

Article

Selection of Reference Genes for Gene Expression Analysis in *Acacia melanoxylon* under Different Conditions

Zhaoli Chen ^{1,†}, Xiaogang Bai ^{2,†}, Xiangyang Li ¹, Bingshan Zeng ^{1,*} and Bing Hu ^{1,*}

¹ Key Laboratory of State Forestry and Grassland Administration on Tropical Forestry, Research Institute of Tropical Forestry, Chinese Academy of Forestry, Guangzhou 510520, China; zlchen0525@163.com (Z.C.); xiangylxiang@163.com (X.L.)

² College of Agriculture and Biology, Zhongkai University of Agriculture and Engineering, Guangzhou 510225, China; bxg469808@163.com

* Correspondence: b.s.zeng@vip.tom.com (B.Z.); binghu@caf.ac.cn (B.H.)

† These authors contributed equally to this study.

Abstract: The research of functional genes in *Acacia melanoxylon*, a precious and fast-growing timber species with wide adaptability, has been greatly limited due to the absence of reliable and suitable reference genes. To fill this gap, five different algorithms (comparative ΔC_t , NormFinder, geNorm, BestKeeper, and RankAggreg) were employed to assess the expression stability of ten candidate genes under nine different experimental sets and their three combined groups. The results showed that *PP2a* and *RPL4* maintained stable expression in all 144 samples and a group of different tissues or organs. *PAT10* and *TIP41* were the best-performing genes in different clonal varieties, pinnate compound leaves at different growth states, salt, and indole acetic acid sets. *PP2a* and *PAT10* were the top two choices for gibberellin and abiotic stress groups. *PP2a* and *UBI11* exhibited stable expression in drought treatment. *UBI3* combined with *OTUD6B*, *RPL4*, or *PP2a* were identified as the optimal reference genes in the heat, ethephon, or exogenous hormone groups, respectively. The reliability of the selected reference genes was further confirmed by evaluating the expression patterns of *AmWRKY6* and *AmWRKY33* genes. This study provides the first comprehensive evaluation of reference gene stability in *A. melanoxylon* and promotes future research on the gene expression analysis of the species.

Keywords: *Acacia melanoxylon*; RT-qPCR; expression stability; normalization



Citation: Chen, Z.; Bai, X.; Li, X.; Zeng, B.; Hu, B. Selection of Reference Genes for Gene Expression Analysis in *Acacia melanoxylon* under Different Conditions. *Forests* **2023**, *14*, 2245. <https://doi.org/10.3390/f14112245>

Academic Editor: Donald L. Rockwood

Received: 9 October 2023

Revised: 7 November 2023

Accepted: 13 November 2023

Published: 14 November 2023



Copyright: © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Acacia melanoxylon, belonging to the Leguminosae family and *Acacia* genus, is an evergreen tree species native to southeastern Australia [1]. It is regarded as an ideal tree species that combines economic, ecological, and greening benefits, leading to its widespread introduction and cultivation across the globe [2–4]. *A. melanoxylon* possesses high-quality timber with reddish brown-colored heartwood, which produces high-end furniture, musical instruments, joinery, flooring, and crafts [2,5]. It belongs to the short and medium management cycle tree species with a primary cutting cycle of 13 to 16 years in South China. Its heartwood is synthesized at an early growth stage, earlier than most other precious tree species [6]. Based on the characteristics of fast growth rate, high proportion of heartwood, and moderate wood density [4], *A. melanoxylon* is expected to be a model for studying heartwood formation in precious tree species. However, the molecular mechanisms of the secondary cell wall thickening, secondary metabolite deposition, and the differences in synthetic pathways between heartwood and sapwood in *A. melanoxylon* are still unclear. Moreover, *A. melanoxylon* is commonly distributed in regions characterized by harsh environmental conditions like drought, high temperature, salinity, and barren land. During these adaptation processes, *A. melanoxylon* has developed diverse intricate mechanisms to resist or acclimate to adverse environmental conditions. Thence, *A. melanoxylon*

is also suitable for studying the abiotic stress resistance mechanism. Notably, during the seedling early growth stage, *A. melanoxylon* produces bipinnately compound “true leaves”. Later, the petiole (or leaf axis) becomes wider and flattened (Phyllode) but with compound leaves attached, forming transition leaves together. Sometimes, it can become phyllodes without compound leaves at all [7]. It has been revealed that this leaf morphology transformation is associated with adaption to changing environmental conditions, such as shade and drought [8,9]. Therefore, it is a special research model for studying the heterophylly of plants to adapt to environmental changes [10,11]. Nevertheless, research pertaining to *A. melanoxylon*’s stress conditions molecular response mechanisms, heteromorphic leaf growth and development patterns, and wood development rules analysis is still relatively limited.

Gene expression analysis is one of the commonly used techniques to investigate gene functionality and provides valuable insights into the molecular processes governing plant organ development and adversity resilience [12,13]. The real-time quantitative polymerase chain reaction (RT-qPCR) technique is universally employed in basic research, molecular medicine, and biotechnology, and it facilitates gene expression study [14–16]. Compared with other gene expression detection techniques, namely microarray, northern blotting, and RT-PCR, RT-qPCR presents many benefits, encompassing rapid reaction, high sensitivity, accurate quantification, strong repeatability, and specificity. However, several factors, including primer specificity, RNA quality and integrity, reverse transcription efficiency, amplification efficiency, and the number of initial materials, can influence the RT-qPCR results. Therefore, to control unnecessary disparities within and between samples, introducing stably expressed housekeeping genes as reference genes is essential for error correction and standardization [17,18].

Generally, housekeeping genes encode proteins that are necessary to maintain fundamental biological processes, such as actin (*ACT*), ubiquitin (*UBI*), elongation factor (*EF*), protein phosphatase 2a (*PP2a*), and tubulin (*TUB*), which are commonly utilized as internal reference genes [19]. However, increasing experimental evidence has shown that housekeeping genes are not as stable as previously believed in different conditions [20,21]. Several studies have reported that some novel characterized genes exhibit more excellent stability than transitional housekeeping genes under certain conditions. For instance, in alkali stress, the expression of the reference gene dimethyladenosine transferase (*DIM1*) in roots of *Nitraria sibirica* was more stable than *ACT7*, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and *EF-1a* [22]. The expression stability of BI1-like protein (*BI1*) and translationally controlled tumor protein homolog (*TCTPH*) was higher than *EF-1a* and *UBI4* in different tissues of soybean [18]. To summarize, no reference gene is universal across all experimental conditions and plant species [23,24]. Therefore, evaluating their expression stability under specific experimental conditions is crucial before employing reference genes. In this context, researchers utilized several statistical algorithms to evaluate the expression stability of candidate reference genes and perform stability ranking, such as comparative ΔC_t [25], geNorm [26], NormFinder [27], BestKeeper [28], and Rank-Aggreg [29]. Previous studies have used these algorithms to identify reference genes for various species effectively [27,28]. To date, the Internal Control Genes Database (ICG, <https://ngdc.cncb.ac.cn/icg/>, accessed on 7 December 2021) [19] has compiled reference genes from over 278 plant species, such as soybean [30], *Arabidopsis* [31], *Eucalyptus grandis* [32], and *Santalum album* [33]. To our knowledge, there is a lack of literature on the appropriate reference genes for gene expression standardization in *A. melanoxylon*.

This research aims to determine the reliable reference genes for RT-qPCR in *A. melanoxylon* under various conditions, including different clonal varieties (DCV), different tissues or organs (DTO), pinnate compound leaves at different growth states (LDGS), heat (HT), salt (ST), drought (PEG), gibberellin (GA), indole acetic acid (IAA), and ethephon (ET). Specifically, we selected eight classic and two novel candidate reference genes from *A. melanoxylon* genomic and transcriptomic data, and five statistical algorithms were employed to evaluate expression stability. To ascertain the reliability of the chosen reference genes, we conducted

normalization analyses of the expression levels of *AmWRKY6* and *AmWRRK33*. Our research provides a series of suitable reference genes for the RT-qPCR of *A. melanoxylon* under various experimental conditions. It establishes a foundation for further research on the molecular mechanisms of this species.

