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Selection of Suitable Reference Genes in *Pinus massoniana* Lamb. Under Different Abiotic Stresses for qPCR Normalization

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Abstract: The normalization of data by choosing suitable reference genes is fundamental for obtaining accurate and reliable results in quantitative real-time polymerase chain reaction (qPCR) analyses. In this study, the expression stability of 12 candidate reference genes of *Pinus massoniana* under different abiotic stresses was evaluated using four statistical algorithms: geNorm, NormFinder, BestKeeper, and RefFinder. The results indicate that the following genes could be used as reference genes under different treatments: Actin 2 (*ACT2*) and F-box family gene (*F-box*) for salinity treatment, cyclophilin (*CYP*) and alpha-tubulin (*TUA*) for ABA treatment, actin 7 (*ACT7*) and *CYP* for drought treatment, actin 1 (*ACT1*) and *ACT7* for cold treatment, *ACT1* and *CYP* for heat treatment, and *TUA* and *ACT2* for the “Total” group. To validate the suitability of the selected reference genes in this study, the Short-Root protein (*SHR*), Alpha-pinene synthase (*APS*), and Pyrabactin resistance-like protein (*PYL*) gene expression patterns were analyzed. The expression patterns had significant biases when the most unstable reference genes were used for normalization, compared with when the optimum reference gene or gene combinations were used for normalization. These results will be beneficial for further studies on gene transcription in early-stage, unignified seedlings of *P. massoniana*.

Keywords: *Pinus massoniana*; qPCR; reference gene; abiotic stresses

1. Introduction

Gene expression profiling maintains an important role in molecular biology research, such as aiding in the elucidation of complex regulatory networks of the genetic, signaling, and metabolic pathway mechanisms [1,2]. Quantitative real-time polymerase chain reaction (qPCR) has become one of the most prevalent technologies for monitoring gene expression patterns [3,4]. Absolute and relative quantification are two methods applied to quantify assays of gene expression [5]. Absolute quantification compares the transforming cycle value (Ct) and provides accurate copy numbers of genes with a standard curve [6]. For the relative quantification method, suitable internal control genes are required as references in qPCR [7], and with this method, researchers are more concerned about discrepancies in gene expression analyses rather than the exact copy numbers of genes. Therefore, relative quantification qPCR has been widely used and plays a significant role in gene expression analysis [8].

Despite being superior in reproducibility, sensitivity, and specificity, the relative quantification method requires stable internal controls as a prerequisite condition for gene expression analyses [1,9]. Typically, reference genes are selected as internal controls for normalizing gene expression data in experiments. The expression levels of ideal reference genes are not influenced by experimental conditions, such as tissues, developmental periods, biological stresses, or abiotic stresses. However,

increasing evidence shows that ideal reference genes are often not available [1,10,11]. Therefore, it is critical to screen and validate suitable reference genes for the normalization of gene expression data under different experimental conditions [12,13].

Housekeeping genes associated with basic cellular processes are commonly chosen as candidate reference genes in plants. *Actin* and *TUB* were the most stable reference genes under abiotic stress and hormone stimuli in carrot [14]. *GAPDH* and *EF-1A* were the most stable reference genes under different abiotic stress in *Caragana korshinskii* [11]. *F-box* and *CYP* were suitable reference genes in soybean [15,16]. *TUA* showed the maximum stability under cold stress in Chinese prickly ash [17]. Besides traditional reference genes, *AQP* was considered as a new candidate gene in *Stevia rebaudiana* and olive [18,19]. One of the important evaluation criteria for selecting suitable reference genes under certain experimental conditions is stable expression of the candidate genes under the expected test conditions [1]. Considering the expression stability of the reference genes under different conditions or cross species are also not the same, thus, the reassessment of the expression stability of candidate reference genes in specific experimental conditions is required and essential to ensure the correctness of the relative quantification results in target genes studies [20,21].

Pinus massoniana (Masson pine) is an economically and ecologically important evergreen conifer in southern China and is one of the most significant forest tree species [22,23]. It is widely used in the production of fiber and solid wood [24,25]. Furthermore, more than 70% of resin and turpentine comes from Masson pines in China, which is the world's largest exporter. Therefore, studying the molecular basis of the economic traits and physiological patterns of Masson pine is of great importance in promoting its breeding process. Illustration of the expression levels of key genes under different conditions is important. Wei et al. selected reference genes for gene expression analysis of *P. massoniana* under nematode-inoculation conditions [26]. Chen et al. selected reference genes in *P. massoniana* for different tissues and abiotic stresses [27]. However, to date, there has been no systematic validation of these selected reference genes for *P. massoniana* in such studies, and there have been no systematic analyses of reference gene screening in early-stage, unignified seedlings of *P. massoniana* under different abiotic stresses.

In this study, 12 common reference genes (actin 1 gene (*ACT1*), actin 2 gene (*ACT2*), actin 4 gene (*ACT4*), actin 6 gene (*ACT6*), actin 7 gene (*ACT7*), F-box family gene (*F-box*), elongation factor-1 α gene (*EF-1A*), alpha-tubulin gene (*TUA*), beta-tubulin gene (*TUB*), aquaporin protein gene (*AQP*), cyclophilin gene (*CYP*), and glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*)) were selected to assess the stability of gene expression under different abiotic stresses in *P. massoniana*. Four common statistical algorithms (geNorm [28], NormFinder [29], BestKeeper [30], and RefFinder [31]) were used to calculate the variability of the candidate reference genes and select the most suitable reference genes. The results will lay a foundation for future gene expression pattern research in unignified seedlings of *P. massoniana*.

2. Methods

2.1. Plant Materials and Treatments

The seeds of *P. massoniana* were collected from the National *P. massoniana* Superior Variety Base of Xinyi, Guangdong, China. The seeds were germinated in pots containing a soil mixture (peat soil:perlite:vermiculite, 3:1:1 v/v) under the conditions of 24 °C and 16 h light/8 h dark. Forty-five-day-old unignified seedlings in similar growth stages were selected for the following experiments.

For cold and heat treatments, seedlings were exposed to high temperature (42 °C) and low temperature (4 °C) for 0.5 h, 3 h, 6 h, 12 h, 24 h, and 48 h. For drought, salinity, and ABA treatments, washed seedlings were immersed in complete medium containing 10% (w/v) PEG₆₀₀₀, 200 mM NaCl, and 100 μ M ABA, respectively, for 0.5 h, 3 h, 6 h, 12 h, 24 h, and 48 h. Drought, salinity, and ABA treatments were performed at room temperature. Untreated seedlings were used as the control.

Each treatment was repeated three times independently as biological replicates. All samples were collected and immediately frozen in liquid nitrogen and then stored at -80°C until RNA isolation.

In total, 93 samples were analyzed in the subsequent experiments, including 90 samples with five different stress treatments and three samples as controls.

2.2. RNA Isolation and cDNA Reverse Transcription

Total RNA was extracted using the RNeasy Pure Kit (DP441, Tiangen Biotech, Beijing, China) with a gDNA-removal step according to the manufacturer's instructions. RNA concentration and purity were measured with a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, Massachusetts, US), and RNA integrity was estimated by 1.2% agarose gel electrophoresis. cDNA (20 μL) was synthesized from 1000 ng of total RNA using the One-step gDNA Removal and cDNA Synthesis Kit (AT311, TransGen Biotech, Beijing, China) with Oligo (dT)₁₈ Primer according to the manufacturer's instructions.

