

Selection of reference genes for gene expression normalization in *Pyropia yezoensis* using quantitative real-time PCR

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Abstract Quantitative real-time PCR (qRT-PCR) is frequently used for gene expression analysis. The selection of reference genes is required for normalization of the variation to avoid misinterpretation of experimental results and erroneous analyses. *Pyropia yezoensis*, growing in the intertidal zone, is an economically important seaweed. Although this intertidal seaweed has been an experimental system for understanding stress tolerance and developmental mechanisms, reference genes suitable for the normalization of qRT-PCR data have not previously been identified. In this study, expression stability of six candidate reference genes, including *ACT*, *eIF4A*, *EF1 α* , *GAPDH*, *TUA*, and *UBQ* (traditional housekeeping genes), has been validated in a diverse set of samples representing different developmental stages and stress conditions of *P. yezoensis*. Three qRT-PCR analysis methods, geNorm, NormFinder, and BestKeeper, were evaluated systematically. The results indicated that *ACT3*, *eIF4A*, and *EF1 α* were the optimal reference genes for *P. yezoensis* under stress conditions; *UBQ*, *EF1 α* , and *eIF4A* were suitable for studying the expression of genes related to *P. yezoensis* development. *TUA* showed the lowest expression stability both under stress conditions and over developmental stages. Our results have provided a reference gene application guideline for *P. yezoensis* gene expression characterization using the qRT-PCR system.

Keywords BestKeeper · GeNorm · NormFinder · Quantitative real-time PCR · Reference gene · *Pyropia yezoensis* · Rhodophyta

Introduction

Gene expression analysis is a very important aspect of functional genomics at the level of messenger RNA. Northern blotting has been widely applied to study the expression characteristics of specific genes. However, this technique is time-consuming and requires a large quantity of RNA. At present, quantitative real-time PCR (qRT-PCR) is the most popular method for quantifying messenger RNA (mRNA) transcription levels, and also for validating digital gene expression profile data, due to its accuracy, sensitivity, specificity, reproducibility, and robustness (Hu et al. 2009; Schmittgen and Livak 2008). When conducting gene expression analysis by RT-qPCR, there are many variable factors, including the RNA quality, reverse transcription of the RNA, and the amplification efficiency. Therefore, the selection of reference genes is a helpful strategy for normalization of the variation arising from the amount and quality of mRNA of different samples (Andersen et al. 2004).

Housekeeping genes refer to those that are required for the maintenance of basic cellular function and are expressed in all cells of an organism under normal and altered physiological conditions (Butte et al. 2001). Those are usually considered to be the most appropriate reference genes for studying gene expression by qRT-PCR, as they are supposed to have a uniform expression in all samples and under all experimental conditions tested. At present, β -actin (*ACT*), α -tubulin (*TUA*), ubiquitin (*UBQ*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), elongation factor 1- α (*EF1 α*), and 18S or 26S ribosomal RNA are commonly used as reference genes or internal controls, for studying gene expression in many plants

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(Chandna et al. 2012; Die et al. 2010; Vandesompele et al. 2002). However, in recent years, many investigations indicate that these genes are not constitutively expressed across all the developmental stages and under various environmental conditions, and thus are unsuitable for gene expression analysis (Chandna et al. 2012; Chang et al. 2012; Chen et al. 2010; Czechowski et al. 2005; de Oliveira et al. 2012). It has also been shown that any single gene is unlikely to completely normalize gene expression data from all types of samples (Li et al. 2011; Die et al. 2010). Moreover, some stress-related genes also can serve as reference genes in some experiments, such as those encoding SKP1/Ask-interacting protein 16 (*SKIP16*), metalloprotease (*MTP*), RNA polymerase subunit (*RPII*), and F-box protein (*F-box*) (Artico et al. 2010; Barsalobres-Cavallari et al. 2009). Therefore, it is important to select multiple stably expressed reference genes, so as to improve the reliability and accuracy of qRT-PCR gene expression data. The validation of multiple reference genes has been performed in many organisms, including animals and plants; however, no suitable internal controls for gene expression studies have been defined for *Pyropia yezoensis*, which limits further studies on this species at the transcript level.

