Reference gene selection in multiple *Passiflora edulis* tissues and under cold stress for RT- qPCR analysis

Zhonghua Tu

Nanjing Forestry University <https://orcid.org/0000-0002-2313-8550>

Hang Fan

Nanjing Forestry University

Anding Li

Institute of Mountain Resources, Guizhou Academy of Sciences

Na Ran

Nanjing Forestry University

Ke Ding

Nanjing Forestry University

Meng Xu (✉ xum@njfu.edu.cn)

<https://orcid.org/0000-0002-6573-9098>

Research article

Keywords: *P. edulis*, reference genes, RT-qPCR, expression stability, data normalization

Posted Date: April 29th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-25297/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: *Passiflora edulis* is a tropical fruit with high edible and medicinal values that is widely cultivated in southern China. The development of new *P. edulis* variety with cold resistance and research on the mechanism of cold resistance are the basis for further promoting the cultivation of this species. In a previous study, our laboratory discovered a cold-resistant variety Pingtang 1, which is used as a material for the study of cold resistance mechanism in *P. edulis*. However, functional genes of *P. edulis* have not been well studied, especially via relative quantitative analysis. In addition, research on reference genes in *P. edulis* has not been reported.

Results: We used three tools to test the expression stability of 10 candidate reference genes in multiple tissue samples and cold-treated samples of *P. edulis*. We found that *Ts* and *EF1* showed the highest expression stability in all samples. Further analysis showed that *HIS* and *EF1* were stably expressed in tissue samples, whereas *UBQ* and *EF1* were stably expressed in cold-treated samples. Interestingly, *EF1* was stably expressed in not only multiple tissue samples but also cold-treated samples. To validate the selected reference genes, we used the target gene *ICE1* and investigated its expression level in cold-treated leaves. Interestingly, the expression of *ICE1* increased with increasing cold treatment times.

Conclusions: In this study, we successfully selected reference genes from among 10 candidates for *P. edulis* RT-qPCR data normalization. We selected *Ts* and *EF1* as reference genes for normalization in all samples. *HIS* and *EF1* were ideal for data normalization in tissue samples, whereas *UBQ* and *EF1* were ideal for cold-treated samples. Our work substantially benefits the study of functional genes in *P. edulis*.

Background

Passiflora edulis (*P. edulis*), or passion fruit, is a perennial vine that belongs to the Passifloraceae family. *P. edulis*, which originated in South America, is now widely cultivated in southern China because of its high edible value, medicinal efficacy and ornamental properties; additionally, *P. edulis* is an important raw material in the juice and pulp industry [1–6]. Passion fruit, a tropical fruit, provides a variety of nutrients, such as carbohydrates, vitamins and minerals, which are essential for biological activities [5]. *P. edulis* was introduced in China at the beginning of the last century [2]. In recent years, researchers have studied the adaptability of *P. edulis* to cold environments, providing a theoretical basis for expanding the cultivation area of *P. edulis* in China [1, 2]. Although the edible value and medicinal efficacy of *P. edulis* have increased, only a few studies on the functional genes of *P. edulis* have been reported, especially on gene expression patterns under cold conditions. Previously, our laboratory had discovered a cold resistant variety Pingtang 1, which has been used as a material for research on cold resistance mechanisms in *P. edulis*. In the future, further research will be focused on the cold resistance mechanism and functional genes in *P. edulis*, that are associated with flavor, color, nutritional elements, medicinal compounds and environmental adaptability. However, before such studies can be performed, suitable internal control genes for reverse transcription quantitative polymerase chain reaction (RT-qPCR) data normalization in *P. edulis* must be selected.

RT-qPCR, as a technology that has high throughput, high accuracy, and high sensitivity, is widely applied in functional gene expression pattern studies [7, 8]. Nonetheless, using RT-qPCR to investigate gene expression levels requires stably expressed reference genes for data normalization, and the expression stability of reference genes directly affects the accuracy of relative quantitative analyses [7, 9–12]. In theory, reference genes should have stable expression patterns in different samples and under different experimental conditions, yet no reference gene has satisfied these conditions simultaneously [8, 11, 13–16]. To our knowledge, a candidate reference gene may exhibit distinct expression patterns in different tissues within a species, and it may also exhibit various expression patterns under different conditions even in the same tissue [15, 17, 18]. For example, *Actin 6* shows distinct expression levels under different types of abiotic stress in *Pinus massoniana* Lamb [18]. In *Liriodendron chinense*, *Ubiquitin 1* exhibits various expression levels in multiple tissues [19]. Therefore, comprehensive evaluation and selection of appropriate internal control genes is a prerequisite for obtaining accurate and reliable relative quantitative results.

To date, many tools have been used to evaluate the expression stability of candidate reference genes. The most commonly used tools are geNorm, NormFinder and BestKeeper [20–22]. These three tools have been successfully used for the selection of reference genes in amaranth (*Amaranthus tricolor*), *Setaria viridis*, *Carex rigescens*, *Lolium multiflorum*, *Euscaphis konishii* Hayata, *P. massoniana*, *Alopecurus aequalis* Sobol, *Carica papaya* L., *Olea europaea* L., *Cucurbita pepo*, *Brassica napus*, *L. chinense* and *Populus* [18, 19, 23–33]. The use of multiple tools to select internal control genes for RT-qPCR data normalization has become a basic and indispensable process in the study of the expression patterns of functional genes. However, the studies on reference gene selection in *P. edulis* have not yet been performed, which has hindered the study of functional gene expression patterns in *P. edulis*.

