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Selection and validation of reference genes for qRT-PCR in cultivated octoploid strawberry

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Abstract

The rapid, reliable, and efficient characteristics of quantitative reverse transcription polymerase chain reaction (qRT-PCR) make it a highly advantageous method for assessing gene expression levels. The identification of stable reference genes is crucial for successful gene expression studies. Cultivated strawberry fruit has been extensively investigated as a model for studying the non-climacteric fruit ripening process. However, more research needs to be conducted on identifying suitable reference genes at different developmental stages of strawberry fruit. We selected the 'Yanli' and 'Chuliandeweidao' cultivars to screen potential reference genes in various tissues, organs, and developmental stages of strawberry fruit. Based on the analysis of high-quality haplotype-resolved genome and transcriptomic FPKM data, *FaADPrf1* (*ADP-ribosylation factor 1*), *FaGAPC2* (*Glyceraldehyde-3-phosphate dehydrogenase*), *FaPPC1* (*Peptidyl-prolyl cis-trans isomerase 1*), and *FaEF1-α* (*Elongation factor 1-alpha*) were selected as candidate reference genes, along with the commonly used *Fa26S rRNA*, for normalization purposes. A qRT-PCR analysis showed 89.21% to 101.51% amplification efficiency for five candidate reference genes, with correlation coefficients (R^2) exceeding 0.99. Reference genes' expression stability was assessed using GeNorm, NormFinder, BestKeeper, and Comparative delta-Ct method. RefFinder analysis determined that *FaGAPC2* and *FaADPrf1* were the most suitable reference genes, considering the results obtained from the abovementioned four methods. The calculated results were validated by studying the expression of *FaMYB10*, *FaUGT1*, and *FaCHS* in different developmental stages of 'Yanli' fruit. This validation confirmed that both *FaGAPC2* and the combination of *FaGAPC2* and *FaADPrf1* could serve as suitable reference genes, with the combination of *FaGAPC2* and *FaADPrf1* being more suitable than the single *FaGAPC2* in certain cases. In summary, we obtained suitable reference genes for research on cultivated strawberry fruit development, which will benefit further study on the ripening of non-climacteric fruits.

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Introduction

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) is a reliable and widely used technique in molecular biology research for quantifying gene expression levels[1]. It offers several advantages over conventional PCR, including high sensitivity, specificity, accuracy, and high throughput capacity[2,3]. The precision of qRT-PCR outcomes relies on numerous elements, such as the stability of reference genes, the cDNA's quality, the efficiency of the cDNA polymerase, and the effectiveness of PCR amplification[4,5]. The consistency of expression levels in reference genes is particularly critical for ensuring qRT-PCR outcomes' reliability[6,7]. To identify appropriate reference genes, researchers have devised numerous techniques. GeNorm, NormFinder, and BestKeeper are software tools that were created utilizing Excel 2003, while the Comparative delta cycle threshold (ΔCt) method represents a conventional computational method[8−11]. Furthermore, RefFinder, an online platform integrating the aforementioned four methodologies, furnishes a ranking system for assessing the stability of candidate gene expression[12].

Housekeeping genes, which play crucial roles in the fundamental life processes of cells, are commonly utilized as reference genes[13,14]. These genes exhibit relatively stable expression levels across different tissues, organs, developmental stages, and under various biotic and abiotic stresses[15,16]. Their expression products are generally essential for maintaining cellular life activities or the cytoskeleton of plant somatic cells[17]. Reference genes such as encoding α, β-tubulin (*TUA* and *TUB*), Actin (*ACT*), Histone B (*H2B*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and elongation factor-1-alpha (*EF1-α*) are commonly selected, along with 18S ribosomal RNA (*18S rRNA*) and 28S ribosomal RNA (*28S rRNA*), for the normalization of qRT-PCR in plants[17−21]. Nonetheless, prior research has provided evidence suggesting the nonexistence of optimal reference genes that demonstrate consistent expression levels in diverse temporal intervals, tissues, and in the presence of various stress-inducing stimuli. This can be attributed to the inherent relativity of gene expression stability, as the expression patterns of genes may exhibit species-specific or even tissue-specific characteristics, thereby challenging the identification of universally stable reference genes[22]. Exposito-Rodriguez et al. found that the expression of some housekeeping genes in tomato was not stable, even at the 6-leaf stage and 7-leaf stage, and from 1 mm to 8 mm in the bud[18]. Therefore, researchers must select the most suitable reference genes based on the specific characteristics of their research materials. Therefore, it is of utmost importance in scientific investigation

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to ascertain the stability of gene expression of internal reference genes under novel experimental circumstances and identify suitable reference genes[23].

