Molecular Biology Reports (2024) 51:1028
https://doi.org/10.1007/s11033-024-09975-8

ORIGINAL ARTICLE

Assessment of housekeeping genes stability for gene transcription regulation analysis of *Spodoptera littoralis* (Lepidoptera: Noctuidae) under Spodoptera littoralis nucleopolyhedrovirus viral infection

Wael Elmenofy[1,2] · Mohamed Abdelsattar[2] · Hosny H. Kesba[1,3] · Reem M. Abd El-Maksoud[2]

Received: 30 April 2024 / Accepted: 25 September 2024

Abstract

Background Normalization with respect to stable housekeeping genes is important to facilitate gene transcription regulation research and acquire more accurate quantitative polymerase chain reaction (qPCR) data. In the current study, five candidates housekeeping genes of the cotton leafworm, *Spodoptera littoralis* encoding for Actin (Actin), elongation factor 1-alpha (EF1α), ribosomal protein S3 (RPS3), ribosomal protein 49 (RP49), and Ubiquitin (Ubi), were evaluated as normalization housekeeping genes under Spodoptera littoralis nucleopolyhedrovirus (SpliNPV) viral infection.

Methods and Results The qPCR results confirmed the expression of all five housekeeping genes in *S. littoralis* viral infected larvae. The expression profiles of the housekeeping genes showed that the EF1α, Actin, and RP49 had the minimum average Ct values of 18.41 ± 0.66, 18.84 ± 0.90 and 19.01 ± 0.87 in all infected samples, respectively. While RPS3 and Ubi showed the maximum average Ct of 21.61 ± 0.51 and 21.11 ± 0.82, respectively. According to the results of ΔCt and geNorm analysis, EF1α was ranked as the most stable housekeeping gene during infection time-course. While by using BestKeeper, geNorm and NormFinder, the Ubi, RP49, and RPS3 showed the most genes transcription stability. The obtained results were also validated using the Cytochrome c oxidase (COX) gene transcripts in response to SpliNPV infection.

Conclusions The results revealed that EF1α and Ubi were the most stable housekeeping genes to be used for normalizing *S. littoralis* gene transcription regulation under SpliNPV infection. These findings, provide a significant addition for gene transcription regulation studies of *S. littoralis* upon infection using SpliNPV as a bio-agent.

Keywords *S. littoralis* · Housekeeping genes · Nucleopolyhedrovirus · Gene transcription regulation · qPCR

Introduction

The Egyptian cotton leafworm, *Spodoptera littoralis*, (Boisd.)((Lep.: Noctuidae) is one of the most devastating polyphagous pests across Africa and the Middle East [1]. More than 60 different cultivated and wild plants are attacked by Egyptian cotton leafworm larvae in Egypt, with cotton, clover, maize, wheat, rice, and barley being the most commonly attacked plants [2, 3]. The damage that happened to cotton plants through this species is most severe and prevalent in North Africa, particularly in Egypt [4]. A pest's biological traits, such as its ability to identify hosts and detoxify, are strongly linked to the damage it can cause. Spodoptera species are often able to feed on a wide range of plants that serve as hosts in a variety of environmental conditions. They are therefore regarded as biological models in the research of host and ecological adaptation [5]. Gene expression is the essential manner and is used to study the adaptation mechanisms of insects to different environmental conditions [6]. Quantitative real-time PCR (qRT-PCR), is considered to be a reliable, measure and evaluate changes in gene expression [7–9]. Because of its high sensitivity, the qRT-PCR can

✉ Wael Elmenofy
welmenofy@kfu.edu.sa

✉ Reem M. Abd El-Maksoud
reem.mohsen@ageri.sci.eg

[1] Department of Arid Land Agriculture, College of Agricultural and Food Sciences, King Faisal University, P.O. Box 420, 31982 Al-Hofuf, Saudi Arabia

[2] Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center (ARC), Giza 12619, Egypt

[3] Department of Zoology and Agricultural Nematology, Faculty of Agriculture, Cairo University, Giza 12613, Egypt

Published online: 30 September 2024

detect changes at a very low level of gene transcription [10, 11]. This technique has been extensively used in different fields, such as biotechnology & basic and applied research [12]. Furthermore, it has developed the advancement of biomedical research and gained comparable significance in the field of entomology [13]. Although this powerful technique has many advantages such as simple operation, ease of analysis, high sensitivity, specificity, accuracy, reproducibility, rapidity, and reliability [14, 15], many factors can cause systematic errors and influence the accuracy and quality of qRT-PCR results such as integrity, and purity of total RNA. Furthermore, pipetting errors, PCR program, primer design, and the efficiency of the reverse transcriptase can add technical variability to the obtained data [16–18]. In addition, because housekeeping genes serve as internal controls for normalizing gene transcription regulation upon qRT-PCR utilization, it is crucial to use relevant and reliable housekeeping genes [17, 19]. Hence, housekeeping gene expression level needs to remain constant across many treatments and/or tissue types [20]. The perfect housekeeping genes should have relatively stable expression in various tissues and be unaffected by changes in different environmental and experimental conditions [21, 22]. Several studies have displayed that the common housekeeping genes differ in their expression among diverse species or under different experimental conditions [23, 24]. In insects, numerous studies have been performed to assess and validate the optimal housekeeping genes upon exposure to different biotic and abiotic stresses [25]. It is well known that, standardizing experimental data with two or more housekeeping genes might further get better accuracy and be recommended [26, 27]. Actually, there is none of the housekeeping genes is applicable to all gene expression analyses which makes q-PCR analysis slightly difficult [28, 29]. Furthermore, variations in gene expression frequently indicate physiologically significant changes in various insect phases of development and tissues alteration [30]. Hence, determining the most stable housekeeping genes under different experimental conditions and/ or different developmental stages is crucial in order to assess gene transcription regulation in an accurate and reliable way [31]. In the same context, Cytochrome c oxidase (COX) plays a crucial role in the host defense mechanism of insects, where it is involved in the activation of apoptosis in response to environmental stressors. Notably, some Lepidopterans have evolved to rely on the mitochondrial apoptosis pathway as a means of protecting themselves against external viral infections [32–34]. As a pathogen of the Egyptian cotton leaf worm *S. littoralis*, Spodoptera littoralis multiple nucleopolyhedrovirus (SpliMNPV) is a member of the Baculoviridae family of insect-specific DNA viruses that pose a serious threat to their insect hosts, which showed to be efficient and environmentally safe bio-control agent for the control of *S. littoralis* population [35].

