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Identification of potential internal control genes for real-time PCR analysis during stress response in *Pyropia haitanensis**

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Abstract *Pyropia haitanensis* has prominent stress-resistance characteristics and is endemic to China. Studies into the stress responses in these algae could provide valuable information on the stress-response mechanisms in the intertidal *Rhodophyta*. Here, the effects of salinity and light intensity on the quantum yield of photosystem II in *Py*. *haitanensis* were investigated using pulse-amplitude-modulation fluorometry. Total RNA and genomic DNA of the samples under different stress conditions were isolated. By normalizing to the genomic DNA quantity, the RNA content in each sample was evaluated. The cDNA was synthesized and the expression levels of seven potential internal control genes were evaluated using qRT-PCR method. Then, we used geNorm, a common statistical algorithm, to analyze the qRT-PCR data of seven reference genes. Potential genes that may constantly be expressed under different conditions were selected, and these genes showed stable expression levels in samples under a salinity treatment, while *tubulin*, *glyceraldehyde-3-phosphate dehydrogenase* and *actin* showed stability in samples stressed by strong light. Based on the results of the pulse amplitude-modulation fluorometry, an absolute quantification was performed to obtain gene copy numbers in certain stress-treated samples. The stably expressed genes as determined by the absolute quantification in certain samples conformed to the results of the geNorm screening. Based on the results of the software analysis and absolute quantification, we proposed that *elongation factor 3* and *18S ribosomal RNA* could be used as internal control genes when the *Py*. *haitanensis* blades were subjected to salinity stress, and that *α-tubulin* and *18S ribosomal RNA* could be used as the internal control genes when the stress was from strong light. In general, our findings provide a convenient reference for the selection of internal control genes when designing experiments related to stress responses in *Py*. *haitanensis*.

Keyword: real-time quantitative PCR; housekeeping genes; internal control genes; stress responding; *Pyropia haitanensis*

Abbreviation: *18S*: 18S ribosomal RNA; *EF3*: elongation factor 3; *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase; *RPS*: 30S ribosomal protein; *TubA*: Alpha-tubulin; *ACT*: actin; *TubB*: beta-tubulin; PAM: pulse-amplitude-modulation fluorometry

1 INTRODUCTION

Pyropia haitanensis, an endemic species of China, belongs to the order Bangiales, the most genetically diverse order of red algae (Ragan et al., 1994). Blades of *Py*. *haitanensis* are found in the upper intertidal

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zones of rocky shores, enduring cycles of desiccation and immergence. Thus, certain stress response mechanisms must have evolved in this seaweed. *Pyropia* is probably of an ancient origin with a long evolutionary history (Blouin et al., 2011). Thus, the stress response mechanisms in these algae might be different from those in higher plants and even in some freshwater algae.

Pyropia haitanensis was first cultivated in Pingtan, Fujian Province, China 1 000 years ago (Zhang et al., 2004). Nowadays, with the establishment and development of modern aquaculture technology, it is an extensively cultivated species in the south of China. The output of *Py. haitanensis* accounts for 85%–90% of the gross production of cultivated *Porphyra* in China (Yan et al., 2005). However, many problems still exist in cultivating *Py. haitanensis*, such as the degeneration of germ quality and a deficiency of high-yield, stress-resistant breeds. Several projects dedicated to seedling cultivation using various methods have been initiated in China. During these projects, real-time quantitative PCR (qRT-PCR) analysis is a technique widely used since gene expression profiles offer information on the regulation of various biological processes (Wise et al., 2007).

