

Characterization of type-2 diacylglycerol acyltransferases in *Haematococcus lacustris* reveals their functions and engineering potential in triacylglycerol biosynthesis and possible roles in astaxanthin esterification

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Abstract

Background: *Haematococcus lacustris* is an ideal source of astaxanthin (AST) which is stored at oil bodies containing esterified AST (EAST) and triacylglycerol (TAG). Diacylglycerol acyltransferases (DGATs) catalyze the last step of the acyl-CoA-dependent TAG biosynthesis and are also considered as the crucial enzymes involving in EAST biosynthesis in *H. lacustris*. Previous studies have identified four putative DGAT2 encoding genes in *H. lacustris* and only the HpDGAT2D allowed the recovery of TAG biosynthesis, but the engineering potential of HpDGAT2s in TAG biosynthesis, especially possible roles in AST esterification, remains ambiguous.

Results: Five putative DGAT2 genes (*HpDGAT2A*, *HpDGAT2B*, *HpDGAT2C*, *HpDGAT2D*, and *HpDGAT2E*) were identified in *H. lacustris*. Transcription analysis showed that the expression levels of *HpDGAT2A*, *HpDGAT2D*, and *HpDGAT2E* genes markedly increased under high light and nitrogen deficient conditions with distinct patterns, which led to significant TAG and EAST accumulation. Functional complementation demonstrated HpDGAT2A, HpDGAT2B, HpDGAT2D, and HpDGAT2E had the capability to restore TAG synthesis in a TAG-deficient yeast strain (H1246) with the large difference in enzymatic activity. Fatty acids (FAs) profiles assays revealed that HpDGAT2A, HpDGAT2D, and HpDGAT2E except for HpDGAT2B preferred monounsaturated fatty acyl-CoAs (MUFAs) for TAG synthesis in yeast cells, and also showed polyunsaturated fatty acyl-CoAs (PUFAs) preference by feeding strategy. The over-expression of *HpDGAT2D* in *Arabidopsis thaliana* and *Chlamydomonas reinhardtii* significantly increased the TAG content and obviously promoted the MUFAs and PUFAs contents. Interestingly, molecular docking analysis implied that HpDGAT2s structures contained AST binding sites, which provides strong evidence for AST esterification function in *H. lacustris*.

Conclusions: Our study represents a pioneering work on the characterization of HpDGAT2s by systematically integrating expression pattern, AST/TAG accumulation, functional complementation, molecular docking, and over-expression in yeast, plants, and algae. These results (1) update the gene models of *HpDGAT2s*, (2) prove TAG biosynthesis capacity of HpDGAT2s, (3) show the strong preference for MUFAs and PUFAs, (4) offer target genes to modulate TAG biosynthesis by genetic engineering method, and (5) provide new evidence for HpDGAT2s roles in AST esterification.

Background

Triacylglycerol (TAG) is the principal storage form of energy in eukaryotic organisms and represents a promising source of biodiesel production [1]. Microalgae can efficiently absorb CO₂ in the atmosphere and turn it into abundant high value products including polysaccharide, lipid, protein, pigment, and biofuel [2-5]. Due to high photosynthetic efficiency, rapid reproduction rate, and short growth cycle, microalgae have been considered as the best candidate to resolve energy crisis and environmental pollution [6]. Further understanding of pathways and regulatory mechanisms involved in TAG accumulation will help benefit the rational genetic manipulation of microalgae [7-9].

Generally, TAG biosynthesis takes place in the endoplasmic reticulum and their assembly can be divided into acyl-CoA-dependent and acyl-CoA independent pathways [10]. Diacylglycerol acyltransferases (DGATs) catalyze 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 pathway [11]. These enzymes represent a bottleneck in TAG biosynthesis in some oilseed crops and algae, and thus have been regarded as a key target in manipulating TAG production [11]. In higher plants and microalgae, there are four major groups of DGATs: (1) membrane bound form of DGAT1, (2) membrane bound form of DGAT2 sharing low sequence similarity with DGAT1, (3) soluble type of DGAT3 which is localized in the cytosol, and (4) dual functional of WS/DGAT which possess both wax ester and TAG biosynthesis activities [12-18]. DGAT1s are considered to play a critical role in TAG accumulation in many higher plants and microalgae, whereas DGAT2s appear to have an important role in the formation of TAG containing unusual fatty acids (FAs) [14]. There is strong evidence supporting the involvement of DGAT3 and WS/DGAT in TAG biosynthesis in microalgae [15, 16]. Usually, only one or two alleles of DGAT1s are identified in a number of microalgae, whereas multiple alleles of DGAT2s are typically present, suggesting that DGAT2s may play an important function in TAG biosynthesis [12-14, 19-27]. Recently, most of the current knowledge about the algal DGATs is derived from the limited algal species including *Chlamydomonas reinhardtii*, *Chlorella ellipsoidea*, *Nannochloropsis oceanica*, *Lobosphaera incise*, *Chlorella/Chromochloris zofingiensis*, *Myrmecia incise*, and *Phaeodactylum tricornutum*, in which DGATs have been focused on the molecular cloning, biochemical identification, functional characterization, and engineering potential in modulating TAG biosynthesis [19-28]. It is not difficult to find some interesting conclusions that the diversity microalgae are prominent candidates for DGATs and the function of distinct DGATs is unique or species-specific. Therefore, it derived the research interest to other industrially special astaxanthin (AST) producing alga such as *Haematococcus lacustris* [29].

H. lacustris is a green microalga widely known for its ability to synthesize the highest amount of AST (4% dry weight) under stress conditions [29, 30]. Natural AST is a red-colored carotenoid with strong antioxidant ability and important commercial value [31], and is stored at oil bodies containing esterified AST (EAST) and triacylglycerol (TAG) in *H. lacustris* [32-35]. Moreover, the previous studies have indicated that the main form of EAST included monoester AST (M-AST, 70%) and diester AST (D-AST, 25%) [36-40]. Although the exact mechanisms of stress-induced TAG and AST accumulation in *H. lacustris* 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 at transcription level [40]. Indeed, the accumulation of AST appears to be dependent on the biosynthesis of FAs and accumulation of TAG [34, 41]. In addition, it has been speculated that certain DGATs are the candidate enzymes catalyzing the esterification of AST in *H. pluvialis* [34]. Recently, although four putative type-2 DGATs (*HpDGAT2A*, *HpDGAT2B*, *HpDGAT2D*, and *HpDGAT2E*) were identified from *H. pluvialis* (*lacustris*), and the only *HpDGAT2D* had the capability of restoring TAG biosynthesis in a TAG-deficient yeast strain [42], but the engineering potential of DGAT2s in TAG biosynthesis, especially roles in AST esterification, remains ambiguous.

By employing the industrially special AST producing alga *H. lacustris*, in the present study, we represented a pioneering work on the characterization of HpDGAT2s by systematically integrating expression pattern, AST/TAG accumulation, functional complementation, molecular docking, and over-expression in yeast, plants, and algae. Five putative *HpDGAT2s* were identified in *H. lacustris*, of which, the transcription levels of *HpDGAT2* genes markedly increased under high light (HL) and nitrogen deficient (ND) conditions with distinct patterns, which led to significant TAG and EAST accumulation. HpDGAT2A, HpDGAT2D, and HpDGAT2E rather than HpDGAT2B had strong TAG biosynthesis activity and preferred monounsaturated fatty acyl-CoAs (MUFAs) and polyunsaturated fatty acyl-CoAs (PUFAs). Over-expression experiments indicated the engineering potential of *HpDGAT2D* in modulating TAG accumulation and FAs composition in algae and plants. We also discussed the roles of HpDGAT2s in AST esterification.

Results

Molecular cloning and bioinformatics analysis of *HpDGAT2* genes

Based on the *H. lacustris* transcriptome database [43], five putative *DGAT2* genes were predicted by BLAST method using other *DGAT2s* from different algal species (Additional file 1: Table S1) as query. The full-length mRNA sequences of the five genes were obtained by rapid amplification of cDNA ends (RACEs) method, and the initiation codon, termination codon, 5'-untranslated region (5'-UTR), 3'-untranslated region (3'-UTR), and poly (A) characteristic tail were determined. Five putative *DGAT2* genes were designed *HpDGAT2A*, *HpDGAT2B*, *HpDGAT2C*, *HpDGAT2D*, and *HpDGAT2E* by multiple sequence alignment with *CrDGAT2s*, four of which, *HpDGAT2A*, *HpDGAT2B*, *HpDGAT2D*, and *HpDGAT2E* contained full-length open reading frame (ORF) while *HpDGAT2C* was partial sequence (Additional file 2: Table S2 and Additional file 3: Table S3). Then, the full-length ORF were cloned and sequenced by PCR with primers (Additional file 4: Table S4), which was renamed and deposited in NCBI GenBank (*HpDGAT2A*: MT875161; *HpDGAT2B*: MT875162; *HpDGAT2C*: MT875163; *HpDGAT2D*: MT875164; *HpDGAT2E*: MT875165). This is so far the highest dose of *DGAT2s* reported in green alga *H. lacustris*. Comparison with gene models of *HpDGAT2s* reported by Nguyen et al. [42], our results confirmed that there were five *HpDGAT2s* members in *H. lacustris*. Generally, only one or two alleles of *DGAT1s* are identified in a number of microalgae, whereas multiple alleles of *DGAT2s* are typically present [14].

To gain insights into the biochemical characteristics of HpDGAT2s, the molecular weight (MW), isoelectric point (pI), sub-cellular location, trans-membrane domain (TM), signal peptide (SP), chloroplast transfer peptide (CTP), and phosphorylation site (Phos) were analyzed. No SP or CTP was present in HpDGAT2s protein sequences except for CTP in HpDGAT2C (Additional file 2: Table S2). There were two TMs in all pDGAT2s protein sequences except for three TMs in HpDGAT2B (Additional file 2: Table S2 and Additional file 5: Figure S1), which is consistent with the membrane bound forms of *DGAT1* and *DGAT2* [14]. In addition, 14-30 phosphorylation sites were predicted in HpDGAT2s protein sequences (Additional file 2: Table S2 and Additional file 6: Figure S2), indicating phosphorylation plays important roles in *DGAT2s* enzymes activity due to it has been indicated that the *DGAT1* enzyme activity was affected by serine phosphorylation sites in mouse *DGAT1* [44], *TmDGAT1* [45], and *BnDGAT1* [46]. It

remains to be determined whether these phosphorylation sites are important for the functional regulation of HpDGAT2 *in vivo*.

