Molecular and Cellular Probes 53 (2020) 101610

Contents lists available at ScienceDirect

Molecular and Cellular Probes

journal homepage: www.elsevier.com/locate/ymcpr

Selection of stable reference genes for gene expression analysis in sweet potato (*Ipomoea batatas* L.)

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ARTICLE INFO

Keywords:
Sweet potato
Quantitative real-time PCR
Reference gene
Gene expression

ABSTRACT

Gene expression analysis is one of the most common and important studies in biology and biomedicine. No matter for traditional blotting analysis or currently commonly used PCR strategy, all need a stable reference gene for normalizing the gene expression. To screen and select housekeeping genes as the most stable reference genes, quantitative real-time PCR (qRT-PCR) was employed to analyze the expression of sixteen commonly used reference genes (*IbelF*, *Ibα-tubulin*, *IbHIS*, *IbCOX*, *IbGAPDH*, *IbH2B1*, *IbARF*, *IbCYC*, *Ibβ-tubulin*, *IbACT*, *IbEFl-a*, *IbG14*, *IbPLD*, *IbRPL2*, *IbUBQ*, *IbUBI*) in five different tissues under two different temperature stresses in sweet potato. Data analysis by the Delta CT, geNorm, NormFinder, and BestKeeper programs revealed that *IbelF* is the most stable gene and *IbUBI* is the least stable gene as reference. Our study also shows that combination of two or more genes as reference is a better choice, rendering more substantiated expression data for comparison. This study provides evidence for selecting reference genes in sweet potato gene expression analysis.

1. Introduction

Quantitative real-time PCR (qRT-PCR) is the most commonly used method for analyzing gene expression with high sensitivity and specificity in a shortest time [1,2]. However, this method and also others, such as Northern blotting, require one or more housekeep genes with stable expression as a standard for normalizing gene expression. The ideal reference genes should be expressed constantly at different samples under various experimental factors. Some housekeeping genes that are thought to have stable expression, such as actin, tubulin, GAPDH and 18SrRNA, are often used as endogenous reference genes for qRT-PCR and other gene expression analysis, including Northern blotting [3]. However, with the wide application of qRT-PCR, it has been reported that the expression of these commonly used reference genes is not stable under certain experimental conditions [4–6]. The constant expression of any housekeeping genes is only constant over a small range of cells under certain experimental conditions [7]. If an inappropriate reference gene is used to normalize the expression of other genes, the results may be misinterpreted [8]. Therefore, many researchers have attempted to find reliable reference genes that have stable expression under specific experimental conditions. The selection of reference genes has an important impact on the quality of quantitative analysis results. At present, several statistical analysis and computational software have been developed, such as geNorm, NormFinder, BestKeeper [6,7,9], for evaluating housekeeping genes as reference genes. These programs base on certain statistical methods to select the reference genes that are least altered under specific experimental conditions from a range of housekeeping genes.

Sweet potato is one of the most important food crops in the world, which is rich in starch and is a good source of soluble sugar, dietary fiber, and vitamins such a beta-carotene [10]. More research in sweet potato requires gene expression analysis for gene function studies and crop improvement. However, the majority of researchers selected *tubulin* and/or *actin* as a reference gene in qRT-PCR and other gene expression analysis [11–13]. This may be misinterpreted the gene expression because all of the housekeeping genes are not constantly expressed in all tissues under all environmental conditions. Therefore, when analyzing the gene expression of different genotypes under different experimental conditions, it is important to choose a reference gene that is stably expressed in the same tissues and conditions. Both abiotic and biotic stresses are major factors affecting gene expression. Among these, temperature is an environmental factor that plays a

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https://doi.org/10.1016/j.mcp.2020.101610
Received 8 April 2020; Received in revised form 6 May 2020; Accepted 27 May 2020
Available online 06 June 2020

decisive role in plant growth and development. High and low temperature stresses hindered the growth and development of sweet potato and further resulted in yield loss. Therefore, in this study, we evaluated the most commonly used reference genes and selected the most reliable reference genes for gene expression analysis in sweet potato using 5 different tissues under two extreme temperature stress (heat and cold stresses) conditions.

