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Evaluation of putative internal reference genes for gene expression normalization in *Nannochloropsis* sp. by quantitative real-time RT-PCR

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ABSTRACT

Quantitative real-time reverse transcription PCR (RT-qPCR), a sensitive technique for quantifying gene expression, depends on the stability of the reference gene(s) used for data normalization. To date, few studies on reference genes have been undertaken for *Nannochloropsis* sp. In this study, 12 potential reference genes were evaluated for their expression stability using the geNorm and NormFinder statistical algorithms by RT-qPCR. The results showed that the best reference genes differed depending on the treatments: different light intensities (DL), the diurnal cycle (DC), high light intensity (HL) and low temperature treatments (LT). A combination of ACT1, ACT2 and TUA would be appropriate as a reference panel for normalizing gene expression data across all the treatments. ACT2 showed the most stable expression across all tested samples but was not the most stable one for individual treatments. Though 18S showed the least stable expression considering all tested samples, it is the most stable one for LT using geNorm. The expression of *Lhc* confirmed that the appropriate reference genes are crucial. These results provide a foundation for more accurate use of RT-qPCR under different experimental conditions in *Nannochloropsis* sp. gene analysis.

1. Introduction

Microalgae are considered one of the most promising potential biofuel feedstocks for containing varying amounts of lipids [1–3]. Several species of *Nannochloropsis* are emerging as models for biofuel production because their lipid content makes up between 10 and 60 wt.% of their dry matter and they also have a high biomass productivity [4,5]. Moreover, the commercial cultivation of *Nannochloropsis* sp. is well understood because they also produce high concentrations of important antioxidants such as zeaxanthin and canthaxanthin, which are high-value nutraceutical products [6]. Although microalgae have long been considered a promising platform for the production of biofuels, earlier studies concluded that the economics of microalgal biofuel production needed to be significantly improved. At present, most research is mainly focused on oil extraction methods and the changes to the algae culture conditions in order to extract or produce more biofuel [3,7–9]. Molecular biology tools, such as new genetic tools, genome sequences, and high-throughput analytical techniques, will allow researchers to analyze and manipulate metabolic pathways with unprecedented precision [10]. With the growing importance of *Nannochloropsis* sp. in the production of biofuels and high value pigments, there is a need to understand the molecular mechanisms behind the metabolic pathways for lipid and pigment accumulation in *Nannochloropsis* sp. so that important new sources of renewable bioenergy can be created [11].

Gene expression analysis is contributing to the understanding of the signaling and metabolic pathways that underlie developmental and cellular processes [12]. Quantitative real-time reverse transcription PCR (RT-qPCR) is an extremely sensitive technique that allows fast and the precise measurement of gene expression, even in transcripts that are not highly expressed [13,14]. The accuracy of the results obtained by this method strongly depends on accurate transcript normalization using stably expressed genes, known as references. Selection and use of appropriate reference genes, as internal controls in RT-qPCR analysis, is very important for accurate quantification of gene expression levels [15]. Identification of candidate genes useful for normalization has become a major task as it has been shown that normalization errors are probably the most common mistake, resulting in significant artifacts that can lead to erroneous conclusions. Statistical algorithms, including the geNorm [16] and NormFinder [17] used in this study,

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have been developed recently to identify the best reference genes for use under a given set of experimental conditions. The program, geNorm, uses pairwise comparisons and geometric averaging across a matrix of reference genes and NormFinder fits data to a mathematical model, which allows comparison of intra- and inter-group variation and the calculation of expression stability [18].