In this study, five candidates housekeeping genes of *S. littoralis* were selected and their expression stabilities were evaluated upon viral infection using SpliNPV in time interval experiments. Hence, the objectives of the current study are to detect the appropriate housekeeping genes for qRT-PCR analysis using of *S. littoralis* as a biological model under viral infection conditions, which might be stringent for confirming the precision of target gene transcription regulation analyses. Moreover, to validate the selected reference genes, the expression profile of target gene cytochrome c oxidase (COX) was investigated.

Material and methods

Insect and virus

Larvae of the cotton leafworm, *S. littoralis* were obtained from the rearing facility of the Agricultural Genetic Engineering Research Institute, Agriculture Research Center. Larvae rearing were performed using semi- artificial diet previously reported by Martins et al. [35] using 26 °C and 65% RH. The *S. littoralis* 4th instars larval were used for virus treatment. The nucleopolyhedrovirus used in this study is the SpliNPV-AN1956 isolate [36].

Treatments and sample collection

Early 4th instar larval of *S. littoralis* were placed in plastic cups with small piece of insect medium inoculated with 10^4 of SpliNPV virus Occlusion Bodies (OBs). One day later, the larvae were transferred to virus-free medium and subsequently reared at 26 °C until larvae death of viral infection. The gut tissue of treated larvae was extracted in a time interval manner at 12, 24, 48 and 72 h post infection (hpi), rinsed in Phosphate-Buffered Saline (PBS), dissected in liquid nitrogen and stored at – 80°C until use. Diet mixed with water instead of SpliNPV virus OBs suspension was used as a negative control (non-infected larvae). Non-infected larvae were sampled at 48 h post treatment to avoid viral cross contamination. Twenty 4th instar larvae were used for each treatment.

Total RNA isolation and cDNA synthesis

Total RNA was extracted from 3 pooled guts tissue, control and all-time interval, using TRIzol reagent following the recommended procedures (ThermoFisher, USA), then total RNA was treated using RNase-free DNase I (ThermoFisher, USA) to get rid of genomic DNA contamination. RNA presence and integrity was checked using 1.0%

agarose gel electrophoresis and quantified by NanoDrop 2000 Spectrophotometer (Thermo Scientific, MA, USA). The first strand of cDNA was synthesized from 1.2 µg of total RNA with RevertAid First Strand cDNA Synthesis kit reverse transcriptase according to the manufacturer's instruction (ThermoFisher, USA). The synthesized cDNA was diluted 1:10 times before use as a template in qPCR analysis.

RT-PCR of polyhedrin gene

The synthesized first-strand cDNA was amplified by PCR using set of specific primers, named Polh560_F (5′-ATC TGGGCAAAACCTATGTAT-3′) and Polh560_R (CTT GGCGAGACTGATGCGGTATTC) designed based on genome sequence of SpliMNPV-AN1956 isolate (accession no. YP_009505893). These primers were used to partially amplify a polyhedrin gene fragment of about 560 bp. A typical PCR program was used as follow; incubation for 3 min at 95 °C, 30 cycles of 95 °C for 30 s, 56 °C for 45 s and at 72 °C for 45 s. The PCR amplicon was electrophoresed in 1% agarose gel and visualized using UV-transilluminator.

Selection of candidate housekeeping gene and primer design

Five genes, Actin, EF1, RPS3, RP49, and Ubi were selected as the candidate housekeeping genes for expression stability in *S. littoralis* during SpliNPV infection. Primers used for Actin, RPS3, RP49, and Ubi qPCR analysis have been designed in previous studies (Table 1) [37–39]. On the other hand, information regarding the primer used for the EF1α and COX (Normalization analysis) have been published by NCBI and designed using the PrimerQuest Tool (https://www.idtdna.com/PrimerQuest/Home/Index) (Table 1).

Quantitative Real-time PCR (qPCR)

Quantitative PCR of the selected genes was assessed by an Agilent Stratagene Mx3005p real-time PCR detection system. PCR reactions were conducted in a total reaction volume of 20 µl. Each reaction contained 10 µl of SYBR™ Green PCR Master Mix (ThermoFisher, USA), 0.3 µl of

Table 1 Primers used for qPCR amplification of housekeeping genes

Gene name	Gene Symbol	Accession	Primer name	Primer sequences (5'-3')	Product length (bp)	Primer efficiency (%)	R2	References
β-actin	ACT	Z46873	Spl-Actin-F	GCGTCGCCCCTG AGGAACAC	100	95.8	0.995	[36]
			Spl-Actin-R	CGACGTACATGG CGGGGGAG				
Elongation factor 1-alpha	EF1α	KP682697.1	Spl- EF1α -F	CTGGTGACTCCA AGAACAAC	100	92.4	0.989	This study
			Spl-EF1α -R	GGTGTGTATCCG TTTGAGATT				
Ubiquitin	UBI	AY149883, AF400203	Spl- Ubi—F	CAAAGATCCAGG ACAAAGAGG GAATCC	80	110	0.997	[36]
			Spl- Ubi—R	CAGGTTGTTGGT GTGTCCACACTT GG				
Ribosomal protein 49	RP49	AY971345	Spl-Rp49-F	AGGTATTGACAA CAGGGTGCG	71	100	0.987	[34] [35]
			Spl-Rp49-R	GGTAGCATGTGA CGGGTCTTC				
Ribosomal protein S3	RPS3	AF429976, U12708, NM_001043788	Spl-RpS3-F	CCAGGCCGAGTC TCTCAGATAC	70	105	0.987	[34] [35]
			Spl-RpS3-F	CTCCAGATTCCA TGATGAAACG				
Cytochrome c oxidase	COX	XM_022966831	COX-F	GTTGTTGCAAGG ATTCATCTC	82	95.8	0.977	This study
			COX-R	CACTCGCTCAAG CAATCT				

each primer at a concentration of 10 µM, 1 µl (1:10 diluted cDNA), and water up to 20 µl total volume. The following program was performed for PCR amplification as follow: 95 °C for 10 min, followed by 45 cycles at 95 °C for 3 s and 60 °C for 30 s, and ending with a melting-curve step.

Using a four-fold dilution series of cDNA as the template, standard curves were created for each primer based on the linear regression model. Three biological and three technical replicates were used for the qPCR analyses for each experiment. To ensure the specificity of the amplifications, melting curve analysis was performed and non-template samples were employed as negative controls. All samples for each housekeeping gene were run on the same plate to avoid between-run variations.

