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Screening of suitable reference genes for gene expression using quantitative real-time PCR in *Gynura bicolor* DC.

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ABSTRACT: Quantitative real-time PCR (qRT-PCR) is a preferred approach for monitoring gene expression levels. Screening of the most stable reference genes is paramount to the accuracy of data generated by qRT-PCR. To date, a systematic exploration of suitable reference genes in different tissues at different developmental stages in *G. bicolor* has not been performed. Thus, in the present study, a systematic reference gene screening was performed by qRT-PCR on 5 candidate genes in *G. bicolor*. All 5 reference genes displayed a wide range of Cq values in all samples, indicating that they were expressed variably. Based on the analysis results from the software programs geNorm, NormFinder and BestKeeper, *GAPDH* was the most stable reference gene in roots and leaves during different developmental stages. *ACTIN* and *GAPDH* were selected as the most suitable reference genes in stems. *ACTIN* was selected as the most suitable gene in all samples, as it was expressed stably in all samples. Overall, this research provides a guideline for the selection of suitable reference genes in qRT-PCR gene expression studies of different tissues at different development stages in *G. bicolor*.

KEYWORDS: gene expression, reference genes, quantitative real-time PCR, *Gynura bicolor* DC.

INTRODUCTION

Gynura bicolor DC. is a perennial plant belonging to Asteraceae. It has high nutritional and medicinal value due to its richness in microelements, anthocyanins, flavonoids, amino acids, and crude protein [1, 2]. Additionally, it possesses high ornamental value, as the adaxial sides of leaves are green and the abaxial sides are reddish purple. It is beneficial for the exploitation and utilization of *G. bicolor* to elucidate the molecular mechanisms underlying various biological processes.

Gene expression analysis promotes our understanding of the molecular mechanisms underlying developmental and cellular processes [3, 4]. Although reverse transcriptase quantitative real-time polymerase chain reaction PCR (qRT-PCR) has become a preferred approach for monitoring gene expression levels in different samples because of its high sensitivity, accuracy, specificity, good reproducibility, and wide dynamic range [5–7], there are several factors (such as template quantity and quality, optimal assay design, appropriate data normalization and analysis) that have a direct impact on the accuracy of the assay [7, 8]. The selection of highly reliable reference genes is essential to ensure the accuracy of qRT-PCR. Hence, reference genes should be evaluated prior to performing qRT-PCR.

ACTIN (*ACTIN*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), elongation factor-1 (*EF1*), a-tubulin (*TUBULIN*), and 60S ribosomal protein (*60S*) are classical reference genes used to normalize gene expression profiles to ensure accurate gene expression results [9, 10]. In recent studies, only a single reference gene (*ACTIN* or *GAPDH*) was used for qRT-PCR in *G. bicolor*, and no preliminary validation was performed [11, 12]. However, previous studies have shown that the expression profiles of reference genes vary across different tissues [13–16] and treatments [17–19]. To the best of our knowledge, a systematic exploration of suitable reference genes in different tissues in *G. bicolor* has not been performed.

Statistical algorithms such as geNorm [20], BestKeeper [21] and NormFinder [22], have been developed to evaluate the most suitable reference genes for normalizing qRT-PCR data from a given set of biological samples. In the present study, 5 candidate reference genes, namely, *ACTIN*, *GAPDH*, *EF1*, *TUBULIN* and *60S* were assessed by qRT-PCR in different tissues of *G. bicolor*. The expression stability of these reference genes was estimated by 3 statistical approaches. The aim of this research was to screen and evaluate the stability of 5 reference genes for the purpose of normalization in studying *G. bicolor* gene expression.

MATERIALS AND METHODS

Plant materials

Seedlings of *G. bicolor* were cultured in Hoagland nutrient solution for 2 weeks. Leaves, stems and adventitious roots were sampled 3 times at 3-day intervals to analyze the expression profiles of the candidate reference genes. Samples were obtained from 5 different plants, 3 independent biological replicates were included for each sample, and each experiment was repeated 3 times. All samples (27 samples collected from roots, stems and leaves at different developmental stages) were immediately frozen in liquid nitrogen and maintained at −80 °C until RNA extraction.