2.3. Candidate Reference Gene Selection, Primer Design, and Gene Cloning

The sequences of 12 candidate reference genes (*ACT1*, *ACT2*, *ACT4*, *ACT6*, *ACT7*, *TUA*, *TUB*, *EF1A*, *F-box*, *AQP*, *GAPDH*, and *CYP*) were acquired from the GenBank database (<https://www.ncbi.nlm.nih.gov/genbank/>). Primers were designed by Primer Premier 5.0 software. The primer sequences of candidate reference genes used in this study are summarized in Table 1. The primer specificities and amplicon sizes were verified by 2% agarose gel electrophoresis. A two-fold cDNA dilution series with three replicates per concentration was used to make standard curves for estimation of amplification efficiency ($E = (10^{[-1/\text{slope}]} - 1) \times 100\%$) and the correlation coefficient (R^2) [11]. The sequences of 12 candidate reference genes from *P. massoniana* were cloned using $2 \times$ Rapid Taq Master Mix (P222, Vazyme Biotech, Nanjing, Jiangsu, China) as the polymerase. The PCR (20 μL) mixture contained 10 μL of $2 \times$ Rapid Taq Master Mix, 7 μL of ddH₂O, 1 μL of the template cDNA, and 1 μL of each primer (10 μM). The amplification conditions were as follows: 3 min at 95°C for denaturation; 35 cycles of 20 s at 95°C (denaturation), 20 s at 58°C (annealing), and 60 s at 72°C (extension); and a final step of 5 min at 72°C for extension.

2.4. Quantitative Real-Time PCR Assay

qRT-PCRs were performed in a StepOne Plus System (Applied Biosystems, Foster City, USA) using SYBR Green Real-time PCR Master Mix (QPK-201, Toyobo Bio-Technology, Shanghai, China). Each PCR mixture (10 μL) contained 1 μL of diluted cDNA ($20 \times$ dilution), 5 μL of SYBR Green Real-time PCR Master Mix, 0.4 μL of each primer (10 μM), and 3.2 μL of ddH₂O. The amplification conditions were as follows: 95°C for 60 s for pre-denaturation, 40 cycles at 95°C for 10 s for denaturation, and 58°C for 30 s for annealing and extension. Melting curves were analyzed from 60°C to 95°C to confirm primer specificity and lack of primer dimers. Each reaction was repeated three times. The negative controls were included on each plate and for each sample, with ddH₂O and total RNA to replace the cDNA.

2.5. Data Analysis

The raw Ct data are shown in Appendix A Table A1. The analysis of the candidate reference gene expression stability was performed by four common software programs: geNorm [28], NormFinder [29], BestKeeper [30], and RefFinder [31]. For analysis with the geNorm and NormFinder algorithms, the raw Ct values were converted into relative quantities. However, for BestKeeper and RefFinder analysis, the Ct data were not transformed.

Table 1. Candidate reference genes and target genes descriptions, primer sequences, and amplicon characteristics in this study.

Gene Symbol	Gene Description	Accession Number	Primer Sequence (5'-3')	Amplicon Size (bp)	PCR Efficiency (%)	Regression Coefficient (R2)	Tm (°C)
Reference genes							
<i>ACT1</i>	Actin 1 gene	KM496527.1	CCGTATGAGCAAGGAAATCAC AGAACCTCCAATCCAGACACT	100	95.679	0.999	85.0
<i>ACT2</i>	Actin 2 gene	KM496525.1	CACGGAATAGGCAGAAGTTGG TGGGCATAAAGTGTTAGAATAGC	97	98.145	0.989	80.8
<i>ACT4</i>	Actin 4 gene	KM496528.1	ATTTATGAGGGATACGCTTTG AGGTGTACCCACGTTCTGTAA	106	97.505	0.995	84.6
<i>ACT6</i>	Actin 6 gene	KM496530.1	AACTCCTGCCATCCTCATCTT CTGTTCCAGCCTTGCTTTCA	99	96.549	0.996	83.1
<i>ACT7</i>	Actin 7 gene	KM496529.1	TGGGATGCTATGGAAGATTTG TACGCCCTTTGGAGTAAGAAG	114	104.801	0.992	82.9
<i>AQP</i>	Aquaporin protein gene	KF582038.1	CACCTTGCCACAATTCCTATCA TCCAATGGTCATCCCAAAACAC	103	95.481	0.998	86.3
<i>CYP</i>	Cyclophilin gene	KM496534.1	CGAGAAGTTTGCCGATGAGAA GAATTGCGAGCCGTTAGTGTT	97	91.042	0.997	87.5
<i>EF1A</i>	Elongation factor 1-alpha gene	KM496532.1	GGATTTGAAACGTGGGTATGT CAGGGTGGTTCATTATGATTACT	97	99.383	0.998	83.5
<i>F-box</i>	F-box family protein gene	KM496542.1	TATTATTGTTGCAGGTGGGTT AGAATGTTGAAGTTCGGCTAT	109	100.101	0.996	81.6
<i>GAPDH</i>	Glyceraldehyde 3-Phosphatase gene	KM496531.1	GGATTTGGTCGTATTGGGAGG TTTGGCATCAATGAAAGGGTC	96	92.132	0.998	82.9
<i>TUA</i>	Tubulin alpha gene	KM496535.1	CAAACTTGGTCCCGTATCCTC CACAGAAAGCTGCTCATGGTAA	95	92.011	0.999	83.7
<i>TUB</i>	Tubulin beta gene	KM496536.1	CTGCGACTATGAGTGGAGTGA AGAAATGAAGACGAGGGAATG	108	98.923	0.993	85.6
Target genes							
<i>SHR</i>	Short-Root gene	MK153765	GCCTGTGAGGATTCTGAAGTT CACTGAAAGCAGCATGTATGA	97	97.636	0.996	85.3
<i>APS</i>	Alpha-pinene synthase gene	KF547035	TGGATCGCCAGTGGTGAGGTG GTCCGTCGTCAGAATGGGTTG	105	94.694	0.991	86.2
<i>PYL</i>	Pyrabactin resistance-like gene	MK953936	GAGTCCGAGTATGTGTGGAGGC ACTAATGACCAAACCAGATGAA	110	100.051	0.997	86.2

GeNorm computes the expression stability measure (M-value), analyzes the pairwise variation (V value) in each candidate reference gene, and then excludes the most unstable genes with the highest M-value. Furthermore, pairwise variation (V_n/V_{n+1} ; 0.15 is recommended threshold) determines the optimal number of reference genes for normalization [20,32].

NormFinder computes the expression stability value (SV) on the basis of intra- and intergroups for each reference gene [29]. A lower SV indicates a higher expression stability of this gene.

BestKeeper computes the stability of candidate reference genes based on standard deviation (SD), Pearson correlation coefficient (r), and coefficient of variation (CV) with the Ct data of all candidate genes. The lowest SD and CV values represent the most stable gene. The range of variation in SD should not surpass 1 [20,32].

RefFinder assigns an appropriate weight to an individual candidate gene and calculates the geometric mean of their weights for the overall final ranking, based on the rankings from the currently available major programs (geNorm, Normfinder, BestKeeper, and the comparative ΔCt method) [31].

