# Validation of reference genes for gene expression studies in nonviruliferous and viruliferous *Frankliniella occidentalis* (Thysanoptera: Thripidae)

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Quantitative real-time PCR (qRT-PCR) is a powerful technique for measuring and evaluating gene expressions during different biological processes. To facilitate gene expression studies, normalization with respect to stable housekeeping genes (HKGs) is mandatory. T he western flower thrips, *Frankliniella occidentalis* (Thysanoptera: Thripidae), the main vector of *Tomato spotted wilt virus* (TSWV), is a very destructive invasive species. In this study, expression profiles of 11 candidate HKGs, including *β-actin* ( *Actin*), *α-tubulin* (*Tubulin*), *elongation factor 1 α* (*EF1A*), *vacuolar-typeH*$^{+}$*-ATPase* (*ATPase*), *NADH-ubiquinone oxidoreductase* (*NADH*), *heat shock protein 60* (*HSP60*), *heat shock protein 70* (*HSP70*), *heat shock protein 90* (*HSP90*), *ribosomal protein l32* (*RPL32*), *28S ribosomal RNA* (*28S*), *and 18S ribosomal RNA* (*18S*), from nonviruliferous and viruliferous *F. occidentalis* were investigated. Four distinct algorithms, *geNorm*, *Normfinder*, *BestKeeper*, and the $\Delta C_t$ method, were employed to determine the performance of these genes as endogenous controls under the virus condition. Based on *RefFinder*, which integrates all four analytical algorithms to compare and rank the candidates, *HSP70* , *HSP60*, *EF1A*, and *RPL32* were the most stable housekeeping genes. This work is the initial first step to establish a standardized qRT-PCR analysis in *F. occidentalis*. Additionally, this study lays a foundation for the research in the interactions between TSWV and *F. occidentalis*.

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## Introduction

The western flower thrips, *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae), is a very destructive invasive species around the world. *F. occidentalis* feeds on plants directly and also can transmit many plant viruses (Brødsgaard, 1994; Jones *et al*., 2005; Brunner & Frey 2010). In China, *F. occidentalis* was first found in Beijing in 2003 (Zhang *et al*., 2003), and a recent investigation revealed that this pest has spread to more than 10 provinces (Chen *et al*., 2011; Zheng *et al*., 2014). *Tomato spotted wilt virus* (TSWV, genus *Tospovirus*, family Bunyaviridae) is transmitted in a circulative and propagative manner by thrips (Ullman *et al*., 1992). TSWV infects and causes disease in hundreds of plant species, including many vegetables and ornamentals (Moyer, 1999). The nearly worldwide occurrence of TSWV epidemics can be related to the worldwide dispersal of its most efficient vector, *F. occidentalis* (Reitz & Funderburk 2012). In China, TSWV was first isolated from peanut (*Arachis hypogaea*) from Guangdong Province in 1984 (Xu *et al*., 1988). During the past one decade, TSWV has spread quickly in China following the introduction and spread of *F. occidentalis*.

In order to better understanding the molecular basis and facilitate the development of integrated pest management strategies of *F. occidentalis* and TSWV, Illumina sequencing platform was used to generate the transcriptome of *F. occidentalis* (Zhang *et al*., 2013). Specifically, a total of 51,391,358 clean reads were generated from *F. occidentalis*, and the reads were *de novo* assembled into 192,285 contigs and 59,932 unigenes (Zhang *et al*., 2013). To take advantage of these genomic resources, establishing a standardized Quantitative real-time PCR (qRT-PCR) procedure in *F. occidentalis* following the MIQE (Minimum Information for publication of Quantitative real time PCR Experiments) guidelines (Bustin *et al*., 2013) will be instrumental for the subsequent genomics and functional genomics studies in this pest.

Although qRT-PCR is one of the most speedy, reliable, and reproducible techniques for measuring and evaluating gene expressions during different biological processes (Vandesompele

*et al*., 2002), limitations still exist, including RNA quality and quantity, efficiency of reverse transcription and PCR reaction which can influence threshold cycle ($C_t$) values (Vandesompele *et al*., 2002; Strube *et al*., 2008; Bustin *et al*., 2005). A commonly used technique in qRT-PCR to normalize the gene expression data is to measure the expression of an internal housekeeping gene (HKG) in the same samples in parallel. Despite the demonstrated necessity for systematic validation of references genes in qRT-PCR studies, normalization procedures have not yet received much attention for this notorious invasive and virus vector species.

