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Analysis of reference gene stability after Israeli acute paralysis virus infection in bumblebees *Bombus terrestris*

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Abstract

To date, there are no validated internal reference genes for the normalization of RT-qPCR data from virus infection experiments with pollinating insects. In this study we evaluated the stability of five candidate internal reference genes: elongation factor-1-alpha (ELF1α), peptidylprolyl isomerase A (PPIA), 60S ribosomal protein L23 (RPL23), TATA-binding protein (TBP) and polyubiquitin (UBI), in relation to Israeli acute paralysis virus (IAPV) infection of *Bombus terrestris*. We investigated the stability of these genes: in whole bodies and individual body parts, as well as in whole bodies collected at different time intervals after infection with IAPV. Our data identified PPIA as the single, most-optimal internal reference gene and the combination of PPAI-RPL23-UBI as a fully-sufficient multiple internal reference genes set for IAPV infection experiments in *B. terrestris*.

Keywords: gene expression; bee virus; RT-qPCR; dicistroviruses; qBase^{PLUS}

1. Introduction

Israeli acute paralysis virus (IAPV), Acute bee paralysis virus (ABPV) and Kashmir bee virus (KBV) constitute a complex of genetically and biologically closely related viruses (de Miranda et al., 2010), capable of inducing rapid and acute mortality of both brood and adult honeybees, in response to unspecified environmental stressors or through active transmission by the parasitic mite *Varroa destructor* (de Miranda et al., 2010; Genersch and Aubert, 2010; Maori et al., 2007). Non-*Apis* hymenopteran pollinators, including bumblebee species, are also susceptible to IAPV and can become naturally infected when living close to IAPV-infected honeybee hives (Singh et al., 2010). Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) is one of the most effective methods for measuring gene transcript abundance (Bustin et al., 2010; Mackay et al., 2002). It is frequently used for the detection and quantification of honeybee viruses (Aubert et al., 2008), and is a valuable method for exploring the relationship between RNA interference and virus infections in insects (Jayachandran et al., 2012; Kemp et al., 2013; Paradkar et al., 2012). Accurate and reliable RT-qPCR-based quantification requires a set of stable internal reference genes for normalizing the raw data to account for sample-specific variation in the quality and quantity of RNA (Bustin et al., 2010). However, the proteins encoded by some widely used reference genes, such as β-tubulin and actin, can be actively involved in virus-host interactions (Han et al., 2012; Roberts and Baines, 2011). Similarly, elongation factor-1 alpha (ELF1α), was found to be inappropriate for normalizing the RT-qPCR data of virus-infected planthoppers (Maroniche et al., 2011). It is therefore crucial to validate the stability of the internal reference genes with respect to the experimental variables investigated, before using them to normalize the actual RT-qPCR data.

Until now, the stability of honeybee reference genes has only been evaluated for bee developmental stages, bee tissues and juvenile hormone exposure (Lourenco et al., 2008), and

for bacterial challenge (Scharlaken et al., 2008). The stability of bumblebees reference genes has only been evaluated for certain tissues: the labial gland and the fat body (Horňáková et al., 2010). In this project we investigated five candidate reference genes, namely ELF1α, peptidylprolyl isomerase A (PPIA), 60S ribosomal protein L23 (RPL23), TATA-binding protein (TBP) and polyubiquitin (UBI), for their stability with respect to virus infection. These candidate genes were chosen based on their performances in several other virus-host interaction experiments (Jorgensen et al., 2006; Liu et al., 2012; Maroniche et al., 2011). The five candidate reference genes were evaluated in two series of *Bombus terrestris* samples. The 1st series consisted of separate extracts of whole bumblebee bodies and isolated bumblebee body parts (head, gut and remnants: the remaining part of bumblebee separated from head and gut). This series was used to select reference genes to normalize gene expression for either whole bodies or the individual body parts. The 2nd series consisted of whole body extracts of bumblebees prepared at different time intervals after injection with IAPV or control solution. This series was used to select the reference genes to normalize gene expression in virus infection experiments.

2. Material and Methods

The sequences of candidate reference genes were obtained from Beebase (http://hymenopteragenome.org/beebase; (Munoz-Torres et al., 2011). The primers for RT-qPCR were designed using "Primer 3.0" (http://frodo.wi.mit.edu/) (Supplementary material). The PCR products amplified by these primers were sequenced to confirm the primers' specificity.

