

THE FATTY ACID ELONGASE OF *PHYSARIA FENDLERI* INCREASES HYDROXY
FATTY ACID ACCUMULATION IN TRANSGENIC CAMELINA

by

Anna Rose Snapp

A thesis submitted in partial fulfillment
of the requirements for the degree

of

Master of Science

in

Plant Sciences

MONTANA STATE UNIVERSITY
Bozeman, Montana

May 2013

©COPYRIGHT

by

Anna Rose Snapp

2013

All Rights Reserved

APPROVAL

of a thesis submitted by

Anna Rose Snapp

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citation, bibliographic style, and consistency and is ready for submission to The Graduate School.

Dr. Chaofu Lu

Approved for the Department of Plant Science & Plant Pathology

Dr. John Sherwood

Approved for The Graduate School

Dr. Ronald W. Larsen

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a master's degree at Montana State University, I agree that the Library shall make it available to borrowers under rules of the Library.

If I have indicated my intention to copyright this thesis by including a copyright notice page, copying is allowable only for scholarly purposes, consistent with "fair use" as prescribed in the U.S. Copyright Law. Requests for permission for extended quotation from or reproduction of this thesis in whole or in parts may be granted only by the copyright holder.

Anna Rose Snapp

May 2013

DEDICATION

I would like to dedicate my thesis work to my family, friends, and fiancé who have always supported and encouraged me to do my best. I would not be where I am today without them.

TABLE OF CONTENTS

1. INTRODUCTION	1
Plant Oil-based Fuels and Materials	1
Camelina: A Promising Industrial Oilseed Crop	4
Modification of Camelina Oils Through Biotechnology	8
2. ENGINEERING INDUSTRIAL FATTY ACIDS IN OILSEEDS - LITERATURE REVIEW	10
An Evolving Model of Triacylglycerol Biosynthesis In Plants	11
Expression of Co-evolved Enzymes to Increase Modified Fatty Acid Content	15
Genomics Approaches to Uncovering Additional Factors in mFA Metabolism	19
Overcoming Bottlenecks - Efficient Removal of mFA from PC	21
Feedback Inhibition Preventing Accumulation of mFA Products	24
3. OBJECTIVES	26
4. MATERIALS AND METHODS	30
Plant Materials	30
Plasmid Constructs	30
Camelina Transformation	34
Screening Procedure to Identify Transformants	35
Advancement and Selection of Transformed Lines	36
Fatty Acid Analysis by Gas Chromatography	37
Harvest of Developing Seeds	39
RNA Extraction and RT-PCR	40
Lipid Extraction and Thin Layer Chromatography	41
Lipase Digest	43
Separation and Analysis of TAG and PC Fractions by TLC	43
PC Fraction	44
Seed Section Analysis of Hydroxy Fatty Acid Accumulation	46
Seed Section Germination Test	46
Germination Test	47
Determination of Transgene Copy Number in T1 Lines	47
5. RESULTS	48
Section 1: Transgenic Line Creation, Confirmation & Analysis	48
A <i>Physaria fendleri</i> Condensing Enzyme Causes an Increase in Very-Long-Chain C20-HFA Content	48

TABLE OF CONTENTS CONTINUED

Expression of LfKCS3 in Addition to RcFAH Causes an Increase in Total HFA.....	50
Section 2: Stereochemical Analyses	57
TLC of Total Oil Separates 1-OH TAG and 2-OH TAG Fractions	57
Composition of HFA Differs Between Oil Fractions	58
Section 3: HFA Accumulation in TAG and PC During Seed Development	63
Section 4: Analysis of Seed Components for Location of Hydroxy Oil Accumulation.....	66
Hydroxy Fatty Acids Accumulate in Both Cotyledons and Seed Coat	66
Section 5: Germination Test & Observations	68
Germination Ability of Top T5 Transgenic RcFAH-LfKCS Lines Unhindered.....	68
6. DISCUSSION.....	72
REFERENCES CITED.....	83

LIST OF TABLES

Table	Page
1. Summary of Previous Co-evolved HFA Experiments.....	18
2. Primer Table.....	33
3. Average and Standard Deviation of T1 RcFAH and RcFAH-LfKCS Lines	54
4. TLC Fraction Oil Composition of T5 RcFAH and T5 RcFAH-LfKCS Lines.....	60
5. Average HFA in Seed Sections.....	66
6. Total Oil Profile of Seed Sections from RcFAH-LfKCS, RcFAH, and MT5	67
7. Germination Test	68
8. Insertion Number Test	71

LIST OF FIGURES

Figure	Page
1. Metabolic Pathway for TAG Synthesis	14
2. Map of pGate-Phaseolin-RcFAH-LfKCS3.....	49
3. Chromatographs of RcFAH, RcFAH-LfKCS3, and MT5 Lines	52
4. RT-PCR of the <i>LfKCS3</i> Gene	53
5. Distribution of HFA Type in T1 Lines	55
6. Comparison of 20 OH Levels to Total HFA.....	55
7. Oleate Derivative Proportion of RcFAH and RcFAH-LfKCS Lines	56
8. TLC Separation of Total Oil	57
9. TLC of Lipase Digested Oil.....	58
10. GC Chromatograph of 1-OH TAG vs 2-OH TAG	61
11. Content of C20 HFA in TLC Oil Fractions	62
12. Comparison of Total HFA Content in TLC Fractions	62
13. GC Graph of OH-MAG	63
14. Changes in HFA Content in PC	64
15. Changes in HFA Content in Total Developing Seed Oil	65
16. Changes in HFA Content in Developing Seed at Specific Times	65
17. Seed Germination Test Results	69
18. White Cotyledon Phenotype	71

NOMENCLATURE

ACP:	acyl carrier protein
CoA:	coenzyme A
CPT:	CDP-choline:DAG cholinephosphotranferase
DAG:	diacylglycerol
DGAT:	acyl-CoA:DAG acyltransferase
ER:	endoplasmic reticulum
FAD:	oleate and linoleate desaturases
FAE1:	Fatty acid elongase 1
G3P:	glycerol-3-phosphate
GPAT:	acyl-CoA:G3P acyltransferase
HFA:	hydroxy fatty acids
LACS:	long-chain acyl-CoA synthetase
LPA:	lysophosphatidic acid
LPAT:	acyl-CoA:LPA acyltransferase
LPC:	lysophosphatidylcholine
LPCAT:	acyl-CoA:LPC acyltransferase
MAG:	monoacylglycerol
mFA:	modified fatty acids
PA:	phosphatidic acid
PAP:	phosphatidic acid phosphatase
PC:	phosphatidylcholine
PDAT:	PC:DAG acyltransferase
PDCT:	PC:DAG cholinephosphotransferase
PLA:	Phospholipase A
PLC:	Phospholipase C
PLD:	Phospholipase D
PUFA:	polyunsaturated fatty acid
TAG:	triacylglycerol

ABSTRACT

Plant oils containing hydroxy fatty acids (HFA) are desirable for a wide variety of applications including lubricants, plasticizers, surfactants, polyesters, paints, sealants, biodiesel, and more. Due to unfavorable agronomic attributes of natural accumulators such as castor and lesquerella, many efforts have been made to produce hydroxyl fatty acids in crop plants. The hydroxy fatty acid synthesis pathway has been extensively studied and key genes such as the castor fatty acid hydroxylase, *RcFAH*, have been discovered. However, insertion of the *RcFAH* gene into various *Arabidopsis* backgrounds under the control of seed specific promoters failed to result in high accumulation of the desired HFA products, highlighting a need for more research to uncover additional constraints and factors affecting the fluxes involved with the accumulation of these unusual fatty acids in seed oil. In this study I investigated the effect of co-expressing a fatty acid elongase gene, *LfKCS3*, from *Lesquerella (Physaria) fendleri* along with the castor hydroxylase gene, *RcFAH*, on accumulation of hydroxyl fatty acids in seed oil of the crop plant *Camelina sativa*. On its own, wild type camelina contains no hydroxy fatty acids but insertion of the *RcFAH* gene results in accumulation of around 15% HFA in transgenic camelina, however, addition of the *LfKCS3* gene resulted in a significant increase in very long chain 20-carbon hydroxyl fatty acids from <2% to 8%; total hydroxyl fatty acids also increased from 15% to 22% in the highest accumulating lines. The presence of the *LfKCS3* enzyme effectively increased total HFA levels at all stages of oil accumulation in developing seeds while also decreasing the amount of these fatty acids left on the phospholipid, phosphatidylcholine. This combination of increased 20-carbon and total hydroxyl fatty acid accumulation along with the decreased HFA levels in phosphatidylcholine indicates that the *LfKCS3* gene helps to enhance the flux of HFA out of phosphatidylcholine for incorporation of HFA into triacylglycerol, aiding in relief of the metabolic bottleneck for engineering economically viable levels of these fatty acids in oilseed crops.

CHAPTER 1

INTRODUCTION

Plant Oil-based Fuels and Materials

Worldwide there is growing interest in the use of bio-based fuels and lubricants to offset dependence on fossil petroleum and its derivatives (Lu et al., 2011). Environmental and economic benefits have been the major reasons fueling the growing popularity of biofuels as an alternative fuel source for the past decade (Durrett et al., 2008), and the development of renewable and environmentally friendly industrial products, such as lubricants (Salimon et al., 2010). Plant oils, one of the most energy-rich and abundant forms of reduced carbon available, have long been used by mankind not only as an essential nutrient but also in many other uses such as illumination and skin care. Recently, vegetable oils are increasingly being used as fuels in the form of straight vegetable oil or processed biodiesel and jet fuels (Huber et al., 2006; Moser, 2010; Steen et al., 2010). Many non-food plant oils contain unusual chemical structures of fatty acids in triacylglycerols (TAG), such as hydroxy, epoxy, or conjugated groups, rendering unique physical and chemical properties (Gunstone, 1998; Jaworski and Cahoon, 2003; Singh et al., 2005; Voelker and Kinney, 2001). This opens up opportunities for the use of vegetable oils in a variety of bio-based industrial formulations that are otherwise petroleum based (Durrett et al., 2008; Gunstone, 1998; Lu et al., 2011; Napier and Graham, 2010).

Plant-based feedstocks for biofuels and biolubricants are a renewable source capable of being domestically produced. This could help relieve dependence on foreign oil markets controlled by relatively few countries. Biofuel is also generally miscible with petrofuels, allowing it to account for a portion of the fuel even if it cannot replace it entirely (Moser, 2010). Other beneficial qualities of biodiesel and biolubricants include greatly increased biodegradability over petroleum sources, high flash point, excellent lubricity, low or no sulfur content, and lower exhaust emissions (Moser, 2010; Salimon et al., 2010). In fact, the greenhouse gas life cycle emissions for green diesel are 80% lower than petroleum derived diesel (Shonnard et al., 2010). The reduced greenhouse gas emissions and increased biodegradability of plant based products are obvious environmental benefits over fossil fuel sources.

Disadvantages of biodiesel still exist though, including poor cold flow properties, poor oxidative stability, elevated nitrogen oxides in exhaust, and insufficient feedstock supply (Moser, 2010). While treatments or additives may help alleviate the former issues, insufficient feedstock is the major issue needing to be addressed (Makareviciene et al., 2013). The feedstocks used for biodiesel generally differ based on world region, with Europe using rapeseed (canola) and sunflower, canola used in Canada, palm oil in Malaysia, coconut in the Phillippines, jatropha in India, and soybean in the United States (Moser, 2010; Soriano and Narani, 2012). However, combined, all of these feedstocks are not capable of replacing a significant portion of the petroleum derived diesel and lubricants consumed worldwide (Moser, 2010). The US is the second largest producer of biodiesel in the world with 85% of that coming from soybean, but even if all of the US soybean were used for biodiesel it has been estimated it would only amount to 6% of

what is needed (Moser, 2010; Soriano and Narani, 2012). There is simply not enough feedstock to meet the growing demand for bio-based products.

Another major issue limiting the availability of feedstock is competition with food uses. Many of the oilseed crops such as sunflower, canola, soy, coconut, and palm oil also have culinary uses as cooking oil or food. There has been much political debate over the use of crops for food versus fuel, and the rising prices that would ensue with increasing industrial use. Public fears of genetically modified plants intended for industrial oil use escaping and contaminating established food sources also cast a negative light on current oil sources. Additionally, the expansion of current oilseed crops to meet demands may be somewhat limited due to food crop rotations already in place and soil suitability concerns (Makareviciene et al., 2013). Thus there is a need for an alternative oil seed crop to be used solely as an industrial oil platform to help meet the feedstock demands while minimizing the impact on food use crops.

Desirable characteristics of a feedstock to consider include: lower life cycle greenhouse emissions compared to fossil fuels, low or no food use competition, adaptability to local growing conditions, high seed oil content and yield, compatibility with existing farm equipment and infrastructure, nutrient efficiency or low agricultural input requirements such as fertilizer and pesticides or water, definable growth season, uniform seed maturation rate, fallow lands compatibility, ease of rotation with conventional cash crops, pathogen tolerance, and commercial outlets for byproducts such as meal (Gehring et al., 2006; Moser, 2010). A low cost feedstock is also preferred to maximize revenue since 80-85% of biodiesel production costs are due to feedstock (Soriano and Narani, 2012). To meet this demand for an industrial oilseed platform we

have focused on the oilseed crop camelina (*Camelina sativa*) which contains many of these desired qualities.

Camelina: A Promising Industrial Oilseed Crop

Camelina [*Camelina sativa* (L.) Crantz], also called false flax, gold of pleasure, or German sesame, is a temperate climate oilseed crop belonging to the mustard family, Brassicaceae (Zubr, 1997). Camelina is an ancient oilseed with a history of cultivation dating to the Bronze Age in Europe, as evidenced by the seeds and capsules found in European and Scandinavian excavation sites (Zubr, 1997). Romans reportedly used camelina oil as massage oil, lamp fuel, and cooking oil while the leftover meal was used for animal feed (Pilgeram et al., 2007). *Camelina linicola* was part of the diet during the Iron Age, eaten along with flax and other cereals in soups and porridges such as that found in the stomach of Tollund man, a preserved man found buried in a peat bog in Denmark (Pilgeram et al., 2007; Zubr, 1997). Grown as an agricultural crop throughout Europe and Russia before World War II, it petered out in the 1950's due in part to subsidies for other commercial crops (Shonnard et al., 2010; Zubr, 1997). Today camelina is not actively used for a specific purpose, although it is generating renewed interest as a second generation oilseed crop and there is some limited interest in it for an omega-3 source as well (Collins-Silva et al., 2011; Pilgeram et al., 2007). Most notably, camelina oil has been processed and used as a new age jet fuel, which has been tested successfully in flights by the U.S. military and by several commercial airliners (Moser, 2010; Shonnard et al., 2010).

Although broadly distributed in its native Europe, camelina is also grown in northerly regions of Asia and has been naturalized in the US and Canada (Moser, 2010; Shonnard et al., 2010). Camelina owes its broad distribution in part to its adaptability. It can be found growing in a wide range of climatic and soil conditions including low fertility or saline soils, and is relatively tolerant of cold weather and drought (Moser, 2010; Zubr, 1997). The possibility of camelina to grow on marginal lands is very useful since it will grow on land that may not support other crops like canola or soybean (Collins-Silva et al., 2011). Camelina is considered to be a low input crop compared to other oilseeds for water usage, fertilizer and pesticide requirements (Moser, 2010; Zubr, 1997). Studies have shown that camelina only needs 80-100kg/ha nitrogen to achieve optimum performance, while other oilseed crops such as canola recommend 134-168kg/ha (Gehringer et al., 2006; NDSU, 2010; Pan et al., 2011). Despite being somewhat drought tolerant, water deficiency can have a larger effect on camelina than nitrogen deficiency (Pan et al., 2011). However, its early maturity means that peak water demands occur early in the season when spring moisture is available and aid in its suitability for dryland applications (Johnson J., 2009; Pan et al., 2011). Pesticide use is also generally lower with camelina because it produces antimicrobial compounds including phytoanticipins and phytoalexins which aid in resistance to a variety of pests and diseases (Gehringer et al., 2006; Sequin-Swartz G., 2009; Zubr, 1997). Camelina is also immune to diseases such as alternaria black spot and blackleg or pests such as flea beetles, which affect other brassica crops like *B. napus* and *B. rapa* (Sequin-Swartz et al., 2009, Johnson et al., 2009). While camelina may not be the top yielding oilseed overall, it has been shown to have the highest dryland production potential and be the most

economical due to lower input costs (Pilgeram et al., 2007, Johnson et al., 2009).

To avoid land use changes it has often been suggested that camelina be used in rotation with crops like winter wheat instead of a fallow year (Moser, 2010; Shonnard et al., 2010). Planting camelina could aid in the disruption of crop specific pest cycles due to its resistance to some known diseases and provide the farmer with the added revenue of a crop in place of his fallow field. Since camelina is a shallow rooted crop with lower moisture and nutrient requirements it will still allow the soil to recharge for the next crop rotation (Shonnard et al., 2010). The short life cycle of camelina at 85-100 days also allows it to easily fit into rotation with crops like winter wheat without disrupting existing planting times. It is estimated that 5-7 million US acres have the potential to grow camelina with no impact on the food supply if it is incorporated into rotations with dryland cereals (Shonnard et al., 2010). Montana alone is estimated to be able to support 2 to 3 million acres per year (Moser, 2010). Summer, Winter, and Intermediate forms of camelina exist so it can be grown as a summer annual or a biannual winter crop adding flexibility to a crop rotation plan (Moser, 2010; Zubr, 1997). Harvesting can be done with existing farm infrastructure as long as combines are adjusted to minimize seed loss (Moser, 2010; Pilgeram et al., 2007; Zubr, 1997). Replacing the fallow year of a cash crop rotation with camelina ensures that the food versus fuel debate is non-existent, no land use changes arise from converting forest into agriculture, and it can maximize the revenue to the farmer by having income from ground that would otherwise be left idle.

The oil content of camelina seeds can vary considerably between genotypes from 30-45% leading to a wide range of potential oil yields reported. This genetic variation is due to the fact that camelina breeding for seed quality characteristics has not been done

extensively in the past, and the germplasm amount is limited compared to other crops (Collins-Silva et al., 2011; Gehringer et al., 2006; Vollmann J., 2007). Due to the lack of applied breeding effort in camelina, variation still exists for agronomic traits such as height, oil content, seed yield, seed mass, pest and disease resistance, and even level of α -linolenic (18:3; number of carbon:number of double bonds) fatty acid (Gehringer et al., 2006; Ghamkhar et al., 2010; Sequin-Swartz G., 2009; Vollmann J., 2007). The presence of variation will allow great advances to be made in breeding through selection of desired traits. In fact, the plant breeding efforts recently underway have already seen yield improvements of ~33% since 2006 and it is believed that further breeding will continue to improve camelina traits (Gehringer et al., 2006). Good camelina lines can give oil yields above those of soybean and sunflower and it has been proposed that yields may approach those of rapeseed with further breeding (Moser et al., 2010). Selecting for plants that are better adapted to certain climatic regions will be beneficial due to the large effect of environment on oil content and seed yield (Moser et al., 2010; Vollman et al., 2007). Efficient improvement of camelina may be hampered by its scarce genetic resources such as limited gene maps (Gehringer et al., 2006), and the allohexaploidy of the camelina genome (Hutcheon et al., 2010). However, the close relationship of camelina to the model plant *Arabidopsis thaliana* (Al-Shehbaz et al., 2006) and the recently developed camelina seed transcriptome database (Nguyen et al., 2013) will aid in gene identification to enhance the ability for camelina improvement.

Modification of Camelina Oils Through Biotechnology

Although camelina has a high oil content in its seeds of 30-45%, it does not lend itself nicely to a single purpose and often requires alteration or additives for industrial applications (Collins-Silva et al., 2011). The major limitation of camelina oil uses is by its high content (over 50%) of polyunsaturated fatty acids that make it prone to oxidation (Collins-Silva et al., 2011; Moser et al., 2010; Vollman et al., 2007).

Traditional breeding, especially mutation breeding, is effective to obtain germplasms that contain altered fatty acid composition (Buchsenschutz-Nothdurft et al., 1998; Kang et al., 2011). However, due to its polyploid nature (Hutcheon et al., 2010), it requires intensive efforts to obtain all alleles of targeted genes and also multiple generations of crossing and selection. Biotechnology offers an efficient alternative approach for camelina breeding. Camelina can be transformed via an *Agrobacterium* mediated inoculation of flowering plants similar to that developed for *Arabidopsis*, and may result in approximately 1% transgenic seeds (Lu and Kang, 2008). The major advantage of vacuum infiltration of floral organs is that the transgenic plants can be acquired in as little as 6 to 8 weeks and most often contain a single transgene copy (Lu and Kang, 2008). Harvested seeds can be easily screened for transgenic plants through the use of a DsRed marker or with herbicide resistance (Kang et al., 2011; Lu and Kang, 2008). Other crops' transformation methods using *Agrobacterium* may involve a lengthy tissue culture stage requiring additional training, expertise, money, and most of all time at 8-10 months to acquire transgenic plants (Collins-Silva et al., 2011). By combining ease of transformation with genetic engineering tools such as a vector system for foreign gene

delivery, selection marker genes, and seed specific promoters it will be possible to modify camelina oil to better fit specific industrial needs beyond its current biodiesel feedstock capabilities (Collins-Silva et al., 2011; Walsh K.D., 2012). It is also possible to reconstruct biochemical pathways in camelina originated in other oilseed plants with valuable oils but unfavorable agronomic qualities (Collins-Silva et al., 2011). In this study, I describe experiments resulting in production of unusual hydroxy fatty acids in camelina.

CHAPTER 2

LITERATURE REVIEW: GENETIC ENGINEERING
OF INDUSTRIAL FATTY ACIDS IN OILSEEDS

Most plant oils are in the form of triacylglycerols (TAG) containing three fatty acids, which are immensely diversified in structures. While the majority of fatty acids in most vegetable oils are 18 carbons with up to three double bonds, there are more than 300 additional modified fatty acids (mFA) found in different plant TAG (Jaworski and Cahoon, 2003; Singh et al., 2005). Many of the unusual structures, such as hydroxy, epoxy, or conjugated groups, render unique physical and chemical properties (Gunstone, 1998; Voelker and Kinney, 2001), that are important for the use of vegetable oils in a variety of bio-based industrial applications, including lubricants and drying oils (Durrett et al., 2008; Gunstone, 1998; Lu et al., 2011; Napier and Graham, 2010).

Above all, the fatty acid composition in triacylglycerols determines the quality and thus the uses of plant oils. While seed oils to be used for human consumption should contain as little saturated fatty acids as possible and a significant proportion of polyunsaturated fatty acids (PUFAs) (Riediger et al., 2009), industrial oils require low polyunsaturated fatty acids for desirable oxidative stability or high homogeneity of certain fatty acids (Dyer and Mullen, 2008; Dyer et al., 2008; Pinzi et al., 2009). Many plants store high levels of industrial mFA in seeds; however most of these plants are not domesticated for agriculture. Molecular biologists have made many attempts over the past 20 years to introduce the capacity for producing specific mFA into crop species (Drexler et al., 2003; Jaworski and Cahoon, 2003; Lu et al., 2006; Napier and Graham,

2010). The results of these experiments have typically been disappointing, producing in most cases, plant lines with very low yields of the desired fatty acids (Broun and Somerville, 1997; Cahoon et al., 1999; Lee et al., 1998; Suh et al., 2002). These results underlie the need to understand the fundamental aspects of how plant fatty acids are synthesized and accumulated in seed oils. Here, I review some important progress made in recent years and discuss how these new discoveries may help achieve the goal of modifying plant oils for industrial uses in a predictable manner.

