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Identification and functional expression of a type 2 acyl-CoA:diacylglycerol acyltransferase (DGAT2) in developing castor bean seeds which has high homology to the major triglyceride biosynthetic enzyme of fungi and animals

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Abstract

Seed oil from castor bean (*Ricinus communis*) contains high amounts of hydroxy fatty acid rich triacylglycerols (TAGs) that can serve as raw material for production of bio-based products such as nylon, cosmetics, lubricants, foams, and surfactants. Diacylglycerol acyltransferase (DGAT) catalyses the terminal reaction in the acyl-CoA dependent Kennedy pathway of triglyceride biosynthesis. There is still some debate whether there are three or four enzymes in yeast that have DGAT activity and catalyse the synthesis of TAG but of these the DGAT2 homologue Dga1 contributes in a major way to TAG biosynthesis. Here we report on the cloning of a cDNA for DGAT2 from castor bean and prove its biological activity following expression in yeast and enzymatic assays using diricinolein as the acceptor and ricinoleoyl-CoA as the donor. Previous reports of DGAT in castor have focussed on DGAT1 which has little amino acid sequence homology to DGAT2. Expressional studies demonstrate that DGAT2 is 18-fold more highly expressed in seeds than in leaves and shows temporal specific expression during seed development. In contrast, DGAT1 shows little difference in expression in seeds versus leaves. We conclude that in castor bean DGAT2 is more likely to play a major role in seed TAG biosynthesis than DGAT1.

Keywords: *Ricinus communis*; Castor bean; Gene expression; Enzyme activity; Triacylglycerol metabolism; Diacylglycerol acyltransferase; Seed oil

1. Introduction

The major storage form of fatty acids in plants, animals and fungi are triacylglycerols (TAGs). Two types of metabolic pathways for the production of TAG have been elucidated, an acyl-CoA dependent pathway and acyl-CoA independent pathways. The acyl-CoA dependent pathway, commonly known as the Kennedy pathway, utilises acyl-CoA as a substrate for successive acylation reactions of the glycerol backbone, with the terminal step being the acylation of diacylglycerol (DAG) by DAG acyltransferases (DGATs) (Kennedy, 1961). The acyl-CoA independent pathway uses an alternative enzyme for the final acylation reaction, phospholipid:diacylglycerol acyltransferase (PDAT). PDAT directly transfers an acyl group from phosphatidylcholine (PC) to DAG in yeast and plants (Dahlqvist et al., 2000). In another acyl-CoA-independent transacylation, it is possible that a DAG transacylase uses two molecules of DAG in TAG formation (Stobart et al., 1997) although no gene encoding such a transacylase has been identified. The PDAT enzyme from *Crepis palaestina* and castor bean preferentially incorporates unusual fatty acids into TAG (Dahlqvist et al., 2000). In yeast the PDAT is a major contributor to TAG accumulation in exponential growth phase, however during late log phase when TAG accumulation is maximal it role is less important

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(Sorger and Daum, 2003). Analysis of mutants of PDAT in *Arabidopsis* (Mhaske et al., 2005; Routaboul et al., 1999) and lipid composition analysis of *Arabidopsis* plants over-expressing AtPDAT (Stahl et al., 2004) plus metabolic control analysis (Ramli et al., 2005) indicate that both PDAT and DAG/DAG acyltransferase do not seem quantitatively important in TAG biosynthesis in tissues of *Brassicaceae*, oil palm or olive calli. The importance of PDAT in TAG biosynthesis may well differ dependent on the biological system and the growth phase. All the acylation reactions are catalysed by membrane bound enzymes that are thought to be located in the endoplasmic reticulum. The first two acylation reactions of TAG biosynthesis are shared with the metabolic pathway for phospholipid biosynthesis and the final acylation reaction is specific to TAG biosynthesis. There is considerable interest in the elucidation of the mechanisms of biosynthesis of TAG, particularly those containing unusual fatty acids which may require appropriate acyl selectivity of the acyltransferases. For *Limnanthes* it is known that the lysophosphatidic acyltransferase (LPAT) has acyl selectivity for erucic acid (Brough et al., 1996). TAG biosynthesis has attracted attention due to concerns of human health and obesity as well as alternative non petroleum-based, renewable plant feed stocks for the oleochemical industry (Dyer and Mullen, 2005; Singh et al., 2005). There is still some debate whether there are three or four enzymes in yeast that have DGAT activity and catalyse the synthesis of TAG (Sorger and Daum, 2003). Two main membrane bound DGAT families designated DGAT1 and DGAT2 exist. The DGAT1 family has high homology to acyl-CoA:cholesterol acyltransferase (ACAT) and indeed was cloned somewhat serendipitously from a mouse cDNA library whilst the authors of the paper were looking for homologues of ACAT (Cases et al., 1998). That work bears testimony to the importance of performing biological activity assays and not relying upon homology based predictions to determine function. Several homologues of DGAT1 have been found in animals and plants and their function proven by both over-expression and deletions (Bouvier-Nave et al., 2000; Hobbs et al., 1999; Katavic et al., 1995; Zou et al., 1999; Routaboul et al., 1999). A second type of membrane bound DGAT gene family, type-2 DGAT or DGAT2, is found in animals, plants and yeast. DGAT2 was characterised following purification of the protein from the fungus *Mortierella ramanniana*, cDNA cloning and biochemical assays of the recombinant protein (Lardizabal et al., 2001). DGAT2 has also been cloned from mouse (Cases et al., 2001) and yeast (Sorger and Daum, 2002) and demonstrated to be a major contributing factor to TAG synthesis in both these organisms. Recently, a third type of DGAT isolated from developing peanut (*Arachis hypogaea*) cotyledons was reported (Saha et al., 2006). However, this DGAT is a cytosolic soluble enzyme and it is possible that the cytosolic DGAT could also be involved in TAG biosynthesis and wax ester synthesis in oilseeds. Members of the membrane bound DGAT1 and DGAT2 gene families exhibit no significant amino acid sequence homologies to each other and are quite distinctive. Whilst only a small number of plant DGAT2 homologues have been identified in data bases, namely from thale cress (*Arabidopsis thaliana*: GenBank Accession No. NM115011; At3g51520), Tung tree (*Vernicia fordii*: GenBank Accession No. ABC94474), barrel medic (*Medicago truncatula*: GenBank Accession No. ABA88819), rice (*Oryza sativa*: GenBank Accession No. BAD33505) and great duckweed (*Spirodela polyrrhiza*: GenBank Accession No. AAQ89590), proof of function is lacking. In oil bearing seeds the level of TAG, and *de novo* fatty acid biosynthetic enzymes plus associated mRNAs, are elevated during castor seed tissue development in comparison to leaves. This increased biosynthetic potential is required to maintain the high rates of synthesis of TAG which can contribute up to 45% of the dry weight of the seed. In *Ricinus communis* the hydroxylated fatty acid, ricinoleic acid, is exclusively made in seeds and accumulates at levels of above 85% in the TAG. We are interested in identifying acyltransferase enzymes which could contribute to the biosynthesis of ricinoleic acid rich TAGs. We instigated an EST based approach to find such genes which are elevated in developing castor seed tissue and thus implicated in TAG assembly. In this study we report on the identification of a cDNA encoding type-2 DGAT from *R. communis* (RcDGAT2). We demonstrate that RcDGAT2 has elevated expression during seed development. The gene product was over-expressed in yeast and assayed to prove biochemical function. A comparison of the expressional pattern of DGAT1 with DGAT2 in both leaf and developing seed material demonstrates that DGAT2 is much more highly expressed in seeds than DGAT1. This implicates DGAT2 as probably the major contributing DGAT to TAG formation in *R. communis*.

