The Selection of Reference Genes for Quantitative PCR and Detection of Transcriptional Variation of Key Genes in Lipid Biosynthesis Pathway under Nitrogen-Starved Condition in Nannochloropsis

Pingguang Chu [1, a], Dongmei Wang [2, b], Yan Wang [1,*]

[1]Research Centre for Harmful Algae and Marine Biology, Jinan University, Guangzhou 510632, China

[2]Single-Cell Center, Qingdao Institute of BioEnergy and Bioprocess Technology, Chinese Academy of Sciences, Qingdao, Shandong 266101, China

[a]chupingguang@ceedi.cn, [b]wangdm@qibebt.ac.cn, *yanwang7018@163.com

Abstract

Oleaginous microalgae Nannochloropsis could accumulate large amounts of lipid under nitrogen-starved condition, and thus be considered to be one of the most promising feedstock of reproducible biodiesel and the ideal research model system for studies on the molecular mechanisms in lipid biosynthesis. The investigation of regulatory mechanism at transcriptional level in lipid biosynthesis in Nannochoropsis relies on the quantification of transcriptional levels of related genes. Real-time fluorescent quantitative-PCR (qPCR) is one of the most popular approaches. Selection of appropriate reference genes for normalization is the key for accurate quantification. To find the reference genes specifically appropriate in the process of nitrogen-induced lipid accumulation in Nannochloropsis, we chose ten candidates that had been reported in literature, including genes encoding Glyceraldehyde-3-phosphate dehydrogenase, Ubiquitin conjugating enzyme, Histone H2A protein, Eukaryotic translation elongation factor 1 alpha, Cyclophilin, Ribulose-1,5-diphosphate shrinkage/oxygenase enzyme, Alpha tubulin, Polyubiquitin, Actin-like protein Actin and 18S rDNA. Then we evaluated their transcriptional stability under Nitrogen-starvation along time courses using geNorm and NormFinder programs by qPCR. Actin, which is traditionally used as reference gene in Nannochloropsis, surprisingly exhibited only moderate transcriptional stability. The analysis indicated that UBCE and HIS as the most appropriate combination of reference genes for Nitrogen-starved condition in Nannochloropsis. Based on this finding, we detected the transcriptional variation of three genes involved in center carbon metabolic pathways. Two of the four homologous genes of phosphatidic acid phosphatase (PAP) exhibited significantly increased transcription at 6hr after nitrogen depletion, suggested their potential involvements in producing sufficient diacyl-glycerol (DAG) for TAG biosynthesis and the two genes could serve as key target genes for further genetic engineering study in the purpose of improving lipid productivity. Moreover, the up-regulation Diacylglycerol acyltransferase 2B gene (DGAT2B), coupled with the down-regulation of light harvesting protein 2 gene (LHP2), pushed the carbon flux flowing into TAG biosynthesis in Nannochloropsis. This work provided the foundation for the most accurate quantification of gene expression under Nitrogen starved condition in Nannochlorpsis. The investigation of transcriptional dynamics of key genes involved in lipid biosynthesis pathway would give researchers new insight of the functional diversity of PAP homologous genes as well as the transcriptional regulation of central carbon metabolic pathway.

Keywords

Oleaginous microalgae; Nannochloropsis oceanica IMET1; Nitrogen-starvation; Real-time fluorescent quantitative PCR; Reference gene; GeNorm; NormFinder.

1. Introduction

In that the oil-producing microalgae as a source of biofuels advantage of high photosynthetic efficiency, large biomass yield, high oil production efficiency, environmental adaptability, carbon sequestration effect is significant, high-quality oil, rich in high-value by-products, comprehensive utilization, etc., Therefore microalgae biofuels have to solve the energy crisis, the enormous potential CO2 emission reduction, environmental governance [1-7]. But industrialized algae biofuels still face enormous challenges costly. Industrial production of microalgae biofuels reduce the cost of the first microalgae fundamentally improve the "germplasm" [8], so the fat accumulation microalgae cells genetic basis and molecular mechanism has been a hot topic internationally. Nannochloropsis oculata manifested in its process of large-scale cultivation of various excellent characteristics, such as high-yield oil [9], the strong growth of power [10-12], can withstand flue gas and waste water [13], and it is considered one of the most promising source of biomass industrialization. Nannocholoropsis oceanica IMET1 nitrogen deficiency induced in 14 days can be accumulated 41.2% of the dry weight of the fat within the cell [14], and have relatively clear genetic basis[15-17], and therefore become a research cell microalgae oil production one biological mechanism model.

