Type-1 Brassica napus Diacylglycerol Acyltransferases: Enzyme Characterization and Molecular Tools for Increasing Storage Lipid Production in Yeast

by

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ABSTRACT

Diacylglycerol acyltransferase (DGAT) catalyzes the final step in the acyl-CoA-dependent biosynthesis of triacylglycerol (TAG). The level of DGAT activity may have a substantial effect on the flow of carbon into TAG in many organisms. In plants, yeast, and animals, two families of membrane-bound DGATs have been identified. In plants, modification of the DGAT-catalyzed step could lead to improved oil seed varieties with either increased nutritional or industrial value. Increased production of oil through manipulation of DGAT activity would be generally beneficial in both plants and yeast. In humans, alteration of DGAT activity through pharmacological intervention could lead to treatments for obesity, type-2 diabetes, and improved cardiovascular health.

Four type-1 *DGAT* genes have been identified in the *Brassica napus* genome which appear to belong to two clades with representatives of each clade in the A and C genome. *B. napus* is of major agricultural and economic importance in Canada. Despite having highly similar amino acid sequences, the DGAT1s encoded by these genes displayed significantly different abilities to catalyze the synthesis of TAG when recombinantly produced in a strain (H1246) of *Saccharomyces cerevisiae* devoid of TAG synthesis. Various modifications to the N-terminal regions and/or the encoding DNA sequences of the four isoforms were shown to have a profound impact on the accumulation of recombinant enzyme polypeptide in this yeast strain. In turn, this information was used as basis for engineering increased oil accumulation in yeast.

Increasing the accumulation of DGAT in yeast cells also facilitated the development of a novel gas chromatography/mass spectrometry-based *in vitro* DGAT assay. This assay circumvents the need for radiolabeled substrates commonly used in DGAT assays and is particularly useful in quickly evaluating substrate selectivity properties.

Phylogenetic analysis of the four *B. napus DGAT1* coding sequences revealed that these genes may have diverged into two separate clades relatively early in Brassicaceae history. Although all four DGAT1s could effectively use a range of molecular species of acyl-CoAs and *sn*-1,2-diacylglycerols, clade II DGAT1s displayed increased preference for substrates containing linoleic acid (18:2 $\Delta^{9cis,12cis}$). In the case of acyl-CoA, α-linolenoyl (18:3$\Delta^{9cis,12cis,15cis}$) was the most effective acyl donor for all four DGAT1s. These differences in substrate specificity occurred despite the relatively high level of amino acid sequence identity between the two clades of DGAT1. These results suggest that the two clades of *B. napus* DGAT1 enzymes have slightly different functional roles in oil formation during seed development.

PREFACE

Some of the research presented in this thesis was produced through collaboration with other graduate students and post-doctoral researchers. I was, however, responsible for the majority of the work including experimental design, data collection, statistical analysis and manuscript preparation. My supervisor, Dr. Randall J. Weselake, provided guidance in experimental design and was involved in editing of the resulting manuscripts and thesis sections.

Chapter three was published in *Applied Microbiology and Biotechnology* (Greer et al., 2015; 99: 2243-2253). Initial identification of *Brassica napus DGAT1*-expressed sequence tag sequences and Southern blot analysis were performed by Dr. Martin Turksa and Shiu-Cheung Lung. Dr. Wei Deng performed full-length gene sequencing. Dr. Guanqun Chen provided support in experimental design and development of the manuscript.

Chapter four was published in *Lipids* (Greer et al., 2014; 49: 831-838). Dr. Ting Zhou helped work out some of the conditions for the method described in this manuscript.

Xue Pan assisted in the phylogenetic analysis of *B. napus* DGAT1 amino acid sequences presented in chapter five.

DEDICATION

This work is dedicated to my parents, Michael and Dianna Greer, for their love and support throughout the many years of my formal education, and for the many lessons they have taught me outside of the classroom.

†

'I have not failed. I've just found 10,000 ways that won't work'
–Thomas Edison

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LIST OF ABBREVIATIONS

ΔF	change in fluorescence
A. thaliana (*At*)	*Arabidopsis thaliana*
ACAT	acyl-CoA: cholesterol acyltransferase
ACC	acetyl-CoA carboxylase
ACP	acyl carrier proteins
ANOVA	analysis of variance
ARE	acyl-CoA: cholesterol acyltransferase-related enzyme
B. napus (*Bna*)	*Brassica napus*
B. oleracea (*Bol*)	*Brassica oleracea*
B. rapa (*Bra*)	*Brassica rapa*
cDNA	complementary DNA
CDS	coding DNA sequence
C. reinhardtii	*Chlamydomonas reinhardtii*
CoA	coenzyme A
CPT	CDP-choline:1,2-diacyl-sn-glycerol cholinephosphotransferase
DAG	diacylglycerol
DCR	defective in cuticular ridges
DGA	diacylglycerol acyltransferase (yeast)
DGAT	acyl-CoA:diacylglycerol acyltransferase (animals, plants)
DHAP	dihydroxyacetone phosphate
ER	endoplasmic reticulum

FA	fatty acid
FAD	fatty acid desaturase
FAE	fatty acid elongase complex
FAS	fatty acid synthase
FAT	fatty acyl-ACP thioesterase
GC/MS	gas chromatography coupled mass spectrometry
G3P	glycerol-3-phosphate
GPAT	acyl-CoA: glycerol-3-phosphate acyltransferase
HT	herbicide tolerant
KAS	3-ketoacyl-ACP synthase
LACS	long-chain acyl-CoA synthetase
LPA	lysophosphatidic acid
LPAAT	acyl-CoA: lysophosphatidic acid acyltransferase
LPCAT	lysophosphatidylcholine acyltransferase
LRO	lecithin cholesterol acyl transferase related open reading frame
MAT	malonyl-CoA: acyl carrier protein malonyltansferase
MT	million tonnes
NADPH	nicotinamide adenine dinucleotide phosphate
NT	N-terminus
N. tabacum	*Nicotiana tabacum*
NTD	N-terminus deleted
OD	optical density
PA	phosphatidic acid

PAP	PA phosphatase
PC	phosphatidylcholine
PDAT	phospholipid: diacylglycerol acyltransferase
PDCT	phosphatidylcholine: diacylglycerol cholinephosphotransferase
PLA2	phospholipase A2
PLC	phospholipase C
PUFA	polyunsaturated fatty acid
S. cerevisiae (*Sc*)	*Saccharomyces cerevisiae*
SAD	stearoyl-ACP desaturase
SIM	selected ion monitoring
SNF	sucrose non-fermenting
S. laevis	*Stokesia laevis*
TAG	triacylglycerol
TUA	technology usage agreement
TLC	thin layer chromatography
V. fordii (*Vf*)	*Vernicia fordii*
V. galamensis (*Vg*)	*Vernonia galamensis*
WS/DGAT	wax synthase/diacylglycerol acyltransferase
Y. lipolytica (*Yl*)	*Yarrowia lipolytica*

CHAPTER 1 - INTRODUCTION

Triacylglycerol (TAG) is a compact, highly reduced, carbon storage compound that is important in both animals and plants as a form of energy reserve. It is composed of three fatty acids esterified to a glycerol backbone. In animals, yeast and plants, acyl-CoA: diacylglycerol acyltransferase (DGAT; EC 2.3.1.20) catalyzes the acyl-CoA-dependent acylation of *sn* 1,2-diacylglycerol to produce TAG. In many cases, the level of DGAT activity may have a substantial effect on the flow of carbon into TAG (Harwood et al., 2013; Weselake et al., 2009). Several unrelated forms of DGAT have evolved through convergent evolution, but the role and relative importance of each of these forms can vary depending on the species (Li et al. 2010). The majority of DGAT studies have focused on the first two forms of the enzyme to be identified, referred to as type 1 and type 2 DGAT (Liu et al., 2012). These enzymes are integral membrane bound proteins which reside in the endoplasmic reticulum (Liu et al., 2012; Shockey et al., 2006).

In humans, drug treatment to reduce DGAT activity has been identified as a means for controlling obesity, diabetes and other metabolic diseases (Cao et al., 2011; Chen and Farese 2005; Zhao et al., 2008). In the model plant *Arabidopsis thaliana* and oil crops, such as *Brassica napus*, over-expression of *DGAT* has been used as means of increasing seed oil content and/or alteration of fatty acid composition for both nutritional and industrial applications, such as production of lubricants and precursors for biofuel production (Weselake et al., 2009). Despite these practical initiatives, the highly hydrophobic characteristics of the two major forms of this enzyme have hindered structure/function analyses (Liu et al., 2012).

Plant DGAT1 was first cloned from developing seeds of *A. thaliana* (Hobbs et al., 1999). Shortly thereafter, Nykiforuk et al. (1999a; 1999b; 2002) cloned and functionally characterized a *DGAT1* cDNA from *B. napus*. This form of *B. napus* DGAT1 (BnaDGAT1) is closely related to *A. thaliana* DGAT1 (Li et al., 2010). In *A. thaliana*, DGAT1 appears to be the major contributor to acyl-CoA-dependent TAG biosynthesis (Zhang et al., 2009; Li et al., 2010). A study by Weselake et al. (2006) suggested that the hydrophilic N-terminal region of BnaDGAT1, identified by Nykiforuk et al. (1999b), may have a regulatory role. In this work, four type-1 *DGAT* genes were identified in the *B. napus* genome, which appear to belong to two clades with a representative of each clade in the A and C genome.

In Canada, *B. napus* is the major oilseed crop (Carré and Pouzet, 2014; Warwick, 2011). The *B. napus* industry generates $19.3 billion (CAD$) in annual economic activity in Canada (LMC International, 2013) accounting for just over one percent of its national gross domestic product (Trading Economics, 2014). The objectives and outcomes of my research on *B. napus* DGAT1s are of direct relevance to the further improvement of this oilseed crop. Strategies for manipulating DGAT activity, however, could further benefit from detailed structure/function information on this class of enzyme.

My research on the further characterization of DGAT1 enzymes is based on the following hypotheses.

1. The amino acid sequence of the N-terminal region of *B. napus* DGAT1 (BnaDGAT1) and/or the encoding DNA sequence can impact enzyme accumulation and activity when BnaDGAT1 is heterologously produced in *Saccharomyces cerevisiae*.

2. High temperature gas-chromatography of TAG produced by DGAT action coupled with selected ion monitoring of TAG ion fragments using mass spectrometry can be used as means of assaying DGAT activity and evaluating enzyme substrate selectivity properties.

3. Four closely related forms of BnaDGAT1 exhibiting a high level of identity can exhibit differences in substrate specificity properties.

Various modifications of the N-terminal amino acid sequence of BnaDGAT1 substantially impacted the accumulation of the enzyme when heterologously produced in *S. cerevisiae* resulting in cultures with significantly altered TAG content. During the investigations of recombinant enzyme production, a novel gas chromatography-mass spectrometry-based *in vitro* DGAT assay was developed. This assay circumvents the need for radiolabeled substrates commonly used in DGAT assays and is particularly useful in quickly evaluating substrate selectivity properties. Despite the high amino acid sequence homology shared by four BnaDGAT1 isoforms, two forms were identified to have a relatively higher preference for substrates containing linoleic ($18{:}2\Delta^{9cis,\ 12cis}$) acid.

CHAPTER 2 - LITERATURE REVIEW

2.1. OILSEED RAPE

The Brassicaceae, or mustard, family includes numerous species of plants vitally important to agriculture across the globe. This family of 3709 species includes *Brassica juncea* (brown mustard), *Sinapis alba* (white mustard), *B. oleracea* (kales, cabbages, Brussels sprouts, cauliflower, broccoli), *B. rapa* (turnip, Pak choi), and *Raphanus sativus* (radish) (Franzke et al., 2011; Warwick, 2011). *Arabidopsis thaliana*, the iconic plant model species, is also a member of the Brassicaceae. Brassicaceae derives its former name, Cruciferae, from its characteristic cross shaped corolla. This family is also defined by six stamens, a capsule often with a septum, and a watery sap famous for its pungent smell (Franzke et al., 2011). Amongst all of the Brassicaceae members, none are as agronomically or economically important as oilseed rape (*B. napus* & *B. rapa*) (Warwick, 2011). In this discussion, oilseed rape is used as an umbrella term for both canola and high erucic acid rapeseed varieties. Canola, which contains low erucic acid and glucosinolates in the seed, was initially developed by Canadian oilseed breeders (Weselake, 2011). Only a small percentage of Canadian acreage is devoted to the production of high erucic acid rapeseed (Scarth and Tang, 2006). Canola is well suited to the western prairies of Canada as it prefers well-draining, nutrient rich soils and cooler temperatures (15-20°C) (Escobar et al., 2009). In reflection of its suitability, and its profitability, the crop has been rapidly adopted in the plains of Canada. In 2012, for the first time, the land area dedicated to seeding canola in all of Canada surpassed that of the long standing incumbent, wheat (excluding durum) (Canola

Council of Canada, 2013). In addition to ideal growth conditions, a considerable amount of canola's success in Canada can be attributed to the global importance of oilseed crops.

Oilseed crops are of paramount importance to many individuals' diets, and many nations' economies. Up to 80% of the oils consumed originate from plants (Yurchenko et al., 2007), accounting for up to 25% of caloric intakes in industrialized nations (Thelen and Ohlrogge, 2002). Among oilseed crops, oilseed rape is the second most produced crop after soybean (*Glycine max*) (Carré and Pouzet, 2014; Scarth and Tang, 2006). In total, *B. napus* and *B. rapa* contribute about 12% of the world's oil and fat production (Weselake et al., 2008). Ninety percent of canola oil is dedicated to human consumption (Taylor et al., 2009), although new opportunities exist for its use in industrial oils and biofuels (Carlsson, 2009). China, India, Canada and the European Union all devote approximately the same acreage to oilseed rape production, but differences are observed in terms of oilseed rape production. Per annum, the European Union produces 20 million tonnes (MT) of oilseed, followed by Canada (15MT) and China (12MT), and India (6MT) (Carré and Pouzet, 2014). Canada, which only crushes approximately half of its annual oilseed rape production, is by the far the world's largest exporter of oilseed rape, followed distantly by Australia and the Ukraine (Carré and Pouzet, 2014). As of 2007/2008, the European Union has emerged at the largest importer of oilseed rape (Carré and Pouzet, 2014).

The value of oilseed rape oil accounts for 82% of the fluctuation in oilseed rape prices, with the remaining variation predominantly influenced by the price of its meal (Carré and Pouzet, 2014). Triacylglycerol (TAG), which is a storage lipid, is composed of three fatty acids

(FAs) esterified to a glycerol back bone and is the predominant component of oilseed rape seed oil. The appeal of an oil for either industrial or nutritional uses varies according the composition and distribution of the FAs contained within its TAG molecules (Scarth and Tang, 2006). Typically, *B. napus* or *B. rapa* seed oil TAG contains appreciable amounts of oleic (18:1 Δ^{9cis}) and linoleic (18:2 $\Delta^{9cis,\ 12cis}$) FAs, and a large amount (45%) of erucic acid (22:1Δ^{13cis}) (Ackman, 1990). Breeding programs initiated in the 1950s, however, successfully reduced the erucic acid content of *Brassicas* to negligible levels to improve the nutritional value of their oils (Scarth and Tang, 2006). By 1975, low erucic varieties (canola) accounted for 95% of the oilseed rape sewn in Canada (Scarth and Tang, 2006). The varieties currently grown in Canada typically contain 4% palmitic (16:0), 2% stearic (18:0), 62% oleic, 22% linoleic, 10% α-linolenic acid (18:3 $\Delta^{9cis,12cis,15cis}$) and less than 2% erucic acid (Yurchenko et al., 2007). With this FA composition, canola oil is the only commercially available vegetable oil which can be legally labelled 'low in saturates' in either Canada or the United States (Scarth and Tang, 2006).

Through selective breeding and genetic engineering, numerous germplasms have been developed that produce oils suited to different applications (Scarth and Tang, 2006; Yurchenko et al., 2007). These include varieties with either low saturated FA content (Dehesh 2004), low α-linolenic acid content (Scarth et al., 1995a), or low erucic acid content (Stephansson and Downey, 1995). Varieties with high levels of erucic (Scarth et al. 1995b), oleic (Scarth and Tang 2006), lauric (12:0) (Voelker et al. 1996), caprylic (8:0) (Dehesh et al. 1996), capric (10:0) (Dehesh et al. 1996), palmitic (Jones et al., 1995), or stearic acid (Knutzon et al., 1992) have also been developed. Attempts have also been made to introduce unique FAs into oilseed rape oil such as hydroxylated and conjugated FAs as well as very long chain FAs (>20C) (Scarth and

Tang, 2006; Snyder et al., 2009; Yurchenko et al., 2007). Some of these programs demonstrate oilseed rape's potential to produce oils for industrial applications, which can be quite appealing. Plant based lubricants are less eco-toxic and are highly bio-degradable compared to mineral oils (Schneider 2006). An emerging market for canola has grown within the 4.4 billion dollar biofuel industry in Canada. In 2013, 26 biofuel plants were commercially operational in Canada. Approximately 30% of their output by volume was biodiesel, which was primarily produced by the methylation of canola oil (Doyletech Corporation, 2013; Escobar et al., 2009). It has been argued, however, that it would be prudent to only develop such industrial oils in crops not currently used for human consumption, thereby mitigating risk to the food chain (Carlsson, 2009). It thus appears that the attractiveness of canola as a feedstock for industrial oils will likely depend upon a complicated mix of economical, ethical, and practical factors.

In addition to modifying the FA composition of seed oil, increasing seed oil content can also significantly enhance the value of an oilseed crop. It has been estimated that a one percent absolute increase in soybean's oil content would increase the crop's value by an estimated $840 million per annum (Lardizabal et al. 2008). Similarly, a one percent absolute increase in canola seed oil content would raise Canadian crusher's profits by $90 million per annum (Taylor et al. 2009). Recently, the canola industry generated $19.3 billion CAD in annual economic activity in Canada (LMC International, 2013), accounting for just over one percent of its national gross domestic product (Trading Economics, 2014). The vast majority of this revenue (90%) was generated in the prairie provinces Alberta, Saskatchewan and Manitoba with the former two provinces accounting for 74% of the revenue generated by the canola industry in Canada (LMC International, 2013). The canola industry supports almost a quarter million jobs in Canada, of

which, only one percent is involved in seed development (LMC International, 2013). This figure may increase in the future as Canada strives to maintain its position as a world leader in canola production.

In addition to breeding seed oil traits, substantial interest lies in developing germplasm with improved agronomic traits. *Brassica* seed siliques are highly susceptible to precocious breakage, ultimately resulting in substantial reduction in recovered yield (Gan et al. 2008). Shatter-resistant germplasm is commercially available, but there remains great interest in further ameliorating this phenotype of *B. napus*. The Canola Council of Canada has cited increased biotic and abiotic stress tolerance, improved nitrogen efficiency, reduced silique shattering and reduced seed chlorophyll content as major areas of interest (Canola Council of Canada, 2014). Improving these traits in canola through genetic engineering or genome editing is likely to be an acceptable means of germplasm development. As of 2010, *B. napus* genetically engineered for herbicide tolerance (HT) (first introduced in 1995) accounted for 93% of canola planted in Canada (Canola Council of Canada, 2010). This stands in notable contrast to wheat, which is 100% non-transgenic in Canada. It has been estimated farmers seeding transgenic Roundup Ready or Liberty Link canola generate greater than 130$CAD/ha in additional revenue compared to farmers growing open pollinated varieties (Brewin and Malla, 2012). These profits are attained in spite of 25-37$CAD/ha technology usage agreements (TUAs) producers must pay, in addition to increased seed costs, to plant these varieties. Canadian farmers' acceptance of high seed prices and TUAs has led to substantial returns on investment for private seed producers. This has led to the private sector investing heavily in canola research and development in Canada, which now dwarfs public investment by approximately 5-fold (Brewin and Malla,

2012). This highly incentivised research may in turn lead to the rapid development of canola varieties which address many of the desired traits requested by the Canola Council of Canada.

Despite widespread acceptance of transgenic HT canola, no commercially available canola variety has been produced which has been genetically engineered to synthesize more seed oil. To date, traditional breeding programs alone have produced varieties with improved seed oil content. Seed oil content, however, is governed by a large number of quantitative trait loci, which hinders selecting for improved oil content while maintaining superior agronomic traits (Rahman et al., 2013). Development of high performance canola cultivars, thus, may require transforming agronomically superior germplasm with transgenes optimized to improve lipid synthesis in seeds.

2.2. FATTY ACID BIOSYNTHESIS

In living systems, FAs and the glycerol backbone represent the building blocks for TAG formation. Since this thesis examines the properties of recombinant plant TAG-biosynthetic enzymes produced in *Saccharomyces cerevisiae*, it is useful to review FA and TAG biosynthesis in plants with some comparisons of these processes with those occurring in *S. cerevisiae*.

2.2.1. Plant fatty acid biosynthesis

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In plants, the process of FA synthesis occurs in the plastid and provides FAs for incorporation into glycerolipids in both the plastid and cytoplasm. The process of FA synthesis

in plants is summarized in FIGURE 2.1. During the early phases of oilseed embryo development, energy stored as sugars is used to produce FAs in plastids (Baud and Lepiniec, 2010). Plants are unique in this regard, as FA synthesis occurs in the cytosol in species from other kingdoms (Chapman and Ohlrogge, 2012). The process of FA synthesis is initiated by the catalytic action of a dissociable complex called acetyl coenzyme A carboxylase (ACC), which extends a two carbon acetyl-CoA into a three carbon malonyl-CoA, consuming bicarbonate and ATP in the process (Li et al. 2011; Nikolau et al. 2003; Roesler et al. 1994). In both plants (Yurchenko et al. 2007) and yeast (Kohlwein, 2010), this reaction exerts substantial control over the amount of carbon flow into FA synthesis. Indeed, it has been shown that deletion of ACC is lethal in *Arabidopsis* as this step is essential for FA synthesis (Sasaki and Nagano 2004). Not surprisingly, the activity of this enzyme is strongly upregulated in *B. napus* shortly before lipid deposition in seeds (Turnham and Northcote, 1983). Following this step, malonyl-CoA:acyl carrier protein malonyltansferase (MAT) catalyzes the transfer of the malonyl groups onto acyl carrier proteins (ACP) (Baud and Lepiniec, 2010). Malonyl-ACP serves as source of two carbon fragments for extending growing FA chains that are attached to the fatty acid synthase (FAS) complex, which has several catalytic sites involved in the process of FA synthesis (Cahoon and Schmid, 2008). In plants this FAS complex is composed of several small dissociable subunits with each subunit responsible for a step in the FA synthesis process (Chapman and Ohlrogge, 2012). The first step is a condensation reaction catalyzed by the FAS subunit 3-ketoacyl-ACP synthase III (KAS III), which consumes malonyl-ACP moiety to extend a bound acetyl group to produce a bound four carbon acyl chain (Clough et al., 1992). Following this condensation reaction, subunits of the FAS complex carry out three additional reactions to regenerate

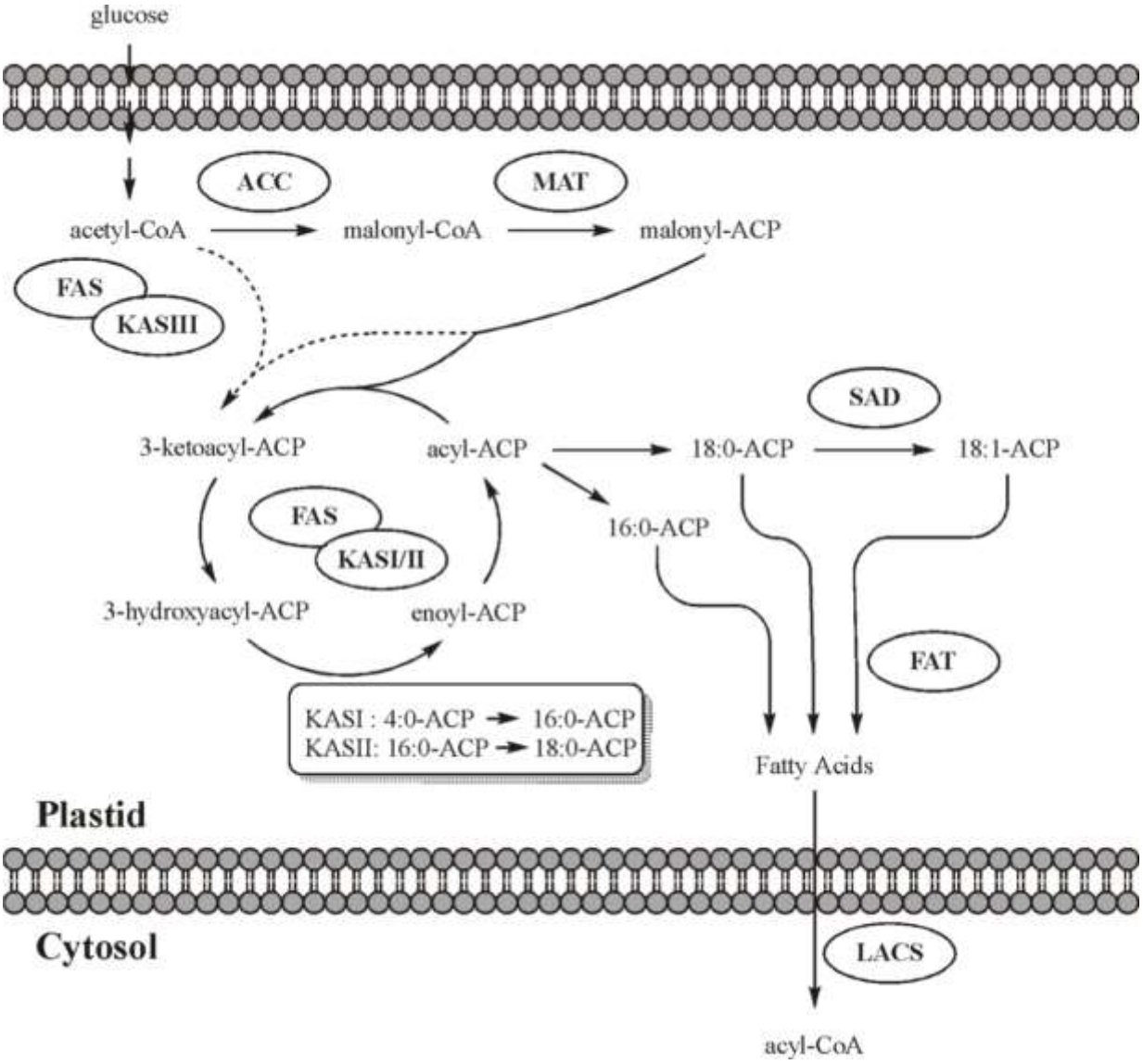

FIGURE 2.1. Schematic representation of fatty acid synthesis in plants. ACC: acetyl Co-enzyme A carboxylase, MAT: malonyl-CoA:acyl carrier protein malonyltansferase, FAS: fatty acid synthase, KAS: 3-ketoacyl-ACP synthase, LACS: long-chain acyl-CoA synthetase, SAD: stearoyl-ACP desaturase, FAT: fatty acyl-ACP thioesterase, ACP: acyl carrier protein.

an acyl-ACP. These reactions are sequentially catalyzed by a 3-ketoacyl-ACP reductase, a 3-hydroxyacyl-ACP dehydratase and an enoyl-ACP reductase (Baud and Lepiniec, 2009). Following this initial condensation/regeneration cycle, KASIII is replaced in the FAS complex by ketoacyl-ACP synthase I (KASI), which through similar cycles catalyzes the extension of the 4 carbon acyl-ACP to 16 carbons. KASI can then be replaced by ketoacyl-ACP synthase II (KASII), which catalyzes the extension of the acyl-ACP by two additional carbons with an additional condensation reaction (Shimakata and Stumpf, 1982). The latter of these products, 18:0-ACP, can be acted upon by a unique soluble desaturase (stearoyl-ACP desaturase) which catalyzes the introduction of a double bond (*cis*) at the Δ9 position. These saturated and monoenoic acyl-ACPs are then liberated as free fatty acids through the catalytic action of fatty acid thioesterase (FAT) A and FATB (Cahoon and Schmid, 2008). The liberated FAs cross the plastidial envelope and are then converted to acyl-coenzymes (CoAs) on the cytosolic side of the plastid through the catalytic action of long-chain acyl-CoA synthetase (LACS) (Baud and Lepiniec, 2010; Schnurr et al., 2002; Li et al., 2015). The transcription factor WRINKLED1 controls expression of *ACC*, and genes encoding subunits of the FAS complex and other enzymes contributing to the production of acetyl-CoA (Chapman and Ohlrogge, 2012). *Arabidopsis wrinkled1* mutants accumulate 50-80% less TAG in seeds compared to wild type plants (Chapman and Ohlrogge, 2012; To et al., 2012).