To further analysis the conserved domains (CDs) and evolutionary relationship between HpDGAT2s and other algal DGAT2s, multiple sequence alignment and phylogenetic tree were reconstructed. CDs analysis showed that HaeDGAT2s contained 7 CDs [26, 47, 48], including YF/YFP block (CD1) which is essential for DGAT2 activity, HPHG/EPHS block (CD2) which is proposed to partially consist of the active site, PxxR (x=random amino acid) block (CD3), xGGxAE block (CD4), RxGFx(K/R)xAxxxGxx(L/V)VPxxxFG block (CD5) which is the longest conserved sequence in plants and animal, PxxxVVGxPlxVP block (CD6), and RHK block (CD7) (Additional file 7: Figure S3). As shown in Additional file 7: Figure S3, there were two completely conserved amino acid residues (proline, P and phenylalanine, F) among all DGAT2s, which is consistent with previous reports that these two highly conserved residues maybe located at the active sites of the enzymes and make significant contribution to the enzymatic activities [49]. The phylogenetic analysis of the HpDGAT2s and other DGATs orthologs from eukaryotic algae and plants was illustrated in Additional file 8: Figure S4, which is consistent with most of previous results [20-26]. Briefly, all HpDGAT2s clustered with the algal DGAT2s orthologs, which are distinct from other DGAT subfamilies including DGAT1, DGAT3, and DGAT/WSD. Of the five HpDGAT2s, HpDGAT2A formed monophyletic subgroup (BS: 100%) with CrDGAT2A, CzDGAT2A, CzDGAT2B, LiDGAT2A, and LiDGAT2B. HpDGAT2B and HpDGAT2E were highly close (BS: 98%) to CrDGAT2B, CzDGAT2E and CrDGAT2C. HpDGAT2C was evolutionary close (BS: 100%) with CzDGAT2C and LiDGAT2C. HpDGAT2D built monophyletic subgroup (BS: 73%) with CrDGAT2D and CzDGAT2D.

AST and TAG accumulation and *HpDGAT2s* genes transcription expression upon high light and nitrogen deficient stresses

High light (HL) and nitrogen deficient (nitrogen-free, ND) stresses can effectively promote the accumulation of AST and TAG in *H. lacustris* [32-34, 50-53]. However, under such circumstances, the growth of algal was completely restricted [51-53]. Recently, our team finished part of the research about the effects of nitrogen deficient degrees (nitrogen content compare to those in control BBM medium e.g., 0, 1/4N, 1/2N, and 3/4N) on algal growth and AST and TAG accumulation. The results indicated that the highest AST productivity arrived at the 1/4N stress due to the certain level of algal growth (data not shown). Therefore, in the current manuscript, 1/4N condition was selected as nitrogen deficient stress in further experiment. To understand the relationship between *HpDGAT2s* transcription expression and TAG and AST biosynthesis, time-course patterns of algal biomass, transcription expression, total AST (T-AST), and total TAG (T-TAG) contents in photoautotrophic cultures of *H. lacustris* under HL, 1/4N, and double HL-1/4N stresses were studied (Fig. 1).

As shown in Fig. 1a, compare to the control, HL, 1/4N, and double HL-1/4N stresses inhibited the algal growth. The T-AST production and composition were summarized in Fig. 1b-1e. From these results we could draw some conclusions that (1) M-AST is the main form, (2) compare to 1/4N stress, HL is more effective in inducing AST accumulation especially under high blue light (HLB) condition, (3) coupled HL

and 1/4N dual stimulation might be better choices in improving AST accumulation. Moreover, T-TAG contents slowly increased from day 1 to 4 day and reached its maximum value of 29.5%, 28.7%, 26.8%, 25.2%, and 24.8% under HLB-1/4N, HLW-1/4N, HLB, 1/4N, and HLW conditions, respectively, which was 159.5%, 155.1%, 144.9%, 136.2%, and 134.1% higher than that of control (Fig. 1f). The effect of HL, 1/4N and double HL-1/4N stresses on TAG and AST accumulation was substantially consistent with previous studies that AST and lipid biosynthesis was enhanced and the former was coordinated with the later biosynthesis under HL and ND conditions [34, 41]. Previous studies have indicated that DGATs enzymes are probably responsible for both AST esterification and TAG biosynthesis in *H. lacustris* [33, 34]. As revealed by qRT-PCR results (Fig. 2), the *HpDGAT2s* genes transcription expression levels exhibited distinct patterns under the HL, 1/4N and double HL-1/4N stresses. Of the five *HpDGAT2s*, the *HpDGAT2B* and *HpDGAT2C* expression levels decreased and maintained constant (Fig. 2b and 2c). The *HpDGAT2A* and *HpDGAT2E* expression levels increased and reached its maximum at 4 d exposure, which were HL and 1/4N stress dependent (Fig. 2a and 2e), respectively, while the *HpDGAT2D* expression level increased and was both stresses dependent (Fig. 2d). These results suggested that these *HpDGAT2A*, *HpDGAT2D*, and *HpDGAT2E* genes were together involved in the AST and TAG biosynthesis under stress.

Functional complementation of *HpDGAT2s* in yeast

To verify the function of the putative *HpDGAT2s* enzymes, the ORF encoding sequences were cloned (Additional file 4: Table S4) into pYES2.0 plasmid and heterologously expressed, respectively, in the quadruple mutant yeast strain *S. cerevisiae* H1246 ($\Delta dga1 \Delta lro1 \Delta are1 \Delta are2$) that lacks the activity of TAG synthesis. The mutant type (H1246) yeast can form TAG when at least one of these four genes was expressed. Furthermore, Wild type (INVSc1) and H1246-EV (H1246 harboring empty vector pYES2.0) yeast strains were used as positive and negative controls, respectively.

The expression of *HpDGAT2A*, *HpDGAT2B*, *HpDGAT2D*, and *HpDGAT2E* restored TAG biosynthesis at different levels in H1246 cells as indicated by the prominent TAG spot on a TLC plate (Fig. 3a). By contrast, *HpDGAT2B* expression in H1246 cells produced un-conspicuous TAG indicating a nonfunctional encoded protein considering the low transcription expression level in H1246 cells (Fig. 3b) and *H. lacustris* cells (Fig. 2b). Nevertheless, the limited FAs composition in *Saccharomyces cerevisiae* might lead to the low TAG content for *HpDGAT2B*. The ability of *HpDGAT2A*, *HpDGAT2B*, *HpDGAT2D*, and *HpDGAT2E* to restore TAG biosynthesis in yeast led us to examine their FAs substrate specificity. As indicated in Fig. 3b and 3c, the *HpDGAT2A*, *HpDGAT2B*, *HpDGAT2D*, and *HpDGAT2E* genes were heterologously expressed in H1246 and INVSc1 cells. The changes of TAG content and FAs composition of TAG extracted from the transformed H1246 and INVSc1 cells were similar (data not shown). As shown in Fig. 3d, the TAG contents of expressed *HpDGAT2A* and *HpDGAT2B* in H1246 cells were 78.3% and 56.5% lower respectively than those of control (INVSc1 and INVSc1+EV). While the TAG contents of expressed *HpDGAT2D* and *HpDGAT2E* were 108.7% and 122.7% higher respectively than control. To further test the FAs substrate specificity, FAs from the transformed H1246 and INVSc1 cells were analyzed by GC. As shown in Fig. 3d, compare to control, the MUFAs palmitoleic acid (C16:1) and oleic acid (C18:1) abundance increased in *HpDGAT2A*, *HpDGAT2D*, and *HpDGAT2E* expressed H1246 cells at

the expense of saturated fatty acids (SFAs) including palmitic acid (C16:0) and stearic acid (C18:0). Such tendency, however at different levels, was observed for almost all transformed lines of H1246 for various DGATs enzymes [20, 23-28].

Considering the limited FAs composition in yeast strains (C16:0, C18:0, C16:1, and C18:1), some PUFA, rich in *H. lacustris*, including linoleic acid (C18:2n6), α -linolenic acid (C18:3n3), γ -linolenic acid (C18:3n6), and parinaric acid (C18:4n3) were tested the substrate specificity for HpDGAT2A, HpDGAT2B, HpDGAT2D, and HpDGAT2E enzymes by feeding strategy. The HpDGAT2A, HpDGAT2D, and HpDGAT2E had similar tendency that these PUFAs were incorporated into TAG on the expense of C16:1 and C18:1 with the following patterns of C18:2n6 > C18:3n3 > C18:3n6 > C18:4n3 (Fig. 3e). Considering that the C18:2n6 and C18:3n3 were rich in *H. lacustris*, it is reasonable to speculate that these HpDGAT2s may have potential in the C18:2n6 and C18:3n3-enriched TAG production [32-34]. The HpDGAT2A, HpDGAT2D, and HpDGAT2E enzymes showed more strong preference for PUFAs than MUFAs, alternative due to the high feeding content of PUFAs than endogenous MUFAs content. This phenomenon was also confirmed by Zienkiewicz et al (2018) that some PUFAs were incorporated into TAG on the expense of 16:1 and 18:1 in *LiDGAT1*, *LiDGAT2.1*, *LiDGAT2.2*, and *LiDGAT2.3* expressing yeast [23] and in *CzDGAT2C* expressing yeast mutant H1246 cells [26] by feeding test. However, FAs profiles of the TAG fraction from yeast cells expressing *HpDGAT2B* showed no obvious changes, implying an un-functional protein (Fig. 3e).

HpDGAT2D* overexpression promotes TAG biosynthesis and its relative MUFAs and PUFAs abundance in *C. reinhardtii

In order to investigate the possible biological role of HpDGAT2s and engineering potential to modulate TAG biosynthesis in algae, we generated *HpDGAT2D* overexpression lines in evolutionary close green algal *C. reinhardtii* CC849. The *HpDGAT2D* was selected in further experiments due to the relative strong TAG biosynthetic activity in yeast cells (Fig. 3) and high transcription expression level in *H. lacustris* under stress condition (Fig. 2d).

