Cloning, Characterization and Functional Analysis of Two Type 1 Diacylglycerol Acyltransferases (DGAT1s) from *Tetraena mongolica*

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Abstract

Two cDNAs encoding putative type 1 acyl-CoA: diacylglycerol acyltransferases (DGAT1, EC 2.3.1.20), were cloned from *Tetraena mongolica* Maxim., an extreme xerophyte with high oil content in the stems. The 1,488-bp and 1,485-bp of the open reading frame (ORF) of the two cDNAs, designated as *TmDGAT1a* and *TmDGAT1b*, were both predicted to encode proteins of 495 and 494 amino acids, respectively. Southern blot analysis revealed that *TmDGAT1a* and *TmDGAT1b* both had low copy numbers in the *T. mongolica* genome. In addition to ubiquitous expression with different intensity in different tissues, including stems, leaves and roots, *TmDGAT1a* and *TmDGAT1b*, were found to be strongly induced by high salinity, drought and osmotic stress, resulting in a remarkable increase of triacylglycerol (TAG) accumulation in *T. mongolica* plantlets. *TmDGAT1a* and *TmDGAT1b* activities were confirmed in the yeast H1246 quadruple mutant (*DGA1, LRO1, ARE1, ARE2*) by restoring DGAT activity of the mutant host to produce TAG. Overexpression of *TmDGAT1a* and *TmDGAT1b* in soybean hairy roots as well as in *T. mongolica* calli both resulted in an increase in oil content (ranging from 37% to 108%), accompanied by altered fatty acid profiles.

Keywords: Diacylglycerol acyltransferase1; *Tetraena mongolica*; triacylglycerol.

Two sequence-unrelated classes of DGAT enzymes, DGAT1 and DGAT2, have been isolated from various plants in previous research (Lardizabal et al. 2001; He et al. 2004; Kroon et al. 2006; Shockey et al. 2006; Xu et al. 2008; Mañás-Fernández et al. 2009). DGAT1s are members of the membrane-bound O-acyltransferase protein superfamily that possess at least six transmembrane domains, while two to four transmembrane domains were found in DGAT2 (Andreia et al. 2011). Moreover, DGAT1 and DGAT2 enzymes were shown to localize to different subdomains of the ER in the tung tree (Vernicia fordii) (Shockey et al. 2006), and were thus suggested to play non-redundant roles in different tissues and species in TAG synthesis (Shockey et al. 2006; Li et al. 2010). Overexpression and mutational downregulation studies of DGAT1 from Arabidopsis thaliana revealed that DGAT1 contributes significantly to seed TAG biosynthesis (Zou et al. 1999; Jako et al. 2001). Contributions of DGAT1 to TAG biosynthesis were also confirmed by studies on soybean (Settlaie et al. 1998), maize (Zheng et al. 2008) and nasturtium (Xu et al. 2008). The emerging role of DGAT2 orthologs appears to be more important for the incorporation of unusual fatty acids in the seed oils of some plants (Kroon et al. 2006; Shockey et al. 2006; Burgal et al. 2008). A third soluble class of DGAT enzyme was reported in peanut (Saha et al. 2006). In Arabidopsis, DGAT3 is involved in a process of recycling linoleic (18:2) and linolenic (18:3) fatty acids into TAG when seed oil breakdown is blocked, and may play a key role in lipid metabolism (Hernández et al. 2012).

In addition to the above, a few workers have also documented that DGAT1 activity and its control over glycerolipid assembly may be influenced by environmental factors. It was demonstrated that an Arabidopsis mutant deficient in DGAT1 displayed heightened sensitivity to abscisic acid (ABA) and osmotic stress (Lu and Hills 2002), while Brassica napus overexpressing DGAT1 led to more seed oil in the mature seed under drought conditions when compared to the control (Weselake et al. 2008). These results indicate the potential use of DGAT1 under stress conditions, and make studying the manipulation of DGAT1 more attractive.

T. mongolica, a member of the narrowly monotypic genus of Zygophyllaceae endemic to the western Gobi desert of Inner Mongolia, China (Qian and Chen 2004), is an extreme xerophyte with a well-developed root system, and can therefore tolerate drought and other harsh natural conditions particularly well (Zhang et al. 2003). Previous work showed that T. mongolica can accumulate large amounts of TAG (approximately 46 mg/g of dry weight (DW)) in stem tissues (Wang et al. 2007). Such a plant might be of potential value to be used as an energy plant. In the present study, we report the cloning, characterization, and functional analysis of two DGAT1s from such a plant, in an attempt to provide a possible means to affect changes in oil content, and as a preliminary study for the exploitation and use of this plant.

Results

TAG content and its fatty acid composition in roots, stems and leaves of T. mongolica plantlets

The TAG content in roots, stems, and leaves of T. mongolica plantlets was determined by gas chromatography (GC) analysis. As shown in Table 1, TAG content in roots was the highest at approximately 1.8 mg/g of DW, followed by stems and then leaves. Table 1 also shows the fatty acid composition of TAG from different tissues of T. mongolica plantlets. The results show that five common fatty acids, including palmitic (16:0), stearic (18:0), oleic (18:1), 18:2 and 18:3, exist as the main constituents. The fatty acid profiles of TAG from different tissues of the plantlets were similar. The most prevalent fatty acid was 18:3, which accounted for 30.6%–38.1% of the total fatty acids in TAG. The polyunsaturated fatty acids (18:2 and 18:3) accounted for about 50% of the total TAG fatty acids. The percentage of 16:0 was 22.7%–27.1% of the total TAG fatty acids, which was second only to 18:3. Moreover, TAG was relatively low in 18:0 and 18:1.

