Available online at www.sciencedirect.com
ScienceDirect
Phytochemistry 69 (2008) 1119–1127
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Cloning and functional analysis of two type 1 diacylglycerol acyltransferases from *Vernonia galamensis*

Keshun Yu [a,1], Runzhi Li [a,1], Tomoko Hatanaka [b], David Hildebrand [a,*]

[a] *Department of Plant and Soil Science, University of Kentucky, 1405 Veterans Drive, Lexington, KY 40546-0091, USA*
[b] *Kobe University, Kobe 657-8501, Japan*

Received 6 September 2007; received in revised form 17 November 2007
Available online 7 January 2008

Abstract

Vernonia galamensis accumulates vernolic acid (*cis*-12-epoxyoctadeca-*cis*-9-enoic acid) as the major fatty acid in its seed oil. Such epoxy fatty acids are useful in a number of industrial applications. Successful genetic engineering of commercial oilseed crops to produce high levels of vernolic acid depends on a better understanding of the source plant enzymes for vernolic acid accumulation. Developing *V. galamensis* seed microsome assays demonstrate that diacylglycerol acyltransferase (DGAT), an enzyme for the final step of triacylglycerol synthesis, has a strong substrate preference for vernolic acid bearing substrates including acyl-CoA and diacylglycerol. There are two classes of DGATs known as DGAT1 and DGAT2. Here we report on the isolation, characterization, and functional analysis of two *DGAT1* cDNAs from *V. galamensis* (*VgDGAT1a* and *VgDGAT1b*). *VgDGAT1a* and *VgDGAT1b* are expressed in all plant tissues examined with highest expression in developing seeds. Enzymatic assays using isolated microsomes from transformed yeast show that VgDGAT1a and VgDGAT1b have the same DGAT activity levels and substrate specificities. Oleoyl-CoA and *sn*-1,2-dioleoylglycerol are preferred substrates over vernoloyl-CoA and *sn*-1,2-divernoloylglycerol. This data indicates that the two VgDGAT1s are functional, but not likely to be responsible for the selective accumulation of vernolic acid in *V. galamensis* seed oil.

Keywords: *Vernonia galamensis*; Asteraceae; Gene expression; Enzyme activity; Triacylglycerol metabolism; Substrate specificity; Vernolic acid; Diacylglycerol acyltransferase

1. Introduction

Vernolic acid (**1**) can accumulate up to 70–80% of total fatty acids in *Vernonia galamensis* seed oil (Carlson et al., 1981). This unusual fatty acid (Fig. 1) bearing an epoxy bond across the 12, 13 carbon positions is a valuable compound in a number of industrial applications (Carlson et al., 1981; Ohlrogge, 1994; Zhu et al., 2002). The current commercial supply of epoxy fatty acids, such as vernolic acid (**1**), is limited. There has been considerable interest in genetic engineering of oil crops to produce high levels of vernolic acid (**1**) (Kinney, 2002). However, there is only limited information as to how plants such as *V. galamensis* accumulate high levels of it. It was found that epoxygenase converts linoleic acid (**2**) in form of *sn*-2-linoleoylphosphatidylcholine (PC) (**3**) into *sn*-2-vernoloyl-PC (**4**) in *V. galamensis* (Bafor et al., 1993). The *V. galamensis* epoxygenase gene was subsequently cloned (Hitz, 1998). Epoxygenase genes from *Crepis* and *Stokesia* have also been cloned (Lee et al., 1998; Hatanaka et al., 2004). Only low levels of vernolic acid (**1**) are found in transgenic Arabidopsis and soybean seeds using epoxygenases alone (Hatanaka et al., 2004; Kinney, 2002). Thus, a better understanding of the mechanism for the effective channeling or selective accumulation of vernolic acid (**1**) into triacylglycerols (TAGs) is needed.

Three enzymes have been either identified or hypothesized for the final step of fatty acid accumulation in

* Corresponding author. Tel.: +1 859 257 5020x80760; fax: +1 859 257 7874.
E-mail address: dhild@uky.edu (D. Hildebrand).
[1] These authors contributed equally to this work.

