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ORIGINAL PAPER

Validation of housekeeping genes for gene expression studies in an ice alga *Chlamydomonas* during freezing acclimation

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Abstract Antarctic ice alga *Chlamydomonas* sp. ICE-L can endure extreme low temperature and high salinity stress under freezing conditions. To elucidate the molecular acclimation mechanisms using gene expression analysis, the expression stabilities of ten housekeeping genes of *Chlamydomonas* sp. ICE-L during freezing stress were analyzed. Some discrepancies were detected in the ranking of the candidate reference genes between geNorm and NormFinder programs, but there was substantial agreement between the groups of genes with the most and the least stable expression. RPL19 was ranked as the best candidate reference genes. Pairwise variation (*V*) analysis indicated the combination of two reference genes was sufficient for qRT-PCR data normalization under the experimental conditions. Considering the co-regulation between RPL19 and RPL32 (the most stable gene pairs given by geNorm program), we propose that the mean data rendered by RPL19 and GAPDH (the most stable gene pairs given by NormFinder program) be used to normalize gene expression values in *Chlamydomonas* sp. ICE-L more accurately. The example of FAD3 gene expression calculation demonstrated the importance of selecting an appropriate category and number of reference genes to achieve an accurate and reliable normalization of gene expression during freeze acclimation in *Chlamydomonas* sp. ICE-L.

Keywords *Chlamydomonas* sp. ICE-L · Freezing acclimation · Reference genes · Real-time RT-PCR · Normalization · FAD3

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Introduction

Freezing tolerance is an important determinant of geographical distribution of plant species, and freezing damage in crop plants leads to severe losses in agriculture. Antarctica constitutes a unique and extreme environment on the earth. On this extraordinary continent, floating sea–ice with a temperature range from 0 to −35 °C is supposed to be one of the coldest habitats on the earth for marine-living creatures (Mock and Thomas 2005). The sea ice interior consists of a two-phase system where ice crystals are interlaced with a network of fluid-filled brine pockets and channels. The salinity within the brine network increase with the decline of ice temperature varies from 0 to over 200 PSU (Gradinger 2001). Consequently, the algae live in the freezing sea ice are exposed to extremes of temperature and salinity.

The survival of microalgae in floating sea–ice matrix requires complex suite of physiological and metabolic mechanisms for acclimatization in this extreme living space. Previous studies indicated that sea ice algae adapt to the extreme condition by producing polyunsaturated fatty acids and cryoprotectants and by having high light-harvesting capacity (Morgan-Kiss et al. 2006). Polar alga inhabit in the sea ice is a good model species for studying low temperature and high salinity acclimation. Of all algae, those in the division Chlorophyta (green algae) display the closest relationship to the vascular plants. Because of the presence of plastids and plant-like cell walls, unicellular green algae may be considered as true plant-like eukaryotic microorganisms (Hicks et al. 2001).

A unicellular green alga *Chlamydomonas* sp. ICE-L was isolated from Antarctic floating ice and kept in an axenic culture in our laboratory (Liu et al. 2006). It is a psychrophilic alga and demonstrates some physiological properties such as freezing and hypersaline adaptations that correlate with Antarctic ice habitats (Kan et al. 2006). Cell division activity has been observed after keeping the alga at −20 °C for several weeks. To study the freezing stress adaptation mechanism of sea ice green alga *Chlamydomonas* sp. ICE-L at gene transcription level, the stability of reference genes needs to be validated prior to real-time reverse transcriptase polymerase chain reaction (real-time RT-PCR) analysis.

Real-time RT-PCR has become a widely used technique for gene expression analysis, and it is a useful method for studying the molecular accumulation mechanism to abiotic stress conditions. Relative quantification methods such as comparative CT ($2^{-\Delta\Delta CT}$) method (Livak and Schmittgen 2001) depend on reference genes for normalization. In order to obtain reliable and repeatable results, the use of appropriate internal reference to normalize the qRT-PCR data is essential (Huggett et al. 2005). Blindly using a housekeeping gene as internal, it might make the small differences in gene expression difficult to find, on the other hand may lead to errors or even opposite conclusions (Guenin et al. 2009).

