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Identification of Suitable Reference Genes for RT-qPCR Assays in *Liriodendron chinense* (Hemsl.) Sarg

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Abstract: The precision and reliability of reverse transcription quantitative polymerase chain reaction (RT-qPCR) depend mainly on suitable reference genes; however, reference genes have not yet been identified for *Liriodendron chinense* (Hemsl.) Sarg. In this study, the expression stability of 15 candidate reference genes, *ACT7*, *ACT97*, *UBQ1*, *eIF2*, *eIF3*, *HIS*, *BIG*, *AGD11*, *EFG*, *GAPDH*, *CYP*, *RPL25*, *UBC*, *RPB1*, and *TUB*, was tested across multiple organs of *L. chinense* using four algorithms, geNorm, NormFinder, BestKeeper, and RefFinder. To understand the difference between the selected reference genes and the unsuitable candidate reference genes, the expression level of a target gene, *LcPAT7*, was normalized across various plant samples. *ACT97* and *eIF3* represented the best combination across all samples tested, while *AGD11* and *UBQ1* were unsuitable for normalization in this case. In the vegetative organ subset, *ACT97*, *ACT7*, and *GAPDH* showed the highest expression stability. For floral organs, *UBC* and *eIF3* were the most stable reference genes. Unsuitable reference genes underestimated the expression levels of a target gene, *LcPAT7*. This study identified two reference genes (*ACT97* and *eIF3*) for the precise and reliable normalization of *L. chinense* RT-qPCR data across various organs. Our work provides an effective framework for quantifying gene expression in *L. chinense*.

Keywords: accuracy of RT-qPCR; internal control genes; gene expression stability

1. Introduction

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) has been extensively applied in gene expression analysis due to its high accuracy, high throughput, and high sensitivity [1–3]. To perform relative quantification via RT-qPCR, normalization is essential to eliminate experimental errors between samples [1], and the selection of appropriate internal genes has a significant influence on the relative quantification results [2]. If the expression pattern of the reference gene is unstable, then the accuracy of the assay will be reduced, and small changes in the expression of the target genes will be impossible to detect [1]. Therefore, to obtain accurate and reliable results, internal control genes must be systematically evaluated to ensure their applicability under different experimental conditions and various species [4,5].

Reference genes usually have basic functions in cells or organisms and are normally expressed at relatively constant rates under different experimental conditions and across different organs [2,6]. Many genes, such as actin, beta-tubulin, elongation factor, polyubiquitin, eukaryotic translation initiation factor, glyceraldehyde-3-phosphate dehydrogenase, histone, ribosomal protein L, cyclophilin,

and 18S RNA have been used as reference genes under different experimental conditions and in different species [2,6–15]

Different statistical algorithms have been developed to assess the expression stability of reference genes under certain experimental conditions for specific species; these algorithms include geNorm, BestKeeper, NormFinder, and RefFinder. These algorithms are widely employed to evaluate the expression stability of reference genes and identify reference genes that are consistent across various species such as *Populus tomentosa* Carr. [16], *Bixa orellana* L. [3] and *Betula luminifera* H.J.P. Winkl. [17]. At least two algorithms were suggested to be used for reference gene stability evaluation [18].

Liriodendron chinense (Hemsl.) Sarg, a broad-leaved deciduous tree species belonging to the magnolia family (Magnoliaceae) that is also called the Chinese tulip tree [19], is naturally distributed in mountainous areas of southern China and northern Vietnam at an elevation of 450–1800 m [20,21]. However, little molecular and gene expression data have been reported for *L. chinense*. Additionally, reference genes have not been properly characterized and identified for *L. chinense*, although a few studies have utilized a limited number of genes (including actin) in *L. chinense* without any systematic evaluation [22]. Therefore, the selection of suitable reference genes in *L. chinense* is very necessary for RT-qPCR analysis.

In this study, to identify suitable reference genes for RT-qPCR analysis in *L. chinense,* we used 15 genes, *ACT7* (*Actin 7*), *ACT97* (*Actin 97*), *GAPDH* (*Glyceraldehyde-3-phosphate dehydrogenase*), *TUB* (*Tubulin beta*), *HIS* (*Histone H3*), *EFG* (*Elongation factor G*), *eIF2* (*Eukaryotic translation initiation factor 2*), *eIF3* (*Eukaryotic translation initiation factor 3*), *BIG* (*Auxin transport protein BIG*), *AGD11* (*ADP-ribosylation factor GTPase-activating protein AGD11*), *UBQ1*(*Ubiquitin extension protein 1*), *CYP* (*Cyclophilin*), *RPL25* (*50S ribosomal protein L25*), *RPB1* (*RNA polymerase II subunit RPB1*) and *UBC* (*Ubiquitin conjugating enzyme ATG10*), as candidate genes and tested their expression stability across multiple organs of *L. chinense* using the geNorm [23], BestKeeper [24], NormFinder [25], and RefFinder [26] programs. In addition, to compare the differences between the selected reference genes and unstable candidate reference genes, the *LcPAT7* (*Liriodendron chinese protein S-acyltransferase 7*) gene was chosen as a target gene, and its expression level was determined by RT-qPCR analysis. In summary, this work aimed to identify appropriate reference genes for RT-qPCR analysis across various organs in *L. chinense.*

2. Materials and Methods

2.1. Plant Materials

Experimental materials were collected from an adult *L. chinense* tree in a provenance trial plantation in Xiashu, Jurong County, Jiangsu Province (119°13′20″ E, 32°7′8″ N) [27]. The sample tree originated from the Danshan Nature Reserve, Xuyong County, Sichuan Province (105°29′13″ E, 28°11′48″ N) [19]. A total of 54 samples were classified into two organ subsets, vegetative organs and flower organs. The vegetative organ subset included leaves, roots, and twigs (see Appendix A: Figure A2a,b). Leaves were harvested at four stages: stage 1 (S1) corresponded to leaf buds, stage 2 (S2) corresponded to newly expanded leaves, stage 3 (S3) corresponded to larger leaves than those in S2, and stage 4 (S4) corresponded to mature leaves (see Appendix A: Figure A2). The flower organs consisted of floral buds, sepals, petals, pistils, and stamens. Flower developmental was also classified into four stages: floral buds (stage 1, S1), inflated buds (stage 2, S2), pre-anthesis (stage 3, S3), and opened flowers (stage 4, S4) (see Appendix A: Figure A2f). Sepals, petals, and pistils were harvested from the S2, S3, and S4 flowers (the S1 flowers were too small for the sepals, petals, stamens, and pistil to be completely separated) (see Appendix A: Figure A2c–e), and stamen materials were collected from the S2 and S3 flowers (see Appendix A: Figure A2c,d). Each sample included three biological replicates. A total of 54 samples were stored at −80 °C in a freezer until RNA extraction.