The nuclear transformation expression vector pDB124 (Additional file 9: Figure S5), characterized in *C. reinhardtii* CC849 and presented by professor Zhangli Hu from Shenzhen University, was used in this study after modified due to it contained overexpression cassettes of the *HpDGAT2D-His* fusion and bleomycin resistance *Ble* genes under the control of the verified endogenous promoter and terminator of *PsaD* and *RBCS2* genes respectively (Fig. 4a). The codon preference (*HpDGAT2D*) was optimized according to the algal *C. reinhardtii* (Additional file 10: Figure S6) before constructing the expression vector. Transformants (screening over 20 putative transformants) were selected on TAP plates supplemented with bleomycin and confirmed by genomic PCR method. The exogenous *HpDGAT2D-His* fusion gene was integrated into the alga chromosome due to the clear band using *HpDGAT2D* gene as primers in transformation lines, whereas no signal was detected in WT cells (Fig. 4b). Three overexpression lines, *HpDGAT2D-4*, *HpDGAT2D-7*, and *HpDGAT2D-9*, exhibited the maximum increase in transcription levels (by ~ 5.5-fold higher than the control) under ND condition at a 4-day batch culture with no significant difference in cell growth between the transgenic lines and control (Fig. 4c and 4d).

Furthermore, in *in vivo* overexpression of the HpDGAT2D protein was validated by using His-tag antibodies to detect the HpDGAT2D-His fusion protein *via* western blot method. The bands were present from the membrane proteins of three overexpression lines (*HpDGAT2D-4*, *HpDGAT2D-7*, and *HpDGAT2D-9*), while were absent from the soluble proteins, which was consistent with that HpDGAT2D was a trans-membrane enzyme (Fig. 4e). The *HpDGAT2D* overexpression led to considerable increases (by ~ 1.4-fold) in TAGs content under ND condition (Fig. 4f). *HpDGAT2D* overexpression also affected the FAs profiles in TAG (Fig. 4f). A significant increase was observed in the MUFAs (C16:1 and C18:1) and PUFAs (C18:2n6 and C18:3n3) relative abundance accompanied by a significant decrease in SFAs (C16:0 and C18:0) and some PUFAs (C16:2, C16:3, C18:3n6, and C18:4n3). These results indicated that (1) HpDGAT2D showed more strong preference for MUFAs and PUFAs than SFAs, (2) of all PUFAs, HpDGAT2D had the first option to C18:2n6 and C18:3n3 rather than C16:2, C16:3, C18:3n6, and C18:4n3, (3) these preferred substrates happened to be the type that is enriched in *C. reinhardtii*. This trend was consistent with results from yeast cells by feeding test (Fig. 3d and 3e) and previous studies of *NoDGAT1A* expression in *C. reinhardtii* UVM4 and *CzDGAT1A* expression in oleaginous alga *N. oceanica* by Wei et al (2017) and Mao et al (2019) respectively [20, 22].

HpDGAT2D* overexpression enhances seed oil content and its relative MUFAs and PUFAs abundance in *A. thaliana

To explore HpDGAT2s as a tool to manipulate acyl-CoA pools and to engineer TAG biosynthesis in higher plants, *HpDGAT2D* was over-expressed in *Arabidopsis thaliana*. Three *A. thaliana* independent expression T2 generation lines (*At-HpDGAT2D-3*, *At-HpDGAT2D-6*, and *At-HpDGAT2D-8*) were selected for further detailed analysis. The transgenic lines did not show any visible morphological difference from untransformed control *A. thaliana* e.g., 1000-seeds weight (Fig. 5a). The qRT-PCR results showed that the *HpDGAT2D* transcript was expressed in transgenic lines at different tissue organs including roots, tubers, leaves, siliques, and seeds with distinct extent (Fig. 5b). The transformation of wild type *A. thaliana* with *HpDGAT2D* resulted in higher (120.0-126.4%) seed TAG content than control (Fig. 5c). Again, further GC analysis of FAs profiles from TAG revealed that PUFAs and MUFAs significantly increased accompanied by a significant decrease in SFAs (Fig. 5c). However, the exact process of change was much more complicated than those in yeast and *C. reinhardtii* cells. Specifically, of SFAs, C16:0 and C22:0 decreased while C18:0 and C20:0 maintained stable. Of MUFAs and PUFAs, HpDGAT2D preferred C18:1, C18:2n6, and C18:3n3 rather than C20:1, C20:2 and C22:1 in TAG biosynthesis. These results were largely in agreement with those in yeast cells (Figs. 3d and 3e) and *C. reinhardtii* cells (Fig. 4c). Guo et al (2017) indicated that the *CeDGAT1* gene can stimulate FAs biosynthesis and enhance seed weight and oil content when expressed in *A. thaliana* and *B. napus* [21].

Molecular docking reveals the binding sites between HpDGAT2s and AST structure

Although some studies have indicated that DGATs are likely to be the crucial enzymes involving in EAST biosynthesis in *H. lacustris* [34], so far there is no direct biochemical evidence. Homology modeling is a useful tool for predicting the 3D structure of proteins [54] and AutoDock tools is a powerful method for

identifying potential binding sites between 3D structures and ligands [55]. In this study, the docking studies were attempted to explore the binding sites between AST structure and 3D models of HpDGAT2s. SWISS-MODEL server was successful in generating 3D structures for HpDGAT2A, HpDGAT2B, HpDGAT2D, and HpDGA2E. All four HpDGAT2s protein structures contained possible AST binding sites (Additional file 11-13: Figure S7-S9). The results from HpDGAT2D were elaborated in detail (Fig. 6). The symmetrical half of the AST molecule (C20) was selected in docking process due to on one hand the oversized C40 structure (compare to C16-C22 fatty acids), on the other hand, in fact, AST esterification mainly occurred on the hydroxyl group of a six-membered ring at both ends (Fig. 6b). The symmetrical half of the AST molecule (C20) got docked onto the predicted 3D model of HpDGAT2D as shown in Fig. 6c. Further, molecular interaction studies showed that 3D model of HpDGAT2D had some potential AST binding sites (amino acids) by van der Waals force, conventional hydrogen bond, alkyl, Pi-alkyl, and Pi-sigma (Additional file 14: Figure S10). Meanwhile, some binding sites between fatty acids (C16:1 and C18:1) and 3D model of HpDGAT2D were also predicted (Fig. 6d and 6e), which verifies the reliability of the AutoDock analysis due to these DGAT2 enzymes should include their binding sites.

Discussion

Usually, the accumulation of AST and TAG is simultaneously significantly enhanced under most of stress conditions in *H. lacustris* e.g., HL and ND conditions [29-35, 50-53]. However, in general, the nitrogen complete deficient seriously limited the algal growth [51-53]. Recently, our results indicated that the highest AST productivity arrived at the 1/4N stress due to the certain level of algal growth (data not shown). Therefore, in the current manuscript, 1/4N condition was selected as ND condition in further experiment. Our results revealed that (1) T-AST and T-TAG contents significantly increased under HL and 1/4N conditions, respectively, which was consistent with previous study [34, 41], (2) M-AST was the main form which is also proven by previous studies [36-39], (3) compared to 1/4N stress, HL was more effective in inducing AST accumulation especially under the high blue light condition, which has been testified in our previous study [50], (4) coupled HL and 1/4N dual stimulation might be better choices in improving AST and TAG accumulation in *H. lacustris* (Fig. 1) [53]. Although the exact mechanisms of stress-induced TAG and AST accumulation in *H. lacustris* are largely unknown, several lines of evidence have implied that the biosynthesis of TAG and AST appears to be linked by the regulation of oil biosynthetic enzymes at transcription level [34, 41]. In fact, the AST accumulation is found to be dependent by the FAs biosynthesis and TAG accumulation in *H. lacustris* [34, 41]. Recently, Zhang et al (2019) reported that synthesized AST was esterified mainly with the fatty acid C18:1 and stored in TAG filled lipid droplets in *C. zofingiensis* [40]. Unlike in *H. lacustris*, although AST accumulated in a well-coordinated manner with TAG, AST is ketolated from zeaxanthin and is independent of FAs synthesis in *C. zofingiensis* [40]. This different result possibly due to the difference in genetic traits of these two organisms. Anyway, the enzymes involving in EAST biosynthesis in both AST producing algae *H. lacustris* and *C. zofingiensis* are unclear.

DGATs catalyze the terminal step in acyl-CoA-dependent TAG production pathway and represent a key target in manipulating TAG production [11]. At present, DGATs from different algal species have been

widely studied, which indicates that the diversity microalgae are prominent candidates for DGATs and the function of distinct DGATs is unique or species-specific [19-28]. Obviously, the *HpDGAT2s* genes were differentially regulated by HL, 1/4N, and double HL-1/4N stresses conditions with distinct patterns, suggesting that these enzymes are together involved in the AST and TAG biosynthesis (Fig. 2). Mao et al (2019) indicated that *CzDGAT1A*, *CzDGTT1*, *CzDGTT5* and *CzDGTT8* were all considerably up-regulated by ND with distinct expression patterns [20]. Chen et al (2015) indicated that the transcript level of *MiDGAT2A* was regulated by ND stress, which lead to TAG accumulation [28]. In addition, the previous studies have indicated that DGATs are the possible candidate enzymes involvement in both TAG and EAST accumulation [34], which makes it more interesting to identify DGATs in AST producing industrially alga *H. lacustris* [29]. Recently, although four putative type-2 DGATs genes were identified from *H. pluvialis* (*lacustris*), and the only HpDGAT2D had the capability of restoring TAG biosynthesis in a TAG-deficient yeast strain [42], but the engineering potential of DGAT2s in TAGs biosynthesis, especially roles in AST esterification, remains ambiguous.

