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Selection and Validation of Reference Genes for the qRT-PCR Assays of *Populus ussuriensis* Gene Expression under Abiotic Stresses and Related ABA Treatment

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Abstract: *Populus ussuriensis* Kom. is one of the most important tree species for forest renewal in the eastern mountainous areas of Northeast China due to its fast growth, high yield, and significant commercial and ecological value. The selection of optimal reference genes for the normalization of qRT-PCR data is essential for the analysis of relative gene expression. In this study, fourteen genes were selected and assessed for their expression stability during abiotic stress (drought, high salinity, and cold stress) and after the treatment with the drought-related hormone ABA. Three algorithms were used, geNorm, NormFinder, and BestKeeper, and a comprehensive ranking of candidate reference genes was produced based on their output. The most appropriate reference genes were *UBQ10* and *RPL24* for drought and ABA treatment, *UBQ10* and *TUB3* for cold stress, and *UBQ10* and *60S rRNA* for high salinity. Overall, *UBQ10* was the most stable reference gene for use as an internal control, whereas *PP2A* was the least stable. The expression of two target genes (*P5CS2* and *GI*) was used to further verify that the selected reference genes were suitable for gene expression normalization. This work comprehensively assesses the stability of reference genes in *Populus ussuriensis* and identifies suitable reference genes for normalization during qRT-PCR analysis.

Keywords: *Populus ussuriensis*; gene expression; qRT-PCR; abiotic stress; abscisic acid

1. Introduction

Gene expression analysis is the basis of modern molecular biology and is widely used in genetic and developmental studies. Gene expression can be analyzed by several RNA quantification methods, including Northern blots, RNase protection assays, semi-quantitative reverse-transcription PCR, and quantitative real-time PCR (qRT-PCR), all of which rely on normalization procedures to quantitatively compare multiple samples [1]. qRT-PCR has become a common technique for gene expression analysis because of its rapidity, high sensitivity, and quantitative accuracy [2,3]. Moreover, qRT-PCR is regarded as the only effective way to measure the expression of genes with low mRNA copy number [4]. However, due to differences in RNA quality and quantity, in the efficiency of DNA synthesis, in PCR amplification, in pipetted volumes, and especially, in the activity of tissues and cells, qRT-PCR results can be severely biased [5]. A set of MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines aimed at improving the reliability and repeatability of research using qRT-PCR, has been published, which refers to a suitable reference gene (RG) for normalization as being essential for obtaining reliable results [6]. RGs whose expression

does not change among tissues or in response to experimental conditions are required to serve as internal controls and avoid bias in qRT-PCR [7]. However, the ideal RG that is stably expressed under all experimental conditions is almost non-existent [8,9]. Therefore, identifying multiple RGs that are stable under specific experimental conditions and using them to normalize target gene expression is essential for obtaining reliable qRT-PCR results.

RGs are generally a set of highly-conserved housekeeping genes (HKGs) whose expression is required to maintain baseline cellular function; they are typically expressed at relatively constant levels in different tissues [10]. Commonly-used housekeeping genes, including *ACT7* (*actin 7*), *β-TUB* (β-tubulin), 18S rRNA (18S ribosomal RNA), *EIF4A* (eukaryotic initiation factor 4AIII), *UBQ* (polyubiquitin), and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), are widely used for gene expression analysis under different experimental conditions in diverse plant species. In previous studies in rice, 18S rRNA and 28S rRNA exhibited the most stable expression when plants were grown under various environmental conditions, including osmotic stress, hormone treatment, and different temperatures. *UBQ5* and *EF1-α* were the most stable in various tissues and developmental stages, including 7-day-old light- and dark-grown seedlings, shoots and roots, mature leaves and seeds, and pre-pollinated or post-fertilized flowers [9]. 18S rRNA expression was the most stable in etiolated rice seedlings after several UV irradiation treatments [11]. Xu et al. [12] reported that *EF1α* and 18S rRNA were the most suitable RGs during adventitious root development, whereas *ACT* was the most unstable gene across multiple developmental stages in hybrid poplar. In soybeans, the expression levels of *ELF1B* and *CYP2* were stable, and these genes could be used as internal controls to normalize gene expression in different tissues [13]. In these studies, the expression of HKGs differed among organ types, developmental stages, and environmental conditions. Therefore, it is necessary to re-screen HKGs to determine which are suitable for specific experimental conditions.

Populus ussuriensis Kom. is one of the most important tree species for forest renewal in the eastern mountainous areas of Northeast China due to its fast growth and high yield. It is an excellent raw material for the production of paper and glued sheets because of its soft wood and high toughness, and it has significant commercial and ecological value [14,15]. Our previous work showed that the expression of genes encoding calmodulin-binding transcription activators differed between *P. ussuriensis* and *Populus trichocarpa* under cold stress, probably due to the differences in cold tolerance between the two species [15]. Ectopic expression of *LbDREB* from *Limonium bicolor* in *P. ussuriensis* has been shown to improve its drought resistance [16]. *PuHSFA4a* overexpression in *P. ussuriensis* increased tolerance to excess Zn by directly regulating *PuGSTU17* to remove reactive oxygen species from roots and by regulating *PuPLA2* to promote root growth [17]. Although several genes and their expression characteristics have been studied in *P. ussuriensis*, a review of the literature suggests that the traditional housekeeping gene *Actin* is the only RG that has been used in poplar (*Populus* spp.) [18–20]. Therefore, the identification of additional RGs in *P. ussuriensis* that are more stable under various stresses is essential for obtaining reliable qRT-PCR results.

Here, we report an experiment designed to select suitable RGs for qRT-PCR assays in *P. ussuriensis* under various abiotic stresses (drought, high salinity, and cold stress) and following treatment with the drought-related hormone ABA. Fourteen candidate RGs were selected from a local *P. ussuriensis* transcriptome database for the evaluation of expression stability with the stem-loop qRT-PCR method. Their expression stability was evaluated using three software programs: geNorm [21], NormFinder [22], and BestKeeper [23]. We also screened out two target genes from previous studies, delta 1-pyrroline-5-carboxylate synthetase 2 (*P5CS2*) and GIGANTEA (*GI*), and used them to assess the suitability of selected RGs. Our results will aid future studies of *P. ussuriensis* gene expression under abiotic stress and phytohormone treatment.

2. Materials and Methods

2.1. Plant Materials and Stress Treatments

Populus ussuriensis clone Donglin plants were grown in vitro on half-strength Murashige and Skoog (1/2 MS) medium (Phytotech) with 0.6% (w/v) agar and 2% (w/v) sucrose [15]. Plants were cultured in 250 mL plastic containers that held 100 mL of medium and were housed in a growth chamber at 25 °C with a 16 h light/8 h dark photoperiod and a light intensity of 46 µmol m^{-2} s^{-1}. For stress treatments, 5-leaf-stage in vitro plants were transferred to 1/2 MS medium supplemented with 7% PEG 6000 (drought), 150 mM NaCl (high salinity), or 100 µM abscisic acid (ABA) medium that had been precooled at 4 °C (cold stress). All media were adjusted to pH 5.8 and sterilized by autoclaving at 121 °C for 20 min. Leaf and root tissues from each treatment were independently collected at six time points (0 h, 6 h, 12 h, 24 h, 48 h, and 72 h). Non-treated plants were used as controls. All samples were immediately frozen in liquid nitrogen and stored at −80 °C for analysis. There were three biological replicates of each experimental condition.