2.2. Total RNA Extraction and cDNA Synthesis

Total RNA was extracted by using the RNAprep Pure Plant Kit (Tiangen, Beijing, China), the specific procedures can be found in the manufacturer's instructions. The concentration and quality of the RNA samples were determined by a NanoDrop 2000 c spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and 1.0 percent (*w/v*) agarose gel electrophoresis. cDNA was synthesized from 1000 ng of total RNA in a 20 µL reaction volume using PrimeScriptTM RT Master Mix (TaKaRa, Dalian, China) according to the manufacturer's instructions.

2.3. Candidate Reference Genes and Primer Design

Based on previous studies of internal control genes for other plants and de novo transcriptome sequencing of *L. chinense* [28], 15 genes were chosen as candidates in this study. The candidate genes were *ACT7*, *ACT97*, *UBQ1*, *eIF2*, *eIF3*, *HIS*, *BIG*, *AGD11*, *EFG*, *GAPDH*, *CYP*, *UBC*, *RPL25*, *RPB1* and *TUB* (Table 1). Using the Oligo 7 algorithm (https://en.freedownloadmanager.org/Windows-PC/OLIGO.html) to design RT-qPCR primers. The melting temperature (TM) of the primers was 58–65 °C, and their lengths were 20–25 bp. The GC contents of the primers were 40–60 percent, and the product lengths were 100–300 bp. The specificity of each amplifications was verified by 2.0 percent (*w/v*) agarose gel electrophoresis and dissociation curve analysis. The LinRegPCR 2014.x algorithm [29] was used to estimate the mean amplification efficiencies of the primer pairs, and the results are shown in Table 1.

Table 1. Primer sequences for reverse transcription quantitative polymerase chain reaction (RT-qPCR).

Gene Name	Primer Sequence (5′-3′)	Tentative Annotation	Amplicon Size (bp)	E	R^2
ACT7	For:TCGAGCAGGAGCTAGAGACA Rev:AAGAGATGGCTGGAAGAGGA	Actin 7	131	1.87	0.99
ACT97	For:TTCCCGTTCAGCAGTGGTCG Rev:TGGTCGCACAACTGGTATCG	Actin 97	187	1.89	0.99
UBQ1	For:ACGGGCAAGACCATTACCTT Rev:GCTTCCCTGCAAAGATCAACC	Ubiquitin extension protein 1	121	1.89	0.99
eIF2	For:ACCTAGAGTGCCGGATGTACGAA Rev:CTTGCTGAGGTCGATGTATCCCTT	Eukaryotic translation initiation factor 2	201	1.88	0.99
eIF3	For:CATCCAATTTCACTTTCCGCTCCAC Rev:AATCACCAGCAGACGAGAAGCA	Eukaryotic translation initiation factor 3	236	1.91	0.99
HIS	For:TCAAGCCCTCTCACCACGGA Rev:GCAAGAGGCAGCAGAGGCAT	Histone H3	245	1.89	0.99
BIG	For:GCCACAGAAGCCAGCGACAACA Rev:ACCCCACCATCTCCTTCACGTT	Auxin transport protein BIG	229	1.81	0.99
AGD11	For:ACCCAGCCTTCGTCCCTTCCAT Rev:TGCCATCCCTCTCACTCTCCCT	ADP-ribosylation factor activating protein AGD11	111	1.86	0.99
EFG	For:TGCATGGGCCTCTTGAATGTCCT Rev:TGGACCTCTTGTTGCATTGGCTT	Elongation factor G	193	1.88	0.99
GAPDH	For:GATCCCTTCATCACGACCGAT Rev:ACAACATATTCAGCGCCAGT	Glyceraldehyde-3-phosphate dehydrogenase	191	1.86	0.99
CYP	For:CAGTCTTCCACCGCATAATCCCA Rev:TGCCATTGATAACATGCCGGGAC	Cyclophilin	152	2.04	0.99
RPL25	For:TCCCGGAACCTACATGCGTCT Rev:ACATTACACCGCAGCAAATTGTCC	50S ribosomal protein L25	258	1.84	0.99
UBC	For:CTTCCTGACGCAATCGCTTTCCACT Rev:CAAATTCTCGCCGCACATCACAACC	Ubiquitin conjugating enzyme ATG10	178	1.86	0.99
RPB1	For:CTGCTGCCCTCATCCTTATTGCT Rev:CATCCAGTCCTTACAGCCCGACA	RNA polymerase II subunit RPB1	259	1.86	0.99
TUB	For:ACGGCAGACGATGAGGAGTATGAG Rev:TCACGCCATAGGATAAGCAAACCA	Tubulin beta	275	1.87	0.99

E = mean amplification efficiency of the primer pair; R^2 = correlation coefficient.

2.4. RT-qPCR Assay

RT-qPCR was performed on a StepOnePlus[TM] System (Applied Biosystems) with a total volume of 10 µL per reaction. Each 10 µL reaction mixture contained 10 ng of cDNA, 10 pmol of each of the

forward and reverse primers, 5 μL of 2 × SYBR Premix Ex Taq and 0.2 μL of 50 × ROX Reference Dye (TaKaRa, Dalian, China). The amplification program was as follows: 95 °C for 30 s and 40 cycles of 95 °C for 5 s and 60 °C for 34 s. After amplification, a thermal denaturing cycle was performed to obtain the dissociation curve of the amplification product to verify the primer's amplification specificity. The dissociation stage was performed as follows: 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s. All RT-qPCRs were carried out in three biological and three technical replicates.

