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Research

Evaluation of Reference Genes for Normalization of RT-qPCR Gene Expression Data for *Trichoplusia ni* Cells During *Antheraea pernyi* (Lepidoptera: Saturniidae) Multicapsid Nucleopolyhedrovirus (AnpeNPV) Infection

Zhenjun Zhao, Linmei Wang, Dongmei Yue, Bo Ye, Peipei Li, Bo Zhang, and Qi Fan[1]

Dalian Institute of Biotechnology, Liaoning Academy of Agricultural Sciences, No.2, Shida Street, Dalian 116024, P. R. China and [1]Corresponding author, e-mail: qifan10001@163.com

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Abstract

Baculovirus infection impacts global gene expression in the host cell, including the expression of housekeeping genes. Evaluation of candidate reference genes during a viral infection will inform the selection of appropriate reference gene(s) for the normalization of expression data generated by ReverseTranscription Quantitative Real-time Polymerase Chain Reaction (RT-qPCR). *Antheraea pernyi* multicapsid nucleopolyhedrovirus (AnpeNPV) is able to infect the High Five cells (Tn-Hi5). In the present study, 10 candidate reference genes were evaluated in AnpeNPV-infected Tn-Hi5 cells. Gene expression data were analyzed using geNorm, NormFinder, BestKeeper, and RefFinder. The candidate genes were further validated as reliable reference genes for RT-qPCR by analyzing the expression of three target genes. The results of data analysis using four statistical methods showed that *RPS18* was the least stable among the candidate reference genes tested. *18S rRNA* and *28S rRNA* were not suitable as reference genes for RT-qPCR analysis in AnpeNPV-infected Tn-Hi5 cells. Comprehensive ranking of the 10 candidate reference genes by RefFinder analysis indicated that *Ann B, c45128_g1,* and *ACT* were the top three genes. Normalization of the expression of three target genes using the candidate reference genes indicated the same expression pattern when *Ann B* and *c45128_g1* were used as reference genes, with slight differences in the relative expression at each infection time point. Overall, *Ann B* and *c45128_g1* were recommended to be more suitable than the most commonly used reference genes, such as *ACT*, *GAPDH*, and *TUB,* for RT-qPCR data normalization in AnpeNPV-infected Tn-Hi5 cells up to 48 hpi.

Key words: RT-qPCR, reference gene, *Antheraea pernyi*, nucleopolyhedrovirus, *Trichoplusia ni* cell

Gene expression analyses have become extremely important for uncovering gene function and the molecular mechanisms that regulate the different responses observed during the insect life cycle or during insect infection by various pathogens. Reverse-transcription quantitative real-time polymerase chain reaction (RT-qPCR) is an accurate and reliable technique for quantifying gene expression (Bustin et al. 2009, Bustin and Nolan 2017, Shakeel et al. 2018). The accuracy of RT-qPCR data is highly dependent on the appropriate reference genes, whose expression has been validated for use for normalization of gene expression of other genes to avoid bias caused by differences in the initial sample amount, quality and integrity of RNA samples, and the efficiency of cDNA synthesis and PCR (Huggett et al. 2005). The ideal reference gene should be stably expressed in all tissues and development stages under different experimental conditions, and its transcript levels should be similar to those of analyzed target genes. Housekeeping genes are commonly validated as reference genes, and a combination of reference genes is recommended to normalize RT-qPCR data of target genes across samples (Bustin et al. 2009, Chapman and Waldenstrom 2015). Housekeeping genes, such as *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), *18S* and *28S ribosomal RNA* (*rRNA*), *ribosomal protein L3* (*RPL3*), α-*tubulin* (*TUB*), and *actin* (*ACT*), have been used as reference genes in different insect cells (Maroniche et al. 2011, Salem et al. 2011, Nguyen et al. 2012, Xue et al. 2012, Fang et al. 2015, Yu et al. 2016b). However, transcript levels of host genes, including housekeeping genes, vary during bacterial or viral infections. For example, infection of Tnms42 and Sf9 cell lines with *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) leads to an increase in viral transcripts and decline of host cell transcripts over the course of infection (Salem et al. 2011, Chen et al. 2014). Similarly, *Bombyx mori* NPV (BmNPV) infection of insect cells leads to a global

down-regulation of host gene expression at later time points (Xue et al. 2012). The expression of commonly used reference genes such as beta-actin (β-actin), *GAPDH*, and tubulin genes, in Sf9 cells is affected by AcMNPV infection (Salem et al. 2014). To accurately determine gene expression patterns by RT-qPCR under new experimental (infection) conditions, the candidate reference genes should be evaluated.

The nucleopolyhedrovirus is a type of baculovirus. Baculoviruses are invertebrate viruses with a narrow host range, generally limited to just one or few closely related insect species. They harbor large, circular, double-stranded, supercoiled DNA genomes with sizes varying from approximately 80 kb to over 180 kb. The genomes encode 90–180 genes with roles in viral replication and assembly during the infection process, and in manipulating cellular processes and host behavior (Braunagel et al. 1998, Clem 2007, Rohrmann 2013). These viruses are widely used as biopesticides in agriculture, as gene expression vectors for the production of recombinant proteins in research and the industry, and as gene delivery systems for transduction of mammalian cells (Szewczyk et al. 2006, Chen et al. 2011, Assenberg et al. 2013). Although extensive research into baculovirus gene functions will help to better understand the virus infection cycle (Rohrmann 2013), knowledge of the host responses to baculovirus infection, such as pathogen recognition, signal transduction, and defensive response, is still at an early stage. Studies of baculovirus-insect interactions at the molecular level will facilitate the engineering of these viruses for their applications and improve understanding of insect antiviral defenses.