An Evolving Model of Triacylglycerol Biosynthesis in Plants

The major pathways and enzymes involved in TAG synthesis have been unveiled in the past decades by biochemical and genetic means, especially by using mutants of *Arabidopsis thaliana*, the model oilseed plant. In plants, fatty acid synthesis occurs exclusively in plastids and produces mostly oleic acid (18:1) and a small amount of palmitic acid (16:0) and stearic acid (18:0) that are esterified to acyl carrier protein (ACP) (Ohlrogge and Browse, 1995). These fatty acids are incorporated into glycerolipids in two subcellular compartments: (1) acyl groups from acyl-ACP are directly used within the plastid by the "prokaryotic" pathway; and (2) fatty acids are removed from acyl-ACP to be exported into the cytosol, and converted into acyl-Coenzyme A (acyl-CoA) to be used in the "eukaryotic" pathway in the endoplasmic reticulum (ER) (Browse and Somerville, 1991; Ohlrogge and Browse, 1995). The proportion of newly synthesized fatty acids that goes into these two pathways varies widely among different plant species and even in different tissues of the same plant. In oilseeds, these acyl groups are used almost entirely (>95%) by the eukaryotic pathway (Browse and Somerville, 1991;

Roughan and Slack, 1982).

In seeds of *Arabidopsis* and some oilseed crops such as *Brassica napus* and *Camelina sativa*, fatty acids exported into the eukaryotic pathway may be modified in two major ways: carbon chain elongation and desaturation (Figure 1; for simplicity, only 18:1 is shown). In the cytosol, 18:1-CoA may be elongated into 20:1 to 22:1 -CoA esters by fatty acid elongase FAE1 (Kunst et al., 1992). These acyl-CoAs, along with other acyl groups derived from *de novo* fatty acid synthesis or from other sources, will be available for incorporation into glycerolipids in the ER. The dominant flux of nascent fatty acids in oilseeds is to enter the membrane lipid phosphatidylcholine (PC) (Roughan and Slack, 1982), where they can be modified by the ER localized fatty acid desaturases (FAD, Fig. 1) including the oleate desaturase FAD2 and the linoleate desaturase FAD3 (Browse et al., 1993; Okuley et al., 1994; Sperling et al., 1993) to produce the polyunsaturated fatty acids (PUFAs) linoleic acid (18:2) and α -linolenic acid (18:3). In plant seeds that accumulate many other modified fatty acids (mFA) with unusual chemical structures, the mFA are also synthesized on PC (Bafar et al., 1991; Jaworski and Cahoon, 2003). Therefore, understanding the mechanisms of fatty acids entering PC and, after they are modified, their subsequent channeling into TAG is critical for successful engineering of the mFA.

As shown in Fig. 1, fatty acids may be incorporated into PC through the *de novo* pathway: Glycerol-3-phosphate (G3P) is sequentially acylated at the *sn*-1 and *sn*-2 positions using acyl-CoA exported from plastids to produce phosphatidic acid (PA). Removing the phosphate group at the *sn*-3 position of PA by PA phosphatases (PAP) produces diacylglycerol (DAG). Finally PC is formed from DAG by a CDP- choline:

diacylglycerol cholinephosphotransferase (CPT) (Goode and Dewey, 1999; Slack et al., 1983). The TAG synthesis pathway shares most of the enzymes in the PC synthesis, and its committed step is the acylation of DAG by the *sn*-3 specific acyl-CoA: diacylglycerol acyltransferases (DGAT) (Hobbs et al., 1999; Routaboul et al., 1999; Zou et al., 1999). The sequential acylations of the *sn*-glycerol-3-phosphate is usually referred to as the Kennedy pathway (Kennedy, 1961). It was later discovered that an acyl-CoA independent phospholipid:diacylglycerol acyltransferase (PDAT) also directly transfers the *sn*-2 acyl group from PC into DAG to form TAG (Dahlqvist et al., 2000). DAG may also be converted into TAG by an elusive DAG:DAG transacylase (Stobart et al., 1997). Recent results demonstrated that DGAT and PDAT are responsible for the majority of TAG synthesized in seeds (Zhang et al., 2009).

However, recent metabolic labeling experiments (Bates et al., 2009; Bates et al., 2007; Williams et al., 2000) demonstrated that the above model should include acyl editing, also termed “remodeling” or “retailoring”, a process that exchanges acyl groups between polar lipids but does not result in the net synthesis of these lipids. The work of Bates and coworkers (Bates et al., 2009; Bates et al., 2007) indicates that the majority of newly synthesized saturated (16:0) and monounsaturated (18:1) fatty acids enter PC by exchanging with polyunsaturated fatty acids (18:2, 18:3) on PC rather than proceeding through the Kennedy pathway. The PUFAs may be released from PC by either the reverse reaction of a lysophosphatidylcholine acyltransferase (LPCAT^F) or by the phospholipase A (PLA). The resulting lysophosphatidylcholine (LPC) can be reacylated

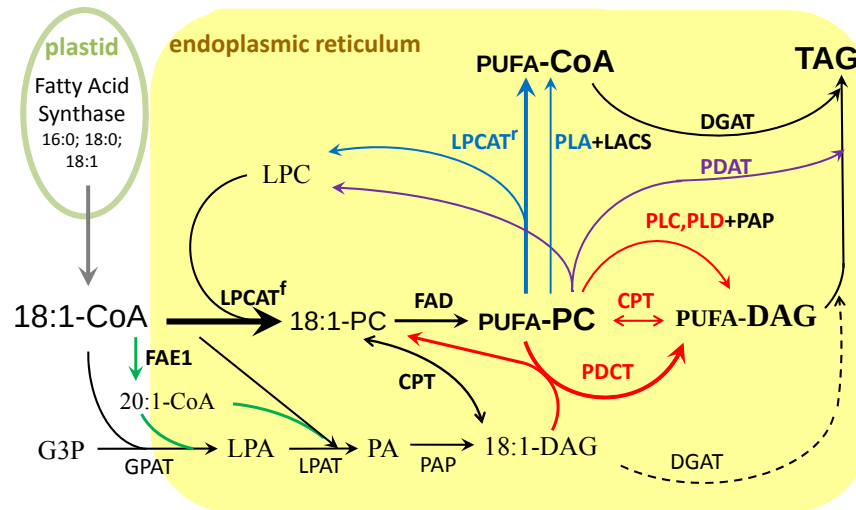


Fig. 1. Metabolic network showing some of the pathway possibilities for the assembly of polyunsaturated fatty acids (PUFA) into TAG in Arabidopsis. Many other modified fatty acids (mFA) use similar mechanisms. Colored arrows denote different pathways for PUFA or other mFAs exiting PC; while heavy-weight arrows indicate major pathways, and dashed arrows indicate alternative or parallel routes for metabolites. Please note that the two 18:1-PCs are actually one pool, just arranged as two due to the spacing required to make different pathway reactions more easily distinguished. Abbreviations: DAG: diacylglycerol; G3P: glycerol-3-phosphate; LPA: lysophosphatidic acid; LPC: lysophosphatidylcholine; PA: phosphatidic acid; PC: phosphatidylcholine; PUFA: polyunsaturated fatty acid; TAG: triacylglycerol.

PLD Phospholipase D

The PUFAs released from PC in the forms of acyl-CoA (via LPCAT) or free fatty acids (via PLA), which are activated by the acyl-CoA synthases (LACS) (Shockey et al., 2002), enter the acyl-CoA pool to be used for phospholipid or TAG synthesis. The acyl editing process thus enriches the PUFA in the acyl-CoA pool, which contribute to high levels of such fatty acids in PC and TAG.

Another breakthrough in understanding the TAG biosynthesis pathway was made by the discovery of a new enzyme, phosphatidylcholine diacylglycerol cholinephosphotransferase (PDCT), encoded by the *ROD1* locus in Arabidopsis (Lu et al., 2009). PDCT catalyzes a previously unknown reaction - the interconversion between DAG and PC by phosphocholine headgroup exchange. This mechanism generates a flux of fatty acids through PC, the site of desaturation and other modifying reactions, and results in the enrichment of PUFA or other mFA in DAG, and subsequently in TAG. The PDCT and the LPCAT pathways are the major mechanisms that direct PUFA accumulation in TAG (Bates et al., 2012).

Expression of Co-Evolved Enzymes to Increase Modified Fatty Acid Content

The above model of TAG synthesis has been derived largely from Arabidopsis; however, it is envisioned that oilseeds which accumulate unusual mFAs in their TAGs also share most of the pathways illustrated in Fig. 1. Genes encoding many enzymes that synthesize mFA have been cloned, which also use PC as the substrate (Bafar et al., 1991; Bao et al., 2002; Cahoon et al., 1999; Cahoon et al., 2002; Jaworski and Cahoon, 2003; Thomæus et al., 2001). However, expressing the single catalytic enzymes required for

mFA biosynthesis is insufficient to create transgenic plants producing large amounts of these fatty acids in seed oils (Jaworski and Cahoon, 2003; Lu et al., 2006). The occurrence of very high mFA content in native seeds is likely the result of collective effects of many genes that have co-evolved to facilitate the processing of mFA and their intermediate metabolites (Lu et al., 2006).

To validate this hypothesis, researchers from John Browse's laboratory initiated a high-throughput approach screening a full-length cDNA library from castor (*Ricinus communis* L.) by shotgun transformation into an engineered *Arabidopsis* line producing hydroxy fatty acids (Lu et al., 2006; Lu et al., 2007); and in parallel, isolated and tested by co-expression of several castor orthologous genes of the acyltransferases (Fig. 1). The hydroxylated ricinoleic acid (12-hydroxyoctadec-*cis*-9-enoic acid; 18:1-OH) accounts for 90% of the total fatty acids in castor oil. The biosynthesis of ricinoleic acid is catalyzed by the oleate Δ 12-hydroxylase (FAH12) using PC as the substrate (van de Loo et al., 1995). Previous heterologous expression of FAH12 in *Arabidopsis* produced only up to 17% hydroxy fatty acids (HFA) in seed oils (Broun and Somerville, 1997; Lu et al., 2006). The efforts in the Browse group identified several genes from castor that boost HFA accumulation in transgenic *Arabidopsis* including an oleosin (Lu et al., 2006) and the DGAT2 and PDAT1A enzymes (Burgal et al., 2008; van Erp et al., 2011).

The acyltransferases DGAT and PDAT catalyze the final step of TAG assembly by the acylation of the *sn*-3 position on DAG (Fig. 1). The two enzymes have overlapping functions for embryo development and TAG biosynthesis in developing seeds and pollen (Zhang et al., 2009), and are considered the two major enzymes for oil accumulation in seeds. In the absence of a functioning DGAT in the *dgat1* mutant, it has

been shown that PDAT1 is responsible for the remaining 65-70% of TAG synthesized in Arabidopsis seeds (Xu et al., 2012). By co-expressing a castor diacylglycerol acyltransferase, RcDGAT2, along with FAH12 in Arabidopsis, the Browse group showed a significant increase in the accumulation of HFA from 17% up to 28% (Burgal et al., 2008). Similar effects were also shown for a PDAT that increased HFA levels in transgenic Arabidopsis (van Erp et al., 2011). The cDNA encoding the castor PDAT1A was transformed into the CL37 Arabidopsis line (also expressing FAH12) both alone and in addition to RcDGAT2. Expression of the castor PDAT1A raised HFA levels from 17 to 27%, similar to the increase observed with the co-expression of FAH12 with RcDGAT2 (Burgal et al., 2008). Expression of RcDGAT2 in the CL37 PDAT1A background increased HFA levels from 25.4% $\pm$ 0.3% to 26.7% $\pm$ 0.2%, a small but statistically significant amount, which translates to an increase of 19.6% in the mass of HFA per seed (van Erp et al., 2011). In contrast, overexpression of the Arabidopsis orthologs of DGAT2 or PDAT1A failed to produce such beneficial effects in both experiments (Burgal et al., 2008; van Erp et al., 2011). These are consistent with results that both RcDGAT2 and RcPDAT1A show a preference for ricinoleic acid-containing substrates, thus facilitating incorporation of high amounts of this fatty acid into TAG in castor (Burgal et al., 2008; Dahlqvist et al., 2000; van Erp et al., 2011). These results fuel the hypothesis that the limited mFA accumulation in seed oils may be due to inefficient utilization of these mFA substrates by the transgenic plant. Enzymes that have co-evolved to utilize mFA substrates for incorporation into TAG in plants such as castor may need to be co-expressed to accumulate these mFAs in the selected crop plant at levels that would be acceptable for industrial uses.

Table 1. Summary of results from past experiments using the castor hydroxylase and genes involved with fatty acid metabolism to accumulate hydroxy fatty acids.

Co-evolved HFA Related Gene Experiments		
Plant	Gene(s) Inserted	% HFA accumulated
Arabidopsis	RcFAH12	<17%
Arabidopsis	RcFAH12 + RcDGAT2	28%
Arabidopsis	RcFAH12 + RcPDAT1A	27%
Arabidopsis	RcFAH12 + RcDGAT2 + RcPDAT1A	26.70%
Arabidopsis	RcFAH12 + RcPDCT	22%
Arabidopsis	RcFAH12 + RcDGAT + RcPDCT	28.50%
Camelina	RcFAH12	15%

The theory of co-evolved enzymes has gained further support from species that accumulate other mFAs. Seeds of the tung tree (*Vernicia fordii*) produce large quantities of TAGs containing ~80% eleostearic acid, a conjugated fatty acid. The tung DGAT1 is expressed at similar levels in various organs, whereas DGAT2 is strongly induced in developing seeds at the onset of oil biosynthesis. Expression in yeast showed the tung DGAT2 possessing an enhanced propensity for the synthesis of trieleostearin, the main component of tung oil (Shockey et al., 2006). It has also been shown that DGATs from *Vernonia galamensis* and *Stokesia laevis* have strong substrate preferences for vernolic acid, an 18-carbon Δ 12-epoxy fatty acid (Yu et al., 2006). Expression of the *Stokesia* epoxygenase alone only resulted in 3-7% epoxide levels. Co-expressing DGAT1 or DGAT2 from *V. galamensis* led to increased accumulation of up to 27.8% vernolic acid (Li et al., 2010). Researchers found that more vernolic acid accumulated in TAG with lower levels of vernolic acid left in PC which was similar to the ratio observed in native *Stokesia* and *Vernonia* seeds. Therefore DGATs from high epoxy fatty acid accumulators may selectively incorporate epoxy fatty acids into TAGs. These experiments indicate that

high-level production of mFA in transgenic seeds requires co-expressing or stacking genes from the original accumulator species.

Genomics Approaches to Uncovering Additional Factors in mFA Metabolism

Greater knowledge has been obtained in recent years on enzyme functions and regulations that are involved in the accumulation of mFA in TAG. However transgenic experiments have shown that a complete understanding of the synthesis, modification and packaging of these mFA in seeds is required to allow for production of these useful fatty acids at commercially viable levels in agricultural crops. Genomics and bioinformatics tools have played important roles in the advancement of plant lipid research (Wallis and Browse, 2010). The most recent examples include the discovery of the PDCT enzyme encoded by the Arabidopsis *ROD1* gene (Lu et al., 2009) and a distinct DGAT with sn-3 acetyltransferase activity that synthesizes the unusual 3-acetyl-1,2-diacyl-sn-glycerols (acTAGs) in seeds of *Euonymus alatus* (Durrett et al., 2010). Advanced genomics tools including next-generation sequencing capabilities and new databases of fatty acid synthesis genes from other mFA accumulators will undoubtedly offer unprecedented opportunities to unravel the molecular and biochemical mechanisms of mFA accumulation, and provide new genes to engineer these mFAs in seed oils of transgenic crops.

Genomics studies in natural mFA-producing species will likely provide valuable insights on why these fatty acids are accumulated at high levels in seeds. Although genome sequences of such species are now only available in castor (Chan et al., 2010),

global analyses of transcriptomes by ultra-deep sequencing are providing increasingly powerful approaches to uncover biochemical functions and regulatory networks in TAG biosynthesis. By the 454 analysis of seed transcriptomes of the bitter melon (*Momordica charantia*) that accumulates conjugated fatty acids, several candidate genes were identified for eleostearic acid metabolism such as DGAT1 and 2, and a PDAT1-related enzyme. Transcripts were also identified for a novel *FAD2* variant gene encoding a functional $\Delta 12$ oleic acid desaturase with potential implications for eleostearic acid biosynthesis (Yang et al., 2010). Another comprehensive RNA-Seq transcriptomic analysis was performed in castor using mRNA isolated from developing seeds and non-oleaginous tissues in order to identify differences in lipid-metabolic pathways and enzyme isoforms which could be important in the biosynthesis of TAG enriched in ricinoleic acid (Brown et al., 2012). This study identified several candidates, including the castor PDCT discussed above (Hu et al., 2012), that might improve the level of ricinoleic acid in transgenic plants.

As discussed above, oilseeds that accumulate unusual mFAs share most of the major pathways illustrated in Fig. 1. It is therefore envisioned that transcriptional and post-translational regulations may also result in improved mFA metabolism. In this regard, a recent study of comparative transcriptomes between mFA- and non-mFA-accumulators yielded some valuable insights (Troncoso-Ponce et al., 2011). A large number of expressed sequence tag (EST) datasets was obtained from four stages of developing seeds of *Ricinus communis*, *Brassica napus*, *Euonymus alatus* and *Tropaeolum majus*, which differ in their storage tissue for oil and in the structure and content of their TAGs. This analysis revealed that while genes encoding core enzymes for

fatty acid synthesis in plastids maintained a conserved stoichiometry and a strong correlation in temporal profiles throughout seed development, enzymes of TAG biosynthesis on ER displayed dissimilar temporal patterns indicative of different regulation. The EST levels for several genes potentially involved in mFA accumulation were also distinct. For example, much higher ESTs were found for orthologs of LPCAT, PLC, and PDAT-like/PDAT2 in castor than *B. napus*, suggesting that these are possible candidates associated with high accumulation of ricinoleate in TAG and its exclusion from membrane lipids in castor. The expression of *FAH* in castor is several fold higher than *FAD2* expression in other oilseeds, suggesting high *FAH* expression may be required to achieve the very high (>90%) ricinoleic acid content of castor oil (Troncoso-Ponce et al., 2011). Future studies of these candidates in transgenic experiments, and up-regulation of *FAH* (e.g., promoter analysis and testing) may reveal their roles in hydroxy fatty acid accumulation.

Overcoming Bottlenecks – Efficient Removal of mFA From PC

The latest model of TAG synthesis highlights the central role of PC as the source of greatly diversified fatty acids in membrane and storage lipids. The metabolic labeling work (Bates et al., 2009; Bates et al., 2007) supports this model by finding that there are two functionally distinct pools of DAG in soybean. One pool of *de novo* DAG is utilized primarily for PC synthesis, while the other PC-derived DAG pool is used for TAG synthesis. It was revealed that 95% of TAG in soybean was synthesized using the PC-derived DAG pool (Bates et al., 2009). Similarly it has been shown that TAG synthesis in

Arabidopsis also primarily utilizes PC-derived DAG (Bates and Browse, 2011). Therefore, the flux of fatty acids through PC and subsequent removal of mFAs from PC has been identified as a major bottleneck for mFA accumulation in TAG (Cahoon et al. 2006; van Erp et al., 2011, Bates and Browse, 2011). In transgenic soybean and Arabidopsis expressing a fatty acid conjugase, transgenic lines accumulated no more than 20% of the total FA as conjugated FAs in TAG, while displaying nearly 25% in PC compared to the <1% typically seen in PC of native accumulating plants (Cahoon et al., 2006). Similar results were also seen in plants transformed with castor FAH12; the amount of HFA that accumulated in PC of the transgenics was more than twice that in castor (van Erp et al., 2011).

The efficient removal of mFA from PC to allow for their incorporation into TAG will be important in the creation of a high mFA accumulating transgenic plant. Enzymes specific for the removal of mFA from PC to break the bottleneck would be of the most use. Expression of a PDAT from castor, RcPDAT1A, was shown to increase HFA levels from 17 to 27% in the Arabidopsis CL37 line (van Erp et al. 2011). This increase in HFA content was most likely due to the direct transfer activity of RcPDAT1A succeeding in removal of HFA from PC, as the percent of HFA left in PC of the CL37 PDAT1A transgenics was lower and more comparable to that seen in castor (van Erp et al., 2011). The newly discovered enzyme, phosphatidylcholine diacylglycerol cholinephosphotransferase (PDCT), offers another possible solution for the removal of mFAs from PC so they can be incorporated into TAG. PDCT functions in the conversion between PC and DAG, enriching the DAG pool with mFAs prior to their incorporation into TAG. Through studies of the Arabidopsis *rod1* mutant that is deficient in the PDCT

enzyme, it was shown that at least 40% of the PUFAs present in TAG are derived from the PDCT-mediated DAG-PC conversions (Lu et al., 2009), so it stands to reason that PDCT could also be an important enzyme in mFA removal from PC. Arabidopsis *rod1* mutants expressing FAH12 only accumulated half the HFA that their wild type counterparts did. When a castor PDCT was co-expressed with FAH12, the RcPDCT enzyme was able to almost double HFA levels (Hu et al., 2012).

The results of PDAT and PDCT demonstrate that efficient metabolism of mFA through PC is an effective strategy to increase mFA accumulation in TAG. Additional enzymes in such roles may include those involved in acyl editing for mFA removal, and phospholipases C and D for PC-DAG conversion (Fig. 1). It is interesting to note that the gene encoding LPCAT2 was upregulated by 50-75% as measured by microarray and quantitative RT-PCR in the *dgat1* mutant, which contains a lesion at the *DGAT1* locus resulting in an oil content decrease by 30-35% compared to wild type levels (Katavic et al., 1995; Xu et al., 2012). Co-incident knockout lines of *dgat1/lpcat2* displayed severe effects including deficient TAG accumulation to levels slightly lower than that of *dgat1*, as well as delayed plant development and seed set. Because PDAT1 was also upregulated by 65% in the *dgat1* mutant, researchers suggested that the LPCAT2 functioned to assist PDAT1 in biosynthesis of TAG by supplying PC as a substrate for the transfer of sn-2 acyl chains to the sn-3 position of the increased DAG pool (Xu et al. 2012). The increased levels of PUFAs and reduced very long chain fatty acids (VLCFA) present at the sn-3 position of TAG are evidence supporting the donation of acyl groups from the sn-2 position of PC to the sn-3 position of DAG for TAG synthesis. While the orthologous LPCATs from other native species remain to be isolated and tested, LPCAT2

shows promise as an enzyme to help increase the ratio of mFA in TAG either through cooperation with a PDAT1, or through the reverse reaction to release mFA from PC before being incorporated into TAG.

Feedback Inhibition Preventing Accumulation of mFA Products

The transgenic lines expressing mFA synthesizing genes fail to accumulate desired levels of products, and also often display decreased oil phenotypes (Dauk et al., 2007; van Erp et al., 2011). Comprehensive investigations of the FAH12 line (Bates and Browse, 2011) suggest that expression of FAH12 results in accumulation of disruptive intermediates and substantial reductions in both HFA and total oil content of seeds. The FAH12-expressing Arabidopsis line CL37 exhibits roughly 30% less oil than its parent *fae1* lines (van Erp et al., 2011). Similar detrimental effects were also observed in soybean expressing epoxy mFA transgenes (Li et al., 2010; Li et al., 2012). This reduced oil accumulation may have been the result of inefficient incorporation or utilization of mFA-containing lipids, which are subsequently degraded since fatty acids cannot accumulate in plant cells. The investigation of FAH12-expressing Arabidopsis lines indicated the fatty acid β -oxidation cycle is present even with low FAH12 gene expression levels (Moire et al., 2004). Similar results were also reported in *Brassica napus* expressing a lauroyl-acyl carrier protein thioesterase (Eccleston et al., 1996; Eccleston and Ohlrogge, 1998). To prevent a futile cycle of mFA synthesis and breakdown, enzymes that are selective for incorporation of mFA-containing DAG and acyl substrates into seed oil will need to be inserted. Utilization of native mFA

accumulator DGAT2 and/or PDAT1A enzymes has been shown to improve the passage of intermediates into TAG and substantially (75-80%) relieves the decline in mFA and oil content (Li et al., 2012; van Erp et al., 2011). The newly discovered enzyme PDCT has also been shown to partially restore low oil phenotype (Hu et al., 2012). Avoiding turnover of mFA lipids will hopefully aid in increasing the flux of mFAs into TAG.

