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IDENTIFICATION OF REFERENCE GENES FOR GENE EXPRESSION ANALYSIS USING RT-QPCR IN *PINELLIA TERNATA*

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ABSTRACT

Pinellia ternata (Thunb.) Briet. is a well-known traditional Chinese herbal medicine that has many beneficial effects such as anti-tumor, anti-fertility, blood fat-reducing, and liver-protective. The comprehensive utilization value of *P. ternata* is high and the development prospect is broad. However, low tuber production resulting from "sprout tumble" (ST) due to high temperature and light intensity has largely reduced its use. Therefore, it is necessary to explore the functional gene responses to high temperature and light. Real-time quantitative PCR (RT-qPCR) is commonly used for accurately gene expression detection during gene function exploration. However, because the RT-qPCR results may vary depending on the conditions and environment, an internal reference gene (IRG) is needed to normalize the RT-qPCR data. Analyzing IRGs in *P. ternata* can help with subsequent functional characterization and verification of the genes regulating *P. ternata* growth. In this study, we screened 8 suitable IRGs, and determined the most suitable IRGs using the Ct value, GeNorm, NormFinder, and BestKeeper under shade and high-temperature conditions, and in different tissues. The results showed that *18S* was a suitable IRG for *P. ternata* studies. This discovery has laid the foundation for further research on *P. ternata*, and is also beneficial for the exploration of more IRGs in medicinal plants.

Keywords: internal reference gene, high temperature, shade, RT-qPCR, *Pinellia ternate*

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INTRODUCTION

Pinellia ternata (Thunb.) Briet. belonging to the Araceae family, is an herbaceous plant that is often used as a popular Chinese medicinal material. Its tuber contains starch, β-sitosterol, glucoside, β- and γ-aminobutyric acid, L-ephedrine, choline, various amino acids and inorganic elements (Xue *et al*. 2021; Gombodorj *et al*. 2017; Wu *et al*. 2015; Iwasa *et al*. 2014). *P. ternata* is derived from dried tubers, and is effective in removing dampness-phlegm, providing relief from vomiting, and dispersing and clearing phlegm. Recently, several important biological effects, such as anti-tumor, anti-fertility, blood fat-reducing and liver-protective, have been discovered (Du *et al*. 2016; Lin *et al*. 2018). The comprehensive utilization value of *P. ternata* is high, and the development prospect is broad, thus the annual demand is large (Zeng and Peng 2008). However, *P. ternata* easily withers in a phenomenon known as "sprout tumble" (ST) in summer, and ST greatly reduces the yield of *P. ternata*, resulting in a shortage (Tian *et al*. 2022; Zhang *et al*. 2021).

Recently, researchers have focused on the effects of light intensity and high temperature on *P. ternata* growth. Currently, most of the studies on the growth of *P. ternata* under conditions of shade and high temperature have focused on physiological and biochemical aspects (Xue *et al*. 2008; Sheng *et al*. 2009). However, the mechanism of ST resulting from strong light intensity and high temperature remains unknown, and needs to be researched. Studies on *P. ternata* at the molecular level are limited. Lu *et al*. used suppression *subtractive hybridization* (SSH) technology to successfully construct a subtractive library of genes related to the ST response to high temperature and verified the expression patterns of some of the candidate genes under heat stress using RT-qPCR (Lu, *et al*. 2013). Likewise, genes involved in the biosynthesis of benzoic acid and ephedrine were identified using de novo sequencing (Zhang *et al*. 2016). Notably, RT-qPCR, a quantitative PCR technology based on traditional PCR, was used for candidate gene identification (Redshaw *et al*. 2013; Mou *et al*. 2013; Eickelberg and Fisher 2013). Because of its quantitative accuracy, high sensitivity, and strong specificity, it is widely used in the identification of new genes and their functions (Huggett *et al*. 2005; De Keyser *et al*. 2013; Ratti, Minguzzi, and Turina 2019). However, the RT-qPCR data may be affected by the sample parameters and to ensure the accuracy of the results it is necessary to select a stably expressed internal

reference gene (IRG) (Lu *et al*. 2018; Tang *et al*. 2017; Yan *et al*. 2018; Qian *et al*. 2018). In their studies on *P. ternata,* Lu *et al*. and Zhang *et al*. used tubulin and actin as IRGs, respectively (Lu *et al*. 2018; Zhang *et al*. 2016). To our best knowledge, there is no comprehensive study on the IRGs in *P. ternata,* which probably affects experimental accuracy. Thus, the identification of IRGs that are expressed constitutively under all condition in *P. ternata* is much needed.

