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Defining Suitable Reference Genes for qRT-PCR in *Plagiodera versicolora* (Coleoptera: Chrysomelidae) under Different Biotic or Abiotic Conditions

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Abstract: *Plagiodera versicolora* (Coleoptera: Chrysomelidae) is one of the most destructive pests of the Salicaceae worldwide, which has established complex interactions with surrounding organisms. Uncovering the molecular mechanisms of some antagonistic interactions would facilitate the development of environmentally friendly pest insect management strategies. Suitable reference genes are essential for reliable qPCR and gene expression analysis in molecular studies; however, a comprehensive assessment of reference genes in *P. versicolora* is still lacking. In this study, the stability of seven housekeeping genes (including *Actin*, *EF1A*, *α-tubulin*, *RPL13a*, *RPS18*, *RPL8* and *UBC*) was investigated under both biotic (developmental stages, tissues, sex and pathogen treatment) and abiotic (RNA interference treatment, temperature treatment) conditions. The geNorm, NormFinder, BestKeeper, and ΔCt programs were used to analyze gene expression data. The RefFinder synthesis analysis was applied to suggest a handful of appropriate reference genes for each experimental condition. *RPS18* and *EF1A* were the most reliable reference genes in different development stages; *RPS18* and *RPL8* were most stable in female and male adults, different tissues, different temperatures, and pathogen treatment; *α-tubulin* and *RPL13a* were most stable after dietary RNAi treatment. The research provides a strong basis for future research into the molecular biology of *P. versicolora*.

Keywords: *Plagiodera versicolora*; qRT-PCR; reference gene; normalization

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1. Introduction

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) is a commonly used method for gene expression quantification owing to its high sensitivity, accuracy, specificity, and rapidity [1–3]. This technique has been extensively used in numerous areas, such as clinical diagnosis [4], the detection of pathogens in a plant [5], the evaluation of RNAi efficiency [6], and the quantification of microbial load in an animal [7,8]. Nevertheless, a series of factors including reference gene selection, RNA quantity or quality, the initial sample size, reverse transcription, PCR efficiency, and primer design can affect the gene expression data produced by qRT-PCR [9–12], among which the reference gene's selection is one of the most prominent and needs systemic evaluation [13,14]. In theory, ideal reference genes must be stably expressed, not influenced by any endogenous or exogenous factors. Basic metabolism genes are generally involved in processes essential for cell survival, and stably expressed at a non-regulated constant level; thus, these housekeeping genes are frequently chosen as reference genes [15]. For example, Luo et al. (15) used the housekeeping gene *ribosomal protein S15* as a reference gene to quantification of microbial load in *Adelphocoris suturalis* (Hemiptera: Miridae). Tang et al. [15] used *β-actin* as a reference gene to quantify odorant receptor protein genes expression in *Sitophilus zeamais* (Coleoptera: Curculionidae) tissues. *Ribosomal protein S3* was adopted to normalize the

expression of *HSP* and *P450* genes in *Tribolium castaneum* (Coleoptera: Tenebrionidae) under UV-A exposure [16].

However, a series of investigations have discovered that the expression of housekeeping genes varies among different insect species and experimental treatments. *RPS15* was shown to be stably expressed in *Spodoptera frugiperda* (Lepidoptera: Noctuidae) after UV-A irradiation, which is not the case when it was treated at 36 °C and after pesticide treatment [17]. *β-actin* was unstably expressed in *Rhopalosiphum padi* (Homoptera: Aphididae) adult samples from different geographic populations, though it had been found stably expressed among different developmental stages [18]. A similar result was obtained for the housekeeping gene *RPS3* in *Lymantria dispar* (Lepidoptera: Erebidae) [19]. Additionally, the expression stability of a reference gene in the same order insects varied [20–23]. Since incorrect reference gene(s) could have a significant impact on quantification results and further lead to misinterpretations [24], a valid and reliable determination of reference genes is a prerequisite before performing qRT-PCR tests.

The leaf beetle *Plagiodera versicolora* (Coleoptera: Chrysomelidae), which mainly feeds on the leaves of willow and poplar, is one of the most notorious herbivorous pest insects of Salicaceae plants [25]. Although chemical pesticides can effectively kill the beetle, a long-term application of pesticides would inevitably lead to increasing resistance and cause negative effects on human and environmental health [26]. In recent years, new strategies have been proposed for the pest's control, e.g., microbial pest control strategy, RNAi or transgenic plant-based techniques [26–29]. Additionally, we found several entomopathogens in the beetle's surroundings (including *Aspergillus nomiae* used in this study), which holds great potential for development as an agent for microbial-based pest management (unpublished data). The development of an effective pest control strategy, along with other scientific goals [30,31], will expand and deepen molecular studies of *P. versicolora*, making gene expression analysis an increasingly deployed technique. Consequently, appropriate reference genes will inevitably be required for accurate interpretation of gene expression in molecular studies of *P. versicolora*, which has yet to be thoroughly examined.

Here, seven commonly used reference genes in other insects were selected, including Actin, Elongation factor 1-α (EF1A), α-tubulin, ribosomal protein L13a (RPL13a), ribosomal protein S18 (RPS18), ribosomal protein L8 (RPL8), and ubiquitin-conjugating enzyme E2 (UBC), as candidate reference genes for *P. versicolora*. The ΔCt method [32], geNorm [33], NormFinder [34], and BestKeeper [35] were used to assess the accuracy and stability of the seven genes under different developmental stages, sexes, tissues, different temperature treatments, pathogenic treatments, and RNAi treatments. We also used online software (RefFinder) to further assay the suitability of reference genes. Finally, the expression patterns of two genes in *P. versicolora* (heat shock cognate protein 70 (HSP70) and odorant blinding protein (OBP7)) were profiled to verify the stability of reference genes.

2. Materials and Methods

2.1. Insect Rearing

P. versicolora adults and larvae were captured from Sha Lake Park in Hubei Province (Wuhan, China). The insects were fed with fresh detached willow leaves, which were collected from Sha Lake Park and reared at 26 ± 1 °C, with 70% ± 5% relative humidity and a 16 h light/8 h dark photoperiod.

2.2. Experimental Treatments

The effects of development stages, sexes, tissues, temperature, pathogen treatment and dsRNA treatment on reference gene expression were measured.

