

# Research Progress on Reference Genes of Insect for Quantitative Real-time Reverse Transcription PCR (RT-qPCR)

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**Abstract** Quantitative real-time reverse transcription PCR (RT-qPCR) has become the most important method for the quantification of mRNA transcription levels owing to its, specificity, sensitivity, reproducibility, and efficiency. In order to avoid sample-to-sample and run-to-run variations particularly in RNA extraction, RNA quality and cDNA reverse transcription level ,etc. it is necessary to use housekeeping gene which stably expressing as reference gene. Ideally, the housekeeping gene should not be regulated or influenced by the experimental procedure or co-regulated with the target gene. Studies insect models have shown that the expression levels of commonly used reference genes can differ among different tissue, organ types or physiological conditions. However, improper selection of reference genes will result in inaccurate calculation results and consequently obscure actual biological differences among samples, even opposite conclusion. Therefore, reference genes which specific stably expression in each experimental system should be selected in different insects and different experiments. This review aims to provide research achievements of domestic and foreign scholars on insect reference genes, which provide great promise for the future.

**Keywords** Reference Gene, Insect, RT-qPCR, Progress

## 1. Introduction

Quantitative real-time reverse transcription PCR (RT-qPCR) is invented by Applied Biosystems company in the United States in 1996, has become the most important method for the quantification of mRNA transcription levels owing to its, specificity, sensitivity, reproducibility, and efficiency. RT-qPCR has turned into a technique extensively employed for quantification of mRNA transcripts. Especially for studying the expression of small set of genes, or no use Northern blot, can be used to complement the above mentioned methods[1].For this reason, RT-qPCR has been widely used in the expression profiling of selected genes in

biological research.

RT-qPCR is divided into absolute quantification and relative quantification. Relative quantification methods depend on reference genes for normalization. However, RT-qPCR measures the mRNA transcript levels differentially contributed by specific biological conditions as well as confounding factors that are non-specific to the biological conditions and non-reproducible in different experiments. Even with careful control of technical variables[2,3], confounding factors may still result from sample-to-sample and run-to-run variations particularly in RNA extraction, RNA quality and cDNA reverse transcription level ,etc. Data normalization using internal reference genes in thus a crucial step necessary to minimize the influence of confounding factors and improve the fidelity of the quantification process with respect to the specific biological conditions[4].Internal reference genes are usually chosen from "housekeeping" genes with abundant and stable expression under various experimental conditions[5,6].

Ideally, the housekeeping gene should not be regulated or influenced by the experimental procedure or co-regulated with the target gene. The housekeeping gene should also be expressed in abundance and have minimal innate variability. Studies insect models have shown that the expression levels of commonly used reference genes can differ among different tissue, organ types or physiological conditions [7,8]. However, improper selection of reference genes will result in inaccurate calculation results and consequently obscure actual biological differences among samples, even opposite conclusion [9]. Gutierrez [10] etc. has shown that incorrect or improper internal reference genes may result in gene expression level in the deviation of 100 times. For this reason, it is very important to select proper internal reference genes.

In current, a few insect's reference genes have been validated and published with RT-qPCR and gene chips used[11]. Aim of the paper is to sum up the reference genes about insect among different tissues, cells and deal with factors, is to provide theoretical reference basis for studying on other insects.

**Table 1.** Commonly used reference genes for insects

Gene symbol	Full gene name	(putative) Function
$\beta$ -actin	Beta actin	Involved in cell motility, structure and integrity
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Glycolytic enzyme
18S rRNA	18S ribosomal RNA	Structural constituent of ribosome
Stx16	Syntaxin 16	Protein involved in exocytosis
rspL40	Ribosomal protein L40	translation of a subset of cellular transcripts
BTF3	Basic Transcription Factor	growth and development
rsp5	Ribosomal protein S5	controls many different cellular processes
RPS18	Ribosomal protein S18	40S ribosome subunit component
$\alpha$ -Tub	Alpha tubulin	Cytoskeleton structural protein
RpL32	Ribosomal protein 49/L32	Structural constituent of ribosome
RPL13a	Ribosomal protein L13	60S ribosome subunit component
EF1-a	Elongation factor 1 alpha	Protein synthesis
SDHA	Succinate dehydrogenase A	Succinate dehydrogenase activity
UBQ	Similar to Ubiquity family member(ubq-1)	Proteolysis
TBP	TATA-Box binding protein	Transcription initiation
GST	Glutathione-S transfers	Antioxidative enzyme
UBI	Ubiquitin conjugating enzyme IO	Protein degradation

