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Expression Profiling in *Bemisia tabaci* under Insecticide Treatment: Indicating the Necessity for Custom Reference Gene Selection

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Abstract

Finding a suitable reference gene is the key for qRT-PCR analysis. However, none of the reference gene discovered thus far can be utilized universally under various biotic and abiotic experimental conditions. In this study, we further examine the stability of candidate reference genes under a single abiotic factor, insecticide treatment. After being exposed to eight commercially available insecticides, which belong to five different classes, the expression profiles of eight housekeeping genes in the sweetpotato whitefly, *Bemisia tabaci*, one of the most invasive and destructive pests in the world, were investigated using qRT-PCR analysis. In summary, elongation factor 1α (*EF1α*), α-tubulin (*TUB1*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were identified as the most stable reference genes under the insecticide treatment. The initial assessment of candidate reference genes was further validated with the expression of two target genes, a P450 (Cyp6cm1) and a glutathione S-transferase (GST). However, ranking of reference genes varied substantially among intra- and inter-classes of insecticides. These combined data strongly suggested the necessity of conducting custom reference gene selection designed for each and every experimental condition, even when examining the same abiotic or biotic factor.


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Introduction

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) is one of the most effective and sensitive techniques for gene expression analysis [1–4]. However, enabling comparisons across different samples, qRT-PCR data must be normalized to correct variations in pipetting, RNA concentration, reverse-transcription, and efficiency of PCR amplification [2,5,6].

The most common normalization method is to compare the mRNA level of the target gene with that of a reference gene whose expression level is considered stable regardless of different experimental conditions [7–9]. However, none of the reference genes discovered thus far is consistently expressed in a universal and invariant way under various experimental conditions [10,11], and recent reference gene selection studies indicate that a single reference gene is generally insufficient to normalize the expression data of all target genes [12–16].

In insect, the reference genes have been validated at least in the desert locust *Schistocerca gregaria* [17], the oriental fruit fly *Bactrocera dorsalis* [18], the fruit fly *Drosophila melanogaster* [19] emerald ash borer *Agrilus planipennis* [20], the diamondback moth *Plutella xylostella* [21], the tobacco whitefly *Bemisia tabaci* [22], and the red imported fire ant, *Solenopsis invicta* [23] under a diverse set of biotic and abiotic conditions. However, no single universal reference was identified, either. Therefore, it is not surprising that no single universal reference is available for four different lepidopteran insect species [24]. In this context, reliable reference genes for gene expression analysis based on different experimental conditions should be selected.

The tobacco whitefly, *B. tabaci*, is an invasive insect pest of agriculture and horticulture worldwide [25]. Because of the application of chemical insecticides has been the primary strategy for the control of *B. tabaci*, this pest has developed different levels of resistance to a wide range of insecticides [26–33]. It has been well documented that insecticide resistance in *B. tabaci* usually is associated with enhanced detoxification by oxidative and hydrolytic pathways [34–36]. Therefore, increasing numbers of studies are using the RT-qPCR techniques to detect the changes of mRNA expression of detoxifying enzymes genes in resistant populations and tried to provide new insights into insecticides resistance mechanisms [37,38].

Though the suitable references genes has been documented in bacterially challenged bees [39], in *Tribolium* beetles infected with fungus [40], in plant virus infected *B. tabaci* [22] and in Bt toxin treated *P. xylostella* [21], this kind of information is lacking for insects stressed by different types of chemical insecticides.

In this study, a set of reliable reference genes for gene expression analysis in the *B. tabaci* biotype *Q*, one of the most invasive and destructive pest in the world, after exposure to eight commonly used insecticides (which belong to five different classes) was...
selected and then valuated with two target genes, a P450 gene \((Cyp6Cm1)\) and glutathione S-transferase gene \((GST)\). Over expression of the P450 has been proved to be responsible for neonicotinoid insecticides resistance in \(B.\ tabaci\) [35,37] while this is not the case for GST. The objective of this work is to provide a set of universal reliable reference genes for research of genes with toxicological function in \(B.\ tabaci\).