For all experiments, we used worker bumblebees from *B. terrestris* colonies obtained from Biobest NV (Westerlo, Belgium) that were fed with pollen and artificial nectar *ad libitum* (Mommaerts et al., 2009). All these bumblebees were confirmed to be free of the

common honeybee viruses by RT-PCR (Meeus et al., 2010) and MLPA (De Smet et al., 2012).

For the 1st series of experiments, we collected five to eight days-old workers, from which four whole body samples were prepared and three samples of each body part (head, gut and remnants; separated under a binocular microscope). For the 2nd series of experiments, we produced an IAPV inoculum by propagating an IAPV reference isolate (Allen and Ball, 1995) in 50 white-eyed honeybee pupae and preparing a chloroform-clarified extract in 10 mM phosphate buffer (pH 7.0)/0.02% diethyl dithiocarbamate (de Miranda et al., 2013). This IAPV inoculum had <0.1% contamination with other common honeybee viruses, as determined by RT-qPCR using previously published assays for IAPV, ABPV, KBV, Chronic bee paralysis virus, Deformed wing virus, Varroa destructor virus-1, Slow bee paralysis virus, Sacbrood virus and Black queen cell virus (Locke et al., 2012). The primers for the above assay are also attached in Supplementary material. The identity of the reference isolate and propagated inoculum was confirmed by sequencing the IAPV PCR product. The exact IAPV particle concentration was determined using transmission electron microscopy, and an IAPV stock inoculum containing $1x10^6$ IAPV particles per µl was prepared. This was diluted to 10 particles per µl for inoculation experiments. Five to eight days-old workers were injected with 2 µl IAPV inoculation solution (20 IAPV particles), or 2 µl of control solution (10 mM phosphate buffer (pH 7.0)/0.02% diethyl dithiocarbamate). Afterwards, four individuals were collected for the IAPV-injected or control samples at each time moment of 8, 24, 48 and 72 h.

RNA was extracted from each sample using the RNeasy mini kit (Qiagen, Germany) and remaining DNA was removed by using TURBO DNA-*free*™ kit (Ambion, USA). RNA concentration and purity were determined using spectrophotometry (260 nm) and the absorption ratios (260/280; 260/230). One microgram RNA was used to synthesize the cDNA by SuperScript® II Reverse Transcriptase (Invitrogen, USA) using Oligo(dT) primers. The absence of contaminating DNA in our samples was confirmed using the RPL23 primers,

which are located on different exons of the gene, producing an extra 452 bp band if DNA was present in the cDNA sample. RT-qPCR was performed on a CFX96™ Real-Time PCR Detection system using SsoFast™ EvaGreen® Supermix (Bio-Rad, USA). Each reaction was performed in duplicate. The primer amplification specificity was checked by both electrophoresis of the RT-PCR products and analysis of the dissociation curve after RT-qPCR. A serial dilution of cDNA was used to obtain standard curves which determined the corresponding primer amplification efficiency (Supplementary data). The quantification cycle (Cq) values were processed into qBase^{PLUS}, and the stability M values for the different reference genes were calculated by GeNorm^{PLUS} (Hellemans et al., 2007). Although different default limits of M values have been used in various studies as ≤1.5 (Zhang et al., 2012), ≤1.0 (Shen et al., 2010), and ≤0.5 (Everaert et al., 2011), we have chosen in our study the strict M value of 0.5 as considering the number of candidate reference genes studied and the number of samples to validate the candidate reference genes. The stability ranking of the candidate reference genes was also obtained by NormFinder, in contrast to GeNorm, it also accounts for optimum reference gene out of a group of genes from diverse samples (Andersen et al., 2004) e.g. from individual body parts, whole bodies, virus infected and non-infected bumblebees in our case.

3. Results and Discussion

For the 1st series of experiments, the M values calculated by GeNorm^{PLUS} were less than 0.5 for all of the five candidate reference genes in whole bodies, guts and remnants, while in the heads the M values of three of the candidate genes (RPL23, UBI and ELF1α) were less than 0.5 (Table 1). When analyzing RT-PCR data from all the samples in 1st series together, all reference genes had an M value higher than 0.5 and ranked from the most stable (lowest M value) to the least stable (highest M value) as: PPIA<TBP<RPL23<UBI<ELF1α (Table 1).