In this work, we used 3 tools, namely, BestKeeper, geNorm, and NormFinder, to comprehensively evaluate the expression stability of *50S* (50S ribosomal protein), *Ts* (threonine synthase), *UBQ* (ubiquitin), *OTU* (OTU domain-containing protein), *18S* (18S ribosomal RNA), *OmpH* (Outer membrane protein), *EF1* (elongation factor 1 alpha), *eIF5A* (eukaryotic translation initiation factor 5A), *HIS* (histone H3), and *Liom* (*Theobroma cacao* L-isoaspartate O-methyltransferase mRNA) and then to select genes with high expression stability for application in RT-qPCR data normalization in *P. edulis*. To further understand the scope of the application of reference genes in *P. edulis*, we classified 60 samples into two subsets, namely, tissue samples and cold-treated samples. Moreover, we used *ICE1* (inducer of C-repeat-binding factor (CBF) expression) as a target gene to validate the internal control genes that we selected. We investigated the expression level of *ICE1* in leaves that were treated with cold stress for different lengths of time. This work will provide reliable internal control genes for the study of functional gene expression levels in *P. edulis*, especially under cold stress.

Results

Amplification Efficiency Calculation and Primer Specificity Verification

Using LinRegPCR 2014.x software, we calculated the mean amplification efficiency of all primers based on row data obtained by RT-qPCR, and the mean amplification efficiencies of the primers ranged from 1.762 (*HIS*) to 1.963 (*18S*) (Table 1) [34]. The primer specificity was validated by melting curve analysis. As shown in Fig. 1, each melting curve had only a single peak. This result indicated that the amplification was specific, and the RT-qPCR data could be used for further analysis.

Table 1
Description of candidate reference genes.

Name	Annotation	Primer sequence (5'-3')	Amplicon length (bp)	Mean PCR efficiency
<i>50S</i>	50S ribosomal protein	F: GCGCATTCGGCGAAACTCAC R: ATCAGGGCCATGACCACCCA	126	1.830
<i>Ts</i>	Threonine synthase	F: CAGTTGGTGCAGCCCATTGC R: GGCCGTAACCTCGCGAATGA	93	1.861
<i>UBQ</i>	Ubiquitin	F: TCAGTCACCTCCGGCCTTCT R: CGAGTGTGGTGCCGGAAGTT	100	1.880
<i>OTU</i>	OTU domain-containing protein	F: TCGTGCTCTGCTTCGGGTTT R: CACCACAGTTCCTCGGCGTA	144	1.819
<i>OmpH</i>	Outer membrane protein	F: AGAGGCAAGATGACACCTCAGGA R: TTCGACCAGGCAACTTGCGA	100	1.834
<i>Liom</i>	<i>Theobroma cacao</i> L-isoaspartate O-methyltransferase mRNA	F: TAAGGCAGCGACGGCATCAG R: TTTCAGCGAGCACGGTCAGG	127	1.829
<i>EF1</i>	Elongation factor 1 alpha	F: GGCTGAGCGTGAACGTGGTA R: CGGCACAATCAGCCTGGGAA	146	1.827
<i>18S</i>	18S ribosomal RNA	F: TTAAGGCCAGGAGCGCATCG R: AGCCGCGGTAATTCCAGCTC	150	1.963
<i>eIF5A</i>	Eukaryotic translation initiation factor 5A	F: TGGGCCGATGTCCTTGAGAGA R: GGATGACTTGAGGCTCCCAACTG	139	1.786
<i>HIS</i>	Histone H3	F: AGAGCCATGCAGTGTTGGCA R: CTTGGCGTGGATGGCACAGA	92	1.762

Ct Values of Candidate Reference Genes

We obtained cycle threshold (Ct) values for the 10 candidate reference genes in 60 samples via RT-qPCR. A low Ct value indicates a high expression level, and a small difference between the maximum Ct value and the minimum Ct value indicates low variation in expression level. The mean Ct values of these genes ranged from 5.99 (*18S*) to 26.75 (*OmpH*) (Fig. 2). The results indicated that *18S* had the highest

expression level among the samples and that *OmpH* had the lowest expression level. Moreover, *18S* showed the least variable expression levels among the 60 samples (maximum Ct value – minimum Ct value = 1.94 Ct), while *OmpH* showed the most variable expression levels (maximum Ct value – minimum Ct value = 12.07 Ct) (Fig. 2). Apparently, *18S* can be used as a reference gene, but this needs further analysis. In studies on rice (*Oryza sativa*) and *Dimocarpus longan* Lour, *18S* also showed small variable expression levels, but it cannot be used as reference gene [35, 36].

Results of the geNorm Analysis

The M value was used to evaluate the expression stability of genes by geNorm software. Genes with relatively small M values have high expression stabilities. An M value greater than 1.5 indicates an unstable gene [20]. In all samples, *Ts* and *EF1* had the most stable expression patterns (Fig. 3a). Among tissue samples, the best choices for normalization were *Ts* and *HIS* (Fig. 3b). For cold-treated samples, *UBQ* and *HIS* showed the most stable expression patterns (Fig. 3c). *OmpH* and *18S* showed the least stable expression patterns under these conditions (Fig. 3a, b, c).

The pairwise variation $V_{n/n+1}$ was calculated to determine the minimum number of reference genes for accurate normalization [20]. If $V_{n/n+1}$ values exceed 0.15, an additional internal control gene must be included for normalization [20]. We found that $V_{2/3}$ was less than 0.15 in all samples, tissue samples, and cold stress-treated samples (Fig. 4). This suggested that the minimum number of reference genes for accurate normalization was two.

Results of the NormFinder Analysis

The stability values were calculated by NormFinder to evaluate gene expression stability. Low stability values indicate high expression stability [21]. We found that *Ts* had the most stable expression level in all samples (Fig. 5a). In tissue samples, *HIS* had the highest expression stability, followed by *EF1* (Fig. 5b). Among cold-treated samples, *UBQ* showed the most stable expression pattern, while *EF1* had the second most stable pattern (Fig. 5c). Notably, *Ts*, *HIS*, and *UBQ* were also recommended as internal control genes by geNorm software in all samples, tissue samples, and cold-treated samples, respectively (Fig. 3a, b, c).