2.3. Development Stage and Sex

The different development stages and sexes of *P. versicolora* included eggs, larvae of different instars, pupae, and male and female adults. Specifically, 45 eggs, 30 first instar

larvae, 15 s instar larvae, 12 third instar larvae, 12 pupae, 12 male adults, and 12 female adults were collected. All samples were randomly chosen and equally distributed in three biological replicates. Each sample was then frozen in liquid nitrogen immediately and kept at −80 °C until further use.

2.3.1. Tissue

Three body regions, including head, thorax and abdomen, were dissected from adults of *P. versicolora*. Each tissue sample was collected from a minimum of 15 insects (n = 3). All the samples were stored at −80 °C after freezing in liquid nitrogen.

2.3.2. Thermal Exposure

After 4 h incubation at 4 °C, 26 °C or 36 °C, 10 first instar larvae of *P. versicolora* were collected and pooled as one sample for RNA extraction (n = 3), respectively.

2.3.3. Pathogen Treatment

The pathogenic fungus *Aspergillus nomiae*, which was isolated from the carcass of *P. versicolora* [36,37], was chosen. The fungus was maintained at 25 °C on Potato dextrose agar (PDA). Conidia were obtained from 1-week-old sporulating cultures. Conidia suspension (0.05% Tween 80 solution at a final concentration of 1×10^7 conidia/mL) was sprayed on first instar larvae, with sterile 0.05% Tween 80 solution used as a control. Fungal infected larvae were collected at 12 h (the time when larvae begin to die) and 24 h (semi-lethal time) after the infection.

2.3.4. dsRNA Treatment

For RNAi treatment, first instar larvae of *P. versicolora* were fed daily with 8 ng/cm^2 of dsRNA soluble N-ethylmaleimide-sensitive fusion attachment protein (SNAP) [28] coated willow leaves. The larvae fed with dsGFP (dsRNA of green fluorescent protein gene) were set as a control. The dsRNA was synthetized in vitro using the T7 RiboMAX™ Express RNAi System (Promega, Madison, WI, USA). After four days' feeding, three individuals from each treatment were collected (n = 3).

2.4. RNA Extraction and cDNA Preparation

Total RNA was extracted from the above samples using RNAiso Plus reagent (TaKaRa, Maebashi, Japan) by following the manufacturer's instructions. The RNA integrity was further assessed by electrophoresis in a 1.5% agarose gel and quantified on a Nano-Drop 2000 (Thermo Scientific, Waltham, MA, USA). cDNA was synthesized from 1 µg total RNA using the Hifair®II 1st Strand cDNA Synthesis SuperMix (Yeasen, Wuhan, China) according to the manufacturer's instructions, which was then stored at –20 °C until further use. The cDNA from each sample was diluted 20 times using nuclease-free water for qPCR.

2.5. Candidate Reference Genes and Primer Design

Using *P. versicolora* transcriptome data [30], sequences matching the seven potential reference genes were identified (*Actin*, *EF1A*, *α-tubulin*, *RPL13a*, *RPS18*, *RPL8* and *UBC*). The genes were PCR-amplified from *P. versicolora* cDNA using the corresponding primers. The obtained sequences were then sub-cloned using the *pEASY*®-T1 Simple Cloning Kit (TransGen Biotech, Beijing, China) and confirmed by Sanger sequencing. The valid gene sequences were deposited in GenBank with accession numbers (see Table 1). After that, an online tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/ (accessed on 25 September 2021)) was used for designing the primers of the genes for the subsequent qRT-PCR analyses. Finally, the primer specificity and the efficiency of PCR amplification were assessed using standard curves, melt curve analyses, and electrophoresis in a 2% agarose gel.

Table 1. Oligonucleotide primers for candidate qRT-PCR reference genes in *Plagiodera versicolora* [(1)].

Gene	Accession Number	Primer Sequence (5′→3′)	Product Length (bp)	R^2	E
Actin	OM885970	F:CGTGACTTGACCGACTACCT R:CGAGAGCGACATAGCAGAGT	118	0.999	103.3%
EF1α	OM885971	F:TGACTCCAAGGGTGAAGGCG R:TCATCGATGCTCCCGGACAC	171	0.998	100.1%
α-tubulin	OM885972	F:TGGTGTCCCACCGGTTTCAA R:TTGTGATCCAGACGTGCCCA	146	0.999	101.6%
RPL13a	OM885973	F:AAGTGGAATGGTCCTCGGGC R:CGTCTTGCGGCAATCGTAGC	167	0.999	99.7%
UBC	OM885974	F:TGGCTACGTTCTCGTGGGTG R:ACTTTTGGCGCTGCGAACTG	150	0.998	105%
RPL18S	OM885975	F:CTTCCTCGTCGGAGCATTCT R:GTTCGCCTTAACTGCCATCAA	110	0.999	102.2%
RPL8	OM885976	F:CGACCACCACCAGCTACGAT R:ACCGTGGTCGATTGGCTAGG	157	0.997	96.6%

This "(1)" is an explanation of the abbreviated portion of the table. E, qRT-PCR efficiency; R2, regression coefficient of the qPCR reaction; F, forward primers; R, reverse primers.

2.6. qRT-PCR Assay

Each amplification reaction (10 μL) contained 5 μL MonAmp™ SYBR® Green qPCR Mix (Monad Biotech, Suzhou, China), 2 μL cDNA, 0.4 μL of each primer (10 ng/μL), and 2.2 μL ddH_2O. The PCR program was as follows: 95 °C for 3 min, 40 cycles of 95 °C for 10 s, 60 °C for 30 s. In each independent sample ($n = 3$), the detection of each gene was performed with three technical replicates. All qPCRs were conducted using the CFX Connect Real-Time System (Bio-Rad, Hercules, CA, USA). Continuous fluorescence measurements were taken when the temperature was ramped up from 55 to 95 °C in 0.5 °C increments every 6 s for melt curve analysis. A standard curve was generated using a serial 5-fold of cDNA template for each gene. Additionally, the gene specific PCR efficiency (*E*) was calculated using the following formula: $E\ (\%) = (10^{(-1/\text{slope})} - 1) \times 100$ [38].

2.7. Stability Analysis of Candidate Reference Genes

Each of the six experimental groups' data was examined separately. The average cycle threshold (Ct) values were calculated using three biological replicates. The stability of a candidate reference gene was evaluated by the ΔCt method (Silver et al., 2006), geNorm [33], NormFinder [34], and BestKeeper [35]. To assay the suitability of reference genes, we also applied an online software RefFinder to analyze the results of the four algorithms [39].

