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Internal Reference Gene Selection for Quantitative Real-Time RT-PCR Normalization in Potato Tissues

Gang Li[1], Yao Zhou[2], Yaqi Zhao[2], Yaxue Liu[2], Yuwei Ke[2], Xiaoqing Jin[1] and Haoli Ma[1,2,*]

[1]Department of Biological Repositories, Zhongnan Hospital of Wuhan University, Wuhan, 430071, China
[2]College of Agronomy, Northwest A&F University, Yangling, Shaanxi, 712100, China
*Corresponding Author: Haoli Ma. Email: mahaoli@whu.edu.cn
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Abstract: Quantitative real-time PCR (qRT-PCR) is widely used for investigating gene expression patterns and has many advantages, including its high sensitivity, fidelity, and specificity. Selecting a satisfactory internal reference gene is crucial for obtaining precise gene expression results in qRT-PCR analyses. In this study, the transcriptomic data of 2 potato varieties were screened for housekeeping genes with stable expression patterns. A total of 77 putative genes were selected, which were highly and stably expressed. Then, qRT-PCR analyses were performed to examine the expression levels of these 77 candidate reference genes in various potato tissues, including leaves, flowers, stolons, and tubers. Gene expression was represented by analyzing the Ct values at given threshold. Through geNorm and NormFinder program analyses, 10 candidate genes with the most stable expression patterns were obtained, including *RPL19*, *RPS15*, *RPS9*, *EF1α*, *TrxP1*, *RPS8*, *NTF*, *CAM*, *AACM*, and *RPS28*. Moreover, through the comprehensive analyses of 4 statistical algorithms (i.e., geNorm, NormFinder, BestKeeper, and RefFinder), results indicated that the most appropriate internal reference genes were *RPL19* and *EF1α*. The obtained stable reference genes will contribute to future qRT-PCR analyses on potato tissue-related gene expression.

Keywords: qRT-PCR; potato tissues; reference genes; geNorm; NormFinder; BestKeeper; RefFinder

1 Introduction

Potato belongs to the *Solanaceae* herbaceous perennial plant tuber and is now the fourth most important food crop in the world, following wheat, rice, and corn. As a result, potato acreage and output have increased dramatically over the past 50 years. Additionally, potatoes provide more food energy and nutrition per unit using the same amount of or fewer fertilizers than other cereal crops. Currently, many genetic studies are being conducted to raise the productivity and quality of potatoes. Quantitative real-time PCR (qRT-PCR) is one of the most available analytical tools used in gene expression analyses; however, its accuracy is heavily dependent on the standardization strategy of unchangeable reference genes [1].

Gene expression analyses have been commonly used in various life science fields, and are deemed as one of the most important methods for predicting new genes and studying gene functions. Compared to

traditional PCR, qRT-PCR is more powerful due to its high sensitivity, specificity, reproducibility, quantitative accuracy, and high-throughput characteristics [2]. It has become one of the most commonly used genomic research techniques in the field of plant sciences [1,3]. However, many factors affect the accuracy of gene expression analysis when using qRT-PCR techniques, such as the productivity and integrity of RNA, efficiency of reverse transcription, and stability of the internal control gene, among other impediments [4]. Therefore, in order to minimize the difference between the samples and obtained data with high accuracy, selecting the appropriate internal reference gene for calibration and standardization is crucial [5]. However, currently, there are no constant internal reference genes. Usually, 1 or more internal reference genes are required to standardize qRT-PCR results [6]. A good combination of internal control genes can minimize abiotic variation [7]. Thus, the choice of a suitable internal control gene is of great importance for qRT-PCR experiments, as an ideal internal reference gene needs to be stably expressed in all tissues [8]. Thus far, several plants have had reference genes identified under different treatments and conditions, such as rice [9], grapevine [10], sugarcane [11], wheat [12], *Arabidopsis thaliana* [13], tomato [14], and tea [15].

Internal reference genes usually use housekeeping genes that are expressed in every cell and whose products are essential for cell structure and metabolism, such as *ubiquitin 6* (*UBQ6*) [16], *beta-tubulin* (β-*TUB*) [17], *actin* (*ACT*) [17,18], *18S rRNA* [19], *cyclophilin* (*CYP*) [20], *elongation factor 1-alpha* (*EF1α*) [18], and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) [10,21], which mostly participate in fundamental cellular activities [19,22]. Currently, most of these genes were commonly used as internal reference genes in genetic research [23]. However, they exhibit different stabilities under different conditions; thus, it is vital in genetic research to choose reference genes that are invariably expressed in any way. In potatoes, *C2*, *exocyst complex component sec3* (*SEC3*), and *ATCUL3/ATCUL3A/CUL3/CUL3A* (*CUL3A*) were thought to be stably expressed in tubers [24]. *EF1α* was demonstrated to be the best reference gene under salt [25], late blight, and drought stress. *EF1α* and *adenine phosphoribosyl transferase* (*APRT*) were stably expressed under cold stress [26]. *SEC3* was confirmed as the optimal reference gene under osmotic stress [27].

To our knowledge, reference gene combinations that are stably expressed in different potato tissues have not been previously reported. Thus, selecting 1 or more than one suitable reference genes is beneficial to the future studies on potatoes using qRT-PCR. RNA-Seq data are subsequently used to select candidate genes and examine the stability of putative reference genes. This study concentrated on candidate genes with relatively high expression levels in various potato tissues to evaluate their expression variability. The qRT-PCR data were analyzed using 4 prevalent programs, including geNorm [28], NormFinder [8], and BestKeeper [29], to determine the appropriate novel genes for gene expression studies under different circumstances. Then, RefFinder was used to order the genes acquired from geNorm, NormFinder, and BestKeeper. The finding of this study will contribute to the accurate and reliable normalization of gene expression studies on potatoes.