The Chinese oak silkworm *Antheraea pernyi* is an economically important insect not only for the production of the tussah silk, but also as a food delicacy. *Antheraea pernyi* multicapsid nucleopolyhedrovirus (AnpeNPV), one of the major pathogens of *A. pernyi*, can cause jaundice of the oak silkmoth and poses a great threat to the tussah industry (Liaoning Institute of Sericulture 2003). The AnpeNPV genome comprises 126, 246 bp encoding 145 predicted open reading frames (ORFs) of more than 50 amino acids each (Fan et al. 2007). Because of their large size, *A. pernyi* pupae have been used as a bioreactor for recombinant protein production using an AnpeNPV-based system (Zhang et al. 1992b, Zhang et al. 1992a, Huang et al. 2001, Wang et al. 2010, Ye et al. 2014). AnpeNPV infects *A. pernyi* and *Philosamia cynthia*, but not *B. mori* or cells of *Spodoptera frugiperda* (e.g., Sf9 and Sf21). Recently, it has been shown that recombinant AnpeNPV, *ApNPV-Δph/egfp*$^+$ (Wang et al. 2010), in which the *polyhedrin* (*ph*) gene of AnpeNPV has been replaced by the *enhanced green fluorescent protein* (*egfp*) gene under the control of the *ph* promoter, is able to infect a non-host cell, High Five (BTI-TN-5B1-4, Tn-Hi5), derived from *Trichoplusia ni* (Zhao et al. 2015). Tn-Hi5 cell is a permissive host for AcMNPV but not for AnpeNPV. Viral and host gene expression for the new virus-host combination of AnpeNPV infection in Tn-Hi5 cells has not yet been investigated.

In the current study, 10 candidate genes of Tn-Hi5 cells were selected for evaluation as reference genes for RT-qPCR data normalization during AnpeNPV infection. Abundances of gene transcripts during the course of viral infection were analyzed. Four data analysis programs, geNorm (Vandesompele et al. 2002), NormFinder (Andersen et al. 2004), BestKeeper (Pfaffl et al. 2004), and RefFinder (Xie et al. 2012) were used to identify the most stably expressed reference gene (s). These genes were further validated by using them in the normalization of the expression of three target genes in AnpeNPV-infected Tn-Hi5 cells.

Materials and Methods

Cell Lines, Virus, and Infection

Alphanodavirus-free Tn-Hi5 cell line (BTI-TN-5B1-4) was purchased from Thermo Fisher Scientific (United States). Cells were cultured in TNM-FH medium (GE Healthcare Life Sciences) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco) containing 0.5% penicillin-streptomycin solution (Gibco) at 27°C. *ApNPV-Δph/egfp*$^+$ (Wang et al. 2010) was propagated in *A. pernyi* pupae and used to infect the cell line. Viral titer was determined by an end-point dilution assay with Tn-Hi5 cells according to a published method (Cha et al. 1997). For the infection, 1×10^6 Tn-Hi5 cells were seeded into wells of a 6-well tissue culture plate (Falcon) and infected with *ApNPV-Δph/egfp*$^+$ (multiplicity of infection: 10). The *ApNPV-Δph/egfp*$^+$ inoculum was removed after 1 h. The cells were rinsed with SF-900TM II medium (Gibco) and cultured in TNM-FH medium supplemented with 10% FBS at 27°C. The time at which the inoculum was removed was considered as 0 hpi. Cells were collected at 6, 12, 18, 24, 36, and 48 hpi. Uninfected cells (mock infection) were collected at 48 h as the control. Three independent biological replicates were prepared at each time point.

Total RNA Extraction, cDNA Synthesis, Library Construction, and RNA-Seq

Total RNA was extracted at each time point and from the control samples immediately after cell collection, using a tissue RNA kit (BIOMIGA), and then treated with DNase I (BIOMIGA) according to the manufacturer's instructions. RNA concentration and quality were checked using the NanoPhotometer spectrophotometer (Implen, CA) and Agilent 2100 Bioanalyzer (Agilent Technologies, CA). OD_{260}/OD_{280} absorption ratio ranged from 1.9 to 2.0, and RNA integrity numbers (RINs) were > 6.0. First-strand cDNA was synthesized using 500 ng of DNase-free total RNA using M-MLV (Invitrogen, Shanghai, China) with oligo-$(dT)_{18}$ primer (TaKaRa, Dalian, China) in a final reaction volume of 20 µl, and stored at –20°C. The cDNA products were diluted 10-fold in nuclease-free water to use as templates in RT-qPCR analysis.

To obtain accurate gene information on Tn-Hi5 cells, mRNA library preparation and RNA-Seq were performed by Novogene Bioinformatics Technology Co., Ltd. (Beijing, China). In brief, 3 µg RNA of Tn-Hi5 cells was used to generate the sequencing library with NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, United States), following manufacturer's recommendations. The mRNA library was sequenced on an Illumina Hiseq 2500 platform and 125 bp/50 bp paired/single-end reads were generated. Clean reads of the data were obtained by filtering out reads containing an adapter or a ploy-N and low-quality reads from the raw data. Transcriptome assembly was accomplished using the Trinity software (Grabherr et al. 2011). The assembled unigenes were annotated based on NCBI nonredundant protein sequences (Nr), NCBI nonredundant nucleotide sequences (Nt), Protein family (Pfam, http://pfam.sanger.ac.uk/), and Gene Ontology (GO, http://www.geneontology.org/) databases (Zhao et al., unpublished data).

Candidate Reference Genes and Primer Design

Based on previous reports concerning insect systems, nine housekeeping genes were initially selected for evaluation as references for RT-qPCR data normalization. These included *28S rRNA* and *ecdysoneless* (*ECD*) genes from Sf21 cells (Salem et al. 2011, Salem et al. 2014), *18S rRNA* and *GAPDH* genes from Bm5 cells (Xue et al. 2012), *ACT* gene from Sf9 cells (Mehrabadi et al. 2015), α-*TUB* gene from *B. mori* (Fang et al. 2015), *ribosomal protein S18* (*RPS18*) gene from *Coleomegilla*

maculata (Maroniche et al. 2011, Yang et al. 2015), *annexin B* (*Ann B*) gene from *Diploptera punctata* (Marchal et al. 2013), and *mitochondrial ribosomal protein L50* (*mRPL50*) genes from *Ericerus pela* (Yu et al. 2016c). In the current study, sequences homologous to *18S rRNA*, *Ann B*, *ECD*, *GAPDH*, *mRPL32*, *RPS18*, α-*TUB*, and *ACT* genes were extracted from the unpublished Tn-Hi5 transcriptome data (Zhao et al., unpublished data). These sequences were deposited in the NCBI GenBank (accession numbers KY514086–KY514092 and MH286943). The *28S rRNA* sequence was from NCBI GenBank (accession numbers EU771090). The *phosphoglycerate kinase 1* (*PGK1*), which encodes a key glycolysis and gluconeogenesis enzyme, has been recently identified as a stable reference gene in whole blood of human and the blood of bottlenose dolphin (Falkenberg et al. 2011, Chen et al. 2015). An uncharacterized gene from the unpublished Tn-Hi5 transcriptome data (*c45128_g1*), annotated as encoding PGK by Pfam, was also selected for evaluation (GenBank accession number KY514093) in the present study. Gene-specific primers were designed; they were 21–23 bp, and with a GC content of 45–55%. Amplicon sizes ranged from 113 to 152 bp (Table 1).

