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Selection and Validation of Appropriate Reference Genes for Real-Time Quantitative PCR Analysis in Needles of *Larix olgensis* under Abiotic Stresses

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Abstract: *Larix olgensis* Henry is an important afforestation species in northeastern China because of its fast juvenile growth, high-quality timber, and significant economic and ecological values. The selection of appropriate reference genes is necessary for the normalization of gene expression determination during quantitative real-time polymerase chain reaction (qRT-PCR) experiments. In this study, qRT-PCR was used to study gene expression. Three software packages geNorm, NormFinder, BestKeeper were used, and a comprehensive ranking of candidate reference genes was produced based on their output to evaluate the expression stability of 16 candidate reference genes from *L. olgensis* under drought, salt, cold, and heat stress. *PP2A-1* and *GAPDH* ranked as the most stable reference genes under drought and cold stress, *PP2A-1* and *UBQ10* were most stable under salt stress, and *TIP41* and *ACT2* were most stable under heat stress. The least stable gene was *ADP*, which ranked the last under all treatments. Expression profile analysis of the antioxidant gene *CAT* using the two most stable and the single least stable reference genes under each stress further verified that the selected reference genes were suitable for gene expression normalization. This study provides an important foundation for the selection of suitable reference genes for the normalization and quantification of *L. olgensis* gene expression under abiotic stress conditions.

Keywords: *Larix olgensis*; reference gene; qPCR; abiotic stress; gene expression

1. Introduction

Gene expression analysis is an important tool for identifying key genes and understanding complex biological processes such as metabolic pathways, signal transduction, and plant development. Quantitative real-time polymerase chain reaction (qRT-PCR) is the most effective, simple, specific, inexpensive, and sensitive method for quantifying the expression of target genes [1,2]. Nevertheless, various factors such as RNA integrity, reverse transcription efficiency, cDNA quality, primer specificity, amplification efficiency, and the selection of reference genes (RGs) may significantly influence the reliability of qRT-PCR results [3,4]. Selection of inappropriate RGs will introduce inaccuracies into the experimental data, and screening for one or more suitable RGs is therefore important for the normalization of gene expression data.

Previous literature has described the selection of RGs for various species under different biotic and abiotic stresses and in different development stages and tissues. These species include *Arabidopsis thaliana* [5], *Oryza sativa* [6], *Solanum lycopersicum* [7], *Malus domestica* [8], and *Populus euramericana cv* [9]. RGs are often housekeeping genes that are associated with basic cellular processes and

therefore expressed at a constant level under different experimental conditions [10]. Glyceraldehyde 3-phosphate (*GAPDH*), actin (*ACT*), ubiquitin (*UBQ*), ubiquitin conjugating enzyme (*UBC*), 18SrRNA, elongation factor 1 alpha (*EF-1α*), eukaryotic translation initiation factor 4*α* (*eIF-4α*), tubulin beta (*TUB*), and alpha-tubulin (*TUA*) genes have frequently been used as RGs to standardize the expression of target genes [11]. In *Lactuca sativa*, *LsPP2A-1* (protein phosphatase 2A-1), *LsPP2AA3* (protein phosphatase 2A regulator subunit A3), and *LsTIP41* (TAP42-interacting protein of 41 kDa) genes were found to be the most suitable RGs in both diurnal and developmental time course experiments [12]. In *Petroselinum crispum*, the most stable genes under abiotic stress were *EF-1α* and *TUB*, whereas the most stable genes under hormone stimulus treatments were *EF-1α*, *GAPDH*, and *TUB* [13]. *ACT2*, *UBC9*, *PP2A-1*, and *PTBP1* were the most stable RGs under a variety of experimental treatments, and *ACT* and *TUB* have been used as RGs for qRT-PCR normalization in *Salix matsudana* under salt and copper stress [14]. In addition, recent studies have indicated that stably expressed RGs in one species may not be suitable for other species and may not be universally suitable under all treatment conditions [15,16]. Although some genes have been selected as good references, the expression of these housekeeping genes in different organs, developmental stages, physiological states, and stress conditions is unknown [17,18]. For example, *TUB* and *TUA* ranked as optimal RGs in *P. crispum* at different development stages [13]; however, in *Cucumis sativus*, *TUA* showed the lowest stability under long-term heavy metal and drought stress [19]. Therefore, the selection of suitable RGs for a specific experimental design or species is very important. However, previous research has focused primarily on herbaceous plants and broadleaf trees; whether universal RGs exist for *Larix olgensis* remains unclear.

L. olgensis Henry is an important timber species that is widely distributed in northeastern China, eastern Russia, and North Korea [20]. It is an important afforestation species because of its fast juvenile growth, high-quality timber, and ability to tolerate severe environmental stresses like drought and extreme temperatures [21,22]. *L. olgensis* produces wood for lumber, paper, pulp, and biofuel and has significant ecological and economic values [21,23]. In recent years, genetic engineering has been applied to coniferous forest species. Genomic and transcriptomic resources for *L. olgensis* have been developed, and several genes related to biotic stress response have been identified [24]. An analysis of the transcriptomes of two hybrid larch (*Larix kaempferi* × *L. olgensis*) clones showed that genes related to stress response may play an important role in adventitious root development [25]. Four *L. olgensis* MYB transcription factors have been identified and shown to have a potential role in xylem development [26]. Expression characteristics of several stress-related *L. olgensis* genes have been the subject of preliminary study. For instance, *LoMYB29* was expressed when induced by MeJA, ABA, NaCl, drought, wounding, and high light [27]. Ectopic expression of *LoERF017* from *L. olgensis* in *A. thaliana* enhanced its salt and osmotic-stress tolerance [28]. In other timber species like *Cunninghamia lanceolata*, Chen et al. selected RGs in roots as material under water, phosphorus, and nitrogen stresses [29]. As we know, nitrogen (N) starvation/toxicity involves important protecting molecules, such as polyamines which influence yield and productivity of timber species [30]. Nevertheless, as these studies show, the traditional housekeeping gene *TUA* is the only RG that has been used to validate *L. olgensis* gene expression. Therefore, more appropriate RGs are required for the normalization of *L. olgensis* gene expression, particularly under different stress conditions.

Recently, the available transcriptome data from *L. olgensis* has increased, providing a foundation from which to identify sets of optimal RGs. In this study, we selected 16 candidate RGs from *L. olgensis* transcriptome sequencing data and evaluated their expression in response to drought, salt, heat, and cold stress. The software packages geNorm, NormFinder, BestKeeper and comprehensive analysis were used to analyze the expression stability of candidate RGs. We then selected a target gene, *CAT*, which was used to evaluate the reliability of candidate RGs under stress treatments [31]. This study will provide useful information for the selection of suitable RGs for normalization of *L. olgensis* gene expression under abiotic stress.

2. Materials and Methods

2.1. Plant Materials and Treatments

Mature seeds of *L. olgensis* Henry were collected from the Qingshan Forestry Bureau Seed Orchard in Heilongjiang Province and sown in plastic pots (11 × 11 cm) containing a vermiculite/soil mixture (1:1) and cultured in a growth chamber with 70% relative humidity under a 16-h/8-h light/dark photoperiod with a light intensity of 150 μmol m^{-2} s^{-1} [27]. Three-month-old seedlings with consistent growth were selected for abiotic treatments. For salt and drought experiments, pots were irrigated with 0.2 M NaCl or 20% PEG 6000 solutions for 0, 2, 6, 12, and 24 h. Plants were irrigated every 12 h during the experiment. For heat and cold stress treatments, three-month-old seedlings were transferred into light incubators and exposed to 40 or 4 °C for 0, 2, 6, 12, and 24 h [32]. All the treatments were performed with three biological replicates. Needles were carefully harvested from treated and untreated plants, frozen immediately in liquid nitrogen, and stored at −80 °C for RNA extraction.

