Selection of stable reference genes for quantitative real-time PCR in *Paeonia ostii* leaves under different drought stress

(Running title: housekeeping genes selection of oil peony)

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**Novelty statement**

1. Screen stable internal reference genes based on high-throughput sequencing
2. The first research on internal reference gene in *Paeonia ostii* under abiotic stress
3. Comprehensive analysis of suitable housekeeping gene based on three public algorithms
4. Provides stable reference gene and a reliable combination of housekeeping gene for precise gene expression level analysis in *Paeonia ostii* under drought stress

**Abstract**

In the present gene expression level analysis, the definition of relatively stable expressed internal reference genes is essential in both traditional blotting quantification and modern data quantitative strategy, and appropriate internal reference genes can accurately standardize the express abundance of target genes, so as to avoid serious experimental errors. In this research, the expression profiles of ten candidate genes, *ACT1*, *ACT2*, *GAPDH*, *eIF1*, *eIF2*, *α-TUB*, *β-TUB*, *TBP*, *RNA Pol II* and *RP II*, were calculated for suitable reference genes selection in the leaves of *Paeonia ostii* under different drought stress. Data was processed by the ΔCt method, geNorm, NormFinder and BestKeeper programs, and then a comprehensive analysis result revealed that *ACT1* and *RNA Pol II* were the most stable genes and *eIF2* was the least stable gene. In addition, geNorm program provided the optimal choice of two reference genes combination, *RNA Pol II* and *β-TUB*,for qRT-PCR normalization in *P. ostii* subjected to different drought stress. Our research provided convenience for gene expression analysis in *P. ostii* under drought stress and promoted research of effective methods to alleviate *P. ostii* drought stress in the future.

**Keywords:** *Paeonia ostii*; Reference gene; qRT-PCR; Drought stress

**Introduction**

The gene expression level analysis has been widely used in different areas and species (Liu et al. 2014; Li et al. 2013; Yu et al. 2016; Tan et al. 2017; Dong et al. 2019; Chen et al. 2019) in order to screen key genes or specific regulatory mechanism. Quantitative real-time PCR (qRT-PCR) is predominant and has been commonly applied due to its accuracy, good specificity and high throughput at mRNA level (Heid et al. 1996; Ginzinger 2002). In order to acquire precise experimental results, the expression levels of target genes to be tested must be corrected with reliable internal reference genes (housekeeping genes) (Richards et al. 2002; Vandesompele et al. 2002; Bustin 2009). Some traditional housekeeping genes have been usually used as reference genes due to their stable expressions in previous studies, such as actin (ACT), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 18S rRNA, elongation factors 1α (EF-1α), ubiquitin (UBQ), α-tubulin (α-TUB) and β-tubulin (β-TUB) (Aoki et al. 2000; Zhang et al. 2014). Theoretically, these traditional reference genes maintain the most basic cellular activities and their expression levels are not affected by the external environments. However, many researches showed that the common housekeeping genes presented different expression modes in different conditions (Vandesompele et al. 2002; Bustin 2009; Mou et al. 2015; Wang et al. 2019; Yang et al. 2020). Using unstable internal reference genes may have great impacts on the target gene expression level analysis and cause erroneous experimental results. Therefore, it is critically important to chose relatively stable housekeeping genes according to the certain experimental conditions before their use in qRT-PCR normalization.

*Paeonia ostii* is a famous traditional ornamental flower in China, which is internationally popular due to its large, showy and fragrance flower. Studies have proved its oil seed enriched a large amount of α-linolenic acid (ALA), which was good for human health. Additionally, as an emerging woody oil crop, it has a lot of advantages such as strong adaption and low planting investment for large-scale cultivation promotion in many areas (Peng et al. 2019b). However, as a common abiotic stress, drought stress greatly restricts the ornamental characteristics and economic values of *P. ostii*, especially in arid areas (Ma et al. 2018). Therefore, it is urgent to find feasible ways to alleviate the damage of *P. ostii* causing by drought stress. At present, there are few literatures on *P.* *ostii* internal reference gene selection, some scholars have studied the reference genes selection of *P*. *ostii* in different tissues or across different developmental stages (Li et al. 2016; Li et al. 2019a). However, there is no literature report on internal reference genes screening under drought conditions in *P.* *ostii*. Thus, when clarifying the underlying molecular mechanism of *P*. *ostii* drought stress and finding effective mitigation routes, there is a lack of stable internal reference genes.

