DGAT2 Is a New Diacylglycerol A cyltransferase Gene Family

PURIFICATION, CLONING, AND EXPRESSION IN INSECT CELLS OF TWO POLYPEPTIDES FROM MORTIERELLA RAMANNIANA WITH DIACYLGlycerol ACYLTRANSFERASE ACTIVITY*

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Acyl CoA:diacylglycerol acyltransferase (EC 2.3.1.20; DGAT) catalyzes the final step in the production of triacylglycerol. Two polypeptides, which co-purified with DGAT activity, were isolated from the lipid bodies of the oleaginous fungus Mortierella ramanniana with a procedure consisting of dye affinity, hydroxyapatite affinity, and heparin chromatography. The two enzymes had molecular masses of 36 and 36.5 kDa, as estimated by gel electrophoresis, and showed a broad activity maximum between pH 6 and 8. Based on partial peptide sequence information, polymerase chain reaction techniques were used to obtain full-length cDNA sequences encoding the purified proteins. Expression of the cDNAs in insect cells conferred high levels of DGAT activity on the membranes isolated from these cells. The two proteins share 54% homology with each other but are unrelated to the previously identified DGAT gene family (designated DGAT1), which is related to the acyl CoA:cholesterol acyltransferase gene family, or to any other gene family with ascribed function. This report identifies a new gene family, including members in fungi, plants and animals, which encode enzymes with DGAT function. To distinguish the two unrelated families we designate this new class DGAT2 and refer to the M. ramanniana genes as MrDGAT2A and MrDGAT2B.

Diacylglycerol acyltransferase (DGAT)‡ is an integral membrane protein that catalyzes the final enzymatic step in the production of triacylglycerols in plants, fungi, and mammals (for reviews, see Refs. 1–3). The enzyme is responsible for transferring an acyl group from acyl-coenzyme-A to the sn-3 position of 1,2-diacylglycerol (DAG) to form triacylglycerol (TAG). As the final step in TAG biosynthesis via the Kennedy pathway, it is the only step not involved in membrane biosynthesis. In plants and fungi, DGAT is associated with the membrane and lipid body fractions, particularly in oilseeds, where it contributes to the storage of carbon used as energy reserves. In animals, the role of DGAT is more complex. Triacylglycerols are synthesized and stored in several cell types including adipocytes and hepatocytes (4), but, in addition, DGAT may play a role in lipoprotein assembly and the regulation of plasma triacylglycerol concentration (4), as well as participate in the regulation of DAG levels (5, 6).

Cases et al. (7) reported the first cloning of a DGAT gene from mouse. Using coding sequences from acyl CoA:cholesterol acyltransferase (ACAT), expressed sequence tag data bases were searched and a gene identified that shared 20% identity with the mouse ACAT. After cloning and expression of the gene in insect cells, no ACAT activity was detected in isolated membranes; however, using [1-14C]oleoyl-CoA as substrate, a range of acceptors was examined and Cases et al. discovered DAG was the acceptor molecule, thus demonstrating DGAT activity. Hobbs et al. (8) reported the cloning of an Arabidopsis homologue of the mouse DGAT gene and confirmed the presence of DGAT activity in insect cells expressing the cDNA. Southern analysis indicated a single gene copy was present in Arabidopsis. Katavic et al. (9) and Kou et al. (10) also implicated this gene in seed oil production when an insertion mutation (AS11) in the gene was found to lower seed oil levels and decrease DGAT activity. The locus, at ~35 cM on chromosome II, was designated TAG1. Routaboul et al. (11) reported similar results identifying an Arabidopsis mutant (ABX45) harboring a frameshift mutation near the 5’ end of the TAG1 reading frame. This mutation resulted in a complete change in coding sequence after the first 60 amino acids. With the identification of a single DGAT gene copy in Arabidopsis and the detection of DGAT activity even after a frameshift mutation disabled gene translation, Routaboul et al. concluded that another protein must be responsible for the remaining DGAT activity.

We chose the oleaginous fungus Mortierella ramanniana as our source material since the organism produces up to 80% of its dry weight as TAG when grown under nitrogen-limiting conditions. M. ramanniana had previously been identified as exhibiting high levels of DGAT activity, and is easily cultured in the laboratory (12, 13). Our approach to the identification of DGAT involved protein purification, peptide sequencing, cloning of the corresponding cDNAs, and testing the gene products for DGAT function.

