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Novel codon-optimization genes encoded in *Chlorella* for triacylglycerol accumulation

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Abstract

Microalgae have been recognized as one of the potential resources for biodiesel production based on its fast growth or its high total lipid content depending on species. Expression of Kennedy pathway genes, which encodes GPAT, LPAAT, PAP, and DGAT for increasing the metabolic flux towards the TAG storage in *Chlorella* sp. from 20 to 46 wt% and total lipid accumulation from 35 to 60 wt.% corresponding to each specific gene combination under autotrophy, compare to the wild type (vector only). The highest TAG content was found in cells expressing a quadruple-gene construct (GPAT-LPAAT-PAP-DGAT) in the Kennedy pathway, corresponding to 46 wt.% of TAG and 60 wt.% of total lipid content. This work provides the optimization of TAG production in *Chlorella* sp. can be achieved by manipulating the selected genes, in turns making commercially producing biodiesel practical.

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Keywords: Kennedy pathway ; glycerol-3-phosphate acyltransferase; lysophosphatidic acid acyltransferase; phosphatidic acid phosphatase; diacylglycerol acyltransferase

1. Introduction

Global petroleum reserves are expected to deplete over the next 50–60 years that lead to the worry of future fuel supply's stability. The search for fuel source is thus an urgent concern to keep future civilization industrialized. Biodiesel, can easily made from a variety of feed stocks (i.e., vegetable oil and animal fat), is deemed as one of the solution to circumvent such problem. However, the limited supply of these feed stocks can not meet the quantity needed for commercialized biodiesel production. Attention is then focus on the use of microalgae of *Chlorella*, which is chlorophyta divisions, that is known for consenting the energy in TAG of good quantity. A unicellular eukaryotic green algae *Chlorella*, known for its high growth rate (up to 0.5 $gL^{-1}d^{-1}$) but moderate TAG content observed 30 wt% in photoautotroph and 50 wt% in mixotrophy [1-3]. It was chose as the test model to study the gene modulation in a specific

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metabolic pathway that can elevate the lipid content to an acceptable level ready for economically use while the high growth rate production can still be maintained. It is known that eukaryotic cells stores energy-rich fatty acids in the molecules form of TAG [4] of which upon isolation can easily convert to biodiesel by simple transesterification reaction [5]. The TAG biosynthesis involves a sequence of steps known as the Kennedy pathway where enzymes GPAT, LPAAT, PAP, and DGAT are responsible for each biotransformation (Fig.1). Indeed, survey on literature reports revealed that overexpressing of single Kennedy pathway gene do increase the rate of TAG synthesis in transgenic plants and yeasts [6-14]. It is reasonable to believe that some of the strategies that result in increased oil seed content in terrestrial plants may be able to increase the lipid content in microalgal cells as well. It would be of great benefit to develop genetic strategies to increase the cellular tolerance to a variety of stress factors. However, there is no related report can be found in *Chlorella*'s example. It might be these four needed gene's sequence of *Chlorella* was yet available to serve such purpose [15]. Nevertheless, gene sequences of *Chlymydomonas reinhardtii* resolved another microalga species of chlorophyta divisions, the genome have been revealed the evolution of key plant function and was a model system, which was suitable for study the TAG synthesis [16]. Since the genome sequence of *Chlorella* sp. remains unknown, the 4 genes necessary for *Chlorella* codon optimization were obtained from *Chlamydomonas reinhardtii* and BLASTed to the reported or predicted genome sequences of *Chlorella variabilis* and/or *Chlorella vulgaris* registered in the National Center for Biotechnology Information (NCBI) database. Thus, the focus of this article is to characterize the complex genes of TAG synthesis, to evaluate enzyme activity under multiple genes combination, and analyzing the effects on TAG accumulation. The *Chlorella*-codon-optimized genes (http://www.kazusa.or.jp/codon/) of heterologous genes (GPAT, LPAAT, PAP, and DGAT) from *Chlamydomonas reinhardtii* were thus obtained and a series of recombinant proteins among these four genes' constructs are designed and synthesized for investigation understating the best gene combine that is able to TAG production.

