Selection and Validation of Suitable Reference Genes for RT-qPCR Analysis in *Bursaphelenchus Xylophilus*

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Abstract

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) is a powerful technique for studying gene expression, and it is widely used in molecular biology and genetic research. The key to quantitative accuracy depends on the stability of the reference genes used for data normalization under different experimental conditions. The pinewood nematode (PWN; *Bursaphelenchus xylophilus*) is the causal agent of a devastating disease, the pine wood disease (PWD), demanding extensive and prompt research to understand the molecular mechanism of PWD, but the identification of reference PWN genes for standardized RT-qPCR has not been reported yet. In this study, we have analyzed eight candidate reference genes of PWN under different temperature conditions and at different developmental stages. Delta Ct method, GeNorm, NormFinder, BestKeeper and RefFinder algorithms were used to evaluate the expression stability of these genes. 18SrRNA and HIS were selected for gene expression research under temperature treatments, while EF1 γ and 18SrRNA for gene expression studies across different developmental stages. In general, the results indicate that 18SrRNA is the most stable gene for RT-qPCR under different conditions. Finally, we use arginine kinase gene (AK) in different temperature and heat shock protein 90 (HSP90) in different developmental stages to confirm the stability of expression. The systematic analysis of qRT-PCR reference gene selection of *B. xylophilus* will be helpful for future functional analysis and exploration of genetic resources of *B. xylophilus*.

Introduction

Pine wilt disease (PWD) has devastated a serious threat to forest safety and ecosystem stability in parts of Asia and Europe and has been quarantined globally ^{1,2}{!!! INVALID CITATION !!! , #0;Rodrigues, 2008 #5;Zhao, 2014 #6}{!!! INVALID CITATION !!! {Rodrigues, 2008#63; *Zhao*, 2014 `#64}, #0;Zhao, 2014 #64}. In China, the disease is caused by an invasive nematode species, the pine wood nematode (PWN), *Bursaphelenchus xylophilus*.

The destructiveness of PWN is usually closely related to its native vectors beetles and ophiostomatoid fungal species ³. PWN feeding on the epithelial tissue of the tree host and ophiostomatoid (blue - stain) fungi. The fungi reproduce on the damaged tree host ^{4,5}. PWN undergoes four propagative larval stages (L1-L4) from development to reproduction ⁶.

However, under adverse conditions, such as scarce food and extreme temperatures , PWN will molt from L₂ into dispersed third juveniles (L_{III}) and enter a dispersal stage of its life cycle ⁷.

When the environmental conditions are suitable, L_{III} are attracted by the insect vector, *Monochamus alternatus*, and gather around their pupal chambers ^{2,5}. Then PWN makes the development of the fourth juveniles (L_{IV}) dispersal stages synchronized with the late pupal or early adult phase of vector beetles, in order to these L_{IV} can enter the tracheal system of *M. alternatus* and spread to new pine hosts ^{2,5}. Since the pathogenesis of *B. xylophilus* is complex and there are strong interactions between vector beetles,

fungi and environmental conditions, detailed study of *B. xylophilus* is necessary to provide an important theoretical basis for the development of new methods in controlling the spread and harm of PWD.

For the control of this destructive disease, a lot of effort has been spent on the control of pine wood nematodes, including the chemical control being one of the most common approach. However, the indiscriminate use of pesticides in addition to posing environmental hazards, have led to the emergence of pesticide resistance in PWN⁸. Developing environmentally friendly pest control methods, such as RNAi or nematophagous fungal formulations, is a necessary alternative solution. Considering the economic importance and the urgency of developing effective and efficient control strategies, further ecological, physiological, and molecular biological explorations on *B. xylophilus* has been extensively conducted over the past decades⁹⁻¹⁴. In the continual research process, further studies involving various aspects of the molecular mechanism of pine wood nematodes, including development, immunity, energy metabolism, reproduction, chemoreception, and other biological process are required¹⁵⁻¹⁸. Analysis of the regulation and expression patterns of functional genes is one of the major approaches in such explorations.