Pyropia yezoensis, growing in the intertidal zone, is an economically important seaweed and is widely cultivated in China, Japan, and Korea. It is periodically exposed to air where it experiences a variety of potentially stressful environmental conditions, including nutrient limitation, high light, high and low temperature, desiccation, and osmotic stress (Davison and Pearson 1996). Members of the genus *Pyropia* have a heteromorphic life cycle with an alternation between a macroscopic foliose thallus, which is the gametophytic phase, and a filamentous sporophyte called the conchocelis phase. It has been used as an experimental system for investigating stress tolerance and developmental mechanisms (Blouin et al. 2010; Sahoo et al. 2002). Although the development of next generation sequencing (NGS) has accelerated studies of gene expression at the transcript level, it is still essential to validate the expression of individual genes of interest using qRT-PCR.

In order to select the most suitable reference genes for gene expression quantification by qRT-PCR, we analyzed different stress factors and developmental stages, including temperature, light intensity, desiccation, vegetative tissue from gametophyte blades, and free-living sporophyte filaments. Six potential reference genes involved in different biological roles, *ACT3*, *UBQ*, *GAPDH*, *eIF4A*, *EF1 α* , and *TUA*, were assessed using several statistical approaches for the normalization of data.

Materials and methods

The pure *Pyropia yezoensis* line RZ58, established by self-fertilization in the laboratory, was used for sampling of

different treatments. Fresh leafy gametophytes were cultured in sterilized seawater at 10 °C and 50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ with a 12:12-h light/dark cycle before use. The filamentous sporophytes were cultured in Provasoli's enriched seawater medium (Provasoli 1968) at 18 °C and 20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ with a 12:12-h light/dark cycle. The seawater was bubbled continuously with filter-sterilized air and renewed every 3 days.

Different abiotic stress factors were applied, including temperature, light intensity, and dehydration. For temperature stress, the gametophytes were held at 4, 10, 15, 20, and 25 °C, for 4 h. For light intensity stress, the samples were placed in the dark and at 50, 150, 300, and 600 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ for 4h. For dehydration stress, the samples were desiccated until the total water content of the thalli reached 30, 50, 70, 90, and 100 % of full hydration. Different developmental stages included vegetative tissue from gametophyte blades and free-living sporophyte filaments. All the collected samples were immersed in liquid nitrogen and stored individually at -70 °C for RNA extraction.

RNA extraction and cDNA synthesis

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. DNase I (RNase free) (Qiagen) was used to remove genomic DNA (gDNA) contamination. The quality of the RNA was assessed from the OD 260/280 ratio (1.9 to 2.1) and the optical density (OD) 260/230 ratio (>2.0). The integrity of RNA samples was also checked by agarose gel electrophoresis. First-strand complementary DNA (cDNA) was synthesized from 1 μg of total RNA in a volume of 20 μL using an MMLV Reverse Transcription kit (Promega). The cDNA was diluted 25 times before being used as templates in qRT-PCR. Successful removal of DNA contamination was confirmed by testing for the presence of a PCR amplification product using the primer pair (5'-GTCAAATACTGGGAAGATC-3' and 5'-TTTGAGGTAGGAAGTGTAGT-3') designed to amplify an intron sequence of the GAPDH gene (GenBank accession no. AB303420.1). RNA extraction and cDNA synthesis from all of the different samples were performed for three biological replicates.

Design of qRT-PCR primers

The six traditional housekeeping genes often used as reference controls in higher plants were selected *ACT3* (Actin3, AB126237.1), *UBQ* (Ubiquitin, FJ232910.1), *eIF4A* (translation initiation factor 4A, FJ407185.1), *EF1 α* (elongation factor, comp149285), *GAPDH* (glyceraldehyde-3-phospho dehydrogenase, AB303420.1), *TUA* (α -tubulin DQ980504.1). Their sequences were downloaded from GenBank, except for

EF1α, which was obtained from the transcriptome sequence obtained by Yang et al. (2011). The primers for six candidate reference genes were designed using Primer 5.0 software (Premier Biosoft International, USA). The design criteria were as follows: annealing temperature of 60 °C, GC content of 42–55 %, and primer length of 19–21 bp. The length of amplicons ranged from 100 to 250 bp (Table 1). All primer pairs were validated by PCR, and amplification products yielding a single band were also sequenced to verify the efficiency and accuracy of the primer pairs.

