Research Article

Requirement for Different Normalization Genes for Quantitative Gene Expression Analysis Under Abiotic Stress Conditions in '*Bangia*' sp. ESS1

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Abstract

Quantitative gene expression analysis is indispensable for accurately characterizing the expression levels of genes involved in the response and acclimation of algae to environmental stresses. However, appropriate reference genes in the genus *Bangia* (order Bangiales) for quantification of stress-inducible gene expression are not well described. Thus, we evaluated the suitability of genes encoding 18S ribosomal RNA (18S rRNA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), *actin*, *tubulin*, elongation factor 1 (EF1), and 60S ribosomal RNA (60S rRNA) as reference genes for real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) under various stress conditions in '*Bangia*' sp. ESS1, classified into the '*Bangia*' 2 group. Our results showed that the 60S rRNA and GAPDH genes were most suitable for gene expression analyses in response to nutrient deficiency, high salinity, and temperature stress, whereas normalization of desiccation-inducible expression required use of the EF1 and *tubulin* genes. These findings indicate that reference genes for analysis of stress-inducible gene expression by qRT-PCR should be selected according to the environmental stress being studied. Our efforts to identify easily amplified reference genes for various stress responses provide novel insights into the selection of reference genes for seaweeds.

Keywords: *Bangia*; Gene Expression; Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR); Red Alga; Reference Gene; Stress Response

Introduction

Members of the order Bangiales live in the intertidal zone where organisms periodically experience abiotic stresses such as aberrant temperature, nutrient deficiency, salinity fluctuation, and long periods of desiccation [1-5]. Compared to the recent progress of molecular biological studies on abiotic stress responses in the genus *Pyropia* [6-11], the study of abiotic stress responses in the genus *Bangia* is not as advanced. However, given its simple body architecture of gametophytic thalli and ease of a laboratory culture, we recently employed *Bangia* species for physiological and molecular biological research to elucidate the regulatory mechanisms of the response and acclimation to abiotic stresses in Bangiales. Promotion of the asexual life cycle, a strategy to increase the number of gametophytic clones by producing asexual spores endowed with the gametophyte identity [12], by high-temperature stress was confirmed in *Bangia fuscopurpurea* [13]. Similar results have been obtained from experiments using *B. fuscopurpurea* collected from different sites [1,14]. In addition, we have successfully expressed foreign reporter genes transiently in gametophytes of *B. fuscopurpurea* using a particle bombardment system [15]. Analyses of abiotic-stress-inducible gene expression have also been performed in several laboratories [16-19]. Thus, *Bangia* species have potential as model red seaweeds for biological research.

Reconstruction of phylogenetic relationships in the genus *Bangia* has been established recently [20]. For the past few decades, all members of the genus *Bangia* have

been classified into only three species: *Bangia atropurpurea*, *B. fuscopurpurea*, and *B. gloiopeltidicola*. However, extensive phylogenetic analysis using sequence information of nuclear small subunit ribosomal RNA (*SSU rRNA*) and plastid Rubisco large subunit (*rbcl*) genes from many representatives of *Bangia* species revealed that the genus *Bangia* could be divided into four groups: *Bangia*, '*Bangia*' 1, '*Bangia*' 2, and '*Bangia*' 3. *B. atropurpurea* and *B. gloiopeltidicola* fall into groups *Bangia* and '*Bangia*' 3, respectively, while *B. fuscopurpurea*, used in numerous previous studies and thought to represent a single species, is classified into '*Bangia*' 1 and '*Bangia*' 2 groups. These findings indicate that specimens previously classified as *B. fuscopurpurea* are a mixture of phylogenetically close but distinct species. Thus, it is necessary to classify our materials collected at Esashi on the northern Hokkaido Island of Japan, which were described as *B. fuscopurpurea* in previous reports [13,15,21].

