RESEARCH LETTER

Selection of proper reference genes for the cyanobacterium *Synechococcus* PCC 7002 using real-time quantitative PCR

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Abstract

Synechococcus sp. PCC 7002 is known to be tolerant to most of the environmental factors in natural habitats of *Cyanobacteria*. Gene expression can be easily studied in this cyanobacterium, as its complete genome sequence is available. These properties make *Synechococcus* sp. PCC 7002 an appropriate model organism for biotechnological applications. To study the gene expression in *Cyanobacteria*, real-time quantitative PCR (qPCR) can be used, but as this is a highly sensitive method, data standardization is indicated between samples. The most commonly used strategy is normalization against internal reference genes. *Synechococcus* sp. PCC 7002 has not yet been evaluated for the best reference genes. In this work, six candidate genes were analyzed for this purpose. Cyanobacterial cultures were exposed to several stress conditions, and three different algorithms were used for ranking the reference genes: geNorm, NormFinder, and BestKeeper. Moreover, gene expression stability value M and single-control normalization error *E* were calculated. Our data provided a list of reference genes that can be used in qPCR experiments in *Synechococcus* sp. PCC 7002.

Introduction

Synechococcus sp. PCC 7002 is a unicellular, photoheterotrophic, and euryhaline cyanobacterium, which can tolerate various light intensities, exposure to oxidative stress, changes in nutrient supply, temperature, and salinity, making it an ideal system for experiments that would be difficult to be accomplished in less robust *Cyanobacteria* (Rippka *et al.*, 1979; Nomura *et al.*, 2006a, b; Zhu *et al.*, 2010). This strain has the highest growth rate among all *Cyanobacteria* investigated so far, with a doubling time of 2.6 h under certain conditions (Frigaard *et al.*, 2004; Ludwig & Bryant, 2012). The complete genome of *Synechococcus* sp. PCC 7002 is available in GenBank – NCBI (accession number CP000951) and encodes around 3200 proteins; transcripts were identified for most of the open reading frames. It is naturally transformable, and a system for genetic complementation and gene overexpression is available, making it a perfect candidate for studies in biotechnological or industrial application, being also a model organism for studies of cyanobacterial metabolism (Ludwig & Bryant, 2011; Xu *et al.*, 2011). To study the global expression patterns in *Cyanobacteria*, microarrays have been extensively used (Postier *et al.*, 2003; Foster *et al.*, 2007; Ostrowski *et al.*, 2010). However, if the expression pattern of a specific set of genes is desired, the use of real-time quantitative PCR (qPCR) is more suitable, as it also involves high accuracy, specificity, and reproducibility at a lower cost (Ludwig & Bryant, 2011). qPCR is a widely used, sensitive, and reproducible technique for gene expression measurements. This fluorescence-based method is used in molecular biology for quantification of transcripts expressed at low levels in many different samples for a various number of genes (Silver *et al.*, 2006; Lee *et al.*, 2007; Bustin *et al.*, 2010; Zhang *et al.*, 2013).

In qPCR, many factors can affect the results (technical and biological variation), thus standardization is needed to reveal significant changes in mRNA levels (Nolan *et al.*, 2006; Derveaux *et al.*, 2010). To enable the comparison of transcript concentrations among different samples, data normalization is required. The most commonly used method is normalizing to an endogenous reference gene, because both gene of interest and reference gene are measured during the same PCR reaction. This involves

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reporting the transcript ratios of the target gene to those of the reference gene (Thellin *et al.*, 1999).

An ideal reference gene is essential for cell function and shows relatively constant expression between samples, experimental conditions, or time points (Bustin *et al.*, 2009; Guénin *et al.*, 2009). Gene pairs that have stable expression patterns relative to each other are considered as suitable control genes (Vandesompele *et al.*, 2002). The use of inappropriate internal reference genes can lead to biased expression profiles especially when a single gene is used for normalization (Pinto *et al.*, 2012). Statistical models with different normalization algorithms are available to identify genes with minimal variance: geNorm, NormFinder, or BestKeeper (Vandesompele *et al.*, 2002; Andersen *et al.*, 2004; Pfaffl 2004; Pfaffl *et al.*, 2004).

