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ORIGINAL ARTICLE

Selection and validation of reference genes for reverse transcription quantitative real-time PCR (RT-qPCR) in silkworm infected with Bombyx mori bidensovirus

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Abstract

Bombyx mori bidensovirus (BmBDV) is a unique bipartite DNA virus that causes chronic disease in silkworms, and its interactions with the host give rise to variations of genes expression. Reverse transcription quantitative real-time PCR (RT-qPCR) has extensive applications in mRNA quantification. To obtain reproducible and meaningful quantification of transcripts, it is crucial to identify the best reference genes in preliminary work. In this work, we analyzed the expression of seven candidate reference genes in silkworm larvae infected or uninfected with BmBDV, and compared their expression stability with three statistical methods (geNorm, NormFinder, and BestKeeper). The results revealed that the *RPL3* and 28S *rRNA* were the stable internal control, and *UBQ* was the least stable gene. Furthermore, the expression of *Bombyx mori serine protease* (*BmSP142*) was assessed using *RPL3* as internal. The expression of *BmSP142* was significantly different between BmBDV-resistant and BmBDV-susceptible silkworm after virus infection, suggesting that *BmSP142* might be associated with the resistance to BmBDV in silkworm.

Keywords Silkwom · Bombyx mori bidensovirus · RT-qPCR · Reference gene

Abbreviations

RT-qPCR	Reverse transcription quantitative real-time polymerase chain reaction
BmBDV	Bombyx mori bidensovirus
BmSP142	*Bombyx mori serine protease*
GAPDH	*3-phosphoric acid glycerol aldehyde dehydrogenase*
UBQ	*ubiquitin*
RPL3	*ribosomal protein L3*
TBP	*TATA response element binding protein*
BmNPV	*Bombyx mori* nucleopolyhedrovirus
TIF-4A	translation initiation factor 4A
Ct	cycle threshold
SD	standard deviation
CV	coefficient of variance
hpi	hours post infection
NCBI	National Center for Biotechnology Information

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Introduction

The silkworm, also called *Bombyx mori* (*B. mori L*), a completely metamorphic insect, has four developmental stages in its life cycle: egg, larva, pupa, and adult. Silkworm larvae molt four times during the larval period, which corresponds to five instars of larval development. The silkworm is an important economic insect for silk production and has become a model organism in the study of life science (Chen et al. 2014). Sericulture is a principal source of income for farmers in many developing countries. However, silkworm pathogens cause losses in cocoon production of almost 20% each year (Jiang et al. 2013). Almost 80% of the total cocoon loss is caused by viral diseases (Guo et al. 2016), one of which is Bombyx mori bidensovirus (BmBDV). BmBDV belongs to *Bidensovirus* in *Bidnaviridae*, which replicates mainly in silkworm midgut columnar cells and causes the fatal flacherie disease. This virus possesses two single-stranded DNA

genome segments in separate capsids and encodes a putative protein-primed DNA polymerase (Hu et al. 2013). Up to date, it is the only bipartite animal virus and is the only species in the *Bidnaviridae* (Hu et al. 2013). However, the interactions between BmBDV and its host at transcription level remain to be detailed.

Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) approaches is the main method used to accurately analyze the transcript abundance of genes involved in viral pathogenesis (Long et al. 2015). Due to its superior speed, sensitivity, and reproducibility, as well as the wide range of commercially available instrumentation and reagents, RT-qPCR has been universally adopted as the choice for transcriptomic analysis (Bustin et al. 2005). To achieve accurate and reliable gene expression analyzed by RT-qPCR, it is essential that one or several reference genes can be used as internal control to normalize experimental variations (Bustin et al. 2005; Huggett et al. 2005). The suitable reference genes used in RT-qPCR should be expressed stably under the research conditions. Housekeeping genes which were considered to be constant expression were commonly used for normalization, such as *3-phosphoric acid glycerol aldehyde dehydrogenase* (*GAPDH*), *28S rRNA* and *atcin*. However, most of these genes displayed variable expression levels under different experimental conditions (Thellin et al. 2009; Lee et al. 2002; Thellin et al. 1999). An erroneously strong specific expression pattern for a given target gene could be obtained, if an inappropriate reference gene was used for normalization (Gutierrez et al. 2008). In silkworm, the candidate reference genes were expressed differentially in different developmental stages and varied in response to experimental conditions (Peng et al. 2012). Thus, it is necessary to verify the expression stability of candidate reference genes before the specific experiment.

