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

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Abstract

Real-time reverse transcription quantitative PCR (RT-qPCR) is an important tool for gene expression analysis. Suitable reference genes are the basis of accurate and reliable RT-qPCR results. Litchi (*Litchi chinensis* Sonn.) is a commercially important tropical and subtropical fruit, but rapid pericarp browning is a substantial negative impact on its commercial use. Reference gene validation could help in the screening for genes involved in the browning mechanism. We assessed 15 new candidate reference genes from litchi transcriptome to determine stable reference genes for RT-qPCR analysis of pericarps from different cultivars, with differing postharvest storage, and under pathogenic stress. *Ct* values, *geNorm*, *Normfinder*, and *RefFinder* algorithms, were used to identify genes with the most stable transcription. *GAGA-25* was the gene with the most stable transcription for comparing different varieties of the fresh pericarp. *HDAC9* was the gene with the most stable transcription for postharvest pericarp. *STAM* was the gene with the most stable transcription for inoculated pericarp. Of the candidate reference genes, *GAGA-25* was the most stable reference gene across the complete sample set. This study evaluated reference gene stability for RT-qPCR in litchi pericarp. This work provides a foundation for using qPCR to study gene function and molecular mechanism studies of litchi pericarp browning.

Keywords: *Litchi chinensis* Sonn., pericarp, reference genes, RT-qPCR, gene validation.

Introduction

Real-time reverse transcription quantitative PCR (RT-qPCR) is a common method for evaluating gene function (Mimida *et al.* 2015), pathogen detection (Lukianova *et al.* 2021), and mutation screening (D'Agostino *et al.* 2015). It uses a fluorescence signal to reflect the gene expression and it is precise, stable, and sensitive (Maroufi 2016). However, working with the same cell number for RT-qPCR is almost impossible due to variation in the biological material and technical difficulties such as storage method, RNA extraction, reverse transcription, PCR, and efficiency (Bustin *et al.* 2009). The use of proper internal reference genes can mitigate these problems and this is the most common method for data normalization (Bustin *et al.* 2009, Chervoneva *et al.* 2010), under strictly controlled

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Abbreviations: CCR4-NOT-TCS - CCR4-NOT transcription complex subunit 11; CDC40 - cell division cycle 40 homolog; CDK5 - CDK5 regulatory subunit associated protein 3; Ct - threshold cycle; E2F-4A - Eukaryotic initiation factor 4A-14; EF-hand - EF-hand calcium binding domain; GAGA-25 - GATA transcription factor 25; HDAC9 - histone deacetylase 9; HLM2B - histone-lysine_N-methyltransferase 2B; NtaA - N(alpha)-acetyltransferase 16, NatA auxiliary; pbP - peroxisome biogenesis protein 22-like; RFU1 - RING finger ubiquitin ligase; RT-qPCR - reverse transcription qPCR; RUB1 - ubiquitin-NEDD8-like protein RUB1; STAM - Stam binding; TL-OEMC - translocon at the outer membrane of chloroplasts 64; UPF3 - UPF3 regulator of nonsense transcripts homolog UPF3; V - variation.
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conditions. The selection of reliable reference genes is indispensable. Ideal reference gene expression should be constant within tissues, organs, development stages, or experimental treatments. No single gene is able to meet all of these criteria of an ideal reference gene (Chandna *et al.* 2012, Li *et al.* 2017, Sun *et al.* 2019a, Duan *et al.* 2020), it is necessary to select and evaluate stable reference genes for different conditions. In most plant species and cultivars, useful reference genes have been selected (Schmidt *et al.* 2010, Gantasala *et al.* 2013, Bevitori *et al.* 2014, De Andrade *et al.* 2017), and these genes vary according to the material or treatments. Suitable reference genes for each experimental set should be identified independently.