Isolation of two cDNA clones encoding DGAT1 from T. mongolica plantlets

For the cloning of T. mongolica DGAT1, conserved regions of DGAT1s were identified by alignment of deduced amino acid sequences from different species. One DNA fragment of 852-bp was amplified from T. mongolica plantlets using a degenerate primers approach, with the primers designed based on DNA sequences of the conserved regions. However, this 852-bp fragment proved to be two different sequences after sequencing. On the basis of the sequence information, gene-specific primers for 3'- and 5'-RACE (rapid amplification of cDNA ends) were generated, yielding two full-length cDNAs named TmDGAT1a and TmDGAT1b, respectively.

Sequence analysis indicates that TmDGAT1a (GenBank accession No. JX163929) contains an ORF of 1,488-bp encoding a protein of 495 amino acids, and the 1,485-bp ORF of TmDGAT1b (GenBank accession No. JX163930) was predicted to encode a protein of 494 amino acids. The predicted Mr and calculated isoelectric points are 57.1 kDa and 9.04 for TmDGAT1a, and 56.8 kDa and 9.19 for TmDGAT1b (Prot- param: http://www.expasy.org), respectively. The two DGAT1s share 82.9% similarity in nucleotide sequence and 82.7% identity in deduced amino acid sequence. Phylogenetic tree analysis of deduced amino acid sequences from TmDGAT1a and TmDGAT1b and other known orthologs indicates that both TmDGAT1a and TmDGAT1b are grouped together with all
known DGAT1s (Figure S1). Alignment of the deduced amino acid sequences of TmDGAT1s and other DGAT1s from Arabidopsis, Nicotiana, Vernicia, maize and castor shows that the proteins share a high identity (44.3%, Figure 1). The C-terminal regions (56.6% identity within 422 amino acids) are much more conserved than the N-terminal regions (0.8% identity in the first 72 amino acids) (Figure 1).

Identification of putative functional motifs in TmDGAT1s

Scanning the amino acid sequences of both TmDGAT1a and TmDGAT1b against the Prosite database (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_proscan.html) revealed a number of putative functional motifs shown in Table S1. The quantity of some functional motifs in TmDGAT1a and TmDGAT1b is different from AtDGAT1. It remains to be determined whether these sites are important in the regulation of the functions of the TmDGAT1s in vivo.

In plants, DGAT1 has been localized to the ER membrane where the Kennedy pathway mainly occurs (Settiage et al. 1995; Lacey and Hills 1996; De Domenico et al. 2011). Nine potential transmembrane helices that likely anchor the two proteins to the ER membrane were strongly predicted by TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) for both TmDGAT1a and TmDGAT1b (Figure S2). Little conservation was found for the N-terminal amino acids, except for the characteristic basic repeat consisting of three arginine residues in TmDGAT1s, which is the same as that in other high plants (Mañas-Fernández et al. 2009; Guihéneuf et al. 2011) (Figure 1). Also detected was the presence of the reported acyl-CoA binding signature (R83-G101 in TmDGAT1a, R82-G100 in TmDGAT1b) (Jako et al. 2001; Mañas-Fernández et al. 2009; Guihéneuf et al. 2011) close to residues (R116–N121 in TmDGAT1a, R115–N120 in TmDGAT1b) that have been involved in the active site, as well as a DAG/phorbol ester binding motif (Jako et al. 2001; Mañas-Fernández et al. 2009; Guihéneuf et al. 2011) (Figure 1). Within the previously reported leucine zipper motif (Lung and Weselake 2006; Mañas-Fernández et al. 2009; Guihéneuf et al. 2011), an invariant proline residue (P191 in TmDGAT1a, P190 in TmDGAT1b) (Figure 1) has been shown critical for DGAT1 activity (Xu et al. 2008). Another conserved feature is an invariant serine among the shown DGAT1s at positions 220 and 219 in TmDGAT1a and TmDGAT1b, respectively (Figure 1). The serine residue has been shown to be essential for the activity of acyl-CoA: cholesterol acyltransferase, an enzyme closely related to DGAT1 (Joyce et al. 2000). In addition, the leucine zipper motif was also found to overlap with a putative thiolase acyl-enzyme intermediate binding motif reported for Arabidopsis, Tropaeolum and Phaeodactylum DGAT1 (Zou et al. 1999; Xu et al. 2008; Guihéneuf et al. 2011). There is also a fatty acid-binding protein signature spanning residues A356-N372 in TmDGAT1a and A355-N371 in TmDGAT1b, which contains a putative tyrosine phosphorylation site, Y367 in TmDGAT1a and Y366 in TmDGAT1b (Xu et al. 2008) (Figure 1). Two C-terminal motifs, YYHD-like and NRKG-like, as the putative ER retrieval motifs, are also present in TmDGAT1s, similar to other DGAT1 plants proteins (Lung and Weselake 2006) (Figure 1).

Gene structure and genomic organization of TmDGAT1a and TmDGAT1b

A comparison between the partial genomic and cDNA sequences allows the identification of 15 introns interrupting the coding region of TmDGAT1a and TmDGAT1b, respectively (Figure S3). To determine the copy number of the TmDGAT1s in the genome, Southern blot analysis was performed. For both TmDGAT1a and TmDGAT1b, a single band was detected with the restriction enzymes HindIII and XbaI (Figure S4, lanes 2, 3, 5 and 6), and more than one band were detected with EcoRI (Figure S4, lanes 1 and 4), probably due to the presence of similar sequences of TmDGAT1s, because there was no EcoRI site in the probes used. These results indicate that both TmDGAT1a and TmDGAT1b are with low copy number in the genome of T. mongolica.