Strawberries are widely cultivated and highly favored by consumers worldwide due to their distinctive flavor and rich nutritional content, including minerals, vitamins, and microelements[24,25]. Some research has been conducted on the selection of internal reference genes in strawberry. Zhang et al. analyzed of the expression stability of seven candidate reference genes in different tissues, various stages of fruit development, and under different light quality and low-temperature conditions in cultivated strawberries. The results revealed that the expression of *DBP*, *HISTH4*, *ACTIN1*, and *GAPDH* genes demonstrated greater stability[26]. Amil-Ruiz et al. tested the expression stability of 13 candidate reference genes in different cultivated strawberry varieties, ripening and senescent conditions, as well as under SA and JA treatments, revealing that *FaRIB413*, *FaACTIN*, *FaEF1a*, and *FaGAPDH2* were suitable reference genes. Stress response constitutes a prominent investigation area within the strawberry research field[27]. Galli et al. investigated the expression stability of seven candidate reference genes under different abiotic stress conditions. The results revealed that the *DBP* gene exhibited the most stable expression under drought stress, while *HISTH4* displayed the most stable expression pattern under osmotic and salt stress. On the other hand, *GAPDH* and *18S* exhibited the least stable expression patterns across all conditions[28].

Non-climacteric fruits, such as strawberries, represent an ideal model for studying the ripening process, which differs significantly from that of climacteric fruits like apples, peaches, bananas, and pears, in which ethylene plays a dominant role[24]. Previous studies suggested that abscisic acid (ABA) may influence strawberry fruit ripening by regulating cell wall degradation, anthocyanin biosynthesis, and growth, but the underlying mechanisms remain to be elucidated[29,30]. In previous studies, *18S rRNA* and *26S rRNA* were used as internal reference genes in qRT-PCR to investigate gene expression during fruit ripening[31,32]. Nevertheless, there is a lack of comprehensive experimental validation regarding the stability of gene expression levels during different developmental stages of strawberry fruit. The precise quantification of target gene expression in qRT-PCR experiments is contingent upon the careful selection of appropriate reference genes, as any variability in the expression levels of these reference genes may introduce inaccuracies that subsequently impact the dependability and authenticity of subsequent research outcomes[33]. Therefore, it is imperative to screen and identify suitable reference genes that exhibit stable expression across various tissues, organs, and developmental stages of cultivated octoploid strawberry. This will greatly facilitate future investigations in this field[34].

Our objective was to identify a reference gene with relatively consistent expression throughout all sampled tissues, organs, and developmental stages of cultivated octoploid strawberry fruit. In a previous investigation, we successfully achieved a high-quality haplotype-resolved genome of cultivated octoploid strawberry and subsequently reannotated the second-generation transcriptome data based on this genome[35]. Based on the analysis of high-quality haplotype-resolved genome and the fragments per kilobase of exon model per million mapped fragments (FPKM) data of transcriptome, we selected candidate reference genes, along with the commonly used *Fa26S rRNA*. Primer3 was used to design primers for qPCR, and the analysis of the melting curve and standard curve showed that the primer design met the primary standard. We employed GeNorm, Normfinder, BestKeeper, and Comparative delta-Ct methods to analyze the Ct values of five candidate reference genes. RefFinder (http://blooge.cn/RefFinder/) calculated that *FaGAPC2* and *FaADPrf1* were suitable reference genes combined with the above four results. Validation using *FaMYB10*, *FaUGT1* and *FaCHS* in different development stages of 'Yanli' fruit supported the calculation results of RefFinder. Further, it determined that *FaGAPC2* and the combination of *FaGAPC2* and *FaADPrf1* could serve as suitable reference genes, with the combination of *FaGAPC2* and *FaADPrf1* being more suitable than the single *FaGAPC2*.

