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Bioengineering Recombinant Diacylglycerol Acyltransferases

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1. Introduction

The complete genomes of many organisms including human, mouse, *Arabidopsis*, and rice have been sequenced. However, the functions of the proteins encoded by a large percentage of the genes in these organisms have not been determined. The immediate challenge of the post-genomic biology is to determine the biological functions of proteins coded for by those unknown genes. Many endogenous proteins occur in extremely low abundance (such as the anti-inflammatory protein tristetraprolin, TTP) (Cao et al., 2004) and are labile (such as omega-3 fatty-acid desaturase, FAD3) (O'Quin et al., 2010), which are major problems inherent to characterization of those proteins.

Recombinant proteins can be used as an alternative source to endogenous proteins. Production of active proteins in large quantities is necessary for the study of protein structure and function (Cao et al., 2003). Purified recombinant proteins are also important for the production of antibodies (Cao 2004; Cao et al., 2008; Cao et al., 2004) and pharmaceutical reagents. Unfortunately, a great number of proteins are difficult to express and purify. Those proteins include membrane proteins, lipid-associated proteins, and low-abundance proteins. The causes of the difficulties in protein expression and purification are various, among which are protein insolubility, protein degradation, and low-level protein expression (Cao 2010). Therefore, production of high-quality recombinant proteins requires optimization of protein expression and purification procedures in each case.

Diacylglycerol acyltransferases (DGATs) catalyze the last and rate-limiting step of triacylglycerol (TAG) biosynthesis in eukaryotic organisms. DGAT genes have been isolated from many organisms. At least two forms of DGATs are present in mammals (Cases et al., 1998; Cases et al., 2001) and plants (Lardizabal et al., 2001; Shockey et al., 2006) with additional forms reported in burning bush (*Euonymus alatus*) (Durrett et al., 2010), peanut (Saha et al., 2006), and *Arabidopsis* (Rani et al., 2010). Plants and animals deficient in DGATs accumulate less TAG (Smith et al., 2000; Stone et al., 2004; Zou et al., 1999). Animals with reduced DGAT activity are resistant to diet-induced obesity (Chen et al., 2004; Smith et al., 2000) and lack milk production (Smith et al., 2000). Over-expression of DGAT enzymes increases TAG content in plants (Andrianov et al., 2010; Bouvier-Nave et al., 2000; Burgal et al., 2008; Durrett et al., 2010; Jako et al., 2001; Lardizabal et al., 2008; Xu et al., 2008), animals (Kamisaka et al., 2010; Liu et al., 2009; Liu et al., 2007; Roorda et al., 2005), and yeast (Kamisaka et al., 2007). DGATs have nonredundant functions in TAG biosynthesis in species

such as mice (Stone et al., 2004) and tung tree (*Vernicia fordii*) (Shockey et al., 2006). Mice deficient in DGAT1 are viable, have modest decreases in TAG, and are resistant to diet-induced obesity (Chen et al., 2002; Smith et al., 2000). In contrast, mice deficient in DGAT2 have severe reduction of TAG and die shortly after birth (Stone et al., 2004). The fact that DGAT1 is unable to compensate for the deficiency in DGAT2 indicates the nonredundant functions of each DGAT isoform in TAG biosynthesis during animal development. Therefore, understanding the roles of DGATs in plants and animals will have tremendous implications in creating new oilseed crops with value-added properties and in providing clues for therapeutic intervention in obesity and related diseases. Over-production of DGATs has been the subject of a number of studies, but progress has been slow in the characterization of the enzymes because DGATs are integral membrane proteins (Shockey et al., 2006; Stone et al., 2006) and difficult to express and purify (Cheng et al., 2001; Weselake et al., 2006). Information regarding the expression of DGAT genes in *E. coli* is limited. The expression of DGAT1 and DGAT2 as full-length proteins in *E. coli* had not been reported. We recently developed a reliable procedure for the expression and purification of tung DGATs in *E. coli* (Cao et al., 2010; Cao et al., 2011).

