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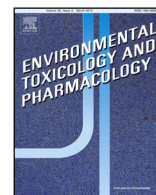

Ribosomal protein L7 as a suitable reference gene for quantifying gene expression in gastropod *Bellamya aeruginosa*

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ABSTRACT

Expression levels of eight candidate reference genes were quantified in tissues of gastropod *Bellamya aeruginosa* exposed for 10 d to various stressors, including fasting, 17β-estradiol, 17α-methyltestosterone, and Cd^{2+}. The results showed that 18s rRNA was the most highly expressed of the candidate reference genes, while H2A was the least expressed. There were no significant changes ($p > 0.05$) in the expression of the eight genes in tissues among the different treatments. Using RefFinder to evaluate the expression stabilities of the eight candidate reference genes, ribosomal protein was shown to be the most stable reference gene, and no effects were observed among the different stressor treatments. These results indicate that RPL 7 is the most suitable reference gene for quantifying gene expression in *B. aeruginosa* under environmental stress, which was verified in *B. aeruginosa* exposed to high doses of E_2 for 24 and 72 h.

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1. Introduction

Quantitative real-time polymerase chain reaction (qPCR) is a powerful tool for analyzing mRNA expression and can assist in the determination of toxicological mechanisms of action and biological pathways in organisms exposed to environmental stressors (Snell et al., 2003). To correct for possible errors and to reduce variation in total RNA content, RNA stability in individual samples, enzymatic efficiencies during reverse transcription, and sample loading volume in PCR amplification systems, a reference gene (i.e., a housekeeping gene) that is "constitutively expressed to maintain cellular function" (Butte et al., 2001) is often used as an internal control to normalize gene expression levels and to improve experimental data accuracy.

The basic requirement of a reference gene is that it has a constant expression profile in an organism, regardless of tissue type or experimental condition. To the best of our knowledge, no "ideal" gene has been reported for use in qPCR assays. Therefore, it is essential to statistically validate the stability of a candidate reference gene before using it to normalize gene expression. Most studies select reference genes based on a conventional reference gene designated in earlier studies (Wu et al., 2010), or from new candidate genes found through transcriptomic data analyses of constitutively expressed genes (Maksup et al., 2013). There are at least nine frequently used reference genes, and more than 90% of gene expression is normalized to beta-actin (β-actin), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), cyclophilin (CYP), elongation factor l alpha (EFlα), or the family of ribosomal genes. However, several studies have reported that β-actin and GAPDH levels vary significantly (Nazari et al., 2015), indicating that they may not be ideal for mRNA transcription analyses. From the perspective of molecular biology, it is impossible for the transcription levels of any gene to be absolutely constant across all cell cycles, developmental stages, and specific physiological statuses (Xu et al., 2011; Cubero-Leon et al., 2012). Moreover, it is impossible for one reference gene to be suitable for all organisms for normalizing mRNA abundance due to differences in expression profiles and experimental conditions. For example, some studies have shown that 18S rRNA has the most stable expression in blue mussel (*Mytilus edulis*) (Cubero-Leon et al., 2012), but other studies have shown that it is unstable in zebrafish (*Danio rerio*) (Tang et al., 2007) and common octopus (*Octopus vulgaris*) (Sirakov et al., 2009). Therefore, it is

Abbreviations: qPCR, quantitative real-time polymerase chain reaction; β-actin, beta-actin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CYP, cyclophilin; EFlα, elongation factor l alpha; TUB, β-tubulin; H2A, histone H2A; DRP II, DNA-directed RNA polymerase II; RPL 7, ribosomal protein L 7; EDCs, endocrine disrupting chemicals; CV, coefficient of variation; OECD, Organisation for Economic Co-operation and Development.

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necessary to screen the stability of potential reference genes in specific organisms under different conditions and in particular tissues.

The river snail or pond snail (*Bellamya aeruginosa*) is a gastropod that is widely distributed in China and other countries. It typically inhabits the bottom of lakes, ponds, and rivers, and feeds on benthic diatoms or organic impurities in the sediment. *B. aeruginosa* is ovoviviparous, meaning that eggs hatch within the mother's body and juveniles are released into the aquatic environment. *B. aeruginosa* has recently been used as a model organism in ecotoxicological studies (Liu et al., 2012; Wang et al., 2014) because of its wide geographical distribution, large populations, high filtration rate, and size. Moreover, a large amount of valuable molecular information has been obtained following construction of its transcriptome database (Li et al., 2012; Lei et al., 2015). Compared with the New Zealand mud snail (*Potamopyrgus antipodarum*), which is used as the ecotoxicological model species in Europe (Zounkova et al., 2014), *B. aeruginosa* is better for evaluating and monitoring endocrine disrupting chemicals (EDCs) in aquatic environments, especially sediments, because of its sexual reproduction and secondary sex characteristics, where the right tentacle in males becomes the copulation organ. However, an optimal reference gene for quantifying gene expression in *B. aeruginosa* has not been identified.

In this study, expression levels of eight reference genes were quantified in *B. aeruginosa* exposed to different stressors, including physiological pressure and chemical stressors, and their stabilities were evaluated using the web-based RefFinder software. Based on the analysis, ribosomal protein L7 (RPL 7) was the most stable reference gene among the examined reference genes, and could be used as the internal reference gene in *B. aeruginosa* for normalizing qPCR assays in future studies.