However, the RT-qPCR approach is underutilized in many species where putative housekeeping genes tend to be used as references without any appropriate validation. Housekeeping genes can vary considerably in response to changes in experimental conditions and/or tissue types and are certainly not the best options [15]. It has been shown that normalization errors are probably the most common mistake in RT-qPCR [18]. Thus, a systematic validation of reference genes is required to ensure proper normalization. A large number of publications describing human, animal, plant and fungal systems, such as in rats [19], *Drosophila melanogaster* [20], *Arabidopsis* [21], rice [22] and *Beauveria bassiana* [23], etc., have concentrated on the identification of genes specific for a certain tissue, developmental stage or environmental condition. The selection of reference genes has also been reported in algae. For example, EF1-α was the most appropriate reference gene for normalization in the brown alga model, *Ectocarpus siliculosus* [24]. The best reference genes for normalization in the green algae, *Ulva linza* [25] and *Chlamydomonas* sp. ICE-L, have also been selected. However, there have been very few studies on the validity of reference genes for the oleaginous *Nannochloropsis* sp.

The aim of the current study was to identify moderately expressed genes that have been commonly used as reference genes and had relatively stable gene expression under all experimental conditions in *Nannochloropsis* sp. For this purpose, genes which have been reported to be good potential candidates in previously published studies, such as ribosomal RNA (18S rRNA), the chloroplast-encoded large subunit of the ribulose-1,5-bisphosphate carboxylase/oxygenase genes (*rbc*L) and a number of housekeeping genes, such as those encoding actin (ACT), tubulin (TUB), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), polyubiquitin (UBQ), elongation factor 1-α (EF1-α), Cyclophilin (CYP) and ubiquitin conjugating enzyme (UBCE) were chosen and their expression stability was tested.

2. Materials and methods

2.1. Sampling and culture conditions

The *Nannochloropsis* sp. cells were grown photoautotrophically at 23 °C and 40 μmol m^{-2} s^{-1} white light, with a 12:12 h light/dark photoperiod, in 500 mL Erlenmeyer flasks containing 300 mL Provasoli seawater medium. To collect samples through the diurnal cycle (DL), cells were kept under 40 μmol photons m^{-2} s^{-1} light density and harvested every 2 h during daytime except the first sample which was taken 1 h after the beginning of the light period. In the high light (HL) stress treatment, algae were exposed to 1200 μmol photons m^{-2} s^{-1} for 0.5, 1, 2, 3, 4, 5 and 6 h to investigate the effects of HL exposure on mRNA expression of reference genes. In different light intensities (DL) experiments, algae were exposed to 40, 80, 100, 120, 250, 500, 1000, 2000 μmol photons m^{-2} s^{-1} for 1 h. In the low temperature (LT) stress treatments, algal cells were kept in 5 °C for 0.5, 1, 2, 3 and 6 h.

2.2. RNA extraction and reverse transcription

The total RNA was extracted using Trizol Reagent according to the user's manual, after which it was dissolved in diethypyrocarbonate-treated water. In this protocol, RNA was digested with DnaseI and no DNA products were visualized after electrophoresis in 1% agarose gel. The primary cDNA used for RT-qPCR was synthesized from equal amounts of purified total RNA using the M-MLV first-strand synthesis system (Promega Biotech Co., Madison, WI, USA) according to the manufacturer's procedure and oligo d(T)18 (TaKaRa Biotech Co., Dalian, China).

2.3. Quantitative real-time PCR

For real-time PCR, 12 pairs of gene- specific primers (ACT1, ACT2, TUA, TUB, UBQ, EF1 - α, GAPDH, His, CPY, UBCE, 18S, *rbc*L) were designed according to the transcriptome of *Nannochloropsis* sp. (Table 1). RT-qPCR was conducted using the ABI Step One Plus Real-Time PCR System (Applied Biosystems) using SYBR Green fluorescence (Takara) according to the manufactoreros instructions. The PCR amplification profile was 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s, 58 °C for 10 s, and 72 °C for 30 s.

2.4. Data analysis

Reference gene expression stability was analyzed using the geNorm (v3.5) and NormFinder (v0.953) software packages. GeNorm derives a stability measure (M), which is the average pairwise variation of a gene compared to the other control genes included in the same analysis and via a stepwise exclusion of the least stable gene, which creates a stability ranking. It also estimates the number of genes required to calculate a robust normalization factor. GeNorm suggests $M = 1.5$ as a cutoff value, meaning that genes with $M > 1.5$ should not be used as reference genes. Gene with the lowest M value is considered to be the most stable one [16]. NormFinder uses an ANOVA-based model to estimate intra- and inter-group variation and combines these estimates to provide a direct measure of the variation in expression for each gene. The lowest stability value is accorded the top rank by the analysis. All other statistical analyses were performed using SPSS (v13, SPSS Inc., Chicago, IL).