In this study, 8 relatively stable candidate genes (18S, EF1α, sec3a, GAPDH, tubulin, actin, APRT and L8) were selected based on the *P. ternata* transcriptome. The RT-qPCR technique was used for screening and detection of the expression of these candidate IRGs in each sample under shade and high temperature conditions. The stability of expression of the candidate IRGs was analyzed using three statistical softwares (geNorm, NormFinder and BestKeeper) (Guo *et al*. 2018; Fei *et al*. 2018; Vera Hernandez *et al*. 2018). The data presented in this paper will facilitate future gene expression studies on *P. ternata,* and also be beneficial for studies on other species of *Araceae*.

MATERIALS AND METHODS

Plant materials, growth conditions and stress treatments: The experiments were carried out with *P. ternata* that were grown in pots in the greenhouse facility of the Huaibei Normal University (Anhui, China) in May. When the plants reached a height of ~15 cm in June, one part was subjected to ~90% shade conditions while the other part grew in natural light as a control. Meanwhile, another seedlings of *P. ternata* were transferred to a growth chamber with a 12 $h.d^{-1}$ illumination at 30 - 40 $\mu mol.m^{-2}.s^{-1}$, and were divided into two groups: one group was placed in 25 °C and the other group was treated with a high temperature of 35 °C. Therefore, this study contains two treatment groups: shading treatment group (the nature light group vs the 90% shade group) and high temperature treatment group (the 25 °C group vs the 35 °C group). The whole seedlings of *P. ternata* including leaf, petiole, and tuber were selected at 20 d in the shading group and 5 d in the high temperature group, respectively, and all the three target tissues were averaged for further experiments. Also, leaf, petiole, and tuber of *P. ternata* grew in the shade conditions were collected. All samples were derived from ten seedlings with three biological repeats.

Reference gene selection and primer design: The 8 genes (18S, EF1α, sec3a, GAPDH, tubulin, actin, APRT and L8) were selected as IRGs based on the third transcriptome sequencing results (Xue *et al*. 2019). Primers were designed from these sequences using the Beacon Designer 7 program (Table 1). Before RT-qPCR analysis, the size of the amplicons was determined by PCR (Table 1), and the target amplicons were sequenced for confirmation of the identity of the PCR product.

RNA extraction and cDNA synthesis: Samples (150 mg each) were thoroughly ground with liquid nitrogen, and 1 mL of Total RNA Extractor (Sangon Biotech, China) was added to completely separate the nucleoprotein and nucleic acid according to the manufacturer's instructions. The RNA concentration and purity were determined using an ultramicro spectrophotometer (Maestro Nano, America) and agarose gel electrophoresis. The RNA samples with a high purity and quality were used as a template for cDNA synthesis. The cDNA synthesis was performed using 5× All-In-One RT MasterMix kit (ABM Company, Vancouver, Canada). The cDNA was diluted 20 times, and stored at -40 °C for RT-qPCR.

Experiments of RT-qPCR: The RT-qPCR was performed with ABI Real-Time 7300 PCR system using SYBR Premix Ex Taq (Vazyme Biotech Co.,Ltd, China) according to the manufacturer's instructions. Each reaction contained 10 µL 2× SYBR Premix Ex Taq, 1 µL cDNA template, 0.75 µL each primer (10 µM), 0.4 µL 50× ROX Reference Dye and 7.1 µL ddH_2O in a total volume of 20 µL. All experiments were repeated in triplicate.

Data processing: The raw Ct values of the selected IRGs generated from the SDS software of ABI 7300 (version 1.4) were used for the stability analysis with the algorithms of geNorm, BestKeeper and NormFinder, strictly following the algorithm manuals.

