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Selection and validation of reference genes for quantitative real-time PCR analysis of Nitraria tangutorum

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Abstract

Background: Suitable reference genes can be used to calibrate the error in quantitative real-time polymerase chain reaction (qRT-PCR) experiments and make the results more credible. However, reference genes suitable for different species and different experimental conditions do not exist. *Nitraria tangutorum* Bobr. is a typical plant in desert areas and desert plains, which is drought-resistant, saline-alkali resistant, barren-resistant, and has extremely strong adaptability. Due to insufficient understanding of the importance of this germplasm in the past, it is still unclear which genes can be used as reference genes to calibrate qRT-PCR data of *N. tangutorum* .

Results: In this study, we analyzed the expression levels of 10 candidate reference genes (*ACT*, *GAPDH*, *TUA*, *TUB*, *CYP*, *UBC*, *His*, *PP2A*, *HSP*, and *EF1-α*) in three tissues (root, stem and leaf) and under five abiotic stresses (salt, drought, heat, cold, and ABA) of *N. tangutorum* seedlings by qRT-PCR. Three analysis software programs (geNorm, NormFinder, and BestKeeper) were used to evaluate expression stability of ten genes. Comprehensive analysis showed that *EF1-α* and *His* had the best expression stability, whereas *HSP* was the least suitable as a reference gene. The expression profile of *NtCER7*, a gene related to the regulation of the waxy synthesis of *N. tangutorum*, verified the accuracy of the experimental results.

Conclusion: Based on this study, we recommend *EF1-α* and *His* as suitable reference genes for *N. tangutorum*. This study provides the first data on stable reference genes in *N. tangutorum*, which will be beneficial to study of the gene expression of *N. tangutorum* and other *Nitraria* species in the future.

Background

Gene expression research has become an important means of revealing gene function and molecular mechanisms. Real-time quantitative PCR, a method that can accurately analyze gene expression, is favored by most scientific researchers [1-3]. An appropriate reference gene can effectively reduce the error in the qRT-PCR experimental process and make the results more credible [4]. Generally, reference genes are various housekeeping genes, which have a stable expression in cells and help maintain cell functions. The expression level of ideal reference genes in plants will not change significantly with the change of tissue locations, life cycle stages, and external environmental conditions [5-7]. However, it was found through research that reference genes do not have universal applicability. That is, reference genes that are stably expressed in different species and under different experimental conditions do not exist [8-10]. Therefore, the key to the analysis of the gene expression level in plants under specific conditions by RT-PCR is to select the suitable reference genes.

Reference genes are often used to calibrate the results of qRT-PCR, so a stable reference gene is the premise for qRT-PCR. The commonly used reference genes are those that constitute the cytoskeleton or are involved in the basic metabolic activities of cells, for example, *ACT*, *GAPDH*, *EF1-α*, *TUA*, *TUB*, and *His*. Most previous studies on selected suitable reference genes focused on model plants [11-15]. However, with our increasing understanding of the agricultural ecological value of non-model plants, it has become important to study their molecular genetic mechanism, and to determine their appropriate reference genes under different experimental conditions. To date, some non-model plants have been selected for internal reference genes, including *Reaumuria* [16], *Caragana intermedia* [17], *Setaria viridis* [18], *Miscanthus lutarioriparia* [19], *Haloxylon ammodendron* [20], pitaya [21], and jute [22].

Nitraria tangutorum Bobr. is an important species of desert, sandy, and saline-alkali vegetation in northwestern China and is the winner of long-term natural selection in harsh habitats because it has good adaptability to the extreme environmental conditions of the desert[23]. In recent years, reports on *N. tangutorum* have mainly focused on the physiological mechanism of drought and salt tolerance [24-27], which indicated that this species has very important ecological functions. With the development of molecular biology, gene expression analysis is helpful to reveal the molecular mechanism of *N. tangutorum* to stress responses, so it is of great significance to screen the suitable reference genes of *N. tangutorum* under different experimental conditions. Unfortunately, there have been no reports in this area so far. In this study, based on our previous transcriptome analysis, 10 commonly used reference genes *ACT*, *UBC*, *TUA*, *TUB*, *GAPDH*, *CYP*(*cytochrome*), *PP2A*, *His*, *HSP*, and *EF1-α* in *N. tangutorum* were cloned. Their expression levels under different abiotic stresses (drought, salt, heat, cold and ABA) and in different tissues (root, stem and leaf) were detected by qRT-PCR. Three kinds of analysis software (geNorm [28], Normfinder [29] and BestKeeper [30]) were used to evaluate expression stability of ten genes. In order to further verify the reliability of the reference gene selection results, we analyzed the expression pattern of *NtCER7*, a gene related to the wax synthesis pathway in *N. tangutorum* under salt stress and in different tissues. This result provides a theoretical basis for subsequent research on the regulation of functional gene expression in *N. tangutorum*.