Results

Screening of candidate reference genes and specificity of primers

We are committed to finding a reference gene with relatively stable expression in different tissues, organs, and fruit development stages of cultivated strawberry. The candidate reference genes were screened using FPKM data from the next-generation transcriptome and the genes whose expression levels were higher than 1,000 respectively in root, shoot, leaf, and five stages of fruit development (SG = Small Green, BG = Big Green, W = White, TR = Turning Red, R = Red) were selected as candidate reference genes. *ADP-ribosylation factor 1* (*ADPrf1*), *glyceraldehyde-3-phosphate dehydrogenase* (*GAPC2*), *peptidyl-prolyl cis-trans isomerase 1* (*PPC1*), and *elongation factor 1-alpha* (*EF1-α*) were selected as candidate reference genes (Supplemental Table S1). We first verified the homology between the alleles of candidate reference genes, respectively. The results showed that each candidate reference gene had at least eight alleles, and the DNA sequence similarity between alleles was more than 95% (Supplemental Figs S1–S4). In addition, previous studies on strawberry always used the *Fa26S rRNA* gene as a reference gene, so *Fa26S rRNA* was also selected as a candidate reference gene[36−38].

Primer3 version 4.1.0 was used to design primers of five candidate reference genes. The lengths of amplification fragments for five candidate reference genes ranged from 132 bp (*Fa26S rRNA*) to 258 bp (*FaPPC1*). In addition, except *FaPPC1* (89.21%), all five candidate reference genes displayed an amplification efficiency exceeding 90%, while the correlation coefficients (R^2) surpassed 0.99 (Table 1, Supplemental Fig. S5). The melting profiles of all potential reference genes displayed a singular peak, validating the specificity of the primer design and the existence of a sole PCR amplification product (Supplemental Fig. S6).

Expression stability analysis of the candidate reference genes

GeNorm software was utilized to calculate the M value, which indicates gene expression stability. Lower M values correspond to higher expression stability. The study showed that *FaGAPC2* and *FaEF1-α* had the lowest M value (0.858), which represented the most stable expression, and *Fa26S rRNA* had the highest M value (1.272), which represented the least stable expression in 'Yanli'. Meanwhile, in 'Chuliandeweidao', *FaGAPC2* and *FaADPrf1* (1.612) had the most stable expression,

Table 1. Primers sequence and amplification characteristics of five candidate reference genes.

Gene symbol	Gene name	Primer sequence (5'-3')	Amplification length (bp)	Amplification efficiency (%)	Correlation coefficiency (R^2)
ADPrf1	ADP–ribosylation factor 1	F: 5'-TGCGAATTCTGATGGTCGGT-3' R: 5'-CTCCACAATGGACGGATCTT-3'	144 bp	95.43%	0.9984
GAPC2	Glyceraldehyde-3-phosphate dehydrogenase	F: 5'-GAATCAACGGATTCGGAAGA-3' R: 5'-ACAATATCGGCACCAACTGA-3'	231 bp	101.51%	0.9996
EF1-α	Elongation factor 1 - alpha	F: 5'-CACATCAACATTGTGGTCAT-3' R: 5'-GTCTCAAACTTCCACAAGGC-3'	187 bp	99.69%	0.9984
PPC1	Peptidyl-prolyl cis-trans isomerase 1	F: 5'-TACAAGGGATCGTCCTTCCA-3' R: 5'-ACCCAACCTTCTCGATGTTC-3'	258 bp	89.21%	0.9955
26S rRNA	26S ribosomal RNA	F: 5'-TAACCGCATCAGGTCTCCAA-3' R: 5'-CTCGAGCAGTTCTCCGACAG-3'	132 bp	95.69%	0.9996

and *FaPPC1* (3.547) had the least stable expression (Fig. 1a). The pairwise variation (V) analysis indicated that increasing the number of genes increases the average stability of reference genes. Therefore, the qRT-PCR results obtained from the analysis of two reference genes will be more accurate in both Yanli and Chuliandeweidao (Fig.1b).