2. Results and discussion

2.1. Cloning and analysis of a cDNA encoding DGAT2 from *Ricinus communis*

A BLAST search of our in house *R. communis* expressed sequence tag (EST) database identified two ESTs which shared homology to *A. thaliana* (At)DGAT2 (GenBank Accession No. NM115011; At3g51520) and rice (*O. sativa* (Os)MGAT-DGAT) (GenBank Accession No. BAD33505). Upon complete sequencing of the EST clones (pRN16N10 and pRN1J09) in both directions, the clones were found to encode full open reading frames (ORFs) for a castor bean (Rc)DGAT2 orthologue. The full length cDNA is 1404 bp long, containing an open reading frame of 1023 bp encoding a protein of 340 amino acids (GenBank Accession No. AQ923083). The RcDGAT2 amino acid sequence has little sequence homology with the type-1 DGATs and ACAT family but the highest identity is shared with the type-2 DGATs in the database confirming

the initial findings with the single-pass EST sequences. The percentages identity at the amino acid level between the RcDGAT2 and the closest related plant DGAT2 orthologues were 65.8% with tung tree (*V. fordii*; GenBank Accession No. ABC94473), 58.3% with thale cress (*A. thaliana*; GenBank Accession No. NM115011, AT3g51520) and 49.7% with rice (*O. sativa* MGAT-DGAT; GenBank Accession No. BAD33505). There is a close phylogenetic relationship between the functionally proven DGAT2 from mouse (*Mus musculus*; GenBank Accession No. BAB22105.1, 20.6%), an oleaginous fungus (*M. ramanniana* 2A; GenBank Accession No. AAK84179, 17.9%) and yeast (*Saccharomyces cerevisiae* Dga1; GenBank Accession No. NP 014888, 17.9%). Alignment of the deduced amino acid sequences of DGAT2 from castor, tung tree, *Arabidopsis*, *Mortierella* and mouse reveals regions of significant homology and illustrates that the proteins share between 20% and 66% identity between the three kingdoms of plants, fungi and animals with the most conserved regions in the C-terminal part of the proteins (Fig. 1a). Notably we observed the presence of similar C-terminal ER retrieval motifs in both the DGAT1 and DGAT2 proteins from