Tissue-specific expression of TmDGAT1s in T. mongolica plantlets

Quantitative real time PCR (qRT-PCR) was performed to quantify TmDGAT1s transcripts in roots, stems and leaves of T. mongolica plantlets. The changes in expression levels of TmDGAT1 transcripts in T. mongolica plantlets were determined relative to the expression level of the TmDGAT1a

### Table 1. Fatty acid composition of triacylglycerol (TAG) extracted from roots, stems and leaves of *Tetraena mongolica* plantlets

<table>
<thead>
<tr>
<th>Sample</th>
<th>TAG content (mg/g DW)</th>
<th>16:0</th>
<th>18:0</th>
<th>18:1</th>
<th>18:2</th>
<th>18:3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Roots</td>
<td>1.8 ± 0.1</td>
<td>24.4 ± 0.4</td>
<td>7.0 ± 0.6</td>
<td>19.1 ± 1.4</td>
<td>16.4 ± 1.0</td>
<td>33.1 ± 1.4</td>
</tr>
<tr>
<td>Stems</td>
<td>1.3 ± 0.1</td>
<td>22.7 ± 1.1</td>
<td>6.6 ± 0.5</td>
<td>13.4 ± 0.9</td>
<td>26.7 ± 0.3</td>
<td>30.6 ± 1.4</td>
</tr>
<tr>
<td>Leaves</td>
<td>1.2 ± 0.1</td>
<td>27.1 ± 1.8</td>
<td>7.9 ± 0.4</td>
<td>11.9 ± 1.1</td>
<td>15.0 ± 1.2</td>
<td>38.1 ± 1.2</td>
</tr>
</tbody>
</table>

Data are the mean ± SD of three independent experiments.
Cloning and Functional Analysis of DGAT1 Genes

Figure 1. Homology comparison of deduced amino acid sequences of TmDGAT1s with their orthologs from GenBank.

The alignment was generated with CLUSTAL W. VfDGAT1 (Vernicia fordii, Accession No. ABC94471.1), RcDGAT1 (Ricinus communis, Accession No. XP002514132.1), AtDGAT1 (Arabidopsis thaliana, Accession No. NP179535.1), NtDGAT1 (Nicotiana tabacum, Accession No. AAF19345.1), ZmDGAT1 (Zea mays, Accession No. ABV91586.1), TmDGAT1a and TmDGAT1b (Tetraena mongolica, Accession Nos. JX163929 and JX163930). Conserved motifs or putative signatures (see text for details) are boxed, such as the N-terminal basic motif RRR in higher plants (I), the acyl-CoA binding signature (II), the fatty acid protein signature (III), which contains a tyrosine phosphorylation site (♦), the DAG-binding site (IV) and two C-terminal motifs –YYHD-like and NRKG-like (V) – as the putative endoplasmic reticulum retrieval motif in the C-terminus. The region containing a conserved leucine repeat (L) in higher plants coincides with a thiolase acyl enzyme intermediate binding signature (marked by a horizontal solid bar) beside the previously-described critical proline and serine residues, which are marked by asterisks. The conserved phenylalanine is designated by an arrow.
transcript in the roots. The transcript abundance was normalized to that of the TmACTIN gene. The two genes with different transcription profiles expressed in all tissues of T. mongolica plantlets. We observed the highest steady-state level accumulation of TmDGAT1b transcripts in roots and stems, which were much higher than that in leaves (Figure 2). Transcript levels of TmDGAT1a in stems were higher than that in roots and leaves, but were all at a much lower level compared to TmDGAT1b (Figure 2). Taken together, these results suggest that the expression level of TmDGAT1b is much higher than that of TmDGAT1a in the whole plant, particularly in the roots of T. mongolica plantlets.

Expression patterns of TmDGAT1s in response to NaCl, PEG and sucrose treatments

Given the fact that T. mongolica was able to survive harsh natural conditions including drought, we first investigated whether expression of TmDGAT1s is induced by stress treatments. One-m-old plantlets were treated with 800 mM NaCl, 20% polyethylene glycol (PEG) and 15% sucrose, respectively, and expression patterns of TmDGAT1s in root tissues were analyzed by qRT-PCR. The results indicated that there was a time-dependent increase in both TmDGAT1a and TmDGAT1b transcripts upon exposure to 800 mM NaCl, 20% PEG and 15% sucrose treatments in roots of the T. mongolica plantlets (Figure 3A–C). As shown in Figure 3A, transcripts of TmDGAT1a were found to be accumulated after 2 h of NaCl treatment, reached a maximum of 10-fold of the control at 24 h, and stayed at this level thereafter. In contrast to TmDGAT1a, transcripts of TmDGAT1b were found to have accumulated after 5 h of exposure to salt stress, and they continued to increase for at least 48 h, reaching a maximum of 5-fold of the control. In addition to salt stress, the expression of TmDGAT1s was also upregulated by PEG-induced drought stress (Figure 3B). TmDGAT1a transcripts began to accumulate after 1 h of treatment by 20% PEG, and exhibited a time-dependent increase, reaching a maximum of 25-fold of the control at 48 h after starting the treatment (Figure 3B). TmDGAT1b transcripts began to accumulate after 5 h of treatment by PEG, and peaked at 24 h (Figure 3B). Moreover, the expression of TmDGAT1s was also induced by exposure to 15% sucrose, and TmDGAT1s expression patterns were similar to those under PEG treatment (Figure 3C). The results showed that expression of both TmDGAT1a and TmDGAT1b was significantly upregulated by NaCl, PEG and sucrose treatment.