2.5. Data Analysis

The expression stability of the 15 candidate genes was valued by four algorithms, geNorm, BestKeeper, NormFinder, and RefFinder. The cycle threshold (Ct) values were converted to relative expression quantities using the formula $E^{-\Delta Ct}$ (ΔCt = each corresponding Ct value—the minimum Ct value, E = mean amplification efficiency of the primer pairs) for geNorm and NormFinder. The geNorm algorithm uses the average expression stability value (M) to evaluate gene stability and uses the pairwise variation ($V_{n/n+1}$) to determine the optimal number of reference genes. The average pairwise variation of a specific candidate gene from all other candidate reference genes is called the M value, and the pairwise variation ($V_{n/n+1}$) is the standard deviation of the logarithmically transformed expression ratios of the combination of two candidate reference genes [23]. The NormFinder algorithm calculates the stability value of each candidate reference gene by combining intragroup and intergroup variations in gene expression [25]. For BestKeeper, the Ct values of candidate reference genes were used to calculate the standard deviation (SD) and the coefficient of variance (CV) [24]. On the basis of rankings from the ΔCt method, geNorm, NormFinder and BestKeeper, the RefFinder program ranks the reference genes comprehensively by calculating the geometric mean of the gene weights [30].

2.6. Comparison of Reference Genes and Unstable Reference Genes

To compare the expression stability of the selected genes to that of the unstable reference genes, the expression profile of the target gene *LcPAT7* was analyzed by using the most stable and least stable reference genes after normalization in S1 leaves, S4 leaves, floral buds, S3 petals, S3 stamens, and S3 pistils. The primer pairs (forward: 5′–CTCGGTAGGCGGAGGTTTCATCA–3′ and reverse: 5′–CTATCTTGCTGCTGCTGCCACTG–3′) of *LcPAT7* were used for RT-qPCR. The relative expression level was calculated by the $2^{-\Delta\Delta CT}$ method and is shown as the fold change.

3. Results

3.1. Primer Specificity and PCR Amplification Efficiency Verification

After PCR amplification of the 15 candidate reference genes, 2.0 percent (*w/v*) agarose gel electrophoresis of each product showed that there was only one amplicon, and its size was consistent with the designed size. The melting curve analysis had the same results and exhibited a single peak in the template (see Appendix A: Figure A1). Furthermore, the mean amplification efficiency (E) of the primer pair ranged from 1.81 for *BIG* to 2.04 for *CYP*, and the correlation coefficient (R^2) of linear amplification was approximately 1 (0.99) (Table 1).

3.2. Candidate Reference Genes Ct Values

We analyzed 54 samples by RT-qPCR assays. The Ct values of the 15 candidate reference genes varied (Figure 1), ranging between 16.47 (for *HIS*) and 29.25 (for *AGD11*), while the average Ct values varied between 17.84 (for *HIS*) and 26.31 (for *UBC*). Moreover, the *ACT97* expression levels were the least variable (3.52 Ct, the maximum and minimum Ct values were 23.28 and 19.76, respectively), while the *RPL25* expression levels were the most variable (5.92 Ct, the maximum and minimum Ct values were 27.80 and 21.88, respectively). Because Ct values are negatively correlated with expression

levels, the results of this study indicated that *HIS* had the highest expression levels and that *UBC* had the lowest expression levels.
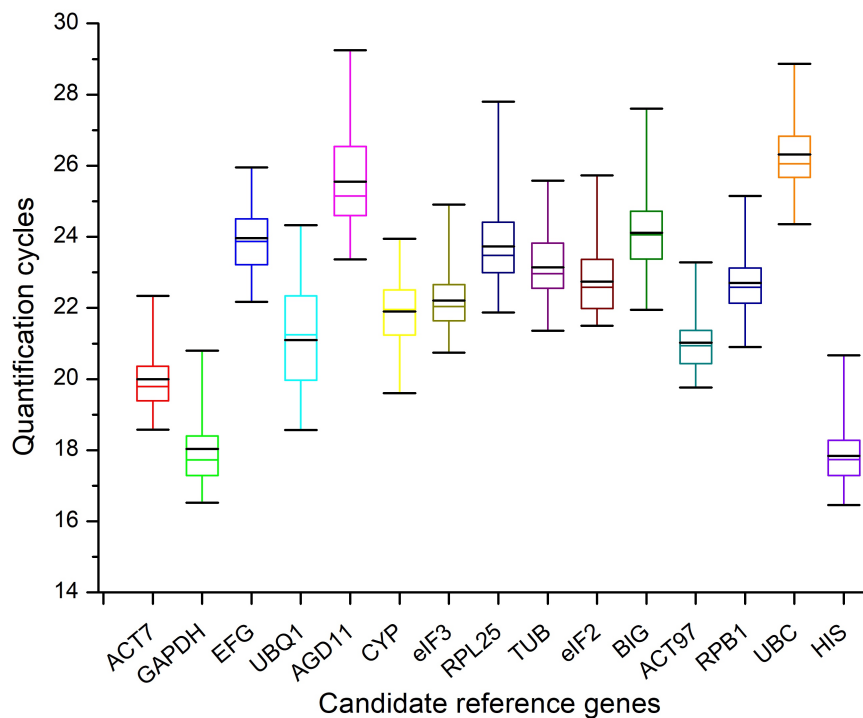

Figure 1. Ct values of 15 candidate reference genes in 54 samples. Boxes indicate the 25th and 75th percentiles. Coloured lines across boxes represent the median Ct values and black lines across boxes indicate the mean Ct values. The maximum and minimum values are represented by whiskers.