In this work, we have evaluated the expression stability of six typical candidate reference genes in *Synechococcus* sp. PCC 7002 by qPCR under three stress treatments which are often used in experiments: microaerobiosis, incubation in darkness, and UV-B stress. Our results provide a valuable starting point for the selection of reference genes that can be used in qPCR experiments concerning *Synechococcus* sp. PCC 7002.

Materials and methods

Strains and culture conditions

Synechococcus sp. strain PCC 7002 was acquired from the Pasteur Culture Collection of *Cyanobacteria* (PCC) and grown at 38 °C under air bubbling in liquid medium A^+ supplemented with vitamin B12 (final concentration of 0.04 mg L^{-1}) under constant cool-white fluorescent illumination at an intensity of 250 μmol of photons m^{-2} s^{-1} (Stevens *et al.*, 1973).

For sample collection, four independent cultures were grown under three experimentally induced stress conditions, until they reached exponential phase ($OD_{600\ nm}$ = 0.7–0.9), microaerobiosis, incubation in darkness, and UV-B stress. The absorption spectra were measured using a Shimadzu UV-1700 spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

Microaerobiosis was induced by bubbling the culture flasks with argon for up to 120 min. To assure the continuous dark condition, the cultures were placed into a Sanyo Versatile Environmental Test Chamber (Sanyo, Osaka, Japan) and grown in standard conditions for 120 min, but without any light source. For the UV-B treatment, cells were exposed for 120 min to 1.3 W m^{-2} UV-B light provided by a Philips TL 20W/12 RS fluorescent lamp, with an emission spectrum of 275–380 nm, and a peak at 310–315 nm (Philips Lighting, Stockholm, Sweden). Culture samples (12 mL of cell suspension) were collected at 15, 30, 60, and 120 min after the stress conditions were initiated. Cells were rapidly chilled on ice and centrifuged (2 min, 12 000 ***g***, 4 °C), and the pellets were suspended in 250 μL TRIzol reagent (Invitrogen, Carlsbad, CA) and stored at −20 °C until further use.

RNA extraction and cDNA synthesis

For total RNA extraction, the TRIzol reagent was used, according to the manufacturer's instruction. RNA concentration was measured with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA). To remove any trace of genomic DNA, samples were treated with 1 U DNase (Ambion Turbo DN-ase, Austin, TX). Synthesis of first strand cDNA was performed using a First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA) using 1.5 μg of purified RNA as template and random hexamer primers.

Primer design and transcription analysis by RT-qPCR

The sequences of the six candidate reference genes were obtained from CYANOBASE (accession numbers in Table 1), and primers were designed using the GENEFISHER tool from the BIBISERV server (Giegerich *et al.*, 1996; Nakao *et al.*, 2010). The length of the amplicons for the designed primers was set around 150 bp (Table 2). The size of the PCR products for each primer pair was verified on 1% agarose gel electrophoresis using 1x TAE buffer. qPCR was performed with a BioRad iQ5 System (BioRad, Berkeley, CA) using domed cap PCR tubes. For the reac-

Table 1. Candidate reference genes tested in this study

Gene symbol	Cyanobase code	Protein description
*rim*M	SYNPCC7002_A1245	16S rRNA processing protein: synthesis and modification
*rnp*A	SYNPCC7002_A0989	Protein subunit of ribonuclease P (RNase P)
*pet*B	SYNPCC7002_A0842	Cytochrome b6: involved in electron transport and ATP generation
16S	SYNPCC7002_A2788	16S ribosomal RNA
*pp*C	SYNPCC7002_A1414	Phosphoenolpyruvate carboxylase (PEPC): central enzyme in carbon concentrating mechanism
*sec*A	SYNPCC7002_A1259	Part of the Sec protein translocase complex

Table 2. Specific primers, melting temperature (Tm), and amplicon length for the reference genes used for RT-qPCR analysis