Estimation of COX gene expression

The relative expression levels of the COX gene were examined to verify the chosen housekeeping genes. In order to assess the impact of various normalization approaches, the COX gene expression was normalized using the $2^{-\Delta\Delta Ct}$ method on each of the three chosen housekeeping genes separately [40]. A calibrator sample (expression = 1) was performed using non-infected insect larvae.

Statistical analysis

Each gene's cycle threshold (Ct) value across all samples was performed via qPCR and the five evaluation approaches of RefFinder [41], DeltaCT [42], BestKeeper [43], geNorm [18], and Norm Finder [44], were applied to evaluate stability of each gene expression. These various approaches concentrate on various elements. RefFinder, accessible at http://www.leonxie.com/referencegene.php, was employed to generate a comprehensive ranking based on the stability evaluation of housekeeping genes. It integrates the results obtained from the four aforementioned software tools. In the Delta CT method, housekeeping gene stability is evaluated by comparing the relative expression of pairs of genes within each sample. Another algorithm used for housekeeping gene stability assessment is BestKeeper, which calculates gene stability based on primer amplification efficiency and the Ct-values of housekeeping genes. GeNorm was employed to assess the stability of potential housekeeping genes and establish the optimal number of housekeeping genes for RT-qPCR. The algorithm places significant importance on housekeeping genes with the lowest Ct value, and the value (M) of stability calculated by geNorm is employed to evaluate the housekeeping genes stability. Moreover, NormFinder identifies the most suitable housekeeping genes for normalization by directly measuring expression variation and confirming their stability. Target gene mRNA transcription was analyzed using one-way analysis of variance (ANOVA) and the significant differences between means were compared using Duncan's multiple range test at $P < 0.05$.

Results

RT-PCR and quantitative real-time RT-PCR were applied using cDNA synthesized from total RNA extracted from pooled larval midguts of three fourth instars *S. littoralis* larvae infected with SpliNPV. Using polyhedrin specific primers, a single band of 560 bp was detected corresponding to the polyhedrin gene in all time intervals but not by non-infected larvae, suggested the successful viral infection of *S. littoralis* larvae (Fig. 1).

Specificity of the candidate housekeeping genes

The qPCR results confirmed the expression of all five housekeeping genes in *S. littoralis* samples. Specificity of housekeeping genes amplification was validated by the presence of only one single peak and the absence of primer dimer peaks upon melting curve analysis (Fig. 2A-F). In this study, four-point standard curves were created using established RNA concentrations to evaluate amplification effectiveness. All housekeeping genes were tested for amplification efficiencies to determine their stability during gene expression analyses, with primers efficiency values ranging from 92.4% to 110% (Table 1).

Expression profiles of housekeeping genes

The five selected housekeeping genes of EF1, Actin, Ubi, RP49, and RPS3 were utilized to evaluate their expression profile upon SpliNPV infection. As shown in (Fig. 3), the Ct values for all housekeeping genes

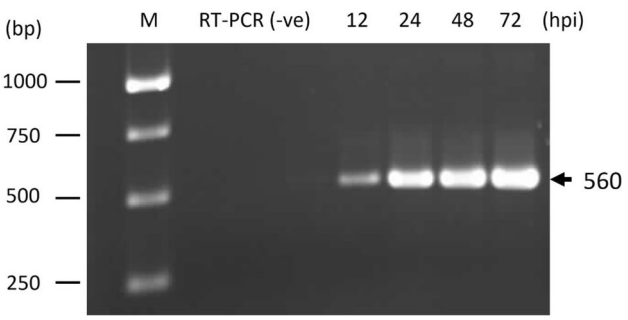

Fig. 1 RT-PCR representing a fragment of of 560 bp coresponding to partial amplification of polyhedrin gene of SpliNPV-AN1956. M: 1kb ladder. RT-PCR -ve: negative control of RT-PCR. Time course of viral infection represents SpliNPV-infected larvae at 12, 24, 48 and 72 hpi. (hpi): hours post infection

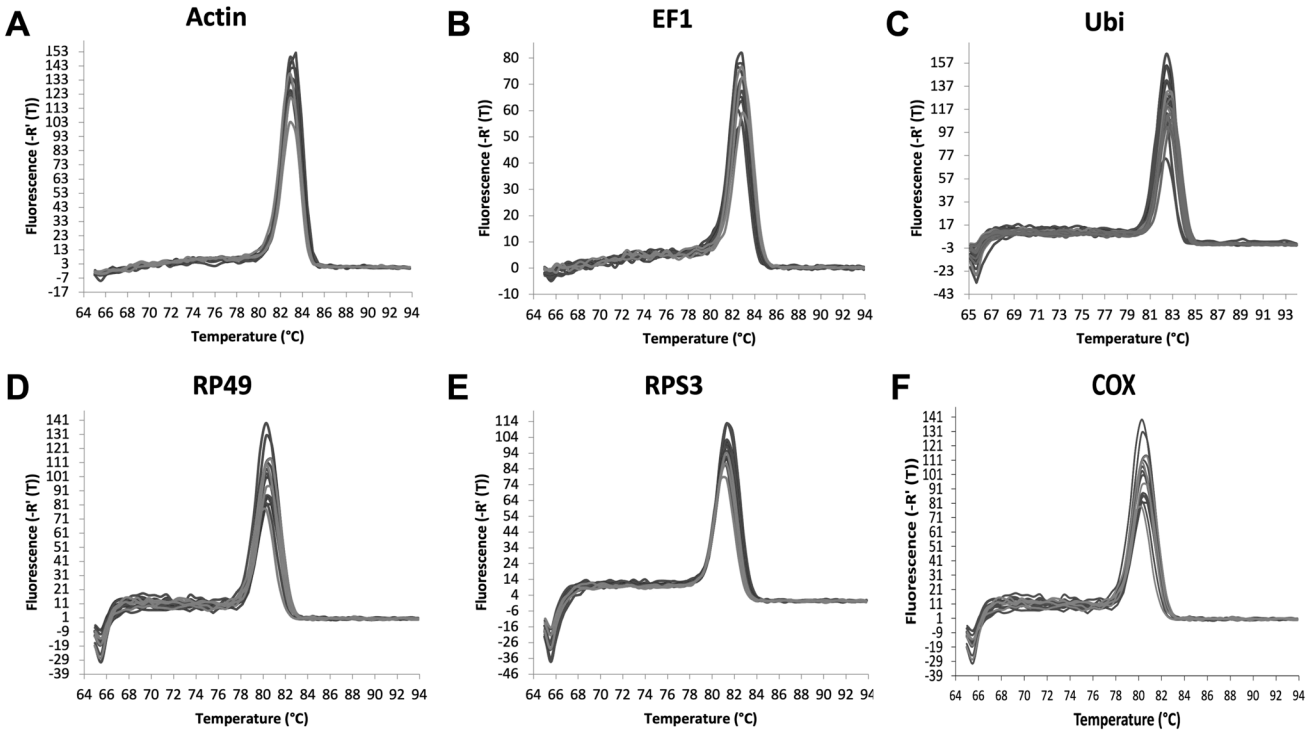

Fig. 2 The melting curve of qPCR analysis. Specificity of qPCR amplification and the melting curve of the five reference genes **A** Actin, **B** EF1, **C** Ubi, **D** RP49, and **E** RPS3 genes and **F** COX gene used for normalization analysis showed one single peak as a T melting point for each housekeeping gene

