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Accumulation of Lipid Production in *Chlorella minutissima* by Triacylglycerol Biosynthesis-Related Genes Cloned from *Saccharomyces cerevisiae* and *Yarrowia lipolytica*

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Discovery of an alternative fuel is now an urgent matter because of the impending issue of oil depletion. Lipids synthesized in algal cells called triacylglycerols (TAGs) are thought to be of the most value as a potential biofuel source because they can use transesterification to manufacture biodiesel. Biodiesel is deemed as a good solution to overcoming the problem of oil depletion since it is capable of providing good performance similar to that of petroleum. Expression of several genomic sequences, including glycerol-3-phosphate dehydrogenase, glycerol-3-phosphate acyltransferase, lysophosphatidic acid acyltransferase, phosphatidic acid phosphatase, diacylglycerol acyltransferase, and phospholipid:diacylglycerol acyltransferase, can be useful for manipulating metabolic pathways for biofuel production. In this study, we found this approach indeed increased the storage lipid content of *C. minutissima* UTEX 2219 up to 2-fold over that of wild type. Thus, we conclude this approach can be used with the biodiesel production platform of *C. minutissima* UTEX 2219 for high lipid production that will, in turn, enhance productivity.

Keywords: microalgae, biodiesel, glycerol-3-phosphate dehydrogenase, glycerol-3-phosphate acyltransferase, lysophosphatidic acid acyltransferase, phosphatidic acid phosphatase, diacylglycerol acyltransferase, diacylglycerol acyltransferase

Introduction

Because it has been projected that the global petroleum reserve in the known oil field reservoir will be depleted in the next 50 to 60 years, urgent attention has been paid recently to searching for an alternative fuel source. Biodiesel is deemed as one solution to this problem, as it can be made from a variety of feedstocks (i.e., vegetable oil, animal fat, and microalgae). Among the range of lipids synthesized in algal cells, triacylglycerols (TAGs) are those of the most value as a biofuel because they can undergo transesterification during biodiesel manufacturing. Microalgae are an extremely diverse group of organisms that are typically found in both freshwater and marine systems. It has been estimated that about 200,000–800,000 microalgae species exist, of which about 35,000 species have been described previously (Cheng and Ogden, 2011). Lipids in microalgae cells have roles both as energy storage molecules and in the formation of biological membranes (Kosa and Ragauskas, 2011; Stephenson *et al.*, 2011). The lipid synthesis pathway of TAG in cells is comprised of three major steps (Bell, 1980; Coleman and Lee, 2004; Rajakumari *et al.*, 2008; Radakovits *et al.*, 2010) (1) carboxylation of acetyl-coenzyme A (CoA) to form malonyl-CoA, the committing step of fatty acid biosynthesis in the plastid; (2) acyl chain elongation in the plastid and cytosol; and (3) TAG formation in the endoplasmic reticulum (ER). However, when a given substrate is limiting-typically nitrogen-TAGs channel carbon sources into lipid formation-balanced nutrient conditions. Promising advances in metabolic engineering allow for not only the increased production of endogenous carbon storage compounds,

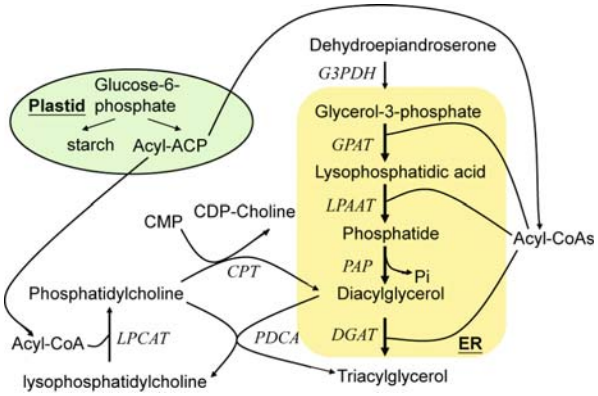

Fig. 1. Metabolic pathway of TAG formation and the Kennedy pathway in eukaryotes. The Kennedy pathway is shown by a broad arrow. CMP, cytidine 5′-monophosphate; CDP-choline, cytidine 5′-diphosphate-choline; CPT, choline phosphotransferase; LPC, lysophosphatidyl choline; LPCAT, lysophosphatidylcholine acyltransferase; DHAE, dehydroepiandrosterone; G3PDH, glycerol-3-phosphate dehydrogenase; G3P, glycerol-3-phosphate; GPAT, glycerol-3-phosphate acyltransferase; LA, lysophosphatidic acid; LPAAT, lysophosphatidic acid acyltransferase; PA, phosphatidic acid; PAP, phosphatidic acid phosphatase; DAG, diacylglycerol; PDAT, phospholipid: diacylglycerol acyltransferase; DGAT, diacylglycerol acyltransferase; and ER, endoplasmic reticulum.

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Table 1. Amino acid similarity and GC contents of genes from *S. cerevisiae* and *Y. lipolytica* related to lipid accumulation

Protein	Gene name	Similarity (%)	GC content (%)	Gene source
G3PDH	*gpd*1	11	44	*S. cerevisiae* INVSC1
	*gpd*2	13	45	*S. cerevisiae* INVSC1
	*gut*2	5	49	*S. cerevisiae* INVSC1
GPAT	*gat*1	–	40	*S. cerevisiae* INVSC1
	*gat*2	–	43	*S. cerevisiae* INVSC1
	YALI0C00209g	–	54	*Y. lipolytica* P01g
LPAAT	*slc*	13	42	*S. cerevisiae* INVSC1
PAP	*dpp*	9	40	*S. cerevisiae* INVSC1
	lpp	13	40	*S. cerevisiae* INVSC1
DGAT	*dga*	13	38	*S. cerevisiae* INVSC1
	YALI0D07986g	5	53	*Y. lipolytica* P01g
	YALI0E32769g	11	53	*Y. lipolytica* P01g
PDAT	*lro*	4	42	*S. cerevisiae* INVSC1

GPAT, glycerol-3-phosphate acyltransferase (*gat1*, *gat2*, and *YALI0C0029g*); G3PDH, glycerol-3-phosphate dehydrogenase (*gpd1*, *gpd2*, and *gut2*); LPAAT, lysophosphatidic acid acyltransferase (*slc*); PAP, phosphatidic acid phosphatase (*dpp* and *lpp*); DGAT, diacylglycerol acyltransferase (*dga*, *YALI0D07986g*, and *YALI0E32769g*).

such as TAGs and starch, but also the direct production, and perhaps secretion, of designer hydrocarbons that may be used directly as fuels.