In this report a new class of proteins involved in TAG production was identified. Two polypeptides from M. ramanniana microsomes that co-purified with DGAT activity were sequenced and their corresponding cDNAs cloned. Expression of the cDNA sequences in insect cells conferred high levels of DGAT activity on membranes isolated from those cells. Although the genes encode proteins with DGAT activity, they are
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TABLE I
Primer sequences used to clone DGAT2 homologues

| Organism       | GenBank™ no. | Primer sequences                  |
|----------------|-------------|-----------------------------------|
| C. elegans     | U64852      | 5'-GCCGCGGCGCCTGCGCAGTCAGGATGAG-3' |
|                |             | 5'-GCCGCGGCGCCTGCGCAGTCAGGATGAG-3' |
|                |             | 5'-GCCGCGGCGCCTGCGCAGTCAGGATGAG-3' |
| S. cerevisiae  | Z81557      | 5'-GCCGCGGCGCCTGCGCAGTCAGGATGAG-3' |
| A. thaliana    | AL133452    | 5'-GCCGCGGCGCCTGCGCAGTCAGGATGAG-3' |

unrelated to the previously identified DGAT gene family (now designated DGAT1), which is related to the ACAT gene family. We designate this new DGAT family DGAT2, and refer to the two genes in *M. ramanniana* as DGAT2A and DGAT2B. Based on nucleic acid comparison, we identified homologues of DGAT2 genes in fungi, plants, and mammals. We describe the cloning of several of these genes, their expression in insect cells, and confirmation of DGAT activity by enzyme assay.

**EXPERIMENTAL PROCEDURES**

**Materials**—Yellow S6 CL-6B-agarose, 1,2–18:1 diacylglycerol (prepared as a 150 mHg stock in 2-methoxyethanol), and L-α-phosphatidic acid (prepared as a 50 mM stock in 1% (w/v) Triton X-100) were obtained from Sigma. Hydroxyapatite was obtained from Bio-Rad. Heparin fraction 28 was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). [1-14C]Oleoyl coenzyme A (50–55 Ci/mol) was obtained from PerkinElmer Life Sciences. Protease inhibitors from Roche Molecular Biochemicals (Mannheim, Germany) were included in all buffers at the following concentrations: 0.1 mM aprotinin, 1 μM leupeptin, and 100 μM Pefabloc. Restriction enzymes were from Roche Molecular Biochemicals.

**Fungal Cultures**—*M. ramanniana* was cultured as described by Kamisaka (12). Cells were harvested by passing 10–13-day-old cultures through Miracloth and removing excess liquid by hand wringing. Wet packed cells were stored at −70 °C.

**Extraction**—Lipid bodies were isolated from 70–75 g of wet packed cells. Immediately prior to use, cells were thawed on ice and resuspended in 200 ml of Buffer A (10 mM potassium phosphate, pH 7.0, 1 mM KCl, 0.5 mM sucrose, 1 mM EDTA). Samples were lysed with an equal fraction of Triton-X100 (pre-dissolved in a buffer containing 10 mM potassium phosphate, pH 7.0, 100–150 mM KCl, and 0.1% Triton X-100 (w/v)). Assay mixtures were incubated at 25 °C for 5 min and reactions terminated by adding 1.5 ml of heptane/isopropanol/0.5 mM H2SO4 (10:40:1, v/v/v). For solubilized samples, 1.5–18:1 DAG was reduced to 0.5 mM, Triton X-100 was increased to 0.2%, and 300 μM L-α-phosphatidic acid was included. The L-α-phosphatidic acid was required to recover activity following solubilization with detergent as described by Kamisaka et al. (13), except we found that 300 μM rather than 500 μM phosphatidic acid gave a greater stimulation of activity. Following solubilization, product formation was dependent on the addition of exogenous DAG. Under these conditions, the reaction rate was linear with respect to time for up to 10 min.