Studies have found that *N.oceanica* IMET1 after nitrogen deficiency induced by central carbon metabolic pathways regulating the expression of related genes at the transcriptional level to control oil synthesis[18]. If, after nitrogen deficiency, glycolysis, PDHC (pyruvate dehydrogenase complex) and PDHC bypass, transcript levels of genes associated mitochondrial citric acid cycle and oxidative pathways, etc. as well as specific transport proteins increase, driven by the carbon flow carbohydrates protein and lipid metabolic pathways flowing synthetic glycerides[19]. Therefore, accurate detection of the key oil-producing related genes expression levels of nitrogen deficiency in oil production process is to resolve the molecular mechanism of microalgae oil production, an important research tool in the monitoring of intracellular lipid synthesis.

Real-time fluorescent quantitative PCR (RT-qPCR) is the most widely used one of quantitative test method of the amount of gene transcription, its accuracy depends on choosing the appropriate internal genes to correct yield and quality of different samples in mRNA, reverse transcription and PCR efficiency and so on various aspects of differences.Traditionally use housekeeping genes as qPCR inside more.But a growing number of studies have found that housekeeping gene in different species, different cell types and physiological state of transcription is not constant.Even some widely used traditionally made in gene transcription level under different environmental conditions have significant differences, adopt the single internal genes to calibration of qPCR process, has a great influence on the accuracy of the results [20-22], therefore, in view of the specific species specific experimental requirements, the need to filter relatively constant gene transcription level as the specific internal genes, in order to improve the accuracy of quantitative target gene transcription level.In micro be found, a study in detail respectively in different intensity, different temperature, as well as the diurnal cycle under the condition of suitable internal genes [23], but for these internal gene in the process of oil production induced transcription expression no changes under nitrogen deficiency

In order to study the mechanism and metabolic pathways under conditions of nitrogen deficiency accumulation of lipids, we first need a clear *N. oculata* dynamic changes in nitrogen and nitrogen deficiency conditions reference gene transcriptional expression. In this paper, real-time PCR technology, for each reference gene transcription level under conditions of nitrogen deficiency and nitrogen were detected using geNorm [24] program. The results were statistically analyzed to determine the most suitable reference gene, so as to accurately study under conditions of nitrogen deficiency N. oculata lipid synthesis pathway transcriptome map key genes to explore the molecular mechanism of the regulation of lipid accumulation in technological foundation.

2. Materials and Methods

2.1 microalgae culture

The *N. oculata* (*Nannochloropsis.oceanica* IMET1) inoculated in 1L nitrogen-rich medium (modified f/2, containing 1000mg/L $NaNO_3$; referred to as N+) in culture, the culture temperature is 22 ℃, 24 hours of continuous light (light intensity 50μmol$m^{-2}s^{-1}$), passed through 1.5% (w/w) CO_2, the initial cell concentration of 3×10^7cells/mL. Culture to the exponential growth phase (cell density of about 2×10^8cells/mL), the collected by centrifugation (3500g, 20 ℃, 5min), were divided into two parts, with the nitrogen-rich medium resuspended algal cells, the cells at a concentration of 2×10^8 cells/mL; another part of the medium with nitrogen deficiency resuspended (a cell concentration of 2×10^8cells/mL). Samples were two treatments are set three biological replicates, after the start of culture, respectively, in 0 h, 0.5 h, 3 h, 6 h, 12 h, 24 h take 100 mL algae solution, collected by centrifugation, algal cells stored at -80 ℃ freezer.

2.2 Total RNA extraction and reverse transcription

For 1.1 collected algal cells were extracted and purified total RNA (with reference to specific steps OMEGA plant RNA kit instructions), end-of 35μL DEPC treated water to dissolve, Qubit 2.0 (Invitrogen) quantitative concentration, -80 ℃ saved for subsequent experiments. Each sample taken 5μg total RNA, the use of the kangwei reagent kit superRT Reverse Transcriptase First-strand cDNA synthesis, PCR amplification products can be used directly or stored at -20 ℃.