Methods

Plant materials

Seeds of *N. tangutorum*, collected from QingHai Province in Northwest China, were used in this study. The seeds were sown in a seedling-raising plate filled with clean river sand and were placed in the artificial climate chamber of Gansu Agricultural University(103°70'E; 36°09' N), Lanzhou, China. The cultivation conditions were 16 hours of light and 8 hours of darkness, and the temperatures under light and dark conditions were 26°C and 22°C, respectively. The relative humidity was 60%. After the seedlings had grown for 4 weeks, seedlings with consistent growth were selected for different experimental treatments. For drought and salt stress, seedlings were treated with 20% polyethylene glycol (PEG) and 300 Mm NaCl, respectively. For heat and cold stress, the seedlings were subjected to 45°C and 4°C, respectively. For hormonal stimulus, the seedlings were sprayed with 100 μM abscisic acid (ABA). All the above samples were collected at 2, 4, 8, 12, and 24 h. At the same time, samples of different tissues (rhizomes and leaves) of untreated seedlings were collected. Untreated seedlings in the incubator were used as blank controls. The collected samples were cleaned with purified water, frozen in liquid nitrogen and placed in an ultra-low temperature refrigerator for subsequent use. The experiment was performed with three biological replicates.

Total RNA extraction and cDNA synthesis

Total RNA was synthesized using the RNAprep pure plant kit (TIANGEN® Biotech, Beijing, China), and RNase-Free DNase I in the kit was used to remove the genomic DNA. The concentration and purity of the total RNA extracted were evaluated by a TGem spectrophotometer (TIANGEN® BIOTECH, Beijing, China). The absorption rate of A260 / A280 was between 1.8 and 2.2, which reverse transcription reaction. The reverse transcription kit (TaKaRa, Japan) was used to reverse transcribe each sample of RNA into first-strand cDNA, and the synthesized product was stored in a refrigerator at -20°C.

Selection of candidate reference genes and the design of qRT-PCR primers

According to the related research literature of the plant reference gene, we obtained candidate reference gene sequences in the transcriptome database of *N. tangutorum*. The obtained sequences were homologously compared in the Arabidopsis database by BLASTX. Finally, 10 candidate reference gene sequences (*ACT*, *GAPDH*, *TUA*, *TUB*, *CYP*, *UBC*, *His*, *PP2A*, *HSP*, and *EF1-α*) with the highest homology were obtained. The quantitative primers of the candidate reference genes were designed by the Primer3Plus online website (www.primer3plus.com/cgi-bin/dev/primer3plus.cgi). The parameters were as follows: the length of the primers and the amplification product size were 18-27 bp and 100-200 bp, respectively, the range of melting temperature (Tm) was 65°C-75°C, and the GC was 40%-60%. Primers were synthesized by Sangon Biotech. (Shanghai) company. The standard curve was constructed with a tenfold dilution of cDNA, and the amplification efficiency (*E*) and correlation coefficient (R^2) of primers were calculated using the standard curve.

qRT-PCR analyses

The qPCR reaction was performed using a QuantStudio®5 real-time fluorescence quantitative PCR system (ABI, USA) and Hieff® qPCR SYBR® Green Master Mix (Yeasen Biotech, Shanghai, China). The 20 μL reaction system consisted of SYBR® Green Master Mix (10 μL), both the forward and reverse primers (0.4 μL), 1 μL of the cDNA template (diluted ten-fold with RNase-free dH_2O) and 8.2 μL of RNase-freed dH_2O. The PCR program was as follows: first step, initial denaturation at 95°C for 5 minutes; second step, amplification at 95°C for 10 seconds, 60°C for 30 s, 40 cycles; third step, dissolution at 95°C for 15 s, 60°C for 1 min and 95°C for 15 s. The relative expression of each gene was calculated by the $2^{-\Delta\Delta CT}$ formula [31].

Stability analysis of candidate reference genes

The stability of candidate reference genes under different abiotic stresses(drought, salt, hot, cold, ABA) and in different tissues(root, stem and leaf) was evaluated using three analysis software programs (geNorm, NormFinder, and BestKeeper) with different algorithms. geNorm can calculate a parameter M that measures the stability of the reference gene expression. The M value is negatively related to the stability of the gene, that is, the smaller the M value, the higher the stability value of the gene. geNorm can also calculate the paired variation V value of the normalized factor after introducing a new internal reference gene, and the number of optimal internal reference genes can be determined according to the Vn/Vn+1 value [28]. The principle of the NormFinder and geNorm programs is similar but the calculation method is different. NormFinder software analyzes the stable value of the expression stability by calculating the variance, including intra group and inter group variance [29]. BestKeeper software analyzes gene stability from the SD (*standard deviation*) and CV (*coefficient of variation*) of the reference gene Ct value. Generally, more stable genes have lower SD and CV values [30].

Validation of reference genes

In order to further verify the reliability of the reference gene screened by the software, the expression pattern of *NtCER7*, a gene related to the regulation of the wax synthesis pathway, was analyzed under salt stress and in different tissues of *N. tangutorum*. The specific primer design is detailed in Table 1.