NormFinder is a Visual Basic application that allows the calculation of reference gene stability, similar to GeNorm. NormFinder first calculates gene expression stability and then outputs specific numbers; the gene expression stability increases proportionally as the numerical value decreases. *FaGAPC2* (0.425 for 'Yanli' and 0.806 for 'Chuliandeweidao') exhibited the lowest stable value, representing the highest expression stability in both varieties. However, the lowest expression stability gene in 'Yanli' and 'Chuliandeweidao' differed. Among the five candidate reference genes, *Fa26S rRNA* showed the lowest level of stability in terms of expression, with a value of 1.442 in 'Yanli'; however, *FaPPC1* (3.455) was the lowest expression stability in 'Chuliandeweidao' (Fig. 2). Considering GeNorm and NormFinder, *FaGAPC2* was a more suitable reference gene among the five candidates.

BestKeeper calculates gene expression stability by combining the coefficient of variation (CV) and standard deviation (SD). The results showed that *FaADPrf1* (0.86) had the highest expression stability due to its minimum SD value in 'Yanli', while *FaEF1-α* (1.52) showed the lowest expression stability (Fig. 3). However, there was little difference in SD value between *FaADPrf1* and *FaEF1-α*. *FaGAPC2* (1.00) had the highest expression stability y due to its minimum SD value, and *FaPPC1* (3.59) displayed the lowest expression stability in 'Chuliandeweidao' (Fig. 3). The stability of expression determined by BestKeeper for the five candidate reference genes differed from that observed through GeNorm and NormFinder analyses.

Comparative ΔCt, another method employed in this study, calculates reference gene stability based on SD values. The lower SD value represented the high expression stability and vice versa. The results demonstrated that *FaGAPC2* was the most stable reference gene with an SD value of 1.076 in 'Yanli' and 2.663 in 'Chuliandeweidao'. At the same time, *FaPPC1* (1.610 in 'Yanli' and 4.145 in 'Chuliandeweidao') showed the least expression stability (Fig. 4).

The expression stability of the five candidate reference genes calculated by different methods was not the same, likely due to different statistical methods. Therefore, we used the online tool RefFinder to analyze all calculation results and get the most appropriate ranking. The lower value of *FaGAPC2* (1.32 for 'Yanli' and 1.00 for 'Chuliandeweidao') and *FaADPrf1* (1.86 for 'Yanli' and 1.68 for 'Chuliandeweidao') indicated that they are more suitable for reference genes. At the same time, the performance of *FaPPC1* (4.00 for 'Yanli' and 5.00 for 'Chuliande weidao') was poor according to all evaluation systems (Table 2). Based on the results from RefFinder and Pairwise variation, we considered the combination of *FaGAPC2* and *FaADPrf1* to be suitable as reference genes for qRT-PCR experiments.

Validation of putative reference genes

To further validate the stability of the selected reference genes, we proceeded to examine the expression of *FaMYB10*, *FaUGT1*, and *FaCHS* genes, which are known for their positive regulatory roles in anthocyanin synthesis (Supplemental Table S2). As the strawberry fruits developed, the expression levels of these three genes exhibited an increasing trend. We utilized the single reference gene *FaGAPC2* and the combination of