Litchi chinensis Sonn. is a fruit tree that grows in tropical and subtropical areas and it is also cultivated in south China. It produces colourful and tasty fruits. Litchi is mainly consumed fresh since pericarp browning occurs rapidly (2 - 3 d) after harvest (Wang *et al.* 2010). The short shelf life reduces its trade value and it is the biggest limitation of the litchi market. Molecular research on fruit postharvest, including related gene screening and gene function analysis, has helped to solve the litchi browning problem. Zhong *et al.* (2011) studied the stability of seven common reference genes in different experiments. The most suitable reference genes were selected from 78 samples. These included preharvest pericarp, 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. Reference genes for mature fruit pericarp and postharvest pericarp were not assessed. Reference gene research in pericarp could provide a basis for gene function analysis.

In this study, we used litchi transcriptome data to select 15 expressed genes. Four statistical algorithms, including *Ct* value, *geNorm*, *Normfinder*, and *RefFinder*, were used to evaluate reference genes for pericarps after different storage times and after *Peronophythora litchii* inoculation. The pathogenesis-related gene *PR5* was assessed to verify the stability of the reference genes. The goal was to identify stable reference genes appropriate for transcript normalization in different pericarps and tissues under various treatments. This work can provide a basis for advanced research on the molecular mechanisms of litchi fruit postharvest browning and fruit pathogenesis.

Materials and methods

Plants and treatments: For reference gene expression, pericarps from mature fruits were sampled from litchi cultivars Feizixiao, Xinqiumili, Dadingxiang, Ziniangxi, Wuheli, Heiye, and Guiwei. Fresh fruits were collected and stored at 25 °C. Pericarps were harvested after 1 and 3 d of storage from 7 cultivars, and after 6 d from Ziniangxi. Fresh fruits of Heiye and Guiwei were immersed in a sporangium suspension of *Peronophythora litchii* and pericarps were collected at 0 and 6 h after inoculation. For gene expression analysis, fresh pericarps of all cultivars were collected separately. All of the pericarp samples were immediately frozen in liquid nitrogen and stored at -80 °C. All experiments were repeated in triplicate independently.

Total RNA isolation and cDNA synthesis: Total RNA was extracted from pericarp samples using a Plant RNA kit (*Aidlab*, Beijing, China). RNA concentration and purity were determined by *NanoDrop 2000* (*Thermo Fisher Scientific*, MA, USA) and electrophoretic separation on 1 % (m/v) agarose gel, respectively. For first-strand cDNA synthesis, 1 µg of total RNA was used with the *PrimeScript 1st Strand cDNA Synthesis* kit (*Thermo Fisher Scientific*) in accordance with manufacturer instructions. For RT-qPCR studies, cDNA products were diluted with nuclease-free water at a 1:10 ratio.

Primer design: Candidate genes were collected from 3 transcriptome results. The first was the transcriptome of 19 Feizixiao tissues and organs, including annual root, bud, autumn shoot, leaf, stalk, axillary bud, inflorescence, anther of male and female flowers, ovaries of male and female flowers, the pericarp of preharvest and postharvest fruits, the pulp of preharvest and postharvest fruits, seed, testa, embryonic calli, and somatic embryos (unpublished data). The second was the transcriptome of 16 Heiye and Guiwei pericarps inoculated with *P. litchii* (Sun *et al.* 2019b). The third was the transcriptome of 17 Xinqiumili and Dadingxiang embryogenic calli which were cultured in a somatic embryo induction medium and held at different development stages (unpublished data). For the candidate reference genes selection, the distribution of Fragments Per Kilobase Million (FPKM) was analyzed based on 52 transcriptomes data, and a set of genes that were situated in the maximum scale range were used for analysis. The variable coefficient and SD value of FPKM were calculated and 15 genes that had variable coefficients within 30 %, and SD values less than 2 in all 3 transcriptomes were selected. Primers for qPCR were designed using the *Beacon Designer 7.91* software (San Francisco**,** USA).

qPCR and PCR amplification efficiency: qPCR reactions were carried out in 384-well plates using the *TB Green Premix Ex Taq* (*Takara Bio*, Kusatsu, Japan) and the *QuantStudio 6 Flex* real-time PCR system (*Thermo Fisher Scientific*, MA**,** USA). Each 10-mm^3 reaction solution contained 0.2 mm^3 of each primer, 5 mm^3 of *TB Green Premix Ex Taq*, 1 mm^3 of diluted cDNA, and 3.6 mm^3 of ddH_2O. The amplification program was as follows: 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 three biological triplicates with three technical replicates. 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 by results from five-fold serial dilutions (1:1 ~ 1:625) of cDNA. The amplification efficiency was calculated using the equation: $E\ [\%] = (10^{-(1/slope)}-1) \times 100$.