Table 1 Detail of primers used in this study

Genes	Primer sequence forward/reverse (5′-3′)	Tm (°C)	Length(bp)	Efficiency(%)	R^2
ACT	TCGTGTTGCCCCTGAAGAACACCCCGT	68.8	129	107.14	0.9911
	/TGGATGGCGACGTACATAGCGGGCA	67.3			
GAPDH	ACCCTGAGGAGATCCCATGGGGTGA	66.34	107	97.45	0.9998
	/TGCACCACCCTTCAAGTGAGCAGCA	65.79			
TUA	ACCAGTGCCTCCACCAACAGCA	64.55	145	101.6	0.9944
	/TGCCGCCAATAACTTTGCCAGAGGA	63.48			
TUB	AGCTCAGGAACAGTGAGGGCCCTGT	67.05	155	101.89	0.9762
	/TGCTGCCTTCGATTCCCTGGTCA	63.64			
CYP	TGGGCCAAATACGAACGGGTCCCA	65.82	162	105.56	0.9975
	/ACAACAGGCTGCGAAGTCCTCCCA	65.67			
UBC	ACCCACCAACTTCATGCAGTGCAGGT	65.47	106	98.72	0.9815
	/ACGAAAACACCTCCGGCGTAGGGGCTA	68.2			
His	AGGAGGCGTCGAGATTGGCGAGGTA	66.5	123	93.12	0.9977
	/TGGTCCCCTCAGAAACAGCGTGCT	65.67			
PP2A	TGGACAGGCAGATCGCGCACTT	64.55	127	109.07	0.9861
	/ACCGGACACTTGACTGGCTGCACGT	67.77			
HSP	TTGTCTGCCTCGGCTCTCTTCCGCA	67.25	177	102.4	0.9902
	/TCCTTGCTGCTTGGTGACCGGA	64.23			
EF1-α	ACGCTCACGCTCGGCCTTAAGCTT	66.03	176	97.9	0.9855
	/TGGTCATTGGCCACGTCGACTCTGG	65.99			
NtCER7	ACCCGACTCACCACGAGGAAGCTGT	66.87	149	96.66	0.9885
	/GGTCCTTGACGCAAGCCGCAAGCAT	67.41			

Results

Selection of reference genes and verification of primer specificity

The nucleic acid sequences of the 10 reference genes were obtained from *N. tangutorum* transcriptome database of our research group, and then their nucleic acid sequences were quickly searched using BLASTX in the *Arabidopsis* database to determine their homology in *N. tangutorum*. Based on the unigene sequences, qRT-PCR primers were designed**.** The amplification efficiency of these 10 pairs of primers ranged from 93.12% to 109.07%, and the correlation coefficients (R^2) ranged from 0.9762 to 0.9998 (Table 1). The melting curve only had a single melting peak, and there was no non-specific amplification (see Additional file 1: Figure S1). The results indicated that the primers of these 10 genes were designed reasonably and with good specificity, which met the qRT-PCR standards and allowed for use in subsequent experiments.

Ct value analyses of reference genes

The Ct value is inversely proportional to the transcription level of a gene, that is, a higher Ct value corresponds to lower expression abundance. A change in the Ct value of the same gene in different samples reflects the variation of the expression level. In this study, the Ct values of the 10 genes changed under different abiotic stresses (drought, salt, heat, cold, and ABA) and in different tissues (root, stem, and leaf), and the change trends were different (Fig. 1). Among the 10 reference genes, the lowest and highest Ct values were found in *HSP* and *UBC*, which were 19.96 and 32.65, respectively, indicating a wide range of expression abundances. The range of Ct values of *ACT*, *GAPDH*, *TUA*, *TUB*, *CYP*, *UBC*, *His*, *PP2A*, *HSP*, and *EF1-α* were 24.30–30.08, 21.92–27.53, 23.62–29.77, 23.50–28.80, 25.50–31.79, 25.72–32.65, 22.21–28.48, 25.54–30.54, 19.96–32.06 and 20.70–26.54, respectively. It can be seen that among the 10 genes, the expression abundances of *EF1-α*, *GAPDH*, *His*, *ACT*, *TUB*, and *TUA* were higher and the range of variation smaller. Followed by *CYP*, and *UBC*, the *PP2A* expression level had low variation, but its expression abundance was also low. The *HSP* expression level was the most variable. It can be preliminarily considered that the expression of *EF1-α*, *GAPDH*, *His*, *ACT*, *TUB*, and *TUA* was relatively stable, and the expression of *HSP* varied greatly. More accurate results need to be evaluated by reference gene analysis software.

GeNorm analysis

Using geNorm analysis, we obtained the stable expression M value of the reference gene, which is negatively correlated with the stability of the gene. That is, a larger M value represents a more unstable gene. When all the samples of different abiotic stresses and tissues were combined, the M value of *His* and *EF1-α* was the lowest and that of *HSP* was the highest, indicating that the expression of *His* and *EF1-α* was the most stable, and the expression of *HSP* was the least stable. For salt, drought, heat, cold, ABA stress, and different tissues, the top two genes were *His* and *EF1-α*, *GAPDH* and *UBC*, *GAPDH* and *PP2A*, *TUA* and *PP2A*, *GAPDH* and *CYP*, and *GAPDH* and *His*, respectively (Fig. 2).

GeNorm can also calculate the best number of genes when selecting multiple reference genes. It uses the relationship between the threshold V and 0.15 to determine whether an additional reference gene needs to be added. The principle is that when a new reference gene is introduced, the paired variation value (V value) will change. By calculating the ratio *Vn/n*+1, we can know whether a new reference gene needs to be introduced; 0.15 is the default *Vn/n*+1 threshold value of the software. If *Vn/n*+1 > 0.15, the *n*+1 reference gene needs to be introduced, otherwise it is not needed. In samples under salt, drought, heat, cold, and ABA stress, as well as in different tissues, the *V2/V3* values were all less than 0.15, indicating that it was not necessary to introduce a third reference gene to calibrate the qRT-PCR data. However, when analyzing the samples of all experimental conditions, the *V2/V3* value was 0.156, which exceeded the threshold of 0.15. The reason may be that the candidate reference genes of *N. tangutorum* changed significantly under different abiotic stresses (drought, salt, heat, cold, and ABA) and in different tissues (root, stem and leaf). At this time, the use of two reference genes cannot meet the needs of calibrating qRT-PCR data (Fig. 3).