An analysis of target gene expression levels can contribute significantly to understanding physiological processes, such as stress responses (Reymond et al., 2000; Setter and Flannigan, 2001). Four commonly used methods for the quantification of transcription are northern blotting, in situ hybridization (Parker and Barnes, 1999), RNase protection assays (Hod, 1992; Saccomanno et al., 1992), and qRT-PCR. The qRT-PCR is a sensitive and flexible method (Giulietti et al., 2001) that can be used to compare mRNA levels in different samples and to characterize mRNA expression patterns (Bustin, 2000; Giulietti et al., 2001; Ginzinger, 2002). A reliable quantification method required eliminating errors derived from the efficiencies of reverse transcription and PCR efficiencies. Indeed, the normalization to an internal control gene would remove the difference in input cDNA amounts derived from the difference of reverse transcription efficiencies among samples (Giulietti et al., 2001). Thus, the $2^{-\Delta\Delta Ct}$ method based on the applying of the internal control gene was the most commonly used quantification method at present. The internal control genes or housekeeping genes which are expressed in a universal and invariant level can serve as the control to correct variations, such as different reverse transcription efficiency, and help to obtain the accurate data. However, the expression levels of these internal control genes usually vary greatly under different experimental conditions (Thellin et al., 1999; Bustin, 2000; Suzuki et al., 2000; Figueroa et al., 2003). More importantly, the peculiar evolutionary status and geographic distribution of *Py. haitanensis* meant that we could not use the internal control genes that are frequently used in higher plants or other algae. To date, there have been no reports, except that of Wu et al. (2012), who focused on the selection of potential internal control genes at different life-history stages in *Py. haitanensis*, concerning the identification of internal control genes for thalli subjected to changing environmental conditions.

In this study, we investigated the effects of high salinity and strong light on the photosynthetic parameters of *Py. haitanensis*. Then, based on the results of pulse-amplitude-modulation fluorometry (PAM), some of the algae samples treated with the different stresses were used for a transcription analysis. The expression level of seven potential reference genes, which were commonly used as internal controls, was assessed by qRT-PCR to determine potential reference gene. A common statistical algorithm, geNorm, was used to identify expression stability. And, the absolute quantification method was applied to further identify the constantly expressed genes. The results will provide useful guidelines for future studies on suitable internal control gene's selection in stress-response research in *Py. haitanensis*.

2 MATERIAL AND METHOD

2.1 *Py. haitanensis* culture

Fresh *Py. haitanensis* leafy gametophytes were collected from Haihu Seaweed Farming Company, Zhejiang Province. Before the stress treatment experiments, the blades were resuscitated at 16°C for 1 day in tanks with a cycle of 50 μmol photons/(m^2·s) cool-white fluorescent irradiance for 10 h and darkness for 14 h. Then, the sea water was replaced with Provasoli's enriched seawater (PES) medium and bubbled continuously with filter-sterilized air through an air inlet at the bottom of the flask. During the experiments, the PES medium was renewed every day.

Table 1 Detail of stress conditions imposed on the samples of *Py. haitanensis*

Treatment / Sample group	Salinity	Light intensity (μmol photons/(m²·s))
C	30	200
S	60	200
	90	
	120	
L	30	400
		800
		1 200

2.2 Treatment of *Py. haitanensis* blades with various stresses

Three groups of healthy *Py. haitanensis* blades were treated separately. For the salinity stress group, the samples were kept in sea water at 16°C with irradiation of 200 μmol photons/(m²·s), and crude salt was added to obtain salinity levels of 30, 60, 90 and 120. For the light-stress group, the samples were maintained in sea water at 16°C with 30 salinity and exposed to 200 (control), 400, 800, and 1 200 μmol photons/(m²·s) illumination. The samples were treated for 4 h and then immediately transferred to the dark. A PAM (Heinz Walz, Effeltrich, Germany) was applied to determine the photosynthetic parameters. For all of the groups, the control sample was *Py. haitanensis* cultured in normal sea water at 16°C with 30 salinity and irradiation of 200 μmol photons/(m²·s) (sample group C in Table 1).

2.3 Measure of photosynthetic parameters

The chlorophyll fluorescence levels of photosystem II (PSII) in vivo in the stress-treated samples were determined using a Dual-PAM fluorometer (Heinz Walz). Three replicates were set for every sample in per groups and each of the sample groups was acclimated to the dark for 10 min before the fluorescence was measured. The intrinsic fluorescence (F_0) of the antennal system of fully oxidized PSII was detected first under a weak measuring light (7 μmol photons/(m²·s)). Then, a 0.8-s high intensity (6 000 μmol photons/(m²·s)) saturating actinic light pulse was applied to obtain the maximal fluorescence (F_m) from the fully reduced PSII reaction centers in the dark-adapted samples (Figueroa et al., 2003). The difference between F_m and F_0 is a measure of the variable fluorescence (F_v), and the maximal quantum yield was obtained as F_v/F_m (Schreiber et al., 1986), which was used to evaluate the potential ability of the algae to transfer light-energy into chemical energy. The effective quantum yield, Y(II), was calculated as described previously (Genty et al., 1989) as:

$$Y(II)=(F_m'-F)/F_m',$$

where F_m' is the maximum fluorescence yield in an illuminated sample. Non-photochemical quenching (NPQ), which denotes the regulated dissipation of photosynthetic energy conversion in PSII (Schreiber et al., 1986).

