RESEARCH ARTICLE

Assessment of suitable reference genes for qRT-PCR analysis in *Adelphocoris suturalis*

LUO Jing[1, 2, 3], MA Chao[1], LI Zhe[1], ZHU Bang-qin[3], ZHANG Jiang[2], LEI Chao-liang[3], JIN Shuang-xia[1], J. Joe Hull[4], CHEN Li-zhen[1, 3]

[1] *National Key Laboratory of Crop Genetic Improvement and National Centre of Plant Gene Research, Huazhong Agricultural University, Wuhan 430070, P.R.China*

[2] *Hubei Collaborative Innovation Center for Green Transformation of Bio-Resources, College of Life Science, Hubei University, Wuhan 430062, P.R.China*

[3] *Hubei Insect Resources Utilization and Sustainable Pest Management Key Laboratory, College of Plant Science and Technology, Huazhong Agricultural University, Wuhan 430070, P.R.China*

[4] *U.S. Arid Land Agricultural Research Center, Agricultural Research Service, U.S. Department of Agriculture, Maricopa 85138, USA*

Abstract

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) is the most commonly-used tool for measurement of gene expression, but its accuracy and reliability depend on appropriate data normalization with the use of one or more stable reference genes. *Adelphocoris suturalis* is one of the most destructive pests of cotton, but until recently knowledge of its underlying molecular physiology had been hindered by a lack of molecular resources. To facilitate research on this pest, we evaluated 12 common housekeeping genes studied in insects (*GAPDH*, *ACT*, *βACT*, *TBP*, *SDH*, *βTUB*, *EF1γ*, *EF1α*, *EF1δ*, *RPL32*, *RPS15*, and *RPL27*) for their expression stability in *A. suturalis* when subjected to various experimental treatments, including three biotic (developmental stage and sex, tissue type, and metathoracic scent gland for varying developmental stages and sexes) and one abiotic (RNA interference injection) conditions. Four dedicated algorithms (ΔCt method, geNorm, BestKeeper and NormFinder) were used to analyze gene expression stability. In addition, RefFinder provided an overall ranking of the stability/suitability of these candidates. This study is the first to provide a comprehensive list of suitable reference genes for gene expression analyses in *A. suturalis*, which can serve to facilitate transcript expression study of related biological processes in this and related species.

Keywords: *Adelphocoris suturalis*, reference gene, qRT-PCR, normalization, expression stability

1. Introduction

Adelphocoris suturalis (Hemiptera: Miridae) is a highly polyphagous pest that can attack a broad range of cultivated crops including cotton, pastures, vegetables, and fruit trees (Jiang *et al.* 2015). This plant bug was originally a secondary pest of cotton, but has since become a significant problem

for cotton growing regions in China due to the reduction in broad-spectrum insecticides that followed the widespread adoption of transgenic *Bacillus thuringiensis* (Bt) cotton (Lu *et al.* 2008a, 2010; Li *et al.* 2010). Currently, control of these plant bugs is largely dependent on the use of broad-spectrum chemical insecticides (Lu and Wu 2008). Although such treatments can be effective, they often threaten human health, adversely affect the environment, and can give rise to resistant populations. Hence, the development of less hazardous, environmentally sound, and sustainable pest management strategies is needed. However, a deeper understanding of the biology of these species is necessary for developing novel alternatives to broad-spectrum chemical control approaches.

In recent years, the ecology and physiology of *A. suturalis* has been expansively studied (Lu *et al.* 2008b; Lu and Wu 2008; Zhang *et al.* 2014; Jiang *et al.* 2015; Zhang *et al.* 2016), but the molecular mechanisms of the biology have largely remained unknown. With the advent of next generation sequencing approaches, there is now an unparalleled opportunity to investigate the genetic basis of its biology and physiology. For this investigation, both the silencing of gene expression to assess gene function as well as quantification of gene expression are required.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) is an indispensable tool for measurement of gene expression (Bustin *et al.* 2010). Older, traditional gene expression measurements such as competitive reverse transcription polymerase chain reaction (RT-PCR), Northern blot analysis, or *in situ* hybridization lacks its high sensitivity, specificity, reproducibility, and high-throughput convenience (Ginzinger 2002; Wong and Medrano 2005; Espy *et al.* 2006; Bustin 2010). However, the accuracy of qRT-PCR is limited by batch to batch variation in RNA extraction and variable efficiency of cDNA synthesis and of PCR reaction (Bustin *et al.* 2005; Huggett *et al.* 2005). To limit variability, a common technique in qRT-PCR is normalizing target gene expression data to reference genes (Vandesompele *et al.* 2002). Consequently, one of the most important considerations in a valid qRT-PCR analysis is the appropriateness of the reference gene(s).

Housekeeping genes, which are expressed constitutively and are essential for survival in all cells, are commonly used as reference genes, under the assumption that their transcript levels will remain constant regardless of experimental treatment and/or physiological condition (Thellin *et al.* 1999; Butte *et al.* 2001). However, these assumptions may not be valid in practice. Numerous reports have demonstrated that some of the most common reference genes (housekeeping genes) undergo significant regulation in response to diverse experimental conditions (Lee *et al.* 2002; Yuan *et al.* 2014; Bansal *et al.* 2016), indicating that these genes are inappropriate for normalization purposes. Indeed, there is no stably expressed reference gene suitable for all cells and experimental conditions. If the reference gene is not selected properly, it will cause erroneous interpretation of the qRT-PCR data (Lee *et al.* 2002; Vandesompele *et al.* 2002; Radonić *et al.* 2004; Huggett *et al.* 2005; Nolan *et al.* 2006; Yuan *et al.* 2014). Therefore, it is essential to select and validate a suite of best-suited qRT-PCR reference gene(s) prior to quantifying transcript levels. However, the expression stability of reference genes in *A. suturalis*, or in any plant bug, has not been systematically assessed to date. Hence, the identification and validation of suitable endogenous reference genes in *A. suturalis* is urgently needed.

The overall goal of this study was to evaluate and select appropriate reference gene(s) for accurate quantification of mRNA transcripts in *A. suturalis*. To achieve this goal, 12 housekeeping genes frequently used in gene expression study of other sap-sucking insects were selected as candidate reference genes: *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), *actin* (*ACT*), *β-actin* (*βACT*), *TATA-box binding protein* (*TBP*), *succinate dehydrogenase* (*SDH*), *β-tubulin* (*βTUB*), *elongation factor-1γ* (*EF1γ*), *elongation factor-1α* (*EF1α*), *elongation factor-1δ* (*EF1δ*), *ribosomal protein L32* (*RPL32*), *ribosomal protein S15* (*RPS15*), and *ribosomal protein L27* (*RPL27*) (Li *et al.* 2013; Bansal *et al.* 2016; Ibanez and Tamborindeguy 2016; Koramutla *et al.* 2016; Zhang *et al.* 2018). These housekeeping genes were chosen from different functional classes and gene families to avoid the effect of co-regulation. We evaluated the expression stability of these candidate genes under varying abiotic (RNA interference *via* dsRNA injection) and biotic (developmental stage and sex, multiple tissue types, and a narrow focus on metathoracic scent glands (MTGs) from different developmental stages and sexes) conditions using the four statistical algorithms geNorm (Vandesompele *et al.* 2002), NormFinder (Andersen *et al.* 2004), BestKeeper (Pfaffl *et al.* 2004), and the ΔCt method (Silver *et al.* 2006). In addition, RefFinder, a comprehensive platform integrating the above-mentioned algorithms, provided an overall ranking of the stability/suitability of these candidates (Yang *et al.* 2015a). Our data provide the first comprehensive assessment of reference genes in *A. suturalis*, and will benefit gene expression studies in this plant bug species.

2. Materials and methods

2.1. Insect rearing

A. suturalis was collected from a Bt cotton field located in Wuhan (Hubei Province, China) in July 2015. Nymphs and

adults were reared in plastic cages (22.5 cm×15 cm×11 cm) and fed a diet of green beans and 5% sugar solution. The green beans were also used to collect eggs (Lu *et al.* 2008b). The newly emerged adults were separated by sex every day and reared in a separate plastic cage at a density of 30 adults per cage (Lu and Wu 2008). All insects were maintained for their entire life cycle at (26±2)°C, (75±5)% relative humidity, and a photoperiod cycle of 16 h L:8 h D.

