INTRODUCTION

Microalgae have been considered as the next-generation biodiesel feedstock because of the robust growth, no competition with food for arable land, efficient solar-driven conversion of carbon dioxide to storage lipids such as triacylglycerol (TAG), and promising lipid yield (Hu et al., 2008; Wijffels & Barbosa, 2010). The realization of commercialized algae-derived biodiesel still faces many challenges in spite of progresses achieved during the past decades (Lenka et al., 2016; Raheem et al., 2018; Zou et al., 2017). From a biorefinery point of view, improving TAG content and yield has great potential to bring down algae-derived biofuels production cost. Here we dissected functional roles and engineering potential of six diacylglycerol acyltransferase (DGAT) genes from the marine alga *Phaeodactylum tricornutum*. *PtDGAT1*, *PtDGAT2B*, and *PtDGAT3*, residing at the chloroplast endoplasmic reticulum (ER) readily for utilizing both chloroplast- and ER-derived diacylglycerol, were transcriptionally correlated with TAG accumulation. Heterologous expression in yeast, in vitro assay and overexpression in *P. tricornutum* all supported that *PtDGAT1* surpassed the other five *PtDGATs* in synthesizing TAG. Compared to wild type *P. tricornutum*, the *PtDGAT1*-overexpressing strain produced more than doubled TAG and total lipids, which reached 57% and 73% of dry weight, respectively, record-high levels ever achieved in this alga. Our results demonstrated the strategy of screening proper engineering targets and manipulating a single gene to pull carbon flux to lipids for TAG hyper-accumulation without growth compromise. The engineered alga with a great trait improvement may serve as a potent lipid producer for production uses.

KEYWORDS
biofuel, diacylglycerol acyltransferase, genetic manipulation, oleaginous alga, *Phaeodactylum tricornutum*, subcellular localization, triacylglycerol

Lipid production is more than doubled by manipulating a diacylglycerol acyltransferase in algae

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Abstract
Oleaginous algae have the ability to synthesize a high level of triacylglycerol (TAG) and are considered as the next-generation feedstock for biofuel production. Manipulating algal lipid biosynthetic pathways has potential to overproduce TAG and represents a feasible way toward bringing down algae-derived biofuels production cost. Here we dissected functional roles and engineering potential of six diacylglycerol acyltransferase (DGAT) genes from the marine alga *Phaeodactylum tricornutum*. *PtDGAT1*, *PtDGAT2B*, and *PtDGAT3*, residing at the chloroplast endoplasmic reticulum (ER) readily for utilizing both chloroplast- and ER-derived diacylglycerol, were transcriptionally correlated with TAG accumulation. Heterologous expression in yeast, in vitro assay and overexpression in *P. tricornutum* all supported that *PtDGAT1* surpassed the other five *PtDGATs* in synthesizing TAG. Compared to wild type *P. tricornutum*, the *PtDGAT1*-overexpressing strain produced more than doubled TAG and total lipids, which reached 57% and 73% of dry weight, respectively, record-high levels ever achieved in this alga. Our results demonstrated the strategy of screening proper engineering targets and manipulating a single gene to pull carbon flux to lipids for TAG hyper-accumulation without growth compromise. The engineered alga with a great trait improvement may serve as a potent lipid producer for production uses.
Enhancing TAG production by algae via genetic engineering can be facilitated by the understanding of pathways and regulatory mechanisms for lipid metabolism (Goncalves et al., 2016; Lenka et al., 2016; Li-Beisson et al., 2019). It is believed in algae that a series of coordinated biological pathways that provide carbon precursors, energy and reductant molecules, pushing and pulling power, and storage sink are stimulated to contribute to TAG synthesis and accumulation (Li et al., 2014; Liu et al., 2019; Park et al., 2015). The acyl-CoA-dependent Kennedy pathway involves several acylation steps that incorporate acyl moiety into the glycerol backbone and plays an important role in abiotic stress-associated TAG synthesis (Lenka et al., 2016; Li-Beisson et al., 2019). Diacylglycerol acyltransferase (DGAT) catalyzes the last acylation step and is considered as a rate-limiting enzyme of TAG synthesis in the Kennedy pathway (Xu et al., 2018). It has been reported that manipulating DGAT via overexpression leads to TAG augmentation in algae to different levels, depending on algal species and gene sources (Cui et al., 2018; Dinamarca et al., 2017; Haslam et al., 2020; Iwai et al., 2014; Manandhar-Shrestha & Hildebrand, 2015; Niu et al., 2013; Wei et al., 2017; Xin et al., 2017; Zienkiewicz et al., 2017; Zulu et al., 2017). TAG productivity, a key characteristic of evaluating algae for biodiesel production, depends on not only TAG content but also biomass productivity (Griffiths & Harrison, 2009). There have been reports about DGAT overexpression that caused a significant decrease in algal growth (Dinamarca et al., 2017; Zienkiewicz et al., 2017; Zulu et al., 2017), which is not desirable for production purposes. It is expected to achieve improved TAG accumulation via manipulation of lipid metabolism yet without compromising algal growth.

The diatom *Phaeodactylum tricornutum* represents a well-established marine alga that plays an important role in the supply of ocean primary productivity (Field et al., 1998). Because of the ease of cultivation, fast growth, and ability to synthesize value-added compounds (e.g., polyunsaturated fatty acids [PUFAs] and fucoxanthin), *P. tricornutum* is considered as a coveted source of natural products for food and pharmaceutical applications (Adarme-Vega et al., 2014; Dambek et al., 2012). This alga is also able to produce abundant intracellular oils under certain conditions and has been cited as a promising feedstock for biofuels (Ge et al., 2014; Yang et al., 2013). The availability of *P. tricornutum* genome sequence (Bowler et al., 2008) aids in identification of six putative DGAT-encoding genes, which are referred to as *PtDGAT1*, *PtDGAT2A* through *PtDGAT2D*, and *PtDGAT3*. Functional complementation of *PtDGAT* has been tested in the TAG-deficient yeast (Cui et al., 2013; Gong et al., 2013; Guiheneuf et al., 2011). Several *PtDGATs* have also been overexpressed to promote TAG accumulation in *P. tricornutum* (Cui et al., 2018; Dinamarca et al., 2017; Haslam et al., 2020; Niu et al., 2013). Nevertheless, these studies conducted by individual groups lack functional comparison among the six *PtDGATs*, and the TAG content achieved in *P. tricornutum* is not satisfactory. Also, the subcellular localization and in vitro activities of these DGATs, which will help understand their functional roles in TAG synthesis, have so far not been touched and remain to be disclosed. Moreover, *PtDGAT1*, the type I DGAT, has only been evaluated in yeast; its functional role and engineering potential in *P. tricornutum* are awaiting clarification.

