Screening Potential Reference Genes in *Tuta absoluta* with Real-Time Quantitative PCR Analysis under Different Experimental Conditions

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Abstract: *Tuta absoluta* is one of the most significant invasive pests affecting tomato plants worldwide. RT-qPCR has emerged as one of the most sensitive and accurate methods for detecting gene expression data. The screening of stable internal reference genes is the most critical step for studying the molecular mechanisms of environmental adaptability. The stable reference genes expressed in *T. absoluta* under specific experimental conditions have not yet been clarified. In this study, seven candidate reference genes (*RPL27*, *RPS13*, *EF1-a*, *TUB*, *TBP*, and *β-actin*) and their optimal numbers were evaluated under biotic (developmental stages and adult tissues) and abiotic (insecticide, temperature, and plant VOC) conditions using four software programs. Our results identified the following reference genes and numbers as optimal: three genes (*EF1-a*, *RPS13*, and *RPL27*) for different developmental stages (egg, larva, pupa, unmated adult), two genes (*RPS13* and *TBP*) for adult tissues (antenna, head, thorax, abdomen, leg), two genes (*TBP* and *RPS13*) for insecticides (*Bacillus thuringiensis*, chlorpyrifos, abamectin-aminomethyl, and chlorantraniliprole), two genes (*RPL27* and *TUB*) for temperature-induced stresses (0, 25, and 40 °C), and two genes (*RPS13* and *TUB*) for VOC-induced stresses (nonanal, α-phellandrene, and tomato leaves). Our results provide a reference for selecting appropriate reference genes for further study of the functional genes of *T. absoluta* under different experimental conditions.

Keywords: biological factors; environmental factors; expression stability; reference genes; RT-qPCR; South American tomato leafminer

1. Introduction

Real-time quantitative polymerase chain reaction (RT-qPCR) is one of the most sensitive, convenient, and widely used methods for detecting gene expression in different biological samples [1–3]. It is also the most reliable method for detecting and quantifying transcriptional abundance [4]. When using RT-qPCR to study the expression levels of target genes, appropriate reference genes must be used to calibrate and standardize the experimental samples [5–7]. The reference genes should not be affected by external environments and should be stably expressed in various tissues or cells and under various experimental conditions [8]. However, many studies have demonstrated that the stability of different reference genes can change in response to different experimental conditions [9,10]. Using different normalization reference genes can lead to significant errors due to differences in sampling methods, total RNA extraction, efficiency of complementary DNA (cDNA)
synthesis, transcriptional activity in biological samples, primer design, and reverse transcription efficiency, with some experimental differences estimated to be as high as 20- to 100-fold [11–14]. Therefore, it is important to identify and evaluate reference genes that are stably expressed under different experimental conditions to obtain more accurate and reliable experimental results [15]. Several genes have been confirmed as stable reference genes and are widely used when studying insects, such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH), actin (ACT), translation elongation factor 1a (TEF-1a), phospholipase A2 (PLA2), arginine kinase (AK), tubulin (TUB), TATA binding protein (TBP), and ribosomal proteins [7,16–20]. However, few reference genes are consistently expressed across all conditions (e.g., tissues, developmental stages, and experimental conditions) [19,21–23]. The screening of reference genes has recently been reported in many Lepidopteran insects, including Plutella xylostella [24,25], Spodoptera litura [22,26], Chilo suppressalis [27], Helicoverpa armigera [28], Spodoptera exigua [29], Sesamia inferens [30], Bicyclus anynana [19], Danaus plexippus [31], Trichoplusia ni [32], and Lymantria dispar [23]. However, reference genes can be expressed differently in different insect species or under different experimental conditions.

The South American tomato leafminer, also known as T. absoluta (Meyrick) (Lepidoptera: Gelechiidae), is one of the most important invasive insect pests globally and has spread to more than 80 countries and regions [33,34]. It has recently invaded China [35] and spread rapidly, becoming well-established in Xinjiang and Yunnan [36] and threatening the growth and development of local tomato plants [33,37,38]. Previous studies assessing T. absoluta have primarily focused on biological characteristics, insecticide resistance, invasiveness and spread pathway, and integrative pest management [33,39]; however, few have assessed the molecular mechanism behind the environmental adaptability of T. absoluta. The screening of stable internal reference genes is the most critical step for studying the molecular mechanisms of environmental adaptability (e.g., temperature, host plant, insecticide).

The ideal reference gene is stably expressed under different conditions [31,40]; however, the stable expression of any reference gene is relative since the perfect reference gene does not exist [23,41]. Therefore, identifying stable reference genes in a specific species under different experimental conditions is important to ensure the accuracy of the expression data of the target genes [27]. In this study, the stability of seven candidate reference genes (ribosomal protein L27 (RPL27), ribosomal protein s13 (RPS13), ribosomal protein s15 (RPS15), elongation factor1-a (EF1-a), TATA binding protein (TBP), tubulin (TUB), and β-actin), all of which are commonly used in other Lepidopteran insects, were evaluated under different environmental conditions and different developmental conditions of T. absoluta. These results will inform subsequent studies of T. absoluta based on gene expression level, including those assessing the olfactory mechanism, environmental adaptability, the mechanism of insecticide resistance, and management using RNAi technology.

2. Materials and Methods

2.1. Insects

The T. absoluta populations were grown in tomato plants under laboratory conditions at 27 ± 2 °C, 50 ± 10% RH, and 14:10 h (light/dark) in Yunnan. Healthy and active insect individuals were used for the experiments.

2.2. Treatments

Experimental treatments included variable T. absoluta conditions and environmental factors. Four different development stages (egg, larva, pupa, and adult) were used for the pest and five adult tissues (antenna, head, thorax, abdomen, and leg) were assessed. For environmental conditions, the insecticides (four insecticides), temperature (three temperature settings), and plant volatile organic compounds (VOCs) (two VOCs and tomato leaves) were assessed. The samples of development stages, temperature, insecticides, and plant
VOC treatment were collected at 11:00 am. The samples of tissues were collected at 10:00 am every time.

Five independent biological replicates were performed for each sample treatment. Each sample was collected and kept in a 1.5 mL centrifuge tube, immediately frozen in liquid nitrogen, and stored at −80 °C for subsequent experiments.

2.2.1. Developmental Stages

Four different developmental stages were included: egg, larva (first, second, third, and fourth instar), pupa (male and female), and adult (male and female). Individuals of *T. absoluta* were randomly selected from the same colony, including the eggs (100 individuals per biological replicate), the 1st instar larvae (30 individuals), the 2nd instar larvae (3 individuals), the 3rd instar larva (1 individual), the 4th instar larva (1 individual), male (1 individual) and female (1 individual) pupae, and unmated male (3 individuals) and female (3 individuals) adults. In total, 45 samples (9 development stages/statuses × 5 biological replicates) were collected.