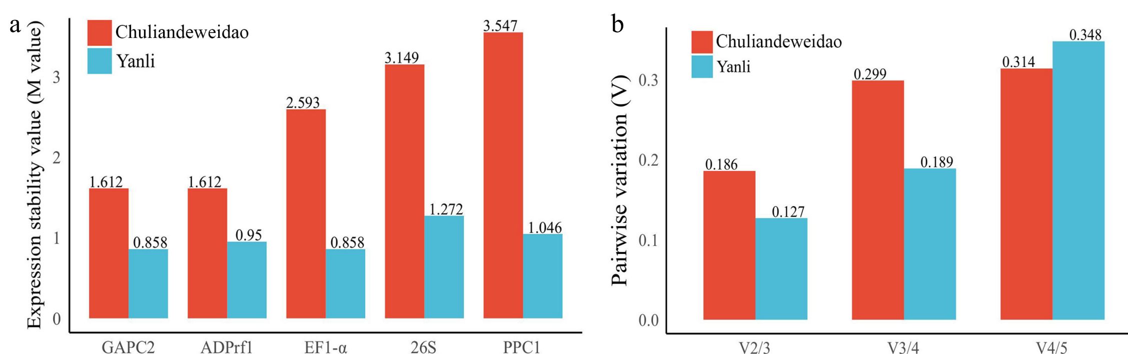

Fig. 1 Expression stability value of five candidate reference genes calculated by geNorm in 'Yanli' and 'Chuliandeweidao'. (a) M value of five candidate reference genes. A lower M-value indicates more stable gene expression. (b) Pairwise variation (V) analysis of five candidate reference genes. A lower value indicates a more stable combination number of reference genes.

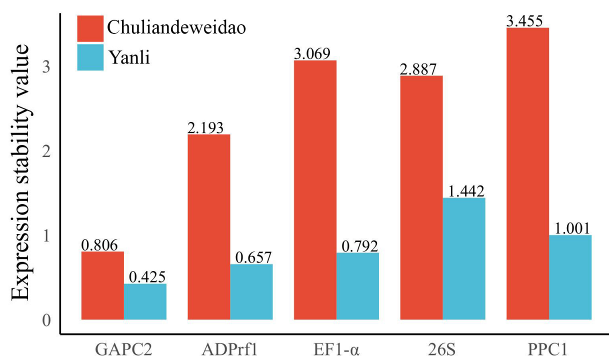

Fig. 2 Expression stability value of five candidate reference genes calculated by NormFinder in 'Yanli' and 'Chuliandeweidao'. A lower value indicates more stable gene expression.

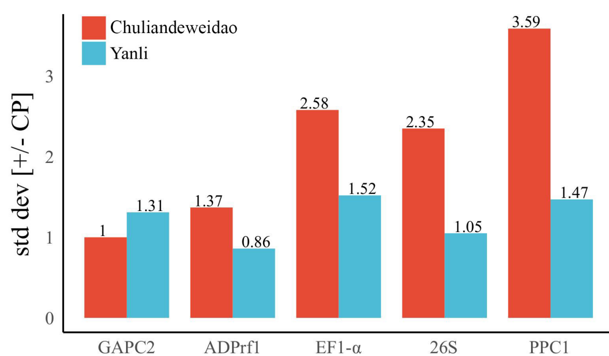

Fig. 3 Expression stability value of five candidate reference genes calculated by BestKeeper in 'Yanli' and 'Chuliandeweidao'. A lower value indicates more stable gene expression.

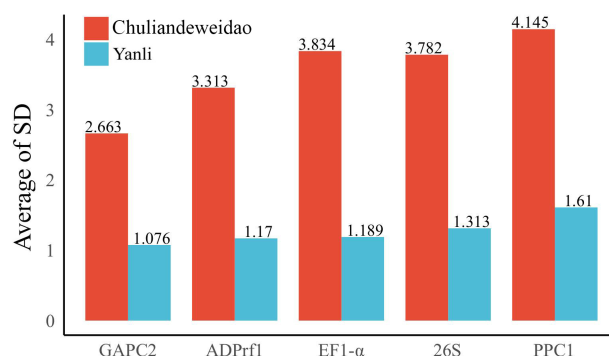

Fig. 4 Expression stability value of five candidate reference genes calculated by delta-CT method in 'Yanli' and 'Chuliandeweidao'. A lower value indicates more stable gene expression.

Table 2. The comprehensive ranking of five candidate reference genes in 'Yanli' and 'Chuliandeweidao' analyzed by RefFinder.

Rank	Yanli		Chuliandeweidao	
	Gene name	Ranking value	Gene name	Ranking value
1	*GAPC2*	1.32	*GAPC2*	1.00
2	*ADPrf1*	1.86	*ADPrf1*	1.68
3	*EF1-α*	2.59	*26S*	3.22
4	*26S*	3.98	*EF1-α*	3.72
5	*PPC1*	4.00	*PPC1*	5.00

FaGAPC2 and *FaADPrf1* to calculate the expression levels of the three genes at different developmental stages of the strawberry fruits. As shown in Fig. 5, regardless of whether a single gene or a combination of genes was used as the reference gene, the expression levels of *FaMYB10*, *FaUGT1*, and *FaCHS* showed an increasing trend during the development of the strawberry fruits. This result suggested that the selected reference genes were suitable for strawberry fruit ripening research.

