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Selection of suitable reference genes in *Paulownia fortunei* (Seem.) Hemsl. under different tissues and abiotic stresses for qPCR normalization

*Jiang Su, Kanghua Xian, Chuanming Fu, Jinxiang He, Baojun Liu, Ningzhen Huang**

Guangxi Key Laboratory of plant conservation and Restoration Ecology in Karst Terrain, Guangxi Institute of Botany, Guangxi Zhuang Autonomous Region and Chinese Academy of Sciences, Guilin, Guangxi, P.R. China

**Corresponding author: hnzhen2002@163.com*

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Abstract: By choosing appropriate candidate reference genes (CRGs) and standardizing qPCR data, more accurate experimental data can be obtained. Herein, the expression stability of *alpha-tubulin1* (*TUA1*), *beta-tubulin* (*TUB*), *beta-tubulin 1* (*TUB1*), *beta-tubulin 5* (*TUB5*), *actin 1* (*ACT1*), *actin 97* (*ACT97*), *molecular chaperone dnaj* (*DNAJ*), *adenine phosphoribosyl transferase* (*APT*), and *histone H4* (*HIS4*) genes from *Paulownia fortunei* (Seem.) Hemsl. under different experimental conditions (different tissues, drought, salinity, Cd, and Cr treatments) was assessed with four statistical tools: RefFinder, BestKeeper, NormFinder, and geNorm. Notably, *TUA1* and *TUB5* were identified as CRGs for different tissues, *ACT97* and *TUB1* for drought treatment, *ACT97* and *APT* for salinity treatment, *TUB1* and *ACT97* for Cd treatment, and *DNAJ*, *TUB1* and *TUB5* for Cr treatment. Furthermore, the results of "total" group, $V_4/V_5 > 0.15$ and $V_5/V_6 < 0.15$ revealed that the CRGs or gene combinations, which could meet all the test conditions, were not easy to identify. To further verify the reliability of CRGs, the expression levels of *paulownia fortunei cellulose synthase A catalytic subunit2* (*PfCesA2*) and *paulownia fortunei glutathione reductase* (*GR*) genes were analysed. The expression patterns were different when the unstable CRGs were used for normalization compared to when the stable CRGs and combination were used for normalization. This study will lay a foundation for study on the expression levels of key genes from *P. fortunei* seedlings.

Keywords: drought treatment; heavy metal; housekeeping genes; qRT-PCR; salinity treatment

Quantitative real-time polymerase chain reaction (qRT-PCR) is one of the most widely used methods for gene quantification due to its high accuracy, sensitivity and specificity (Shen et al. 2020). Absolute and relative quantification are two approaches used for the analysis of gene expression (Zhu et al. 2019). Absolute quantification can accurately determine the copy numbers of genes (Leong et al. 2007). Different from the absolute real-time quantitative technique, the relative quantification method is used to detect

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discrepancies in gene expression under different experimental conditions rather than the exact copy numbers of genes (Livak & Schmittgen 2001). For the relative quantification method, in order to reduce deviations in the processes of initial template quantity between cells, RNA preparation and reverse transcription under different conditions (e.g., different tissues, stress treatments, and developmental stages), suitable reference genes are required as internal controls in qPCR (Ye et al. 2018).

The expression patterns of candidate reference genes (CRGs) should not be affected by experimental conditions, including different tissues, development stages or stress treatments. The *alpha-tubulin* (*TUA*), *beta-tubulin* (*TUB*), *actin* (*ACT*), *molecular chaperone dnaj* (*DNAJ*), *adenine phosphoribosyl transferase* (*APT*), and *histone H4* (*HIS4*) genes as traditional reference genes have been widely analysed and used as internal controls for gene expression analyses in many plants (Wei et al. 2012; Jain et al. 2015; Zhuang et al. 2015; Chen et al. 2017b; Fei et al. 2018; Wang et al. 2020). For instance, *Carex duriuscula* subsp. *rigescens E3 ubiquitin protein ligase UPL7 mRNA* (*UPL7*) was the least stable CRG in leaves treated with high salinity, while excellent stability was achieved in response to Cd treatment (Zhang et al. 2019). *Elongation factor 1 alpha* (*EF-1α*), *translation elongation factor* (*TEF*), *actin* (*ACT*), and *beta-tubulin* (*TUB*) were the most stable CRGs for different birch tissues, salinity treatment, and osmotic stress treatment (Gururani et al. 2019). The most stable gene combinations were *ACT* and *U-box domain-containing protein* (*U-box*) for NaCl treatment, *heat shock protein 70* (*HSC70*) and *TUB* for PEG treatment, α-tubulin (*TUA-1726*) and *ACT* for methyl viologen (MV) treatment (Wang & Zhang 2022). However, a CRG with stable expression under all experimental conditions has not yet been discovered (Yang et al. 2014; Chen et al. 2017a; Ye et al. 2018). Generally, those CRGs are differentially expressed in different species or under different experimental conditions. Therefore, identifying appropriate CRGs for a particular species under different experimental conditions is essential for normalizing target gene (TG) expression (Huggett et al. 2005).

Paulownia fortunei (Seem.) Hemsl. is an important fast-growing tree. This species is usually used for greening and improving the ecological environment due to its excellent resistance to salt, drought, and other extreme environments (Clemens et al. 2002; Wu et al. 2014; Tzvetkova et al. 2015; Wang et al. 2018). Its molecular resistance mechanisms to salt, drought, and heavy metal stress have been reported (Dong et al. 2014; Wang et al. 2010). Systematically monitoring the expression profiles of target genes involved in resistance to abiotic stress can help us to effectively analyse various physiological and molecular pathways. At present, the most common method to analyse the expression level of genes is the use of qRT-PCR. Meanwhile, screening stable reference genes is necessary for qPCR. However, there is a lack of research on the screening of CRGs in early-stage *P. fortunei* seedlings under different tissues and abiotic stresses.

Therefore, the aims of this research were: (1) to obtain the sequences of ten CRGs based on our transcriptome database (unpublished), (2) to screen and evaluate the stability of CRGs for gene expression in *P. fortunei* under different tissues and abiotic stresses and (3) to verify the appropriateness of the selected CRGs with *glutathione reductase* (*GR*) and *cellulose synthase A catalytic subunit 2* (*PfCesA2*) in *P. fortunei.*

MATERIAL AND METHODS

Plant materials and treatment groups. The excellent individual of *P. fortunei* (wild-type, diploid, height: 20 m, diameter at breast height (DBH): 54.1 cm) derived from Daxu Town (110°24'39''E, 25°11'8''N), Lingchuan County, Guilin City, Guangxi Province, China was selected for the experiment. Stem segments with axillary buds from current-growth branches were harvested for tissue culture. The stem segments were sterilized with 0.1% $HgCl_2$ for 7 min and rinsed with sterile water, then transferred to MS medium for culture. After two weeks, sterile materials were transferred to MS medium (pH = 5.8) containing 0.4 mg/L 6-benzylaminopurine (6-BA), 0.04 mg/L indole-3-butyric acid (IBA), 3.5 g/L agar, 30 g/L sucrose for multiplication culture with a temperature of 26 ± 3 °C, a light duration of 12 h/day, and a light intensity of 40 μmol/m^2/s. The culture cycle was 35 days. After two generations of multiplication culture, tissue culture seedlings with height of 3–4 cm were selected for rooting culture (1/2 MS medium (pH = 5.8) containing 0.2 mg/L 1-naphthaleneacetic acid (NAA), 3.5 g/L agar, 20 g/L sucrose; culture room temperature 26 ± 3 °C, light duration 12 h/day, light intensity 40 μmol/m^2/s). After the rooting culture, tissue culture seedlings with 6–8 roots were selected. They were washed gently and transplanted into pots supplemented with peat soil-perlite mixture (4 : 1, v/v). After growing for 45 days at 24 °C under a 16 : 8 h light/dark cycle,

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seedlings at the same growth stage were chosen for the subsequent analyses.

