

Identification and characterization of a novel type-2 DGAT homologue gene from the green microalga *Haematococcus pluvialis*

Hongjiang Zhang

Shanxi Agricultural University

Wei Hang

Shanxi Agricultural University

Chunchao Zhao

Shanxi Agricultural University

Wenxin Xu

Shanxi Agricultural University

Xiaoli Zhu

Shanxi Agricultural University

Chunli Ji

Shanxi Agricultural University

Jinai Xue

Shanxi Agricultural University

Chunhui Zhang

Shanxi Agricultural University

Runzhi Li

Shanxi Agricultural University

Hongli Cui (✉ cuihongli@sxau.edu.cn)

Shanxi Agricultural University <https://orcid.org/0000-0002-2908-2711>

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Abstract

Background: The unicellular green microalga *Haematococcus pluvialis* is an ideal source of astaxanthin (AST) which is stored at oil bodies contain both esterified astaxanthin (EAST) and triacylglycerol (TAG). Diacylglycerol acyltransferase (DGAT) catalyzes the last step of the acyl-CoA-dependent TAG biosynthesis and is considered as the crucial enzyme involving in EAST biosynthesis in *H. pluvialis*. However, the function of DGAT in *H. pluvialis* has not been reported. **Results:** A full-length cDNA sequences encoding a putative DGAT2 (HaeDGAT2E) was obtained from *H. pluvialis*. It contained an open reading frame (ORF) of 1,017-bp encoding a protein of 338 amino acid residues. The isolated HaeDGAT2E protein shared high identity of 57.6% and 54.1% with DGAT2E from *Chlamydomonas reinhardtii* and *Chromochloris zofingiensis* respectively. There were 7 conserved motifs and 3 trans-membrane regions in HaeDGAT2E. The phylogenetic analysis suggested that HaeDGAT2E belonged to DGAT2E subfamily. HaeDGAT2E activity was confirmed in the TAG deficient yeast strain (H1246) by restoring its ability to produce TAG. Upon expression of HaeDGAT2E, C16:0 and C18:1 fatty acid contents were 190.2% and 132.4% higher respectively than that of the H1246 strain. In addition, over-expression of HaeDGAT2E in transgenic *Nicotiana Benthamiana* resulted in increased contents of C16:0 (113.5%) and C18:1 (234.5%). **Conclusions:** A novel gene encoding HaeDGAT2E was identified in *H. pluvialis*. This is the first functional analysis of DGAT2 in *Haematococcus*. This information is important for understanding TAG accumulation and for further elucidating EAST biosynthesis in *H. pluvialis*.

Background

Microalgae can efficiently absorb carbon dioxide in the atmosphere and turn it into abundant high-value products including proteins, astaxanthin, polysaccharides, biodiesel, etc [1–3]. Triacylglycerols (TAG), which is the principal storage form of energy in eukaryotic organisms, represents a promising source of biodiesel production [4]. Due to high photosynthetic efficiency, rapid reproduction rate, and short growth cycle [5], microalgae have been considered as the best candidate to resolve energy crisis and environmental pollution.

Generally, TAG biosynthesis takes place in the endoplasmic reticulum and its assembly can be divided into acyl-CoA-dependent and acyl-CoA independent pathways [6]. Diacylglycerol acyltransferase (DGAT) catalyzes the final acylation of sn-1,2-diacylglycerol (DAG) to form TAG, which is the last and limiting step in the acyl-CoA dependent TAG formation [7]. This enzyme represents a bottleneck in TAG biosynthesis in some oilseed crops and algal species, and thus has been regarded as a key target in manipulating oil production [7]. In higher plants and microalgae, there are three major groups of DGATs: (1) membrane bound forms of DGAT1 and DGAT2 which shares no sequence similarity; (2) soluble type of DGAT3 which is localized in the cytosol; and (3) dual functional of WS/DGAT which possess both wax ester and DGAT biosynthesis activities [8–11]. DGAT1 is considered to play a critical role in TAG accumulation in many higher plants and microalgae, whereas DGAT2 appears to have an important role in the formation of TAG containing unusual fatty acids. There is strong evidence support the involvement of DGAT3 and WS/DGAT in TAG biosynthesis in microalgae [12–14]. Interesting, only one copy of DGAT1

has been identified in a number of microalgae, whereas multiple copies of DGAT2 genes are typically present, suggesting that DGAT2 may play an important function in TAG biosynthesis and algal growth [8].

Haematococcus pluvialis is a green microalga widely known for its ability to synthesize the highest amount of astaxanthin (4% dry weight) under stress conditions [15–17]. Natural astaxanthin (AST) is a red-colored carotenoid with strong antioxidant ability and important commercial value [18–20]. This microalga also represents a potential source of TAG, since a considerable increase in TAG content accompanies the accumulation of AST [21–24]. Moreover, the previous studies have indicated that the main form of AST is esterified astaxanthin (EAST), which includes astaxanthin monoester and diester, and which is stored in TAG rich cytosolic oil bodies (OBs) [26]. Although the exact mechanisms of stress-induced TAG and AST accumulation in *H. pluvialis* are not well understood, several lines of evidence have suggested that the biosynthesis of both compounds appears to be linked through the regulation of oil biosynthetic enzymes [26]. Indeed, the accumulation of AST appears to be dependent on the accumulation of TAG. In addition, it has been speculated that certain DGAT is the candidate enzyme catalyzing the esterification of AST in *H. pluvialis* [26]. Although DGATs from different microalgae had been confirmed that have the ability to catalyze TAG biosynthesis [27–29], there are few studies on the cloning and functional identification of DGAT from the green alga *H. pluvialis*.

In the present study, homologous cloning coupled with the rapid amplification of complementary DNA ends (RACEs) was applied to clone the full-length cDNA sequences of HaeDGAT2E. The sequence analysis for DGAT from green algae and higher plants were finished, focusing on their phylogeny, evolution, and conserved domains. The function of HaeDGAT2E in TAG biosynthesis was confirmed in the TAG deficient yeast strain (H1246). The total lipids accumulation and fatty acid composition of yeast and tobacco were studied according to the over expression of HaeDGAT2E respectively. These results lay the fundament for elucidating TAG biosynthesis and provide evidence for illuminating EAST biosynthesis in future in *H. pluvialis*.

