Analysis of reference gene expression for real-time PCR based on relative quantitation and dual spike-in strategy in the silkworm Bombyx mori

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In general, for real-time quantitative polymerase chain reaction (qPCR), normalization strategies use a reference gene as a control and to avoid the introduction of experimental errors expression of this gene should not vary in response to changing conditions. However, the expression of many reference genes has been reported to vary considerably and, without appropriate normalization, the expression profile of a target gene can be misinterpreted. In this study, the expression levels of seven commonly used reference genes (ACT, GAPDH, 28srRNA, RPL3, α-tubulin, UBC, and TBP) were detected at different development time points and in response to treatment with 20-hydroxyecdysone (20E) and with rutin. The expression stability was analyzed using geNorm and NormFinder software. Significant variations were found among normal tissues and between experimentally treated tissues. The dual spike-in strategy also revealed significant variations of the expression levels of the reference genes among normal tissues and between experimentally treated tissues. Glutathione-S-transferase sigma 1 (GSTs1), which has a high expression level in fat body and is related to the mechanism of resistance, was used as a target gene to validate the feasibility and difference of these two approaches.

Keywords real-time quantitative PCR; reference gene; normalization; dual spike-in strategy; silkworm Bombyx mori

Introduction

The analysis of gene expression level is increasingly popular in many research fields [1]. Real-time quantitative polymerase chain reaction (qPCR) has been universally adopted as the transcriptional method of choice due to its superiority with regard to speed, sensitivity, and reproducibility as well as the wide range of commercially available instrumentation and reagents [2]. This approach, like other methods of mRNA quantitation such as northern blot, ribonuclease protection assay, and microarray, requires normalization [3]. It is now generally accepted that gene expression level that reflects differences in cellular input, RNA quality, and reverse transcription efficiency should be normalized to that of an invariably expressed internal control gene [4]. Relative quantitation, the most widely adopted approach, requires the use of a constitutively expressed housekeeping gene as an internal control to normalize differences in starting complementary DNA (cDNA) template between samples [5]. The key to the reliable determination of gene expression is the choice of control gene; however, doubts have been expressed about the reliability and appropriateness of this approach. It has become increasingly obvious that there is no mRNA with a constant expression level and that the mRNA level of housekeeping gene can vary quantitatively in response to numerous factors, such as the stage of development and the cell cycle, and different experimental conditions [6–10]. Multiple reference genes have been used because it is difficult to find a universal gold standard that suits all experimental samples [11]. The geNorm and NormFinder algorithms have been used in many studies to estimate the variability of the candidate reference genes [12,13]. Some researchers have established guidelines to encourage better experimental practice, allowing more reliable and unequivocal interpretation of qPCR results [14].

The economically important silkworm Bombyx mori (Lepidoptera: Bombycidae) has become a useful model organism in the study of lepidopteran and arthropod biology. To our knowledge, there is no report of any investigation on the variation in expression of housekeeping genes in treated silkworm tissues. In this study, the seven candidate reference genes ACT, GAPDH, 28srRNA,
RPL3, α-tubulin, UBC, and TBP were evaluated at different development stages in three normal tissues and in fat body (FB) treated with 20-hydroxyecdysone (20E) and with rutin. Special attention was paid to selecting genes that belong to different functional classes to reduce the chance that they might be co-regulated. Two popular programs, geNorm and NormFinder, were used to analyze the stability of the reference gene. The dual spike-in qPCR strategy was used to measure the transcriptional level per gene copy in which an exogenous reference mRNA is adopted as a ‘spike’ to minimize the variability between samples [15]. To validate the differences between relative quantitation and a spike-in qPCR technique, glutathione-S-transferase sigma 1 (GSTs1), with high expression level in FB was used.

Materials and Methods

Bombyx mori

Bombyx mori strain P50, a Chinese silkworm strain, provided by the Sericulture Institute of Soochow University, was reared on mulberry (Morus alba) leaves at 25 ± 1°C and 80% ± 5% relative humidity with a 12 h light/12 h dark photoperiod. Ten animals were used for each experimental group.