2.2. Experimental treatments

Developmental stage and sex The different developmental stages and sexes of *A. suturalis* included eggs, second instar nymphs, fifth instar nymphs, sexually immature (1-d-old) male and female adults, and sexually mature (8-d-old) male and female adults. A total of 100 eggs, 30 second instar nymphs, 6 fifth instar nymphs, 3 male adults (1-d-old), 3 female adults (1-d-old), 3 male adults (8-d-old) and 3 female adults (8-d-old) were collected, then immediately frozen in liquid nitrogen and stored at −80°C until use. The samples were collected in three biological replicates.

Tissue Five body regions, including head, MTG, gut, ovary and fat body, were dissected from 8-d-old female adults of *A. suturalis*. Three independent biological replicates were collected and each tissue group was derived from a minimum of 25 insects. All the samples were handled and stored as described above.

MTG at different developmental stages and sexes The MTGs of 1-d-old male, 1-d-old female, 8-d-old male, and 8-d-old female adults of *A. suturalis* were dissected. Three independent biological replicates were collected with each group corresponding to a minimum of 30 insects. All the samples were handled and stored as described above.

dsRNA injection For RNAi treatments, 1-d-old female adults of *A. suturalis* were microinjected with 100 nL dsRNA (10 µg µL^{-1}) against *fatty acyl-CoA reductase* (*FAR*), encoded an NAD(P)H-dependent oxidoreductase that catalyzes the reduction of fatty acyl-CoA precursors into fatty alcohols, using a micro-injector (World Precision Instruments, Sarasota, FL, USA). Control injections consisted of H$_2$O and *dsGFP* (dsRNA of *green fluorescent protein* gene). The dsRNA was synthetized using the corresponding primers (Appendix A) as described (Liu *et al.* 2016). At 3 days post-injection, 3 individuals from each treatment were collected with 3 independent biological replicates performed. All the samples were handled and stored as described above.

2.3. Total RNA isolation and cDNA synthesis

Total RNA was extracted using RNAiso Plus reagent (TaKaRa, Kyoto, Japan) according to the manufacturer's protocol. The RNA integrity was confirmed by 1.5% agarose gels electrophoresis and quantified on a Nano-Drop 2000 (Thermo Scientific, Wilmington, DE, USA). Total RNA (1 µg) was depleted of residual genomic DNA and then reverse transcribed using the PrimeScript®RT Reagent Kit with gDNA Eraser (TaKaRa, Kyoto, Japan) according to the manufacturer's protocol. The synthesized cDNA was stored at –20°C until use. For qRT-PCR analysis, each cDNA sample was diluted 20 times with nuclease-free water.

2.4. Candidate reference genes and primer design

Using *A. suturalis* transcriptomic data (Luo *et al.* 2014), sequences corresponding to 12 candidate reference genes commonly used in qRT-PCR analyses in other insect species were selected (Appendix B). All of the candidate reference genes were PCR amplified from *A. suturalis* cDNA using the corresponding primers (Appendix C), sub-cloned using the pEASY-T1 Simple Cloning Kit (TransGen, Beijing, China) and sequenced. The corresponding gene sequences were deposited in GenBank (the accession numbers are listed in Table 1). After confirmation of candidate reference genes, primers for the subsequent qRT-PCR analyses were designed using an online tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The PCR amplification efficiency and primer specificity were assessed using standard curves, melt curve analyses, and 2% agarose-gel electrophoresis.

2.5. qRT-PCR

All of the qRT-PCR reactions were performed using a Bio-Rad iQ2 Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA) following the MIQE (Minimum Information for publication of Quantitative real time PCR Experiments) guidelines (Bustin *et al.* 2010). Reactions were carried out in a final volume of 10 µL containing 2 µL diluted cDNA template, 5 µL 2×SYBR® Premix *ExTaq*™ II (TaKaRa, Kyoto, Japan) and 400 nmol L^{-1} of each gene-specific primer (Table 1). The reaction cocktails were set up in 96-well format Microseal PCR plates (Bio-Rad Hercules, CA, USA) in triplicate. The PCR program consisted of 95°C for 30 s, followed by 40 cycles of dissociation at 95°C for 5 s, and then annealing and extension at 62°C for 30 s. For melt curve analysis, continuous fluorescent measurements were made as the temperature was ramped up from 55 to 95°C in increments of 0.5°C every 6 s. A serial 5-fold dilution of cDNA template was used to generate standard curves and the gene specific PCR efficiency (E) of each gene were calculated according to previously described methods (Yang *et al.* 2015a; Koramutla *et al.* 2016). Three biological replicates were performed for individual treatment.

Table 1 Oligonucleotide primers for candidate qRT-PCR reference genes in *Adelphocoris suturalis*[1]

Gene	Accession number	Primer sequence (5´→3´)	Product length (bp)	Tm (°C)	E (%)	R^2
GAPDH	KY038867	F: GACCGTCGTTTCCAATGCTTCCTGC	120	62	102.4	0.999
		R: GTAGCAGTAACGGCGTGGACAGTAG				
ACT	KY038868	F: CTAGAGGAGGCCTTGTGGGCA	153	62	101.9	0.999
		R: GCCGCTCAGGGAAGCCTTGTA				
βACT	KY038869	F: CGGCAACGAGAGGTTCAGGTG	144	62	100.3	0.997
		R: ACGGTGTTGGCGTACAGGTC				
TBP	KY027055	F: ATGACTCCCGCCTTGCAGCTA	111	62	91.7	0.998
		R: TGACATCGCAGCTTCCCACCA				
SDH	KY027056	F: GGCCATGAGGTTGCCCGGTA	114	62	103.4	0.998
		R: GGCACTCCGCCCATGTTGTA				
βTUB	KY027057	F: GCGGCTCACAGCAGTATCGC	104	62	91.3	0.996
		R: GTAGCGTCCGTGTCTGGGGT				
EF1γ	KY027058	F: TTGGCCCTTGCTGCAGAACC	170	62	91.0	0.998
		R: TCTCCGAGCCAGATGGAGTAGTT				
EF1α	KY027060	F: CCTTCGCCCCAGTCAACCTG	138	62	92.6	0.997
		R: CGTATCCTCGGCGCAACTCC				
EF1δ	KY027059	F: TCTGAGGAAGACGAAGAAGCCAACA	168	62	93.1	0.998
		R: TAGACGAACGCGCCTCTCCA				
RPL32	KY027061	F: AGGGCCAGTACCTGATGCCA	159	62	93.7	1.000
		R: CGGCATGAGCGATTTCTGCG				
RPS15	KY027062	F: TGGTTGGCAGCATTGTCGGTG	137	62	92.6	0.997
		R: ACCAATACCGGGCCTTCCGT				
RPL27	KY027063	F: GGACTCTCGTCACCGAGCAA	93	62	92.6	0.998
		R: TGTAGTAACCCGCTCGGACG				

[1] Tm, melting temperature; E, qRT-PCR efficiency; R^2, regression coefficient of the qPCR reaction; F, forward primers; R, reverse primers.

2.6. Stability analysis of candidate reference genes

The Bio-Rad iQ5 Optical System software (ver. 2.1.94.617) (Bio-Rad, Hercules, CA, USA) was used to analyze the qRT-PCR data. Three biological replicates were used to calculate the average cycle threshold (Ct) values. The stabilities of the 12 candidate reference genes were evaluated by geNorm (Vandesompele *et al.* 2002), NormFinder (Andersen *et al.* 2004), BestKeeper (Pfaffl *et al.* 2004), and the ΔCt method (Silver *et al.* 2006). GeNorm calculates an average expression stability value (M) in which lower values indicate more stable expression or lower variation (Vandesompele *et al.* 2002). M is calculated by a geometric averaging of the mean pairwise variation of a candidate reference gene to all the other candidate reference genes. An M value less than 1.5 is recommended to identify stably expressed gene. NormFinder determines the expression stability by considering intra- and inter-group variations for candidate reference genes (Andersen *et al.* 2004). NormFinder provides the stability value (SV) for each candidate reference gene. Genes with a lower SV are considered to be more stably expressed and are ideal to select as reference gene for that particular experimental conditions (Andersen *et al.* 2004). The BestKeeper program determines the stability of a candidate reference gene based on the standard deviations (SD) of the Ct values. SD values below 1 are recommended for stably expressed genes, and the lower the SD, the better the gene is as a reference (Pfaffl *et al.* 2004). In the ΔCt method, rank order is determined based on pair-wise comparisons of gene-sets using mean ΔCt values within a particular treatment. Therefore, the average standard deviation of each gene-set is inversely proportional to the gene-expression stability (Silver *et al.* 2006). Finally, RefFinder, a comprehensive software platform integrating all four algorithms, provides an overall ranking of the stability/suitability of the candidates (Yang *et al.* 2015a). Furthermore, geNorm performs a stepwise calculation of the pairwise variation (V_n/V_{n+1}) between sequential normalization factors (NF_n and NF_{n+1}) to determine the optimal number of reference genes required for accurate normalization. A threshold value below 0.15 suggests no additional reference genes are necessary for normalization (Vandesompele *et al.* 2002). BestKeeper and RefFinder use raw Ct values, whereas geNorm and Normfinder use expression values calculated as $2^{-\Delta\Delta C_T}$.

