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Selection and validation of stable reference genes for RT-qPCR analysis across seven *Musa* genomes during early flowering development

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[1]Department of Agricultural Science, Faculty of Agriculture, Natural Resources and Environment, Naresuan University. 99 Moo 9, Mueang Phitsanulok 65000, Phitsanulok, Thailand. Tel.: +66 55 962 736, ♥email: thanitab@nu.ac.th
[2]Center of Excellence in Research for Agricultural Biotechnology, Naresuan University. 99 Moo 9, Mueang Phitsanulok 65000, Phitsanulok, Thailand
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Abstract. *Sujipuli K, Mingmanit Y, Inthima P, Laywisadkul S, Ratanasut K, Premjet D, Premjet S, Pongcharoen P, Pathaichindachote W, Phinyo M, Boonsrangsom T. 2025. Biodiversitas 26: 134-144.* Banana (*Musa* spp.) is a globally important fruit crop, with most edible varieties resulting from hybridizations between *Musa acuminata* (AA genome) and *Musa balbisiana* (BB genome). Seed formation in hybrid bananas is undesirable for both fresh consumption and processing, making it crucial to understand the genetic mechanisms controlling pollen viability and development to prevent seed set in commercial cultivation. However, comprehensive evaluations of reference genes for gene expression studies in various *Musa* genomic groups are still lacking. This study evaluated five candidate reference genes-*ACT2*, *CAC*, *RPS4*, *RPL4*, and *SAMDC1*-across seven *Musa* genomes using reverse transcriptase quantitative real-time PCR (RT-qPCR). Four of these genes (*CAC*, *RPS4*, *RPL4*, and *SAMDC1*) demonstrated high primer specificity, with single PCR amplification peaks and melting curve Tm values ranging from 75.42 ± 0.03 to 82.51 ± 0.03. Expression abundance varied, with Ct values between 21.58 ± 0.26 and 24.68 ± 0.20. Using five stability analysis programs, *CAC* was identified as the most stable reference gene across all seven *Musa* genomes, making it the optimal candidate for normalizing gene expression data in banana studies. This study provides a valuable tool for enhancing the accuracy of gene expression analysis in banana breeding programs targeting seedless fruit production, thereby demonstrating the practical relevance of reference gene evaluation in banana genomic studies.

Keywords: Banana genomes, gene expression, *Musa* spp., reference gene, RT-qPCR

Abbreviations: Ct: Threshold cycle; RT-qPCR: Reverse transcriptase quantitative real-time polymerase chain reaction

INTRODUCTION

Banana (*Musa* spp.) is one of the most important fruit crops globally, widely consumed as both a staple food and for commercial processing (FAO 2023). Primarily cultivated in subtropical and tropical regions, including Thailand, the genus *Musa* comprises over 1,000 species. *Musa acuminata* Colla (AA genome; 2n=2x=22) and *Musa balbisiana* Colla (BB genome; 2n=2x=22) serve as primary wild species for many edible banana cultivars. Interspecific and intraspecific hybridization between these species has resulted in several genome groups with various polyploid levels: AA, AB, BB, AAA, AAB, ABB, AAAB, AABB, and ABBB (Akech et al. 2024). Previous studies highlight considerable morphological and genetic diversity among cultivars across these genome groups (El-Shahed et al. 2017; Boonsrangsom et al. 2020, 2023, 2024; Premjet et al. 2022; Safhi et al. 2023; Slameto 2023), while recent findings reveal significant differences in pollen viability, especially between AA and BB genomes (Mingmanit et al. 2023). This genetic diversity is crucial for breeding programs focused on improving crop traits.

Typically, most banana cultivars flower 7-9 months after planting, with an additional 2-3 months required for fruit development and harvest (Chaurasia et al. 2017). However, abiotic stresses, such as temperature fluctuations and water deficits, and biotic stresses like *Fusarium* wilt (caused by *Fusarium oxysporum* f. sp. *cubense*) frequently cause yield losses during this critical stage (Zheng et al. 2018a). Understanding the genetic mechanisms that regulate flowering and stress responses is essential for developing new genotypes with improved fruit quality, shorter life cycles, and enhanced stress resistance, ultimately increasing banana production resilience against environmental challenges.

Reverse transcription-quantitative real-time PCR (RT-qPCR) is a robust and precise technique widely used for investigating gene expression in *Musa* species. This method provides high sensitivity, specificity, and reproducibility, even for detecting low-abundance mRNA transcripts. RT-qPCR is particularly valuable for elucidating genetic mechanisms at the cellular level and identifying candidate genes critical for genetic improvement (Yu et al. 2021). For example, the expression profiles of *CAT*, *PR10*, and *PAL* genes were analyzed in response to *Fusarium oxysporum* f.

sp. *cubense* tropical race 4 in *Musa acuminata* cv. Berangan (genome AAA) (Munusamy et al. 2019). Similarly, the *TPD1A* gene was found to have high expression levels, playing a critical role in pollen formation and fruit development in the diploid *Musa itinerans* cv. Yunnan (genome AA) is a close relative of *M. acuminata* (Hu et al. 2020). Additionally, *MYB* genes from *Musa acuminata* cv. DH-Pahang (genome AA) was shown to regulate secondary wall deposition and respond to root-knot nematode infestation (Castañeda et al. 2017; Pucker et al. 2020).

Reference genes, also known as housekeeping genes, are crucial for normalizing gene expression levels in RT-qPCR assays. Ideally, these genes should maintain stable expression across various tissues and experimental conditions, with consistent threshold cycle (Ct) values typically within the range of 15 to 30. However, identifying appropriate reference genes can be particularly challenging in polyploid plants like bananas, where expression levels often vary across tissues and conditions. Previous studies have identified reference genes for specific *Musa* cultivars, such as *RPS2* and *UBQ2* in *Musa acuminata* cv. Cavendish (genome AAA) (Chen et al. 2011) and *APT* and *UBQ2* in *Musa acuminata* cv. Calcutta 4 (genome AA) (Rego et al. 2019). However, research on other banana genome groups remains limited, and traditional reference genes like *β-actin* have shown variable expression across different species and conditions (Tian et al. 2015; Li et al. 2016; Wang et al. 2016). This variability highlights the need for a comprehensive evaluation of reference genes in polyploid plants like *Musa*.

Due to the genetic diversity and complexity of bananas, identifying stable reference genes for accurate RT-qPCR normalization across all *Musa* genome groups is essential. Although several reference genes have been validated in diploid (AA) bananas (Hu et al. 2020) and triploid (AAA) bananas (Chen et al. 2011; Rego et al. 2019), no comprehensive study has evaluated suitable reference genes across the full spectrum of *Musa* genomes. This study addresses this critical gap by identifying the most stable reference genes for RT-qPCR normalization during the early flowering stage across seven *Musa* genome groups (AA, BB, AAA, BBB, AAB, ABB, and ABBB). These findings will improve the precision of gene expression analyses, facilitating the development of superior banana genotypes with traits such as enhanced fruit quality, shorter life cycles, and increased disease resistance.