NormFinder analysis

The NormFinder software directly evaluated the stability of reference genes according to the variance within and between groups. Generally, the smaller the stability value, the better the stability of the corresponding gene expression. As shown in Table 2, in all samples, *EF1-α*, *His*, and *GAPDH* had the lowest stability values, that is, 0.068, 0.080, and 0.080, respectively, This showed that among the 10 reference genes, *EF1-α*, *His*, and *GAPDH* had the best stability. *HSP* had the highest stability value, reaching 0.161, indicating that it had the highest variability. This coincides with the geNorm analysis. The highest ranked pairs of genes under different experimental conditions were not completely consistent. For example, *PP2A* and *HSP*, *UBC* and *EF1-α*, *ACT* and *PP2A*, *EF1-α* and *CYP*, *GAPDH* and *His*, and *EF1-α* and *TUA* were the top ranked genes under salt, drought, heat, cold, ABA stress, and in different tissues, respectively.

Table 2 Expression stability of the ten candidate reference genes calculated by NormFinder

Rank	Salt treatment		Drought treatment		Heat treatment		Cold treament		ABA treatment		Differe
	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability	Gene
1	*PP2A*	0.06	*UBC*	0.033	*ACT*	0.008	*EF1-α*	0.018	*GAPDH*	0.017	*EF1-α*
2	*HSP*	0.066	*EF1-α*	0.047	*PP2A*	0.01	*CYP*	0.037	*His*	0.041	*TUA*
3	*EF1-α*	0.09	*GAPDH*	0.061	*TUA*	0.024	*GAPDH*	0.07	*PP2A*	0.049	*GAPDH*
4	*CYP*	0.096	*PP2A*	0.064	*GAPDH*	0.03	*His*	0.075	*EF1-α*	0.049	*TUB*
5	*GAPDH*	0.099	*His*	0.067	*CYP*	0.036	*ACT*	0.077	*CYP*	0.056	*His*
6	*TUB*	0.1	*TUB*	0.079	*TUB*	0.037	*UBC*	0.089	*TUB*	0.061	*UBC*
7	*TUA*	0.102	*ACT*	0.095	*His*	0.038	*PP2A*	0.094	*TUA*	0.095	*HSP*
8	*His*	0.106	*TUA*	0.142	*EF1-α*	0.039	*TUA*	0.117	*ACT*	0.112	*CYP*
9	*UBC*	0.108	*CYP*	0.156	*HSP*	0.133	*TUB*	0.159	*UBC*	0.115	*ACT*
10	*ACT*	0.151	*HSP*	0.198	*UBC*	0.139	*HSP*	0.217	*HSP*	0.14	*PP2A*

BestKeeper analysis

The BestKeeper algorithm calculates SD and CV using Excel software based on the Ct value of the gene. The smaller the SD and the smaller the CV, the better the stability of the gene. If SD> 1, the gene is considered unstable. The analysis results are shown in Table 3. In all samples, only *ACT* and *PP2A* showed SD values <1, which met the requirements of reference genes. However, this is different from the analysis results of geNorm and NormFinder. There were differences in the number of genes that met the SD <1 condition under different experimental conditions. Under the salt, drought, heat, cold, ABA and in different tissues respectively, the highest ranked pair of genes were *ACT* and *PP2A*, *TUA* and *ACT*, *UBC* and *CYP*, *TUB* and *TUA*, *PP2A* and *TUB*, and *CYP* and *TUB*, respectively. Interestingly, three different analysis software programs showed the worst expression stability for *HSP*.

Table 3 Expression stability of the ten candidate reference genes calculated by BestKeeper analysis

Rank	Salt treatment		Drought treatment		Heat treatment		Cold teratment		ABA treatment	
	Gene	CV±SD	Gene	CV±SD	Gene	CV±SD	Gene	CV±SD	Gene	CV±SD
1	*ACT*	1.46±0.38	*TUA*	2.10±0.56	*UBC*	2.6±0.74	*TUB*	3.18±0.84	*PP2A*	2.48±0.67
2	*PP2A*	2.42±0.65	*ACT*	2.63±0.70	*CYP*	2.66±0.77	*TUA*	3.44±0.97	*TUB*	3.05±0.75
3	*GAPDH*	4.41±1.08	*TUB*	2.75±0.78	*TUB*	2.72±0.71	*ACT*	3.66±1.02	*EF1-α*	3.13±0.68
4	*TUA*	4.23±1.11	*PP2A*	2.94±0.78	*PP2A*	2.75±0.78	*PP2A*	3.62±1.04	*His*	3.43±0.81
5	*TUB*	4.34±1.12	*EF1-α*	3.48±0.82	*ACT*	3.17±0.85	*CYP*	3.77±1.11	*CYP*	3.49±0.95
6	*UBC*	4.28±1.23	*UBC*	3.37±0.97	*GAPDH*	3.32±0.83	*His*	5.67±1.41	*ACT*	3.54±0.90
7	*CYP*	4.49±1.30	*GAPDH*	3.53±0.87	*His*	3.67±0.95	*UBC*	5.58±1.57	*GAPDH*	4.15±0.97
8	*His*	5.26±1.31	*His*	4.74±1.19	*TUA*	3.71±0.96	*GAPDH*	5.71±1.44	*UBC*	3.8±1.07
9	*EF1-α*	5.73±1.36	*CYP*	4.96±1.41	*EF1-α*	4.26±1.01	*EF1-α*	5.78±1.38	*TUA*	4.02±1.01
10	*HSP*	6.50±1.88	*HSP*	5.91±1.67	*HSP*	9.99±2.50	*HSP*	5.74±1.60	*HSP*	8.29±2.21

