Selection and validation of reference genes for RT-qPCR analysis in Litchi chinensis Sonn. pericarp

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Selection and validation of reference genes for RT-qPCR analysis in *Litchi chinensis* Sonn. pericarp

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Abstract

Background: Quantitative real time PCR (qRT-PCR) is an important tool for gene expression analysis and function identification. Suitable reference genes are the basis of accurate and reliable qPCR results. Litchi (*Litchi chinensis* Sonn.) is a commercially important tropical and subtropical fruit crop, rapid pericarp browning is the major negative impact on the industry. Reference gene validation would help screen for genes involved in the browning mechanism.

Results: In this study, fifteen new candidate reference genes, identified with transcriptome data, were assessed to determine stable reference genes for qRT-PCR analysis of litchi pericarps from different varieties, with differing postharvest storage, and under pathogenic inoculation. Ct values, Genorm, Normfind, and Reffinder algorithms, were used to identify the most stable genes. The results showed that *GAGA-25* was the most stable gene for comparing different varieties of fresh pericarp, *HDAC9* was the most stable gene for postharvest pericarp, *STAM* was the most stable for inoculated pericarp. Among the candidate reference genes, *GAGA-25* was the most stable reference gene across the complete sample set.

Conclusion: This study evaluated reference gene stability for qRT-PCR with litchi pericarp. This work supplies a foundation for qPCR use in future gene function and molecular mechanism studies of litchi pericarp browning.

Keywords: *Litchi chinensis* Sonn., Pericarp, Reference genes, RT-qPCR, Gene validation

Background

Gene expression is a significant approach for identifying gene function and analyzing molecular mechanism. Real-time PCR (qPCR) is a precise, stable, and sensitive technique that uses fluorescence signal accumulation to monitor gene expression. Gene expression analysis with qPCR requires strict control and the initial sample amount is needed to evaluate results. Even when the whole process, including sample selection and preparation, RNA extraction, reverse transcription, and qPCR, are strictly performed, errors are hard to avoid [6]. Selection of proper internal reference genes could mitigate problems [8]. Reference genes are used to correct data for template quantity variance. The expression of

a suitable reference gene should be constant with different experiment conditions and at different points of time. Hence, evaluation and selection of stable reference genes is necessary. For most plant varieties, stable reference genes, such as *Actin*, *GAPDH*, *UBQ*, *RPII*, or *18s rRNA*, have been selected [3,10,12,13, 32].

However, multiple studies have shown that the optimal reference gene is not a constant, as it depends on cultivar, tissue, or experimental conditions used [7, 24, 35, 37]. Suitable reference genes for each experimental set should be identified independently. In grapevine, reference genes have been evaluated for different varieties [31], developmental stages [14], and physiological conditions [4], such as biotic and abiotic stress. This need to optimize reference genes for different conditions arises in studies of other plants, such as rice [18, 30, 45], potato [27,40], apple [5,21], and strawberry [12].

Litchi chinensis Sonn. is a fruit tree which grows in tropical and subtropical areas and is mainly cultivated in southern China. It produces a precious, juicy fruit with delicious taste and brilliant color. Litchi is mainly consumed fresh and pericarp browning occurs 2–3 days after picking [44]. The short shelf-life seriously affects its trade value and is the most important limitation of the litchi industry. Molecular research on fruit postharvest, including related gene screening and gene function analysis, plays a positive role in solving the litchi browning problem. In litchi, Zhong et al [50] studied the stability of seven common reference genes in different experiment sets. The most suitable reference genes were selected using 78 samples, including preharvest pericarp from different varieties, pericarp samples treated with 1-naphthylacetic acid, pericarp samples with shading and girdling plus defoliation treatments, ovaries at different developmental stages, fruitlets, and pericarp tissue samples. However, reference genes for mature fruit pericarp and postharvest pericarp were not assessed. Reference gene research in pericarp would be a basis for efficient gene function analysis.