2.5. Impact of using inappropriate reference genes on the expression analysis of Lhc gene

The *Lhc* gene, which encodes a major light-harvesting protein used in photosystems and is essential in light capture and energy transfer within antenna complexes, was used for validating the impact of the use of inappropriate reference genes on the gene expression analysis. Gene expression levels of *Lhc* were quantified (Forward: GGTACATTGTCCAGGGGCTG and reverse: ATGGCAATGC CGATCTGCA) using the most stable/unstable reference genes as determined by geNorm and NormFinder under HL treatment. The PCR amplification profile was 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s, 58 °C for 10 s, and 72 °C for 30 s. The $2^{-\Delta\Delta Ct}$ method [26] was used to analyze quantitative real-time PCR data.

3. Results

3.1. Expression profiling of candidate reference genes

C_t values were calculated using the raw expression data varied within the range 7.82–28.76 among the 12 reference genes across all the treatments. Compared with other genes, 18S was highly expressed (Fig. 1). The lowest transcriptional level was for UBCE, with C_t values ranging from 23.47 to 28.76. The individual reference genes had different C_t value ranges across all the samples studied. Variation was lower in UBCE and TUB (<6 cycles), but much higher in 18S (>10 cycles). No single reference gene had a constant expression level in different samples of *Nannochloropsis* sp.

Table 1
Genes and primers.

Gene name	Molecular function	Primer sequences (forward/reverse)
ACT1	Actin	F:GGATACTCCTTCACTACCACG R:CCAAGCTGGAAGACTCCTCC
ACT2	Actin	F:ACCTTCTACAACGAGCTGC R:GAACGTCTCAAACATAATCTGG
TUA	Alpha-tubulin	F:TGACCTGCTGCCTCATGTACC R:TTGATGCCGCACTTGAAGC
TUB	Beta-tubulin	F:AGCATGGCATTGACTCCACC R: AACGGCCTCGTTGTAGTACACG
EF1-α	Elongation factor 1-alpha	F:TCAGATCCAAAACGGCTACTGC R:GGTACGTCGGTCAATCTTTTCC
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	F:TGACTTCATCACGGACTCC R:GTCATACCACGACACCAGC
UBQ	Polyubiquitin	F:CGCTTTCTGACTACAACATCC R:TCCAGCGTGATGGTCTTGCC
HIS	Histone	F:CGCATTATTCCTCGCCACG R:ACACCACCGGAAGCAATGG
CYP	Cyclophilin	F:GCAATTCTTCCTTTGCACG R:TCAATGGCTTTTACGACATCC
UBCE	Ubiquitin conjugating enzyme	F:CTCCATCCTCAACGAAGAC R:TCCAACAGGTCCTGAATGC
18S	18s rDNA	F: AGGGGACCGTACTATTGTTGG R: AATGTATTCAGGGCCTAAGC
rbcL	Ribulose-1,5-bisphosphate carboxylase/oxygenase gene	F:ATCCATTGTGGACAAATGCACC R:CACCAGAAGCGATACCATCAGG

3.2. Expression stability of reference genes

The average expression stability (*M*) values (Table 2) of the 12 reference genes were calculated using geNorm. The most stably expressed gene throughout the HL and DC treatments was the ACT2 gene. For the LT treatment, 18S was the most stable gene with $M = 0.953$ while for the DL treatment, ACT1 ($M = 0.510$) showed the highest expression stability. When all samples were considered, the ACT2 gene showed the most stable expression with $M = 1.008$, which is lower than the recommended cutoff value of 1.5, and therefore other genes may be applicable as a single reference gene for multiple treatments, with the exception of the 18S and rbcL in *Nannochloropsis* sp. In addition, it was notable that GAPDH in the LT treatment had $M > 1.5$ (the cutoff value) and less stable expression, which indicated that it was not suitable for expression analysis under these experimental conditions.