Many methods of identifying normalization genes, such as geNorm (Vandesompele et al. 2002) and NormFinder (Andersen et al. 2004) have been developed. Many reports on the identification of the best normalization genes from a broad range of species and specific tissues of a variety of animals and plants have been published. But so far as we are aware, the experimental reference gene studies performed with algae was only on a brown alga *Ectocarpus siliculosus* (Le Bail et al. 2008) and a dinoflagellate *Symbiodinium* (Rosic et al. 2010). Even the references gene used in gene expression normalization of the model species *Chlamydomonas reinhardtii* have not been validated. In this study, to understand the freeze adaptation mechanism of ice alga at gene transcription level, the selection of suitable housekeeping genes for real-time PCR experiments under freezing conditions is essential. The expression variability of ten housekeeping genes (ALDA, EF-1α, PGK, GAPDH, H2B, RPL19, RPL32, RPS10, TUBA, and TUBB) was studied in *Chlamydomonas* sp. ICE-L exposed to freezing conditions to assess their value as internal controls in gene expression normalization.

Materials and methods

Culture conditions and treatments

The water samples containing Antarctic ice algae were provided by the Polar Research Institute of Shanghai, China. They were collected from the floating ice near the Zhongshan Research Station of Antarctica (69.8 °S, 77.8 °E) during the 18th Antarctic expedition of China. A unicellular ice alga was isolated and identified as *Chlamydomonas* sp. ICE-L (Liu et al. 2006). The algae were axenic cultured at light intensity of 40 μmol photons m^{-2} s^{-1}, 12L: 12D cycle, temperature of 6 °C, in the Provasoli seawater medium (Provasoli 1968). Seawater used for ice algae culture was taken from the coast of Qingdao (Shandong, China). To investigate the effects of freezing stress on the expression of the housekeeping genes, *Chlamydomonas* sp. ICE-L samples were kept at −20 °C for 0.5, 1.5, 3, 6 and 12 h, respectively, in a dark cycle. Another group of samples were kept at 6 °C at the same time points as controls. After treatment, the alga samples were collected by centrifugation (4 °C, 5 min, 3,000×*g*), then immediately frozen in liquid nitrogen, and stored at −80 °C before RNA extraction.

RNA extraction and cDNA synthesis

Frozen algae samples were ground independently in liquid nitrogen. Total RNA was extracted using CTAB method according to Wu et al. (2010). Residual genomic DNA was removed by RNase free-DNase (Invitrogen, Carlsbad, CA, USA). The purity of prepared RNA was detected by the absorption at 260 nm and the ratio of absorption at 260:280 nm (A260/A280). RNA was reverse transcribed to produce cDNA using PrimeScript™ Reverse Transcriptase (TaKaRa, Dalian, China) with anchored oligo $(dT)_{18}$ primers in total reaction volumes of 20 μl.

PCR primer design and qRT-PCR

The candidate reference genes were obtained from the *Chlamydomonas* sp. ICE-L expressed sequence tag (EST) databases. Primer pairs designed using Primer Premier 5.0 (Premierbiosoft, Palo Alto, CA, USA) are described in Table 1. To ensure the maximum specificity and efficiency during PCR amplification, a stringent set of criteria was used for primer design (Udvardi et al. 2008). This included predicted melting temperatures (T_m) of ~60 °C, primer lengths of 18–25 nucleotides, guanine–cytosine contents of 40–60 %, and PCR amplicon lengths of 60–150 base pairs. All primers were custom ordered from a commercial supplier (Biosune, Shanghai, China). QRT-PCR was performed in an Mx3005P real-time PCR machine (Stratagene, USA) using a SYBR® PrimeScript™ RT-PCR Kit (TaKaRa, Dalian, China).