MATERIALS AND METHODS

Plant materials

Twelve Thai banana cultivars, representing seven genome groups-2 AA, 2 BB, 2 AAA, 1 BBB, 2 AAB, 2 ABB, and 1 ABBB-were sourced from the *Musa* germplasm collection at the Phitsanulok Agricultural Extension and Development Center in Phitsanulok Province, Thailand (Table 1). For each cultivar, the youngest rolled leaf was harvested during the early flowering stage, rapidly frozen in liquid nitrogen, and stored at -80°C until further analysis.

Procedures

Total RNA isolation and cDNA synthesis

Total RNA was extracted from each young leaf sample (100 mg) by grinding the tissue into a fine powder in liquid nitrogen and using the cetyl-trimethylammonium bromide (CTAB) method (Khairul-Anuar et al. 2019), with slight modifications. A 24:1 ratio of chloroform to isoamyl alcohol (without phenol) was used during extraction. Next, to remove residual DNA, each RNA sample (1 µg) was treated with two units of DNase I (Thermo Fisher Scientific, Baltics UAB, Lithuania). RNA concentration and purity were then assessed by measuring absorbance at 260 and 280 nm using a NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Only RNA samples with an $A_{260}/_{280}$ ratio between 1.8 and 2.0 were used for further analysis. The integrity of the RNA was verified via 1.2% agarose gel electrophoresis, requiring clear, enriched bands for the 28S (~4.5 kb) and 18S (~1.9 kb) ribosomal RNAs (rRNAs), with the 28S rRNA band being approximately twice the intensity of the 18S rRNA band and no evidence of smearing.

First-strand cDNA was synthesized by reverse-transcribing 500 ng of total RNA with the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. Briefly, the 20 µL reaction mixture included 1 µL of Oligo $(dT)_{18}$ primers, 2 µL of 10 mM dNTP mix, 4 µL of 5X Reaction Buffer, 1 µL of RiboLock RNase Inhibitor (20 U/µL), and 1 µL of RevertAid M-MuLV Reverse Transcriptase (200 U/µL), adjusted to a final volume of 20 µL with nuclease-free water. The reaction was incubated at 25°C for 5 minutes, 42°C for 60 minutes, and then terminated at 70°C for 5 minutes. The resulting first-strand cDNA was either immediately used for RT-qPCR amplification or stored at -20°C for future use.

Table 1. List of twelve *Musa* cultivars representing seven genome groups used in this study

Botanical name 'Cultivar'	Genome
Musa cv. 'Kluai Nam Thai'	AA
Musa cv. 'Kluai Leb Mu Nang'	AA
Musa cv. 'Tani Mo'	BB
Musa cv. 'Tani Nuea'	BB
Musa cv. 'Kluai Hom Khieo'	AAA
Musa cv. 'Kluai Hom Thong'	AAA
Musa cv. 'Kluai Lep Chang Kut'	BBB
Musa cv. 'Kluai Nang Klai Surin'	AAB
Musa cv. 'Kluai Nang Paya'	AAB
Musa cv. 'Kluai Namwa Mali-Ong'	ABB
Musa cv. 'Hak Muk Khieo'	ABB
Musa cv. 'Theparod'	ABBB

Quantification of reference gene expression using RT-qPCR assay

In this study, five candidate reference genes, including *ACT2* (actin), *CAC* (clathrin adaptor complexes medium), *RPS4* (ribosomal protein S), *RPL4* (ribosomal protein L), and *SAMDC1* (S-adenosyl methionine decarboxylase), were selected to validate stable gene expression across seven *Musa* genomes (Table 2). These genes were identified based on prior studies that highlighted stable reference genes in *Musa acuminata* cv. Cavendish bananas (Chen et al. 2011; Rego et al. 2019). The selected genes are involved in key cellular functions, including cytoskeletal maintenance (*ACT2*), intracellular transport (*CAC*), protein synthesis (*RPS4* and *RPL4*), and polyamine biosynthesis (*SAMDC1*), making these candidates suitable for normalizing gene expression.

Besides the five selected genes, *UBQ2* (ubiquitin) and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) were also tested as potential reference genes. However, non-specific amplification, as indicated by multiple bands in gel electrophoresis, led to their exclusion from further analysis. The five selected reference genes were subsequently validated for specificity and stability across the seven *Musa* genomes included in this study.

RT-qPCR analysis for the expression of all candidate reference genes was performed using the Thermo Scientific Maxima SYBR Green qPCR Master Mix (2X) (No ROX) (Thermo Fisher Scientific, Waltham, MA, USA). Each 12.5 µL reaction mixture contained 6.5 µL of SYBR Green Master Mix (2X), 0.5 µL of each 10 µM forward and reverse primer, 1 µL of first-strand cDNA template, and nuclease-free water to reach the final volume of 12.5 µL. The reactions were run on the Eco48 Real-Time PCR system (Eco™48, PCRmax Limited, UK) using the following cycling conditions. An initial denaturation was performed at 95℃ for 10 minutes, followed by 40 cycles of 95℃ for 10 seconds, 55℃ for 30 seconds, and 72℃ for 30 seconds. A final extension was carried out at 72℃ for 5 minutes. A melting curve analysis was performed afterward to verify amplification specificity. Each reaction included three biological replicates, with a minimum of two technical duplicates per replicate.

Data analysis

Expression data for each candidate reference gene were visualized as threshold cycle (Ct) values from each RNA sample, directly obtained using the Eco™48 Study Software installed on the instrument. The Ct value represents the number of amplification cycles required to reach a predetermined threshold detection level during the exponential phase of the PCR reaction.

The arithmetic means of the Ct values were calculated using Microsoft Excel 2010. Next, to assess the expression stability (M value) of each candidate reference gene, five algorithm-based software programs were used: geNorm v3.4 (Vandesompele et al. 2002), NormFinder v20 (Andersen et al. 2004), BestKeeper (Pfaffl et al. 2004), the Delta Ct method (Silver et al. 2006), and RefFinder (Xie et al. 2012). Additionally, pairwise variation (V value) results from the geNorm analysis were further examined using the qBase^{PLUS} program (Hellemans et al. 2007) to determine the optimal number of reference genes needed for accurate normalization across all tested samples.