Experiments were carried out using the automated procedures provided by the Dual-PAM software at room temperature, and the above mentioned parameters can be read directly in the report window on computer. Values of the measurements were expressed as means±standard deviations.

2.4 RNA isolation and reverse transcription

Samples under different stress treatment were collected to extract RNA. Based on the manufacturer's protocols, we used an RNAprep Pure plant kit (Tiangen Biotech Co., Beijing, China) to extract the total RNA. The concentration was quantified with a Qubit fluorometer (Invitrogen Corp., Carlsbad, CA, USA) using the corresponding RNA Assay Kit (Invitrogen Corp.). The RNA yield was calculated as RNA quantity/sample weight. A statistical analysis was performed using a one way ANOVA and the values were deemed to be significantly different when $P<0.05$.

RNase-free DNase I (Promega Biotech Co., Madison, WI, USA) was added to the isolated RNA solution to eliminate genomic DNA contamination and then the effect of digestion was checked with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) primers using the total RNA as the template prior to qRT-PCR. Reverse transcription was carried out in a PCR apparatus (Eppendorf, Hamburg, Germany) according to the manufacturer's instructions using M-MLV reverse transcriptase (Promega Biotech Co.) and random primers. The amplified cDNA was stored at -80°C for later use.

2.5 Genomic DNA extraction

To eliminate possible errors caused by different degree of fragmentation in different samples during RNA isolation, we also set aside part of the sample powder for genomic DNA extraction using a Universal Genomic DNA Extraction Kit (TaKaRa, Dalian,

Table 2 Primer sequences and PCR product size of selected target genes

Gene name	Gene description	Primer sequence 5′→3′	Product size (bp)
18S rRNA	18S ribosomal RNA	CCGTTACTCCTGTGGACCTG TCGTTCAGGACGCAGCAGT	223
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	AAGGACCGAACAGCGTAGGA GGCCAGCAAGTGGGAGTAAG	103
EF3	Elongation factor 3	GACGAGCCGACCAACTACCT ACCACACCGCCGTCAAA	80
RPS	30S ribosomal protein	TCTTGGTCATCCTCTGGTGCT ACCTTGATCCATTGCCTGTTTA	108
TubA	α-tubulin	GCGGTCGTGGAGCCATACA TGCCCAGGCGGTTCTTG	118
ACT	Actin	CGAGCTGGACAAGGTCAACA ATGAGGGCGAGGAAACGA	83
TubB	β-tubulin	GGCTACGCACCGCTTTCTG CATCTGCTCCTCCACCTCCTT	186

China) following the manufacturer's instructions. Then, the genomic DNA concentration was quantified using a Qubit fluorometer with the corresponding DNA Assay Kit (Invitrogen Corp.). Finally, we used the DNA quantity/sample weight to calculate the DNA yield.

2.6 Normalization of RNA yield by DNA

To minimize the inaccuracies in RNA yields among individual extractions, the quantity of RNA was normalized using the genomic-DNA yield derived from the same batch of ground algae. The relative RNA yield was expressed as RNA quantity/DNA quantity.

2.7 Primer design

The genes that we selected as potential internal control genes were 18S ribosomal RNA (*18S*), *GAPDH*, elongation factor 3 (*EF3*), 30S ribosomal protein (*RPS*), α-tubulin (*TubA*), actin (*ACT*) and β-tubulin (*TubB*). The gene sequences were obtained from our in-house EST database. Primers were designed using Primer Premier 5.0 (Premier Biosoft International, Palo Alto, CA, USA) and evaluated with Oligo Primer Analysis software (Wojciech and Piotr Rychlik Copyright, version 6.31). The primer sequences and other details are shown in Table 2.