## 2. Selection of Reference Genes for Insects

When studying gene expression patterns in different tissues, a commonly used reference gene may be not stable under all experimental conditions[12]. Therefore, the expression levels of commonly used reference genes can differ among different tissue/organ types or physiological conditions. Ideally, the reference gene should not be false gene, should be expressed in abundance and have minimal innate variability, Ct value of the reference gene should be between 15 to 30 [13,14]. In current, a few commonly used reference genes in insects such as: EF1-a (elongation factor-1 alpha), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), SDHA (succinate dehydrogenase complex subunit A) and so on[15], have relatively stable expression level in cell metabolism (Table 1).

Each candidate reference gene should be evaluated under specific experimental conditions for gene expression profiling to make sure expression occurs at a constant level. Furthermore, researchers have documented, to improve robustness of the experiment, it is recommended to use more than one reference genes and to verify that their transcriptional activity are stable across conditions and tissue types.

## 3. Research of Reference Genes for Insects

Although reference genes commonly have been as the

standard of goal genes, a large number of studies have shown that any kind of "housekeeping gene" expresses constantly only on a special condition. The reference genes at the same treat factor may be different because of their species difference. The reference genes at the same species may also be different because of different experiment condition. At present, the study on reference genes as follows:

Li-hua Chen[16] et al. evaluated four genes in different organs and developmental stages of *Philosamia Cynthia ricini* and identified that the best reference gene varied according to the tissue and physiological condition,  $\beta$ -actin in blood, fat body, midgut, silk gland and larvae, pupae, adult, egg of eri-silkworm was more stable as a suitable reference gene.

Miao Yuan[17] evaluated eight candidate genes of *Nilaparvata lugens*, RPS15, TUB, 18S, and EF should be required for a suitable normalization in the different developmental stages; RPS11, 18S, and RPS15 in the different body parts; RPS11, EF, and RPS15 in the two different geographic populations; RPS15, TUB, and EF in the different temperature treatment samples; RPS11, EF, and TUB in the pesticide-stressed samples; RPS 15, TUB, EF, and RPS11 in the different diets treatments; RPS11, AK, and EF in the starvation treatments.

Sun[18] et al. evaluated eight commonly used candidate genes of *Tetranychus cinnabarinus*, RPS18 and 5.8SrRNA had the most stable expression regardless of the four different strains, whereas RPS18 and  $\alpha$ -TUB were expressed most stably in different developmental stages.

Jia Wang[19] et al. evaluated ten candidate genes in different organs and developmental stages of *Bactrocera*

minax, based on the comprehensive analysis of stability ranking, UBC, GAPDH and GST were recommended as the reference genes for different developmental stages, and TUB, GAPDH and GST for different body parts of adults.

Lü[20] et al. research on *Bactrocera minax* in different developmental stages, RPL 32 was expressed most stably as a suitable reference gene.

Liu-hao Wang[21] research on *Bactrocera minax* in different developmental stages,  $\alpha$ -TUB, RPL32 and EF1- $\alpha$  were expressed most stably as a suitable reference gene.

Wang Song[22] et al. evaluated ten candidate genes in different survival time of *Philosamia Cynthia ricini*, RPS, GAPDH and  $\alpha$ -TUB were recommended as the most suitable reference genes.

Raman Bansal[23] et al. research on *Aphis glycines* in different developmental stages, TBP was expressed most stably as a suitable reference gene.

Chang-ning Guo[24] evaluated seven candidate genes of phyllonorycter *Ringoniella*,  $\beta$ -TUB and  $\beta$ -actin should be required for a suitable normalization in the different developmental stages;  $\beta$ -actin and 18S in the different body parts;  $\beta$ -actin and  $\beta$ -TUB in the pesticide-stressed samples.

Su-ping Xu[25] evaluated six traditional (18SrRNA, ACTB, RPL3, PPI, TBP, RPII) and one novel (DIMT) candidate reference genes of *Aphis gossypii* Glover, PPI and DIMT were the most reliable reference genes in different morphs, hosts, and tissues; PPI, RPII and DIMT are suitable reference genes for different developmental stages samples.