Results

Cloning, sequence, and phylogenetic analysis of HaeDGAT2E

A full-length cDNA sequences of *HaeDGAT2E* (GenBank MN073495) was obtained by the strategy of combining homologous cloning and rapid amplification of cDNA ends (RACEs) method. The *HaeDGAT2E* cDNA sequence was 1,193 base pairs (bp) in length, which contained a 1,017 bp open reading frame, a 133-bp 5'-untranslated region (UTR), and a 43-bp 3'-UTR with the characteristic of the poly (A) tail (Fig. 1a). The deduced protein had a calculated molecular mass of 38.83 kDa with an estimated isoelectric point of 9.7. The putative HaeDGAT2E protein shared 54.1% and 57.6% identities with DGAT2E from green algae *Chromochloris zofingiensis* (QB0559.1) and *Chlamydomonas reinhardtii* (XP_001694904.1), respectively. However, it shared low identity with DGAT2 from higher plants, such as *Brassica napus* (37.2%, XP_013737142.1), *Gossypium mustelinum* (32.2%, TYI54295.1), and *Arabidopsis thaliana* (31.9%, NP_566952.1). HaeDGAT2E possessed 3 strongly hydrophobic trans-membrane regions (Fig. 1b)

and also had the LPLAT superfamily and DAGAT functional domain, which was consistent with DGAT2s from other species (Fig. 1c).

Conserved domain analysis showed that HaeDGAT2E contained 7 conserved motifs, including YFP block, PR block, PHG block, GGE block, RGFA block, VPFG block, and G block (Fig. 2). In the first block, only CzDGAT2D and CrDGAT2D had the complete Tyr-Phe-Pro (YFP) block like those of higher plants (AtDGAT2 and GmDGAT2), which suggested that these two proteins were plant-type DGAT2, while HaeDGAT2E from *H. pluvialis* was not. The first two amino acids (YF) were highly conserved among all DGAT2s examined, while the third residue was variable in microalgae. Interestingly, in the PHG block, the first two continuous residues Pro-His (PH) were obviously conserved among all DGAT2s examined, whereas the third residue was Gly (G) or Ser (S). It was worth mentioning that the Tyr-Ile-Phe (YIF) motif was conserved in this region and was replaced by Leu-Val-Met (LVM) in HaeDGAT2E. In the following PR block, HaeDGAT2E had a conserved PxxR motif as well as other DGAT2s examined. Similar with PR block, the core GGxxE motif in the GGE block was highly conserved. Basing on this block, CzDGAT2D and CrDGAT2D proteins belonged to plant-type DGAT2. The RGFA block and VPFG block were also conserved among all DGAT2s. In the last block, the alignment result showed that NoDGAT2B was special because it had no G block.

In order to further investigate the evolutionary relationship of HaeDGAT2E, phylogenetic analysis was performed using proteins of DGATs (DGAT1, DGAT2, DGAT3, and WS/DGAT) from different higher plants and microalgae (Fig. 3). Four groups including DGAT1, DGAT2, DGAT3, and WS/DGAT were clustered in this tree. As expected, HaDGAT2E was separated into DGAT2 subgroup with other DGAT2 from algae, and it was clearly separated with those from fungal and higher plants. In addition, HaeDGAT2E also had a close evolutionary relationship with CrDGAT2B, CrDGAT2C, and CzDGAT2E, which implied that they have the same origin and function.

Recovery the TAG synthesis in quadruple mutant yeast strain H1246 with HaeDGAT2E

To verify the function of the putative HaeDGAT2E enzyme, the ORF encoding sequences was heterologously expressed in the quadruple mutant yeast strain *S. cerevisiae* H1246 ($\Delta dga1 \Delta lro1 \Delta are1 \Delta are2$) that lacks the activity of TAG synthesis. This yeast mutant strain contains knockout mutations in four TAG biosynthesis-related genes (*dga1*, *lro1*, *are1*, and *are2*) and is unable to synthesize TAG. The mutant type (MT) yeast can formed TAG when at least one of these four genes was expressed. Furthermore, WT (INVSc1) and H-EV (H1246 harboring empty vector *pYES2.0*) yeast strains were used as positive and negative controls, respectively.

As shown in Fig. 4, HaeDGAT2E was able to restore yeast TAG biosynthesis. There was a prominent TAG spot on the TLC plate from WT and the H-HaeDGAT2E strains respectively, but no TAG spot was detected in both H-EV and MT strains (Fig. 4a). Nile red can specifically stain the lipid molecule in cells, resulting in an orange fluorescence that can be used to quantify the lipid accumulation. As shown in Fig. 4b, the fluorescence in the cells of H-EV and MT strains was difficult to observe and exhibited a shaded orange. However, the lipid droplets were easier to observe, and they were large, clear, and bright in the MT and H-

HaeDGAT2E strains. These results suggested that expression of *HaeDAGT2* in the quadruple mutant strain H1246 can recover its ability to form neutral lipids through interaction with yeast lipids biosynthesis pathway and confirm that HaeDGAT2E indeed encoded a functional protein with DGAT activity.

Analysis of total lipids and fatty acid composition in H-HaeDGAT2E yeast strain

The changes of total lipids content and fatty acid composition were studied in different yeast strains. As shown in Fig. 5a, the total lipids content of MT strain still remained as low as that in H-EV strain, whereas the total lipids content in the yeast transformed with *HaeDGAT2E* significantly increased and was 404.5% higher than that of the H-EV or MT strain. However, the total lipids content of H-HaeDGAT2E strain is still lower (77.6%) than that of the WT yeast INVSc1.

To further test the change of fatty acid composition in different yeast strains, the TAG extracted from different cells was analyzed by GC-MS. Since MT and H-EV had almost the same fatty acid composition, we only chose H-EV as the negative control for the subsequent analysis, and selected WT as the positive control (Fig. 5b). In addition, the C18:2 and C18:3 were fed to test the substrate specificity of HaeDGAT2E to polyunsaturated fatty acids. As shown in Fig. 5b, the C16:1 and C18:1 fatty acid was principle component which was 58%, 48%, and 45% in WT, MT, and H-EV strains respectively. However, in H-HaeDGAT2E strain, the principle component was C16:0 and C18:1 fatty acid and reached 55%. Moreover, compared to WT, MT, and H-EV strains, the polyunsaturated fatty acid (C18:2 and C18:3) content decreased.

Transient expression of HaeDGAT2E in *Nicotiana benthamiana*

To explore HaeDGAT2E as a tool to manipulate acyl-CoA pools and to engineer TAG in higher plants, HaeDGAT2E was over-expressed in the leaves of *Nicotiana benthamiana* by injecting the *Agrobacterium* (GV3101) strain harboring binary vector (pCAMBIA1303) with the targeted gene. RT-PCR results showed that the HaeDGAT2E transcript was expressed (6.7 fold) in transgenic lines (Fig. 6a). The total lipids content significantly increased and was 138.9% higher than that in WT (Fig. 6b). Transgenic *N. benthamiana* lines did not show any visible difference on the total starch and protein contents from wild-type plants (Fig. 6c and 6d). In addition, the overexpression of HaeDGAT2E resulted in increased C16:0 and C18:1 fatty acid content, which was consistent with results from yeast strain (Fig. 6e).

