Effect of β-Cypermethrin Exposure on the Stability of Nine Housekeeping Genes in Bactrocera dorsalis (Diptera: Tephritidae)

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EFFECT OF β-CYPERMETHRIN EXPOSURE ON THE STABILITY OF NINE HOUSEKEEPING GENES IN *BACTROCERA DORSALIS* (DIPTERA: TEPHRITIDAE)

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ABSTRACT

Housekeeping genes are thought to be consistently expressed in different tissues, and therefore they are commonly used as references to normalize qPCR data. But the expression of these genes has proved to be affected by certain experimental conditions. In this study, we evaluated the stability of 9 housekeeping genes of economically important pest, *Bactrocera dorsalis,* under the stress of β-Cypermethrin insecticide. Variations in gene expression were identified both in the whole bodies of larvae and in their midguts. The expression level of each housekeeping gene was shown to be quite different in different tissues, and suggested that the expression stabilities of these genes were differentially affected by toxicity stress. The stability of EF1α was evaluated both in the whole body and in the midgut by *geNorm* and *Normfinder* and in both analyses it proved to be the best reference gene. The folds of changes of expression of the housekeeping genes normalized by EF1α were in accordance with the evaluation results. Furthermore, the variations in expression of these genes were found to be tissue specific. Based on this work we selected EF1α as a reliable reference for data normalization in qPCR studies, and concluded that it will be helpful in studies of expression of genes related to the insecticide target or its detoxification under toxicity stimulation.

Key Words: oriental fruit fly, housekeeping gene, reference evaluation, quantitative real-time PCR, toxicity stimulation

RESUMEN

Se cree que los genes constitutivos son expresados consistentemente en diferentes tejidos y por lo tanto se utilizan comúnmente como referencias para normalizar los datos de qPCR. Sin embargo, se ha demostrado que la expresión de estos genes esta afectada por ciertas condiciones experimentales. En este estudio, se evaluó la estabilidad de 9 genes constitutivos de *Bactrocera dorsalis* (Tephritidae)*,* una plaga de importancia económica, bajo el estrés del insecticida β-cipermetrina. Se identificó variaciones en la expresión de los génes tanto en los cuerpos enteros de larvas y en sus intestinos medios. Se encontró que el nivel de expresión de cada gen constitutivo fue muy distinto en los diferentes tejidos y hemos probado que la estabilidad de la expresión de estos genes fue afectada en maneras diferentes por el estrés de toxicidad. Se evaluó la estabilidad de EF1α tanto en todo el cuerpo y en el intestino medio con *geNorm* y *Normfinder* y en ambos análisis se demostró que es el mejor gen de referencia. Los pliegues de los cambios de expresión de los genes constitutivos normalizado por EF1α estan de acuerdo con los resultados de la evaluación. Además, se han encontrado que las variaciones en la expresión de estos genes son específicas según el tejido. Basándose en este trabajo se seleccionó EF1α como una referencia fiable para la normalización de datos en estudios de qPCR y se llegó a la conclusión de que esto será útil en estudios de la expresión de genes relacionados con el insecticida enfocado o su desintoxicación bajo estimulación tóxica.

Palabras Clave: mosca oriental de la fruta, limpieza de genes, evaluación de referencia, cuantitativos PCR en tiempo real, estimulación toxicidad

Housekeeping genes are genes that are expressed at fairly consistent levels across different tissues. The products of these genes are usually proteins or enzymes typically needed for the maintenance of the cells. Some well-known housekeeping genes, such as actin, tubulin, ribosomal RNA, and elongation factor 1 alpha, are necessary for cell survival, and highly abundant proteins in all cell types. Previously, housekeeping genes were thought to be expressed consistently and stably under all kinds of experimental conditions. For this reason, these genes were commonly used as

references to normalize qPCR data in gene expression studies. However, in a number of studies the expressions of certain housekeeping genes proved to vary under different experimental conditions. The products of housekeeping proteins are not only basic cell components, but they also participate in cellular functions. The number of housekeeping genes may vary with differences in function of different tissues or with different stimulations by experimental conditions (Rubie et al. 2005; Thellin et al. 1999). Thus, when using a housekeeping gene as a reference, evaluation of its expression stability usually is required.