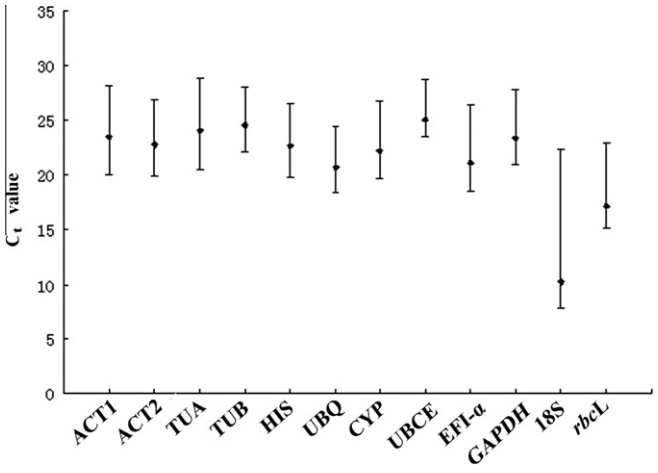

Fig. 1. The range of expression level of the 12 genes for all tested samples is expressed in C_t values. The black diamond represents the arithmetic mean. The bar indicates the minimal to maximal value.

Table 2
The expression stability values (M) estimated using geNorm algorithm of the candidates genes in all tested samples.

Gene	All samples	HL	LT	DC	DL
ACT1	1.068	0.932	1.209	0.748	0.510
ACT2	1.008	0.826	1.052	0.704	0.549
TUA	1.080	0.850	1.181	0.752	0.589
TUB	1.220	0.847	1.005	0.929	0.549
HIS	1.201	1.016	1.380	0.872	0.797
UBQ	1.090	0.886	0.963	0.724	0.734
CYP	1.318	1.012	1.296	1.169	0.722
UBCE	1.073	0.888	1.040	0.818	0.702
EF1-α	1.112	0.906	1.002	0.962	0.531
GAPDH	1.358	0.855	2.003	1.195	0.620
18S	2.700	4.863	0.953	0.818	0.580
*rbc*L	2.030	0.853	3.813	1.062	0.886

ACT2 was the best reference gene (and had the lowest M of 0.232) across all the samples in the NormFinder analysis (Table 3). The genes showing the highest stability under the LT, HL, DC and DL treatments were UBQ, TUA, ACT2 and ACT1, respectively. Therefore, with the exception of the HL and LT treatments, NormFinder gave the same results as geNorm.

3.3. Determination of the optimal number and most reliable combination of reference genes

Although most studies have used only one single gene as an internal control for normalization, the use of two or more reference genes for normalization may produce more reliable results [27,28]. The geNorm algorithm also calculated the pairwise variation, $Vn/Vn+1$, which determines the ideal number of reference genes required for accurate normalization. *Vn* values were calculated by stepwise inclusion of more reference genes until the $(n+1)$ gene made no significant contribution to the newly calculated normalization factor. A cutoff value of 0.15 has been proposed as the variation below which the inclusion of an additional reference gene was not required. The V2/3 values in the HL, LT and DL treatments all had *V* values <0.15 (Fig. 2), which suggested that two reference genes were enough for quantitative analysis. The *V* values for DC were only slightly higher than the threshold and a third gene did lead to decrease in the *V*3/4 value compared to the *V*2/3 value (from 0.163 to 0.117). Therefore, it was unnecessary to use the third reference gene in quantitative analysis under single experimental conditions. However, for all tested samples, $V3/4\ (0.143) < 1.5 < V2/3\ (0.162)$, which suggested that three reference genes were required.

Using geNorm establishes a rank order of gene stability via stepwise exclusion of the least stable gene, which allows identification of the remaining genes with the lowest *M* values (Fig. 3). UBQ and rbcL, UBQ and 18S, together with ACT1 and ACT2, were the best

Table 3
Stability values of candidate reference genes as calculated by NormFinder.