Thirty-five cycle reactions were conducted (95 °C for 10 s; 60 °C annealing for 10 s; 72 °C for 20 s). To evaluate the amplification of primers, standard curves of each pair of primers were constructed and PCR efficiencies were

Table 1 Primer pairs used in qRT-PCR

Gene name	Symbol	Primer sequence
Aldolase A	ALDA	5′ ACGTCAAGGAGAAGAAGGGTCTGT 3′
		5′ TCCTCCGTGGAAAACAAAGTAGAT 3′
Translation elongation factor 1 alpha	EF-1α	5′ GCTGATGTCTGTCTGCTGATGGT 3′
		5′ CTTCTGGATGGCAGTGGTAAAGT 3′
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	5′ GCAGCGACATCCCTACCT 3′
		5′ CCCTGACAATGCCGAACT 3′
Histone H2B	H2B	5′ TGGGCATCCTGAACTCCTTTAT 3′
		5′ GTGACAGTGGGCTTCTTGTTGT 3′
60S ribosomal protein L19	RPL19	5′ CCAATGAGGTCAACGAGATTTCC 3′
		5′ ATCACAAATCCGTCCTTCACCAG 3′
60S ribosomal protein L32	RPL32	5′ CTCGCTTCAAACGCTTCCAGAG 3′
		5′ CCAACATTAGGCATGGCAATCAT 3′
40S ribosomal protein S10	RPS10	5′ AGAGGTTTGCATGGAGGCACTAC 3′
		5′ GCACAATATCATCAGGCAGGTTC 3′
Alpha tubulin	TUBA	5′ ACTACACCATCGGCAAGGAGATT 3′
		5′ TGGAGACCAGTACAGTTGTCAGC 3′
Beta tubulin	TUBB	5′ AGGAGGCCGAGTCTTGC 3′
		5′ AGGGTGGCGTTGTAGGG 3′
Phosphoglycerate kinase	PGK	5′ GTCTGCGGATTCCTGATGC 3′
		5′ AACAATGGCGACGAAAGG 3′
Ω-3 fatty acid desaturases	FAD	5′ CCGTTACTGATTCTTGTTTGGGCTAC 3′
		5′ TCGGCTGTATTTGAATGCTCGTCT 3′

calculated from the slopes of the curves. Relative gene expression determinations were made with the comparative delta–delta C_T method ($2^{-\Delta\Delta CT}$) described by Livak and Schmittgen (2001). Results were given as the mean of three biological replicates.

Results

QRT-PCR amplification of housekeeping genes

The expression of ten potential housekeeping genes, i.e., ALDA, EF-1α, PGK, GAPDH, H2B, RPL19, RPL32, RPL10, TUBA, and TUBB, from *Chlamydomonas* sp. ICE-L under freezing conditions was analyzed by qRT-PCR. The specificities of PCR amplifications were confirmed by melting curve analysis with the appearance of a single peak. The PCR products were also examined by agarose gel electrophoresis, which showed that the products from each amplification appeared as a single band of the expected size. PCR efficiency (E) and correlation coefficients (R_2) were determined based on the slopes of the standard curves generated from the *Ct* values of serial dilutions of the cDNA. The E values of the ten genes ranged from 93 to 102 %, and the R_2 values ranged from 0.991 to 0.998.

Expression levels of the housekeeping genes

To give an overview of the relative abundance of the candidate reference genes in *Chlamydomonas* sp. ICE-L, the calculated cycle threshold (*Ct*) values were determined for each gene across all of the experimental samples and scatter plots were constructed and shown in Fig. 1. Gene expression analyses of the ten reference genes exhibited a narrow range of mean *Ct* value across all experimental samples (Fig. 1). The *Ct* values ranged from 18 to 25. The least abundant transcripts were TUBB with a *Ct* value around 23. RPS10, RPL19 and GAPDH were abundantly transcribed than others, reaching the threshold fluorescence peak after 19 amplification cycles.

Gene expression stability analysis

The expression stabilities (M) of each reference gene were determined using software geNorm based on the average pairwise variation between all of the genes tested. Genes with the lowest M value are the most stably expressed, while a higher M value is indicative of less stable expression. All of the genes in this study had an M value below the geNorm threshold of 1.5 (Fig. 2). According to our tests, RPL19 was the most stably expressed reference gene in freezing treatment conditions. TUBA was the least stable