In this study, we demonstrated that there were five DGAT2s genes in the alga and renamed as *HpDGAT2A*, *HpDGAT2B*, *HpDGAT2C*, *HpDGAT2D* and *HpDGAT2E* according to sequence alignment and phylogenetic analysis results (Additional file 3: Table S3 and Additional file 8: figure S4), which replenished the previous report of four putative type-2 DGATs in *H. pluvialis* (*lacustris*) transcriptome database [42]. Generally, only one or two copies of DGAT1s are identified in a number of microalgae, whereas multiple copies of DGAT2s are typically present [14]. The numbers of DGAT2s were species-specific in various algal organisms, e.g., *Chlamydomonas reinhardtii* (5), *Nannochloropsis oceanica* (13), *Lobosphaera incise* (3), *Chlorella zofingiensis* (8), *Myrmecia incise* (2), and *Phaeodactylum tricornutum* (4) [20, 23, 24, 26-28]. The subcellular localization prediction revealed the different sub-location of HpDGAT2s (Additional file 2: Table S2), which is in consistence with the subcellular localization prediction of DGATs from the green alga *C. reinhardtii* [24] and *C. zofingiensis* [20]. Two or three TMs were present in all HpDGAT2s (Additional file 2: Table S2 and Additional file 5: Figure S1) implying the members of membrane bound forms of DGAT1 and DGAT2 [14]. Interestingly, abundant phosphorylation sites were predicted in all HpDGAT2s (Additional file 2: Table S2 and Additional file 6: Figure S2), indicating phosphorylation plays important roles in DGAT2s enzymes activity due to it has been proven that the DGAT1 enzyme activity was affected by phosphorylation in mouse DGAT1 [44], BnaDGAT1 [46] and TmaDGAT1 [45]. It remains to be determined whether these phosphorylation sites are important for the functional regulation of HpDGAT2 *in vivo*. The CDs previously identified in DGAT2 enzymes from higher plants and microalgae [26, 47, 48] were also present in HpDGAT2s but with varying degrees of conservation (Additional file 7: Figure S3) including YF/YFP block (CD1) which is essential for DGAT2 activity, HPHG/EPHS block (CD4) which is proposed to partially consist of the active site, and RxGFx(K/R)xAxxxGxx(L/V)VPxxxFG block (CD5) which is the longest conserved sequence in plant and animal. Some putative lipid binding motifs (FLxLxxx and FVLf blocks) in the mouse DGAT2 were not conserved among HpDGAT2s and algal DGAT2s [47, 48, 56]. Moreover, there were two completely conserved amino acid residues (proline, P and phenylalanine, F) among all DGAT2s, which is consistent

with previous reports that these two highly conserved residues maybe located at the active sites of the enzymes [49].

To characterize the roles of HpDGAT2s, we cloned and identified the four *HpDGAT2s* genes with confirmed full-length coding sequence (Additional file 2: Table S2). Expression in the TAG-deficient yeast strain H1246, a commonly used system for DGAT functional complementation [57], confirmed that all of the *HpDGAT2s* genes are functional, despite the large difference in enzymatic activity (Fig. 3a). Further functional characterization in yeast showed that HpDGAT2D and HpDGAT2E can increase the TAG content more than HpDGAT2A and HpDGAT2B, resulting in a significant increase in the TAG content of yeast of 108.7%-122.7% (Fig. 3d). Its higher activity provides an alternative candidate of DGAT2 to modulating TAG accumulation in algae. However, previous study detected that the only HpDGAT2D had the capability of restoring TAG biosynthesis in a TAG-deficient yeast strain [42]. By contrast, in our study, *HpDGAT2B* expression in H1246 cells produced un-conspicuous TAG possible due to the limited FAs in *Saccharomyces cerevisiae*. This holds true, at least for *CzDGTT1* expressed in yeast, the TAG content increased when feeding of the two other free FAs [20]. It is also possible that HpDGAT2B may not a real DGAT but other types of transferase, which cannot be distinguished based only on the sequence data [20]. This phenomenon is usually present in green algae, e.g., CrDGTT1 through CrDGTT3 are functional while CrDGTT4 is not [24], NoDGAT1A and CzDGTT1 rather than NoDGAT1B is functional [20, 22].

DAGs and acyl-CoAs are essential substrates for TAG biosynthesis under the catalysis of DGAT enzymes [20, 22, 24]. The acyl-CoAs substrate specificity was determined by FAs profiles analysis. HpDGAT2s showed their strong preference for MUFAs (C16:1 and C18:1) in yeast cells. Such tendency, however at different levels, was observed for almost all transformed lines of H1246 for various DGATs enzymes [20, 23, 24, 26-28]. Considering the limited FAs in yeast cells, some PUFAs (e.g., C18:2n6, C18:3n3, C18:3n6, and C18:4n3) that are present in *H. lacustris* but not in yeast cells were selected to test the acyl-CoAs substrate specificity by feeding strategy. Interestingly, all HpDGAT2s except for HpDGAT2B showed their wide range preference for PUFAs with distinct patterns in yeast cells, especially for C18:2n6 and C18:3n3 that also rich in *H. lacustris*, indicating that these HpDGAT2s may have potential in the engineering of these PUFAs-enriched TAG production. This phenomenon was also confirmed by Zienkiewicz et al (2018) that some PUFAs were incorporated into TAG on the expense of C16:1 and C18:1 in *LiDGAT1*, *LiDGAT2.1*, *LiDGAT2.2*, and *LiDGAT2.3* expressing yeast [23] and *CzDGAT2C* expressing yeast mutant H1246 cells [26] by feeding test respectively. Consistent with the low transcription expression of *HpDGAT2B* in algal and yeast cells, feeding test demonstrated the low preference of PUFAs, again indicating a nonfunctional encoded protein. Although the acyl-CoAs substrate preference was characterized, the DAGs (prokaryotic and eukaryotic) substrate specificity needed to be elucidated in future.

In order to evaluate the possible biological function and engineering potential to modulate TAG biosynthesis for *HpDGAT2s* in algae and plants, in the present study, we generated overexpression lines in evolutionary close green algal *C. reinhardtii* CC849 and model plants *A. thaliana*, respectively. It is not surprising that *HpDGAT2D* overexpression enhanced TAG contents in both *C. reinhardtii* CC849 (by ~ 1.4-fold) and *A. thaliana* (by ~ 1.2-fold). Guo et al (2017) indicated that the *CeDGAT1* gene can stimulate FAs

biosynthesis and enhance seed weight and oil content when expressed in *A. thaliana* and *B. napus* [21]. Compared to control, under 1/4N stress condition, it was also worth noting that the TAG content was significantly increased at a 4 day batch culture for *HpDGAT2D* overexpression lines under the same stress condition (Fig. 4b), possibly due in part to the high transcription expression level (Fig. 4d). Wei et al (2017) detected that *NoDGAT1A* expression in *C. reinhardtii* UVM4 had no effect on TAG accumulation under nitrogen-replete condition but TAG enhancement was observed under nitrogen-depleted condition [22], which possibly due to the difference in genetic traits of distinct organisms. However, Mao et al (2019) declared that *CzDGAT1A* expression in oleaginous alga *N. oceanica* resulted in a considerable increase (~ 2.8-fold) in TAGs level in the linear growth stage [20]. Consistent with the strong preference for MUFAs and PUFAs rather than SFAs in yeast cells, *HpDGAT2D* also showed similar trend in *C. reinhardtii*. Specifically, *HpDGAT2D* had the first option to C16:1, C18:1, C18:2n6 and C18:3n3 rather than C16:2, C16:3, C16:4, C18:3n6, and C18:4n3. Interestingly, these preferred substrates happened to be the type that is enriched in *C. reinhardtii*, indicating the potential in the engineering of *C. reinhardtii* for MUFAs and PUFAs-enriched TAG production. This trend was also consistent with results from yeast cells by feeding test (Figs. 3d and 3e) and also consistent with previous studies of *NoDGAT1A* expression in *C. reinhardtii* UVM4 and *CzDGAT1A* expression in oleaginous alga *N. oceanica* by Wei et al (2017) and Mao et al (2019) respectively [20, 22]. In higher plants, the expression of *DGATs* generally enhances the oil deposition in developing seeds [58]. For example, a stronger expression of *DGAT1* was found in developing seeds than in other tissues in soybeans [59]. However, *DGAT1* transcripts were also detected in other plant tissues but most strongly in developing embryos and flower petals [60]. In the current study, *HpDGAT2D* transcript was expressed in transgenic lines at different tissue organs including roots, tubers, leaves, siliques, and seeds with distinct extent (Fig. 5b). However, the exact process of FAs change was much more complicated than those in yeast and *C. reinhardtii* cells (Fig. 5c). The *HpDGAT2D* showed strong preference for C18:1, C18:2n6, and C18:3n3 rather than C20:1, C20:2 and C22:1 in TAG biosynthesis, which were largely in agreement with those in yeast cells (Figs. 3d and 3e) and *C. reinhardtii* cells (Fig. 4c). Previous studies have indicated that seed-specific overexpression of *EgDGAT2* in *A. thaliana* enhanced the content of PUFAs C18:2n6 and C18:3n3 in seed TAG, when compared to that from wild-type *Arabidopsis*. In turn, the proportion of C18:0 and C20:0 SFAs in seed TAG from *EgDGAT2* transgenic lines decreased accordingly [61]. In *Thraustochytrium aureum*, *DGAT2* expressing under a strong seed-specific promoter in wild-type *A. thaliana* increased C18:2n6 content [62]. In addition, transgenic plants showed no other phenotypic differences. Therefore, *HpDGAT2D* should have great potential for increasing the specific oil production in other oil crops.

Although it has been early suggested that *DGATs* may be involved in the esterification of AST in *H. lacustris* [34], so far there is no direct biochemical evidence to support this hypothesis. Recently, all ten *CzDGATs* were expressed in a reconstructed AST-producing yeast strain [63] to examine if these enzymes were responsible for EAST biosynthesis. However, no EAST was detected, indicating the null function of *CzDGATs* in AST esterification [20]. Considering the difference in genetic traits and AST biosynthetic pathway of both AST-producing algal strains *C. zofingiensis* and *H. lacustris*, it inspires us to study the possible roles of *HpDGAT2s* in AST esterification. The combination of homology modeling and AutoDock

is a powerful tool to identify potential binding sites between HpDGAT2s 3D structures and AST molecular [54, 55]. Some possible binding sites were predicted as shown in Fig. 6 (HpDGAT2D) and Additional file 11-13: Figure S7-S9 (HpDGAT2A, HpDGAT2B, and HpDGAT2E), which provides another key clue for functional roles of HpDGAT2s in AST esterification, however, further biological experiments, e.g., the expression of *HpDGAT2s* in AST-producing yeast, algal, and bacteria strains, are necessary to support this hypothesis in future. Recently, binding sites of potential protease inhibitors of COVID-19 and 3D structure of COVID-19 has been detected using AutoDock [64, 65]. However, compare to the presence of cryo-electron microscopy structure of human DGAT1 in complex with an oleoyl-CoA substrate [66, 67], the absence 3D structures of DGAT2s hampers the accuracy of AutoDock results.