RESULTS

Determination of primer specificity and Ct values of IRGs: The sequences of 18S, EF1α, sec3a, GAPDH, tubulin, actin, APRT and L8 in *P. ternata* were first amplified with their respective primers using a standard template. The primers were evaluated through the solubility curve of RT-qPCR, and all the target amplicons were found to have a specific peak, indicating that all the primers were specific. Ct values represent the expression of the gene in different tissues of the plants. The smaller the Ct value, the higher the level of gene expression and vice versa. Ct values of the 8 genes mentioned above were obtained by RT-qPCR, and the average Ct values were between 13 and 35. *18S* had the highest expression in different tissues, and under the two stress conditions with a Ct value of around 13-14 cycles. *Actin*, *sec3a* and *APRT* had high Ct values in shading and high temperature stress, and also in different tissues (between 33 and 36, Figure. 1).

GeNorm analysis: The stability of the above candidate IRGs was first evaluated using the geNorm method, which is based on the geometric means of IRGs.

According to the statistical analysis of the average expression stability coefficient M1 by geNorm software, the cut-off value of stability (M1) is < 1.5. It was found that the M1 value of all the candidate IRGs was < 1.0, indicating that they all exhibited high stability. For shading treatment, *18S* and *actin* were the best IRGs with equal M1 values of < 0.1. *L8*, *sec3a* and *tubulin* followed closely with M1 values between 0.1 and 0.5. *GAPDH*, *APRT* and *EF1a* were the least stably expressed genes, with the M1 values > 0.5 (Figure 2A). For the high-temperature treatment, *sec3a* and *APRT* showed best stability, followed by *18S*, *tubulin*, *GAPDH*, *L8*, *EF1a* and *actin* (Figure 2B). For different tissues, the stabilities of the candidate IRGs were in the following order: *EF1a = L8 = 18S > GAPDH > tubulin > APRT > actin > sec3a* (Figure 2C).

NormFinder analysis: Like the geNorm, the NormFinder software is also based on the expression stability (M2) value of each candidate IRG, and evaluates its M2 value through variance. Genes with lower M2 value represent a higher stability. For shading treatment, *18S* and *actin* were the most stable IRGs, while *EF1α* and *APRT* were the least stable genes. For high-temperature treatment, the two most stable genes were *18S* and *APRT,* while the most unstable genes were *actin* and *EF1α*. Among the selected IRGs, the stabilities of the candidate IRGs were in the following order: *L8 > EF1α > 18S > tubulin > GAPDH > actin > APRT > sec3a* for different tissues (Table 2).

BestKeeper analysis: The BestKeeper program directly calculates the coefficient of variation (CV) and standard deviation (SD) according to the Ct values of each gene. Usually, stable IRGs have a smaller SD value and genes with SD greater than 1 are considered unacceptable. The best correlations were obtained for *L8* (0.08), *18S* (0.14) and *tubulin* (0.31) in shading treatment, and for *18S* (0.10), *APRT* (0.12), *tubulin* (0.21) and *sec3a* (0.23) in high temperature treatment (Table 3). As for different tissues, except for *18S* (0.40) and *actin* (0.82), the SD values of the other IRGs were all > 1, indicating that *18S* and actin had the best stability.

Table 1. Candidate reference genes and primer sequences

Gene	Primer sequences (forward/reverse)	Amplification length (bp)	Tm (°C)
18S	CGCATATAAATAAACGGAGGAA GACGCTTCTACAGACTACA	125	60.5
tubulin	TGATGGTGCTCTGAATGT CGCAGTGTTTGTGATCTC	154	60
actin	GCCAATCGTGAGAAGATG TGGACTTGCTTACCTGTT	122	59.7
EF1α	CCAACTCCAAGGATGACC ATATCCGTTGCCGATCTG	92	61.2
GAPDH	ACTGTTGATGGACCTTCTG TTGGAACTCGGAATGACAT	151	60.6
sec3a	CTGATGGAGATGATAGCAATG CCTGGATACCGTCAAGTAA	135	60
APRT	GAGGCAAGAGGTTTCATAT AATTACCAGGCAGTTTCC	95	60.3
L8	ATCGTCATCAGCCACAAT AGCAGTTTCTCTTCACTCT	184	60.5

Table 2. Gene expression stability of the candidate reference genes using the NormFinder for shade, high temperature, and tissues.