qRT-PCR

The qRT-PCR reactions were carried out in a total volume of 20 μL containing 10.0 μL of 2× SYBR Green PCR Master Mix (Bio-Rad), 1 μL (10 pmol) of each primer, 1 μL 1:5 diluted template, and 7.0 μL of sterile distilled water. The thermal cycling consisted of 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. The dissociation curve was generated by heating from 60 to 95 °C to check for specificity of amplification. All reactions were performed in 96-well reaction plates, in triplicate for both technical and biological replicates, using an iQ5 instrument (Bio-Rad). Each PCR run included a no-template negative control using sterilized water. The standard curve for each gene was generated by using a 10-fold dilution series of one sample over at least six dilution points measured in triplicate.

Determination of reference gene expression stability

To identify the most appropriate reference genes in different experimental treatments, three different software packages, geNorm, NormFinder, and BestKeeper, were applied to assess

the stability of the mRNA expression of each reference gene. All three software packages were used according to the manufacturer’s instructions. The geNorm and NormFinder rank the candidate reference gene according to expression stability (*M*). The geNorm software also calculates the optimal number of reference genes needed for normalization. BestKeeper performs numerous pairwise correlation analyses and estimates intergene relationships of possible reference gene pairs (Andersen et al. 2004; Pfaffl 2001; Vandesompele et al. 2002).

Results

Verification of primer specificity and PCR efficiency analysis

In order to determine the specificity and efficiency of primers in this study, agarose gel electrophoresis analyses were performed to check the amplicons of the candidate reference genes derived from all templates in RT-PCR. All the primer pairs amplified a single PCR product of expected size from the cDNA template. Also, the specificity of the amplicon was confirmed by the presence of a single peak in the melting curve and further confirmed by amplicon sequencing. Before carrying out the qRT-PCR, all of the RNA samples were shown to be clean by the PCR results for the *PyGAPDH* gene. There was no gDNA contamination in the cDNA templates, and no amplicons were detected in the negative controls. A standard curve was generated using 10-fold serial dilutions of plasmids, and the slopes of standard curves were used to check the correlation coefficient (*R*²) and PCR efficiency. The *R*² and PCR efficiency ranged from 0.994 to 0.995 and from 98.0 to 105 %, respectively, which are well within the acceptable range of qRT-PCR and suitable for further gene expression analysis by qRT-PCR (Table 1).

Table 1 Information about the six candidate reference genes and primers used in this study

Gene abbreviation	GenBank accession number	Gene function description	Primer sequences (5′–3′)	Product size (bp)	Efficiency (%)	Correlation coefficient (<i>R</i> ²)
<i>ACT3</i>	AB126237.1	Actin3	F-CAAGCAGAAGGGCATCAT R-CCGAGTAGAAAGCGTGGT	164	102	0.9995
<i>UBQ</i>	FJ232910.1	Ubiquitin	F-TTCCAAGGTGCTCCTCTCCATC R-CGTCTCTCATAGCGACTGCGGTT	116	99.5	0.9961
<i>GAPDH</i>	AB303420.1	Glyceraldehyde-3-phospho dehydrogenase	F-GGTGCGTCCAAGCATCTGA R-TGGGTGTAGTCTGGTTCGTTT	113	99	0.9884
<i>eIF4A</i>	FJ407185.1	Translation initiation factor 4A	F-GCTTTCTGTCTGGACGAGG R-TCTTACAAGGATGCGGAT	181	101	0.9992
<i>EF1α</i>	comp149285	Elongation factor1-α	F-TTTTGGTGGCACTGCTGAT R-TACACGCACAAGTTGGGGA	141	98	0.9975
<i>TUA</i>	DQ980504.1	α-Tubulin	F-ATGGCGTGCTGCCTGATGT R-TCGGGGGCTGGTAGTTGAT	145	105	0.9946

Expression levels of candidate reference genes

In order to provide an overview of the expression stability of the six candidate reference genes in all cases, the Cq values of these genes from five different treatments across all samples were calculated. The Cq values of reference genes showed a range of variation from 18.34 to 36.93, and most Cq values were between 20.64 and 29.08. The *eIF4A* gene displayed the highest expression level, while *TUA* had the lowest expression. In addition, each candidate gene showed a specific Cq value variation range under the conditions tested. The calculated coefficient of variance (CV) of the Cq values was further used to assess the expression stability of these particular genes, and the results indicated that *GAPDH* and *eIF4A* showed stable gene expression (below 3 cycles), while *EF1 α* and *TUA* had obvious expression variation as shown in Fig. 1. However, simple evaluation of the raw Cq values for selection of reference genes with stable expression could not provide sufficient information. So, we used the following three different statistical algorithms for reference gene validation.