2.8. Validation of Reference Genes

The *P. versicolora heat shock cognate protein 70* (*HSP70*) gene and *odorant binding protein 7* (*OBP7*) [31] gene were selected to validate the stability of reference genes in different tissues. *HSP70* is a component of folding and signal transduction pathways that have housekeeping roles in cells and is usually expressed under normal settings [40,41]. *OBPs* are small soluble proteins released in the sensillar lymph of insect chemosensory sensillae [42,43], many of which serve as important components in insects' chemosensory systems and are highly expressed in the antenna, leg, wing, head, and thorax of insects [44]. We used the best reference gene pair *RPL8/RPS18* (ranked by geNorm), the single best reference gene *RPS18* (identified by RefFinder), and the least stable reference gene *UBC* (evaluated by all five algorithms) to normalize the relative expression level of *HSP70* and *OBP7*, respectively. The qRT-PCR reactions were carried out as described above, and qRT-PCR data were analyzed via the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak 2008). One-way analysis of variance (ANOVA) followed by Tukey's HSD test were used to test gene expression.

3. Results

3.1. Evaluation of Primer Specificity and Amplification Efficiency

All amplicons have 99–100% homology with the corresponding sequences obtained from the transcriptome. The specificity of gene amplification of all candidate reference genes was confirmed as only one single band with expected length using 2% agarose (Figure 1A). Melting curve analysis showed a single peak for each primer pair, indicating the high specificity of the primers (Figure 1B). The PCR efficiency (*E*) and correlation coefficient (R^2) of the standard curve are calculated (Table 1). The PCR efficiencies of primers ranged from 96.6–105% with high R^2 values (0.997–0.999).

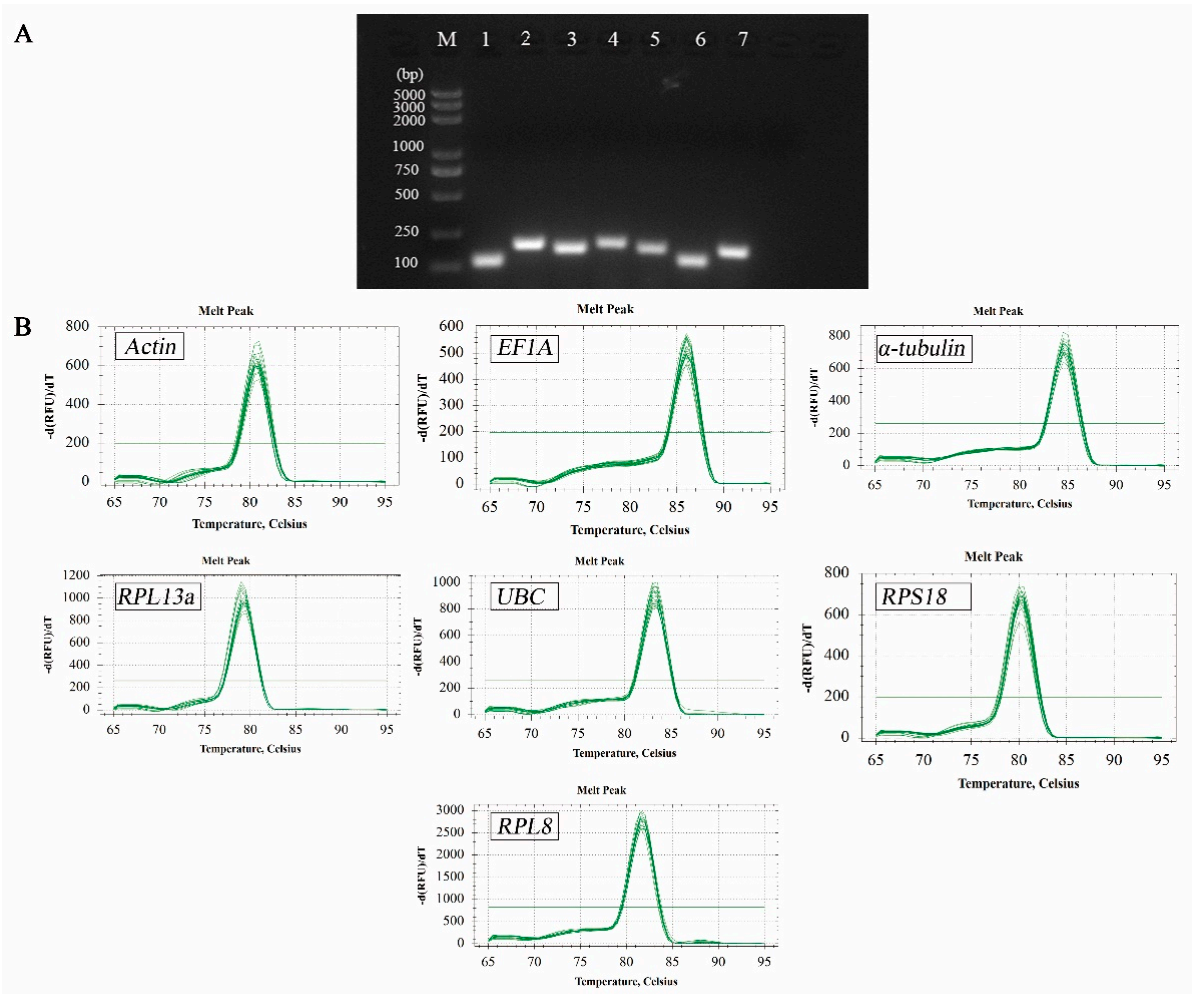

Figure 1. Amplification specificity of primers. (**A**) Single amplicon with an expected length for each gene was visualized in a 2% agarose gel. 1, *Actin*, 2, *EF1A*, 3, *α-tubulin*, 4, *RPL13a*, 5, *UBC*, 6, *RPS18*, 7, *RPL8*; M, marker. (**B**) Melt curve analysis identifies a single peak for each gene.

3.2. Expression Patterns of Candidate Reference Genes

The expression patterns of the candidate reference genes were investigated to offer an overall representation of primer variability under various experimental settings (Figure 2). Under the six experimental conditions, the mean Ct values of the seven potential reference genes ranged from 18.12 to 24.96 cycles. Analysis of the overall sample data showed that *ACT* had the highest expression level (lowest mean Ct value), followed by *RPL8*, *EF1A*, *α-tubulin*, *RPS18*, *RPL13a*, and *UBC*. Additionally, the extent of expression changes of certain reference genes varied with experimental settings. For example, *UBC* varied more (~5 cycle) between samples across tissue types than before and after RNAi treatment (~2 cycles) (Figure 2C,E).