Previous studies have used *actin A3* (Kong et al. 2011) or *GAPDH* (Bao et al. 2013) as internal reference genes in BmBDV-infectied silkworm. Nevertheless, whether these reference genes keep stable expression remains to be determined in silkworm infected by BmBDV. In this study, we have selected and validated the most suitable internal control gene(s) for the normalization of RT-qPCR data upon BmBDV infection in silkworms. Using statistical algorithm programs (geNorm, Normfinder, and BestKeeper), we evaluated the expression stability of seven candidate reference genes: *28S rRNA*, *actin A3*, *ubiquitin* (*UBQ*), *GAPDH*, *ribosomal protein L3* (*RPL3*), *TATA response element binding protein* (*TBP*), and *a-tubulin*. *RPL3* and *28S rRNA* were identified as the best reference genes. The expression of *Bombyx mori serine protease* (*BmSP142*) varied significantly during BmBDV infection (Li et al. 2017). The reliability of *RPL3* and *28S rRNA* were validated by expression analysis of *BmSP142*.

Materials and methods

Silkworm and virus

The BmBDV susceptible *Bombyx mori* strains Jingsong and Huaba35 were bred by our laboratory. The near isogenic line HuabaBC7 which is resistant to BmBDV was constructed by our laboratory (Chen et al. 2011). The silkworm larvae were reared on fresh mulberry (*Morus alba*) leaves at 25 ± 1 °C and 80% ± 15% relative humidity with a 12 h light/12 h dark cycle. Thirty animals were used for each experimental group. Newly exuviated fifth-instar larvae were used for this experiment. BmBDV was propagated in fifth instar susceptible lavae and the virus supernatant was prepared as described previously (Hu et al. 2016).

Midgut collection, RNA extraction, quality controls, and cDNA synthesis

The BmBDV-infected fifth instar larvae were dissected, and their midguts were collected at 0, 12, 24, 36, 48, 72, 84 and 96 h post-infection (hpi). One hundred milligrams of the midgut was ground into powder in liquid nitrogen. Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The quality and concentration of each of the RNA samples was determined with a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and an Agilent 2100 Bioanalyzer. Only those RNA samples having A260 to A280 ratios of 1.9 to 2.1 and A260 to A230 ratios of 2.0 to 2.5, as well as an RNA integrity number greater than 7.0, were used for the analysis. The integrity of the RNA was also checked by agarose gel electrophoresis. Approximately 500 ng of RNA was reverse transcribed using the PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa, Dalian, China) according to the manufacturer's instructions, and the product was used as the template in RT-qPCR.

Primer design and qPCR

In this study, The sequences of seven reference genes and the target gene were obtained from NCBI database by BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.

cgi). Primer 5.0 software (PREMIER Biosoft International, Palo Alto, CA, USA) was used to design the primers. Primers were synthesized by Generay Biotech Co, Ltd. (Shanghai, P.R. China). The primers of seven reference genes and the target gene for RT-qPCR were shown in Table 1. The fluorescent RT-qPCR reaction system consisted of 5 μL 2 × SYBR Premix Ex Taq™ II (Takara, Dalian, China), 0.2 μL upstream and downstream primers (10 mM), 0.2 μL ROX Reference Dye II (50 × Conc.), 1 μL template cDNA, and 3.4 μL double-distilled H_2O in a total volume of 10 μL. The RT-qPCR

Table 1 Primer pairs for qPCR. List and description of the genes used in this study

Gene name	Gene symbol	Primer sequence (5′–3′)	Amplicon size (bp)	Tm (°C)	GenBank accession no.
actin A3	*actin*	F: TTGCGTCTGGACTTGGC R: TTTCGTTTCCGATGGTGA	235	88	NM_001126254.1
28S ribosomal RNA	*28S rRNA*	F: CTCAGAACTGGCACGGACA R: TTAGATGACGAGGCATTTGG	195	85	AY038991.1
α-tubulin	*tubulin*	F: CCAGATGCCCACAGACAAGA R: AAGGAAGTGAACCCAGAGCC	354	86	NM_001043419.1
3-phosphoric acid glycerol aldehyde dehydrogenase	*GAPDH*	F: CCATGTTTGTTGTGGGTGTTA R: AGAGGCAGGAATGATGTTTTG	245	83	NM_001043921.1
ribosomal protein L3	*RPL3*	F: CAAAGTGAAATGGGCCAGAG R: AGCACGAGCTACAGTGAACGA	232	84	AY769270.1
ubiquitin	*UBQ*	F: ATTCCTCCAGACCAACAACG R: TGAAGACGGGCATAGCATTT	206	82	AF308163.1
TATA response element binding protein	*TBP*	F: TAAGGGAACCGAGAACAACAG R: GGGAACTTCACATCGCAACTA	190	81	NM_001043594.1
serine protease142	*BmSP142*	F: TACTACAACGACACCGCACAG R: TCGGCTTCAGGTCCTCACT	229	89	AF309500.1
Translation initiation factor 4A	*TIF-4A*	F: GAATGGACCCTGGGACACTT R: CTGACTGGGCTTGAGCGATA	186	85	NM_001043911.1

conditions were as follows: an initial denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 55 °C 27 s, and 72 °C for 31 s, and finally, PCR amplification specificity was verified by a dissociation curve (75–94 °C). The RT-qPCR analysis was performed for three biological replicates for each sample, and three technical replicates were analyzed for each biological replicate.