2.2. RNA Extraction and cDNA Preparation

Total RNA was isolated using the CTAB (cetyltrimethylammonium bromide) method [33]. The concentration of extracted RNA was quantified using a P300 ultramicro spectrophotometer (IMPLEN, Munich, Germany) and evaluated by 0.8% agarose gel electrophoresis. Next, cDNA synthesis was performed using RNA samples with A260/A280 ratios between 1.8 and 2.0 and A260/A230 ratios higher than 2.0. Approximately 1000 ng of total RNA was used to be reversely transcribed into cDNA with the *TransScript*® II One-Step RT-PCR SuperMix (TransGen Biotech, Beijing, China). The cDNA was serially diluted (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}) for determination of the correlation coefficient (R^2), R^2 and slope values were obtained from the standard curves, and the amplification efficiency (E) calculated according to the formula: $E\% = (10^{-1/slope} - 1) \times 100\%$; it was diluted 10-fold for qPCR analysis and stored at −20 °C until further use.

2.3. Selection of Candidate Reference Genes and Primer Design

This study took advantage of the unpublished *L. olgensis* genome information generated by our team: we had previously analyzed transcriptome for this species with and without watering (unpublished). We screened candidate reference genes according to the following criteria: the fragments per kilobase of exon model per million mapped reads (FPKM values) were appropriating in all samples, and the coefficient of variation (CV) of FPKM value was cut off less than 0.5. Based on previous studies the criteria, 16 commonly used reference genes including glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), 18S ribosomal RNA (*18S*), actin 2 (*ACT2*), tubulin beta-6 (*TUB*), eukaryotic translation initiation factor 4α-1 (*eIF-4α*), Ef 1alpha (*EF-1α*), tubulin alpha-2 (*TUA*), ubiquitin-conjugating enzyme 9 (*UBC9*), TIP41-like protein (*TIP41*), protein phosphatase 2A-1 (*PP2A-1*), polyubiquitin 10 (*UBQ10*), polypyrimidine tract binding protein (*PTBP1*), ADP-ribosylation factor (*ADP*, or ARF), histone (*HIS*), ubiquitin-like protein RUB2 (*UBQ7*), and actin protein coding 12 (*ACT12*) genes were used as candidate genes to identify the most stable RGs under different treatments. Detailed information on each gene is presented in Table 1. The coding sequences (CDs) of the 16 *Arabidopsis* genes were downloaded from the TAIR database (http://www.arabidopsis.org) to identify their homologs in the *L. olgensis* transcriptome. The Bioedit Sequence Alignment Editor was used to perform a local BLAST to conduct the blastn search of the *L. olgensis* transcriptome using the *Arabidopsis* query sequences. We identified 16 candidate RGs and one target gene, *CAT* (Table 2). The CDs sequences from the *L. olgensis* transcriptome were submitted to GenBank. Primer5 was used to design specific primers for each gene using the following criteria: GC content 44%–60%, optimal Tm 58–60 °C, primer length 20–22 bp, and amplicon length 80–220 bp (Table 2). The specificity of all primer pairs was checked by standard PCR using cDNA as a template with KOD FX (Toyobo, Osaka, Japan), and amplified products were verified with 2% agarose gel and sequenced to confirm their identity.

Table 1. Selected candidate reference genes used in quantitative real-time polymerase chain reaction (qRT-PCR) analysis, with information on their *Arabidopsis* homologs.

Gene	Accession Number	Description	Arabidopsis Homolog Locus	E-Value	Identities
GAPDH	MN905721	Glyceraldehyde 3-phosphate dehydrogenase	AT1G13440	4×10^{-81}	80%
18S	MN905722	18S ribosomal RNA	AT2G47420	0.005	88%
ACT2	MN905723	Actin 2	AT3G18780	4×10^{-141}	80%
TIP41	MN905724	TIP41-like protein	AT4G34270	4×10^{-15}	84%
EF-1a	MN905725	Elongation factor 1-alpha	AT5G60390	4×10^{-101}	81%
TUB	MN905726	Tubulin beta-6	AT5G12250	0	82%
eIF-4α	MN905727	Eukaryotic translation initiation factor 4α-1	AT3G13920	0	81%
PP2A-1	MN905728	Protein phosphatase 2A-1	AT1G59830	2×10^{-31}	82%
UBC9	MN905729	Ubiquitin-conjugating enzyme 9	AT4G2796	6×10^{-60}	81%
UBQ10	MN905730	polyubiquitin 10	AT4G05320	10^{-164}	80%
PTBP1	MN905731	Polypyrimidine tractbinding protein	AT3G01150	7×10^{-18}	80%
TUA	MN905732	Tubulin alpha-2	AT1G50010	0	99%
ADP	MN905733	ADP-ribosylation factor	AT1G02430	10^{-102}	84%
HIS	MN905734	Histone	AT5G10980	2×10^{-52}	81%
UBQ7	MN905735	Ubiquitin-like protein RUB2	AT2G35635	3×10^{-33}	79%
ACT12	MN905736	Actin protein coding 12	AT3G46520	10^{-124}	80%
CAT	MN905737	Target gene catalase	AT4G21120	0.14	85%

Table 2. Primers, amplicon characteristics and qRT-PCR parameters for candidate reference genes and target gene.

Gene Symbol	Primer Sequence (5′–3′) Forward/Reverse	Amplicon Length (bp)	R2	PCR Efficiency (%)	SD	CV
GAPDH	ATTGGAAGACTCGTCGCTCG/ACCGAAAACAGCCACAGGTT	201	0.9949	104.13	1.10	5.11
18S	CAGCGCCATCAAGGAGGAAT/ACCATGCGAGGATCCAACC	209	0.9997	103.43	0.92	3.18
ACT2	TGAGCTACGAGTTGCTCCAG/GGCGACATACATTGCAGGTG	130	0.9982	100.53	0.64	2.56
TIP41	ATGCCCGTCAAGAATGGGAG/TCAACGGGTGGTAAGGCTTC	166	0.9922	99.79	0.83	3.06
EF-1a	TGTGTTGGACTGCCACACTT/TGGGTTTGGAGGGCATCATC	152	0.9972	103.43	1.40	6.12
TUB	TGGTACCATGGATAGCGTGC/TGCCCCTTAGCCCAATTGTT	105	0.9924	100.07	1.23	4.36
eIF-4α	GCTCTTTGCAAGCTATGATG/CACATCAAGACCCTTGCAGA	151	0.9958	99.93	1.40	6.12
PP2A-1	GGAGACATCCATGGGCAGTT/ACGACACGGTCTCAACAGG	130	0.9933	99.98	0.66	2.47
UBC9	TCCCTATGCAGGGGGTGTAT/GGATCCGTCAACAAGGAGCA	210	0.9918	98.35	1.15	4.95
UBQ10	GATGGACGTACTCTCGCTGA/AAAATCGCCACCACGAAGAC	81	0.9992	99.29	1.04	4.55
PTBP1	CCCGTCGAAGGTTTTGCATC/AGCCTGGTTATGGTTGGCTC	130	0.9994	101.12	0.71	2.78
TUA	ATAAGACAGTTGGCGGTGGG/TGCTCTGGGTGAAAGAGCTG	157	0.9992	105.62	1.23	4.98
ADP	ACCAAGCTCTTTCAGCGTCT/GGTCGTCTTACCAGCAGCAT	81	0.9957	108.72	1.33	5.94
HIS	CGAGGCTTACCTTGTAGGGC/CCCTTTCACCGCGAATCCTT	116	0.9978	99.5	1.06	4.77
UBQ7	CTCCTGTGCAACAGAGGCTT/TAATGACCACCACGTAGGGC	124	0.9978	99.66	0.86	3.54
ACT12	CTTGCCGGTCGGGATTTAAC/TTCCAGGGAGGAACTGGTCT	174	0.9963	96.85	0.89	3.70
	Target gene					
CAT	TGCTCACCGTGCTGCATCTA/GCGGCATTGAACACCCCATT	148	0.9918	100.38		