2.2. Total RNA Extraction and cDNA Synthesis

RNA extraction was carried out according to the MIQE guidelines to ensure the reliability of the results [6]. Total RNA from each sample (approximately 100 mg of leaf or root tissue) was isolated using the CTAB (cetyltrimethylammonium bromide) method [24] and treated with DNaseI to remove genomic DNA contamination. The concentration and quality of each RNA sample were measured spectrophotometrically using the OD260/OD280 and OD260/OD230 absorption ratios, and all RNA samples were verified by 1% (*w/v*) agarose gel electrophoresis. cDNA was synthesized using the HiScript II Q RT SuperMix for qPCR (+gDNA wiper) (Vazyme, Nanjing, China) in a 20 µL reaction system according to the manufacturer's instructions. The cDNA was diluted 50-fold with ddH$_2$O and used as the template for PCR amplification.

2.3. Candidate Reference Gene Selection and PCR Primer Design

Fourteen candidate genes from a local *P. ussuriensis* transcriptome database were selected for assessment of their stability and suitability as RGs for the normalization of qRT-PCR data under various experimental treatments. Primers were designed based on the cDNA sequences of the candidate reference genes using Primer Premier 6 software (http://www.premierbiosoft.com/) with the following criteria: primer length 17–25 bp (optimum 20), product size 75–300 bp, melting temperature 50–65 °C (optimum 60 °C), and GC content 40%–60% (optimum 50%). The specificity of all selected primer pairs was assessed by PCR using the cDNA of control groups at 0 h as the template, and gene fragments were visualized following 2.0% (w/v) agarose gel electrophoresis to ensure reliability. All primers mentioned above are listed in Table 1.

2.4. qRT-PCR

TransStart$^®$ Top Green qPCR SuperMix (TransGen, Beijing, China) was used to perform qRT-PCR in 96-well optical reaction plates (Applied Biosystems, Foster City, CA, USA). Reactions were prepared in 20 µL volumes containing: 10 µL of 2 × TransStart$^®$ Top Green qPCR SuperMix, 7.2 µL of ddH$_2$O, 2 µL of 50-fold diluted cDNA, and 0.4 µL of each specific primer, prepared to a final concentration of 10 µM. The qRT-PCR program consisted of an initial step at 94 °C for 30 s, followed by 40 denaturation cycles at 94 °C for 5 s and primer annealing at 60 °C for 30 s. Next, melting curves ranging from 60 °C to 95 °C with an increment of 0.2 °C were recorded in each reaction to check the specificity of the amplicons. Three technical replicates were analyzed for each biological sample. The threshold cycle (Ct) was automatically measured. A total of 48 cDNA samples from six time points in the control groups were used to determine the mean amplification efficiency (*E*) of each primer pair using the LinRegPCR program. The relative expression levels of two target genes (*PuP5CS2* and *PuGI*) were calculated with the equation 2$^{-\Delta\Delta Ct}$ under abiotic stress and ABA treatment, respectively [25].

Table 1. Description of candidate reference genes (RGs) and target genes, primer sequences, product sizes, and amplicon characteristics.

Gene Symbol	Accession Number	Gene Name	Primer Sequence (5′–3′)	Amplicon Size (bp)	Amplification Efficiency (*E*)	Correlation Coefficient (R^2)
			Reference Genes			
ACT7	MN872586	Actin7	ATGGAACTGGAATGGTGAAGGCTGG CCGTGCTCAATGGGGTATTTCAAGGTC	187	1.85	0.9971
TUA2	MN872587	Tubin alpha 2	TTCAAGTCGGAAATGCCTGCTGGGAAC GTTGGCAGCATCTTCCTTGCCGC	263	1.90	0.9968
TUB3	MN872588	Tubulin beta chain 3	ATGAGGGAAATCCTTCACATTCAAGGAGG CCAGGCTCAAGATCCATAAGCACAGC	212	1.99	0.9927
GAPDH	MN872589	Glyceraldehyde 3-phosphate dehydrogenase	GGAAGCACGGAGATATCAA ACCACCCTTCAAATGAGCA	187	2.08	0.9936
UBQ10	MN872590	Polyubiquitin	GGAGTCAACCCTTCACTTGGTGC GAGGACAAGGTGAAGGGTGGACTCC	253	1.97	0.9925
EF1-α	MN872591	Elongation factor 1-alpha	CACTGGTCACTTGATCTACAAGCTTG TGACAGTGCAGTAGTACCTGG	200	1.82	0.998
SAND	MN872592	SAND family	ATGTCCTCATCCGATTCCAACTCCTC CACGACACTCCTGACGAGGCC	240	2.00	0.9963
60S rRNA	MN872593	60S acidic ribosomal protein P0-A	CTTCAGCCCTGAGGTGCTGGACC GCACCCCCAGAAGCAGCAGC	276	2.05	0.9959
PP2A	MN872594	Protein phosphatase	CAGCTAAGGTTAAACTCAATCCGTAGAC CCTCGACAGTGCAAAGAGTCTCC	226	1.98	0.9935
CYP2	MN872595	Cyclophilin	ATTGGCAAGATGAAAGCAGGTAGGATTG CTTTGCGCCATAGATTGATTCTCCTC	234	1.99	0.9986
F-box	MN872596	F-box family protein	GGGGCTGGAATCAGTGGGAG GCAGAAAGATCAAGATCTTGACGGC	162	2.01	0.9918
RPL24	MN872597	60S ribosomal protein L24	CCTCGACTGTATACCCGGGGC GATTCCTATCATATCTCTCAGGCC	225	1.99	0.9996
RPL25	MN872598	Large subunit ribosomal protein L23Ae	GGCTGATGCAAAGACACAGGCAC GGTTCCTTTCCTTCTTCAATGTCCTGG	134	1.94	0.9985
UBC	MN872599	Ubiquitin-protein ligase	GTCAACCCCAGCCAGGAAGAGG CTTAAACGTCCCTCCATCCCATGG	160	1.99	0.9974
			Target Genes			
P5CS2	MN872600	Delta1-pyrroline-5-carboxylate synthase 1	CACGGATCCTTCTCGTGGTT TTTTTGGAGATCGGCGAAGC	247	1.99	0.9991
GI	MN872601	GIGANTEA protein	GCAGCCCTCCATTTGCTTCT TTGCCATCACTGCTGCTTCT	178	1.97	0.9942

2.5. Statistical Analysis to Determine Expression Stability with geNorm, NormFinder, and BestKeeper

To analyze the expression stability of 14 candidate RGs, the cycle thresholds (Cts) from different tissues under different stresses and treatments were analyzed using box plots and three different algorithms of the statistical tools geNorm [21], NormFinder [22], and BestKeeper [23]. The geNorm tool calculates the average expression stability value (*M* value) of RGs based on the average pairwise variation (*V*) value. With 1.5 as the threshold, a lower *M* value corresponds to a more stable gene. The *V* value calculated by geNorm determines the optimal number of RGs required to accurately normalize the data and is based on a threshold value of *Vn+1* < 0.15. The NormFinder tool analyzes the stability of candidate RG expression based on the results of intra- and inter-group variation analysis and automatically ranks the genes according to their stability value (SV) [22]. A lower SV corresponds to higher stability. BestKeeper assesses the stability of RGs based on the standard deviation (SD) and coefficient of variation (CV) of the Ct values. An RG with an SD less than 1 is considered to be stably expressed. The CV decreases with the SD, indicating that the RG is more stable [23,26].