2. Results

2.1. Primer Specificity and Amplification Efficiency Analysis

We first identified ten candidate reference genes from the *A. melanoxylon* genome and transcriptome. The RT-qPCR primers were designed based on the sequences associated with these genes. Gel electrophoresis showed that the sizes of all primer pairs were in line with expectations, and the bands were evident (Figure S1). The melting curve assays indicated a single peak for each gene, further confirming the specificity of the primer pairs (Figure S2). The E-values of the ten reference genes ranged from 97.01% (protein S-acyltransferase 10, *PAT10*) to 106.15% (*EF1a*), and the R^2 values were higher than 0.99. Table 1 provides the details of the ten genes. The results demonstrate that the primer pairs meet the criteria for subsequent RT-qPCR analysis.

2.2. Expression Levels of Candidate Reference Genes

The cycle threshold (Ct) value variation coefficient can be used to assess the gene expression stability. The expression levels of ten candidate reference genes were determined using RT-qPCR under DCV, DTO, LDGS, HT, ST, PEG, GA, IAA, and ET treatments (Figure 1). The Ct values of the ten genes ranged from 14.99 (*UBI11*) to 32.86 (*OTUD6B*), indicating significant variability among these gene expression levels. And *UBI11* (17.67) and *ACT7* (27.15) had the highest and lowest mean expression levels, respectively. Additionally, it was observed that *OTUD6B* (17.98–32.86) had the largest variation in Ct values, whereas *PP2a* (18.83–23.55) showed relatively moderate variation. The distribution of Ct values revealed that all ten genes exhibited varied expression levels in different conditions (Figure S3). Thus, it is essential to screen the internal reference genes under specific conditions in *A. melanoxylon*.

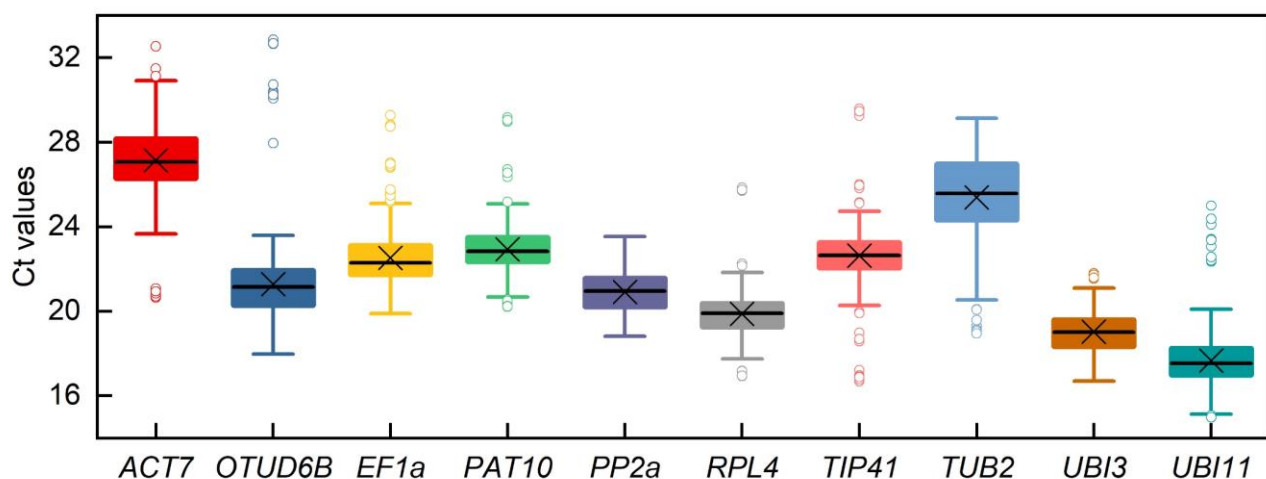


Figure 1. The Ct values of the ten candidate reference genes in all 144 samples. The box indicates the 25th to 75th percentiles. The cross sign in the box shows the mean values. The line across the box represents the median. The whisker caps indicate the maximum and minimum values. The dots represent outliers. The raw Ct values are shown in Table S1. Gene name abbreviations are listed in Table 1 or the Abbreviations.

Table 1. Details of the ten candidate reference genes used for RT-qPCR.

Gene Symbol	Gene ID	Gene Description	Forward/Reverse Primer (5'-3')	Amplicon Length (bp)	Primers TM (°C)	E (%)	R ²
<i>ACT7</i>	evm.model.Chr8.816	actin 7	F:AGATTCCGCTACCCAGAAG R:AGCCGCCACTTAGAACAAT	148	56.94/ 57.44	103.47	0.991
<i>OTUD6B</i>	evm.model.Chr7.1946	deubiquitinase OTUD6B	F:TCCTTCCCAGATGTTGAGAT R:TAGTCCAAATGCGTGCTTAT	105	57.06/ 56.02	105.49	1.000
<i>EF1a</i>	evm.model.Chr7.4292	elongation factor1-alpha	F:AAGTATGCCTGGGTCTTGA R:TGATGAAGTCTCTGTGCCTG	136	57.27/ 56.29	106.15	0.998
<i>PAT10</i>	evm.model.Chr3.3020	protein S-acyltransferase 10	F: CTGGTCTGTGTAGCCGTCT R:GGAGGAAATGGAGGTAACAA	138	57.96/ 56.57	91.07	1.000
<i>PP2a</i>	evm.model.Chr3.536	protein phosphatase 2a	F:AAGAGTTTGGTCCTGAGTGG R:CAAGCAGAGAGACAGCGTTA	114	56.78/ 56.96	105.96	0.999
<i>RPL4</i>	evm.model.Chr8.89	60S ribosomal protein L4-like	F:AAAGGCAAGATGAGAAATCG R:ATAACGAACCTCCCAAGATG	179	57.03/ 56.6	103.59	0.999
<i>TIP41</i>	evm.model.Chr10.2641	tonoplast intrinsic protein	F:TAGGCACAGAGCGAAGAAAT R:TCAAAGTCTCAATCTCCCAAC	156	57.73/ 56.81	106.11	1.000
<i>TUB2</i>	evm.model.Chr3.2570	beta-tubulin	F:CACCATCCAGTTTGTGACT R:ACAGCCCTCTGAACCTTG	108	55.94/ 56.2	103.02	0.999
<i>UBI3</i>	evm.model.Chr3.1463	ubiquitin 3	F:AGCAGCGTCTCATCTTCG R:ATCTTCTTGGGCTTGGTGTA	154	57.71/ 57.27	103.88	0.998
<i>UBI11</i>	evm.model.Chr10.2426	ubiquitin 11	F:AGATTCCGCTACCCAGAAG R:AGCCGCCACTTAGAACAAT	148	56.5/ 56.6	104.81	1.000

2.3. Expression Stability of the Candidate Reference Genes

This paper employed four different statistical algorithms (comparative ΔC_t , NormFinder, geNorm, and BestKeeper) to assess and rank the expression stability of selected reference genes from *A. melanoxylon* under various experimental conditions. Each candidate reference gene was initially evaluated under nine experiment sets analyzed individually. To achieve a more comprehensive result, these individual sets were then split up into groups of three: abiotic stresses (ASs, including HT, ST, and PEG), exogenous hormone treatments (ETHs, including GA, IAA, and ET), and all samples (All, including all 144 samples of the nine experimental sets).

2.3.1. ΔC_t Algorithm

Figure 2a and Table S2 show that *PP2a* exhibited the highest stability in the DCV, DTO, ST, GA, ET, EHTs, and All groups. For HT, PEG, and ASs groups, *UBI3* was found to be the most stable gene. For LDGS and IAA sets, *UBI11* and *RPL4* emerged as the most stable genes, respectively. In addition, *ACT7* was identified as the least stable reference gene in multiple groups, including DTO, LDGS, HT, ET, and EHTs groups.