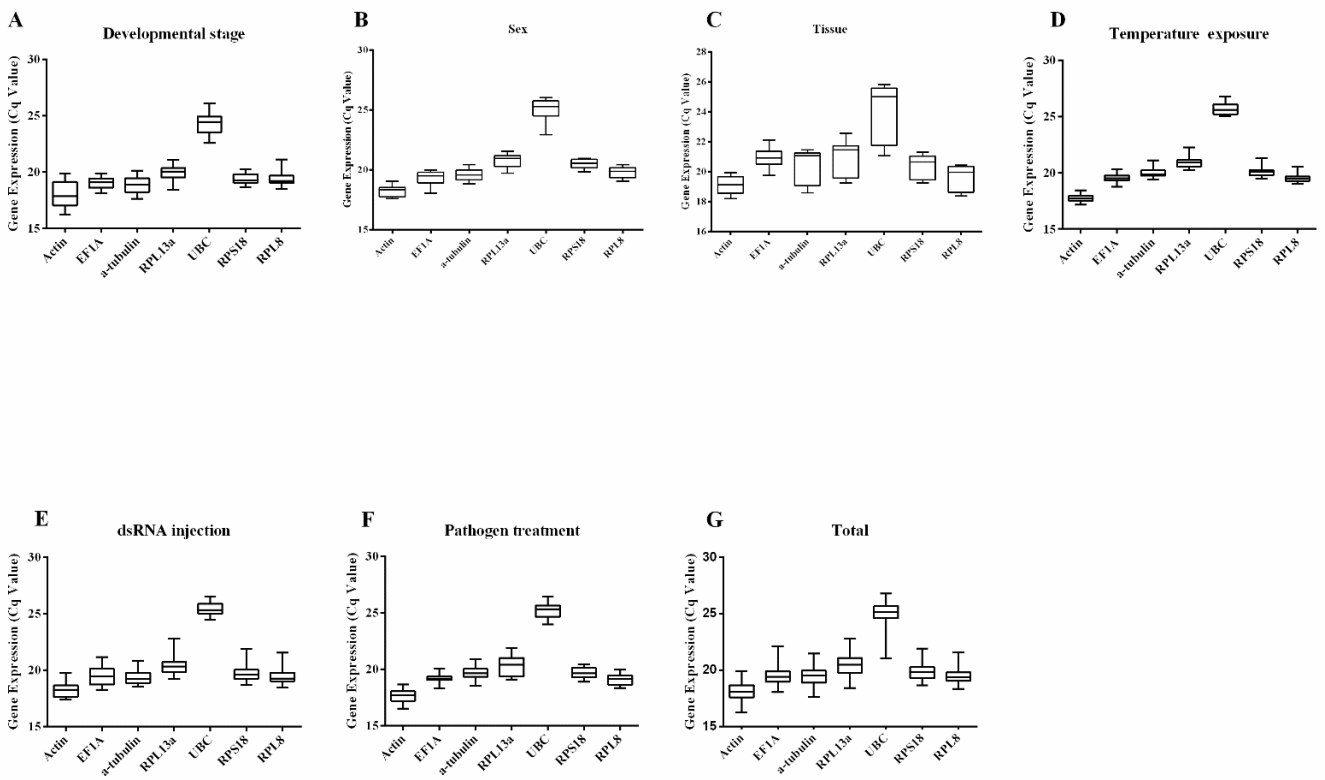

Figure 2. Candidate reference genes expression profiles in *P. versicolora*. (**A**), different developmental stages. (**B**), sexes. (**C**), different tissues. (**D**), temperature exposure. (**E**), dsRNA treatment. (**F**), pathogen treatment. (**G**), total samples. The expression levels of candidate reference genes are shown as Ct values. The line in the box represents the median. The upper and lower edges of the interquartile range indicate the 75th and 25th percentiles, respectively. The minimum and maximum values are shown by the whisker caps.

3.3. Stability of Candidate Reference Genes

The expression stabilities of the seven candidate genes in the distinct experimental settings were analyzed using the ΔCt method, BestKeeper, NormFinder, and geNorm to select the most stable reference gene(s). RefFinder was used to determine the overall stability ranking.

Developmental stages: For different developmental stages, the ΔCt method and NormFinder indicated that *RPL13a*, *RPS18* and *EF1A* were the most stable genes, while *UBC* and *Actin* presented the greatest variation (Table 2). *RPS18* was the most stable reference gene based on BestKeeper. The *RPL13a/EF1A* pair had the lowest M value (0.368) in GeNorm, indicating that they are the most stable transcripts. From most stable to least stable, RefFinder ranked the genes as follows: *RPS18*, *EF1A*, *RPL13a*, *α-tubulin*, *RPL8*, *UBC* and *Actin* (Figure 3A).

Sexes: *RPS18* was identified as the least stable reference gene when calculated by all four algorithms (Table 2). Based on geNorm data, the pair-wise value of V2/3 was 0.128, and *RPL8/RPS18* were considered the most stable reference genes across sexes (Table 2). The sex-based ranking of reference gene stability, according to RefFinder (from most to least stable) was *RPS18*, *RPL8*, *RPL13a*, *α-tubulin*, *EF1A*, *Actin* and *UBC* (Figure 3B).

Table 2. Rank order of the candidate *Plagiodera versicolora* reference genes under different experimental conditions.