Validation of reference gene stability: To validate the reliability of the optimal qPCR reference genes, the relative expression of the pathogenesis-related 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 one most unstable reference genes. The relative expression data were calculated by the $2^{-\Delta\Delta Ct}$ method and three technical replicates were performed for each sample.

Statistics: The expression stability of candidate genes across samples was statistically analyzed using *geNorm* (Vandesompele *et al.* 2002), *Normfinder* (Andersen *et al.* 2004), and *RefFinder* (Xie *et al.* 2012) programs, in accordance with their instruction manuals. The *geNorm* analysis was based on the average pairwise variation (V) between all genes, and 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 data from *delta-Ct* values, *geNorm* (M-values), and *Normfinder* (stability values).

Results

Specific primers were designed for each target gene. In PCR and RT-qPCR, all amplification products showed a single target-sized band in gel electrophoresis and a single melting curve peak, respectively (Fig. 1 Suppl.). Amplification efficiencies and R^2 values were calculated from the slopes of standard curves. The RT-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 seemed suitable for RT-qPCR analysis.

The expression stability of 15 candidate reference genes was established *via* RT-qPCR *Ct* values corresponding to 16 samples, which were divided into 4 treatment groups. The *Ct* value of all candidate genes varied from 20.71 to 38.22 in all tested samples (Fig. 1). *CDC 40* had the lowest

Table 1. Details of the primers used in this study.

Gene name	Description	Forward primer	Reverse primer	Product length [bp]	R^2	Efficiency [%]
CCR4-NOT-TCS	CCR4-NOT transcription complex subunit 11	TTGTGGTGGA GTTAGCGAAT	GGTAAGGACAT CAACAGCAATC	113	0.996	109.236
CDC40	cell division cycle 40 homolog	TCTCGCTCTC CTGAATAAG	CTCATTGTCTTC TTGGTCTG	142	0.984	104.600
CDK5	CDK5 regulatory subunit associated protein 3	GGATACGGAGT ACAGGAATAGG	GGAGCAACTGA CTGGACTT	129	0.995	110.640
E2F-4A	eukaryotic initiation factor 4A-14	GATGTCCAG CAAGTCTCT	ACCTTCCACTAC GACCTA	88	0.995	105.376
EF-hand	EF-hand calcium binding domain	GCTCCTCCTT CAACTTCAAC	TGCTCCATGTAC TGCTTCT	115	0.990	118.311
GAGA-25	GATA transcription factor 25	CCAAGAGGCA AGACTAGAT	ACCACAGGCAT TACAGAG	130	0.987	106.770
HDAC9	histone deacetylase 9	GGTGCCATAGT TCTTCAATGT	ACCTCCAGTAA CCAGTAACG	138	0.983	109.589
HLM2B	histone-lysine_N-methyltransferase 2B	GTCAACATCCT CCTCACAA	CTTCCACAAGC ATCACAAC	148	0.993	105.003
NtaA	N(alpha)-acetyltransferase 16, NatA auxiliary	CGAGCCTGAT AAGAATTGGT	GAGGTAGTGCT GAAGAAGAAG	82	0.998	98.915
pbP	peroxisome biogenesis protein 22-like	TCACGGTCAA GATGTCCAAT	CCTGGTCAACC TCCAAGTAA	116	0.992	98.221
RFU1	RING finger ubiquitin ligase	GTATAGAAGC TGCACACTTG	TAACTGTCTCC TCCTCCA	106	0.997	109.360
RUB1	ubiquitin-NEDD8-like protein RUB1	GAGGCTTAGAG GAGGAACTATG	ATCCGCTCAAT GGTATCAGT	93	0.994	103.877
STAM	stam binding	TGCCGAGGAG TTATGTCATC	GGAGGTGGAG AAGGTTGATT	87	0.985	101.453
TL-OEMC	translocon at the outer membrane of chloroplasts 64	TCTGTGTCC TTCATAGCC	CTTGTTCCTGG TTGATAGC	139	0.990	105.233
UPF3	UPF3 regulator of nonsense transcripts homolog UPF3	AAGCAGGAAA GGCGTACCAGAA	ACAGTGAAGG TTGAGAAGCAGTTG	124	0.989	99.243