RESULTS AND DISCUSSION

Primer specificity verification of the reference genes

Five candidate reference genes and their respective primer pairs (listed in Table 2) were selected based on previous validations in the *Musa* AA and AAA genomes (Chen et al. 2011; Rego et al. 2019). Prior to RT-qPCR analysis, the specificity of each primer pair was evaluated by PCR amplification using cDNA templates from the seven *Musa* genome groups. PCR products were visualized via agarose gel electrophoresis. The results showed that all five primer pairs (*ACT2*, *CAC*, *RPS4*, *RPL4*, and *SAMDC1*) produced a single band of the expected size: 137, 146, 113, 150, and 172 bp, respectively, for the *Musa* AA genome (Figure 1.A). Interestingly, only cultivars with the A genome (AA, AAA, AAB, and ABB genomes) exhibited the expected 137-bp PCR product for *ACT2*. At the same time, no amplification was detected in cultivars containing the B genome (BB, BBB, and ABBB genomes) (Figure 1.B).

Table 2. Validation of candidate reference genes in this study

Gene	Accession no.	Function	Primer sequence (5'- 3')	Amplicon size (bp)
ACT2	HQ853238	Actin 2	F: CTTAGCACTTTCCAGCAGATG R: ACACCAAAAAACTACCCCGAC	137
CAC	HQ853240	Clathrin adaptor complex	F: CTCCTATGTTGCTCGCTTATG R: GGCTACTACTTCGGTTCTTTC	146
RPS4	HQ853247	40S Ribosomal protein S4	F: TGAGAGTGGCTTGACCCTGA R: GTGACATTTAGTCGTCTGCTGG	113
RPL4	HQ853245	40S Ribosomal protein L4	F: TTCTCCAAGTGGCTAGGTGTG R: CCCAACAATCATGTCCATAGGT	150
SAMDC1	HQ853248	s-Adenosyl methionine decarboxylase 1	F: CCACTGGGAAAATGAAGAAAC R: CAGACACAGCAAGCCACCTA	172

In this study, *UBQ2* and *GAPDH* were initially tested as potential reference genes alongside the five candidates. However, both exhibited non-specific amplification, with multiple bands observed in gel electrophoresis. As a result, these findings indicated that the primer pairs for *UBQ2* and *GAPDH* failed to produce single-band amplifications, potentially compromising downstream analyses. Therefore, these genes were excluded. Only the five selected genes (*ACT2*, *CAC*, *RPS4*, *RPL4*, and *SAMDC1*) were retained for further analysis.

Further verification of primer specificity was performed through melting curve analysis during RT-qPCR. The melting curve analysis for primers *CAC*, *RPS4*, *RPL4*, and *SAMDC1* revealed a single peak at the predicted primer annealing temperatures of 75.42 ± 0.03, 82.51 ± 0.03, 75.90 ± 0.03, and 77.82 ± 0.03, respectively (Figure 2). No amplification signals were detected in the negative controls, which lacked the cDNA template. These results confirm that the primer sets are highly specific and suitable for use in RT-qPCR analysis.

Expression abundance of candidate reference genes

The reference genes should exhibit consistent and high expression levels to serve as effective internal controls for RT-qPCR studies. In this experiment, the expression levels of the four selected reference genes (*CAC*, *RPS4*, *RPL4*, and *SAMDC1*) were assessed using threshold cycle (Ct) values, which correspond to the transcriptional mRNA levels of each gene. The results revealed varying Ct values for the four reference genes across the seven *Musa* genomes: *CAC* (23.13 ± 0.14), *RPS4* (21.58 ± 0.26), *RPL4* (24.68 ± 0.20), and *SAMDC1* (21.69 ± 0.26) (Figure 3).

Among these, *RPS4* and *SAMDC1* displayed similarly low Ct values, with no significant difference ($p \leq 0.01$), suggesting they had the highest mRNA transcript abundances across the *Musa* genomes. In contrast, *RPL4* had the highest Ct value, significantly different from the other reference genes ($p \leq 0.01$), indicating it had the lowest expression abundance. All reference genes exhibited Ct values below 30, indicating relatively high expression abundance, as recommended by Yu et al. (2021). Based on these findings, the four candidate reference genes were further evaluated for stable expression levels using RT-qPCR analysis across the seven *Musa* genomes.

Expression stability analysis of reference genes

The expression stability and ranking of the four candidate reference genes, amplified from cDNA templates of the seven *Musa* genomes, were evaluated using five software programs: geNorm v3.4, NormFinder v20, Delta Ct, BestKeeper, and RefFinder. These analyses helped identify the most suitable reference genes for normalizing target gene expression in RT-qPCR experiments. The combined results are shown in Figure 4 and Table 3.

geNorm analysis

The geNorm analysis revealed that the four reference genes (*CAC*, *RPS4*, *RPL4*, and *SAMDC1*) exhibited high expression stability, with M values below 0.5 in two *Musa* genome groups (BBB and ABBB). The lowest M values (≤0.5) indicated the most stable reference genes. Among these, the *CAC* gene demonstrated the highest expression stability, with M values ranging from 0.21 to 0.47 in most of the *Musa* genomes, except for AAA and ABB. The reference genes *SAMDC1*, *RPS4*, and *RPL4* also exhibited stable expression, with M values ranging from 0.13 to 0.44 across three genomes (BBB, AAB, and ABBB), 0.21 to 0.48 across four genomes (BBB, AAB, ABB, and ABBB), and 0.13 to 0.48 across five genomes (AA, BB, BBB, ABB, and ABBB).