2. Materials and methods

2.1. Plant materials and stress treatment

Sweet potato (*Ipomoea batatas* L.) seedlings at the same growth stage were cultivated in the greenhouse with regular agronomic practices, including daily watering. After two weeks, when they reached a steady state, the seedlings were transferred to an incubator for temperature stress treatment. The plants were divided into three groups. For the heat treatment group, sweet potato seedlings were cultivated in an incubator at a temperature of 47 °C. At the same time, for the cold treatment group, seedlings were cultivated in a 4 °C incubator. Plants maintained at 25 °C were used as controls. After 6 and 48 h of temperature treatment, the leaves were randomly sampled from three plants of each treatment group [35]. Additionally, leaves, fibrous roots, storage roots, ovary and petals of sweet potato mature plants were also sampled from the greenhouse. All materials were immediately frozen in liquid nitrogen and then stored at −80 °C until RNA extraction and gene expression analysis.

2.2. RNA isolation and cDNA synthesis

The samples were kept in liquid nitrogen and ground into powders using a mortar. Total RNAs were extracted from each collected sample using the mirPremier® microRNA Isolation Kit (Sigma, US) following the protocols suggested by the suppliers. The quality of the extracted RNAs was measured by using an Nanodrop 1000 spectrophotometer (Thermo SCIENTIFIC, US). Only RNA samples with high quality (260/280 ratio: 1.8 to 2.1 and a 260/230 ratio: 1.8 to 2.1) were used for subsequent gene expression analysis. A total of 1000 ng RNAs were reverse-transcribed to cDNA by using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems).

2.3. Primer design and quantitative real time PCR (qRT-PCR)

A total of 16 potential reference genes were selected for this study. Their sequences were extracted from the GenBank or from the deep sequenced RNA-seq data in a previous report [13]. NCBI primer-blast program was employed to design the primers for each gene with a manual inspection. The designed primers (Table 1) were synthesized by Sigma (Houston, TX, US). The specificity of each primer pair was tested by melting curve analysis; the amplicon size was checked by gel electrophoresis. The primer efficiency was tested using the diluted cDNA.

qRT-PCR was performed by an ABI 7300 Real Time PCR System (Applied Biosystems). Each reaction (final volume of 20 μl) included 1.0 μl diluted cDNAs, 10 μl of 2 × SyberGreen PCR Master Mix (Applied Biosystems), and 1 μl of each primer. The PCR amplification was as follows: 95 °C for 10 min, followed by the 45 amplification cycles at 95 °C for 15 s and 60 °C for 60 s.

2.4. Determination of expression stability of potential reference genes

Four different statistical algorithms (Delta CT, geNorm, NormFinder, and Bestkeeper) were employed to determine the expression stability of 16 candidate reference genes in different tissues under different experimental conditions. The Delta CT software is able to calculate the SD value of the CT value of each candidate reference gene. The smaller the SD value, the higher the stability of the reference

Table 1
Primer for sweet potato reference genes.