In this study, we selected 10 commonly used candidate reference genes, and used ΔCt method, geNorm, NormFinder and BestKeeper to test their expression stability under different drought stress degrees in *P*. *ostii*. The identified internal reference genes provide valuable information for further qRT-PCR analysis and drought stress alleviation methods of *P*. *ostii*.

**Materials and methods**

**Plant materials and drought treatment**

The three-year-old potted seedlings of *P. ostii* were used as plant materials. *P. ostii* were with uniform growth condition, and cultivated in the same experimental environment with regular agronomic practices. Before drought treatment, watering was normally conducted at 17:00 in three consecutive days. These plants were then divided into three groups of 12 plants in each group and subjected to natural drought treatment. The materials that were not subjected to drought treatment were used as control group. The leaves were randomly collected after the drought treatment on the 4th, 8th, and 12th day from each treatment group. Simultaneously, the leaves were collected from 2 to 4 pairs of leaves from top to bottom, and then were quickly frozen in liquid nitrogen and store at -80 ℃ until RNA extraction and further analysis.

**RNA extraction and cDNA synthesis**

Total RNA was extracted using TRIzol reagent (Invitrogen, USA) and genomic DNA was removed using DNase I (Takara, Japan). The RNA integrity was analyzed by 1.0% agarose gels, and the RNA purity was assessed using BioPhotometer D30 (Eppendorf AG, Hamburg, Germany). Only qualified RNA samples (260/280 ratio ranged from 1.8 to 2.0; 260/280 ratio > 2.0) could be used for subsequent experiments. The first-strand cDNA was synthesized using PrimeScript RT reagent Kit (Takara, Dalian, China).

**Candidate gene selection and primer design**

The following 10 potential genes were selected for this study including *ACT*, *GAPDH*, eukaryotic translation initiation factor (*eIF*), *α-TUB*, *β-TUB*, TATA-box binding protein (*TBP*), RNA polymerase II (*RNA Pol II*) and RNA polymerase II transcription factor (*RP II*). These sequences were obtained from the RNA-seq data with accession number SRP161474. All of these candidate genes were chosen for their essential functions in maintaining the most basic cellular activities. Using the Primer Premier 5 software, we designed qRT-PCR primers for each of the 10 reference genes from *P. ostii*. The specific sequence of each primer was shown in Table 1 and the design standard rules were as follows: the amplification product length ranged from 100 bp to 200 bp; primer length ranged from 18 bp to 22 bp; melting temperature (TM) ranged from 45 ℃ to 55 ℃; GC content ranged from 40% to 60%. Additionally, we used the mixed cDNA sample of each sample as cDNA template, and the diluted cDNA template was used to calculate PCR efficiency (E) and correlation coefficients (R2). Finally, the amplified products of each reference genes were visualized on 1.0% agarose gels.

**Cloning of candidate reference genes**

For visualization, the PCR products of cDNA of the 10 candidate genes were purified from agarose gels using Agarose Gel DNA Recovery Kit (Qingke, Beijing, China), and then purified DNA products were ligated with the pMD18-T simple Vector (Qingke, Beijing, China). Recombinant vectors were transformed into DH5α chemically competent cells (Qingke, Beijing, China), and positive recombinant clones were sequenced by Qingke Biotechnology Co., Ltd.

**qRT-PCR analysis**

qRT-PCR reactions were conducted with a BIO-RAD PCR CFX96 system (Bio-Rad, USA). The candidate genes were amplified by qRT-PCR from four different drought stress using SYBR Premix Ex Taq (Takara, Dalian, China). The volume of the amplifications was 25.0 μL, containing 12.5 µL 2 × SYBR Premix Ex Taq, 2 µL cDNA template, 2 µL each gene-specific primer (10 μM) and 8.5 µL ddH2O, and the reactions without cDNA were generated as the negative controls simultaneously. The qRT-PCR system was as follows: 95 ℃ for 30 s, followed by 40 cycles of 5 s at 95 ℃, 30 s at 55 ℃, and 30 s at 72 ℃. Each reaction was conducted in triplicate.

