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Original Research

Assessment of Appropriate Reference Genes for Quantitative Real-Time Polymerase Chain Reaction Normalisation in *Magnolia sieboldii* K. Koch across Various Experimental Conditions

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Abstract

Magnolia sieboldii K. Koch is a famous ornamental plant. The deep dormancy of the seeds makes breeding difficult and prevents cultivation for economic purposes. Gene expression profiles of germination-related genes can provide clues for understanding the molecular mechanism of dormancy breaking. The accuracy of results acquired through quantitative real-time polymerase chain reaction (RT-qPCR), which was developed for investigating the gene expression profiles, is determined by the suitability of the selected reference gene. Nevertheless, appropriate RT-qPCR reference genes have not been determined in *M. sieboldii*. In the present study, seven potential reference genes in diverse *M. sieboldii* sample subgroups of different organ tissues, seed developmental stages, and seed subjected to imbibition and stratification treatments were checked through RT-qPCR. The gene expression stability was assessed and analysed through three statistical algorithms, namely, geNorm, NormFinder, and BestKeeper. *Cyclophilin* (*CYC*) and *ubiquitin-conjugating enzyme* (*UCE*) were identified as the best reference genes for diverse organs. *CYC* and *UCE* were evaluated as suitable reference genes in seeds in diverse periods of development. *Copper/zinc superoxide dismutase* (*Cu/Zn-SOD)* and *manganese superoxide dismutase* (*Mn-SOD*) served as optimal reference genes during seed imbibition treatment, whereas *Cu/Zn-SOD* and *30S ribosomal protein S13* (*RPS13*) were optimal during seed stratification treatment. In *M. sieboldii*, *Cu/Zn-SOD* was recommended for RT-qPCR normalisation, whereas *DBP* was inappropriate for gene expression analysis. The expression pattern of DELAY OF GERMINATION1 (*DOG1*) was analysed for validating reference gene creditability better. The present study provides

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a useful guideline for appropriate reference gene selection for RT-qPCR at diverse experiment conditions for *M. sieboldii*.

Keywords: *Magnolia sieboldii* K. Koch, reference gene, RT-qPCR normalisation, gene expression, *DOG1* gene

Introduction

Magnolia sieboldii K. Koch, a member of the family Magnoliaceae, is a Chinese deciduous tree that has been listed as a national endangered species due to its rarity worldwide. *M. sieboldii* had great economical potential because its flowers are highly ornamental and have medical applications, and the timber of the tree can be utilised for furniture-making. However, the seeds of this species are unable to germinate without the help of cold stratification or gibberellic acid treatment [1, 2], causing breeding difficulty due to weak natural regeneration. The molecular mechanisms underlying seed germination must be thoroughly understood for facilitating and optimising *M. sieboldii* growth.

The gene expression analysis is crucial in revealing the mechanisms of different biological processes at the molecular level [3]. Quantitative real-time polymerase chain reaction (RT-qPCR) is frequently used for detecting and measuring gene expression levels. This method has several advantages such as accuracy, specificity, sensitivity, reproducibility, and high throughput capability [4, 5]. Many factors such as the quantity and quality of mRNA, efficiency of cDNA preparation and PCR, heterogeneiy of organ tissues and developmental stages, sample genotypes, and biotic and abiotic stress can greatly affect the RT-qPCR accuracy [6-8]. Currently, gene expression normalisation based on reference genes can minimise such factors [9, 10]. Nevertheless, using unsuitable reference genes may result in misinterpreted data, eventually resulting in inaccurate, even erroneous results [11, 12]. Therefore, appropriate reference genes are vital for the RT-qPCR normalisation analysis.

An ideal reference gene must exhibit steady expression between all sample tissues and across various experimental conditions. Reference genes such as *actin* (*ACT*), *tubulin* (*TUB*), *ubiquitin* (*UBQ*), *18S ribosomal RNA* (*18S rRNA*), and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) are often selected for the normalisation of target gene expression levels without assessment of the expression stability due to their essential roles in primary metabolism, maintenance of cell structure, and basic cellular processes [13]. These selected reference genes are considered to display the properties of ideal reference genes. Nonetheless, such gene transcript levels have been found to alter at different development periods [14-16], tissues [17, 18], cultivars [19, 20] and treatments [21, 22]. If the reference gene was variant for RT-qPCR normalisation, it will resulted in inaccurate result [11, 23]. Furthermore, the optimal reference gene number should be identified because some gene expression analyses demand two or more reference genes [24, 25, 26]. Therefore, reference genes must be assessed in each experimental system prior to gene expression studies.

The usable algorithms for identifying the appropriate reference genes for subsequent RT-qPCR are geNorm, NormFinder and BestKeeper [24, 27, 28]. Optimal reference gene validations have been reported for several plants such as oil palm [29], *Caragana microphylla* [30], *Jatropha curcas* [17], longan [31], *Nitraria tangutorum* [32], *Petunia hybrida* [33], apple [34], *Carex rigescens* [35], pigeonpea [22], *Lilium* spp. [36], buffalo grass [21], *Sanionia uncinata* [37], rice [38], *Caragana korshinskii* [39], strawberry [16], *Betula platyphylla* [40], switchgrass [41, 42], *Lycoris aurea* [43], zucchini [44], *Codonopsis pilosula* [45], *Oxytropis ochrocephala* Bunge [46] and celery [47] with these programmes. However, to the best of our knowledge, no research has been conducted for the selection of the appropriate reference genes for *M. sieboldii* RT-qPCR analysis.

In the present work, seven potential reference genes, namely *actin* (*ACT*), *cyclophilin* (*CYC*), *putative DNA binding protein* (*DBP*), *copper/zinc superoxide dismutase* (*Cu/Zn-SOD*), *manganese superoxide dismutase* (*Mn-SOD*), *30S ribosomal protein S13* (*RPS13*), and *ubiquitin-conjugating enzyme* (*UCE*) were selected on the basis of stably expressed evidence in previous studies [15, 16, 31] and evaluated through RT-qPCR among diverse organs, seeds in five development periods, seeds under imbibition, and stratification treatments. Reference gene expression stability was calculated by three statistical programmes, namely geNorm, NormFinder, and BestKeeper while obtaining appropriate reference genes to normalise gene expression level. Moreover, the DELAY OF GERMINATION1 (*DOG1*) gene expression in the given experimental backgrounds was subjected to normalisation based on the reference genes with the highest and lowest stability for verifying appropriate reference gene validity. Our results offer valuable reference to choose appropriate reference genes across various experimental conditions in *M. sieboldii*.