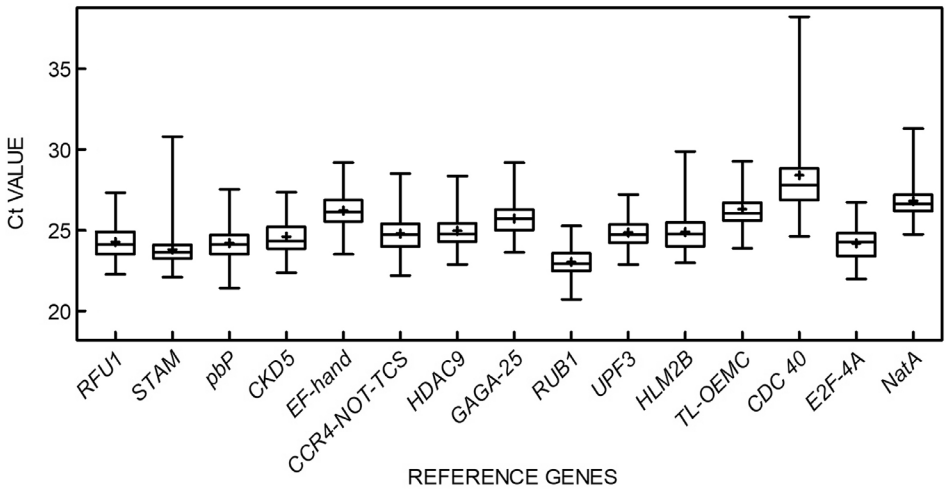

Fig. 1. Ct value of candidate reference genes. Boxplots represent the 25th and 75th percentiles; whisker up-limit and floor-limit show the maximum and minimum values; plot represents the mean value; '+' in the box depicts the median.

Table 2. Ranking of the candidate reference genes and their expression stability values calculated by *NormFinder*.

Pericarp		Pericarp post-harvest		Pericarp infection		Total	
gene	stability	gene	stability	gene	stability	gene	stability
GAGA-25	0.125	*GAGA-25*	0.341	*STAM*	0.079	*UPF3*	0.309
NatA	0.192	*pbP*	0.369	*GAGA-25*	0.107	*GAGA-25*	0.373
RFU1	0.203	*RFU1*	0.382	*CKD5*	0.125	*NatA*	0.374
UPF3	0.210	*EF-hand*	0.414	*E2F-4A*	0.138	*HDAC9*	0.380
STAM	0.282	*UPF3*	0.425	*RFU1*	0.139	*RFU1*	0.386
CCR4-NOT-TCS	0.299	*CKD5*	0.439	*HLM2B*	0.151	*RUB1*	0.406
TL-OEMC	0.299	*HDAC9*	0.461	*HDAC9*	0.160	*pbP*	0.412
CKD5	0.300	*CCR4-NOT-TCS*	0.475	*UPF3*	0.201	*CCR4-NOT-TCS*	0.416
HDAC9	0.304	*E2F-4A*	0.523	*pbP*	0.219	*STAM*	0.423
RUB1	0.305	*HLM2B*	0.588	*CDC 40*	0.220	*TL-OEMC*	0.443
pbP	0.313	*NatA*	0.636	*TL-OEMC*	0.230	*CKD5*	0.446
HLM2B	0.314	*TL-OEMC*	0.752	*RUB1*	0.242	*EF-hand*	0.467
EF-hand	0.405	*STAM*	0.988	*NatA*	0.252	*E2F-4A*	0.475
CDC 40	0.439	*CDC 40*	2.616	*EF-hand*	0.293	*HLM2B*	0.495
E2F-4A	0.502	*RUB1*	4.615	*CCR4-NOT-TCS*	0.330	*CDC 40*	1.277
GAGA-25 and *UPF3*	0.095	*pbP* and *EF-hand*	0.319	*STAM* and *GAGA-25*	0.051	*CKD5* and *UPF3*	0.225