2.8 Optimal reference genes' screening by geNorm

All of the fragments of the seven selected genes were amplified by conventional PCR and purified using an EZNA TM Gel Extraction kit (OMEGA Bio-Tech, Doraville, GA, USA). The purified fragments were then cloned into the PMD19-T vector (Tiangen Biotech Co.) and transformed into *Escherichia coli* competent cells. Subsequently, the cloned plasmids were purified using the EZNA TM plasmid mini kit (OMEGA Bio-Tek) and quantified using a Qubit fluorometer with the corresponding dsDNA Assay Kit (Invitrogen Corp.). Based on the concentration (g/mL) and length of the vector, the copy number was calculated as described previously (Whelan et al., 2003):

Copy number=[6.02×10^{23} (copies/mol)×DNA amount (g)]/[DNA length (bp)×660 (g/(mol·bp))].

We run the qRT-PCR in triplicate on a BIO-RAD IQ5 RT-PCR detection system (Bio-Rad, Hercules, California, USA) using SYBR Green Master Mix (Tiangen, Beijing, China). The PCR reaction mixture was prepared in a total volume of 20 μL containing 9 μL SYBR Green Master Mix, 2 μL 1:10 diluted template (plasmid or cDNA), 5 μL of sense and antisense primers (2 μmol/L) and 4 μL RNase-free water. The reaction conditions were: 3 min at 94°C, followed by 40 cycles of 15 s at 94°C, 40 s at 60–64°C and 30 s at 72°C.

To validate the specificity of the amplification, the melting curve was generated by heating for 30 s from 55 to 95°C. A 10-fold serial dilution of each plasmid was prepared and a standard curve was constructed (Whelan et al., 2003). The amplification efficiency (E) was calculated as follows:

Efficiency= [$10^{(-1/\text{slope})}$–1]×100%.

Considering that different stresses would lead to different effects on gene expression levels, the samples were divided into two groups, salinity and strong light treatments. Based on the photosynthetic parameters, the samples whose F_v/F_m was significantly

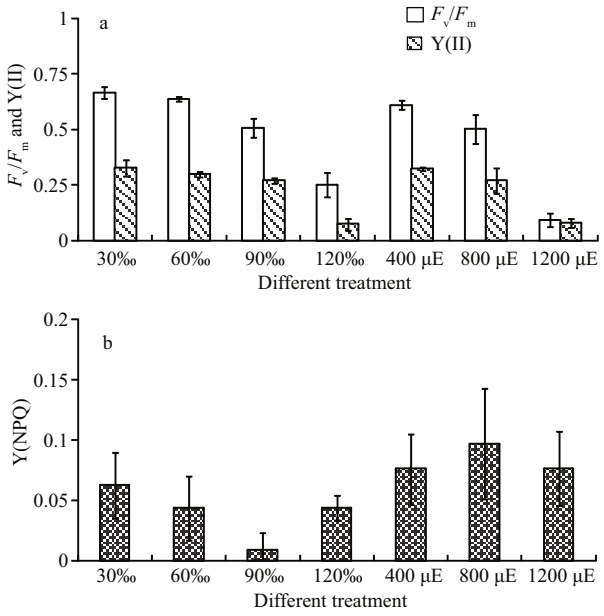

Fig.1 Variation of the optimum quantum yield, F_v/F_m, effective PSII quantum yield, Y(II), and non photochemical quantum yield of PSII, Y(NPQ) in *Py. haitanensis*

The algae were treated with high salinity, strong light. The control sample was *Py. haitanensis* cultured with 30 salinity and irradiation of 200 μmol photons/(m²·s) (sample 30 salinity in Fig.1). The data were the mean of three independent determinations (±SD).

decreased but Y(II) showed relatively stable were selected for absolute quantification analyses. The mRNA abundance levels of the seven candidate reference genes in all of the samples were determined by the C_t values obtained. Then, the stability parameters of these genes in each group were analyzed using geNorm software following the manual. By calculating gene expression stability (M), this software created a stability ranking. The gene with the lowest M value was the most stable (Vandesompele et al., 2002). Usually, if the M value < 1.5, then the gene could be regarded as a potential reference gene.