Gene symbol	Primer name	Primes sequence 5′–3′	Tm (°C)	Amplicon length (bp)
*rim*M	7002_rimM_F	GATCGCCCCGAACTCGAAGC	58	152
	7002_rimM_R	TTCTGGTTGGCATCGGTGACTTC	58	
*rnp*A	7002_rnpA_F	GCCCCAACCAAAATTGGCATCAG	58	149
	7002_rnpA_R	TACCGCACAGCAATGACGATGTG	58	
*pet*B	7002_petB_F	GGGTGGTTGATCCGTTCGATCC	58	151
	7002_petB_R	CGGTGATCGTCGCCATGATGAC	58	
16S	7002_16S_F	CGGGTTTGATGAGATTCGCTTGC	58	157
	7002_16S_R	AGTTGGGCACTCTAGGGAGACTG	58	
*pp*C	7002_ppc_F	CACCCTGCCCGAATTATCGGTAC	58	151
	7002_ppc_R	CCACGTAACGTCAGGAGTGACAG	58	
*sec*A	7002_secA_F	GCCGAAATGAGAACCGGGGAAG	58	150
	7002_secA_R	GAAACGGTGTACCTGCCCCATC	58	

tions, SensiFast SYBR & Fluorescein Kit (Bioline, London, UK) was used, with the reaction mix containing both forward and reverse primers at a concentration of 400 nM each, 10 μL of SensiFAST SYBR & Fluorescein Mix to a final concentration of 1x, 4 μL of 5x diluted cDNA and H_2O added up to a final volume of 20 μL. The PCR profile contained: one cycle of 95 °C for 3 min, followed by 45 cycles of 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s. Negative controls (reactions without cDNA template) were included to detect the presence of any DNA contamination. All the reactions were performed in triplicate for each cDNA sample. C_t (crossing thresholds) values were exported and gene-stability measure (*M* value) and single-control normalization error (*E* value) were calculated according to Vandesompele *et al.* (2002). The equation model for *E* value calculation was extended, taking into account the three abiotic stress conditions that were used to verify expression stability of the candidate genes. Moreover, the gene stability was approached using three different algorithms: geNorm, NormFinder, and BestKeeper (Vandesompele *et al.*, 2002; Andersen *et al.*, 2004; Pfaffl *et al.*, 2004).

Results and discussion

Choice of candidate reference genes

The candidate reference genes were selected based on previously reported genes that have been used as controls in qPCR and microarray studies in *Synechococcus* sp. strain PCC 7002 and other *Cyanobacteria* (Sakamoto *et al.*, 1997; Engelbrecht *et al.*, 1999; Hihara *et al.*, 2001; Price *et al.*, 2004; Balasubramanian *et al.*, 2006; Nomura *et al.*, 2006b; Woodger *et al.*, 2007; Cumino *et al.*, 2010; McNeely *et al.*, 2011; Pinto *et al.*, 2012). Six candidate reference genes belonging to independent metabolic pathways (to minimize the effect of co-regulation) were selected for further investigation: *rim*M (GI: 170077861, locus tag: SYNPCC7002_A1245), *rnp*A (GI: 169885279, locus tag: SYNPCC7002_A0989), *pet*B (GI:169885133, locus tag: SYNPCC7002_A0842), 16S (GI: 169884305, locus tag: SYNPCC7002_A2788), *pp*C (GI: 169885692, locus tag: SYNPCC7002_A1414), and *sec*A (GI: 169885544, locus tag: SYNPCC7002_A1259).

qPCR amplification specificity, efficiency and analysis of C_t values

A standard PCR amplification has confirmed that all six primer pairs target single DNA fragments for each gene. In the electrophoresis gel, single bands of the desired molecular weight were observed (Supporting Information, Fig. S1). qPCR was used to measure RNA transcription variations of the candidate genes, in all the samples. After 45 cycles of amplification, melting curves were analyzed. They displayed distinct peaks, suggesting specific melting temperature and unique amplicons (data not shown). Three replicates for each stress condition were examined, with abnormal plots being excluded from further data analysis. Control samples lacking cDNA did not produce amplification, indicating that the template was not contaminated.

Raw C_t values were extracted from BioRad iQ5 System and analyzed. C_t values varied between 10 and 37, the lowest value being observed in case of the microaerobiosis, and the highest in the samples irradiated with UV-B. The gene coding for 16S rRNA consistently displayed the lowest median C_t values (10–18), while the highest median value was obtained for the *pp*C gene, although it did not show a significant difference when compared to other median C_t values. The raw expression values of candidate genes across different experimental conditions were calculated using the comparative C_t method ($2^{-\Delta\Delta C_t}$) (Livak & Schmittgen, 2001), and they can be observed in Table S1.