2.4. Reverse Transcription Quantitative PCR (qRT-PCR) Analysis

Quantitative RT-PCR reactions were conducted in 96-well plates with a qTOWER 3G Cycler and qPCR software (Analytik Jena, Jena Germany) using the TransStart Top Green qPCR SuperMix (TransGen Biotech, Beijing, China). The 20 µL reaction mix contained 10 µL TransStart Top Green qPCR SuperMix, 7 µL nuclease-free water, 1 µL diluted cDNA, and 1 µL of each specific primer (final concentration 10 µM). PCR conditions were 94 °C for 30 s, followed by 45 cycles of 95 °C for 5 s, 59 °C for 15 s, and 72 °C for 10 s. Three technical replicates were performed for each sample to ensure the accuracy of the results. All primers used in the study are listed in Table 2.

2.5. Gene Expression Stability Analysis

Three different Microsoft Excel-based software programs, geNorm [3], NormFinder [15], and BestKeeper [13,34], were used to analyze the expression stability of the candidate RGs under different experimental conditions. The Cq (PCR cycle threshold) data were used directly in the BestKeeper program, but for geNorm and NormFinder, Cq values were converted into relative values and imported into geNorm and NormFinder program analyzed as described previously [13]. The output of each software program permitted us to rank the expression stability of RGs in different treatment groups. A comprehensive ranking of RGs was also generated as described previously [35,36].

2.6. Validation of RGs by qRT-PCR

QRT-PCR was also performed to analyze the expression levels of the target gene *CAT* under different experimental conditions. The top two best RGs and worst ranked RG for each experimental condition were selected normalization of *CAT* expression in *L. olgensis*. The quantitative variation between replicates was calculated with the relative quantification method ($2^{-\Delta\Delta CT}$) [37]. Graphs were generated using Excel and GraphPad Prism7. The primer for *CAT* is listed in Table 2.

3. Results

3.1. Selection of Candidate Reference Genes

Based on sequence homology with Arabidopsis genes, 16 candidate RGs were identified from the *L. olgensis* transcriptome (unpublished) (Table 1). Subsequently, primer specificity was confirmed based on agarose gel electrophoresis. Amplicons of a single band with the expected size indicate good primer specificity (Figure S1). In qRT-PCR reactions, melting curve analysis of every candidate RG showed a single peak. Amplification efficiency (E) and correlation coefficient values (R^2) of the RG standard curves varied from 96.85 to 108.72% and from 0.9918 to 0.9997, respectively (Table 2 and Figure S2).

3.2. Expression Profiles of Candidate RGs

The distribution of raw Cq values for the 16 candidate RGs in 20 *L. olgensis* samples (five samples from each stress condition) is shown in Figure 1. The RGs exhibited a relatively wide range of Cq values, from 13.07 (*ADP*) to 31.75 (*18S*). The mean Cq values of the RGs varied from 21.63 to 28.85, their SDs varied from 0.64 to 1.4, and their coefficients of variation (CVs) varied from 2.47% to 6.12%. As Cq values are negatively related to gene expression levels, the highest maximum expression level was detected in *ADP*, whereas the lowest minimum level was detected in *18S* (Figure 2). *ADP* exhibited the highest variation in expression, and *PTBP1* exhibited the lowest variation, with Cq values ranging from 24.20 to 27.62. *eIF-4α* showed relatively moderate variation, with Cq values ranging from 22.27 to 26.72. A small CV of the Cq values indicates that a given gene is more stably expressed. The lowest and highest CV values under the four treatments were *GAPHH* (0.79%) and *ADP* (15.3%) under drought stress, *PP2A-1* (0.65%) and *ADP* (14.06%) under salt stress, *PP2A-1* (1.51%) and *ADP* (14.77%) under cold stress, and *UBQ7* (1.22%) and *ADP* (15.22%) under heat stress. The ranking of gene stability by

average CV values across all treatments was *PP2A-1* > *ACT2* > *PTBP1* > *TIP41* > *18S* > *UBQ7* > *ACT12* > *eIF-4α* > *TUB* > *UBQ10* > *HIS* > *UBC9* > *TUA* > *GADPH* > *ADP* > *EF-1α* (Table 2).