Discussion

H. pluvialis is not only able to produce a substantial amount of TAG but also the highest content of AST under stress conditions, which has great potential as an alternative source of industrial oil production [21-24]. In addition, the previous studies have implied that the main form of AST is esterified astaxanthin (EAST), which includes astaxanthin monoester and diester, and which is stored in TAG-rich cytosolic oil bodies (OBs) [25]. Although the exact mechanisms of stress-induced TAG and AST accumulation in *H. pluvialis* are largely unknown, the AST accumulation is found to be dependent by the TAG biosynthesis

[26]. Therefore, elucidating the stress induced TAG and EAST biosynthetic pathway is crucial to the improvement in the production of both TAG and AST in *H. pluvialis*. DGAT catalyzes the terminal step in acyl-CoA-dependent TAG production, and the expression of four DGAT2 isoforms is found to be up-regulated in *H. pluvialis* under stress conditions [21], which indicates that DGAT2 is the possible candidate enzyme involvement in TAG and EAST accumulation [26]. Recently, DGATs from different oil-accumulating species have been widely reported [29-33]. To our knowledge, there is no report about the function of DGAT2 in *H. pluvialis*. In the current study, a novel DGAT2 cDNA was isolated from the green algae *H. pluvialis* and its function was characterized using a yeast system. In addition, the potential of HaeDGAT2E as a tool to manipulate acyl-CoA pools for improving TAG accumulation was also explored.

One DGAT2 isoform (HaeDGAT2E) was identified from *H. pluvialis* (Fig. 1a). According to the sequences analysis result, the HaeDGAT2E protein shared high identity with DGAT2E of *C. zofingiensis* and *C. reinhardtii*, which implied that the isolated gene belonged to DGAT2E subfamily and might have the same function. Trans-membrane (TM) represents the typical property of DGAT1 and DGAT2. Generally, DGAT1 contains 8-10 predicted TMs, while DGAT2 contains 2 TMs [34, 35]. HaeDGAT2E protein had 3 putative TMs which was consistent with previous study (Fig. 1b). The conserved motifs determine the potentially important functions, which has been previously identified in DGAT2 enzymes from plants, animals, fungi, and microalgae [34, 35]. These typical conserved motifs were also present in HaeDGAT2E but with varying degrees of conservation (Fig. 2). The origin and evolution of multi-copies DGAT2 members is still an interesting and puzzling topic [8]. The HaeDGAT2E was found to be clustered into DGAT2 subfamily and constructed a monophyletic subgroup with CrDGAT2B, CrDGAT2C, and CzDGAT2E (Fig. 1), which was different from the classification of multiple sequences alignment. Together, these results indicate that this isolated DGAT2 might have important physiological functions in *H. pluvialis*.

Heterologous expression in *S. cerevisiae* H1246 strain, which lacks the activity of neutral lipid biosynthesis, can intuitively detect the function of target gene in TAG biosynthesis [28]. This method has been widely used in DGATs from higher plants, fungi, and microalgae [34-40]. In this study, we also introduced HaeDGAT2E gene into this mutant type H1246 strain. Nile red staining and TLC analysis results indicated that HaeDGAT2E encode a protein with DGAT activity (Fig. 4). Recently, the function of different members of DGAT2 remains unclear since the seed oil content of type DGAT2 gene mutant has no significant decrease compared with that of wild type [36]. Moreover, CrDGAT2D failed to accumulate TAG in H1246 yeast [36, 37]. The CzDGAT2D, which was highly close to CrDGAT2D, produced a trace amount of TAG in H1246 yeast [38]. All these previous studies have indicated that different members of DGAT2 family in distinct organisms even in same organism are various. It was further noted that HaeDGAT2E has a close evolutionary relationship with CrDGAT2B which has the ability of restored TAG biosynthesis in H1246 yeast strains [37]. As expected, the HaeDGAT2E was able to restore the TAG biosynthesis ability of yeast mutant H1246 (Fig. 4). Two possible reasons are responsible for the failure to restore yeast TAG biosynthesis by expressing an algal or plant DGAT2 [41]. The first is the differences in codon usage between yeast and algae or plants, and the second is the limited fatty acid composition of yeast, which does not contain the diverse species of fatty acids as algae and thus cannot provide appropriate substrates for DGAT [41]. Therefore, it will be interesting to further test the function of

HaeDGAT2E in yeast strain feeding with exogenous fatty acid. Unfortunately, the substrate specificity of HaeDGAT2E to polyunsaturated fatty acids (C18:2 and C18:3) was weak (Fig. 5b). The C18:2 and C18:3 polyunsaturated fatty acids are rich in *H. pluvialis*.

The fatty acid composition of the resulting yeast TAG appears to be related to the substrate specificity of the introduced DGAT2 toward the four dominant fatty acids in yeast [34-40]. As such, the introduction of DGAT gene from microalgae into yeast cells has also caused changes in fatty acid composition [5, 30, 34, 38, 41]. For instance, yeast cells harboring DGAT2A from *Nannochloropsis oceanica* led to the accumulation of about 40% of C16:0 in TAG, which may derive from its preference for 16:0-CoA [34]. In the present study, the TAG isolated from yeast producing HaeDGAT2E contained ~ 23% of C16:0 and ~ 35% C18:1 (Fig. 5b) suggesting that HaeDGAT2E may have a lower preference for C16:0 containing substrate than the two DGAT2 from *N. oceanica*, but a higher preference for monounsaturated fatty acid C18:1. Indeed, DGAT2 from *C. reinhardtii* have been demonstrated to contribute to the synthesis of diverse TAG species in algal cells by displaying distinct specificities toward acyl-CoA and DAG [36]. Therefore, it is interesting to conduct a comprehensive in vitro characterization of HaeDGAT2E with different substrates including both acyl-CoA and DAG in future.

Algal DGAT is a potential target to engineer improved oil rich biomass accumulation [33]. For instance, genetic engineering of *Arabidopsis thaliana* and *Brassica napus* by expressing a DGAT1 cDNA from *Chlorella ellipsoidea* led to increases in the contents of lipids and polyunsaturated fatty acid [33]. In this present work, HaeDGAT2E was over-expressed in the leaves of *Nicotiana benthamiana*. The over-expression of HaeDGAT2E significantly increased the total lipids content and did not show any visible difference in total starch and protein contents (Fig. 6a-6d). Similarly, the over-expression of DGAT gene in tobacco increased the total lipids accumulation [42-46]. In addition, the change of fatty acid composition from *Nb-HaeDGAT2E* tobacco leaves indicated that HaeDGAT2E has a higher preference for fatty acid C16:0 and C18:1, which was consistent with results from yeast strain.