2 Materials and Methods

2.1 Plant Materials and Growth Conditions

Experiments were performed using the "Atlantic" potato cultivar. Plants were cultivated in a greenhouse facility at Northwest A&F University (Yangling, China) from March to June, 2017. All plant materials were cultivated at 23 ± 2°C under a 16/8 h light/dark photoperiod. Different potato tissues were collected at different growing times; leaves and flowers were collected after flowering, stolons were sampled 10 days after flowering, and tubers were collected at 90 days after sprouting. Then, the tissues were quickly frozen in liquid nitrogen and stored at −80°C for future analyses.

2.2 Total RNA Isolation and cDNA Synthesis

Total RNA was isolated from 3 biological replicates of each potato tissue, including leaves, flowers, stolons, and tubers, using an RP1202 high purity total RNA rapid extraction kit (BioTeke Co., Beijing, China). Then, 1.5% agarose gel electrophoresis was used to examine the quality of RNA products. The

concentration and quality of RNA samples were detected using a NanoDrop 2000C ultraviolet spectrophotometer (Thermo Fisher Scientific, MA, USA). First-strand cDNA was synthesized using an FSK-100 ReverTra Ace kit (Toyobo, Osaka, Japan) with the addition of 2 μg of RNA from each reaction. Finally, the cDNA solutions were diluted (1:10) with ddH_2O and stored at −20°C for subsequent qRT-PCR reactions.

2.3 Potential Reference Gene Selection

To ensure the correctness and reliability of potential reference gene selection, the fragments per kilobase per million reads (FPKM) values of 2 potato strains, the doubled monoploid DM1-3 (DM) and heterozygous diploid RH89-039-16 (RH), were obtained from Potato Genome Sequencing Consortium (PGSC) database. First, genes with an FPKM value >50 in all tissues of both potato strains were selected. In total, 39,000 genes were identified. Then, the average expression (AVE) and standard deviation (SD) of selected genes from various tissues were calculated. The coefficient of variation (CV) was computed as SD/AVE. Genes with CV values < 0.5 were reserved from the DM and RH strains, where DM had 151 genes and RH had 147 genes. Afterward, Venn diagrams were constructed to determine the number of overlapping genes between the DM and RH strains; 77 genes were shared between the 2 strains. Subsequently, qRT-PCR was conducted to test the expression levels of the candidate reference genes. The selected genes were further screened using geNorm and NormFinder to identify the top 10 internal reference genes (Fig. 1).

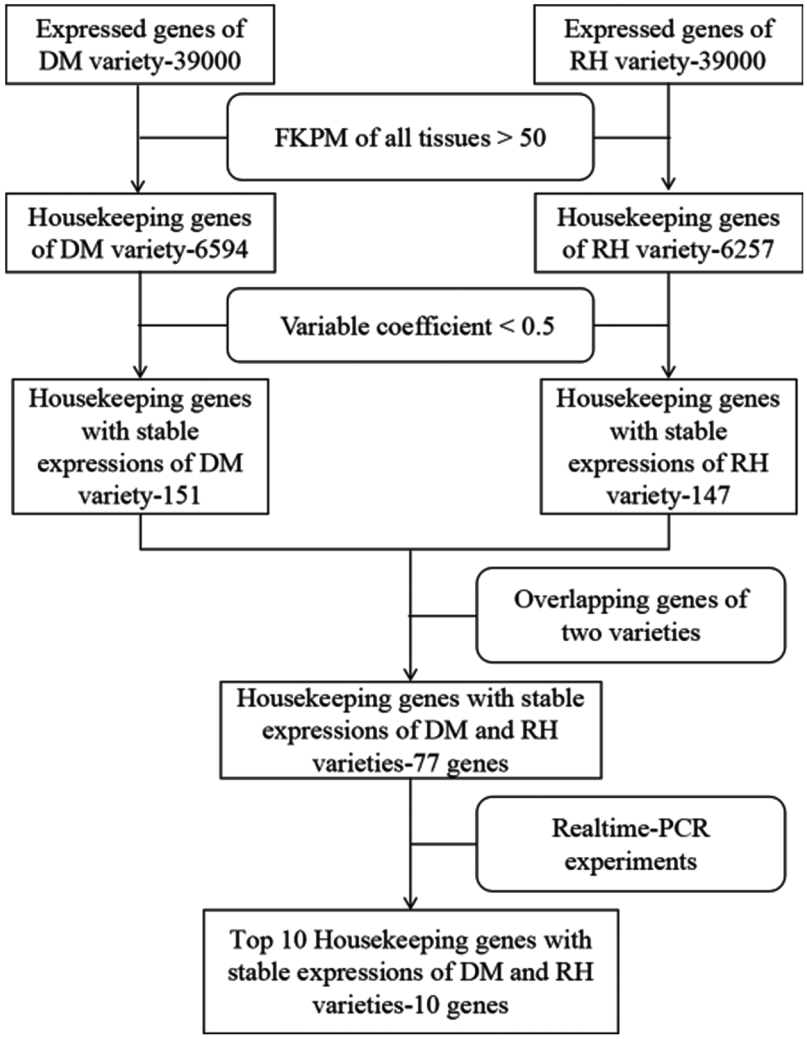

Figure 1: The workflow diagram of selecting reference genes

2.4 Primer Design and qRT-PCR

Forward and reverse primers were searched in the qPCR Primer Database [30,31]. A total of 66 pairs of suitable primers corresponding to 66 genes were found. For the remaining 11 genes, the Primer v6.0 program was used to design primers. The 77 selected genes and primers were synthesized by GenScript Corporation (Nanjing, China) (Tab. S1).

qRT-PCR experiments were conducted to estimate the expression levels of the candidate genes on 96-well plates using SYBR Green-based PCR assays, which were fulfilled using the KK4601 SYBR green mix (Kapa Biosystems, MA, USA) in a CFX96 RT-PCR machine (BioRad Laboratories, CA, USA). Experiments were conducted with at least 3 biological repeats, and each biological repeat was performed with 3 technical duplicates. Each PCR mixture (20 μL) contained 1 μL cDNA,10 μL SYBR green mix, 1 μL forward primer, 1 μL reverse primer, and 7 μL ddH_2O. The PCR procedures were as follows: 95°C for 2 min, 39 cycles at 95°C for 5 s, 60°C for 30 s, and a final melting curve between 65°C and 95°C (Δ0.5°C/s) [18].