2. Materials and Methods

2.1 Materials and cultivation condition

A domestic algal strain of *Chlorella* sp. was kindly provided by Dr. Huei-Meei Su of Tungkang Marine Laboratory, Taiwan Fisheries Research Institute. The tris-acetate-phosphate (TAP) medium was stored for pre-cultivation of *Chlorella* at 25 °C under light with continuous standing, and the cells were transferred to MES-volvex (MV) medium without vitamin supply for two-stage cultivation. The MV medium was used for TAG production at 25 °C under 8,000 lux light intensity, with 0.2 vvm of 2% CO_2 gas mixture supply. The growth was estimated spectrophotometrically (Beckman DU 530, USA) at 660 nm and converted to biomass by an appropriate calibration curve.

2.2 Construction of genes in the Kennedy pathway

The *Chlorella*–codon–optimized genes (GPAT, LPAAT, PAP and DGAT) from *Chlamydomonas reinhardtii* were obtained through artificial oligonucleotide synthesis from GeneScript (USA) and transformed into *Chlorella* by electroporation. The resulting chimeric genes were re-isolated as *Bgl*II/*Eco*RI fragments and cloned into the binary vector pAlgae (Table. 1). The left and right borders of pAlgae are homologous fragments of *Chlorella*, that allowing specific gene exchange into the *Chlorella* chromosome. Kanamycin and geneticin were used as the selection markers in *E. coli* and *Chlorella*, respectively (Fig.2).

2.3 Electroporation

An algal culture grown to a cell density of 10^6 cell mL^{-1} was harvested by centrifugation (3000 rpm) at room temperature and washed once with culture medium. Washed cells were resuspended in 10^{-2} volume of sterilized distilled water. The aliquot of resuspended cells (80 μL) was mixed with plasmid (>5 μg) and transferred to an ice-cold electroporation cuvette and treated in a Bio-Rad electroporator (Bio-Rad, USA). Electrotransformation was conducted at 25 μF and 200 ohm at field strength of 2000 Vcm^{-1} using a Gene Pulser. Electroporated cells were incubated on ice for 5 min. A 5-mL aliquot of culture medium was added, and the cells were allowed to grow in the dark at room temperature for 24 h. The efficiency of transformation was determined by plating the culture on agar plates supplemented with geneticin (500 μgmL^{-1}).

2.4 RNA extraction

The culture (100 mL) was transferred to centrifuge tube and centrifuged at 3000 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. Nucleic acid concentrations were measured by spectrophotometer at 260 nm. The 260 to 280 nm ratios were determined and referred as the purity of the total RNA extracted.

2.5 Real-time PCR

To facilitate the real-time PCR analysis of the selected genes under the same reaction conditions, primers were designed by Primer3 input (version 0.4.0) with the set criteria of melting point similarity. The 18S rRNA gene was selected as the housekeeping gene (Table. 2). Reverse transcription was carried out using a reverse transcriptase kit (Qiagen, USA). The real-time quantitative PCR was performed on a PCR instrument (Bio-Rad MyiQ5, USA). A reaction mixture for each PCR run was prepared in the presence of SYBR Green PCR Core Reagents (Bio-Rad, USA). The cycle parameters consist of 1 cycle of 3 min at 95 °C, and 40 cycles of 10 s at 95 °C, followed by 30 s at 55 °C. Data were collected at the end of each extension step. The relative amounts of gene expression among the treatment groups were analyzed, where *Ct* is the cycle number at which the fluorescent signal rises statistically above the background.

2.6 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, USA) measures the phosphate producing of the enzyme samples of PAP. The phosphate standard curve: $Y_{650nm} = 0.3534X$ (nmol) + 0.1588 ($R^2 = 0.9995$).

2.7 Lipid extraction and gas chromatography analysis

The total lipids were estimated as fatty acid methyl esters (FAME) by the direct transesterification method was carried out as described previously [17] and modified by [18]. Fatty acid methyl esters were analyzed by a gas chromatography (Agilent VARIAN 3900, USA) equipping with a flame ionization detector (FID) and a Stabilwax column. Nitrogen (1.5 $mLmin^{-1}$) was used as the carrier gas. Temperature

was programmed increasement from 130 °C to 180 °C with a 10 °C min^{-1} and thereafter to 210 °C with a 15 °Cmin^{-1}. Injector and detector were maintained at 220 °C and 250 °C, respectively. FAME contents were determined from their corresponding peak areas using methyl pentadeconate as the internal standard. The data presented are the average of three estimations.