Conclusion

A novel HaeDGAT2E gene was obtained from *H. pluvialis*. HaeDGAT2E activity was confirmed in the TAG deficient yeast strain (H1246) by restoring its ability to produce TAG. HaeDGAT2E has a higher preference for fatty acid C16:0 and C18:1 in both yeast strain and higher plants. This is the first functional analysis of DGAT2 in *Haematococcus*. This information is important for understanding TAG accumulation and for further elucidating EAST biosynthesis in *H. pluvialis*.

Methods

Strains, plasmid, and growth conditions

Haematococcus pluvialis (Flotow 1844) strain was obtained from Culture Collection of Algae and Protozoa (Dunstaffnage Marine Laboratory) and maintained at the Institute of Molecular Agriculture and

Bioenergy (IMAB), Shanxi Agricultural University. *H. pluvialis* was cultivated on 100-mL BBM medium in 250-mL Erlenmeyer flasks. These Erlenmeyer flasks were placed in a plant growth chamber under the culture conditions of $25 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light intensity with a diurnal cycle of 12 h light /12 h dark at the temperature 23 ± 1 °C. Shake the culture solution in the flasks at the fixed time and twice a day. The *Saccharomyces cerevisiae* wild type strain INVSc1, quadruple mutant type strain H1246, and *Nicotiana benthamiana* were stored in our laboratory. The plasmids pYES2.0 and pCAMBIA1303 were used to transformation of *S. cerevisiae* and *N. benthamiana* respectively.

Extraction of total RNA and synthesis of cDNA and RACEs templates

The total RNA was extracted using the *H. pluvialis* cells at the phase of exponential growth according to the EasySpin RNA Extraction Kit (Aidlab Biotech, Beijing, China). The total RNA concentration was quantified by NanoDrop 2000c (Thermo Scientific, USA). Totally, 2 μg RNA was used to synthesize the first-strand cDNAs by the PrimeScript® RT Enzyme Mix I (TaKaRa DRR047A, China) Kit. It is worth to note that RNA solution should be store at -80 °C if not use immediately. The RACE cDNA template was made using the SMARTer™ RACE cDNA Amplication Kit (Clontech) according to the manufacturer's instructions.

Gene cloning of *HaeDGAT2E*

The core sequence design was based on the highly conserved regions of DGAT2 from some green algae (*Chlamydomonas reinhardtii*, *Chlorella zofingiensis*, and *Phaeodactylum tricornutum*). Two pair homologous cloning degenerate primers (F1/R1 and F2/R2) were designed by CODEHOP software [47]. Moreover, the 5'- and 3'-RACEs gene specific primers were designed based on the homologous cloning sequences obtained in the previous step (5'RACE R3, R4 and 3'RACE F3, F4). All primers were showed in Table 1 and were synthesized by Sangon Biotech (China) company.

First-strand cDNA was used as template and PCR amplification was conducted with TaKaRa LATaq® (TaKaRa DRR002A, China) according to the manufacturer's instructions. The PCR was processed with the following parameters: initial denaturation at 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min (according to the length of product, $1,000 \text{ bp min}^{-1}$), with a final extension at 72 °C for 7 min and cooling to 4 °C. Then the RACEs template was used as template and nested PCR was carried out using the nested universal primers and gene specific primers for 5'- and 3'-RACE reaction. The PCR products were resolved by electrophoresis on 1 % agarose gel. Then, the fragment of interest was excised and purified using an agarose gel DNA fragment recovery kit (TaKaRa D823A, China). Finally, the fragment was cloned into pMD-18T vector (TaKaRa D101A, China) and sequenced (Invitrogen, China).

Bioinformatics analysis

The full-length cDNA sequence of *HaeDGAT2E* was obtained by splicing the middle, 5'- and 3'-RACE fragments with DNASTar 7.1 (DNASTAR Inc., USA) software. The molecular weight (Mw), isoelectronic point (pI), sub-cellular localization, signal peptides, and trans-membrane regions of Hae-P-CRY were

predicted by ExPASy [48]. Hae-P-CRY and other CRYs were aligned using ClustalX [49]. Maximum likelihood trees (Le and Gascuel evolutionary model) of some CRYs proteins were constructed using PhyML [50]. Bootstrap (BS) values were inferred from 400 replicates. Graphical representation and edition of the phylogenetic tree were performed with TreeDyn (v198.3) [51].

Heterologous expression in yeast

The *S. cerevisiae* wild type strain INVSc1 and quadruple mutant H1246 strain, which lacks the ability of TAG biosynthesis, were used to determine the function of *HaeDGAT2E* by heterologous expression. The yeast expression vector pYES2.0, which is controlled by the inducible promoter *GAL1*, was selected to complete this test. The primers information of pYES2.0 vector was showed in Table 1 (pYES2-F/pYES2-R). The mutant type yeast strain H1246 was cultivated on YPD medium until the OD value reached to 0.6-0.8, and then the construction of HaeDGAT2E-pYES2.0 was transformed using the method of LiAc [52]. Selection of the transformants was finished by using the synthetic medium without uracil (SC-ura). For the transformed strains, we selected individual colonies from petri dishes and added them into a 250-mL conical flask with 100-mL liquid SC-ura medium containing 2% (w/v) glucose. Then it was cultured at 30 °C with the shaker speed of 150 RPM for 24 hours. The yeast cells were collected by centrifugation and imported them into a 500-mL conical flask with 200-mL liquid SC-ura medium containing 2% (w/v) galactose to induce. After 72 hours cultivation, yeast cells were collected again and freeze-dried for subsequent experiments. The expression of *HaeDAGT2* in H1246 yeast strain was verified at the transcript level by qRT-PCR method.

Nile Red staining and microscopy

Nile red fluorescent staining was used for the lipid qualitative analysis. Firstly, the collected yeast cells were diluted with sterile water to $OD_{600}=0.2-0.3$. Then, 200 μ L of dimethyl sulfoxide and 10 μ L of Nile Red acetone solution (0.1 mg/mL) were added to 800 μ L of yeast cells solution. The solution was then mixed, heated in a water bath at 42 °C for 5 min, and viewed under an inverted fluorescence microscope at an excitation of 543 nm. Finally, each cell sample was counted, and images of each sample were captured during microscopy.