2.3 The internal gene specific primer design and amplification detection efficiency

For the selected 10 internal candidate genes, in *nannochloropsis oculata* genome database access to the full length cDNA sequence, using Primer Premier5.0 the software design of a pair of specific primers, Primer sequence information shown in table 1.

Table 1 Primer sequences for the candidate reference genes.

Gene name	Gene ID	Forward primer(5'-3')	Reverse primer(5'-3')
GAPDH	g10357	GGTCGCCTTGTTGCCCGTATC	GAGTCGTATTTGAACTGGTA
UBCE	g2049	CCTTCCAGACGGAGATGTG	TCTGCTGTTTCTTGGTCGTTGA
HIS	g4682	GGGCGTTTCCCGTTCCTCCA	GGGATGATGCGAGACTTTTT
EF1-a	g7073	GAAGCCTGGCATGGTTGT	CTGGGCAGTAGCCGTTTT
CYP	g3597	GTAGTGCCCAAGACTGCTGA	CGTGCTTTCCGTCCAACC
RBCL	-------	TCCGTATCACTCCACAAC	TATGCAACGTAACCGAAG
18S rDNA	-------	GTCGGACGGGATGTATTT	CTGCCTTCCTTGGATGTG
TUA	g5608	TGGCATGCTGCCTCATGTACC	TTGATGCCGCACTTGAAGC
UBQ	g6899	CCACCATACACCGACCAA	CGCCTACGGACACTTTCCTCAA
Actin	g9477	CATAACAAACCAGCCCATAGT	CTTGCCAATCTACCACCATC

Amplification of cDNA full length of each candidate gene, is connected to the TOPO-TA vector (Invitrogen), transformed into *E.coli* TOP10 cells, plasmid concentration was measured. Initial plasmid as a template, gradient serially diluted 7, each 10-fold dilution, real-time quantitative PCR analysis, analysis of the standard curve calculated correlation coefficient, slope(S) and other parameters, primer efficiency (E) the formula $E = 10^{-1/S}$.

2.4 Real-time fluorescent quantitative PCR

For the sample of the N+ and N- all time points, respectively, for each internal candidate genes with SYBR Green Ⅰ fluorescence quantitative PCR kit (Roche) in ABI prism 7500 PCR (Applied Biosystems) on fluorescence quantitative experiments.Fluorescence quantitative PCR reaction

system is as follows: 2×SYBR Green Ⅰ mix 10μL;CDNA template 1μL (0.5 g/L);0.5μL upstream primer (10 m mol/L) and downstream primers 0.5μL (10mmol/L); ddH_2O 8μL.qPCR amplification conditions is as follows: after 95 ℃ for 10 min pre degeneration;95 ℃ for 15 s degeneration, 60 ℃ for 1 min annealing, 40 repeat cycle;Melting curve 97 ℃ and 40 ℃ cooling 30 s.Through Light cycler R480 (Roche) analysis of each candidate genes of Ct value can be obtained.

2.5 Data processing and analysis

According to the 10 strategists gene in the N+ and N- handle the relative expression at different time points, in ten internal gene expression through geNorm program stability through statistical analysis, select the optimal internal genes.

Set an internal genes in different samples under the condition of a certain Ct value in the expression of minimum quantity is 1, the rest of the sample relative expression for $2^{-\Delta Ct}$, will the data import geNorm program, calculate each internal genetic stability of the M value [25] (M value is the number of individual values transformed from .M reference gene and reference gene expression levels of other pairwise ratio higher after smaller stability, M> 1.5 stability of the worst.)

2.6 Central carbon metabolism in the transcription of related gene expression level analysis

According to Phosphatidic acid phospholipase (Phosphatidic acid phosphatase, PAP) of the four genes (*PAP1 PAP2 PAP3 and PAP4*), depleting compound protein gene *LHP2* and fatty acyl glycerin: length of the DGAT2B acyltransferase gene cDNA sequence, design used by qPCR primers, primer sequence information such as table 2.Internal gene using the most stable genetic screening in 1.5.The reaction conditions of qPCR: 95 ℃ for 10 min after pre degeneration;95 ℃ for 15 s degeneration, 60 ℃ for 1 min annealing, 40 repeat cycle;Melting curve 97 ℃ and 40 ℃ cooling 30 s.