In the present study, we confirmed the full-length cDNA and updated the gene model of *PtDGAT1*, comparatively evaluated the expression pattern of all six *P. tricornutum* DGAT genes under TAG induction conditions, compared their functional role both in vivo and in vitro, and unraveled the subcellular localization of functional DGATs. Our results demonstrated that *PtDGAT1*, likely localized at the chloroplast endoplasmic reticulum (ER), surpassed the other DGATs in synthesizing TAG. Moreover, overexpressing *PtDGAT1* allowed *P. tricornutum* to produce more than doubled TAG, which reached 57.0% of dry weight, much higher than that achieved in the previously engineered *P. tricornutum* (Cui et al., 2018; Dinamarca et al., 2017; Haslam et al., 2020; Niu et al., 2013; Zulu et al., 2017). We also evaluated the performance of manipulating DGAT gene for TAG enhancement by *P. tricornutum* and discussed their biotechnological implications.

## 2 MATERIALS AND METHODS

### 2.1 Algal culture conditions

*P. tricornutum* (CCMP2561), obtained from the culture collection of the Provasoli-Guillard National Center for Culture of Marine Phytoplankton, Bigelow Laboratory for Ocean Sciences, was maintained and cultured in F/2 medium containing 20 g/L sea salt. The strain was denoted as Pt1 with a fusiform morphotype (De Martino et al., 2007). Briefly, 10 ml of liquid F/2 medium was inoculated with cells from agar plates and the alga was grown aerobically in 50 ml flasks at 23°C for 6 days (hand shaking twice per day) illuminated with continuous light of 30 µE m⁻² s⁻¹ (cool-white fluorescent tube light, from the top). The algal cells were then inoculated at 10% (v/v) into 250 ml flasks for growth with orbital shaking at 150 rpm in an orbital shaker (Zhichu) and constant illumination of 30 µE m⁻² s⁻¹. Algal cells grown to late exponential phase were used as seeds for experiments.

For the treatment of nitrogen deprivation (ND), the seeds were harvested, washed twice with F/2 medium lacking nitrogen, and re-suspended in this medium. Cells re-suspended in F/2 medium (nitrogen replete, NR) were used as the control. Both had a starting cell number of 1.5 × 10⁶ ml⁻¹ and were grown for 4 days with orbital shaking at 150 rpm and constant illumination of 30 µE m⁻² s⁻¹. For the comparison between the
wild type (WT) and transgenic strains of *P. tricornutum*, cells were inoculated to a starting cell number of $5 \times 10^5$ ml$^{-1}$ and allowed to grow for 12 days with orbital shaking at 150 rpm and constant illumination of 30 $\mu$E m$^{-2}$ s$^{-1}$.

2.2 Cloning and in silico analysis of *P. tricornutum* DGATs

To obtain the full-length coding sequence of *PtDGAT1*, its untranslated regions were first determined by 5' and 3' rapid amplification of cDNA ends (RACE)-PCR using the SMARTer RACE 5'/3' Kit (TaKaRa). Then, primer pairs were used to amplify the full-length coding sequence, which was verified by sequencing and deposited into NCBI GenBank (accession number MN061782). The sequences of *PtDGAT2A* through *PtDGAT2D* and *PtDGAT3* were retrieved from NCBI GenBank (JX469835, JQ837823, JX469836, JX469837, and XM_002184438).

Sequence alignment of DGAT polypeptides from various organisms was conducted using ClustalX2.1 (http://www.clustal.org/clustal2/) and the phylogenetic tree was generated using MEGA6 (Tamura et al., 2013). Transmembrane helices of *PtDGAT* were predicted using TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/).

2.3 RNA isolation and quantitative real-time PCR

Total RNA extraction (from around $10^7$ algal cells) and removal of contaminated DNA were conducted by using the plant RNA extraction kit (TaKaRa). After the synthesis of cDNA, quantitative real-time PCR (qPCR) was conducted on a 7500 Fast Real-Time PCR System (Applied Biosystems) with SYBR® Premix Ex Taq™ II (TaKaRa), following our previously described procedures (Wei et al., 2017). Primer sequences used for qPCR are listed in Table S1. The mRNA expression level of *PtDGAT* genes was normalized using the actin gene as the internal control.

2.4 Functional complementation of *PtDGAT* in the TAG-deficient yeast

The six *PtDGAT* genes were PCR amplified using cDNA as template and cloned into the yeast expression vector pYES2-CT (Invitrogen), using the In Fusion Advantage PCR Cloning Kit (TaKaRa). PCR primers for the cloning are listed in Table S1. The recombinant plasmids, once confirmed by sequencing, were each introduced into the TAG-deficient quadruple mutant strain H1246 of *Saccharomyces cerevisiae* (Sandager et al., 2002). The empty vector (EV) pYES2-CT was used as the negative control. The yeast transformants, after verified by Colony PCR, were cultured in S/−Ura medium containing 2% (w/v) galactose to induce transgene expression. For the free fatty acid experiment, linoleic acid (C18:2), α-linolenic acid (C18:3n3), and eicosa-pentaenoic acid (C20:5n3) were each fed to yeast cultures upon galactose induction at a concentration of 100 µM, as described by Mao et al. (2019). After 2 days of cultivation, the yeast cultures were harvested for lipid analysis and fluorescent staining.