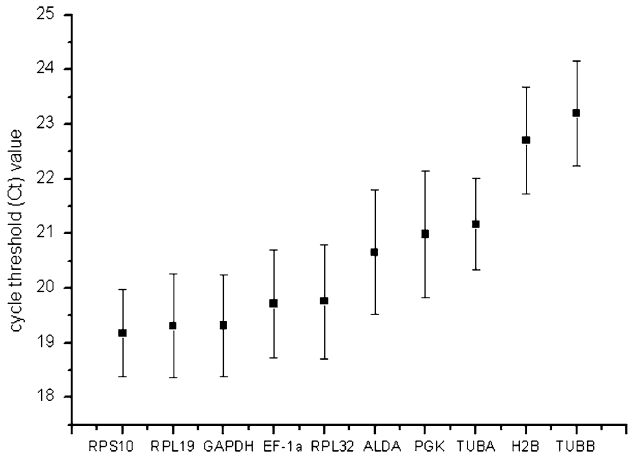

Fig. 1 Expression levels of candidate reference genes in tested alga samples. The *scatter plots* exhibit the expression levels of candidate reference genes in the tested *Chlamydomonas* sp. ICE-L samples ($n = 33$). Values are given as cycle threshold numbers (*Ct* values) with a mean of duplicate samples

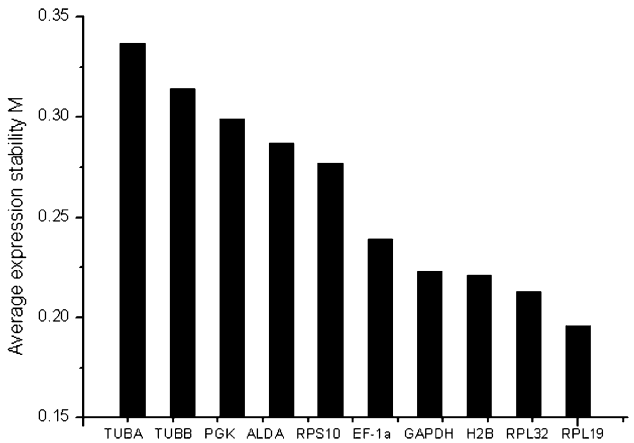

Fig. 2 Average expression stability values (*M*) of the candidate reference genes. The least stable gene is on the *left*, and the most stable gene is on the *right*

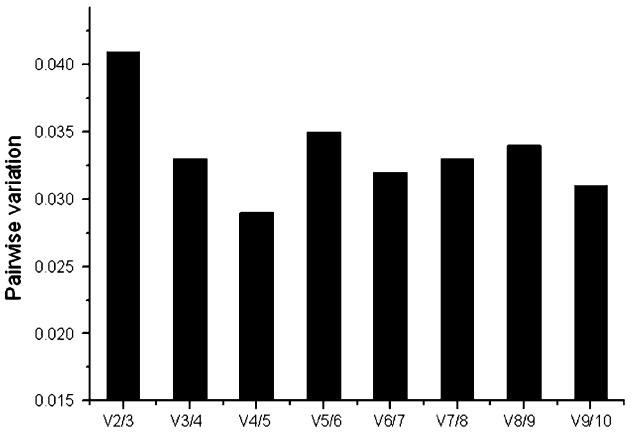

Fig. 3 Optimal number of reference genes required for effective normalization. The pairwise variation ($Vn/Vn + 1$) between the normalization factors NFn and NFn + 1 was analyzed by the geNorm program to determine the optimal number of reference genes required for qRT-PCR data normalization

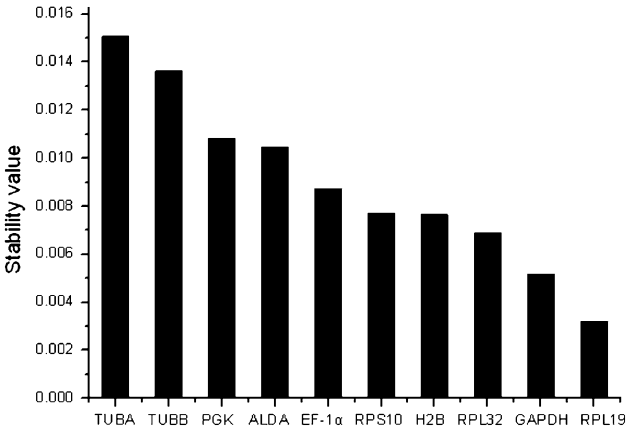

Fig. 4 Reference genes for normalization and their expression stability order a calculated by NormFinder

one. To determine the optimal number of reference genes required for normalization, pairwise variation (*V*) analysis was performed with the software geNorm. A threshold *V* value of 0.15 was adopted to determine whether the inclusion of an additional reference gene was necessary. A V2/3 score lower than the threshold of 0.15 was achieved, indicating that the combination of two reference genes was sufficient for qRT-PCR data normalization under the experimental conditions (Fig. 3).