Materials and Methods

Ethics Statement

\(Bemisia\ tabaci\) biotype \(Q\) strains used in this study were initially collected in the field at Beijing in 2010, and have been maintained in our laboratory at the China Agricultural University for three years without exposure to any insecticide. No specific permit was required for the described field collections, and the location is not privately-owned or protected in any way. The species in the genus of \(Bemisia\) are common agricultural pests and are not included in the “List of Endangered and Protected Animals in China”.

Leaf-dip bioassay and \(B.\ tabaci\) susceptibility to various insecticides

A total of eight kinds of insecticides commonly been used in the management of \(B.\ tabaci\) were used in this study, including chlorpyrifos, beta-cypermethrin, carbosulfan, abamectin, buprofezin and three neonicotinoids \((imidacloprid,\ acetamiprid,\ nitenpyram)\.

All insecticides were used of technical grade with purity greater than 95%.

First, the LC50 of each insecticide to \(Bemisia\ tabaci\) biotype \(Q\) was determined using the leaf-dip method [41]. Briefly, the stock solution of insecticide was diluted to five to seven concentrations with 0.02% Triton X-100, then eggplant leaf discs (33 mm in diameter) were dipped in aqueous solutions of insecticides for 10 s. After being air-dried for 1 h, the leaf disc was placed abaxial side down on the bed of agar (2%) within the plug seal cap of a 100 ml centrifuge tube (the bottom of the tube was cut off and covered with a piece of black cotton cloth, the tube was 8 cm in height and 3.8 cm in diameter). Approximately 30–50 adult whiteflies were transferred into the tubes and the tubes were thereafter covered with the caps (with leaf disc inside). Adult whiteflies treated with 0.02% Triton X-100 were used as control. Four replicates of each concentration were carried out. The adults were allowed to feed on the treated disc for 48 h or 72 h (depending on classes of insecticide) at 25±2°C, 75% RH, and a 16:8-h (light:dark) photoperiod. LC50 values and their virulence regression equation slope were calculated using PoloPlus™ software (LeOra Software, Berkeley, USA). The bioassay results were listed in Table S1.

And then the similar leaf-dip method was used for treatment of \(B.\ tabaci\) except that the eggplant leaf discs were dipped at a concentration corresponding to the LC50 of each insecticide, respectively. About 300 adults were treated for each insecticide. The adults were allowed to feed on the treated disc for 48 h at 25±2°C, 50–70% RH, and a 12:12 h (light:dark) photoperiod. Adult whiteflies treated with 0.02% Triton X-100 were used as control. The surviving insects were collected for subsequent RNA extraction. Three biological replicates were performed for each insecticide treatment.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from 100 to150 \(B.\ tabaci\) adults using Trizol Reagen (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. RNA concentration and quality were measured according to the optical density at 260 nm and the A260/A280 absorption ratio using a NAS-99 spectrophotometer (ACTGene, USA). RNA samples with an A260/A280 ratio ranging from 1.8 to 2.0 and A260/A230 >2.0 were used for analysis. All RNA samples were adjusted to the same concentration to homogenize RNA input in the subsequent reverse-transcription reaction. One microgram of RNA was reverse transcribed into first-strand cDNA using a Thermo Scientific Verso™ cDNA Synthesis Kit (Thermo Scientific, Wilmington, DE, USA). The cDNA was stored at −20°C until use.

Primer design and quantitative real-time PCR

A total of eight candidate reference genes, including five commonly used reference genes, \(\beta\)-actin \((ACT)\), \(\alpha\)-tubulin \((TUB\beta)\), elongation factor 1\(\alpha\) \((EF\beta)\), glyceroldehyde-3-phosphate dehydrogenase \((GAPDH)\) and 18S ribosomal RNA \((18S\ rRNA)\), as well as three rarely used reference genes ribosomal protein \(L15a\) \((RPL15A)\), cyclphilin \(1\) \((CYP7)\), and TATA box binding protein-associated factor \((TBP\-AF)\) in \(B.\ tabaci\) were chosen for valuation of their expression stability in \(B.\ tabaci\). The sequences, length of products, and source of these candidate genes were listed in Table 1.

