Identification and Validation of Reference Genes for Gene Expression Analysis Using Quantitative PCR in *Spodoptera litura* (Lepidoptera: Noctuidae)

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**Abstract**

Reverse transcription quantitative polymerase chain reaction (qRT-PCR) has rapidly become the most sensitive and accurate method for the quantification of gene expression. To facilitate gene expression studies and obtain more accurate qRT-PCR data, normalization relative to stable housekeeping genes is required. These housekeeping genes need to show stable expression under the given experimental conditions for the qRT-PCR results to be accurate. Unfortunately, there are no studies on the stability of housekeeping genes used in *Spodoptera litura*. In this study, eight candidate reference genes, elongation factor 1 alpha (EF1), gyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal protein L10 (RPL10), ribosomal protein S3 (RPS3), beta actin (ACTB), beta FTZ-F1 (FTZF1), ubiquinol-cytochrome c reductase (UCCR), and arginine kinase (AK), were evaluated for their suitability as normalization genes under different experimental conditions using the statistical software programs, BestKeeper, geNorm and Normfinder, and the comparative ACT method. We determined the expression levels of the candidate reference genes for three biotic factors (developmental stage, tissue, population), and four abiotic treatments (temperature, insecticide, food and starvation). The results indicated that the best sets of candidates as reference genes were as follows: GAPDH and UCCR for developmental stages; RPL10, AK and EF1 for different tissues; RPL10 and EF1 for different populations in China; GAPDH and EF1 for temperature-stressed larvae; AK and ACTB for larvae treated with different insecticides; RPL10, GAPDH and UCCR for larvae fed different diets; RPS3 and ACTB for starved larvae. We believe that these results make an important contribution to gene analysis studies in *S. litura* and form the basis of further research on stable reference genes in *S. litura* and other organisms.

**Introduction**

Reverse transcription quantitative polymerase chain reaction (qRT-PCR) has rapidly become the most sensitive, accurate and widely used method for gene expression analysis in order to understand biological processes and physiological functions, as well as for validation of the results of microarray analysis and other techniques [1,2]. One of the critical challenges of qRT-PCR analysis for reliable mRNA quantification in any biological system is the availability of appropriate normalization genes, the expression level of which is considered stable, regardless of cell type and across various experimental conditions [3]. However, several studies have revealed that using different normalization genes which show variations in a biological system can result in appreciable errors owing to different treatments, sampling methods, total RNA extraction, reverse-transcription, etc., even up to 20-fold by some estimations [4–6]. Hence, the use of normalization genes should be experimentally validated for different developmental stages, tissues and specific experimental designs [3,6,7]. Furthermore, at least two or three reference genes should be used for accurate normalization based on the studies of Thellin et al. [8] and Vandesompele et al. [6]. In most studies, normalization has been described for certain systems but is frequently applied to other systems without an appropriate validation of their stability in that particular system. Therefore, it is necessary to select the most suitable genes for normalization from a panel of candidate genes in a given set of biological samples from a specific organism.

*Spodoptera litura* is an important polyphagous insect pest that causes widespread economic damage to vegetables and other crops, including ornamental plants in tropical and subtropical regions [9,10]. As a polyphagous species, this pest has the potential to invade new areas and to adapt to new host plants. In recent years, molecular technology, particularly qRT-PCR for gene expression, has been widely used in genetic studies on *S. litura* [11,12]. Changes in gene expression can often reflect biologically significant changes across insect developmental stages, tissues and other samples from different experimental conditions. Therefore, it is important to establish normalization genes so that specific changes in gene expression can be evaluated. However, no experimental data are available on the most appropriate
transcription factor beta FTZ-F1 (β-actin (ACTB), β-actin (ACTB), ribosomal protein L10 (RPL10), beta-actin (ACTB), ubiquinol-cytochrome c reductase (UCCR), transcription factor beta FTZ-F1 (FT2F1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), elongation factor (EF1) and arginine kinase (AK)). These genes are commonly used as single normalization genes in gene expression studies of S. litura and other insects.