Fang Chen[26] et al. evaluated six candidate reference genes ( $\alpha$ -TUB, GAPDH, rpl32,  $\beta$ -TUB, SDHA and TBP) in different developmental stages of *P. solenopsis* under seven different temperature regimes by RT-qPCR method,  $\alpha$ -TUB can be used as a reference gene for the 2nd and 3rd instar nymph, and rpl32 could be used as the reference gene for female adult under different temperature regimes.

Niu[27] research on *Panonychus citri* (McGrego) by RT-qPCR method, RPII was expressed most stably in different developmental stages as a suitable reference gene;  $\alpha$ -TUB and RPII could be used as the reference gene under different temperature regimes. Niu[54], et al. research on the expression stability reference genes in different tissues of pesticide-susceptible and pesticide-exposed in *Panonychus citri* (McGrego). EF-1 $\alpha$  and GAPDH exhibited the most stable.

Lu[28] et al. evaluated candidate reference genes of *Nilaparvata lugens*, GAPDH and UCCR should be required for a suitable normalization in the different developmental stages; RPL10, AK and EF-1 $\alpha$  in the different body parts; RPL10 and EF-1 $\alpha$  in the different geographic populations; GAPDH and EF-1 $\alpha$  in the different temperature treatment samples.

Matthias[29] et al. have identified seven orthologs of commonly used housekeeping genes (actin, EF-1 $\alpha$ , GAPDH, RP49, TubA1, UBI, and CG13220) in the desert locust, the most preferred reference genes were RP49, EF-1 $\alpha$  and ACT for studies of fifth instar nymphs; however, GAPDH, UBI and EF-1 $\alpha$  in adult brains.

TOUTGES[30] et al. researched on the candidate reference genes in *T. castaneum*, rps6, rpl13a, rps3, and rps18 were the most stably in different developmental stages as a suitable reference gene.

N.H.BAGNALL[31] et al. researched on the candidate reference genes in *Lucilia cuprina*, 18SrRNA, 28SrRNA, GST1,  $\beta$ -TUB and RPLPO were the most stably in different developmental stages as a suitable reference gene.

Mamidala[32] et al. research on the expression stability of eight reference genes in different tissues and developmental stages of pesticide-susceptible and pesticide-exposed in *Cimex lectularius* by using qRT-PCR. RPL18 exhibited the most stable gene expression across all the tissue and developmental-stage samples.

Zhang[33] et al. have identified twelve candidate reference genes of *H. armigera* and evaluated for their expression stability under different biotic and abiotic conditions. The most suitable candidate combinations of reference genes were as follows: 28S and RPS15 for developmental stages; RPS15 and RPL13 for larvae tissues; EF and RPL27 for adult tissues; GAPDH, RPL27, and  $\beta$ -TUB for nuclear polyhedrosis virus infection; RPS15 and RPL32 for insecticide treatment; RPS15 and RPL27 for temperature treatment; and RPL32, RPS15, and RPL27 for all samples.

Muhammad Shakeel[34] et al. evaluated the stability of expression of nine potential reference genes under different experimental conditions including temperature, mechanical injury, starvation, photoperiod, and developmental stages of *Helicoverpa armigera*, RPL28 and RPS15 were found to be the most stable reference genes in case of starved larvae, temperature stressed larvae, and different developmental stages. HSP90 and TUBB proved to be highly stable in case of photoperiod stressed larvae. TUBB and GAPDH were the most stable reference genes in case of larvae subjected to mechanical injury.

Cardoso[35] et al. evaluated the expression stability of six genes (Actin, Gapdh, Rp49, Rps17,  $\alpha$ -tubulin, and GstD1) among species within the same life stage and between life stages within each species of the Calliphoridae family. Actin, Gapdh, and Rp49 were the most stable among the selected genes.

Jia-hong Wu[36] et al. evaluated six housekeeping genes in *Ae. albopictus*, rspL40 and BTF3a has the most stable expression in different tissues, whereas rspL40 and rsp5 stably in different blood feeding phases.

Ran Peng[37] et al. detected seven commonly used reference genes (ACT, GAPDH, 28SrRNA, RPL3,  $\alpha$ -Tubulin, UBC and TBP) in different development stages of *Bombyx mori*. ACT3, GAPDH and  $\alpha$ -Tubulin in the mid-gut,  $\alpha$ -tubulin, UBC and TBP in the fat body as well as  $\alpha$ -Tubulin, ACT3 and UBC in the Malpighian tubule were identified as the most stable genes.