	Rank	GeNorm		NormFinder		BestKeeper		ΔCt		RefFinder	
		Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability
Developmental stage	1	*RPL13a* *RPS18*	0.368 0.368	*RPS18*	0.065	*RPS18*	0.370	*RPS18*	0.714	*RPS18*	1.000
	2	-	-	*EF1A*	0.131	*EF1A*	0.380	*EF1A*	0.718	*EF1A*	2.213
	3	*EF1A*	0.407	*RPL13a*	0.258	*RPL8*	0.415	*RPL13a*	0.785	*RPL13a*	2.449
	4	*α-tubulin*	0.542	*α-tubulin*	0.403	*RPL13a*	0.498	*α-tubulin*	0.878	*α-tubulin*	4.229
	5	*RPL8*	0.673	*RPL8*	0.511	*α-tubulin*	0.616	*RPL8*	0.977	*RPL8*	4.401
	6	*UBC*	0.764	*UBC*	0.647	*UBC*	0.748	*UBC*	1.092	*UBC*	6.000
	7	*Actin*	0.930	*Actin*	0.872	*Actin*	0.954	*Actin*	1.343	*Actin*	7.000
Sex	1	*RPS18* *RPL8*	0.277 0.277	*RPS18*	0.096	*RPS18*	0.287	*RPS18*	0.508	*RPS18*	1.000
	2	-	-	*RPL8*	0.125	*RPL8*	0.341	*RPL13a*	0.514	*RPL8*	1.861
	3	*RPL13a*	0.365	*RPL13a*	0.148	*Actin*	0.364	*RPL8*	0.525	*RPL13a*	3.080
	4	*EF1A*	0.381	*α-tubulin*	0.243	*α-tubulin*	0.407	*EF1A*	0.570	*α-tubulin*	4.472
	5	*α-tubulin*	0.398	*EF1A*	0.243	*RPL13a*	0.469	*α-tubulin*	0.580	*EF1A*	4.681
	6	*UBC*	0.530	*UBC*	0.553	*EF1A*	0.527	*UBC*	0.880	*Actin*	5.664
	7	*Actin*	0.644	*Actin*	0.600	*UBC*	0.836	*Actin*	0.931	*UBC*	6.236
Thermal exposure	1	*RPL13a* *RPS18*	0.255 0.255	*RPS18*	0.114	*Actin*	0.218	*RPS18*	0.294	*RPS18*	1.414
	2	-	-	*RPL8*	0.146	*RPL8*	0.247	*RPL8*	0.318	*RPL8*	2.515
	3	*UBC*	0.286	*RPL13a*	0.153	*EF1A*	0.267	*RPL13a*	0.321	*RPL13a*	2.711
	4	*α-tubulin*	0.296	*EF1A*	0.164	*18S*	0.278	*EF1A*	0.332	*EF1A*	4.120
	5	*RPL8*	0.303	*α-tubulin*	0.167	*α-tubulin*	0.321	*α-tubulin*	0.334	*Actin*	4.304
	6	*EF1A*	0.315	*UBC*	0.180	*RPL13a*	0.401	*UBC*	0.343	*α-tubulin*	4.729
	7	*Actin*	0.329	*Actin*	0.203	*UBC*	0.422	*Actin*	0.364	*UBC*	5.244
dsRNA treatment	1	*RPL13a* *α-tubulin*	0.454 0.454	*α-tubulin*	0.146	*α-tubulin*	0.457	*α-tubulin*	0.510	*α-tubulin*	1.000
	2	-	-	*RPL8*	0.269	*UBC*	0.464	*RPL13a*	0.580	*RPL13a*	2.449
	3	*RPL8*	0.470	*RPL13a*	0.275	*Actin*	0.477	*RPL8*	0.586	*RPL8*	3.080
	4	*EF1A*	0.497	*Actin*	0.299	*RPS18*	0.501	*Actin*	0.610	*Actin*	3.936
	5	*Actin*	0.524	*EF1A*	0.352	*RPL8*	0.557	*EF1A*	0.642	*UBC*	5.118
	6	*RPS18*	0.584	*RPS18*	0.360	*RPL13a*	0.603	*RPS18*	0.655	*EF1A*	5.144
	7	*UBC*	0.607	*UBC*	0.372	*EF1A*	0.661	*UBC*	0.665	*RPS18*	5.422
Pathogen treatment	1	*RPS18* *RPL8*	0.234 0.234	*RPS18*	0.116	*EF1A*	0.232	*RPS18*	0.384	*RPS18*	1.316
	2	-	-	*UBC*	0.153	*α-tubulin*	0.410	*RPL8*	0.412	*RPL8*	2.213
	3	*UBC*	0.284	*RPL8*	0.161	*RPS18*	0.420	*UBC*	0.412	*UBC*	3.224
	4	*Actin*	0.338	*Actin*	0.207	*RPL8*	0.446	*Actin*	0.453	*EF1A*	3.344
	5	*EF1A*	0.376	*EF1A*	0.265	*Actin*	0.484	*EF1A*	0.496	*Actin*	4.229
	6	*α-tubulin*	0.431	*α-tubulin*	0.326	*UBC*	0.536	*α-tubulin*	0.563	*α-tubulin*	4.559
	7	*RPL13a*	0.469	*RPL13a*	0.335	*RPL13a*	0.752	*RPL13a*	0.566	*RPL13a*	7.000
Tissue	1	*RPL8* *RPS18*	0.099 0.099	*RPS18*	0.034	*Actin*	0.465	*RPL8*	0.609	*RPL8*	1.189
	2	-	-	*RPL8*	0.034	*EF1A*	0.554	*RPS18*	0.622	*RPS18*	1.565
	3	*RPL13a*	0.254	*RPL13a*	0.140	*18S*	0.676	*RPL13a*	0.687	*RPL13a*	3.409
	4	*α-tubulin*	0.330	*α-tubulin*	0.188	*RPL8*	0.720	*α-tubulin*	0.714	*EF1A*	3.976
	5	*EF1A*	0.441	*EF1A*	0.297	*RPL13a*	0.998	*EF1A*	0.816	*Actin*	4.304
	6	*UBC*	0.646	*UBC*	0.842	*α-tubulin*	1.001	*UBC*	1.262	*α-tubulin*	4.427
	7	*Actin*	0.885	*Actin*	1.002	*UBC*	1.696	*Actin*	1.482	*UBC*	6.236

Tissues: In our analysis of multiple tissue types, ΔCt, GeNorm, and NormFinder all suggested *RPS18* and *RPL8* as the most appropriate reference genes. BestKeeper, on the other hand, deemed *Actin* and *EF1A* to be the most stable genes (Table 2). For different tissues, the overall RefFinder stability ranking was: *RPL8*, *RPS18*, *RPL13a*, *EF1A*, *Actin*, *α-tubulin*, and *UBC* (in order of most to least stable) (Figure 3C).