Conclusion

Here, we performed an in-depth characterization of HpDGAT2s by systematically integrating expression pattern, AST/TAG accumulation, functional complementation, molecular docking, and over-expression in yeast, plants, and algae. Five putative *DGAT2s* genes (*HpDGAT2A*, *HpDGAT2B*, *HpDGAT2C*, *HpDGAT2D*, and *HpDGAT2E*) were identified in *H. lacustris* by BLAST and CD analysis. These *DGAT2s* genes markedly increased at transcription levels under stress condition, which led to significant TAG and EAST accumulation. Functional complementation demonstrated HpDGAT2A, HpDGAT2B, HpDGAT2D, and HpDGAT2E had the capability to restore TAG synthesis in a TAG-deficient yeast strain (H1246) with the large difference in enzymatic activity. FAs profiles assays revealed that HpDGAT2A, HpDGAT2D, and HpDGAT2E except for HpDGAT2B preferred MUFAs for TAG synthesis in yeast cells, and also showed PUFAs preference by feeding strategy. The over-expression of *HpDGAT2D* in wild type *A. thaliana* and *C. reinhardtii* significantly increased the TAG content and also showed strong preference for MUFAs and PUFAs, indicating the engineering potential for increasing the specific TAG production in plants and algae. Interestingly, molecular docking analysis implied that HpDGAT2s protein structures contained binding sites for symmetrical half of the AST, which provide strong evidence for AST esterification function in *H. lacustris*.

Methods

Algal strain and growth condition

The unicellular algal *Haematococcus lacustris/pluvialis* (FACHB-712) strain was obtained from Freshwater Algae Culture Collection at the Institute of Hydrobiology and maintained at the Institute of Molecular Agriculture and Bioenergy (IMAB), Shanxi Agricultural University. *H. lacustris* 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 $\mu\text{mol}/\text{m}^2/\text{s}$ light intensity with a diurnal cycle of 12 h light /12 h dark at the temperature 23 ± 1 °C. The culture solution in the flasks was shaken at the fixed time and twice a day. For the HL treatment, after the cultures were dark-adapted for 48 h, the later exponentially growing cultures (biomass content of approximately 200 mg/L) were further transferred into fresh medium under continuous white light (390-770 nm) or blue light (420-500 nm) with a light

intensity of 500 $\mu\text{mol}/\text{m}^2/\text{s}$ without a light/dark cycle, respectively. For the 1/4N nitrogen deficient treatment, these pre-cultured and dark induced cells were collected and washed with nitrogen-free BBM medium, and then further transferred into fresh BBM medium with 1/4 nitrogen content (as those in BBM medium) under the control culture conditions without a light/dark cycle. For the HL and 1/4N double stresses (HL-1/4N) treatment, these pre-cultured and dark induced cells were transferred into fresh medium with 1/4 nitrogen content under the same continuous white light or blue light, respectively. The cultures under control conditions were used as the control. These pre-cultured and dark induced cells, after centrifugation and washing with sterilized water, were sampled as the starting point (N-0 day). The cultures were sampled N-1, N-2, N-3, and N-4 day after treatment. The cells were harvested by centrifugation (13,100 g at 4 °C for 5 min) and were washed with PBS prior to storage in liquid nitrogen. For cell dry biomass determination, 20 mL cells culture was collected and washed three times, and then the EP tubes containing cells were dried in a DW3 freeze-drier (Heto Dry Winner, Denmark).

Cloning and bioinformatics analysis of HpDGAT2s

The genes encoding putative HpDGAT2s were predicted and cloned as follow three steps: (1) the local BLAST program was used to predict *DGAT2s* genes based on the *H. lacustris* transcriptome database by the annotated CzDGAT2s and CrDGAT2s (Additional file 1: Table S1), (2) the rapid amplification of cDNA ends (RACEs) method was used to obtain the full-length mRNA sequences and then determine their transcription start sites, stop sites, and encoding sequences, (3) the open reading frame (ORF) for each *HpDGAT2s* gene was obtained by PCR method again to construct distinct expression plasmids. All the primers used in this study were listed in Additional file 4: Table S4. The molecular weight (Mw), isoelectronic point (pI), sub-cellular localization, signal peptides (SP), chloroplast transfer peptides (CTP), trans-membrane regions (TM), and phosphorylation site (Phos) of HpDGAT2s were predicted by Compute pI/MW, TargetP, ChloroP, SignalP, TMHMM, and NetPhos tools respectively in ExPASy [68]. HpDGAT2s and other DGATs from plants and algae were aligned using ClustalX [69]. Maximum likelihood trees (Le and Gascuel evolutionary model) of HpDGAT2s and other DGATs proteins were constructed using PhyML [70, 71]. Bootstrap (BS) values were inferred from 400 replicates. Graphical representation and edition of the phylogenetic tree were performed with MEGA5 [72] and TreeDyn (v198.3) [73].

RNA isolation and quantitative real-time PCR

The total RNA was extracted 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 qRT-PCR was performed as described by our previous study using a 7500 Fast Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) with SYBR Green PCR Master Mix (Invitrogen). The mRNA expression level was normalized using the *actin* gene as the internal control. All analyses were based on the CT values of the PCR products. The comparative CT method was used to investigate the transcriptional expression levels of *HpDGAT2s* genes [74].

Functional complementation of HpDGAT2s in the TAG-deficient yeast H1246

The ORFs of *HpDGAT2A*, *HpDGAT2B*, *HpDGAT2D*, and *HpDGAT2E* were PCR amplified using cDNA as template and cloned into the yeast expression vector pYES2.0 (Invitrogen). After confirmation by restriction enzyme digestion and sequencing, the recombinant pYES2.0-HpDGAT2s plasmids were transformed into the *S. cerevisiae* TAG-producing strain INVSc1 or TAG-deficient quadruple mutant strain H1246 by means of S.c. EasyComp Transformation Kit (Invitrogen). Selection of the transformants was finished by using the synthetic medium without uracil (SC-ura). *HpDGAT2s* expression induction by galactose was performed as previously described [20]. The expression of *HpDGAT2s* genes in yeast strain was verified at the transcript level by qRT-PCR method. For the feeding experiments, yeast cultures were induced as described above but in presence of 1% (w/v) Tergitol NP-40 (Sigma Aldrich, St. Louis, MO, USA) in the medium. At the beginning of induction, the appropriate FAs (C18:2n6, C18:3n3, C18:3n6, and C18:4n3) were added to the culture to a final concentration of 100 μ M. Samples at OD600 of 2.5 were harvested for lipid extraction, separation by TLC and analysis by GC.

Over-expression of *HpDGAT2D* in *C. reinhardtii*

The nuclear transformation expression vector pDB124 (Additional file 9: Figure S5), characterized in *C. reinhardtii* CC849 and presented by professor Zhangli Hu from Shenzhen University [75], was used in this study after modified. The codon preference (*HpDGAT2D*) was optimized according to the algal *C. reinhardtii* (Additional file 10: Figure S6) before constructing the expression vector. The codon preference optimized coding sequence of *HpDGAT2D* was amplified and cloned into *Pml* and *BmtI* sites of pDB-124, followed by sequencing for verification. The resulting plasmid was linearized by *XbaI* and transformed into the *C. reinhardtii* cc849 strain via the glass beads method [76]. Transformants were selected on Tris-acetate-phosphate (TAP) plates with 10 μ g/mL bleomycin (Sigma-Aldrich). The integration of *HpDGAT2D* into *Chlamydomonas* genome was verified by genomic PCR, and its expression was determined by qRT-PCR. For ND stress, the later exponentially growing *C. reinhardtii* cc849 cells (biomass content of approximately 420 mg/L) were collected and washed with nitrogen-free TAP medium, and then further transferred into fresh TAP medium without nitrogen, and cultured under the conditions of 25 μ mol/m²/s light intensity with a diurnal cycle of 12 h light /12 h dark at the temperature 23 $\pm$ 1 $^{\circ}$ C. The integration of the transforming cassette into the algal chromosome was validated by genomic PCR method. The transcription and protein expression levels of *HpDGAT2D-His* fusion gene were validated via qRT-PCR and western blotting methods, respectively. His-tag antibodies were used to detect the protein expression fusion protein of HpDGAT2D-His. To determine whether HpDGAT2D has a trans-membrane motif, the *HpDGAT2D* overexpression *C. reinhardtii* cells at the log phase were harvested and soluble and membrane proteins were used for (previously described) immunodetection [19]. Briefly, algal cells were re-suspended in 10 mL extraction buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10 mM KCl, 1 mM MgCl₂, 1 mM β -mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 1 mg/mL leupeptin, and 0.25 M sucrose) and broken by an ultrasonic cell disrupter at 120 W for 15 min. The soluble and membrane proteins were separated via centrifugation at 18,000 g for 20 min.

Over-expression of *HpDGAT2D* in *A. thaliana*

The coding sequence of *HpDGAT2D* was amplified and cloned into *EcoRI/XbaI* sites of pCAMBIA1303 to yield pCAMBIA1303-*HpDGAT2D*. The final binary vector was verified by restriction enzyme digestion and sequencing, and then transferred into *Agrobacterium tumefaciens* strain GV3101 by the freeze-thaw method [77]. *A. thaliana* plants were transformed by vacuum infiltration [78]. T1 generation seeds were selected on hygromycin (50 mg/L), and then the selected transformed plants were transferred to soil. T2 transgenic *A. thaliana* lines were used for seed and oil analyses. The stable integration of the pCAMBIA1301-*HpDGAT2D* into the genome of transgenic *A. thaliana* was checked by genomic PCR amplification. In the meantime, the expression of *HpDGAT2D* was determined by qRT-PCR.