TAG accumulation in roots of T. mongolica plantlets in response to NaCl, PEG and sucrose treatments

Since the transcripts of TmDGAT1s were significantly increased by NaCl, PEG and sucrose treatment, TAG content in roots of T. mongolica plantlets under stress were further examined. As expected, oil deposition in roots of T. mongolica plantlets exhibited a typical time-dependent increase pattern under applied stress (Figure 3D–F). After exposure to 800 mM NaCl for 2 h, a consistent increase in TAG accumulation in roots of the plantlets was observed, which finally reached a level 3-fold higher than that of the control at 72 h (Figure 3D). TAG content increased much more slowly under 20% PEG treatment, with the largest increase occurring during the last 24 h. The amount of TAG finally reached 7.73 mg/g of DW, approximately 6-fold higher than that of the control (Figure 3E). Similar to NaCl treatment, 15% sucrose treatment could also result in an increase of TAG deposition in the roots of the plantlets. However, TAG accumulation was found to increase after 10 h of sucrose treatment—a delay of 8 h compared with NaCl treatment (Figure 3F). The results indicated that the increase of TAG content in the roots of plantlets might be mainly due to the increase of the expression level of TmDGAT1s induced by stress.

Heterologous expression of TmDGAT1a and TmDGAT1b in Saccharomyces cerevisiae

To assess TmDGAT1s activity, a complementation assay was carried out using a Saccharomyces cerevisiae neutral lipid (NL)-deficient quadruple mutant strain (H1246) (Sandager et al. 2002). Mutant strain H1246 transformed with pYES2-TmDGAT1a or pYES2-TmDGAT1b was designated as TD1a/H1246 or TD1b/H1246, respectively. SCY62 (wild-type) and H1246 cells harboring an empty pYES2 vector were used as positive and negative controls, designated pYES2/SCY62 and pYES2/H1246, respectively. GC analysis showed that the content of TAG extracted from TD1a/H1246 and TD1b/H1246 was approximately 18 mg/g and 20 mg/g, resulting in increases.
Figure 3. Analysis of TmDGAT1s expression and triacylglycerol (TAG) content in roots of Tetraena mongolica plantlets in response to applying stress.

(A) Quantitative real time PCR (qRT-PCR) analysis of TmDGAT1s expression in response to 800 mM NaCl treatment for varying periods.

(B) qRT-PCR analysis of TmDGAT1s expression in response to 20% PEG6000 treatment for varying periods.

(C) qRT-PCR analysis of TmDGAT1s expression in response to 15% sucrose treatment for varying periods.

(D) TAG content in roots of T. mongolica plantlets in response to 800 mM NaCl treatment for varying periods.

(E) TAG content in roots of T. mongolica plantlets in response to 20% PEG6000 treatment for varying periods.

(F) TAG content in roots of T. mongolica plantlets in response to 15% sucrose treatment for varying periods.

Data are the mean ± SD of three independent experiments. **P < 0.01
of 28% and 42%, respectively, compared to the positive control (Figure 4). These results suggested that both TmDGAT1a and TmDGAT1b encode proteins with DGAT1 activities.

Expression of TmDGAT1s in Glycine max hairy roots

A non-seed system, Glycine max hairy root, was used to better understand the function of TmDGAT1s. We generated the transgenic G. max hairy roots by the overexpression constructs pEGAD-TmDGAT1a and pEGAD-TmDGAT1b (Figure S5A). The phosphinothricin (PPT)-resistant hairy roots were examined by reverse transcription PCR (RT-PCR). Amplification with primers for TmDGAT1a and TmDGAT1b, respectively, resulted in a single band with the expected size of approximately 1500 bp (Figure S5B, C). Four transgenic hairy root lines were maintained for TmDGAT1a (designated TD1a/HR-1, TD1a/HR-2, TD1a/HR-3 and TD1a/HR-4), and another four transgenic hairy root lines were maintained for TmDGAT1b (designated TD1b/HR-1, TD1b/HR-2, TD1b/HR-3, and TD1b/HR-4). Hairy root lines obtained from the explants transformed by K599 containing empty pEGAD were used as the control for GC analysis. As shown in Figure 5A and B, on a residue DW basis, the transgenic hairy root lines of TmDGAT1a and TmDGAT1b both exhibited an increase in TAG content, compared with the control. Moreover, the increase of TAG content generally ranged from 37% to 67% of TmDGAT1a transgenic lines, and from 53% to 85% of TmDGAT1b transgenic lines. The fatty acid composition of TmDGAT1 transgenic hairy root lines were both significantly affected, with an increase of 19% to 37% for 18:2 as well as a decrease of 24% to 32% for 18:3 in TD1a/HRs (Figure 5C), an increase of 22% to 44% for 18:2, and a decrease of 12% to 23% for 18:3 in TD1b/HRs (Figure 5D). In addition, all of the transgenic hairy root lines of TmDGAT1a exhibited an increase of 15% to 33% for 16:0, compared with the control (Figure 5C). The results indicate that both TmDGAT1a and TmDGAT1b are functional in G. max hairy roots. Besides an increase in TAG content, there was also an alteration of fatty acid composition in the transgenic lines of both TmDGAT1a and TmDGAT1b.