2.6. Validation of Reference Genes

To validate the reliability of selected reference genes, the two most stable and two unstable reference genes and the optimum internal reference gene combinations were used to normalize the relative expression patterns of *SHR*, *APS*, and *PYL* in each experimental condition, according to the rankings from RefFinder and geNorm, respectively. The relative expression levels were calculated by the $2^{-\Delta\Delta\text{Ct}}$ method [8].

3. Results

3.1. Selection of Reference Genes, Amplification Specificity, and Efficiency

Based on the GenBank database, 12 candidate reference genes (*ACT1*, *ACT2*, *ACT4*, *ACT6*, *ACT7*, *TUA*, *TUB*, *EF1A*, *F-box*, *AQP*, *GAPDH*, and *CYP*) were cloned from *P. massoniana*. The primer pairs of all candidate reference genes and target genes were designed for qPCR, and the amplicon lengths were between 95 and 114 bp. Agarose gel electrophoresis (Appendix A Figure A1) and melting curve analysis (Appendix A Figure A2) were used to determine primer specificity. The amplification efficiency of qPCR across all 12 reference genes and three validated genes varied from 91.0% to 104.8%, with R^2 varying from 0.989 to 0.999 (Table 1 and Appendix A Figure A3).

3.2. Expression Profiles of the Reference Genes

The expression levels of candidate reference genes were quantified by Ct values; the high expression stability of the genes was reflected by the low SV. The raw Ct values for all samples in this study are listed in Appendix A Table A1 (there were no Ct values in the negative controls), and a box and whiskers plot was used to describe the raw Ct value distribution (Figure 1 and Appendix A Table A1). The 12 candidate reference genes had a wide range of expression across all samples in this study ($20.89 \leq \text{Ct} \leq 33.96$). The results indicate that there were five genes (*ACT1*, *CYP*, *TUA*, *GAPDH*, and *AQP*) with average Ct values between 23 and 25 cycles that displayed high expression levels; the other seven genes (*EF1A*, *ACT4*, *TUB*, *F-box*, *ACT7*, *ACT6*, and *ACT2*), which presented Ct values between 25 and 31, displayed intermediate expression levels. *ACT1* was the most highly expressed gene among the 12 candidate genes (mean Ct of 23.32). On the other hand, *ACT2* was the least highly expressed gene (mean Ct of 30.86). In addition, every reference gene had different coefficients of variation (lower values represent less variability) in this study, as shown in Figure 1; *TUB* had the most variation, whereas *F-box* had the least variation, which indicated that *TUB* was the most unstable gene and *F-box* was the most stable gene of all the reference genes.

Figure 1. Distribution of transforming cycle (Ct) values of the twelve candidate reference genes across all experimental samples of *P. massoniana*. The outside box is determined from 25th to 75th percentiles, and the inside '+' represents the mean value. The line across the box is the median. The whiskers represent the 5th to 95th percentiles, and the asterisks represent the outliers.

3.3. Expression Stability Analysis of the Reference Genes

In this study, five experimental conditions were performed, including cold, heat, drought, ABA, and salinity treatments. Furthermore, these experimental conditions were sorted into the “Total” group (all experimental conditions). To select appropriate reference genes for these experimental conditions, four software programs (geNorm [28], NormFinder [29], BestKeeper [30], and RefFinder [31]) were used to evaluate the stability of the 12 candidate reference genes.

3.4. geNorm Analysis

The geNorm analysis results are presented in Table 2. Under different experimental conditions, the reference genes with the highest stability were different. The twelve reference genes in the five experimental conditions and “Total” group showed high expression stabilities with threshold values below 1.5. For cold treatment, *ACT1* and *TUA* ($M = 0.157$) were the most stable genes, while *EF1A* ($M = 0.634$) was the most variable gene. For heat treatment, *CYP* and *GAPDH* ($M = 0.309$) were identified as the most stable genes, whereas the *ACT6* ($M = 0.47$) gene was the least stable. For salinity treatment, *ACT2* and *F-box* ($M = 0.275$) were the most stable genes, while *AQP* ($M = 0.787$) was the most variable gene. For drought treatment, *ACT4* and *TUB* ($M = 0.280$) were identified as the most stable genes, whereas *ACT6* ($M = 0.785$) was the least stable. For ABA treatment, *CYP* and *TUA* ($M = 0.246$) were the most stable genes, while *TUB* ($M = 0.663$) was the most variable gene. A combination of individual samples in the “Total” group showed that *ACT1* and *TUA* ($M = 0.437$) featured the most stable expression, whereas the most variable genes included *AQP* ($M = 0.882$) and *ACT6* ($M = 0.932$).

The optimal number of reference genes for normalization depends on pairwise variation ($V_n/n+1$). When $V_n/n+1 < 0.15$, it suggests that an additional reference gene is not necessary for normalization. For four experimental conditions (cold, salinity, drought, and ABA), two reference genes were sufficient for accurate normalization (Figure 2); the most stable gene pairs for cold, salinity, drought, and ABA conditions were *ACT1* and *TUA*, *ACT2* and *F-box*, *ACT4* and *TUB*, and *CYP* and *TUA*, respectively (Appendix A Figure A4). For heat treatment and the “Total” group, $V_{2/3} > 0.15$, and $V_{3/4} < 0.15$ (Figure 2). Therefore, the genes *CYP*, *GAPDH*, and *ACT2*, and *ACT1*, *TUA*, and *ACT7* were chosen, respectively (Appendix A Figure A4).

Table 2. The stability ranking of candidate reference genes by geNorm, NormFinder, BestKeeper, and RefFinder. SD [$\pm$ Ct], standard deviation of the Ct; CV [%Ct], coefficient of variance expressed as a percentage of the Ct level.

