mohammed et al., 2017). Optimum amount of nitrogen (N) application is 45 kg/ha; however, 60 kg/ha N or 134–22–22–28 kg/ha N–P_2O_5–K_2O–S fertilizer combination gave maximum seed and oil yields in Montana (Mohammed et al., 2017).

Total seed oil content of Camelina (35-45%) is slightly lower than rapeseed (40-44%) and sunflower (39-49%), but twice that of soybean (18-22%) (Moser, 2010). The

major fatty acids in Camelina oil are 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 – 1.9 %), eicosatrienoic (20:3, 0.8 – 1.7 %), behenic (22:0, 0.2 – 0.3 %), erucic (22:1, 2.3 – 2.9%), and others (1.2 – 3.7%) (Moser, 2010). The ratio of ALA (18:3)/ LA (18:2) is about 2.43 for Camelina which is higher than canola (~0.47), walnut (~0.22), soybean (~0.15); however, it is lower than flax (~3.31) and chia (~3.15) (Abedi & Sahari, 2014). Camelina meal is a leftover product that is obtained after oil extraction, and it contains 45% protein, 13% fibers, 10% residual oil, 5% minerals, glucosinolates and vitamins (Moser, 2010). Moreover, it contains at least 18 types of amino acids (Zubr, 2003), and essential amino acids in camelina are significantly higher than those of flax (Pekel et al., 2009).

Polyunsaturated fatty acids (PUFAs), constituting more than 50% in Camelina oil, causes oxidative instability; however, Camelina oil contains some antioxidants called α-tocopherols (Vitamin E) in the range of 806 – 1008 mg/kg so that the rancidity problem is improved in Camelina (Zubr, 2009). For instance, PUFA containing flax and fish oils are less stable than camelina oil, on the other hand, some crop oils such as sunflower, olive, sesame, and corn have a better stability towards oxidation since their oils are mostly monounsaturated (Ni Eidhin et al., 2003). In terms of biodiesel production from Camelina oil, oxidation instability along with high iodine number are the major setbacks (Petcu et al., 2016).

Although Camelina is a good source of omega-3, α-tocopherols, and essential amino acids, it also contains anti-nutritive compounds called glucosinolates that cause

goiter depending on the high consumption since it eliminates iodine uptake. Glucosinolates are common in mustard family, including mustard, rapeseed, canola, crambe, and camelina, and in cabbage family, including cabbage, cauliflower, broccoli, and brussels sprouts (Fahey et al., 2001). High amounts of glucosinolates are found in crambe (118 μmol/g) and mustard (130 μmol/g), but camelina contains relatively low glucosinolates (14.5 – 23.4 μmol/g) (Matthaus & Zubr, 2000). Studies showed when hens were fed with 10% camelina meal, their eggs did not contain glucosinolates, therefore, it is unlikely to observe adverse effects if consumption is minimal (Kakani et al., 2012).

Various diseases and pests that reduce yield in other oilseed crops have less effect on Camelina. For example, Camelina has significant resistance to diseases such as alternaria black spot and blackleg, and some accessions are tolerant to sclerotinia stem rot, brown girdling root rot, and downy mildew (Seguin-Swartz et al., 2009). On the contrary, Camelina is susceptible to clubroot, white rust, and aster yellow disease (Seguin-Swartz et al., 2009). Furthermore, insect pests targeting canola on Canadian prairies such as flea beetles, root maggots, diamondback moths, bertha armyworms, leafhoppers, grass hoppers, cutworms, and lygus bugs do not cause significant harm in Camelina (Soroka et al., 2015).

In genomics perspective, Camelina belongs to mustard family, therefore it is closely related to *Arabidopsis thaliana*. Hutcheon et al. (2010) showed the first evidence that Camelina has three redundant copies of *FAD2* and *FAE1* genes, suggesting hexaploidy nature of Camelina genome. Kang et al. (2011) also confirmed the polyploidy in Camelina by characterizing three copies of *FAD2* genes. Another supporting findings by Kwak et al. (2013) demonstrated that glycine-rich RNA-binding proteins (GRPs), control stress

adaptation process, had three isoforms in Camelina genome. Camelina genome was sequenced (Kagale et al., 2014), and findings showed that Camelina is an allohexaploid crop having 3 sub genomes with 20 chromosomes; two of them contain quite similar seven chromosomes each, suggesting autopolyploidy and third sub genome consists of slightly different six chromosomes. Since Camelina genome (785 Mb) (Kagale et al., 2014) is about six times bigger than Arabidopsis genome (135 Mb) (Kaul et al., 2000), whole genome triplication event in Camelina is a strong possibility. Developmental transcriptome atlas of camelina was published (Kagale et al., 2016). Expression levels of 12 different tissues (germinating seed, cotyledon, young leaf, senescing leaf, bud, flower, root, stem, seed developmental stages: early, early-mid, late-mid, and late seed) covering major developmental stages during life cycle of Camelina can be found on Camelina sativa electronic Fluorescent Pictograph (eFP) Browser (http://bar.utoronto.ca/efp_camelina/cgi-bin/efpWeb.cgi).

Camelina is generally accepted as a self-pollinating crop though cross-pollination may occur through bees (Zubr, 1997). In this regard, camelina outcrossing rate is between 0.09 – 0.28% (Walsh et al., 2012) which is much smaller than flax (1.85%) (Jhala et al., 2011) and soybean (1.22%) (Ahrent & Caviness, 1994). Close proximity to one another, such as 20 cm, and flowering at the same time are the contributing factors for high outcrossing. Camelina is regarded as a low outcrossing plant; however, there is still a small possibility due to pollinators, synchronous flowering and close distance to each other.

Engineering Fatty Acids in Camelina through Biotechnology

Rather than time-consuming tissue culture procedures, Camelina can be transformed *in planta* with agrobacterium-mediated floral dip method in the presence (Lu & Kang, 2008) or absence (X. J. Liu et al., 2012) of vacuum pressure. To identify transgenic seeds, reporter genes can be introduced such as DsRed for red fluorescent seed coat (Lu & Kang, 2008), basta for herbicide resistance (Y. Zhang et al., 2012), and hygromycin for bacterial selection. Lu and Kang (2008) emphasized that transformation efficiency, a ratio of transgenic red seeds to normal seeds, was 1.3% using vacuum-assisted agro-infiltration.

Camelina is a good example of surrogate plant whose oil profile can be modified through various genetic tools such as antisense, RNA interference (RNAi), microRNA (miRNA), enzyme overexpression, and CRISPR technologies. In this regard, the major obstacle is the polyploidy nature of Camelina genome (Kagale et al., 2014) since it complicates the process with three redundant sub-genomes. Trends showed that enzyme overexpression was the most preferred way for Camelina modification in last 6 years (Table 1). It is important to mention that the number of paper published has significantly increased after Camelina genome is published in 2014 (Kagale et al., 2014). Enzyme silencing through antisense, RNAi, and miRNA is the second modification type, however, the new technology CRISPR/Cas9 silencing, more specifically knock out, has been accepted as a norm due to its efficiency.

Table 1. Transgenic Camelina Papers Published through 2012-2017

	Number of transgenic camelina papers					
Modification Type	2012	2013	2014	2015	2016	2017
Enzyme Silencing (Antisense, RNAi, or microRNA)	-	2	-	4	1	1
Enzyme Overexpression	1	1	10	15	4	7
CRISPR/Cas9 silencing	-	-	-	-	-	3

The first fatty acid modification in camelina was done by overexpressing the fatty acid hydroxylase gene from castor, *Ricinus communis*, (RcFAH12) driven by seed-specific phaseolin promoter, so that camelina plant, that cannot produce hydroxy fatty acids (HFAs) by itself, produced 15% HFAs (Lu & Kang, 2008). Secondly, Camelina fatty acid desaturase 2 (CsFAD2) enzyme expression was reduced by antisense silencing so that desaturation pathway was blocked and more oleic acid (18:1) was accumulated (Kang et al., 2011). In this study, 18:1 levels were increased from 15.5% to 51.2%, while 18:2 and 18:3 levels were reduced from 16.8% and 33.2% to 6.3% and 11% (Kang et al., 2011). Another study showed that oleic acid content was improved even more through RNAi silencing of *FAD2* and *FAE1* in that 18:1 accumulation reached up to 70% and 18:2 and 18:3 levels are diminished from 17% and 36% to 4% and 8% (Nguyen et al., 2013). In addition, an elongated fatty acid, 20:1, was reduced from 12% to 3% since *FAE1* was targeted by RNAi, as well (Nguyen et al., 2013). Similar approach has been applied to target *FAD3* and *FAE1* through RNAi silencing, and results indicated that 18:3 and 20:1

accumulation were significantly reduced from 33% and 12% to 5% and 2%, and 18:2 level was increased from 20% to 54% (Horn et al., 2013).

A group of researchers focus on medium chain fatty acid productions (MCFAs) in Camelina seed oil by expressing gene combinations of *FatB* from *Cuphea* and California bay and *LPAAT* from coconut, along with endogenous RNAi silencing of *KASII*. For instance, *Cuphea pulcherrima FATB* (*CpuFatB1*) overexpression in Camelina increased 16:0 levels from 6.6% in WT to 41% (Horn et al., 2013). Another study confirmed the same results that *CpuFatB1* and *CpuFatB4* overexpression in Camelina increased 16:0 levels from 8.9% in WT to 43.5% and 42.7%, respectively (Kim et al., 2015). 14:0 levels were increased by overexpression of *Cuphea palustris FatB* (*CpFatB2*) from 0% in WT to 23.8%, and co-expression of *CpFatB2* and coconut, *Cocos nucifera, LPAAT* (*CnLPAAT*) accumulated as high as 36.9% 14:0 fatty acids (Kim et al., 2015). Same study was, also, achieved high 12:0 accumulation in Camelina by overexpressing California bay, *Umbellularia californica*, *FatB* (*UcFatB1*) from 0% in WT to 18.4%, and co-expression of *UcFatB1* and *CnLPAAT* increased 12:0 levels even more from 18.4% to 28.4% (Kim et al., 2015). 10:0 levels, also, increased by individual overexpressing *Cuphea viscosissima FatB* (*CvFatB1*), or *Cuphea hookeriana FatB* (*ChFatB2*) from 0% in WT to 8.7% and 10.3% (Kim et al., 2015).

Camelina fatty acid metabolism can be engineered to produce omega-3 fatty acids found in fish oils such as eicosapentaenoic acid (20:5, EPA) and docosahexaenoic acid (22:6, DHA) fatty acids. Ruiz-Lopez et al. (2014) demonstrated that overexpression of an EPA construct (*Phytophthora sojae* Δ12-desaturase (des), *Phytophthora infestans* ω3-des,

Ostreococcus tauri Δ6-des, *Physcomitrella patens* Δ6-elongase (elo), *Thraustochytrium sp.* Δ5-des) can produce 24% EPA, and a DHA construct (EPA construct plus *O. tauri* Δ5-elo and *Emiliania huxleyi* Δ4-des) can produce 11% EPA and 8% DHA in Camelina seed oil. Petrie et al. (2014) showed 12.4% DHA accumulation by overexpression of the Mod-F construct (*Lachancea kluyveri* Δ12-des, *Pichia pastoris* ω3-des, *Micromonas pusilla* Δ6-des, *Pyramimonas cordata* Δ6-elo, *Pavlova salina* Δ5-des, *P. cordata* Δ5-elo and *P. salina* Δ4-des) in Camelina seed oil. In aquaculture, the feedstock for fish is usually fish oil due to EPA and DHA content; however, transgenic camelina plant, capable of producing EPA and DHA in its oil, can replace fish oil. Betancor et al. (2015) confirmed that consumption of Camelina containing more than 20% EPA did not cause any adverse effect on Atlantic salmon growth.

For industrial applications, Camelina was engineered to produce acetyl-TAGs that have lower viscosity and freezing point than regular TAG so that it is valuable product for lubricant applications (J. Liu et al., 2015). *Euonymus alatus* diacylglycerol acetyltransferase (EaDAcT) was overexpressed along with RNAi silencing of *DGAT1* so that 85% acetyl-TAG was achieved (J. Liu et al., 2015). Another industry related example is hydroxy fatty acid production in Camelina oil. *Ricinus communis* fatty acid hydroxylase (*RcFAH12*) overexpression generated 15% HFAs in Camelina seed oil (Lu & Kang, 2008). Co-expression of *RcFAH12* and *Physaria fendleri* 3-ketoacyl-CoA synthase (*PfKCS18*) increased total HFAs from 15% to 22% in Camelina seed oil (Snapp et al., 2014).

CRISPR/Cas9-mediated Mutagenesis

Desired traits for plants have been attained by selective breeding through the ages; however, it is not an efficient way for a single gene modification since traditional breeding depends on random recombination and integration. Recent advances in genetics have contributed to the precise plant genome editing so that researchers can pinpoint specific gene(s) for specific purposes. Modification tools in genetic editing toolbox consist of meganucleases, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced palindromic repeats and its associated protein (CRISPR/Cas9). Meganucleases such as I-*Sce*I recognize 18 bp targets and cause double stranded breaks (DSBs) in the DNA; however, they are rare cutters due to its natural target site specificity, and its application is limited (Puchta et al., 1993, 1996).

To address this limited specificity problem, a non-specific nuclease, *Fok*I, was fused with three to six zinc fingers, specific DNA-binding sites, to form a zinc finger nuclease (ZFN) (Urnov et al., 2010). Each zinc finger recognizes 3 base pairs (bp), and successful DSB is achieved through the simultaneous action of two independent ZFNs targeting the same site from complementary and non-complementary strands through *Fok*I nuclease (Fichtner et al., 2014). Major problems of ZFNs are time-consuming and the costly assembly process due to the difficulty in zinc finger engineering, not-so-specific triplet recognition, and potential off-targets (Fichtner et al., 2014).

Although nuclease specificity problem was solved by using *Fok*I, the binding specificity for target DNA was still immature for ZFN technology so that TALENs overcame this issue by binding to a single nucleotide using TALE repeats rather than triplet

recognition in ZFNs (Mahfouz et al., 2011). TALE repeats, discovered in *Xanthomonas* pathogens, contain 34 amino acids (aa) and their variation only occurs in every 12th and 13th positions, which is called repeat variable di-residue (RVD) (Moscou & Bogdanove, 2009). To bind DNA, a series of TALE repeats, known as DNA-binding domain (DBD), are required since they are designed to have corresponding RVD for each base on target DNA. RVD specificity in 34 aa-length TALE repeat determines DNA binding site. For example, if 12th aa is H and 13th aa is D (RVD is HD) in TALE repeat, then it binds to Cytosine (C) in target DNA, and, if RVD is NG, NI, or NN, then they bind T, A, and G, respectively (Cermak et al., 2011). Briefly, one TALE repeat domain including one RVD (NI, NG, NN, or HD) can bind one nucleotide (A, T, G, or C) on DNA. TALEN with *Fok*I nuclease works as a dimer in the case of ZFNs with *Fok*I nuclease, therefore another TALEN pair is needed. TALEN is a much better alternative to ZFN since DNA binding is more specific, off-targets are relatively low, and construct design is easier. Nonetheless, there are still some drawbacks such as redundancy of TALE repeats increases the size of construct so that delivery and expression become difficult (Gupta & Musunuru, 2014).

The CRISPR and its associated protein (CRISPR/Cas9) system is one of the latest genome editing technique that is more precise and effective than ZFNs and TALENs (Gaj et al., 2013). The CRISPR/Cas9 system is based on the adaptive immune system of bacterial cells against invading bacteriophages (Jinek et al., 2012). As indicated in Fig. 4, a seek and destroy mechanism of CRISPR/Cas9 system is carried out by acquisition of foreign DNA sequence, integration of this short sequence as “protospacer” into CRISPR array region, amplification of protospacer as guide RNA (gRNA), also known as CRISPR

RNA (crRNA), hybridization of crRNA with trans activating crRNA (tracrRNA) and Cas9 protein, recognition of target DNA though guide RNA (protospacer) on Cas9 and protospacer adjacent motif (PAM) on target DNA, and destruction of target DNA via Cas9 nuclease domains: HNH and RuvC (F. G. Jiang & Doudna, 2017).

RNA-guided Cas9 protein makes DSB at 3 nucleotides upstream of the PAM. By customizing 20 nt gRNA sequence, Cas9 protein can virtually target all genes having NGG PAM sites (N, any nucleotide; G, Guanine). There are two ways of repairing DSBs: nonhomologous end joining (NHEJ) that can cause small insertion, deletion, or substitution and homology directed repair (HDR) that enables large insertion (Fig. 4). Causing insertion and/or deletion (InDel) or substitution in the target gene leads to frameshift mutation so that the protein loses its unique 3D structure, along with functionality.

Designing guide RNAs in Camelina is easier than other crops due to its small genome (785 Mb) (Kagale et al., 2014) compared to wheat (>15,000 Mb) (Zimin et al., 2017), maize (>3,200 Mb) (Schnable et al., 2009), soybean (>1,100 Mb) (Schmutz et al., 2010), and canola (>1,100 Mb) (Chalhoub et al., 2014), low percentage of repetitive DNA (28%) compared to wheat (90%), maize (85%), soybean (57%), and low percentage of GC content, indicating PAM sites are less frequent and target region is more unique (Zhu et al., 2017). There are various online tools for gRNA design (See Appendix 1), however, only 4 websites (Cas-Designer, Cas-OFFinder, CRISPOR, and ChopChop) include *Camelina sativa* genome so that these websites are important to minimize off-targets. I chose ChopChop web-based tool (Labun et al., 2016; Montague et al., 2014) to design my guide RNA for *Camelina sativa FAE1* alleles. My selection criteria include targeting the

5’ end of the gene to increase the rate of frameshift mutations, covering of all three *CsFAE1* copies for complete knockout, choosing highest efficiency score and lowest possible off-targets that is predicted by algorithm, selecting minimum number for self-complementary regions to keep efficiency high, managing GC content around 40 to 70% (Tsai et al., 2015; T. Wang et al., 2014). To make sure about the absence of possible off-targets, I also double check my gRNA with another web-based tool, Cas-OFFinder (Bae et al., 2014).

The Cas9-induced mutations have been made in various plants such as *Arabidopsis thaliana, Brassica oleracea, Camelina sativa, Citrus sinensis, Cucumis sativus, Glycine max, Gossypium hirsutum, Hordeum vulgare, Lotus japonicus, Marchantia polymorpha, Medicago truncatula, Nicotiana benthamiana, Nicotiana tabacum, Oryza sativa, petunia hybrid, Populus tomentosa, Solanum lycopersicum, Solanum tuberosum, Sorghum bicolor, Triticum aestivum, Vitis vinifera, Zea Mays,* and details can be found in those review papers (Arora & Narula, 2017; X. Liu et al., 2017; Weeks, 2017). Specifically, three CRISPR papers about Camelina were published in 2017 (Table 2), targeting desaturase enzyme *CsFAD2* (W. Z. Jiang et al., 2017; Morineau et al., 2017) and rate limiting enzymes *CsPDAT1* and *CsDGAT1* (Aznar-Moreno & Durrett, 2017).

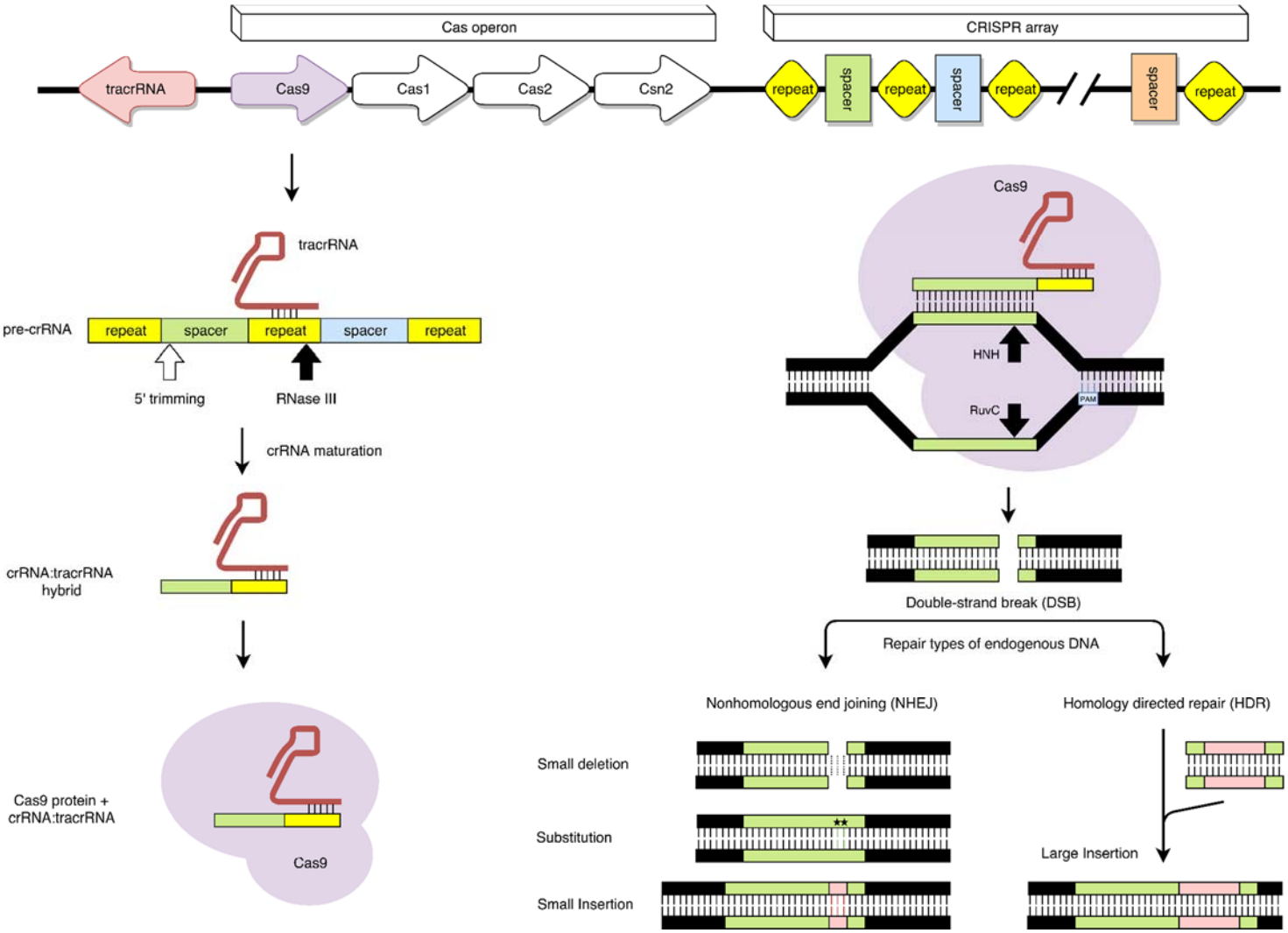

Figure 4. The CRISPR/Cas9 Mechanism in Bacterial Immune System.

