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Characterization of a broad substrates specificity acyl-CoA: diacylglycerol acyltransferase 1 from the green tide alga *Ulva prolifera*

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Abstract

Triacylglycerols (triglycerides, TAGs) are the major carbon and energy storage forms in various organisms, and important components of cellular membranes and signaling molecules; they have essential functions in multiple physiological processes and stress regulation. Acyl-CoA: diacylglycerol acyltransferase (DGAT) catalyzes the final and only committed acylation step in the synthesis of TAGs in eukaryotes. The present work identified and isolated a novel gene, *UpDGAT1*, from the green tide alga *Ulva prolifera*. The activity of *UpDGAT1* was confirmed by heterologous expression in a *Saccharomyces cerevisiae* TAG-deficient quadruple mutant. Results of thin-layer chromatography and BODIPY staining indicated that UpDGAT1 was able to restore TAG synthesis and lipid body formation in the yeast. Lipid analysis of yeast cells revealed that UpDGAT1 showed broad substrate specificity, accepting saturated as well as mono- and polyunsaturated acyl-CoAs as substrates. High salinity and high temperature stresses increased *UpDGAT1* expression and TAG accumulation in *U. prolifera*. The present study provides clues to the functions of UpDGAT1 in TAG accumulation in, and stress adaptation of, *U. prolifera*.

Key words: *Ulva prolifera*, diacylglycerol acyltransferase, triacylglycerol, stress

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1 Introduction

Lipids are the major carbon and energy storage forms in various organisms, including vertebrates, oilseed plants, oleaginous fungi, yeasts, and algae. They are also important components of cellular membranes and signaling molecules, with essential functions in multiple physiological processes and stress regulation (Chen et al., 2011). As sessile organisms, plants and maroalgae are exposed to many types of biotic and abiotic stresses under their natural conditions. Plants can sense these stimuli and use lipids as substrates for the generation of numerous signalling lipids, such as phosphatidic acid, phosphoinositides, sphingolipids, oxylipins, free fatty acids and others, and transduce these signals into downstream biological responses (Hou et al., 2016).

Neutral lipid triacylglycerols (triglycerides, TAGs) are mainly assembled by the enzymes of Kennedy pathway in the endoplasmic reticulum (ER), where acyl chains are sequentially transferred from -CoAs to the sn-1, -2 and -3 positions of a glycerol backbone (Ohlrogge and Browse, 1995). Diacylglycerol acyltransferase (DGAT, EC 2.3.1.20), which is thought to be the primary and rate-limiting enzyme for de novo TAG biosynthesis in all organisms studied so far, catalyzes the last and committed step in TAG production by transferring an acyl group to diacylglycerol (Chen and Smith, 2012). There are at least two major families of DGATs, named Type 1 (DGAT1) and Type 2 (DGAT2), which are membrane-bound and catalyze the same reaction, but have no clear sequence similarities. A cytosolic DGAT Type 3 that is thought to be involved in the cutin biosynthesis pathway has also been reported in several plant species (Rani et al., 2010), but its role in TAG biosynthesis is still unclear. DGAT1 shows high sequence homology with mammalian acyl-CoA/cholesterol acyl-

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transferases (EC 2.3.1.26). It was first described in mouse, and subsequently in several plant and algal species (Chen and Smith, 2012; Roesler et al., 2016). Though DGAT1 and DGAT2 catalyze the same reaction, the two enzymes have no redundant functions in TAG biosynthesis (Guihéneuf et al., 2011). In plants, several studies showed that DGAT1 plays a dominant role in the determination of oil accumulation and fatty acid composition (Xu et al., 2008; Taylor et al., 2009; Savadi et al., 2016; Wang et al., 2019; Kim et al., 2016). In *Arabidopsis thaliana*, mutations in *DGAT1*, but not *DGAT2*, affected seed oil levels (Xu et al., 2008), and overexpression of *DGAT1* led to increased seed oil content in *Brassica napus* (Weselake et al., 2008; Taylor et al., 2009), *B. juncea* (Savadi et al., 2016), soybean *(Glycine max*) (Roesler et al., 2016), maize (*Zea mays*) (Zheng et al., 2008), tobacco (*Nicotiana tabacum*) (Andrianov et al., 2010), and *A. thaliana* (Misra et al., 2013).

The basic mechanism of lipid synthesis in algae was assumed to be the same as that in land plants, which is supported by in silico analysis as many sequences in algal genomes seem likely to encode the relevant enzymes for TAG biosynthesis (Hu et al., 2008; Miller et al., 2010). However, algae are phylogenetically very diverse, and it is becoming clear that for most algal species, even for those that fall into the green algal lineage, there are some notable differences (Chen and Smith, 2012; Brodie et al., 2017). With regard to the DGATs, the majority of algal species encode at least one *DGAT1* gene, but it appears to be absent from *Ostreococcus* and *Micromonas* (Chen and Smith, 2012). Several biochemical and functional studies on DGATs have been carried out in unicellular microalgae, such as *Phaeodactylum tricornutum* (Niu et al., 2013), *C. reinhardtii* (Russa et al., 2012), *Chlorella zofngiensis* (Mao et al., 2019), *Ostreococcus tauri* (Wagner et al., 2010), *Tetraselmis chui* (Úbeda-Mínguez et al., 2017) and *Nannochloropsis oceanica* (Li et al., 2016), *Myrmecia incisa* (Chen et al., 2015). However, the mechanism of TAG synthesis in seaweed remains poorly understood.