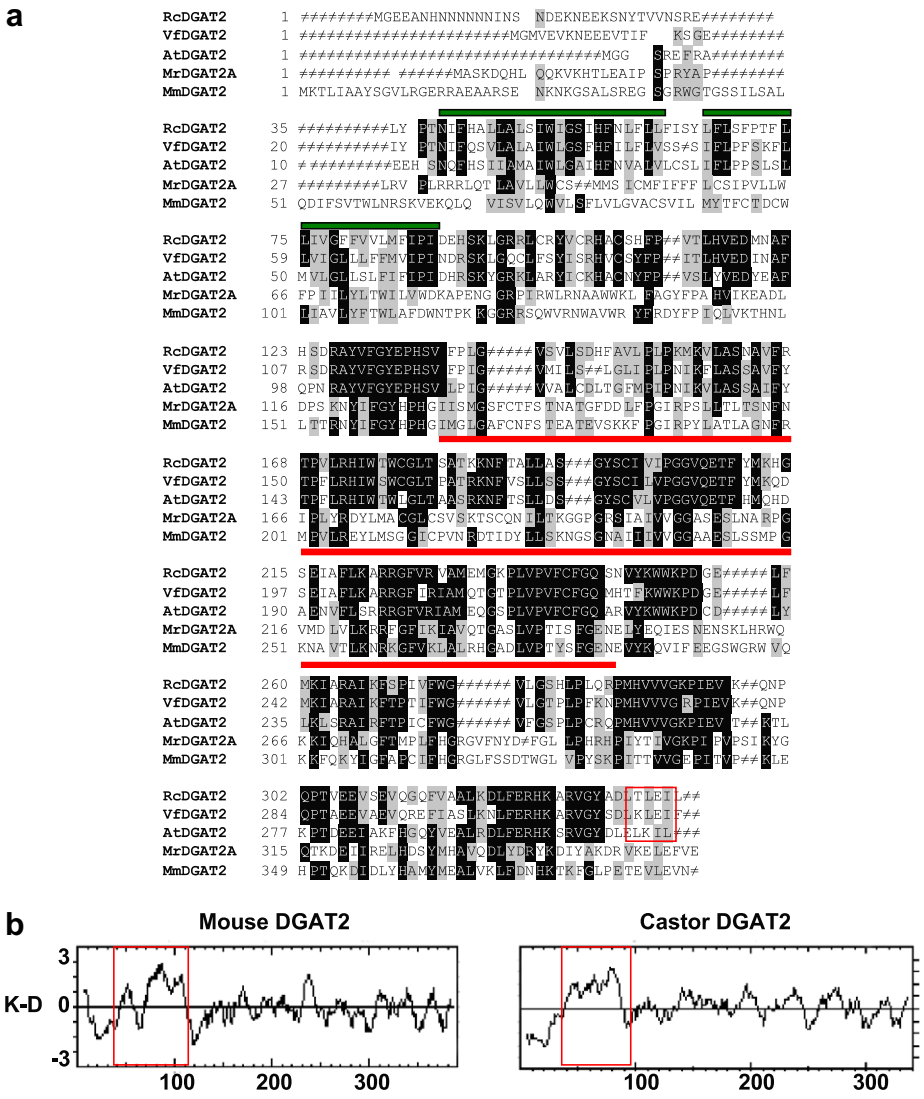

Fig. 1. Sequence analysis of deduced amino acids of type-2 acyl-CoA:diacylglycerol acyltransferases. (a) ClustalW alignment using the multiple sequence alignment program (http://align.genome.jp) and the output was decorated with Boxshade (http://www.ch.embnet.org). The identical amino acid residues between the five proteins are shaded black and gray shading is the consensus of two or more sequences. The Genbank accession numbers for these type-2 DGATs are *Ricinus communis* RcDGAT2: GenBank Accession No. AQ923083, *Arabidopsis thaliana* AtDGAT2: GenBank Accession No. NM115011, *Mus musculus* MmDGAT2: GenBank Accession No. BAB22105, *Mortierella ramanniana* 2A MrDGAT2A: GenBank Accession No. AAK84179, *Vernicia fordii* VfDGAT2: GenBank Accession No. ABC94473. The two transmembrane domains as predicted by TMpred software at The Eukaryotic Linear Motif resource (http://elm.eu.org/) are overlined in green (amino acid residues 39–61 and 66–88), the phospholipid/glycerol acyltransferase domain (PlsC; InterPro IPR002123) is underlined in red and the C-terminal ER retrieval motif in the plant DGAT2 sequences are boxed in red. (b) Hydrophobicity plots of mouse and castor DGAT2 as assessed by Kyte–Doolittle (K–D) analysis (Kyte and Doolittle (1982)). Hydrophobic regions are above the K–D = 0 line. The main transmembrane segment as predicted by TMpred software at The Eukaryotic Linear Motif resource (http://elm.eu.org/) is boxed in red.

plants (*Arabidopsis* AtDGAT1, rape BnDGAT1, nasturtium TmGDAT1, castor RcDGAT1 and rape BnDGAT2; -YYHDL-, tung tree VfDGAT2; -LKLEI-, Arabidopsis AtDGAT2; LELKI and castor RcDGAT2; -LTLEI-). These putative ER retrieval motifs (-Φ-X-X-K/R/D/E-Φ-COOH, in which Φ is any large hydrophobic amino acid residue) were positioned at the extreme C-terminus and very likely serve as general ER localisation signals (McCartney et al., 2004). The type-2 RcDGAT protein has an estimated molecular mass of 38.7 kDa with an isoelectric point at pH 8.94 (predicted by Protparam at http://www.expasy.ch) and therefore will be positively charged in neutral pH environments. Kyte and Doolittle hydrophobicity plot analysis of type 2 DGAT proteins, such as the mouse and castor DGAT2 (Fig. 1b) is consistent with the presence of at least one membrane spanning domain (amino acids 40–90) and also a short (~5 kDa) hydrophilic domain in the N-terminal part of the protein. The amino acid sequence was also analysed using a TMpred program which predicts transmembrane helices by comparison with a database of naturally occurring membrane bound proteins The main transmembrane segments as predicted by software at The Eukaryotic Linear Motif resource (http://elm.eu.org/) suggests two transmembrane domains, however some caution needs to be exerted here as only four amino acids separate these predicted transmembrane domains and thus there might be only one. Another suggested model for transmembrane topology (http://www.ch.embnet.org/software) strongly predicts three transmembrane helices (amino acid residues 54–82, 188–211, 265–283) with the N-terminal domain lying on the ER lumen side of the membrane. The model for the plant DGAT1 proteins strongly predicts the hydrophilic N-terminal domain to lie on the cytoplasmic side of the membrane. The type-2 RcDGAT amino acid sequence contains two potential N-linked glycosylation sites (residues 26–29; -NYTV- and 187–190; -NFTA-) in contrast to the type-1 RcDGAT, which is predicted to have three

