Cytosolic Triacylglycerol Biosynthetic Pathway in Oilseeds. Molecular Cloning and Expression of Peanut Cytosolic Diacylglycerol Aeryltransferase1[W]  

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Triacylglycerols (TAGs) are the most important storage form of energy for eukaryotic cells. TAG biosynthetic activity was identified in the cytosolic fraction of developing peanut (Arachis hypogaea) cotyledons. This activity was NaF insensitive and acyl-coenzyme A (CoA) dependent. Acyl-CoA:diacylglycerol acyltransferase (DGAT) catalyzes the final step in TAG biosynthesis that acylates diacylglycerol to TAG. Soluble DGAT was identified from immature peanuts and purified by conventional column chromatographic procedures. The enzyme has a molecular mass of 41 ± 1.0 kDa. Based on the partial peptide sequence, a degenerate probe was used to obtain the full-length cDNA. The isolated gene shared less than 10% identity with the previously identified DGAT1 and 2 families, but has 13% identity with the bacterial bifunctional wax ester/ DGAT. To differentiate the unrelated families, we designate the peanut gene as AhDGAT. Expression of peanut cDNA in Escherichia coli resulted in the formation of labeled TAG and wax ester from [14C]acetate. The recombinant E. coli showed high levels of DGAT activity but no wax ester synthase activity. TAGs were localized in transformed cells with Nile blue A and oil red O staining. The recombinant and native DGAT was specific for 1,2-diacylglycerol and did not utilize hexadecanol, glycerol-3-phosphate, monoacylglycerol, lysophosphatic acid, and lysophosphatidylcholine. Oleoyl-CoA was the preferred acyl donor as compared to palmitoyl- and stearoyl-CoAs. These data suggest that the cytosol is one of the sites for TAG biosynthesis in oilseeds. The identified pathway may present opportunities of bioengineering oil-yielding plants for increased oil production.

Oils and fats are glycerol triesters of fatty acids (triacylglycerols [TAGs]) and are mainly derived from plant and animal sources, respectively. Vegetable oils are the major source of edible lipids, accounting for more than 75% of the total lipids consumed across the world (Brown et al., 1999). The global demand for plant oils has intensified our efforts to genetically modify the organism to enhance oil yield.

De novo biosynthesis of TAG has been shown to occur by the sequential acylation of glycerol-3-P (Kennedy, 1961; Ohlrogge et al., 1991). The first enzyme in this pathway, glycerol-3-P acyltransferase, catalyzes the formation of lysophosphatidic acid (LPA) that can be acylated to give phosphatidic acid (PA) by LPA acyltransferase. PA is the precursor for diacylglycerol (DAG) and anionic phospholipids. PA phosphatase catalyzes the dephosphorylation of PA to form DAG, which is an immediate precursor for TAG, phosphatidylcholine, and phosphatidylethanolamine.

The possible routes of TAG formation are (1) acyl-CoA-dependent acylation of DAG by DAG acyltransferases (DGATs), (2) acyl-CoA-independent transacylation using two molecules of DAG by DAG transacylase (Stobart et al., 1997), and (3) acyl-CoA-independent acylation using phospholipids as acyl donors and DAG as an acyl acceptor catalyzed by phospholipid:DGAT (Dahlqvist et al., 2000).

DGAT1 encoding DGAT (EC 2.3.1.20) was identified in mice (Cases et al., 1998), and the gene is homologous to acyl-CoA:cholesterol acyltransferase (ACAT). The isolated gene shared significant sequence homology with an Arabidopsis (Arabidopsis thaliana) expressed sequence tag (EST), and the sequence information was used to isolate Arabidopsis (Hobbs et al., 1999; Zou et al., 1999; Bouvier-Nave et al., 2000) and rapeseed (Brassica napus) DGAT1 (Nykvforuk et al., 2002). ACAT-unrelated DGAT2 gene has been isolated from Saccharomyces cerevisiae (Oelkers et al., 2000) and Mortierella ramanniana (Lardizabal et al., 2001). Recently, a novel bifunctional DGAT/wax ester synthase was described in Acinetobacter calcoaceticus (Kalscheuer and Steinbüchel, 2003). All the acyltransferases in these pathways are membrane bound.

Here, we describe the identification and molecular cloning of a cytosolic DGAT from developing peanut (Arachis hypogaea) cotyledons. The gene encoding soluble DGAT was expressed in Escherichia coli and characterized. This study provides evidence for the presence of an additional TAG biosynthetic pathway in plants.