Reference gene validation

In order to verify the screening results of geNorm and NormFinder, we used the two genes (*EF1-α* and *His*) with the best ranking stability and the least stable gene (*HSP*) as reference genes and analyzed the expression pattern of *NtCER7* related to the regulation of waxy synthesis of *N. tangutorum* under salt stress and in different tissues by qRT-PCR (Fig. 4). When *EF1-α* and *His* were used as reference genes under salt stress, the trend of *NtCER7* expression showed an inverted V shape from 2 h to 24 h, with a peak at 4 h. When the data of *NtCER7* expression were normalized with these two reference gene combinations, consistent results were obtained. When using *HSP*, the results showed that the lowest value appeared at 4 h, and the trend increased from 4 to 12 h. On the other hand, in different tissues, when *EF1-α*, *His*, and *EF1-α*+*His* were used to calibrate the expression data of *NtCER7*, they all showed consistent results. The expression level of *NtCER7* showed a trend of stem> leaf> root. However, the expression pattern of *NtCER7* normalized by *HSP* in different tissues showed the trend of being highest in roots, followed by stems, and lowest in leaves. It is clear that the expression of *NtCER7* in the stem was underestimated. Therefore, based on the above experimental results, we believe that the results of geNorm and NormFinder were reliable.

Discussion

qRT-PCR is one of the commonly used techniques for studying gene expression. [32]. However, the results of quantitative experiments are easily affected by the specificity of primers, the length of amplification products, the quality of RNA samples and so on. The most important factor is the stability of the reference gene[33]. Selecting a suitable gene as a reference is the key to study the expression pattern of the target gene. In general, some housekeeping genes are used as reference genes, such as *ACT*, *EF1-α*, and *TUB*, which are expressed in various types of cells in an organism, and the expression level is less affected by environmental factors. However, the stability of their expression changes greatly in different species, under different abiotic stresses, and in different tissues [34, 16, 21]. In other words, the selection of suitable reference genes under specific experimental conditions has become an important prerequisite for the qRT-PCR process.

N. tangutorum is a pioneer plant for soil desertification and salinization control due to its strong resistance to stress[35]. Therefore, it is of great significance to develop and utilize the anti-stress gene resources of *N. tangutorum* and to study its molecular mechanisms. Unfortunately, due to the lack of genetic information, the research progress on the molecular mechanism of the stress resistance of *N. tangutorum* is very slow; there is no research on a reference gene suitable for *N. tangutorum* so far. In our study, 10 typical candidate reference genes (*ACT*, *GAPDH*, *TUA*, *TUB*, *CYP*, *UBC*, *His*, *PP2A*, *HSP*, and *EF1-α*) were selected from the transcriptome database of *N. tangutorum*, and their expression stability under salt, drought, heat, cold, ABA, and in the root, stem and leaf was evaluated using geNorm, NormFinder and BestKeeper software. As shown in the analysis results of Tables 2 and 3, Figs. 1 and 2, the optimal gene ranking order obtained by the three software programs was not exactly the same under different experimental conditions. First, from the perspective of the transcriptional abundance and variation of candidate genes, *EF1-α*, *GAPDH* , *His*, *ACT*, *TUB*, and *TUA* met the requirements for ideal reference gene conditions. geNorm analysis suggested that *EF1-α* and *His* were more suitable as reference genes among the 10 genes from all samples of *Nitraria tangutorum* Bobr.. Similar results were obtained from NormFinder analysis. However, *PP2A*, and *ACT* showed better stability for use in the BestKeeper analysis in all samples. This is not surprising, because the algorithms and procedures used by the three analysis software programs are different, which leads to inconsistent results. The BestKeeper algorithm provided comprehensive analysis of the stability of the reference gene based on its SD and CV between the Ct values. [30]. The geNorm and NormFinder algorithms focused more on the variation of reference genes [28, 29]. This situation also appeared with geNorm and NormFinder analysis in *Pyrus pyrifolia*[36], longan[37], and *Miscanthus lutarioriparia*[19]. It has been reported that BestKeeper can obtain the most unstable gene stability ranking results [38], and this is consistent with our experiment results. Therefore, from the above analysis results, we suggest that *EF1-α* and *His* are the best reference genes for the expression study of *N. tangutorum* under salt, drought, heat, cold, ABA stress and in different tissues. In addition, three different algorithms all showed that *HSP* had the worst stability among the 10 reference genes. Therefore, we do not recommend this as a reference gene for *N. tangutorum*.