Gene name	GenBank ID	Gene abb	Forward/Reversed Primer (5′- 3′)	Melting Temp (°C)
actin	EU250003.1	ACT	GCTTGCATATGTGGCCCTTG	59.9
			CAGCGGAACCCTTCTCTTGTTAC	61.41
ADP-ribosylation factor	JX177359.1	ARF	GGGATGCTGTGTTGCTTGTG	60.04
			TGACGTTGACGGAGAGAGTG	59.41
cytochrome coxidase subunit Vc	S73602.1	COX	CTCCCAGTGGCGGTGTTATG	60.74
			GGATGTTCTTGAGCCGGTCG	61.08
cyclophilin	EF192427.1	CYC	CCAACACCAACGGCTTTCAG	59.97
			CCACCTTCTTCACCACGTCC	60.00
glyceraldehyde-3-phosphate	JX177362.1	GAPDH	TGGCGAGAAGTCAGTTAAGGTC	60.03
			CCTTCTTGGCACCAGCCTTC	61.25
	JX177361.1	H2B1	GGTGCCGGAGACAAGAAGAAG	60.94
			GCCTTGCTGGAGATTCCGATG	61.41
phospholipase D1 alpha	JX177360.1	PLD	TGATGGCGACGTAACAGAGC	60.46
			ATGATGAGGCAAGCAGTGTGG	60.95
ribosomal protein L2	AY596742.1	RPL2	ATTAGCTAGAGCAGCGGGTG	59.61
			CTTGTCCGACTGTTGCTGAG	58.86
Alpha tubulin	BM878762.1	α-tubulin	CCAACCGGCTTCAAATGTGG	60.04
			TGTGGTCGATGCGTGAGAAC	60.67
ubiquitin extension protein	JX177358.1	UBI	CGAGGTTGAATCGTCAGACAC	59.01
			CTCCTTCTGGATGTTGTAGTCG	58.22
tubulin alpha-3 chain-like	XM-019297562.1	β-tubulin	TGCTGAGAGTGCTTTGCCTTC	61.15
			CGGGCAGACATTACACAAACAC	60.35
metallothionein-like protein	AF242374.1	G14	AGGAAGGGAAGCAAGATGGAC	59.72
			CACTTGCACCCATCATTCTC	56.78
translation initiation factor eIF-2B	XM-019343175.1	eIF	CCATCTCTTTGACGGCTGGTTG	61.76
			TCTCTGCACGCTCAAGAAGG	60.04
subunit alpha-like	XM-019297116.1	His	ATGGCTCGTACCAAGCAGAC	60.11
histone H3.2			CAACGATGCGGCTTCTTCAC	60.18
elongation factor 1-alpha	XM-019307199.1	EF1-a	CGCAGGTTTCTAGGCTCTCG	60.53
			CACACGCTTGTCTATACCTCC	58.19
polyubiquitin	XM-019327923.1	UBQ	TGCAGGGAAGCAATTGGAAG	58.74
			TCAGAAACCACCACGGAGAC	59.61

gene; conversely, the larger the SD value, the lower the stability of the gene. The geNorm program algorithm examines the optimal number of reference gene expression stability and standardization. It first calculates the stable expression of each gene and then calculates the pairwise variation of the gene with other genes. The lowest stable value represents the most stable expression of this reference gene in the detected genes. The NormFinder program algorithm determines the expression stability of the candidate reference gene and the optimal gene or combination of genes for standardization purposes. The principle for NormFinder program analysis is similar to that of geNorm. By calculating the stable value of the candidate reference gene, the lower the stable value, the more stable the expression of the gene. The BestKeeper program algorithm can determine the stability of the reference genes by comparing the coefficient of variation, standard deviation, and the value of the correlation coefficient between the genes for each selected gene. The higher the value of the correlation coefficient of the selected gene, the smaller the value of the coefficient of variation and the value of the standard deviation, indicating that the expression stability of the candidate reference gene is higher.

3. Results

3.1. Expression analysis of candidate reference genes

We calculated the Ct values of 16 candidate reference genes in all tested materials (Fig. 1 and Table 2). *IbRPL2* had the smallest of Ct value and *IbEFl-a* had the largest Ct value. The expression variation of each candidate reference gene was different, and the Ct changes of *IbPLD* and *IbHIS* were small with no more than three cycles. The Ct changes in the expression of *IbUBQ* and *IbGAPDH* were larger with more than four cycles of difference. Moreover, the Ct value of *IbEFl-a* changed the least, and the Ct value of *IbG14* changed the most. Obviously, not every reference gene has the same expression stability, so screening for reference genes is necessary.

3.2. Expression stability analysis of 16 reference genes using Delta Ct program

In order to find the most stable reference genes, we performed qRT-PCR analysis on 16 candidate reference genes, and calculated and sorted the obtained Ct values using the Delta Ct program. The results are shown in Fig. 2. Based on the Delta Ct analysis, the order of gene expression stability was as follows: *IbeIF* > *Ibα-tubulin* > *IbHIS* > *IbCOX* > *IbGAPDH* > *IbH2B1* > *IbARF* > *IbCYC* > *Ibβ-tubulin* > *IbACT* > *IbEFl-a* > *IbG14* > *IbPLD* > *IbRPL2* > *IbUBQ* > *IbUBI*. Based on this analysis, we can calculate that *IbeIF* gene was the most stable reference gene whereas *IbUBI* gene was the worst one.