The objective of this study is to select suitable HKGs with stable expression in *F. occidentalis*. Here, 11 candidate HKGs (Zhang *et al*., 2013), including *β-actin* (*Actin*), *α-tubulin* (*Tubulin*), *elongation factor 1 α* (*EF1A*), *vacuolar-type $H^+$-ATPase* (*ATPase*), *NADH-ubiquinone oxidoreductase* (*NADH*), *heat shock protein 60* (*HSP60*), *heat shock protein 70* (*HSP70*), *heat shock protein 90* (*HSP90*), *ribosomal protein l32* (*RPL32*), *28S ribosomal RNA* (*28S*), *and 18S ribosomal RNA* (*18S*), from the nonviruliferous and viruliferous *F. occidentalis* were tested. To validate the selected reference genes, the effectiveness of these candidates was further examined by qRT-PCR analysis of a TSWV-receptor gene in both nonviruliferous and viruliferous *F. occidentalis*.

## Materials and Methods

### Insect rearing, plant cultures and TSWV inoculation

*Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) used in current study was collected from clover plants, *Trifolium repens* L. at the Experimental Station of Qingdao Agricultural University. The colony was reared on the common bean, *Phaseolus vulgaris* and maintained in MGC-250BP-2 incubators (Shanghai Yiheng Instruments, China) at 55% to 60% RH and 16: 8 h L: D. *F. occidentalis* adults were allowed to lay eggs on the *P. vulgaris* for one day, and then adults were removed. After that, the first instar larvae were obtained in a few days.

Pepper (*Capsicum annuum* L., cv. Zhongjiao 6) plants were grown in soil mixed vermiculite and organic fertilizer in 1.5-L pots (one plant per pot) under natural light and controlled temperatures (30 ± 2°C) within a glasshouse.

TSWV was maintained on *Datura stramonium* L. (Solanaceae). The virus inoculum was prepared by grinding infected plant material in chilled 0.01 M phosphate buffer, pH7, containing 1% (wt:vol) sodium sulfite and 2% (wt:vol) PVP (Boonham *et al*., 2002; Mandal *et al.,* 2008). The inoculum was mechanically transmitted by rubbing the ground material onto the upper leaves of the experimental pepper plants, which had been dusted with diatomaceous earth. Pepper plants were inoculated at the three true-leaf stage (Pan *et al*., 2013). After two weeks, infection was determined for inoculated by the molecular methods (Mason *et al*., 2003). Control plants handled with the inoculum grinding health plant material.

### Nonviruliferous and viruliferous *Frankliniella occidentalis*

Healthy and TSWV-infected pepper leaf discs (diameter, 26mm) were obtained using a cork borer. Each leaf was kept in a ventilated vial covering 3 ml 1.0% agar on the bottom to keep the leaves fresh. 30 of the first instar larvae maintained on the healthy and TSWV-infected discs in the vial for 24 h to obtain the nonviruliferous and viruliferous insects, respectively (Boonham *et al*., 2002; Wijkamp & Peters 1993). There are four replicates for each of the treatments. The samples were frozen in liquid nitrogen and stored at -80°C.

### Total RNA extraction and cDNA synthesis

Total RNA was extracted using TRIzol® (Invitrogen, Carlsbad, CA) following the manufacturer's instruction. First-strand cDNA was synthesized from 1.0 μg of total RNA using PrimeScript™ RT reagent kit with gDNA Eraser (TaKaRa, Shiga, Japan) according to the manufacturer's recommendations.

**Reference gene selection**

Eleven HKGs with previous record as the reference genes were selected as the candidate reference genes (Table 1). PCR amplifications were performed in 25μl reactions containing 2.5μl 10×PCR Buffer (Mg2+ Plus), 0.5μl dNTP mix (10 mM of each nucleotide), 0.5μl of each primer (10μM each), and 0.25μl of TaKaRa Taq (5u/μl) (TaKaRa). The PCR parameters were as follows: one cycle of 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 59 °C for 45 s and 72 °C for 1 min; a final cycle of 72 °C for 10 min. The resultant PCR products were electrophoresed on a 2.0% agarose gel in a 1.0×TBE buffer.