2.5 In vitro assay of *PtDGAT*

The induction of heterologous expression of *PtDGAT* genes in H1246 and the microsome preparation from these yeast strains containing *PtDGAT* were conducted according to our previously described procedures (Liu et al., 2017). The prepared microsomal fractions were each re-suspended in the storage buffer (50 mM Tris-HCl, pH 7.5, 10% glycerol) for subsequent in vitro assay.

The in vitro DGAT assay was performed in a 200 µl reaction system, which consists of 20 µg microsomal protein, 250 µM of both substrates acyl-CoA and DAG, and 10 mM MgCl$_2$ in potassium phosphate buffer (Liu et al., 2017). DAG (16:1/16:1) was used as the acyl acceptor, while 16:0-CoA, 16:1-CoA, and C20:5-CoA were used as the acyl donor; all were purchased from Larodan Fine Chemicals.

2.6 Overexpression of *PtDGAT* in *P. tricornutum*

The coding sequences of *PtDGAT1*, *PtDGAT2A* through *PtDGAT2D* and *PtDGAT3* were each subcloned into the *P. tricornutum* expression vector peGFP (Zhang & Hu, 2014), using the In Fusion Advantage PCR Cloning Kit (TaKaRa). PCR primers for the cloning are listed in Table S1. The transformation of *P. tricornutum* via electroporation followed our previously described protocols (Zhang & Hu, 2014). Putative transformants selected on F/2 solid medium containing 75 µg/ml zeocin were verified by genomic PCR. Then, qPCR was performed to determine the expression level of transgenes in the selected transformants. Two strains with the highest expression levels for each transgene were chosen for further experiments.

2.7 Fluorescent and confocal microscopy analyses

For the observation of neutral lipids in *P. tricornutum* and yeast cells, they were first stained with the fluorescence
BODIPY™ 505/515 (Invitrogen) with a 1 µg/ml working concentration for 10 min at room temperature, and then visualized under an Olympus BX51 Fluorescence Microscope (Olympus) with excitation at 488 nm and emission between 505 and 530 nm. For subcellular localization of PtDGATs, the corresponding transgenic algal strains were visualized under a Leica TCS SP8 laser scanning confocal microscope. GFP fluorescence was observed with excitation at 488 nm and emission between 505 and 530 nm. For subcellular localization of PtDGATs, the corresponding transgenic algal strains were visualized under a Leica TCS SP8 laser scanning confocal microscope. GFP fluorescence was observed with excitation at 488 nm and emission at 500–525 nm, and chlorophyll autofluorescence was observed with excitation at 488 nm and emission at 650–750 nm. To observe the nucleus of algal cells, they were stained with Hoechst 33342 (Invitrogen) at a concentration of 5 µM for 30 min at room temperature and then visualized with excitation at 405 nm and emission at 425–475 nm.

2.8 | Analytical methods

Cell counting using a hemocytometer under a light microscope and gravimetrical determination of dry weight for *P. tricornutum* followed our previously described procedures (Liu et al., 2019). The maximum quantum yield of PSII, $F_v/F_m$ or $F_m - F_o/F_m$, was measured on a pulse amplitude-modulated (PAM) fluorometry (Water-PAM, Walz), using 2 hr dark-adapted algal cell (Liu et al., 2019): $F_o$ was recorded under a weak light (<10 µmol m$^{-2}$ s$^{-1}$, peaking at 650 nm) and $F_m$ was under a saturating pulse (0.8 s) of red light (3,000 µmol m$^{-2}$ s$^{-1}$, peaking at 660 nm). Protein content in *P. tricornutum* cells was determined according to Meijer and Wijffels (1998), using lyophilized algal samples. Carbohydrate content in *P. tricornutum* cells was determined by the colorimetric method (Renaud et al., 1999), after hydrolysis of the lyophilized algal samples with 4 M H$_2$SO$_4$.

Lipids from yeast and *P. tricornutum* cells were extracted with chloroform–methanol (2:1, v/v) as previously described by Mao et al. (2019). For thin-layer chromatography (TLC) analysis, the extracted lipids were separated on a Silica gel 60 TLC plate (Merck) using a mixture of hexane/tert-butylmethyl ether/acetic acid (80/20/2, by vol) as the mobile phase (Liu et al., 2016). After separation, TAG on the TLC plate was visualized with iodine vapor, recovered, and transesterified with 1.5% sulfuric acid in methanol at 85°C for 2.5 hr. Total lipids from *P. tricornutum* and yeast cultures and the reaction products from in vitro DGAT assays were directly transesterified.

Fatty acid methyl esters prepared from the transesterification of lipids were analyzed by using an Agilent 7890 capillary gas chromatograph equipped with a 5975 C mass spectrometry detector and a HP-88 capillary column (60 m x 0.25 mm; Agilent Technologies), following our previously described procedures (Liu et al., 2016).

3 | RESULTS

3.1 | Bioinformatic analysis of PtDGATs

When blasted with the genome database of *P. tricornutum* (https://mycocosm.jgi.doe.gov/Phatr2/Phatr2.home.html), we noticed that, of the six *PtDGAT* genes, the *PtDGAT1* coding sequence from Guiheneuf et al. (2011) was incomplete. With assistance of 5’ and 3’ RACEs, we obtained the full-length cDNA of *PtDGAT1* gene, which consists of a 265-bp 5’ untranslated region, a 511-bp 3’ untranslated region, and a 2,271-bp coding sequence interrupted by three introns (Figure 1a). Sequence analysis suggested that the encoded protein differs from that reported by Guiheneuf et al. (2011): the latter has a truncation of 169 amino acid residues at the N-terminus leading to the loss of a pleckstrin homology (PH) domain, and a small truncation at the C-terminus without interference in the
membrane-bound O-acyltransferase domain (Figure 1a). The PH domain is commonly present in the proteins involved in cell signaling and helps protein binding to membranes (Lemmon & Ferguson, 2000). It has been reported that deletion of the PH domain resulted in severely attenuated ability of an algal DGAT1 in restoring TAG synthesis in the TAG-deficient yeast (Chen et al., 2015), indicative of an important contribution of the PH domain to the enzymatic activity of DGAT1. Interestingly, the PH domain is present in many algal DGAT1 proteins but not the counterparts from plants or yeast. TMHMM analysis suggested the presence of transmembrane domain in all six PtDGATs, with PtDGAT1 containing the largest number (Figure S1). To understand the evolution of PtDGATs, a cladogram was constructed using the DGAT homologs from algae, higher plants, fungi, and animals (Figure 1b). PtDGAT1, closely related to TpDGAT1 from the diatom Thalassiosira pseudonana, is clustered within the type I algal DGATs. PtDGAT2A through PtDGAT2D, distinct from PtDGAT1, are clustered within the type II algal DGATs. PtDGAT3, on the other hand, is clustered within the type III DGATs.