2.5 Statistical Analyses

The Ct values were analyzed to obtain the stable levels and ranks of the putative reference genes in each potato tissue using geNorm [32], NormFinder [8], and BestKeeper [29] algorithms. The web-based algorithm, RefFinder, which integrates these widely used computational programs, was utilized to assess a recommended comprehensive ranking, as it is convenient method for evaluating the appropriate reference genes for qRT-PCR analysis [27].

The geNorm program was also used to evaluate the reference genes, as it can screen any amount of internal reference genes and select 2 or more internal reference genes to form a combination that corrects the data, thereby making the relative quantification results more accurate. The $2^{-\Delta Ct}$ (ΔCt = the Ct value of each gene–the lowest Ct value of all genes) was used to convert the original Ct values of different genes under different samples [27]. Then, the obtained results were imported into the geNorm program to obtain the gene expression stability value (M). The smaller the M value, the more stable the reference gene. Thus, the gene with the largest M value was the most unstable gene. The program also calculated the paired variation values (V) of the normalization elements to assess the number of optimal internal reference genes [32].

The NormFinder program calculated the M value of each candidate reference gene and the V values among sample groups. However, the program only selects the most appropriate internal reference genes. The method was the same as that of geNorm [8].

The BestKeeper program is relatively simple, as it only calculates the geometric mean (CP) of the fluorescence values obtained from qRT-PCR analyses repeated 3 times then inputs the CP value into the table. When the last CP value was input, the BestKeeper program automatically calculated the correlation coefficient (R), SD, and CV. Based on these 3 values, the stability of the reference genes was estimated [29].

The RefFinder program, which integrates the other 3 programs is a website tool that analyzes the stability of the reference genes by combining the results of the 3 current computing programs to obtain an integrated ranking [33].

3 Results

3.1 Validation of Reference Genes Using geNorm and NormFinder

After performing the qRT-PCR experiments, the geNorm program was used to evaluate the expression levels of the candidate genes and obtained 14 stable putative reference genes with M values < 0.5. These genes included *40S ribosomal protein S15* (*RPS15*), *40S ribosomal protein S9* (*RPS9*), *transcription factors* (*TFs*), *60S ribosomal protein L18-2* (*RPL18*), *40S ribosomal protein S14* (*RPS14*), *ribosomal protein L19* (*RPL19*), *ubiquitin extension protein1* (*UEP1*), *40S ribosomal protein S28* (*RPS28*),

calmodulin-5/6/7/8 (*CAM*), *EF1α*, *40S ribosomal protein S8* (*RPS8*), *thioredoxin peroxidase 1* (*TrxP1*), *actin1* (*ACT1*), *nuclear transport factor* (*NTF*), *ADP, ATP carrier proteins mitochondrial* (*AACM*) (Tab. 1). Moreover, NormFinder was used to analyze the stability values of putative genes to ensure accurate and precise results. A total of 15 genes with an M values < 0.17 were obtained, including *RPL19*, *RPS15*, *CAM*, *RPS9*, *TFs*, *EF1α*, *mRNA-the RTFL01-30-A06 clone* (a predicted protein with an unknown function), *NTF*, *AACM*, *TrxP1*, *ubiquitin* (*UBI*), *RPS8*, *tubulin alpha-1 chain* (*TUB*), *histone H3.3* (*His-H3.3*), and *RPS28* (Tab. 1). These results were obtained from the geNorm and NormFinder algorithms, which revealed that there were 10 candidate genes with high M values in both assessing systems (Tab. 1).

Table 1: Stability of reference genes tested by geNorm and NormFinder

geNorm		NormFinder		Consensus
Gene	M-value	Gene	M-value	
RPS15[a]	0.268358	*RPL19*	0.097	*RPS15*
RPS9	0.268358	*RPS15*	0.106	*RPS9*
TF	0.296171	*CAM*	0.111	*RPL19*
RPL18	0.364888	*RPS9*	0.117	*RRPS28*
RPS14	0.390116	*TF*	0.123	*CAM*
RPL19	0.414952	*EF1α*	0.126	*EF1α*
UEP1	0.440746	*Unknown*	0.128	*RPS8*
RPS28	0.463827	*NTF*	0.129	*TrxP1*
CAM	0.491967	*AACM*	0.142	*NTF*
EF1α	0.527389	*TrxP1*	0.159	*AACM*
RPS8	0.551129	*UBI*	0.160	
TrxP1	0.569221	*RPS8*	0.162	
ACT1	0.584119	*TUB*	0.162	
NTF	0.59835	*His-H3.3*	0.164	
AACM	0.61186	*RPS28*	0.167	

[a]Gene descriptions are as follows: RPS15 (40S ribosomal protein S15); RPS9 (40S ribosomal protein S9); TF (transcription factor); RPL18 (60S ribosomal protein L18-2); RPS14 (40S ribosomal protein S14); RPL19 (Ribosomal protein L19); UEP1 (Ubiquitin extension protein 1); RPS28 (40S ribosomal protein S28); CAM (Calmodulin-5/6/7/8); EF1α (Elongation factor 1 alpha); RPS8 (40S ribosomal protein S8); TrxP1 (Thioredoxin peroxidase 1); ACT1 (Actin1); NTF (Nuclear transport factor); AACM (ADP, ATP carrier protein, mitochondrial); Unknown (mRNA, clone: RTFL01-30-A06); UBI (Ubiquitin); TUB (Tubulin alpha-1 chain); His-H3.3 (Histone H3.3).