We used the software NormFinder (http://www.mdl.dk/publicationsnormfinder.htm) to further confirm the results obtained by the geNorm program. NormFinder is an algorithm based on the variance estimation and identifies the optimal normalization gene among a set of candidate genes under a given set of experimental conditions. Although the ranking generated by this approach differed somewhat from that determined by geNorm, RPL19 was identified as the most stable gene and TUBA as the least stable gene with both software programs (Figs. 2, 4).

Normalization of CiFAD3 expression

The effects of freezing treatment on the expression of FAD3 (GQ888689), an ω-3 fatty acid desaturases encoding gene, were analyzed using the most stable (RPL19) and most unstable (TUBA) housekeeping genes for expression normalization (Fig. 5). For freezing treatment, ice alga *Chlamydomonas* sp. ICE-L in exponential growth status were treated at −20 °C for 0.5, 1.5, 3, 6, and 12 h, respectively, and that under the normal culture temperature 6 °C was set as a control. Similar expression patterns were obtained when either one or two of the most stable genes (RPL19 and RPL19 + GAPDH, as identified by geNorm)

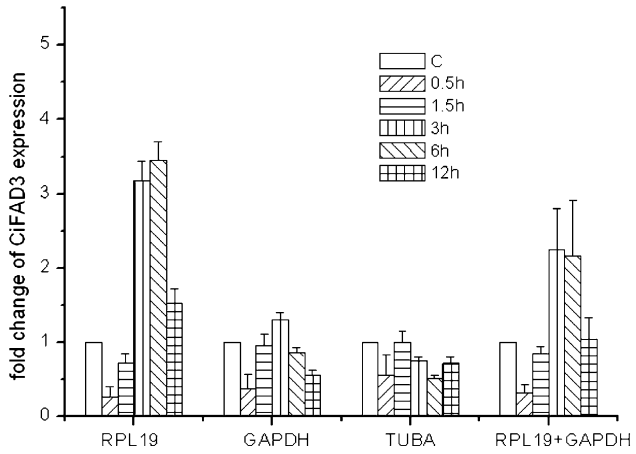

Fig. 5 Relative quantification of CiFAD3 gene expression in *Chlamydomonas* sp. ICE-L normalized to reference genes with different stability. Results are given as the mean of three biological replicates ±SD

were used for normalization. The expression of FAD3 declined significantly at the first 0.5 h when exposed to freezing stress, then increased after freezing treatment for 3–6 h to more than twofold when compared with the initial 6 °C. However, the estimated transcript abundances were not significantly changed when normalized against the most unsuitable reference genes (TUBA). Therefore, the selection of appropriate reference gene is critical for accurately evaluating the expression of a gene of interest.

Discussion

In this study, we sought suitable reference genes for the normalization of relative gene expression data in *Chlamydomonas* sp. ICE-L under freezing stress conditions. The expression stability of the ten candidate genes was tested by the computer programs geNorm and NormFinder. Some discrepancies were detected in the ranking of the candidate reference genes, but there was substantial agreement between the groups of genes with the most and least stable expression. RPL19 and RPL32 were ranked as the most stable housekeeping genes tested by geNorm. It has been argued that co-regulation of genes may confound geNorm analysis, because of the software's tendency to select the genes with a similar expression profile (Andersen et al. 2004). Of all our tested housekeeping genes, RPL19 and RPL32 belong to a particular gene family, and thus may be prone to co-regulation. GAPDH was the second suitable gene followed RPL19 by Normfinder testing. Our results lead us to propose that the mean data rendered by the combination of RPL19 and GAPDH be used to normalize gene expression values in *Chlamydomonas* sp. ICE-L more accurately.