2.7. Validation of reference genes

Validation of the selected reference genes was performed using various tissues. The *A. suturalis vacuolar-type H+-ATPase* (*V-ATPase*) (accession no. MF102282) gene and the *fatty acid synthase* (*FAS*) (accession no. MG520370)

gene were selected as target genes for stability validation (*V-ATPase*, F: 5′-ACCCTCCATCAGCGTCCCAT-3′, R: 5′-AGGCGCCAAAGGAGTATCGAC-3′; *FAS*, F: 5′-ACTGGGG CGAATGTGGATGGTTAC-3′, R: 5′-GGTCTCCTACCTTG GTTCCTGTTC-3′). The relative expression level of *V-ATPase* and *FAS* were normalized using the best reference gene pair (*RPL32/RPS15* identified by geNorm), the single best reference gene (*RPS15* determined by RefFinder), and the least stable reference gene (*βACT* determined by all five algorithms), respectively. The qRT-PCR reactions were carried out as described above and qRT-PCR data were analyzed *via* the $2^{-\Delta\Delta C_T}$ method (Schmittgen and Livak 2008). One-way ANOVA followed by Tukey's HSD Multiple Comparison was used to determine statistical significance. Statistical differences are shown as different letters.

3. Results

3.1. Performance of qRT-PCR primers

We confirmed specific PCR amplification by testing the primer specificity for each candidate reference gene with RT-PCR. PCR amplifications for each primer pair showed single bands of the anticipated sizes on the 2.0% agarose gel (Fig. 1-A). All amplicons were sequenced and confirmed to exhibit 99–100% identity with the corresponding transcriptomic sequences. Melt curve analyses revealed
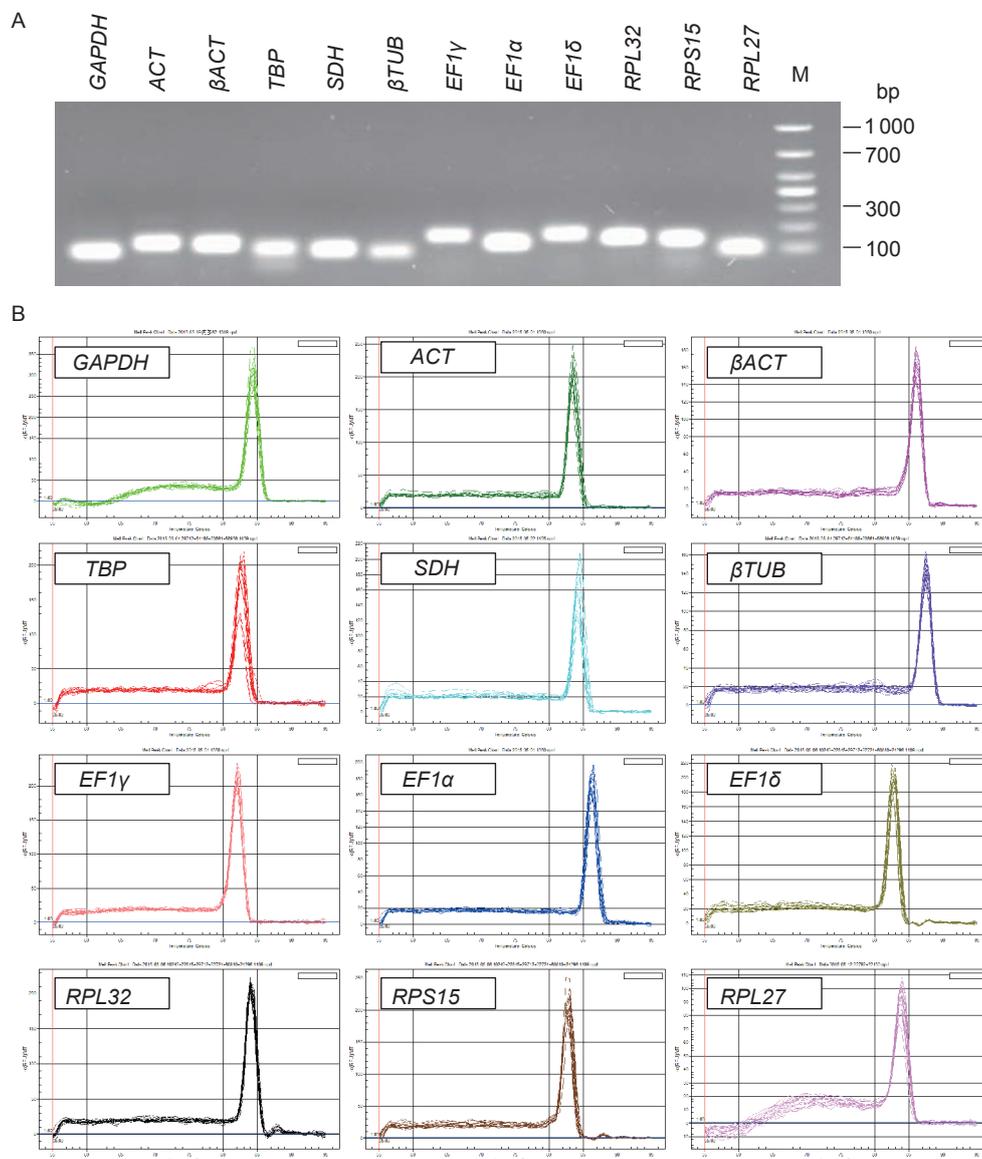

Fig. 1 Amplification specificity of primers in RT-PCR and qRT-PCR. A, single amplicon of the expected size for each gene was visualized on a 2% agarose gel. M, marker. B, single peaks in melt curve analysis.

single peaks for each primer pair, suggesting the absence of non-specific amplification (Fig. 1-B). The PCR efficiency (E) and correlation coefficient (R^2) for each standard curve are shown in Table 1. The PCR efficiencies for all tested primer pairs varied between 91.0 and 103.4%, with associated R^2 values of 0.996–1.000.

3.2. Expression profiles of candidate reference genes

To provide an overall representation of primer variability under varying experimental conditions, the expression profiles of the candidate reference genes were examined (Fig. 2). Ct values of the 12 candidate reference genes under the four experimental conditions spanned a range of 14.53–29.27 cycles. *ACT* and *TBP* had average Ct values >24 cycles, while the average Ct value of the other candidate reference genes (*GAPDH*, *βACT*, *SDH*, *βTUB*, *EF1γ*, *EF1α*, *EF1δ*, *RPL32*, *RPS15* and *RPL27*) ranged between 16–24 cycles (Fig. 2-A–D). Furthermore, we found that experimental treatment influenced the degree of variability in candidate reference gene expression. For example, *βTUB* varied less (~1 cycle) between samples before and after dsRNA injection than across tissue types (>6 cycles). When

considering all the experimental conditions, we found that a limited number of the candidate genes (e.g., *EF1γ*, *EF1α*, *EF1δ*, *RPS15* and *RPL27*) were relatively stable (<4 cycle difference), whereas the others exhibited greater variation in expression (e.g., *βACT* at ~11 cycles). Although variation was observed in all treatments, it was less pronounced following dsRNA injection (Fig. 2-D).

3.3. Stability of candidate reference genes

Performance of the 12 candidate genes was assessed in four experimental sets, including different developmental stages and sexes, across multiple tissues, in MTGs from different developmental stages and sexes, and after dsRNA injection. To identify the most stable reference gene(s) for these different experimental conditions, the expression stabilities were evaluated using the ΔCt method, BestKeeper, NormFinder, and geNorm. The overall stability ranking was obtained by RefFinder.