Standard curve

Seven reference gene segments were PCR-amplified, and cloned into the pMD18-T vector (Takara, Dalian, China). The recombinant plasmids were extracted using the E.Z.N.A Plasmid Mini Kit I (Takara, Dalian, China) and sequenced as the initial templates. The plasmid concentrations were converted into copies of the reference genes, and the standard templates were diluted to 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, and 10^2 copies/mL. Then, the 7300 Fast system (Applied Biosystems, Foster City, CA, USA) was used to draw a standard curve.

Data calculation and analysis of gene stability

Three Excel-based softwares the BestKeeper, NormFinder and geNorm programs were used to measure the expression stability of the seven candidate genes. The geNorm was used to calculate the expression stability (M) based on the raw expression data (http://medgen.ugent.be/~jvdesomp/genorm/), and NormFinder uses raw data as an input in the form of expression values generated using the comparative Ct (cycle threshold) values (http://www.mdl.dk/publicationsnormfinder.htm). While BestKeeper evaluated the expression stability based on the SD (standard deviation) and CV (coefficient of variance) for the target genes in all the samples (http://www.genequantification.de/bestkeeper.html). Statistical comparisons were made using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA).

Results

Analysis of the expression levels of the reference genes

The expression of seven reference genes were evaluated in BmBDV-infected or non-infected silkworm at different time points by RT-qPCR. The primer specificities were confirmed by the single bands of the expected sizes on 1% agarose gel and the melting curves with a single distinctive peak (Fig. 1). Standard curves of the seven candidate genes are shown in Table 2. The linear correlation coefficient (R^2) ranged from 0.997 to 0.999, and the PCR efficiency commonly varied from 90 to 120%. The regression coefficient values were greater than 0.99. The absolute values of the straight-line gradient were controlled within approximately three to four.

We used the value of the absolute copies to analyze the expression of the control genes at the various time points (Fig. 2). The results showed that the expressions of *GAPDH*, *RPL3*, *UBQ* and *28S rRNA* were relatively stable across all the tested samples, while the expressions of *actinA3* and *a-tubulin* exhibited larger variation. *TBP* was the least expressed gene.