Gene	Shade Stability value	High temperature Stability value	Tissues Stability value
18S	0.018	0.023	0.197
tubulin	0.405	0.042	0.429
actin	0.018	0.792	0.626
EF1α	0.972	0.372	0.117
GAPDH	0.508	0.086	0.521
sec3a	0.152	0.136	0.781
APRT	0.879	0.023	0.729
L8	0.053	0.267	0.103

Table 3. Ranking of candidate reference genes based on stability values calculated by BestKeeper analysis for shade, high temperature, and different tissues.

Gene	Shade (CV±SD)	High temperature (CV±SD)	Tissues (CV±SD)
18S	0.96±0.14	0.72±0.10	2.83±0.40
tubulin	1.21±0.31	0.78±0.21	7.74±2.23
actin	6.10±2.02	2.41±0.84	2.32±0.82
EF1α	1.53±0.39	1.48±0.36	7.15±1.93
GAPDH	2.02±0.55	1.53±0.40	8.22±2.48
sec3a	3.80±1.23	0.71±0.23	4.35±1.47
APRT	2.34±0.80	0.35±0.12	3.84±1.29
L8	0.25±0.08	1.24±0.35	5.29±1.59

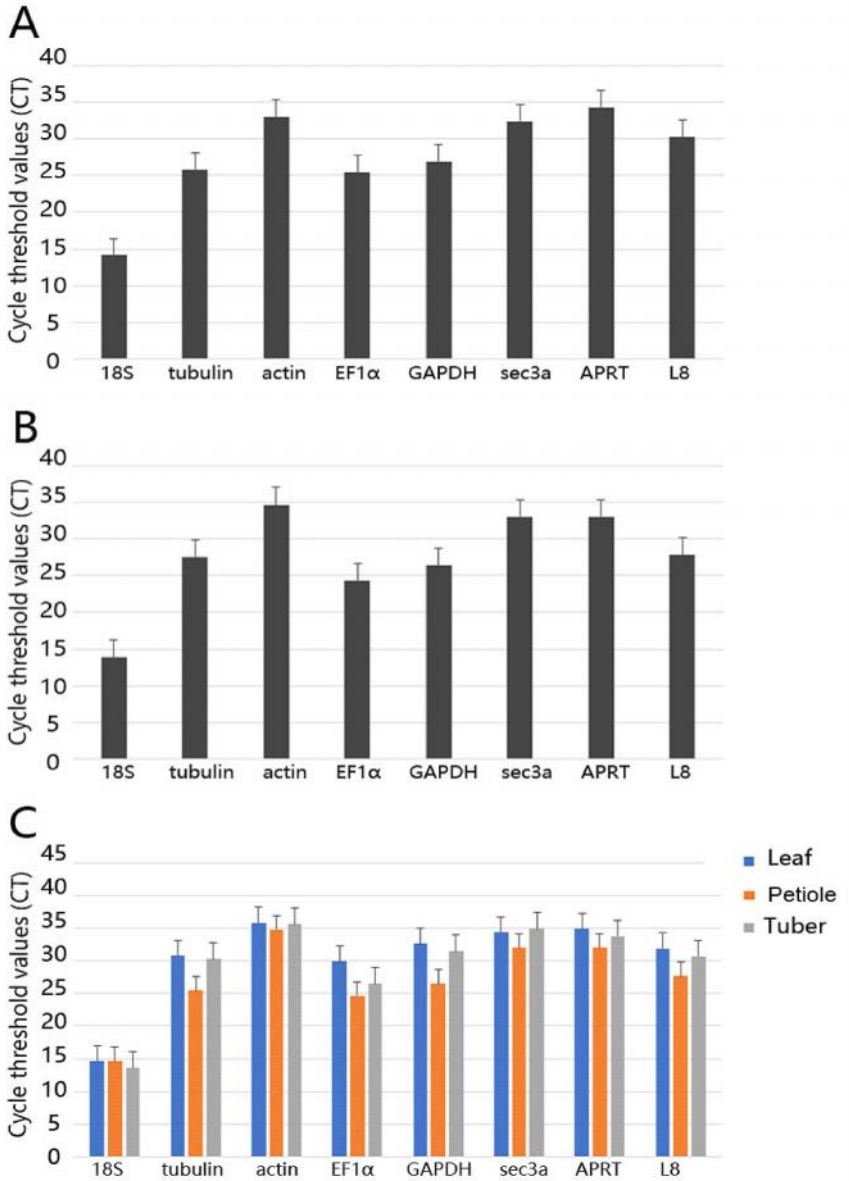

Figure 1 Expression levels of candidate reference genes in experimental samples. Expression data are displayed as Ct values for each reference gene in all samples. A. shading, B. high temperature, C. different tissues