Ulvophyte green seaweeds represent attractive model systems for understanding growth, development, and evolution. They are untapped resources for production of food, fuel, and high-value compounds, but they can also cause significant environmental problems in the form of green tides and biofouling (Vesty et al., 2015; Callow and Callow, 2006a, b; Smetacek and Zingone, 2013). Massive green tides, which are mainly caused by *Ulva prolifera* have occurred for 13 successive years (2007–2019) in the Yellow Sea (Zhang et al., 2019). Unlike land plants and unicellular green algae, mechanistic studies of growth and development at the molecular level in green seaweeds are severely limited. In the present study, we report the identification and characterization of a DGAT1-encoding gene (*UpDGAT1*) from *U. prolifera*, whose function was confirmed by expression in the yeast *Saccharomyces cerevisiae*.

2 Materials and methods

2.1 *Sample collection and cultivation*

Floating samples of *U. prolifera* were collected in July 2017 from the Zhanqiao Wharf (35°35′N, 119°30′E), Qingdao, China. In the laboratory, the intact samples were washed several times with sterile seawater, and then rinsed with autoclaved seawater. The *U. prolifera* samples were then placed into an aquarium containing enriched seawater (500 mmol/L $NaNO_3$ and 50 mmol/L NaH_2PO_4), and maintained at 15°C under a cycle comprising 12 of light alternating with 12 h of darkness. The light intensity was 100 μmol/(m^2·s) provided by cool-white fluorescent tubes. Samples used for real-time PCR were treated with high light (2 000 μmol/(m^2·s) for 1 h), high temperature (30°C for 24 h), high salinity (66 for 24 h) and low salinity (16 for 24 h).

2.2 *Identification of upDGAT cDNA and protein sequence analysis*

The cDNA sequences used in this study came from *U. prolifera* transcriptome (SRR3524808, SRR3504905). The amino acid sequences of the five *DGAT* genes of *Ulva mutabilis* (UM011_0232.1, UM022 0045.1, UM020_0139.1, UM020_0128.1, UM059_0034.1) were used as query to search for homologous genes from the *U. prolifera* transcriptome sequences. The deduced amino-acid sequence of *UpDGAT* gene was examined for homology with other known sequences by using the Muscle of the MEGA 7.0 program (Kumar et al., 2016). Its domain was predicted in Pfam HMM (http://pfam.sanger.ac.uk/search), and whether there is a membrane-spanning helix structure in this sequence was predicted by HMMTOP (http://www.enzim.hu/hmmtop/html/submit.html). The phylogenetic tree was constructed by the neighbor-joining algorithm of the MEGA 7.0 program (Kumar et al., 2016). A total of 1 000 bootstrap replicates were performed.

2.3 *Expression of upDGAT1 under different stress conditions*

Total RNA of *U. prolifera* samples exposed to each form and level of stress was extracted using Trizol Reagent (Invitrogen) as specified in the user's manual and dissolved in diethylpyrocarbonate (DEPC)-treated water. The genomic DNA was removed by DNase I (RNase free) (NEB) from the total RNA samples. The cDNA used for real-time PCR was synthesized from the total RNA by Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Promega).

The real-time quantitative PCRs were performed with ABI StepOne Plus Real-Time PCR System (Applied Biosystems) using SYBR Green fluorescence (Takara) according to the manufacturer's instructions. A 111 bp product of *UpDGAT1* was amplified with DGATF1 (TGA TGC TGT TCC TGG TGG AC) and DGATR1 (GGG AGA GCT TCA GGA TAC GC). Primers of Tub1F (CCG ATG GGC AAG TAA TCAC) and Tub1R (TGA AGG TTG TAT CAT GGA CTCC) were used to amplify a 139 bp fragment of tubulin as an internal control (Dong et al., 2012). The thermal profile for real-time PCR was 30 s followed by 40 cycles at 95°C for 5 s, 55°C for 10 s, and 72°C for 30 s. Dissociation curve analysis of the amplification products was performed at the end of each PCR to confirm that only one specific PCR product was amplified and detected. Triplicate qPCRs were performed for each sample.