Reverse Transcription Quantitative Real-time Polymerase Chain Reaction (RT-qPCR)

RT-qPCR was performed using SYBR Green-based detection and a 7300 real-time PCR system (Applied Biosystems, United States), and analyzed using 7300 System SDS software. Each reaction mixture contained 2 µl of 10-fold diluted cDNA, 0.4 µM of each gene-specific forward and reverse primer, 10 µl of 2×SYBR Premix Ex Taq II (Tli RNaseH Plus) PCR master mix (TaKaRa, Dalian, China), and nuclease-free water in a total volume of 20 µl. The thermal cycling conditions were as follows: 30 s at 95°C, followed by 40 cycles of 5 s at 95°C and 31 s at 60°C, and a final dissociation-curve step of 15 s at 95°C, 60 s at 60°C, and 15 s at 95°C, to verify the consistency and specificity of each reaction. Three technical replicates were performed for each biological sample. Nuclease-free water without the template was assayed as the negative control for each primer pair.

To determine the RT-qPCR efficiency of each primer pair, 10-fold serially diluted cDNA samples (10^0 to 10^{-5} dilutions) of pooled cDNA were used as templates to construct the standard curves. Each reaction was performed in triplicate under the conditions described above. The standard curve of each primer pair was generated based on the linear relationship between the mean threshold cycle (Ct) values and serial dilutions ($-\log_{10}$ concentration) by using the 7300 System SDS software. The amplification efficiency (E) was determined using the slopes of the standard curves: $E\ (\%) = 10^{(-1/\text{slope})} - 1$ (Bustin et al. 2009).

Data Statistics and Expression Stability Analysis

Expression stability of each candidate reference gene was analyzed using four different algorithms: geNorm (http://medgen.ugent.be/~jvdesomp/genorm/), NormFinder_0953 (http://moma.dk/normfinder-software), BestKeeper version 1 (http://www.gene-quantification.de/bestkeeper.html), and RefFinder (http://150.216.56.64/reference-gene.php), following the developers' instructions. The average Ct values from each sample of all assayed candidate reference genes were converted into relative quantities using the formula $2^{-\Delta Ct}$, where ΔCt is obtained after subtracting the lowest Ct value from the Ct value of the candidate reference gene from the corresponding sample (ΔCt = each sample Ct value − the lowest Ct value). The obtained relative quantities were then used for geNorm and NormFinder analyses. For BestKeeper and RefFinder analysis, the average Ct values from each sample were directly used. The relative transcript level of each target gene was calculated according to the $2^{-\Delta\Delta Ct}$ method for gene normalization to each candidate reference gene (Schmittgen and Livak 2008). The homologs of *caspase-4*, *caspase-5*/*dronc*, and *inhibitor of apoptosis 2* (*iap2*) genes from *B. mori*, *Spodoptera exigua*, and *Drosophila melanogaster*, respectively, identified in Tn-Hi5 cells (GenBank accession numbers KY514094–KY514096) were selected as target genes for normalization. Specific primers for the three target genes are provided in Supp. Table S1. The relative transcript levels in the mock-infected samples were set as 1 and the relative transcript levels in the 6–48 hpi samples were calculated accordingly. All other statistical analyses were performed using Microsoft Excel 2007. Student's *t*-test was performed on the relative transcript levels of the samples with the lowest and the highest $2^{-\Delta\Delta Ct}$ value at different infection time points for each candidate reference gene. P-value < 0.05 was considered to indicate significant differences between samples.

Table 1. Candidate reference genes in expression studies by RT-qPCR in Tn-Hi5 cells

Gene symbol	Accession number	Primer Sequences (5′–3′)	Amplicon length (bp)	PCR efficiency (E)	R^2
18S rRNA	KY514086	F: TGAGAAACGGCTACCACATCC R: GATTACGAGGCCTCGTAAGAG	115	0.93	0.9946
28S rRNA	EU771090	F: GATGGAGCGTCGATCTAGGT R: GCATTCACGGATTGGAAACGAG	66	1.04	0.9803
ACT	MH286943	F1: ATCGCTGACCGTATGCAGAAG R1: TTCGAGATCCACATCTGCTGG	152	0.94	0.9989
Ann B	KY514087	F: TCATGACTTCTCTTTCCATGGG R: AAGACGGATTCGTCTGTACCGAG	127	1.03	0.9901
ECD	KY514088	F: ACACAGGCAGCACATAGTATTG R: GCTAATGTAGAGGGCACCTTGAC	113	1.04	0.9868
GAPDH	KY514089	F: CATACAAGGTCATCTCCAACGC R: AAGGTCCGTCAACAGTCTTCTG	147	0.96	0.9974
mRPL32	KY514090	F: TGTTCCGAAATTCCGTCGCACC R: ATGATGATGGCCACAATCCTGAC	127	0.95	0.9953
RPS18	KY514091	F: GGACTTGGAAAGGCTCAAGAAG R: ACACCAACAGTTCTTCCTCTCC	117	0.97	0.9952
α-*TUB*	KY514092	F: TTACCGAGTTCCAGACCAAC R: AAGCAGGCGTTGGTGATCTC	133	1.03	0.9985
c45128_g1	KY514093	F: ACGATGCCTGTGCCGTATGTAC R: AAGGGTAGAGCGCAGTCATTCG	122	1.10	0.9994

Results

Analysis of Primer Specificity and PCR Efficiency

The specificity of primers for 10 candidate reference genes was determined by examining the dissociation curves after RT-qPCR of serially diluted cDNA samples. A single peak for each primer set was observed after 40 cycles (Fig. 1). All primer pairs produced unique products without primer dimers or other nonspecific amplification products. Visualization of the amplification products in the agarose gel further confirmed that all unique products were of the expected length (Fig. 2). The amplification efficiency (E) of these candidate reference genes varied from 0.93 for *18S rRNA* to 1.10 for *c45128_g1*. Correlation coefficient (R^2) values ranged from 0.9803 to 0.9994 (Table 1).

