Journal of Applied Biology & Biotechnology Vol. 12(4), pp. 219-226, Jul-Aug, 2024
Available online at http://www.jabonline.in
DOI: 10.7324/JABB.2024.163935

Standardization of reference genes for real-time PCR analysis of *Vigna radiata* L. under *Agrobacterium tumefaciens* infection

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ARTICLE INFO

Article history:
Received on: January 05, 2024
Accepted on: April 07, 2024
Available online: May 20, 2024

Key words:
Real-time polymerase chain reaction,
Vigna radiata,
Agrobacterium tumefaciens,
Reference gene,
Normalization,
Cyclophilin.

ABSTRACT

Gene expression analysis using real-time polymerase chain reaction (PCR) requires the validation of a stable reference gene for normalization. Previously reported candidates may not be suitable for novel crops. *Vigna radiata* is a protein-rich legume crop used in many countries across the world. As this crop is recalcitrant to transformation such as most legumes, we were interested in *V. radiata-Agrobacterium* interaction studies. Toward this end, we wanted to identify a suitable reference for normalization under *Agrobacterium*-infections conditions. We selected seven candidate genes (*Actin*, *Tubulin*, *Glyceraldehyde 3-phosphate dehydrogenase*, *Ubiquitin 10*, *Cyclophilin*, *18S Ribosomal RNA*, and *F-box protein*) and performed temporal-expression analysis using real-time PCR at 3 h, 24 h, and 4-day post-infection. When their Ct values were compared using the routinely used four algorithms, Normfinder, geNorm, Bestkeeper, and RefFinder, each suggested a different candidate gene. We narrowed-down to *Cyclophilin* by taking the statistical approach of using analysis of variance and Tukey honestly significant difference. Thus, our report signifies the use of additional statistical analyses in case of situations of discrepancies due to variable suggestions by software.

1. INTRODUCTION

Vigna radiata (mung bean) is an important dietary pulse crop with about 62.62 g of carbohydrates and 28.86 g of protein per 100 g, along with fiber, vitamins, minerals, and antioxidants. This crop is cultivated in arid regions of Asia. Currently, India is the largest producer of mung bean, which is about 60% of the global [1]. However, there is economic loss and yield reduction because of various diseases and pests globally [2]. Mung bean yellow mosaic disease caused up to 85% economic loss in India [3]. Insect spotted pod borer caused 2–85% yield loss in Asia, Australia, Africa, and the USA [4]. As mung bean, such as most other legumes, is recalcitrant to transformation, reports on the improvement of this crop using genetic engineering are meager [5-7]. Traditional breeding of mung bean is also limited as it is mostly confined to its small germplasm due to the incompatibility with its wild relatives [8]. Interspecific crosses are succumbed to pre- and post-fertilization barriers such as structural abnormality of stigma/style [9] and seed non-viability [10], respectively. The availability of *V. radiata* whole genome sequence [11] can be effectively tapped to improve this crop using genome editing, provided more efficient transformation techniques are developed [5,12]. Though various approaches, such as trying various explants and *Agrobacterium* strains have been previously tried to improvise transformation efficiency, the lack of reproducibility and lower efficiency has barricaded the improvement of mung bean through molecular techniques [6]. As both genome editing and genetic engineering rely on transformation, it is important to understand the genetic response of mung bean plant during transformation.

Real-time polymerase chain reaction (PCR) is a widely used technique to understand the expression pattern of genes. Normalization of genes and finding an apt candidate as a reference gene are crucial steps for better analysis using real-time PCR [13]. Although the quality of data relies on multiple factors such as experimental design, sample purity, primers, machine, and the software accuracy, for a reliable comparative-expression analysis, normalization using stably expressing endogenous control is inevitable [13]. Normally, housekeeping genes (HKGs) are used as candidates for standardization because of their constitutive expression, irrespective of the tissue used [14]. Although HKGs genes seem to be consensus in their expression, they may behave differently under different conditions of stress and development [15], especially during real-time PCR. Hence, we cannot use a universal internal control because endogenous control used in one particular experiment may not work well in another set of experiments, even within the same species. Thus, the identification of a suitable endogenous control is important for gene expression analyses.

Different software tools are used to analyze the stability of expression of these reference genes, aiding in solving the problem of selection of good endogenous control. NormFinder, geNorm, Bestkeeper, and

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RefFinder are widely used for this purpose. Since each web tool follows a particular algorithm, using only one software may not select the best reference gene. A combination of two or more software can promote a better selection of a particular gene as internal control under different experimental conditions [16].

Previously, though *Actin* was used as a reference gene during the expression analysis of differentially expressed genes after whole transcriptome sequencing of *V. radiata*, those reports did not describe the rationale for selecting *Actin* [17,18]. It could be possible that a previously reported reference gene under one condition may not be suitable for other conditions. For instance, Ke *et al*. [19] identified *UBC* as stable gene under drought and *Cercospora canescens* infection, *EF1α* under water-logging, and *ACT* under salinity, for standardization of real-time PCR of *V. radiata.*

Most of the previous studies on plant-*Agrobacterium* interaction are in model systems such as *Arabidopsis thaliana* [20,21] and other crops like maize [22]. There are hardly any reports on reference gene standardization under *Agrobacterium tumefaciens* infection in *V. radiata* or any other legume species. As *Vigna* and many other legumes are recalcitrant to *Agrobacterium*-mediated transformation [7], such gene expression study will help to find out the probable role of genes involved during the plant-*Agrobacterium* interaction. Hence, we aimed to standardize reference gene for real-time PCR of *V. radiata*, under the biotic stress of *A. tumefaciens* infection.

2. MATERIALS AND METHODS

2.1. Plant Material and Growth Conditions

V. radiata, Co8 variety, seeds were obtained from the Regional Agricultural Research Station, Pattambi, Kerala. Mature seeds (harvested from approximately 60–75 days-old plants and dried) were treated with 0.1% mercuric chloride for 10 min. Seeds were then rinsed with 70% ethanol for 30 s and washed well with sterile distilled water 5 times (modified method of [23]). Sterilized seeds were kept for germination in ½ MS liquid medium, under shaking at 110 rpm. One-day-old germinated seedlings were de-coated under aseptic conditions and used for further experiments.

