Cloning and Characterization of a cDNA Encoding Type 1 Diacylglycerol Acyltransferase from Sunflower (*Helianthus annuus* L.)

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A full-length cDNA encoding a putative diacylglycerol acyltransferase (DGAT; EC 2.3.1.20) was obtained from sunflower (*Helianthus annuus* L.) seeds. The 1524-bp open reading frame of this cDNA, designated as *HaDGAT1*, encodes a protein of 507 amino acids with a molecular mass of 58.5 kDa showing high homology to DGAT1 enzymes of other plants. The protein characters, such as a predicted structure with a long *N*-terminal hydrophilic domain followed by 9 transmembrane domains, acyl-CoA-binding signature, diacylglycerol (DAG)-binding and putative endoplasmic reticulum retrieval motifs (ER-DIR), also indicated that *HaDGAT* belongs to the DGAT1 family. *HaDGAT1* is expressed in all plant tissues especially in developing seeds. Expression of recombinant *HaDGAT1* in yeast showed an 1.76-fold increase of total fatty acids, especially unsaturated fatty acids such as palmitoleic acid (enhanced by 86.6%) and oleic acid (enhanced by 81.6%).

Key words: Helianthus annuus L., Diacylgycerol Acyltransferase, Overexpression

Introduction

Plant oil is an important renewable resource for human dietary consumption and industrial uses (Durrett *et al*., 2008; John *et al*., 2008). According to the increasing demand for vegetable oil production, metabolic pathway engineering in oil seed crops is burgeoning to produce a desirable oil quality or composition.

The main storage lipids in plants are triacylglycerols (TAGs). Two types of metabolic pathways for the production of TAGs have been found, an acyl-CoA-dependent pathway (Kennedy pathway) and an acyl-CoA-independent pathway. In the Kennedy pathway, TAG synthesis uses acyl-CoA as acyl donor and diacylglycerol (DAG) as acceptor. The process begins with sn-glycerol-3-phosphate (G3P), which is first catalyzed by the glycerol-3-phosphate acyltransferase (GPAT; EC 2.3.1.15), followed by a second acylation step catalyzed by the acyl-CoA:lysophosphatidate acyltransferase (LPAT; EC 2.3.1.51). The phosphatidic acid obtained is then dephosphorylated by the phosphatidate phosphatase (PAP; EC 3.1.3.4) to generate DAG. In the final step acyl-CoA:diacylglycerol acyltransferase (DGAT; EC 2.3.1.20) uses DAG as substrate to produce TAG (Kennedy, 1961; Anders *et al*., 2000; Coleman and Lee, 2004); it is the only enzyme exclusively committed to TAG biosynthesis using acyl-CoA as acyl donor in the Kennedy pathway. DGAT also has roles in leaf senescence and germination (Kaup *et al*., 2002; He *et al*., 2006), while in the acyl-CoA-independent pathway the TAG synthesis reaction uses phospholipids as acyl donors and DAG as acceptor; it is catalyzed by the enzyme phospholipid:diaylglycerol acyltransferase (PDAT; EC 2.3.1.158) and DAG/DAG transacylase (DGTA) (Anders *et al*., 2000; Stobart *et al*., 1997). But both PDAT and DGTA do not seem quantitatively important in TAG biosynthesis in plants by mutants (Mhaske *et al*., 2005; Routaboul *et al*., 1999), overexpression (Stahl *et al*., 2004) and metabolic control analysis (Ramli *et al*., 2005).

Three families of DGAT genes have been identified in plants, fungi, and mammals. The first DGAT gene was cloned from mouse (Cases *et al*., 1998) and is a member of the DGAT1 gene family, which is closely related to the acyl-

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CoA:cholesterol acyltransferase (ACAT) enzyme. Subsequently, homologous DGAT1 genes were cloned and characterized from *Arabidopsis* (Hobbs *et al*., 1999) and other plants (Bouvier-Nave *et al*., 2000; Nykiforuk *et al*., 2002; He *et al*., 2004; Milcamps *et al*., 2005; Wang *et al*., 2006; Mañas-Fernández *et al*., 2009). The second family of DGAT genes (DGAT2) has been identified in different plant species (Kroon *et al*., 2006; Shockey *et al*., 2006), after being first cloned from *Mortierella ramanniana* (Lardizabal *et al*., 2001). Although the DGAT1 and DGAT2 gene families exhibit no significant amino acid sequence homologies to each other and are quite distinctive, they all encode membrane-bound proteins that catalyze the committed step in TAG biosynthesis (Shockey *et al*., 2006). Most recently, a new DGAT gene encoding a soluble cytosolic enzyme was isolated from developing peanut (*Arachis hypogaes*) cotyledons (Saha *et al*., 2006), which is not related to the DGAT1 or DGAT2 gene families described above. This protein is closely related to bacterial bifunctional wax ester/DGAT, thus representing the type 3 family of DGAT (DGAT3), and it is possible that the DGAT3 could also be involved in TAG biosynthesis and wax ester synthesis in oil seeds.