In this research, we used litchi transcriptome data [38] to select fifteen stably expressed genes. Four statistical algorithms, Ct value, geNorm, NormFinder, and refFinder, were used to evaluate reference genes for pericarps after different storage times and under *Peronophythora litchii* inoculation. Further, the disease gene *PR5* was assessed to verify the stability of reference genes. This study aimed to identify stable reference genes appropriate for transcript normalization in different pericarps, tissues, and under various treatments. This work will provide a basis for further research on molecular mechanisms of litchi fruit postharvest browning and fruit pathogenesis.

Methods

Plant Materials and Treatments

For reference gene expression, litchi cultivars ‘Feizixiao’, ‘Xinqiumili’, ‘Dadingxiang’, ‘Ziniangxi’, and ‘Wuheli’ were selected from orchards in Haikou, China (19°44′N; 110°11′E). ‘Heiye’ and ‘Guiwei’ were selected from an orchard in Guangzhou, China (23°09′N; 113°22′E). The fresh pericarps of all varieties were collected, separately. Fresh fruits were stored at 25°C, then pericarps were harvested after 1 and 3 days of storage for ‘Ziniangxi’, ‘Wuheli’, ‘Heiye’ and ‘Guiwei’ and after 6 days for ‘Ziniangxi’. Fresh ‘Heiye’ and ‘Guiwei’ fruit were inoculated with *P. litchii* and pericarps were collected at 0 h and 6 h after inoculation. All samples were immediately frozen in liquid nitrogen and stored at -80°C.

Total RNA Isolation and cDNA Synthesis

Total RNA was extracted from samples using a Plant RNA Kit (Aidlab, China). RNA concentration and purity were determined by NanoDrop 2000 (Thermo Scientific, USA) and 1% agarose gel, respectively. For first-stand cDNA synthesis, 1 μg of total RNA was used with the PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Japan) in accordance with manufacturer instructions. For RT-qPCR studies, cDNA products were diluted with nuclease-free water at a 1:10 ratio.

Primer Design and PCR Amplification Efficiency

Fifteen stably expressed genes were selected from litchi transcriptome data [37]. Amplification primers for real-time PCR were designed using Beacon Designer 7.91 software (USA). Primer specificity using 'feizixiao' litchi cDNA template was tested by visualizing PCR bands and analyzing qPCR melting curves. The PCR amplification efficiency (E) and the correlation coefficient (R^2) were determined using standard curves generated from results with five-fold serial dilutions (1:1–1:625) of cDNA. The amplification efficiency was calculated using the equation: $E\ (\%) = (10^{-(1/slope)}-1) \times 100$ (da Costa et al., 2015).

Real-Time Quantitative PCR

Real-time amplification reactions were carried out in 384-well plates using TB Green Premix Ex Taq (TaKaRa, Japan) with the QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific, USA). Reactions were performed in a total volume of 10 μL comprised of 0.2 μL each primer, 5 μL TB Green Premix Ex Taq, 1 μL diluted cDNA, and 3.6 μL ddH_2O. The amplification program was: 95°C for 5 min and 40 cycles of 95°C for 10 s and 55°C for 20 s. The melting curve was analyzed at 65–95°C. All RT-qPCR reactions were carried out in biological triplicates with three technical replicates.

Data Statistics

The expression stability of candidate genes across samples was statistically analyzed using geNorm [42], NormFinder [2], and RefFinder [33] programs, in accordance with their instruction manuals. For geNorm analysis, based on the average pairwise variation (V) between all genes, the expression stability value (M) of each candidate reference gene was calculated. For NormFinder analysis, an ANOVA-based model of each candidate reference gene was used to calculate the expression stability value from intergroup and intragroup variation, in which the gene with the lowest value had the most stable expression. For RefFinder analysis, a comprehensive ranking was generated with the data from delta-Ct values, GeNorm (M-values), and NormFinder (stability values).