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) has been widely used for gene expression analysis owing to its high specificity, rapidity, reproducibility and low costs¹⁹⁻²¹. An indispensable factor for the RT-qPCR method is to select a reference gene with stable expression and use it as a reference to reduce the differences between samples through analysis and calculation to ensure the confidence of the final result²². Common internal reference genes generally include elongation factor (EF), actin (ACT), 18srRNA, etc.²³⁻²⁵. However, the stability of the reference gene is a relative phenomenon that is affected by various factors including temperature, development stage, tissue type etc. Previous studies have shown that with the test conditions change, the expression of a reference gene in different cell types and different physiological states is not always constant²⁶⁻²⁸. Therefore, screening appropriate internal reference genes under different test conditions is the basis for accurately judging RT-qPCR test results. Although qPCR has been widely used in the study of *B. xylophilus*, studies on the selection of internal reference genes under different development stages and different temperatures are still not well-known.

Therefore, to investigate a stable and appropriate reference gene, we have evaluated nine commonly used reference genes, including elongation factor-1A (EF1A), elongation factor-1 γ (EF1 γ), actin (ACT), β -tubulin (β TUB), ubiquitin conjugating enzyme(UBCE), ubiquitin(UBQ), histone H2A(HIS), 18S ribosomal RNA (18srRNA) and peroxisomal membrane protein(PMP) as candidate reference genes for *B. xylophilus*. To obtain the available reference genes for qPCR, several statistical algorithms have been established to identify reference genes with stable expression levels, such as the delta Ct method, BestKeeper, geNorm, NormFinder and RefFinder²⁹⁻³³. We used these 5 tools to investigate the expression of these nine internal reference genes in different development stages and different temperatures of pine wood nematodes.

Materials And Methods

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Nematodes material. The *B. xylophilus* isolate used for this study was isolated from wilted pine trees or *Monochamus alternatus* beetles found in Zhejiang province, China. The laboratory cultures of PWN were reared on *Botrytis cinerea* growing on PDA plates at 25°C.

Collection of second-stages reproductive juveniles (L₂). Laboratory reared *B. xylophilus* on 8th day of culturing were isolated using a modified Baermann funnel technique for 2 hours at 25°C³⁴. The collected nematodes were cleaned by sucrose flotation and phosphate-buffered saline Tween 20 (PBST)³⁵. The washed isolates of nematodes were poured into a glass dish and allowed to stand for half an hour. When the nematode eggs got settled at the base, the excess liquid was drained off and PBST containing streptomycin sulfate and chloramphenicol were added and the glass dishes were incubated for 24h at 25°C to hatch the eggs and get L₂ larvae.

Collection of third-stages reproductive juveniles (L₃). The L₂ nematodes were cultured on *B. cinerea* - PDA culture plates and incubated for 18 hours at 25°C. The L₃ nematodes were isolated from the cultures using the Baermann funnel technique and cleaned as described earlier followed by freezing in liquid nitrogen and storage at -80°C for later use.

Collection of fourth-stages reproductive juveniles (L₄). The earlier prepared L₂ nematode cultures were incubated for 42 hours to get them developed into fourth-stage reproductive juveniles (L₄). Once isolated and cleaned as described earlier, the L₄ nematodes were immediately frozen in liquid nitrogen and stored at -80°C.

Collection of third-stages dispersal juveniles (L_{III}). The wild wilted pine wood was cut into 35 cm thin strips, and the nematodes were separated by the Baermann funnel technique³⁶, whereas any of the non-L_{III} nematode isolated, were removed during microscopic observations. To further purify the isolates, sucrose flotation centrifugation method was used to remove fungal hyphae and impurities³⁷. These purified supernatants containing L_{III} nematodes were frozen in liquid nitrogen and stored at -80°C for later use.

Collection of fourth-stages dispersal juveniles (L_{IV}). The newly eclosing *M. alternatus* beetles were dissected and placed in a 35 mm Petri dish with sterilized ddH₂O for 30 min. After half an hour, a large number of L_{IV} nematode were clearly visible floating in the solution. these were isolated and cleaned before freezing in liquid nitrogen and storage at -80°C.

Collection of reproductive adult. L₂ nematodes as collected earlier, were inoculated to *B. cinerea* cultures and kept for 72 hours. Once the larval stages developed in to adults, nematodes were isolated by Baermann funnel method and cleaned as previously described. The adult nematodes were stored in 1.5 mL centrifuge tubes and stored at -80°C.

Collection of nematodes treated with different temperatures. To study the effect of different temperatures, randomly collected nematodes from *B. cinerea* culture plates were isolated and cleaned as

described earlier. These cultures were exposed to 4°C, 10°C, 15°C, 20°C, 25°C, 30°C and 35°C for 48 hours, respectively. Another dish of random culture of nematodes was incubated at 50°C for 20 min and then shifted at 25°C for next 24 hours. Each temperature treatment was replicated thrice and after incubation all samples were stored in liquid nitrogen at -80°C.