After the assay was stopped, radiolabeled glycerolipids were isolated by adding 0.1 ml 1 M NaHCO3 and 1 ml of heptane containing 15 mg/ml triolein as a carrier. The mixture was vortexed and the upper organic phase was removed to a new glass vial. The organic extract was back-extracted with 1 ml 1 M NaCl. Forty percent of the final organic phase was removed for liquid scintillation counting and the remaining organic phase evaporated to dryness under nitrogen gas. The residue was resuspended in hexane and subjected to TLC on Silica gel-G with a preadsorbent loading zone (model 31011; Analtech, Newark, DE). The TLC plate was developed in hexane:diethyl ether:acetic acid (50:50:1, v/v/v). For solubilized samples, 1.5–18:1 DAG was reduced to 0.5 mM, Triton X-100 was increased to 0.2%, and 300 μM L-α-phosphatidic acid was included. The L-α-phosphatidic acid was required to recover activity following solubilization with detergent as described by Kamisaka et al. (13), except we found that 300 μM rather than 500 μM phosphatidic acid gave a greater stimulation of activity. Following solubilization, product formation was dependent on the addition of exogenous DAG. Under these conditions, the reaction rate was linear with respect to time for up to 10 min.

The TLC plate was developed in hexane:diethyl ether:acetic acid (50:50:1, v/v/v), dried, and scanned by a radiomage analyzer (model 3000; AMBIS, San Diego, CA) to determine the portion of radioactivity incorporated into TAG. Confirmation of TAG identity on the TLC plate was determined by comigration of the unlabeled triolein carrier and the [1-14C]TAG following exposure to iodine vapor.

**DGAT Purification**—DGAT activity in the Triton X-100 extract was further purified by dye-binding chromatography on a Yellow S6-agarose column (2.5 cm × 6.4 cm) equilibrated with 75 mM KCl in Buffer D (10 mM potassium phosphate, pH 7.0, 0.1% (w/v) Triton X-100, 10% (v/v) glycerol). The column was washed with five volumes of equilibration buffer at 2 ml/min, and then activity was eluted with 500 mM KCl in Buffer D. The eluate was stable to freeze/thaw at this stage of purification so eluted fractions were assayed immediately and active fractions stored at −70 °C. Four preparations of Yellow 86-agarose-purified activity were combined and concentrated 12-fold by ultrafiltration (YM-30 membrane, Amicon, Beverly, MA). The activity was further purified by hydroxyapatite chromatography on a 1.0 cm × 25.5 cm column equilibrated with 500 mM KCl in Buffer D. The column was washed with 40 ml of equilibration buffer before bound proteins were
bound proteins were eluted in a 10-ml linear gradient of 150 mM KCl followed by 10 ml of 500 mM KCl in Buffer D at 0.25 ml/min. The column was washed with equilibration buffer, and DGAT2 activity was eluted in buffer A containing 75 mM KCl. DGAT2 activity was eluted in buffer A containing 500 mM KCl. Protein content was determined according to the method of Bradford (14) and is reported as milligrams of protein per fraction. DGAT2 activity is reported as nanograms of TAG formed per minute per fraction. Active fractions present in the flow-through were pooled and diluted 1:3.3 in Buffer D containing 500 mM KCl. The column was washed with equilibration buffer, and bound proteins were eluted with 0.1M potassium phosphate in equilibration buffer. Fractions containing DGAT activity were pooled and diluted 1:3.3 in Buffer D to reduce the KCl concentration to 150 mM. C, heparin chromatography. The diluted hydroxyapatite flow-through was applied to a heparin column (0.55 × 4.7 cm) equilibrated with 150 mM KCl in Buffer D. The column was washed with five volumes of equilibration buffer at 0.5 ml/min, and bound proteins were eluted in a 10-ml linear gradient of 150–500 mM KCl followed by 10 ml of 500 mM KCl in Buffer D at 0.25 ml/min. Fractions of 1.1 ml were collected.

Protein Determination—The protein concentration of extracts was determined according to Bradford (14) using bovine serum albumin as standard.