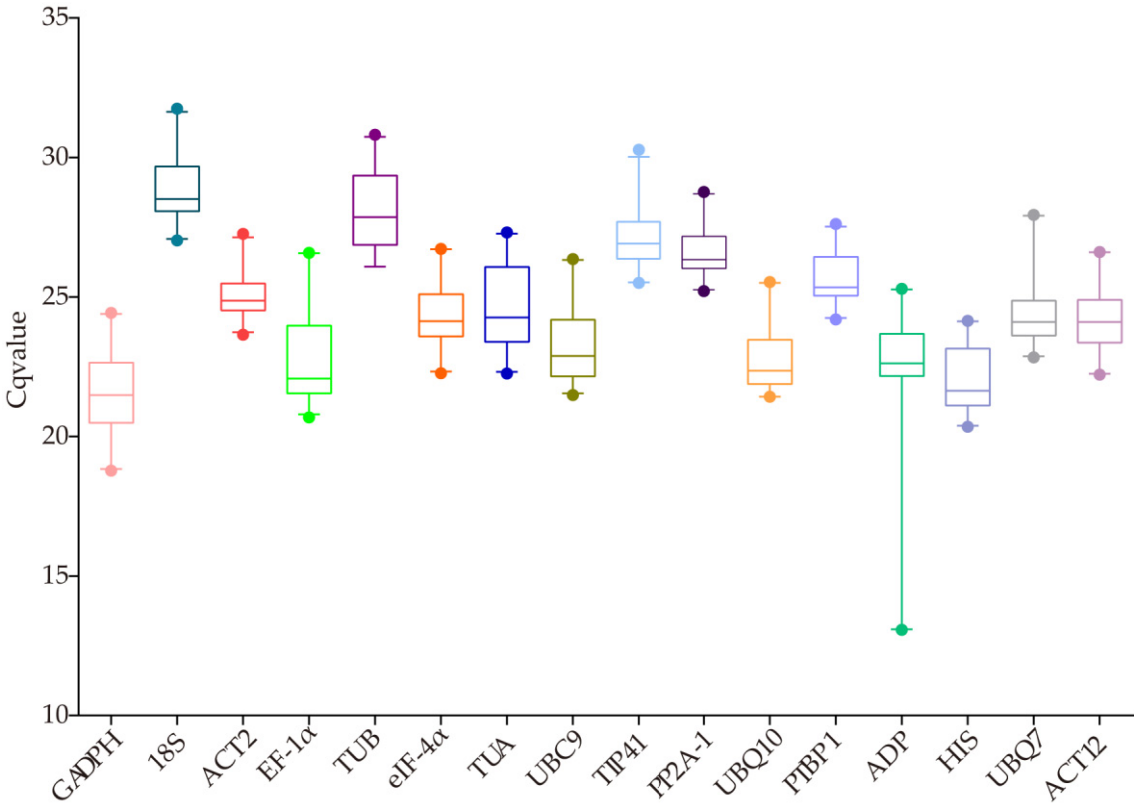
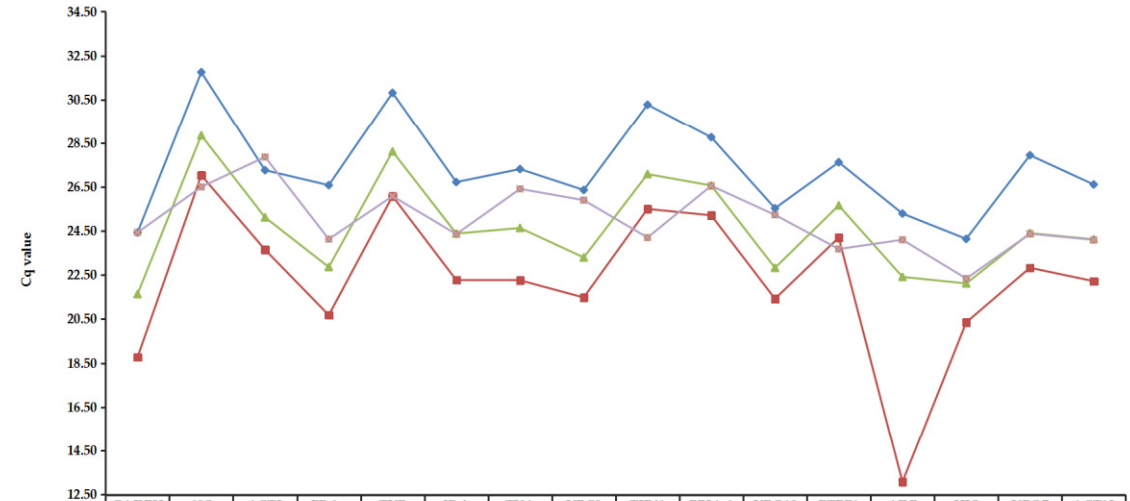


Figure 1. Distribution of raw Cq values for the 16 candidate reference genes (RGs) across all *Larix olgensis* samples. The lines inside each box represent the medians. The outside box is determined from 25th to 97.5th percentiles. The top and bottom whiskers represent the highest and lowest Cq values, respectively.



	GADPH	18S	ACT2	EF-1α	TUB	eIF-4α	TUA	UBC9	TIP41	PP2A-1	UBQ10	PTBP1	ADP	HIS	UBQ7	ACT12
Maximum	24.43	31.75	27.26	26.58	30.81	26.72	27.31	26.36	30.27	28.76	25.53	27.62	25.29	24.14	27.94	26.61
Minimum	18.77	27.03	23.65	20.68	26.08	22.27	22.26	21.48	25.50	25.21	21.42	24.20	13.07	20.35	22.83	22.22
Mean	21.63	28.85	25.12	22.86	28.11	24.38	24.64	23.30	27.08	26.56	22.82	25.66	22.42	22.12	24.40	24.12
Median	24.43	26.50	27.86	24.13	26.08	24.36	26.41	25.90	24.20	26.55	25.23	23.68	24.10	22.34	24.37	24.10

Figure 2. Maximum, minimum, mean, and median Cq values of the 16 candidate reference genes across all samples.

3.3. Analysis of Gene Expression Stability

The software programs geNorm, NormFinder, and BestKeeper were used to analyze the expression stability of the 16 candidate RGs in *L. olgensis* plants that had been subjected to four experimental treatments. Data from each treatment were analyzed separately and in combination.

geNorm analysis: for geNorm analysis, the *M*-value of candidate RGs was calculated and used to rank RG stability. A gene with an *M*-value below 1.5 is considered to be stably expressed, and the smaller the *M*-value, the more stable the gene [3]. In our study, the *M*-values of all candidate RGs were lower than 1.5 in individual treatments and when the treatments were combined, with the exception of *ADP* under heat stress. The most suitable RG differed among treatments. As shown in Table 3 and Figure S3, *GAPDH* and *18S* under drought stress, *UBQ10* and *PP2A-1* under salt stress, *PP2A-1* and *GAPDH* under cold stress, and *TIP41* and *ACT2* under heat stress were ranked as the most stable genes with the lowest *M*-values. *PP2A-1* and *ACT2* had the most stable expression when data from all samples were combined, whereas, *ADP* was the least stable gene in all treatment conditions.

Table 3. Expression stability of candidate reference genes calculated by geNorm.

Rank	Drought		Salt		Cold		Heat		Total	
	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability
1	*GAPDH*	0.25	*PP2A-1*	0.14	*PP2A-1*	0.20	*TIP41*	0.48	*PP2A-1*	0.47
2	*18S*	0.25	*UBQ10*	0.14	*GAPDH*	0.20	*ACT2*	0.48	*ACT2*	0.47
3	*PP2A-1*	0.31	*UBC9*	0.27	*TIP41*	0.22	*PTBP1*	0.51	*TIP41*	0.53
4	*UBQ10*	0.35	*18S*	0.29	*PTBP1*	0.24	*eIF-4α*	0.66	*PTBP1*	0.57
5	*UBC9*	0.39	*TIP41*	0.31	*18S*	0.26	*18S*	0.73	*18S*	0.61
6	*TIP41*	0.41	*eIF-4α*	0.34	*ACT2*	0.28	*HIS*	0.79	*eIF-4α*	0.63
7	*EF-1α*	0.42	*EF-1α*	0.37	*UBQ10*	0.29	*UBC9*	0.86	*UBC9*	0.67
8	*eIF-4α*	0.45	*TUB*	0.40	*ACT12*	0.33	*UBQ10*	0.92	*UBQ10*	0.71
9	*ACT12*	0.48	*PTBP1*	0.42	*eIF-4α*	0.36	*TUB*	0.96	*GAPDH*	0.73
10	*ACT2*	0.50	*ACT2*	0.45	*UBC9*	0.40	*PP2A-1*	1.03	*HIS*	0.75
11	*PTBP1*	0.53	*GAPDH*	0.47	*EF-1α*	0.45	*UBQ7*	1.07	*ACT12*	0.77
12	*TUB*	0.56	*UBQ7*	0.50	*UBQ7*	0.51	*GAPDH*	1.12	*UBQ7*	0.80
13	*TUA*	0.61	*ACT12*	0.53	*HIS*	0.55	*TUA*	1.17	*TUA*	0.84
14	*HIS*	0.67	*TUA*	0.59	*TUA*	0.66	*ACT12*	1.22	*EF-1α*	0.88
15	*UBQ7*	0.72	*HIS*	0.66	*TUB*	0.75	*EF-1α*	1.28	*TUB*	0.93
16	*ADP*	1.11	*ADP*	1.06	*ADP*	1.15	*ADP*	1.64	*ADP*	1.10

For qRT-PCR, the selection of a greater number of RGs can permit more accurate quantification of target gene expression. geNorm was used to calculate the pairwise variation (Vn/n+1), which permits the determination of an optimal number of RGs for each treatment group using a threshold value of 0.15. There is no need for additional RGs if the variation value is below 0.15 [3]. As shown in Figure 3, the pairwise variation values V_2/V_3 for drought, salt, and cold stress samples were all less than 0.15, indicating that two suitable RGs were adequate for the normalization of data from these treatments. For the heat stress samples, V4/5 was 0.1498, indicating that four RGs (*TIP41*, *ACT2*, *PTBP1*, and *eIF-4α*) were required. When all samples were combined, V3/4 was 0.1315, indicating that three RGs (*ACT2*, *PP2A-1*, and *TIP41*) were required.