For different developmental stages and sexes, the ΔCt method and NormFinder indicated that *EF1δ*, *EF1γ* and *RPS15* were the most stable, whereas *EF1α* and *βACT* exhibited the greatest variation (Table 2). Based
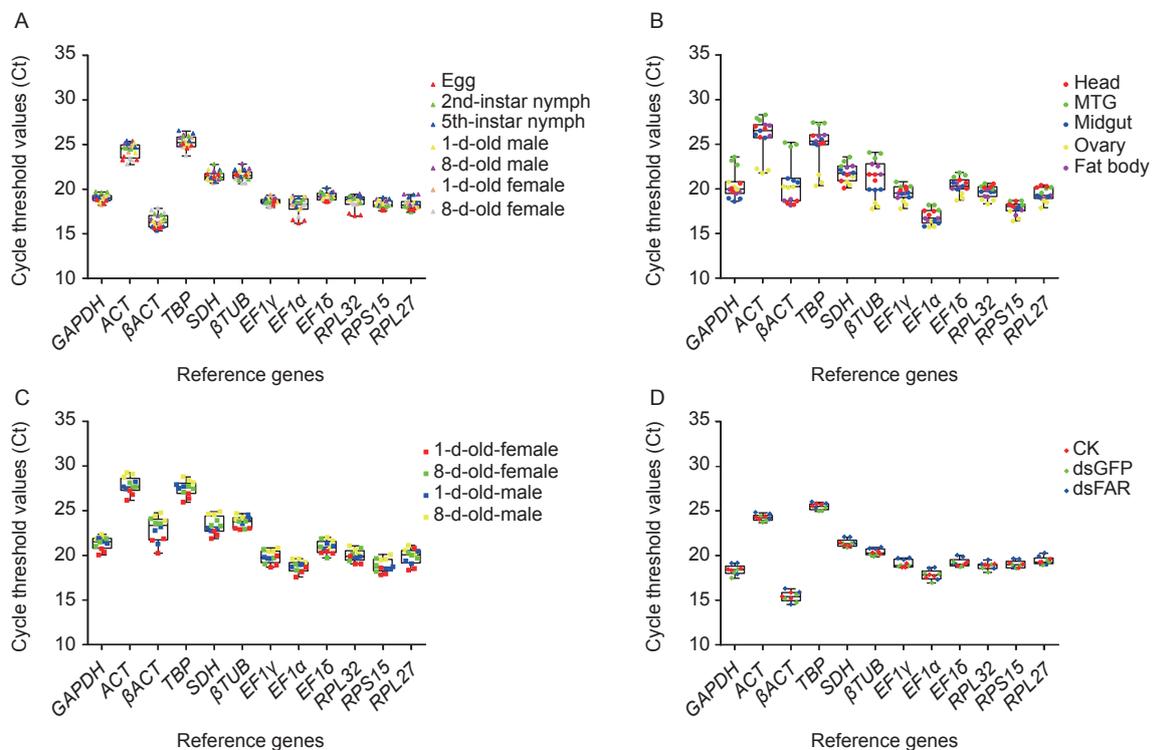

Fig. 2 Box-and-whisker plots of expression profiles of candidate reference genes under four experimental conditions. A, different developmental stages and sexes. B, different tissues. C, metathoracic scent glands (MTGs) from different developmental stages and sexes. D, dsRNA injection. The expression levels of candidate reference genes are shown as Ct values. Each data point represents the Ct values of each biological replicate in each treatment. The median is represented by the line in the box. The interquartile range is bordered by the upper and lower edges, which indicate the 75th and 25th percentiles, respectively. The whisker caps indicate the minimum and maxium values.

on BestKeeper, *EF1γ* and *GAPDH* were the most stable reference genes (Table 2). Similarly, GeNorm calculated the lowest M value for the *EF1γ*/*GAPDH* pair (0.051), suggesting that they are the most stable transcripts. The M value of all candidate reference genes exhibited little variation and remained >0.3 (Table 2). RefFinder ranked the genes from most to least stable as: *EF1δ*>*EF1γ*>*RPS15*>*GAPDH*>*βTUB*>*SDH*>*RPL27*>*RPL32*>*TBP*>*ACT*>*βACT*>*EF1α* (Fig. 3-A).

In our analysis of multiple tissue types, both the ΔCt method and NormFinder suggested *EF1δ*, *EF1γ*, and *EF1α* were the most suitable reference genes (Table 2). In contrast, *RPL32*/*RPS15* and *RPS15* were considered the most stable genes by geNorm and BestKeeper, respectively (Table 2). Furthermore, all four algorithms identified *βACT* as the least suitable reference gene (Table 2). For tissues, the overall RefFinder stability ranking (from most to least stable) was: *RPS15*>*EF1δ*>*RPL32*>*EF1γ*>*EF1α*>*RPL27*>*SDH*>*GAPDH*>*βTUB*>*ACT*>*TBP*>*βACT* (Fig. 3-B).

For MTGs from different developmental stages and sexes, both the ΔCt method and NormFinder suggested *RPS15* and *RPL32* were the most stable genes (Table 2), whereas BestKeeper ranked *EF1α* and *RPL32* as the most stable and geNorm ranked *EF1γ* and *EF1δ* as the best pair (Table 2). *βACT* was again identified by all four algorithms as the least stable reference gene (Table 2). For the MTG analyses, the RefFinder ranking was: *RPL32*>*RPS15*>*EF1α*>*EF1γ*>*EF1δ*>*GAPDH*>*βTUB*>*ACT*>*TBP*>*SDH*>*RPL27*>*βACT* (Fig. 3-C).

In the last set of experiments, which assessed the effect of dsRNA-mediated RNAi on reference gene expression, *ACT* was identified by all four analyses as one of the most stable genes (Table 2). In addition, *RPS15* (ΔCt method and NormFinder), *RPL32* (BestKeeper), and *βTUB* (geNorm) were also identified as having a calculated stability value equivalent to that of *ACT* (Table 2). The stability values (calculated by BestKeeper and geNorm) for all candidate genes were lower than the recommended threshold for reference gene suitability with SD<0.460 for BestKeeper and M<0.160 for geNorm (Table 2). This is consistent with the smaller variation in RNAi-injected treatments (Fig. 2-D). For the dsRNA injection study, the RefFinder ranking was: *ACT*>*RPS15*>*RPL27*>*βTUB*>*RPL32*>*EF1δ*>*TBP*>*SDH*>*GAPDH*>*EF1γ*>*EF1α*>*βACT* (Fig. 3-D).

3.4. Determination of the optimal number of reference genes for normalization

Established methods of qRT-PCR often use a single reference gene with sufficient expression for analysis, though use of more than one reference gene strengthens analysis (Vandesompele *et al.* 2002). Thus, we used

geNorm to estimate the pairwise variation (V_n/V_{n+1}) to determine the optimal number of reference genes required for normalization of samples under a given experimental condition. All pairwise variations were determined to be below 0.15 (the recommended threshold of cut-off) for all treatments, indicating that no additional genes are required for the normalization (Fig. 4). Thus, the use of only the top two reference genes for each experimental set is sufficient for normalization.

3.5. Validation of selected reference genes in *A. suturalis*

To validate the reference genes selected, we assessed the relative expression of *A. suturalis V-ATPase* and *FAS* in various tissues. The best reference gene pair determined by geNorm, *RPL32*/*RPS15*, the single best reference gene determined by RefFinder, *RPS15*, and the most variable reference gene determined by all algorithms, *βACT*, were used to normalize the expression levels of those two target genes.

V-ATPase is a highly conserved evolutionarily ancient enzyme that functions in cellular homeostasis, found at the plasma membrane of cells lining the gut and the Malpighian tubules of many insects, where it regulates pH, energizes ion transport, and modulates fluid secretion (Nelson *et al.* 2000; Wieczorek *et al.* 2009). Normalization of transcripts using *RPL32*/*RPS15* and *RPS15* alone revealed peak *V-ATPase* expression in the gut (Fig. 5-A). In contrast, normalization with *βACT* suggested the highest expression in MTG. *FAS* is a multi-enzyme protein that catalyzes the *de novo* synthesis of long-chain fatty acids, and plays an important role in energy production and storage, cellular structure, and the biosynthesis of pheromone in insects (Volpe and Vagelos 1973; Tillman *et al.* 1999). We saw peak expression in the MTG where *A. suturalis* synthesizes and releases pheromones (Zhang *et al.* 2014) regardless of the reference gene used. Although the trend was similar, normalization with an unsuitable reference gene such as *βACT* not only increased gene expression levels, but also resulted in larger standard error values (Fig. 5-B). Therefore, our results support the importance of the selection and validation of accurate reference genes RT-qPCR to avoid misinterpretation of the expression data.

