University of Alberta

Recombinant expression of plant diacylglycerol acyltransferases from tissues that accumulate saturated fatty acids

by

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Abstract

Vegetable oils enriched in saturated fatty acids (SFAs) could provide solid-fat functionality and decrease our reliance on hydrogenated oils. This thesis focuses on the characterization of enzymes that can preferentially incorporate SFAs into triacylglycerol (TAG). Acyl-CoA:diacylglycerol acyltransferase (DGAT) catalyzes the final reaction in acyl-CoA-dependent TAG biosynthesis, and may have a substantial effect on the flow of carbon into TAG. *DGAT* cDNAs encoding type-1 or 2 enzymes were isolated from sea buckthorn (*Hippophae rhamnoides*) pulp and cocoa (*Theobroma cacao*) beans, both of which contain oils enriched in SFAs. Expression of the *HrDGATs* or *TcDGATs* resulted in different ratios of palmitic to stearic acid in the TAG of yeast mutant strain deficient in TAG synthesis. Furthermore, expression of *HrDGAT1* in developing seeds of the Arabidopsis *DGAT1* knockout mutant restored TAG accumulation and altered the fatty acid composition. These results suggest that *HrDGATs* or *TcDGATs* could potentially be used to increase the SFA content of vegetable oils.

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List of symbols, nomenclature, and abbreviations

aa: amino acid

acyl-CoA: acyl-coenzyme A

ACCase: acetyl-CoA carboxylase

ACAT: acyl-CoA:cholesterol acyltransferase

ACP: acyl carrier protein

ACS: acyl-CoA synthetase

ANOVA: analysis of variance

AtDGAT: *Arabidopsis thaliana* DGAT

AtPDAT: *Arabidopsis thaliana* PDAT

BnDGAT: *Brassica napus* diacylglycerol acyltransferase

BSA: bovine serum albumin

℃: degree celsius

cDNA: complementary DNA

CoA: coenzyme A

CPT: CDP-choline:diacylglycerol cholinephosphotransferase

CHD: coronary heart disease

CTAB: cetyltrimethylammonium bromide

DAG: *sn*-1,2-diacylglycerol

DGAT: diacylglycerol acyltransferase

DGTA: DAG:DAG transacylase

DHAP: dihydroxyacetone phosphate

DTT: dithiothreitol

DNA: deoxyribonucleic acid

EAR: enoyl-ACP reductase

EDTA: ethylene diamine tetraacetate

EMS: ethyl methanesulfonate

EST: expression sequence tag

ER: endoplasmic reticulum

ΔF: Δ Florescence

FAMEs: fatty acid methyl esters

FAS: fatty acid synthase

FatA: fatty acyl thioesterase A

FatB: acyl thioesterase B

g: gram

g: gravitational force

G3P: *sn*-glycerol-3-phosphate

GC/MS: gas chromatography-mass spectrometry

GPAT: glycerol-3-phosphate acyltransferase

HAD: *β*-hydroxyacyl-ACP dehydrase

HrDGAT: sea buckthorn (*Hippophae rhamnoides*) pulp DGAT

KAR: *β*-ketoacyl-ACP reductase

KAS: *β*-ketoacyl-ACP synthase

Kb: Kilobase

kDa: Kilo Dalton

L: liter

LB: Luria-Bertani broth

LPA: lysophosphatidic acid

LPAAT: lysophosphatidic acid acyltransferase

LPC: lysophosphatidylcholine

LPCAT: lysophosphatidylcholine acyltransferase

MAG: monoacylglycerol

MBOAT: membrane-bound *O*-acyltransferase

MUFA: monounsaturated fatty acid

mg: microgram

mg: milligram

mL: microliter

mL: millilitre

mm: micrometer

mM: micromolar

mM: millimolar

mRNA: messenger RNA

MS: Murashige Skoog

MtDGAT: *Medicago truncatula* DGAT

ORF: open reading frame

PA: phosphatidic acid

PAP: phosphatidic acid phosphatase

PC: phosphatidylcholine

PCR: polymerase chain reaction

PDAT: phospholipid:diacylglycerol acyltransferase

PDCT: phospholipid:diacylglycerol cholinephosphotransferase

PDHC: pyruvate dehydrogenase complex

PLA_2: phospholipase A_2

PUFA: polyunsaturated fatty acid

PPT: phosphinothricin

PVP: Polyvinylpyrrolidone

RcDGAT: castor (*Ricinus communis*) DGAT2

RcFAH: castor (*Ricinus communis*) fatty acid hydroxylase

RNA: ribonucleic acid

RT-PCR: reverse transcription-PCR

SAD: stearoyl-ACP desaturase

SD: standard deviation

SDS: sodium dodecyl sulfate

SFA: saturated fatty acid

TAG: triacylglycerol

TcDGAT: cocoa (*Theobroma cacao*) bean DGAT

TLC: thin layer chromatography

TFA: *trans* fatty acid

TMDs: transmembrane domains

UFA: unusual fatty acid

VfDGAT: tung tree (*Vernicia fordii*) DGAT

VgDGAT: *Vernonia galamensis* DGAT

WT: wild type

sn-1,2-dioctanoyl: di-8:0-DAG

sn-1,2-dipalmitin: di-16:0-DAG

sn-1,2 diolein: di-18:1-DAG

palmitoyl-CoA: 16:0-CoA

palmitoleoyl-CoA: 16:1-CoA

stearoyl-CoA: 18:0-CoA

oleoyl-CoA: 18:1-CoA

palmitic acid: 16:0

palmitoleic acid: $16{:}1^{cis\Delta 9}$

stearic acid: 18:0

oleic acid: $18{:}1^{cis\Delta 9}$

vaccenic acid: $18{:}1^{cis\Delta 11}$

ricinoleic acid: 12-hydroxy-9-*cis*-octadecenoic acid

vernolic acid: 12,13-epoxy, 9-*cis*-octadecenoic acid:

linoleic acid: $18{:}2^{cis\Delta 9,12}$:

α-linolenic acid: $18{:}3^{cis\Delta 9,\ 12,\ 15}$

α-eleostearic acid: $18{:}\ 3^{cis\Delta 9,\ trans\Delta 11,\ 13}$

eicosenoic acid: $20{:}1^{cis\Delta 11}$

Chapter 1. Introduction

Solid fat functionality is required for many food products, including margarines, shortenings, baked and fried goods. In addition to the animal fats and highly saturated vegetable oils from tropical regions (such as, palm oil and coconut oil), solid fats may be obtained from the hydrogenation of liquid lunsaturated oils (primarily vegetable oils). These hydrogenated oils are used to improve the oxidative stability of the oils and raise their melting points, which allows for the modification of physical properties of oils for a broader range of food applications (Clemente and Cahoon, 2009). However, during the hydrogenation process, *trans* fatty acids (TFAs) are formed, which can have harmful effects on human health, such as causing coronary heart disease (CHD) (Mozaffarian et al., 2006; Pedersen and Kirkhus, 2008). The adverse effects of TFAs prompted food manufacturers to look for substitutes for these hydrogenated vegetable oils (Uauy et al., 2009). Therefore, there is particular interest in using transgenic plants to produce high stability oils enriched in saturated fatty acids (SFAs) (Wang et al., 2001).

Previous attempts to engineer oilseeds to accumulate SFAs targeted the early stages of fatty acid metabolism. In the current study, our focus is on latter stages of storage lipid assembly with particular attention to enzymes that can preferentially incorporate SFAs into triacylglycerol (TAG). In plants, TAG is the main storage lipid, required for seed germination and plant development. Acyl-CoA:diacylglycerol acyltransferase (DGAT) catalyzes the final reaction in acyl-CoA-dependent TAG biosynthesis, transferring an acyl group to the *sn*-3 position of *sn*-1,2-diacylglycerol (DAG) to form TAG; DGAT is believed to exert control

over the carbon flow into TAG (Perry et al., 1999; Stals et al., 1994). There are at least two types of DGATs, designated DGAT1 and DGAT2, which are non-homologous polypeptides, present in distinctive endoplasmic reticulum (ER) domains. DGAT1 plays an important role in seed oil accumulation in most common oilseed crops, while DGAT2 appears to incorporate unusual fatty acids (e.g. epoxy, conjugated and hydroxy fatty acids) into seed oil (Li et al., 2010[a]).

Recent studies have confirmed the importance of using DGATs with specific substrate preferences in combination with auxiliary approaches to increase oil content and desired fatty acids products (Xu et al., 2008; Burgal et al., 2008). Therefore, in this study, we focus on DGATs to determine if the enzyme can preferentially incorporate SFAs in TAG.

Two plant materials, sea buckthorn (*Hippophae rhamnoides*) pulp and cocoa (*Theobroma cacao*) bean, were selected for this study due to their SFA-enriched oils. Sea buckthorn is considered an important natural resource in the high altitude mountainous regions of China and Russia for soil erosion protection, food and medicinal products (Zeb, 2004). The pulp oil of sea buckthorn is mainly composed of palmitic acid (16:0), palmitoleic acid ($16:1^{cis\Delta 9}$) and oleic acid ($18:1^{cis\Delta 9}$) (Kaminskas et al., 2006). Cocoa bean fat contains a high level of stearic acid (18:0), along with palmitic acid, oleic acid and several polyunsaturated fatty acids (PUFAs) (Rucker, 2009).

The ultimate goal of this thesis was to investigate the characteristics of DGATs for potential use in modifying the fatty acid composition of oilseed crops

using molecular genetic strategies. Specifically, this project had the following objectives:

(1) To isolate complementary DNA (cDNA) encoding *DGATs* from sea buckthorn pulp and cocoa bean, based on the hypothesis that DGATs from SFA-rich species would possess the appropriate substrate preferences.

(2) To evaluate DGAT activity, substrate specificity and selectivity in *Saccharomyces cerevisiae* mutant H1246 expressing the *DGATs* cDNAs.

(3) To characterize lipid production in *Arabidopsis thaliana* mutant AS11 expressing the recombinant *DGATs*.

This study contributes to our understanding of the molecular and functional characteristics of sea buckthorn pulp DGATs (HrDGATs) and cocoa bean DGATs (TcDGATs), and sheds insight into their role in oil deposition.

Chapter 2. Literature Review

Triacylglycerol (TAG) is the major class of storage lipid in oilseeds, consisting of a glycerol backbone and three fatty acids attached by ester bonds. In most plants, TAGs are a highly reduced form of carbon, which serve as a chemical energy reserve for seed germination and subsequent plant development (Lung and Weselake, 2006). In addition, these storage lipids have tremendous value for use as food, feed and petrochemical alternatives. In seed oils, the fatty acid composition of TAG can be influenced by several factors including the availability of fatty acids and the substrate preferences of acyltransferases involved in TAG biosynthesis. Many biotechnology approaches have been applied to modify seed oil content and composition, and have resulted in the identification of metabolic "bottlenecks" in the oil formation process (Snyder et al., 2009). In this Chapter, background knowledge related to the research of my thesis is presented in the following three sections: lipid biosynthesis in plants, the DGAT family in plants, and using DGAT to increase SFA content in seed oil.

2.1 Lipid biosynthesis in plants

The biosynthesis of TAG in plant cells is a complex process consisting of three phases: *de novo* synthesis of fatty acids in the plastid, formation of the acyl-CoA pool in the cytosol, and TAG assembly in the ER of developing seeds (Ohlrogge, 1997).

2.1.1 *de novo* fatty acid synthesis

In plants, *de novo* fatty acid biosynthesis occurs in the plastid (Fig. 2.1) (Ohlrogge and Browse, 1995). The initial substrate for plastidial fatty acid synthesis is acetyl-CoA. Acetyl-CoA carboxylase (ACCase) catalyzes the formation of malonyl-CoA from acetyl-CoA (Sasaki and Nagano, 2004), and the malonyl-CoA is then converted to malonyl-acyl carrier protein (ACP) by the catalytic action of malonyl-CoA:acyl carrier protein transferase (Ohlrogge and Browse, 1995). Plant plastidial fatty acid synthase (FAS) complex catalyzes elongation of the growing fatty acid chain through addition of two-carbon units, which are donated from malonyl-CoA, to the growing acyl chain, to reach 16:0 and 18:0 (Slabas et al., 2001).

Plant plastidial FAS complex (type II) is a dissociable enzyme, which has an individual polypeptide for each enzyme activity (Slabas et al., 2001). The elongation of malonyl-ACP to a saturated acyl chain involves repeated cycles of condensation, reduction, dehydration, and reduction (Ohlrogge and Browse, 1995). The initial step of condensation is acetyl-CoA with malonyl-ACP to form 3-ketobutyryl-ACP and CO_2, catalyzed by β-ketoacyl-ACP synthase III (KASIII).

Subsequent condensation from 4:0-ACP to 16:0-ACP and 16:0-ACP to 18:0-ACP are catalyzed by *β*-ketoacyl-ACP synthase I (KAS I) and *β*-ketoacyl-ACP synthase II (KAS II), respectively (Harwood, 2005). Within each condensation cycle, three additional reactions (reduction, dehydration, and reduction) are required to convert the initial product of each condensation reaction, 3-ketoacyl-ACP, to the saturated acyl-ACP (Ohlrogge and Browse, 1995). These additional reactions are sequentially catalyzed by *β*-ketoacyl-ACP reductase (KAR), *β*-hydroxyacyl-ACP dehydrase (HAD), and enoyl-ACP reductase (EAR) (Ohlrogge, 1997).

In most plants, *de novo* fatty acid synthesis is terminated when the acyl chains reach 16-18 carbons in length. Some 16:0-ACP can enter the prokaryotic glycerolipid pathway in the inner envelope of plastids, in which the acyl-ACPs are used directly by plastidial acyltransferases to produce glycerolipids (Ohlrogge and Browse, 1995); the remaining 16:0-ACP can be hydrolyzed by fatty acyl thioesterase B (FatB), and the resultant free fatty acids are exported out of the plastids and re-esterified to form acyl-CoA through the catalytic action of acyl-CoA synthetase (ACS) on the outside of plastid (Ohlrogge and Browse, 1995; Li-Beisson et al., 2010). Although some 18:0-ACP is released from the plastids, most is efficiently desaturated through the catalytic action of a soluble plastidial stearoyl-ACP desaturase (SAD), which introduces a double bond at the Δ9 position of 18:0-ACP to form $18:1^{cis\Delta 9}$-ACP (Li-Beisson et al., 2010). The stearoyl-ACP desaturase is an important desaturase in the plant kingdom, because it is located in the plastid stroma, while most of desaturases are membrane-

localized proteins (Ohlrogge and Browse, 1995). The resulting 18:1-ACP can either be hydrolyzed via the catalytic action of fatty acyl thioesterase A (FatA) to form free fatty acids, which are then released from the plastid, or enter the prokaryotic glycerolipid pathway (Li-Beisson et al., 2010).

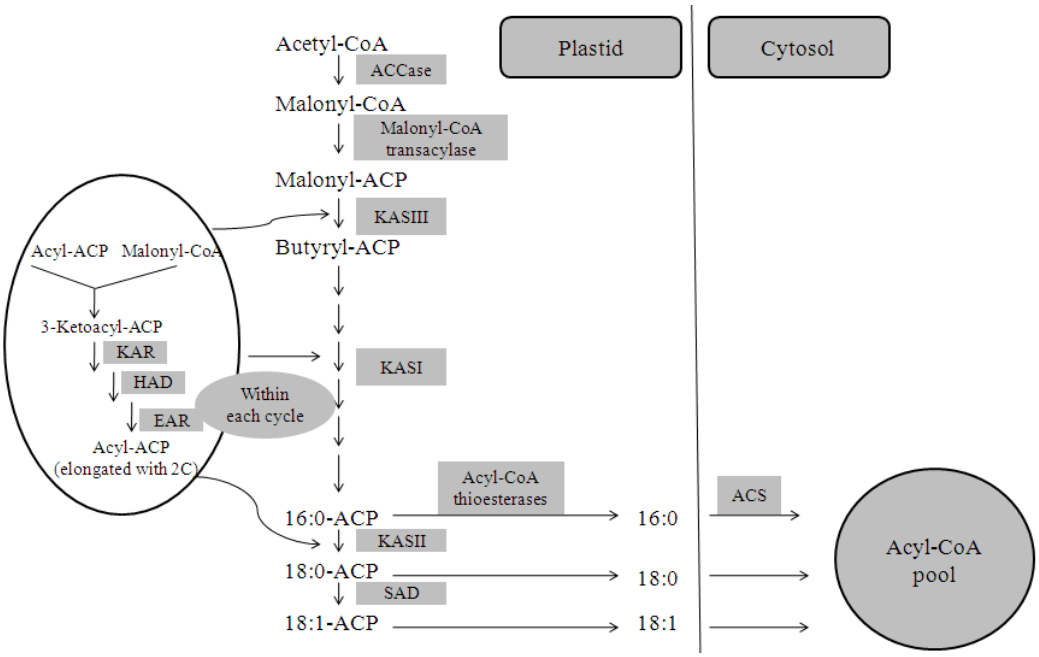

Fig. 2.1: *de novo* fatty acid synthesis in plastids and export to the cytosol
The first step of *de novo* fatty acid synthesis is the conversion of acetyl-CoA to malonyl-CoA, catalyzed by ACCase. Malonyl-CoA is converted to malonyl-ACP, by malonyl-CoA: ACP transacylase. Malonyl-CoAs donate two carbon units in repeated cycles for elongation of the acyl chain to reach 16 and 18 carbons. Seven repeated cycles are required to synthesize 16:0-ACP. The first cycle is the condensation of acetyl-CoA with malonyl-ACP to form 3-ketobutyryl-ACP, catalyzed by KAS III. For the subsequent six cycles, the condensation reaction is catalyzed by KAS I. Then KAS II converts 16:0-ACP to 18:0-ACP. These elongation steps all require the reduction, dehydration, and reduction step, which are catalyzed by KAR, HAD, and EAR, sequentially. 18:0-ACP may be desaturated to 18:1-ACP by SAD. Once the acyl chains reach16-18 carbons, they are hydrolyzed to form free fatty acids by acyl-ACP thioesterase. Free fatty acids are then activated to acyl-CoA by ACS, and form a cytosolic acyl-CoA pool feeding other lipid biosynthesis reactions. The diagram is modified based on Li-Beisson et al., 2010.