Firstly, all the seedlings were transferred into 1/2 Hoagland's medium for 3 days, and then into Hoagland's medium for 4 days. For the treatment of different tissues, the third leaves from the base, barks, trunks without barks, fibril roots of the seedlings were harvested. For drought, salinity, cadmium (Cd), and chromium (Cr) treatments, the seedlings were immersed in Hoagland's medium containing 400 mol/L D-mannitol, 200 mmol/L NaCl, 70 μmol/L $CdSO_4 \cdot 8/3\ H_2O$, 100 μmol/L $K_2Cr_2O_7$, respectively, at room temperature for 0, 0.5, 3, 6, 12, and 24 h. The seedlings without treatment were employed as controls. All treatments were repeated three times independently. The leaf blade specimens were separately harvested, snap-frozen in liquid nitrogen, and then kept at –80 °C until RNA extraction.

RNA extraction and cDNA synthesis. The RNAprep Pure Kit (DP432, Tiangen Biotech, Beijing, China) with a gDNA-removal step was used to isolate the total RNA. The RNA integrity was assessed by 1.2% agarose gel electrophoresis, while the RNA yield and purity were determined with a NanoDrop 2000 spectrometer (Thermo Fisher Scientific, USA). The cDNA for cloning and sequencing was synthesized from total RNA (1μg) with the PrimeScript™ 1st Strand cDNA Synthesis Kit (6210A, Takara Biotech, Nanning, China). The cDNA (10μL) for real-time PCR was synthesized from total RNA (500 ng) with the PrimeScript™ RT Master Mix (RR036Q, Takara Biotech, Nanning, China).

Gene selection and cloning. The sequences of ten CRGs (*TUA1*, *TUB*, *TUB1*, *TUB5*, *ACT1*, *ACT97*, *DNAJ*, *APT*, *HIS4*, and *18S rRNA*) and 1 TG (*GR*) were originated from our transcriptome (unpublished), while another TG (*PfCesA2*, MK340935) has been cloned earlier. The primers were designed at both ends of the genes (*TUA1*, *TUB*, *TUB1*, *TUB5*, *ACT1*, *ACT97*, *DNAJ*, *APT*, *HIS4*, *18S rRNA*, and *GR*) using the Primer Premier 5.0 software, and the sequence accuracy of those genes was verified by sequencing. The primer sequences are summarized in Table 1. TaKaRa Ex Tap® DNA Polymerase (RR001A, Takara Biotech, Nanning, China) was selected as the polymerase. The PCR mixture (50 μL) was consisted of TaKaRa Ex *Taq*® DNA Polymerase (0.25 μL), 20 mmol/L 10× Ex *Taq* Buffer (5 μL), 25 mmol/L dNTP (4 μL), cDNA template (2 μL), 10 μmol/L forward/reverse primers

Table 1. Descriptions and primer sequences of candidate reference genes (CRGs) and target genes (TGs) in this study

Gene symbol	Primer sequence (5'–3')	Product size (bp)	Accession No.
TUA1	CCTCTGGTCTTTCTCCTTCTTT GCTTGTAAACACACACTGGCTT	1 464	MZ821053
TUB	ACCCGAAGTGAGATACCCAGA CAGTGAAAAATAAGAACCCCCA	1 419	MZ821054
TUB1	CTGGAAAACCCCTTCCCCTATATAA AGAAACACAGAACCCAATCCCAAA	1 583	MZ821055
TUB5	TAGGGCCAACATTTTTCACAC CCAGACTTTCTTTTGGCTACTACT	1 573	MZ821057
ACT1	CTCACTGGAGGTTGGTTTTCG TAATAAGCCGAAGCACCCACA	1 256	MZ821058
ACT97	AGGGCGGCACCTCAGACA GTGCCCTTCATCCAAAACTCA	1 502	MZ821059
DNAJ	CATTTTCAGGTTAAACAGTCCCCC ACCCAACAAAGGGAAGGTACAAAA	1 631	MZ821060
APT	CCCTCCTCACTTCATCACCC CAAGCATTGTCTTTCCGCAT	979	MZ821062
HIS4	CACGTCTATTTTAAACCTCCCC CATACATTCCATTGAACCAGCA	485	MZ821063
18S rRNA	CTACTCGGATAACCGTAGTA GGACCATTCAATCGGTAGGAG	1 538	MZ823806
GR	AAAAAAGATGAATAGACAATATAATG AACGTCTTTTACAAATGGATTACGA	2 204	MZ848185
PfCesA2			MK340935

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(2 µL each), and ddH_2O (34.75 µL). The following amplification conditions were used: 5 min at 94 °C; 35 cycles of 10 s at 98 °C, 30 s at 58 °C (*TUA1*, *TUB*, *TUB1*, *TUB5*, *ACT1*, *ACT97*, *DNAJ*, *18S rRNA*, *GR*, and *PfCesA2*)/56 °C (*APT*, *HIS4*), and 90 s at 72 °C; and 10 min at 72 °C. After ligated transformation, PCR products were sequenced by Springen Biotechnology corp. (Nanjing, China).

Primer design and qRT-PCR assay. The primers for qRT-PCR were designed with Primer Premier 5.0 software and embodied in Table 2. The amplicon size and primer specificity were verified by 2% agarose gel electrophoresis. Premix *Taq*^{TM} (R004A, Takara Biotech, Nanning, China) was used as the polymerase. The PCR (25 µL) mixture contained Premix *Taq* (12.5 µL), template cDNA (1 µL), 10 µmol/L each primer (1 µL), and ddH_2O (9.5 µL). The following amplification conditions were used: 25 cycles of 98 °C for 10 s, 58 °C for 30 s, and 72 °C for 30 s; and 72 °C for 10 min.