Expression Profiling of Candidate Reference Genes

The overall variability of expression of each candidate reference gene in the tested samples was determined based on the average Ct values and SD analysis (Fig. 3, Table 2). According to the analysis of the mean Ct values of 10 candidate reference genes, the minimum Ct value of the *28S rRNA* gene was 13.43 ± 0.22, indicating the highest transcript abundance, whereas that of the *c45128_g1* gene was 26.71 ± 1.11, indicating the lowest transcript abundance (Table 2). The Ct values of the *28S rRNA* and *18S rRNA* genes were relatively constant (13.17–13.59 and 14.14–14.52, respectively), with low SD values throughout the infection and in the mock-infected sample (Table 2). For the other eight candidate reference genes, the mean Ct values ranged from 15.48 ± 0.37 to 24.22 ± 0.39 in the mock-infected samples but increased (21.90 ± 0.85 to 27.09 ± 0.64) at 6 hpi, and remained relatively stable up to 48 hpi (Table 2). This indicated that transcript abundances of these genes declined after 6 hpi. Of these, mean Ct values of *c45128_g1* changed over a narrow range, from 24.22 ± 0.39 to 27.36 ± 0.36, whereas those of *RPS18* ranged widely from 15.48 ± 0.37 to 23.75 ± 0.62, during viral infection and in mock-infected samples (Table 2).

Evaluation of the Expression Stability of Candidate Reference Genes

geNorm Analysis

The geNorm software identifies the most stable reference gene based on the average pairwise variation between a particular gene and all other genes whose expression stability (M-value) is analyzed

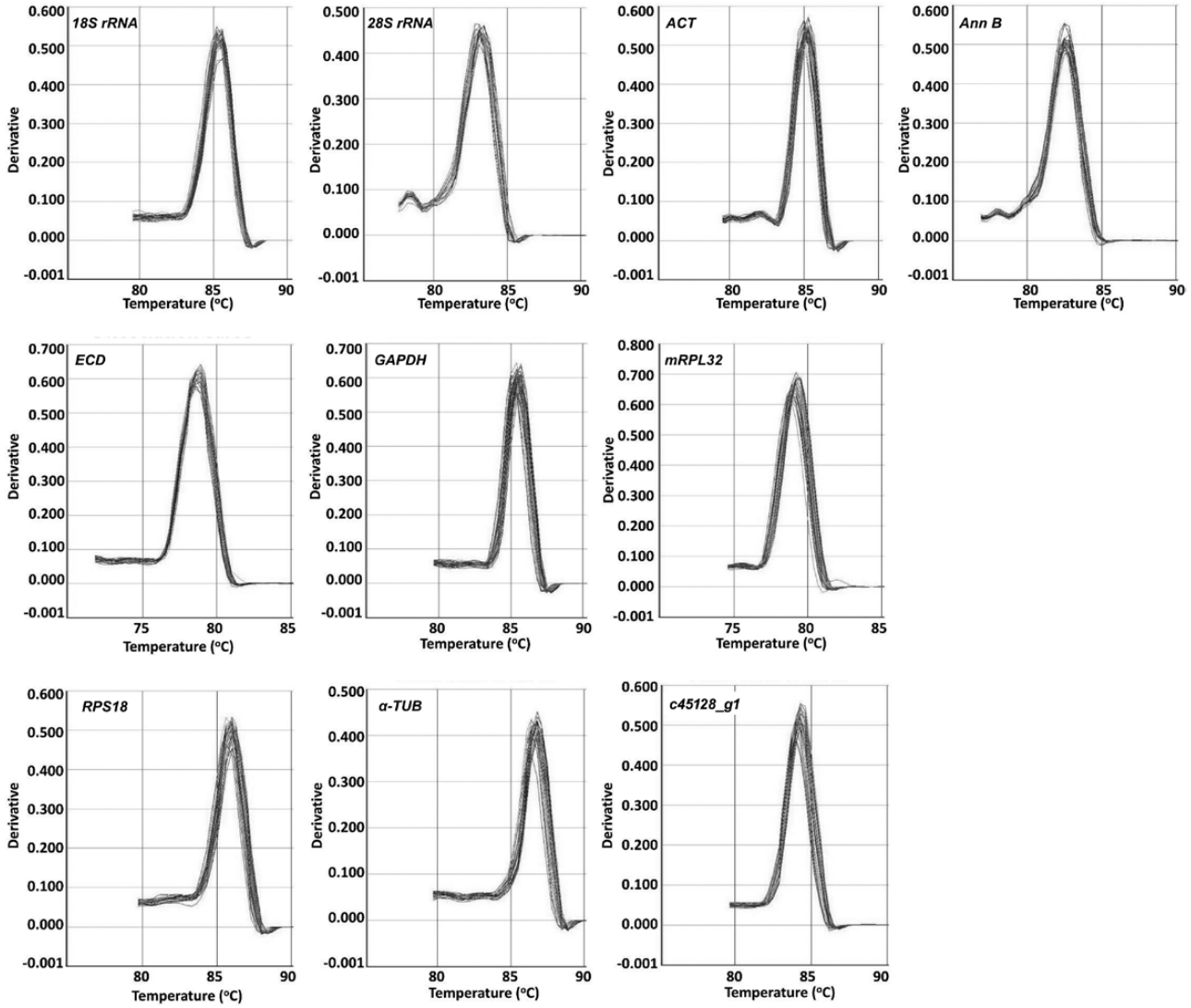

Fig. 1. Dissociation curves of 10 candidate reference genes of Tn-Hi5 cells used for RT-qPCR.