One kind of microalgae, *Chlorella*, is the most popular in several applications, such as in biofuel, health food, cosmetics, and bioremediation. While autotrophic growth of *Chlorella vulgaris* has been shown to provide high cellular lipid content (38%), the lipid productivity (54 mg/L/day) of this organism was attained using 1% (w/v) glucose under heterotrophic growth conditions (Liang *et al.*, 2009). In addition, the lipid content of *Chlorella sorokiniana* reached 51% during mixotrophy, with expression of *accD* (heteromeric acetyl-CoA carboxylase beta subunit), *acc1* (homomeric acetyl-CoA carboxylase), and *rbcL* (ribulose 1, 5-bisphosphate carboxylase/oxygenase large subunit) under mixotrophic conditions (Wan *et al.*, 2011). In this study, we showed that the modulation of several enzymes (Fig. 1), including glycerol-3-phosphate dehydrogenase (G3PDH), glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidic acid acyltransferase (LPAAT), phosphatidic acid phosphatase (PAP), diacylglycerol acyltransferase (DGAT), or phospholipid:diacylglycerol acyltransferase (PDAT), in the Kennedy pathway (CDP-ethanolamine and CDP choline branches, respectively) effectively increased the storage lipid content up to 2-fold more than that of the wild type during 14 days of cultivation.

Materials and Methods

Materials and cultivation conditions

The yeast strain *Saccharomyces cerevisiae* INVSC1 was provided by Professor Chien-Chia Wang of National Central University. The yeast strain *Yarrowia lipolytica* Po1g was purchased from Yeastern Biotech Co. (Taiwan) and cultured with a rich medium (YPD) at 28°C. The algal strain *C. minutissima* UTEX 2219 was kindly provided by Professor Hung-Non Chou of Taiwan University. The MES-volvox medium included a thiamine (which plays a pivotal role in intermediary carbon metabolism), biotin (a cofactor for several essential carboxylase enzymes), and cobalamin (a cofactor for enzymes that catalyze either rearrangement-reduction reactions or methyl-transfer reactions) vitamin solution, and was used for cultivation of *C. minutissima* UTEX 2219 at 25°C in continuous light with stirring (250 rpm). Culture densities were monitored using a spectrophotometer at 640 nm (y=0.8982x-0.0189).

Construction of genes in the Kennedy pathway

The G3PDH (*gpd1*, *gpd2*, *gut*2), GPAT (*gat1*, *gat2*, *YALI0C00209g*), LPAAT (*slc*), PAP (*dpp*, *lpp*), DGAT (*dga*), and PDAT (*lro*) genes were derived from *S. cerevisiae*, and DGAT (*YALI0E32769p* and *YALI0E07986p*) were derived from *Y. lipolytica*, because they were thought to be involved in the TAG accumulation process (Table 1). The resulting chimeric genes were re-isolated as *Bgl*II/*Eco*RI fragments and cloned into the binary vector pAlgae (pBI121 binary vector and pBluescript II SK$^+$ plasmid combination in our

Table 2. Triacylglycerol accumulation by bacterial strain

Strain	Relevant genotype or sequences	Source
E. coli DH5α	*end*A1 *rec*A1 *rel*A1 *gyr*A96 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 Δ(*lac*ZYA-*arg*F)U169 Φ80 Δ(*lac*Z)M15 F$^-$	ECOS^{TM}101
Algae-gpd1	*p*Algae, Gen^R	This study
Algae-gpd2	*p*Algae, Gen^R	This study
Algae-gut2	*p*Algae, Gen^R	This study
Algae-gat1	*p*Algae, Gen^R	This study
Algae-gat2	*p*Algae, Gen^R	This study
Algae-yali0C00209g	*p*Algae, Gen^R	This study
Algae-slc	*p*Algae, Gen^R	This study
Algae-dpp	*p*Algae, Gen^R	This study
Algae-lpp	*p*Algae, Gen^R	This study
Algae-dga	*p*Algae, Gen^R	This study
Algae-yali0D07986g	*p*Algae, Gen^R	This study
Algae-yali-0E32769g	*p*Algae, Gen^R	This study
Algae-lro	*p*Algae, Gen^R	This study
Algae-quintuple	*p*Algae, Gen^R	This study

GPAT, glycerol-3-phosphate acyltransferase (*gat1*, *gat2*, and *YALI0C0029g*); G3PDH, glycerol-3-phosphate dehydrogenase (*gpd1*, *gpd2*, and *gut2*); LPAAT, lysophosphatidic acid acyltransferase (*slc*); PAP, phosphatidic acid phosphatase (*dpp* and *lpp*); DGAT, diacylglycerol acyltransferase (*dga*, *YALI0D07986g*, and *YALI0E32769g*).