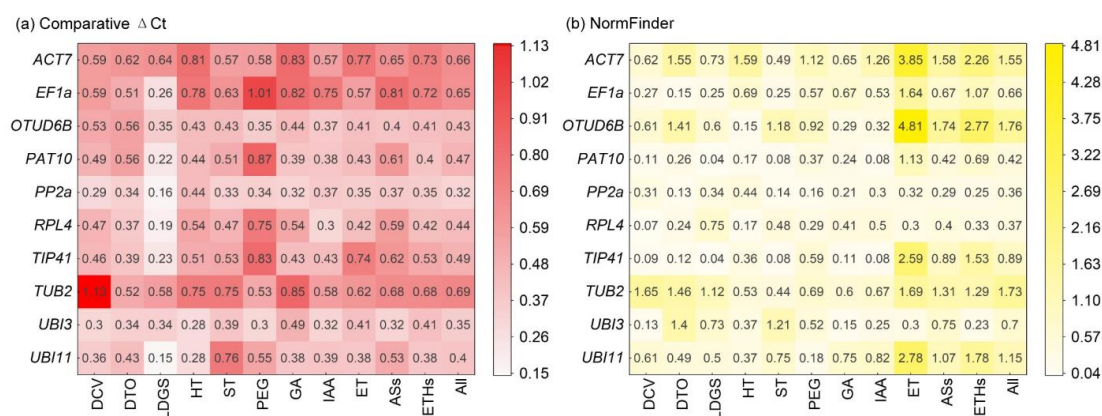


Figure 2. The heat map presents the stability of the ten candidate reference genes calculated by comparative ΔC_t and NormFinder. (a) Comparative ΔC_t ; (b) NormFinder. The color of a pane becomes lighter as the value reduces, indicating a higher stability of the candidate reference genes. Gene name and group name abbreviations are listed in the Abbreviations.

2.3.2. NormFinder Algorithm

NormFinder evaluates the stability of gene expression by calculating the stability (S) values. As shown in Figure 2b and Table S3, *RPL4* and *UBI3* (S values = 0.3) showed the highest stability in the ET treatment, while *PAT10* and *TIP41* showed the least variation in LDGS (0.04), ST (0.08), and IAA (0.08) sets. *TIP41* was a suitable reference gene for the DTO set (0.12) and GA (0.08) treatment, while *PP2a* was ranked as the top gene for the PEG (0.16), ASs (0.29), and All (0.36) groups. In addition, *RPL4*, *OTUD6B*, and *UBI3* were identified as the most stable genes for DCV (0.07), HT (0.15), and ETHs (0.23) groups, respectively.

2.3.3. GeNorm Algorithm

The M values of the ten genes from eight sets (expected for the ET set), as well as ASs and All groups, were less than the threshold of 1.5, which indicated that the vast majority of genes remain stable across various treatments (Figure 3). *PAT10* and *TIP41* were the most stable genes for DCV (M value = 0.04), LDGS (0.07), ST (0.15), PEG (0.19), and ASs (0.37) groups; *PP2a* and *RPL4* showed good stability for DTO (0.27) set; for HT treatment, *OTUD6B* and *UBI3* were the most stable genes (0.22); *PP2a* and *OTUD6B* were the most stable genes for GA (0.22) and IAA (0.03) treatments; *UBI3* (0.6) and *RPL4* (0.46) were the two best reference gene in ET and ETHs groups, respectively; and *RPL4* and *PAT10* had the highest stability with M values of 0.53 in All groups.

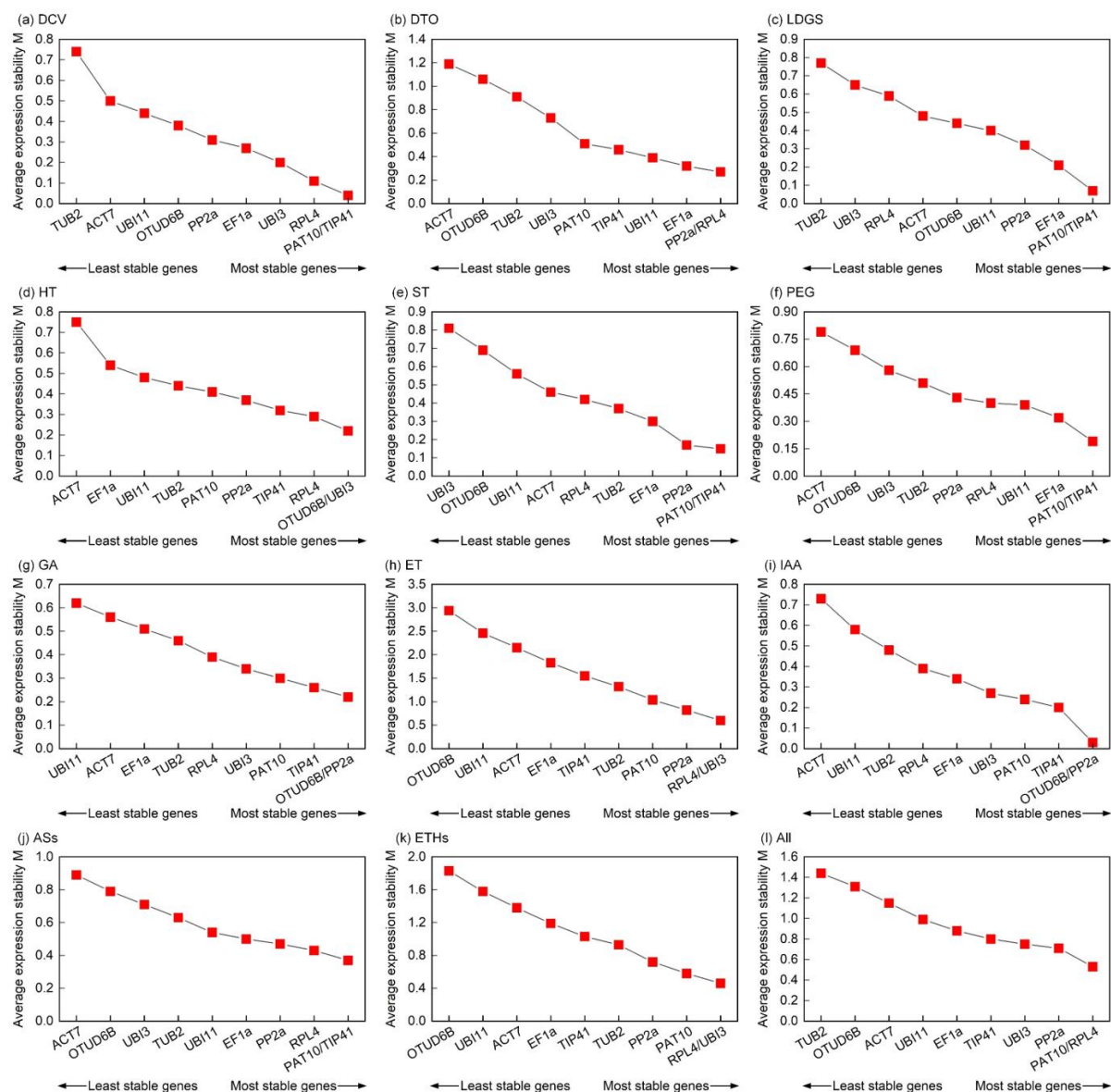


Figure 3. The M values of ten candidate reference genes calculated by geNorm. The genes were arranged from left to right according to the order of stability. The smaller the M values, the higher the stability of the genes. (a) Different clonal varieties; (b) different tissues or organs; (c) pinnate compound leaves at different growth states; (d) heat; (e) salt; (f) drought; (g) gibberellin; (h) ethephon; (i) indole acetic acid; (j) abiotic stresses; (k) exogenous hormone treatments; (l) all 144 samples of nine experimental sets. Gene name and group name abbreviations are listed in the Abbreviations.