In insects, the variation of housekeeping genes has been already realized, and the effect of experimental conditions on the stability of housekeeping genes was evaluated to select a most stable one as a reference (Bansal et al. 2012). These studies showed that the stability of housekeeping genes was affected by both endogenous and exogenous factors, such as cell types, developmental stages, diets, and insecticides or environmental stresses (Jiang et al. 2010; Niu et al. 2012; Peng et al. 2012; Ponton et al. 2011). The oriental fruit fly, *Bactrocera dorsalis* (Hendel), is a worldwide agriculture pest of great economic importance (Clarke et al. 2005). Its molecular characterization on resistance, development, and antioxidant ability were well studied (Cong et al. 2012; Hsu et al. 2012; Jia et al. 2011; Shen et al. 2011). As an important tool of gene expression study, qPCR was commonly used in these researches. The stability of housekeeping genes used as the reference was a key point to guarantee the accuracy of the results. In previous observations of housekeeping gene stability of *B. dorsalis*, no single gene was suitable as a reference under all kinds of experimental conditions (Shen et al. 2010; Shen et al. 2012). Therefore, the evaluation of stability of putative reference genes is an important and basic work for gene expression studies.

The midgut is the second largest organ in the insect body (Hakim et al. 2010). It is also a major place for xenobiotic (insecticides or natural toxins) metabolism. In this study, the reaction of housekeeping genes of *B. dorsalis* to the stimulation of stomach poison toxicity was clarified. The most stable one either in the whole body or midgut after treatment was selected by both analyses of *geNorm* and *Normfinder*. This work improved our understanding on housekeeping genes and will be helpful in expression studies of target or detoxification related genes under toxicity stimulation.

Materials and Methods

Insects

Specimens of *B. dorsalis* were originally collected in Haikou, Hainan Province, People's Republic of China. The adults were reared at 27 ± 1 °C, 70 ± 5% RH, 14:10 h L:D, and the larvae were kept under the same conditions but without light. The artificial diet for adults consisted of water, yeast, honey, vitamin C in the ratio 500:15:15:1. Eggs were collected with a Petri dish full of artificial larval diet, which consisted of corn flour, agar, wheat-germ flour, yeast, sugar, nipagin, sorbic acid, vitamin C, and linolenic acid in the ratio 600: 60: 250: 200: 200: 14: 7: 10: 1. The Petri dish was kept in a pot filled with sand until the larvae had pupated. After pupation, pupae were sieved out, placed into a plastic cups and covered with sand. The plastic cups were placed in a cage to collect adults.

Exposure to β-Cypermethrin

Different doses of β-Cypermethrin were dissolved in 200 μL acetone, and then all mixed into 20 g artificial larval diet at a final concentration of 0.33, 1.0, and 33 μg/g, respectively. The appropriate doses (200 μL) of acetone added in diets were used as controls. The newly hatched larvae were reared on these diets until the third instar (5 days). An apparent body weight growth of larvae was observed in this process to ensure that the mixed diet was consumed. Each treatment contained 50 individuals and 4 replicates. The relative survival rates were normalized by the untreated groups to find the best concentration. The β-Cypermethrin was provided by Institute for Control of Agrochemicals of Sichuan Province, Chengdu, China, and the purity was 95%.

RNA Extraction and cDNA Synthesis

After treatment with β-Cypermethrin, the larvae were dissected individually in physiological saline using a dissection needle under a stereomicroscope (Olympus SZX12, Japan). The midgut was collected and stored in RNAstore Reagent (Tiangen Biotech, China). The midguts from 20 larvae were used as one replicate, and 3 replicates were prepared. Collected tissues were homogenized immediately using liquid nitrogen in a mortar. RNA was extracted following the manufacturer's instruction for the RNeasy plus Micro Kit (Qiagen, Germany). Also, the RNA from the whole bodies of larvae was extracted using Trizol (Biomed, China) following the manufacturer's instruction. Five intact larvae were homogenized together for RNA extraction as one sample.

RNA was quantified by measuring the absorbance at 260 nm using a NanoVue UV-Vis spectrophotometer (GE Healthcare Bio-Science, Sweden). The purity of all RNA samples was assessed at an absorbance ratio of $OD_{260/280}$ and at $OD_{260/230}$, and the integrity of RNA was checked with 1% agarose gel electrophoresis. To exclude the inter-

ference of genomic DNA in qPCR, RNA extraction of the midguts included a genomic DNA elimination step by using a genomic DNA elimination column, which could efficiently remove genomic DNA. For RNA extracted by Trizol reagent, DNase I (Promega, USA) was used to remove the genomic DNA.