Table 3. Plasmid information in transgenic *C. minutissima* UTEX2219

Plasmid	Relevant genotype or sequences	Source
pBI121	14.758-kb cloning or sequencing vector; Kan^R	Clontech
pBluescript II SK^+	2.9-kb cloning or sequencing vector; Am^R	Stratagene
pAlgae	pBI121 binary vector and pBluescript II SK^+ plasmid combination	This study
pGpd1	*S. cerevisiae* INVSC1; Kan^R	This study
pGpd2	*S. cerevisiae* INVSC1; Kan^R	This study
pGut2	*S. cerevisiae* INVSC1; Kan^R	This study
pGat1	*S. cerevisiae* INVSC1; Kan^R	This study
pGat2	*S. cerevisiae* INVSC1; Kan^R	This study
pYali0C00209g	*Y. lipolytica* P01g; Kan^R	This study
pSlc	*S. cerevisiae* INVSC1; Kan^R	This study
pDpp	*S. cerevisiae* INVSC1; Kan^R	This study
pLpp	*S. cerevisiae* INVSC1; Kan^R	This study
pDga	*S. cerevisiae* INVSC1; Kan^R	This study
pYali0D09786g	*Y. lipolytica* P01g; Kan^R	This study
pYali0E32769g	*Y. lipolytica* P01g; Kan^R	This study
pLro	*S. cerevisiae* INVSC1; Kan^R	This study

GPAT, glycerol-3-phosphate acyltransferase (*gat1*, *gat2*, and *YALI0C0029g*); G3PDH, glycerol-3-phosphate dehydrogenase (*gpd1*, *gpd2*, and *gut2*); LPAAT, lysophosphatidic acid acyltransferase (*slc*); PAP, phosphatidic acid phosphatase (*dpp* and *lpp*); DGAT, diacylglycerol acyltransferase (*dga*, *YALI0D07986g*, and *YALI0E32769g*).

study (Table 2) with two different promoters: CaMV (cauliflower mosaic virus) 35s and RbcS (ribulose-1,5-bisphosphate carboxylase small subunit). The left and right borders of pAlgae were homologous fragments of *C. minutissima* UTEX 2219, allowing exchange into the *Chlorella* chromosome. Kanamycin and geneticin (G418) were the selection markers in *Escherichia coli* and *C. minutissima* UTEX 2219, respectively. The detail information of single and quintuple-gene construct was designed and shown in Table 3, Figs. 2A, and 4A.

Electroporation

A suitable cell density (10^6 cells/ml) was harvested (3,000 rpm) at room temperature and washed once with culture medium. Eighty microliters of the resuspended cells (1 per 100 volume of sterilized distilled water) was mixed with approximately 5 μg of plasmid, and the mixture was transferred to a sterile electroporation cuvette with aluminum electrodes spaced 0.2 cm apart. Electrotransformation (field strength of 2000 V/cm) was carried out using a Gene Pulser (Bio-Rad) and electroporated cells incubated on ice for 5

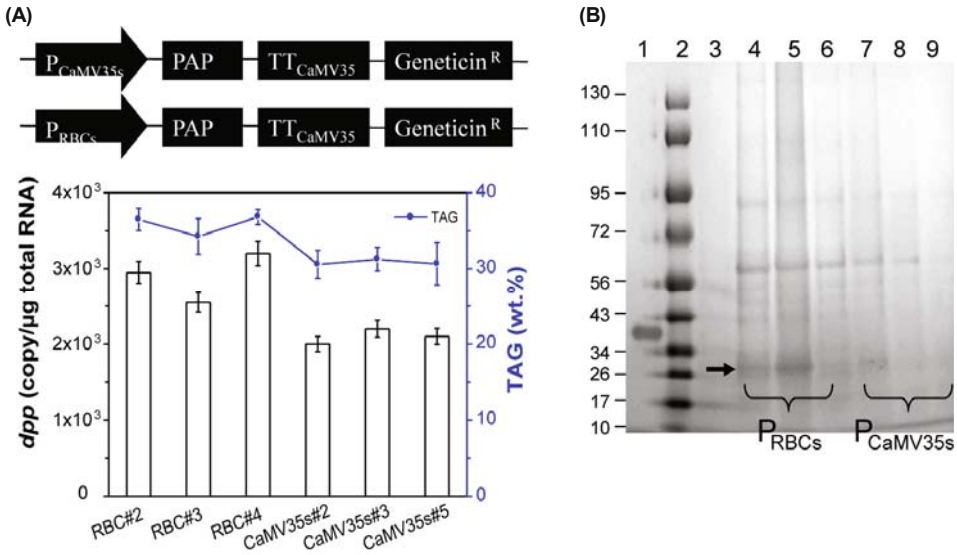

Fig. 2. Real-time and protein expression analyses of the TAG synthesis genes using 2 different promoters in transgenic *C. minutissima*. (A) Real-time expression analyses of TAG synthesis genes. Promoter of cauliflower mosaic virus 35s ($P_{CaMV35s}$), promoter of ribulose-1,5-bisphosphate carboxylase small subunit$_{(PRBCs)}$, phosphatidic acid phosphatase (PAP), terminator of cauliflower mos$_{aic}$ virus 35s ($TT_{CaMV35s}$), and geneticin (G418). (B) Western blotting of *dpp* genes. Lanes: 1, $His_{s\text{-}tagged}$ protein; 2, marker; 3, *C. minutissima* wild type; 4, *dpp*-rbc#2; 5, *dpp*-rbc#3; 6, *dpp*-rbc#4; 7, *dpp*-CaMV35s#2; 8, *dpp*-CaMV35s#3; 9, *dpp*-CaMV35s#5.

min. The cells (in a 5-ml aliquot of culture medium) were grown in the dark at room temperature for 24 h.