4. Discussion

qRT-PCR is the most widely used molecular technique for gene expression analysis, but the accuracy and reliability of the results are critically dependent on appropriate data normalization with the use of stable reference gene(s) (Vandesompele *et al.* 2002; Bustin *et al.* 2010). Using inappropriate reference gene(s) can significantly impact

Table 2 Rank order of the candidate *Adelphocoris suturalis* reference genes under different experimental conditions

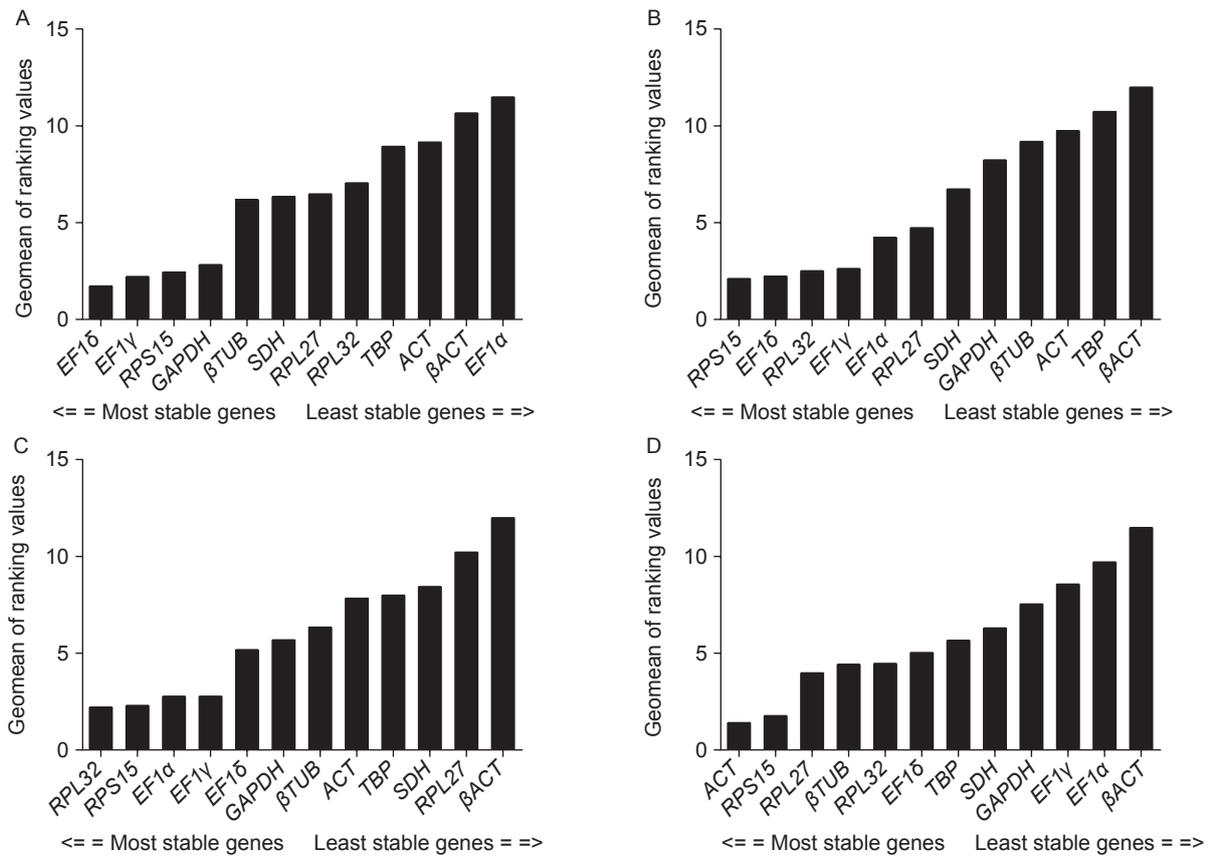| Experimental conditions	Rank	ΔCt		BestKeeper		NormFinder		geNorm	
		Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability
Developmental stage and sex	1	EF1δ	0.579	EF1γ	0.246	EF1δ	0.258	RPS15	0.051
								GAPDH	0.051
	2	EF1γ	0.620	GAPDH	0.314	EF1γ	0.350	–	–
	3	RPS15	0.633	EF1δ	0.346	RPS15	0.370	EF1δ	0.075
	4	GAPDH	0.635	RPS15	0.354	GAPDH	0.372	SDH	0.113
	5	RPL32	0.664	SDH	0.394	RPL32	0.432	βTUB	0.124
	6	RPL27	0.682	βTUB	0.405	RPL27	0.456	EF1γ	0.139
	7	βTUB	0.715	RPL27	0.456	βTUB	0.521	RPL27	0.156
	8	ACT	0.742	TBP	0.539	ACT	0.562	TBP	0.180
	9	SDH	0.755	RPL32	0.556	SDH	0.571	βACT	0.200
	10	TBP	0.758	βACT	0.592	TBP	0.584	ACT	0.232
	11	EF1α	0.827	ACT	0.662	EF1α	0.676	RPL32	0.255
	12	βACT	1.879	EF1α	0.703	βACT	0.812	EF1α	0.288
Tissue	1	EF1δ	0.946	RPS15	0.532	EF1δ	0.171	RPL32	0.130
								RPS15	0.130
	2	EF1γ	0.956	RPL32	0.552	EF1γ	0.380	–	–
	3	EF1α	1.020	RPL27	0.594	EF1α	0.475	EF1γ	0.149
	4	RPL32	1.030	EF1γ	0.621	RPS15	0.631	RPL27	0.175
	5	RPS15	1.037	EF1δ	0.687	RPL32	0.638	EF1δ	0.190
	6	RPL27	1.098	EF1α	0.699	SDH	0.690	EF1α	0.213
	7	SDH	1.207	SDH	0.754	RPL27	0.753	SDH	0.227
	8	βTUB	1.513	GAPDH	1.163	GAPDH	1.166	GAPDH	0.386
	9	GAPDH	1.519	ACT	1.562	βTUB	1.177	βTUB	0.573
	10	ACT	1.543	TBP	1.618	ACT	1.288	ACT	0.718
	11	TBP	1.631	βTUB	1.710	TBP	1.402	TBP	0.824
	12	βACT	2.280	βACT	1.864	βACT	2.121	βACT	0.949
Metathoracic scent glands of developmental stage and sex	1	RPS15	0.494	EF1α	0.473	RPS15	0.205	EF1γ	0.116
								EF1δ	0.116
	2	RPL32	0.511	RPL32	0.508	RPL32	0.271	–	–
	3	EF1γ	0.518	βTUB	0.536	EF1α	0.284	RPL32	0.120
	4	EF1α	0.524	EF1δ	0.547	EF1γ	0.286	RPS15	0.129
	5	GAPDH	0.577	EF1γ	0.551	GAPDH	0.332	EF1α	0.148
	6	ACT	0.592	GAPDH	0.560	SDH	0.359	βTUB	0.157
	7	SDH	0.597	RPS15	0.594	ACT	0.386	GAPDH	0.168
	8	TBP	0.598	TBP	0.620	TBP	0.397	TBP	0.181
	9	EF1δ	0.635	ACT	0.714	βTUB	0.464	RPL27	0.196
	10	βTUB	0.641	RPL27	0.719	EF1δ	0.482	ACT	0.211
	11	RPL27	0.793	SDH	0.766	RPL27	0.672	SDH	0.229
	12	βACT	1.108	βACT	1.144	βACT	1.044	βACT	0.303
dsRNA injection	1	RPS15	0.277	ACT	0.281	RPS15	0.126	ACT	0.061
								βTUB	0.061
	2	ACT	0.281	RPL32	0.285	ACT	0.141	–	–
	3	RPL27	0.287	TBP	0.290	RPL27	0.168	RPS15	0.077
	4	EF1δ	0.307	SDH	0.296	EF1δ	0.187	SDH	0.091
	5	RPL32	0.308	RPS15	0.300	RPL32	0.192	RPL27	0.096
	6	GAPDH	0.312	βTUB	0.301	GAPDH	0.211	EF1δ	0.102
	7	TBP	0.329	RPL27	0.335	TBP	0.229	EF1γ	0.110
	8	βTUB	0.334	EF1δ	0.344	βTUB	0.237	TBP	0.120
	9	EF1α	0.341	EF1γ	0.369	EF1α	0.256	RPL32	0.126
	10	EF1γ	0.375	GAPDH	0.386	EF1γ	0.298	GAPDH	0.138
	11	SDH	0.470	EF1α	0.416	βACT	0.356	EF1α	0.145
	12	βACT	0.662	βACT	0.450	SDH	0.425	βACT	0.153

Fig. 3 Expression stability and relative ranking of candidate reference genes calculated by the Geomean method of RefFinder. A, different developmental stages and sexes. B, different tissue. C, metathoracic scent glands (MTGs) from different developmental stages and sexes. D, dsRNA injection. A lower Geomean ranking indicates more stable expression.

quantification results, leading to false inferences or misinterpretations (Radonić *et al.* 2004; Huggett *et al.* 2005; Nolan *et al.* 2006; Yuan *et al.* 2014). Furthermore, numerous qRT-PCR studies have revealed that most reference/housekeeping genes exhibit variable expression depending on the organism and experimental conditions, which suggests that there is no suitable "universal" reference gene (Lee *et al.* 2002). Therefore, it is essential to validate reference gene(s) prior to examining the effects of different experimental conditions on target gene expression.