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RESULTS

Identification of a Cytosolic TAG Biosynthetic Pathway in Developing Peanut Cotyledons

Subcellular distribution of DGAT in fresh cotyledons was obtained by differential centrifugation. The TAG biosynthetic capacity was found to be high (70%) in membrane fraction, and 31% of the total activity was found in cytosolic fraction (Fig. 1A). The effect of [14C]oleoyl-CoA concentration on the biosynthesis of TAG was studied in cytosolic and membrane fractions. The maximum amount of TAG formation was observed with 10 µM oleoyl-CoA in both the fractions (Fig. 1B). Addition of 1,2-DAG in cytosolic fraction did not alter the rate and the pattern of incorporation of [14C]oleoyl-CoA into TAG.

TAG can be synthesized either by dephosphorylation of PA followed by acylation of DAG or by the successive acylation of monoacylglycerol (MAG). To find out the contribution of each pathway to the total TAG pool, peanut cytosolic fraction was treated with 20 mM NaF to inhibit PA phosphatase activity, and the incorporation of [14C]oleoyl-CoA into TAG and its biosynthetic intermediates was studied in the presence of NaF. As shown in Figure 1C, there was about 28% to 34% decrease in the incorporation of [14C]oleoyl-CoA into TAG, suggesting the presence of a PA dephosphorylation-independent pathway. To rule out the hydrolysis as well as transacylation of fatty acids either from TAG or from phosphatidylcholine, the cytosolic fraction was incubated either with [3H]phosphatidylcholine or with [3H]TAG followed by separation of lipids by thin-layer chromatography (TLC). There was no formation of labeled mono-, di-, and triacylglycerols from labeled phosphatidylcholine and there was no mono- and diacylglycerol formed from labeled TAG, indicating the absence of transacylation and hydrolysis reactions in the cytosol (data not shown).

Purification of Cytosolic DGAT from Developing Peanut Cotyledons

A summary of purification of DGAT is presented in Table I. Solid ammonium sulfate was added to bring cytosolic fraction to 1 M and then loaded onto an octyl-Sepharose column. The column was eluted with 1 to 0 M linear-reversed gradient of ammonium sulfate. This was an efficient step resulting in a 62-fold purification of DGAT. The active fractions from the octyl-Sepharose were loaded onto a blue-Sepharose column and eluted with a linear NaCl gradient. The activity was eluted between 0.35 and 0.4 M NaCl. The recovery of DGAT activity from the blue-Sepharose column was nearly 48% of that applied. The pooled active fractions were applied to a preparative Superdex 75 column. The DGAT activity was eluted between 27 to 31 fractions. The active fractions were pooled and applied to a heparin-agarose column as the final step. As summarized in Table I, an overall purification of 1,724-fold was obtained, and the specific activity of acyltransferase was 7.1 nmol min⁻¹ mg⁻¹.

Figure 1. Generation of TAG. A, Fresh developing peanut cotyledons (50 g) were used for obtaining various subcellular fractions by differential centrifugation. TAG formation was monitored using [14C]oleoyl-CoA in the absence of exogenously added acyl acceptors. Values are mean ± SD of three independent determinations. Homo, Homogenate; Plas, plastidal fraction; Mito, mitochondrial fraction; Cyto, cytosolic fraction; Memb, membrane fraction. B, Acylation of [14C]oleoyl-CoA into TAG was carried out for 15 min at 30°C in the presence of 50 µM 1,2-DAG in cytosolic (28 to 36 µg; ●) and in membrane (40 to 45 µg; ○) fractions of developing peanut cotyledons. Each point is the average of two independent experiments. C, Time-dependent formation of TAG. Incorporation of [14C]oleoyl-CoA was performed with cytosolic fraction of developing peanut cotyledon into TAG in the absence of added acyl acceptors. The formation of TAG was monitored either in the presence (○) or in the absence (●) of 20 mM NaF. Each point is mean ± SD of four independent determinations.
The preparation from each step of purification was resolved on a 12% (w/v) SDS-PAGE, which showed two major proteins (41 and 37 kD) upon silver staining (Fig. 2A). The final preparation was unstable. Despite our effort, we could not purify the enzyme to homogeneity. We therefore electroblotted the final enzyme preparation onto a nitrocellulose membrane and performed immunoblot analysis with peptide antibodies raised against the oleaginous yeast cytosolic DGAT. The immunoblot revealed a strong cross-reactivity with only 41.6 kD protein (Fig. 2B). It is possible that the internal amino acid sequence of *Rhodotorula glutinis* DGAT (Gangar et al., 2001) may have a significant similarity with soluble peanut DGAT, and this could be the reason for peptide antibody cross-reactivity.