**Data analysis**

The Data were processed by Bio-Rad CFX Manager software. Four different methods based on statistical algorithms including ΔCt method, geNorm (Vandesompele et al. 2002), NormFinder (Andersen et al. 2004) and BestKeeper (Pfaffl et al. 2004) were used to evaluate candidate reference genes’ expression stability and availability in various treatments. The ΔCt method has the ability to calculate each gene’s ΔCt value and standard deviation (SD) based on the relative pair-wise comparisons. The lower the SD value, the smaller the change in gene expression, and the higher the stability of expression. The calculated results are used for ranking. The geNorm program define the stable genes by calculating the expression stability value (M) of each gene. The final criterion identified genes with the lowest M value as the most appropriate reference gene. geNorm can also calculate the pair-wise variation (Vn/Vn+1) as to define the optimal numbers of reference genes. The NormFinder’s reference gene definition principles are consistent with geNorm which are based on the variance between samples. The BestKeeper program generates the pair-wise correlation coefficient (r), coefficient of variation (CV) and standard deviation (SD) by comparing Ct values of each selected reference gene. The r values, CV values, and SD values determine the stable genes. The selected gene with the higher r value, lower CV value and SD value is more stable, conversely, it has the lower stability.

**Results**

**Validation of PCR assays**

The primers of all candidate genes were designed based on the gene sequences obtained from *P. ostii* RNA-seq data (Zhao et al. 2019). The fragment length of PCR amplifications ranged from 104 to 199 (Fig. 1), then the PCR amplification results were verified by sequencing, and the sequencing results were consistent with the original RNA-seq results. In addition, quality analysis result of each primer showed that the melting curve of each candidate gene showed a single sharp peak, indicating good specificity (Fig. 2). The PCR efficiencies (E) of the 10 candidate reference genes ranged from 92.1% to 108.4% and the correlation coefficients (R2) varied from 0.9826 to 0.9999 (Table 1).

**Expression analysis of 10 candidate reference genes using ΔCt method**

To assess the gene expression stability of 10 candidate genes, we calculated the mRNA expression levels of different genes under different drought stress. The average Ct values of ranged from 25.30 to 29.85 in various drought environments (Fig. 3 and Table 2). Among them, *RNA Pol II* had the smallest average Ct value, followed by *α-TUB*, *ACT1*, *TBP*, *eIF2*, *β-TUB*, *eIF1*, *GAPDH*, *RP II* and *ACT2*. Using the ΔCt method, we gained the SD values of 10 candidate genes based on the Ct values, and the analysis results determined the stability order of 10 candidate genes as follows: *ACT1* > *β-TUB* > *RNA Pol II* > *GAPDH* > *ACT2* > *α-TUB* > *eIF1* > *TBP* > *RP II* > *eIF2*.

**Expression analysis of 10 candidate reference genes using geNorm program**

The analysis results of the geNorm program indicated that the M values of the 10 candidate genes were all less than 1.5. geNorm ranked the stability of 10 candidate genes as follows: *RNA Pol II* ≥ *β-TUB* > *TBP* > *ACT1* > *α-TUB* > *eIF1* > *GAPDH* > *RP II* > *ACT2* > *eIF2* (Fig. 4a).

**Expression analysis of 10 candidate reference genes using NormFinder program**

The analysis results of the NormFinder program indicated that the order of 10 candidate reference genes is: *TBP* > *RNA Pol II* > *β-TUB* > *ACT1* > *α-TUB* > *eIF1* > *GAPDH* > *RP II* > *ACT2* > *eIF2* (Fig. 4b).

**Expression analysis of 10 candidate reference genes using BestKeeper program**

According to the analysis results of the BestKeeper program, BestKeeper combined the r value, CV value, and SD value, and ranked the stability of reference genes as follows: *ACT1* > *TBP* > *GAPDH* > *RNA Pol II* > *β-TUB* > *RP II* > *α-TUB* > *ACT2* > *eIF1 > eIF2*.