Gene	All samples	HL	LT	DC	DL
ACT1	0.391	0.416	0.621	0.239	0.162
ACT2	0.263	0.221	0.406	0.134	0.217
TUA	0.343	0.059	0.499	0.273	0.257
TUB	0.516	0.059	0.158	0.498	0.208
HIS	0.523	0.472	0.677	0.416	0.460
UBQ	0.365	0.290	0.070	0.228	0.416
CYP	0.649	0.365	0.728	0.712	0.413
UBCE	0.333	0.250	0.219	0.361	0.396
EF1-α	0.366	0.185	0.141	0.515	0.163
GAPDH	0.631	0.117	1.100	0.723	0.296
18S	1.784	3.362	0.185	0.355	0.227
*rbc*L	1.254	0.208	2.602	0.632	0.543
Best gene	ACT2	TUA	UBQ	ACT2	ACT1

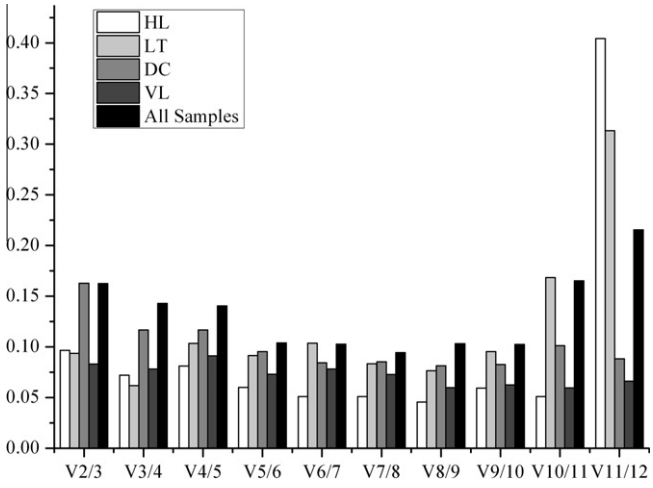

Fig. 2. Determination of the optimal number of reference genes. The pair-wise variation (*Vn*/*Vn* + 1) was analyzed between the normalization factors NFn and NFn + 1 using geNorm software to determine the optimal number of reference genes required for qRT-PCR data normalization in various sample pools.

combinations of reference genes for the HL, LT and DL treatments, respectively. For the DC treatments, the best combinations were UBQ, ACT2 and TUA. Over all the treatments, three reference genes (ACT1, ACT2 and TUA) were required for accurate normalization.

3.4. Quantification of Lhc expression with the most stable / unstable reference genes

The expression levels of a target gene, *Lhc*, were used as an example to show the effect of using different reference genes for normalization. The most and the least stable reference genes selected by geNorm were ACT2 and 18S for the HL treatment, while, TUA and 18S were selected by NormFinder (Tables 2 and 3; Fig. 4). The most appropriate gene combination (UBQ + *rbc*L) and the second best reference gene (TUB) according to geNorm and NormFinder. The expression of *Lhc* under normal conditions was set to 1. When 18S was used as the reference gene, the expression level of *Lhc* gene decreased during the first hour and then increased and peaked at 2 h, after which it declined. The expression profile clearly differed from the other three reference genes and the combination of UBQ and *rbc*L, which the gene's expression continuously decreased. These results further confirmed that the use of different reference genes will lead to differences in the quantification profile and quantification accuracy could be influenced by the inadequate selection of reference genes.