Abbreviations: ACCase, acetyl-CoA carboxylase; ACP, acyl carrier protein; EAR, enoyl-ACP reductase; HAD, hydroxyacyl-ACP dehydrase; KAR, ketoacyl-ACP reductase; KAS, ketoacyl-ACP synthase; CoA, coenzyme A; ACS, acyl-CoA synthetase.

2.1.2 Acyl-CoA pool formation and function of acyl-CoA

To form the cytosolic acyl-CoA pool (see Fig. 2.1), most of the newly formed acyl-ACP in the plastid are hydrolyzed by an acyl-ACP thioesterase to form free fatty acids. As indicated in section 2.1.1, ACS then catalyzes the formation of acyl-CoAs from free fatty acids as they are exported from the plastid (Ohlrogge and Browse, 1995). In addition to *de novo* fatty acid biosynthesis, which contributes saturated and monounsaturated moieties to acyl-CoA pool, acyl-editing pathways lead to the exchange of acyl-groups between acyl-CoA and phospholipids, providing a mechanism to provide polyunsaturated acyl groups to the acyl-CoA pool (Bates et al., 2007).

Phosphatidylcholine (PC), which is the major eukaryotic membrane lipid, is the major substrate for 18:1 desaturation to linoleic acid ($18:2^{cis\Delta 9,12}$) and α-linolenic acid ($18:3^{cis\Delta 9,12,15}$) (Lu et al., 2009), catalyzed by oleoyl desaturase and linoleoyl desaturase, respectively (Stymne and Appelqvist, 1978). One possible mechanism for releasing newly desaturated acyl groups is catalyzed by the reverse reaction of lysophosphatidylcholine acyltransferase (LPCAT) (Bates et al., 2007). Another acyl-editing mechanism for releasing PUFA into the acyl-CoA pool is the hydrolysis of an acyl-group from the *sn*-2 position of PC catalyzed by phospholipase A_2 (PLA_2). Then, the free fatty acids are reactivated by an ACS (Das et al. 2001). The acyl-editing pathways for releasing acyl-CoAs and free fatty acids from PC are shown in Fig. 2.2. Other pathways for moving acyl groups from PC directly to storage lipids are discussed in section 2.1.3.

In the cytosol, newly synthesized acyl-CoAs can be used in several reactions, including elongation, desaturation and further modifications, or the thioesters can provide fatty acyl groups for incorporation into glycerolipids catalyzed by acyltransferases in the ER.

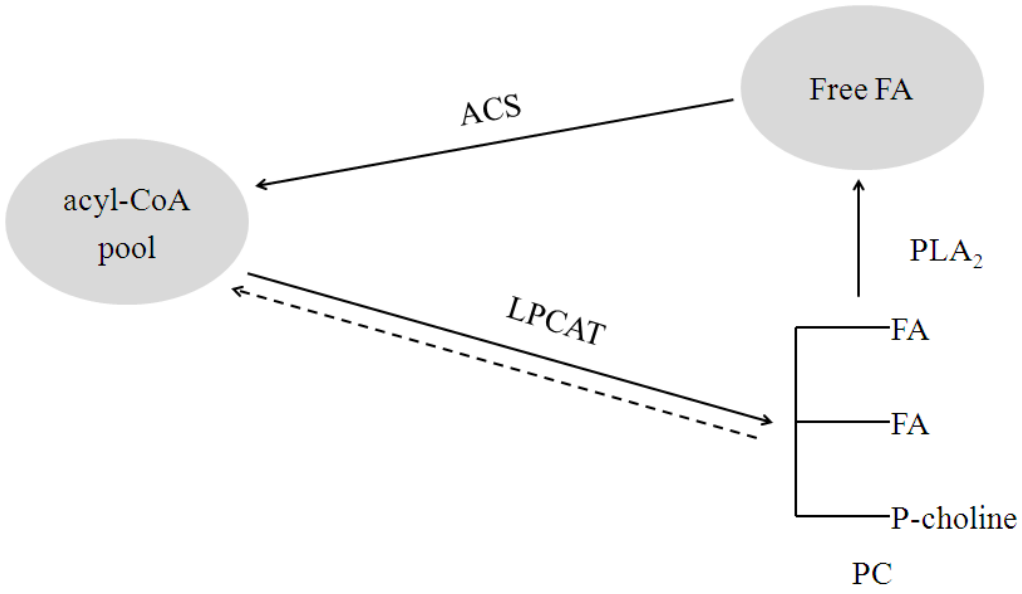

Fig. 2.2: **Acyl-editing pathway for releasing acyl-CoAs and free fatty acids from PC.**
Newly desaturated acyl-CoAs can either be released by the reverse reaction of LPCAT or be hydrolyzed by PLA_2 action from *sn*-2 position of PC. The black arrow represents the forward reaction and the dashed arrow is reverse reaction. The figure was drawn based on the description by Bates et al., 2007 and Das et al. 2001.

Abbreviations: LPCAT, lysophosphatidylcholine acyltransferase; P-choline, phosphocholine; PC, phosphatidylcholine; PLA_2, phospholipase A_2; ACS, acyl-CoA synthetase; FA, fatty acid; CoA, coenzyme A.

2.1.3 Triacylglycerol assembly

The classic acyl-CoA-dependent pathway for TAG biosynthesis is known as Kennedy pathway, and involves the transfer of acyl group to the glycerol backbone by three sequential acylation steps.

The Kennedy pathway (shown in Fig. 2.3) starts with the production of *sn*-glycerol-3-phosphate (G3P), which comes from the reduction of dihydroxyacetone phosphate (DHAP) in the cytosol. Glycerol-3-phosphate acyltransferase (GPAT) catalyzes the acylation of an acyl group from the acyl-CoA pool to the *sn*-1 position of G3P to yield lysophosphatidic acid (LPA). Lysophosphatidic acid acyltransferase (LPAAT) then catalyzes the transfer of an acyl group to the *sn*-2 position of LPA to form phosphatidic acid (PA). The cleavage of the final phosphate group from PA, catalyzed by phosphatidic acid phosphatase (PAP), leads to the formation of *sn*-1,2-diacylglycerol (DAG). In the acyl-CoA-dependent pathway, DGAT catalyzes the acylation at *sn*-3 position of DAG, resulting in the production of TAG (Kennedy, 1961). As DGAT is a major focus of the current study, it will be discussed in greater detail in section 2.2.

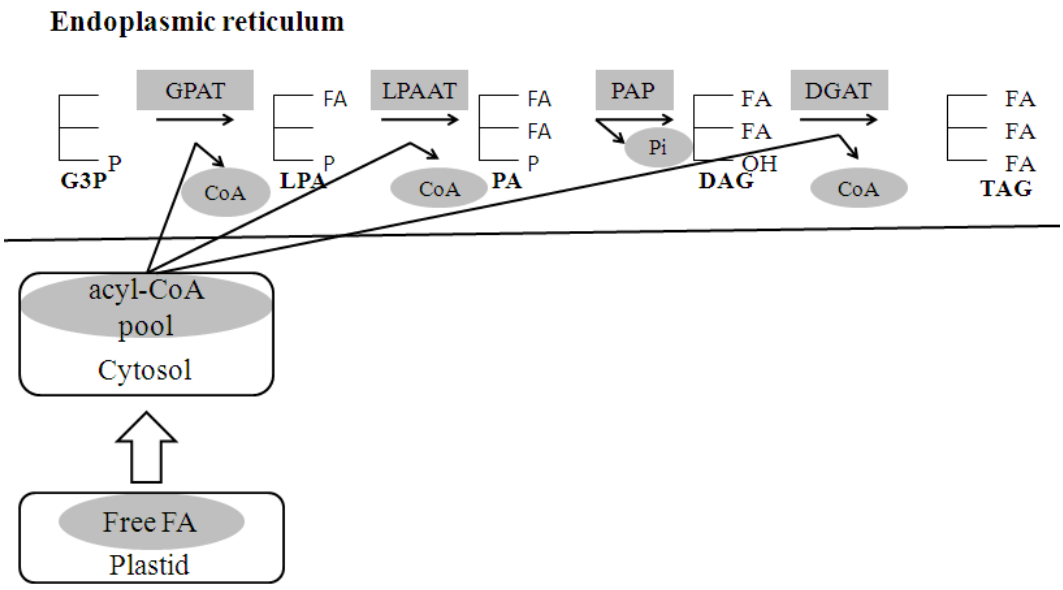

Fig. 2.3: Acyl-CoA-dependent synthesis of triacylglycerol (TAG) in the ER
This diagram illustrates the incorporation of fatty acids (FAs) from acyl-CoA into TAGs by three sequential acylation steps. This diagram is based on the description of Kennedy et al., (1961).

Abbreviations: ER, endoplasmic reticulum; G3P, glycerol-3-phosphate; GPAT, glycerol-3-phosphate acyltransferase; LPA, lysophosphatidic acid; LPAAT, lysophosphatidic acid acyltransferase; PA, phosphatidic acid; PAP, phosphatidic acid phosphatase; DAG, diacylglycerol; DGAT, diacylglycerol acyltransferase; CoA, coenzyme A.

In addition to the classic Kennedy pathway, a number of alternative routes have been proposed for TAG biosynthesis (Fig. 2.4). Bates et al. (2009) suggested that only 17% of newly synthesized acyl-CoA is involved in the Kennedy pathway. Sixty percent of the nascent acyl groups were directly acylated to lysophosphatidylcholine (LPC) to form PC (called "bulk" PC) through the forward reaction catalyzed by LPCAT (Bates et al., 2009). Therefore, there are some acyl remodeling processes alter the acyl composition at the *sn*-2 position of PC.

Meanwhile, only a small portion of *de novo* synthesized DAG is used for TAG; most of the *de novo* DAG is converted to *de novo* PC by CDP-choline: diacylglycerol cholinephosphotransferase (CPT) (Vogel and Browse, 1996). Then, the acyl groups of *de novo* synthesized PC (active) can become substrates for desaturation (as described in the previous section), participating in acyl-editing with "bulk" PC, or converting to "PC-derived" DAG by phospholipid:diacylglycerol cholinephosphotransferase (PDCT) (Bates et al., 2009; Lu et al., 2009). The "PC-derived" DAG subsequently becomes an acyl acceptor for TAG synthesis. In soybean, more than 95% of TAG is synthesized from PC-derived DAG (Bates et al., 2009).

The "PC-derived" DAG could be used for TAG formation by the catalytic actions of (1) DGAT (as shown in Fig 2.4) (Kennedy, 1961), (2) DAG:DAG transacylase (DGTA), catalyzing the transfer of an acyl moiety between two DAG molecules to produce TAG and monoacylglycerol (MAG) (Stobart et al., 1997) (Fig. 2.4), or (3) phospholipid: diacylglycerol acyltransferase (PDAT), which

catalyzes the transacylation of acyl groups from *sn*-2 position of PC to DAG, forming TAG and LPC (Dahlqvist et al., 2000). Ghosal et al. (2007) have demonstrated, however, that PDAT possesses some DGTA activity.

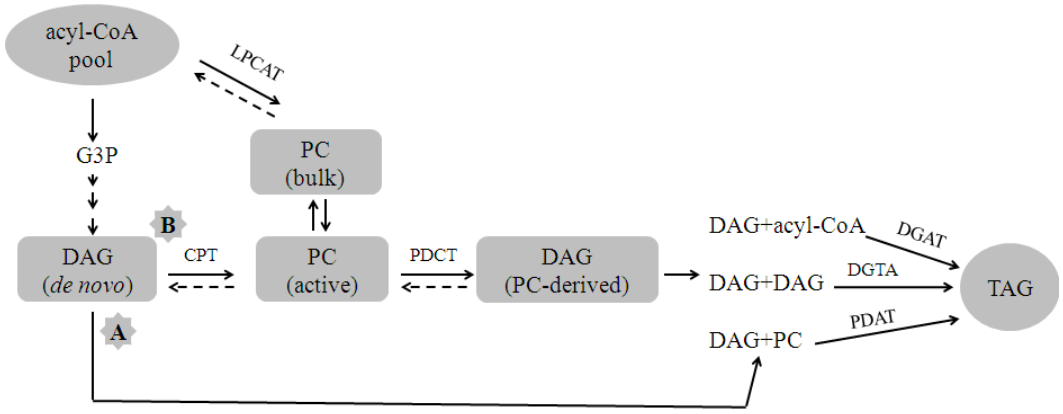

Fig. 2.4: The flow of glycerol into triacylglycerol (TAG).
There are at least two pathways for DAG synthesis, and each pathway can provide different molecular species of DAG, which will affect the fatty acid composition of TAG. Pathway A, *de novo* synthesis of DAG starts from G3P by Kennedy pathway. Pathway B, the production of TAG through PC, which requires synthesis of DAG twice, *de novo* DAG is synthesized and converted to active PC, then active PC is back to bulk DAG. The terminal step of TAG synthesis can be catalyzed by DGAT, DGTA or PDCT using different substrates. The model is based on Bates et al., (2009).

Abbreviations: CoA, coenzyme A; CPT, diacylglycerol cholinephosphotransferase; DAG, diacylglycerol; DGAT, acyl-CoA: diacylglycerol acyltransferase; DGTA, diacylglycerol transacylase; G3P, glycerol-3-phosphate; LPCAT, lysophosphatidylcholine acyltransferase; PC, phosphatidylcholine; PDAT, phospholipid:diacylglycerol acyltransferase; PDCT, phosphatidylcholine: diacylglycerol cholinephosphotransferase; TAG, triacylglycerol.

As TAG continues to be synthesized by the catalytic action of membrane-bound acyltransferases, it accumulates between the two phospholipid layers of the ER membrane (Huang, 1994). TAG droplets or oil bodies eventually bud off of the ER to form oil bodies ranging in size from 0.5 to 2 micrometers in diameter (Huang, 1994; Tzen et al., 1993). Each oil body consists of a matrix of TAG coated by single layer of phospholipid with embedded structural proteins called oleosins (Siloto et al., 2006). In seeds, oleosins are important proteins for stabilizing oil bodies preventing coalescence of the lipid particles during seed dehydration and germination (Cummins et al., 1993; Leprince et al., 1998).

2.2 The diacylglycerol acyltransferase family in plants

The activities of DGAT, DGTA and PDAT catalyzing the final reaction of TAG synthesis have been described (section 2.1.3), but the specific physiological roles of the PDAT and DGTA remain to be elucidated. Previous studies showed that over-expression of Arabidopsis *PDAT* (*AtPDAT1*) increased PDAT activity 10-fold in Arabidopsis plants, but the fatty acid composition in transgenic plants was not significantly different from the wild type (Stahl et al., 2004). Moreover, no significant changes in fatty acid content or composition were reported in Arabidopsis *PDAT* knockout mutants (Mhaske et al. 2005). PDAT was originally proposed to be involved in transfer of unusual acyl groups from phospholipids for TAG synthesis (Dahlqvist et al., 2000). These results suggest that while PDAT may not be a major factor for TAG biosynthesis in some plants, it might be important in altering the fatty acid composition in phospholipids (Mhaske et al. 2005; Lung and Weselake, 2006).

Although the biosynthetic activity of DGTA has been reported in animals (Lehner and Kuksis, 1993) and plants (Stobart et al., 1997), including Arabidopsis (Stahl et al., 2004), neither the biochemical characterization of DGTA enzyme nor the corresponding gene have been reported (Hildebrand et al., 2008).

Together, these observations suggest that DGATs, which have strict substrate preferences, is likely to be a more important consideration in the design of molecular biology strategies to modify the fatty acid composition of seed oils. Furthermore, Bates et al. (2009) suggested that the synthesis of TAG rich in

saturates at the *sn*-3 position is most likely to require DGAT, since it uses newly synthesized acyl-CoAs directly.

At least two types of DGATs, designated as DGAT1 and DGAT2, are present in distinctive ER domains, and have two distinct polypeptides. In 1998, the first *DGAT1* cDNA was cloned from mouse liver based on sequence similarity with the expressed sequence tag (EST) of acyl-CoA:cholesterol acyltransferase (ACAT), an enzyme that uses acyl-CoA and cholesterol as substrates (Cases et al., 1998; Chang et al., 1997). Expression of a mouse cDNA encoding *DGAT1* in insect cells resulted in high levels of DGAT activity (Cases et al., 1998). The deduced amino acid sequence of mouse DGAT1 is 20% identical to the mouse ACAT1 with the most conserved region in the C-terminus (Farese et al., 2000). DGAT1 and ACAT belong to the superfamily of membrane-bound *O*-acyltransferases (MBOAT) (Hofmann, 2000).

Several years later, *DGAT2* from *Umbelopsis ramanniana* was identified by Lardizabal et al. (2001). The deduced amino sequences of DGAT2 shared little or no homology with the polypeptide of DGAT1. The polypeptide of DGAT2 has fewer amino acid residues, is less hydrophobic, and has higher sequence divergence compared to DGAT1 (Siloto et al., 2009[a]). The involvement of DGAT2 in TAG biosynthesis varies among organisms. DGAT2 from yeast was reported to play an important role in TAG synthesis, and it is the only DGAT that contributes to TAG synthesis in yeast (Sandager et al., 2002). Arabidopsis DGAT2, however, showed little activity in a yeast recombinant system (Shockey et al., 2006). In 2006, two plant *DGAT2* cDNAs from castor bean (*Ricinus*

communis) and tung tree (*Vernicia fordii*) were cloned and characterized in yeast cells (Kroon et al., 2006; Shockey et al., 2006). When expressing these plant *DGAT2* cDNAs in yeast cells, the two enzymes showed a preference for incorporation of unusual fatty acids into the TAG fraction.