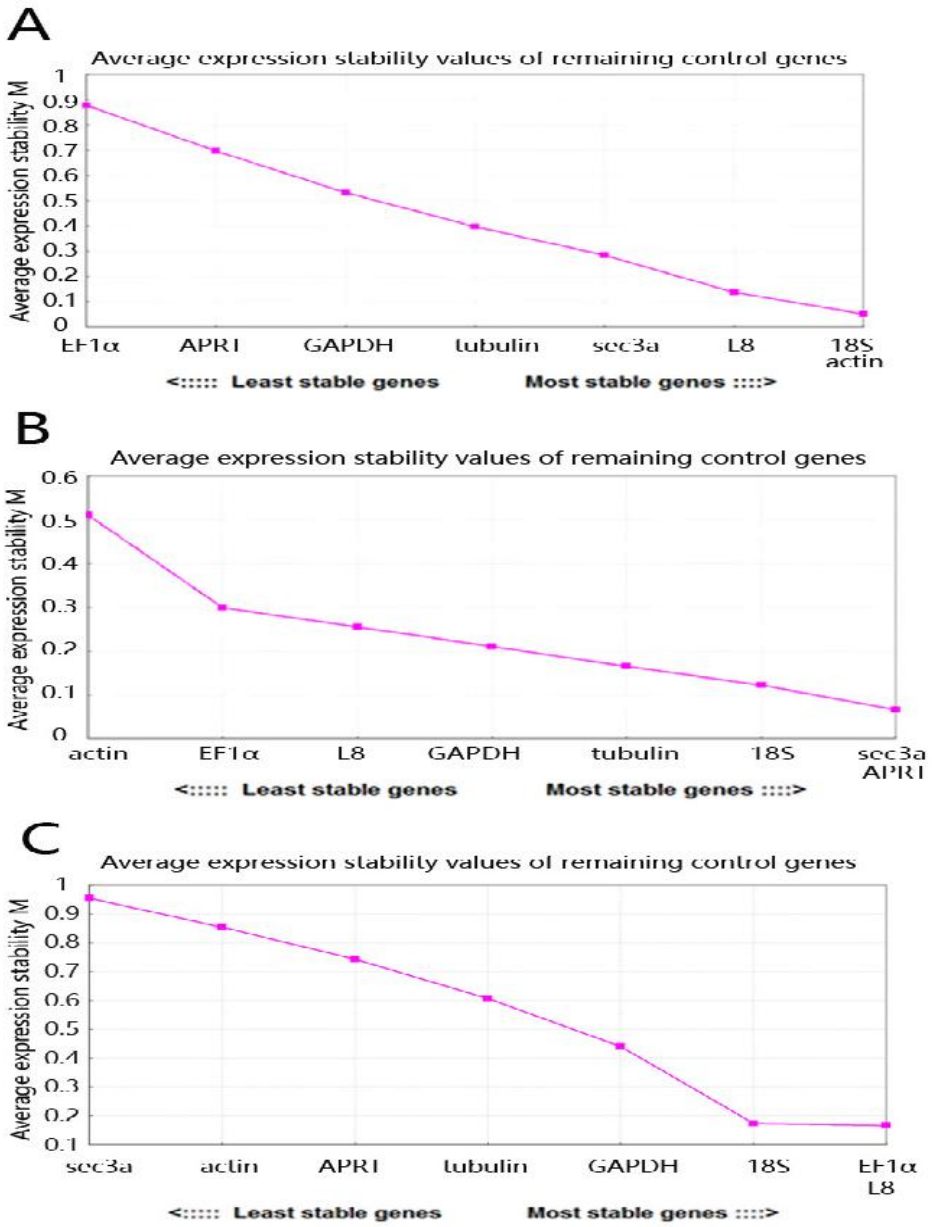

Figure 2 Stability of optimal number of reference genes using geNorm. A, B and C represent rankings based on average expression stability value (M) for shading (A), high temperature (B) and different tissues (C).