2.9 Absolute quantification

The copy numbers of the internal control genes in different samples were calculated by relating threshold cycle (C_t) values to the standard curves (Yu et al., 2005; Lee et al., 2006). After normalization against the genomic-DNA yield in each group, the expression levels of the tested genes in the different samples were compared. We selected the copy number of the genes in sample C (leafy gametophyte cultured in 30 salinity at 16°C with 200 μmol photons/(m²·s) light-intensity) as the control, against which the behavior of each of the tested genes under the various experimental stress treatments was evaluated. The expression stability of the tested genes in response to experimental stress treatments was evaluated by comparing the observed copy numbers among the different samples. The relative transcriptional level was deemed to be stably expressed when the ratio was between 0.5 and 2.

3 RESULT

3.1 Effects of stress conditions on the photosynthetic parameters

The variations in *Py. haitanensis* treated with the different stresses are summarized in Fig.1. For the salt-stress group, the values of F_v/F_m and the effective PSII quantum yield [Y(II)] declined as the salinity increased. When the salinity reached 120, both the potential and the effective quantum yield of PSII declined sharply (Fig.1a). However, the lowest Y(NPQ) was recorded at 90 salinity in the salinity stressed samples (Fig.1b). This might contribute to the operation of stress response mechanisms rather than thermal dissipation. The increase of Y(NPQ) as the quantum yield at 120 salinity decreased, indicated that the photosynthetic physiology of PSII was affected and that the extra energy absorbed by the algae would be dissipated through heat. Thus, the sample treated with 90 salinity was selected for an absolute quantification analysis.

Similar to the results of the salinity stressed samples, the values of F_v/F_m and Y(II) decreased with increasing irradiance (Fig.1a), especially in the algae treated with 1 200 μmol photons/(m²·s). The value of Y(NPQ) reached a maximum in the 800 μmol photons/(m²·s) treated sample. The decrease of Y(NPQ) meant a change in the regulated thermal dissipation to non-regulated energy dissipation, which denoted a loss of photoprotection to PSII. Thus, the sample under 800 μmol photons/(m²·s) was selected for an absolute quantification analysis.

3.2 Yields of total RNA and genomic DNA

Gene transcript number is ideally normalized to cell number, but this approach cannot be applied to the tissues composed of multicellular mass (Bustin, 2000), therefore, it could not be used for *Py. haitanensis*. Normalization to the total RNA concentration has been proposed as an acceptable alternative method. Here, using a Qubit fluorometer and the corresponding RNA Assay Kit (Invitrogen Corp.), we obtained an

Table 3 Summarization of RNA and DNA extraction from *Py. haitanensis*

Sample group	RNA yield (μg/mg)	DNA yield (μg/mg)	RNA yield normalization against DNA (μg/μg)
C	0.25±0.0243	0.008±0.0012	31.3
60	0.242±0.063 5	0.006 4±0.000 5	37.8
90	0.212±0.019 2	0.013 2±0.001 2	16.1
120	0.077±0.008 5*	0.012 5±0.000 7	6.2
400 μE	0.063±0.006 1*	0.005 8±0.000 2	10.9
800 μE	0.074±0.016 1*	0.009 8±0.001 4	7.6
1 200 μE	0.051±0.005 8*	0.008 8±0.002 2	5.8

* The yield of total RNA was statistically significant different from the control (P<0.001).

accurate measurement of total RNA based on sample weight (Table 3). Total RNA and genomic DNA of the different stressed samples were quantified by Qubit fluorometer, and the yields were calculated based on sample weight (Table 3). For different treatment of samples, three replicates were included for the calculation of the parameter below. However, we found that the RNA yield varied among the different samples. Except for the samples treated with 60 and 90 salinity, the stress treatments usually led to the significant down-regulation of RNA transcription. In addition, the relative RNA yields were also affected more dramatically under strong light than under salinity treatments (Table 3). Thus, to find a more reliable way of normalizing the transcript numbers of the tested genes, we extracted genomic DNA using the same batch grinding algae as used in the RNA isolation and then determined the quantity. We assumed that the amount of DNA in the different tissues of a species is constant. Moreover, the samples used for DNA and mRNA extractions were ground to similar levels. To confirm the accuracy of the expression levels of the seven tested genes, their copy numbers should be normalized against the genomic DNA quantity.

