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Measuring *Symbiodinium* sp. gene expression patterns with quantitative real-time PCR

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Abstract Quantitative real-time PCR is a popular method for measuring gene expression in many biological organisms. The dinoflagellate *Symbiodinium* spp. lives in eukaryotic symbioses with reef-building corals and other marine invertebrates and protists. The use of quantitative real-time PCR to measure gene expression levels of the dinoflagellate symbiont has been limited by the lack of validated normalisation genes and the inability to purify mRNA from the alga without significant host contamination. Normalisation genes to correct for errors inherent to this technique are essential and as yet no validated normalisation genes have been identified for use in *Symbiodinium* gene expression studies. Three commonly used normalisation genes, β-actin, proliferating cell nuclear antigen (PCNA) and 18S rRNA, are tested for use as internal standards in light manipulation experiments of the major light harvesting protein complexes (chlorophyll *a* chlorophyll c_2 peridinin protein complexes - acpPC). Using the algorithm software program geNorm, β-actin and PCNA were found to be more stably expressed than the more highly expressed 18S rRNA genes, and result in similar gene expression profiles when used to normalise gene expression data for three different acpPC genes.

Key words: qRT-PCR, normalisation genes, *Symbiodinium* sp, light harvesting protein complexes

Introduction

The use of reverse transcription in conjunction with quantitative real-time polymerase chain reaction (qRT-PCR) is now a fundamental technique for measuring transcript levels of genes in a wide variety of organisms. The most commonly used approach involves the quantitation of transcript levels in relation to the level of an experimentally stable internal reference or normalisation gene. Alternatively absolute copy numbers of the transcript of interest can be calculated from a standard curve. qRT-PCR, while relatively straight forward to perform, has technical problems. These include RNA variability, variability in extraction protocols and different reverse transcription and PCR efficiencies (Bustin and Nolan 2004), while analysis of results is complicated by the method used for normalisation (Dheda et al. 2004). Appropriate selection of normalisation genes is important in controlling for these inherent problems, but the selection of an unstable gene may statistically influence results by either masking the detection of small changes or providing an incorrect result (Dheda et al. 2005). While growing numbers of studies test the potential of normalisation genes for use with organisms such as plants (Olbrich et al. 2008), Chromalveolata (Siaut et al. 2007) and Cnidarians (Rodriguez-Lanetty et al. 2008), the vast majority continue to use plasmid DNA or cell number (Demir et al. 2008) to quantify absolute transcript levels (Table 1). Use of qRT-PCR to measure gene expression where both partners are symbiotic eukaryotes is significantly more complicated than studies with single organisms or symbioses between eukaryote and prokaryote partners. The greatest challenge with symbioses between two eukaryote partners involves differentiating RNA contribution of the host organism from the symbiont and determining variability in the RNA contribution between samples.

In dinoflagellate symbioses studies qRT-PCR is primarily used to determine or compare the presence of different algal populations (example Loram et al. 2007) within scleractinian corals. In these studies use of normalisation genes or absolute transcript copy numbers is not necessary. Future approaches to eukaryotic symbioses studies are expected to incorporate exogenous RNA spikes. This enables quantification of symbiont contribution to RNA extracts, as was elegantly demonstrated for anthozoans harbouring endosymbiotic dinoflagellates (Mayfield et al. 2008). While utilisation of qRT-PCR to measure gene expression of coral or their single-celled phototrophic dinoflagellate of the genus *Symbiodinium* is still in the initial stages, it will become an important tool for elucidating the effects of increasing anthropogenic and environmental stresses on this important symbiotic relationship.

Here validation of three commonly used normalisation genes, 18S rRNA, β-actin and proliferating cell nuclear antigen (PCNA) are tested for stability under varying light conditions for future gene expression experiments with *Symbiodinium* sp. light harvesting chlorophyll *a* chlorophyll c_2 peridinin protein complex (acpPC) genes.

Table 1: Common normalisation methods used in plant, algae, symbiosis and bacteria.