Larvae were dissected on each day (24 h period) of the fifth instar and the mid-gut, FB and Malpighian tubule were collected. Day 2 of the fifth instar stage (48 h after the first feeding of the fifth instar larvae) was chosen for feeding with 20E at 2 × 10⁻³ μg/μl (6 μg; Sigma Aldrich, St Louis, USA) and rutin (C₂₇H₃₀O₁₆) at 5 × 10⁻² μg/μl (150 μg; Sigma Aldrich). The control larvae were fed with 20E at 2°C and the remainder was used to extract DNA. The tissues were ground in liquid nitrogen. A total of 1 ml of extraction solution was transferred to a tube containing ~100 mg of sample. The mixtures were kept at room temperature for 10 min and then centrifuged for 15 min at 12,000 g at 4°C. The supernatant (800 μl) was decanted and discarded, and 0.2 ml of chloroform was added. The tubes were shaken vigorously for 15 s, kept at room temperature for 3 min and then centrifuged for 15 min at 12,000 g at 4°C. The supernatant (500 μl) was decanted and discarded, 0.5 ml of isopropanol was added and the tubes were shaken for 15 s, kept at room temperature for 10 min and then centrifuged for 10 min at 12,000 g at 4°C. The supernatant was discarded; the pellet was washed in cold 70% (v/v) ethanol and then centrifuged as described above. The ethanol supernatant was removed, the nucleic acid pellet was allowed to dry and then suspended in 30 μl of double distilled H₂O followed by treatment with RNase-free DNase I (Sangon, Shanghai, China) to eliminate potential genomic DNA in the samples and the RNA samples were stored at −80°C. RNA was quantified by measuring ultraviolet absorbance at 260 nm (A₂₆₀) and 280 nm (A₂₈₀) with a Thermo NanoDrop 1000 spectrophotometer (Nanodrop 1000, Thermo Scientific, Wilmington, USA). The concentration of total RNA must be within an A₂₆₀/A₂₈₀ ratio of 1.80–2.00 to ensure the integrity of the mRNA. The total RNA was used as a template to synthesize the primary cDNA chain by reverse transcription with a PrimeScript RT reagent Kit (TaKaRa, Dalian, China) following the manufacturer’s instructions. The cDNA was stored at −20°C and the remainder was used to extract DNA. The DNA portion was treated with phenol/chloroform and then precipitated in ethanol by standard procedures.

Exogenous RNA and DNA references

In this work, in vitro transcribed IFP2 (piggyback transposon) mRNA (provided by Prof. Lu CD, the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences) was used as the spike reference for RNA sample, and plasmid pPigT7 (4983 bp) was used as the spike reference for DNA sample. The 1:1 (w/w) mixture of exogenous RNA and DNA references was added to each sample homogenate before the simultaneous extraction of RNA and DNA so that the exogenous RNA reference underwent the same process as the sample RNA and the exogenous DNA reference underwent the same process as the sample DNA.

The treatment of pPigT7 and the in vitro transcription of IFP2 mRNA were done as described [15]. Equal amounts of IFP2 mRNA and IFP2 DNA were mixed, and the copy ratio of IFP2 mRNA:IFP2 DNA in the mixture was 4.09.

Sample preparation

Procedures for RNA preparation conform to the Minimum Information for Publication of Quantitative Real-time PCR Experiments (MIQE) guidelines [14]. In this section, the essential information required to allow reliable interpretation of the corresponding qPCR results was indicated.