However, there were some differences between the geNorm analysis and NormFinder analysis. For example, in all samples, *EF1* ranked first in geNorm but fourth in NormFinder (Fig. 3a and Fig. 5a). In tissue samples and cold-treated samples, *EF1* ranked second in NormFinder and ranked fourth and fifth in geNorm, respectively (Fig. 3b, c and Fig. 5b, c).

Results of the BestKeeper Analysis

In the BestKeeper algorithm, genes with a standard deviation (SD) value more than 1 are regarded as unacceptable [22]. In addition, r^2 (r represents the Pearson correlation coefficient) values close to 1 indicate a gene with an increasingly stable expression pattern [22]. In all samples, *50S*, *HIS*, *Liom* and

OmpH were unsuitable for data normalization (SD value > 1), while *Ts* exhibited the most stable expression level followed by *EF1* (Table 2). Similarly, *50S*, *Liom* and *OmpH* were unsuitable for data normalization in tissue samples, while *EF1* and *HIS* showed the highest expression stability in this case (Table 2). For the cold-treated samples, *OmpH* was unsuitable for normalization (SD value > 1), while *UBQ* showed the highest expression stability, followed by *Ts* (Table 2).

We found that *Ts* was suggested as a reference gene by BestKeeper, NormFinder and geNorm in all samples (Fig. 3a, Fig. 5a, Table 2), and *UBQ* had the highest expression stability calculated by the three tools in cold-treated samples (Fig. 3c, Fig. 5c, Table 2). Among tissue samples, the expression stability of *HIS* ranked first in geNorm and NormFinder, and second in BestKeeper (Fig. 3b, Fig. 5b, Table 2). Notably, *OmpH* showed unstable expression levels in all samples, tissue samples and cold-treated samples (Fig. 3, Fig. 5, Table 2). However, the rankings of these 10 genes were slightly different among the three algorithms. For example, in tissue samples, the *Ts* gene ranked first in geNorm, third in NormFinder and fifth in BestKeeper (Fig. 3b, Fig. 5b, Table 2). Among cold-treated samples, the *EF1* gene ranked fifth in geNorm, second in NormFinder and third in BestKeeper (Fig. 3c, Fig. 5c, Table 2). *EF1* also ranked differently in the analysis results of the three tools among all samples (Fig. 3a, Fig. 5a, Table 2). The reason for this phenomenon is that these tools use different algorithms to evaluate the expression stability of genes.

Table 2
Results of the BestKeeper tool analysis.

Rank	All samples			Cold-treated samples			Tissue samples		
	Gene name	SD	r ²	Gene name	SD	r ²	Gene name	SD	r ²
1	<i>Ts</i>	0.84	0.85	<i>UBQ</i>	0.48	0.78	<i>EF1</i>	0.70	0.74
2	<i>EF1</i>	0.79	0.83	<i>Ts</i>	0.48	0.68	<i>HIS</i>	0.59	0.70
3	<i>eIF5A</i>	0.76	0.75	<i>EF1</i>	0.55	0.66	<i>OTU</i>	0.89	0.69
4	<i>OTU</i>	0.91	0.74	<i>Liom</i>	0.58	0.62	<i>eIF5A</i>	0.99	0.68
5	<i>UBQ</i>	0.50	0.62	<i>50S</i>	0.59	0.60	<i>Ts</i>	0.72	0.65
6	<i>18S</i>	0.28	0.62	<i>HIS</i>	0.68	0.55	<i>UBQ</i>	0.42	0.55
7	<i>50S</i>	1.33	0.87	<i>18S</i>	0.22	0.48	<i>18S</i>	0.24	0.49
8	<i>HIS</i>	1.27	0.86	<i>eIF5A</i>	0.40	0.46	<i>50S</i>	1.11	0.74
9	<i>Liom</i>	1.33	0.86	<i>OTU</i>	0.56	0.38	<i>Liom</i>	1.14	0.72
10	<i>OmpH</i>	2.21	0.77	<i>OmpH</i>	1.12	0.44	<i>OmpH</i>	2.90	0.69

Table 3
Top five genes ranked by the three tools.

Tools	All samples		Tissue samples		Cold-treated samples	
	Rank	Gene name	Rank	Gene name	Rank	Gene name
geNorm	1	<i>Ts</i>	1	<i>Ts</i>	1	<i>UBQ</i>
	1	<i>EF1</i>	1	<i>HIS</i>	1	<i>HIS</i>
	3	<i>eIF5A</i>	3	<i>UBQ</i>	3	<i>OTU</i>
	4	<i>OTU</i>	4	<i>EF1</i>	4	<i>eIF5A</i>
	5	<i>HIS</i>	5	<i>OTU</i>	5	<i>EF1</i>
NormFinder	1	<i>Ts</i>	1	<i>HIS</i>	1	<i>UBQ</i>
	2	<i>OUT</i>	2	<i>EF1</i>	2	<i>EF1</i>
	3	<i>eIF5A</i>	3	<i>Ts</i>	3	<i>eIF5A</i>
	4	<i>EF1</i>	4	<i>UBQ</i>	4	<i>Liom</i>
	5	<i>50S</i>	5	<i>OTU</i>	5	<i>OTU</i>
BestKeeper	1	<i>Ts</i>	1	<i>EF1</i>	1	<i>UBQ</i>
	2	<i>EF1</i>	2	<i>HIS</i>	2	<i>Ts</i>
	3	<i>eIF5A</i>	3	<i>OTU</i>	3	<i>EF1</i>
	4	<i>OTU</i>	4	<i>eIF5A</i>	4	<i>Liom</i>
	5	<i>UBQ</i>	5	<i>Ts</i>	5	<i>50S</i>

Reference Gene Determination

Through the analyses of the three tools, two reference genes were found to be sufficient for accurate normalization in all samples, tissue samples and cold-treated samples (Fig. 4). Our results suggested that *Ts* and *EF1* was the best combination for accurate normalization in all samples (Table 3). Moreover, *HIS* and *EF1* were selected as reference genes in tissue samples (Table 3). Under cold stress, *UBQ* and *EF1* were the best choices for accurate normalization (Table 3). Notably, among all samples, tissue samples and cold-treated samples, *OmpH* was always the least stably expressed gene, indicating that *OmpH* was unsuitable for RT-qPCR data normalization under these conditions (Fig. 3, Fig. 5, Table 2). Although the *18S* gene possessed least variable expression levels among the 60 samples (Fig. 2), geNorm and NormFinder analysis showed that *18S* had an unstable expression pattern (Fig. 3 and Fig. 5). We suggest that *18S* should not be used as a reference gene.