Normalisation Standard	Organism
β-actin	Cnidaria (example Deboer et al. 2007) Dinoflagellate (Kobiyama et al. 2005) Plant (Jurca et al. 2008)
18S rRNA	Plant (Giorio et al. 2007)
GAPDH	Plant (Jurca et al. 2008)
Plasmid DNA or cell numbers	Dinoflagellates (example Demir et al. 2008) Raphidophytes (example Handy et al. 2008) Pelagophytes and haptophytes (John et al. 2007) Cyanobacteria (John et al. 2007)

Material and Methods

Symbiodinium *samples*

Coral branches from three colonies of *Acropora aspera* hosting *Symbiodinium* (clade C3) were collected from Heron Island (Great Barrier Reef (23°33'S, 151°54'E) in June 2005 and placed in four flow through aquaria. Light levels in two aquaria were reduced by shading (average daily light dosage 0.7 mol quanta m^{-2} d^{-1}), while the two remaining tanks were left exposed (average daily light dosage of 17.4 mol quanta m^{-2} d^{-1}). Over the course of the experiment the maximum irradiance recorded in the shaded tanks ranged between 27.3 and 98.9 µmol quanta m^{-2} s^{-1}, while the exposed tanks ranged from 573 to 1540 µmol quanta m^{-2} s^{-1}. One sample from each colony was collected from each tank at 1300 h on days 1, 3, 5, 7 and 9 of the experiment for RNA extraction.

RNA isolation

Symbiodinium were removed from the coral skeleton using an oral irrigator (WaterPik™) into 70 ml of 0.45 µm filtered sea water (FSW). The resulting homogenate was centrifuged for 2 min at 5000 g, the supernatant discarded and pellet resuspended and washed in 5 ml of FSW. This protocol was repeated a second time before the pellet was transferred to a clean tube and frozen in liquid nitrogen and stored at -80°C. Algal pellets were ground under liquid nitrogen and total RNA isolated using a RNeasy Plant Mini kit (Qiagen, Valencia USA). RNA was qualitatively assessed by a 0.9% formaldehyde agarose gel and quantified using a NanoDrop-1000 (NanoDrop Technologies, Wilmington USA).

qRT- PCR analysis

Reverse transcription and genomic DNA elimination was performed with QuantiTect Reverse Transcription kit (Qiagen, Valencia USA) in a 20 µl reaction using 500 ng of total RNA as template and a RT primer mix consisting of random primers and oligo-dT. Template dilution series were prepared to optimize quantification accuracy. After 4 fold dilution with ddH_2O, 3 µl of diluted template was analysed using the Rotor-Gene™ 6000 (Corbett Life Science, Australia). The PCR was performed with 7.5 µl of Platinium® SYBR® Green qPCR SuperMix-UDG (Invitrogen Corp, Carlsbad, USA) and gene specific primers (200 nM) for either β-actin, PCNA, 18S rRNA or one of three acpPC genes. The protocol was 95°C for 2 min, followed by 45 cycles of 15 s at 95°C and 30 s at 60°C with the temperature increasing 1°C every 5 s from 66°C to 95°C in a final melt stage. Each GeneDisc™-100 (Corbett Life Sciences, Australia) included reactions for one normalisation and one *Symbiodinium* acpPC gene, with samples run in triplicate and non-template controls in duplicate. Standard curves for each normalisation and *Symbiodinium* acpPC gene were generated on every run with five duplicates using a single template sample. An additional PCR efficiency check using four different RNA templates was conducted to ensure inter-disc comparability.

Primers for β-actin, PCNA and 18S rRNA were designed using combinations of MacVector Inc (USA) and DNASTAR Lasergene Primer Select (USA) based on alignments of multiple *Symbiodinium* cDNA sequences obtained from the NCBI GenBank database (www.ncbi.nlm.nih.gov) (Table 2). Alignments were constructed in BioEdit Sequence Alignment Editor (Hall 1999) using ClustalW Multiple Alignment (Thompson et al. 1994) and checked for specificity against nucleotide expressed sequence tags in NCBI GenBank database. Primer specificity was checked using coral cDNA extracted from *Acropora millepora* at both the prawn chip and donut developmental stages and *Symbiodinium* clade C3 cDNA extracted from *A. aspera*. *Symbiodinium* C3 acpPC primers were designed to an expressed sequence tag library (Leggat et al. 2007). acpPC primers were designed and checked as per normalisation genes (Table 2).