Expression of TmDGAT1s in T. mongolica calli

T. mongolica calli cells were also used to further verify the functions of the two genes. The pEGAD-TmDGAT1a and pEGAD-TmDGAT1b constructs were introduced into Agrobacterium tumefaciens EHA105 and used to transform T. mongolica calli cells induced from stems of plantlets, and the progeny were analyzed as described in the Materials and Methods section of this paper. TmDGAT1s-overexpressed calli generated by PPT selection and confirmed by RT-PCR for the presence of Bar gene (data not shown) were obtained and named TD1a/TmC and TD1b/TmC, respectively. qRT-PCR analysis indicated that the expression of TmDGAT1a in TD1a/TmC increased by 77%, and TmDGAT1b in TD1b/TmC increased by 151%, compared to the control (Figure 6A,B). On a residue DW basis, the TAG content of TD1a/TmC and TD1b/TmC increased by 59% and 108%, respectively, compared to the control (Figure 6C). The fatty acid composition of the TAG in transgenic calli was also significantly affected by overexpression of the TmDGAT1s. In TD1a/TmC, there was a significant increase in 18:2 of 80%, as well as a decrease of 25% in 18:3. 18:1 was also greatly enriched with an increase of 57%. Similar changes of the fatty acid profiles also occurred in TD1b/TmC; 18:1 and 18:2 increased by 100% and 92%, respectively. In addition, 18:3 decreased by 30%. Slight changes were also observed in 16:0 and 18:0 in both TD1a/TmC and TD1b/TmC (Figure 6D).

Discussion

We cloned and characterized two DGAT1 genes (TmDGAT1a and TmDGAT1b) from T. mongolica, which encode proteins with high homologies and significant primary structural similarities to other previously identified DGAT1 genes in different plant species (Figure 1, Figure S1). Protein hydrophobicity analysis predicted that both TmDGAT1a and TmDGAT1b possess nine putative transmembrane domains, a structural feature, which correlates with other plant DGAT1s and is

![Figure 4. Gas chromatography (GC) analysis of triacylglycerol (TAG) content obtained from yeast cells.](image-url)
Cloning and Functional Analysis of DGAT1 Genes

Figure 5. Gas chromatography (GC) analysis of triacylglycerol (TAG) content in transgenic soybean hairy roots of TmDGAT1s.

(A) TAG content of transgenic hairy root lines of TmDGAT1a.
(B) TAG content of transgenic hairy root lines of TmDGAT1b.
(C) Fatty acid compositions of TAG extracted from transgenic hairy root lines of TmDGAT1a.
(D) Fatty acid compositions of TAG extracted from transgenic hairy root lines of TmDGAT1b.
TD1a/HR, transgenic hairy roots of TmDGAT1a; TD1b/HR, transgenic hairy roots of TmDGAT1b; control, hairy roots containing empty pEGAD. Data are the mean ± SD of three independent experiments. **P < 0.01

consistent with its role as an integral membrane protein that has been shown to be localized to the ER (Zou et al. 1999; Nykiforuk et al. 2002; He et al. 2004; Shockey et al. 2006).

Southern blot analysis indicated that both TmDGAT1a and TmDGAT1b were with low copy number in the T. mongolica genome (Figure S4). Quantification of TmDGAT1s transcripts by qRT-PCR showed that both TmDGAT1a and TmDGAT1b ubiquitously expressed in all tissues of T. mongolica plantlets (Figure 2), which is the same situation as in A. thaliana DGAT1 (Zou et al. 1999). TmDGAT1b transcript intensity was much stronger than TmDGAT1a in all tissues, and particularly in roots, which correlates quite well with TAG content from different tissues (Figure 2; Table 1). These results indicate that TAG accumulation in different tissues of T. mongolica plantlets may largely be due to TmDGAT1b.

However, TAG content reported here was much lower than that of T. mongolica growing in the local environment (Wang et al. 2007). The TAG content difference may be due to different periods the plants were in and different environments in which they survived. T. mongolica is a typical xerophytic plant in Inner Mongolia, and could not survive successfully outside its local environment, which makes it unqualified for study in the laboratory. Therefore, we had to choose tissue culture plantlets as research materials in this paper. Normally, TAG is an efficient high density carbon and energy source that is important in transferring reduced carbon from one generation to the next. Thus, it is found mainly in seeds, pericarps and pollen (Lin and Oliver 2008).

T. mongolica plantlets from tissue cultures had higher water content and a lower lignified degree, and were in a strong vegetative growth stage, which was not an ideal storage mode for large amounts of TAG, compared with the woody stems of the shrub. Wang et al. (2007) conjectured that high levels of TAG in stems are advantageous to survival in harsh natural surroundings, especially drought. However, the plantlets used in this study were cultured in the greenhouse where conditions are stable and unchallenged. It seems that there is no need for the plantlets to accumulate large amounts of TAG under such conditions. When the plantlets were treated by applying stress, including high salinity, drought and osmotic stress; TAG accumulation in T. mongolica plantlets exhibited a typical time-dependent increase pattern (Figure 3D–F), providing optimal adaptation to environmental factors. An increased
expression level of both TmDGAT1a and TmDGAT1b could thus provide an explanation, at the molecular level, for the rapid induction of TAG biosynthesis in T. mongolica plantlets under applied stress (Figure 3A–C). Our results are consistent with the fact that DGAT1 activity and its control over TAG assembly may be influenced by environmental factors (Weselake et al. 2008). Similar to microalgae, TAG is mainly accumulated under stressful conditions in extra-plastidial oil bodies (Guihénéuf et al. 2011). The main work to be done in the future is to investigate the molecular mechanism of large amounts of TAG accumulation in stems in order to exploit and use this plant.