Materials and Methods

Insects

The laboratory strain of S. litura was established from field collections in June 2012 obtained from a lotus field at the Agricultural Experiment Station, Huazhong Agricultural University (Wuhan, Hubei, China). The larvae were reared on lotus leaves, and the adults were fed 10% honey solution in the laboratory. They were reared at a temperature of 30°C, under a photoperiod of 16:8 h L: D, and relative humidity of 70%. Other populations used in this experiment were collected from lotus fields at Jiangsu, Zhejiang, Jiangxi, Anhui and Shandong provinces. The laboratory strain and other populations used in this experiment were from different fields. No specific permissions were required as these fields are experimental plots that belong to Huazhong Agricultural University, Wuhan, Hubei in China.

Biotic Factors

Developmental stage. Samples used comprised 300 first-day eggs, 50 first-instar larvae, 30 second-instar larvae, five third-instar larvae, five fourth-instar larvae, five fifth-instar larvae, five sixth-instar larvae, five pre-pupae, five first-day male and female pupae, five six-day male and female pupae, five 12-day-old male and female pupae, five first-day male and female adults, and five 7-day-old male and female adults for each replication. All the samples were collected in 1.5 mL microcentrifuge tubes, which were immediately frozen in liquid nitrogen and stored at −80°C.

Tissue. Tissue from the brain, midgut, fat body, epidermis and hemolymph were obtained from third-instar larvae using dissection needle in PBS solution on ice [13].

Population. One laboratory S. litura strain and five field collected populations from Jiangsu, Zhejiang, Jiangxi, Anhui and Shandong provinces were used. The laboratory strain was maintained without exposure to any insecticide within the laboratory setting.

Abiotic Stresses

Temperature-induced stress. Each group of five third-instar larvae were exposed to temperatures of 15°C (cold), 25°C (room temperature) or 35°C (hot) for 1 h in a glass tube placed in a water bath. Five insects in each temperature were then collected for RNA extraction.

Insecticide-induced stress. The insecticides used were chlorpyrifos, diafenthiuron, spinosad, indoxacarb and chlorantraniliprole, which are often used in Lepidopteran pest management programs. The leaf-dip bioassay method reported by Shelton et al. [14] and Liang et al. [15] was adopted for insecticide bioassay. Cabbage discs (6.5 cm diameter) were cut and dipped in various concentrations of insecticides prepared with distilled water containing 0.1% Triton X-100. Each disc was dipped for 10 s and allowed to air dry at room temperature. The discs were then placed individually inside plastic petri dishes (7.0 cm diameter). A total of 10–15 third-instar larvae were confined to each dish, and three replications were prepared. Controls were cabbage discs treated with distilled water containing 0.1% Triton X-100. The treated larvae were reared routinely and mortality was checked after 48 h. The 48-h LC15 (sublethal dose) values for the insecticides were estimated by probit analysis (Table 1). Third-instar larvae were then treated using the LC15 value of each insecticide. The surviving insects after 48 h were collected for RNA extraction.

Food. The newly hatched larvae were reared on an artificial diet [16], lotus leaves, taro leaves or water oats, until they reached third-instar stage. Then, the third-instar larvae were collected for RNA extraction.

Starvation-induced stress. Thirty third-instar larvae were starved for 6 h, and were collected for RNA extraction.

Reference Gene Selection and Primer Design

Eight commonly used reference genes were selected (Table 2). Based on the described insect reference genes in literature, the NCBI database (http://www.ncbi.nlm.nih.gov) was searched for available S. litura sequences: ACTB [17], UCCR, FT2F1, GAPDH [6], EF1 [23, 24] and AK [18]. We explored whether UCCR and FT2F1 could be used as reference genes. However, RPS3 [1, 19] and RPL10 (unpublished data from our laboratory) sequences were amplified based on the sequences from Spodoptera frugiperda and Spodoptera exigua (SfRPS3, accession no. AF429976; SfRPL10, accession no. EU250622). We have submitted RPS3 (accession No., KCB866374) and RPL10 (accession No., KCB866373) gene sequences from S. litura to GenBank. However, 28S rRNA was omitted in our study although it has been used in several qRT-PCR studies, because many literatures [3] suggested that 28S gene may not be an ideal gene for qRT-PCR due to its high expression level. All gene-specific primers were designed using Beacon Designer 8.0 software (Premier Biosoft International, Palo Alto, CA, USA; Table 2).