An additional primer check and negative control to determine the effect of coral contamination on *Symbiodinium* relative expression was performed. A total of 30 ng of template was used with decreasing amounts of *Symbiodinium* cDNA template (30 ng to 0 ng) mixed with increasing concentrations of coral cDNA from the donut developmental stage (0 ng to 30 ng of coral). qPCR was performed according to the above protocol using β-actin, PCNA, acpPCSym_1 and acpPCSym_10 primers and relative gene

expression determined using the $\triangle\triangle C_t$ method (Livak and Schmittgen 2001).

Table 2: Normalisation genes and acpPC genes tested in qRT-PCR assays

Gene	Primers	Product size
β-actin	F1: TGG ACA ACG GAA GCG GAA TG B1: GCC AAC AAT GGA TGG GAA AAC T	80 bp
PCNA	F1: GAG TTT CAG AAG ATT TGC CGA GAT B1: ACA TTG CCA CTG CCG AGG TC	113 bp
18S rRNA	F3: GTC TAA CGC AAG GAA GTT TGAG B3: CAG GAC ATC TAA GGG CATC A	57 bp
acpPC Sym_10	F1: TTC GCC GAT GTG CCT AAT GG B1: TTC CTG GGA GAC TTC GCA GAA A	102 bp
acpPC Sym_1	F1: AGT GGA GTG AAC CAG GAA GCA A B1: AAC CAA TCG CAC CGA CCA AGA G	54 bp
acpPC Sym_9	F1: CGA ATG GAA GTT GGT GGT AAC B2: GTG CTC AAC CCA CTG TCT TTT	51 bp

Statistical analysis

Results were analysed using the geNorm (http://medgen.ugent.be/~jvdesomp/genorm/) statistical algorithm software to calculate gene expression stability (*M*) for the three normalisation genes tested. The gene or genes with the lowest *M* value is considered the most stably expressed (Vandesompele et al. 2002). All target samples collected on Day 7, which were determined to be the most stably expressed, and normalised to the two highest ranking normalisation genes were subsequently analysed using geNorm to identify a calibration sample. The three *Symbiodinium* acpPC gene expression profiles were analysed in the relative expression software REST© (http://www.gene-quantification.de/rest-2005.html) (Pfaffl et al. 2002). PCR efficiencies were calculated for each standard curve generated and threshold fixed at 0.0859 for all C_t calculations. This threshold value represented the average detection threshold across the 15 runs with the upper and lower bounds used to scan for an optimal threshold set between 1 and a value necessary to exclude background noise. Melting curve analysis was performed for each assay to check reaction specificity.

Results

Primer specificity and coral contamination

Specificity of primer design for *Symbiodinium* sp. normalisation genes was examined using coral cDNA isolated and transcribed from prawn chip and donut developmental stages, both early post fertilisation aposymbiotic stages. qPCR primers designed for clade C3 *Symbiodinium* and amplified according to either standard PCR conditions or qPCR conditions outlined above, failed to amplify coral cDNA. Glyceraldehye-3-phosphate dehydrogenase (GAPDH) qRT-PCR primers designed for *A. millepora* were included as a positive control with pawn chip cDNA. When tested with C3 *Symbiodinium* cDNA, single amplicons of expected size were detected (data not shown).

