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Identification of Optimal Reference Genes for qRT-PCR Normalization in Foxtail Millet (*Setaria italica* L.)

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Abstract: Foxtail millet (*Setaria italica* L.) is an ancient C_4 crop that recently emerged as a model crop for genetic and genomic studies. To explore the biological function of genes and regulatory mechanisms in plants, quantitative real-time PCR (qRT-PCR) has proven to be an effective method. However, the accuracy of the results obtained by this method requires reference genes with stable and constant expression to normalize the data. In this study, eight candidate genes, namely ACT-7, α-TUB, β-TUB, GAPDH, EF-1α, RNA POL II, APRT and TLF, were selected, and their expression levels were detected by qRT-PCR in two foxtail millet cultivars, including Yugu1 and Yangu 22. NormFinder and geNorm softwares were used to assess the expression stability of these reference genes. The results revealed that ACT-7, APRT, and TLF were the most stable reference genes. The stability of these three candidate genes was confirmed by analyzing the relative expression levels of C_4 genes in foxtail millet. The mRNA levels of C_4 genes were more abundant in flag leaves than in ears, roots, and developing seeds. Taken together, the results of this study provide a basis for future research exploring functional genes and their associated expression profiles in foxtail millet. We therefore suggest conducting field trials to validate these results under real-world conditions.

1. Introduction

Foxtail millet (*Setaria italica* L.) is a diploid C_4 panicoid crop species, originating from Northern China and widely cultivated as a food and fodder crop in Asia and Europe (Cheng, 2005, Doust *et al.*, 2009). Some cultivars of foxtail millet are extremely drought tolerant, making them important crops in the arid regions of Northern China, where annual rainfall amounts can be as low as 100-400 mm (Vivitha and Vijayalakshmi, 2015). Foxtail millet is increasingly recognized as a model cereal crop for sustainable agriculture due to its remarkable climate resilience (Li *et al.*, 2019, Sood *et al.*, 2020). With advances in sequencing technologies, the genome of the C_4 model species, foxtail millet (Yugu1) has been sequenced (Bennetzen *et al.*, 2012, Zhang *et al.*, 2012), thus, offering a good opportunity to dissect the C_4 pathway using system biology approaches. The C_4 photosynthetic pathway is characterized by

the Kranz Structure of bundle sheath cells and C_4 acid shuttling-related enzymes, such as malate dehydrogenase (MDH), phosphoenolpyruvate carboxylase (PEPC) and pyruvate orthophosphate dikinase (PPDK). Based on the different carboxylases present in the bundle sheath, C_4 species are classified into NADP-ME (NADP-malic enzyme), NADME (NAD-malic enzyme), and PEPCK (phosphoenolpyruvate carboxykinase) types (Sommer *et al.*, 2012). Several studies have reported the expression of C_4 genes in crops such as maize (Hahnen *et al.*, 2003, Ding *et al.,* 2015, Dong *et al.,* 2016, sorghum (Ding *et al.*, 2015), transgenic rice (Ku *et al.*, 2000, Zhang *et al.,* 2003, Taniguchi *et al.,* 2008, Emarkova *et al.,* 2021), and wheat (Lin *et al.*, 2012, Zhang *et al.,* 2014, Rangan *et al.,* 2016) to improve photosynthesis, nutrient use efficiency, and stress tolerance.

Quantitative real-time PCR (qRT-PCR) is an efficient, fast, sensitive and reliable method widely used to quantify messenger RNA (mRNA) expression patterns in many different samples (Galli *et al.*, 2015, Machado *et al.,* 2015, Chapman and aldenström, 2015). It involves the use of appropriate normalization methods to ensure reliable measurements of target gene expressions. However, the results of qRT-PCR are often affected by many factors including RNA quality and quantity, reverse transcription efficiency, amplification efficiency, primer characteristics, and experimental process (Nestorov *et al.*, 2013, You *et al.,* 2021). To avoid potential experimental errors and improve the reliability and reproducibility of qRT-PCR, it is necessary to design an appropriate normalization strategy for the analysis of target genes using the most stable reference genes under different experimental conditions (Huggett *et al.*, 2005, Morales *et al.,* 2016). Previous studies have shown that no single reference gene can remain reliable under different experimental conditions; therefore, suitable reference genes are needed for each specific situation (Podevin *et al.*, 2012, Wan *et al.,* 2017). Several reference genes involved in basic, ubiquitous cellular functions have been used to normalize gene expression (Adeyinka *et al.*, 2019, Li *et al.,* 2021). For example, Actin encodes a cytoskeleton structural protein, TUB (β-Tubulin) is mainly involved in cell growth, and EF-1α (eukaryotic elongation factor-1α) is involved in transcriptional extension. Numerous studies on screening stable-expression reference genes have been conducted in many plants such as Arabidopsis (Remans *et al.,* 2008), rice (Jain, 2009), pearl millet (Saha and Blumwald, 2014), maize (Dong *et al.,* 2016), soybean (Wan *et al.,* 2017), strawberry (Zhang *et al.,* 2018), wheat (Dudziak *et al.,* 2020), peach (You *et al.,* 2021), okra (Zhu *et al.,* 2023), and sweet potato (You *et al.,* 2025). To our knowledge, only the study of Kumar *et al.* (2013) reported on the suitability of reference genes for normalization of gene expression in foxtail millet under abiotic stress conditions.

In this study, eight candidate reference genes were selected and assessed by qRT-PCR in different organs of two foxtail millet cultivars under greenhouse conditions, aiming to obtain the appropriate reference genes for normalization of gene expression to understand C_4 photosynthetic-related gene expression patterns.

2. Methodology

2.1 Plant material and growth conditions

Two summer-cultivated foxtail millet cultivars, Yugu1 and Yangu 22 were used this study. Seeds of the two cultivars were sown up to maturity in greenhouse conditions. Samples from four organs including flag leaves (heading and grain filling), ears (heading), roots (heading) and developing seeds (21 days after anthesis) were harvested, frozen immediately in liquid nitrogen and stored in -80°C freezer until RNA isolation.

