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Selection of Reference Genes for Expression Studies in *Diaphorina citri* (Hemiptera: Liviidae)

Meire Menezes Bassan,[1,*] Jéssika Angelotti-Mendonça,[1,*] Gustavo Rodrigues Alves,[2] Pedro Takao Yamamoto,[2] and Francisco de Assis Alves Mourão Filho[1,3]

[1]Departamento de Produção Vegetal, Universidade de São Paulo, Escola Superior de Agricultura "Luiz de Queiroz", Av. Pádua Dias, 11 - Cx. Postal 9, Piracicaba, São Paulo, Brazil, [2]Departamento de Entomologia e Acarologia, Universidade de São Paulo, Escola Superior de Agricultura "Luiz de Queiroz", Av. Pádua Dias, 11 - Cx. Postal 9, Piracicaba, São Paulo, Brazil, and [3]Corresponding author, e-mail: francisco.mourao@usp.br

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*These authors had equal contribution for the development of this research work.

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Abstract

The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), is considered the main vector of the bacteria associated with huanglongbing, a very serious disease that has threatened the world citrus industry. The absence of efficient control management protocols, including a lack of resistant cultivars, has led to the development of different approaches to study this pathosystem. The production of resistant genotypes relies on *D. citri* gene expression analyses by RT-qPCR to assess control of the vector population. High-quality, reliable RT-qPCR analyses depend upon proper reference gene selection and validation. However, adequate *D. citri* reference genes have not yet been identified. In the present study, we evaluated the genes *EF 1-α*, *ACT*, *GAPDH*, *RPL7*, *RPL17*, and *TUB* as candidate reference genes for this insect. Gene expression stability was evaluated using the mathematical algorithms deltaCt, NormFinder, BestKeeper, and geNorm, at five insect developmental stages, grown on two different plant hosts [*Citrus sinensis* (L.) Osbeck (Sapindales: Rutaceae) and *Murraya paniculata* (L.) Jack (Sapindales: Rutaceae)]. The final gene ranking was calculated using RefFinder software, and the *V-ATPase-A* gene was selected for validation. According to our results, two reference genes are recommended when different plant hosts and developmental stages are considered. Considering gene expression studies in *D. citri* grown on *M. paniculata*, regardless of the insect developmental stage, *GAPDH* and *RPL7* have the best fit as reference genes in RT-qPCR analyses, whereas *GAPDH* and *EF 1-α* are recommended as reference genes in insect studies using *C. sinensis*.

Key words: developmental stages, host, normalizers, psyllid, RT-qPCR

The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), is the most important vector of "*Candidatus* Liberibacter asiaticus" (CLas) and "*Candidatus* Liberibacter americanus" (CLam), bacteria known to be associated with huanglongbing (HLB). HLB, considered a highly destructive citrus disease, and already reported in very important citrus growing regions around the world, has threatened the citrus industry because of its significant associated losses and lack of efficient management protocols. Moreover, there are no citrus genotypes considered resistant to HLB (Bové 2006). The spread of HLB has been very fast. In some groves, significant plant losses and consequent rapid increases in production costs have been reported. In Florida, for instance, approximately 70% of all citrus plants are contaminated by HLB, which has resulted in one of the lowest harvest seasons (2015/2016) within the last 50 yr (USDA 2015). In Brazil, since the first report of HLB about 12 yr ago (Teixeira et al. 2008) until the first semester of 2016, more than 44 million citrus plants in São Paulo state, the main producing region, have been destroyed because of the disease (Coordenadoria de Defesa Agropecuária 2016).

D. citri has a considerable host range, especially among plants from the Rutaceae family (Sapindales), including the *Citrus* genus and *Murraya paniculata* (L.) Jack (Sapindales: Rutaceae). This last species is considered the preferential host (Halbert and Manjunath 2004, Teck et al. 2011). Although *D. citri* is able to exploit a large number of plant hosts, insect development can vary considerably among them. The choice of a potential host depends on different

factors such as nutrient availability and acquisition, and the ability to overcome plant defenses and avoid intoxication. These factors may directly affect insect feeding and fitness (Simon et al. 2015).

There are no curative methods for HLB, and disease management is based on preventative actions, such as the use of healthy nursery trees, the eradication of symptomatic plants in the field, and control of the vector population by spraying with insecticides (Gottwald 2010). Considering the severity of HLB, the lack of curative methods and the lack of citrus resistant cultivars, the application of molecular biology tools may help in the development of new biotechnological approaches for the management of the insect vector *D. citri* in the field.