Cloning of the candidate reference genes

To acquire the *G. bicolor* orthologs of the reference genes previously reported in other plant species, publicly available gene fragments, including *ACTIN*, *GAPDH*, *EF1*, *TUBULIN* and *60S*, from species in Asteraceae (*Chrysanthemum*, *Helianthus annuus*, *Cynara cardunculus* and *Lactuca sativa*) were downloaded. The abbreviation of the species name *Gynura bicolor*, "Gb", was added as a prefix to the name of each selected gene to specify that this was an orthologous gene in *G. bicolor*. Primers for the cloning of these genes were designed as follow: *GbACTIN*, according to the conserved regions among *H. annuus* (XM_022126129.2), *Chrysanthemum* (LC195832.1) and *C. cardunculus* (XM_025113656.1); *GbGAPDH*, according to the conserved regions between *H. annuus* (XM_022164985.2) and *L. sativa* (XM_023882460.1); *GbEF1*, according to the conserved regions between *Chrysanthemum* (KF305681.1) and *H. annuus* (XM_022145511.2); *GbTUBULIN*, according to the conserved regions between *H. annuus* (XM_022172599.2) and *C. cardunculus* (XM_025108072.1); and *Gb60S*, according to the conserved regions between *H. annuus* (XM_022121993.2) and *C. cardunculus* (XM_025108052.1).

Total RNA was extracted using an RNAprep Pure Plant kit (Tiangen, Beijing, China), and cDNA was synthesized using the TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (TRAN, Beijing, China).

The *GbACTIN*, *GbGAPDH*, *GbEF1*, *GbTUBULIN* and *Gb60S* gene fragments in *G. bicolor* were amplified by RT-PCR. The primers used are shown in Table 1. RT-PCR was performed using the cDNA of leaves of *G. bicolor* as the template. The RT-PCR program was as follows: (1) 95 °C for 5 min, (2) 35 cycles of 95 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min, and (3) maintenance at 72 °C for 2 min.

Analysis of gene expression by qRT-PCR

qRT-PCR was performed using TB Green® premix Ex Taq (Takara, Dalian, China). The qRT-PCR primers used to amplify the 5 candidate reference gene fragments are shown in Table 1. The qRT-PCR program was as follows: (1) 95 °C for 30 s; (2) 40 cycles of 95 °C for 5 s, 58 °C for 20 s, and 72 °C for 20 s; (3) a melt curve programme (95 °C, 1 s, 20 °C/s; 65 °C, 15 s; 95 °C, 0 s, 0.1 °C/s). The signals were monitored using a LightCycler® 96 System (Roche, USA). For each primer pair, the amplification efficiency was derived from the standard curve generated from 5-fold serial dilutions of the cDNA from all the samples. The specificity of the qRT-PCR amplification product for each primer pair was further determined by electrophoresis in a 2% (w/v) agarose gel and by melting-curve analysis. Cq values were obtained for the baseline and quantification cycles. The expression stability of the reference genes was analyzed using the software programs geNorm [20], BestKeeper [21] and NormFinder [22]. The average Cq value was calculated from 3 biological and 3 technical replicates.

RESULTS AND DISCUSSION

Cloning and sequence analysis of *G. bicolor* orthologs for candidate reference genes

A total of 5 genes were selected for use as candidate reference genes for *G. bicolor*. The following fragments were obtained: 642-bp *GbTUBULIN*, 1089-bp *GbEF1*, 365-bp *GbGAPDH*, 878-bp *GbACTIN* and 346-bp *Gb60S* fragments (Fig. 1). A BLASTX search of the sequence results was conducted, and the search results showed that the *GbACTIN*, *GbGAPDH*, *GbEF1*, *GbTUBULIN* and *Gb60S* amplification fragments displayed high similarity with their corresponding homologous genes (Table 2). *GbACTIN* showed 88.1% similarity with *Actin-7* in *H. annus*, whereas *GbGAPDH*, *GbEF1*, *GbTUBULIN* and *Gb60S* exhibited 99.12%, 97.24%, 100% and 92.16% similarity to their corresponding homologous genes in *H. annuus*, respectively. These results showed that the homologous *ACTIN*, *GAPDH*, *EF1*, *TUBULIN*, *60S* gene fragments were amplified successfully in *G. bicolor*.