Real-time PCR was conducted using an ABI 7500 Real Time PCR System (Applied Biosystems, Foster, CA) and the ROX’s Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen, Carlsbad, CA). The reactions were performed in a 20 \(\mu\L\) mixture contained 1 \(\mu\L\) of cDNA template, 10 \(\mu\L\) of SYBR Green qPCR SuperMix-UDG, 1 \(\mu\L\) of each primer and 7 \(\mu\L\) of nuclease-free water. The optimized real-time PCR program consisted of an initial step at 50°C for 2 min, 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Relative standard curves for the transcripts were generated with serial dilutions of cDNA \((1/5, 1/15, 1/45, 1/135,\ and\ 1/405)\). The corresponding qRT-PCR efficiencies \((E)\) were calculated according to the equation: 

\[ E = (10^{-1/\text{slope}}) - 1 \times 100 \]

Three independent biological duplications were performed for all the reference genes studied and data for each biological duplicate were carried out in triplicate. The dissociation curves were obtained after amplification to inspect the specificity of the primer sets.

Statistical analysis

The raw data of qPCR were analyzed using ABI 7500 SDS System software (versio 2.0) (Applied Biosystems) and the threshold cycle \((Ct\ value,\ the\ cycle\ at\ which\ the\ fluorescent\ signal\ was\ first\ significantly\ different\ from\ the\ background)\ for each gene. cDNA sample was determined automatically. All biological replicates (each contained three technical replications) were used to calculate the average \(Ct\) values using geNorm and NormFinder software packages as described in their manuals. \(Ct\) values were converted into relative quantities and imported into the geNorm and the NormFinder software for further analysis. The geNorm provides a measure of gene expression stability \((M)\), and creates stability ranking via a stepwise exclusion of the least stable gene. Genes with the lowest M values have the most stable expression. The geNorm also calculates a serial values of \(\text{Vn/}\text{Vn}+1\), which indicates the pairwise variation between two sequential normalization factors and determines the optimal number of reference genes required for accurate normalization. A value below 0.15 indicates that an additional reference gene will not significantly improve normalization. Ct values were converted into relative quantities and further analyzed by RefFinder, a user-friendly web-based comprehensive tool [http://www.leonxie.com/referencegene.php], to evaluate the expression stability of candidate reference genes. The RefFinder was developed for evaluating and screening reference genes from the extensive experimental datasets. It integrated the currently available major computational programs [geNorm, Normfinder, BestKeeper, and the comparative \(\Delta C\) method]
levels of selected target genes (Cyp6cm1 gene, and the generally adopted threshold of V = 0.15 was used for
tion factors (NF) that result from stepwise introduction of another
calculate the pair-wise variation V of two consecutive normaliza-
amine in B. tabaci
expression level of candidate reference genes among samples was produced respectively, from
correlation of each standard curve
pooled cDNA generated from each experiment. The correlation
specificity of all primer sets (data not shown). A standard curve was
generated for each gene, using three-fold serial dilutions of the

expression profiles of the eight candidate reference genes were assessed in B. tabaci treated with eight different insecticides belong to five classes. For the whiteflies treated by LC50 of
abamectin, according to the ranking of stability generated with
RefFinder, RPL13A was the most stable gene with the lowest ranking
value (1.8) followed by CYP1, TUB1a, ACT, GADPH, EF1a, TBP-
AF and the 18S RNA was the most unstable one (Figure 2A). If we
consider the generally adopted threshold of V = 0.15, the geNorm
analysis revealed that two most stable genes are needed for a
reliable normalization, because of the addition of a third gene does
not result in any appreciable improvement of the normalization
factor (Figure 3). Thus the RPL13A and CYP1 were identified as
the most stable pair of genes.