Although breakdown of fatty acids by β -oxidation may contribute to the low oil content of mFA-transgenic seeds, recent results indicate that a reduction in fatty acid synthesis (FAS) rates is the main consequence of the bottleneck in TAG synthesis. This feedback inhibition of FAS may have a major role in limiting the productivity of plants engineered to produce mFA. FAS is believed to be controlled by activity of plastid ACCase which is a complex of four subunits in dicots and non-graminaceous monocots. The feedback involves inhibition of this complex by acyl-ACP (the end-products of fatty acid synthesis) (Andre et al., 2012; Shintani and Ohlrogge, 1995). Overexpression of an *Arabidopsis* cytosolic ACCase, which is not subject to inhibition by acyl-ACP, in the plastid of *Brassica napus* resulted in a substantial increase in ACCase activity (Roesler et al., 1997). In native oilseeds, TAG synthesis is highly efficient so that feedback inhibition is possibly almost negligible. Therefore oil accumulation in the ACCase over-expressing lines increased by no more than 5% (Roesler et al., 1997). By contrast, the fatty acid synthesis rate is >30% reduced in FAH12 plants (Bates and Browse, 2011). If this is due to feedback down-regulation of the plastid ACCase, expression of cytosolic ACCase in the plastid will restore fatty acid synthesis and may increase mFA accumulation.

CHAPTER 3

OBJECTIVES

My objectives are to further understand the metabolism of hydroxy fatty acids (HFA) and to successfully produce HFAs in transgenic camelina. Plant oils containing hydroxy fatty acids are desirable for a wide variety of applications including lubricants, plasticizers, surfactants, polyesters, paints, sealants, biodiesel, and more (Chen et al., 2011; Dyer et al., 2008). Recently, research revealed that using hydroxy fatty acids as a base stock for the creation of estolides led to a superior product able to out-compete commercial synthetic and petroleum derived oil products in regards to biodegradability and cold temperature properties such as pour point, viscosity index, and cloud point (Cermak et al., 2006). With so many possible high value applications it is clear that hydroxy fatty acids are a very desirable commercial product. There are at least 14 known plant species that produce hydroxy fatty acids, however, most of these plants have very limited oil yields or are unsuitable for large scale agronomic production (Bates and Browse, 2011; van de Loo et al., 1993). The main commercial source of hydroxy oil is the castor plant, *Ricinus communis*, whose seed oil contains 80-90% ricinoleic acid, Δ -12-hydroxyoctadec-*cis*-9-enoic acid, 18:1OH (Severino et al., 2012). Castor is mainly grown in tropical regions of India, China, Brazil, and Thailand, but is considered by many to be unsuitable for large scale agricultural use due to the presence of the seed toxin ricin and highly allergenic 2S albumins in its seed (Chan et al., 2010). The United States imports the majority of its castor oil from India, which makes the multi-billion dollar import vulnerable to foreign market fluctuations in price and supply.

In the past few years there has been increased interest in replacing castor oil with lesquerella oil, an alternative source of hydroxy fatty acids. Lesquerella (*Physaria fendleri*) is a small, slow growing, arid region plant native to Mexico and the South Western United States. Lesquerella does not contain the toxins seen in castor. The seed accumulates up to 60% of its oil as hydroxy fatty acid, 20:1OH, Δ -14-hydroxyeicos-*cis*-11-enoic acid (Dierig et al., 2011). Lesquerella oil is desirable for some of the same reasons as castor oil, the presence of the hydroxy group and monounsaturations of the oil, but additionally, the fatty acid chains of lesquerella oil are 2 carbons longer which can impart better qualities to the oil such as increased resistance to friction and wear when (Cermak et al., 2006). When compared to castor, soybean, and rapeseed oil, lesquerella oil has been shown to have superior performance at much lower concentrations for reducing wear and damage to diesel engines with fuel injections (Dierig et al., 2011). Despite the desirable qualities in its oil, lesquerella is not yet a commercial crop and more breeding efforts and research are ongoing to improve the oil profile, address its outcrossing habit, breed for traits such as salinity and heat stress, quicker maturity time, increased branching, as well as solve agronomic questions like identification of usable herbicides, pest management, soil type, and irrigation management (Dierig et al., 2011).

The desire to synthesize hydroxy (HFA) and other modified fatty acids (mFA) in a more agronomically favorable oilseed crop has been the focus of research in the past decades. The HFA synthesis pathway has been extensively studied and key genes such as the castor and lesquerella fatty acid hydroxylase, *RcFAH* and *LfFAH*, have been discovered (Broun et al., 1998; van de Loo et al., 1995). However, insertion of these hydroxylase genes into various *Arabidopsis* backgrounds under the control of seed

specific promoters failed to result in high accumulation of HFA products, yielding disappointing levels of 17% or less (Lu et al., 2006). Next, researchers tried increasing the level of HFA by incorporating additional fatty acid synthesis genes from castor including diacylglycerol acyltransferase (*RcDGAT*), phospholipid:diacylglycerol acyltransferase (*RcPDAT1A*), and phosphatidylcholine:diacylglycerol cholinephosphotransferase (*RcPDCT*) (Burgal et al., 2008; Hu et al., 2012; van Erp et al., 2011). The highest accumulating transgenic combinations were the *Arabidopsis* lines containing RcFAH and RcDGAT2 + RcPDCT which accumulated 28.5% HFA and the *Arabidopsis* line containing RcFAH + RcDGAT2 + RcPDAT1A which accumulated 27% HFA (Hu et al., 2012; van Erp et al., 2011). While these new genes succeeded at increasing the level of hydroxy fatty acids in seed oil, they still failed to match the levels seen in native accumulator plants. Recent radio labeling studies implicated phosphatidylchoine (PC) as a bottleneck for the accumulation of modified fatty acids that results in increased turnover of HFA containing lipids and potential feedback inhibition leading to a reduced oil phenotype (Bates and Browse, 2011). While the incorporation of DGAT or PDAT was shown to be able to help recover the reduced oil phenotype, it also highlighted a need for more investigation into the TAG synthesis process to uncover additional constraints and factors affecting the fluxes through competing pathways involved with accumulation of HFA in TAG.

In this study, I will create transgenic camelina plants to produce HFA in its seed oils. Although *Camelina sativa* and *Lesquerella (Physaria) fendleri* both belong to the Brassica family, camelina does not contain a hydroxylase gene and does not produce hydroxy fatty acids. Previously, our lab has shown that camelina seeds accumulated

HFAs by expressing a castor hydroxylase gene, *RcFAH* (Lu and Kang, 2008). To further understand HFA accumulation in camelina, I will introduce another gene from *P. fendleri*. The *LfKCS* gene encodes a fatty acid condensing enzyme that specifically recognizes 18:1OH and elongates it to 20:1OH, the major component of lesquerella oil (Moon et al., 2001). After the castor *RcFAH* acts to hydroxylate 18:1 on the PC, 18:1OH that has been removed from PC can be elongated in the cytosol by *LfKCS3* to 20:1OH. It has been speculated that removal of HFA-CoA's from the acyl-CoA pool may cause a net movement of HFA out of PC and into TAG due to the nature of the dynamic flux of between PC and the acyl-CoA pool (van Erp et al., 2011). Very long chain fatty acids (VLCFA) are generally excluded from the membrane lipids of developing *Arabidopsis* seeds (Norton and Harris, 1983; Smith et al., 2003), so elongating the HFA from 18 to 20 carbons may place them in a different modified acyl-CoA pool that could be more readily incorporated into TAG. Increased flux of HFA off PC and into TAG has been previously shown to increase HFA accumulation and relieve potential feedback inhibition (Bates et al, 2011; Hu et al., 2012; van Erp et al., 2011). Additionally, occupation of HFA by the *LfKCS3* enzyme could potentially make them unavailable to the degradation enzymes involved in FA turnover and signal less of a product buildup that could cause inhibition of FA synthesis. Here I investigate whether modification of HFA content through addition of a *Lesquerella (Physaria) fendleri* ricinoleic acid specific elongase, *LfKCS3*, will impact the hydroxy fatty acid accumulation in transgenic camelina containing the castor *RcFAH* gene. I expect that new results will shed more light on the TAG synthesis pathway interactions as they pertain to mFA, as well as the ability to successfully reconstruct the HFA pathway in a Brassica oilseed crop plant.

CHAPTER 4

MATERIALS AND METHODS

Plant Materials

A *Camelina sativa* variety, Suneson, released by MSU and designated MT5, was used as the wild type plant line. Plants were germinated as 5 seeds to a 6" or 8" pot and grown in a 1:1 mix of MSU soil (equal parts by volume of loam soil : washed concrete sand : Canadian sphagnum peat moss with AquaGro 2000 G wetting agent blended in at 1lb/cubic yard of soil. Aerated steam pasteurized at 70°C for one hour) and Sunshine Mix #1 (Bellevue, WA, USA). Greenhouse conditions were 70°/60°F +/- 7 ° for day/night temperatures, a relative humidity of 30%, and a 14 hour photoperiod of natural lighting supplemented when necessary by season. Plants were watered daily and fertilized once a week with an all-purpose 24-8-16 Miracle Grow fertilizer (Maysville, OH, USA) as recommended. Plants were staked using bamboo stakes and twist ties as needed. The same growth conditions were applied to transgenic plants and wild type plants. All plant pots contained color coded stakes labeled as to the background, transformation treatment, or transformed line.

Plasmid Constructs

A binary plasmid vector, pGATE-PHAS-RcFAH-LfKCS3, was constructed containing the *Ricinus communis* fatty acid hydroxylase gene, *RcFAH*, and the *Physaria* (formerly *Lesquerella*) *fendleri* elongase gene, *LfKCS3*. Spectinomycin resistance was

used for bacterial selection, and glufosinate resistance through the *bar* gene for plant selection. The *LfKCS3* gene was expressed under the control of its native *Lesquerella* promoter and terminator, while the *RcFAH* gene was inserted under the control of the seed specific phaseolin promoter and terminator. *Lesquerella* genomic DNA for the *KCS3* gene was obtained from Dr. Ljerka Kunst (University of British Columbia), as part of construct MHS15: pGEM7-Lf in XL1-Blue *E. coli* cells (Moon et al., 2001). MHS15 plasmid was extracted from overnight culture in LB broth using the Qiagen miniprep kit (Qiagen, Germantown, Maryland, USA), then digested for 1 hour at 37°C with the restriction enzyme *EcoRI* (New England Biolabs, Ipswich, MA, USA) to excise the *LfKCS3* gene. The digestion reaction was then run on a 0.7% agarose gel with ethidium bromide to separate and visualize the plasmid fragments. The gel was viewed under UV light and the band corresponding to the 4313bp *LfKCS* gene was cut out of the gel and recovered using the QIAquick gel extraction kit (Qiagen, Germantown, Maryland, USA).

The previously created vector backbone, pGate-Phas-RcFAH (Lu et al., 2006) was prepared by digestion with *EcoRI* for 1 hour at 37°C to open the backbone then the enzyme was deactivated for 15 minutes at 65°C. The backbone was then dephosphorylated with shrimp alkaline phosphatase (Roche, Indianapolis, IN, USA) to prevent self-ligation. The *EcoRI* digested, dephosphorylated backbone and *EcoRI* digested *LfKCS3* insert DNA were ligated overnight at 4°C using T4 DNA ligase (Promega, Madison, WI, USA). Electroporation transformation of competent DH10B *E. coli* cells was performed using 1µl of ligation mixture and 0.2 cm cuvettes (Bio-Rad, Hercules, CA, USA) at a field strength of 2.5 kv/cm, resistors of 600 Ω, and a capacitance of 25µF. An outgrowth period of 1 hour in LB culture tubes on a shaker set

at 37°C and 250rpm was performed prior to spreading cell transformation mixture on LB agar plates containing spectinomycin. Parts of single, isolated colonies were tested using colony PCR (Promega Go-Taq, Madison, WI, USA) and gene specific primers to ascertain transformation events with a successful ligation product, pGP-RcFAH-LfKCS. PCR primers used: LfKCS3-1 direct 5'-TTACGTCCCGGATCTTAAGC-3' paired with LfKCS3-2 complement 5'- ATGAATAAACGGCCTGCCTG-3'. PCR products were separated by 100 volt gel electrophoresis on a 0.7% agarose gel with ethidium bromide and viewed under UV light. Colony PCR samples containing the correct sized band on the gel were grown overnight at 37°C and 250 rpm in culture tubes of LB broth plus the antibiotic spectinomycin. Plasmid from positive colonies was purified using the Qiagen QIAprep Spin Miniprep Kit (Qiagen, Germantown, Maryland, USA) and digested with restriction enzymes *EcoRI*, *HpaI* or *PstI* to affirm correct banding pattern expected for the desired 16,343bp plasmid, pGP-RcFAH-LfKCS as determined by the plasmid map (Figure 1). Positive cultures were labeled and saved in the -80°C freezer in a 500:500ul solution of culture and 50% glycerol.

Plasmid sample was sent for sequencing to UC Berkley using gene specific sequencing primers for both the *RcFAH* and *LfKCS3* gene, and the resulting sequences compared to the Vector NTI program gene sequences using the NCBI alignment Blast tool. Primers used to sequence can be found in table 1. 2µl of positive, sequenced pGP-RcFAH-LfKCS plasmid construct #14 was used to transform electrocompetent *Agrobacterium tumefaciens* strain GV3101 using the electroporation methods described above, followed by outgrowth at 28°C. After one hour outgrowth, 100µl of the

transformed *Agrobacterium* solution was plated onto pre-warmed LB agar + spectinomycin + rifampicin plates and grown at 28°C for

Table 2. Primers used with plasmid pGP-RcFAH-LfKCS. Primers were created using the Vector NTI program and synthesized by Invitrogen.

Primers for pGP-RcFAH-LfKCS					
Primer #	Primer Name	Sequence	Use	Orientation	Location
205	EV-CHB3 (RcFAH)	5'-TCGATATCTTAATACTTGTTCCGGTACC-3'	PCR	Sense	Exon 2
206	EI-CBH5 (RcFAH)	5'-TCGAATTCATGGGAGGTGGTGGTCGCAT-3'	PCR	Antisense	Exon 3
351	LfKCS3-1	5'-TTACGTCCCGGATCTTAAGC-3'	PCR	Sense	Exon 3
352	LfKCS3-2	5'-ATGAATAAACGGCCTGCCTG-3'	PCR	Antisense	Intron after exon 3
358	LfKCS 3-3	5'-CTTTTTTCGCGGACAATGAC-3'	PCR	Antisense	Intron after exon 3
361	LfKCS3-4	5'-GGAGGAATGGGTTGTAGTGC-3'	PCR	Sense	Exon 2
362	LfKCS3-5	5'-CGAACTCCTACTTTACCATCTC-3'	PCR	Antisense	Exon 3
357	LfKCS seq1	5'-ACATCTCATCCCTTAGTACC-3'	Sequencing	Sense	Exon 1
359	LfKCS seq2	5'-GTTTGTATCCTTTATTTTTTC-3'	Sequencing	Sense	Exon 2
360	LfKCS seq3	5'-GAGATAAGAGAAGGTCTAAGTAC-3'	Sequencing	Sense	Exon 3
365	LfKCS seq 4	5'-ACTTGTTGATCTTCCATCG-3'	Sequencing	Sense	Intron after exon 3
366	LfKCS seq5	5'-AGTCTAGTTAAAGTTGCCAA-3'	Sequencing	Sense	Intron before exon 1
428	LfKCS exon 2	5'-GAATGTTAGTAATTGCTTGTTCG-3'	RNA analysis	Sense	Exon 2
429	LfKCS exon 3	5'-GCCGAAGCTTTAACATTACGTA-3'	RNA analysis	Antisense	Exon 3

36-48 hours or until colonies appeared. Colony PCR of *Agrobacterium* from the transformation plate was done starting with 10µl of water and the colony swab heated at 100°C for 10 minutes, cooled, then the rest of the PCR solution including the gene specific primers was added (Invitrogen, Carlsbad, CA, USA) and PCR was resumed with the following parameters: 30 cycles of denaturation of 94°C for 15 seconds followed by

annealing at 55°C for 30 seconds and extension of 72°C for one minute, ending with a final extension of 72°C for seven minutes. Positive colony #5 was selected to be grown in a 3ml overnight culture of LB + Spectinomycin + Rifampicin so 1ml aliquots could be saved as a stock in the -80°C as a 1:1 ratio of culture to 50% glycerol. This stock would be used to start larger 500ml culture flasks containing LB + spectinomycin and rifampicin antibiotics for use in plant transformation.

Camelina Transformation

Camelina plants were transformed following the established protocol in our lab (Lu and Kang, 2008). MT5 wild type camelina plants at the early flowering stage were used for transformation. This stage was identified as having the first flowers blooming and many large buds present. The day before the transformation 1 ml of starter culture from the agrobacterium pGP-RcFAH-LfKCS stock in the -80°C freezer was thawed and used to inoculate a flask containing 500ml of LB broth plus 500ul each of 100mg/ml spectinomycin and 50mg/ml rifampicin antibiotics. The culture flask was grown at 28°C with shaking at 250rpm for 24 hours or overnight. The day of transformation the culture was spun down at 5000rpm for 10 minutes and agrobacterium cell pellets re-suspended into the infiltration solution of 500ml double de-ionized water, half strength MS salts, 50g/L sucrose, and 0.05% (v/v) silwet L77 surfactant (Lehle Seeds, Round Rock, TX, USA). Plants in 6" pot size were placed into a 310mm high vacuum desiccator chamber (Bel-Art, Pequannock, NJ, USA) with the inflorescences immersed into a container of the re-suspended agrobacterium inoculum. The vacuum was applied to the chamber and held

for 3 minutes at 85kPa, then let out quickly. The treated plants were covered with white plastic bags for 24 hours before resuming normal greenhouse growth to maturity.

Screening Procedure to Identify Transformants

Seed from mature *Agrobacterium* treated plants was harvested in bulk by hand using a 1.7mm sieve (No. 12 Fischer Scientific, Pittsburgh, PA, USA). 3-4 grams of seed was then planted out directly in flats (Kadon Corp, Dayton, OH, USA) of pre-moistened 50:50 mix of MSU soil to sunshine mix (Bellevue, WA, USA) and grown in the greenhouse. Once the majority of seeds had germinated and were starting their first true leaves, about 6-7 days, the flat was sprayed with glufosinate herbicide at a concentration of 23.4ml/L (5.78% Glufosinate ammonium, Power Force Grass and Weed Killer, Bayer, Birmingham, AL, USA). The herbicide was allowed to take effect for three days then any surviving plants or newly emerged seedlings were re-sprayed with herbicide. Any seedlings proving to have survived herbicide treatment were transplanted into 6" pots of a 50:50 mix of soil to sunshine mix and allowed to establish for one week before another reapplication of herbicide treatment.

To further ensure the herbicide selection process was selecting for plants containing the plasmid a polymerase chain reaction, PCR, was performed using genomic DNA from leaf tissue of the surviving transgenics. DNA was extracted from two week old plants using 1" leaf pieces following the procedure modified by (Lukowitz et al., 2000). Leaf tissue was ground by pestle in a 1.5ml reaction tube containing 300ul 2x CTAB buffer, incubated at 65°C for 10 minutes, then cooled to room temp. 300μl chloroform was added and the sample vortexed and then spun to separate phases. The

upper aqueous phase was added to 300µl of 2-propanol, mixed gently by inversion, and incubated on ice 5 min to precipitate DNA. DNA was pelleted by centrifuging for 5 minutes then washed with 500µl of 70% Ethanol. Pellets were air dried before resuspending in 100µl sterile double de-ionized water. 2µl of extracted DNA was used in a PCR reaction with *RcFAH* gene specific primers 205 and 206 (Table 2). PCR parameters were an initial denaturation of 3 minutes at 94°C followed by 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1:20 and a final extension time of 7 minutes at 72°C. PCR results comparing the plant DNA to the PCR of the plasmid gene were run on a 0.7% agarose gel with ethidium bromide at 100V and viewed under UV light to evaluate band sizes. Positive, surviving T₀ seedlings were assigned a transgenic event number and grown to maturity. T₁ seed was harvested individually using the sieve method in order to retain individual event line identity.

Advancement and Selection of Transformed Lines

The herbicide selection of seedling flats described earlier was used to obtain 22 individual transgenic T₀ RcFAH-LfKCS lines. Mature T₁ seed was harvested from each of the 22 transgenic plants individually using a sieve and saved in labeled coin envelopes. T₁ seed was re-planted to advance to the T₂ generation by planting 6 seeds per 6" pot for each line. Pots were filled with a 50:50 mix of soil to sunshine mix and grown in the greenhouse. The emerging seedlings were sprayed with herbicide after one week to eliminate any non-transgenic plants segregating out. Plants of each line were harvested individually using a sieve with the number of each individual harvested plant of each transgenic event line marked on the envelope. Six single seeds of each individual harvest

plant of each transgenic event line were run through the GC using 96 well plates and the TMSH derivitization method (Lu et al., 2006). For this method the seeds are crushed, 100µl of TMSH added, and after 10 minutes 200µl of methanol is added. The individual plant showing the highest hydroxy fatty acid amount was kept from each T₁ derived event line and re-planted to advance to the T₃ generation.

As with the T₁ seed, 6 seeds of each individual T₂ event line were planted out in 6" pots filled with 50:50 mix of soil to sunshine mix then grown in the greenhouse. Seedlings were sprayed with herbicide to eliminate any non-transgenic seedlings segregating out. T_{2:3} seed was individually harvested from each plant and designated with an event # - individual plant # system such as line 23-3. Six seeds of each individual plant were mass screened through the GC using 96 well plates and the TMSH method. Presence or absence of hydroxy fatty acids in the individual seed fatty acid profiles and herbicide spraying survival were used to determine which plants were potentially homozygous (Figure 2). Homozygous plants were those characterized as having hydroxy fatty acids in all profiles of the six single seeds tested and no seedling death from herbicide spraying. Best individual harvest plants of lines identified as homozygous were selected and replanted. The selection process for T_{2:3} seeds was repeated for T_{3:4} lines. Homozygous T₄ and homozygous, same event line bulked T₅ seed were used for testing.

Fatty Acid Analysis by Gas Chromatography

Two different methods were used to create fatty acid methyl esters (FAMES) from total lipid samples to separate and identify fatty acids by gas-chromatography (GC) and gas-chromatography mass spectrometry (GC-MS). (1) TMSH method:

Trimethylsulfonium hydroxide (TMSH) preparation was used for the 96-well plate screenings. Preparation involved crushing single seeds in 96 well metal plates using a metal crusher (custom made by Automation by Design, Valencia, CA, USA) prior to addition of 100µl of TMSH. After letting samples rest at room temperature for 15 minutes, 200µl of methanol was added. Plates were prepared in the fume hood and kept covered with saran plastic wrap to prevent evaporation or inhalation of fumes. (2) Acid derivation method: FAMES were prepared by crushing the single seeds in glass tubes with a metal rod and adding 1ml of 2.5% H₂SO₄ in methanol prior to incubating at 90°C for 60-90 minutes. Samples were then cooled to room temperature before addition of 250µl of hexane and 1.5ml of 0.9% NaCl. After mixing vigorously samples were spun down for 3 minutes to separate phases, and the top hexane phase transferred to a glass insert fitted in a vial. For analysis involving quantification a set amount of 17:0 standard (10mg/ml) was added to tubes prior to FAME derivatization.