Cytoplasmic acyl-CoAs are used to support glycerolipid synthesis in the endoplasmic reticulum (ER), which is described in section 2.3.1. The enzymes of glycerolipid metabolism can influence the FA composition of different lipid classes through their substrate selectivity properties (Weselake, 2005). The composition of the acyl-CoA pool can also have an impact on

the FA composition of glycerolipids which are synthesized using these thioesters. For example, suppression of plant stearoyl-ACP desaturase can increase stearic levels of up to 40% in seed oil (Knutzon et al., 1992; Thelen and Ohlrogge, 2002). Expressing cDNAs encoding thioesterases from *Cuphea hookeriana* or *Umbellularia californica* in canola has been shown to result in seed oil containing 12% caprylic and 48% lauric acid, respectively (Hildebrand and Yu, 2003). These thioesterases catalyzed the release of FAs from the FAS complex at a shorter chain length. FA synthesis provides the building blocks to support TAG synthesis, making it a crucial piece of the overall lipid biosynthetic picture.

2.2.2. Fatty acid biosynthesis in *Saccharomyces cerevisiae*

Yeast has the ability to uptake FAs from the growth medium (Tehlivets et al., 2007). When exogenous FAs are not available, however, *de novo* FA synthesis generally follows the same progression as in plants. In *S. cerevisiae*, a highly regulated cytosolic ACC catalyzes the production of malonyl-CoA which then supplies the FAS complex with two carbon fragments for FA synthesis (Kohlwein, 2010). Unlike the multimeric plastidial FAS of plants, the cytosolic *S. cerevisiae* FAS complex is made up of two subunits, which catalyze the same series of reactions (Tehlivets et al., 2007). It should be noted that *S. cerevisiae* does possess a multimeric FAS complex, similar to that of plants, which resides in mitochondria (Hiltunen et al., 2005). The purpose of FA synthesis in *S. cerevisiae* mitochondria is still not well understood, but evidence suggests this process occurs to produce octanoyl-ACP, which is used to synthesize a co-factor (lipoic acid) necessary for oxidative decarboxylation reactions (Tehlivets et al., 2007). At the termination of FA synthesis, the *S. cerevisiae* cytosolic FAS complex directly catalyzes

the transfer of the acyl chain from ACP to CoA (Tehlivets et al., 2007). By and large, the products of *S. cerevisiae* FA synthesis are 16:0 and 18:0, of which 80% are converted to monoenoic FAs by the catalytic action of Δ9 desaturase Ole1 (Tehlivets et al., 2007).

2.3. TRIACYLGLYCEROL BIOSYNTHESIS

2.3.1. Fatty acid modification, acyl-editing and triacylglycerol assembly in developing seeds of oleaginous plants

FA chains derived from cytoplasmic acyl-CoAs are used to form TAG in a process called the Kennedy or *sn*-glycerol-3-phosphate pathway (Kennedy, 1961). Most of the enzymes catalyzing FA modification, TAG assembly, and associated membrane metabolism, are bound to the ER. As shown in FIGURE 2.2, TAG assembly is initiated by acyl-CoA: *sn*-glycerol-3-phosphate acyltransferase (GPAT) which catalyzes the acylation of *sn*-glycerol-3-phosphate (G3P) at the *sn*-1 position, yielding lysophosphatidic acid (LPA) (Chen et al., 2011). LPA is then further acylated at the *sn*-2 position to form phosphatidic acid (PA) through the catalytic action of acyl-CoA:lysophosphatidic acid acyltransferase (LPAAT) (Maisonneuve et al., 2010). The phosphate group of PA is then released through the catalytic action of phosphatidic acid phosphatase (PAP) to produce *sn*-1,2-diacylglycerol (DAG) (Mietkiewska et al., 2011). Finally, acyl-CoA: diacylglycerol acyltransferase (DGAT) catalyzes the acylation of DAG at the *sn*-3 position to produce TAG (Jako et al., 2001). TAG can also be synthesized in a non-acyl-CoA dependant manner by the action of phospholipid: diacylglycerol acyltransferase (PDAT),

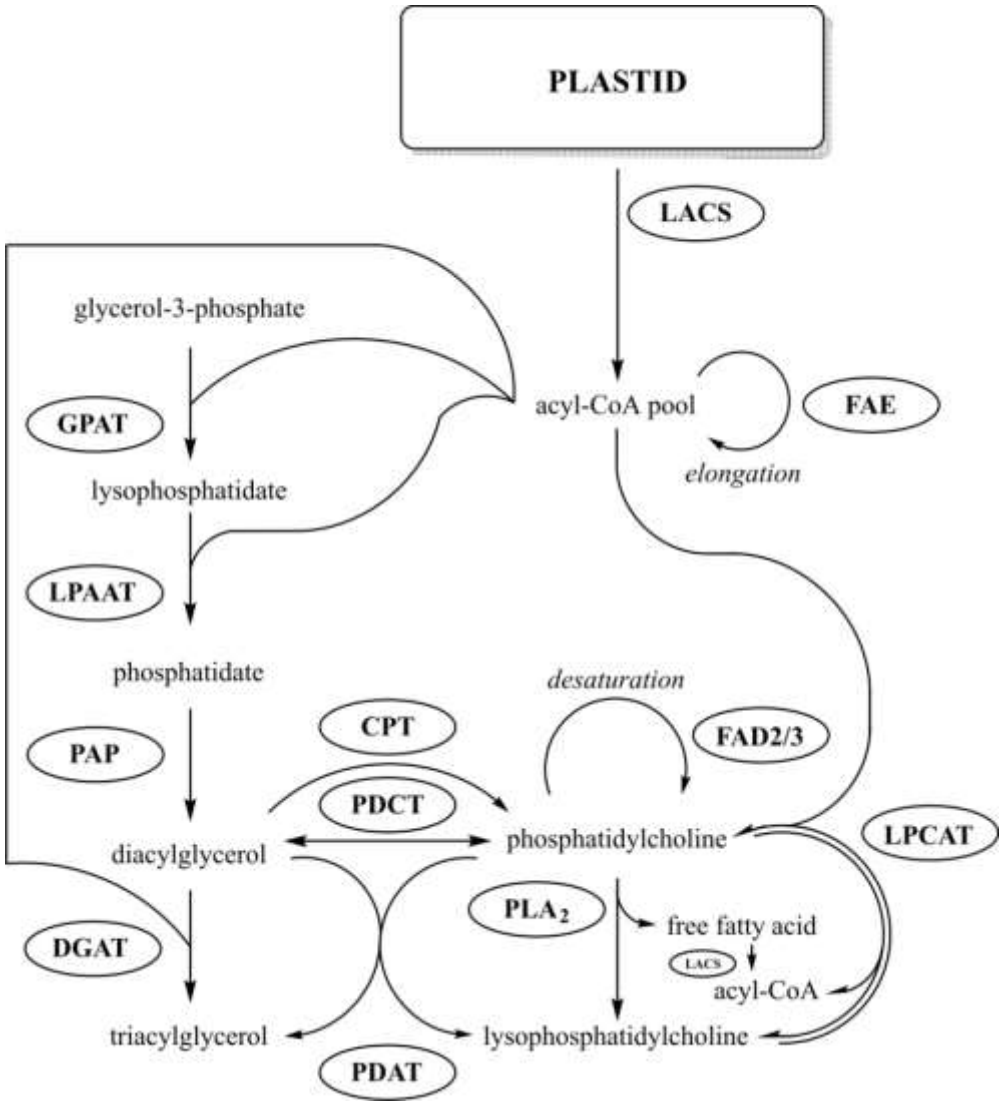

FIGURE 2.2. Schematic representation of fatty acid modification, acyl-editing and triacylglycerol assembly in plants. GPAT: acyl-CoA:*sn*-glycerol-3-phosphate acyltransferase, LPAAT: acyl-CoA:lysophosphatidic acyltransferase, PAP: Phosphatidic acid phosphatase, DGAT: acyl-CoA:diacylglycerol acyltransferase, CPT: CDP-choline:1,2-diacyl-*sn*-gycerol cholinephosphotransferase, PDCT: phosphatidylcholine: diacylglycerol cholinephosphotransferase, PDAT: phospholipid: diacylglycerol acyltransferase, PLA_2: phospholipase A2, FAD: fatty acid desaturase, LPCAT: acyl-CoA:lysophosphatidylcholine acyltransferase, FAE: fatty acid elongase, LACS: long-chain acyl-CoA synthetase.

which catalyzes the transfer of a fatty acid from the *sn*-2 position of phosphatidylcholine (PC) to DAG (Ståhl et al., 2004). FAs exported from the plastid into the cytoplasm can be further modified at the level of acyl-CoA and PC. Acyl-CoAs can also be further elongated by the action of a fatty acid elongase (FAE) complex which is located in the ER (Ghanevati and Jaworski, 2001; Rossak et al., 2001). Unlike the action of stearoyl-ACP desaturase, FA desaturation outside the plastid targets FAs bound to phospholipids. Fatty acid desaturase (FAD) 2 and FAD3 enzymes, which sequentially catalyze the introduction of double bonds at the Δ12 and Δ15 carbons of fatty acid chains, respectively, primarily act upon PC (Browse et al., 1993; Ohlrogge and Browse, 1995; Okuley et al., 1994). FAs which are modified at the level of PC can eventually enter TAG through various routes of acyl-editing (Weselake et al., 2009; Bates and Browse, 2012). One route involves the transfer of PUFA from the *sn*-2 position of PC to DAG through the catalytic action of PDAT to form PUFA-enriched TAG. A second possibility involves the action of the Lands cycle (Lands, 1958), wherein PUFA are liberated from PC through the catalytic action of phospholipase A_2 (PLA_2) followed by formation of PUFA-CoA catalyzed by LACS. The vacant *sn*-2 position on the resulting lysophosphatidylcholine (LPC) is then re-acylated through the catalytic action of acyl-CoA: lysophosphatidylcholine acyltransferase (LPCAT). Though seemingly futile, this cycle may yield a PC with an oleoyl moiety at the *sn*-2 position and a PUFA-CoA for the cytoplasmic acyl-CoA pool. These newly added PUFA-CoAs could then be incorporated into TAG via the Kennedy pathway. Indeed, *lpcat* double mutants were observed to exhibit slightly reduced PUFA content in their seed oil, but not enough to suggest the Lands cycle plays a significant role in shuttling PUFAs into TAG in *Arabidopsis* (Wang et al., 2012). This appears to be in contradiction with other findings published the same year. Xu et al. (2012) found that the *Arabidopsis dgat1/lpcat2* double mutant

accumulates 65% less seed oil than the wild type, a far lower amount than the 30% reduction in seed oil content observed in the *dgat1* mutant. As *PDAT* transcription is upregulated by 65% in the absence of *DGAT1*, it was suggested that LPCAT2-catalyzed reaction may play a role in providing PDAT with PC to produce TAG. There is also some evidence to suggest that PUFA-derived PC may enter the acyl-CoA pool through the combined reverse/forward reactions catalyzed by LPCAT in a process referred to as acyl-exchange (Yurchenko et al., 2009).

In addition to the uncertainty surrounding the transfer of PUFA and other ER-modified FAs from the *sn*-2 position of PC to the acyl-CoA pool, an additional layer of complexity in TAG assembly has recently been identified. Labelling experiments conducted with developing soybean or *Arabidopsis* seeds have identified that the majority of *de novo* DAG is converted into PC by CDP-choline:1,2-diacyl-*sn*-gycerol cholinephosphotransferase (CPT), which then serves as a substrate for FA modification to produce PUFA or unusual FAs such as ricinoleic acid (OH-18:1Δ^{9cis}). This PC is then converted into DAG skeletons which are used for TAG synthesis (Bates et al., 2009; Bates et al., 2012). It is unclear how this second pool of modified DAG is created *in vivo*. The modified DAG pool may be created through the catalytic action of phospholipase C (PLC), which catalyzes the removal of the phosphocholine head group of PC to produce DAG (Bates et al., 2009). An alternative route involves the action of phosphatidylcholine: diacylglycerol cholinephosphotransferase (PDCT), which catalyzes the transfer of the phosphocholine head group of PC to a DAG. Knock out of the *PDCT* gene resulted in 40% less 18:2 and 18:3 FA contents in *Arabidopsis* seed oil which correlated with decreased 18:1 levels in PC (Lu et al., 2009). PDCT appeared to be involved transferring phosphocholine from modified PC to produce modified DAG and adding phosphocholine to *de*

novo DAG to form PC for further FA modification. There is still much to learn about acyl editing as it relates to TAG biosynthesis in plants. For example, the extent to which various routes for acyl editing are operative may vary among different plant species (Mietkiewska et al., 2014).

TAG produced in the ER eventually leads to the formation of oil bodies or lipid droplets which vary in size from about 0.2 to 2 microns (Weselake, 2005). These oil bodies are composed of a phospholipid monolayer surrounding an inner core of TAG and sterol-esters, and can occupy 60% of the volume of an *Arabidopsis* seed at maturity (Baud and Lepiniec, 2009). To date, little is known about how oil bodies develop and disassociate from the ER (Chapman et al., 2012). It is believed, that TAG accumulates within the leaflets of the ER membrane, eventually forming a bulge. This bulge then grows into an emerging droplet which pinches off of the ER. Oil bodies are coated with oleosin proteins which assist in regulating their size and prevent their coalescing once free of the ER. Oleosin may also assist in recruiting lipases during lipid breakdown in germinating seedlings (Chapman et al., 2012). Once created, however, the oil bodies are thought to be metabolically inert until they are acted upon by lipases.

2.3.2. Storage lipid biosynthesis in *Saccharomyces cerevisiae*

S. cerevisiae generally assembles and packages TAG in a similar fashion to oil-forming plants (Kohlwein, 2010; Tehlivets et al., 2007). A notable caveat, however, is that *S. cerevisiae* has the ability to generate LPA through a second pathway. Here, dihydroxyacetone phosphate (DHAP) is acylated through the catalytic action of the same enzymes which catalyze the acylation of *sn*-glycerol-3-phospate, to produce 1-acyl-DHAP. This compound is then reduced

in a reaction which consumes NADPH to produce LPA (Czabany et al., 2007). In plants, however, DHAP is directly converted to *sn*-glycerol-3-phospate by the catalytic action of a cytosolic dehydrogenase, which consumes NADH in the process (Weselake et al., 2009). This unique pathway may not play a major role in TAG formation *in situ,* because blocking the oxidation of G3P to DHAP was shown to increase TAG production (Dulermo and Nicaud, 2011) Interestingly, it has been shown that TAG synthesis is non-essential for survival and growth of yeast cells, though growth penalties and increased sensitivity to free FAs are incurred (Petschnigg et al., 2009; Sandager et al., 2002; Siloto et al., 2009b). It has also been shown that yeast *pdat* mutant strains accumulate 40% less TAG (Sorger and Daum, 2002). The majority of PDAT's action contributing to TAG biosynthesis occurs during the exponential growth phase. DGAT activity increases from 25% during the exponential growth phase, to eventually representing half of all TAG biosynthetic activity during the stationary phase (Oelkers et al., 2002). Two sterol acyltransferase have been demonstrated to have small amounts of DGAT activity as well, but 90% of acyl-CoA-dependent TAG synthesis in microsomal fractions is the product of DGAT action in *S. cerevisiae* (Oelkers et al., 2002) .

2.4. PROPERTIES AND REGULATION OF DIACYLGLYCEROL ACYLTRANSFERASE

2.4.1. Identification of plant *DIACYGLYCEROL ACYLTRANSFERASE* genes

Nearly 50 years after the first reports of DGAT activity, the first gene encoding a DGAT was identified in the murine (*Mus musculus*) genome, aided by its sequence homology to an

enzyme of similar activity called acyl-CoA: cholesterol acyltransferase (ACAT) (Cases et al., 1998; Liu et al., 2012). Shortly thereafter, four groups reported on the functional characterization of a cDNA encoding *Arabidopsis* DGAT (Bouvier-Nave et al., 2000; Hobbs et al., 1999; Routaboul et al., 1999; Zou et al., 1999). A cDNA (GenBank ID: AF164434) encoding an isoform *B. napus* DGAT1 was first cloned by Nykiforuk et al. (1999b; 2002). Recently, another isoform of recombinant *B. napus* DGAT1, systematically described as BnaC.DGAT1.a (corresponding to GenBank ID: JN224473), was purified to apparent homogeneity in an active form (Caldo et al. 2015).

Two years following the initial identification of *AtDGAT1*, protein sequencing of a fungal enzyme isolated from lipid bodies led to the discovery of *DGAT2* (Lardizabal et al., 2001). This same group was then able to identify a *DGAT2* homolog in *Arabidopsis*. More recently, nucleic acid sequences encoding a cytosolic DGAT3 were discovered first in peanut (*Arachis hypogaea*), and then in *Arabidopsis* (Hernandez et al., 2012; Saha et al., 2006). Analysis of an *Arabidopsis* mutant defective in cuticular ridges (DCR) led to the discovery of a gene encoding a second soluble DGAT (Rani et al., 2010). A bi-functional enzyme with both wax ester synthase and DGAT activity (known as WS/DGAT) was discovered in bacteria (Kalscheuer and Steinbuchel, 2003), and subsequently found to also be present in *Arabidopsis* (Li et al., 2008a). Surprisingly, although the *Arabidopsis* genome contains only one copy of a *DGAT1* gene, it possesses 11 putative copies of *WS/DGAT* (Li et al., 2008a).

Identification of these *DGAT* coding sequences allowed for the analysis of the evolutionary relationship between the genes. Interestingly, the genes *DGAT1*, *DGAT2* and

WS/DGAT, which encode membrane-bound enzymes, share no sequence homology and have evolved through convergent evolution (Cao, 2011; Liu et al., 2012). The peanut *DGAT3* gene shares less than ten percent DNA sequence homology with *DGAT1* or *DGAT2*, and only marginally higher sequence homology (13%) with *WS/DGAT* (Saha et al., 2006). DCR is a member of the BAHD (BEAT, AHCTs, HCBT and DAT- enzymes within the family) acyltransferase family (Luo et al., 2007; Panikashvili et al., 2009) and is distinct from the other DGAT enzymes as well.

2.4.2. Localization and topology of diacylglycerol acyltransferases

As mentioned previously, DCR and DGAT3 are soluble cytosolic enzymes (Chi et al., 2014; Rani et al., 2010), whereas the other three DGAT families are composed of integral membrane bound proteins. Both DGAT1 and DGAT2 localize to the ER, and may also localize to lipid droplets to some extent (Aymé et al., 2014; Bouvier-Nave et al., 2000). Interestingly, epitope-tagged DGAT1 and DGAT2 from tung tree (*Vernicia fordii*) appear to localize to different micro-domains within the ER when heterologously expressed in tobacco (*Nicotiana tabacum*) leaf cells (Shockey et al., 2006). The significance of this precise targeting has yet to be uncovered. Although analyses are limited, at least one *Arabidopsis* WS/DGAT isoform has been confirmed by confocal microscopy to localize to the ER as well (Li et al., 2008a).

Using a mobility shift assay, alternatively known as the scanning cysteine accessibility method, it was demonstrated that *S. cerevisiae* DGAT2 possess four transmembrane domains (Liu et al. 2011). These results, however, stand in contradiction to earlier work by Stone et al.

(2006), which found that murine DGAT2 possesses two transmembrane domains. Potentially, this discrepancy results from the Stone group cloning epitopes into the DGAT2 amino acid sequence to permit their protease protection assay. The introduction of these epitopes may have caused the murine DGAT2 to incorrectly insert into the ER membrane. Both authors agree, however, that the amino and carboxyl terminal ends of DGAT2 are located in the cytosol.

In silico analysis of the type-1 DGATs generally predict these enzymes have between 8-10 transmembrane domains (Liu et al., 2011). Previously, it was reported that, similarly to DGAT2, both termini of *Vf*DGAT1 face the cytosolic side of the ER (Shockey et al., 2006). This may also, however, be of some controversy. Protease protection analyses of murine DGAT1 suggested that the majority of the enzyme, including the C-terminus, is present in the ER lumen (McFie et al., 2010). Further confounding the subject, work using membrane permeases found that DGAT1 activity and inhibitor sensitivity can be detected on both sides of the ER membrane, suggesting this DGAT1 adopts a dual topology in the membrane (Wurie et al., 2011). Additional investigation will be required to reveal the natural conformation DGAT1 adopts within the ER membrane.

In silico analyses suggest WS/DGAT contains at least four transmembrane domains (Li et al., 2008a). This prediction, however, still requires experimental validation. The difficulties encountered assigning reliable topologies to membrane-bound DGATs serves as an example of the typical challenges faced when studying structure/function in this class of enzymes (Liu et al., 2012).

2.4.3. Expression of plant *DIACYLGLYCEROL ACYLTRANSFERASE* genes

Northern blot analysis of *DGAT1* expression in *Arabidopsis* revealed this gene is expressed at a high level in germinating seeds and seedlings, and at lower levels in leaf, root, flowers and siliques (Zou et al., 1999). Similar analysis by Hobbs et al. (1999) found the same general expression pattern, but with the highest *AtDGAT1* expression present in embryos at the cotyledonary stage. Later analysis of *Arabidopsis* lines transformed with *DGAT1* promoter::*GUS* fusion constructs would support Hobbs' expression pattern (Lu et al., 2003). The discrepancy between the *DGAT1* expression patterns found by Zou et al. (1999) and Hobbs et al. (1999) may result from the latter isolating developing embryos from the silique prior to RNA isolation. Alternatively, the authors may have isolated RNA from the *Arabidopsis* seeds or siliques at different developmental stages. Indeed, later work by Li et al. (2010) showed that *AtDGAT1* expression levels in developing embryos are not static. *DGAT1* expression steadily increases from a base level to a maximum in late embryo development, followed by a sharp decline in late seed maturation (Li et al., 2010).

The same study by Li et al. (2010) observed that *AtDGAT2* expression increases during embryo development, but far below the levels observed for *AtDGAT1*. This study also found that *AtPDAT* transcription was only slightly induced as well, but to a higher extent than that of *AtDGAT2*. In contrast to the low *DGAT2* transcription observed in *Arabidopsis* embryos, *DGAT2* expression increased more dramatically than *DGAT1* in developing tung tree and castor bean (*Ricinis communis*) seeds (Kroon et al., 2006; Shockey et al., 2006). Thus, it appears that the relative contributions of different TAG biosynthetic enzymes can be species specific (Liu et

al. 2012). Li et al. (2010) also observed *AtDGAT2* transcript abundance was higher in roots, stems and leaves than in embryos.

The mRNA product of *WSD1*, a WS/DGAT-encoding gene from *Arabidopsis*, was detected most strongly in flower petals, and to a lesser extent in stem tops and leaves. Significantly lower levels were detected in roots and seeds (Li et al., 2008a).

The expression patterns of the soluble DGATs may suggest these enzymes perform different biological roles *in situ*. The expression of the peanut *DGAT3* varies through different stages of seed maturation. This gene is most strongly expressed in leaf and flower organs (Chi et al., 2014). In contrast, *GUS*/promoter fusion assays indicate *AtDCR* is strongly expressed throughout embryo development (Panikashvili et al., 2009). Its expression can also be detected in numerous tissues including root caps and lateral root initiation sites, in trichomes, cotyledons and in young developing leaves. Although seed FA content was not measured, *Atdcr* mutants produced shrunken seeds (Panikashvili et al., 2009), a characteristic phenotype of reduced TAG production (Rani et al., 2010).

The above expression patterns appear to suggest that different DGAT types may perform different biological roles in plants. In some instances, however, individual DGATs may perform more than one biological role. For example, in addition to having an important role in seed oil accumulation, *DGAT1* transcription can be upregulated by high salt or low nitrogen, and by the stress hormones jasmonic acid, salicylic acid, and abscisic acid (Kong et al., 2013; Yang et al., 2011). *DGAT1* expression has also been detected in senescing leaves (Kaup et al., 2002). A

putative roll for DGAT activity during stress is to safely sequester acyl-chains liberated during rapid membrane remodelling. In a similar scenario, *DGAT3* is expressed in germinating seeds, where lipases rapidly catalyze the hydrolysis of TAG to fuel seedling growth. If the activity of are not balanced with beta-oxidation, toxic levels of free FAs can accumulate in the cytosol. *DGAT3*, which is expressed at higher levels than *DGAT1* or *DGAT2* in germinating *Arabidopsis* seedlings has thus been suggested to play a 'housekeeping' role in maintaining cellular acyl-CoA pool size and composition (Hernandez et al., 2012).

2.4.4. Post-transcriptional control of diacylglycerol acyltransferases

It has been noted in plants heterologously expressing *DGAT* coding sequences that the observed increases in *DGAT* transcript abundance are disproportionate to observed increases in cellular DGAT activity. For example, over-expression of *DGAT1* in *B. napus* produced a twenty-fold increase in *DGAT1* seed transcript levels, but only five- to ten-fold higher DGAT activity in seeds, and only two to seven percent more seed oil (Taylor et al., 2009). A similar phenomenon was observed in mouse adipocytes, where increasing *DGAT1* expression by 20-fold only increased DGAT activity by two-fold (Yu et al., 2002). The suppression of DGAT activity in these transgenic lines may suggest that DGAT can be directly, or indirectly, regulated at the post-transcriptional level.

Despite substantial interest in studying the mechanism(s) of DGAT regulation, the highly hydrophobic nature of ER-bound DGAT often complicates structure/function analyses (Liu et al., 2012). In spite of these difficulties, however, progress has been made in identifying

important regions in DGAT1 polypeptide sequences. Two amino acid stretches within bovine DGAT1 polypeptide sequence have been shown to change conformation in the presence of oleoyl-CoA or *sn*-1,2-dioleoylglycerol, suggesting they may be substrate binding sites (Lopes et al., 2014). The hydrophilic N-terminal region of DGAT1 has been identified as a region of interest as well. Analysis of truncated DGAT1s from mammalian and plant sources has demonstrated that this domain is unnecessary for catalysis (McFie et al., 2010; Siloto et al., 2009a). Furthermore, this region of mouse and *B. napus* DGAT1s has been demonstrated to bind different acyl-CoAs with different affinities, suggesting a possible regulatory role for this domain (Siloto et al. 2008; Weselake et al. 2006). The N-terminal domain of *B. napus* DGAT1 has also been shown to self-associate (Weselake et al., 2006), which is in agreement with homo-dimerization of the mammalian enzyme (Cheng et al., 2001; McFie et al., 2010). Interestingly, deletion of the amino acids within the N-terminus which promote homo-dimerization led to a 14-fold increase in DGAT activity, suggesting self-association through the N-terminus may regulate this enzyme's activity (McFie et al., 2010). DGAT1 may also be capable of self-associating through a second binding site located outside of the N-terminus, allowing for the formation of homo-tetramers (Cheng et al. 2001; McFie et al., 2010). It has been suggested that the active form of DGAT1 may be the dimer structure, whereas the tetramer may be a down-regulated form (McFie et al., 2010).