2.4 *Heterologous expression of UpDGAT1 in yeast*

The coding sequences of *UpDGAT1* was amplified with gene specific primers (DAGTSEf: TCGAATTCATGACACAAGCTCTT; DAGTSEr: TCTCTAGACTATGCCATCGCTGA) containing the appropriate restriction sites to facilitate cloning into the yeast expression vector pYES2/CT (Invitrogen). The UpDGAT1 cds sequence was cloned into the pYES2 plasmid and was then transformed into the yeast quadruple mutant H1246MATa (dga1Δ lroΔ are1Δ are2Δ) which is deficient in oil synthesis by poly (ethylene glycol)/lithium acetate method according to the manual (Invitrogen). The auxotrophic Saccharomyces cerevisiae strain INVSc1 (MATa his3-Δ1 leu2 trp1-289 ura3-52) was used as positive control. Yeast cells transformed with an empty pYES2 plasmid were used as negative control. Yeast transformants were selected by growth on synthetic complete medium lacking uracil (SC-ura), supplemented with 2% glucose. The colonies were transferred into liquid SC-ura with 2% glucose and grown at 28°C overnight. The overnight cultures were diluted to A=0.4 in induction medium (SC-ura+2% galactose+1% raffinose) and were induced by incubating at 28°C overnight. Cells were harvested by

centrifugation, washed three times with double-distilled water and used for extraction of total lipids.

2.5 *The microscopic observation of intracellular lipid bodies*

The BODIPY505/515 (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene) (Invitrogen, USA) staining method described by Mou et al. (2012) was used to visualize the intracellular lipid bodies as an indicator of TAG formation. The yeast cells in the culture medium was stained with 2 μmol/L BODIPY 505/515 within 1 min at room temperature, and then immediately observed by fluorescence microscopy (Nikon Eclipse 80i, Japan). A filter allowing maximum excitation at 450–490 nm was used to stimulate the stained the yeast cells.

2.6 *Lipid extraction and analysis*

To extract total lipids from yeast, harvested cell pellets were lyophilized, ground with a mortar and then incubated in 7 mL methanol/chloroform (2:1, v/v) at 50°C for 10 min. After transferring lipid extract to a fresh tube, re-extract tissue with 1.5 mL methanol/chloroform (2:1, v/v). This operation was repeated two times. Combined all of the lipid extracts, and then add 2.5 mL chloroform and 3 mL 1% NaCl. Transfer the organic phase (lower phase) to a fresh glass tube, dried them under N2 and then dissolved in hexane. TAGs were separated from total lipids by thin layer chromatography (TLC) using a solvent system of hexane: ether:acetic acid (70:30:1, v/v/v). Individual lipid spots were visualized by exposing the silica gel plates to primuline vapor. Total fatty acids were extracted and transmethylated with methanolic HCl from yeast cells according to Browse et al. (1986). All samples were analyzed using a 7890A/5975C gas chromatography (Agilent Technologies, California, USA) and high purity nitrogen was used as the carrier gas. Measurements were performed using peak height area integrals expressed as a percentage of the total of all integrals. The experiment was carried out in triplicate, and the data subjected to analysis of variance using DPS software (Zhejiang University, China) Version 7.05. Duncan's multiple range test was employed to determine the statistical significance (p<0.05) of the differences between the means.