3.2 Expression Profiles of the Reference Genes

The average Ct values of the 10 putative reference genes from different samples are summarized (Fig. 2). These results indicated that the average Ct values of all candidate genes varied from 20 to 27. The Ct values of *EF1α* and *RPS28* were the lowest, ranging from 20.04 to 22.41 and 20.31 to 24.42, respectively, indicating that the expression abundances of these 2 genes were the highest. *NTF* had the highest Ct value, ranging from 22.67 to 25.28, indicating that it had the lowest expression abundance. Thus, it was concluded that the changes and differences in the mean Ct values were related to the M values of different genes. Interestingly, the same gene had different M values in different tissues. Thus, it is clearly vitally to select stable candidate reference genes for the data normalization in different tissues.

The line across the box represents the median values. Whiskers indicate the maximum and minimum values, respectively. The box represents the 25th and 75th percentiles.

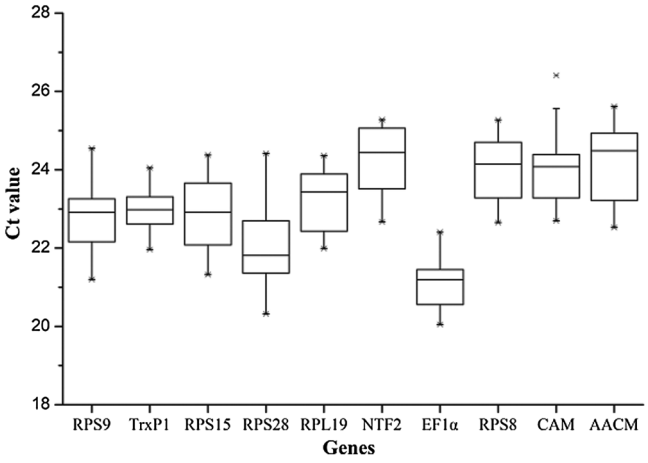

Figure 2: Average Ct values represent RNA transcription levels of reference genes

3.3 geNorm Analysis

The 10 putative reference genes were evaluated to obtain M values using the geNorm program, which corrects multiple internal reference genes [28]. The average M values were computed by Ct-converted data. According to the selection principles of the geNorm program, the candidate gene will be regarded as a stable reference gene if M value < 1.5. Genes with the lower M values represent a more stable expression. Results revealed that the M values of the 10 internal reference genes were all < 0.65, much less than the standard 1.5 threshold, indicating that they were relatively stable. Among these genes, *RPS28* was the least stable, and the combination of RPS15 and RPS9 was the best gene combination, and both genes were stably expressed. The remaining 7 candidate genes' M values were located between these 2 genes (Fig. 3).

The results revealed that there was more than 1 internal reference gene, which may be a combination of multiple genes depending on the V values [32]. According to the fundamental concept, it is not necessary to

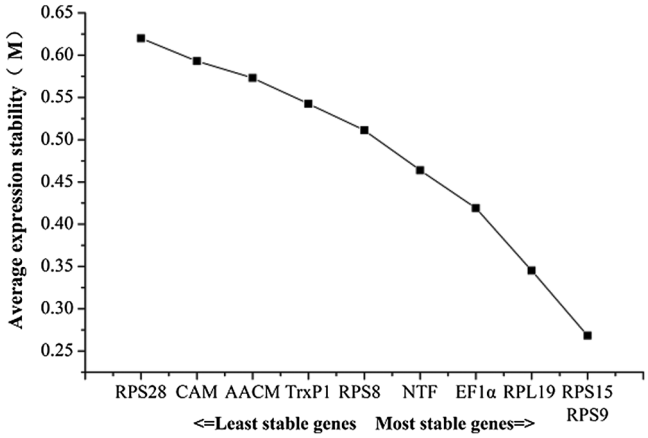

Figure 3: Average expression stability (M) and ranking order of 10 reference genes by geNorm program analysis

add extra genes for reliable normalization unless the value of Vn/n + 1 is > 0.15. In this study, V2/3 indicated the lowest pairwise variation was 0.093, which was < 0.15; thus, for the optimal internal reference genes, 2 should be selected without appending additional genes to normalize accurate results (Fig. 4).

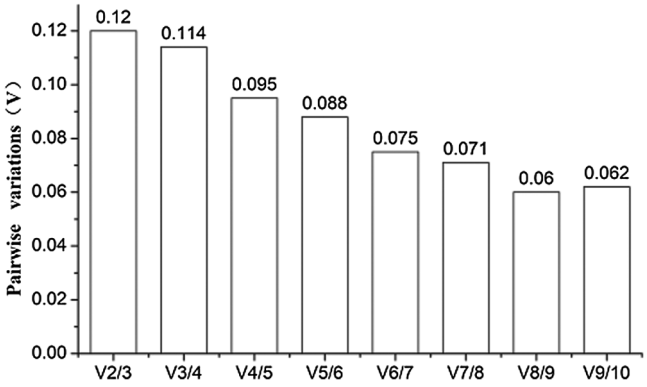

Figure 4: Pairwise variation (V) for the optimal number of candidate genes by geNorm software

3.4 NormFinder Analysis

The M values of all 10 candidate reference genes were analyzed using the NormFinder program, which can compare the expression differences of putative reference genes. Additionally, it can calculate the variation between sample groups, but it can only select the most appropriate candidate reference gene [34]. Based on the results, the reference gene with the lowest M value was regarded as the most stable reference gene. *RPL19* had the lowest M value and was ranked first for expression stability. *CAM* ranked second, and *EF1α* ranked third. *TrxP1* was the most unstable gene with the largest M value (Fig. 5). It is worth noting that *RPL19* was ranked second by the geNorm program, indicating that there were some differences between the 2 programs.

