Screening for stable internal reference genes for quantitative PCR analysis of Wolbachia-host interactions in whitefly Bemisia tabaci (Homoptera: Aleyrodidae)

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Abstract. Stable reference genes (RGs) determine the reliability of quantitative polymerase chain reaction (qPCR) analyses and it is recommended that different reference genes are used for different types of DNA and tissues. The present study aimed to screen for stable RGs for the qPCR analysis of the immune responses of the whitefly Bemisia tabaci to the Wolbachia wMel strain from Drosophila melanogaster. A total of eight candidate RGs were evaluated using five different methods, i.e., Coefficient of Variation analysis, GeNorm, NormFinder, BestKeeper and ΔCt. The stability of these RGs was assessed for both genomic DNA (gDNA) and complementary DNA (cDNA). The results indicate that β-actin (Actin) and elongation factor 1 alpha (EF-1α) were the most stable RGs for gDNA, whereas 18S rRNA (18S) and glyceraldehyde phosphate dehydrogenase (GAPDH) were the least stable; in contrast, Actin and GAPDH were the most stable for cDNA, whereas RPL29 and ATPase were the least stable. The effectiveness of the most stable RGs was then validated against the least stable using qPCR analysis of the titre of wMel (gDNA) and the transcriptional responses of the antimicrobial peptide Alo-3-like and the phosphatidylinositol-bisphosphate 3-kinase catalytic subunit delta isofrom (cDNA) to wMel transfection. The results support the notion that reliable RGs are essential for a qPCR analysis of samples of both gDNA and cDNA.

INTRODUCTION

Real-time quantitative PCR (qPCR) is a commonly used technique for gene quantitation at both genomic DNA (gDNA) and complementary DNA (cDNA) levels, which recently has been used in many studies (Artico et al., 2010; Derveaux et al., 2010; Hindson et al., 2013). Nevertheless, the reliability of qPCR is affected by many different factors (Bustin, 2000; Derveaux et al., 2010) and the use of reference genes (RGs) can greatly improve the accuracy of qPCR results by standardizing or normalizing experimental data from different developmental stages, organs or tissues (Artico et al., 2010; Arya et al., 2017). Screening for stable RGs is reported for different species of insects, such as Aphis glycines (Bansal et al., 2012), Nilaparvata lugens (Yuan et al., 2014), Bactericera cockerelli (Ibanez & Tamborindegy, 2016) and Lipaphis erysimi (Koramutla et al., 2016). The RGs have to be carefully evaluated for particular situations, as it is unlikely that the same RGs (non-evaluated) are generally suitable (Thellin et al., 1999; Vandesompele et al., 2002; Ponton et al., 2011; Zhou & Li, 2016). Several algorithms have been developed for evaluating the stability of RGs, including the Coefficient of Variation (Boda et al., 2009), GeNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004), BestKeeper (Pfaffl et al., 2004) and the ΔCt method (Silver et al., 2006). These algorithms have proved to be helpful in the screening for stable RGs in a variety of organisms (Freetig et al., 2018; Meng et al., 2019; Xie et al., 2019).

Whitefly Bemisia tabaci is a destructive agricultural pest and causes serious damage to vegetables, ornamental plants and other crops worldwide (De Barro et al., 2011; Navas-Castillo et al., 2011). B. tabaci is often infected with Wolbachia (Li et al., 2007), a group of maternally inherited intracellular Gram-negative bacteria frequently found in arthropods and filarial nematodes (Hilgenboecker et al., 2008; Zug & Hammerstein, 2012). Wolbachia can manipulate host reproduction in different ways, block the transmission of human diseases and control insect pests (Bourtzis & O’Neill, 1998; Stouthamer et al., 1999; Werren et al., 2008). However, the interactions between Wolbachia and their hosts are still poorly understood (Lemaitre & Hoffmann, 2007). In our research group, several exo-
enous Wolbachia strains were successfully transferred into *B. tabaci* by microinjection and transinfected isofemale lines were established in the laboratory (Zhong & Li, 2014; Hu & Li, 2015; Zhou & Li, 2016). Recently, we sequenced the transcriptomes of *B. tabaci* in response to Wolbachia transfection using the RNA-seq technique, which is necessary for a detailed investigation of the host-Wolbachia interaction, including qPCR analysis of the titre of Wolbachia and expression levels of differentially expressed genes (DEGs) and functional analysis of candidate genes via RNA interference (RNAi). All of these studies require the selection of suitable RGs. In a previous study, 15 candidate RGs were evaluated for *B. tabaci* using the algorithms geNorm and NormFinder (Li et al., 2013), which revealed that the stability of RGs was greatly affected by both biotic and abiotic factors, and that different RGs should be used depending on the species and conditions. The purpose of the present study was to screen for suitable RGs for qPCR analysis of both gDNA and cDNA from *B. tabaci* artificially transinfected with exogenous Wolbachia. Based on our studies, different RGs are needed for determining the titre of Wolbachia or level of expression of functional genes in different generations when *B. tabaci* is transinfected with the wMel Wolbachia strain from the fruit fly *Drosophila melanogaster*.