Results from heterologous expression studies on the S. cerevisiae NL-deficient quadruple mutant confirmed that the TmDGAT1 genes encode proteins that function as DGAT1 enzymes. When the recombinant TmDGAT1a and TmDGAT1b proteins were expressed in the NL-deficient mutant strain H1246 devoid of all enzymes, which can contribute to TAG synthesis, it was shown that DGAT activity of the mutant host was restored to produce TAG (Figure 4). In plant transformation studies, T. mongolica calli and soybean hairy roots were used as explants for further functional verification of TmDGAT1a and TmDGAT1b in this study. Overexpression of TmDGAT1a and TmDGAT1b in soybean hairy roots resulted in an increase in oil content of 37% to 85% (Figure 5A, B). Similar experiments conducted using TmDGAT1a and TmDGAT1b overexpression in T. mongolica calli showed that transgenic calli exhibited an oil content increase of 59% and 108%, respectively (Figure 6C). These results further confirmed that both TmDGAT1a and TmDGAT1b encode proteins to function as DGAT1 enzymes. In addition, our results also support earlier biochemical evidence indicating that DGAT is rate-limiting for the production of TAG (Perry et al. 1999; Lung and Weselake 2006).

Besides an increase in oil content, the fatty acid composition of TAG was also significantly affected in all transgenic T. mongolica calli and transgenic hairy root lines by overexpression of TmDGAT1a and TmDGAT1b, with an increase of 18:2 and 16:0 as well as a dramatic decrease of 18:3 (Figure 5C,D; Figure 6D). Moreover, transgenic T. mongolica calli also exhibited an
increase of 18:1 concomitant with a decrease of 18:0 in TAG compared to the control (Figure 6D). The observed changes in overexpression of TmDGAT1s are not very surprising. Similar results have been obtained before. For overexpression of Arabidopsis DGAT1 in tobacco, the percent of 18:3 in leaf TAG was reduced from 20% to 12%, and 18:1 increased from 18% in wild-type (WT) to 44% in the analyzed transgenic line (Andrianov et al. 2010). Even more profound, a single amino acid insertion in DGAT resulted in significantly increased oil and oleic acid content in maize seeds (Zheng et al. 2008). DGATs typically exhibit a strong capacity for incorporating the most prevalent acyl moieties into the sn-3 position (Xu et al. 2008). Evidence from this work indicated that there seems to be more 18:1 and 18:2 in the T. mongolica calli as well as in soybean hairy roots. In addition, both TmDGAT1a and TmDGAT1b appeared to prefer 18:1 and 18:2 rather than 18:0 and 18:3. Therefore, manipulation of the expression of these two genes may both increase oil content and alter fatty acid composition.

In the present study, transgenic soybean hairy roots and transgenic T. mongolica calli were used as two quick and efficient systems for TmDGAT1s function verification, which offer rapid techniques to introduce foreign genes in plant cells (Aarrouf et al. 2011). Production of transgenic hairy roots with A. rhizogenes only takes a few weeks (Guillon et al. 2006). Genetically engineered root cultures have been used as a model system to modulate the metabolism and regulation of several natural product pathways (Zhang et al. 2009; Park et al. 2011). Similar to soybean hairy roots, it also took only a few weeks to harvest transgenic calli. In many high plant species, calli were widely used as explants for plant transformation and regeneration of transgenic plantlets because of their high efficiency, including such species as Ponkan mandarin (Li et al. 2002) and apple trees (Dong et al. 2011). Transgenic calli could be preserved over the long-term, and be induced to regenerate transgenic plants when necessary. Moreover, the genetically identical background of the material, easy proliferation and rare occurrence of chimeras during regeneration are other positive features of this transformation system. In this paper, the two systems were used for the first time in studying DGAT1 functions, and performed very well in both transformation and TmDGAT1s functional identification.

Materials and Methods

Plant materials and growth conditions

Tetraena mongolica Maxim. seeds were collected from several isolated populations in the Wuhai Conservation Zone of T. mongolica, Inner Mongolia, China, and were grown on MS medium (Murashige and Skoog 1962) after being sterilized in 70% (v/v) alcohol for 3 min followed by a second sterilization in 10% (w/v) NaClO for 15 min. All of the T. mongolica plantlets used in this work were regenerated by stem segments cut from 2-month-old seedlings and cultured on 1/2 MS medium containing 1 mg/L indolebutyric acid. After root formation, plantlets were transferred to containers filled with vermiculite containing MS liquid medium for subsequent growth. All media had 7 g/L agar, and the pH was adjusted to 5.8 using 1 N NaOH or HCl, before autoclaving at 121 °C, 105 kPa for 20 min. Cultures were incubated at 26 ± 2 °C, 100 µmol/m² per s, with a 16 h photoperiod.

Stems of T. mongolica plantlets were used to establish T. mongolica calli on modified MS medium containing 0.5 mg/L 2,4-D and 0.1 mg/L 6-BA, and were incubated at 26 ± 2 °C, 100 µmol/m² per s, with a 16-h photoperiod. The cultures were sub-cultured every 28 d and used for experiments 20–25 d after sub-culturing.

Soybean (Glycine max (L.) Merrill, cv. Heinong44) seeds were surface-sterilized in a hermetically sealed desiccator with chlorine gas produced by mixing 100 mL bleach (5.25% sodium hypochlorite) and 4 mL 12 N HCl for 14 h. Sterilized seeds were germinated on MS medium containing 3% sucrose and 0.8% agar at 26 ± 2 °C, 100 µmol/m² per s, with a 16 h photoperiod.

Cloning of T. mongolica DGAT1 cDNAs

Degenerate primers were designed according to amino acid sequences conserved in Arabidopsis and other known plant DGAT1s. Total RNA was isolated from 1-month-old T. mongolica plantlets with RNAiso Plus (TaKaRa, Dalian, LiaNing, China), and treated with RNase free DNase I (TaKaRa). A single-stranded cDNA template for RT-PCR was synthesized at 42 °C from plantlets poly (A) RNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA). A 50 µl PCR contained single-stranded cDNA derived from 40 ng of poly (A) RNA, 12.5 nmol of each primer (DF and DRr, see Table S2), and 2.5 U of TransTag DNA Polymerase High Fidelity (HFi) (TransGen Biotech, Beijing, China) under standard conditions. One internal part of the DGAT sequence was amplified in a thermocycler for 30 cycles of the following program: 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min. The sequence of a 852-bp PCR product was used to design gene-specific primers (GSPs) to amplify the 5′ and 3′ ends of the cDNA as described by Scotto-Lavino et al. (2006, 2007), and finally yielded two full-length cDNAs named TmDGAT1a and TmDGAT1b, respectively. The GSPs used for RACE are displayed in Table S2.