2.2 Sequence identification and primer design

The CDS sequences encoding the isoforms of PEPC (Si016228m, Si028826m and Si005789m), NADP-ME (Si021600m and Si000774m), MDH (Si030117m, Si022252m and Si013877m) and PPDK (Si021174m and Si034163m) of foxtail millet were retrieved from the Phytozome v11.0 (www.phytozome.jgi.doe.gov/) and EnsemblPlants (http://plants.ensembl.org/Setaria_italica/Info/Index). Eight commonly used reference genes, including actin-7 (ACT-7), alpha tubulin (α-TUB), beta tubulin (β-TUB), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), elongation factor 1-alpha (EF-1α), RNA polymerase II (RNA POL II), adenine phosphoribosyl transferase (APRT), and translation factor (TLF), were chosen as candidates to identify the most stably expressed reference genes for qRT-PCR studies of the C_4 pathway genes. The CDS sequences encoding the eight reference genes of foxtail millet were also downloaded from Phytozome v11.0. Specific primers for the C_4 pathway genes and the eight reference genes were designed using the qPCR assay design tool (http://www.idtdna.com/scitools/applications/primerquest/) and PCR Primer Stats (Stothard, 2000). The specificity of the primers was verified through BLAST searches of public databases (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Details of the primers are provided in **Table 1** and **2**.

Table 1. Primer sequences of C_4 pathway genes used for qRT-PCR analysis

Primer	Accession number	Sequence (5'-3')	Amplicon length (bp)
SiPEPC_1	Si016228m	F: CCATCAAAGAGGAAGCCTAGTG R: CTTGAATGCTGCGCCAAATC	120
SiPEPC_2	Si028826m	F:ATCGAGTCACTTCGTGCTATTC R:GTGGAGATTCCTGATGTCCTTC	126
SiPEPC_4	Si005789m	F:CAGAGGTGACAAGGGATGTATG R:TCTCTGTGAAGAAGCGTGAAG	155
SiNADP-ME_3	Si021600m	F:AAGTACAGCAAGAGCCATCTC R:GTAGGTGTGGTCAGCAAGAG	120
SiNADP_ME_5	Si000774m	F:CTGAATGCACTGCTGAACAAG R:GCCAAACCCTGGGAAGATATAG	146
SiMDH_2	Si030117m	F:CCCTATCCTTCGCTTCAGTTC R:GGTCCTCCCGATTTCAGATTG	127
SiMDH_3	Si022252m	F: CTCGTGACTGTGCAGACC R:AGAGTACCTTGTGACACCAT	124
SiMDH_6	Si013877m	F:GGTTGTGTCTGTACTCTGTAGG R:ATTGGTAAACAGCAGGCAATAC	137
SiPPDK_3	Si021174m	F:GATGCACTGACAGCAAGAAAC R:ATCTGCCTCACAGCCTTAATC	104
SiPPDK_9	Si034163m	F:AATGATGCTGAGAAGGTGGTAG R:CTAAGTGTTGGCAGGGATAGAG	125

2.3 RNA isolation and cDNA synthesis

Total RNA was extracted from collected samples using a three-step modified Hot Phenol method (Verwoerd *et al.*, 1989, Shinmachi *et al.*, 2010). Initial extraction was carried out in 1 mL (80°C) 1:1 Phenol/ Extraction buffer (0.1 M Tris-HCL, pH 8.0, 0.1 M LiCl, 1% (w/v) SDS and 10 mM EDTA). Afterwards, two phenol/chloroform/isoamyl (25:24:1) extractions were conducted. To avoid genomic DNA contamination, the isolated RNA was treated with DNase using DNA-Free Kit (Thermo Fisher

Scientific, China) according to the manufacturer's instructions. Integrity of the RNA was determined by gel electrophoresis and absorbance was measured at 260 and 280 nm. Only RNA samples with absorbance A260/A280 ratios between 1.8 and 2.2 were used for further analysis. First strand cDNA was synthesized using RT Enzyme Mix and FastQuant-RT primer Mix (Tiangen Biotech, China) in a final reaction volume of 20 µL. cDNA was stored at –20°C for further use.

Table 2. Primer sequences of candidate reference genes used for qRT-PCR

Genes	Description	Accession number	Primer pair (5'-3')	Amplicon size (bp)
ACT-7	Actin-7	Si026509m.g	F: CAGGGAGAAGATGACCCAAATC R: CACCAGAGTCCAGCACAATAC	125
α -TUB	Alpha tubulin	Si029822m	F: TGATGTACCGTGGTGATGTTG R: GATGGTGGCTGGTAGTTGATAC	133
β-TUB	Beta tubulin	Si035709m	F: TGGTGACCTGAACCATCTTATC R: AAGAAATGGAGACGAGGGAAC	132
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	Si013613m	F: CTGTCTGAGTTCCAAGATACCC R: CTATTAGTGCTGGGCCAATCT	101
EF-1α	Elongation factor 1-alpha	Si022045m	F: GGAAGTTTGAGACCACCAAGTA R: GTGGAGTCAATGATGAGGACAG	124
RNA POL II	RNA polymerase II	Si033113m	F: GCCAAGAATTGAGGCTTCGT R: TGTCAGGCTCATACATGAAAGTC	102
APRT	Adenine phosphoribosyl transferase	Si023070m	F: AGGTGAGGTGATCTCTGAAGAA R: AGTGTTCCACCAGTAGCAATAAG	129
TLF	Translation factor	Si000298m	F: CCCTCAGTGTGTGTTTGACC R: CTTGAGACCCTTCCTCTTGC	109

2.4 Quantitative real time PCR analysis

qRT-PCR reactions were performed in a 96 well plate using a 7300 Real-Time PCR system (Applied Bio systems, USA). Reaction cocktail contained 10µL Syber Green 2 x SuperReal PreMix Plus (Tiangen Biotech, China), 2 µL of 50 x ROX reference dye 0.8 µL each of the forward and reverse primers (100 µM), 2 µL of diluted cDNA, and ddH_2O up to 20µL total volume. Amplification conditions were 2 min of initial denaturation at 50ºC, 10 min at 95ºC, and 40 cycles of 15 s at 95ºC and 1 min at 60ºC. After the 40 cycles were completed, the specificity of the amplification was carried out by heating at 95 ºC for 15 s followed by constant increase of temperature between 95ºC and 60ºC, resulting in melting curves. Each reaction was performed in three technical replicates and samples of all reference genes were evaluated using four independent biological replicates. All samples were normalized to the most suitable reference genes and the relative expression levels of the target genes were calculated according to the $2^{-\Delta\Delta C_T}$ method (Livak *et al.*, 2001).