Although *A. suturalis* is one of the most destructive pests in major cotton growing regions (Jiang *et al.* 2015), its molecular physiology had not been actively explored due to a lack of molecular resources. However, recent transcriptomic advances (Luo *et al.* 2014; Tian *et al.* 2015) have opened the door for functional genomics research and associated gene-expression studies. However, the lack of dependable information regarding reference genes for *A. suturalis* can limit data normalization and lead to false inferences or misinterpretations (Huggett *et al.* 2005; Ferguson *et al.* 2010). In this study, we used abiotic and biotic conditions to evaluate the expression stability of 12 housekeeping

genes frequently used in expression studies of other sap-sucking insects (Li *et al.* 2013; Bansal *et al.* 2016; Ibanez and Tamborindeguy 2016; Koramutla *et al.* 2016).

To better evaluate the candidate reference genes and to avoid analysis errors caused by selecting co-regulated transcripts, four statistical models (ΔCt method, geNorm, BestKeeper and NormFinder) were used to evaluate gene expression stability. We found that stability rankings frequently varied with the analysis package utilized. For example, when compared across multiple tissues, *RPS15* had the best BestKeeper and geNorm scores, but was not prioritized by NormFinder. These variations can be attributed to differences in the scaling system utilized by the algorithms (Zhai *et al.* 2014; Sagri *et al.* 2017). Although the ranking orders fluctuated according to the analysis software used, the general trends were similar. Genes among the diverse tissues were largely split into two groups by all four algorithms (Table 2), those that were stably expressed (*EF1δ*, *EF1γ*, *EF1α*, *RPL32*, *RPS15*, *RPL27* and *SDH*) and those which exhibited variable expression patterns (*GAPDH*, *ACT*, *TBP*, *βTUB* and *βACT*). Thus, we used RefFinder, a comprehensive platform that integrates all four algorithms
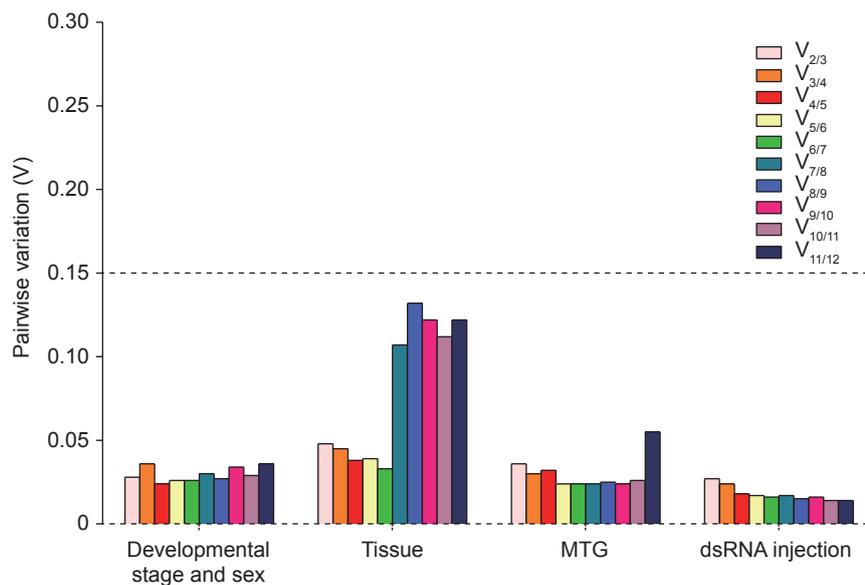
Fig. 4 Determination of the optimal number of reference genes for normalization by geNorm analysis. MTG, metathoracic scent gland. Average pairwise variations (V_n/V_{n+1}) were calculated by geNorm between the normalization factors NF_n and NF_{n+1} to indicate whether inclusion of an extra reference gene would add to the stability of the normalization factor. A value below 0.15 indicates that an additional reference gene will not significantly improve normalization.

to rank the overall stability of candidate genes.

Reference gene suitability can vary in response to diverse biotic and abiotic factors (Table 2; Fig. 3). This result is consistent with previous studies. For example, *EF1α* was stably expressed in *A. suturalis* MTGs at different developmental stages and sexes, whereas its expression was highly variable at the whole-body level under similar conditions. The variation of *EF1α* in different developmental stages and sexes may only occur in certain tissues, e.g., eggs (Fig. 2). This has also been observed in many other species (Pan *et al.* 2015; Tan *et al.* 2015; Bansal *et al.* 2016; Koramutla *et al.* 2016). For example, the expression of *αTUB1* in *Colaphellus bowringi* varied across different developmental stages, though it is stably expressed under different photoperiods (Tan *et al.* 2015).

Furthermore, our verification results showed that normalization of transcripts using *RPL32/RPS15* and *RPS15* alone revealed peak *V-ATPase* expression in the gut (Fig. 5-A), consistent with results from *Culex quinquefasciatus* (Filippova *et al.* 1998), *Holotrichia parallela* (Wei *et al.* 2016), and *Nasutitermes takasagoensis* (Kumara *et al.* 2015). In contrast, normalization with *βACT* suggested the highest expression in MTG. The *FAS* showed a peak expression in the MTG regardless of the reference gene used. The same expression pattern was also observed in *Spodoptera litura* (Lin *et al.* 2018), *Agrotis ipsilon* (Gu *et al.* 2013) and Bumblebee (Zacek *et al.* 2013). Although the trend was similar, normalization with an unsuitable reference gene such as *βACT* not only increased gene expression

levels, but also resulted in larger standard error values (Fig. 5-B). When the target gene was normalized to different reference genes that were either the most or least suitable for different tissue types, there was a dynamic shift in gene expression levels (Fig. 5). This shift in gene expression levels is driven by varied expression of the selected reference gene under varying experimental conditions, and contributes to biased conclusions that are based on calculations utilizing a faulty reference gene dataset (Vandesompele *et al.* 2002; Radonić *et al.* 2004; Huggett *et al.* 2005; Nolan *et al.* 2006; Ling and Salvaterra 2011). Previous studies have demonstrated that this variability under diverse conditions can affect transcript quantification results, leading to false inferences or misinterpretations (Lee *et al.* 2002; Yuan *et al.* 2014; Wang *et al.* 2015; Koramutla *et al.* 2016). All the above underscore the need for validation of reference genes prior to their experimental use.