Verification of Reference Genes

To verify the reference genes *Ts* and *EF1*, we used *ICE1* as a target gene and *Ts* and *EF1* as internal control genes. *ICE1* is a transcription factor that can regulate the expression of the downstream *CBF* gene, thereby improving plant resistance to cold stress [37]. In the leaves of *P. edulis*, when we used *Ts* and *EF1* as internal control genes, we found that the expression level of *ICE1* improved with increasing cold treatment times (Fig. 6). This phenomenon was consistent with the findings of a previous study [37]. This suggests that *ICE1* may increase the stability of *P. edulis* to cold stress.

Discussion

Reference genes are used for RT-qPCR data normalization. The expression stability of reference genes can directly influence the accuracy of RT-qPCR, and reference genes should sustain stable expression levels in various tissues and under distinct experimental conditions [7, 38]. To our knowledge, the expression levels of genes vary with changes in tissue, species and experimental conditions [11]. Therefore, a gene must be strictly screened before being used as a reference gene to ensure the accuracy of RT-qPCR analysis. In addition, functional genes of *P. edulis* have not been well studied, especially by relative quantitative analysis of functional genes, and research on reference genes in *P. edulis* has not been reported. [1, 2]. To enhance the reliability of functional gene expression research, we systematically selected reliable internal control genes for RT-qPCR data normalization in multiple tissue samples and cold-treated samples of *P. edulis*.

In this work, we selected 10 candidate reference genes, namely, *50S*, *Ts*, *UBQ*, *OUT*, *OmpH*, *EF1*, *18S*, *eIF5A*, *HIS*, and *Liom*, and we used RT-qPCR to obtain their Ct values in 60 samples. Through analysis based on Ct values, we found that *18S* had the highest expression level (5.99 Ct) and the smallest expression variation (1.94 Ct), while *OmpH* had the lowest expression level (26.75 Ct) and the largest expression variation (12.07 Ct) (Fig. 2). However, we could not use *18S* as a reference gene due to its low expression variation. According to previous studies, genes with low expression variation may not be suitable for data normalization, and reference genes must be evaluated comprehensively by using different tools [39, 40]. For example, research conducted on rice and *D. longan* showed that *18S* cannot be used as an internal control gene [35, 36]. In particular, in research conducted on *Bixa orellana*, although *18S* had the highest expression level and the smallest expression variation, further analysis results showed that *18S* was the most unstable gene in multiple tissues and at different seed development stages [8]. This result indicated that Ct values of candidate reference genes were not enough to determine the expression stability.

Through further analysis by geNorm, NormFinder, and BestKeeper, *Ts* and *EF1* were found to be ideal for data normalization in all samples (Table 3). The protein encoded by *Ts* can catalyze the last step of threonine biosynthesis, and there are many reports about its structure, expression and function [41, 42]. However, there are few reports about *Ts* as an internal control gene. *EF1* is stably expressed in not only *P. edulis*, but also other plants. For example, *EF1* was used as an internal control gene in *Ilex paraguariensis* leaves and potato (*Solanum tuberosum* L.) under abiotic stress [43, 44]. To our knowledge, the expression level of reference genes may change with changes in species, experimental conditions, and tissue types.

For example, although *EF1* was suitable for data normalization in *P. edulis*, potato under abiotic stress, drought-treated leaves of *I. paraguariensis*, and *Betula luminifera*, this gene was unsuitable for data normalization in *Camellia sinensis* under metal stress and in leaves of *Camellia sinensis* [38, 43–46]. Overall, *Ts* and *EF1* have the highest expression stability, but if we classify all samples into different subsets, there may be other genes in each subset with higher expression stability than *Ts* and *EF1*.

To determine whether there were other genes that had higher expression stability than *Ts* and *EF1* in different sample subsets, we classified the 60 samples into two subsets, namely, tissue samples and cold-treated samples. In previous studies on reference gene selection in different species, we found that the expression patterns of candidate reference genes may differ between nonstress and abiotic stress conditions [23, 47]. Under nonstress conditions, *HIS* and *EF1* was the best combination for data normalization in tissue samples (Table 3). In *L. multiflorum*, *HIS* also showed a stable expression pattern under acidic aluminum stress and heavy metal stress, while it could not be selected as an internal control gene in *Populus tomentosa* due to its poor expression stability [26, 48]. Under cold stress conditions, *UBQ* and *EF1* were sufficient for data normalization in *P. edulis* (Table 3). *UBQ* was also used as a reference gene in cotyledons of *Cunninghamia lanceolata* [11]. However, this gene was unsuitable for data normalization in *Bursaphelenchus mucronatus* and lettuce (*Lactuca sativa*) [49, 50]. Compared with *Ts*, *HIS* had higher expression stability in tissue samples and *UBQ* had higher expression stability in cold-treated samples. This finding indicates that *HIS* and *UBQ* can be used as internal control genes in only specific samples and experimental conditions. Moreover, *18S* and *L18m* were unstably expressed in this study, and *18S* also showed unstable expression patterns in amaranth, *B. orellana* and *Stellera chamaejasme* (Fig. 3, Fig. 5, Table 2) [8, 23, 51]. However, *18S* could be used for data normalization in *Bursaphelenchus mucronatus* and *C. sinensis* under metal stress [45, 49].