The reaction products formed at each step of purification were analyzed on silica-TLC and autoradiographed (Fig. 2C). When the cytosol was incubated with labeled oleoyl-CoA in the presence of DAG, TAG and other biosynthetic intermediates were formed, suggesting the presence of many different acylation activities.

After electrophoresis proteins were electroblotted onto a polyvinylidene difluoride membrane; the poly-peptide corresponding to molecular size of 41 kD was excised and treated with cyanogen bromide to cleave Met residues. The major peptide was taken for sequencing and the sequence (NH$_2$-GNKCKKSGLALLQE-FERVVGA) did not match with any known sequence in the database. This was suitable for designing a degenerate primer for gene isolation.

### Cloning of a Gene Encoding DGAT

To obtain the full-length cDNA clone encoding putative DGAT, a seed-specific cDNA library was screened with the oligonucleotide primer 5'-GGCAACAAGTGCAAGAAAGCGGAAGTATCGCTTTATTGCAGGATTCGAGAGGGTCGTGGGGGCC-3' (66-mer), based on the internal amino acid sequence of the protein (KCKKSGLALLQEFERVVGA). The nucleotide probe was radiolabeled at the 3' end using TdT and [$\gamma$-32P]ATP. A large number of positive clones were isolated from the screen, and the plasmid from clones containing the longest inserts (approximately 1.6 kb) was sequenced on both strands using an automated sequencer. The sequence (1,592 bp) was submitted to GenBank with the identification as *AhDGAT* (accession no. AY875644). Analysis of the nucleotide sequence revealed an open reading frame of 345 amino acids with translation initiating at the $^{360}$ATG$^{362}$ codon (Supplemental Fig. 1).

### Table 1. Purification of cytosolic DGAT from developing peanut cotyledons

The results are the summary of a typical purification of DGAT. Frozen immature peanut cotyledons (100 g) were used for preparing the cytosol. Enzyme activity measurements and the purification steps are described in “Materials and Methods.”

<table>
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<th>Fraction</th>
<th>Protein nmol min$^{-1}$</th>
<th>Total Activity pmol min$^{-1}$</th>
<th>Specific Activity pmol min$^{-1}$ mg$^{-1}$</th>
<th>Purification Fold</th>
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<td>7,069.5</td>
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</table>

Figure 2. Purification of cytosolic DGAT from developing peanut cotyledons. A, SDS-PAGE protein profile. Samples from each purification step were separated on a 12% (w/v) SDS-PAGE and stained with silver nitrate. Lane 1 is standard molecular mass marker, and lanes 2 to 6 correspond to the pooled active fractions from steps 1 to 5 (Table I). B, Western-blot analysis. Samples from each step of purification were separated on 12% (w/v) SDS-PAGE and electroblotted onto a nitrocellulose membrane, followed by probing with anti-DGAT antibodies from *R. glutinis*. Lane 1, Prestained protein molecular mass marker. Lanes 2 to 6 correspond to the pooled active fractions from steps 1 to 5 (Table I). C, Autoradiography of the reaction products formed at each step of the purification. DGAT assays were performed using [14C]oleoyl-CoA and DAG for 15 min. The products formed were separated on a TLC using petroleum ether:diethyl ether:acetic acid (70:30:1, v/v). Lanes 1 to 5 correspond to the active fractions from steps 1 to 5 of purification Table I.
The 239-bp 3′-untranslated region contained a noncanonical polyadenylation signal (AATAT) near the poly(A) tail, indicating that the isolated clone represented full-length cDNA. The calculated molecular mass of the open reading frame was 37.5 kD. When the sequence of internal amino acid was aligned with the deduced amino acid sequence, there was a complete match, confirming that the cloned cDNA encoded DGAT. Hydropathy plot of the predicted protein indicated the absence of any transmembrane domains (Fig. 3A). Absence of signal sequence also confirms its cytosolic nature.