Figure 4 Continued. There are three loci in *Streptococcus pyogenes* (*Spy*) genome for adaptive immunity; CRISPR array that stores viral genes as protospacer, spacer for short, between repeat sequences, Cas operon that consists of Cas9 protein (*SpyCas9*) that makes double stranded break (DSB) and other proteins, Cas1, Cas2 and Csn2 that helps incorporation of viral gene into CRISPR array, and trans-activating CRISPR RNA (tracrRNA) that is important for CRISPR RNA (crRNA) maturation. The first step starts with integrating viral gene into CRISPR array by Cas1, Cas2, and Csn2 genes. When the same virus invades bacteria, precursor CRISPR RNA (pre-crRNA) from CRISPR array and tracrRNA on adjacent sequence are transcribed. tracrRNA binds on repeat, then a unknown enzyme cuts 5' end of spacer and RNase III cuts 3' end of repeat, so that pre-crRNA becomes mature crRNA. The hybrid crRNA:tracrRNA makes a complex with *SpyCas9* protein, thus Cas9 complex searches for target DNA depending on the crRNA sequence or guide RNA (gRNA). Cas9 protein also surveillances NGG sequences (N, any nucleotide) for protospacer adjacent motif (PAM). When Cas9 complex binds on target DNA, DSB occurs 3 nucleotides before NGG PAM site. HNH cuts complementary strand, and RuvC cuts non-complementary strand. DSB is repaired randomly through nonhomologous end joining (NHEJ) such as, small insertion, deletion, or substitution. Another deliberate repair mechanism is called homology directed repair (HDR). In this case, the gap after DSB is filled with desired complementary sequence so that it allows large insertion.

In recent CRISPR studies, two Camelina cultivars, Celine and Suneson, were used for Cas9-induced mutations. The major differences in construct design include *Cas9* promoters, either *Caulifower Mosaic Virus 35S* (*CaMV35S*) or *Petroselinum crispum polyubiquitin* (*PcUBI4-2*), Cas9 optimization, based on *A. thaliana*, *C. reinhardtii*, or *Z. mays*, and single guide RNA (sgRNA) promoters, either *A. thaliana U6* or *Camelina sativa U3* and *U6*. One of the most critical factors that affect mutation frequencies is the promoter that drives Cas9 protein expression (Bortesi et al., 2016). For instance, homozygous mutations didn't occur at 1st transgenic generation (T_1) since *CaMV35S* and *PcUBI4-2* promoters cause somatic mutations rather than germline mutations. Nevertheless, promoters that target germlines allow homozygous mutations in T_1 generation such as

Table 2. Examples of CRISPR/Cas9 Modification in *Camelina sativa*

Crop	Cas9 promoter	Cas9 optimization	sgRNA promoter	Target Gene(s) Name	Modification type	Trait	References
Camelina cv. Suneson	*Caulifower Mosaic Virus 35S* (*CaMV35S*) *promoter*	*Chlamydomonas reinhardtii* codon optimized Cas9	*AtU6*	**3 alleles of *CsFAD2*** *Csa19g016350* *Csa01g013220* *Csa15g01600*	NHEJ	High oleic (16% vs 50%)	*(W. Z. Jiang et al., 2017)*
Camelina cv. Celine	*Petroselinum crispum polyubiquitin* (*PcUBI4-2*) *promoter*	*Arabidopsis thaliana* codon optimized Cas9	*CsU3* and *CsU6*	**3 alleles of *CsFAD2*** *Csa19g016350* *Csa01g013220* *Csa15g01600*	NHEJ	High Oleic (10% vs 62%)	*(Morineau et al., 2017)*
Camelina cv. Suneson	*Caulifower Mosaic Virus 35S* (*CaMV35S*) *promoter*	*Zea mays* codon optimized Cas9	*AtU6*	**3 alleles of *CsDGAT1*** *Csa19g056370* *Csa01g042590* *Csa15g084220* **3 alleles of *CsPDAT1*** *Csa13g016300* *Csa20g019000* *Csa08g005560*	NHEJ	Reduced Oil content	*(Aznar-Moreno & Durrett, 2017)*
Camelina cv. Suneson	*Egg cell-specific promoter* (*EC1.1pro*) *and enhancer* (*EC1.2en*)	*Zea mays* codon optimized Cas9	*AtU6*	**3 alleles of *CsFAE1* (A, B, and C)** *Csa11g007400* *Csa10g007610* *Csa12g009060*	NHEJ	Reduced VLCFAs (22% vs <2%)	*(Ozseyhan et al., 2018)*

egg cell-specific (EC) promoter (Z. P. Wang et al., 2015), meristematic tissue-specific YAO (Yan et al., 2015) and INCURVATA2 promoter (Hyun et al., 2015), and anther-specific DD45 promoter (Mao et al., 2016). Efficiency of egg cell specific (EC) promoters in T_1 generation was tested in Arabidopsis, and results showed 1.8% and 8.3% homozygous efficiencies for EC1.1 and EC1.2, respectively. It is important to mention that the same study indicated 0% efficiency for 35S promoter in T_1 lines. Interestingly, 35S enhancer and EC promoter combination increased the efficiency in the range of 3.5% - 4.5%. The study, also, demonstrated that the highest efficiency (17%) was obtained in EC1.2 enhancer and EC1.1 promoter combination, therefore, I chose this combination.

CHAPTER TWO

OBJECTIVES

The objective of my study is to decrease very long chain fatty acids (VLCFAs) in Camelina seed oil using guide RNA-directed Cas9 endonuclease to target the VLCFA-producing *Fatty Acid Elongase1* (*FAE1*) genes and concomitantly enhance polyunsaturated fatty acids (PUFAs). Among other plants, Camelina has some special features such as high seed and oil yield, high omega-3 content, low input requirements, short growth season, easy transformation, drought and cold tolerance, and similar protein content as soybean. In contrast, camelina oil has VLCFAs that may not be desirable as in the case of rapeseed oil (Lin et al., 2013) so that VLCFAs are needed to be diminished. Application of plant oils is determined by their properties such as chain length, presence of double bond(s), and functional group(s). Some oils can be used as feedstocks for food application such as cooking and salad oils, or non-food application such as lubricants, biofuels, surfactants, drying oils, and so on. Although camelina oil was re-introduced in the last decade since it was an alternative bio-source to the fossil fuels, it is still not considered as generally accepted as safe (GRAS) plant by the U.S. Food & Drug Administration (FDA). The major underlying reasons are VLCFAs and glucosinolates, therefore my project is focused on tackling VLCFAs.

Gene modification in plants has never been easier any time in history due to the discovery and advancements in CRISPR/Cas9 technology (Lander, 2016). Previous gene editing technologies did not have flexibility on cutting specificity (meganucleases) and on

target specificity (ZFNs), and their delivery and expression on plants was hard due to large construct size (TALENs). In contrast, Cas9 protein can easily find the target DNA due to the specificity of guide RNA (gRNA), causing a double stranded break (DSB) on target site. Moreover, using a Cas9 protein driven by a promoter that targets germlines helps achieve homozygous lines in the first generation. Therefore, CRISPR/Cas9 is one of the best editing tool discovered so far.

Camelina genome contains three redundant copies of *FAE1* genes since its genome is allohexaploid (Kagale et al., 2014), and all three *FAE1* alleles are active and contribute to VLCFA production (Hutcheon et al., 2010; Kagale et al., 2016). Successful *FAE1* silencing requires knocking out all three *FAE1* alleles. Kang et al. (2011) identified *FAE1* mutants caused by ethyl methanesulfonate (EMS), and observed more than 60% reduction in VLCFAs. Sequencing results indicate that highly expressed *FAE1-B* copy was the only one mutated copy out of three *FAE1* copies. Thus, other two copies are still active. To address this multiple-allele-knockout-at-once problem, here I investigate whether CRISPR/Cas9 system can effectively target all *FAE1* copies without causing any off-target, and minimize VLCFA accumulation in Camelina seed oil.

CHAPTER THREE

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Camelina (*Camelina sativa*) cv. Suneson, released by Montana State University, was selected as a starting point for further experiments. Wild type plants were grown in a 1:1 mix of Plant Growth Center (PGC) soil mix (equal parts, by volume, of loam soil : washed concrete sand : Canadian sphagnum peat moss with AquaGro 2000 G wetting agent is blended in at one pound per cubic yard of soil mix. Aerated steam pasteurized at 70°C for 60 minutes) and Sunshine mix #1 (Canadian sphagnum peat moss, perlite, vermiculite, starter nutrient charge, wetting agent, and dolomitic lime.). For every 6" pot, 5 seeds were distributed equal distance from one another. Experiments were undertaken at greenhouse 179F where temperature conditions were at 22°/18ºC ±1ºC for day/night, relative humidity was at 30%, and 16 hours photoperiod of natural lighting supplemented when necessary by season. Daily watered plants were fertilized every two weeks with Miracle-Gro® water soluble all-purpose (24-8-16) plant food (Marrysville, OH, USA). To enable plants to grow straight, bamboo stakes and twist ties were used when needed. Details about plant background, agrobacteria containing gene-of-interest, and the date were written in the color-coded tags in every pot.

CRISPR/Cas9 Vector Construction

The single guide RNA (sgRNA) targeting all three alleles of *Camelina sativa Fatty Acid Elongase1* (*FAE1-A*, *Csa11g007400*; *FAE1-B*, *Csa10g007610*; and *FAE1-C*, *Csa12g009060*) (Hutcheon et al., 2010) was designed by using gRNA prediction algorithms of Harvard CHOPCHOP website (http://chopchop.cbu.uib.no) (Labun et al., 2016; Montague et al., 2014). The gRNA was selected according to the following criteria: (1) The proximity to the 5' end, (2) the coverage of all three FAE1 genes, (3) the higher efficiency score, (4) the absence of predicted possible off-targets, (5) minimum self-complementary regions, (6) higher GC content, preferably, between 40 and 70% (Tsai et al., 2015; T. Wang et al., 2014). The best gRNA that obeys the criteria above then tested with Cas-OFFinder web-based tool (http://www.rgenome.net/cas-offinder) for off-target possibility (Bae et al., 2014). *FAE1* sgRNA was synthesized by Eurofins MWG Operon USA (Louisville, KY) as two oligonucleotides; gRNA-F (5'-ATTGTTGGAGA TGGGAATAGAAG-3') and gRNA-R (5'-AAACCTTCTATTCCCATCTCCAA-3'), (gRNA is underlined). Then, two oligos were mixed in a microcentrifuge tube and kept in thermocycler at 95^{o}C for 5 minutes and at 4^{o}C for 5 minutes. The newly generated gRNA was then cloned into the binary transformation vector pHEE401E (Z. P. Wang et al., 2015; Xing et al., 2014) (obtained from Addgene; www.addgene.org/71827) by the Golden Gate cloning method (Engler et al., 2008) (Table 3). Since digestion and ligation can be carried out concomitantly in golden gate cloning, Spectinomycin gene in pHEE401E was removed and replaced with a gRNA that targets all Camelina *FAE1* alleles.

Table 3. Golden Gate Cloning Protocol

PCR Protocol	Volume	PCR Conditions
ddH_2O	6 µL	5 hours @ 37°C 5 min @ 50°C 10 min @ 80°C
pHEE401E (AddGene) (100 ng/µL)	2 µL	
CsFAE1 gRNA (50 µmol/L)	2 µL	
10X T4 DNA Ligase Buffer (NEB)	1.5 µL	
10X Bovine Serum Albumin	1.5 µL	
BsaI enzyme (NEB)	1 µL	
T4 DNA Ligase (NEB)	1 µL	
Total Volume	15 µL	

New pHEE401E construct containing *CsFAE1* gRNA was then transformed into competent *E. coli* K12 JM109 cells (New England Biolabs, Ipswich, MA, USA). Transformation protocol was initiated with thawing 50 µL competent cells in an ice bucket. Then, 3 µL of new pHEE401E construct was added and gently mixed by pipetting up and down 2-3 times. The mixture was placed on ice for 20 minutes and heat shocked at 42°C water bath for 30 seconds. Later, 200 µL LB medium was included and the tube was placed in a rotating shaker at 37°C for 1 hour with 250 RPM. While waiting, a Kanamycin LB agar plate was taken from the refrigerator and located in an incubator at 37°C to warm it. One hour later, the tube was taken from the rotating shaker, centrifuged for 5 seconds, the supernatant part was removed, and new 200 µL LB medium was added. After homogenizing by pipetting up and down, 50 µL *E.coli* cells were spread on LB agar plate and incubated overnight at 37°C. Next day, the colonies appeared on plate were tested with gene specific primers (Eurofins MWG Operon, Louisville, KY, USA) U6-26p-F, 5'-TGTCCCAGGATTAGAATGATTAGGC-3', and U6-26t-R, 5'-CGGAACTGCAAA ACTCAACTAACTG-3' (Table 4).

Table 4. Oligonucleotide Table

Oligo Name	Oligo Sequence (5' to 3')
gRNA-F	ATTGTTGGAGATGGGAATAGAAG
gRNA-R	AAACCTTCTATTCCCATCTCCAA
U6-26p-F	TGTCCCAGGATTAGAATGATTAGGC
U6-26t-R	CGGAACTGCAAAACTCAACTAACTG
FP_CsFAE1-A	GTCCGGATAGTTTGATGCAATTAAATGATT
FP_CsFAE1-B	GAGCACCACTCATAAACTATTTTTTTTTTGTT
FP_CsFAE1-C	AGCAGCAACATATTGTAGTTTGTAGG
RP_CsFAE1	CTTTGAGATGCGGTGGTGGAA

For colony PCR, parameters were arranged as following: initial denaturation at 95^{o}C for 5 minutes, 32 cycles of denaturation at 95^{o}C for 30 seconds, annealing at 53^{o}C for 30 seconds, extension at 68^{o}C for 90 seconds, and final extension at 68^{o}C for 7 minutes. Since DNA consists of negatively charged sugar-phosphate backbone, gel electrophoresis (125 volts) was used to separate bands. Colony PCR products were run on a 1% agarose gel with ethidium bromide and seen under UV light. The colony corresponding to the correct sized band on the gel were grown overnight in LB medium with kanamycin at 37^{o}C in a rotating shaker with 250 RPM. Next day, cloudy LB medium was purified by using Zyppy^{TM} Plasmid Miniprep Kit (Zymo Research, Irvine, CA, USA), then new pHEE401E plasmid was digested for 1 hour at 37^{o}C with the restriction enzyme *EcoRI* and *HindIII* (New England Biolabs, Ipswich, MA, USA) to excise the CRISPR/Cas9 cassette (Figure

7). Simultaneously, pBinGlyRed2 plasmid (Lu *et al*., 2006) was digested with same two enzymes at 37°C to open space for CRISPR/Cas9 cassette. Then, to visualize the plasmid fragments, all the digestion reactions were run on 1% agarose gel with ethidium bromide. The gel results were analyzed under UV light and the bands corresponding to the 8879bp for pBinGlyRed2 backbone and 7003bp for CRISPR/Cas9 cassette were excised and recovered by using Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA). Furthermore, pBinGlyRed2 backbone and CRISPR/Cas9 cassette including *CsFAE1* gRNA ligated.

Ligation protocol starts with mixing the following ingredients on ice; 2 µL of thawed and resuspended T4 DNA ligase buffer (10X) (New England Biolabs, Ipswich, MA, USA), 3 µL double digested pBinGlyRed2 fragment, 9 µL double digested CRISPR/Cas9 fragment, 5 µL nuclease-free water, and 1 µL T4 DNA ligase (New England Biolabs, Ipswich, MA, USA). The reaction tube was kept at 4°C refrigerator overnight to maximize the ligation. Next day, the tube was place at 65°C water bath for 10 minutes to inactivate the enzyme. Later, 3 µL was aliquoted and transferred into thawed 50 µL competent *E. coli* K12 JM109 cells (New England Biolabs, Ipswich, MA, USA) on ice. The tube was kept on ice for 20 minutes and subsequently heat shocked at 42°C water bath for 30 seconds. LB medium (200 µL) was added to support optimum growth condition and then the tube was located in a rotating shaker at 37°C for 1 hour with 250 RPM. Meanwhile, LB agar plate (Kanamycin resistant) was taken out of refrigerator and placed in an 37°C incubator. After 1 hour, the tube was taken out of the rotating shaker and snap centrifuged for 5 seconds. The upper layer was discarded and new LB medium (200 µL) was included.

The solution was mixed by pipetting up and down until the precipitate disappears. A plenty of amount of *E.coli* (50 µL) were spread on LB agar plate and placed in 37°C incubator for overnight. The following day, many colonies were seen on plate and a couple of them was selected for colony PCR. Same primers and PCR settings were used as before. Colony #2 showing positive result was grown in LB medium with kanamycin at 37°C in a rotating shaking for overnight. Next day, the blurry mixture containing pHEE401E Red2 Cas9 CsFAE1 plasmid was extracted with Zyppy™ Plasmid Miniprep Kit (Zymo Research, Irvine, CA, USA). To make sure about gRNA, newly purified plasmid was sent for sequencing with gRNA specific primers to MCLAB (South San Franscisco, CA, USA). Sequencing results were analyzed by Molecular Evolutionary Genetics Analysis (MEGA) software, and 100% match was observed with desired gRNA and actual sequencing results. Then, the positive E. coli culture was labeled and stored in a 50% glycerol mix at -80°C freezer.

In the follow-up phase of the experiment, the confirmed plasmid pHEE401E Red2 Cas9 *CsFAE1* was transferred into *Agrobacterium tumefaciens* strain GV3101 (pMP90) using the electroporation method. First, 100 µL *Agrobacterium* was thawed on ice and transferred into pre-chilled 0.2 cm gene pulser electroporation cuvette (Bio-Rad, Hercules, CA, USA). Then, 5 µL of pHEE401E Red2 Cas9 *CsFAE1* plasmid was added into cuvette. Electroporator was set up for a field strength of 2.5 kV/cm, a restorer of 600 Ω, and a capacitance of 25 µF. All moisture outside of cuvette was wipe down by Kimwipes, and later, the cuvette was placed in electroporator and pulse button was pushed and hold 1 second. Immediately, 200 µL LB medium was poured into cuvette and mixed by pipetting

up and down 3-4 times. Agrobacterium solution was transferred into microcentrifuge tube and incubated in a rotating shaker at 28°C for 1 hour. Then, tube was snap centrifuged for 5 seconds, supernatant part was discarded, and new LB medium media (200 µL) was added and mixed gently. After that, 50 µL of *Agrobacterium* solution was spread onto pre-warmed LB agar plate with kanamycin (50 µg/ml) and gentamycin (40µg/ml) and plate was kept in 28°C incubator for 2 days or until colonies are visible. Extracting plasmid DNA from *Agrobacterium* requires one more step which is freeze-thaw method. *Agrobacterium* colony was mixed with 10 µL autoclaved water and microwaved 90 seconds, cooled at -80°C freezer for 10 minutes, and microwaved 90 seconds again to extract plasmid DNA from *Agrobacterium*. Then, this plasmid DNA was used as a template DNA in Agro colony PCR. When PCR product was run on 1% agarose gel, the band size was same as positive control so that it was confirmed that *Agrobacterium* contains expected plasmid. Positive Agro colony was grown in LB media (3 mL) with kanamycin (3 µL) and gentamycin (3 µL) antibiotics at 28°C incubator overnight. Next day, the positive *Agrobacterium* culture was labeled and stored in a 50% glycerol mix at -80°C freezer.