Table 2 Primer sequences for the six genes involved in center carbon metabolic pathways

Gene name	Forward primer(5'-3')	Reverse primer(5'-3')
LHP2	GGACGGACTGGGTCATTTTC	GATGTGGTCGTGGGGTGCGG
DGAT2B	TTTCGCCCTGTATGTCCGC	TCCAATCCACCACACGCCT
PAP1	GAACTCCGACATCGCAACT	GGAGACTGGAAATGGGTAAG
PAP2	CACTCCTCCCGCCTTCAA	CCATACCAGGGCATCTCAAT
PAP3	CTCGGCAACGGTGGGATA	CCGCTTCTGCTGCTGTAGGT
PAP4	CCACAACCAACAGCCTCA	AGCCCAGAGCAAACCTAAC

3. The results

3.1 Internal candidate genes

We collect the literature of internal genes, through Blast method in *N. oculata* genome identified its homologous genes, and based on screen at the beginning of the following two criteria: 1, in micro quasi found no gene in the genome sequence is highly similar to the homologous genes, in order to ensure the specificity of the PCR amplification;2, in micro be found under the condition of nitrogen deficiency whole transcriptome data, its transcription (FPKM value) is higher, at the same time in different time points after nitrogen deficiency had no significant change.Finally selected 10 have different biological functions of housekeeping gene as the candidate genes inside.They include Actin-like protein (Actin)[26], Glyceraldehyde 3-phosphate dehydrogenase (GADPH)[27], Alpha tubulin (TUA), the Eukaryotic translation elongation factor 1 Alpha (α-EF1) [28], Polyubiquitin (UBQ), Cyclophilin (CYP) [29], Histone H2A protein (His) and Ubiquitin conjugating enzyme (UBCE) [23], 18 SrDNA with Ribulose 1,5-diphosphate shrinkage/oxygenase enzyme (RBCL).

3.2 Internal candidate gene primer amplification efficiency analysis

Amplifying the internal candidate gene cDNA and build plasmid, diluted samples of plasmid gradient as the template to complete the qPCR experiment, and draw standard curve, the calculation of the related parameters in the internal candidate gene specific primers (table 3).Results show that the

correlation coefficient R^2 10 candidate genes in the range between 0.9917~0.9994, the amplification efficiency between 2.017~2.126, the dissolution curve of internal gene are for a single signal peak, there is no nonspecific amplification, duplicate samples and the curve between the repeatability is better.

Table 3 The amplification parameters in PCR experiments for candidate reference genes.

Gene name	Correlation(R2)	Slope	PCR efficiency
Actin	0.9942	-3.1318	2.085956
18S rDNA	0.9985	-3.0905	2.106552
UBQ	0.9924	-3.1351	2.084343
CYP	0.9979	-3.0836	2.110067
HIS	0.9965	-3.0537	2.125551
UBCE	0.9994	-3.1375	2.083172
RBCL	0.998	-3.0853	2.109199
TUA	0.9953	-3.2825	2.016721
GAPDH	0.9937	-3.0768	2.113552
EF1-α	0.9917	-3.1601	2.072267

3.3 Total RNAs extracted from algal cells under the condition of nitrogen deficiency induced

Growth phase of *N. oculata* after nitrogen deficiency treatment, respectively, at 6 different time points (0 h, 0.5 h, 3 h, 6 h, 12 h and 24 h) extracting total RNA samples collected cells, and synchronous sample collection of nitrogen.In order to ensure the accuracy of qPCR results, we first detect total RNA the yield and the integrity of the sample.By agarose gel electrophoresis detection, the RNA samples showed clear ribosomal RNA bands (figure1).By scanning the UV/visible spectrophotometer (NanoVue) of each sample measured OD260 / OD280 is 2.00~2.10, the total RNA sample quality is good, meet the requirements of subsequent experiments.