3.2  PtDGAT1 enables the TAG-deficient yeast to produce the highest TAG level

To validate the function of PtDGATs, their coding sequences were introduced into the TAG-deficient yeast strain H1246, with the EV pYES2-CT being the negative control. Obviously, the yeast cells carrying PtDGAT1, PtDGAT2B, or PtDGAT3 accumulated the TAG-filled lipid droplets (LDs, indicated by green fluorescence); by contrast, the yeast cells carrying PtDGAT2A, PtDGAT2C, or PtDGAT2D, similar to EV, failed to accumulate detectable LDs (Figure 2a), indicating the functional role of PtDGAT1, PtDGAT2B, or PtDGAT3 in TAG synthesis. This is further confirmed by the TLC separation analysis of lipids and GC–MS quantification of TAG: the yeast cells carrying EV, PtDGAT2A, PtDGAT2C, or PtDGAT2D synthesized only a trace amount of TAG with a very low TAG/TFA ratio (below 2%), while those expressing PtDGAT1, PtDGAT2B, or PtDGAT3 accumulated considerably higher amount of TAG (~4.3 μg OD600 cells) with the TAG/TFA ratio up to 43% (Figure 2b,c). Considering the greater TAG content and TAG/ratio, PtDGAT1 may have a stronger enzymatic activity than PtDGAT2B or PtDGAT3. The expression of the three functional PtDGATs also affected the relative abundance of fatty acids in TAG, leading to the increase of unsaturated fatty acids (C16:1 and C18:1) at the expense of saturated ones (C16:0 and C18:0; Figure 2d). This suggests that PtDGAT1, PtDGAT2B, or PtDGAT3 may all prefer C16:1 over C16:0 and C18:1 over C18:0 for TAG synthesis, at least in yeast cells.

It is worth noting that P. tricornutum synthesizes many PUFAs not present in yeast cells, such as C18:2, C18:3, and C20:5 (Abida et al., 2015; Ge et al., 2014). To see if the feeding of these PUFAs could affect TAG synthesis in yeast, they were supplemented in the culture medium at a concentration of 100 μM. H1246 cells carrying PtDGAT2A, PtDGAT2C, or PtDGAT2D, which failed to restore TAG synthesis (Figure 2a–c), showed no difference in TAG level between without (control) and with the feeding of PUFAs (Figure 2e), further suggesting that the three DGATs may have no activity in TAG synthesis. The feeding of C18:2 or C18:3 had no effect on the TAG accumulation in H1246 cells carrying PtDGAT1, PtDGAT2B, or PtDGAT3 either (Figure 2e), indicative of their poor activity in incorporating C18:2 or C18:3 into TAG, which was also indicated by the presence of only a trace amount of the fed PUFAs in TAG (Figure 2f). This is somewhat inconsistent with the results in a previous study where the expression of PtDGAT1 in yeast cells could incorporate C18:3 into TAG (Guiheneuf et al., 2011). Such a difference may be attributed to the fact that the latter used a truncated version of PtDGAT1 for expression and were supplemented with a much higher PUFA concentration (250 μM) than us. On the other hand, C20:5 could stimulate TAG accumulation slightly (Figure 2e) and was incorporated into TAG with a percentage of ~5.3% (Figure 2f), suggesting the activity of PtDGAT1, PtDGAT2B, and PtDGAT3 on C20:5.

3.3  PtDGAT1 has the highest in vitro activity in synthesizing TAG

According to the TAG profiles of P. tricornutum (Abida et al., 2015), the acyl acceptor DAG (16:1/16:1) and the acyl donors C16:0-CoA, C16:1-CoA, and C20:5-CoA were used to evaluate the in vitro activities of PtDGATs. Unlike PtDGAT2A, PtDGAT2C, or PtDGAT2D, PtDGAT1, PtDGAT2B, and PtDGAT3 showed obvious in vitro activities in utilizing the three tested acyl-CoAs for TAG synthesis (Table 1), consistent with the results of functional complementation experiments in yeast (Figure 2a–c). Clearly, regardless of acyl-CoAs used, PtDGAT1 had the highest in vitro activity (Table 1). Moreover, PtDGAT1 preferred 16:1-CoA over C16:0-CoA (Table 1), agreeing with the yeast experiments where PtDGAT1 expression led to increased C16:1 percentage and decreased C16:0 percentage (Figure 2d). The in vitro activities of PtDGAT1, PtDGAT2B, and PtDGAT3 on C20:5-CoA were also consistent with the fatty acid feeding experiments in yeast where heterologous expression of these three genes led to the incorporation of C20:5 into TAG (Table 1; Figure 2f).
3.4 PtDGAT1 has the highest transcript abundance and is most up-regulated among the six PtDGATs under lipid induction conditions

Nitrogen is an essential nutrient for both algal growth and lipid metabolism and ND has been demonstrated as an effective strategy to induce the accumulation of lipids particularly TAG in various algae (Abida et al., 2015; Klaitong et al., 2017; Liu et al., 2016; Mao et al., 2019; Zienkiewicz et al., 2017). Compared to under the NR condition, the growth of *P. tricornutum* was impaired under the ND condition, as suggested by the considerably lower cell density.
(Figure 3a). \( F_{v}/F_{m} \), the maximum quantum yield of PSII (Rohacek et al., 2008), also showed a decrease in response to ND (Figure 3b). While maintaining a relatively stable level of total fatty acids (TFA) under the NR condition, *P. tricornutum* showed a rapid increase in response to ND (Figure 3c). Similarly, TAG exhibited a drastic increase in response to ND and reached a much higher level than that under the NR condition (Figure 3d,e). The level of polar lipids, on the other hand, was considerably lower under the ND condition than that under the NR condition (Figure 3f), pointing to the contribution of recycle of polar lipids to TAG synthesis, in agreement with the results observed in many other algae (Liu et al., 2016, 2019; Wei et al., 2017).