Total lipids extraction and fatty acids analysis

Extraction of total lipids was finished according to the method of Bligh and Dyer [53]. The TAG was separated and recovered from the total lipid extracts by thin-layer chromatography (TLC) on Silica Gel plates (Merck, Darmstadt, Germany) in petroleum ether/diethyl ether/glacial acetic acid (80: 20: 2, v/v). The liquid volume ratio was 16 mL: 4 mL: 0.4 mL, and the total volume was 20.4 mL. After mixing, the liquid was added to the 200 \times 100 TLC expansion cylinder. Then, after the solvent on the silica gel plate had completely evaporated, put it in an iodine tank for staining and analyze it. The TAG was recovered from the TLC plate and was transferred into a straw with glass fiber. Then, the TAG was trans-esterified with 10% H₂SO₄ in methanol at 80 °C for 2 hour. Lastly, fatty acid methyl esters (FAMES) were extracted

with hexane and analyzed by GC according to the previous method [54]. The total fatty acid content was quantified with the internal standard of heptadecanoic acid (C17:0).

Transient expression of HaeDGAT2E in *Nicotiana benthamiana*

The complete HaeDGAT2E was cloned into the plant expression vector pCAMBIA1301 under the control of the nopaline synthase (NOS) promoter and nos terminator, yielding pCAMBIA1301-HaeDGAT2E. The final binary vector was verified and then transferred into *Agrobacterium tumefaciens* strain GV3101 by the freeze-thaw method [55]. The strong tobacco plants cultured in an artificial climate incubator at 24 °C with a diurnal cycle of 16 h light (light intensity is 4000 lx) and 8 h dark were selected. The tobacco leaves were infected by the *Agrobacterium* strain which harbors binary vector pCAMBIA1303-HaeDGAT2E according to the method [56]. The transformants were selected according to the expression of target gene. The total RNA was extracted from tobacco leaves 2 days after infiltration according to the above method. The qRT-PCR technology was used to analyze the *HaeDGAT2E* gene expression. The *NbActin* gene was used as internal reference. Five days after infiltration, the tobacco leaves were harvested and subsequently extracted for total lipids (TL), total protein (TP), and total starch (TS). All primers used were shown in Table 1.

Statistical analysis

All experiments were performed three times, and the data were analyzed using a one-way analysis of variance (ANOVA) with the Social Sciences (SPSS) software, and the statistically significant at P values of < 0.05. Furthermore, the software of GraphPad Prism 8 was used to draw charts.

Abbreviations

AST: Astaxanthin; EAST: Esterified astaxanthin; TAG: Triacylglycerol; DGAT: Diacylglycerol acyltransferase; ORF: Open reading frame; RACEs: Rapid amplification of complementary DNA ends; UTR: Untranslated region.

Declarations

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Authors' contributions

HZ carried out the experiments, analyzed the data and drafted the manuscript. WH and CZ participated in the experimental design and performed phylogenetic analyses of DGAT. WX and XZ performed phylogenetic analyses of DGAT and detected the expression of DGAT1s in yeast. CJ, JX, and CZ gave

some good advice for writing the manuscript. RL and HC conceived the study, participated in its design and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Datasets used in the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

Institute of Molecular Agriculture and Bioenergy, Shanxi Agricultural University, Taigu 030801, China.

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Tables

Table 1 Primers used in experiment.

Primers	Sequence (5'-3')
Homology cloning F1	GGTATTCAGCACATGNCYCAA
Homology cloning R1	TTGCCAAAGTGGTACACGSGCKC
Homology cloning F2	TGAGTTCCTCACGGNGTGTGCC
Homology cloning R2	CCACCTCCACAAAGCCTNTGSGT
5'-RACE-R3	TGGTACACGGGCACCACT
5'-RACE-R4	CTCCACTGCCACCTCCACA
3'-RACE-F3	TGTTCCCGGACTTCAACAT
3'-RACE-F4	CATCCTCAGTGTTCTCAGT
ORF-F5	ATGGGTGTCGCAACGAATGCGA
ORF-R5	TCACTGGATCTCCAGCGGCTTGT
pYES2-F	GCATAACCACTTTAACTAATAC
pYES2-R	TCGGTTAGAGCGGATGTG
P1303-M13F	TGTAAAACGACGGCCAGT
P1303-M13R	CAGGAAACAGCTATGACC
<i>NbActin</i> -F	CAGTGGCCGTACAACAGGTA
<i>NbActin</i> -R	AACCGAAGAATTGCATGAGG
<i>HaeDGAT2E</i> -q-F6	GGGCRCCGTGGCGGTGATAG
<i>HaeDGAT2E</i> -q-R6	GCCTCGTTGCGGCTCGTCTT

Table 2 DGAT genes and GenBank accession numbers.

Gene name	Accession NO	Gene name	Accession NO
CzDGAT1A	QBG05553.1	CrDGAT1	XP_001692975.1
CzDGAT1B	QBG05554.1	CrDGAT2A	AGO32156.1
CzDGAT2A	QBG05555.1	CrDGAT2B	AGO32157.1
CzDGAT2B	QBG05556.1	CrDGAT2C	AGO32158.1
CzDGAT2C	QBG05557.1	CrDGAT2D	AGO32159.1
CzDGAT2D	QBG05558.1	CrDGAT2E	XP_001701667.1
CzDGAT2E	QBG05559.1	PtDGAT1	ADY76581.1
CzDGAT2F	QBG05560.1	PtDGAT2A	AFQ23659.1
CzDGAT2G	QBG05561.1	PtDGAT2B	AFM37314.1
CzDGAT2H	QBG05562.1	PtDGAT2C	AFQ23660.1
NoDGAT1A	ASL69957.1	PtDGAT2D	AFQ23661.1
NoDGAT1B	ASL69958.1	PtWSD	XP_002180007.1
NoDGAT2A	ATB53137.1	AtDGAT1	CAB45373.1
NoDGAT2B	ATB53138.1	AtDGAT2	NP_566952.1
NoDGAT2C	ATB53139.1	AtDGAT3	sp Q9C5W0.2
NoDGAT2D	ATB53140.1	AtWSD	sp Q93ZR6.1
NoDGAT2E	ATB53141.1	GmDGAT1A	BAE93460.1
NoDGAT2F	ATB53142.1	GmDGAT1B	NP_001237684.2
NoDGAT2G	ATB53143.1	GmDGAT1C	NP_001242457.1
NoDGAT2H	ATB53144.1	GmDGAT2D	K7K424.1
NoDGAT2I	ATB53145.1	GmDGAT3	XP_003542403.1
NoDGAT2J	ATB53146.1	GmWSD	XP_003552517.1
NoDGAT2K	ATB53136.1	UrDGAT2A	AAK84179.1
NoWSD	EWM29694.1	UrDGAT2B	AAK84180.1
Af293DGAT	EAL93134.1	AfDGAT	RMZ41827.1
TmDGAT	EEA25986.1	RmDGAT2B	CEG77579.1
MaDGAT	AQX34626.1		