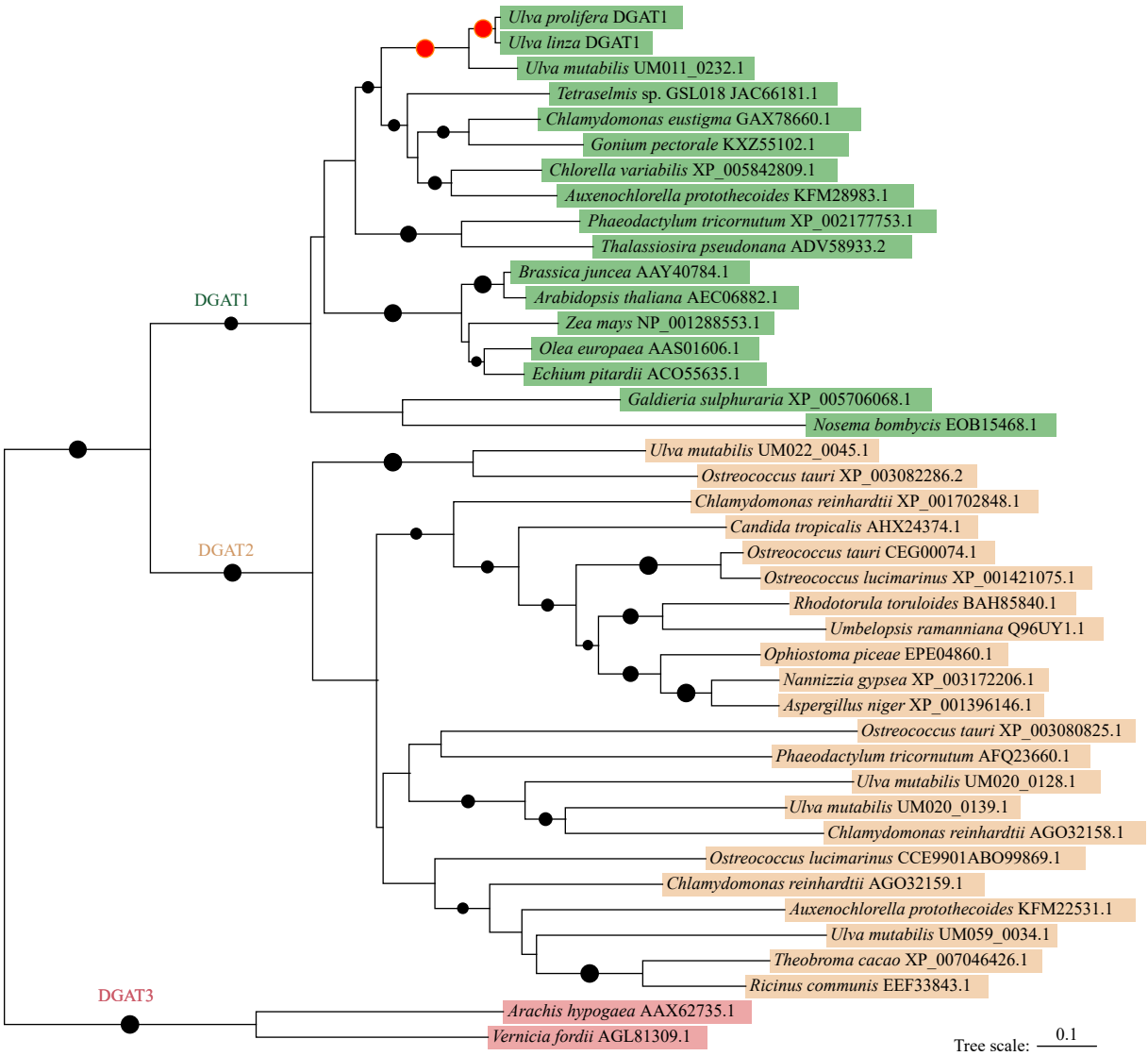

Fig. 1. Phylogenetic tree of DGAT gene families reconstructed by the neighbor-joining (NJ) method. Gene sequences other than *Ulva* DGATs were shown by their nomenclatures found in NCBI. Colored branches indicate different groups of proteins. Green: DGAT1, yellow: DGAT2, red: DGAT3. Bootstrapping with 1 000 replicates was used to establish the confidence limits of the tree branches.

3 Results

3.1 *Characterization of UpDGAT1 from U. prolifera*

Only one acyl-CoA: diacylglycerol acyltransferase-encoding gene was found in the *U. prolifera* transcriptome which was designated *UpDGAT1* (Fig. 1). The open reading frame was 1 653-bp long, encoding a protein of 551 amino acids with a predicted molecular weight of 62.1 kDa and an isoelectric point of 8.85. Its amino acid sequence exhibited pairwise distance of 0.16 with DGAT1 from *U. mutabilis* (UM011_0232.1), but 0.71 and 0.89 with DGAT1 from *C. variabilis* (XP_005842809.1) and *A. thaliana* (AEC06882.1) respectively. This result indicates a deep evolutionary history of DGAT1-encoding genes. Analysis using TMpred software suggested that UpDGAT1 has nine transmembrane domains, located in the region of amino acids 93–111, 142–163, 194–216, 229–248, 333–351, 382–401, 439–463, 476–495, and 506–528, respectively.

Multiple sequence alignment showed that UpDGAT1 contains seven conserved motifs, namely Motif 1 (GL Block), Motif 2 (KSR Block), Motif 3 (PTR Block), Motif 4 (QP Block), Motif 5 (LWLFFEFDRFYWWNWWNPPFSHP Block), Motif 6 (FQL Block), and Motif 7 (NGQPY Block). These motifs are characteristic sequences in DGAT1s (Cao et al., 2011). Meanwhile, UpDGAT1 has a YYHD box in Motif 7, a putative ER retrieval motif, suggesting probable ER localization of the protein (Fig. 2).