3.3 Optimal reference gene screening using geNorm

The amplification specificity for the seven tested genes was determined by analyzing the dissociation curves of the PCR products. Only when a single peak was found in the dissociation curve for a gene, was the amplification of that gene deemed to be specific. At the end of every reaction cycle, the copy numbers of all the unknown samples were calculated automatically by the Bio-Rad optical system software.

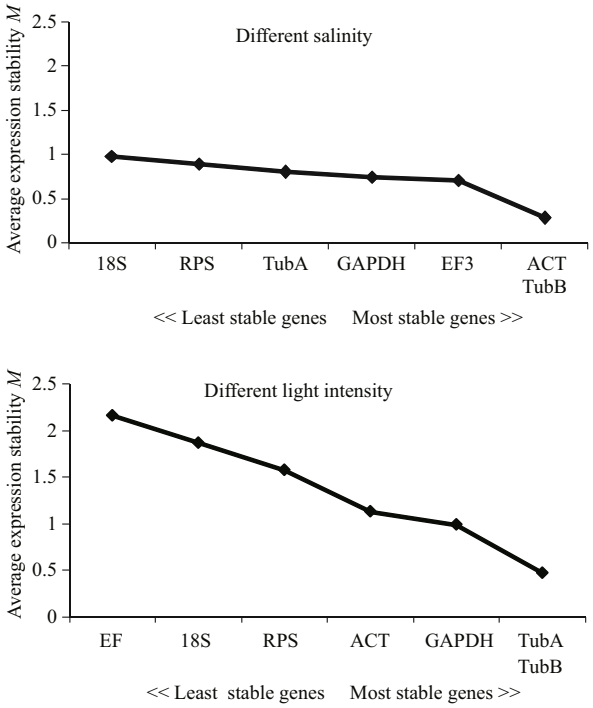

Fig.2 Expression stability of the candidate reference genes analyzed by geNorm

Expression stability values (M) of the seven candidate reference genes are shown for different salinity samples and different light intensity treatment respectively.

The E values of the seven candidate genes ranged from 0.962 for *β-TUB* to 1.03 for *α-TUB* and the correlation coefficients (R^2) were from 0.992 for *β-TUB* to 0.998 for *α-TUB* and 18S rRNA.

Here, the stress treatments were divided into two sample pools and the potential internal control genes were analyzed separately. In each group, all of the candidate genes were ranked based on the M values calculated from geNorm (Fig.2). Under salinity stress conditions, the seven tested genes presented high expression stabilities with M values of less than 1.5 (Vandesompele et al., 2002). The statistical algorithms used in the geNorm software showed that the degree of sample variation when treated with salinity was less than what treated with strong light. Under the strong light conditions, only *α-TUB*, *β-TUB*, *GAPDH*, and *ACT* belonged to the stably expressed genes.

3.4 Absolute quantification of the tested genes in certain samples

Based on standard curves derived from plasmids containing the test genes, the copy numbers of the candidate genes in different samples were calculated by relating threshold cycle (C_t) values to the standard

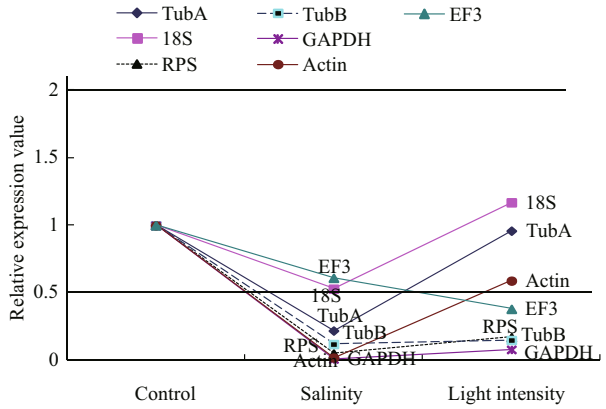

Fig.3 Transcript level of tested genes under different stresses treatment in *Py. haitanensis*

All the genes whose relative transcript level observed was between 0.5 and 2 were designated as the potential internal control genes.

curves. After normalization against the genomic-DNA yield in each group, the expression levels of the tested genes in the different samples were compared (Fig.3).