Figure 3. DGAT gene sequence analyses. A, Hydropathy plot of cytosolic peanut DGAT. No transmembrane domain was predicted. B, Alignment of AhDGAT and homolog genes. Sequence alignment was performed using the http://www.ch.embnet.org/software/BOX_form.html program. AhDGAT, peanut DGAT (AY875644); G. max, soybean (BM187962); nucleotide sequence was retrieved from the National Center for Biotechnology Information (NCBI) and then translated; longest reading frame was selected for alignment; At1g48300, Arabidopsis 1g48300 (AAD49767); O. sativa, rice (XP_475575). Conserved amino acids are shaded black.
Figure 4. Conserved acyltransferase domains across acyltransferase families and phylogenetic analysis. Sequence alignment of peanut DGAT (AY875644) and other known acyltransferase family members was performed using the http://www.ch.embnet.org/software/BOX_form.html sequence alignment program. Identical amino acid residues are highlighted in black. Conserved acyltransferase domains are underlined. Catalytic site conserved residues are marked with stars. A, Alignment of the catalytic domains of members of the acyltransferase family. Sequences aligned are designated as follows: E. coli GPAT and LPEAT, EcGPAT and EcLPEAT; Mus musculus GPAT and LPAAT, MmGPAT and MmLPAAT; S. cerevisiae GPAT1, GPAT2, and LPAAT are ScGAT1, ScGAT2, and ScLPAAT; Homo sapiens LPAAT1 to 6 and DHAPT are HsLPAAT1 to 6 and HsDHAPT; Arabidopsis GPAT, AtGPAT; A. calcoaceticus wax synthase/DGAT, AcWS/DGAT; and peanut DGAT, AhDGAT. B, Similar alignment of catalytic domain of
BLAST analyses have identified likely orthologs in Arabidopsis, rice (*Oryza sativa*), and soybean (*Glycine max*), and closely related genes are represented in EST collections from diverse plant species. Soybean showed the identity of 50% (63% similarity) to peanut DGAT. Similarly, rice showed 44% identity (53% similarity) and Arabidopsis showed 33% identity (similarity 48%) to peanut DGAT (Fig. 3B). Therefore, the isolated DGAT gene is unique and might constitute a different family of cytosolic DGAT.

When the deduced amino acid sequence of 38 kD was examined for a number of structural motifs, we identified potential DGAT motifs at 54HVQYYGD (Fig. 4A) and 205HHNAVELFRRNND (Fig. 4B), and a partially conserved DGAT active site at 81KKRVLFDDL (Fig. 4C), which matched with reported members of the acyltransferase family. Insertion of a few amino acids between critical His and Asp residues has also been reported in *Mycobacterium tuberculosis* bifunctional wax ester synthase/DGAT.
(Daniel et al., 2004). In addition, Tyr kinase phosphorylation site (PCDOC0007) KETMIL showed 100% identity. There are several protein kinase C and casein kinase 2 phosphorylation sites present in the protein. Phosphopantetheine attachment site (PCDOC0012) displayed 75% similarity between residues RGGDSCVTVPVRMRK. Thiolase acyl enzyme intermediate signature (PCDOC0092) displayed 68% similarity between residues TNPDCESSSSSSSESES. An invariant Pro is present in the hydrophobic block at position 34 in peanut DGAT between a putative phosphopantetheine attachment site and a thiolase acyl enzyme intermediate signature and therefore may be responsible for presenting the fatty acyl group to the active site for esterification to the glycerol backbone. Fatty-acid binding protein signature pattern (PCDOC00188) displayed 64% similarity between residues KSGSIALLQEFERVVGAEG.

An alignment with known acyltransferases confirmed the presence of several acyltransferase motifs, including the DGAT catalytic motif present in cytosolic peanut DGAT. In addition, we used the ClustalW algorithm to obtain an optimized multiple sequence alignment with known DGAT1 and 2 family members and found the identity to be <10% (<30% similarity) and 13% (53% similarity), respectively, with the bacterial bifunctional wax ester synthase/DGAT. Therefore, the isolated DGAT gene is unique and might constitute a different family of cytosolic DGAT. Dendrogram representing the phylogenetic relationship of AhDGAT with DGAT from many different organisms across the species was performed. The analysis revealed that AhDGAT is closely related to bacterial bifunctional DGAT/wax ester synthase (Fig. 4D).

**Expression of AhDGAT in E. coli**

A pBluescript SK- vector containing the peanut DGAT full length (345 amino acids) was subcloned in pRSET A by BamHI and HindIII digestion. The transformed E. coli BL21 (DE3) cells were induced with 0.5 mM isopropyl-1-thio-D-galactopyranoside (IPTG), and the induced cells were used for preparing cell extracts for enzyme activity and the intact cells used for [14C]acetate labeling. Studies on the incorporation of [14C]acetate into lipids of transformed E. coli showed a time-dependent increase in the incorporation of label into TAG and wax ester as compared to vector-transformed cells (Fig. 5A). In vitro DGAT assay with AhDGAT-transformed cell lysates showed a time-dependent increase in incorporation of [1-14C]oleoyl-CoA into TAG (Fig. 5B). Immunoblot analysis of the transformed cells with anti-His-tag (Fig. 5C) and peptide anti-DGAT (Fig. 5D) antibodies showed an intense signal at around 42 kD, but no signal was detected in the vector-transformed cells. The internal amino acid sequence of R. glutinis (Gangar et al., 2001) was aligned with the deduced amino acid sequence of AhDGAT gene revealed a significant homology (Fig. 5E), which could be the reason for antibody cross-reactivity.