Validation of Reference Gene Stability

To validate the reliability of the optimal qPCR reference genes, the relative expression level of the resistance gene *PR5* was used. The relative expression of *PR5* in 'heiye' and 'guiwei' pericarp inoculated with *P. litchii* was calculated from RT-qPCR using the two most stable and the most unstable reference genes. The relative expression data was calculated by the $2^{-\triangle\triangle Ct}$ method, and three technical replicates were performed for each sample.

Results

Assessment of primer specificity and PCR amplification efficiency

Specific primers were designed for each target gene. In PCR and qPCR, all amplification products showed a single target-sized band in gel electrophoresis and a single melting curve peak (Additional file

1; Fig. 1). Amplification efficiencies and R^2 values were calculated from slopes of standard curves. The qPCR amplification efficiencies of candidate genes ranged from 98% to 110%, and R^2 ranged from 0.983 to 0.997 (Table 1). These primers were deemed suitable for qPCR analysis.

Expression stability of candidate reference genes

Expression stability of 15 candidate reference genes was established via qPCR Ct values with 16 samples, which were divided into four experimental treatment groups. The Ct value of all candidate genes varied from 20.71 to 38.22 in all test samples (Fig. 2). *CDC 40* had the lowest gene expression and the highest Ct value (38.22). *RUB 1*, *STAM*, and *pbP* had higher gene expression and Ct values ranging from 22.72 to 23.98. Among the various individual candidate reference genes, *CDC 40* showed the most abundant expression with the largest SD value, while *RFU1*, *HDAC9*, *UPF3*, *HLM2B*, and *GAGA-25* showed the most stable expression with lower SD values. Across different experimental sets, Ct values for all genes differed. This result suggested that the selected genes had variable expression levels with different samples, and it would be indispensable to evaluate suitable reference genes for each experimental condition.

GeNorm Analysis

GeNorm evaluates candidate gene stability, identifying the most suitable gene pairs by pairwise variation (*V*). The *V* value of usable gene pairs should below 0.15. Based on geNorm analysis, reference gene pairs were identified for specific experimental treatments and the complete sample set (Fig. 3). *RFU1*/*GAGA-25* was the most stable reference gene pair for fresh pericarp with an M value of 0.095. *GAGA-25*/ *RUB1* was the most stable reference gene pair for post-storage pericarp with an M value of 0.131. *RFU1*/ *STAM* was the most stable reference gene pair for inoculated pericarp with an M value of 0.076. *RFU1*/*GAGA-25* was the most stable reference gene combination for the complete sample set with an M value of 0.123. The most unstable gene pair varied for different experimental groups. From the M values, *CDC40* was the most unstable gene for fresh pericarp and the complete sample set. *RUB1* was the most unstable gene for post-storage pericarp. *CCR4-NOT-TCS* was the most unstable gene for inoculated pericarp.

NormFinder Analysis

The NormFinder program selects reference genes by ranking candidate genes on the basis of a stability value. More stable genes possess higher stability values. NormFinder results are shown in Table 3. *GAGA-25* was the most stable gene with pericarp and postharvest pericarp, *STAM* was the most stable gene for inoculated pericarp, and *RFU1* was the most stable gene over all samples. In addition, NormFinder can suggest stable gene pairs. The most stable reference gene pair was *GAGA-25* and *UPF3* for fresh pericarp, *pbP* and *EF-hand* for postharvest pericarp, *STAM* and *GAGA-25* for inoculated pericarp, and *CKD5* and *UPF3* for the complete sample set.

RefFinder analysis

Because gene ranking resulting from Ct value, GeNorm, and NormFinder analyses differed, RefFinder analysis was used to provide a comprehensive evaluation. On the basis of the above results, RefFinder analyzed stability and ranked reference genes. The comprehensive ranking is shown in Table 4. The gene ranking for various experimental sets differed. The five most stable genes were *UFP3*, *E2F-4A*, *STAM*, *RUB1*, *HDAC9*, and *GAGA-25*. For pericarp, *RUB1* and *GAGA-25* displayed the most stability, while *HDAC9* and *STAM* displayed the most stability for the postharvest and inoculated groups, respectively.