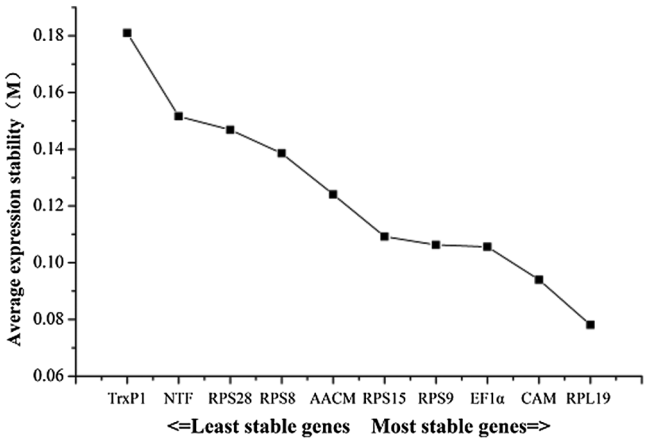

Figure 5: Expression stability value of ten candidate genes by NormFinder analysis

3.5 BestKeeper Analysis

BestKeeper is an excel program used for analyzing candidate reference gene expression variation. It is used to compare acceptable internal reference genes and identify the expression degree of interesting genes. However, it could only calculate the expression level of 10 reference genes and 10 target genes, which amounted to 100 sample groups. Afterwards, the R, SD, and CV values between each gene were obtained. The larger the R value and the smaller the SD and CV, the better the stability of the internal reference gene. However, when SD > 1, it indicates that the reference gene is unstable. The SD of the 10 selected genes were all < 1 (Tab. 2); thus, they were considered acceptable internal reference genes. The

Table 2: Expression stability values of ten candidate genes by BestKeeper analysis

	TrxP1	*EF1α*	*RPS8*	*RPL19*	*CAM*	*NTF*	*RPS9*	*RPS15*	*AACM*	*RPS28*
Geo Mean [CP]	22.92	21.06	24.06	23.23	24.12	24.25	22.84	22.86	24.23	22.02
Ar Mean [CP]	22.93	21.07	24.07	23.24	24.14	24.26	22.86	22.88	24.25	22.04
Min [CP]	21.96	20.05	22.65	21.99	22.70	22.67	21.20	21.32	22.53	20.32
Max [CP]	24.05	22.41	25.27	24.36	26.41	25.28	24.55	24.38	25.62	24.42
SD [± CP]	0.44	0.53	0.69	0.69	0.75	0.80	0.76	0.78	0.85	0.85
CV [% CP]	1.93	2.51	2.86	2.96	3.12	3.28	3.34	3.42	3.49	3.85
R	0.81	0.86	0.84	0.97	0.89	0.87	0.95	0.96	0.87	0.87

Geo Mean: Geometric mean; Ar Mean: Arithmetic mean; CP: Confidence parameter; SD: Standard deviation; CV: Coefficient of variation; R: the pairwise correlation; coefficient, n = 18.

ranking order was as follows: *TrxP1* > *EF1α* > *RPS8* > *RPL19* > *CAM* > *NTF* > *RPS15* > *AACM* > *RPS28*. Therefore, the least stable gene was *RPS28*, while the most stable gene was *TrxP1*.

3.6 RefFinder Analysis

RefFinder is an online analytical tool that integrates the existing Delta Ct, geNorm, NormFinder, and BestKeeper analytical procedures. By computing the geometric mean of the stable value weights, a comprehensive ranking of each internal reference gene was obtained. The results of RefFinder program are provided (Fig. 6). The ranking in descending order was as follows: *RPL19* > *RPS15* > *RPS9* > *EF1α* > *TrxP1* > *RPS8* > *NTF* > *CAM* > *AACM* > *RPS28*. Therefore, *RPL19* was the most suitable gene, matching the results of NormFinder. In contrast, *RPS28* was the least stable gene.

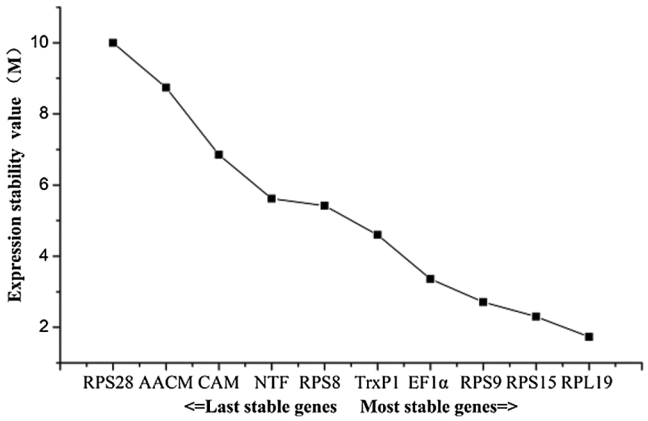

Figure 6: Expression stability values (M) of candidate genes by RefFinder analysis

4 Discussion

In the qRT-PCR analyses, the stability of the same reference gene in the same species under different experimental conditions may differ considerably. The stability of reference genes is an important precondition for gene expression research [2,8,35]. Notably, various tissues in the same species may require different internal reference genes. For instance, *ACT* was the most stable reference gene in different tissues of eucalyptus tereticornis. However, in leaf and internode tissues, *cellulose synthase*

4 (*CesA4*), *cellulose synthase 5* (*CesA5*), and *cellulose synthase 6* (*CesA6*) were the most stable reference genes but they were unstable in growing and mature xylem tissues [36].

Unlike conventional verification experiments for internal reference genes, this study did not directly select commonly used housekeeping genes as experimental genes. Instead, the FPKM algorithm was used to calculate gene expression stability in the transcriptome data of various potato tissues [37]. Based on the transcriptome data, genes were relatively, moderately, or highly expressed in the DM and RH strains. According to the CVs, the genes of DM and RH were screened separately.