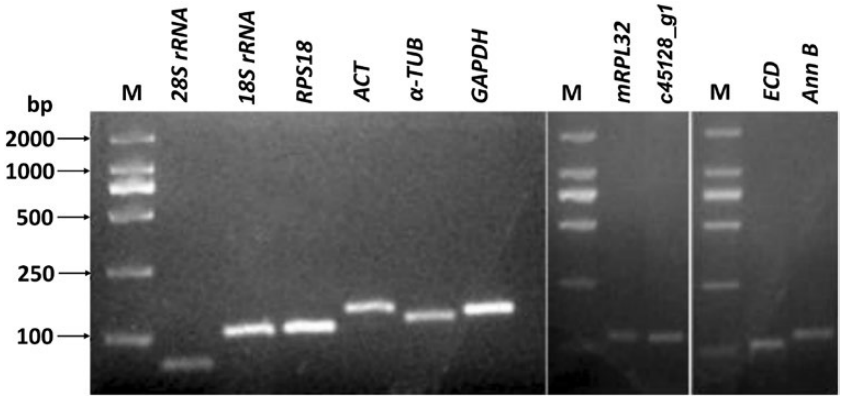

Fig. 2. Agarose gel electrophoresis of RT-qPCR products amplified using primers of the ten candidate reference genes and DL 2,000 DNA marker.

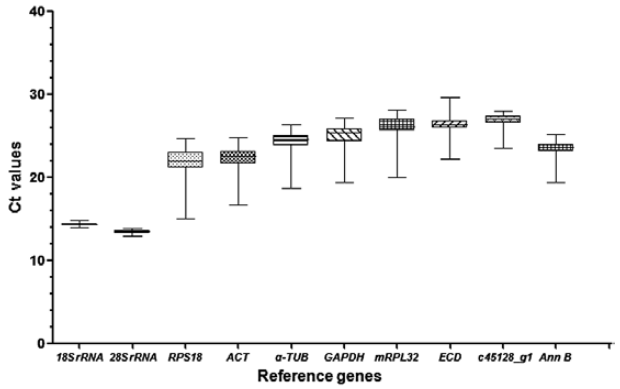

Fig. 3. Expression levels of 10 candidate reference genes during infection of Tn-Hi5 cells with *ApNPV-Δph/egfp*- presented as mean Ct value. Lines across the boxes show the median values.The whiskers represent the minimum and maximum values.

(Vandesompele et al. 2002). The gene with the lowest *M*-value (<0.5) is considered to exhibit the most stable expression, while *M* values up to 1 are acceptable for more difficult samples. The software also calculates a pairwise comparison (Vn/n+1) between two sequential normalization factors to determine the optimal number of reference genes required for accurate normalization. The *V*-value cut-off is 0.15, below which it is not necessary to include additional reference genes (Vandesompele et al. 2002). The results of geNorm analysis revealed that *ACT/α-TUB* had the lowest *M*-value (0.546) (Fig. 4A, Table 3), suggesting their high expression stability, and were the best pair for sample set normalization; on the other hand, *28S rRNA* was ranked as least stable, with the highest *M*-value (1.259) (Fig. 4A, Table 3). The pairwise variation data indicated that the *V*-value of V2/3 was 0.173 (>0.15) and that of V5/6 was 0.121, i.e., below the cut-off value of 0.15 (Fig. 4B). This suggested that at least five reference genes were required for reliable normalization. *GAPDH*, *Ann B*, and *c45128_g1* ranked in the top five positions based on the *M*-value, they were also considered acceptable candidate reference genes (Fig. 4A, Table 3).

NormFinder Analysis

Similarly to the geNorm algorithm, NormFinder identifies the reference gene that exhibits the lowest variation in expression, by combining the intragroup and intergroup variations in given sample sets (Andersen et al. 2004). The program ranks the candidate reference genes based on their stability value (SV), with the lowest SV representing the highest gene expression stability. The top three most stable genes determined in the current study using NormFinder were *ECD*, *Ann B*, and *c45128_g1*, with SV of 0.195, 0.300, and 0.400, respectively. The best pair for a reliable normalization was *GAPDH /c45128_g1* (SV = 0.160) (Table 3). Similar to the results of geNorm analysis, *28S rRNA*, *18S rRNA*, and *RPS18* showed the highest variation of expression, with SV of 1.148, 1.345, and 1.439, respectively (Table 3).

BestKeeper Analysis

The BestKeeper program identifies the most stable reference genes by analyzing the correlation coefficients of all possible pairs of candidate reference genes (Pfaffl et al. 2004). The gene with the lowest SD and coefficient of variation (CV) with the highest correlation coefficient (*r*) is the most stable reference gene. Genes with SD values above 1 are unacceptable. The results of BestKeeper analysis revealed that the SD values were below 1 for only five candidate reference genes; the SD values were above 1 for other genes (Table 3). The three most stable candidate reference genes were *18S rRNA*, *28S rRNA*, and *c45128_g1*, with SD of 0.15, 0.18, and 0.77, respectively. *Ann B* and *ECD*, with SD values below 1, were also acceptable as stable candidate reference genes. *RPS18* was the least stable candidate reference gene with the highest SD (1.76), followed by *ACT*, *α-TUB*, *GAPDH*, and *mRPL32*, all with SD values above 1 (Table 3).

RefFinder Analysis

RefFinder is a comprehensive online analysis tool, consisting of four major computational programs, including geNorm, NormFinder, BestKeeper, and the comparative delta Ct method (Silver et al. 2006). It provides a comprehensive ranking of candidate reference genes based on the ranking results from each algorithm. Candidate reference genes with lower geometric mean (GM) values are considered stable and suitable as ideal reference genes. The comprehensive ranking of the candidate reference genes based on RefFinder analysis revealed that *Ann B* was the most stable candidate reference gene, in terms of expression, with the lowest GM value (2.00), followed by *c45128_g1*, *ACT*, *GAPDH*, and *α-TUB*, while *ECD* was not among the top five positions. *RPS18*, *mRPL32*, and *28S rRNA* were the most unstable genes (Table 3).