Temperature exposure: For different temperatures, *RPS18*, *RPL13a*, and *RPL8*, were the most stable reference genes (analyzed by Normfinder and the ΔCt method); *RPL13a*, and *RPS18* were the most stable (suggested by geNorm); and *Actin* and *RPL8* were the most stable (determined by BestKeeper) (Table 2). The ranking of reference genes based on RefFinder across photoperiod treatments was: *RPS18*, *RPL8*, *RPL13a*, *EF1A*, *Actin*, *α-tubulin*, and *UBC* (in order of most to least stable) (Figure 3D). The geNorm analysis found a value of less than 0.15 for V2/3 (Figure 4). Consequently, we suggested *RPS18* and *RPL8* as the most stable reference genes at various temperatures (Table 2).

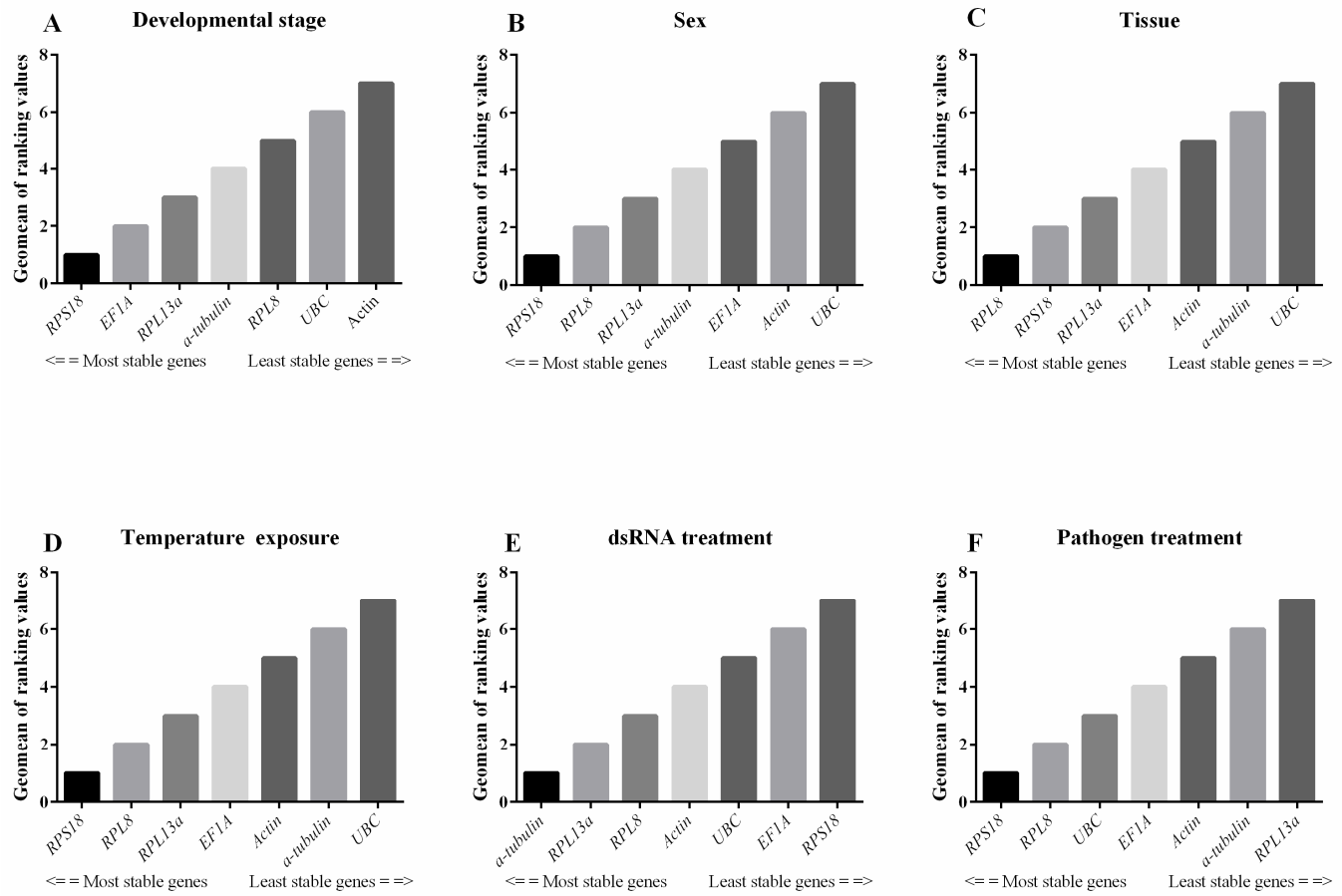

Figure 3. Stability of candidate reference genes in *P. versicolora* under various experimental conditions. The expression stability and relative ranking of candidate reference genes were determined by RefFinder. (**A**) Different developmental stages; (**B**) sexes; (**C**) different tissues; (**D**) temperature exposure; (**E**) dsRNA treatment; (**F**) pathogen treatment.

dsRNA treatment: In an experiment to assess the effect of RNAi on reference gene stability, *α-tubulin* was identified as one of the most stable genes by all four analyses (Table 2). Furthermore, *RPL13a* (ΔCt method and GeNorm), *RPL8* (NormFinder), and *UBC* (BestKepper) were also identified as having a similar stability value to that of *α-tubulin* (Table 2). For the dsRNA treatment study, the RefFinder ranking was: *α-tubulin*, *RPL13a*, *RPL8*, *Actin*, *UBC*, *EF1A*, and *RPS18* (Figure 3E).

Pathogen treatment: In this set of experiments, *RPS18* was ranked first according to NormFinder and ΔCt, whereas *EF1A* was the best gene in BestKeeper. GeNorm identified that *RPS18* and *RPL8* were the most appropriate reference genes (Table 2). The most unstable reference gene calculated by the four different algorithms was *RPL13a*. According to RefFinder analysis, the ranking order was *RPS18*, *RPL8*, *UBC*, *EF1A*, *Actin*, *α-tubulin*, and *RPL13a*. We chose *RPS18* and *RPL8* as the most credible reference genes by combining the findings of pairwise values by GeNorm (Figures 3F and 4).