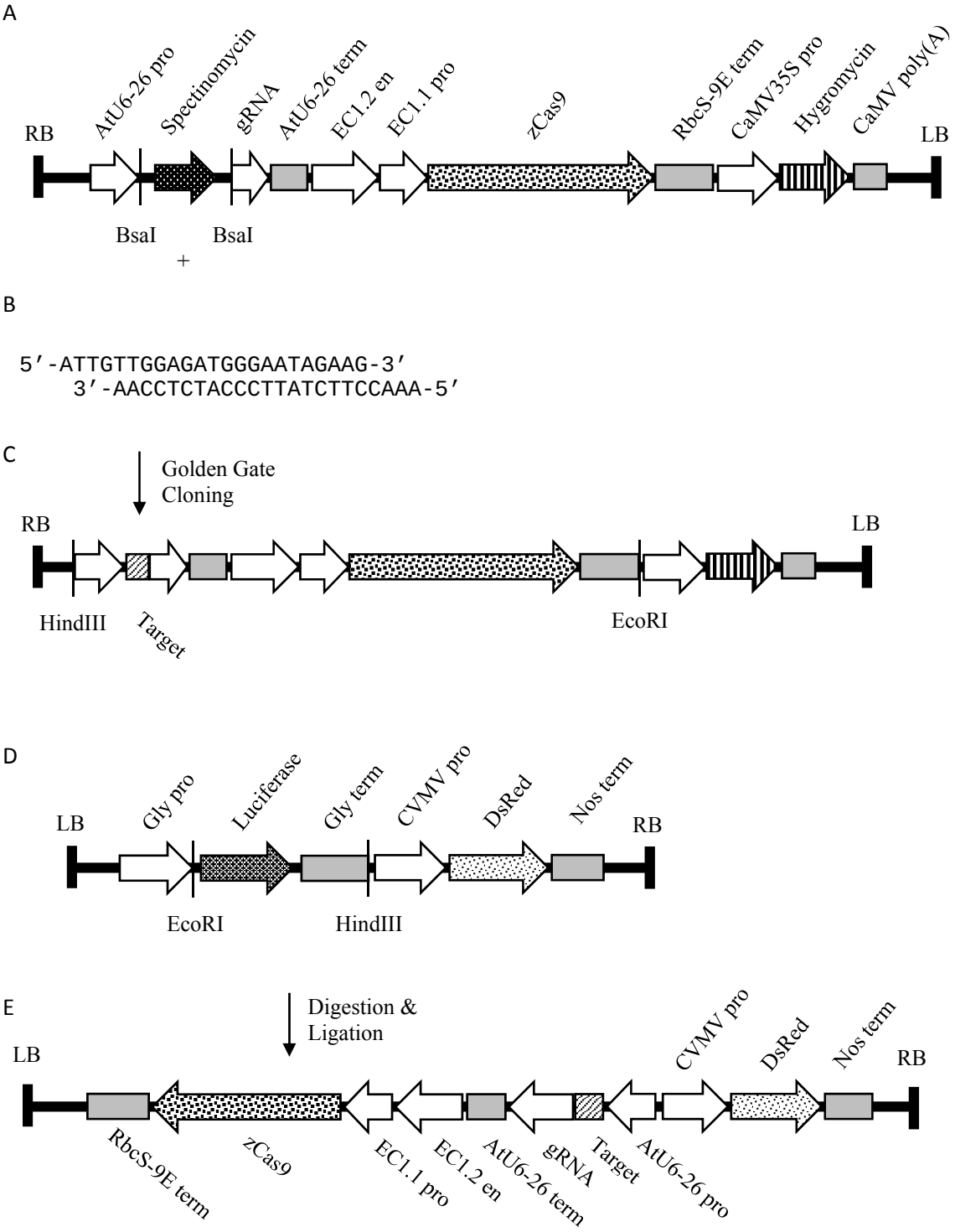

Figure 5. CRISPR/Cas9 Construct Design.

Figure 5 Continued. **A.** pHEE401E plasmid (obtained from Addgene; *www.addgene.org/71827*) contains *Arabidopsis thaliana U6-26* small nuclear RNA promoter (U6-26 pro), Spectinomycin, guide RNA (gRNA) scaffold, *Arabidopsis thaliana U6-26* small nuclear RNA terminator (U6-26 term), egg cell-specific 1.2 enhancer (EC1.2 en), egg cell-specific 1.1 promoter (EC1.1 pro), CRISPR associated protein 9 (Cas9) from *Zea mays*, *Pea sativum* Rubisco small subunit 9E terminator (RbcS-9E term), Cauliflower mosaic virus 35S promoter (CaMV35S pro), Hygromycin, and CaMV poly (A). **B.** The target sequence design for knocking out three *FAE1* copies in *Camelina sativa.* Also, both ends include *BsaI* restriction sites for Golden Gate cloning. **C.** After Golden Gate cloning, Spectinomycin gene was replaced with the target sequence in the new pHEE401E construct. **D.** pBinGlyRed2 construct includes *Glycine max* Glycinin promoter (Gly pro), Luciferase, *Glycine max* Glycinin terminator (Gly term), CaMV35S pro, *Discosoma sp.* red reporter gene (DsRed), Nopaline synthase terminator (Nos term). **E.** Newly generated pHEE401E and pBinGlyRed2 constructs were digested with EcoRI and HindIII, then CRISPR/Cas9 cassette was ligated into digested pBinGlyRed2 construct in the final construct.

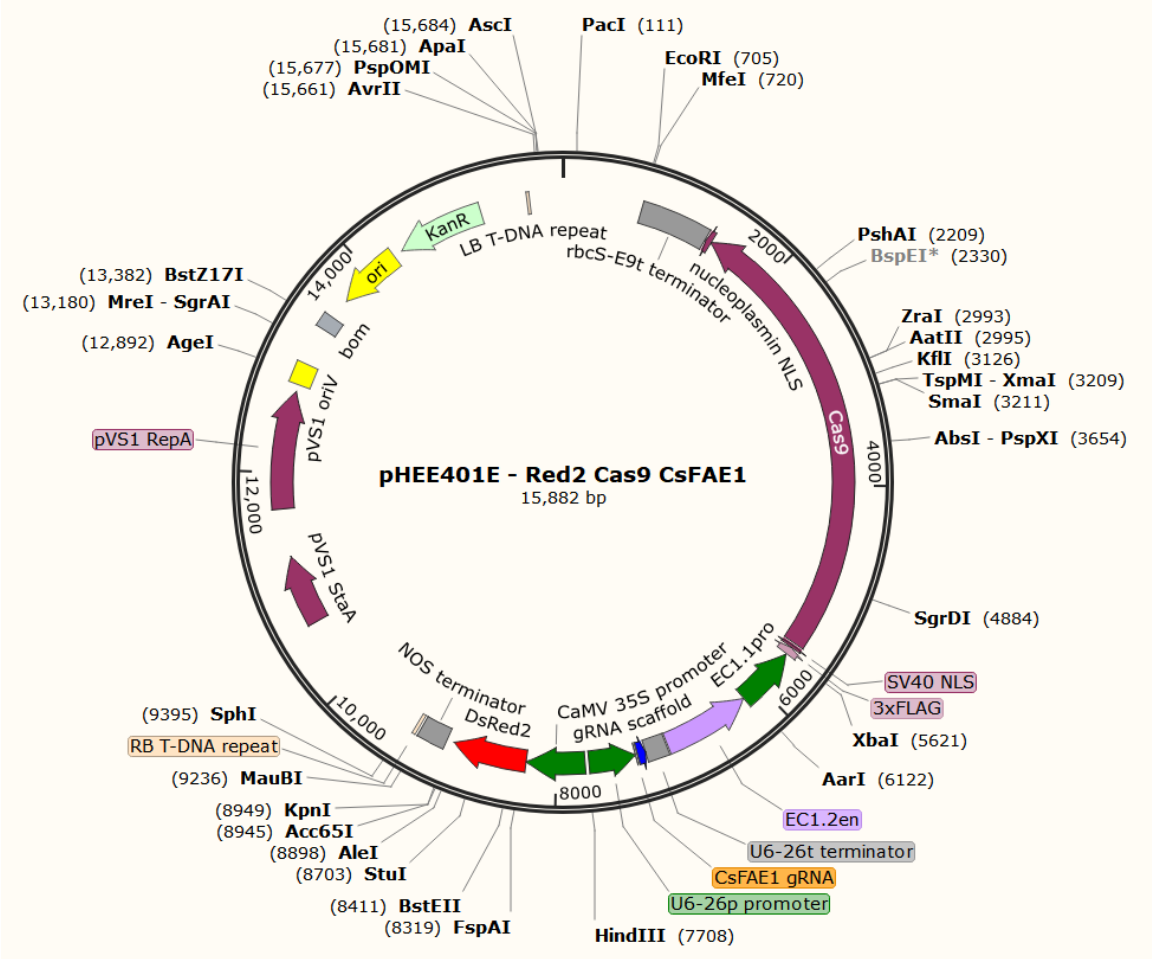

Figure 6. The Map of pHEE401E Red2 Cas9 CsFAE1 Construct.

Camelina Transformation

A well-established camelina transformation protocol, first published by the Lu Lab, was followed to generate transgenic plants (Lu & Kang, 2008). Wild type Camelina, Suneson, plants were planted one month before the transformation. The optimum transformation happens at early flowering stage when the first flowers are blooming and many large buds are present (Dalal, 2016). Historically, the transformation efficiency was recorded around 1% (X. J. Liu et al., 2012; Lu & Kang, 2008). After decided that plants were ready for transformation, 1 µL of *Agrobacterium* containing pHEE401E Red2 Cas9 CsFAE1 plasmid was grown in 3 mL of LB medium with 3 µL kanamycin (100mg/mL) and 3 µL rifamycin (50mg/mL) at 28^{o}C rotating shaker overnight. Next day, 1 mL aliquot from cloudy Agro solution was transferred into 500 mL LB medium for mass production and the large flask was kept in a rotating incubator at 28^{o}C overnight again. The following morning, OD_{600} value was measured 1.0, which should be in the range of 0.8-1.5, by spectrophotometers. Then, 500 mL Agro solution was equally divided into three 200 mL plastic tubes and they spun down at 4500 RPM for 10 minutes. Pink *Agrobacterium* residues were observed for each tube and the supernatant parts were discarded. To resuspend *Agrobacterium*, infiltration solution was prepared and mixed thoroughly. Infiltration solution contains 500 mL double de-ionized water with 5% (w/v) sucrose and 0.05% (v/v) Silwet L77 surfactant (Lehle Seeds, round Rock, TX, USA). Later, 6” pots having ready-for-transformation Camelina plants were placed in a techni-dome vacuum desicator chamber (Bel-Art - SP Scienceware, Wayne, NJ, USA) and a container having resuspended *Agrobacterium* was placed in the middle of vacuum chamber to submerge all

inflorescences during transformation. Plants were held inside the chamber with sustained vacuum pressure for 8 minutes, then pressure valve was opened. After this treatment, tall kitchen bags were used to cover the plants and then they stored in room temperature overnight. Next day, plastic bags were removed, and plants were placed in same greenhouse, and carefully watered without wetting flowering buds. The same transformation protocol was repeated 10 days later to increase efficiency.

Figure 7. Agrobacterium-mediated Camelina Transformation.

Screening and Confirmation of Transgenic Plants

Camelina seed capsules turned from green to light brown when they were completely mature. Harvesting of Agrobacterium treated Camelina plants was done by hand using 1.7 mm sieve (No. 12 Thermo Fischer Scientific, Waltham, MA, USA). After bulk harvesting, all seeds were collected in a spacious envelope and screened under a green light source while looking at them from a red filter covered magnifier. Transgenic seeds were determined based on red fluorescence on seed coat. All red seeds were grown for next generation, and 1" leaf tissue was collected from all plants after 15 days to extract genomic DNA from leaf. CTAB method for DNA extraction (Lukowitz et al., 2000) started with grounding leaf samples by pestle in 1.5 mL microcentrifuge tube, then 300 µL CTAB buffer was added and the tube was incubated at 65°C for 30 minutes. Later, the tube was cooled at room temperature for 5 minutes, 300 µL chloroform was added and vortexed for 10 seconds. Then, the tube was centrifuged for 2 minutes at 12,500 RPM to separate phases. Next, the upper aqueous layer was transferred into a new tube and 300 µL isopropanol (2-propanol) was added and gently mixed by inversion. To help precipitate DNA, the tube was kept on ice for 5 minutes and then centrifuge for 5 minutes at 12,500 RPM. Supernatant part was discarded and the residue part was washed with 500 µL of 70% ethanol. The tube was spun again for 5 minutes at 12,500 RPM, and upper layer was discarded and pellets were air dried until no visible ethanol left. Finally, 100 µL of double de-ionized water was added to resuspend genomic DNA. For leaf DNA PCR protocol, 1.5 µL newly produced leaf DNA templates were used with CsFAE1 gRNA specific primers (U6-26p-F and U6-26t-R). To confirm transgene, the band sizes of PCR products using genomic DNA and

plasmid DNA were compared. Every PCR positive and red seedling was considered as transgenic T_1 events and advanced for next generation. In order to track individual event line, every T_1 and next generations were harvested and stored individually.

Advancing Transformed Lines Towards Homozygosity

The first bulk harvest of transformed plants was analyzed with red film covered magnifier under green light source and 14 T_1 red seeds were obtained. All of these T_1 red seeds were grown and leaf samples were collected per plant. After using leaf genomic DNA of these T_1 plants as a template for PCR, all 14 plants were confirmed that they contain pHEE401E Red2 Cas9 *CsFAE1* construct. These 14 T_1 plants were individually harvested using a sieve so that they produced T_2 seeds from 14 lines and kept in separate envelopes labeled as line 1 to 14. After the seeds from T_1 lines harvested, their fatty acid profiles were analyzed by TMSH derivatization method (Lu et al., 2006). Specifically, 5 seeds from each 14 individual T1 lines were placed in 96-well plate and crushed by metal seed crusher (custom made by Automation by Design, Valencia, CA, USA). Later, 200 µL of TMSH was added to all wells and waited for 15 minutes, then 200 µL of methanol was included for all wells and 96-well plate was cover with plastic wrap to prevent evaporation of methanol. According to GC results, all 14 lines showed some degree of reduction in very long-chain fatty acids (VLCFAs) compared to WT Suneson, therefore, all T_2 events were advanced to the T_3 generation.

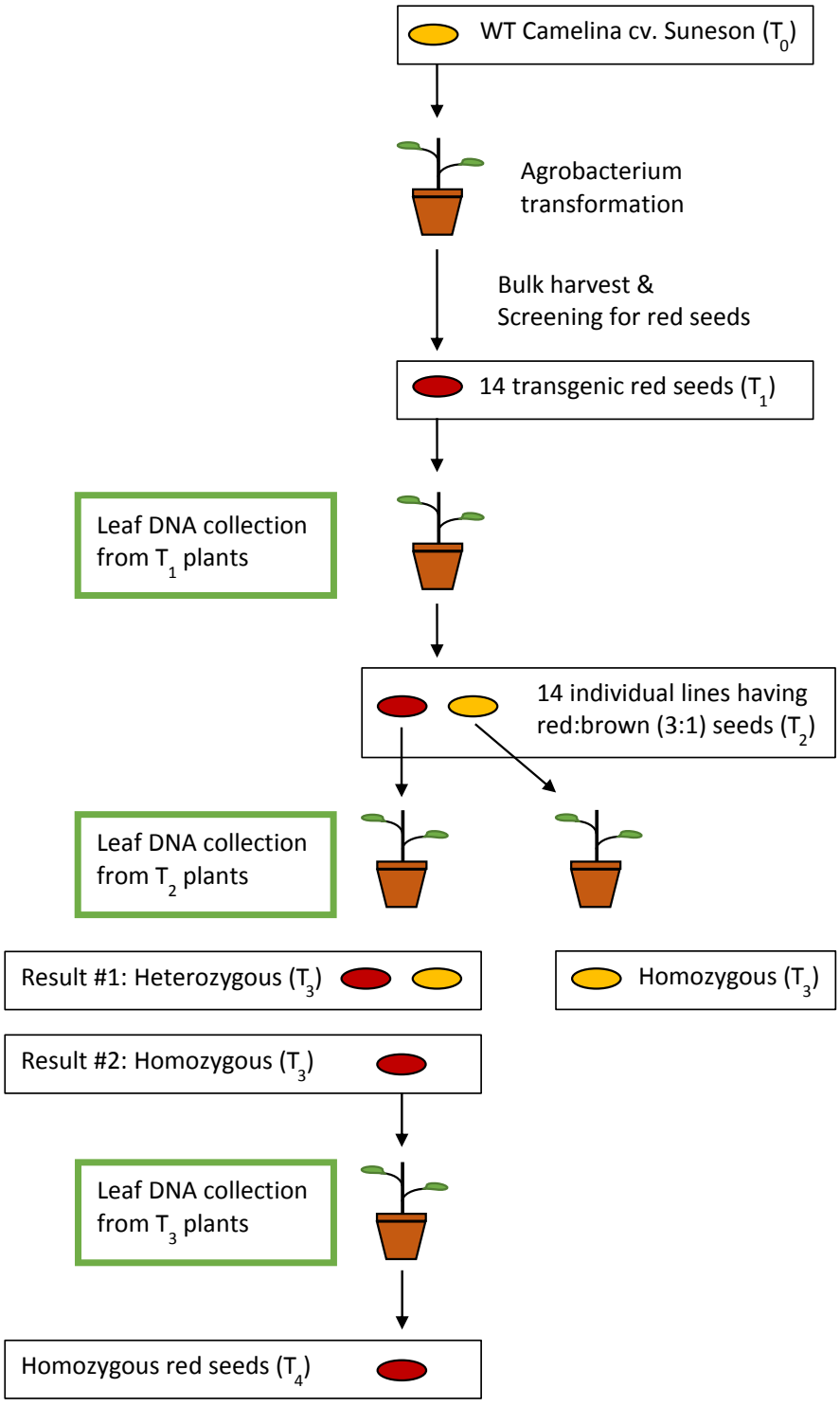

Figure 8. Advancing CRISPR/Cas9 Lines Towards Homozygosity.

Seed and Fatty Acid Analyses

For fatty acid analysis, single camelina seeds were placed in 96-well plate and crushed by metal seeds crusher (custom made by Automation by Design, Valencia, CA, USA). Later, 200 µL of trimethylsulfonium hydroxide (TMSH) was added to all wells and waited for 15 minutes. Then, 200 µL of methanol was included to all wells and 96-well plate was cover with plastic wrap to prevent evaporation of methanol. The resulting fatty acid methyl esters (FAMEs) were analyzed in a gas chromatograph GC-2010 (Shimadzu Scientific Instruments, Columbia, MD, USA) fitted with a 15m × 0.25mm column (DB-23; Agilent, Lexington, MA) and an FID detector. The GC was programmed for an initial temperature of 180°C for 1 min followed by an increase of 10°C/min to 230°C and maintained for a further 1 min.

Seed weight was determined by weighing 100 seeds. Seed oil content was determined by a bench-top NMR seed analyzer (MQC23, Oxford Instruments, Concord, MA, USA) and by GC analysis using heptadecanoic acid (17:0) as internal standard (10 mg/ml), which was added to test tubes prior to FAMEs derivatization. All seeds used for comparison of oil content and seed traits were harvested from plants grown at the same time in the greenhouse.

Amplification of Individual *FAE1* alleles

Camelina sativa consists of three sequence-closely related Fatty Acid Elongase1 (FAE1) enzymes. These isoforms are, namely, *FAE1-A*, *Csa11g007400*; *FAE1-B*, *Csa10g007610*; and *FAE1-C*, *Csa12g009060* (Hutcheon et al., 2010). The main purpose of amplifying individual *FAE1* alleles is to identify modification (insertion, deletion, and substitution) in each of *CsFAE1* genes due to CRIPSR/Cas9 modification. Although coding regions of three *FAE1* loci are the same size (1518bp), their 5' untranslated regions (5' UTR) vary in sequences so that forward primers were chosen at 5' UTR. For amplifying fragments, single reverse primer RP_CsFAE1 was used a combination of forward primers FP_CsFAE1-A, -B, and -C (Table 4). Forward primers were designed in a way that they can uniquely amplify copy A, B, or C (Figure 9).

After guide RNA was confirmed in transgenic line, every line was analyzed regarding to the changes in FAE1 alleles. Thermocycle parameters were as follows: initial denaturation at 95^{o}C for 5 minutes, 32 cycles of denaturation at 95^{o}C for 30 seconds, annealing at 53^{o}C for 30 seconds, extension at 68^{o}C for 90 seconds, and final extension at 68^{o}C for 7 minutes. PCR products were run on 1% agarose gel with ethidium bromide at 125 V and band sizes were analyzed under UV light. To confirm transgene, the band sizes of PCR products using genomic DNA and plasmid DNA were compared.