2.2. *Agrobacterium* Strain and Infection

A. tumefaciens strain LBA4404, containing pCAMBIA 1301 vector, was used for transformation. Cultures were initiated in AB minimal media containing kanamycin 50 mg/L. Three colonies of 3-day-old bacteria were inoculated into 3 mL Yeast Extract Peptone (YEP) media with kanamycin 25 mg/L and grown overnight at 28°C at 220 rpm. The next day, 2 mL of overnight culture was inoculated in 20 mL of fresh YEP containing kanamycin 25 mg/L and grown at the same conditions until the O.D. reached 0.8. The bacterial culture was then centrifuged at 6000 rpm at 4°C and the pellet was washed with sterile water. Bacteria were resuspended in a co-cultivation medium with 100 µM acetosyringone in ½ MS. Infection was performed using the previous method [24] with slight modifications.

De-coated seedlings were pricked gently 10–12 times with a fine needle, especially at the embryogenic region to facilitate *VIR* gene induction. Seedlings were then soaked in bacterial co-cultivation media for 1 h. Simultaneously, seedlings soaked in the same media without bacteria, were taken as control for the experiment. After co-cultivation, seedlings were bloat-dried and placed on solid co-cultivation media. Seedlings were then taken for RNA isolation at different time intervals of 3 h, 24 h, and 4 days post-infection.

2.3. Total RNA Isolation and cDNA Synthesis

Total RNA was isolated from six seedlings, each for a particular time interval, using Plant RNA kit (Sigma), along with DNase I (Sigma) treatment. RNA integrity was checked through gel electrophoresis by running on a 1.5% agarose gel. RNA of purity ranging from 1.9 to 2.2 (260/280 values) and 1.8–2.1 (260/230 values) were used for further experiments. Two micrograms of RNA were normalized for cDNA synthesis using ORIGIN cDNA synthesis kit.

2.4. Selection of Candidate Reference Genes, Primer Designing, and Amplification Efficiency

Candidate reference genes were selected based on the previous reports in various plants [19,21,25-27], and their homologs were identified from *V. radiata* transcriptome data (unpublished). Primers were designed [Table 1] using IDT (Integrative DNA technology) primer quest tool, following the default criteria of the website. Exons flanking an intron region were selected for the primer designing wherever possible, which helped to rule out the probable genomic DNA contamination in the sample. Primers were standardized under normal gradient PCR to check specificity.

2.5. Real-time PCR

Real-time experiments were carried out to check primer efficiency and compare the gene expressions, in which 10-fold diluted pooled cDNA samples in the ratio 1: 0.1: 0.001 were used for the former case. Experiments were carried out using Roche-Light Cycler® 480 II system, following the manufacturer's instructions. For each experiment, 20 µL sample mixture contained 10 µL of SYBR green master mix (Roche), forward and reverse primer (10 pmol/µL each), and cDNA. Each experiment started with an initial denaturation of 95°C for 10 min, followed by 40 cycles of 95°C denaturation for 30 s, 60°C annealing for 15 s, and 72°C extension for 15 s. Melting curve was generated from temperature ranging from 60°C to 95°C, to check specificity. Assays were repeated with three technical replicates and three biological replicates. Amplification efficiency and regression coefficient (R^2) were calculated from the slope of the standard curve of serial dilution of cDNA. The efficiency of the primers (in %) was calculated using the formula,

$$E = [10 (-1/\text{slope}) - 1] \times 100 \text{ (as given by [28])}$$

2.6. Box Plot Analysis

To check the variation in cycle threshold and thereby the stability of the candidate genes, a box plot was constructed in MS Excel.

2.7. Software-based Analysis

Expression stability was assessed using four different software tools. They are Norm Finder, geNorm, Bestkeeper, and RefFinder. NormFinder calculates a gene expression stability value based on intra- and inter-group variation among the sample subgroups and combines both the results into a stability index for each of the tested reference genes [29]. The test values are inversely proportional to the stability of reference genes. geNorm calculates a gene expression stability measure '*M*', which is calculated by the average pairwise variation of a single gene with all other candidate reference genes [30]. Genes with the lowest *M* value show the highest stability. Bestkeeper analyses based on the Ct values and calculates the geometric mean, arithmetic mean, minimum and maximum value, standard deviation, and coefficient of variation. On the basis of the calculated variation

Table 1: Primer details of seven candidate reference genes.

Gene symbol	Description	Primer sequences (F/R)	Tm (°C)	Amplicon length
ACT	*Actin 3*	5'GGATGAGCAAGGAGATTAC3' 5'GGGCCAGATTCATCATAC3'	57	166
UBQ10	*Ubiquitin-conjugating enzyme E2 10*	5'GATGATCCTTTGGTCCCTGAAA3' 5'TCGCTCTCCCTCGTACTATATG3'	60	128
CYP	*Cyclophilin 1*	5'CCAACGGATCTCAGTTCTTCAT3' 5'GATCTCCTTCACCACGTTCAG3'	60	107
18SrRNA	*18S ribosomal RNA gene*	5'GCGCGCAAATTACCCAATC3' 5'CCTCCAATGGATCCTCGTTAAG3'	60	122
GAPDH	*Glyceraldehyde-3- phosphate dehydrogenase, cytosolic*	5'CCTTCATCACCACCGATTACA3' 5'GACTGGCTTCTCACCAAAGA3'	60	122
TUB	*Tubulin beta chain*	5'GAGTGGAGTTACTTGCTGTCTAC3' 5'GGTGCAAACCCAACCATAAAG3'	60	117
FBOX	*F-box protein SKIP16*	5'GTTGGAATCGGTCGGAGATTTA3' 5'ATCGGATGCAGAAGACCTAAAC3'	60	112

(SD and CV), genes are ranked [31]. Further, these data were compared using another web tool, RefFinder [32]. Here, the ranking order is created based on the geomeans of the values.