**MATERIAL AND METHODS**

**Insect rearing and transfection through microinjection**

The whitefly *B. tabaci* (Q cryptic species) was collected in tomato greenhouses of Jinan Academy of Agricultural Sciences, Shandong, China, and then maintained on pot-grown plants of cotton in a laboratory (14L: 10D, RH 60–80%, 27 ± 1°C). The 4th-instar nymphs (pseudopupae) of *B. tabaci* were artifically transinfected with exogenous Wolbachia. Based on our studies, different RGs are needed for determining the titre of Wolbachia or level of expression of functional genes in different generations when *B. tabaci* is transinfected with the wMel Wolbachia strain from the fruit fly *Drosophila melanogaster*.

**Extraction of genomic DNA (gDNA) and synthesis of complementary DNA (cDNA)**

Twenty fresh whiteflies (adults) were used for extraction of gDNA from samples of Gp, Gs, Gf, G and G, individuals using the KAC method as described by Zhong & Li (2013) and the purity and concentration of gDNA were checked using a Nanodrop (Thermo Scientific, Wilmington, DE). Samples of thirty fresh whiteflies were used for the extraction of total RNA in a 1.5-mL centrifuge tube containing TRIzol reagent (TransGen Biotech, Beijing, China), which were from Gp, Gs, Gf and G, of the transinfected isofemale line, with the wild type as the control. The first-strand cDNA was synthesized from 500 ng of total RNA using the PrimeScript™ 1st strand cDNA Synthesis Kit (TaKaRa, Beijing, China) according to the supplier’s instructions.

**Selection of RGs**

Eight RGs were selected: heat shock protein 20 (*HSP20*), heat shock protein 70 (*HSP70*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), 60S ribosomal protein L29 (*RPL29*), β-actin (*Actin*), 18S rRNA (18S), elongation factor 1 alpha (*EF-1α*), and adenosine triphosphate enzyme (*ATPase*) (Table 1). Most of these genes have been used as internal references for real-time qPCR analysis in previous studies (Zhou et al., 1998; Li et al., 2013; Zhou & Li, 2016). These RGs were evaluated against both gDNA and cDNA in the present study, but *HSP20* and *HSP70* were not used for cDNA samples as it is likely they are induced by Wolbachia transfection.

**Quantitative PCR analysis**

The specificity of the primers for the eight RGs (Table 1) was examined using PCR in a total reaction volume of 25 μL containing 2 μL of 10X DNA template, 1 μL of each primer (10 μM), 2 μL of dNTPs (2.5 mM), 0.5 μL of *Taq* DNA polymerase (5.0 U/μL) and 16 μL ddH2O. The thermocycling program was: 94°C for 5 min, 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s and a final 10 min extension at 72°C. PCR products were analysed using 1% agarose gel electrophoresis. For the qPCR analysis, the DNA templates (gDNA or cDNA) were successively diluted by 5x, 5x, 5x, 5x and 5x times for construction of standard curves and melting curves. A suitable pair of primers for qPCR analysis should have a correlation coefficient (**r**²) > 0.99, an amplification efficiency (**E**) > 90% and a unimodal melting curve (Livak & Schmittgen, 2001). The reaction was performed in a total volume of 20 μL containing 10 μL of AceQ™ SYBR® Green Master Mix (Vazyme, Nanjing, China), 0.4 μL of each primer (10 μM), 1 μL DNA template and 8.2 μL ddH2O on ABI 7500 platform (Applied Biosystems, Foster City, California, USA); the primer pair wspQ384/wspQ513 (Table 1) was used to detect the titre of wMel strain in *B. tabaci* after transfection. The thermocycling program was 50°C for 2 min, 95°C for 5 min, 40 cycles of 95°C for 10 s and 60°C for 30 s. The program used for determining the melting curve was 95°C for 15 s, 65°C for 60 s and 95°C for 15 s. DNase/-RNase-free water was used as the negative control. Each treatment was performed in triplicate.

**Stability of RGs**

The cycle threshold (Ct) values of RGs for different generations were compared in order to evaluate their stability. The working concentration of gDNA for the qPCR analysis was 20 ng/μL and that of cDNA was 100 ng/μL. The stability of RGs was evaluated using four different algorithms: GeNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004), BestKeeper (Pfaffl et al., 2004) and ΔAct method (Silver et al., 2006). The four algorithms rated the stability according to different variables: GeNorm provides an M-value based on the average paired expression ratio. The lower the M-value, the more stable the expression: M ≤ 1.5 was considered to be stable. NormFinder calculates stability based on the inter- and intra-group variance of each candidate RG; the lower the NormFinder value, the more stable the RG. BestKeeper determines the stability of RG according to the standard deviation (SD): The higher the SD, the more unstable the expression. ΔAct method performs pairwise multiple comparisons between the expression levels and identifies the most stable RGs. Each algorithm sorts RGs according to their stability and assigned the RGs a series of consecutive integers (starting at one) and the geometric average (geomean) of the weights based on the four algorithms was then calculated for each RG. The RG with the lowest geomean value was considered to be the most stable and the RGs were ranked accordingly. Subsequently, the Coefficient of Variation (CV) was used to measure the population variance of each gene as it is the only method that is not affected by other factors (Boda et al., 2009). Here a threshold of CV = 50% was defined: genes with a CV value above this threshold were
considered highly variable and excluded from further analyses. Finally, a corrected ranking of the RGs was obtained.