Gene expression analysis through real-time quantitative polymerase chain reaction (RT-qPCR) has been extensively utilized in entomological studies in order to understand insect behavior under different biotic and abiotic conditions, as well as at different developmental stages (Shakeel et al. 2015, Ibanez and Tamborindeguy 2016, Koramutla et al. 2016, Nakamura et al. 2016). RT-qPCR allows precise expression quantification of a given gene by comparison with the expression of reference genes that are known to be stably expressed.

Although great precision and sensitivity of gene expression analysis is possible using RT-qPCR, some limitations in the technique may lead to variations in cycle threshold (Ct) values. The Ct is defined as the PCR cycle at which the fluorescent signal of the reporter dye crosses an arbitrarily placed threshold. The numerical value of the Ct is inversely related to the amount of amplicon in the reaction (i.e., the lower the Ct, the greater the amount of amplicon; Schmittgen and Livak 2008). These limitations include variations among samples, variations among reverse transcription reactions, and PCR efficiency (Bustin et al. 2005). Therefore, the precise results obtained from this analysis depend upon an adequate selection of reference genes for each experimental condition.

For most studies involving gene expression analysis, constitutive genes are used as reference genes, as their expression is assumed to be constant and independent of the physiological condition of the samples and the applied treatments (Vandesompele et al. 2002, Kozera and Rapacz 2013, Li et al. 2013). However, several studies have questioned the universal use of these genes as reference genes, because most of them have shown variation in their expression depending on the type of organism, its developmental stage, and the applied treatment (Kozera and Rapacz 2013, Yang et al. 2015a, Ma et al. 2016). Therefore, for gene expression studies, it is important to select and validate reference genes according to the organism and to the experimental conditions.

The aim of this study was to select appropriate reference genes to develop an accurate and comprehensive qRT-PCR method for use in *D. citri* gene expression analyses. Six candidates were examined as potential reference genes: elongation factor 1-α (*EF 1-α*), actin (*ACT*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), ribosomal protein L7 (*RPL7*), ribosomal protein L17 (*RPL17*), and α-tubulin (*TUB*). The stability of expression of these genes was evaluated using four different mathematical algorithms at various developmental stages on two different hosts.

Materials and Methods

Experimental Conditions

The insects were collected from the maintenance collection at the Integrated Management of Pests Laboratory, Department of Entomology and Acarology, Escola Superior de Agricultura 'Luiz de Queiroz', Univerdade de São Paulo, and reared on orange jasmine (*M. paniculata*) according to adapted methodology (Parra et al. 2016).

In the study of reference genes in *D. citri* at different developmental stages and on different hosts, adult insects were placed in cages (35 by 35 by 53 cm) for 48 h to allow oviposition on two different hosts: sweet orange plants [*Citrus sinensis* (L.) Osbeck (Sapindales: Rutaceae)] cultivar Valencia and orange jasmine (*M. paniculata*). After that time, the adults were removed and the plants with eggs were kept in the same cages. The study was conducted under controlled conditions of 25 ± 2°C; 60 ± 10% relative humidity and with a photoperiod of 14:10 (L:D) h.

The experiment was conducted with three biological replicates composed of a pool of 150 first and second instar nymphs (N12), 30 third instar nymphs (N3), 10 fourth and fifth instar nymphs (N45), three newly emerged adults (collected 24 h after emergence; A1D), and three adults collected 10 d after emergence (A10D). The insect specimens were not sexed nor segregated by color morph.

The expression of candidate reference genes was analyzed at different developmental stages of *D. citri* reared on two hosts. The samples were flash frozen in liquid nitrogen and stored at −80°C until RNA isolation.

Selection of Reference Genes and Primer Design

Six commonly reference genes used in hemipteran studies were selected: elongation factor 1-α (*EF 1-α*), actin (*ACT*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), ribosomal protein L7 (*RPL7*), ribosomal protein L17 (*RPL17*), and α-tubulin (*TUB*). Their gene sequences in *D. citri* were obtained from the NCBI database and primers were designed using the RT-PCR Tool (http://www.idtdna.com/scitools/Applications/RealTimePCR/; Table 1). The primers were subsequently evaluated using NetPrimer (http://www.premierbiosoft.com/netprimer/index.html) in order to avoid designs that are likely to form secondary structures.