0031-9422/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2007.11.015

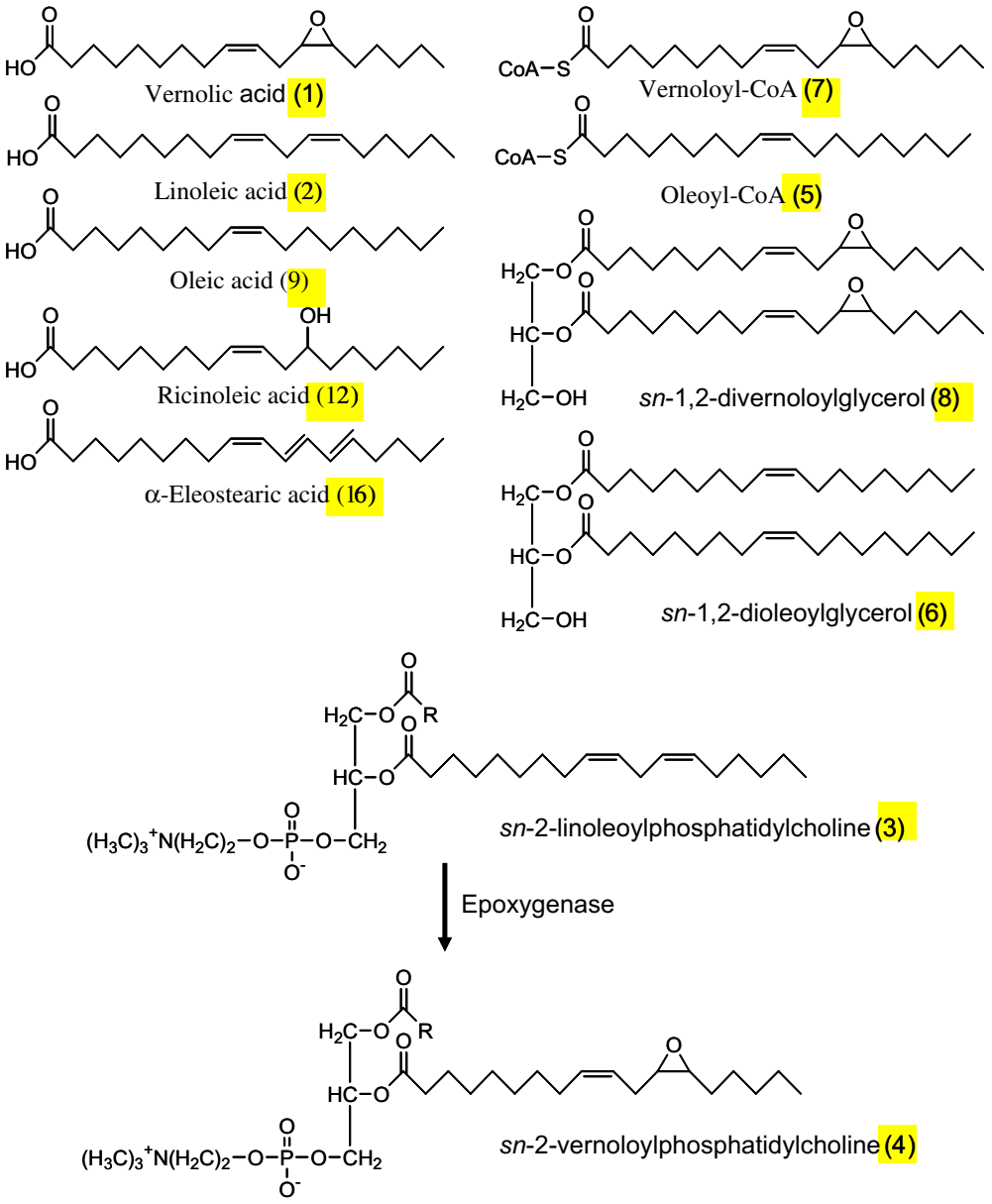

Fig. 1. Chemical structures of vernolic acid (**1**), linoleic acid (**2**), oleic acid (**9**), ricinoleic acid (**12**), α-eleostearic acid (**16**), vernoloyl-CoA (**7**), oleoyl-CoA (**5**), *sn*-1,2-diovernoloylglycerol (**8**), *sn*-1,2-dioleoylglycerol (**6**) and the epoxygenase catalyzed reaction from *sn*-2-linoleoylphosphatidylcholine (**3**) to *sn*-2-vernoloylphosphatidylcholine (**4**). R: fatty acyl chain.

TAG:acyl-CoA:diacylglycerol acyltransferase (DGAT, EC 2.3.1.20), phospholipid:diacylglycerol acyltransferase (EC 2.3.1.158) and diacylglycerol transacylase (Weselake, 2002). Our previous studies using developing seed microsomal assays showed that DGATs from *V. galamensis* and *Stokesia laevis* have strong substrate preferences for vernolic acid (**1**) bearing substrates including acyl-CoA and diacylglycerols (DAGs) (Yu et al., 2006). There are two types of non-homologous DGAT genes designated as *DGAT1* and *DGAT2* encoding endoplasmic reticulum (ER) membrane-bound enzymes in plants (He et al., 2004; Kroon et al., 2006; Lardizabal et al., 2001; Shockey et al., 2006; Zou et al., 1999) and animals (Cases et al., 1998, 2001). Other *DGAT* genes found to date include *DGAT2* from yeast (Sorger and Daum, 2002) and a *DGAT* gene from peanuts encoding a cytosolic soluble enzyme (Saha et al., 2006).

In the present study, we report the cloning, characterization, and functional analysis of two *V. galamensis DGAT1* genes.

2. Results and discussion

2.1. Isolation of two cDNA clones encoding type 1 DGAT from Vernonia galamensis

For the cloning of *V. galamensis DGAT*, conserved regions of *DGAT1*s were identified by alignment of

deduced amino acid sequences from different species, and degenerate primers were designed based on DNA sequences of the conserved regions. Two DNA fragments of 384 and 278 bp were amplified from *V. galamensis* developing seeds by degenerate PCR and sequenced. Based on the sequence information, gene-specific primers for 3′- and 5′-RACE were generated, yielding two full-length cDNAs named as *VgDGAT1a* and *VgDGAT1b*, respectively.

Sequence analysis indicates that *VgDGAT1a* is 1828 bp in length with 130 bp 5′- and 126 bp 3′-untranslated regions (GenBank EF653276). This cDNA contains an ORF of 1572 bp encoding a protein of 523 amino acids. The full-length of *VgDGAT1b* is 1738 bp containing 46 bp of the 5′-leader sequence and 138 bp of the 3′-untranslated region (GenBank EF653277). The 1554 bp ORF of *VgDGAT1b* is predicted to encode a protein of 517 amino acids. The predicted *Mr* and calculated isoelectric points are 60.2 kDa and 8.1 for *VgDGAT1a*, 59.6 kDa and 8.2 for *VgDGAT1b* (Protparam: http://www.expasy.ch), respectively. The two *DGAT1*s share 92% similarity in nucleotide sequence and 94% identity in deduced amino acid sequence. Phylogenetic tree analysis of deduced amino acid sequences from *VgDGAT1a* and *VgDGAT1b* and other known cDNA clones of *DGAT1* isolated from different organisms after a BLAST search indicates that both VgDGAT1a and VgDGAT1b are grouped together with all known DGAT1s except OsDGAT1 (*Oryza sativa*, rice) which is on the root of the tree (Fig. 2). Alignment of the deduced amino acid sequences of VgDGAT1s and other DGAT1s from *Arabidopsis*, tobacco and castor showed that the proteins share high identity (50% or more) (Fig. 3). The C-terminal regions (65.1% identity within 409 amino acids) are much more conserved than the N-terminal regions (6.3% identity in the first 112 amino acids) (Fig. 3).

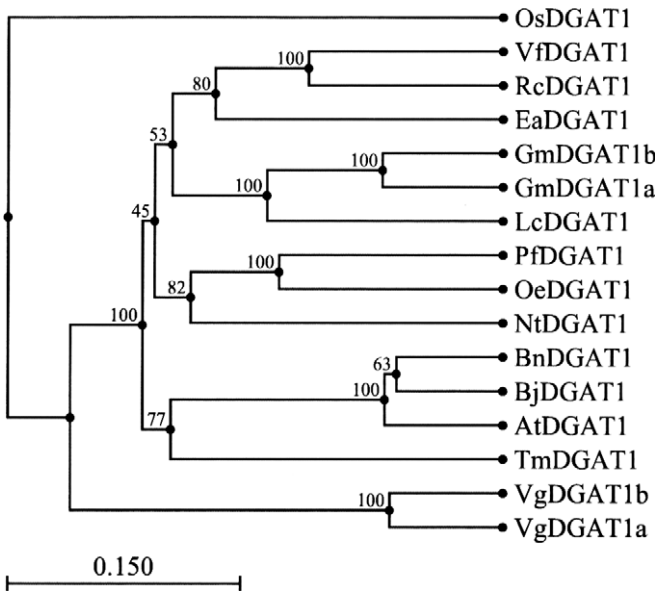

Fig. 2. Phylogenetic tree showing relationships among predicted protein sequences from full-length *DGAT1 cDNA*s of various plant species. The tree was generated by CLC Combined Workbench 2 using Unweighted Pair Group Method using Arithmetic averages (UPGMA) after a slow (accurate) alignment procedure. Bootstrap values are shown on the corresponding nodes. The GeneBank accession numbers for the listing DGAT1s are as follows: AtDGAT1 from *Arabidopsis thaliana*, AJ131831; BnDGAT1 from *Brassica napus*, AAF64065; BjDGAT1 from *Brassica juncea*, DG016106; TmDGAT1 from *Tropaeolum majus*, AY084052; PfDGAT1 from *Perilla frutescens*, AF298815; OeDGAT1 from *Olea europaea*, AAS01606; NtDGAT1 from *Nicotiana tabacum* (tobacco) AAF19345; VfDGAT1 from *Vernicia fordii* (tung), DQ356680; RcDGAT1 from *Ricinus communis* (castor), AY366496; EaDGAT1 from *Euorymus alatus*, AY751297; GmDGAT1a and 1b from *Glycine max* (soybean), AB257589 and AF257090; LcDGAT1 from *Lotus corniculatus*, AAW51456; OsDGAT from *Oryza sativa* (rice), AAW47581; VgDGAT1a and 1b from *Vernonia galamensis*, EF653277 and EF653276.