**Comprehensive analysis of the expression stability of 10 candidate reference genes**

The four algorithms as above were based on their respective algorithms to sort the 10 candidate reference genes, but the sorting results caused by each algorithm were somewhat different. Generally, the results of geNorm and NormFinder were roughly the same, while the results of ΔCt method and BestKeeper were very similar. Therefore, we conducted a comprehensive analysis of the four sorting results in order to normalize this rank. Based on the comprehensive analysis, the re-rank order of the 10 candidate gene stability was: *ACT1* ≥ *RNA Pol II > β-TUB > TBP > GAPDH > α-TUB > eIF1 > RP II* ≥ *ACT2 > eIF2* (Table 3).

**Optimal numbers of reference genes for qRT-PCR normalization**

The threshold of 0.15 was proposed by geNorm as to determine optimal reference gene numbers, and if Vn/Vn+1 value < 0.15, the number of optimal reference genes is n; in contrast, the optimal reference gene number is n+1. In this study, V2/3 value was 0.085 in various drought stress (Fig. 5), which indicated that the *RNA Pol II* and *β-TUB* were the optimal combination for accurate qRT-PCR analysis in *P. ostii*.

**Discussion**

Compared with conventional quantitative methods, qRT-PCR was superior in high sensitivity and good specificity, which makes qRT-PCR the most important tool for precise gene expression levels. However, studies have shown that no gene express in the same abundance under any conditions, and a single gene may have different expression levels in different tissues, growth stages, and experimental treatments (Bustin 2002; Bowen et al. 2014; Li et al. 2017). Therefore, it is critically necessary to screen the relatively stable expression internal reference genes according to specific experimental treatments before qRT-PCR.

At present, with the wide application of qRT-PCR in many areas (An 2018) such as molecular biology (Pabuayon et al. 2016), modern medicine (Gebeh et al. 2012; Sahoo et al. 2019), food detection (Ding et al. 2020) and environmental monitoring (Chen et al. 2020), some evaluation methods for judging gene stability such as ΔCt method, geNorm, NormFinder and BestKeeper have gradually emerged simultaneously, which improved the efficiency of gene stability analysis and suitable reference genes selection. However, the results of each algorithm are different because of the unique algorithm of each program. Therefore, a comprehensive analysis needs to be synthesized according to the analysis results of each statistical algorithm.

*P. ostii* is a traditional famous flower in China, and its related research is very rich from multiple levels, such as antioxidant activity research (Zhang et al. 2016), seed dormancy (Xue et al. 2015), oil resource promotion (Li et al. 2015), ornamental traits regulation (Gao et al. 2015), growth and ecological adaptation resistance (Peng et al. 2019a). In recent years, as the medicinal value and economic benefits of *P. ostii* have become increasingly prominent, the planting scale has expanded in many areas. However, in arid and semi-arid areas, due to the lack of water resources, the use of *P. ostii* and the orderly development of *P. ostii* industry has been greatly restricted. Therefore, many scholars have conducted numerous drought-related researches on *P. ostii*, such as water physiology (Fang et al. 2020), cell membrane system (Li et al. 2019b), osmotic system (Li et al. 2014), photosynthetic mechanism (Fang et al. 2020), etc.

In our previous research, some relevant indicators used to clarify the damage extent of drought stress to *P. ostii* were measured, and we have tried to find ways to alleviate drought stress on *P. ostii* (Zhao et al. 2020; Zhang et al. 2019). At the same time, with the development of transcriptome sequencing, the research on drought stress has been gradually advanced and deepened from the physiological level to the molecular level (Guo et al. 2018; Qu et al. 2019). Therefore, it is essential to verify the expression of key genes involved in *P. ostii* drought stress.