Experimental evidence suggests that DGAT1 and DGAT2 are the major isoenzymes acting in the synthesis of TAG in seed plants (Hobbs *et al*., 1999; Shockey *et al*., 2006). The important role of DGAT1 in TAG synthesis in seeds has been well documented in *Arabidopsis thaliana* (Katavic *et al*., 1995; Routaboul *et al*., 1999; Zou *et al*., 1999; Lu *et al*., 2003; Jako *et al*., 2001). Seed-specific overexpression of *Arabidopsis* DGAT1 cDNA enhances the seed oil content and seed weight (Jako *et al*., 2001), while silencing of DGAT1 in tobacco causes a reduction in seed oil content (Zhang *et al*., 2005). A study of castor bean (*Ricinus communis*) showed that the DGAT1 activity pattern matches in time with that of TAG accumulation (Chen *et al*., 2007). Another study on castor bean suggested that DGAT2 was the main enzyme for TAG synthesis in seeds (Kroon *et al*., 2006). But expression of *Vernicia fordii* DGAT1 in yeast was more efficient in producing TAG than that transformed with DGAT2, and DGAT2 may play a more important role in channelling unusual fatty acids into seed storage oils (Shockey *et al*., 2006). Moreover, unsuccessful search for DGAT2 in *Tropaeolum majus* suggested that DGAT1 may be the sole DGAT in this plant species (Xu *et al*., 2008). Such results show that both DGAT1 and DGAT2 may play distinct roles in different tissues and plant species (Shockey *et al*., 2006; Chen *et al*., 2007), and further functions of DGAT enzymes in triacylglycerol synthesis in higher plants still have to be established.

In the present paper, we show the cloning, characterization, and heterologous expression analysis in a yeast system of a DGAT1 gene from sunflower (*Helianthus annuus* L.) and demonstrate that overexpression of sunflower *DGAT1* gene in yeast results in yeast oil deposition.

Material and Methods

Biological materials

Sunflower (*Helianthus annuus* L.) hybrid line XKH5 (Shihezi, Xinjiang, China) was used in this work. Seeds were collected from plants located in their natural habitat. Plants were cultivated in growth chambers at 20/10 °C (day/night), with a 16-h photoperiod and a photon flux density of 300 μmol m^{-2} s^{-1}. The different tissues and seeds at various stages of development were harvested, the seed coats were removed, and embryos were frozen in liquid nitrogen and stored at −80 °C for RNA extraction.

The yeast *Saccharomyces cerevisiae*, strain INVSc1 (genotype, *MATa*/a his3Δ1 leu2 trp1-289, ura3-52, Invitrogen, Shanghai, China) was used to assay the function of *HaDGAT1* by heterologous expression.

Total RNA extraction

Approx. 0.5 g of developing sunflower seeds, 15 d after flowering, were ground in liquid nitrogen with a precooled sterile mortar and pestle. RNA was extracted by RNAprep plant RNA isolation kit (Tiangen, Beijing, China). The RNA samples were stored at −80 °C prior to rapid amplification of cDNA ends (RACE) and reverse transcription-polymerase chain reaction (RT-PCR) analyses after removal of DNA.

Cloning of Helianthus annuus L. DGAT1 full-length cDNA

First-strand cDNA was synthesized using the first-strand cDNA synthesis kit (Takara, Dalian, China). A cDNA fragment was amplified using the degenerated oligonucleotide primers SL1

(5'-GGAGAGYCCGCTYAGCTCYGA-3') and SL2 (5'-CATCCAYTTTGRACAGGC-3') designed based on the conserved regions of plant DGAT1 from *Arabidopsis thaliana* (GenBank accession No. NP179535), *Vernicia fordii* (GenBank accession No. ABC94471), *Nicotiana tabacum* (GenBank accession No. AF129003), and *Ricinus communis* (GenBank accession No. AAR11479). RT-PCR reactions generated an internal 920-bp cDNA fragment exhibiting high homology to plant DGAT1. With gene-specific primers designed on the basis of the obtained 920-bp fragment, 3'- and 5'-RACE generated the cDNA ends, using SMARTer™ RACE cDNA amplification kit (Clontech, Inc., Palo Alto, CA, USA). Briefly, first-strand cDNA for RACE reactions was reverse-transcribed from 1 μg RNA extracted from sunflower developing seeds using the SMARTer II oligonucleotide and 5'- or 3'-RACE cDNA synthesis primer provided with the kit. The 5'- and 3'-RACE were performed by nested PCR amplification. The specific primers of sunflower DGAT1 were designed as follows: RGSP1 (5'-GAGCAGAAGAGAGAGGCAGCACATTA-3'), RNGSP1 (5'-TGAGCCTACTATTTACAGCAACAAGC-3'), RGSP2 (5'-TTGCTCCTACTTTGTGTTACCAGA-3'), and RNGSP2 (5'-CTGGAGACTTTGGAATATGCCTGT-3'). The 5'-RACE amplification was performed in 50 μl of final volume using 2.5 μl of 1/100 diluted 5'-RACE-ready cDNA template, 1 μl RGSP1 (10 μM), 5 μl Universal Primer Mix (10x), and Advantage 2 Polymerase Mix (Clontech). The 3'-RACE amplification was performed similarly by using 3'-RACE-ready cDNA and RGSP2. PCR was conducted as follows: five cycles of 94 °C for 30 s and 72 °C for 3 min, followed by five cycles of 94 °C for 30 s, 70 °C for 30 s, 72 °C for 3 min, and ending with 30 cycles of 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 3 min. The nested PCR was used with gene-specific primers RNGSP1 for 5'-RACE products, and RNGSP2 for 3'-RACE products, respectively. The positive DNA fragments were subcloned into the pMD19-T vector (Takara) for sequencing using an ABI 3730 DNA analyzer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) at Invitrogen Corporation.