**Quantitative real-time PCR**

Quantitative real-time PCR were performed on a qTOWER 2.2 real-time Thermal Cycler system (Analytik Jena, Germany). PCR reactions (20 μl) contained 7.2 μl of $ddH_2O$, 10.0 μl of 2×SYBR® Premix Ex Taq (TaKaRa), 0.4 μl of each specific primer (10 μM), and 2.0 μl of first-strand cDNA template. The qPCR program included an initial denaturation for 3 min at 95 °C followed by 40 cycles of denaturation at 95 °C for 15 s, annealing for 30 s at 60 °C. For melting curve analysis, a dissociation step cycle (55 °C for 10 s, and then 0.5 °C for 10 s until 95 °C) was added. The reactions were set up in 96-well format Microseal PCR plates (Sangon, Shanghai, China) in triplicate. A 5-fold dilution series of cDNA (1/5, 1/25, 1/125, 1/625, and 1/3125) was used to construct a standard curve, and the qRT-PCR efficiency was calculated according to the equation: $E= (10^{[-1/slope]} -1)\times 100$.

## Validation of selected reference genes

One TSWV-receptor gene of *Frankliniella occidentalis* was used to evaluate the candidate housekeeping genes (GenBank No. AF247969). TSWV-receptor gene expression levels were investigated in nonviruliferous and viruliferous *F. occidentalis*. Two normalization factors (NFs) were calculated based on (1) geometric mean of genes with lowest *Geomean* values (as determined by *RefFinder*), and (2) a single reference with lowest or highest *Geomean* value. Relative quantification of TSWV-receptor gene in different samples was performed using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001).

## Data analysis

All biological replicates were used to calculate the average $C_t$ value. The stability of candidate HKGs was evaluated by algorithms *geNorm* (Vandesompele *et al*., 2002), *NormFinder* (Andersen *et al*., 2004), *BestKeeper* (Pfaffl *et al*., 2004), and the $\Delta C_t$ method (Silver *et al*., 2006). Finally, we compared and ranked the tested candidates based on a web-based comprehensive analysis tool *RefFinder* (http://www.leonxie.com/referencegene.php). One-way ANOVA was used to compare the expression levels of the TSWV-receptor gene in the viruliferous and nonviruliferous *F. occidentalis.*

## Results

### Transcriptional profiling of candidate reference genes

All genes tested were visualized as a single amplicon with expected size on a 2.0% agarose gel (Figure S1). Furthermore, gene-specific amplification of these genes was confirmed by a single peak in real-time melting curve analysis (Figure S2). A standard curve was generated for each gene, using a five-fold serial dilution of the pooled cDNAs. The correlation coefficient and PCR efficiency for each standard curve were shown in Table 1.

The mean and the standard derivation (SD) of the $C_t$ values were calculated for all the samples (Table S1). *EF1A* (SD=0.68) had the least variable expression level and it was reflected in its low SD values. By contrast, *28S* (SD=1.35) had the most variable expression levels, and it was shown in its high SD values. Additionally, *18S* had the lowest $C_t$ values ($C_{tavg}$=10.09), suggesting that it had the highest expression level, whereas, *Actin* was the least expressed gene among the candidates ($C_{tavg}$=31. 62) (Figure 1; Table S1).

### Quantitative analysis of candidate reference genes based on *geNorm*

To decide the minimal number of genes required for normalization, the V-value was computed by the *geNorm*. Starting with two genes, the software continuously adds another gene and recalculates the normalization factor ratio. If the added gene does not promote the normalization factor ratio over the proposed 0.15 cut-off value, then the starting pair of genes is enough for the normalization. If not, more genes should be incorporated. Here, the first V-value less than 0.15 were after V2/3 (Figure 2). This means that two reference genes were sufficient for reliable normalization no matter what the virus infection status of the insect.

### Selection of the best candidate reference genes

*GeNorm* bases its ranking on the geometric mean of the SD of each transformed gene set of pair combinations (M-value). The lower the M-value is, the higher the ranking. *EF1A* and *HSP70* were co-ranked as the most stable genes (M=0.270). The overall order based on *geNorm* from most stable to least stable reference genes was: *EF1A* = *HSP70*, *RPL32*, *HSP60*, *ATPase*, *HSP90*, *NADH*, *28S*, *18S*, *Actin*, and *Tubulin* (Table 2).

The $\Delta C_t$ method relies on relative pair-wise comparisons. Using raw $C_t$ values, the average SD of each gene set is inversely proportional to its stability. As shown in Tables 2 and S2, *HSP60* (0.69) was the top-ranked gene. The overall order from most stable to least stable reference genes based on the $\Delta C_t$ method was: *HSP60*, *HSP70*, *RPL32*, *HSP90*, *EF1A*, *ATPase*, *NADH*, *28S*, *18S*, *Actin*, and *Tubulin* (Table 2; Table S2).

A low stability value suggests a more stable gene by *NormFinder*. *HSP60* (0.152) was the most reliable and stable reference gene. The overall order from most stable to least stable reference genes based on *NormFinder* was: *HSP60*, *HSP70*, *RPL32*, *EF1A*, *ATPase*, *HSP90*, *NADH*, *28S*, *18S*, *Actin*, and *Tubulin* (Table 2).