**Validation of RGs**

The reliability of candidate RGs was validated by a qPCR analysis of the titre of wMel (gDNA) and the transcriptional response of two genes (antimicrobial peptide Alo-3-like; phosphatidylinositol-bisphosphate 3-kinase catalytic subunit delta isoform, Pl3K) to wMel transfection (cDNA) (the primer sequences are included in Table 1). Alo-3-like is an antimicrobial peptide involved in immune response of the host to wMel transfection, and Pl3K is involved in the immune-related signalling pathway in B. tabaci based on our transcriptome sequencing data (Tables S1 and S2; Figs S1 and S2). Here we used the two most stable and two least stable RGs based on their geomean values to normalize the qPCR analysis before the results were compared. The procedures for qPCR analysis were the same as described above.

**Data analysis**

The statistical differences were analysed using One-way ANOVA followed by Student Newman Keuls (SNK) test at 0.05, 0.01 and 0.001 levels of probability on SPSS v.20.0 (SPSS Inc., Chicago, IL, USA).

**RESULTS**

**Specificity, amplification efficiency and cycle thresholds of RG primers**

The specificity of the RG primers (Table 1) was determined using PCR and agarose gel electrophoresis, which indicated that all primer pairs resulted in single specific bands of expected size. In addition, the melting curves of all the primer pairs had single peaks, and the correlation coefficients and amplification efficiencies of the standard curves were all within the range of $R^2 > 0.99$ and $90\% < E < 110\%$ (Table 1; Fig. S3). Moreover, the determination of RG using qPCR revealed that the Ct-value of 18S was the lowest (the log of starting template copies) for both gDNA and cDNA templates, while that of GAPDH was the highest for cDNA (Fig. 1) and that of RPL29 was the most variable (Fig. 1A).

**Stability of candidate RGs**

Based on qPCR analysis of gDNA and cDNA samples taken from transfected B. tabaci adults and the different algorithms (GeNorm, NormFinder, BestKeeper or ΔCt method), stabilities were assigned to each RG and the geomeans of these stabilities calculated, which were used to determine their combined ranking (Table 2). The results indicated that ATPase and EF-1α were the two most stable RGs for gDNA templates and RPL29 and GAPDH the two least stable RGs; in contrast, 18S and Actin were the two most stable RGs for cDNA templates, whereas EF-1α and ATPase were the two least stable RGs.

The CV analysis was the most appropriate method for identifying the most variable RGs, which were excluded from further assessments. The analysis showed that RPL29 and EF-1α were the most variable RGs for both gDNA and cDNA samples (Table 3). After removing RPL29 and EF-1α from the ranking analysis, a corrected stability ranking value was generated for each RG using the different algorithms and finally all RGs were ranked according to their geomean values (Table 4). The results indicate that Actin and EF-1α were the two most stable RGs for gDNA samples, whereas Actin and GAPDH were the two most stable for cDNA samples. Interestingly, 18S/GAPDH and 18S/RPL29/ATPase were identified as the least stable RGs for both gDNA and cDNA samples.

Table 1. Primer sequences used in this study.