Generally, it is more reliable to use multiple reference genes than one gene to make a quantitative analysis. With this in mind, we calculated the pairwise variation (V) values for the reference genes, and determined the optimal number of reference genes based on these values. As shown in Figure 4, the $V_{2/3}$ values under DCV, DTO, LDGS, PEG, HT, ST, GA, IAA, and ASs groups were all below the cut-off values of 0.15, indicating that two reference genes were necessary for the reliable normalization of the gene expression data. For ET, ETHs, and All samples, the $V_{n/n+1}$ is greater than 0.15, which means that no candidate reference gene combination could be used in those groups within this experiment. These results further emphasize the importance of screening internal reference genes in specific experimental conditions.

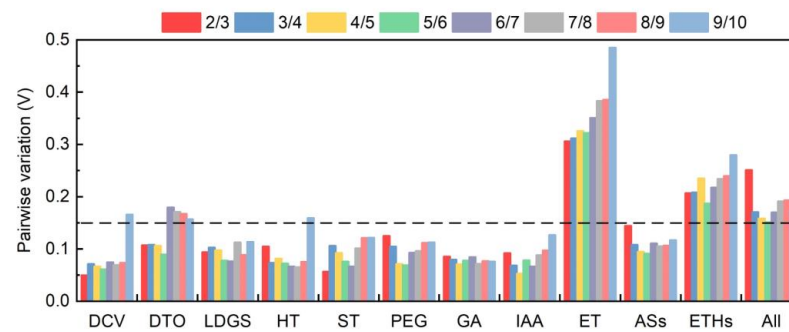


Figure 4. Pairwise variations for the ten candidate reference genes using geNorm to determine the suitable number of reference genes. A threshold of 0.15 was used to determine the optimal number of reference genes required for accurate normalization. Group name abbreviations are listed in the Abbreviations.

2.3.4. BestKeeper Algorithm

For BestKeeper, a gene with a *p*-value less than 0.05 and a standard deviation (SD) value less than 1 was stable. Then, the ranking of gene stability was based on the principle that the lower the $SD \pm CV$ (coefficient of variance) value, the higher the stability. Table 2 shows *PAT10* was most stably expressed in DCV and IAA sets. *RPL4* was the most stable gene in DTO, GA, and All groups. *TIP41* showed the highest stability in LDGS and ST groups. *UBI11* exhibited the fluctuation of a few expressions in PEG, HT, and ASs groups. *UBI3* emerged as the most stable gene in ET and ETHs groups.

Table 2. Expression stability of the ten candidate reference genes as calculated by BestKeeper.

Rank	DCV	SD ± CV	DTO	SD ± CV	LDGS	SD ± CV	PEG	SD ± CV
1	<i>PAT10</i>	0.43 ± 1.90	<i>RPL4</i>	0.51 ± 2.57	<i>TIP41</i>	0.47 ± 2.12	<i>UBI11</i>	0.64 ± 3.75
2	<i>TIP41</i>	0.43 ± 1.93	<i>UBI11</i>	0.61 ± 3.34	<i>PAT10</i>	0.47 ± 2.14	<i>UBI3</i>	0.68 ± 3.63
3	<i>UBI3</i>	0.45 ± 2.38	<i>PP2a</i>	0.65 ± 3.08	<i>PP2a</i>	0.47 ± 2.25	<i>TUB2</i>	0.81 ± 3.16
4	<i>RPL4</i>	0.48 ± 2.43	<i>TIP41</i>	0.72 ± 3.14	<i>EF1a</i>	0.48 ± 2.19	<i>PP2a</i>	0.82 ± 3.96
5	<i>ACT7</i>	0.49 ± 1.81	<i>EF1a</i>	0.77 ± 3.37	<i>UBI11</i>	0.63 ± 3.58	<i>ACT7</i>	0.87 ± 3.13
6	<i>OTUD6B</i>	0.57 ± 2.68	<i>PAT10</i>	0.83 ± 3.55	<i>OTUD6B</i>	0.66 ± 3.12	<i>OTUD6B</i>	0.89 ± 4.26
7	<i>PP2a</i>	0.59 ± 2.80	<i>UBI3</i>	1.01 ± 5.09	<i>UBI3</i>	0.69 ± 3.73	<i>PAT10</i>	0.93 ± 4.02
8	<i>EF1a</i>	0.63 ± 2.85	<i>ACT7</i>	1.24 ± 4.53	<i>RPL4</i>	0.77 ± 4.06	<i>EF1a</i>	0.93 ± 4.16
9	<i>UBI11</i>	0.78 ± 4.36	<i>OTUD6B</i>	1.33 ± 6.35	<i>ACT7</i>	0.97 ± 3.77	<i>RPL4</i>	1.04 ± 5.19
10	<i>TUB2</i>	1.79 ± 8.04	<i>TUB2</i>	1.43 ± 5.84	<i>TUB2</i>	1.27 ± 5.22	<i>TIP41</i>	1.07 ± 4.67
Rank	HT	SD ± CV	ST	SD ± CV	GA	SD ± CV	ET	SD ± CV
1	<i>UBI11</i>	0.39 ± 2.22	<i>TIP41</i>	0.66 ± 2.89	<i>RPL4</i>	0.73 ± 3.59	<i>UBI3</i>	0.46 ± 2.38
2	<i>PAT10</i>	0.63 ± 2.70	<i>ACT7</i>	0.70 ± 2.61	<i>PAT10</i>	0.77 ± 3.32	<i>RPL4</i>	0.62 ± 3.12
3	<i>TIP41</i>	0.66 ± 2.93	<i>PAT10</i>	0.70 ± 3.04	<i>UBI11</i>	0.83 ± 4.84	<i>PP2a</i>	0.69 ± 3.23
4	<i>UBI3</i>	0.68 ± 3.65	<i>RPL4</i>	0.71 ± 3.53	<i>UBI3</i>	0.86 ± 4.50	<i>EF1a</i>	1.39 ± 5.94
5	<i>RPL4</i>	0.72 ± 3.57	<i>TUB2</i>	0.74 ± 2.74	<i>ACT7</i>	0.87 ± 3.08	<i>TUB2</i>	1.45 ± 5.74
6	<i>OTUD6B</i>	0.74 ± 3.52	<i>PP2a</i>	0.80 ± 3.80	<i>TIP41</i>	0.88 ± 3.85	<i>UBI11</i>	1.92 ± 10.30
7	<i>PP2a</i>	0.83 ± 4.04	<i>EF1a</i>	0.83 ± 3.70	<i>PP2a</i>	0.92 ± 4.36	<i>PAT10 *</i>	0.88 ± 3.90
8	<i>EF1a</i>	0.97 ± 4.29	<i>UBI11</i>	0.98 ± 5.67	<i>OTUD6B</i>	1.01 ± 4.67	<i>TIP41 *</i>	1.88 ± 8.70
9	<i>TUB2 *</i>	0.78 ± 2.89	<i>UBI3</i>	1.01 ± 5.15	<i>TUB2</i>	1.23 ± 4.66	<i>ACT7 *</i>	2.45 ± 9.10
10	<i>ACT7 *</i>	1.20 ± 4.34	<i>OTUD6B</i>	1.45 ± 7.15	<i>EF1a</i>	1.36 ± 5.91	<i>OTUD6B *</i>	3.19 ± 13.77
Rank	IAA	SD ± CV	ASs	SD ± CV	ETHs	SD ± CV	All	SD ± CV
1	<i>PAT10</i>	0.50 ± 2.16	<i>UBI11</i>	0.65 ± 3.78	<i>UBI3</i>	0.65 ± 3.40	<i>RPL4</i>	0.55 ± 2.75
2	<i>TIP41</i>	0.52 ± 2.24	<i>PAT10</i>	0.74 ± 3.19	<i>RPL4</i>	0.69 ± 3.45	<i>PP2a</i>	0.59 ± 2.80
3	<i>OTUD6B</i>	0.58 ± 2.72	<i>TIP41</i>	0.79 ± 3.47	<i>PP2a</i>	0.74 ± 3.51	<i>PAT10</i>	0.60 ± 2.61
4	<i>PP2a</i>	0.60 ± 2.88	<i>RPL4</i>	0.82 ± 4.09	<i>PAT10</i>	0.75 ± 3.27	<i>TIP41</i>	0.62 ± 2.74
5	<i>UBI3</i>	0.66 ± 3.39	<i>UBI3</i>	0.83 ± 4.38	<i>TIP41</i>	1.07 ± 4.74	<i>UBI3</i>	0.66 ± 3.49
6	<i>UBI11</i>	0.66 ± 3.78	<i>PP2a</i>	0.84 ± 4.04	<i>EF1a</i>	1.19 ± 5.17	<i>UBI11</i>	0.78 ± 4.44

Table 2. Cont.