Early findings showing that incubating mammalian DGAT1 with ATP and cytosolic fractions could inactivate the enzyme supported *in silico* analyses that identified potential phosphorylation sites in the enzyme (Lau and Rodriguez, 1996). To date, however, no further evidence has supported this mode of DGAT1 regulation, and site directed mutagenesis of

potential phosphorylation sites has not resulted in altered mammalian DGAT activity (Yen et al., 2008).

2.4.5. Regulation and properties of diacylglycerol acyltransferases from *Saccharomyces cerevisiae*

S. cerevisiae possess a type-2 *DGAT* in its genome, known as *DGA1*, but not a *DGAT1* (Oelkers et al., 2002). DGA1 has been the subject of numerous studies focused on detailing its regulation and biochemical properties. *In vitro* DGAT assays performed using microsomal extracts from *S. cerevisiae* triple mutant lines with no other TAG synthetic enzymes (Δ*are1,* Δ*are2,* Δ*lro1, DGA1*) suggest DGA1 prefers using oleoyl- or palmitoyl-CoA as a substrate when *sn*-1-steroyl-*sn*-2-arachidonyl--glycerol is provided as an acyl-acceptor (Oelkers et al., 2002). DGA1 has a molecular mass of 47 kDa and an isoelectric point of 10.4 (Czabany et al., 2007). *In vitro* activity assays performed using lipid particles recovered Δ*dga1* mutants, and strains over-expressing *DGA1*, produced strong evidence that DGA1 is present in both the ER and lipid droplets (Sorger and Daum, 2002). Lipid particles derived from the *dga1* strain of *S. cerevisiae* only possess two percent of the DGAT activity of lipid particles recovered from wild type *S. cerevisiae*.

Some progress has been made in understanding the post-translational control of DGA1. *S. cerevisiae* Δ*snf2* cultures over-expressing *DGA1* produced significantly more TAG than wild-type cultures expressing the same transgene (Kamisaka et al., 2007). *SNF* encodes a DNA-dependent ATPase component the SWI/SNF (switching/sucrose non-fermenting) chromatin remodeling complex, and thus regulates the expression of many genes. Following the discovery

of its importance to lipid metabolism, ensuing studies demonstrated that the N-terminal region of DGA1 is cleaved in the Δ*snf2* background, resulting in a more active (four-fold) form of the enzyme (Kamisaka et al., 2010). Truncated versions of DGA1 produced through genetic engineering mimicked the naturally cleaved DGA1's high activity. The protease controlled by SNF2 was not, however, identified (Kamisaka et al., 2010). In a phenomenal achievement, the lipid titer of *Y. lipolytica* was increased by 60-fold using a combination of genetic engineering and culture condition optimization (Blazeck et al., 2014). Here, combining the over-expression of *DGA1,* blocking peroxisomal oxidation of fatty acids, introducing a leucine biosynthetic pathway, and promoting growth through genetic engineering raised the lipid content of this yeast from 17 to 74 percent. Optimization of culture conditions further increased this genetically engineered strain's lipid content up to ninety percent. It would be of great interest to observe if this strain's lipid content could be increased even higher by replacing the over-expressed *DGA1* with a truncated form of *DGA1*.

In contrast to *S. cerevisiae*, some oleaginous yeasts, such as *Y. lipolytica*, produce a type-1 DGAT, known as DGA2 (Beopoulos et al., 2012). Over-expressing *YlDGA2* in a *Y. lipolytica* quadruple mutant, which is endogenously devoid of DGAT activity, produces cultures with significantly more oil than those over-expressing *YlDGA1* (*DGAT2*) or *YlLRO1* (*PDAT*). The Δ*dga2* single mutant also produced significantly less TAG than Δ*dga1* or Δ*lro1* mutants after 11 hours of incubation. After 48 hours of incubation, however, Δ*dga2* mutants contained more TAG then Δ*dga1* or Δ*lro1* strains, suggesting yeast DGAT1 may play a more predominant role in TAG formation at earlier stage of the stationary phase, but less of a role compared to DGAT2 in the later stationary phase (Beopoulos et al., 2012).

2.5. *IN VITRO* DIACYLGLCYEROL ACYLTRANSFERASE ACTIVITY ASSAYS

2.5.1. Conventional *in vitro* diacylglycerol acyltransferase activity assays

In vitro assays have been one of the most successfully employed techniques to study the properties of DGAT enzymes. These kinetic assays are often used to compare substrate preferences of DGATs from different sources. They can also assist in describing how different molecules, mutations or conditions affect DGAT activity. Typical *in vitro* DGAT assays are performed by incubating microsomes in the presence of ^{14}C-labeled substrates (Little et al., 1994). In many cases, 100 000g microsome fractions are isolated and used for DGAT reactions. These membrane fractions often contain mixtures of DGAT1, DGAT2 and PDAT proteins which can complicate the enzymatic analysis (Liu et al., 2012). Recently, yeast expression systems which are devoid of endogenous TAG-biosynthetic activity have been developed rendering the mutant yeast a suitable host for heterologous expression of cDNAs encoding DGATs from various sources (Beopoulos et al., 2012; Sandager et al., 2002).

No set of parameters has been universally accepted as standard conditions for DGAT assays. Wide variation in acyl-CoA concentration and DAG reaction mixture content are seen in the literature. Routine DGAT analyses have been reported using 5 to 500 µM acyl-CoA (McFie and Stone, 2011; Yu et al., 2008) and 10-400 µM bulk DAG (Bouvier-Nave et al., 2000; Hobbs et al., 1999). Both radiolabeled acyl-CoAs and radiolabeled DAGs have been employed for

DGAT assays, but typically the former is preferred. Radiolabeled acyl-CoA can be readily synthesized from less expensive radioactive free FA (Taylor et al., 1990). Assays using radiolabeled DAG are potentially subject to interference by PDAT activity which can also utilize DAG to synthesize TAG. DGAT enzymes have also been shown to acylate DAG endogenous to microsomal membranes, thus interfering with the use of exogenously supplied DAG (Valencia-Turcotte and Rodríguez-Sotres, 2001). The inclusion of the surfactant Tween-20 in reaction mixtures has been shown to increase *in vitro* DGAT activity (Cao and Huang, 1986). The detergent has proven useful in the dispersion of DAG, making the substrate more accessible for DGAT action (Lung and Weselake, 2006).

Lung and Weselake (2006) have surveyed the literature summarizing the effects of a number of metabolites and reaction conditions on DGAT activity. The addition of increasing concentrations of magnesium chloride or free CoA have been shown to dramatically increase the *in vitro* DGAT activity of microsomes isolated from *B. napus* microspore-derived cell suspension cultures (Byers et al., 1999). It is unclear, however, which enzyme forms (DGAT1 versus DGAT2) were affected by these co-factors. Byers et al. (1999) reported that a low molecular weight organic molecule found in the cytosolic fraction of these cells stimulated DGAT activity in microsomes from *B. napus* microspore-derived cell suspension cultures. Further research is required to identify this stimulatory molecule and to determine whether it acts on DGAT1 or DGAT2 or both isozymes.

Quantifying the incorporation rate of radiolabeled acyl group into TAG requires that the product be separated from the other radiolabeled species. These additional radioactive species

consist primarily of the unreacted substrate, but may also include glycerolipids and free FAs created by the activities of other enzymes in the microsomal fraction. The isolation of TAG is often accomplished by thin layer chromatography (TLC) (Little et al., 1994) which is sometimes preceded by a lipid extraction step (Cao and Huang, 1986). Once isolated, TAG produced by DGAT activity can be quantified by scintillation or imaging techniques. TLC, however, cannot separate TAGs of similar acyl composition. More cumbersome protocols, therefore, are required to analyze DGAT substrate selectivity properties in reactions where more than one acyl-CoA or DAG is made available to DGAT. Most commonly, this is performed by enzymatically or chemically removing acyl chains from the TAG backbone, followed by a second round of TLC to separate acyl chains of different carbon length and desaturation. Once isolated, these acyl chains can then be quantified by the same means described above (Oo and Chew, 1992).

2.5.2. Development of novel *in vitro* diacylglycerol acyltransferase activity assays methods

To avoid the high cost and potential dangers presented by using radiolabeled substrates, fluorescence-based DGAT assays have been developed. These assays use either fluorescently-labeled substrate analogs or fluorescent dyes which measure the release of CoA from acyl-CoA (Cao et al., 2011; McFie and Stone, 2011; Sanderson and Venable, 2012). As an additional advantage to improved safety and cost, many devices capable of quantifying fluorescent emissions are capable of rapidly measuring numerous samples, allowing for so called 'high-throughput' DGAT assays. The variety of fluorescently labeled analogs which are commercially available, however, is limited (CHAPTER 4). Measuring the release of free CoA with fluorescent dyes avoids this issue, but numerous side reactions which release free CoA can occur

during the assay, thus interfering with the quantification of DGAT activity. DGAT enzymes also have notoriously low activities, which are more readily quantified using scintillation detection of radiolabeled compounds (Siloto and Weselake, 2010). Fluorescence-based DGAT assays make them more amenable to high throughput analyses rather than for accurate determination of DGAT specific activity.

An adaptation of the classical radiolabel-based DGAT assay which is amenable to high-throughput has recently been developed (Seethala et al., 2008). This assay uses scintillation beads which bind to TAG, leading to a close association of scintillant and the tritium labeled TAG. A similar function can be accomplished using specially designed microplates which mimic the function of the scintillation beads, also producing a high-throughput assay (Qi et al., 2010). Although these methods do not avoid the use of radioactive species, they considerably increase the speed at which assessment of DGAT activity can be performed.

At the present time, no single method eliminates all the shortfalls of *in vitro* DGAT assays. Future work in DGAT assay development will likely improve the accuracy and speed of high-throughput methods.

CHAPTER 3 - ENGINEERING INCREASED TRIACYLGLYCEROL ACCUMULATION IN *SACCHAROMYCES CEREVISIAE* USING A MODIFIED TYPE-1 PLANT DIACYLGLYCEROL ACYLTRANSFERASE

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3.1. ABSTRACT

Diacylglycerol acyltransferase (DGAT) catalyzes the acyl-CoA-dependent acylation of *sn*-1, 2 diacylglycerol to produce triacylglycerol (TAG). This enzyme, which is critical to numerous facets of oilseed development, has been highlighted as a genetic engineering target to increase storage lipid production in microorganisms designed for biofuel applications. Here, four transcriptionally active *DGAT1* genes were identified and characterized from the oil crop *Brassica napus*. Over-expression of each *BnaDGAT1* in *Saccharomyces cerevisiae* led to an increase TAG biosynthesis. Further studies showed that adding an N-terminal tag could mask the deleterious influence of the DGAT native N-terminal sequences, resulting in increased *in vivo* accumulation of the polypeptides and an increase of up to about 150-fold in *in vitro* enzyme activity. The levels of TAG and total lipid fatty acids in *S. cerevisiae* expressing the N-terminally-tagged BnaDGAT1.b at 72 hours were 53% and 28% higher than those in cultures producing untagged BnaA.DGAT1.b, respectively. These modified DGAT catalyzed the synthesis of up to 453 mg fatty acid/L by this time point. The results will be of benefit in the biochemical analysis of recombinant DGAT1 produced through heterologous expression in yeast, and offer a new approach to increase storage lipid content in yeast for industrial applications.

Keywords: Yeast, Diacylglycerol acyltransferase, Triacylglycerol, *Brassica napus*, Biofuel, Lipid

3.2. INTRODUCTION

Triacylglycerol (TAG) serves as feedstock for the production of biodiesel, which is composed of fatty acid alkyl esters (Knothe, 2005). Biodiesel derived from plant TAG is nearly neutral with regard to production of carbon dioxide because the hydrocarbon chains of TAG (and biodiesel) are derived from the photosynthetically driven capture of carbon dioxide (Durrett et al., 2008; Lackey and Paulson, 2011). There have been, however, serious concerns expressed about plant-derived biodiesel leading to increased food prices or environmental damage due to increased oilseed production (Durrett et al., 2008). Currently, seeds of oleaginous crops serve as the major feedstock for biodiesel production, but reduced land requirements, weather independence and lower labor inputs have made microorganisms such as yeast an attractive alternative (Li et al., 2008b). Although yeast do not rely on photosynthesis for carbon, these microorganisms can be engineered to utilize various carbon sources such as those found in agricultural waste (Yu et al., 2013).

Metabolic engineering of yeast to feed acyl chains into TAG can create a 'pull' effect on fatty acid synthesis, leading to greater cellular fatty acid content (Tai and Stephanopoulos, 2013). As a result, diacylglycerol acyltransferase (DGAT) has been identified as a promising target for engineering increased oil content (Liang and Jiang, 2013; Runguphan and Keasling, 2014). DGAT is a membrane-bound enzyme which catalyzes the acyl-CoA-dependent acylation of *sn*-1, 2-diacylglycerol (DAG) to produce TAG (Liu et al., 2012). Plants, animals and some yeast species possess two unrelated forms of DGAT referred to as DGAT1 and DGAT2 (Liu et al., 2012; Lung and Weselake, 2006). DGAT1 enzymes belong to a family of membrane-bound O-

acyltransferases and have a typical mass of 58 kDa. These enzymes have been predicted to have between 6-12 transmembrane domains (McFie et al., 2010). DGAT2, conversely, belong to a family of DGAT2/acyl-CoA: monoacylglycerol acyltransferase (McFie et al., 2010), and are typically smaller than DGAT1 enzymes. DGAT2 has been demonstrated to have 4 transmembrane domains (Liu et al., 2011). It has been observed in plants that DGAT1 and DGAT2 localize to different subdomains of the ER, suggesting these enzymes may have evolved non-redundant roles (Shockey et al., 2006). Coding DNA sequences (CDS) of eukaryotic DGAT of either family have been over-expressed both in plants and microorganisms as a means of increasing TAG content (Andrianov et al., 2010; Kamisaka et al., 2007; Tai and Stephanopoulos, 2013; Taylor et al., 2009; Yu et al., 2013).

Although there are numerous species of oleaginous yeasts which naturally produce high TAG content, *Saccharomyces cerevisiae* features numerous advantages for engineering increased oil production, including established genetic resources and molecular tools, availability of a large assortment of deletion strains, short generation time, ease of culturing and a proven track record in industry (Runguphan and Keasling, 2014; Tang et al., 2013). Additionally, plant lipid research can directly contribute to the metabolic engineering of *S. cerevisiae* as this species is commonly used as model system for the study of liposynthetic enzymes from plants. The first demonstration of a plant DGAT used to increase oil content in *S. cerevisiae* was by Bouvier-Navé et al. (2000) where the yeast was transformed with a CDS encoding an *Arabidopsis thaliana DGAT*. The primary focus of that study, however, was to functionally evaluate the plant DGAT. Contrary to *S. cerevisiae* which possesses only DGAT2, DGAT1 has been shown to play a predominant role in TAG synthesis in plants (Liu et al., 2012).

Here, we report on the identification of four actively transcribed *Brassica napus DGAT1 (BnaDGAT1)* genes and use of their CDS in increasing oil content in *S. cerevisiae*. We also identified a polymorphism in the second codon of the *BnaDGAT1* CDS which can substantively affect the accumulation of the recombinant BnaDGAT1 polypeptides and result in large differences in TAG production. We further demonstrate that modification of the N-terminal sequence of BnaDGAT1 is a simple method of circumventing the effects of native sequences of BnaDGAT1 when expressed in yeast, which does not impact cellular growth rates. This modification represents an additional tool to those currently available to produce greater TAG content in *S. cerevisiae*.

3.2. METHODS AND MATERIALS

3.2.1. Yeast culture conditions

Two yeast strains, a TAG deficient quadruple mutant *S. cerevisiae* H1246 (*MATα are1-Δ::HIS3 are2- Δ::LEU2 dga1- Δ::KanMX4 lro1- Δ::TRP1 ADE*) and its parental strain *S. cerevisiae* SCY 62 (*MAT a ADE2 can 1-100 his3-11,15 leu2-3 trp1-1 ura3-1*), were used in this study (Sandager et al., 2002). Cultures of *S. cerevisiae* H1246 were initiated in 2% (w/v) glucose synthetic liquid media lacking uracil (0.67% (w/v) yeast nitrogen base, 0.2% (w/v) synthetic complete drop out mix (SC synthetic minimal media). After overnight growth, cultures were inoculated into synthetic media containing 2% (w/v) galactose and 1% (w/v) raffinose to an optical density of 0.4. Cultures were rotated at 250 rotations per min at 30°C. *S. cerevisiae* SCY

62 was grown similarly, unless otherwise indicated, with the exception that they were all initiated in synthetic media containing 1% (w/v) raffinose as the carbon source.

3.2.2. Cloning of *BnaDGAT1* cDNA and genes

A *cDNA* library representing a full range of seed developmental stages was constructed using the double haploid *B. napus* line DH12075 (Séguin-Swartz, 2003). With information obtained from the *B. napus* EST database (National Research Council of Canada, Saskatoon, Saskatchewan), the library was used to isolate full length *BnaDGAT1* cDNA using the Marathon cDNA amplification kit (Clonetech) and primers indicated in Table 2.1-A. Genomic DNA was isolated from *B. napus* line DH12075 seedlings and used for isoform-specific *BnaDGAT1* amplification (Table 2.1-B). Amplified products were then cloned into the pCR4-TOPO TA vector (Invitrogen) as per the manufacturer's instructions. The 3Kb genes were sequenced (Table 2.1-C, or manufacturer's primers) and results were assembled using Seqman (DNAstar).

3.2.3. Generation of yeast expressing *BnaDGAT1*

Full length *BnaDGAT1* CDS were amplified by PCR (Table 2.1-C) and cloned into the pYES2.1/V5-His TOPO yeast expression vector (Invitrogen), under the control of the *Gal1* promoter and with the addition of a 3' V5 epitope. *BnaDGAT1* fragments were amplified by PCR and used as templates to construct full length chimera constructs by the overlapping PCR method (Table 2.1-D). Chimera constructs were cloned into the pYES2.1/V5-His TOPO yeast expression vector (Invitrogen) as described above. *BnaDGAT1* coding DNA sequences were

Table 3.1. Primers used for amplification of DGAT1 for identification, sequencing, and construct design.

Construct	Oligonucleotide sequences (5'-3' orientation)
A. Primers used for BnaDGAT1 cDNA cloning	
BnaC.DGAT1.a	TCTGGAGGCGTCACTATGC, CCCGCTTCTCCACCG
BnaA.DGAT1.a	TCTGGAGGCGTCACTATGC, CTTCTCCGCCGCCTC
BnaA.DGAT1.b	TCGCTGTACCGCCGAC, TAAACCTTACATCGCCTCCG
BnaC.DGAT1.b	AGGCGTCGCTGTACCGA, TACATCGCCGTTTCCTCCT
B. Primers used for genomic BnaDGAT1 sequence isolation	
BnaC.DGAT1.a	TTGGATTCTGGAGGCGTCACTATG, ACATCTATGACATCTTTCCTTTGC
BnaA.DGAT1.a	TTGGATTCTGGAGGCGTCACTATG, ACATCTATGACATCTTTCCTTTGC
BnaA.DGAT1.b	TTGGATTCTGGAGGCGTCGCTGTA, TGTGGAGTAAAAAGTCCTTCTCAG
BnaC.DGAT1.b	TTGGATTCTGGAGGCGTCGCTGTA, TGTGGAGTAAAAAGTCCTTCTCAG
C. Primers used for sub-cloning BnaDGAT1s	
BnaC.DGAT1.a	ACCATGGAGATTTTGGATTCTGGAGGCG, TGACATCTTTCCTTTGCGGTTC
BnaA.DGAT1.a	ACCATGGAGACTTTGGATTCTGGAGGCG, TGACATCTTTCCTTTGCGGTTC
BnaA.DGAT1.b	ACCATGGCGGTTTTGGATTCTGGAGGCG,

	GGACATGGATCCTTTGCGGTTC
BnaC.DGAT1.b	ACCATGGCGGTTTTGGATTCTGGAGGCG, GGACATGGATCCTTTGCGGTTC
D. Primers used for Chimeric DGAT1s	
	ACCATGGAGATTTTGGATTCTGGAGGCG,
	GATACGTAAACCTTACATCGCCTCCCGCTTCTCCACCGGAT
BnaC.DGAT1.a$_{(NT)}$::	TCCCTAATT,
BnaA.DGAT1.b$_{(NTD)}$	TCTCCACCGGATTCCCTAATT,
	AATTAGGGAATCCGGTGGAGAAGCGGGAGGCGATGTAAGG
	TTTACGTATC
	ACCATGGCGGTTTTGGATTCTGGAGGCG,
	TGTACCTTACATCCACGTTTCCCCCGGCGGATTCCCTAGTTT
BnaA.DGAT1.b$_{(NT)}$::	CGGCATCT,
BnaC.DGAT1.a$_{(NTD)}$	AGATGCCGAAACTAGGGAATCCGCCGGGGGAAACGTGGAT
	GTAAGGTACA,
	TGACATCTTTCCTTTGCGGTTC
E. Primers used for site directed mutations	
BnaC.DGAT1.a$_{(E2A)}$	ACCATGGAGATTTTGGATTCTGGAGGCGTCAC, TGACATCTTTCCTTTGCGGTTC
BnaA.DGAT1.b$_{(A2E)}$	ACCATGGAGGTTTTGGATTCTGGAGGCGTCGC, GGACATGGATCCTTTGCGGTTCAT
BnaA.DGAT1.b$_{(AV/EI)}$	ACCATGGAGATTTTGGATTCTGGAGGCGTCGC, GGACATGGATCCTTTGCGGTTCAT

F. Primers used for N-terminal tagging	
NT::BnaC.DGAT1.a	TAAAATGCGGCCGCATGGAGATTTTGGATTCTGGAG,TAAA ATTCTAGATGACATCTTTCCTTTGCGGTTC
NT::BnaA.DGAT1.b	TAAAACGCGGCCGCATGGCTGTTTTGGATTCTGG,TAAATTC TAGAGGACATGGATCCTTTGCGGTTC

also amplified by PCR with forward primers designed to introduce site-directed mutations at the +5 and +7 nucleotides (Table 2.1-E) before cloning into pYES2.1/V5-His Topo (Invitrogen). Restriction sites *NotI* and *XbaI* were added to *BnaDGAT1* CDS by PCR amplification (Table 2.1-F). Resulting PCR products were digested and cloned in frame into pYES2/NT-B (Invitrogen) to produce NT-DGAT1. The N-terminal tag of this vector is composed of 6x polyhistidine linked to an 'Xpress' epitope. It, and all other constructs, were cloned in frame with 3' V5 epitope coding sequences, and were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies) to ensure the fidelity of the coding sequences. Vectors were transformed into the *S. cerevisiae* strains by polyethylene glycol-mediated transformation (Gietz and Schiestl, 2007).

3.2.4. Analysis of neutral lipid content in yeast

Nile red analysis of TAG accumulation was performed as described previously (Siloto et al., 2009a) using either a Fluoroskan Ascent (Thermo Electron Company) or a Synergy H4 hybrid reader (Biotek). Briefly, 95 µl of yeast culture was incubated with 5 µl of 0.8 µg/ml Nile red solution (suspended in methanol). Fluorescent emission was assayed before and 5 minutes after addition of Nile red solution. Change in fluorescence over optical density of the cultures ($\Delta F/OD_{600}$) was used as a measure of cellular neutral lipid content. Three technical replicates of three biological replicates were performed for each BnaDGAT1 isoform and construct.

3.2.5. Preparation of microsomes and Western blotting

Microsomal fractions were isolated from *S. cerevisiae* H1246 cultures expressing *DGAT1* constructs according to Siloto et al. (2009b). For Western blotting, twenty-five micrograms of isolated protein were separated by 10% (w/v) denaturing SDS-PAGE. Proteins were electrophoretically transferred to polyvinylidene difluoride membranes which were then blocked with 2% (w/v) milk fat before incubation with V5-HRP conjugated antibody (1:10,000). BnaDGAT1 content was visualized using ECL Advance Western Blotting Detection Kit (Amersham) with the aid of a variable mode imager (Typhoon Trio+, GE Healthcare).

3.2.6. *In vitro* DGAT activity assays

DGAT assays were conducted in a similar fashion to the procedure described by Byers et al. (1999). Microsomes containing recombinant BnaDGAT1 were incubated in the presence of 200 mM Hepes-NaOH (pH 7.4), 3.2 mM $MgCl_2$, 333 µM *sn*-1,2-dioleoyl-glycerol dispersed in 0.2% Tween-20 (Avanti Polar Lipids) and 15 µM [1-^{14}C] oleoyl-CoA (55uCi/µmol) (Perkin-Elmer). DGAT reactions were allowed to proceed at 30°C for 2-5 min before quenching with sodium dodecyl sulfate. Entire reactions were then spotted onto G25 silica plates and TAGs were isolated by thin layer chromatography using 80:20 hexane: ether (v/v) as a mobile phase. After air-drying, silica containing TAG spots were detected using an imaging plate (FujiFilm) in combination with a Typhoon Trio+ variable mode imager (Amersham Biosciences). Identified spots were scraped from the TLC plate, combined with Ecolite scintillation cocktail (MP Biomedicals) and radioactivity was quantified using an LS 6500 multi-purpose scintillation counter (Beckman Coulter).

3.2.7. Yeast fatty acid analysis

Wild type *S. cerevisiae* cells were harvested 72 h post induction, flash frozen, and freeze dried. Approximately 60 mg of sample were rehydrated and lipids were extracted using the Folch method, with minor modifications (Christie, 2010). Isolated lipids were re-suspended in chloroform and split into two equal portions: one portion was used for TLC isolation of TAG (80:20:1 hexane: diethyl ether: acetic acid mobile phase) and the remaining portion was used for determination of total lipid content. TAG spots on TLC plates (silica G25) were identified using primulin staining. Both isolated TAG and dried down total lipid samples were derivatized by incubation in 2 mL methanolic HCl for 3 hours at 80°C. Reactions were quenched by addition of 2 mL of saline, and methyl esters were isolated by extraction twice with hexane. Isolated fatty acid methyl esters were separated by chromatography on a 30 meter DB23 column (Agilent Technologies) using a 6896 N network GC system (Agilent Technologies) and quantified using a 5975 inert XL mass selection detector (Agilent Technologies). Triheptadecanoin and heneicosanoic methyl esters (Nu-Chek Prep.) were used as external and internal controls, respectively. Three biological replicates were tested for each construct.

3.2.8. Visualization of yeast fat pads

S. cerevisiae H1246 cells were cultured as described above for 24 h. Cells were lysed and centrifuged as described for the isolation of microsomes. The resulting fat pads in the centrifuge tubes were photographed using a Canon EOS 60D camera (with a Canon EF 100mm f/2.8 Macro USM lens).

3.2.9. Statistical analysis

Analysis of *in vivo* TAG accumulation and fatty acid composition of yeast producing DGAT1 enzymes was performed using one-way analysis of variance (ANOVA; SAS 9.3, SAS Institute Inc.), with $\alpha = 0.05$.