GAGA-25 was the most stable reference gene for the complete sample set.

Reference Gene Validation

Plants respond to pathogens with up- or down-regulated expression of resistance genes. To validate the selected reference genes, the expression of *PR5* gene was analyzed in 'Heiye' and 'Guiwei' pericarp that were inoculated with *P. litchii.* In accordance with the comprehensive RefFinder analysis, two sets of reference genes were selected. The most stable candidate reference genes were *STAM* and *E2F-4A* and the least stable gene was *CCR4-NOT-TCS*. *PR5* expression in 'Guiwei' pericarp at 0 min post-inoculation was normalized as '1', and $2^{-\Delta\Delta Ct}$ values were used to calculate the relative expression in other treatment samples. As shown in Figure 4, when the most stable reference genes (*STAM* and *E2F-4A*) were used for normalization, the relative *PR5* expression in *P. litchii* inoculated 'Guiwei' pericarp gradually increased for 180 min and was relatively lower in 'Heiye' pericarp at 180 min. The relative expression was higher in 'Guiwei' pericarp than in 'Heiye', which is consistent with transcriptome data. However, when the least stable reference gene *CCR4-NOT-TCS* was used for normalization, the relative expression of *PR5* gene was different, especially at 180 mins, and the relative expression was lower in 'Guiwei' than in 'Heiye'.

Discussion

Pericarp browning is one of the most significant limiting factors of the litchi industry. There are multiple causes of pericarp browning: polyphenol oxidase reaction [19, 26, 36, 48], pigment degradation [49], water loss [34], energy reduction [46], and post-harvest pathogen invasion [44, 47]. Enzymatic and non-enzymatic pericarp browning severely inhibits litchi transportation, especially long-term transportation. Several mitigation methods have been developed, such as SO_2 fumigation [20], controlled atmosphere treatments [41], radiation in combination with low temperature storage [28], and dip treatments [1,22, 23]. However, a long-term and effective method remains lacking. In molecular biology research, several genes have been cloned that have a confirmed relationship with browning, such as *LcGOX2* [17], *LcAsr* [25], *LcPPO* [43], and *ADE/LAC* [11]. But direct evidence about gene functions remains lacking. With the development of genomic data and transcriptome data, additional relevant genes will be verified. The selection of novel reference genes for pericarp sets in this study could help elucidate post-harvest related gene functions and pathogenic mechanisms. For the reference genes of fruit storage research, 'Ziniangxi', 'Wuheli', 'Heiye' and 'Guiwei' were selected for their storage tolerance or more rapid pericarp browning. 'Ziniangxi' pericarps were studied after half or whole of the pericarp had browned at 3 and 6 days after postharvest storage, respectively. 'Wuheli', 'Heiye', and 'Guiwei' pericarps were analyzed after the entire pericarp had browned after 3 days of postharvest storage.

qPCR was used to effectively analyze plant gene expression. Suitable reference genes were strictly assessed on the basis of our experiment set. In this study, we selected 15 stable expression genes indicated by transcriptome data and analyzed their expression level. All genes displayed different expression patterns in pericarps, and different suitable reference genes were selected for different experiment treatments.

Many typical reference genes such as, *UBQ*, *Actin*, *RPII*, and *CYP2,* were isolated homologous sequences in plants [9, 16, 21]. In litchi, Zhong et al [50] reported the first litchi reference gene selection study.

Several typical reference genes, including *ACTIN*, *GAPDH*, *EF-1α*, *UBQ*, *TUA*, *TUB*, and RPII, were assessed for various tissues, varieties, treatments, and developmental stages, including pericarp development. But those reference genes and gene combinations were not suitable for this experimental treatment. Suitable reference genes vary for different experimental sets. In this study, we investigated genes with low variable coefficients in transcriptome data. Pericarps under different treatments were assessed using 15 candidate reference genes. The results showed that none of the genes were uniformly expressed across different treatments. The suitable reference gene changed depending on the treatment. The results demonstrate the necessity to assess reference genes for specific experiments.