Table 1. The toxicity of insecticides to the third-instar larvae of S. litura.

| Insecticides     | N*         | Slope ± SEa | LC15 ± SEa | LC50 ± SEa | χ² d | 95% fiducial limits (LC15, LC50) |
|------------------|------------|-------------|------------|------------|------|---------------------------------|
| Chlorpyrifos     | 240        | 1.71±0.20   | 8.08 (4.91–11.46) | 32.69 (24.83–43.45) | 1.38 |                                |
| Diatethionuron   | 210        | 1.90±0.25   | 7.69 (5.07–10.35) | 27.07 (20.74–37.56) | 1.06 |                                |
| Spinosad         | 210        | 1.85±0.24   | 10.64 (6.05–15.41) | 38.68 (29.18–50.61) | 2.26 |                                |
| Indoxacarb       | 210        | 2.21±0.27   | 10.28 (6.40–14.18) | 30.26 (23.46–38.27) | 2.35 |                                |
| Chlorantraniliprole | 210       | 1.63±0.23   | 0.36 (0.18–0.56) | 1.58 (1.15–2.13) | 0.54 |                                |

*N = Number of tested larvae.

SE = standard error.

*Expressed in mg/L. 95% fiducial limits (FL) of LC15, LC50 are given in parenthesis, respectively.

*χ² = chi-square testing linearity of dose-mortality responses.

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Total RNA Isolation and cDNA Synthesis

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the recommended procedures. The purity of all RNA samples was assessed at absorbance ratios of A260/A280 and A260/A230 with a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan), and the integrity of the RNA was immediately checked using 1.0% agarose gel electrophoresis.

Then, the RNA was treated with DNaseI (Fermentas, Glen Burnie, MD, USA) according to the manufacturer’s instructions, and the first-strand cDNA template was synthesized from 1.0 μg of total RNA using the First-Strand cDNA Synthesis Kit (Fermentas, Glen Burnie, MD, USA) following the recommended procedures. The concentration and purity of total RNA isolated from different samples were determined using the UV-1800 spectrophotometer.

Table 2. Primer pairs used for quantitative real-time PCR.

| Gene name (Abbreviation) | Accession No. | Primer Name | Sequence (5’-3’) | Product length (bp) | Tm (°C) | Primer efficiency (%) | R²b |
|--------------------------|---------------|-------------|------------------|---------------------|--------|-----------------------|-----|
| Elongation factor-1 (EF1) | DQ192234      | SIN-F1      | CTCTCATACTCAAGAGATC | 295                 | 55     | 96.7                  | 0.997 |
| Ribosomal protein L10 (RPL10) | KC866373 | SIN-F2      | GACTTGTTGTTAGAGAAAG | 189                 | 55     | 107.9                 | 0.998 |
| Actin (ACT8) | DQ494753      | SIN-R2      | GATGACCAGGATTGAGATG | 55                 |        |                       |      |
| Glycerinaldehyde-3-phosphate dehydrogenase (GAPDH) | HQ012003 | SIN-R3      | GGTATTCCTGTACTACAC | 184                 | 55     | 109.6                 | 0.996 |
| Beta FTZ-F1 | HQ260326      | SIN-F5      | CTGATGAGACTACCTTC | 297                 | 55     | 107.9                 | 0.998 |
| Ubiquinol-cytochrome c reductase (UCCR) | HQ599193 | SIN-R5      | CAGGAATCATTACTAG | 55                 |        |                       |      |
| Ribosomal protein S3 (RPS3) | KC866374 | SIN-R7      | GATGACCAGGATTGAGAC | 55                 |        |                       |      |
| Arginine kinase (AK) | HQ840714      | SIAK-F      | CTGAGAAGATACCTACC | 80                  | 55     | 105.2                 | 0.989 |

| Gene name (Abbreviation) | Accession No. | Primer Name | Sequence (5’-3’) | Product length (bp) | Tm (°C) | Primer efficiency (%) | R²b |
|--------------------------|---------------|-------------|------------------|---------------------|--------|-----------------------|-----|
| ACTB                     |              |             |                  |                     |        |                       |      |
| RPL10                    |              |             |                  |                     |        |                       |      |
| EF1                      |              |             |                  |                     |        |                       |      |
| GAPDH                    |              |             |                  |                     |        |                       |      |
| FTZF1                    |              |             |                  |                     |        |                       |      |
| UCCR                     |              |             |                  |                     |        |                       |      |
| RPS3                     |              |             |                  |                     |        |                       |      |
| AK                       |              |             |                  |                     |        |                       |      |

*F and R refer to forward and reverse primers, respectively; *R² refers to the coefficient of determination.