Ribosomal protein encoding genes have been suggested to be good housekeeping genes as they are expressed in all cell types to direct biogenesis of new ribosomes. Ribosomal protein encoding genes have been widely used as references in qRT-PCR experiments in both human and animals (Hsiao et al. 2001; de Jonge et al. 2007). More and more ribosomal protein genes have been validated to be a suitable reference gene in plants and algae. The stability and suitability of a RPL7 gene as reference genes were validated in *Coffea arabica* (Barsalobres-Cavallari et al. 2009) and *Oryzias latipes* (Zhang and Hu 2007). A RPS4 gene of *Symbiodinium* was proved to be the highest expression stability among the nine selected putative housekeeping genes during thermal stress (Rosic et al. 2010). In our study, RPL19 gene was proved to be the best housekeeping gene. While to our knowledge, there was not any report on RPL19 as a calibrated gene been tested or used in the gene expression normalization in plants or algae during abiotic stress studies.

According to our results, the gene encoding glyceraldeyde-3-phosphate dehydrogenase (GAPDH), an enzyme involved in glycolysis, could be considered as a suitable reference for gene expression studies in *Chlamydomonas* sp. ICE-L. This observation corroborates the quantification of GAPDH expression in *Coffea Arabica* (Barsalobres-Cavallari et al. 2009), sugarcane (Iskandar et al. 2004) and *Eucalyptus* (Vicentini et al. 2005). Czechowski et al. (2005) compared traditional and novel reference genes in *Arabidopsis* and found that *GAPDH* was ranked among the 100 most stably expressed genes after omission of seed and pollen samples. In contrast, several reports on validation of reference genes have shown that *GAPDH* is not stable in different development processes or environmental conditions (Lovdal and Lillo 2009; Exposito-Rodriguez et al. 2008).

The elongation factor EF-1α has been tested as one of the most widely used reference gene in normalizing gene expression of many plant species under abiotic stress, such as *Arabidopsis* in freezing conditions (Cuevas et al. 2008), potato during cold and salt stresses (Nicot et al. 2005), *Lolium temulentum* under varies abiotic stress conditions including salt and cold (Dombrowski and Martin 2009) and *Brachypodium distachyon* during cold/heat stress (Hong et al. 2008). However, in these internal control gene evaluation studies, ribosomal protein genes have not been included. According to our results, EF-1α was deemed moderately stable in *Chlamydomonas* sp. ICE-L by geNorm and normfinder analysis. Although TUBA and TUBB are widely used in qRT-PCR analysis as references genes, they performed poorly as reference genes in this study as well as they did in potato (Nicot et al. 2005), grape (Reid et al. 2006), soybean (Jian et al. 2008) and ryegrass studies (Martin et al. 2008).

The ability to adjust membrane lipid fluidity by changing the levels of unsaturated fatty acids is a feature of cold responsive organisms, and is provided mainly by the regulated activity of fatty acid desaturases (FADs) (Upchurch 2008). Acclimation to low-temperature stress via an increase in expression of desaturases has been documented in poikilothermic organisms such as bacteria, algae, plants, and animals (Morgan-Kiss et al. 2006). Ω-3 fatty acid desaturases *FAD3* catalyze the conversion of linoleic acid (18:2) to linolenic acid (18:3). A previous study in our lab has indicated that the contents of linolenic acid (C18:3) in *Chlamydomonas* sp. ICE-L are increased from 19.2 % at 6 °C to 19.7 % at −2 °C, while the contents of linoleic acid (C18:2) are decreased from 8.1 % at 6 °C to 7.4 % at −2 °C (Hou et al. 2002). FAD3 mRNA transcript has been proved to be 2.6-fold increase after freezing and thawing the alga based on the result of qRT-PCR analysis (Zhang et al. 2010). The comparison of the relative FAD3 mRNA expression using different normalization approaches (Fig. 5) showed that α-tubulin, the most frequently used in gene profiling studies was less effective than the best reference genes identified in this study (Fig. 5). The example of expression level analysis of FAD3 shows that the selection of stable reference genes represents a crucial issue for the correct normalization of qRT-PCR data.

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