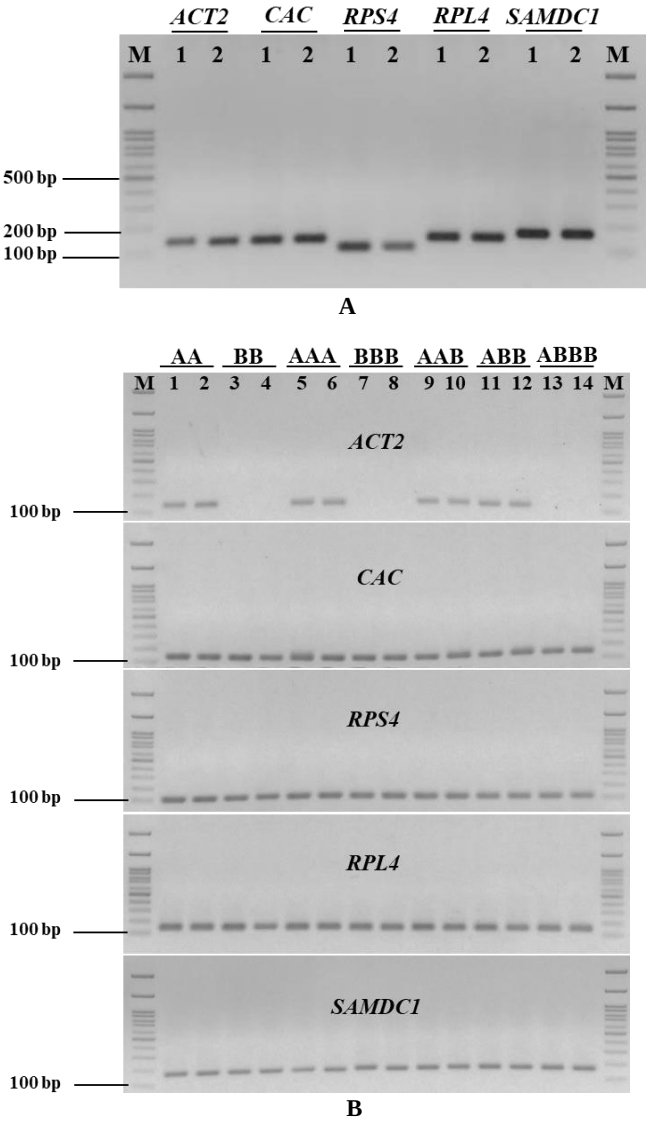

Figure 1. PCR amplification results demonstrating primer specificity for five candidate reference genes-*ACT2*, *CAC*, *RPS4*, *RPL4*, and *SAMDC1*-tested in the *Musa* AA genome (A) to confirm amplicon precision with gene-specific primers and across seven *Musa* genomes (B). Each RT-qPCR product was generated using primer pairs listed in Table 2 with cDNA templates from the seven *Musa* genomes. Lanes M: 100bp Marker (One MARK 100 DNA Ladder, Bio-Helix (GeneDirex)). Lane 1: 'Kluai Nam Thai'; Lane 2: 'Kluai Leb Mu Nang'; Lane 3: 'Tani Mo'; Lane 4: 'Tani Nuea'; Lane 5: 'Kluai Hom Khieo'; Lane 6: 'Kluai Hom Thong'; Lanes 7-8: 'Kluai Lep Chang Kut'; Lane 9: 'Kluai Nang Klai Surin'; Lane 10: 'Kluai Nang Paya'; Lane 11: 'Kluai Namwa Mali-Ong'; Lane 12: 'Hak Muk Khieo'; Lanes 13-14: 'Theparod'

Table 3. Stability analysis of four candidate reference genes (M Values) across seven *Musa* genome groups using five algorithms

Genome	Rank	geNorm		NormFinder		BestKeeper		Delta Ct		RefFinder	
		Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability
AA	1	*CAC*	0.28	*RPL4*	0.14	*RPS4*	0.46	*RPL4*	0.52	*RPL4*	1.32
	2	*RPL4*	0.28	*CAC*	0.20	*CAC*	0.88	*CAC*	0.56	*CAC*	1.68
	3	*SAMDC1*	0.51	*SAMDC1*	0.66	*RPL4*	0.94	*SAMDC1*	0.76	*RPS4*	2.83
	4	*RPS4*	0.67	*RPS4*	0.76	*SAMDC1*	1.17	*RPS4*	0.83	*SAMDC1*	3.22
BB	1	*CAC*	0.42	*RPS4*	0.37	*SAMDC1*	0.51	*RPS4*	1.07	*RPS4*	1.57
	2	*RPL4*	0.42	*CAC*	0.60	*RPS4*	0.95	*CAC*	1.08	*CAC*	1.86
	3	*RPS4*	1.01	*RPL4*	1.15	*CAC*	1.96	*RPL4*	1.29	*RPL4*	2.45
	4	*SAMDC1*	1.22	*SAMDC1*	1.32	*RPL4*	2.28	*SAMDC1*	1.42	*SAMDC1*	2.83
AAA	1	*RPS4*	0.52	*RPL4*	0.28	*RPS4*	0.77	*RPL4*	0.64	*RPS4*	1.19
	2	*RPL4*	0.52	*CAC*	0.49	*RPL4*	0.87	*CAC*	0.72	*RPL4*	1.73
	3	*CAC*	0.66	*RPS4*	0.60	*CAC*	0.95	*RPS4*	0.76	*CAC*	2.45
	4	*SAMDC1*	0.73	*SAMDC1*	0.64	*SAMDC1*	1.19	*SAMDC1*	0.79	*SAMDC1*	4.00
BBB	1	*CAC*	0.21	*RPL4*	0.12	*RPS4*	0.16	*RPL4*	0.32	*RPS4*	1.41
	2	*RPS4*	0.21	*RPS4*	0.18	*RPL4*	0.18	*RPS4*	0.33	*RPL4*	1.57
	3	*RPL4*	0.28	*CAC*	0.27	*CAC*	0.20	*CAC*	0.35	*CAC*	2.28
	4	*SAMDC1*	0.36	*SAMDC1*	0.41	*SAMDC1*	0.20	*SAMDC1*	0.45	*SAMDC1*	4.00
AAB	1	*RPS4*	0.44	*CAC*	0.15	*CAC*	0.19	*CAC*	0.52	*CAC*	1.32
	2	*SAMDC1*	0.44	*SAMDC1*	0.24	*RPL4*	0.30	*SAMDC1*	0.53	*SAMDC1*	1.86
	3	*CAC*	0.47	*RPS4*	0.51	*SAMDC1*	0.38	*RPS4*	0.62	*RPS4*	2.45
	4	*RPL4*	0.60	*RPL4*	0.66	*RPS4*	0.53	*RPL4*	0.73	*RPL4*	3.36
ABB	1	*RPS4*	0.48	*CAC*	0.34	*CAC*	0.91	*CAC*	0.85	*SAMDC1*	1.19
	2	*RPL4*	0.48	*RPL4*	0.58	*RPS4*	1.00	*RPL4*	0.87	*RPS4*	1.86
	3	*CAC*	0.71	*RPS4*	0.59	*RPL4*	1.06	*RPS4*	0.88	*CAC*	2.83
	4	*SAMDC1*	0.94	*SAMDC1*	1.08	*SAMDC1*	1.15	*SAMDC1*	1.18	*RPL4*	3.22
ABBB	1	*RPL4*	0.13	*SAMDC1*	0.16	*SAMDC1*	0.16	*SAMDC1*	0.36	*SAMDC1*	1.00
	2	*SAMDC1*	0.13	*RPS4*	0.19	*RPL4*	0.17	*RPS4*	0.41	*RPL4*	2.06
	3	*RPS4*	0.34	*RPL4*	0.38	*RPS4*	0.31	*RPL4*	0.44	*RPS4*	2.45
	4	*CAC*	0.44	*CAC*	0.49	*CAC*	0.35	*CAC*	0.54	*CAC*	4.00