First strand cDNA was synthesized from 1 µg of DNA-free RNA using PrimeScript® RT reagent Kit (Takara, Japan). Briefly, the 20 µL reaction system consisted of 1 µg RNA, 400 pmol Random 6 mers, 4 µL reverse transcription buffer, 2 µL PrimerScript® RT Enzyme Mix I and RNase free dH$_2$O. The reverse transcription reaction was performed on a C1000TM Thermal Cycler (Bio-Rad, USA). The reaction conditions included a step of 37 °C for 15 min and 85 °C for 5 sec. After the reverse transcription, synthesized cDNA was stored at -20 °C for future use.

Quantitative Real-Time PCR

Nine housekeeping genes of *B. dorsalis*, including ribosomal protein L13 (RPL13), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), elongation factor-1α (EF1α), 18S ribosomal RNA (18S), succinate dehydrogenase (SD), α-tubulin (α-TUB), β-tubulin (β-TUB), RNA polymerase II (RPE), and actin 2 (ACT) were evaluated and primers were designed by Primer 3 software (version 0.4.0) [http://frodo.wi.mit.edu/primer3/]. All primers were placed in continuous exons. Gene

accession numbers, primer sequences, amplicon sizes, PCR efficiencies and coefficients of determination are presented in Table 1.

All qPCR reactions were performed on the StepOne Plus Real-Time PCR System (ABI, USA). The 20 µL reaction system consisted of 1 µL of diluted cDNA, 10 µL GoTaq® qPCR Master Mix (Promega, USA) and 0.2 mM of each primer. Thermal cycling conditions consisted of an initial denaturation at 94 °C for 2 min, followed by 40 cycles of 94 °C for 15 sec, and 60 °C for 30 sec. After reaction, a melting curve analysis from 60 °C to 95 °C was applied to all reactions to ensure consistency and specificity of the amplified product. A 5-fold dilution series of cDNA was employed as a standard to construct a relative standard curve and determine the PCR efficiency that would be used in converting quantification cycle (*Cq*-values) into raw data (relative quantities).

Gene Expression Stability Evaluation

The expression stability of housekeeping genes was evaluated by *geNorm*[PLUS] [http://www.biogazelle.com/genormplus], and by *Normfinder* [http://www.mdl.dk/publicationsnormfinder.htm] programs. *geNorm*[PLUS] software determined the stability of candidate genes with parameter *M* as the average pairwise variation between a single gene and all other genes in a given set of samples. The specific gene associated with the lowest *M* value was considered to be the most stably ex-

TABLE 1. DETAILS OF THE PRIMER PAIRS USED FOR REAL-TIME PCR OF 9 HOUSEKEEPING GENES OF *BACTROCERA DORSALIS*.

Gene name	GenBank accession number	Primer sequences (forward/reverse)	Amplicon length (bp)	Efficiency (%)	R^2
GAPDH	GU269901	GACGCCTACAAGCCTGACAT GTTGAAGCGGGAATGATGTT	221	103.2	0.996
EF1α	GU269900	CGTTGGTGTCAACAAGATGG TGCCTTCAGCATTACCTTCC	230	107.6	0.957
18S	AF033944	GCGAGAGGTGAAATTCTTGG CGGGTAAGCGACTGAGAGAG	191	100.3	0.999
SD	HM236868	CAAGTGCAAAGGTGTGTTGG GTCGCAATGACGGTGTTATG	81	96.8	0.961
α-TUB	GU269902	CGCATTCATGGTTGATAACG GGGCACCAAGTTAGTCTGGA	184	104.8	0.994
β-TUB	EU938673	TTACATTTCTTTATGCCTGGTTTC CATTTGTTCGTCCACTTCCTTC	204	104.0	0.985
RPE	HM236867	GTTACGGCGAGGACAGTTTG CACGCAATGCTTCACGACC	234	101.0	0.927
ACT	L12254	GTGTGATGGTTGGTATGGGA GGCTGGGGAGTTGAAGGTTT	269	89.5	0.998
RPL13	HM236866	CAGTTGTACGTTGCGAGGAATT TCTTGATGGAGCACGGGAG	134	110.0	0.923

R^2, coefficients of determination.

pressed one. For the optimal number of reference genes for normalization, a value of Vn/Vn+1 was provided to show the pair-wise variation between 2 sequential normalization factors containing an increasing number of genes (Vandesompele et al. 2002). The strategy of *Normfinder* is rooted in a mathematical model of gene expression (Andersen et al. 2004). Better stability was reflected by a lower parameter value.