Semi-quantitative RT-PCR

A semi-quantitative RT-PCR method was used to evaluate *HaDGAT1* mRNA levels. Total RNA was extracted from young roots, stems, young leaves, flowers, and developing seeds at an early age, middle and late developmental stages, using RNAprep plant RNA isolation kit (Tiangen). 1 μg of total RNA from different tissues was used to synthesize the first-strand cDNA. A pair of specific primers for *HaDGAT1* cDNA were designed to amplify the target cDNA at approx. 730 bp. The primers were SL51 (5'-ATTGAGAATCTGATGAAGTATGG-3') and SL52 (5'-CGAAGAAGCTCAGCAAGTATATT-3'). Sunflower 18S rRNA gene (GenBank accession No. AF107577) was used as an internal control with the primers SL7 (5'-CTACCACATCCAAGGAAGGCAG-3') and SL8 (5'-CGACAGAAGGGACGAGTAAACC-3'). PCR was conducted as follows: 95 °C for 3 min, then 35 cycles at 95 °C for 30 s, 53 °C for 30 s, and 72 °C for 1 min, followed by 72 °C for 10 min. PCR products were electrophoresed using 1% agarose gel.

Yeast vector construction and transformation

The ORF-encoding *HaDGAT1* gene was amplified by PCR with primers specific to BamHI (5'-CGCGGATCCATGGCGTTATTAGATCCGCCT-3') and XhoI (5'-CCGCTCGAGTCACTTGCCATTATTCACCT-3') sites. The fragment was subcloned into the yeast expression vector pYES2.0 (Invitrogen, Carlsbad, CA, USA) to obtain the recombinant plasmid pYE-HaD1, and was transformed into the yeast *Saccharomyces cerevisiae*, strain INVSc1, with S. c. EasyComp™ transformation kit (Invitrogen). An empty pYES2.0 vector was transformed into yeast as a negative control. The yeast transformants were selected and cultured by plating on SC-Ura selective medium [0.2% (w/v) yeast synthetic dropout medium without uracil (Sigma, Ronkonkoma, NY, USA), 0.17% yeast nitrogen base without amino acids and ammonium sulfate (Sigma), 0.5% $(NH_4)_2SO_4$, 2% glucose, and 2% agar] at 30 °C for 3 d. Subsequently, the recombinant protein was induced with SC-Ura medium supplemented with the auxotrophic requirement of the strain plus 1% (w/v) raffinose, and expression was further induced on a 0.4-OD_{600} culture by the addition of 2% (w/v) galactose (Mañas-Fernández *et al.*, 2009). Incubation under inductive conditions was prolonged for 48 h at the same temperature. Yeast cells were harvested and stored at −25 °C until processed for fatty acid analysis.

Fatty acids analysis

Total fatty acids of yeast cells were extracted and transmethylated as described by Tonon *et al.* (2002) and Yamamoto *et al.* (1978). Fatty acid methyl esters (FAMEs) were dissolved in hexane for GC (Agilent 6890 plus GC system, Agilent, Palo Alto, CA, USA) analysis with a 25 m × 0.53 mm × 1.0 μm (film thickness) capillary column (Varian, Palo Alto, CA, USA). The initial column temperature was 100 °C for 2 min, then was raised at 10 °C min^{-1} to 180 °C, and 4 °C min^{-1} to 260 °C, and finally held at 260 °C for 12 min. A FAME standard mixture was ordered from Sigma. Methyl pentadecanoate was used as an internal standard.

GenBank accession numbers

The GenBank accession numbers of proteins described in this study are as follows: *Arabidopsis thaliana* DGAT1 (NP179535), DGAT2 (NP_566952) and putative orthologues DGAT3 (AAK06873); *Oryza sativa* DGAT1 (AAU10815), DGAT2 (BAD33251) and putative orthologues DGAT3 (AAS98422); *Vernicia fordii* DGAT1 (ABC94471) and DGAT2 (ABC94473); *Ricinus communis* DGAT1 (AAR11479) and DGAT2 (XP_002528531); *Brassica napus* DGAT1 (AAD45536) and DGAT2 (ACO90187); *Nicotiana tabacum* DGAT1 (AF129003); *Vernonia galamensis* DGAT1 (ABV21945); *Olea europaea* DGAT1 (AAS01606); *Jatropha curcas* DGAT1 (ABB84383); *Glycine max* DGAT1 (AAS78662); *Euonymus alatus* DGAT1 (AAV31083); *Tropaeolum majus* DGAT1 (AAM03340); *Brassica juncea* DGAT1 (AAY40784); *Zea mays* DGAT1 (ABV91586); *Elaeis oleifera* DGAT2 (ACO35365); *Medicago truncatula* DGAT2 (ACJ84867); and *Arachis hipogaea* DGAT3 (AAX62735).

Results and Discussion

Cloning of a cDNA encoding DGAT1 from Helianthus annuus L.

By RT-PCR amplification with degenerate primers, using total RNA isolated from developing seeds as a template, a DNA fragment of about 920 bp was amplified showing sequence similarity to plant DGAT1s. 3'- and 5'-RACE, using gene-specific primers, yielded a full-length cDNA, named *HaDGAT1*. Sequence analysis indicated that *HaDGAT1* is 1936 bp in length with a 167-bp 5'-leader sequence and 245-bp 3'-untranslated regions (GenBank accession No. HM015632). The cDNA contains an ORF of 1524 bp encoding a protein of 507 amino acids. The predicted molecular mass and calculated isoelectric points are 58.5 kDa and 9.17 (Protparam: http://www.expasy.ch). The deduced amino acid sequence of *HaDGAT1* shows high identity to DGAT1 of higher plants such as *Vernonia galamensis* (80%), *Olea europaea* (69%), *Jatropha curca*s (69%), *Vernicia fordii* (68%), *Ricinus communis* (66%). Comparision with the deduced amino acids sequence of different plant DGAT1s showed that the *C*-terminal regions are much more conserved than the *N*-terminal regions among known plants DGAT1s (Fig. 1). Phylogenetic tree analysis of *HaDGAT1* against the three families of plant DGATs (membrane-bound DGAT1 and DGAT2, and cytosolic DGAT3) showed that *HaDGAT1* is most closest to the DGAT1 family (Fig. 2).