In addition, a soluble DGAT (DGAT3) was observed in peanut (*Arachis hypogaea*) cotyledons, but the role of this soluble DGAT3 in storage oil production is still not clear (Saha et al., 2006; Cahoon et al., 2007).

2.2.1 The importance of diacylglycerol acyltransferase as a regulator of carbon flow into triacylglycerol

Both *DGAT1* and *DGAT2* have been successfully over-expressed in crops as a means of increasing seed oil content. Arabidopsis mutant AS11 has an ethyl methanesulfonate (EMS)-induced mutation, resulting in modified fatty acid composition, decreased DGAT activity and TAG content in the seed oil (Katavic et al., 1995). In comparison to wild type (WT) Arabidopsis (ecotype Columbia), the seed oil of Arabidopsis AS11 has reduced amounts of eicosenoic acid ($20:1^{cis\Delta 11}$) and oleic acid, and increased levels of α-linolenic acid (Katavic et al., 1995). Over-expression of WT Arabidopsis *DGAT1* cDNA in the Arabidopsis mutant AS11 resulted in an increase in seed oil content and average seed weight (Jako et al., 2001). *B. napus*, expressing *DGAT1* from Arabidopsis WT or *Brassica napus* L. cv Jet Neuf, exhibited an increase in seed oil content when tested under greenhouse or field conditions (Taylor et al., 2009; Weselake et al., 2008). Expression of *DGAT2* from the soil fungus (*Umbelopsis ramanniana*) in

soybean (*Glycine max*) resulted in a 1.5% (by weight) increase in seed oil content and larger oil body formation under field conditions (Lardizabal et al., 2008).

Over-expression of *DGATs* encoding enzymes with specific substrate preferences can increase unusual fatty acid content in transgenic plants. Burgal et al. (2008) observed that castor bean (*Ricinus communis)* DGAT2, designated RcDGAT2, prefers acyl-CoA and DAG containing ricinoleic acid (12-hydroxy-9-*cis*-octadecenoic acid). The co-expression of castor fatty acid hydroxylase (*RcFAH*) and *DGAT2* resulted in a 30% increase in ricinoleic acid, the level of which was two times more than for expression of *RcFAH* alone (Burgal et al., 2008). Moreover, the cDNA encoding tung tree (*Vernicia fordii*) *DGAT2* (*VfDGAT2*) was cloned and overexpressed in yeast cells, and showed high preferences for synthesis of TAG containing eleostearic acid (18:3$^{cis\Delta 9,\ trans\Delta 11,\ trans\Delta 13}$) (Shockey et al., 2006). These studies demonstrated the importance of DGAT activity in affecting fatty acid composition in addition to TAG accumulation.

Taken together, DGAT1 and DGAT2 play an important role in TAG biosynthesis, but the relative substrate preferences and expression pattern of *DGAT1* versus *DGAT2* varies. In most plant species, DGAT1 appears to play a more important role in seed oil accumulation (Li et al., 2010[a]). In plants accumulating unusual fatty acids, such as epoxy and hydroxy fatty acids, DGAT2 appears to have a more important role in incorporation of these unusual fatty acids into TAG (Li et al., 2010[a]). The different expression patterns of *DGAT1* and *DGAT2* in developing seeds might also have a role in determining the ultimate

fatty acid composition of seed oil at maturity. During seed development of tung tree, *DGAT1* expression was slightly increased, whereas the expression of *DGAT2* was strongly induced before the onset of TAG accumulation (Shockey et al., 2006). The expression of *DGAT1* is consistent in TAG biosynthesis in various tissues, and DGAT2 appears to be more directly involved in TAG synthesis in seeds (Shockey et al., 2006). It is difficult, however, to extrapolate the regulatory role of DGAT from one crop species to another. For example, the activity of DGAT has a major influence on TAG formation in olive (*Olea europaea*) and *B. napus* (Weselake et al., 2008), but the DGAT activity exerts only minor control over TAG formation in palm (*Elaeis guineensis*) (Ramli et al., 2005). Therefore, a better understanding of *DGAT* expression and enzyme action is needed.

2.2.2 Substrate specificity and selectivity of diacylglycerol acyltransferase

The biochemical properties of DGAT have been widely investigated by *in vitro* and *in vivo* complementation studies. Early *in vitro* studies on DGAT substrate specificity and/or selectivity did not recognize the differences between DGAT1 and DGAT2. The discovery of genes encoding these two DGAT polypeptides has allowed a more detailed analysis of TAG biosynthesis. Now, over-expression of *DGAT* cDNAs in the organisms deficient in endogenous DGAT and TAG biosynthesis provides an effective tool to characterize the substrate specificity and selectivity of DGATs.

A comparative analysis of plant DGAT1 and DGAT2 has been performed in tung tree (*Vernicia fordii*), sunflower (*Vernonia galamensis*) and castor bean

(*Ricinus communis*). Expression of tung tree DGATs (*VfDGATs*) in yeast cells, followed by *in vitro* enzyme assay, revealed that VfDGAT1 and VfDGAT2 showed only minor differences in substrate preference, and VfDGAT2 had lower levels of activity than VfDGAT1 (Shockey et al., 2006). However, *in vivo*, VfDGAT2 showed five-fold higher activity for incorporation of α-eleostearic acid into TAG than VfDGAT1 (Shockey et al., 2006). Similarly, seed-specific expression of sunflower *DGAT1* and *DGAT2* (*VgDGAT1* and *VgDGAT2*) in soybean seeds showed that VgDGAT2 was more selective than DGAT1 for substrates with vernolic acid (12,13-epoxy, 9-*cis*-octadecenoic acid), an unusual fatty acid containing an epoxy bond (Li et al., 2010[b]). Moreover, the castor bean DGAT2 (RcDGAT2) was also found to prefer unusual fatty acids substrates such as ricinoleic acid (Burgal et al., 2008; Kroon et al., 2006). Overall, these studies pointed out the preference of DGAT2 to catalyze the production of TAG species with a rather defined fatty acid composition.

2.3 Using diacylglycerol acyltransferase to increase saturated fatty acid content in seed oil

Introduction of SFAs in vegetable oils may decrease the utilization of hydrogenated vegetable oils and also improve the stability of oils, used for the production of margarines, baked goods and fried foods. Previous genetic engineering approaches for increasing the levels of stearic acid in seed oils were focused on the early stages of fatty acid synthesis. Seed-specific expression of the *FatA* gene from mangosteen (*Garcinia mangostana*) in *B. napus* resulted in a stearic acid content exceeding 20% in seed oil (Hawkins and Kridl, 1998; Facciotti et al., 1999). Moreover, the "anti-sense" suppression of stearoyl-ACP desaturase, which converts stearoyl-ACP to oleoyl-ACP, increased the availability of stearic acid in transgenic plants (Knutzon et al., 1992). However, transgenic high-stearate (greater than 30%) *B. napus* had increased dormancy and grew more slowly than its untransformed parent and similar problems were not observed in high-laurate hybrids *B. napus* (Linder and Schmitt, 1995).

The greatest advances in engineering oil crops to produce SFAs were in the area of lauric acid (12:0) production in *B. napus*. Voelker et al. (1996) introduced the California bay (*Umbellularia californica*) lauroyl (12:0)-ACP thioesterase into *B. napus* and it resulted in the production of lauric acid up to 50% in the seed oil. However, the incorporation of lauroyl moieties was almost exclusively at the *sn*-1 and *sn*-3 positions of the TAG due to the strict substrate preferences of the *B. napus* LPAAT for C18 fatty acids (Cao and Huang, 1987; Sun et al., 1988). Coexpression of the cDNA encoding the California bay lauroyl-

ACP thioesterase in combination with a cDNA encoding a laurate-CoA-preferring LPAAT from coconut (*Cocos nucifera*) in *B. napus* seeds led to the accumulation of lauric acid at the *sn*-2 position and above 50% further increases of total lauric acid (Knutzon et al., 1999).

Therefore, it is important to ensure SFA are efficiently incorporated into TAG (Hildebrand et al., 2008). In the current study, we focused on identifying DGATs, which exhibit enhanced preference for saturated acyl chains and catalyze the final and dedicated step in the synthesis of TAG. Two plant materials, sea buckthorn (*Hippophae rhamnoides*) pulp and cocoa (*Theobroma cacao*) bean were selected as potential sources of desirable DGATs due to their high SFA content. The fatty acid profile, physical properties, and economic application of the two plant species are briefly described in the next section.

2.3.1 Sea buckthorn

Sea buckthorn is a deciduous spiny shrub, which bears yellow or orange fruits, and is popular in the temperate and sub-tropical zones of world, especially at high altitude, to prevent soil erosion and produce food and medicinal products (Zeb, 2004).The qualitative and quantitative proportions of sea buckthorn oil vary in the seeds, peel, and pulp of berry. In sea buckthorn, the proportion of seed oil never exceeded that of pulp oil (Zeb, 2004). The seed oil constitutes only 10%-20% of the total oil (Zeb, 2004). Sea buckthorn pulp accumulates higher proportion of palmitoleic acid compared to most plants (Yang and Kallio, 2002). Other major fatty acids in pulp include palmitic acid and oleic acid. Conversely,

sea buckthorn seed oil is rich in linoleic acid and α-linolenic acid. Oleic acid, palmitic acid, stearic acid and vaccenic acid (18:1 $^{cis\Delta 11}$) also are found in sea buckthorn seed oil. The fatty acid compositions of sea buckthorn pulp oil and seed oil are presented in Table 2.1.

2.3.2 Cocoa beans

Cocoa is an important evergreen tree fruit crop that is the source of chocolate. It is in the family of *Sterculiaceae*, and native to the deep tropical region of the Americas (Rusconi and Cont, 2010). Cocoa is particular rich in proteins, oils and biologically active compounds such as polyphenols, methylxanthines and caffeine (Schenker, 2000).

The carbon and energy reserves for germinating cocoa embryos are provided by storage lipids (triacylglycerols). In cocoa seed, storage lipids are 50% of the dry seed weight (Griffiths and Harwood, 1991). The composition of cocoa butter is mainly composed of stearic acid, palmitic acid and oleic acid**,** with a smaller amount of several other fatty acids (Kritchevsky, 1994). The fatty acid profile of cocoa bean oil is summarized in Table 2.1. The high level of stearic acid (30–37%) gives cocoa butter its relatively high melting point (34–38 °C), which is similar to the temperature of human body (Gilabert-Escriva et al., 2002). One can experience this property first hand when chocolate melts in the mouth! The unique fatty acid profile of cocoa butter enhances the qualities of chocolate, and coffee, and makes it valuable addition to other many other products, including cosmetics and pharmaceuticals.

Although there is great interest in high-quality cocoa, good quality cocoa production is less than 5% of the world cocoa production, due to the poor agronomic performance and disease susceptibility of cocoa tree (Argout et al., 2011). In 2011, the genome of *Theobroma cacao* was sequenced and assembled (Argout et al., 2011). This will be a great contribution for investigating cocoa lipid biosynthesis.

Table 2.1: Fatty acid composition (mol%) of oil from sea buckthorn pulp, sea buckthorn seed, and cocoa bean

	Sea buckthorn Seed Oil[1]	Sea buckthorn Pulp Oil[1]	Cocoa Bean Oil[2]
16:0	8.7	28.2	30.6
16:1^{cis9}	nd	31.3	0.6
18:0	2.7	1.1	33.9
18:1^{cis9}	18.0	17.2	31.4
18:1^{cis11}	2.5	8.6	Nd
18:2 ^{cis9,12}	39.5	10.9	2.5
18:3 ^{cis9,12,15}	28.6	5.2	Nd

nd, Fatty acid not detected
[1], Fatty acid compositions of sea buckthorn seed oil and pulp oil are cited from Yang and Kallio (2001).
[2], Fatty acid composition of cocoa bean oil is cited from Gilabert-Escriva *et al.*, 2002.

2.4 Closing comments and hypothesis

Understanding the mechanism of lipid biosynthesis and characterization of DGATs are important for developing genetic strategies for modifying fatty acid composition and oil content in oilseed crops. The major focus of this study is to determine if sea buckthorn pulp DGATs (HrDGATs) and cocoa bean DGATs (TcDGATs) can preferentially incorporate SFAs into TAG. Results from this study will provide information on the enzymatic characterization and regulation of DGATs for further metabolic engineering of TAG biosynthesis in oilseed crops to increase levels of desirable fatty acids.

Chapter 3. Materials and Methods

3.1 Cloning of *DGAT1* and *DGAT2* genes from sea buckthorn (*Hippophae rhamnoides*) pulp and cocoa (*Theobroma cacao*) bean

The cDNA clone of *HrDGAT1* and *HrDGAT2* was obtained from Drs. T. Fatima and P. Krishna of the University of Western Ontario (London, ON). The *TcDGAT1* and *TcDGAT2* sequences were identified by BLAST searching the cocoa expressed sequence tag (EST) database (http://www.cacaogenomedb.org/user) using the Arabidopsis *DGAT1* and *DGAT2* nucleotide sequences (Accession numbers AJ238008.1 and AT3G51520, respectively) as probes. By analyzing the top hits in alignment with other plant DGATs, we identified one cocoa *DGAT1* and one *DGAT2*. Gene-specific primer pairs (Table 3.1) were then designed based on these known sequences to amplify the full-length cDNAs of *TcDGAT1* and *TcDGAT2*. For cDNA amplification, total RNA prepared from the developing cocoa bean was used in reverse transcription-polymerase chain reaction (RT-PCR).

The RNA extraction method was based on the cetyltrimethylammonium bromide (CTAB)-based protocol described by Gambino et al. (2008). Nine hundred microlitres of extraction buffer (2% CTAB, 2.5% PVP-40, 2 M NaCl, 100 mM Tris-HCl pH 8.0, 25 mM EDTA pH 8.0 and 2% of β-mercaptoethanol added just before use) were added to a microcentrifuge tube, which contained several 0.5mm glass beads and approximately 50 mg grounded developing cocoa beans, which were obtained from the reddish cocoa fruit in greenhouse (Department of Agricultural, Food and Nutritional Science, University of Alberta,

AB, Canada). The samples were then homogenized in a bead beater homogenizer (Biospec, Bartlesville, USA), followed by heating at 65°C for 10 min. An equal volume of chloroform: isoamyl alcohol (24:1 v/v) was added, and then the mixture was centrifuged at 11,000*g* for 10 min at 4°C. The supernatant was recovered and a second chloroform:isoamyl alcohol (24:1 v/v) extraction was performed above. The collected supernatant was precipitated with 3M LiCl, and the mixture was incubated on ice for 30 min, followed by centrifugation at 21,000*g* for 20 min at 4°C to pellet the RNA. The pellet was resuspended in 500 µL of buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA pH 8.0, 1% SDS, 1 M NaCl), and 500 µL of chloroform:isoamyl alcohol (24:1 v/v), and then the mixture was centrifuged at 11,000*g* for 10 min at 4°C. The supernatant was recovered and the RNA was precipitated with 0.7 volumes of cold isopropanol and immediately centrifuged at 21,000*g* for 15 min at 4°C. The pellet was precipitated with 0.5 volumes of 100% ethanol, and mixed well by pipetting. To avoid any ethanol or salt carryover and contamination, the RNA purification step was performed based on the protocol of Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The first-strand cDNA was then synthesized using total RNA isolated from cocoa bean as template and Superscript III (Invitrogen, Carlsbad, CA, USA) as reverse transcriptase.

3.2 Expression of *DGAT* cDNAs in yeast (*Saccharomyces cerevisiae*) mutant H1246

3.2.1 Construction of plasmids containing *HrDGATs* and *TcDGATs* for overexpression in yeast

The full-length *HrDGAT* and *TcDGAT* open reading frames (ORFs) were subcloned into yeast expression vector pYES2.1/V5-His-TOPO (Invitrogen, Carlsbad, CA, USA). The primers for constructing the plamids pYES-HrDGAT1, pYES-HrDGAT2, pYES-TcDGAT1 and pYES-TcDGAT2 are listed in Table 3.1. The vector pYES2.1/V5-His-TOPO carries the *GAL1* promoter for galactose-inducible expression and glucose repression, a V5 epitope for detection and polyhistidine (6xHis) tag for purification. The constructs were sequenced using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) to verify the fidelity of PCR.

Table 3.1: Oligonucleotides used for cloning full-length cDNA into yeast expression vector pYES2.1

Cloning full-length cDNA into yeast expression vector pYES2.1		
	Forward primer	**Reverse primer**
HrDGAT1	5'- GGGATGGCGATTTCGGAATCC-3'	5'-TTCTGCTAGCCCTTTCCGGTTC-3' **(no stop codon)**
HrDGAT2	5'-AGTTGGATCCTACACAATGTCAAAAAGGGATAAAAGTGAAAACGG-3'	5'-AAAACTCGAGTCAGAGAATTTGCAATTGGAGATCTG-3'
TcDGAT1	5'-GACATGGGGATATCCGACTCA-3'	5'-ATCTGCTTTCCCTTTGCGATTC-3' **(no stop codon)**
TcDGAT2	5'-TACACAATGGGGGAAGAAATGGAGG-3'	5'-TCAAAGAATCTTTAATGGAAGGTCAG-3'

3.2.2 Preparation of yeast stain and culture

Plasmids pYES-HrDGAT1, pYES-HrDGAT2, pYES-TcDGAT1 and pYES-TcDGAT2 were transformed into the yeast mutant H1246 using the method described by Gietz and Schiestl (2007). The yeast mutant H1246 has quadruple knockout of the *DGA1, LRO1, ARE1* and *ARE2*; therefore, it is deficient in DGAT activity and TAG accumulation (Sandager et al., 2002). The mutant yeast strain H1246 bearing pYES2.1-LacZ plasmid was used as a control. The transformants were selected on plates lacking uracil and cultivated in 10 mL of minimal media containing 0.67% (w/v) yeast nitrogen base, 20 mg/L of adenine, arginine, tryptophan, methionine, histidine, and tyrosine, 30 mg/L of lysine and 100 mg/L of leucine, 2% (w/v) glucose, and grown at 30°C for 24 h. This culture was subsequently centrifuged at 3,000*g* for 5 min and washed twice with sterile water and resuspended in induction media, where the glucose of minimal media was replaced by 2% (w/v) galactose and 1% (w/v) raffinose, to the starting OD600 of 0.4 for induction. The yeast was grown at 30^{o}C (250 rpm) to induce the expression of recombinant genes. After induction, the cells were harvested by centrifugation (3,000 *g* for 5 min), washed twice with water, and used for lipid analysis and Nile red assay.