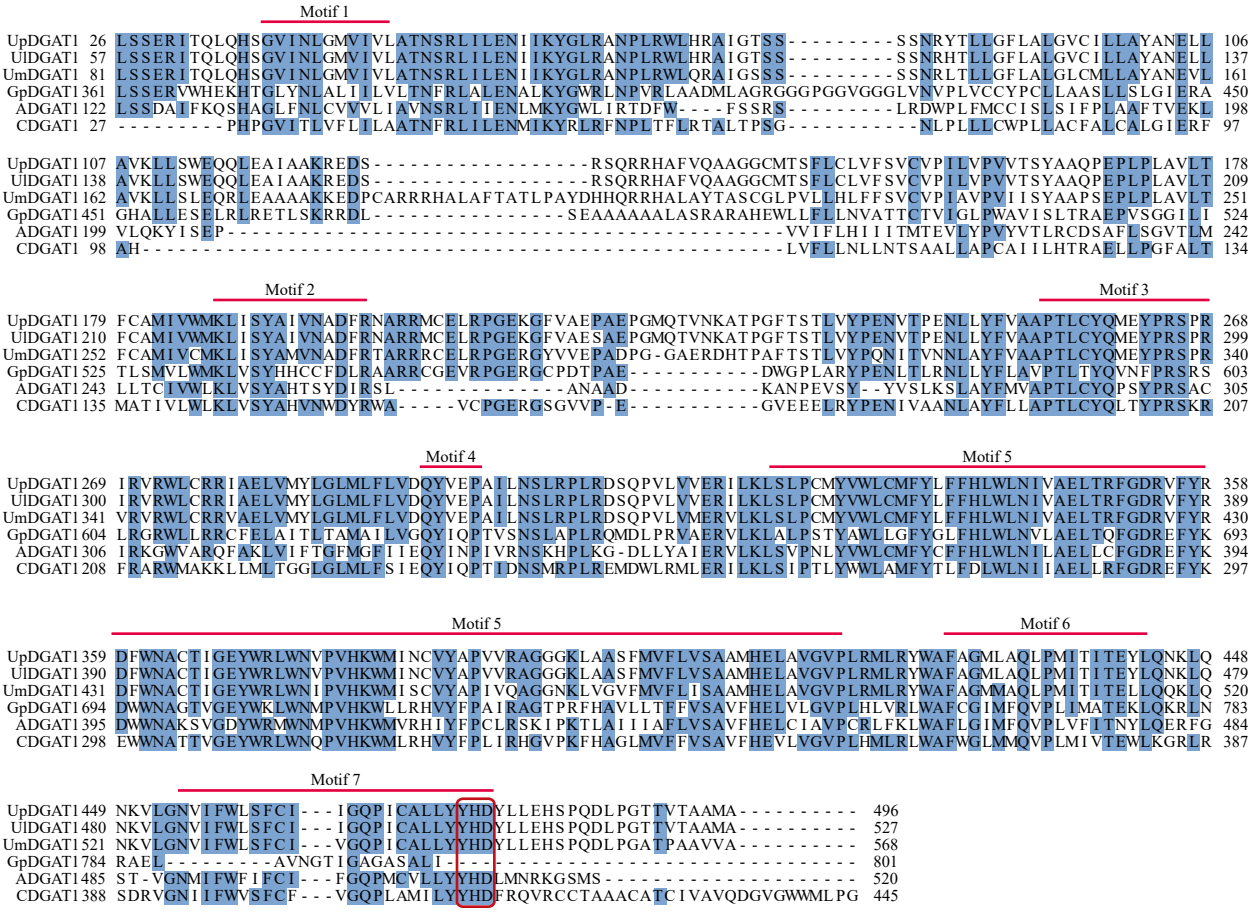

Fig. 2. Homology comparison of the amino acid sequences of *UpDGAT1* with DGAT1s from other algal species. Identical amino acid residues are highlighted in blue. UlDGAT, UmDGAT, GpDGAT, ADGAT and CDGAT were obtained from *Ulva linza*, *U. mutabilis*, *Gonium pectorale*, *Auxenochlorella protothecoides* and *Chlorella variabilis*, respectively.

3.2 *Heterologous expression of UpDGAT1 in TAG-deficient yeast mutant*

To verify whether *UpDGAT*1 indeed encodes a protein with DGAT activity, the putative *U. prolifera DGAT1* gene was expressed in a TAG-deficient *S. cerevisiae* quadruple mutant strain (H1246) (Burgal et al., 2008). INVSc1 was used as a positive control, and the empty vector pYES2 was transformed into the mutant strain as a negative control. Following expression, total lipids were extracted from the yeast cells in late stationary phase. Thin-layer chromatography (TLC) analysis of total lipids showed a prominent spot corresponding to TAG accumulation upon expression of *UpDGAT1*, whereas no TAG was detected in the quadruple mutant strain carrying the empty expression vector (Fig. 3).

In yeast cells, storage lipids, in the form of TAGs and steryl esters in lipid bodies, can be visualized using the fluorescent dye BODIPY505/515. To visualize the lipid bodies generated by the expression of *UpDGAT1*, yeast transformants were stained with BODIPY505/515 (Fig. 4). We found that lipid bodies were abundant in the wild-type and the mutant strain transformed with *UpDGAT1*, whereas they were absent from the mutant transformed with empty vector (pYES2). This result suggests that expression of *UpDGAT1* in the yeast quadruple mutant strain can completely restore its ability to form lipid bodies through interaction with the