2.2.2. Adult Tissues

Male and female adults were sampled separately. The adults were dissected to obtain samples of five different tissues, including the antenna (200 pairs per biological replicate), head (without antennae, 50 individuals), thorax (without wings and legs, 20 individuals), abdomen (10 individuals), and leg (50 individuals). Five types of different adult tissues were collected and were separately placed in 1.5 mL centrifuge tubes. In total, 25 samples (5 tissues × 5 biological replicates) were collected.

2.2.3. Insecticides

Healthy, one-day-old third-instar larvae were treated with four common insecticides, specifically one microbial formulation BT (G033A) (provided by the Department of Biological Insecticide Research, IPP, CAAS, Beijing, China) and three chemical insecticides: chlorpyrifos (99.0%), abamectin-aminomethyl (98.5%), and chlorantraniliprole (97.2%) (Beijing Qincheng Yixin Technology Co., Ltd., Beijing, China). First, the chlorpyrifos and abamectin-aminomethyl were dissolved in acetone (w/v, 1:25) and chlorantraniliprole in dimethyl sulfoxide (DMSO) (1:250). Based on the results of previous studies [42–44], the concentration multiples of the four insecticides in this study were slightly changed. The concentrations of the three chemical insecticides were then diluted into 28.34 g/L, 0.5 mg/L, and 1.4 mg/L samples using distilled water containing 0.1% Triton X-100. The BT was diluted to a concentration of 1 g/L using distilled water containing 0.1% Triton X-100. The tomato leaves were each dipped into four insecticide solutions for 10 s and air-dried. The third instar larvae were starved for 12 h and individually fed with the dipped tomato leaves for 36 (BT) or 24 h (chlorpyrifos, abamectin-aminomethyl, and chlorantraniliprole), while distilled water containing 0.1% Triton X-100 was used as the control. In total, 20 samples/individuals (4 insecticides × 5 biological replicates) were collected.

2.2.4. Temperatures

Three temperature treatments were designed based on previous studies, including cold-shock temperature treatment (0 °C) [45], heat-shock temperature treatment (40 °C) [46], and moderate temperature treatment (25 °C) [46]. The living, one-day-old third-instar larvae were treated for 1 h at all three setting temperatures. Five larval individuals were separately placed in five glass tubes to serve as five replicates, after which they were placed at setting temperatures for 1 h. For the 0 °C treatment, a heating refrigeration type circulator (CC-K6-NR, Peter Huber Kältemaschinenbau GmbH, Offenburg, Germany) was used; for 40 °C, a thermostat water bath (HH.S11-Ni2, Beijing Changan Scientific Instrument Factory) was used; and for 25 °C (as the control), a constant-temperature incubator (KRQ-250A, Shanghai Qixin Scientific Instrument Co., Ltd., Shanghai, China) was used. In total, 15 samples (3 temperatures × 5 biological replicates) were collected.
2.2.5. Plant Volatile Organic Compounds (VOCs)

We used two kinds of plant VOCs: nonanal (96%, Beijing Bingda Biotechnology Co., Ltd., Beijing, China) and α-phellandrene (85%, Sigma, and tomato leaf (mature leaf) (Solanum lycopersicum cv. Shouhefenguan). The plant VOCs were diluted in hexane (5%, v/v), while the tomato leaflets (about 20 mm × 30 mm) were directly used as a control. Fifty newly emerged (one-day-old) male and female adults (sex ratio, 1:1) were placed in a 50 mL centrifuge tube and considered one biological replicate. Two hours later, one piece of filter paper (20 mm × 30 mm) was dripped with 20 µL of diluted plant VOC (nonanal or α-phellandrene) or a tomato leaflet, which was placed in the above centrifuge tube to excite the adults [47,48]. Five hours later, the heads of the excited adults were dissected and placed in a 1.5 mL centrifuge tube. In total, 15 samples (3 VOCs/tomato leaf × 5 biological replicates) were collected.

2.3. Total RNA Extraction and cDNA Synthesis

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions. To reduce deviation, the purity and concentration of each RNA sample were determined twice using a NanoPhotometer™ p330 (Implen, Munich, Germany). The A260/A280 ratios of all the RNA samples ranged from 1.8 to 2.2. The RNA integrity was tested using 1% agarose gel electrophoresis. Total RNA was used to synthesize cDNA (1 µg) using the TransScript All-In-One First-Strand cDNA Synthesis SuperMix for qPCR and PCR (Transgen Biotech, Beijing, China) according to the manufacturer’s instructions. The cDNA of each sample treatment had five biological replicates and was stored at −80 °C.

2.4. Candidate Reference Gene Selection and Specific Primer Design

Seven candidate reference genes (i.e., RPL27, RPS13, RPS15, EF1-α, TUB, TBP, and β-actin) commonly used in other Lepidoptera were selected based on the transcriptome data of T. absoluta (GeneBank accessions: MZ357900–357906). Primer pairs for each candidate reference gene were designed using the Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA), and the target candidate reference genes were amplified with optimal parameters (Table 1). The seven amplified reference genes were then amplified, sequenced (Sangon Biotechnology Co., Ltd., Shanghai, China), and validated. The specific RT-qPCR primer pairs targeted to T. absoluta were designed (Beacon designer 8.0 software (BioRad, Hercules, CA, USA) based on the validated sequences (Table 2). All primers were synthesized by Sangon Biotechnology Co., Ltd. (Shanghai, China).

| Gene Name | Primer | Primer Sequences (5’-3’) | Tm (°C) | Amplicon Length (bp) |
|-----------|--------|--------------------------|---------|----------------------|
| TUB       | TUB-F  | GAGTGCATCTCAGTCCACGTT    | 55      | 417                  |
|           | TUB-R  | GAAGGAGTGGAAGATGAGGAAT   |         |                      |
| β-actin   | β-actin-F | TCCCTGGAGATCCACATCTG     | 53      | 590                  |
|           | β-actin-R | CCCATCTACGAAGGTTACCG     |         |                      |
| EF1-α     | EF1-α-F | GCCCTCTCTGCCTTCACCCTT    | 62      | 381                  |
|           | EF1-α-R | GGGCAGGGTTCCAATACCAACC  |         |                      |
| RPL27     | RPL27-F | GTAAGATTATGAAACCTGGGAA   | 51      | 395                  |
|           | RPL27-R | ACCAAGCTTTGGGAAGAACC     |         |                      |
| RPS13     | RPS13-F | CCCCACCTGTTGAAACTGAC     | 51      | 376                  |
|           | RPS13-R | TGAAGCCGCGTGAGACTCATA    |         |                      |
| TBP       | TBP-F  | TCCCTCACACCTGTGCGGCT     | 58      | 374                  |
|           | TBP-R  | GTGTCGGCTGGCTCTCCTGAT    |         |                      |
| RPS15     | RPS15-F | CGAGAATACACCACAGAATGT    | 51      | 429                  |
|           | RPS15-R | TTCTTGGCTTGTGTTGATT      |         |                      |
Table 2. Reference genes and specific primers used for RT-qPCR analysis in *T. absoluta*.