3.2.3 Nile red assay

The yeast mutant H1246 cells expressing the recombinant *DGATs* were induced for 24 h in 2% (w/v) galactose induction medium, prior to the Nile red assay (Siloto et al., 2009[b]). The amount of intracellular TAG in yeast was estimated using Nile red dye (9-diethylamino-5*H*-benzo[α]phenoxazine-5-one).

Nile red was dissolved with methanol to reach the concentration 0.8 mg/mL. The Nile red assay was conducted by aliquoting 100 μL of yeast culture in a 96-well dark plate. Fluorescence spectra were measured with the fluorometer (Fluoroskan Ascent, Thermo, USA), and the data processing and analysis was performed by the Ascent software (version 2.6). The baseline fluorescence of the yeast without Nile red was measured for 100 ms with the excitation filters of 485 nm and the emission filters of 538 nm, and was recorded as Florescence1. Two microliters of Nile red solution were added and mixed the sample well to eliminate the sedimentation of cells. Five minutes later, the fluorescence in the same wavelength was measured and recorded as Florescence2. Fluorescence was corrected by subtracting the fluorescence before and after the Nile red addition, designated as Δ Florescence. The Nile red results were expressed as ΔF divided by OD600 to normalize the effects of cell density.

3.2.4 Yeast lipid analysis

The yeast cultures were induced for 24 h in 2% (w/v) galactose induction medium for lipid analysis. The yeast lipid extraction method followed the chloroform/methanol method described by Bligh and Dyer (1959). Ten milliliters of yeast culture were harvested by centrifugation for 2 min at 2,000*g*. The harvested cells were then mixed with 1 mL of 0.9% NaCl and glass beads (0.30 mm diameter), and vortexed for 2 min. Then lipids were extracted with chloroform-methanol (1:2, vol/vol). The lipids recovered from the extractions were evaporated under nitrogen. The total extracted lipids were separated by thin layer chromatography (TLC, SIL G25, 0.25mm, Macherey-Nagel, Germany),

developed with hexane/diethyl ether/acetic acid 70:30:1 (v/v/v). TAG bands were visualized with 0.05% primuline spray solution (in acetone: H_2O, 80:20, v/v). Fatty acid methyl esters (FAMEs) were prepared by direct methylation of TAG scraped from the TLC, by adding 1mL of 3N methanolic HCl and incubating at 80°C for 1 h. The reaction was stopped by the addition of 1 mL 0.9% NaCl, and the FAMEs were then extracted twice with 4 mL hexane. The resulting FAMEs were dried down under nitrogen and resuspended in 0.25 mL iso-octane containing C21:0 methyl ester (0.1 mg/mL) for gas chromatography-mass spectrometry (GC/MS) analysis.

Gas chromatography-mass spectrometry analysis was conducted using Agilent 6890N chromatographic system and Agilent 5975 Inert Mass Selective Detector. A DB-23 capillary column (30 m x 250 μm i.d. x 0.25 μm) was used for chromatographic separation, with helium as the carrier gas at 1.1 mL/min, and the following temperature program: 165-180°C at 10°C/min for 5 min, then 180-230 °C at 10°C/min, holding for 5 min. The injection volume was 1 μL and the inlet was separated in split-less mode at 250°C. The mass spectrometer was operated in electron impact ionization mode (70 eV), scanning mass ranges from 30-350 amu. The temperature zones were: 230°C (source), 150°C (quad) and 250°C (transfer line). Peak identification was determined on the retention time against a NuChek 421A gas–liquid chromatography standard (NuChek, Elysian, MN, USA) and the spectral matching using the NIST Mass Spectral Search Program 2.0.

3.2.5 *In vitro* DGAT assay

The yeast cultures expressing *HrDGAT1*, *HrDGAT2*, *TcDGAT1*, and *TcDGAT2*, were induced for 16 h in 2% (w/v) galactose induction medium, and then were used for *in vitro* DGAT assay. The microsome preparation and the procedure of DGAT assay was based on the description of Siloto et al. (2009[b]). Induced yeast cells were harvested by centrifugation, washed with water and resuspended in 1 mL extraction buffer containing 20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM EDTA, 5% (v/v) glycerol, 300 mM ammonium sulfate and 2 mM dithiotreitol (DTT). Yeast cells were lysed with 0.5 mm glass beads in a bead beater cell homogenizer (Biospec, Bartlesville, USA). The lysate was centrifuged at 10,000*g* for 10 min at 4^{o}C to remove the cell debris. The resulting supernatant was recovered and centrifuged at 100,000*g* at 4^{o}C for 60 min to obtain the microsomal fraction. The microsomal fraction was resuspended in 3 mM imidazole buffer pH 7.4 with 125 mM sucrose. The microsomal protein was quantified using the Bio-Rad protein assay (Bio Rad, Hercules, CA, USA) according to Bradford (1976). The DGAT assay mixture contained 25 mM sucrose, 0.1 mM EDTA, 15 mM Tris-HCl pH 7.4, 125 μg/mL BSA (fatty acid-free), various concentrations and species of DAG and [^{14}C] acyl-CoA (55mCi/mmol), and different amounts of microsome. The 4mM DAG was resuspended in 0.2% Tween, and sonicated before use. After adding microsome, the reaction mixture (60 μL) was incubated at 30^{o}C. The reaction was stopped by adding 10 μL of 10% sodium dodecyl sulfate (SDS). The “Results section” describes the effects of different protein amounts, reaction times, concentration

and species of acyl-CoA and DAG on the enzyme reaction. Total lipids were extracted, spoted on a TLC plate and separated as previously described. TAG spots were detected with iodine vapor, scraped off the plate, and the radioactivity was measured in a Beckman-Coulter LS6500 liquid scintillation counter.

3.3 Expression of *HrDGAT1* cDNA in Arabidopsis mutant AS11

3.3.1 Construction of plasmids containing *HrDGAT1* for overexpression in Arabidopsis mutant AS11

The restriction sites (*BamHI* and *XbaI*) were added to 5'- and 3'- end of *HrDGAT1* cDNA, respectively, to facilitate the cloning of *HrDGAT1* into pGreen vector. pGreen is a plant transformation binary vector, containing the *napin* promoter, kanamycin resistance gene (*npt*II) for both *Escherichia coli* and *Agrobacterium tumefaciens* selection, and phosphinothricin resistance gene (*bar*) for plant selection. The primers used for PCR amplification were *HrDGAT1fwdBamHI* (5'-AATTGGATCCATGTCGATTTCTGAGTCCTCTGA-3') and *HrDGAT1revXbaI* (5'-TATATCTAGACTATTCTGCTAGCCCTTTCCG GTTCATC-3'). The PCR amplification system was: 94°C for 5 min; 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 2 min; and 72°C for 7 min. The purified *HrDGAT1* cDNA fragment was then digested by *B*am*HI/XbaI*, and ligated into *B*am*HI/XbaI*-digested pGreen. The resulting plasmid was designated as *pGreen-HrDGAT1*. The construct integrity was confirmed by sequencing.

3.3.2 Transformation of *pGreen-HrDGAT1* into *Agrobacterium*

Electrocompetent *Agrobacterium* cells, GV3101 strain, were prepared using the following protocol. The *Agrobacterium* culture was grown 16 h at 28°C (250 rpm) in 100 mL Luria-Bertani media containing 25 μg/mL gentamycin and 10 μg/mL rifampicin. Fifty milliliters were transferred into 500 mL fresh Luria-Bertani media, and allowed to grow at 28°C (250 rpm) until the OD_{600} reached 1 to 1.5, which takes 4 h. The cells were chilled on ice for 20 min and pelleted by centrifugation (4,000*g*, 15 min at 4°C). The pellet was washed in 0.5 volumes of cold sterile water four times and 0.01 volumes of cold sterile 10% glycerol once. Finally, the cells were resuspended in 0.004 volumes of cold 10% (v/v) glycerol. The electrocompetent *Agrobacterium* cells were then frozen in liquid nitrogen and stored at -80°C until use. The thawed *Agrobacterium* cells (50 μL) were mixed with 20 to 50 ng of pGreen-derived plasmids (*pGreen-HrDGAT1* and *pGreen*) and pSoup, which provides replication function for the *pSa* replication origin of pGreen. After incubation on ice for 5 min, the plasmid mixture was transformed into *Agrobacterium* cells by electroporation. The transformed cells were grown for 16 h (28°C, 250 rpm) in 5 mL Luria-Bertani media containing 50 μg/mL kanamycin and 25 μg/mL gentamycin. The presence of the plasmids of pGreen-HrDGAT1 and pGreen in *Agrobacterium* were confirmed by PCR with vector specific primers, *pGreen5401fwd* (5'-GAGGTCGACGGTATCGATAAGC-3') and *pGreen5531rev* (5'-TCAGTAGGATTCTGGTGTGT-3').

3.3.3 Transformation of Arabidopsis by the floral dipping method

All the Arabidopsis wild type and transgenic lines were grown simultaneously at the same growth chamber under 22°C (16 h) and 20°C (8 h) with continuous light. The plant transformation was performed by floral dipping method as described by Zhang et al. (2006). To maintain the humidity, the dipped plants were covered with a dome for 16 h to 24 h. When the siliques were mature and dry, seeds were harvested and selected for positive transformants.

3.3.4 Selection of transgenic plants

The T_1 seeds were harvested and dried prior to storage and further use. Seeds were surface-sterilized by submerging in 70% (v/v) ethanol for 30 sec, followed by rinsing in 20% (v/v) bleach and 0.01% (v/v) Triton X-100 for 20 min, and then washing three times with sterile water containing 0.01% (v/v) Triton X-100. The seeds were spread onto selection plates, containing 1/2 Murashige Skoog (MS) media, with 0.8% (w/v) phytagar, 3% (w/v) sucrose, 80 μM phosphinothricin, 50 μg/mL timentin, and adjusted pH 5.8 with 1N KOH. Timentin is an antibiotic used to suppress *Agrobacterium* in plant transformation without adverse effects on plant tissues and regeneration (Cheng et al., 1998). After 7 to 10 days, herbicide-resistant plants that had green leaves and well established roots within the medium were identified as T_1 plants. When the transformants had two true leaves, the selected T_1 plants were transplanted into soil and grown individually in a growth chamber to produce mature T_2 seeds. Ten T_2 plants per line were selected on plates containing phosphinothricin and the

herbicide-resistant plants were transferred to soil to produce T_3 seeds. The ARASYSTEM (Lehle Seeds, Tuscon, AZ, USA) was used in T_2 and T_3 generations to collect seeds from individual transgenic lines.

3.3.5 DNA and RNA isolation from transgenic lines

Following the protocol by Kasajima et al. (2004), genomic DNA was isolated from individual T_1 plants. PCR amplification, using the paired primers *pGreen5401fwd* and *pGreen5531rev* (mentioned in 3.3.3), was performed to confirm the presence of the *HrDGAT1 i*n the T_1 transgenic plants.

Total RNA was isolated from the developing seeds, which are produced from the T_1 plants, based on the protocol described by Suzuki et al. (2004). The total RNA was used as a template to synthesize single-stranded cDNA with the Superscript III reverse transcriptase followed by RNAse H treatment (Invitrogen, Carlsbad, CA, USA). The specific primers *HrDGAT1fwdBamHI* and *HrDGAT1revXbaI* (previously described in section 3.3.1) were also used for RT-PCR.

3.3.6 Determination of Arabidopsis lipid content and fatty acid profile by direct transmethylation

Dried T_2 and T_3 Arabidopsis seeds were completely cleaned from plant debris and around 10 mg were weighed on an analytical balance. The seeds were placed in Teflon-lined screw capped glass tubes, which were pre-rinsed thoroughly with chloroform and dried to remove any contaminating lipid residues.

To this tube was added 100 μL of triheptadecanoylglycerol (2 mg/mL C17:0 TAG in Chloroform) as internal standard. The internal standard was used to calculate the sample loss associated with the experimental procedures and to estimate the total fatty acid content by GC/MS. The mixtures were dried down under nitrogen. Two millilitres of 3N methanolic HCl were added into the mixture followed by incubation at 80°C for 16 h. After removing the tubes from the 80°C incubator, the tubes were cooled on ice for 5 min, followed by addition of 2 mL of 0.9% NaCl and 2mL of hexane twice to extract FAME. Pooled extracts were evaporated under nitrogen gas and then dissolved in 1 mL of isooctane containing C21:0 methyl ester (0.1 mg/ml) and analyzed by GC/MS. Gas chromatography-mass spectrometry analysis was performed with the same equipment and method as the one used in analysis fatty acid composition in yeast (see section 3.2.4).

Chapter 4. Results

The main focus of my research was to characterize DGATs from the pulp of sea buckthorn because these enzymes might be useful in enhancing the accumulation of SFAs in the seed oil of *Brassica napus*. Previous analysis of the fatty acid composition of sea buckthorn seed oil versus pulp oil suggested that pulp DGATs may have increased preference for incorporating saturated fatty acids into TAG (unpublished data, Fatima et al., 2011). For comparative purposes, some characterization studies were also performed with cocoa DGATs. Cocoa seed is known to be enriched in palmitic acid (30.6%) and stearic acid (33.9%) (Gilabert-Escriva et al., 2002) and therefore would likely contain DGATs with enhanced preference for palmitoyl moieties and stearoyl moieties (Griffiths and Harwood, 1991).

In this Chapter, the results of my thesis are presented in the following sections: (1) identification of *HrDGATs* and *TcDGATs* cDNAs encoding active DGAT enzymes for TAG biosynthesis (section 4.1- 4.2); (2) characteristics of HrDGATs in a recombinant yeast system (section 4.3 - 4.4); (3) seed-specific expression of *HrDGAT1* in Arabidopsis AS11 (section 4.5); (4) characteristics of TcDGATs in a recombinant yeast system (section 4.6 – 4.7).

4.1 The molecular characterizations of HrDGATs and TcDGATs

The deduced amino acid sequences of *HrDGAT1*, *HrDGAT2*, *TcDGAT1* and *TcDGAT2* cDNAs were aligned and compared with other plant DGATs enzymes. The polypeptide sequences of HrDGAT1 and TcDGAT1 share high similarity with other plant DGAT1s (Fig. 4.1A). HrDGAT2 and TcDGAT2 have more than 60% identity with Arabidopsis DGAT2 (*Arabidopsis thaliana*, AtDGAT2) or castor bean DGAT2 (*Ricinus communis*, RcDGAT2), and over 50% identity with tung tree DGAT2 (*Vernicia fordii*, VfDGAT2). A phylogenetic tree was also created to evaluate the evolutionary relationships within the DGAT family (Fig. 4.1B). The phylogenetic tree indicated that HrDGAT1 and TcDGAT1 are from plant DGAT1 family and HrDGAT2 and TcDGAT2 are from plant DGAT2. A Kyte Doolittle hydropathy plot (http://gcat.davidson.edu/DGPB/kd/kyte-doolittle.htm) predicted that HrDGAT1 (535 a.a.) and TcDGAT1 (502 a.a.) have potential nine and eight transmembrane domains (TMDs), respectively, while HrDGAT2 (331 a.a.) and TcDGAT2 (326 a.a.) have two putative TMDs (Fig. 4.2).

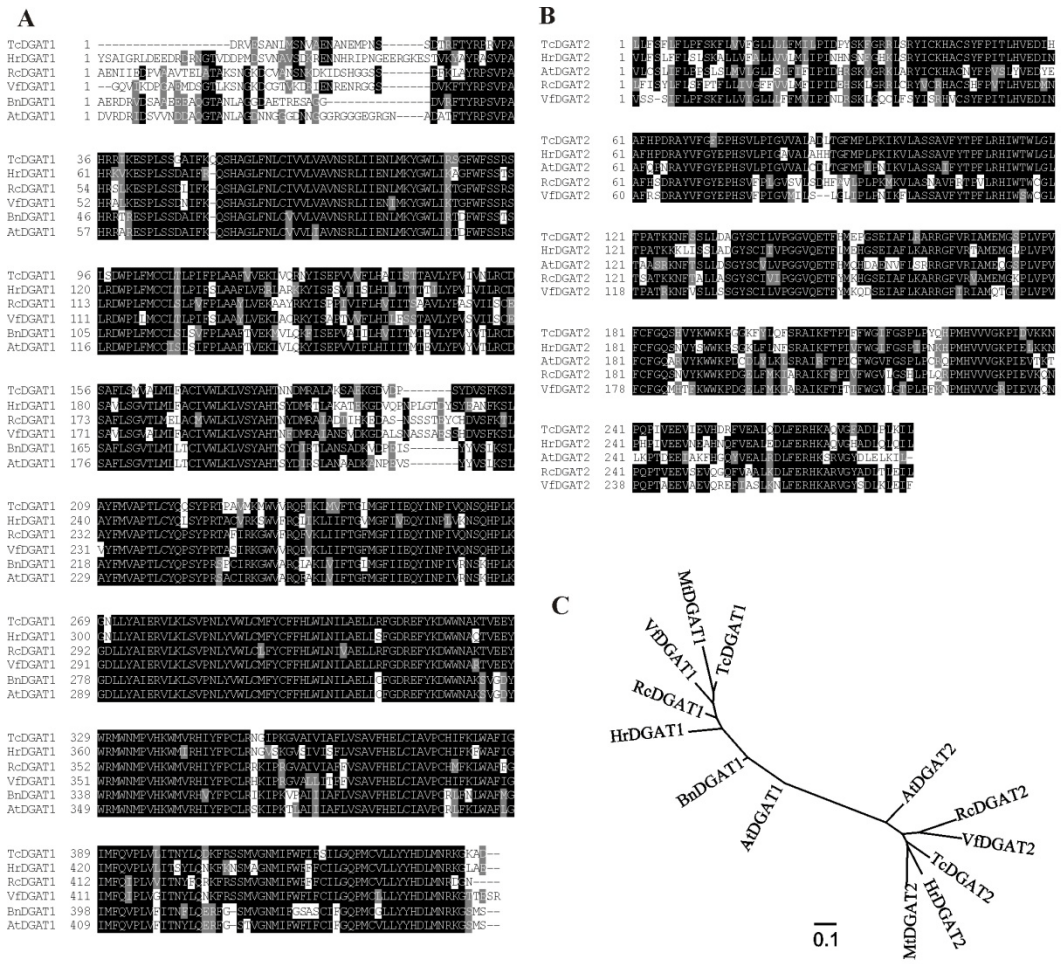

Fig. 4.1: Sequence analysis of deduced amino acids of HrDGATs and TcDGATs.