NormFinder analysis: the NormFinder approach provides a stability value for each gene based on inter- and intra-group variation in expression [15]. As shown in Table 4, the stability ranks calculated in NormFinder were consistent with those calculated in geNorm under cold stress (*GAPDH*). The top three most stable genes were *GAPDH*, *18S*, and *PP2A-1* under drought stress; *ACT2*, *18S*, *UBC9*, *PPA2-1*, and *UBQ10* under salt stress; and *18S*, *PTBP1*, and *ACT2* under heat stress. These rankings were broadly similar to those of geNorm, although there were some differences. For instance, in the stability rankings for the combined samples, NormFinder suggested that *18S* and *UBC9* were the two most suitable two RGs, a result that differed from that of geNorm. Similar to geNorm, NormFinder indicated that *ADP* was the least stable gene in all treatments. Although there were some differences in

rankings between geNorm and NormFinder, the top five most stable genes were relatively consistent between them.

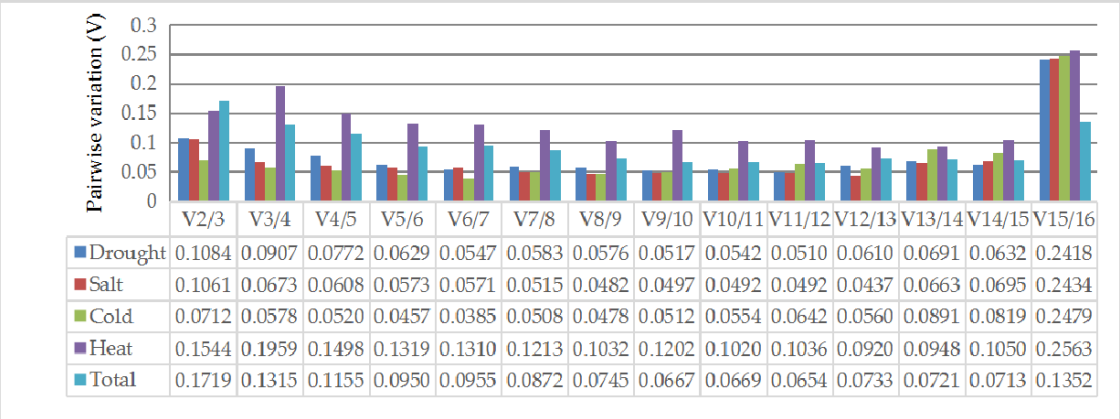


Figure 3. Pairwise variation (Vn/Vn+1) values calculated by geNorm for samples from drought, salt, cold, and heat stress, and for the combination of all samples. The threshold value to determine the optimal number of RGs for qRT-PCR normalization is 0.15.

Table 4. Gene expression stability in *L. olgensis*. under multiple stress treatments, as ranked by the software program NormFinder.

Rank	Drought		Salt		Cold		Heat		Total	
	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability
1	*PP2A-1*	0.08	*ACT2*	0.06	*GAPDH*	0.07	*18S*	0.20	*18S*	0.15
2	*18S*	0.09	*18S*	0.07	*18S*	0.10	*PTBP1*	0.24	*UBC9*	0.27
3	*GAPDH*	0.09	*UBC9*	0.07	*UBC9*	0.12	*ACT2*	0.41	*PTBP1*	0.28
4	*eIF-4α*	0.13	*PP2A-1*	0.07	*PP2A-1*	0.12	*UBC9*	0.41	*TIP41*	0.38
5	*UBQ10*	0.17	*UBQ10*	0.14	*ACT2*	0.18	*TUB*	0.45	*eIF-4α*	0.39
6	*UBC9*	0.22	*eIF-4α*	0.15	*UBQ10*	0.23	*eIF-4α*	0.48	*UBQ10*	0.39
7	*TIP41*	0.25	*GAPDH*	0.16	*TIP41*	*0.24*	*TIP41*	0.60	*ACT2*	0.39
8	*ACT2*	0.29	*TIP41*	0.20	*eIF-4α*	0.25	*UBQ10*	0.61	*PP2A-1*	0.42
9	*EF-1α*	0.31	*EF-1α*	0.28	*PTBP1*	0.28	*HIS*	0.68	*GAPDH*	0.46
10	*TUA*	0.41	*TUB*	0.31	*EF-1α*	0.35	*TUA*	0.68	*HIS*	0.47
11	*ACT12*	0.42	*PTBP1*	0.39	*ACT12*	0.44	*GAPDH*	0.76	*ACT12*	0.57
12	*TUB*	0.43	*ACT12*	0.48	*UBQ7*	0.65	*PP2A-1*	0.79	*TUA*	0.59
13	*PTBP1*	0.44	*UBQ7*	0.49	*HIS*	0.71	*UBQ7*	0.95	*UBQ7*	0.61
14	*UBQ7*	0.72	*TUA*	0.55	*TUA*	0.73	*EF-1α*	1.04	*EF-1α*	0.66
15	*HIS*	0.79	*HIS*	0.87	*TUB*	0.74	*ACT12*	1.05	*TUB*	0.71
16	*ADP*	2.68	*ADP*	2.70	*ADP*	2.75	*ADP*	2.84	*ADP*	1.50

BestKeeper analysis: another method for detection of suitable RGs is BestKeeper. Unlike the other two programs, BestKeeper ranks RGs based on the standard deviation (SD) and coefficient of variation (CV) of the Cq values from the qRT-PCR assay. The smaller the CV, the better the stability of the gene was [13,34]. The results of BestKeeper analysis are also listed in Table 5. *PPA2-1* was ranked first under salt stress and cold stress, with CV ± SD values of 0.17 ± 0.65 and 0.40 ± 1.51, respectively. *GAPDH* was ranked first under drought stress, with a CV ± SD of 0.17 ± 0.79 and *UBQ7* was ranked first under heat stress, with a CV ± SD of 0.29 ± 1.22. BestKeeper suggested that *ACT2* was the most suitable RG when data from all samples were combined. Few genes had an SD greater than 1.0, indicating that most of the candidate RGs were relatively stable. Similar to the results of geNorm and NormFinder, *ADP* was the least stable RG for all sample sets, with the exception of the combined samples, in which *EF-1α* was the least stable.

Table 5. Gene expression stability in *L. olgensis*. under multiple stress treatments, as ranked by the software program BestKeepers.