| Primer | Primer Sequences (5′-3′, F/R) | Amplicon Length (bp) | Tm (°C) | Efficiency (%) | R² |
|--------|-------------------------------|---------------------|---------|----------------|----|
| RPL27  | CGTTACTCCGTAGACTTCA GTCTTITCACAATGTTGATCG | 126 | 60 | 92.7 | 0.992 |
| RPS13  | AGTTGCCCAAGTCAAGTT ACCAATGTTCACTTCAT | 133 | 60 | 102.0 | 0.999 |
| RPS15  | ATACGAAATGAACAGTTACC AGCTCCATTCTTCACAACT | 92 | 60 | 103.8 | 0.990 |
| EF1-α  | AGTCTCTCTACATCAAAAG GTCTCCGCTTACCAACAAG | 143 | 60 | 100.7 | 0.998 |
| TBP    | GTCTACTCCACACCTCAAA CTGCCACTTGTATCATG | 77 | 60 | 110.2 | 0.993 |
| TUB    | GCTGAGAAGGTTGTGAAC CTGAGAAGGTTGTAC | 126 | 60 | 95.9 | 0.998 |
| β-actin| CAGTCTTTAGGGTGCTTTC GCTCTTACGCTCAACAG | 99 | 60 | 104.4 | 0.996 |

2.5. Standard Curve Construction and RT-qPCR

Relative standard curves for the transcripts were generated with 2-fold serial dilutions of cDNA (1/2, 1/4, 1/8, 1/16, 1/32, and 1/64) extracted from the second instar larvae of *T. absoluta*. The corresponding RT-qPCR efficiencies (E) were determined for each gene and calculated according to the following equation: 

\[ E = \left(10^{-\frac{1}{\text{slope}}} - 1\right) \times 100 \]  

RT-qPCR was performed on the ABI 7500 PCR Detection System (Applied Biosystems, Waltham, MA, USA). The RT-qPCR reactions consisted of 10 µL Hieff® qPCR SYBR® Green Master Mix (Low Rox) (Yeasen Biotech Co., Ltd., Shanghai, China), 0.4 µL each forward and reverse primers (10 µM), and 1 µL the cDNA template. Thermal cycling conditions were as follows: an initial cycle at 95 °C (30 s), followed by 40 cycles of 10 s at 95 °C and 30 s at 60 °C. After all reactions, a melting curve analysis from 65 to 95 °C was used to ensure the consistency and specificity of the amplified product. According to the results of the standard curve, the cDNA template of all samples was diluted 5 times in the reference gene expression experiment. Each treatment included five biological replicates, and each reaction was performed in triplicate.

2.6. Statistical Analysis

Relative cycle threshold (C_t) values are widely used to identify stably expressed genes by RT-qPCR analysis. The average C_t value was calculated based on five biological replicates. Two commonly used software tools, geNorm version 3.5 [13] and NormFinder version 0.953 [50], were used to evaluate the raw average C_t values of the seven selected reference genes, according to the manufacturer’s instructions. For both geNorm and NormFinder, the 2^{-ΔΔC_t} value (ΔΔC_t = the corresponding C_t value—minimum C_t value) was used to calculate the stability value of the reference genes. The values of the expression stability measure (M value) and the pair-wise variation (V) of the target gene were evaluated using the geNorm software. An M value below 1.5 indicates that the gene can be used as a reference gene, while the gene with the lowest M value should be the most stable [13]. When V_{n/n+1} is below 0.15, it indicates that the most suitable number of reference genes for normalization is n; when the V_{n/n+1} is above 0.15, it indicates that the most suitable number is n+1 [13]. The stability value (SV) was evaluated by the NormFinder software and was used to rank the expression stability of the suitable reference genes. The gene with the lowest value is typically the most stable. The standard deviation (SD) was calculated according to the average C_t value using BestKeeper [51]. The lower the standard deviation, the better the stability of the reference gene. However, if the SD > 1, this indicates that the expression of the reference gene is unstable. Finally, the comprehensive and user-friendly web-based comprehensive tool RefFinder (https://localhost/RefFinder-master/RefFinder-master/?type=reference (accessed on 15 March 2021)) [52] was used to calculate the geometric mean (GM) values according to the results of these algorithms, after which
the candidate reference genes were ranked based on GM. The most suitable reference genes under each condition/treatment were identified according to the GM value and the number of optimal reference genes determined by the V value. The difference of gene expression was analyzed by post-hoc testing using IBM SPSS statistics 25.0 (SPSS Inc., Chicago, IL, USA) with a threshold of $p < 0.05$.

3. Results

3.1. Acquisition of Candidate Reference Gene Sequences

The sequences of the seven candidate genes, namely RPL27, RPS13, RPS15, EF1-α, TUB, TBP, and β-actin, were obtained using the transcriptome database of the antenna of T. absoluta (unpublished data). The sequences were validated by PCR (Figure S1) and then sequenced and confirmed to exhibit 100% identity with the corresponding transcriptome sequence data (Table 1).

3.2. Specificity and Amplification Efficiency of Specific RT-qPCR Primers

Based on the validated sequences of the seven reference genes, we designed the specific RT-qPCR primers (Table 2). The accuracy of the RT-qPCR primers was confirmed by the presence of a single peak in the melting curve analyses (Figure S2) and a single amplicon on 1% agarose gel. The sizes of the amplified products were consistent with the expected sizes, indicating that each primer pair was specific. The amplification efficiency of each primer pair under RT-qPCR analysis for the seven candidate reference genes ranged from 92.7 to 110.2%, while the linear regression coefficient $R^2$ ranged from 0.99 to 1.00 based on the standard curve (Table 2). This demonstrated that all seven primer pairs met the requirements of RT-qPCR.

3.3. Expression Levels of the Candidate Reference Genes

The transcription levels of the seven candidate reference genes were evaluated using the $C_t$ values obtained from the RT-qPCR analysis. The $C_t$ values of the seven candidate reference genes varied under the five experimental conditions (Table 3). Under the different developmental stages, β-actin ($C_t = 18.11$) showed the highest expression level, and RPL27 ($C_t = 27.49$) showed the lowest expression level, but the gap between the maximum ($C_t = 28.39$) and the minimum values ($C_t = 15.03$) of β-actin was the largest (Figure 1A). This could be related to the expression of the β-actin in the eggs ($C_t = 27.43$), which was significantly lower than in other developmental stages ($C_t = 16.85$) (data not shown). In different adult tissues, the expression level of EF1-α ($C_t = 20.30$) was the highest, while RPL27 ($C_t = 28.88$) was the lowest (Figure 1B). For the insecticide-induced stresses, the expression level of β-actin ($C_t = 16.33$) was the highest, and the expression level of RPL27 ($C_t = 27.63$) was the lowest (Figure 1C). For the expression levels of all samples of temperature-induced stresses, β-actin was the highest ($C_t = 16.71$), and TBP was the lowest ($C_t = 27.80$) (Figure 1D). Under plant VOC exposure conditions, the expression level of EF1-α ($C_t = 22.74$) was the highest, and the expression level of RPL27 ($C_t = 27.45$) was the lowest (Figure 1E). For the total $C_t$ values of the five experimental conditions, the lowest $C_t$ value was detected in β-actin ($C_t = 14.26$), and the highest was in RPL27 ($C_t = 33.11$). The $C_t$ value of β-actin varied most due to differences between the eggs and other treatments. The expression levels of both β-actin and EF1-α were the highest, with average $C_t$ values of 19.36 and 20.19, respectively. The lowest expression level was RPL27, with an average $C_t$ value of 27.47 (Table 3, Figure 1F).
Table 3. Distribution of the Ct values of each candidate reference gene in *T. absoluta* (mean ± SD).