2.5 Stability analysis of gene expression

Gene expression stability of the candidate reference genes was estimated using Microsoft Excel based software packages: geNorm v3 (Vandesompele *et al.*, 2002) and NormFinder (Andersen *et al.*, 2004). The geNorm program calculates an expression stability value (*M*) for each gene based on geometric mean, where the lower the *M* value, the higher is the expression stability and the *M* value less than 1.5 is regarded as acceptable level of expression stability. It also estimates the number of genes that is required for normalization of appropriate controls, by the evaluation of variation in pairs (V values),

checking the variation of the expression of every two possible genetic combinations between two consecutively ranked normalization factors. A threshold value below 0.15 suggests the requirement of no additional reference gene for normalization (Vandesompele *et al.,* 2002). NormFinder determines the stability of expression of candidate reference genes according to their group origin (tissue, organ etc.) and compares expression variation between groups, and genes with the lowest rank are considered to be most stably expressed.

3. Results and Discussion

3.1 Expression levels of candidate reference genes

Expression levels of the eight candidate reference genes were measured by monitoring the Ct values in the qRT-PCR reactions. Gene-specific amplification of each candidate gene was confirmed by the presence of a single peak in melting curve analysis and agarose gel electrophoresis **(Figure 1a, b)**, indicating that there was no non-specific amplifications. The eight reference genes showed different levels of expression, with TLF (mean Ct = 16.92) and EF-1α (mean Ct = 19.03) displaying the highest transcript levels, while RNA POL II and β-TUB, with respective Ct means of 22.89 and 22.47, showed lower transcript expression (**Figure 2**).

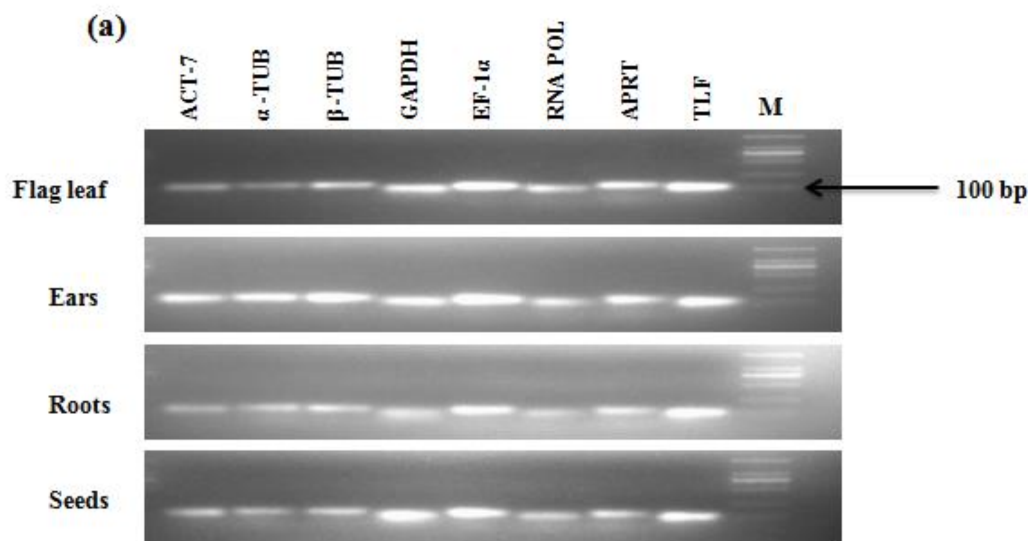

Figure 1a. Specificity of qRT-PCR and amplicon size of the candidate reference genes. Amplified fragment for each gene with the expected size shown on agarose gel (2.5%)

3.2 Gene expression stability

Selecting reference genes with adequate expression in different samples can improve the accuracy and reliability of expression analysis obtained by qRT-PCR (Radonic *et al.,* 2004). In our study, two different programs, geNorm and NormFinder, were used to calculate the expression stability of all candidate reference genes. The results of the geNorm analysis showed that the *M* values of the eight reference genes were all less than 1.5, indicating that they all met the basic requirements for a reference gene. ACT-7 and APRT were ranked as the most stable reference genes in different organs (Flag leaf, ears, roots and developing seeds), followed by TLF and RNA POL II. GAPDH and β-TUB were the least stable genes, exhibiting the highest *M* values **(Figure 3a)**. The optimal number of reference genes was determined by assessing the pairwise variation (V_n/V_{n+1}) between two sequential normalization factors (NF_n and NF_n+1) **(Figure 3b)**. The results revealed that the pairwise variation $V_{2/3}$ was 0.155 > 0.15, indicating that the two most stable reference genes were deficient in determining variations of

the normalization factor, while three stable reference genes (ACT-7, APRT and TLF; $V_{3/4}$ = 0.123 <0.15) would be sufficient to normalize the expression of the target gene.