SDS-PAGE—Polyacrylamide gradient gel electrophoresis (10–13%) was carried out according to the method of Laemmli (15) with some of the modifications of Delepelaire (16). The resolving gel contained a 10–13% linear gradient of acrylamide stock stabilized by a 0–10% linear gradient of sucrose. Proteins were visualized by staining with silver according to the method of Blum et al. (17) or with Coomassie Blue (0.1% Coomassie Blue R-250, 50% methanol (v/v), 10% acetic acid (v/v)).

Partial Amino Acid Sequence Determination—Proteins in active fractions eluting from the heparin step were precipitated with 10% trichloroacetic acid, washed with ice-cold acetone, and resuspended in SDS sample buffer. Samples were subjected to SDS-PAGE, and the gel was stained with Coomassie Blue. Protein bands at apparent molecular masses of 36 and 36.5 kDa were excised from the gel and sent to a commercial laboratory (Argo Bioanalytica, Morris Plains, NJ) for analysis. Gel slices were digested in situ with trypsin, and the resulting peptides were separated by reversed-phase HPLC. Amino acid sequencing was performed on a model 473 Protein Sequencer (Applied Biosystems, Foster City, CA).

Polymersase Chain Reaction—Degenerate oligonucleotides were synthesized on an oligonucleotide synthesizer (Applied Biosystems model 394) and used as primers in polymerase chain reaction. The peptide sequences used for synthesizing the corresponding coding and complementary oligonucleotides were designed according to the partial amino acid sequence obtained. The Marathon cDNA was used as a template. The amplification mixture consisted of template, polymerase chain reaction buffer, 200–300 ng of each primer, 2.5 mM dNTP, and 1 unit of AmpliTaq Gold polymerase (PerkinElmer Life Sciences) in 50 ul. The amplification program consisted of one 10-min hold at 95 °C, 10 cycles of denaturation (94 °C, 10 s), annealing (55 °C, 10 s), and primer extension (72 °C, 1 min). Products of the reaction were separated on a 7% agarose gel, excised, and then purified according to the QIAPREP DNA extraction handbook (Qiagen, Santa Clarita, CA). The purified products were cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA).

Complete Amplification of cDNA Ends (RACE)—RACE reactions were completed according to the instruction manual for Marathon cDNA amplification kit using oligonucleotides designed from the products of the degenerate PCR. Gel-purified RACE products were cloned into the pCR2.1-TOPO vector.

Cloning of DGAT2 Homologs—Data base searches of the predicted proteins from the public genomic data bases of Caenorhabditis elegans yielded three similar sequences. Searches of the public Saccharomyces cerevisiae predicted protein data base yielded one sequence. Searches of proprietary Arabidopsis expressed sequence tag data bases yielded partial sequences that were sufficient for PCR primer design. Total RNA was collected from these three organisms, and first strand cDNA libraries were created using the Marathon cDNA library kit (CLON-
TECH). The primers in Table I were used to PCR-amplify each of the sequences. The PCR products were cloned into the pCR2.1-TOPO vector.

DNA Sequence Determinations—DNA sequence determinations were carried out using a modified protocol from Applied Biosystems. Sequence analyses were carried out using software of the Gen Codes Corp. (Ann Arbor, MI).

Expression of DGAT2 Genes in Insect Cells—The commercial BAC-to-BAC baculovirus expression system (Life Technologies, Inc.) was used to express full-length proteins in cultured insect (Sf9) cells. Full-length DGAT2 open reading frames were amplified by PCR employing primers containing restriction sites at the 5’ ends (NotI and SpeI to the sense primers and PstI to the antisense primers). The PCR products were cloned into the pCR2.1-TOPO vector.

Insect cells (1 × 10^6 cells/ml) were infected at a multiplicity of infection of 0.05–0.1 and harvested after 5 days at 27 °C by centrifugation. Pelleted cells were resuspended in Buffer E (100 mM Tricine-NaOH, pH 7.8, 10% glycerol, 100 mM NaCl) and lysed by sonication (2 × 10 s). Cell walls and other debris were pelleted by centrifugation and