Quantitative Real-time PCR

Reverse transcription quantitative PCR (qRT-PCR) was performed using SsoFast™ EvaGreen® Supermix (Bio-Rad, Hercules, CA, USA) on a Bio-Rad iQ2 Optical System (Bio-Rad) based on the method of Giulietti et al. [20]. The amplification conditions were as follows: 95°C for 30 s followed by 40 cycles of 95°C for 5 s and 55°C for 10 s. After the reaction, a melting curve analysis from 65°C to 95°C was applied to all reactions to ensure consistency and specificity of the amplified product. A 10-fold dilution series of cDNA from the whole body of five third-instar larvae was used to create the standard curve, and the qRT-PCR efficiency was determined for each gene and each treatment using the linear regression model [21]. The corresponding qRT-PCR efficiencies (E) were calculated according to the equation: \( E = \left(10^{\text{1/efficiency} - 1}\right) \times 100 \) [22].

Statistical Analysis

Expression levels were determined as the number of cycles needed for the amplification to reach a fixed threshold in the exponential phase of the PCR reaction [23]. The threshold was set at 500 for all genes to determine the Ct values. Gene stabilities of the eight candidate reference genes were evaluated using the software tools BestKeeper [21], geNorm version 3.5 (http://medgen.ugent.be/~jvdesomp/genorm/) [6] and NormFinder version 0.953 (http://www.mdl.dk/publications/normfinder.htm) [24]. BestKeeper uses raw data and PCR amplification efficiency to determine the best-suited standards and combines them to create an index. Ct values were converted into relative quantities and imported into the geNorm and NormFinder software programs. The geNorm algorithm first calculates an expression stability value (M) for each gene and then compares the pair-wise variation (V) of this gene with the others. Using microarray data as a training set for the algorithm, a threshold of V < 0.15 was suggested for valid normalization [6]. NormFinder also ranks the stability of the tested genes independently from each other. We also used a user-friendly web-based comprehensive tool, RefFinder (http://www.leonxie.com/referencegene.php?type = reference), including the comparative ΔCt method [21], to compare and rank the tested candidate reference genes. Based on the rankings from each program, RefFinder assigns an appropriate weight to an individual gene and calculates the geometric mean of their weights for the overall final ranking. The lower ranking indicated genes with more stable gene expression.

Results

Total RNA Quality and PCR Amplification Efficiencies

The concentration and purity of total RNA isolated from different samples were determined using the UV-1800 spectrophotometer. The A260/A280 ratios ranged from 1.80 to 2.20 for most RNA samples, indicating a high purity of total RNA for all samples. The integrity of all total RNA samples was confirmed using 1.0% agarose gel electrophoresis.

For each of the primer pairs, the single peak qPCR melting curves suggested that each of the primer pairs amplified a unique
product. The products were sequenced and showed 100% identity with the fragment sequences on which the primer design was based (Fig. S1). The linear regression coefficients ($R^2$) of each standard curve for PCR efficiency, which was determined for each gene using 10-fold serial dilutions of the cDNA generated from third-instar larvae of the laboratory strain, were 0.989 (AK), 0.996 (GAPDH), 0.997 (EF1 and RPS3) and 0.998 (RPL10, ACTB, FTZF1 and UCCR) (Table 2). The PCR efficiency of the eight candidate reference genes was excellent, ranging from the lowest for EF1 (96.7%) to the highest for RPL10 and UCCR (109.7%; Table 2).