RNA Extraction and cDNA Synthesis

Total RNA was isolated using TRIzol® reagent (Invitrogen, Carlsbad, CA), according to manufacturer's protocol. RNA yield and purity were determined by Nanodrop (Thermo Scientific, Waltham, MA), and RNA integrity was verified by electrophoresis in 1.0% agarose gels. DNase I Amplification Grade (Invitrogen) was used to remove genomic DNA from the samples. First strand cDNA was synthesized from 500 ng of DNA-free RNA using M-MLV reverse transcriptase (Fermentas, Waltham, MA) according to the manufacturer's instructions, and stored at −20°C until use.

Expression Analysis by Real-time Quantitative PCR (RT-qPCR)

Quantitative real-time PCR was performed using Sybr Master Mix Fast (Invitrogen) on an Applied Biosystems 7500 Real-Time PCR instrument (Applied Biosystems, Foster City, CA). The thermocycling program was as follows: one denaturation step at 95°C for 20 s, followed by 40 cycles, each consisting of denaturation at 95°C for 3 s and annealing and extension at 60°C for 30 s. At the end of each PCR run, a melting curve analysis from 60 to 95°C was applied to all reactions to ensure the specificity of the amplified product. The reactions were performed in 10 µl volumes containing 6 µl of Sybr Master Mix Fast (Invitrogen), 0.15 µl of each primer, 2.7 µl of RNase free water, and 1 µl of cDNA template. Standard curves were created based on a fivefold dilution series of cDNA (1:5, 1:25, 1:125, 1:625). The gene-specific PCR efficiency (E) of the primer pairs was calculated using a linear regression model. The amplification efficiency for each gene specific primer was calculated according to the equation: $E = (10^{(-1/\text{slope})} - 1 \times 100)$ (Pfaffl 2001). Three biological

Table 1. Primers used for qRT-PCR analysis of candidate reference genes and *V-ATPase-A*, a target gene

Gene symbol	Gene name	Accession number (NCBI)	Function	Primer sequence (5′-3′)	Product length (bp)	Efficiency (%)	Tm (°C)	R^2
ACT	*Actin*	DQ675553.1	structural constituent of cytoskeleton	F: CCATCTTGGCTTCTCTGTCTAC R: CATTTGCGGTGAACGATTCC	91	101.62	60	0.966
α-TUB	*Alpha-tubulin*	DQ675550.1	structural constituent of cytoskeleton	F: GGTTCAAGGTGGGTATCAACTAT R: TAGCGGTGGTGTTGGAAAG	102	105.77	60	0.977
EF 1-α	*Elongation factor 1 alpha*	XM_008479903.1	translation elongation factor activity	F: ACCACCACCAACACATCTAC R: ACTTCTTCTCCTCCTCTCATCT	119	92.83	60	0.998
GAPDH	*Glyceraldeyde-3-phopate dehydrogenase*	XM_008481619.1	Glycolytic enzyme	F: GACACTCACTCCTCCATCTTTG R: GTATCCGTACTCGTTGTCATACC	96	95.03	60	0.995
RPL7	*Ribosomal protein L7*	XM_008476740.1	structural constituent of ribosome	F: GAGGTGTTCACTGTTGGAGATAA R: TCTTACGCCATCCTCCAGTA	91	94.87	60	0.952
RPL17	*Ribosomal protein L17*	NM_001297694.1	structural constituent of ribosome	F: CCAGGAATCCCACCAAATCA R: AAGAACTTGATGGCTCGTCTC	121	98.85	60	0.991
V-ATPase -A	*Vacuolar ATPase subunit A*	FK254156.1	organelles acidification; pH homeostasis; membrane energization	F: GGAAGTCATATCACTGGTGGAG R: AAGTGACAGTACCCTTAGCTTTAG	94	82.54	60	0.950

F, forward primer; R, reverse primer; Tm, melting temperature; R^2, coefficient of correlation.

replicates were used for each condition, and each reaction was analyzed with three technical replicates.