Total RNA and DNA were extracted simultaneously from various tissues of B. mori larvae by using TRNzol-A + Total RNA reagent (Tiangen, Beijing, China) following the manufacturer’s instructions. The tissues were ground in liquid nitrogen. A total of 1 ml of extraction solution was transferred to a tube containing ~100 mg of sample. The mixtures were kept at room temperature for 10 min and then centrifuged for 15 min at 12,000 g at 4°C. The supernatant was decanted and discarded, and 0.2 ml of chloroform was added. The tubes were shaken vigorously for 15 s, kept at room temperature for 3 min and then centrifuged for 15 min at 12,000 g at 4°C. After the supernatant was decanted and discarded, 0.5 ml of isopropanol was added and the tubes were shaken for 15 s, kept at room temperature for 10 min and then centrifuged for 10 min at 12,000 g at 4°C. The supernatant was discarded; the pellet was washed in cold 70% (v/v) ethanol and then centrifuged as described above. The ethanol supernatant was removed, the nucleic acid pellet was allowed to dry and then suspended in 30 μl of double distilled H₂O followed by treatment with RNase-free DNase I (Sangon, Shanghai, China) to eliminate potential genomic DNA in the samples and the RNA samples were stored at −80°C. RNA was quantified by measuring ultraviolet absorbance at 260 nm (A₂₆₀) and 280 nm (A₂₈₀) with a Thermo NanoDrop 1000 spectrophotometer (Nanodrop 1000, Thermo Scientific, Wilmington, USA). The concentration of total RNA must be within an A₂₆₀/A₂₈₀ ratio of 1.80–2.00 to ensure the integrity of the mRNA. The total RNA was used as a template to synthesize the primary cDNA chain by reverse transcription with a PrimeScript RT reagent Kit (TaKaRa, Dalian, China) following the manufacturer’s instructions. The cDNA was stored at −20°C and the remainder was used to extract DNA. The DNA portion was treated with phenol/chloroform and then precipitated in ethanol by standard procedures.

Real-time qPCR

The primers were designed with the Primer Premier (version 5.0) software (Premier Biosoft International, San Diego, USA). The PCR primer sequences used for quantitation of the candidate reference were given in Table 1. The same primer pair was used when determining the mRNA level and DNA copy number of a certain gene. A pair of primers was designed within an exon to ensure that the lengths of amplions cons aged from cDNA template and DNA template were the same. Gene-specific amplification was confirmed.
by a single peak in melt-curve analysis. The PCR products were subjected to electrophoresis in 2% (w/v) agarose gels to confirm the specificity of amplification and the absence of primer dimer formation. For each assay, primer efficiency was determined by a standard curve of cDNA samples according to the MIQE guidelines for qPCR. Amplification was followed by melting-curve analysis to check the specificity of the PCR product. The linear correlation coefficient ($R^2$) ranged from 0.991 to 0.998 and the PCR efficiency varied from 91.2% to 106%. These findings show that these assays are suitable for quantitative purposes.

**Table 1 Primer pairs for real-time PCR**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequence (5’–3’)</th>
<th>GenBank accession no.</th>
<th>Amplicon size (bp)</th>
<th>Tm of primer (°C)</th>
<th>Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin-3 (cytoplasmic actin)</td>
<td>CGGCTACTCGTTCACTACC U49854</td>
<td>147</td>
<td>59.7</td>
<td>102</td>
<td></td>
</tr>
<tr>
<td>28srRNA (28S ribosomal RNA)</td>
<td>CCCAGTGCTCTGAATGTCAC AY038991</td>
<td>150</td>
<td>59.9</td>
<td>105</td>
<td></td>
</tr>
<tr>
<td>GAPDH (glyceraldehyde-3-phosphate dehydrogenase)</td>
<td>TGGTAAAGGGCTTGATGC ACY038991</td>
<td>150</td>
<td>55.0</td>
<td>106</td>
<td></td>
</tr>
<tr>
<td>RPL3 (ribosomal protein L3)</td>
<td>ACCTAACCACAGCTTTG ATC76270</td>
<td>231</td>
<td>55.4</td>
<td>97.2</td>
<td></td>
</tr>
<tr>
<td>α-tubulin</td>
<td>CTTCCCTCCACATACATT ACT0143419</td>
<td>186</td>
<td>59.5</td>
<td>94.5</td>
<td></td>
</tr>
<tr>
<td>TBP (TATAbox binding protein)</td>
<td>GGTGTGGCTGGGACTGTC TAC014359</td>
<td>179</td>
<td>59.5</td>
<td>91.2</td>
<td></td>
</tr>
<tr>
<td>UBC (ubiquitin)</td>
<td>GTGTCCGACCTTGTCTT CAT014363</td>
<td>115</td>
<td>55.0</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>IFP2</td>
<td>CTGTGACGATGGATGTC TCA014364</td>
<td>155</td>
<td>55.0</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>GSTs1</td>
<td>GACATGGGGTGATTCTTG TCA014362</td>
<td>149</td>
<td>57.3</td>
<td>101</td>
<td></td>
</tr>
</tbody>
</table>