A possible confounding factor in using qPCR to quantify symbiont gene expression is the unknown contribution of host RNA. To test for this the relative expression of acpPCSym_1 and acpPCSym_10 was examined with increasing concentrations of coral cDNA. The concentration of *Symbiodinium* RNA (which would probably include approximately 10-20% host contamination (Leggat et al. 2007)) used in the assay was decreased from 100% to 0.5%, while the coral cDNA was increased. The expression of acpPCSym_1 and acpPCSym_10 was then compared across this concentration range. A variation from a relative expression of 1, when compared to a sample with 100% *Symbiodinium* cDNA (30 ng) would indicate an effect of coral contamination. Only when *Symbiodinium* cDNA composed 0.5% (0.15 ng) of the total cDNA was there a significant deviation ($p = 0.02$) from a relative expression value of 1 (Fig. 1). No fluorescence was recorded in samples without *Symbiodinium* template (30 ng of coral cDNA only).

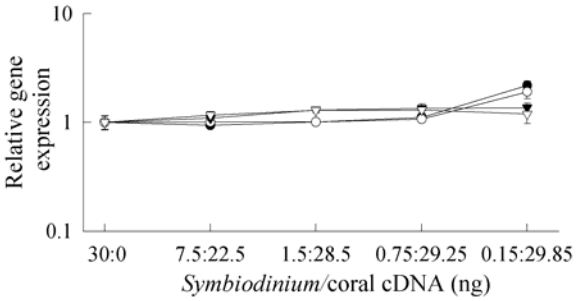

Figure 1: Effect of increasing the proportional coral cDNA concentration on relative expression of acpPCSym_1 normalised to β-actin (-●-) and PCNA (-o-) and acpPCSym_10 normalised to β-actin (-▼-) and PCNA (-▽-). Total template concentration is 30 ng for each sample and error bars indicating the standard error.

qRT-PCR efficiency

Amplification plots were analysed for the three normalisation and three *Symbiodinium* acpPC genes. Assay validations performed confirmed PCR efficiency and optimisation of procedure. Samples from Day 5 were removed from all expression profile analyses due to three samples being compromised prior to RNA extraction. Comparison of C_t values for each of the six genes on all sample days, including four additional templates run across multiple discs to ensure inter-disc comparability, confirmed minimal variation between PCR efficiencies, permitting comparison of genes and samples performed on different GeneDisc™-100 discs (data not shown).

C_t values for 18S rRNA were considerably lower than those of β-actin, PCNA and the three *Symbiodinium* acpPC genes across all sampling days (Fig. 2) indicating high transcript levels and the need for further optimisation of this gene.

Normalisation gene stability

β-actin (M = 0.467) was marginally more stable than PCNA (M = 0.498) with 18S rRNA the least stable (M = 0.687) under the specific experimental conditions used here. Target samples normalised to β-actin and PCNA from Day 7 of the experiment expressed the greatest stability. The most stably expressed target sample from Day 7 was used as the calibration sample and PCR efficiencies for each of the six genes from Day 7 were used to qualitatively compare the expression of the three acpPC genes subjected to varying light levels.

Relative gene expression levels for the three *Symbiodinium* light harvesting genes were calculated using the REST© method (Pfaffl et al. 2002) (Fig. 3). The profile of all three genes was similar when normalised to β-actin (Fig. 3a, c, e) with those samples shaded from light showing increased expression compared with light exposed samples. Light exposed samples exhibited minimal expression change across the nine day experiment and these results were replicated when acpPC genes were normalised to PCNA (Fig. 3b, d, f). acpPCSym_1 normalised to PCNA (Fig. 3f) were the only shaded samples not to increase expression.

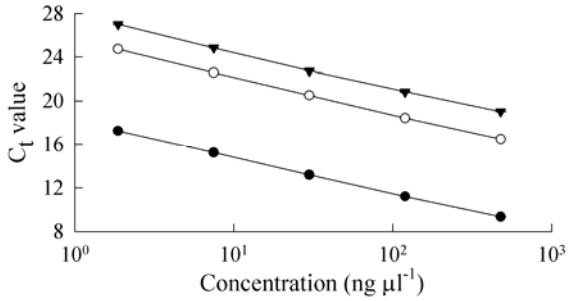

Figure 2: β-actin (-o-), PCNA (-▼-) and 18S rRNA (-●-) standard curves generated from amplification plots of serial four-fold dilution series. The plotted C_t is the average of triplicate samples and error bars (hidden by symbols) indicating the standard error.