Identification of putative functional motifs in HaDGAT1

In plants, DGAT1 has been found localized at the membrane of endoplasmic reticulum (ER) (Shockey *et al.*, 2006) where the Kennedy pathway mainly occurs. A Kyte and Doolittle hydrophobicity plot analysis (Kyte and Doolittle, 1982) suggested that *HaDGAT1* contains a large hydrophilic domain of about 110 amino acids at the *N*-terminus to lie on the cytoplasmic side of the membrane, followed by a long hydrophobic stretch at the *C*-terminus (about 385 residues) (Fig. 3). Using a program of transmembrane alpha-helices predictor software (Localizome, http://localodom.kobic.re.kr/LocaloDom/index.htm), nine potential transmembrane domains (from amino acid resides 109 to 492) are strongly predicted (Fig. 3), which are likely to anchor protein at the ER membrane. The highest similarity of plant DGAT1s are the multiply transmembrane domains in the *C*-terminal conserved regions, consistent with an integral membrane enzymes.

Using the Prosite database (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_proscan.html), 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 were found.

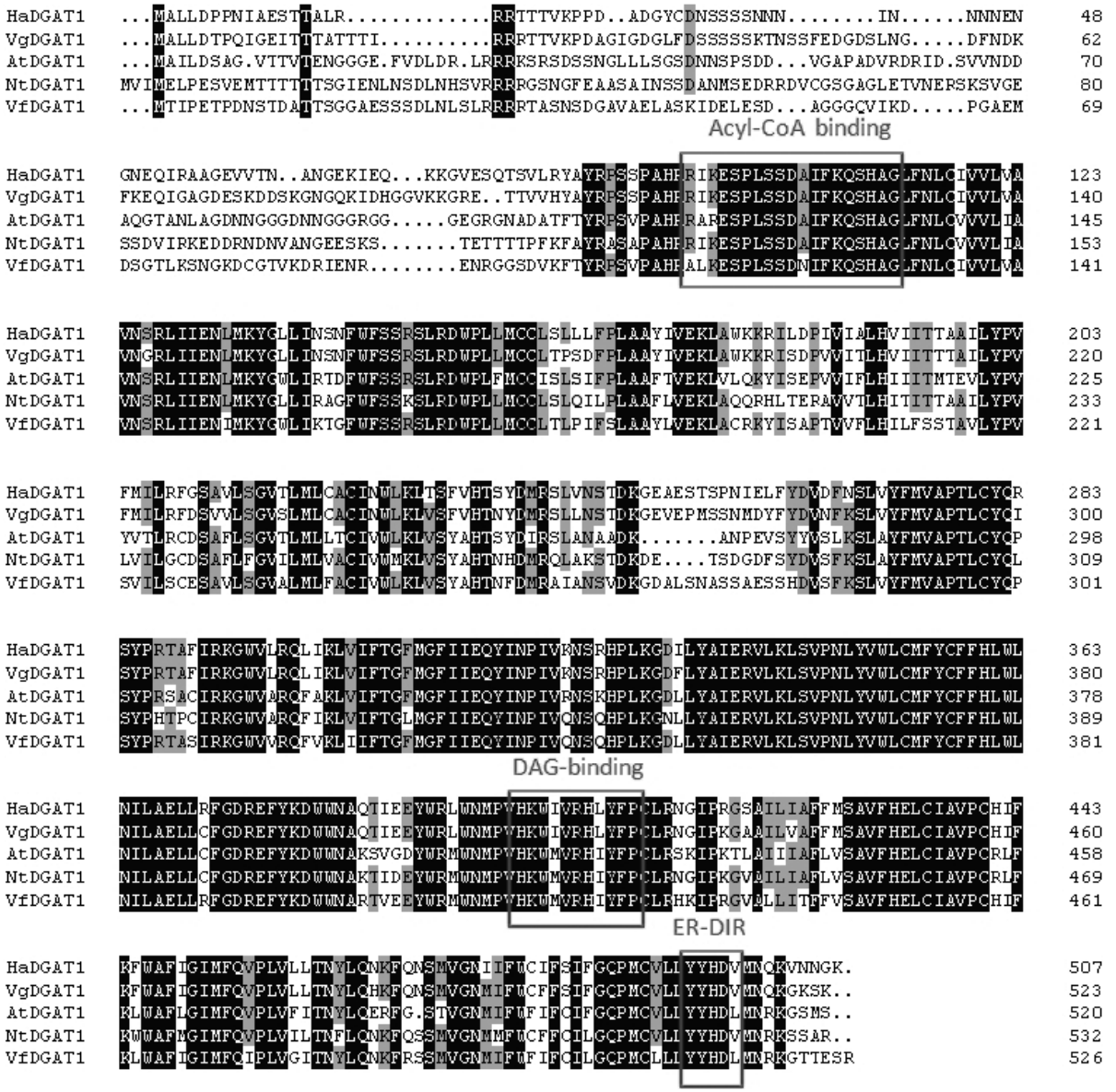

Fig. 1. Sequence comparison of *HaDGAT1* with related DGAT1 enzymes from higher plants. The amino acid sequence of *HaDGAT1* (GenBank accession No. HM015632) was aligned, using the software DNAMAN6.0, together with those of characterized plant DGAT1 from *Vernonia galamensis* (Vg, GenBank accession No. ABV21945), *Arabidopsis thaliana* (At, GenBank accession No. NP179535), *Nicotiana tabacum* (Nt, GenBank accession No. AF129003), and *Vernicia fordii* (Vf, GenBank accession No. ABC94471). Conserved motifs or putative signatures are boxed, such as the acyl-CoA binding signature, DAG-binding, and putative ER retrieval motifs (ER-DIR).