3. Discussion

3.1 mRNA expressions in single and multiple-gene constructs

The *Chlorella*-codon-optimized genes of GPAT, LPAAT, PAP, and DGAT were synthesized artificially and designed for TAG production. The analysis of the mRNA expression levels of single-gene and multiple-gene constructs in *Chlorella* sp. (Fig. 3). The BLAST result of codon-optimized GPAT, LPAAT, PAP, and DGAT showed amino acid similarity to *C. variabilis* or *C. vulgaris*, and it caused lower gene expression (3.2×10^3 copy/μg total RNA) in the transgenic *Chlorella* sp. The codon-optimized PAP has 35% similarity to *C. variabilis*, exhibits the highest mRNA expression of 9×10^3 copies/μg total RNA. The codon-optimized LPAAT and DGAT showed 29% and 26% similarity to *Chlorella variabilis*, and the mRNA expression level of LPAAT was 1.2-fold higher than that of DGAT (Fig. 3A).

Among these four genes, GPAT and DGAT have been proposed as the 2 enzymes involved in rate-limiting steps, and DGAT is generally recognized as the key enzyme for TAG synthesis [6]. This might explain why a single-gene construct has little effect on enhancing TAG production. Therefore, the focus is on the possibility of creating a double-gene construct, especially GPAT–DGAT, which can act in concert to enhance TAG synthesis. In addition, the double-gene construct of LPAAT–PAP was also prepared for the purpose of comparison (Fig. 3B). The result of mRNA expression analysis of the double-gene construct showed that the relative expression level of GPAT (57%) was higher than that of DGAT (43%) within the DGAT–GPAT combination and PAP (58%) was higher than that of LPAAT (42%). This result revealed that DGAT, together with GPAT, might induce a synergistic effect and produce higher expression than the single-gene construct of GPAT.

However, these findings prompted us to carry out further studies on the effect of non-rate-limiting steps genes' combinations on *Chlorella* sp. Moreover, we hoped to understand not only the effect of rate-limiting genes but also the other regulatory factors in manipulating the expression of Kennedy pathway genes. Thus, triple-gene constructs were designed to investigate these effects. The triple-gene constructs were devised, consisting of GPAT-LPAAT-DGAT, GPAT-PAP-DGAT, LPAAT-PAP-DGAT, and GPAT-LPAAT-PAP with the aim of increasing FAME accumulation by expression of Kennedy pathway genes in *Chlorella* sp. In the first triple-gene construct, GPAT-LPAAT-DGAT, LPAAT was combined with two rate-limiting enzymes to investigate its effect on TAG synthesis. The results indicated that the order of relative mRNA expression was GPAT>DGAT>LPAAT (Fig. 3C) and suggested that LPAAT and DGAT might increase expression of GPAT in the GPAT-LPAAT-DGAT combination. In the second triple-gene construct, PAP was combined with two rate-limiting enzymes to investigate its effect on TAG synthesis. GPAT-PAP-DGAT showed that the order of relative mRNA expression was PAP>DGAT>GPAT (Fig. 3C). In the third triple-gene construct, LPAAT and PAP were each combined with one rate-limiting enzyme of DGAT to investigate their effect on TAG synthesis. The results showed that the order of relative mRNA expression was PAP>DGAT>LPAAT (Fig. 3C). In the last triple-gene construct, LPAAT and PAP were each combined with one rate-limiting enzyme of GPAT to investigate their effect on TAG synthesis. GPAT-LPAAT-PAP was designed to investigate the effect of substituting PAP for LPAAT and DGAT in the regulation of TAG synthesis. The results showed that the order of relative mRNA expression was PAP>GPAT>LPAAT (Fig. 3C).