2.6. Validation of Candidate Reference Genes

To assess the accuracy of the expression stability of candidate RGs, the geometric means of the ranking results from geNorm, NormFinder, and BestKeeper for each stress or treatment were used to calculate a comprehensive ranking of candidate genes. The lower the comprehensive ranking, the better the stability of gene expression. Finally, the two best RGs for each experimental condition were used to normalize the expression of two target genes, *PuP5CS2* and *PuGI*, under that condition.

3. Results

3.1. Selection, Amplification Efficiency, and Ct Value Range of Candidate Reference Genes

We compared RGs used previously in other plant species, such as *Arabidopsis thaliana*, with a local *P. ussuriensis* transcriptome database using a local Blast search in Bioedit. Fourteen candidate RGs were selected for use in the gene normalization studies (Table 1). To evaluate the amplification efficiency of primer pairs, PCR amplification specificities were assessed using 2.0% agarose gel electrophoresis (Figure S1), melting curves (Figure S2), and direct sequencing. The results showed that we obtained specific target fragments of the expected lengths and sequences. The qRT-PCR amplification efficiency (*E*) and correlation coefficient (R^2) were obtained from the slope of the calibration curves (Table 1). For the 14 candidate RGs and two target genes, the *E* values ranged from 1.85 to 2.08 and the R^2 values ranged from 0.9918 to 0.9996 (Table 1). The results showed that all 16 pairs of primers met the requirements for use in qRT-PCR experiments.

3.2. Expression Profiles of Candidate Reference Genes under Abiotic Stresses and Hormone Treatment

The Ct value is the cycle number at which the reaction curve intersects the threshold line; it indicates the number of cycles required to detect a real signal from the sample. High abundance cDNA samples reach this threshold at a lower Ct value during PCR amplification, indicating a higher gene expression level [27]. The transcript abundances of 14 RGs were determined based on Ct values from the qRT-PCR analysis of 48 samples, including six time points for roots and leaves under four treatment conditions (drought, high salinity, cold stress, and ABA treatment). Mean Ct values ranged from 15.15 (*UBQ10*) to 29.86 (*SAND*) (Figure 1). Under all stresses and treatments, *UBQ10* had the highest expression and *SAND* had the lowest expression. The coefficient of variation (CV) is related to the degree of fluctuation in Ct values. The highest and lowest CVs were 7.25% (*TUA2*) and 0.85% (*UBQ10*) under drought, 9.4% (*TUA2*) and 0.94% (*F-box*) under ABA treatment, 13.30% (*UBC*) and 1.80% (*UBQ10*) under cold stress, and 11.07% (*RPL25*) and 1.18% (*SAND*) under high salinity (Figure 1). The average CVs of the candidate RGs were ranked as *UBQ10* < *CYP2* < *SAND* < *GAPDH* < *F-box* < *RPL24* < *60S rRNA* < *PP2A* < *UBC* < *TUB3* < *ACT7* < *RPL25* < *EF1-α* < *TUA2*.
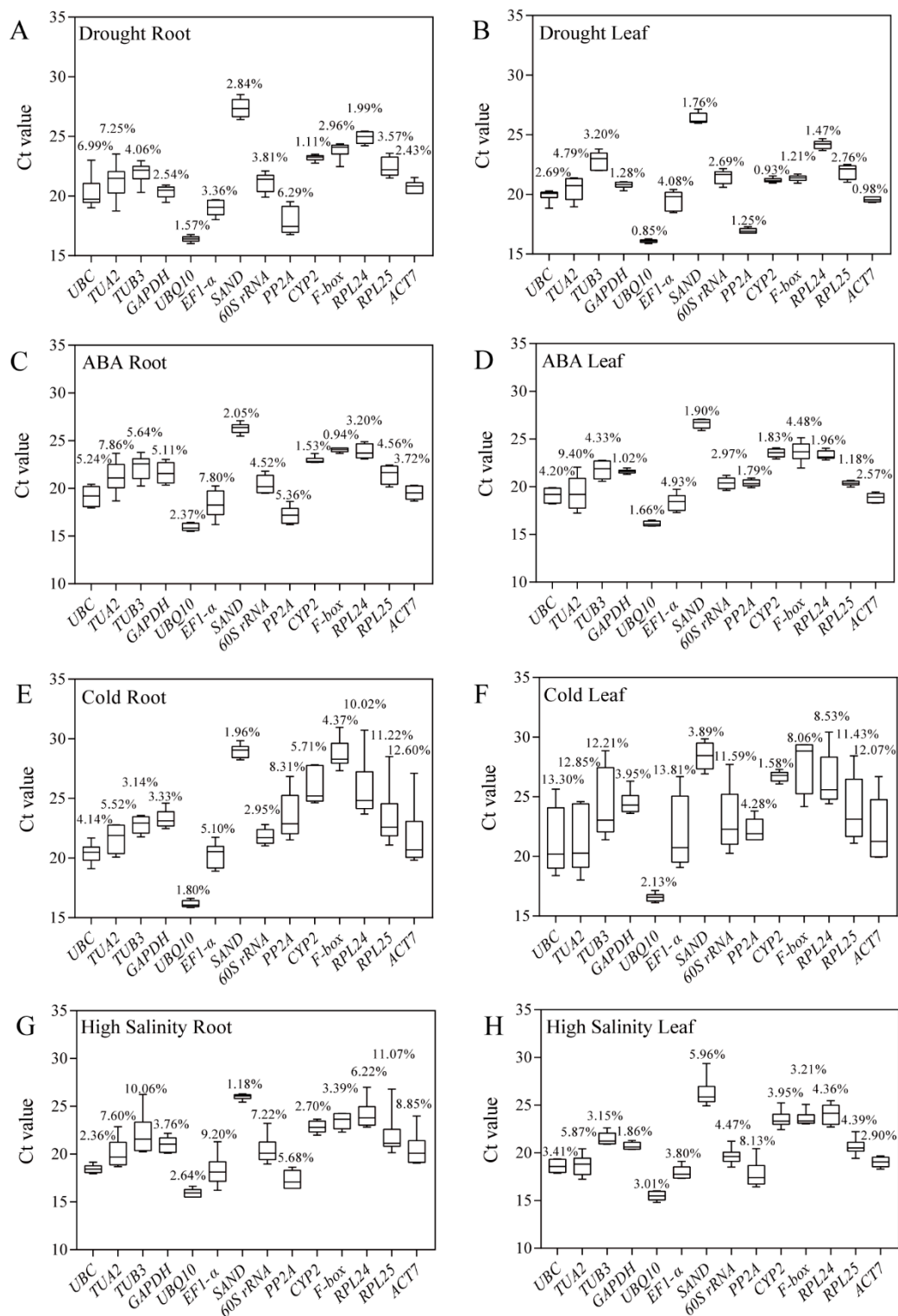
Figure 1. Threshold cycle (Ct) values of fourteen candidate reference genes in roots and leaves under various stresses and ABA treatment. Boxplot of Ct values of fourteen candidate reference genes from qRT-PCR analysis of root and leaf tissues. (**A**,**B**) Drought stress; (**C**,**D**) ABA treatment; (**E**,**F**) cold stress; and (**G**,**H**) high salinity stress. For each reference gene, the line inside the box is the median. The top and bottom lines of the box are the first and third quartiles, respectively. The values on top of box represent the coefficient of variation (CV) values.

3.3. Expression Stability of Candidate Reference Genes under Abiotic Stress and Hormone Treatment

To identify the most suitable RGs for gene expression analysis under drought, high salinity, cold stress, and ABA treatment in *P. ussuriensis*, the expression stability of candidate RGs was evaluated by three different algorithms in geNorm, NormFinder, and BestKeeper.