Data analysis

On the basis of the standard curve method, the threshold cycle ($C_t$) model was used for relative quantitation in this study. The number of threshold cycles is referred to as the quantification cycle ($C_q$) value that is transformed into quantity via the standard curve using PCR efficiency. Two freely available software tools, geNorm (http://medgen.ugent.be/~javdesomp/genorm/index.php) and NormFinder (http://www.mdl.dk/publications/normfinder.htm), were used to evaluate gene expression stability and quantities transformed to a linear scale (the highest relative quantity for each gene was set to 1) were used as input data.

The transcription level per gene copy of a target mRNA is calculated from $C_t$ for mRNA (cDNA) and DNA templates. IFP2, which is not inherently possessed by *B. mori*, was used as an exogenous reference in this work. Exogenous DNA was added as the spike reference for target DNA in addition to the exogenous RNA used as the reference for target RNA. After the mRNA/DNA ratio of a target gene was estimated by qPCR, it was normalized to the mRNA/DNA ratio of the exogenous reference and the transcription level per gene copy was estimated.

The qPCR data were processed with ABI PRISM 7300 sequence detection software (version 1.3.1) and were regulated as described [15].

Statistical analysis

The SPSS statistical software (SPSS 17.0) was used to analyze statistical significance. To analyze variance between the gene expressions in the different days of
sample, one-way ANOVA was used. The t-test for equality of means was used to analyze the gene expression of two groups (controlled and stimulated) of samples. A P value of $\leq 0.05$ was deemed to be significant.

Results

Stability ranks of candidate reference genes using geNorm and NormFinder

The stability of candidate reference genes was analyzed using geNorm and NormFinder. The amplification efficiency-corrected relative quantities from raw fluorescence data were calculated. An average expression stability measure ($M$) of $<1.5$ calculated for a gene using geNorm analysis indicates that the expression is relatively stable. A lower $M$ value of average expression stability or a lower stability value indicates more stable expression. We found that the most stable reference genes were different among the different tissues and between the different treatments. The ranks produced by the geNorm and NormFinder approaches are different. In three normal tissues, ACT3 and GAPDH in the mid-gut, $\alpha$-tubulin, and UBC in the FB and the Malpighian tubule were identified as the most stable genes using geNorm software [Fig. 1(A)]. However, NormFinder analysis revealed ACT3 in the mid-gut and the Malpighian tubule, and TBP in the FB were the most stable genes [Fig. 1(B)]. Using geNorm, we found that in treated FB $\alpha$-tubulin and UBC for 20E and GAPDH and ACT3 for rutin were established as the most stable genes [Fig. 2(A)]. UBC for 20E and GAPDH for rutin were established as the most stable genes by NormFinder analysis [Fig. 2(B)]. To determine the optimal number of reference genes, the pairwise variation ($V_n/V_{n+1}$) was analyzed by the geNorm software. According to Vandesompele et al. [3], the cut-off value for such significance is 0.15. A pairwise variation of $<0.15$ indicates that the suitable number of reference genes to normalize the target is ‘$n$’ [13]. Figure 3 showed that across the different tissues or treatments two reference genes might be enough to normalize expression values of target genes.