TAG synthesis in the Kennedy pathway involves a set of enzymes, of which DGAT is believed to catalyze the rate-limiting committed step and thus plays a crucial role in TAG accumulation (Xu et al., 2018). It has been suggested that *P. tricornutum* harbors a total of six putative DGAT-encoding genes, namely, *PtDGAT1*, *PtDGAT2A*-*PtDGAT2D*, and *PtDGAT3* (Cui et al., 2013; Gong et al., 2013; Guiheneuf et al., 2011). Our interest in the role of *PtDGATs* in TAG synthesis necessitates the transcriptional profiling of these genes. Therefore, the transcriptional dynamics of *PtDGAT* genes during a 48 hr period under the NR and ND conditions were recorded by qPCR (Figure 4). Unlike the stable transcriptional expression under the NR condition, *PtDGAT1* was up-regulated under the ND condition in a time-dependent manner and reached ca. threefold increase of the transcript level at 48 hr, considerably higher than that under the NR condition. Similar to *PtDGAT1*, *PtDGAT2B* showed a gradual increase in its transcript level upon ND, which was substantially greater than that under the NR condition. *PtDGAT3* was also up-regulated by ND, but reached its maximum at 12 hr and then declined to the same level as that under the NR condition. *PtDGAT2A*, *PtDGAT2C*, and *PtDGAT2D*, on the other hand, showed little difference at their transcript levels between the NR and ND conditions. In this context, the up-regulation of *PtDGAT1*, *PtDGAT2B*, and *PtDGAT3* are likely involved in the ND-induced TAG synthesis.

**FIGURE 3** Growth and lipid profiles of *Phaeodactylum tricornutum* cells under NR and ND conditions. (a) Cell number. (b) \( F_{v}/F_{m} \). (c) TFA content. (d) TAG content. (e) Microscopic observation of algal cells stained by Bodipy. Green fluorescence indicates the Bodipy-bound TAG-filled lipid droplets. (f) Polar lipid content. Data in (a–d) and (f) are expressed as mean ± SD (n = 3). An asterisk indicates significant difference (Student’s *t* test, *p* < .01) between ND and NR.
Considering that PtDGAT1 has the highest transcript abundance and is most up-regulated, it may have a greatest contribution to stress-associated TAG production in *P. tricornutum*.

### 3.5 PtDGAT1 overexpression shows little effect on growth of *P. tricornutum*

To investigate the *in vivo* function of PtDGATs, the six genes were each introduced into *P. tricornutum* for overexpression. Over 10 transformants for each transgene were analyzed and two with the highest gene expression (ranging from 5- to 12-fold increase) were selected for further study (Figure 5a). Overall, the overexpression of PtDGATs in *P. tricornutum* exhibited slight effect on cell growth, which entered the stationary growth phase after 7 days of cultivation (Figure 5b). This is consistent with the phenomenon previously observed for algae overexpressing DGATs (Cui et al., 2018; Haslam et al., 2020; Klaitong et al., 2017; Niu et al., 2013; Wei et al., 2017). Nevertheless, some reports indicated that DGAT overexpression caused a significant decrease in algae growth (Dinamarca et al., 2017; Zienkiewicz et al., 2017; Zulu et al., 2017), pointing to the difference that may be associated with algal species, DGAT sources, promoters used for driving DGAT expression, culture conditions, etc.

### 3.6 PtDGAT1 overexpression causes more than doubled lipid production in *P. tricornutum*

Cell staining with Bodipy, a specific fluorescent dye binding to neutral lipids, indicated the accumulation of more lipids in the transgenic strains carrying PtDGAT1, PtDGAT2B, or PtDGAT3, as compared to the WT strain (Figure 5c). To confirm this, GC–MS quantification of lipids was conducted using samples from three different time points, namely day 4 (linear growth phase), day 7 (early stationary growth phase), and day 12 (late stationary growth phase). Albeit having little effect on TAG synthesis on day 4, overexpressing PtDGAT1, PtDGAT2B, and PtDGAT3 each enabled *P. tricornutum* to produce considerably greater amount of TAG on both day 7 and day 12; by contrast, overexpression of PtDGAT2A, PtDGAT2C, or PtDGAT2D had little impact on TAG accumulation (Figure 5d). Generally, the overexpression results in *P. tricornutum* agree with the functional
FIGURE 5 Characterization of transgenic strains of *Phaeodactylum tricornutum* overexpressing various PtDGAT genes. (a) Relative expression of the transgenes to WT, which was set as 1. Five-day cultures were used for analysis; (b) cell growth dynamics; (c) microscopic observation of the transgenic strains (day 12) stained by Bodipy. Green fluorescence indicates the Bodipy-bound TAG-filled lipid droplets. A representative cell was shown for each transgene; (d) TAG content; (e) TFA content; (f) TAG and TFA yields. WT strain was used as the control. Data in (a), (b) and (d)–(f) are expressed as mean ± SD (n = 3). An asterisk indicates significant difference (Student’s t test, p < .01) between PtDGAT-overexpressing strains and WT complementation experiments in yeast (Figure 4) and the in vitro assays (Table 1). It is worth noting that PtDGAT1-overexpressing strains synthesized the highest level of TAG, which reached 31.0% of dry weight (DW) on day 7 and was 2.3-fold higher than the WT did (Figure 5d). The enhanced TAG content caused by PtDGAT1 overexpression was still maintained in the late stationary growth phase (day 12), which reached 57.0% of DW and was 2.1-fold higher than that in the WT (Figure 5d). Similarly, overexpression of PtDGAT1, PtDGAT2A, and PtDGAT3 but not PtDGAT2A, PtDGAT2C, or PtDGAT2D promoted TFA accumulation, which was up to more than doubled of that achieved in the WT (Figure 5e). Accordingly, the PtDGAT1-overexpressing algal strains gave rise to the highest yields of both TAG and TFA, over twofold greater than the WT did (Figure 5f). Nevertheless, overexpressing PtDGAT1, PtDGAT2B, and PtDGAT3 showed little impact on the relative abundance of fatty acids in TAG or TFA (Figure S2). In agreement with our results, Cui et al. (2018) reported that overexpression of PtDGAT3 in *P. tricornutum* had only a marginal effect on the relative abundance of fatty acids in TAG. These appear to be inconsistent with the in vitro assays, in which PtDGATs exhibited distinct preference on acyl-CoAs (Table 1). One possible explanation is the difference in reaction environment between in vitro and in vivo: the enzyme and substrates (DAG and acyl-CoA) for the in vitro assays are unique (set artificially), whereas they are much more complex in *P. tricornutum* (in vivo).