Similar analyses were conducted for the remaining treatments. For the whiteflies treated by LC50 of imidacloprid, the eight
candidate reference genes were ranked (from the highest to lowest
stability) by the
RefFinder as TUB1a<RPL13A<TBP- AF<

**Results**

**Amplification specificity and efficiency of the primer sets**

Single peaks in the dissociation curves further demonstrated the
specificity of all primer sets (data not shown). A standard curve was
generated for each gene, using three-fold serial dilutions of the
poled cDNA generated from each experiment. The correlation
coefficient and PCR efficiency characterizing each standard curve
are given in Table 1. Box plots of raw Ct values of the candidate
reference genes among samples were produced respectively, from
which the expression stability of the candidate reference genes can
be told intuitively (Fig. 1). The eight candidate reference genes
expressed different transcription levels from each other, with Ct
values spanning 14.23–26.91, in which the lowest Ct (and highest
expression) corresponded to 18SrRNA and the highest Ct to
EF1a.

**Expression stability analysis of the candidate genes and optimum number of genes for normalization**

The expression profiles of the eight candidate reference genes were assessed in B. tabaci treated with eight different insecticides belong to five classes. For the whiteflies treated by LC50 of


<table>
<thead>
<tr>
<th>Gene</th>
<th>Molecular function</th>
<th>Accession No.</th>
<th>Primer sequences (5’ to 3’)a</th>
<th>Product length (bp)</th>
<th>Tmbb (°C)</th>
<th>Ec</th>
<th>R2d</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACT</td>
<td>♂-actin</td>
<td>AF071908</td>
<td>F:ACCGCAAGATCCATACCCA RGCGTCGCCCTCAACTCATT</td>
<td>127</td>
<td>60</td>
<td>103.5</td>
<td>0.996</td>
</tr>
<tr>
<td>TUB1a</td>
<td>Tubulin alpha-1 chain</td>
<td>EE598061</td>
<td>F:CACTTGTTTGCTGTTGCGG RAGTGCCAGAAACGCACCTTG</td>
<td>140</td>
<td>60</td>
<td>93.6</td>
<td>0.999</td>
</tr>
<tr>
<td>EF1a</td>
<td>elongation factor1-alpha</td>
<td>EE650059</td>
<td>F:GATGCGACGGAGACAAATATG RTTTCAGTGCTGGCTCTAGG</td>
<td>138</td>
<td>60</td>
<td>94.5</td>
<td>0.995</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
<td>JU470454</td>
<td>F:AAATGAGCTTTTACCTACGCT RATTAGGGCGGTGAGGCGC</td>
<td>82</td>
<td>60</td>
<td>90.2</td>
<td>0.996</td>
</tr>
<tr>
<td>18S RNA</td>
<td>18S ribosomal RNA</td>
<td>Z15051</td>
<td>F:CGGCTACCATCCTCAAGGAA RGGCTGAATTACCCGGCCT</td>
<td>112</td>
<td>60</td>
<td>92.6</td>
<td>0.998</td>
</tr>
<tr>
<td>RPL13A</td>
<td>ribosomal protein L13a</td>
<td>EE596312</td>
<td>F:CAITCCACTACAGCTCCA RTTTCAGGTGGCAGGGCTT</td>
<td>101</td>
<td>60</td>
<td>99.2</td>
<td>0.996</td>
</tr>
<tr>
<td>CYP1</td>
<td>cyclophilin 1</td>
<td>EE596217</td>
<td>F:CAACCTGTCATTCCCAACATT RGTGCTGGTGGAGGTTAAGT</td>
<td>118</td>
<td>60</td>
<td>88.5</td>
<td>0.997</td>
</tr>
<tr>
<td>TBP- AF</td>
<td>TATA box binding protein-associated factor</td>
<td>EE596204</td>
<td>F:GTGCGGACACCCATTACAG RTGTGCAAGCCAGGAATAAG</td>
<td>162</td>
<td>60</td>
<td>96.5</td>
<td>0.995</td>
</tr>
<tr>
<td>CYP6CM1</td>
<td>cytochrome P450 CYP6cm1</td>
<td>GQ214539</td>
<td>F:GCACTCGGTGATAAAGGGA RAAACTCGTTCTCCTCATCGT</td>
<td>128</td>
<td>60</td>
<td>91.4</td>
<td>0.996</td>
</tr>
<tr>
<td>GSTa</td>
<td>Glutathione S-transferase gene</td>
<td>EU723684</td>
<td>F:GTGAGGAGAAAACACCTCCT RAGTCGTTTCCGGCTT</td>
<td>97</td>
<td>60</td>
<td>90.8</td>
<td>0.995</td>
</tr>
</tbody>
</table>

a: F, forward primer; R, reverse primer.
b: Tm, Melting temperature.
c: E, Efficiency.
d: R2, Coefficient of correlation.
doi:10.1371/journal.pone.0087514.t001