Figure 9. Alignment of Partial Nucleotide Sequences of Three *Camelina sativa FAE1* Genes.

Figure 9 Continued. Greater sequence variation in upstream of the translation start ATG allowed for designing primers (shown as **bold**) to distinguish three homologs of the FAE1 genes in the camelina sub genomes, and to detect mutations at the sgRNA targets (shown as underlined)

Germination Test

For germination test, 100 T_3 seeds of best CRISPR/Cas9 FAE1 line were used and compared with control wild type MT5 line with triple replicates. Seeds were placed on filter paper dampened with tap water in a covered petri plate 2.5 mm tall x 14 mm wide. Water was replenished as needed when filter paper dried and seeds were left to germinate a couple of days at room temperature.

CHAPTER FOUR

RESULTS

Section 1: Transgenic Line Creation, Confirmation & Analysis

Knocking out *FAE1* alleles via CRISPR/Cas9 reduces VLCFA content in Camelina seeds

Fatty Acid Elongase1 from *Camelina sativa* (CsFAE1) catalyzes the rate limiting step in very long-chain fatty acid (VLCFA) production by extending the length of fatty acids from 18 carbons up to 22 carbons. Malonyl-Coenzyme A (Malonyl-CoA), 2 carbon molecules, is used to elongate oleic acid (18:1) into eicosenoic acid (20:1) by the action of FAE1 enzyme in the acyl-CoA pool. Further extension may happen using malonyl-CoA and FAE1 combination to generate erucic acid (22:1). Previously, the Lu Lab screened a M2 population of EMS-treated seeds to isolate high-oleic camelina mutants (Kang et al., 2011). Interestingly, one mutant line contained low level of VLCFAs such as 0.8% 20:0, 6.1% 20:1, 0.7% 20:2, 0.5% 20:3, 0.3% 22:0, and 0.4% 22:1. Sequencing results indicated that *FAE1-B* copy was mutated, and caused reduced VLCFAs. To achieve even lower amount of VLCFA accumulation in Camelina seeds, *FAE1* alleles were simultaneously targeted by single guide RNA (sgRNA) in WT Camelina, cv. Suneson (Figure 10). The 5'-end of these *FAE1* sequences were deliberately chosen to increase frameshift mutation, and consequently inactivate the FAE1 enzyme. *Arabidopsis* U6-26 promotor (*At*U6-26pro) driven gRNA and egg cell-specific enhancer (EC1.2en) and promoter (EC1.1pro) driven Cas9 gene were used for transformation. The final construct *pHEE401E Red2 Cas9*

CsFAE1 contains cassava vein mosaic virus promoter driven DsRed reporter gene as a selection marker. This construct was transformed into WT camelina, cv. Suneson, by using a vacuum-assisted agroinfiltration method. T_1 plants were harvested at bulk, 14 red seeds were obtained in T_1 generation and were successfully germinated in soil for further analysis.

Seeds were harvested from all 14 independent T_2 lines that showed DsRed segregation indicating single transgene (Table 5). To evaluate the Cas9-induced germline mutations on *FAE1* genes, all 14 T_2 lines containing red and brown seeds were analyzed by gas chromatography (GC). Table 6 shows the fatty acid profile of five individual brown seeds (mean ± SD) from heterozygous 14 T_2 lines. Red seeds from the same heterozygous T_2 lines were analyzed with five seeds per line and listed on Table 7. Since the most abundant VLCFA synthesized by the *FAE1* enzyme is 20:1, the distribution of 20:1 content of all red seeds (Fig. 11A) and brown seeds (Fig. 11B) from heterozygous T_2 lines is compared with non-transgenic seeds of WT Suneson that had been grown together with the transgenic plants in the greenhouse. GC results showed that WT seeds contain 14% 20:1 of total fatty acids (Table 6, Table 7). As a sign of a germline mutation, 4/14 brown lines (#3-3, #4-5, #8-2, and #14-1) have significantly reduced 20:1 content, especially, the brown line #3-3 has less than 1% 20:1 (Table 6). Furthermore, all red lines (14/14) have some degree of reduction in 20:1, and they could be categorized in three groups based on their 20:1 levels: 5-12% (#1-1, #5-1, #6-2, #7-3, #8-2, #9-3, #10-4, #11-4, #12-4, #13-5, #14-1), 2-5% (#2-3, #4-5), and <2% (#3-3) (Table 7). More specifically, individual seeds were compared with wild type seeds (the first five seeds) in Fig.11. Analysis of individual

70 red seeds (Fig. 11A) indicates that 51 of them have low 20:1 content ranging from 0.6 to 11.4%, while other 19 seeds have 12-14% of 20:1 so that they are appeared to be wild type. In this regard, the correlation between reduced 20:1 levels and Cas9-induced mutation at *FAE1* loci is significant, therefore, 20:1 accumulation is directly related to *FAE1* genes and mutations at *FAE1* loci result in low 20:1.

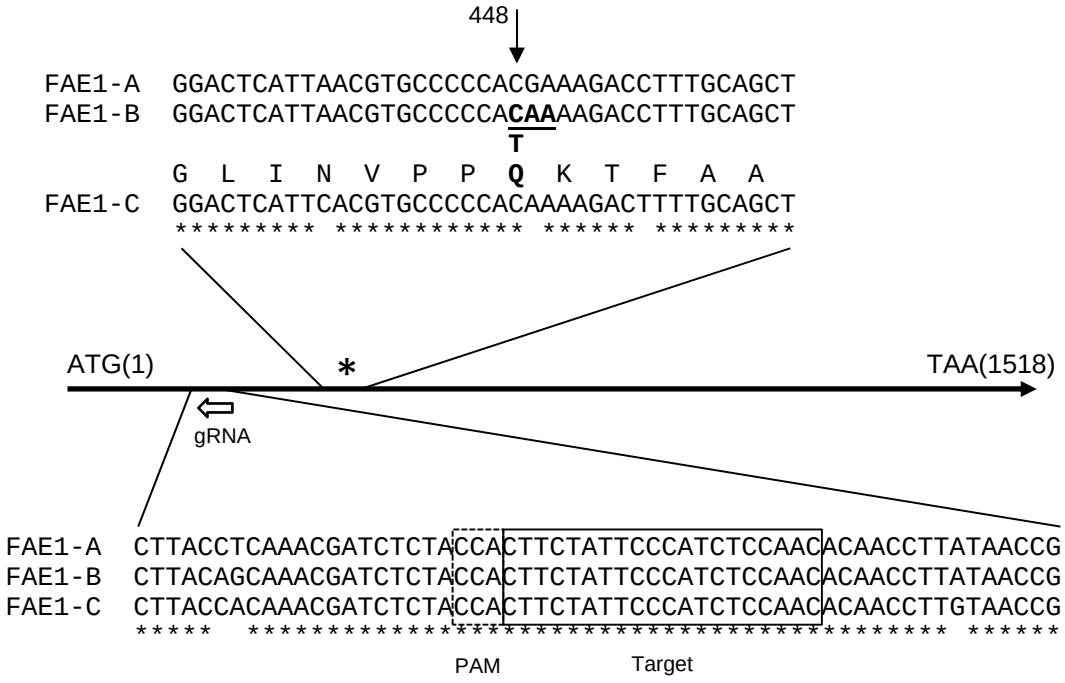

Figure 10. Mutagenesis in the *FAE1* Alleles in *Camelina sativa.* **(A)** An EMS-induced mutation is shown on top at nucleotide no. 448 from the start codon ATG in the FAE1-B gene. Single-letter amino acids are encoded by FAE1-B. The sgRNA sequence targeting all three FAE1 genes (on the reverse strands) is shown at the bottom. Asterisks indicate identical nucleotides. PAM: protospacer adjacent motif. **(B)** T-DNA region (between the left/right borders) of the CRISPR/cas9 construct. The FAE1 sgRNA gene is bracketed by the Arabidopsis thaliana U6-26 small nuclear RNA promoter (U6-26 pro) and terminator (U6-26 term). zCas9 (Zea mays codon-optimized) is driven by an egg cell-specific 1.2 enhancer (EC1.2 en) and an egg cell-specific 1.1 promoter (EC1.1 pro) and the translation stopped by a Pea sativum Rubisco small subunit 9E terminator (RbcS-9E term). The selection marker DsRed is driven by the cassava vein mosaic virus promoter (CVMV pro).

Individual brown seed analysis (Fig. 11B) showed that half of 14 lines have at least one seed with decreased 20:1 levels, however the ratio of low 20:1 seeds is relatively low (20/70). This expected result is due to the egg cell-specific promoter that drives Cas9 protein. It is important to mention that 20:1 contents were less than 1% in both red and brown seeds of line #3, thus homozygosity had been achieved in T_2 generation and all copies of *FAE1* gene were successfully mutated. My results are in agreement with previous Arabidopsis study that homozygosity can be obtained in first generation since target genes had been mutated with same egg cell-specific driven Cas9 gene (Z. P. Wang et al., 2015).

Table 5. Segregation of Red and Brown Seeds in the T2 Lines

Line no.	Red seeds	Brown seeds	χ^2 value[a]
1	133	46	0.047
2	115	41	0.137
3	47	16	0.005
4	109	35	0.037
5	61	22	0.100
6	89	33	0.273
7	76	27	0.081
8	63	22	0.035
9	69	22	0.033
10	140	45	0.045
11	40	15	0.152
12	260	84	0.062
13	76	26	0.013
14	99	34	0.023

[a] $df = 1$, P = 0.05 of $\chi^2 = 3.84$

Table 6. Fatty Acid Composition of Brown Seeds of 14 T2 lines

	Fatty Acid Composition (%)												
Lines	16:0	18:0	18:1	18:2	18:3	20:0	20:1	20:2	20:3	22:0	22:1	Total Elongation	Total Unsaturation
T2 b #1-1	7.6	4.6	14.1	20.0	30.7	4.3	12.5	1.8	1.1	0.4	3.0	23.1	64.8
stdev	1.5	1.4	2.0	3.1	5.0	1.2	1.4	0.3	0.5	0.3	0.5	2.3	3.2
T2 b #2-3	7.0	2.6	11.1	18.9	37.6	3.5	12.6	1.9	1.3	0.2	3.3	22.8	67.7
stdev	0.2	0.5	0.9	0.4	1.6	0.6	0.3	0.1	0.1	0.3	0.1	1.0	1.4
T2 b #3-3	9.4	4.2	17.0	24.7	42.5	1.6	0.5	0.0	0.0	0.0	0.0	2.1	84.3
stdev	1.9	1.0	5.3	4.7	4.0	0.2	0.5	0.0	0.0	0.0	0.0	0.3	2.4
T2 b #4-5	6.8	4.2	17.3	21.4	40.7	1.9	6.1	0.8	0.5	0.2	0.1	9.5	79.5
stdev	0.4	1.6	2.2	0.8	3.5	0.3	1.4	0.2	0.3	0.2	0.2	2.0	1.9
T2 b #5-1	6.4	3.1	13.4	21.5	32.0	3.7	13.2	1.8	1.0	0.5	3.3	23.5	67.0
stdev	0.3	0.9	1.7	3.2	5.9	1.0	0.4	0.1	0.3	0.1	0.4	0.6	1.4
T2 b #6-2	6.7	3.0	12.8	20.5	33.3	3.7	13.1	1.8	1.1	0.5	3.5	23.7	66.6
stdev	0.3	0.6	2.2	1.3	4.2	0.5	0.5	0.2	0.3	0.1	0.1	0.5	0.9
T2 b #7-3	6.7	3.1	12.2	20.7	34.0	3.7	13.1	1.8	1.1	0.5	3.3	23.4	66.9
stdev	0.5	0.4	1.2	2.1	2.4	0.4	0.7	0.1	0.2	0.1	0.4	0.5	0.5
T2 b #8-2	7.2	4.1	13.7	20.6	35.2	3.2	10.5	1.5	1.0	0.4	2.5	19.2	69.5
stdev	0.5	1.7	2.0	1.4	3.9	1.0	1.5	0.3	0.2	0.2	0.7	2.7	2.9
T2 b #9-3	8.0	3.4	10.2	23.7	32.6	3.9	11.9	2.0	1.0	0.1	3.3	22.1	66.5
stdev	0.7	0.9	0.6	2.9	3.1	0.9	1.1	0.1	0.5	0.2	0.4	1.5	0.8
T2 b #10-4	7.2	4.1	11.7	20.1	34.0	4.1	12.2	1.8	1.2	0.4	3.2	23.0	65.7
stdev	0.2	1.6	1.4	1.4	4.0	1.4	0.5	0.2	0.2	0.4	0.2	1.5	2.9
T2 b #11-4	6.5	2.7	12.6	20.9	33.9	3.3	13.3	1.8	1.1	0.4	3.5	23.4	67.4
stdev	0.4	0.3	1.1	2.9	3.8	0.3	0.7	0.1	0.3	0.0	0.2	0.8	0.6
T2 b #12-4	6.8	2.6	12.2	19.4	37.0	3.0	12.5	1.7	1.2	0.2	3.2	21.9	68.7
stdev	0.4	0.4	0.8	1.9	1.7	0.3	1.3	0.1	0.2	0.2	0.3	2.1	1.6
T2 b #13-5	6.5	3.0	12.6	21.6	33.4	3.6	12.9	1.8	1.0	0.5	3.1	22.9	67.6
stdev	0.2	0.4	1.2	2.1	3.3	0.7	0.5	0.1	0.2	0.1	0.2	0.8	1.1
T2 b #14-1	7.8	4.4	15.3	25.5	33.1	2.9	7.8	1.1	0.6	0.2	1.2	13.9	73.9
stdev	1.0	1.2	3.7	2.3	5.1	0.6	1.0	0.2	0.2	0.3	0.4	1.8	1.3
WT	5.5	2.8	14.3	16.5	37.9	2.3	13.9	1.8	1.6	0.4	3.0	23.1	68.7
stdev	0.1	0.2	0.6	0.6	1.1	0.2	0.1	0.1	0.1	0.0	0.1	0.3	0.4

Table 7. Fatty Acid Composition of Red Seeds of 14 T2 lines

	Fatty Acid Composition (%)												
Lines	16:0	18:0	18:1	18:2	18:3	20:0	20:1	20:2	20:3	22:0	22:1	Total Elongation	Total Unsaturation
T2 r #1-1	6.7	3.0	15.1	20.5	40.8	2.1	8.5	1.1	0.7	0.2	1.3	13.9	76.4
stdev	0.4	0.6	1.8	2.0	2.2	0.4	3.5	0.5	0.5	0.2	1.3	6.2	5.3
T2 r #2-3	7.3	3.4	16.2	20.7	44.8	1.6	4.5	0.5	0.4	0.1	0.4	7.6	81.8
stdev	0.2	1.2	1.2	2.3	5.2	1.1	2.6	0.4	0.4	0.2	0.4	4.8	5.5
T2 r #3-3	8.2	4.5	18.3	24.4	42.6	1.2	0.7	0.0	0.0	0.0	0.0	1.9	85.4
stdev	1.3	1.3	2.8	3.0	5.0	0.6	0.4	0.0	0.0	0.0	0.0	0.6	2.6
T2 r #4-5	7.0	4.0	18.8	22.6	43.4	1.5	2.4	0.2	0.2	0.0	0.0	4.3	84.7
stdev	0.0	0.8	3.1	1.9	4.7	0.1	1.9	0.3	0.2	0.0	0.0	2.5	2.4
T2 r #5-1	6.6	2.8	14.6	19.8	40.3	2.1	9.8	1.3	0.9	0.1	1.7	16.0	74.6
stdev	0.3	0.5	2.7	2.2	3.8	0.5	3.8	0.5	0.4	0.2	1.6	6.9	6.4
T2 r #6-2	6.9	3.0	14.3	21.2	40.6	2.1	8.6	1.2	0.8	0.0	1.1	13.9	76.2
stdev	0.1	0.4	1.4	1.5	2.3	0.3	1.6	0.3	0.2	0.0	0.5	2.8	2.7
T2 r #7-3	7.0	3.0	12.9	20.7	39.0	2.4	10.4	1.5	1.0	0.1	2.1	17.4	72.6
stdev	0.8	0.5	1.8	3.0	1.7	0.3	3.0	0.4	0.4	0.2	1.2	5.4	4.4
T2 r #8-2	7.2	3.0	13.9	20.3	39.1	2.3	10.1	1.4	1.0	0.1	1.7	16.6	73.3
stdev	0.4	0.4	1.2	3.1	3.7	0.2	1.4	0.2	0.2	0.1	0.4	2.0	1.6
T2 r #9-3	6.8	2.7	13.0	18.0	41.0	2.4	11.1	1.5	1.2	0.1	2.2	18.5	71.9
stdev	0.4	0.3	1.4	0.9	3.4	0.4	3.0	0.4	0.2	0.2	1.1	5.0	4.5
T2 r #10-4	6.9	3.0	13.0	19.8	38.0	2.7	11.1	1.6	1.0	0.2	2.7	19.3	70.8
stdev	0.5	0.5	1.5	2.5	4.5	0.6	4.0	0.5	0.4	0.2	1.3	6.9	6.2
T2 r #11-4	6.7	3.1	13.1	20.7	36.1	2.9	12.0	1.6	1.0	0.2	2.6	20.2	70.0
stdev	0.3	0.5	2.0	1.7	2.3	0.4	2.1	0.3	0.3	0.2	0.8	3.7	3.1
T2 r #12-4	7.0	3.4	19.9	19.8	38.2	1.9	7.4	0.8	0.5	0.0	1.0	11.7	77.9
stdev	0.6	0.5	5.9	1.5	4.4	0.6	2.9	0.6	0.3	0.0	1.1	5.4	4.8
T2 r #13-5	6.5	2.9	13.8	20.0	38.2	2.6	11.1	1.5	1.0	0.3	2.1	18.6	72.0
stdev	0.3	0.9	2.2	3.2	5.4	0.7	3.0	0.4	0.4	0.2	1.3	5.5	5.0
T2 r #14-1	7.8	4.5	16.3	26.2	33.5	2.4	6.8	1.1	0.5	0.2	0.8	11.7	76.0
stdev	0.3	1.0	2.6	1.8	4.6	0.5	1.7	0.4	0.3	0.2	0.6	3.2	3.2
WT	5.5	2.8	14.3	16.5	37.9	2.3	13.9	1.8	1.6	0.4	3.0	23.1	68.7
stdev	0.1	0.2	0.6	0.6	1.1	0.2	0.1	0.1	0.1	0.0	0.1	0.3	0.4

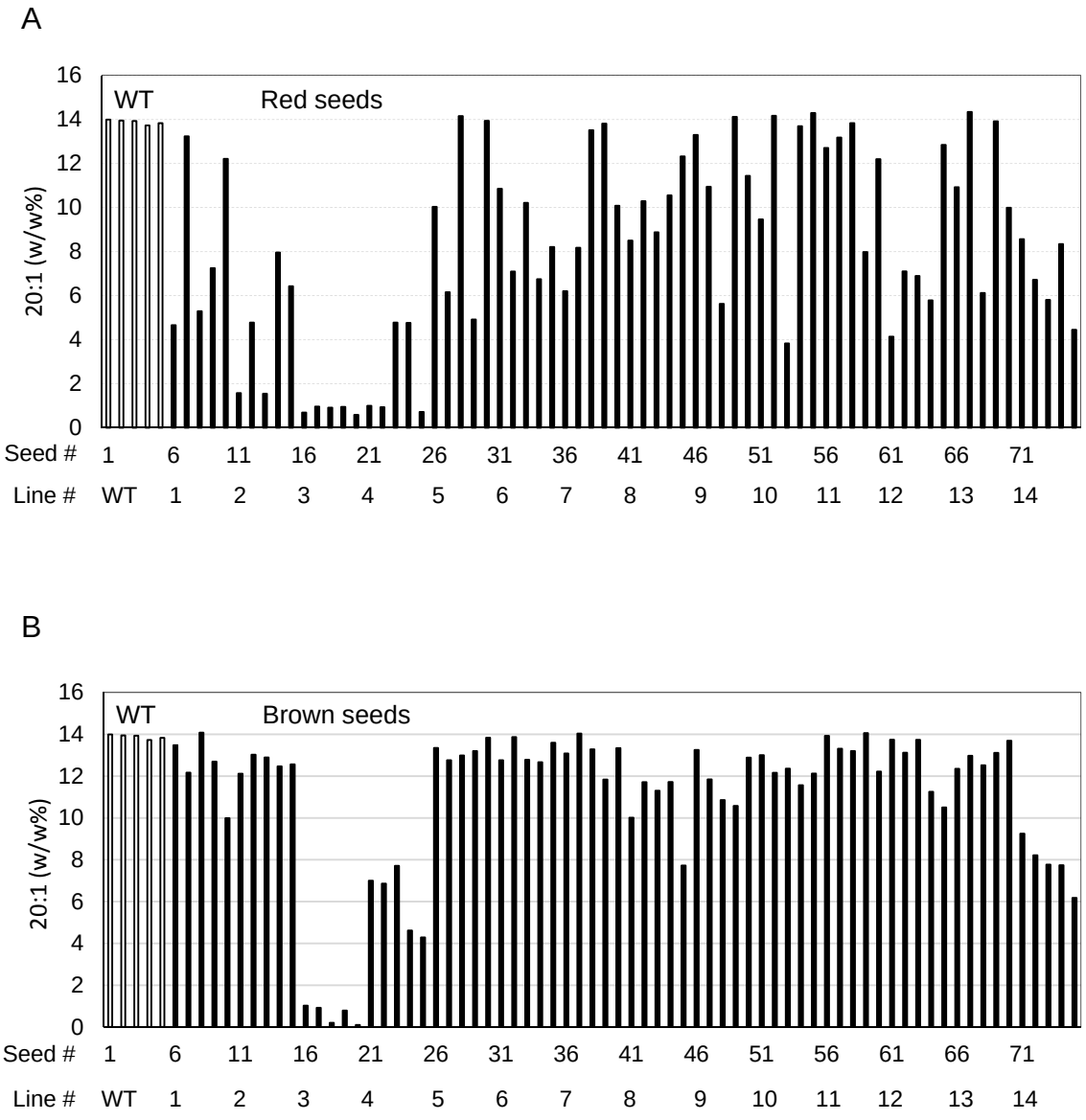

Figure 11. Eicosenoic acid (20:1) Content in Single Seeds of Wild Type and Cas9-modified Camelina. Seeds no. 1-5 (white bars) show wild type. Red (A) and brown (B) seeds from 14 T_2 transgenic lines are shown in black bars.