2.2. Identification of putative functional motifs in VgDGAT1s

In plants, DGAT1 has been localized to the ER (Lacey and Hills, 1996; Settlage et al., 1995) where the Kennedy pathway mainly occurs. A Kyte and Doolittle hydrophobicity plot suggests that VgDGAT1s contain a number of membrane spanning domains with a large hydrophilic domain at the N-terminus (Fig. 4). Using a program of Transmembrane Helices Prediction (http://npsa-pbil.ibcp.fr), nine potential transmembrane helices are strongly predicted (Fig. 4). It also predicts a large N-terminal domain lying on the cytoplasmic side of the membrane (Fig. 4). The most notable structures that VgDGAT1s share in common with other plant DGAT1s cloned to date are the multiple transmembrane domains in the C-terminal conserved regions, consistent with an integral membrane enzyme.

Scanning the protein sequence against the Prosite database (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_proscan.html) identified a number of putative functional motifs including *N*-glycosylation, cAMP- and cGMP-dependent protein kinase phosphorylation, protein kinase C phosphorylation, casein kinase II phosphorylation and *N*-myristoylation sites as well as leucine zipper pattern (Table 1). It remains to be determined whether these sites are important in the regulation of the functions of the enzyme *in vivo*. By comparing the VgDGAT1s with other plant DGAT1s, a MBOAT (membrane bound *O*-acyltransferase) domain (amino acid 211–496 in VgDGAT1a and 197–482 in VgDGAT1b) was found. This domain is possibly involved in acyl-transfer (Hofmann, 2000). Also detected is the presence of the putative C-terminal ER retrieval motifs in VgDGAT1s (–YYHDV–, YYHEV–) and as was found in other plant DGAT1s (tobacco DGAT1, –YYHDV–; Arabidopsis DGAT1 and castor DGAT1, –YYHDL–). These putative ER retrieval motifs (–Φ–X–X–K/R/D/E–Φ–COOH, where Φ is any large hydrophobic amino acid residue) are positioned at the extreme C-termini and very likely serve as general ER localization signals (McCartney et al., 2004). Another conserved feature is an invariant serine among the shown DGAT1s at positions 249 and 244 in VgDGAT1a and

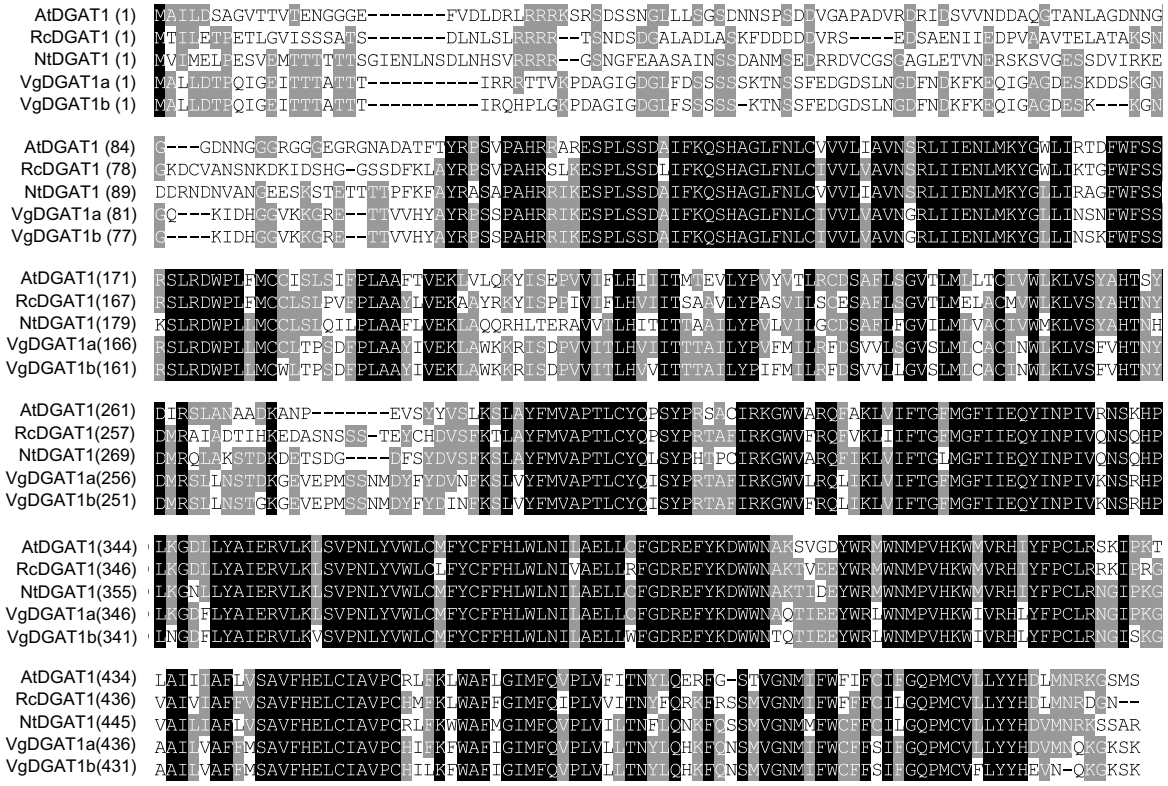

Fig. 3. Alignment of deduced amino acid sequences of type 1 diacylglycerol acyltransferases (DGAT1s). Alignments were generated by Vector NTI (v. 9) (Invitrogen). The identical amino acid residues between the five DGAT1s are shaded black and gray shading is the consensus of two or more sequences. The Genbank accession numbers for the DGAT1s are *Arabidopsis thaliana* AtDGAT1, NM_127503; *Nicotiana tabacum* NtDGAT, AF129003; *Ricinus communis* RcDGAT1, AY366496; *Vernonia galamensis* VgDGAT1a, EF653276 and VgDGAt1b, EF653277.

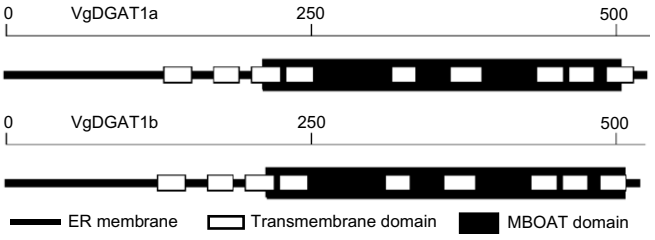

Fig. 4. Putative transmembrane domains in *VgDGAT1*s. The main transmembrane segments were predicted by Transmembrane alpha-helix predictor software (Localizome, http://localodom.kobic.re.kr/LocaloDom/index.htm) (Lee et al., 2006). MBOAT: membrane bound *O*-acyltransferase.