2. Bioengineering recombinant diacylglycerol acyltransferases

2.1 DGAT genes have been identified in a wide range of organisms

Database search identified at least 115 DGAT sequences from 69 organisms including plants (such as *Arabidopsis*, barley, castor bean, cauliflower, corn, rape, rice, sorghum, soybean, tobacco, tung tree), animals (such as bird, chimpanzee, cow, dog, fish, fly, frog, monkey, mosquito, mouse, pig, rabbit, rat, sheep, worm), fungi (such as yeast), and human. The names of organisms, the subfamilies of DGATs (DGAT1 and DGAT2) and the GenBank accession numbers are listed in Table 1. Although more than two isoforms of DGATs are found in some species, most of them could be classified into the DGAT1 or DGAT2 subfamily according to their sequence similarities and phylogenetic analysis (data not shown). However, DGAT3 (Saha et al., 2006) and DGAT4 (Rani et al., 2010) were reported recently which have very different sequences with those of DGAT1 and DGAT2. DGAT1 and DGAT2 subfamilies have many conserved residues among the diverse species. However, addition of DGAT3 and DGAT4 from *Arabidopsis* (GenBank accession number: AAN31909.1), castor bean (GenBank accession number: XP_002519339.1), peanut (GenBank accession number: AY875644.1), and yeast (GenBank accession number: DG315417.1) to the multiple sequence alignment completely destroyed all the conserved residues (data not shown), which is contrary to the general belief that the active sites of the enzymes should have certain degree of conservation during the evolution because all are supposed to catalyze the same/similar biochemical reaction.

No.	Organism	DGAT	GenBank accession number	No.	Organism	DGAT	GenBank accession number
1	<i>Aedes aegypti</i> (A)	1	XP_001658299	59	<i>Medicago truncatula</i> (P)	2	ACJ84867.1
2	<i>Ajellomyces capsulatus</i> (F)	1	EGC41804.1	60	<i>Nicotiana tabacum</i> (P, tobacco)	1	AAF19345.1

3	<i>Anolis carolinensis</i> (A)	2	XP_003225477.1	61	<i>Nematostella vectensis</i> (A, worm)	2a	XP_001630435.1
4	<i>Ashbya gossypii</i> (F)	2	NP_983542.1	62	<i>Nematostella vectensis</i> (A, worm)	2b	XP_001633322.1
5	<i>Arthroderma otae</i> (F)	1	EEQ31683.1	63	<i>Nematostella vectensis</i> (A, worm)	2c	XP_001635548.1
6	<i>Arabidopsis thaliana</i> (P)	1	NP_179535.1	64	<i>Ovis aries</i> (A, sheep)	1	NP_001103634.1
7	<i>Arabidopsis thaliana</i> (P)	2	NP_566952	65	<i>Ovis aries</i> (A, sheep)	2	XP_001518899.1
8	<i>Bubalus bubalis</i> (A, buffalo)	1	AAZ22403.1	66	<i>Oryctolagus cuniculus</i> (A, rabbit)	1	XP_002724427.1
9	<i>Brassica juncea</i> (P)	1a	AAAY40784.1	67	<i>Olea europaea</i> (P, tree)	1	AAS01606.1
10	<i>Brassica juncea</i> (P)	1b	AAAY40785.1	68	<i>Olea europaea</i> (P, tree)	2	ADG22608.1
11	<i>Brassica napus</i> (P)	1a	AAD45536.1	69	<i>Oryza sativa</i> (P, rice)	1	NP_001054869.2
12	<i>Brassica napus</i> (P)	1b	AAD40881.1	70	<i>Oryza sativa</i> (P, rice)	2a	NP_001047917
13	<i>Brassica napus</i> (P)	2	ACO90187	71	<i>Oryza sativa</i> (P, rice)	2b	NP_001057530
14	<i>Brassica napus</i> (P)	2	ACO90188	72	<i>Ostreococcus tauri</i> (algae)	2	XP_003083539.1
15	<i>Bos taurus</i> (A, cow)	1	NP_777118.2	73	<i>Pongo abelii</i> (A)	2	XP_002822304.1
16	<i>Bos taurus</i> (A, cow)	2a	DAA21853.1	74	<i>Paracoccidioides brasiliensis</i> (F)	1	EEH17170.1
17	<i>Bos taurus</i> (A, cow)	2b	XP_875499.3	75	<i>Perilla frutescens</i> (P)	1	AAG23696.1
18	<i>Bos taurus</i> (A, cow)	2c	XP_002683800.1	76	<i>Polysphondylium pallidum</i> (F)	1	EFA85004.1
19	<i>Caenorhabditis elegans</i> (A, worm)	2a	NP_505413.1	77	<i>Polysphondylium pallidum</i> (F)	2	EFA83646.1
20	<i>Caenorhabditis elegans</i> (A, worm)	2b	NP_872180.1	78	<i>Physcomitrella patens</i> (P, moss)	1	XP_001770929.1
21	<i>Canis familiaris</i> (A, dog)	1b	XP_849176.1	79	<i>Physcomitrella patens</i> (P, moss)	1	XP_001758758.1
22	<i>Canis familiaris</i> (A, dog)	1c	XP_858062.1	80	<i>Physcomitrella patens</i> (P, moss)	2b	XP_001777726.1
23	<i>Capra hircus</i> (A, sheep)	1	ABD59375.1	81	<i>Picea sitchensis</i> (P, tree)	2	ABK26256.1