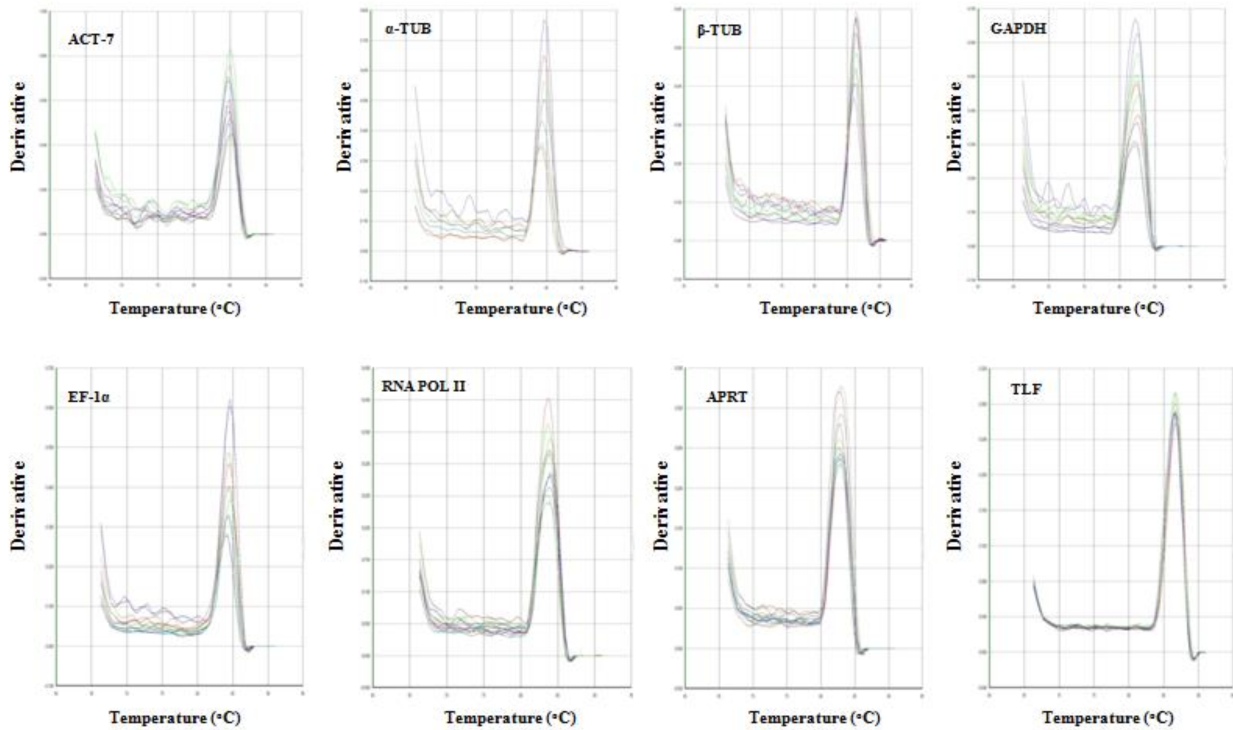

Figure 1b. Specificity of the qRT-PCR and melting curve generated with a single peak

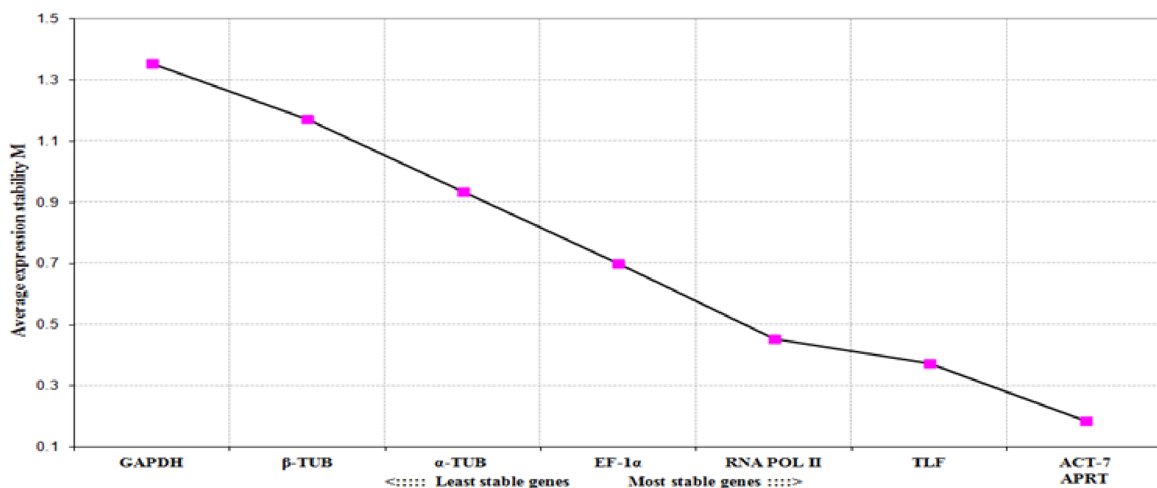

Figure 3a. Average expression stability (M) values and ranking of the 8 candidate reference genes calculated by geNorm. Lower average expression stability levels (M value) denote more stable expression.

The results from Normfinder revealed that RNA POL II, ACT-7 and TLF were the most stable genes, with respective stability values of 0.366, 0.485 and 0.515 **(Figure 4)**. The use of ACT as a reference gene in plants is widespread due to its essential role in cytoskeleton formation and cellular metabolism (Xia *et al.,* 2023). However, its expression can vary considerably depending on the tissue, developmental stage, and environmental conditions. ACT-1 has been identified as a suitable reference

gene in different organs (roots, stems, leaves and seeds) and has been used to normalize the expression of PPDK and PCK in maize (Dong *et al.,* 2016). The study by Kumar *et al.* (2013) showed that ACT-2 (with the same sequence as ACT-7 in the present study) and RNA POL II were suitable reference genes for salinity stress-related studies in foxtail millet. It has been previously reported that ACT-7 was the most appropriate reference gene in jute (Hossain *et al.,* 2019), pear (Xu *et al.,* 2015), and leaf of okra (Zhu *et al.,* 2023), while being highly variable in Rhododendron (Xiao *et al.,* 2016) and Arabidopsis (Remans *et al.,* 2008). ACT11 was identified as the most stably expressed reference genes under nitrogen stress in both shoots and roots of different cultivars of soybean (Wan *et al.,* 2017). In the present study, the least stable reference genes were GAPDH and β-TUB. It has been found that GAPDH was unstable in pearl millet (Saha and Blumwald, 2014), brachypodium (Hong *et al.,* 2008), Glehnia perennial herb (Li *et al.,* 2020), and Arabidopsis (Chen *et al.,* 2024), whereas it was much more stable in strawberry (Zhang *et al.,* 2018). β-TUB was reported unstable in radish (Xu *et al.,* 2012), Rhododendron (Xiao *et al.,* 2016), and Barnyard millet (Renganathan *et al.,* 2023).

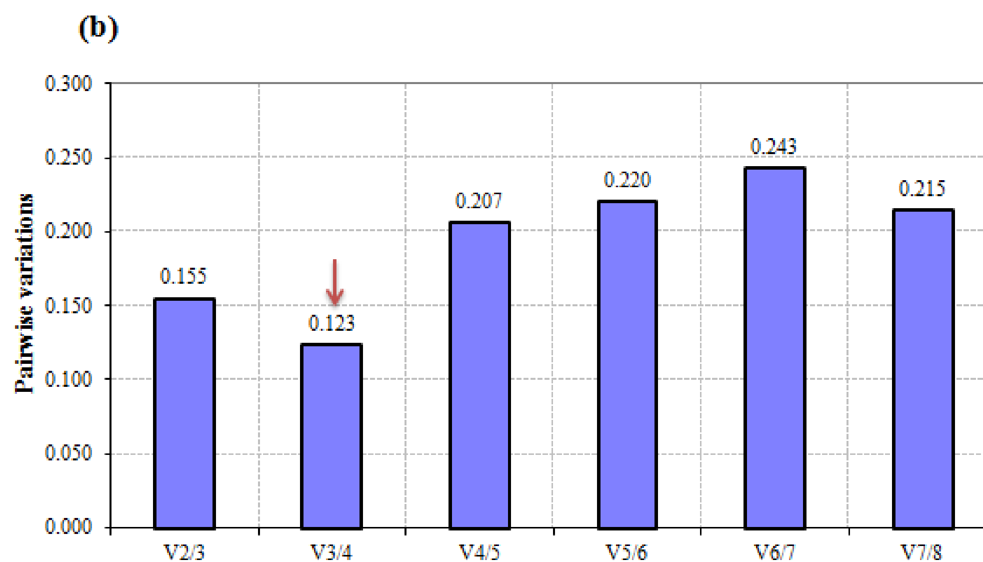

Figure 3b. Pairwise variation (V) calculated by geNorm to determine the optimal number of reference genes for accurate normalization. Arrow indicates the optimal number of genes for normalization.