1b, respectively (Fig. 3). The serine residue has been shown to be essential for the activities of acyl-CoA: cholesterol acyltransferases, a closely related enzyme to DGAT1 (Joyce et al., 2000).

2.3. Genomic organization and expression analysis of *VgDGAT1s*

To determine the copy number of the *VgDGAT1* gene in *V. galamensis* plants, we performed genomic Southern blot analysis under high-stringency hybridization conditions. The results from the restriction enzyme digestion pattern of BamHI, EcoRI, HindIII and NotI suggest that *VgDGAT1* is a multiple-copy gene in this genome, at least two (Fig. 5). Since *VgDGAT1a* and *VgDGAT1b* do not have any BamHI site, it is possible that another *VgDGAT1* is present in *V. galamensis*. However, during the cloning of *VgDGAT1a* and *VgDGAT1b*, we amplified and sequenced one additional fragment and did not detect any other unique sequences indicating the presence of additional *VgDGAT1*s. The one additional fragment amplified (degenerate PCR product) is 296 bp in length. It shares 59% and 63% identity with *VgDGAT1a* and *VgDGAT1b* and the sequence differences with *VgDGAT1a* and *VgDGAT1b* are 123 and 111 bp. Further studies are needed to determine whether there are additional *DGAT1*s in the *V. galamensis* genome and contributions of specific DGATs in seed oil biosynthesis.

To further investigate the potential role of *V. galamensis* DGAT1s in TAG biosynthesis we analyzed the temporal and tissue-specific expression patterns of both *VgDGAT1a* and *VgDGAT1b* transcripts. Semi-quantitative reverse transcription (RT)-PCR was employed to monitor the level of *VgDGAT1a* and *VgDGAT1b* expression using an equal amount of total RNA from the sample tissues. The *actin* gene, a housekeeping gene was used as an internal control. The levels of both *VgDGAT1a* and *VgDGAT1b* mRNAs are much higher in embryo tissue than in root, stem, leave and fruit coat (pericarp) (FC) where their expression levels are lower, except for a slightly higher expression of *VgD-*

Table 1
Putative functional motifs in VgDGAT1s

Functional site	VgDGAT1a		VgDGAT1b	
	Position	Amino acid	Position	Amino acid
N-Glycosylation	46–49	NSSF	45–48	NSSF
	262–265	NSTD	257–260	NSTG
cAMP-/cGMP-dependent protein kinase phosphorylation [RK](2)-x-[ST]	21–24	RRRT	192–195	RIS
	22–25	RRTT		
	197–200	KRIS		
Protein kinase C phosphorylation [ST]-x-[RK]	19–21	TIR	19–21	TIR
	25–27	TVK	41–43	SSK
	42–44	SSK	72–74	SKK
	164–166	SSR	159–161	SSR
	167–169	SLR	162–164	SLR
	264–266	TDK	259–261	TGR
Casein kinase II phosphorylation [ST]-x(2)-[DE]	47–50	SSFE	46–49	SSFE
	48–51	SFED	47–50	SFED
	73–76	SKDD	162–165	SLRD
	167–170	SLRD	174–177	TPSD
	179–182	TPSD	248–251	TNYD
	253–256	TNYD	269–272	SNMD
	274–277	SNMD	398–401	TIEE
	403–406	TIEE		
N-Myristoylation G-{EDRKHPFYW}-x(2)-[STAGCN]-{P}	31–36	GIGDGL	31–36	GIGDGL
	35–40	GLFDSS	35–40	GLFSSS
	88–93	GVKKGR	83–88	GVKKGR
	154–159	GLLINS	149–154	GLLINS
	431–436	GIPKGA	426–431	GISKGA
	503–508	GQPMCV	498–503	GQPMCV
Leucine zipper pattern L-x(6)-L-x(6)-L-x(6)-L	217–238	LYPVFM-ILRFDS-VVLSGV-SLML	212–233	LYPIFM-ILRFDS-VVLLGV-SLML
	224–245	LRFDSV-VLSGVS-LMLCAC-INWL	219–240	LRFDSV-VLLGVS-LMLCAC-INWL

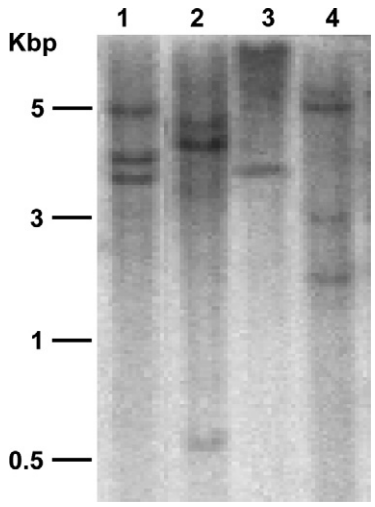

Fig. 5. Southern blot analysis of *Vernonia galamensis* genomic DNA. *V. galamensis* genomic DNA (7 μg/lane) was digested with BamH1 (1), EcoR1 (2), HindIII (3) and Not1 (4). The DNA blot was hybridized with a dioxigenin-labeled *cDNA* encoding the ORF of *VgDGAT1* as a prob. The blot was washed at high stringency after hybridization at 0.1× SSC/0.1% SDS at 65 °C.

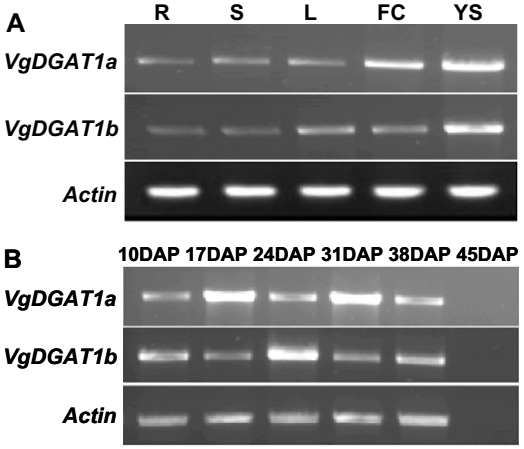

Fig. 6. Semi-quantitative RT-PCR analysis of *VgDGAT1* gene expression in different organs and seed developmental stages of *Vernonia galamensis* plants. (A) *VgDGAT1* gene expression in root (R), stem (S), leaf (L), fruit coat (FC) and young seeds (YS) at 20 days after pollination (DAP). (B) *VgDGAT1* gene expression during seed development. Total RNA were extracted from different organs and developing seeds at 10 days DAP, 17 DAP, 24 DAP, 31 DAP, 38 DAP and 45 DAP. The first strain *cDNA* was used as template to amplify the target gene. The *actin* gene was amplified as an internal control. The developmental stages of seeds are indicated in DAP.