24	<i>Ciona intestinalis</i> (A)	2	XP_002120879.1	82	<i>Pan troglodytes</i> (A, chimpanzee)	1	XP_520014.2
25	<i>Chlamydomonas reinhardtii</i> (algae)	2a	XP_001694904.1	83	<i>Pan troglodytes</i> (A, chimpanzee)	2	XP_527842.2
26	<i>Chlamydomonas reinhardtii</i> (algae)	2b	XP_001693189.1	84	<i>Phaeodactylum tricornutum</i> (F)	1	XP_002177753.1
27	<i>Chlorella variabilis</i> (algae)	1	EFN50697.1	85	<i>Populus trichocarpa</i> (P, tree)	1a	XP_002308278.1
28	<i>Chlorella variabilis</i> (algae)	2	EFN51306.1	86	<i>Populus trichocarpa</i> (P, tree)	1b	XP_002330510.1
29	<i>Dictyostelium discoideum</i> (mold)	1	XP_645633.2	87	<i>Populus trichocarpa</i> (P, tree)	2	XP_002317635.1
30	<i>Dictyostelium discoideum</i> (mold)	2	XP_635762.1	88	<i>Ricinus communis</i> (P, castor bean)	1	XP_002514132.1
31	<i>Drosophila melanogaster</i> (A, fly)	1a	NP_609813.1	89	<i>Ricinus communis</i> (P, castor bean)	1	XP_002528531.1
32	<i>Drosophila melanogaster</i> (A, fly)	1d	NP_995724.1	90	<i>Rattus norvegicus</i> (A, rat)	1	NP_445889.1
33	<i>Danio rerio</i> (A, zebrafish)	1a	NP_956024.1	91	<i>Rattus norvegicus</i> (A, rat)	2	NP_001012345.1
34	<i>Danio rerio</i> (A, zebrafish)	1b	NP_001002458.1	92	<i>Sorghum bicolor</i> (P, sorghum)	1a	XP_002437165.1
35	<i>Danio rerio</i> (A, zebrafish)	2	NP_001025367.1	93	<i>Sorghum bicolor</i> (P, sorghum)	1b	XP_002439419.1
36	<i>Euonymus alatus</i> (P)	1	AAV31083.1	94	<i>Sorghum bicolor</i> (P, sorghum)	2	XP_002452652.1
37	<i>Euonymus alatus</i> (P)	2	ADF57328.1	95	<i>Saccharomyces cerevisiae</i> (F, yeast)	2	NP_014888.1
38	<i>Elaeis oleifera</i> (P)	2	ACO35365.1	96	<i>Saccoglossus kowalevskii</i> (A, worm)	1	XP_002736160.1
39	<i>Echium pitardii</i> (P)	1	ACO55635.1	97	<i>Selaginella moellendorffii</i> (P)	1	XP_002964165.1
40	<i>Glycine max</i> (P, soybean)	1a	AAS78662.1	98	<i>Selaginella moellendorffii</i> (P)	2	XP_002972054.1

41	<i>Glycine max</i> (P, soybean)	1b	BAE93461.1	99	<i>Spirodela polyrhiza</i> (P)	2	AAQ89590.1
42	<i>Glycine max</i> (P, soybean)	2	ACU20344.1	100	<i>Schizosaccharomyces pombe</i> (F, yeast)	2	XP_001713160.1
43	<i>Helianthus annuus</i> (P)	2	ABU50328.1	101	<i>Sus scrofa</i> (A, pig)	1	NP_999216.1
44	<i>Homo sapiens</i> (human)	1	NP_036211.2	102	<i>Tribolium castaneum</i> (A)	1	XP_975142.1
45	<i>Homo sapiens</i> (human)	2a	AAQ88896.1	103	<i>Tribolium castaneum</i> (A)	2	XP_975146.1
46	<i>Homo sapiens</i> (human)	2b	NP_835470.1	104	<i>Toxoplasma gondii</i> (A)	1	AAP94209.1
47	<i>Hordeum vulgare</i> (P, barley)	2	BAJ85730.1	105	<i>Taeniopygia guttata</i> (A, bird)	2	XP_002187643.1
48	<i>Ictalurus punctatus</i> (A, catfish)	2b	NP_001188005.1	106	<i>Tropaeolum majus</i> (P)	1	AAM03340.2
49	<i>Jatropha curcas</i> (P)	1	ABB84383.1	107	<i>Vernicia fordii</i> (P, tung tree)	1	DQ356680.1
50	<i>Lotus japonicas</i> (P)	1	AAW51456.1	108	<i>Vernicia fordii</i> (P, tung tree)	2	DQ356682
51	<i>Metarhizium acridum</i> (F)	1a	EFY86774.1	109	<i>Vernonia galamensis</i> (P)	1	ABV21945.1
52	<i>Metarhizium anisopliae</i> (F)	1b	EFY97444.1	110	<i>Vernonia galamensis</i> (P)	2	ACV40232.1
53	<i>Monodelphis domestica</i> (A)	1	XP_001371565.1	111	<i>Vitis vinifera</i> (P, grape)	1	XP_002279345.1
54	<i>Monodelphis domestica</i> (A)	2	XP_001365685.1	112	<i>Vitis vinifera</i> (P, grape)	2	XP_002263626
55	<i>Mus musculus</i> (A, mouse)	1	NP_034176.1	113	<i>Xenopus tropicalis</i> (A, frog)	2	NP_989372.1
56	<i>Mus musculus</i> (A, mouse)	2	NP_080660.1	114	<i>Zea mays</i> (P, corn)	1b	EU039830
57	<i>Macaca mulatta</i> (A, monkey)	1	XP_001090134.1	115	<i>Zea mays</i> (P, corn)	2	NP_001150174.1
58	<i>Medicago truncatula</i> (P)	1	ABN09107.1				