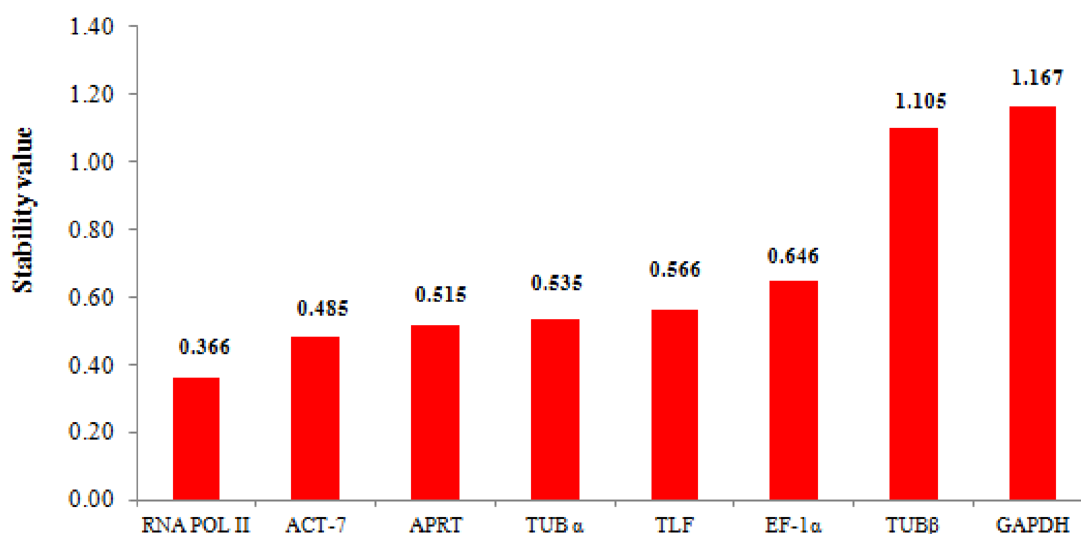

Figure 4. NormFinder ranking of reference genes based on their stability values.

3.3 Relative quantification of C4 gene expression using selected references genes as internal controls

A Search on the genome database of foxtail millet in Phytozome (www.phytozome.jgi.doe.gov/) revealed that the isoforms of *SiPEPC*, *SiNADP-ME*, *SiMDH* and *SiPPDK* are located respectively on chromosome 1, 2 and 3 (*SiPEPC*), chromosome 3 and 5 (*SiNADP-ME*), chromosome 2, 3 and 6 (*SiMDH*) and chromosome 3 and 9 (*SiPPDK*). To determine whether the genes that encode the different isoforms of the C_4 pathway enzymes are functioning or not, their expressions were assessed using qRT-PCR in each of the different organs of two foxtail millet cultivars (Yugu 1 and Yangu 22), respectively, including flag leaves, ears, roots and developing seeds. The selected reference genes, ACT-7, APRT and TLF, were used as internal controls to normalize potential variations in mRNA quality across different organs. All isoforms of the C_4 pathway genes showed different expression profiles in different organs of the two foxtail millet cultivars (**Table 3**). The highest expression of the isoforms was observed in flag leaves at the heading and grain filling stages, compared to the ears, roots and developing seeds. *SiPEPC_4* expression was higher in flag leaves of both cultivars than that of *SiPEPC_2* and *SiPEPC_1*. *SiPEPC_4* was also more abundant in the ears and developing seeds of cultivar Yugu 1. *SiMDH_6*, *SiNADP-ME_5* and *SiPPDK_3* showed higher abundance in flag leaves with the highest expression levels in the Yugu 1 cultivar, whereas *SiMDH_3* was almost not detected in any organs of either cultivar. *SiMDH_2* and *SiPPDK_9* exhibited a similar expression profile, while displaying the lowest expression levels in all organs. These results indicate that isoforms of C_4 genes are expressed differently in different organs during foxtail millet development, suggesting that these isoforms may play distinct roles in the photosynthesis of foxtail millet. The study by Dong *et al.* (2016) showed that mRNA levels of C_4 genes were higher in maize leaves than in seeds 25 days after pollination. Bachir *et al.* (2017) reported the expression of the C_4-like genes in the flag leaves of bread wheat genotype at three growth stages. Engineering of maize C_4-specific genes into the leaves of transgenic rice plants resulted in the overexpression of PEPC, PPDK and NADP-ME genes (Taniguchi *et al.,* 2008). A high accumulation of PPDK transcripts was observed in maize leaves and C_4 mesophyll cells (Hahnen *et al.,* 2003). It has been reported that NADP-ME transcripts were more highly expressed in the leaves of sugarcane plants, whether irrigated or water-deprived (Sales *et al.,* 2018; Cacefo *et al.,* 2019; Laita *et al.*, 2024; Dhansu *et al.*, 2026).

Table 3. Relative expression of the isoforms of C_4 genes in different organs of two foxtail millet cultivars

C_4 genes	Yugu 1					Yangu22				
	FLH	Ears	Roots	FLGF	Seeds	FLH	Ears	Roots	FLGF	Seeds
SiPEPC_1	0.112	0.219	0.125	0.514	0.523	0.112	0.279	0.128	0.422	0.428
SiPEPC_2	0.691	0.506	0.604	0.723	0.327	0.260	0.166	0.178	0.143	0.109
SiPEPC_4	7.963	1.425	0.819	3.323	1.281	3.448	0.355	0.213	1.940	0.548
SiNADPME_3	0.903	0.841	0.364	0.538	0.128	0.158	0.234	0.088	0.125	0.096
SiNADPME_5	1.175	1.053	0.856	1.550	0.536	0.219	0.050	0.138	0.591	0.009
SiMDH_2	0.316	0.243	0.048	0.371	0.015	0.212	0.170	0.054	0.240	0.023
SiMDH_3	0.047	0.019	0.073	0.045	0.041	0.012	0.023	0.065	0.016	0.013
SiMDH_6	0.850	0.531	0.191	0.469	0.520	0.462	0.428	0.131	0.343	0.478
SiPPDK_3	1.063	0.525	0.151	0.671	0.119	0.341	0.154	0.062	0.255	0.092
SiPPDK_9	0.310	0.210	0.065	0.180	0.087	0.128	0.081	0.075	0.150	0.046