Variation of Housekeeping Genes in the Midgut under Toxicity Stress

According to the evaluation, all housekeeping gene transcriptions in the midgut were normalized to the transcription of EF1α. The fold of change of expression between the control and the treatment were calculated using Pfaffl's method based on the *Cq* value (Pfaffl 2001).

RESULTS

Effect of β-Cypermethrin Exposure on Larval Survival

To determine the appropriate concentration of β-Cypermethrin against larvae in the exposure experiment, different doses of β-Cypermethrin were mixed into the diet that was fed to the larvae. The survive rate and the relative mortality were normalized by a control group. The results (Fig. 1) show that the relative survival rate of the control (acetone) was more than 99%, which suggested that as a solvent, acetone was not a lethal factor. 0.33 µg/g (β-Cypermethrin/artificial diet) was determined as a suitable concentration to stimulate the larvae. Such a concentration would not cause an apparent death as 1 µg/g and 33 µg/g, and most individuals could normally develop under the toxicity stress.
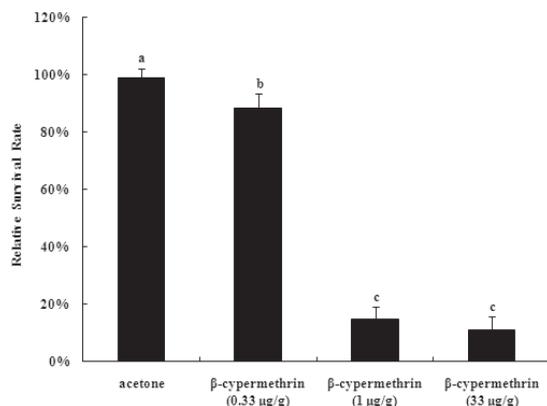

Fig. 1. Relative survival rate of *Bactrocera dorsalis* larvae after treatment by different concentrations of β-Cypermethrin.

Expression Differences of Housekeeping Genes in the Larvae and their Midguts

The variation of *Cq* values of the 9 housekeeping genes was reflected by the box plot graph (Fig. 2). In the larval whole bodies, the median *Cq* values were distributed from lowest in the case of 18S (9.83) to highest in the case of RPE (30.19). In the midgut, the median *Cq* values were distributed from lowest in the case of 18S (7.78) to highest in the case of β-TUB (26.52). In both whole bodies of the larvae and in the midgut, 18S was the most abundant protein with the lowest *Cq* value, and followed by EF1α. In contrast, the least abundant proteins were RPE and β-TUB. Interestingly, α-TUB and β-TUB are both tubulin genes, but the abundance of their proteins in the whole body or midgut samples were significantly different.

Analysis of Gene Expression Stability

The *geNorm*[PLUS] program was applied to evaluate the variations of the 9 housekeeping genes under β-Cypermethrin stomach toxicity stress in the whole bodies and midguts of larvae. According to this program, the stability of housekeeping genes was reflected by an internal control gene-stability measure value (*M* value), and genes with the lowest *M* values have the most stable expression. The stability rankings of expression of the housekeeping genes of larval whole bodies with judging from the *M* values calculated by *geNorm*[PLUS] from the lowest to highest were: EF1α (0.25) > α-TUB (0.27) > 18S (0.288) > SD (0.383) > RPL13 (0.432) > GAPDH (0.475) > RPE (0.567) > ACT (0.651) > β-TUB (0.754). EF1α expression was ranked the highest in the whole bodies under the toxicity stress, but the *M* values for the 3 best genes (EF1α, α-TUB, and 18S) showed no significant differences (Fig. 3A), which means the stability of these 3 genes were nearly at the same level. The pairwise variation value provided the information for the optimum number when multiple genes were used as reference. Vandesompele et al. (2002) suggested a threshold value at 0.15, below which, the inclusion of an additional reference gene was not required. Therefore, the pairwise variation value of $V_{2/3}$ was below 0.15, which suggested that when using multiple genes as references for normalization, 2 genes were enough (Fig. 3B).