GAT1a in FC (Fig. 6a). During *V. galamensis* seed development, *VgDGAT1a* and *VgDGAT1b* mRNA expression levels are highest at intermediate stages of development (Fig. 6b). No transcript was detected for both *VgDGAT1*s and *actin* at either stage 6 or 45 days after pollination (DAP) when the seeds were desiccating. TAG synthesis mainly occurs in seeds although fatty acid synthesis also occurs in other plant tissues. The expression analysis suggests that *VgDGAT1*s are important for TAG synthesis in developing *V. galamensis* seeds.

2.4. Yeast expression of VgDGAT1a and VgDGAT1b

VgDGAT1a and *VgDGAT1b* were expressed in yeast along with a vector control. From our preliminary study, *VgDGAT1a* and *VgDGAT1b* transformed yeast showed DGAT activity well above the vector control and the highest DGAT activities for VgDGAT1a and VgDGAT1b are from the substrate combination of [^{14}C]oleoyl-CoA (**5**) with *sn*-1,2-dioleoylglycerol (*sn*-DODAG) (**6**). In order to get more accurate microsomal DGAT assay results, the substrate combination of [^{14}C]oleoyl-CoA (**5**) and *sn*-DODAG (**6**) were used for both VgDGAT1a and VgDGAT1b to determine the linear range of microsomal protein levels for yeast microsomal assays (Fig. 7). At the levels of microsomal protein from 5 to 80 ng, VgDGAT1a

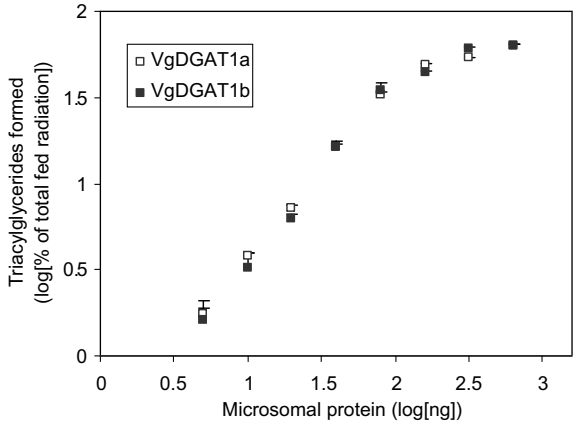

Fig. 7. [^{14}C]Triglycerides formed in yeast microsomal assays expressing VgDGAT1a and VgDGAT1b were examined at various microsomal protein levels to determine the linear range of activity. Yeast microsomes (5–640 ng protein equivalents) were administered 5 μM of [^{14}C]oleoyl-CoA together with 100 μM of *sn*-1,2-dioleoylglycerol (DODAG). Bars are means ± STD of two replicates.

and VgDGAT1b activities proportionally increased as the microsomal protein level increased as shown on a logarithmic scale on both *X* and *Y* axes. To leave some margin, the 40 ng microsomal protein level (data point #4 in Fig. 7) was used for the subsequent DGAT activity and substrate specificity analyses on VgDGAT1a and VgDGAT1b along with the vector control. This level is three magnitudes lower than the 50–100 μg used by other studies (Bouvier-Nave et al., 2000; He et al., 2004; Kroon et al., 2006). The DGAT assays using 40 ng microsomal protein equivalents have greatly reduced the background on the phosphorimages. Also the microsomes needed for each reaction is greatly reduced. The DGAT specific activities as shown in Fig. 8 are also two to four magnitudes higher than those reported in other yeast expression studies for plant DGATs. The DGAT specific activities in the present

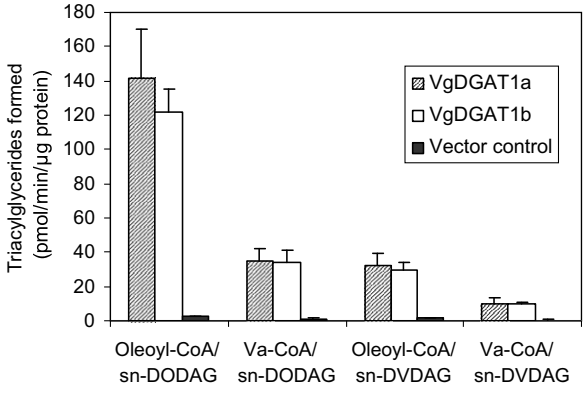

Fig. 8. [^{14}C]Triglycerides formed when microsomes from yeast expressing VgDGAT1a, VgDGAT1b and vector control (40 ng microsomal protein equivalents) were administered 5 μM of [^{14}C]oleoyl-CoA or [^{14}C]vernoloyl-CoA (Va-CoA) together with 100 μM of *sn*-1,2-dioleoylglycerol (DODAG) or *sn*-1,2-divernoloylglycerol (DVDAG). Bars are means ± STD ($n = 6$).

study are also two magnitudes higher than those from *V. galamensis* developing seed microsomes that were up to ca. 1 pmol/min/μg protein (or 10 pmol/min/nmol PC) for the optimum substrate combination of vernonoyl-CoA (Va-CoA) (**7**) with *sn*-1,2-divernoloylglycerol (*sn*-DVDAG) (**8**) (Yu et al., 2006) which is probably due to much higher proportion of VgDGAT1a and VgDGAT1b in the overall microsomal proteins when over-expressed in yeast.

The DGAT assay results in Fig. 8 shows that VgDGAT1a and VgDGAT1b exhibits much higher activity relative to the vector control. Also, no difference was found in the DGAT activity between VgDGAT1a and VgDGAT1b. The substrate specificities of VgDGAT1a and VgDGAT1b are very similar to the vector control. Specifically, the substrate combination of oleoyl-CoA (**5**) and *sn*-DODAG (**6**) has the highest activity and Va-CoA (**7**)/*sn*-DVDAG (**8**) has the lowest activity. The substrate combination of Va-CoA (**7**) and *sn*-DODAG (**6**) and the substrate combination of oleoyl-CoA (**5**) and *sn*-DVDAG (**8**) have similar activities at an intermediate level.

Our previous studies show that *V. galamensis* microsomes exhibit a substrate preference with *sn*-DVDAG (**8**) over *sn*-DODAG (**6**) and Va-CoA (**7**) over oleoyl-CoA (**5**) (Yu et al., 2006). The high preference of *V. galamensis* microsomes for Va-CoA (**7**) and *sn*-DVDAG (**8**) may be very important for vernolic acid (**1**) accumulation in its seed oil. The present study indicates that DGAT1s from *V. galamensis* have substrate specificities different from what we found from the developing seed microsome assays. Thus, barring dramatically different results from other VgDGAT1 isoforms not cloned so far, DGAT1s are not likely to have substrate selectivity for vernolic acid (**1**) accumulation into TAG in *V. galamensis*. Then, what might be the biological functions of VgDGAT1s *in vivo*? It will be interesting to see if a knockout of the VgDGAT1s would have much effect on the seed oil accumulation and other growth characteristics of the plants.