Rank	IAA	SD $\pm$ CV	ASs	SD $\pm$ CV	ETHs	SD $\pm$ CV	All	SD $\pm$ CV
7	<i>RPL4</i>	0.71 $\pm$ 3.48	<i>TUB2</i>	0.91 $\pm$ 3.44	<i>UBI11</i>	1.19 $\pm$ 6.72	<i>EF1a</i>	0.80 $\pm$ 3.55
8	<i>EF1a</i>	0.80 $\pm$ 3.54	<i>EF1a</i>	0.96 $\pm$ 4.29	<i>TUB2</i>	1.29 $\pm$ 4.94	<i>OTUD6B</i>	0.90 $\pm$ 4.23
9	<i>TUB2</i> *	0.65 $\pm$ 2.42	<i>ACT7</i>	0.98 $\pm$ 3.59	<i>ACT7</i>	1.34 $\pm$ 4.86	<i>ACT7</i>	1.00 $\pm$ 3.67
10	<i>ACT7</i> *	0.81 $\pm$ 2.94	<i>OTUD6B</i>	1.02 $\pm$ 4.92	<i>OTUD6B</i>	1.44 $\pm$ 6.54	<i>TUB2</i>	1.54 $\pm$ 6.05

* indicates *p*-value greater than 0.05, gray shading indicates SD greater than 1, SD $\pm$ CV (%) means standard deviation $\pm$ coefficient of variance (%). Gene name and group name abbreviations are listed in the Abbreviations.

2.4. Comprehensive Stability Ranking of Reference Genes

We have analyzed the expression stability ranking of the 10 genes through four algorithms. The stability rankings differed due to the diverse principles employed by these algorithms. To obtain a comprehensive ranking, we employed the RankAggreg algorithm to calculate the order of the ten genes under each experimental condition. The principle of this method is to generate and classify all possible ranking lists by an unweighted ranking aggregation of brute force methods. As shown in Figure 5, *PAT10* and *TIP41* were identified as the most stable genes in DCV, LDGS, ST, and IAA sets; *PP2a* and *RPL4* were optimal combinations in DTO and All groups; *PP2a* and *PAT10* exhibited stable expression in GA and ASs groups; *PP2a* and *UBI11* were identified as the two most stable genes in PEG treatment; *UBI3* combined with *OTUD6B*, *RPL4* or *PP2a* were the two most stable genes in HT, ET, or ETHs, respectively.

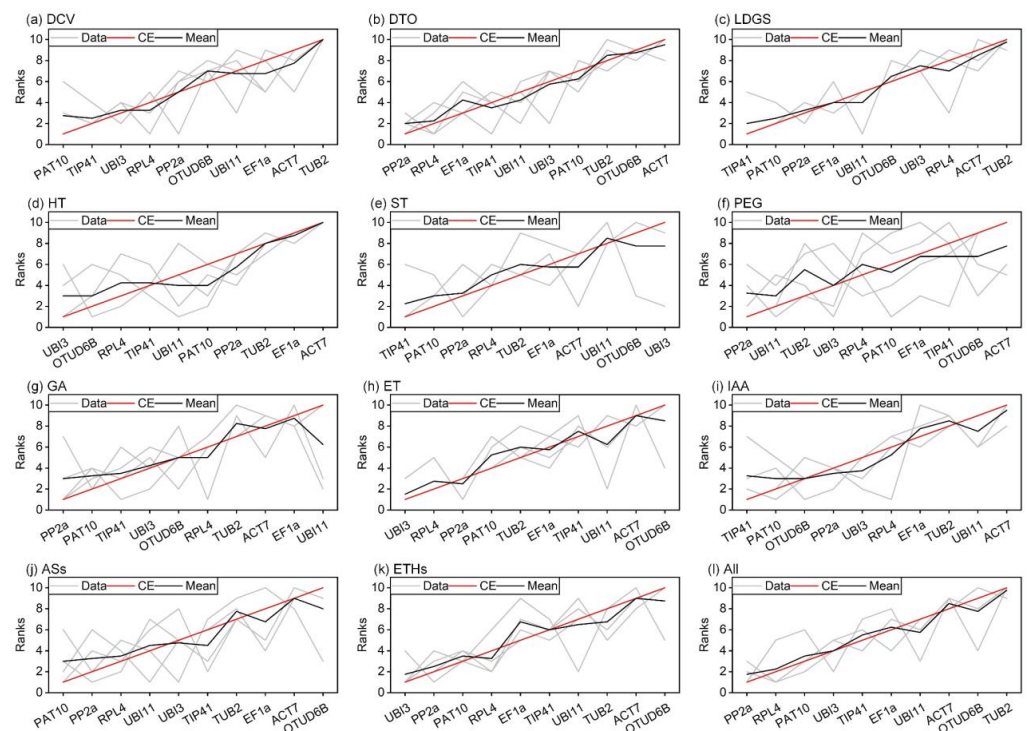


Figure 5. Expression stability of the ten candidate reference genes as calculated by Rank aggregation. (a) Different clonal varieties; (b) different tissues or organs; (c) pinnate compound leaves at different growth states; (d) heat; (e) salt; (f) drought; (g) gibberellin; (h) ethephon; (i) indole acetic acid; (j) abiotic stresses; (k) exogenous hormone treatments; (l) all 144 samples of nine experimental sets. Visual representation of rank aggregation using RankAggreg with the Cross-Entropy Monte Carlo algorithm and Spearman foot rule distances. Different lines in the plot represent the following: gray lines mean stability ranking according to Δ Ct method, geNorm, NormFinder, BestKeeper; black lines mean rank position; and red lines mean model computed using the Cross-Entropy Monte Carlo algorithm. Gene name and group name abbreviations are listed in the Abbreviations.

2.5. Validation of the Stability of Reference Genes

The expression stability of the selected reference genes was verified by measuring *AmWRKY6* or *AmWRKY33* expression patterns using either the two most stable reference genes (individually or combined) or the least stable gene. In the DCV group, the relative expression of *AmWRKY6* was similar whether standardized using the two most stable genes (*PAT10* and *TIP41*) separately or together. However, the relative expression of *AmWRKY6*'s in SR17 samples was abnormally increased when normalized with the least stable gene *TUB2*. In the DTO and LDGS groups, *AmWRKY6*'s expression level and trends were relatively consistent when relative quantification was implemented using the top two stable genes or their combination. However, utilizing the least stable genes in relative quantification elicited variations in expression levels and trends of *AmWRKY6*. As shown in Figure 6, for HT, ST, ET, and IAA treatments, the expression pattern of *AmWRKY33* remained consistent when the most stable genes and their combination were used as the reference genes. When relative quantification was carried out using the least stable gene, the expression levels showed significant differences in some samples. In PEG treatment, when the most stable genes and their combinations were used as the reference genes, *AmWRKY33*'s relative expression level was highest at 24 h. However, the relative expression of *AmWRKY33* peaked at 6 h and dropped to the lowest level at 24 h when the least stable gene was used as the reference gene. A similar phenomenon was observed under GA treatment, the expression patterns of *AmWRKY33* were very similar when using the top two stable reference genes and their combinations for standardization, but the expression levels and expression trends had a significant variation when the least stable gene was used.