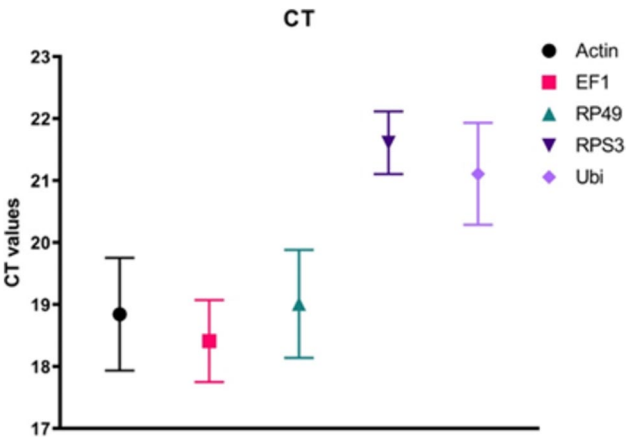

Fig. 3 Variations in cycle threshold (Ct) values of each reference gene. Ct values for all housekeeping genes under study were evaluated during a time course of SpliNPV viral infection at 12, 24, 48 and 72 hpi. Symbol for each gene chart is located to the right. The whiskers represent the minimum and maximum values

evaluated during a time course of viral infection ranged from 17.71 to 22.21. The EF1, Actin, and RP49 housekeeping genes had the minimum Ct values average of 18.41 ± 0.66, 18.84 ± 0.90 and 19.01 ± 0.87, respectively. While RPS3 and Ubi showed the maximum Ct values average of 21.61 ± 0.51 and 21.11 ± 0.82, respectively. The transcription profiles of the housekeeping genes in all time interval showed significant variation. The qPCR analysis showed that the Ct values for all housekeeping genes ranged from 17.93 to 19.72, 17.71 to 19.04, 18.11 to 19.86, 21.10 to 22.10 and 20.25 to 21.92 for Actin, EF1, RP49, RPS3 and Ubi, respectively (Fig. 3).

Determine of gene transcription stability

To analyze the expression stability of the five housekeeping genes in stages under SpliNPV infection of *S. littoralis*, the five algorithms of ΔCt, RefFinder, geNorm, NormFinder, and BestKeeper were used. According to the results of ΔCt and geNorm analysis, *EF1* was ranked as the most stable housekeeping gene during infection times, while the analysis showed that Ubi, *RP49, and RPS3* were the most stable genes using geNorm, NormFinder, and BestKeeper analyses. The following order was displayed by RefFinder's stability rankings: EF1 > Ubi > RPS3 > RP 49 > Actin. Generally, the results of the various algorithms verified that *EF1* and Ubi exhibited more stable expression. However, under SpliNPV infection of *S. littoralis* larvae, Actin and RP49 were consistently regarded as the lowest stable genes in various time intervals (Fig. 4 A-E).