3.3. Analysis of Gene Expression Stability

The geNorm analysis. The geNorm algorithm valuates the expression stability of the candidate reference gene by the M value. A smaller M value indicates higher expression stability, if the M value of a candidate reference gene is higher than 1.5, then the candidate reference gene is considered unstable. As measured by geNorm, *ACT7* (M value 0.33) and *GAPDH* (M value 0.33) were the most suitable genes for normalization across all samples of *L. chinense* (Figure 2a). The third to fifth most suitable were *eIF3* (M value 0.39), *ACT97* (M value 0.40) and *EFG* (M value 0.44). In contrast, *UBQ1* and *AGD11* were the least stable reference genes. In the vegetative organ subset, the top five candidate reference genes were *UBC* (M value 0.35), *HIS* (M value 0.35), *ACT97* (M value 0.55), *RPB1* (M value 0.61), and *BIG* (M value 0.74) (Figure 2b). In the flower organ subset, *HIS* (M value 0.33) and *eIF2* (M value 0.33) showed the most stable expression pattern, followed by *UBC* (M value 0.37), *ACT97* (M value 0.39), and *eIF3* (M value 0.41) (Figure 2c).

The pairwise variation ($V_{n/n+1}$) was used to determine the optimal number of internal control genes. If $V_{n/n+1}$ values below 0.15, it was not required to include additional reference genes [23]. Across all samples and in the flower organ subset, the $V_{2/3}$ value were less than 0.15 (0.126 and 0.119, respectively), which indicated that two reference genes were sufficient for normalization. In the vegetative organ subset, the $V_{3/4}$ value was below 0.15, which suggested that three reference genes were the best choice for normalization (Figure 3).

NormFinder analysis. NormFinder ranks candidate reference genes based on their stability values. Stable reference gene expression has a low stability value. Among all the samples, *eIF3*, *ACT97*, *UBC*, *ACT7* and *GAPDH* were the top five candidate reference genes. When the vegetative organ samples were evaluated, *TUB*, *eIF3* and *UBC* were the most suitable genes for normalization, followed by *ACT97* and *ACT7*. For the flower organ subset, *eIF3*, *eIF2*, *ACT97*, *UBC* and *EFG* were the top-ranked

genes (Table 2). In contrast, *UBQ1* and *AGD11* were unsuitable for normalization across all samples, and *UBQ1* was also the least stable gene evaluated within the vegetative organ samples and flower organ samples (Table 2).
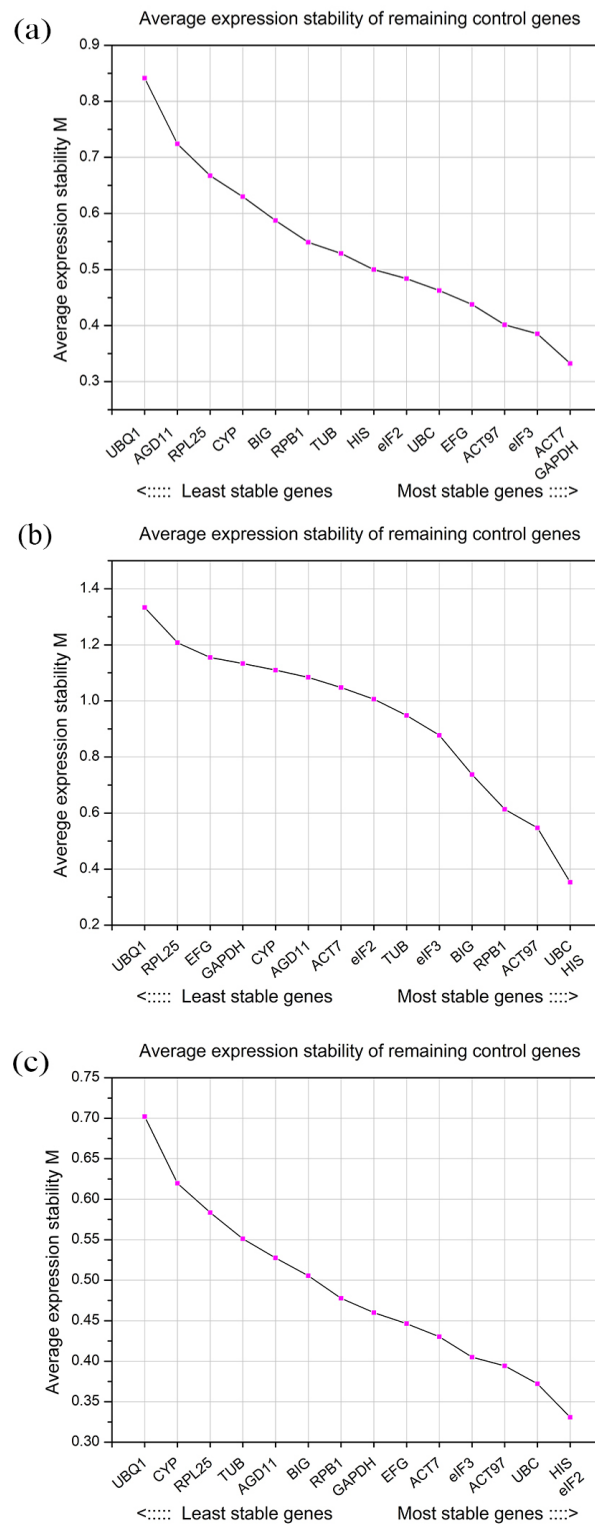

Figure 2. Average expression stability (M) values and ranking of 15 candidate reference genes determined by geNorm. The least stable genes are on the left, and the most stable genes are on the right. (**a**) All tested samples; (**b**) vegetative organ subset; and (**c**) flower organ subset.
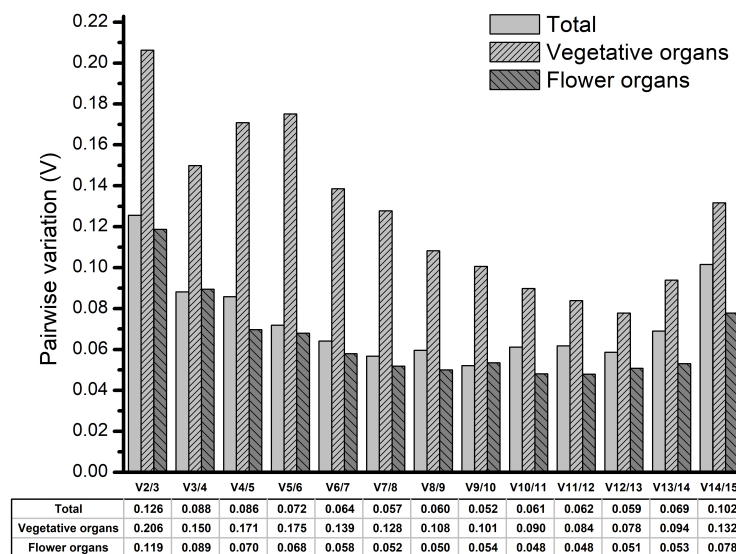
Figure 3. Pairwise variation (V) of 15 candidate reference genes as measured by the geNorm algorithm.