Rank	Drought			Salt			Cold			Heat			Total		
	Gene	SD	CV	Gene	SD	CV	Gene	SD	CV	Gene	SD	CV	Gene	SD	CV
1	*GADPH*	0.17	0.79	*PP2A-1*	0.17	0.65	*PP2A-1*	0.40	1.51	*UBQ7*	0.29	1.22	*ACT2*	0.54	2.17
2	*PTBP1*	0.63	2.43	*UBQ10*	0.19	0.84	*ACT12*	0.42	1.74	*TIP41*	0.43	1.60	*PP2A-1*	0.58	2.18
3	*HIS*	0.66	2.90	*PTBP1*	0.23	0.93	*PTBP1*	0.44	1.72	*ACT2*	0.60	2.42	*PTBP1*	0.67	2.60
4	*ACT2*	0.69	2.74	*TIP41*	0.23	0.88	*ACT2*	0.46	1.84	*PP2A-1*	0.66	2.52	*TIP41*	0.77	2.84
5	*ACT12*	0.85	3.43	*ACT12*	0.30	1.24	*TIP41*	0.47	1.73	*PTBP1*	0.81	3.14	*UBQ7*	0.79	3.23
6	*TUB*	0.87	3.12	*UBC9*	0.35	1.54	*18S*	0.53	1.88	*GAPDH*	0.93	4.51	*ACT12*	0.85	3.54
7	*PP2A-1*	0.88	3.26	*18S*	0.36	1.29	*eIF-4α*	0.54	2.23	*eIF-4α*	0.99	4.13	*GAPDH*	0.86	4.02
8	*UBQ10*	0.90	3.91	*UBQ7*	0.36	1.52	*UBQ10*	0.56	2.49	*ACT12*	1.05	4.43	*18S*	0.90	3.11
9	*18S*	1.09	3.74	*EF-1α*	0.42	1.90	*GAPDH*	0.60	2.89	*HIS*	1.06	4.73	*eIF-4α*	0.90	3.70
10	*UBQ7*	1.13	4.46	*eIF-4α*	0.42	1.77	*UBQ7*	0.76	3.11	*TUA*	1.14	4.65	*HIS*	1.02	4.59
11	*EF-1α*	1.15	4.96	*TUB*	0.52	1.93	*HIS*	0.76	3.40	*TUB*	1.19	4.14	*UBQ10*	1.02	4.48
12	*eIF-4α*	1.16	4.60	*ACT2*	0.53	2.14	*UBC9*	0.94	4.09	*18S*	1.32	4.59	*UBC9*	1.15	4.94
13	*TIP41*	1.18	4.24	*HIS*	0.57	2.67	*EF-1α*	0.95	4.24	*UBC9*	1.55	6.67	*TUB*	1.20	4.25
14	*UBC9*	1.20	5.06	*GAPDH*	0.60	2.84	*TUA*	1.07	4.48	*UBQ10*	1.73	7.48	*TUA*	1.22	4.93
15	*TUA*	1.39	5.56	*TUA*	0.87	3.67	*TUB*	1.65	5.86	*EF-1α*	2.15	9.18	*ADP*	1.26	5.62
16	*ADP*	3.28	15.30	*ADP*	2.87	14.06	*ADP*	3.10	14.77	*ADP*	3.25	15.22	*EF-1α*	1.39	6.07

3.4. Comprehensive Analysis

To reduce the effect of any limitations and biases associated with individual algorithms, a comprehensive stability analysis was performed by taking the geometric mean of the geNorm, NormFinder, and BestKeeper rankings in order to identify the best RGs [38]. For comprehensive analysis, two RGs were selected for further normalization. As shown in Table 6 and Figure 4, *PP2A-1* was ranked among of the top two most stable RGs under drought, salt, and cold stress, and *GAPDH* under cold stress, *UBQ10* under salt stress were also the most stable RGs, respectively. *TIP41* and *ACT2* were the most stable RGs under heat stress, and *ACT2* and *PP2A-1* were the most stable RGs when all samples were combined. The comprehensive RG rankings for single treatments (drought, salt, and cold) and for combined samples were consistent with the results obtained by geNorm and BestKeeper. *ADP* was the least stable RG under all experimental conditions (Table S1).

Table 6. Gene expression stability ranked by the comprehensive ranking method in *L. olgensis*.

Rank	Drought		Salt		Cold		Heat		Total	
	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability
1	*GAPDH*	1.44	*PP2A-1*	2.00	*PP2A-1*	1.59	*TIP41*	2.41	*ACT2*	2.41
2	*PP2A-1*	2.76	*UBQ10*	2.15	*GAPDH*	2.62	*ACT2*	2.62	*PP2A-1*	2.52
3	*18S*	3.30	*UBC9*	3.78	*18S*	3.91	*PTBP1*	3.11	*PTBP1*	3.30
4	*UBQ10*	5.43	*18S*	3.83	*TIP41*	4.72	*18S*	3.91	*18S*	3.42
5	*PTBP1*	6.59	*ACT2*	4.93	*PTBP1*	4.76	*UBQ7*	5.23	*TIP41*	3.63
6	*ACT2*	6.84	*TIP41*	5.43	*ACT2*	4.93	*eIF-4α*	5.52	*UBC9*	5.52
7	*eIF-4α*	7.27	*PTBP1*	6.67	*ACT12*	5.60	*UBC9*	7.14	*eIF-4α*	6.46
8	*UBC9*	7.49	*eIF-4α*	7.11	*UBQ10*	6.95	*PP2A-1*	7.83	*UBQ10*	8.08
9	*ACT12*	7.91	*EF-1α*	8.28	*UBC9*	7.11	*HIS*	7.86	*GAPDH*	8.28
10	*TIP41*	8.17	*ACT12*	9.21	*eIF-4α*	7.96	*TUB*	7.91	*ACT12*	8.99
11	*HIS*	8.57	*TUB*	9.58	*EF-1α*	11.27	*GAPDH*	9.25	*UBQ7*	9.21
12	*EF-1α*	8.85	*GAPDH*	10.25	*UBQ7*	11.29	*UBQ10*	9.64	*HIS*	10.00
13	*TUB*	9.52	*UBQ7*	10.77	*HIS*	12.30	*TUA*	10.91	*TUA*	12.97
14	*TUA*	12.49	*HIS*	14.30	*TUA*	14.00	*ACT12*	11.89	*TUB*	14.30
15	*UBQ7*	12.81	*TUA*	14.33	*TUB*	15.00	*EF-1α*	14.66	*EF-1α*	14.64
16	*ADP*	16.00	*ADP*	16.00	*ADP*	16.00	*ADP*	16.00	*ADP*	15.66