Total astaxanthin analysis

For carotenoids analysis, the HPLC method was applied to quantify the contents of all the carotenoids [50, 79]. Briefly, the freeze-dried cells (0.01 g) were ground with liquid nitrogen and extracted with acetone until the cells became colorless. After centrifugation at $13100 \times g$ at 4 °C for 15 min, the supernatant was collected and evaporated under nitrogen gas. Finally, the residue was re-dissolved in 1 mL acetone and filtered through a 0.22-mm Millipore organic membrane (Millipore Co., USA) prior to HPLC analysis. Carotenoids were eluted at a flow rate of 1.2 mL/min with a linear gradient from 100 % solvent A [acetonitrile/methanol/0.1 M Tris-HCl (84:2:14), pH 8.0] to 100 % solvent B [methanol/ethyl acetate (68:32)] for 15 min, followed by 12 min of solvent B. The absorption spectra of the carotenoids ranged from 300 nm to 700 nm (Thermo Scientific UltiMate 3000 HPLC, USA). Peaks were measured at 450 nm. The contents of F-AST, M-AST, and D-AST were determined using the standard curves of standard carotenoids at known concentrations. The carotenoids standards were purchased from Sigma-Aldrich.

Lipid extraction and fatty acids analysis

The lipid extraction and FAs analysis were all performed according to previously described procedures [21, 80-82]. Briefly, cellular FAs were extracted by incubating 50 mg of yeast cells or 10 mg freeze-dried algae cells or 10 mg of dried seeds of control and transformed plants in 3 mL of 7.5% (w/v) KOH in methanol for saponification at 70 °C for 4 h. Then the pH was adjusted to 2.0 with HCl. The total lipids were extracted according to a modified version of the Bligh and Dyer method [81], and TAG was separated from the total lipids by thin-layer chromatography (TLC) on Silica Gel 60 plates. The solvents that were used were hexane/diethyl ether/glacial acetic acid (70:30:1, v/v). The lipids were visualized by spraying Primuline (Sigma, 10 mg/100 mL acetone: water (60:40 v/v)) and exposing the plate to UV. Triolein (Sigma) was used as the standard. TAGs were recovered from the TLC plates and then transesterified with 5% H₂SO₄ in methanol at 85 °C for 1 h. The fatty acid methyl esters (FAMES) were extracted with hexane and analyzed by gas chromatography. For quantification of the FAs, an appropriate amount of C17:0 FAME (Sigma) was added as internal standard. FAMES were analyzed by Agilent GC equipped with a flame ionization detector (FID) and a capillary column (HP-88 100 m × 0.25 mm × 0.2 mm). Nitrogen was used as the carrier gas at a flow rate of 2.64 mL/min. The injection and detector

temperatures were both set as 250 °C. The gas chromatograph oven was programmed at an initial temperature of 140 °C for 5 min, and then increased up to 250 °C at a rate of 15°C/min. FA component was estimated according to the retention time of fatty acid standard and data was collected by peak area normalization with C17:0 internal standard.

Molecular docking

Homology modeling is a useful tool for predicting the 3D structure of proteins [54] and AutoDock tools is a powerful method for identifying potential binding sites between 3D structures and ligands [55]. SWISS-MODEL server was used to generate 3D structures for HpDGAT2A, HpDGAT2B, HpDGAT2D, and HpDGAT2E [54]. The output of the predicted model generated as pdb file was downloaded for further analysis and visualized using SPDBV 4.10 [83]. The symmetrical half of the AST molecule (C20) was selected in docking process due to on one hand the oversized C40 structure (compare to C16-C22 fatty acids), on the other hand, in fact, AST esterification mainly occurred on the hydroxyl group of a six-membered ring at both ends. In addition, fatty acids CoA (C16:1 and C18:1) were also used to AutoDock analysis as positive control due to these DGAT2 enzymes should include their binding sites.

Statistical analysis

All experiments were repeated three times to ensure the reproducibility. The data were obtained as the mean value $\pm$ SD. Statistical analyses were performed using the SPSS statistical package (SPSS Inc., Chicago, IL, USA). Significant differences between treatments were statistically analyzed by paired-samples t-test. The statistical significances are achieved when $P < 0.01$.

Abbreviations

AST: Astaxanthin; E-AST: Esterified astaxanthin; F-AST: Free AST; M-AST: Monoester AST; D-AST: Diester AST; TAG: Triacylglycerol; DGAT: Diacylglycerol acyltransferase; ORF: Open reading frame; RACEs: Rapid amplification of complementary DNA ends; UTR: Untranslated region; HL: High light; ND: Nitrogen deficient; MUFAs: Monounsaturated fatty acyl-CoAs; PUFAs: Polyunsaturated fatty acyl-CoAs.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent to publish

Not applicable.

Availability of data and materials

The datasets supporting the results of this article are included within the article and its additional files. More datasets used in the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

HC and CZ carried out the experiments, analyzed the data and drafted the manuscript. WX and HZ performed the over-expression of *HpDGAT2s* in yeast, algae and plants. WH and XZ participated in gene cloning and sequence analysis of *HpDGAT2s*. CJ and JX participated in transcription expression of *HpDGAT2s* in *H. lacustris* under stress condition. CZ participated in growth and TAG and AST accumulation for *H. lacustris* under stress condition. RL conceived the study, participated in its design and revised the manuscript. All authors read and approved the final manuscript.

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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 marneffei ATCC*), Rm (*Rhizopus microsporus*), Ma (*Mortierella alpina*).

Figures

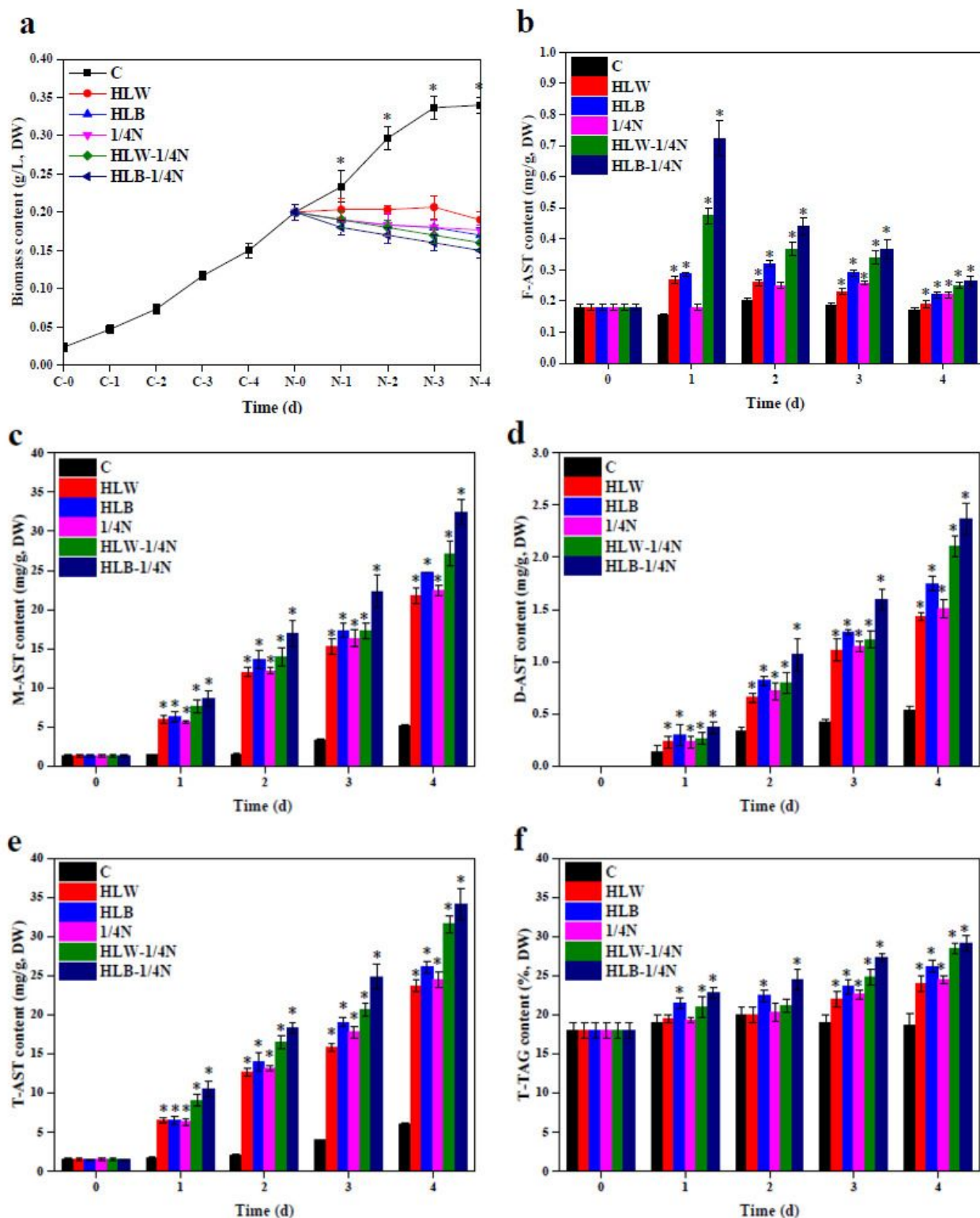


Figure 1

Growth, astaxanthin, and triacylglycerol profiles of *Haematococcus lacustris* under HLW, HLB, 1/4N, HLW-1/4N, and HLB-1/4N conditions after 1, 2, 3, and 4 day. (a) Time course of biomass content. (b) Free astaxanthin content. (c) Monoesterified astaxanthin content. (d) Diesterified astaxanthin content. (e) Total astaxanthin content. (f) Total triacylglycerol content