Ct value comparisons, Genorm, Normfinder, and Reffinder are common methods and software for assessing reference gene stability. This study assessed 15 candidate reference genes using Ct value, geNorm, and NormFinder. The most stable reference gene varied for Ct value (Fig. 2), geNorm (Fig. 3), and NormFinder software (Table 2) analyses, though a consensus group of five relatively stable reference genes were repeatedly identified. The least stable reference gene was consistently ranked across analyses. Using different software with various calculation methods, candidate reference gene rankings were somewhat variable. Inconsistent results between software analyses also appear with other plants [15,24]. Thus, we used refFinder for comprehensive analysis of multiple software results and reference gene ranking (Table 3). The use of multiple software analyses to assess reference genes allows for better results [29, 39]. In addition, although only one reference gene will be identified as the best for analyzing a specific sample, combinations of two or three reference genes should be used to attain the most reliable results.

Conclusion

In this study, we selected 15 candidate reference genes indicated by transcriptome data to investigate gene expression stability. Stable reference genes were identified for different experimental conditions using Ct values and geNorm, NormFinder, and refFinder software. This is the first report of new reference gene stability screening and validation in litchi. This work reinforces the importance of establishing proper reference genes for specific experimental conditions. The results also highlight the idea that transcriptome sequencing can inform screens for reliable reference genes. The identified reference genes will facilitate future gene expression and functional analysis studies.

Additional files

Additional file 1. The sequences of 15 candidate genes.

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Not applicable.

Authors' contributions

Fang Li and Jiabao Wang designed the experiments. Fang Li, Jiali Men, Huanling Li, Guo Wang and Shujun Wang performed the experiments. Fang Li, Jiabao Wang and Jinhua Sun analyzed the data, Fang Li and Jiabao Wang wrote the paper. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets supporting the conclusions and description of a complete protocol are included within the article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors have consented for publication.

Competing interests

The authors declare that they have no competing interests.