| Gene Name | Total Samples | Developmental Stage | Tissue | Insecticide | Temperature | Plant VOC |
|-----------|---------------|---------------------|--------|-------------|-------------|-----------|
|           |               |                     |        | Treatment   | Control     | Treatment | Control |
| TBP       | 25.84 ± 1.28 b| 25.14 ± 1.15 b      | 25.45 ± 0.98 b | 26.92 ± 0.51 a | 27.64 ± 0.52 a | 27.80 ± 0.65 a | 27.84 ± 0.64 a | 25.61 ± 0.39 b | 25.54 ± 0.35 b |
| TUB       | 21.14 ± 2.22 d| 19.31 ± 1.27 d,e    | 22.81 ± 1.16 c | 20.41 ± 2.29 b,c | 21.34 ± 0.44 d | 21.78 ± 0.60 d | 21.63 ± 0.74 d | 24.09 ± 0.51 c,d | 23.88 ± 0.47 d |
| EEF1-α    | 20.19 ± 1.50 d,e| 19.80 ± 1.04 d    | 20.30 ± 1.47 d | 19.22 ± 1.04 c | 20.12 ± 0.83 e | 19.74 ± 0.89 e | 19.92 ± 0.85 e | 22.74 ± 0.69 e | 22.35 ± 0.37 e |
| β-actin   | 19.36 ± 3.68 e| 18.11 ± 3.69 e     | 22.42 ± 1.41 c | 16.33 ± 1.70 d | 16.50 ± 0.15 f | 16.71 ± 0.25 f | 16.58 ± 0.21 f | 23.83 ± 0.63 d | 23.69 ± 0.59 d |
| RPS13     | 22.61 ± 1.22 c| 22.30 ± 1.00 c     | 22.83 ± 0.95 c | 22.16 ± 0.49 b | 21.28 ± 0.46 d | 21.44 ± 0.55 d | 21.46 ± 0.58 d | 24.72 ± 0.50 c | 24.51 ± 0.41 c |
| RPS15     | 26.08 ± 1.62 b| 25.35 ± 1.31 b     | 26.94 ± 1.14 b | 27.39 ± 1.91 a | 26.57 ± 0.53 b | 26.93 ± 0.70 b | 26.69 ± 0.53 b | 24.44 ± 0.63 c,d | 24.08 ± 0.41 c,d |
| RPL27     | 27.47 ± 1.84 a| 27.49 ± 1.15 a     | 28.88 ± 2.34 a | 27.63 ± 0.74 a | 24.17 ± 0.57 c | 24.37 ± 0.61 c | 24.38 ± 0.69 c | 27.45 ± 0.76 a | 26.99 ± 0.42 a |

Lowercase letters indicate the Ct value of each reference gene was significant different under the same treatment (*p* < 0.05, one-way ANOVA followed by LSD test).
Figure 1. Expression levels of seven candidate reference genes in *T. absoluta*. Line across the box shows the median value, while inside the box indicates Ct values. The top and bottom whiskers represent the 25th and 75th percentages, respectively. Developmental stages (A), adult tissues (B), insecticides (C), temperatures (D), plant VOCs (E), and total (F) based on Ct values of all samples exhibited in the five experimental conditions.

3.4. Expression Stability and Ranking of the Candidate Reference Genes

3.4.1. Developmental Stages

Analysis using the geNorm software determined that the M values of *β-actin* exceeded 1.5, indicating that *β-actin* could not be used as the stable reference gene in this treatment (Figure 2(A1)). Both *EF1-α* and *RPS13* were the most stable reference genes as indicated by the geNorm, NormFinder, and BestKeeper software programs (Figure 2(A1–A3)). RefFinder was used to comprehensively rank the highest to the lowest stability: *EF1-α*, *RPS13*, *RPL27*, *RPS15*, *TBP*, *TUB*, and *β-actin* (Figure 2(A4)). The pairwise variation value V2/3 (0.259) exceeded 0.15, according to analysis using geNorm software (Figure 3), suggesting that the optimal number of reference genes for normalization is three genes at different developmental stages: *EF1-α*, *RPS13*, and *RPL27* (Figure 2(A3,A4)).
Figure 2. Stability analysis of the seven candidate reference genes expression of *T. absoluta* under the following experimental conditions: developmental stage (A), adult tissue (B), insecticide (C), temperature (D), and plant VOC (E), as calculated by four different software programs: geNorm (1), NormFinder (2), BestKeeper (3), and RefFinder (4).

3.4.2. Adult Tissues

Based on analysis using the geNorm software, *TUB* and *RPS13* were the most stable reference genes (Figure 2(B1)). *RPS13* and *RPL27* were the most and least stable reference genes, respectively, based on analysis using the NormFinder software (Figure 2(B2)). Based on analysis using the BestKeeper program, *TBP* was the most stable reference gene, and *RPL27* was the least stable reference gene (Figure 2(B3)). Based on analysis using the RefFinder program, the reference genes ranked from the most to least stable were: *RPS13*, *TBP*, *TUB*, *EF1-α*, *RPS15*, β-actin, and *RPL27* (Figure 2(B4)). Analysis using the geNorm program indicated that the pairwise variation value V2/3 (0.149) was below 0.15, meaning that both *RPS13* and *TBP* could be used for normalization in the different adult tissues of *T. absoluta* (Figures 2(B4) and 3).
Reference genes ranked from most to least stable were based on analysis using the BestKeeper software. Using the geNorm software, the reference genomes ranked from the most to least stable were indicated that the pairwise variation value $V_{2/3} = 0.149$ was below 0.15, meaning that the optimal number of reference genes for normalization is $n+1$. TBP was the most stable reference gene according to all three programs (Figure 2(C1–C3)). Based on analysis using the geNorm software, the reference genomes ranked from most to least stable were TBP, RPS13, EF1-α, β-actin, RPL27, RPS15, and TUB. Both TBP and RPS13 could be selected for normalization under insecticide-induced stresses of T. absoluta, based on analysis using the RefFinder and geNorm programs (Figures 2(C4) and 3).