The expression levels of all of the tested genes were down-regulated in samples under salinity stress (90 salinity). The mean expression level of *18S* was the highest among all of the genes, and it was stably expressed in all the samples tested here. The expression levels of *RPS*, *EF3* and *ACT* under the different stresses varied greatly. *GAPDH* showed considerable down-regulation across all stressed samples. The expression levels of *TubA* and *TubB* had low abundance but were more stable than *RPS*, *EF3* and *ACT* in the tested samples. Our results suggest that *EF3* and *18S* could be used as internal control genes when *Py*. *haitanensis* blades were salt-stressed and *TubA*, *ACT* and *18S* were stable when *Py*. *haitanensis* blades were stressed by strong light.

3.5 Screening of potential internal control genes

While evaluating the results from the absolute quantification and the geNorm software analysis, we found that there were some differences between these two methods. The geNorm analysis proposed all the seven tested genes can be selected as the potential internal control genes under salinity stress but only *18S* and *EF3* were identified with the absolute quantification. Under the strong light conditions, *α-TUB*, *β-TUB*, *GAPDH*, and *ACT* belonged to the stably expressed genes determined by the geNorm analysis and *18S*, *α-TUB* and *ACT* were recommended as the internal control genes according to the absolute quantification. Considering together with the results from two methods, *α-TUB* and *β-TUB* were the best candidate reference genes under strong light stress while *18S* and *EF3* could be selected as the internal control genes under salinity stress in *Py*. *haitanensis*.

4 DISCUSSION

4.1 Determination of conditions for stress responses in *Py. haitanensis*

PAM chlorophyll fluorescence is a powerful tool used to assess the macro-algal physiological state under environmental stresses (Franklin and Badger, 2001; Figueroa et al., 2003; Prasil et al., 2007). In this study, we used PAM fluorometry to evaluate the effects of stresses on *Py*. *haitanensis*. Each measurement could be completed quickly in situ (Beer and Björk, 2000), which is extremely convenient. The value of Y(II) was regarded as an indicator of the photo-inhibition levels and the value of Y(NPQ) indicated the regulated dissipation of photosynthetic energy. The closing of PSII reaction centers usually caused a decrease in Y(NPQ) (Gao and Wang, 2012). Based on PAM, the physiological status of the sample was easily and accurately identified. Some stress-response-related protective mechanisms were activated and other metabolic pathways were reduced. The expression levels of genes involved in those pathways was changed greatly through certain regulatory mechanisms. Thus, based on the results of PAM, the samples (treated with 90 salinity and 800 μmol photons/(m^2·s) light intensity) with obvious stress responses were collected to perform the absolute quantification analysis.

4.2 Screening of potential internal control genes using different methods

The method of qRT-PCR is increasingly used for the high-throughput evaluation of gene expression levels where relative quantification is estimated by normalizing target gene expression against the expression of one or two stable internal control genes. A comprehensive analysis of expression studies in the literature indicated that, in more than 90% of cases, *18S*, *28S* or *GAPDH* have been used as the single internal control gene for normalization (Suzuki et al., 2000). Ideally, the internal control gene for quantitative studies should not be influenced greatly by the conditions of the experiment. However, no single gene is stably expressed under every experimental condition and numerous studies have reported that the expression

of constitutive genes can vary considerably (Schena et al., 1995; Parker and Barnes, 1999; Vaidyanathan et al., 1999; Vandesompele et al., 2002; Schmid et al., 2005). Therefore, it is crucial to verify the expression stability of a control gene under the specific experimental conditions prior to its use for normalization.

With the development of statistical analysis assisted with computer, several softwares were programmed to detect the stably expressed genes. One of them is the geNorm, which ranks the expression stability by stepwise exclusion of the gene with the highest expression stability measure—*M* (Vandesompele et al., 2002). Only the stability value of the tested gene is lower than 1.5 can be recommended as a potential reference gene (Vandesompele et al., 2002). GeNorm caculates the stability value through relative quantities transformed from C_t values, while the absolute quantification requires standardization.