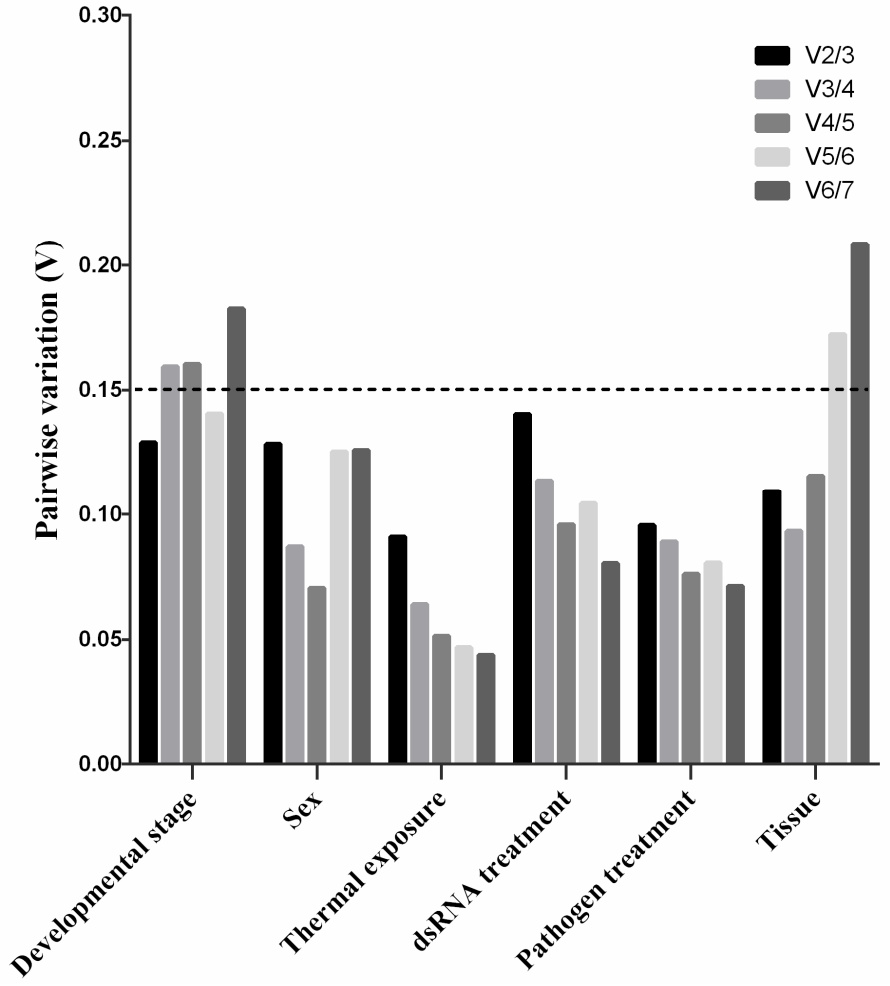

Figure 4. Determination of optimal number of reference genes for different *P. versicolora* samples. Pairwise variation (V) value below 0.15 suggests that an additional reference gene will not improve normalization.

3.4. The Optimal Number of Reference Genes for Normalization in P. versicolora

The conventional use of a single gene for data of qRT-PCR normalization leads to relatively large errors, and the application of more than one reference gene can strengthen the analysis [33]. Therefore, geNorm was applied to calculate the pairwise variation (Vn/Vn+1) to further determine the optimal number of reference genes. Generally, a number of *n* reference genes is sufficient to normalize the target gene once the value of (Vn/Vn+1) is below 0.15 [24]. The $V_{2/3}$ value was first lower than 0.15 in all pairwise variants in development stages, sexes, tissues, temperature, pathogen treatment and dsRNA treatment (Figure 4), indicating that the optimal number of reference genes for normalization was two for each experimental set.

3.5. Validation of Reference Genes in P. versicolora

The relative expression of *P. versicolora HSP70* and *OBP7* in diverse tissues was examined to validate the reference genes. Here, reference genes *RPL8/RPS18* (determined by geNorm), *RPS8* (suggested by RefFinder), and *UBC* (determined by all algorithms) were chosen and used to normalize the expression levels of the two above genes.

The normalization of transcripts using *RPL8/RPS18* and *RPL8* alone revealed there were no differences in expression of *HSP70* in the three groups. In contrast, normalization with *UBC* suggested there was a significant difference in *HSP70* gene expression between the groups, with the highest expression in the thorax (Figure 5A). This indicates that using

the inappropriate reference genes may lead to incorrect conclusions that are completely different from the facts. When the most stable reference gene, *RPL8*, was used, the relative expression of *OBP7* in the head and thorax was significantly higher than that in the abdomen (Figure 5B). Similar results were obtained using *RPS18* and *RPL8*. Notably, normalization with an unsuitable reference gene such as *UBC* leaded to raised expression levels though the trend of gene expression was similar (Figure 5B). As a result, our findings emphasize the need for choosing and confirming accurate RT-qPCR reference genes in order to avoid misinterpretation of expression data.

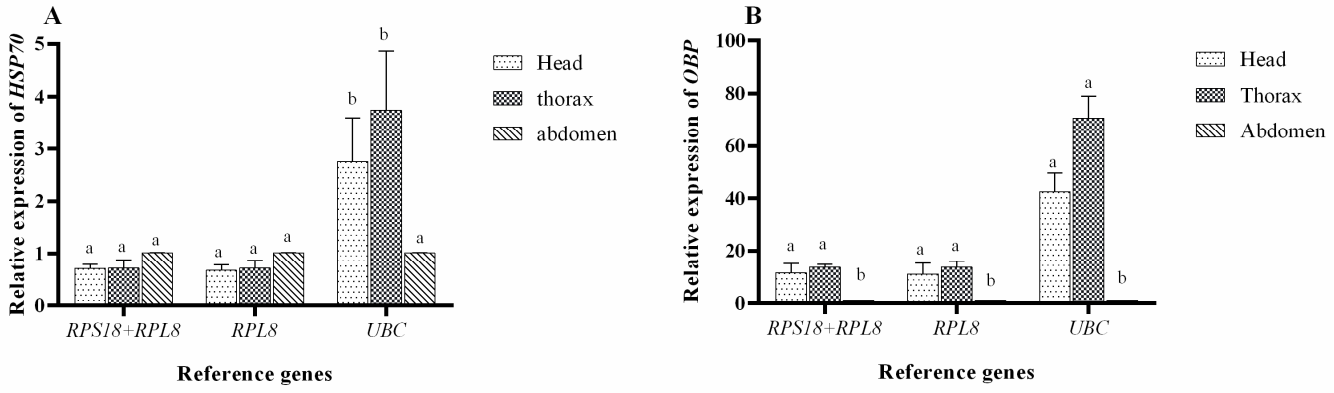

Figure 5. Validation of reference genes. The relative expression level of *HSP70* (**A**) and *OBP7* (**B**) in different tissues of *P. versicolora* were normalized using *RPL8/RPS18*, *RPL8*, or *UBC*, respectively. Data represent mean values ± SE (n = 3). Different letters indicate statistical differences ($p < 0.05$, one-way ANOVA).