| Gene name | GenBank acc. no. | Primer sequence (5’→3’) | Expected size (bp) | gDNA $E\%$ | gDNA $R^2$ | cDNA $E\%$ | cDNA $R^2$ | References |
|-----------|-----------------|-------------------------|-------------------|-----------|----------|-----------|----------|------------|
| HSP20     | EU934239        | F: AAGAAGTCAGCAGGAAAGTCG R: GTACCTCCTAGGAAAGATCGG | 107          | 103.8    | 0.9986   | —         | —        | Li et al., 2013 |
| HSP70     | EU934240        | F: AGCCTCGGCGCTTACG R: CGAACATGCGGACCAGACCC | 134          | 108.4    | 0.9966   | —         | —        | Li et al., 2013 |
| GAPDH     | JU470454        | F: GGCACGGGAAGAGCCATACCG R: ACCACGCTACCCAAAGAGCC | 166          | 102.7    | 0.9971   | 109.9     | 0.9965   | Li et al., 2013 |
| RPL29     | EE596314        | F: TCTGAAAATATCCGTTGAG R: GAACCTTGTGATCTACCTCGTG | 144          | 107.4    | 0.9984   | 92.9      | 0.9939   | Li et al., 2013 |
| Actin     | AF071908        | F: TGGTTCCAGCACCCTCTTGTG R: CGTGAGTTTGCTCTGATT | 174          | 96.75    | 0.9996   | 102.6     | 0.9999   | Zhou & Li, 2016 |
| 18S       | U20401          | F: CGGCTACACATTCCAGAAAAA R: GCTGGAATTTACCGGCGT | 187          | 94.62    | 0.9941   | 104.9     | 0.9913   | Li et al., 2013 |
| EF-1α     | EE600682        | F: ATGCCATGTCCAAGGGATGGA R: ACATCTGGATGGAACAGCGG | 134          | 97.69    | 0.9951   | 110.0     | 0.9971   | Designed by the authors |
| ATPase    | JU470453        | F: CGTACTCCCCCTTTCGGCTG R: CAGAAGACGGGCGATCGGAGA | 122          | 108.5    | 0.9943   | 107.7     | 0.996   | Designed by the authors |
| wsp       | KU870673        | F: TGGTCCAATAAGTGAAGGAAAC R: AAAATTIAACCCGTCTCGCA | 632          | —        | —        | —        | —        | Zhou et al., 1998 |
| wspQ384   | KU867063        | F: TGTTGCCAGCTGTTGGAATGAT R: GCACCTAAGAAGACCCGAATAAGCG | 130          | 93.76    | 0.9976   | 95.46     | 0.9957   | Zhou & Li, 2016 |
| Pl3K      | LOC109034225    | F: TGTTGCAACGTATGTGCTTGG R: TAAGAATGCGGCGCTTGGAC | 146          | 97.51    | 0.9987   | 93.14     | 0.9989   | Designed by the authors |
| AMP       | LOC109033344    | F: TCTCCAAGCCAAAACCAACACCAACAC R: CGCTGCGAGGAGGACATTGCAG | 110          | 106.5    | 0.9961   | 108.2     | 0.9916   | Designed by the authors |

$E\%$ – amplification efficiency; $R^2$ – correlation coefficient.
Validation of stable RGs

The qPCR analysis of the relative titre (gDNA) of the Wolbachia wMel strain was normalized by using a combination of the two most stable (Actin and EF-1α) and two least stable (18S and GAPDH) RGs. The results revealed that the measurement of Wolbachia titre was indeed affected by the nature of the RG in all of the five generations assessed in this study (Fig. 2). Specifically, the relative titres normalized by unstable RGs varied dramatically between generations, especially in G6 for which the titre was several-fold higher than in that recorded in other generations. In contrast, the titres normalized by stable RGs looked more normal.

The qPCR analysis of the relative expression (cDNA) of two immune-related genes (AMP and PI3K) was also normalized using a combination of the two most stable (Actin and GAPDH) and two least stable (RPL29/ATPase) RGs. The results indicate that the relative level of expression of AMP (Alo-3-like gene) when normalized by unstable RGs was very high in the wild type, while there were no significant differences between G0 and G4 and the control (G1) (Fig. 3A). In the case of PI3K, the relative level of expression normalized by unstable RGs were exceptionally high in the wild type and in G0 of the transinfected lines (Fig. 3B). Moreover, the relative levels of expression of both the Alo-3-like gene and PI3K when normalized using stable RGs were generally consistent with the transcriptome data, whereas those normalized using unstable RGs were not (Tables S1 and S2).

DISCUSSION

Our studies confirmed that the reference genes significantly affected the accuracy of qPCR analysis of both gDNA and cDNA samples. Moreover, the selection of candidate RGs based on the algorithms used in this study seemed very effective: the scoring and ranking procedures ensured the selection of suitable RGs for the qPCR analysis of both gDNA and cDNA templates. Our results support
the notion that the use of suitable RGs is essential for a qPCR analysis.

The wMel Wolbachia strain from D. melanogaster is able to establish and induce strong cytoplasmic incompatibility (CI) in B. tabaci (Zhou & Li, 2016), an important worldwide agricultural pest, which indicates it might be possible to use it to control this pest. Nevertheless, the titre of wMel fluctuated during transgenerational transmission in B. tabaci. For instance, the titre of wMel was extremely low after transfection in G1, and G2 and higher in G4-6. This observation prompted us to speculate that the titre of Wolbachia might be modulated by the interaction between the host and Wolbachia. Obviously, clarifying the quantitative relationship between the titre of Wolbachia and the levels of expression of the candidate genes involved in the host-Wolbachia interaction would help us understand the mechanisms underlying the fluctuations in the Wolbachia titre, especially when the titre is potentially related to the pest control capability of Wolbachia (Breeuwer & Werren, 1993; Noda et al., 2001). Therefore, it is essential to quantitatively measure both the titre of Wolbachia (gDNA) and the levels of expression (cDNA) of functional genes, and thus the screening for stable RGs for both gDNA and cDNA samples is a prerequisite for a reliable qPCR analysis. In the present study, a combination of algorithms was used to evaluate the candidate RGs, which circumvented the drawbacks of using a single algorithm, and the results indicate that use of the stable RGs rather than unstable RGs could result in more reliable qPCR data. Indeed, the stable RGs specifically identified in this study were successfully used in the qPCR analysis of both gDNA and cDNA samples from B. tabaci artificially transfected with Wolbachia, which will facilitate the study of the host insect-Wolbachia interaction.