Fatty acid methyl ester (FAME) samples were run on a GC-2010 (Shimadzu, Kyoto, Japan) using an SHX20 wax column (7.0m x 1.0µm x 0.53µm, max temperature 260°C). Split injection mode was used and helium was the carrier gas. The GC was programmed for oven temp of 190°C increasing to 250°C after 4 minutes and holding at 250°C for 5 minutes for a total run time of 9 minutes (method designated HFAm8min).

FAMES were also run on GC-MS comprising an Aglient 7890A GC fitted with an HP-Innowax 19091N-133 (30m x 250µm x 0.25µm, 260°C max temp.) and a 5975C inert MSP with triple ONS detector. GC was programmed for a 12 minute method designated HFA.m: starting oven temperature of 190°C and hold for 0.6 minutes then increase to

250°C with a ramp time of 25°C/min and hold at 250°C for 7 minutes. A split splitless inlet and helium carrier gas was used to run samples.

GC (GC Solutions, Franklin, MA, US) and GC-MS Chemstation (Agilent, Santa Clara, CA, USA) software was used to measure the ratio of peak areas and identities. GC analysis files were exported into Microsoft Excel for further analysis of percentages and ratios of peaks corresponding to fatty acids of interest.

Harvest of Developing Seeds

Homozygous T_{2:3} RcFAH-LfKCS line numbers 12, 14, 15, 17, 19, and 23 along with control T₅ RcFAH 7-1 and 7-2 lines were used for lipid analysis during seed development. Ten seeds of each line were planted in two 8" pots filled with a 50:50 mix of soil and sunshine mix then grown in both the west wing and 177D greenhouses. Growth conditions were day/night temperatures of 72°/65°F +/- 7° for the west wing and 70°/60°F +/- 7° for 177D, both had a 14 hour photoperiod and relative humidity of 30%. Seedlings were thinned 6 to a pot, and sprayed with herbicide as a further check for homozygosity. When beginning to form inflorescences, plants were staked and numbered. The label tape marker on the stake contained both the transgenic line number, individually assigned plant number, and the day point to be harvested, example: line 23, plant #3, 8 days. Plants to be used for developing seed for 8, 12, 16, 20, and 24 days after flowering were labeled. In addition to the main inflorescence, side branches were also assigned numbers with labeling tape as they emerged. The number of newly opened flowers on each plant's main inflorescence or branch was recorded daily and kept track of in separate log book pages corresponding to the line # and developmental day point

targeted for harvest. At the specified days after flowering the recorded number of pods relating to the developmental time frame desired were individually harvested off each plant daily. Seeds were removed from the pod and immediately frozen in liquid nitrogen to preserve the fatty acid profile and prevent degradation of any fatty acid components by lipases. Frozen seed was transferred to 1.5ml eppendorf tubes labeled with the line, plant #, and developmental time point then stored in the -80°C freezer. Tubes were filled with seed from the same line, plant #, and developing time point until they were half full, then a new tube was started. Care was taken to ensure the seed stayed frozen and did not thaw in order to preserve the sample.

RNA Extraction and RT-PCR

Developing T₄ seed at 16 days old from T₃ RcFAH LfKCS lines 14 and 17 and control line T₃ RcFAH line 7-1 was used for RNA extraction. RNA was extracted by grinding seed samples with pestle and mortar in liquid nitrogen to form a paste. RNA extraction was then carried out as described in the Qiagen RNeasy plant mini kit using buffer RLC (Qiagen, Germantown, MD , USA). RNA concentration was tested using a nanodrop 2000 (Thermoscientific, Pittsburgh, PA, USA). Samples were diluted to equal concentrations prior to cDNA creation using the Superscript III First Strand Synthesis System kit with oligo(dT) primers (Invitrogen, Carlsbad, CA, USA). Genomic DNA was extracted from leaf samples of the corresponding plants via the method described earlier. PCR was run using gene specific primers for the *LfKCS3* gene located in two different exons of the native gene: *LfKCS3* exon2 sense orientation primer 5'-GAATGTTAGTAATTGCTTGTTCG-3' and *LfKCS3* exon 3 antisense orientation

primer 5'-GCCGAAGCTTTAACATTACGTA-3'. Primers were paired with both the cDNA and genomic DNA of RcFAH-LfKCS lines 14 and 17 as well as control line RcFAH 7-1. PCR was run using Taq enzyme and buffer from GBiosciences (St. Louis, MO, USA), and the following cycles: initial denaturation of 94°C for 2 min followed by 30 cycles of 94°C for 30 seconds to denature, 55°C for 30 seconds to anneal, and 72°C for 1 minute to extend, then a final extension of 72°C for 7 minutes. PCR results were run on a 0.7% agarose gel plus 4µl ethidium bromide at 100V until the ladder was separated. The Vector NTI (Invitrogen, Carlsbad, CA, USA) plasmid map was referenced to determine the expected DNA vs cDNA band size and compared to the gel results.

Lipid Extraction and Thin Layer Chromatography

Total lipids were extracted using a modified Blight and Dyer method as previously described (van Erp et al., 2011). Twenty dry T₄ seeds of each line were placed in a glass tube and crushed with a metal rod. The rod was cleaned with chloroform between uses. Four ml of 2:1 v/v chloroform:methanol was added to the crushed seeds and the tubes were capped and mixed and left at room temp for 10 minutes to extract. After 10 minutes 1.5ml of 0.9% NaCl was added and the samples were vortexed at medium speed. The samples phases and seed debris were then separated by centrifuging at 4,000rpm for 10 minutes. The bottom chloroform layer was carefully transferred via glass Pasteur pipette to a new tube. Samples were then dried down under a stream of nitrogen gas to a small volume for loading onto TLC plates.

Thin layer chromatography (TLC) plates (silica gel 60, 20x20cm, EMD Chemicals, Darmstadt, Germany) were used to separate the TAG from the OH-TAG oil.

Prior to the run the plates were baked at 100°C for a period of 30 minutes then cooled to room temperature. Samples were loaded onto the plates at a distance of 1.5cm up from the edge of the plate. Capillary tubes were used to load the samples in a thin line. For methods requiring recovery of the band the entire sample was loaded, with only two samples and one castor oil standard per plate to allow enough running room. The TLC tank was washed and a new filter paper backing placed in the tank to ensure no contamination from different buffer solutions or runs. The tank atmosphere was allowed to develop until at least half of the filter paper backing had absorbed running buffer. The buffer was then quickly replaced with a fresh solution prior to insertion of the plate to run. The running buffer used for separation of TAG from OH TAG was 70:30:1 v/v Hexane : anhydrous ethyl ether : formic acid. Plates were developed until the solution reached the top of the plate, then removed to air dry before being sprayed with 0.0005% primulin in acetic acid. Once plates had re-dried they were viewed under UV light and the placement of bands was marked with pencil.

Bands corresponding to TAG and OH-TAG were scraped off the plate into glass wool stoppered glass Pasteur pipettes. Samples were then eluted off the silica gel by running 3ml of 2:1 v/v chloroform:methanol over the gel. To recover the lipids the samples proceeded through the Blight and Dyer extraction by addition of 1.5ml of 0.9% NaCl, mixing, centrifugation at 4000rpm for 10 minutes, and recovery of the bottom CHCl_3 phase to dry down under nitrogen. Samples were stored at -20°C until use, but not more than 2 days.

Lipase Digest

Lipase digest of the OH-TAG band fractions was carried out using dry seed samples of all T₄ RcFAH-LfKCS lines, lesquerella oil, castor oil, and dry seed of wild type MT5 extracted as described above or dissolved in chloroform in the case of the oil samples. All samples were completely dried down under nitrogen gas to remove chloroform, then resuspended in 300µl anhydrous ethyl ether. One ml of Tris buffer pH 7.8 with 500mM CaCl₂ was added to all samples. Quickly, 200µl of *Rhizomucor miehei* lipase (Sigma, St. Louis, MO, USA) was added to the tubes and they were placed on a vortex shaker (Vortex Genie 2, Scientific Industries, Bohemia, NY, USA) at speed of 3 for 20 minutes. After 20 minutes samples were promptly removed and the digestion stopped by the addition of 3ml of 2:1 v/v chloroform:methanol. To continue Blight and Dyer extraction, 1.5ml of 0.9% NaCl solution was added, samples were mixed, spun down at 4,000rpm for 10 minutes, and the bottom chloroform phase moved to a new tube using glass Pasteur pipettes. The samples were dried down under N₂ and loaded onto a TLC plate as described previously. Non-digested castor and lesquerella oil as well as a non-digested T₄ oil sample were run as standards to aid in band identification. Samples were run using the buffer 35:70:1 v/v/v of hexane : anhydrous ethyl ether : formic acid. Plates were run to the top, removed to air dry, then sprayed with 0.0005% primulin in acetone. Dried plates were viewed under UV light to see band locations.

Separation and Analysis of TAG and PC Fractions by TLC

Total oil TAG fractions of 8, 12, 16, 20 and 24 day old developing seed from T_{3,4} RcFAH-LfKCS lines 12, 15, and 23 were analyzed using lipid extraction, TLC and GC

analysis. Developing seeds previously frozen in liquid nitrogen and stored at -80°C were quickly ground in a glass tube containing 0.5ml of 2:1 (v/v) ratio of chloroform:methanol using a metal rod or Polytron PT 10-35 homogenizer (Kinematica AG) to break seeds. The rod and machine were cleaned with chloroform between uses to prevent contamination. The tube solution was made up to 4ml of 2:1 CHCl_3 :MeOH, capped, and allowed to sit for 10 minutes. Samples were briefly vortexed at medium speed then 1.5ml of 0.9% NaCl solution was added to the tubes. Samples were then mixed and spun down at 4,000rpm for 10 minutes. The bottom chloroform layer was transferred to a clean glass tube using a glass Pasteur pipette and dried down under a stream of nitrogen gas. Samples were re-suspended in 100 μl of chloroform, then 20 μl of sample was transferred to a new tube and combined with 100 μl of 10mg/ml 17:0 standard. The standard containing sample fraction was then dried down under nitrogen and re-suspended in 1ml of 2.5% H_2SO_4 in MeOH. Samples were cooked for 30 minutes at 90°C and cooled to room temp. After the addition of 250 μl of hexane and 1.5ml of 0.9% NaCl solution, samples were mixed and spun down for 3 minutes to separate phases. The top hexane phase containing the methylated fatty acids was removed via pipette to a GC tube insert and capped.

PC Fraction

Total lipids were extracted as described above from a range of staggered general seed development stages identified visually as 12-16 day old light green seed, 16-20 day old plump green green seed, non-plump 22-24 day old green seed, 28-30 day old seed just beginning to turn yellow, 33-35 day old yellow seed, and mature dry seed. Capillary tubes were used to load the sample in a thin line located 1.5cm up from the bottom of a

20x20 TLC silica gel 60 plates (EDT chemicals, Darmstadt, Germany). Two to three samples were loaded per plate along with a sample of soy phosphatidylcholine (PC) standard. Prior to loading, plates were pre-baked at 100°C for 30 minutes and cooled to room temp. The TLC tank was filled with running buffer solution of 70:30:1ml (v/v/v) hexane:anhydrous ethyl ether: formic acid, and the new filter paper backing allowed to develop at least halfway up the paper. The running buffer solution was quickly replaced with fresh solution prior to placing the plate in the tank. The TLC plate was allowed to develop to the top of the plate, then removed and air dried. The plate was then sprayed with 0.0005% primulin, air dried briefly, and viewed under UV light. The polar lipid band near the origin corresponding to PC was scraped off the plate using a razor blade and added to a glass tube. 1-2ml of 2.5% H₂SO₄ in methanol was added to the tube depending on the volume of silica gel and the tubes were capped and cooked at 90°C for 30 minutes. After cooling to room temperature, 1.5ml of 0.9% NaCl solution and 250µl of hexane was added. The samples were mixed and spun down at 3000rpm for 3 minutes to induce phase separation. The top hexane phase was transferred to a GC glass tube insert for analysis.

Both the PC fraction from the TLC run and the total oil fraction comprised of mostly TAG were run on the GC using the SHX20 wax column (Shimadzu, Kyoto, Japan) and the HFAM8m method described above. The samples were also run on the GC-MS for more accuracy of dilute samples using the Aglient HP-Innowax 1909 1N-133 column and HFA method.

Seed Section Analysis of Hydroxy Fatty Acids Accumulation

T₄ RcFAH-LfKCS seeds of lines 12, 14, 15, 17, 19, 23, and control T₅ RcFAH line 7-2 were allowed to imbibe sterile water for 30 minutes to soften the seedcoat and swell the embryo. Seeds were then dissected under a Leica MZ6 microscope (Leica, Buffalo Grove, IL, USA) to separate the cotyledons, hypocotyl, and seed coat components. Four samples of hypocotyl or cotyledon and 6 of seed coat from the same line were combined in a tube to ensure a strong enough signal for the GC analysis. FAMES were prepared using the acid derivatization method, and analyzed on GC using the HFAm8m method.

Seed Section Germination Test

Seeds of selected higher hydroxy fatty acid T₁ RcFAH-LfKCS lines 26, 24, 27, 28, 9 and 7 were sterilized in a 70% v/v ethanol solution for 2 minutes. The ethanol was then poured off and replaced by a 22% v/v bleach solution with a drop of tween 20 added and sterilized with shaking for 20 minutes. The seeds were then rinsed with 5 washes of sterile double deionized water. In the hood a section of the seed cotyledon was dissected off using a sterilized scalpel and tweezers and a dissecting microscope. The seed piece with the embryo was placed on a sterile MS media plate with 2% sucrose. The cotyledon piece was added into a glass tube for fatty acid methyl ester preparation using the acid derivatization method and GC analysis using the HFAm8m method. The GC results for HFA content were then compared to germination ability of seeds.

Germination Test

For the germination test, 100 T₅ seeds of each of the six RcFAH-LfKCS lines were used and compared to the control T₅ RcFAH 7-2 line. Seeds were placed on filter paper dampened with sterile water in a covered petri plate 2.5mm tall x 14mm wide. Water was replenished as needed when filter paper dried and seeds were left to germinate for 2 weeks at room temperature. Ratios of germinated to ungerminated seeds were counted, and notes recorded for oddly germinated seeds.

Determination of Transgene Copy Number in T₁ Lines

To estimate transgene copy number in the T₁ RcFAH-LfKCS lines, a scattering of roughly 50-150 seeds of seeds were sown in 6" pots filled with 50:50 mix of soil to sunshine potting mix and grown in the 177D and West Wing greenhouse at MSU. Growth conditions were 30% relative humidity, a 14 hour photoperiod, and day/night temperatures of 70°/60°F +/-7° for 177D and 72°/65°F +/-7° for the west wing. The number of seedlings that emerged in each pot was counted and recorded for each line. Glufosinate herbicide (2% Glyphosate, isopropylamine salt, Powerforce grass and weed killer, Bayer chemicals) was sprayed on the seedlings when the first true leaves started to appear. The seedlings were re-sprayed 2 days later. The ratio of survival to germination was compared to the expected ratios corresponding to insertion number with one insertion expected to yield a 75% ratio of survival to seedling death, two or more insertions would be expected to yield a 94% or greater survival.

CHAPTER 5

RESULTS

Section 1: Transgenic Line Creation, Confirmation & Analysis*A Physaria fendleri* Condensing Enzyme Causes an Increase in Very-Long-Chain C20-HFA Content

The *Physaria (Lesquerella) fendleri* *LfKCS3* gene that was previously isolated encodes a condensing enzyme which specifically elongates the hydroxy fatty acid 18:1OH into the very-long-chain hydroxy fatty acid, 20:1OH. To determine the effect of 18:1OH elongation on the accumulation of hydroxy fatty acids in camelina seed oil, *LfKCS* was inserted into variety MT5 camelina along with a castor hydroxylase gene, *RcFAH* (Figure 2). For comparison, transgenic plants containing the *RcFAH* gene only were also created using the construct pGate-Phas-RcFAH (Lu et al., 2006). The *RcFAH* gene was expressed under seed specific phaseolin promoter; the *LfKCS3* was under its native promoter, which is also seed-specific (Moon et al., 2001). A total of 25 *RcFAH*-*LfKCS* and 10 *RcFAH* transgenic lines were obtained via the vacuum floral infiltration method and glufosinate herbicide (basta) selection.

The seed fatty acid profiles were determined by gas chromatography (GC) analysis. Both groups of transgenic seeds showed distinct peaks on the chromatographs compared to wild type seeds, which were identified as hydroxy fatty acids, ricinoleic acid (18:1OH), densipolic acid (18:2OH), lesquerolic acid (20:1OH), and auricolic acid (20:2OH). The transgenic *RcFAH* and *RcFAH*-*LfKCS* lines also displayed an altered

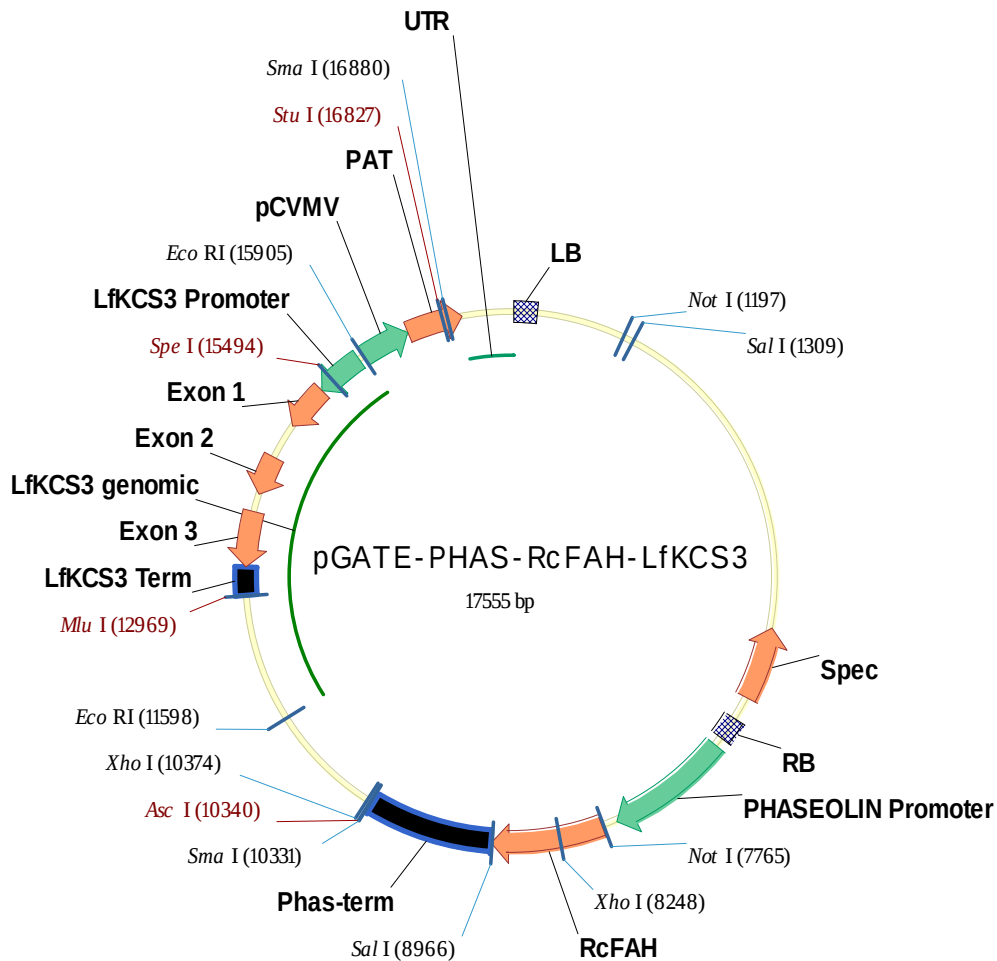


Figure 2. Map of plasmid pGate-Phaseolin- RcFAH-LfKCS3 used to transform MT5 camelina plants for creation of transgenic lines.

profile of usual fatty acids, e.g., 18:1, 18:2, and 18:3 from the wild type MT5 (Figure 3 a, b, c). In the wild type, the 18:3 was the highest (33%) followed by 18:2 (17%) then 18:1 (15%) (Kang et al., 2011), but in the transgenics the 18:1 was the highest with lower 18:2 and 18:3 amounts (Table 3).

The transgenicRcFAH-LfKCS lines contained a higher percentage of 20-carbon (C20) hydroxy fatty acids 20:1OH and 20:2OH than the control RcFAH only lines, with the majority of lines containing greater than twice the C20 HFA levels seen in RcFAH

only plants (Table 3; Figure 3). The highest average C20-OH level from the RcFAH lines was measured at 1.4%, whereas the highest averaged T1 line was over five times greater at 7.5% very-long-chain hydroxy FA (Table 3). To determine whether the difference in C20 HFA between the RcFAH and the RcFAH-LfKCS lines was attributed to the presence of the *LfKCS3* gene, I did the RT-PCR analysis of the RcFAH-LfKCS transgenic lines using 16 day old developing seeds. The primers were specific for the *Physaria LfKCS3* gene and did not recognize the native camelina *FAE1* gene. This distinction was important to confirm positive expression of *LfKCS3* due to the fact that many oil synthesis enzymes can show sequence homology to one another and because camelina and lesquerella are in the same family, Brassicaceae. Positive expression of the *LfKCS3* gene was confirmed in RcFAH-LfKCS transgenics but not in the RcFAH only control lines (Figure 4). These results showed that the *LfKCS3* gene was expressed in transgenic camelina at the beginning stages of oil filling and resulted in a much higher accumulation of C20 hydroxy fatty acids in the oil than is seen in lines lacking a *LfKCS3* gene. The native camelina elongase *FAE1* gene does not have significant effects as very low amounts of C20 HFA were measured in RcFAH only lines (Table 3, Figure 3 a, b).

Expression of LfKCS3 in Addition to RcFAH Causes an Increase in Total HFA

As seen previously (Lu and Kang, 2008), expression of RcFAH alone in camelina resulted in accumulation of ~15% HFA, which consisted almost entirely of 18:1OH and 18:2OH (Table 3). Addition of the *LfKCS3* gene resulted in total HFA measures of up to ~20-22% in the highest HFA accumulating T1 and T2 RcFAH-LfKCS lines, yielding measureable amounts of ricinoleic acid (18:1OH), densipolic acid (18:2OH), lesquerolic

acid (20:1OH), and auricollic acid (20:2OH) (Tables 3). These four hydroxy fatty acids are obvious in the GC profiles of the RcFAH-LfKCS lines (Figure 3 b). While total HFA levels differed, the amount of 18:1OH was relatively similar between the highest RcFAH-LfKCS lines and the control RcFAH-only lines (Figure 5). The transgenic lines did however differ greatly in the amount of C20 HFA they accumulated and the distribution of hydroxy fatty acids contributing to the total HFA measurement (Figure 6). RcFAH-LfKCS lines that had a similar very-long-chain C20 HFA content to the RcFAH control lines displayed lower total HFA levels than their control counterparts, whereas lines that contained a similar total HFA content to the RcFAH control lines accumulated a higher amount of that total HFA as very-long-chain hydroxy fatty acids 20:1OH and 20:2OH (Figure 6). RcFAH-LfKCS lines with a similar amount of C18 HFA to the RcFAH lines contained higher total HFA levels due to the addition of the extra C20 HFA to the hydroxy fatty acid profile.