Notes: Expression stability and ranking of the four candidate reference genes, amplified from cDNA templates of the seven *Musa* genomes, were analyzed using the following programs: geNorm v3.4 (Vandesompele et al. 2002), NormFinder v20 (Andersen et al. 2004), BestKeeper (Pfaffl et al. 2004), Delta Ct method (Silver et al. 2006), and RefFinder (Xie et al. 2012). A lower stability value, as calculated by these programs, indicates greater gene expression stability. Each stability value is derived from the analysis of tetra-replicates

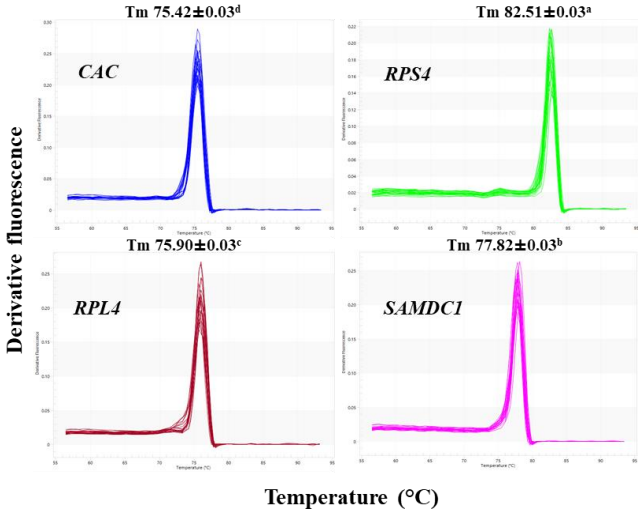

Figure 2. Melting curve analysis of four candidate reference genes across seven *Musa* genomes (n=24; 1 gene × 2 replicates × 12 genotypes): *CAC*, *RPS4*, *RPL4*, and *SAMDC1*. Dissociation curves of RT-qPCR products were analyzed using two biological replicates per gene across the seven *Musa* genomes. A single peak at the melting temperature (Tm) confirms a single PCR product per sample. Data represent mean ± SE from four biological replicates. Different letters indicate significant differences among genes based on DMRT analysis at $p \leq 0.01$

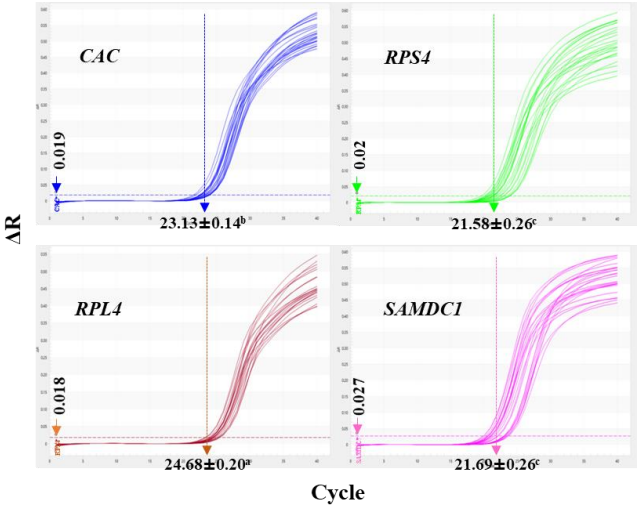

Figure 3. Threshold cycle (Ct) values for candidate reference genes across seven *Musa* genomes (n=24; 1 gene x 2 replicates x 12 genotypes): *CAC*, *RPS4*, *RPL4*, and *SAMDC1*. Ct values were analyzed using RT-qPCR software with two biological replicates for each candidate reference gene. The Ct value represents the cycle number at which the amplification reaction enters the early exponential phase, correlating with the amount of PCR product generated; lower Ct values indicate higher mRNA abundance. Data represent mean ± SE of four biological replicates. Different letters denote significant differences among genes as determined by DMRT analysis at $p \leq 0.01$

To ensure accurate normalization, pairwise variation (V) was calculated using qBase^{PLUS} software (Hellemans et al. 2007). If the Vn/Vn+1 value (where n represents the number of reference genes) is less than or equal to 0.15, then n reference genes are considered sufficient for normalization. In all genomes except for BB, the V2/3 pairwise variation was below this threshold, confirming that the top two reference genes identified by geNorm were sufficient for normalization (Figure 5).

NormFinder analysis

For the NormFinder analysis, the expression stability (M value) of each reference gene was calculated based on its expression variation, preventing the selection of co-regulated genes (Andersen et al. 2004). A smaller M value indicates better stability. The results showed that all candidate reference genes (*CAC*, *RPS4*, *RPL4*, and *SAMDC1*) exhibited stable gene expression, with M values ranging from 0.12 to 0.49 across two *Musa* genome groups (BBB and ABBB) (Figure 4).