The final design was the quadruple-gene construct followed the directed design of the GPAT-LPAAT-PAP combination. The quadruple-gene construct was planned as GPAT-LPAAT-PAP-DGAT for this purpose (Fig. 3D). The relative mRNA expression levels of GPAT, LPAAT, PAP, and DGAT in this combination were 23%, 35%, 28%, and 14%, respectively. The order of the relative mRNA expression levels was LPAAT>PAP>GPAT>DGAT. This construct entirely changed the order of the expression level observed in single-gene constructs (PAP>LPAAT>DGAT>GPAT). Thus, it appears that LPAAT and DGAT contribute to increasing the expression of GPAT and that PAP might assist the expression of LPAAT and DGAT in the GPAT-LPAAT-PAP-DGAT combination. In summary, the multiple genes approach to analysis of the Kennedy pathway has already shown how to tune the metabolic flux leading to TAG synthesis. Future studies will focus on ways to improve expression, for example, by promoter replacement, increasing the copy number, and optimal cultivation for enhancing the TAG accumulation in transgenic *Chlorella* sp.

3.2 Protein expression in single and multiple-gene constructs

This effect of differences in the mRNA expression level compared to the vector only was a response to enzyme activity based on the production of the byproducts—coenzyme A and inorganic phosphate. The analysis of the enzyme activity of single-gene and multiple-gene constructs in *Chlorella* sp. (Fig. 4). The codon-optimized PAP exhibits the highest enzyme activity is 2.2-fold greater than that of the vector only. The codon-optimized LPAAT, DGAT, and GPAT showed the enzyme activity correlating with the mRNA expression level. This agreed with the enzyme activity level in which PAP>LPAAT>DGAT>GPAT and correlated well with the corresponding expression of each mRNA (PAP>LPAAT>DPAT>GPAT). In addition, the double-gene construct of GPAT-DGAT and LPAAT–PAP were also prepared for the purpose of comparison. The result of this incidental result showed that the relative enzyme activity of PAP with the LPAAT–PAP combination (3.6-fold greater than that of the vector only) was higher than that with the GPAT–DGAT combination (1.2-fold greater than that of the wild type) or LPAAT with the LPAAT-PAP combination (2.2-fold higher than that of the vector only). To our surprise, PAP activity of the LPAAT-PAP combination was greatly increased. The triple-gene constructs were devised, consisting of GPAT-LPAAT-DGAT, GPAT-PAP-DGAT, LPAAT-PAP-DGAT, and GPAT-LPAAT-PAP with the aim of increasing FAME accumulation by expression of Kennedy pathway genes in *Chlorella* sp.. In the first triple-gene construct, GPAT-LPAAT-DGAT showed the enzyme activities was 1.2-fold more than that of the vector only. This result also supported the hypothesis that DGAT modulates the expression of GPAT, as seen in the double-gene constructs of DGAT-GPAT, and that LPAAT plays a role in assisting TAG synthesis in the GPAT-LPAAT-DGAT combination. In the second triple-gene construct, GPAT-PAP-DGAT showed that the relative enzyme activities of PAP with the GPAT-PAP-DGAT combination was 1.4-fold greater than that of the wild type and GPAT-DGAT with the GPAT-PAP-DGAT combination was 1.3-fold higher than that of the wild-type strain. In the third triple-gene construct, LPAAT- PAP - DGAT showed that 1.5-fold higher relative enzyme activity in LPAAT-DGAT of the LPAAT-PAP-DGAT and PAP of the LPAAT-PAP-DGAT combination. In the last triple-gene construct, GPAT-LPAAT-PAP showed that 1.8-fold higher relative enzyme activity in LPAAT-DGAT and 2.2-fold higher relative enzyme activity in PAP of the LPAAT-PAP-DGAT combination. It indicated the very importance to characterize the synergistic effect observed with the triple-gene construct and should be understand the effect of inserting the fourth gene, DGAT, into the GPAT-LPAAT-PAP combination. The quadruple-gene construct followed the directed design of the GPAT-LPAAT-PAP-DGAT combination. The result showed the equal relative activity of GPAT-LPAAT-DGAT and PAP in the GPAT-LPAAT-PAP-DGAT combination.