However, whether these sites are important in the regulation of the functions of the enzyme *in vivo* remains unknown.

Analysis using the pfam programme, an MBOAT (membrane-bound *O*-acyltransferase) domain (amino acid resides 220 to 495 of *HaDGAT1*) was found which is possibly involved in acyltransfer (Hofmann, 2000).

A putative acyl-CoA binding motif ($_{94}$RIKESPLSSDAIFKQSHAG$_{112}$) was found in the *HaD-*

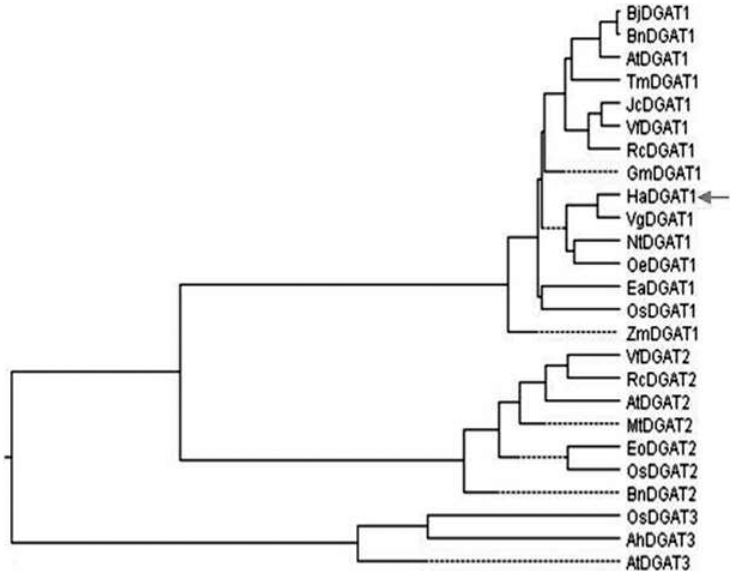

Fig. 2. Phylogenetic tree showing relationships among *HaDGAT1* and diverse DGAT enzymes from higher plants. Amino acid sequences for three types of plant DGAT enzymes (membrane-bound DGAT1 and DGAT2, and cytosolic DGAT3) were obtained from the GenBank, aligned with that of *HaDGAT1* (marked by an arrow), and the tree was constructed using the DNASTAR software. *Arabidopsis thaliana* (At), *Oryza sativa* (Os), *Vernicia fordii* (Vf), *Ricinus communis* (Rc), *Brassica napus* (Bn), *Nicotiana tabacum* (Nt), *Vernonia galamensis* (Vg), *Olea europaea* (Oe), *Jatropha curcas* (Jc), *Glycine max* (Gm), *Euonymus alatus* (Ea), *Tropaeolum majus* (Tm), *Brassica juncea* (Bj), *Zea mays* (Zm), *Elaeis oleifera* (Eo), *Medicago truncatula* (Mt), and *Arachis hipogaea* (Ah).

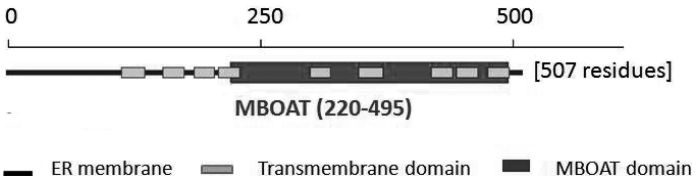

Fig. 3. Putative transmembrane domains in *HaDGAT1* protein. The main transmembrane segments were predicted by transmembrane alpha-helix predictor software (Localizome, http://localodom.kobic.re.kr/LocaloDom/index.htm) (Lee *et al.*, 2006).

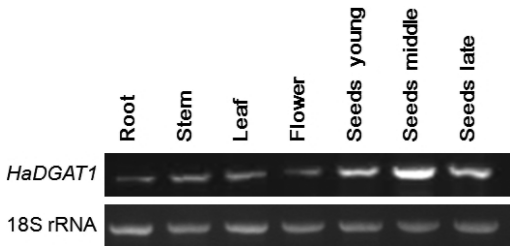

Fig. 4. Semi-quantitative RT-PCR analysis of *HaDGAT1* gene expression in different organs and different developmental stages of seeds in sunflower. The 18S rRNA gene was amplified as an internal control.

GAT1 protein (Fig. 1). These amino acids may be involved in acyl-CoA binding, and the homology sequence was also found in *Arabidopsis thaliana* (Jako *et al.*, 2001), *Brassica napus* (Nykiforuk *et al.*, 2002), and *Euonymus alatus* (Milcamps *et al.*, 2005).

A putative diacylglycerol-binding motif (HKW-X-X-RH-X-Y-X-P), observed to be unique amongst the DGATs while absent in the ACATs (Oelkers *et al.*, 1998), was also found in *HaDGAT1* amino acids sequence ($_{399}$HKWIVRHLYFP$_{409}$) (Fig. 1), and the similarity sequence was also found in *Arabidopsis thaliana* (Zou *et al.*, 1999) and *Brassica napus* (Nykiforuk *et al.*, 2002).