Total RNA isolation and cDNA synthesis. Total RNA from each sample was extracted using Trizol reagent (Invitrogen, Carlsbad CA, USA) according to the manufacturer’s instructions. RNA integrity was confirmed by 1% agarose gel electrophoresis and RNA concentration was determined using a NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific, West Palm Beach, FL, United States). CDNAs for quantitative RT-PCR (qRT-PCR) were synthesized from 2µg of total RNA using the Primescript™ RT reagent Kit with genomic DNA eraser (perfect real time) (Tiangen, Beijing, China), according to the manufacturer’s instructions.

Selection of candidate reference genes and qPCR primer design. Nine candidate reference genes were selected which had previously been reported to be suited to transcript normalization in other organisms under different experimental conditions. The sequences of candidate reference genes were obtained from the transcriptome *de novo* assembly sequences of *B. xylophilus*³⁸. Primers were designed using Primer Premier 6 software (Premier Biosoft International, Palo Alto, CA, United States) according to the SYBR Green Master Mix (Tiangen, Beijing, China) manufacturer’s instructions.

Quantitative reverse transcription-polymerase chain reaction. RT-qPCR was carried out using the StepOne Plus Real-time PCR System (Applied Biosystems, Foster City, CA, United States) in which amplification, detection and analysis steps were combined. Reactions were performed using the SYBR Green Master Mix (Tiangen, Beijing, China) in a 20 µL reaction volume, containing 10 µL SYBR Green Master Mix, 0.4 µL 10 pmol/L of each primer (Table 1) and 2 µL diluted cDNA. The following program parameters were used for all amplifications: 95°C for 5 mins followed by 40 cycles at 95°C for 15s each, 60°C for 32s, and finally one cycle at 95°C for 15s, 60°C for 1 min, 95°C for 15s, and 60°C for 15s to generate dissociation curves³⁹. The amplification efficiency (E) was calculated from standard curves, according to the SYBR Green Master Mix manufacturer’s instructions. All assays were performed using three biological replicates, each consisting of technical triplicates, and a non-template control³⁹.

Table 1. Primers for candidate reference genes investigated in *B. xylophilus* by qRT-PCR

Gene symbol	Primer sequence(5' to 3')	Amplicon length(bp)	E%	R ²
Actin	F:GACTTGACCGATTACTTGAT R:GTAGGTGGTCTCGTGGATA	255	119.89	0.9951
TUB	F:TCTTACTTCGCTGAATGGAT R:CGCTTCTTGATATTGTTGGT	270	131.56	0.9979
UBCE	F:GTGGATGCCTCAGTTATGTA R:TTGTGTTCCGATGCCTTC	170	106.43	0.9968
PMP-2	F:GCCATTCCAATCCTCTTCA R:CTTGTTCTCCTTCACTTCTTC	225	152.33	0.9900
HIS	F:GCTCGTGACAACAAGAAGT R:CCTTGAGCGATGGTGACT	107	219.36	0.9831
EF1 γ	F:GTCGCATTGAACCTCCTC R:CATCTTCTTCTGGCTCTTGA	283	142.21	0.9965
18SrRNA	F:AGTCGTCTCGCATTGTTC R:AGTTCAGCAGGTAGTCATAC	298	142.88	0.9975
UBQ	F:TTGGTGTTGAGGTTGAGAG R:GATGTTGTAGTCCGAGAGG	207	141.53	0.9948

E, qRT-PCR efficiency; R², Regression coefficient of the qPCR reaction; F, forward primers; R, reverse primers; TUB, tubulin; UBCE, ubiquitin conjugating enzyme; PMP-2, peroxisomal membrane protein-2; HIS, histone; EF1 γ , elongation factor-1 γ ; 18SrRNA, 18S ribosomal RNA; UBQ, ubiquitin.

Assessing the expression stability of reference genes. The expression stabilities of the candidate reference genes were estimated and ranked using four different statistical algorithms, geNorm, NormFinder, BestKeeper, and the delta Ct method, and a web-based analysis tool, RefFinder. The geNorm evaluates the most stable reference genes and determines the optimal number of reference genes required for normalization ³¹. When the expression stability value (MV) is less than 1.5, the expression of this gene is stable ⁴⁰. The NormFinder ranks candidate reference genes by calculating their stability value (SV) ³². The BestKeeper calculates the stability of the candidate genes based on the standard deviation (SD) and coefficient of variation (CV), the smaller SD and CV, the more suitable they are as reference genes ³⁰. The comparative delta Ct method calculates the delta Ct of the candidate reference genes, and smaller Δ Ct means more stable gene expression ²⁹. Finally, we comprehensively ranked the candidate

reference genes based on the geomean (GM) values of the above results from the four different statistical algorithms, using the web-based analysis tool RefFinder³³.