3.4.3. Insecticide-Induced Stress

TBP and RPS13 were the most stable reference genes, based on analysis using the geNorm software (Figure 2(C1)). TBP was the most stable reference gene, based on analysis using the NormFinder software (Figure 2(C2)). EF1-α and RPS13 were the most stable reference genes, based on analysis using the BestKeeper software (Figure 2(C3)). TUB was the most unstable reference gene according to all three programs (Figure 2(C1–C3)). Based on analysis using the RefFinder software (Figure 2(C4)), the reference genomes ranked from most to least stable were TBP, RPS13, EF1-α, β-actin, RPL27, RPS15, and TUB. Both TBP and RPS13 could be selected for normalization under insecticide-induced stresses of T. absoluta, based on analysis using the RefFinder and geNorm programs (Figures 2(C4) and 3).

3.4.4. Temperature-Induced Stress

Based on analysis using the geNorm software, the reference genes with the most stable expression were β-actin and RPS15, while EF1-α was the most unstable reference gene (Figure 2(D1)). RPL27 was the most stable reference gene, and EF1-α was the least stable gene, based on analysis using the NormFinder and BestKeeper software (Figure 2(D2,D3)). Based on analysis using the RefFinder software, the reference genomes ranked from most to least stable were RPL27, TUB, β-actin, RPS13, RPS15, TBP, and EF1-α (Figure 2(D4)). Both RPL27 and TUB could be selected for normalization under cold- and heat-shock treatments for T. absoluta, based on analysis using the RefFinder and geNorm programs (Figures 2(D4) and 3).

3.4.5. Plant VOC-Induced Stress

Both TUB and RPS13 were the most stable reference genes based on analysis using the geNorm and NormFinder programs (Figure 2(E1,E2)). TBP was the most stable reference gene based on analysis using the BestKeeper software (Figure 2(E3)). However, RPL27 was the least stable reference gene based on analysis using the geNorm, NormFinder, and BestKeeper programs (Figure 2(E1–E3)). Based on analysis using the RefFinder software, the reference genomes ranked from most to least stable were RPS13, TUB, EF1-α, TBP, RPS15, TBP, and EF1-α (Figure 2(E4)).
β-actin, RPS15, and RPL27 (Figure 2(E4)). Both RPS13 and TUB could be selected and used for normalization under the condition of plant VOC exposure of T. absoluta, based on analysis using the geNorm and RefFinder programs (Figures 2(E4) and 3).

4. Discussion

Quantitative real-time polymerase chain reaction is an important technique for analyzing gene expression in various biological systems [2,3,53] and has become the standard technique for detecting mRNA levels in gene expression. RT-qPCR uses a relative quantitative method to evaluate the expression level of the target gene based on a stable reference gene and is both highly sensitive and highly accurate [5]. The stability of the reference genes directly affects the accuracy of the expression of a target gene. The reference genes (housekeeping genes) are expressed in all kinds of cells and play an important role in maintaining the basic life activities of cells. The expression of reference genes varies greatly in different types of cells, tissues, and under various experimental conditions [11–13]. Therefore, selecting suitable and stable reference genes is a crucial first step to study gene expression and ensure the reliability of gene expression data.

The stability value and rank of each reference gene were all evaluated with three commonly used software programs: BestKeeper, geNorm, and NormFinder. However, the stability ranks of the analyzed genes were inconsistent due to different algorithms used by each of these analysis tools, an anomaly that has been identified in previous studies [22,26,28]. Therefore, the comprehensive ranking of the stability of the reference genes was evaluated using the RefFinder software. Furthermore, selecting more than one reference gene at the same time, under certain conditions, can improve both the accuracy and reliability of the resulting data [26]. Therefore, the appropriate number of reference genes should be determined according to the pair mutation value (\(V_{n/n+1}\)) using the geNorm software. If the \(V_{n/n+1}\) is less than 0.15, the optimal number of reference genes is \(n\); if the \(V_{n/n+1}\) is greater than 0.15, the optimal number of reference genes is \(n+1\). In this study, it is most appropriate to select at least two reference genes under specific experimental conditions.

The classical reference genes, including ACT, TBP, TUB, EF1-α, and ribosomal proteins, are widely expressed in organisms and are typically used in data normalization. Ribosomal proteins are the primary components of ribosomes and play an important role in protein biosynthesis in cells [54,55]. Elongation factors (EFs) promote polypeptide chain elongation during mRNA translation and are highly conserved in organisms [56]. TUB and ACT are skeleton proteins. These are often used as reference genes [57]. However, these reference genes are not consistently expressed under all conditions [26,29], so the stability of the reference genes should be evaluated under specific conditions.

In this study, the expression stability of seven candidate reference genes (RPL27, RPS13, RPS15, EF1-α, TUB, TBP, and β-actin) were evaluated with various biological factors (development stages, adult tissues) and environmental factors (insecticides, temperatures, plant VOCs). To our knowledge, this is the first study to evaluate the expression stability of the reference genes used in T. absoluta. Our results demonstrate that the most suitable candidate combinations of reference genes were EF1-α, RPS13, and RPL27 for different developmental stages; RPS13 and TBP for adult tissues; TBP and RPS13 for insecticide-induced stresses; RPL27 and TUB for temperature-induced stresses; and RPS13 and TUB for plant VOC exposure. No reference genes were stably expressed under all experimental conditions. For example, TBP was only stable for adult tissues and insecticide; TUB was stable for plant VOC exposure and temperature treatments but unstable for insecticide-induced stresses; RPL27 was stable for temperature stresses and developmental stages but unstable for adult tissues and plant VOC exposure; and EF1-α was stable for developmental stages but unstable for temperature-induced stresses. Our results align with those obtained in previous studies. EF1-α is the most stable gene for the different developmental stages of S. inferens [30], P. xylostella [24], S. exigua [29], and C. suppressalis [27]; β-actin is the least stable gene in L. dispar [23]. For different insect tissues, RPS13 is the optimal reference gene.
for *S. inferens* [30]. *RPL27* is the optimal reference gene under temperature-induced stresses in *H. armigera* [28].

However, different insect species had different results: *β*-actin is the most stable reference gene for the developmental stages of *Phyllonorycter ringoniell* [58], *P. xylostella* [24], *S. exigua* [29], *Mythimna separata* [59], and *S. inferens* [30]; *TUB* is the most stable reference gene for *L. dispar* [23]. *EF1* is the most stable reference gene for different insect tissues of *S. litura* [22], *P. xylostella* [24], and *S. inferens* [30]; and *ACT* is the most stable reference gene for *C. suppressalis* [27]. *β*-actin is the most stable reference gene for insecticide-induced stresses on *S. litura* (insecticides: chlorpyrifos, diafenthiuron, spinosad, indoxacarb, or chlorantraniliprole, treated for 48 h) [22]; *RPS15* is the most stable reference gene for *H. armigera* (BT, *β*-cypermethrin, and Spinetoram; treated for 24 and 48 h, respectively) [28]. *RPS15* is the most stable reference gene for temperature-induced stresses of *H. armigera* (*T* = 4, 27, and 40 °C; treated for 2, 6, and 12 h, respectively) [28]. These differences could be caused by differences across species (such as development stages and adult tissues) and/or differences in treatment conditions (such as insecticides and temperatures). In the case of plant VOCs, Liu used *β*-actin as a reference gene under plant VOC treatment in *Diaphorina citri* [48].