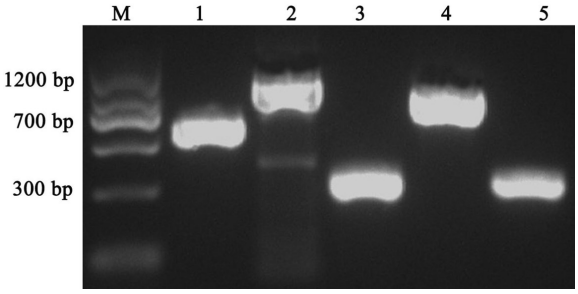

Fig. 1 RT-PCR products of the 5 reference gene fragments in *G. bicolor*. M, Marker; Lane 1–5, RT-PCR products of: 1, *TUBULIN* gene; 2, *EF1* gene; 3, *GAPDH* gene; 4, *ACTIN* gene; 5, *60S* gene. All primers are shown in Table 1.

Assessment of primer specificity and expression levels of candidate reference genes

For identification of qRT-PCR primer specificity, the PCR-amplified products were analyzed by agarose gel electrophoresis and melting curve analysis. Electrophoretic separation of the amplicons generated signal bands with expected sizes in all cases (Fig. 2A-1, A-2, and A-3), and nucleotide sequencing further confirmed the identity of these amplicons. Additionally, melting curve analysis also yielded a single peak with no visible primer-dimer formation (Fig. 2B). The above two results indicated that the 5 reference gene primer pairs yielded specific amplification of the reference genes in *G. bicolor*. The correlation coefficients (R^2)

Table 1 Primers used for qRT-PCR analyses.

Gene symbol	Primer name	Primer sequence (5′–3′)	Product length (bp)	R^2	E %
ACTIN	Gb-YG-ACTIN-F	CCTGCCATGTATGTTGCCATCCA	160	0.99	1.05
	Gb-YG-ACTIN-R	ACAGAGACAAGTGGCTCGTCACA			
GAPDH	Gb-YG-GAPDH-F	TCGCTCTTCGTGTACCAACTCCTA	160	1.0	1.08
	Gb-YG-GAPDH-R	TCGCTCTTCGTGTACCAACTCCTA			
EF1	Gb-YG-EF1-F	CAGCACAATCAGCCTGAGAAGTC	145	0.99	1.10
	Gb-YG-EF1-R	GCAGAGCGTGAACGTGGTATC			
TUBULIN	Gb-YGTUBULIN-F	ACATCACCACGGTACATGAGACAG	95	0.99	1.08
	Gb-YG-TUBULIN-R	ACCAACAGTGCCTTTGAGCCTTC			
60S	Gb-YG-60S-F	CGAAGAAACCGCTCATTGGAAGG	164	0.99	1.06
	Gb-YG-60S-R	GGCACAACTTGACCTTGAACCTG			

Table 2 Candidate reference genes used for gene screening in *G. bicolor*.

Gene symbol	Ortholog locus	Locus description	E value	Identity (%)
GbACTIN	XP_021981820.1	Actin-7	0	88.10
GbGAPDH	ABW89104.1	Glyceraldehyde-3-phosphate dehydrogenase	5e-49	99.12
GbEF1	AHE76183.1	Elongation factor 1-alpha	0	97.24
GbTUBULIN	PWA94154.1	Alpha tubulin	7e-157	100
Gb60S	XP_021977685.1	60S ribosomal protein-13-1	3e-60	92.16

of the standard curve were higher than 0.99 (Table 1), suggesting good linear relationships among all samples. The amplification efficiencies ranged from 105% for *ACTIN* to 110% for *EF1*, suggesting that the reference gene primer pairs are suitable for further gene expression analysis (Table 1).

The expression profiles of the 5 candidate reference genes are presented as raw Cq values. The results showed that the selected reference genes exhibited relatively wide expression abundance and variation. The mean Cq values for the reference genes ranged from 17.29 (*GbTUBULIN*) to 24.71 (*Gb60S*) (Fig. 3). None of the candidate reference genes were constitutively expressed in the tested samples. The results indicated that screening for suitable reference genes was necessary for the characterization of gene expression in *G. bicolor*.

Reference gene expression stability analysis

The stability of the reference genes in all samples was evaluated separately using geNorm, NormFinder and BestKeeper.