Discussion

qRT-PCR is widely regarded as the most robust and efficient technique for detecting gene expression levels and patterns in plants[39]. Researchers widely use it because of its specificity, high sensitivity, and simple operation. However, the accuracy of qRT-PCR is influenced by various factors, including RNA quality and integrity, instrument fluorescence detection sensitivity, and appropriate selection of reference genes[40,41]. In this investigation, reference genes for different tissues, organs, and developmental stages of cultivated octoploid strawberry were identified using two cultivars, 'Yanli' and 'Chuliandeweidao', as experimental materials. Subsequently, stable reference gene expressions were obtained for subsequent qRT-PCR experiments.

The amalgamation of transcriptome databases, facilitated by advancements in sequencing technology, has emerged as a mature approach for reference gene screening[42]. A previous study showed that the expression stability of reference genes *UXS3*, *SAP5*, and *ARFA1E* mined from transcriptome data was better than that of the traditional reference gene *Actin7* in *Brassica napus*[43]. This study selected five housekeeping genes as candidate reference genes combined with the second-generation transcriptome data and high-quality haplotype-resolved genome. The expression stability of five candidates in different tissues and organs and five fruit development stages were evaluated by qRT-PCR and calculated by geNorm, NormFinder, BestKeeper, and Comparative delta-Ct. Owing to differences in operational logic and statistical methodologies, the ranking of candidate reference gene expression stability varied slightly across different analysis tools. For example, the analysis results of geNorm, NormFinder, and Comparative delta-Ct showed that the expression stability of *FaGAPC2* was the best. In contrast, BestKeeper analysis showed that *FaADPrf1* had the most stable expression level in 'Yanli'. This phenomenon also occurred in the study of reference genes in poplar (*Populus deltoides*), strawberry (*Fragaria vesca*), apple (*Malus domestic*), and other plants. RefFinder, a widely utilized program for comprehensive stability analysis of candidate reference genes, was employed for reference gene screening. To conduct a comprehensive evaluation of reference gene stability, RefFinder was utilized to thoroughly assess the stability of five selected reference genes. The expression stability ranking from high to low was *FaGAPC2* (1.32) > *FaADPrf1* (1.86) > *FaEF1-α* (2.59) > *Fa26S rRNA* (3.98) > *FAPPC1* (4.00) in 'Yanli' and *FaGAPC2* (1.00) > *FaADPrf1* (1.68) > *Fa26S rRNA* (3.22) > *FaEF1-α* (3.72) > *FaPPC1* (5.00) in 'Chuliandeweidao', which demonstrated that *FaGAPC2* and *FaADPrf1* were suitable reference gene for qRT-PCR in cultivated octoploid strawberry. Previous studies have investigated suitable reference genes under different stress conditions, but the ideal reference genes for distinct stress were not the same. While *GAPDH* demonstrates stable expression under low-temperature conditions, it is not a