To validate the accuracy of the experimental results, we used *EF1-α*, *His* and *HSP* as reference genes and analyzed the expression patterns of *NtCER7*, which regulates the waxy synthesis-related gene of *N. tangutorum* under salt stress and in different tissues by qRT-PCR. The results showed that when *EF1-α*, *His*, and *EF1-α+His* were used to calibrate the expression of *NtCER7*, the results obtained were similar. However, when *HSP* was used to calibrate the expression data, there were serious variations (Fig. 4). Previous studies have suggested that unstable internal control genes will introduce significant errors in the qRT-PCR analysis, leading to misinterpretation of expression data [39-42]. Our results further confirm these views. Consequently, reliable reference genes are a necessary prerequisite for obtaining the correct results in qRT-PCR experiments. In general, *EF1-α*, and *His* have the best stability under different abiotic stress and in different tissues from the results of our study. *EF1-α*, and *His* can be used as internal reference genes of *N. tangutorum* for subsequent experimental research.

Conclusion

As far as we know, this study is the first time to reveal the most suitable reference gene for *N. tangutorum*. The expression stability of 10 typical candidate reference genes was evaluated by the geNorm, NormFinder and BestKeeper programs. We suggested that *EF1-α*, and *His* are the most ideal reference genes; *HSP* showed lower expression stability under different abiotic stresses (salt, drought, heat, cold, and ABA) and in different tissues (root, stem and leaf). At the same time, the target gene *NtCER7* was used to validate the analysis results. This work will be beneficial for the study of gene expression of *N. tangutorum* and other *Nitraria* species in the future.

Abbreviations

ACT: actin; *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase; *TUA*: alpha -tubulin; *TUB*: beta-tubulin; *UBC*: ubiquitin-conjugating enzyme; *CYP*: cytochrome; *PP2A*: Protein phosphatase 2A; *His*: histone; *HSP*: heat shock protein; *EF1-α*: eukaryotic elongation factor 1-alpha; Ct: cycle threshold.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors have consented to this publication.

Availability of data and materials

The data and materials supporting the conclusions of this study are included within the article.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Authors' contributions

B.W., Y.L. and L.W conceived and designed the experiments. B.W. and H.D. performed the experiments. B.W., H.D. and P.C. analyzed the data. S.S., L.S. and D.Y. contributed reagents/materials/analysis tools. B.W., Y.L. and L.W wrote and revised the paper. All authors read and approved the final manuscript.