Materials and Methods

Plant Materials and Treatments

Three 15-year-old *M. sieboldii* trees were selected from the botanical garden of Shenyang Agricultural

University (41º82'N, 123º56'E) for analysing the potential reference gene expression stability, and each tree was utilised as a biological replicate. The root, stem, leaf, petiole, and flower were collected in the flowering period of *M. sieboldii* tree. The seeds were also harvested at 0, 30, 60, 90, and 120 days after pollination (DAP). Mature *M. sieboldii* seeds (120 DAP) were collected and divided into two parts; one part was utilised for imbibition, and the other part was prepared for stratification. Full seeds were chosen, and seed testas were removed manually.

For imbibition treatment, the seeds were dried for one week at ambient temperature (20ºC-25ºC) and humidity (50%-60%) and were soaked in 1500 μM of GA_3 (Solarbio, Beijing, China) solution in the culture dish at 4ºC under dark. The seeds were harvested at diverse time points (0, 6, 12, 24, and 48 h) after the treatment. For stratification treatment, the seeds were mixed with wet sand harboring a 20% moisture content at the mass ratio of 1:10 and maintained at 0ºC-4ºC under dark in the non-woven bag. The seeds were collected at 0, 15, 30, 45, and 60 days from the onset of treatment. The obtained samples were then subjected to liquid nitrogen freezing at once, followed by storage under −80ºC before subsequent use.

Total RNA Isolation as well as cDNA Synthesis

The RNAprep Pure Plant Kit with gDNA Eraser (Tiangen, Beijing, China) was used for isolating the total RNA in accordance with specific protocols. Then, 1% (w/v) agarose gel electrophoresis (AGE) was used in assessing RNA integrity. The extracted RNA content and purity were evaluated through the NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Afterwards, oligo dT primer was utilised with the PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara, Beijing, China) to reversely transcribe 1 μg total RNA according to instruction manual. Then, nuclease-free water was used to dilute the obtained cDNA fivefold, followed by storage under −80ºC for RT-qPCR.

Candidate Reference Gene Selection, RT-qPCR Primer Design and Verification

On the basis of previous studies, we selected seven candidate *M. sieboldii* reference genes to compare the stability in their expression levels among different samples. Nucleotide sequences of *CYC* (GenBank number MN707546), *DBP* (GenBank number MN707548), *Cu/Zn-SOD* (GenBank number MN707549), *Mn-SOD* (GenBank number MN707550), *RPS13* (GenBank number MN707551), *UCE* (GenBank number MN707552), and *ACT* (GenBank number MN707553) were obtained from our transcriptome dataset [48] and uploaded to National Center for Biotechnology Information (NCBI). The Primer Premier 5.0 software (http://www.premierbiosoft.com/) was used to design RT-qPCR primers (Table 1). The primer design criteria were set as follows: melting temperature (T_m) 55ºC-65ºC; primer length 15-25 nucleotides; guanine-cytosine (GC) concentration 40%-60%; and amplicon length 100-300 bp. Primer specificity was evaluated with AGE, sequencing and melting curve analyses.

Analysis of RT-qPCR and Gene Expression Stability

The RT-qPCR was performed in a 10-μL system on the Monad Selected q225 RT-PCR system (Monad,

Table 1. Selected candidate reference genes, primers, and PCR amplification characteristics.

Gene	Accession number	Forward (F) and reverse (R) primer sequence (5'-3')	Amplicon length (bp)	Product TM (ºC)	R^2	E (%)
ACT	MN707553	F: GCACCACTCAATCCCAAGGC R: TGCGACCACTGGCATAAAGG	121	81.94	0.999	98.977
CYC	MN707546	F: GACCTGGAACGAACGGCTCT R: GACCGCAATCGGCAATCA	177	86.79	0.999	101.914
Mn-SOD	MN707550	F: GTTGGGCTATTGACACTCATTTTG R: GCTCCTTTAGTTACCAGCGGATC	172	82.46	0.999	100.424
Cu/Zn-SOD	MN707549	F: GTGGCAAGGAGGGTGTTTGTG R: CATGGAGCCCAGGTTGAAGG	102	83.62	0.999	100.800
RPS13	MN707551	F: AGTCAGCAAAGGAGGAAACCCT R: CCGCCCTATGCCGTGTATG	187	82.88	0.997	107.074
DBP	MN707548	F: CCCAACGCACTCCGTAGCC R: CCCGTCAGGGACAGTATCTCAAA	206	89.29	0.997	101.341
UCE	MN707552	F: AGGACCCTCCCACATCTTGC R: CGGGGCTCCACTGCTCTTT	243	84.69	0.997	96.068
DOG1	MN707547	F: CTTAGCGGCTCTGGCGAATC R: TGCTGACGGTCCCTCCTTTT	168	86.04	0.998	96.515

Wuhan, China). The reaction system consisted of MonAmp™ SYBR® Green qPCR Mix (5 μL, Monad, Wuhan, China), nuclease-free water (3 μL), 0.5 μL of each primer (10 μM), and cDNA (1 μL). The reaction was conducted under the following three-step cycling conditions: 3 min under 95ºC; 10 s under 95ºC, 10 s under 58ºC and 30 s under 72ºC for 40 cycles in the 96-well plate (Monad, Wuhan, China). Melting curves were examined after 40 cycles under 60ºC-95ºC (increment 0.5ºC/5 s) to verify primer specificity. The no-template control was also used to monitor the potential reagent contamination. Furthermore, the whole samples for each gene across the same experimental condition were run on the same plate to avoid any interplate differences and technical changes. Each reaction was conducted for three biological and three technical replicates. The standard curve obtained from the tenfold serial dilutions of cDNA pools (10^{-0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) was used to assess the amplification efficiency (E) and correlation coefficient (R^2).