Here, we found that the quantification results of candidate reference genes varied across tissue types and developmental stages, and that exogenous dsRNA injection has minor impact on gene expression (Fig. 2). This type of variation has also been described in *Halyomorpha halys* (Bansal *et al.* 2016) and *Coleomegilla maculate* (Yang *et al.* 2015b). We speculated that this variation of gene expression in the different tissue types and developmental stages is partly caused by variable RNA extraction efficiencies from different tissues of insects (Huggett *et al.* 2005). To minimize the experimental errors caused by the variability of RNA extraction, a similar sample size with similar tissue volume
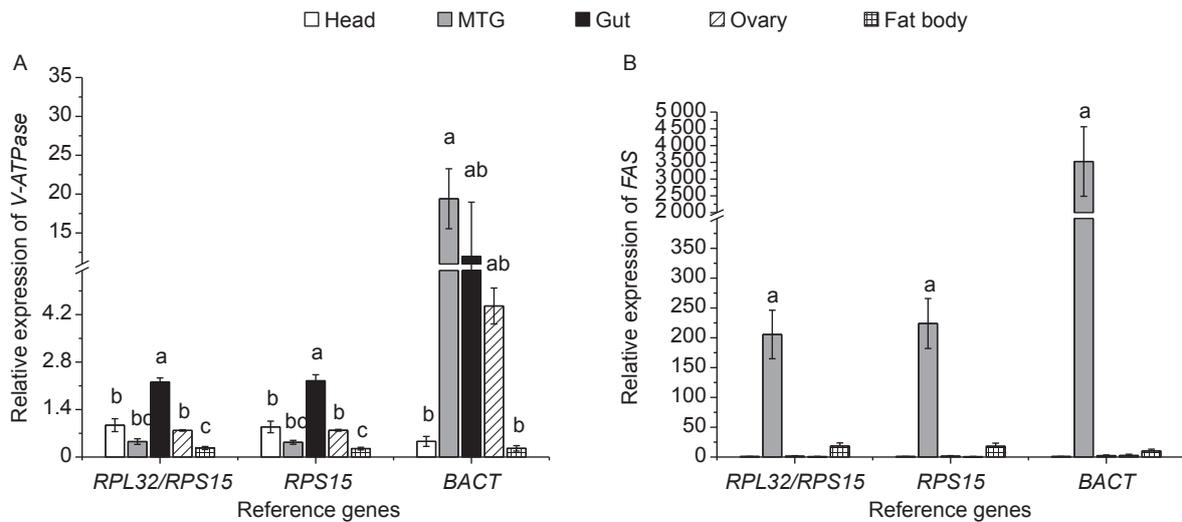
Fig. 5 Validation of reference genes. The relative expression level of *vacuolar-type H⁺-ATPase* (*V-ATPase*, A) and *fatty acid synthase* (*FAS*, B) were normalized using the best reference pair (*RPL32/RPS15* identified by geNorm), the single best reference gene (*RPS15* determined by RefFinder), and the least stable reference gene (*βACT* determined by all five algorithms). The results are depicted as the mean±SE based on three independent biological replicates. Different letters indicate significant differences (*P*<0.05, one-way ANOVA followed by Tukey's HSD Multiple Comparison).

and weight is needed. Then, RNA extraction steps should be optimized to accommodate different type of samples. Finally, it is essential to accurately quantify and assess the quality of RNA prior to reverse transcription (Bustin and Nolan 2004; Huggett *et al.* 2005). RNAi is an effective tool to assess gene function that utilizes introduced dsRNAs to trigger a sequence-specific knockdown of gene expression at the post-transcriptional level (Hannon 2002). Measuring gene expression is thus critical for confirming the reduction in target transcript levels following RNAi-mediated silencing. Many dsRNA delivery systems have been used successfully for this purpose. Among these, injection remains the most frequently used method due to its high efficiency and accuracy (Hughes and Kaufman 2000). The mechanical stress exerted by puncturing the cuticle, or the stress associated with the introduction of exogenous dsRNA, could alter housekeeping gene expression, and affect study of target gene silencing. Therefore, it is crucial to identify and validate reference gene expression stability under variable conditions in order to develop RNAi. Our study found that all used candidate reference genes remained stable under the experimental treatments (Table 2, Fig. 3). However, it is possible that the stability of these reference genes could change when injected with other dsRNAs, and further study on this topic is required.

5. Conclusion

Given the economic importance of *A. suturalis* as an agricultural pest, the application of gene expression analyses and functional genomics can further facilitate basic and applied research on this species. Hence, it is critical to establish a standardized qRT-PCR analysis. The current study identified stable reference genes in *A. suturalis* under an array of biotic and abiotic conditions. Based on a comprehensive analysis integrating five commonly used methods to compare and rank the expression stability of candidate reference genes, we recommend the following genes as the best suited for use in *A. suturalis*: 1) *EF1δ* and *EF1α* for gene expression studies across different developmental stages and sexes; 2) *RPS15* and *EF1δ* for tissue profiling; 3) *RPL32* and *RPS15* for MTGs at different developmental stages and sexes; and 4) *ACT* and *RPS15* for dsRNA-mediated RNAi studies. This is the first study to evaluate suitable reference genes for gene expression analyses in *A. suturalis* and the results will facilitate future research into the transcriptional changes associated with various biological processes.

provider and employer.

Appendices associated with this paper can be available on http://www.ChinaAgriSci.com/V2/En/appendix.htm

References

Andersen C L, Jensen J L, Ørntoft T F. 2004. 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 Research*, **64**, 5245–5250.

Bansal R, Mittapelly P, Chen Y, Mamidala P, Zhao C, Michel A. 2016. Quantitative RT-PCR gene evaluation and RNA interference in the brown marmorated stink bug. *PLoS ONE*, **11**, e0152730.

Bustin S A. 2010. Developments in real-time PCR research and molecular diagnostics. *Expert Review of Molecular Diagnostics*, **10**, 713–715.

Bustin S A, Beaulieu J F, Huggett J, Jaggi R, Kibenge F S B, Olsvik P A, Penning L C, Toegel S. 2010. MIQE précis: Practical implementation of minimum standard guidelines for fluorescence-based quantitative real-time PCR experiments. *BMC Molecular Biology*, **11**, 1–5.

Bustin S A, Benes V, Nolan T, Pfaffl M W. 2005. Quantitative real-time RT-PCR — a perspective. *Journal of Molecular Endocrinology*, **34**, 597–601.

Bustin S A, Nolan T. 2004. Pitfalls of quantitative real-time reverse transcription polymerase chain reaction. *Journal of Biomolecular Technology*, **15**, 155–166.

Butte A J, Dzau V J, Glueck S B. 2001. Further defining housekeeping, or "maintenance," genes Focus on "A compendium of gene expression in normal human tissues". *Physiological Genomics*, **7**, 95–96.

Espy M J, Uhl J R, Sloan L M, Buckwalter S P, Jones M F, Vetter E A, Yao J D, Wengenack N L, Rosenblatt J E, Cockerill F R, Smith T F. 2006. Real-time PCR in clinical microbiology: Applications for routine laboratory testing. *Clinical Microbiology Reviews*, **19**, 165–256.

Ferguson B S, Nam H, Hopkins R G, Morrison R F. 2010. Impact of reference gene selection for target gene normalization on experimental outcome using real-time qRT-PCR in adipocytes. *PLoS ONE*, **5**, e15208.

Filippova M, Ross L S, Gill S S. 1998. Cloning of the V-ATPase B subunit cDNA from *Culex quinquefasciatus* and expression of the B and C subunits in mosquitoes. *Insect Molecular Biology*, **7**, 223–232.

Ginzinger D G. 2002. Gene quantification using real-time quantitative PCR: An emerging technology hits the mainstream. *Experimental Hematology*, **30**, 503–512.

Gu S H, Wu K M, Guo Y Y, Pickett J A, Field L M, Zhou J J, Zhang Y J. 2013. Identification of genes expressed in the sex pheromone gland of the black cutworm *Agrotis ipsilon* with putative roles in sex pheromone biosynthesis and transport. *BMC Genomics*, **14**, 636–636.

Hannon G J. 2002. RNA interference. *Nature*, **418**, 244–251.

Huggett J, Dheda K, Bustin S, Zumla A. 2005. Real-time RT-PCR normalisation; strategies and considerations. *Genes and Immunity*, **6**, 279–284.

Hughes C L, Kaufman T C. 2000. RNAi analysis of deformed, proboscipedia and sex combs reduced in the milkweed bug *Oncopeltus fasciatus*: Novel roles for *Hox* genes in the Hemipteran head. *Development*, **127**, 3683–3694.

Ibanez F, Tamborindeguy C. 2016. Selection of reference genes for expression analysis in the potato psyllid, *Bactericera cockerelli*. *Insect Molecular Biology*, **25**, 227–238.

Jiang Y Y, Lu Y H, Zeng J. 2015. *Forecast and Management of Mirid Bugs in Multiplie Agroecosystems of China*. China Agriculture Press, Beijing. pp. 16–31. (in Chinese)

Koramutla M K, Aminedi R, Bhattacharya R. 2016. Comprehensive evaluation of candidate reference genes for qRT-PCR studies of gene expression in mustard aphid, *Lipaphis erysimi* (Kalt). *Scientific Reports*, **6**, 25883.

Kumara R P, Saitoh S, Aoyama H, Shinzato N, Tokuda G. 2015. Predominant expression and activity of vacuolar H(+)-ATPases in the mixed segment of the wood-feeding termite *Nasutitermes takasagoensis*. *Journal of Insect Physiology*, **78**, 1–8.

Lee P D, Sladek R, Greenwood C M T, Hudson T J. 2002. Control genes and variability: Absence of ubiquitous reference transcripts in diverse mammalian expression studies. *Genome Research*, **12**, 292–297.

Li G P, Feng H Q, Chen P Y, Wu S Y, Liu B, Qiu F. 2010. Effects of transgenic Bt cotton on the population density, oviposition behavior, development, and reproduction of a nontarget pest, *Adelphocoris suturalis* (Hemiptera: Miridae). *Environmental Entomology*, **39**, 1378–1387.