*BestKeeper* provides a two-way ranking: Pearson's correlation coefficient [r] value and *BestKeeper* computed SD value. The stability of a gene is directly proportional to the [r] value, while it is inversely proportional to the SD value. *HSP60* (r=0.924) had the highest [r] value, and *EF1A* (SD=0.499) had the least variable expression levels across all the samples (Table 2; Table S3).

**Comprehensive ranking of best reference genes using *RefFinder***

According to *RefFinder*, which integrates the above-mentioned four software tools to compare and rank the candidates, the comprehensive ranking of candidate reference genes from the most to the least stable was: *HSP70*, *HSP60*, *EF1A*, *RPL32*, *ATPase*, *HSP90*, *NADH*, *18S*, *Actin*, *28S*, and *Tubulin* (Table 2). Among them, *Tubulin* had GM value almost 10.0 (Table 2), it had the lowest ranking and less suitable to serve as reliable reference gene for normalizing gene expression.

**Validation of selected reference genes**

Expression profile of one TSWV-receptor gene was evaluated in the nonviruliferous and viruliferous *F. occidentalis* in order to validate the selected reference genes (genes with the low *Geomean* value are considered stable). Using one, two or three best reference gene combinations for normalization, two genes [*HSP70* and *HSP60*; NF (1-2)] or three genes [*HSP70*, *HSP60*, and

171 *EF1A*; NF (1-3)], the TSWV-receptor gene expression levels were significantly higher in the
172 viruliferous than in the nonviruliferous *F. occidentalis* ($P<0.05$) (Figure 3).

## Discussion

The large body of recent works clearly suggested that there are no "universal" reference genes that are stably expressed and applicable for all the cell and tissue types and various experimental conditions (Li *et al*., 2013; Zhu *et al*., 2014; Fu *et al*., 2013; Sinha &Smith, 2014; Shi *et al*., 2013; Yuan *et al*., 2014; Shen *et al*., 2010; Galetto *et al*., 2014; Wu *et al*., 2013; Yang *et al*., 2014). Each candidate reference gene, however, should be evaluated under specific experimental conditions for gene profiling to ensure a constant level of expression (Thellin *et al*., 1999). For examples, our study demonstrated that *Tubulin* was the least appropriate reference gene in *F. occidentalis* (Table 2), whereas, *Tubulin* was a suitable reference gene in *F. occidentalis* across different developmental stage and different temperatures (Zheng *et al*., 2014). Zheng *et al*., (2014) focused on the selection of reference genes under the impacts of development and temperature in *F. occidentali*, while our study was mainly to investigate the expression profiles of 11 housekeeping genes in both the nonviruliferous and viruliferous *F. occidentalis*. In addition, our study demonstrated that the TSWV-receptor gene expression levels were significantly higher in the viruliferous than in the nonviruliferous *F. occidentalis*. Therefore, our study not only provides a standardized procedure for quantification of gene expression in *F. occidentalis*, but also lays a solid foundation for the study in the interactions between TSWV and *F. occidentalis*.

There has been ongoing discussion about the optimal number of reference genes demanded for qRT-PCR analysis. Previously, gene expression studies have mainly used a single endogenous control, however, this will observably influence the statistical outcome and may result in inaccurate data interpretation (Ferguson *et al*., 2010) or it is simply insufficient to normalize the expression of target genes (Veazey *et al*., 2011). To avoid biased normalization, more and more researchers have moved away from a single endogenous control and started to embrace the idea of using multiple reference genes to analyze gene expression (Li *et al*., 2013; Zhu *et al*., 2014; Fu *et al*., 2013). Results from our validation study with a targeted TSWV-receptor gene are consistent with the multi-gene normalize concept, suggesting that the use of

two reference genes is sufficient to normalize the expression data and provides more conservative estimation of target gene expression (Figure 2; Figure 3). As a result, we recommend that two reference genes are adequate for investigating gene expressions when we do the research upon the interactions between TSWV and *F. occidentalis*.