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Figures

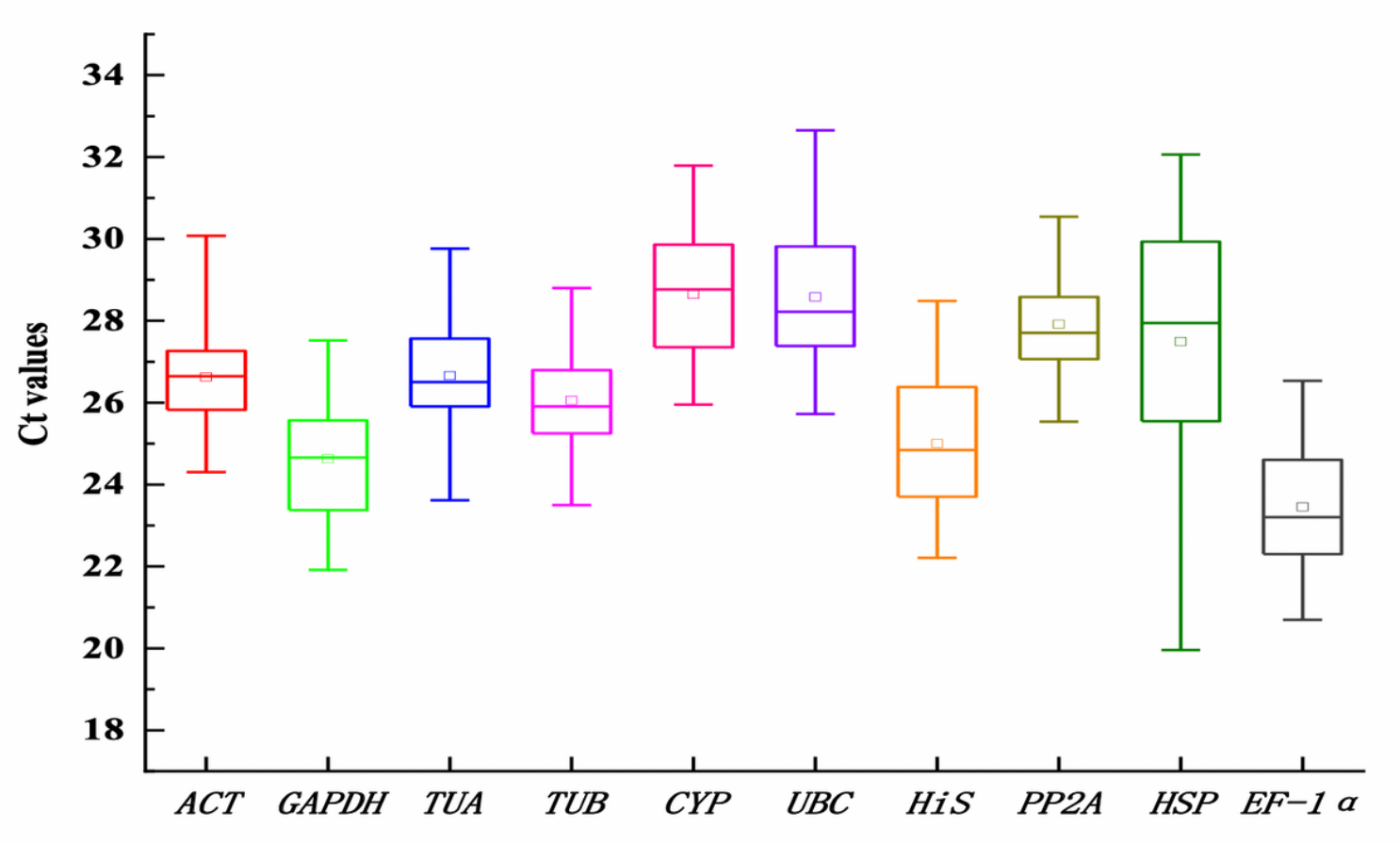

Figure 1

Ct values of 10 candidate reference genes in all N. tangutorum samples. The block diagram shows the quartile range. The outer box indicates the 25th to 75th percentile, and the inner box indicates the average. The top and bottom horizontal lines depict the maximum and minimum values, respectively, and the horizontal lines inside the box are the median lines.

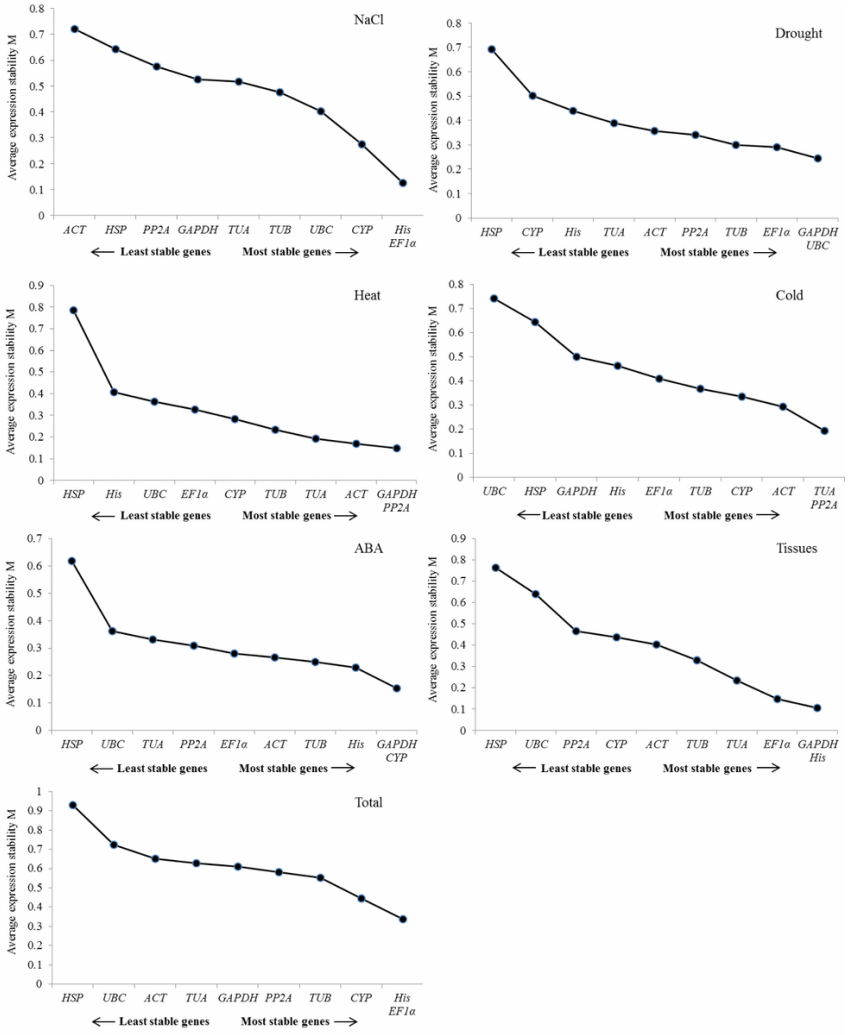

Figure 4

Average expression stability values (M) of the ten candidate reference genes based on the geNorm algorithm. The lower the M value, the more stable the expression. The most stable genes are on the right and the most unstable genes are on the left.