Reverse transcription and Real-time PCR

The culture (100 ml) was transferred to centrifuge tube and centrifuged at 3,000 rpm for 5 min. The isolated algal cells were disrupted in liquid nitrogen in a ceramic mortar, and the RNA was isolated using a MasterPure Plant RNA purification kit (Epicentre, USA) according to the manufacturer's instructions. cDNA synthesis from each nucleic acid by using QuantiTect Reverse Transcription kit (QIAGEN, USA). cDNA synthesis with integrated genomic DNA removal by gDNA Wipeout Buffer. Incubate for 2 min at 42°C and then place immediately on ice. Prepare the reverse-transcription master mix (including quantiscript reverse transcriptase, quantiscript RT Buffer, and a unique RT Primer Mix) on ice and further incubates for 15 min at 42°C. After transcription, continues incubation for 3 min at 95°C to inactivate quantiscript reverse transcriptase. Add an aliquot of each finished reverse-transcription reaction to real-time PCR mix. To facilitate real-time PCR analysis of the selected genes under the same reaction conditions, primers were first designed by Primer3 input (version 0.4.0), as shown in Table 4. The 18S rRNA gene was selected as a housekeeping gene. The cycle parameters of real-time quantitative PCR consisted of 1 cycle of 3 min at 95°C, and 40 cycles of 10 sec at 95°C, followed by 30 sec at 55°C. Data were collected at the end of each extension step. The relative amounts of gene expression between the treatment groups were analyzed, where *Ct* was the cycle number at which the fluorescent signal increased statistically above the background.

Enzyme activity assays

Enzymes assays were conducted in triplicate with a background control in which the protein was inactivated at 100°C for 10 min. The Coenzyme A assay (Biovision, USA) was used for measuring the Coenzyme A producing of the enzyme samples of GPAT, LPAAT, and DGAT. Coenzyme A standard curve: Y_{570nm}=0.0151X (nmol)+0.0496 (R^2=0.9999). The phosphate colorimetric assay (Biovision) measures the phosphate producing of the enzyme samples of PAP. The phosphate standard curve: Y_{650nm}=0.3534X (nmol)+0.1588 (R^2=0.9995). The NADH colorimetric quantification (Biovision) measures the NADH producing of the enzyme samples of G3PDH. The NADH standard curve: Y_{450nm}=0.0139X (nmol)+0.0237 (R2=0.9996).

Western blot analysis

A His-tag–purified (Strategy, USA) protein (50 μg) from the algal extract was homogenized, separated by a 4–20% (gradient) precast acrylamide gel of SDS-PAGE (Expedeon, UK) under reducing conditions, and transferred to polyvi-

Table 4. Oligonucleotide primers used for real-time PCR detection of the expressions of genes related to lipid accumulation in *C. minutissima*

Target ORF	Primer sequence (5′–3′)	Subcellular localization	Length (base pairs) of production
G3PDH (*gpd*1)	F: ggtctaggctggggtaacaa R: gcaggtggtgatcaaatcag	ER	150
NM_001183314 (*gpd*2)	F: accgtggcttaccaactacc R: tgttaccccatcccatacct	ER	199
YIL155C (*gut*2)	F: aagacgtgctaagtgcatgg R: ttgtcgactgtttcctcagc	Mit	199
GPAT XM_501275 (YALI0C00209g)	F: catgaaccaggtcaaacagg R: tgcgtcgacgtaaatctttc	Lipid particle, ER	169
AJ311354(*gat*1)	F: gtagccgctgatcctaccat R: ctcacgtggggagtccttat	LP, ER	156
AJ314608 (*gat*2)	F: ggtgaccccattgaaatacc R: cgtttgaaccaccatcaaag	ER	159
LPAAT YSCPATFS (*slc*)	F: gcacgttaatcggtaagcaa R: atccaaggtggattggtgat	ER	161
Pap YDR284C (*dpp*)	F: ggtggcctttctaccactgt R: aatggtttgaacggaagagg	Vac, Gol, Mem, ER	174
YDR503C (*lpp*)	F: ggtttttggcttggacattt R: tcttgtattgcgtgtggtga	Vac, Gol	151
DGAT YOR245C (*dga*)	F: tggcgctagggaatctttat R: tgcattttacccaggactga	LP, ER	186
XM_502557 (YALI0D07986g)	F: gtcggtggtggttttctttt R: gaatgcacaagtgggttgtc	LP, ER	220
XM_504700 (YALI0E32769g)	F: aagggttttgttcgacttgg R: gcatcaaaggaagggtgaat	LP, ER	169
PDAT YNR008W (*lro*)	F: agctacaccacaagcactgg R: cctcggtgaggaatacaggt	ER	192
18S rDNA X13688	F: ttgacggaagggcacca R: caccacccatagaatcaagaaagag	Rib	127

LP, lipid particle; ER, endoplasmic reticulum; Vac, vacuole; Mem, membrane; Gol, Golgi; Mit, mitochondria; Rib, ribosome; F, forward; R, reverse.