Treatment	Rank	geNorm		NormFinder		BestKeeper		RefFinder		
		Gene	Stability	Gene	Stability	Gene	SD(±Ct)	CV(%Ct)	Gene	Stability
Salinity	1	ACT2	0.275	F-box	0.145	EF1A	0.30	1.24	ACT2	1.41
	2	F-box	0.275	ACT2	0.164	ACT2	0.34	1.13	F-box	1.68
	3	CYP	0.315	ACT4	0.177	CYP	0.39	1.69	CYP	3.46
	4	ACT4	0.410	CYP	0.264	F-box	0.49	1.74	ACT4	4.36
	5	ACT7	0.453	ACT1	0.266	ACT7	0.54	1.91	ACT1	5.48
	6	ACT1	0.502	ACT7	0.347	ACT1	0.58	2.53	ACT7	5.48
	7	ACT6	0.549	TUA	0.398	GAPDH	0.60	2.58	EF1A	6.04
	8	TUA	0.583	ACT6	0.405	ACT6	0.66	2.15	ACT6	7.48
	9	GAPDH	0.633	TUB	0.501	TUA	0.76	3.28	TUA	7.97
	10	TUB	0.687	GAPDH	0.572	ACT4	0.78	2.81	GAPDH	8.91
	11	EF1A	0.734	EF1A	0.588	TUB	1.03	3.75	TUB	9.72
	12	AQP	0.787	AQP	0.656	AQP	1.12	4.67	AQP	12.00
ABA	1	CYP	0.246	CYP	0.085	GAPDH	0.42	1.78	CYP	1.41
	2	TUA	0.246	TUA	0.142	F-box	0.42	1.51	TUA	2.38
	3	ACT6	0.319	AQP	0.193	ACT1	0.47	2.07	ACT1	3.94
	4	ACT7	0.345	ACT1	0.202	CYP	0.53	2.29	AQP	4.24
	5	ACT1	0.361	ACT7	0.257	EF1A	0.61	2.52	GAPDH	4.30
	6	AQP	0.375	ACT6	0.267	AQP	0.62	2.67	ACT7	5.14
	7	GAPDH	0.423	GAPDH	0.282	ACT7	0.69	2.43	ACT6	5.73
	8	ACT2	0.466	ACT2	0.321	TUA	0.70	3.03	F-box	7.18
	9	ACT4	0.507	ACT4	0.400	ACT2	0.75	2.49	ACT2	8.24
	10	EF1A	0.556	EF1A	0.453	ACT6	0.75	2.44	EF1A	8.41
	11	F-box	0.611	F-box	0.578	ACT4	0.75	2.74	ACT4	9.46
	12	TUB	0.663	TUB	0.580	TUB	0.99	3.67	TUB	12.00
Drought	1	ACT4	0.280	ACT7	0.093	F-box	0.24	0.84	ACT7	1.57
	2	TUB	0.280	CYP	0.223	ACT7	0.31	1.06	CYP	2.63
	3	ACT7	0.360	TUA	0.264	CYP	0.34	1.43	ACT4	3.36
	4	CYP	0.427	ACT4	0.296	AQP	0.39	1.56	TUA	4.24
	5	ACT2	0.458	ACT2	0.328	ACT2	0.45	1.45	TUB	4.36
	6	TUA	0.490	TUB	0.344	TUA	0.50	2.16	F-box	4.45
	7	F-box	0.557	GAPDH	0.398	EF1A	0.51	2.04	ACT2	5.00
	8	AQP	0.600	F-box	0.403	ACT4	0.52	1.84	AQP	7.14
	9	EF1A	0.640	AQP	0.466	GAPDH	0.56	2.36	GAPDH	8.43
	10	GAPDH	0.679	ACT1	0.512	TUB	0.60	2.16	EF1A	9.34
	11	ACT1	0.722	EF1A	0.555	ACT1	0.74	3.21	ACT1	10.49
	12	ACT6	0.785	ACT6	0.692	ACT6	0.83	2.71	ACT6	12.00
Cold	1	ACT1	0.157	GAPDH	0.111	ACT1	0.24	0.99	ACT1	1.57
	2	TUA	0.157	ACT7	0.133	TUA	0.29	1.20	ACT7	2.34
	3	ACT7	0.232	ACT1	0.145	ACT4	0.30	1.04	TUA	2.51
	4	GAPDH	0.281	ACT2	0.150	F-box	0.30	1.06	GAPDH	2.91
	5	ACT4	0.325	TUA	0.215	ACT7	0.35	1.18	ACT4	4.82
	6	ACT2	0.350	ACT4	0.224	GAPDH	0.37	1.54	ACT2	5.89
	7	ACT6	0.394	AQP	0.283	AQP	0.39	1.64	F-box	6.93
	8	AQP	0.427	F-box	0.284	CYP	0.40	1.72	AQP	7.24
	9	F-box	0.453	ACT6	0.330	ACT6	0.42	1.34	ACT6	8.45
	10	CYP	0.476	CYP	0.395	ACT2	0.45	1.43	CYP	9.46
	11	TUB	0.523	TUB	0.455	TUB	0.70	2.47	TUB	11.00
	12	EF1A	0.634	EF1A	0.782	EF1A	0.87	3.41	EF1A	12.00

Table 2. Cont.

Treatment Rank	geNorm		NormFinder		BestKeeper			RefFinder	
	Gene	Stability	Gene	Stability	Gene	SD($\pm$ Ct)	CV(%Ct)	Gene	Stability
Heat	1	CYP	0.309	ACT1	0.187	TUA	0.50	ACT1	2.30
	2	GAPDH	0.309	TUA	0.275	ACT4	0.55	CYP	2.63
	3	ACT2	0.416	CYP	0.284	ACT1	0.58	TUA	3.13
	4	ACT7	0.496	TUB	0.325	TUB	0.89	GAPDH	3.64
	5	AQP	0.589	GAPDH	0.329	AQP	0.94	ACT2	5.05
	6	TUB	0.635	ACT2	0.356	GAPDH	0.96	TUB	5.18
	7	ACT1	0.658	AQP	0.405	ACT2	0.99	F-box	5.62
	8	TUA	0.686	ACT4	0.419	F-box	1.13	AQP	6.19
	9	ACT4	0.724	ACT7	0.482	ACT6	1.23	ACT4	6.45
	10	F-box	0.798	F-box	0.682	ACT7	1.32	ACT7	7.54
	11	EF1A	0.874	EF1A	0.847	EF1A	1.38	EF1A	11.24
	12	ACT6	1.019	ACT6	1.145	CYP	1.46	ACT6	11.74
Total	1	ACT1	0.437	ACT2	0.267	F-box	0.44	TUA	2.11
	2	TUA	0.437	TUA	0.306	CYP	0.59	ACT2	2.30
	3	ACT7	0.625	ACT1	0.316	GAPDH	0.65	ACT1	2.71
	4	ACT2	0.650	CYP	0.335	ACT4	0.72	CYP	3.72
	5	GAPDH	0.671	ACT7	0.358	TUA	0.75	ACT7	5.10
	6	CYP	0.686	ACT4	0.372	ACT1	0.77	GAPDH	5.21
	7	ACT4	0.703	GAPDH	0.394	ACT2	0.77	F-box	5.62
	8	TUB	0.747	TUB	0.508	ACT6	0.85	ACT4	5.63
	9	EF1A	0.778	EF1A	0.541	ACT7	0.89	TUB	8.66
	10	F-box	0.827	F-box	0.580	EF1A	1.00	EF1A	9.24
	11	AQP	0.882	AQP	0.698	TUB	1.16	ACT6	10.84
	12	ACT6	0.932	ACT6	0.711	AQP	1.22	AQP	11.24

Figure 2. Pairwise variation (V) of the candidate reference genes analyzed by geNorm. Pairwise variation (V_n/V_{n+1}) was analyzed between the normalization factors (NF_n and NF_{n+1}) by geNorm to determine the optimal number of reference genes. V_n/V_{n+1} values below 0.15 suggest that there was no need to introduce another gene.

3.5. NormFinder Analysis

The SV of each candidate reference gene was also analyzed by NormFinder, wherein a lower SV indicates a higher expression stability. In this study, the results of NormFinder analysis were similar to those of geNorm analysis (Table 2). For cold treatment, *GAPDH* was the most stable reference gene, and *EF1A* was the most variable gene. For heat treatment, *ACT1* was the most stable reference gene, and *ACT6* was the most variable gene. For salinity treatment, *F-box* was the most stable reference gene, and *AQP* was the most variable gene. For drought treatment, *ACT7* was the most stable reference gene, and *ACT6* was the most variable gene. For ABA treatment, *CYP* was the most stable reference gene, and *TUB* was the most variable gene. For the “Total” group, *ACT2* was the most stable reference gene, and *ACT6* was the most variable gene.