Shen[38] et al. validated the suitable reference genes for gene expression profiling in different tissues of *B. dorsalis*. Moreover, appropriate reference genes were selected out for gene expression profiling of the same tissues taking the

sexual differences into consideration. ACT2 and  $\alpha$ -TUB are the best choice for both males and females in the midgut and Malpighian tubules. However,  $\alpha$ -TUB and ACT1 are the best pair for fat body.

Yu Wu[39] et al. detected the expression level of seven commonly used reference genes (Actin3, GAPDH, 28SrRNA, RPL3,  $\alpha$ -Tubulin, UBC and TBP) in *Bombyx mori*.  $\alpha$ -TUB and 28S rRNA were the most stable expression in middle silkgland, GAPDH and 28SrRNA in rear silkgland,  $\alpha$ -TUB and UBC in fat body.

Rafaela[40] et al. evaluated the expression stability of five candidate reference genes (18S rRNA, GAPDH, b-actin,  $\alpha$ -tubulin and ribosomal protein L26) in two tissues (salivary gland and intestine) and under different physiological conditions (before and after blood feeding and after infection with *T. cruzi* or *T. rangeli*) of *Rhodnius prolixus*. 18S rRNA, GAPDH and  $\alpha$ -tubulin showed acceptable stability for studies in all of the tissues and experimental conditions evaluated.  $\beta$ -actin, one of the most widely used reference genes, was confirmed to be one of the most suitable reference genes in studies with salivary glands.

D. Majerowicz[41] et al. compared the expression of seven genes in organs of *Rhodnius prolixus* under diverse conditions, Rp18S and EF-1 $\alpha$  were the most reliable genes for normalization, Rp18S was also the best reference gene in the fat bodies of unfed and fed insects. EF-1 $\alpha$  was found to be the best reference gene for comparison between posterior midguts, and MIP or Actin should be used to compare gene expression in the ovaries.

Hornakova[42] et al. assessed the stability of eight reference genes in the labial gland and fat body of the bumblebees *Bombus terrestris* and *Bombus lucorum* of different ages. AK and PLA2 were the most stable genes in both tissues of *B. terrestris*. EF-1 $\alpha$  and PLA2 were the most stable genes for the labial gland and fat body of *B. lucorum*.

Swapna Priya Rajarapu[43] et al. validated six *A. planipennis* reference genes (ACT; $\beta$ -TUB, GAPDH; RPL7; EF-1 $\alpha$ ; and UBQ) in different larval tissues, developmental stages and two treatments. TEF-1 $\alpha$  is the most appropriate reference gene.

Ponton[44] et al. analyzed the expression levels of seven candidate reference genes (Actin, EF1, Mnf, Rps20, Rpl32, Tubulin and 18S) in *Drosophila melanogaster* that were injured, heat-stressed, or fed different diets. Actin, Mnf and TUB were the most stable genes in heat-stressed treatments, RPL32 and  $\alpha$ -TUB in fed different diets, ACT, TUB and EF-1 $\alpha$  in injured.

Scharlaken B[45] et al. analyzed the expression of eleven candidate reference genes in the honeybee head, for their potential use in the analysis of differential gene expression following bacterial challenge. actin, RPS18 and GAPDH

were found suitable reference genes in the honeybee head in the context of bacterial infection.

Maroniche[46] et al. used reference genes ACT, TUB, GAPDH, EF-1 $\alpha$ , RPS18 and UBI from *Delphacodes kuscheli*, UBI, followed by RPS18 and ACT, are the most suitable genes as internal controls for quantitative gene expression studies in MRCV-infective planthoppers.

Xue[47] et al. had found the use of 28S rRNA as a housekeeping gene in real-time quantitative PCR analysis of gene transcription in insect cells infected by viruses.

Jeffrey C. Lord[48] et al. evaluated reference genes expression in *Tribolium castaneum* exposed to *Beauveria bassiana*. The most stable were ribosomal protein genes, RPS3, RPS18, and RPL13a.

Niu[49] et al. evaluated the stability of five candidate internal reference genes: EF-1 $\alpha$ , PPIA, RPL23, TBP and UBI, in relation to Israeli acute paralysis virus (IAPV) infection of *Bombus terrestris*. PPIA as the single, most-optimal internal reference gene and the combination of PPAI-RPL23-UBI as a fully-sufficient multiple internal reference genes set for IAPV infection experiments.