4. Discussion

In recent years, it has become clear that no single gene is expressed the same way in all cell types and under all experimental conditions and the most suitable reference genes are conditional, tissue-specific, developmental and cultivar-dependent [12]. This

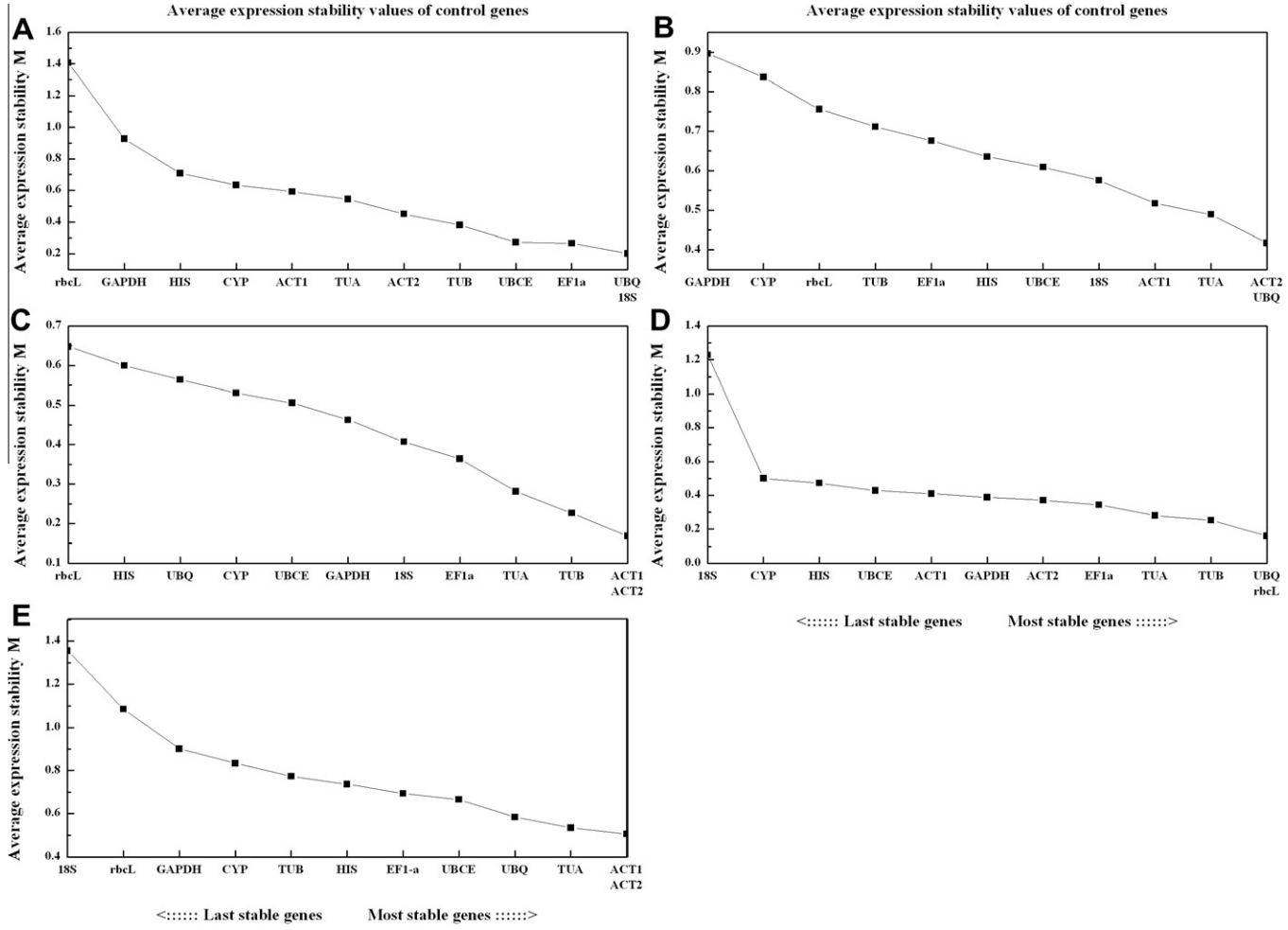

Fig. 3. Gene expression stability and ranking of the ten reference genes as calculated by geNorm in all tested samples and different experiment conditions. (A) High light (Hl), (B) low temperature (LT), (C) diurnal cycle (DC), (D) different light intensity (DL), (E) all tested samples.