FLH: flag leaf at heading, FLGF: flag leaf at grain filling. Ears and roots: at heading, Seeds: developing seeds

Conclusion

This study assessed the stability of eight candidate reference genes and analyzed the expression profile of the isoforms of normalized C_4 pathway genes relative to appropriate reference genes. *SiPEPC_4*, *SiNADP-ME_5*, *SiPPDK_3* and *SiMDH_6* were the most highly expressed and most abundant isoforms in the flag leaves of foxtail millet. The results of this study provide a basis for the selection of suitable reference genes in different cultivars of foxtail millet, and can also served as a reference for the exploration of related functional genes. However, we suggest further field evaluation to validate the results under real-world conditions for a better understanding of C_4 genes expression profiles.

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Compliance with Ethical Standards: This article does not contain any studies involving human or animal subjects.

References

Adeyinka O. S., Tabassum B., Nasir I. A., Yousaf I., Sajid I. A., Shehzad K., Batcho A., Husnain T. (2019) Identification and validation of potential reference gene for effective dsRNA knockdown analysis in *Chilo partellus*. *Sci. Rep.,* 9(1), 13629. doi.10.1038/s41598-019-49810-w.

Andersen C. L., Jensen J. L., Ørntoft T. F. (2004) Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res.,* 64, 5245-5250. doi.10.1158/0008-5472.CAN-04-0496.

Bachir D. G., Saeed I., Song Q., Linn T. Z., Chen L., Hu Y. (2017) Characterization and expression patterns of key C_4 photosynthetic pathway genes in bread wheat (*Triticum aestivum* L.) under field conditions. *J. Plant Physiol.,* 213, 87-97. doi.10.1016/j.jplph.2017.03.002.

Bennetzen J. L., Schmutz J., Wang H., Percifield R., Hawkins, J., Pontaroli, A. A., Estep, M., *et al.* (2012) Reference genome sequence of the model plant Setaria. *Nat. Biotechnol.,* 30(6), 555-561. doi:10.1038/nbt.2196.

Cacefo, V., Ribas, A. F., Zilliani, R. R., Neris, D. M., Domingues, D. S., Moro, A. L., Vieira, L. G. E. (2019) Decarboxylation mechanisms of C_4 photosynthesis in Saccharum spp.: increased PEPCK activity under water-limiting conditions. *BMC Plant Biol.,* 19(1), 144. doi: 10.1186/s12870-019-1745-7.

Chapman J.R, Waldenström J. (2015) With reference to reference genes: A systematic review of endogenous controls in gene expression studies. *PLoS ONE*, 10(11), e0141853. doi: 10.1371/journal.pone.0141853.

Chen, S., Cai, Q., Liu, P., Liu, J., Chen, G., Yan, H., Zheng, H. (2024) Identification and Validation of qRT-PCR Reference Genes for Analyzing Arabidopsis Responses to High-Temperature Stress. *Curr. Issues Mol. Biol.,* 46(12), 14304-14320. doi.org/10.3390/cimb46120857.

Cheng R. H. (2005) The present situation of foxtail millet (*Setaria italica* Beauv) breeding and production as well as the further research directions in China. *J. Herbei Agric. Sci.,* 9(04), 86-90. https://doi.org/10.16318/j.cnki.hbnykx.2005.04.022.

Dhansu P., Kaushik S., Kumar R., Kumar A., Mishra D.S., Kaushik P., Tunç Y., Khadivi A. (2026) Salinity responses and tolerance mechanisms in sugarcane (Saccharum officinarum L.): Recent updates and future perspectives, *Industrial Crops and Products,* 239, 122556, ISSN 0926-6690, https://doi.org/10.1016/j.indcrop.2025.122556

Ding Z., Weissmann S., Wang M., Du B., Huang L., Wang L., Tu X., Zhong S., Myers C., Brutnell T. P., Sun Q., Li P. (2015) Identification of Photosynthesis-Associated C_4 Candidate Genes through Comparative Leaf Gradient Transcriptome in Multiple Lineages of C_3 and C_4 Species. *PLoS ONE*, 10(10), e0140629. doi.10.1371/journal.pone.0140629.

Dong X.M., Yuan L., Qing C., Jie S., Xiu-jie G., Biligen-gaowa Z., Bai-chen W. (2016) Analysis of gene expression and histone modification between C_4 and non-C_4 homologous genes of PPDK and PCK in maize. *Photosynth Res.,* 129:71-83. doi.10.1007/s11120-016-0271-9.

Doust, A. N., Kellogg, E. A., Devos K. M., Bennetzen J. L. (2009) Foxtail millet: a sequence-driven grass model system. *Plant Physiol.,* 149(1), 137–141. doi: 10.1104/pp.108.129627.

Dudziak, K., Sozoniuk, M., Szczerba, H., Kuzdraliński, A., Kowalczyk, K., Börner, A., Nowak, M. (2020) Identification of stable reference genes for qPCR studies in common wheat (*Triticum aestivum* L.) seedlings under short-term drought stress. *Plant Methods.,* 16, 58. doi: 10.1186/s13007-020-00601-9.

Ermakova, M., Arrivault, S., Giuliani, R., Danila, F., Alonso-Cantabrana, H., Vlad, D., Ishihara, H., *et al.* (2021) Installation of C_4 photosynthetic pathway enzymes in rice using a single construct. *Plant Biotechnol J.,* 19(3), 575-588. doi: 10.1111/pbi.13487.

Galli V., Borowski J., Perin E., Messias R., Labonde, J., Pereira, I., Silva, S., *et al.* (2015) Validation of reference genes for accurate normalization of gene expression for real-time quantitative PCR in strawberry fruits using different cultivars and osmotic stresses. *Gene*., 554(2), 205-214. doi.10.1016/j.gene.2014.10.049.

Hahnen S., Joeris T., Kreuzaler F., Peterhänsel C. (2003) Quantification of photosynthetic gene expression in maize C(3) and C(4) tissues by real-time PCR. *Photosynth. Res.,* 75(2), 183-192. doi.10.1023/A:1022856715409.

Han X., Lu M., Chen Y., Zhan Z., Cui Q., Wang Y. (2012) Selection of reliable reference genes for gene expression studies using real-time PCR in tung tree during seed development. *PLoS ONE.,* 7(8), e43084. doi.10.1371/journal.pone.0043084.