2. Materials and methods

2.1. Animals and treatments

B. aeruginosa specimens were cultured continuously for more than one year in a laboratory-based flow-through system using tap water treated with activated carbon (hardness, 81.1 ± 1.2 mg/L calcium carbonate; dissolved oxygen, 7.8 ± 0.3 mg/L; temperature, 23 ± 1 °C, photoperiod, 10 h light/14 h dark). Snails were fed freshly frozen *Chlorella vulgaris* (a single-celled algae) twice daily.

Snails of similar size (body weight, 1.72 ± 0.48 g; shell height, 29.4 ± 2.4 mm) were selected and randomly divided into experimental groups, with five males and five females in each group. Based on aquatic environmental concentrations of the target chemicals (Chang et al., 2008), the experimental groups consisted of a solvent control group (acetone), a fasting group (no food), a 17β-estradiol (E_2) (10 ng/L) group, a 17α-methyltestosterone (MT) (10 ng/L) group, and a Cd^{2+} ($CdCL_2$) (0.1 mg/L) group, which were chosen to yield molecular responses that would reflect actual environmental stress responses. Each treatment was performed with two replicates, and 10 μL acetone was added to 2 L of water for the exposure, resulting in a final concentration of 0.01‰ acetone based on the OECD guideline (OECD, 2008). Acetone was used only in the control, E_2, and MT groups; no acetone was added to the fasting and Cd^{2+} groups. During the treatment period, animals were fed 2 mL of fresh *C. vulgaris* ($\sim 1 \times 10^7$/mL) twice daily, except for the fasting group. Water was completely renewed every 24 h, with a constant temperature (24 ± 1 °C). After treatment for 10 d, which was based on an earlier study (Ciocan et al., 2010), different tissues (foot, hepatopancreas, testis, ovary, penis, and antenna) were dissected and frozen in liquid nitrogen until further experimentation. No individuals died throughout the experimental period.

The second experiment was then performed, in which ten females and ten males from each group were exposed to 10, 100, or 1000 ng E_2/L. Each treatment was performed with two replicates. After 24 and 72 h, the hepatopancreases of five females and five males were sampled to evaluate reference gene expression stability. The other conditions and treatments were carried out as described above.

2.2. Total RNA extraction and cDNA synthesis

Total RNA was isolated from each sample (the penis and antenna were pooled in each group) using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA was purified after DNAse digestion (TaKaRa Ltd., Dalian, China) with two chloroform extractions and ethanol precipitation, and dissolved in diethylpyrocarbonate-treated distilled water. RNA quality was assessed based on the $Abs_{260/280}$ ratios and a 1.2% agarose-formaldehyde gel stained with ethidium bromide. RNA concentrations were measured with a Nano 200 microspectrophotometer (BIORISE Group, Shanghai, China), and the concentration was adjusted to 1 μg/μL. Then, 1 μL of RNA was mixed with 0.25 μL dNTPs (2.5 mM each) and 0.25 μL of Oligo(dT) 15 (0.5 μg/μL). The mixture was incubated at 70 °C for 10 min and then chilled on ice. Next, 4 μL of 5× first-strand buffer, 0.5 μL of dithiothreitol (0.1 M), and 0.25 μL of M-MLV RT (Promega, USA) were added. Finally, the reaction mixture was incubated at 25 °C for 5 min and 42 °C for 60 min, and then inactivated at 70 °C for 10 min. The synthesized cDNA was diluted 100 times and stored at −20 °C until qPCR analysis.

2.3. qPCR analysis of the candidate reference genes

To reduce possible errors due to the co-regulation of different genes, eight candidate reference genes involved in different cellular functions were intentionally selected: β-actin, ribosomal protein L7 (RPL 7), 18S ribosomal RNA (18S rRNA), EFlα, GAPDH, β-tubulin (TUB), histone H2A (H2A), and DNA-directed RNA polymerase II (DRP II). The primers used to quantify gene expression were designed using the primer design software Primer3 Plus (Rozen and Skaletsky, 2000) based on sequences obtained from the river snail transcriptome database (Table 1) (Lei et al., 2015). A qPCR was conducted in 96-well plate with a 7500 Real-Time PCR System (Applied Biosystems, Alameda, CA, USA), and the data were analyzed using 7500 System Software (Applied Biosystems). Each reaction consisted of 12.5 μL of SYBR® Green PCR master mix (Applied Biosystems), 10 μL of cDNA, and 0.75 μL of each specific primer (10 μM each), with a final volume of 25 μL. The thermal profile was programmed using default parameters: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s. To ensure amplification specificity, the dissociation curve of the PCR product was investigated by heating from 60 °C to 95 °C at the end of each reaction. To obtain the amplification efficiencies of the target genes, standard curves were constructed using 10-fold serially diluted cDNA as a template before candidate gene expression analysis, and each analysis was performed in triplicate. A negative control without any reverse transcriptase was included.

2.4. Data processing

RefFinder was used to evaluate the expression stability of the reference genes. This program is a user-friendly, web-based, comprehensive tool developed for evaluating and screening reference genes from extensive experimental datasets. RefFinder integrates the outcomes of the four major statistical algorithms (geNorm, NormFinder, Delta Ct, and Bestkeeper), assigns an appropriate weighting to an individual gene, and then calculates the

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