The RT-qPCR data obtained from the Monad Selected q225 manager were entered into the Excel datasheet. Gene expression stability across various experimental conditions was ranked by geNorm [24], NormFinder [27], and BestKeeper [28]. These Excel-based tools were run according to their manuals.

Verification of Reference Genes

DOG1 (*DELAY OF GERMINATION1*) plays a vital role during seed dormancy, seed chilling response and plant growth [49, 50]. For the important regulatory effects, *MsDOG1* (GenBank number MN707547) was selected for verifying the identified reference gene creditability. Nucleotide sequence of *MsDOG1* was acquired based on our transcriptome dataset [48] and uploaded to NCBI. Specific primers for *MsDOG1* are shown in Table 1. Primer design, verification and RT-qPCR was conducted similar to the abovementioned methods. The reference genes with the highest stability among diverse organ tissues (*CYC*, *UCE*), seed developmental stages (*CYC*, *UCE*), seed imbibition (*Cu/Zn-SOD*, *Mn-SOD*), and seed stratification (*RPS13*, *Cu/Zn-SOD*) subsets, and those with the lowest stability among diverse organ tissues (*RPS13*, *DBP*), seed developmental stages (*Mn-SOD*, *DBP*), seed imbibition (*RPS13*, *DBP*), and seed stratification (*CYC*, *DBP*) subsets were selected as calibrators to evaluate the *MsDOG1* expression level.

Results

Evaluation of Amplification Specificity and Efficiency

Specific primers were screened for the seven potential reference genes to carry out RT-qPCR, with the length of amplicon varying between 102 and 243 bp (Table 1). Amplicon specificity was typically verified with the presence of a single band that exhibited the designed size on 2% AGE (Fig. 1a). Single-peak melting curves for the seven potential reference genes exhibited the specific amplifications of all primers (Fig. 1b). In the meantime, the no-template control, where cDNA was replaced by nuclease-free water in equal volume, did not exhibit amplicons for each selected reference gene. Additionally, the sequence analysis demonstrated that the amplified sequences were aligned to the sequences used for primer design. The melting temperature of RT-qPCR products spanned from 81.94ºC for *ACT* to 89.29ºC for *DBP* (Table 1). The amplification efficiency was between 96.068% for *UCE* and 107.074% for *RPS13*, and the standard curve R^2 values changed between 0.997 and 0.999 (Table 1).

Expression Profiles for the Potential Reference Genes

Transcript abundances for the seven potential reference genes obtained through RT-qPCR were presented as values of cycle threshold (Ct). All Ct values for these potential reference genes are tabulated in Table 2. Ct values for the potential reference genes varied greatly between 14.16 (*CYC*) and 33.27 (*DBP*) (Fig. 2, Table 3). *CYC* showed the highest expression with the lowest average Ct value (16.50), whereas *DBP* showed the lowest expression with the highest average Ct value (29.79). The transcript abundance of the *DBP* gene exhibited the greatest variation (11.13 Ct, with the maximum and minimum Ct values being 33.27 and 22.14, respectively), whereas that of the *UCE* gene exhibited the smallest variation (3.12 Ct, with the maximum and minimum Ct values being 22.39 and 19.27, respectively).

Examination of Potential Reference Gene Expression Stability

The expression stability of the seven potential reference genes was determined among diverse experiment sets, followed by separate ranking using the three statistical algorithms (geNorm, NormFinder, and BestKeeper) (Table 4).

The reference gene expression stability can be ranked using the geNorm algorithm in accordance with the values of mean expression stability (M) from mean pairwise expression ratio [24]. Typically, the threshold M is <1.5, and potential reference genes having low M values exhibit stable expression [51]. For seeds under imbibition treatment, *Cu/Zn-SOD* and *Mn-SOD* genes exhibited the highest stability, and the M values were both 0.13. *CYC* and *UCE* genes exhibited the highest suitability for diverse organ tissues as well as seeds at different developmental stages, and the M values were 0.51 and 0.26, respectively. For seeds during stratification treatment, the gene pairs with the highest

expression stability were *RPS13* and *Cu/Zn-SOD* with an M value of 0.23. After pooling each sample into the geNorm analysis, *UCE* and *Cu/Zn-SOD* (M = 0.50) were identified as the genes with the highest expression stability, whereas *DBP* (M = 1.48) and *Mn-SOD* (M = 1.00) were identified as genes with the lowest expression stability.

The geNorm algorithm may be further utilised for identifying the best reference gene number for accurate gene expression normalization through computing the pairwise variation (V_n/V_{n+1}). The V_n/V_{n+1} value of <0.15 reveals that the best reference gene number identical to the n value is adequate [24]. In the present study, the V_2/V_3 values for seeds under imbibition