Sequence alignment of the amino acid sequence of HrDGAT1 and TcDGAT1 with other plant DGAT1 family members (A), and amino acid sequence of HrDGAT2 and TcDGAT2 with other plant DGAT2 family members (B). DGAT polypeptides from plants were aligned using the CLUSTALW multiple sequence alignment program (http://www.genome.jp/tools/clustalw/) and the output was shaded with Boxshade (http://www.ch.embnet.org). The identical amino acid residues are shaded black and gray shading is the consensus of two or more sequences, showing HrDGAT1 and TcDGAT1 share high similarity with other plant DGAT1 family members, and HrDGAT2 and TcDGAT2 share high similarity with other plant DGAT2 family members.

(C): Phylogenetic analysis of various cloned DGATs sequences from plants. The branch lengths of the tree are proportional to divergence.

Accession numbers for the DGAT1 and DGAT2 polypeptides are: *Arabidopsis thaliana*, AtDGAT1 (AAF19262.1), AtDGAT2 (NP_566952.1); *Brassica napus*, BnDGAT1 (AAD45536); *Ricinus communis*, RcDGAT1 (ACB30543), RcDGAT2 (ACB30544); *Vernicia fordii*, VfDGAT1 (ABC94472), VfDGAT2 (ABC94473); *Medicago truncatula*, MtDGAT1 (ABN09107), MtDGAT2 (ACJ84867).

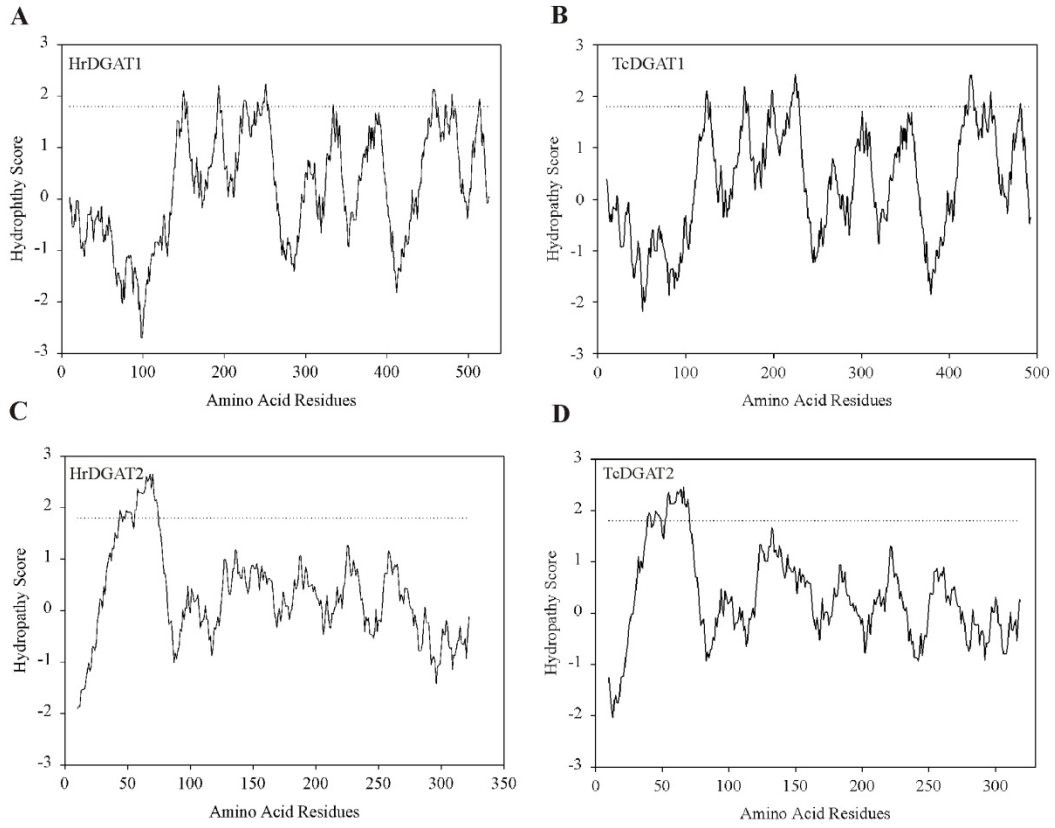

Fig. 4.2: Kyte–Doolittle hydropathy plots of HrDGAT1 (A), TcDGAT2 (B), HrDGAT2 (C), and TcDGAT2 (D).
Plots were generated by Kyte Doolittle Hydropathy plot (http://gcat.davidson.edu/DGPB/kd/kyte-doolittle.htm) using a window size of 19. Cutoff value (line) is 1.8 and peaks with score greater than 1.8 indicate possible transmembrane regions.

4.2 *HrDGATs* and *TcDGATs* encode active DGAT enzymes

To further characterize the function of *HrDGAT1*, *HrDGAT2*, *TcDGAT1*, or *TcDGAT2*, the corresponding cDNAs were cloned and over-expressed in quadruple knockout yeast mutant H1246, which is deficient in DGAT activity and TAG biosynthesis (Sandager et al., 2002). Because this yeast strain cannot synthesize neutral lipids, TAG produced by transformed yeast originates solely from the activity of recombinant enzymes expressed. The amount of neutral lipids produced by yeast cultures can be quickly estimated using Nile red assay, a live-cell-based method that utilizes the fluorescent properties of Nile red dye (Siloto et al., 2009[b]). As shown in Fig. 4.3A, expression of recombinant *HrDGAT1*, *HrDGAT2*, *TcDGAT1*, and *TcDGAT2* in yeast mutant H1246 resulted in significantly higher fluorescence values comparing to cells producing LacZ, an unrelated protein to lipid biosynthesis used as a negative control. To validate our experiment we also expressed a *Brassica napus DGAT1* (*BnDGAT1*) as a positive control which produced comparable fluorescence levels to yeast cells expressing *HrDGAT1*. For *HrDGAT2*, the fluorescence values observed were about 13-fold lower compared to the cultures producing BnDGAT1 and HrDGAT1 (Fig. 4.3A). Similarly, yeast cells expressing *TcDGAT1* accumulated much higher TAG content, when compared with the yeast expressing *TcDGAT2* (Fig. 4.3A). TLC analysis of lipids extracted from yeast cells expressing *HrDGAT1*, *HrDGAT2*, *TcDGAT1*, *TcDGAT2*, and *BnDGAT1* also confirmed the observations in Nile red assay. Triacylglycerol could be detected in yeast expressing *HrDGAT2* and *TcDGAT2* when up to 10 times more culture was extracted (Fig. 4.3B).

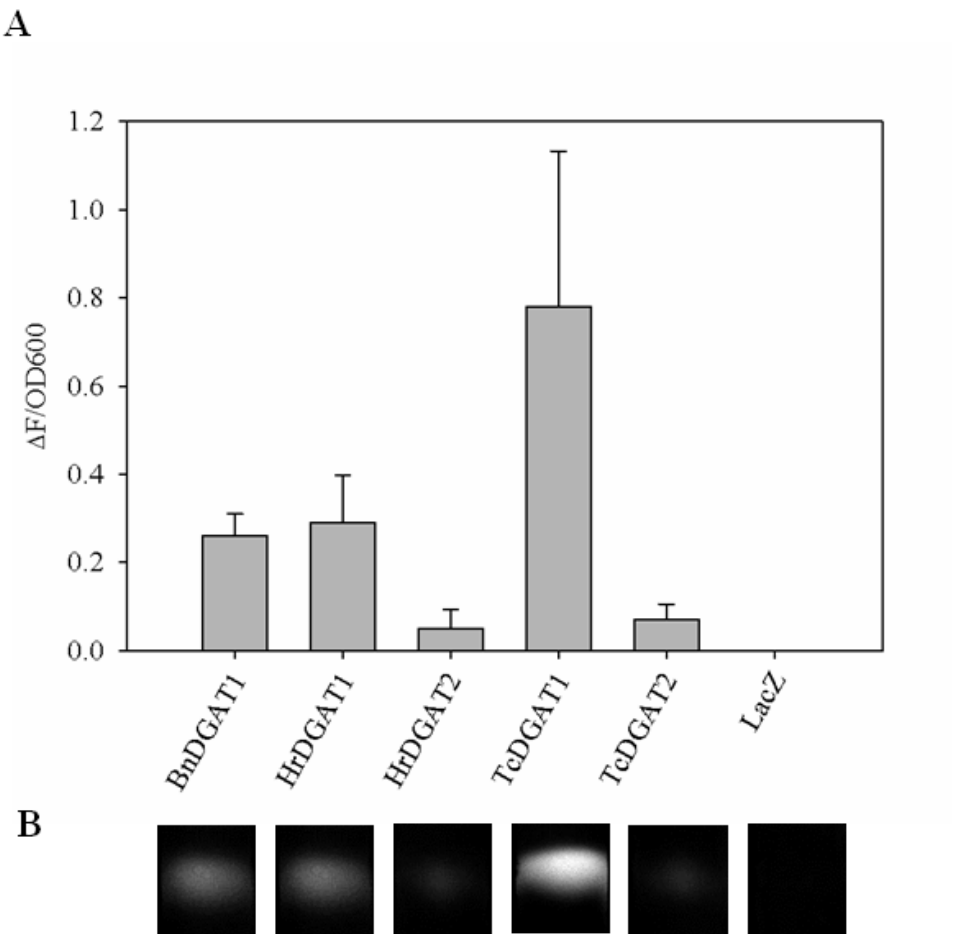

Fig. 4.3: Triacylglycerol (TAG) accumulation was detected by (A) Nile red assay and (B) thin layer chromatography (TLC).
(A) A Nile red assay was performed with yeast mutant H1246 expressing *BnDGAT1*, *HrDGAT1*, *HrDGAT2*, *TcDGAT1*, *TcDGAT2* or *LacZ* under 24 h of galactose induction. TAG accumulation in the H1246 yeast culture expressing *LacZ* was not detected. Values are mean of three replicates. (B) The TAG accumulation was resolved by TLC after 24 h induction in 2% galactose media at 30^{o}C (250 rpm).

4.3 The enzymatic activities of HrDGAT1 in the yeast recombinant system

The enzymatic activities of HrDGAT1 and HrDGAT2 were investigated in the microsomal fraction of yeast mutant H1246 expressing the recombinant *DGATs*. Since the yeast mutant H1246 is entirely devoid of background DGAT activity, it is an ideal system for examining the activities of recombinant DGATs. The microsomal fraction from the recombinant yeast cells, which was induced for 16 h in 2% galactose, was assayed for DGAT activity using different radiolabeled acyl-CoA donors, and unlabelled DAG as the acyl acceptor. The activity of HrDGAT2, however, could not be detected *in vitro*. Therefore, we only conducted HrDGAT1 *in vitro* assay in the following experiments. As shown in Fig. 4.4, the HrDGAT1 activity was characterized in terms of dependence of TAG production on protein quantities, reaction time course, and *sn*-1,2 diolein (di-18:1-DAG) concentration.

To determine the relationship between HrDGAT1 activity and protein quantity, the DGAT *in vitro* assay was performed using 20-70 μg crude HrDGAT1 microsomal fraction, with 320 μM di-18:1-DAG, 7.5 μM [^{14}C] oleoyl (18:1)-CoA and reaction buffer containing 25 mM sucrose, 0.1 mM EDTA, 15 mM Tris-HCl pH 7.4, and 125 μg/mL BSA (fatty acid-free), at 30°C for 15 min incubation. The synthesis rate of TAG was used as an index for analyzing the effect of protein quantities. As shown in Fig. 4.4A, the enzymatic activities (pmol TAG/min) of HrDGAT1were linear in response to protein quantities in the reaction mixture (R^2=0.99) within the range of 20 to 70 μg.

To determine the reaction velocity of the HrDGAT1-catalyzed reaction, the DGAT assays was conducted for different reaction times ranging from 5 to 15 min. The assays were performed using 320 μM di-18:1-DAG, 7.5 μM [^{14}C] 18:1-CoA, 40 μg protein and reaction buffer containing 25 mM sucrose, 0.1 mM EDTA, 15 mM Tris-HCl pH 7.4, and 125 μg/mL BSA (fatty acid-free) at 30°C. The analysis revealed that the TAG produced increased gradually from 5 min to 15 min, and that the HrDGAT1-catalyzed reaction was linear for at least 15 min (Fig. 4.4B).

To determine the HrDGAT1 activity in response to different concentrations of di-18:1-DAG, the enzyme assay was performed from 0-400 μM di-18:1-DAG, using 7.5 μM [^{14}C] 18:1-CoA, 40 μg protein, and reaction buffer containing 25 mM sucrose, 0.1 mM EDTA, 15 mM Tris-HCl pH 7.4, and 125 μg/mL BSA (fatty acid-free), at 30°C for 15 min incubation. HrDGAT1 activity was substantially increased when the di-18:1-DAG concentration was increased from 0 to 80 μM (Fig. 4.4C). About two thirds of the maximum enzyme activity occurred in the absence of exogenous DAG suggesting that the microsomes contained endogenous DAG. The enzyme activity decreased as the DAG concentration was increased beyond 80 μM. Therefore, 80 μM of di-18:1-DAG was optimal for the assay of HrDGAT1 activity. The reaction conditions for DGAT enzyme assays have been long established and reproduced by several different research groups using 320 μM of di-18:1-DAG (Little et al., 1994; Soensen et al., 2005; Weselake, et al., 1991; Weselake et al., 1993; Weselake et al., 1998; Weselake et al., 2000). In order to consistent and compare with other

microsomal plant DGAT assay, 320 uM of di-18:1-DAG was used for the other microsomal recombinant yeast DGAT assay.

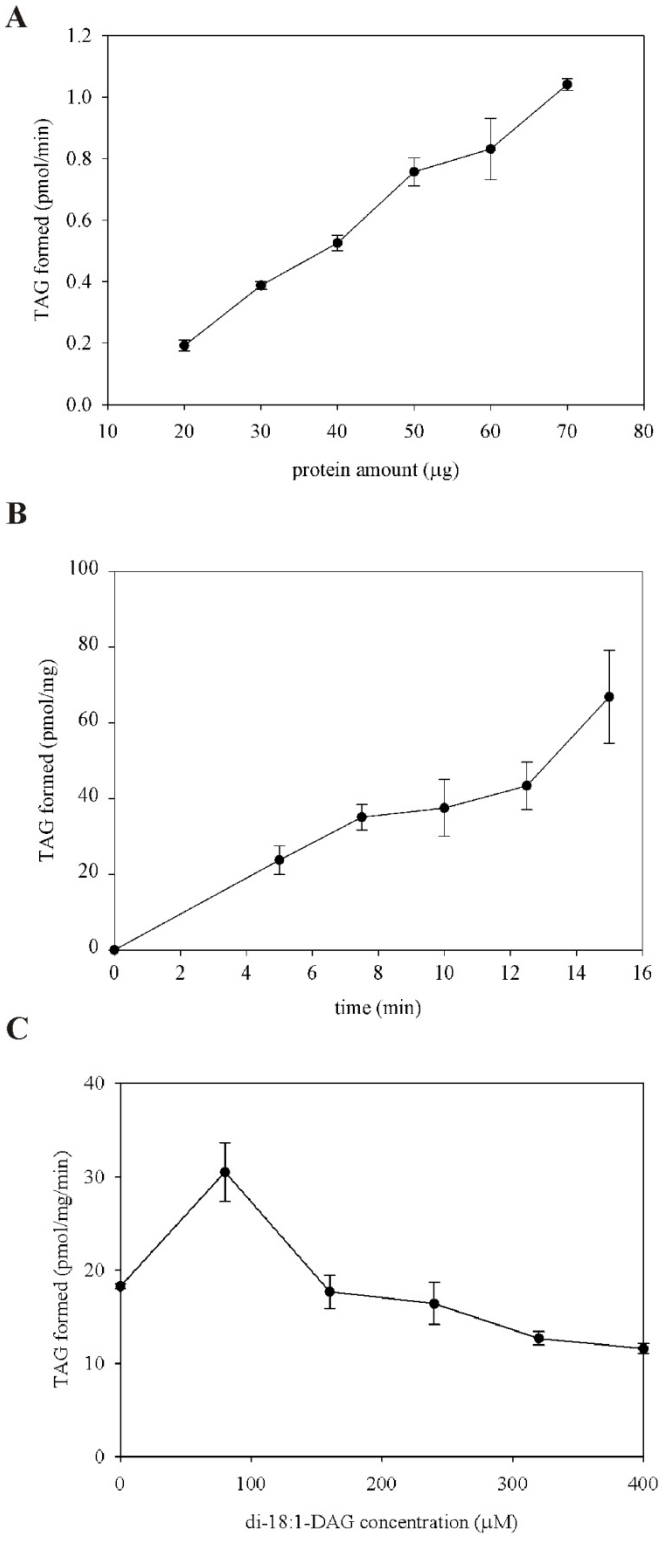

Fig. 4.4: Dependence of HrDGAT1 activity on (A) different quantities of protein, (B) reaction time, and (C) different concentrations of di-18:1-DAG.