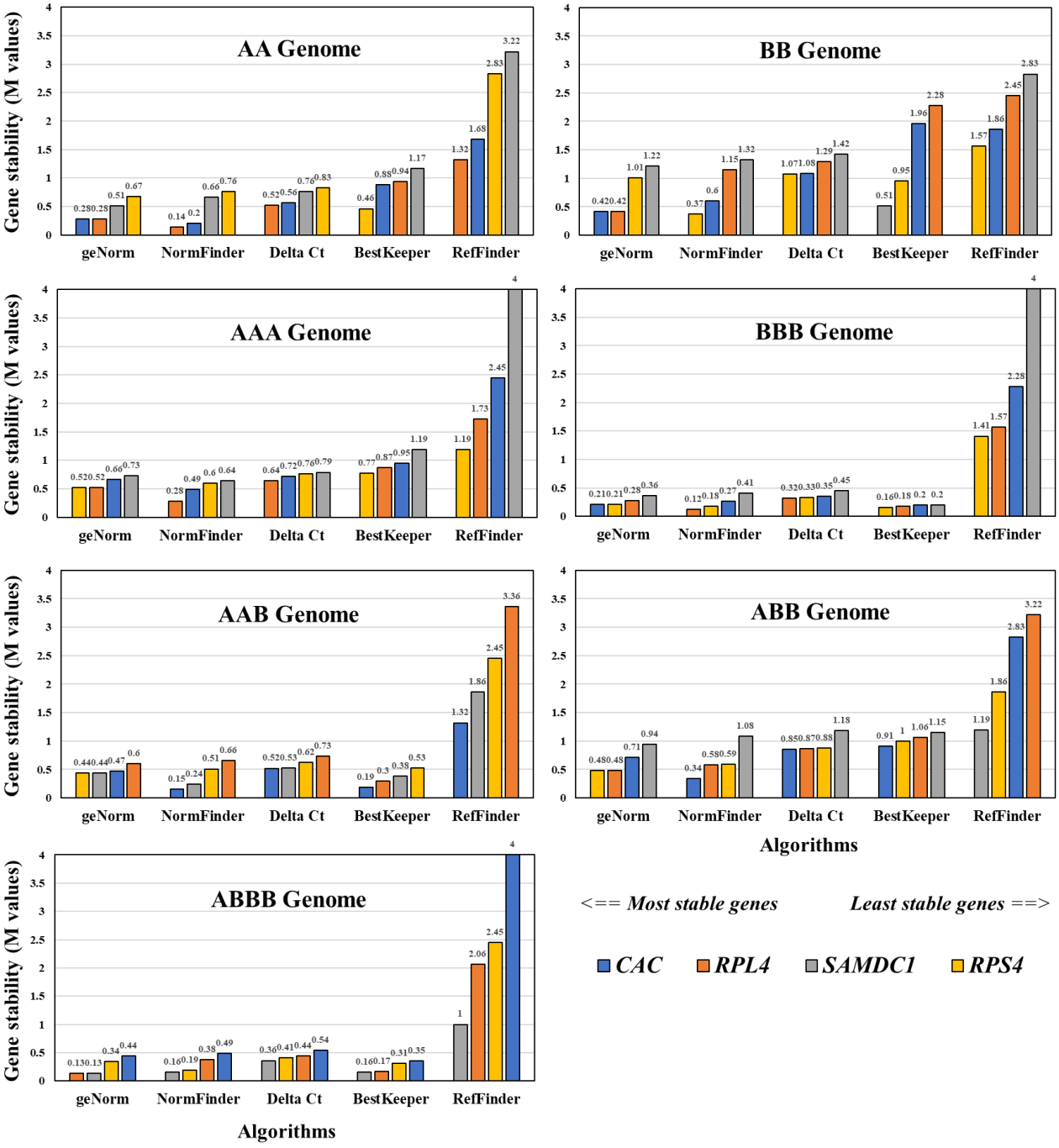

Figure 4. Gene stability (M values) and stability ranks of four candidate reference genes (*CAC*, *RPS4*, *RPL4*, and *SAMDC1*) across seven *Musa* genome groups, evaluated using five algorithms: geNorm, NormFinder, Delta Ct, BestKeeper, and RefFinder

Among these, *CAC* displayed the most stable expression, with M values ranging from 0.20 to 0.49 across six *Musa* genomes (AA, AAA, BBB, AAB, ABB, and ABBB). *RPL4* also demonstrated high expression stability, with M values ranging from 0.12 to 0.38 in four genomes (AA, AAA, BBB, and ABBB). *RPS4* showed stable expression in three B genome-containing groups (BB, BBB, and ABBB), with M values between 0.18 and 0.37. Finally, *SAMDC1* exhibited the most stable expression in three genomes (AAB, BBB, and ABBB), with M values ranging from 0.16 to 0.41. In summary, *CAC* was identified as the most suitable reference gene across all *Musa* genomes studied, except for the BB genome, where *RPS4* was the more stable option.

Delta Ct analysis

The Delta Ct (ΔCt) analysis produced results consistent with those from the NormFinder tool (Figure 4) in evaluating the expression stability of all reference genes. In this analysis, the stability of housekeeping genes was assessed by comparing the relative expression of gene pairs within each sample. A constant ΔCt value between two genes across different RNA samples indicated stable expression of both genes. In contrast, variations in the ΔCt value suggested inconsistent expression of one or both genes (Silver et al. 2006). A smaller ΔCt value indicated greater expression stability.

BestKeeper analysis

The BestKeeper software tool identifies the most suitable reference genes by creating an index from four housekeeping genes in each *Musa* genome, calculated using crossing point (CP) values as previously described (Pfaffl et al. 2004). This index is compared with the tested reference genes to assess expression stability based on two key variables: standard deviation (SD) and CP value (Pfaffl et al. 2004). An SD value of less than 1.0 is considered optimal for expression stability, with smaller SD values indicating greater stability (Zhou et al. 2022). The results from the BestKeeper analysis (Figure 4) differed from those obtained using the other three software tools (geNorm, NormFinder, and Delta Ct). Notably, the *RPS4* gene exhibited the most stable expression across all *Musa* genomes, with SD values ranging from 0.16 to 1.00. The *CAC* gene demonstrated high stability in six *Musa* genomes (AA, AAA, BBB, AAB, ABB, and ABBB), with an SD range of 0.19 to 0.95. Likewise, the *RPL4* gene showed stable expression across five *Musa* genomes (AA, AAA, BBB, AAB, and ABBB), with an SD range of 0.17 to 0.94. Finally, the *SAMDC1* gene displayed stability in four *Musa* genomes (BB, BBB, AAB, and ABBB), with an SD range of 0.16 to 0.51.

RefFinder analysis

The stability values from the RefFinder analysis, ranging from 1.00 to 1.86, revealed that the expression of several genes was highly consistent across different *Musa* genomes. The *RPL4* gene showed lower stability values in the AA, AAA, and BBB genomes, while the *CAC* gene exhibited consistent expression in the AA, BB, and AAB genomes. The *RPS4* gene demonstrated stable expression in the BB, AAA, BBB, and ABB genomes, and the *SAMDC1* gene was stable in the AAB, ABB, and ABBB genomes (Figure 4). Of these, *SAMDC1* exhibited the highest stability, ranking 1st with a stability value of 1.00 in the ABBB genome and 2nd with a value of 1.19 in the ABB genome. Similarly, the *RPS4* gene ranked 2nd with a stability value of 1.19 in the AAA genome.

Overall, the results from all software analyses are summarized in Figure 6 and Table 4. The expression stability of each candidate reference gene, evaluated from pooled data across the seven *Musa* genomes (related to Figure 4), was assessed using five algorithms: geNorm, NormFinder, Delta Ct, BestKeeper, and RefFinder. The four candidate reference genes exhibited varying stability values, with some genes falling below the accepted threshold in certain *Musa* genomes.

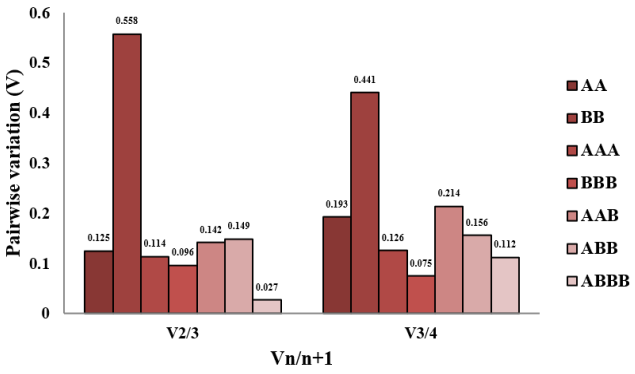

Figure 5. Pairwise variations (V) analysis was performed using qBase^{PLUS} to identify the optimal number of reference genes required for accurate normalization across all tested samples. V values below 0.15 suggest that no additional reference genes are necessary to calculate a reliable normalization factor