FIG. 3. Sequence alignment of derived DGAT2 polypeptide sequences. The amino acid sequences of the predicted DGAT2 polypeptides were aligned using the Clustal multiple sequence alignment program. Totally conserved residues are shaded black; gray shading is the consensus of three or more sequences. All sequences are full-length. Residues shown above the alignment are highly conserved signature amino acids found in the motifs D and E of the acyltransferase superfamily (Ref. 20, and our own alignments). In this area DGAT2 and the acyltransferase superfamily sequences co-align; only the shared conserved amino acid residues are shown. Sources are as follows: M. ramanniana (MrDGAT2A, accession no. AF391089; MrDGAT2B, accession no. AF391090), S. cerevisiae (ScDGAT2, accession no. YOR245C), C. elegans (CeDGAT2A, accession no. CAB04533; CeDGAT2B, accession no. AAB04969; CeDGAT2C, accession no. AAD45832), A. thaliana (AtDGAT2, accession no. T45783), and Mus musculus (MmDGAT2, accession no. BAB22105).
In order to maintain maximal activity, subsequent chromatography was performed and fractions assayed on the same day. Significant purification was achieved using hydroxypatite chromatography (Fig. 1B). Although DGAT activity did not bind the column, 64% of the protein present bound the column and was removed. Active fractions from the flow-through of the hydroxypatite column were purified on heparin CL 6B-agarose (Fig. 1C). Two activity peaks eluted from the heparin column, one during the 100–500 mM KCl gradient and one during the 500 mM KCl wash. Several protein bands (36.5, 36, 35, and 34 kDa) were associated with the first peak of activity (Fig. 2, fraction 22). The 34-kDa band did not correlate with DGAT activity in all chromatographic steps so it was eliminated (data not shown). The second peak had a higher specific activity (Table II) and contained a major protein band at 36 kDa by SDS-PAGE (Fig. 2, fraction 28). Three proteins (36.5, 36, and 35 kDa) were identified from the purification as potential DGAT candidates.

Partial Amino Acid Sequence Determination and Cloning of Purified DGAT2 Polypeptides—The three proteins associated with DGAT activity were gel-purified by SDS-PAGE, stained with Coomassie Blue, and then excised for protein sequencing. In-gel digestion of the proteins was performed using trypsin, and peptides were purified using reversed-phase HPLC. Examination of the peptide maps revealed that the 36.5-kDa map and the 35-kDa map were identical. Only peptides from the 36.5-kDa band were sequenced. The peptide map of the 36-kDa protein was significantly different than that of the 36.5/35-kDa proteins, and several of these peptides were sequenced.

Degenerate primers (Fig. 3), designed from the amino acid sequences generated from the 36-kDa peptide, were constructed in both sense and antisense orientations. These primers were employed in different combinations to amplify cDNA produced from M. ramanniana total RNA. PCR products were cloned into pCR2.1-TOPO and analyzed by DNA sequencing. Comparisons between peptide sequences obtained by Edman degradation not used to design the primers and the deduced amino acid sequences of PCR products were used to confirm the identity of the fragments. RACE using primers specific to these fragments was performed to yield a 1280-bp cDNA. This cDNA, which was designated DGAT2A (accession no. AF391089), contains a large open reading frame starting at bp 1. The most 5’ ATG codon of this reading frame is located at bp 26, allowing for the translation of a 355-amino acid polypeptide (DGAT2A, Fig. 3).2

A similar strategy was employed to clone the cDNA encoding the 36.5-kDa protein. We observed similarities between peptide sequences obtained from the 36- and 36.5-kDa polypeptides. Therefore, degenerate oligonucleotide primers were designed to the sequenced fragments of the 36.5-kDa peptide that had the least homology to the 36-kDa protein (Fig. 3). Evolutionary PCR, combined with RACE using primers specific to these fragments, was performed to yield a 1133-bp cDNA. This cDNA, which was designated DGAT2B (accession no. AF391090), contains a single large open reading frame starting at bp 1. The most 5’ ATG codon of this reading frame is located at bp 26, allowing for the translation of a 349-amino acid polypeptide (DGAT2B, Fig. 3).2

Sequence is also present in Patent Application WO 00/01713.
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**Fig. 5.** DGAT activity in insect cells expressing selected DGAT2 genes. Activity is expressed as the nanomoles of TAG produced per minute per milligram of membrane protein. Data are not normalized for the amount of gene product produced.