3.6. BestKeeper Analysis

The results of the BestKeeper analysis are also shown in Table 2. The results indicated that all candidate reference genes were markedly stable when expressed under three experimental conditions (cold, drought, and ABA) in this study. The rankings by BestKeeper analysis showed that the most stable reference genes were *ACT1* ($CV \pm SD = 0.99 \pm 0.24$) and *TUA* ($CV \pm SD = 1.20 \pm 0.29$) for cold treatment. *F-box* ($CV \pm SD = 0.84 \pm 0.24$) and *ACT7* ($CV \pm SD = 1.06 \pm 0.31$) were the most stable genes for drought treatment. *GAPDH* ($CV \pm SD = 1.78 \pm 0.42$) and *F-box* ($CV \pm SD = 1.51 \pm 0.42$) were the most stable genes for ABA treatment. For salinity treatment and the “Total” group, 10 reference genes were expressed stably; the most stable genes were *ACT2* ($CV \pm SD = 1.13 \pm 0.34$) and *EF1A* ($CV \pm SD = 1.24 \pm 0.30$) for salinity treatment. *F-box* ($CV \pm SD = 1.57 \pm 0.44$) and *CYP* ($CV \pm SD = 2.53 \pm 0.59$) showed the most stable expression under cold treatment. For heat treatment, only seven reference genes were expressed stably; the most stable genes were *ACT4* ($CV \pm SD = 1.92 \pm 0.55$) and *TUA* ($CV \pm SD = 2.06 \pm 0.50$) (Table 2).

3.7. RefFinder Analysis

The geometric mean rankings were estimated from geNorm, NormFinder, and BestKeeper programs by RefFinder algorithms [31]. This allowed us to generate a recommended comprehensive ranking of reference genes for accurate transcript normalization in each experimental set. The rankings by RefFinder analysis showed that *ACT2* and *F-box* were the most stable genes for salinity treatment, that *CYP* and *TUA* were the most stable genes for ABA treatment, that *ACT7* and *CYP* were the most stable genes for drought treatment, that *ACT1* and *ACT7* were the most stable genes for cold treatment, that *ACT1* and *CYP* were the most stable genes for heat treatment, and that *TUA* and *ACT2* were the most stable genes for the “Total” group (Table 2).

3.8. Reference Gene Validation

To validate the accuracy of selected reference genes, the relative expression levels of *SHR*, *APS*, and *PYL* were analyzed in all the experimental conditions involved in this study. For each experimental condition, the two most stable and two unstable reference genes according to the combined analytic results obtained by geNorm, NormFinder, and BestKeeper, and the reference gene combination according to geNorm, were selected for normalization.

Under cold treatment, *SHR* was upregulated, and the highest expression levels were observed at 3 h (3.91-fold change) (Figure 3A). *APS* was downregulated at 3 h and 48 h and upregulated at 0.5 h, 6 h, 12 h, and 24 h, especially at 6 h (2.28-fold change) (Figure 3B). There was no significant difference in the expression of *PYL*, and *PYL* had the highest expression at 3 h (1.56-fold change) (Figure 3C).

Under drought treatment, *SHR* was downregulated at 6 h and upregulated at 0.5 h, 3 h, 12 h, 24 h, and 48 h, especially at 24 h (3.34-fold change) (Figure 3D). *APS* was upregulated, and the highest expression levels were observed at 24 h (21.02-fold change) (Figure 3E). *PYL* was downregulated at 0.5 h, 3 h, 6 h, and 48 h and upregulated at 12 h and 24 h, and the highest expression levels were observed at 24 h (3.47-fold change) (Figure 3F).

Under ABA treatment, *SHR* was downregulated at 6 h and 12 h and upregulated at 0.5 h, 3 h, 24 h, and 48 h, especially at 48 h (2.68-fold change) (Figure 3G). *APS* was upregulated, and the highest expression levels were observed at 3 h (7.56-fold change) (Figure 3H). *PYL* was downregulated at 0.5 h, 3 h, 6 h, and 12 h and upregulated at 24 h and 48 h, and the highest expression levels were observed at 48 h (2.45-fold change) (Figure 3I).

Under salinity treatment, *SHR* was upregulated, and the highest expression levels were observed at 3 h (4.26-fold change) (Figure 3J). *APS* was upregulated, and the highest expression levels were observed at 3 h (15.58-fold change) (Figure 3K). *PYL* was downregulated at 6 h and upregulated at 0.5 h, 3 h, 12 h, 24 h, and 48 h, and the highest expression levels were observed at 24 h (3.21-fold change) (Figure 3L).

Figure 3. Effect of different reference genes for normalization on the relative expression of the *SHR*, *APS*, and *PYL* genes. (A–C) cold treatment; (D–F) drought treatment; (G–I) ABA treatment; (J–L) salinity treatment; and (M–O) heat treatment.

Under heat treatment, *SHR* was upregulated, and the highest expression levels were observed at 48 h (6.03-fold change) (Figure 3M). *APS* was downregulated at 6 h and upregulated at 0.5 h, 3h, 12 h, 24 h, and 48 h, especially at 0.5 h (4.06-fold change) (Figure 3N). *PYL* was upregulated, and the highest expression levels were observed at 24 h (3.11-fold change) (Figure 3O).

Our results confirm that using different reference genes for normalization causes great differences among the expression patterns of *SHR*, *APS*, and *PYL*. When the stable reference genes and optimum reference gene combinations were used for normalization, the expression patterns of *SHR*, *APS*, and *PYL* were similar. However, the expression patterns of *SHR*, *APS*, and *PYL* had significant biases when the most unstable reference genes were used for normalization compared with when the optimum reference gene combinations were used for normalization. The results illustrate that a stably-expressed reference gene was essential for the accurate normalization of target gene expression.

4. Discussion

Level and pattern analyses of gene expression is essential for the functional analysis of genes in different organisms and conditions [33]. Although gene expression levels can be obtained using different technologies, qPCR is currently the prevailing tool for the analysis of gene expression patterns in molecular biology research because of its speed, reproducibility, sensitivity, and specificity [34–36].

The use of reliable reference genes with steady expression levels and proper expression abundance is necessary for target gene expression pattern evaluation in different species or under different conditions by correcting the errors of RNA quantity and reverse transcription efficiency [2,37]. Ideal reference genes should be expressed at a steady and continuous level across different experimental conditions. An increasing number of studies have indicated that it is important to select an appropriate reference gene or gene combination using a systematic experiment due to the lack of available ideal reference genes [32]. Many studies on reference gene selection have been reported in plant organisms under different cultivars, developmental stages, biotic stresses, and abiotic stresses; for instance, rice [38], wheat [39], peach [40], poplar [41], *Prunus mume* [42], *Coffea Arabica* [43], *Metasequoia* [44], *P. pinaster* [45], and *Picea abies* [45], among others. However, to the best of our knowledge, the selection of reference genes has been performed in only nematode-inoculated and lignified materials of *P. massoniana*.