3.3. RESULTS

3.3.1. Identification of BnaDGAT1 genes and coding sequences

Four different *DGAT1* mRNA sequences were identified in cDNA amplified from developing *B. napus* line DH12075 seeds (Séguin-Swartz, 2003), and the presence of the four *BnaDGAT1* genes in the *B. napus* genome were confirmed by full gene sequencing and Southern blot analysis (SUPPLEMENTAL FIGURE 3.1). The genes were named according to the nomenclature suggested for *Brassica* species (Ostergaard and King, 2008). *B. napus* is an allotetraploid containing two (A & C) of the three ancestral *Brassica* genomes (Liu and Wang, 2006). Two of the *BnaDGAT1*s, *BnaA.DGAT1.a* (GenBank ID: JN224474) and *BnaA.DGAT1.b* (GenBank ID: JN224475), displayed high sequence homology to *DGAT1* genes identified in *B. rapa*, a species containing only the *Brassica* A genome (BRAD, 2011). The coding sequence of *BnaA.DGAT1.b* was previously reported by our group (GenBank ID: AF164434.1) (Nykiforuk et al., 1999b) whereas the sequence of *BnaA.DGAT1.a* has not been previously reported. The remaining two *BnaDGAT1* genes showed high sequence homology to *DGAT1* nucleotide sequences present in EST libraries derived from *B. oleracea*, which possesses only the *Brassica* C genome. The mRNA sequence of *BnaC.DGAT1.a* (Genbank ID: JN224473) was registered

into the NCBI database by Brown et al. (1998) as GenBank ID: AF251794.1, whereas *BnaC.DGAT1.b* (Genbank ID: JN224476) has not been previously reported. All four genes appear to have similar organization, common to *DGAT1* genes, with a large first exon followed by 15 smaller exons.

The *BnaDGAT1* coding sequences could be divided into two groups based on homology (FIGURE 3.1.a), with *BnaC.DGAT1.a* and *BnaA.DGAT1.a* falling into one clade and *BnaA.DGAT1.b* and *BnaC.DGAT1.b* into a second clade. The first and second clades share 98.6 and 96.2 % pairwise identity, respectively, compared to 91.0 % pairwise identity for all four BnaDGAT1 amino acid sequences. The amino acid sequence differences among the encoded enzymes reside primarily in the hydrophilic polypeptide segment encoded by the first exon (74.2% amino acid pairwise identity) (FIGURE 3.1.b).

3.3.2. Functional characterization of *BnaDGAT1* coding sequences

To initially characterize the four BnaDGAT1, their coding sequences were first expressed in the quadruple knock-out *S. cerevisiae* strain H1246 (*Δare1 Δare2 Δlro1 Δdga1*) which lacks the genes necessary to synthesize neutral lipids (Sandager et al., 2002). As a result, the neutral lipid content of these cultures directly relates to the amount of TAG produced by the introduced *DGAT1* CDSs. Nile red fluoresces at a unique wavelength in the presence of neutral lipids, and thus, under the above conditions, provides for a rapid method to quantify TAG produced by the

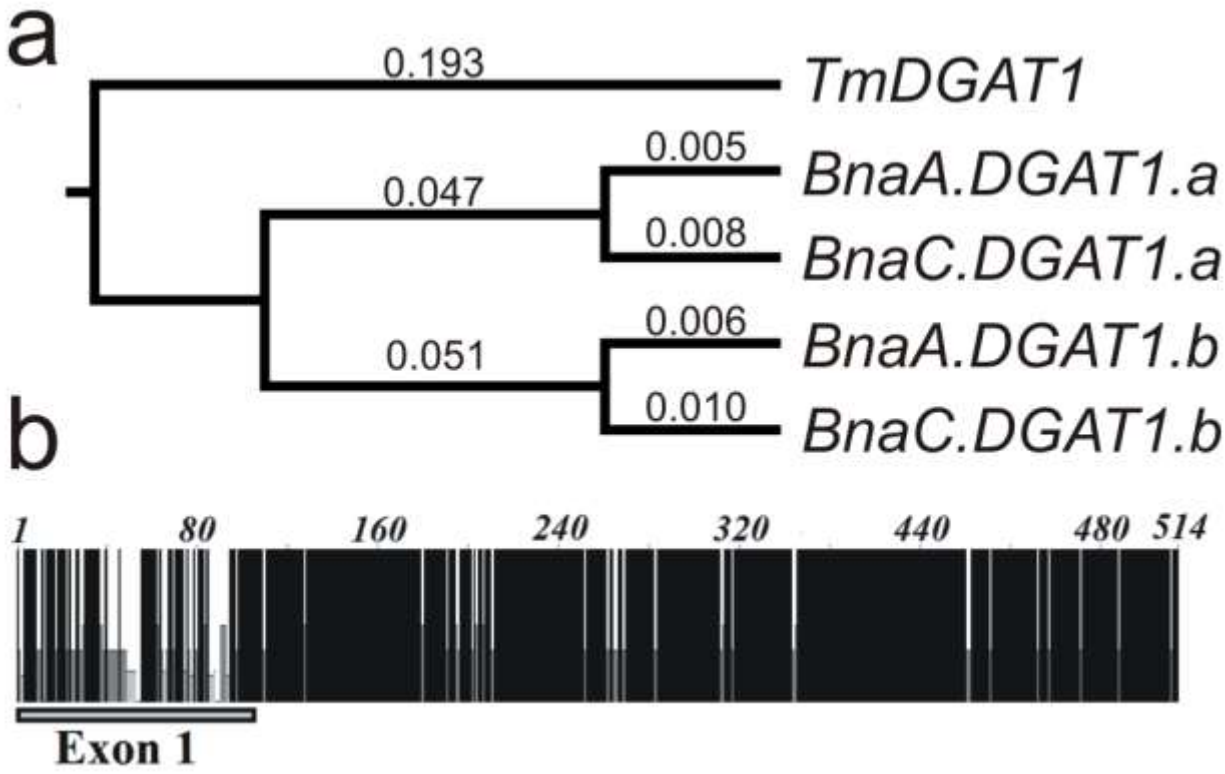

FIGURE 3.1. BnaDGAT1 sequence homology. (a) Phylogenetic tree of *BnaDGAT1* coding sequences. Numbers indicate substitutions per site. (b) Homology alignment of BnaDGAT1 polypeptides. Higher bar height and darker shade indicates higher amino acid residue identity consensus. Shaded rectangle indicates amino acid residues encoded by the first exon of *BnaDGAT1*.

catalytic action of recombinant DGATs (Siloto et al., 2009a). Using this method, it was demonstrated that all four *BnaDGAT1* CDS encoded active DGAT enzymes. Large differences in TAG contents were observed, however, despite the high degree of homology shared between these coding sequences. BnaA.DGAT1.a generated far greater TAG content compared to yeast producing BnaA.DGAT1.b (FIGURE 3.2.a). Similarly, cultures producing BnaC.DGAT1.a generated significantly more TAG than those producing BnaC.DGAT1.b. Western blot of microsomal protein extracts revealed that the divergent *in vivo* DGAT activities were largely a product of differential accumulation of the polypeptides (FIGURE 3.2.b). Differences in DGAT1 content appeared to be of a larger scale than those observed for the TAG content of these cultures. This discrepancy may result from the yeast being incapable of providing sufficient substrates or environment to highly accumulating DGAT1s, leading to proportionately smaller returns in TAG contents over time.

Following these initial results, we subsequently observed that expressing coding sequences of two candidate BnaDGAT1 variants (*BnaC.DGAT1.a* and *BnaA.DGAT1.b*) in frame with 5' epitopes from the pYES2.1-NT vector produced enzymes (NT::BnaDGAT1) which accumulated significantly more neutral lipids than their equivalents with no N-terminal tag (FIGURE 3.3.a). Furthermore, *in vitro* assays with radiolabeled acyl-CoA and exogenous DAG indicated that the tagged polypeptides displayed high enzyme specific activity (FIGURE 3.3.b).

As little as 150 ng of microsomal protein could produce a relatively large quantity of TAG in a two-minute assay. The high DGAT activity observed was almost completely dependent on exogenous DAG; enzyme activity was reduced by approximately 98% when

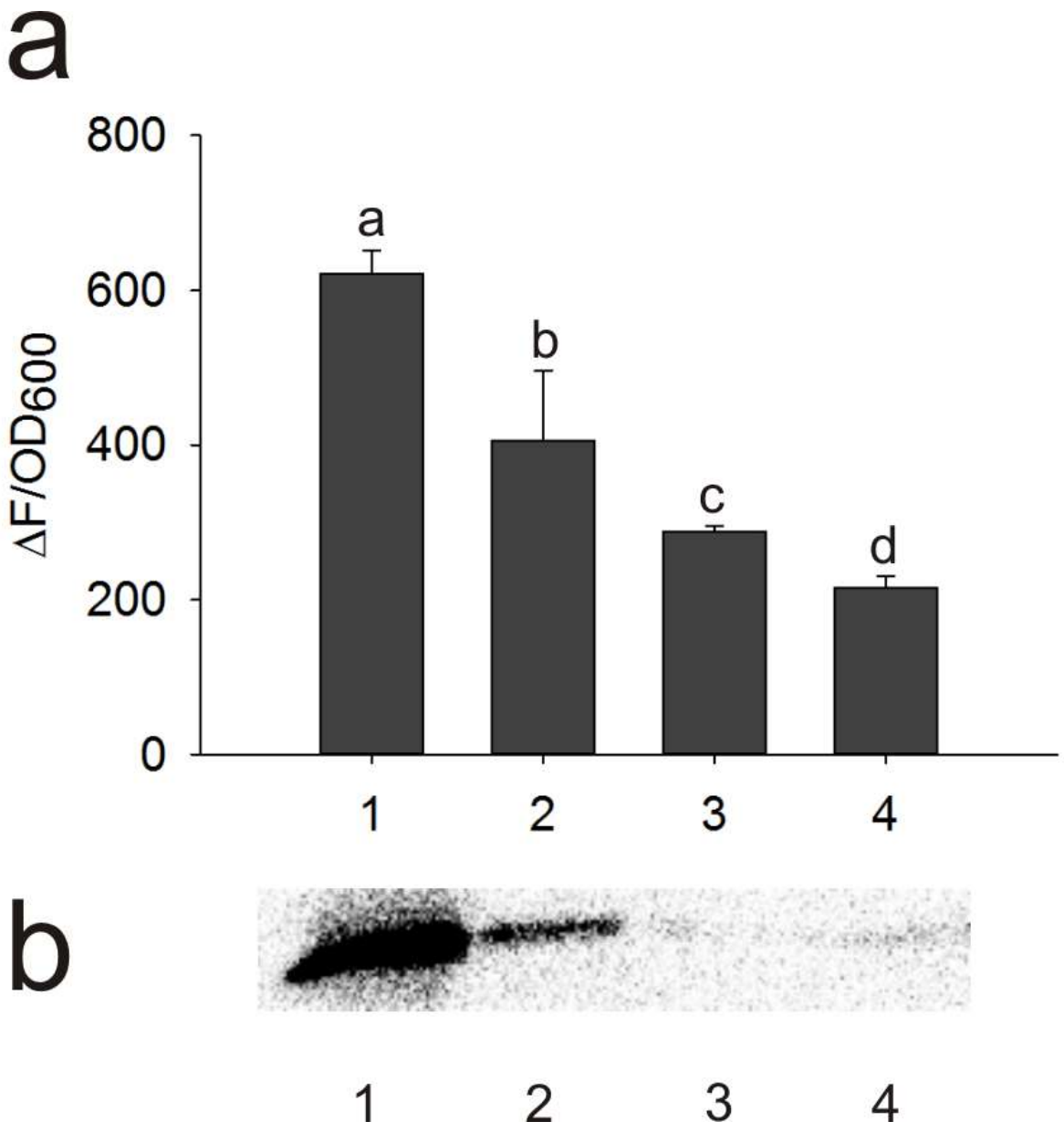

FIGURE 3.2. ***In vivo*** **TAG (a) and BnaDGAT1 (b) accumulation in** ***S. cerevisiae*** **strain H1246 cultures producing different forms of BnaDGAT1.** Nile red readings and microsomal protein extractions were performed with cultures of similar optical densities (OD_{600}) 24 hours post-induction. 1. BnaC.DGAT1.a, 2. BnaA.DGAT1.a, 3. BnaA.DGAT1.b, 4. BnaC.DGAT1.b. Error bars indicate +/- 1 standard deviation. Letters indicate statistical grouping.

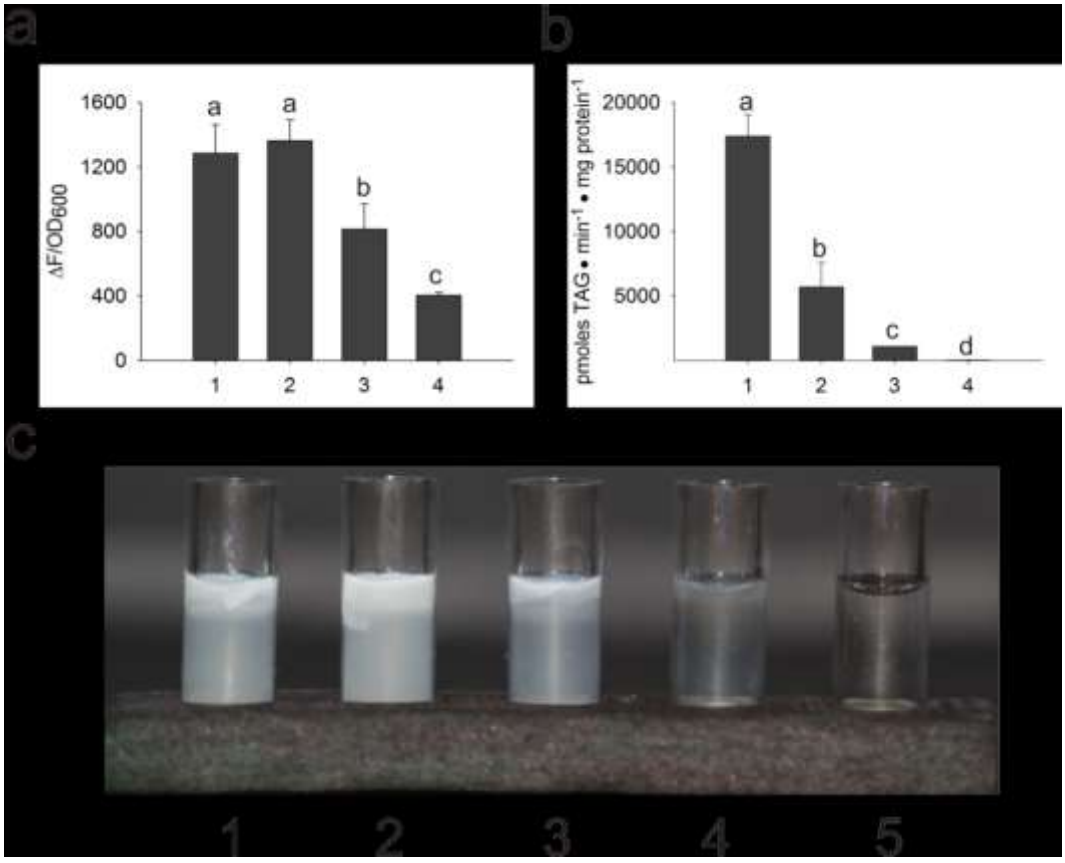

FIGURE 3.3. Effect of N-terminal modification of BnaDGAT1 on lipid accumulation and microsomal DGAT activity for recombinant enzymes produced in *S. cerevisiae* H1246. Nile red analysis of *in vivo* lipid accumulation (a) was performed on cultures of similar optical densities (OD_{600}) 24 hours post-induction. *In vitro* DGAT enzyme assays (b) were conducted using [1-^{14}C] oleoyl-CoA in the presence of exogenous *sn*-1,2-diolein. Error bars indicate +/- 1 standard deviation. Fat pads (c) were isolated from 50 ml cultures 24 hours post induction from cultures grown at 30°C, 250 rpm. 1. N-terminally tagged BnaC.DGAT1.a (NT::BnaC.DGAT1.a); 2. NT::BnaA.DGAT1.b; 3. BnaC.DGAT1.a; 4. BnaA.DGAT1.b. 5. LacZ. Letters indicate statistical grouping.

exogenous DAG was not made available. Under optimized conditions, the specific activities of microsomal DGAT for oleoyl-CoA in the presence of *sn*-1,2-diolein were 17.4 and 6.76 nmol TAG • min^{-1} • mg $protein^{-1}$ for NT::BnaC.DGAT1.a and NT::BnaA.DGAT1.b producing cultures, respectively. These activities represented increases of approximately 16 and 149 fold, respectively, over their untagged equivalents. The impact of expressing BnaDGAT1 enzymes with and without the N-terminal tag is significant enough that differences in fat pad accumulation can be observed visually (FIGURE 3.3.c).

To understand how the N-terminal tag was increasing DGAT1 activity, we attempted to elucidate why the enzymes without N-terminal tags displayed such different *in vivo* activities. We hypothesized that the differences in BnaDGAT1 activity may be a product of the divergent amino acid sequences present in their N-terminal domain, as the remainder of their polypeptide sequences displayed very high similarity. To test this hypothesis, yeast cultures expressing chimeric versions of the candidate CDS with interchanged N-terminal domains (i.e., BnaC.DGAT1.$a_{(\underline{N}\text{-}\underline{T}\text{erminus})}$::BnaA.DGAT1.$b_{(\underline{N}\text{-}\underline{T}\text{erminus }\underline{D}\text{eleted})}$ and BnaA.DGAT1.$b_{(NT)}$::BnaC.DGAT1.$a_{(NTD)}$) were subjected to Nile red analysis. The N-terminal domains of these chimeras represent the amino acids encoded by the first exons of their parental gene (FIGURE 3.1.b). TAG content in cultures producing these chimeric proteins accumulated to levels equal to the native *B. napus* enzyme with which they shared N-terminal homology (FIGURE 3.4.a). BnaC.DGAT1.$a_{(NT)}$::BnaA.DGAT1.$b_{(NTD)}$ generated an equal amount of TAG to cultures producing BnaC.DGAT1.a, which was up to two-fold more TAG than that generated by cultures producing BnaA.DGAT1.b. The opposite was also observed for cultures producing BnaA.DGAT1.$b_{(NT)}$::BnaC.DGAT1.$a_{(NTD)}$, which generated far less TAG than BnaC.DGAT1.a

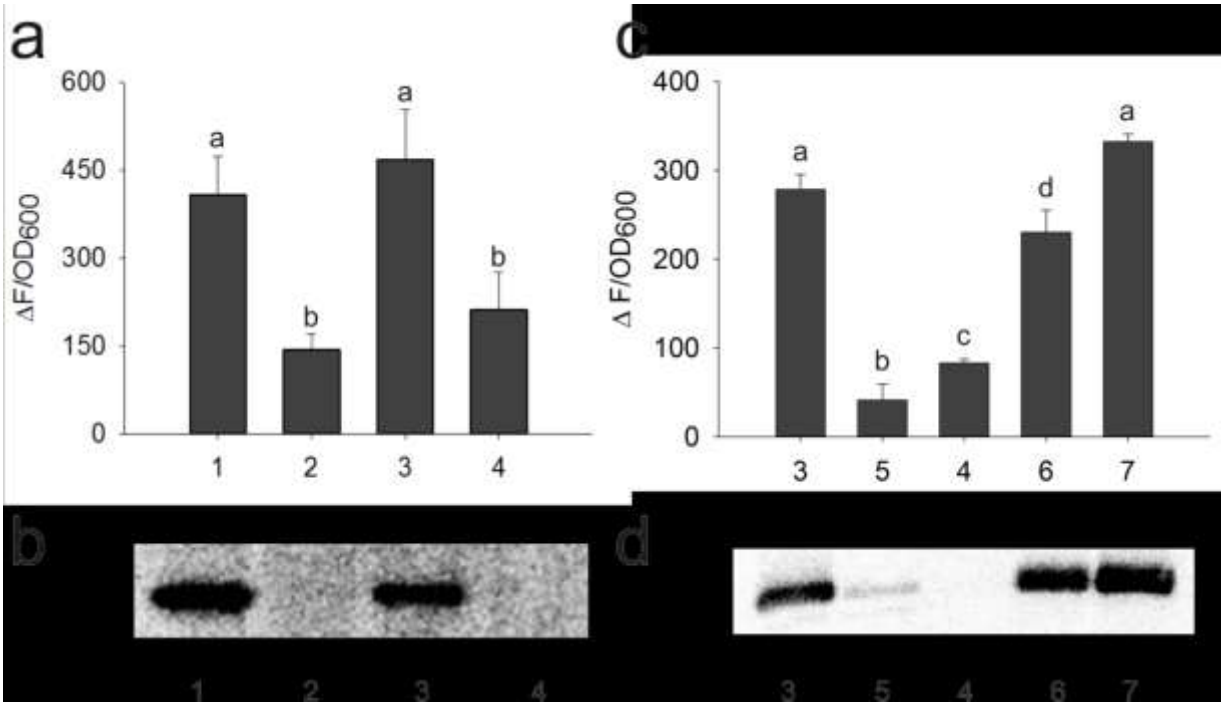

FIGURE 3.4. Impact of BnaDGAT1 N-terminal form on enzyme activity and accumulation in *S. cerevisiae strain* H1246. *In vivo* neutral lipid (a) and BnaDGAT1 accumulation (b) in H1246 *S. cerevisiae* cultures expressing chimeric DGAT1 coding sequences. *In vivo* neutral lipid (c) and BnaDGAT1 accumulation (d) in H1246 *S. cerevisiae* cultures expressing mutated *BnaDGAT1* coding sequences. Nile red readings and microsomal protein isolations were performed on cultures at similar optical densities (OD_{600}) 24 hours post-induction. Error bars indicate +/- 1 standard deviation. 1. BnaC.DGAT1.$a_{(\underline{N}\text{-}\underline{T}\text{erminus})}$::BnaA.DGAT1.$b_{(\underline{N}\text{-}\underline{T}\text{erminus }\underline{D}\text{eleted})}$, 2. BnaA.DGAT1.$b_{(NT)}$::BnaC.DGAT1.$a_{(NTD)}$, 3. BnaC.DGAT1.a, 4. BnaA.DGAT1.b., 5. BnaC.DGAT1.a $_{(\Delta E2A)}$, 6. BnaA.DGAT1.$b_{(\Delta A2E),}$ 7. BnaA.DGAT1.$b_{(\Delta AV/EI).}$ Letters indicate statistical grouping.

cultures. Western blots performed on microsomal protein extracts from the yeast cultures demonstrated the TAG content phenotype was dependent upon the variant form of the N-terminal region of these DGATs (FIGURE 3.4.b).

In silico analysis of the N-terminal domains of the BnaDGAT1 CDS identified a potential phosphorylation site (BnaA.DGAT1.b$_{S30}$), and a potential ubiquitination site (BnaA.DGAT1.b$_{R27}$) which theoretically could lead to the differential accumulation of the enzymes. Replacement of these amino acids through site directed mutagenesis, however, had no effect on the enzyme's activity, suggesting the accumulation of the enzyme was not affect (data not shown). Mutation of the second amino acid residue in the BnaDGAT1 polypeptide sequence, conversely, was shown to have a large effect on enzyme activity (FIGURE 3.4.c). Yeast producing BnaC.DGAT1.a with its second amino acid residue (glutamic acid) converted to an alanine residue produced significantly less oil than cultures producing BnaC.DGAT1.a. The reverse was observed for cultures producing BnaA.DGAT1.b with its second amino acid residue (alanine) replaced with a glutamate residue, which produced significantly more oil than cultures producing unmodified BnaA.DGAT1.b. Replacing both the second and third amino acid residues of BnaA.DGAT1.b led to even greater oil accumulation. Mutation of these amino acid residues was also shown to dramatically alter the accumulation of these polypeptides *in vivo* (FIGURE 3.4.d) as well. Interestingly, BnaC.DGAT1.a$_{(E2A)}$ produced less oil than BnaA.DGAT1.b, despite it apparently accumulating to a higher degree. This may suggest that mutating the second amino acid can affect both DGAT accumulation and its enzymatic activity. It is possible, however, that some discrepancy can be expected between western blot and Nile red analysis as these assays are performed at different time points in the yeast's growth curve. In

either scenario, however, producing BnaDGAT1 enzymes with an N-terminal epitope in yeast appears to mask the impact of deleterious nucleotide or amino acid residues present at native N-terminus of these enzymes, leading to increased enzyme production and, subsequently, increased *in vivo* TAG synthesis.

3.3.3. Potential use of tagged BnaDGAT1 for industrial biofuel applications

Placement of an N-terminal tag onto DGAT1 enzymes represents a simple method of increasing *in vivo* DGAT activity which could easily be combined, with many other methods shown to increase yeast cellular TAG content. To provide insight as to how useful these higher accumulating DGAT1 enzymes could be for yeast based fatty acid production and biofuel applications, the performance of the enzymes was followed when produced in wild type *S. cerevisiae* cells (SCY 62). First, the impact of increasing BnaDGAT1 content upon cellular growth was monitored. As shown in FIGURE 3.5.a, cultures expressing *BnaDGAT1* constructs, with or without coding segments for N-terminal epitopes, grew identically to cultures expressing *LacZ*. Having ensured N-terminal tagging did not impede yeast growth, the accumulation of neutral lipids was then followed over time in cultures expressing *NT::BnaA.DGAT1.b* (FIGURE 3.5.b). Cultures producing this enzyme were observed to immediately produce significantly more neutral lipid relative to both *LacZ* and *BnaA.DGAT1.b* -expressing cultures. At approximately 28 hours, the yeast cells achieved their maximal content of neutral lipids. At this point, the *NT::BnaA.DGAT1.b* cultures had 115% greater neutral lipid content than cultures expressing *LacZ*, and 54% more neutral lipid than cultures expressing BnaA.DGAT1.b. The

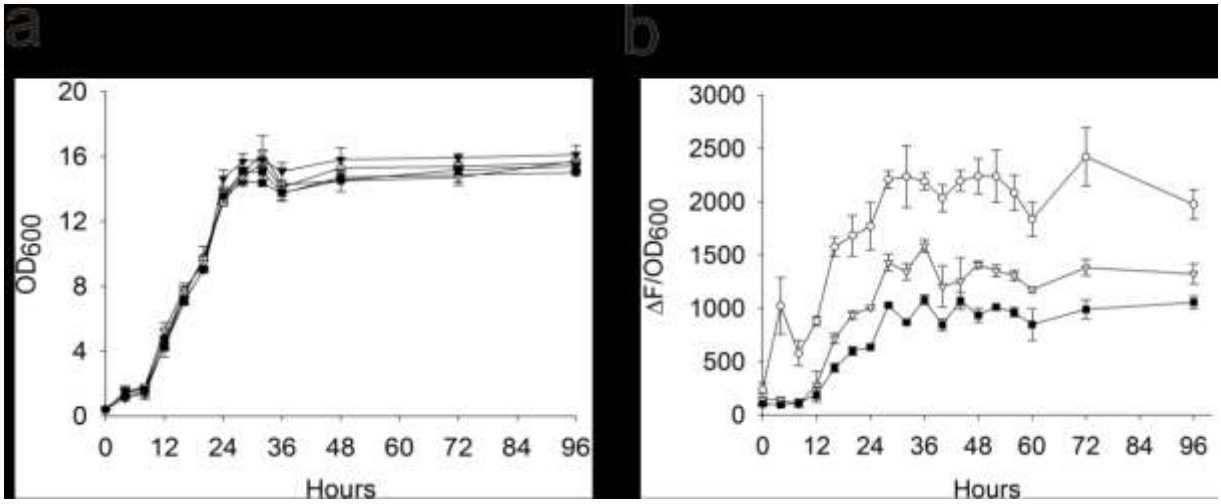

FIGURE 3.5. Growth and relative neutral lipid content of wild type *S. cerevisiae* producing N-terminally modified BnaDGAT1 enzymes. (a) Growth rates of cultures producing BnaDGAT1 polypeptides. Determination of optical densities began with inoculation of 50 mL of induction media to OD_{600} of 0.4. Cultures were grown at 30°C in 250 mL Erlenmeyer flasks, rotated at 250 rotations per minute. Accumulation of neutral lipids over time (b) in cultures expressing *BnaDGAT1* constructs. Error bars indicate +/- 1 standard deviation. ● N-terminally tagged BnaC.DGAT1.a (NT::BnaC.DGAT1.a), ○ *NT::BnaA.DGAT1.b,* ▼ *BnaC.DGAT1.a,* ∇ *BnaA.DGAT1.b,* ■ *LacZ.*

increased cellular neutral lipid levels of *NT::BnaA.DGAT1.b* -expressing cultures was not limited to a particular window of time, and consistently remained higher than the BnaA.DGAT1.b and *LacZ* -expressing cultures.