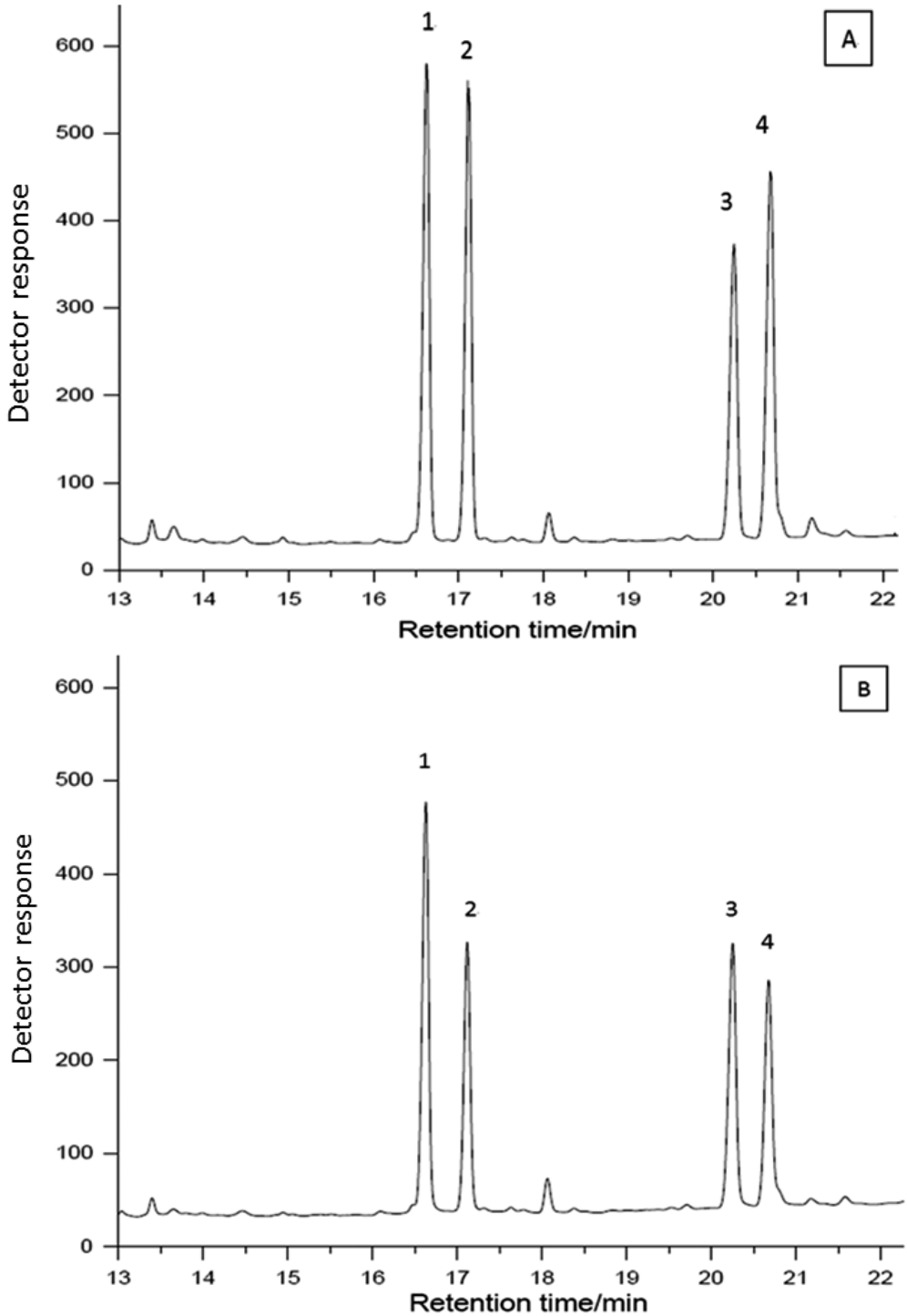

Fig. 5. GC-MS assay of the fatty acid methyl esters of transformed yeast. (A) Yeast transformed with vector pYES2.0 containing the *HaDGAT1* gene. (B) Yeast transformed with empty vector pYES2.0 (negative control). The peaks 1, 2, 3, 4 are palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), and oleic acid (C18:1), respectively.

Another motif was detected in the presence of the putative *C*-terminal ER retrieval motifs in *HaDGAT1* (-YYHDV-) (Fig. 1), and it was also found in other plant DGAT1 s (*Arabidopsis* and Castor, -YYHDL-; tobacco, -YYHDV-). 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).

Expression of HaDGAT1 mRNA in different tissues

To further investigate the potential role of *HaDGAT1* in TAG biosynthesis, a semi-quantitative RT-PCR method was used to monitor the gene expression pattern of *HaDGAT1*. An equal amount of total RNA from young root, stem, young leaf, flower, and developing seeds at different developmental stages (early, middle, and old stage) was used. The sunflower 18S rRNA, a housekeeping gene, was used as an internal control. The results from sunflower 18S rRNA (Fig. 4) suggested that the efficiencies of RT-PCR among samples are uniform in this system. The results showed that *HaDGAT1* was transcriptionally active in all tested tissues (Fig. 4). This generalized expression level was also found for DGAT1 genes of other plants such as *Arabidopsis* (Hobbs *et al*., 1999), *Echium* (Mañas-Fernández *et al*., 2009), and soybean (Wang *et al*., 2006). However, the transcript levels are relatively high in developing seeds, while lower in roots and flowers. TAG synthesis mainly occurs in seeds, although fatty acid synthesis also occurs in other plant tissues. The tissue and temporal expression pattern of *HaDGAT1* suggests that *HaDGAT1* is important for TAG synthesis in sunflower seeds.