A study with castor (*Ricinus communis*) DGAT1 expressed in yeast had seven-fold higher DGAT activity compared with controls and it showed a greater preference to catalyze the transfer of oleic acid (**9**) from oleoyl-CoA (**5**) to *rac*-1,2-diricinoleoylglycerol (**10**) than to *sn*-DODAG (**6**) and *sn*-1,2-dipalmitoleoylglycerol (**11**) (He et al., 2004) which suggest that DGAT1 is important for accumulation of ricinoleic acid (**12**) into TAG in castor. However, another study in castor showed that *DGAT2* has high expression in developing seeds than in leaves, whereas *DGAT1* is evenly expressed in developing seeds and leaves suggesting a more important role for DGAT2 in TAG synthesis in the developing seeds than DGAT1 (Kroon et al., 2006). When castor DGAT2 is expressed in yeast it is able to incorporate ricinoleoyl-CoA (**13**) and *rac*-1,2-diricinoleoylglycerol (**10**) into triricinolein (**14**) as compared to the vector control although the substrate specificities were not tested for castor DGAT2 in the study. A recent study with tung tree (*Vernicia fordii*) triacylglycerol synthesis also shows that *DGAT1* is expressed at similar levels in

various organs but *DGAT2* is strongly expressed in developing seeds (Shockey et al., 2006). When expressed in yeast tung DGAT2 enhances the synthesis of trieleostearin (**15**). These results indicate that in tung, DGAT2 is of greater importance for α-eleostearic acid (**16**) accumulation in TAG. The present study on VgDGAT1a and VgDGAT1b also implicate a possible important role of DGAT2 in the synthesis of epoxy TAG in *V. galamensis*.

Although VDGAT1s showed no apparent preference for vernolic acid (**1**), they may have important functions in the developing seeds. *VgDGAT1a* and *VgDGAT1b* may not be very useful for the genetic engineering of oilseed crops for high level accumulation of vernolic acid (**1**) based on our current findings, but the very high activities exhibited by these two genes especially for oleoyl-CoA (**5**), linoleoyl-CoA (**17**) (unpublished data) and *sn*-DODAG (**6**) when expressed in yeast suggest that they might be of value for increasing oil accumulation of common oilseed crops. Seed-specific overexpression of DGAT1 from *Arabidopsis thaliana* has been reported to increase DGAT activity in developing seeds, seed oil content and seed weight (Jako et al., 2001).

3. Conclusions

Two *DGAT1 cDNA*s were cloned from *V. galamensis* and very high DGAT specific activities are shown when expressed in yeast. Expression data also support their function in fatty acid metabolism in the developing seeds. Since the two VgDGAT1s do not show any selectivity towards incorporating vernolic acid (**1**) into TAGs, they are not likely to be responsible for vernolic acid (**1**) accumulation in the seed oil.

4. Experimental

4.1. General experimental procedures

Trizol used for RNA isolation, SuperScript II RT Kit for RT-PCR analyses and yeast expression vector pYES2 were from Invitrogen, CA. Smart RACE cDNA Amplification Kit for RACE (rapid amplification of cDNA ends) was from BD Biosciences, NJ. A Gel Extraction Kit for DNA extraction from agarose gels was from Qiagen Inc., CA. The pGEM-T Easy vector for sub-cloning of cDNAs was from Promega, WI. The Big Dye Terminators v3.1 Cycle Sequencing Kit for DNA sequencing was from Applied Biosystems, CA. The PCR DIG Probe Synthesis Kit for Southern blot analyses was from Roche Applied Science, IN.

4.2. Biological materials

V. galamensis seeds were planted in a soil tray in April each year in a greenhouse and transplanted to a soil bed at the University of Kentucky in Lexington, KY with little control over weather conditions except daily watering when needed. The yeast strain INVSc1 and *Escherichia coli* strain DH5α for yeast and *E. coli* transformation were from Invitrogen, CA.

4.3. Cloning of V. galamensis DGAT1 cDNAs

We have previously cloned a full-length *DGAT1* from *V. galamensis* designated *VgDGAT1a* (Hatanaka et al., 2003). Another partial *DGAT1* cDNA from *V. galamensis* was obtained and the full-length *DGAT1* that we term *VgDGAT1b* from *V. galamensis* was also cloned. Total RNA was isolated from developing *V. galamensis* seeds at stage from 24 DAP to 31 DAP at which *DGAT1* is at its highest expression shown by expression analysis. Primers were designed from the sequence information of a partial *DGAT* cDNA fragment. The 5′- and 3′-end RACE of the cDNAs were performed using the PCR conditions described in the user manual of the kit. The amplified products were fractionated on an agarose gel, extracted from the gel and sub-cloned into the pGEM-T Easy plasmid according to the manufacturer's instructions. The cDNA inserted was sequenced in both directions. Gene-specific primers were designed and PCR was employed to clone the full-length cDNA sequence of *VgDGAT1b*. Database searches were done using the BLAST program at the National Center of Biotechnology Information. Phylogenetic tree analysis was performed by using CLC Combined Workbench 2 (CLC Bio, Aarhus, Denmark). For the phylogenetic tree generation, an Unweighted Pair Group Method using Arithmetic averages (UPGMA) was used after a slow (accurate) protein sequence alignment. DNA sequence alignment and similarity amongst species were determined by ClustalW (http://clustalw.genome.jp/) while protein sequence alignment and similarity were determined by Vector NTI (v.9) (Invitrogen). The protein motifs were identified using PROSITE scan at http://ca.expasy.org/tools/#pattern and Localizome at http://localodom.kobic.re.kr/LocaloDom/index.htm.

4.4. Southern blot analysis

Genomic DNA of *V. galamensis* was isolated from young leaves using a modified CTAB (*N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide) procedure as described previously (Hatanaka et al., 2004). Aliquots of genomic DNA (10 μg) was digested overnight with four restriction enzymes, BamH1, EcoRI, HindIII and Not1, individually. The digested DNA was fractionated in a 0.8% (w/v) agarose gel and transferred to a positively charged nylon membrane (Hybond N+, Amersham Biosciences, NJ) overnight in 20× SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0). The membrane was hybridized to a digoxigenin (DIG)-labeled probe representing the protein encoding region of *VgDGAT1* cDNA. The membrane was washed with 2× SSC, 0.1% SDS; 0.2× SSC, 0.1% SDS and 0.1× SSC, 0.1% SDS for 15 min at 65 °C. The hybridized DNA was

detected with alkaline phosphatase conjugated anti-DIG antibody and its chemiluminescent substrate, CDP-Star, following the manufacturer's protocol.