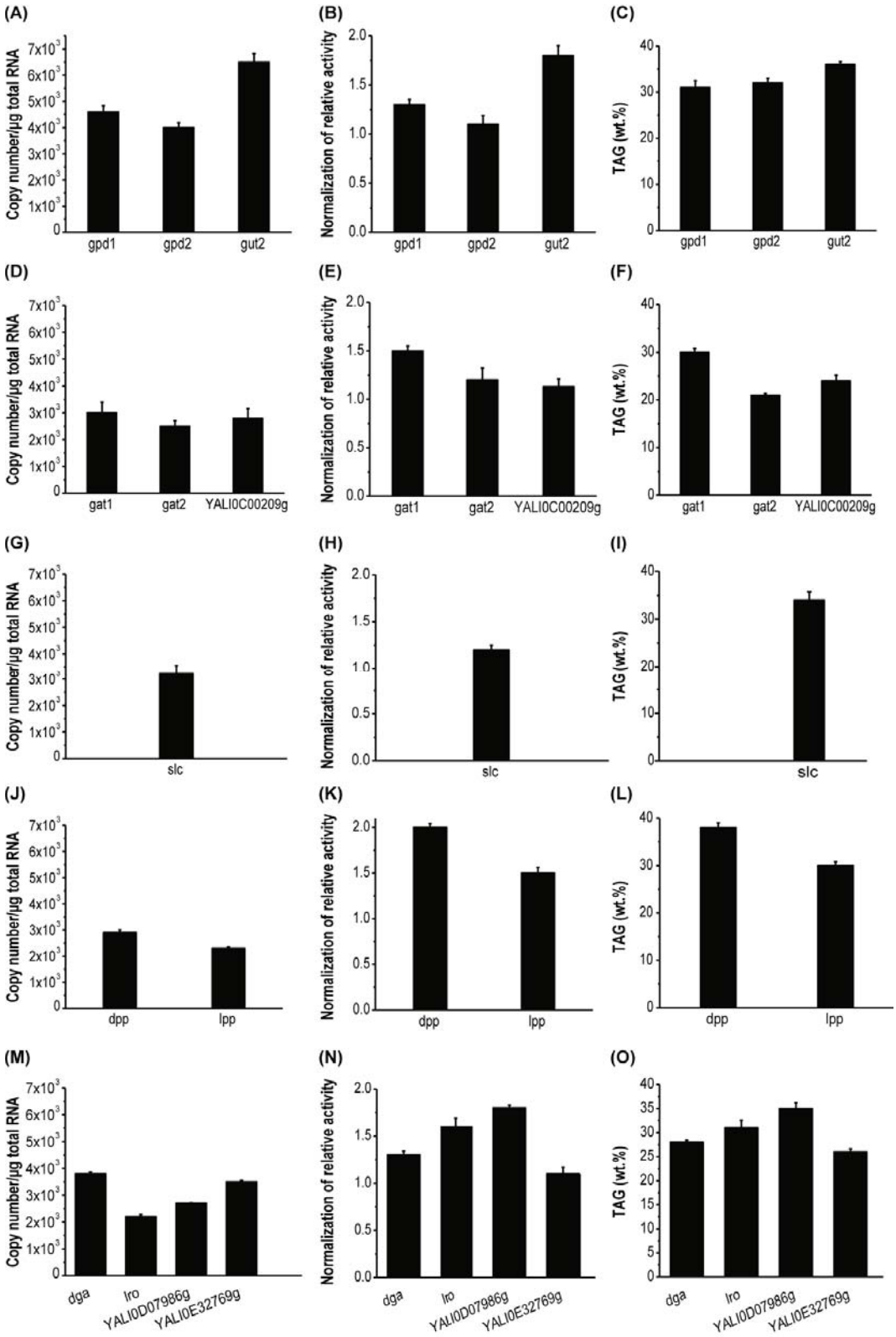

Fig. 3. Biochemical analyses of TAG synthesis genes expressed in transgenic *C. minutissima*. (A, D, G, J, and M) The real-time expression analyses; (B, E, H, K, and N) the normalization of the relative enzyme activity; (C, F, I, L, and O) the TAG contents. LPAAT, lysophosphatidic acid acyltransferase (*slc*); PAP, phosphatidic acid phosphatase (*dpp* and *lpp*); G3PDH, glycerol-3-phosphate dehydrogenase (*gpd1*, *gpd2*, and *gut2*); GPAT, glycerol-3-phosphate acyltransferase (*gat1* and *gat2*, *YALI0C0029g*); DGAT, diacylglycerol acyltransferase (*dga*, *YALI0D07986g*, and *YALI0E32769g*); and TAG, triacylglycerol.

nylidene fluoride (PVDF) membranes using a semi-dry apparatus V10-SDB (Scie-plas, UK). Membranes were blocked with rapid blocking buffer (Amresco, USA) for 5 min at room temperature. Membranes were then incubated with anti-pentamidine (1:10000) (QIAGEN) and phosphatase-labeled goat anti-mouse IgG (1:5000) (PerkinElmer^{TM}, USA) for 90 min. All membranes were visualized using a BCIP/NBT alkaline phosphatase color development kit (Sigma, USA).

Rapid lipid extraction and gas chromatography analysis

The method used was a modified version of that used by Wawrik and Harriman (2010). Briefly, the process involved saponification of cellular lipids, neutralization, extraction, and colorimetric detection. A control sample was added to 2-butanol to replace the color developer to avoid the co-extracted chlorophyll. The samples for gas chromatography analysis were pulverized and subjected to saponification, neutralization, transesterification with Boron-trifluoride, and extraction using n-Hexane. Samples were injected into a VARIAN 3900 (Agilent, USA) equipped with a flame ionization detector (FID) using a Stabilwax column. Injection and detector temperatures were 220°C and 250°C, respectively. The column temperature, which was initially 130°C, was increased to 210°C by a temperature gradient of 15°C/min.

Results

Gene expression of TAG-synthesis genes by two promoters in transgenic *C. minutissima*

The single-gene construct *dpp* was derived from the commonly used 35S promoter of CaMV (*Cauliflower mosaic* virus) or RbcS (ribulose-1,5-bisphosphate carboxylase small subunit). The mRNA expression levels revealed the copy numbers of single genes involved in TAG synthesis (Fig. 2A). Our results showed that the copy number corresponded with gene expression based on total RNA according to real-time PCR quantification. The average gene expression (3 random colonies) for the RbcS promoter was better than that for the CaMV promoter, which indicates the drive strength of the RbcS promoter was larger than that of the CaMV promoter, at least based on the *dpp* genes from *S. cerevisiae*. The protein expression of Dpp (molecular weight, 32 kDa) agreed with the result of gene expression, as revealed by western blot (Fig. 2B). Discussion of protein expression level correlates with the mRNA expression level, it implied for the production of target compounds. The relationship of RbcS and CaMV promoters and the TAG content were shown in Fig. 2B. The extracts were prepared and purified with a His-tag purification assay, and subsequently identified by western blotting. In the figure, lanes 4 to 6 show the expression derived by the RbcS promoter, whereas lanes 7 to 9 show the expression carried by the CaMV promoter. These findings demonstrate the TAG-synthesis gene was successfully expressed in proteins in *C. minutissima* UTEX 2219.