To data, the evaluation of internal reference gene in *P.* *ostii* is only reported in the different tissues across different developmental stages without environmental stresses (Li et al. 2016; Li et al. 2019), and the most two stable reference genes were identified in *P.* *ostii* flowers, indicating that it is essential to chose reference genes in different cultivars, even they are the same species (Li et al. 2016). Based on the special experimental needs to solve corresponding scientific problems, some scholars screened the reference genes under different abiotic stresses such as plant hormone stress (Qu et al. 2019), light stress (Wan et al. 2019), cold or high temperature stress (Li et al. 2019c; Yu et al. 2020), salt stress (Ma et al. 2020), etc. Similarly, due to the high industrial values of *P. ostii*, the research on *P*. *ostii* drought stress and its mitigation methods is also very significant. In deep, screening the key genes, explaining the key mechanism of drought stress requires suitable reference genes which can correct the expression levels of drought-related genes.

Leaf is the main organ of plants for photosynthesis and transpiration, and also the most sensitive part to drought stress (Otto et al. 2017). Drought stress can cause leaf wilting and withering, reducing the leaf water content in *P.* *ostii*. In the drought research of plants, leaves are often measured for drought-related physical and chemical indicators to measure the drought degree of the overall plant. Therefore, in this study, we used leaves as experimental materials to screen the stable internal reference genes of *P.* *ostii* under different drought conditions as to provide reference and help for *P.* *ostii* drought research.

In this study, we evaluated the expression stability of 10 commonly used internal reference genes of *P.* *ostii* under different drought stresses. After a comprehensive evaluation by ΔCt method, geNorm program, NormFinder program and BestKeeper program, *ACT1* and *RNA Pol II* were identified as the most stable genes, and they could be used as internal reference genes under drought stress in *P.* *ostii*. Simultaneously, *ACT1* had a moderate to high expression abundance, which could be used to quantify genes with moderate to high expression levels, while *RNA Pol II* had a high expression abundance among these ten candidate genes which could be used for high expression target genes normalization. MIQE guideline for qPCR methods suggested that there is no perfect gene which is constantly expressed regardless of the external environment under any conditions (Bustin et al. 2009). Additionally, the optimal numbers of reference genes were evaluated by geNorm program, and the results showed that using two genes as internal reference corrections was more reliable than using one gene. Therefore, a combination of two internal reference genes (*RNA Pol II* and *β-TUB*) could be used for qRT-PCR analysis under drought conditions. This study provided convenience for qRT-PCR analysis, and also provided useful reference data for screening key genes to alleviate drought stress of *P*. *ostii* in the future.

**Conclusions**

Here, we evaluated 10 candidate reference genes in the leaves of *P. ostii* under different drought stress, including *ACT1*, *ACT2*, *GAPDH*, *eIF1*, *eIF2*, *α-TUB*, *β-TUB*, *TBP*, *RNA Pol II* and *RP II*. The final comprehensive ranking results analyzed by four algorithms recommended that *ACT1* and *RNA Pol II* were the most stable reference genes, and could be used to relatively quantify the expression levels of target genes. geNorm program defined *RNA Pol II* and *β-TUB* as the best combination for qRT-PCR normalization in *P. ostii* exposed to drought conditions. Our research facilitated the expression analysis of target genes in *P. ostii* under drought stress, and also promoted the establishment of optional internal reference gene library in *P. ostii*.

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**Authors’ contributions**

J.T. and D.Z. designed the experiments. C.X., Y.L., and X.W. performed the experiments. C.X., Y.L. and X.W. analyzed the data, wrote and revised the manuscript. All authors have carefully read and approved the final manuscript.

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**Fig. 1** PCR amplification of 10 candidate reference genes of *P. ostii*

M, 2000 marker; 1, *ACT1*; 2, *ACT2*; 3, *GAPDH*; 4, *eIF1*; 5, *eIF2*; 6, *α-TUB*; 7, *β-TUB*; 8, *TBP*; 9, *RNA Pol II*; 10, *RP II*



**Fig. 2** Melting curves of 10 candidate reference genes of *P. ostii*



**Fig. 3** Comparativeexpression profiles of 10 candidate reference genes in *P. ostii* under different drought stress



**Fig. 4** Expression stability analysis of 10 candidate reference genes in *P. ostii* by geNorm program (a) and NormFinder program (b)