4.5. Semi-quantitative RT-PCR

Total RNA was isolated from roots, stems, leaves, fruit coats and developing seeds at six developmental stages using the Trizol reagent according to manufacturer's instructions. After extraction, RNA samples were treated with DNaseI (Promega) to remove contaminating DNA. First-strand cDNA was synthesized using equal amounts of RNA as templates following the manufacturer's instructions. PCR controls were performed in the absence of added reverse transcriptase to ensure RNA samples were free of DNA contamination. The first-strand cDNA (5 μl) was used to amplify the target cDNA. The primers for specific amplification of each *VgDGAT1* cDNA were designed to amplify the target cDNA at approximately 500 bp in length. The primers for *VgDGAT1a* were 5′-CCACCACAACTATAAGACGGCGGACCACTGT-3′ (forward) and 5′-CTGAATCGAACCTCAGAATCATGAAGACCGG-3′ (reverse). The primers for *VgDGAT1b* were 5′-CGGCTGTGGTTTCCTTTCCAACATTTCTACG-3′ (forward) and 5′-GGCGAGGGGGAAGTCGGAGGGGGTCAGCCAA-3′ (reverse). The primers for the *actin* gene were 5′-AGGGGATAACCACCCCATGAATCCA-3′ (forward) and 5′-TGCATGGTCTCCTGATACGGCCAAG-3′ (reverse). RT-PCR was performed for 30 cycles with an annealing temperature of 63 °C. One-tenth of the RT-PCR product was analyzed on a 1% agarose gel.

4.6. Yeast vector construction and transformation

VgDGAT1a and *VgDGAT1b* were cloned into pYES2 as follows: primers containing the restriction sites in pYES2 multiple cloning sites in the correct direction were designed to amplify the genes of interest by PCR. The yeast plasmid and the amplified gene fragments were digested with the same pairs of restriction enzymes. The digested plasmid and gene fragments were gel purified and used for ligation reactions. The ligation mixtures were used to transform *E. coli*. The recovered constructs from *E. coli* were sequenced to confirm the inserted genes of interest. The constructs were used to transform yeast according to Gietz and Woods (2002) or Dohmen et al. (1991). The transformed yeast was confirmed using a plasmid rescue technique according to Jones (2001).

4.7. Yeast microsome extraction

Yeast microsomal fractions from transformed yeast including VgDGAT1a and VgDGAT1b along with the vector control were prepared according to Bouvier-Nave et al. (2000) and Urban et al. (1994) with some modifications. A single colony from a spread culture plate in selective media was picked to inoculate 2 ml of Yeast Minimum Medium with glucose and incubated at 28 °C with shaking at 270 rpm for 2 days. One milliliter of the culture was used to inoculate 100 ml of the same medium and incubated for 3 days at the same conditions. The cells were harvested, washed with autoclaved Milli-Q H_2O (150 ml) and used to inoculate Yeast Complete Medium (200 ml) with galactose which was subsequently incubated at the same conditions for 22 h to obtain large cell mass. The cells were harvested, washed with Buffer A (50 mM Tris–HCl, pH 7.4, 2 mM EDTA), re-suspended in buffer A with 100 mM 2-mercaptoethanol (0.5 g wet cells/ml) and left at room temperature for 10 min. The cells were harvested and re-suspend in 5 ml ice-cold Buffer B (50 mM Tris–Cl pH 7.4, 2 mM EDTA, 0.6 M sorbitol) in a 50 ml centrifuge tube and 1% BSA was added based on the total volume of the cells and buffer. Glass beads (ca. 8 ml) were added and each centrifuge tube was vigorously vortexed for 20 min in 30 s time periods. The centrifuge tubes were kept in ice for at least 30 s between two vortexing periods. The lysate was transferred to a new centrifuge tube using Buffer B washes to obtain a combined volume of ca. 40 ml and was centrifuged at 12,000*g* for 30 min at 4 °C. The resulting supernatant was centrifuged at 100,000*g* for 60 min at 4 °C and the pellet was re-suspended and homogenized in Buffer C (100 mM Tris–Cl pH 7.0, 20% glycerol) at ca. 1 ml/1 g cell pellet. The microsomes were stored in 50 μl aliquots at −80 °C. Microsomal protein concentrations were determined by a modified Lowry method according to Wang (2005).

4.8. Yeast microsomal assay for DGAT activity

Yeast microsomal DGAT assay materials, equipment, and conditions were as previously described for the developing seed microsomal assays (Yu et al., 2006) with some modifications described as follows. The substrate combinations used for the yeast microsomal assays were [^{14}C]oleoyl-CoA (**5**) or [^{14}C]Va-CoA (**7**) with either *sn*-DODAG (**6**) or *sn*-DVDAG (**8**). From a preliminary study, we found that the highest DGAT activity for VgDGAT1a and VgDGAT1b was from the substrate combination of [^{14}C]oleoyl-CoA (**5**) with *sn*-DODAG (**6**). Therefore this substrate combination was used to determine the linear range of microsomal protein levels for our yeast microsomal assays. The following microsomal protein levels for each assay were used for the linear range determination: 5, 10, 20, 40, 80, 160, 320 and 640 ng. Since we found that the level of 40 ng microsomal protein was within the linear range of DGAT activity response for the highest substrate combination, it was used for all the other microsomal DGAT assays. The lipids from microsomal assay reactions were extracted and analyzed as previously described (Yu et al., 2006). For the linear range of microsomal protein level analysis, there were two replicates for each treatment. For all other microsomal DGAT assays, there were two

replicates for each treatment and the assays were performed three times. Statistical analyses were as previously described (Yu et al., 2006).

Acknowledgements

This work was supported by the United Soybean Board, the Kentucky Soybean Promotion Board, the Kentucky Science & Engineering Foundation, Ashland Inc., and the Consortium for Plant Biotechnology Research.

References

Bafor, M., Smith, M.A., Jonsson, L., Stobart, K., Stymne, S., 1993. Biosynthesis of vernoleate (*cis*-12-epoxyoctadeca-*cis*-9-enoate) in microsomal preparations from developing endosperm of *Euphorbia lagascae*. Arch. Biochem. Biophys. 303, 145–151.

Bouvier-Nave, P., Benveniste, P., Oelkers, P., Sturley, S.L., Schaller, H., 2000. Expression in yeast and tobacco of plant cDNAs encoding acyl CoA:diacylglycerol acyltransferase. Eur. J. Biochem. 267, 85–96.

Carlson, K.D., Schneider, W.J., Chang, S.P., Princen, L.H., 1981. *Vernonia galamensis* seed oil: a new source for epoxy coatings. Am. Oil Chem. Soc. Monogr. 9, 297–318.

Cases, S., Smith, S.J., Zheng, Y., Myers, H.M., Lear, S.R., Sande, E., Novak, S., Collins, C., Welch, C.B., Lusis, A.J., Erickson, S.K., Farese Jr., R.V., 1998. Identification of a gene encoding an acyl CoA:diacylglycerol acyltransferase, a key enzyme in triacylglycerol synthesis. Proc. Natl. Acad. Sci. U.S.A. 95, 13018–13023.

Cases, S., Stone, S.J., Zhou, P., Yen, E., Tow, B., Lardizabal, K.D., Voelker, T., Farese Jr., R.V., 2001. Cloning of DGAT2, a second mammalian diacylglycerol acyltransferase, and related family members. J. Biol. Chem. 276, 38870–38876.

Dohmen, R.J., Strasser, A.W.M., Hoener, C.B., Hollenberg, C.P., 1991. An efficient transformation procedure enabling long-term storage of competent cells of various yeast genera. Yeast 7, 691–692.