gene expression and the highest *Ct* value (38.22). *RUB1*, *STAM*, and *pbP* had higher gene expression and *Ct* values ranging from 22.72 to 23.98. Across different experimental sets, *Ct* values for all genes differed. This result suggested that the selected genes had variable expressions in different samples and that it would be best to evaluate suitable reference genes for all experimental conditions.

The *geNorm* evaluates candidate gene stability, identifying the most suitable gene pairs by pairwise variation (*V*). The *V* value of usable gene pairs should be below 0.15. Based on *geNorm* analysis, reference gene pairs were identified for specific experimental treatments and the complete sample set (Fig. 2). *RFU1*/*GAGA-25* was the most stable reference gene pair for fresh pericarp with an M value of 0.28. *RFU1*/*UPF3* was the most stable reference gene pair for post-storage pericarp with an M value of 0.37. *GAGA-25*/*E2F-4A* was the most stable reference gene pair for inoculated pericarp with an M value of 0.18. *GAGA-25*/*UPF3* was the most stable reference gene combination for the complete sample set with an M value of 0.54. The most unstable gene pair varied for different experimental groups. From the M values, *E2F-4A* was the most unstable gene for fresh pericarp. *RUB1* was the most unstable gene for post-storage pericarp. *CCR4-NOT-TCS* was the most unstable gene for inoculated pericarp. *CDC40* was the most unstable gene for the complete sample set.

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 2. *GAGA-25* was the gene with the most stable transcription with pericarp