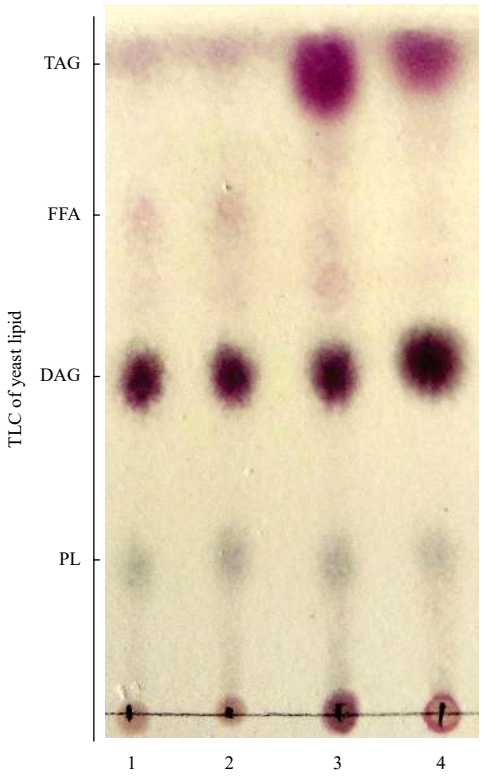

Fig. 3. Evaluation of TAG biosynthesis in the yeast quadruple mutant (H1246) complemented with *UpDGAT* genes. Lipid extracts from the yeast cells were separated by TLC. The neutral lipid-deficient quadruple mutant strain H1246 (1) and the mutant harboring the empty vector (pYES2) (2) were used as the negative controls, the wild-type strain INVSc1 was used as the positive control (3), and the quadruple mutant expressing *UpDGAT1* (4) was analyzed. TAG, triacylglycerol; FFA, free fatty acid; DAG, diacylglycerol; PL, phospholipid. All experiments were carried out at least three times.

yeast lipid synthesis pathway, which is consistent with the TLC results (Fig. 3).

3.3 *Fatty acid profile of yeast TAG-deficient mutant expressing UpDGAT1*

We investigated whether expression of *UpDGAT1* had any effect on the fatty acid composition of cellular lipids in *S. cerevisiae*. The expression of *UpDGAT1* in the quadruple *S. cerevisiae* mutant resulted in a differential fatty acid composition of lipids compared with the mutant strain or the strain transformed with empty vector (pYES2) (Fig. 5). Fatty acids C16:1 and C18:0 decreased by 62.3% and 64.6% respectively, compared with the non-transgenic control strain. Expression of *UpDGAT1* in the quadruple mutant led to a more than 1.2- and 1.05-fold increase in C16:0 and C18:1 compared with the control, respectively. There was a low proportion of C17:1 and C18:2 in the quadruple mutant, and their proportions in the lipids of *UpDGAT1* transgenic yeast were increased 1.7-fold and 14-fold respectively. These results suggest that expression of *UpDGAT1* in yeast can increase the incorporation and transfer of endogenous unsaturated fatty acids into lipids.

3.4 *Lipid analysis and expression pattern of UpDGAT1 in U. prolifera under abiotic stresses*