Validation of reference genes. To confirm the stability of expression of the selected reference genes, verification experiments were carried out in samples from different temperature and different developmental stages. For the developmental stage studies, we quantified the relative expression of arginine kinase genes (AK), which have been shown to be differentially expressed at different developmental stages^{41,42}. For the different temperatures studies, we quantified the relative expression of the thermotolerance-related gene heat shock protein 90 (HSP90), which is essential for the survival of *B. xylophilus*⁴³. Normalization of the two target genes (AK, HSP90) was conducted using the most stable gene combinations (EF1 γ /18SrRNA or HIS/18SrRNA) and the least stable combinations (TUB/HIS or EF1 γ /PMP-2) as determined by geNorm and RefFinder. Relative quantification of these two target genes was calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). The primers for validation of selected reference genes were as follows: *Bx-AK-F*, 5'-CATCTCCGAATCATCTCCAT-3' and *Bx-AK-R*, 5'-GCCTTGAAGTTGTCCTTATC-3'; *Bx-HSP90-F*, 5'-GAAGGTCATCAGGAAGAACT-3' and *Bx-HSP90-R*, 5'-TGTGGTAACGGAGGAACT-3'. Statistical analysis was performed by SPSS 22.0 (SPSS Inc., Chicago, IL, United States) based on the independent sample t-test.

Results

Performance of RT-qPCR primers. In order to determine the availability of reference genes, first verify the specificity and efficiency of its amplification primers. The 2.0% agarose gel image showed that the PCR amplifications of each primer pair had a single expected size (Fig. 1A), and the melting curves showed a single peak (Fig. 1B; Fig.S1). The primer efficiency (E) was between 106.43% and 219.36%, and the linear regression coefficient (R^2) values were between 0.983 and 0.998 (Table 1). The comprehensive results showed that the designed primers could accurately amplify the candidate reference genes.

Expression profiles of candidate reference genes. We examined the expression profiles of the candidate reference genes to have an overall representation of primer variability under different experimental conditions (Fig. 2). All the Ct values of the candidate reference genes were between 13 and 26, which indicated that the eight reference genes expressed high level under different experimental conditions. PMP-2 and HIS had average Ct values >23 cycles, followed by UBCE, TUB, EF1 γ , UBQ, Actin and 18SrRNA whose average Ct values ranged between 13-23 cycles (Fig. 2). Meanwhile, it was obviously that the degree of variation in the expression of some reference genes varies with experimental conditions. For example, UBQ varied less (~1 cycle) between temperature treatments than across developmental stages (>4 cycles).

Stability of candidate reference genes. To further evaluate the expression stability of the eight candidate reference genes under two experimental conditions (temperature and developmental stage), ΔC_t method, BestKeeper, NormFinder, and geNorm were employed, and then RefFinder was used to calculate an overall

For different temperature treatments, the analysis of the result of ΔC_t method, BestKeeper, and NormFinder obviously showed that HIS, 18SrRNA and TUB were the most stable reference genes (Table 2). The geNorm showed that all eight candidate genes were suitable for reference genes ($MV < 1.5$), while 18SrRNA and UBQ were the most stable genes (Fig. 3A). EF1 γ and PMP-2 were the most unstable reference genes in the results of the four statistical algorithms. The stability of genes from most to least stable ranked by RefFinder was HIS>18SrRNA>TUB>UBQ>UBCE>Actin>PMP-2>EF1 γ (Fig. 4A). Pairwise variations values of the expression stability of candidate reference genes calculated by geNorm was V-value < 0.15 at V2/3, which indicated that only two references genes were required to normalize the target gene data (Fig. 5). Thus, the best combination of reference genes for temperature treatments of *B. xylophilus* was HIS and 18SrRNA.