For T_3 generation, 10 red seeds and 10 brown seeds from each 14 T_2 were grown (Fig. 8), along with WT control plants. T_2 red seeds produced high percentage of all red T_3 seeds in next generation (8 all red lines/10); however, some lines (#4, #5, #8, #9, #10) have higher ratio of mixed lines (3-7 all red lines/10) since they have still segregating. Then, I compared fatty acid profiles of brown seeds (Table 8) and red seeds (Table 9) from T_3 heterozygous lines. Brown seeds from T_3 heterozygous lines (Fig. 8, Result#1: Heterozygous (T_3)) had even lower 20:1 than brown seeds from T_2 heterozygous, thus it demonstrated highly effective inheritance of CRISPR/Cas9 induced mutations. Most of the brown seeds from T_3 heterozygous lines showed a degree of reduction in 20:1 levels: 4 lines have 10-14%, 6 lines have 4-10%, 1 line has 2-4%, and 2 lines have <2%. Also, there is no brown seeds observed in line #13. Table 9 shows the fatty acid profile of red seeds from T_3 heterozygous lines, and 20:1 levels are groups as 10-14% (1 line), 4-10% (9 lines), 2-4% (2 lines) and <2% (2 lines). Therefore, it is obvious that red seeds have better efficiency than brown seeds in T_3 heterozygous lines. Red seeds from T_3 homozygous lines (Fig. 8, Result #2: Homozygous (T_3)) were analyzed (Table 10) and results indicated that 20:1 levels are lower than red seeds from T_2 heterozygous lines such as, 10-14% (0 lines), 4-10% (8), 2-4% (3 lines), <1% (3 lines). Especially, line #3, #8, and #9 have desired FA profiles with lowest elongation. I continued with line #3 and selected the best lines (#3-3-1, #3-3-3, #3-3-4, #3-3-14) (Table 11). Total elongation of these 4 T_3 lines were less than 2%, and also, 20:1 was less than around 1%. T_4 generation of those lines had even better results with 20:1 is less than 1% and total elongation was less 1.5% (Table 12). Furthermore, brown seeds of 14 T_3 homozygous lines had the similar FA profile as brown

seeds from T_2 heterozygous lines (Table 13). Since germline mutation affected fatty acid profiles in T_1 generation and Cas9-protein was segregated, brown lines showing WT profile in T_2 continued to show WT profile in T_3. In other words, no improvement regarding VLCFAs reduction was observed in T_3 homozygous lines.

Table 8. Fatty Acid Composition of Brown Seeds from Heterozygous T3 Lines

	Fatty Acid Composition (%)												
Lines	16:0	18:0	18:1	18:2	18:3	20:0	20:1	20:2	20:3	22:0	22:1	Total Elongation	Total Unsaturation
T3 b #1-1-6	9.3	4.8	23.5	17.3	31.0	2.5	7.9	0.0	0.0	0.8	1.2	12.5	71.8
stdev	0.7	1.0	1.9	0.7	1.4	0.2	0.7	0.0	0.0	0.8	1.2	1.3	0.2
T3 b #2-3-9	8.2	3.8	16.2	16.5	31.4	3.9	14.1	1.8	0.7	0.6	1.7	22.9	64.1
stdev	1.8	3.2	1.3	3.4	7.4	3.9	1.1	1.4	0.5	0.5	1.2	6.3	11.9
T3 b #3-3-10	7.7	4.4	25.3	20.0	37.3	1.1	1.8	0.6	0.8	0.6	0.8	4.5	82.6
stdev	1.1	1.2	2.6	1.9	4.7	0.7	0.9	0.3	0.5	0.4	0.5	2.5	5.1
T3 b #4-5-1	9.7	5.3	20.0	15.9	32.4	3.1	4.7	7.6	3.5	0.4	0.6	16.2	68.3
stdev	1.3	1.1	1.6	3.1	9.9	2.4	3.0	5.9	2.6	0.2	0.4	10.0	11.4
T3 b #5-1-3	8.2	5.0	20.8	17.2	36.7	2.4	7.1	1.1	0.2	0.9	0.0	11.1	74.8
stdev	1.4	1.5	1.1	3.0	2.5	1.0	0.3	0.6	0.1	0.9	0.0	2.8	6.4
T3 b #6-2-5	8.4	4.2	14.1	16.0	31.0	5.4	13.8	2.3	0.9	1.1	2.0	25.1	61.0
stdev	2.1	3.7	1.7	3.7	8.9	4.4	1.5	1.6	0.7	0.7	1.2	7.2	14.0
T3 b #7-3-4	8.3	4.3	13.9	17.8	32.2	2.9	12.6	2.2	0.8	1.0	3.0	22.0	63.9
stdev	1.7	2.3	0.9	1.4	4.9	0.7	1.3	0.5	0.5	0.6	0.1	0.9	5.8
T3 b #8-2-5	6.9	3.5	17.1	17.9	38.3	1.4	10.9	1.2	1.0	0.0	1.8	16.3	73.3
stdev	1.5	1.7	2.3	1.0	1.8	0.8	0.5	0.7	0.6	0.0	1.1	3.6	1.2
T3 b #9-3-1	8.1	4.5	26.3	15.7	30.9	3.0	8.6	0.9	0.2	0.8	0.5	13.7	72.9
stdev	1.4	1.6	7.6	3.6	5.5	2.3	1.0	1.1	0.3	0.7	0.3	5.4	9.6
T3 b #10-4-2	7.4	3.4	22.1	20.6	42.6	1.2	2.3	0.0	0.0	0.0	0.0	3.4	85.3
stdev	0.8	0.7	1.8	1.3	3.1	0.4	2.2	0.0	0.0	0.0	0.0	2.5	3.1
T3 b #11-4-7	7.5	2.6	17.9	15.7	38.2	1.6	7.6	5.6	3.6	0.2	1.0	17.7	71.8
stdev	0.9	1.7	1.8	3.2	7.9	0.6	2.5	4.3	3.0	0.1	0.8	9.9	10.0
T3 b #12-4-5	7.8	4.7	44.3	11.1	28.6	0.8	1.1	0.0	0.6	1.0	0.0	3.1	84.0
stdev	1.9	1.8	3.2	1.8	2.7	0.8	0.9	0.0	0.4	0.7	0.0	3.2	7.5
T3 b #14-1-4	9.9	6.2	19.4	22.0	36.6	0.7	4.0	0.0	0.0	1.0	0.0	5.3	78.0
stdev	4.1	3.6	0.8	2.8	7.5	0.7	2.3	0.0	0.0	0.6	0.0	2.3	9.7
WT	5.5	2.9	14.9	16.8	36.7	2.4	14.1	1.8	1.5	0.4	2.9	23.2	68.4
stdev	0.3	0.3	1.0	0.8	1.6	0.3	0.4	0.1	0.1	0.0	0.2	0.6	0.9

Table 9. Fatty Acid Composition of Red Seeds from Heterozygous T3 Lines

	Fatty Acid Composition (%)												
Lines	16:0	18:0	18:1	18:2	18:3	20:0	20:1	20:2	20:3	22:0	22:1	Total Elongation	Total Unsaturation
T3 r #1-1-6	6.1	2.6	20.6	20.5	40.0	1.6	7.0	1.0	0.7	0.0	0.0	10.3	81.0
stdev	0.2	0.2	0.5	0.7	0.8	0.1	0.2	0.0	0.1	0.0	0.0	0.3	0.4
T3 r #2-3-9	6.6	2.7	19.1	20.4	39.6	1.7	7.2	1.0	0.7	0.0	0.8	11.4	79.1
stdev	0.3	0.2	1.3	1.4	2.1	0.3	2.9	0.4	0.3	0.0	0.7	4.6	4.3
T3 r #3-3-10	6.5	2.6	25.0	21.7	42.2	0.9	1.1	0.0	0.0	0.0	0.0	2.1	88.9
stdev	0.5	0.4	1.9	0.7	1.8	0.5	0.0	0.0	0.0	0.0	0.0	0.4	0.4
T3 r #4-5-1	6.5	3.1	20.2	20.5	41.5	1.6	5.2	0.7	0.4	0.1	0.1	8.1	82.3
stdev	0.3	0.2	1.2	1.3	1.7	0.2	2.3	0.4	0.3	0.1	0.1	3.2	3.0
T3 r #5-1-3	6.8	2.7	25.7	21.4	39.1	1.2	2.8	0.2	0.2	0.0	0.0	4.4	86.2
stdev	0.1	0.1	7.8	3.5	3.8	0.2	1.4	0.2	0.2	0.0	0.0	1.9	1.9
T3 r #6-2-5	6.7	2.5	17.7	21.1	39.6	1.7	7.8	1.2	0.8	0.0	0.8	12.3	78.4
stdev	0.4	0.3	1.1	1.9	1.7	0.2	2.6	0.4	0.3	0.0	0.7	4.2	3.7
T3 r #7-3-4	7.3	2.7	15.9	21.3	34.5	2.2	11.2	1.7	1.0	0.1	1.8	17.9	71.7
stdev	0.3	0.3	1.1	1.1	1.1	0.3	1.5	0.2	0.1	0.1	0.7	2.7	2.6
T3 r #8-2-5	6.4	2.6	18.0	20.3	39.3	1.9	8.2	1.2	0.8	0.1	0.9	13.1	77.7
stdev	0.3	0.1	1.6	1.7	1.5	0.2	1.4	0.2	0.2	0.1	0.4	2.5	2.4
T3 r #9-3-1	6.2	2.6	23.1	18.4	37.1	1.8	8.4	1.0	0.8	0.0	0.5	12.5	78.7
stdev	0.2	0.2	1.8	1.1	1.8	0.1	0.7	0.1	0.1	0.1	0.3	1.1	1.0
T3 r #10-4-2	6.5	3.0	22.8	22.9	41.3	1.1	2.3	0.2	0.1	0.0	0.0	3.6	86.9
stdev	0.3	0.1	1.2	0.4	2.3	0.5	1.0	0.2	0.1	0.0	0.0	1.6	1.8
T3 r #11-4-7	6.3	3.3	18.4	19.2	42.0	1.9	6.6	0.8	0.7	0.2	0.5	10.7	79.7
stdev	0.3	0.3	1.5	1.3	1.0	0.2	2.3	0.3	0.2	0.0	0.3	3.3	2.7
T3 r #12-4-5	6.5	2.6	45.4	12.4	31.4	0.9	0.8	0.0	0.0	0.0	0.0	1.7	89.1
stdev	0.4	0.1	1.8	0.8	1.7	0.0	0.1	0.0	0.0	0.0	0.0	0.1	0.3
T3 r #13-5-5	6.8	3.1	15.8	22.1	40.5	2.1	6.9	1.0	0.6	0.1	0.9	11.5	78.4
stdev	0.2	0.3	1.1	1.1	5.4	0.5	3.7	0.5	0.4	0.1	0.8	5.9	6.0
T3 r #14-1-4	7.1	3.3	17.0	22.5	42.2	1.7	5.0	0.7	0.4	0.0	0.2	7.8	81.8
stdev	0.3	0.4	1.3	1.1	2.3	0.3	2.3	0.4	0.2	0.0	0.2	3.3	3.4
WT	5.5	2.9	14.9	16.8	36.7	2.4	14.1	1.8	1.5	0.4	2.9	23.2	68.4
stdev	0.3	0.3	1.0	0.8	1.6	0.3	0.4	0.1	0.1	0.0	0.2	0.6	0.9

Table 10. Fatty Acid Composition of Red Seeds from Homozygous T3 Lines

	Fatty Acid Composition (%)												
Lines	16:0	18:0	18:1	18:2	18:3	20:0	20:1	20:2	20:3	22:0	22:1	Total Elongation	Total Unsaturation
T3 r #1-1-1	6.4	2.1	20.8	19.8	41.8	1.5	6.3	0.8	0.6	0.0	0.0	9.2	82.3
stdev	0.2	0.4	2.0	0.7	1.8	0.1	0.2	0.1	0.0	0.0	0.0	0.4	0.4
T3 r #2-3-1	6.1	2.3	19.0	18.2	43.4	1.6	6.8	1.0	0.8	0.2	0.7	11.0	80.6
stdev	0.3	0.1	1.0	0.9	0.7	0.2	1.6	0.2	0.2	0.1	0.3	2.5	2.3
T3 r #3-3-1	6.2	2.8	24.5	21.3	43.6	0.9	0.8	0.0	0.0	0.0	0.0	1.6	89.5
stdev	0.2	0.2	0.7	1.1	2.2	0.4	0.0	0.0	0.0	0.0	0.0	0.4	0.7
T3 r #4-5-6	5.8	3.3	21.4	19.3	43.2	1.0	4.7	0.6	0.5	0.1	0.3	6.9	83.9
stdev	0.3	0.2	0.9	1.1	1.3	0.1	1.0	0.1	0.1	0.1	0.1	1.8	1.6
T3 r #5-1-1	6.1	3.6	25.8	17.4	42.2	0.9	3.1	0.3	0.4	0.1	0.2	4.9	85.4
stdev	0.1	0.3	8.9	3.4	5.2	0.1	0.6	0.2	0.1	0.1	0.1	1.1	1.0
T3 r #6-2-1	6.5	2.9	17.4	18.4	46.3	1.0	5.3	0.7	0.9	0.1	0.6	8.5	82.1
stdev	0.4	0.2	1.3	1.1	2.1	0.2	2.3	0.3	0.3	0.1	0.4	3.5	3.1
T3 r #7-3-1	7.3	2.6	18.0	21.5	38.2	1.9	8.2	1.1	0.7	0.0	0.7	12.4	77.7
stdev	0.6	0.5	2.0	2.3	4.0	0.4	4.2	0.6	0.4	0.0	0.6	6.1	5.4
T3 r #8-2-1	6.4	2.8	20.6	19.9	49.2	0.3	0.8	0.0	0.0	0.0	0.0	1.1	89.8
stdev	0.3	0.2	1.2	1.4	1.2	0.5	0.1	0.0	0.0	0.0	0.0	0.5	0.6
T3 r #9-3-2	6.3	3.5	22.4	20.4	45.8	0.7	0.9	0.0	0.0	0.0	0.0	1.6	88.5
stdev	0.2	0.2	0.5	0.5	0.7	0.0	0.1	0.0	0.0	0.0	0.0	0.1	0.4
T3 r #10-4-1	7.0	3.3	19.4	20.2	43.3	1.0	4.4	0.5	0.6	0.0	0.4	6.9	82.8
stdev	0.3	0.3	1.9	1.5	1.5	0.1	1.9	0.3	0.3	0.0	0.3	2.8	2.5
T3 r #11-4-1	6.6	2.9	19.4	20.9	43.5	1.5	4.1	0.6	0.4	0.0	0.2	6.7	83.8
stdev	0.1	0.3	0.4	0.2	1.5	0.2	1.3	0.2	0.2	0.0	0.2	1.9	1.4
T3 r #12-4-1	5.9	2.4	20.8	20.0	43.3	1.5	4.7	0.7	0.5	0.0	0.3	7.6	84.0
stdev	0.4	0.2	0.7	2.3	1.2	0.1	1.4	0.2	0.3	0.0	0.2	2.2	1.8
T3 r #13-5-1	6.9	3.2	19.1	22.5	45.5	0.6	2.4	0.1	0.0	0.0	0.0	2.9	87.1
stdev	0.2	0.4	1.3	1.7	4.6	0.8	2.1	0.3	0.0	0.0	0.0	3.4	3.6
T3 r #14-1-1	7.1	3.2	18.8	23.2	41.9	1.6	3.8	0.3	0.2	0.0	0.0	5.8	83.9
stdev	0.3	0.6	1.1	1.1	0.8	0.2	1.6	0.2	0.1	0.0	0.0	1.9	1.6
WT	5.5	2.9	14.9	16.8	36.7	2.4	14.1	1.8	1.5	0.4	2.9	23.2	68.4
stdev	0.3	0.3	1.0	0.8	1.6	0.3	0.4	0.1	0.1	0.0	0.2	1.2	3.4

Table 11. Fatty Acid Composition of Best T3 Lines

	Fatty Acid Composition (%)												
Lines	16:0	18:0	18:1	18:2	18:3	20:0	20:1	20:2	20:3	22:0	22:1	Total Elongation	Total Unsaturation
T3 r #3-3-1	6.2	2.8	24.5	21.3	43.6	0.9	0.8	0.0	0.0	0.0	0.0	1.6	89.5
stdev	0.2	0.2	0.7	1.1	2.2	0.4	0.0	0.0	0.0	0.0	0.0	0.4	0.7
T3 r #3-3-3	6.1	3.6	26.5	19.2	42.9	0.7	1.1	0.0	0.0	0.0	0.0	1.8	88.5
stdev	0.1	0.1	0.9	0.5	0.7	0.0	0.1	0.0	0.0	0.0	0.0	0.1	0.2
T3 r #3-3-4	6.5	4.0	18.1	19.7	49.7	0.8	1.1	0.0	0.0	0.0	0.0	1.9	87.6
stdev	0.3	0.3	0.4	0.5	0.6	0.0	0.1	0.0	0.0	0.0	0.0	0.1	0.5
T3 r #3-3-14	6.2	3.6	21.2	21.4	45.8	0.8	1.1	0.0	0.0	0.0	0.0	1.8	88.4
stdev	0.3	0.2	1.9	0.6	1.7	0.1	0.2	0.0	0.0	0.0	0.0	0.3	0.6
WT	5.5	2.9	14.9	16.8	36.7	2.4	14.1	1.8	1.5	0.4	2.9	23.2	68.4
stdev	0.3	0.3	1.0	0.8	1.6	0.3	0.4	0.1	0.1	0.0	0.2	1.2	3.4

Table 12. Fatty Acid Composition of Best T4 Lines

	Fatty Acid Composition (%)												
Lines	16:0	18:0	18:1	18:2	18:3	20:0	20:1	20:2	20:3	22:0	22:1	Total Elongation	Total Unsaturation
T4 r #3-3-1-2	6.8	2.7	17.8	20.5	51.3	0.0	0.9	0.0	0.0	0.0	0.0	0.9	89.6
stdev	0.3	0.1	1.2	1.0	1.8	0.0	0.1	0.0	0.0	0.0	0.0	0.1	0.3
T4 r #3-3-2-2	7.1	2.6	19.4	18.7	51.0	0.3	0.9	0.0	0.0	0.0	0.0	1.2	89.1
stdev	0.4	0.1	1.5	1.7	2.2	0.6	0.1	0.0	0.0	0.0	0.0	0.6	0.9
T4 r #3-3-4-2	7.2	2.8	16.9	21.5	50.7	0.3	0.7	0.0	0.0	0.0	0.0	1.0	89.0
stdev	0.2	0.4	1.4	1.4	3.1	0.5	0.1	0.0	0.0	0.0	0.0	0.6	0.5
T4 r #3-3-14-2	6.8	2.4	19.1	21.1	49.3	0.5	0.8	0.0	0.0	0.0	0.0	1.3	89.5
stdev	0.3	0.4	1.0	1.6	3.0	0.6	0.0	0.0	0.0	0.0	0.0	0.6	0.7
WT	6.4	1.7	12.6	17.9	39.0	2.5	13.3	1.9	1.4	0.1	3.1	22.3	69.5
stdev	0.4	0.2	0.8	1.5	1.6	0.1	0.7	0.1	0.2	0.1	0.3	1.2	0.6