Different algorithms were developed for selecting RGs in the past, but as mentioned above, each algorithm has advantages and disadvantages, which potentially generate biased results. The use of a combination of algorithms may hopefully counteract any bias. For instance, GeNorm and BestKeeper are based on paired comparisons, and the selection of the most appropriate RGs are based on the change in the expression of genes, which does not eliminate the influence of co-regulation; on the other hand, NormFinder and the ΔCt method can neutralize this influence. Here we used geomeans to aggregate the RGs and generated an integrated ranking for the candidate RGs and the most variable RGs based on the CV analysis were not used in this study. Our corrected results indicate that the combined analysis worked well in selecting stable RGs.

The overall design of our experiments was based on the results of our previous study of the wMel Wolbachia strain after transfection by microinjection, which revealed the titre of wMel differed greatly in the different generations. As noted above, the change in titre was most marked from G0 to G1; hence, the samples (both gDNA and cDNA) were collected during this period of time. Hopefully, the change in the expression of the functional genes selected and the titre of Wolbachia were effectively detected. In addition, the selection of immune-related genes for validating the RGs was based on those genes that were identified by the transcriptome sequencing, which revealed that an active immune response was induced after infection with Wolbachia, and thus they will be functionally analysed in the next step. The antimicrobial peptide Alo-3 was greatly

### Table 3. Stability of internal reference genes based on a Coefficient of Variation (CV) analysis.

| Ref. gene | CV% | Ranking |
|-----------|-----|---------|
| Actin     | 11.71 | 1       |
| HSP70     | 26.26 | 2       |
| 18S       | 28.99 | 3       |
| EF-1α     | 33.5  | 4       |
| ATPase    | 35.4  | 5       |
| HSP20     | 39.89 | 6       |
| GAPDH     | 43.62 | 3       |
| RPL29     | 43.91 | 4       |
| 18S       | 43.91 | 5       |
| EF-1α     | 108.11| 6       |
| Actin     | 28.27 | 1       |
| GAPDH     | 34.17 | 2       |
| RPL29     | 43.62 | 3       |
| ATPase    | 43.91 | 4       |
| 18S       | 43.91 | 5       |
| EF-1α     | 108.11| 6       |

### Table 4. Corrected rankings of reference genes.

| Ref. gene* | GeNorm M-value | NormFinder Stability | BestKeeper SD ± CP | ΔCt method | Geomean | Ranking |
|------------|----------------|----------------------|--------------------|------------|---------|---------|
| gDNA       |                |                      |                    |            |         |         |
| Actin      | 0.528(4)       | 0.231(1)             | 0.138(1)           | 0.536(4)   | 2       | 1       |
| EF-1α      | 0.253(1)       | 0.271(3)             | 0.503(6)           | 0.518(1)   | 2.06    | 2       |
| HSP20      | 0.346(2)       | 0.268(2)             | 0.393(4)           | 0.534(3)   | 2.63    | 3       |
| ATPase     | 0.253(1)       | 0.279(5)             | 0.458(5)           | 0.532(2)   | 2.66    | 4       |
| HSP70      | 0.474(3)       | 0.276(4)             | 0.317(3)           | 0.549(5)   | 3.66    | 5       |
| 18S        | 0.631(6)       | 0.462(7)             | 0.298(2)           | 0.685(7)   | 4.92    | 6       |
| GAPDH      | 0.578(5)       | 0.402(6)             | 0.504(7)           | 0.637(6)   | 5.95    | 7       |
| cDNA       |                |                      |                    |            |         |         |
| Actin      | 0.297(1)       | 0.444(3)             | 0.394(2)           | 0.72(2)    | 1.86    | 1       |
| GAPDH      | 0.297(1)       | 0.614(4)             | 0.373(1)           | 0.923(4)   | 2.21    | 3       |
| 18S        | 0.636(2)       | 0.158(1)             | 0.704(4)           | 0.812(3)   | 2.21    | 3       |
| RPL29      | 0.774(3)       | 0.358(2)             | 0.604(3)           | 0.716(1)   | 2.21    | 3       |
| ATPase     | 0.841(4)       | 0.738(5)             | 0.805(5)           | 0.973(5)   | 4.4     | 4       |

*RPL29 and EF-1α are excluded from gDNA and cDNA samples, respectively, in the corrected ranking analysis based on their highest CV values (Table 3).
CONCLUSIONS

We developed a set of suitable RGs for studying the host insect-Wolbachia interaction using the agricultural pest B. tabaci artificially transfected with an exogenous strain of Wolbachia. The RGs are suitable for both gDNA and cDNA templates. A more reliable qPCR analysis of the titre of Wolbachia and the expression of functional genes will increase our understanding of the infection dynamics of Wolbachia in this pest insect, which might provide a scientific basis for the development of a CI-based control strategy for this pest.