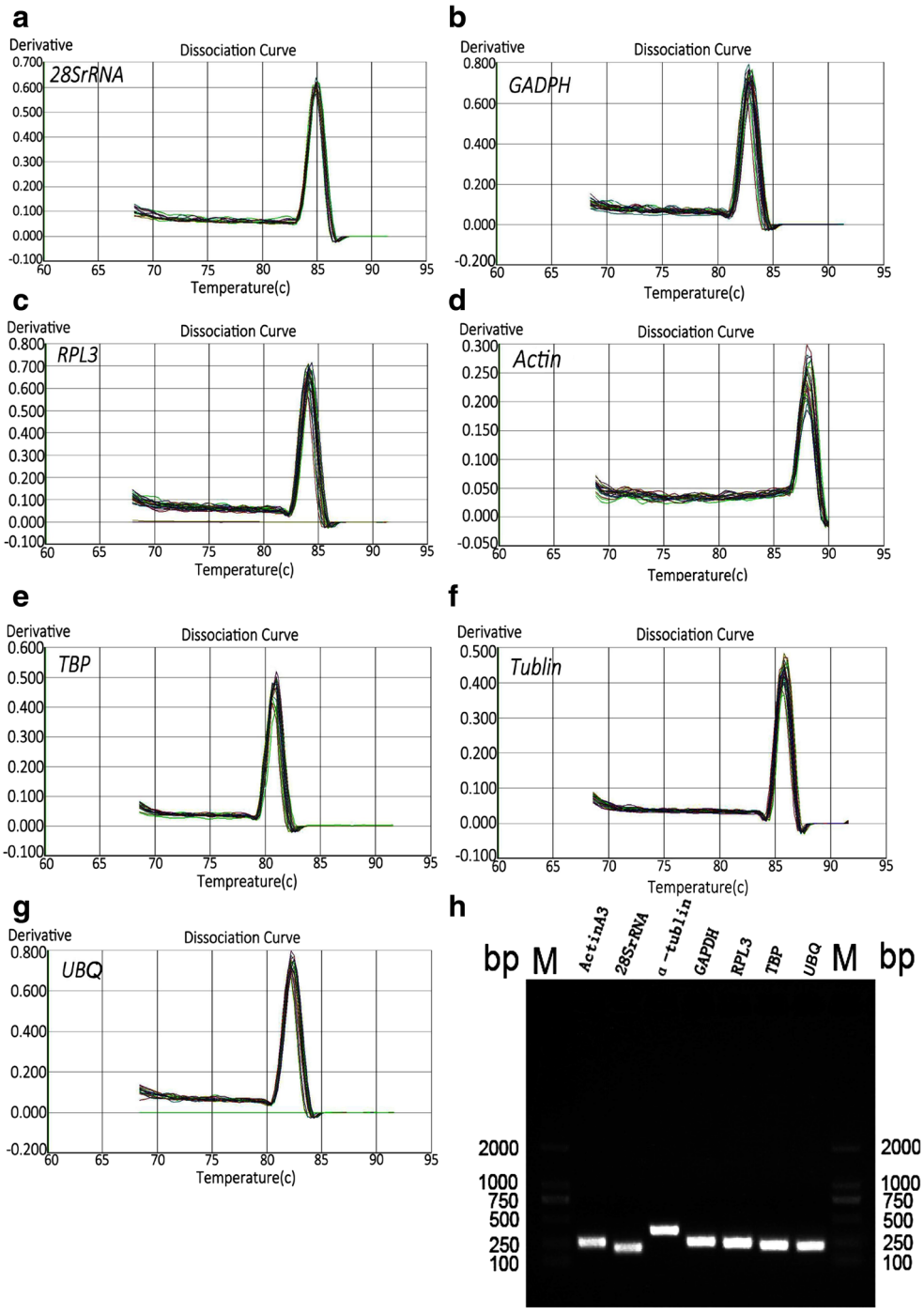

Fig. 1 Confirmation of primer specificity and amplicon size. a-g Melting curves of seven candidate reference genes and one target gene showing single peaks. **h** Agarose gel (1%) showing specific RT-qPCR products of the expected size for each gene. M represent a 2000-bp DNA marker

Assessment of the expression stability of the reference genes

We calculated the mean and the standard deviation (SD) of the cycle threshold (Ct) values for all the samples. The number of Ct values was transformed into a quantity via the standard curve based on the PCR efficiency, which allowed the initial copies of the cDNA to be obtained. Three different programs were applied to evaluate the expression stability of the selected reference gene candidates: geNorm, NormFinder, and BestKeeper. To select stably expressed genes, we collected Ct values across all the samples. These raw Ct values were used directly for stability calculations for the BestKeeper analysis, and then they were transformed to relative quantities using the $-\Delta\Delta$Ct method for geNorm, while they transformed to absolute quantities by the standard curves for NormFinder (Sang et al. 2013).

Table 2 Standard curve equations for the seven reference genes

Genes	The equation of standard curves	Slope	Amplification efficiency(E%)	Regression Coefficient(R^2)
RPL3	y = −3.5879× + 39.658	3.59	108.23	0.99735
28SrRNA	y = −3.6348× + 40.309	3.63	102.45	0.99883
actin A3	y = −4.0484× + 43.575	4.05	98.65	0.99703
GAPDH	y = −3.6443× + 39.781	3.64	99.01	0.99716
TBP	y = −3.7644× + 41.673	3.76	92.98	0.99792
α-tubulin	y = −3.0139× + 36.777	3.01	101.25	0.99662
UBQ	y = −3.6029× + 39.539	3.60	100.02	0.99709

geNorm analysis geNorm allows the most appropriate mean of the expression of a candidate cDNA to be determined (Jain et al. 2006). The expression stability *M* for all the reference genes were calculated by the geNorm program based on the average pairwise variation between all the tested reference genes (Bustin et al. 2009). The most stable reference gene has the lowest *M* value, whereas the least stable one has the highest *M* value (Bustin et al. 2009). In our analysis, all the reference genes of the BmBDV-infected and uninfected silkworms at different time points had an *M* value less than the geNorm threshold of 1.5, which is recognized as stability (Fig. 3). The results showed that *RPL3* and *28S rRNA* had the lowest *M* values among the seven candidate genes, implying that these two candidate genes are the appropriate reference genes for researching BmBDV infection of silkworms. The data also showed that *UBQ* and *TBP* were the least stable control genes.