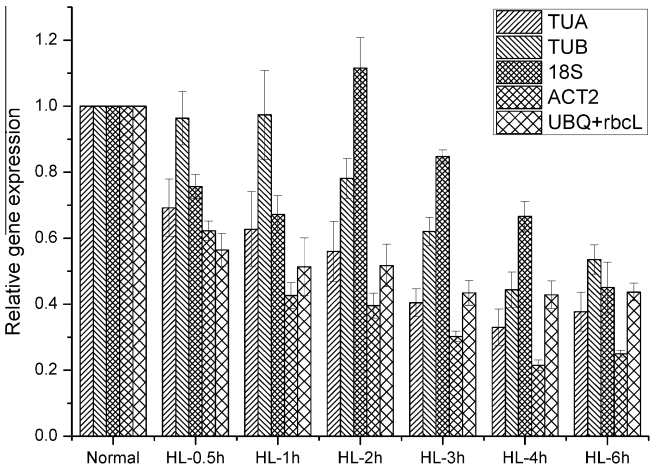

Fig. 4. Relative quantification of the target gene LHC in HL treatment using different reference genes.

implies that the expression stability of a putative control gene must be verified before each RT-qPCR experiment and that one reference gene is generally not enough to normalize the expression data of target genes. This study pre-tested the stability of 12 candidate reference genes to determine the most appropriate reference genes and the most reliable combination of reference genes for the oleaginous *Nannochloropis* sp. using geNorm and NormFinder. As far as can be ascertained, this was the first attempt to select a set of candidate reference genes in *Nannochloropis* sp. for the normalization of gene expression analysis using RT-qPCR.

Current RT-qPCR studies in *Nannochloropis* sp. had used 18S as the reference gene [29], which was in accordance with this study's results in that 18S was the most stable gene at low temperatures. As a classic housekeeping gene, 18S has been widely used as a reference gene for a number of species in gene expression analysis [30–32]. However, this study found that it displayed very poor expression stability, being the lowest ranked gene averaged across all the samples, although it was the best gene for the low temperature analysis. Furthermore, its C_t value was much lower than that of the other candidates tested, which implied that its expression level was several orders of magnitude higher than the other genes and so might deviate from the confidence interval of the standard curve. Therefore, the results of this study suggested that 18S should not be used as a reference for RT-qPCR in *Nannochloropis* sp.

This study used geNorm and NormFinder to determine the most stable gene(s) under different experimental conditions. A major conclusion arising from geNorm was that universally suitable reference genes for RT-qPCR analyses could not be identified because the best-ranked reference genes differed across the treatments. ACT2 was the most reliable candidate gene (i.e. lowest M value) when averaged across all the samples tested. However, under LT and DL experimental conditions, ACT2 did not have the lowest M value. Similarly, ACT2 was identified as the best reference gene using NormFinder when averaged across all the samples but under the DL, LT and HL treatments, it did not have the best stability. When quantifying *Lhc* expression, the expression level and the trend under high light differed between 18S and TUA/TUB/ACT2 and UBQ + *rbc*L. These results further confirmed the importance of validating reference gene stability. For *Nannochloropis* sp., different reference genes should be selected depending on the experimental conditions. In addition, the lack of a systematic validation of reference genes is a serious problem when undertaking RT-qPCR with algae and so there needs to be more research conducted into the validation of reference genes for algae.

Normalization is a major issue when analyzing gene expression in response to various experimental conditions and the use of incorrect normalization targets could lead to erroneous interpretation of quantification data. Through stability analysis, we identified several genes suitable for normalization of RT-qPCR data under multiple experimental conditions. Knowledge of *Nannochloropis* sp. development, metabolism and stress responses is still limited, so the reasons for the stability of some genes and the variability of others still need to be determined. This study was based on RT-qPCR, so these findings will provide direct guidance for future RT-qPCR experiments and may be of use in the Northern blot technique, which also requires reference genes for normalization. Moreover, these findings will improve the understanding of the mechanisms behind development, metabolism and stress response in *Nannochloropis* sp.

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