Determination of Gene Expression Stability

The expression stability of the selected candidate reference genes was evaluated using geNorm (Vandesompele et al. 2002), Normfinder (Andersen et al. 2004), BestKeeper (Pfaffl et al. 2004), and the deltaCt method (Silver et al. 2006). BestKeeper uses raw data (Ct values) and PCR efficiency (E) to calculate the suitable standards and combines them into an index. The geNorm algorithm calculates an expression stability value (M) for each gene for a given set of samples and ranks them. The lower the M value, the higher the expression stability, and an M value less than 1.5 is recommended for the identification of stably expressed genes. The R-based SLqPCR package was used to calculate the pairwise expression variation (V_n/V_{n+1}) of these genes. This index was used to determine the appropriate number of reference genes necessary to increase the normalization accuracy of the RT-qPCR. A threshold value below 0.15 suggests that no additional reference genes are required for normalization (Vandesompele et al. 2002). This statistical algorithm starts the analysis with two genes and consecutively adds further genes, recalculating the ratio normalization factor. If the added gene does not increase the ratio of the normalization factor above the threshold value suggested (0.15), the pair of genes is considered sufficient to normalize the data; otherwise, more genes must be incorporated. NormFinder calculates gene expression stability for all samples in any number of groups based on intra and inter group variations and combines these values to provide a gene rank order based on the variation in gene expression. The candidate gene with the lowest value is considered to be the most stable reference gene (Andersen et al. 2004). In the deltaCt method, a rank order is determined based on pairwise comparisons of gene-sets using mean deltaCt values within a particular treatment. Therefore, the stability of a candidate reference gene is inversely proportional to its average standard deviation value, and the gene with the lowest value is considered to be the most stable (Silver et al. 2006).

Finally, the RefFinder tool (http://www.leonxie.com/referencegene.php) was used to determine an overall ranking of the candidate genes. RefFinder integrates the previous four methods (geNorm, Normfinder, BestKeeper, and the comparative deltaCt method) to compare and rank the tested candidate reference genes. The software assigns a weight to each gene and calculates their geometric mean to determine an overall final ranking (Xie et al. 2012).

Validation of Reference Gene Selection

To validate the selection of reference genes, the expression levels of *vacuolar ATPase subunit A* (*V-ATPase-A*; GenBank FK254156.1) of *D. citri* were analyzed at different developmental stages and on two hosts. The genes recommended by RefFinder and the number of genes indicated by pairwise variation analysis were used for data normalization.

For each host, the expression profiles of the *V-ATPase-A* gene were normalized using the most stable reference gene (NF1), the least stable reference gene (NF8), and a combination of the two most stable reference genes (NF1 and NF2). The N12 stage was used as a control for the calculation of gene expression.

Amplification efficiencies and relative expression levels of the target gene in different samples were calculated according to the method described by Pfaffl (Pfaffl 2001). The significance of changes in target gene expression normalized using the most stable reference gene, the least stable reference gene and the recommended combination of reference genes were calculated using Dunnett's test implemented in SAS software (Statistical Analysis System, V. 9.3, Cary, NC) with a significance level set at $P = 0.05$.

Results

Performance of RT-qPCR Primers

For all of the designed primers, one single amplicon of the expected size was detected. A standard curve for each candidate gene was generated using a fivefold cDNA dilution series. Primer specificity was confirmed by the presence of a single peak in melting curve analysis. The primer efficiency varied from 82.54 to 105.77% and correlation coefficients (R^2) ranged from 0.950 to 0.998 (Table 1). The cDNA dilution used for RT-qPCR analyses was 1:5.

Expression Profiles of Candidate Reference Genes

Average Ct values varied from 17.91 (*ACT*) to 29.37 (*TUB*). The lowest expression variation was recorded for *GAPDH* (21.27 ± 0.59) and *ACT* (17.91 ± 0.72). *TUB* and *RPL17* showed the greatest variations in expression, with average Ct values of 20.37 ± 3.01 and 22.26 ± 1.00, respectively (Fig. 1).

Stability of Candidate Reference Genes

Gene classification varied according to the algorithm used. However, *EF 1-α* was ranked among the three most stable genes by all five of the softwares used, whereas *TUB* was classified as the least stable gene at all developmental stages on both hosts (Table 2). For a better understanding of the reference gene ranking, each statistical method is considered below.

DeltaCt method

This method identified *EF 1-α*, *GAPDH*, and *RPL7* as the three most stable genes at all developmental stages of *D. citri* reared on *M. paniculata* and *C. sinensis*. Under the same conditions, *TUB* was reported as having the lowest expression stability (Table 2).