Another method to confirm the identity of putative TAG bio-

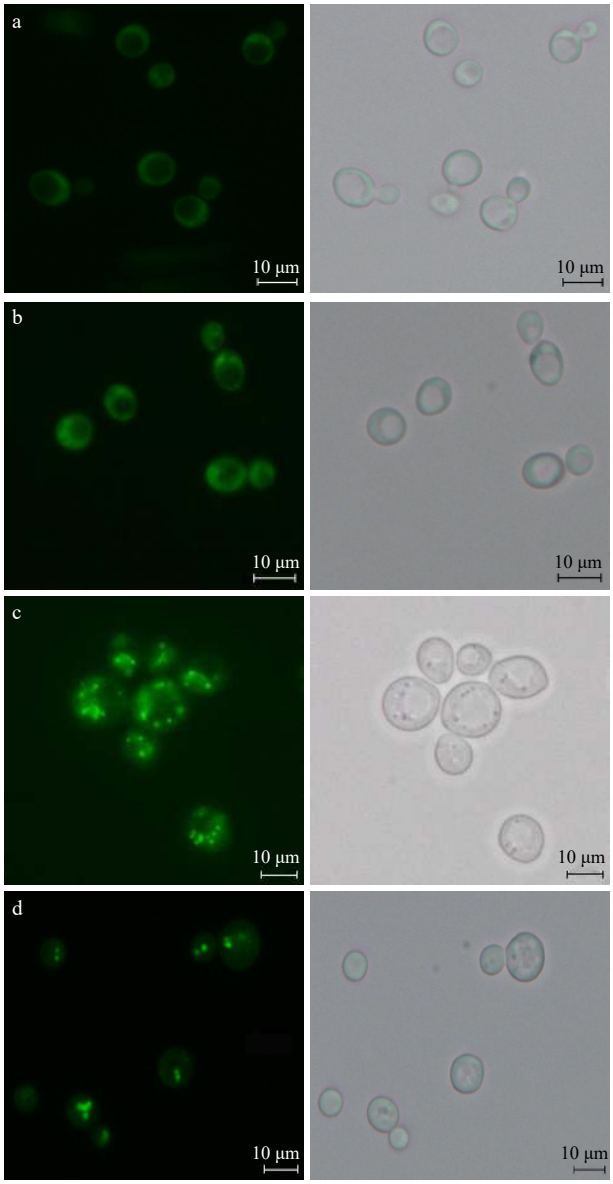

Fig. 4. Lipid body formation is restored upon expression of *UpDGAT1* in the yeast strain H1246. Neutral lipid accumulation in lipid bodies was visualized in yeast cells with the fluorescent dye BODIPY505/515. The neutral lipid-deficient quadruple mutant strain H1246 (a) and the mutant harboring the empty vector (pYES2) (b) were used as the negative controls. The wild-type strain INVSc1 was used as a positive control (c). The quadruple mutant expressing *UpDGAT1* (d) was analyzed.

synthesis enzymes is to monitor their expression patterns to see if gene expression is correlated with increased TAG productivity in algae in certain growth conditions. To understand the relationship between *UpDGAT1* expression and lipid synthesis, we investigated the expression pattern of *UpDGAT1* and the TAG content of *U. prolifera* in different stress conditions (Fig. 6). Increased light intensity and salinity significantly down-regulated the expression of *UpDGAT1*, by 22.5- and 45.2-fold respectively. On the contrary, increased temperature and decreased salinity significantly upregulated the expression of *UpDGAT1*, by 4.79- and 7.34-fold respectively. The TAG contents were accordingly increased by 2.65- and 2.69-fold in high temperature and low salt

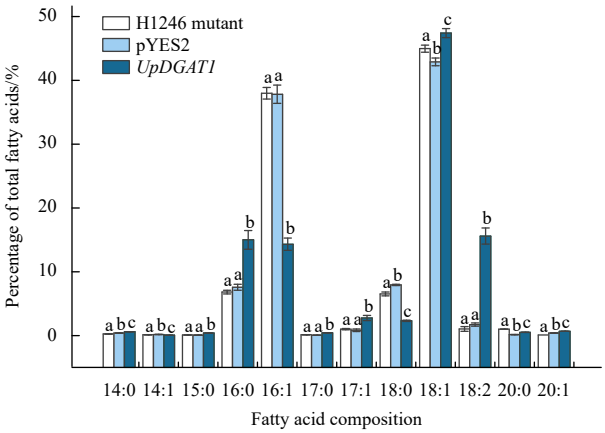

Fig. 5. Impact of *UpDGAT1* expression on fatty acid profiles of yeast. The bars are standard deviations (SD) of three technical repeats. Different letters on error bars indicate statistically significant differences (Duncan's multiple range test, $p<0.05$).

conditions, respectively (Fig. 6). This result suggests that expression of *UpDGAT1* in *U. prolifera* was very sensitive to environmental stresses, and that the enzyme may play an important role in stress acclimation.

4 Discussion

There are at least two major families of DGATs, named Type 1 and Type 2, both of which are membrane-bound. Type 1 and Type 2 DGATs do not share any significant amino acid sequence similarity, although both catalyze the same reaction. In animals, DGAT2 seems to be the more potent enzyme, with higher affinity for its substrates and higher rates of TAG accumulation compared with DGAT1 (Yen et al., 2008). In higher plants, the functional difference between the two DGAT isozymes is not as clear (Shockey et al., 2006). Moreover, there is clear evidence that there are spatial and temporal differences in DGAT expression patterns. In olive, DGAT1 seems to play a more prominent role in olive oil accumulation compared with DGAT2 (Banilas et al., 2011). In contrast, DGAT2 may play a more important role in oil production compared with DGAT1 in oleogenic seed crops such as the tung tree and castor oil plant, which contain unusual fatty acids (Kroon et al., 2006; Shockey et al., 2006). Further evidence for this divergence in functionality between plant DGAT1 and DGAT2 can be seen from differences in their biochemical characteristics (Li et al., 2010). In *P. tricornutum*, *Pt*DGAT1 tends to incorporate saturated C16:0 and C18:0 fatty acids into TAGs, whereas *Pt*DGAT2 has a preference for unsaturated fatty acids (Guihéneuf et al., 2011; Gong et al., 2013). In *O. tauri* and *Chlamydomonas reinhardtii*, DGAT2 exhibited broad substrate specificity, preferring long-chain acyl groups (Wagner et al., 2010; La Russa et al., 2012). In *U. prolifera*, UpDGAT1 showed broad substrate specificity, accepting saturated (C16:0) as well as mono- (C18:1, C17:1) and polyunsaturated (C18:2) acyl-CoAs as substrates. In addition, TAG formation in algae is dependent on environmental conditions, which also regulate carbon metabolism. In *U. prolifera*, we found that both the expression level of *UpDGAT1* and the TAG content was increased in low salt and high temperature conditions. We suggest that *U. prolifera* modifies its carbon metabolism to energy storage pathways in these conditions compared with high salt and high light intensity conditions.