Expression Profiles of Candidate Reference Genes

Expression levels were determined as the number of cycles needed for the amplification to reach a fixed threshold (500) in the exponential phase of the PCR reaction [23]. The gene expression analysis of seven candidate reference genes, with the exception of FTZF1, displayed a narrow range of mean Ct values across all experimental samples (Fig. 1). The raw Ct values ranged from 14.58 (ACTB) to 38.59 (FTZF1) in all samples, from 15.92 (ACTB) to 33.89 (FTZF1) in the developmental stages, from 14.58 (ACTB) to 33.59 (FTZF1) in the different tissues, from 16.08 (ACTB) to 33.14 (FTZF1) in the different populations, from 15.58 (ACTB) to 33.92 (FTZF1) in the different temperature treatments, from 14.92 (ACTB) to 38.59 (FTZF1) in the insecticide treatments, 14.95 (ACTB) to 32.43 (FTZF1) in the third-instar larvae reared on different diets, and from 17.48 (ACTB) to 35.92 (FTZF1) in the third-instar larvae starved for 6 h. The fluorescence peak after about 15 cycles showed that ACTB was the most abundantly transcribed, whereas FTZF1 was the least abundant transcript with a Ct value of 35 or higher. All candidate genes except FTZF1 exhibited relatively small variations in Ct values.

Analysis of Gene Expression Stability

Biotic Factors

Developmental stage. The stability rankings generated by the Delta Ct method were the same as those generated by NormFinder. Additionally, the stability rankings generated by geNorm were largely similar with the results obtained from Act and NormFinder methods, even though the ranking order of the genes was different to some extent. However, the gene stability rankings by BestKeeper analysis were different to the results generated by the other three methods. All four programs, except for BestKeeper, identified GAPDH and UCCR as the most stable genes (Fig. S2). According to the results of RefFinder, the stability rankings across the developmental stages were in decreasing order of GAPDH, UCCR, AK, RPL10, ACTB, EF1, RPS3, and FTZF1 (Fig. 2A). For geNorm, the V value of 0.160 obtained by the RPS3-ACTB pair was close to the proposed 0.15 cut-off. Moreover, the inclusion of additional reference genes did not lower the V value below the proposed 0.15 cut-off until the eighth gene was added (Fig. 3).

Tissue. The stability rankings generated by the Delta Ct method and NormFinder identified RPL10 and UCCR as the most stable pair of genes. However, gene stability, as ranked by BestKeeper and geNorm, differed from the results generated by the Act and NormFinder methods (Fig. S3). According to the results of RefFinder, the stability rankings from the most stable to the least stable gene in different tissues were RPL10, AK, EF1, GAPDH, RPS3, UCCR, FTZF1 and ACTB (Fig. 2B). GeNorm analysis revealed that the pair-wise variation value V3/4 was below the proposed 0.15 cut-off (Fig. 3). An increase in variation in this value was related to a decrease in expression stability, because of the inclusion of a relatively unstable fourth gene (GAPDH). The inclusion of a fourth reference gene did not improve the statistical significance for each of the candidate reference gene pair groups.

Population. The stability rankings generated by BestKeeper and geNorm identified RPL10 and EF1 as the most stable pair of genes. However, gene stability as ranked by the Delta Ct method and NormFinder was different to the results generated by the BestKeeper and geNorm methods (Fig. S4). According to the RefFinder results, the stability rankings from the most stable to the least stable gene in the different populations were as follows: RPL10, EF1, UCCR, GAPDH, AK, ACTB, FTZF1, and RPS3 (Fig. 2C). GeNorm analysis revealed that the pair-wise variation value V4/5 was below the proposed 0.15 cut-off (Fig. 3). This result suggests that the inclusion of a fifth reference gene would not provide any additional improvement to the statistical significance for each of the candidate reference gene pair groups.

Abiotic Stresses

Temperature. All four programs, with the exception of geNorm, identified GAPDH as the most stable gene in the third-instar larvae treated at different temperatures. From the results of RefFinder, the stability rankings from the most stable to the least stable gene in the temperature-stressed samples were GAPDH, EF1, ACTB, AK, RPL10, UCCR, FTZF1 and RPS3 (Fig. 2D). However, GeNorm analysis revealed that all the pair-wise variation values were above the proposed 0.15 cut-off (Fig. 3) and identified EF1 as the most stable gene (Fig. S5). These results indicate that normalization with three stable reference genes was required (as suggested by the geNorm manual).

Insecticide. All four programs except for geNorm identified AK as the most stable gene in the third-instar larvae treated with different insecticides (Fig. S6). According to RefFinder, the stability rankings from the most stable to the least stable gene in the insecticide-stressed samples were AK, ACTB, GAPDH, FTZF1, RPL10, UCCR, EF1 and RPS3 (Fig. 2E). However, geNorm identified RPL10 as the most stable gene (Fig. S6). GeNorm analysis also revealed that all the pair-wise variation values were above the proposed 0.15 cut-off (Fig. 3). These results indicate that normalization with three stable reference genes was required (as suggested by the geNorm manual).