For the different developmental stages, the ΔC_t method and NormFinder represented that 18SrRNA and EF1 γ were the most stable genes, whereas TUB and HIS exhibited the greatest variation (Table 2). Based on BestKeeper, 18SrRNA and Actin were the most stable reference genes (Table 2). Whereas, the analysis result of BestKeeper indicated that HIS and UBQ were relatively unstable ($SD > 1$) (Table 2). Additionally, GeNorm calculated the lowest M value for the 18SrRNA/EF1 γ pair, suggesting that they are the most stable reference genes (Fig. 3B). RefFinder ranked the genes from most to least stable as: EF1 γ >18SrRNA>UBQ>actin>UBCE>PMP-2>TUB>HIS (Fig. 4B). The geNorm pairwise variation analysis also revealed that the first V-value <0.15 emerged at V2/3 (Fig. 5). Thus, the best combination of reference genes for developmental stages samples of *B. xylophilus* was EF1 γ and 18SrRNA.

Validation of selected reference genes in *B. xylophilus*. To validate the reference genes selected in *B. xylophilus* under different experimental conditions, the expression of HSP90 and AK were checked under different temperature treatments and developmental stages. The proposed stable gene combinations (HIS/18SrRNA or EF1 γ /18SrRNA, respectively) (Fig. 6) and the least stable combinations (EF1 γ /PMP-2 or TUB/HIS, respectively) (Fig. 7) were used to normalize the expression levels of those two target genes.

The expression patterns of *HSP90* under different temperature treatments were inconsistent when normalized with the two most stable or two least stable reference genes. *HSP90* expression was found to be 2.6 fold ($p < 0.05$) higher at 50°C than 25°C when the proposed gene combination (HIS/18SrRNA) was used (Fig. 6A). When the least stable combination (EF1 γ /PMP-2) was used, *HSP90* expression was only 1.5 fold ($p > 0.05$) higher at 50°C than in the controls under the same conditions (Fig. 6B).

At different developmental stages, *AK* expression was sharply up regulated at L₄ stage and adult stage. However, when *AK* expression at L₄ stage was 214 fold higher than at L_{III} stage when it was normalized to the two most stable reference genes, and only 80 fold higher when normalized to the two least stable reference genes (Fig. 7).

Discussion

B. xylophilus a serious pest of pine trees causing Pine wilt disease (PWD), which has been considered as the most serious forestry disaster in China in recent years. Due to the significance of PWD and the economic losses occurring due to this pest, many in depth studies are being initiated to understand the disease cycle and most of them involve the qRT-PCR technique to quantify the expression of target genes efficiently and accurately. This technique relies on stable expression of reference genes to eliminate the impact of differences in RNA quality, reverse transcription efficiency, and human factors in different samples on quantitative results. Hence, selection of the most appropriate and stable reference genes is the basis of qRT-PCR success ⁴⁴. Since, the expression of reference genes of interest varies with varying experimental conditions⁴⁵⁻⁴⁷, screening of reference genes is particularly necessary for quantitative study of genes under certain conditions to get authentic results. Based on the transcriptome data of pine wood nematodes, in this study, we have screened candidate reference genes suitable for studying the effect of different temperature region and at different development stages of *B. xylophilus*.

The reference genes are diverse in various organisms at different developmental stages and at different temperatures, indicating that there is no absolute universality between the homologous reference genes of different insect species ⁴⁸⁻⁵¹. In order to remedy this limitation some research have reported on the screening of internal reference genes in insects, such as the selecting of reference genes in different tissues and geographical populations of *Nilaparvata lugens* ⁵²; screening of reference genes in *Helicoverpa armigera* at different temperatures ⁵³. In this study, we have evaluated different internal reference genes of *B. xylophilus* under different development stages and different temperatures, and have reported their relative stability for the first time.

Regarding the stability of eight reference genes selected reported by different analyses, different results were demonstrated (Table 2). It is difficult to identify reference genes which are generally stable across these four algorithms, as each program has its own strengths and appropriate application conditions ^{29,54,55}. When compared across different temperature treatments, the results of geNorm is quite distinct from those of delta Ct, BestKeeper, and NormFinder (Table 2), nonetheless, there also a partial consistency was noted. As shown in Table 2, the stable combination of reference genes for temperature treatments was HIS and 18SrRNA, whereas EF1 γ and PMP-2 were the most unstable reference genes in the results of the four statistical algorithms. In the analysis of the developmental stages, the Δ Ct method and NormFinder represented that 18SrRNA and EF1 γ were the most stable genes. Based on BestKeeper, 18SrRNA and Actin were the appropriate reference genes (Table 2).