BestKeeper analysis. BestKeeper ranks candidate reference genes according to the CV and standard deviation (SD) of the average Ct values [24]. The most stable gene exhibits the lowest CV ± SD value. If the SD value was more than 1, then the candidate reference gene was regarded as unacceptable. Across all samples, *ACT97* (2.93 ± 0.62) and *HIS* (3.45 ± 0.62) were identified as stable genes. In contrast, *UBQ1* (5.90 ± 1.25) and *AGD11* (4.40 ± 1.12) were unsuitable genes. In the vegetative organ samples, *UBC* (2.50 ± 0.67) and *ACT97* (3.17 ± 0.68) were the top-ranked genes. In the flower organ subset, *UBC* (1.92 ± 0.50) and *eIF3* (2.28 ± 0.50) were suitable genes for normalization (Table 3).

The ranks determined by geNorm had a certain similarity to the ranks from NormFinder. Among all samples, *GAPDH*, *ACT97*, *ACT7*, and *eIF3* showed better stability in both geNorm and NormFinder (Table 4). For the vegetative organs, *ACT97* ranked in the top five in geNorm and NormFinder (Table 4). For the flower organ subset, *eIF3* and *ACT97* had better performance in geNorm and NormFinder (Table 4). The ranks from geNorm, NormFinder, and BestKeeper also showed some similarity. In all samples, *eIF3* and *ACT97* were always ranked in the top five by the above three algorithms (Table 4). In addition, *ACT97* and *eIF3* showed better stability in the vegetative organs and flower organs, respectively (Table 4).

Table 2. Rankings of 15 candidate reference genes by the NormFinder algorithm.

Ranks	All Samples Gene Names	Stability Value	Vegetative Organs Gene Names	Stability Value	Flower Organs Gene Names	Stability Value
1	*eIF3*	0.16	*TUB*	0.46	*eIF3*	0.15
2	*ACT97*	0.18	*eIF3*	0.47	*eIF2*	0.16
3	*UBC*	0.22	*UBC*	0.49	*ACT97*	0.18
4	*ACT7*	0.24	*ACT97*	0.50	*UBC*	0.20
5	*GAPDH*	0.26	*ACT7*	0.55	*EFG*	0.23
6	*eIF2*	0.26	*BIG*	0.55	*HIS*	0.24
7	*EFG*	0.26	*RPB1*	0.60	*ACT7*	0.28
8	*HIS*	0.28	*HIS*	0.61	*GAPDH*	0.30
9	*RPB1*	0.38	*eIF2*	0.62	*RPB1*	0.31
10	*TUB*	0.42	*AGD11*	0.62	*BIG*	0.34
11	*BIG*	0.42	*CYC*	0.64	*AGD11*	0.34
12	*CYC*	0.50	*GAPDH*	0.66	*TUB*	0.44
13	*RPL25*	0.55	*EFG*	0.70	*RPL25*	0.47
14	*AGD11*	0.65	*RPL25*	0.89	*CYC*	0.49
15	*UBQ1*	1.05	*UBQ1*	1.36	*UBQ1*	0.80

Table 3. Rankings of 15 candidate reference genes by the BestKeeper algorithm.

Ranks	Total Samples Gene Name	SD	CV	Vegetative Organs Gene Name	SD	CV	Flower Organs Gene Name	SD	CV
1	ACT97	0.612	2.92	UBC	0.67	2.50	UBC	0.50	1.92
2	HIS	0.62	3.45	ACT97	0.68	3.17	eIF3	0.50	2.28
3	RPB1	0.67	2.96	RPB1	0.70	3.05	ACT7	0.52	2.63
4	eIF3	0.68	3.05	HIS	0.72	3.94	GAPDH	0.53	2.99
5	UBC	0.68	2.59	BIG	0.73	2.95	HIS	0.54	3.04
6	ACT7	0.71	3.53	CYP	0.74	3.35	ACT97	0.54	2.58
7	CYP	0.73	3.35	eIF3	0.80	3.48	eIF2	0.54	2.42
8	TUB	0.74	3.20	TUB	0.80	3.41	AGD11	0.56	2.26
9	eIF2	0.77	3.40	ACT7	0.84	4.07	RPB1	0.62	2.76
10	GAPDH	0.78	4.32	AGD11	0.85	3.12	TUB	0.63	2.76
11	EFG	0.79	3.28	eIF2	0.87	3.70	EFG	0.64	2.71
12	BIG	0.81	3.37	RPL25	0.88	3.63	BIG	0.69	2.90
13	RPL25	0.86	3.61	GAPDH	0.91	4.86	RPL25	0.71	3.02
14	AGD11	1.12	4.40	UBQ1	0.93	4.20	CYP	0.72	3.30
15	UBQ1	1.25	5.90	EFG	0.95	3.85	UBQ1	1.02	4.93

Table 4. Top five candidate reference genes ranked by the four algorithms.