Table 1. DGAT1 and DGAT2 sequence information (DGAT3 and DGAT4 are not included in the Table because of their divergent sequences). A: animal, F: fungus, P: plant.

2.2 Literature survey of DGAT expression

A literature survey was performed to find out how many publications related to DGATs have been collected by the two most popular databases, PubMed and Scopus. The data in Table 2 indicate that approximately 1000 papers had been collected by the two databases during the past 28 years when using DGAT and diacylglycerol acyltransferase as search terms in title/abstracts/keywords. Approximately four times of publications were obtained when using the full name of the enzyme “diacylglycerol acyltransferase” as a search term

instead of using the abbreviation “DGAT” in the database search. More than half of the publications were from animals and approximately one quarter of the publications were from plants. Less than half of those publications dealt with expression of DGATs at the RNA and protein levels. Some of the publications reported of using more than one organism in the same paper, resulting in the total number of publications less than the number of publications from plants, animals, and human adding together (Table 2). Similarly, the total expression papers are less than the combination because more than one expression methods were used in the same paper. Approximately 5% of the publications were related to heterologous expression. However, only a few papers were from *E. coli* expression system.

Database	PubMed	PubMed	Scopus	Scopus
Search terms in title/abstracts/keywords	DGAT	diacylglycerol acyltransferase	DGAT	diacylglycerol acyltransferase
Total publications	216	817	255	1102
Plant	57	118	60	137
Human	74	203	72	316
Animal	138	588	164	760
Total expression papers	90	225	122	322
Plant expression	31	50	34	62
Human expression	31	85	42	131
Animal expression	53	144	78	220
<i>E. coli</i> expression	4	8	1	6
Yeast expression	17	32	17	33
Insect expression	5	12	7	15

Table 2. Literature survey of publications related to DGAT expression in PubMed and Scopus databases (1982-2010).

2.3 Recombinant DGAT expression update

Expression and purification of recombinant DGATs from any source represents a challenge because DGATs are integral membrane proteins (Hobbs et al., 1999; Siloto et al., 2008; Weselake et al., 2006). In addition, more than 40% of the total amino acid residues are hydrophobic (Table 3). Yeast was the preferred host for DGAT expression (Bouvier-Nave et al., 2000; Bursal et al., 2008; Cao et al., 2010; He et al., 2004; Kalscheuer et al., 2004; Kalscheuer & Steinbuchel 2003; Kroon et al., 2006; Liu et al., 2011; Liu et al., 2010; Manas-Fernandez et al., 2009; Mavraganis et al., 2010; Milcamps et al., 2005; Nykiforuk et al., 2002; Quittnat et al., 2004; Shockey et al., 2006; Siloto et al., 2009; Wagner et al., 2010; Xu et al., 2008; Yu et al., 2008) followed by insect cells (Buszczak et al., 2002; Cases et al., 1998; Cases et al., 2001; Lardizabal et al., 2001). A limited number of reports used other host cells including *E. coli* (Saha et al., 2006; Siloto et al., 2008; Weselake et al., 2006) and human cells (Cheng et al., 2001). The great majority of the yeast and insect cell expression studies were designed to confirm the functions of full-length cloned genes. A few studies were directly related to the expression and purification of recombinant DGATs using *E. coli* expression system for functional and structural studies. The recombinant N-terminal region of *Brassica napus* DGAT1 was purified from *E. coli* with a predicted molecular mass of 13,278 Da which was confirmed by MALDI-TOF mass spectrometry. However, the apparent molecular mass on SDS-PAGE was doubled and the native size was four times of the size of the monomer

due to self-association (Weselake et al., 2006). The N-terminal region of mouse DGAT1 was also studied in a similar way (Siloto et al., 2008). Full-length DGAT1 or DGAT2 from any organism was, however, not successfully expressed in *E. coli* (Hobbs et al., 1999; Weselake et al., 2006). The exceptional case was that expression of soluble peanut DGAT (DGAT3) in *E. coli* resulted in high levels of DGAT activity and the formation of labeled TAG (Saha et al., 2006), although its sequence is very different from those of DGAT1 or DGAT2.