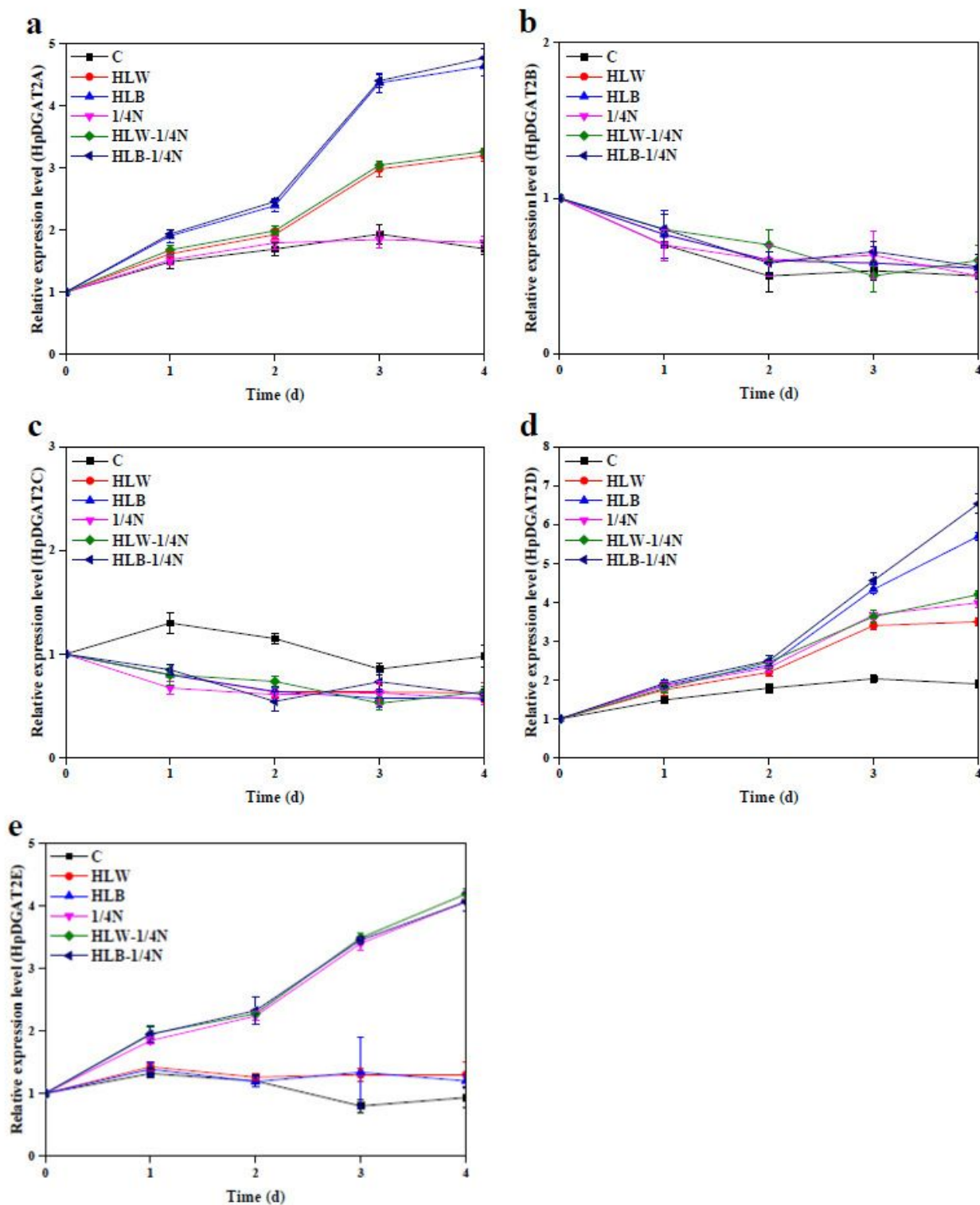


Figure 2

The transcriptional expression levels of HpDGAT2s in *Haematococcus lacustris* under HLW, HLB, 1/4N, HLW-1/4N, and HLB-1/4N conditions after 1, 2, 3, and 4 day. (a) HpDGAT2A. (b) HpDGAT2B. (c) HpDGAT2C. (d) HpDGAT2D. (e) HpDGAT2E. The gene expression levels were normalized to the endogenous actin gene

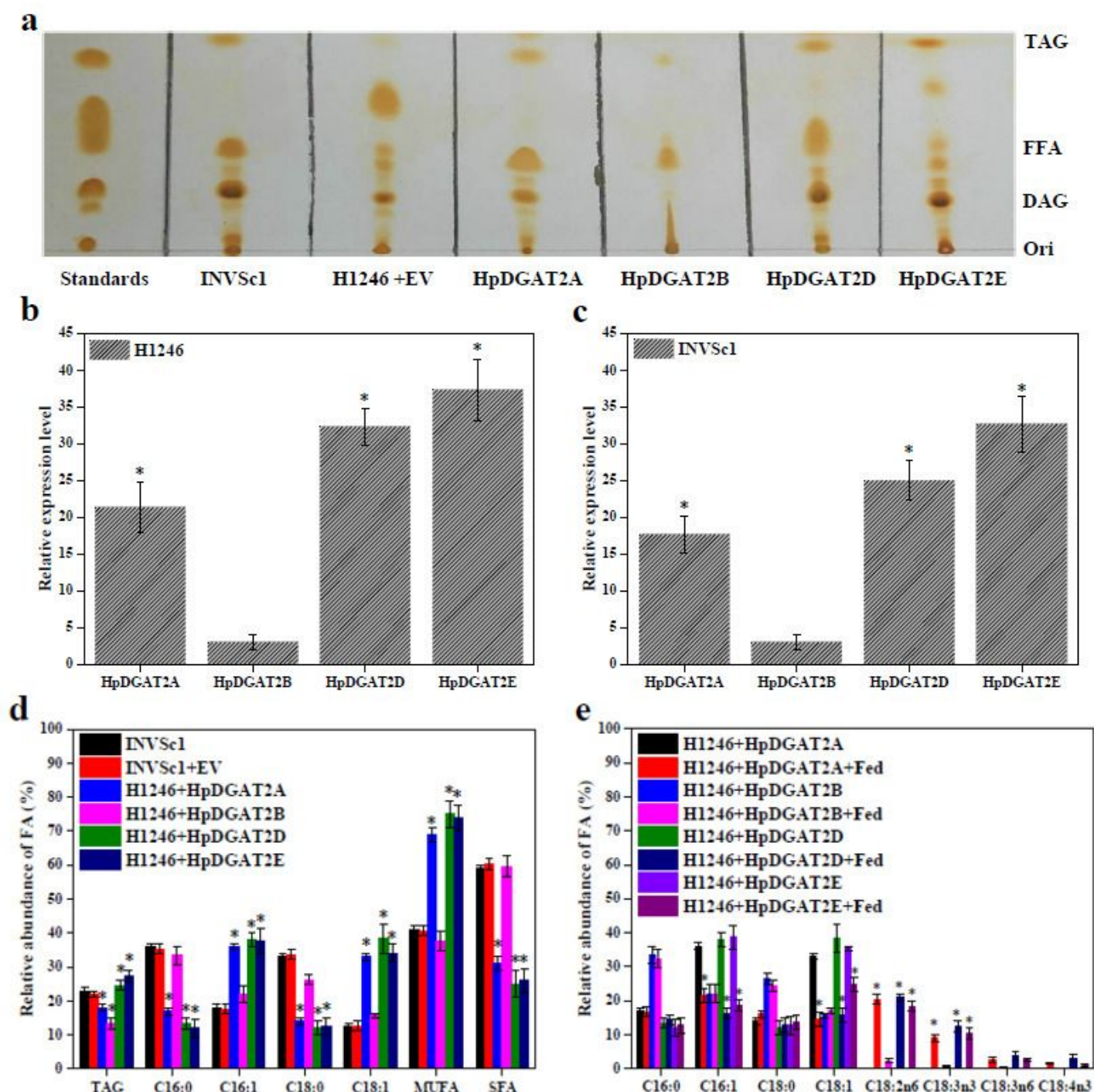


Figure 3

Functional characterization of HpDGAT2s in *Saccharomyces cerevisiae* cells. (a) TLC analysis of total lipids extracted from control *S. cerevisiae* (INVSc1), TAG-deficient *S. cerevisiae* (H1246), and H1246 cells transformed with HpDGAT2s and Empty vector (EV). (b) and (c) The transcriptional expression levels of HpDGAT2s in H1246 and INVSc1 cells transformed with HpDGAT2s. The gene expression levels were normalized to the endogenous actin gene. (d) TAG contents and relative abundance of fatty acids in INVSc1 and H1246 cells transformed with HpDGAT2s. (e) Relative abundance of fatty acids in H1246

cells transformed with HpDGAT2s by the feeding of free fatty acids of C18:2n6, C18:3n3, C18:3n6, and C18:4n3 after a 24 h cultivation