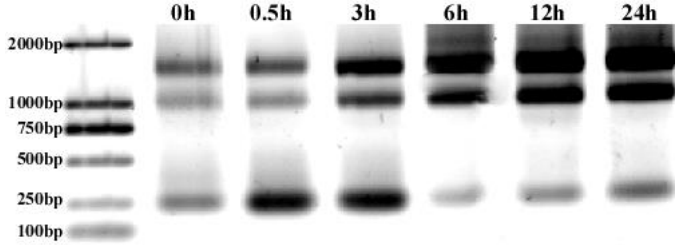

Fig. 1 Total RNAs extracted from algal cells under N- conditions at each time point

3.4 Internal genes stability analysis

3.4.1 Expression of each internal genes in all samples

For micro quasi found nitrogen (N+) and nitrogen (N-) under the condition of different points in time samples, respectively using specific primers by qPCR experiments to investigate the internal candidate genes in nitrogen deficiency induced on the different time points after the stability of transcriptional expression level.Gene transcription level with qPCR results of Ct values to characterize, Ct value is smaller, shows that the higher the transcription.The 10 candidate genes in various samples of qPCR Ct value in 12.89-12.89 range, including *18 s rDNA* of Ct value in 12.89-17.30, express the amount the highest (figure 2).The Actin, *UBQ* and *UBCE* transcription are relatively low.

3.4.2 GeNorm and NormFinder calculates the stability of internal genes

For N+ and N- two kinds of culture conditions, respectively with 10 geNorm calculates internal candidate genes expressed in different time points of the average stable value of M (M value is smaller, which indicates that more stable) gene expression.Results show that, under the condition of nitrogen *UBCE* (M = 0.861) in the expression of the most stable, followed by *HIS* (M = 0.875);Under the condition of nitrogen deficiency, *CYP* (M = 0.957) in the most stable, followed by *UBCE* (M =

1.018), while *TUA* and *GAPDH* M value is greater than 1.5, the stability of the worst (table 4).Comprehensive N+ and N- all samples under the condition of two kinds of statistics, *CYP* stability is best in all processing of samples (with a 0.945 M value).At the same time with NormFinder[30] program calculation and the stability of internal genes in value (the smaller the stable value, show that the greater the stability of the gene expression), is one of the most stable *UBCE* and *CYP*, followed by *HIS*, and the most unstable is *18 s rDNA* and *TUA* (table 5).

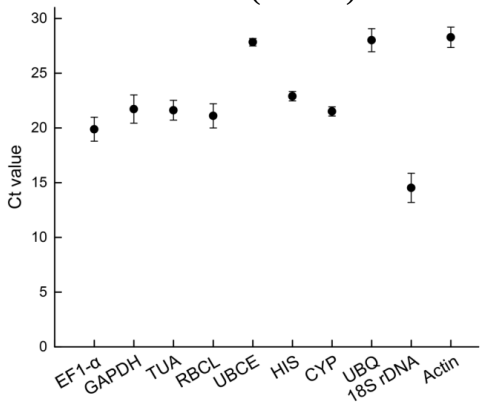

Fig.2 The range of qPCR Ct values of the ten candidate genes in all tested samples.

Table 4 The expression stability values (M) calculated using geNorm program for the candidate reference genes in all tested samples.

Gene name	All sample	N+	N-
Actin	1.083	0.989	1.288
18S rDNA	1.261	1.383	1.292
UBQ	1.400	1.442	1.411
CYP	0.945	0.925	0.957
HIS	1.059	0.875	1.180
UBCE	0.947	0.861	1.018
RBCL	1.011	0.989	1.134
TUA	1.340	1.032	1.673
GAPDH	1.209	0.935	1.517
EF1-α	1.083	1.169	1.098

Table 5 The expression stability values of the candidate reference genes calculated by the NormFinder program.