Discussion

Three normalisation genes, β-actin, PCNA and 18S rRNA, were tested for stability in light manipulation experiments with *Symbiodinium* major light harvesting protein complexes (acpPC). Using the statistical algorithm software geNorm, β-actin was found to be marginally more stable than PCNA, and gene expression profiles for three acpPC genes normalised to β-actin and PCNA exhibited similar trends (Fig. 3).

PCNA was tested here rather than the more commonly used GAPDH to complement the use of β-actin and because synthesis of chloroplastic GAPDH is regulated over a diel cycle and exhibits greater than 50% identity in the region of overlap with the cytosolic isoform (Fagan et al. 1999). PCNA has been

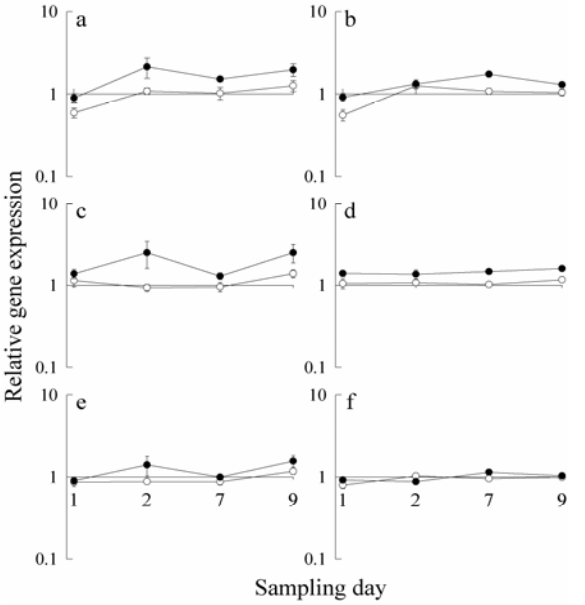

Figure 3: Expression profiles of *Symbiodinium* gene acpPCSym_10 (a, b), acpPCSym_9 (c, d) and acpPCSym_1 (e, f) normalised to β-actin (a, c, e) and PCNA (b, d, f) exposed to light (-o-) or shade from sunlight (-●-). Error bars represent the standard error for the averaged C_t values used to calculate relative expression for six biological replicates.

used as a normalisation gene in a range of animal species and humans (Schiller et al. 2003), is present in plant genomes (Suzuka et al. 1989) and has been isolated from a dinoflagellate where it was found not to significantly alter expression levels during cell cycle (Zhang et al. 2006), although this differs from other algal studies (example Wei et al. 2004).

Although using total RNA for normalisation is not always reflective of the mRNA fraction, it does enable the testing of 18S rRNA stability. In this study 18S rRNA was found to be unsuitable and would require additional optimisation. Compared with target mRNA transcripts the abundance of 18S rRNA is much higher (C_t value of approximately 10 compared to β-actin 17 and PCNA 19 indicating a 128-512 fold greater representation) (Fig. 2) and this causes difficulties when analysing data and determining the background baseline to subtract from the data (Vandesompele et al. 2002).

The overwhelming issue when using qRT-PCR with organisms such as *Symbiodinium* isolated from coral is whether varying RNA contamination influences the relative expression of the target genes. Results here suggest coral contamination is not a major factor and does not influence relative gene expression of *Symbiodinium* acpPC genes (Fig. 1) until transcript levels become very low, at which stage the variation between replicates increases. This suggests if valid normalisation genes can be identified for specific experimental conditions, the use of mixed

RNA populations will not confound results.

Microarray studies to date suggest dinoflagellate genes express small changes and that photosynthetic genes may only vary 2 – 3.4 fold (example Van Dolah et al. 2007). The identification of suitable normalisation genes is important if such minor changes in expression are to be detected and quantified. This study determined the stability of two normalisation genes and is an initial step towards validating a suite of genes with potential for use in *Symbiodinium*-coral gene expression work.

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