Ranking and determination of optimal reference genes

For each stress condition the C_t values were used to analyze gene expression stability. To minimize bias, three different algorithms were used for this purpose: geNorm (Vandesompele *et al.*, 2002), NormFinder (Andersen *et al.*, 2004), and BestKeeper (Pfaffl *et al.*, 2004). They rank the candidate reference genes based on distinct statistical algorithms using stability value – *M* value (geNorm and NormFinder); or Pearson's correlation coefficient – *r* value (BesKeeper). This resulted in some discrepancies between the rankings, but usually the same genes were identified as having the most stable expression under the tested conditions. Moreover, the *M* and *E* parameters (Vandesompele *et al.*, 2002) were separately calculated to verify data generated by the three algorithms and also for an easier understanding and interpretation of the results.

The gene-stability parameter (*M*) is based on geometric averaging of multiple control genes and it is calculated through pairwise comparison and stepwise exclusion of least stable gene (Jain *et al.*, 2006; Pinto *et al.*, 2012). GeNorm determined a default limit $M = 1.5$, below which candidate reference genes can be classified as stably expressed, while lower value indicates an even greater stability of the gene expression (Vandesompele *et al.*, 2002; Ohl *et al.*, 2005; Han *et al.*, 2012). *M* values for most of the expression stability calculated were under the limit 1.5 attesting the possibility of using them for normalization. The only gene with the *M* value above 1.5 was *pet*B: 1.53 under dark incubation in one of the three replicates. The *M* values varied, from 0.37 for *sec*A gene in UV-B irradiation conditions to 1.53 for *pet*B in dark incubation. The most constant values were observed for *sec*A (*M* value varying between 0.38 and 0.84) and *pp*C (*M* value varying between 0.45 and 0.84). Under microaerobiosis, the *rim*M gene displayed the lowest *M* value (0.45) (Fig. 1a). In dark incubation, the lowest *M* value was 0.57 in case of *pp*C gene (Fig. 1b), while under irradiation with UV-B, this parameter was 0.45 for the *sec*A gene (Fig. 1c).

Under microaerobiosis, the three algorithms ranked the *rim*M gene in the top position, suggesting that it could be used for normalization under this particular stress condition (Table 3). Under dark incubation, the *pp*C, *rnp*A, and *sec*A were shown to be the most suitable reference genes, with *pp*C being ranked the first according to two of three algorithms (Table 3). For UV-B irradiation, the algorithms ranked *sec*A, *pp*C, and *rnp*A as the most stable genes to be used for normalization (Table 3). For more detailed information concerning the results acquired with the three algorithms, see Fig. S2.

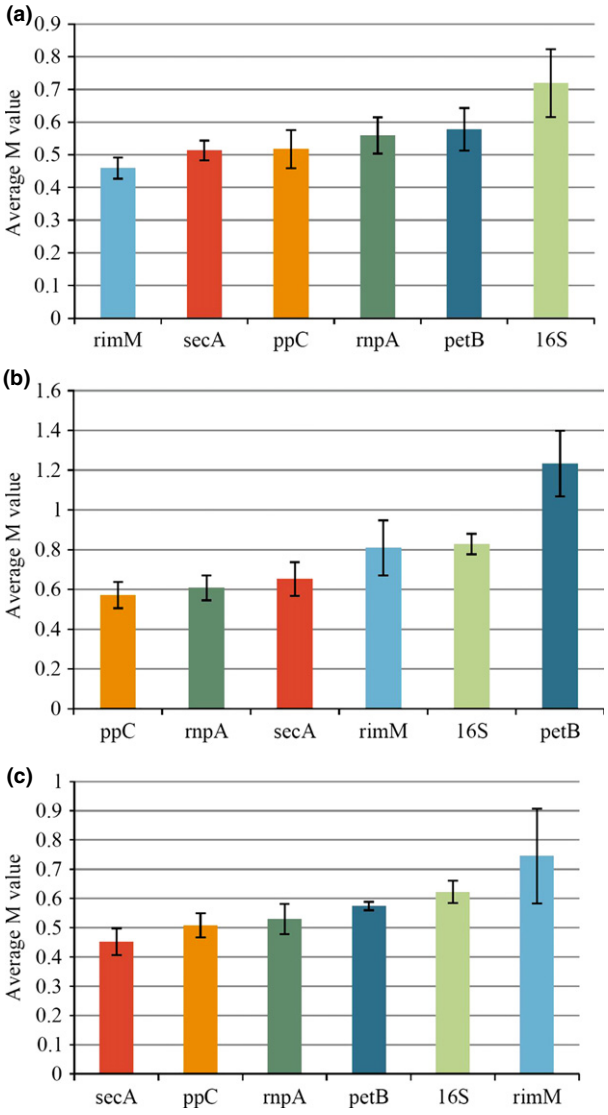

Fig. 1. Average expression stability value *M* under: (a) microaerobiosis (b) dark incubation and (c) UV-B irradiation, and ranking of the candidate reference genes.