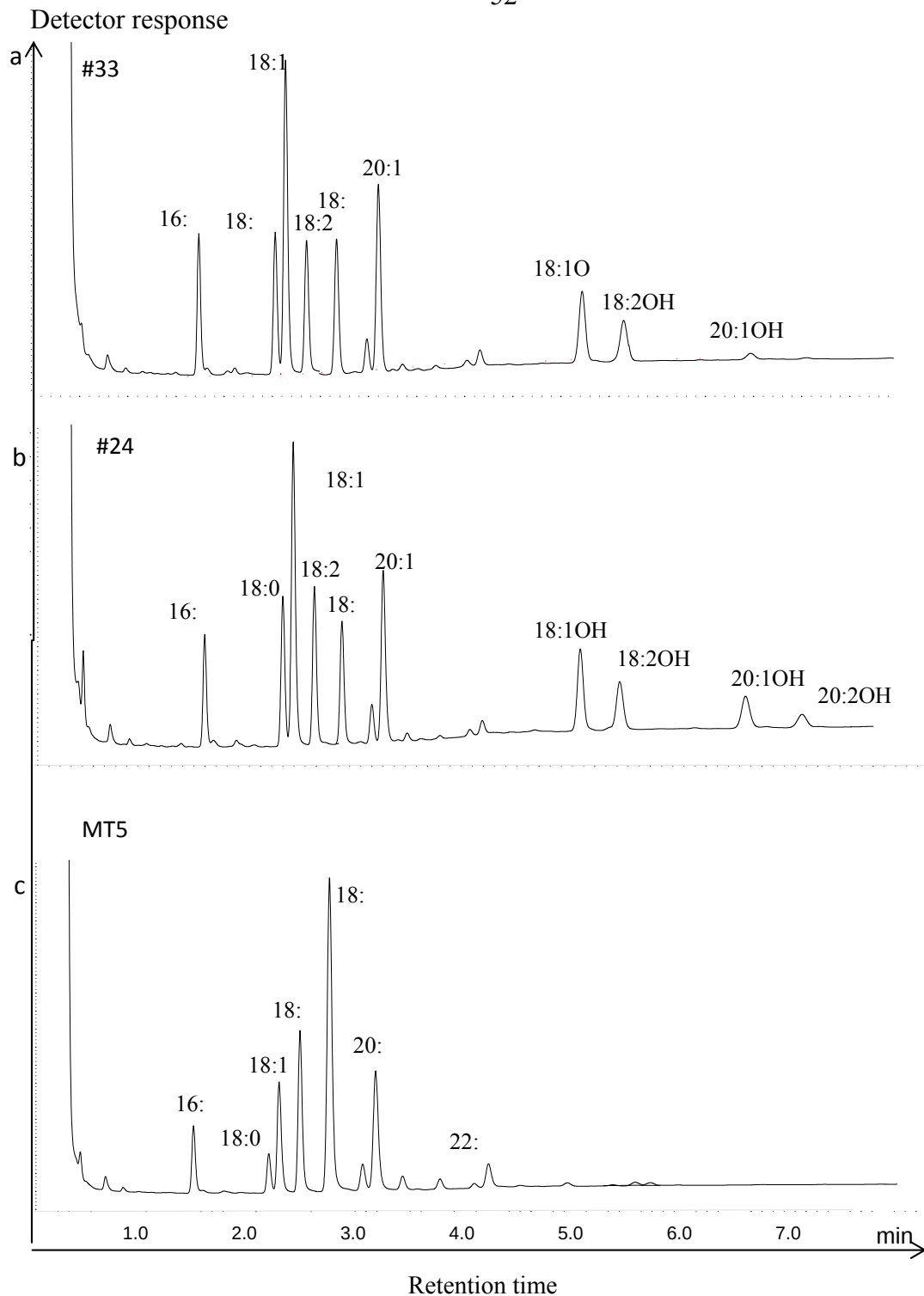


Figure 3. Chromatographs of total fatty acid profile from a single seed of the highest HFA accumulating lines. **a.** Transgenic control T1 RcFAH line #33. **b.** T1 RcFAH-LfKCS line #24. **c.** Wild type MT5. Note the HFA presence and the altered ratios of usual FA 18:1, 18:2 and 18:3 in the transgenic profiles as compared to the wild type MT5.

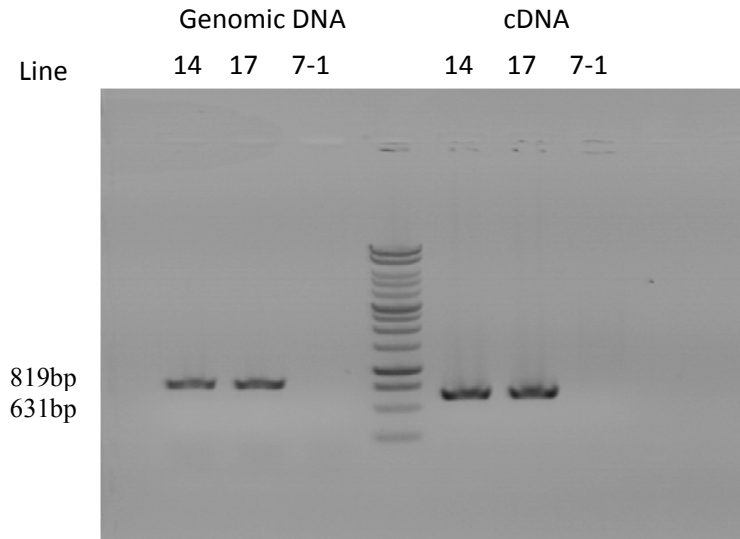


Figure 4. RT-PCR of the *LfKCS3* gene to test expression in 16 day old seed. The gene specific primer PCR product of the three left lanes used genomic DNA template and three on the right side of ladder used cDNA template. Bands are present for both RcFAH-LfKCS line 14 and 17 samples, but absent for control RcFAH 7-1 line in each instance. Difference in PCR band size corresponded to positive removal of the intron from the cDNA product compared to the genomic DNA product. Expression was positive showing the expected 819bp band for genomic DNA and 631bp band for cDNA.

Table 3. Average and Standard Deviation of Highest HFA Accumulating T1 RcFAH-LfKCS vs T1 RcFAH lines. Previously made T5 RcFAH is used as a control.

Highest HFA Accumulating T1 RcFAH-LfKCS and T1 RcFAH Line Averages													
Fatty Acid Composition (% of total)													
Transgenic line	Ave & St. Dev	16:0	18:0	18:1	18:2	18:3	20:0	20:1	18:1 OH	18:2 OH	20:1 OH	20:2 OH	Total OH
T1 RcFAH-LfKCS #24	Average	8.4	12.5	20.2	12.1	7.3	3.5	12.8	9.4	4.6	5.8	1.7	21.5
	St. Deviation	1.7	2.2	3.8	2.6	1.4	0.7	0.7	0.4	1.6	0.6	0.7	1.7
T1 RcFAH-LfKCS #28	Average	7.5	10.5	21.7	12.8	7.6	3.5	13.6	9.0	4.8	6.0	1.6	21.3
	St. Deviation	1.3	2.3	2.3	1.8	2.9	0.9	0.5	1.3	2.4	1.2	0.7	0.6
T1 RcFAH-LfKCS #27	Average	8.2	8.7	21.4	12.5	9.8	2.6	15.5	7.7	5.3	4.5	1.7	19.1
	St. Deviation	0.6	0.7	2.0	0.7	0.5	0.1	1.1	1.0	0.3	0.6	0.3	1.2
T1 RcFAH-LfKCS #26	Average	6.5	7.0	25.6	11.2	10.9	2.3	16.2	5.7	7.0	3.4	2.9	18.9
	St. Deviation	0.2	1.1	1.6	0.5	0.4	0.4	0.5	0.6	1.5	0.7	0.9	3.7
T1 RcFAH-LfKCS #9	Average	7.0	7.8	23.8	11.8	10.7	2.4	16.0	6.8	6.4	3.8	1.8	18.8
	St. Deviation	1.4	2.4	3.4	2.0	2.1	0.4	2.2	2.6	1.9	1.9	0.6	3.5
T1 RcFAH #22	Average	8.7	8.4	23.0	12.7	11.2	2.8	15.8	7.5	5.5	1.4	0.0	14.3
	St. Dev	0.2	0.2	0.9	1.7	0.3	0.1	0.7	0.6	0.9	0.1	0.0	0.5
T1 RcFAH #33	Average	9.7	9.5	22.3	11.1	11.7	2.7	15.5	8.3	5.6	1.1	0.0	14.9
	St. Dev	0.9	0.7	1.4	0.7	1.0	0.1	0.4	0.4	0.1	0.2	0.0	0.5
T1 RcFAH #34	Average	8.7	8.2	22.5	11.6	11.5	3.0	17.3	7.6	5.5	1.1	0.0	14.3
	St. Dev	0.4	0.7	1.6	0.5	0.1	0.2	0.5	0.1	0.2	0.0	0.0	0.2
T1 RcFAH #15	Average	9.7	8.5	21.9	12.6	9.9	2.9	17.7	8.0	4.7	1.2	0.0	13.9
	St. Dev	1.6	1.7	5.1	2.1	1.6	0.1	1.8	0.8	1.0	0.1	0.0	0.5
T1 RcFAH #39	Average	9.4	8.0	19.8	14.5	12.4	2.9	17.0	7.6	4.6	1.3	0.0	13.4
	St. Dev	2.0	1.6	3.3	2.1	3.5	0.2	0.5	2.0	0.9	0.4	0.0	1.6
T5 RcFAH #7-1	Average	10.9	10.9	22.0	12.2	10.5	2.5	14.0	8.9	4.9	1.1	0.0	14.8
	St. Dev	0.6	0.2	1.0	1.0	0.4	0.2	0.5	0.3	0.2	0.1	0.0	0.3

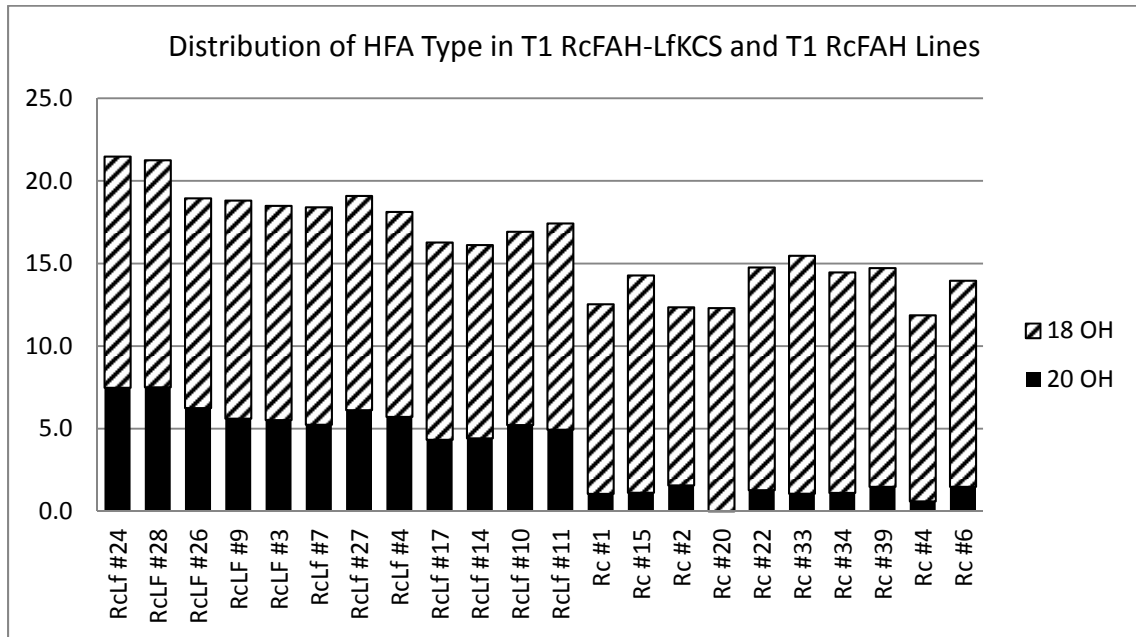


Figure 5. Graph highlighting the ratio of C20 to C18 hydroxy fatty acids in the overall HFA content for averaged highest HFA accumulator T1 RcFAH-LfKCS lines compared to highest single measure from each T1 RcFAH control line.

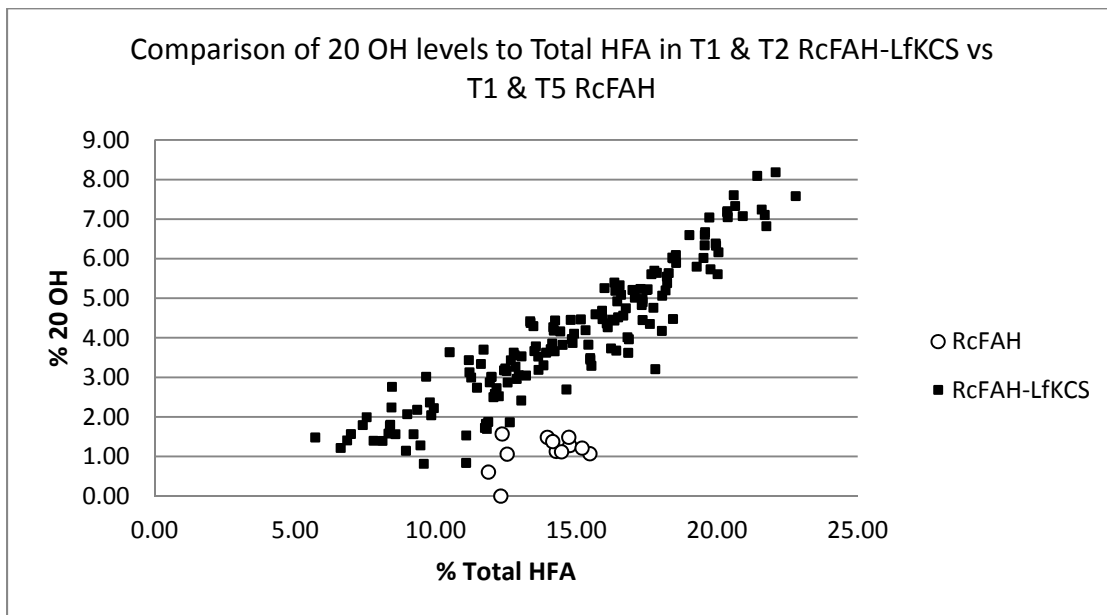


Figure 6. Graph comparing C20 HFA content to total HFA content in all T1 and T2 RcFAH-LfKCS lines compared to top single measure from each control T1 or T5 RcFAH line.

To examine the relationship of oleic acid (18:1) and its derivatives, desaturated and hydroxylated fatty acids, the oleate derivative proportion (ODP) was calculated. The ODP of the RcFAH-LfKCS lines was similar to that of the RcFAH lines, indicating there was not an increase in the amount of oleate used to make HFA, but rather a more efficient incorporation without degradation of current HFA product (Figure 7).

Taken together, it is clear that the addition of the C20 hydroxy fatty acids helps achieve higher total content of hydroxy fatty acids in the oil profile of transgenic T1 and T2 RcFAH-LfKCS lines.

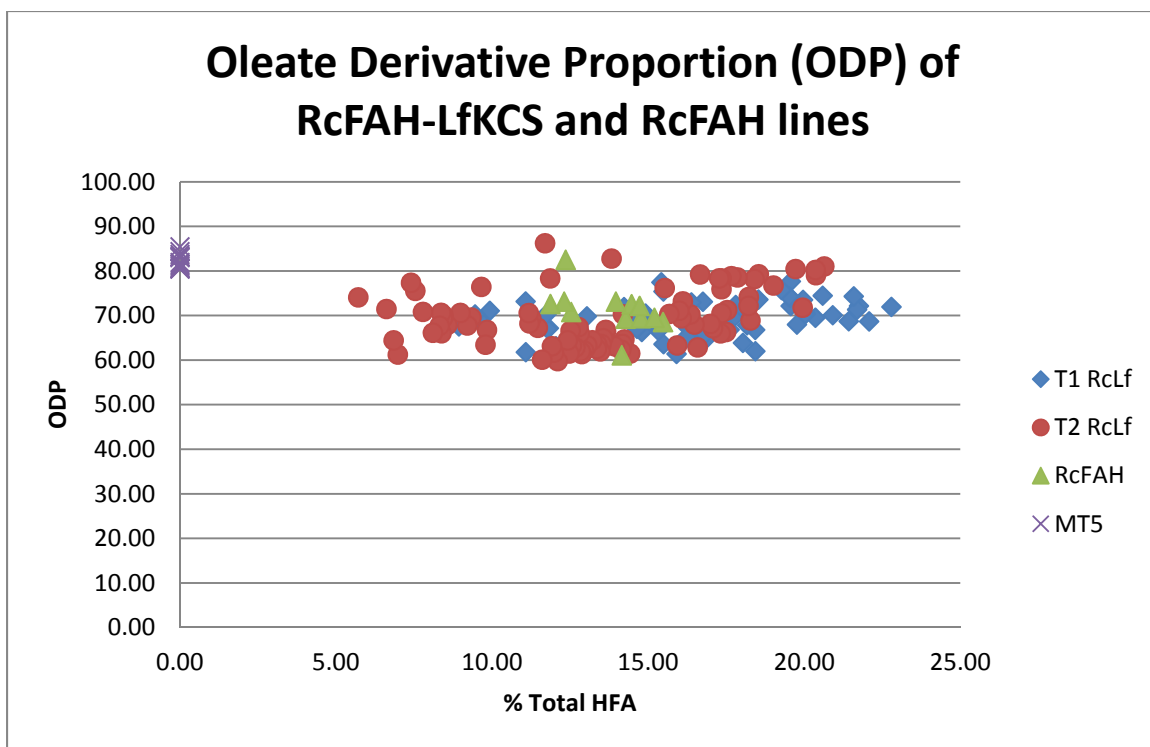


Figure 7. Oleate Derivative Proportion (ODP) of RcFAH-LfKCS vs. RcFAH lines. ODP = Sum of the derivatives of oleate (18:2 + 18:3 + 20:1 + 18:1OH + 18:2OH + 20:1OH + 20:2OH) divided by the sum of the derivatives of oleate plus oleate (18:1 + 18:2 + 18:3 + 20:1 + 18:1OH + 18:2OH + 20:1OH + 20:2OH).

Section 2: Stereochemical Analyses

TLC of Total Oil Separates 1-OH TAG and 2-OH TAG Fractions

Thin layer chromatography (TLC) was used to separate total oil of six T4 RcFAH-LfKCS lines, confirming the presence of 1-hydroxy triacylglycerol (1-OH TAG) and a low amount of 2-OH TAG in the oil (Figure 8). The 1-OH TAG band was recovered from the plate and lipase digested to further elucidate the position of the HFA in TAG. Digestion with an sn-1 and sn-3 position specific lipase revealed free HFA and the presence of sn-2 OH-MAG in all RcFAH-LfKCS samples (Figure 9).

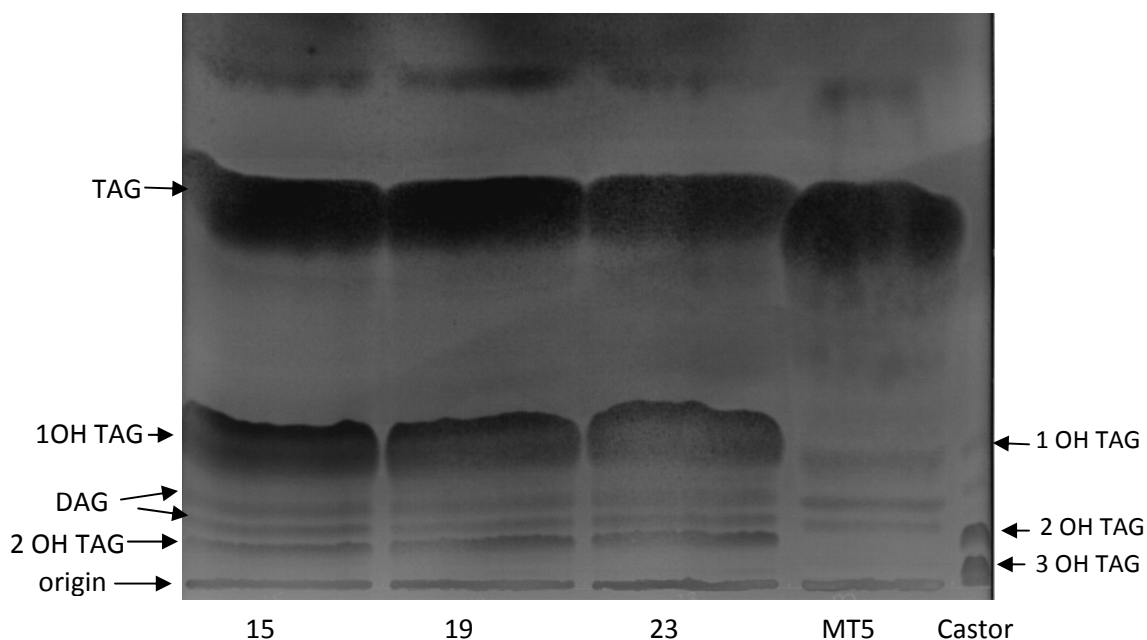


Figure 8. Separation of total oil. Samples from left to right: T4 RcFAH-LfKCS lines 15, 19, 23, MT5 control, and Castor oil control.

Composition of HFA Differs Between Oil Fractions

Recovery and analysis of the 1- and 2-OH TAG, and non-HFA containing TAG bands from the TLC plate revealed a differing composition of hydroxy fatty acids between oil fractions. A higher level of C20 HFA was seen in the 2-OH TAG as compared to the 1-OH TAG for both lines (Figure 10, Table 4). The T5 RcFAH-LfKCS

lines also showed over twice the amount of incorporation of C20 HFA in 2-OH TAG as compared to the T5 RcFAH lines (Figure 11). Due to similar background, this difference in 2-OH TAG makeup is assumed to be due to the increased availability of C20 HFA present in the RcFAH-LfKCS lines because of the action of *LfKCS3* and not due to a difference in ability of the lines to incorporate C20 HFA. The band from the RcFAH-LfKCS digestion plate corresponding to 1-OH MAG was also analyzed via GC-MS and found to contain mainly C18 HFA with little to no C20 HFA (Figure 13). This result is consistent with similar findings in *Arabidopsis* and the knowledge that the RcFAH hydroxylates sn-2 position fatty acids on the PC (Bafor et al., 1991; Smith et al., 2003). This finding indicates a location preference for incorporation of C20 HFA into the sn-1 and sn-3 positions. The percent of total HFA present in the 1- and 2-OH TAG fractions was closer to the 33% and 66% expected in the RcFAH-LfKCS lines than the RcFAH-only lines (Table 4, Figure 12). A lower expected HFA content for 1- and 2-OH TAG in RcFAH lines was noted previously (Smith et al., 2003).

Table 4. GC analysis of the fatty acid composition of three different TLC oil fractions, TAG, 1-OH TAG, and 2-OH TAG. Samples runs were done with combination of seed from T4 LfKCS lines.