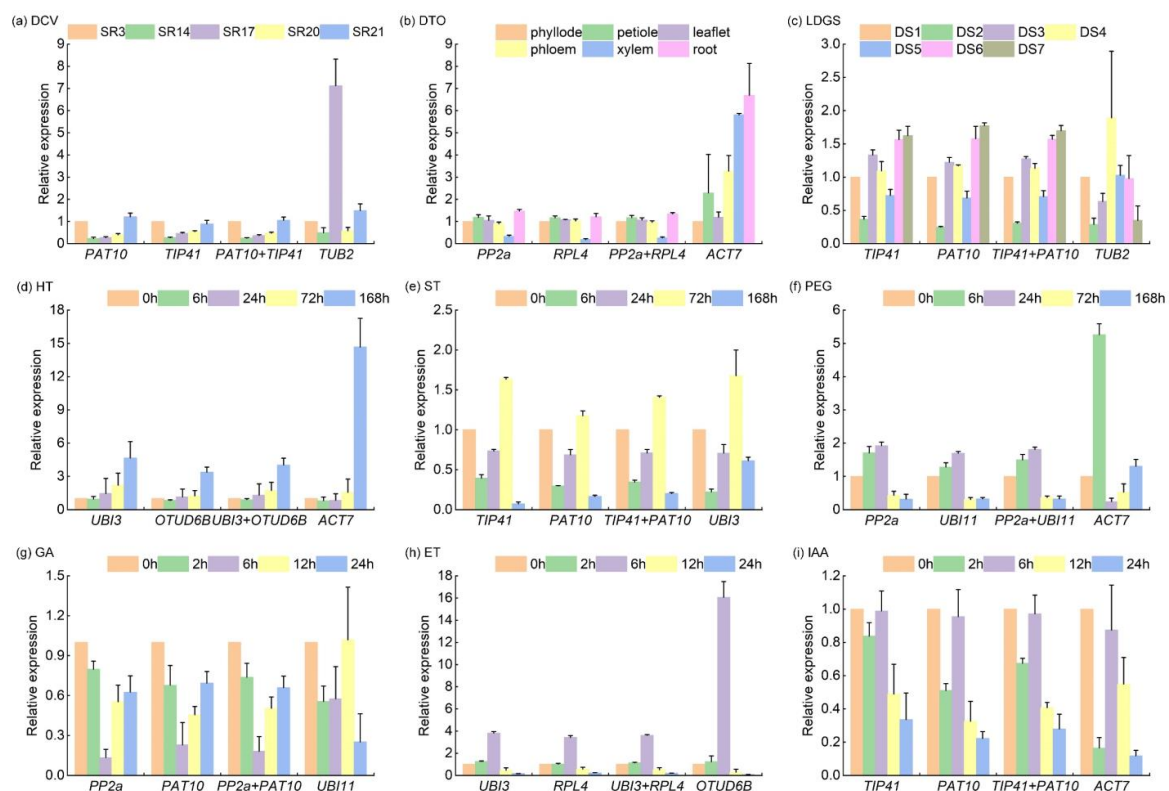


Figure 6. Relative expression patterns of *AmWRKY6* and *AmWRKY33*. The two most stable reference genes (alone or in combination) and an unstable gene were employed for normalization. (a) Different clonal varieties; (b) different tissues or organs; (c) pinnate compound leaves at different growth states; (d) heat; (e) salt; (f) drought; (g) gibberellin; (h) ethephon; (i) indole acetic acid. Gene name and group name abbreviations are listed in the Abbreviations.

3. Discussion

RT-qPCR is a widely used technology in molecular biology for investigating gene expression patterns and biological regulatory mechanisms [34]. To ensure the reliability of gene expression data obtained by RT-qPCR analysis [35], it is essential to select suitable reference genes under specific experimental conditions. Ideally, a reference gene should exhibit stable expression across all experimental conditions and remain consistent across different tissues and growth stages of the organism. However, such genes almost do not exist [17].

This paper selected ten candidate reference genes from the *A. melanoxylon* transcriptome and genome database and evaluated their expression stability under DCV, DTO, LDGS, HT, ST, PEG, GA, IAA, and ET conditions. Experiment findings indicated that each reference gene's Ct value exhibited obvious variation under different experimental conditions (Figure 1 and Figure S3), which implies that the candidate reference genes require specific selection according to different experimental conditions. To that end, four common algorithms based on statistical analysis were employed to determine the expression stability of the reference genes. Interestingly, the ranking results obtained through comparative Δ Ct, geNorm, and NormFinder analyzed showed greater similarities to each other compared to the results obtained from BestKeeper. For instance, *RPL4* was the most stable reference gene in GA treatment according to BestKeeper, while it was ranked low in comparative Δ Ct, geNorm, and NormFinder results. Previous studies conducted on *N. sibirica* [22], *Rubus* [36], and *Toona ciliata* [37] have also reported similar discrepancies between BestKeeper and other algorithms. These differences in results can be attributed to the distinct screening principles and emphases employed by each algorithm [20]. Each program had potential advantages and limitations, complementing them if properly utilized and analyzed. In this paper, to consolidate the results obtained from the four algorithms, RankAggreg was employed for calculating the overall ranking.

Utilizing more than one reference gene is necessary to prevent erroneous interpretations when analyzing changes in target genes [12,38,39]. In this study, the threshold value of 0.15 ($V_{n/n+1}$) obtained from geNorm was utilized to determine the optimal number of reference genes for normalization. For ET, ETHs, and All samples, the $V_{n/n+1}$ values were greater than 1.5, indicating that even ten reference genes cannot accurately normalize the target gene. It is important to note that the threshold value of 0.15 ($V_{n/n+1}$) obtained from geNorm, while commonly used, should not be considered a strict restriction [40,41]. In addition, we consider the ranking results obtained by the other three algorithms and the M value of geNorm, which all meet the criteria of reference gene screening. Therefore, it is unnecessary to employ multiple reference genes to replace two during the verification process. We selected the top two stable reference genes for each group according to the results of the RankAggreg comprehensive ranking. The normalization results in Figure 6 show that the relative expression levels and trends of *AmWRKY33* had small changes when using the most stable reference genes to normalize the ET group.

PP2a and *RPL4* were the top two stable reference genes for DTO and All groups in *A. melanoxylon*. *RPL4* is responsible for encoding a ribosomal protein, which functions as a component of the 60S subunit [42]. A previous study has reported the suitability of *RPL4* as a reference gene in diverse plant tissues and under various stress conditions of mulberry. In the tissue and abiotic groups of mulberry, the *RPL4* ranked fourth and sixth among the 20 candidate reference genes, respectively [43]. *PP2a*, a key enzyme in the reversible protein phosphorylation regulatory mechanism [44], has been reported to exhibit stable expression in different okra tissues and cranberry cultivars [45,46]. Existing research has stated that the *ACT* and *TUB* genes encoding cytoskeletal proteins exhibit high stability and are extensively used as reference genes for multiple plants. Wang et al. (2019) reported that *ACT* and *TUB* had the highest stability in drought and ABA treatments of *Polygonum cuspidatum* [20]. However, in this work, the expression stability of *ACT7* and *TUB2* was poor in most experimental conditions. Specifically, the expression level of *ACT7* was greatly affected under DTO, HT, PEG, and IAA sets. And *TUB2* performed less well in DCV, LDGS,

and All groups (Figure 4). *EF1a* encodes a eukaryotic translation elongation factor during protein synthesis, a classic and frequently used reference gene for RT-qPCR. For example, previous research has indicated that *EF1a* serves as a suitable reference gene in various leaves of *Cannabis* [47], different tissues of *Cymbidium sinense* [48], and drought treatment of flax plants [49]. However, in most cases, the expression stability of *EF1a* was poor in *A. melanoxylon* samples (Figures 5 and 6). According to this result, there may be differences in the expression stability of the same gene among different species. *TIP41* [50] has been suggested as a reference gene. For instance, *TIP41* proved extremely stable under abiotic stress conditions of wild chickpeas [51]. In this study, *TIP41* was assessed as one of the highly stable internal reference genes across multiple sample groups, including DCV, LDGS, ST, and IAA sets. Even so, *TIP41* is not always suitable for use as an internal reference gene. In the PEG set of *A. melanoxylon*, heat stress of bermudagrass [52], and various tissue sets of *Isatis indigotica fortune* [53], *TIP41* was the unstable gene. Ubiquitin, a small regulatory protein found in diverse eukaryotic tissues, is widely employed as a reference gene for RT-qPCR due to its high expression stability in plants [54,55]. In previous work, *UBI3* exhibited the highest stability under hormone stimuli treatments in *Scutellaria baicalensis* [56]. In HT and ET treatments of *A. melanoxylon*, *UBI3* was the top-two stable reference gene, while the expression stability of *UBI11* was poor. This indicates that there may be diversity in the stability among individual members of the internal reference gene family, and the stability of other members cannot be judged only by the stability of a specific member.