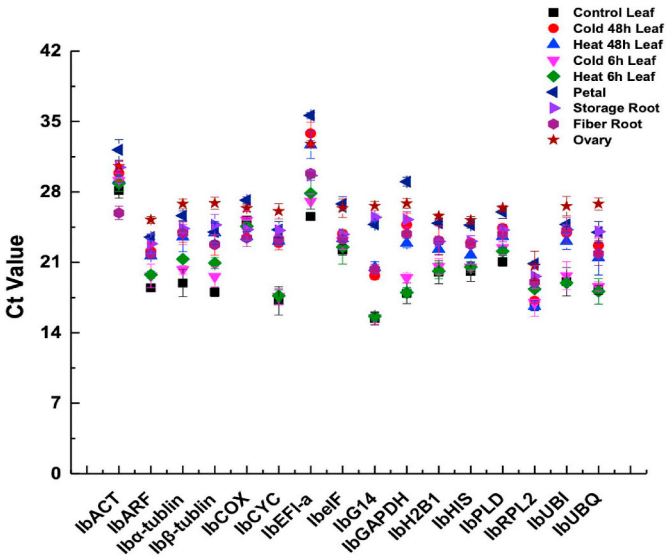

Fig. 1. Ct value of candidate reference gene cross all experiment samples.

Table 2
The Ct values of 16 candidate reference genes.

Gene Name	Total number of Samples	Average Ct value	SD	Maximum Ct value - minimum Ct value
IbACT	58	29.36	0.75	2.32
IbARF	78	21.67	1.11	3.13
Ibα-tublin	78	23.20	1.54	2.80
Ibβ-tublin	78	22.62	1.72	4.14
IbCOX	73	24.92	0.23	2.61
IbCYC	75	21.80	2.37	3.17
IbEFl-a	56	30.53	2.38	1.79
IbeIF	73	23.92	0.608	2.97
IbG14	76	20.42	3.418	6.94
IbGAPDH	76	23.11	2.928	4.30
IbH2B1	75	22.54	0.99	2.42
IbHIS	78	22.39	0.82	2.20
IbPLD	77	23.78	0.75	2.03
IbRPL2	78	18.44	0.67	3.69
IbUBI	77	22.72	1.78	2.66
IbUBQ	77	21.75	2.00	4.15

3.3. Expression stability analysis of 16 reference genes using geNorm program

Based on the geNorm program, the expression stability of 16 tested candidate genes were from high to low: *IbARF/Ibα-tubulin* > *Ibβ-tubulin* > *IbCOX* > *IbeIF* > *IbCYC* > *IbGAPDH* > *IbG14* > *IbH2B1* > *IbHIS* > *IbACT* > *IbRPL2* > *IbPLD* > *IbEFl-a* > *IbUBQ* > *IbUBI* (Fig. 3). Among the 16 candidate genes, *IbARF* and *Ibα-tubulin* genes were the most stable reference genes, while the *IbUBI* gene was the least stable one.

3.4. Expression stability analysis of 16 reference genes using NormFinder program

Based on NormFinder program, the order of gene stability calculated is: *IbHIS* > *IbeIF* > *IbGAPDH* > *IbCOX* > *IbACT* > *IbH2B1* > *Ibα-tubulin* > *IbCYC* > *IbEFl-a* > *IbARF* > *Ibβ-tubulin* > *IbPLD* > *IbG14* > *IbRPL2* > *IbUBQ* > *IbUBI* (Fig. 4). Among the 16 tested genes, *IbHIS* gene is the most stable reference gene, and the *IbUBI* gene is the least stable gene.