In the midgut, the stability rankings of housekeeping genes from the most to the least were: EF1α (0.406) > RPL13 (0.431) > GAPDH (0.454) > SD (0.597) > ACT (0.597) > α-TUB (0.679) > 18S (0.73) > β-TUB (0.762) > RPE (0.803) (Fig. 4A). With the lowest *M* value (0.406), EF1α was considered to be the most stable gene. The *M* values of the 3 best genes (EF1α, RPL13, and
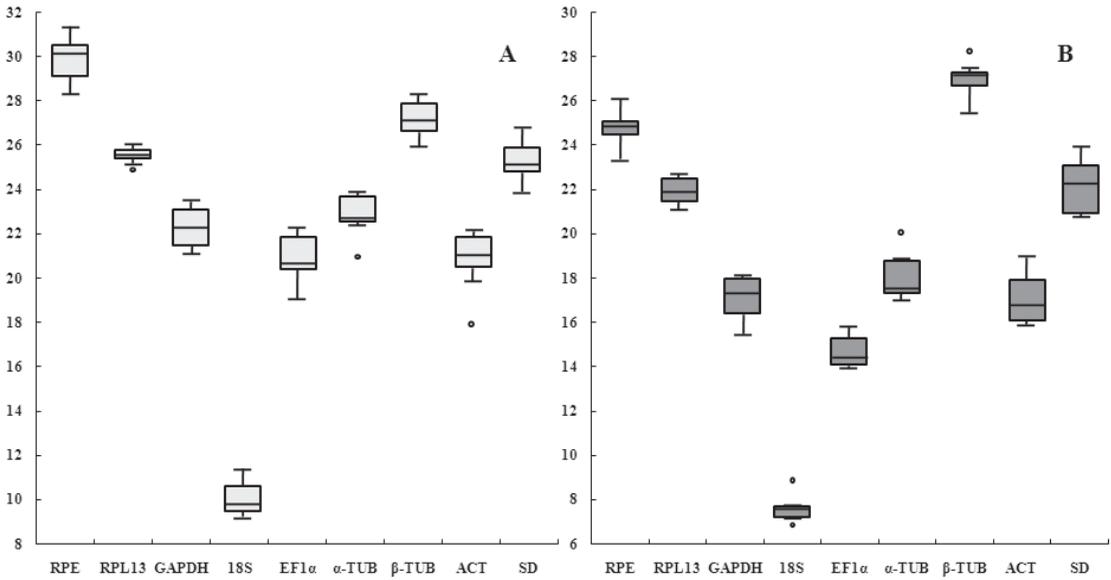
Fig. 2. Distribution of *Cq* values for housekeeping genes of *Bactrocera dorsalis* obtained using qPCR in the whole body (A) and midgut (B).

GAPDH) were also not significantly different. The pairwise variation value, $V_{2/3}$, was 0.157, just near 0.15, so when used as reference gene in normalization of a qPCR study, 2 genes may be enough (Fig. 4B).

Furthermore, to decide whether the housekeeping genes were appropriate as references under our experimental conditions, a threshold value (0.5) for homogeneous tissues was considered as a standard (Vandesompele et al. 2002). The results showed that the most stable gene, either in the whole bodies or midguts, was EF1α, and its respective *M* values were much lower than 0.5. When estimating the expressions of certain genes under stomach toxicity stress with qPCR, the use of EF1α as a reference was reasonable and acceptable.