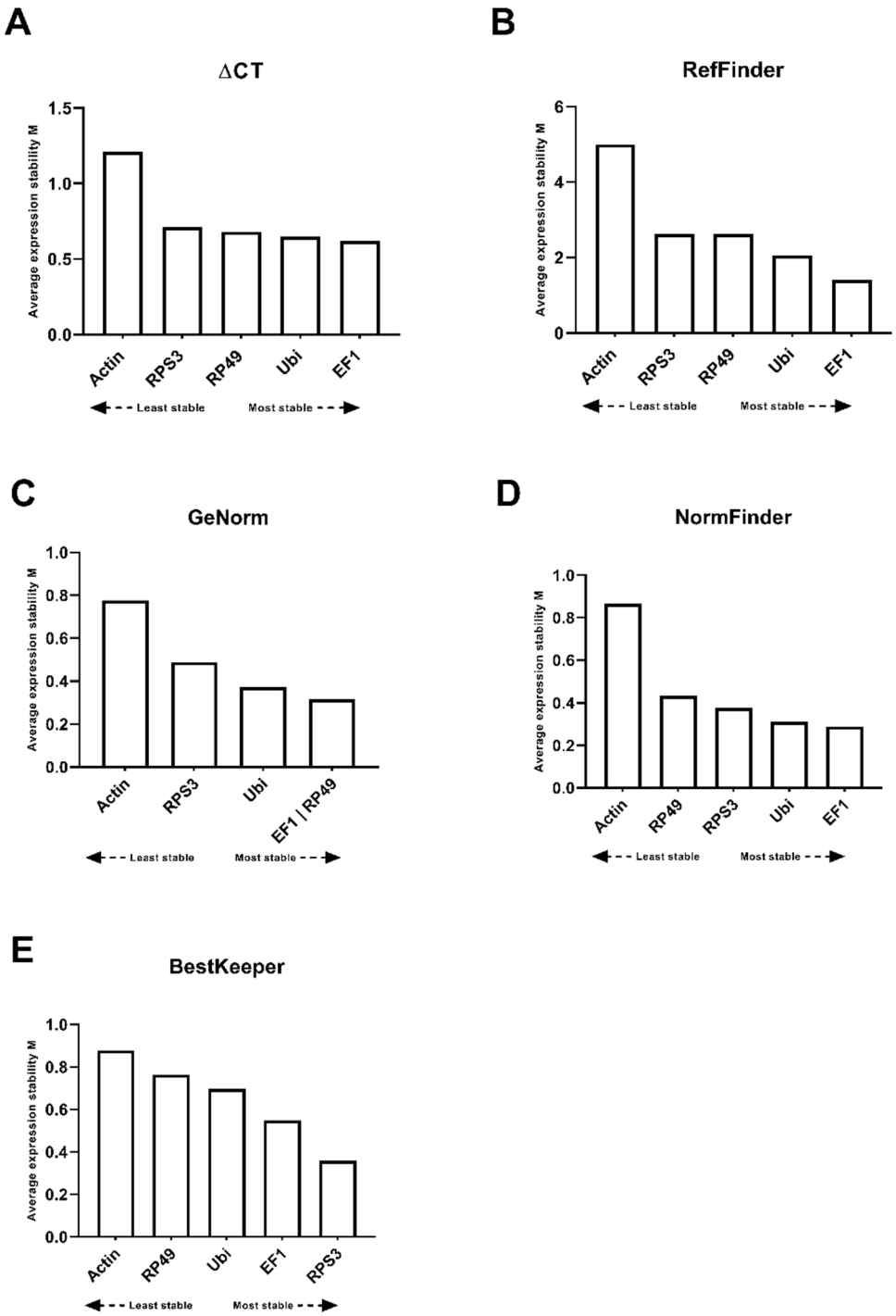

Fig. 4 A column chart representing average expression stability values of candidate reference genes (Actin, RP49, Ubi, EF1 and RPS3) of *S. littoralis* under SpliNPV infection using different algorithms **A** ΔCt, **B** RefFinder, **C** geNorm, **D** NormFinder, and **E** BestKeeper

Determining the expression of COX in response to viral infection

To further assess the reliability of the most stable housekeeping genes (EF1 and Ubi) and the least stable gene (*Actin*), we selected the Cytochrome c oxidase (COX) gene for qPCR normalization analysis. This selection was validated using different methods; ΔCt, RefFinder, geNorm, NormFinder, and BestKeeper, as previously described (Fig. 5). The transcription level of COX was not significantly different at 12- and 24-h post-infection (h.p.i.) when EF1 was used as a housekeeping gene for normalization, compared to the control. However, when Ubi was used for normalization at 24 h.p.i, COX expression was significantly decreased compared to the control. However, when Ubi was used for normalization at 24 h.p.i, COX expression decreased compared to the control. At 48 h.p.i., COX expression was similarly downregulated compared to the control when Actin, EF1, and Ubi

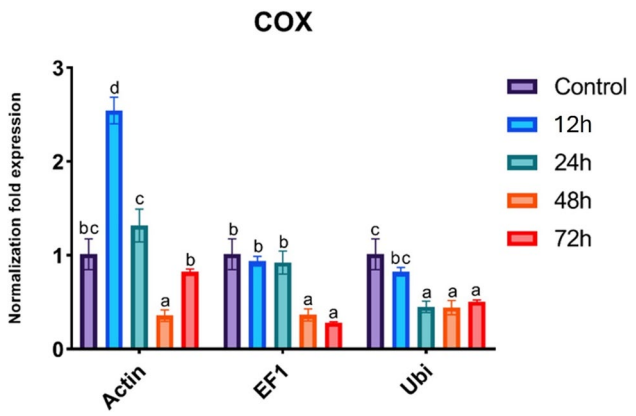

Fig. 5 A column chart showing normalized expression of the COX gene at different time-interval upon SpliNPV infection of S. *littoralis* using validated reference genes (Actin, EF1, and Ubi). Results are represented as mean ± SD. Different letters (a, b) show the statistical difference ($P < 0.05$) among the normalization strategies. Biological replicates $n = 3$

were used as housekeeping genes. We also observed a slight reduction in COX expression patterns at 72 h.p.i. when EF1 and Ubi were used for normalization. Notably, at 12 h.p.i., COX expression was significantly upregulated when *Actin* was used as housekeeping gene, while EF1 and Ubi showed no significant changes compared to the control at the same time of infection. Hence, these results suggested that *Actin* may not be suitable gene for normalization analysis due to its instability.

Discussion

Due to its fast rate of reproduction and significant crop losses, the cotton leafworm, *S. littoralis*, is considered as one of the most damaging insect pests worldwide. The most popular molecular method for examining target gene mRNA expression patterns and confirming RNA interference effectiveness is qPCR, which were extensively employed in *S. littoralis* studies [45]. The primary challenge with qPCR lies in selecting the most stable housekeeping gene (s). No single gene consistently exhibits stable expression in insects across various stresses, including different developmental stages, tissues, temperatures, and exposure to pesticides [46]. Consequently, it is highly important to confirm the housekeeping genes' expression stability prior to conducting qPCR tests. This study was conducted in order to assess the suitable housekeeping genes of the cotton leafworm *S. littoralis* for RT-qPCR investigation under viral infection stress. For this, and in order to define the most stable and representable housekeeping gene, five candidate housekeeping genes of *S. littoralis* encoding for Actin (Actin), elongation factor 1-alpha (EF1 α), ribosomal protein S3 (RPS3), ribosomal protein 49 (RP49), and Ubiquitin (Ubi), were selected. The expression stability of the candidate genes was determined using *S. littoralis* SpliNPV-infected larvae in a time interval infection experiment ranged from 12 to 72 hpi.

For this, five alternative algorithms (ΔCt, BestKeeper, geNorm, NormFinder, and RefFinder) that are commonly used for evaluating the expression stability of housekeeping genes in insects, were utilized to assess the given data [46, 47]. Furthermore, the stability of these housekeeping genes' expression under various SpliNPV infection time interval was assessed, and the best housekeeping gene (s) were suggested. The obtained results showed that the order sequence of the five housekeeping genes shown to be differed within the various experimental conditions. EF1α and Ubi were determined to be the most stable housekeeping genes, while Actin and RP49 exhibited the least stability.

To the best of our knowledge, the current study represents the first study to evaluate the stability of a set of housekeeping genes expression in *S. littoralis* under baculovirus infection.

Overall, the results from the various algorithms indicated that, under SpliNPV infection of *S. littoralis*, EF1 and Ubi showed more stable transcription, while Actin and RP49 were consistently thought to be the lowest stable genes at all time interval.