BestKeeper

This mathematical model classified *GAPDH*, *EF 1-α*, and *RPL7* as the three most stable reference genes expressed in insects reared on *C. sinensis*. On the other hand, for insects reared on *M. paniculata*, *GAPDH*, *ACT*, and *EF 1-α* were reported as the most stably expressed genes. The BestKeeper algorithm also classified *TUB* as the least recommended gene for data standardization in expression studies of *D. citri* at different developmental stages (Table 2).

NormFinder

Based on the ranking indicated by this algorithm, the levels of gene expression were more consistent for the *GAPDH*, *ACT*, and *EF 1-α*

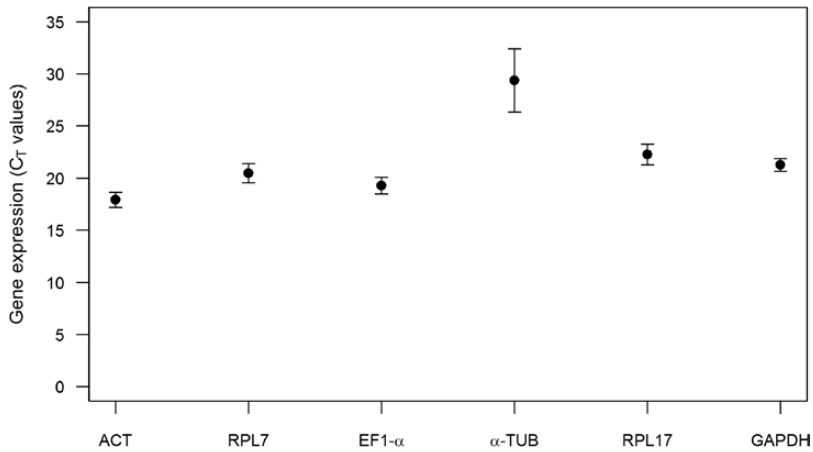

Fig. 1. Expression profiles of six candidate reference genes of *D. citri* at five developmental stages and on two different hosts. The expression levels of candidate reference genes in the samples are documented by the Ct values. The 'black dots' indicate the mean value of replicated samples, and the whiskers indicate the standard deviation of the mean.

Table 2. Ranking of the six reference genes of *D. citri* using four different algorithms at five developmental stages and on two different hosts

Host	Reference gene	ΔCT		BestKeeper		Normfinder		geNorm	
		Stability	Rank	Stability	Rank	Stability	Rank	Stability	Rank
Citrus sinensis	*ACT*	1.265	4	0.606	4	0.279	2	0.682	4
	α-TUB	3.35	6	2.682	6	3.324	6	1.442	5
	EF 1-α	1.143	1	0.547	2	0.368	3	0.453	2
	GAPDH	1.149	2	0.439	1	0.279	1	0.580	3
	RPL7	1.223	3	0.620	3	0.784	4	0.371	1
	RPL17	1.319	5	0.780	5	1.006	5	0.371	1
Murraya paniculata	*ACT*	1.414	5	0.508	2	0.277	2	0.804	4
	α-TUB	3.396	6	2.571	6	3.363	6	1.552	5
	EF 1-α	1.292	2	0.714	3	0.731	3	0.445	2
	GAPDH	1.220	1	0.422	1	0.277	1	0.635	3
	RPL7	1.336	3	0.836	4	0.914	4	0.382	1
	RPL17	1.383	4	0.891	5	1.031	5	0.382	1

genes at all insect developmental stages on both plant hosts. The *TUB* gene was, again, the least recommended for data standardization of gene expression in *D. citri* in all experimental conditions used in this study (Table 2).

geNorm

This statistical algorithm classified the ribosomal protein genes *RPL7* and *RPL17*, followed by *EF 1-α* and *GAPDH* as the most stably expressed genes, in the experimental conditions tested. *TUB* showed greater variation in gene expression, and was, therefore, ranked lowest for insect gene expression studies on both hosts (Table 2). Pairwise variation analysis indicated that, for the five developmental stages investigated in this study, two reference genes should be recommended for data standardization on both hosts because the V2/3 values were less than 0.15 (Fig. 2).

RefFinder

This software was used to produce a general ranking of all candidate reference genes, considering the results from the four previously used statistical algorithms. For the different developmental stages of *D. citri*, reared on either host, the three most stably expressed genes were *GAPDH*, *RPL7*, and *EF 1-α*, whereas *TUB* was the least stably expressed gene (Fig. 3).