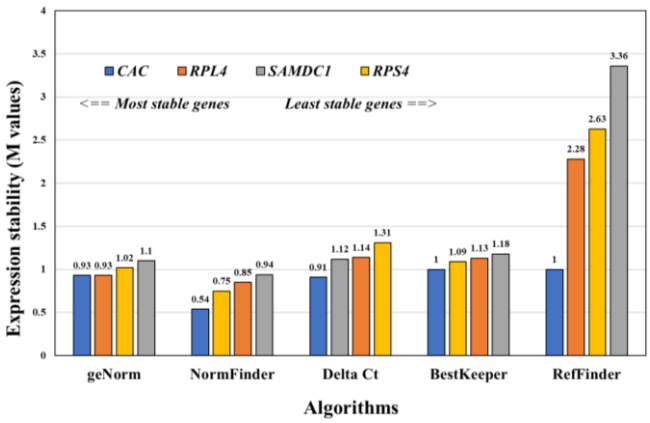

Figure 6. Summary of the stability and ranking of candidate reference genes across seven *Musa* genomes, evaluated using geNorm, NormFinder, Delta Ct, BestKeeper, and RefFinder

Table 4. Expression stability of candidate reference genes across seven *Musa* genomes, assessed by five algorithms: geNorm, NormFinder, BestKeeper, Delta Ct, and RefFinder

Rank	geNorm		NormFinder		BestKeeper		Delta Ct		RefFinder	
	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability
1	*CAC*	0.93	*CAC*	0.54	*CAC*	1.00	*CAC*	0.91	*CAC*	1.00
2	*RPL4*	0.93	*RPS4*	0.75	*RPS4*	1.09	*SAMDC1*	1.12	*RPL4*	2.28
3	*RPS4*	1.02	*RPL4*	0.85	*RPL4*	1.13	*RPL4*	1.14	*RPS4*	2.63
4	*SAMDC1*	1.10	*SAMDC1*	0.94	*SAMDC1*	1.18	*RPS4*	1.31	*SAMDC1*	3.36

Notes: A lower stability value indicates greater gene expression stability. Each stability value was calculated from 48 replicates (1 gene × 4 biological replicates × 12 genotypes)

Among these, *CAC* emerged as the most stable reference gene in the AA and AAB genomes, showing consistent expression stability across multiple analysis tools. *RPL4* demonstrated the highest expression stability in the AA and BBB genomes, while *RPS4* was identified as the most stable in the BBB genome. Lastly, *SAMDC1* displayed highly stable expression in the AAB genome. These findings confirm the suitability of these reference genes for normalizing gene expression in *Musa* using RT-qPCR across diverse genomic backgrounds.

Discussion

Reverse transcription-quantitative real-time PCR (RT-qPCR) is essential for evaluating gene expression profiles by quantifying mRNA transcript levels. Selecting appropriate reference genes is essential to account for technical variations between samples (Lü et al. 2018). Reliable reference genes help to reduce expression variability, thereby improving the precision of target gene quantification (Pinheiro and Siegfried 2020). In *Musa* species, reference genes have proven invaluable for RT-qPCR normalization across diverse genotypes (Yang et al. 2015; Costa et al. 2024), various tissues and developmental stages (Chen et al. 2011; Hu et al. 2020), and in response to abiotic and biotic stressors (Lee et al. 2015; Zhang et al. 2017; Rego et al. 2019; Nocum et al. 2022). However, most research has primarily focused on the A genome (AA and AAA), with a limited investigation into stability across other *Musa* genomic groups.

Therefore, to address this gap, this study assessed the primer specificity and stability of five reference genes (*ACT2*, *CAC*, *RPS4*, *RPL4*, and *SAMDC1*) across seven *Musa* genome groups (AA, BB, AAA, BBB, ABB, AAB, and ABBB) using a multi-algorithm approach. Results showed that *ACT2* was effectively amplified only in samples containing the A genome, indicating its specificity for conserved sequences unique to *M. acuminata* (A genome), consistent with prior validations in *Musa acuminata* cv. Cavendish (AAA genome) (Chen et al. 2011; Rego et al. 2019). In contrast, *CAC*, *RPS4*, *RPL4*, and *SAMDC1* demonstrated high primer specificity, yielding single amplicons in both PCR and RT-qPCR assays across all genome groups, indicating their stability and suitability for broad application. Chen et al. (2011) suggested that *ACT* and *GAPDH* may lack stability for RT-qPCR normalization in *Musa acuminata*, particularly under diverse ripening and stress conditions. In contrast, alternatives such as *βTUB3*, *L2*, *EF1α*, *UBQ2*, and *ACTA1* have demonstrated better consistency, especially under stress conditions like *Fusarium* infection (Costa et al. 2024). Although *UBQ2* has shown utility in other studies, it was unsuitable in this study due to issues with non-specific amplification, highlighting the necessity of rigorous validation under varying conditions. The validated reference genes *CAC*, *RPS4*, *RPL4*, and *SAMDC1*, all transcribed by RNA polymerase II, provide a stable basis for RT-qPCR normalization, allowing more accurate insights into gene function across diverse *Musa* genotypes and conditions.

Selecting suitable housekeeping genes is critical for accurate normalization in RT-qPCR, as factors like sample type and genetic background significantly influence expression stability (Qi et al. 2016; Zheng et al. 2018b). For example, during the development of *Chrysanthemum lavandulifolium* (Fisch. ex Trautv.) Makino, the *SAND* gene exhibited the highest expression stability across all tissues. In Chinese large-flowered chrysanthemum cultivars, *SAND* and *PGK* exhibited high stability, whereas in potted chrysanthemums, only *PGK* was consistently stable (Qi et al. 2016). In this study, *CAC* emerged as the most consistent gene across *Musa* genomes, likely due to its role in clathrin adaptor complexes, which are essential for vesicle formation and intracellular transport in eukaryotes (Jackson et al. 2010). Previous studies further demonstrate the validity of *CAC* as a reference gene across various stages of banana fruit development (Chen et al. 2011) and in other plant species. For instance, *CAC* maintained stability under postharvest treatments in plums (You et al. 2016) and during reproductive stages in *Salvia hispanica* (Gopalam et al. 2017). Additionally, *CAC* and *ClACT* showed stability during watermelon fruit development (Kong et al. 2015); *CAC* remained robust under diverse environmental conditions in *Aegilops tauschii*, a resilient annual weed (Abbas et al. 2021). The role of *CAC* as a stable reference gene was further corroborated by its ability to account for expression variability across diverse conditions and tissues, as discussed in prior research.