To confirm our results, another program, *Normfinder*, was also used to evaluate the stability of housekeeping genes under the experimental conditions. The final ranking of housekeeping genes was in accordance with *geNorm*[PLUS]. The 3 best genes in the larval whole bodies were EF1α, α-TUB, and 18S, and in their midguts, RPL13, EF1α, and GAPDH were the best. In contrast, *Normfinder* and *geNorm*[PLUS] both estimated β-TUB and RPE as the least stable gene in whole bodies and midguts (Table 2).
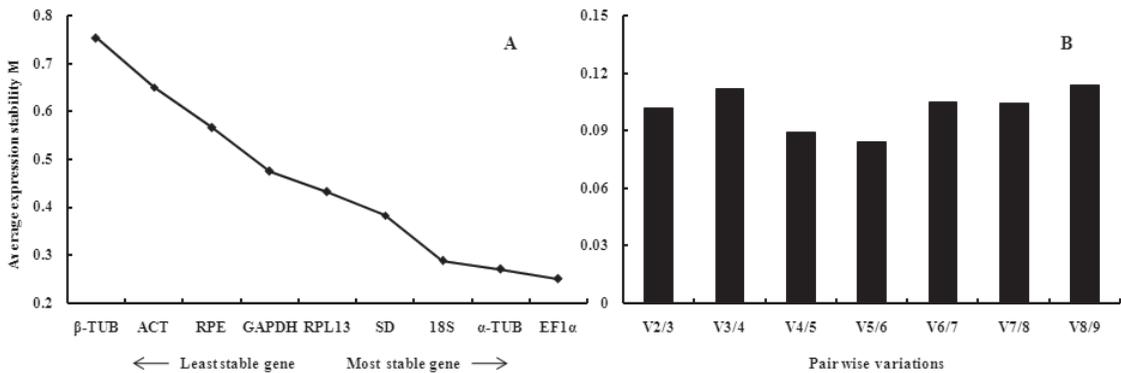

Fig. 3. Stability of housekeeping genes of *Bactrocera dorsalis* in the whole bodies of larvae under stomach toxicity stress (A), and optimal number of reference genes for normalization (B) evaluated by *geNorm*[PLUS].
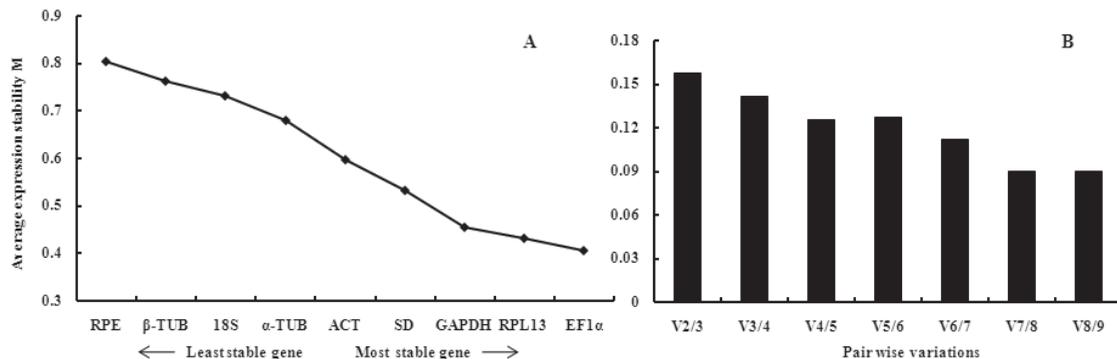
Fig. 4. Stability of housekeeping genes in the midguts of *Bactrocera dorsalis* larvae under stomach toxicity stress (A), and optimal number of reference genes for normalization (B) evaluated by *geNorm*[PLUS].

Variation of Housekeeping Gene Expression under Stomach Toxicity Stress

To show the variation of each housekeeping gene under the toxicity stress in whole bodies or midguts, the most stable gene, EF1α, was used as the reference to normalize the expressions and to calculate the fold of change of expression of each gene. In the whole bodies of larvae, the fold of change of expression of housekeeping genes was in accordance with their expression stability evaluation (Fig. 5). The fold change of the stably expressed genes (i.e., α-TUB, 18S) was close to "1", which suggested that their expression did not change or changed only slightly among the tested samples. With increases in M value, such variation was more apparent. The least stable housekeeping gene, β-TUB, was 2-fold up-regulated under toxicity stress of β-Cypermethrin, while most of the other genes were down-regulated. Among these genes, the expression of SD was quite different from the stability evaluation result. Although SD was listed as one of the stable genes by *geNorm*[PLUS]; and therefore the observed significant variation was unexpected.

In the midgut, the least stable genes (i.e., RPE, β-TUB) were significantly down-regulated,

and the fold of change of expression of the most stable gene (i.e., RPL13) was close to "1" (Fig. 6). The tendency of variation was still a reflection of stability evaluation (Fig. 4). Interestingly, unexpected variation of SD was observed again. The expression of this gene was apparently affected by the β-Cypermethrin toxicity stress, because it was highly up-regulated in both the whole body and the midgut.