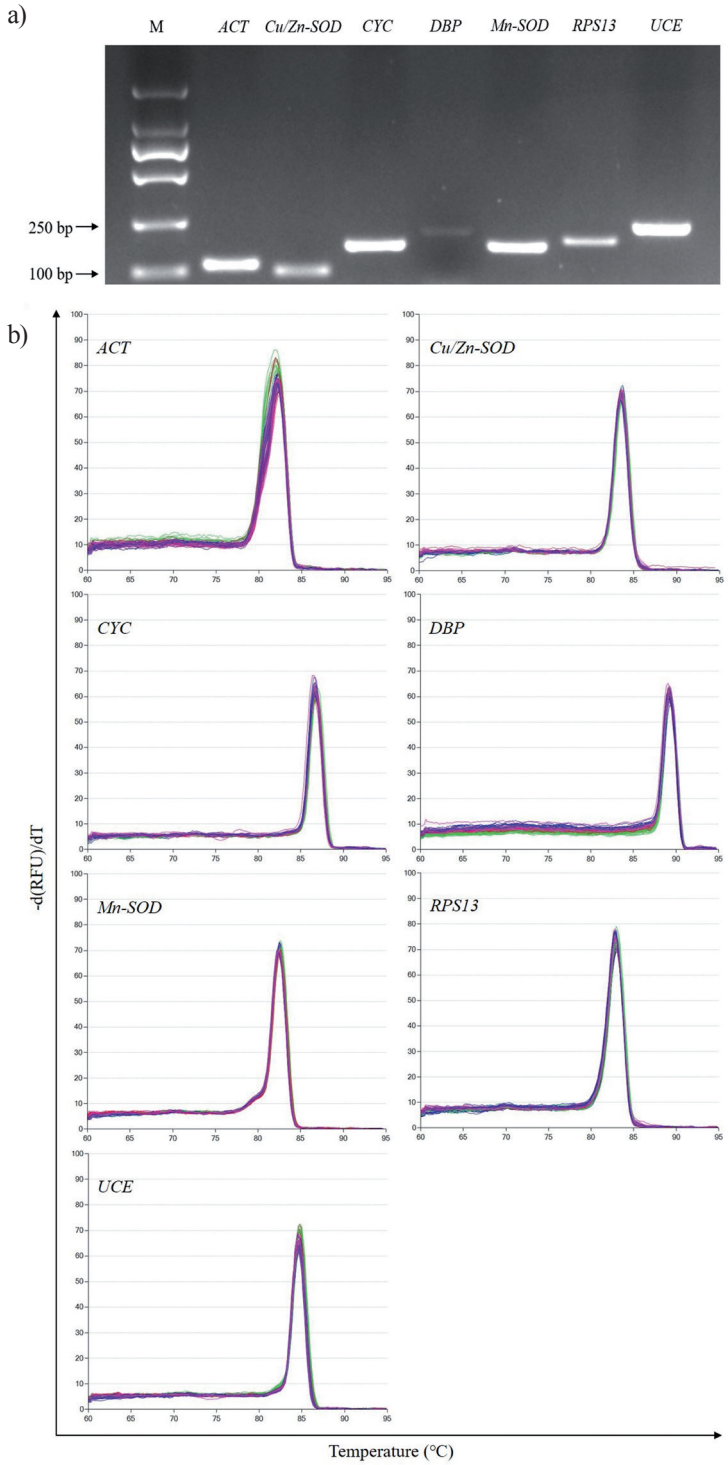

Fig. 1. Primer amplification specificity for RT-qPCR analysis. a) PCR amplifications of seven candidate reference genes on 2% agarose gel. A single band with the expected size represents the amplicon specificity. b) Melting curves for the seven potential reference genes in all samples with three biological replicates. A single peak represents the primer specificity.

Table 2. Ct values of all samples in *M. sieboldii*.

		ACT	*CYC*	*DBP*	*Mn-SOD*	*Cu/Zn-SOD*	*RPS13*	*UCE*
Different developmental stages	0 DAP	18.776	14.131	24.806	19.604	16.392	21.796	19.196
		18.826	14.234	24.955	19.695	16.764	21.913	19.262
		18.974	14.125	25.018	19.730	16.767	21.913	19.355
	30 DAP	19.362	15.597	30.640	17.225	17.836	21.582	20.047
		19.423	15.679	30.579	17.240	17.833	21.561	20.173
		19.374	15.652	30.487	17.234	17.968	21.586	20.113
	60 DAP	19.727	15.762	29.256	17.588	17.813	22.195	20.718
		19.777	15.623	29.531	17.686	17.836	22.223	20.587
		19.636	15.660	29.271	17.681	17.878	22.212	20.694
	90 DAP	19.987	15.621	29.546	18.327	18.410	22.560	20.597
		20.074	15.598	29.425	18.335	18.472	22.446	20.613
		20.180	15.719	29.781	18.407	18.538	22.476	20.659
	120 DAP	20.770	15.715	29.690	18.999	18.021	22.785	20.378
		20.673	15.695	29.600	19.057	18.040	22.830	20.287
		20.709	15.699	29.982	19.078	18.052	22.821	20.543
Different organ tissues	Root	20.158	16.560	22.031	22.719	18.308	24.249	21.555
		20.190	16.598	22.142	22.735	18.349	24.358	21.857
		20.222	16.623	22.238	22.767	18.352	24.364	21.570
	Stem	19.404	15.378	27.778	19.287	18.164	21.900	20.434
		19.429	15.433	27.854	19.259	18.156	21.951	20.455
		19.128	15.432	27.836	19.239	18.156	21.907	20.418
	Leaf	21.527	16.223	26.864	22.167	19.994	20.619	21.988
		21.733	16.247	27.052	22.204	20.056	20.688	21.975
		21.675	16.310	27.014	22.218	20.075	20.714	21.985
	Petiole	20.584	17.211	28.548	23.391	19.648	23.813	22.074
		20.642	17.258	28.767	23.594	19.667	23.964	22.159
		20.695	17.339	28.902	23.686	19.690	24.036	22.073
	Flower	18.712	16.083	28.989	19.896	18.727	22.265	20.234
		18.657	16.117	29.276	20.205	18.809	22.266	20.228
		18.788	15.815	28.945	20.182	18.863	22.299	20.494
Imbibition (GA3)	0 h	22.260	17.910	33.085	20.578	20.122	23.855	22.323
		22.293	18.030	33.214	20.625	20.184	23.924	22.419
		22.306	18.067	33.496	20.644	20.171	23.940	22.277
	6 h	22.402	17.787	32.583	20.091	19.757	24.490	22.347
		22.404	17.897	32.747	20.198	19.956	24.502	22.412
		22.377	17.718	32.332	20.066	19.791	24.489	22.396
	12 h	21.702	16.856	32.805	19.706	19.328	23.238	21.696
		21.945	16.894	32.421	19.688	19.338	23.415	21.560
		21.856	16.657	32.392	19.736	19.266	23.409	21.682
	24 h	21.326	16.605	31.157	18.982	18.662	23.290	21.017
		21.489	16.667	31.204	19.069	18.785	23.230	21.154
		21.565	16.700	31.102	19.053	18.769	23.307	21.053
	48 h	21.903	16.553	32.199	19.539	19.400	22.904	20.986
		21.759	16.650	32.441	19.631	19.614	22.893	21.043
		21.740	16.537	32.453	19.666	19.475	22.876	20.969

Table 2. Continued.