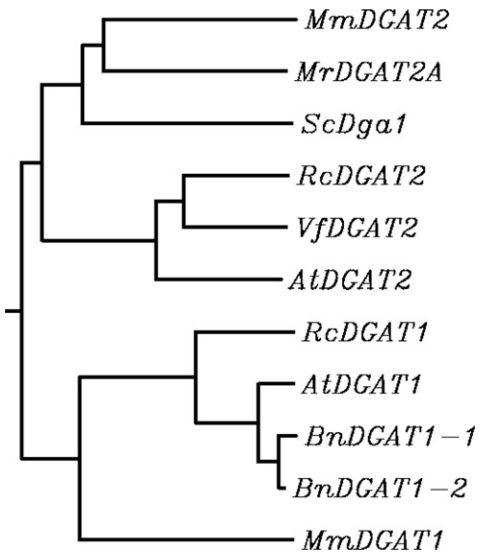

Fig. 2. The membrane bound type-1 and type-2 DGATs are related but belong to different protein families. A rooted dendrogram of the predicted protein sequences of various full length cDNAs of the DGAT1 and DGAT2 gene family was generated using PHYLIP. The protein sequences that were aligned with the ClustalW program were the DGAT1 and DGAT2 from mouse (*Mm*), the DGAT2A from the fungus *Mortierella ramanniana* (*Mr*), the DGAT2 (ScDga1) from yeast (*Sc*), the DGAT 2 from tung (*Vf*), the DGAT1 from rape (*Bn*) and the DGAT1 and DGAT2 from *Arabidopsis* (*At*) and castor bean (*Rc*).

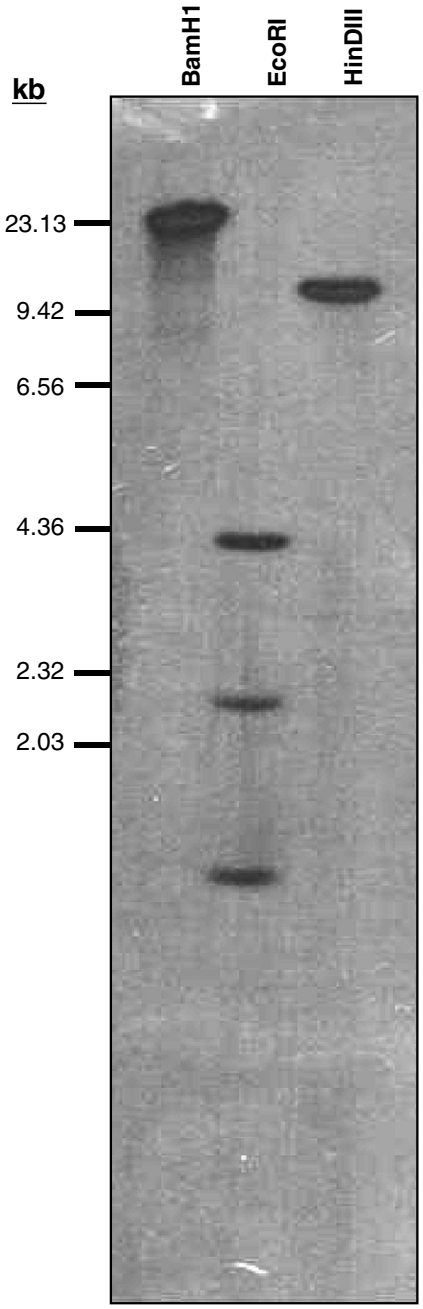

Fig. 3. Southern blot analysis of *Ricinus communis* genomic DNA. Castor bean (*Ricinus communis, cv. 99N89I*) genomic DNA (10 μg/lane) was digested with the indicated restriction enzymes and after Southern blotting the membrane was hybridized with ^{32}P dCTP-labelled cDNA encoding the ORF of RcDGAT2 as a probe. The blot was washed at high stringency after hybridisation at 0.1 × SSC/0.1% SDS at 65 °C.