Out of the five housekeeping genes, Ubi and EF1α were the most suitable housekeeping genes for normalizing the transcripts from baculovirus-infected *S. littoralis*. The transcription elongation factors (EF1 α) and Ubiquitin (Ubi) are frequently used as a housekeeping gene. The EF1 α is considered as highly conserved gene with its ability to combine with RNA polymerase and ensure effective transcription across the nucleosome [48]. Among the top 10 most commonly used housekeeping genes, EF1α was one of the most commonly used housekeeping genes for normalization of qPCR data with respect to its stability in lepidopteran insects under various situations [49]. The EF1α was identified as the most stable gene for different insect developmental stages, such as; *Sesamia inferens*, *Spodoptera exigua*, and *Danaus plexippus* [21, 50, 51]. In addition to EF2 for *Thitarodes armoricanus* and EF1-β for *Phenacoccus solenopsis* [49, 50]. The stability of EF1α was also reported for *Mythimna separata* using different tissue, in addition to EF2 and EIF4A for samples of *Thitarodes armoricanus* upon fungal infections [51, 52]. Hence, in the current study, the obtained results showed that out of the candidate housekeeping genes, EF1α ranked as the most stable genes of *S. littoralis*, under baculovirus infection stress.

An another highly conserved protein is Ubiquitin, which has homologous protein sequences in all animals and is conserved in all eukaryote cells [51]. The Ubi tagging directs the movement of important proteins in the cell, for proteolytic

degradation especially by a proteasome. In the current study, Ubi found to be the most stably expressed genes in the different developmental stages under viral infection-stressed larvae. The results showed that, Ubi was identified as the most stable expressed protein in geNorm, NormFinder, and BestKeeper analyses in conjugation with RP49, and RPS3. Analysis of the expression profile of Ubi showed that it has the maximum average Ct values in most time-interval. This in accordance with southern corn rootworm in which Ubi exhibited the highest Ct values for all treatments [53]. On the other hand, a study in brown marmorated stink bug (BMSB) to identify the best genes to be used as a housekeeping in qPCR, across dsRNA treatments in nymphs and adults of BMSB, Ubiquitin showed lower variation in its Ct values when compared to other common housekeeping genes such as EF1-β and β-Tubulin. Hence, Ubi might be the most stable and reliable gene to target during insect developmental phases [54]. In order to validate the candidate housekeeping genes, expression of COX in response to SpliNPV infection was determined using the most stable housekeeping genes (EF1 and Ubi) and the most unstable gene (Actin), using algorithms ΔCt, RefFinder, geNorm, NormFinder, and BestKeeper as mentioned before. The validation using COX gene confirmed the obtained results, as the COX gene expressions over time were consistent when using the two most stable housekeeping genes for the normalization (EF1 and Ubi), while it was different when using the less stable housekeeping gene (actin). This indicates that the Actin gene may not be suitable for normalization analysis due to its instability upon SpliNPV infection. In different studies, a single housekeeping gene was suggested to standardize the obtained results of qPCR. However, different studies on housekeeping genes stability elucidated that more than one housekeeping genes normally used to avoid incorrect results. Furthermore, utilizing multiple housekeeping genes enhance the normalization of qPCR results [55]. Hence, define the suitable number of housekeeping genes in qPCR studies is highly recommended. Q-PCR are in general acknowledged and broadly used in different biological species, including both animals and plants [56]. Likewise, additional research is required to assess the stability of housekeeping gene expression in insects, particularly lepidopterans.

Conclusion

The expression profile analysis of five candidate housekeeping genes; EF1α, Actin, RPS3, RP49, and Ubi of *S. littoralis* under SpliNPV infection were carried out by qPCR and the transcription regulation stability were analyzed by five different algorithms (ΔCt, BestKeeper, geNorm, NormFinder, and RefFinder). Out of the five housekeeping genes analyzed, Ubi and EF1α were the most stable housekeeping genes for gene transcription normalization of *S. littoralis* under SpliNPV infection. These results also were validated using the COX gene transcription in response to SpliNPV infection. The current study provides an important fundamental step toward establishment of qPCR for normalizing *S. littoralis* gene transcription analysis under SpliNPV infection, which lays the foundation in the future for more research taking into consideration these results.

Acknowledgements All authors extend their gratitude to Deanship of Scientific Research, Vice Presidency for Graduate Studies and Scientific Research, King Faisal University, Saudi Arabia for funding this research work (Grant Number: KFU241570). Furthermore, authors are thankful to Agricultural Genetic Engineering Research Institute, Agricultural Research Center for providing facilities and equipment to finalize this research work.

Authors' contributions RA and MA collected and analyzed the data, WE and RA reviewed and edited this manuscript, HK and WE designed this research and drafted this manuscript. All authors contributed to the analysis and evaluation of the results. The authors RA and WE have contributed equally to this work. All authors contributed to the article and approved the submitted version.

Funding This research was funded by Deanship of Scientific Research, Vice Presidency for Graduate Studies and Scientific Research, King Faisal University, Saudi Arabia, Grant Number: KFU241570.

Data availability No datasets were generated or analysed during the current study.

Declarations

Conflict of interest The authors declare no competing interests.

Ethics approval and consent to participate Not applicable.

Consent for publication Informed consent was obtained from all individual participants included in the study.

References

1. Brown ES, Dewhurst CF (1975) The genus Spodoptera (Lepidoptera: Noctuidae) in Africa and the Near East. Bull Entomol Res 65:221–262
2. Pineda S, Schneider MI, Smagghe G, Martinez AM, Del Estal P, Vinuela E, Valle J, Budia F (2007) Lethal and sublethal effects of methoxyfenozide and spinosad on *S. littoralis* (Lepidoptera: Noctuidae). J Econ Entomol 100:773–780
3. Abd El Mageed AEM, Shalaby SM (2011) Toxicity and biochemical impacts of some new insecticide mixtures on cotton leafworm *S. littoralis* (Boisd.). Plant Prot Sci 47:166–175
4. Hosny MM, Topper CP, Moawad GM, El-Saadany GB (1986) Economic damage thresholds of *S. littoralis* (Boisd.)(Lepidoptera: Noctuidae) on cotton in Egypt. Crop Prot 5:100–104
5. Chao W, Zhang L, Liu B, Gao B, Huang C, Zhang J, Jin M, Wang H, Peng Y, Rice A, Hegazi E, Wilson K, Pengjun X, Xiao Y (2022) Genomic features of the polyphagous cotton leafworm S littoralis. BMC Genom 23:353
6. Lv Z, Jian-Yu M, Hong-Yun R, Chang-Li Y, Chang-Yu Z (2021) Expression stability of candidate RT-qPCR housekeeping genes