Hong S. Y., Seo P., Yang M. S., Xiang F., Park C. M. (2008) Exploring valid reference genes for gene expression studies in *Brachypodium distachyon* by real-time PCR. *BMC Plant Biol.,* 8(1), 112. doi.10.1186/1471-2229-8-112.

Hossain S., Ahmed R., Haque S., Alam M., Islam S. (2019) Identifcation and validation of reference genes for real-time quantitative RT-PCR analysis in jute. *BMC Mol. Biol.,* 20(1), 13. doi.10.1186/s12867-019-0130-2.

Huggett J., Dheda K., Bustin S., Zumla A. (2005) Real-time RT-PCR normalization; strategies and considerations. *Genes Immun.,* 6(4), 279-284. doi.10.1038/sj.gene.6364190.

Jain M. (2009) Genome-wide identification of novel internal control genes for normalization of gene expression during various stages of development in rice. *Plant Sci.,* 176(5), 702-706. doi.10.1016/j.plantsci.2009.02.001.

Ku M. S., Cho D., Ranade U., Hsu T. P., Li X., Jiao D. M., Ehleringer J., Miyao M. (2000) Photosynthetic performance of transgenic rice plants overexpressing maize C_4 photosynthesis enzymes. *Stud. Plant Sci.,* 7, 193-204. doi.10.1016/S0928-3420(00)80015-4.

Kumar K., Mehanathan M., Manoj P. (2013) Reference genes for quantitative real-time PCR analysis in the model plant foxtail millet (*Setaria italica* L.) subjected to abiotic stress conditions. *Plant Cell Tiss. Organ. Cult.,* 115, 13-22. doi.10.1007/s11240-013-0335-x.

Li, L., Li, N., Fang, H., Qi, X., Zhou, Y. (2020) Selection and Validation of Reference Genes for Normalisation of Gene Expression in *Glehnia littoralis*. *Sci. Rep.,* 10(1), 7374. doi.org/10.1038/s41598-020-63917-5.

Li M., Li X., Wang C., Li Q., Zhu S., Zhang Y., Li X., Yang F., Zhu X. (2021) Selection and Validation of Reference Genes For qRT-PCR Analysis of *Rhopalosiphum padi* (Hemiptera: Aphididae). *Front Physiol.,* 12, 663338. doi.10.3389/fphys.2021.663338.

Li P., Li B., Seneweera S., Zong Y., Li F.Y., Han Y., Hao X. (2019) Photosynthesis and Yield Response to Elevated CO_2, C_4 Plant Foxtail Millet Behaves Similarly to C_3 Species. *Plant Sci.,* 285, 239-247. doi: 10.1016/j.plantsci.2019.05.006.

Lin H., Yan L., WeiGang X., QingChen Z., Lei Z., Xueli Q., Haibin D. (2012). Improvement of the photosynthetic characteristics of transgenic wheat plants by transformation with the maize C_4 phosphoenolpyruvate carboxylase gene. *Plant Breed.,* 131(3), 385-391. doi.10.1111/j.1439-0523.2012.01960.x.

Livak K. J., Schmittgen T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}{}_{T}$ method. *Methods.,* 25(4), 402-408. doi.10.1006/meth.2001.1262.

Machado R., Christoff A., Loss-Morais G., Margis-Pinheiro M., Margis R., Körbes A. (2015) Comprehensive selection of reference genes for quantitative gene expression analysis during seed development in *Brassica napus*. *Plant Cell.,* 34(7), 1139-1149. doi.10.1007/s00299-015-1773-1.

Moraes, M. B. F., Daúde, M. M., de Oliveira, K. K. P., Gonçalves, R. C., Ságio, S. A., Lima, A. A., Chalfun-Junior, A., *et al.* (2025) Evaluation and validation of reference genes for RT-qPCR normalization in different sweet potato tissues. *Sci Rep.,* 15(1), 39899. doi: 10.1038/s41598-025-22650-7.

Morales M. A., Mendoza, B. M., Lavine L. C., Lavine M. D., Walsh D. B., Zhu F. (2016) Selection of Reference Genes for Expression Studies of Xenobiotic Adaptation in Tetranychus urticae. *Int. J. Biol. Sci.,* 12(9):1129-1139. doi.10.7150/ijbs.16319.

Matsuoka M., Fukayama H., Ku M. S. B., Miyao M. (2008) High-level expression of C_4 photosynthetic genes in transgenic rice. *Rice Genetics IV.,* 42, 439-447. doi.org/10.1142/9789812814296_0029.

Nestorov J., Matic G., Elakovic I., Tanic N. (2013) Gene expression studies: How to obtain accurate and reliable data by quantitative real-time RT-PCR. *J. Med. Biochem.,* 32(4), 325-338. doi.10.2478/jomb-2014-0001.

Podevin N, Krauss A, Henry I, Swennen R, Remy S (2012) Selection and validation of reference genes for quantitative RT-PCR expression studies of the non-model crop Musa. *Molecular Breed.,* 30(3), 1237-1252. doi.10.1007/s11032-012-9711-1.

Radonic A, Thulke S, Mackay IM, Landt O, Siergert W, Nitsche A (2004) Guideline to reference gene selection for quantitative real-time PCR. *Biochem. Biophys. Res. Commun.,* 313(4), 856-862. doi.10.1016/j.bbrc.2003.11.177.

Remans T., Smeets K., Opdenakker K., Mathijsen D., *et al.* (2008) Normalisation of real-time RT-PCR gene expression measurements in Arabidopsis thaliana exposed to increased metal concentrations. *Planta.,* 227(6), 1343-1349. doi.10.1007/s00425-008-0706-4.

Renganathan, V.G., Renuka, R., Vanniarajan, C., Raveendra, M., Elangovan, A. (2023) Selection and validation of reliable reference genes for quantitative real-time PCR in Barnyard millet (*Echinochloa* spp.) under varied abiotic stress conditions. *Sci. Rep.,* 13(1), 15573. doi.org/10.1038/s41598-023-40526-6.

Saha P., Blumwald E. (2014) Assessing Reference Genes for Accurate Transcript Normalization Using Quantitative Real-Time PCR in Pearl Millet (*Pennisetum glaucum* (L.) R. Br.). *PLoS ONE.,* 9(8), e106308. doi.10.1371/journal.pone.0106308.