Biochemistry analyses of TAG-synthesis genes expressed in transgenic *C. minutissima*

In the literature, the rate-limiting enzymes of TAG biosynthesis are reported as GPAT and DGAT (Radakovits *et al.*, 2010). However, three other important enzymes, G3PDH, LPAAT, and PAP, still participate in TAG synthesis. The synergistic contribution of acyltransferase gene expression to glycerol-3-phosphate (G3P) synthesis involves G3PDH (*gut2*) required for TAG biosynthesis, which can reach 42% of lipid accumulation in *Y. lipolytica* (Thierry and Jean-Marc, 2011). When the LPAAT gene (*slc1*) cloned originally from *S. cerevisiae* was introduced into *Brassica napus* under the constitutive promoter of CaMV 35s, it showed substantial increases of 8% to 48% in lipid content in previous studies (Zou *et al.*, 1997). The best regulatory function of PAP in *S. cerevisiae* is as a signaling molecule in the transcriptional regulation of glycerophospholipid synthesis (Carman and Henry, 2007). Based on previous studies in the literature, the single-gene constructs for G3PDG (*gpd1*, *gpd2*, *gut2*), GPAT (*gat1*, *gat2*, *YALI0C00209g*), LPAAT (*slc*), PAP (*dpp*, *lpp*), DGAT (*dga*, *YALI0D07986g*, *YALI0E32769g*), and PDAT (*lro*) were designed for biochemistry analysis to investigate the individual effect of the single-gene constructs (Fig. 3). Figs. 3(A), (D), (G), (J), and (M) show the mRNA expression level; Figs. 3(B), (E), (H), (K), and (N) show the normalization of the relative enzyme activity. Because of the genome sequence of *Chlorella minutissima* UTEX2219 is not disclosure, the absolutely enzyme activity should using radioisotope to measure the real enzyme activity. Besides the radioisotope, the partial enzyme activity is our alternative way to calculate the normalization of relative enzyme activity. Comparison of the crude enzyme of wild type and his-tag purification enzyme of transgenic Chlorella, the quintuple gene construct accumulated higher TAGs production than that of the wild type. The NADH product corresponded to G3PDH activity; the CoA product was represented as the partial enzyme activity of GPAT, LPAAT, DGAT, and PDAT; and the PA (phosphatidic acid) product was based on phosphate incorporation synthesis during the conversion of PA to DAG. Figs. 3(C), (F), (I), (L), and (O) show the FAME (fatty acid methyl ester) content, which was correlated to the amount of TAG accumulation in transgenic *C. minutissima* UTEX 2219. Based on the mRNA expression level, *gut2* showed the highest levels, followed by *gpd1* and *gpd2* in G3PDH genes, and had the potential to enhance TAG accumulation based on its unique gene sequence. The protein similarity of Gpd1-Gpd2, Gpd1-Ggut2, and Gpd2-Gut2 was 63%, 7%, and 10%, respectively. The same profile was revealed in the analysis of relative enzyme activity and TAG content. On the other hand, among the series genes of GPAT, *gat1* revealed the highest expression level and achieved the highest TAG content relative to the levels of *gat2* and *YALI0C00209g*. We speculated that *gat1* was not specific to the acyl-substrate, unlike *gat2*, which had high specificity. Otherwise, the protein similarity of Gat1-Gat2, Gat1-YALI0C00209g, and Ggat2-YALI0C00209g was 34%, 36%, and 41%, respectively. The results showed that the single-construct of *gat1* also presented higher relative enzyme activity and TAG content in transgenic *C. minutissima* UTEX 2219. Regarding the single-gene constructs of the members of the series of PAP, *dpp* showed higher expression and TAG content than *lpp* (20% similarity to *dpp*). In the series of DGAT/PDAT, YALI0D07986g showed the highest

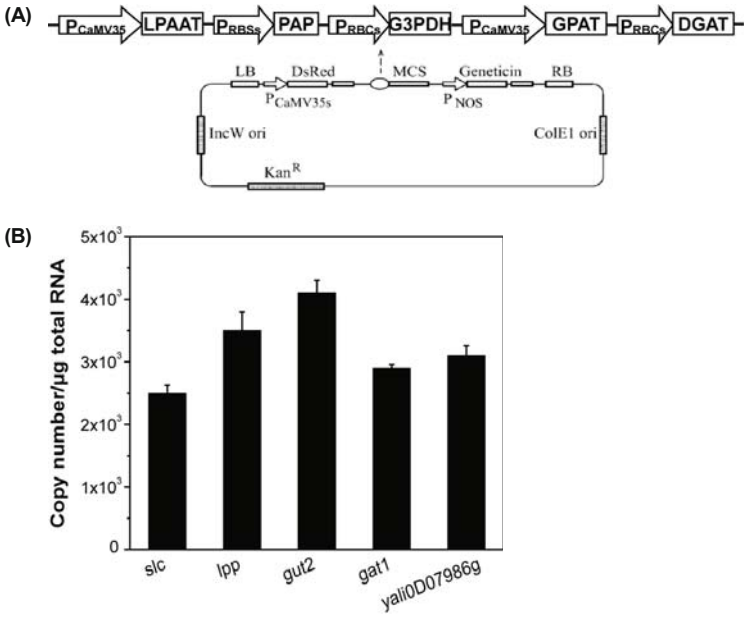

Fig. 4. Relative mRNA expression levels of quintuple-gene construct in transgenic *C. minutissima*. (A) Simplified schematic diagram of quintuple gene construction; (B) real-time expression analysis of quintuple-genes. $P_{CaMV35s}$, promoter of cauliflower mosaic virus 35s; P_{RBCs}, promoter of ribulose-1,5-bisphosphate carboxylase small subunit; PAP, phosphatidic acid phosphatase; LPAAT, lysophosphatidic acid acyltransferase (*slc*); PAP, phosphatidic acid phosphatase (*dpp*); G3PDH, glycerol-3-phosphate dehydrogenase (*gut2*); GPAT, glycerol-3-phosphate acyltransferase (*gat1*); DGAT, diacylglycerol acyltransferase (*YALI0D07986g*).

expression level, relative enzyme activity, and TAG content among the four genes. It should be mentioned that these four genes belonged to different yeast strains of *S. cerevisiae* and *Y. lipolytica*. In addition, the protein similarity of Dga-Lro and YALI0D07986g-YALI0E32769g was 10% and 12%, respectively. In summary, we can see *gpd1*, *gat1*, *lpp*, and YALI0D32769g showed the highest expression and TAG accumulation in G3PDH, GPAT, PAP, and DGAT/PDAT.