NormFinder analysis Another adopted program, NormFinder, which is one of the Visual Basic application tools for Microsoft Excel, is used to calculate the stability value of reference genes based on their intra- and inter-group variations, and it combines both results into a consistent value for each investigated gene (Andersen et al. 2004; Jian et al. 2008). NormFinder not only measures the variation, but it also ranks the potential reference genes by how they differ between studies (Chuaqui et al. 2002). Genes exhibiting lower average expression stability values are regarded as being more stably expressed reference genes (Vandesompele et al. 2002). The ranking generated by this approach (Fig. 4) was approximately similar to that determined by geNorm. Based on our data, NormFinder identified *RPL3* and *28S rRNA* as the best reference genes, while *UBQ* and *a-tubulin* were the least stable control genes for the gene analysis of BmBDV infected-silkworms.

BestKeeper analysis The BestKeeper program is used to evaluate the expression stability of reference genes using the raw Ct values based on their correlation coefficient (*r*) to the BestKeeper Index by calculating the SD and coefficient of variance (CV) (Tang et al. 2015). Surprisingly, the BestKeeper analysis revealed that the best correlations were obtained for *actin A3* (0.92), *a-tubulin* (0.90), and *RPL3* (0.85) with a *P*-value of 0.001 across all the samples (Table 3). Because of the high correlation value and low BestKeeper index, *actin A3*, *a-tubulin*, and *RPL3* were selected as the three likely reference genes. The results varied slightly from the previously mentioned programs, which might result from the distinct statistical algorithms of BestKeeper.

Basing on above analysis and considering that *RPL3* and *28S rRNA* had the lower SD and CV among these genes, we suggest that *RPL3* and *28S rRNA* may be the appreciate control genes.

Expression profile of *BmSP142* in BmBDV-infected silkworm

The previous studies demonstrated that the expression of *BmSP142* varied greatly during BmBDV infection (Li et al. 2017). To verify the reliability of the selected reference genes, we analyzed the expression patterns of *BmSP142* by RT-qPCR in BmBDV-infected Huaba35 (BmBDV-sensitive) and HuabaBC7 (BmBDV-resistant) silkworm at different infection times. In consideration of the previous report that *TIF-4a* (translation initiation factor 4A) was an appropriate reference gene for studies of the effects of BmBDV on silkworms (Guo et al. 2016), the relative expression levels of *BmSP142* at different time points across different strains were normalized by *RPL3*, *28S rRNA* and *TIF-4A*, respectively. Similar expression pattern of *BmSP142* was displayed using these there genes as internal control respectively (Fig. 5). The expression levels of *BmSP142* were remarkably up-regulated at 24 hpi in resistant silkworm, and significantly higher compared with the sensitive silkworm. Meanwhile, the transcription levels of *BmSP142* were markedly up-regulated at 84 hpi in sensitive silkworm, and remarkably higher than that in resistant silkworm.