3.3.1. geNorm Analysis

The geNorm tool calculates an RG stability value (*M* value) based on the average pairwise variation *V* value [21]. The more stable the gene, the lower the *M* value, and vice versa. As shown in Figure 2 and Table 2, all 14 RGs showed average *M* values less than 1.5 under drought, high salinity, cold stress, and ABA treatment. *UBQ10* and *ACT7* (*M* = 0.300) were the most stable RGs under drought stress. *RPL24* and *ACT7* were the most stable RGs under ABA treatment (*M* = 0.22). *F-box* was the least stable RG under drought stress and ABA treatment, with *M* values of 0.88 and 0.94, respectively. Under cold stress, the stability of *F-box* was again the lowest (*M* = 1.88), consistent with the results of the drought and ABA treatment. *TUB3* and *EF1-α* were the most stable RGs with *M* values of 0.36. Under high salinity stress, *TUA2* and *RPL24* were the most stable RGs with *M* values of 0.54, and *PP2A* was the least stable RG with an *M* value of 1.28. For all conditions except cold stress, *ACT7* and *RPL24* were among the top three stable RGs. By contrast, *F-box* was the least stable RG in all conditions except high salinity, in which *PP2A* was the least stable RG.
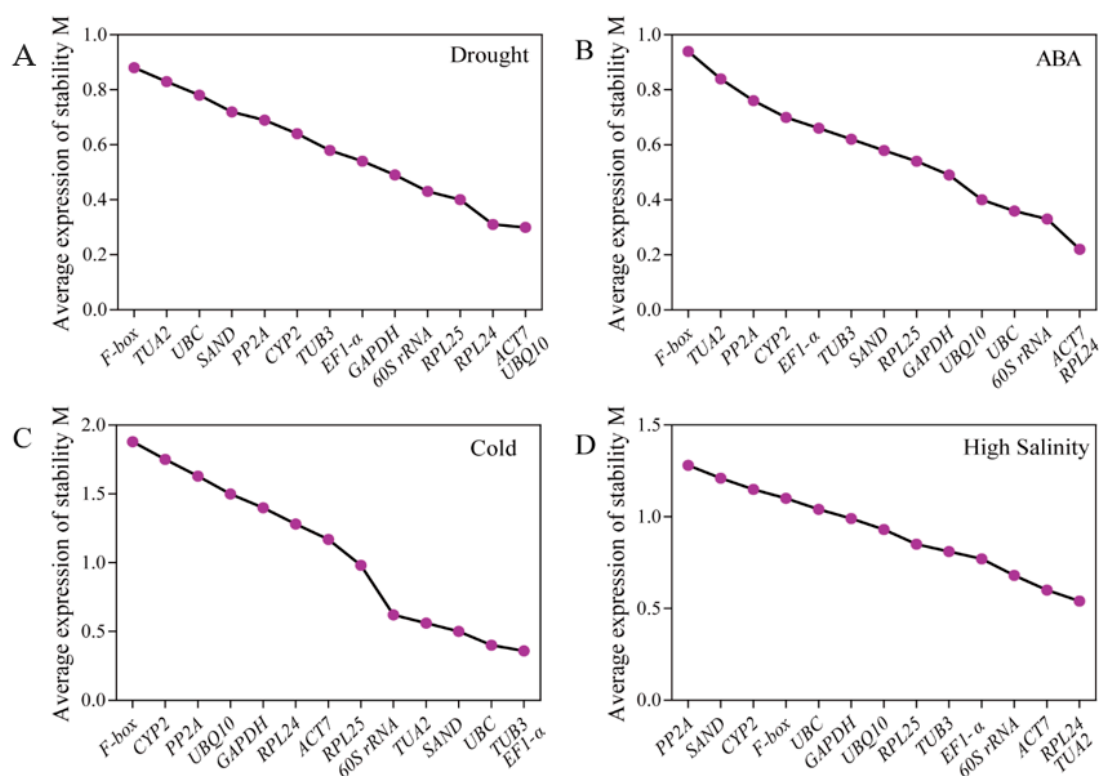

Figure 2. Average expression stability value (M) and ranking of the fourteen RGs across all stresses and ABA treatment calculated using geNorm. (**A**) Drought stress, (**B**) ABA treatment, (**C**) cold stress, and (**D**) high salinity stress. The least stable genes are listed on the left, and the most stable genes are listed on the right.

Sometimes a single RG does not meet the stability requirements for use in normalization, and two or more RGs are therefore needed to reduce error and obtain more accurate results [28]. geNorm can determine the optimal number of candidate RGs based on the calculation of pairwise variation (Vn/Vn+1). The program suggests a threshold of 0.15 as a standard for selecting the best pairwise variation value (V value) for normalization. There is no need to add more candidate RGs if the V

value is lower than 0.15. As shown in Figure 3, the value of V5/V6 among 14 candidate RGs under salt stress was 0.125, which is smaller than 0.15, suggesting that the most suitable RG combination contained four genes, namely *60S rRNA*, *ACT7*, *TUA2*, and *RPL24*. Similarly, the most appropriate RG combinations under drought, ABA, and cold stress were *ACT7* and *UBQ10*, *ACT7* and *RPL24*, and *TUB3* and *EF1-α*, respectively.

Table 2. Expression stability of candidate reference genes calculated by geNorm.

Rank	Drought		ABA		Cold		High Salinity	
	Gene Name	***M***	**Gene Name**	***M***	**Gene Name**	***M***	**Gene Name**	***M***
1	*UBQ10*	0.30	*RPL24*	0.22	*TUB3*	0.36	*TUA2*	0.54
2	*ACT7*	0.30	*ACT7*	0.22	*EF1-α*	0.36	*RPL24"*	0.54
3	*RPL24*	0.31	*60S rRNA*	0.33	*UBC*	0.40	*ACT7*	0.60
4	*RPL25*	0.40	*UBC*	0.36	*SAND*	0.50	*60S rRNA*	0.68
5	*60S rRNA*	0.43	*UBQ10*	0.40	*TUA2*	0.56	*EF1-α*	0.77
6	*GAPDH*	0.49	*GAPDH*	0.49	*60S rRNA*	0.62	*TUB3*	0.81
7	*EF1-α*	0.54	*RPL25*	0.54	*RPL25*	0.98	*RPL25*	0.85
8	*TUB3*	0.58	*SAND*	0.58	*ACT7*	1.17	*UBQ10*	0.93
9	*CYP2*	0.64	*TUB3*	0.62	*RPL24*	1.28	*GAPDH*	0.99
10	*PP2A*	0.69	*EF1-α*	0.66	*GAPDH*	1.40	*UBC*	1.04
11	*SAND*	0.72	*CYP2*	0.70	*UBQ10*	1.50	*F-box*	1.10
12	*UBC*	0.78	*PP2A*	0.76	*PP2A*	1.63	*CYP2*	1.15
13	*TUA2*	0.83	*TUA2*	0.84	*CYP2*	1.75	*SAND*	1.21
14	*F-box*	0.88	*F-box*	0.94	*F-box*	1.88	*PP2A*	1.28