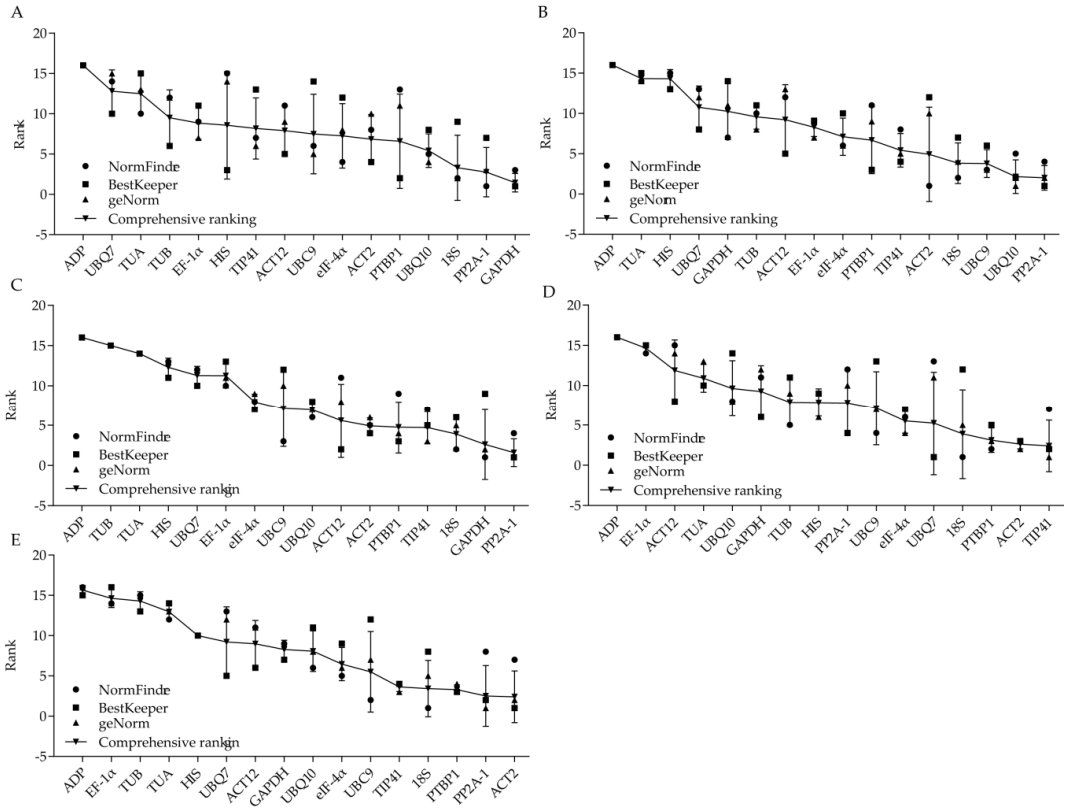


Figure 4. Comprehensive ranking of the 16 RGs in *L. olgensis* calculated as the geometric mean of three types of ranking (geNorm, NormFinder, and BestKeeper) for each sample group. (**A**) Drought stress. (**B**) Salt stress. (**C**) Cold stress. (**D**) Heat stress. (**E**) All samples.

3.5. Validation of CAT Reference Gene

To verify the reliability of the selected reference genes, *CAT* was selected as a target gene. The expression of the antioxidant *CAT* gene is induced by many abiotic stresses, including chilling, drought, osmotic stress, and salt stress [39–41]. The *CAT* sequence was obtained from *L. olgensis* transcriptome data and showed 48.75% nucleotide identity with *CAT* from *Arabidopsis*. We used the comprehensive ranking results to select the top two stable RGs for normalization of *CAT* expression in *L. olgensis* needles. The selected RGs were *PP2A-1* and *GAPDH* for drought and cold stress, *PP2A-1* and *UBQ10* for salt stress, and *TIP41* and *ACT2* for heat stress. As shown in Figure 5, the expression patterns of *CAT* showed few differences when different stable RGs were used. Similar expression patterns were obtained under drought and cold stress when *GAPDH* and *PP2A-1* were used for normalization. *CAT* expression reached a peak at 12 h for both drought stress (<29-fold) and cold stress (<5-fold). When using the most stable RGs for salt stress *PP2A-1* and *UBQ10*, *CAT* expression patterns were consistent, and the highest expression was observed at 24 h (no more than 10-fold). Under heat stress, *CAT* expression levels reached a similar peak at 6 h when normalized with *TIP41* and *ACT2*. However, large differences in expression patterns were detected when the least stable RG, *ADP*, was used for normalization under the same stresses. Specifically, the expression levels of *CAT* were overestimated.

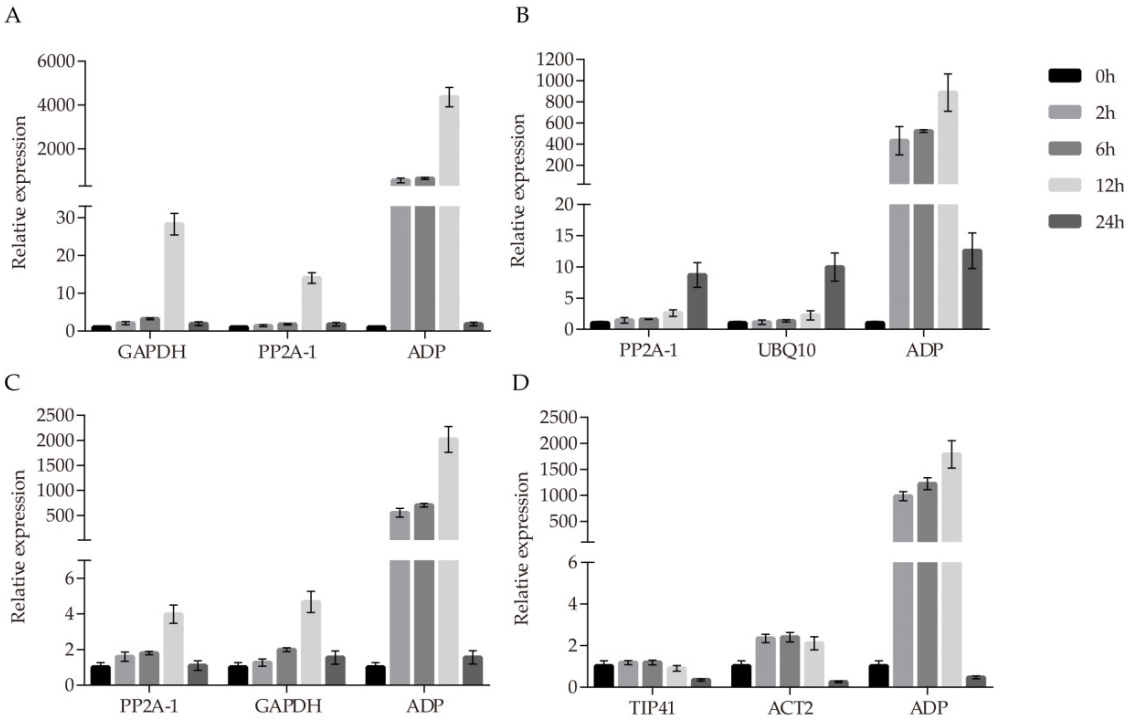


Figure 5. Relative expression of *LoCAT* gene under abiotic stresses normalized using the top two stable RGs and one unstable RG. (**A**) Drought stress. (**B**) Salt stress. (**C**) Cold stress. (**D**) Heat stress.

4. Discussion

Quantitative RT-PCR is an important technology that permits rapid and reliable quantification of gene expression [41]. Due to its high sensitivity, many experimental variables can easily affect the reliability of qRT-PCR gene expression results [4]. Therefore, to obtain more reliable results, an appropriate normalization strategy is very important. Among several methods [1,2], selection of one or more RGs as normalization factor(s) is the most common approach for different experimental conditions such as abiotic stress [38,42,43]. However, no research on RGs for *L. olgensis* under abiotic stress has been reported. In this study, we identified appropriate *L. olgensis* RGs under different abiotic stress conditions using qRT-PCR and parallel calculations in geNorm, Normfinder, Bestkeeper, and comprehensive analysis.