2.8. One-way Analysis of Variance (ANOVA) and *post-hoc* Tukey Honest Significant Difference (HSD)

To analyze the statistical difference between Ct values of different treatments, a one-way ANOVA was conducted, followed by *post-hoc* Tukey HSD. Genes which showed $P > 0.05$ were considered to be stable. The *post hoc* Tukey HSD tests would identify which pair of treatments are significantly different from each other.

3. RESULTS AND DISCUSSION

Real-time expression analysis of genes has become an inevitable part of functional genomics studies. The reliability of real-time PCR data depends on the quality of RNA and cDNA used, PCR setup and experimental conditions, efficiency and specificity of primers, use of a stable endogenous control, and precision in analysis [13]. Validation of expression stability of the internal control has to be conducted thoroughly [33].

3.1. Selection of Candidate Reference Genes, Specificity, and Efficiency of Primers

HKGs (e.g., *18S ribosomal RNA [18SrRNA*, *TUB*, and *ACT*) are believed to be expressing stably in different cell conditions, and therefore HKGs are commonly selected for reference gene standardization [16]. However, while validating reference genes for real-time PCR, it has been proven that these commonly used HKGs show variation in expressions under different experimental conditions [34]. Based on the previous reports and transcriptome data (unpublished), seven frequently used HKGs, *actin* (*ACT*) [19,27] *tubulin* (*TUB*) [21,35], *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) [26,36], *ubiquitin 10* (*UBQ10*) [21,37], *cyclophilin* (*CYP*) [25], *18SrRNA* [27,36], and *F-box protein* (*FBOX*) [26,38], were short-listed for reference gene selection. In our study, we validated these genes in *V. radiata* under the biotic stress of *Agrobacterium* infection. Previously 24-h infection of *A. thaliana* with *A. tumefaciens* altered DNA repair gene expression [39]. We also intend to perform similar studies in *V. radiata*. Therefore, we checked the expression pattern of these genes with and without *A. tumefaciens* infection (infected or mock-inoculated control) at 3-time intervals, 3 h, 24 h, and 4 d. The annealing temperature (Tm) of all seven genes was identified, and their amplicon lengths were confirmed [Table 1] using gradient PCR. While maximum amplification was obtained at Tm of 60°C for most genes, 57°C was more suitable for *ACT*.

Real-time PCR of all the seven genes resulted in single peaks in the melting curve, which confirmed the primer specificity [Figure 1]. The primer efficiency ranged from 94.86 to 111.29 %, which lies in the acceptable efficiency range (E = 1.60–2.10 or 90–110%) explained by Pfaffl [31], except for *F-BOX* with a slight increase as 111.29. According to Auler *et al.* [40] and Ke *et al.* [19], efficiency ranging between 80 and 120% is also acceptable. The regression coefficient, R^2 of the primers ranged from 0.9939 (*FBOX* and *ACT*) to 0.9999 (*GAPDH*), in which values are closer to 1 indicating that the data fits into the linear regression [Table 2].

3.2. Cycle Threshold Value Distribution

When the cDNA samples from control and infected tissue under three different time intervals (3 h, 24 h, and 4 D) were subjected to real-time PCR analysis, the average Ct value ranged from 12.74 (*18SrRNA*) to 29.09 (*GAPDH*) [Supplementary Table 1]. The box plot analysis [Figure 2] indicated that *ACT* and *CYP* showed the least variation across different samples that varied for their infection time. *TUB*, *GAPDH*, *18S*, and *FBOX* showed more variations in the Ct values across different samples, possibly indicating that their expression was altered by the experimental conditions of infection.

3.3. Expression Stability Evaluation using Software

The use of different software programs and statistical analysis help to identify the best or most stable genes among candidate genes. Four different statistical programs, such as Normfinder, geNorm, Bestkeeper, and RefFinder, were used to evaluate the stability of the seven candidate genes in their real-time PCR expression.

NormFinder showed stability values ranging from 0.020 to 0.069 [Table 3]. As *FBOX* showed the lowest value (0.020), it was suggested as the most stable gene compared to others by this algorithm [Figure 3].

The expression stability value *M* obtained during geNorm analysis ranged from 0.034 to 0.085 [Table 3]. Since both *GAPDH* and *FBOX*