The expression stability and ranking of the four candidate reference genes, amplified from cDNA templates of the seven *Musa* genomes, were evaluated using five software programs. In this study, RT-qPCR software analysis showed that Ct values directly reflected mRNA abundance, where higher Ct values indicated lower gene expression levels. Specifically, Ct values for the four selected reference genes (*CAC*, *RPS4*, *RPL4*, and *SAMDC1*) ranged from 21.58 ± 0.26 to 24.68 ± 0.20, suggesting that each gene could reliably normalize target gene expression across

Musa genomes. Significantly, these Ct values align with the ideal range (15-30) recommended for accurate quantification (Yu et al. 2021; Zhou et al. 2022). Therefore, the four selected reference genes meet stability criteria suitable for RT-qPCR analysis.

Because environmental factors significantly affect the stability of gene expression, potential variability was taken into account by validating candidate genes across a range of genetic backgrounds and experimental conditions. Ideally, a reference gene should demonstrate consistent expression across different genotypes and experimental conditions, and it should be involved in fundamental cellular processes (Pinheiro and Siegfried 2020). To ensure this, *CAC*, *RPS4*, *RPL4*, and *SAMDC1* were evaluated across seven *Musa* genomes using five algorithms: geNorm, NormFinder, Delta Ct, BestKeeper, and RefFinder. However, stability results from geNorm differed slightly from those of the other software (Figure 4 and Table 3). This discrepancy likely arises because geNorm relies on the comparative Ct method for co-regulated genes, while the other algorithms calculate stability independently for each gene (Sagun et al. 2020). As a result, many studies combine geNorm with additional algorithms (e.g., NormFinder, BestKeeper, Delta Ct, or RefFinder) to increase accuracy, as seen in studies on banana (Chen et al. 2011; Zhang et al. 2017; Rego et al. 2019), rapeseed (Han et al. 2017), rice (Sagun et al. 2020), and kiwifruit (Zhou et al. 2022). As summarized in Figure 6 and Table 4, each gene's stability across the seven *Musa* genomes was calculated from pooled sample results using Microsoft Excel 2010. Importantly, *CAC* exhibited the lowest expression stability values across all algorithms, confirming it as the optimal reference gene for *Musa* genomes. Moreover, *RPL4* and *RPS4* ranked second in stability, while *SAMDC1* showed the least stability, as confirmed by all algorithms except BestKeeper. The observed variation in stability across algorithms underscores the importance of employing a multi-faceted evaluation approach to ensure robustness. The stability and accuracy of *CAC* as a reference gene were further validated in a related study by our research team (Mingmanit et al. 2023). In this study, *CAC* was used to normalize three target genes associated with pollen formation (*TPD1A*, *PTC1*, and *MYB80*) across six *Musa* cultivars with varying pollen viability levels and genomic compositions (AA, BB, AAA, and ABB genomes). Despite differences in genomic background and physiological traits, *CAC* consistently demonstrated stable expression, confirming its reliability as a robust reference gene under diverse experimental conditions.

This study is the first to confirm that *CAC* is the preferred and reliable reference gene for RT-qPCR normalization across *Musa* genomes A and B, encompassing diverse genotypes such as AA, BB, AAA, BBB, AAB, ABB, and ABBB. In contrast, previous research primarily focused on reference genes for *M. acuminata* with the A genome; our findings expand the scope to include a more comprehensive range of genomic compositions. Future studies should aim to evaluate additional genotypes, tissues, and experimental conditions, addressing potential limitations such as environmental variability, which can affect reference gene stability. These findings underscore the broad applicability of *CAC* as a reliable reference gene for gene expression analysis across both species and environmental contexts, providing a solid foundation for future studies.

Moreover, this comprehensive validation of *CAC*, along with *RPL4* and *RPS4*, establishes a robust foundation for more accurate and consistent gene expression studies in *Musa*. It also highlights the potential for these findings to be applied to other plant species under diverse experimental conditions and genomic compositions. Numerous studies have emphasized the importance of identifying reference genes with consistent expression across closely related species, as well as across varying genotypes and experimental conditions. For instance, the discovery of stable reference genes has significantly improved gene expression analysis in monocotyledonous species such as *Oryza sativa* and *Zea mays*, particularly under diverse biotic and abiotic stresses (Sagun et al. 2020; Oliveira et al. 2021). Similarly, the validation of *CAC* in this study underscores its potential as a universal reference gene for other members of the *Musa* genus, including *Musa balbisiana* and *Musa acuminata*, as well as hybrids with distinct genome compositions, such as ABB and AAB genomes.

In particular, the validated reference genes hold significant promise for banana breeding programs. They enhance gene expression analysis, enabling more precise selection for critical traits such as disease resistance, stress tolerance, and fruit quality. Furthermore, their application in marker-assisted selection (MAS) allows for the more accurate screening of genetic markers linked to these traits, thus accelerating the development of improved banana cultivars. Investing in the validation of these reference genes is crucial for accurate normalization in gene expression studies, improving data interpretation and boosting breeding program efficiency. This facilitates the identification of genetic markers related to adaptive traits, supporting the development of high-quality banana varieties better suited to evolving environmental conditions, climate change, and new pathogens. These efforts represent a strategic investment in the long-term resilience and success of banana breeding programs. Additionally, these findings align with previous studies highlighting the importance of reference genes involved in essential physiological processes, which tend to exhibit stable expression across species with shared evolutionary histories (Wang et al. 2016; You et al. 2021).

By incorporating stability assessments under varying environmental conditions, this study addresses critical limitations in current methodologies, paving the way for improved applications in breeding programs and stress physiology studies. The consistent performance of *CAC* across diverse *Musa* genotypes and experimental conditions, including its successful application in pollen development studies (Mingmanit et al. 2023), further solidifies its utility as a universal reference gene within the genus. Additionally, *RPL4* and *RPS4* demonstrated exceptional stability in specific genomes, such as AA and BBB, suggesting their potential as genome-specific reference genes. This genome-specific stability could prove particularly advantageous for designing RT-qPCR assays tailored to various *Musa* species or hybrids with differing genomic compositions. Extending these validations to additional *Musa* species and

hybrids will further enhance the reliability of these reference genes, ultimately advancing gene expression normalization across diverse genomic backgrounds.

In conclusion, this study identifies *CAC* as the most stable reference gene for RT-qPCR normalization across seven *Musa* genome groups (AA, BB, AAA, BBB, ABB, AAB, and ABBB), addressing a key gap beyond the commonly studied *Musa acuminata* A genome. *CAC*, along with *RPL4* and *RPS4*, demonstrated strong stability across diverse genome combinations. Using five computational algorithms (geNorm, NormFinder, Delta Ct, BestKeeper, and RefFinder) for rigorous cross-validation, these findings support reliable gene expression analysis for applications in fruit development, stress response, and breeding for seedless bananas. This validated set of reference genes provides a reliable basis for consistent, reproducible insights into gene functions across *Musa* species, advancing research in banana biology and breeding.

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