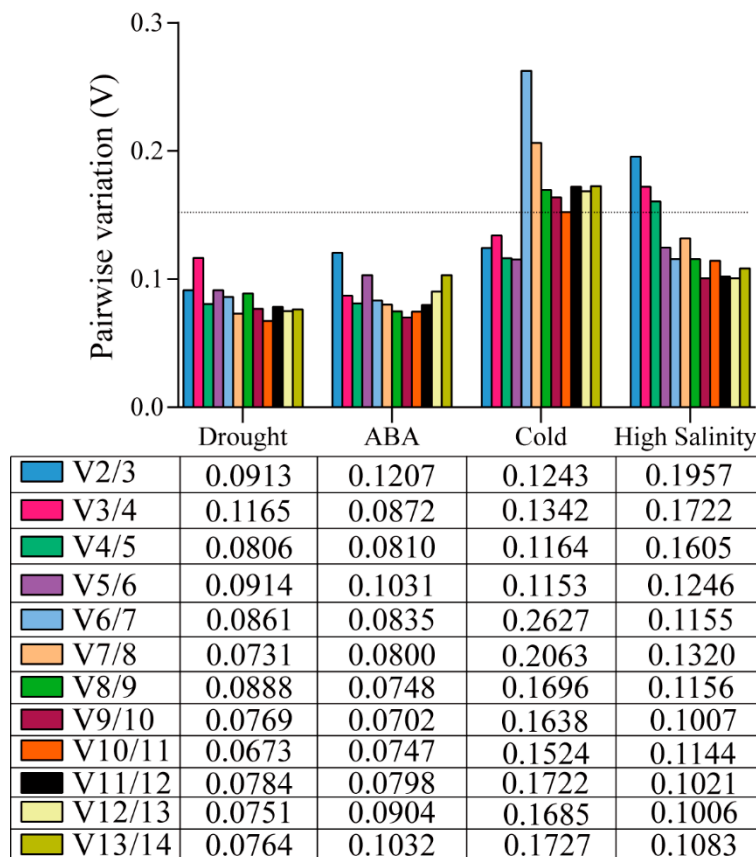|	Drought	ABA	Cold	High Salinity
V2/3	0.0913	0.1207	0.1243	0.1957
V3/4	0.1165	0.0872	0.1342	0.1722
V4/5	0.0806	0.0810	0.1164	0.1605
V5/6	0.0914	0.1031	0.1153	0.1246
V6/7	0.0861	0.0835	0.2627	0.1155
V7/8	0.0731	0.0800	0.2063	0.1320
V8/9	0.0888	0.0748	0.1696	0.1156
V9/10	0.0769	0.0702	0.1638	0.1007
V10/11	0.0673	0.0747	0.1524	0.1144
V11/12	0.0784	0.0798	0.1722	0.1021
V12/13	0.0751	0.0904	0.1685	0.1006
V13/14	0.0764	0.1032	0.1727	0.1083

Figure 3. Pairwise variation (Vn/Vn+1) values of the fourteen experimental subsets calculated using geNorm. The cut-off value to determine the optimal number of RGs for qRT-PCR normalization is 0.15.

3.3.2. NormFinder Analysis

The NormFinder program analyzes the stability of candidate RG expression based on the results of variance analysis and ranks the genes according to their stability [22]. As shown in Table 3, *UBQ10* was the most stable candidate RG under drought, similar to the results of geNorm. But unlike the results of geNorm, *UBQ10* was also ranked first in cold stress and ABA treatment. However, *TUB3* (ranked first by geNorm) in cold stress and *RPL24* and *ACT7* (ranked first by geNorm) in ABA treatment had stabilities similar to that of *UBQ10*, a result that differed from the geNorm analysis. For high salinity, *60S rRNA* was the most stable RG, which is again different from the result of the geNorm analysis.

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

Rank	Drought		ABA		Cold		High Salinity	
	Gene Name	**SV [1]**	**Gene Name**	**SV**	**Gene Name**	**SV**	**Gene Name**	**SV**
1	*UBQ10*	0.08	*UBQ10*	0.05	*UBQ10*	0.12	*60S rRNA*	0.15
2	*ACT7*	0.10	*RPL24*	0.12	*TUB3*	0.14	*RPL25*	0.17
3	*RPL24*	0.10	*ACT7*	0.12	*SAND*	0.16	*UBQ10*	0.17
4	*GAPDH*	0.19	*60S rRNA*	0.13	*UBC*	0.17	*ACT7*	0.19
5	*60S rRNA*	0.19	*UBC*	0.17	*EF1-α*	0.18	*RPL24*	0.22
6	*TUB3*	0.20	*SAND*	0.19	*TUA2*	0.20	*UBC*	0.23
7	*EF1-α*	0.20	*TUB3*	0.20	*60S rRNA*	0.21	*EF1-α*	0.25
8	*RPL25*	0.22	*EF1-α*	0.20	*GAPDH*	0.24	*TUB3*	0.25
9	*TUA2*	0.23	*GAPDH*	0.23	*RPL25*	0.24	*SAND*	0.28
10	*CYP2*	0.24	*RPL25*	0.25	*RPL24*	0.26	*TUA2*	0.28
11	*UBC*	0.25	*TUA2*	0.25	*ACT7*	0.27	*GAPDH*	0.31
12	*F-box*	0.26	*CYP2*	0.28	*F-box*	0.34	*CYP2*	0.32
13	*SAND*	0.27	*PP2A*	0.33	*PP2A*	0.35	*F-box*	0.32
14	*PP2A*	0.28	*F-box*	0.43	*CYP2*	0.40	*PP2A*	0.39

[1] SV represents stable value.

3.3.3. BestKeeper Analysis

BestKeeper assesses the stability of RGs by calculating the standard deviation (SD) and coefficient of variation (CV) of the Ct values. An RG with an SD of less than 1.0 is considered to be stably expressed. The CV decreases with the SD, indicating that the RG is more stable [23,26]. As shown in Table 4, *UBQ10* was the most stable RG with the lowest SD and CV values under drought, high salinity, cold stress, and ABA treatment. Additionally, the SD and CV values of twelve candidate genes were less than 1.0 under drought stress, and only a few genes had SD and CV values greater than 1.0 under salinity stress and ABA treatment, indicating that the expression of most candidate RGs was relatively stable.

Table 4. Expression stability of candidate reference genes calculated by BestKeeper.

Rank	Drought			ABA			Cold			High Salinity		
	Gene Name	SD[1]	CV[2]	Gene Name	SD	CV	Gene Name	SD	CV	Gene Name	SD	CV
1	*UBQ10*	0.21	1.28	*UBQ10*	0.28	1.75	*UBQ10*	0.30	1.86	*UBQ10*	0.39	2.49
2	*GAPDH*	0.34	1.65	*SAND*	0.41	1.55	*SAND*	0.70	2.42	*UBC*	0.42	2.26
3	*RPL24*	0.45	1.85	*CYP2*	0.42	1.80	*GAPDH*	0.79	3.30	*GAPDH*	0.49	2.36
4	*RPL25*	0.53	2.39	*F-box*	0.50	2.10	*CYP2*	0.94	3.56	*F-box*	0.55	2.33
5	*60S rRNA*	0.56	2.61	*ACT7*	0.55	2.85	*F-box*	1.26	4.46	*SAND*	0.58	2.22
6	*UBC*	0.59	2.93	*GAPDH*	0.55	2.56	*PP2A*	1.16	5.08	*CYP2*	0.58	2.50
7	*ACT7*	0.60	2.99	*RPL24*	0.55	2.35	*60S rRNA*	1.27	5.68	*EF1-α*	0.83	4.58
8	*PP2A*	0.63	3.63	*60S rRNA*	0.62	3.04	*TUB3*	1.47	6.23	*60S rRNA*	0.84	4.17
9	*EF1-α*	0.64	3.30	*RPL25*	0.74	3.52	*RPL24*	1.75	6.71	*RPL24*	0.92	3.80
10	*SAND*	0.66	2.45	*UBC*	0.76	3.96	*UBC*	1.41	6.77	*PP2A*	0.93	5.28
11	*TUB3*	0.68	3.02	*EF1-α*	0.89	4.86	*EF1-α*	1.62	7.67	*ACT7*	0.94	4.78
12	*TUA2*	0.97	4.63	*TUB3*	0.93	4.21	*TUA2*	1.66	7.73	*TUA2*	1.05	5.41
13	*CYP2*	1.01	4.55	*TUA2*	1.52	7.49	*RPL25*	1.97	8.34	*TUB3*	1.06	4.89
14	*F-box*	1.27	5.63	*PP2A*	1.58	8.42	*ACT7*	2.01	9.16	*RPL25*	1.08	5.07

[1] SD represents standard deviation. [2] CV represents coefficient of variation.