To complement the Nile red analysis of wild type cultures expressing *NT:BnaA.DGAT1.b*, GC-MS was used to analyze the fatty acid content of TAG and total lipid from these cultures 72 hours post induction. These results were quite consistent with data obtained from Nile red analysis for this time point. Expression of *BnaA.DGAT1.b* increased the cellular TAG content of yeast by 50%, and total lipid by 30%, relative to cultures expressing *LacZ* (FIGURE 3.6.a). Modification of the N-terminus of BnaA.DGAT1.b, however, resulted in significantly greater lipid content. Wild type cultures expressing *NT::BnaA.DGAT1.b* produced 9.9% total lipid and 6.4% TAG, respectively, on a dry cell weight basis. These results translate into a 130% increase in TAG content, and a 67% increase in total lipid, relative to cultures expressing only *LacZ*. These levels of TAG and total lipid fatty acids were 53% and 28% higher than those observed in cultures producing untagged BnaA.DGAT1.b. The mass of fatty acids not esterified into TAG molecules (i.e., sterol esters, polar lipids) appeared to be equivalent in all of the samples, suggesting the increase in total lipids was solely a product of increased TAG production.

Interestingly, the fatty acid composition of lipid fractions from yeast cells appeared to be influenced by the degree to which BnaA.DGAT1.b accumulated. Cultures producing NT::BnaA.DGAT1.b generated TAG that contained 11.2% less unsaturated fatty acids on an absolute basis than cultures expressing LacZ (FIGURE 3.6.b). Relative to the LacZ control,

however, cultures producing BnaA.DGAT1.b generated TAG reduced in unsaturated fatty acids by only 6.4%. The changes in TAG fatty acid composition were mirrored in the total fatty acid composition (FIGURE 3.6.c). Primarily, increasing BnaA.DGAT1.b content resulted in increased palmitic acid and reduced oleic acid content in either lipid fraction. Small differences in palmitoleic and steric acid contents were observed, however, between total lipid fractions of tagged *BnaA.DGAT1.b* and *LacZ* expressing cultures. For the enzyme BnaC.DGAT1.a, which naturally displays relatively high activity, no significant differences in fatty acid profiles were observed between cultures expressing tagged and untagged versions of this enzyme.

Increasing the raffinose content of the standard media (2% galactose, 1% raffinose), while maintaining galactose and nitrogen contents constant, led to a small increase in cellular growth without penalty to neutral lipid accumulation. Under these conditions, the tagged and untagged versions of BnaC.DGAT1.a produced 453 and 370 mg/L of fatty acids, respectively, in 72 hours (data not shown). This translates to an increase of 22% in total lipid content. In the same time period, tagged and untagged versions of BnaA.DGAT1.b produced 416 and 252 mg/L fatty acids respectively, an increase of 65%. The wild type control produced 242 mg/L total fatty acids under the same conditions. Higher concentrations of raffinose were observed to either provide no improvement, or to be deleterious.

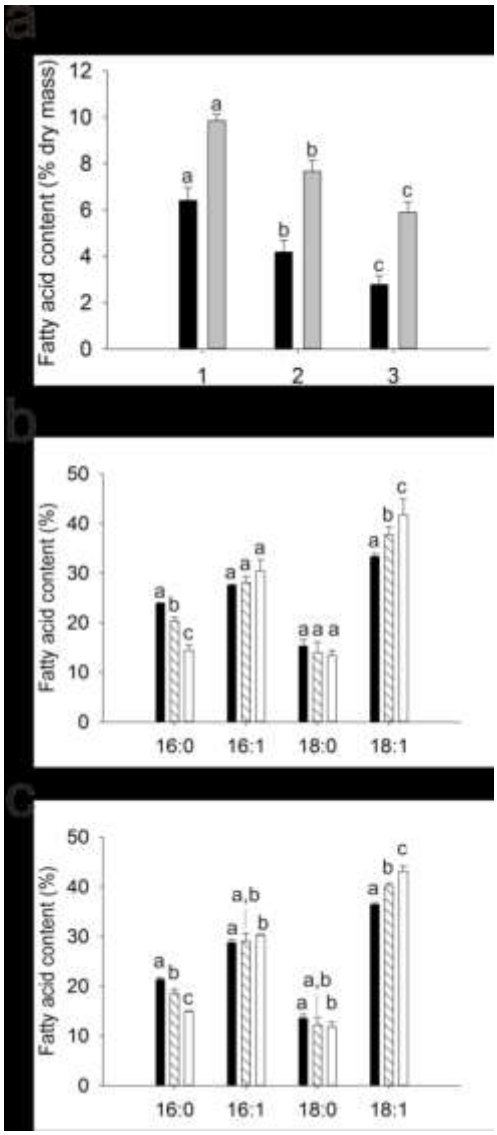

FIGURE 3.6. Fatty acid accumulation (a) and composition of triacylglycerol (b) and total lipid fractions (c) isolated from wild type *S. cerevisiae* cultures expressing *BnaDGAT1* constructs with and without coding segments for the N-terminal tag 72 hours post induction. In panel (a), N-terminally tagged BnaA.DGAT1.b (1), *BnaA.DGAT1.b* (2) and *LacZ* (3) are indicated with numbers, and TAG (■) and total lipids (■) are indicated by color. In panels (b) and (c), above enzymes are indicated with black, striped, and white bars respectively. Error bars indicate +/- 1 standard deviation. Letters indicate statistical grouping.

3.4. DISCUSSION

Here, we report the identification of four transcriptionally active *DGAT1* genes from the major oilseed crop *B. napus*. To initially characterize the products of these genes, we expressed their CDSs in *S. cerevisiae*, a model organism commonly used for plant lipid research (Liu et al., 2011; Sandager et al., 2002; Siloto et al., 2009a; Turchetto-Zolet et al., 2011; Yu et al., 2008; Zhang et al., 2013). This initial characterization, however, was hindered by the large differences observed in the accumulation of the BnaDGAT1 isoforms when produced in this species. We then identified that placement of a tag on the N-termini of the BnaDGAT1 could dramatically increase their *in vivo* accumulation, ultimately leading to increased TAG accumulation. Interestingly enough, a similar effect was observed by O'Quin et al. (2009), who demonstrated placement of Myc or hemagglutinin epitopes at the N-termini of plant desaturases could increase the accumulation of these polypeptides in *S. cerevisiae*. We probed the mechanism further and observed that the N-terminal tag of pYES2-NT appeared to increase DGAT1 accumulation by masking deleterious polymorphisms present at the +5 nucleotide position of the four *DGAT1* CDSs. It would be interesting to investigate how this polymorphism can account for a 149 fold difference in *in vitro* activity. Speculatively, it has been demonstrated that the identity of the amino acid residues present at the N-terminus of proteins can significantly influence their turnover rate (Sriram et al., 2011; Tasaki et al., 2012), which is a possibility given that the BnaDGAT1 CDS, and the N-terminal tag, encode different amino acid residues at the N-termini. An alternative possibility may be that this polymorphism affects the rate at which this CDS is translated. Analysis of Kozak sequences from highly expressed yeast genes suggest that the +5 cytosine of BnaA.DGAT1.b and BnaC.DGAT1.b would actually be favored, and not detrimental

(Hamilton et al., 1987). A third possibility worthy of future exploration is that N-termini of DGAT1 are involved in a regulatory mechanism specific to this enzyme. Indeed, it has been suggested that DGAT1 may be regulated through the interaction of their N-terminal domains with acyl-CoAs (Siloto et al., 2008).

Irrespective of the underlying mechanism, addition of an N-terminal epitope was shown to dramatically increase the activity of all four BnaDGAT1 enzyme forms expressed in yeast. Identifying a simple method that can increase the accumulation of at least some DGATs when produced in yeast should be of great value to researchers studying the biochemical properties of these enzymes. It could also have benefits for researchers attempting to increase lipid content in yeast for biofuel and other industrial applications. To our knowledge, there is only one report of *in vitro* DGAT1 activity using a *S. cerevisiae* extract as high as we observed with the tagged BnaDGAT1 enzymes (Yu et al., 2008), whereas other reports are several orders of magnitude lower. The specific activity of microsomal NT::BnaC.DGAT1.a observed here was approximately 11-fold higher than that reported for lipid body fractions containing a genetically improved version of DGA1, despite the latter being expressed in a wild type background (Kamisaka et al., 2010). In the SCY 62 background, placing an N-terminal tag on BnaA.DGAT1.b resulted in a 67% increase in total fatty acid content for the DGAT-producing cultures, which corresponded to approximately 10% of the dry weight of the cells. This level of lipid accumulation is similar to that achieved by overexpressing *DGA1* under the control of the *Gal1* promoter in *S. cerevisiae* (Runguphan and Keasling, 2014). That study, however, reported a much lower production value of 168 mg/L of fatty acids compared to the 453 mg/L reported here. Kamisaka et al. (2013) reported a similar total fatty acid production value (450mg/L) when

expressing *DGA1* under the control of *ADH1* for 7 days in the presence of 10% glucose. Thus, it appears that production of tagged BnaDGAT1 enzymes under the control of *GAL1* can provide equivalent or better fatty acid production results relative to those achieved by the overexpression of *DGA1* in *S. cerevisiae*. It should be noted, however, that Kamisaka et al. (2013) were able to achieve much greater yield (up to 930mg/L) by expressing an N-terminally truncated *DGA1* in a Δ*dga* background. In light of these findings, it would be of great interest to observe how N-terminally tagged BnaDGAT1 performed when expressed in a Δ*dga* background, or when co-expressed with the improved *DGA1* variant.

Although *S. cerevisiae* is known to produce lower lipid content then many oleaginous yeast strains, it has many genetic tools available and is familiar to industry, and thus has been suggested to be a good model strain for yeast-based biofuel production (Runguphan and Keasling, 2014). Further genetic modification of yeast metabolism to increase substrate production for the tagged DGAT1 enzymes may further maximize the potential effectiveness of these modified TAG-biosynthetic enzymes. The *in vitro* activity increases observed for these enzymes were significantly greater than those observed for TAG accumulation, and the extracts required addition of exogenous DGAT for maximal performance. This suggests that DAG availability may be limiting in the synthesis of TAG.

We noted the fatty acid profiles of both TAG and total lipid fractions were significantly different between cultures expressing tagged and untagged versions of *BnaA.DGAT1.b*. This observation may have important implications for the engineering for yeast to produce unsaturated fatty acids, as there was a negative relationship between DGAT1 accumulation and

unsaturated fatty acid content. Although the metabolic 'pull' created by the catalytic action of DGAT is generally accepted to promote TAG accumulation in yeast, it may be that in certain circumstances that reducing DGAT activity to a moderate level may increase the relative content of unsaturated fatty acids in TAG. Conversely, it may be necessary to up-regulate Δ9-desaturase activity or increase the abundance of this enzyme when increasing DGAT accumulation in yeast in order to achieve a higher proportion of unsaturated fatty acids in TAG. Indeed, both DGAT and Δ9-desaturases of yeast are present in the ER (Stukey et al., 1990) and presumably compete for cytosolic acyl-CoA as substrates.

In summary, placement of an N-terminal epitope on DGAT1 enzymes can provide a facile method to eliminate the influence of native N-terminal sequences during heterologous expression in *S. cerevisiae*. This discovery could potentially benefit researchers involved in studying the biochemical properties of recombinant DGAT produced through heterologous expression in yeast and has implications for optimization of plant *DGAT* to facilitate effective production of the encoded recombinant polypeptides in this microbial organism. In addition, this discovery offers a new approach to increase lipid content in yeast for industrial applications such as producing feedstock for biodiesel production.

3.5. ACKNOWLEDGEMENTS

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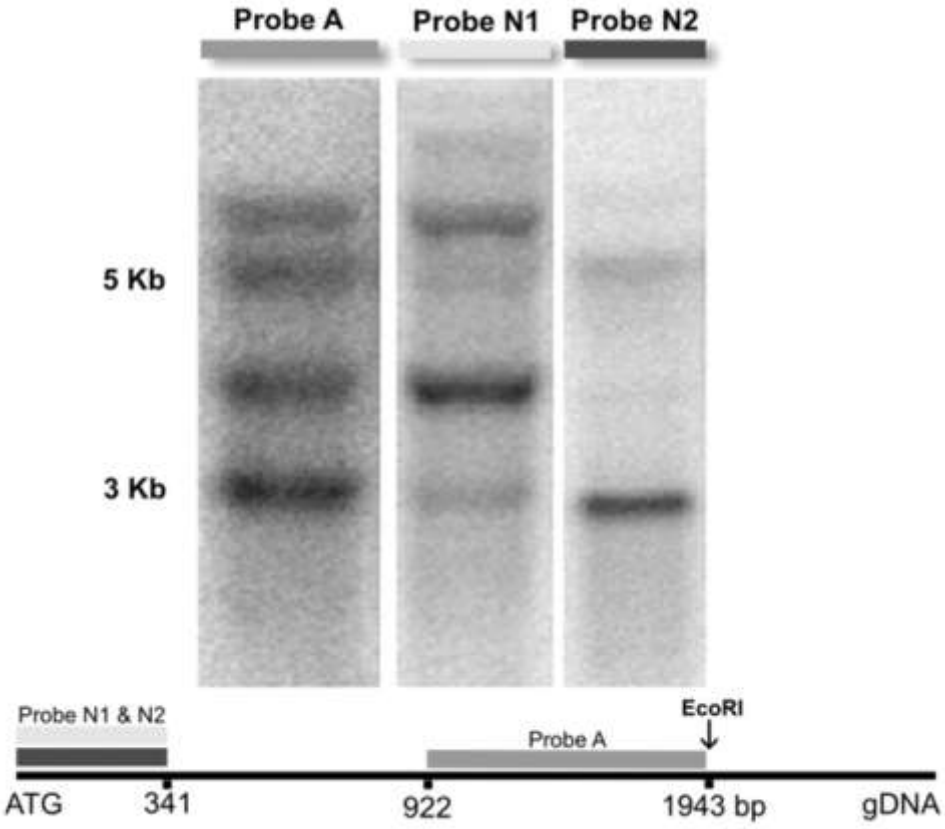
3.6. CONFLICT OF INTEREST

The authors have no conflicts of interests to declare.

3.7. SUPPLEMENTAL DATA



SUPPLEMENTAL FIGURE 3.1. Southern blot analysis of the *BnaDGAT1* gene variants in the *B. napus* DH12075 genome. Fragments from *EcoRI* digestions were separated by gel electrophoresis before transfer to a nylon membrane. Radiolabelled probes were designed to hybridize to a conserved region (Probe A, ATGACCGAGGTCTTGTATCCAG, CACGGTCCCCGAAGCAGAGGAGCTCTG) or a variable N-terminal region *Bna*DGAT1s (ATGGAGATTTTGGATTCTGGAGGCG, GCGGCCGCATGGCTTTGTTTGAAGAT). These N-terminal probes were generated using BnaC.DGAT1.a or BnaA.DGAT1.b as templates. A conserved region of BnaDGAT1.a served as template to generate a probe capable of hybridizing to all four BnaDGAT1 genes. Hybridization and washing of the blot was performed at 55°C. This figure was modified from a version produced by Dr. Martin Truksa.

CHAPTER 4 - A NOVEL ASSAY OF DGAT ACTIVITY BASED ON HIGH TEMPERATURE GC/MS OF TRIACYLGLYCEROL

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Abbreviated Title: GC/MS Quantification of DGAT Activity

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4.1. ABSTRACT

Diacylglycerol acyltransferase (DGAT) catalyzes the final step in the acyl-CoA-dependent biosynthesis of triacylglycerol (TAG), a high energy compound composed of three fatty acids esterified to a glycerol backbone. *In vitro* DGAT assays, which are usually conducted with radiolabeled substrate using microsomal fractions, have been useful in identifying compounds and genetic modifications that affect DGAT activity. Here, we describe a high-temperature gas chromatography (GC)/mass spectrometry (MS)-based method for quantifying molecular species of TAG produced by the catalytic action of microsomal DGAT. This method circumvents the need for radio-labeled or modified substrates, and only requires a simple lipid extraction prior to GC. The utility of the method is demonstrated using a recombinant type-1 *Brassica napus* DGAT produced in a strain *Saccharomyces cerevisiae* which is deficient in TAG synthesis. The GC/MS-based assay of DGAT activity was strongly correlated with the typical *in vitro* assay of the enzyme using [1-^{14}C] acyl-CoA as an acyl donor. In addition to determining DGAT activity, the method is also useful for determining substrate specificity and selectivity properties of the enzyme.

Key Words: diacylglycerol acyltransferase; recombinant; acyl-CoA; selective ion monitoring; *Brassica napus*

4.2. INTRODUCTION

Triacylglycerol (TAG) is a high energy storage compound composed of three fatty acids esterified to a glycerol backbone. The carbon chain length and number of double bonds in these three fatty acids can impact the industrial or nutritional value of this storage lipid (Food and Agriculture Organization of the United Nations, 2010; Dyer et al., 2008; Innis, 2011; Yu et al., 2011). In many plants, animals and micro-organisms, TAG is synthesized by the acyl-CoA-dependent action of diacylglycerol acyltransferase (DGAT) (FIGURE 4.1) (Liu et al., 2012; Lung and Weselake, 2006). Both membrane-bound and cytosolic forms of this enzyme have been cloned and partially characterized (Hernandez et al., 2012; Liu et al., 2012; Lung and Weselake, 2006; Rani et al., 2012; Saha et al., 2006; Shockey et al., 2006; Turchetto-Zolet et al., 2011; Waltermann and Steinbuchel, 2005). Increasing the abundance of DGAT during seed development has been shown to result in increased seed oil content in some plant species add (Taylor et al., 2009; Weselake et al., 2008; Weselake et al., 2009). Increasing seed oil content is of paramount importance with a growing global population that has a high demand for vegetable oil for both food and industrial applications (Durrett et al., 2008; Rahman et al., 2013; Weselake et al., 2009). In terms of human health, the inhibition of DGAT activity is a promising strategy for treating obesity and other lipid-related disorders (Cao et al., 2011; Chen and Farese, 2005; Zhao et al., 2008).

Typically, *in vitro* DGAT assays are based on the quantifying the incorporation of radio-labeled acyl groups into TAG (Liu et al., 2012; Siloto and Weselake, 2010). These DGAT assays routinely use TLC to separate TAG from other lipid classes, thereby increasing the

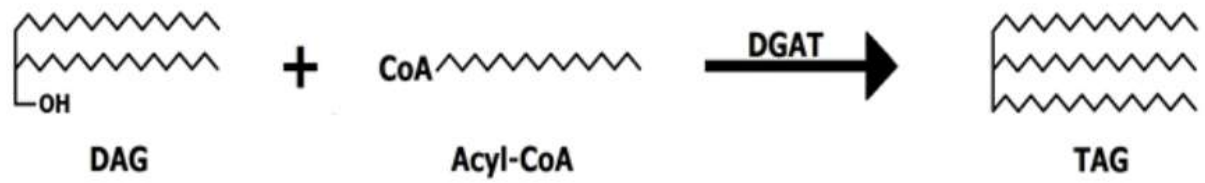

FIGURE 4.1. DGAT catalyzes the acyl-CoA-dependent acylation of *sn*-1,2-diacylglycerol (DAG) to produce triacylglycerol (TAG) and CoA.

amount on time spent on assaying the enzyme. Seethala et al. (2008) have circumvented the use of TLC in the *in vitro* DGAT assay by capturing radiolabeled TAG with polylysine SPA beads, which results in the production of a signal that can be measured by an imaging system. Radiolabeled substrates, however, are expensive and heavily regulated due to their potential health risks. Recently, fluorescence- based techniques for assaying DGAT activity have been developed (McFie and Stone, 2011; Sanderson and Venable, 2012). These methods use fluorescent substrate analogs which are limited to only a few commercially available compounds.

In early studies, Ichihara et al. (1982; 1988) used flame ionization detection to quantify molecular species of intact TAG resolved by high-temperature gas chromatography (GC) in order to gain insight into the substrate specificity and selectivity properties of DGAT activity in microsomes from developing safflower (*Carthamus tinctorius*) seed. The method, however, had to be conducted at a relatively large scale and involved lipid extraction coupled with TLC prior to GC. For routine *in vitro* DGAT assays, the investigators resorted to using the typical DGAT assay involving radiolabeled acyl-CoA as the donor substrate. More recently, mass spectrometry (MS) has been used to analyze and quantify molecular species of TAG resolved by high-temperature GC (Evershed, 1996; Kemppinen and Kalo, 2006; Ruiz-Samblás et al., 2010). Here, we report on the use of high-temperature GC/MS to resolve and quantify molecular species of TAG as a basis for the determination of DGAT activity, substrate specificity and substrate selectivity for recombinant *Brassica napus* DGAT1 produced in a strain (H1246) of *Saccharomyces cerevisiae* deficient in TAG synthesis. TAG quantification is based on the selected analysis of ion fragments produced by electron impact fragmentation. The GC/MS-

based DGAT assay is highly correlated with the typical method implementing radiolabeled acyl-CoA over a wide range of microsomal protein content. The method is comparable in cost to fluorescence-based methods and can use a wide range of commercially available, non-radioactive and unmodified substrates. The method also eliminates the use of TLC which can add time to *in vitro* DGAT assays and produce substantial amounts of organic solvent waste. As well, this method has been optimized using equipment far lower in cost than recently developed methods which use LC/MS/MS to quantify TAG production (Qi et al., 2010; Zhang et al., 2010). These features make GC/MS-based analysis DGAT activity analysis a highly attractive alternative to competing methods.

4.3. METHODS AND MATERIALS

4.3.1. Culture conditions and microsome preparation

TAG deficient quadruple knock out *S. cerevisiae* strain H1246 (Sandager et al., 2002) was transformed with pYES-NT vector (Invitrogen) carrying the coding sequence of *B. napus* DGAT1.a from the C genome (BnaC.DGAT1.a; Genbank ID: JN224473) in frame with the vector's N-terminal epitope, as per Gietz and Schiestl (2007). Cultures were induced and microsomes were isolated according to Siloto et al. (2009a). Quantification of protein content in yeast microsomes was performed using the Bradford assay (Bradford, 1976) with BSA as a standard.

4.3.2. GC-MS-based DGAT assay

Unless otherwise stated, approximately 3 μg microsomal protein was incubated in the presence of 198 mM Hepes-NaOH (pH 7.4), 3.2 mM $MgCl_2$, 333 μM *sn*-1, 2-dioctanoylglycerol (Avanti Polar Lipids Inc.; sonically dispersed in 0.2% Tween 20 (v/v) and 15 μM acyl-CoA (Avanti Polar Lipids Inc.). The total reaction volume was 120 μL. Microsomes were used to initiate the enzyme reaction. Reactions were allowed to proceed at 30°C for one minute, unless otherwise indicated, before quenching with 1mL chloroform methanol 2:1 (v/v). Two hundred microliters of 150 mM acetic acid and 80 μl of distilled H_20 were then added to the mixture (yielding a 10:5:6 choloroform:methanol:aqueous, v/v/v) prior to vortexing and centrifugation at 1,500g for 5 minutes. The non-polar phase was transferred to a 2 mL glass vial and dried down under nitrogen gas. The isolated lipids were resuspended in 60 μL of isooctane followed by vortexing. This solution was transferred to a 2 mL glass GC vile with 0.3 mL inserts. Care was taken to avoid the use of even chemically resistant plastic pipette tips or tubes during lipid extraction since even minimal exposure to the organic solvents leads to mass spectra with large contamination peaks. Selectivity assays were set up identically with the exception that reactions contained 15μM of palmitoyl (16:0)-CoA and 15μM oleoyl ($18:1\Delta^{9cis}$)-CoA.

Lipids were separated using a 6890N Network GC system (Agilent Technologies) along a 30m x 0.32 mm I.D. HP-5 column (5% Phenyl Methyl Siloxane, 0.25 μm film thickness) at a constant helium flow of 4 mL/min. The initial oven temperature of 150°C was raised at 15°C/min to a final temperature of 335°C and held there for an additional 18 minutes. Electron impact (70 eV) TAG fragmentation ions were produced and identified using a 5975 inert XL Mass Selective

Detector (Agilent Technologies) operating in selected ion monitoring mode, set to high resolution with 100 ms ion dwell time. Ions with *m/z* of 439, 327 and 127 were monitored for DGAT reactions using palmitoyl-CoA, and 465, 327 and 127 for those using oleoyl-CoA. MS data was acquired and quantified with the aid of MSD Chemstation Data Analysis software (version D.02.00.271, Agilent Technologies). Selectivity assays were performed using a 7890A GC system (Agilent Technologies) with the aid of MSD Chemstation Data Analysis software (version F.01.00.1903, Agilent Technologies).

4.3.3. DGAT assay using radio-labeled substrate

Conventional *in vitro* DGAT assays were performed similarly but acyl-CoA was replaced with 55 μCi/μmol [1-^{14}C] oleoyl-CoA (Perkin-Elmer). After phase extraction, TAGs suspended in isooctane were spotted on G25 silica plates (Macherey-Nagel) and isolated by TLC using 80:20:1 hexane: diethyl ether: acetic acid (v/v/v) as a mobile phase. Silica plates were imaged using a storage phosphor screen (Fuji Film) and Typhoon Trio+ variable mode imager (GE Healthcare). Identified TAG spots were scraped, submerged in Ecolite (MP Biomedicals), and quantified using an LS 6500 multi-purpose scintillation counter (Beckman Coulter). In some cases, the phase extraction procedure was omitted and reaction mixtures quenched with a solution of sodium dodecyl sulfate were spotted directly onto G25 silica plates (Byers et al., 1999).