A five-fold cDNA dilution series with 3 replicates/concentration was employed to construct a standard curve for estimating correlation coefficient (R^2) and amplification efficiency ($E = (10^{[-1/slope]} - 1) \times 100\%$) (Yang et al. 2014). qRT-PCR assays were conducted with Line Gene 9600 Plus (Bier Technology, Hangzhou, China) and TB Green®Premix Ex *Taq*^{TM} (2×) (Tli RNaseH Plus) (RR820A, Takara Biotech, Nanning, China). The PCR reaction (20 µL) comprised of 2 × TB Green Premix Ex *Taq* (10 µL), 10 × dilution cDNA (1 µL), 10 µmol/L forward/reverse primers (0.8 µL), 50 × Rox Reference Dye (0.4 µL), and ddH_2O (7 µL). The following amplification conditions were used: 95 °C for 20 s; 40 cycles of 95 °C for 10 s and 58 °C for 30 s. The melting curve was determined at a range of 60–95 °C. All assays were repeated 3 times. Negative controls using total RNA or ddH_2O instead of cDNA for all samples.

Data analysis. Table S1 in Electronic Supplementary Material (ESM) shows the raw cycle threshold (Ct) data. The expression stability of CRGs was analysed by 4 widely used software programs: geNorm (Vandesompele et al. 2002), NormFinder (Andersen

Table 2. Primer sequences and related information of the 10 candidate reference genes (CRGs) and 2 target genes (TGs) for quantitative real-time polymerase chain reaction (qRT-PCR) analysis in *Paulownia fortunei*

Gene symbol	Primer sequence (5'–3')	Amplicon size (bp)	E (%)	R^2
TUA1	TAGGTGGCGGTGATGATGC TGAGTTGTTCTGGGTGGAATAGC	152	103.96	0.998
TUB	CAGTCAGGTGCGGGGAATAA GAACCTGTGCCTCCTCCAAGT	154	99.35	0.995
TUB1	TGTTGTGAGGAAAGAGGCGGA TGACACCTTGGGGGATGGG	174	99.60	0.997
TUB5	TTCTCTGTGTTCCCTTCGCCT TAACCCCACTCATAGTTGCCG	210	92.87	0.989
ACT1	ACATTGTCCTCAGTGGTGGTTCA TCTGTTGGAAGGTGCTTAGGGAT	177	105.11	0.995
ACT97	GCAAATCGTGAAAAAATGACT AG- ATGGGGACTGTATGGCTGA	157	99.17	0.998
DNAJ	AGAGGGTAGTGAGAGGGACGAA ACGGAACACTGGGTCTTCTCTG	163	106.73	0.996
APT	TCCTCCTCCATTCGGGTCATA TAAAACCTCTTGCCTCAACACC	171	96.23	0.996
HIS4	AGAAAAATGTCAGGGCGGG TCCAGGAAAATCTTCAGCACG	196	109.1	0.998
18S rRNA	ACCATAAACGATGCCGACC GCCTTGCGACCATACTCCC	108	99.81	0.999
GR	GAAGGACTTTGCTTGATACGCC AAGAGAGGAAAGAAACGGGAA	171	98.29	0.991
PfCesA2	TTATTGGAGTCGTAGTTGGGGT GTGGGAAGTCGGTCCTGTTT	154	100.69	0.997

E – amplification efficiency; R^2 – correlation coefficient

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et al. 2004), BestKeeper (Pfaffl et al. 2004), and RefFinder (Chen et al. 2011). For the NormFinder and geNorm algorithm analysis, the raw Ct values were converted into relative quantification data.

Using GeNorm we calculated the expression stability value (M), determined the pairwise variation (V) for each gene, and identified the stable genes with a low M-value. Pairwise variation (V_n/V_{n+1}; cutoff value of 0.15) was used to determine the optimal number of reference genes for normalization (Petriccione et al. 2015; Li et al. 2016).

Using NormFinder we analysed the expression stability value (SV) based on the inter- and intra-groups for each reference gene (Andersen et al. 2004). The high expression stability of a gene was indicated by a low SV.

Using BestKeeper and Ct data we evaluated the stability of each gene according to the coefficient of variation (*CV*), Pearson's correlation coefficient (*r*), and standard deviation (SD). The genes with low SD and *CV* values were considered stable. The range of SD variation needed to be lower than 1 (Petriccione et al. 2015; Li et al. 2016).

Using RefFinder we generated a comprehensive ranking of CRGs under different experimental conditions (Chen et al. 2011).

To verify the robustness of selected candidate genes, the optimal internal reference genes and stable/unstable CRGs (2 each) were used for expression normalization of *PfCesA2* and *GR* under different experimental conditions. The relative expression level of each gene was calculated by $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001).

RESULTS

Selection and cloning of reference and TGs. Ten CRGs (*TUA1*, *TUB*, *TUB1*, *TUB5*, *ACT1*, *ACT97*, *DNAJ*, *APT*, *HIS4*, and *18S rRNA*) and 1 TG (*GR*) were cloned from *P. fortunei*. The sequencing results showed that the amplicon lengths of *TUA1*, *TUB*, *TUB1*, *TUB5*, *ACT1*, *ACT97*, *DNAJ*, *APT*, *HIS4*, *18S rRNA*, and *GR* genes were 1 464, 1 419, 1 583, 1 573, 1 256, 1 502, 1 631, 979, 485, 1 538, and 2 204 bp, respectively. Their GenBank accession numbers were MZ821053, MZ821054, MZ821055, MZ821057, MZ821058, MZ821059, MZ821060, MZ821062, MZ821063, MZ823806, and MZ848185, respectively.

Design, amplification specificity, and efficiency of qRT-PCR primers. The primer sequences of 10 CRGs and 2 TGs, with amplicon sizes of 108 to 210 bp, were designed for qRT-PCR. Based on the results of melting curve analysis (Figure S1 in ESM) and 2% agarose gel electrophoresis (Table 2, Figure 1), the primers were specific. And their amplification efficiency ranged from 92.87% to 109.1%, with R^2 ranging from 0.991 to 0.999 (Table 2, Figure 2).

Preliminary expression analysis of the reference genes. The expression level of each gene was quantified by Ct values. Lower Ct values indicate higher expression levels. The raw Ct values of all specimens are shown in Table S1 in ESM. The Ct values of 10 CRGs across all specimens widely distributed from 8.82 to 29.98. Distinctively, the Ct values of *18S rRNA* gene ($8.82 \leq Ct \leq 12.06$) were very low, indicating the expression level of *18S rRNA* gene was extremely abundant compared with that of other 9 CRGs. Therefore, the *18S rRNA* gene was excluded from CRGs in this study.