Food. The stability rankings generated by the Delta Ct method and geNorm identified RPL10 and GAPDH as the most...
Figure 2. Expression stability of the candidate reference genes as calculated by the Geomean method of RefFinder (http://www.leonxie.com/referencegene.php?type=reference). A lower Geomean ranking indicates more stable expression. Expression stability of reference
stable pair of genes in the third-instar larvae reared on different diets (Fig. S7). Moreover, the results determined by NormFinder also identified RPL10 as the most stable gene. However, the stability ranking generated by BestKeeper and NormFinder identified UCCR as the most stable gene. Based on the RefFinder results, the stability rankings from the most stable to the least stable gene in the third-instar larvae reared on different diets were as follows: RPL10, GAPDH, UCCR, AK, RPS3, EF1, ACTB and FTZF1 (Fig. 2F). GeNorm analysis revealed that the pair-wise variation value V2/3 was below the proposed 0.15 cut-off (Fig. 3). This result suggests that the inclusion of a third reference gene would not improve the statistical significance of each of the candidate reference gene pair groups.

Discussion

This study was conducted to identify the optimal reference genes for qRT-PCR studies. To our knowledge, this is the first study to evaluate the expression stability of different candidate reference genes for qRT-PCR in S. litura.

A major conclusion of this study is that few, if any, universally suitable reference genes in S. litura can be used for qRT-PCR analyses under various experimental conditions, as the candidate reference genes showed too much variation in expression among the different treatments (Table 3). RPL10 exhibited the most stable expression in different tissues, populations, and in the third-instar larvae reared on different diets. GAPDH displayed the most stable expression in different developmental stages and in samples treated at different temperatures. AK and RPS3 showed the most stable expression in samples treated with insecticides and starved for 6 h. These results indicate that the stability of reference gene expression in S. litura needs be investigated for each experimental treatment. These results were similar to the reference gene analysis of Drosophila [3].

Table 3. Preferable reference genes across different experimental conditions according to the software analysis.

| Experimental conditions | Preferable reference genes |
|-------------------------|---------------------------|
| Biotic factors          | Developmental stage       |
| Tissue                  | GAPDH, UCCR               |
| Population              | RPL10, AK, EF1            |
| Abiotic factors         | Temperature               |
| Insecticide             | GAPDH, EF1                |
| Food                    | RPL10, GAPDH, UCCR        |
| Starvation              | RPS3, ACTB                |

Figure 3. Determination of the optimal number of reference genes as calculated by geNorm for accurate normalization of gene expression. Average pairwise variations (V) were calculated by geNorm between the normalization factors NFn and NFn−1 to indicate whether inclusion of an extra reference gene would add to the stability of the normalization factor. Values <0.15 indicate that additional genes are not required for the normalization of gene expression.

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**Supporting Information**

**Figure S1** Primer positions and amplicon sequences used for qRT-PCR. The DNA sequences are shown from the 5’ to 3’ end, and the primer positions are shaded. The products were first amplified by regular PCR and then sent to Invitrogen for sequencing. (TIF)

**Figure S2** Expression stability of the candidate reference genes across different developmental stages of *S. litura*. The expression stability of the reference genes in *S. litura* across developmental stages was measured using the ΔCt method, BestKeeper, NormFinder and geNorm. A lower average stability value indicates more stable expression. (TIF)
The expression stability of the reference genes in the different tissues of *S. litura* was also measured using the Ct method, BestKeeper, NormFinder and geNorm. A lower average stability value indicates more stable expression.

**Figure S6 Expression stability of the candidate reference genes in insecticide-stressed samples of *S. litura***

The expression stability of the reference genes in the insecticide-stressed samples of *S. litura* was also measured using the Ct method, BestKeeper, NormFinder and geNorm. A lower average stability value indicates more stable expression.

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**Author Contributions**

Conceived and designed the experiments: YL MY XG HW JL. Performed the experiments: YL MY TK SZ. Analyzed the data: YL MY XG JL. Wrote the paper: YL MY XG JL.
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