General expression levels of reference genes in normal tissues

Total RNA and DNA were extracted simultaneously from the mid-gut, FB and Malpighian tubule of *B. mori* larvae, and the RNA and DNA copy numbers of seven reference genes and the exogenous reference IFP2 in all of these tissues were detected by qPCR. The transcription levels per gene copy of the seven reference genes were estimated as described in Materials and Methods. The expression levels of the reference genes determined in three different silk-worm tissues were shown as a logarithmic histogram in Fig. 4. For all seven genes, the transcription levels were quite different at different stages of development. TBP has the lowest levels of expression among these genes, which indicates that it might not be qualified as an internal control (Fig. 4).

Different expression levels of reference genes in experimentally treated FB

The expression levels of these genes in treated FB tissue were differentially regulated, even in control tissues (Fig. 5). For example, reference gene expression levels under stimulated conditions are prone to various degrees of up-regulation or down-regulation (e.g. ACT3 in FB with 20E stimulation shows up-regulation of 194.6 fold relative to the control at 2 h and down-regulation of 20.4 fold relative to the control at 8 h). Figure 5 suggested profiling of the seven reference genes treated with 20E differed from that after treatment with rutin. Periods of change in response to different types of stimulation show different trends (e.g. in the FB, UBC up-regulation is 1.9-fold at 24 h after 20E induction, but down-regulation is 4.7-fold after induction by rutin at the same time). UBC and $\alpha$-tubulin are relatively stable compared with other candidate reference genes after induction.

Target gene expression

Two normalization methods, the relative quantitation method and the dual spike-in qPCR method were used to assess the profiling of GSTs1 in FB tissue treated with 20E and with rutin. The most stable reference genes $\alpha$-tubulin and UBC for 20E and ACT3 and GAPDH for rutin selected by geNorm were used as internal controls for relative quantitation. The use of different reference genes led to different experimental results (Fig. 6). GSTs1 with 20E treatment had the highest expression at 2 h relative to UBC or $\alpha$-tubulin [Fig. 6(A)]. After the treatment with rutin, GSTs1 had the highest expression at 24 h relative to ACT3, and at 12 h to GAPDH [Fig. 6(B)], which seems relevant to the lowest transcription level per gene copy expression at 24 h of ACT3 (Fig. 5), but not the highest expression of the target gene GSTs1 at that time. Dual spike-in qPCR analysis demonstrated the transcription level per gene copy of GSTs1 had the highest expression level at 12 h after the treatment with 20E [Fig. 7(A)] and at 4 h with rutin treatment [Fig. 7(B)]. The profiling of GSTs1 varied with the normalization methods used; the relative quantitation and dual spike-in qPCR techniques yielded different results.

Discussion

Relative quantitation is the most widely adopted approach and, as the name suggests, quantitation of gene expression is based on the analysis of a target gene whose expression is relatively normalized to the expression of a control gene [2].
If the expression profile of the reference gene is changed, the expression profile of the target gene is likely to be misinterpreted. It is necessary and urgent to identify some stable reference genes if we are to make relative quantitative measurement more accurate at the mRNA level. The silkworm *B. mori* is an economically important insect and has become a model organism in the study of lepidopteran and arthropod biology. The differences in the levels of
functional gene expression result in the changes of signal transduction, protein synthesis, and metabolism among different development time points. This means that the reference genes in the silkworm cannot be stable, unlike mammalian reference genes. Usually, reference genes in the silkworm are selected by consensus and experience of other organisms instead of empirical normalization.

Accurate normalization of gene expression level is an absolute prerequisite for reliable results, especially when the biological significance of subtle differences of gene expression is studied [3]. geNorm and NormFinder were used to calculate amplification efficiency-corrected relative quantities from raw fluorescence data. We found that the most stable reference genes were different among the different tissues and between different treatments. In three normal tissues, ACT3 and GAPDH in the mid-gut, α-tubulin, and UBC in the FB and the Malpighian tubule were identified as the most stable genes using geNorm.
software [Fig. 1(A)]. However, NormFinder analysis revealed \textit{ACT3} in the mid-gut and the Malpighian tubule, and \textit{TBP} in the FB were the most stable genes [Fig. 1(B)]. Using geNorm, we found that in treated FB, \textit{\alpha-tubulin}, and \textit{UBC} for 20E and \textit{GAPDH} and \textit{ACT3} for rutin were established as the most stable genes [Fig. 2(A)]. \textit{UBC} for 20E and \textit{GAPDH} for rutin were established as the most stable genes by NormFinder analysis.