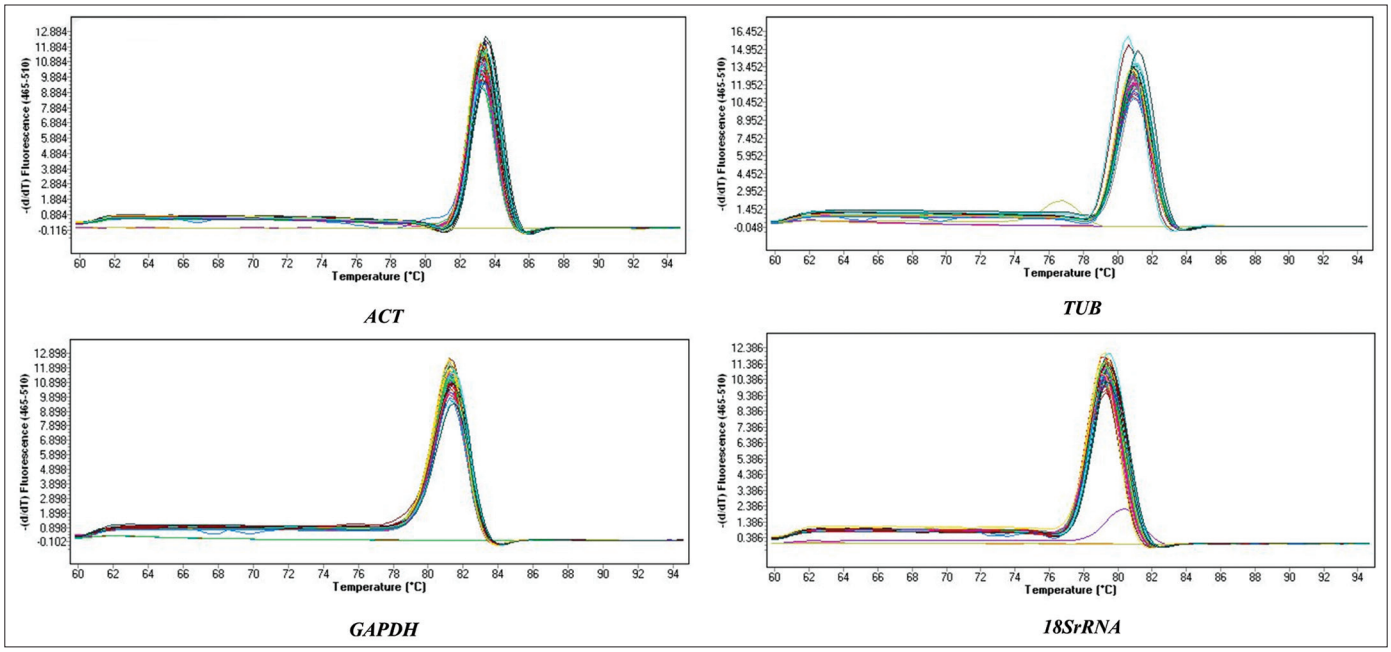

Figure 1: Melting curves of some candidate genes, in which single peak indicates the primer specificity.

Table 2: Primer efficiencies and regression coefficients of seven reference genes.

Gene symbol	Amplification efficiency (%)	Regression coefficient (R^2)
ACT	105.97	0.9939
UBQ10	101.85	0.9993
CYP	94.86	0.9952
18SrRNA	101.78	0.9988
GAPDH	104.36	0.9999
TUB	98.84	0.9989
FBOX	111.29	0.9939

ACT: Actin 3, UBQ10: Ubiquitin-conjugating enzyme E2 10, CYP: Cyclophilin 1, 18SrRNA: 18S ribosomal RNA gene, GAPDH: Glyceraldehyde-3- phosphate dehydrogenase, cytosolic, TUB: Tubulin beta chain, FBOX: F-box protein SKIP16

Table 3: Expression stability measure values of reference genes based on four software tools.

Gene symbol	NormFinder (Stability measure)	geNorm (M value)	Bestkeeper (SD)	RefFinder (Geomean of ranking values)
ACT	0.063	0.076	0.72	3.83
UBQ10	0.022	0.045	0.95	3.31
CYP	0.069	0.085	0.79	5.12
18SrRNA	0.064	0.055	1.38	1.41
GAPDH	0.026	0.034	1.70	5.14
TUB	0.022	0.035	1.69	2.63
FBOX	0.020	0.034	1.49	3.41

ACT: Actin 3, UBQ10: Ubiquitin-conjugating enzyme E2 10, CYP: Cyclophilin 1, 18SrRNA: 18S ribosomal RNA gene, GAPDH: Glyceraldehyde-3- phosphate dehydrogenase, cytosolic, TUB: Tubulin beta chain, FBOX: F-box protein SKIP16

showed the lowest *M* value, they were categorized as the most stable genes by geNorm [Figure 4].

In Bestkeeper analysis, SD ranged from 0.72 to 1.70 [Table 3]. *ACT*, *CYP*, and *UBQ10* showed SD<1 [Table 4], with the values 0.72, 0.79, and 0.95, respectively, indicating that *ACT* and *CYP* could be the best candidate reference genes.

RefFinder is a comprehensive analysis tool that combines the algorithm of NormFinder, geNorm, Bestkeeper, and Delta Ct method. In this study, RefFinder created a comprehensive ranking order using the geomeans of ranking values. The values ranged from 1.41 for *18SrRNA* to 5.14 for *GAPDH* [Table 3], and *18SrRNA* was suggested as the best gene according to this tool [Figure 5].

Although the box plot analysis indicated that *ACT* and *CYP* show least variation among different treatments, the gene order ranking [Table 5] gave varied results for each software tool. Only the results of Bestkeeper matched with the box plot analysis. While Normfinder identified *FBOX*, geNorm identified *FBOX* and *GAPDH.* Previously Ke *et al.* [19] analyzed 10 HKGs in *V. radiata*, and they got different genes as stable under different stress conditions, using NormFinder, geNorm, and Bestkeeper. Hence, they used the comprehensive tool, RefFinder for selecting the best genes. In our study, RefFinder shortlisted *18SrRNA* as the best among seven candidates. Recently, Zhou *et al*. [41] analyzed five candidate reference genes in *V. radiata* under four different pathogenic infections and hormonal treatments. While *TUA* was stable under various hormonal treatments and biotic stress (SA, ABA, GA3, and *Pythium myriotylum*), while different genes (*ACT* and *EF1α*) showed stability under different biotic stresses when analyzed using the same four software.

FBOX was considered a stable gene in *Vigna ungiculata*, under salt stress [37], and in *V. angularis*, under saline and waterlogging stress [26] also. Previously, *ACT* was regarded as a stable reference gene under salinity and biotic stress in *V. mungo* [27], in *Hibiscus hamabo* [42], and in Pear under hormonal treatment [43]. *CYP* was the most stable reference gene for leaf tissue (tissue specific-stability) under iron-deficiency stress in *V. angularis* [38] and infected root tissue in tomato [44].

Table 4: Descriptive statistics of seven candidate genes using Bestkeeper tool.

Gene name descriptive statistics	*ACT*	*CYP*	*UBQ10*	*18SrRNA*	*FBOX*	*TUB*	*GAPDH*
Geometric mean (CP)	23.50	22.87	24.08	14.10	25.70	24.45	25.78
Arithmetic mean (CP)	23.52	22.88	24.10	14.17	25.76	24.51	25.85
Min (CP)	21.90	21.44	22.73	12.74	23.52	22.62	23.61
Max (CP)	24.80	23.93	25.34	15.77	27.84	26.79	29.09
Standard deviation (±CP)	0.72	0.79	0.95	1.38	1.49	1.69	1.70
Covariance (% CP)	3.05	3.44	3.94	9.75	5.78	6.90	6.58
Power (x-fold)	1.25	1.14	1.69	2.21	2.54	2.73	3.14

ACT: Actin 3, UBQ10: Ubiquitin-conjugating enzyme E2 10, CYP: Cyclophilin 1, 18SrRNA: 18S ribosomal RNA gene, GAPDH: Glyceraldehyde-3- phosphate dehydrogenase, cytosolic, TUB: Tubulin beta chain, FBOX: F-box protein SKIP16

Table 5: Ranking order of reference genes based on different software tools.