	Tung tree DGAT1	Tung tree DGAT2	DGAT1 - DGAT2
Length (aa)	526	322	204
Molecular weight	59773.84	36726.20	23047.64
Isoelectric point (PI)	8.91	9.24	- 0.33
Charge at pH 7	11.78	8.44	3.34
Charged (RKHYCDE) (%)	27.00	23.60	3.40
Acidic (DE) (%)	7.98	7.14	0.84
Basic (KR) (%)	10.08	9.63	0.45
Polar (NCQSTY) (%)	25.86	21.74	4.12
Hydrophobic (AILFWV) (%)	41.06	43.48	-2.42

Table 3. Tung DGATs properties and amino acid composition.

2.4 Bioengineering recombinant DGAT for expression in bacteria

We recently described a procedure for over-expression of recombinant full-length DGAT1 and DGAT2 in a bacterial expression system (Cao et al., 2010; Cao et al., 2011). DGAT1 is much larger than DGAT2, although they are similar in other properties and amino acid composition (on % of frequency basis) (Table 3). The two DGAT isoforms have only limited sequence identity and similarity (Figure 1). We were able to express both proteins in *E. coli* as full-length recombinant proteins. In our study, we engineered a maltose binding protein (MBP) tag at the amino terminus and 6 histidine residues (His-tag) at the carboxyl terminus of full-length tung DGATs (Table 4).

Primer	Sequence (5' to 3')	Comments
DGAT1 forward	AATATTGGTACCCTGTTTCAGGGTCC GACAATCCTTGAAACGCCG	<i>Kpn</i> I site underlined Codons for PreScission protease site Colored
DGAT1 reverse	CGATTAACTAGTAGCTAGCTCAATG ATGATGATGATGATGCTTTGATTCCG TAGTCCC	<i>Spe</i> I site underlined Codons for 6 His Colored
DGAT2 forward	AATATTGGTACCCTGTTTCAGGGTCC GGGGATGGTGGAAGTTAAG	<i>Kpn</i> I site underlined Codons for PreScission protease site Colored
DGAT2 reverse	CGATTAACTAGTAGCTAGCTCAATG ATGATGATGATGATGAAAAATTTC AGTTTAAG	<i>Spe</i> I site underlined Codons for 6 His Colored

Table 4. Primers for PCR-amplification of the full-length DGAT1 and DGAT2 insert sequences.

We engineered plasmids pMBP-DGAT1-His and pMBP-DGAT2-His for expressing the full-length tung tree type 1 and type 2 diacylglycerol acyltransferases (DGAT1 and DGAT2, GenBank Accession No. DQ356680 and DQ356682, respectively (Shockey et al., 2006) as fusion proteins in *E. coli*. The recombinant proteins contained MBP at the amino terminus and His-tag at the carboxyl terminus. The cloning vector pMBP-hTTP (Figure 2) was