3.5. Expression stability analysis of 16 reference genes using BestKeeper program

Based on the BestKeeper program, the order of gene expression stability is: *IbCOX* > *IbeIF* > *IbHIS* > *Ibα-tubulin* > *IbARF* > *Ibβ-tubulin* > *IbACT* > *IbH2B1* > *IbPLD* > *IbCYC* > *IbRPL2* > *IbGAPDH* > *IbEFl-a* > *IbG14* > *IbUBI* > *IbUBQ* (Fig. 5). Among the 16 tested genes, *IbCOX* gene is the most stable gene, and the *IbUBQ* gene is the least stable gene.

3.6. Comprehensive analysis of the expression stability of 16 candidate reference genes

The four specialized programs, Delta CT, NormFinder, GeNorm, and BestKeeper, gave the similar ranks of the 16 tested candidate reference genes with a slight difference. To normalize this rank, a comprehensive analysis was performed to re-rank the expression stability of 16 tested genes (Fig. 6 and Table 3). Based on this comprehensive analysis, the order of candidate reference gene stability was: *IbeIF* > *Ibα-tubulin* > *IbCOX* > *IbHIS* > *IbARF* > *IbGAPDH* > *Ibβ-tubulin* >

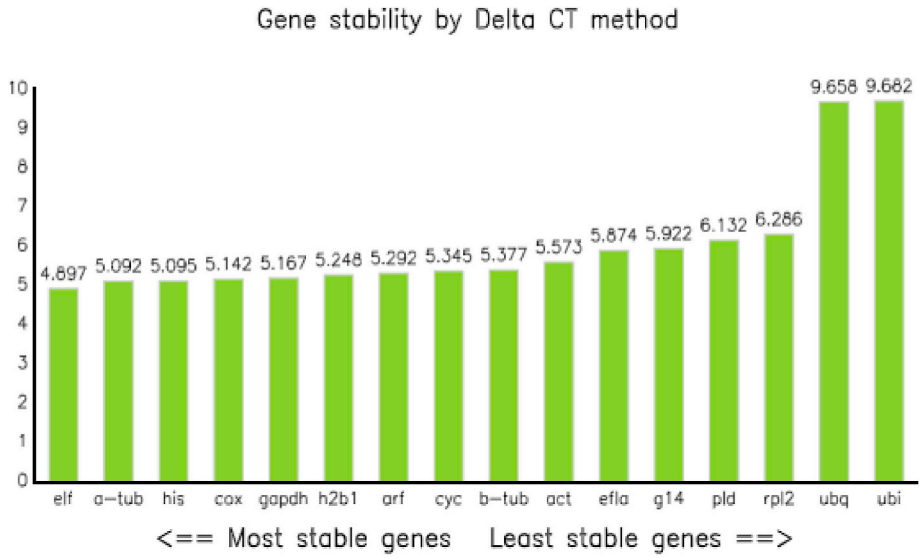

Fig. 2. Expression stability and ranking of reference genes based on Delta CT.

IbH2B1 > *IbCYC* > *IbACT* > *IbG14* > *IbEFl-a* > *IbPLD* > *IbRPL2* > *IbUBQ* > *IbUBI*. Among the 16 genes, *IbeIF* gene is the most stable reference gene followed by *tubulin*, which can be selected as the reference genes for subsequent experiments, while the *IbUBI* gene is the least stable gene.

4. Discussion

For gene expression analysis, qRT-PCR has a number of advantages over conventional methods, such as semi-quantitative PCR or Northern blot. These advantages include high sensitivity, good reproducibility, and high specificity, that make qRT-PCR the most important tool for gene expression analysis. However, in order to obtain very accurate results using qRT-PCR, as suggested in MIQE guidelines for qPCR methods [14], in addition to the rationality of the experiment and primer design, it is necessary to handle carefully each step of the experiment, including the quality of extracted RNA, synthesis of cDNA, prevention of various contaminations during PCR preparation, and accurate operation as well as selecting the right reference genes. The appropriateness of selected reference genes will have a great impact on interpretation of the gene expression results. Previous studies have shown that if a single reference gene is used in any case without selecting it, it will cause a large error in the experiment and affect the experimental results. For example, Vandesompele et al. (2002) found that using only one reference gene resulted in a three-fold higher expression error in the 25% range of samples and a 6.4-fold expression error in the 10% range of samples. Many studies also confirmed that there is no optimal reference gene, and the expression stability of the reference gene is not always consistent under different processing conditions and in different plant tissues [15–18]. Therefore, when selecting a standardized gene, the candidate gene should be carefully analyzed and verified according to different experimental conditions. Before using quantitative real-time PCR to perform gene expression analysis, the first step is to select the appropriate housekeeping gene as a reference gene according to the established experimental materials and conditions.