DISCUSSION

Housekeeping genes serve a series of basic functions, which are of great importance to cell structure and the life activity of cells. Their expression is essential and necessary for normal cellular physiology. Housekeeping genes are presumed to be expressed at the same level throughout all organs. For this reason, in our gene expression study, housekeeping genes were used as a reference for normalization in qPCR. However, recent research had revealed that the expressions of housekeeping genes were not continuously stable across tissues or under certain experimental conditions. In some experiments, the variation of expression of a housekeeping gene was not suitable for it to be used as a reference. In this case,

TABLE 2. GENE EXPRESSION STABILITY OF 9 HOUSEKEEPING GENES OF *BACTROCERA DORSALIS* EVALUATED AS REFERENCE CANDIDATES BY *NORMFINDER*.

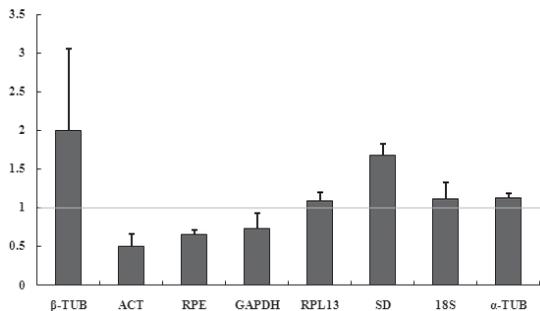| Ranking order	Gene name	Stability value in larvae	Gene name	Stability value in midgut
1	EF1α	0.075	RPL13	0.122
2	α-TUB	0.075	EF1α	0.147
3	18S	0.188	GAPDH	0.380
4	RPL13	0.294	β-TUB	0.428
5	GAPDH	0.307	18S	0.440
6	SD	0.354	ACT	0.462
7	RPE	0.528	α-TUB	0.467
8	ACT	0.570	SD	0.473
9	β-TUB	0.696	RPE	0.545

Fig. 5. The variation of expression of housekeeping genes in the whole bodies of *Bactrocera dorsalis* larvae under toxicity stress, and normalized by EF1α.

the definition of "housekeeping gene" and "reference gene" is modified. "Housekeeping gene" has been defined functionally as a gene that is constitutively expressed to maintain cellular function, and "reference gene" has been defined as one that is expressed at a constant level across all experimental conditions, tissues or cell lines. A reference gene usually is a housekeeping gene, but not all housekeeping genes can be used as a reference (Butte et al. 2001; Folkersen et al. 2009; Ruan & Lai 2007; Selvey et al. 2001; Thorrez et al. 2008).

The evaluation of expression of certain housekeeping genes of *B. dorsalis* under toxicity stress served to elucidate the influence of environmental stress on the stabilities of expressions of various genes and to identify the best gene to serve as a reference for a qPCR study under β-Cypermethrin treatment. As the result, 9 housekeeping genes showed different variations in the larval whole body and midgut samples. EF1α is one of the most abundant proteins, and responsible for the delivery of all aminoacyl-tRNAs to the ribosome, aside from initiator and selenocysteine tRNAs (Mateyak & Kinzy 2010). Its expression showed high stability under experimental stress, and was identified as the best reference gene under injury,
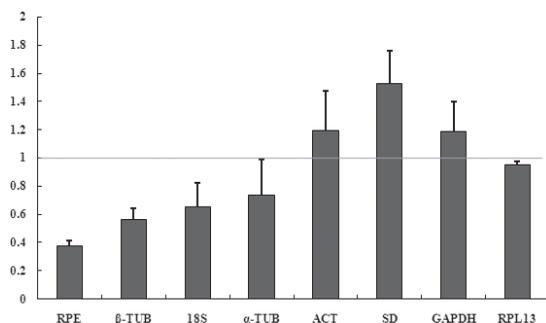

Fig. 6. The variation of expression of housekeeping genes in the midguts of *Bactrocera dorsalis* larvae under toxicity stress, and normalized by EF1α.

heat shock, and diet stresses in *Drosophila melanogaster* (Ponton et al. 2011). The EF1α gene of *B. dorsalis* also expressed stably under stomach toxicity stress of β-Cypermethrin either in the whole body or midgut. However, in our previous study, EF1α was variable across different tissues of *B. dorsalis* (Shen et al. 2010). Compared with these results, an ideal reference gene for all experimental conditions, especially across tissues, may not exist (Folkersen et al. 2009; Zhu et al. 2008). We assume that the transcript levels of EF1α are different across tissues, but it is very stable under stress, and will not be affected by environmental changes.