Samples	Ranks	geNorm	NormFinder	BestKeeper	RefFinder
Total samples	1	GAPDH	eIF3	ACT97	eIF3
	2	ACT7	ACT97	HIS	ACT97
	3	eIF3	UBC	RPB1	ACT7
	4	ACT97	ACT7	eIF3	GAPDH
	5	EFG	GAPDH	UBC	UBC
Vegetative organs	1	HIS	TUB	UBC	ACT97
	2	UBC	eIF3	ACT97	ACT7
	3	ACT97	UBC	RPB1	GAPDH
	4	RPB1	ACT97	HIS	UBC
	5	BIG	ACT7	BIG	eIF3
Flower organs	1	eIF2	eIF3	UBC	eIF3
	2	HIS	eIF2	eIF3	UBC
	3	UBC	ACT97	ACT7	eIF2
	4	ACT97	UBC	GAPDH	ACT7
	5	eIF3	EFG	HIS	ACT97

3.4. Reffinder Analysis and Reference Gene Selection

Because of the differences in the evaluation results of the 15 candidate reference genes by geNorm, BestKeeper and NormFinder, we also used the RefFinder program to comprehensively evaluate the ranks of the candidate reference genes. To the best of our knowledge, the use of at least two reference genes represents a realistic calculation basis in a typical laboratory and the minimal number necessary for an accurate and reliable analysis of RT-qPCR [9,23,24]. Therefore, according to the optimal number of reference genes for normalization (Figure 3) and the results of the RefFinder analysis (Table 5), we selected *eIF3* and *ACT97* as the most suitable reference genes for RT-qPCR analysis across all samples. *ACT97*, *ACT7* and *GAPDH* were collectively the best choice for normalization in the vegetative organ subset (Table 5). For the flower organ samples, *eIF3* and *UBC* were the most stable reference genes (Table 5). In addition, *AGD11* and *UBQ1* were the least stable reference genes across all samples (Table 5).

We found that *eIF3* and *ACT97* ranked among the top four in the geNorm, NormFinder and BestKeeper algorithms for all samples (Table 4). For the vegetative organ subset, *ACT97* showed better stability in the same three algorithms (Table 4). In the flower organs, *eIF3* and *UBC* had better performance in the same three algorithms (Table 4). These results were similar to those of RefFinder.

Table 5. The 15 candidate reference genes ranked by the RefFinder program.

Ranks	All Samples Geo-Mean of Ranking Values	Gene Name	Vegetative Organs Geo-Mean of Ranking Values	Gene Name	Flower Organs Geo-Mean of Ranking Values	Gene Name
1	1.86	*eIF3*	1.57	*ACT97*	1.57	*eIF3*
2	2.00	*ACT97*	2.45	*ACT7*	3.25	*UBC*
3	2.91	*ACT7*	3.46	*GAPDH*	3.44	*eIF2*
4	4.33	*GAPDH*	3.96	*UBC*	3.48	*ACT7*
5	4.36	*UBC*	4.28	*eIF3*	3.66	*ACT97*
6	5.66	*HIS*	5.63	*HIS*	4.00	*GAPDH*
7	6.59	*eIF2*	5.89	*TUB*	6.22	*EFG*
8	6.93	*EFG*	7.95	*RPB1*	6.45	*HIS*
9	7.02	*RPB1*	8.13	*CYP*	9.00	*RPB1*
10	8.91	*TUB*	9.03	*EFG*	9.69	*AGD11*
11	10.61	*CYP*	9.64	*BIG*	11.45	*TUB*
12	11.49	*BIG*	10.24	*eIF2*	11.49	*BIG*
13	13.00	*RPL25*	12.17	*AGD11*	12.74	*CYP*
14	14.00	*AGD11*	14.00	*RPL25*	14.00	*RPL25*
15	15.00	*UBQ1*	15.00	*UBQ1*	15.00	*UBQ1*

3.5. Comparison of Reference Genes and Unstable Candidate Reference Genes

The expression level of *LcPAT7* among the different samples was evaluated to compare the differences between the selected reference genes and unstable candidate reference genes. The results are shown in Figure 4. When the combination of *ACT97* and *eIF3* was used for normalization, the expression levels of *LcPAT7* were upregulated in the floral buds, S3 petals, S3 stamens, and S3 pistils (Figure 4) but downregulated in S4 leaves. When the combination of *AGD11* and *UBQ1* was used as an internal control, *LcPAT7* expression was underestimated (Figure 4). There were significant differences between the cases where the selected reference genes and the unstable candidate reference genes were used as internal controls for normalization in different samples of *L. chinense*. This result means that *AGD11* and *UBQ1* should be avoided when selecting internal controls for RT-qPCR in *L. chinense*.
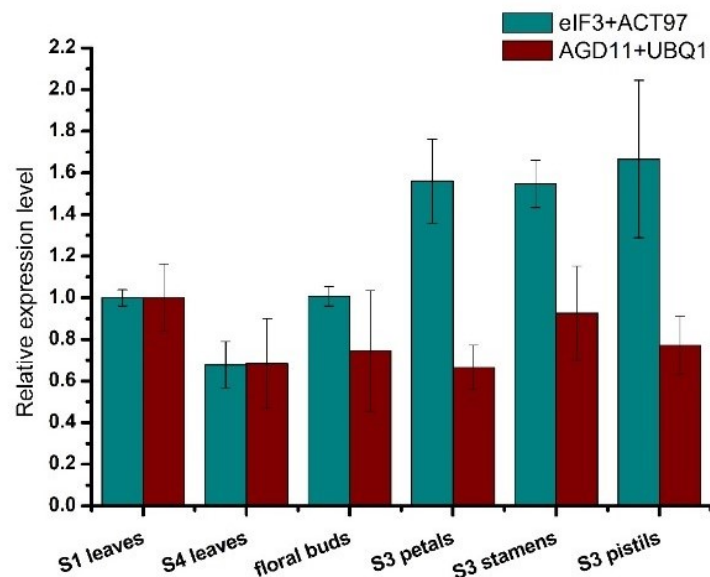

Figure 4. Relative expression levels of *LcPAT7*. According to the results of analyses of 15 candidate reference genes using four programs, *eIF3* and *ACT97* were the selected reference genes, and *AGD11* and *UBQ1* were unstable candidate reference genes. Both sets were as internal control in six organs.

4. Discussion

As a high throughput and accurate method of gene expression level analysis, RT-qPCR has been extensively applied in studies of functional genes across various samples and under different experimental conditions [2,3]. However, the accuracy of RT-qPCR analyses relies on the selection of suitable genes as internal control, because if an unstable gene is chosen as the internal control, the analysis yields unreliable results [31,32]. However, no report exist of systematically selecting suitable reference genes for RT-qPCR analysis in *L. chinense*. Therefore, suitable internal control genes for RT-qPCR analysis must be carefully identified in *L. chinense*. To the best of our knowledge, this study is the first to systematically and comprehensively select internal control genes for RT-qPCR analysis in *L. chinense*.