Jiang[50] et al. evaluated the stability of four candidate internal reference genes from *Liposcelis bostrychophila*, 18S rRNA was the most stability for deltamethrin induction and the different developmental stages.

Katina [51] et al. tested six candidate reference genes for normalizing transcription levels of *D. pulex* genes. Xbp1, Tbp, CAPON and Stx16 were suitable reference genes for accurate normalization in qRT-PCR studies using *Chaoborus*-induced.

Marie-Pierre Chapuis[52] et al. assessed a suite of reverse transcription-quantitative PCR reference genes for analyses of density-dependent behavioural plasticity in the Australian plague locust, Arm and EF1 $\alpha$  are the most stably expressed combination of two reference genes of the eight examined.

Wang[53] et al. chose ten genes as candidate reference genes in *Mylabris cichorii* L. (Coleoptera: Meloidae), recommended UBE3A and RPL22e as suitable reference genes in females and UBE3A, TAF5, and RPL22e in males.

To collected the results of previous scholars show that the reference genes at the same insect may be different because of different physiological conditions. The reference genes at the same physiological conditions or treat factor may also be different because of their species difference (Table 2). Therefore, the reference genes of stable expression absolutely in all conditions don't exist. To select suited reference genes for different experiment when we study different goal genes, can't blindly reference.

**Table 2.** The reference genes of insects in different experiment conditions

Insect name	Different treatment									
	Organ	Developmental stage	Geographic population	Temperature	Pesticide stressed	Diets treatment	Starvation treatment	Virus infection	Photoperiod stressed	Mechanical injury
Philosamia Cynthia ricini	β-actin	β-actin	—	—	—	—	—	—	—	—
Nilaparvata lugens	RPS11 18S rRNA RPS15	RPS15 TUB 18S rRNA EF	RPS11 EF-1a RPS15	RPS15 TUB EF-1a	RPS11 EF-1a TUB	RPS 15 TUB EF-1a RPS11	RPS11 AK EF-1a	—	—	—
Tetranychus cinnabarinus	RPS18 5.8S rRNA	RPS18 α-TUB	—	—	—	—	—	—	—	—
Bactrocera minax	TUB GAPDH GST	UBQ GAPDH GST	—	—	—	—	—	—	—	—
Bactrocera minax	—	α-TUB RPL32 EF1-α	—	—	—	—	—	—	—	—
Philosamia Cynthia ricini	—	RPS GAPDH α-TUB	—	—	—	—	—	—	—	—
Aphis glycines phyllonorycter Ringoniella	— β-actin 18S rRNA	TBP β-TUB β-actin	—	—	— β-actin β-TUB	—	—	—	—	—
Aphis gossypii Glover	PPI DIMT	PPI RPII DIMT	—	—	—	PPI DIMT	—	—	—	—
P. solenopsis	—	α-TUB	—	rp132 α-TUB RPII	— EF-1α GAPDH	—	—	—	—	—
Panonychus citri	—	RPII	—	—	—	—	—	—	—	—
Nilaparvata lugens	RPL10 AK EF-1α GAPDH	GAPDH UCCR RP49	RPL10 EF-1α	GAPDH EF-1α	—	—	—	—	—	—
Desert locust	UBI EF-1α	EF-1α ACT rps6 rp113a rps3 rps18	—	—	—	—	—	—	—	—
T. castaneum	—	18SrRNA 28SrRNA GST1 β-TUB RPLPO	—	—	—	—	—	—	—	—
Lucilia cuprina	—	—	—	—	—	—	—	—	—	—
Cimex lectularius	RPL18	RPL18	—	—	—	—	—	—	—	—
H. armigera	RPS15 RPL13	28S rRNA RPS15	—	RPS15 RPL27	RPS15 RPL32	—	—	GAPDH RPL27 β-TUB	—	—
Helicoverpa armigera	—	RPL28 RPS15 Actin Gapdh Rp49	—	RPL28 RPS15	—	—	RPL28 RPS15	—	HSP90 TUBB	TUBB GAPDH
Calliphoridae	—	—	—	—	—	—	—	—	—	—
Ae. albopictus	rspL40 BTF3a	—	—	—	—	rspL40 rsp5	—	—	—	—
B. dorsalis	a-TUB	—	—	—	—	—	—	—	—	—
Bombus terrestris	AK	—	—	—	—	—	—	—	—	—
Bombus lucorum	PLA2 EF-1a	—	—	—	—	—	—	—	—	—
A. planipennis	TEF-1α	TEF-1α	—	—	—	—	—	—	—	—
Drosophila melanogaster	—	—	—	Actin Mnf TUB	—	RPL32 α-TUB	—	—	—	ACT TUB EF-1α
Honeybee	—	—	—	—	—	—	—	actin RPS18 GAPDH	—	—
Delphacodes kuscheli	—	—	—	—	—	—	—	UBI	—	—