3.4. Comprehensive Stability Analysis of the Reference Genes

The final comprehensive RG ranking is shown in Table 5 and Figure 4, based on the results from geNorm, NormFinder, and BestKeeper. *UBQ10* and *RPL24* were the most stable RGs under drought stress and ABA treatment, *UBQ10* plus *TUB3* was the best combination under cold stress, and *UBQ10* plus *60S rRNA* was the best combination under high salinity stress. *UBQ10* was the most stable RG under all experimental conditions.
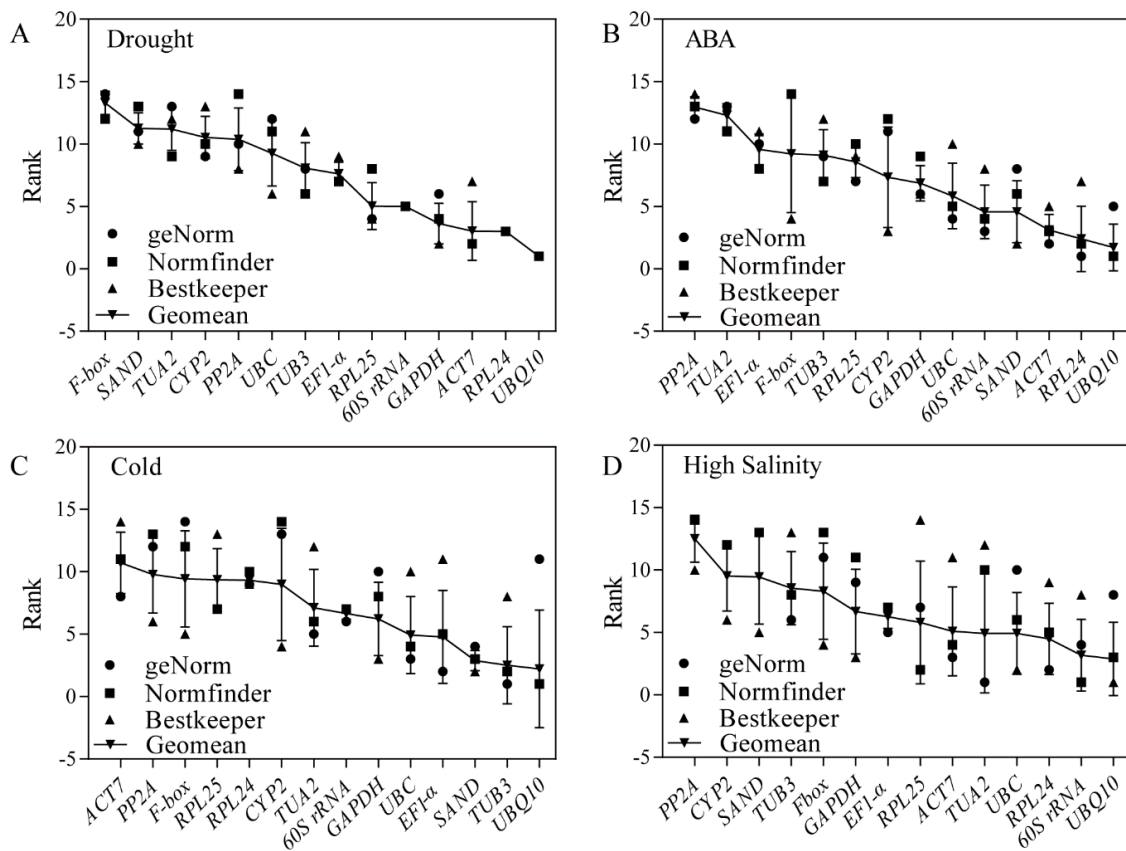

Figure 4. Comprehensive ranking of the fourteen RGs across all stresses and ABA treatment calculated as the geometric mean (Geomean) of three types of ranking (geNorm, NormFinder, and BestKeeper) for each sample group. (**A**) Drought stress, (**B**) ABA treatment, (**C**) cold stress, and (**D**) high salinity stress.

Table 5. Expression stability ranking of the 14 candidate reference genes.

Method	1	2	3	4	5	6	7	8	9	10	11	12	13	14
A. Ranking order under drought stress														
geNorm	UBQ10	ACT7	RPL24	RPL25	60S rRNA	GAPDH	EF1-α	TUB3	CYP2	PP2A	SAND	UBC	TUA2	F-box
NormFinder	UBQ10	ACT7	RPL24	GAPDH	60S rRNA	TUB3	EF1-α	RPL25	TUA2	CYP2	UBC	F-box	SAND	PP2A
BestKeeper	UBQ10	GAPDH	RPL24	RPL25	60S rRNA	UBC	ACT7	PP2A	EF1-α	SAND	TUB3	TUA2	CYP2	F-box
Comprehensive Ranking	UBQ10	RPL24	ACT7	GAPDH	60S rRNA	RPL25	EF1-α	TUB3	UBC	PP2A	CYP2	TUA2	SAND	F-box
B. Ranking order under ABA treatment														
geNorm	RPL24	ACT7	60S rRNA	UBC	UBQ10	GAPDH	RPL25	SAND	TUB3	EF1-α	CYP2	PP2A	TUA2	F-box
NormFinder	UBQ10	RPL24	ACT7	60S rRNA	UBC	SAND	TUB3	EF1-α	GAPDH	RPL25	TUA2	CYP2	PP2A	F-box
BestKeeper	UBQ10	SAND	CYP2	F-box	ACT7	GAPDH	RPL24	60S rRNA	RPL25	UBC	EF1-α	TUB3	TUA2	PP2A
Comprehensive Ranking	UBQ10	RPL24	ACT7	60S rRNA	SAND	UBC	GAPDH	CYP2	RPL25	TUB3	F-box	EF1-α	TUA2	PP2A
C. Ranking order under cold stress														
geNorm	TUB3	EF1-α	UBC	SAND	TUA2	60S rRNA	RPL25	ACT7	RPL24	GAPDH	UBQ10	PP2A	CYP2	F-box
NormFinder	UBQ10	TUB3	SAND	UBC	EF1-α	TUA2	60S rRNA	GAPDH	RPL25	RPL24	ACT7	F-box	PP2A	CYP2
BestKeeper	UBQ10	SAND	GAPDH	CYP2	F-box	PP2A	60S rRNA	TUB3	RPL24	UBC	EF1-α	TUA2	RPL25	ACT7
Comprehensive Ranking	UBQ10	TUB3	SAND	EF1-α	UBC	GAPDH	60S rRNA	TUA2	CYP2	RPL24	RPL25	F-box	PP2A	ACT7
D. Ranking order under high salinity stress														
geNorm	TUA2	RPL24″	ACT7	60S rRNA	EF1-α	TUB3	RPL25	UBQ10	GAPDH	UBC	F-box	CYP2	SAND	PP2A
NormFinder	60S rRNA	RPL25	UBQ10	ACT7	RPL24	UBC	EF1-α	TUB3	SAND	TUA2	GAPDH	CYP2	F-box	PP2A
BestKeeper	UBQ10	UBC	GAPDH	F-box	SAND	CYP2	EF1-α	60S rRNA	RPL24	PP2A	ACT7	TUA2	TUB3	RPL25
Comprehensive Ranking	UBQ10	60S rRNA	RPL24″	TUA2	UBC	ACT7	RPL25	EF1-α	GAPDH	F-box	TUB3	SAND	CYP2	PP2A