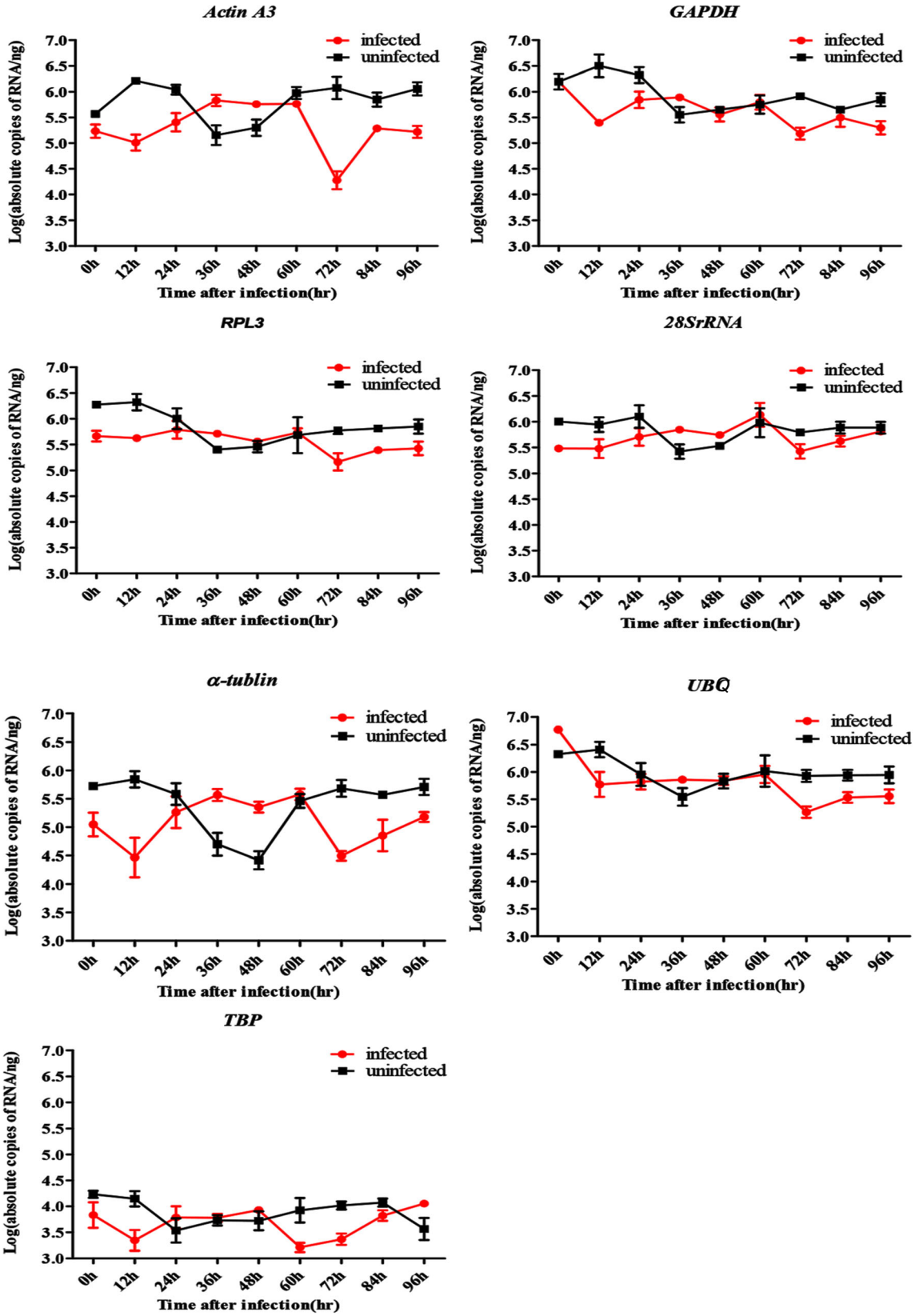

Fig. 2 The transcript abundance of the candidate reference genes at the indicated time points in BmBDV-infected and healthy silkworm. We used the raw Ct values to calculate the initial copies of the respective cDNAs according to the standard curves. The error bars represent the standard deviation (SD). The experiments were performed in triplicate

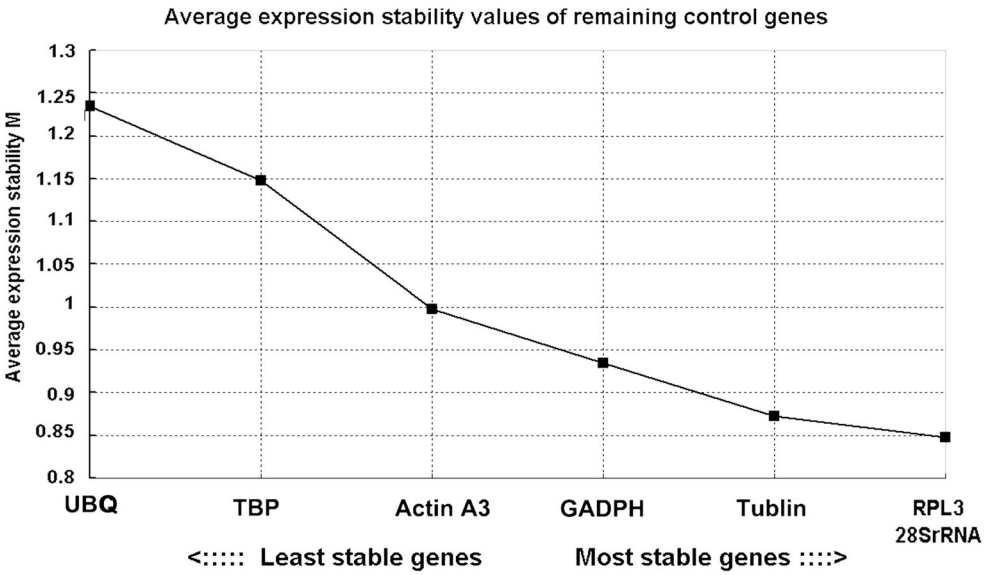

Fig. 3 Expression stability (*M*) of the seven control genes across all tested samples calculated with geNorm. The average value of the expression stability, *M*, for each gene was determined using geNorm software. Genes on the *x*-axis are ordered from the left to the right according to their expression stability from the least to the most stable