Method	Ranking order						
	← Most stable Least stable →						
	1	2	3	4	5	6	7
NormFinder	*FBOX*	*UBQ10*	*TUB*	*GAPDH*	*ACT*	*18S*	*CYP*
geNorm	*FBOX*	*GAPDH*	*TUB*	*UBQ10*	*18S*	*ACT*	*CYP*
Bestkeeper	*ACT*	*CYP*	*UBQ10*	*18S*	*FBOX*	*TUB*	*GAPDH*
RefFinder	*18S*	*TUB*	*UBQ10*	*FBOX*	*ACT*	*CYP*	*GAPDH*

ACT: Actin 3, UBQ10: Ubiquitin-conjugating enzyme E2 10, CYP: Cyclophilin 1, 18SrRNA: 18S ribosomal RNA gene, GAPDH: Glyceraldehyde-3- phosphate dehydrogenase, cytosolic, TUB: Tubulin beta chain, FBOX: F-box protein SKIP16

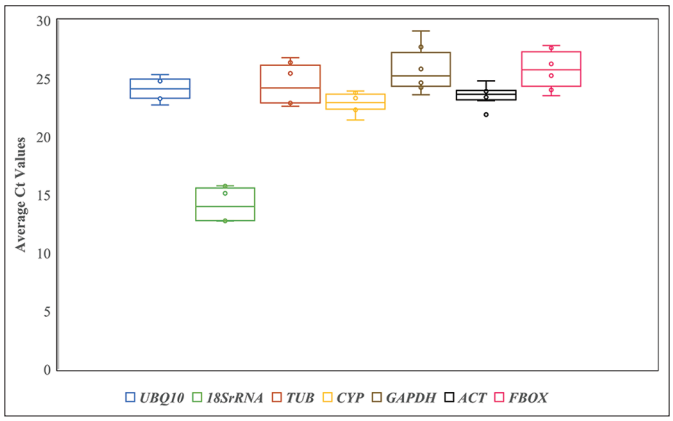

Figure 2: Box plot, showing the average Ct values of candidate reference genes across samples. Each box represents the 25% of the first and 75% of the third quartiles, whiskers are the maximum and minimum Ct values, while the line across the box signifies the median values.

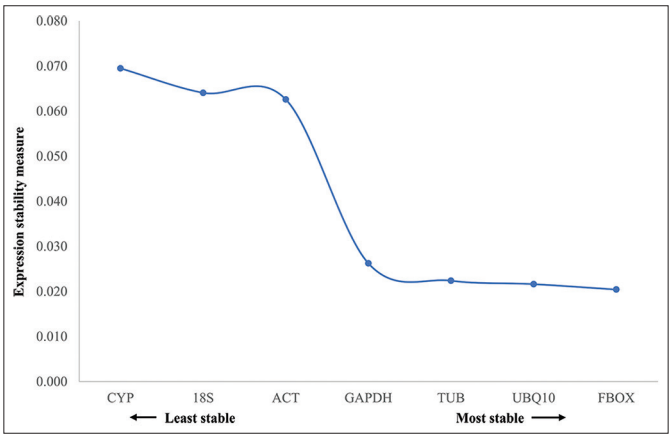

Figure 3: Expression stability measure value of candidate reference genes based on NormFinder software.

It is to be noted that the software that we used was based on different algorithms. The principle of Normfinder is that the stability of the reference gene depends on the least expression variation among different samples and sample groups. In our work, control and infected tissue under three different time intervals were regarded as two different sample subsets. In this tool, the Ct values are log-transformed and then used as input values on the basis of amplification efficiency [29]. On the other hand, geNorm follows the determination of pairwise variation of a particular reference gene with all other candidate genes and gives the expression stability measure, denoted as '*M*'. Based on this *M* value, genes are ranked such that the lowest *M* value will be the most stable reference gene. According to Sinha *et al.* [36], *M* value of <1.5 can be recognized as stable genes. In our study, the *M* values of all genes were <1.5 and thus may be considered as stable genes. But, that criterion alone is insufficient because such genes may not show minimum expression variations among samples. Thus, even though *FBOX* and *GAPDH* had the least *M* value, their expression variation among various treatments was high. Therefore, *FBOX* and *GAPDH* cannot be considered stable reference genes.

Bestkeeper gives descriptive statistics of the candidate reference genes and compares the expression stability with an index formed by the software. Compared to geNorm and Normfinder, Bestkeeper uses raw Ct values as input [31]. Genes with the least SD (SD<1) are considered to be the most stable as internal controls as per this tool. Hence, this tool identified *ACT* and *CYP* as the most stable genes. RefFinder selected candidates with the least Ct values (as it incorporates the delta CT method), irrespective of their variation among treatments. Thus, *18SrRNA*, which is a highly expressed gene, thus exhibiting less Ct values (12.74–15.74) was chosen by RefFinder. However, a gene is ideally regarded as a stable reference gene if its expression is unaltered, irrespective of the treatment or conditions applied. In this regard, Bestkeeper showed SD > 1 (1.38) for *18SrRNA* and hence, it is not consistent in its stability.

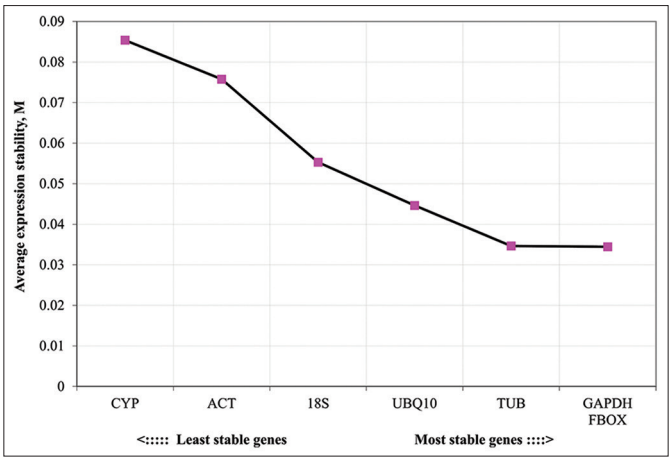

Figure 4: Average expression stability value, M of candidate reference genes based on geNorm software.