At present, due to the popularization and application of qRT-PCR technology and the increasing requirements for scientific research accuracy, researchers are paying more and more attention to the housekeeping genes that can be used as a reference gene. At the same time, a series of programs for screening the most stable reference genes, such as Delta CT, NormFinder, geNorm and BestKeeper, have been developed. The operation of these programs is based on the Ct value of the candidate reference genes to screen the appropriate reference genes, using

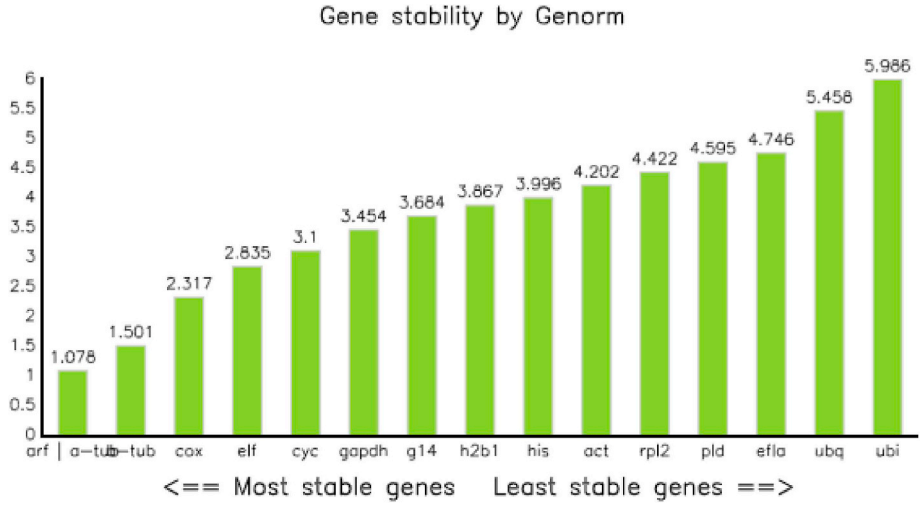

Fig. 3. Expression stability and ranking of reference genes based on geNorm.

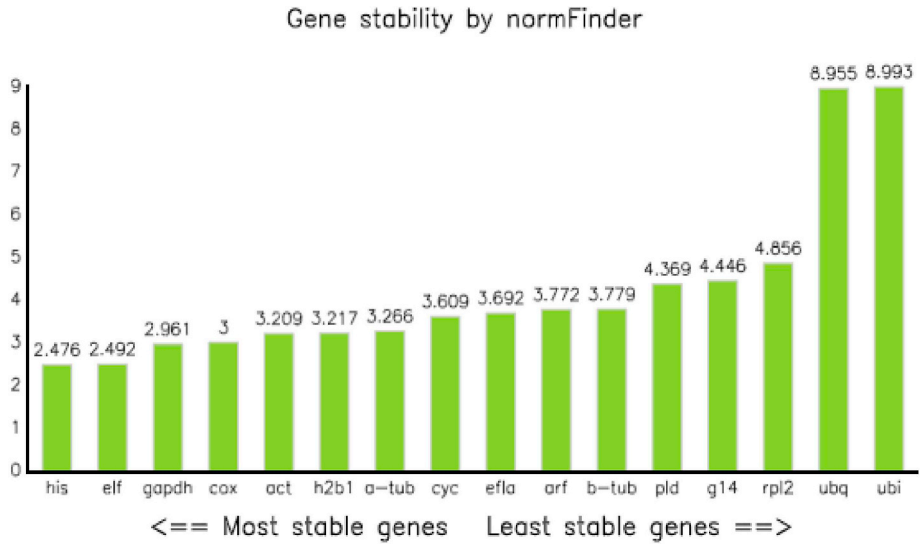

Fig. 4. Expression stability and ranking of reference genes based on NormFinder.