such sites. It has been reported that it appears that the type-1 RcDGAT is not glycosylated (He et al., 2004). Since the recombinant RcDGAT2 protein expressed in a yeast system corresponds to the predicted size, it is unlikely that RcDGAT2 is glycosylated. Scanning the RcDGAT2 protein sequence against the Prosite database (http://expasy.hcuge.ch/cgi-bin/scanprosite) identified a number of putative phosphorylation sites of; (i) protein kinase C ([ST]-x-[RK]), amino acid residues 124–126 and 184–186; casein kinase II ([ST]-x(2)-[DE]), amino acid residues 16–19 and 304–307; and, tyrosine kinase ([RK]-x(2,3)-[DE]-x(2,3)-Y), amino acid residues 20–27. Also five N-myristoylation sites (G-{EDRKHPFYW}-x(2)-[STAGCN]-{P}), amino acid residues 179–184, 204–209, 214–219, 275–280, 313–318, and an amidation site (x-G-[RK]-[RK]), amino acid residues 94–97 were predicted. The possible evolutionary relationships between the membrane bound type-1 and type-2 DGATs were investigated via a phylogenetic comparison between a set of protein sequences from organisms representative of three different kingdoms (Fig. 2). The dendrogram reveals two main branches representing either entirely type-1 or entirely type-2 DGAT sequences. The plant type-2 DGATs form a tight cluster different from that of the fungi and mammals. It is also revealed that the BnDGAT1-1 and BnDGAT1-2, previously reported to be called BnGDAT1 and BnDGAT2, respectively, are both members of the type-1 DGAT cluster, in contrast to what the reported name might suggest (Nykiforuk et al., 2002). It is also apparent that both types of DGAT have no similarity and therefore we believe that the enzymes with DGAT activities belong to functionally convergent protein classes.

2.2. The RcDGAT2 is a single-copy gene and is highly expressed during castor seed development and oil biosynthesis

We determined the copy number of RcDGAT2 in the castor plant by performing genomic Southern hybridisation under low and high stringency conditions, which indicates that only a single copy exists in this genome (Fig. 3). To further investigate the potential role of castor type-2 DGAT in TAG biosynthesis we analysed the temporal and tissue-specific expression pattern of both types of DGAT genes. In order to quantify the level of RcDGAT1 and RcDGAT2 gene expression, we performed real time PCR, in triplicate, on RNA isolated from leaves and various stages of development of castor seed tissue derived from castor seed. The main phase of increase in TAG accumulation in *Ricinus* seeds occurs from 30 DAP (Greenwood and Bewley, 1982). TAG biosynthesis is highly upregulated in seeds versus leaves. There is little difference in the steady state expression of RcDGAT1 between leaves and developing seeds and maximal expression of DGAT1 is at 10 DAP and then decreases (Fig. 4a). In marked contrast, the expression of RcDGAT2 is highly elevated in seed versus leaves (Fig. 4b) showing 18-fold higher levels of expression at 25 DAP. During seed development expression of RcDGAT2 moderately increased at the early stages (from 10 to 15 DAP) and after 15 DAP there was a sharp rise reaching the highest expression at 25 DAP. After that the expression drops quickly and at 35 DAP it was at about the same level as in leaves. The tissue and temporal expression pattern of DGAT1 and DGAT2 are consistent with DGAT2 playing a more important role in TAG biosynthesis in seeds. In yeast, DGAT2 (Dga1p) is a major contributor to total cellular TAG synthesis (Sorger and Daum, 2002). If DGAT2 is an important contributor to TAG biosynthesis in *Ricinus* it would be expected to have enzyme activity with diricinolein as acceptor and ricinoleoyl-CoA as donor since triricinolein is the major TAG in *Ricinus*. In order to investigate this we overexpressed the DGAT2 in yeast and assayed for this biological activity.

2.3. Assay of RcDGAT2 activity in yeast microsomes

In order to determine if the isolated RcDGAT2 cDNA encodes a biologically active DGAT enzyme, we cloned the cDNA encoding the protein RcDGAT2 into the pYES2.1/V5-His-TOPO vector and transformed the yeast *S. cerevisiae* strain INVSc-1 with this plasmid. The determination of the optimal time of expression of the recombinant proteins was performed using SDS-PAGE analysis of microsomal membranes (Fig. 5). The expression was monitored via Western blotting using an antibody against the engineered C-terminal V5-epitope fused to the recombinant

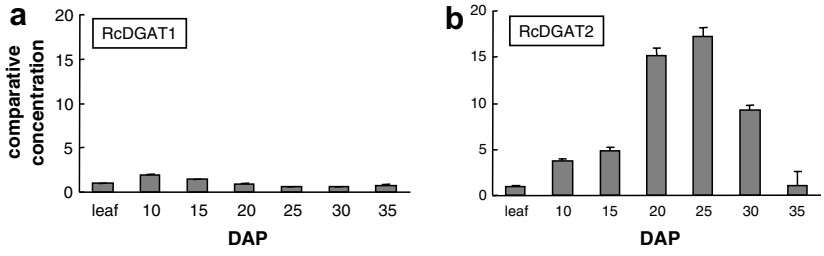

Fig. 4. Expression of RcDGAT1 and RcDGAT2 in castor leaf and developing endosperm of castor seeds monitored by quantitative Real-Time PCR. The developmental stages are indicated in days after pollination (DAP) on the *X*-axis. On the *Y*-axis, the comparative concentration represents the relative amount of PCR product formed in the developing endosperm samples compared to the leaf. Values are means ± standard deviation (SD) of three replicates. (a) RcDGAT1. (b) RcDGAT2.