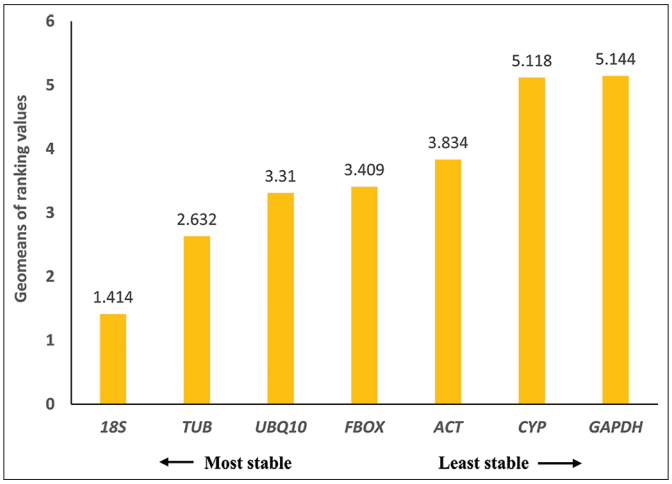

Figure 5: Comprehensive ranking order of candidate reference genes using geomeans of ranking values by RefFinder.

3.4. Statistical Analyses

Since each software tool identified different candidate as the most stable reference gene, we analyzed the data statistically by one-way ANOVA and Tukey HSD. Except *CYP*, all other candidate reference genes had $P < 0.01$ when compared using ANOVA, indicating that their Ct values among various treatments varied significantly. The data were further analyzed by a *post hoc* Tukey HSD, which analyses the values by performing multiple pairwise comparisons among the treatments. In this study, only *CYP* showed statistically insignificant *P* values for the 15 possible comparisons [Supplementary Tables 2-4]. The Ct values of other six genes, *ACT*, *GAPDH*, *18SrRNA*, *TUB, UBQ10*, and *FBOX* were significantly different in at least one of the comparisons [Figure 6].

In our experiment, it was important that the Ct values of the reference gene should not be influenced by infection conditions. Hence, mock-inoculated values should not be significantly different from infected. Our analysis showed that of all the software that we used, this criterion was satisfactorily addressed by Bestkeeper, because its results abided by box plot analysis and *post hoc* Tukey HSD test as well. Thus, taken together, we selected *CYP* as the stable reference gene for real-time PCR of *V. radiata* under *A. tumefaciens* infection.

There are many previous reports on the selection of candidate reference genes in different plants under the treatment of various biotic/abiotic stresses [43,45]. All these reports rely on the same software that we used. Many of these reports indicate that they also faced situations, where the results from each software did not match. For example, Duan *et al.* [46] used Bestkeeper, Normfinder, geNorm, and Delta Ct method. They got inconsistency in the case of most stable genes. Similarly, in a study conducted on wheat seedlings, four software tools showed differences in stability ranking [47]. It is believed that the statistical analysis chosen here (ANOVA and Tukey HSD tests) is a feasible tool for circumventing the aforementioned limitations when choosing an appropriate set of genes. Previously, the study on *Arabidopsis-Agrobacterium* interaction reported that as the software-selected candidate genes identified by three software, geNorm, Normfinder, and BestKeeper did not satisfy certain criteria of a stable

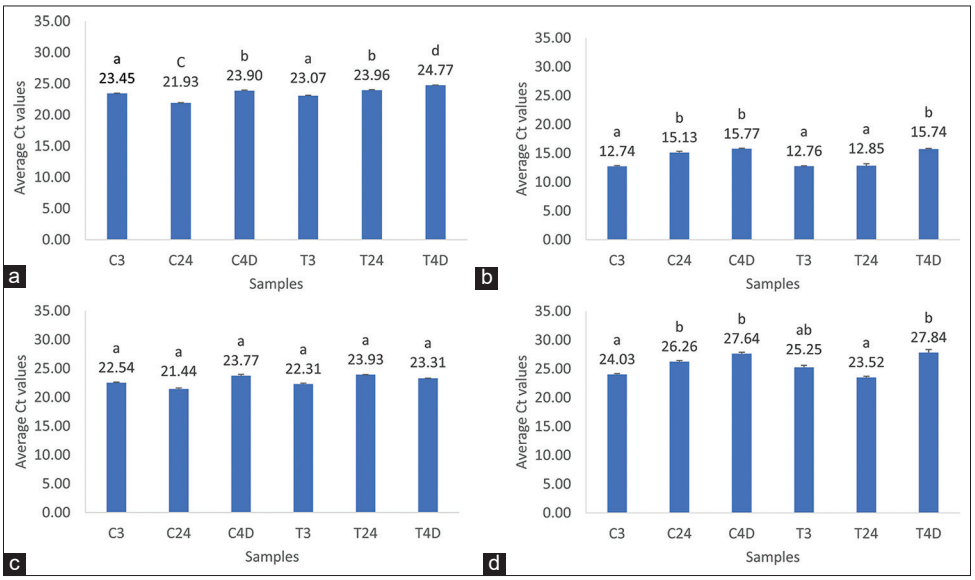

Figure 6: Ct values of four genes, A. ACT, B. 18S, C. CYP, D. FBOX. C3, C24, and C4D represent control samples, and T3, T24, and T4D represent treated samples, at three-time intervals, 3 h, 24 h and 4D, respectively. The letter a, b, c, and d indicate significantly different values at $P < 0.05$. The letters ab indicates that these values are similar to both a and b.

reference gene, they also took the aid of Student's t-test, ANOVA, and Tukey-HSD test [21]. The reliability of the results obtained from software was confirmed by ANOVA and Tukey HSD in the analysis of reference genes in *Passiflora edulis* Sims [48]. Hence, similarly, we hereby recommend statistical re-analysis of Ct values in case of discrepancy among the results suggested by multiple software.