DISCUSSION

Detection and quantification of gene transcript abundance is an important task in molecular studies. RT-qPCR is a frequently-used and convenient technique for gene expression analysis. However, it is important to select an appropriate internal gene for normalizing the gene expression. An IRG is a gene that has relatively stable expression regardless of the changes in conditions or in different tissues or parts of the organism (Jain, Vergish, and Khurana 2018; Shabrangy *et al*. 2018). An ideal IRG generally possesses the characteristic of stable expression under different physiological conditions (Vera Hernandez *et al*. 2018; Kumar, Das, and Sarmah 2018). Unfortunately, each gene is often affected by many factors, and expressed differently under different experimental conditions or in different tissues (Park *et al*. 2018; Walling, Zalapa, and Vinje 2018). There is no versatility in the selection of IRGs. It is important to select an IRG with stable expression in RT-qPCR based on the specific experimental conditions (Sun *et al*. 2019). The commonly used IRGs are either essential components of the organelle skeleton (ACT, TUA, TUB etc.), or they participate in basic biochemical and metabolic processes in the organism (GAPDH, EF-1α, UBQ etc.) (Huggett *et al*., 2005), and are expressed

relatively stably in the corresponding tissues and organs (Takamori *et al*. 2017; Zhou, Niu, and Quan 2018). Due to the randomness and uncertainty of IRG selection in the study of *P. ternata*, it is necessary to identify the appropriate IRG in *P. ternata*.

In this study, 8 relatively stable IRGs that are commonly used in other plants and the samples of *P. ternata* treated with high temperature, shading, together with different tissues were selected for IRG filtration. Mathematical methods such as NormFinder (Vandesompele *et al*. 2002), geNorm (Vandesompele *et al*. 2002), BestKeeper (Andersen, Jensen, and Orntoft 2004), ΔCt approach (Silver *et al*. 2006), and stability index are often used to assess the stability of IRGs (Brunner, Yakovlev, and Strauss 2004). GeNorm and NormFinder have similar algorithms, but geNorm screens the most appropriate internal parameters under different conditions, choosing a pair of optimal combinations instead of a single gene (Zhang *et al*. 2019). NormFinder will only select the appropriate IRG (Rudus and Kepczynski 2018). In contrast, BestKeeper differs from geNorm and NormFinder in assessing the relative stability of genes by directly calculating the Ct values (Hossain *et al*. 2019). Therefore, we used geNorm, NormFinder and BestKeeper to assess the stability of the IRG. For each analytical method, the three most stable genes were selected, and the genes common to all three methods were selected as the most stable genes. Hence, for shading treatment, *18S* and *L8* were stable IRGs; for high temperature treatment, *18S* and *APRT* were stable IRGs; and for different tissues, *18S* was the stable IRG. Through this comprehensive assessment, *18S* was identified as the most suitable IRG for *P. ternata*. 18S has not been used as an IRG in *P. ternata* before, which further illustrates the significance of this paper. However, unlike in *P. ternata*, the *18S* gene proved unsuitable as an IRG in *Stevia rebaudiana* (Lucho *et al*. 2018) and *Zanthoxylum bungeanum* (Fei *et al*. 2018), which demonstrates that IRGs differ between species.

Therefore, it is important to identify the appropriate IRG before carrying out RT-qPCR in any species. In this study, the identification of the IRGs in *P. ternata* provides the foundation for accurate gene expression analysis under different environmental conditions, and using different sources of tissues. This will enable further research into the mechanism of shading and high temperature on the growth of *P. ternata*, which will serve to improve production in future.

Conclusion: This study attempted to identify for the first time a set of candidate IRGs in *P. ternata* under shade and high-temperature conditions, and in different tissues, for the normalization of gene expression data from RT-qPCR. Based on the geNorm, NormFinder and BestKeeper analyses, it was showed that the IRGs *18S* and *L8* were stable in shaded environment, *18S* and *APRT* were stable in high temperature environment, and *18S* was stable in the different tissues. Taken together, the most appropriate IRG in all the three biological contexts is *18S*.

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