The screened genes were considered putative reference genes for qRT-PCR experimental validation and the stabilities of their expression were analyzed using 4 common internal reference gene analysis programs, NormFinder, geNorm, BestKeeper, and RefFinder. NormFinder, geNorm, and BestKeeper are 3 programs that analyze putative reference genes based on different algorithms to select a suitable internal reference gene for expression studies under different experimental conditions. NormFinder and geNorm process data by using the $2^{-\Delta Ct}$ method to convert the original Ct values of different genes from different samples. Moreover, geNorm, also selects the best number of genes based on V values. According to previous studies, the number of internal reference genes should be based on the Vn/n + 1 formula. If only 1 internal reference gene is selected, it may cause errors in the experimental results [6]. Although NormFinder can compare the expression differences of candidate internal reference genes and calculate the variation between sample groups, this program can only select the most suitable internal reference gene [34]. BestKeeper implemented calculations directly using the Ct values obtained from the qRT-PCR analyses, which were different from geNorm and NormFinder [29]. Although previous studies have shown that different analytical methods can produce the same results [38], the results of the analysis are not always the same [27,39,40], which may be because the stability of gene expression was based on different foundations. Therefore, based on the differences in the results obtained by different analytical algorithms in this study, the web-based tool RefFinder, was used to comprehensively evaluate and screen ideal internal reference genes to ensure accuracy and dependability.

The 4 programs used in this study have been widely used for selecting internal reference genes in plants, animals and microorganisms [40]. In this study, it was not always appropriate to select *EF1α* as the reference gene in the qRT-PCR. Based on the results, although *EF1α* ranked in the top 5 genes based on the 4 programs, it was not always ranked first, indicating that it was not the most appropriate internal reference gene. geNorm revealed that 2 genes, *RPS15* and *RPS9*, were stably expressed in potato leaves, flowers, stolons, and tubers. Based on the V values, the appropriate number of reference genes was 2. The NormFinder results revealed that the best gene combination was *RPL19* and *CAM*. BestKeeper determined that the 2 most stably expressed genes in the 4 tissues were *TrxP1* and *EF1α*. Based on the RefFinder analysis, *RPL19* and *RPS15* were the most stable reference genes in the potato tissues. It should be noted that the integrated results obtained by these different analysis methods were different from one another. However, based on a comprehensive analysis of the 4 programs, *RPL19* and *EF1α* were better than the other genes under different analyses and ranked in the top 4 genes in all 4 programs (Tab. 3). Previous studies have suggested that when performing gene expression analyses, compared to using a single reference gene to normalize the expression value of target genes, selecting 2 or more internal reference genes is conducive to obtaining accurate and reliable results [19]. Therefore, based on the findings of this study, *RPL19* and *EF1α* are recommended as internal reference genes for the future qRT-PCR analyses on potato tissues.

Table 3: Ranking of 10 reference genes calculated using all four software

Ranking	geNorm	NormFinder	BestKeeper	RefFinder
1	*RPS15*[a]	*RPL19*	*TrxP1*	*RPL19*
2	*RPS9*	*CAM*	*EF1α*	*RPS15*
3	*RPL19*	*EF1α*	*RPS8*	*RPS9*
4	*EF1α*	*RPS9*	*RPL19*	*EF1α*
5	*NTF*	*RPS15*	*CAM*	*TrxP1*
6	*RPS8*	*AACM*	*NTF*	*RPS8*
7	*TrxP1*	*RPS8*	*RPS9*	*NTF*
8	*AACM*	*RPS28*	*RPS15*	*CAM*
9	*CAM*	*NTF*	*AACM*	*AACM*
10	*RPS28*	*TrxP1*	*RPS28*	*RPS28*

[a]The expression value of RPS15 and RPS9 were the same, so they both ranked first.

5 Conclusions

In this study, the stability of reference genes in various potato tissues were analyzed, and *Ribosomal protein L19* (*RPL19*) and *Elongation factor 1-alpha* (*EF1α*) were selected to normalize the gene expression results of qRT-PCR on potato tissues. It provides suitable internal reference genes for the future research of potato and also lays indirect foundation for the study of potato genetics.

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Appendix A

Table S1: General information, primer sequences, and amplification length of candidate reference genes

Transcript_ID	Name	Primer sequence (5'–3')	Amplicon length (bp)
PGSC0003DMT400013068	Thioredoxin peroxidase 1	F: CAATGACCCCTTTGTGATGAAG R: CATTGAGGATTTCATCAGCACC	245
PGSC0003DMT400081472	ADP,ATP carrier protein, mitochondrial	F: GACAATTTGATGGGTTGGTTGA R: CATGAAATGTTAAACCCACGGT	88
PGSC0003DMT400061684	Yth domain-containing protein	F: ATGCTGATGCCAAGTTCTT R: TGCCAATACTCCACATTCTT	250
PGSC0003DMT400076016	40S ribosomal protein S6	F: ACTGAGAAACCAAGGATGAGAG R: ATACAGAACAGACTAGGGACCT	161
PGSC0003DMT400058435	Calmodulin-5/6/7/8	F: GCAAGATGAAGGACACTGATTC R: TCATCAGTAAGCTTTTCGCCTA	136
PGSC0003DMT400080373	4F5 protein family protein	F: TTGAACTATCTCGTCTTCCT R: AACGCTCCACTAATGACAT	179
PGSC0003DMT400042276	ADP-ribosylation factor	F: GAATGCTGCTGAAATAACCGAT R: GATCAATCCGCAACAACTCTAC	173
PGSC0003DMT400009623	Thioredoxin	F: TATTGTGACTGCTTGAGGCTTA R: ACAAATGTCAGATTCCAAGCAC	163
PGSC0003DMT400042183	Ribosomal protein S27	F: AGTTGAGCAGGAGAAGAGA R: ACACCGTTGTTATGTTGAAG	107
PGSC0003DMT400073070	Sec61 transport protein	F: GGTGACGCTTACACATAAATCC R: AATTATCTAGAAGCAGCGACGA	156
PGSC0003DMT400049116	Glutaredoxin	F: AACTGTGCCAAACGTCTTCATA R: AGTTAGCAGAGGAACAAGCTTC	97
PGSC0003DMT400052308	CBS1	F: GCAAGGAGGAGGATTCAAG R: GTTCTTCTATACGAGCAGTTG	152
PGSC0003DMT400046201	Ribosomal protein L19	F: CCCGTCAAAACATCAGGAAATT R: GTACCTTTGCGCTTACCATATC	142
PGSC0003DMT400089729	Protein C6orf115	F: CGTTGAAGAAGAGGTTGAAC R: GTGGTGGCTTGAGAATGAT	239
PGSC0003DMT400056871	Phosphoglycerate kinase	F: TCATCCTTGCCTCTCATCT R: GGTCATTCTTCTCCTCTTCTT	219
PGSC0003DMT400002454	Phosphoglycerate mutase	F: TCTTTTGGAATGGAAACCGTTC R: ATTAGGTATGTTCACCCGTACC	185
PGSC0003DMT400076528	GTP-binding nuclear protein Ran1	F: TTTGTCAAAAGGCATCTCACTG R: CTGTATCCCAGCAATAAAAGCG	124
PGSC0003DMT400072220	Alpha chain of nascent polypeptide associated complex	F: GCAGAACTTGGAGATGGTA R: GCTCTTCTTGACAGTTACAC	126
PGSC0003DMT400058429	Cystatin	F: GAAAGCATATGAAGCCAAGGTC R: ACATCATTCAGCGCTTGTTAAG	111