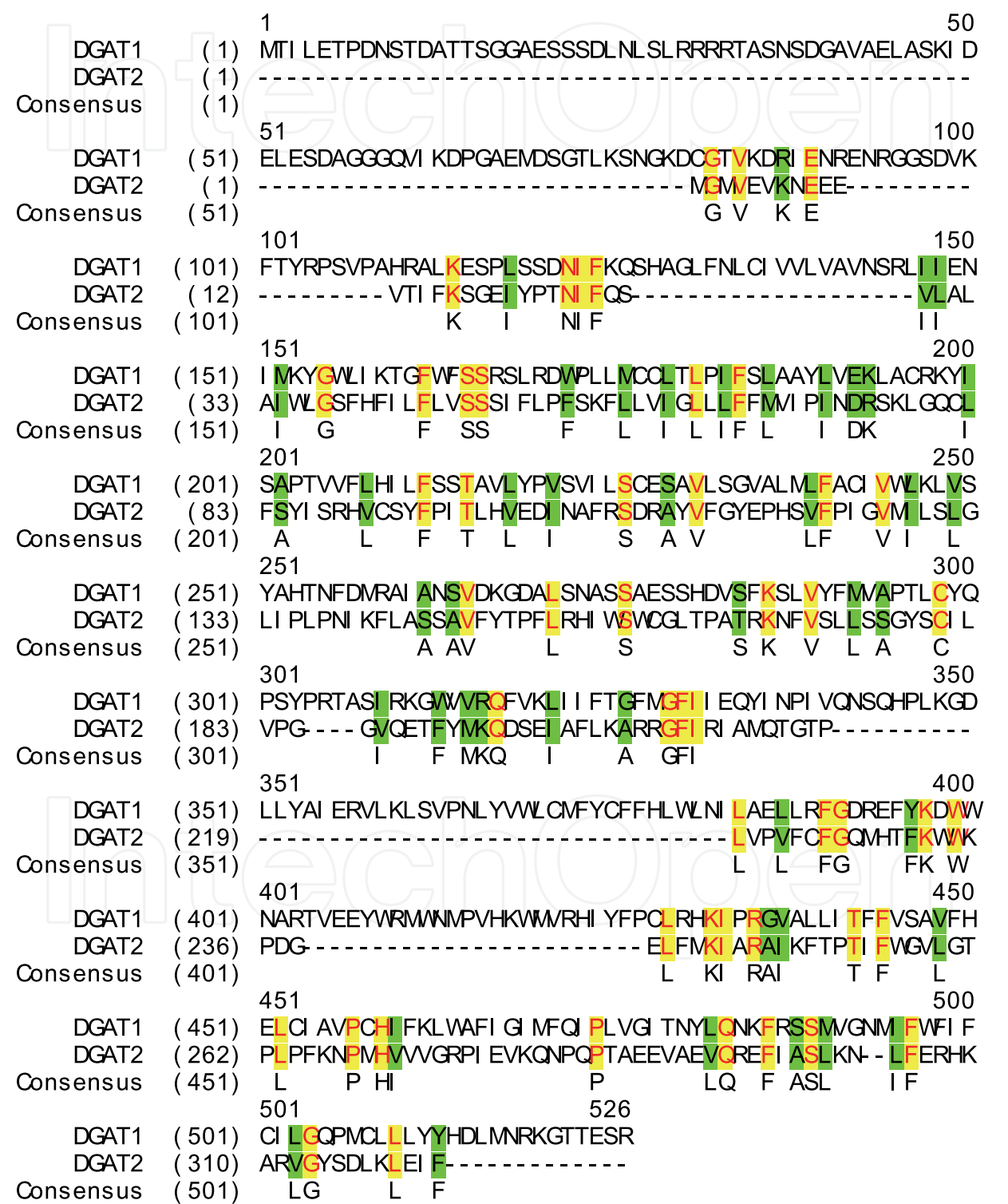


Fig. 1. Alignment of tung tree DGAT1 and DGAT2 amino acid sequences.

reported previously (Cao et al., 2003). Plasmids pMBP-DGAT1-His (Figure 3) and pMBP-DGAT2-His (Figure 4) were constructed by replacing the hTTP fragment in plasmid pMBP-hTTP (Figure 2) with the PCR-amplified DGAT1 and DGAT2 fragments at the *KpnI* and *SpeI* sites (Table 4). Existing DGAT plasmid DNAs were used as the templates for PCR-amplification of the DGAT DNA open reading frames (Shockey et al., 2006). DGAT forward primers contained DNA sequence for a *KpnI*/*Asp718I* restriction enzyme recognition site followed by a PreScission protease cleavage site (5'-CTGTTTCAGGGTCCG-3') (Cao et al., 2003) which codes for 5 amino acid residues (LFQGP) between MBP and DGAT protein sequences (Table 4). DGAT reverse primers contained sequence for a His-tag (5'-ATGATGATGATGATGATG-3') coding for 6 histidine residues at the carboxyl terminus of DGATs (Table 4).

The successful expression of full-length recombinant DGATs was probably due to the fusion to MBP, which was shown to increase the solubility of target proteins such as human and mouse TTP (Cao et al., 2003; Cao et al., 2008; Kapust & Waugh 1999). Although we engineered double affinity tags for facilitating purification of recombinant DGAT from *E. coli*, recombinant DGATs were only partially purified from the extract by either type of affinity beads [amylose resin and nickel-nitrilotriacetic agarose (Ni-NTA) beads] or both kinds of beads in tandem. Our data, together with the various published reports cited in the previous section, underline the tremendous challenges that exist for the purification of recombinant full-length DGAT proteins.