As a nutritious tropical fruit, *P. edulis* is widely cultivated in southern China [2]. However, cold resistance has been an important factor limiting the expansion of the *P. edulis* cultivation area. Our laboratory discovered a cold-resistant variety, Pingtang 1, which is used as sample material for the study of cold resistance mechanisms. Our previous research has shown that the ICE1-CBF-COR pathway plays a crucial role in the cold tolerance of *P. edulis* [2]. *ICE1* is a transcription factor that can regulate the expression of the downstream *CBF* gene, thereby improving plant resistance to cold stress [37]. To validate the reference genes that we selected, we used *ICE1* as a target gene and *Ts* and *EF1* as internal control genes, to investigate the expression level of *ICE1* in cold-treated leaves of *P. edulis*. We found that the expression level of *ICE1* increased with increasing cold-treatment times (Fig. 6). In previous studies, increased expression of *ICE1* enhanced plant resistance to cold stress [37, 52]. Therefore, the increase in *ICE1* expression may enhance the adaptability of *P. edulis* to cold stress.

Our work will facilitate the study of functional gene expression levels in *P. edulis*. We selected stable and reliable internal control genes and applied these genes in RT-qPCR data normalization in different tissue samples and under cold stress conditions in *P. edulis*.

Conclusions

In this study, we successfully selected reference genes from among 10 candidates for *P. edulis* RT-qPCR data normalization. In all samples, *Ts* and *EF1* were the best reference genes for data normalization; *HIS* and *EF1* were ideal for tissue samples, and *UBQ* and *EF1* were the best internal control genes for cold-treated samples. In conclusion, this work provides reliable and effective reference genes for gene expression level analysis in *P. edulis*.

Methods

Plant Materials and Stress Treatment

Plant materials were sampled from adult *P. edulis* “Pingtang 1” (a new *P. edulis* variety with cold resistance was developed by Institute of Mountain Resources in Pingtang County, Guizhou Province, and the voucher specimen was deposited in the Herbarium of Guizhou Academy of Sciences, voucher no. A.L2008001) individuals planted in Pingtang County, Guizhou Province (25°44'77" N, 106°48'45" E). “Pingtang 1” survived the 2008 Snow Disaster in China (average temperature below -2 °C), and our laboratory completed its transcriptome sequencing in 2017 [2]. A total of 60 samples were classified into two subsets, tissue samples and cold stress-treated samples. Tissue samples included stems, leaves (mature leaves and immature leaves), fruits (young, adult, and old), flowers (buds and blooming flowers) and roots. Annual *P. edulis* plants were treated at 4 °C for 72 h (cold treatment) and then treated at 25 °C for 72 h (recovery treatment). The stress-treated samples (leaves) included samples collected at 1 h, 4 h, 8 h, 24 h and 72 h during cold treatment and recovery treatment, with one control sample. Each sample had three biological replicates. A total of 60 samples were stored at -80 °C.

RNA Isolation and cDNA Synthesis

Total RNA was isolated from 60 samples by using the RNeasy Pure Plant Kit (Qiagen, Beijing, China) according to the steps in the manufacturer’s protocol. Two percent (w/v) agarose gel electrophoresis was performed to validate the RNA quality, and a NanoDrop 2000 c spectrophotometer (Thermo Scientific, Wilmington, USA) was used to determine the RNA concentration. Then 1 µg of total RNA was used to synthesize cDNA by applying the PrimeScriptTM RT Master Mix system (TaKaRa, Dalian, China), following the steps described in the manufacturer’s instructions.

Primer Design for Candidate Reference Genes

According to previous studies, we selected 10 candidate reference genes: *50S*, *Ts*, *OTU*, *OmpH*, *Liom*, *EF1*, *UBQ*, *18S*, *eIF5A*, and *HIS*. Oligo 7 software (<https://en.freedownloadmanager.org/Windows-PC/OLIGO.html>) was used to design primers for RT-qPCR with the following parameters: the lengths of primers were 17–25 bp, the GC content of the primers was 40–60%, the melting temperature was 58–62 °C, and the lengths of the products were 80–210 bp (Table 1). The mean amplification efficiency of the primers was calculated with LinRegPCR 2014.x software [34].

Expression Levels of Candidate Reference Genes Detected by RT-qPCR

RT-qPCR was performed on an ABI ViiA 7 platform (Applied Biosystems, Foster City, USA). Each reaction had a final volume of 10 μ L, which included 1 μ L of cDNA, 5 μ L of 2 $\times$ PowerUp™ SYBR™ Green Master Mix (TaKaRa, Dalian, China), 0.5 μ L of forward primer (10 μ M) and reverse primer (10 μ M) and 3 μ L of ddH₂O. The amplification was performed under the following standard thermal profile: 95 °C for 20 s and 40 cycles of 95 °C for 1 s and 60 °C for 20 s, followed by a melt curve step at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. All the reactions had three technical replicates.

Data Analysis

Three tools, namely, BestKeeper, NormFinder and geNorm were used to evaluate gene expression stability based on the RT-qPCR results [20–22]. The formula $E^{-\Delta Ct}$ (ΔCt = each Ct value – minimum Ct value, where E represents mean efficiency of primer) was used to transform Ct values to relative expression quantities, which were needed for geNorm and NormFinder analyses. However, the Ct values were directly imported into BestKeeper. The geNorm tool uses the average expression stability value (M) to select internal control genes. Candidate reference genes have a relatively small M value, which indicates that these genes have relatively high expression stabilities [20]. At the same time, the pairwise variation ($V_{n/n+1}$) value given by geNorm is useful for determining the minimum number of internal control genes [20]. By combining intergroup and intragroup variations in gene expression levels, NormFinder evaluates genes based on stability values [21]. A low stability value indicates high expression stability. In the BestKeeper algorithm, the coefficient of determination (r^2 , r represents the Pearson correlation coefficient) and the SD are used to select reference genes [22].