Regulation of genes involved in acquisition of stress tolerance is fundamental for intertidal seaweeds to have normal development and growth under strict living conditions [22]. Thus, gene expression analysis is important for understanding the mechanisms of response to environmental stress in intertidal seaweeds. Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) allows monitoring of relative changes in gene expression [23] using a normalization strategy that relies on comparison of the target gene with an endogenous control (reference gene). At present, housekeeping genes such as *actin*, *tubulin*, and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) as well as ribosomal RNA genes are commonly used as reference genes. These genes are thought to be universally required for basic cellular functions and to be constitutively and stably expressed under different experimental conditions in land plants [24-26]; fungi [27-29]; the green algae *Volvox carteri* [30], *Ulva linza* [31], and *Chlamydomonas* sp. ICE-L [32]; the brown alga *Ectocarpus siliculosus* [33]; and the red algae *Porphyra yezoensis* [8,34,35], *Pyropia haitanensis* [9], and *Gracilaria lemaneiformis* [36]. Indeed, these housekeeping genes have been employed as reference genes in *Chlamydomonas*, *Volvox*, *Pyropia*, and *Porphyra* species. However, little is known about appropriate reference genes in *Bangia* species to date.

In this study, we aimed to identify reference genes suitable for gene expression analysis under various abiotic stresses in *Bangia* species. Based on our unpublished transcriptome data and information from previous studies [16,18], the six housekeeping genes *18S ribosomal RNA* (*18S rRNA*), *GAPDH*, *EF1*, *60S ribosomal RNA* (*60S rRNA*), *actin*, and *tubulin* were chosen as candidates for reference genes to evaluate their applicability in quantitative gene expression analysis under four types of stress: desiccation, nutrient deficiency, high salinity, and temperature changes. In addition, a cold-stress-inducible gene encoding delta-12 fatty acid desaturase in *B. fuscopurpurea* [18] was employed for further validation of the selected reference genes.

Materials and Methods

Algal strain and culture conditions

Thalli of *Bangia* species used in this study were originally collected at Esashi, Hokkaido, Japan, on May 17, 2010 and maintained clonally in the laboratory as an experimental line. Filamentous gametophytes were cultured in sterilized artificial seawater (SEALIFE; Marineteck, Tokyo, Japan) containing ESS₂ with NaNO₃ as a nitrogen source, vitamins, and trace metal elements [37] under 60 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ in a short-day photoperiod (10 h light/14 h dark) at 15 °C with air filtered through a 0.22- μm filter (Whatman; Maidstone, UK). The culture medium was changed weekly.

Nutrient deficiency, high salinity, desiccation, cold, and heat were selected as abiotic stresses (Table 1). For nutrient deficiency treatment, 0.1 g of thalli was treated with ESS₂-less seawater for 24, 48, 72, 96, 120, 144, and 168 h. For high-salinity stress, thalli were incubated in medium containing 25, 50 or 100 mM NaCl for 24, 48, and 72 h. For desiccation stress, 0.1 g of thalli were desiccated in a clean bench until the weight of samples reached 75, 50 or 25% of the initial weight (W_i). To examine recovery from desiccation, thalli weighing 25% of W_i were incubated in normal seawater medium for 1h. For temperature treatment, 0.1 g of thalli were exposed to 5°C or 30°C for 24, 48, and 72 h. All samples were immersed in liquid nitrogen and stored at -80°C prior to RNA extraction.

Phylogenetic analysis

The *rbcl* gene of our material was partly amplified with gene-specific primers (5'-AAGTGAACGTTACGAATCTGG-3' and 5'-GATGCTTTATTTACACCCT-3') [38] using Ex taq polymerase (TaKaRa Bio, Kusatsu, Japan) and sequenced on an ABI Model 3130 Genetic Analyzer (Life Technologies, Carlsbad, USA). The nucleotide sequence of the amplified DNA fragment was deposited in DDBJ/EMBL/GenBank under accession number MN052802. Specimens and accession numbers of their *rbcl* gene sequences are listed in Table 2. A neighbor-joining phylogenetic tree was reconstructed with MEGA 7 software (<https://www.megasoftware.net>), using ClustalW to align the *rbcl* nucleotide sequences.