**Fig. 6.** Effect of temperature on MrDGAT2A and MrDGAT2B activity in insect cell membranes.

We also investigated some of the enzymological properties of the expressed *M. ramanniana* DGAT2A and DGAT2B genes. The effect of pH on DGAT activity was evaluated from 4.0 to 11.0. The pH optimum for both enzymes was observed at 6.8. No differences were detected between the two polypeptides with respect to pH (data not shown). A difference was observed in their response to temperature. The temperature optimum for DGAT2A was 37 °C, whereas DGAT2B did not demonstrate an optimum temperature (Fig. 6). The polypeptides were also characterized with respect to their ability to utilize two different acyl-coenzyme A donors, 18:1 and 12:0, and a range of diacylglycerol acceptors (6:0 through 18:0, even numbers, and 18:1) (Figs. 7, A and B). We detected an enhanced capacity for the utilization of medium-chain substrates (6:0 to 10:0) for both DGAT2A and DGAT2B proteins. Since we did not determine the specificity constants (V<sub>max</sub>/K<sub>m</sub>) for the various substrates supplied, these data are preliminary and should be substantiated by further investigation.

**DISCUSSION**

We have isolated novel DGAT proteins from cells of the oleaginous fungus *M. ramanniana*. Following cell lysis, DGAT activity was associated with the lipid body fraction and detergent solubilization was required to release the membrane-bound proteins to permit their purification using traditional chromatographic techniques. A stimulation of DGAT activity in the homogenate was observed following the addition of the detergent Triton X-100. Using a five-step protocol, two proteins, 36 and 36.5 kDa by SDS-PAGE, were identified as being associated with DGAT activity. Final specific activity recoveries of 1.6 and 4.2%, respectively, were reported for the purest, most active fractions containing each protein. Expression of the cloned cDNAs in insect cells allowed the unambiguous confirmation of DGAT activity as being associated with the two polypeptides. Alignment of the two protein sequences indicates they share only 54% sequence similarity (Fig. 3, top two lines).

Our purification of *M. ramanniana* DGAT differs from that reported by Kamisaka and co-workers (19), who identified a 53-kDa protein (by SDS-PAGE) as DGAT. The two polypeptides we identified corresponded to 36- and 36.5-kDa (by SDS-
PAGE). In addition, all other identified DGAT2 polypeptides from other species (Fig. 3) are approximately in the 33–42-kDa range. Since apparent and predicted molecular mass values match approximately, it is likely that the proteins we isolated represent unprocessed DGAT2 polypeptides. It is noteworthy that using our assay, the 36- and 36.5-kDa polypeptides were the only protein bands we observed that correlated with DGAT activity throughout purification.

An unexpected observation of the characterization of *M. ramanniana* DGAT2 proteins isolated from insect cells was the enhanced activity with medium-chain substrates. *M. ramanniana* produces TAG comprising primarily C18 acyl groups, yet more activity was detected when C6-C10 DAGs were provided as the acyl acceptor, especially when a medium chain donor (12:0-CoA) was used. Although absolute activity values cannot be compared between samples because of differences in the level of protein expression in different insect cell lines, DGAT2A appears to have greater specificity for medium-chain substrates relative to long-chain substrates than does DGAT2B. Whether the observed activities with medium-chain substrates are a unique feature of the *M. ramanniana* enzymes or an artifact due to differing solubilities of the hydrophobic substrates remains to be determined. If true, these findings offer intriguing possibilities for the use of *M. ramanniana* genes in the engineering of unusual fatty acids in plant seed oils.

A search of the sequence databases using the deduced amino acid sequences of the two *M. ramanniana* clones revealed no homology with the previously identified DGAT1 gene family that is sequence-related to the ACAT gene family. Unidentified DGAT2 homologues were found in many eukaryotic species, namely animals, plants, fungi, and *Leishmania*, but were absent from the prokaryotes (Fig. 4). However, it is noteworthy to mention that several conserved signature amino acid residues of motifs D and E of the previously proposed acyltransferase superfamily (20) and motif IV of sn-glycerol-3-phosphate acyltransferase consensus (21) are also conserved in DGAT2 (see the sequence above the alignments in Fig. 3). Since acyl-CoA is the shared substrate used by all these diverse enzymes, we can only speculate that this motif might be related to acyl-CoA binding and might indicate a common origin.