Note: Cz (*Chromochloris zofingiensis*), Cr (*Chlamydomonas reinhardtii*), Pt (*Phaeodactylum tricornutum*), No (*Nannochloropsis oceanica*), At (*Arabidopsis thaliana*), Gm (*Glycine max*), Ur (*Umbelopsis ramanniana*), Af (*Aspergillus fumigatus Af293*), Tm (*Talaromyces marneffeii ATCC*), Rm (*Rhizopus microsporus*), Ma (*Mortierella alpina*).

Figures

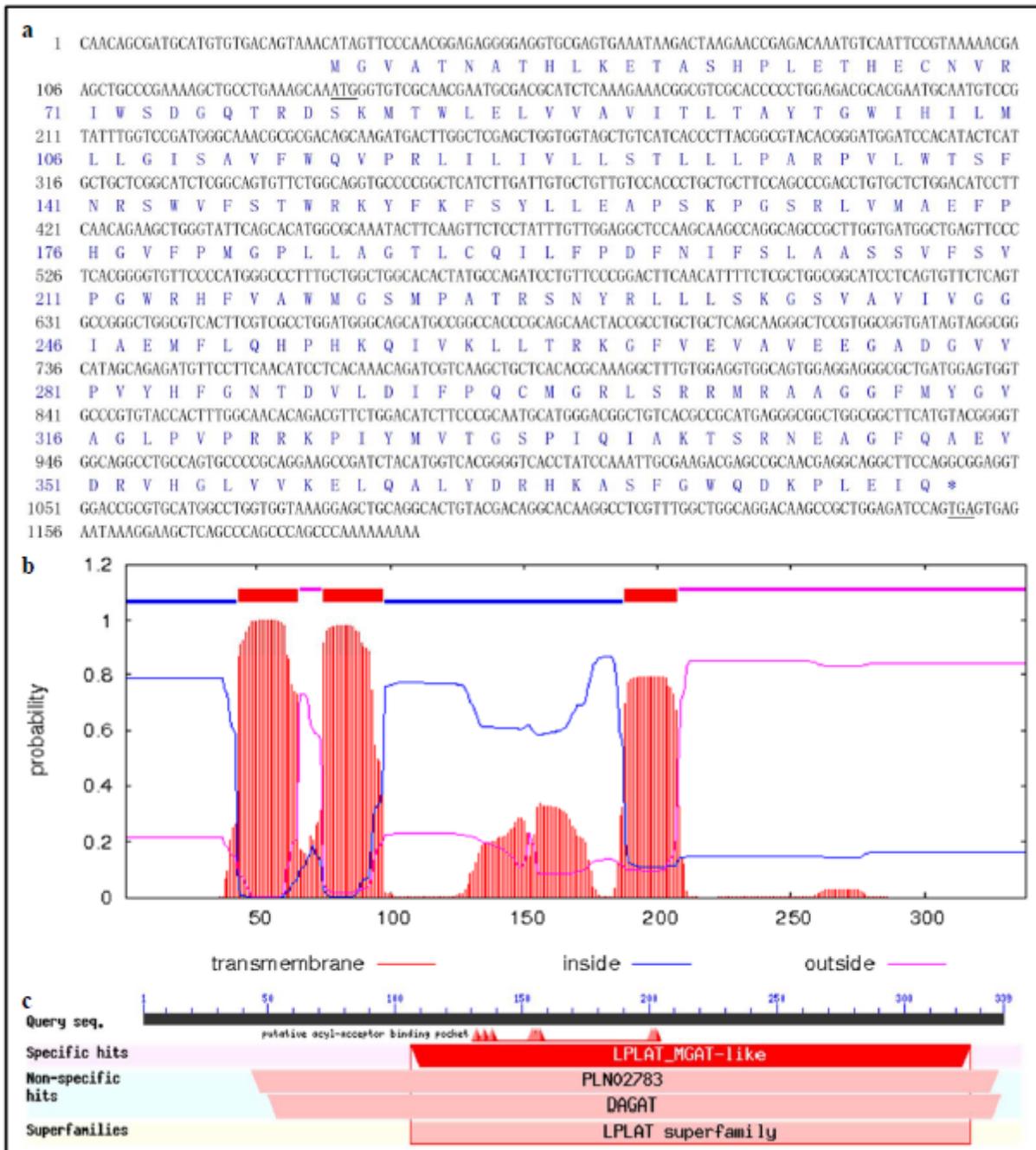


Figure 1

Bioinformatics analysis of HaeDGAT2E. (a) Nucleotide sequence and amino acid sequence of HaeDGAT2E in *H. pluvialis*, (b) transmembrane domain analysis, and (c) blastp analysis.