The roles of lipids in both abiotic and biotic stresses are mainly as intermediates of signal transduction pathways in plant which is of rising interest (Berkey et al., 2012; Hou et al., 2016; Zhao, 2015). In algae, however, environmental stresses are mostly binding to TAG accumulation in various microalgae in light of current environmental and energy supply concerns (Arora et al., 2018). Various stress factors, especially nutrient-starvation conditions are known in inducing an increased formation of TAG in microalgae, such as *Coccomyxa subellipsoidea* (Allen et al., 2015) and *Chlorella* (Goncalves et al., 2013). Except for the nutrient stress, many studies have reported that non-chemical based stresses lead to the formation of TAG in microalgae including unfavorable temperature, high salinity, light intensity and dehydration. For example, increased temperature results in the elevation of lipid content in the freshwater phytoflagellate *Ochromonas danica* (Aaronson, 1973), the marine alga *Nannochloropsis salina* (Boussiba et al., 1987) and *Chlamydomonas* (Hemme et al., 2014); high light intensity increases TAG content in such as marine *Nannochloropsis oculata* (Ma et al., 2016) and

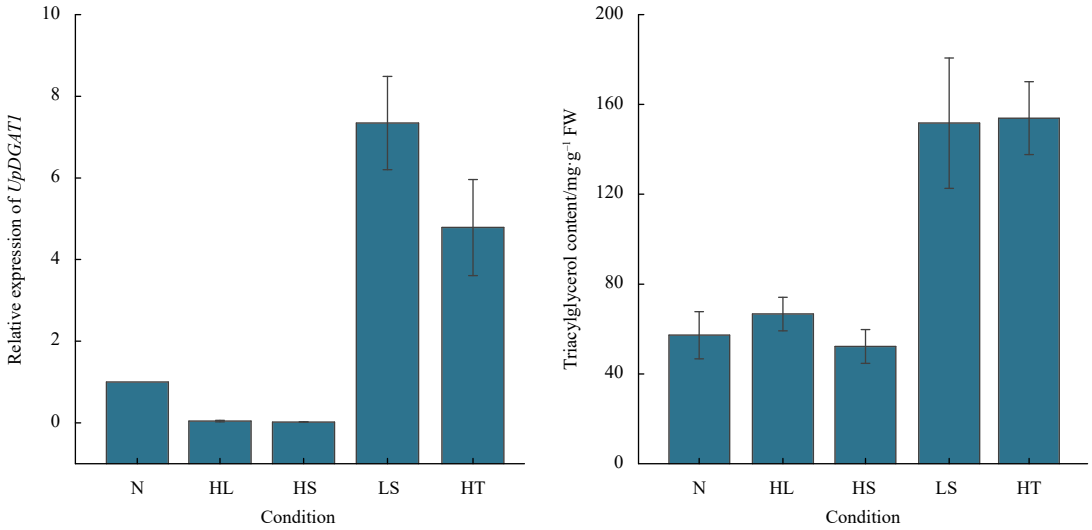

Fig. 6. Expression pattern of *UpDGAT1* gene and TAG accumulation under different stress conditions. N, control; HL, high light; HT, high temperature; HS, high salt; LS, low salt.

Thalassiosira pseudonana (Brown et al., 1996). Synthesis of TAG can also be induced by high salinity as seen in the green algae *Dunaliella salina* (Takagi et al., 2006) and *Chlamydomonas* (Siaut et al., 2011). Similar studies was limitied in macroalgae as most of them contain comparable low lipid contents. One result came from the red alga *Tichocarpus crinitus* whose TAG content was increased by high light intensity (Khotimchenko and Yakovleva, 2005). Besides as energy content, lipid could also participate in rapid response to stresses by the generation of numerous signalling lipids, such as the rapid releasing of inositol 1,4,5-trisphosphate (InsP3) and diacylglycerol in model unicellular alga *Chlamydomonas* under osmotic stress (Meijer et al., 2017). In macroalgae, lipid peroxidation was confirmed partcipated in both the short term rapid defense reactions and long term cascade reactions under abiotic stress in kelp (Cosse et al., 2007). In our study, two specific stress conditions, low salinity and high temperature, could increase the TAG contents in accrodance with the increased *UpDGAT1* expression level, while high light intensity and high salinity have no obvious impact on TAG accumulation. These results indicated that there is an increased TAG requirement when *U. prolifera* under low salinity and high temperature than under high salinity and light intensity, at least under our experimental conditions. We proposed that these unique characters of *U. prolifera* comparing to the unicellular microalgae, especially the response to different salinity, may be related to its special coastal conditions. However, the exact function of the *UpDGAT1* gene and TAG, as energy store or participating in signal transduction, in stress response and acclimation in *U. prolifera* need more experimental proofs.

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