The relative RNA yield index showed a close relationship to the photosynthetic activity in each treatment group. This meant that the responding mechanisms between different stress treatments were different and that the gene expression level under certain stresses would decrease with an increase in the degree of stress. However, the consistency between the relative RNA yield index and the photosynthetic activity indicated the validity of our results. This was very important to accurately evaluate the expression levels of the seven designated genes.

During the calculation of the stability value *M* of different tested genes, it was ideally to assume that the amplification efficiency is 100%. However, not all the primers and the templates quality satisfy the criteria of PCR amplification of 100%. On the other hand, the minor differences in input of mRNA will result in unacuracy of PCR product (Freeman et al., 1999). So, the different results obtained from the geNorm analysis and the absolute quantification seemed understandable. Thus, to avoid the systematical error caused by the method applied here, we took into consideration of the results from both methods and proposed that *EF3* and *18S* could be used as internal control genes when the *Py. haitanensis* blades were subjected to salinity stress, and that *TubA* and *18S* could be used as the internal control genes when the stress was from strong light.

4.3 Determination of constitutively expressed genes under light and salt stress conditions

We have demonstrated that the expression levels of the seven commonly used internal control genes were influenced differently by stress treatments. *GAPDH*, which is involved in the glycolysis pathway, is commonly used as an internal control gene. Iskandar et al. (2004) used *GAPDH* as a suitable control to measure gene expression in different tissues/organs of sugarcane. During the salinity-induced transition from C3 photosynthesis to CAM photosynthesis in *Mesembryanthemum crystallinum*, *GAPDH* expression was found to increase rapidly in leaf tissue (Ostrem et al., 1990). We found that, in *Py. haitanensis*, *GAPDH* expression was down-regulated to half that of the control in the stressed samples. We deduced, therefore, that *GAPDH* should not be used as an internal control in quantitative gene expression studies in *Py. haitanensis* under stress treatments.

The *18S* gene was the most stable one of the seven tested genes in the absolute quantification. Recently, *18S* has been proposed as the calibration gene and the present study confirms its applicability in some cases. However, the expression level of *18S* was usually high due to the abundance of rRNA.

The other candidate genes which we tested in this study displayed high expression variability in different stress treatments, limiting their universal use as internal control genes in *Py. haitanensis*. Elongation factor was reported to be conserved in growing cells (Axelos et al., 1989), and it was related to growth, translation regulating and stress responding reaction (Dharmawardhane et al., 1991). In our determination, it showed little relative variation during the salinity treatment. Thus, it belonged to another potential internal control in future salinity stressed experiments besides *18S*. In the high light stressed samples, both actin and tubulin played an essential role in cellular structure and kinetics of the cytoskeleton. Tubulin not only took part in the formation of spindle fibres and phragmoplasts, but also functioned in cellulose deposition in dividing cells (Setter and Flannigan, 2001). The variation ranges of actin and tubulin mRNA level were quite stable and could be selected as the internal control genes when the samples were subjected to high light stress.

Proper selection of the internal control gene was prerequisite for accurate transcript quantification. To investigate the subtle expression variability, Vandesompele et al. (2002) recommended the use of three control genes at least to calculate the normalization factor to decide how many control genes required. Tricarico et al. (2002) advocated measurements of the internal control gene simultaneously during qRT-PCR experiments. We

had screened the stably expressed genes through the absolute quantification and the geNorm analysis. The recommended internal control genes expressed stably in both determinations. Moreover, considering the possibility of co-regulation of the genes, we suggested the use of two genes belonged to different function classes as the internal controls in qRT-PCR analysis.

5 CONCLUSION

The relative quantification of target gene using $2^{-\Delta\Delta Ct}$ method has become the most common way to characterize mRNA expression pattern, which contribute significantly to understanding physiological processes. However, none of the genes showed stable expression levels under all of the experimental conditions tested. We suggested that the most reliable internal control genes were *EF3* and *18S* in salinity-treated samples, and *18S* and *TubA* under strong light conditions. The results provide a first report of reference genes selection for qRT-PCR experiments related to the stresses responses in *Py*. *haitanensis*.

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