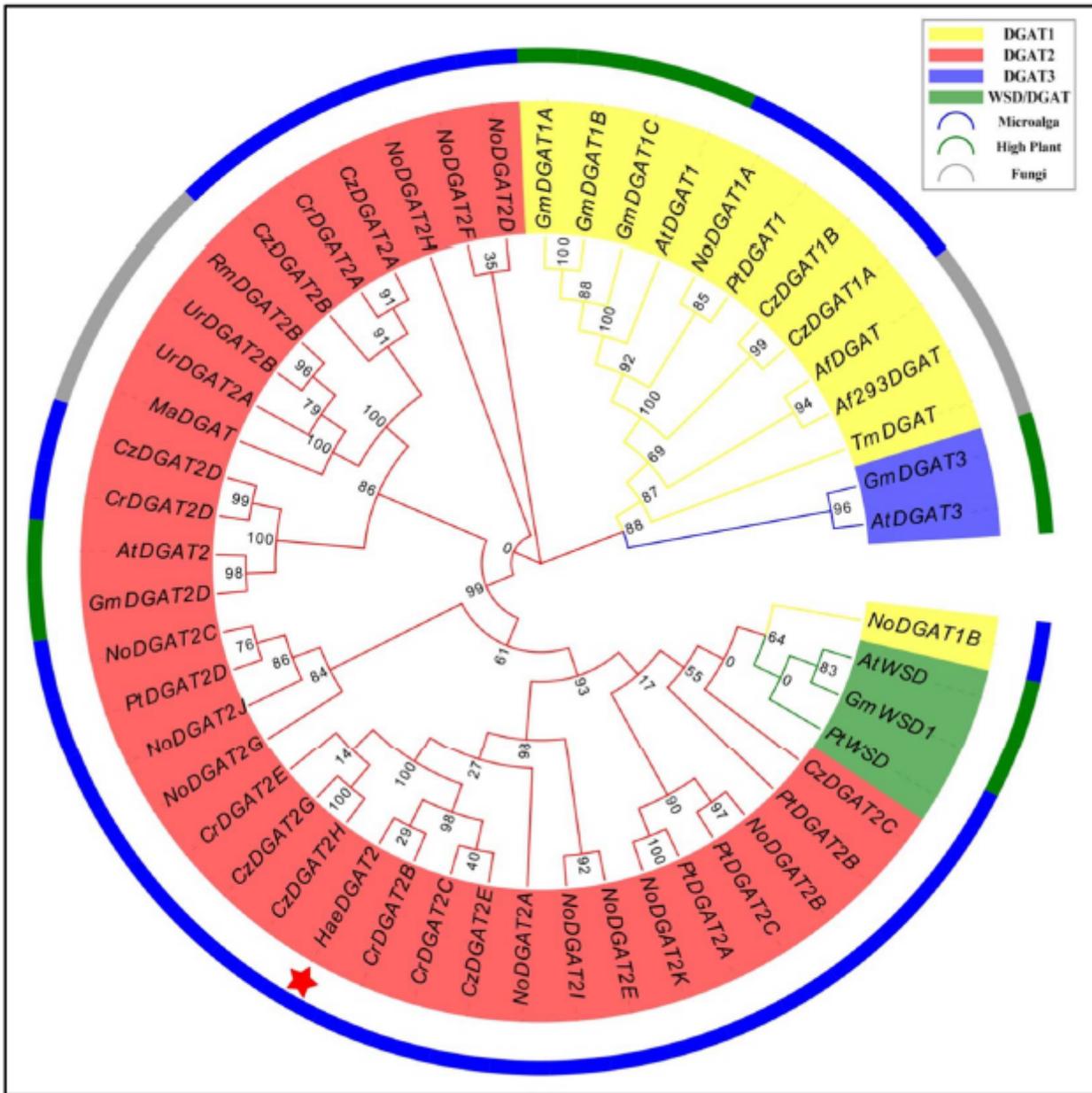


Figure 3

Phylogenetic analysis of HaeDGAT2E and other DGATs from higher plants, fungi and microalgae. Yellow background, red background, blue background, and green background represent DGAT1, DGAT2, DAGT3, and DGAT/WSD, respectively. Blue circle, green circle, and gray circle represent microalgae, higher plants, and fungi, respectively. Red pentacle represents the target gene HaeDGAT2E. The GenBank accession numbers were showed in Table 2.

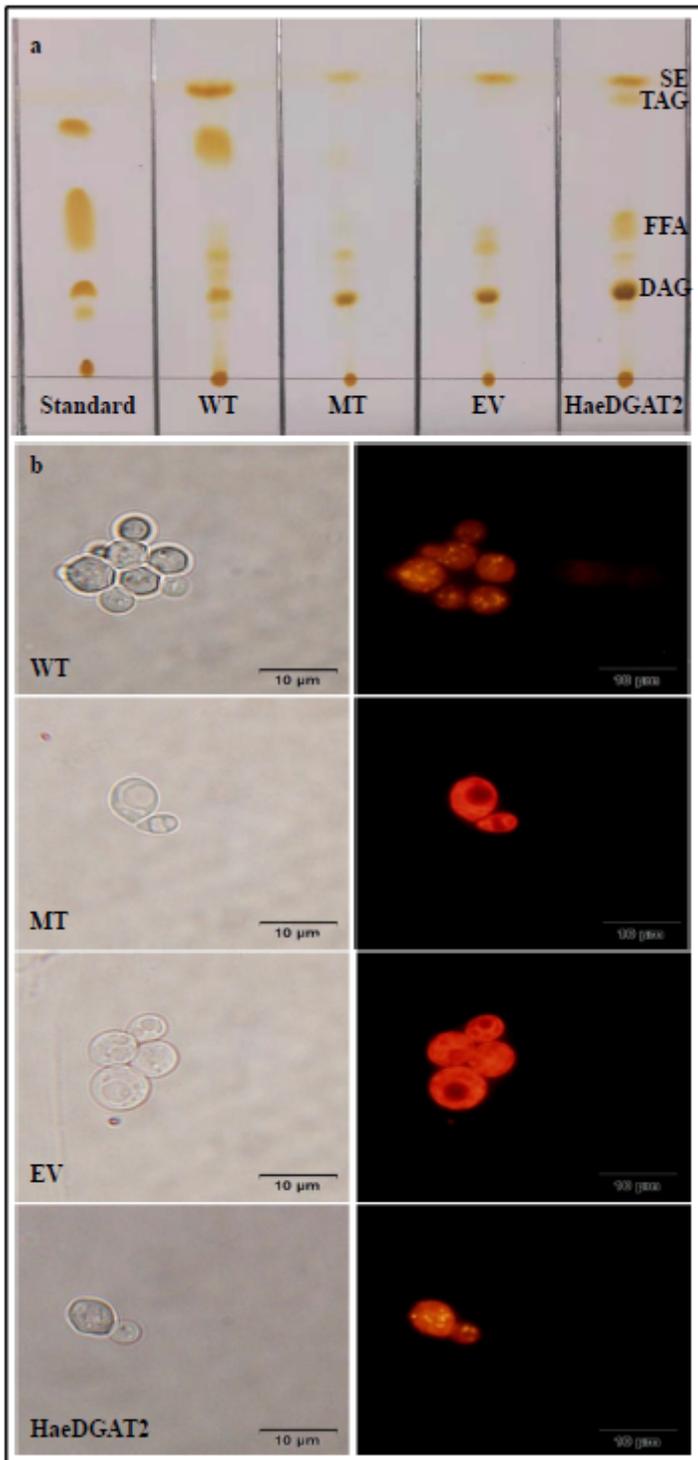


Figure 4

TLC and Nile Red staining analysis of transformed yeast strain H1246. Standard: TAG standard sample; WT: the wild type yeast strain INVSc1. MT: The mutant type yeast strain H1246. EV: the TAG-deficient mutant type yeast strain H1246 transformed with empty vector pYES2.0. H-HaeDGAT2E: the TAG-deficient type H1246 harboring the target gene HaeDGAT2E. SE: steryl ester. TAG: Triacylglycerol. FFA: free fatty acid. DAG: diacylglycerol.