Total lipids extracted from E. coli cells expressing AhDGAT gene were separated by silica-gel column, and the purified TAG and wax ester were subjected to structural analysis using 1H-NMR and infrared spectroscopy. These analyses confirmed the molecular identity of TAG (data not shown) and wax ester (Supplemental Fig. 2). Cells were viewed under confocal microscope after staining with Nile blue A and oil red O. Localization studies revealed that cells harboring AhDGAT gene showed intense Nile blue A and oil red O.  

**Figure 6.** Purification of recombinant AhDGAT. A, SDS-PAGE profile of recombinant DGAT purification. Samples from each fraction were separated by 12% (w/v) SDS-PAGE and stained with Coomassie Blue R-250. Lane 1 represents vector IPTG uninduced; lane 2, vector IPTG induced; lane 3, molecular mass marker; lane 4, AhDGAT IPTG uninduced; and lane 5, AhDGAT IPTG induced. Lanes 6 to 7 correspond to 250 mM imidazole eluted fractions from Ni-nitrilotriacetic acid agarose chromatography. B, Time course of DGAT activity using purified recombinant enzyme. Lane 1 represents no-enzyme control. Lanes 2 to 6 represent 0, 5, 10, 15, and 30 min, respectively.
O staining as compared to vector-transformed cells (Supplemental Fig. 3).

The full-length AhDGAT cDNA was subcloned into pRSET A and expressed in E. coli BL21 (DE3) cells as an N-terminal fusion protein of oligo-His. The His-6-AhDGAT was purified by a nickel (Ni)-affinity column and shown to have a molecular mass of 42 kD (Fig. 6A). Recombinant DGAT is of a higher molecular mass as compared to the calculated molecular mass, and this could be due to the insertion of a few amino acids at the N terminus from the parent vector.

Characterization of Recombinant AhDGAT

To determine the acylation kinetics, the purified recombinant acyltransferase was incubated with DAG and labeled oleoyl-CoA for various time intervals, and maximum acylation was observed in 30 min (Fig. 6B). The recombinant enzyme was specific for DAG and did not utilize hexadecanol, MAG, LPA, and lysophosphatidylcholine. Even though the isolated gene has 13% identity with the bacterial bifunctional wax ester synthase/DGAT, we were unable to demonstrate this activity under the standard assay conditions. This could be due to the inactivation of enzyme activity during isolation procedure. Effect of 1,2-DAG on the DGAT activity was studied using oleoyl-CoA as an acyl donor (Fig. 7A). A concentration-dependent increase in DGAT activity was observed, and the highest activity was found at 30 μM DAG. Further increase in DAG concentration led to a significant reduction in activity. Native and the recombinant AhDGAT showed the highest activity with oleoyl-CoA as compared to palmitoyl- and stearoyl-CoAs (Fig. 7B).

Tissue-Specific Expression of AhDGAT in Peanut

We examined the expression of AhDGAT with Actin2 as a positive control by reverse transcription (RT)-PCR as shown in Figure 8. AhDGAT mRNA was detected only in immature seeds between 8 to 14 d after flowering (DAF) and 15 to 24 DAF. At the third stage of seed development (between 25 and 30 DAF), the transcript was barely detectable and no transcript was detected in late stage of seed development, leaf, and root (Fig. 8A).

Western-blot analysis was performed using AhDGAT antibodies with soluble extracts from various developmental stages and tissues of peanut. The immunoblot indicated that AhDGAT protein (approximately 41 kD) was detected in developing seed but not in other tissues examined (Fig. 8B).
DISCUSSION

The biosynthesis of TAG is shown to occur in microsomal membranes (Ohlrogge et al., 1991; Hobbs et al., 1999; Zou et al., 1999; Bouvier-Nave et al., 2000; Oelkers et al., 2000; Lardizabal et al., 2001; Nykiforuk et al., 2002; Kalscheuer and Steinbuchel, 2003). We report here the presence of TAG biosynthetic machinery in cytosol of developing peanut cotyledons. The existence of cytosolic enzymes that provide important precursors for TAG biosynthesis has been well documented in plant systems. Soluble glycerol-3-P acyltransferase has been identified and purified from several sources (Murata and Tasaka, 1997). In developing rapeseed, the formation of PA has been demonstrated in cytosolic fraction (Murphy, 1988), and it has been proposed that the cytosolic PA phosphatase could be metabolically inactive (Ichihara et al., 1988). Cytosolic LPA phosphatase (Shekar et al., 2002) and MAG acyltransferase (Tumaney et al., 2001) have been reported in developing peanut cotyledons. Several independent lines of evidence indicate that the identified DGAT is cytosolic in nature, and this is based primarily on the following observations. (1) The activity is associated with 150,000 g supernatant. (2) The enzyme is purified by successive column chromatographic separations without detergent. (3) The isolated enzyme (AhDGAT) has neither membrane spanning region nor signal sequence (GenBank accession no. AY875644).