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REFERENCES

ANDERSEN C.L., JENSEN J.L. & ØRNTOFT T.F. 2004: Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. — Cancer Res. 64: 5245–5250.

ARTICO S., NARDELI S.M., BRILHANTE O., ROSSI-DE-SA M.F. & ALVES-FERREIRA M. 2010: Identification and evaluation of new reference genes in Gossypium hirsutum for accurate normalization of real-time quantitative RT-PCR data. — BMC Plant Biol. 10: 49, 12 pp.

ARYA S.K., JAN G., UDHIYAY S.K., SINGH H., DIXIT S. & VERMA P.C. 2017: Reference genes validation in Phenacoccus solenopsis under various biotic and abiotic stress conditions. — Sci. Rep. 7: 13520, 12 pp.

BANSAL R., MAMIDALA P., MIAN M.R., MITTAPALLI O. & MICHEL A.P. 2012: Validation of reference genes for gene expression studies in Aphis glycines (Hemiptera: Aphididae). — J. Econ. Entomol. 105: 1432–1438.

BODA E., PINI A., HENXA E., PAROLISI R. & TEMPIA F. 2009: Selection of reference genes for quantitative real-time RT-PCR studies in mouse brain. — J. Mol. Neurosci. 37: 238–253.
Bourtiz K. & O'Neill S. 1998: “Wolbachia” Infections and arthropod reproduction. — Biology 48: 287–293.

Breeuwer J.A. & Werren J.H. 1993: Cytoplasmic incompatibility and bacterial density in Nasonia vitripennis. — Genetics 135: 565–574.

Bustin S.A. 2000: Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. — J. Mol. Endocrinol. 25: 169–193.

De Barro P.J., Liu S.S., Boykin L.M. & Dinsdale A.B. 2011: Bemisia tabaci: a statement of species status. — Annu. Rev. Entomol. 56: 1–19.

Deraveux S., Vandesompele J. & Hellemans J. 2010: How to do successful gene expression analysis using real-time PCR. — Methods 50: 227–230.

Freitag D., Koch A., McLean A.L., Kalf R. & Walter J. 2018: Validation of reference genes for expression studies in human meningiomas under different experimental settings. — Mol. Neurobiol. 55: 5787–5797.

Hilgenboecker K., Hammerstein P., Schlattmann P., Telschow A. & Werren J.H. 2008: How many species are infected with Wolbachia? A statistical analysis of current data. — FEMS Microbiol. Lett. 281: 215–220.

Hindson C.M., Chevllet J.R., Briggs H.A., Gallicchio E.N., Ruf I.K., Hindson B.J. & Tewari M. 2013: Absolute quantification by droplet digital PCR versus analog real-time PCR. — Nat. Methods 10: 1003.

Hornakova D., Matouskova P., Kindl J., Valterova I. & Pichova I. 2010: Selection of reference genes for real-time polymerase chain reaction analysis in tissues from Bombus terrestris and Bombus lucorum of different ages. — Anal. Biochem. 397: 118–120.

Hu H.Y. & Li Z.X. 2015: A novel Wolbachia strain from the rice moth Corcyra cephalonica induces reproductive incompatibility in the whitfly Bemisia tabaci: sequence typing combined with phenotypic evidence. — Environ. Microbiol. Rep. 7: 508–515.

Ibanez F. & Tamborindeguy C. 2016: Selection of reference genes for expression analysis in the potato psyllid, Bactericera cockerelli. — Insect Mol. Biol. 25: 227–238.

Koramulata M.K., Amnini R. & Bhattacharya R. 2016: Comprehensive evaluation of candidate reference genes for qRT-PCR studies of gene expression in mustard aphid, Lipaphis erysimi (Kalti). — Sci. Rep. 6: 25883, 10 pp.

Lemaitre B. & Hoffmann J. 2007: The host defense of Drosophila melanogaster. — Annu. Rev. Immunol. 25: 697–743.

Li Z.X., Lin H.Z. & Guo X.P. 2007: Prevalence of Wolbachia infection in Bemisia tabaci. — Curr. Microbiol. 54: 467–471.

Li R., Xie W., Wang S., Wu Q., Yang N., Yang X. & Zhou X. 2013: Reference gene selection for qRT-PCR analysis in the sweetpotato whitefly, Bemisia tabaci (Hemiptera: Aleyrodidae). — PLoS ONE 8: e53006, 8 pp.

Livak K.J. & Schmittgen T.D. 2001: Analysis of relative gene expression data using real-time quantitative PCR and the 2(−ΔΔCt) method. — Methods 25: 402–408.

Martins P.K., Mafra V., De Souza W.R., Ribeiro A.P., Vinecky F., Basso M.F. & Molinari H.B.C. 2016: Selection of reliable reference genes for RT-qPCR analysis during developmental stages and abiotic stress in Setaria viridis. — Sci. Rep. 6: 28348, 10 pp.

Meng H., Yang Y., Gao Z.H. & Wei J.H. 2019: Selection and validation of reference genes for gene expression studies by RT-PCR in Dalbergia odorifera. — Sci. Rep. 9: 3341, 10 pp.