The distribution of raw Ct values is presented in a box and whiskers plot (Figure 3). Excluding *18S rRNA*, the results displayed that there were five genes (*TUA*, *TUB5*, *ACT1*, *ACT97*, and *APT*) with mean Ct values of 22–25 cycles which indicated high expression levels and the others had Ct values of 25–27 which suggested moderate expression levels. Among these genes, the expression of *TUA* gene was highest (mean Ct of 22.39), while that of *TUB* was lowest (mean Ct of 26.99). All CRGs had different *CV* values (less variability is represented by low *CV* value), as displayed in Figure 3. *DNAJ* exhibited the least variation, while *TUB5* exhibited the greatest

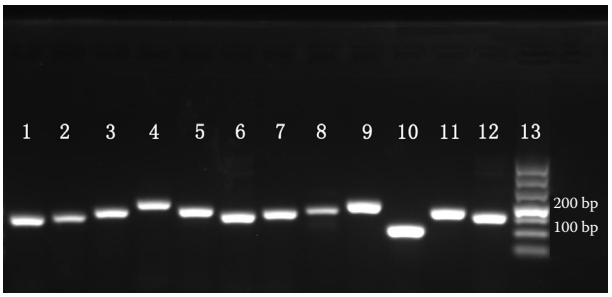

Figure 1. Amplified fragments of 10 candidate reference genes (CRGs) and 2 target genes (TGs) using quantitative real-time polymerase chain reaction (qRT-PCR) specific primers were separated by 2% agarose gel electrophoresis
Lanes: 1 – *TUA1* (152 bp); 2 – *TUB* (154 bp); 3 – *TUB1* (174 bp); 4 – *TUB5* (210 bp); 5 – *ACT1* (177 bp); 6 – *ACT97* (157 bp); 7 – *DNAJ* (163 bp); 8 – *APT* (171 bp); 9 – *HIS4* (196 bp); 10 – *18S rRNA* (108 bp); 11 – *GR* (171 bp); 12 – *PfCesA2* (154 bp); 13 – DL500 DNA Marker (3590Q, Takara Biotech, Nanning, China)

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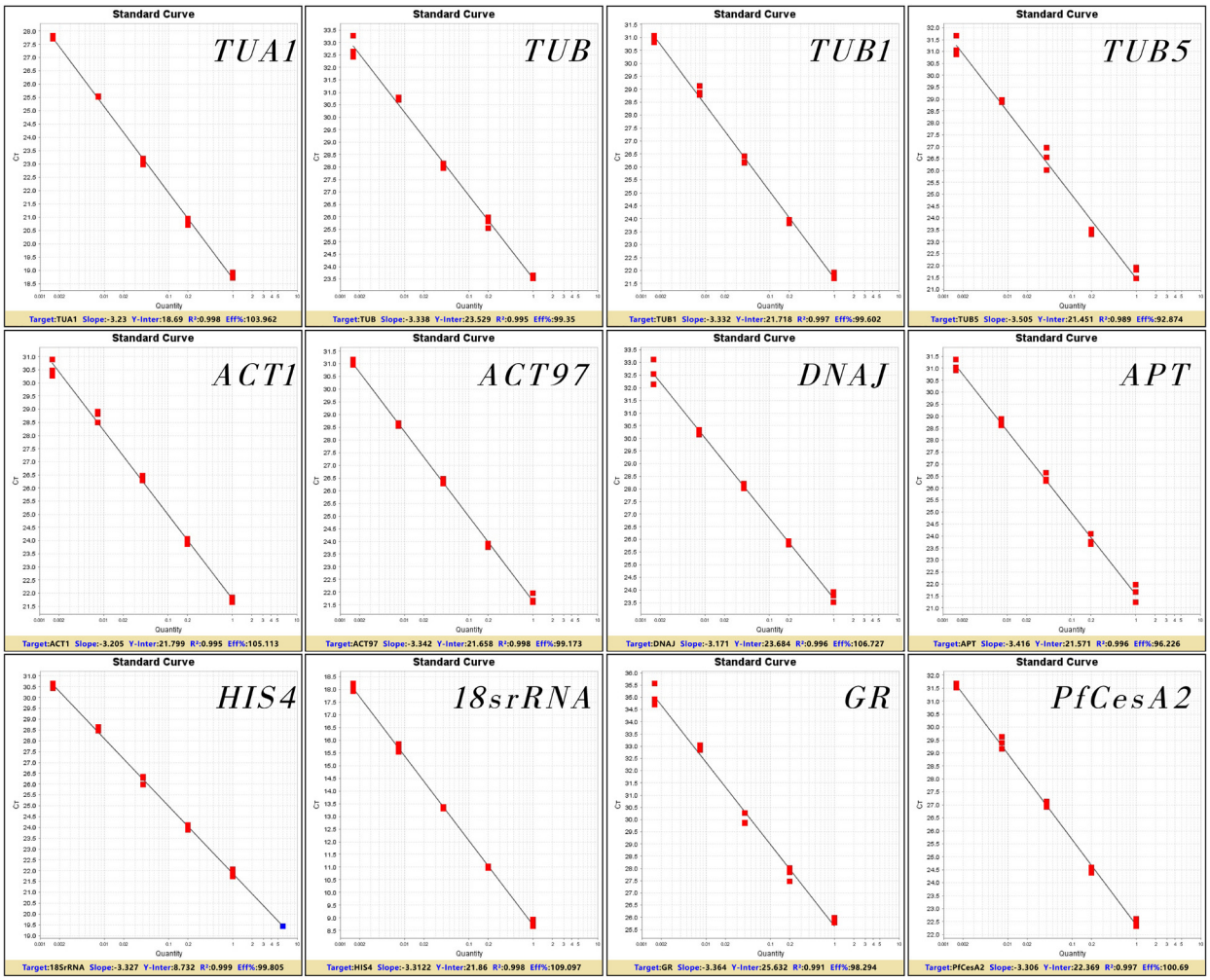

Figure 2. Standard curves of the 10 candidate reference genes (CRGs) and 2 TGs in *Paulownia fortune*; the amplification efficiency (E = $(10^{[-1/\text{slope}]} - 1) \times 100\%$) and determination coefficient (R^2) were calculated from the standard curve

variation, indicating that *DNAJ* and *TUB5* were the most stable and unstable genes, respectively.

Expression stability analysis. In this study, 5 experimental conditions which were different tissues, drought, salinity, Cd and Cr treatments were set, and then sorted into the "total" group. To select appropriate reference genes for different experimental conditions, the stability of the 9 CRGs was evaluated

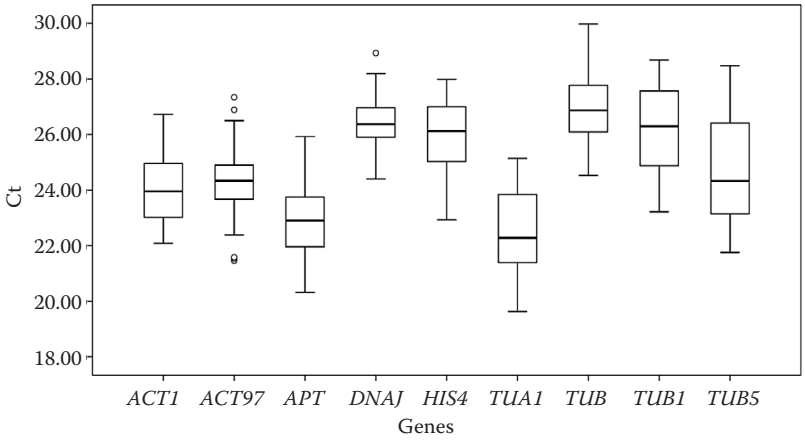

Figure 3. Cycle threshold (Ct) values distribution of *TUA1*, *TUB*, *TUB1*, *TUB5*, *ACT1*, *ACT97*, *DNAJ*, *APT*, and *HIS4* genes across all experimental samples
The outside boxes are determined from 25th to 75th percentiles and the lines across the boxes are the median; the whiskers represent the 5th to 95th percentiles, and the circular symbols represent the outliers