(Continued)

Table S1 (continued).

Transcript_ID	Name	Primer sequence (5'–3')	Amplicon length (bp)
PGSC0003DMT400065149	Protein C6 orf115	F: ATTAAGGGCTGCAAAGAAAAGG R: TCCAGTAACTTGTACACACTCC	174
PGSC0003DMT400069911	Ribosomal protein L33	F: GAGTTTTCATGTACCCAAGCAA R: TGACCTCGATAGACAAACGTAG	85
PGSC0003DMT400033268	BI1	F: CGCAACAAACCTTATTCATTGC R: CAAACTCGTGAATCCGTACATC	83
PGSC0003DMT400041634	14-3-3 protein	F: GTTCATGGACAAGCTCGTAATC R: CGATAACGTTCTTATACGCCAC	92
PGSC0003DMT400061066	TGB12K interacting protein 3	F: GGATATGGTAGGAAGGAGTGTG R: CATGACCAAACATCTATGCCAG	176
PGSC0003DMT400016576	GTP-binding nuclear protein Ran1	F: TTTGTCAAAAGGCATCTCACTG R: CTGTATCCCAGCAATAAAACCG	124
PGSC0003DMT400066245	Poly(A)-binding protein	F: GACGATGCTAGGTGAAAATCTG R: GAGACTCAAGCAAGTGCAATAC	122
PGSC0003DMT400038856	Aspartic protease	F: AGAAAATGAGAGTTGGTTTCGC R: CTCGTTCAAAAGACAACGTGAT	95
PGSC0003DMT400028926	Eukaryotic translation initiation factor 5A-3	F: GGTTACATCGTTATCAAAGGCC R: TCAATCAGCTGATAGTCGGTAC	194
PGSC0003DMT400004661	60S ribosomal protein L37a	F: TAAAGAGGAAAGCAGTGGGAAT R: ACTCCTAACTGTAACAGCACTC	104
PGSC0003DMT400018728	mRNA, clone: RTFL01-30-A06	F: AAAGAATTAGCTGCAAAGGTCG R: CACTTTGCAAAGAGGACATTCA	126
PGSC0003DMT400007088	NtK-1	F: CAGGCAAAATGCTTAGAGACTG R: TGGAAAAGAAGCAGTGTTTCAG	142
PGSC0003DMT400011939	Ubiquitin extension protein	F: CCAAGAAGCGTAAGAAGAAG R: TGCCAGAATCATCAACCTT	108
PGSC0003DMT400036714	TMS membrane family protein	F: CATCGGTAGGTGGAAGTATT R: AGCAGAGTAGACAACAGATAG	191
PGSC0003DMT400047481	Actin1	F: TCTCTACATACTTGTGAGGTGC R: CCTAAAAGAGACACTTCGACCA	83
PGSC0003DMT400054361	60S ribosomal protein L32-1	F: AGAGTGAGGAGAAAGTTCAAGG R: TGGAACTAGGGTTTCTGCTAAG	125
PGSC0003DMT400039497	P40	F: TCTCATTCTCACTGACCCTAGA R: ACCATCCTTGCTAAGATCCAAA	183
PGSC0003DMT400020252	Eukaryotic translation initiation factor 5A-1/2	F: CATGCAAAATGTCACTTTGTGG R: CACCTTTAACCTGGTTCAACAG	226
PGSC0003DMT400035158	ADP-ribosylation factor	F: CACTCAGGGTCTCATTTTTGTG R: ATCAGTTATTTCGGCAGCATTC	172
PGSC0003DMT400069948	Iron transport protein 2	F: ATTAGATCTGATCTGAGCGCAT R: ATCATCACATTCACAAACCGTC	201

Table S1 (continued).