Functional expression of HaDGAT1 in yeast

In order to analyse the function of the *HaDGAT1* gene, the cDNA was cloned into the yeast vector pYES2.0 and transformed into the yeast *Saccharomyces cerevisia*, strain INVSc1. As control, the yeast was also transformed with the empty pYES2.0 vector. Cells were cultured overnight in a medium containing 1% raffinose, then were induced by the addition of 2% galactose to overexpress the *HaDGAT1* protein. Growth of the cultures was continued for 48 h, until aliquots were removed for the analysis of fatty acids content by gas chromatography-mass spectrometry (GC-MS). GC-MS analyses of the fatty acid methyl esters of empty vector and *HaDGAT1*-transformed yeast are shown in Fig. 5. The fatty acid composition of yeast is predominantly a mixture of palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), and oleic acid (C18:1). The *HaDGAT1* gene provided an 1.76-fold increase in total fatty acids, with increases in palmitic acid (enhanced by 37.8%), palmitoleic acid (enhanced by 86.6%), stearic acid (enhanced by 34.2%), and oleic acid (enhanced by 81.6%), relative to the empty vector control, respectively. Most or all of the increase in fatty acids content can be ascribed to the increase in TAG content. Compared with the empty vector control, the percentage of saturated fatty acids among total fatty acids in the *HaDGAT1*-transformed yeast line (C16:0 and C18:0) was decreased, while the percentage of undersaturated fatty acids (C16:1 and C18:1) was increased (Fig. 6). These results indicate that *HaDGAT1* encodes a protein with TAG biosynthetic activity.

An important role for DGAT in TAG biosynthesis has been established by cloning DGAT genes from different species. However, the gene for DGAT in sunflower has never been cloned. Here we identified a cDNA encoding a protein that shares high sequence similarity with the plant DGAT1 gene family. Expression of *HaDGAT1* in wild-type yeast resulted in a distinct increase of total fatty acid content. The present study provides fundamental information for future studies of the regulatory mechanism of TAG synthesis in plants.

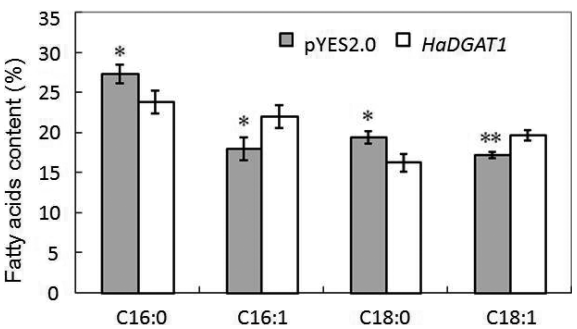

Fig. 6. Fatty acid composition in INVSc1 yeast cells transformed with the empty vector (pYES2.0) or the vector containing *HaDGAT1*. Values correspond to percentages on the total fatty acids in each fraction. Each error bar indicates S.D. ($n = 3$). * $p < 0.05$, ** $p < 0.01$ (Student's t test).

Anders D., Ulf S., Marit L., Antoni B., Michael L., Line S., Hans R., and Sten S. (2000), Phospholipid:diacylglycerol acyltransferase: An enzyme that catalyzes the acyl-CoA-independent formation of triacylglycerol in yeast and plants. Proc. Natl. Acad. Sci. USA **97**, 6487–6492.

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

Cases S., Smith S. J., Zheng Y. W., Myers H. M., Lear S.R., and Sande E. (1998), Identification of a gene encoding an acyl CoA:diacylglycerol acyltransferase, a key enzyme in triacylglycrol synthesis. Proc Natl. Acad. Sci. USA **95**, 13018–13023.

Chen G. Q., Turner C., He X., Nguyen T., McKeon T. A., and Laudencia C. D. (2007), Expression profiles of genes involved in fatty acid and triacylglycerol synthesis in castor bean (*Ricinus communis* L.). Lipids **42**, 263–274.

Coleman R. A. and Lee D. P. (2004), Enzymes of triacylglycerol synthesis and their regulation. Prog. Lipid Res. **43**, 134–176.

Durrett T., Benning C., and Ohlrogge J. (2008), Plant triacylglycerols as feedstocks for the production of biofuels. Plant J. **54**, 593–607.

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

He X. H., Chen G. Q., Lin J. T., and McKeon T. (2006), Diacylglycerol acyltransferase activity and triacylglycerol synthesis in germinating castor seed cotyledons. Lipids **41**, 281–285.

Hobbs D. H., Lu C., and Hills M. J. (1999), Cloning of a cDNA encoding diacylglycerol acyltransferase from *Arabidopsis thaliana* and its functional expression. FEBS Lett. **452**, 145–149.

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

John M. D., Sten S., Allan G. G., and Anders S. C. (2008), High-value oils from plants. Plant J. **54**, 640–655.

Katavic V., Reed D. W., Taylor D. C., Giblin E. M., Barton D. L., Zou J., MacKenzie S. L., Covello P. S., and Kunst L. (1995), Alteration of seed fatty acid composition by an ethyl methane sulfonate-induced mutation in *Arabidopsis thaliana* affecting diacylglycerol acyltransferase activity. Plant Physiol. **108**, 399–409.

Kaup M. T., Froese C. D., and Thonpson J. E. (2002), A role for diacylglycerol acyltransferase during leaf senescence. Plant Physiol. **129**, 1616–1626.

Kennedy E. (1961), Biosynthesis of complex lipids. Fed. Proc. **20**, 934–940.