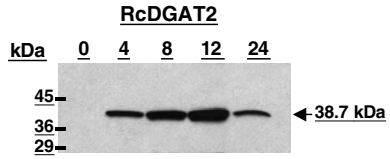

Fig. 5. Expression of recombinant RcDGAT2 in yeast. Western blot of microsomal protein (20 μg/lane) probed with anti-V5-HRP antibodies. Aliquots of yeast cultures were harvested at the different time points (indicated in hours above the lanes) after addition of the cells to the induction medium. The optimal expression of the recombinant RcDGAT2 proteins was around 12 h after induction.

DGATs and it was clear that optimal expression levels were achieved around 12 h after induction with 2% galactose. Enzyme assays were conducted to determine the biological activity of the RcDGAT2 in the microsomal fraction of the transformed yeast. *Sn*-1,2-diricinolein was used as acyl-CoA acceptor and [1-^{14}C]-ricinoleoyl-CoA as donor. Under the assay conditions investigated the DGAT2 activity is linear for the first 15 min of the reaction and then deviates from linearity with time (Fig. 6a). We used a 15 min time point to look at the products of the reaction (Fig. 6b and c). Without adding exogenous diricinolein to the assay reaction, triricinolein (RRR) is not detectable. Triricinolein was produced at a threefold greater extent in the assay containing added 0.5 mM diricinolein with the yeast microsomes from transformed cells expressing the RcDGAT2 gene, compared to the empty vector control. The DGAT2 expression profile and biological activity clearly places this type-2 DGAT enzyme as a potentially important protein in storage lipid biosynthesis in seeds of *Ricinus*.

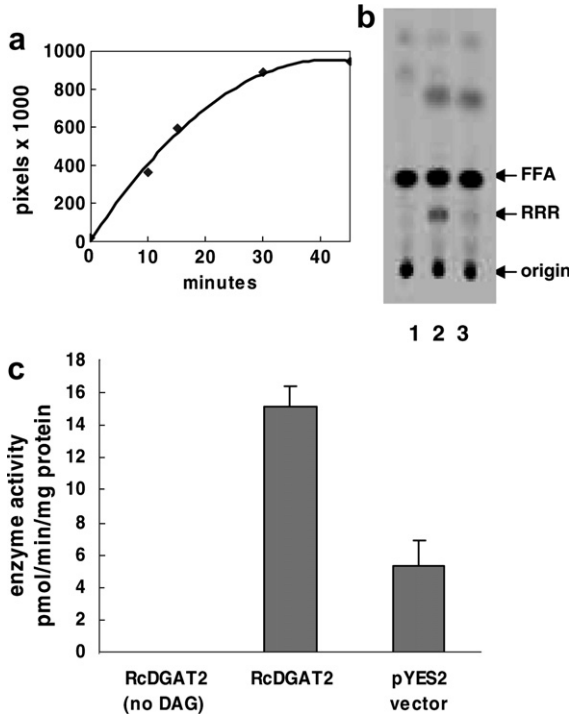

Fig. 6. Biochemical enzyme activity of DGAT *in vitro*. (a) Determination of linearity of the DGAT2 assay using 50 μg microsomal protein isolated from the yeast strain INVSc1 transformed with the castor DGAT2 gene. Time points were taken between 0 and 45 min. The density of the product RRR was measured in pixels. (b) TLC analysis of products of DGAT assays after 15 min (lane 1 + 2) and the empty PYES2 vector (lane 3). The enzyme assays were performed in the presence of sn-1,2-diricinolein (except lane 1) and ^{14}C-ricinoleoyl-CoA and the products analysed by TLC using hexane/diethylether/acetic acid (50/50/1, v/v/v). The origin of sample application, the free fatty acid (FFA) and triricinolein (RRR) products are indicated by arrows. (c) RcDGAT2 activity expressed as picomoles of TAG produced per minute per milligram of yeast microsomal protein. Values are means ± standard deviation (SD) of four replicate measurements.

3. Experimental

3.1. Plant material

Castor bean (*R. communis*, 99N89I) was grown in soil under 16 h photoperiod at 23 °C and 8 h dark period at 18 °C in a Sanyo growth chamber. The first real leaf at the stage of 10 days old and developing endosperm of castor seed at stages of 10, 15, 20, 25, 30 and 35 days after pollination (DAP) (Greenwood and Bewley, 1982) were harvested and immediately frozen in liquid nitrogen and stored at −80 °C.

3.2. Isolation of cDNA clones encoding the DGAT1 and DGAT2 enzyme

The cDNA (E6B2T7) clone corresponding to the *A. thaliana* expressed sequence tag (EST) (GenBank Accession No. AA042298) with sequence homology to the mouse DGAT protein sequence (Cases et al., 1998), was obtained from the Arabidopsis Biological Resource Center, Columbus, OH, USA. Upon complete sequencing, the 849 bp E6B2T7 cDNA was labeled with [^{32}P]dCTP using a random primer kit (Rediprime Random Primer Labeling System; Amersham) according to the manufacturer's instructions. A directionally cloned cDNA library (OdT primed, *Eco*RI–*Xho*I) from castor bean developing endosperm (cv. Hale; mix of stage 15–30 DAP), was constructed in λUniZapII (Stratagene) and a RcDGAT1 cDNA clone was isolated via heterologous screening of 2×10^5 plaque forming units (pfu). The cDNA clone represented a partial protein and the full length cDNA for RcDGAT1 from the castor bean variety 99N89I was obtained via a combination of 5′ RACE (rapid amplification of cDNA ends) and end-to-end amplification (KOD Hot Start polymerase; Novagen) using RACE ready cDNA template prepared according to the manufacturer's instruction (Invitrogen, UK) using Random Primers (N_6). The 5′ RACE oligonucleotide primers used in this study were RcDGAT1 GS P1 (5′-d(GGATAACTTGGCTGGTAACATAATGTG)-3′) and nested primer RcDGAT1 nGSP2 (5′-d(GCTGGTAACATAATGTGGGTGCGAC)-3′). The primers used in the end-to-end amplification were RcDAT1 5.1