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Table 3. The stability analysis results of candidate reference genes (CRGs) by geNorm, NormFinder, BestKeeper and RefFinder

Treatment	Rank	geNorm		NormFinder		BestKeeper			RefFinder	
		gene	stability	gene	stability	gene	SD (± Ct)	*CV* (% Ct)	gene	stability
Total	1	*ACT97*	0.82	*ACT97*	0.26	*DNAJ*	0.63	2.37	*ACT97*	1.19
	2	*TUB*	0.88	*TUB1*	0.35	*ACT97*	0.88	3.62	*TUB*	2.71
	3	*TUB1*	0.88	*TUB*	0.39	*APT*	0.95	4.18	*TUB1*	3.36
	4	*ACT1*	0.93	*TUA1*	0.42	*ACT1*	0.98	4.09	*DNAJ*	4.14
	5	*TUA1*	0.94	*APT*	0.44	*HIS4*	1.02	3.92	*TUA1*	4.28
	6	*APT*	0.94	*ACT1*	0.45	*TUB*	1.03	3.82	*ACT1*	4.95
	7	*DNAJ*	0.98	*DNAJ*	0.48	*TUA1*	1.20	5.35	*APT*	5.01
	8	*HIS4*	1.05	*HIS4*	0.58	*TUB1*	1.24	4.73	*HIS4*	7.11
	9	*TUB5*	1.33	*TUB5*	0.85	*TUB5*	1.64	6.63	*TUB5*	9.00
Differnet tissues	1	*TUA1*	0.48	*TUA1*	0.06	*TUA1*	0.11	0.55	*TUA1*	1.19
	2	*TUB5*	0.48	*TUB5*	0.06	*TUB5*	0.12	0.52	*TUB5*	1.41
	3	*DNAJ*	0.53	*DNAJ*	0.20	*DNAJ*	0.15	0.57	*DNAJ*	3.00
	4	*ACT1*	0.61	*ACT1*	0.26	*TUB1*	0.21	0.89	*TUB1*	4.47
	5	*TUB1*	0.63	*TUB1*	0.31	*ACT1*	0.36	1.56	*ACT1*	4.68
	6	*TUB*	0.73	*TUB*	0.41	*ACT97*	0.43	1.93	*ACT97*	6.62
	7	*ACT97*	0.77	*HIS4*	0.44	*HIS4*	0.44	1.83	*TUB*	6.70
	8	*HIS4*	0.78	*ACT97*	0.46	*TUB*	0.49	1.95	*HIS4*	7.24
	9	*APT*	0.89	*APT*	0.56	*APT*	0.53	2.54	*APT*	9.00
Drought	1	*ACT97*	0.54	*ACT97*	0.14	*ACT97*	0.47	1.90	*ACT97*	1.00
	2	*TUB1*	0.55	*TUB5*	0.19	*TUB1*	0.48	1.73	*DNAJ*	2.45
	3	*TUB5*	0.55	*DNAJ*	0.20	*TUB5*	0.49	1.77	*TUB5*	2.63
	4	*DNAJ*	0.57	*TUB1*	0.21	*DNAJ*	0.50	1.83	*TUB1*	3.56
	5	*TUB*	0.61	*TUB*	0.30	*HIS4*	0.51	1.94	*TUB*	4.61
	6	*HIS4*	0.64	*HIS4*	0.31	*TUB*	0.61	2.21	*HIS4*	5.96
	7	*ACT1*	0.75	*ACT1*	0.40	*APT*	0.70	2.98	*TUA1*	7.44
	8	*TUA1*	0.81	*TUA1*	0.51	*TUA1*	0.75	3.17	*ACT1*	7.71
	9	*APT*	0.88	*APT*	0.57	*ACT1*	0.79	3.24	*APT*	8.45
Salinity	1	*ACT97*	0.45	*ACT97*	0.09	*HIS4*	0.25	1.01	*ACT97*	1.32
	2	*APT*	0.52	*TUB5*	0.17	*APT*	0.35	1.58	*TUB5*	2.34
	3	*TUB5*	0.52	*APT*	0.18	*ACT97*	0.36	1.50	*APT*	2.45
	4	*ACT1*	0.53	*ACT1*	0.20	*DNAJ*	0.51	1.96	*HIS4*	3.83
	5	*DNAJ*	0.63	*DNAJ*	0.33	*TUB5*	0.51	2.20	*ACT1*	4.43
	6	*HIS4*	0.66	*HIS4*	0.36	*ACT1*	0.53	2.25	*DNAJ*	4.73
	7	*TUA1*	0.73	*TUA1*	0.42	*TUA1*	0.57	2.60	*TUA1*	7.24
	8	*TUB*	0.74	*TUB*	0.43	*TUB1*	0.58	2.29	*TUB*	7.97
	9	*TUB1*	0.78	*TUB1*	0.47	*TUB*	0.60	2.28	*TUB1*	8.74
Cd	1	*TUB1*	0.58	*ACT97*	0.03	*APT*	0.37	1.57	*TUB1*	1.68
	2	*ACT97*	0.59	*TUB1*	0.09	*HIS4*	0.42	1.55	*ACT97*	1.73
	3	*DNAJ*	0.59	*DNAJ*	0.09	*TUB1*	0.53	1.98	*DNAJ*	3.08
	4	*TUB5*	0.74	*TUB5*	0.33	*DNAJ*	0.53	2.00	*TUB5*	4.60
	5	*ACT1*	0.77	*ACT1*	0.37	*ACT97*	0.53	2.13	*APT*	4.76
	6	*TUA1*	0.89	*TUA1*	0.47	*ACT1*	0.54	2.20	*ACT1*	5.48

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Table 3 to be continued

Treatment	Rank	geNorm		NormFinder		BestKeeper			RefFinder	
		gene	stability	gene	stability	gene	SD (± Ct)	*CV* (% Ct)	gene	stability
	7	*TUB*	0.89	*TUB*	0.55	*TUB5*	0.70	2.80	*TUA1*	6.16
Cd	8	*APT*	0.94	*APT*	0.56	*TUA1*	0.80	3.51	*HIS4*	6.18
	9	*HIS4*	1.06	*HIS4*	0.66	*TUB*	1.00	3.65	*TUB*	7.45
	1	*DNAJ*	0.61	*DNAJ*	0.09	*HIS4*	0.29	1.07	*DNAJ*	1.68
	2	*TUB1*	0.69	*TUB5*	0.28	*DNAJ*	0.69	2.60	*TUB1*	2.34
	3	*TUB5*	0.70	*TUB1*	0.29	*APT*	0.76	3.20	*ACT97*	3.31
	4	*TUB*	0.71	*TUB*	0.32	*TUB5*	0.94	3.83	*TUB5*	3.31
Cr	5	*ACT1*	0.74	*ACT97*	0.33	*TUB1*	0.95	3.55	*TUB*	4.68
	6	*ACT97*	0.77	*ACT1*	0.34	*ACT97*	1.05	4.19	*HIS4*	5.20
	7	*APT*	0.86	*APT*	0.45	*ACT1*	1.07	4.30	*APT*	5.86
	8	*TUA1*	0.90	*TUA1*	0.49	*TUB*	1.16	4.22	*ACT1*	6.24
	9	*HIS4*	0.94	*HIS4*	0.59	*TUA1*	1.32	5.77	*TUA1*	7.97