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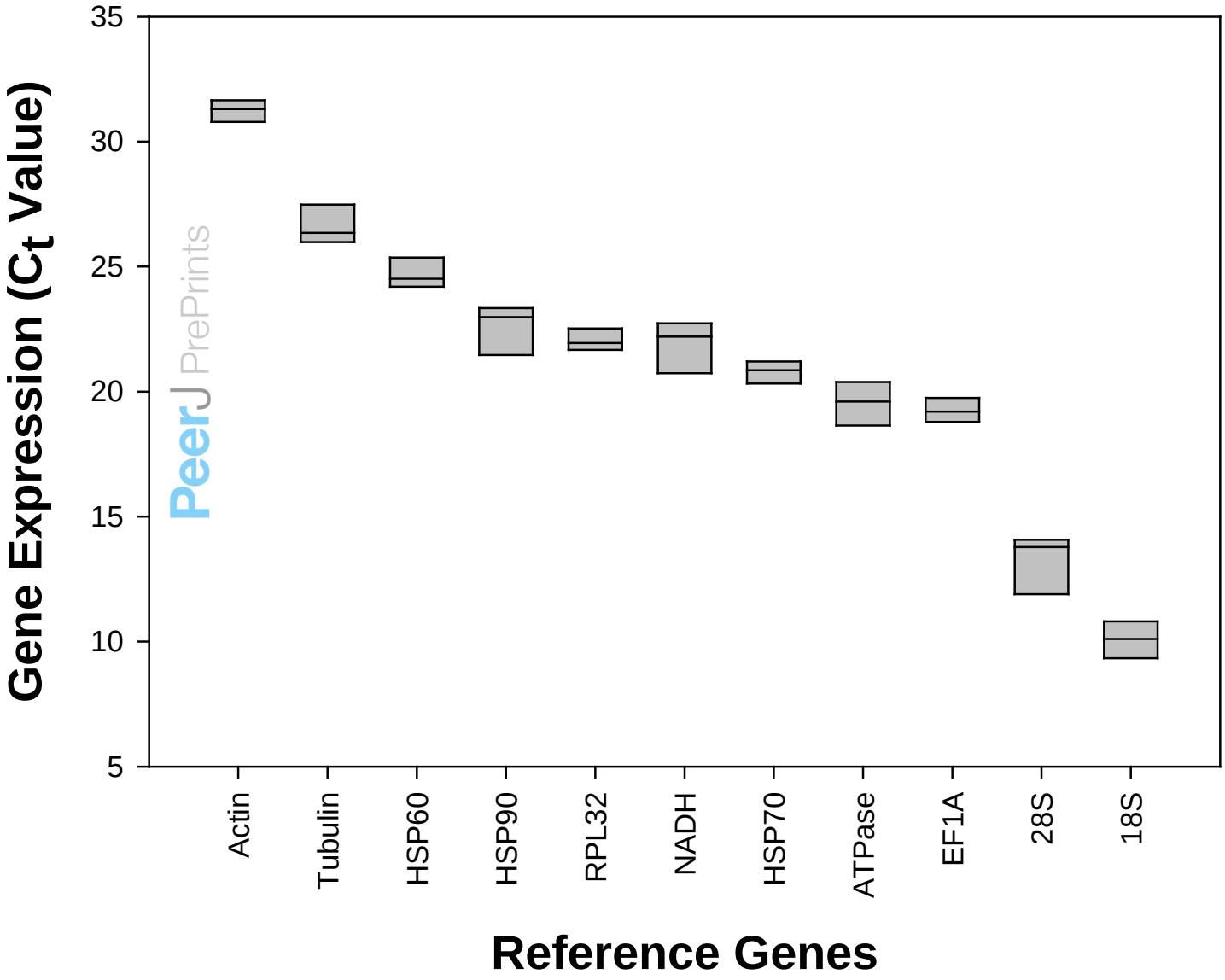
## Figure 1(on next page)

Expression profiles of candidate 11 reference genes in *Frankliniella occidentalis*.

**Expression profiles of candidate 11 reference genes in *Frankliniella occidentalis*.**
The expression level of candidate reference genes are documented in $C_t$-value. The median is represented by the line in the box. The interquartile rang is bordered by the upper and lower edges, which indicate the 75$^{th}$ and 25$^{th}$ percentiles, respectively.