GeNorm analysis

Before investigating the gene expression stability, all samples were divided into two groups according to the stress factors and developmental stages. The raw Cq values were transformed into the relative expression quantity, and the gene *M* measure was ranked, based on the average pairwise variation among all candidate reference genes. A lower *M* value represented a more stable expression. Hellemans et al. (2007) recommended a stability measure threshold lower than 1.5 to ensure that the most stable genes are selected. In this study, for different stress treatments and different developmental stages, both of the *eIF4A* and *EF1 α* genes were most stably

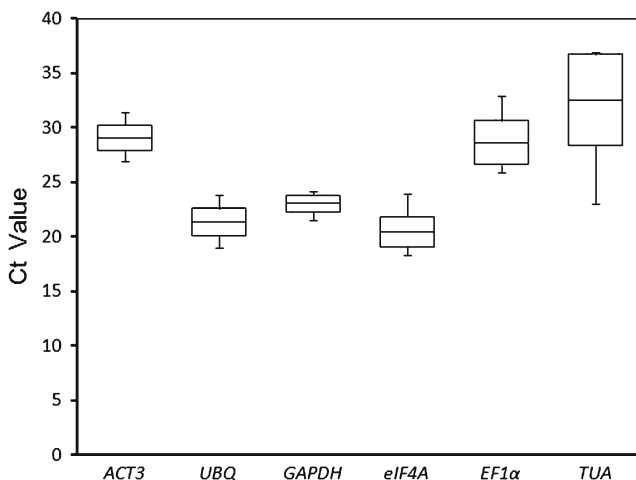


Fig. 1 The mean Cq values of six candidate reference genes in all *P. yezoensis* samples. The boxes represent mean Cq values. The bars indicate the maximum and minimum values

expressed, with an *M* value of 0.64 and 0.26, followed by *UBQ*, *ACT3*, and *GAPDH* for stress treatments, *UBQ*, *GAPDH*, and *ACT3* for developmental stages. Their *M* values were also lower than 1.5. *TUA* was the least stably expressed, whether under different treatments or developmental stages, with an *M* value of 2.0 and 1.8 (Fig. 2a, b). Pairwise variation (*V*) was applied to assess the optimal number of genes when using multiple reference genes. When analyzing all six genes with a pair variation under stress treatment and developmental stages, there was no significant difference in the *V* numbers. All the $V_{n/n+1}$ values from two different treatment groups were above 0.15, which is the default limit of 0.15 by geNorm (Fig. 3). Adding additional reference genes will increase the instability, and also the complexity of the experimental work. Considering these factors, only the two reference genes, *eIF4A* and *EF1 α* combination, should be used in qRT-PCR experiments examining different stress and developmental stages.

NormFinder analysis

NormFinder ranks the set of candidate normalization genes according to the expression stability based on the average pairwise variation to identify the optimal normalization gene in a given experimental treatment (Andersen et al. 2004). The lower the average expression stability value is, the more stable the gene expression. The stability value of each gene in different experimental groups is listed in Table 2. Only limited differences were found between the geNorm and the NormFinder analysis results. For different stress treatments, *ACT3* ranked as the most stable gene (*M* value of 0.029), while for developmental stages, *UBQ* had the most stable expression (*M* value of 0.006). *TUA* was the most unstable gene, both for different stress treatments and developmental stages. *EF1 α* and *eIF4A* occupied the second and third positions under stress treatment with lower stability values of 0.034 and 0.069, respectively. *GAPDH* and *EF4A* had stable expression and ranked second and third for developmental stages of *P. yezoensis* (*M* values of 0.049 and 0.052). As different softwares apply different statistical algorithms, these slight differences are acceptable.