Gene name	All sample	N+	N-
GAPDH	0.043	0.023	0.054
TUA	0.050	0.034	0.066
RBCL	0.029	0.030	0.029
UBCE	0.021	0.020	0.026
HIS	0.032	0.021	0.043
CYP	0.021	0.027	0.018
UBQ	0.046	0.050	0.048
18S rDNA	0.078	0.098	0.056
Actin	0.025	0.021	0.027
EF1-α	0.036	0.044	0.030

3.4.3 Best combination of internal genes with geNorm program calculation

In gene expression analysis, compared with single internal genes, made of two or more genes can be better deviation correction system.Aiming at the 10 internal candidate genes, we geNorm program is

used to calculate them in nitrogen and nitrogen deficiency average express stable value of different point in time.The results show that the micro be found under the condition of nitrogen deficiency in the optimal internal genes for *UBCE* and *HIS* (figure 3), so can use *UBCE* and *HIS* as double internal to study target gene transcription profile after nitrogen deficiency.

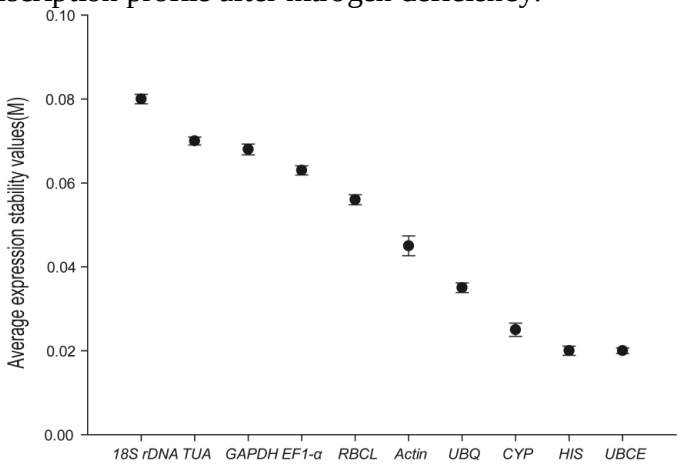

Fig.3 The Average expression stability values of reference genes analyzed by geNorm program.

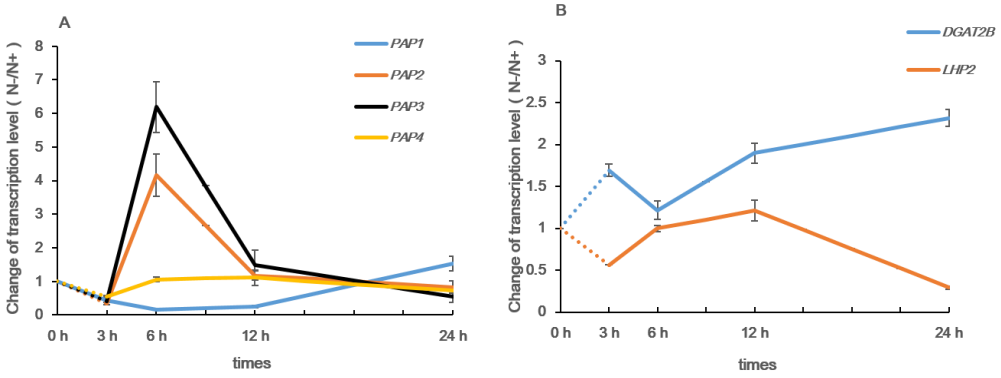

Fig.4 Transcriptional profile of genes in center carbon metabolic pathways under nitrogen-depleted (N-) conditions. (A) The transcriptional variation of homologous genes encoding the Phosphatidic acid phosphatase under nitrogen starvation; (B) The transcriptional variation of LHP2 and DGAT2B under nitrogen starvation

3.5 The center carbon metabolic pathway genes transcription map under nitrogen deficiency with selected internal genes

For exploring the mechanism of micro algae oil production, identification of micro algae nitrogen deficiency in the process of oil production key genetic components and control factors, we first explore the micro be found when nitrogen deficiency, carbon metabolic pathways of key gene transcription profile.We adopt UBCE and HIS as the double internal, was investigated by means of qPCR. N.o ceanica IMET1 nitrogen deficiency induced by 3 h, 6 h ,12 h and 24 h when multiple gene transcription dynamics, including coding three fatty acyl glycerin (TAG) synthetic ways of Phosphatidic acid phospholipase (Phosphatidic acid phosphatase, PAP) and direct catalytic three fatty acyl glycerol synthesis of the key enzymes of two fatty acyl glycerin: acyltransferase 2B (Diacylglycerol acyltransferase, DGAT2B), and the photosynthetic pathways of depleting compound protein 2 (light harvesting protein 2, LHP2) gene.Phosphatidic acid phospholipase PAP catalytic phosphatidic acid PA to phosphorylation to generate two fatty acyl glycerin DAG, and DAG is the direct precursor of TAG synthesis [31].In micro quasi found identified four PAP gene in the genome, PAP1, PAP2, PAP3 and PAP4. from the figure 4A,we can see the four homologous genes in nitrogen deficiency in the process of oil production transcription profile present diversity, including PAP2 and PAP3 in nitrogen deficiency in a significant rise in 6 hours ago, and decreased significantly after 6 hours, not stable;And PAP1 transcription has been stabilized, PAP4 continued to fall.Photosynthetic