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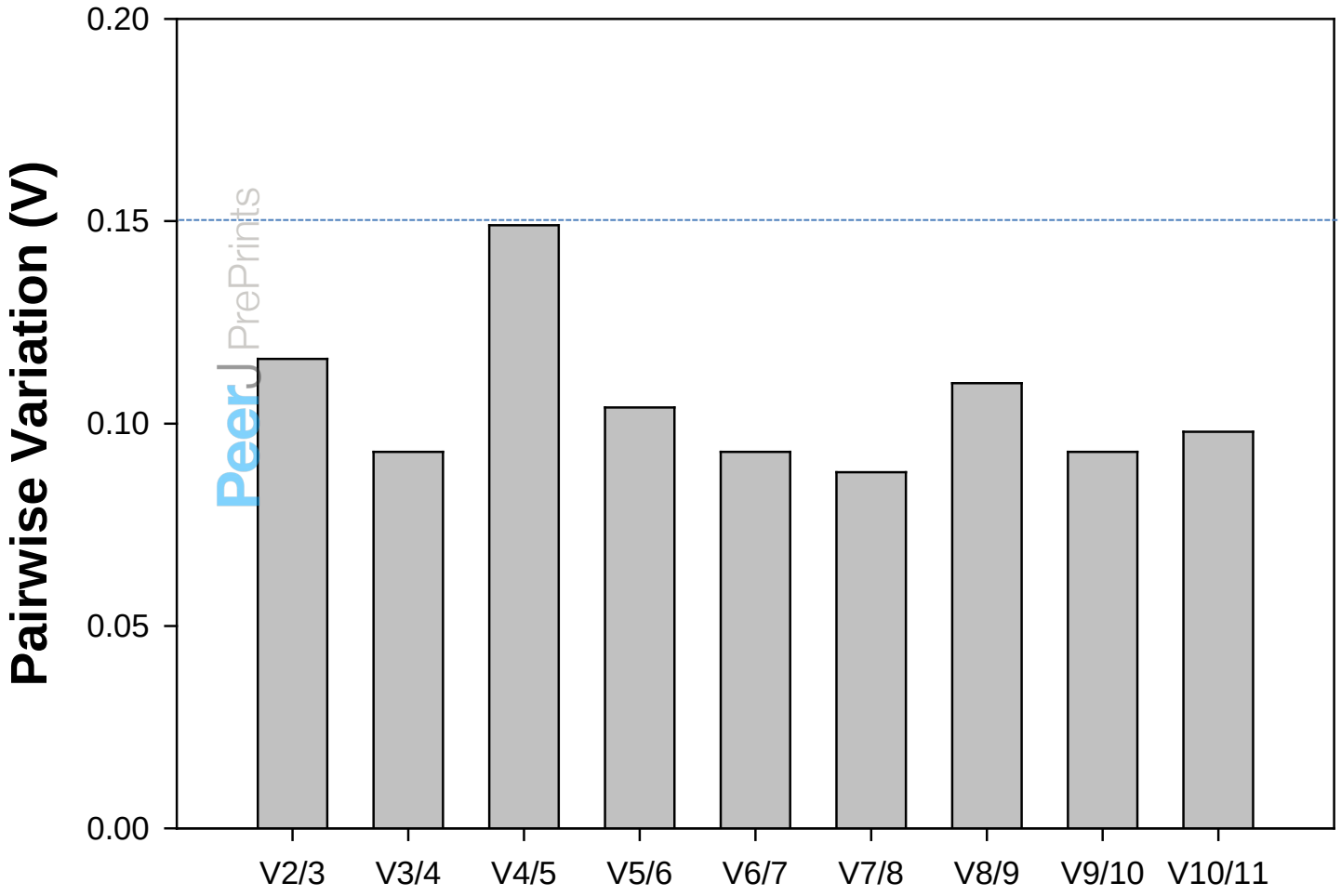
## Figure 2(on next page)

Pairwise variation analysis of the candidate 11 reference genes in *Frankliniella occidentalis*

**Pairwise variation analysis of the candidate 11 reference genes in *Frankliniella occidentalis***. The *geNorm* first calculates an expression stability value (M) for each gene and then compares the pair-wise variation (V) of this gene with the others. A threshold of V<0.15 was recommended for valid normalization.