3.3 Starch content and TAG synthesis in multiple-gene constructs

A distinctive feature of the photosynthetic oleaginous algae is starch and TAG accumulation. The regulation of oil synthesis in developing algae can occur at multiple levels in the biochemical conversion of photosynthetically fixed carbon into TAG. One way to generate high oil content that are economically viable is to redirect carbon flux from starch to oil [19]. ADP-glucose synthesis through ADP-glucose pyrophosphorylase defines the major rate-controlling step of storage polysaccharide synthesis in both bacteria and plants. The preliminary results showed isolated mutant strains defective in the STA6 locus of the monocellular green alga *Chlamydomonas reinhardtii* that fail to accumulate starch and lack ADP-glucose pyrophosphorylase activity [20-21]. Therefore, how to regulate the starch synthesis is a key factor for TAG synthesis in *Chlorella* sp. The single-gene construct results indicated that the TAG content of the single-gene constructs of GPAT, LPAAT, PAP, and DGAT was 25%, 27%, 28%, and 26%, respectively (data not showed), comparison of the vector only levels of 20 wt%. In addition to the TAG content of double-gene (GPAT-DGAT and LPAAT-PAP), triple-gene (GPAT-LPAAT-DGAT, GPAT-PAP-DGAT, LPAAT-PAP-DGAT, and GPAT-LPAAT-PAP) and quadruple-gene constructs (GPAT-LPAAT-PAP-DGAT) was 28%, 32%, 35%, 36%, 42%, 37%, and 45% (Table 2). Analysis of the starch content of the double-gene (GPAT-DGAT and LPAAT-PAP), triple-gene (GPAT-LPAAT-DGAT, GPAT-PAP-DGAT, LPAAT-PAP-DGAT, and GPAT-LPAAT-PAP) and quadruple-gene constructs (GPAT-LPAAT-PAP-DGAT) were 19%, 18%, 15%, 16%, 16%, 20%, and 14% (Table 2). These transgenic strains did accumulate amount of lipids more than the vector only by heterologous Kennedy pathway's gene expression and also decreased the starch synthesis in our results. The TAG content of the LPAAT–PAP combination was 32 wt%, which was higher than the GPAT–DGAT combination of 28 wt%. In the first triple-gene construct, GPAT-LPAAT-DGAT, the TAG content was increased reaching 35 wt% (1.8-fold more than that of the vector only) in these combination. This result also supported the hypothesis that DGAT modulates the expression of GPAT, as seen in the double-gene constructs of DGAT-GPAT, and that LPAAT plays a role in assisting TAG synthesis in the GPAT-LPAAT-DGAT combination. In the second triple-gene construct, GPAT-PAP-DGAT showed that the TAG content was 36 wt%, it indicated that not only LPAAT but also PAP has a positive effect on the controlling of the ate-limiting step. In the third triple-gene construct, LPAAT-PAP-DGAT resulted in the 42 wt% content of TAG; directed design successfully increased the TAG content in transgenic *Chlorella* sp., by increasing the metabolic flux toward TAG synthesis. In the last triple-gene construct, GPAT-LPAAT-PAP observed that the TAG content was achieved to 37 wt%. The final design was the quadruple-gene construct followed the directed design of the GPAT-LPAAT-PAP-DGAT combination, the TAG content of the quadruple-gene construct was 46 wt%, to levels 2.3-fold more than that of the vector only. We proposed to increase the TAG content with the quadruple-gene construct (GPAT-LPAAT-PAP-DGAT) by manipulating heterologous expression and examining the effect on TAG synthesis to understand fully the expression of Kennedy pathway genes in enhancing TAG biosynthesis. In summary, the multiple genes approach to analysis of the Kennedy pathway has already shown how to tune the metabolic flux leading to TAG synthesis. Future studies will focus on ways to improve expression, for example, by promoter replacement, increasing the copy number, and optimal cultivation for enhancing the TAG accumulation in transgenic *Chlorella* sp.