pathways of *LHP2* transcription cut after nitrogen deficiency, and *DGAT2B* transcription level during the initial phase, the showed a trend of increase, nitrogen deficiency after 6 hours while down slightly, but over the next 12, 24 hours were significantly increased nitrogen samples over the same period (Fig.4B).

4. Conclusion

Experimental analysis results showed that that *UBCE* and *HIS* as the most appropriate combination of reference genes for Nitrogen-starved condition in *Nannochloropsis*. Based on this finding, we detected the transcriptional variation of three genes involved in center carbon metabolic pathways. Two of the four homologous genes of phosphatidic acid phosphatase (PAP) exhibited significantly increased transcription at 6 hr after nitrogen depletion, suggested their potential involvements in producing sufficient diacyl-glycerol (DAG) for TAG biosynthesis and the two genes could serve as key target genes for further genetic engineering study in the purpose of improving lipid productivity. Moreover, the up-regulation Diacylglycerol acyltransferase 2B gene (*DGAT2B*), coupled with the down-regulation of light harvesting protein 2 gene (*LHP2*), pushed the carbon flux flowing into TAG biosynthesis in *Nannochloropsis*. This work provided the foundation for the most accurate quantification of gene expression under Nitrogen starved condition in *Nannochlorpsis*. The investigation of transcriptional dynamics of key genes involved in lipid biosynthesis pathway would give researchers new insight of the functional diversity of *PAP* homologous genes as well as the transcriptional regulation of central carbon metabolic pathway.

Acknowledgements

Supported by the national natural science youth fund of China (31401116); National natural science Foundation of China (41276155).

References

[1] Chisti, Y. Biodiesel from microalgae[J]. Handbook of Hydrocarbon & Lipid Microbiology, 2010. 26(3):2827-2839.
[2] Hu, Q., et al. Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances[J]. Plant J, 2008. 54(4): p. 621-39.
[3] Wijffels, R.H. and M.J. Barbosa. An Outlook on Microalgal Biofuels[J]. Science, 2010. 329(5993): p. 796-799.
[4] Wu, X.D., et al. Current Status and Prospects of Biodiesel Production from Microalgae[J]. Energies, 2012. 5(8): p. 2667-2682.
[5] Pereira, C.M.P., et al. Biodiesel Derived from Microalgae: Advances and Perspectives[J]. Quimica Nova, 2012. 35(10): p. 2013-2018.
[6] Lim D K Y, Garg S, Timmins M, et al. Isolation and evaluation of oil-producing microalgae from subtropical coastal and brackish waters[J]. Plos One, 2012, 7(7):266-266.
[7] Li, Y.G., et al. Microalgal biodiesel in China: Opportunities and challenges[J]. Applied Energy, 2011. 88(10): p. 3432-3437.
[8] Wang, D., et al. Establishing oleaginous microalgae research models for consolidated bioprocessing of solar energy[J]. Adv Biochem Eng Biotechnol, 2012. 128: p. 69-84.
[9] Tredici, M.R., et al. Microalgae for Oil: Strain Selection, Induction of Lipid Synthesis and Outdoor Mass Cultivation in a Low-Cost Photobioreactor[J]. Biotechnology and Bioengineering, 2009. 102(1): p. 100-112.
[10] Su, C.H., et al. Factors affecting lipid accumulation by Nannochloropsis oculata in a two-stage cultivation process[J]. Journal of Applied Phycology, 2011. 23(5): p. 903-908.
[11] Rodolfi, L., et al. Growth medium recycling in Nannochloropsis sp mass cultivation[J]. Biomolecular Engineering, 2003. 20(4-6): p. 243-248.