Ct – cycle threshold; SD – standard deviation; *CV* – coefficient of variation

with 4 software (RefFinder, BestKeeper, NormFinder and geNorm).

geNorm analysis. The results of geNorm analysis indicated that the most stable reference genes were differential under different experimental conditions (Table 3). For "total" group, *ACT97* and *TUB5* were the most and least stable genes with M-values of 0.817 and 1.325, respectively. For different tissues, *TUA1* and *APT* were the most and least stable genes with M-values of 0.483 and 0.890, respectively. Under drought treatment, *ACT97* and *APT* were the most and least stable genes with M-values of 0.535 and 0.878, respectively. Under salinity treatment, *ACT97* and *TUB1* were the most and least stable genes with M-values of 0.452 and 0.776, respectively. Under Cd treatment, *TUB1* and *HIS4* were the most and least stable genes with M-values of 0.576 and 1.062, respectively. Under Cr treatment, *DNAJ* and *HIS4* were the most and least stable genes with M-values of 0.606 and 0.943, respectively.

The optimal number of reference genes for expression normalization is dependent on pairwise variation (V_n/V_{n+1}). When $V_n/V_{n+1} < 0.15$, no additional reference genes are required for normalization. For different tissues, drought, salinity and Cd treatment, only 2 reference genes were needed for accurate normalization (Figure 4). The most stable reference gene pairs for 4 treatments were *TUA1* and *TUB5*, *ACT97* and *TUB1*, *ACT97* and *APT*, *TUB1* and *ACT97*, respectively (Table 3, Figure S2 in ESM). For Cr treatment, $V_2/V_3 > 0.15$ and $V_3/V_4 < 0.15$. Therefore, the genes *DNAJ*, *TUB1*, and *TUB5* were chosen. For "total" group, $V_4/V_5 > 0.15$, and $V_5/V_6 < 0.15$. Therefore, the genes *ACT97*, *TUB*, *TUB1*, *ACT1*, and *TUA* were chosen.

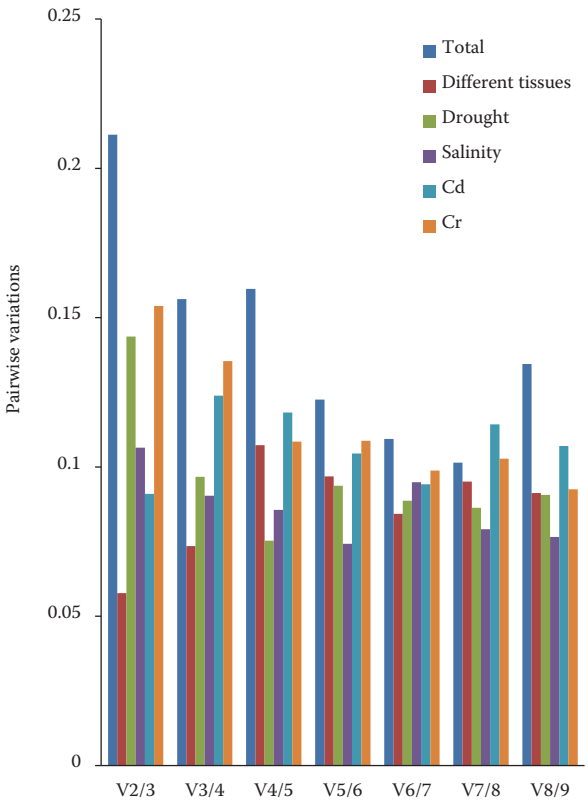

Figure 4. Pairwise variation (V_n/V_{n+1}) was analysed between the normalization factors (NF_n and NF_{n+1}) by geNorm to determine the optimal number of reference genes

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NormFinder analysis. The stability value of each CRG under different experimental conditions was also analysed by NormFinder, in which a high expression stability is represented by a lower stability value. The analysis results of NormFinder were consistent with that of geNorm (Table 3). For "total" group, drought, salinity, and Cd treatment, *ACT97* was the most stable CRG. For different tissues, *TUA* was the most stable CRG. For Cr treatment, *DNAJ* was the most stable CRG.

BestKeeper analysis. The data obtained from BestKeeper analysis are presented in Table 3. All CRGs were stably expressed under 3 experimental conditions (different tissues, drought, and salinity treatment). The most stable CRGs were *TUA* and *TUB5* (*CV* ± SD = 0.55 ± 0.11 and 0.52 ± 0.12, respectively) in different tissues. The most stable CRGs were *ACT97* and *TUB1* (*CV* ± SD = 1.90 ± 0.47 and 1.73 ± 0.48, respectively) under drought treatment. The most stable CRGs were *HIS4* and *APT* (*CV* ± SD = 1.01 ± 0.25 and 1.58 ± 0.35, respectively) under salinity treatment. For Cd treatment, only *TUB* gene was expressed unstably, and the most stable CRGs were *APT* and *HIS4* (*CV* ± SD = 1.57 ± 0.37 and 1.55 ± 0.42, respectively). For Cr treatment, 5 CRGs were stably expressed and the most stable CRGs were *HIS4* and *DNAJ* (*CV* ± SD = 1.07 ± 0.29 and 2.6 ± 0.69, respectively). For "total" group, only 4 CRGs were stably expressed, and the most stable CRGs were *DNAJ* and *ACT97* (*CV* ± SD = 2.37 ± 0.63 and 3.62 ± 0.88, respectively).

RefFinder analysis. Through RefFinder analysis, rankings of CRGs for accurate normalization were obtained (Table 3). As shown in the Table 3, *TUA* and *TUB5* were the most stable CRGs in different tissues, *ACT97* and *DNAJ* were the most stable CRGs under drought treatment, *ACT97* and *TUB5* were the most stable CRGs under salinity treatment, *TUB1* and *ACT97* were the most stable CRGs under Cd treatment, *DNAJ* and *TUB1* were the most stable genes for Cr treatment, and *ACT97* and *TUB* were the most stable CRGs in the "total" group.

Verification of CRGs. To verify the accuracy of selected CRGs, the expression levels of *PfCesA2* in different tissues were analysed with the most stable/unstable CRGs and optimal CRG combination. Meanwhile, the expression levels of *GR* were analysed under different experimental conditions (drought, salinity, Cd and Cr treatment) with the most stable/unstable CRGs and optimal CRG combination.

In different tissues, the expression patterns of *PfCesA2* were similar when *TUA1*, *TUB5* and *TUA1/TUB5* were used for normalization respectively. *PfCesA2* had higher expression levels in "root" and "bark", lower expression levels in "xylem" and "leaf" (Figure 5E). When the least stable reference genes *HIS4* and *APT* were used for normalization, the expression patterns of *PfCesA2* were very different (Figure 5E). When the optimal reference gene combination *TUA1/TUB5* was used for normalization, the expression levels of *PfCesA2* were significantly down-regulated in "xylem" and "leaf" (Figure 5E).