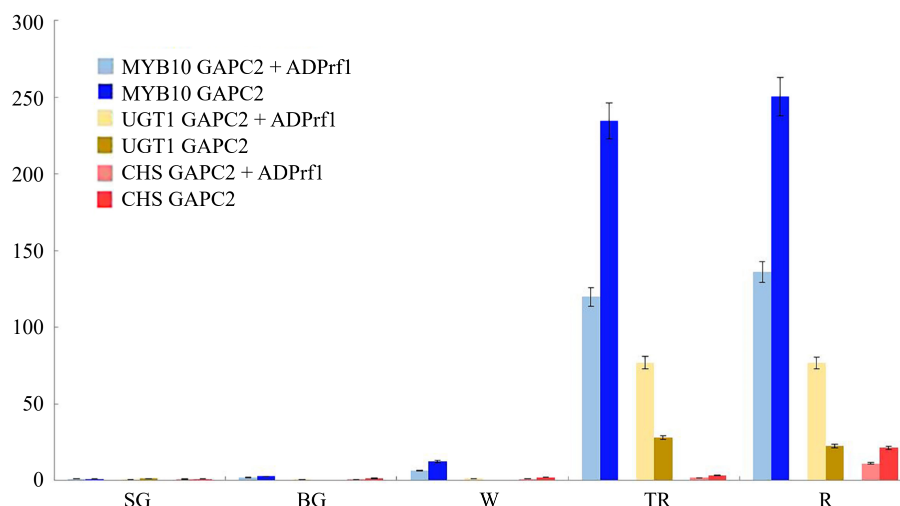

Fig. 5 Relative expression levels of *FaMYB10*, *FaUGT1*, and *FaCHS* in five different development stages of 'Yanli' fruit. The bars of different colors represent the relative expression levels of the validation genes calculated using different reference genes. (SG = Small Green, BG = Big Green, W = White, TR = Turning Red, R = Red).

suitable reference gene under drought and salt stress. Additionally, the suitable reference genes under these two stress conditions are also different[28]. Therefore, further research is needed to select suitable reference genes under different stress conditions.

FaMYB10, a member of the R2R3-MYB transcription factor family, exerts a pivotal role in anthocyanin biosynthesis in strawberry fruit[44,45]. Previous studies and transcriptome data revealed that FaMYB10 predominantly exhibits expression during the TR and R stages of strawberry fruit development, thereby verifying the reliability of the selected reference genes. Our study demonstrated that the expression trend of *FaMYB10* calculated using either *FaGAPC2* or the combination of *FaGAPC2* and *FaADPrf1* as reference genes aligned with the FPKM values from the transcriptome. According to the FPKM values from the transcriptome, the expression level of *FaUGT1* gradually increased during fruit development and reached its highest expression level at the R stage. However, when using *FaGAPC2* as a single reference gene to calculate the expression trend of *FaUGT1*, the expression level was shown to be higher at the TR than at the R stage, which is inconsistent with the FPKM values. Nevertheless, when we used the combination of *FaGAPC2* and *FaADPrf1* as the reference genes to calculate the expression trend, it aligned with the FPKM values. This suggests that the combination of *FaGAPC2* and *FaADPrf1* was more suitable than *FaGAPC2* alone when calculating the expression level of *FaUGT1* gene. The *Fa26S rRNA* was always used to study the strawberry gene expression pattern. The Ct value analysis demonstrated that the expression level of the *Fa26S rRNA* gene was the highest among the five candidate genes in different tissues and organs and five development stages of cultivated strawberry. However, the expression pattern of *Fa26S rRNA*, with Ct values ranging from 7.99 to 13.28, displayed instability, highlighting its unsuitability as a reference gene for normalizing target gene expression across various tissues, organs, and fruit development stages (Fig. 6).

Conclusion

FaGAPC2 and *FaADPrf1* are suitable reference genes for qRT-PCR.

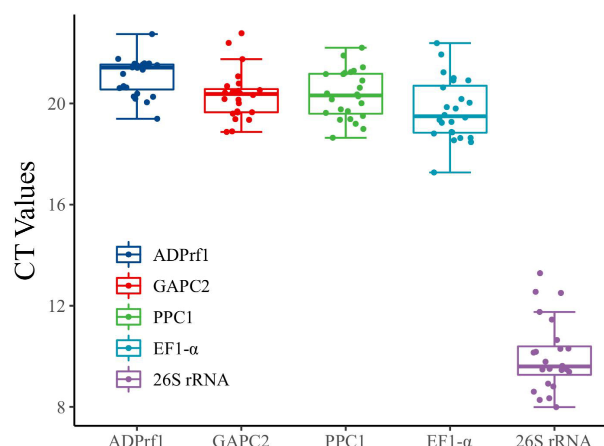

Fig. 6 Boxplot analysis of the expression profiles of *FaADPrf1*, *FaGAPC2*, and *Fa26S rRNA* in root, crown stem, leaf, flower, and five fruit developmental stages. Solid dots represent the expression cycle threshold (CT) values of candidate reference genes in different organs. The line across the box represents the median. The boxes represent the 25/75 percentiles.