Total RNA extraction and cDNA synthesis

Total RNA was separately extracted from each sample using a RNeasy Plant Mini kit (Qiagen, Hilden, Germany) and then treated with DNase (TURBO DNA-free TM kit, Invitrogen, Carlsbad, USA) to remove genomic DNA contamination. Purity and concentration of RNA samples were calculated using a GeneQuant pro spectrophotometer (UK), and the integrity of RNA samples was checked using agarose gel electrophoresis. RNA samples of good quality with A_{260}/A_{280} ratios ranging from 1.9 to 2.1 were used. First-strand complementary DNA (cDNA) was synthesized from 300 ng of total RNA in a volume of 20 μl using a Prime Script 1st strand cDNA synthesis kit (TaKaRa Bio, Kusatsu, Japan)

Factor	Treatment	Duration
Desiccation	percentage of initial weight	75%
		50%
		25%
		25% seawater 1h
Nutrient-deficiency	ESS2-less	0d
		1d
		2d
		3d
		4d
		5d
		6d
		7d
High salinity	25 mM	1d
	25 mM	2d
	25 mM	3d
	50 mM	1d
	50 mM	2d
	50 mM	3d
	100 mM	1d
	100 mM	2d
	100 mM	3d
Temperature changes	5°C	1d
	5°C	2d
	5°C	3d
	30°C	1d
	30°C	2d
	30°C	3d

Table 1: Experimental conditions

Taxon	Collection location	Accession No.
' <i>Bangia fuscopurpurea</i> ' BB Bf 1	Bolinas Bay, CA, USA	EU289018
' <i>Bangia fuscopurpurea</i> ' CMNH UM BF1	Banda, Tateyama, Chiba, Japan	HQ687502
' <i>Bangia fuscopurpurea</i> ' France	Nice, France	AF168659
' <i>Bangia fuscopurpurea</i> ' Taiwan	Taiwan	AF168654
' <i>Bangia fuscopurpurea</i> ' WA	Fisherman's Bay, WA, USA	AF169329
' <i>Bangia</i> ' <i>maxima</i>	Bolinas Bay, CA, USA	EU289020
' <i>Bangia</i> ' sp. BC Can	Ogden Point, Victoria, BC, Canada	AF043376
' <i>Bangia</i> ' sp. BCH	Taylor's Mistake, Christchurch, South I, NZ	HQ687504
' <i>Bangia</i> ' sp. BFK	Frank Kitts Lagoon, Wellington, North I, NZ	HQ687505
' <i>Bangia</i> ' sp. BGA	Gentle Annie, Westland, South I, NZ	HQ687506
' <i>Bangia</i> ' sp. BMW	Makawhio, North I, NZ	HQ687508
' <i>Bangia</i> ' sp. BNS	Bawley Point, N. of Bateman's Bay, NSW, Australia	HQ687509
' <i>Bangia</i> ' sp. BPL	Maketu, Bay of Plenty, North I, NZ	HQ687510
' <i>Bangia</i> ' sp. BRM	Kaka Point, Otago, South I, NZ	HQ687511
' <i>Bangia</i> ' sp. BWP	Woodpecker Bay, Paparoa, Westland, South I, NZ	EU570051
' <i>Bangia</i> ' sp. CH620	Supseom, Jeju-do, Korea	HQ728203
' <i>Bangia</i> ' sp. MA	Woods Hole, MA, USA	AF043369
' <i>Bangia</i> ' sp. NthBC Can	Triple Island, BC, Canada	AF043372
' <i>Bangia</i> ' sp. OR	Lincoln City, OR, USA	AF043367
' <i>Bangia</i> ' sp. SB Bf 1	Solana Beach, CA, USA	EU289019
' <i>Bangia</i> ' sp. TX	Port Aransas, TX, USA	AF043377
' <i>Bangia</i> ' <i>vermicularis</i>	Golden Gate, San Francisco Bay, CA, USA	EU289022
' <i>Bangia</i> ' sp. OUCPT-01	Putian, Fujian, China	KP747609.1

Table 2: Species and GenBank accession numbers of their *rbcL* gene sequences used for the phylogenetic analysis