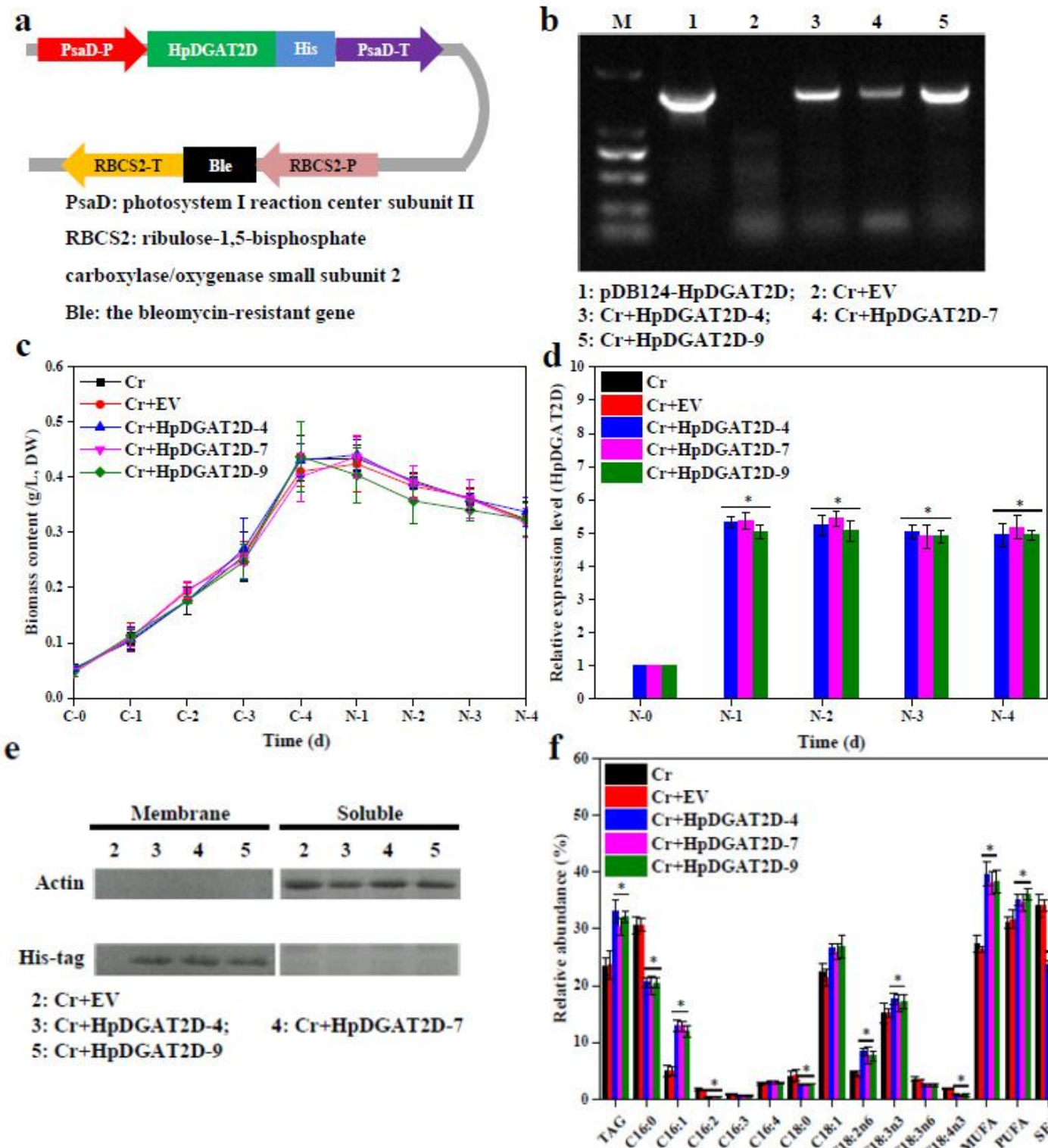


Figure 4

Overexpression of HpDGAT2D in *Chlamydomonas reinhardtii* cells. (a) Construct of the expression vector. PsaD-P, promoter of PsaD gene; His tag, 6-His encoding gene; PsaD-T, terminator of PsaD gene; RBCS2-P, promoter of RBCS2 gene; Ble, the bleomycin-resistant gene; RBCS-T, terminator of RBCS gene. (b)

Genomic level of HpDGAT2D in *C. reinhardtii* cells. (c) Time course of biomass content under control and 1/4N conditions. (d) The transcriptional expression levels of HpDGAT2D in *C. reinhardtii* cells. (e) Western blotting of HpDGAT2D-6-His tag fusion protein with His-tag antibodies. Soluble and membrane proteins were separated and used for blotting. Actin which was known soluble protein was used as controls. (f) TAG contents and relative abundance of fatty acids in *C. reinhardtii* cells transformed with HpDGAT2D.

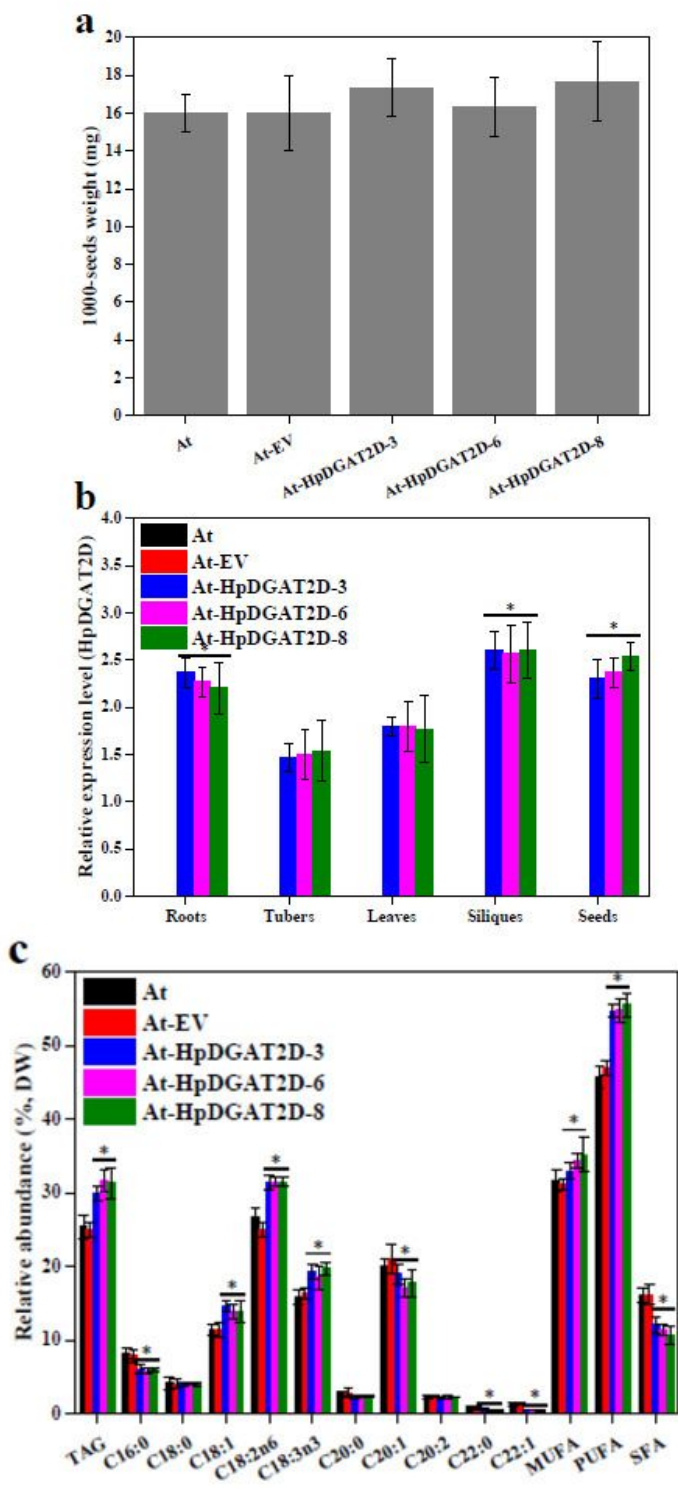


Figure 5

Overexpression of HpDGAT2D in *Arabidopsis thaliana*. (a) Average 1,000-seed weight (expressed as milligrams of weight/1,000 seeds) of transgenic *Arabidopsis* T2 seeds. (b) The transcriptional expression levels of HpDGAT2D in *A. thaliana*. The gene expression levels were normalized to the endogenous actin gene. (c) TAG contents and relative abundance of fatty acids in *A. thaliana* transformed with HpDGAT2D

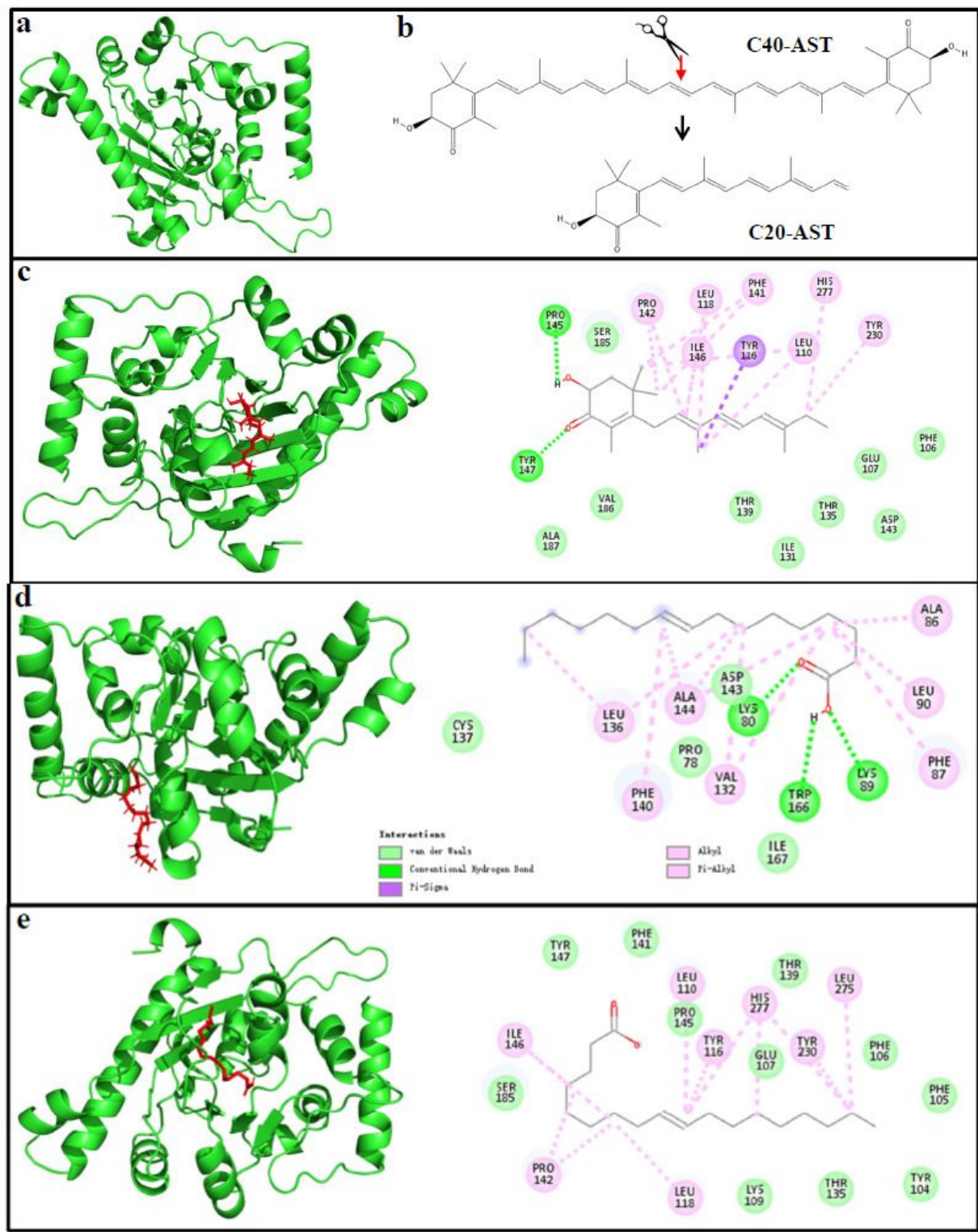


Figure 6

Docking of AST molecular and 3D model of HpDGAT2D using AutoDock software. (a) The 3D model of HpDGAT2D. (b) AST molecular. (c) Binding sites between AST and HpDGAT2D. (d) Binding sites between C16:1 and HpDGAT2D. (e) Binding sites between C18:1 and HpDGAT2D

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