Table 13. Fatty Acid Composition of Brown Seeds from Homozygous T3 Lines

	Fatty Acid Composition (%)												
Lines	16:0	18:0	18:1	18:2	18:3	20:0	20:1	20:2	20:3	22:0	22:1	Total Elongation	Total Unsaturation
T3 b #1-1-1	6.0	2.0	17.1	18.3	35.6	2.3	13.3	1.9	1.3	0.0	2.1	21.0	71.0
stdev	0.2	0.2	0.5	0.8	1.1	0.1	0.4	0.1	0.1	0.0	0.1	0.6	0.5
T3 b #2-3-1	5.7	2.0	16.2	18.2	35.7	2.4	13.9	2.0	1.3	0.1	2.4	22.2	70.1
stdev	0.3	0.1	0.7	1.2	1.0	0.1	0.2	0.0	0.1	0.1	0.1	0.6	0.6
T3 b #3-3-1	6.2	2.8	23.5	21.3	44.9	0.2	1.0	0.0	0.0	0.0	0.0	1.2	89.8
stdev	0.3	0.3	0.6	0.5	1.3	0.5	0.1	0.0	0.0	0.0	0.0	0.5	0.9
T3 b #4-5-6	6.3	3.0	22.1	21.7	41.2	1.4	3.6	0.4	0.3	0.0	0.0	5.7	84.9
stdev	0.3	0.3	0.7	0.9	0.6	0.1	1.3	0.2	0.1	0.0	0.0	1.8	1.5
T3 b #5-1-1	5.8	3.0	15.4	17.4	35.1	2.3	14.4	1.8	1.5	0.3	2.9	23.3	67.9
stdev	0.4	0.3	0.7	1.7	1.9	0.3	0.4	0.1	0.1	0.2	0.2	0.8	0.6
T3 b #6-2-1	5.5	2.8	16.5	17.6	35.0	2.2	14.1	1.8	1.5	0.3	2.7	22.6	69.0
stdev	0.2	0.1	0.7	0.7	0.9	0.1	0.1	0.0	0.1	0.0	0.2	0.5	0.5
T3 b #7-3-2	5.9	2.8	20.6	20.3	40.1	1.7	6.6	0.9	0.6	0.2	0.1	10.2	81.0
stdev	0.0	0.0	0.5	0.4	1.1	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.1
T3 b #8-2-1	5.8	2.1	13.4	16.4	39.8	2.6	13.2	1.9	1.6	0.3	2.9	22.5	69.5
stdev	0.3	0.1	1.3	0.5	1.4	0.2	0.4	0.0	0.1	0.0	0.3	0.7	0.9
T3 b #9-3-2	5.8	2.7	14.4	17.2	35.4	2.2	14.8	2.1	1.7	0.4	3.4	24.5	67.0
stdev	0.5	0.1	1.0	1.0	0.8	0.1	0.2	0.0	0.1	0.0	0.2	0.3	0.7
T3 b #10-4-1	5.8	3.2	16.4	17.9	33.4	2.4	14.4	1.8	1.4	0.4	2.9	23.3	67.7
stdev	0.2	0.2	1.0	1.1	2.1	0.1	0.3	0.0	0.1	0.0	0.1	0.3	0.2
T3 b #11-4-1	6.0	2.6	17.0	18.6	35.7	2.4	12.5	1.7	1.2	0.3	2.0	20.2	71.2
stdev	0.2	0.0	0.6	0.8	0.8	0.1	0.4	0.1	0.1	0.0	0.3	0.9	0.8
T3 b #12-4-1	5.9	2.4	14.8	17.7	36.3	2.6	13.8	1.9	1.3	0.3	3.0	22.9	68.8
stdev	0.0	0.1	0.4	0.5	0.2	0.2	0.2	0.0	0.0	0.0	0.1	0.6	0.6
T3 b #13-5-2	6.1	2.4	14.6	18.5	35.7	2.5	13.9	2.0	1.3	0.8	2.1	22.6	68.9
stdev	0.2	0.1	0.8	1.1	1.0	0.2	0.9	0.1	0.1	1.0	1.1	1.5	1.7
T3 b #14-1-1	5.8	2.3	14.1	16.9	38.1	2.5	13.8	1.9	1.5	0.3	2.7	22.8	69.2
stdev	0.2	0.1	0.5	0.4	0.9	0.2	0.5	0.1	0.0	0.0	0.2	0.9	1.2
WT	5.5	2.9	14.9	16.8	36.7	2.4	14.1	1.8	1.5	0.4	2.9	23.2	68.4
stdev	0.3	0.3	1.0	0.8	1.6	0.3	0.4	0.1	0.1	0.0	0.2	0.6	0.9

Reduction of VLCFA helps increase PUFA content

Elongated fatty acids (C20-C24) were decreased by Cas9-induced mutations at three copies of Camelina *FAE1* genes. The fatty acid metabolism was diverted towards desaturation pathway rather than elongation, so that mono and polyunsaturated fatty acids were further accumulated in Camelina seeds. Comparison between wild type and CRISPR/Cas9-modified transgenic Camelina in that WT Camelina produces C20 and higher fatty acids in its seeds, while T_3 *FAE1* knockout Camelina had almost entirely lost its C20 fatty acids (Fig.12). When two graphs are overlaid, it is easily seen that reduction in C20 FAs diverted in 18:1, 18:2, and 18:3 FAs.

Best four T_3 CRISPR/Cas9 FAE1 lines were compared with WT camelina (Table 13). Cas9-induced plants had less amount of 20:0 (2.5% vs 0-0.51%), 20:1 (13.3% vs 0.74-0.94%), 20:2 (1.85% vs 0%), 20:3 (1.44% vs 0%), 22:0 (0.08% vs 0%) and 22:1 (3.11% vs 0%), and also, accumulated more 16:0 (6.43% vs 6.75-7.21%), 18:0 (1.73% vs 2.37-2.76%), 18:1 (12.6% vs 16.9-19.4%), 18:2 (18% vs 18.7-21.5%), and 18:3 (39% vs 49.35-51.29%) (Table 13A). While the total amount of elongation and desaturation did not change (~90%), elongation was decreased from 22.3% in WT to 0.94-1.28%, and total desaturation was increased from 69.54% in WT to 89.03-89.64% (Table 13B).

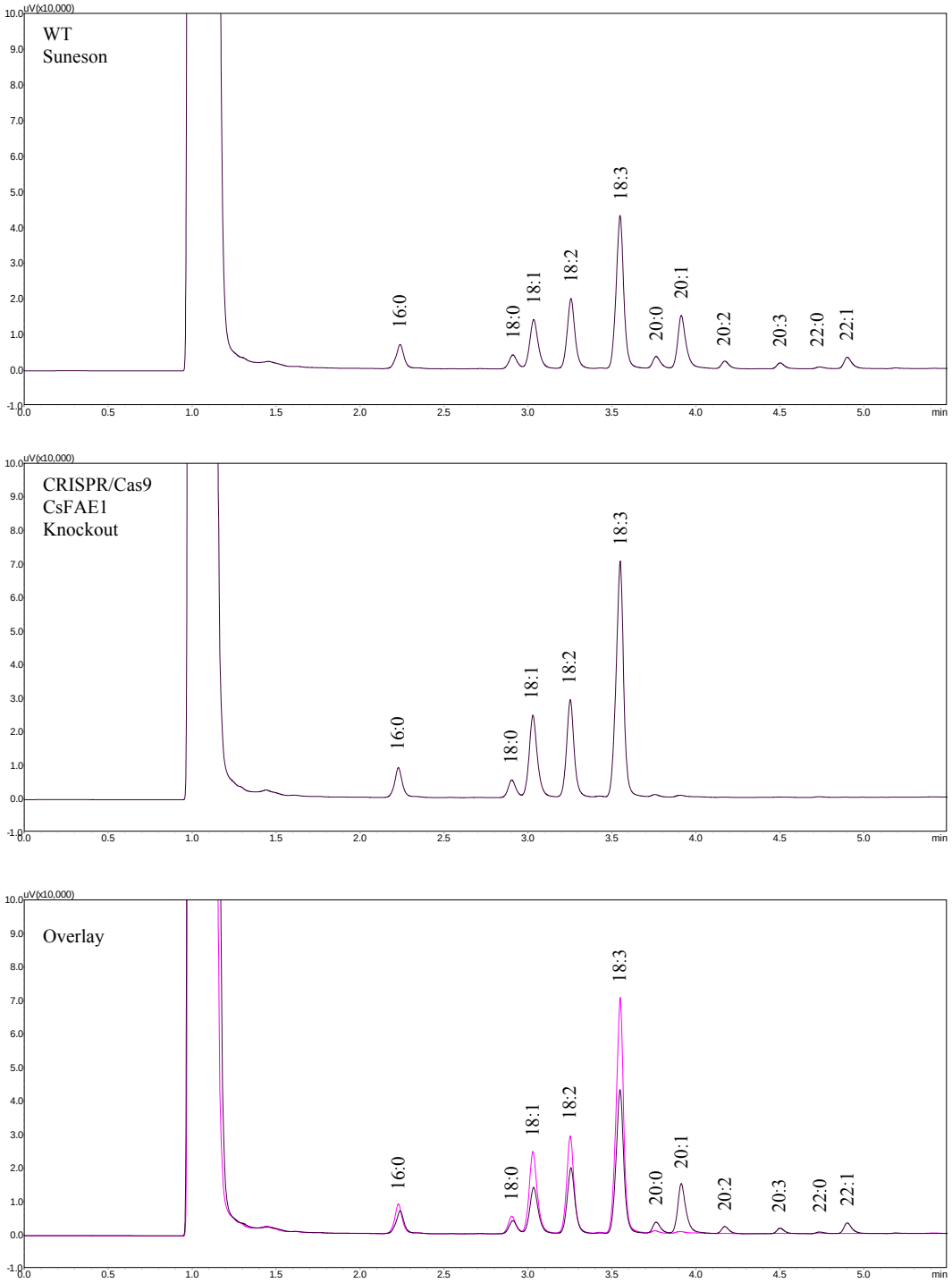

Figure 12. Chromatographs of Wild Type and Cas9-modified Best T3 Lines. A. Single seed of wild type Camelina cv. Suneson (MT5). B. Single seed of CRISPR/Cas9-modified best T_3 line. C. Juxtaposition of wild type and Cas9-modified seeds.

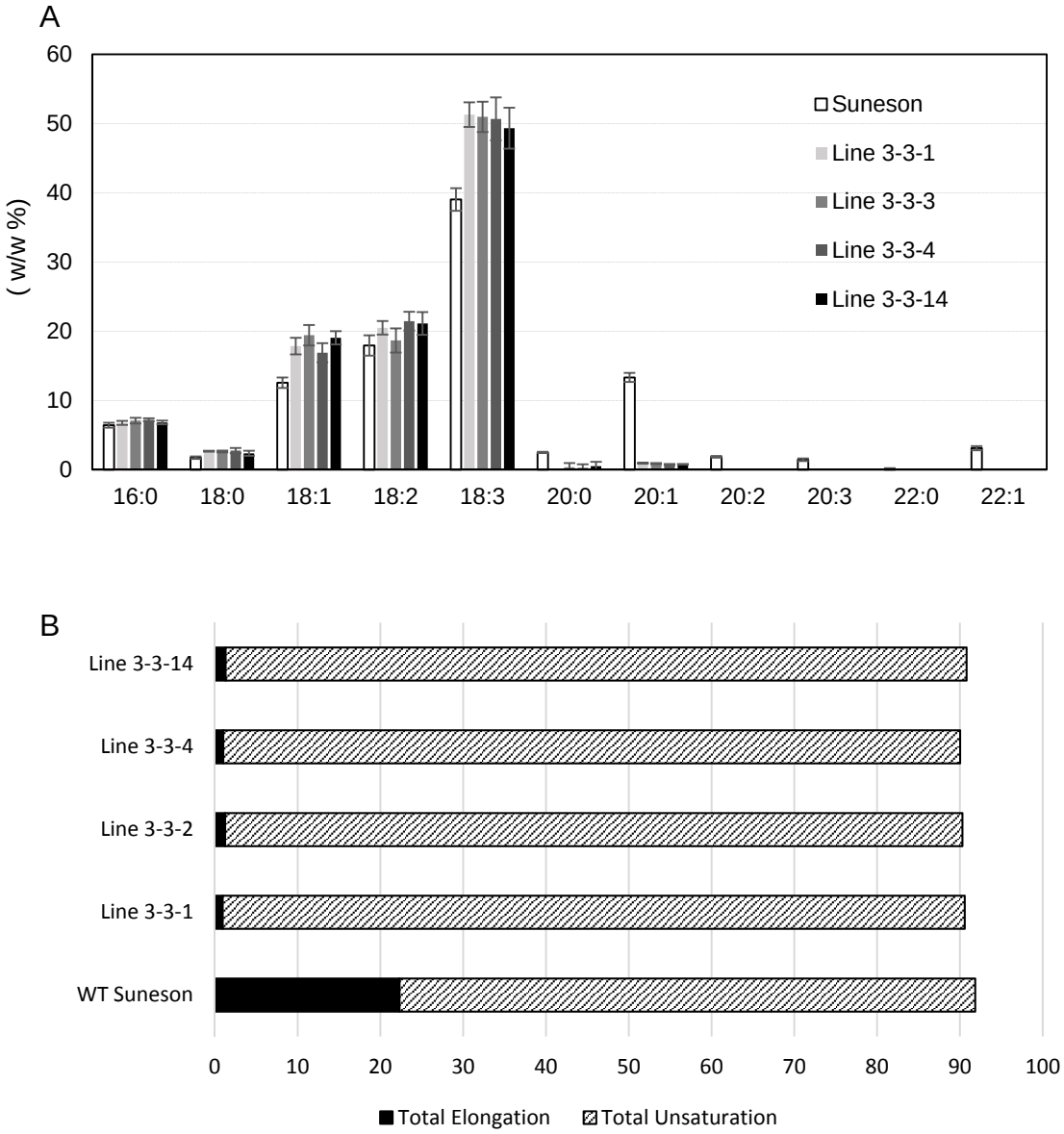

Figure 13. Changes in Fatty Acid Profile Between Wild Type and Cas9-modified Camelina seeds. A. Fatty acid composition between wild type Suneson and Cas9-modified Camelina seeds. B. Comparison of total elongation (20:0 + 20:1 + 20:2 + 20:3 + 22:0 +22:1) and total unsaturation (18:1 + 18:2 + 18:3) between wild type and Cas9-modified Camelina seeds.

Section 2: *FAE1* Expression during Seed Development

All three copies of *Camelina FAE1* genes are shown to be expressed in early and mid-seed developmental stages (Kagale et al., 2016). Data from Camelina eFP website showed that *CsFAE1* expression level is in the order of *FAE1-B* > *FAE1-C* >>> *FAE1-A* (Table 14), so that each *FAE1* copy is active and has its own contribution to VLCFAs production. The highest expression occurs in early-mid stage of seed development, then early and late-mid stages (Fig. 14). Other tissues have no *FAE1* expression so that *FAE1* modification should only affect Camelina seed oil composition.

Table 14. Expression Pattern of *FAE1* Genes in *Camelina sativa*. Data are obtained from Camelina eFP Browser (*http://bar.utoronto.ca/efp_camelina/cgi-bin/efpWeb.cgi*).

	CsFAE1-A (Csa11g007400.1)		**CsFAE1-B (Csa10g007610.1)**		**CsFAE1-C (Csa12g009060.1)**	
Tissue	Expression Level	Standard Deviation	Expression Level	Standard Deviation	Expression Level	Standard Deviation
Germinating Seed	0.0	0.0	0.0	0.0	0.0	0.0
Cotyledon	0.0	0.0	0.0	0.0	0.0	0.0
Young Leaf	0.0	0.0	0.0	0.0	0.0	0.0
Senescing Leaf	0.0	0.0	0.0	0.0	0.0	0.0
Root	0.0	0.0	0.0	0.0	0.0	0.0
Stem	0.0	0.0	0.0	0.0	0.0	0.0
Buds	0.0	0.0	0.0	0.0	1.49	0.71
Flower	0.0	0.0	0.0	0.0	0.38	0.13
Early Seed Development	0.51	0.12	14.34	1.84	5.33	0.28
Early-mid Seed Development	1.51	0.25	34.01	3.97	18.96	1.96
Late-mid Seed Development	0.55	0.2	8.23	2.52	5.22	1.5
Late Seed Development	0.0	0.0	0.0	0.0	0.0	0.0

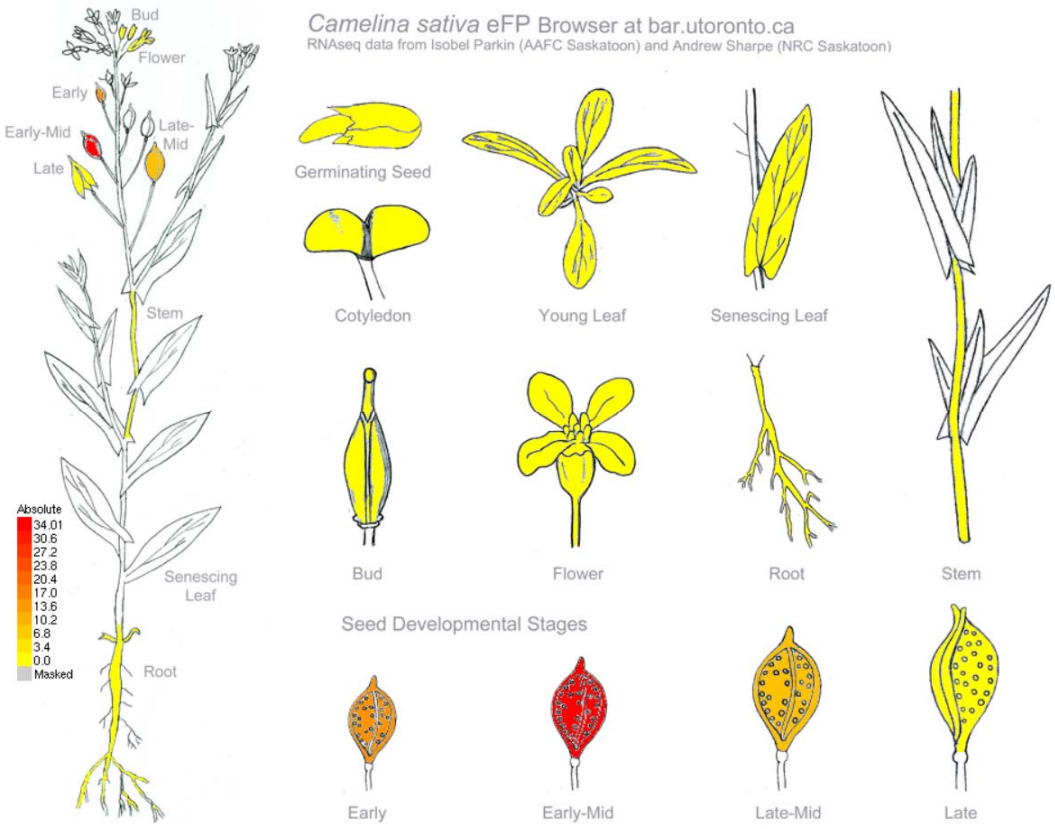

Figure 14. Expression of *FAE1* Genes in Various Parts of *Camelina sativa*. Data are obtained from Camelina eFP Browser *(http://bar.utoronto.ca/efp_camelina/cgi-bin/efpWeb.cgi).*

Section 3: Mutation at the *FAE1* Alleles of Cas9 Transgenic Plants

Genomic sequences close to the guide RNA were analyzed in transgenic Camelina plants to evaluate the effectiveness of Cas9-induced mutagenesis at the *FAE1* gene sequences. Allohexaploid Camelina contains three *FAE1* genes (A, B, and C) so that unique sequences at upstream were selected to individually amplify three *FAE1* copies (Fig. 9). PCR products from transgenic and wild type plants showed similar sizes, therefore, mutation(s) at target site are quite small rather than large insertion or deletion. As expected, sequences of all three *FAE1* alleles of wildtype plant are identical to the published *FAE1* sequence (Hutcheon et al., 2010; Kagale et al., 2014).

T_1 sequencing results indicate that at least one of the three *FAE1* genes were mutated at the guide RNA target sites through deletion and substitution in half of the transgenic 14 T_1 plants, while the other half did not show any sign of nucleotide changes at the target sites (Fig. 15). For T_2 generation, 10 red seeds from each T_1 line were planted, and leaf samples of each plant were collected and stored at -80°C for further analysis by extracting genomic DNA and sequencing. After harvesting, seed color analysis showed that some T_2 lines produced all red seeds indicating putative homozygous lines. To investigate the Cas9-induced mutations at the *FAE1* genes, I selected one homozygous plant from each of the T_2 lines that I stored as plant leaves from each line at -80°C. It was confirmed by PCR using U6 promoter-specific primers that these plants had Cas9 transgene cassette (Table 4). Moreover, sequencing results demonstrated that target regions near PAM sites were mutated either nucleotide deletion (13/14 lines) or substitution (10/14 lines) for each of the *FAE1* genes (Fig. 16).