Real-time expression analysis and lipid accumulation of quintuple-gene construct in transgenic *C. minutissima*

A previous study proposed that, in the TAG synthesis-related genes, GPAT and DGAT are the two enzymes involved in the rate-limiting steps, and DGAT is generally recognized as the key enzyme for TAG synthesis (Radakovits *et al.*, 2010). In our previous study, G3PDH, LPAAT, and PAP were also shown to play an important role by participating in TAG synthesis. This might explain why a single-gene construct has little effect on enhancing TAG production. However, it is reasonable to speculate that the combination of these five genes should have the potential to enhance TAG accumulation in transgenic *C. minutissima* UTEX 2219. Therefore, the focus of this study was on the possibility of creating a quintuple-gene construct to enhance TAG synthesis. The quintuple-gene construct was shown in Fig. 4A. The analysis showed that the relative expression levels of G3PDH, GPAT, LPAAT, PAP, and DGAT were 27%, 17%, 16%, 22%, and 18% (the sum of five genes is 100%), respectively (Fig. 4B). Furthermore, G3PDH showed the highest mRNA expression level, and thus more G3P should be introduced into the acyl pool to increase the metabolic flux for GPAT, LPAAT, PAP, and DGAT for use. The TAG accumulation and lipid productivity of the quintuple-gene construct are shown in Fig. 5. After 14 days of cultivation in MES-volvox medium including vitamin solution in a 1-L bioreactor, the TAG accumulation of *C. minutissima* UTEX 2219 with the construct achieved 44 wt%, which was enhanced 2-fold relative to that of wild-type *C. minutissima*. The lipid productivity results showed that TAG and crude lipid level was 0.16 g/L/d and 0.17 g/L/d, respectively. These results indicate that the multiple-gene approach was the most effective for achieving maximal TAG accumulation in this complex system.

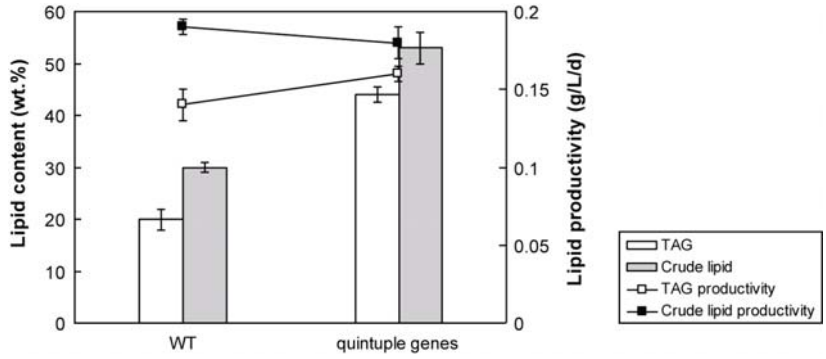

Fig. 5. Lipid content and productivity of the cells expressing the quintuple-gene construct in transgenic *C. minutissima*. 1, *Chlorella minutissima* wild type (wt); 2, quintuple-gene construct: *slc-dpp-gut2-gat1-YALI0D07986g*. LPAAT, lysophosphatidic acid acyltransferase (*slc*); PAP, phosphatidic acid phosphatase (*dpp*); G3PDH, glycerol-3-phosphate dehydrogenase (*gut2*); GPAT, glycerol-3-phosphate acyltransferase (*gat1*); DGAT, diacylglycerol acyltransferase (*YALI0D07986g*); and TAG, triacylglycerol.