BestKeeper analysis

In order to fully determine the optimal reference genes for *P. yezoensis*, BestKeeper software was also applied, which is based on the coefficient of correlation (*r*) to the BestKeeper index by calculating the standard deviation and CV (de Oliveira et al. 2012). Initial analysis of the data was performed by variations (SD [\pm Ct] and CV [%Ct]). Reference genes with SD values greater than 1 should be excluded; thus, *EF1 α* and *TUA* were excluded from stress treatment analysis, and *TUA* was excluded from developmental stage analysis. The final

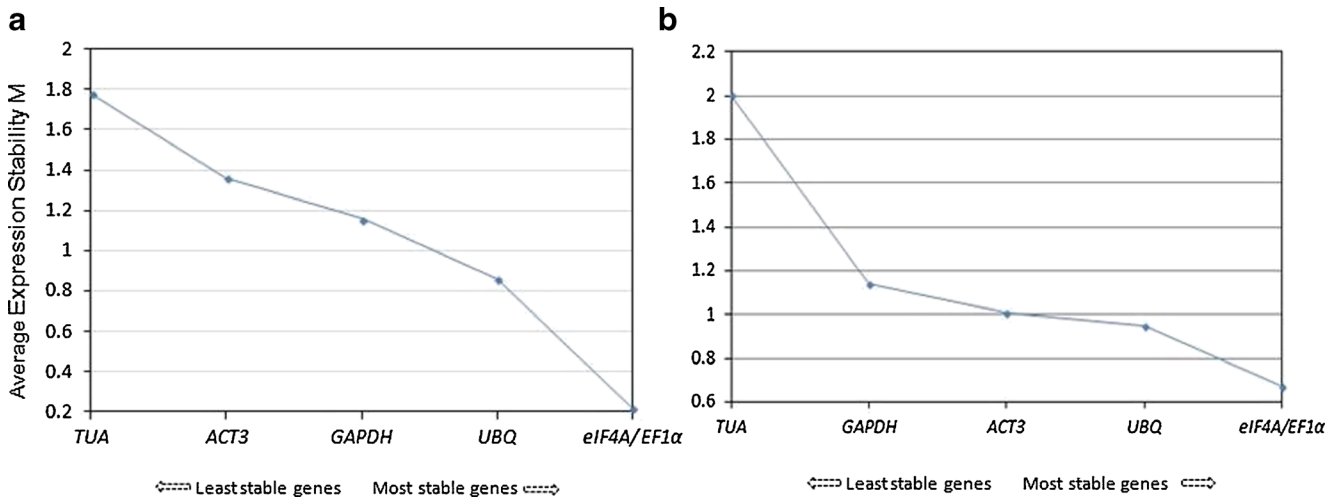


Fig. 2 Expression stability values (*M*) of six candidate reference genes in different sample groups (**a**, **b**) by geNorm software. **a** different stress factors, **b** different developmental stages

results revealed that under different stress treatments, *ACT3* was the most stable gene, with the highest correlation coefficient ($r=0.81$). *eIF4A* was the second most stable gene with $r=0.66$. However, *eIF4A* showed the most stable expression ($r=0.84$) for different developmental stages. The SD value of *TUA* under either condition was greater than 1, which further showed that it was not appropriate as a reference gene in qRT-PCR analysis (Table 3).

In summary, the rank orders were slightly different among the three methods, and no gene was listed to be the most stable reference gene by the three software packages, whether for stress treatments or developmental stages. For stress treatments, both NormFinder and BestKeeper analyses revealed that *ACT3* was the most stable reference gene, and geNorm analyses revealed that *eIF4A* and *EF1α* combination can be adequate for our qRT-PCR normalization of different stress treatments. So, overall, we accepted that *ACT3*, *eIF4A*, and

EF1α are appropriate reference genes when considering stress factors. For the same reasons, *eIF4A*, *EF1α*, and *UBQ* can be used for studying gene expression in developmental stages. *TUA* was evaluated by three methods as the least stable gene for both stress factors and developmental stages in *P. yezoensis* in this study.