[12] Zittelli, G.C., R. Pastorelli, and M.R. Tredici. A Modular Flat Panel Photobioreactor (MFPP) for indoor mass cultivation of Nannochloropsis sp. under artificial illumination[J]. Journal of Applied Phycology, 2000. 12(3-5): p. 521-526.
[13] Lim, D.K.Y., et al. Isolation and Evaluation of Oil-Producing Microalgae from Subtropical Coastal and Brackish Waters[J]. Plos One, 2012. 7(7).
[14] Jia, J., et al. Molecular mechanisms for photosynthetic carbon partitioning into storage neutral lipids in Nannochloropsis oceanica under nitrogen-depletion conditions[J]. Algal Research, 2015. 7: p. 66-77.
[15] Vieler, A., et al. Genome, functional gene annotation, and nuclear transformation of the heterokont oleaginous alga Nannochloropsis oceanica CCMP1779[J]. PLoS Genet, 2012. 8(11): p. e1003064.
[16] Radakovits, R., et al. Draft genome sequence and genetic transformation of the oleaginous alga Nannochloropis gaditana[J]. Nat Commun, 2012. 3: p. 686.
[17] Wang, D., et al. Nannochloropsis genomes reveal evolution of microalgal oleaginous traits[J]. PLoS Genet, 2014. 10(1): p. e1004094.
[18] J, L., et al. Choreography of Transcriptomes and Lipidomes of Nannochloropsis Reveals the Mechanisms of Oil Synthesis in Microalgae[J]. Plant Cell, 2014. 26(4): p. 1645-1665.
[19] Corteggiani Carpinelli, E., et al. Chromosome Scale Genome Assembly and Transcriptome Profiling of Nannochloropsis gaditana in Nitrogen Depletion[J]. Molecular Plant, 2014.7 (2): 323- 335.: p. 323-335.
[20] Tricarico, C., et al. Quantitative real-time reverse transcription polymerase chain reaction: normalization to rRNA or single housekeeping genes is inappropriate for human tissue biopsies[J]. Analytical biochemistry, 2002. 309(2): p. 293-300.
[21] Lin, Y. and Z. Lai. Reference gene selection for qPCR analysis during somatic embryogenesis in longan tree[J]. Plant Science, 2010. 178(4): p. 359-365.
[22] Radonić, A., et al. Guideline to reference gene selection for quantitative real-time PCR[J]. Biochemical and biophysical research communications, 2004. 313(4): p. 856-862.
[23] Cao, S., et al. Evaluation of putative internal reference genes for gene expression normalization in Nannochloropsis sp. by quantitative real-time RT-PCR[J]. Biochem Biophys Res Commun, 2012. 424(1): p. 118-23.
[24] Vandesompele, J., et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes[J]. Genome biology, 2002. 3(7): p. research0034.
[25] Taylor, S., et al. A practical approach to RT-qPCR—publishing data that conform to the MIQE guidelines[J]. Methods, 2010. 50(4): p. S1-S5.
[26] Reid, K.E., et al. An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development[J]. BMC plant biology, 2006. 6(1): p. 27.
[27] Le Bail, A., et al. Normalisation genes for expression analyses in the brown alga model Ectocarpus siliculosus[J]. BMC Molecular Biology, 2008. 9(1): p. 75.
[28] Nicot, N., et al. Housekeeping gene selection for real-time RT-PCR normalization in potato during biotic and abiotic stress[J]. Journal of experimental botany, 2005. 56(421): p. 2907-2914.
[29] Jian, B., et al. Validation of internal control for gene expression study in soybean by quantitative real-time PCR[J]. Bmc Molecular Biology, 2008. 9(25): p.: 59.
[30] Andersen, C.L., J.L. Jensen, and T.F. Ørntoft. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets[J]. Cancer research, 2004. 64(15): p. 5245-5250.
[31] Liang, M.H. and J.G. Jiang. Advancing oleaginous microorganisms to produce lipid via metabolic engineering technology[J]. Progress in Lipid Research, 2013. 52(4): p. 395-408.