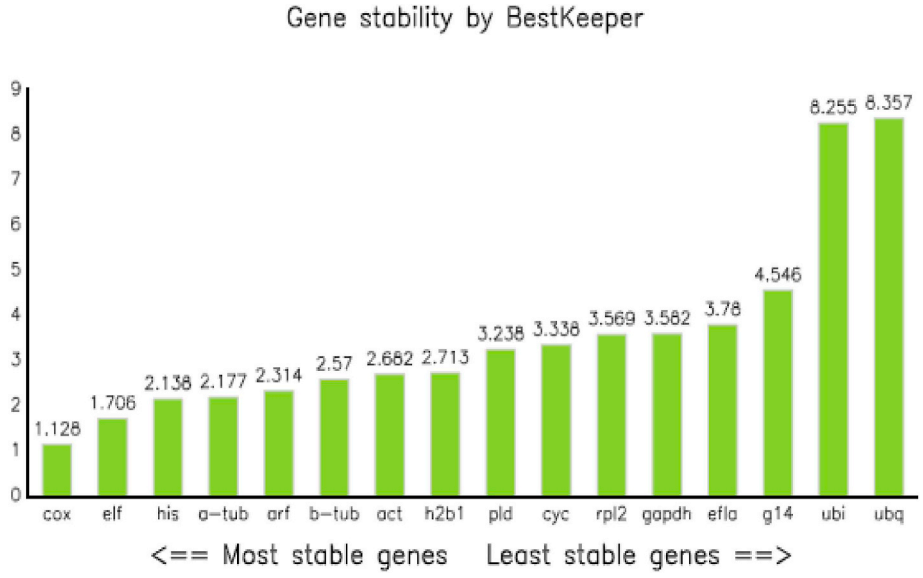

Fig. 5. Expression stability and ranking of reference genes based on BestKeeper.

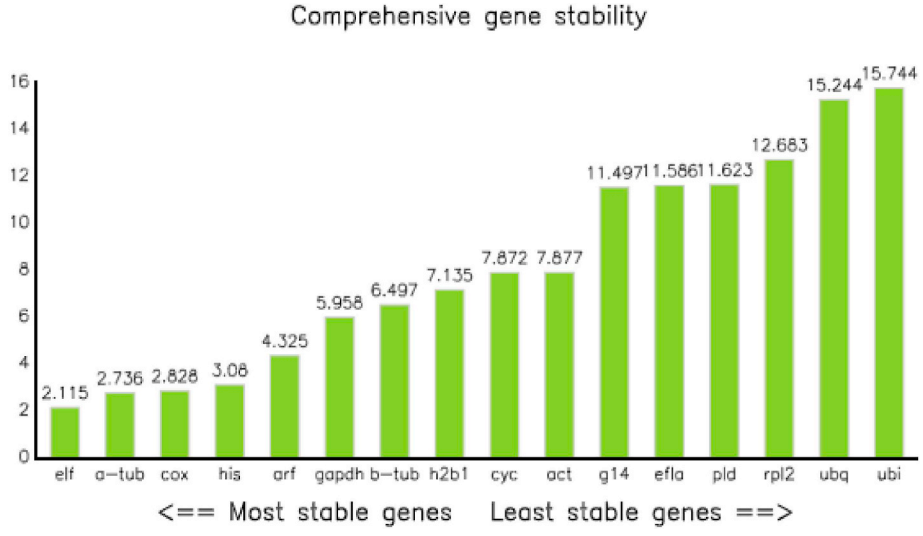

Fig. 6. Comprehensive ranking of candidate reference gene stability.

Table 3
The Rank order of 16 candidate reference genes.