TLC Fraction Oil Composition of T5 RcFAH (7-1) and T5 RcFAH-LfKCS (LfKCS) Lines															
TLC Sample	Ave & St.Dev	Fatty Acid Composition (%)										18:1	18:2	20:1	20:2
		16:0	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1	OH	OH	OH	OH	Total HFA
7-1 TAG	Average	9.2	8.9	32.4	12.9	15.1	2.9	16.0	0.4	1.6	0.1	0.1	0.0	0.0	0.2
	St.														
7-1 1-OH TAG	Deviation	0.5	0.5	1.9	1.4	3.3	0.4	1.4	0.1	0.2	0.1	0.2	0.0	0.0	0.3
	Average	7.8	9.6	20.2	4.3	4.9	3.1	17.2	0.4	1.6	15.6	12.3	1.8	1.0	30.6
7-1 2-OH TAG	St.														
	Deviation	0.1	0.0	0.1	0.2	0.2	0.0	0.0	0.0	0.0	0.4	0.1	0.0	0.1	0.2
LfKCS TAG	Average	4.8	5.3	15.6	3.8	6.4	1.5	8.8	0.4	1.0	27.1	14.6	7.4	3.4	52.5
	St.														
LfKCS 1-OH TAG	Deviation	0.4	0.6	1.9	1.0	1.4	0.3	1.0	0.5	0.3	6.3	1.8	0.5	0.3	7.4
	Average	9.5	7.7	27.5	15.9	16.8	2.7	16.9	0.4	1.8	0.1	0.2	0.0	0.0	0.3
LfKCS 2-OH TAG	St.														
	Deviation	0.5	0.4	1.7	1.3	3.2	0.3	1.4	0.1	0.2	0.1	0.3	0.0	0.0	0.4
LfKCS 2-OH TAG	Average	6.8	7.9	18.1	5.6	5.7	2.9	18.6	0.4	1.8	13.6	11.7	4.3	2.3	31.8
	St.														
LfKCS 2-OH TAG	Deviation	0.4	0.0	0.1	0.3	0.7	0.1	0.3	0.0	0.0	0.7	0.2	0.1	0.0	0.8
	Average	4.0	3.7	13.0	4.5	6.3	0.5	7.1	0.2	0.4	19.9	15.1	16.4	8.8	60.2
	St.														
	Deviation	0.1	1.2	2.3	0.5	0.0	0.8	2.6	0.2	0.6	3.9	2.3	0.8	1.3	8.3

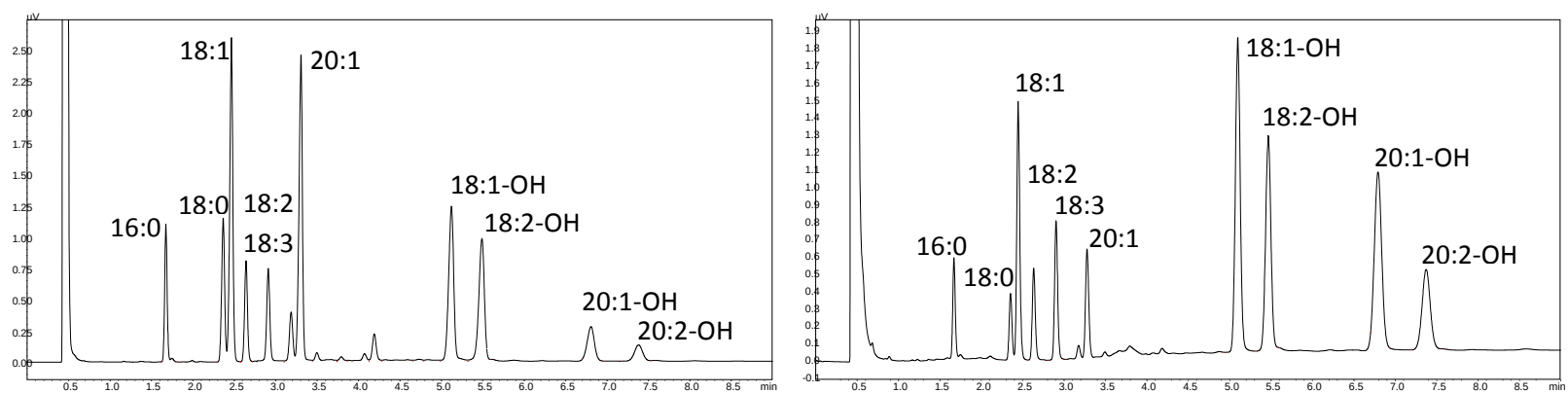


Figure 10. Chromatograph comparing the T5 RcFAH-LfKCS 1-OH TAG fraction (left) and 2-OH TAG fraction (right).

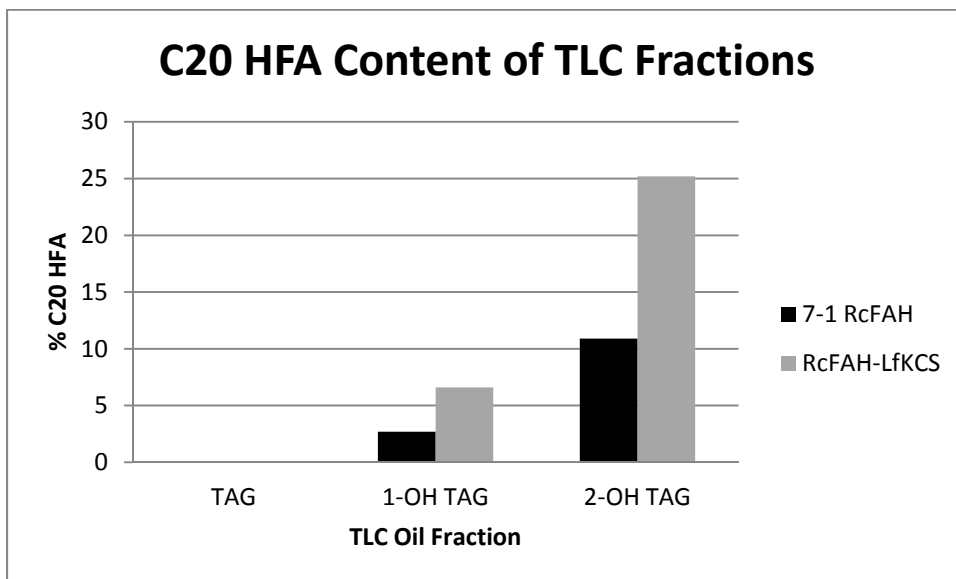


Figure 11. Content of C20 hydroxy fatty acids in TLC oil fractions of T5 RcFAH and T5 RcFAH-LfKCS.

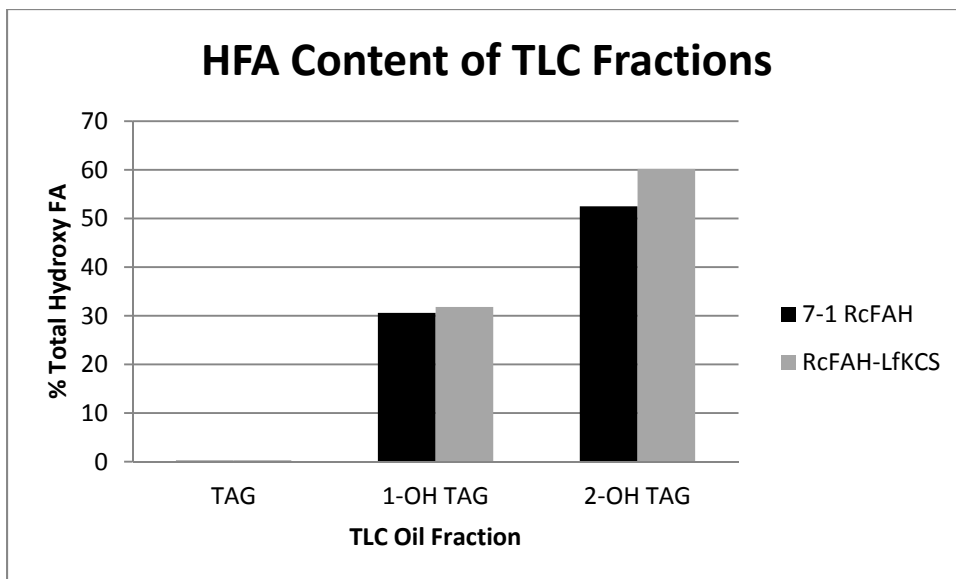


Figure 12. Comparison of total HFA content in TLC oil fractions from T5 RcFAH and T5 RcFAH-LfKCS.

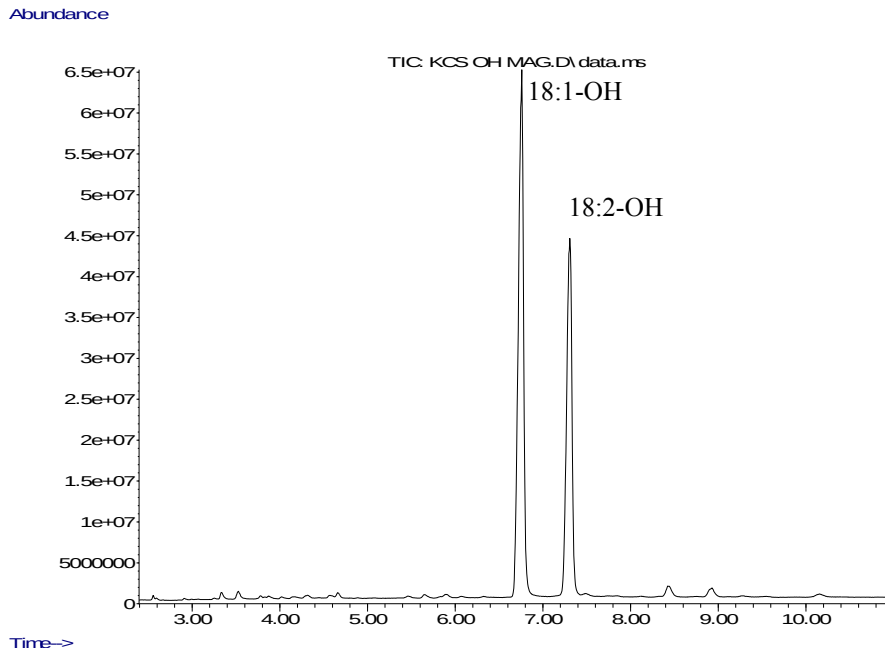


Figure 13. GC-MS chromatograph of OH-MAG from RcFAH-LfKCS lines.

Section 3: HFA Accumulation in TAG and PC During Seed Development

Changes in the hydroxy fatty acid content of both total oil and the polar PC fraction were measured via GC-MS analysis of transgenic seed at six different developmental stages previously defined. An increase in HFA content was observed in the PC fraction up until the stage 5 yellow seed stage, after which it was decreased in the mature seed. The RcFAH line 7-2 seeds displayed more HFA in PC at every level from stage 2 on, and almost twice the level of HFA present in PC compared to the RcFAH-LfKCS seed line at stage 5 (Figure 14). Conversely, the RcFAH-LfKCS lines displayed a higher level of HFA in the total oil fraction from stage 2 on when compared to the RcFAH 7-1 line (Figure 15). Similar to the PC HFA accumulation, the total oil samples also showed a peak HFA content at the stage 5 range followed by a slight decrease in

HFA content in the mature seed oil. A measure of the HFA content in total oil of specific early time points indicated that HFA doesn't start to accumulate at measureable levels until between the 12 and 16 day time point, after which it increases rapidly (Figure 16).

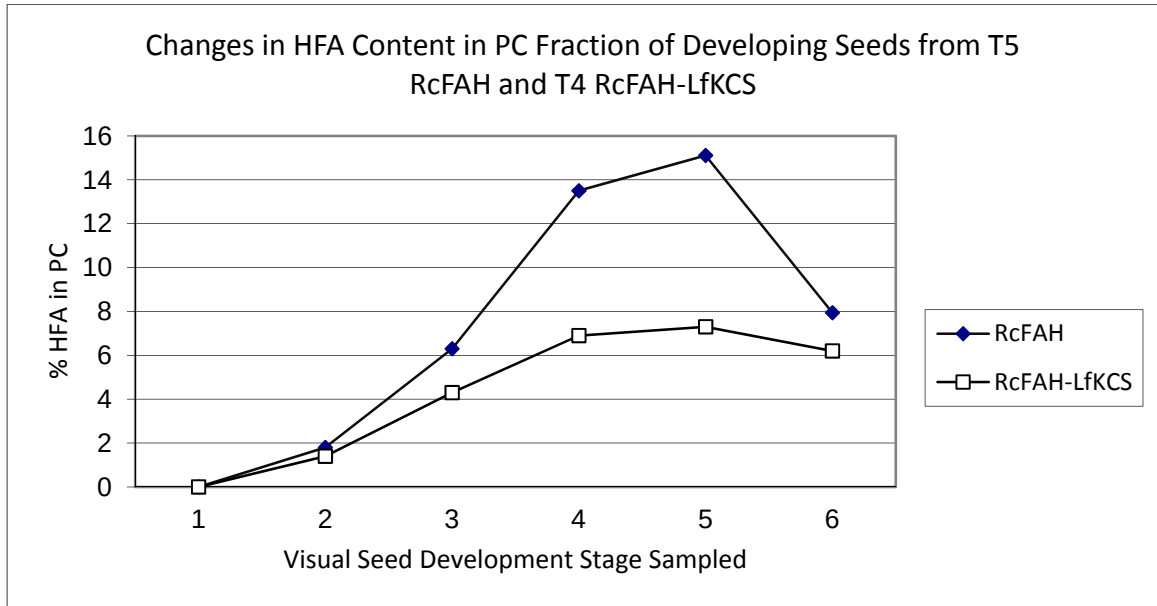


Figure 14. Changes in HFA content in PC of developing seeds from lines T5 RcFAH 7-1 and T4 RcFAH-LfKCS. Six seed stages sampled: approximately 12-16 day old light green seeds for stage 1, ~16-20 day old plump green seed for stage 2, ~ 20-24 day old green seeds for stage 3, beginning to yellow ~30 day seed for stage 4, yellow ~35 day old seed for stage 5, and mature dry seed for stage 6.

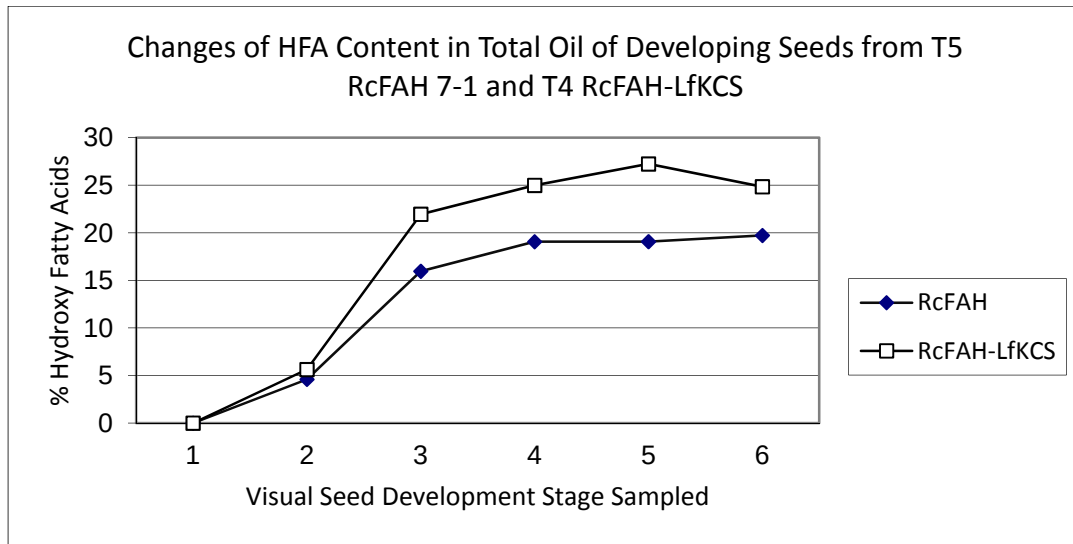


Figure 15. Changes in the measured hydroxy fatty acid content of total oil from various developing seed stages in lines T5 RcFAH 7-1 and T4 RcFAH-LfKCS . Same seed stages as Figure 12: light green, green 1, green 2, start to yellow, yellow, mature dry.

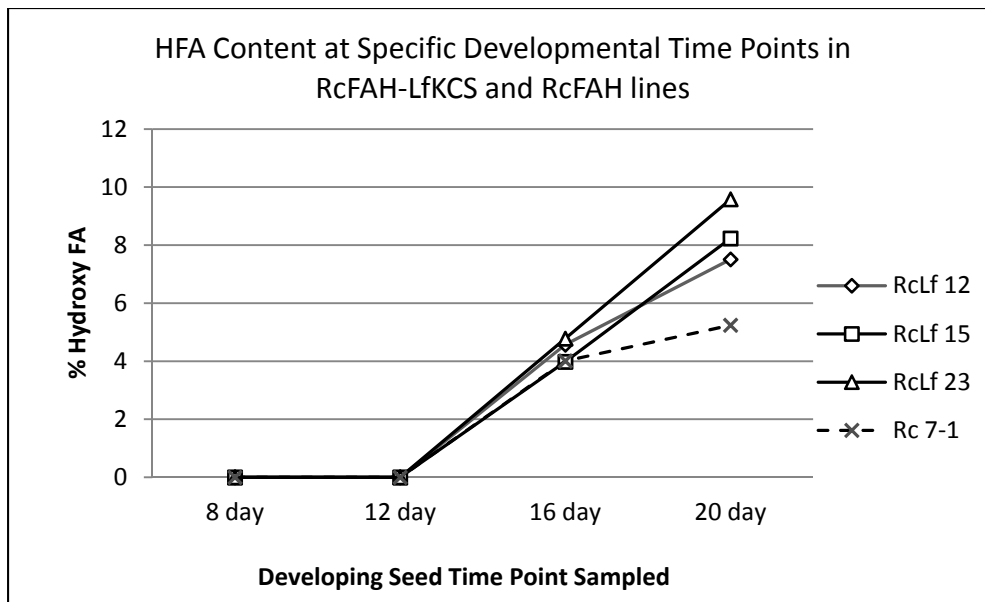


Figure 16. Measurement of HFA content at specific developmental time points in RcFAH-LfKCS (RcLf) and RcFAH (Rc) seed lines. A more specific measurement representation of stages 1-3 in Figure 13.

Section 4: Analysis of Seed Components for Location of Hydroxy Oil Accumulation

Hydroxy Fatty Acids Accumulate in Both Cotyledons and Seed Coat

Dissected mature seed sections of hypocotyl and cotyledon of six T4 RcFAH-LfKCS line seeds and two T4 RcFAH line seeds were run separately on the GC to measure the oil profile. Hydroxy fatty acids were observed in both the hypocotyl and cotyledon sections of seeds (Tables 5, 6). More hydroxy fatty acids were measured in the cotyledons than in the hypocotyls for both the control RcFAH-only lines and the RcFAH-LfKCS lines, with the difference of the averaged line hydroxy measures being 4-5% higher in the cotyledons (Table 5, 6). Long chain HFA were also measured in both the hypocotyl and cotyledon fractions, but similar to the pattern for total HFA levels, they appeared at higher amounts in the cotyledon sections by an average of 1.37% for the combined average of the six lines (Table 5).

Table 5. Average GC analysis of hydroxy fatty acids present in cotyledon and hypocotyl seed fractions as measured by GC analysis. T4 RcFAH-LfKCS is an average of 6 different line measures, RcFAH is an average of 2 lines.

Average % HFA Present In T4 Seed Section				
	C20 HFA		Total HFA	
Line	cotyledon	hypocotyl	cotyledon	hypocotyl
RcFAH	0.9	0	15.9	11.1
RcFAH-LfKCS	3.2	1.8	15.5	10.4

Table 6. Fatty acid composition and location of hydroxy fatty acids in dissected T4 RcFAH-LfKCS, T4 RcFAH 7-2 and 23, and MT5 line seed. H = hypocotyl, C = cotyledon. Arranged by decreasing FA content for each plant line group.

Total Oil Profile of Dissected Seed Sections from RcFAH-LfKCS, RcFAH and MT5 Lines														
Fatty Acid Composition (%)														
Seed piece	Line	16:0	18:0	18:1	18:2	18:3	20:0	20:1	18:1OH	18:2OH	20:1OH	20:2OH	total HFA	Total C20 HFA
C	RcLf 19	7.5	7.5	21.9	10.2	9.1	2.4	18.4	9.0	6.8	2.7	1.0	19.4	3.7
C	RcLf 23	5.7	7.4	28.4	8.2	8.7	2.8	19.5	6.1	6.5	2.4	1.2	16.2	3.6
C	RcLf 12	7.8	8.5	16.8	12.0	10.9	2.8	16.0	8.4	4.7	2.2	0.5	16.2	2.7
C	RcLf 17	6.2	7.4	26.7	8.3	8.5	2.7	17.6	5.8	5.5	2.2	1.4	15.1	3.7
C	RcLf 15	7.4	7.0	18.8	13.0	13.5	2.9	19.5	6.3	4.2	2.0	0.7	13.5	2.6
C	RcLf 14	5.6	6.7	30.1	7.3	10.8	2.6	19.0	4.3	5.5	1.3	1.3	12.6	2.7
C	Rc 7-2	9.0	11.4	22.6	9.3	9.0	2.5	15.9	10.2	5.3	0.9	0.0	17.3	0.9
C	Rc 23	9.8	11.5	19.1	9.9	8.0	3.2	16.5	9.1	4.2	0.9	0.0	14.4	0.9
C	MT5	6.0	4.0	12.0	18.7	30.3	3.3	14.8	0.0	0.0	0.0	0.0	0.0	0.0
H	RcLf 23	12.5	8.4	23.6	17.1	13.1	1.8	11.8	6.3	3.1	2.5	0.0	11.8	2.5
H	RcLf 17	12.7	8.7	21.4	17.7	12.7	1.9	12.3	5.7	2.3	2.6	0.0	11.6	2.6
H	RcLf 19	14.0	8.9	19.6	19.0	14.0	2.0	11.3	6.4	2.9	2.0	0.0	11.3	2.0
H	RcLf 12	14.7	8.6	14.6	22.0	17.1	2.0	10.1	6.1	2.9	2.0	0.0	11.0	2.0
H	RcLf 14	12.0	7.4	22.9	17.1	16.2	1.8	12.5	4.2	2.8	1.6	0.0	8.6	1.6
H	RcLf 15	15.3	7.9	13.3	24.2	18.9	2.0	10.4	5.6	2.4	0.0	0.0	8.0	0.0
H	Rc 7-2	19.0	10.2	15.3	19.4	13.8	1.7	9.2	8.9	2.5	0.0	0.0	11.4	0.0
H	Rc 23	18.0	10.1	14.8	18.1	14.1	2.0	11.	8.3	2.6	0.0	0.0	10.8	0.0
H	MT5	10.4	6.2	11.3	29.8	24.1	1.8	7.6	0.0	0.0	0.0	0.0	0.0	0.0

Section 5: Germination Test & Observations

Germination Ability of Top T5 Transgenic RcFAH-LfKCS Lines Unhindered

Transgenic T5 RcFAH-LfKCS lines were tested for germination ability by plating 100 seeds on damp filter paper. After one week all six of the homozygous transgenic lines displayed a germination ratio of 88% or higher, with two lines at 100% (Table 7). The control line, T5 RcFAH 7-2, displayed much delayed germination relative to the RcFAH-LfKCS lines or wild type MT5 lines and took longer than one week to achieve full germination results (Figure 17). RcFAH seed that had been stored for a period of time was very difficult to germinate with less than 5% of the seeds proving viable (data not shown). New seed had to be collected from a single successfully germinated plant for use in this germination test in an attempt to gain back viability. It remains to be seen whether long term storage will affect the RcFAH-LfKCS lines' viability in the same way. It should be noted that seeds were stored at room temperature and not at colder conditions which could have an effect on viability in the long term.

Table 7. Germination ratios after 1 week.
100 seeds of each line were tested.