4. CONCLUSION

We identified *CYP* as a candidate reference gene of normalization for real-time PCR experiments of *V. radiata* infected with *Agrobacterium*. In addition, our work conveys the importance of statistical analysis such as ANOVA and *post hoc* tests to solve the discrepancy of results suggested by multiple software for reference gene standardization. Although previous studies on plant-*Agrobacterium* interaction aided in improving the transformation of many crops, there is much less understood information underlying the transformation-recalcitrant crops such as most legumes. Identifying suitable reference gene for expression analyses is the stepping stone toward *V. radiata*-*Agrobacterium* interaction studies, which could pave the way for crop improvement using genetic engineering and genome editing.

5. AUTHORS' CONTRIBUTION

All authors made substantial contributions to conception and design, acquisition of data, analysis, and interpretation of data, drafting the article or revising it for important intellectual content, agreed to submit to the current journal, agreed final approval of the article to be published, and approved to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/ guidelines.

6. ACKNOWLEDGMENTS

NVM acknowledges DST (Department of Science and Technology)-INSPIRE for providing her doctoral fellowship.

7. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

This article does not involve experiments on animal or human subjects.

10. DATA AVAILABILITY

All the data generated and analyzed are included in this article and for further details the corresponding author may be accessed.

11. PUBLISHER'S NOTE

This journal remains neutral with regard to jurisdictional claims in published institutional affiliation.

REFERENCES

1. GlobeNewswire by Notified. Available from: https://www.globenewswire.com/en/news-release/2021/06/14/2246352/28124/en/global-mung-beans-market-2021-to-2026-industry-trends-share-size-growth-opportunity-and-forecasts.html [Last accessed on 2023 May 10].
2. Nair RM, Pandey AK, War AR, Hanumantharao B, Shwe T, Alam AK, *et al*. Biotic and abiotic constraints in mungbean production-progress in genetic improvement. Front Plant Sci 2019;10:1340.
3. Karthikeyan A, Shobhana VG, Sudha M, Raveendran M, Senthil N, Pandiyan M, *et al*. Mungbean yellow mosaic virus (MYMV): A threat to green gram (*Vigna radiata*) production in Asia. Int J Pest Manag 2014;60:314-24.
4. Zahid MA, Islam MM, Begum MR. Determination of economic injury levels of *Maruca vitrata* in mungbean. J Agric Rural Dev 2008;6:91-7.
5. Baloglu MC, Celik Altunoglu Y, Baloglu P, Yildiz AB, Türkölmez N, Özden Çiftçi Y. Gene-editing technologies and applications in legumes: Progress, evolution, and future prospects. Front Genet 2022;13:859437.
6. Tripathi A, Debnath S, Shukla S. Review on biotechnological advances in *Vigna radiata* and their future prospects. J Crop Sci Biotechnol 2021;24:245-58.
7. Nivya VM, Shah JM. Recalcitrance to transformation, a hindrance for genome editing of legumes. Front Genome Ed 2023;5:1247815.
8. Aasim M, Barpete S, Usta A, Sevinc C. An insight into *Agrobacterium tumafacıens*-mediated genetic transformation studies in mungbean (*Vigna radiata* L. Wilczek). J Glob Innov Agric Soc Sci 2019;7:47-51.
9. Thiyagu K, Jayamani P, Nadarajan N. Pollen pistil interaction in interspecific crosses of *Vigna* sp. Cytologia 2008;73:251-7.
10. Basavaraja T, Murthy N, Kumar LV, Mallikarjun K. Studies on cross compatibility in interspecific crosses of *Vigna radiata* × *Vigna umbellata* species. Legume Res Int J 2019;42:699-704.
11. Kang YJ, Kim SK, Kim MY, Lestari P, Kim KH, Ha BK, *et al*. Genome sequence of mungbean and insights into evolution within *Vigna* species. Nat Commun 2014;5:5443.
12. Rasheed A, Barqawi AA, Mahmood A, Nawaz M, Shah AN, Bay DH, *et al*. CRISPR/Cas9 is a powerful tool for precise genome editing of legume crops: A review. Mol Biol Rep 2022;49:5595-609.
13. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, *et al*. The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. Clin Chem 2009;55:611-22.
14. Joseph JT, Poolakkalody NJ, Shah JM. Plant reference genes for development and stress response studies. J Biosci 2018;43:173-87.
15. Thellin O, Zorzi W, Lakaye B, De Borman B, Coumans B, Hennen G, *et al*. Housekeeping genes as internal standards: Use and limits. J Biotechnol 1999;75:291-5.
16. Kozera B, Rapacz M. Reference genes in real-time PCR. J Appl Genet 2013;54:391-406.
17. Tian X, Li S, Liu Y, Liu X. Transcriptomic profiling reveals metabolic and regulatory pathways in the desiccation tolerance of mungbean (*Vigna radiata* [L.] R. Wilczek). Front Plant Sci 2016;7:1921.
18. Liu C, Fan B, Cao Z, Su Q, Wang Y, Zhang Z, *et al*. A deep sequencing analysis of transcriptomes and the development of EST-SSR markers in mungbean (*Vigna radiata*). J Genet 2016;95:527-35.
19. Ke XW, Yin LH, Xu J, Sun WN, Xu XD, Guo YX, *et al*. Reference genes for quantitative real-time PCR analysis of gene expression in mungbean under abiotic stress and *Cercospora canescens* infection. Legume Res Int J 2021;44:646-51.
20. Duan K, Willig CJ, De Tar JR, Spollen WG, Zhang ZJ. Transcriptomic analysis of *Arabidopsis* seedlings in response to an *Agrobacterium*-mediated transformation process. Mol Plant Microbe Interact 2018;31:445-59.