Validation of the Reference Genes

The consolidated data from *RefFinder* indicated that for *C. sinensis* the two most stable genes are *GAPDH* and *EF 1-α*, whereas for *M. paniculata GAPDH* and *RPL7* are recommended as reference genes. The *TUB* gene had the lowest expression stability on both hosts. The validation of the reference genes was realized by using them for the normalization of *V-ATPase-A* gene expression, either alone or in combination.

In gene expression studies in *D. citri* reared on *C. sinensis*, the *V-APTase-A* gene expression results at each developmental stage were similar when the most stable reference gene or the combination of the two most stably expressed genes was utilized as normalizers (Fig. 4B). With *M. paniculata* as plant host, when the most stably expressed reference gene was used as a normalizer, only young adults of *D. citri* showed significant differences in *V-ATPase-A* gene expression compared with insects at the N12 developmental stage ($P < 0.05$). On the other hand, when the calculation of the target gene expression utilized the least stably expressed reference gene (*TUB*), significant differences ($P < 0.05$) in *V-ATPase-A* gene

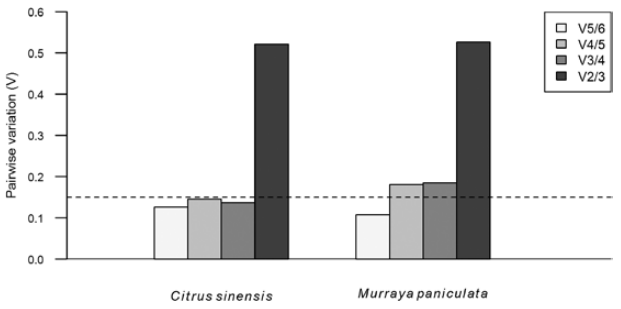

Fig. 2. Optimal number of reference genes for accurate normalization in *D. citri*. The pairwise variation (V_n/V_{n+1}) between the normalization factors NF_n and NF_{n+1} was analyzed using the R-based SLqPCR package, to determine the optimal number of reference genes required for accurate normalization in a given class of experiment. A value below 0.15 indicated that the additional reference gene has no significant improvement on normalization in qRT-PCR data.

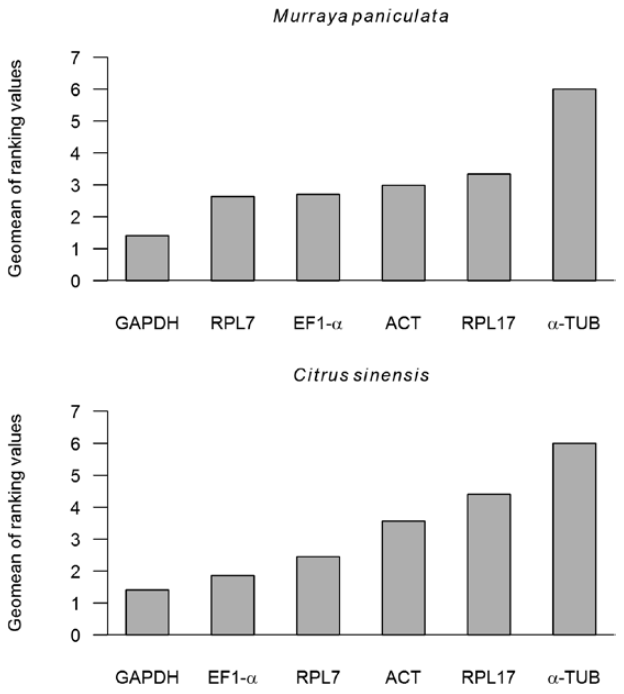

Fig. 3. Expression stability of six candidate reference genes of *D. citri* at five developmental stages and on two different hosts. The average expression stability of the reference genes was calculated using the Geomean method in RefFinder. A lower Geomean ranking value indicates more stable expression: (A) *M. paniculata* and (B) *C. sinensis*.