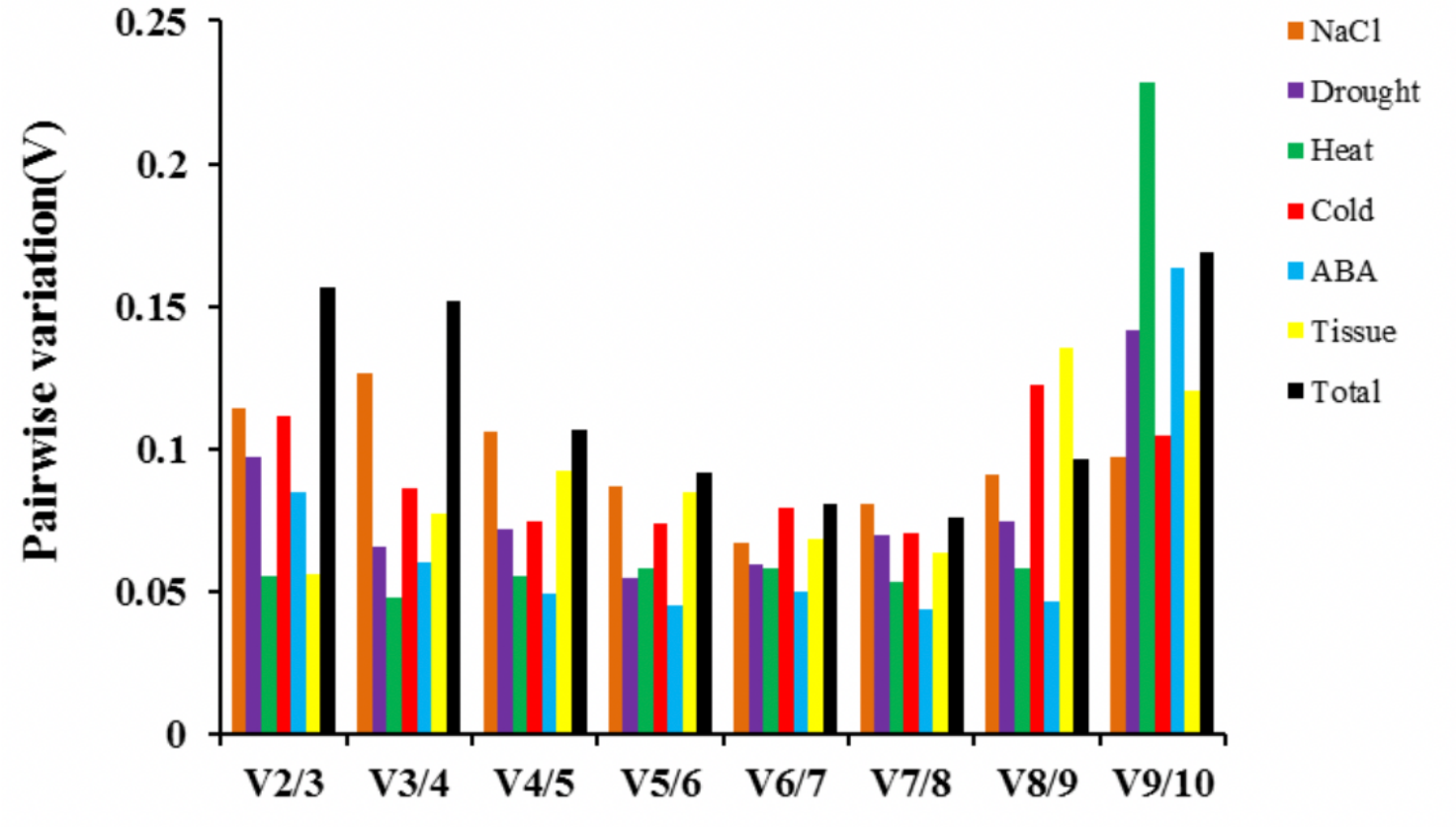

Figure 6

Pairwise variation (V) of the ten candidate reference genes calculated by geNorm to determine the optimal number of reference genes for accurate normalization. The threshold used was 0.15.

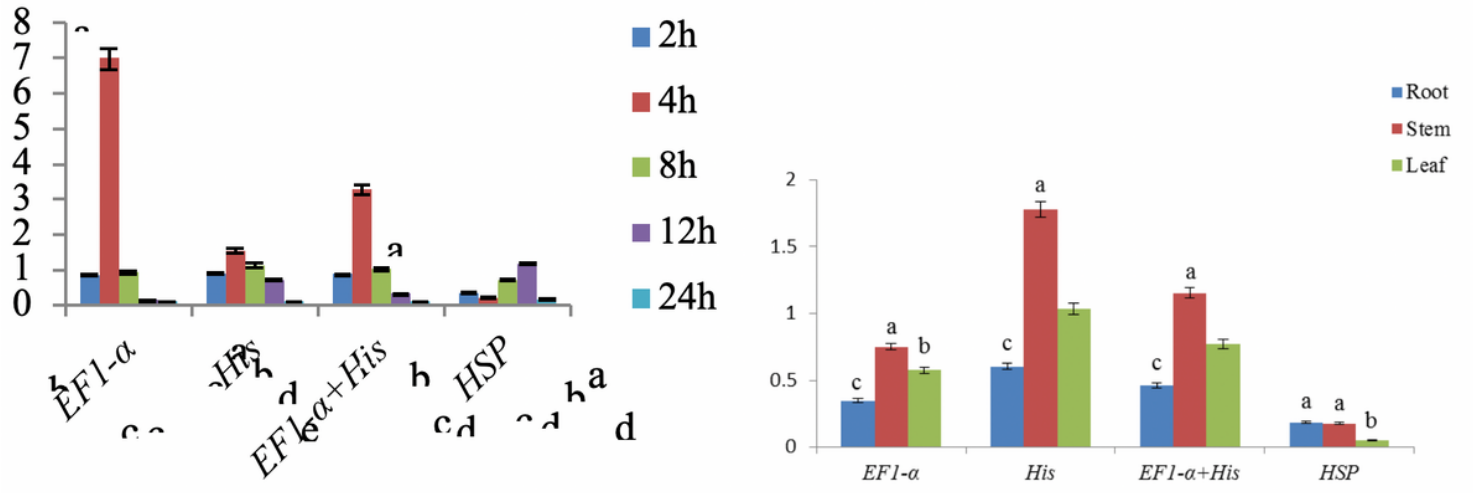

Figure 8

Relative expression of NtCER7 under salt stress and in different tissues of N. tangutorum. EF1-α and His and EF1-α + His were used as one or two of the most stable reference genes; HSP was used as the least stable reference gene. Different letters indicate significant differences in the expression of the target gene based on three biological replications ($P < 0.05$).

Supplementary Files

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