21. Joseph JT, Poolakkalody NJ, Shah JM. Screening internal controls for expression analyses involving numerous treatments by combining statistical methods with reference gene selection tools. Physiol Mol Biol Plants 2019;25:289-301.
22. Liu Y, Zhang Z, Fu J, Wang G, Wang J, Liu Y. Transcriptome analysis of maize immature embryos reveals the roles of cysteine in improving *Agrobacterium* infection efficiency. Front Plant Sci 2017;8:1778.
23. Mekala GK, Juturu VN, Mallikarjuna G, Kirti PB, Yadav SK. Optimization of *Agrobacterium*-mediated genetic transformation of shoot tip explants of green gram (*Vigna radiata* (L.) Wilczek). Plant Cell Tissue Organ Cult 2016;127:651-63.
24. Jaiwal PK, Kumari R, Ignacimuthu S, Potrykus I, Sautter C. *Agrobacterium tumefaciens*-mediated genetic transformation of mungbean (*Vigna radiata* L. Wilczek)-a recalcitrant grain legume. Plant Sci 2001;161:239-47.
25. Bansal R, Mittapelly P, Cassone BJ, Mamidala P, Redinbaugh MG, Michel A. Recommended reference genes for quantitative PCR analysis in soybean have variable stabilities during diverse biotic stresses. PLoS One 2015;10:e0134890.
26. Chi C, Shen Y, Yin L, Ke X, Han D, Zuo Y. Selection and validation of reference genes for gene expression analysis in *Vigna angularis* using quantitative real-time RT-PCR. PLoS One 2016;11:e0168479.
27. Kundu A, Patel A, Pal A. Defining reference genes for qPCR normalization to study biotic and abiotic stress responses in *Vigna mungo*. Plant Cell Rep 2013;32:1647-58.
28. Rasmussen R. Quantification on the Lightcycler. In: Meuer S, Wittwer C, Nakagawara KI, editors. Rapid Cycle Real-time PCR: Methods and Applications. Berlin, Heidelberg: Springer Berlin Heidelberg; 2001. p. 21-34.
29. Andersen CL, Jensen JL, Ørntoft TF. 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 2004;64:5245-50.
30. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, *et al*. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002;3(7):research0034.1–0034.11.
31. Pfaffl MW. Quantification strategies in real-time PCR. In: A-Z of Quantitative PCR. La Jolla: International University Line; 2004. p. 87-113.
32. RefFinder. Available from: https://www.heartcure.com.au/for-researchers [Last accessed on 2022 Feb 02].
33. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. Methods 2001;25:402-8.
34. Guenin S, Mauriat M, Pelloux J, Van Wuytswinkel O, Bellini C, Gutierrez L. Normalization of qRT-PCR data: The necessity of adopting a systematic, experimental conditions-specific, validation of references. J Exp Bot 2009;60:487-93.
35. Gutierrez N, Giménez MJ, Palomino C, Avila CM. Assessment of candidate reference genes for expression studies in *Vicia faba* L. By real-time quantitative PCR. Mol Breed 2011;28:13-24.
36. Sinha R, Sharma TR, Singh AK. Validation of reference genes for qRT-PCR data normalisation in lentil (*Lens culinaris*) under leaf developmental stages and abiotic stresses. Physiol Mol Biol Plants 2019;25:123-34.
37. Amorim LL, Ferreira-Neto JR, Bezerra-Neto JP, Pandolfi V, de Araújo FT, da Silva Matos MK, *et al*. Cowpea and abiotic stresses: Identification of reference genes for transcriptional profiling by qPCR. Plant Methods 2018;14:88.
38. Wang J, Yang K, Zhao B, Li YS, Wan P. Identification and validation of reference genes in the adzuki bean (*Vigna angularis*) under iron deficiency using quantitative real-time PCR. Plant Mol Biol Rep 2020;38:250-61.
39. Joseph TJ, Chandhini S, Das S, Mysore SK, Shah MJ. Methylation status of *Arabidopsis* DNA repair gene promoters during *Agrobacterium* infection reveals epigenetic changes in three generations. Plant Mol Biol Rep 2021;39:773-91.
40. Auler PA, Benitez LC, do Amaral MN, Vighi IL, dos Santos Rodrigues G, da Maia LC, *et al*. Evaluation of stability and validation of reference genes for RT-qPCR expression studies in rice plants under water deficit. J Appl Genet 2017;58:163-77.
41. Zhou Y, Liu H, Wu T, Zheng Y, Wang R, Xue D, *et al*. Screening of reference genes under biotic stress and hormone treatment of mungbean (*Vigna radiata*) by quantitative real-time PCR. Genes 2023;14:1739.
42. Ni L, Wang Z, Liu L, Guo J, Li H, Gu C. Selection and verification of candidate reference genes for gene expression by quantitative RT-PCR in *Hibiscus hamabo* Sieb.et Zucc. Trees 2019;33:1591-601.
43. Zhou P, Huang L, Wang Y, Li X, Feng X, Li L. Stepwise optimization of the RT-qPCR protocol and the evaluation of housekeeping genes in pears (*Pyrus bretschneideri*) under various hormone treatments and stresses. Horticulturae 2023;9:275.
44. Mascia T, Santovito E, Gallitelli D, Cillo F. Evaluation of reference genes for quantitative reverse-transcription polymerase chain reaction normalization in infected tomato plants. Mol Plant Pathol 2010;11:805-16.
45. Guo L, Li Y, Wei Z, Wang C, Hou X. Reference genes selection of *Paeonia ostii* 'Fengdan' under osmotic stresses and hormone treatments by RT-qPCR. Mol Biol Rep 2023;50:133-43.
46. Duan M, Wang J, Zhang X, Yang H, Wang H, Qiu Y, *et al*. Identification of optimal reference genes for expression analysis in radish (*Raphanus sativus* L.) and its relatives based on expression stability. Front Plant Sci 2017;8:1605.
47. Dudziak K, Sozoniuk M, Szczerba H, Kuzdraliński A, Kowalczyk K, Börner A, *et al*. Identification of stable reference genes for qPCR studies in common wheat (*Triticum aestivum* L.) Seedlings under short-term drought stress. Plant Methods 2020;16:58.
48. Zhao M, Fan H, Tu Z, Cai G, Zhang L, Li A, *et al*. Stable reference gene selection for quantitative real-time PCR normalization in passion fruit (*Passiflora edulis* Sims.). Mol Biol Rep 2022;49:5985-95.

How to cite this article:
Nivya VM, Shah JM. Standardization of reference genes for real-time PCR analysis of *Vigna radiata* L. under *Agrobacterium tumefaciens* infection. J App Biol Biotech. 2024;12(4):219-226. DOI: 10.7324/JABB.2024.163935