**Table 1. Candidate reference genes and primers used for qRT-PCR analysis.**

![Graph showing the expression levels of candidate reference genes](image-url)
Figure 2. Expression stabilities of the candidate reference genes. The average expression stability of the reference genes was measured using Geomean method by RefFinder. A lower Geomean of ranking value indicates more stable expression. CK: Treated with 0.02% Triton X-100 only. Other 5 insecticides: treated by chlopyrifos, beta-cypermethrin, carbosulfan, abamectin and buprofezin, respectively. Eight insecticides: treated with each of eight insecticides used in this work.
doi:10.1371/journal.pone.0087514.g002
And the TUB1-GeNorm reference gene pair according to the V values calculated with the CYP1 genes from the most stable to the least stable was: RPL13A.

The stability order of the eight genes expressed in the control (untreated) group, from the most stable to the least stable, was ranked as ACT<TUB1z<GADPH<TBP-1z<18S rRNA(AF) (Figure 2). And the GeNorm analysis revealed that EF1z and GADPH was the optimum gene pair for a reliable normalization.

When the imidacloprid, acetamiprid and nitenpyram treated group was combined as neonicotinoid treated group, the stability of the eight candidate genes (from the highest to the lowest) was ranked as ACT<TUB1z<GADPH<RPL13A<EF1z<CYP1<18S rRNA(AF) (Figure 2I). And according to the V values, ACT, TUB1z and GADPH was considered as the most stable reference gene set for normalization.

Finally, all whitefly groups treated with eight insecticides were analyzed together and designated as the comprehensive group. According to RefFinder, from the most to the least stable reference genes, the overall ranking of the eight candidates across different developmental stages was: EF1z<TUB1z<GADPH<CYP1<TBP-1z<ACT<18S rRNA(AF) (Figure 2L). As a result, EF1z, TUB1z and GADPH were considered as the most stable reference genes for qRT-PCR normalization (Table 2).

Validation of selected reference genes in B. tabaci

The relative expression levels of two target genes, Cyp6CM1 and GST, were analyzed after whiteflies treated with three neonicotinoid insecticides. No significant differences were found among the expressions of Cyp6CM1 and GST using five different sets of reference genes (P>0.05) (Fig. 4 A–C). Similarly, when normalized with their respective optimal reference gene sets for each of the five non-neonicotinoid insecticides, expression levels of Cyp6CM1 and

Table 2. Selected reference genes under different insecticide treatments.

<table>
<thead>
<tr>
<th>Insecticide class</th>
<th>Insecticide</th>
<th>Reference genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avermectins</td>
<td>Abamectin</td>
<td>RPL13A and CYP1</td>
</tr>
<tr>
<td>Neonicotinoids</td>
<td>Imidacloprid</td>
<td>TUB1z, RPL13A and TBP-AF</td>
</tr>
<tr>
<td></td>
<td>Acetamiprid</td>
<td>ACT and CYP1</td>
</tr>
<tr>
<td></td>
<td>Nitpyram</td>
<td>EF1z and RPL13A</td>
</tr>
<tr>
<td>Carbamates</td>
<td>Carbosulfan</td>
<td>TUB1z and EF1z</td>
</tr>
<tr>
<td>Organophosphates</td>
<td>Cholorpyrifos</td>
<td>TUB1z and CYP1</td>
</tr>
<tr>
<td>Pyrethroids</td>
<td>Beta-cypermethrin</td>
<td>CYP1 and EF1z</td>
</tr>
<tr>
<td>Chitin synthesis inhibitors</td>
<td>Buprofezin</td>
<td>CYP1 and EF1z</td>
</tr>
<tr>
<td>Neonicotinoid</td>
<td>Neonicotinoid</td>
<td>ACT, TUB1z and GADPH</td>
</tr>
<tr>
<td>Comprehensive</td>
<td></td>
<td>EF1z, TUB1z and GADPH</td>
</tr>
</tbody>
</table>