Materials and methods

Plant materials

The cultivated strawberry (*Fragaria* × *ananassa*) cultivars 'Yanli' and 'Chuliandeweidao' were grown in the solar greenhouse of Shenyang Agriculture University (China). Root, crown stem, leaf, flower, and fruits at five different development stages (Small Green = SG, Big Green = BG, White = W, Turning Red = TR, and Red = R) were collected in June 2023. All materials were stored at −80°C.

Total RNA extraction and cDNA synthesis

We used the CTAB method to extract the total RNA of the materials. The brief steps were: The samples were ground into powder form and transferred into a 1.5 ml RNase-free centrifuge tube. After adding 588 μl CTAB extraction solution and 12 μl β-mercaptoethanol, the samples were put into a 65 °C water bath for 30 min and shaken violently for 1 min every 5 min. Samples were shaken violently for 5 min after adding

600 μl chloroform/isoamyl alcohol (volume ratio = 24:1) and then centrifuged at 12,000 rpm at 4 °C for 10 min. Four hundred μl supernatant was extracted before adding 400 μl chloroform/isoamyl alcohol (volume ratio = 24:1), shaken violently for 5 min, then centrifuged at 12,000 rpm at 4 °C for 10 min. After absorbing 300 μl supernatant, adding 75 μl of 10 M $LiCl_2$, and precipitating RNA overnight at −20 °C, the total RNA was subjected to two rounds of cleansing using absolute ethanol and subsequently solubilized in DEPC-treated water. NanoDrop 2000 and 1% agarose gel electrophoresis were utilized to assess the purity and integrity of the RNA samples.

Complementary DNA (cDNA) was reverse transcribed using the PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China) following the manufacturer's protocol.

Quantitative PCR

The qPCR was performed on QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems). A total volume of 10 μl containing 0.5 μl cDNA, 1 μl gene-specific primers, 3.5 μl ddH_2O, and 5 μl UltraSYBR Mixture was mixed and carried out qPCR using UltraSYBR Mixture (CWBio, Beijing, China). The PCR program was set based on the description provided by Zhang et al[46]. The relative mRNA levels were determined by employing the $2^{-\Delta\Delta C_T}$ approach. Each sample was examined in triplicate with three biological replicates. Primer sequences of candidate reference genes for qPCR are listed in Table 1 and *FaMYB10* for qPCR are as follows: forward (5'-ACAGATGCAG GAAGAGCTGT-3') and reverse (5'-GTTCTTCCTGGCAATCGTCC-3'). Primer sequences of *FaCHS* are as follows: forward (5’-TCAACGGCCCAAACTATCCT-3’) and reverse (5’-TTAGCCTCAAC CTGGTCCAG-3’). Primer sequences of *FaUGT1* are as follows: forward (5’-CAGTAACAAGACCATCGCCG-3’) and reverse (5’-GAGTTCCAACCGCAATGTGT-3’). The design of all candidate genes and validation genes quantitative PCR primers was based on the high-quality haploid genome sequence of 'Yanli' (www.rosaceae.org/Analysis/14723107).

Statistical analysis

GeNorm, NormFinder, BestKeeper, and Comparative delta-Ct were used to calculate the expression stability of five candidate reference genes. The cycle threshold (Ct) value was first converted to the appropriate format and then used to analyze geNorm, Comparative delta-Ct, and NormFinder. BestKeeper is Excel spreadsheet software and can directly input Ct value for calculation. RefFinder is an online tool that can synthesize the above four results and rank candidate gene expression stability.

Author Contributions

The authors confirm contribution to the paper as follows: carry out experiments and data analysis: Mao J; analyzed the transcriptome data analysis: Li J; helped ensure the completion of the experiment: Wang Y; draft manuscript preparation: Zhang Z. All authors reviewed the results and approved the final version of the manuscript.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Conflict of interest

The authors declare that they have no conflict of interest. Zhihong Zhang is the Editorial Board member of *Fruit Research* who was blinded from reviewing or making decisions on the manuscript. The article was subject to the journal's standard procedures, with peer-review handled independently of this Editorial Board member and the research groups.

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