Discussion

In this study, three approaches were used to investigate seven candidate genes for their potential to be used as reference genes in silkworms after BmBDV infection. *RPL3* and *28S rRNA*, were found as the most stable genes by geNorm and Normfinder analysis (Figs. 3 and 4). Meanwhile, Bestkeeper has ranked the *actin A3*, *a-tubulin* and *RPL3* as the three most suitable reference genes, and *RPL3* and *28S rRNA* had the lower SD and CV among these genes (Table 3). *UBQ* was consistently identified as the least stable gene by all three algorithms. The analysis of transcript abundant showed that *actin A3* and *a-tubulin* were more variable comparing with the other candidates (Fig. 1). Previous studies reported an interesting property of the absence of the 28S rRNA band usually present in eukaryotic cells when viewed on non-denaturing agarose gels and the dissociation of the 28S rRNA molecule into two 18S rRNA components upon exposure to a brief heat treatment in dipteran species (Balazs and Agosin 1968). Because the expression level of *28S rRNA* was significantly higher than that of other genes, *28S rRNA* gene may not be ideal for some studies in insect, e.g. in *L.cuprina* (Bagnall and Kotze 2010). *RPL3*, which encodes ribosomal protein L3, is expressed stably at different development stages in *Rhododendron molle G. Don* (Xiao et al. 2016). *RPL3* has also been found to be the most stable gene in wing discs of

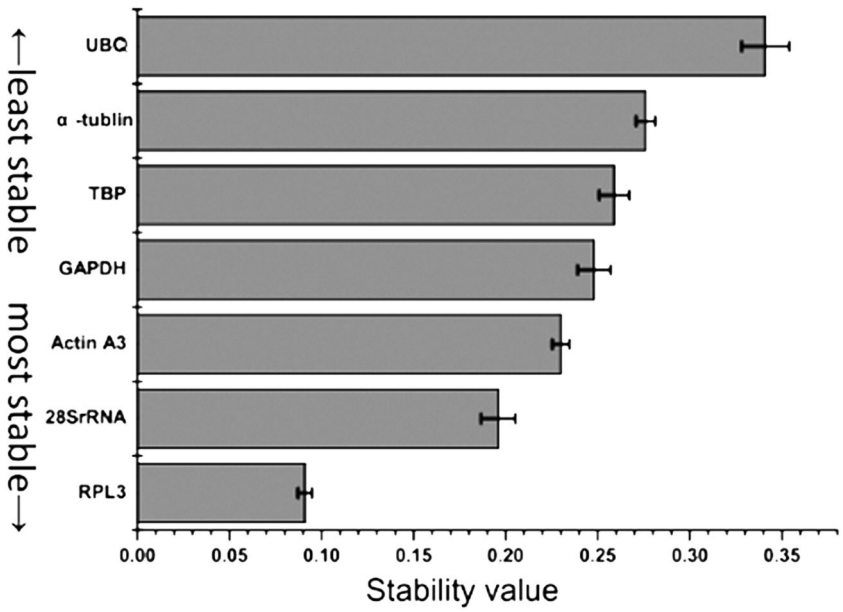

Fig. 4 Ranking of the candidate reference genes in order of their expression stability calculated by NormFinder. Genes on the *y*-axis are ordered from top to bottom according to their expression stability from the least to the most stable

Table 3 Expression stablility for the seven reference genes calculated by BestKeeper based on Ct values

Genes	Rank	*r*	*P*-value	CV (%)	SD
actin A3	1	0.92	0.001	6.52	2.53
α-tubulin	2	0.90	0.001	5.15	2.10
RPL3	3	0.85	0.001	2.94	1.49
GAPDH	4	0.80	0.001	5.36	2.01
28S rRNA	5	0.68	0.002	3.02	1.67
TBP	6	0.46	0.053	2.50	1.61
UBQ	7	0.16	0.525	3.73	1.62

butterfly *Heliconius numata* at different developmental stages (Prunier et al. 2016). *UBQ* has also been commonly used as the appropriate internal control for gene expression analysis in different genotype of *Vitis viniferarice* infected with downy mildew (Monteiro et al. 2013) and in turbot gonad (Robledo et al. 2014). In present study, *UBQ* was found to be the least stable gene in BmBDV-infected silkworm.

Li et al. (2017) assessed the expression of *BmSP142* in BmBDV-infected silkworm by absolute quantification, and their results showed that the expression was significantly up-regulated at 24 hpi in resistant silkworm and up-regulated at 72 hpi in sensitive silkworm. Guo et al. (2016) suggested that the expression level of *TIF-4A* was stable for BmCPV or BmBDV challenge in silkworms using transcriptome data analysis and RT-qPCR verification. To validate *RPL3, 28S rRNA* and *TIF-4A* being the appropriate reference genes in BmBDV-infected silkworm, we evaluated the relative expression levels of *BmSP142* in silkworm infected with BmBDV normalized by *RPL3, 28S rRNA* and *TIF-4A*, respectively (Fig. 5). Similar expression pattern was observed using these three genes as reference genes respectively. Consistent with the findings of Li et al. (2017), the mRNA levels of *BmSP142* changed significantly after virus infection, and the expression patterns of *BmSP142* were significantly different between BmBDV-sensitive and BmBDV-resistant silkworm. These results further indicated that *RPL3, 28S rRNA* and *TIF-4A* were the reliable reference genes. The expression level of *BmSP142* was remarkably up-regulated in resistant silkworm and markedly higher at early stage of infection (24 hpi) than that in sensitive silkworm, suggesting that *BmSP142* may play an important role in resistance to BmBDV in silkworm. Genome-wide identification indicate that silkworm possesses 51 serine proteases (SPs) and 92 serine protease homolog genes, which may participate in biological processes such as digestion, development and defence (Ping et al. 2010). *BmSP142* was a 35 KDa protein which interacted with non-structural protein NS1 and NS2 of BmBDV (Bao et al. 2013). Over expression of *BmSP142* in BmN cells inhibited the propagation of Bombyx mori nucleopolyhedrovirus (BmNPV),