For the 2nd series of experiments, concerning the virus-infection time course, we analyzed the stability of the reference genes at 8, 24, 48, 72 h after injecting the bumblebees with IAPV. The IAPV transcript levels in the 8 h and 24 h post-inoculation samples were below the detection limit of RT-PCR and comprised the 'low IAPV' samples, while the 48 h and 72 h post-inoculation samples comprised the 'high IAPV' samples (Fig. 1A – IAPV inoculation). The IAPV detection limit ($1x10^5$ particles/bee) was determined by spiking a virus-free bumblebee extract with 1×10^7 IAPV particles, purifying the RNA and preparing a ten-fold serial dilution of the RNA prior to RT-PCR (Fig. 1A – IAPV particles). This detection limit corresponds to ~2 IAPV cDNA molecules per PCR reaction (*i.e.* close to the theoretical detection limit of PCR), after accounting for the different dilution factors associated with RNA extraction, DNAse treatment, cDNA synthesis and PCR. As shown in Table 1, at 8, 48 and 72 h post-inoculation, most of the candidate reference genes were relatively stable, with M values less than 0.5. Although at 24 h post-inoculation the M values of all candidate reference genes were elevated relative to the other time-points, that of PPIA stayed below 0.5. When combining all IAPV- and control-injected samples into a single analysis, PPIA, RPL23 and UBI were the most stable reference genes, all with M values below 0.5 (Table 1). To assess the effect of virus infection on reference gene stability, we combined only the virus infected samples from all time points into a single analysis. These results indicated that RPL23 and PPIA were the two most stable internal reference genes.

The results from the data analysis using GeNorm^{PLUS} were compared with those obtained using NormFinder, an alternative program for analyzing the stability of potential internal reference genes. The rankings of the five candidate internal reference genes for individual body parts (1^{st} series) and individual post-inoculation time points (2^{nd} series) slightly differ between the two analyses except the whole body group (Table 1). This in itself is not unexpected, since the two analyses use different algorithms. However, both algorithms

identify PPIA and RPL23 as the best two internal reference genes for IAPV infection studies in bumblebees and these are also among the top three genes identified by both algorithms for body parts-specific studies. One remarkable observation for the body parts-specific studies (1st series) was the stability values were (with a few exceptions) well below 0.5 for individual tissues or whole body extracts, but above 0.5 when the data was pooled into a single analysis indicated by GeNorm^{PLUS}. We therefore evaluated, using GeNorm^{PLUS}, whether the use of multiple reference genes could improve the normalization in such cases. This was done by calculating the pairwise variation $V_n/_{n+1}$, which measures the effect of adding extra reference gene on the normalization factor (the geometric mean of the expression values of selected reference genes). So the value of $V_n/_{n+1}$ was equal to the ratio of two sequential normalization factors through stepwise inclusion of more reference genes. None of the $V_n/_{n+1}$ values dropped below the recommended upper threshold of 0.15 (Vandesompele et al., 2002), suggesting that extra candidate reference genes are needed for stabilizing the normalization of RT-qPCR in gene expression analysis when grouping the data from various body parts and whole body extracts together. We also calculated the $V_n/_{n+1}$ for all samples of the 2nd series, where bumblebees were analyzed at different time intervals after IAPV infection. The combined data of all IAPV-infected and control samples suggested that just three internal reference genes (PPIA, RPL23 and UBI) were sufficient to normalize the data for all samples and that including a fourth reference gene would not improve normalization.

In conclusion, PPIA was the single most optimal internal reference gene for IAPV infection studies in bumblebees, due to its good stability at individual time intervals after virus infection, as well as for all time intervals combined, while the PPIA-RPL23-UBI combination was optimal and fully sufficient for normalization of IAPV infection experiments when using multiple reference genes.