Gene name	Gene ID / Reference	Gene function description	Primer sequence	Amplicon length (bp)	Efficiency (%)	Correlation coefficient (R^2)
<i>18S rRNA</i>	[16]	small subunit ribosomal RNA	ACAGGACTTGGGCTCTATTTTG	135	99	0.9932
			AGATGCTTTCGCAGTGGTTTCG			
<i>GAPDH</i>	[18]	glyceraldehyde 3-phosphate dehydrogenase	CTGGTGAGGCACTTTGGAA	102	105	0.9915
			AAGGAGGAGGACTGATGGG			
<i>actin</i>	CL1634.Contig3	actin	TCAACCCCAAGGCCAACC	150	98	0.9930
			TCACGCCGTCCCCAGAAT			
<i>tubulin</i>	CL70.Contig4	alpha-tubulin	ACTCGGTACATCAACGTTCA	131	99	0.9925
			TATCTGCCGCCGCTCTCT			
<i>EF1</i>	Unigene64554	enlongation factor 1	ATCTCGTTGTAGCGGGACTC	86	101	0.9933
			AAGCAGCTGATCGTTGGC			
<i>60S rRNA</i>	Unigene3570	large subunit ribosomal protein	CCACGTTGTCCATTTACGC	98	102	0.9940
			CACCTCCTCCAAGTTCGG			
<i>BE1Des12</i>	[18]	delta12-fatty-acid desaturase	CACCCAGAGGCAAACCC	180	96	0.9911
			ATGGACGGGCAGTAGGG			

Table 3: Sequences of the primers used for qRT-PCR in this study.

according to the manufacturer's instructions. The cDNA was diluted 20 times before use as template in qRT-PCR.

Design and evaluation of primers for candidate reference genes

Six housekeeping genes encoding 18S rRNA, 60S rRNA, GAPDH, EF1, actin, and tubulin were selected as candidates for reference genes. Primers for qRT-PCR of *18S rRNA* and *GAPDH* genes were available from previous studies [16,18]. Nucleotide sequences of the other four candidate genes were obtained from our unpublished transcriptome analysis: *EF1* (Unigene64544), *actin* (CL1634.Contig3), *tubulin* (CL70.Contig4), and *60S rRNA* (Unigene3570). Primers for these genes were designed using Primer Premier 5.0 software (<http://www.premierbiosoft.com>) with the following conditions: annealing temperature of 60°C, DNA G+C content of 40 to 60%, and amplicon length of 80 to 200 bp (Table 3). The specificity of primer pairs for each candidate gene to produce PCR fragments of a single expected size was validated by agarose gel electrophoresis of products from PCR reactions performed using Phusion high-fidelity DNA polymerase and GC buffer (Biolabs, Massachusetts, USA) according to the manufacturer's instructions. A standard curve for each gene was generated using a 5-fold dilution series of one sample over six dilution points measured in three technical replicates, and PCR amplification efficiency was also calculated.

Quantitative gene expression analysis

Reactions of qRT-PCR were carried out in a total volume of 20 µl containing 10 µl of 2 × SYBR Premix Ex Taq GC, 0.4

µl of ROX Reference Dye, 2 µl of cDNA template, and 0.4 µl (10 µM) of each primer, using the SYBR Premix Ex Taq GC kit (TaKaRa Bio, Kusatsu, Japan). The thermal cycling parameters were 95°C for 5 min and 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 20 s. A dissociation curve was generated by heating from 60 to 95°C to check for specificity of amplification using an Applied Biosystems 7300 real-time PCR system (Life Technologies, Carlsbad, USA). Each reaction was triplicated, and the data were analysed using one-way ANOVA, where the level of significance was defined at $P < 0.05$.

Evaluation of gene expression stability and its validation using the delta-12 fatty acid desaturase gene

Two software packages, geNorm [39] and NormFinder [40], were used to assess the stability of the expression level of each reference gene under different experimental treatment conditions. geNorm ranks the candidate reference genes according to calculation of an expression stability value (M). A lower M value reflects higher stability of gene expression. geNorm also determines the optimal number of reference genes required for accurate normalization of target gene expression by a pairwise variation ($V_{n/n+1}$). Generally, a $V_{n/n+1}$ value less than 0.15 indicates no need for an additional reference gene for normalization. NormFinder produces expression stability values (SVs) to evaluate candidate reference genes in a given experimental design and considers intra- and intergroup variations, enabling an estimate of candidate gene stability values for normalization without any influence of coregulated candidate genes. Low SV indicates high stability in normalization.

Figure 1: Phylogenetic identification of the specimen used in the study.

Neighbor joining-based phylogenetic tree reconstructed using sequences of *rbcl* genes from different specimens from the genus *Bangia* whose DDBJ/EMBL/GenBank accession numbers are listed in Table 2. The specimen used in this study is boxed. Bootstrap values over 50% from 1000 replicates are indicated at nodes. Bar, 0.01 substitutions per site.