Our results demonstrate that the isolated cDNA encodes a DGAT. (1) Although E. coli cells do not synthesize TAG, AhDGAT-transformed cells showed significant amounts of TAG and wax ester accumulation as compared to vector-transformed cells. (2) E. coli cells expressing AhDGAT gene were capable of incorporating radiolabeled acetate into TAG and wax ester. (3) The expressed recombinant protein cross-reacted with R. glutinis peptide DGAT polyclonal antibodies and the same protein also cross-reacted with monoclonal antibody against His-tag. (4) A higher level of DGAT activity was obtained from cells expressing the isolated cDNA than from cell-free extract from vector-transformed E. coli cells. The acyltransferase activity depended on the presence of oleoyl-CoA and was specific for DAG. We propose that the isolated enzyme could be involved in yet another TAG and wax ester biosynthetic pathway in plants.

Four independent gene families (DGAT1, DGAT2, PDAT, and bifunctional WS/DGAT) have been confirmed the ability to synthesize TAG. Recent reports suggest, apart from unspecific bifunctional WS/DGAT (Uthoff et al., 2005), that DGAT1 has wax ester synthase and acyl CoA:retinol acyltransferase activities when expressed in COS 7 cells (Yen et al., 2005). It has been speculated that these gene families may play different roles across species, in a tissue- and time-dependent manner during development (Cases et al., 1998; Hobbs et al., 1999; Zou et al., 1999; Bouvier-Nave et al., 2000; Dahlqvist et al., 2000; Oelkers et al., 2000; Lardizabal et al., 2001; Nykiforuk et al., 2002; Kalscheuer and Steinbuchel, 2003). Therefore, the isolated AhDGAT may be involved in a unique pathway of TAG and wax ester synthesis. Further studies are required to understand the roles of various gene families in various organisms.

DGAT1 mRNA from Arabidopsis was present at high levels in many different tissues, including germinating seeds, young seedlings, roots, and leaves. However, our results from peanut indicated that AhDGAT mRNA is detected only in immature seeds. During embryo development, AhDGAT protein is present at 8 DAF, increases dramatically at 15 to 24 DAF, and then gradually declines in the mature seeds. A recent microarray study of tissue-specific Arabidopsis ESTs has determined that Arabidopsis homolog of AhDGAT is highly (>2-fold) expressed in the early stages of seed development (Yamada et al., 2003). Arabidopsis homolog (AtH48300) is highly expressed in cork, xylem, hypocotyl, and senescent leaf, and moderately expressed in pollen and stem (Supplemental Table I). DGAT homolog is shown to be ubiquitously expressed in various developmental stages, and very highly expressed in leaf and siliques (Zimmermann et al., 2004). Therefore, it is likely that AhDGAT and AtDGAT expression is regulated in a tissue-specific and time-dependent manner. Hence, the identification of AhDGAT and AtDGAT has significant implications in understanding the regulation of TAG biosynthesis in plants.

In addition to the membrane-bound pathway for TAG synthesis, an alternate pathway was proposed that involved the formation of MAG from LPA by the enzyme LPA phosphatase (Shekar et al., 2002), which in turn gets converted to DAG by MAG acyltransferase (Tumaney et al., 2001). The further acylation of DAG to TAG involves another enzyme, DGAT, thus completing the biosynthesis of TAG in oilseeds. Genes involved in this pathway could find application in improving the quantity of vegetable oils.