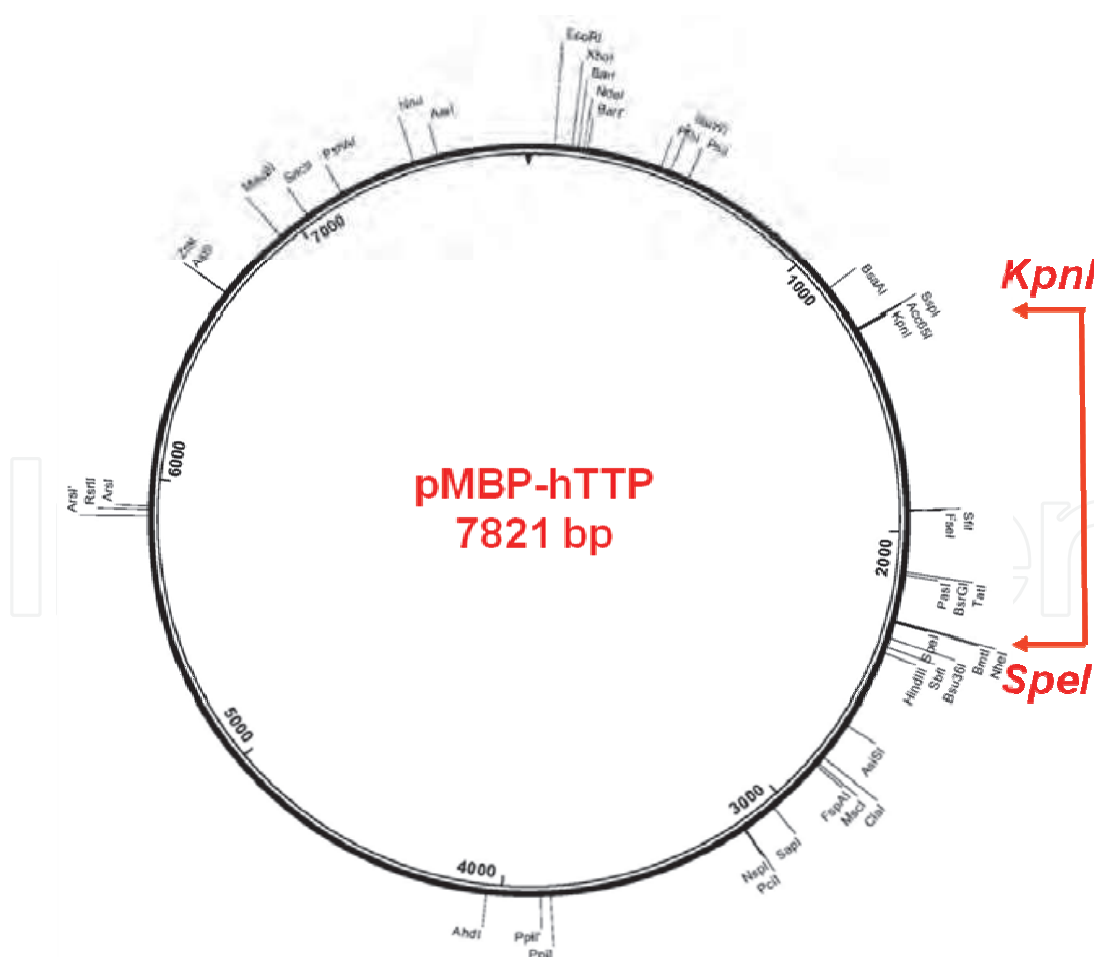


Fig. 2. Plasmid map of *E. coli* expression vector pMBP-hTTP.