The research has found that some novel genes exhibit higher expression stability than classic housekeeping genes [22,57]. Therefore, besides traditional housekeeping genes, we also selected two novel genes exhibiting stable expression in the transcriptome as candidate internal reference genes, namely *OTUD6B* and *PAT10*. As we all know, *OTUD* encodes a deubiquitinating enzyme and plays an important role in removing ubiquitin from target proteins in mammals [58]. Moreover, *OTUD* is also involved in numerous essential cellular processes in *Arabidopsis* [59]. However, research has yet to investigate their potential as plant reference genes. *OTUD6B* exhibited the highest stability under HT treatment in our work. It was ranked fourth and third in terms of stability in the IAA and GA treatments, respectively (Figure 5), but had to be discarded under the other individual experimental sets and the three combination groups due to its high variation. *PATs* are enzymes responsible for catalyzing protein S-acylation, a reversible post-translational modification observed in a diverse range of cellular proteins [60]. Specifically, *PAT10* performed well (top three) under DCV, LDGS, HT, ST, GA, IAA, ASs, and All groups. The findings indicate that *PAT10* is suitable as an internal reference gene of *A. melanoxylon* in various cases. The high expression stability of *PAT10* under different conditions in this research may be attributed to the fact that *PATs* catalyze protein S-acylation, a fundamental cellular process that is critical for the proper function and localization of proteins within cells, implying that this process must be tightly regulated to ensure proper cellular function [61].

To validate the reliability of the chosen reference genes, we analyzed the expression patterns of two specific genes, namely *AmWRKY6* and *AmWRKY33*. WRKY proteins, belonging to one of the largest transcription factor families in plants, play critical roles in diverse aspects, such as plant growth, development, and response to biotic and abiotic stresses [62,63]. Figure 6 shows that when the top two stable reference genes were used for normalization, the expression trends were almost similar, but their expression levels were slightly different. As described in previous studies, relying on only one reference gene for standardization in gene expression analysis cannot guarantee the accuracy of the experimental results, and it is necessary to incorporate two or more reference genes for standardization to ensure the acquisition of reliable results [17,36]. Moreover, target genes' expression levels and trends would deviate significantly if unstable reference genes were used for standardization correction. These results demonstrate the accuracy and reliability of the reference genes identified in this paper.

4. Materials and Methods

4.1. Plant Materials and Treatments

A. melanoxylon plants were cultivated in a greenhouse at the Research Institute of Tropical Forestry, Chinese Academy of Forestry in Guangzhou, China (23°20' N, 113°39' E). The DCV, DTO, and LDGS samples were collected from one-year-old plants cultivated in containers with soil. The one-year-old plants exhibit two types of foliage: bipinnate compound leaves and vertically oriented phyllodes. The DCV samples consist of foliage with phyllodes from *A. melanoxylon* clones SR3, SR14, SR17, SR20, and SR21. The DTO samples included the root, xylem, phloem, phyllode, compound leaves, and petiole. As shown in Figure S4, the LDGS samples were collected from pinnate compound leaves with petiole at seven different growth states. To obtain leaves in different growth states, samples were collected every 4 days. We collected samples from three-month-old plants grown in plastic containers containing 1/2 Murashige and Skoog (MS) liquid medium for HT, ST, PEG, GA, IAA, and ET treatments. Plants only exhibit pinnate compound leaf type at this age. For the HT treatment, which simulates temperatures during hot weather in Australia (Bureau of Meteorology, Melbourne, Australia, <http://www.bom.gov.au/>), the plants were placed in an illumination box with temperature cycles of 40/25 °C, following a photoperiod of 14 h of light and 10 h of darkness. For ST and PEG treatments, the plants were immersed in the MS liquid medium with 200 mM NaCl or 15% PEG. For GA, IAA, and ET treatment, the plants were sealed in white transparent plastic bags and sprayed evenly with 0.5 mM GA, 0.1 mM IAA, or 0.2 mM ET. Leaf samples with the same growth status were collected at different time points: 0, 6, 24, 72, and 168 h under HT, ST, or PEG treatments; and at 0, 2, 6, 12, and 24 h under GA, IAA, or ET treatments. Three biological replicates, each with three plants, were collected for all the above samples. Note that all samples are from *A. melanoxylon* SR17, except for the DCV set. All samples were rapidly frozen in liquid nitrogen and subsequently stored at −80 °C until RNA extraction.

4.2. RNA Extraction and cDNA Synthesis

Total RNA was extracted from a total of 144 samples using the RNeasy Pure Plant Plus Kit (Qiagen, Tianjin, China). The RNA integrity was verified through 1.5% (*w/v*) agarose gel electrophoresis. The quality and purity of RNA were evaluated utilizing a NanoDrop spectrophotometer 2000 (Thermo, Waltham, MA, USA). Both mean absorbance ratios of all RNA samples at A260/280 and A260/230 are around 2.0, indicating suitable quality for subsequent cDNA synthesis. The cDNA synthesis utilized the Prime-Script™ RT reagent Kit and gDNA Eraser (Takara, Kusatsu, Japan), according to the manufacturer's instructions.

4.3. Candidate Reference Genes Selection and Primer Design

In this study, we performed BlastP queries on the *A. melanoxylon* genome database to obtain the highest orthologous sequences of the classic and frequently used reference genes of forest trees [37,64,65], which were downloaded from the ICG database. The candidate reference genes were selected based on their stable expression and appropriate fragments per kilobase of exon model per million mapped fragments (FPKM) values in *A. melanoxylon* transcriptome data (unpublished). Finally, eight classic candidate reference genes were screened out, namely *ACT7*, *EF1a*, *PP2a*, *RPL4*, *TIP41*, *TUB2*, *UBI3*, and *UBI11*. In addition, two novel candidate genes, *OTUD6B* and *PAT10*, were also selected due to their stable expression pattern and appropriate FPKM values (as shown in Table S4). The primers were designed using the Primer3 software program, version 4.1.0 (<https://primer3.ut.ee/>, accessed on 7 December 2021). The design criteria for the primers included the following ranges: product lengths ranging from 100 to 200 bp, primer lengths of 18 to 24 bp, melting temperature within 55 to 60 °C, and GC content between 40 and 60%. To verify the specificity of each primer pair, PCR products were visualized using 2.0% agarose gel electrophoresis. The genomic DNA sequences of these candidate reference genes can be

found in Table S5, while the details of primer sequences for the candidate reference gene are presented in Table 1.

4.4. RT-qPCR and Amplification Efficiency Analysis

The RT-qPCR reactions were performed using a 96-well plate format on LightCycler480II instrument (Roche, Basel, Switzerland), with SYBR Premix Ex Taq™ II (Takara, Kusatsu, Japan). Each 20 µL reaction consists of 1 µL of cDNA template, 2 µL of each primer pair (10 µM), 10 µL of SYBR Premix Ex Taq™ II (2X), and 7 µL ddH₂O. The amplification conditions were as follows: predenaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s, and then a melting curve was produced. The RT-qPCR assays were performed with three biological replicates, each containing three technical replicates. A no-template control (NTC) reaction was performed for each primer pair in each RT-qPCR process. To calculate the amplification efficiency (E) and correlation coefficient (R^2) of the primer pair for each candidate reference gene, the standard curve was generated using a five-fold dilution (1, 1/5, 1/25, 1/125, and 1/625) of the mixed cDNA [66].