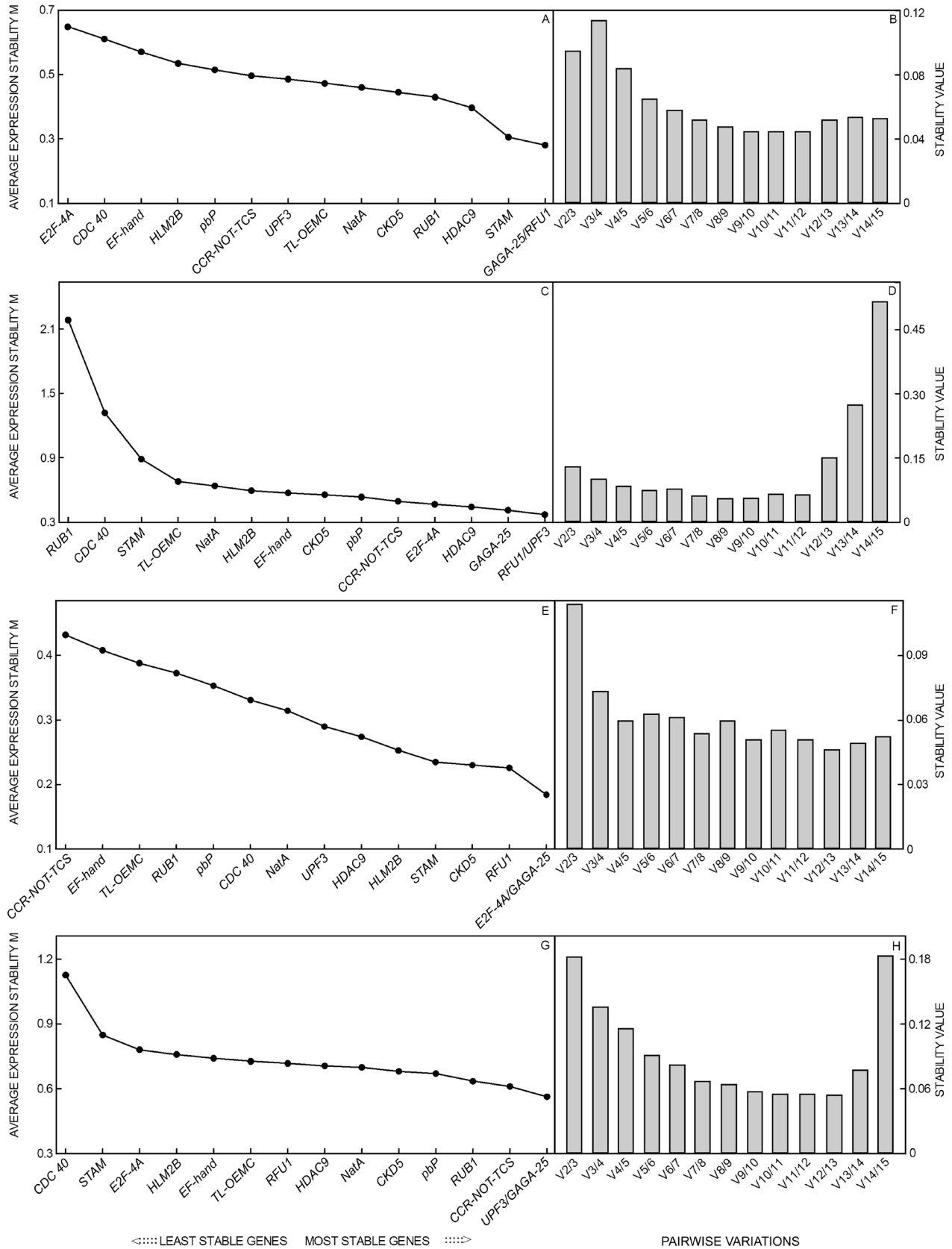

Fig. 2. Expression stability value (M) of candidate reference genes calculated by *geNorm*. *A,B* - fresh pericarps, *C,D* - post-storage pericarps, *E,F* - inoculated pericarps, *G,H* - all samples. A lower value of average expression stability (M) indicates a more stable expression level.

Table 3. Ranking of the candidate reference genes calculated by *RefFinder*.

Ranking	Pericarp	Postharvest	Inoculated	Total
1	*GAGA-25*	*HDAC9*	*STAM*	*UPF3*
2	*RFU1*	*RUB1*	*E2F-4A*	*GAGA-25*
3	*STAM*	*UPF3*	*RFU1*	*NatA*
4	*NatA*	*E2F-4A*	*GAGA-25*	*CCR4-NOT-TCS*
5	*UPF3*	*GAGA-25*	*CKD5*	*HDAC9*
6	*HDAC9*	*RFU1*	*HLM2B*	*RUB1*
7	*RUB1*	*CCR4-NOT-TCS*	*HDAC9*	*RFU1*
8	*TL-OEMC*	*NatA*	*CDC 40*	*pbP*
9	*CKD5*	*CKD5*	*UPF3*	*STAM*
10	*HLM2B*	*HLM2B*	*NatA*	*CKD5*
11	*CCR4-NOT-TCS*	*EF-hand*	*pbP*	*EF-hand*
12	*pbP*	*pbP*	*RUB1*	*TL-OEMC*
13	*EF-hand*	*TL-OEMC*	*TL-OEMC*	*E2F-4A*
14	*CDC 40*	*STAM*	*EF-hand*	*HLM2B*
15	*E2F-4A*	*CDC 40*	*CCR4-NOT-TCS*	*CDC40*

and postharvest pericarp, *STAM* was the gene with the most stable transcription for inoculated pericarp and *UPF3* was the gene with the most stable transcription 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.