3.7 PtDGAT1 is likely localized at the chloroplast ER for TAG synthesis in *P. tricornutum*

In yeast and higher plants, DGATs are membrane-bound proteins and reside at the ER for TAG synthesis (Chapman
& Ohlrogge, 2012; Sorger & Daum, 2002). In spite of several reports about the localization of algal DGATs (Liu et al., 2016; Manandhar-Shrestha & Hildebrand, 2015; Wei et al., 2017; Zienkiewicz et al., 2017), the subcellular compartmentalization of DGATs at the ER remains ambiguous in algae and needs to be fully explored. To investigate the subcellular localization of PtDGAT1, PtDGAT2B, and PtDGAT3, the three functional DGATs, we fused their coding sequences each upstream the eGFP and had them introduced into *P. tricornutum* individually. The eGFP alone was used as the control, in which the GFP signal (green) was present in the cytosol, distinct from the plastid autofluorescence (red; Figure 6a). By contrast, in the algal cells expressing *PtDGAT1-GFP*, the green signal formed irregular layers and surrounded the plastid (red) and nucleus (blue, stained with the nucleus specific dye Hoechst 33342; Figure 6a,b). It is believed that *P. tricornutum* plastid evolves via secondary endosymbiosis and consists of four membranes (Hempel et al., 2009; Stork et al., 2013). The outermost membrane fuses with the ER and is referred to as the chloroplast ER (cER), which is continuous with the outer membrane of the nuclear envelope (Gibbs, 1979). Obviously, the localization pattern of PtDGAT1 resembles hDer1-2, a cER-localized protein confirmed by GFP fusion experiments (Hempel et al., 2009), suggesting that PtDGAT1 resides at the cER in *P. tricornutum*. PtDGAT2B and PtDGAT3 showed a similar localization pattern (Figure 6a) and thus should also be localized at the cER. It is likely in *P. tricornutum* that PtDGATs reside at the cER, which is in contact with both plastid and ER, and readily access DAG from both organelles for TAG synthesis, similar to NoDGAT1A in *Nannochloropsis oceanica* (Wei et al., 2017).

### 3.8 | PtDGAT1 overexpression diverts more carbon flux to lipid production in *P. tricornutum*

To assess the effect of *PtDGAT* overexpression on macromolecules other than lipids, for example, protein and carbohydrate, *PtDGAT1*-overexpressing strains and WT were comparatively analyzed. Obviously, as the culture time extended, protein content showed a severe decrease regardless of algal strains; by contrast, carbohydrate content increased (Table 2). It is worth noting that, compared to WT, *PtDGAT1*-overexpressing strains had significantly lower (Student’s *t* test, *p* < .05) contents of protein and carbohydrate, particularly carbohydrate on late culture days (e.g., day 12; Table 2), while producing a more than doubled amount of lipids (Figure 5). These results indicate that overexpression of *PtDGAT1* shifts carbon flux from protein and carbohydrate, probably more from the latter, to lipids and thereby promoting lipid production in *P. tricornutum*.
TABLE 2 The effect of PtDGAT1 overexpression on protein and carbohydrate levels in Phaeodactylum tricornutum cells

| Culture time | WT     | PtDGAT1-OE11 | PtDGAT1-OE22 |
|--------------|--------|--------------|--------------|
| Day 4        | 41.2 ± 3.1 | 43.5 ± 2.1*  | 37.4 ± 1.4   |
| Day 7        | 32.5 ± 1.4 | 26.7 ± 1.9*  | 27.8 ± 1.6*  |
| Day 12       | 17.5 ± 0.9 | 12.5 ± 0.5*  | 11.3 ± 0.7*  |

Protein content (% of DW)

| Carbohydrate content (% of DW) |
|-------------------------------|
| Day 4 | 15.8 ± 0.5 | 18.2 ± 1.6* | 14.4 ± 0.8 |
| Day 7 | 24.6 ± 1.5 | 18.1 ± 0.8* | 16.3 ± 1.1* |
| Day 12| 34.4 ± 1.6 | 21.3 ± 0.9* | 19.5 ± 1.8* |

Note: Data are expressed as mean ± SD (n = 3). An asterisk indicates significant difference (Student’s t test, p < .05) as compared to WT.

Abbreviations: DW, dry weight; WT, wild type.