Verification of Reference Genes

To test the reference genes *Ts* and *EF1*, we chose *ICE1* as a target gene and investigated the expression pattern of the *ICE1* gene in the leaves of *P. edulis* under cold stress. The leaves were collected from *P. edulis* treated with cold stress (4 °C) for 0 h (CK), 1 h, 4 h, 8 h, 24 h, and 72 h. The primers for *ICE* for RT-qPCR were 5′–AAGCCACAGACAACAAGGCA–3′ (forward) and 5′–AGCACTGCGGACATTCTTCA–3′ (reverse). All further steps are described above.

Abbreviations

RT-qPCR, reverse transcription quantitative polymerase chain reaction; 50S, 50S ribosomal protein; Ts, threonine synthase; UBQ, ubiquitin 1; OTU, OTU domain-containing protein; 18S, 18S ribosomal RNA; OmpH, outer membrane protein; EF1, elongation factor 1 alpha; eIF5A eukaryotic translation initiation factor 5A; HIS, histone H3; Liom, *Theobroma cacao* L-isoaspartate O-methyltransferase mRNA; ICE1, inducer of CBF expression; CBF, C-repeat-binding factor.

Declarations

Ethics approval and consent to participate: Not applicable.

Consent to publish: Not applicable.

Availability of data and materials: The raw CT values for analysis can be found in the Supplementary Material 1.

Competing interests: The authors declare that they have no competing interests.

Funding: This study was financially funded by the following programs: the National Natural Science Foundation of China (Gran no. 31960576; for collecting materials and conducting experiment), the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD; for manuscript preparation) and the Collaborative Innovation Plan of Jiangsu Higher Education (CIP; for data analysis and interpretation).

Authors' Contributions: Z.T. and H.F. contributed equally to this work. Experimental design: M.X., H.F. and Z.T. Collected plant materials and performed the experiments: H.F., A.L., N.R. and K.D. Data analysis: Z.T. and H.F. Manuscript writing: Z.T. and M.X. All authors approved the final draft.

Acknowledgments: We wish to thank our colleagues in the laboratory, namely, Haoran Qi, Tengfei Shen, Ling Wu, Heng Cai, Hao Rong, and Xin Han, for their contributions to conducting the experiments and writing the article.

References

1. Xu M, Li A, Teng Y, Sun Z, Xu M. Exploring the adaptive mechanism of *Passiflora edulis* in karst areas via an integrative analysis of nutrient elements and transcriptional profiles. *BMC Plant Biol.* 2019;19(1):185–200.
2. Liu S, Li A, Chen C, Cai G, Zhang L, Guo C, Xu M. **De Novo Transcriptome Sequencing in *Passiflora edulis* Sims to Identify Genes and Signaling Pathways Involved in Cold Tolerance.** *Forests.* 2017;8:435–48.
3. Dos Reis LCR, Facco EMP, Salvador M, Flores SH, de Oliveira Rios A. Antioxidant potential and physicochemical characterization of yellow, purple and orange passion fruit. *J Food Sci Technol.* 2018;55(7):2679–91.
4. Rosado RDS, Rosado LDS, Borges LL, Bruckner CH, Cruz CD, dos Santos CEM. **Genetic Diversity of Sour Passion Fruit Revealed by Predicted Genetic Values.** *Agron J* 2018, 111(1).
5. Mattar GS, Moraes CCd, Meletti LMM, Purquerio LFV. **Accumulation and exportation of nutrients by yellow Passion fruit cv. IAC 275.** *Rev Bras Frutic* 2018, 40(3).
6. Oliveira DA, Angonese M, Ferreira SRS, Gomes CL. Nanoencapsulation of passion fruit by-products extracts for enhanced antimicrobial activity. *Food Bioprod Process.* 2017;104:137–46.

7. Dheda K, Huggett JF, Chang JS, Kim LU, Bustin SA, Johnson MA, Rook GA, Zumla A. The implications of using an inappropriate reference gene for real-time reverse transcription PCR data normalization. *Anal Biochem.* 2005;344:141–3.
8. Moreira VS, Soares VLF, Silva RJS, Sousa AO, Otoni WC, Costa MGC. **Selection and validation of reference genes for quantitative gene expression analyses in various tissues and seeds at different developmental stages in *Bixa orellana* L.** *Physiol Mol Biol Plants.* 2018;24(3):369–78.
9. Lee PD, Sladek R, Greenwood CMT, Hudson TJ. Control Genes and Variability: Absence of Ubiquitous Reference Transcripts in Diverse Mammalian Expression Studies. *Genome Res.* 2002;12:292–7.
10. Hong S-Y, Seo PJ, Yang M-S, Xiang F, Park C-M. **Exploring valid reference genes for gene expression studies in *Brachypodium distachyon* by real-time PCR.** *BMC Plant Biol.* 2008;8:112–22.
11. Bao W, Qu Y, Shan X, Wan Y. **Screening and Validation of Housekeeping Genes of the Root and Cotyledon of *Cunninghamia lanceolata* under Abiotic Stresses by Using Quantitative Real-Time PCR.** *Int J Mol Sci.* 2016;17(8):1198–214.
12. Liu YK, Li HP, Huang T, Cheng W, Gao CS, Zuo DY, Zhao ZX, Liao YC. Wheat-specific gene, ribosomal protein l21, used as the endogenous reference gene for qualitative and real-time quantitative polymerase chain reaction detection of transgenes. *J Agric Food Chem.* 2014;62(43):10405–13.
13. Die JV, Roman B, Nadal S, Gonzalez-Verdejo CI. **Evaluation of candidate reference genes for expression studies in *Pisum sativum* under different experimental conditions.** *Planta.* 2010;232:145–53.
14. Bustin SA. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *J Mol Endocrinol.* 2002;29:23–9.
15. Gutierrez L, Mauriat M, Guenin S, Pelloux J, Lefebvre JF, Louvet R, Rusterucci C, Moritz T, Guerineau F, Bellini C, et al. The lack of a systematic validation of reference genes: a serious pitfall undervalued in reverse transcription-polymerase chain reaction (RT-PCR) analysis in plants. *Plant Biotechnol J.* 2008;6:609–18.
16. Mehennaoui K, Legay S, Serchi T, Guerold F, Giamberini L, Gutleb AC, Cambier S. **Identification of reference genes for RT-qPCR data normalization in *Gammarus fossarum* (Crustacea Amphipoda).** *Sci Rep.* 2018;8(1):15225–32.
17. Huggett J, Dheda K, Bustin S, Zumla A. Real-time RT-PCR normalisation; strategies and considerations. *Genes Immun.* 2005;6(4):279–84.
18. Zhu P, Ma Y, Zhu L, Chen Y, Li R, Kongshu J. **Selection of Suitable Reference Genes in *Pinus massoniana* Lamb. Under Different Abiotic Stresses for qPCR Normalization.** *Forests* 2019, 10(8).
19. Tu Z, Hao Z, Zhong W, Li H. **Identification of Suitable Reference Genes for RT-qPCR Assays in *Liriodendron chinense* (Hemsl.) Sarg.** *Forests* 2019, 10(5).
20. Vandesompele J, Preter KD, Pattyn F, Poppe B, Roy NV, paepe AD, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal.pdf. *Genome Biol.* 2002;3(7):0034.0031–0034.0011.