MATERIALS AND METHODS

Materials

[1-14C]oleoyl-CoA (54 mCi mmol⁻¹), [1-13C]acetate (2 mCi mmol⁻¹), [9,10-3H(N)]triolaoylglycerol (10 Ci mmol⁻¹), [1-14C]acetate (2 mCi mmol⁻¹), [9,10-3H(N)]triolaoylglycerol (10 Ci mmol⁻¹), [2-palmitoyl-9,10-3H]phosphatidylcholine (92.3 Ci mmol⁻¹), and [γ-32P]ATP (3,000 Ci mmol⁻¹) were obtained from Perkin Elmer Biosystems. Superdex 75 (26/60 prep grade) FPLC column, octyl-Sepharose, blue-Sepharose, heparin-agarose, and gel-filtration molecular mass standards were purchased from Amersham. Silica-TLC plates were from Merck. Hexadecanol, 1,2-dipalmitoyl-sn-glycerol (1,2-DAG), 1,3-dipalmitoyl-sn-glycerol (1,3-DAG), Nile blue A, oil red O, and other chemicals were from Sigma-Aldrich. Field-grown developing peanut (Arachis hypogaea; Jl. 24) cotyledons were harvested at 20 to 24 DAF and used either fresh or stored at –80°C until further use.

Subcellular Fractionation

Frozen immature seeds (50 g) were ground in a prechilled mortar and pestle with 5 g of acid-washed sand and 125 mL of extraction buffer containing 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10 mM KCl, 1 mM MgCl₂, 1 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg mL⁻¹.
leupeptin, and 0.25 mM Suc. The extract was passed through two layers of cheesecloth and the filtrate was differentially centrifuged to fractionate intracellular components. The filtrate was centrifuged at 3,000 × g for 10 min and the supernatant was centrifuged at 18,000 × g for 15 min. The 18,000 × g supernatant was further centrifuged at 150,000 × g for 1.5 h. The pellet thus obtained was resuspended in a small volume of buffer containing 20 mM Tris-HCl, pH 7.5, and 1 mM β-mercaptoethanol. Cytosol (150,000 × g supernatant, soluble fraction) was used as the enzyme source. All the operations were performed at 4°C. Protein concentrations were determined by the bicinchoninic acid method (Smith et al., 1985) using bovine serum albumin (BSA) as the standard.

**DGAT Assay**

The assay mixtures consisted of 50 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, 10 μM [1-14C]oleoyl-CoA (2 × 10⁶ cpm), 15 to 40 μg of protein, and 30 μM sonicated suspension of 1,2-DAG in a total volume of 100 μL. The incubation was carried out at 30°C for 15 min and stopped by the addition of 400 μL of CHCl₃:CH₃OH (1:2, v/v). Lipid extraction was carried out and chloroform-soluble material was separated by silica-TLC using petroleum ether:diethyl ether:acetic acid (70:30:1, v/v) as a solvent system. The lipids were visualized with iodine vapor and identified by their migration with standards. The spots of TAG were scraped off from TLC for determination of radioactivity by liquid scintillation counting. Control incubations were carried out for zero time, in the absence of enzyme and heat-inactivated enzyme source. The control values were subtracted from the actual assay value, and enzyme activity was calculated after correction. Wax ester synthase activity was monitored under the standard DGAT assay conditions. Acyl acceptor in this assay was hexadecanol instead of DAG.

**Purification of DGAT**

We previously reported the purification of MAG acyltransferase from cytosol (soluble fraction) of immature peanut (Tumaney et al., 2001). During purification, both MAG and DGAT activities coeluted, and the separation of these activities was achieved in heparin-agarose column. The DGAT activity was eluted at 0.25 M salt.

**Screening of a cDNA Library**

A seed-specific cDNA library was constructed in a-ZAP II (Rudrabhatla and Rajasekharan, 2002). The library was screened using oligonucleotide generated from the internal amino acid sequence of the purified peanut DGAT. A total of 2 × 10⁶ plaques were screened. Positive plaques were purified by three additional screenings with a homologous oligonucleotide probe.

**Sequence Analysis**

Plasmid DNA isolated from the positive clone was sequenced on both strands using the Biotech Taq cycle sequencing kit on an automated sequencer (Applied Biosystems 377). Sequence data were analyzed and compared using the following programs and software. Sequence analysis was performed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST). Multiple sequence alignment was done with ClustalW (http://www.ebi.ac.uk). Pedro’s Biomolecular Research Tool (http://www.public.iastate.edu/~pedro) and ExPASy Molecular Biology Server (http://www.expasy.org) were used for domain and motif identification. Protein hydropathy plot was generated by http://www.bio. davidson.edu/~courses/compbio/flc/home.html. The phylogenetic tree was generated using bootstrapping for 10,000 trees, using ClustalW version 1.82. The bootstrap tree was converted to figure format using Njplot (Perriere and Gouy, 1996).