Method	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Delta CT	elF	α-tublin	HIS	COX	GAPDH	H2B1	ARF	CYC	β-tublin	ACT	EFl-a	G14	PLD	RPL2	UBQ	UBI
BestKeeper	COX	elF	HIS	α-tublin	ARF	β-tublin	ACT	H2B1	PLD	CYC	RPL2	GAPDH	EFl-a	G14	UBI	UBQ
Normfinder	HIS	elF	GAPDH	COX	ACT	H2B1	α-tublin	CYC	EFl-a	ARF	β-tublin	PLD	G14	RPL2	UBQ	UBI
Genorm	ARF/	α-tublin	β-tublin	COX	elF	CYC	GAPDH	G14	H2B1	HIS	ACT	RPL2	PLD	EFl-a	UBQ	UBI
Final ranking	elF	α-tublin	COX	HIS	ARF	GAPDH	β-tublin	H2B1	CYC	ACT	G14	EFl-a	PLD	RPL2	UBQ	UBI

different statistical analysis methods under different treatment conditions, different plant growth stages, and different plant tissue types. Its expression is used as a standard to ensure the reliability of the expression levels of other genes. Each program has its own advantages and disadvantages. The algorithms of these programs are not the same, and may result in differences in their analysis results [6,7,9,19]. Therefore, when screening stable reference genes, these statistical programs can be comprehensively used.

Although ribosomial RNA (rRNA) has been successfully used in many studies, there are still many debates about its use in qRT-PCR due to the fact that the expression level of rRNA is too high compared with the tested genes [20,21]. In some cases, the ratio of rRNA to total RNA changes, and expression at various stages of tissue development is not stable enough [22]. Additionally, since there is no polyA tailor at rRNA ends, the purified mRNA samples usually do not contain rRNAs.

Research on the selection of reference genes is mainly focused on biomedical and animal research, such as immunology and diagnostics [23–26]. Under different experimental conditions, the expression stability of reference genes was not consistent. At present, there are a few reports on the stable screening and evaluation of reference genes in plants such as apples, longan, peach, grape, and *Populus* [27–30]. Different tree species, different tissues, different stress conditions, and different developmental stages were selected to have relatively stable expression of reference genes. With the release of sweet potato genome, the current research focus has been shifted to functional genomics, and qRT-PCR is a powerful tool for gene expression analysis which can be combined with the research of gene chips to verify the results of gene chips. At present, the research on the function of certain genes in sweet potato that control important agronomic traits is receiving attention. Therefore, it is very urgent to select the most stable reference genes in different varieties to perform gene expression analysis. At present, there is only one similar report in sweet potato, in which. the authors evaluated 10 housekeeping genes at 4 different abiotic stress condition (cold, drought, salt and oxidative stress) [31]. However, no any study to evaluate the reference genes among different tissues and organs. In our study, we evaluated 16 commonly used housekeeping genes as reference genes not only under stress conditions but also among different tissues and organs in sweet potato. Our results show that *IbelF* had the most stable expression, which can serve as suitable reference gene for sweet potato gene expression analysis. Interestingly, the widely used *IbARF* and *IbTUB* genes were not the most stable reference genes in different stress treatments and among different tissues in this study. Once again, it is proved that there is no ideal reference gene suitable for all experiments. When selecting a reference gene, it should be first determined according to the specific experiment.

MIQE guidelines for qPCR methods [14] and other studies [32–34] also suggest that the use of multiple reference genes may be better to interpret the gene expression results. The major reason is that the expression of all genes is affected by developmental time, tissue type, and/or environmental condition, there is no perfect gene with constant expression levels although some genes may be more stable than others. Based on this factor, we also tested the stability of two or more housekeeping genes as reference genes, our results show that the stability of two or more candidate reference genes was better than one. Thus, it may consider to use two or more reference genes during gene expression analysis.

Acknowledgment

This work was funded by the National Key R&D Program of China (2018YFD1000704 and 2018YFD1000700), the National Natural Science Foundation of China (31771367). China Agriculture Research System (CARS-10-B3). The Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD) and 2018 Graduate research innovation project (KYCX18_2128). This work is also partially support by the National Science Foundation (#1658709) to BZ.

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