Transcript_ID	Name	Primer sequence (5'–3')	Amplicon length (bp)
PGSC0003DMT400036088	SUMO	F: CAGGATCAAAAGAAGCACTCAG R: GACCATCAAACAAGAAGGCAAT	201
PGSC0003DMT400030110	Tubulin alpha-1 chain	F: TCATTTCAGCTGAGAAGGCTTA R: CATAGAAGAGGGTTCAAATGCG	80
PGSC0003DMT400007062	60S ribosomal protein L24	F: CAAGAAGCTGTTAGGAAGAGGA R: CTTTCAGCTCTCTTCTTCTGGA	156
PGSC0003DMT400053626	40S ribosomal protein S8	F: ACTCGTATCCTTGATGTGGTTT R: GGTGCAGCATCAACTTGAATAA	225
PGSC0003DMT400041105	Ubiquinol-cytochrome c reductase iron-sulfur subunit	F: CTTTCACCGTTACCGATGATTC R: CTAGGATCACCAGGTGGATAAC	196
PGSC0003DMT400041662	Histone H3.3	F: CTTCAAGACTGATCTGCGTTTC R: CTGAATGTCCTTGGGCATAATG	145
PGSC0003DMT400053553	Elongation factor 1-alpha	F: ATTGGAAACGGATATGCTCCA R: TCCTTACCTGAACGCCTGTCA	101
PGSC0003DMT400027156	Polyubiquitin 10	F: TGATAATGTGAAGGCGAAGA R: ACGAAGAACAAGGTGAAGA	148
PGSC0003DMT400000264	Eukaryotic translation initiation factor 1A	F: CGGTGTGAAGCTATGTGTATTG R: TGTCATCCTGATAATCACGGAG	127
PGSC0003DMT400035380	ADP,ATP carrier protein	F: CTTATGGCAACTACTCCAATGC R: GGATCAATAGTTTCACACGCTC	210
PGSC0003DMT400023419	Ubiquitin-conjugating enzyme E2	F: AATGGTCATTTTTCCTTGACGG R: AACTCCACCAGAATAAGGACTG	259
PGSC0003DMT400078234	Peptidyl-prolyl cis-trans isomerase	F: TGACACCAATGGTTCACAATTC R: GTGTTCCACTTTGTCTTCCTTC	140
PGSC0003DMT400010174	Actin1	F: CATCACTTAGCACCTTCCA R: AATAGCAGCAGCACCTTAG	104
PGSC0003DMT400018921	40S ribosomal protein S15	F: TCTTGACATGTCTACTGACGAG R: GCTTCTTGATCAAAGCCATAGG	153
PGSC0003DMT400007431	40S ribosomal protein S9	F: TGAAGCGAAAGAACCAAAAGTC R:ACAAGCCAAATAGACCAAACAC	157
PGSC0003DMT400007183	Small GTP-binding protein Sar1BNt	F: AGATGAACTGCGTTACCATTTG R: TTGATGTACTGAGACATCCACC	162
PGSC0003DMT400061896	Conserved gene of unknown function	F: CGTGATCGGACCAAGGATATTA R: TCATCCACAACTTTAGTCGGAA	227
PGSC0003DMT400016513	Acireductone dioxygenase	F: CTACATTAAGGCAATGCGTCTC R: GTAAGGCACACATCAAGAACAG	237
PGSC0003DMT400017277	Mitosis protein YLS8	F: TGTTGCTTTCAAGATCCAAGTG R: ATTAGCATTCTGAAGCGGAAAC	138
PGSC0003DMT400064031	Chloroplast heat shock protein 70-2	F: GGGACAACAAATCTTTAGGCAG R: GTCACGGAAAGAATACCATTGG	115

(Continued)

Table S1 (continued).

Transcript_ID	Name	Primer sequence (5'–3')	Amplicon length (bp)
PGSC0003DMT400079216	Histone H3.3	F: TACAAATCTGTGTGCCATTCAC R: GCTTTAGCAATCTAAGCAACGA	127
PGSC0003DMT400024783	SKP1	F: TTTTGTCAAAGTTGATCAGGGC R: CCTCAGGAGTGAAGTCATTCTT	173
PGSC0003DMT400006183	60S ribosomal protein L10	F: TAGAAGCTGCGAATTGAAGTTG R: CTTTGAAAAGGCAAACGTTCAG	136
PGSC0003DMT400058781	Transcription elongation factor 1 homolog	F: CTTCAGTACCACTGTCACAGAT R: GTTTCGTCTCGAGACTCAAAAC	153
PGSC0003DMT400022864	40S ribosomal protein S14	F: CTGGCATGAAAATTGGACGTAT R: AAGAAACAGTTTTCACAGCCTC	104
PGSC0003DMT400011736	Ubiquitin extension protein	F: AAGGTTCAGAGACTTCGTAAGG R: TCTGGTAAACATAGGTAAGCCC	120
PGSC0003DMT400022663	Ferritin	F: CGAAACAATGATGTGCAATTGG R: TCAGCTGAGCAACATATTCTGA	196
PGSC0003DMT400001468	Hydrogen peroxide-induced 1	F: TGGTGCTACTGCTGAATCTATT R: GAGACTCGCATAAACACAAACA	165
PGSC0003DMT400073918	transcription factor	F: GAATAGGTGTCAATGCCATTCC R: AACTTTGGGGTTAACGAATTGG	175
PGSC0003DMT400065401	J1P	F: CGTATGAGGTTTTGAATGACCC R: TGACTGGAATATGTCAAACGGA	122
PGSC0003DMT400052213	Nuclear transport factor	F: GATCACTACTACTCCACCTTCG R: GACAATCGACGGTGTTGATATG	175
PGSC0003DMT400021531	40S ribosomal protein S28	F: GATACACAGCAGCAACATACAG R: AGAAACTTGACTCTCACCTGAG	176
PGSC0003DMT400063593	60S ribosomal protein L18-2	F: AGTAAGAAGACTAAGCGTACCG R: CCTCTTAAGAATCACCGCATTG	192
PGSC0003DMT400089149	Polyubiquitin 10	F: CCATCACTTTGGAGGTTGAAAG R: ACAAAAATCTGCATACCACCAC	208
PGSC0003DMT400052032	Mitochondrial phosphate carrier protein	F: TATTGTATCCCATCCTGCTGAC R: TATCTTCTTTACGGCATCACCA	85
PGSC0003DMT400008913	Actin depolymerizing factor 3	F: TGAAAACTAAAAGGGCTTTCCG R: TACACTGCATAACGACACTCAT	142
PGSC0003DMT400048091	High mobility group protein 2 HMG2	F: ATAACAAGCTCGGAGTGAAGAA R: AAGCTTCTCATACTCAGCCTTT	269
PGSC0003DMT400037403	Ubiquitin	F: ATCCAAAAGGAGTCTACTCTGC R: TCTTCTAGACTCTCTTGGGACA	151