Because the gene ranking resulting from *Ct* value, *geNorm*, and *Normfinder* analyses differed, *RefFinder* analysis was used to provide a comprehensive evaluation. Based on the above results, *RefFinder* analyzed stability and ranked reference genes. The comprehensive ranking is shown in Table 3. The gene ranking for various experimental sets differed. The five genes with the most stable transcription were *UPF3*, *E2F-4A*, *STAM*, *RUB1*, *HDAC9*, and *GAGA-25*. For pericarp, *RFU1* and *GAGA-25* displayed the most stability, while *HDAC9* and *STAM* displayed the most stability for the postharvest and inoculated groups, respectively. *UPF3* and *GAGA-25* was the most stable reference gene for the complete sample set.

Plants respond to pathogens with up- or down-regulated expression of resistance genes. To validate the selected reference genes, expression of the *PR5* gene was analyzed in Heiye and Guiwei pericarps that were inoculated with *P. litchii.* In accordance with the comprehensive analysis, two sets of reference genes were selected. The most stable candidate reference genes were *UPF3* and *GAGA-25* 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 time-points. Fig. 3 shows that when the most stable reference genes (*UPF3* and *GAGA-25*) were used for normalization, the relative *PR5* expression trends were similar. It gradually increased within 180 min in Guiwei or Heiye pericarp inoculated *P. litchii*, and the expression changed little in pericarp without inoculation. The relative expression of the *PR5* gene in inoculated pericarp was significantly higher than in pericarp without inoculation. The relative gene expression increased 180 min after inoculation about 5 times in Geiwei pericarp and in Heiye pericarp when *UPF3* and *GAGA-25* were used. However, when the least stable reference gene *CCR4-NOT-TCS* was used for normalization, the relative gene expression of pericarp inoculated slowly increased and had a downward trend in Guiwei pericarp. At 180 min after inoculation, the relative *PR5* expression of inoculated pericarp increased 1-time and 3-times in Guiwei and Heiye pericarps, respectively. A large difference was evident in the change patterns when the least stable reference gene was used for normalization.

Discussion

The RT-qPCR was used to effectively analyze plant gene expression. Suitable reference genes were strictly assessed on the basis of our experimental set. We selected 15 genes expressed in litchi, indicated by transcriptome data, including 54 tissues, organs, and treatments and then analyzed their expression level. All genes displayed different expression patterns in pericarps, and suitable reference genes were selected for different treatments.

Many typical reference genes, such as *UBQ*, *Actin*, *RPII*, and *CYP2,* were identified according to homologous sequences in various plants species (Condori *et al.* 2011, Hossain *et al.* 2019). In litchi, Zhong *et al.* (2011) 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, cultivars, treatments, and developmental stages, including pericarp development. But those reference genes and gene combinations were not suitable for this experimental treatment. Zhong *et al.* (2011) evaluated *ACTIN* as the most suitable reference gene for different cultivars, our