To our knowledge, this is the first report of the validation of reference genes with

respect to virus infections in bumblebees and pollinating insects. We only validated the five candidate internal reference genes for whole-body extracts of IAPV-infected samples. The results from the 1st series, on uninfected body parts and tissues, suggest that these reference genes should also function well for virus-infected body parts or tissues, but this should be established independently prior to any experiments aiming to analyze individual IAPV-infected bumblebee tissues. The optimal reference genes could be used for gene expression normalization of healthy tissues but also for normalizing gene expression differences as a consequence of viral infection. Our results also serve as an additional resource for selecting candidate reference genes in other insect-virus interaction studies. Finally, these results underline the importance of validating reference gene stability before using these in gene expression and virus infection studies.

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278 Figure legend

279 Fig. 1. IAPV detection and determination of optimal number of reference genes. (A) left:
280 IAPV detection threshold determination using dilution series of IAPV particles; right: IAPV
281 detection at different post-injection time points. (B) Determination of optimal number of
282 reference genes for all samples of each series.

Tables

Table 1. Ranking of the stability for the five candidate internal reference genes investigated in two different series of samples. The data were a nalyzed by both GeNorm^{PLUS} and Normfinder, The stability values are given between parentheses.

Method	First series: Samples of virus-free whole body and body parts of *Bombus terrestris*					
	Ranking	**Whole Body**	**Head**	**Gut**	**Remnants**	**Whole Body/Head/Gut/Remnants**
GeNorm^{PLUS}	1	PPIA (0.194)	RPL23 (0.300)	RPL23 (0.145)	PPIA (0.195)	PPIA (0.652)
	2	TBP (0.196)	UBI (0.346)	PPIA (0.151)	TBP (0.213)	TBP (0.655)
	3	ELF1α (0.220)	ELF1α (0.400)	TBP (0.164)	RPL23 (0.244)	RPL23 (0.696)
	4	RPL23 (0.282)	PPIA (0.542)	UBI (0.196)	UBI (0.389)	UBI (0.857)
	5	UBI (0.401)	TBP (0.704)	ELF1α (0.347)	ELF1α (0.493)	ELF1α (1.326)
Normfinder	1	TBP (0.115)	RPL23 (0.005)	RPL23 (0.057)	PPIA (0.027)	TBP (0.173)
	2	UBI(0.144)	TBP (0.018)	ELF1α (0.061)	RPL23 (0.027)	RPL23(0.241)
	3	RPL23 (0.207)	PPIA (0.036)	PPIA (0.071)	TBP (0.155)	PPIA (0.339)
	4	ELF1α (0.235)	UBI(0.159)	TBP (0.081)	UBI(0.398)	UBI(0.357)
	5	PPIA (0.260)	ELF1α (0.706)	UBI(0.123)	ELF1α (0.400)	ELF1α (0.669)
	Second series: Samples of whole bodies of *Bombus terrestris* at different time intervals after IAPV infection					
	Ranking	**8 h**	**24 h**	**48 h**	**72 h**	**8/24/48/72 h**
GeNorm^{PLUS}	1	UBI (0.278)	PPIA (0.426)	PPIA (0.400)	UBI (0.316)	PPIA (0.483)
	2	ELF1α (0.301)	TBP (0.511)	RPL23 (0.400)	RPL23 (0.319)	RPL23 (0.491)
	3	RPL23 (0.309)	RPL23 (0.565)	ELF1α (0.402)	PPIA (0.359)	UBI (0.498)
	4	PPIA (0.388)	UBI (0.632)	UBI (0.473)	TBP (0.468)	ELF1α (0.566)
	5	TBP (0.516)	ELF1α (0.722)	TBP (0.520)	ELF1α (0.622)	TBP (0.651)
Normfinder	1	RPL23 (0.075)	PPIA(0.016)	RPL23 (0.059)	PPIA (0.129)	PPIA (0.138)
	2	PPIA (0.079)	RPL23(0.089)	PPIA (0.061)	RPL23 (0.192)	RPL23 (0.262)
	3	UBI(0.150)	UBI(0.106)	UBI(0.180)	ELF1α (0.210)	UBI(0.289)
	4	ELF1α (0.164)	ELF1α (0.331)	ELF1α (0.209)	UBI(0.213)	ELF1α (0.385)
	5	TBP (0.274)	TBP (0.344)	TBP (0.345)	TBP(0.575)	TBP (0.496)

Figure
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