Discussion

The promoter CaMV 35S is one of the most widely used, general-purpose constitutive promoters that can be used to cause high levels of gene expression in all areas of plant molecular biology research. The expression pattern of this promoter will be of interest to those researchers involved in field-release studies. The activities of CaMV 35S promoters in pollen in populations of stably transformed plants and in transient expression analysis have been described previously (Wilkinson *et al.*, 1997). The other promoter used in this study, RbcS, has also been commonly used in transgenic plants, and is increasingly used as a production platform for various proteins. The RbcS gene family of *Chrysanthemum morifolium* Ramat was previously engineered to drive heterologous expression of various genes, and was found to cause 7- to 8-fold higher levels of gene expression than the commonly used CaMV 35S promoter (Outchkourov *et al.*, 2003). In our present study, expression of *dpp* presented a stable transformation using CaMV 35s and RbcS promoters (Fig. 2). Our finding reproduced the expression drive of *dpp* with the RbcS promoter, which enhanced 32% expression level that is more than that of the CaMV 35s promoter drive. Moreover, analysis of the different genes involved in glycerol metabolism indicated that *Y. lipolytica* has a modified and unique metabolism of glycerol that is dedicated to G3P synthesis and to TAG synthesis, which may contribute to its oleaginous character. The synergistic contribution of acyltransferase gene expression to G3P synthesis is by *gut2* required for high levels of TAG synthesis and lipid accumulation in *Y. lipolytica* (Thierry and Jean-Marc, 2011). In our experiment, upon cloning G3PDH from *S. cerevisiae* and transforming it into *C. minutissima*, mRNA expression of *gut2* was higher (44–50%) than that of *gpd1* and *gpd2* (Figs. 3A, 3B, and 3C). Comparison of the similarity of Gpd1, Gpd2, and Gut2 was carried out by comparison to *C. variabilis* in the NCBI database, which revealed results of 11%, 13%, and 5%, respectively. This reveals the unique sequence of *gut2* can reach 35 wt.% of the TAG content, which is better than that of the wild type (20 wt.%), which explains its importance in lipid accumulation. The Gat1 protein does not exhibit particular preference for acyl-CoA substrates yielding LA (lysophosphatidic acid), and shows minor specificity of cellular fatty acid profiles. It is the prevailing enzyme for the acylation of G3P/DHAP and is dually localized in the LP (lipid body) and ER. In contrast, localization of Gat2 is restricted to microsomes; its apparent preference for C-16 saturated fatty acids plays a major role in the specific fatty acid composition of phospholipids (Zheng and Zou, 2001). Based on the results from cloning *gat1* and *gat2*, the nonspecific *gat1* presents a higher mRNA expression level, crude enzyme activity, and TAG content in transgenic *C. minutissima* UTEX 2219 (Figs. 3D, 3E, and 3F). The direct precursor of TAG synthesis is DAG. DAG can be formed by dephosphorylation of *de novo*-synthesized PA; degradation of glycerophospholipids occurs through the action of phospholipase C, or of phospholipase D in combination with PAP; and/or deacylation of TAG (Carman and Henry, 2007). PA was converted by LPAAT, and *slc* had been identified as a suppressor of a defect in sphingolipid biosynthesis. Dpp and Lpp are members of the PAP2 class of enzymes, which are characterized by their Mg^{2+} independence. Dpp and Lpp are integral membrane proteins with six transmembrane spanning regions, are localized to the vacuoles and Golgi compartment, and are assumed to play a role in both phospholipid metabolism and cell signaling (Furneisen and Carman, 2000). The results of *dpp* and *lpp* cloning showed that Dpp expression can enhance lipid content up to 37% wt, which is higher than that of Lpp (Figs. 3J, 3K, and 3L). This finding implies the LPAAT and PAP could improve the TAG content by cloning heterologous genes from eukaryotic microorganisms into microalgae. This approach attains more TAG accumulation than that from one of the rate-limiting step enzyme, GPAT (Fig. 3). This finding suggests that the other key factor can regulate the flux direction in TAG biosynthesis. The truly major enzyme catalyzing acyl-CoA–dependent DAG acylation is DGAT. Dga belongs to the family of DGAT2 proteins, which harbor transmembrane regions and lack classical signal peptides. Lro (LCAT-related open reading frame, PDAT) also converts DAG to TAG in an acyl-CoA–independent esterification reaction using the sn-2 acyl group from glycerophospholipids as co-substrate for the third acylation step. Lro is believed to be mainly active in the logarithmic growth phase, and Dga activity is more pronounced in the stationary phase. Using molecular methods, a previous literature study included changing non-oleaginous *S. cerevisiae* to oleaginous yeast with approximately 30% lipid content, thus allowing investigation of the mechanisms of lipid accumulation (Yasushi *et al.*, 2007). In addition, another previous study showed a transgenic soybean seed, containing DGAT from *Y. lipolytica* upregulated for increased seed storage of lipid production and altered fatty acid profiles, also had increased total fatty acid content (of at least 10%), especially increased oleic acid content (of at least 25%), compared to corresponding contents of a nonransgenic, null sergeant soybean seed (Knut *et al.*, 2009). Our data shows the expression of *dga* and *lro* indeed increased TAG accumulation by about 25–50% compared to the expression of wild type (Figs. 3M, 3N, and 3O). In the cloning of DGAT, we also select two genes, YALI0D07986g and YALI0E32769g from *Y. lipolytica*, which improved the TAG content up to 35 wt.%, which is higher than that resulting from *dga* or *lro* in *C. minutissima* UTEX 2219. Comparison of all the single-gene constructs showed that relative enzyme activity was correlated to TAG content. It is thought that if we can enhance higher enzyme activity we should also improve (i.e., increase) the TAG content using the methods described in this study. In the quintuple-gene construct, the highest relative mRNA expression level was *gut2*, followed by *dpp*, YALI0D07986, *gat1*, and *slc* (Fig. 4). In the previous study, the literature identified 26 genes encoding 6 distinct classes of enzymes involved in TAG biosynthesis (Cagliari *et al.*, 2010). *In silico* characterization and sequence analysis allowed the identification of of GPAT and LPAAT enzyme families form a cluster apart from the cytoplasmic isoforms, involved in the eukaryotic pathway. In addition, two distinct membranes bound DGAT enzymes were quantitative expression pattern analyses demonstrated variations in gene expressions during castor seed development. The agreement of GPAT, LPAAT, and DGAT were benefited for TAG bio-

synthesis in eukaryotic cell. In summary, this gene combination indeed reached 2-fold TAG content (also 1.8-fold TAG productivity) relative to that of the wild-type *C. minutissima* UTEX 2219 (Fig. 5). This confirms that this multiple gene system enhances TAG accumulation in *C. minutissima* UTEX 2219. Determinations of the unique phycological properties and optimal cultivation with variable simulation of the transgenic *C. minutissima* UTEX 2219 are the next steps to further investigating the findings in our study. It expects that the combination strategy of genetic engineering and bioprocess engineering will facilitate the development of biomass capacity convert into economically biodiesel producing by the transgenic microalgae.

Conclusions

Expression of G3PDH, GPAT, LPAAT, PAP, and/or DGAT increased the storage lipid content of *C. minutissima* UTEX 2219 up to 2-fold over that of wild type after 14 days of cultivation. The highest lipid content was found in cells expressing a quintuple-gene construct for the enzymes in this pathway. These results indicate that the multiple-gene approach is the most effective method for achieving maximal enzymatic activity and thus maximal lipid production in this complex system. Using a metabolomic approach, it should be possible to successfully boost TAG storage levels in microalgae, thus rendering them suitable resources for biofuel production. Future work on the responses to stress and nutrient availability via the major signaling pathways should provide new insights in the algal cell.

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