Sales C.R.G., Ribeiro R.V., Hayashi A.H., Marchiori P.E.R., Silva K.I., Martins M.O., Silveira J.A.G., Silveira N.M., Machado E.C. (2018). Flexibility of C4 decarboxylation and photosynthetic plasticity in sugarcane plants under shading, *Environmental and Experimental Botany*, 149, 2018, 34-42, https://doi.org/10.1016/j.envexpbot.2017.10.027

Shinmachi F., Buchner P., Stroud J. L., Parmar S., Zhao F. J., McGrath S. P., Hawkesford M. J. (2010) Influence of sulfur deficiency on the expression of specific sulfate transporters and the distribution of sulfur, selenium and molybdenum in wheat. *Plant Physiol.,* 153(1), 327–336. doi.10.1104/pp.110.153759.

Sommer M., Bräutigam A., Weber A. P. M. (2012) The dicotyledonous NAD malic enzyme C_4 plant Cleome gynandra displays age-dependent plasticity of C_4 decarboxylation biochemistry. *Plant Biol.,* 14(4), 621-629. doi.10.1111/j.1438-8677.2011.00539.x.

Sood, P., Singh, R. K., Prasad, M. (2020) An efficient Agrobacterium-mediated genetic transformation method for foxtail millet (*Setaria italica* L.). *Plant Cell Rep.,* 39(4), 511–525. doi.org/10.1007/s00299-019-02507-w.

Stothard P. (2000) The Sequence Manipulation Suite. JavaScript programs for analyzing and formatting protein and DNA sequences. *Biotechniques.,* 28(6), 1102-1104. doi.10.2144/00286ir01.

Taniguchi Y., Ohkawa H., Masumoto C., Fukuda T., Tamai T., Lee K., Sudoh S., Tsuchida H., Sasaki H., Fukayama H., Miyao M. (2008) Overproduction of C_4 photosynthetic enzymes in transgenic rice plants: an approach to introduce the C_4-like photosynthetic pathway into rice. *J. Exp. Bot.,* 59(7), 1799-1809. doi.10.1093/jxb/ern016.

Vandesompele J., De Preter , Pattyn F., Poppe B., Van Roy N., De Paepe A., Speleman F. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple control genes. *Genome Biol.,* 3(7), research0034.1-0034.11. doi.10.1186/gb-2002-3-7-research0034.

Verwoerd T. C., Dekker B. M., Hoekema A. (1989) A small-scale procedure for the rapid isolation of plant RNAs. *Nucleic Acids Res.,* 17(6), 2362-2362. doi.10.1093/nar/17.6.2362.

Vivitha P., Vijayalakshmi D. (2015) Minor millets as model system to study C_4 photosynthesis - A review. *Agric. Rev.,* 36(4), 296-304. doi: 10.18805/ag.v36i4.6666.

Wan, Q., Chen, S., Shan, Z., Yang, Z., Chen, L., Zhang, C., Yuan, S., *et al.* (2017) Stability evaluation of reference genes for gene expression analysis by RT-qPCR in soybean under different conditions. *PLoS ONE.*, 12(12), e0189405. doi: 10.1371/journal.pone.0189405.

Wang M., Lu S. (2016) Validation of suitable reference genes for quantitative gene expression analysis in *Panax ginseng*. *Front. Plant Sci.,* 6, 1259. doi.10.3389/fpls.2015.01259.

Xia, S., Zhang, H., He, S. (2023) Genome-Wide Identification and Expression Analysis of ACTIN Family Genes in the Sweet Potato and Its Two Diploid Relatives. *Int. J. Mol. Sci.,* 24(13), 10930. doi: 10.3390/ijms241310930.

Xiao Z., Sun X., Liu X., Li C., He L., Chen S., Su J. (2016) Selection of Reliable Reference Genes for Gene Expression Studies on *Rhododendron molle* G. Don. *Front. Plant Sci.,* 7, 1547. doi.10.3389/fpls.2016.01547.

Xu Y., Zhu X., Gong Y., Xu L., Wang Y., Liu L. (2012) Evaluation of reference genes for gene expression studies in radish (*Raphanus sativus* L.) using quantitative real-time PCR. *Biochem. Biophys. Res. Commun.,* 424(4), 398-403. doi.10.1016/j.bbrc.2012.06.119.

Xu Y., Li H., Li X., Lin J., *et al.* (2015) Systematic selection and validation of appropriate reference genes for gene expression studies by quantitative real-time PCR in pear. *Acta Physiol. Plant.,* 37, 40. doi.org/10.1007/s11738-015-1784-0.

You S.H., Cao K., Chen C.W., Li Y., Wu J.L., Zhu G.R., et al. (2021) Selection and validation reference genes for qRT-PCR normalization in different cultivars during fruit ripening and softening of peach (*Prunus persica*). *Sci. Rep.,* 11(1), 7302. doi.10.1038/s41598-021-86755-5.

Zhang F., Chi W., Wang Q., Zhang Q., Wu N.H. (2003) Molecular cloning of C_4- specific *Ppc* gene of sorghum and its high level expression in transgenic rice. *Chinese Sci. Bull.,* 48, 1835-1840. doi.org/10.1007/BF03184064.

Zhang G., Liu X., Quan Z., Cheng S., Xu X., Pan S., *et al.* (2012) Genome sequence of foxtail millet (*Setaria italica*) provides insights into grass evolution and biofuel potential. *Nat. Biotechnol.,* 30, 549-554. doi.org/10.1038/nbt.2195.

Zhang H., Xu W., Wang H., Hu L., Li Y., Qi X., Zhang L., Li C., Hua X. (2014) Pyramiding expression of maize genes encoding phosphoenolpyruvate carboxylase (PEPC) and pyruvate orthophosphate dikinase (PPDK) synergistically improve the photosynthetic characteristics of transgenic wheat. *Protoplasma.,* 251(5), 1163-1173. doi.10.1007/s00709-014-0624-1.

Zhang Y. T., Peng X. R., Liu Y., Li Y. L., Luo Y., Wang X. R., Tang H. R. (2018) Evaluation of suitable reference genes for qRT-PCR normalization in strawberry (Fragaria × ananassa) under different experimental conditions. *BMC Mol. Biol.,* 19(1), 8. doi.org/10.1186/s12867-018-0109-4.

Zhu, Z., Yu, J., Tang, X., Xiong, A., Sun, M. (2023) Selection and validation of reference genes in different tissues of Okra (*Abelmoschus esculentus* L.) under different abiotic stresses. *Genes.,* 14(3), 603. doi.org/10.3390/genes14030603.

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