Tribolium castaneum	—	—	—	—	—	—	—	RPS3 RPS18 RPL13a EF-1 $\alpha$ PPIA	—	—
Bombus terrestris	—	—	—	—	—	—	—	RPL23 TBP UBI	—	—
Liposcelis bostrychophila	—	18S rRNA	—	—	18S rRNA	—	—	—	—	—

## 4. Analysis of Reference Genes Expression Stability

As the current, three freely available software tools, for example GeNorm[54], NormFinder[55], and BestKeeper [56], were usually comprehensively used to evaluate reference gene expression stability.

GeNorm program was compiled by Jo Vandesompele in 2002, was used to identify genes that were the most stably expressed for all of the experimental conditions. The program defines two parameters to quantify the housekeeping gene stability: M (the arithmetic mean of the pairwise variations between a particular gene and all other candidate control genes) and V (the pairwise variation). The least stable genes have the highest M values and are successively excluded. Furthermore, To obtain the optimal number of reference genes for data normalization, we calculated the pairwise variation ( $V_{n/n+1}$ ) of serial log-transformed NF ratios using N relative to N+1 reference genes as previously described. The  $V_{n/n+1}$  value reflects NF stability across samples. While individual reference genes have considerably differential expression levels across samples, NF will be sensitive to stepwise inclusion of these reference genes resulting in an increase or decrease in  $V_{n/n+1}$  value. If inclusion of more or less reference genes has little or no effect on  $V_{n/n+1}$  value, NF will become insensitive to stepwise inclusion of these reference genes and approach a relatively stable status with a minimal  $V_{n/n+1}$  value, which was below the default cut-off value of  $V = 0.15$ . GeNorm address: <http://medgen.ugent.be/~jvdesomp/genorm/index.php>

NormFinder program was compiled by Claus in 2004, applies a model-based approach, which in contrast to geNorm allows the assignment of groups to the samples (treatment vs. control). NormFinder approach attempts to compensate for expression differences between treatment and control by selecting combinations of genes with opposite expression and as little intra- and intergroup variation as possible. Inter- and intragroup variations are used for the calculation of a stability value i.e., candidates with minimal combined intra- and intergroup variation are ranked as the most stable genes. This approach has advantages over the pairwise comparison approach of geNorm if coregulated genes, but only selects one gene as optimal reference genes[57]. NormFinder address:

<http://www.mdl.dk/publicationsnormfinder.htm>

BestKeeper Excel tool was compiled by Pfaffl in 2004,

analyzes each gene's expression variability by calculating the Ct set standard deviation (SD) and coefficient of variance (CV) and then by pair-wise comparison calculates the correlation between the genes and with the Bestkeeper index. The most stable genes have the lowest CV values. BestKeeper address: <http://www.gene-quantification.de/best-keeper.html>

## 5. Conclusions

On the basis of the research results of the insects internal reference genes in domestic and foreign, have shown that the optimum internal reference genes is not identical among different tissue, organ types, physiological conditions, or species, can't be used directly before don't be tested and verified. In addition, the different internal reference genes of the same species or dealing factors also are related with the selection of candidate housekeeping genes.

For reliable results, must select the appropriate internal reference genes to deter error such as inaccurate quantification of RNA, the quality of the RNA, and difference during cDNA synthesis that can trigger variations in PCR reactions[58,59]. From the existing reports, the optimum internal reference genes is not identical in different species or dealing factors[60,61,62]. For example, ACT was used commonly as internal reference gene for researching the whiteflies[63,64],but Su[7] had shown that ACT is not stable in many conditions. Therefore, can't blindly select internal reference genes. Internal reference genes are stable expression only in certain types of cells, or specific experimental factors. It will be more several times or even one hundred times differences if select improper Internal reference genes[65,66]. At the same time, it is best to use two or more internal reference genes to adjust the system deviation, especially outstanding in genetic subtle expression.

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