3.4 Growth rate and TAG productivity in multiple-gene constructs

The transgenic strain did accumulate amount of lipids in multiple-gene constructs significantly more than the vector only. To further fine-tune the relative TAG productivity compassion of growth rate, we designed the nitrogen starvation condition for TAG productivity incensement (Table 2). The result of the double-gene construct showed that the growth rate of GPAT-DGAT and LPAAT-PAP (0.24, 0.25 $gL^{-1}d^{-1}$)

was lower than that of the vector only (0.25 $gL^{-1}d^{-1}$). In addition, the TAG productivity of GPAT-DGAT and LPAAT-PAP (0.129, 0.129 $gL^{-1}d^{-1}$) was higher than that of the vector only (0.087 $gL^{-1}d^{-1}$). The growth rate of triple-gene constructs (GPAT-LPAAT-DGAT, GPAT-PAP-DGAT, LPAAT-PAP-DGAT, and GPAT-LPAAT-PAP) were 0.22, 0.24, 0.21, 0.21 $gL^{-1}d^{-1}$, which were lower than that of the vector only. However, the compassion of these triple-gene constructs, the TAG productivity was higher than that of the vector only. Moreover, the profile of growth rate and TAG productivity of quadruple-gene construct (GPAT-LPAAT-PAP-DGAT) was similar to the triple-gene constructs. It indicated that the defined culture condition (2.1 Materials and culture condition) showed that the high TAG productivity even in transgenic *Chlorella* sp. The future work should improve the growth rate of the transgenic *Chlorella* sp. for TAG productivity incensement for more economic-cost production in large-scale culture system.

4. Conclusions

Expression of GPAT, LPAAT, PAP, and DGAT individually or in combination, can increase the storage TAG content in *Chlorella* in different expends. The highest TAG content of 2.3-fold incensement was found in cells expressing a quadruple-genes construct (GPAT-LPAAT-PAP-DGAT) in Kennedy pathway. These results suggested the multiple-genes approach is the most effective for achieving maximal enzymatic activity and thus maximal TAG production in this complex system. By taking the advantage of metabolic approach, it is possible to boost TAG storage levels in other famous chlorophyta divisions of microalgae (e.g., *Nannochloropsis oculata*, *Dunaliella salina*, *Tetraselmis chuii*, *Micractinium reisseri*) thus rendering it become a suitable resources for biofuel production.

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Table1. Plasmid information in transgenic *Chlorella sp.*

Plasmid	Relevant genotype or sequence	Sources
pAlgae	pBI121 binary vector and pBluescript II SK^+ plasmid combination	This study
pGPAT	*Chlorella*-codon-optimized gene	This study
pLPAAT	*Chlorella*-codon-optimized gene	This study
pPAP	*Chlorella*-codon-optimized gene	This study
pDGAT	*Chlorella*-codon-optimized gene	This study

GPAT, glycerol-3-phosphate acyltransferase; G3PDH, glycerol-3-phosphate dehydrogenase; LPAAT, lysophosphatidic acid acyltransferase; PAP, phosphatidic acid phosphatase; DGAT, diacylglycerol acyltransferase

Table 2. Growth rate and TAG content in multiple-gene constructs

Transgenic algae strains	Biomass (g/L/d)	TAG content (%)	Starch content (%)	Crude Lipid (%)
Host (vector only)	0.25	20.13±0.30	25	34.91
GPAT	0.24	25.33±0.11	21	47.98
LPAAT	0.19	27.51±0.40	22	49.56
PAP	0.28	28.44±0.13	21	42.78
DGAT	0.17	26.51±0.19	19	49.57
GPAT-DGAT	0.24	28.21±0.12	19	54.11
LPAAT-PAP	0.25	32.99±0.31	18	51.99
GPAT-LPAAT-DGAT	0.22	35.89±0.27	15	53.56
GPAT-PAP-DGAT	0.24	36.97±0.12	16	48.11
LPAAT-PAP-DGAT	0.21	42.99±0.31	16	51.99
GPAT-LPAAT-PAP	0.21	37.20±0.21	20	53.26
GPAT-LPAAT-PAP-DGAT	0.22	45.89±0.27	14	59.56