According to the results acquired by all three algorithms and to the *M* values, the most suitable reference genes for *Synechococcus* sp. strain PCC 7002 gene expression stability under all the tested conditions are *pp*C, *sec*A, and *rnp*A. Under microaerobiosis, *sec*A and *rim*M genes are recommended to be used for RT-qPCR normalization. In dark incubation, the most stable reference genes are *pp*C and *rnp*A, while for UV-B treatment, *sec*A, *rnp*A, and *pp*C are also stable (Table 3). To display small variance among conditions, the use of two reference genes is recommended. The *E* value (single-control normalization error) was calculated for all the 15 pair combinations of the six analyzed genes, to determine the most suitable

Table 3. Genes ranked in order of their expression stability using the three algorithms: geNorm, NormFinder, and BestKeeper and calculated *M* values under each experimentally induced stress condition. Bold characters = the top two positions for each treatment

	GeNorm			NormFinder			BestKeeper			*M* value		
Gene symb.	Micro-oxic	Dark	UV-B	Micro-oxic	Dark	UV-B	Micro-oxic	Dark	UV-B	Micro-oxic	Dark	UV-B
rimM	**1**	4	6	3	5	6	**2**	4	4	**1**	4	6
rnpA	3	**1**	3	**1**	3	**2**	6	**2**	**1**	4	**2**	**2**
petB	5	6	5	5	6	5	5	6	6	5	6	5
16S	6	5	4	6	4	3	4	5	5	6	5	4
ppC	**2**	**2**	**1**	4	**1**	4	6	**1**	3	3	**1**	3
secA	4	3	**2**	**2**	**2**	**1**	**1**	3	**2**	**2**	3	**1**

Table 4. Ranking of the 15 pairs of genes according to the *E* value. In the far-right column, the results of the adapted equation for all the three stress conditions are displayed

Gene combination	Micro-oxic- Dark	Dark-UV-B	Micro-oxic-UV-B	Micro-oxic-Dark-UV-B
ppC + secA	1	1	1	2
rnpA + secA	4	3	2	3
rnpA + ppC	3	2	7	1
rimM + rnpA	5	5	10	4
rimM + ppC	2	8	15	5
rimM + secA	6	6	9	6
16S + secA	8	7	5	9
rnpA + 16S	7	9	6	13
16S + ppC	10	4	13	10
petB + secA	11	10	8	7
rnpA + petB	12	11	4	8
rimM + 16S	9	15	12	15
petB + 16S	14	14	3	11
petB + ppC	13	12	11	12
rimM + petB	15	13	14	14

reference gene combination. For two ideal control genes, the *E* parameter equals 1, although in practice it is generally larger than 1 (Vandesompele *et al.*, 2002). *E* values were calculated for all two-by-two combinations of candidate genes (Table 4). According to these calculations, *pp*C + *sec*A is the best choice as reference for normalizing gene expression data under all three stress treatments, as it had the best *E* values ranging from 1.4 to 1.5 (Table S2). *rnp*A + *pp*C and *rnp*A + *sec*A were also found to be optimal combinations to be used in pairwise normalization with *E* values of 1.5–1.9. In general, combinations including 16S or *pet*B genes were ranked the lowest, as the highest *E* values for every condition was calculated for *rim*M + *pet*B, with values ranging between 2.7–8.6. This underlines our previous indications that 16S and *pet*B genes and their combinations are not the best choice as reference genes in our own experiment.

As the experimental procedure included three stress conditions, we adapted the *E* equation which calculates the value of different gene combinations under two different conditions, for the calculation of pairwise variation under three conditions. The results were consistent, confirming the previous findings, the combinations with the best *E* values being, in this order, *rnp*A + *pp*C (1.74), *pp*C + *sec*A (1.77), and *rnp*A + *sec*A (2.13) (Table 4). The exact *E* values are presented in Table S2.