According to the results for gene expression per gene copy, seven reference genes were expressed differentially at different development stages and changed in response to experimental treatment in \textit{B. mori} larvae. The transcription level of gene expression per gene copy varied greatly; genes are expressed in different phases and tend to change with development stages so these genes are not suitable for use as internal references for the estimation of relative gene expression.
expression levels. Further, the expression levels of these potential reference genes can change in response to different stimulus conditions.

The fact that expression of these genes does vary greatly in given situations may be explained, in part, by the fact that housekeeping proteins are implicated in the basal cell metabolism and participate in other functions [9,16]. The essential functions of these genes are variable. ACT3 is an actin cytoskeleton gene that can control the formation of microtubules. The level of GAPDH mRNA reflects energy metabolizing capability, and that of the 28srRNA gene indicates protein synthesis activity. The characteristic feature of aging organisms is the loss of homeostatic functions, including the levels of mRNA and protein synthesis; the profiling of constitutively housekeeping genes is not immutable [17,18]. More importantly, however, we found no consistent pattern: for example, GAPDH mRNA levels were higher in FB and lower or not different in other tissues. It has become clear that no single gene is constitutively expressed, and mRNA expression of housekeeping genes can vary considerably among different tissues and disease stages.

Furthermore, the classic reference genes include ACT3, GAPDH, HPRT, and 18srRNA, which have been used as references in semi-quantitative northern blots and RNase protection assays for many years. Their use was acceptable because these genes are expressed at relatively high levels in all cells and are ideal positive controls if the gene of interest is switched off. However, the advent of real-time qPCR that is proven more sensitive than northern blotting places the emphasis on quantitative change, and should have resulted in re-evaluation of the use of these reference genes. This has not been done, however, and workers have continued to use arbitrarily chosen reference genes.

The profiling of target gene GSTs1 varied with the different methods used; the relative quantitation and dual-spark PCR techniques yielded different results. On the basis of the earlier relative quantitative methods, researchers measured only RNA samples of different genes and reduced the errors, with different calculation of efficiency or other methods. The correct choice of reference genes depends on the cells or tissues under study and researchers should look carefully at their experimental system for stable expression of the chosen reference gene. Unfortunately, these types of errors (coming from the selection of different genes as suitable reference genes, etc.) cannot be eliminated completely.

To quantitate the same template, the relative quantitation can give reliable results whether using an internal or an external reference. In different tissues, different developmental time points or different stimulus conditions, the stability analysis of reference gene expression should be considered in designing the experimental scheme.

The dual-spark PCR technique uses exogenous reference mRNA along with mRNA absolute standard curves and appears to give reliable results. It is a direct and intuitive result that ‘per gene copy transcription level’ measures the RNA/DNA ratio of the copy number of the target gene in the cell. The DNA sample and the RNA sample for the same gene were measured with the same primer pair, so that variation between different genes attributed to PCR conditions, primer characteristics, and amplicon length was eliminated, unlike relative quantitative methods that measure only RNA samples of different genes using different primer pairs [15,19,20]. Accordingly, this method does not need different references to detect target genes, thus avoiding errors that might be introduced by using different reference genes.

Dual-spark technology is focused more on the transcriptional activity of a gene or regulation of the activity of the promoter. In the experimental operation of dual spark-in technology, we need to extract DNA samples, when RNA is extracted. In addition, although the dual-spark qPCR technique described here doubles the workload of relative quantitative one; it is still a good choice for quantitative analysis.

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References