4.4. RESULTS AND DISCUSSION

The specificity and selectivity properties of microsomal DGAT activity in safflower microsomes were previously examined using high-temperature GC coupled to flame ionization detection (Ichihara et al., 1988; Ichihara and Noda, 1982). These early investigations used *rac*-1,2-dihexanoylglycerol as an acyl donor to increase the volatility of the TAG generated through the catalytic action of DGAT. We also used a DAG consisting of short acyl chains for this purpose. In the process of designing the GC/MS-based assay of DGAT activity, we first demonstrated that microsomes containing recombinant BnaC.DGAT1.a could effectively use *sn*-1, 2-dioctanoylglycerol as an acyl-acceptor in the *in vitro* enzyme assay using [1-^{14}C] oleoyl-CoA as an acyl donor. Radiolabeled products resulting from DGAT action are shown for *sn*-1, 2-dioctanoylglycerol and *sn*-1, 2-dioleoylglycerol, respectively, in FIGURE 4.2. Both molecular species of DAG served as effective acyl acceptors for BnaC.DGAT1.a. Molecular species of TAG produced by acylation of this low molecular mass DAG had considerably shorter retention times than larger molecular species of TAG which were not as volatile. Thus, the use of a short chain DAG eliminated interference by long chain endogenous TAGs which may be present in microsomal fractions (Liu et al., 2012). Since the TAGs of most organisms are devoid of TAG containing octanoyl chains, the GC/MS-based assay of DGAT activity should be widely applicable providing the DGAT in question can utilize *sn*-1, 2-dioctanoylglycerol as an acyl acceptor. Recently, *sn*-1, 2-dihexanoylglycerol was effectively used as a substrate to assay recombinant *Arabidopsis* DGAT2 activity in leaf microsomes of tobacco (*Nicotiana benthamiana*) (Zhou et al., 2013). In earlier work, *sn*-1, 2-dioctanoylglycerol was also shown to

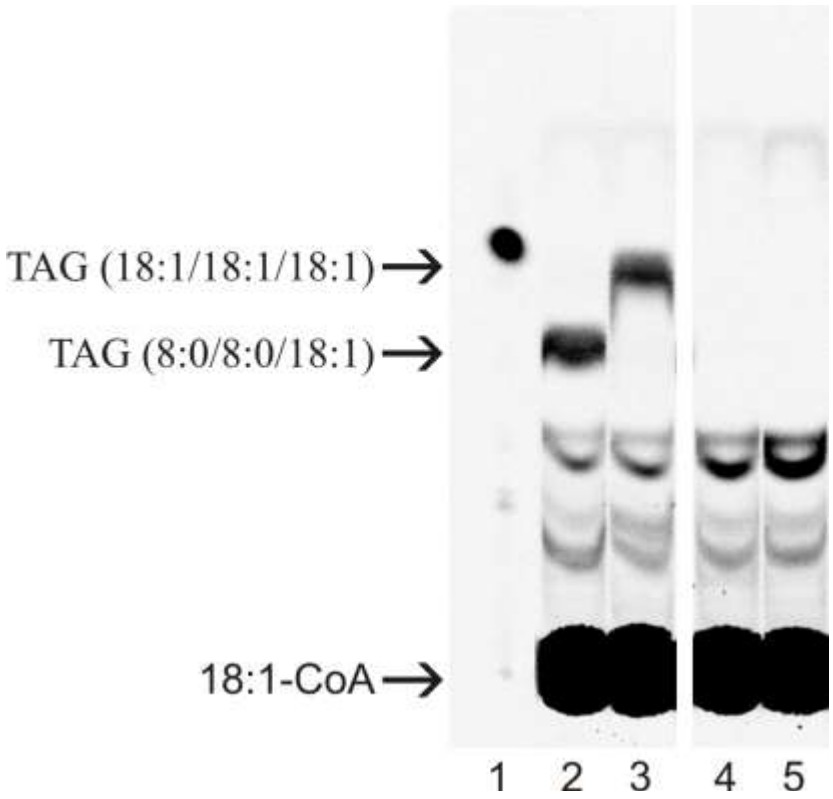

FIGURE 4.2. BnaC.DGAT1.a catalyzes the acylation of *sn*-1,2-dioctanoylglycerol or *sn*-1,2-dioleoylglycerol. [1-^{14}C] oleoyl-CoA was used as an acyl donor and reactions were quenched using a solution of sodium dodecyl sulfate, and then applied directly to the TLC plate as described by Byers et al. (34). Lipid classes were resolved using 80:20:1 hexane:diethyl ether: acetic acid (v/v/v). Lane 1, radiolabeled trioleoylglycerol standard; lane 2, microsomes from yeast cultures producing recombinant *B. napus* DGAT1.a assayed using *sn*-1, 2-dioctanoyl-glycerol; lane 3, assay of microsomal DGAT1 using *sn*-1, 2-dioleoylglycerol; lane 4, assay control with *sn*-1,2-dioctanoylglycerol and LacZ microsomes; lane 5, assay control with *sn*-1, 2-dioleoylglycerol and LacZ microsomes.

be an effective acyl-acceptor in the assay of mammalian microsomal DGAT activity (Lehner and Kuksis, 1993).

It has been demonstrated that the bonds within TAG molecules do not break randomly during electron impact ionization, and thus the production of certain fragment ions can be predicted. For example, $[RCO]^+$ and $[M\text{-}RCO_2]^+$, where R represents a hydrocarbon chain, are known to be abundant fragment ions in mass spectra produced by TAG compounds at 70 *e*V (Kemppinen and Kalo, 2006; Ruiz-Samblás et al., 2010). For DGAT reactions using oleoyl-CoA as a substrate (which produces *sn*-1,2-dioctanoyl-3-oleoylglycerol), the *m/z* ratios of these diagnostic fragment ions can be calculated to be 465, 327, 265 and 127. All of these ions are abundant in the total ion chromatogram of the mass spectra peak corresponding to *sn*-1, 2-dioctanoyl-3-oleoylglycerol (FIGURE 4.3). Each of the multiple ion peaks shown in FIGURE 4.4.a are based on the sum of the three most abundant ions which include127 $[C_8H_{15}O]^+$, 327 $[M\text{-}C_{18}H_{33}O_2]^+$ and 465 $[M\text{-}C_8H_{15}O_2]^+$. Each peak corresponds to a different microsomal protein content in the reaction mixture. Virtually no background noise was detected at this retention time, and large easily quantifiable peaks were produced by this mode of data acquisition. Integrating the area of these peaks demonstrated a linear dependence of enzyme activity on protein content of the reaction mixture between 0.75- 15µg of microsomal protein (FIGURE 4.4.b). In addition, the appearance of product as function of time was linear for 3 minutes where protein content was held constant (FIGURE 4.5). DGAT assays were routinely conducted using a reaction time of one minute. Furthermore, the enzyme activity data generated by the GC/MS-based method showed a high correlation (R^2=0.990) with the typical *in vitro* activity assay using

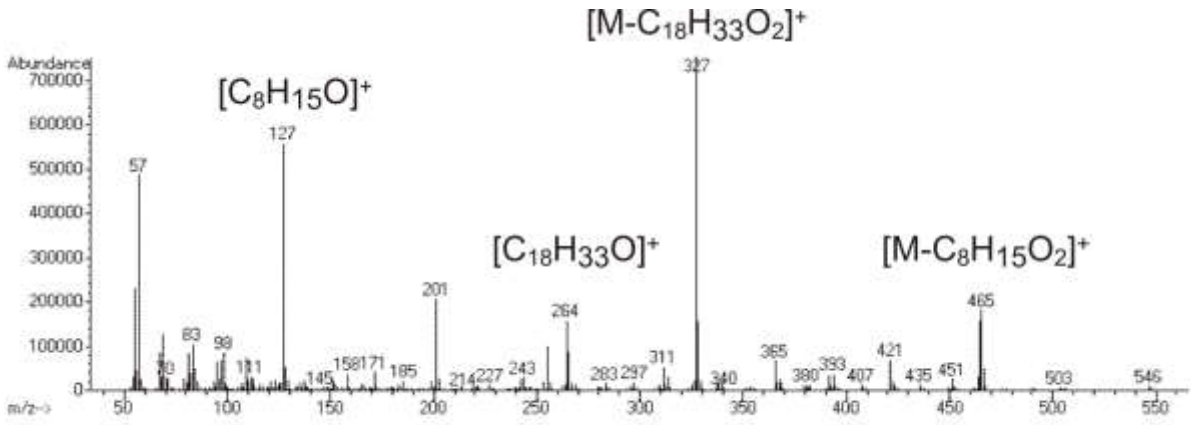

FIGURE 4.3. Total ion chromatogram of a *sn*-1, 2-dioctanoyl-3-oleoylglycerol peak produced by the *in vitro* assay of BnaC.DGAT1.a.

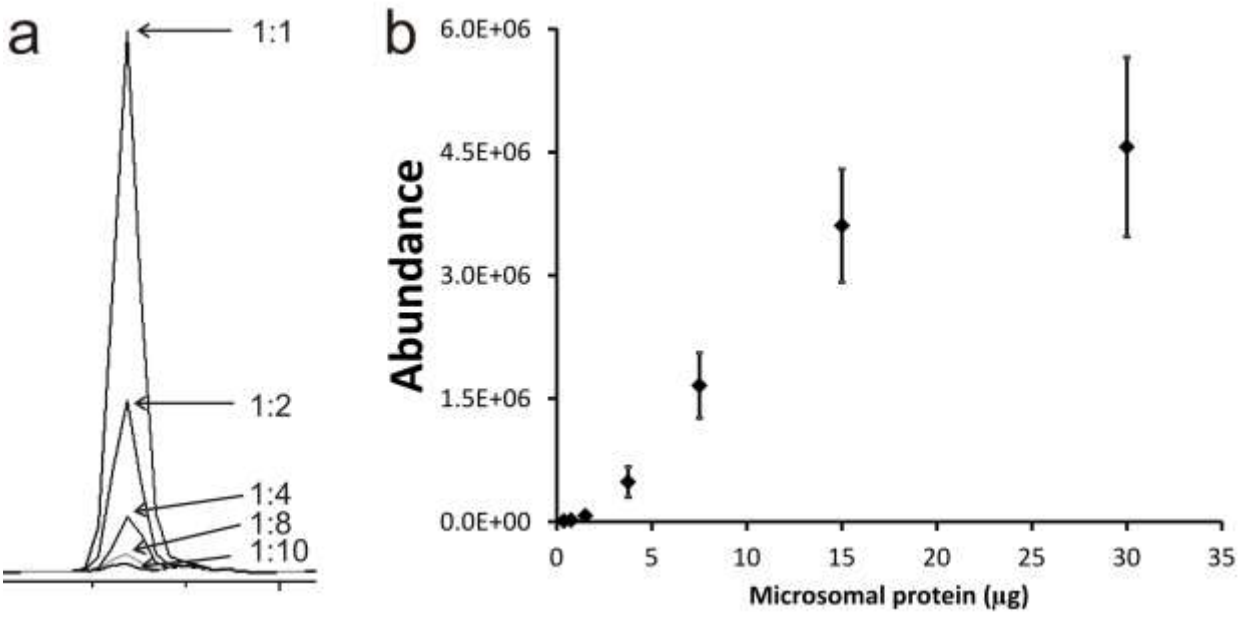

FIGURE 4.4. Analysis of triacylglycerol (TAG) produced by BnaC.DGAT1.a action using GC/MS. a) Overlay of selected ion monitoring abundance peaks corresponding to *sn*-1, 2-dioctanoyl-3-oleoylglycerol generated by *in vitro* DGAT assays with serially diluted microsomes. b) Dependence of TAG production (abundance) on microsomal protein content in the reaction mixture. Oleoyl-CoA (15 µM) was used as an acyl donor and *sn*-1, 2-dioctanoylglycerol (333 µM) was used as an acyl acceptor. Reactions were allowed to proceed for 2 minutes at 30˚C. Quantification of *sn*-1, 2-dioctanoyl-3-oleoylglycerol was performed using selective ion monitoring of m/z 465, 327 and 127.

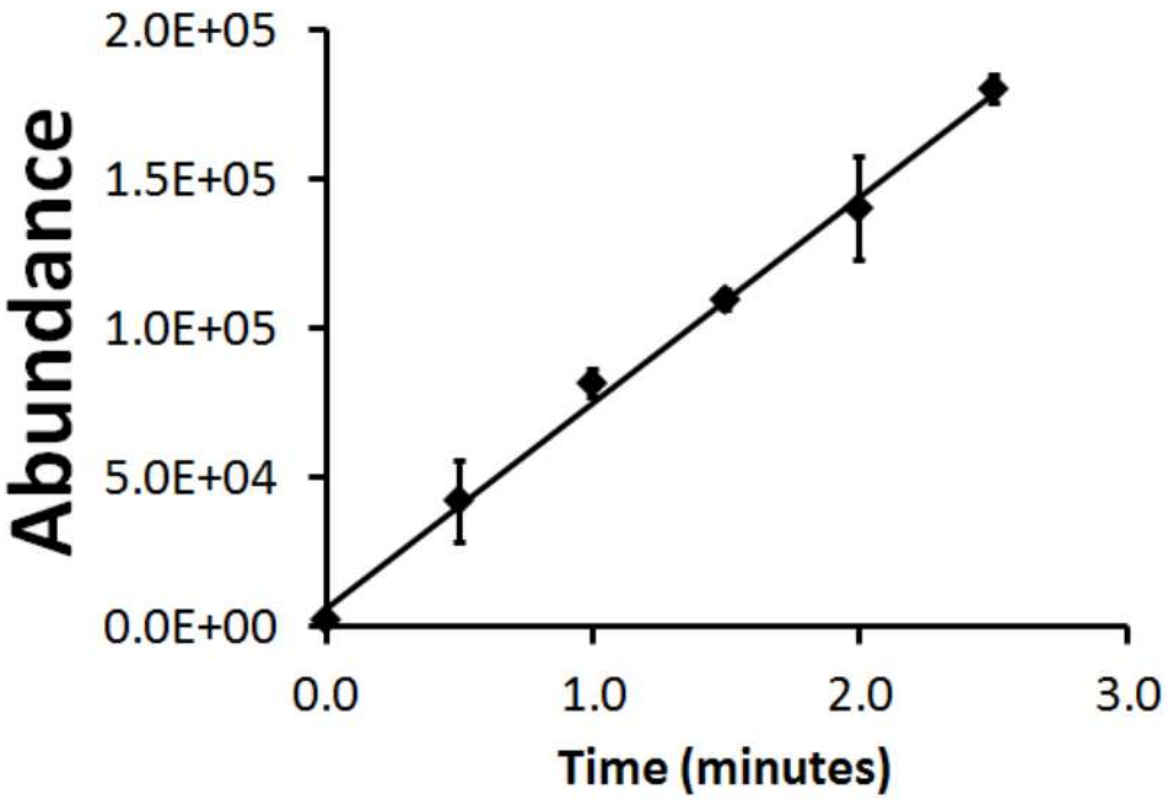

FIGURE 4.5. Dependence of triacylglycerol production (abundance) on reaction time for GC/MS-based assay of BnaC.DGAT1.a. Reactions were performed with 3 µg of microsomal protein at 30°C using oleoyl-CoA (15 µM) and *sn*-1,2-dioctanoyl glycerol (333 µM) as enzyme substrates. Quantification of *sn*-1,2-dioctanoyl-3-oleoylglycerol was performed using selective ion monitoring of m/z 465, 327 and 127.

radioactive acyl-CoA over a wide range of microsomal protein content in the reaction mixture (FIGURE 4.6). In cases where DGAT activity is substantially lower than that of microsomal BnaC.DGAT1.a, the lipid extraction step of the method allows for an increase in the volume of DGAT reaction mixture, which ultimately results in an increased concentration of TAG product (dissolved in isooctane) in the solution used for GC/MS. Increasing reaction time and microsomal protein content in the reaction mixture can also be used to produce detectable amounts of TAG in the DGAT assays. Based on the correlation plot shown in FIGURE 4.6, we estimate that our method can detect as little as 2.5 picomoles of *sn*-1,2-dioctanoyl-3-oleoylglycerol. This amount of TAG could be produced in 15 min by 3 μg of microsomal protein from H1246 yeast cultures producing a typical recombinant plant DGAT (Siloto et al., 2009a).

If DGAT specific activity is to be reported as the quantity of TAG produced per minute per mg protein then it should be possible to calibrate the GC/MS system by injecting known quantities of TAG standard. When these standards are unavailable, however, a calibration plot, such as the one shown in FIGURE 4.6, can be used to convert abundance to enzyme specific activity. Expressing TAG production rates in terms of MS response, however, will often be sufficient to assess DGAT activity. Indeed, both LC-MS/MS (Qi et al., 2010; Sharma et al., 2008) and fluorescent-based (McFie and Stone 2011) DGAT activity assay methods which do not rely on such calibration plots have been recently published.

A major advantage of GC/MS-based quantification of TAG is the ability to distinguish different molecular species of TAG produced in DGAT reaction mixtures. In contrast, liquid scintillation counting of radiolabeled TAG cannot distinguish between different molecular

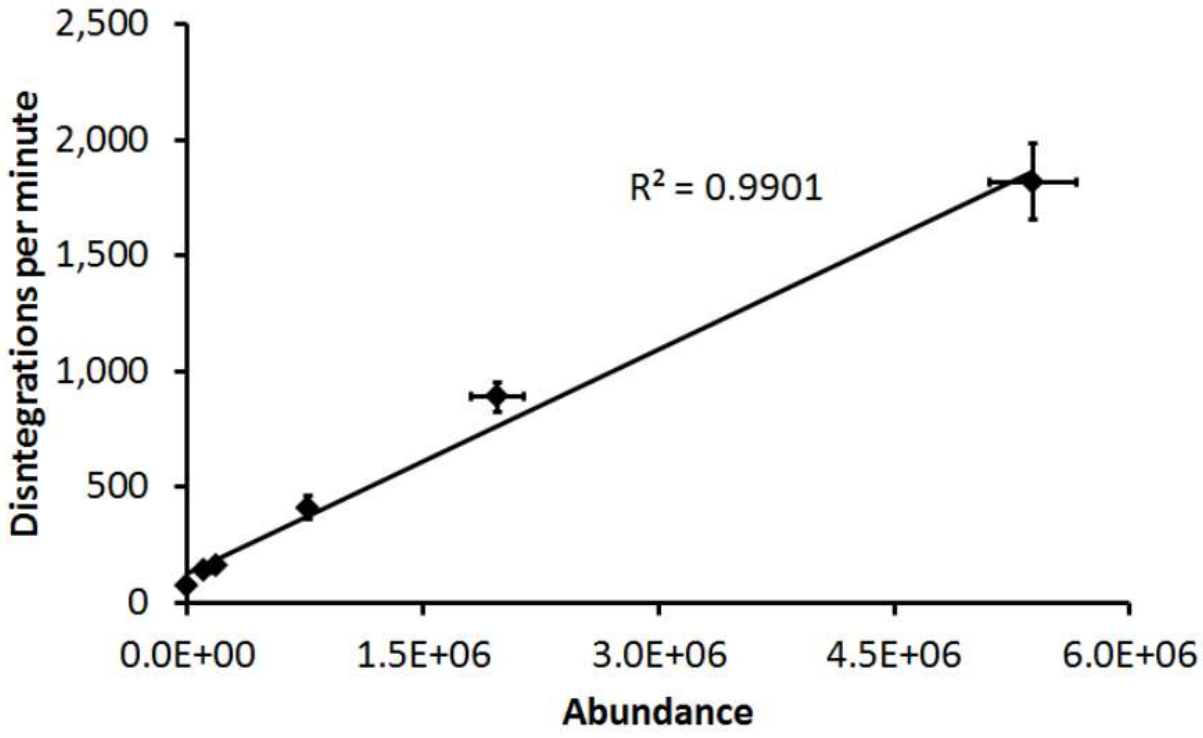

FIGURE 4.6. The GC/MS-based method for determining DGAT activity is highly correlated with the typical method using radio-labeled acyl-CoA over a wide range of microsomal protein content. Reactions were allowed to proceed for 2 minutes at 30°C using a microsomal protein content in the reaction mixture ranging from 0 to 30 µg.

species of TAG and TLC is often necessary to isolate TAG from other reaction mixture lipids prior to determination of radioactivity. DGAT selectivity assays based on the use of radioactive substrates require a laborious process of isolating TAG species by TLC, converting the fatty acyl chains of isolated TAG into fatty acid methyl esters, and then resolving the fatty acid methyl esters of different chain length and degree of saturation by a second round of chromatography (Oo and Chew, 1992). Using the method presented here, however, DGAT selectivity analysis requires no more extra work or time than for routine enzyme activity analysis. We have successfully analyzed TAG produced in enzyme reactions which utilized 16:0-, 18:0-, 18:2$\Delta^{9cis,12cis}$ or 18:3$\Delta^{9cis,12cis,15cis}$-CoA as acyl donors using the same conditions described for reactions using oleoyl-CoA as a substrate, with the exception that the MS/selected ion monitoring was adjusted to follow ions $[RCO]^+$ and $[M\text{-}RCO_2]^+$ which correspond to the new TAG species. It is important to note, however, fatty acid chain length, degree of saturation and regiospecificity in TAG can influence the proportion of each fragment ion produced during electron ionization, thereby producing different MS response factors per unit TAG (Kemppinen and Kalo, 2006). As a result, molar correction factors need to be empirically determined for each new acyl-CoA tested to determine the exact molar ratio of one molecular species of TAG to another. In many instances, however, molar quantification of TAG may not be required.

Results from an experiment which combines analysis of specificity and selectivity of BnaC.DGAT1.a is shown in FIGURES 4.7 and 4.8. The GC/MS profiles shown in FIGURE 4.7 demonstrate that *sn*-1,2-dioctanoyl-3-palmitoylglycerol produced by the catalytic action of DGAT can be effectively resolved from *sn*-1,2-dioctanoyl-3-oleoylglycerol to allow for independent detection of these molecular species of TAG by selective ion monitoring. Excess

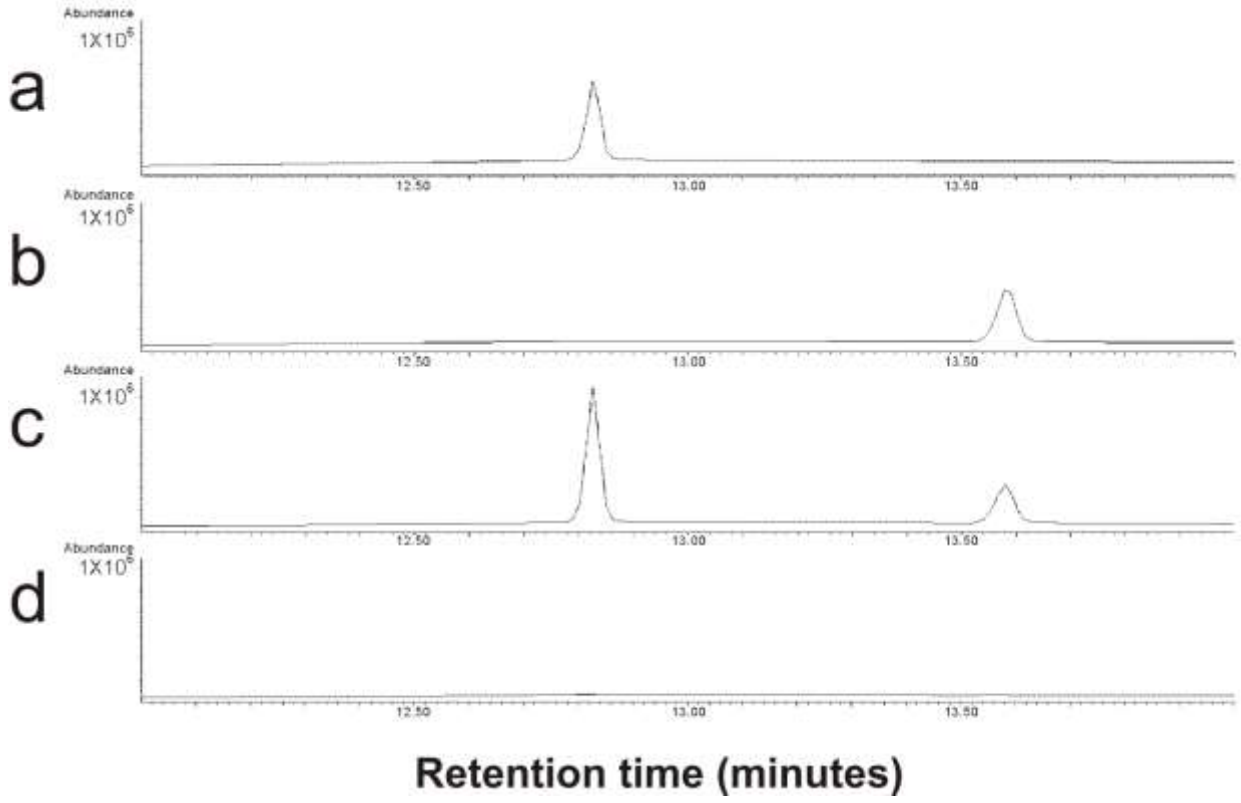

FIGURE 4.7. GC/MS separation and detection of molecular species of triacylglycerol produced by BnaC.DGAT1.a action. a) 15 µM palmitoyl-CoA used as the acyl donor; b) 15 µM oleoyl-CoA used as the acyl donor; c and d) 15 µM of each acyl-CoA Reactions for a, b and c were carried out using 3 µg of microsomal protein for 1 minute at 30˚C, whereas reaction d was terminated at t = 0 minutes.

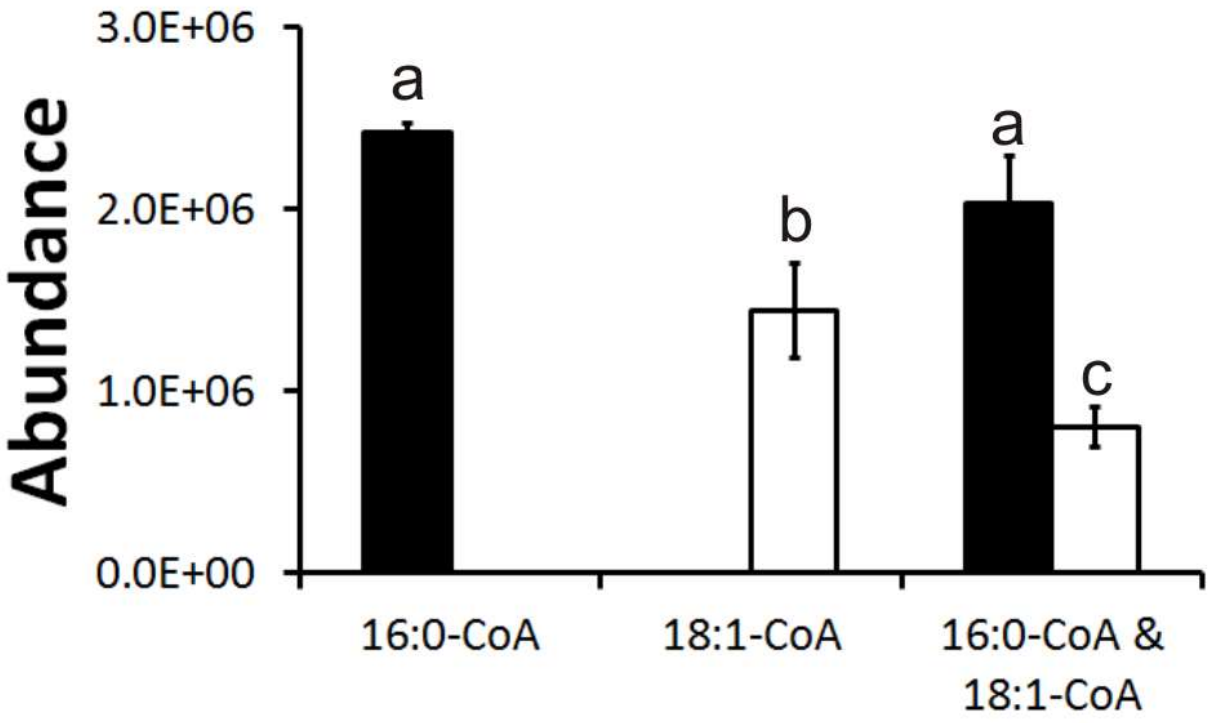

FIGURE 4.8. Acyl-CoA specificity and selectivity of BnaC.DGAT1.a using two molecular species of acyl-CoA. Enzyme reactions were carried out using 15 µM of palmitoyl-CoA or oleoyl-CoA, or in combination, with 3 µg of microsomal protein for 1 minute at 30˚C. Letters indicate statistical grouping.

sn-1, 2-dioctanoylglycerol remaining in the injected sample is released from the column at about 6 minutes (not shown). It is important to note, however, that complete separation of TAG products is not always required, depending upon the fragments ions selected for monitoring.

Specificity and selectivity results are summarized in FIGURE 4.8. Here, it was shown that when BnaC.DGAT1.a can select either palmitoyl-CoA or oleoyl-CoA as an acyl donor, the enzyme's preference for palmitoyl-CoA was substantially higher than the level of preference based on activity assays performed individually with the two acyl donors. In the specificity assay where substrates were not combined, the ratio of enzyme activity supported by palmitoyl-CoA to activity supported by oleoyl-CoA was 1.7. In contrast, under conditions where both substrates were available to the enzyme in equimolar proportions, this ratio increased to about 2.6. In this competitive situation, palmitoyl-CoA may inhibit the incorporation of oleoyl moieties into TAG. Selectivity data such as this can aid investigators to better understand the response of a DGAT to combinations of different molecular species of acyl-CoA. Indeed, under cellular conditions DGATs are exposed to such mixtures of acyl donors.