Fifteen candidate reference genes were selected for this study, and most of them had been studied previously in different plants such *Oryza sativa* L. [9], *Pinus massoniana* L. [11], citrus [33], *Pisum sativum* [34], *Petunia hybrida* [35], *Linum usitatissimum* L. [36], *Nicotiana tabacum* [37], *Camellia sinensis* [38], and *Populus* [39]. In terms of standardization and quality, amplification efficiencies of the candidate reference gene's primer pairs varied from 1.81 to 2.04 (Table 1). The correlation coefficient (R^2) of linear amplification was approximately 1 (0.99) (Table 1). These results revealed that the primer pairs for the candidate reference genes were highly accurate, efficient, and sensitive.

Under any experimental conditions, an appropriate reference gene should exhibit a steady expression pattern across different samples [38]. However, these 15 candidate reference genes had variable expression in all samples (Figure 1). The variation ($Ct_{max} - Ct_{min}$) in the 15 candidate reference genes was between 3.52 Ct (for *ACT97*) and 5.92 Ct (for *RPL25*). This result strongly indicated that *ACT97* and *RPL25* were the most stably and the least stably expressed genes, respectively. The mean Ct values of candidate reference genes varied from 17.84 (for *HIS*) to 26.31 (for *AGD11*). In a study of the *Vigna unguiculata* L. [40], *Setaria virdis* [41], *Euscaphis konishii* Hayata [42] and *Solanum tuberosum* L. [43], candidate reference genes also had variable expression among tested materials, and the average Ct values of the candidate reference genes were between 15 and 27. These results suggested that it is necessary to evaluate the stability of reference genes systemically before selecting them as internal controls for RT-qPCR analysis [40].

Four methods (geNorm, NormFinder, BestKeeper and RefFinder) were used to evaluate the expression stability of the 15 candidate reference genes in our study. Among all samples, *eIF3* and *ACT97* ranked third and fourth in geNorm, first and second in NormFinder, and fourth and first in BestKeeper (Table 4). The different ranks of the candidate reference genes between geNorm and NormFinder and BestKeeper was expected because these three methods use different algorithms [23–25]. In addition, recent studies have noted that there is no universal internal control gene applicable to all experimental conditions and that using a single reference gene as internal control could introduce errors in the results [9,23,25,44]. According to the analysis results of RefFinder and the optimal number of reference genes given by geNorm analysis, we selected *eIF3* and *ACT97* as the most suitable reference genes for RT-qPCR analysis in *L. chinense* (Figure 3, Table 5).

In general, *eIF3* and *ACT97* showed the highest expression stability among all samples of *L. chinense* (Tables 4 and 5). *eIF* (*Eukaryotic translation initiation factor*) and *ACT* (*actin*) also showed good expression stability in different plants across different sample sets. For example, *eIF* was chosen as the internal control gene for RT-qPCR analysis under drought stress in *Lolium multiflorum* [45], and it also showed the most stable expression pattern in *Linum usitatissimum* L. stems [36]. *ACT* was the most suitable internal control for RT-qPCR analysis in *Pinus massoniana* L. [11], and it was still the most stably expressed reference gene in *Daucus carota* L. leaves [7], stem segments of *Populus tomentosa* [16], and *Glycine max* L. Merr. [44]. In addition, in a study of *Gossypium hirsutum*, *ACT* presented a more stable expression pattern during flower development than other candidate reference genes [31]. However, in a study of *Euscaphis konishii* Hayata [42] and *Linum usitatissimum* L. [36], *ACT* was unsuitable for normalization. In contrast, *UBQ1* and *AGD11* were unstably expressed genes in *L. chinense* (Table 5). As a member of the *UBQ* family, *UBQ1* did not have good expression stability in

our study, and it also showed an unstable expression pattern in lettuce (*Lactuca sativa*) [8] and in leaves of *Ilex paraguariensis* under drought treatment [46]. However, *UBQ1* had better expression stability in the parasitic life cycle of *Striga hermonthica* [10]. These results can be clarified by the fact that reference genes exhibited species-specific expression.

Generally, reference genes exhibit organ-specific expression as well as species-specific expression [12]. We classified 54 samples into two subsets: flower organs and vegetative organs. In the flower organ subset, *eIF3* and *UBC* were the best choice for normalization (Table 5). Consistent with this result, *UBC* had the highest expression stability among different organs of the same development stage in *Glycine max* [L.] Merr. [44], and *UBC* was identified as a suitable reference gene across all *Brachypodium distachyon* samples [5]. In the vegetative organs, *ACT97*, *ACT7*, and *GAPDH* were the best combination for RT-qPCR analysis (Figure 3, Table 5). Similarly, *ACT7* had the highest expression stability under heat treatment in *Stipa grandis* [47]. However, compared to *ACT* in a previous study in tea plants, *ACT7* was regarded as an unsuitable internal control [40]. *GAPDH* is commonly used as a reference gene and has been chosen as an internal control gene in the cotyledons of *Cunninghamia lanceolata* [12], different samples of *Vigna unguiculata* L. [40], cold-treated and GA-treated leaves of *Daucus carota* L. [7], and cold-treated and salt-treated leaves of *Carex rigescens* [48]. However, *GAPDH* was unsuitable for normalization in *Pisum sativum* and *Petunia hybrida* [34,35].

In addition, *RPL25*, *CYP*, and *TUB* did not demonstrate high expression stability in *L. chinense* (Table 5). However, *RPL25* demonstrated the highest expression stability across stress-treated samples of *Nicotiana tabacum* [37]. *CYP* was identified as the most stable internal control gene in *Petunia hybrida* [35], insect-resistant leaves of *Pinus massoniana* L. [11], and GA-treated samples of *Stellera chamaejasme* [2]. In a study of *Pisum sativum*, *TUB* was suitable for normalization under abiotic stress and biotic stress [34].