Cas9-induced mutations were gradually increased in T_2 plants compared to T_1 which was consistent with previous studies (Jiang et al., 2017; Aznar-Moreno & Durrett, 2017; Morineau et al., 2017), indicating progressive Cas9/gRNA action while transgenic plants were advanced to the next generations (Fig. 11). In addition, my sequencing results show that all three *FAE1* alleles had already been mutated in the T_2 generation so that homozygous Cas9-transgenic camelina plants were obtained. Interestingly, those frameshift mutations did not always cause *FAE1* inactivation even if all *FAE1* genes of 13 T2 lines have mutated (Fig. 11). Some mutations result in 1-2 amino acids deletion or substitution (Fig. 16) in the translated *FAE1* genes; however, enzyme activity could be sustained as unaltered *FAE1* genes, thus 20:1 contents in some mutated lines were measured as near or equal to those found in wild type.

There was a variation in C20 fatty acids in T_2 plants (Fig. 11), so that I selected the line that had lowest amounts of VLCFAs for next generation and tracked the mutations at *FAE1* alleles. After GC analysis of all T_2 lines, I decided to select line #3-3 since this line has very low amounts of 20:1 compared to other T_2 lines. 20 red seeds were selected from line #3-3 and small chips were excised from the cotyledon part of each seed, then analyzed for fatty acid composition. The seeds containing less than 1% of 20:1 fatty acids were selected to germinate and advances to T_3 (#3-3-1, #3-3-3, #3-3-4, #-3-3-14) for sequence analysis. Predictably, mutations were detected at the gRNA target sites of all three *FAE1* alleles, and deletions near the PAM sites were the majority type of mutations that would cause translational frameshift and consequently inactivate *FAE1* enzyme (Fig. 17). As an exception, *FAE1-A* gene in line #3-3-4 had insertion of two nucleotides that might still be

translated into an intact protein. Nonetheless, amino acid frameshift was changed at the 5' end such as Tyr-His-Phe in the wildtype *FAE1-A* with Ser-Leu-Leu in mutated line #3-3-4, thus could render enzyme inactive.

FAE1-A

```
WT       CCTCAAACGATCTCTACCACTTCTATTCCCATCTCCAACACAACCTTATAACCGTAATTT
Line 1   CCTCAAACGATCTTTTCCACTTCTATTCCCATCTCCAACACAACCTTATAACCGTAATTT S
Line 2   CCTCAAACGATCTCTACCACTTCTATTCCCATCTCCAACACAACCTTATAACCGTAATTT
Line 3   CCTCAAACGATCTTTTCCCCTT-TATTCCCATCTCCAACACAACCTTATAACCGTAATTT SD
Line 4   CCTCAAACGATCTCTACCACTT-TATTCCCATCTCCAACACAACCTTATAACCGTAATTT D
Line 5   CCTCAAACGATCTCTACCACTTCTATTCCCATCTCCAACACAACCTTATAACCGTAATTT
Line 6   CCTCAAACGATCTCTACCACTTCTATTCCCATCTCCAACACAACCTTATAACCGTAATTT
Line 7   CCTCAAACGATCTCTACCACTTCTATTCCCATCTCCAACACAACCTTATAACCGTAATTT
Line 8   CCTCAAACGATTTTTTCCCCTCTATTCCCATCTCCAACACAACCTTATAACCGTAATTT S
Line 9   CCTCAAACGATCTCTACCACTTCTATTCCCATCTCCAACACAACCTTATAACCGTAATTT
Line 10  CCTCAAACGATCTCTACCACTTCTATTCCCATCTCCAACACAACCTTATAACCGTAATTT
Line 11  CCTCAAACGATCTCTACCACTTCTATTCCCATCTCCAACACAACCTTATAACCGTAATTT
Line 12  CCTCAAACGATCTCTACCACTTCTATTCCCATCTCCAACACAACCTTATAACCGTAATTT
Line 13  CCTCAAACGATCTCTACCACTTCTATTCCCATCTCCAACACAACCTTATAACCGTAATTT
Line 14  CCTCAAACGATCTCTACCACTTCTATTCCCATCTCCAACACAACCTTATAACCGTAATTT
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FAE1-B

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WT       CAGCAAACGATCTCTACCACTTCTATTCCCATCTCCAACACAACCTTATAACCGTAATTT
Line 1   CAGCAAACGATCTCTACCACTTCTATTCCCATCTCCAACACAACCTTATAACCGTAATTT
Line 2   CAGCAAACGATCTCTACCACTTCTATTCCCATCTCCAACACAACCTTATAACCGTAATTT
Line 3   CAGCAAACGATCTCTACCACTT-TATTCCCATCTCCAACACAACCTTATAACCGTAATTT D
Line 4   CAGCAAACGATTTTTTCCCCCT-TATTCCCATCTCCAACACAACCTTATAACCGTAATTT SD
Line 5   CAGCAAACGATCTCTACCACTTCTATTCCCATCTCCAACACAACCTTATAACCGTAATTT
Line 6   CAGCAAACGATCTCTACCACTTCTATTCCCATCTCCAACACAACCTTATAACCGTAATTT
Line 7   CAGCAAACGATCTCTACCACTTCTATTCCCATCTCCAACACAACCTTATAACCGTAATTT
Line 8   CAGCAAACGATCTCTACCACTTCTATTCCCATCTCCAACACAACCTTATAACCGTAATTT
Line 9   CAGCAAACGATCTCTCCCCCTTTTATTCCCATCTCCAACACAACCTTATAACCGTAATTT S
Line 10  CAGCAAACGATCTCTACCACTTCTATTCCCATCTCCAACACAACCTTATAACCGTAATTT
Line 11  CAGCAAACGATCTCTACCACTTCTATTCCCATCTCCAACACAACCTTATAACCGTAATTT
Line 12  CAGCAAACGATCTCTACCCCTTCTATTCCCATCTCCAACACAACCTTATAACCGTAATTT S
Line 13  CAGCAAACGATCTCTACCACTTCTATTCCCATCTCCAACACAACCTTATAACCGTAATTT
Line 14  CAGCAAACGATCTCTACCCCTTCTATTCCCATCTCCAACACAACCTTATAACCGTAATTT S
```

FAE1-C

```
WT       CCACAAACGATCTCTACCACTTCTATTCCCATCTCCAACACAACCTTGTAACCGTAATTT
Line 1   CCAC---CGATGTTTTCCACTTCTATTCCCATCTCCAACACAACCTTGTAACCGTAATTT SD
Line 2   CCACAAACGATCTCTACCACTTCTATTCCCATCTCCAACACAACCTTGTAACCGTAATTT
Line 3   CCACAAACGTTTTTTCCCCCT--TATTCCCATCTCCAACACAACCTTGTAACCGTAATTT SD
Line 4   CCACAAACGATATCTTCCCCTT-TATTCCCATCTCCAACACAACCTTGTAACCGTAATTT SD
Line 5   CCACAAACGATCTCTACCACTTCTATTCCCATCTCCAACACAACCTTGTAACCGTAATTT
Line 6   CCACAAACGATCTCTACCACTTCTATTCCCATCTCCAACACAACCTTGTAACCGTAATTT
Line 7   CCACAAACGATCTCTACCACTTCTATTCCCATCTCCAACACAACCTTGTAACCGTAATTT
Line 8   CCACAAACGATCTCTCCC---TTTATTCCCATCTCCAACACAACCTTGTAACCGTAATTT SD
Line 9   CCACAAACGATCTCTACCACTTCTATTCCCATCTCCAACACAACCTTGTAACCGTAATTT
Line 10  CCACAAACGATCTCTACCACTTCTATTCCCATCTCCAACACAACCTTGTAACCGTAATTT
Line 11  CCACAAACGATCTCTACCACTTCTATTCCCATCTCCAACACAACCTTGTAACCGTAATTT
Line 12  CCACAAACGATCTCTACCCCTTTTATTCCCATCTCCAACACAACCTTGTAACCGTAATTT S
Line 13  CCACAAACGATCTCTACCACTTCTATTCCCATCTCCAACACAACCTTGTAACCGTAATTT
Line 14  CCACAAACGATCTCTACCACTTCTATTCCCATCTCCAACACAACCTTGTAACCGTAATTT
```

Figure 15. Cas9/sgRNA-induced Nucleotide Changes in T1 Plants.
S, substitution; D, deletion

FAE1-A

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WT        CCTCAAACGATCTCTACCACTTCTATTCCCATCTCCAACACAACCTTATAACCGTAATTT
Line 1-1  CCTCAAACGATTTTTTCCCCTT-TATTCCCATCTCCAACACAACCTTATAACCGTAATTT S,-1
Line 2-3  CCTCAAACGATCTTTTTCCCCT-TATTCCCATCTCCAACACAACCTTATAACCGTAATTT S,-1
Line 3-3  CCTCAAACGATCTCTACCACTTT-TATTCCCATCTCCAACACAACCTTATAACCGTAATTT -1
Line 4-5  CCTCAAACGATCTCTACCCCTT-TATTCCCATCTCCAACACAACCTTATAACCGTAATTT S,-1
Line 5-1  CCTCAAACGATCTCTACCACTT-TATTCCCATCTCCAACACAACCTTATAACCGTAATTT -1
Line 6-2  CCTCAAACGATTTTTACCCCT--TATTCCCATCTCCAACACAACCTTATAACCGTAATTT S,-2
Line 7-3  CCTCAAACGATTTCTCCCCC---TATTCCCATCTCCAACACAACCTTATAACCGTAATTT S,-3
Line 8-2  CCTCAAACGATCTCTACCACTT-TATTCCCATCTCCAACACAACCTTATAACCGTAATTT -1
Line 9-3  CCTCAAACGATCTCTACCACTT-TATTCCCATCTCCAACACAACCTTATAACCGTAATTT -1
Line 10-4 CCTCAAACGATTTCTACCCCT--TATTCCCATCTCCAACACAACCTTATAACCGTAATTT S,-2
Line 11-4 CCTCAAACGATCTCTACCACTT-TATTCCCATCTCCAACACAACCTTATAACCGTAATTT -1
Line 12-4 CCTCAAACGTTTTTTCCCCCT--TATTCCCATCTCCAACACAACCTTATAACCGTAATTT S,-2
Line 13-5 CCTCAAACGATTTTTTCCCCTTCTATTCCCATCTCCAACACAACCTTATAACCGTAATTT S
Line 14-1 CCTCAAACGATCTCTACCACCT-TATTCCCATCTCCAACACAACCTTATAACCGTAATTT S,-1
```

FAE1-B

```
WT        CAGCAAACGATCTCTACCACTTCTATTCCCATCTCCAACACAACCTTATAACCGTAATTT
Line 1-1  CAGCAAACGATGTTTTCCACCT-TTTTCCCATCTCCAACACAACCTTATAACCGTAATTT S,-1
Line 2-3  CAGCAAACGATTTTTTCCCCTT-TATTCCCATCTCCAACACAACCTTATAACCGTAATTT S,-1
Line 3-3  CAGCAAACGATCTCTACCACTT-TATTCCCATCTCCAACACAACCTTATAACCGTAATTT -1
Line 4-5  CAGCAAACGATCTCTACCACTT--ATTCCCATCTCCAACACAACCTTATAACCGTAATTT -2
Line 5-1  CAGCAAACGATCTCTCCC----TTTTTCCCATCTCCAACACAACCTTATAACCGTAATTT S,-4
Line 6-2  CAGCAAACGATCTCTCCC---TCTTTTCCCATCTCCAACACAACCTTATAACCGTAATTT S,-3
Line 7-3  CAGCAAACGATGTTTTCCACCT-TTTTCCCATCTCCAACACAACCTTATAACCGTAATTT S,-1
Line 8-2  CAGCAAACGATGTTTTCCACCA-TATTCCCATCTCCAACACAACCTTATAACCGTAATTT S,-1
Line 9-3  CAGCAAACGATCTCTCCC---TTTATTCCCATCTCCAACACAACCTTATAACCGTAATTT S,-3
Line 10-4 CAGCAAACGATCTCTCCC------ATTCCCATCTCCAACACAACCTTATAACCGTAATTT S,-6
Line 11-4 CAGCAAACGATCTTT----------TTCCCATCTCCAACACAACCTTATAACCGTAATTT S,-10
Line 12-4 CAGCAAACGATCTCTACCACTT-TATTCCCATCTCCAACACAACCTTATAACCGTAATTT -1
Line 13-5 CAGCAAACGATCTCTACCACTTCTATTCCCATCTCCAACACAACCTTATAACCGTAATTT
Line 14-1 CAGCAAACGATCTCTACCACTT-TATTCCCATCTCCAACACAACCTTATAACCGTAATTT -1
```

FAE1-C

```
WT        CCACAAACGATCTCTACCACTTCTATTCCCATCTCCAACACAACCTTGTAACCGTAATTT
Line 1-1  CCACAAACGATCTCTCCC---TTCATTCCCATCTCCAACACAACCTTGTAACCGTAATTT S,-3
Line 2-3  CCACAAACGATCTCTCCC-----TATTCCCATCTCCAACACAACCTTGTAACCGTAATTT S,-5
Line 3-3  CCACAAACGATCTCTACCACTT-TATTCCCATCTCCAACACAACCTTGTAACCGTAATTT -1
Line 4-5  CCACAAACGATCTCTACCACT-CTATTCCCATCTCCAACACAACCTTGTAACCGTAATTT -1
Line 5-1  CCACAAACGATCTCTCCCTC---TATTCCCATCTCCAACACAACCTTGTAACCGTAATTT S,-3
Line 6-2  CCAC----------TCCCGTTTTCAACACAACCTTGTAACCGTAATTT S,-22
Line 7-3  CCACAAACGATCTCTACCTCC--TATTCCCATCTCCAACACAACCTTGTAACCGTAATTT S,-2
Line 8-2  CCACAAACGATCTCTACCACTT-TATTCCCATCTCCAACACAACCTTGTAACCGTAATTT -1
Line 9-3  CCACAAACGATTTTTTCCCCTTTTATTCCCATCTCCAACACAACCTTGTAACCGTAATTT S
Line 10-4 CCACAAACGATCTCTCCCT----TATTCCCATCTCCAACACAACCTTGTAACCGTAATTT S,-4
Line 11-4 CCACAAACGATCTTT----------TTCCCATCTCCAACACAACCTTGTAACCGTAATTT S,-10
Line 12-4 CCACAAACGATTTTTCCCCCT--TATTCCCATCTCCAACACAACCTTGTAACCGTAATTT S,-2
Line 13-5 CCACAAACGATTTTTCCCCCTT-TATTCCCATCTCCAACACAACCTTGTAACCGTAATTT S,-1
Line 14-1 CCACAAACGATCTCTACCACT----TTCCCATCTCCAACACAACCTTGTAACCGTAATTT -4
```

Figure 16. Cas9/sgRNA-induced Nucleotide Changes in T2 Plants. S, nucleotide substitution; - deletions.

FAE1-A (Csa11g007400)

```
WT              CCTCAAACGATCTCTACCACTTCTATTCCCATCTCCAACACAACCTTATAACCGTAATTT
                            Y  H  F  Y  S
Line 3-3-1      CCTCAAACGATCTCTACCAC-----TTCCCATCTCCAACACAACCTTATAACCGTAATTT       -5
Line 3-3-3      CCTCAAACGATCTCTACCC--TTTATTCCCATCTCCAACACAACCTTATAACCGTAATTT       -2
Line 3-3-4      CCTCAAACGATCTCT-CC-CTTCTTTATTCCCATCTCCAACACAACCTTATAACCGTAATTT     -2,+2
                          S    L  L  Y  S
Line 3-3-14     CCTCAAACGATCTCTACCAC--CTATTCCCATCTCCAACACAACCTTATAACCGTAATTT       -2
```

FAE1-B (Csa10g007610)

```
WT              CAGCAAACGATCTCTACCACTTCTATTCCCATCTCCAACACAACCTTATAACCGTAATTT
Line 3-3-1      CAGCAAACGATCTCTACCACTT-TATTCCCATCTCCAACACAACCTTATAACCGTAATTT       -1
Line 3-3-3      CAGCAAACGATCTCT--------------CATCTCCAACACAACCTTATAACCGTAATTT       -14
Line 3-3-4      CAGCAAACGATCTCTACCCCT--TATTCCCATCTCCAACACAACCTTATAACCGTAATTT       -2
Line 3-3-14     CAGCAAACGATCTCTA-------------CATCTCCAACACAACCTTATAACCGTAATTT       -13
```

FAE1-C (Csa12g009060)