Under drought treatment, when the most unstable reference genes *ACT1* and *APT* were employed for normalization, the expression levels of *GR* had great biases compared to that when the optimal reference genes (*ACT97*, *DNAJ*) and combination (*ACT97/TUB1*) were employed (Figure 5A). When the optimal reference gene combination *ACT97/TUB1* was used for normalization, the expression levels of *GR* had no significant difference at 0, 0.5, 3, 6 and 24 h, and was significantly up-regulated at 12 h (2.29-fold change) (Figure 5A).

Under salinity treatment, when the most unstable reference genes *TUB* and *TUB1* were employed for normalization, the expression levels of *GR* had great biases compared to that when the optimal reference genes (*ACT97*, *TUB5*) and combination (*ACT97/APT*) were employed (Figure 5B). When the optimal reference gene combination *ACT97/APT* was used for normalization, the expression levels of *GR* were significantly up-regulated at all time points, especially at 6 h (2.0-fold change) (Figure 5B).

Under Cd treatment, when *TUB1*, *ACT97* and *TUB1/ACT97* were used for normalization respectively, the expression patterns of *GR* were similar. The expression levels of *GR* were down-regulated at all time points (Figure 5C). When the least stable reference genes *HIS4* and *TUB* were used for normalization, the expression patterns of *GR* were very different (Figure 5C). When the optimal reference gene combination *TUB1/ACT97* was used for normalization, the expression levels of *GR* were down-regulated at all time points, significantly at 0.5 and 24 h (Figure 5C).

Under Cr treatment, when the most unstable reference genes *ACT1* and *TUA1* were employed for normalization, the expression levels of *GR* had great biases compared to that when the optimal reference genes (*DNAJ*, *TUB1*) and combination (*DNAJ/TUB1/TUB5*) were employed (Figure 5D). When the optimal reference gene combination *DNAJ/TUB1/TUB5* was used for normalization, the expression levels of *GR*

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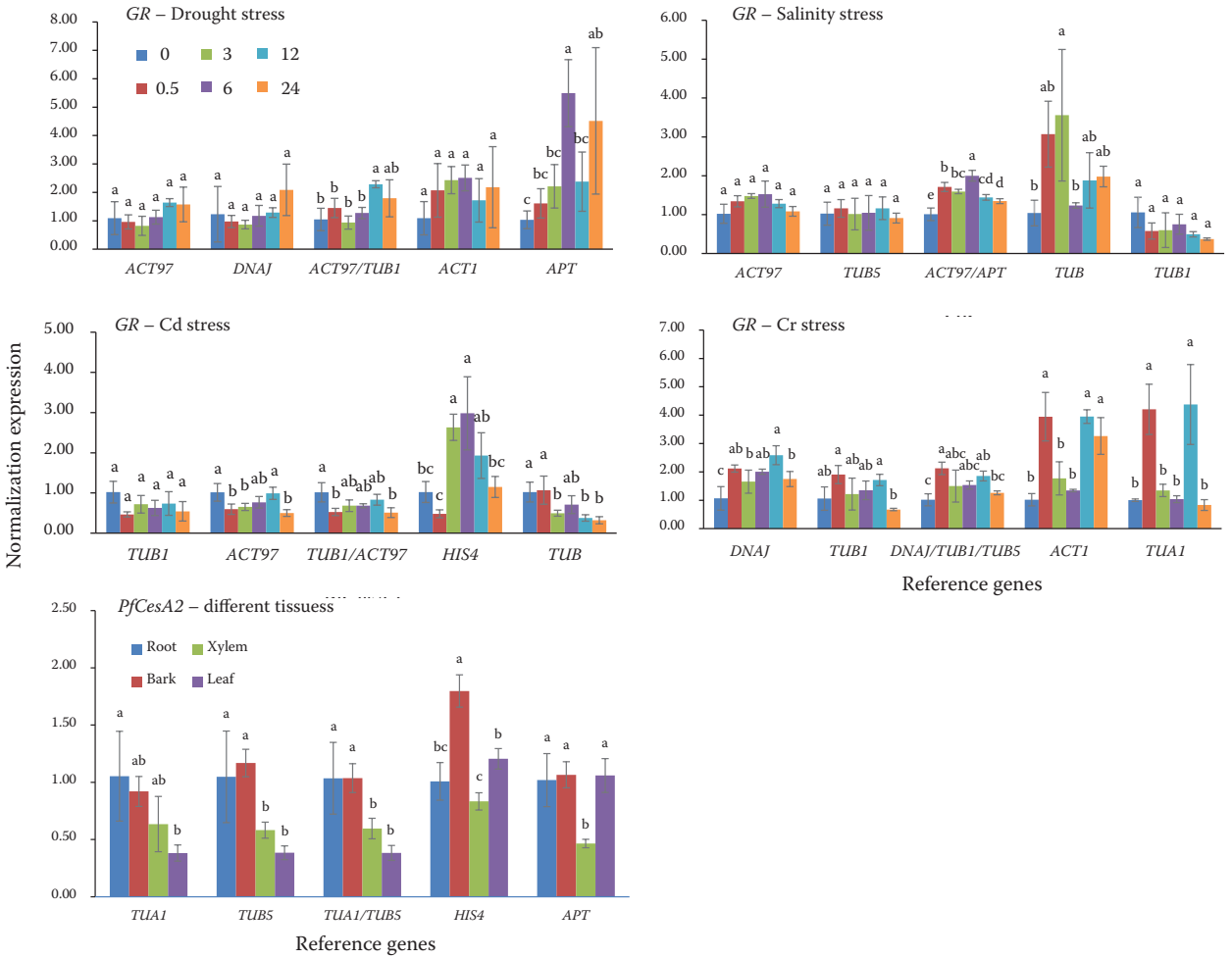

Figure 5. Effect of different reference genes for normalization on the relative expression of the *PfCesA2* and *GR* genes: the relative expression of *GR* was analysed using stable (*ACT97*, *DNAJ*, *ACT97/TUB1*) and unstable (*ACT1*, *APT*) reference genes as internal controls under drought stress after 0, 0.5, 3, 6, 12, and 24 h of treatment (A), the relative expression of *GR* was analysed using stable (*ACT97*, *TUB5*, *ACT97/APT*) and unstable (*TUB*, *TUB1*) reference genes as internal controls under salinity stress after 0, 0.5, 3, 6, 12, and 24 h of treatment (B), the relative expression of *GR* was analysed using stable (*TUB1*, *ACT97*, *TUB1/ACT97*) and unstable (*HIS4*, *TUB*) reference genes as internal controls under Cd stress after 0, 0.5, 3, 6, 12, and 24 h of treatment (C), the relative expression of *GR* was analysed using stable (*DNAJ*, *TUB1*, *DNAJ/TUB1/TUB5*) and unstable (*ACT1*, *TUA1*) reference genes as internal controls under Cr stress after 0, 0.5, 3, 6, 12, and 24 h of treatment (D), the relative expression of *PfCesA2* was analysed using stable (*TUA1*, *TUB5*, *TUB1/TUB5*) and unstable (*HIS4*, *APT*) reference genes as internal controls for different tissues after root, bark, xylem, and leaf (E) Different colours represent different levels of treatment; the height of the columns represents the relative expression level; the whiskers represent standard deviation; different letters indicate significant differences in the expression of the target gene based on three biological replications ($P < 0.05$)

were up-regulated at all time points, significantly at 0.5 and 12 h (Figure 5D).