Navas-Castillo J., Fiallo-Olive E. & Sanchez-Campos S. 2011: Emerging virus diseases transmitted by whiteflies. — Ann. Rev. Phytopathol. 49: 219–248.

Noda H., Koizumi Y., Zhang Q. & Deng K. 2001: Infection density of Wolbachia and incompatibility level in two planthopper species, Laodelphax striatellus and Sagotella fuscifera. — Insect Biochem. Mol. Biol. 31: 727–737.

Olsvik P.A., Lie K.K., Jordal A.E.O., Nilsen T.O. & Hordvik I. 2005: Evaluation of potential reference genes in real-time RT-PCR studies of Atlantic salmon. — BMC Mol. Biol. 6: 21, 9 pp.

Paffl M.W., Tichopad A., Prgomet C. & Neuvians T.P. 2004: Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper-Excel-based tool using pair-wise correlations. — Biotechnol. Lett. 26: 495–515.

Ponton F., Chapuis M.P., Pernice M., Sword G.A. & Simpson S.J. 2011: Evaluation of potential reference genes for reverse transcription-qPCR studies of physiological responses in Drosophila melanogaster. — J. Insect Physiol. 57: 840–850.

Scharlaken B., de Graaf D.C., Goossens K., Bruinain M., Pelham I.J. & Jacobs F.J. 2008: Reference gene selection for in-sent expression studies using quantitative real-time PCR: The head of the honeybee, Apis mellifera, after a bacterial challenge. — J. Insect Sci. 8: 33, 10 pp.

Shen Y., Li Y., Ye F., Wang F., Lu W. & Xie X. 2010: Identification of suitable reference genes for measurement of gene expression in human cervical tissues. — Anal. Biochem. 405: 224–229.

Silver N., Best S., Jiang J. & Thein S.L. 2006: Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. — BMC Mol. Biol. 7: 33, 9 pp.

Stouthamer R., Breeuwer J.A. & Hurst G.D. 1999: Wolbachia piipiens: microbial manipulator of arthropod reproduction. — Annu. Rev. Microbiol. 53: 71–102.

Thellin O., Zorzi W., Lakaye B., de Borman B., Commins B., Hennen G. & Heinen E. 1999: Housekeeping genes as internal standards: use and limits. — J. Biotechnol. 75: 291–295.

Tong Z., Gao Z., Wang F., Zhou J. & Zhang Z. 2009: Selection of reliable reference genes for gene expression studies in peach using real-time PCR. — BMC Mol. Biol. 10: 71, 13 pp.

Vandesompele J., De Preter K., Pattyn F., Poppe B., Van Roy N., De Paepe A. & Speleman F. 2002: Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. — Genome Biol. 3: re-search0034-1, 12 pp.

Van Hiel M.B., Van Wielendaele P., Temmerman L., Van Soest V., Vuereinckx K., Huybrechts R. & Simonet G. 2009: Identification and validation of housekeeping genes in brains of the desert locust Schistocerca gregaria under different development conditions. — BMC Mol. Biol. 10: 56, 10 pp.

Werren J.H., Baldo L. & Clark M.E. 2008: Wolbachia: master manipulators of invertebrate biology. — Nat. Rev. Microbiol. 6: 741–751.

Xie L.H., Quan X., Zhang J., Yang Y.Y., Sun R.H., Xia M.C. & Yang L.R. 2019: Selection of reference genes for real-time quantitative PCR normalization in the process of Gaeumannomyces graminis var. tritici infecting wheat. — Plant Pathol. J. 35: 11–18.

Yuan M., Lu Y., Zhu X., Wan H., Shakteel M., Zhan S. & Li J. 2014: Selection and evaluation of potential reference genes for gene expression analysis in the brown planthopper, Nilaparvata lugens (Hemiptera: Delphacidae) using reverse-transcription quantitative PCR. — PLoS ONE 9: e86503, 10 pp.

Zhong Y. & Li Z.X. 2013: Influences of tetracycline on the reproduction of the B biotype of Bemisia tabaci (Homoptera: Aleyrodidae). — Appl. Entomol. Zool. 48: 241–246.
ZHONG Y. & Li Z.X. 2014: Bidirectional cytoplasmic incompatibility induced by cross-order transfection of Wolbachia: Implications for control of the host population. — *Microb. Ecol.* 68: 463–471.

ZHOU X.F. & Li Z.X. 2016: Establishment of the cytoplasmic incompatibility-inducing *Wolbachia* strain *w*Mel in an important agricultural pest insect. — *Sci. Rep.* 6: 39200, 9 pp.

ZHOU W., ROUSSET F. & O’NEILL S. 1998: Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. — *Proc. Biol. Sci.* 265: 509–515.

ZUG R. & HAMMERSTEIN P. 2012: Still a host of hosts for *Wolbachia*: analysis of recent data suggests that 40% of terrestrial arthropod species are infected. — *PLoS ONE* 7: e38544, 3 pp.