For validation of these evaluations, the expression of the *delta-12 fatty acid desaturase* gene was normalized using the reference genes evaluated as suitable by geNorm combined with NormFinder under different abiotic stresses. The primer sequence of *delta-12 fatty acid desaturase* [18] is indicated in Table 3.

Results

Phylogenetic classification of the specimen used in this study

A DNA fragment corresponding to the *rbcl* gene was amplified from our *Bangia* material, sequenced, and used for phylogenetic analysis. Results indicated that the sample was classified into the '*Bangia*' 2 group established by Sutherland et al. [20], with the closest relationship to '*B. fuscopurpurea*' CMNH UM BF1 collected at Banda, Tateyama, Chiba, Japan (Figure 1). We designated our experimental material '*Bangia*' sp. ESS1 (ESS was derived from Esashi).

Amplification efficiency and cycle threshold values of PCR reactions using candidate reference genes

Specificity and efficiency of primers were confirmed by agarose gel electrophoresis of amplicons of the candidate reference genes derived from all cDNA libraries. All the primer pairs amplified single fragments of the expected size (Table 3) that gave rise to one single peak in the melting curve (data not shown). These primer pairs were therefore considered specific and used for evaluation of gene expression levels by qRT-PCR. In addition, the correlation coefficient (R^2) and PCR efficiency of each gene were analysed using standard curves. The R^2 value and efficiency ranged from 0.991 to 0.994 and 91.1% to 105%, respectively (Table 3), both of which were within the acceptable range (0.9910 to 0.9998 for R^2 and 90–105% for PCR efficiency), indicating the suitability of all primer sets for further gene expression analysis by qRT-PCR.

Figure 2: Cycle threshold (Ct) values of candidate reference genes across conditions tested.

Boxes show the median values (central lines), Q1 (lower outline), and Q3 (upper outline), and whiskers. The whiskers are set at 1.5 times interquartile range (IQR) above Q3 and 1.5 times IQR below Q1. Data were derived from 27 culture samples and repeated for 3 times.

Desiccation		Nutrient deficiency		Salinity		Temperature		Total	
Ranking ^a	SV ^b	Ranking	SV	Ranking	SV	Ranking	SV	Ranking	SV
tubulin	0.397	GAPDH	0.137	GAPDH	0.132	60S	0.164	GAPDH	0.392
EF1	0.505	actin	0.217	60S	0.319	actin	0.279	tubulin	0.551
actin	0.617	60S	0.511	actin	0.324	GAPDH	0.390	EF1	0.604
GAPDH	0.705	18S	0.705	EF1	0.494	EF1	0.503	actin	0.809
18S ^c	1.073	EF1	0.750	18S	0.679	tubulin	0.548	18S	0.962
60S ^d	1.112	tubulin	1.143	tubulin	1.000	18S	0.862	60S	0.876

a: Ranking indicates the stability of gene expression from the most stable to the least stable.

b: SV represents stability value.

c: 18S represents 18S rRNA.

d: 60S represents 60S rRNA.

Table 4: Expression stability analysis of six reference genes using NormFinder.

Cycle threshold (Ct) values represent relative expression levels; low and high values indicate high and low levels of gene expression, respectively. The Ct values of the six candidate reference genes from four different treatments across all samples in '*Bangia*' sp. ESS1 were calculated as shown in Figure 2. The Ct values of the reference genes varied from 11.5 to 30.7, with the majority between 18 and 28. The *18S rRNA* gene showed the highest expression level with Ct values ranging from 11.5 to 13.0, while *GAPDH* displayed the lowest expression level with Ct values ranging from 26.1 to 30.7. Based on the comparative ranges of Ct values for each reference gene, *18S rRNA* showed the most stable gene expression, while *60S rRNA* had the most obvious expression variation.

However, a simple evaluation of the raw Ct values could not provide sufficient information to determine expression stability of the candidate reference genes. We therefore used

geNorm and NormFinder to provide more information on expression stability.

Expression stability of candidate reference genes

The expression stability values (*M*) produced by geNorm software allow ranking of the expression stability of each reference gene. Figure 3 ranks the reference genes for effectiveness under each stress treatment based on *M*, with the least and most stable genes plotted at the left and right, respectively. Other points represent genes with intermediate expression stabilities compared to those of the least and most stable genes. All *M* values were lower than 1.5, indicating that the expression levels of these genes were relatively stable under all experimental conditions. Among the genes, *60S rRNA* and *actin* were the most stable under nutrient deficiency, salinity, or temperature treatment, whereas *EF1*

Figure 3: Gene expression stability values (M) and ranking of six reference genes in all samples under different treatments calculated using geNorm software.