21. Andersen CL, Jensen JL, Orntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res.* 2004;64(15):5245–50.
22. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper – Excel-based tool using pair-wise correlations. *Biotechnol Lett.* 2004;26:509–15.
23. Vera Hernandez FP, Martinez Nunez M, Ruiz Rivas M, Vazquez Portillo RE, Bibbins Martinez MD, Luna Suarez S, Rosas Cardenas FF. Reference genes for RT-qPCR normalisation in different tissues, developmental stages and stress conditions of amaranth. *Plant Biol (Stuttg).* 2018;20(4):713–21.
24. Nguyen DQ, Eamens AL, Grof CPL. **Reference gene identification for reliable normalisation of quantitative RT-PCR data in *Setaria viridis*.** *Plant methods.* 2018;14:24–35.
25. Zhang K, Li M, Cao S, Sun Y, Long R, Kang J, Yan L, Cui H. **Selection and validation of reference genes for target gene analysis with quantitative real-time PCR in the leaves and roots of *Carex rigescens* under abiotic stress.** *Ecotoxicol Environ Saf.* 2018;168:127–37.
26. Liu Q, Qi X, Yan H, Huang L, Nie G, Zhang X. **Reference Gene Selection for Quantitative Real-Time Reverse-Transcriptase PCR in Annual Ryegrass (*Lolium multiflorum*) Subjected to Various Abiotic Stresses.** *Molecules.* 2018;23(1):172–88.
27. Liang W, Zou X, Carballar-Lejarazu R, Wu L, Sun W, Yuan X, Wu S, Li P, Ding H, Ni L, et al. **Selection and evaluation of reference genes for qRT-PCR analysis in *Euscapis konishii* Hayata based on transcriptome data.** *Plant methods.* 2018;14:42–50.
28. Zhao N, Yan Y, Wang H, Bai S, Wang Q, Liu W, Wang J. **Acetolactate Synthase Overexpression in Mesosulfuron-Methyl-Resistant Shortawn Foxtail (*Alopecurus aequalis* Sobol.): Reference Gene Selection and Herbicide Target Gene Expression Analysis.** *J Agric Food Chem.* 2018;66(37):9624–34.
29. Wei J, Li F, Guo J, Li X, Xu J, Wu G, Zhang D, Yang L. Collaborative Ring Trial of the Papaya Endogenous Reference Gene and Its Polymerase Chain Reaction Assays for Genetically Modified Organism Analysis. *J Agric Food Chem.* 2013;61:11363–70.
30. Nonis A, Vezzaro A, Ruperti B. **Evaluation of RNA extraction methods and identification of putative reference genes for real-time quantitative polymerase chain reaction expression studies on olive (*Olea europaea* L.) fruits.** *J Agric Food Chem.* 2012;60(27):6855–65.
31. Obrero A, Die JV, Roman B, Gomez P, Nadal S, Gonzalez-Verdejo CI. **Selection of reference genes for gene expression studies in zucchini (*Cucurbita pepo*) using qPCR.** *J Agric Food Chem.* 2011;59(10):5402–11.
32. Wu G, Zhang L, Wu Y, Cao Y, Lu C. **Comparison of five endogenous reference genes for specific PCR detection and quantification of *Brassica napus*.** *J Agric Food Chem.* 2010;58(5):2812–7.
33. Xu M, Zhang B, Su X, Zhang S, Huang M. Reference gene selection for quantitative real-time polymerase chain reaction in *Populus*. *Anal Biochem.* 2011;408(2):337–9.
34. Ruijter JM, Ramakers C, Hoogaars WMH, Karlen Y, Bakker O, Hoff MJBvd, Moorman AFM. Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. *Nucleic*

Acids Res. 2009;37(6):e45.