Discussion

An appropriate reference gene is required for normalizing qRT-PCR data prior to analyzing the expression of a target gene. However, many researchers have indicated that the traditional reference genes are not always stably expressed in different species, developmental stages, and environmental influences (Artico et al. 2010). The selection and evaluation of reference genes have been a prerequisite for studying gene expression using RT-PCR (Chen et al. 2011). To screen for reference genes suitable for studies on developmental and stress-responsive gene expression in *P. yezoensis*, six candidate genes (*ACT3*, *UBQ*, *eIF4A*, *EF1α*, *GAPDH*, and *TUA*)

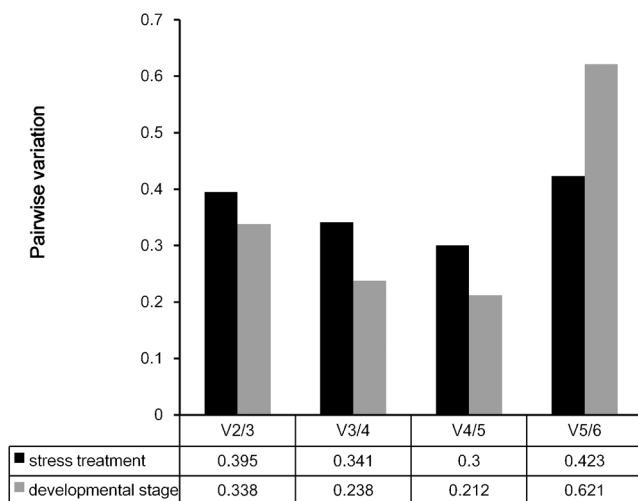


Fig. 3 Pairwise variation (*V*) analysis of six candidate reference genes of *P. yezoensis*, in different sample groups

Table 2 Expression stability analysis of six reference genes by NormFinder

Rank	Stress treatment		Developmental stage	
	Gene name	Stability value	Gene name	Stability value
1	<i>ACT3</i>	0.029	<i>UBQ</i>	0.006
2	<i>EF1α</i>	0.034	<i>GAPDH</i>	0.049
3	<i>eIF4A</i>	0.069	<i>EF1α</i>	0.052
4	<i>GAPDH</i>	0.076	<i>ACT3</i>	0.118
5	<i>UBQ</i>	0.087	<i>eIF4A</i>	0.13
6	<i>TUA</i>	0.343	<i>TUA</i>	0.257

Table 3 Expression stability values for candidate reference genes calculated using BestKeeper

Gene name	Stress treatment			Developmental stage			
	SD [\pm Ct]	CV [%Ct]	Coefficient of correlation (<i>r</i>)	SD [\pm Ct]	CV [%Ct]	Coefficient of correlation (<i>r</i>)	
<i>ACT3</i>	0.8	2.77	0.81	<i>eIF4A</i>	0.84	4.3	0.84
<i>eIF4A</i>	0.65	3.22	0.66	<i>EF1α</i>	0.85	3.13	0.7
<i>UBQ</i>	0.65	3.13	0.44	<i>ACT3</i>	0.97	3.37	<0.4
<i>GAPDH</i>	0.65	2.81	<0.4	<i>UBQ</i>	0.21	0.99	<0.4
<i>EF1α</i>	1.17	4.15	0.71	<i>GAPDH</i>	0.5	2.12	<0.4
<i>TUA</i>	2.33	6.9	0.39	<i>TUA</i>	1.41	5.62	0.43

were selected to assess their expression using three software packages: geNorm, NormFinder, and BestKeeper. Some reports suggested that different software would result in different conclusions due to their distinct statistical algorithms and analytical procedures (Hong et al. 2008; Hu et al. 2009; Wan et al. 2010). Our research also showed that slight differences occurred among different experimental groups using these three analysis approaches. Under different stress treatments, the stabilities of *eIF4A* and *EF1 α* were ranked as the highest by geNorm, while NormFinder and BestKeeper analyses found *ACT3* to be expressed most stably, followed by *eIF4A*. When considering different developmental stages, geNorm and BestKeeper both found *eIF4A* to be expressed most stably, whereas *UBQ* was ranked as the most suitable candidate reference gene by NormFinder. Increasing the number of reference genes is helpful for normalization when analysis variation is large, but it is expensive and time-consuming (Maroufi et al. 2010). Use of two stable reference genes is acceptable for studying gene expression in most cases and has already resulted in more accurate and reliable normalization compared to the use of a single reference gene. GeNorm software can list the optimal number of reference genes. In this research, the value of pairwise variation $V_{n/n+1}$ from two group samples is all above 0.15, which is the threshold in the software. So, based on our research and considering the results of three programs, we recommend *eIF4A* and *EF1 α* genes as the reference gene combination to analyze gene expression, either for different stress factors or developmental stages of *P. yezoensis*, when using qRT-PCR.