**Fig. 5** Analysis of the optimal choice of reference gene numbers using geNorm program

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Gene symbol | Gene name | Forward primer (5’—3’) | Reverse primer (5’—3’) | Amplicon length(bp) | Amplicon Tm (°C) | E (%) | R2 |
| *ACT1* | Actin | CTTCTTGCTACGACATCC | AGCTAAGGTATTGCCACT | 188 | 51.5 | 98.7 | 0.9949 |
| *ACT2* | Actin | GCAGAGGGGTATGTTATT | CTTCACTTTGCGAGCTAC | 143 | 51.5 | 99.1 | 0.9874 |
| *GAPDH* | Glyceraldehyde-3-phosphate dehydrogenase | ATGACCACTGTCCACTCC | TTCCAGTCAACTTCCCAT | 168 | 52.6 | 108.4 | 0.9826 |
| *eIF1* | Eukaryotic translation initiation factor | ACGATTACGAAGACAGACC | CAAAGGACTTGACAGAGTC | 104 | 53.0 | 99.0 | 0.9978 |
| *eIF2* | Eukaryotic translation initiation factor | GGAAGTGGAAAGGAAGAC | ATCCATAATAGCAGCTCC | 146 | 51.5 | 92.1 | 0.9942 |
| *α-TUB* | α-Tubulin | CTGGGAACTTTACTGTCTC | TCCTGACTTCATCAATGAC | 178 | 51.9 | 107.7 | 0.9887 |
| *β-TUB* | β-Tubulin | GTATTACAACGAGGCTTCT | CAGCATCAATCAACTCAG | 199 | 50.6 | 98.7 | 0.9999 |
| *TBP* | TATA-box binding protein | GTAATGCTGAATACAATCCC | ATACTTCCGTGCTGCTAA | 148 | 50.8 | 99.1 | 0.9891 |
| *RNA Pol II* | RNA polymerase II | GGTTTGTGACATTTCCTG | ATAGTTTGGTGCATCTCC | 190 | 50.3 | 108.4 | 0.9929 |
| *RP II* | RNA polymerase II transcription factor | ACGGAGATAGGGTTTGCC | CCCAAAGTTCTTATCGCATT | 135 | 53.1 | 99.0 | 0.9921 |

**Table 1:** Primers and PCR efficiencies for *P. ostii* reference genes.

**Table 2:** The Ct values of 10 candidate reference genes in *P. ostii*.

|  |  |  |  |
| --- | --- | --- | --- |
| Gene symbol | Average Ct value | SD value | Maximum Ct value - minimum Ct value |
| *ACT1* | 27.20 | 0.20 | 0.69 |
| *ACT2* | 29.85 | 0.64 | 2.03 |
| *GAPDH* | 29.78 | 0.58 | 2.18 |
| *eIF1* | 28.99 | 1.01 | 2.97 |
| *eIF2* | 27.93 | 1.29 | 3.57 |
| *α-TUB* | 25.65 | 0.99 | 3.89 |
| *β-TUB* | 28.65 | 0.39 | 1.09 |
| *TBP* | 27.53 | 1.02 | 2.42 |
| *RNA Pol II* | 25.30 | 0.50 | 1.53 |
| *RP II* | 29.85 | 1.21 | 4.62 |

**Table 3:** Ranking of 10 candidate reference genes of *P*. *ostii* under drought stress.

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Method | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| ΔCt | *ACT1* | *β-TUB* | *RNA Pol II* | *GAPDH* | *ACT2* | *α-TUB* | *eIF1* | *TBP* | *RP II* | *eIF2* |
| geNorm | *RNA Pol II / β-TUB* | *TBP* | *ACT1* | *α-TUB* | *eIF1* | *GAPDH* | *RP II* | *ACT2* | *eIF2* |
| NormFinder | *TBP* | *RNA Pol II* | *β-TUB* | *ACT1* | *α-TUB* | *eIF1* | *GAPDH* | *RP II* | *ACT2* | *eIF2* |
| BestKeeper | *ACT1* | *TBP* | *GAPDH* | *RNA Pol II* | *β-TUB* | *RP II* | *α-TUB* | *ACT2* | *eIF1* | *eIF2* |
| Final ranking | *ACT1/RNA Pol II* | *β-TUB* | *TBP* | *GAPDH* | *α-TUB* | *eIF1* | *RP II / ACT2* | *eIF2* |