4. Discussion

Although *P. versicolora* is one of the most destructive pests of the Salicaceae [45], its molecular physiology has not been rigorously explored due to incomplete background genetic information. Fortunately, recent developments in transcriptomics research have paved the way for functional genomics and associated gene expression studies [28,31,46]. However, a previous study demonstrated that incorrect reference gene(s) could have a significant impact on quantification results and further lead to incorrect inferences and misinterpretations [24]. In line with the conclusion, our experimental results revealed that *α-tubulin* could be expressed stably after dsRNA treatment, but its expression varied among different developmental stages, tissues, sexes, and other treatments (Table 2). It is therefore essential to assess suitable reference genes in the *P. versicolora* under various biotic and abiotic settings.

Ribosomal protein genes were consistently expressed in several insect species: for instance, *RPS8*, *RPL13*, and *RPL28* showed high stability across tissues, sexes and developmental stages in *Harmonia axyridis* [47]; *RPL13a*, *RPS3* and *RPL18* in *Holotrichia oblita* (Coleoptera: Scarabaeidae), *RPL13a* in *Anomala corpulenta* (Coleoptera: Scarabaeidae) have a similar patten [20,48]. Moreover, similar results were obtained in several Coleoptera insects including *Agasicles hygrophila* (Coleoptera: Chrysomelidae), *Anthonomus eugenii* (Coleoptera; Curculionidae), *Propylea japonica* (Coleoptera: Coccinellidae), and *Harmonia axyridis* (Coleoptera: Coccinellidae), among others [21–23,47]. In line with these conclusions, we found that ribosomal protein genes are relative suitable reference genes for gene expression studies of *P. versicolora* in the experimental situation described above.

Our overall analysis revealed that *α-tubulin* and *EF1A* ranked high in only one experimental setting (dsRNA treatment and development stage, respectively) (Figure 3A,E). Similarly, *α-tubulin* was identified as a stable reference gene only when it was used to normalize target gene expression in RNAi treatment of *Coccinella septempunctata* (Coleoptera: Coccinellidae) [49]. *EF1A* is not stably expressed under some occasions and could not be set as a suitable reference gene in many insect species, such as *Sesamia inferens* (Lepidoptera:

Noctuidae) [50], *Phaedon brassicae* (Coleoptera: Chrysomelidae) [51], *Bradysia odoriphaga* (Diptera: Sciaridae) [52], and *Harmonia axyridis* (Coleoptera: Coccinellidae) [47]. Thus, the reliability of the above two reference genes may be context dependent. *Actin* is another common reference gene in many insects, encoding a major structural protein which is involved in the maintenance of the cytoskeleton and basic nuclear processes from gene expression to DNA repair [53]. Nevertheless, we found that the expression of *Actin* was very unstable compared to other studies, especially in *P. versicolora* samples of different developmental stages (Figure 3A). Several other investigations have found that *Actin* expression varies depending on the sample type, which is consistent with our findings [21,54]. Collectively, these results indicate that the stability of reference genes varies and is easily influenced by a handful of biotic and abiotic factors. Thus, no one universal reference gene exists that is suitable for all insects and under all situations; even the most used housekeeping genes respond differentially to diverse experimental settings. As a result, it is critical to select the most accurate normalization approach in order to obtain the best gene expression data and exclude non-biological variance from the biological results [55].

The ΔCt method, GeNorm, NormFinder and BestKeeper are often used in selection of reference genes [56–58]. Although some reference genes were ranked in the same position by the four algorithms under certain conditions, in general, there was some variation in the stability rankings produced by these algorithms. For example, the ranking of *Actin* varied among the four algorithms under different tissues in our experiments (Table 2). In many studies, the variations in ranking order of reference genes can be linked to the algorithm's various statistical methodologies [59,60]. To solve this problem, RefFinder can construct a composite rating of reference genes based on the ranking values provided by the four methods described above [61]. Furthermore, a great number of experimental results suggest that selecting two or more reference genes is more accurate and reliable than using a single reference gene for rectification. As a result, we propose that the findings of the pairwise variation (Vn/Vn+1) of the geNorm can be used to calculate the number of reference genes that normalize the target genes. Then, the results of the comprehensive RefFinder ranking are combined to determine the best combination of reference genes that can accurately analyze the expression of the target genes.

In general, *HSP70* is stably expressed across a variety of experimental conditions. For example, *HSP70*, which served as a reference gene in *Coleomegilla maculate* (Coleoptera: Coccinellidae) [62], is stably expressed in different developmental stages and in different sexes of *Chilo partellus* (Lepidoptera: Crambidae) [63]. Thus, the gene was often chosen as a target to assay the stability of candidate reference genes [59]. Here, the gene, together with *OBP7*, was applied to assay the stability of the seven candidate reference genes in *P. versicolora*. We showed that the *HSP70* gene expression was stable in different tissues when normalized with *RPL8/RPS18* or *RPL8* alone. In contrast, normalization with *UBC* suggested there was a significant difference in *HSP70* gene expression between the groups, with the highest expression in the thorax (Figure 5A). These findings suggest that using the wrong reference gene can result in radically different experimental results, highlighting the necessity of screening for reference genes.

The independent normalization of qRT-PCR data using either a stable reference combination (*RPS18/RPL8*) or the most stable reference gene (*RPL8*) indicated that *OBP7* was expressed 11 and 13-fold higher in the head and thorax, respectively, than in the abdomen. Overall, the *OBP7* is highly expressed in the head and thorax in *P. versicolora*, which is consistent with the result in *Bactrocera dorsalis (Diptera: Tephritidae)* and *Bemisia tabaci (Hemiptera: Aleyrodidae)* [64,65]. However, we have to mention that *P. versicolora* adults were dissected and separated into three segments to represent head, thorax, and abdomen, respectively, and the thorax contains thoracic legs and wings. In previous research, *OBPs* have been found to be expressed in a variety of insect tissues, including antennae [66], legs [67], and wings [68]. Thus, more specific expression profiles about *OBP7* need to be explored further on the basis of this experiment.

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