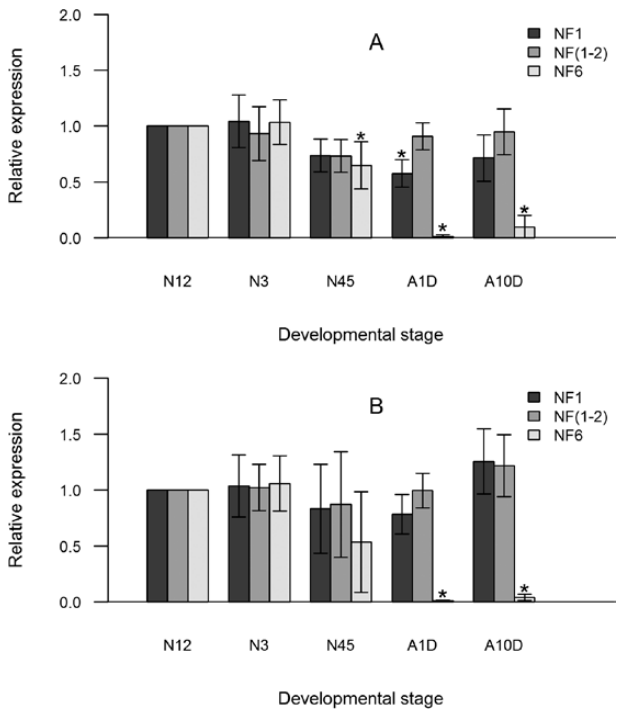

Fig. 4. Variation in *V-ATPase-A* gene expression data normalized by different reference genes and their combinations for five developmental stages and two hosts: (A) *M. paniculata* and (B) *C. sinensis*. The most stable reference gene *GAPDH* (NF1); the combinations of *GAPDH* and RPL7; and *GAPDH* and EF 1-α (NF1-2), respectively, and the least stable reference gene *TUB* (NF6) were independently used as normalizers. Bars represent the means and standard deviations of three biological replicates, and comparison of means was carried out using the Dunnett test ($P < 0.05$). * indicates significantly different values.

expression were recorded at the N45, A1D, and A10D developmental stages in insects reared on *M. paniculata* and at the A1D and A10D developmental stages on *C. sinensis* (Fig. 4A).

Discussion

In the present study, six candidate reference genes were evaluated and validated for gene expression analyses using RT-qPCR in *D. citri* at different developmental stages, reared on two different hosts. As previously reported for the lepidoptera species *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae) (Chandra et al. 2014, Shakeel et al. 2015), *Thitarodes armoricanus* (Oberthür) (Lepidoptera: Hepialidae) (Liu et al. 2016), *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) (Teng et al. 2012), and *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae) (Lu et al. 2013), and for the aphid *Lipaphis erysimi* (Kaltenbach) (Hemiptera: Aphididae) (Koramutla et al. 2016), the reference gene with the most stable expression at all different developmental stages of *D. citri* on both hosts was *GAPDH*. This gene is important for energy metabolism, encoding enzymes of the glycolytic pathway (Ponton et al. 2011). However, *GAPDH* did not demonstrate adequate expression stability in experiments carried out with the hemiptera species *Aphis gossypii* Glover (Hemiptera: Aphididae) (Ma et al. 2016) and *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) (Kang et al. 2017). Low expression stability of *GAPDH* was also reported in *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) (Rodrigues et al. 2014) and in the fruit fly, *Anastrepha obliqua* (Macquart) (Diptera: Tephritidae) (Nakamura et al. 2016). These different results in different species may be due to the fact that the *GAPDH* gene is directly involved in metabolic processes. Considering that such processes can be very dynamic and may be differentially regulated according to the physiological stage of the insect, *GAPDH* should not be used as a reference gene for normalization in all insect species (Ponton et al. 2011).

EF 1-α was the second and the third most stably expressed gene at all different developmental stages of *D. citri* reared on *C. sinensis* and *M. paniculata,* respectively. This gene plays an important role in the translation of gene to proteins, by catalyzing the binding of GTP-dependent aminoacyl tRNA site to its receptor in the ribosome (Ponton et al. 2011, Lu et al. 2013, Shang et al. 2015). Stable expression of this gene seems to be common in insects of the order Hemiptera, and our results are in agreement with those from other hemipteran species such as *Toxoptera citricida* (Kirkaldy) (Hemiptera: Aphididae) (Shang et al. 2015), *Bactericera cockerelli* (Sulc) (Hemiptera: Triozidae) (Ibanez and Tamborindeguy 2016), and *Nilaparvata lugens* (Stal) (Hemiptera: Delphacidae) (Yuan et al. 2014). However, in *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae), *EF 1-α* did not demonstrate adequate expression stability at different developmental stages (Collins et al. 2014). Low expression stability of *EF 1-α* was reported in *M. persicae* (Kang et al. 2017) and in species of the order Lepidoptera, such as *H. armigera* (Chandra et al. 2014) and *S. litura* (Lu et al. 2013).