In this study, we assessed seven stably selected reference genes (*RPL27, RPS13, RPS15, EF1-α, TUB, TBP*, and *β*-actin) in *T. absoluta* under different biotic factors (development stage and adult tissue) and abiotic factors (insecticide, temperature, and plant VOC) to normalize the expression of the target genes during RT-qPCR analysis. Our results demonstrated that the stability of the reference genes was inconsistent under different experimental conditions. No single gene was stably expressed across different developmental stages, adult tissues, and experimental conditions [21], and the stability of the reference genes varied among different insect species. Therefore, multiple reference genes should be used to obtain reliable and accurate results in quantitative expression studies of functional genes when study gene expression using RT-qPCR analysis.

5. Conclusions

This experiment was the first in-depth study to identify and validate reference genes for RT-qPCR data normalization under different biotic and abiotic stresses in *T. absoluta*. Our results indicate that *EF1-α, RPS13*, and *RPL27* had the greatest stability across various developmental stages, while *RPS13 and TBP* had the greatest stability across various adult tissues of *T. absoluta*. *TBP* and *RPS13* were the most stable genes under insecticide treatments; *RPL27 and TUB* had the greatest stability under thermal (heat- and cold-shock) treatments; and *RPS13 and TUB* had the greatest stability under plant VOC treatments. Based on all the experimental conditions, *RPS13 can be used under all conditions except temperature treatment, while RPL27 and TUB can be used as supplements under temperature treatment. The expression stability of a putative reference gene is typically verified prior to each RT-qPCR experiment by setting the experimental conditions. To obtain reliable and accurate results, multiple reference genes should be used when analyzing quantitative gene expression. Our results provide comprehensive information on the reference genes needed to study gene expression in *T. absoluta* and other invasive Lepidopteran pests.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10.3390/genes12081253/s1, Figure S1: Single amplicon of desired size for the seven candidate reference genes in *T. absoluta* visualized on 1% agarose gel, Figure S2: Melting curves and melting peaks of the seven candidate reference genes in *T. absoluta.*

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References
1. Bustin, S.A. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J. Mol. Endocrinol. 2000, 25, 169–193. [CrossRef] [PubMed]
2. Kubista, M.; Andrade, J.M.; Bengtsson, M.; Forootan, A.; Jonák, J.; Lind, K.; Sindelka, R.; Sjöback, R.; Sjögreen, B.; Strömbom, L.; et al. The real-time polymerase chain reaction. Mol. Asp. Med. 2006, 27, 95–125. [CrossRef]
3. Derveaux, S.; Vandesompele, J.; Hellemans, J. How to do successful gene expression analysis using real-time PCR. Methods 2010, 50, 227–230. [CrossRef] [PubMed]
4. Bustin, S.A. Quantity and real-time reverse transcription PCR (RT-PCR): Trends and problems. J. Mol. Endocrinol. 2002, 29, 23–39. [CrossRef] [PubMed]
5. Radonić, A.; Thulke, S.; Mackay, I.; Landt, O.; Siegert, W.; Nitsche, A. Guideline to reference gene selection for quantitative real-time PCR. Biochem. Biophys. Res. Commun. 2004, 313, 856–862. [CrossRef] [PubMed]
6. Huggett, J.; Dheda, K.; Bustin, S.; Zumla, A. Real-time RT-PCR normalization; strategies and considerations. Genes. Immun. 2005, 6, 279–284. [CrossRef]
7. Bai, Y.; Lü, Y.N.; Zeng, M.; Jia, P.Y.; Lu, H.N.; Zhu, Y.B.; Li, S.; Cui, Y.Y.; Luan, Y.X. Selection of reference genes for normalization of gene expression in Thermonema domestica (Insecta: Zygentoma: Lepismatidae). Genes 2021, 12, 21. [CrossRef]
8. Thellin, O.; Zorzi, W.; Lakaye, B.; De Borman, B.; Coumans, B.; Hennen, G.; Grisar, T.; Igout, A.; Heinen, E. Housekeeping genes as internal standards: Use and limits. J. Biotechnol. 1999, 75, 291–295. [CrossRef]
9. de Jonge, H.J.M.; Fehrmann, R.S.N.; de Bont, E.S.J.M.; Hofstra, R.M.W.; Gerbens, F.; Kamps, W.A.; de Vries, E.G.E.; van der Zee, A.G.J.; te Meeran, G.J.; ter Elst, A. Evidence based selection of housekeeping genes. PLoS ONE 2007, 2, e998. [CrossRef] [PubMed]
10. Piazza, V.G.; Bartke, A.; Miquet, J.G.; Sotelo, A.I. Analysis of different approaches for the selection of reference genes in RT-qPCR experiments: A case study in skeletal muscle of growing mice. Int. J. Mol. Sci. 2017, 18, 1060. [CrossRef]
11. Schnittgen, T.D.; Zakrajsek, B.A. Effect of experimental treatment on housekeeping gene expression: Validation by real-time, quantitative RT-PCR. J. Biochem. Biophys. Meth. 2000, 46, 69–81. [CrossRef]
12. Lee, P.D.; Sladek, R.; Greenwood, C.M.; Hudson, T.J. Control genes and variability: Absence of ubiquitous reference transcripts in diverse mammalian expression studies. Genome Res. 2002, 12, 292–297. [CrossRef] [PubMed]
13. Vandesompele, J.; Preter, K.D.; Pattyn, F.; Poppe, B.; Roy, N.V.; Paepe, A.D.; Speleman, F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 2002, 3, 467–470. [CrossRef]
14. Gutierrez, L.; Mauriat, M.; Pelloux, J.; Bellini, C.; Wuytswinkel, O.V. Towards a systematic validation of references in real-time RT-PCR. Plant Cell Physiol. 2008, 20, 1734–1735. [CrossRef]
15. Ren, H.; Wu, X.Q.; Lyu, Y.P.; Zhou, H.Z.; Xie, X.Y.; Zhang, X.J.; Yang, H.T. Selection of reliable reference genes for gene expression studies in Botrytis cinerea. J. Microbiol. Meth. 2017, 142, 71. [CrossRef] [PubMed]
16. Scharlaken, B.; de Graaf, D.C.; Goossens, K.; Brunain, M.; Peelman, L.J.; Jacobs, F.J. Reference gene selection for insect expression studies using quantitative real-time PCR: The head of the honeybee, Apis mellifera, after a bacterial challenge. J. Insect Sci. 2008, 8, 33. [CrossRef]
17. de Boer, M.E.; de Boer, T.E.; Mariën, J.; Timmermans, M.J.T.N.; Nota, B.; van Straalen, N.M.; Ellers, J.; Roelofs, D. Reference genes for qRT-PCR tested under various stress conditions in Folsomia candida and Orchesella cirrata (Insecta, Collembola). BMC Mol. Biol. 2009, 10, 54. [CrossRef] [PubMed]
18. Jiang, H.B.; Liu, Y.H.; Tang, P.A.; Zhou, A.W.; Wang, J.J. Validation of endogenous reference genes for insecticide-induced and developmental expression profiling of Liposcelis bostrychophila (Psocoptera: Liposcelididae). Mol. Biol. Rep. 2010, 37, 1019–1029. [CrossRef] [PubMed]
19. Arun, A.; Baumlé, V.; Amelot, G.; Nieberding, C.M. Selection and validation of reference genes for qRT-PCR expression analysis of candidate genes involved in olfactory communication in the butterfly Bicyclus anynana. PLoS ONE 2015, 3, e0120401. [CrossRef]
20. Koramulla, M.K.; Amined, R.; Bhattacharya, R. Comprehensive evaluation of candidate reference genes for qRT-PCR studies of gene expression in mustard aphid, Lipaphis erysimi (Kalt.). Sci. Rep. 2016, 6, 25883. [CrossRef]
21. Ponton, E.; Chapuis, M.P.; Fernice, M.; Sword, G.A.; Simpson, S.J. Evaluation of potential reference genes for reverse transcription-qPCR studies of physiological responses in Drosophila melanogaster. J. Insect Physiol. 2011, 57, 840–850. [CrossRef] [PubMed]
22. Lu, Y.H.; Yuan, M.; Gao, X.W.; Kang, T.H.; Zhan, S.; Han, H.; Li, J.H. Identification and validation of reference genes for gene expression analysis using quantitative PCR in Spodoptera littura (Lepidoptera: Noctuidae). PLoS ONE 2013, 8, e68059. [CrossRef] [PubMed]
23. Yin, J.J.; Sun, L.L.; Zhang, Q.H.; Cao, C.W. Screening and evaluation of the stability of expression of reference genes in *Lymantria dispar* (Lepidoptera: Erebidae) using qRT-PCR. *Gene* 2020, 749, 144712. [CrossRef] [PubMed]