Li R, Xie W, Wang S, Wu Q, Yang N, Yang X, Pan H, Zhou X, Bai L, Xu B, Zhou X, Zhang Y. 2013. Reference gene selection for qRT-PCR analysis in the sweetpotato whitefly, *Bemisia tabaci* (Hemiptera: Aleyrodidae). *PLoS ONE*, **8**, e53006.

Lin X, Wang B, Du Y. 2018. Key genes of the sex pheromone biosynthesis pathway in female moths are required for pheromone quality and possibly mediate olfactory plasticity in conspecific male moths in *Spodoptera litura*. *Insect Molecular Biology*, **27**, 8–21.

Ling D, Salvaterra P M. 2011. Robust RT-qPCR data normalization: Validation and selection of internal reference genes during post-experimental data analysis. *PLoS ONE*, **6**, e17762.

Liu W, Li Y, Zhu L, Zhu F, Lei C L, Wang X P. 2016. Juvenile hormone facilitates the antagonism between adult reproduction and diapause through the methoprene-tolerant gene in the female *Colaphellus bowringi*. *Insect Biochemistry and Molecular Biology*, **74**, 50–60.

Lu Y H, Qiu F, Feng H Q, Li H B, Yang Z C, Wyckhuys K A G, Wu K M. 2008a. Species composition and seasonal abundance of pestiferous plant bugs (Hemiptera: Miridae) on Bt cotton in China. *Crop Protection*, **27**, 465–472.

Lu Y H, Wu K M. 2008. *Cotton Mirid Bugs and its Prevention*. Golden Shield Press, Beijing. pp. 18–19. (in Chinese)

Lu Y H, Wu K M, Cai X M, Liu Y Q. 2008b. A rearing method for mirids using the green bean, *Phaseolus vulgaris* in the laboratory. *Acta Phytophysiologica Sinica*, **35**, 251–269.

Lu Y H, Wu K M, Jiang Y Y, Xia B, Li P, Feng H Q, Wyckhuys K

A G, Guo Y Y. 2010. Mirid bug outbreaks in multiple crops correlated with wide-scale adoption of Bt cotton in China. *Science*, **328**, 1151–1154.

Luo J, Liu X Y, Liu L, Zhang P Y, Chen L J, Gao Q, Ma W H, Chen L H, Lei C L. 2014. *De novo* analysis of the *Adelphocoris suturalis* Jakovlev metathoracic scent glands transcriptome and expression patterns of pheromone biosynthesis-related genes. *Gene*, **551**, 271–278.

Nelson N, Perzov N, Cohen A, Hagai K, Padler V, Nelson H. 2000. The cellular biology of proton-motive force generation by V-ATPases. *Journal of Experimental Biology*, **203**, 89–95.

Nolan T, Hands R, Bustin S. 2006. Quantification of mRNA using real-time RT-PCR. *Nature Protocols*, **1**, 1559–1582.

Pan H P, Yang X W, Siegfried B D, Zhou X G. 2015. A comprehensive selection of reference genes for RT-qPCR analysis in a predatory lady beetle, *Hippodamia convergens* (Coleoptera: Coccinellidae). *PLoS ONE*, **10**, e0125868.

Pfaffl M W, Tichopad A, Prgomet C, Neuvians T P. 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper-Excel-based tool using pair-wise correlations. *Biotechnology Letters*, **26**, 509–515.

Radonić A, Thulke S, Mackay I M, Landt O, Siegert W, Nitsche A. 2004. Guideline to reference gene selection for quantitative real-time PCR. *Biochemical and Biophysical Research Communications*, **313**, 856–862.

Sagri E, Koskinioti P, Gregoriou M E, Tsoumani K T, Bassiakos Y C, Mathiopoulos K D. 2017. Housekeeping in Tephritid insects: The best gene choice for expression analyses in the medfly and the olive fly. *Scientific Reports*, **7**, 45634.

Schmittgen T D, Livak K J. 2008. Analyzing real-time PCR data by the comparative C_T method. *Nature Protocols*, **3**, 1101–1108.

Silver N, Best S, Jiang J, Thein S L. 2006. Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. *BMC Molecular Biology*, **7**, 33.

Tan Q Q, Zhu L, Li Y, Liu W, Ma W H, Lei C L, Wang X P. 2015. A *de novo* transcriptome and valid reference genes for quantitative real-time PCR in *Colaphellus bowringi*. *PLoS ONE*, **10**, e0118693.

Thellin O, Zorzi W, Lakaye B, De Borman B, Coumans B, Hennen G, Grisar T, Igout A, Heinen E. 1999. Housekeeping genes as internal standards: Use and limits. *Journal of Biotechnology*, **75**, 291–295.

Tian C H, Tek Tay W, Feng H Q, Wang Y, Hu Y M, Li G P. 2015. Characterization of *Adelphocoris suturalis* (Hemiptera: Miridae) transcriptome from different developmental stages. *Scientific Reports*, **5**, 11042.

Tillman J A, Seybold S J, Jurenka R A, Blomquist G J. 1999. Insect pheromones ─ an overview of biosynthesis and endocrine regulation. *Insect Biochemistry and Molecular Biology*, **29**, 481–514.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N,

De Paepe A, Speleman F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology*, **3**, 0034.1.

Volpe J J, Vagelos P R. 1973. Saturated fatty acid biosynthesis and its regulation. *Annual Review of Biochemistry*, **42**, 21–60.

Wang X, Xiong M, Wang J, Lei C, Zhu F. 2015. Reference gene stability of a synanthropic fly, *Chrysomya megacephala*. *Parasit Vectors*, **8**, 565.

Wei W, Wei G, Dan Z, Xiaoping Y, Yakun Z. 2016. Characterization of a novel Cry8Ea3-binding V-ATPase subunit A in *Holotrichia parallela*. *Genetics and Molecular Research*, **15**, 15038994.

Wieczorek H, Beyenbach K W, Huss M, Vitavska O. 2009. Vacuolar-type proton pumps in insect epithelia. *Journal of Experimental Biology*, **212**, 1611.

Wong M L, Medrano J F. 2005. Real-time PCR for mRNA quantitation. *BioTechniques*, **39**, 75.

Yang C X, Pan H P, Liu Y, Zhou X G. 2015a. Stably expressed housekeeping genes across developmental stages in the two-spotted spider mite, *Tetranychus urticae*. *PLoS ONE*, **10**, e0120833.

Yang C X, Pan H P, Noland J E, Zhang D Y, Zhang Z H, Liu Y, Zhou X G. 2015b. Selection of reference genes for RT-qPCR analysis in a predatory biological control agent, *Coleomegilla maculata* (Coleoptera: Coccinellidae). *Scientific Reports*, **5**, 18201.

Yuan M, Lu Y, Zhu X, Wan H, Shakeel M, Zhan S, Jin B R, Li J. 2014. Selection and evaluation of potential reference genes for gene expression analysis in the brown planthopper, *Nilaparvata lugens* (Hemiptera: Delphacidae) using reverse-transcription quantitative PCR. *PLoS ONE*, **9**, e86503.

Zacek P, Prchalova-Hornakova D, Tykva R, Kindl J, Vogel H, Svatos A, Pichova I, Valterova I. 2013. *De novo* biosynthesis of sexual pheromone in the labial gland of bumblebee males. *ChemBioChem*, **14**, 361–71.

Zhai Y, Lin Q, Zhou X, Zhang X, Liu T, Yu Y. 2014. Identification and validation of reference genes for quantitative real-time PCR in *Drosophila suzukii* (Diptera: Drosophilidae). *PLoS ONE*, **9**, e106800.

Zhang B Z, Liu J J, Yuan G H, Chen X L, Gao X W. 2018. Selection and evaluation of potential reference genes for gene expression analysis in greenbug (*Schizaphis graminum* Rondani). *Journal of Integrative Agriculture*, **17**, 2054–2065.

Zhang Z, Luo J, Wang Y, Chen L, Chen L, Lei C. 2014. Morphology and chemical analysis of the metathoracic scent glands system in *Adelphocoris suturalis* (Hemiptera: Miridae). *Journal of Insect Science*, **14**, 293.

Zhang Z, Zhang T, Zhang A, Luo J, Chen L, Wang M, Ning J, Lei C. 2016. Identification and field verification of sex pheromone from the mirid bug, *Adelphocoris suturalis*. *Chemoecology*, **26**, 25–31.