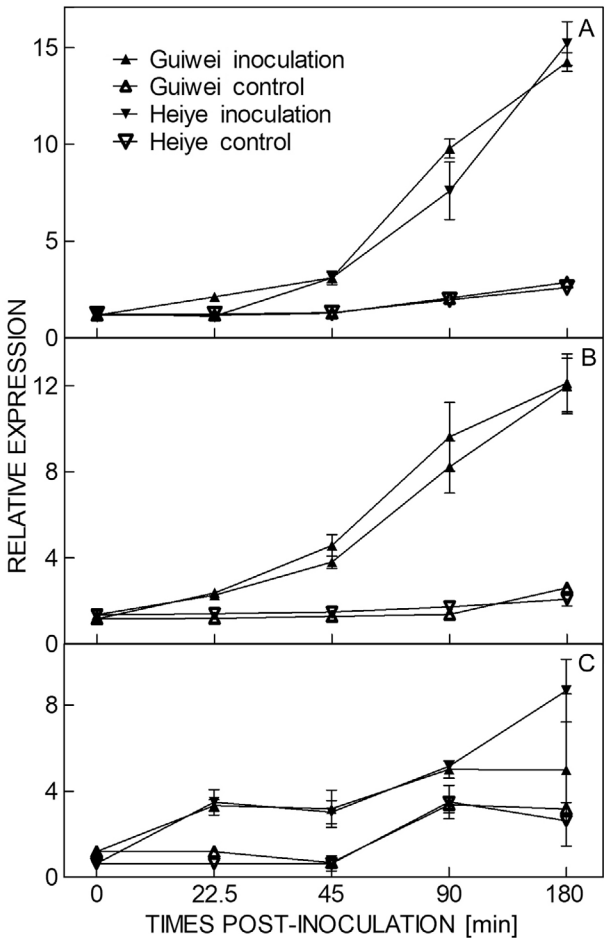

Fig. 3. The *PR5* gene relative expression using stable reference genes (*A* - *UPF3*, *B* - *GAGA-25*) and least stable reference gene (*C* - *CCR4-NOT-TCS*). Guiwei inoculation represents Guiwei pericarp inoculated with *P. litchii*, Heiye inoculation represents pericarp inoculated with *P. litchii*. Guiwei control represents Guiwei pericarp that was not inoculated as control. Heiye control represents Heiye pericarp that was not inoculated and used as the control. Means ± SEs, $n = 3$ biological replicates.

study showed its lower stability than that of *GAGA-25* for pericarp of our litchi cultivars. Reference genes vary in their suitability for different experimental data sets. We investigated genes with low variation coefficients in transcriptome data. Pericarps, collected under different treatments, were assessed using 15 candidate reference genes. None of the genes was uniformly expressed across different treatments. The most suitable reference gene depended on the treatment. These results demonstrate the necessity to assess the suitability of reference genes for specific experiments.

Ct value comparisons, *geNorm*, *Normfinder*, and *RefFinder* are common methods and tools for assessing reference gene stability. We assessed 15 candidate reference genes using *Ct* value, *geNorm*, and *Normfinder*. The most stable reference gene varied for *C*t value (Fig. 1), *geNorm* (Fig. 2), and *Normfinder* (Table 2) analyses, though a consensus group of five relatively stable reference genes were repeatedly identified. The least stable reference gene was consistently ranked lowest across analyses. Using different software programs with various calculation methods, candidate reference gene rankings were variable. Inconsistent results between software analyses have also been reported in other plants (He *et al.* 2016, Li *et al.* 2017). Thus, we used *RefFinder* for a comprehensive analysis of multiple software results and reference gene ranking (Table 3). The use of multiple software analyses to assess reference genes produces better results (Niu *et al.* 2015, Tang *et al.* 2019). In addition, although only one reference gene will be identified as the best for analyzing a specific sample, a combination of two or three reference genes should be used to attain the most reliable results.

Pericarp browning remains one of the most significant limiting factors of the litchi market. There are multiple causes of pericarp browning. These include water loss (Sun *et al.* 2010), polyphenol oxidase reaction (Jiang 2000, Liu *et al.* 2010), pigment degradation (Zhang *et al.* 2004), energy deficiency (Yang *et al.* 2009), and post-harvest pathogen invasion (Wang *et al.* 2010, Ye *et al.* 2016). Enzymatic and non-enzymatic pericarp browning severely limits litchi storage and the possibility of especially long-range transport. Corresponding to the above pericarp browning factors, several mitigation methods have been developed, such as SO_2 fumigation (Kore *et al.* 2014), controlled atmosphere (Tian *et al.* 2005), radiation in combination with low-temperature storage (Mishra *et al.* 2012), and chemical treatments (Kumari *et al.* 2015, Ali *et al.* 2019). However, a long-term and effective method is still needed. The molecular mechanisms of browning have not been systematically studied. Several genes with a confirmed relationship with browning, such as *LcGOX2* (Hu *et al.* 2008), *LcAsr* (Liu *et al.* 2013), *LcPPO* (Wang *et al.* 2014), and *ADE/LAC* (Fang *et al.* 2015) have been cloned, but direct evidence about gene functions remains lacking. The development of litchi genomic data and transcriptome data should verify additional relevant genes. Identification of reference genes of RT-qPCR for pericarp sets in this study may help reveal the functions of the genes related to pericarp browning and resistance genes.

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