Southern blot analysis

Genomic DNA was isolated from T. mongolica plantlets using a modified CTAB-based extraction procedure (Murray and Thompson 1980). Southern blot analysis was conducted according to the methods described by Sambrook and Russell.
(2001). Aliquots of genomic DNA (10 μg) were digested overnight with three restriction enzymes, HindIII, EcoRI and XbaI, individually. Digoxigenin (DIG)-labeled probes were generated by PCR spanning 291-bp of the 5’ coding sequence of TmDGAT1a, and 288-bp of the 5’ coding sequence of TmDGAT1b, and the corresponding primers are displayed in Table S2.

**Stress treatments of *T. mongolica* plantlets**

For the treatment of high salinity, drought and osmotic stresses, one-month-old plantlets grown in vermiculite containing MS liquid medium were transferred to vermiculite watered with MS liquid medium supplemented with 800 mM NaCl or 20% PEG 6000 or 15% sucrose, and roots of the plantlets were sampled at 0.5 h (used as control), 1 h, 2 h, 5 h, 10 h, 24 h, 48 h and 72 h for analysis of gene expression and TAG content.

**RNA isolation and quantitative PCR**

Total RNAs were extracted from root tissues of treated *T. mongolica* plantlets with RNAiso Plus (TaKaRa), and treated with RNase free DNase I (TaKaRa). The total RNAs were reverse-transcribed into first-strand cDNA in a 20-μL volume with M-MLV reverse transcriptase (Promega). The samples were diluted to 100 μL with water and 1 μL of each sample (~10 ng RNA equivalent) was amplified using TransStart Green qPCR SuperMix UDG (TransGen Biotech) in a 20 μL reaction, containing 1 μL of diluted cDNA, 10 μL TransStart Green qPCR SuperMix UDG, 0.4 μL Passive Reference Dye II, 1 μL of 10 μM forward primer, 1 μL of 10 μM reverse primer and 6.6 μL of water. The thermal cycle used was as follows: 50 °C for 2 min; 95 °C for 10 min; 40 cycles of 95 °C for 5 s, 57 °C for 15 s and 72 °C for 10 s. The corresponding specific primers are displayed in Table S2. The relative expression levels were determined as described by Livak and Schmittgen (2001).

**Expression of *TmDGAT1s* in yeast**

Saccharomyces cerevisiae strains SCY62 (Relevant genotype: MATaADE2), H1246 (Relevant genotype: MATaare1-Δ::HIS3 are2-Δ::LEU2 dga1-Δ::KanMX4 iro1-Δ::TRP1ADE2) (Sandager et al. 2002) and the pYES2 expression vector were kindly provided by S. Stymne (Scandinavian Biotechnology Research, Alnarp, Sweden).

The full-length cDNA of *TmDGAT1s* was amplified by PCR with the corresponding primers (see Table S2), and subsequently cloned behind the GAL1 inducible promoter in the respective sites of the pYES2 vector to create the pYES2-TmDGAT1a and pYES2-TmDGAT1b constructs. The resulting plasmids were transformed to H1246 strain cells according to the methods described by Elble (1992). H1246 and SCY62 cells harboring the empty pYES2 vector were used as negative and positive controls, respectively. Transformants were selected by growth on synthetic complete medium lacking uracil (SC-ura), supplemented with 2% (w/v) glucose.

For functional expression, the colonies were transferred into liquid SC-ura with 2% (w/v) glucose and grown at 30 °C overnight. The overnight cultures were diluted to an OD 600 of 0.2 in induction medium (SC-ura + 2% galactose), and were induced for 36 h at 30 °C. Cells were collected for lipid analysis.

**Plant transformation vectors**

The full-length ORF of *TmDGAT1s* was amplified by PCR with the corresponding primers (see Table S2), and subsequently cloned behind the cauliflower mosaic virus (CaMV) 35S promoter in the respective sites of the pEGAD vector to create the pEGAD-TmDGAT1a and pEGAD-TmDGAT1b constructs.

**Plant transformation and selection**

The pEGAD-TmDGAT1a and pEGAD-TmDGAT1b constructs were introduced into *T. mongolica* callus cultures using *A. tumefaciens* strain EHA105, as reported by Li et al. (2002). After 3 d of co-culture in darkness at 22 °C, the calli were washed three to four times, and then transferred to selection medium (MS medium containing 0.1 mg/L 6-BA, 0.25 mg/L 2, 4-D, 5 mg/L PPT and 500 mg/L carbenicillin). Four weeks later, most of the calli had become necrotic and died. The calli that did proliferate on the selection medium were sub-cultured on selection medium three or four times at 3 week intervals. Transgenic calli were further confirmed by PCR amplification of cDNA using primers of *Bar* gene (see Table S2), and were then used for further expression of *TmDGAT1s* and TAG analysis.

*Agrobacterium rhizogenes* strain K599 with binary vector pEGAD-TmDGAT1a or the pEGAD-TmDGAT1b construct was used to obtain transgenic hairy roots according to Cho et al. (2000). Established root cultures were further detected by PCR analysis of cDNA (primers used are displayed in Table S2), and then used for further TAG analysis.