Stratification	0 d	21.417	16.581	30.928	19.420	19.125	22.973	21.184
		21.417	16.580	31.268	19.484	19.198	23.093	21.206
		21.299	16.610	31.347	19.479	19.262	23.208	21.122
	15 d	21.090	17.209	29.585	18.630	18.938	22.611	20.258
		21.182	17.489	29.952	18.993	19.136	22.742	20.384
		21.101	17.416	29.734	19.016	19.188	22.746	20.291
	30 d	20.897	17.541	31.629	19.506	19.134	22.438	20.645
		20.956	17.240	31.975	19.225	19.126	22.325	20.393
		20.874	17.139	32.045	19.443	19.141	22.414	20.613
	45 d	21.331	16.938	31.138	19.071	18.840	22.995	21.146
		21.365	16.943	31.284	19.073	18.902	23.067	21.144
		21.451	16.973	31.422	19.112	18.992	23.083	21.222
	60 d	20.336	17.518	30.803	20.001	18.765	22.291	20.668
		20.383	17.742	30.780	20.115	18.952	22.543	20.926
		20.354	17.630	30.800	20.166	18.868	22.471	20.981

Ct value of each biological replicate is the mean of three technical replicates.

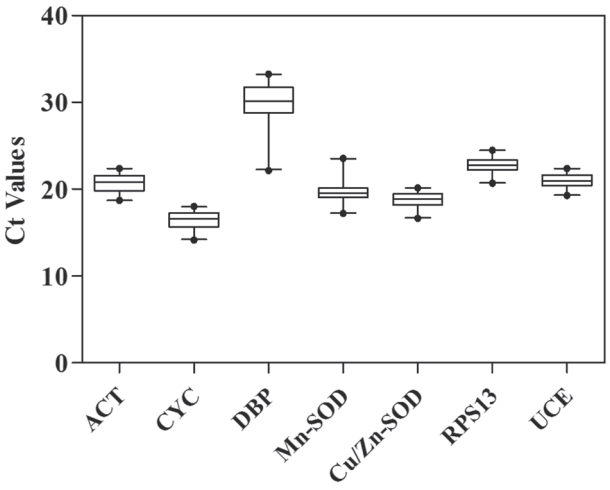

Fig. 2. Ct values for the seven potential reference genes across all *M. Sieboldii* samples. Boxes depict the 25th and 75th percentiles. Lines across the boxes depict the median values. Whiskers indicate the maximum and minimum values. Black dots indicate outliers. The higher boxes and whiskers indicate the larger variations.

treatment, during stratification treatment, and at different developmental stages were lower than 0.15 (0.084, 0.112, and 0.080, respectively), suggesting the necessity of two reference genes to normalise the target genes. However, all V_n/V_{n+1} values among diverse organs and total samples were higher than 0.15 (Fig. 3). Hence, the threshold of 0.15 was slightly rigorous in these experimental conditions. Numerous studies have indicated that the application of a threshold of 0.15 is merely a suggestion, and whether it is practically applied is determined by the data [52-54]. In general, a reliable normalisation could also be performed with one reference gene in most of experiments [16, 31, 36]. In view of this, one reference gene was adopted for each of the experimental conditions.

NormFinder algorithm can be used to rank the gene expression stability through the calculation of the mean pairwise variation of one reference gene with the rest of genes [27]. Genes that possess the minimum expression stability value are featured to be the tested reference genes with the highest stability [27]. The stability ranking for the seven genes obtained through NormFinder analysis was relatively identical to the data array of geNorm. *CYC* and *UCE* were

Table 3. Ct value characteristics of the seven potential reference genes in *M. Sieboldii*.

	ACT	*CYC*	*DBP*	*Mn-SOD*	*Cu/Zn-SOD*	*RPS13*	*UCE*
Minimum	18.72	14.16	22.14	17.23	16.64	20.67	19.27
Maximum	22.39	18.00	33.27	23.56	20.16	24.49	22.39
Mean	20.71	16.50	29.79	19.79	18.83	22.78	21.00
Median	20.81	16.59	30.16	19.54	18.89	22.76	20.93

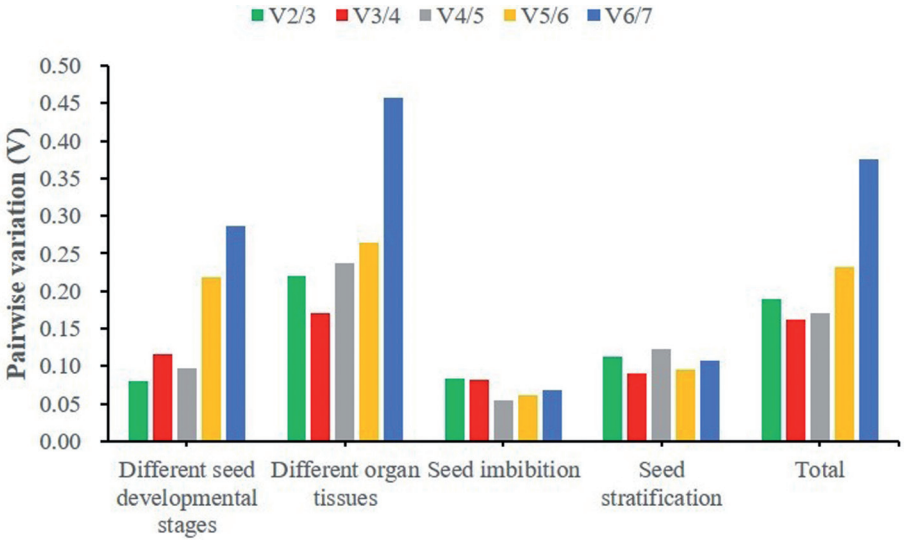

Fig. 3. Pairwise variation (V) for the seven potential reference genes. The geNorm algorithm was utilised to analyse the pairwise variation (V_n/V_{n+1}) of normalisation factors (NF_n and NF_{n+1}). The best reference gene number was determined to accurately normalise gene expression according to V<0.15.