3.5. Reference Gene Validation

To verify the accuracy of the stable expression of the RGs, two marker genes (*PuP5CS2* and *PuGI*) were used as positive controls in roots and leaves under drought, high salinity, cold stress, and ABA treatment (Figures 5 and 6). *P5CS2* is a regulatory enzyme in proline biosynthesis and plays a crucial role in abiotic stress resistance and tolerance [29–32]. *GI* encodes a nuclear-localized protein that regulates both freezing tolerance and various development processes such as photoperiod-mediated flowering [33]. We selected two RGs based on the comprehensive ranking results for each stress and used them to normalize the *PuP5CS2* and *PuGI* expression data. The selected RGs were *UBQ10* and *RPL24* for drought, ABA, *UBQ10*, and *TUB3* for cold stress, and *UBQ10* and *60S rRNA* for high salinity. When the two optimal RGs were used for normalization, the expression levels of *PuP5CS2* and *PuGI* differed among treatments (Figures 5 and 6). Under drought stress, *PuP5CS2* was induced in roots and reached a maximum expression value at 48 h (Figure 4). *PuP5CS2* was also upregulated by ABA treatment and reached a maximum value at 24 h (Figure 4). Under cold stress, *PuP5CS2* was upregulated in both roots and leaves and was significantly induced at 48 h and 72 h. *PuGI* was significantly upregulated (>20 fold) in leaves under cold stress, and its expression also responded to drought and high salinity (Figure 5). Similar expression patterns were observed when we used the two single appropriate genes as RGs. However, the expression patterns of *PuP5CS2* and *PuGI* showed significant fluctuations when the least stable gene, *PP2A*, was selected as the RG, and their expression patterns were not consistent with those observed when more appropriate RGs were used in specific experimental conditions (Figures 5 and 6).
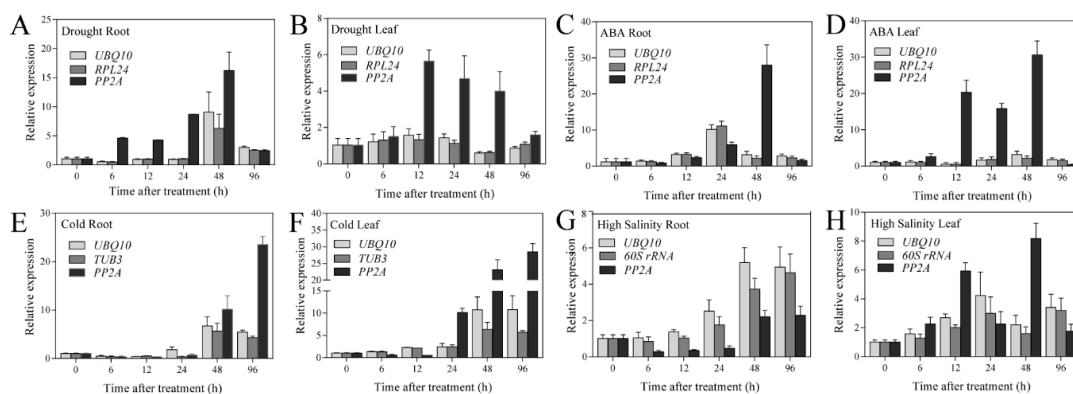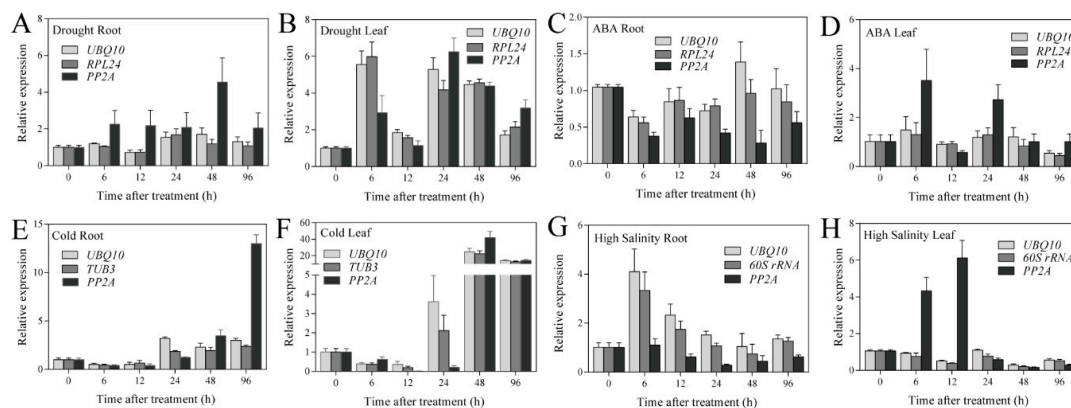

Figure 5. Relative expression levels of *PuP5CS2* under different experimental conditions normalized by the top two stable genes and an unstable gene. (**A**,**B**) Drought stress; (**C**,**D**) ABA treatment; (**E**,**F**) cold stress; and (**G**,**H**) high salinity stress.

Figure 6. Relative expression levels of *PuGI* under different experimental conditions normalized by the top two stable genes and an unstable gene. (**A**,**B**) Drought stress; (**C**,**D**) ABA treatment; (**E**,**F**) cold stress; and (**G**,**H**) high salinity stress.

4. Discussion

To improve forest productivity and maintain the forest's economic benefits, it is necessary to identify stably-expressed RGs for use in qRT-PCR normalization when studying transcriptional regulatory pathways and the functions of key genes in *P. ussuriensis*. Abiotic stresses such as drought, high salinity, and cold temperatures are the main environmental factors that limit forest growth. ABA is a key signaling molecule during abiotic stress response and has particularly important biological functions in the response to drought [34], high salinity [35], and low temperature [36]. Under these stresses, increased levels of endogenous ABA can upregulate many stress-related genes in an ABA-dependent manner [37]. Numerous studies have identified and validated RGs in rice [38], maize [39], soybean [40,41], and carrot [42] under abiotic stress. However, there are currently no reports of RGs that are universally and stably expressed under abiotic stress in *P. ussuriensis*. The purpose of our study was therefore to select suitable RGs for qRT-PCR in *P. ussuriensis* under various abiotic stresses.