Sixteen potential RGs were identified from *L. olgensis* transcriptome data generated in our laboratory. Three statistical algorithms, geNorm, NormFinder, and BestKeeper, were used to assess the expression stability of candidate RGs for accurate normalization in gene expression studies [20]. In the geNorm analysis, *M*-values of the RGs under different experimental conditions were all less than 1.5 (except *ADP* in heat stress), indicating their potential expression stability [44]. The candidate RGs exhibited differential stability and relative rankings in response to different stresses. In two treatments, the most stable genes calculated by geNorm and Bestkeeper were consistent, namely *GAPDH* in drought and *PP2A-1* in cold (Tables 3 and 5). In salt and heat stress, the most stable genes calculated by the three algorithms differed. For heat stress, *TIP41* and *ACT2* were ranked as the most stable RGs according to the *M*-values calculated in geNorm. NormFinder regarded *18S* as the most stable RG, and BestKeeper identified *UBQ7* as the most stable RG; however, *UBQ7* was ranked lower by geNorm and Normfinder (Tables 3 and 4). The differences in ranking were mainly caused by variations in the algorithms of the three programs. Previous work has also shown that the three programs generate different results under abiotic stress in *Vitis vinifera* [45]. A comprehensive analysis is therefore necessary to provide ultimate stability rankings for RGs under different treatments, as reported previously [46,47].

Our comprehensive ranking results showed that *PP2A-1* and *GAPDH* were the top two most stable RGs under drought and cold stress. *PP2A-1* also ranked first under salt stress in the comprehensive analysis. Our findings confirm several previous studies in which *PP2A-1* was also shown to be the most stable RG. These include studies of *Pennisetum glaucum* under different abiotic stresses and hormonal stimuli [48], *Agrostis stolonifera* under different abiotic stresses [49], *P. euphratica* under drought stress [50], and *M. charantia* under salt stress [51]. *GAPDH* also ranked as a highly suitable RG under drought and cold stress in this study. This result is in agreement with previous research on *Caragana korshinskii* under heat stress [46] and *Coffea arabica* leaves under drought stress and GA, SA, and MeJA treatments [52]. However, *GAPDH* was ranked as the least stable RG in various tissues and under abiotic stresses in *Sorghum bicolor* and in various tissues and under PEG6000 and MeJA treatments in *Peucedanum praeruptorum* [53,54]. These results demonstrate that there is no universal RG that is stably expressed in all treatments and tissues.

UBQ10 was also the most stable RG pair with *PP2A-1* under salt stress. *UBQ10* was also the most constitutively expressed polyubiquitin gene in *A. thaliana* across all development processes [44], in *Prunus persica* fruit at multiple developmental stages [55] and in *Platycladus orientalis* under NaCl and ABA treatments [56]. However, in *O. sativa*, *UBQ10* was the least stable RG in different tissues, cell types, and developmental stages, a result similar to that observed in *Glycine max* [57]. In the present study, *TIP41* and *ACT2* showed highly stable expression under heat stress. In a previous work, *TIP41* was a stable RG in *M. charantia* under UV and $CuSO_4$ treatments [51] and in *P. praeruptorum* under NaCl stress [54]. No previous research has shown that *TIP41* is the most stable RG under heat stress. *ACT2* is a traditional housekeeping gene widely used in many species, such as *Betula platyphylla* under salt stress and *Panicum virgatum* L. leaves and roots [58,59]. In this study, *ACT2* was an appropriate RG under heat stress, consistent with results from *P. praeruptorum* under heat stress [54]. Nonetheless, geNorm, NormFinder, and Bestkeeper all showed that *ADP* was the least stable RG under different experimental conditions. Previous studies have shown that *ADP* is the most stably RG in many species, such as Triticeae [60], *Triticum aestivum* L [61], and *Swingle citrumelo* [62]. However, *ADP* was recognized as the least stable gene by three algorithms for all samples in this study. Several papers have reported similar results. For example, *GAPDH* ranked the worst in *T. aestivum* but showed the most stability in *V. vinifera* cv. *Cabernet Sauvignon* [63,64]; *UBI* and *ACT* showed instability in *S. lycopersicum* [7], but performed suitably in *T. aestivum* [63]. These results indicate that the expression stabilities of RGs vary in different tissues and under environmental stresses.

Plant growth and development are generally affected by abiotic stresses (cold, heat, drought, and high salinity), and previous research has shown that *CAT* is the predominant enzyme controlling H_2O_2 levels. *CAT* has often been used as an abiotic-stress-inducible gene, upregulated by drought [40], cold [65], and salt [66] treatments. *CAT1* exhibited increased expression in response to exogenous ABA and osmotic stress in maize [39], and its expression was activated by drought, ABA and salt stress in *A. thaliana* [67]. In this study, *CAT* expression was upregulated gradually and reached a maximum value at 24 h (≈10-fold upregulated) under salt stress when using the two most stable RGs, *PP2A-1* and *UBQ10*, for normalization. The *CAT* expression pattern was consistent with previously reported responses of *PgCAT1* to salt stress, which showed the highest *PgCAT1* expression at 24 h [66] and the highest transcript levels as 10-fold [14]. However, the expression level of *CAT* was overestimated when the least stable RG, *ADP*, was used for normalization (Figure 5). Like salt stress, large differences were detected in *CAT* expression pattern when the least RG rather than the two most stable RGs was used for normalization under drought, cold, and heat stress (Figure 5). These results demonstrate that it is necessary to select a suitable stable RG for normalization of target gene expression under different conditions.

In this study, needles were used as the single material to test RGs in *L. olgensis*. The identified RGs may not apply to a broad range of developmental tissue samples of *L. olgensis*. For further studies, we will focus on exploring appropriate reference genes among different tissues and different developmental stages for accurate determination of gene expression in *L. olgensis* in further related research.

5. Conclusions

We selected 16 candidate RGs to validate suitable RGs using three statistical algorithms (geNorm, NormFinder, BestKeeper, and comprehensive analysis) for gene expression normalization in *L. olgensis* under drought, salt, cold, and heat stress. The results were compared and ranked using a comprehensive analysis. Based on the comprehensive analysis of gene stability, we identified *PP2A-1* and *GAPDH* as the most stable RGs under drought and cold stresses and *PP2A-1* and *UBQ10* as the two most stable RGs under salt stress. *TIP41* and *ACT2* were the most stable RGs under heat stress, and *ADP* was the least stable RG under all stresses. Furthermore, the expression profiles of *CAT* confirmed the importance of using two suitable RGs rather than one unstable RG under different stresses. Selection of suitable RGs provides a foundation for functional genomic studies in *L. olgensis* and other woody plants under abiotic stress conditions.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/11/2/193/s1, Figure S1: Agarose gel electrophoresis of PCR products for each of the 16 RGs and the one target genes in *Larix olgensis*., Figure S2: Melting curve for 16 RGs and one target gene *CAT* in *L. olgensis*, Figure S3: Average expression stability values (*M*-values) of the 16 RGs in *L. olgensis* calculated by geNorm. Table S1: Expression stability ranking of the 16 candidate reference genes under different abiotic stresses in *L. olgensis*.

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