For the buprofezin treated group, the expression stability of the eight genes were ranked as CYP1<TBP-1z<TUB1z<RPL13A<EF1z<GADPH<18S rRNA(AF) by RefFinder from the highest to the lowest (Figure 2H). And the GeNorm analysis revealed that EF1z and GADPH was the optimum gene pair for a reliable normalization.

For the buprofezin treated group, the expression stability of the eight genes were ranked as CYP1<TBP-1z<TUB1z<RPL13A<EF1z<GADPH<18S rRNA(AF) by RefFinder from the highest to the lowest (Figure 2H). And the GeNorm analysis revealed that EF1z and GADPH was the optimum gene pair for a reliable normalization.
GST also exhibited no apparent differences (P>0.05) (Table 2; Fig. 4 D–H). However, significant expression differences were found between the most stable reference genes for all eight insecticides, *EF1α, TUB1α* and *GADPH*, and *18S rRNA*, the optimal reference gene recommended previously Fig. 4 I, [22].

**Discussion**

There exists no doubt that the qRT-PCR technology has made the quantitative determination of gene expression more convenient than ever before. However, extreme care must be taken for the selection of internal reference genes before their application of qRT-PCR. It has been documented that the selection of suitable reference genes is very important to obtain reliable and accurate data [2,3,46]. Currently, however, many common reference genes are used as internal controls without any evaluation of their variance and instability, and most publications only use a single internal control for normalization. These un-validated single reference genes, however, were proved to be not always reliable under various experimental conditions [12–16]. Therefore, more and more biologists pay their attention to the selection and validation of reliable reference gene(s) expressed stably regardless of different experimental conditions from the species they are interested in, in order to avoid unnecessary errors in qRT-PCR analysis.

Insecticide resistance is becoming a serious barrier for the sustainable control of pest insects. And the identification of insecticide resistance mechanisms would provide ways of detection and management of resistance [47]. The qPCR has been extensively used to uncover the mechanisms of insecticide resistance, and it has been proven by a lot of publications that the overexpression of detoxifying enzymes and the reduced expression of insecticide target genes were responsible for insecticide resistance [48–51]. Up to date, however, no universal and reliable reference genes were selected and evaluated in insects stressed with different classes of insecticides.

In the present work, a total of eight candidate reference genes were validated in the tobacco whitefly treated with eight commonly used insecticides for the control of this pest. According to the final ranking order calculated by RefFinder, the five most stable reference genes for the eight tested insecticides treated whitefly were selected (Fig. 2A–H). Even for imidacloprid,
acetamiprid and nitenpyram which belong to the same class of insecticides, the most stable reference genes were also different from each other (Fig. 2B–D). These results further proved that no single universal reference is available under different experiment conditions, and made the stability evaluation of reference gene necessary before the quantification of gene expression by qPCR.

Very interestingly, the relative expression level of two detoxifying enzymes, Cyp6m1 and GST from each of eight insecticides treated B. tabaci groups showed no significant difference between calculations using the selected reference gene set for each insecticide and calculations using the selected reference gene set for all eight insecticides (EF1a, TUB1a and GAPDH). Combined with the results of Li et al. [22] that the expression of EF1a and GAPDH was stable between the thiamethoxam (a neonicotinoid insecticide) susceptible and resistant B. tabaci strains, we recommended that our selected reference gene set (EF1a, TUB1a and GAPDH) can be used as reliable internal reference for the data normalization in qRT-PCR experiments using B. tabaci treated with different insecticides.

Table S1 Toxicity of eight insecticides to adults of Bemisia tabaci Mediterranean. (DOC)

Author Contributions
Conceived and designed the experiments: PL XZ XG. Performed the experiments: PL YG. Analyzed the data: PL YG. Contributed reagents/materials/analysis tools: XG. Wrote the paper: PL XZ.

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