Average expression stability value (M) was calculated by stepwise exclusion of the least stable gene compared across different desiccation, nutrient deficiency, salinity, and temperature conditions, and the combination of all conditions (Total). In each plot, the least stable gene is on the left, and the most stable gene is on the right. Data were derived from 27 culture samples and repeated for 3 times.

and *tubulin* showed the best stability under desiccation treatment and conditions combining all treatments (total).

To provide an independent assessment, the expression stabilities of reference genes were also ranked by NormFinder. Table 4 shows that *tubulin* and *60S rRNA* were the most stable genes under desiccation and temperature treatments, respectively, while *GAPDH* was highly stable under nutrient deficiency and salinity conditions. *GAPDH*, *tubulin*, and *EF1* were the most stable genes under conditions combining all treatments.

The geNorm software also produced information on pairwise variation value ($V_{n/n+1}$), which was used to assess the optimal number of reference genes for accurate normalization (Figure 4). In this analysis, a $V_{n/n+1}$ value below 0.15 indicates that an additional reference gene is not required. The $V_{2/3}$ for each single treatment was below 0.15, implying that use of two stable reference genes is acceptable for normalizing gene expression within each single treatment (Table 5). In addition, $V_{2/3}$ and $V_{3/4}$ were above 0.15 and below 0.15, respectively, under treatment combining all conditions, revealing that three reference genes are required for gene expression analysis under combined stress conditions (Table 5).

These findings, presented in Figures 3 and 4 and Tables 4 and 5, indicated that use of two or three reference genes ranked as the top two or three in Table 5 is suitable for normalization of stress-inducible gene expression in '*Bangia*' sp. ESS1. For example, *EF1* and *tubulin* are suitable for desiccation treatment, whereas nutrient deficiency and salinity treatments require use of *60S rRNA*, *actin*, and *GAPDH*. Combination of all treatments requires *EF1*, *tubulin*, and *GAPDH* as reference genes.

Validation of reference gene suitability by quantification of expression levels of the *delta-12 fatty acid desaturase* gene under different stress conditions

To evaluate the suitability of reference genes selected by our analyses, we quantified the expression levels of the *delta-12 fatty acid desaturase* gene from '*Bangia*' sp. ESS1, designated *BE1Des12*, under normal (15°C), cold stress (5°C), and heat stress (30°C) conditions for 24, 48, and 72 h by qRT-PCR using the most stable (*60S rRNA* and *actin*) and least stable (*18S rRNA*) reference genes. When *60S rRNA*, *actin*, and the combination of both were used as reference genes for normalization, expression levels of *BE1Des12* were

Figure 4: Determination of the optimal number of reference genes for normalizing gene expression data. Pairwise variation ($V_{n/n+1}$), where n represents number of genes) was analysed between normalization factors (NFn and $NFn+1$) by geNorm software for all different conditions examined (desiccation, nutrient deficiency, salinity, temperature, and all these factors combined). The dotted line at 0.15 is a cut off value, below which an additional reference gene is not needed. Data were derived from 27 culture samples and repeated for 3 times.

Ranking ^a	Desiccation		Nutrient deficiency		Salinity		Temperature		Total	
	geNorm	NormFinder	geNorm	NormFinder	geNorm	NormFinder	geNorm	NormFinder	geNorm	NormFinder
1	EF1	tubulin	60S	GAPDH	60S	GAPDH	60S	60S	EF1	GAPDH
2	tubulin	EF1	actin	actin	actin	60S	actin	actin	tubulin	tubulin
3	GAPDH	actin	GAPDH	60S	GAPDH	actin	GAPDH	GAPDH	GAPDH	EF1
4	actin	GAPDH	18S	18S	18S	EF1	EF1	EF1	actin	actin
5	18S ^b	18S	EF1	EF1	EF1	18S	tubulin	tubulin	18S	18S
6	60S ^c	60S	tubulin	tubulin	tubulin	tubulin	18S	18S	60S	60S

a: Ranking indicates the stability of gene expression from the most stable to the least stable.

b: 18S represents 18S rRNA.

c: 60S represents 60S rRNA.