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**Table S1.** DEGs involved in the immune-related signalling pathways.

| Pathway / Gene ID | Annotated function | GenBank acc. no. | log2(FC) |
|-------------------|--------------------|-----------------|----------|
| Recognition / gene5108 | Peptidoglycan recognition protein 2-like (PGRP) | LOC109032651 | –1.96 |
| IMD / gene1970 | RING-box protein 1A | LOC109044484 | –1.22 |
| TOLL / gene7989 | RING-box protein 2 | LOC109035809 | –1.96 |
| | Mitogen-activated protein kinase kinase kinase (MAP3K) | LOC109041956 | 2.26 |
| | Putative serine protease K12H4.7 | LOC109030575 | –2.15 |
| | Putative serine protease F56F10.1 | LOC109036101 | –2.15 |
| | Venom serine protease Bi-VSP-like | LOC109037519 | –1.71 |
| | Transmembrane protease, serine 9 | LOC109037575 | –2.13 |
| | Serine protease ruedel | LOC109038630 | 3.83 |
| | Serpin B3-like | LOC109030665 | –3.16 |
| AMPK/TOR / gene3222 | Protein spätzle-like | LOC109040362 | –3.84 |
| | Toll-like receptor 7 | LOC109033743 | –1.47 |
| | Toll-like receptor | LOC109042320 | 1.03 |
| PI3K/AKT/mTOR / gene13005 | Phosphatidylinositol-bisphosphate 3-kinase cataytic subunit delta isoform (PI3K)* | LOC109034225 | 2.83 |
| | 5'-AMP-activated protein kinase subunit gamma-2 (AMPK) | LOC109040764 | 1.09 |
| | DNA-dependent protein kinase cataytic subunit (mTOR) | LOC109043096 | 1.19 |
| | Ribosomal protein S6 kinase beta-1 | LOC109037417 | 1.42 |

* Only the genes with q value < 0.005 and |log2(FC)|>1 (significantly regulated) are listed. FC – fold change. The same below. * The gene in bold was used in the validation of the RGs.

**Table S2.** DEGs involved in the immune responses of the host.

| Category/Gene ID | Annotated function | GenBank acc. no. | log2(FC) |
|------------------|--------------------|-----------------|----------|
| AMP / gene553 | Antimicrobial peptide Alo-3-like* | LOC109033344 | –3.02 |
| | Lysozyme-like | LOC109040865 | 2.11 |
| | Lysozyme C, milk isoyme-like | LOC109042207 | –6.47 |
| Phagocytosis / gene12425 | Down syndrome cell adhesion molecule-like protein Dscam2 | LOC109030752 | 1.38 |
| | Down syndrome cell adhesion molecule-like protein Dscam2 | LOC109036103 | 1.11 |
| |cdc42 homolog | LOC109037334 | 1.28 |
| |cdc42 homolog | LOC109043068 | 1.17 |
| | Partitioning defective 3 homolog | LOC109043237 | 1.38 |
| | Epidermal growth factor receptor (Egfr) | LOC109031082 | 1.44 |
| | Epidermal growth factor receptor substrate 15-like | LOC109044512 | 1.33 |
| | Mucin | LOC109039701 | 1.70 |
| | Protein-disrupted (Dab1) | LOC109032491 | 1.65 |
| | G protein-coupled receptor kinase 1 | LOC109037372 | 1.26 |
| | Protein-disrupted (Dab1) | LOC109037388 | 1.43 |
| | Tyrosine-protein kinase Src64B | LOC109043668 | 1.19 |
| | Ras-like GTP-binding protein Rho1 | LOC109040974 | 1.02 |
| | Ras-related protein Rad-11A | LOC109037003 | 1.04 |
| | EH domain-containing protein | LOC109038437 | 1.20 |
| | EH domain-containing protein 1-like | LOC109040805 | 1.26 |
| | Rab11 family-interacting protein | LOC109031749 | 1.06 |
| Encapsulation / gene7278 | Integrin alpha-PS2-like | LOC109035063 | 1.24 |
| | Integrin beta-PS | LOC109031639 | 1.47 |
| | Rho GTPase-activating protein gacZ-like | LOC109034894 | 1.95 |
| | Rho GTPase-activating protein 10-like | LOC109035840 | 1.16 |

* The gene in bold was used in the validation of the RGs.
Fig. S1. Distribution of UniGenes per FPKM interval (A) and Volcanoplot of DEGs (B). The up- and down-regulated genes are indicated by red and green dots, respectively. Blue dots indicate no significant difference.

Fig. S2. GO (A) and KEGG (B) pathway enrichment analyses of DEGs. q value: the corrected $P$ value, indicating a significant difference between WT and Ti. Rich factor: The ratio of DEG numbers annotated in a given pathway to all the genes annotated in that pathway.
Fig. S3. Standard curves and melting curves of the reference genes. (A) Standard curves for gDNA templates; (B) Standard curves for cDNA templates; (C) Melting curves for gDNA templates and (D) Melting curves for cDNA templates.