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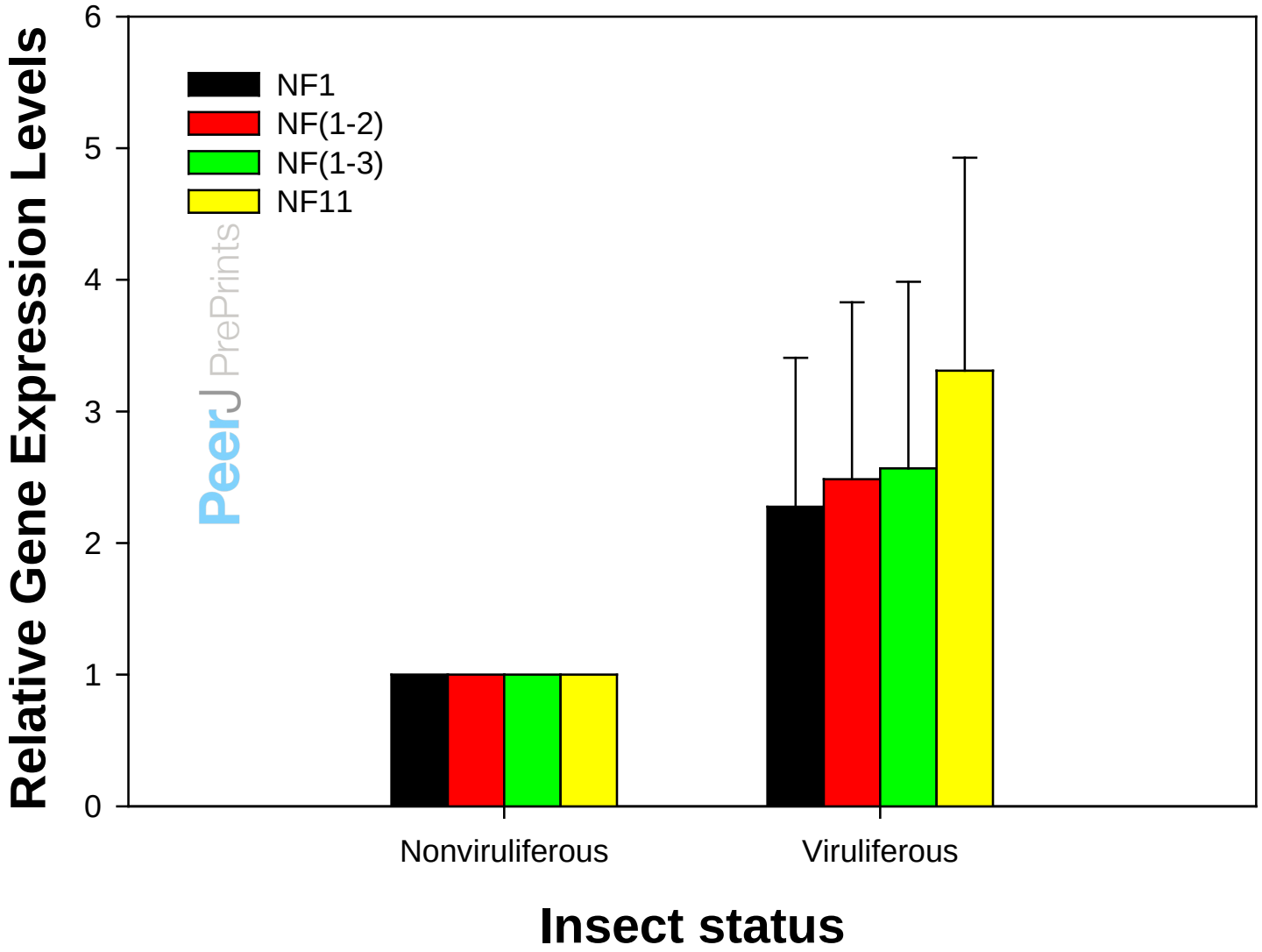
## Figure 3(on next page)

Validation of recommended reference gene in *Frankliniella occidentalis*

**Validation of recommended reference gene in *Frankliniella occidentalis*** . Expression profiles of TSWV-receptor gene in both the nonviruliferous and viruliferous *Frankliniella occidentalis* were investigated using different combinations of reference genes. NF1, NF (1-2), NF (1-3), and NF11 indicate that the expression of TSWV-receptor gene was normalized using the best, the top two, the top three, or, the worst reference genes, respectively. Bar represents the mean and standard error of four biological replicates.

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## Table 1(on next page)