24. Fu, W.; Xie, W.; Zhang, Z.; Wang, S.; Wu, Q.; Liu, Y.; Zhou, X.; Zhou, X.G.; Zhang, Y. Exploring valid reference genes for quantitative real-time PCR analysis in *Plutella xylostella* (Lepidoptera: Plutellidae). *Int. J. Biol. Sci.* 2013, 9, 792–802. [CrossRef]

25. Zhang, L.L.; Jing, X.D.; Chen, W.; Bai, J.L.; Vasveer, L.; He, W.Y.; You, M.S. Selection of reference genes for expression analysis of plant-derived microRNAs in *Plutella xylostella* using qRT-PCR and ddPCR. *PLoS ONE* 2019, 14, e0220475. [CrossRef]

26. Shu, B.; Zhang, J.J.; Cui, G.F.; Sun, R.R.; Sutharman, V.; Yi, X.; Zhong, G.H. Evaluation of reference genes for real-time quantitative PCR analysis in larvae of *Spodoptera litura* exposed to azadirachtin stress conditions. *Front. Physiol.* 2018, 9, 372. [CrossRef]

27. Xu, H.X.; Wang, G.R.; Lu, Y.H.; Yang, Y.J.; Zheng, X.S.; Tian, J.C.; Lü, Z.X. Screening reference genes and evaluating of their expression stability for qRT-qPCR normalization in *Chilo suppressalis* (Lepidoptera: Pyralidae). *Chin. J. Rice Sci.* 2019, 33, 75–84.

28. Zhang, S.D.; An, S.H.; Li, Z.; Wu, F.M.; Yang, Q.P.; Liu, Y.C.; Cao, J.J.; Zhang, H.J.; Zhang, Q.W.; Liu, X.X. Identification and validation of reference genes for normalization of gene expression analysis using qRT-PCR in *Helicoverpa armigera* (Lepidoptera: Noctuidae). *Genes* 2015, 555, 393–402. [CrossRef] [PubMed]

29. Pan, H.P.; Yang, X.W.; Bidne, K.; Hellmich, R.L.; Siegfried, B.D.; Zhou, X.G. Selection of reference genes for RT-qPCR analysis in a biparental beetle, *Lethrus apterus* (Coleoptera: Nitidulidae). *Insect Sci.* 2016, 30, 197–215. [CrossRef]

30. Zhao, Z.J.; Wang, L.M.; Yue, D.M.; Ye, B.; Li, P.P.; Zhang, B.; Fan, Q. Evaluation of reference genes for normalization of RT-qPCR gene expression data for *Trichoplusia ni* cells during *Antheraea pernyi* (Lepidoptera: Saturniidae) multicapsid nucleo polyhedrovirus (AnpeNPV) infection. *J. Insect Sci.* 2019, 19, 1–9. [CrossRef]

31. Biondi, A.; Guedes, R.N.C.; Wan, F.H.; Desneux, N. Ecology, worldwide spread, and management of the invasive South American tomato pinworm, *Tuta absoluta*: Past, present, and future. *Annu. Rev. Entomol.* 2018, 63, 239–258. [CrossRef] [PubMed]

32. Sun, M.; Lu, M.X.; Tang, X.T.; Du, Y.Z. Exploring valid reference genes for quantitative real-time PCR in *Plutella xylostella*. *PLoS ONE* 2015, 10, e0115979. [CrossRef]

33. Pan, H.P.; Yang, X.W.; Bidne, K.; Hellmich, R.L.; Siegfried, B.D.; Zhou, X.G. Selection of reference genes for RT-qPCR analysis in the monarch butterfly, *Danaus plexippus* (L.), a migrating bioindicator. *Trichoplusia ni* cells during *Antheraea pernyi* (Lepidoptera: Saturniidae) multicapsid nucleo polyhedrovirus (AnpeNPV) infection. *J. Insect Sci.* 2019, 19, 1–9. [CrossRef]

34. Zhang, G.F.; Liu, W.X.; Wan, F.H.; Xian, X.Q.; Zhang, Y.B.; Guo, J.Y. Bioecology, damage and management of the tomato leafminer *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), a worldwide quarantine pest. *J. Biosaf.* 2018, 27, 155–163.

35. Zhang, G.F.; Ma, D.Y.; Wang, Y.S.; Gao, Y.H.; Liu, W.X.; Zhang, R.; Fu, W.J.; Xian, X.Q.; Wang, J.; Meng, K.; et al. First report of the South American tomato leafminer, *Tuta absoluta* (Meyrick), in China. *J. Integr. Agric.* 2020, 19, 1912–1917. [CrossRef]

36. Zhang, G.F.; Xian, X.Q.; Zhang, Y.B.; Zhang, R.; Ma, D.Y.; Liu, W.X.; Gao, Y.H.; Wang, J.; Yang, Z.L.; Li, Q.X.; et al. Warning of the dispersal of a newly invaded alien species, tomato leafminer *Tuta absoluta* (Meyrick), in China. *Plant Prot.* 2020, 46, 281–286.