Table 4. The seven potential reference gene expression stability determined using the geNorm, NormFinder, and BestKeeper algorithms.

Group	Rank	geNorm		NormFinder		BestKeeper		
		Gene	Stability	Gene	Stability	Gene	SD [±CP]	CV [% CP]
Different seed developmental stages	1	*CYC*	0.26	*CYC*	0.090	*RPS13*	0.37	1.69
	2	*UCE*	0.26	*UCE*	0.090	*UCE*	0.42	2.07
	3	*Cu/Zn-SOD*	0.27	*Cu/Zn-SOD*	0.091	*Cu/Zn-SOD*	0.45	2.55
	4	*ACT*	0.38	*ACT*	0.125	CYC	0.48	3.13
	5	*RPS13*	0.44	*RPS13*	0.244	*ACT*	0.52	2.62
	6	*Mn-SOD*	0.73	*Mn-SOD*	1.082	*Mn-SOD*	0.77	4.21
	7	*DBP*	1.10	*DBP*	1.383	*DBP*	1.56	5.43
Different organ tissues	1	*CYC*	0.51	*CYC*	0.176	*CYC*	0.50	3.06
	2	*UCE*	0.51	*UCE*	0.176	*Cu/Zn-SOD*	0.68	3.60
	3	*Cu/Zn-SOD*	0.64	*Cu/Zn-SOD*	0.233	*UCE*	0.74	3.47
	4	*ACT*	0.69	*ACT*	0.569	*ACT*	0.87	4.31
	5	*Mn-SOD*	0.92	*Mn-SOD*	0.936	*RPS13*	1.20	5.32
	6	*RPS13*	1.17	*RPS13*	1.133	*Mn-SOD*	1.51	7.02
	7	*DBP*	1.77	*DBP*	2.203	*DBP*	1.92	7.14
Seed imbibition	1	*Cu/Zn-SOD*	0.13	*Mn-SOD*	0.089	*ACT*	0.31	1.40
	2	Mn-SOD	0.13	*Cu/Zn-SOD*	0.131	*Cu/Zn-SOD*	0.39	2.01
	3	*ACT*	0.22	*ACT*	0.139	*Mn-SOD*	0.44	2.22
	4	*CYC*	0.28	*UCE*	0.147	*RPS13*	0.49	2.09
	5	*UCE*	0.30	*CYC*	0.161	*DBP*	0.49	1.52
	6	*RPS13*	0.33	*RPS13*	0.288	*UCE*	0.54	2.48
	7	DBP	0.38	*DBP*	0.320	*CYC*	0.59	3.42

Table 4. Continued.

Seed stratification	1	*ACT*	0.23	*Cu/Zn-SOD*	0.102	*Cu/Zn-SOD*	0.12	0.64
	2	*RPS13*	0.23	*UCE*	0.163	*RPS13*	0.27	1.18
	3	*Cu/Zn-SOD*	0.31	RPS13	0.215	*UCE*	0.31	1.47
	4	*UCE*	0.35	*ACT*	0.315	*ACT*	0.32	1.51
	5	*Mn-SOD*	0.46	*Mn-SOD*	0.324	*Mn-SOD*	0.32	1.65
	6	*CYC*	0.53	*CYC*	0.395	*CYC*	0.32	1.86
	7	*DBP*	0.61	*DBP*	0.513	*DBP*	0.56	1.82
Total	1	*Cu/Zn-SOD*	0.50	*UCE*	0.157	*UCE*	0.65	3.09
	2	*UCE*	0.50	*Cu/Zn-SOD*	0.173	*Cu/Zn-SOD*	0.66	3.49
	3	*CYC*	0.58	*CYC*	0.191	*RPS13*	0.73	3.20
	4	*ACT*	0.65	*ACT*	0.234	CYC	0.75	4.55
	5	*RPS13*	0.77	*RPS13*	0.533	*ACT*	0.91	4.41
	6	*Mn-SOD*	1.00	*Mn-SOD*	1.161	*Mn-SOD*	1.09	5.50
	7	*DBP*	1.48	*DBP*	1.808	*DBP*	1.97	6.62