In this study, 12 commonly used reference genes (*ACT1*, *ACT2*, *ACT4*, *ACT6*, *ACT7*, *TUA*, *TUB*, *EF1A*, *F-box*, *AQP*, *GAPDH*, and *CYP*) were selected as candidate reference genes for analysis under five abiotic stress conditions for the lignified seedlings of *P. massoniana* (45-days-old). The twelve candidate reference genes used in this study achieved an appropriate expression abundance ($20.89 \leq Ct \leq 33.96$), which allowed further assessment of their expression stability. To date, this study is the first report of a systematic analysis of reference genes in different abiotic stress conditions in lignified plant materials of *P. massoniana*. Many researchers have chosen several statistical methods simultaneously to assess the optimal reference genes under different experimental conditions and avoided a one-sided analysis of the expression stability of reference genes that may occur when using only one algorithm [46,47]. In the present study, four of the most popular statistical programs (geNorm, NormFinder, BestKeeper, and RefFinder) were used to screen and determine appropriate reference genes. The four statistical programs generated inconsistent stability rankings in each experimental condition, since these algorithms had different sensitivities to coregulation genes [28–31]. Many published studies indicated that the stability ranking results from different statistical algorithms were approximately the same, and the most divergent ranking of gene stability was achieved with BestKeeper [32]. In this study, for the ABA treatment, *CYP* and *TUA* were identified as the most stable genes by geNorm, NormFinder, and RefFinder, while BestKeeper indicated that *F-box* and *GAPDH* were the best reference genes, although *F-box* and *GAPDH* were ranked as the 11th and seventh genes by both geNorm and NormFinder.

There is a possibility to obtain declinational gene expression analysis for normalization by a single reference gene [28,48]. Therefore, the experimental error could be reduced using two or more internal control genes as references [49]. In this study, the optimal number of reference genes for gene expression analysis under different abiotic stress conditions was determined using the geNorm program. Our results showed that under cold, drought, salinity, and ABA treatments, the pairwise variation was $V2/3 < 0.15$, which indicated that two reference genes were sufficient for optimal normalization. However, in the heat treatment and “Total” groups, $V2/3 > 0.15$, which indicates that more genes were needed. However, although the use of two or more reference genes can make gene expression analyses more exact, this practice is not an essential standard [28].

The suitability of the selected reference genes was assessed by analyzing the expression levels in three target genes related to a GRAS family transcription factor (*SHR*), alpha-pinene biosynthesis (*APS*), and an ABA receptor (*PYL*). The *SHR* protein, as a member of the GRAS transcription factor family, is related to the process of Masson pine root development; *APS* is a key enzyme-coding gene related to alpha-pinene biosynthesis; and *PYL* is an ABA receptor in the ABA signaling pathway. Their expression levels may be directly related to the process of growth and development, resin biosynthesis, and response to drought stress. In our study, the expression levels of *SHR*, *APS*, and *PYL* were different under different abiotic stresses.

Furthermore, we compared the most stable, unstable, and optimal reference gene combinations in transcriptional expression analysis of *SHR*, *APS*, and *PYL*, and the results were quite different. The results indicate that when the most stable reference genes and gene combinations were used for

normalization, the transcriptional expression patterns of *SHR*, *APS*, and *PYL* were similar. However, the expression patterns of *SHR*, *APS*, and *PYL* had significant biases when the least stable reference genes were used for normalization compared with when the most stable reference genes and gene combinations were used for normalization. These results indicate that the reference genes screened in this study are reliable.

Significantly, the expression stability of these candidate reference genes had not been evaluated for various tissues from other developmental stages of *P. massoniana* or other plant species under different abiotic stresses in our study. Some reported findings suggest that the stability of reference genes exhibited organ-specificity as well as species-specificity in plants, generally [50]. Tu et al. reported that the suitable reference genes for flower organs and vegetative organs were not consistent [51]. A similar result had been found in *Petunia hybrid* [52]. Yang et al. reported *GAPDH* was the most stable reference gene under heat stress in *Caragana korshinskii* [11]. By contrast, *GAPDH* had a low expression stability in *pisum sativum* [53]. Similarly, *GAPDH* was an unsuitable reference gene under different abiotic stresses in *P. massoniana* in our study.

The selected stable reference genes in this study will be beneficial for more accurate quantification of gene expression levels in early-stage, unignified seedlings of *P. massoniana* under different abiotic stresses.

5. Conclusions

In this study, we selected specific reference genes for qPCR analysis of unignified seedlings in *P. massoniana* different abiotic stresses. The most suitable combination for qPCR analysis across all samples was *TUA* and *ACT1*. The following genes could be used as reference genes under different abiotic stresses: *ACT2* and *F-box* for salinity treatment, *CYP* and *TUA* for ABA treatment, *ACT7* and *CYP* for drought treatment, *ACT1* and *ACT7* for cold treatment, and *ACT1* and *CYP* for heat treatment. In conclusion, this study provides an important framework for quantifying reference gene expression levels in *P. massoniana*.

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Conflicts of Interest: The authors declare that they have no competing interests.

Appendix A

Figure A1. Amplification of q-PCR specific primers. Amplified fragments of 12 candidate reference genes and three validate genes in *Pinus massoniana* were separated by 2.0% agarose gel electrophoresis. Lane 1 and 17 (GsDL2502, Shanghai Generay Biotech, Shanghai, China); Lane 2: *ACT1*(100bp); Lane 3: *ACT2*(97bp); Lane 4: *ACT4*(106bp); Lane 5: *ACT6*(99bp); Lane 6: *ACT7*(114bp); Lane 7: *AQP*(103bp); Lane 8: *CYP*(97bp); Lane 9: *EF1A*(97bp); Lane 10: *F-box*(109bp); Lane 11: *GAPDH*(96bp); Lane 12: *TUA*(95bp); Lane 13: *TUB*(108bp); Lane 14: *SHR*(97bp); Lane 15: *APS*(105bp); Lane 16: *PYL*(110bp).

Figure A2. Melting curve for 12 candidate reference genes and three validate genes in *P. massonina*. The melting curve for *ACT1*, *ACT2*, *ACT4*, *ACT6*, *ACT7*, *AQP*, *CYP*, *EF1A*, *F-box*, *GAPDH*, *TUA*, *TUB*, *SHR*, *APS*, and *PYL*, with single peak obtained from three technical replicates of different cDNA with no template control.

Figure A3. Standard curves of 12 candidate reference genes and three validate genes in *P. massonina*. Standard curves of *ACT1*, *ACT2*, *ACT4*, *ACT6*, *ACT7*, *AQP*, *CYP*, *EF1A*, *F-box*, *GAPDH*, *TUA*, *TUB*, *SHR*, *APS*, and *PYL*. The linear correlation (R^2) and PCR efficiencies ($E = (10^{[-1/\text{slope}]} - 1) \times 100\%$) were calculated from the standard curve.

Figure A4. The average expression stability values (M value) of the candidate reference genes analyzed by geNorm. Expression stability was evaluated in samples from *P. massoniana* submitted to cold stress, ABA stress, heat stress, drought stress, salinity stress, and total group.

Table A1. Raw Ct values of 12 reference genes in three validate genes in *P. massoniana*.