T5 Line	Total % germination
RcFAH-LfKCS 12	93
RcFAH-LfKCS 14	88
RcFAH-LfKCS 15	100
RcFAH-LfKCS 17	91
RcFAH-LfKCS 19	100
RcFAH-LfKCS 23	98
RcFAH 7-1	70

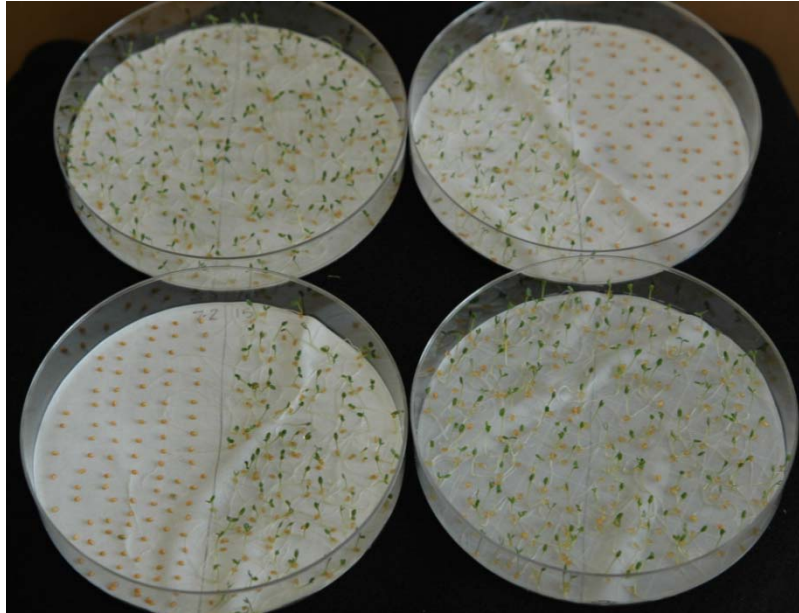


Figure 17. T5 Seed germination testing results after 1 week. Top left RcFAH-LfKCS line 23 and 19, top right RcFAH-LfKCS line 12 and RcFAH 7-2, bottom left line RcFAH 7-2 and RcFAH-LfKCS line 15, bottom right RcFAH-LfKCS line 14 and 17. RcFAH-LfKCS3 lines germinated on a timeline similar to what had been previously observed for wild type seeds, but RcFAH 7-2 lines were delayed and barely starting to show signs of germination at 1 week.

A peculiar white cotyledon phenotype was observed on some of the T1 RcFAH-LfKCS and T₅ RcFAH seeds (Figure 18). Tentative testing of cotyledon slices of seeds prior to plating on MS media to germinate the seedlings indicated that this phenotype might be linked to the seeds containing the highest HFA content (data not shown). Previous testing of seed embryo components indicated that 4-5% more HFA accumulated in the cotyledons than the hypocotyls, so it is possible that too high of free hydroxy levels could cause the white cotyledons (Table 5). While this was not pursued further in this project, the effects causing the white cotyledons and failure of some seedlings to survive warrants further investigation as it could potentially lead to an inadvertent selection for lower HFA lines. Seedling die-off could also have altered calculations for insertion

number by giving lower results than expected. An example of this can be seen from one of the T1 RcFAH-LfKCS lines tested, Line 28, which yielded only 39% survival on insertion copy number testing (Table 8), much lower than the expected 75% with one gene insertion and a dominant gene effect for herbicide resistance. Most RcFAH-LfKCS lines were in the 60-70's range for percent survival indicating a single copy gene insert. Three other T1 RcFAH-LfKCS lines, 14, 17, and 26 gave survival ratios of 89, 91, and 92% which is much closer to the expected 94% survival ratio of 2 segregating genes (Table 8). A lower seed planting to seedling germination ratio was also observed for T₀ RcFAH seedlings containing the DsRed marker, with ~100 red seeds planted and only 43 sprouting from soil media (results not shown). Although the cause for the low germination rate is unknown, theories include an unfavorable insert location resulting in seedling death during germination, or the possibility that too high of hydroxy levels may disrupt chloroplast membranes during germination yielding the white cotyledons and seedling death. If increased levels of HFA not stored in TAG are causing disruption during germination the process, decreasing the level of HFA left in PC could aid in germination. Reducing HFA remaining in PC could be accomplished by incorporation of genes encoding co-evolved enzymes that specifically deal with utilization and proper storage of hydroxy fatty acids.



Figure 18. White cotyledons and lack of a radicle on a germinating T1 transgenic seed. This phenotype proved lethal as seedlings were unable to recover and survive even when placed on MS + 2% sucrose media.

Table 8. Transgene insertion number testing of different transgenic event lines. Seedlings were counted then sprayed with herbicide to determine % survival.

T1 plant line	total # germinated	total # survived	ratio %
RcFAH-LfKCS #1	82	57	69.5
RcFAH-LfKCS #3	59	45	76
RcFAH-LfKCS #4	72	49	69
RcFAH-LfKCS #5	81	58	71.6
RcFAH-LfKCS #7	39	20	51.3
RcFAH-LfKCS #8	118	86	72.9
RcFAH-LfKCS #9	86	46	53
RcFAH-LfKCS #10	78	52	67
RcFAH-LfKCS #11	51	33	65
RcFAH-LfKCS #12	111	80	72
RcFAH-LfKCS #13	94	74	79
RcFAH-LfKCS #14	65	58	89
RcFAH-LfKCS #15	76	51	67
RcFAH-LfKCS #17	80	73	91
RcFAH-LfKCS #18	68	46	68
RcFAH-LfKCS #19	81	59	73
RcFAH-LfKCS #22	104	85	82
RcFAH-LfKCS #23	115	79	69
RcFAH-LfKCS #24	28	23	82
RcFAH-LfKCS #25	79	57	72
RcFAH-LfKCS#26	53	49	82
RcFAH-LfKCS #27	85	51	60
RcFAH-LfKCS #28	61	24	39
RcFAH-LfKCS #29	144	100	69

CHAPTER 6

DISCUSSION

Synthesis of modified fatty acids (mFA) such as those containing hydroxy, epoxy, or conjugated groups, has been the focus of many researchers in the past decades as these specialized structures render unique properties for industrial uses of the plant oil. However, despite detailed knowledge of the genes responsible for the synthesis of these modified fatty acids in their native accumulator plants, researchers have been unsuccessful in their attempts to duplicate similar levels of mFA in *Arabidopsis* or oilseed crops solely through insertion of the genes responsible for their creation. Multiple efforts to co-express additional genes such as *PDCT*, *DGAT2*, or *PDAT1A*, to aid in increasing mFA accumulation were met with some success, albeit limited in most cases when compared to the native accumulator mFA levels (van Erp et al., 2011; Hu et al., 2012; Bates et al., 2011).

Recent studies have highlighted the importance of phosphatidylcholine (PC) and the dynamic role it plays in the flux between the de novo and modified DAG pools used for TAG synthesis (Bates et al., 2012; Bates & Browse, 2011). Since many fatty acid modifying enzymes, such as the castor hydroxylase, are derived from desaturases that work on fatty acids while they are on the PC, efficient removal of the mFA from PC for TAG synthesis has been shown to be a major limiting factor for the accumulation of mFA in high amounts in TAG (Van Erp., 2011; Bates and Browse, 2011; Hu et al., 2012; Bates et al., 2011). This inefficient removal of mFA from PC leads to a bottleneck effect that

reduces mFA accumulation in TAG and increases HFA turnover through β -oxidation and feedback inhibition (Bates & Browse, 2011). A clearer understanding of how modified fatty acids are metabolized and how different genes affect their flux through PC for incorporation into TAG is needed in order to increase the level of mFA production in transgenic seed oil.

In this study I investigated the effect of co-expressing a fatty acid elongase gene, *LfKCS3*, from *Lesquerella (Physaria) fendleri* along with the castor hydroxylase gene, *RcFAH*, on accumulation of HFA in TAG oil of the crop plant *Camelina sativa*. On its own, the *RcFAH* gene results in accumulation of around 15% HFA in camelina (Table 3). Addition of the *LfKCS3* gene resulted in significant increase of HFA accumulation, especially the C20 very-long-chain HFA. The *LfKCS3* gene has been previously shown to encode an 18:1OH substrate specific fatty acid elongase responsible for the high amount of 20:1OH present in lesquerella oil (Moon et al 2001). After the hydroxylase modifies the sn-2 18:1 on the PC, the 18:1OH may exit PC to enter the acyl-CoA pool, where 18:1OH-CoA is elongated to 20:1OH-CoA by the LfKCS enzyme. Due to the fact that very long chain fatty acids (VLCFA) are generally excluded from the membrane lipids (Norton & Harris, 1983; Smith et al., 2003), it is possible that elongating the 18:1OH to 20:1OH may effectively place them in a different modified acyl-CoA pool that could be more readily incorporated into TAG. Removal of these very-long-chain HFA-CoA's from the modified acyl-CoA pool may cause a net movement of HFA out of PC and into TAG due to the dynamic flux between the PC and acyl-CoA pools (van Erp et al., 2011). This increased flux of HFA would help alleviate the amount of HFA stuck in the PC

bottleneck. Additionally, HFA that is occupied by the LfKCS3 enzyme is unavailable for turnover by FA degradation mechanisms and may increase the amount of FA available for incorporation into TAG. In the following discussion I put forth results supporting the above hypotheses by showing that incorporation of the LfKCS3 enzyme effectively increased the amount of both total hydroxy fatty acids and long chain hydroxy fatty acids in transgenic camelina lines while helping to relieve the PC bottleneck.

Incorporation of LFKCS3 along with RcFAH resulted in increased accumulation of very-long-chain HFA from less than 2% in RcFAH-only lines to 8.2% in the highest RcFAH-LfKCS line and raised the overall HFA accumulation in transgenic camelina lines from 15% to a peak measure of 22.8% in the highest RcFAH-LfKCS line, a nearly 50% increase. In total, ten independent transgenic lines were identified that were able to accumulate higher than 19% total HFA and 6% or more C20 HFA, differences of 4% or more each depending on the line (Table 3). This increase in both total and C20 HFA was significant because the higher accumulation of 20:1OH and 20:2OH seen in the RcFAH-LfKCS lines was not simply a shift in types of HFA product, but an incorporation of more total HFA product into the plant oil. The higher total HFA accumulating RcFAH-LfKCS lines actually displayed similar C18 HFA levels to the RcFAH only lines at 13-15%, but were set apart by their additional accumulation of C20 HFA. This pattern of the highest HFA accumulators also containing the highest C20 HFA content was consistent in each generation of RcFAH-LfKCS and indicated that successful accumulation of C20 HFA was key to obtaining higher total HFA in the transgenic lines (Figure 6). These results support the hypothesis that creation and incorporation of C20 HFA into TAG may

increase the flux of HFA out of the PC and into the acyl-CoA pool where they would be available for incorporation into TAG and lead to higher total HFA levels. If there were no increase in the flux of HFA out of PC we would not expect to see an increase in the total HFA amount simply from the accumulation of more C20 HFA. Since the C20 HFA product is derived from C18 HFA, if flux of HFA out of the PC were unchanged it would be expected that only a difference in the distribution of type of HFA, but not the overall amount would be observed. Therefore, the increase in not only C20 HFA content, but overall HFA accumulation indicates that the LfKCS3 enzyme alters the flux of HFA product out of PC for incorporation into TAG and increases the availability of C20 HFA.

Thin layer chromatography (TLC) of total oil from the RcFAH-LfKCS lines revealed the presence of 1-OH TAG and 2-OH TAG in the oil (Figure 8). The ability of the plant to accumulate 2-OH TAG is encouraging since levels of the hydroxy fatty acids would not be expected to accumulate to more than roughly 33% if constrained to occupying only one of three *sn* positions in TAG at any given time. Digestion of the 1-OH TAG fraction by a *sn*-1/3 specific lipase revealed the presence of free hydroxy fatty acids and 1-OH MAG in the oil (Figure 9). This result confirms that the HFA must be accumulating at both the *sn*-2 position, as evidenced by the presence of OH-MAG, as well as the *sn*-1 or *sn*-3 position in order to yield free hydroxy fatty acids. The ability to accumulate HFA in multiple positions of TAG confirms the observation of 2-OH TAG presence and raised the question of whether measurable amount of 3-OH TAG would be possible with better HFA recruitment into TAG. When the composition of the 1-OH MAG was investigated it was found to contain almost exclusively 18:1-OH and 18:2-OH

(Figure 13). This result is consistent with previous findings of OH-MAG in *Arabidopsis* and knowledge that the RcFAH acts on FA in the sn-2 position of PC (Smith et al. 2003, Bates & Browse 2011), leading PC derived DAG to contain mostly C18 HFA in the sn-2 position. From this result we can assume most of the C20 HFA is preferentially incorporated into the sn-1 or sn-3 positions.

Investigation of the 1-OH TAG and 2-OH TAG bands by GC analysis yielded peaks for all four types of HFA, 18:1OH, 18:2OH, 20:1OH, and 20:2OH, but the ratios of each differed between the 1-OH to 2-OH TAG fractions (Table 4). The 1-OH TAG derived most of its HFA content from 18:1OH and 18:2OH at 13.6% and 11.7% respectively. The 20:1OH and 20:2OH fatty acids only made up a combined 6.6% of the HFA possible in the 1-OH TAG fraction. This suggests that the 1-OH TAG fraction could be made from a variety of sources including acylating an sn-2 HFA DAG with a non-hydroxy FA at the sn-3 position by either DGAT or PDAT. Alternatively, either DGAT or PDAT could add a C18 HFA originating from the acyl CoA pool or the sn-2 position of another PC-DAG to the sn-3 position of a 0-HFA DAG. A third scenario would entail the incorporation of C18 HFA into either the sn-1 or sn-2 position of DAG during the de-novo pathway followed by addition of a FA through the action of PDAT or DGAT to form TAG. Compared to the 1-OH TAG fraction, the 2-OH TAG fraction showed decreased levels of the usual fatty acids such as 18:1 and 20:1, which were decreased by 4.9% and 11.46% respectively. This decrease in monounsaturates intuitively makes sense because the usual fatty acids have been replaced with hydroxy fatty acids at two of three positions of the TAG molecule. The higher decrease in 20:1 as

compared to 18:1 could be due to competition between the RcFAH and the native elongase (FAE1) for the same 18:1 product, or modified acyl pool competition between the 20:1 and 20:1OH fatty acids for incorporation into 2-OH TAG. While a rise in HFA is to be expected in 2-OH TAG compared to 1-OH TAG, the amount of increase was not equal between the C18 and C20 HFAs. Incorporation of 18:1OH and 18:2OH into 2-OH TAG was measured at just under 1.4 times higher, but the incorporation of 20:1OH and 20:2OH more than quadrupled to a combined total of 25.2% (Table 4). Total HFA content of the TAG increased from 32% in 1-OH TAG lines to 60% in 2-OH TAG lines as expected, but a higher amount of that HFA increase consisted of C20 hydroxy moieties for the RcFAH-LfKCS lines. A similar trend was observed in the RcFAH 7-1 line where the C18 HFA level in 2-OH TAG was 1.5 times higher than in 1-OH TAG, and the C20 HFA level was 4 times higher despite only accumulating to 10.9% of the total HFA level present in 2-OH TAG (Table 4). Due to the similar plant background between the RcFAH and RcFAH-LfKCS lines, the difference in their ability to accumulate long chain HFA in 2-OH TAG can be attributed solely to the presence or absence of the *LfKCS3* gene and resulting availability of C20 HFA product for incorporation. These results also point to a preference for utilization of the longer chain HFA to make 2-OH TAG as compared to 1-OH TAG. The synthesis of 2-OH TAG can proceed via two main routes: long chain HFA can be pulled from the modified acyl Co-A pool and assimilated into the sn-3 position of TAG by DGAT, or it can be incorporated into the sn-1 or sn-2 position of DAG via the de-novo synthesis pathway before a second HFA is added by PDAT or DGAT (Figure 1). However, based on recent research suggesting that most of the DAG

used for TAG synthesis is PC derived and not de novo derived (Bates et al., 2009; Bates & Browse, 2011), the finding that the OH-MAG sn-2 position contained almost entirely C18 HFA, and that the levels of C18 HFA are still higher than C20 HFA in the 2-OH TAG, it is more likely that the 2-OH TAG results from a PC derived DAG containing an sn-2 18:1/2-OH that has a 20:1/2-OH added onto the sn-3 position by DGAT. It is also interesting to note that the HFA levels in RcFAH-LfKCS lines for 1 and 2-OH TAG were closer to the expected amounts of 33% and 66% at 31.8% and 60.2% than the RcFAH only lines that were only 30.6% and 52.5%. This lower than expected HFA percentage for RcFAH lines was also observed in *Arabidopsis* but not fully explained (Smith et al 2003). Increasing the flux of HFA out of PC by elongating some of the HFA product to 20-carbon chains increases the availability of HFA in the modified acyl-CoA pool for enzymes such as DGAT to utilize in the formation of 2-OH TAG, resulting in an enrichment of C20 HFA in 2-OH TAG of RcFAH-LfKCS lines as compared to RcFAH only lines and adding to the increase in total HFA content.

Another line of evidence that supports the increased flux of hydroxy fatty acids out of PC for incorporation into TAG in the RcFAH-LfKCS lines is the measurement of HFA present at six different seed developmental stages starting with the beginning of oil accumulation and ending with mature seed. For each developmental time point tested past the ~16 day after flowering, the RcFAH lines displayed a higher amount of HFA present in the PC than the RcFAH-LfKCS lines (Figure 14). For the same developmental stage, the RcFAH-LfKCS lines displayed a higher accumulation of HFA in the total oil fraction when compared to the RcFAH lines (Figure 15). This observation is consistent

with those of other studies stating that decreased accumulation of HFA in the PC is correlated with relieving the bottleneck and increasing HFA accumulation in TAG (van Erp et al., 2011; Hu et al., 2012). It is probable that *LfKCS3* both helps to reduce accumulation of HFA in PC by increasing the flux of C18 HFA products out of PC and into the acyl-CoA pool, while also preventing it from re-entering PC due to the elongation to C20 HFA by *LfKCS3*. This combination would be expected to result in a higher accumulation of HFA in TAG through their greater presence in the modified acyl-CoA pool and increased availability for utilization by TAG synthesizing enzymes such as DGAT. Here we observed a difference in HFA accumulation in both PC and total oil fractions throughout the developing seed stages and in the final mature seed oil of RcFAH and RcFAH-*LfKCS* lines.

Finally, the spatial storage of HFA and germination ability of the two lines, RcFAH and RcFAH-*LfKCS*, were compared. Comparison of six T₄ RcFAH-*LfKCS* lines with two different T₄ RcFAH lines showed similar spatial accumulation of hydroxy fatty acids within the seeds. In each line HFA accumulated in both the cotyledons and hypocotyls (Table 6). On average the cotyledons accumulated 4-5% higher total HFA than the hypocotyls did and a slightly higher proportion of long chain HFA as well (Table 5). Thus, aside from the increased proportion of C20 HFA accumulation seen in the RcFAH-*LfKCS* lines, the pattern of HFA accumulation in T₄ lines seems unchanged by the additional *LfKCS3* gene. A major change in germination ability was noted between the two lines when the T₅ generation was tested. Difficulties with the RcFAH 7-1 and 7-2 lines were encountered as longer term storage had decreased the seed viability

dramatically, with only around 5% or less of seeds germinating. It is not clear whether the RcAFH-LfKCS lines would display the same issues with viability retention as the RcFAH lines after a period of time in storage. However, even the newly harvested T₅ RcFAH 7-1 seed displayed much delayed germination compared to the T₅ RcFAH-LfKCS lines. By 5 days after imbibition, all of the viable RcFAH-LfKCS seed had germinated, with germination ratios of 88% to 100% (Table 7). This time range is the same as what has been previously observed for untransformed camelina MT5 germinating in soil. After one week only a few of the 200 plated RcFAH 7-1 seeds were beginning to show signs of germination (Figure 17). The newly harvested T₅ RcFAH 7-1 seeds took twice as long as the RcFAH-LfKCS lines to germinate and still did not reach 100% germination. The difference in delayed germination between the two lines is very obvious, but the cause is difficult to determine. One plausible explanation is the difference in HFA remaining in PC between the two lines. Though the levels in RcFAH-LfKCS lines were lowered by only 2% in the final mature seed, it is not precisely known how much of an effect small differences in PC could have on germination. It has also been hypothesized that seed viability may be determined during seed maturation (Rajjou et al., 2012), when there is a greater difference in accumulation of HFA in PC between the two lines.

The results presented here, along with previous reports by other researchers, highlight the need for increased knowledge of the interactions involving fatty acid flux and metabolism in seed oil plants that accumulate modified fatty acids (mFA). It has been made clear that insertion of a single gene is not enough to achieve high accumulation of

mFA in plants that are not native accumulators (Lu et al., 2006; van Erp et al., 2011; Bates & Browse, 2011; Hu et al., 2012). Through investigation of which co-evolved genes impart the greatest influence or contribution to accumulation of mFA, we can ascertain the most useful combination of genes to create mFA in alternative plant backgrounds. Here I investigated the effect on total HFA accumulation in camelina of the incorporation of a hydroxy substrate specific elongase, *LfKCS3*, from the native HFA accumulator *Lesquerella (Physaria) fendleri*. The presence of the *LfKCS3* enzyme effectively increased total HFA levels at all stages of oil accumulation and the mature seed while decreasing the amount of HFA left in the PC. The increased incorporation of C20 HFA did not appear to replace the C18 HFA in the total oil sample, but added to the overall HFA accumulation. The combination of increased total and C20 HFA accumulation along with the decreased HFA levels in PC indicates that the *LfKCS3* gene helps to enhance incorporation of HFA into TAG through a modification to C20 HFA. This may potentially increase HFA availability in the acyl-CoA pool by preventing them from being re-incorporated into the PC or membranes while aid in the flux of HFA out of PC. While an increase to 22% HFA is not the highest transgenic level achieved, it indicates that there is still much we do not know about the interactions governing accumulation of HFA in crop plants such as camelina. The main factors limiting accumulation have been theorized to be accumulation of product in PC due to inefficient removal, and low incorporation of HFA into TAG (Bates and Browse, 2011). Due to its ability to help relieve accumulation in the PC bottleneck, incorporation of *LfKCS3* along with other co-evolved genes responsible for incorporation of HFA into TAG, such as

RcPDCT, *RcDGAT2*, and *RcPDAT1A*, would be expected to result in even higher levels of HFA, especially C20 HFA.

LITERATURE CITED

- Al-Shehbaz, I.A., Beilstein, M.A. and Kellogg, E.A. (2006) Systematics and phylogeny of the Brassicaceae (Cruciferae): an overview. *Plant Syst Evol* **259**, 89-120.
- Andre, C., Haslam, R.P. and Shanklin, J. (2012) Feedback regulation of plastidic acetyl-CoA carboxylase by 18:1-acyl carrier protein in *Brassica napus*. *Proc Natl Acad Sci USA* **109**, 10107-10112.
- Bafor, M., Smith, M.A., Jonsson, L., Stobart, K. and Stymne, S. (1991) Ricinoleic acid biosynthesis and triacylglycerol assembly in microsomal preparations from developing castor bean (*Ricinus communis*) endosperm. *Biochem J* **280**, 507-514.
- Bao, X., Katz, S., Pollard, M. and Ohlrogge, J. (2002) Carbocyclic fatty acids in plants: biochemical and molecular genetic characterization of cyclopropane fatty acid synthesis of *Sterculia foetida*. *Proc Natl Acad Sci USA* **99**, 7172-7177.
- Bates, P.D. and Browse, J. (2011) The pathway of triacylglycerol synthesis through phosphatidylcholine in *Arabidopsis* produces a bottleneck for the accumulation of unusual fatty acids in transgenic seeds. *Plant J* **68**, 387-399.
- Bates, P.D., Durrett, T.P., Ohlrogge, J.B. and Pollard, M. (2009) Analysis of acyl fluxes through multiple pathways of triacylglycerol synthesis in developing soybean embryos. *Plant Physiol* **150**, 55-72.
- Bates, P.D., Fatihi, A., Snapp, A.R., Carlsson, A.S., Browse, J. and Lu, C. (2012) Acyl editing and headgroup exchange are the major mechanisms that direct polyunsaturated fatty acid flux into triacylglycerols. *Plant Physiol* **160**, 1530-1539.
- Bates, P.D., Ohlrogge, J.B. and Pollard, M. (2007) Incorporation of newly synthesized fatty acids into cytosolic glycerolipids in pea leaves occurs via acyl editing. *J Biol Chem* **282**, 31206-31216.
- Broun, P., Boddupalli, S. and Somerville, C. (1998) A bifunctional oleate 12-hydroxylase: desaturase from *Lesquerella fendleri*. *Plant J* **13**, 201-210.
- Broun, P. and Somerville, C. (1997) Accumulation of ricinoleic, lesquerolic, and densipolic acids in seeds of transgenic *Arabidopsis* plants that express a fatty acyl hydroxylase cDNA from castor bean. *Plant Physiol* **113**, 933-942.
- Brown, A.P., Kroon, J.T., Swarbreck, D., Febrer, M., Larson, T.R., Graham, I.A., Caccamo, M. and Slabas, A.R. (2012) Tissue-specific whole transcriptome sequencing in castor, directed at understanding triacylglycerol lipid biosynthetic pathways. *PLoS One* **7**, e30100.