(A) The assay was performed with 20-70 µg of microsome from yeast mutant H1246 expressing *HrDGAT1*, 320 µM di-18:1-DAG, 7.5 µM [^{14}C] 18:1-CoA and reaction buffer at pH 7.4 for 15 min. (B) The assays were conducted with different incubation time range from 5-15 min, using 320 µM di-18:1-DAG, 7.5 µM [^{14}C]18:1-CoA, 40 µg protein and reaction buffer at pH 7.4. (C) The assay was performed using 0-400 µM di-18:1-DAG, 7.5 µM [^{14}C]18:1-CoA, 40 µg protein, reaction buffer at pH 7.4, and with 15 min incubations. The microsomal fraction used in this analysis was isolated from the cells incubated at 30^{o}C, 250 rpm for 16 h galactose induction. Error bars represent standard deviation (SD) of triplicate assays.

To determine the substrate specificity, the microsomal fraction with HrDGAT1 was incubated with one of four [^{14}C] acyl-CoAs including palmitoyl (16:0)-, palmitoleoyl (16:1)-, stearoyl (18:0)-, 18:1-CoA, at concentrations ranging from 2.5 to 15 μM. The assays were performed using 320 μM di-18:1-DAG, 40 μg protein and reaction buffer containing 25 mM sucrose, 0.1 mM EDTA, 15 mM Tris-HCl pH 7.4, and 125 μg/mL BSA (fatty acid-free), at 30°C for 15 min incubation. HrDGAT1 exhibited relatively higher specificity for 16:0-CoA and 18:1-CoA when compared with enzyme activities observed with the other molecular species of acyl-CoAs. In general, the highest activities of HrDGAT1 were observed with the different species of acyl-CoAs at a concentration of 10 μM (Fig. 4.5A).

To determine the HrDGAT1 activity in response to different types of DAGs, including 320 μM exogenous di-18:1-DAG and *sn*-1,2-dioctanoyl (di-8:0-DAG), and endogenous DAG. The enzyme assay was performed with 7.5 μM [^{14}C]18:1-CoA, 40 μg protein, and reaction buffer containing 25 mM sucrose, 0.1 mM EDTA, 15 mM Tris-HCl pH 7.4, and 125 μg/mL BSA (fatty acid-free), at 30°C for 15 min incubation. The amount of radioactivity in the TAG fractions derived from di-8:0-DAG was much higher (more than 3-fold) than when using di-18:1-DAG as an acyl acceptor (Fig. 4.5B).

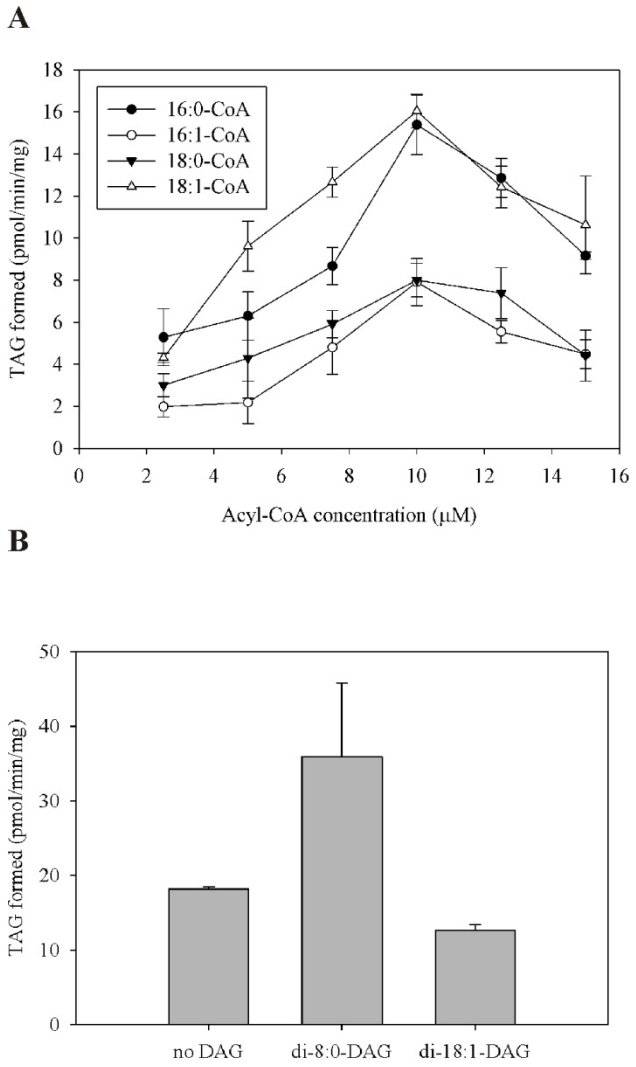

Fig. 4.5: Substrate specificity of HrDGAT1 for different molecular species of (A) acyl-CoA and (B) DAG.

(A) The HrDGAT1 was assayed with different types of [^{14}C] acyl-CoAs including 16:0, 16:1, 18:0, and 18:1-CoAs, at concentrations ranging from 2.5 to 15 μM. The assays were performed using 320 μM di-18:1-DAG, 40 μg protein and reaction buffer containing 25 mM sucrose, 0.1 mM EDTA, 15 mM Tris-HCl pH 7.4, and 125 μg/mL BSA (fatty acid-free), for 15 min incubation. (B) The assays were conducted without exogenous DAG, or with 320 μM exogenous di-8:0-DAG or di-18:1- DAG. The assay was performed using 7.5μM [^{14}C] 18:1-CoA, 40 μg protein and reaction buffer containing 25 mM sucrose, 0.1 mM EDTA, 15 mM Tris-HCl pH 7.4, and 125 μg/mL BSA (fatty acid-free), for 15 min incubation. The microsomal fraction used in this analysis was isolated from the cells incubated at 30^{o}C, 250 rpm for 16 h galactose induction. For the plots in panels (A) and (B), error bars represent SD of triplicate assays.

4.4 Fatty acid compositions of TAG from yeast producing expressing *BnDGAT1, HrDGAT1* or *HrDGAT2*

Analysis of the fatty acid composition of TAG isolated from yeast mutant H1246 cells producing HrDGAT1 or HrDGAT2 was conducted in order to gain insight into the substrate preferences of these recombinant enzymes under physiological conditions in yeast cells. Yeast strain contains four major fatty acids (16:0, 16:1$^{cis\Delta 9}$, 18:0 and 18:1$^{cis\Delta 9}$), a profile that resembles the acyl-CoA substrates used for *in vitro* assays. BnDGAT1 was included in the study for comparative purposes since this enzyme is found in a plant species which only accumulates 7% of SFA in the seed oil (Omidi et al., 2010). Generally, the TAG fraction of yeast expressing *BnDGAT1* or *HrDGAT1* contained greater proportions of monounsaturated fatty acids (MUFAs) than SFAs. The TAG from *HrDGAT1*-expressing cells exhibited a higher proportion of palmitic acid and lower proportion of oleic acid compared to the TAG from *BnDGAT1*-expressing cells (Fig. 4.6A). In contrast, expression of *HrDGAT2* in yeast mutant H1246 resulted in a greater proportion of stearic acid in the TAG fraction when compared to yeast transformed with *BnDGAT1* or *HrDGAT1* (Fig. 4.6A). The yeast expressing *HrDGAT1* and *HrDGAT2* both exhibited enhanced accumulations for SFAs, including palmitoleic acid and stearic acid, in the TAG fraction, in comparison with *BnDGAT1*-expressing cells (Fig. 4.6B). Since no other TAG-synthesizing enzyme is present in H1246 these results suggested that HrDGAT1 and HrDGAT2 prefer substrates containing palmitic and stearic acids in yeast, respectively.

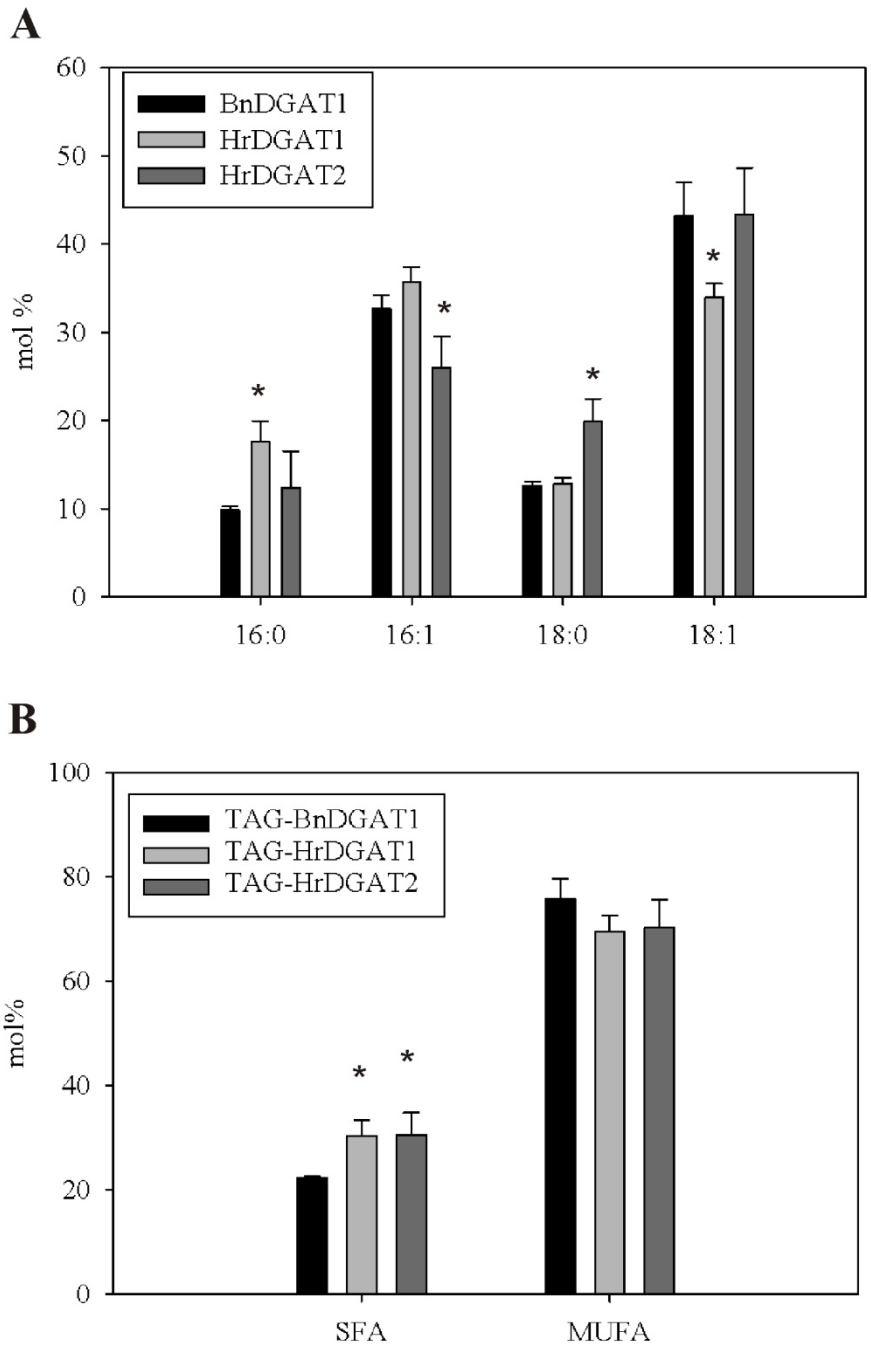

Fig. 4.6: Fatty acid profile (A) and composition of fatty acid classes (B) of the triacylglycerol (TAG) fractions from yeast mutant H1246 cells expressing *BnDGAT1, HrDGAT1* or *HrDGAT2*.

The cells used in this analysis were incubated at 30^{o}C, 250 rpm for 24 h galactose induction. The results were expressed as mean mol% of total fatty acid methyl esters ± SD (n=3). Fatty acid composition of yeast cells expressing *HrDGAT1* or *HrDGAT2* were compared to the the yeast cells expressing *BnDGAT1* using analysis of variance (ANOVA) with Holm-Sidak test. (*****) values represent results are significantly different from the yeast cells expressing *BnDGAT1* at α=0.05.

4.5 Seed-specific expression of *HrDGAT1* in Arabidopsis AS11

Based on the results of *in vitro* and *in vivo* assay in yeast mutant H1246 expressing *HrDGAT1*, the cDNA encoding this enzyme form may be useful in engineering of plants to accumulate increased amounts of SFAs in the seed oil. It was hypothesized that overexpression of *HrDGAT1* cDNA during seed development in Arabidopsis mutant AS11 may lead to both an increase in seed TAG with enhanced content of SFAs. Because Arabidopsis AS11 is a *dgat1* knockout mutant (Katavic et al., 1995) and there are DGAT2 activities in the plants, the overexpression of *HrDGAT2* cDNA in Arabidopsis mutant AS11 is not included in this study. In addition to decreased DGAT activity, TAG content and abnormal seed development, Arabidopsis AS11 exhibited higher levels of α-linolenic acid and lower accumulation of eicosenoic acid (20:1$^{cis\Delta 11}$**)** and oleic acid than that of Arabidopsis wild type (WT, ecotype Columbia) (Katavic et al., 1995; Jako et al., 2001). This may be due to PDAT utilizing acyl groups from *sn*-2 position of PC, which is the major substrate for desaturation to α-linolenic acid (Lu et al., 2009), for TAG biosynthesis.

The *HrDGAT1* cDNA was cloned under the control of a seed specific *napin* promoter into the pGreen plasmid. The resulting vectors were named *pGreen-HrDGAT1*, and the null vector *pGreen* as negative control. It has been shown that the null vector in WT and AS11 transgenics displayed no significant increase of oil content compared with the WT and AS11 lines, respectively (Jako et al., 2001). In this study, the phenotypes (including oil content and seed oil fatty

acid composition) from different lines of AS11:*pGreen* are very similar (Fig. 4.8 and Table 4.1).

All transgenic Arabidopsis plants were selected by plates containing phosphinothricin, and analyzed by PCR to confirm the presence of *pGreen* and *pGreen-HrDGAT1*. As shown in Fig. 4.7, *HrDGAT1* was successfully expressed in the transgenic plants of AS11:*pGreen-HrDGAT1*.

Dried T_3 transgenic seeds were collected individually from each plant and the oil content and fatty acid composition were determined. Analysis of T_3 seeds of AS11: *pGreen-HrDGAT1* indicated that HrDGAT1 was able to complement the reduced oil content of the AS11 mutant, and the oil content in some T_3 transgenic seeds was restored to a level similar to that of WT (Fig. 4.8). Analysis of oil from T_3 seeds of AS11: *pGreen-HrDGAT1* showed an elevation of oleic acid and α-linolenic acid in some lines, and a reduction of eicosanoic acid in most of transgenic lines, compared with WT (Table 4.1A). In relation to the three lines of AS11:*pGreen*, transgenic plants overexpressing *HrDGAT1* accumulated increased levels of eicosanoic acid and had a lower proportion of α-linolenic acid (Table 4.1A). In terms of SFAs contents, most AS11: *pGreen-HrDGAT1* transgenic lines did not show a significant increase in comparison with the SFA content in WT (Table 4.1).

In addition, the AS11: *pGreen-HrDGAT1* appeared to have decreased rate of seedling development compared to the WT, and the lowest rate of seedling growth was seen in AS11: *pGreen* (Fig. 4.9).

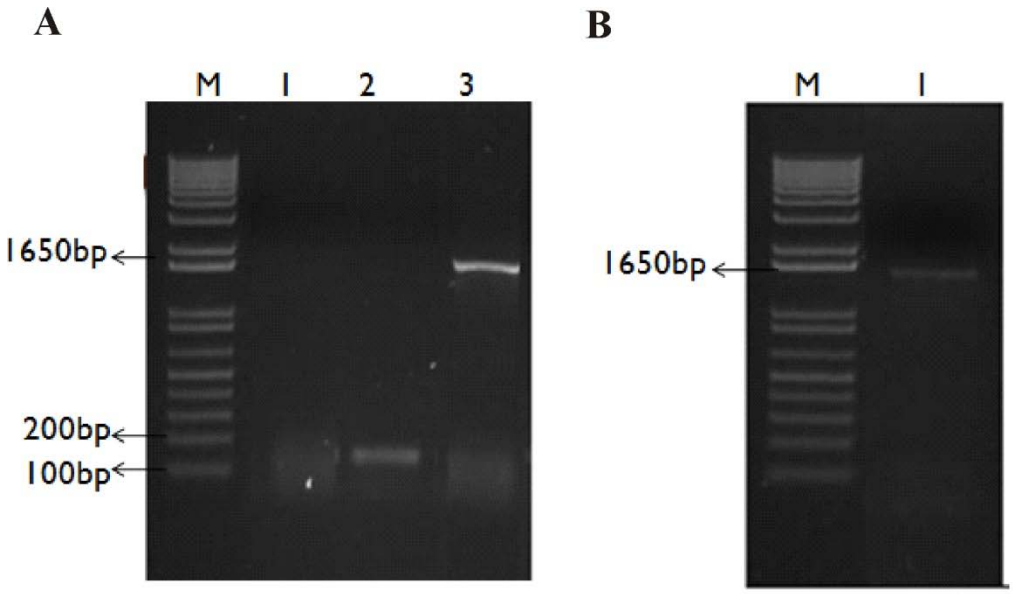

Fig. 4.7: Expression of *HrDGAT1* cDNA in T_2 developing seeds of transgenic Arabidopsis.
(A) DNA (25-50ng) isolated from leaves of wild type Arabidopsis (lane 1), *Arabidospsis* AS11: *pGreen* (lane 2), or AS11: *pGreen-HrDGAT1* (lane 3). (B) Lane 1: RT-PCR amplification of *HrDGAT1* using the total RNA isolated from T_2 mid-green developing seeds of Arabidopsis AS11: *pGreen-HrDGAT1* as a template. The primers *HrDGAT1fwdBamHI* and *HrDGAT1revXbaI* were used. Lanes M: molecular weight marker.