		ACT-1	ACT-2	ACT-4	ACT-6	ACT-7	AQP	CYP	EF1A	F-box	GAPDH	TUA	TUB	SHR	APS	PYL
Control	0h	23.93	30.57	28.69	31.83	29.96	24.71	23.93	22.77	28.81	23.84	23.87	27.82	30.40	27.88	27.86
		23.83	30.49	28.79	31.63	29.76	24.92	23.81	22.90	28.40	24.08	23.99	27.93	30.27	27.87	27.62
		23.78	30.86	28.54	31.72	29.80	22.84	23.79	24.90	28.38	23.87	23.80	28.52	30.17	27.72	27.54
ABA	0.5h	22.55	29.77	27.96	29.97	27.74	23.50	22.84	23.86	28.85	23.93	23.41	25.76	29.62	24.55	27.83
		22.88	30.61	28.16	30.29	28.19	23.80	23.31	24.40	28.79	24.34	23.54	26.84	28.84	25.86	27.48
		22.94	30.39	27.49	30.46	28.38	23.93	23.44	24.41	28.31	23.63	22.96	26.53	28.79	24.74	27.02
	3h	23.22	31.19	26.75	30.49	28.27	24.27	23.23	24.57	28.07	23.29	22.89	26.56	28.90	24.36	27.90
		22.90	28.40	25.85	30.81	28.63	23.01	22.76	24.29	27.94	22.91	22.86	26.42	28.36	23.70	27.58
		23.18	28.87	26.10	30.68	28.86	23.29	22.98	24.51	28.01	23.07	22.88	26.52	28.55	23.90	27.72
	6h	22.76	28.47	26.27	30.46	28.41	23.25	22.66	23.67	28.16	22.40	21.94	26.37	28.91	23.65	26.95
		22.84	28.89	26.83	30.34	28.83	23.12	22.58	24.14	28.66	23.69	22.64	26.83	29.30	24.07	27.61
		21.84	29.26	26.66	29.39	27.08	22.30	21.80	22.94	28.24	22.92	22.29	25.78	29.02	23.88	27.26
	12h	21.54	29.02	26.57	28.84	26.93	21.89	21.64	22.72	27.86	22.86	21.91	25.85	28.68	23.81	26.92
		21.89	29.04	26.01	29.04	27.09	22.07	21.91	23.07	27.52	22.43	21.34	25.83	27.90	22.80	26.40
		21.70	28.92	26.86	28.95	27.01	21.96	21.82	22.92	27.14	22.49	21.54	25.73	28.25	23.06	26.72
	24h	22.85	29.80	27.66	30.75	27.96	22.68	22.92	24.05	27.85	23.19	23.44	26.83	29.01	24.89	26.87
		22.65	29.62	27.85	30.61	27.88	22.36	22.41	23.97	27.76	23.52	23.62	26.57	28.97	25.42	27.00
		23.60	30.47	27.18	31.50	28.91	23.48	23.55	25.05	27.22	22.78	22.96	27.38	28.33	24.75	26.39
	48h	23.00	31.88	27.99	31.76	29.23	23.94	23.76	24.95	27.85	23.66	24.51	29.42	29.12	28.27	26.80
		23.50	31.65	27.44	31.98	29.79	24.19	23.90	25.65	27.04	23.41	23.84	29.82	28.83	26.09	26.20
		23.05	31.40	27.78	31.53	29.18	23.70	23.54	25.10	27.53	23.55	24.21	29.39	29.04	26.90	26.56
Cold	0.5h	22.96	29.99	28.37	31.67	30.30	22.90	23.45	24.76	27.88	22.89	24.56	27.95	29.73	28.80	28.15
		23.59	30.80	28.68	30.38	29.01	23.55	22.34	25.20	28.52	23.80	23.17	27.93	28.55	26.77	26.79
		23.74	30.93	28.95	30.73	29.49	23.65	22.64	25.41	28.61	23.89	23.66	28.23	28.96	27.42	27.26
	3h	24.89	32.25	29.87	31.72	30.25	23.70	22.86	25.71	28.71	24.88	24.95	29.87	29.01	29.99	27.58
		23.86	30.77	28.68	31.85	30.40	22.86	23.02	24.92	27.62	23.65	24.82	28.89	28.89	29.35	27.86
		24.46	31.40	29.25	31.68	30.34	23.45	22.88	25.44	28.16	24.33	24.73	29.48	28.94	29.40	27.68
	6h	23.89	30.88	28.30	31.79	29.53	23.30	23.82	25.83	28.91	23.88	23.92	26.63	29.86	25.91	28.37
		23.36	30.64	27.69	31.75	29.94	22.77	23.46	25.40	28.39	23.55	23.81	27.83	29.47	27.88	27.85
		23.55	30.48	27.87	31.70	29.45	22.92	23.34	25.49	28.48	23.62	23.71	27.02	29.36	26.21	27.66
	12h	23.90	31.01	28.85	31.91	30.57	23.88	23.91	26.30	28.08	24.38	24.44	27.93	29.91	27.87	27.77
		23.69	30.51	28.40	31.32	30.00	23.82	23.25	25.90	27.61	23.76	23.84	27.40	28.88	26.40	27.51
		23.68	31.63	28.65	31.40	29.84	23.66	23.15	26.01	27.74	23.90	23.77	27.34	28.97	26.67	27.34
	24h	24.85	32.94	29.88	32.85	30.79	25.30	24.13	27.89	29.89	25.47	24.94	29.83	29.79	28.87	28.94
		23.65	31.64	28.55	31.67	30.47	23.88	23.17	27.07	28.75	24.87	23.76	28.86	28.84	26.22	27.65
		23.75	31.36	28.66	32.10	30.51	23.90	23.53	26.99	28.87	24.87	24.13	28.85	29.31	27.06	28.04
	48h	23.94	31.64	29.35	30.52	29.73	23.89	22.75	26.88	28.82	23.92	23.89	28.99	28.86	29.82	27.80
		23.59	31.08	28.66	30.64	29.17	23.82	22.63	26.39	28.42	23.45	23.67	28.72	28.39	29.77	27.72
		23.65	31.71	28.93	30.62	29.44	23.76	22.54	26.65	28.53	23.65	23.61	28.77	28.61	29.62	27.69

Table A1. Cont.