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Figures

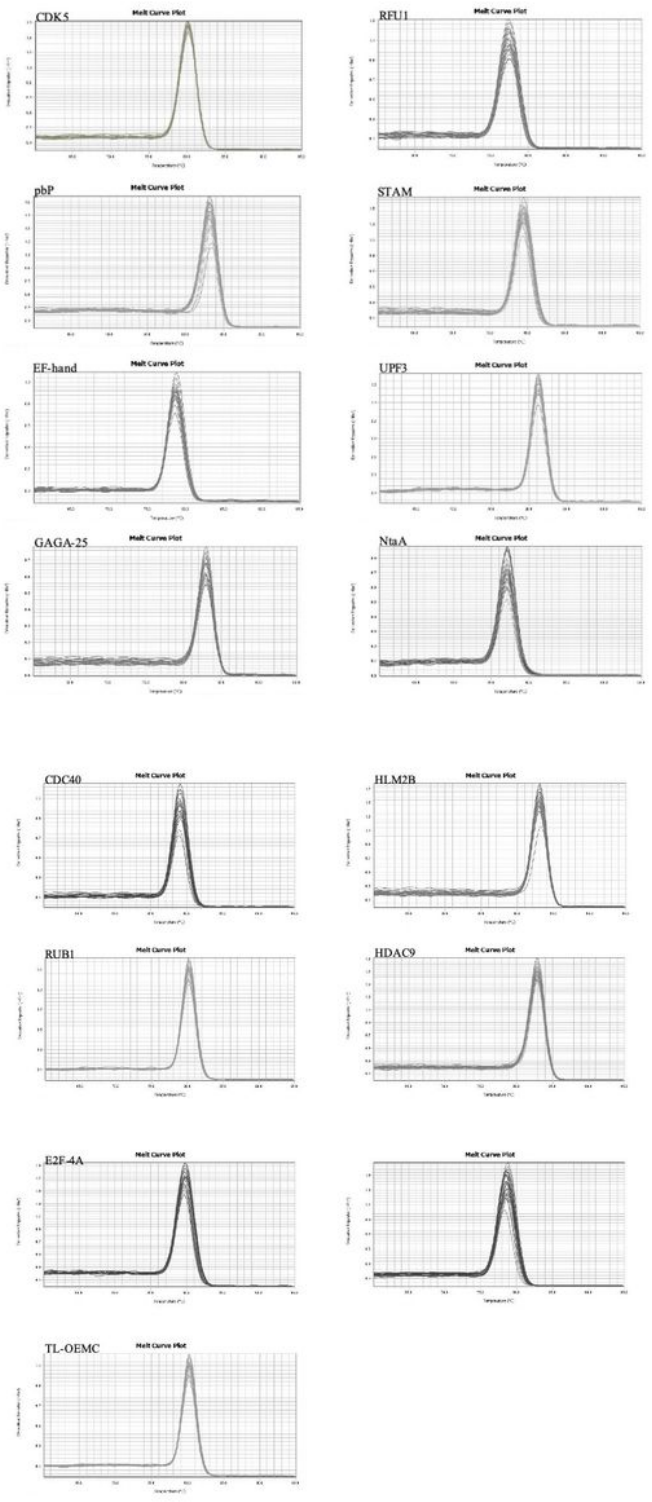

Figure 1

Melting curves of 15 reference genes.

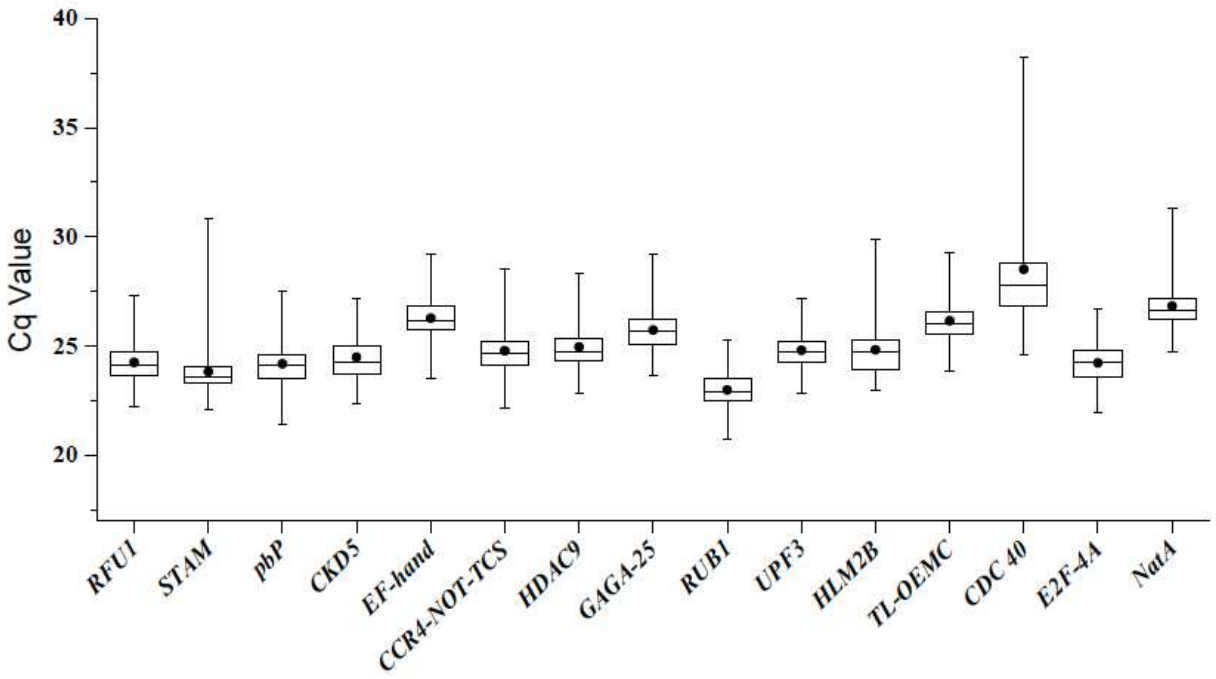

Figure 2

Ct value of candidate reference genes. Boxplots represent the 25th and 75th percentiles. whisker up-limit and floor-limit show the maximum and minimum values. Plot represent the mean value. The spot in the box depicts the median.