Gietz, R.D., Woods, R.A., 2002. Transformation of yeast by the Liac/SS carrier DNA/PEG method. Method Enzymol. 350, 87–96.

Hatanaka, T., Shimizu, R., Hildebrand, D.F., 2004. Expression of a *Stokesia laevis* epoxygenase gene. Phytochemistry 65, 2189–2196.

Hatanaka, T., Yu, K., Hildebrand, D.F., 2003. Cloning and expression of a *Vernonia* and *Euphorbia* diacylglycerol acyltransferase cDNAs. In: Murata, N., Yamada, M., Nishida, I., Okuyama, H., Sekiya, J., Hajime, W. (Eds.), Advanced Research on Plant Lipids. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 155–158.

He, X., Turner, C., Chen, G.Q., Lin, J.T., McKeon, T.A., 2004. Cloning and characterization of a cDNA encoding diacylglycerol acyltransferase from castor bean. Lipids 39, 311–318.

Hitz, W.D., 1998. Fatty acid modifying enzymes from developing seeds of *Vernonia galamensis*. United States Patent 5,846,784, December 8.

Hofmann, K., 2000. A superfamily of membrane-bound *O*-acyltransferases with implications for wnt signaling. Trends Biochem. Sci. 25, 111–112.

Jako, C., Kumar, A., Wei, Y., Zou, J., Barton, D.L., Giblin, E.M., Covello, P.S., Taylor, D.C., 2001. Seed-specific over-expression of an Arabidopsis cDNA encoding a diacylglycerol acyltransferase enhances seed oil content and seed weight. Plant Physiol. 126, 861–874.

Jones, M., 2001. Isolation of plasmid DNA from yeast using the QIAPrep Spin Miniprep Kit. QIAGEN. http://www1.qiagen.com/literature/protocols/pdf/PR04.pdf (cited April 6, 2007).

Joyce, C.W., Shelness, G.S., Davis, M.A., Lee, R.G., Skinner, K., Anderson, R.A., Rudel, L.L., 2000. ACAT1 and ACAT2 membrane topology segregates a serine residue essential for activity to opposite sides of the endoplasmic reticulum membrane. Mol. Biol. Cell 11, 3675–3687.

Kinney, A.J., 2002. Perspectives on the production of industrial oils in genetically engineered oilseeds. In: Kuo, T.M., Gardner, H.W. (Eds.), Lipid Biotechnology. Marcel Dekker Inc., New York, pp. 85–93.

Kroon, J.T.M., Wei, W., Simona, W.J., Slabas, A.R., 2006. 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. Phytochemistry 67, 2541–2549.

Lacey, D.J., Hills, M.J., 1996. Heterogeneity of the endoplasmic reticulum with respect to lipid synthesis in developing seeds of *Brassica napus* L.. Planta 199, 545–551.

Lardizabal, K.D., Mai, J.T., Wagner, N.W., Wyrick, A., Voelker, T., Hawkins, D.J., 2001. DGAT2 is a new diacylglycerol acyltransferase gene family. Purification, cloning, and expression in insect cells of two polypeptides from *Mortierella ramanniana* with diacylglycerol acyl transferease activity. J. Biol. Chem. 276, 38862–38869.

Lee, M., Lenman, M., Banas, A., Bafor, M., Singh, S., Schweizer, M., Nilsson, R., Liljenberg, C., Dahlqvist, A., Gummeson, P.-O., Sjodahl, S., Green, A., Stymne, S., 1998. Identification of non-heme diiron proteins that catalyze triple bond and epoxy group formation. Science 280, 915–918.

Lee, S., Lee, B., Jang, I., Kim, S., Bhak, J., 2006. Localizome: a server for identifying transmembrane topologies and TM helices of eukaryotic proteins utilizing domain information. Nucl. Acids Res. 34, W99–W103.

McCartney, A.W., Dyer, J.M., Dhanoa, P.K., Kim, P.K., Andrews, D.W., McNew, J.A., Mullen, R.T., 2004. Membrane-bound fatty acid desaturases are inserted co-translationally into the ER and contain different ER retrieval motifs at their carboxy termini. Plant J. 37, 156–173.

Ohlrogge, J.B., 1994. Design of new plant products: engineering of fatty acid metabolism. Plant Physiol. 104, 821–826.

Saha, S., Enugutti, B., Rajakumari, S., Rajasekharan, R., 2006. Cytosolic triacylglycerol biosynthetic pathway in oilseeds: molecular cloning and expression of peanut cytosolic diacylglycerol acyltransferase. Plant Physiol. 141, 1533–1543.

Settlage, S.B., Wilson, R.F., Kwanyuen, P., 1995. Localization of diacylglycerol acyltransferase to oil body associated endoplasmic reticulum. Plant Physiol. Biochem. 33, 399–407.

Shockey, J.M., Gidda, S.K., Chapital, D.C., Kuan, J.-C., Dhanoa, P.K., Bland, J.M., Rothstein, S.J., Mullen, R.T., Dyer, J.M., 2006. Tung tree DGAT1 and DGAT2 have nonredundant functions in triacylglycerol biosynthesis and are localized to different subdomains of the endoplasmic reticulum. Plant Cell 18, 2294–2313.

Sorger, D., Daum, G., 2002. Synthesis of triacylglycerols by the acyl-coenzyme A:diacyl-glycerol acyltransferase Dga1p in lipid particles of the yeast *Saccharomyces cerevisiae*. J. Bacteriol. 184, 519–524.

Urban, P., Werck-Reichhart, D., Teutsch, H.G., Durst, F., Regnier, S., Kazmaier, M., Pompon, D., 1994. Characterization of recombinant plant cinnamate 4-hydroxylase produced in yeast. Kinetic and spectral properties of the major plant P450 of the phenylpropanoid pathway. Eur. J. Biochem. 222, 843–850.

Wang, Y.L., 2005. Lowry protein assay. http://ylwang.umassmed.edu/protocol/pa/lowry.htm (cited December 8, 2005).

Weselake, R.J., 2002. Biochemistry and biotechnology of triacylglycerol accumulation in plants. In: Kuo, T.M., Gardner, H.W. (Eds.), Lipid Biotechnology. Marcel Dekker Inc., New York, pp. 27–56.

Yu, K., McCracken Jr., C.T., Li, R., Hildebrand, D.F., 2006. Diacylglycerol acyltransferases from *Vernonia* and *Stokesia* prefer substrates with vernolic acid. Lipids 41, 557–566.

Zhu, J., Garg, A., Mekhissi, K., Chandrashekhara, K., Flanigan, V., Kapila, S., 2002. Curing and mechanical characterization of soy-based epoxy resin system. In: Proceedings of the International SAMPE Symposium and Exhibition, Long Beach, CA, pp. 1209–1219.

Zou, J., Wei, Y., Taylor, D.C., 1999. The *Arabidopsis thaliana* TAG1 mutant has a mutation in a diacylglycerol acyl transferase gene. Plant J. 19, 645–654.