The geNorm program is used to rank the stability of the expression of the tested genes by calculating

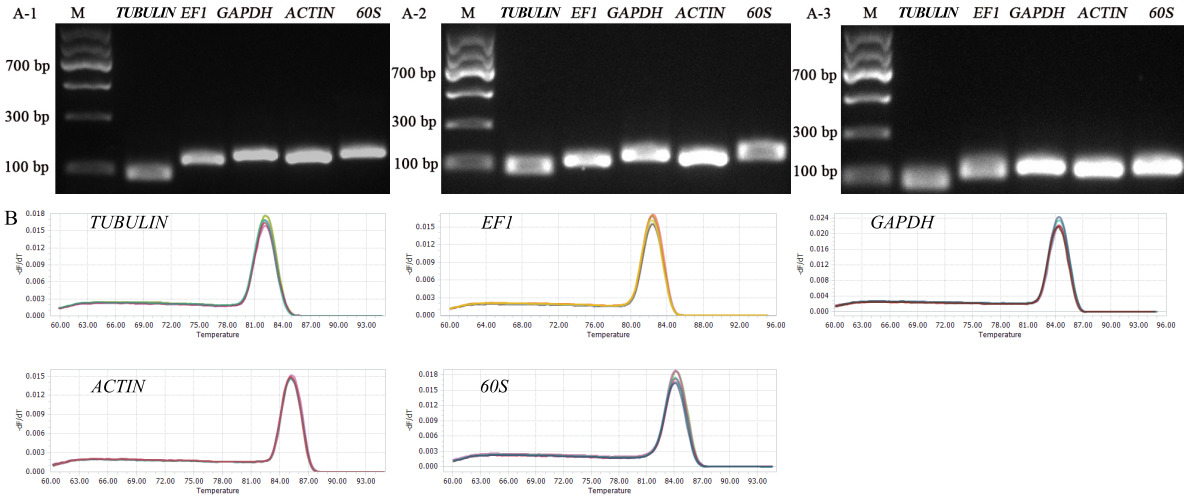

Fig. 2 Assessment of the specificity of primers for qRT-PCR amplification. (A) RT-PCR products of the expected size obtained for the 5 candidate genes on a 2% agarose gel using cDNA from (A-1) roots, (A-2) stems and (A-3) leaves. (B) Melting curves of 5 reference genes.

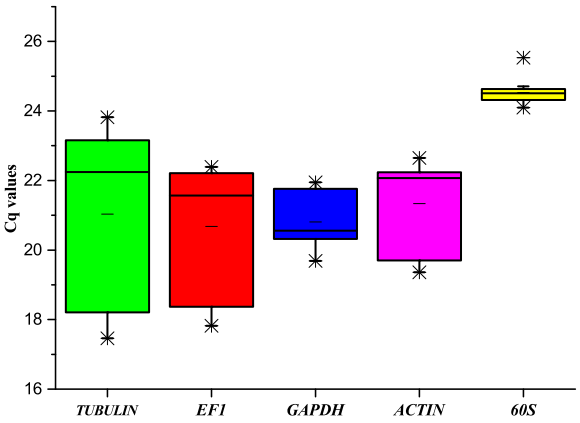

Fig. 3 Distribution of Cq values of the 5 candidate reference genes in different experimental samples. A line across the box depicts the median values. The box indicates the 25th and 75th percentiles, and the whiskers represent the maximum and minimum values.

Table 3 Rating of candidate reference genes in order of their expression stability calculated by NormFinder.

Ranking	All samples	Roots	Stems	Leaves
1	*ACTIN*	*GAPDH*	*ACTIN*	*GAPDH*
2	*EF1*	*EF1*	*GAPDH*	*TUBULIN*
3	*GAPDH*	*ACTIN*	*EF1*	*60S*
4	*60S*	*60S*	*TUBULIN*	*ACTIN*
5	*TUBULIN*	*TUBULIN*	*60S*	*EF1*

Table 4 Ranking of candidate reference genes in order of their expression stability calculated by BestKeeper.