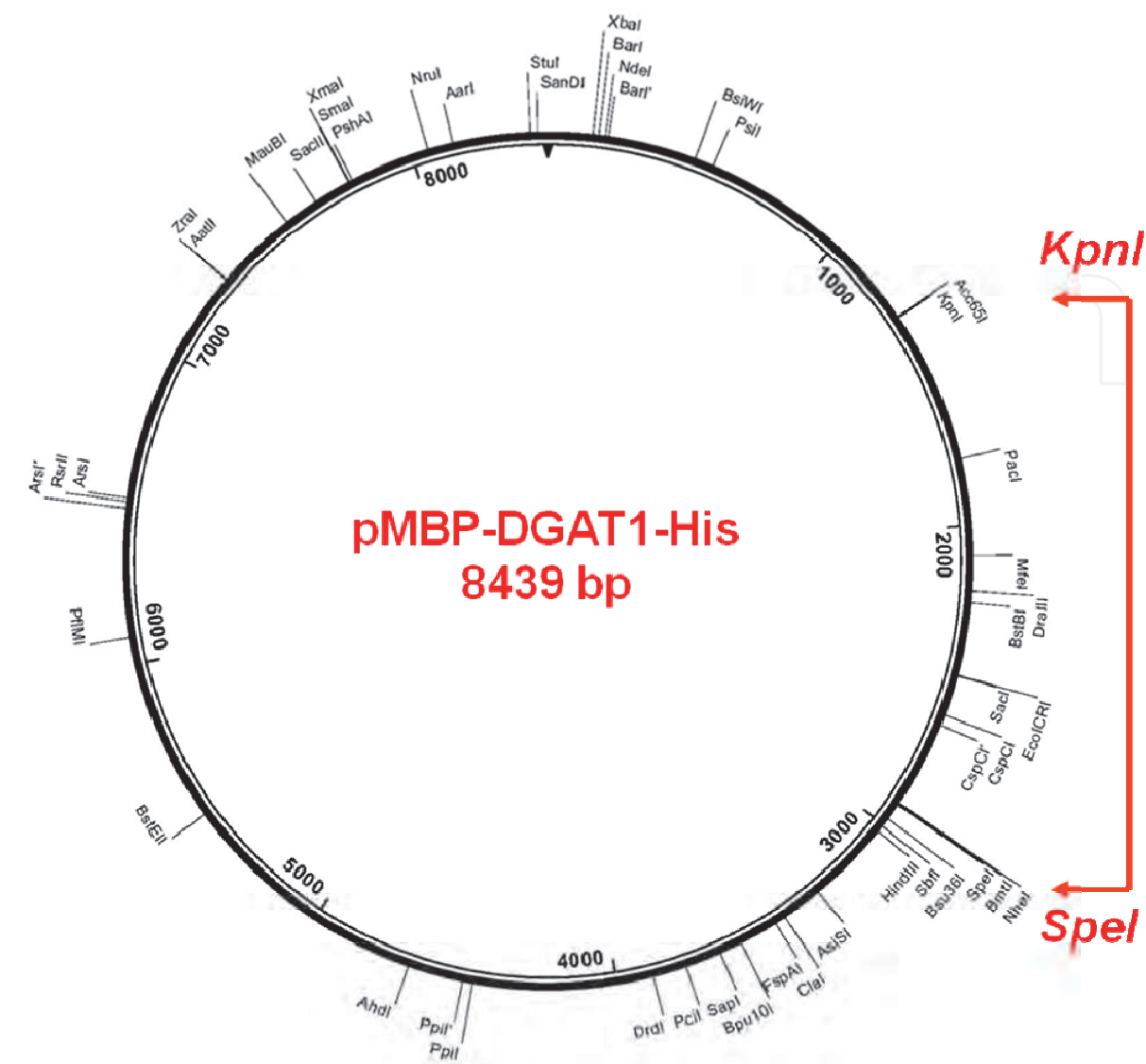


Fig. 3. Plasmid map of *E. coli* expression vector pMBP-DGAT1-His.

3. Conclusion

Diacylglycerol acyltransferases (DGATs) catalyze the last and rate-limiting step of triacylglycerol (TAG) biosynthesis in eukaryotic organisms. At least 115 DGAT sequences are identified from 69 organisms in the GenBank databases. Only a few papers have been published in the last 28 years on the expression of the recombinant DGAT proteins in a bacterial expression system. None of the full-length DGAT1 or DGAT2 had been expressed in *E. coli* expression system. The difficulties in DGAT expression and purification are due to the nature of these proteins being integral membrane proteins with more than 40% of the total amino acid residues being hydrophobic. Therefore, progress in characterization of the enzymes has been slow. We recently developed a procedure for full-length DGAT expression in *E. coli*. Expression plasmids were engineered to express tung DGATs fused to maltose binding protein and poly-histidine. The development of the technique should help to purify full-length DGATs for further studies such as raising high-titer antibodies and studying the structure-function relationship. Understanding the roles of DGATs in plant oil

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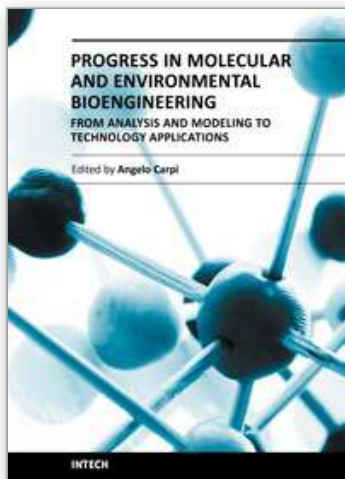
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Progress in Molecular and Environmental Bioengineering - From Analysis and Modeling to Technology Applications

Edited by Prof. Angelo Carpi

ISBN 978-953-307-268-5

Hard cover, 646 pages

Publisher InTech

Published online 01, August, 2011

Published in print edition August, 2011

This book provides an example of the successful and rapid expansion of bioengineering within the world of the science. It includes a core of studies on bioengineering technology applications so important that their progress is expected to improve both human health and ecosystem. These studies provide an important update on technology and achievements in molecular and cellular engineering as well as in the relatively new field of environmental bioengineering. The book will hopefully attract the interest of not only the bioengineers, researchers or professionals, but also of everyone who appreciates life and environmental sciences.

How to reference

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Heping Cao (2011). Bioengineering Recombinant Diacylglycerol Acyltransferases, Progress in Molecular and Environmental Bioengineering - From Analysis and Modeling to Technology Applications, Prof. Angelo Carpi (Ed.), ISBN: 978-953-307-268-5, InTech, Available from: <http://www.intechopen.com/books/progress-in-molecular-and-environmental-bioengineering-from-analysis-and-modeling-to-technology-applications/bioengineering-recombinant-diacylglycerol-acyltransferases>

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