Table 5: Expression stability ranking results from geNorm and NormFinder software.

increased by exposure to cold stress, reaching the highest level at 72h, while expression levels were not changed under heat stress (Figure 5). By contrast, when the *18S rRNA* gene was used as the reference gene, normalized expression levels of *BE1Delta12* under both cold and heat stress were not significantly different from those under normal conditions (Figure 5). These experiments confirmed the suitability of *actin* and *60S rRNA* as reference genes for examination of cold-inducible *BE1Delta12* expression in '*Bangia*' sp. ESS1.

These findings also indicated the suitability of the two top-ranked genes in Table 5 as reference genes under the corresponding stress conditions. As *BE1Des12* induction by stresses other than cold has not been examined to date, we quantified *BE1Des12* expression levels under various stress conditions by normalization with the most suitable reference genes shown in Table 5. Desiccation, nutrient deficiency, and high salinity induced expression of *BE1Des12*, although the kinetics of induction varied among these stresses (Figure 6), suggesting that modifications of membrane fatty acid composition probably occurred following *BE1Des12* expression in response to various stresses in '*Bangia*' sp. ESS1.

Discussion

We classified the material used in this study as a member of the '*Bangia*' 2 group based on phylogenetic analysis (Figure 1). Since this indicated the difficulty of recognizing this material as *B. fuscopurpurea*, as in our previous study [13], we renamed it '*Bangia*' sp. ESS1. The plastid genome of a member of the '*Bangia*' 2 group, designated '*Bangia*' sp. OUCPT-01 and collected at Putian, Fujian, China, was recently sequenced completely [41]; however, '*Bangia*' sp. OUCPT-01 is different from '*Bangia*' sp. ESS because it forms a clade with '*Bangia*' sp. BGA collected at Gentle Annie, Westland, New Zealand, while '*Bangia*' sp. ESS1 and '*B. fuscopurpurea*' CMNH UM BF1 form a distinct sister clade (Figure 1). These findings make the results of biological studies using *Bangia* species highly confusing because experimental results are partially derived from different *Bangia* species; thus, it is difficult to obtain a unified view of physiological regulations in a certain species or among species. Therefore, physiological and molecular biological research in *Bangia* must be performed using several specimens classified phylogenetically and maintained in laboratories to examine biological characteristics of each and among the four '*Bangia*'

Figure 5: Gene expression patterns of *Delta12-fatty-acid desaturase* under temperature stress normalized by the two most stable reference genes, *60S rRNA* and *actin*, and the least stable reference gene, *18S rRNA*.

Relative expression values shown are mean fold changes compared to samples at 15°C (Control samples, C), with error bars representing standard deviation. Data are from triplicate samples, each with triple technical replicates for qRT-PCR. Different letters above the bars indicate significant differences from the control at 15°C ($P < 0.05$).

Figure 6: Gene expression patterns of *Delta12-fatty-acid desaturase* (BE1Des12) under desiccation, nutrient deficiency, and high salinity, normalized using the two most stable reference genes for each treatment.

Relative expression values are mean fold changes compared to control (C) samples, with error bars representing standard deviation. Data are from triplicate samples, each with triple technical replicates for qRT-PCR. Different letters above the bars indicate significant differences from the control at 15°C ($P < 0.05$). NaCl was used for the high salinity treatment. The label of "25%→1h" in desiccation experiments means that thalli weighing 25% of the initial weight were incubated in normal seawater medium for 1 h.

groups. This in turn will provide evidence for the current phylogenetic classification of *Bangia* species.