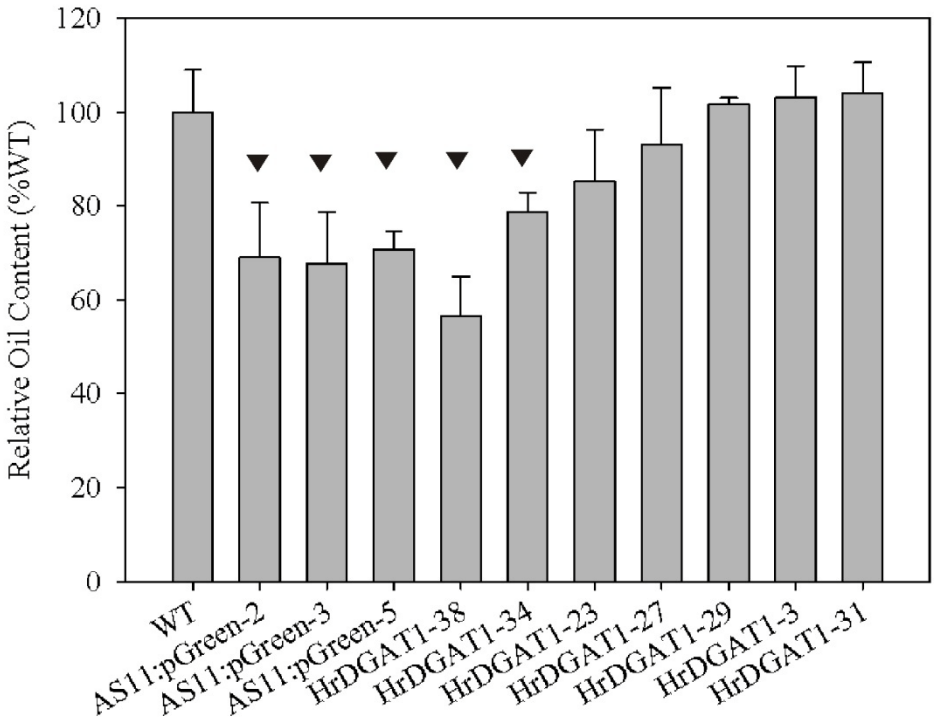

Fig. 4.8: Relative oil content from Arabidopsis AS11 T_3 seeds transformed with *pGreen* (null vector) or *HrDGAT1*.
Values are represented relative to the wild type (WT) oil content (measured as a percentage of seed weight on a dry matter basis) set at 100%. The results are expressed as mean % mol ± SD (n=3) and analyzed by ANOVA with the Holm-Sidak test. (▲/▼) values significantly greater/smaller than WT at α=0.05.

Table 4.2: Fatty acid profile (A) and composition of fatty acid class (B) of the total acyl lipids from Arabidopsis AS11 T_3 seeds transformed with *pGreen* (null vector) or *HrDGAT1*.

Fatty acid composition (mol%) of the total acyl lipids of seed oil extracted from Arabidopsis wild type (WT) seeds, T_3 seeds of Arabidopsis AS11 transformed with *pGreen* or *HrDGAT1*. The results were expressed as mean mol% ± SD (n=3) and analyzed by ANOVA with Holm-Sidak test. (▲/▼) values significantly greater/smaller than WT at α=0.05.

A

	16:0	18:0	18:1 $^{cis\Delta 9}$	18:3	20:1
WT	9.44±0.43	3.36±0.21	12.05±0.95	23.94±0.53	16.18±0.48
AS11:*pGree*n-2	8.97±0.24	1.78±0.45▼	4.59±0.45▼	46.11±4.81▲	2.76±0.77▼
AS11:*pGree*n-3	9.09±0.19	2.13±0.11▼	5.09±0.17▼	44.56±1.51▲	3.55±0.34▼
AS11:*pGree*n-5	9.92±0.57	2.46±0.34▼	4.89±0.54▼	44.59±3.74 ▲	3.69±0.91 ▼
HrDGAT1-38	10.49±0.38▲	2.88±0.41	11.43±0.33	27.42±0.45	9.88±0.57▼
HrDGAT1-34	9.63±0.22	2.81±0.28	15.89±0.22▲	26.47±1.14	10.99±0.84▼
HrDGAT1-23	9.61±0.40	2.85±0.47	11.78±0.72	29.59±3.34▲	10.08±2.05▼
HrDGAT1-27	9.81±0.78	2.42±0.32▼	12.39±0.61	30.67±2.42▲	9.08±0.89▼
HrDGAT1-42	9.40±0.19	2.73±0.24	13.26±0.79	29.58±2.66▲	10.25±1.28▼
HrDGAT1-29	10.24±0.79	2.92±0.56	6.28±4.87	33.02±3.14	9.58±1.93▼
HrDGAT1-3	9.23±0.21	2.79±0.21	14.20±0.24▲	27.86±1.00▲	11.57±0.10▼
HrDGAT1-31	9.69±0.13	3.01±0.14	15.02±0.68▲	26.03±1.43	10.99±1.10▼

B

	SFA	MUFA	PUFA
WT	14.63±0.12	28.23±0.90	56.28±0.65
AS11:*pGree*n-2	12.37±0.99	7.35±1.04▼	77.61±5.29▲
AS11:*pGree*n-3	13.20±0.48	8.64±0.50▼	76.97±1.10▲
AS11:*pGree*n-5	15.10±1.54	8.72±1.74▼	75.45±3.72▲
HrDGAT1-38	16.22±1.06	21.31±0.88▼	61.16±0.48
HrDGAT1-34	14.44±0.70	27.57±1.80	57.54±1.33
HrDGAT1-23	15.00±1.65	21.86±2.77▼	62.12±4.25▲
HrDGAT1-27	11.13±0.73▼	29.86±0.35	45.47±1.55▼
HrDGAT1-42	14.38±0.68	23.51±2.07▼	60.70±2.87
HrDGAT1-29	16.02±1.90	16.69±2.71▼	65.85±3.48▲
HrDGAT1-3	13.91±0.61	26.36±1.16	56.34±0.34
HrDGAT1-31	15.13±0.65	26.71±0.82	56.57±0.61

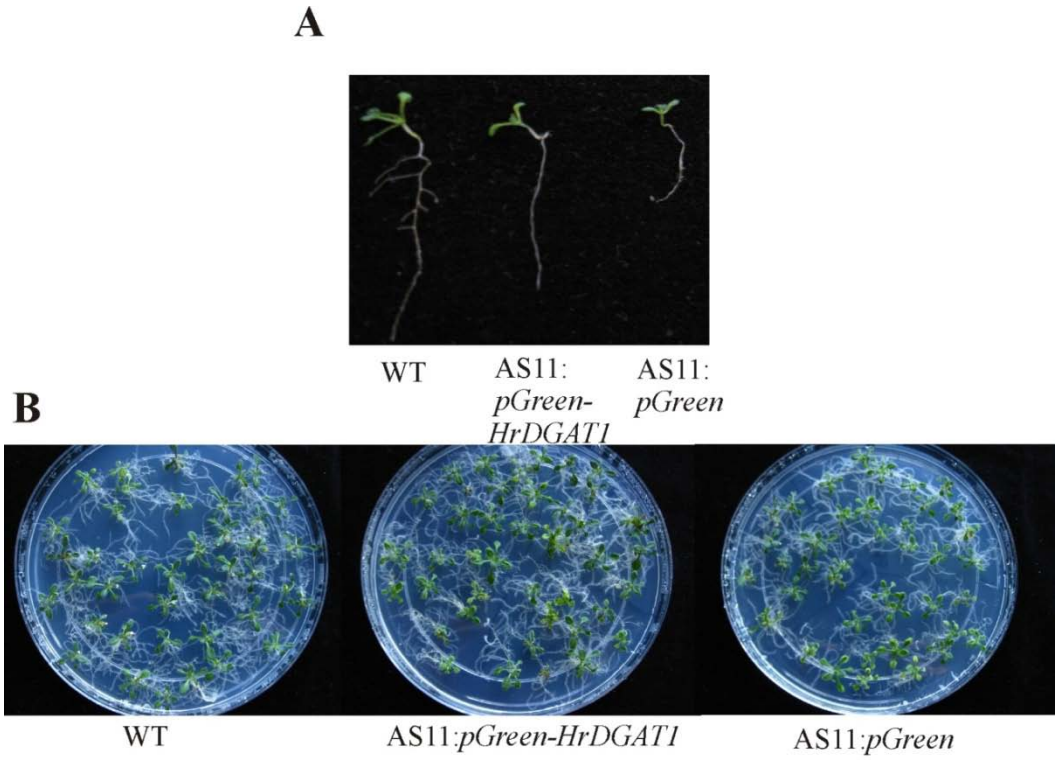

Fig. 4.9: Seedling development (A) after 7 days[a] sowing and (B) after 14 days[a] sowing for Arabidopsis wild type (WT), Arabidopsis AS11 transformed with *HrDGAT1* or *pGreen* (null vector).
(A) Root development comparisons among WT, AS11: *pGreen-HrDGAT1* and AS11: *pGreen*. (B) Each plate contains 40 healthy plants. For WT and AS11: *pGreen-HrDGAT1*, more than 85% of the seedlings have 8 rosette leaves>1 mm in length. For AS11: *pGreen*, 50% of the seedlings have 8 rosette leaves>1 mm in length.

[a] The day from date of sowing, not including a 5-day stratification at 4°C to synchronize germination.

4.6 The substrate specificity of TcDGAT1

The enzymatic activities of TcDGAT1 and TcDGAT2 were also investigated using microsomal fractions prepared from yeast recombinant cells. Similar to the situation for HrDGAT2, the presence of TcDGAT2 could not be detected based on the *in vitro* enzyme assay. Therefore, *in vitro* enzyme assays were only performed using recombinant TcDGAT1. The acyl substrate specificity of the TcDGAT1 was determined, using 7.5 μM of [^{14}C] 16:0-, 16:1-, 18:0- or 18:1-CoA. TcDGAT1 generally exhibited high specificity to [^{14}C] 16:0-CoA and [^{14}C] 18:1-CoA and similar specificity for the other two types of [^{14}C] acyl-CoAs (16:1-CoA or 18:0-CoA) (Fig. 4.10).

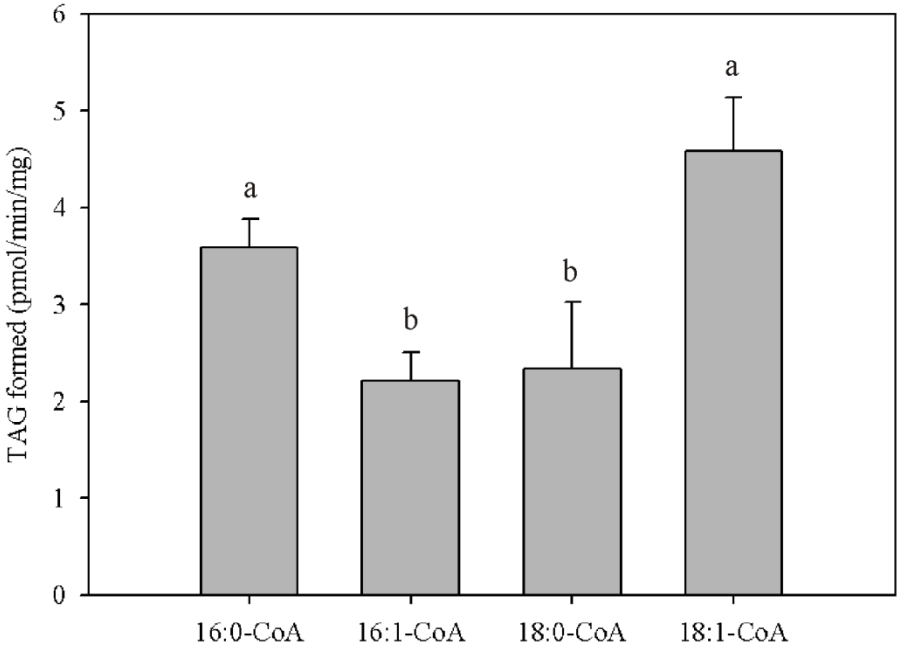

Fig. 4.10: Acyl-CoA substrate specificity of TcDGAT1.
Microsomes isolated from yeast expressing *TcDGAT1* were assayed with 7.5 μM of [^{14}C] acyl-CoA, including 16:0, 16:1, 18:0 or 18:1-CoA. The assays were performed using 320 μM di-18:1-DAG, 40 μg protein and reaction buffer containing 25 mM sucrose, 0.1 mM EDTA, 15 mM Tris-HCl pH 7.4, and 125 μg/mL BSA (fatty acid-free), at 30^{o}C for 15 min incubation. TcDGAT1 generally exhibited the highest specificity for 18:1-CoA and similar specificity for 16:0-CoA, 16:1-CoA or 18:0-CoA. The results were expressed as mean ± SD (n=3) and analyzed by ANOVA with Tukey test. $^{a, b}$ Mean values within unlike superscript letters were significantly different at α=0.05.

4.7 Fatty acid composition of TAG from H1246 yeast expressing *TcDGAT1* and *TcDGAT2*

The TAG fraction from H1246 yeast cells transformed with *BnDGAT1*, *TcDGAT1*or *TcDGAT2* was analyzed for fatty acid composition. Transformation with *BnDGAT1* was included as a control because *B. napus* is one of the most important oil seeds and has been known to accumulate a relatively low content of SFA (7%; Omidi et al., 2010). Yeast expressing *TcDGAT1* accumulated a greater proportion of palmitic acid than the yeast expressing *BnDGAT1* (Fig. 4.11A). Moreover, when calculating the total accumulation of SFAs, the *TcDGAT1*-expressing cells exhibited higher levels than the *BnDGAT1*-expressing cells, although the *TcDGAT1*-expressing cells did not show significant preference for stearic acid (Fig. 4.11B). On the other hand, yeast expressing *TcDGAT2* accumulated greater proportions of palmitic acid and stearic acid in TAG fraction than the yeast expressing *BnDGAT1* (Fig. 4.11). Yeast expressing *TcDGAT2* also displayed a significant decrease in the proportion of palmitoleic acid in TAG compared to yeast expressing *BnDGAT1* (Fig. 4.11A).

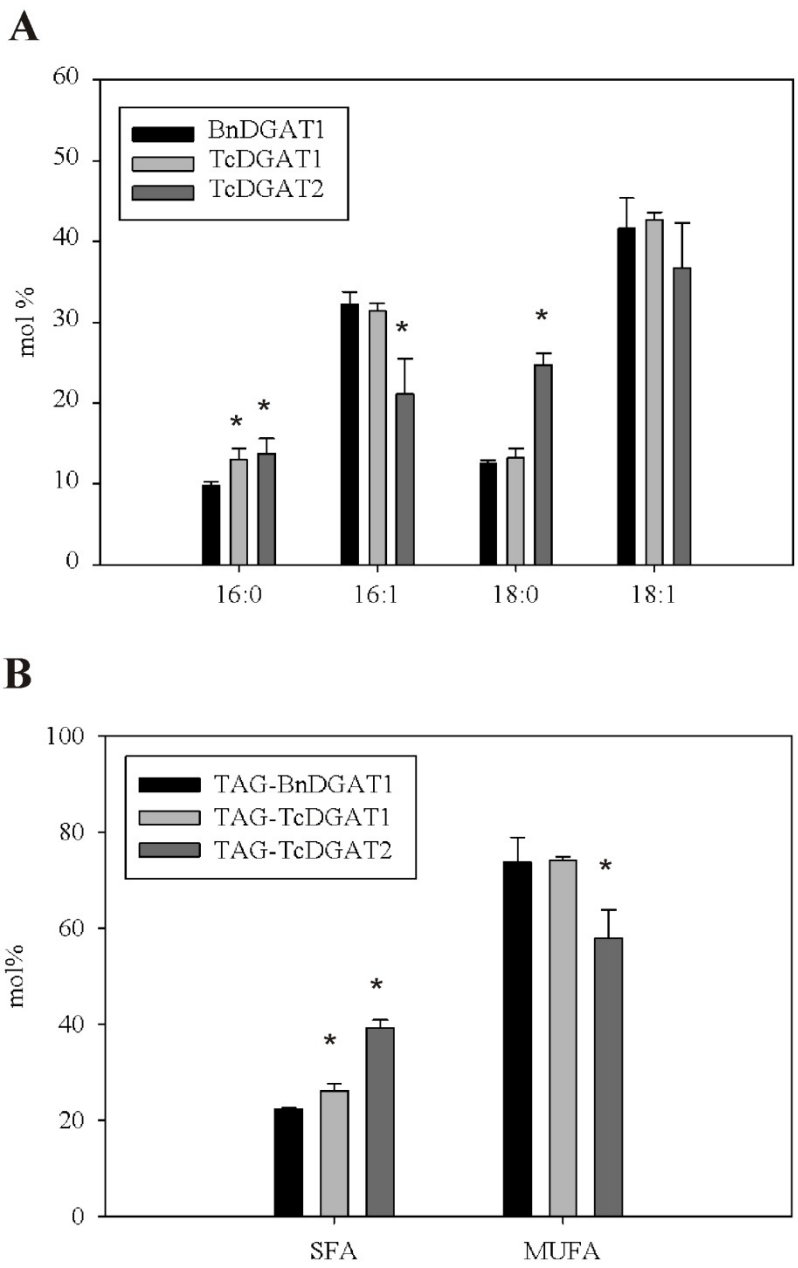

Fig. 4.11: Fatty acid profile (A) and composition of fatty acid class (B) in TAG fraction from yeast cells expressing *BnDGAT1*, *TcDGAT1* or *TcDGAT2*. The cells used in this analysis were incubated at 30^{o}C, 250 rpm for 24 h galactose induction. Results were expressed as mean mol% of total fatty acid methyl esters ± SD (n=3). Fatty acid compositions of yeast cells expressing *TcDGAT1* or *TcDGAT2* were compared to the the yeast cells expressing *BnDGAT1* using ANOVA analysis with the Holm-Sidak test. (*****) values represent results that are significantly different from the yeast cells expressing *BnDGAT1* at α=0.05.