Tubulins are a major component of ciliary microtubules and the principal protein subunit of microtubules in the cell cytoplasm. Tubulin genes are primarily divided into 2 families, α and β (Cleveland & Sullivan 1985). Later, a new member of tubulin superfamily was discovered and named as α-tubulin (Oakley & Oakley 1989). Because tubulin is a basic component of cells, tubulin genes were considered consistently expressed in all type of tissues, and α- and β-tubulins were commonly used as reference genes in qPCR studies. But our evaluation of gene expression stability revealed that the expression levels of α and β-tubulins were quite different, and they were also variable under some experimental conditions (Cao et al. 2012; Spanier et al. 2010; Zhong et al. 2011). In this study, we found that the differences of mRNA abundances between α and β-tubulins were significant. The variation of β-tubulin was the most apparent of all either in the whole body or midgut. The α-tubulin gene was stably expressed across different tissues of *B. dorsalis* (Shen et al. 2010), but its expression might be affected by toxicity stress in the midgut. Interestingly, α-tubulin and EF1α were 2 opposite housekeeping genes: EF1α was stable under environmental stress, but variable across tissues, whereas, α-tubulin showed high stability across tissues, but was variable when under β-Cypermethrin toxicity stress.

Another housekeeping gene, actin, has been commonly used as a reference. It was thought to have more stable expression levels compared with other internal controls, but later, researchers suggested that actin was unsuitable as an endogenous internal control for gene expression studies under a variety of conditions. Various other housekeeping genes, such as GAPDH and 18S, have also been found to be unsuitable (Ruan & Lai 2007; Selvey et al. 2001). The abundance of actin of *B. dorsalis* was at same level as EF1α and α-tubulin, but its variation of expression was similar to β-tubulin, and opposite to EF1α.

Although high abundance is a feature of housekeeping genes, no significant relation between the abundance of a gene and its expression stability was identified in our study. 18S was the most abundant housekeeping gene in all samples.

Its *Cq* values were around 10, while the *Cq* values other genes were mostly above 20. The expression stability of 18S was not reliable as a reference (Mehta et al. 2010). 5.8S rRNA was also highly abundant but unstably expressed under environmental stresses (Niu et al. 2012). We propose that rRNA is not a good choice as a reference gene, but if an rRNA gene is used to normalize qPCR data, its stability should first be evaluated.

Among the evaluated housekeeping genes, the expression changes of SD were unique. According to our calculations by *geNorm* and *Normfinder*, EF1α was suitable for use as a reference to normalize the change of expression of each gene under β-Cypermethrin toxicity stress. Fold changes of most genes reflected variation, except for SD. Although the stability of SD was evaluated as one of the best genes in both cases, the variations of its expression were close to the worst (i.e., β-TUB or RPE). In some studies, SD was considered to be a good reference, and when used in normalization, it was also reliable (Lardizábal et al. 2012). However, in *B. dorsalis*, this gene was not a good choice, and more attention should be paid to validation of its reliability.

The variations of the tested housekeeping genes showed that their expressions would be affected by stomach toxicity stress. Most gene expressions were suppressed, but the expression of some genes were induced by β-Cypermethrin. Furthermore, this revealed that the variations of gene expression were tissue specific. In the case of β-TUB, its expression was highly up-regulated in the whole body under the toxicity stress of β-Cypermethrin, but significantly down-regulated in the midgut. This fact suggested that in certain tissues, the expression of this gene was opposite to that in the midgut. Similarly divergent regulation can be seen in the expression of ACT and GAPDH.

In conclusion, evaluation of the stability of housekeeping genes of *B. dorsalis* under the toxicity stress of β-Cypermethrin showed that even the expression of housekeeping genes would be affected under toxicity stress, and the variations of these genes were tissue specific. EF1α was shown to be the most stable in the whole body and in the midgut by both *geNorm* and *Normfinder*. EF1α can be used as a reference for data normalization of qPCR study in the reaction of target or detoxification related genes under toxicity stimulation.

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