```
WT              CCACAAACGATCTCTACCACTTCTATTCCCATCTCCAACACAACCTTGTAACCGTAATTT
Line 3-3-1      CCACAAACGATCTCTACCACT-----TCCCATCTCCAACACAACCTTGTAACCGTAATTT       -5
Line 3-3-3      CCACAAACGATCTCTACCACTT-TATTCCCATCTCCAACACAACCTTGTAACCGTAATTT       -1
Line 3-3-4      CCACAAACGATCTCTACCACTT-TATTCCCATCTCCAACACAACCTTGTAACCGTAATTT       -1
Line 3-3-14     CCACAAACGATCTCTACCACT--TATTCCCATCTCCAACACAACCTTGTAACCGTAATTT       -2
```

Figure 17. Cas9/sgRNA-induced Nucleotide Changes in Best T3 Plants. In WT sequences, bold letters distinguish nucleotide polymorphism between different FAE1 alleles. The gRNA sequences are underlined. PAM sites on reverse strands are italicized. In transgenic lines, red letters indicate nucleotide substitution; green letters show nucleotide insertion. Deletions are indicated by dashes and the number of deleted nucleotides, and insertions are indicated by + number. Partial sequences of amino acids of the translated FAE1-A proteins are also shown under the nucleotide sequences.

Section 4: Seed Weight, Plant Height, and Total Oil Content

Seed physiology and plant growth did not show any undesirable characteristics in *FAE1* knockout mutants according to my greenhouse study (Fig. 18). My best transgenic T_3 red homozygous lines were compared with control groups including WT Suneson, and brown seeds from T_3 homozygous lines. Seed weight comparison indicates that all three lines are statistically the same. Seed weight for WT, brown, and transgenic red seeds are 0.115±0.006 g, 0.115±0.006 g and 0.103±0.008 g, respectively (Fig. 18A). Also, p value is 0.083 which is higher than 0.05. In case of plant height, there is no significant difference among WT (64.5±3.2 cm), brown (66.2±4.8 cm), and transgenic red seeds (67.1±4.6 cm) and p value (0.158) is higher than 0.05 (Fig. 18B). Total oil content is statistically same in that WT seeds have 36.9±0.7%, brown seeds have 37.3±0.4%, and transgenic red seeds have 36.8±1.6% and p value is 0.902 that is higher than 0.05 (Fig. 18C). The only statistical change was observed in 20:1 fatty acids. Transgenic red seeds have less than 1% 20:1, while WT and brown seeds have 14.1±0.2% and 14.1±0.1% 20:1 (Fig. 18D). Also, p value is less than 0.001.

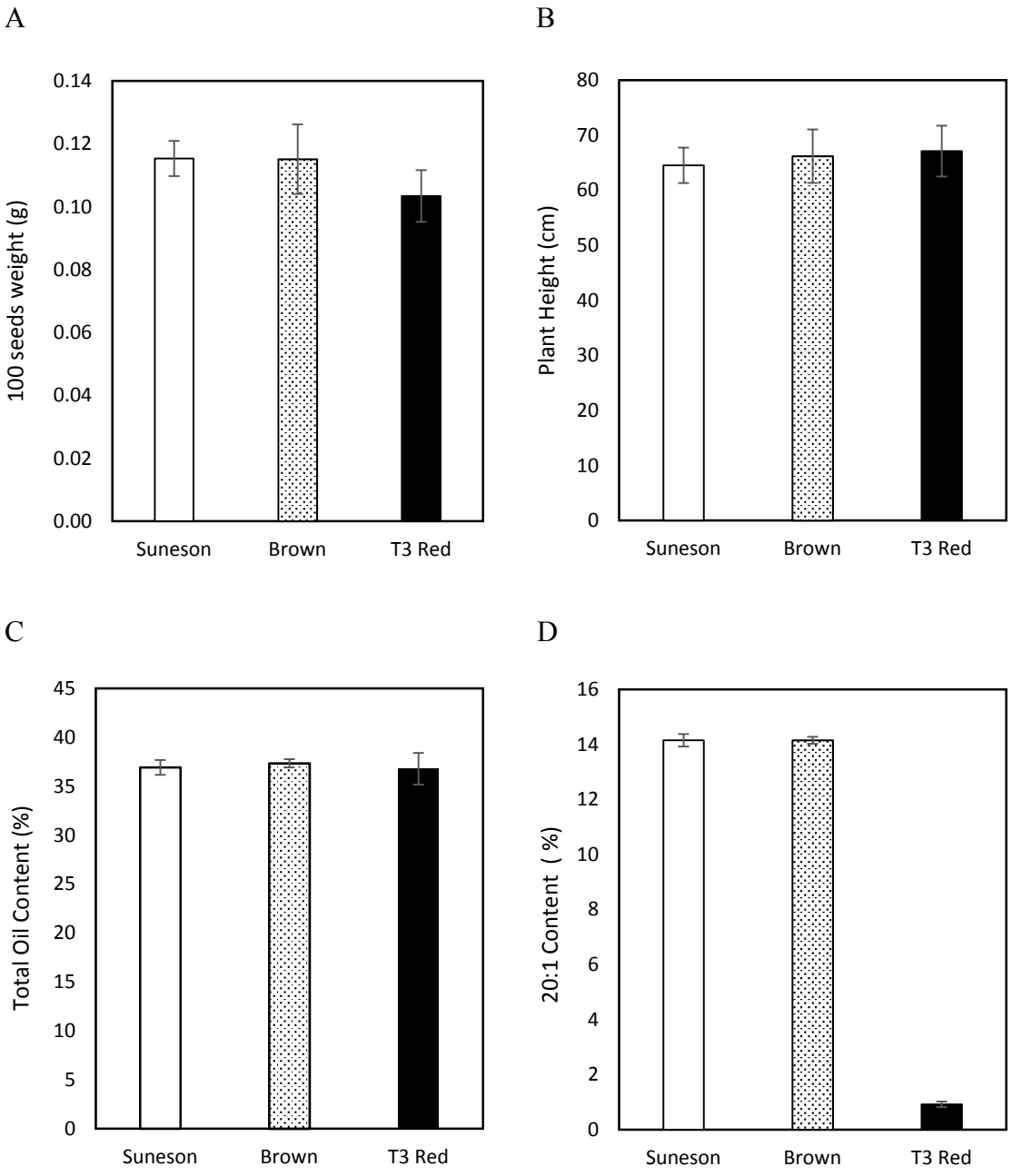

Figure 18. Comparison of Various Traits Between Wild Type and Cas9-modified Camelina Seeds. A. Seed weight (g), B. Plant height (cm), C. 20:1 fatty acid content, D. Total oil content (w/w %).

Section 5: Germination Test

Transgenic T_3 CRISPR/Cas9 FAE1 lines were tested for their germination ability by planting 3 replicates of 100 seeds on damp filter papers (Fig. 19). Seeds were incubated at the room temperature. One day later, CRISPR lines displayed a germination rate of 97% while WT Suneson had 99% germination. This difference was disappeared, and all CRISPR lines germinated within three days. These germination tests confirmed that *FAE1* knock-out by CRISPR/Cas9 lines showed no germination delay.

A. WT Suneson

B. T_3 CRISPR/Cas9 FAE1 line 3-3-4

Figure 19. Seed Germination Test Results. A. 100 seeds of wild type camelina with triple replicates. B. 100 seeds of T3 CRISPR/Cas9 FAE1 line 3-3-4 with triple replicates.

CHAPTER FIVE

DISCUSSION

Camelina sativa is a promising crop due to its high omega-3 content in seed, low input requirements and facile transformation. Nonetheless, some undesired features such as very long chain fatty acids (VLCFAs) and glucosinolate should be eliminated. In this study, I concentrated on the VLCFAs reduction by targeting FAE1 enzymes which elongate the 18:1 substrate to 20:1 and 22:1. Nascent 18:1 fatty acids can be either elongated in the acyl-CoA pool by FAE1 or desaturated on PC pool by FAD2 and FAD3 enzymes. *FAE1* locus was first isolated in Arabidopsis by mutant characterization (James et al., 1995). Its expression is specific to developing seeds (Rossak et al., 2001). A study showed that artificial microRNA can be used to target the *FAE1* gene in Arabidopsis, and 20:1 fatty acids were reduced from 15.4% to 1.9%. (Belide et al., 2012). Hutcheon et al. (2010) discovered three copies of *FAE1* in Camelina. Low 20:1 (6.1%) Camelina line was isolated from EMS-mediated mutant lines (Kang et al., 2011). Nguyen et al. (2013) co-silenced *FAD2* and *FAE1* genes using RNAi, and obtained high oleic (70% 18:1) camelina with decreased level of PUFAs (4% 18:2 and 8% 18:3) and VLCFAs (3% 20:1). Horn et al. (2013) co-targeted *FAD3* and *FAE1* genes using RNAi, and indicated low 18:3 (5%) and 20:1 (2%) and high 18:2 (54%) contents in Camelina seed oil.

Allohexaploid nature of Camelina complicates the complete knockout of *FAE1* genes since there are three active *FAE1* copies which contribute in the order of *FAE1-B* > *FAE1-C* >>> *FAE1-A*. To accomplish successful knockout of *FAE1* genes, all alleles

should be targeted at the same time because silencing a single copy of *FAE1* gene did not cause complete knockout. For example, sequencing results of *fae1* EMS mutant showed that only *FAE1-B* was mutated, however, other two copies were still active, so that Camelina continued to produce VLCFAs. I chose CRISPR/Cas9 tool for targeting multiple-alleles-at-once problem because previous genetic tools have limited abilities for this purpose. For example, meganucleases have cutting specificity problem, ZFNs are target specificity problem since they are recognizing the target DNA as triplet, and TALEN constructs are bigger and their delivery and expression is hard.

Recently, three papers were published about CRISPR/Cas9-induced modification in Camelina. Two groups of researchers target the same *FAD2* enzyme and obtained high oleic camelina oil, and another group of researchers individually targeted *DGAT1*, and *PDAT1* enzymes and obtained reduced oil contents. None of these papers used promoter that targets germline cells for Cas9 protein so that their results in first transgenic generation (T_1) were heterozygous. Even if the Cas9 protein makes highly efficient double strand breaks and efficient enzyme knockout, the homozygosity of these transgenic lines takes at least 3 generation. The underlying reason behind heterozygous plants in T_1 lines is directly related to promoter selection. Desired traits can be obtained in the first generation as long as the promoter targets germline. In my case, I selected egg cell-specific promoter (EC1.1) and enhancer (EC1.2) combination to drive Cas9 protein because this combination led to 17% homozygosity in an Arabidopsis study (Z. P. Wang et al., 2015).

In T_1 generation, 14 red transgenic seeds were obtained, and successfully germinated (Fig. 8). Seeds were harvested from all 14 independent T_1 lines that showed

DsRed segregation indicating single-transgene inserts (Table 5). Brown seeds from heterozygous 14 T_2 lines (Table 6) were analyzed on GC, and 4 lines (#3-3, #4-5, #8-2, and #14-1) were identified with low 20:1 levels. Especially, brown seeds from line #3-3 had less than 1% 20:1. Sequencing results for line #3-3 (Fig. 16) also confirmed that all three *FAE1* copies had deletion at target site, thus *FAE1* was inactive. Gas chromatography results of red seeds from heterozygous 14 T_2 lines (Table 7) indicated that the frequency of mutation was higher, as expected. The majority of red seeds (51/70) had reduced 20:1 ranging from 0.6 to 11.4% (Fig. 11A), and other red seeds (19/70) appeared to be wild type in 20:1 content (12-14%). Furthermore, individual brown seed analysis (Fig. 11B) showed that half of 14 lines had at least one seed with decreased 20:1 levels, however the ratio of low 20:1 seeds was relatively low (20/70). Mutagenesis in brown seeds from heterozygous T_2 lines was expected due to Cas9-driven egg cell-specific promoter. Both red and brown seeds of line #3 reached homozygosity in T_2 generation because their 20:1 content was less than 1% (Fig. 11) and all three *FAE1* copies were successfully mutated (Table 16). My results confirmed the efficiency of egg cell-specific promoter and enhancer that were first tried in Arabidopsis (Z. P. Wang et al., 2015).

To demonstrate the heritability of Cas9-induced *FAE1* mutations, ten red seeds and ten brown seeds from each 14 T_2 lines were advanced to T_3 generation (Fig. 8). High percentage of all red T_3 seeds was obtained such as, 8 replicates out of 10 are all red seeds in a single line; however, some lines (#4, #5, #8, #9, #10) contained higher ratio of red and brown mixed lines such as, 3-7 replicates out of 10 are all red seeds in a single line. Next, brown (Table 8) and red (Table 9) seeds from T_3 heterozygous lines were analyzed.

Advancing to next generation enhanced the VLCFAs reduction in brown and red seeds from T_3 heterozygous lines (Table 8, Table 9). More importantly, all red homozygous T_3 lines (Table 10) showed lower 20:1 levels are than red seeds from T_2 heterozygous lines (Table 9). Line #3, #8, and #9 demonstrated lowest elongation fatty acid profile, and I continued with the line #3 and selected the best lines (#3-3-1, #3-3-3, #3-3-4, #3-3-14) (Table 11). Total elongation of these 4 T_3 lines were less than 2%, and 20:1 was less than 1%. T_4 generation of those lines had even better results with 20:1 is less than 1% and total elongation was less 1.5% (Table 12). Advancement of brown seeds from T_2 heterozygous lines did not decrease 20:1 level in the next generation (Table 13). Since germline mutation affected fatty acid profile in T_1 generation and Cas9-protein was segregated, lines of brown seeds showing WT profile in T_2 continued to show WT profile in T_3. In other words, no improvement regarding to VLCFAs reduction was observed in T_3 homozygous lines.

Knocking out *FAE1* significantly altered fatty acid metabolism in camelina seed. Comparison of my best CRISPR/Cas9 and WT lines indicated that 20:1 and 22:1 levels were decreased from 13.3% and 3.11% to 0.74% and 0%, and concomitantly increase in 18:1, 18:2, and 18:3 from 12.6%, 18%, 39% to 19.4%, 21.5%, and 51.3% (Table 13A). Strikingly, total elongation was decreased from 22.3% in WT to 0.94% in transgenic lines, and total desaturation was increased from 69.5% in WT to 89.6% transgenic lines (Table 13B). This might suggest that the fatty acid metabolism pathway is directly towards desaturation rather than elongation since the total amount of elongation and desaturation did not changed (~90%).

Efficacy of Cas9-induced *FAE1* mutations was confirmed at the molecular level. DNA sequencing results from T_1 to T_4 generation were consistent with previously published *FAE1* sequences (Hutcheon et al., 2010). My single guide RNA design destined to target 5' ends of three *FAE1* genes. Leaf DNA sequencing from T_1 plants indicated that at least one of the three *FAE1* genes were mutated at the guide RNA target sites through deletion and substitution in half of the transgenic 14 T_1 plants. In contrast, other 7 lines out of 14 did not show any sign of nucleotide changes at the target sites (Fig. 15). Harvested seeds showing red fluorescence and brown at the 3:1 ratio indicating single transgene inserts (Table 5). T_2 generation seeds showed much higher mutation frequency as 13 lines out of 14 had nucleotide deletion and 10 lines out of 14 had nucleotide substitution of each of the *FAE1* genes (Fig. 16). Line advancement caused even lower VLCFAs such as 20:1, and this result was consistent with previous studies (Jiang et al., 2017; Aznar-Moreno & Durrett, 2017; Morineau et al., 2017), indicating progressive Cas9/gRNA action while transgenic plants were advanced to the next generations (Fig. 11). Homozygous lines were already obtained in seeds from T1 plants. Surprisingly, even if all *FAE1* genes of 13 T_2 lines had been mutated, those frameshift mutations did not always cause FAE1 inactivation. (Fig. 11). Enzyme activity could be sustained as unmodified FAE1 when some mutations resulted in 1-2 amino acids deletion or substitution (Fig. 16) in the translated FAE1 proteins. Therefore, 20:1 levels in some mutated lines were near or equal to those found in wild type. Best 4 lines in T_3 generation (less than 1% 20:1) were advanced to further generation, and sequence results of all those lines indicated deletions at the target site, as expected (Fig. 17). This confirmed that Cas9-induced mutations are heritable

through 4 generations. Interestingly, 2 nucleotide insertions were occurred in line #3-3-4; however, frameshift was changed from Ser-Leu-Leu in WT to Tyr-His-Phe in transgenic so that it might render the mutant enzyme inactive.

In conclusion, the oilseed crop camelina has great potentials in food and non-food applications. It is necessary to improve fatty acid composition in its oils to meet different requirements. In this study, I investigated the usage of the fatty acid elongase 1 (FAE1) in camelina by the CRISPR/Cas9 approach. A significant decrease in very long-chain fatty acids in a previously isolated *FAE1-B* mutant suggested that the three *FAE1* genes in camelina are responsible for synthesizing these VLCFAs and they may act in an additive fashion. By designing a sgRNA targeting all three *FAE1* genes using the CRISPR/Cas9 technology, I obtained the knock-out mutants that accumulated very small amounts of VLCFAs without causing deleterious effects on seed traits and plant growth. Homozygous mutant plants were successfully obtained in a single generation by using egg cell-specific expression of the Cas9/sgRNA transgenes. My results demonstrate the high efficiency of the CRISPR/Cas9 technology in a hexaploidy oilseed crop to simultaneously edit the homologous genes. The *FAE1* knock-out camelina seeds had significantly changed fatty acid profiles especially C18 unsaturated fatty acids, thus provide ways to breed high oleic acid or α-linolenic acid varieties for food or industrial applications.

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APPENDICES

APPENDIX A

Guide RNA Design Websites and Their Plant Databases

Online Tool	Website URL and Plant Genome(s)		
ATUM gRNA Design Tool	https://www.atum.bio/eCommerce/cas9/input/		
	A. thaliana		
Benchling CRISPR	https://benchling.com/crispr/		
	A. thaliana, *B. distachyon*, *B. napus*, *B. oleracea*, *B. rapa*, *C. clementina*, *C. sinensis*, *C. melo*, *E. tef*,	*G. max*, *G. hirsutum*, *H. vulgare*, *L. perenne*, *M. acuminata*, *M. domestica*, *M. esculenta*, *M. polymorpha*, *N. benthamiana*,	*N. tabacum*, *O. sativa*, *P. trichocarpa*, *S. bicolor*, *S. moellendorffii*, *S. lycopersicum*, *V. vinifera*, *Z. mays*
Cas-Designer	http://www.rgenome.net/cas-designer/		
	A. duranensis, *A. ipaensis*, *A. thaliana*, *A. chinensis*, *B. distachyon*, *B. napus*, *B. oleracea*, *B. rapa*, *B. vulgaris*, *C. annuum*, *C. cajan*, *C. canephora*, *C. clementina*, *C. melo*, *C. lanatus*, *C. reinhardtii*, ***C. sativa***, *C. sativus*,	*C. sinensis*, *D. carota*, *E. grandis*, *E. tef*, *F. vesca*, *G. max*, *G. hirsutum*, *H. annuus*, *H. vulgare*, *K. fedtschenkoi*, *K. laxiflora*, *L. sativa*, *M. acuminata*, *M. domestica*, *M. esculenta*, *M. truncatula*, *N. benthamiana*, *N. tabacum*,	*N. obtusifolia*, *O. sativa*, *P. axillaris*, *P. inflate*, *P. patens*, *P. tremula*, *P. tremuloides*, *P. trichocarpa*, *P. virgatum*, *P. vulgaris*, *S. bicolor*, *S. lycopersicum*, *S. tuberosum*, *T. aestivum*, *T. cacao*, *V. vinifera*, *Z. mays*

Cas-OFFinder	http://www.rgenome.net/cas-offinder/		
	A. duranensis,	*C. sinensis*,	*N. obtusifolia*,
	A. ipaensis,	*D. carota*,	*O. sativa*,
	A. thaliana,	*E. grandis*,	*P. axillaris*,
	A. chinensis,	*E. tef*,	*P. inflate*,
	B. distachyon,	*F. vesca*,	*P. patens*,
	B. napus,	*G. max*,	*P. tremula*,
	B. oleracea,	*G. hirsutum*,	*P. tremuloides*,
	B. rapa,	*H. annuus*,	*P. trichocarpa*,
	B. vulgaris,	*H. vulgare*,	*P. virgatum*,
	C. annuum,	*K. fedtschenkoi*,	*P. vulgaris*,
	C. cajan,	*K. laxiflora*,	*S. bicolor*,
	C. canephora,	*L. sativa*,	*S. lycopersicum*,
	C. clementina,	*M. acuminata*,	*S. tuberosum*,
	C. melo,	*M. domestica*,	*T. aestivum*,
	C. lanatus,	*M. esculenta*,	*T. cacao*,
	C. reinhardtii,	*M. truncatula*,	*V. vinifera*,
	C. sativa,	*N. benthamiana*,	*Z. mays*
	C. sativus,	*N. tabacum*,	
CCTop	https://crispr.cos.uni-heidelberg.de/		
	A. thaliana,	*M. truncatula*,	*S. italica*,
	C. arietinum,	*N. benthamiana*,	*S. lycopersicum*,
	C. papaya,	*N. tabacum*,	*S. tuberosum*,
	E. grandis,	*P. axillaris*,	*S. viridis*
	G. max,	*P. trichocarpa*,	
	L. sativa,	*S. bicolor*,	
CRISPRdirect	https://crispr.dbcls.jp/		
	A. lyrata	*L. japonicus*,	*O. sativa*,
	A. thaliana,	*L. perrieri*,	*P. patens*,
	B. distachyon,	*L. usitatissimum*,	*P. persica*,
	B. napus,	*M. acuminata*,	*P. trichocarpa*,
	B. oleracea,	*M. domestica*,	*R. sativus*,
	C. clementina,	*M. esculenta*,	*R. communis*,
	C. grandiflora,	*M. guttatus*,	*S. bicolor*,
	C. melo,	*M. trancatula*,	*S. italica*,

	C. reinhardtii, *C. papaya*, *C. rubella*, *C. sativus*, *C. sinensis*, *E. grandis*, *F. vesca*, *G. max*, *G. raimondii*, *H. vulgare*, *I. nil*,	*N. benthamiana*, *N. tabacum*, *O. barthii*, *O. brachyantha*, *O. glaberrima*, *O. glumaepatula*, *O. longistaminata*, *O. meridionalis*, *O. nivara*, *O. punchata*, *O. rufipogon*,	*S. lycopersicum*, *S. tuberosum*, *S. polyrhiza*, T. aestivum, T. cacao, T. urartu, V. angularis, V. vinifera, Z. mays
CRISPR-P	http://crispr.hzau.edu.cn/CRISPR2/		
	A. comonus, *A. duranensis*, *A. ipaensis*, *A. lyrata*, *A. thaliana*, *B. distachyon*, *B. napus*, *B. oleracea*, *B. rapa*, *C. canephora*, *C. melo*, *C. merolae*, *C. lanatus*, *C. reinhardtii*, *C. rubella*, *C. sativus*,	*C. sinensis*, *F. vesca*, *G. max*, *G. hirsutum*, *G. raimondii*, *L. edodes*, *L. japonicus*, *M. acuminata*, *M. esculenta*, *M. polymorpha*, *M. truncatula*, *N. benthamiana*, *O. brachyantha*, *O. glaberrima*, *O. indica*, *O. sativa*,	*P. patens*, *P. trichocarpa*, *P. virgatum*, *R. communis*, *S. bicolor*, *S. italica*, *S. miltiorrhiza*, *S. moellendorffii*, *S. lycopersicum*, *S. tuberosum*, *S. viridis*, *U. gibba*, *V. vinifera*, *Z. mays*
CRISPR-PLANT	http://www.genome.arizona.edu/crispr/CRISPRsearch.html/		
	A. thaliana, *B. distachyon*, *G. max*,	*M. truncatula*, *O. sativa*, *P. trichocarpa*,	*S. lycopersicum*, *Z. mays*
CRISPOR TEFOR	http://crispor.tefor.net/		

	A. lyrata, *A. thaliana*, *B. distachyon*, *B. napus*, *B. rapa*, *C. clementina*, *C. melo*, *C. papaya*, *C. reinhardtii*, *C. rubella*, ***C. sativa***, *C. sativus*, *C. sinensis*, *E. grandis*, *G. max*,	*G. raimondii*, *H. annuus*, *H. vulgare*, *L. sativa*, *L. usitatissimum*, *L. perenne*, *L. japonicus*, *M. domestica*, *M. esculenta*, *M. polymorpha*, *M. truncatula*, *N. benthamiana*, *N. tabacum*, *O. sativa*, *P. axillaris*,	*P. inflate*, *P. patens*, *P. trichocarpa*, *R. communis*, *S. bicolor*, *S. italica*, *S. lycopersicum*, *S. tuberosum*, *S. viridis*, *T. aestivum*, *T. cacao*, *V. vinifera*, *Z. mays*
DESKGEN	https://www.deskgen.com/guidebook/		
	A. thaliana, *H. vulgare*,	*O. sativa*, *P. patens*,	*T. aestivum*, *Z. mays*
E-CRISPR	http://www.e-crisp.org/E-CRISP/		
	A. thaliana, *B. distachyon*, *C. reinhardtii*, *H. vulgare*,	*O. indica*, *O. nivara*, *O. sativa*, *P. patens*,	*P. trichocarpa*, *T. aestivum*, *V. vinifera*, *Z. mays*
GT Scan	http://gt-scan.csiro.au/		
	A. thaliana, *G. max*, *H. vulgare*,	*P. patens*, *S. italica*, *S. lycopersicum*,	*S. viridis*, *O. sativa*, *Z. mays*
Harvard CHOPCHOP	http://chopchop.cbu.uib.no/		
	A. duranensis, *A. ipaensis*, *A. thaliana*,	*C. arietinum*, ***C. sativa***, *E. grandis*,	*E. tef*, *E. salsugineum*, *S. lycopersicum*

Synthego	https://design.synthego.com/	
	V. vinifera,	*Z. mays*