Chapter 5. Discussion

Oilseed crops are important commodities on the marketplace due to their value as dietary oils and as raw materials for industrial applications. It has been demonstrated that increasing seed oil content and modifying fatty acid composition can be achieved by biotechnological approaches (Snyder et al., 2009). At least two groups of enzymes are needed for specific fatty acids to accumulate in seed oil. One is for the synthesis of the desired fatty acids, and the other is for specifically incorporating the target fatty acids into TAG. Several genes encoding enzymes involved in fatty acid synthesis and modification reactions have been identified (Jaworski and Cahoon, 2003). For example, epoxygenase genes responsible for Δ^{12}-desaturase-like enzymes have been cloned from plant species enriched in vernolic acid, an unusual 18-carbon Δ^{12}-epoxy fatty acid. Expression of these genes in transgenic plants, however, resulted in only low levels of the unusual fatty acids accumulation in seed oil (Cahoon et al., 2002; Hatanaka et al., 2004). Low-level accumulation of unusual fatty acids in transgenic plants could be due to the inefficient incorporation fatty acids into TAGs because the unusual fatty acids are rapidly degraded via β-oxidation (Moire et al., 2004). As well, enzymes catalyzing the TAG bioassembly might represent additional control points for selective accumulation of specific fatty acids in native plant seed oil (Li-Beisson et al., 2010).

The substrate preferences of acyltransferases involved in TAG biosynthesis, are important for accumulation of specific fatty acids in transgenic oilseeds (Snyder et al., 2009). The identifications of the genes encoding many of

these enzymatic activities have been reported. For instance, the Arabidopsis GPAT and LPAAT, which catalyze the first two acylation reactions of the acyl-CoA-dependent pathway, are encoded by large gene families (Zheng et al., 2003; Xu et al., 2006; Kim et al., 2005). Therefore, there are many possibilities for GPAT and LPAAT participating in seed oil biosynthesis.

With regard to the activities of PDAT, DGTA and DGAT, which catalyze the final reaction of TAG synthesis, the specific physiological roles of the PDAT and DGTA are in need of more investigation (mentioned in section 2.2). On the other hand, the DGAT, catalyzing the final reaction in acyl-CoA-dependent TAG biosynthesis, is believed to exert control over the carbon flow into TAG (Perry et al., 1999; Stals et al., 1994; Weselake et al., 2009). Previous studies also indicated that the overexpression of *DGATs* in new host species could influence the TAG content and the fatty acid composition of TAG (Jako et al., 2001; Shockey et al., 2006; Xu et al., 2008; Burgal et al., 2008; Lardizabal et al., 2008). Therefore, DGATs with specific substrate preferences should be considered in the design of molecular biology strategies to increase the proportions of desired fatty acids in the seed oil.

Examination of the fatty acid composition of sea buckthorn seed and pulp oil indicates that the pulp oil fraction contains a relatively high proportion of palmitic acid (Yang et al., 1999) suggesting that the developing pulp may contain DGATs with enhanced preference for SFAs. Thus, cDNAs encoding sea buckthorn pulp DGAT may prove useful in generating transgenic oilseed crops with increased SFA content in the seed oil. *HrDGAT1* and *HrDGAT2* cDNAs

were cloned from the developing fruit of sea buckthorn pulp. The substrate specificity properties of microsomal recombinant HrDGAT1, produced in yeast mutant H1246, were examined using various molecular species of acyl-CoAs. Insight into the substrate specificity properties of HrDGAT1 and HrDGAT2 under *in vivo* conditions was obtained by examining the fatty acid composition of TAG extracted from yeast transformed with the cDNAs encoding these enzyme forms. Further insight into the *in vivo* effect of HrDGAT1 on TAG production came from experiments in which Arabidopsis mutant AS11 (a *dgat1* knockout) was transformed with *HrDGAT1*. Some characterization studies were also conducted with DGATs from cocoa bean, which accumulates high amounts of palmitic acid and stearic acid, to compare the enzyme activities and substrate preferences in the recombinant yeast cells.

5.1 *HrDGAT1* and *TcDGAT1* are functionally expressed in yeast

Expression of *HrDGAT1* and *TcDGAT1* in a quadruple knockout yeast mutant strain H1246, which lacks the genes (*DGA1*, *LRO1*, *ARE1* and *ARE2*) required for TAG and sterol esters synthesis, and performing the *in vitro* enzyme assay, revealed that the *HrDGAT1* and *TcDGAT1* gene encodes a protein that functions as an acyl-CoA-dependent DGAT. Although the expression of *HrDGAT2* and *TcDGAT2* in yeast mutant cells could also restore TAG accumulation, which was detected *in vivo* by the Nile red assay (see Fig. 4.3), the activity of HrDGAT2 and TcDGAT2 could not be detected using *in vitro* enzyme assays (data not shown).

An *in vitro* enzyme assay was also performed by Liu (2011) to investigate the activities of Arabidopsis DGAT2 (AtDGAT2) and *B. napus* DGAT2 (BnDGAT2). The soluble and microsomal fraction from the yeast cells expressing *AtDGAT2* and *BnDGAT2*, however, did not show any DGAT activity using *in vitro* enzyme assay (data not shown). Moreover, a double mutant of *AtDGAT1* and *AtDGAT2* did not result in a significant additional reduction of oil content in comparison with the *DGAT1* single mutant (Zhang et al., 2009). Shockey et al. (2006), however, were able to detect DGAT activity in microsomes from yeast, deficient in DGAT and PDAT activities, which were transformed with tung tree *DGAT1* (*VfDGAT1*) or tung tree *DGAT2* (*VfDGAT2*), but VfDGAT2 was produced at lower level compared to VfDGAT1. It is may be that the steady state of VfDGAT2 is lower than VfDGAT1 in the yeast system (Shockey et al., 2006). DGAT2s from certain plant species, such as Arabidopsis and *B.napus*, may require unidentified *in planta* factors for manifestation of enzyme activity and/or there may be issues associated with different codon usage preferences between the yeast and plant organisms (Liu, 2011; Angov et al., 2008; Chiapello et al., 1998).

5.2 HrDGAT1 exhibits relatively high activities with 16:0 and 18:1-CoA, and di-8:0-DAG

Several reports in the literature using plant microsomes describe plant DGAT substrate specificity (Vogel and Browse, 1996; Lin et al., 2002; Wiberg et al., 1994). It is difficult, however, to conclude that the substrate specificity is

solely due to a single enzyme in the plant microsomes, since a number of activities are present that can contribute to the end product. In this study, over-expression of *DGAT* cDNAs in a yeast mutant deficient in endogenous DGAT activity provides an effective tool to characterize the substrate specificity of microsomal recombinant HrDGAT1.

Microsomal HrDGAT1 showed enhanced substrate specificity for [^{14}C]16:0 and 18:1-CoA (Fig. 4.5A) with optimal activity occurring at 10 μM [^{14}C] acyl-CoAs. Higher concentrations of acyl-CoAs led to enzyme inhibition. The enhanced activity observed using [^{14}C]16:0-CoA is in agreement with enhanced incorporation of palmitic acid in the pulp oil of sea buckthorn.

Evaluating the effect of exogenous DAGs on microsomal HrDGAT1 activity proved difficult because about two thirds of the maximum enzyme activity could be detected without adding exogenous DAG (Fig. 4.5B). It was assumed, therefore, that the microsomes contained substantial quantities of endogenous DAG of underfined acyl composition. The degree of microsomal DGAT activity dependence on exogenous DAG is known to be attributable to the endogenous DAG content, which can vary among plant species and seed development stages (Cao and Huang, 1986; Ichihara and Noda, 1982; Oo and Chew, 1992; Valencia-Turcotte and Rodriguez-Sotres, 2001; Weselake, 2005), and the accessibility of the exogenous DAG to the enzyme (Kamisaka et al., 1993). Although complicated by the presence of endogenous DAG in the yeast cells, the absolute incorporation of [^{14}C]18:1-CoA into di-8:0-DAG catalyzed by microsomal HrDGAT1 was much higher than when using di-18:1-DAG (Fig.

4.5B). Unlike di-18:1-DAG, di-8:0-DAG is soluble and also known to be a cell-permeable DAG (Gorodeski, 2000). The greater microsomal HrDGAT1 activity observed with di-8:0-DAG suggests that this acyl acceptor could be used to increase the sensitivity of the DGAT assay providing acyl-CoAs specificity issues were not a prime concern. In some cases, organic solvent can be used to remove endogenous DAG from microsomes without compromising DGAT activity (Valencia-Turcotte and Rodriguez-Sotres, 2001).

5.3 HrDGAT1 shows increased preference for substrates containing palmitic acid under *in vivo* conditions in yeast

The analysis of the TAG fraction from yeast H1246 expressing *HrDGAT1* and *HrDGAT2* indicated that TAG bioassembly was restored. The recombinant HrDGAT1 or HrDGAT2 utilized endogenous DAG and acyl-CoA pools for acylation. As presented in Fig. 4.6A, HrDGAT1 showed a clear preference for endogenous palmitic acid as compared with the yeast expressing *BnDGAT1*. Once again, this is consistent with the fatty acid composition of oil from sea buckthorn pulp, which has a relatively high proportion of of palmitic acid. Moreover, the expression of *HrDGAT2* resulted in a significantly higher proportion of stearic acid compared to yeast expressing *BnDGAT1* (Fig. 4.6A). The preference of HrDGAT2 for stearic acid observed in the yeast system, however, was not reflected in the oil of sea buckthorn fruit pulp which only had a low proportion of stearic acid. It is possible that the plastidial 18:0-CoA desaturase in the pulp cells catalyzes the desaturation of most of the available 18:0-CoA making the stearoyl

moieties largely unavailable for incorporation into TAG. With regard to total SFA accumulation, the recombinant HrDGAT1 or HrDGAT2 showed higher preferences for these fatty acids than yeast producing BnDGAT1 (Fig. 4.6B). This indicates that HrDGAT1 or HrDGAT2 is potential enzyme for increasing SFA content in *B.napus*.

5.4 Expression of *HrDGAT1* restores WT levels of TAG accumulation and fatty acid composition in the Arabidopsis AS11 mutant

Arabidopsis AS11 expressing *HrDGAT1* resulted in an approximately 35% further increase in the seed oil content compared with the AS11-*pGreen* control lines (Fig. 4.8). The seed oil content of some transgenic lines was restored to near WT levels. These findings strongly support previous research, wherein seed-specific expression of *DGAT1* was able to restore TAG accumulation in Arabidopsis mutant AS11 (Jako et al., 2001).

The overexpression of *HrDGAT1* in Arabidopsis developing seeds also affected the fatty acid composition of the seed oil (Table 4.1). Gas chromatography analysis showed that seed-specific expression of *HrDGAT1* in AS11 was able to complement the fatty acid compositional mutant phenotype, restoring the proportions of oleic acid, eicosenoic acid and α-linolenic acid seen in the WT (Table 4.1A). Jako et al. (2001) and Xu et al. (2008) also showed that seed-specific expression of *DGAT1* from Arabidopsis or *Tropaeolum majus* (garden nasturtium) in Arabidopsis AS11 was able to increase the proportions of

oleic acid and eicosenoic acid, and decrease the proportion of α-linolenic acid in the seed oil.

The T_3 seeds of AS11: *pGreen-HrDGAT1*, however, did not show increased proportions of SFAs accumulation compared to the WT (Table 4.1B). There was only one line of AS11: *pGreen-HrDGAT1* T_3 seeds with a significant increase of palmitic acid (Table 4.1A). This is not consistent with the observation in the yeast recombinant system where HrDGAT1 exhibited increased preference for substrates containing palmitoyl moieties. The limited incorporation of palmitic acid into Arabidopsis AS11: *pGreen-HrDGAT1* seed oil may be attributable to the nature of the endogenous DAG acceptor because Larson and Graham (2001) have demonstrated that 16:0-CoA is present at much higher concentrations in the acyl-CoA pool of Arabidopsis developing seeds than other molecular species of acyl-CoAs.

Storage TAGs in seeds also provide energy to support germination (Hsieh and Huang, 2004). The AS11 lines expressing *HrDGAT1* showed more rapid germination than AS11: *pGreen* line, which was in agreement with increased TAG levels in the seed of AS11:*pGreen-HrDGAT1* lines.

5.5 TcDGATs display enhanced selectivity for SFAs under *in vivo* conditions in yeast

As indicated at the beginning of the discussion, some comparative studies were conducted with cocoa DGATs. In *in vitro* assays using microsomes from transformed yeast, recombinant *TcDGAT1* showed increased specificity for

[^{14}C]16:0-CoA and [^{14}C]18:1-CoA when compared to [^{14}C]16:1-CoA and [^{14}C]18:0-CoA (Fig. 4.10).

The recombinant TcDGAT1 and TcDGAT2 could both effectively utilize the yeast endogenous DAG and acyl-CoA pool for TAG synthesis when their encoding cDNAs were expressed in yeast mutant H1246. As shown in Fig. 4.11A, TcDGAT1 showed a clear preference for endogenous palmitic acid as compared with the yeast expressing *BnDGAT1*. Although the stearic acid content in the TAG fraction of *BnDGAT1* or *TcDGAT1*-expressing cells was similar, total SFA content in *TcDGAT1*-expressing cells producing was still significantly higher than for *BnDGAT1*-expressing cells (Fig. 4.11B). Moreover, the TAGs formed by the action of TcDGAT2 were substantially more saturated than those formed by the catalytic action of BnDGAT1 (Fig. 4.11B). TcDGAT2 was more selective towards palmitic acid and stearic acid, collectively, in the recombinant yeast cells (Fig. 4.11A). The high preference of TcDGAT2 for SFAs (palmitic acid and stearic acid) is consistent with the high accumulation of SFAs in cocoa bean.

Different ratios of palmitic acid to stearic acid in oils can influence the end use application of the oil. For example, oil enhanced in palmitic acid is more useful for applications in margarine. Differential co-expression of sea buckthorn pulp and cocoa *DGATs* may be potentially useful for generating plant oils with different ratios of palmitic acid to stearic acid.

Chapter 6. Conclusions and future directions

Overall, in this study, cDNAs encoding forms of DGAT1 and DGAT2 were identified in sea buckthorn pulp and cocoa bean that share high sequence similarity with the other plant type 1 and type 2 DGATs and possess DGAT activity. The cloned *HrDGAT1*, *HrDGAT2*, *TcDGAT1* and *TcDGAT2* catalyzed oil biosynthesis *in vivo* in a yeast recombinant system, but the yeast expressing *HrDGAT2* and *TcDGAT2* accumulated much lower TAG content than yeast expressing *HrDGAT1* or *TcDGAT1*. HrDGAT2 or TcDGAT2 activities were not detectable using *in vitro* assays using yeast microsomes. The absence of activity for these type-2 DGATs as observed using *in vitro* assays may be due to some disruption of favourable *in vivo* conditions and/or differences in codon usage between yeast and plant species.

One of the major issues in plant lipid biochemistry and biotechnology is the understanding of how plants assemble TAG and, specifically, how a single fatty acid accumulates to high proportions in the oil of an oleaginous plant species. The cloning of *HrDGATs* and *TcDGATs* make it possible to elucidate the individual role of these particular enzymes in sea buckthorn pulp and cocoa bean oil biosynthesis. The current *in vivo* studies provide evidence that HrDGATs and TcDGATs have higher preferences for SFAs than BnDGAT1. HrDGAT1 or TcDGAT1 exhibit increased preferences for palmitic acid in TAG biosynthesis, whereas HrDGAT2 or TcDGAT2 preferentially utilized stearic acid as a substrate.

Hence, the functional and cellular aspects of HrDGATs and TcDGATs presented here have at least two important implications for the genetic engineering of crop plants that are designed for production SFA-enriched oils. Firstly, the expression of the *HrDGATs* and *TcDGATs* could be coupled with the enzymes responsible for SFA synthesis from sea buckthorn or cocoa bean, respectively, to promote the utilization of saturated acyl-CoAs by the acyl-CoA-dependent acyltransferases. Although this hypothesis has not been supported in transgenic plant lines enriched in SFAs, parallel observation have been made with regard to the incorporation of unusual fatty acids into oil. For instance, the co-expression of castor bean DGAT2 with castor *fatty acid hydroxylase* (*RcFAH*) resulted in a 30% increase in ricinoleic acid, which is two times more than with the expression of *RcFAH* alone (Burgal et al., 2008). Secondly, in light of the current results with *HrDGAT1* and *TcDGAT1*, and previous findings with other plant *DGAT1*, the general utility of the over-expression of the *DGAT1* genes in high yielding oilseed crops to increase oil contents will be corroborated.

Additional research will be needed in the future. Firstly, the cDNA encoding *TcDGAT1* could be expressed in Arabidopsis WT and mutant AS11. It was hypothesized that overexpression *DGAT1* cDNAs in Arabidopsis WT also could result in the increase of seed oil content. Secondly, site-directed mutagenesis (SDM) and chemical modification could be used in combination to probe the structure and function of HrDGAT1 and TcDGAT1 (Liu, 2011). Lastly, the relative contribution of HrDGAT2 and TcDGAT2 in the TAG biosynthesis could also be investigated by the genetic and biochemical studies, including

multiple gene disruptions and searching for protein partners that potentially interact with and stimulate these type-2 DGATs.

Results from these studies will also provide valuable information for better understanding the roles of HrDGATs or TcDGATs in TAG biosynthesis. The expression of *HrDGATs* and *TcDGATs* in oilseed crops, such as *B. napus*, is the subject of future investigation.

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