Reference Gene Validation in a Gene Expression Study

To select the best reference gene in Tn-Hi5 cells infected with *ApNPV-Δph/egfp*$^{+}$, *Ann B*, *c45128_g1*, *ACT*, *GAPDH*, *α-TUB*, and *ECD* were further validated by using them to normalize the relative expression levels of three target genes. *28S rRNA* was selected as the reference gene control (Salem et al. 2011). *RPS18* was also selected for validation because it was identified as the least stable gene among the 10 candidate reference genes in the current study. The relative expression patterns of three Tn-Hi5 host genes (*caspase-4*,

Table 2. Comparison of average Ct ± SD of the 10 candidate reference genes during *ApNPV*Δ*ph/egfp*$^{+}$ infection

	18S rRNA	*28S rRNA*	*ACT*	*Ann B*	*ECD*	*GAPDH*	*mRPL32*	*RPS18*	*α-TUB*	*c45128_g1*
Mock	14.23 ± 0.09	13.59 ± 0.12	17.30 ± 0.66	20.45 ± 0.51	23.05 ± 0.51	20.17 ± 0.44	21.09 ± 0.74	15.48 ± 0.37	19.06 ± 0.42	24.22 ± 0.39
6	14.25 ± 0.19	13.48 ± 0.29	22.35 ± 0.75	23.39 ± 0.69	26.54 ± 0.75	25.08 ± 0.72	26.17 ± 0.96	21.90 ± 0.85	25.02 ± 0.84	27.09 ± 0.64
12	14.52 ± 0.14	13.54 ± 0.12	22.69 ± 0.32	24.13 ± 0.58	26.57 ± 0.35	25.83 ± 0.25	26.00 ± 0.57	21.85 ± 0.40	24.72 ± 0.24	27.36 ± 0.36
18	14.45 ± 0.12	13.51 ± 0.12	23.02 ± 0.66	23.87 ± 0.33	26.54 ± 0.99	25.48 ± 0.23	26.05 ± 0.40	21.82 ± 0.88	24.83 ± 0.41	27.10 ± 0.23
24	14.14 ± 0.06	13.17 ± 0.16	22.14 ± 0.74	23.39 ± 0.18	26.39 ± 0.33	24.64 ± 0.43	26.24 ± 0.33	21.64 ± 0.65	23.86 ± 0.59	26.75 ± 0.21
36	14.28 ± 0.10	13.26 ± 0.16	22.82 ± 0.70	23.74 ± 0.35	26.84 ± 1.17	25.24 ± 0.78	27.04 ± 0.56	23.01 ± 0.43	24.56 ± 0.68	27.17 ± 0.48
48	14.41 ± 0.19	13.48 ± 0.20	23.63 ± 0.59	23.85 ± 0.25	26.82 ± 0.33	26.38 ± 0.50	27.33 ± 0.16	23.75 ± 0.62	25.36 ± 0.66	27.32 ± 0.36
Avg.	14.33 ± 0.18	13.43 ± 0.22	21.99 ± 2.08	23.26 ± 1.26	26.11 ± 1.44	24.69 ± 1.99	25.70 ± 2.04	21.35 ± 2.59	23.93 ± 2.08	26.71 ± 1.11

caspase-5/dronc, and *iap2*) were normalized to the expression level of the selected candidate reference genes. The results of the analysis are shown in Fig. 5.

Compared with mock infection, transcript levels of *Tncaspase-4* with *28S rRNA* as the reference gene declined between 6 and 48 hpi, with the lowest levels at 6 hpi. Although the transcript levels of *Tncaspase-4* between 12 and 48 hpi were higher than those at 6 hpi, they showed a decreasing trend after 36 hpi (Fig. 5A1). When *Ann B*, *c45128_g1*, and *ECD* were used, respectively, as the reference genes, the transcript levels of *Tncaspase-4* showed a similar trend, with the lowest levels at 6 hpi, and relatively constant (variation range below twofold) and close to those in the mock-infected samples between 12 and 48 hpi. Reduction of expression of the *Tncaspase-4* gene at 48 hpi was also observed, similarly to *28S rRNA* data (Fig. 5A1). By contrast, the relative transcript levels of *Tncaspase-4* with *RPS18* as the reference gene were increasing between 6 to 48 hpi, with the highest levels at 48 hpi. The levels were elevated 29-fold at 48 hpi in comparison with mock infection (Fig. 5A2). The normalization curves of *Tncaspase-4* expression using either *ACT*, *GAPDH*, or *α-TUB* as the reference gene almost overlapped and indicated that the relative transcript levels of *Tncaspase-4* increased from 6 to 48 hpi, with the highest levels at 48 hpi (Fig. 5A2).

For *Tncaspase-5/dronc* and *Tniap2*, the relative transcript levels determined using *28S rRNA* as the reference gene dropped appreciably at 6 hpi, and then minimally declined between 6 and 48 hpi (Fig. 5B1 and 5 C1). When *Ann B, c45128_g1*, or *ECD* were used as the reference gene, the transcript levels of *Tncaspase-5/dronc* increased 3- to 5-fold at 6 hpi (*ECD* and *c45128_g1*) or 12 hpi (*Ann B*) in comparison with mock-infection levels, and minimally reduced through the remainder of the infection (Fig. 5B1). The transcript levels of *Tniap2* were elevated at 6 hpi (*c45128_g1* and *ECD*) or 12 hpi (*Ann B*) in comparison with those during mock infection, and then declined during the remainder of the infection (Fig. 5C1). When *ACT*, *GAPDH*, or *α-TUB* were used as the reference gene, the transcript levels of *Tncaspase-5/dronc* or *Tniap2* increased more than fivefold at 6 hpi, and reduced between 12 and 24 hpi, and then kept an elevating trend between 24 and 48 hpi (Fig. 5B2 and 5 C2). On the other hand, the transcript levels of *Tncaspase-5/dronc* or *Tniap2* with *RPS18* as the reference gene were elevated at 6 hpi, relatively stable between 12 and 24 hpi, and notably increased after 36 hpi, reaching the highest levels at 48 hpi (Fig. 5B2 and 5 C2).

Discussion

Baculovirus infection impacts gene expression of the host cell (Nobiron et al. 2003, Nguyen et al. 2012, Xue et al. 2012, Yu et al. 2016a, Yu et al. 2016b). AnpeNPV could infect nonspecific host cells, Tn-Hi5, with reduced infectivity in our previous study (Zhao et al. 2015). To enable the exploration of expression patterns of individual genes of Tn-Hi5 cells and virus during AnpeNPV infection, and evaluation of the differences in gene expression in comparison with AcMNPV infection, in the current study, the utility of 10 candidate reference genes was examined for RT-qPCR data normalization in AnpeNPV-infected Tn-Hi5 cells.