(5′-d(TTCATTGAATCCTCCTTCTTAGCGTG)-3′) and nested RcDAT1 *n*5.2 (5′-d(TCCTCCTTCTTAGCGTGTCTTTGTCC)-3′) and RcDAT1 3.1 (5′-d(CTGAAACTGACGATTAACCTACTCTG)-3′) and nested RcDAT1 *n*3.2 (5′-d(TGACGATTAACCTACTCTGTCAATGG)-3′). The DNA product of 1743 bp was cloned into pCR 2.1 – TOPO according to the manufacturer's instructions. The full-length cDNA was completely sequenced in both directions using Big Dye Terminators v3.1 Cycle Sequencing Kit (Applied Biosystems). Database searches were done using the BLAST algorithm (Altschul et al., 1997) and sequence alignments and similarity amongst species were determined using the ClustalW program in the DNA STAR software package. The protein motifs were identified using ScanProsite at http://www.expasy.ch/tools/scnpsit1.html. A castor bean expressed sequence tag (EST) database was generated by single-pass sequencing of 10,000 cDNAs, specifically reflecting gene expression during seed development of castor bean. Total RNA was extracted from metabolically active developing castor bean endosperm (cv. 99N89I; mix of stage 15–30 DAP) using TriZol reagent (BRL) followed by on column DNAse treatment using the RNeasy Plant Mini Kit (Qiagen). cDNA was oligo-dT primed, size selected at >500 bp and directionally cloned in the pCMV-Sport 6.1 vector. The library was normalized (Invitrogen, patent pending, Sankhavaram et al., 1991) and subtracted for 12S-Albumin, 2S-Albumin and the castor seed toxin/allergen ricin. Effectiveness of normalization for 12S-Albumin was a 138-fold reduction (from 10.8% down to 0.078%), for 2S-Albumin a 51-fold reduction (from 15.8% down to 0.31%) and for castor bean ricin a 34-fold reduction (from 0.3% to 0.0088%). This reflects a good degree of normalization. A RcDGAT2 orthologue was identified from the in house *R. communis* EST database via BLAST searches against public databases. EST clones with grid number pRN16N10 and pRN1J09 were completely sequenced in both directions and the open reading frame (ORF) prediction confirmed the initial EST search results in that the encoded product is structurally similar to *A. thaliana* DGAT2 (At3g51520).

3.3. Southern blot analysis

Castor bean genomic DNA was isolated from young leaves using a modified CTAB (*N*-acetyl-*N*,*N*,*N*-trimethylammonium bromide) procedure as follows: 100 μl of 5% SDS, 500 μl of extraction buffer (0.2 M Tris–HCl, pH8.5; 2.5 mM NaCl; 25 mM EDTA, pH 8.0; 0.5% SDS) and 5 μl β-mercaptoethanol were added to 0.5 g frozen fine powder of young leaf followed by addition of 500 μl of phenol/chloroform/iso-amylalcohol (25:24:1) with vigorous shaking. The mixture was centrifuged for 20 min at 13,000 rpm. An equal volume of iso-propanol was added to the aqueous phase and maintained on ice for 30 min. After centrifugation for 10 min at 13,000 rpm., the pellet was dissolved in 600 μl CTAB buffer (100 mM Tris–HCl, pH 7.5; 0.7 M NaCl; 10 mM EDTA, pH 8.0; 1% CTAB; 1.4 mM β-mercaptoethanol). After addition of 600 μl of chloroform/iso-amylalcohol (24:1) and centrifugation, the aqueous phase was mixed with 200 μl distilled H_2O and 10 μl RNase A and incubated at 40 °C for 30 min followed by chloroform/iso-amylalcohol (24:1) and iso-propanol procedures to purify DNA. Castor genomic DNA was digested by restriction enzymes and was separated on a 0.8% Tris–acetic acid EDTA (TAE) agarose gel followed by blotting to a positively charged nylon membrane (Hybond-N; Amersham). The filter was hybridized to a ^{32}P-labeled DNA probe representing the protein encoding region of RcDGAT2 cDNA by PCR using primers, pW1 (5′-d(TAGCGTTGAGCATATGGATTGG)-3′) and pW2 (5′-d(CTTTAATGTCCGCAACAAACTG)-3′). Southern blot analysis was conducted according to Sambrook et al. (1989) followed by high stringency washes (0.1 × SSC/0.1% SDS, 65 °C).