EF1 α and *eIF4A* encode important protein factors involved in the initiation and elongation processes of eukaryotic translation. Studies in various species suggested that their expression patterns show significant differences. In *Arabidopsis*, *eIF4A* is a less reliable reference gene, and the relative amount of *EF1 α* mRNA declined from 40 to 15 % in different organs (Gutierrez et al. 2008). In contrast, *eIF4A* is suitable as the reference gene for studying gene expression of red cover root tissues (Mehdi Khanlou and Van Bockstaele 2012). In this research, we found that *eIF4A* is also a useful reference gene for studying gene expression in *P. yezoensis*

thalli responding to different stresses or at different developmental stages.

Actin is the principle constituent of the cytoskeleton and is involved in cell motility and structure. The *ACT* gene is a commonly used reference gene for gene expression in many species including plants and animals. However, *ACT* did not appear to be the best choice as reference gene when examining different treatments of potato (Nicot et al. 2005). The same conclusion was reached in a study of various developmental stages in *Arabidopsis* (Gutierrez et al. 2008). Yet, when studying different tissues of chicory, *ACT* was assigned as the most stable reference gene (Maroufi et al. 2010). In this paper, analyses conducted with both NormFinder and BestKeeper software indicated that *ACT3* in *P. yezoensis* was expressed very stably under different stress treatments. However, when examining different developmental stages of the alga, *ACT3* was expressed more variably. This is the same as found in the brown alga *Ectocarpus siliculosus*, where *ACT* had the least stable expression (Le Bail et al. 2008).

GAPDH was ranked among the 100 most stably expressed genes of *Arabidopsis*, but only after omission of seed and pollen samples (Czechowski et al. 2005). It is also one of the most suitable reference genes for normalization of barley yellow dwarf virus (BYDV) quantification in barley tissues (Jarosova and Kundu 2010). In contrast to the above, our results showed that *GAPDH* was not one of the most stable genes when examining different stress treatments and developmental stages in *P. yezoensis*, making it less suitable as a reference gene for our purposes.

Tubulin is a globular protein that constitutes the building block of microtubules. *TUA* and *TUB* encode α -tubulin and β -tubulin, respectively, which together as heterodimers are the major component of microtubules (Dutcher 2001). In many higher plants, such as *Eucalyptus globules*, *Brassica juncea*, *Cucumis sativus*, and *Platygladus orientalis*, they are considered to be the most suitable reference genes for studying the expression of specific developmental processes and responses to abiotic stresses (Chandna et al. 2012; Chang et al. 2012; Wan et al. 2010). However, in this research, we found *TUA* to

be the least stable gene when examining gene expression for different stresses and developmental stages.

UBQ was found to be the most appropriate reference gene when examining gene expression impacted by various stresses and different developmental stages in *B. juncea*, *Chrysanthemum* sp., and *Musa* sp., while *UBQ* and its homologs were the least stably expressed genes during total developmental stages in *Oryza sativa*, *Glycine max*, *Cichorium intybus*, and *Vitis vinifera* (Gu et al. 2011; Jian et al. 2008; Maroufi et al. 2010; Reid et al. 2006). In our findings, the analysis using NormFinder software suggested that *UBQ* was expressed stably in different developmental stages, which also demonstrated that *UBQ* can be the candidate reference gene for studying gene expression in *P. yezoensis*.

The literature contains many reports of analyses for different organisms where the most stably expressed genes are different under various stress factors or developmental stages. The varied expression profiles of housekeeping genes in different organisms may be because they are involved in different cellular processes besides their basic cellular metabolic functions (Chandna et al. 2012). In the present study, we have reported the first systematic analysis for the selection of superior reference genes for use in qRT-PCR normalization with respect to different stress factors and developmental stages in *P. yezoensis*. The data analysis from three different software packages identified *ACT3*, *eIF4A*, and *EF1 α* as appropriate reference genes, while *TUA* showed low expression stability for such studies in *P. yezoensis*. These results will be a solid starting point for identifying additional reference genes in studying *P. yezoensis* by qRT-PCR, and also for reference gene selection for other *Pyropia* or *Porphyra* species.

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