It should be noted that the described GC/MS-based DGAT assay could potentially be subject to interference by phospholipid: diacylglycerol acyltransferase (PDAT) depending on the source of enzyme. PDAT catalyzes the transfer of a fatty acyl chain from phosphatidylcholine to DAG to produce TAG (Dahlqvist et al., 2000). Our assay is particularly suited to determining the activity of recombinant DGAT produced in yeast strain H1246 which is devoid of PDAT activity (Sandager et al., 2002), and should also be applicable to the assay of DGAT activity from mammalian tissues which are not known to exhibit PDAT activity (Lung and Weselake,

2006). Thus far, PDAT activity is known to be present in higher plants (Dahlqvist et al., 2000), algae (Yoon et al., 2012), yeast (Sandager et al., 2002) and in *Streptomyces coelicolor* (Arabolaza et al., 2008). Our GC/MS-based DGAT assay, however, would be applicable to analyzing the activity of a recombinant DGAT from any source produced in a PDAT-free yeast such as strain H1246. Acyl-CoA-dependent activity should indicate DGAT rather than PDAT activity.

In summary, high-temperature GC/MS-based analysis of DGAT activity is a relatively rapid assay procedure for this enzyme which eliminates the need for radioactive substrates. In addition, TLC separation of lipid classes is not required thereby shortening assay time and reducing the production of organic solvent waste. The assay is highly correlated with the typical assay using radio-labeled acyl-CoA as a substrate over a range of microsomal protein content and can be used to quickly acquire substrate selectivity data.

4.5. ACKNOWLEDGEMENTS

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CHAPER 5 – TWO CLADES OF TYPE-1 *BRASSICA NAPUS* DIACYLGLYCEROL ACYLTRANSFERASE EXHIBIT DIFFERENCES IN SUBSTRATE PREFERENCE

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Running Title: Divergent selective properties of four BnaDGAT1

Keywords: DGAT, MBOAT, triacylglycerol biosynthesis, substrate specificity, oilseed rape

5.1. ABSTRACT

Globally, oilseed rape (*Brassica napus* and *Brassica rapa*) is the third largest source of vegetable oil. Diacylglycerol acyltransferase (DGAT) catalyzes the acyl-CoA dependent acylation of *sn*-1,2 diacylglycerol to produce TAG, which is the main component of the seed oil of *Brassica* oilseed species. In *B. napus*, the level of DGAT activity has been shown to have a substantial effect on the flow of carbon into seed oil. Within the genome of *B. napus*, we identified four actively transcribed *DGAT1* genes. Phylogenetic analysis of the amino acid sequences encoded by these genes suggests they diverged over time into two clades (I and II), with representative members in each genome (A and C) of *B. napus*. The majority of amino acid sequence differences in these DGAT1 isoforms reside outside of motifs suggested to be involved in catalysis. Despite this, the clade II enzymes displayed a significantly enhanced preference for substrates containing linoleic acid when assessed using *in vitro* enzyme assays with yeast microsomes containing recombinant plant enzyme. Although the four enzymes could use a range of molecular species of acyl-CoA as substrates, α-linolenoyl-CoA was the most effective acyl donor in each case. *sn*-1,2-dioleoylglycerol was the most effective acyl acceptor of the three molecular species of *sn*-1,2-diacylglycerol evaluated as substrates. These findings contribute to our understanding of TAG biosynthesis in *B. napus* oilseeds, and may advance our ability to engineer DGAT1s with desired substrate selectivity properties.

5.2. INTRODUCTION

The Brassicaceae, or mustard family, contains many agronomically- important species (Warwick, 2011). Of these, oilseed rape (*Brassica napus* and *B. rapa*) represents the crop of greatest economic value, particularly in the temperate climate regions of northern Europe and Canada (Weselake et al., 2008). In Canada, most of the oilseed rape is low in erucic acid ($22{:}1\Delta^{13cis}$) and glucosinolate content, and known as canola (Synder and Weselake, 2012). The Canadian canola industry adds 19.3 billion dollars (CAN$) annually to the national economy (Canola Council of Canada, 2013), accounting for just over one percent of the national gross domestic product (Trading Economics, 2014). Canola primarily derives its value from its nutritionally superior seed oil, which has been bred to contain low levels of saturated fatty acids (FA), high levels of oleic acid ($18{:}1\Delta^{9cis}$) and relatively high levels of α-alpha-linoleic acid ($18{:}2\Delta^{9cis,12cis}$) (Scarth and Tang, 2006; Yurchenko et al., 2007). These FA are esterified in triacylglycerol (TAG), which is the primary component of canola seed oil. Both increasing overall TAG content and modifying the proportion of desirable FAs in TAG could potentially increase the economic value of this crop (Yurchenko et al., 2007).

During seed oil development in oilseed species such as *B. napus,* diacylglycerol acyltransferase (DGAT; EC 2.3.1.20) catalyzes the acyl-CoA-dependant acylation of *sn*-1,2 diacylglycerol (DAG) to produce TAG (Liu et al., 2012; Lung and Weselake, 2006). The level of DGAT activity during seed development appears to have a substantial effect on the flow of carbon into TAG in *B. napus* and other oleaginous plant species (Harwood et al., 2013; Jako et al., 2001; Misra et al., 2013; Weselake et al., 2009). In addition, maximum DGAT activity in *B.*

napus has been shown to occur during the active phase of seed lipid accumulation and decline with seed maturity (Tzen et al., 1993; Weselake et al., 1993). Two unrelated membrane-bound DGAT families (DGAT1 and DGAT2) with gene orthologs in numerous organisms have been identified (Liu et al., 2012). A membrane-bound bi-functional wax ester synthase/DGAT, which appears to function in wax ester formation in stem epidermis, has also been reported in *Arabidopsis thaliana* (*Arabidopsis*) (Li et al., 2008a). In addition, soluble DGATs have been characterized in peanut (*Arachis hypogaea*) (Chi et al., 2014; Saha et al., 2006) and *Arabidopsis* (Hernandez et al., 2012; Rani et al., 2010). Seed TAG can also be produced through the transfer of a FA from phosphatidylcholine to *sn*-1,2-DAG in a reaction catalyzed by membrane-bound phospholipid:diacylglycerol acyltransferase (PDAT; EC 2.3.1.158) (Dahlqvist et al. 2000; Ståhl et al., 2004; Zhang et al., 2009). Experiments on transcript accumulation in various oil-producing species have suggested that the relative contributions of DGAT1, DGAT2 and PDAT to TAG production are species-specific (Li et al., 2010), but DGAT1 is probably the major DGAT contributing to TAG formation in *Arabidopsis* (Li et al., 2010). Additional research is required to clarify the possible role of DGAT2 in seed oil formation in *Arabidopsis* and other Brassicaceae. Recently, *Arabidopsis DGAT2* was functionally expressed in tobacco (*Nicotiana benthamiana*) leaves (Zhou et al., 2013) and codon-optimized for functional expression in *Saccharomyces cerevisiae* mutant strain H1246 which is defective in TAG biosynthesis (Aymé et al., 2014). Previous attempts at functionally expressing *Arabidopsis DGAT2* in *S. cerevisiae* were not successful (Liu et al., 2012; Zhang et al., 2009). In contrast, tung tree (*Vernicia fordii*) *DGAT2* (Shockey et al., 2006) and castor (*Ricinus communis*) *DGAT2* (Burgal et al., 2008; Kroon et al., 2006) could be functionally expressed in yeast. Kroon et al. (2006), however, incorporated a yeast consensus sequence in the construct.

Earlier studies with microsomes from developing seeds of oleaginous plants suggested that DGATs generally have broad substrate specificities although there appeared to be a few exceptions (Lung and Weselake, 2006; Weselake, 2005). As examples of the latter, microsomal DGAT activity from *B. napus* (Lenora X CHR 1775/82) containing 54% erucic acid displayed enhanced specificity for 22:1-CoA (Bernerth and Frentzen, 1990), whereas microsomal cocoa (*Theobroma cacao*) DGAT activity showed strong selectivity for stearoyl (18:0)-CoA (Griffiths and Harwood, 1991). In addition, in a more recent study, Yu et al. (2006) demonstrated that microsomes from *Vernonia galamensis* or *Stokesia laevis* exhibited highest DGAT activities with substrate combinations of vernoloyl (*cis*-12-epoxy-octadeca-*cis*-9-enoyl)-CoA and *sn*-1,2-divernoloylglycerol. Both of these plant species accumulate vernolic acid as the major FA in the seed oil. The eventual recombinant production of different DGAT families in yeast and other expression systems revealed new insights into DGAT specificity and selectivity that could be attributed to specific isoenzymes (i.e., DGAT1 versus DGAT2). Tung tree DGAT2, which accumulated high levels of α-eleostearic acid ($18{:}3\Delta^{9cis,11trans,13trans}$) in the seed oil exhibited an enhanced preference for producing trieleostearin (Shockey et al. 2006). In addition, castor *DGAT2* expressed in *Arabidopsis* transformed with *FATTY ACID HYDROXYLASE* resulted in a near doubling of the ricinoleic acid (12-OH $18{:}1\Delta^{9cis}$) content of the seed oil (Burgal et al., 2008), ricinoleic acid being the main FA of castor oil. Li et al. (2010) further demonstrated that co-expression of *S. laevis EXPOXYGENASE* and *V. galamensis DGAT2* in petunia (*Petunia* x *hybrida*) or soybean (*Glycine max*) somatic embryos resulted in enhanced production of vernolic acid when compared to a combination of *EPOXYGENASE* and *DGAT1* expression. Recently, the substrate preferences of *Arabidopsis* DGAT1 versus DGAT2 were compared in microsomes from tobacco leaves and *S. cerevisiae* strain H1246 expressing cDNAs encoding these

isoenzymes. Zhou et al. (2013) demonstrated that microsomal recombinant *Arabidopsis* DGAT2 preferred oleoyl-CoA and α-linolenoyl (18:3$\Delta^{9cis,12cis,15cis}$)-CoA as acyl donors whereas DGAT1 exhibited a strong preference for oleoyl-CoA when *sn*-1,2-Dihexanoyl (6:0) glycerol was used as an acceptor substrate. Analysis of the FA composition of TAG in yeast strain H1246 expressing codon optimized *Arabidopsis DGAT1* or *DGAT2* indicated that DGAT1 preferentially incorporated palmitic acid (16:0) whereas DGAT2 exhibited a preference for palmitoleic acid (16:1Δ^{9cis}) (Aymé et al. 2014). Maize *DGAT1-2* is the first example of plant *DGAT1* alleles encoding DGAT1 enzymes with different substrate preferences (Zheng et al., 2008). Ectopic expression in maize of the allele with a phenylalanine insertion at position 469 (F469) resulted in transgenic lines with both enhanced oil and oleic acid content. The F469 form of DGAT1-2 also resulted in increased microsomal DGAT activity and TAG content when the cDNA representing the allele was expressed in yeast.

B. napus DGAT1 (*BnaDGAT1*; GenBank ID: AF155224 and AF1644341) was first cloned and functionally expressed in *Pichia pastoris* by Nykiforuk et al. (1999a; 1999b; 2002). cDNA AF155224 appears to be a truncated form of cDNA AF1644341, encoding an N-terminally truncated DGAT1 which is still active. Seed-specific over-expression of *BnaDGAT1* AF1644341 *in B. napus* has been shown to increase seed oil content (Weselake et al., 2008; Taylor et al., 2009). In addition, a recent report has shown that expression of the truncated *BnaDGAT1* cDNA (AF155224) in *Chlamydomonas reinhardtii* results in increased lipid production (Ahmad et al., 2014). In addition, saturated FA content decreased by about 7%, whereas polyunsaturated FA content was increased by about 12% (especially α-linolenic acid), suggesting that the *B. napus* enzyme has increased selectivity for substrates containing

polyunsaturated FAs. Recently, we reported the identification of four transcriptionally active *BnaDGAT1* genes in the *B. napus* genome (CHAPTER 3). In the current study, we describe the phylogeny of these *BnaDGAT1* gene forms and characterized the substrate specificity and selectivity properties of the recombinant enzymes. Two clades of DGAT1, which appear to have evolved relatively early in Brassicaceae's history, differ in their preference for linoleoyl-CoA and *sn*-1,2-dilinoleoylglycerol. These findings contribute to our understanding of oil synthesis in *B. napus*, and may ultimately aid in the development of DGAT1 enzymes with desired substrate specificity properties.

5.3. METHODS AND MATERIALS

5.3.1. Protein alignment and phylogenetic analysis

B. rapa and *B. oleracea DGAT1* coding DNA sequences (CDS) were obtained from the Brad.org and Bolbase (version 1.0) databases, respectively. The identification and sequencing of *B. napus DGAT1* CDS is described in chapter 3. For phylogenetic analysis, the full-length CDS were aligned based on their corresponding amino acid translations using the TranslatorX server ((Abascal, et al., 2010); http://translatorx.co.uk/). Subsequently, maximum likelihood (ML) phylogenetic analysis was constructed via the CIPRES Web Portal http://www.phylo.org/portal/Home using MrBayes 3.2.2 (Huelsenbeck and Ronquist, 2001). The phylogenetic tree was visualized using FigTree (http:/tree.bio.ed.ac.uk/software/figtree/).

5.3.2. Transformation of *S. cerevisiae* with *BnaDGAT1*

Generation and culture conditions of *S. cerevisiae* H1246 cultures expressing N-terminally tagged *BnaDGAT1* cDNAs is described in chapter 3.

5.3.3. *In vitro* DGAT activity assays

Microsomal fractions were isolated from *S. cerevisiae* H1246 cultures expressing *DGAT1* constructs according to Siloto et al. (2009a). Acyl-CoA specificity assays were conducted in a similar fashion to the procedure described by Byers et al. (1999). Unless indicated otherwise, microsomes containing 150 to 300 ng of protein were incubated in the presence of 200 mM Hepes-NaOH (pH.74), 3.2 mM $MgCl_2$, 333 µM *sn*-1,2-dioleoyl glycerol (Avanti Polar Lipids) and 15 µM [1-^{14}C]acyl-CoA (51-55uCi/µmol). Acyl-CoAs were prepared according to Taylor et al. (1990) using FA purchased from Nu-Chek Prep. DGAT reactions were allowed to proceed at 30°C for 2-3 minutes before quenching with sodium dodecyl sulfate. DAG specificity reactions were carried out similarly for 3-5 minutes using 1 µg of microsomal protein in the presence of indicated DAG (Avanti Polar Lipids) and 15 µM [1-^{14}C] oleoyl-CoA (Perkin Elmer). Reactions were then spotted on G25 silica plates and TAGs were isolated using 80:20:1 hexane: ether: acetic acid (v/v/v) as a mobile phase. After air-drying, TAG spots were scraped, submerged in Ecolite (MP Biomedicals), and radioactivity was quantified using an LS 6500 multi-purpose scintillation counter (Beckman Coulter).

In vitro selectivity assays were performed as described in chapter 4, with some modifications. Lipid extracts from selectivity reactions were resuspended in hexane and molecular species of TAG were resolved by GC on a CP-TAP column (Agilent Technologies). Quantification of TAG fragmentation ions (m/z 127, 328, 253 and 251) was performed using an Agilent 7890A GC/MS system (Agilent Technologies) with the assistance of MSD Chemstation Data Analysis software (version F.01.00.1903, Agilent Technologies).

5.3.4. Statistical analysis

Analysis of DGAT-specific activity was performed using one-way ANOVA, $\alpha = 0.05$. Analysis of DGAT1 selectivity was performed by t-test, $\alpha = 0.05$.

5.4. RESULTS

5.4.1. Phylogenetic analysis of BnaDGAT1 genes and deduced amino acid sequences encoded by the open reading frames

Earlier work describing the identification of four *B. napus DGAT1* genes demonstrated that these sequences could be subdivided into two clades based upon polypeptide sequence homology (CHAPTER 3). Here, so called clade I contained BnaA.DGAT1.a and BnaC.DGAT1.a, and clade II contained BnaA.DGAT1.b and BnaC.DGAT1.b. A more comprehensive analysis of DGAT1 polypeptide sequences from Brassicaceae species suggests the DGAT1s of these clades may have diverged before the separation of the *Arabidopsis* and

Brassica linages (FIGURE 5.1.a). *Arabidopsis*, *Eutrema* and *Capsella* possess single copy *DGAT1* genes, which were found to be more closely related to clade II than they are to clade I BnaDGAT1. As expected, the progenitors of *B. napus*, *B. rapa* (A genome) and *B. oleracea* (C genome), have two copies of *DGAT1* genes, which are each present in one of the two DGAT1 clades. These genes did not group with any other (n=47) plant *DGAT1* sequences. The *BnaDGAT1* genes encoding clade I and clade II enzymes possess highly conserved gene organization, with 16 similarly sized and spaced exons (FIGURE 5.1.b). The deduced gene architecture of all four genes displayed high similarity to that reported for AtDGAT1 (Liu et al., 2012). In all cases, the genes' first exon was the largest, encoding a hydrophilic N-terminal domain with lower sequence homology than the following coding nucleotide sequence.

Alignment of the amino acid sequences encoded by the *B. napus*, *B. rapa* and *B. oleracea* *DGAT1* amino acid sequences (FIGURE 5.2) highlights that these enzymes share a high degree of homology (FIGURE 5.3), particularly within their respective clades. The eight enzymes share 91% pairwise identity with each other, and all have deduced isoelectric points and molecular masses close to 8.4 and 57.4kDa, respectively. Even greater similarity can be observed within the aforementioned sub-clades, with clade I enzymes sharing 98% pairwise homology, and clade II enzymes sharing 97%. As an example, BnaA.DGAT1.b and *B. rapa* DGAT1.b differ by only 2 amino acids in their 503 amino acid sequences. The majority of the amino acid sequence variations found in these polypeptides reside outside of motifs identified to be common in

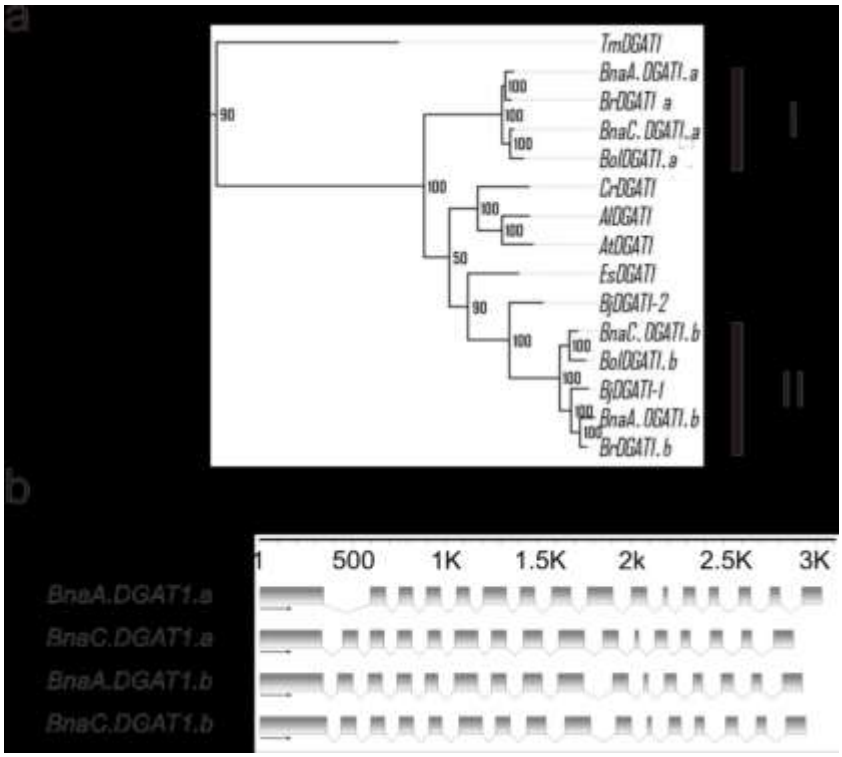

FIGURE 5.1. Analysis of *Brassica napus DGAT1* coding DNA sequences (a) Phylogenetic tree of Brassicaceae DGAT1 coding sequences. The maximum likelihood tree was generated using the MrBayes program, and the clade support values at the internal nodes are Bayesian posterior probabilities (shown as percentage). *Brassica napus DGAT1* clades A (I) and B (II) are denoted with solid black bars. *Arabidopsis lyrata- AlDGAT1* (XM 002885993), *Arabidopsis thaliana- AtDGAT1* (NM 127503), *Brassica juncea- BjDGAT1-1* (DQ 016106.1), *BjDGAT1-2* (DQ 016107.1), *Brassica napus- BnaA.DGAT1.a* (JN 224474), *BnaA.DGAT1.b* (JN 224475), *BnaC.DGAT1.a* (JN 224473), *BnaC.DGAT1.b* (JN 224476), *Brassica oleracea- BolDGAT1.a* (Bol 022722), *BolDGAT1.b* (Bol 029796), *Brassica rapa- BrDGAT1.a* (Bra 039003), *BrDGAT1.b* (Bra 036722), *Capsella rubella- CrDGAT1* (XM 006299234), *Eutrema salsugineum- EsDGAT1* (XM 006408994), *TmDGAT1* (XM 007012718). (b) Gene organization of *B. napus* DGAT1 genes. Exons are represented with shaded boxes and introns by dotted lines.

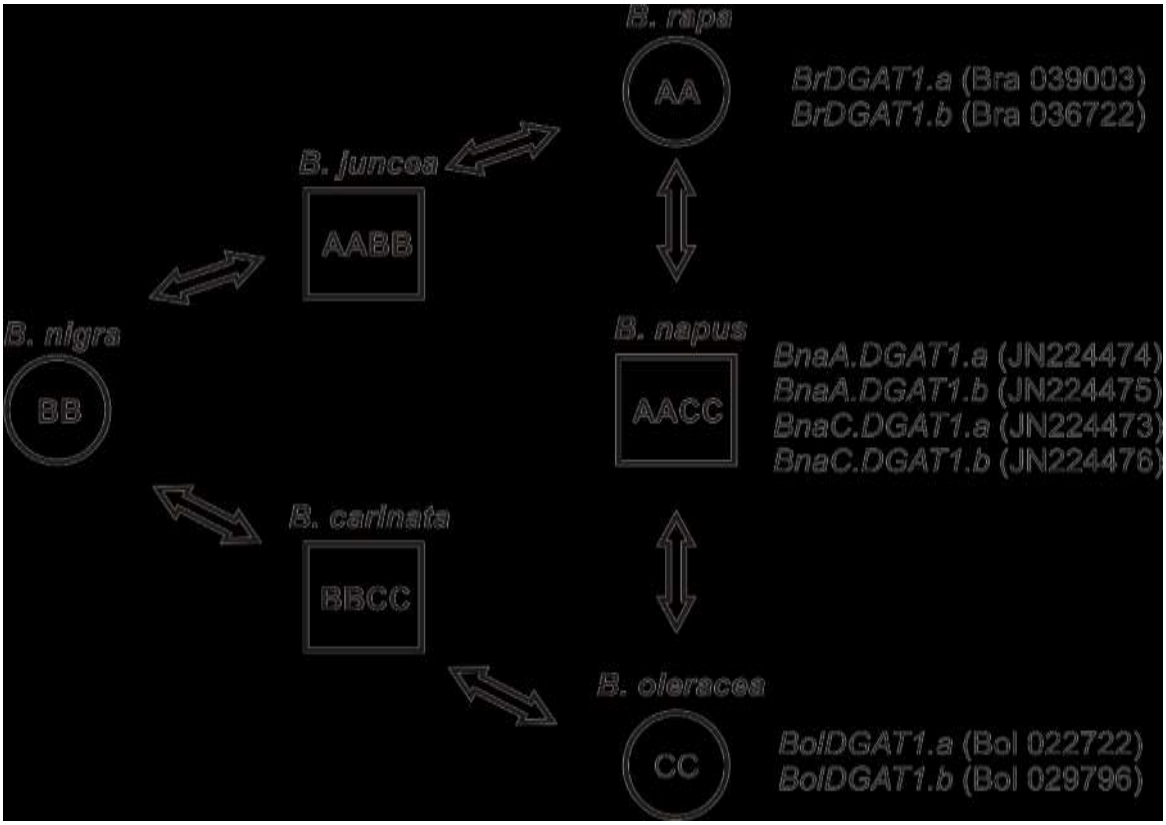

FIGURE 5.2. Triangle of U diagram demonstrating the genetic relationship of *Brassica* DGAT1 genes. BolBase, BRAD and GENBANK database reference numbers are provided in parenthesis for *B. oleracea*, *B. rapa* and *B. napus DGAT1* genes, respectively.

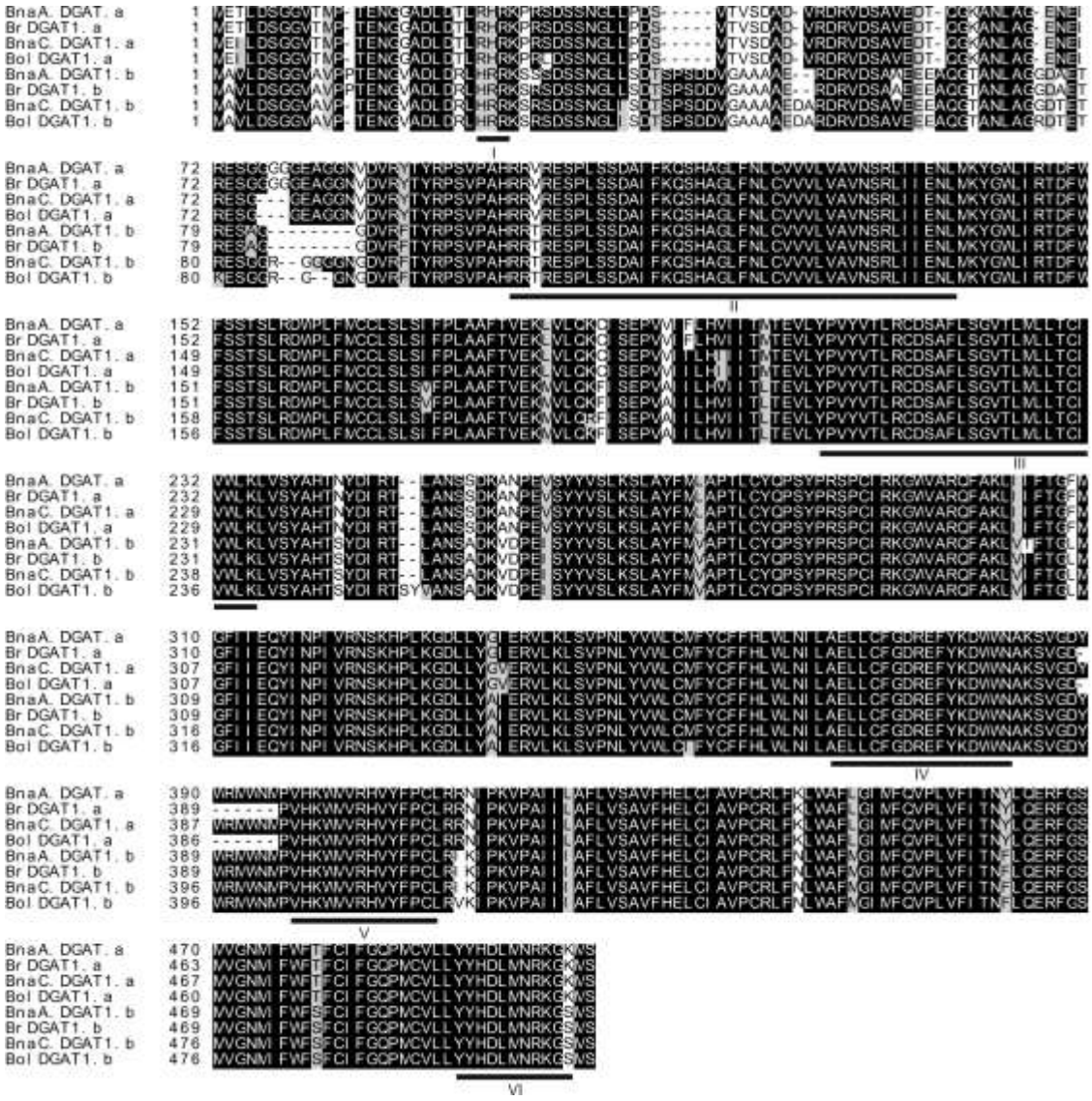

FIGURE 5.3. Homology comparison of deduced *Brassica* DGAT1 amino acid sequences. Alignment of BnaA.DGAT1.a (JN224474), BnaC.DGAT1.a (JN224473), BnaA.DGAT1.b (JN224475), BnaC.DGAT1.b (JN224476), BolDGAT1.a (Bol 022722), BolDGAT1.b (Bol 029796), BrDGAT1.a (Bra 039003) and BrDGAT1.b (Bra 036722) sequences was performed using Clustal W2. Conserved motifs of putative basic RRR motif (I), acyl-CoA binding signature (II), thiolase acyl enzyme intermediate binding signature (III), fatty acid protein signature (IV), DAG binding site (V) and ER-retrieval motif (VI) are highlighted with solid black bars.

type-1 DGATs. Interestingly, a histidine/arginine conversion in the basic RRR motif, conversion of a valine to a threonine in the acyl-CoA binding signature, and a lysine to serine mutation in the C-terminal ER retrieval motif are notable exceptions where clade II enzymes differ from clade I enzymes within known motifs.