35. Jain N, Vergish S, Khurana JP. Validation of house-keeping genes for normalization of gene expression data during diurnal/circadian studies in rice by RT-qPCR. *Scientific reports*. 2018;8:3203.
36. Wu J, Zhang H, Liu L, Li W, Wei Y, Shi S. Validation of Reference Genes for RT-qPCR Studies of Gene Expression in Preharvest and Postharvest Longan Fruits under Different Experimental Conditions. *Frontiers in plant science*. 2016;7:780.
37. Xiang D, Chai Y, Man L, Sun Y, Zhang T, Wei C, Xie Z, Li H, Zhang W, Liu D, et al. Overexpression of a heading Chinese cabbage ICE1 gene confers freezing tolerance in transgenic rice. *Plant Cell Tissue Organ Cult*. 2016;128(1):43–54.
38. Wu J, Zhang J, Pan Y, Huang H, Lou X, Tong Z. **Identification and evaluation of reference genes for normalization in quantitative real-time PCR analysis in the premodel tree *Betula luminifera***. *J For Res*. 2016;28(2):273–82.
39. Wan D, Wan Y, Yang Q, Zou B, Ren W, Ding Y, Wang Z, Wang R, Wang K, Hou X. **Selection of Reference Genes for qRT-PCR Analysis of Gene Expression in *Stipa grandis* during Environmental Stresses**. *PloS one*. 2017;12(1):e0169465.
40. Jacob F, Guertler R, Naim S, Nixdorf S, Heinzelmann-Schwarz V. Careful Selection of Reference Genes Is Required for Reliable Performance of RT-qPCR in Human Normal and Cancer Cell Lines. *PloS one*. 2013;8(3):1–8.
41. Avraham T, Amir R. The expression level of threonine synthase and cystathionine- γ -synthase is influenced by the level of both threonine and methionine in Arabidopsis plants. *Transgenic Res*. 2005;14(3):299–311.
42. Garrido-Franco M, Ehlert S, Messerschmidt A, Marinkovic S, Huber R, Laber B, Bourenkov GP, Clausen T. Structure and function of threonine synthase from yeast. *J Biol Chem*. 2002;277(14):12396–405.
43. Acevedo RM, Avico EH, Ruiz OA, Sansberro PA. Assessment of reference genes for real-time quantitative PCR normalization in *Ilex paraguariensis* leaves during drought. *Biol Plant*. 2018;62(1):89–96.
44. Tang X, Zhang N, Si H, Calderon-Urrea A. Selection and validation of reference genes for RT-qPCR analysis in potato under abiotic stress. *Plant methods*. 2017;13:85–92.
45. Wang ML, Li QH, Xin HH, Chen X, Zhu XJ, Li XH. Reliable reference genes for normalization of gene expression data in tea plants (*Camellia sinensis*) exposed to metal stresses. *PloS one*. 2017;12(4):e0175863.
46. Wu ZJ, Tian C, Jiang Q, Li XH, Zhuang J. **Selection of suitable reference genes for qRT-PCR normalization during leaf development and hormonal stimuli in tea plant (*Camellia sinensis*)**. *Scientific reports*. 2016;6:19748.
47. Schmidt GW, Delaney SK. Stable internal reference genes for normalization of real-time RT-PCR in tobacco (*Nicotiana tabacum*) during development and abiotic stress. *Molecular genetics genomics: MGG*. 2010;283(3):233–41.

48. Wang Y, Chen Y, Ding L, Zhang J, Wei J, Wang H. Validation of Reference Genes for Gene Expression by Quantitative Real-Time RT-PCR in Stem Segments Spanning Primary to Secondary Growth in *Populus tomentosa*. *PloS one*. 2016;11(6):e0157370.
49. Zhou L, Chen F, Ye J, Pan H. **Selection of Reliable Reference Genes for RT-qPCR Analysis of *Bursaphelenchus mucronatus* Gene Expression From Different Habitats and Developmental Stages.** *Front Genet*. 2018;9:269–79.
50. Sgamma T, Pape J, Massiah A, Jackson S. Selection of reference genes for diurnal and developmental time-course real-time PCR expression analyses in lettuce. *Plant methods*. 2016;12:21–9.
51. Liu X, Guan H, Song M, Fu Y, Han X, Lei M, Ren J, Guo B, He W, Wei Y. **Reference gene selection for qRT-PCR assays in *Stellera chamaejasme* subjected to abiotic stresses and hormone treatments based on transcriptome datasets.** *PeerJ* 2018:4535–4555.
52. Wang DZ, Jin YN, Ding XH, Wang WJ, Zhai SS, Bai LP, Guo ZF. Gene Regulation and Signal Transduction in the ICE-CBF-COR Signaling Pathway during Cold Stress in Plants. *Biochemistry*. 2017;82(10):1103–17.

Figures

Figure 1

Melt curve plots of ten candidate reference genes. (a) – (j) Melt curve plots of 18S, 50S, EF1, eIF5A, HIS, Liom, OmpH, OTU, Ts, and UBQ.

Figure 2

Box map of Ct values for the 10 candidate internal control genes. The 25th, 50th and 75th percentiles are indicated by boxes. Red triangles represent the mean Ct values of the candidate reference genes. The maximum and minimum Ct values are indicated by whiskers.

Figure 3

Ranking of the gene expression stability calculated by geNorm. Ranking of the gene expression stability in all samples (a), tissue samples (b), and cold-treated samples (c). A low average expression stability value, M, indicates high expression stability.

Figure 4

Pairwise variation (V) of ten candidate reference genes calculated by geNorm. If $V_n/n+1$ values exceed 0.15, an additional internal control gene must be included for normalization. In total samples, tissue samples, and cold-treated samples, V2/3 values were less than 0.15, indicating that two reference genes were sufficient for RT-qPCR data normalization.

Figure 5

Ranking of the gene expression stability calculated by NormFinder. A low stability value indicates increased expression pattern stability. Ranking of the expression stability of genes in all samples (a), tissue samples (b), and cold stress-treated samples (c).

Figure 6

Investigation of the expression levels of ICE1 under cold stress in *P. edulis*. *P. edulis* was treated at 4 °C for 0 h (CK), 1 h, 4 h, 8 h, 24 h, and 72 h.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryMaterial1.xlsx](#)