In the current study, the oligo-dT primer was used for cDNA synthesis because oligo-dT–primed cDNA provides a more accurate estimation of the mRNA pool than that primed using random primers (Bustin et al. 2005). Genes for rRNA, such as *18S rRNA* and *28S rRNA*, are transcribed by RNA polymerase I and cannot be reverse-transcribed by oligo-dT–priming (Radonic et al. 2004). Nevertheless, data presented in the current study indicated that *18S rRNA* and *28S rRNA* transcripts were characterized by the lowest

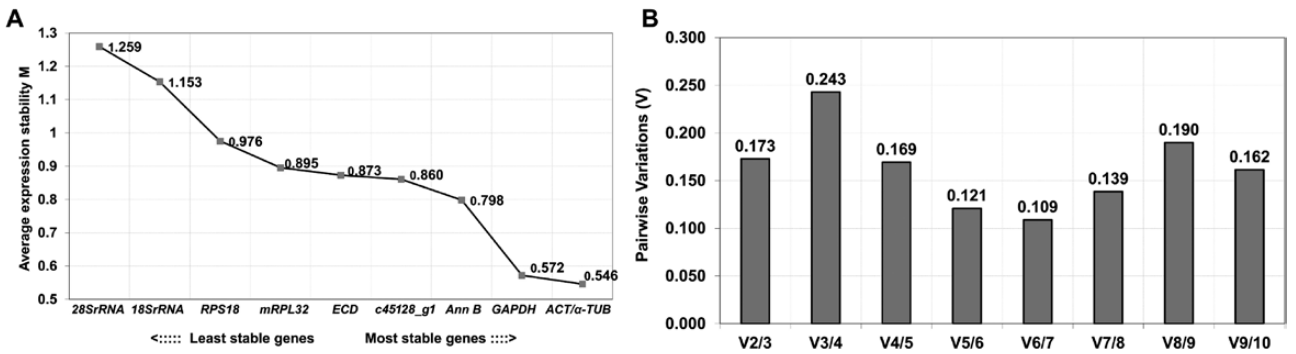

Fig. 4. Average expression stability and pairwise variation of 10 candidate reference genes calculated by the GeNorm algorithm. (A) The gene with the lowest *M*-value (<1.5) is considered to have the most stable expression. (B) The Vn/n+1 parameter (<0.15) defines the number of reference genes required for a reliable normalization in each dataset.

Table 3. Expression stability values and overall rankings for the ten candidate reference genes calculated by geNorm, NormFinder, BestKeeper, and RefFinder, respectively

Ranking	geNorm		NormFinder		BestKeeper		RefFinder	
	Gene symbol	Stability value (*M*)	Gene symbol	Stability value (SV)	Gene symbol	SD	Gene symbol	GM
1	*ACT*	0.546	*ECD*	0.195	*18S rRNA*	0.15	*AnnB*	2.00
2	*α-TUB*	0.546	*Ann B*	0.300	*28S rRNA*	0.18	*c45128_g1*	2.78
3	*GAPDH*	0.572	*c45128_g1*	0.400	*c45128_g1*	0.77	*ACT*	3.87
4	*Ann B*	0.798	*GAPDH*	0.576	*Ann B*	0.87	*GAPDH*	3.98
5	*c45128_g1*	0.860	*α-TUB*	0.686	*ECD*	0.97	*α-TUB*	4.12
6	*ECD*	0.873	*mRPL32*	0.686	*mRPL32*	1.40	*ECD*	4.36
7	*mRPL32*	0.895	*ACT*	0.691	*GAPDH*	1.40	*18SrRNA*	5.20
8	*RPS18*	0.976	*RPS18*	1.148	*α-TUB*	1.45	*28SrRNA*	6.69
9	*18S rRNA*	1.153	*18S rRNA*	1.345	*ACT*	1.45	*mRPL32*	6.74
10	*28S rRNA*	1.259	*28S rRNA*	1.439	*RPS18*	1.76	*RPS18*	8.46
Best pair	*ACT/α-TUB*	0.546	*GAPDH/c45128_g1*	0.160				

Ct value (<15) and the lowest variation in mRNA levels among the 10 candidate reference genes tested during viral infection, even though oligo-dT primer was used for cDNA synthesis (Table 2). Although *18S rRNA* and *28S rRNA* are used as reference genes for data normalization in various studies focussing on virus-infected insect, vertebrate, and plant cells (Klok et al. 2002, Xue et al. 2010, Wang et al. 2011, Kuchipudi et al. 2012), their high abundance compared to that of the potential target mRNA transcripts makes it difficult to accurately subtract the baseline value in RT-qPCR data analysis (Vandesompele et al. 2002). A gene with a very low (>30) or very high (<15) transcript Ct value would not be an optimal reference gene (Wan et al. 2010). The results from RefFinder analysis and reference gene validation further indicated that *18S rRNA* and *28S rRNA* were not suitable as reference genes in this study.

Four different statistical algorithms were used to evaluate the suitability of 10 candidate reference genes for RT-qPCR data normalization. Considering the results from the four algorithms, the most suitable reference genes among the 10 evaluated candidate reference genes for data normalization in Tn-Hi5 cells during AnpeNPV infection appeared to be *Ann B* and *c45128_g1* (Table 3). Similar findings were reported previously. Validation of candidate reference genes using geNorm, NormFinder, and BestKeeper revealed that the expression of *Ann* was more stable than that of *ACT* and *TUB* in wing discs in the late larval and pupal stages of *Heliconius numata* (Piron Prunier et al. 2016). Further, when the blood of bottlenose dolphin was analyzed, the three programs indicated that the expression of *PGK1* (encoding a member of the PGK family) was less variable than that of *ACT* and *GAPDH* (Chen et al. 2015). Although *ACT*, *GAPDH*, and *α-TUB* ranked in the top five positions according to both geNorm and RefFinder, they were unacceptable as candidate reference genes in AnpeNPV-infected Tn-Hi5 cells because their SD values were above 1 based on the BestKeeper analysis (Table 3). In a previous study, *ECD* transcript levels were shown to be stable between 6 and 48 hpi when *28S rRNA* was used as an internal normalization control in AcMNPV-infected Sf21 cells (Salem et al. 2014). In our study, *ECD* was the most stable according to NormFinder analysis, whereas it was unreliable in terms of the stability of its expression according to geNorm and RefFinder (Table 3). The analyses conducted using all four algorithms consistently found that *RPS18* and *mRPL32* were the most unstable candidate reference genes in Tn-Hi5 cells during AnpeNPV infection (Table 3).