Kroon J. T., Wei W., Simon W. J., and 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.

Kyte J. and Doolittle R. F. (1982), A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157**, 105–131.

Lardizabal K. D., Mai J. T., Wagner N. W., Wyrick A., Voelker T., and 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 acyltransferase activity. J. Biol. Chem. **276**, 38862–38869.

Lee S., Lee B., Jang I., Kim S., and Bhak J. (2006), Localizome: a server for identifying transmembrane topologies and TM helices of eukaryotic proteins utilizing domain information. Nucl. Acids Res. **34**, 99–103.

Lu C. L., de Noyer S. B., Hobbs D. H., Kang J. L.,Wen Y. C., Krachtus D., and Hills M. J. (2003), Expression pattern of diacylglycerol acyltransferase-1, an enzyme involved in triacylglycerol biosynthesis, in *Arabidopsis thaliana*. Plant Mol. Biol. **52**, 31–41.

Mañas-Fernández A., Vilches-Ferrón M., Garrido-Cárdenas J. A., Belarbi E. H., Alonso1 D. L., and García-Maroto F. (2009), Cloning and molecular characterization of the acyl-CoA:diacylglycerol acyltransferase 1 (DGAT1) gene from *Echium*. Lipids **44**, 555–568.

McCartney A. W., Dyer J. M., Dhanoa P. K., Kim P. K., Andrews D. W., McNew J. A., and 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.

Mhaske V., Beldjilali K., Ohlrogge J., and Pollard M. (2005), Isolation and characterisation of an *Arabidopsis thaliana* knockout line for phospholipid:diacylglycerol transacylase gene (At5g13640). Plant Physiol. Biochem. **43**, 413–417.

Milcamps A., Tumaney A. W., Paddock T., Pan D. A., Ohlrogge J., and Pollard M. (2005), Isolation of a gene encoding an 1,2-diacylglycerol-sn-acetyl-CoA acetyltransferase from developing seeds of *Euonymus alatus*. J. Biol. Chem. **280**, 5370–5377.

Nykiforuk C. L., Furukawa-Stoffer T. L., Huff P. W., Sarna M., Laroche A., Moloney M. M., and Weselake R. J. (2002), Characterization of cDNAs encoding diacylglycerol acyltransferase from cultures of *Brassica napus* and sucrose-mediated induction of enzyme biosynthesis. Biochim. Biophys. Acta **1580**, 95–109.

Oelkers P., Behar A., Cromley D., Billheimer J. T., and Sturley S. T. (1998), Characterization of two human genes encoding acyl coenzyme A:cholesterol acyltransferase-related enzymes. J. Biol. Chem. **273**, 26765–26771.

Ramli U. S., Salas J. J., Quant P. A., and Harwood J. L. (2005), Metabolic control analysis reveals an important role for diacylglycerol acyltransferase in olive but not in oil palm lipid accumulation. FEBS J. **272**, 5764–5770.

Routaboul J. M., Benning C., Bechtold N., Caboche M., and Lepiniec L. (1999), The TAG1 locus of *Arabidopsis* encodes for a diacylglycerol acyltransferase. Plant Physiol. Biochem. **37**, 831–840.

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

Shockey J. M., Gidda S. K., Chapital D. C., Kuan J. C., Dhanoa P. K., Bland J. M., Rothstein S. J., Mullen R. T., and 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.

Stahl U., Carlsson A. S., Lenman D. A., Huang B. Q., and Banas W. (2004), Cloning and functional characterization of a phospholipid:diacylglycerol acyltransferase from *Arabidopsis*. Plant Physiol. **135**, 1324–1335.

Stobart A. K., Mancha M., Lenman M., Dahlqvist A., and Stymne S. (1997), Triacylglycerols are synthesized and utilized by transacylation reactions in microsomal preparations of developing safflower (*Carthamus tinctorius* L.) seeds. Planta **203**, 58–66.

Tonon T., Harvey D., Larson T. R., and Graham I. A. (2002), Long chain polyunsaturated fatty acid production and partitioning to triacylglycerols in four microalgae. Phytochemistry **61**, 15–24.

Wang H. W., Zhang J. S., Gai J. Y., and Chen S. Y. (2006), Cloning and comparative analysis of the gene encoding diacylglycerol acyltransferase from wild type and cultivated soybean. Theor. Appl. Genet. **112**, 1086–1097.

Xu J., Francis T., Mietkiewska E., Giblin E. M., Barton D. L., Zhang Y., Zhang M., and Taylor D. C. (2008), Cloning and characterization of an acyl-CoA-dependent diacylglycerol acyltransferase 1 (DGAT1) gene from *Tropaeolum majus*, and a study of the functional motifs of the DGAT protein using sitedirected mutagenesis to modify enzyme activity and oil content. Plant Biotechnol. J. **6**, 799–818.

Yamamoto A., Yano I., Masui M., and Yabuuchi E. (1978), Isolation of a novel sphingoglycolipid containing glucuronic acid and 2-hydroxy fatty acid from *Flavobacterium devorans* ATCC 10829. J. Biochem. **83**, 1213–1216.

Zhang F. Y., Yang M. F., and Xu Y. N. (2005), Silencing of DGAT1 in tobacco causes a reduction in seed oil content. Plant Sci. **169**, 689–694.

Zou J., Wei Y., Jako C., Kumar A., Selvaraj G., and Taylor D. C. (1999), The *Arabidopsis thaliana TAG1* mutant has a mutation in a diacylglycerol acyltransferase gene. Plant J. **19**, 645–653.