The genes that code for ribosomal proteins *RPL7* and *RPL17*, both constituents of the 60S subunit, showed differences in their expression stability when used as reference genes at different developmental stages of *D. citri*. On both hosts, better performance of *RPL7* compared with *RPL17* was recorded.

Genes that code for ribosomal proteins have been reported as adequate reference genes for RT-qPCR analyses, at different developmental stages, for several insect species, for example, *RPL7* in *A. gossypii* (Ma et al. 2016), *RPS18* in *L. erysimi* (Koramutla et al. 2016), *RPL5* and *RPS18* in *B. cockerelli* (Ibanez and Tamborindeguy 2016), and *RPL32* and *L17* in *M. persicae* (Kang et al. 2017). Although these proteins are constituents of the same ribosomal subunit, they may exhibit variations in their gene expression related to the experimental conditions and to the organism (Yuan et al. 2014, Sun et al. 2015, Yang et al. 2015b).

ACT is the most abundant protein in eukaryotic cells and is an essential part of cytoskeleton filaments. It is involved in many cellular processes, such as cell motility, muscle contraction, cytokinesis, and cell division (Niu et al. 2012, Lu et al. 2013). Although commonly used as a gene normalizer in RT-qPCR analyzes, *ACT* has not demonstrated adequate performance regarding gene expression stability at different insect developmental stages, for many species. Because the *ACT* gene is involved in many cellular processes, it is expected that its transcription level may vary depending on the insect developmental stage, as reported in this study for *D. citri*. The instability of its expression has also been reported at different developmental stages of *H. armigera* (Shakeel et al. 2015, Zhang et al. 2015), *N. lugens* (Yuan et al. 2014), *B. tabaci* (Li et al. 2013), *S. litua* (Lu et al. 2013), *Frankliniella occidentalis* (Pergande) (Zheng et al. 2014), and *Sesamia inferens* Walker (Lepidoptera: Noctuidae) (Sun et al. 2015).

The four statistical algorithms used in this study ranked *TUB* as the reference gene with the least stable expression in *D. citri* on both hosts. Tubulin is a constituent of microtubules that comprise the cytoskeleton and are involved in cell division processes and organization of the cytoplasm, including the positioning of organelles (Gonzalez et al. 1998). The role of the TUB protein in cell division may explain the variable expression of this gene at different developmental stages, because cell division occurs at different rates at different stages (Gonzalez et al. 1998). The results reported herein for *D. citri* corroborate this variation in *TUB* gene expression, as higher expression of this gene was observed at the nymphal stage compared with adults (Fig. 4). Similar results were reported for *A. gossypii* (Ma et al. 2016), *H. armigera* (Shakeel et al. 2015, Zhang et al. 2015), *S. inferens* (Sun et al. 2015), and *T. citricida* (Shang et al. 2015). However, *TUB* was adequate for normalization of RT-qPCR data under experimental conditions designed to induce temperature stress (Zheng et al. 2014, Shang et al. 2015, Sun et al. 2015), in populations from different sources (Yuan et al. 2014), under different photoperiods (Shakeel et al. 2015), and for *M. persicae* reared in different hosts (Kang et al. 2017).

The use of different statistical algorithms for evaluation of the reference genes led to changes in their ranking, according to the different mathematical models considered in each algorithm. Therefore, the use of the software that combines these results into a single rank can help with the selection of reference genes for data normalization. Reference gene studies have indicated that the use of more than one gene is recommended for data normalization in order to obtain more accurate results (Kozera and Rapacz 2013). According to our results for *D. citri*, the use of two reference genes is also recommended in this species when considering different hosts and distinct insect developmental stages, as was indicated by data normalization of *V-ATPase-A* gene expression.

The results from the different reports presented and discussed herein demonstrate that there is variation in the expression stability of reference genes between different insect orders and even within the same order, such as in hemipterans. Therefore, it is important to consider adequate reference gene selection for each species and for each experimental condition to be considered in a given study. Our results strongly suggest that for gene expression analysis in *D. citri* reared on *M. paniculata*, regardless of the insect developmental stage, *GAPDH* and *RPL7* are recommended as reference genes, whereas *GAPDH* and *EF 1-α* are indicated as the best reference genes in *D. citri* gene expression studies, when the insect is reared on *C. sinensis*.

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