**Expression of a His-6-AhDGAT Fusion Protein**

The cDNA spanning region of AhDGAT was subcloned into His-tagged fusion protein expression vector pRESET A (Invitrogen) by BamHI and HindIII digestion. The constructs were expressed in *Escherichia coli* BL21 (DE3). The fusion protein was induced with 0.5 mM IPTG for 12 h at 22°C and purified by Ni-nitrilotriacetic acid agarose chromatography (Qiagen). Expression was verified by immunoblotting with oleaginous yeast anti-DGAT and His-tag antibodies.

**Immunoblotting**

Proteins were separated by 12% (w/v) SDS-PAGE and transferred onto a nitrocellulose membrane by a semidry transfer apparatus. Upon transfer, the membrane was blocked with 0.5% (w/v) BSA in phosphate-buffered saline for 1 h. The peptide corresponding to *Rhodotorula glutinis* cytosolic DGAT was conjugated to BSA and antiserum was raised as described (Gangar et al., 2001). Primary antibodies were diluted (1:500, v/v) in phosphate-buffered saline containing 0.1% (w/v) BSA, added to the blocked membrane, and incubated for 1 h. The unbound antibodies were washed with phosphate-buffered saline containing 0.05% (v/v) Tween 20, and goat anti-rabbit IgG-alkaline phosphatase conjugate was added to the membrane and incubated for 45 min. The membrane was washed and the immunoreactive bands were visualized using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate.

**Nile Blue A and Oil Red O Staining**

A smear of *E. coli* cells was prepared on a glass slide and heat fixed. The slides were immersed in 1% (w/v) aqueous solution of Nile blue A stain for 10 min at 55°C. Excess stain was removed, air-dried, and visualized under confocal microscope (Gangar et al., 2001). Oil red O was dissolved to a stock solution by adding 500 mg oil red O to 100 mL of 60% (v/v) triethyl phosphate. For staining, 36% (v/v) triethyl phosphate working solution containing 12 mL of oil red O stock solution and 8 mL of water, was prepared, and cells were stained for 30 min and washed three times in water, air-dried, and visualized under confocal microscope on 100 × magnification.

**Incorporation of [1-14C]Acetate into TAG**

*E. coli* cells (10⁶ cells mL⁻¹) were incubated with [1⁴C]acetate (1.5 μCi mL⁻¹ Luria-Bertani medium) and grown for 2 h at 37°C. Cells were harvested by centrifugation, and the cell pellet was washed twice with ice-cold water. To the pellet (0.5 mL of 10% (v/v) acetic acid in isopropyl alcohol was added and boiled for 3 min. One milliliter of hexane was added to the mixture to extract lipids. Hexane layer was removed and concentrated. The extracted lipids were separated on a silica-TLC using petroleum ether:diethyl ether:acetic acid (70:30:1, v/v) as the solvent system. Lipids were identified by their migration with standards. The TLC plate was phosphorimaged, and individual spots were scraped off from the plate and the radioactivity was measured in a liquid scintillation counter.

**RNA Isolation and RT-PCR**

Total RNA was isolated from four different seed development stages based on DAF (stage 1, 8–15 DAF; stage 2, 16–24 DAF; stage 3, 25–30 DAF; stage 4, 30–35 DAF), leaf, and root from soil-grown wild-type plants using TRI reagent (Sigma) as described by the manufacturer. One microgram of RNA was reverse transcribed at 43°C using Moloney murine leukemia reverse transcriptase and the RevertAid H Minus first-strand cDNA synthesis kit (Fermentas GmbH) with oligo(dT) as primer according to the protocol provided by the supplier. The resulting cDNA was diluted 10-fold and 1 μL used as template for 30 cycles of PCR amplification using Taq DNA polymerase with the specific oligonucleotides for AhDGAT (forward 5'-AGGTTTCACGCGCCCGTCTC-3'; reverse 5'-TTCATAGTACTTCTCAGTTCTCAGTCCCAAG-3'). Amplification of the Actin2 control transcript was done using the primers Actin2-1 (5'-TCCCTCAATCTCATCTTCTTCC-3') and Actin2-1 (5'-GACCTGCTCTATCACTTTTCTTCCTTCC-3'). The PCR products were analyzed on ethidium bromide-stained agarose gels.

**Other Methods**

Samples were resuspended in CdCl₂, and NMR spectra of the purified lipid were recorded using the JEOL JNM-LA 300 FT NMR system. Infrared spectra were recorded on the JASCO FT/IR-410 system with neat samples. Samples to be analyzed were converted to fatty-acid methyl esters and purified by preparative TLC, and then analyzed under GC-EIMS using the VG AutoSpecM mass spectrometer equipped with HP 5890 series II gas chromatography fitted with a HP-5 capillary column (Tumaney and Rajasekharan, 1999).
Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number AY875644.

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LITERATURE CITED