3.4. Quantitative real time PCR

Total RNA from leaves and endosperm from castor seed at different developmental stages was extracted and purified with the RNeasy Mini Kit (Qiagen, UK). First strand cDNA synthesis was performed in a standard reaction using 5 μg of total RNA in combination with oligo(dT)$_{12–18}$ primer (Promega, UK) in a 20 μl volume using Superscript III at 50 °C for 1 h. Real time PCR was performed at least in triplicate using the Roto-Gene RG-3000 (Corbett Research) with 2 μl of a 1:4 dilution of a standard cDNA synthesis reaction in combination with SYBR Green I dye as fluorescence indicator. Gene specific primers were designed to generate PCR products of lengths between 150 and 250 bp. The primers for RcDGAT1 were 5′-(dGAGTTGTGCATTGCTGTTCCTTGCC)-3′ and 5′-(dCTGGACTGAGCCCATGGTTCAGTTCC)-3′, for RcDGAT2 were 5′-(dACGTCCGATGCATGTTGTCGTCG)-3′ and 5′-(dTTCAAGTGTAAGGTCTGCATAGC)-3′.

3.5. Expression of DGAT1 and DGAT2 in yeast cells

The RcDGAT1 and RcDGAT2 coding region was subcloned into the pYES2.1/V5-His-TOPO vector (Invitrogen) and transformed into *S. cerevisiae* strain INVSc1. The primers used were pRcD1-F (5′-d(G<u>ACC**ATGG**</u>GGATTCTCGAAACGCC)-3′) and pRcD1-R (5′-d(GTTCCCATCGCGATTCATTAGG)-3′) for RcDGAT1 and pRcD2-F (5′-d(G<u>ACCATGG</u>GGGAAGAAGCGAATCAT)-3′) and pRcD2-R (5′-d(AAGAATTTCAAGTGTAAGGTCTGC)-3′) for RcDGAT2. In the amplified DNA we incorporated a yeast consensus sequence (the Kozak sequence (G/A)NNATGG is underlined) for initiation of translation and this led to a change of the second amino acid in RcDGAT1 (T–G). The translation stop sites (TGA) were removed to clone in frame with the C-terminal V5 epitope and polyhistidine tag for

detection and purification of the fusion PCR product. The expression of the recombinant proteins in yeast cells was carried out according to the manufacturer's instructions and cells were harvested at 14–16 h after induction and cell pellets were snap-frozen in N_2(l) followed by storage at −80 °C. Microsomes were isolated from harvested yeast cells grown in 1400 ml induced cultures. The cells were resuspended in HB buffer containing 0.1 M Tris–HCl, pH 7.0, 20% glycerol and EDTA-free protease inhibitor cocktail (complete min; Roche) and disrupted using 0.5 mm beads according to operating instructions of the Bead-Beater (BioSpec Products, Inc.).The beads were washed with HB buffer and the suspension was centrifuged at 12,000*g* for 10 min. Microsomal membranes were prepared by ultracentrifugation of the supernatant at 100,000*g* for 1 h and resuspension of the pellet in HB buffer or alternatively HB buffer without glycerol. Protein concentrations were measured using the Bio-Rad protein assay kit (Bio-Rad).

3.6. Western blot analysis

The recombinant proteins were detected by Western blotting of the yeast microsomal protein samples (usually 20 μg), by separation via SDS-PAGE (10% acrylamide) and electroblotting onto Hybond ECL nitrocellulose membranes (Amersham). For immunodetection the membranes were incubated with the Anti-V5-HRP Antibody (Invitrogen) at 1:5000. The horseradish peroxidise (HRP)-conjugated primary antibody allows one-step detection using chemiluminescent detection with Supersignal West Pico Chemiluminescent Substrate (Pierce).

3.7. Assay for DGAT2 activity

DGAT activity was assayed by incorporation of [1-^{14}C]-ricinoleoyl-CoA into *sn*-1,2-diricinolein to form triricinolein. [1-^{14}C]-ricinoleoyl-CoA was synthesised in by using Acyl-Coenzyme A Synthetase (ACS) (Sigma, UK) in a reaction with [1-^{14}C]-ricinoleic acid (American Radiolabeled Chemicals Inc.) and Coenzyme A. *Sn*-1,2-diricinolein was prepared by lipase catalyzed methanolysis of tricinolein (Turner et al., 2003). The microsomes were freeze-dried after removal of the glycerol via ultra-centrifugation, followed resuspension in benzene solution containing *sn*-1,2-diricinolein. This mixture was sonicated for 30 s on ice cold water and the benzene was evaporated immediately under a N_2 flow. The assay reaction volume (100 μl) includes 50 μg of a microsome/0.5 mM diricinolein mixture, 100 mM Tris–HCl, pH 7.5; 1 mg/ml BSA; 8 mM $MgCl_2$; 20% glycerol. After sonication for 60 s, the reaction was started by adding 20 μM [1-^{14}C]-ricinoleoyl-CoA. Throughout the assay was conducted at 30 °C in a water bath with constantly shaking for 15 min and terminated by adding 720 μl of chloroform/methanol (1:1) and 280 μl of 1 M KCl/0.2 M H_3PO_3. The entire organic phase was dried under N_2 flow and resuspended in 30 μl of chloroform. Lipids were separated by thin-layer chromatography (TLC) with hexane/diethyl ether/acetic acid (50:50:1, v/v/v). The TLC plates were analysed by exposure to Phospho-imaging screens and the image was scanned and quantified (pixel density) using the Typhoon 9400 scanner and Imagequant software (GE Healthcare). The radioactive triricinolein (RRR) product bands were localised by alignment with validated lipid standards after iodine spray. The RRR bands were scraped off the TLC plate and the amount of radioactivity was determined via liquid scintillation counting.

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