in *Spodoptera frugiperda* (Lepidoptera: Noctuidae). Arch Insect Biochem Physiol 108:e21831
7. Gibson UE, Heid CA, Williams PM (1996) A novel method for real time quantitative RT-PCR. Genome Res 6:995–1001
8. Heid CA, Stevens J, Livak KJ, Williams PM (1996) Real time quantitative PCR. Genome Res 6:986–994
9. Huggett J, Dheda K, Bustin S, Zumla A (2005) Real-time RT-PCR normalization: strategies and considerations. Genes Immun 6:279–284
10. Nolan T, Hands RE, Bustin SA (2006) Quantification of mRNA using real-time RT-PCR. Nat Protoc 1:1559–1582
11. Bustin SA, Benes V, Nolan T, Pfaffl MW (2005) Quantitative real-time RT-PCR – a perspective. J Mol Endocrinol I34:597–601
12. Shu B, Zhang J, Zeng J, Cui G, Zhong G (2019) Stability of selected reference genes in Sf9 cells treated with extrinsic apoptotic agents. Sci Rep 9:1–9
13. Cusson M (2008) The molecular biology toolbox and its use in basic and applied insect science. Bioscience 58:691–700
14. Park Y, Kim J, Choi JR, Song J, Chung JS, Lee K-A (2008) Evaluation of multiplex PCR assay using dual priming oligonucleotide system for detection mutation in the Duchenne muscular dystrophy gene. Korean J Lab Med 28:386–391
15. Valasek MA, Repa JJ (2005) The power of real-time PCR. Adv Physiol Educ 29:151–159
16. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL et al (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55:611–622
17. Bustin SA (2002) Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J Mol Endocrinol 29:23–29
18. 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 Biol 3:34
19. Pinheiro DH, Siegfried BD (2020) Selection of reference genes for normalization of RT-qPCR data in gene expression studies in Anthonomus eugenii Cano (Coleoptera: Curculionidae). Sci Rep 10:5070
20. Ponton F, Chapuis MP, Pernice M, Sword GA, Simpson SJ (2011) Evaluation of potential reference genes for reverse transcription-qPCR studies of physiological responses in Drosophila melanogaster. J Insect Physiol 57:840–850
21. Pan H, Yang X, Bidne K, Hellmich RL, Siegfried BD, Zhou X (2015) Selection of reference genes for RT-qPCR analysis in the monarch butterfly, Danaus plexippus (L.), a migrating bio-indicator. PLoS ONE 10:e0129482
22. Chen H, Yang Z, Hu Y, Tan J, Jia J, Xu H et al (2016) Reference genes selection for quantitative gene expression studies in Pinus massoniana L. Trees-Struct Func 30:685–696
23. Mughal BB, Leemans M, Spirhanzlova P, Demeneix B, Fini JB (2018) Reference gene identification and validation for quantitative real-time PCR studies in developing Xenopus laevis. Sci Rep 8:1–9
24. Radonic A, Thulke S, Mackay IM, Landt O, Siegert W, Nitsche A (2004) Guideline to reference gene selection for quantitative real-time PCR. Biochem Biophys Res Commun 313:856–862
25. Zhang S, An S, Li Z, Wu F, Yang Q, Liu Y, Cao J, Zhang H, Zhang Q, Liu X (2015) Identification and validation of reference genes for normalization of gene expression analysis using qRT-PCR in Helicoverpa armigera (Lepidoptera: Noctuidae). Gene 555:393–402
26. Nagy NA, Németh Z, Juhász E, Póliska S, Rácz R, Kosztolányi A et al (2017) Evaluation of potential reference genes for real-time qPCR analysis in a biparental beetle, Lethrus apterus (Coleoptera: Geotrupidae). Peer J 5:e4047
27. Shi C, Yang F, Zhu X, Du E, Yang Y, Wang S et al (2016) Evaluation of housekeeping genes for quantitative real-time PCR analysis of Bradysia odoriphaga (Diptera: Sciaridae). Int J Mol Sci 17:E1034
28. Eisenberg E, Levanon EY (2013) Human housekeeping genes, revisited. Trends Genet 29:569–574
29. Adeyinka OS, Tabassum B, Nasir IA, Yousaf I, Sajid IA, Shehzad K et al (2019) Identification and validation of potential reference gene for effective dsRNA knockdown analysis in Chilo partellus. Sci Rep 9:13629
30. Kubista M, Andrade JM, Bengtsson M, Forootan A, Jonák J, Lind K et al (2006) The real-time polymerase chain reaction. Mol Aspects Med 27:95–125
31. Li M, Li X, Wang C, Li Q, Zhu S, Zhang Y, Li X, Yang F, Zhu X (2021) Selection and validation of reference genes For qRT-PCR analysis of rhopalosiphum padi (Hemiptera: Aphididae). Front Physiol 12:1–13
32. Lu Y, Yuan M, Gao X, Kang T, Zhan S, Wan H, Li J (2013) Identification and validation of reference genes for gene expression analysis using quantitative PCR in Spodoptera litura (Lepidoptera: Noctuidae). PLoS ONE 8:1–9
33. Shan S, Liu K, Peng J, Yao H, Li Y, Hong H (2009) Mitochondria are involved in apoptosis induced by ultraviolet radiation in lepidopteran Spodoptera fitura cell line. Insect Sci 16:485–549
34. Huang JF, Tian M, Lv CJ, Li HY, Muhammad RUH, Zhong GH (2011) Preliminary studies on induction of apoptosis by abamectin in Spodoptera frugiperda (Sf9) cell line. Pestic Biochem Phys 99:256–263
35. Wang XY, Wu KH, Pang HL, Xu PZ, Li MW, Zhang GZ (2019) Study on the role of cytc in response to BmNPV infection in Silkworm, Bombyx mori (Lepidoptera). Int J Mol Sci 20:4325
36. Martins T, Montiel R, Medeiros J, Oliveira L, Simões N (2005) Occurrence and characterization of a nucleopolyhedrovirus from *S. littoralis* (Lepidoptera: Noctuidae) isolated in the azores. J Invertebr Pathol 89:185–192
37. Breitenbacha JE, El-Sheikha A, Rb H, Rowleyb DL, Sparksb ME, Gundersen RindalbDE PHJR (2013) Determination and analysis of the genome sequence of *S. littoralis* multiple nucleopolyhedrovirus. Virus Res 171:194–208
38. Apone F, Ruggiero A, Tortora A, Tito A, Grimaldi MR, Arciello S, Andrenacci D, Lelio ID, Colucci G (2014) Targeting the diuretic hormone receptor to control the cotton leafworm. S littoralis J Insect Sci 14:87
39. Iga M, Smagghe G (2010) Identification and expression profile of Halloween genes involved in ecdysteroid biosynthesis in *S. littoralis*. Peptides 31:456–467
40. Lemeire E, Vanholme B, Van Leeuwen T, Van Camp J, Smagghe G (2008) Angiotensin-converting enzyme in *S. littoralis*: molecular characterization, expression and activity profile during development. Insect Biochem Mol Biol 38:166–175
41. Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative CT method. Nat Protoc 3:1101–1108
42. Xie F, Xiao P, Chen D, Xu L, Zhang B (2012) miRDeepFinder: a miRNA analysis tool for deep sequencing of plant small RNAs. Plant Mol Biol 80:75–84
43. Silver N, Best S, Jiang J, Thein SL (2006) Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. BMC Mol Biol 7:33
44. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP (2004) Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper—Excel-based tool using pair-wise correlations. Biotechnol Lett 26:509–515
45. Andersen CL, Jensen JL, Ørntoft TF (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 Res 64:5245–5250
46. Hebert PDN, Cywinska A, Ball SL, deWaard JR (2003) Biological identiÞcations through DNA barcodes. Proc R Soc Lond B 270:313D321
47. Kang ZW, Liu FH, Tian HG, Zhang M, Guo SS, Liu TX (2017) Evaluation of the reference genes for expression analysis using quantitative real-time polymerase chain reaction in the green peach aphid. Myzus persicae Insect Sci 24:222–234
48. Zhang BZ, Liu JJ, Chen XL, Yuan GH (2018) Selection and evaluation of reference genes for gene expression using quantitative real-time PCR in Mythimna separata Walker (Lepidoptera: Noctuidae). Entomol Res 48:390–399
49. Ehara H, Kujirai T, Fujino Y, Shirouzu M, Kurumizaka H, Sekine SI (2019) Structural insight into nucleosome transcription by RNA polymerase II with elongation factors. Science 363:744–747
50. Lü J, Yang C, Zhang Y, Pan H (2018) Selection of reference genes for the normalization of RT-qPCR data in gene expression studies in insects: a systematic review. Front Physiol 9:1560
51. Sun M, Lu MX, Tang XT, Du YZ (2015) Exploring valid reference genes for quantitative real-time PCR analysis in Sesamia inferens (Lepidoptera: Noctuidae). PLoS ONE 10:e0115979
52. Zheng L, Zhang ZJ, Zhang JM, Li XW, Huang J, Lin WC, Li WD, Li CR, Lu YB (2019) Selection of reference genes for RT-qPCR analysis of Phenacoccus solenopsis (Hemiptera: Pseudococcidae) sex-dimorphic development. J Integr Agric 18:854–864
53. Liu G, Qiu X, Cao L, Zhang Y, Zhan Z, Han R (2016) Evaluation of reference genes for reverse transcription quantitative PCR studies of physiological responses in the ghost moth, Thitarodes armoricanus (Lepidoptera, Hepialidae). PLoS ONE 11:e0159060
54. Basu S, Pereira AE, Pinheiro DH, Wang H, Valencia-Jiménez A, Siegfried BD, Louis J, Zhou X', Vélez AM (2019) Evaluation of reference genes for real-time quantitative PCR analysis in southern corn rootworm, Diabrotica undecimpunctata howardi (Barber). Sci Rep 9:10703
55. Mogilicherla K, Howell JL, Palli SR (2018) Improving RNAi in the brown marmorated stink bug: identification of target genes and reference genes for RT-qPCR. Sci Rep 8:3720
56. Ling D, Salvaterra PM (2011) Robust RT-qPCR data normalization: validation and selection of internal reference genes during post-experimental data analysis. PLoS ONE 6:e17762