Therefore, when diverse methods are used, a comprehensive ranking must be made based on the results of different methods. In the latest research, in order to overcome the differences in the results of different algorithms and obtain the final ranking, the web-based analysis tool RefFinder was used. RefFinder obtained the comprehensive overall ranking based on the Geomean of the ranking values from above-mentioned algorithms ⁵⁶. However, one disadvantage of RefFinder is that the results of these algorithms are not weighted according to the unavailability of their cut-offs and appropriate weights ⁵⁷.

Based on the results from the comprehensive analysis using the five algorithms, HIS and 18SrRNA, EF1 γ and 18SrRNA are pairs of the reliable combination, which will facilitate the molecular mechanisms conferring functional and development genomics studies of the nematode in future studies.

Further, according to the results of gene expression studies, we found that traditional reference genes are not always stable and consistent with earlier studies. Many studies have shown that some common reference genes have different expression stability under different experimental conditions^{52,58-61}. This study further emphasizes the shortcomings of simple use of commonly reference genes without a comprehensive evaluation (Table 2; Fig. 4). For example, the commonly used internal reference gene ACT has unstable expression under different temperature treatments and different developmental stages, especially different temperature treatments. Similarly, HIS gene expression is stable under different temperature treatments, but extremely unstable at different developmental stages (Table 2; Fig. 2). These data indicate that under different conditions, the expression levels of traditional reference genes may vary significantly, which requires that all reference genes should be validated.

Conclusions

Under a series of experimental conditions, we identified suitable and reliable reference genes for the normalization of gene expression in *B. xylophilus* in conclusion, 18SrRNA and HIS can give stable gene expressions under temperatures while, EF1 γ and 18SrRNA were found a good selection as reference gene in expression studies across different developmental stages. This study identified several suitable internal reference genes for the standardized RT-qPCR analysis of pine wood nematode, which will contribute to further explorations of molecular mechanism and functional genomics of pine wood nematode with pest control implications.

Declarations

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Author contributions

Conceptualization, J.F. and L.R.; Methodology, Y.L., J.Z., H.Z., and F.A.; Formal analysis, Y.L. and J.Z.; Investigation, Y.L. and H.Z.; Resources, Y.L. J.Z. J.F. and L.R.; Data curation, Y.L., J.Z.; Writing—original draft preparation, Y.L., J.Z.; Writing—review and editing, Y.L., J.Z., H.Z., F.A., J.F. and L.R.; Supervision, J.F. and L.R.; Funding acquisition, J.F. and L.R. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

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Figures

Figure 1

Amplification specificity of primers in RT-PCR and qRT-PCR. A) Single amplicon of the desired size for each candidate reference gene was visualized on a 2% agarose gel and B) melting curves of the eight candidate reference genes. M, marker.

Figure 2

Expression profiles of the eight candidate reference genes in different experimental conditions. A) Different temperature treatment; B) Different developmental stages. The expression levels of the candidate reference genes are shown as Ct values. The box represents the 75th to 25th percentiles and the line in the box represents the median. The whiskers on each box represent the minimum and maximum Ct values

Figure 3

Average expression stability values (MV) of the candidate reference genes calculated by geNorm at (A) different temperature treatment and (B) different developmental stages.

A

B

Figure 4

Expression stability of the candidate reference genes determined by RefFinder analysis. The average expression stability of the reference genes was calculated by the Geomean method of RefFinder. A lower Geomean value indicates more stable expression. A) different temperature treatment and B) different developmental stages.

Figure 5

Pairwise variations (V) of the expression stability of candidate reference genes calculated by geNorm in two experimental sets. Pairwise variations ($V_n/n + 1$) were analyzed to determine the optimal number of the reference genes for normalizing RT-qPCR data.

Figure 6

Relative expression levels of HSP90 under different temperature treatment. The relative mRNA expression levels of HSP90 were normalized to the most suited (A, 18S rRNA and HIS) and the least suited (B, EF1 α and PMP-2) reference genes, respectively. Values are means $\pm$ SE. Different letters indicate significant differences ($p < 0.05$, one-way analysis of variance followed by LSD Multiple Comparison).

Figure 7

Relative expression levels of AK in different developmental stages. The relative mRNA expression levels of AK were normalized to the most suited (A, EF1 γ and 18SrRNA) and the least suited (B, TUB and HIS) reference genes, respectively. Values are means SE. Different letters indicate significant differences ($p < 0.05$, one-way analysis of variance followed by LSD Multiple Comparison)

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