		ACT-1	ACT-2	ACT-4	ACT-6	ACT-7	AQP	CYP	EF1A	F-box	GAPDH	TUA	TUB	SHR	APS	PYL
Salinity	0.5h	22.62	30.31	26.61	30.82	27.58	22.89	22.66	23.73	27.86	23.66	22.84	25.37	28.65	24.67	26.60
		22.82	30.54	27.84	30.90	28.61	23.23	23.01	24.01	28.21	24.53	23.15	27.10	28.82	25.39	26.47
		22.60	30.08	26.92	30.58	27.94	22.70	22.54	23.50	27.75	23.92	22.88	25.94	28.65	24.90	26.33
	3h	21.73	30.04	26.68	29.81	28.40	22.89	22.89	24.36	27.81	22.02	21.62	26.54	27.76	23.76	26.35
		20.91	29.22	26.33	29.09	27.87	22.32	22.46	23.88	27.28	21.76	20.89	25.91	26.93	22.40	25.78
		21.08	29.93	26.81	29.39	28.52	22.87	22.94	24.46	27.67	21.71	21.12	26.36	27.14	22.82	25.87
	6h	22.51	29.79	27.02	30.63	27.85	22.77	22.92	24.01	27.93	23.51	22.59	26.81	28.73	23.94	27.10
		22.89	29.78	26.90	30.75	27.53	22.85	22.73	23.94	27.71	23.88	22.90	26.90	29.81	24.54	27.55
		22.57	30.50	27.49	30.95	27.99	23.26	23.43	24.56	28.29	23.50	22.62	27.15	28.97	24.08	27.21
	12h	22.85	30.57	27.90	29.87	28.45	23.92	23.39	24.80	27.89	23.28	22.91	27.38	28.98	23.95	26.91
		22.57	30.04	27.29	29.78	27.97	23.44	22.90	23.95	27.28	22.36	22.75	26.99	28.31	23.76	26.96
		22.77	30.24	27.57	29.69	28.30	23.56	23.09	24.30	27.48	22.47	22.75	27.17	28.55	23.85	27.00
	24h	23.42	31.12	28.91	30.89	28.92	25.98	23.85	24.88	29.17	23.75	23.80	29.42	29.67	25.79	26.80
		22.55	30.80	28.49	29.88	28.70	26.04	24.19	24.46	28.83	22.47	22.92	28.93	28.59	23.85	26.11
		22.92	30.95	28.81	30.26	28.90	25.88	24.05	24.63	28.96	22.84	23.22	29.00	28.98	24.45	26.30
	48h	23.45	30.70	28.69	31.45	28.77	25.87	22.95	23.95	28.79	22.84	24.55	28.85	29.81	26.96	27.71
		23.83	30.67	28.57	31.69	28.87	25.80	23.19	23.83	28.69	23.20	24.72	29.07	29.46	27.89	27.89
		23.85	30.90	28.87	31.35	29.06	26.16	23.50	24.15	28.84	23.06	24.80	29.12	29.63	27.62	27.80
Drought	0.5h	22.94	31.58	27.86	30.91	29.28	25.66	23.79	25.46	29.42	23.91	22.82	27.55	29.91	24.95	27.30
		22.19	30.87	27.34	27.79	28.53	24.91	23.59	24.95	28.90	23.59	22.63	26.82	28.84	23.98	27.70
		22.18	31.66	27.92	27.78	28.94	25.19	23.92	25.40	29.39	23.47	22.59	27.28	28.95	24.14	27.37
	3h	23.03	30.84	27.78	31.05	28.90	25.21	22.93	24.86	28.88	23.85	23.14	26.87	28.93	24.88	27.84
		21.85	28.97	26.08	30.48	28.02	24.40	22.40	23.89	28.17	22.51	21.87	26.00	28.83	23.38	26.88
		22.23	29.87	26.81	30.71	28.71	24.89	22.92	24.63	28.79	22.90	22.29	26.59	28.87	23.83	27.19
	6h	23.37	30.69	27.82	30.91	29.12	25.54	23.90	25.47	29.04	24.09	22.96	26.70	29.80	24.17	28.25
		23.75	30.58	28.00	31.54	29.22	25.38	23.86	24.88	28.95	24.27	23.77	27.90	30.19	24.84	27.97
		23.33	30.36	27.46	30.92	28.92	24.93	23.46	24.68	28.54	23.99	23.19	26.81	29.86	24.37	27.97
	12h	22.49	30.46	27.89	29.82	28.73	24.78	22.93	24.74	28.65	22.88	22.79	26.88	28.87	22.56	26.65
		22.88	31.28	28.47	30.16	29.35	25.28	23.56	24.91	28.76	23.10	23.46	28.32	28.68	24.94	26.82
		22.46	31.22	28.50	29.70	29.25	25.41	23.61	24.99	28.88	22.64	22.88	27.91	28.59	23.29	26.45
	24h	22.30	31.22	28.81	30.24	29.07	25.66	23.92	25.56	28.66	23.05	22.91	27.89	28.85	23.64	26.58
		21.89	31.81	28.46	30.31	29.31	25.56	23.97	25.80	28.85	22.70	23.33	28.49	28.57	23.70	25.79
		21.34	31.52	28.78	29.69	29.36	25.90	24.20	25.95	29.00	22.09	22.86	28.59	28.17	22.96	25.38
	48h	24.54	31.72	28.89	31.93	29.71	24.96	23.67	25.49	28.34	24.75	25.10	29.41	29.92	27.72	27.88
		23.86	30.47	27.88	31.21	29.24	24.31	22.96	24.86	27.93	24.03	23.76	27.88	29.61	25.58	27.75
		23.92	31.43	28.34	31.39	29.68	24.81	23.46	25.22	28.15	23.97	23.97	28.38	29.41	25.94	27.38
Heat	0.5h	23.95	31.52	27.82	29.53	29.46	25.92	23.92	25.90	29.24	22.94	22.74	28.40	28.47	25.47	26.67
		23.43	30.66	27.67	29.49	28.96	24.74	23.23	25.01	28.52	22.92	22.87	27.84	29.00	25.36	26.52
		23.58	30.75	27.98	29.94	28.84	24.95	23.39	25.25	28.72	23.08	23.04	27.93	28.93	25.53	26.86
	3h	24.55	30.89	28.19	31.92	29.94	25.51	23.47	25.87	28.72	23.87	24.69	29.72	29.86	29.25	26.53
		24.61	31.15	27.52	31.15	29.40	26.20	23.54	25.63	28.64	22.71	23.90	29.76	28.78	26.65	25.98
		24.90	31.48	27.63	31.11	29.57	26.34	23.88	25.96	28.95	23.03	24.05	30.39	29.08	27.35	26.15
	6h	23.78	30.59	28.78	32.62	29.62	25.70	23.94	25.78	27.66	24.38	24.84	28.63	29.88	28.78	27.60
		23.88	31.50	28.43	32.14	29.79	25.87	23.78	25.89	28.13	23.88	23.85	29.08	29.40	29.26	27.00
		23.90	31.06	28.65	32.58	29.87	25.67	23.83	25.90	27.96	23.98	24.20	29.08	29.57	28.95	27.12
	12h	24.32	31.95	27.65	28.33	31.81	26.56	24.02	27.92	28.38	23.87	23.88	29.76	28.59	26.92	26.72
		23.90	31.63	27.87	27.74	30.89	25.95	23.64	27.36	27.86	23.80	23.82	29.08	28.95	26.63	27.17
		24.30	31.96	27.61	27.86	31.48	26.52	24.02	27.89	28.08	23.74	23.74	29.60	28.73	26.85	26.92
	24h	25.91	33.44	29.89	32.81	32.93	28.37	26.43	28.82	29.31	26.75	25.61	31.56	30.22	28.16	28.44
		25.34	33.59	28.73	31.96	31.83	27.90	25.84	27.99	28.81	25.79	24.78	30.61	29.98	30.09	27.89
		25.20	32.89	28.96	32.50	31.97	27.58	25.85	27.95	28.67	25.86	24.87	30.90	30.00	28.86	27.94
	48h	24.67	33.64	28.79	31.05	32.03	26.87	25.94	27.86	28.18	25.86	24.31	30.01	29.93	29.32	27.83
		25.60	33.96	29.40	31.20	32.62	27.86	26.66	28.23	28.49	25.73	24.88	30.61	29.52	30.24	28.46
		24.90	33.72	29.01	31.12	32.01	27.07	26.04	27.63	28.01	25.72	24.57	30.11	29.80	29.84	28.05

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