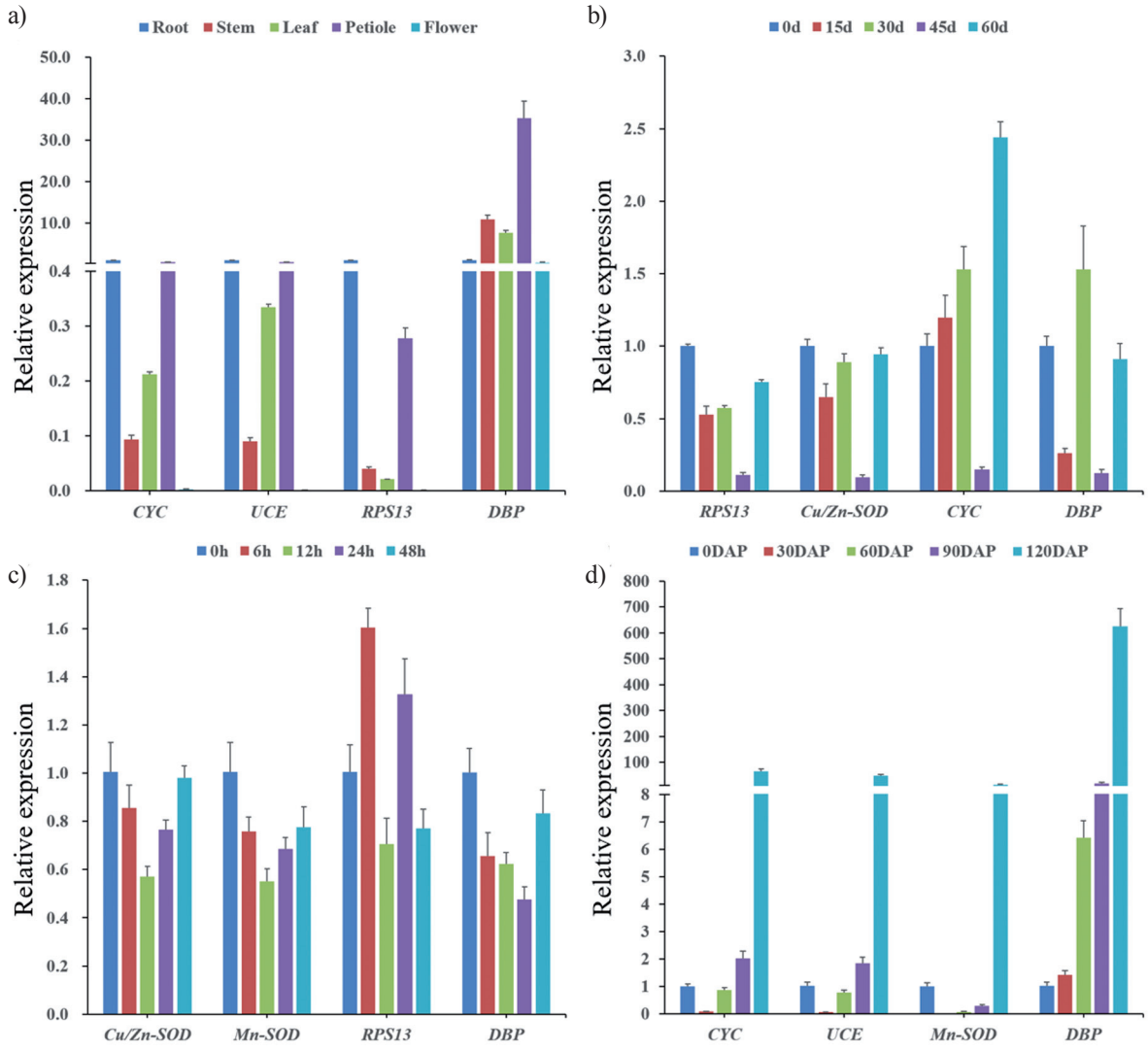

Fig. 4. Normalised expression pattern of *MsDOG1* using the validated reference genes under certain experimental backgrounds. These experimental series contain a) different organ tissues, b) seed stratification treatment, c) seed imbibition treatment, and d) different seed developmental stages. Mean Ct value was determined based on three technical and three biological duplicates and used in subsequent gene expression analyses. Error bar stands for standard deviation.

the two genes having the highest expression stability, and the lowest expression stability values were both 0.176 for different organ tissues and both 0.090 for seeds at different developmental stages. *Mn-SOD* exhibited the highest expression stability (0.089) for seeds under imbibition treatment, whereas *Cu/Zn-SOD* exhibited the highest expression stability (0.102) for seeds during stratification treatment. Finally, *UCE* was identified as the potential gene with the highest stability (0.157), whereas *DBP* was identified as the potential gene with the lowest stability (1.808) in total samples.

The BestKeeper algorithm is also an Excel-based approach for identifying gene stability according to standard deviation (SD) and coefficient of variation (CV) from the quantification cycle (Cq) values [28]. Candidate genes with the highest expression stability exhibited the lowest SD and CV [28]. In the present work, *CYC* (0.50±3.06) was determined as the most stably expressed gene for different organ tissues, *ACT* (0.31±1.40) for seeds under imbibition treatment, *Cu/Zn-SOD* (0.12±0.64) for seeds during stratification treatment, *RPSI3* (0.37±1.69) for seeds at different developmental stages, and *UCE* (0.65±3.09) in total samples.

Verification of Those Determined Reference Genes

For verifying the suitability of the screened reference gene for normalising RT-qPCR data under diverse experiment conditions, *MsDOG1* expression was investigated using the reference genes with the highest and lowest stability as internal controls (Fig. 4). For diverse organ tissues, *MsDOG1* was highly expressed in root, followed by petiole, leaf, stem, and then in flower when applying the two most stable reference genes (*CYC*, *UCE*). However, a different bias was observed when applying the two least stable reference genes (*RPSI3*, *DBP*) (Fig. 4a). The normalisation results of the *MsDOG1* expression in seeds were identical when selecting the two stable genes (*RPSI3* and *Cu/Zn-SOD*) as internal controls during stratification, whereas major differences were displayed when normalising target genes against reference genes with the lowest stability (*CYC* and *DBP)* (Fig. 4b). For seeds under imbibition treatment, *MsDOG1* was highly expressed in each imbibed time point across the reference genes. Nonetheless, the variation patterns normalised with reference genes exhibiting the highest stability (*Cu/Zn-SOD*, *Mn-SOD*), and the reference genes exhibiting the lowest stability (*RPSI3*, *DBP*) apparently differed (Fig. 4c). In seeds at different developmental stages, similar *MsDOG1* expression tendencies were observed when using *CYC* and *UCE* (the most stable) as reference genes. The utilisation of *Mn-SOD* and *DBP* (the least stable) as reference genes led to the obvious underestimation of *MsDOG1* expression at two developmental stages (60 and 90 DAP) and overestimation at four stages (30, 60, 90, and 120 DAP), respectively (Fig. 4d). Thus, suitable reference genes must be selected to accurately normalise target gene expression.

Discussion

The gene expression analysis could result in a comprehensive understanding of gene functions. Although the accuracy and sensitivity of RT-qPCR makes it an ideal method for analysing the gene expression, a reliable and veracious outcome depends on whether a suitable reference gene is applied [6, 13]. An ideal reference gene must have unchanged expression under different conditions. Nonetheless, there may be no such an optimal reference gene [23]. For example, *RPSI3* displayed high stability during seed development, but ranked poorly during flower development for Sacha inchi [15]. *DBP* was reported to be homogeneously expressed under light quality treatment, but possessed a relatively unstable expression under low temperature treatment in strawberry [16]. Therefore, it is crucial to validate the suitable reference genes prior to RT-qPCR analysis at certain experimental conditions [31, 55]. To the best of our knowledge, the present study pioneers the systematic investigation of the choice of suitable reference genes for RT-qPCR analysis within *M. sieboldii*.