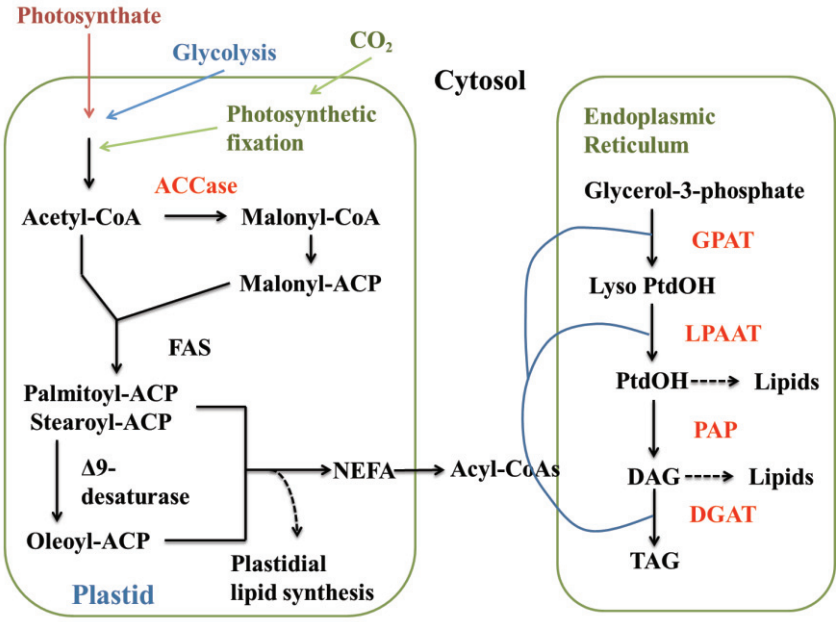

Figure 1. Metabolic pathway of TAG formation and the Kennedy pathway in eukaryotes.

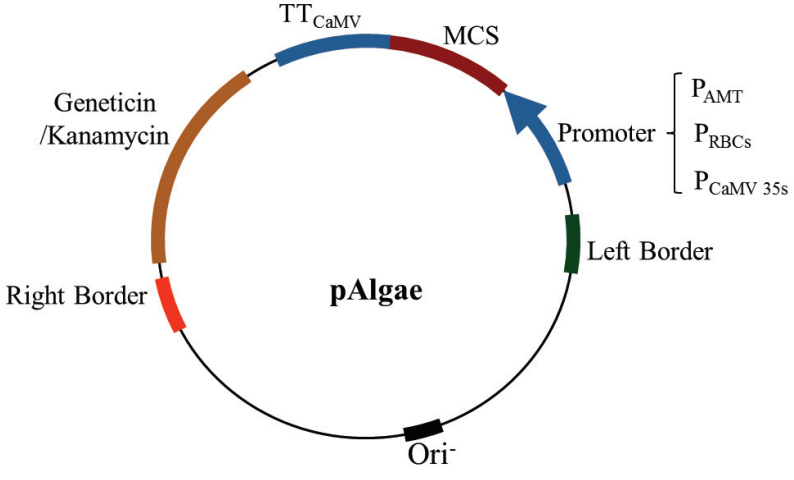

Figure 2. Simplified schematic diagram of the pAlgae plasmid..