Full-length clones were obtained for several homologues, and the expressed proteins were evaluated in insect cells. All of the homologues tested exhibited some level of DGAT activity, demonstrating that the genes in this family are related by function. These data confirm our discovery of a second DGAT gene family. The identification of a new DGAT gene family is consistent with previous biochemical observations (9, 11). First, gene disruptions of *DGAT1* (*TAG1* locus) in *Arabidopsis* did not abolish DGAT activity completely or eliminate TAG production in seeds. Second, Smith et al. (22), working with *DGAT1* knockout mice, concluded there may be an additional DGAT gene present in mammals when experimental data showed that TAG production still occurred in these animals. These data collectively supported the presence of an additional source for DGAT in plants and mammals. Cases et al. (35) report the cloning of a mouse *DGAT2* cDNA, verify DGAT enzyme function in insect cells, and describe the *DGAT2* mRNA distribution in mouse.

In addition to our discovery of a second *DGAT* gene family, a novel, alternative mechanism for the production of TAG has recently been reported in yeast (23, 24). This pathway utilizes phospholipid, rather than acyl-coenzyme A, as a substrate for acyl transfer to DAG to produce TAG. The acyl-CoA-independent production of TAG during exponential growth in yeast was associated with the *LOR1* gene (25, 26). A knock-out of *LOR1* resulted in the complete removal of the acyl-CoA-independent activity and a significant reduction in TAG accumulation. Dahlqvist designated this enzyme phospholipid:diacylglycerol acyltransferase (PDAT) since the enzyme apparently does not discriminate between phospholipid species supplying the acyl group. PDAT is structurally related to the lecithin:cholesterol acyltransferase family, and homologues of *LOR1* appear to be common in eukaryotes. With this discovery, the contribution of PDAT as well as the newly discovered DGAT2 family to the overall production of TAG must be determined.

To date, three independent gene families (*DGAT1, DGAT2,* and *PDAT*) have been described that encode unique proteins with the capacity to form TAG, and all three are present in genomes of eukaryotes. It is possible the three gene families may play different roles in different species, in different tissues, or at different times during development. In yeast, for example, all three genes are present but their expression levels vary during different phases of the life cycle (26). In mice in which the *DGAT1* gene was disrupted, certain tissues appeared to be more affected than others (22). For example, although the *Dgat1*–/– mice showed only a 20% reduction in total carcass triglyceride, the female mice lost the ability to lactate. Examination of the breast tissue showed a severe reduction in lipid droplets, indicating DGAT1 plays a key role in this specific tissue. Dahlqvist et al. (26) proposed, in plant seeds, PDAT may be responsible for the selective shunting of unusual fatty acids out of membrane lipids and into TAG. Microsomes isolated from developing seeds of species that produce large amounts of unusual fatty acids in their oil, such as ricinoleic acid in castor and vernolic acid in *Crepis palaestina*, preferentially incorporate these fatty acids into TAG. Further research is needed to elucidate the roles these three gene families play in different organisms.

TAG is an abundant molecule found in many forms of life most likely because of its high energy density. The ability to alter oil levels either up or down, depending on the species, is of commercial interest. For example, in humans fat storage has many implications in health maintenance and well-being and drug therapies are being developed to reduce its accumulation. In oilseeds, which are economically and nutritionally significant crops, increasing seed value by increasing stored TAGs is an important goal in agriculture. In this regard, the study of
TAG synthesis is of consequence as we consider ways to manipulate the production of TAG. Researchers have successfully altered fatty acid composition of seed oils through biotechnology (28–31); however, increasing fatty acid content has proved more elusive, although several reports have appeared in the literature containing preliminary evidence of success (32–34).

The manipulation of oil levels in model organisms can be achieved by expression of genes that increase DGAT activity. Expression of DGAT1 genes in insect cells (8), yeast (27), and tobacco leaf (28) all resulted in an increase in TAG accumulation. We observed that expression of M. ramanniana DGAT2A in insect cells increased the total amount of TAG 2–3-fold in those cells. All of these genes show great potential as tools to increase TAG levels in oilseeds.

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