16S rRNA gene has been used in several qPCR studies for data normalization so far (Sakamoto *et al.*, 1997; Engelbrecht *et al.*, 1999; Hihara *et al.*, 2001; Schafer *et al.*, 2006; Pinto *et al.*, 2012). However, our results showed that the expression rate of 16S rRNA gene is not stable, and it has significantly higher copy number than other genes. Therefore, it can be concluded that this gene is not the best choice to be used for normalization in experiments with *Synechococcus* sp. strain PCC 7002.

It should be noted that there are concerns with using rRNA genes as control as rRNA may not be influenced by the degradation machinery in a matter similar to mRNA. rRNA genes are not always a good internal control as the rRNA is not representative of the mRNA and there is a significant imbalance between the rRNA and mRNA fractions (Solanas *et al.*, 2001; Ludwig & Bryant, 2011). It had been suggested that this is not associated with a certain condition but to a more general phenomenon that would affect all the mRNA populations. For example, a differential regulation of the RNA polymerases

I and II, responsible for transcription of genes encoding rRNA and mRNA, could produce an rRNA/mRNA imbalance (Solanas *et al.*, 2001). For accurate quantification by qPCR, it is important to choose a reference target whose transcription is regulated in a similar manner (Radonic *et al.*, 2004). Certain experiments on *Synechocystis* sp. PCC 6803 showed that stress conditions could induce the expression of genes coding for proteins involved in translation of other proteins. In *Synechocystis,* genes for ribosomal proteins are located in a putative ribosomal-protein operon, thus stress can enhance their expression, and this could explain the low C_t values observed during our experiments. Moreover, the crystallographic structure of the large subunit of ribosomes has revealed that ribosomal proteins are located near the peptidyltransferase center, suggesting that some stress conditions (e.g. salt stress) might destabilize ribosomes and that *de novo* synthesis of these proteins might be necessary to maintain the activity of ribosomes (Kanesaki *et al.*, 2002).The *pet*B gene was previously recommended as reference gene in *Cyanobacteria* as it displayed stability in microarray studies (Hihara *et al.*, 2001; Pinto *et al.*, 2012). However, according to our findings, this gene is not suitable for qPCR analyses in *Synechococcus* sp. strain PCC 7002, as in this study, the *M* values and also the three algorithms have placed it among the weakest reference genes. The diversity of these results suggests the importance of validating reference genes under multiple experimental conditions. Experiments demonstrate that statistical significance is based on correct reference gene selection, even when changes in reference gene expression are minor, while use of unsuitable references can lead to over- or underestimation of relative transcript abundance. Even though a 'universal' set is difficult to be identified, our experiments have shown that the most suitable reference genes for qPCR in *Synechococcus* sp. PCC 7002 are *ppC, sec*A, and *rnp*A. However, it is highly recommended the use of gene pairs, the most stable combination being *ppC* + *sec*A. Genes *pet*B and 16S rDNA are not recommended to be used as reference genes in this organism.

To our knowledge, this is only the second paper about the selection of proper reference genes in *Cyanobacteria* (the other one being Pinto *et al.*, 2012), and the first one concerning the strain *Synechococcus* sp. PCC 7002. Considering the combined results, our findings emphasize the fact that one reference gene which is proper for a certain cyanobacterium may not be suitable for another one (for example, gene 16S is recommended as a suitable reference gene in *Synechocystis* and *Nostoc*, while in *Synechococcus* sp. PCC 7002 it has one of the weakest scoring). Moreover, the gene *rim*M has never been analyzed before with respect to its quality of reference gene in *Cyanobacteria*, even if it was used for this purpose in some occasions. Thus, we conclude that certain genes should be used with caution in studies on *Cyanobacteria*, and only after verifying their quality as reference gene.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Agarose gel (1%) showing amplification of specific PCR products of expected size for the genes tested in this study.

Fig. S2. Comprehensive gene stability value graphic obtained by combining values calculated by the three algorithm (geNorm, NormFinder, BestKeeper) used for accurate ranking and normalization under: (a) microaerobiosis (b) dark incubation (c) UV-B irradiation.

Table S1. Average C_t values of the candidate reference genes under the three stress condition.

Table S2. Average E values for the 15 pairs of genes.