Our aim here was to identify reference genes suitable for qRT-PCR analysis in studies on the regulation of stress response and tolerance in '*Bangia*' sp. ESS1. We thus evaluated six candidate reference genes under cold, heat, nutrition deficiency, desiccation, and high salinity, the most common environmental factors influencing growth and development of algae. Stability of candidate gene expression was assessed using two widely used analysis programs, geNorm and NormFinder (Figures 2 and 3; Tables 4 and 5). These programs determined the order of candidate genes in terms of suitability as reference genes under each stress condition and the numbers of reference genes required for accurate normalization. As shown in Tables 4 and 5, *GAPDH* is a suitable reference gene for gene expression analysis under nutrient deficiency and salinity treatments, whereas normalization of gene expression levels under desiccation stress requires *ELF1*. *GAPDH* and *ELF1* are necessary for normalization under stress conditions combining all treatments. In addition, *actin* is a suitable reference for examination of gene expression under nutrient deficiency, temperature changes, and high salinity, while *tubulin* is a highly stable reference gene under desiccation and combined-stress conditions. Moreover, *60S rRNA* is suitable for normalization of gene expression under temperature changes, nutritional deficiency, and high salinity. The suitability of *60S rRNA* and *actin* was indeed validated by quantitative gene expression analysis of low-temperature-inducible expression of *BE1Des12* (Figure 5), although Cao et al. [18] employed *tubulin* and *GAPDH* genes were employed as references. Therefore, we conclude that there is no perfect reference gene suitable for gene expression analysis under a variety of stress conditions; thus, selection of reference genes appropriate for the desired stress conditions is necessary for performing accurate qRT-PCR analyses in '*Bangia*' sp. ESS1.

Our findings are in part consistent with previous knowledge. For instance, *GAPDH* has been established as a suitable reference gene for gene expression studies in the green alga *Chlamydomonas* sp. ICE-L under different light intensities [42] and in the red alga *G. lemaneiformis* under temperature stress and at different life cycle stages [36]. *EF1* is also recognized as a highly suitable reference gene, showing stable expression, for instance, under the diurnal cycle, high light, high salinity, and UV-B irradiation in the green alga *Chlamydomonas* sp. ICE-L [42] and under certain stress conditions and at different developmental stages in the red alga *P. yezoensis* [34]. In addition, the *actin* gene is useful for studying gene expression responses to temperature changes in *G. lemaneiformis* [36]; however, it is suitable as a reference gene for studying responses to abiotic stress in *P. yezoensis* but not gene expression changes at different life cycle stages [34]. The *tubulin* gene is generally used as a reference gene in the brown alga *E. siliculosus*, the dinoflagellate *Prorocentrum minimum*, the green alga *U. linza*, and the red alga *P. yezoensis* [31,33,34,43]. Moreover, ribosomal protein

genes have been reported as reference genes in the green algae *Chlamydomonas* sp. ICE-L [32] and *V. carteri* [44] as well as the diatom *Phaeodactylum tricornutum* [45]. It is not known how these reference genes were selected in these reports, so we suggest reconfirming the suitability of these reference genes to allow more accurate quantification of stress-inducible gene expression in these algae.

It is notable that *18S rRNA* was not suitable as a reference gene for analysis of stress-inducible gene expression in '*Bangia*' sp. ESS1. The *18S rRNA* gene is commonly used as a reference gene in algae [46,47]. For instance, *18S rRNA* showed outstanding stability under different light intensities [31] and among life cycle stages [30] in green algae and has been used extensively to normalize gene expression during development and under temperature changes and nutrient deficiency in red algae [9,16]. However, *18S rRNA* showed moderate stability across all the treatments we examined in this study. The median Ct value of 12.3 for *18S rRNA* was much lower than those for the other reference genes (Figure 2), meaning that the abundance of *18S rRNA* mRNA transcripts was much higher. The high abundance of *18S rRNA* makes it difficult to reliably subtract the background baseline value when normalizing the mRNA transcripts of target genes. In addition, *18S rRNA* did not rank among the three most stable reference genes in any treatment (Table 5). These findings suggest that experiments using *18S rRNA* as a reference gene should be revised by identifying more suitable reference genes in algae. For instance, *18S rRNA* has been employed as a reference gene for gene expression analyses in the red alga *P. yezoensis* [10,11,48]; however, it is necessary to find more suitable reference genes for studies of stress-inducible and life cycle generation-dependent gene expression in this organism.

As far as we know, this work is the first to evaluate candidate reference genes for environmental stress-inducible gene expression analysis in *Bangia* species. Our results indicate that reference genes used in qRT-PCR of stress-inducible gene expression should be employed separately according to differences in environmental stresses to avoid misinterpretation of results in '*Bangia*' sp. ESS1. The identification of reference genes in this study will facilitate future studies on gene expression in *Bangia* species to improve our understanding of the molecular mechanisms of responses to environmental stress.

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