DISCUSSION

In the present study, the expression stability of 10 commonly used CRGs (*TUA1*, *TUB*, *TUB1*, *TUB5*, *ACT1*, *ACT97*, *DNAJ*, *APT*, *HIS4*, and *18S rRNA*) was analysed under different experimental conditions. To our knowledge, this is the first study on CRGs in *P. fortunei* seedlings under different tissues and abiotic stress conditions.

Distinctively, the Ct values of *18S rRNA* gene ($8.82 \leq Ct \leq 12.06$) were lower than that of the other 9 CRGs,

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indicating its expression was extremely abundant compared with that of the other 9 genes. Genes showing high-level variation are not suitable as CRGs (Wei et al. 2012; Chen et al. 2017b). Therefore, the *18S rRNA* gene was not considered as a CRG in this study. Except for the *18S rRNA* gene, the other nine genes obtained an ideal expression abundance ($19.63 \leq Ct \leq 29.98$), which enabled further analysis of their expression stability (Hu et al. 2009).

Four statistical programs (RefFinder, BestKeeper, NormFinder, and geNorm) were employed to identify appropriate CRGs. For all treatments, the CRGs' stability ranking data obtained from RefFinder, NormFinder, and geNorm were similar. However, in some treatments, the stability ranking results from BestKeeper were different from that from geNorm, NormFinder, and RefFinder. For example, under Cd treatment, *TUB1*, *ACT97*, and *DNAJ* were identified as the most stable CRGs by RefFinder, NormFinder, and geNorm, while *APT*, *HIS4*, and *TUB1* were regarded as the most stable CRGs by BestKeeper. Therefore, using multiple statistical algorithms to analyse the expression stability of CRGs was beneficial to eliminate error and avoid one-sided analysis that may occur via only one algorithm. Previous research also indicated that BestKeeper might generate the most divergent ranking of expression stability compared to other statistical programs (Zhu et al. 2019).

Using a single reference gene for normalization might lead to a deviation in the results (Vandesompele et al. 2002; Zhu et al. 2008). Adopting two or more internal control genes as reference could reduce the experimental error (Schmid et al. 2003). In the present study, the optimal number of CRGs for expression normalization under different tissues and abiotic stresses was identified with the geNorm algorithm. The data indicated that under different tissues, salinity, drought and Cd treatments, the pairwise variation was $V_2/V_3 < 0.15$, indicating that 2 CRGs were appropriate for normalization. However, for Cr treatment, $V_2/V_3 > 0.15$, and $V_3/V_4 < 0.15$, suggesting that 3 CRGs were needed. Furthermore, the results of "total" group ($V_4/V_5 > 0.15$, and $V_5/V_6 < 0.15$) demonstrated that the CRGs or gene combinations which could meet all. the test conditions were not easy to identify. Many results have also confirmed that optimal reference genes were inconsistent under different experimental conditions (Wei et al. 2012; Fei et al. 2018; Balestrini et al. 2022). Our results also suggested that *TUA1* was the most stable reference gene for different tissues, but not in drought and Cr treatment. Although *ACT97* and *TUB1* was a suitable reference gene combination for drought and Cd treatment, but not for other treatments. Therefore, it is necessary to screen optimal reference genes for different experimental conditions.

TUA, *TUB*, *ACT*, *DNAJ*, *APT*, and *HIS4* genes are widely used reference genes for qPCR (Wei et al. 2012; Jain et al. 2015; Zhuang et al. 2015; Chen et al. 2017b; Fei et al. 2018; Wang et al. 2020). But they also have their scope of application in species (Hu et al. 2009; Balestrini et al. 2022). For example, *TUA* has been demonstrated as a suitable reference gene for different tissues in *Ziziphus jujuba* Mill. and cucumber (Wan et al. 2010; Pang et al. 2016). However, it was not suitable for different tissues in *Passiflora edulis* and *Zanthoxylum bungeanum* Maxim (Fei et al. 2018; Wu et al. 2020). In the present study, the final ranking showed *TUA1* was the most stable gene for different tissues in *Paulownia fortunei* (Seem.) Hemsl. Therefore, it is necessary to screen suitable reference genes for different plant species under certain conditions.

For different tissues, the appropriateness of the selected CRGs was evaluated by detecting the expression level of *PfCesA2* gene. The *CesA* gene belongs to membrane-bound glycosyltransferase family II (GT-2), it plays a key role in the cellulose biosynthesis pathway (Desprez et al. 2007; Mutwil et al. 2008; Maleki et al. 2020). For other abiotic stress treatments, the appropriateness of the selected CRGs was evaluated by determining the expression level of *GR* gene, which has been confirmed to have a remarkable response to abiotic stresses (Romero-Puertas et al. 2006; Gill et al. 2013; Lin et al. 2018; Hasanuzzaman et al. 2019; Raja et al. 2021). In our study, the expression level of *PfCesA2* differed greatly under different tissues and the expression level of *GR* differed greatly under different abiotic stresses.

Additionally, the most stable/unstable CRGs and optimal CRG combination were employed to normalize the expression levels of *PfCesA2* and *GR*, the data were greatly different. When the stable CRGs and optimal CRG combination were employed for normalization, the expression levels of *PfCesA2* and *GR* were consistent. However, while the most unstable CRGs were employed, the expression levels of *PfCesA2* and *GR* had remarkable biases. *GR* can respond to more abiotic stresses, and it expression is firstly up-regulated in most cases (Romero-Puertas et al. 2006; Sun et al. 2011). In this study, the expression level of *GR* was analyzed by optimal reference gene combinations. The findings revealed that the

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expression levels of *GR* were remarkably up-regulated in some term under drought, salinity, and Cr treatments. However, for Cd treatment, the expression levels of *GR* were markedly down-regulated. This might be because Cd concentration in this study was at highly toxic level for *P. fortunei* seedlings, which led to the decrease of *GR* expression (Ekmekçi et al. 2008). These results indicate that the CRGs screened in our study were robust.

The stable CRGs and combination selected in the study have important implications for analysis of the expression profiles of target genes in *P. fortunei.*

CONCLUSION

Through stability and pairwise variation analysis, *TUA1* and *TUB5*, *ACT97* and *TUB1*, *ACT97* and *APT*, *TUB1* and *ACT97* were recommended as qPCR reference gene combinations for different tissues, drought treatment, salinity treatment, and Cd treatment respectively. *DNAJ*, *TUB1*, and *TUB5* were selected for Cr treatment. The findings will lay a foundation for the qPCR expression analysis of key genes from *P. fortunei* seedlings.

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