5.4.2. Clade II BnaDGAT1 enzymes exhibited increased preference for linoleoyl-CoA when compared to clade I enzymes

To investigate the acyl-CoA substrate preferences of the BnaDGAT1, *in vitro* assays were performed using H1246 yeast microsomes containing recombinant BnaDGAT1 in the presence of radiolabeled acyl-CoA and exogenously supplied *sn*-1,2-dioleoylglycerol. Specific activities observed for the four recombinant enzyme forms ranged from 1 to 35 nmol•min^{-1}•mg^{-1}, but in all cases α-linolenoyl-CoA was the preferred substrate (FIGURE 5.4.a). To overcome the large differences in absolute specific activities and observe the relative preference of different acyl-CoAs for each BnaDGAT1, DGAT activities were normalized relative to activities obtained using α-linolenoyl-CoA as an acyl donor (FIGURE 5.4.b). The clade II enzymes (BnaA.DGAT1.b and BnaC.DGAT1.b) were observed to have a significantly higher relative preference for linoleoyl-CoA compared to the clade I enzymes BnaA.DGAT1.a and BnaC.DGAT1.a. In addition, BnaC.DGAT1.b exhibited significantly increased preference for stearoyl-CoA relative to the three other BnaDGAT1 variants.

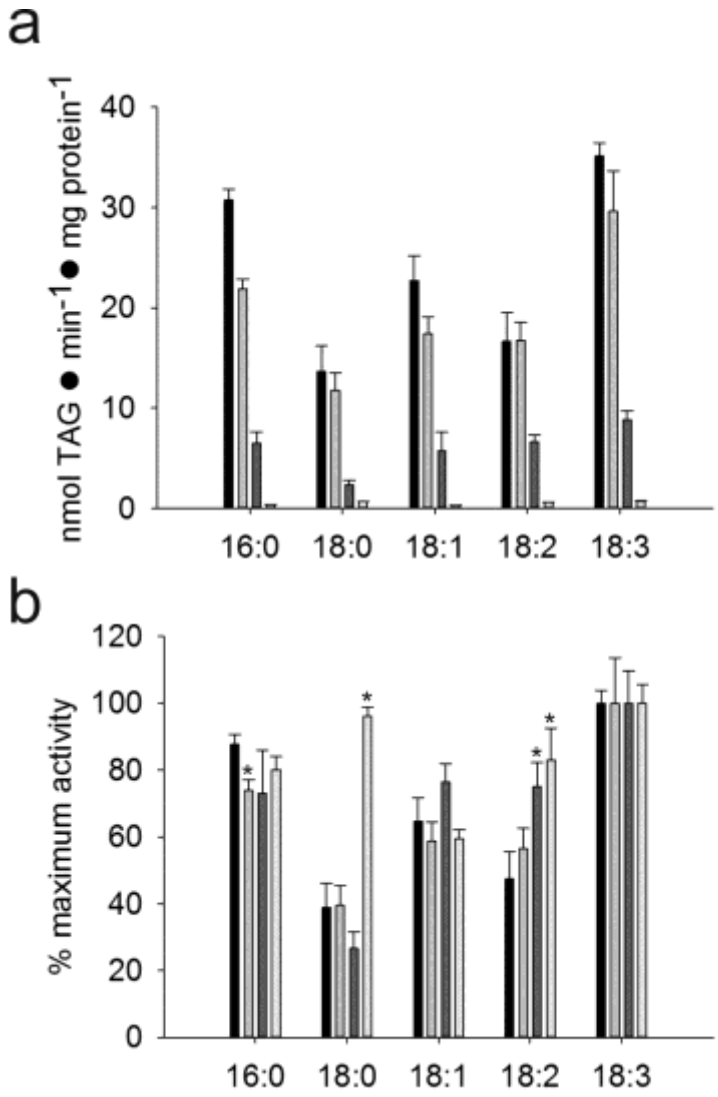

FIGURE 5.4. Acyl-CoA substrate specificities of the four recombinant BnaDGAT1 variants. Panel a: *In vitro* DGAT assays were conducted in triplicate using microsomes isolated from *S. cerevisiae* H1246 cultures producing different recombinant BnaDGAT1s, various radiolabeled acyl-CoAs, and 333 mM exogenous *sn*-1,2 diolein. Panel b: Specific activity results, normalized for percent maximum activity. Error bars indicate +/- 1 standard deviation * indicates significant difference from BnaA.DGAT1.a. ■BnaA.DGAT1.a, ■BnaC.DGAT1.a, ■BnaA.DGAT1.b, ■BnaC.DGAT1.b

An enzyme form from each clade was selected for substrate selectivity assays using equimolar concentrations of linoleoyl- and α-linolenoyl-CoA in the reaction mixture (FIGURE 5.5). In this competitive scenario, the clade II enzyme BnaA.DGAT1.b incorporated relatively more linoleic acid into TAG than its clade I counterpart (BnaC.DGAT1.a).

5.4.3. Clade II BnaDGAT1 enzymes exhibited increased preference for *sn*-1,2-dilinoleoylglycerol when compared to clade I enzymes

To expand upon the substrate specificity analysis, DGAT assays were performed using radiolabeled oleoyl-CoA and three molecular species of DAG (FIGURE 5.6.a and 5.6.b). To observe the contributions of endogenous DAG in support of DGAT activity, control reactions were performed without the addition of exogenous DAG. Without exogenous DAG, enzyme activities were about 20% of the rates observed in the presence of exogenous DAG (FIGURE 5.6b). All the DGAT1s appeared to prefer *sn*-1,2- dioleoylglycerol whereas *sn*-1,2-dipalmitoylglycerol was a poor acyl acceptor. The data, however, suggested that BnaA.DGAT1.a and BnaA.DGAT1.b may be relatively more efficient at using this substrate than BnaC.DGAT1.a or BnaC.DGAT1.b. Clade II BnaDGAT1 enzymes preferred *sn*-1,2-dilinoleoylglycerol when compared with clade I enzymes.

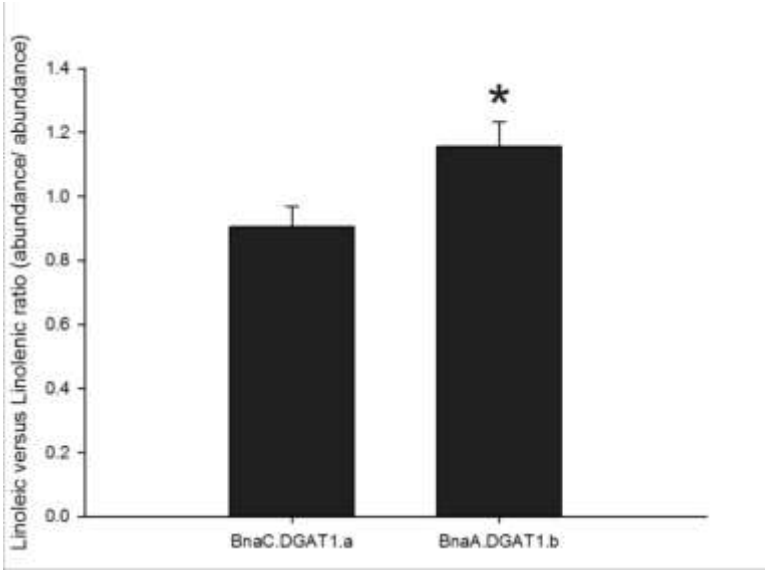

FIGURE 5.5. Linoleoyl-CoA versus linolenoyl-CoA selectivity analysis of recombinant BnaDGAT1 enzymes produced in *S. cerevisiae* H1246. DGAT selectivity assays were conducted in triplicate using microsomes isolated from *S. cerevisiae* H1246 cultures producing recombinant BnaC.DGAT1.a or BnaA.DGAT1.b. Error bars indicate +/- 1 standard deviation, * indicates significant difference between means.

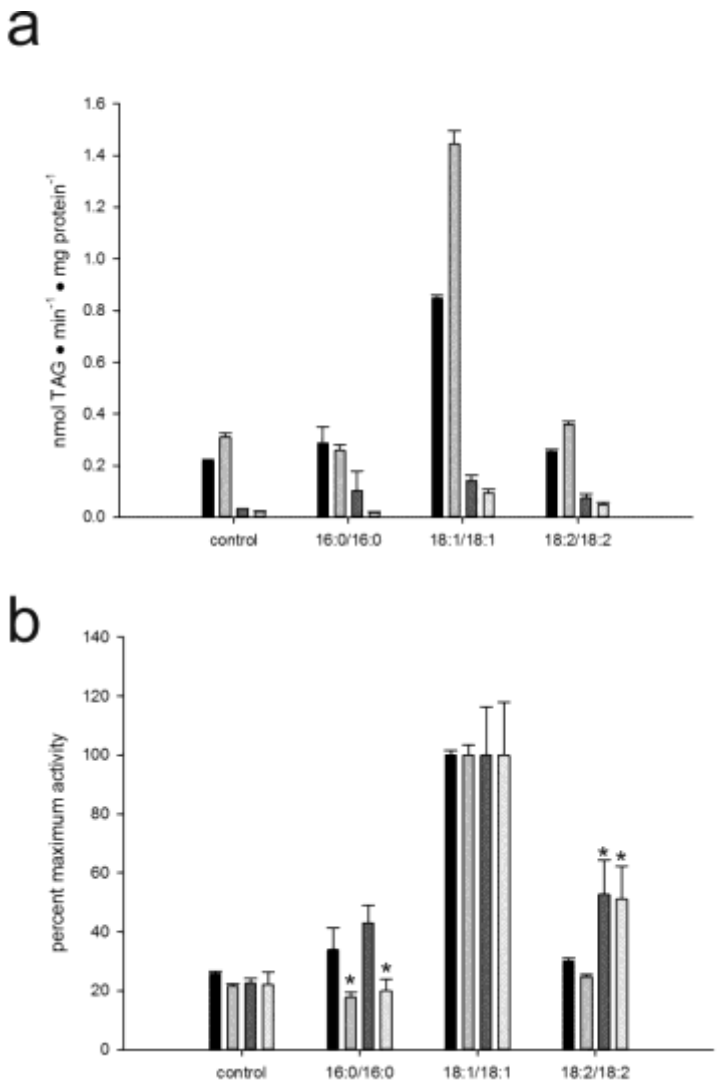

FIGURE 5.6. *sn*-1,2-Diacylglycerol substrate specificities of the four recombinant BnaDGAT1 variants. a. *In vitro* DGAT assays were conducted in triplicate using microsomes isolated from *S. cerevisiae* H1246 cultures producing different recombinant BnaDGAT1, radiolabeled oleoyl-CoA, and different molecular species of 333mM exogenous *sn*-1,2 DAGs. Reactions were carried out for 3-5 minutes1 µg of microsomal protein. b. Specific activity results normalized for percent maximum activity. Error bars indicate +/- 1 standard deviation. * indicates significant difference from BnaA.DGAT1.a. ■BnaA.DGAT1.a, ■BnaC.DGAT1.a, ■BnaA.DGAT1.b, ■BnaC.DGAT1.b

5.5. DISCUSSION

Although genome and EST sequence information is limited for many Brassicaceae family members, our phylogenetic analysis of available Brassicaceae DGAT1 polypeptide sequences suggests two clades of DGAT1 were produced prior to the *Brassica* tribe diverging from the *Arabidopsis* line, which has been estimated to have occurred approximately 20-24 million years ago (Ziolkowski et al., 2006). Indeed, numerous duplication events followed by significant interspersed whole gene deletions have been demonstrated to have occurred in the evolution of the Brassicaceae species (Town et al., 2006). This history of genomic re-arrangements may explain how an ancestral species may have acquired two forms of DGAT1, one of which (clade I) being subsequently lost during *Arabidopsis*' evolution. While the BnaDGAT1 enzyme forms share high amino acid sequence homology, the enzymes appear to have differences in amino acid sequence within possible functional DGAT1 motifs. Additionally, the diversification of these genes occurring early in Brassicaceae evolution provided additional time for the enzymes to adopt different biological roles, or adapt selective preferences which better fit the environment they are expressed in. Indeed, it is well known that gene duplication provides flexibility for genes to evolve new roles without compromising essential biological functions (Qian and Zhang, 2014). As additional sequence information becomes available, it will be interesting to see how many members of the Brassicaceae family possess a clade I DGAT1.

For the first time, we have described substrate specificity differences among four closely related BnaDGAT1 enzymes. Previous studies of BnaDGAT specificity utilized microsomes (or solubilized fractions) from developing seeds and embryo/cell cultures as a source of enzyme

activity (Bernerth and Frentzen, 1990; Byers et al., 1999; Cao and Huang, 1986; Little et al., 1994; Vogel and Browse, 1996; Weselake et al., 1993; Weselake et al., 1991) and thus observations on substrate specificity would have been based on the combined activities of various forms of BnaDGAT1 and possibly further confounded by the presence of BnaDGAT2 activity. Interestingly, however, the order of specific preferences for palmitoyl-, stearoyl- and oleoyl-CoA observed are in agreement with previous *in vitro* assays of DGAT activity in a solubilized fraction prepared from microspore-derived cell suspension cultures of *B. napus* L. cv Jet Neuf (Little et al., 1994). Another study using microsomes of developing cotyledons of *B. napus* indicated nearly equal preference of microsomal DGAT activity for *sn*-1,2-dioleoylglycerol or *sn*-1,2-dilinoleoylglycerol (Vogel and Browse, 1996). In the current study, the *in vitro* substrate specificity and selectivity assays of recombinant BnaDGAT1 isoforms suggest that the A and C genomes of *B. napus* each contain two closely related *BnaDGAT1* genes which encode two clades of DGAT1 which differ in their preference for substrates containing linoleic acid. Ahmad et al. (2014) recently reported that the expression of a truncated *BnaA.DGAT1.b* cDNA (AF155224) in *C. reinhardtii* results in increased polyunsaturated FA content (especially α-linolenic acid) in the algal lipid. The fact that this DGAT1 was essentially devoid of its hydrophilic N-terminus suggests that enhanced selectivity for substrates containing polyunsaturated FAs resides in the region of the enzyme beyond the hydrophilic N-terminal region. It should be noted, however, that Ahmad et al. (2014) have mistakenly referred to this enzyme as a type-2 DGAT, apparently based on an earlier attempt at DGAT nomenclature (Nykiforuk et al., 2002) which was not adopted by the scientific community.

The discovery of differences in substrate preferences among highly similar enzyme isoforms can be of potential value in the identification of amino acid residues or regions of enzyme governing substrate selectivity. As previously indicated, the basic RRR motif, acyl-CoA binding signature and C-terminal ER retrieval motif represent potential sites in the BnaDGAT1 isoforms which could be potentially be modified by site-directed mutagenesis to alter substrate specificity so as to convert a clade I substrate preference to a clade II substrate preference, and vice-versa. It is also interesting to note that the recombinant hydrophilic N-terminal region of BnaA.DGAT1.b was able to interact with acyl-CoA and display an increased affinity for erucoyl-CoA over oleoyl-CoA (Weselake et al., 2006). The study by Zheng et al. (2008) on maize DGAT1-2 showed that a single amino acid substitution at position 469 (found in an ancestral allele encoding the enzyme) resulted in both a more active enzyme with increased preference for substrate containing oleic acid. The study on maize DGAT1-2 thus shows that it is possible for highly identical DGATs to exhibit marked differences in catalytic efficiency and substrate preference. Site-directed mutagenesis of a serine residue in a putative protein kinase site in a *Tropaeolum majus* DGAT1 has been shown to result in a substantial increase in seed oil content when the recombinant enzyme was introduced into *Arabidopsis* in a seed-specific fashion (Xu et al., 2008), once again demonstrating that modification of a single amino acid residue in a DGAT can have a profound impact on enzyme activity. In addition, the expression of two forms of *Tetraena mongolica DGAT1*, encoding enzymes sharing 82% amino acid sequence identity, in *T. mongolica* calli produce cells differing in FA compositions (Li et al., 2013). Further investigation, however, will be necessary to establish if the modified FA compositions observed in *DGAT1* expression experiments are due solely to changes in enzyme substrate preference or also involve increases in carbon flux into TAG due to increased DGAT

activity in the system (CHAPTER 3). Indeed, in the case of *T. mongolica TmDGAT1* over-expression in *T. mongolica* calli, it was noted that *DGAT1-b* transcripts were elevated two-fold over *DGAT1-a* transcripts resulting in fifty percent more cellular TAG content (Li et al., 2013).

In conclusion this is the first study reporting differences in substrate preferences among four DGAT1 enzymes encoded by the *B. napus* genome. Our discovery represents an important step in developing a more in depth understanding how DGAT activity contributes to TAG production in developing *B. napus* seed. In turn, this fundamental information will be critical in the engineering of DGAT1 enzymes with desired substrate specificity properties.

5.6. ACKNOWLEDGEMENTS

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CHAPTER 6 - CONCLUSION

Diacylglycerol acyltransferase (DGAT) plays an important part in the biosynthesis of triacylglycerol (TAG) in many oilseed species, including *Brassica napus* (*B. napus*) (Liu et al., 2012; Lung and Weselake, 2006). As a result, this class of enzyme has been the target of numerous studies attempting to identify strategies for modifying seed lipid content and fatty acid composition (Weselake et al., 2009). Over-expression of open reading frames encoding DGAT polypeptides has successfully increased seed oil content in many species (Andrianov et al., 2010; Taylor et al., 2009; Weselake et al., 2008), but the observed gains in seed oil content and DGAT activity were not proportionate to the increases in *DGAT1* transcripts (Nykiforuk et al., 2002; Taylor et al., 2009). These results suggest that plant DGAT1 activity may be regulated at the post-transcriptional level. Structure/function analyses which might identify mechanisms of DGAT regulation, however, have had limited success due in part to the highly hydrophobic nature of the two major forms of this enzyme (Liu et al., 2012). These difficulties place a premium on any form of DGAT characterization which might assist in understanding how this enzyme functions and how it can be engineered to produce greater lipid content in biological system.

The first goal of this thesis was to gain insight into the functional importance of the N-terminal region of BnaDGAT1. Placement of an N-terminal epitope tag on any four of the BnaDGAT1 isoforms eliminated the influence of native N-terminal sequence during heterologous production of the enzyme in *S. cerevisiae*. Genetic engineering of *BnaDGAT1* coding sequences to change amino acid residues in the N-terminus of BnaDGAT1 isoforms also

affected accumulation of the enzyme forms which was reflected in significantly altered TAG accumulation. Thus, modification of the N-terminal region of BnaDGAT1 proved to be a valuable tool for producing yeast microsomes highly enriched in recombinant BnaDGAT1 polypeptide as a basis for biochemical characterization of the isoforms. Additionally, these findings provide a new approach to increase lipid content in yeast for industrial applications such as providing feed stocks for biodiesel.

It would be of great interest to observe if seeds producing DGAT1 isoforms with engineered N-terminal regions can accumulate more TAG than seeds producing the wild type isoforms. Such a discovery would have the potential to greatly increase the value of numerous oilseed crops (Jako et al., 2001). Similarly, it would be of interest to evaluate the effect of N-terminally-modified BnaDGAT1 isoforms in further boosting oil content in yeast strains which naturally produce high oil. As mentioned in chapter 3, yeast- based production of fatty acids can be an attractive alternative to plant based production of biofuel feedstock (Li et al., 2008).

Investigating the mechanism of how the N-terminal region of BnaDGAT1 influences enzyme accumulation may be of great value as well. Such knowledge could potentially contribute to our understanding how DGAT1 activity is regulated in plants, and may lead to novel strategies to modify DGAT action. The N-terminal region of DGAT1 has been shown to interact with acyl-CoAs and can facilitate self-association of enzyme subunits (Caldo et al., 2015; McFie et al., 2010; Siloto et al. 2008; Weselake et al., 2006). This region of the enzyme has also been shown to be non-essential for catalysis (Siloto et al., 2009), further suggesting this domain has been retained in DGAT1 enzymes to perform a regulatory function.

Research guided by the second hypothesis of this thesis found that TAG produced by *in vitro* DGAT1 reactions could be determined by high temperature gas chromatography (GC) coupled to selective ion monitoring mass spectrometry (MS) to produce results nearly identical to those produced by conventional *in vitro* DGAT assays methods. Using GC/MS to detect *in vitro* TAG production eliminates the need of radiolabeled substrates and large amounts of organic solvents. Additionally, this method is useful for analyzing DGAT selectivity properties. Selectivity data can be useful in understanding how DGAT responds to the presence of different molecular species of acyl-CoA in the reaction mixture. Indeed, DGAT enzymes are exposed to mixtures of acyl-CoAs under cellular conditions. It would be interesting to use this method to analyze TAG produced by DGAT in *in vitro* reactions which have been designed to mimic cellular compositions of acyl species. DGAT assays performed under these conditions may better represent how the enzyme will perform within oil-forming plant cells.

Phylogenetic analysis suggested two clades of DGAT1 evolved relatively early in Brassicaceae's history, with representatives of each clade in the A and C genome. Despite the high degree of amino acid sequence homology shared by the four BnaDGAT1 isoforms, *in vitro* enzyme activity assays revealed BnaDGAT1 of the two clades differed in their substrate preferences. Identifying means to 'tailor' the fatty acid composition of TAG can significantly increase the value of oilseed crops (Scarth and Tang, 2006). The limited number of amino acid sequence variations which exist among the BnaDGAT1 isoforms may serve as a basis for identifying regions of the polypeptides which influence substrate specificity properties. Site directed mutagenesis could be used as a tool to probe the role of candidate amino acid residues in determining substrate specificity.

This is the first study reporting differences in substrate preferences among four DGAT1 enzymes encoded by the *B. napus* genome. In addition to improving our understanding of TAG synthesis in *B. napus*, these results suggest there is value to probing if these DGATs have adopted different biological roles. Initial investigation in this area may entail studying the expression pattern of each *BnaDGAT1* gene.

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APPENDIX 1

FIGURE A.1 Drawing of *Brassica napus DIACYLGLYCEROL ACYLTRANSFERASE* yeast expression cassettes. Vectors carrying expression cassettes contained nucleotide sequences encoding ampicillin resistance, a uracil selection marker (*URA3*) and a 2µ origin of replication. Expression of all cassettes was control in yeast by *GALACTOSE 1* (*GAL1*) promoter and *CYTOCHROME C1* (*CYC1*) terminator. *Brassica napus DGAT1*s (*BnaDGAT1*s) open reading frames were cloned in frame with nucleotide sequences encoding polyhistidine (6xHIS), X press, or V5 epitopes, as indicated.

APPENDIX 2

The following list describes contributions in addition to the preceding chapters, which I have made to the field of lipid metabolism during my doctoral studies.

2.1. Published manuscripts

- Pan X, Zou J, Qiu X, Chen C, Kazachkov M, **Greer MS**, Lager I, Weselake RJ (2015) *In vivo* and *In vitro* evidence for biochemical coupling of reactions catalyzed by lysophosphatidylcholine acyltransferase and diacylglycerol acyltransferase *J. Bio. Chem.* (*in press*).
    - For this work, I assisted in development of coupled and un-coupled *in vitro* DGAT activity assay conditions, and assisted in manuscript preparation.

- Caldo KMP, **Greer MS**, Chen G., Lemieux MJ, Weselake RJ (2014) Purification and Properties of recombinant *Brassica napus* diacylglycerol acyltransferase 1. *FEBS Lett.* 589: 773-8
    - For this work, I assisted in experimental design and optimization of protein expression conditions. I also critically reviewed the manuscript.

- Mietkiewska E, Miles R, Wickramarathna A, Sahibollah AF, **Greer MS**, Chen G, Weselake RJ (2014) Combined transgenic expression of *Punica granatum conjugase* (*FADX*) and *FAD2* desaturase in high linoleic acid *Arabidopsis* thaliana mutant leads to increased accumulation of punicic acid. *Planta* 240:575-83

- For this work, I assisted in numerous experiments and data analysis, in addition to critical review of the manuscript.

- Chen G, **Greer MS**, Weselake RJ (2013) Plant Phospholipase A: Advances in molecular biology, biochemistry and cellular function. *Biomolecular concepts* 4: 527–532
    - For this work, I assisted the first author in literature research, manuscript development, and final proofreading of the manuscript.

- Singer, SD, **Greer, MS**, Mietkiewska, E, Pan, X, Weselake, RJ (20130 Genetic engineering of lipid biosynthesis in the seeds of crucifer species. *Biotechnology of Crucifers* Gupta S.K. ed. July 31 2013
    - For this work, I contributed the chapter introduction and sections on triacylglycerol biosynthesis and non-acyl lipid biosynthesis. I also contributed two illustrations to the chapter as well.

- Chen G, **Greer MS**, Lager I, Yilmaz JL, Mietkiewska E, Carlsson AS, Stymne S, Weselake RJ (2012) Identification and characterization of an LCAT-like *Arabidopsis* thaliana gene encoding a novel phospholipase A. *FEBS Lett.* 586: 373-7
    - For this work, I analyzed yeast lipid contents through gas chromatography coupled mass spectrometry, and analyzed yeast fractions for presence of heterologously expressed LCAT constructs through western blotting.

- Chen G, Snyder CL, **Greer MS**, Weselake RJ (2010) Biology and Biochemistry of Plant Phospholipases. *Critical Rev. Plant Sci.* 30: 239-258
    - For this work, I authored one of three chapters, reviewing Phospholipase Cs.

2.2. Manuscripts in preparation

- Chen G, Tian B, **Greer MS.**, Caldo K, Singer S, Mietkiewska E, Dyer J, Smith M, Xiao Q, Stymne, S, Weselake RJ *Lesquerella* LCAT-like phospholipase facilitates the channeling of hydroxy fatty acid from PC to TAG in a hydroxylase over-expressed strain of *Saccharomyces cerevisiae*
    - For this work, I generated site directed mutations to analyze the catalytic active site of *Lesquerella* LCAT, and assisted in manuscript preparation.

- Mietkiewska, E., **Greer, M. S.**, Weselake, R. J. FADX RELATED MANUSCRIPT
    - For this work, analyzed the capacity the ability of pomegranate DGATS to utilize the products of FAD2 and FADX enzymes when expressed in yeast.