In the present work, seven potential reference gene expression stability was evaluated within *M. sieboldii* under different experimental subgroups such as 'different organ tissues,' 'seed developmental stages,' 'seed imbibition,' and 'seed stratification' treatments. The primers possessed excellent specificity because a single peak existed in the analysis of the melting curve. Additionally, RT-qPCR displayed high efficiency for all candidate reference genes (nearly 100%). These findings suggested the suitability of our primers to analyse reference gene expression stability.

The seven potential reference gene expression stability was estimated by three computational methods (geNorm, NormFinder, and BestKeeper). The ranking order for the identified reference genes produced through various statistical algorithms displayed certain divergences. For example, according to the three algorithms in the 'different organ tissues' set, *CYC*, *UCE,* and *Cu/Zn-SOD* were the three most stable genes, whereas *UCE* and *Cu/Zn-SOD* altered positions in the BestKeeper analysis. In the 'seed developmental stages' set, *CYC* was the best reference gene according to the geNorm and NormFinder estimation, whereas it ranked 4th in the BestKeeper estimation. For the 'seed imbibition' set, *Cu/Zn-SOD*, *Mn-SOD,* and *ACT* were the best reference genes for geNorm, NormFinder, and BestKeeper analysis, respectively. For the "seed stratification" set, *ACT* exhibited the highest stability according to geNorm, whereas *Cu/Zn-SOD* was predicted by NormFinder and BestKeeper as the optimal reference gene. Stability ranking discrepancies of these

methods are also observed in other studies [18, 36, 56, 57], most likely due to the various statistical models and analytical procedures that they adopt.

Mn-SOD was the most stable reference gene for the 'seed imbibition' group; however, it did not rank high in the 'seed developmental stages', 'different organ tissues', 'total', and 'seed stratification' sets. Similarly, *Mn-SOD* might not be the appropriate reference gene in longan during somatic embryogenesis [58]. In the current study, *UCE* was suggested to be the optimal candidate reference gene among the tested genes under seed development, total and different organ tissues. This finding was concurrent with that reported in *Plukenetia volubilis,* demonstrating that *UCE* was the best reference gene in seed development, total and different organ tissues [15]. However, *UCE* was demonstrated to be one of the most variable reference genes in tung tree and *Jatropha curcas* [17, 59]. *ACT* was widely applied as reliable reference gene for RT-qPCR normalization [60, 61]. This study indicated that *ACT* was the best reference gene but only under seed imbibition and not in other conditions. In line with this, *ACT* was reported to be the worst reference gene in pear [62] and apple [34]. *DBP* was considered an appropriate reference gene in strawberry in the different organ tissues, fruit developmental stages and fruit under red light treatment [16]. Nevertheless, *DBP* performed poorly as the reference gene in the strawberry fruit's response to osmotic stress [63]. Identical to this, in this study, *DBP* always occupied the bottom position (Table 4), indicating that it might be inappropriate for gene expression analysis in *M. sieboldii*. Overall, these results show that the reliable reference genes differ among species, tissues or experimental conditions. Therefore, the reference gene choice is extremely vital.

In our study, the *CYC* gene was ranked higher in different organ tissues and seeds at different developmental stages, which is similar to the findings in bamboo and *Plukenetia volubilis* [15, 64, 65]. The *RPS13* gene, which was adopted for RT-qPCR normalisation in *Petunia hybrida* [33], might also act as a suitable reference gene for the investigation of *M. sieboldii* seed stratification. *Cu/Zn-SOD* was ranked within the top three best reference genes for all subset samples (Table 4). Thus, *Cu/Zn-SOD* was recommended for RT-qPCR normalisation in *M. sieboldii*. *Cu/Zn-SOD* was also identified as the most stable reference gene during somatic embryogenesis cultured under different temperatures in longan [58].

DOG1 negatively modulates the seed germination and flowering time [49, 66]. Apart from the effects on plant development, DOG1 also participate in seed response to chilling [50]. To verify the appropriateness of the determined reference genes, the relative *MsDOG1* gene expression was detected via reference genes exhibiting the highest and lowest stability for all subgroups with the exception of 'total' (Fig. 4). *MsDOG1* gene expression patterns exhibited high identity when normalising expression using reference genes with the highest stability. However, the normalisation results of *MsDOG1* were significant different via application of the most variable genes. The reference genes played a crucial role in the normalisation of RT-qPCR data. Additionally, adopting the unsuitable reference genes can elicit inaccurate results. Hence, reliable reference genes must be selected for gene expression studies.

Gene expression analysis is essential for clarifying the molecular mechanisms underlying various biological processes [3]. The deep seed dormancy, which is a hamper for *M. sieboldii* breeding, is a crucial field of biological research. In our study, the optimal reference genes were identified in *M. sieboldii* under different experimental conditions to ensure the accurate RT-qPCR results. Among these, gene expression levels related to seed imbibition and stratification may contribute to elucidate the potential molecular mechanisms of seed dormancy breaking, which provide a guideline for optimising *M. sieboldii* growth.

Conclusions

To the best of our knowledge, the present work pioneers the systematic investigation of the choice of appropriate reference genes for the RT-qPCR within *M. sieboldii*. The *CYC* and *UCE* genes were recommended as reference genes within diverse organs as well as seeds in diverse development periods. The *Cu/Zn-SOD* and *Mn-SOD* genes were identified as the optimal reference genes under seed imbibition treatment, and the *Cu/Zn-SOD* and *RPS13* genes exhibited the highest stability under seed stratification treatment. *Cu/Zn-SOD* was suitable for gene expression analysis in *M. sieboldii*. In contrast, *DBP* was inappropriate for RT-qPCR normalisation in *M. sieboldii*. *MsDOG1* gene expression analysis proved that the utilisation of the appropriate reference genes was critical for obtaining precise RT-qPCR results. Thus, the results from this study provide a framework for selecting suitable reference genes for the accurate RT-qPCR analysis of *M. sieboldii*.

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Conflict of Interest

The authors declare no conflict of interest.

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