Terms and Conditions

Springer Nature journal content, brought to you courtesy of Springer Nature Customer Service Center GmbH (“Springer Nature”).
Springer Nature supports a reasonable amount of sharing of research papers by authors, subscribers and authorised users (“Users”), for small-scale personal, non-commercial use provided that all copyright, trade and service marks and other proprietary notices are maintained. By accessing, sharing, receiving or otherwise using the Springer Nature journal content you agree to these terms of use (“Terms”). For these purposes, Springer Nature considers academic use (by researchers and students) to be non-commercial.
These Terms are supplementary and will apply in addition to any applicable website terms and conditions, a relevant site licence or a personal subscription. These Terms will prevail over any conflict or ambiguity with regards to the relevant terms, a site licence or a personal subscription (to the extent of the conflict or ambiguity only). For Creative Commons-licensed articles, the terms of the Creative Commons license used will apply.
We collect and use personal data to provide access to the Springer Nature journal content. We may also use these personal data internally within ResearchGate and Springer Nature and as agreed share it, in an anonymised way, for purposes of tracking, analysis and reporting. We will not otherwise disclose your personal data outside the ResearchGate or the Springer Nature group of companies unless we have your permission as detailed in the Privacy Policy.
While Users may use the Springer Nature journal content for small scale, personal non-commercial use, it is important to note that Users may not:

1. use such content for the purpose of providing other users with access on a regular or large scale basis or as a means to circumvent access control;
2. use such content where to do so would be considered a criminal or statutory offence in any jurisdiction, or gives rise to civil liability, or is otherwise unlawful;
3. falsely or misleadingly imply or suggest endorsement, approval , sponsorship, or association unless explicitly agreed to by Springer Nature in writing;
4. use bots or other automated methods to access the content or redirect messages
5. override any security feature or exclusionary protocol; or
6. share the content in order to create substitute for Springer Nature products or services or a systematic database of Springer Nature journal content.

In line with the restriction against commercial use, Springer Nature does not permit the creation of a product or service that creates revenue, royalties, rent or income from our content or its inclusion as part of a paid for service or for other commercial gain. Springer Nature journal content cannot be used for inter-library loans and librarians may not upload Springer Nature journal content on a large scale into their, or any other, institutional repository.
These terms of use are reviewed regularly and may be amended at any time. Springer Nature is not obligated to publish any information or content on this website and may remove it or features or functionality at our sole discretion, at any time with or without notice. Springer Nature may revoke this licence to you at any time and remove access to any copies of the Springer Nature journal content which have been saved.
To the fullest extent permitted by law, Springer Nature makes no warranties, representations or guarantees to Users, either express or implied with respect to the Springer nature journal content and all parties disclaim and waive any implied warranties or warranties imposed by law, including merchantability or fitness for any particular purpose.
Please note that these rights do not automatically extend to content, data or other material published by Springer Nature that may be licensed from third parties.
If you would like to use or distribute our Springer Nature journal content to a wider audience or on a regular basis or in any other manner not expressly permitted by these Terms, please contact Springer Nature at

onlineservice@springernature.com