37. Desneux, N.; Wajnberg, E.; Wyckhuys, K.A.G.; Burgiog, G.; Arpaia, S.; Narváez-Vasquez, C.A.; González-Cabrera, J.; Ruescas, D.C.; Tabone, E.; Frandon, J.; et al. Biological invasion of European tomato crops by *Tuta absoluta*: Ecology, geographic expansion and prospects for biological control. *J. Pest Sci.* 2010, 83, 197–215. [CrossRef]

38. Campos, M.R.; Biondi, A.; Adiga, A.; Guedes, R.N.C.; Desneux, N. From the Western Palaearctic region to beyond: *Tuta absoluta* ten years after invading Europe. *J. Pest Sci.* 2017, 90, 789–796. [CrossRef]

39. Guedes, R.N.C.; Roditakis, E.; Campos, M.R.; Haddi, K.; Bielza, P.; Siqueira, H.A.A.; Tsagkarakou, A.; Vontas, J.; Nauen, R. Insecticide resistance in the tomato pinworm *Tuta absoluta*: Patterns, spread, mechanisms, management and outlook. *J. Pest Sci.* 2019, 92, 1329–1342. [CrossRef]

40. Chen, H.; Yang, Z.Q.; Hu, Y.; Tan, J.H.; Jia, J.; Xu, H.L.; Chen, X.H. Reference genes selection for quantitative gene expression studies in *Pinus massoniana* L. *Peer J.* 2016, 4, e4047. [CrossRef] [PubMed]

41. Nagy, N.A.; Németh, Z.; Juhász, E.; Poliska, S.; Rácz, R.; Kosztolányi, A.; ZoltánBarta, Z. Evaluation of potential reference genes for real-time qPCR analysis in a biparental lettuce, *Lethrus apterus* (Coleoptera: Geotrupidae). *Peer J.* 2017, 5, e4047. [CrossRef] [PubMed]

42. Feng, B.; Liang, P.; Gao, X.W. Cloning and evaluation of U6 snRNA gene as a reference gene for miRNA quantification in *Plutella xylostella* (Lepidoptera: Plutellidae). *Acta Anim. Sin.* 2015, 14, 252–296.

43. Ma, L.; Li, X.W.; Wang, S.M.; Wang, T.Z.; Lü, Y.B. Toxicity of six insecticides to tomato leaf miner moth in Yunnan. In Proceedings of the 2019 Annual Conference of Plant Protection Society of China, Guiyang, China, 23 October 2019.

44. Zhang, G.F.; Zhang, Y.B.; Zhang, J.; Liu, W.X.; Wang, Y.S.; Wan, F.H.; Shu, C.L.; Liu, H.; Wang, F.L.; Zhao, L.; et al. Laboratory Toxicity and Field Control Efficacy of Biopesticide Bacillus thuringiensis G033A on the South American Tomato Leafminer *Tuta absoluta* (Meyrick), a New Invasive Alien Species in China. *Chin. J. Biol. Control.* 2020, 36, 175–183.

45. Cuthbertson, A.G.S.; Mathers, J.J.; Blackburn, L.F.; Korycinska, A.; Luo, W.Q.; Jacobson, R.J.; Northing, P. Population development of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) under simulated UK glasshouse conditions. *Insects* 2013, 4, 185–197. [CrossRef] [PubMed]

46. Martins, J.C.; Picanço, M.C.; Bacci, L.; Guedes, R.N.C.; Santana, P.A.; Ferreira, D.O.; Chediak, M. Life table determination of thermal requirements of the tomato borer *Tuta absoluta*. *J. Pest Sci.* 2016, 89, 897–908. [CrossRef]

47. Weid, D.; Rossier, D.; Lindup, M.; Tuberosa, J.; Widmer, A.; Col, J.D.; Kan, C.; Carleton, A.; Rodriguez, I. Large-scale transcriptional profiling of chemosensory neurons identifies receptor-ligand pairs in vivo. *Nat. Neurosci.* 2015, 18, 1455. [CrossRef] [PubMed]
48. Liu, X.Q. Identification of Odorant Receptors Responding to Myrcene and Ethyl Butyrate in *Diaphorina citri*. Master’s Thesis, Southwest University, Chongqing, China, 2018.

49. Pfaffl, M.W. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **2001**, *29*, 45. [CrossRef] [PubMed]

50. Andersen, C.L.; Jensen, J.L.; Orntoft, T.F. Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res.* **2004**, *64*, 5245–5250. [CrossRef] [PubMed]

51. Pfaffl, M.W.; Tichopad, A.; Prgomet, C.; Neuviens, T.P. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper-Excel-based tool using pair-wise correlations. *Biotechnol. Lett.* **2004**, *26*, 509–515. [CrossRef] [PubMed]

52. Andersen, C.L.; Jensen, J.L.; Orntoft, T.F. Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res.* **2004**, *64*, 5245–5250. [CrossRef] [PubMed]

53. Provenzano, M.; Mocellin, S. Complementary techniques: Validation of gene expression data by quantitative real time PCR. *Adv. Exp. Med. Biol.* **2007**, *593*, 593–66. [CrossRef] [PubMed]

54. Ladror, D.T.; Frey, B.L.; Scalf, M.; Levenstein, M.E.; Artymiuk, J.M.; Smith, L.M. Methylation of yeast ribosomal protein S2 is elevated during stationary phase growth conditions. *Biochem. Biophys. Res. Commun.* **2014**, *445*, 535–541. [CrossRef] [PubMed]

55. Zhou, X.; Liao, W.J.; Liao, J.M.; Liao, P.; Lu, H. Ribosomal proteins: Functions beyond the ribosome. *J. Mol. Cell Biol.* **2015**, *7*, 92–104. [CrossRef]

56. Jank, T.; Belyi, Y.; Wirth, C.; Rospert, S.; Hu, Z.; Dengjel, J.; Tzivelekidis, T.; Andersen, G.R.; Hunte, C.; Schlosser, A.; et al. Protein glutaminylation is a yeast-specific posttranslational modification of elongation factor 1A. *J. Biol. Chem.* **2017**, *292*, 16014–16023. [CrossRef] [PubMed]

57. Sun, W.; Jin, Y.; He, L.; Lu, W.C.; Li, M. Suitable reference gene selection for the different strains and developmental stages of the carmine spider mite, *Tetranychus cinnabarinus*, using quantitative real-time PCR. *J. Insect Sci.* **2010**, *10*, 208. [CrossRef] [PubMed]

58. Guo, C.N. Cloning and Stability Analysis of Reference Gene from *Phyllonorycter ringoniell*. Master’s Thesis, Northwest A&F University, Xianyang, China, 2014.

59. Li, H.B.; Dai, C.G.; Zhang, C.R.; He, Y.F.; Ran, H.Y.; Chen, S.H. Screening potential reference genes for quantitative real-time PCR analysis in the oriental armyworm, *Mythimna separata*. *PLoS ONE* **2018**, *13*, e0195096. [CrossRef] [PubMed]