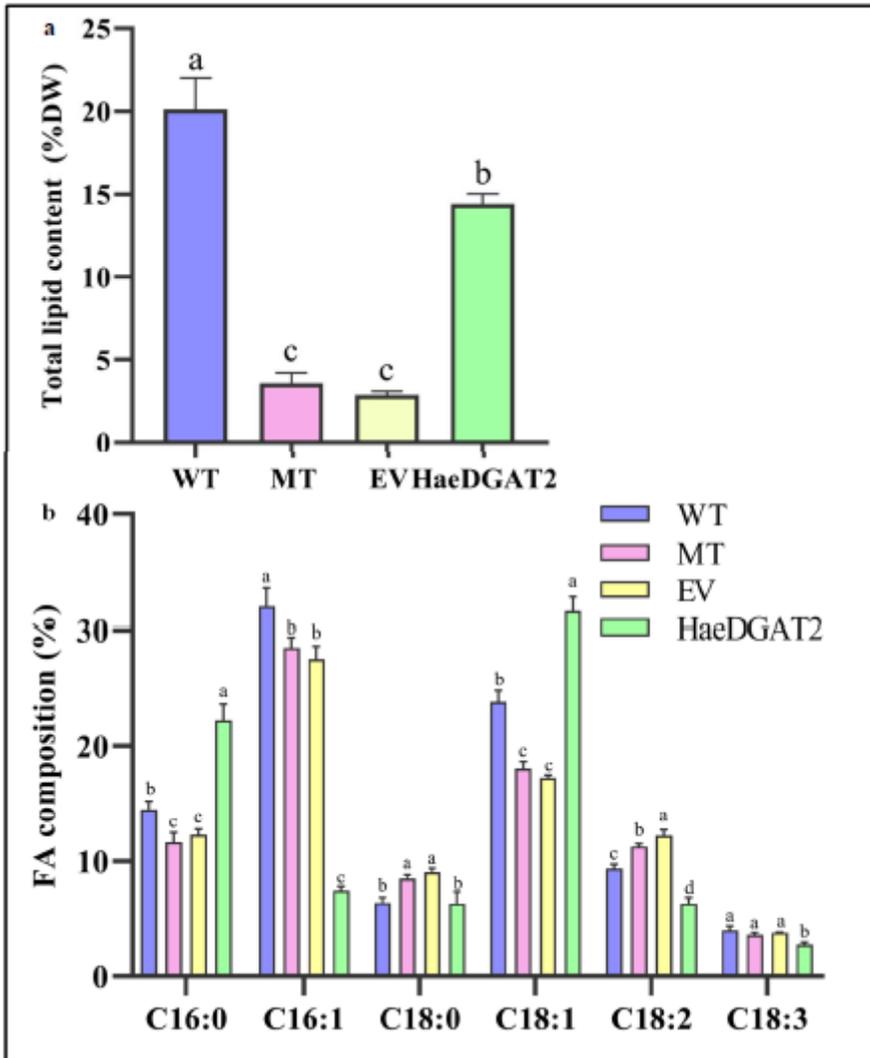


Figure 5

Analysis of total lipids and fatty acid composition in yeast. (a) Total lipids content, (b) Fatty acid composition of different yeast strains. WT: the wild type yeast strain INVSc1. MT: The mutant type yeast strain H1246. EV: the TAG-deficient mutant type yeast strain H1246 transformed with empty vector pYES2.0. H-HaeDGAT2E: the TAG-deficient type H1246 harboring the target gene HaeDGAT2E. C16:0: palmitic acid. C16:1: palmitoleic acid. C18:0: stearic acid. C18:1: oleic acid. C18:2: methyl linoleate. C18:3: methyl linolenate. The bars reveal an average value of three independent technical repeats. The numbers with different letters indicate a statistically significant ($P < 0.05$).

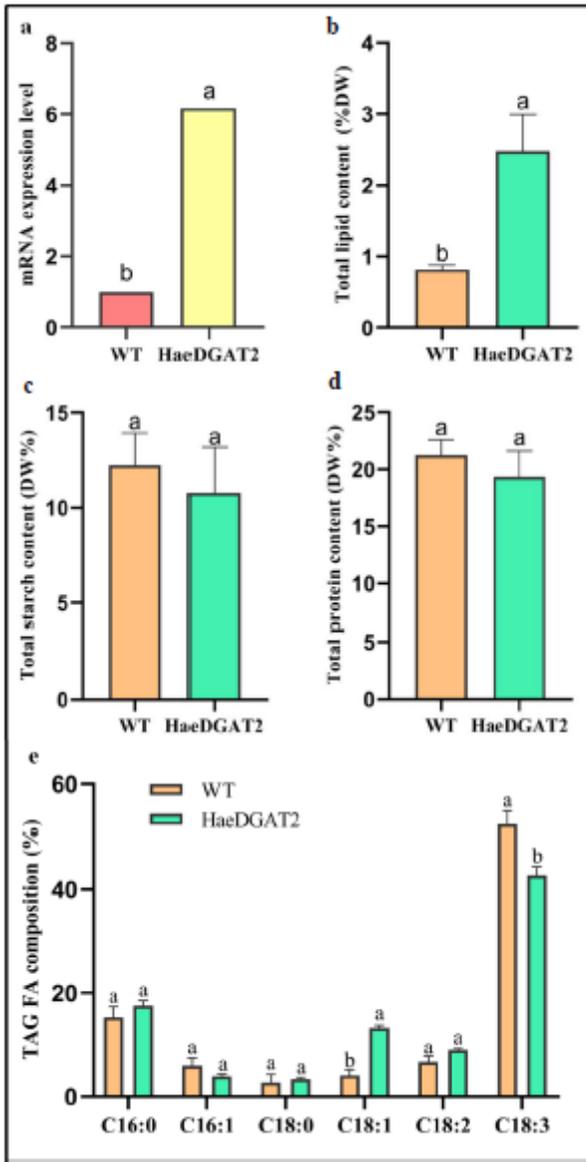


Figure 6

Transient expression of HaeDGAT2E in *Nicotiana benthamiana*. (a) Relative transcript abundance of HaeDGAT2E in tobacco leaves, (b) total lipid content, (c) total protein content, (d) total starch content, (e) fatty acids composition of tobacco leaves upon expressing with HaeDGAT2E. The bars reveal an average value of three independent technical repeats. The numbers with different letters indicate a statistically significant ($P < 0.05$).