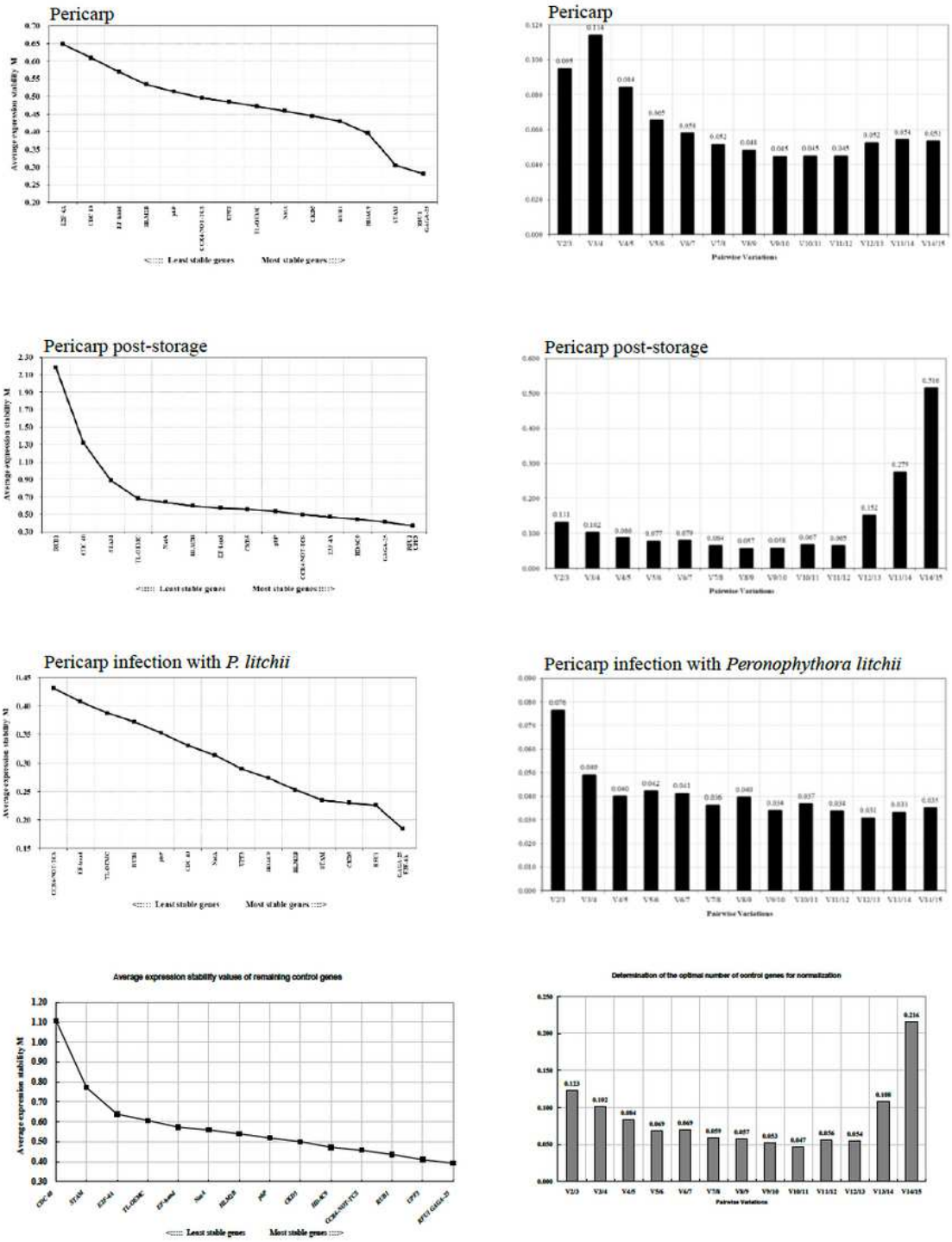

Figure 3

Expression stability value (M) of candidate reference genes calculated by geNorm. A lower value of average expression stability (M) indicates a more stable expression level.

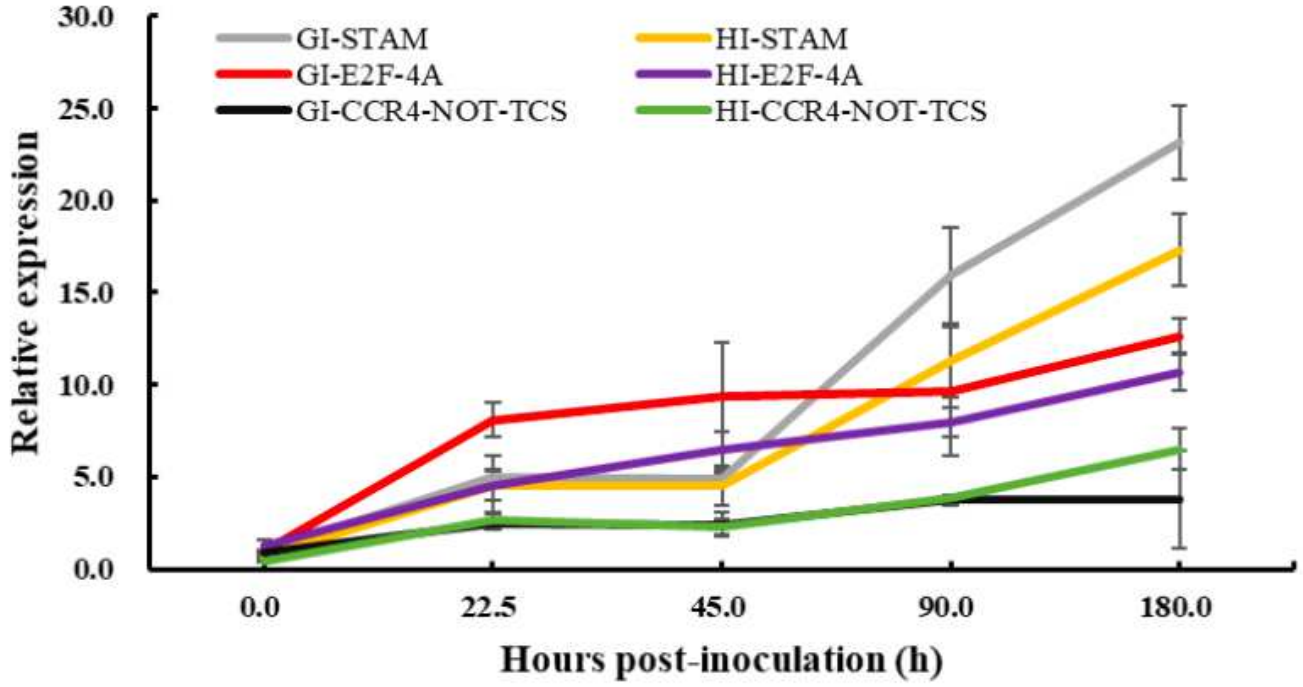

Figure 4

PR5 gene relative expression level using stable reference genes (STAM and E2F-4A) and least stable reference gene (CCR4-NOT-TCS). GI represents ‘guiwei’ pericarp inoculated with P. litchii, HI represents pericarp inoculated with P. litchii. Error bars show the mean standard error calculated with three biological replicates.

Supplementary Files

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- Additionalfile1.pdf