LcPAT7 in *L. chinense* is a member of the Asp–His–His–Cys (DHHC) protein family, and it has a significant influence on development and morphogenesis [49]. To compare the differences between the selected reference genes and unsuitable candidate reference gene, the expression profile of *LcPAT7* was investigated in different organs. The results showed that using unsuitable candidate reference genes as internal controls resulted in underestimates of expression(Figure 4), which indicated that suitable reference genes must be selected for RT-qPCR analysis in *L. chinense*.

5. Conclusions

In this work, we identified specific reference genes for RT-qPCR analysis of different samples in *L. chinense*. The most suitable combination for RT-qPCR analysis across all samples was *eIF3* and *ACT97*. Notably, *AGD11* and *UBQ1* should be eliminated when selecting suitable internal control genes for RT-qPCR analysis in all samples of *L. chinense*. In the vegetative organs, *ACT7*, *ACT97*, and *GAPDH* were the most stably expressed reference genes. *eIF3* and *UBC* were the most suitable reference genes for normalization in flower organs. In conclusion, this study provides an effective framework for quantifying reference gene expression levels in *L. chinense*.

Abbreviations

The following abbreviations are used in this manuscript:

|	
ACT7	actin 7
ACT97	actin 97
UBQ1	ubiquitin extension protein1
eIF2	eukaryotic translation initiation factor 2
eIF3	eukaryotic translation initiation factor 3
HIS	histone H3
BIG	auxin transport protein BIG
TUB	tubulin beta
AGD11	ADP-ribosylation factor activating protein AGD11
EFG	elongation factor G
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
CYP	cyclophilin
RPL25	50S ribosomal protein L25
UBC	ubiquitin conjugating enzyme ATG10
RPB1	RNA polymerase II subunit RPB1

Appendix A
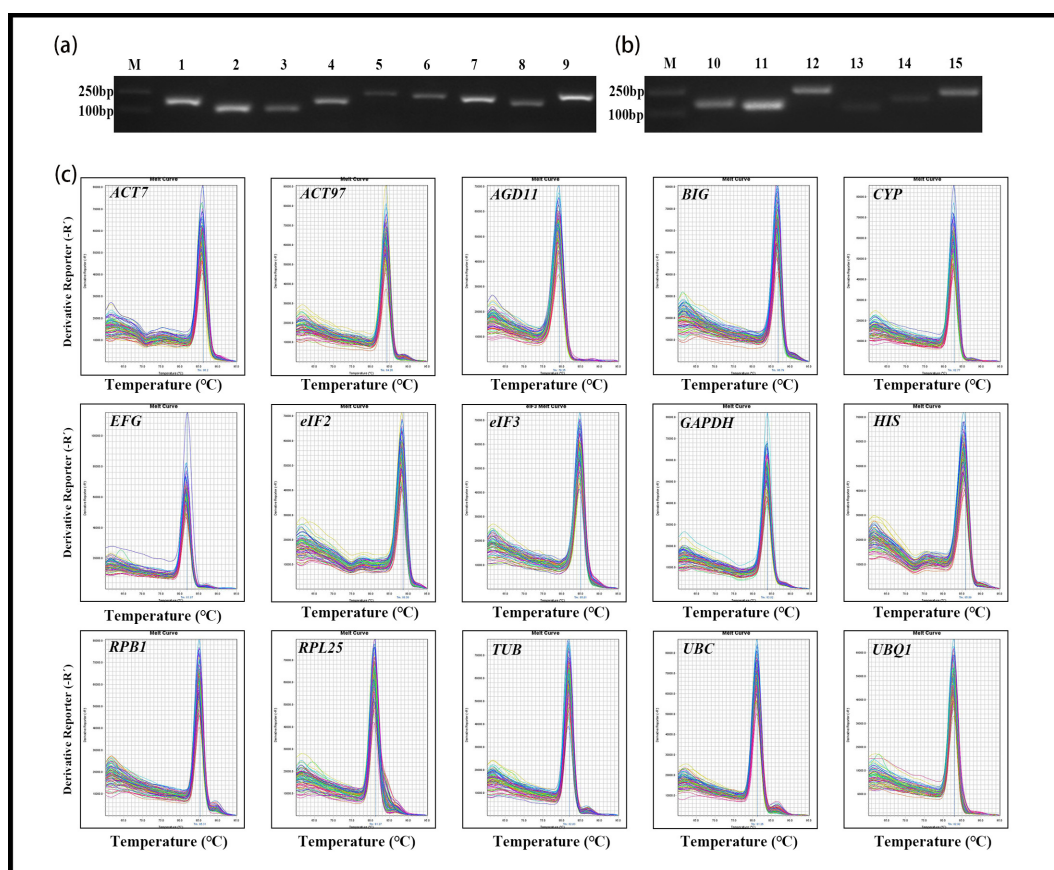
Figure A1. Specificity of each candidate reference gene primer pair. (**a**,**b**) 2% agarose gel showing that all primer pairs have specificity and the size of the amplification products are consistent with the expected sizes. M, marker; 1, *GAPDH*; 2, *ACT7*; 3, *UBQ1*; 4, *ACT97*; 5, *eIF2*; 6, *eIF3*; 7, *TUB*; 8, *EFG*; 9, *RBP1*; 10, *CYP*; 11, *HIS*; 12, *PRL25*; 13, *AGD11*; 14, *UBC*; 15, *BIG*. (**c**) Melt curve analysis of the 15 candidate reference genes tested across different samples of *L. chinense* showed a single peak for each primer pair.

Figure A2. Plant materials used in this experiment. (**a**) The leaf stages are leaf bud (stage 1, S1); young, newly expanded leaf (stage 2, S2); larger leaf than that in S2 (stage 3, S3); and mature leaf (stage 3, S4), ranging from left to right. (**b**) Twigs (left) and roots (right). (**c**) Materials from S2 flower. From top to bottom, materials are sepals, petals, stamens, and pistil. (**d**) Materials from S3 flower. From top to bottom, materials are sepals, petals, stamens, and pistil. (**e**) Materials from the S4 flower. From top to bottom, materials are sepals, petals, stamens, and pistil. (**f**) Flowers at four different developmental stages. From left to right, flowers are a floral bud (stage 1, S1), an inflated bud (stage 2, S2), pre-anthesis (stage 3, S3), and an opened flower (stage 4, S4).

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