Summary of the 11 housekeeping genes tested in this study

Table 1. Summary of the 11 housekeeping genes tested in this study

| Gene | Description | Accession No. | Primer sequences (5'-3') | Length (bp) | E (%)* | $R^{2,**}$ |
|---|---|---|---|---|---|---|
| *HSP70* | *heat shock protein 70* | KC148536 | F: GTCACCGTACCCGCATATTT<br>R: GCAGTGGGCTCGTTGATAATA | 104 | 0.95 | 0.9845 |
| *HSP60* | *heat shock protein 60* | JX967580 | F:CTGGACTGTAAGCGTGCTATAA<br>R: GGCACGATGAACACCTATGA | 80 | 0.91 | 0.9903 |
| *EF1A* | *elongation factor 1 α* | AB277244 | F: AAGGAACTGCGTCGTGGATA<br>R: AGGGTGGTTCAGGACAATGA | 99 | 1.05 | 0.991 |
| *RPL32* | *ribosomal protein l32* | AB572580 | F:CTGGCGTAAACCTAAGGGTATT<br>R: GTCTTGGCATTGCTTCCATAAC | 96 | 0.98 | 0.9998 |
| *ATPase* | *vacuolar type $H^+$-ATPase* | JN835456 | F:TACCAAATGGGACTCCAATACC<br>R:GTAAGTAAGAGGTGGCCAGATAC | 130 | 0.90 | 0.9970 |
| *HSP90* | *heat shock protein 90* | JX967579 | F: CTCGCAACCAGGACGATATTAG<br>R: CTGACCCTCCACAGAGAAATG | 110 | 0.96 | 0.9918 |
| *NADH* | *NADH-ubiquinone oxidoreductase* | YP_006576366 | F: AGCTACTAAACCGCCTCATAAA<br>R:GGTGGTTATGGTATTTATCGTTTGT | 99 | 0.95 | 0.9656 |
| *18S* | *18S ribosomal RNA* | JX002704 | F: CTGCGGAAATACTGGAGCTAATA<br>R: AAGTAGACGATGGCCGAAAC | 109 | 1.09 | 0.9960 |
| *Actin* | *β-actin* | AF434716 | F:CCTCATCCCTAGTTGTCTTGTG<br>R: TTCTCGCTCAGCTGTAATTGT | 96 | 0.86 | 0.9788 |
| *28S* | *28S ribosomal RNA* | GU980314 | F: GGGTGGTAAACTCCATCTAAGG<br>R:CACGTACTCTTGAACTCTCTCTTC | 108 | 0.97 | 0.9969 |
| *Tubulin* | *α-tubulin* | KC513334 | F: GTGGACAACGAAGCCATCTA<br>R: CGGTTCAGGTTGGTGTAGG | 77 | 1.04 | 0.9900 |

"*": PCR efficiency (calculated from the standard curve)
"**": Regression coefficient

## Table 2(on next page)

Ranking of the 11 housekeeping genes using five different algorisms

Table 2. Ranking of the 11 housekeeping genes using five different algorisms

| *RefFinder* | | *geNorm* | | *NormFinder* | | $\Delta C_t$ | | | *BestKeeper* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Genes | GM | Genes | SV | Genes | SV | Genes | SV | Genes | [r] | Genes | SD |
| *HSP70* | 1.68 | *EF1A* | 0.270 | *HSP60* | 0.152 | *HSP60* | 0.69 | *HSP60* | 0.924 | *EF1A* | 0.499 |
| *HSP60* | 2.00 | *HSP70* | 0.270 | *HSP70* | 0.299 | *HSP70* | 0.71 | *HSP90* | 0.917 | *HSP70* | 0.521 |
| *EF1A* | 2.11 | *RPL32* | 0.335 | *RPL32* | 0.398 | *RPL32* | 0.75 | *28S* | 0.900 | *RPL32* | 0.556 |
| *RPL32* | 3.00 | *HSP60* | 0.375 | *EF1A* | 0.507 | *HSP90* | 0.80 | *HSP70* | 0.882 | *HSP60* | 0.560 |
| *ATPase* | 5.89 | *ATPase* | 0.537 | *ATPase* | 0.531 | *EF1A* | 0.80 | *NADH* | 0.870 | *Actin* | 0.566 |
| *HSP90* | 6.16 | *HSP90* | 0.592 | *HSP90* | 0.547 | *ATPase* | 0.82 | *ATPase* | *0.860* | *18S* | 0.595 |
| *NADH* | 7.45 | *NADH* | 0.627 | *NADH* | 0.638 | *NADH* | 0.85 | *RPL32* | 0.858 | *Tubulin* | 0.784 |
| *18S* | 8.13 | *28S* | 0.667 | *28S* | 0.852 | *28S* | 1.00 | *EF1A* | 0.748 | *ATPase* | 0.809 |
| *Actin* | 8.41 | *18S* | 0.754 | *18S* | 0.878 | *18S* | 1.06 | *Actin* | 0.379 | *NADH* | 0.894 |
| *28S* | 8.66 | *Actin* | 0.815 | *Actin* | 0.893 | *Actin* | 1.08 | *18S* | 0.355 | *HSP90* | 0.915 |
| *Tubulin* | 9.82 | *Tubulin* | 0.888 | *Tubulin* | 1.060 | *Tubulin* | 1.22 | *Tubulin* | 0.276 | *28S* | 1.069 |

The parameters include Geometric mean (GM); Stability Value (SV); Pearson's correlation coefficient ([r]); Standard Deviation (SD)