Fourteen candidate RGs were selected, including traditional housekeeping genes (*ACT7*, *UBQ10*, *GAPDH*, *TUA2*, *TUB3*, and *EF1-α*) and widely-used RGs (*60S rRNA*, *SAND*, *CYP2*, *PP2A*, *F-box*, *RPL24*, *RPL25*, and *UBC*). The suitability of these candidate RGs was then evaluated using three algorithms including geNorm, NormFinder, and BestKeeper. Under drought, all algorithms ranked *UBQ10* as having the highest stability. However, in other treatments, differences were observed in gene stability values and relative rankings generated by the different algorithms. For example, *ACT7* was ranked second by geNorm and NormFinder, but it was ranked in the middle of the 14 candidate RGs by BestKeeper. Under ABA treatment, *RPL24* and *ACT7* were ranked among the top three most stable genes by geNorm and NormFinder, but they were ranked in intermediate positions by BestKeeper. These differences may be due to the different algorithms used to calculate stability. Previous studies have reported similar differences in stability ranking [43]. In general, the use of more than two algorithms is recommended to screen for stably-expressed RGs [21].

In our study, we calculated a comprehensive ranking of candidate genes based on the geometric mean of the rank results from three algorithms in each experimental condition. *UBQ10* ranked as the most stable RG under all stresses and ABA treatment in *P. ussuriensis*. Our results were consistent with the stable expression of *UBQ* reported in *Platycladus orientalis* under NaCl and ABA treatments [44], white clover leaves and stolons under water-limited and well-watered conditions [45], *Brachypodium distachyon* in different tissues and under various hormone treatments [46] and *A. thaliana* green siliques and seeds under hormone treatment [47]. However, *UBQ* showed less-stable expression in salt-stressed *Suaeda aralocaspica* seeds [48] and in different developmental stages and photoperiodic treatments in soybean [13]. *UBQ*, which is primarily involved in proteolysis in the ubiquitin–proteasome system, appears to be regulated differently in different plant species and cell types and in response to different experimental conditions [46,49]. Therefore, although *UBQ10* was a universally reliable RG in our study, further verification is needed to determine its suitability for use as an RG under other experimental conditions.

According to the comprehensive ranking, *RPL24* emerged as a relatively-stable choice for drought and high salinity stresses, and ABA treatment. *RPL24*, named as 60S ribosomal protein L24, is one of several large ribosomal subunit proteins widely used as an RG in many studies. For example, *60S rRNA* was stably expressed among different developmental stages in *Panax ginseng* [50]. Others include *RPL30*, which has been used as an RG in roots and shoots of soybean seedlings under cold stress and ABA treatment [40], and *RPL2*, which is stably expressed under nitrogen, cold, and light stress in tomato [8]. Ribosomal protein genes are also widely used as RGs in animals [51–53]. In our study, *RPL25*, which encodes a different large ribosomal subunit protein, was also evaluated. However, *RPL25* was not an optimal RG under any condition in *P. ussuriensis*. In our study, the performances of *RPL24* and *60S rRNA* were within an acceptable range in all stresses and ABA treatments. *Actin* has been used extensively as a traditional HKG for normalization of gene expression data [54]. In our study, geNorm and NormFinder ranked *ACT7* among the top four stable genes in roots and leaves

under drought, high salinity, and ABA treatment. As noted previously, the expression of *ACT7* is relatively stable under ultraviolet treatment in rice, drought, high salinity, and cold stress in common bean, and high salinity and cold stress in peanut [11,55,56]. However, its expression varies in pollen, seeds, and roots in *A. thaliana* [47]. Therefore, the expression of traditional housekeeping genes is not always stable under all experimental conditions.

　　SAND and *TUB3* were two of the top three stable genes under cold stress. Han et al. [57] and Reid et al. [58] reported that *SAND* was a suitable RG for gene expression analysis in iron-deficient *A. thaliana* and in developing grape berry tissues. However, *SAND* has not been reported previously as a stable RG under low-temperature stress in any species. Similar to our results, previous studies have reported that *TUB* was stably expressed as an RG in various developmental stages of soybean [13], different tissues of poplar [59], and the barks of *Populus yunnanensis* cuttings [60]. The expression of *TUB* was relatively stable in leaves from powdery mildew-infected wheat [61] and in *P. orientalis* seedlings under cold, heat, salinity, PEG, and ABA treatment [44]. *TUB* was also reported as a suitable RG in all tissues (root, stem, and leaf) under multiple abiotic stress (cold, heat, salinity, and drought), and hormone treatments (ABA, SA, ET, and GA3) in maize [62]. These results suggest that the expression of the same RG varies among organs, developmental stages, and environmental conditions.

　　We analyzed the expression levels of two target genes (*PuP5CS2* and *PuGI*) to validate the applicability and stability of suitable, high-ranking RGs. Consistent results were obtained by comparing the top two high-ranking RGs in roots and leaves under drought, high salinity, cold stress, and ABA treatment. The expression of *PuP5CS2* was significantly induced in roots under drought, high salinity, and ABA treatment. Similar expression patterns of *SbP5CS1* and *SbP5CS2* under drought and high salinity were reported in sorghum [29]. In *A. thaliana*, *AtP5CS1* responded to ABA and salt stress through controlling proline accumulation [63]. The expression of *PuGI* was upregulated significantly in leaves during cold stress but not following ABA treatment, which is consistent with the behavior of *GI* in *A. thaliana*. In addition, *GI* was not induced by drought stress or ABA treatment in *A. thaliana*, but *PuGI* was upregulated under drought stress and ABA treatment in our study. This result may be because different species and RGs were used in the experiments.

　　We compared the results of *PuP5CS2* and *PuGI* expression analysis performed using appropriate and inappropriate RGs. When the appropriate RGs were used for normalization, the expression patterns of *PuP5CS2* and *PuGI* were similar, but some differences still emerged. As previous studies have noted, more RGs are often required to correct for non-specific experimental variation and small differences in qRT-PCR conditions [27]. Nevertheless, when the expression data for *PuP5CS2* and *PuGI* were normalized using inappropriate RGs, there were significant biases. These results indicate that the RGs screened in this study were reliable.

　　In summary, we evaluated and validated stable RGs in *P. ussuriensis* under abiotic stresses (drought, high salinity, and cold stress) and following treatment with the drought-related hormone ABA. The appropriate RGs for the qRT-PCR normalization of target gene expression data were *UBQ10* and *RPL24* for drought and ABA treatment, *UBQ10* and *TUB3* for cold stress, and *UBQ10* and *60S rRNA* for salinity stress. Suitable RGs differed among various stresses and treatments. However, *UBQ10* appeared to be the most reliable RG across all experimental conditions in this study.

5. Conclusions

　　We selected fourteen candidate RGs to validate their expression stability using three algorithms, namely geNorm, NormFinder, and BestKeeper during abiotic stress (drought, high salinity, and cold stress) and after the treatment with the drought-related hormone ABA in *P. ussuriensis*. The results of the three algorithms were analyzed using comprehensive ranking. We identified the most appropriate reference genes, which were *UBQ10* and *RPL24* for drought and ABA treatment, *UBQ10* and *TUB3* for cold stress, and *UBQ10* and *60S rRNA* for high salinity. *UBQ10* was the most stable reference gene for use as an internal control, whereas *PP2A* expression was the least stable. Furthermore, the expression of two target genes (*P5CS2* and *GI*) confirmed the importance of selecting appropriate RGs

in gene expression studies. This study provides a guideline for molecular studies of gene expression in *P. ussuriensis* under various abiotic stresses and provides the foundation for genomic researches in woody species.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/11/4/476/s1, Figure S1: Agarose gel electrophoresis of PCR products for each of the fourteen RGs and the two target genes; Figure S2: Dissociation curves of the qRT-PCR amplicons for 14 RGs.

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Conflicts of Interest: The authors declare no conflict of interest.

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