- Browse, J., McConn, M., James, D., Jr. and Miquel, M. (1993) Mutants of Arabidopsis deficient in the synthesis of alpha-linolenate. Biochemical and genetic characterization of the endoplasmic reticulum linoleoyl desaturase. *J Biol Chem* **268**, 16345-16351.
- Browse, J. and Somerville, C. (1991) Glycerolipid Synthesis - Biochemistry and Regulation. *Annu Rev Plant Phys* **42**, 467-506.
- Buchsenschutz-Nothdurft, A., Schuster, A. and Friedt, W. (1998) Breeding for modified fatty acid composition via experimental mutagenesis in *Camelina sativa* (L.) Crtz. *Ind Crop Prod* **7**, 291-295.
- Burgal, J., Shockey, J., Lu, C., Dyer, J., Larson, T., Graham, I. and Browse, J. (2008) Metabolic engineering of hydroxy fatty acid production in plants: RcDGAT2 drives dramatic increases in ricinoleate levels in seed oil. *Plant Biotechnol J* **6**, 819-831.
- Cahoon, E.B., Carlson, T.J., Ripp, K.G., Schweiger, B.J., Cook, G.A., Hall, S.E. and Kinney, A.J. (1999) Biosynthetic origin of conjugated double bonds: Production of fatty acid components of high-value drying oils in transgenic soybean embryos. *Proc. Natl. Acad. Sci. USA* **96**, 12935-12940.
- Cahoon, E.B., Dietrich, C.R., Meyer, K., Damude, H.G., Dyer, J.M. and Kinney, A.J. (2006) Conjugated fatty acids accumulate to high levels in phospholipids of metabolically engineered soybean and Arabidopsis seeds. *Phytochem* **67**, 1166-1176.
- Cahoon, E.B., Ripp, K.G., Hall, S.E. and McGonigle, B. (2002) Transgenic production of epoxy fatty acids by expression of a cytochrome P450 enzyme from *Euphorbia lagascae* seed. *Plant Physiol* **128**, 615-624.
- Cermak, S.C., Brandon, K.B. and Isbell, T.A. (2006) Synthesis and physical properties of estolides from lesquerella and castor fatty acid esters. *Ind Crop Prod* **23**, 54-64.
- Chan, A.P., Crabtree, J., Zhao, Q., Lorenzi, H., Orvis, J., Puiu, D., Melake-Berhan, A., Jones, K.M., Redman, J., Chen, G., Cahoon, E.B., Gedil, M., Stanke, M., Haas, B.J., Wortman, J.R., Fraser-Liggett, C.M., Ravel, J. and Rabinowicz, P.D. (2010) Draft genome sequence of the oilseed species *Ricinus communis*. *Nat Biotechnol* **28**, 951-956.
- Chen, G., Lin, J. and Lu, C. (2011) Hydroxy fatty acid synthesis and lipid gene expression during seed development in *Lesquerella fendleri*. *Ind Crop Prod* **34**, 1286-1292.
- Collins-Silva, J.E., Lu, C. and Cahoon, E.B. (2011) Camelina: A designer biotech oilseed crop. In: *INFORM* pp. 610-613.

- Dahlqvist, A., Stahl, U., Lenman, M., Banas, A., Lee, M., Sandager, L., Ronne, H. and Stymne, S. (2000) Phospholipid : diacylglycerol acyltransferase: An enzyme that catalyzes the acyl-CoA-independent formation of triacylglycerol in yeast and plants. *Proc Natl Acad Sci USA* **97**, 6487-6492.
- Dauk, M., Lam, P., Kunst, L. and Smith, M. (2007) A FAD2 homologue from *Lesquerella lindheimeri* has predominantly fatty acid hydroxylase activity. *Plant Sci* **173**, 43-49.
- Dierig, D.A., Wang, G., W.B. McCloskey, W.B., Thorp, K.R., Isbell, T.A., Ray, D.T. and Foster, M.A. (2011) *Lesquerella*: New crop development and commercialization in the U.S. *Ind Crop Prod* **34**, 1381-1385.
- Drexler, H., Spiekermann, P., Meyer, A., Domergue, F., Zank, T., Sperling, P., Abbadi, A. and Heinz, E. (2003) Metabolic engineering of fatty acids for breeding of new oilseed crops: strategies, problems and first results. *J Plant Physiol* **160**, 779-802.
- Durrett, T.P., Benning, C. and Ohlrogge, J. (2008) Plant triacylglycerols as feedstocks for the production of biofuels. *Plant J* **54**, 593-607.
- Durrett, T.P., McClosky, D.D., Tumaney, A.W., Elzinga, D.A., Ohlrogge, J. and Pollard, M. (2010) A distinct DGAT with sn-3 acetyltransferase activity that synthesizes unusual, reduced-viscosity oils in *Euonymus* and transgenic seeds. *Proc Natl Acad Sci USA* **107**, 9464-9469.
- Dyer, J.M. and Mullen, R.T. (2008) Engineering plant oils as high-value industrial feedstocks for biorefining: the need for underpinning cell biology research. *Physiol Plant* **132**, 11-22.
- Dyer, J.M., Stymne, S., Green, A.G. and Carlsson, A.S. (2008) High-value oils from plants. *Plant J* **54**, 640-655.
- Eccleston, V.S., Cranmer, A.M., Voelker, T.A. and Ohlrogge, J.B. (1996) Medium-chain fatty acid biosynthesis and utilization in *Brassica napus* plants expressing lauroyl-acyl carrier protein thioesterase. *Planta* **198**, 46-53.
- Eccleston, V.S. and Ohlrogge, J.B. (1998) Expression of lauroyl-acyl carrier protein thioesterase in *brassica napus* seeds induces pathways for both fatty acid oxidation and biosynthesis and implies a set point for triacylglycerol accumulation. *Plant Cell* **10**, 613-622.
- Gehringer, A., Friedt, W., Luhs, W. and Snowdon, R.J. (2006) Genetic mapping of agronomic traits in false flax (*Camelina sativa* subsp *sativa*). *Genome* **49**, 1555-1563.

- Ghamkhar, K., Croser, J., Aryamanesh, N., Campbell, M., Kon, rsquo, kova, N. and Francis, C. (2010) Camelina (*Camelina sativa* (L.) Crantz) as an alternative oilseed: molecular and ecogeographic analyses. *Genome* **53**, 558-567.
- Goode, J.H. and Dewey, R.E. (1999) Characterization of aminoalcoholphosphotransferases from *Arabidopsis thaliana* and soybean. *Plant Physiol Biochem* **37**, 445-457.
- Gunstone, F.D. (1998) Movements towards tailor-made fats. *Prog Lipid Res* **37**, 277-305.
- Hobbs, D.H., Lu, C. and Hills, M.J. (1999) Cloning of a cDNA encoding diacylglycerol acyltransferase from *Arabidopsis thaliana* and its functional expression. *FEBS Lett* **452**, 145-149.
- Hu, Z., Ren, Z. and Lu, C. (2012) The phosphatidylcholine diacylglycerol cholinephosphotransferase is required for efficient hydroxy fatty acid accumulation in transgenic *Arabidopsis*. *Plant Physiol* **158**, 1944-1954.
- Huber, G.W., Iborra, S. and Corma, A. (2006) Synthesis of transportation fuels from biomass: chemistry, catalysts, and engineering. *Chem Rev* **106**, 4044-4098.
- Hutcheon, C., Ditt, R., Beilstein, M., Comai, L., Schroeder, J., Goldstein, E., Shewmaker, C., Nguyen, T., De Rocher, J. and Kiser, J. (2010) Polyploid genome of *Camelina sativa* revealed by isolation of fatty acid synthesis genes. *BMC Plant Biol* **10**, 233.
- Jaworski, J. and Cahoon, E.B. (2003) Industrial oils from transgenic plants. *Curr Opin Plant Biol* **6**, 178-184.
- Johnson J., E.N., Schneekloth J., Helm A., Malhotra R. Coonrod D. (2009) Development of Oilseed Crops for Biodiesel Production Under Colorado Limited Irrigation Conditions. Completion Report 211, Colorado Water Institute, Colorado State University, Fort Collins, CO, 51 pages.
- Kang, J., Snapp, A.R. and Lu, C. (2011) Identification of three genes encoding microsomal oleate desaturases (FAD2) from the oilseed crop *Camelina sativa*. *Plant Physiol Biochem* **49**, 223-229.
- Katavic, V., Reed, D.W., Taylor, D.C., Giblin, E.M., Barton, D.L., Zou, J., Mackenzie, S.L., Covello, P.S. and Kunst, L. (1995) Alteration of seed fatty acid composition by an ethyl methanesulfonate-induced mutation in *Arabidopsis thaliana* affecting diacylglycerol acyltransferase activity. *Plant Physiol* **108**, 399-409.
- Kennedy, E.P. (1961) Biosynthesis of complex lipids. *Fed Proc Amer Soc Exp Biol* **20**, 934-940.

- Kunst, L., Taylor, D.C. and Underhill, E.W. (1992) Fatty acid elongation in developing seeds of *Arabidopsis thaliana*. *Plant Physiol Biochem* **30**, 425-434.
- Lee, M., Lenman, M., Banas, A., Bafor, M., Singh, S., Schweizer, M., Nilsson, R., Liljenberg, C., Dahlqvist, A., Gummesson, P.O., Sjodahl, S., Green, A. and Stymne, S. (1998) Identification of non-heme diiron proteins that catalyze triple bond and epoxy group formation. *Science* **280**, 915-918.
- Li, R., Yu, K., Hatanaka, T. and Hildebrand, D.F. (2010) Vernonia DGATs increase accumulation of epoxy fatty acids in oil. *Plant Biotechnol J* **8**, 184-195.
- Li, R., Yu, K., Wu, Y., Tateno, M., Hatanaka, T. and Hildebrand, D.F. (2012) Vernonia DGATs can complement the disrupted oil and protein metabolism in epoxygenase-expressing soybean seeds. *Metab Eng* **14**, 29-38.
- Lu, C., Fulda, M., Wallis, J.G. and Browse, J. (2006) A high-throughput screen for genes from castor that boost hydroxy fatty acid accumulation in seed oils of transgenic *Arabidopsis*. *Plant J* **45**, 847-856.
- Lu, C. and Kang, J. (2008) Generation of transgenic plants of a potential oilseed crop *Camelina sativa* by *Agrobacterium*-mediated transformation. *Plant Cell Rep* **27**, 273-278.
- Lu, C., Napier, J.A., Clemente, T.E. and Cahoon, E.B. (2011) New frontiers in oilseed biotechnology: meeting the global demand for vegetable oils for food, feed, biofuel, and industrial applications. *Curr Opin Biotechnol* **22**, 252-259.
- Lu, C., Wallis, J.G. and Browse, J. (2007) An analysis of expressed sequence tags of developing castor endosperm using a full-length cDNA library. *BMC Plant Biol* **7**, 42.
- Lu, C., Xin, Z., Ren, Z., Miquel, M. and Browse, J. (2009) An enzyme regulating triacylglycerol composition is encoded by the *ROD1* gene of *Arabidopsis*. *Proc Natl Acad Sci USA* **106**, 18837-18842.
- Lukowitz, W., Gillmor, C.S. and Scheible, W.R. (2000) Positional cloning in *Arabidopsis*. Why it feels good to have a genome initiative working for you. *Plant Physiol* **123**, 795-805.
- Makareviciene, V., Skorupskaite, V. and Andruleviciute, V. (2013) Biodiesel fuel from microalgae-promising alternative fuel for the future: a review. *Rev Environ Sci Biotechnol*, 1-12.
- Moire, L., Rezzonico, E., Goepfert, S. and Poirier, Y. (2004) Impact of unusual fatty acid synthesis on futile cycling through beta-oxidation and on gene expression in transgenic plants. *Plant Physiol* **134**, 432-442.

- Moon, H., Smith, M.A. and Kunst, L. (2001) A condensing enzyme from the seeds of *Lesquerella fendleri* that specifically elongates hydroxy fatty acids. *Plant Physiol* **127**, 1635-1643.
- Moser, B.R. (2010) Camelina (*Camelina sativa* L.) oil as a biofuels feedstock: Golden opportunity or false hope? *Lipid Technology* **22**, 270-273.
- Napier, J.A. and Graham, I.A. (2010) Tailoring plant lipid composition: designer oilseeds come of age. *Curr Opin Plant Biol* **13**, 330-337.
- NDSU (2010) NDSU extension canola crop fertilization suggestions. NDSU Extension.
- Nguyen, H., Silva, J., Podicheti, R., Macrander, J., Yang, W., Nazarens, T., Nam, J.-W., Jaworski, J., Lu, C., Scheffler, B., Keithanne, M. and Cahoon, E. (2013) Camelina seed transcriptome: A tool for meal and oil improvement and translational research *Plant Biotechnol J* **in press**.
- Norton, G. and Harris, J. (1983) Triacylglycerols in oilseed rape during seed development *Phytochem* **22**, 2703-2707.
- Ohlrogge, J. and Browse, J. (1995) Lipid Biosynthesis. *Plant Cell* **7**, 957-970.
- Okuley, J., Lightner, J., Feldmann, K., Yadav, N., Lark, E. and Browse, J. (1994) *Arabidopsis* Fad2 gene encodes the enzyme that is essential for polyunsaturated lipid synthesis. *Plant Cell* **6**, 147-158.
- Pan, X., Lada, R., Caldwell, C.D. and Falk, K.C. (2011) Photosynthetic and growth responses of *Camelina sativa* (L.) Crantz to varying nitrogen and soil water status. *Photosynthetica* **49**, 316-320.
- Pilgeram, A.L., Sands, D.C., Boss, D., Dale, N., Wichman, D., Lamb, P., Lu, C., Barrows, R., Kirkpatrick, M., Thompson, B. and Johnson, D.L. (2007) *Camelina sativa*, A Montana Omega-3 and Fuel Crop. In: *Issues in new crops and new uses* (Janick, J. and Whipkey, A. eds), pp. 129-131. Alexandria, VA: ASHS Press.
- Pinzi, S., Garcia, I.L., Lopez-Gimenez, F.J., Luque de Castro, M.D., Dorado, G. and Dorado, M.P. (2009) The Ideal Vegetable Oil-based Biodiesel Composition: A Review of Social, Economical and Technical Implications. *Energy & Fuels* **23**, 2325-2341.
- Rajjou, L., Duval, M., Gallardo, K., Catusse, J., Bally, J., Job, C. and Job, D. (2012) Seed germination and vigor. *Annu Rev Plant Biol* **63**, 507-533.
- Riediger, N.D., Othman, R.A., Suh, M. and Moghadasian, M.H. (2009) A systemic review of the roles of n-3 fatty acids in health and disease. *J Am Diet Assoc* **109**, 668-679.

- Roesler, K., Shintani, D., Savage, L., Boddupalli, S. and Ohlrogge, J. (1997) Targeting of the Arabidopsis homomeric acetyl-coenzyme A carboxylase to plastids of rapeseeds. *Plant Physiol* **113**, 75-81.
- Roughan, P.G. and Slack, C.R. (1982) Cellular-organization of glycerolipid metabolism. *Annu Rev Plant Phys* **33**, 97-132.
- Routaboul, J.M., Benning, C., Bechtold, N., Caboche, M. and Lepiniec, L. (1999) The TAG1 locus of Arabidopsis encodes for a diacylglycerol acyltransferase. *Plant Physiol Biochem* **37**, 831-840.
- Salimon, J., Salih, N. and Yousif, E. (2010) Biolubricants: Raw materials, chemical modifications and environmental benefits. *Eur J Lipid Sci Technol* **112**, 519-530.
- Sequin-Swartz G., E.C., Gugel R.K., Strelkov S.E., Oliver C.Y., Li J.L., Klein-Gebbinck H., Borhan H., Caldwell C.D., Falk K.C. (2009) Diseases of *Camelina sativa* (false flax) *Can. J. Plant Pathol.* **31**, 375-386.
- Severino, L.S., Auld, D.L., Baldanzi, M., Candido, M.J.D., Chen, G., Crosby, W., Tan, D., He, X.H., Lakshamma, P., Lavanya, C., Machado, O.L.T., Mielke, T., Milani, M., Miller, T.D., Morris, J.B., Morse, S.A., Navas, A.A., Soares, D.J., Sofiatti, V., Wang, M.L., Zanotto, M.D. and Zieler, H. (2012) A Review on the Challenges for Increased Production of Castor. *Agron J* **104**, 853-880.
- Shintani, D.K. and Ohlrogge, J.B. (1995) Feedback inhibition of fatty acid synthesis in tobacco suspension cells. *Plant J* **7**, 577-587.
- Shockey, J.M., Fulda, M.S. and Browse, J.A. (2002) Arabidopsis contains nine long-chain acyl-coenzyme a synthetase genes that participate in fatty acid and glycerolipid metabolism. *Plant Physiol* **129**, 1710-1722.
- Shockey, J.M., Gidda, S.K., Chapital, D.C., Kuan, J.C., Dhanoa, P.K., Bland, J.M., Rothstein, S.J., Mullen, R.T. and Dyer, J.M. (2006) Tung tree DGAT1 and DGAT2 have nonredundant functions in triacylglycerol biosynthesis and are localized to different subdomains of the endoplasmic reticulum. *Plant Cell* **18**, 2294-2313.
- Shonnard, D., Williams, L. and Kalnes, T. (2010) Camelina-Derived Jet Fuel and Diesel: Sustainable Advanced Biofuels. *Environmental Progress & Sustainable Energy* **29**, 382-392.
- Singh, S.P., Zhou, X.R., Liu, Q., Stymne, S. and Green, A.G. (2005) Metabolic engineering of new fatty acids in plants. *Curr Opin Plant Biol* **8**, 197-203.

- Slack, C.R., Campbell, L.C., Browse, J.A. and Roughan, P.G. (1983) Some evidence for the reversibility of cholinephosphotransferase-catalyzed reaction in developing linseed cotyledons *in vivo*. *Biochim Biophys Acta* **754**, 10-20.
- Smith, M.A., Moon, H., Chowrira, G. and Kunst, L. (2003) Heterologous expression of a fatty acid hydroxylase gene in developing seeds of *Arabidopsis thaliana*. *Planta* **217**, 507-516.
- Soriano, N., Jr. and Narani, A. (2012) Evaluation of Biodiesel Derived from Camelina sativa Oil. *J Amer Oil Chem Soc* **89**, 917-923.
- Sperling, P., Linscheid, M., Stocker, S., Muhlbach, H.P. and Heinz, E. (1993) In vivo desaturation of cis-delta 9-monounsaturated to cis-delta 9,12-diunsaturated alkenylether glycerolipids. *J Biol Chem* **268**, 26935-26940.
- Steen, E.J., Kang, Y., Bokinsky, G., Hu, Z., Schirmer, A., McClure, A., Del Cardayre, S.B. and Keasling, J.D. (2010) Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. *Nature* **463**, 559-562.
- Stobart, K., Mancha, M., Lenman, M., Dahlqvist, A. and Stymne, S. (1997) Triacylglycerols are synthesised and utilized by transacylation reactions in microsomal preparations of developing safflower (*Carthamus tinctorius* L) seeds. *Planta* **203**, 58-66.
- Suh, M.C., Schultz, D.J. and Ohlrogge, J.B. (2002) What limits production of unusual monoenoic fatty acids in transgenic plants? *Planta* **215**, 584-595.
- Thom  us, S., Carlsson, A.S. and Stymne, S. (2001) Distribution of fatty acids in polar and neutral lipids during seed development in *Arabidopsis thaliana* genetically engineered to produce acetylenic, epoxy and hydroxy fatty acids. *Plant Sci* **161**, 997-1003.
- Troncoso-Ponce, M.A., Kilaru, A., Cao, X., Durrett, T.P., Fan, J., Jensen, J.K., Thrower, N.A., Pauly, M., Wilkerson, C. and Ohlrogge, J.B. (2011) Comparative deep transcriptional profiling of four developing oilseeds. *Plant J* **68**, 1014-1027.
- van de Loo, F.J., Broun, P., Turner, S. and Somerville, C. (1995) An oleate 12-hydroxylase from *Ricinus communis* L is a fatty acyl desaturase homolog. *Proc Natl Acad Sci USA* **92**, 6743-6747.
- van de Loo, F.J., Fox, B.G. and Somerville, C. (1993) Unusual fatty acids. In: *Lipid metabolism in plants* (Moore, T.S.J. ed) pp. 91-126. Boca Raton: CRC Press.
- van Erp, H., Bates, P.D., Shockey, J., Burgal, J. and Browse, J. (2011) Castor phospholipid:diacylglycerol acyltransferase facilitates efficient metabolism of hydroxy fatty acids in transgenic Arabidopsis. *Plant Physiol* **155**, 683-693.

- Voelker, T. and Kinney, A.T. (2001) Variations in the biosynthesis of seed-storage lipids. *Annu Rev Plant Phys* **52**, 335-361.
- Vollmann J., M.T., Kargl C., Baumgartner S., Wagentristl H. (2007) Agronomic evaluation of camelina genotypes selected for seed quality characteristics *Ind Crop Prod* **26**, 270-277.
- Wallis, J.G. and Browse, J. (2010) Lipid biochemists salute the genome. *Plant J* **61**, 1092-1106.
- Walsh K.D., P.D.M., Hills M.J., Yang R., Topinka K.C., Hall L.M. (2012) First report of outcrossing rates in camelina [*Camelina sativa* (L.) Crantz], a potential platform for bioindustrial oils *Can. J. Plant Sci.* **92**, 681-685.
- Williams, J.P., Imperial, V., Khan, M.U. and Hodson, J.N. (2000) The role of phosphatidylcholine in fatty acid exchange and desaturation in *Brassica napus* L. leaves. *Biochem J* **349**, 127-133.
- Xu, J., Carlsson, A.S., Francis, T., Zhang, M., Hoffmann, T., Giblin, M. and Taylor, D.C. (2012) Triacylglycerol synthesis by PDAT1 in the absence of DGAT1 activity is dependent on re-acylation of LPC by LPCAT2. *BMC Plant Biol* **12**, 4.
- Yang, P., Li, X., Shipp, M.J., Shockey, J.M. and Cahoon, E.B. (2010) Mining the bitter melon (*Momordica charantia* L.) seed transcriptome by 454 analysis of non-normalized and normalized cDNA populations for conjugated fatty acid metabolism-related genes. *BMC Plant Biol* **10**, 250.
- Yu, K., McCracken, C.T., Jr., Li, R. and Hildebrand, D.F. (2006) Diacylglycerol acyltransferases from *Vernonia* and *Stokesia* prefer substrates with vernolic acid. *Lipids* **41**, 557-566.
- Zhang, M., Fan, J., Taylor, D.C. and Ohlrogge, J.B. (2009) DGAT1 and PDAT1 acyltransferases have overlapping functions in *Arabidopsis* triacylglycerol biosynthesis and are essential for normal pollen and seed development. *Plant Cell* **21**, 3885-3901.
- Zou, J., Wei, Y., Jako, C., Kumar, A., Selvaraj, G. and Taylor, D.C. (1999) The *Arabidopsis thaliana* TAG1 mutant has a mutation in a diacylglycerol acyltransferase gene. *Plant J* **19**, 645-653.
- Zubr, J. (1997) Oil-seed crop: *Camelina sativa*. *Ind Crop Prod* **6**, 113-119.