4 | DISCUSSION

Similar to land plants, algae harbor two distinctive pathways for TAG synthesis, that is, the acyl-CoA-dependent Kennedy pathway and the acyl-CoA-independent pathway mediated by phospholipid:diacylglycerol acyltransferase (PDAT; Xu et al., 2018). In algae such as Chlamydomonas reinhardtii, while PDAT pathway contributes to basal TAG synthesis under favorable growth conditions and early stage of stress conditions (Yoon et al., 2012), DGAT in the Kennedy pathway is believed to play pivotal roles in stress-induced TAG synthesis (Liu et al., 2016). Unlike land plants that typically contain a low dose of DGAT copies (e.g., one type I and one type II in Arabidopsis; Xu et al., 2018), algae have a high dose of copies particularly for the type II DGAT, for example, one type I and five type II in C. reinhardtii (Liu et al., 2016), two type I and eight type II in Chromochloris zofingiensis (Mao et al., 2019), two type I and eleven type II in N. oceanica (Li et al., 2014), and one type I (referred to as PtDGAT1) and four type II (referred to as PtDGAT2A-2D) in P. tricornutum (Gong et al., 2013; Guiheneuf et al., 2011). Moreover, P. tricornutum has an additional DGAT, designated as PtDGAT3, which shows low similarity to the type I and type II DGATs (Cui et al., 2013). The presence of high DGAT gene copies may point to a more complex TAG synthesis network in algae than in land plants. We cloned the full-length coding sequence of PtDGAT1 gene (Figure 1) and dissected the transcriptional expression patterns of the six PtDGAT genes upon ND, a condition well studied for inducing TAG synthesis (Figure 4). As PtDGAT1, PtDGAT2B, and PtDGAT3 genes were up-regulated when lipid accumulation was induced (Figures 3 and 4), they may be of importance to TAG synthesis in P. tricornutum and are worth functional validation to screen best ones for subsequent trait improvements.

Functional complementation in a TAG-deficient yeast strain, for example, H1246, represents a fast and convenient way to screen DGATs’ function. Our functional assays in H1246 demonstrated that three out of the six of PtDGATs, namely, PtDGAT1, PtDGAT2B, and PtDGAT3, were able to restore TAG synthesis (Figure 2a–c). In addition to agreeing with the functional complementation results performed by previous studies (Cui et al., 2013; Gong et al., 2013; Guiheneuf et al., 2011), our data revealed that PtDGAT1 was the best one in synthesizing TAG in yeast (Figure 2b). PtDGAT2A, PtDGAT2C, and PtDGAT2D, albeit predicted to be DGAT family, were not able to restore TAG synthesis in yeast (Figure 2b). This seems to be unexceptional, because several DGATs from such algae as C. reinhardtii (Hun et al., 2013; Liu et al., 2016), C. zofingiensis (Mao et al., 2019; Xu et al., 2019), and N. oceanica (Wei et al., 2017; Xin et al., 2017) failed to enable H1246 to produce detectable levels of TAG. The functional failure of certain DGATs in yeast may be due to the fact that yeast differs from algae in the fatty acid composition and lacks PUFAs that are present in algae, for example, C20:5 in P. tricornutum, and may be the substrate of these DGATs. However, neither our free fatty acid feeding experiments with C18:2, C18:3, and C20:5 nor the in vitro assay using C20:5-CoA as the acyl donor supported the DGAT functional of PtDGAT2A, PtDGAT2C, and PtDGAT2D (Figure 2; Table 1).

It is also possible that these PtDGATs may not be properly expressed, processed, or need certain yeast-lacking cofactors and thus cannot catalyze TAG synthesis in yeast. According to Gruber et al. (2015), PtDGAT2D is predicted to possess a plastid transit peptide and be plastid-localized with high confidence. Probably, the plastid transit peptide cannot be properly processed to form a mature protein in yeast leading to the functional failure in TAG assembly. To avoid these issues, we introduced each of the six PtDGAT genes into P. tricornutum itself to evaluate their function in vivo. Consistent with the heterologous expression experiments in yeast (Figure 2) and in vitro enzymatic assay (Table 1), overexpression experiments in P. tricornutum demonstrated that PtDGAT1, PtDGAT2B, and PtDGAT3, but not PtDGAT2A, PtDGAT2C, or PtDGAT2D, enabled the alga to produce more lipids including TAG (Figure 5). Taken these in vivo and in vitro results together, PtDGAT2A, PtDGAT2C, and PtDGAT2D may not function in synthesizing TAG. Probably, the duplication events during evolution introduce variations to essential positions thus causing the null function of these DGATs. In support of our results, Haslam et al. (2020) reported that overexpression of PtDGAT2B rather than PtDGAT2A promoted TAG accumulation in P. tricornutum. Nevertheless, we cannot rule out that PtDGAT2A, PtDGAT2C, and PtDGAT2D do function but their activities are too low to be observed even overexpressed or they were overexpressed at the mRNA level but not at the protein level in P. tricornutum.
On the other hand, considering that PtDGAT1, PtDGAT2B, and PtDGAT3 have the same subcellular localization at cER (Figure 6) and are all functional on certain acyl-CoAs (Table 1), they may have overlapping functions in TAG biosynthesis in *P. tricornutum*. Lipid content is a key parameter to evaluate the potential of algae for lipid production. Algae with high lipid content are desired, which can be obtained via screening naturally occurring strains and/or manipulating lipid biosynthetic pathways in certain strains of interest (Hu et al., 2008). *DGAT* is a promising gene target and has been overexpressed to manipulate TAG biosynthetic pathway for enhanced TAG production in several algae including *C. reinhardtii*, *Scenedesmus obliquus*, *Neochloris oleoabundans*, *N. oceanica*, *T. pseudonana*, and *P. tricornutum*, which are summarized in Table 3. It is worth noting that *DGAT* overexpression does not always guarantee the enhancement of TAG in algae. For example, efforts to overexpress the endogenous *DGTT1*, *DGTT2*, and *DGTT3* individually in *C. reinhardtii* failed to result in appreciable difference in TAG content; by contrast, overexpressing the low-abundance endogenous *DGTT4* or the heterologous *DGAT* promoted TAG accumulation (Ahmad et al., 2015; Iwai et al., 2014). It is possible that in *C. reinhardtii* certain regulatory loops or negative feedback inhibition occur for the overexpression of high-abundance endogenous *DGAT* genes (Liu et al., 2016). Nevertheless, *C. reinhardtii* accumulates only a moderate level of lipids and is considered as a model alga for studying lipid metabolism but generally not an oleaginous strain for oil production (Hu et al., 2008). Recently, *N. oceanica* has been emerging as an oleaginous alga for both fundamental study and TAG production (Li et al., 2014). Of the 13 *N. oceanica* *DGAT*-encoding genes, four were able to restore TAG synthesis in the TAG-deficient yeast and overexpressing each of them promoted TAG synthesis in *N. oceanica*, yet the increase in TAG content remained below 50% (Wei et al., 2017; Xin et al., 2017; Zienkiewicz et al., 2017). Similar to *N. oceanica*, *P. tricornutum* is a marine alga rich in oils (Abida et al., 2015; Ge et al., 2014). In our study, the overexpression of *PtDGAT1* allowed *P. tricornutum* to produce 110% more TAG, which reached 57% of DW and was considerably higher than that achieved in previously reported *DGAT*-overexpressing *P. tricornutum* and other algae (Table 3). According to Heydarizadeh et al. (2017), the light intensity (30 µE m⁻² s⁻¹) used in our study may