Ranking	All samples	Roots	Stems	Leaves
1	*GAPDH*	*GAPDH*	*GAPDH*	*GAPDH*
2	*ACTIN*	*ACTIN*	*60S*	*60S*
3	*60S*	*60S*	*ACTIN*	*EF1*
4	*EF1*	*EF1*	*EF1*	*TUBULIN*
5	*TUBULIN*	*TUBULIN*	*TUBULIN*	*ACTIN*

their expression stability values (M) based on the average pairwise expression ratio. The M value of a suitable reference gene should be less than 1.5, and the gene with the lowest M value is considered to be a more stably expressed reference gene [20]. The M values of the tested genes evaluated by geNorm are shown in Fig. 4. All the M values of the 5 genes were less than 1.5, indicating that the 5 genes could be used as suitable reference genes. When all of the samples were taken together (Fig. 4A), the average M value of *ACTIN* was the lowest and that of *TUBULIN* was the highest, indicating that *ACTIN* had the most stable expression and that *TUBULIN* was expressed most variably. The M value of *GAPDH* was the lowest in roots and leaves (Fig. 4B,D), suggesting that *GAPDH* had the most stable expression in roots and leaves. However, the M value of *TUBULIN* was the highest in roots and that of *EF1* was the highest in leaves (Fig. 4B,D), indicating that *TUBULIN* was expressed most variably in roots, while *EF1* was expressed most variably in leaves. Additionally, the M value of *ACTIN* was the lowest and that of *TUBULIN* was the highest in stems (Fig. 4C), suggesting that *ACTIN* was the most stable reference gene and *EF1* was the least stable reference gene in stems.

NormFinder software is another approach to identify the optimal normalization genes among a set of candidates [22]. Genes with the lowest expression stability value were considered the most stable. The ranking of the values calculated by NormFinder is shown in Table 3. For all samples, the ranking order of the values was *ACTIN* < *EF1* < *GAPDH* < *60S* < *TUBULIN*, indicating that *ACTIN* and *TUBULIN* were the most and the least stable gene in all samples, respectively. For values in roots, the lowest was for *GAPDH* and the highest was for *TUBULIN*, thus the most stable reference gene was *GAPDH* and *TUBULIN* the least stable. For leaves, the ranking order of the values was *GAPDH* < *TUBULIN* < *60S* < *ACTIN* < *EF1*, indicating that the genes showing the highest and lowest expression stability were *GAPDH* and *EF1*, respectively. For stems, *ACTIN* possessed the lowest value, and *60S* possessed the highest value, suggesting that the most stable reference gene was *ACTIN* and the least stable was *60S*. Interestingly, the most stable gene calculated by NormFinder was in agreement with the results calculated by geNorm software.

BestKeeper software is also frequently used to determine expression stability by calculating the coefficient of variation (CV), the Pearson correlation coefficient (r) and the standard deviation (SD) using raw Cq values [21]. The lowest SD and CV values and the highest r value indicated the most reliable reference gene. The results of the software analysis are presented in Table 4. For all samples, the most stable gene was *GAPDH*, followed by *ACTIN* and *60S*. For roots and leaves, *GAPDH* was also the most stable reference gene. For stems, *GAPDH* was the most stable reference gene, followed by *60S* and *ACTIN*.

Taken together, *GAPDH* showed the most stable performance analyzed by geNorm, NormFinder and Bestkeeper in roots and leaves; thus, *GAPDH* was selected as the most stable reference gene in roots and leaves in the present study. For stems, there were some differences among the 3 software programs. The top 2 ranked genes were *ACTIN* and *GAPDH* in the analyses by geNorm and NormFinder, while the best performing reference genes in the analysis by Bestkeeper were *GAPDH*, *60S*, and *ACTIN*. As the mean Cq value of *60S* reached 24.98 (Fig. 3), it was too high to be used as a reference gene. Therefore, *ACTIN* and *GAPDH* were selected as the most suitable genes in stems. For all samples, the 2 best performing reference