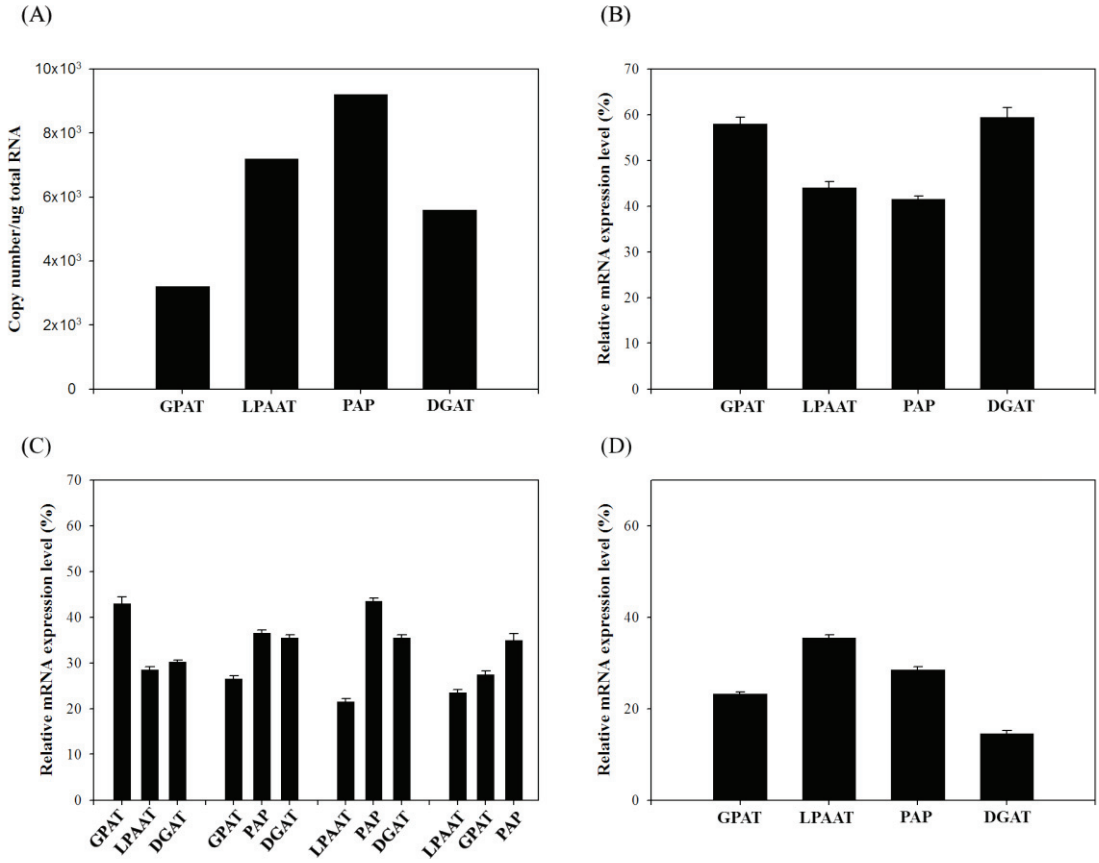

Figure 3. The analysis of the mRNA expression levels of single-gene and multiple-gene constructs in *Chlorella* sp.

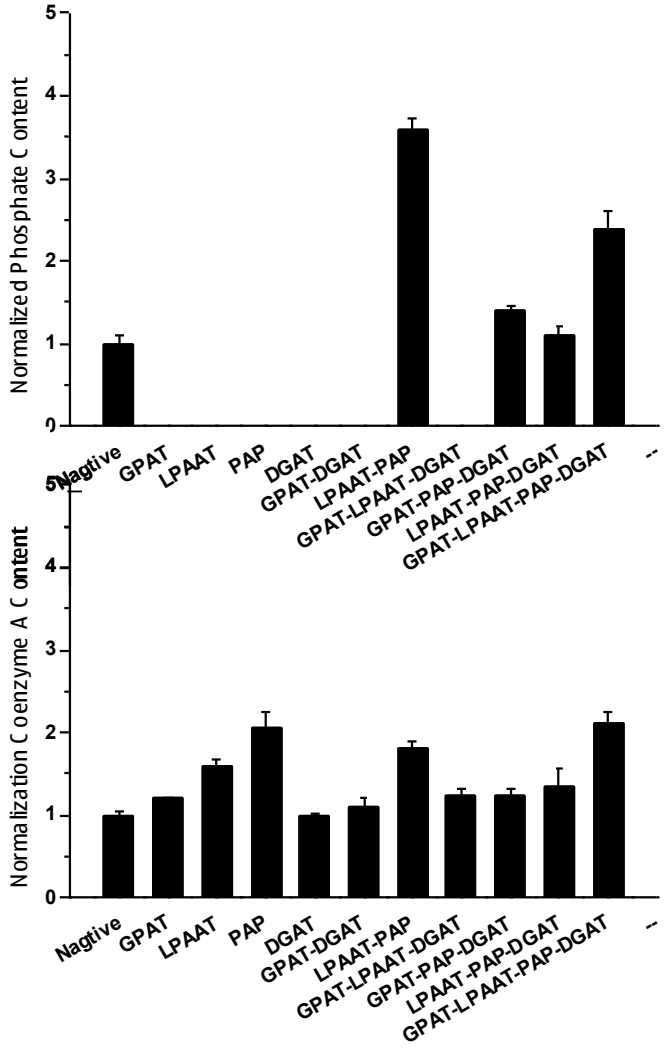

Figure 4. The analysis of the enzyme activity of single-gene and multiple-gene constructs in *Chlorella* sp.