To identify the most suitable reference genes, three *T. ni* genes were selected as target genes to verify the performance of the candidate reference genes. Two different gene expression patterns were observed upon data normalization. When *Ann B*, *c45128_g1*, or *ECD* were used for data normalization, three target gene expression levels appeared to decline at 48 hpi in AnpeNPV-infected Tn-Hi5 cells (Fig. 5A1, 5B1, and 5C1). The gene expression patterns derived from normalization to *ACT*, *GAPDH*, *α-TUB*, and *RPS18* were not consistent with those after normalization to *Ann B*, *c45128_g1*, or *ECD*. The expression of the three target genes was elevated at 48 hpi in AnpeNPV-infected Tn-Hi5 cells. Appreciable differences were noted for target gene expression when the least stable gene, *RPS18*,

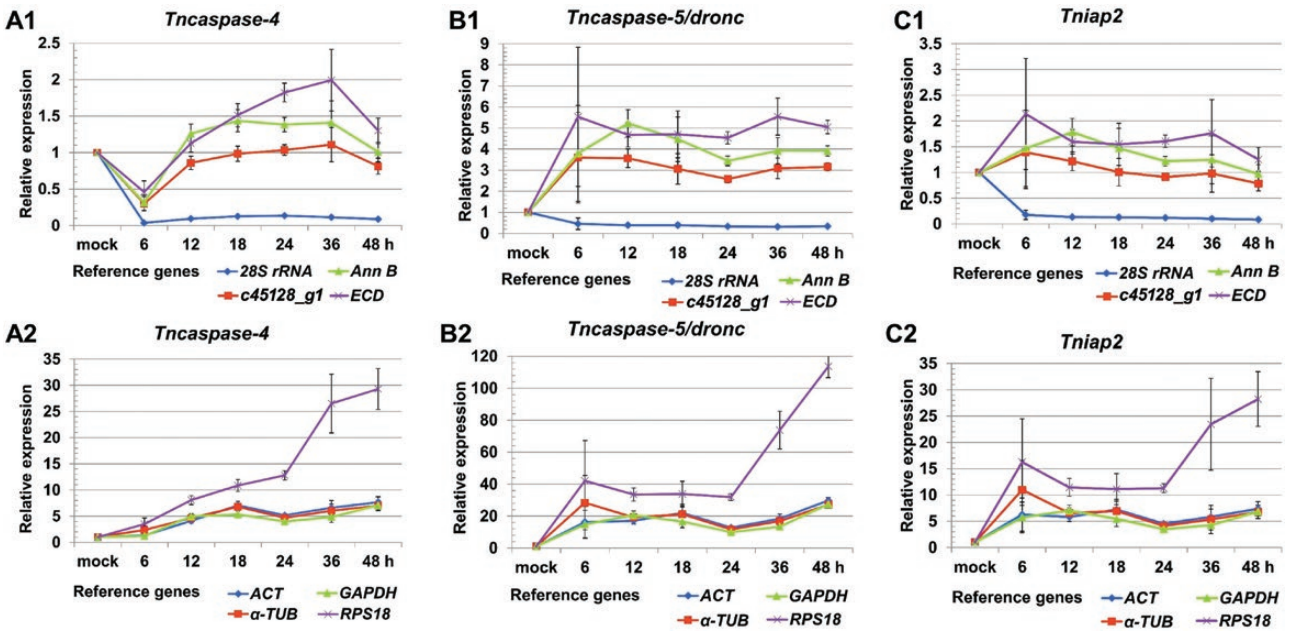

Fig. 5. Normalization of the expression of three target genes during infection of Tn-Hi5 cells with *ApNPV-Δph/egfp*$^{+}$ to the expression of eight selected reference genes. Relative expression of the three target genes derived from RT-qPCR data is shown. (A) Relative expression of *Tncaspase-4* normalized to *28S rRNA, Ann B, ECD,* and *c45128_g1* (A1); and to *GAPDH, ACT,* α*-TUB,* and *RPS18* (A2). (B) Relative expression of *Tncaspase-5/dronc* normalized to *28S rRNA, Ann B, ECD,* and *c45128_g1* (B1); and to *GAPDH, ACT,* α*-TUB,* and *RPS18* (B2). (C) Relative expression of *Tniap2* normalized to *28S rRNA, Ann B, ECD,* and *c45128_g1* (C1); and to *GAPDH, ACT,* α*-TUB,* and *RPS18* (C2).

was used for data normalization (Fig. 5A2, 5B2, and 5C2). A global down-regulation of the host genome at late time points of infection has been observed in several studies (Salem et al. 2011, Xue et al. 2012, Chen et al. 2014). A microarray approach with RT-qPCR showed that transcripts for the majority of host genes declined substantially by 12 h in AcMNPV-infected Sf21 cells (Salem et al. 2011). In *T. ni* and Tnms42 cell lines, AcMNPV infection causes down-regulation of the majority of the host genes, including *GAPDH*, *ACT*, and α*-TUB*, beyond 6 hpi, as determined by RNA sequencing. Only 5.7% of *T. ni* unigenes is up-regulated between 0 and 6 hpi, and this is followed by a reduction in expression during the remainder of the infection cycle (Chen et al. 2014). For example, a relatively dramatic increase in the transcript levels of *caspase-5* was observed in Tnms42 cells, after addition of the virus and through 6 hpi, followed by a reduction between 6 and 48 hpi. The transcript levels of *iap2* and *caspase-4* were minimally increased at 6 hpi and reduced through the remainder of the infection (Chen et al. 2014). Similar results were found for *iap2* in BmNPV-infected Bm5 cells (Xue et al. 2012). Hence, the expression levels of the three target genes in AnpeNPV-infected Tn-Hi5 cells appeared to be more consistent when determined after normalization to *Ann B* and *c45128_g1*, respectively, than after normalization to other genes. Thus, a comprehensive analysis of results of the current study as well as published reports suggests that *Ann B* and *c45128_g1* may be more suitable than the most commonly used reference genes, such as *ACT*, *GAPDH*, and *TUB,* for RT-qPCR data analyses in AnpeNPV-infected Tn-Hi5 cells up to 48 hpi.

Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

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