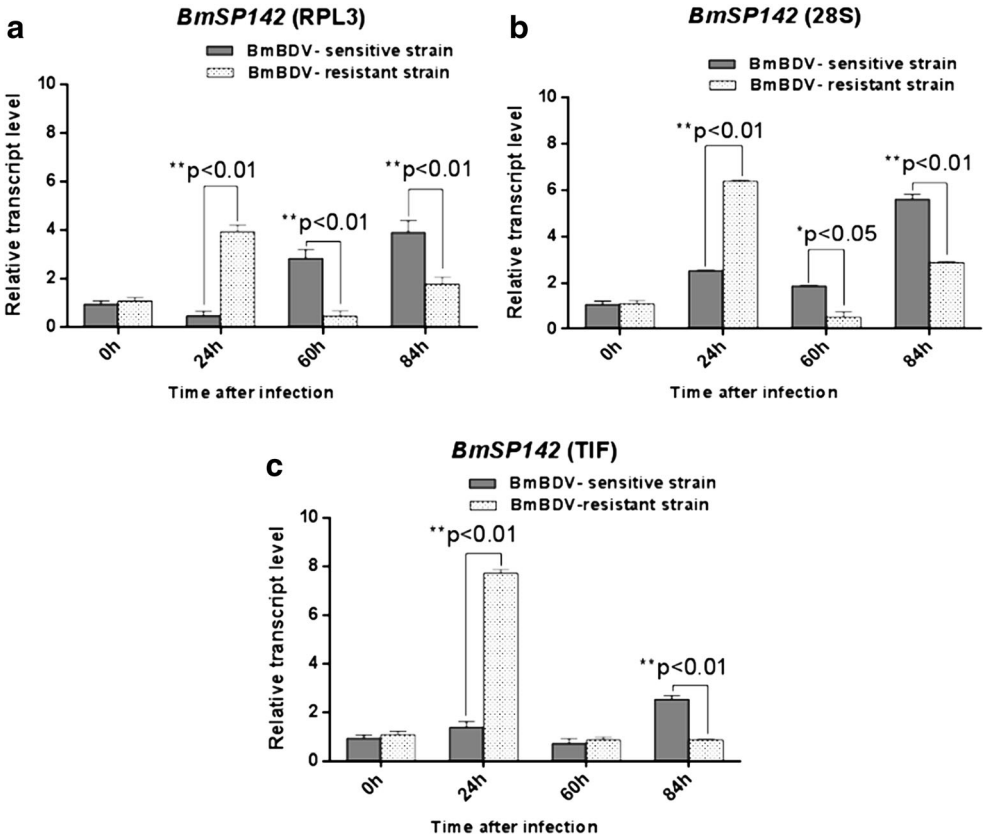

Fig. 5 The relative expression levels of *BmSP142* normalized by different reference genes in different strains at different infection periods. The relative expression levels of *BmSP142* normalized by *RPL3* (**a**), *28S rRNA* (**b**) and *TIF-4A* (**c**) in sensitive silkworm (Huaba35) and resistant silkworm (HuabaBC7) infected with BmBDV. ** indicates remarkable difference ($P < 0.01$), * indicates significant difference ($P < 0.05$)

and the replication efficiency of BmBDV and BmNPV treated with purified *BmSP142* decreased in silkworm, indicating that *BmSP142* may be involved in defence response to virus infection (Li et al. 2017).

Overall, the present study evaluated the expression stability of seven candidate reference genes for RT-qPCR normalization in BmBDV-infected silkworm. *RPL3*, and *28Sr RNA* were identified as the suitable internal control genes under BmBDV challenge. And we further verified that *TIF-4A* was also a reliable reference gene. The expression patterns of *BmSP142* were significantly different in susceptible and resistant silkworm after BmBDV infection, indicating that *BmSP142* may be involved in the resistance to virus. This study will be beneficial for investigating the gene expression in silkworm under the BmBDV infection.

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