**Lipid extraction and separation**

The collected yeast cells were broken in a glass-bead shaker, and extracted into chloroform: MeOH (1:2, v/v). Other samples, including different tissues separated from *T. mongolica* plantlets, *T. mongolica* calli, and soybean hairy roots, were ground to a fine powder under liquid nitrogen, and subsequently dumped into chloroform: MeOH (1:2, v/v) immediately. Total lipids were extracted from the samples by methods described by Bligh and Dyer (1959), and TAG was separated from the total lipids by thin-layer chromatography (TLC). The plates were developed in hexane:Et2O (70:30:1, v/v). Spots
were made visible by spraying the plates with 0.01% primuline in acetone/H₂O (60:40; v/v) and examining the plates under ultraviolet (360 nm) light. Triolein was used as the standard. Residue of the samples after extracting was rinsed five times with distilled water and dried at 80 °C for 24 h to determine the DW of residue.

**Fatty acid analysis**

Fatty acid analysis was carried out following the method described by Xu et al. (2003). Briefly, the TAGs separated by TLC were transesterified with 5% H₂SO₄ in MeOH at 68-90 °C for 1 h, and the fatty acid methyl esters (FAMES) were extracted with hexane and separated on a Hewlett-Packard 6890 gas chromatography apparatus supplied with a hydrogen flame ionization detector and a capillary column HP INNOWAX (30 m; 0.25 mm i.d.) with N₂ carrier at 20 mL/min. The oven temperature was maintained at 170 °C for 5 min, and then increased in steps to 210 °C, with the temperature being raised by 5 °C every min. FAMES from TAG were identified by comparing their retention times with known standards (37-component FAME mix, Supelco47885-U). Heptanoic acid (17:0, from Sigma) served as the internal standard to quantify the amounts of TAG.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. A phylogram showing relationships among TmDGAT1a and TmDGAT1b proteins from other plants.

The alignment was generated with CLUSTAL W, and the phylogram was constructed by the neighbor-joining method with MEGA.4: OeDGAT1 (Olea europaea, Accession no. AAS01606.1), PIDGAT1 (Perilla frutescens, Accession no. AAG23696.1), NiDGAT1 (Nicotiana tabacum, Accession no. AAF19345.1), GmDGAT1a and GmDGAT1b (Glycine max, Accession nos. AAF19366.1 and AAS78662.1), LjDGAT1 (Lotus japonica, Accession no. AAW51456.1), EaDGAT1 (Eucalyptus alatus, Accession no. AAV31083.1), JcDGAT1 (Jatropha curcas, Accession no. ABB84383.1), VIJDGAT1 (Vernicia fordii, Accession no. ABC94471.1), RcDGAT1 (Ricinus communis, Accession no. XP00251432.1), BjDGAT1a and BjDGAT1b (Brassica juncea, Accession nos. AAY40784.1 and AAY40785.1), AtDGAT1 (Arabidopsis thaliana, Accession no. NP179535.1), TmDGAT1 (Tropaeolum majus, Accession no. AAM03340.2), ZmDGAT1 (Zea mays, Accession no. ABV91586.1), OsDGAT1 (Oryza sativa, Accession no. NP001054869.2), SmDGAT1 (Selaginella moellendorfii hypothetical protein, Accession no. XP002994237.1), VgDGAT1a and VgDGAT1b (Vernonia galamensis, Accession nos. ABV21945.1 and ABV21946.1), EpDGAT1 (Echium pitardii, Accession no. ACO55634.1), TmDGAT1a and TmDGAT1b (Tetraena mongolica, Accession nos. JX163929 and JX163930).

Figure S2. Putative transmembrane domains of TmDGAT1a and TmDGAT1b.

The main transmembrane segments were predicted by TMHMM (http://www.cbs.dtu.dk/services/TMHMM/). Nine predicted transmembrane helices were identified for both TmDGAT1a and TmDGAT1b.

Figure S3. Genomic structure of TmDGAT1s.

(A) Genomic structure of TmDGAT1a.

(B) Genomic structure of TmDGAT1b. An examination of partial genomic clone of TmDGAT1s (8 043 bp for TmDGAT1a and 7 588 bp for TmDGAT1b) allows the identification of 15 introns interrupting the corresponding coding region, respectively.

Figure S4. Southern blot analysis of Tetraena mongolica genomic DNA.

(A) Analysis of the genomic organization of TmDGAT1a by Southern blot.

(B) Analysis of the genomic organization of TmDGAT1b by Southern blot.

T. mongolica genomic DNA (10 ug/lane) was digested with restriction enzymes EcoRI (lane 1 and lane 4), HindIII (lane 2 and lane 5) and XbaI (lane 3 and lane 6). The DNA blot was hybridized with a dioxigenin-labeled partial cds of TmDGAT1a or TmDGAT1b as a probe, respectively. Marker sizes (Kb) are indicated.

Figure S5. Induction and examination of soybean hairy roots.

(A) Induction of hairy roots on soybean cotyledons 3 weeks after infection with A. rhizogenes K599 containing empty pEGAD or pEGAD-TmDGAT1a or pEGAD-TmDGAT1b.

(B) The PPT resistant hairy roots were examined by RT-PCR with primers responsible for TmDGAT1a. Lane 2 to lane 5, transgenic lines of TmDGAT1a; lane 1, negative control; Lane 6, positive control.

(C) The PPT resistant hairy roots were examined by RT-PCR with primers responsible for TmDGAT1b. Lane 8 to lane 11, transgenic lines of TmDGAT1b; lane 7, negative control; lane 12, positive control.

M, Marker.

Table S1. Putative functional motifs in TmDGAT1s and AtDGAT1.

Table S2. A list of oligonucleotides used in this study.