**TABLE 3** Lipid production by engineered *Phaeodactylum tricornutum* in comparison with previously reported *DGAT*-overexpressing algae

| Transgenic alga | Gene source | TAG | Total lipids |
|-----------------|-------------|-----|--------------|
|                 |             | Content (%) of DW | Fold change | Content (%) of DW | Fold change |
| *C. reinhardtii* | *DGTT4* from *C. reinhardtii* | — | 2.5 | — | — |
| *C. reinhardtii* | *DGAT2* from *Brassica napus* | — | — | 18.8¹ | 1.5 |
| *Scenedesmus obliquus* | *DGTT1* from *C. reinhardtii* | — | — | 18.1¹ | 1.8 |
| *Neochloris oleoabundans* | *DGAT2* from *N. oleoabundans* | 46.1c | 3.2 | 74.5a | 2.3 |
| *N. oceanica* | *DGAT1* from *N. oceanica* | 11.9c | 1.5 | 18.9b | 1.2 |
| *N. oceanica* | *DGAT2* from *N. oceanica* | 31.0c | 1.4 | 42.2b | 1.3 |
| *Thalassiosira pseudonana* | *DGAT2* from *T. pseudonana* | — | 1.95 | — | — |
| *P. tricornutum* | *DGAT2* from *P. tricornutum* | — | — | 37.5b | 1.4 |
| *P. tricornutum* | *DGAT2* from *P. tricornutum* | — | 1.8 | — | 2.0 |
| *P. tricornutum* | *DGAT1* from *S. cerevisiae* | 13.3c | 2.0 | 17.8b | 2.0 |
| *P. tricornutum* | *DGAT3* from *P. tricornutum* | 22.8c | 1.3 | 36.2b | 1.1 |
| *P. tricornutum* | *DGAT2* from *P. tricornutum* | 15.4d | 1.4 | — | — |
| *P. tricornutum* | *DGAT1* from *P. tricornutum* | 57.0c | 2.1 | 73.3b | 2.3 |

*Note:* Fold change, the fold change of TAG or total lipid content compared to WT or empty vector control. —, not available. ¹Determined by gravimetric analysis. ²Expressed as total fatty acids determined by GC-MS. ³Determined by GC-MS. ⁴Determined by ESI-MS/MS.
be weak for supporting fast growth of *P. tricornutum* and impact TAG accumulation in WT and transgenic lines. We increased the light intensity from 30 to 100 µE m\(^{-2}\) s\(^{-1}\) and observed faster algal growth and TAG accumulation (Figure S3). Nevertheless, there was little difference in growth between WT and the *PtDGAT1*-overexpressing line (Figure S3). Furthermore, similar to under the light intensity of 30 µE m\(^{-2}\) s\(^{-1}\), *PtDGAT1* overexpression gave rise to more than doubled TAG content as compared with WT under the light intensity of 100 µE m\(^{-2}\) s\(^{-1}\) (Figure S3), suggesting that the great increase of TAG caused by *PtDGAT1* overexpression is maintained in *P. tricornutum* regardless of the light intensities (or growth conditions). The hyper-accumulation of TAG needs input of sufficient acetyl-CoA, the precursor of de novo fatty acid synthesis, which can be converted from pyruvate. Pyruvate, the intermediate involved in multiple biological pathways, can also enter TCA cycle to form 2-oxoglutarate for amino acid synthesis, or enter gluconeogenesis to form glucose-6-phosphate for subsequent synthesis of the major storage carbohydrate chrysolaminarin. Likely, *PtDGAT1* overexpression provides a potent pulling power for enhanced TAG synthesis, which diverts carbon flux away from carbohydrate and protein to lipid metabolism, leading to the decrease of carbohydrate and protein contents (Table 2).

Combined, our screening of six *PtDGAT* genes via both in vivo and in vitro assays strongly support that *PtDGAT1* surpasses the other DGATs for TAG synthesis. Overexpression of *PtDGAT1* enables *P. tricornutum* to produce more than doubled and a record-high level of TAG, yet without compromising biomass production. *PtDGAT1*, residing at cER for easily accessing DAGs from both plastid and ER, likely provides a strong pulling power to divert carbon from carbohydrates and protein to lipids. We demonstrate, using a marine alga as the cell factory, that screening proper engineering targets and manipulating a single gene can pull carbon flux to lipid production for TAG hyper-accumulation, and the *PtDGAT1*-overexpressing *P. tricornutum* may serve as a promising feedstock for oil production. In addition to DGAT, there have been many reports about the manipulation of other genes for improved TAG production in *P. tricornutum*, such as genes involved in lipid metabolism (Balamurugan et al., 2017; Barka et al., 2016; Hamilton et al., 2014; Hao et al., 2018; Li et al., 2018; Niu et al., 2016; Wang et al., 2020), NADPH generation (Xue et al., 2015, 2017), carbon precursor production (Daboussi et al., 2014), and transcription factor (Kumar Sharma et al., 2019). The manipulation of multiple genes that provides the combination of pulling, pushing, and protection may have the potential to achieve even higher lipid level and production, which remains yet to be fully explored in the future.

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**DATA AVAILABILITY STATEMENT**

The data that support the findings of this study are available in the Supporting Information of this article.

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