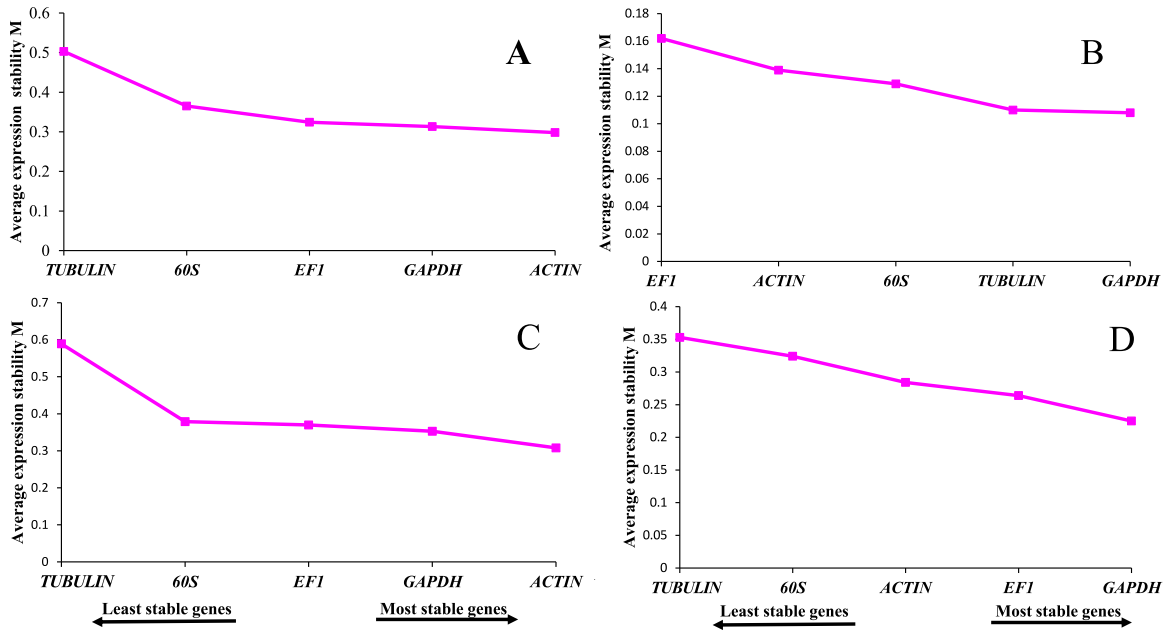

Fig. 4 Assessment of the M values of 5 candidate reference genes calculated by geNorm. (A) All samples, (B) leaves, (C) stems, and (D) roots, at different developmental stages.

genes by BestKeeper analysis were *GAPDH* and *ACTIN*, while the top 2 ranked genes assessed by geNorm and NormFinder were *ACTIN* and *EF1*. *ACTIN* was selected as the most suitable reference gene in all samples, because it was always classified between the 2 best performing reference genes analyzed by the 3 software programs in all the samples. The above differences might be due to the different algorithms and analytical procedures of the 3 programs. Differences in the ranking orders obtained with these algorithms have also been observed in other characterizations [23–25].

G. bicolor possesses high nutritional, medicinal and ornamental value. It is of great significance to investigate the molecular mechanism underlying developmental and cellular processes in *G. bicolor*. qRT-PCR is a popular approach to assess gene expression profiles. However, the stability of reference genes affected the accuracy of qRT-PCR. Thus, it is necessary to screen for a suitable reference gene prior to assessing gene expression levels. To our knowledge, the present study is the first systematic survey on the stability of reference genes for qRT-PCR analysis of various tissues in *G. bicolor*.

CONCLUSION

We successfully cloned 5 reference gene fragments in *G. bicolor* and screened 5 pairs of specified primers for qRT-PCR analysis in roots, stems and leaves at different developmental stages. *GAPDH* was identified as the most stable reference gene in roots and leaves. *ACTIN* and *GAPDH* were screened as the most suitable genes in stems. Additionally, *ACTIN* was selected as the most suitable gene in all samples, as it was expressed stably in all samples. These results lay the foundation for gene expression analysis in roots, stems and leaves at different developmental stages in *G. bicolor*.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2022.124.

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Appendix A. Supplementary data

Table S1 Primers used to amplify reference gene fragments.

Gene symbol	Primer name	Primer sequence (5′–3′)
ACTIN	GbACTIN-F	CGTCTGCGATAATGGAACT
	GbACTIN-R	CCACCACTGAGAACAATGT
GAPDH	GbGAPDH-F	AACAATGACCACGACACA
	GbGAPDH-R	CCACAGTTGAGGAGACATC
EF1	GbEF1-F	TTAAGGCAGAGCGTGAAC
	GbEF1-R	TAACACCGACAGCAACAG
TUBULIN	GbTUBULIN-F	TACCTACACCAACCTCAATC
	GbTUBULIN-R	CCAACCTCCTCATAATCCTT
60S	Gb60S-F	ATTGCTGTTGACCATCGT
	Gb60S-R	CACTCTACTTCTTCTCTTCCT