4.5. Stability Assessment of Candidate Reference Genes

Based on the Ct values obtained using RT-qPCR, the expression stability of candidate reference genes was evaluated using four algorithms (comparative Δ Ct, geNorm, NormFinder, and BestKeeper) and a comprehensive sorting tool (RankAggreg) in different experimental conditions.

The mean standard deviation (mSD) was calculated using the comparative Δ Ct method [25]. This method analyzed the relative expression levels of candidate reference genes in all pairwise combinations, with the aim to assess and rank their stability. The candidate reference gene with the lowest mSD value was determined to be the most stable reference gene in different sample sets.

In geNorm analysis [26], an average expression stability value M was calculated for each gene, which represented the average pairwise variation (V) of a specific gene with all other tested genes. Lower M-values indicated higher expression stability and genes with M-values below 1.5 were considered stable. The two most stable expression reference genes were determined by iteratively excluding the least stable gene. Moreover, the pairwise variation of one gene with others was utilized to ascertain the number of reference genes needed for optimal data normalization. If the metric values of $V_{n/n+1}$ are less than 0.15, the addition of further reference genes is no longer a significant contribution to the normalization process. In this case, the appropriate number of reference genes is 'n'. Otherwise, the appropriate number is 'n + 1'. The data input to geNorm is the relative quantities transformed from raw Ct values.

Similarly, in NormFinder [27], the input data are the log of raw Ct values. NormFinder utilized a one-way analysis of variance (ANOVA)-based mathematical analysis to calculate gene expression stability, considering intra- and inter-group differences. The gene exhibiting the lowest stability (S) value exhibits the highest stability in different experiment conditions.

The BestKeeper software (version 1, München, Germany) [28] analyzes the gene expression stability by comparing the *p*-value, SD, CV, and coefficient of correlation (*r*) among genes within each group. A smaller SD ± CV value indicated more stability of gene expression. If SD > 1 or *p*-value > 0.05, the gene was considered unreliable. Note that BestKeeper can only be used to compare the expression levels of up to 10 internal reference genes and 10 target genes in a maximum of 100 samples.

Finally, the RankAggreg algorithmic package of R software version 4.3.0 (R Foundation for Statistical Computing, Vienna, Austria) [29] was utilized to combine the stability metrics obtained from the above four algorithms. This process was achieved by aggregating ordered rank lists with unweights using a Cross-Entropy Monte Carlo algorithm. The ranking lists generated from the Δ Ct, NormFinder, geNorm, and BestKeeper were used as the input with the following parameters: the distance calculated using Spearman's Footrule function, rho with 0.1, the seed with 100, and the 'convIn' argument with 50.

4.6. Validation of Reference Genes

To validate the dependability of selected reference genes, the expression patterns of *AmWRKY33* (Primer F: 5'-CTTCTCTCCCAATTCCTG-3'; Primer R: 5'-CATCATCCCCCATC GATA-3') and *AmWRKY6* (Primer F: 5'-CCGTCTCCGCCGAAGATT-3'; Primer R: 5'-AGCC ACAAGTGCTGCAGT-3') were analyzed under six treatments (HT, ST, PEG, GA, IAA, and ET) and three experimental conditions (DCV, DTO, and LDGS), respectively. Specifically, the relative expression levels of the putative *AmWRKY33* and *AmWRKY6* with the two most stable (alone and in combination) and the least stable reference genes were calculated using the $2^{-\Delta\Delta C_t}$ method [67].

5. Conclusions

In this study, we comprehensively evaluated the expression stability of reference genes used for RT-qPCR analysis across different treatments of *A. melanoxylon*. Our results show that *PAT10* and *TIP41* emerged as the most stable reference genes across the DCV, LDGS, ST, and IAA groups; *PP2a* and *PAT10* were the top two choices for GA and ASs groups; *PP2a* and *RPL4* maintained stable expression in DTO and All samples; in PEG treatment, both *PP2a* and *UBI11* exhibited stable expression; *UBI3* and *OTUD6B* were identified as stable reference genes in HT treatment; and *UBI3* combined with *RPL4* or *PP2a* exhibited the highest stability in ET or ETHs groups, respectively. This research provides a basis for performing a quantification and expression analysis of target genes under nine experimental conditions in *A. melanoxylon*. Furthermore, it will also facilitate deeper investigations into the molecular mechanisms of this species.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/f14112245/s1>. Figure S1: Amplification products of the ten candidate reference genes. Figure S2: Melting curves of ten candidate reference genes. Figure S3: Box-and-whisker plot depicting the cycle threshold (C_t) value range of the 10 candidate reference genes in 12 set samples. Figure S4: Morphological of different developmental stages of *Acacia melanoxylon* leaves. Table S1: The raw C_t values of ten genes across all samples. Table S2: Stability of ten candidate reference genes by comparative ΔC_t algorithm. Table S3: Stability of ten candidate reference genes by NormFinder algorithm. Table S4: The fragments per kilobase of exon model per million mapped fragments values of ten candidate reference genes. Table S5: The genomic DNA sequences of ten candidate reference genes.

Author Contributions: Conceptualization, B.Z., X.L. and B.H.; methodology, Z.C. and B.H.; software, Z.C.; validation, X.B.; formal analysis, Z.C. and X.B.; resources, B.H.; writing—original draft preparation, Z.C. and X.B.; writing—review and editing, B.H.; visualization, Z.C.; project administration, B.H.; funding acquisition, B.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Guangzhou Science and Technology Planning Project (202201011180); National Key Research and Development Program of China (2022YFD2200205); Forestry Science and Technology Innovation Project of Guangdong Province (2020KJCX014).

Data Availability Statement: Data is contained within the article or Supplementary Materials.

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

Abbreviations

DCV: different clonal varieties; DTO: different tissues or organs; LDGS: pinnate compound leaves at different growth states; HT: heat; ST: salt; PEG: drought; GA: gibberellin; IAA: indole acetic acid; ET: ethephon; ASs: abiotic stresses; EHT: exogenous hormone treatments; All: all 144 samples of nine experimental sets; *ACT7*: actin 7; *OTUD6B*: deubiquitinase OTUD6B; *EF1a*: elongation factor1-alpha; *PAT10*: protein S-acyltransferase 10; *PP2a*: protein phosphatase 2a; *RPL4*: 60S ribosomal protein L4-like; *TIP41*: tonoplast intrinsic protein; *TUB2*: beta-tubulin; *UBI3*: ubiquitin 3; *UBI11*: ubiquitin 11.

References

1. Wujeska-Klaue, A.; Bossinger, G.; Tausz, M. The Concentration of Ascorbic Acid and Glutathione in 13 Provenances of *Acacia melanoxylon*. *Tree Physiol.* **2016**, *36*, 524–532. [\[CrossRef\]](#) [\[PubMed\]](#)
2. Bradbury, G.J.; Potts, B.M.; Beadle, C.L. Genetic and Environmental Variation in Wood Properties of *Acacia melanoxylon*. *Ann. For. Sci.* **2011**, *68*, 1363–1373. [\[CrossRef\]](#)
3. Kull, C.A.; Shackleton, C.M.; Cunningham, P.J.; Ducatillon, C.; Dufour-Dror, J.-M.; Esler, K.J.; Friday, J.B.; Gouveia, A.C.; Griffin, A.R.; Marchante, E.; et al. Adoption, Use and Perception of Australian Acacias around the World: Adoption, Use, and Perception of Australian Acacias. *Divers. Distrib.* **2011**, *17*, 822–836. [\[CrossRef\]](#)
4. Machado, J.S.; Louzada, J.L.; Santos, A.J.A.; Nunes, L.; Anjos, O.; Rodrigues, J.; Simões, R.M.S.; Pereira, H. Variation of Wood Density and Mechanical Properties of Blackwood (*Acacia melanoxylon* R. Br.). *Mater. Design* **2014**, *56*, 975–980. [\[CrossRef\]](#)
5. Searle, S.D. *Acacia melanoxylon*—A Review of Variation among Planted Trees. *Aust. For.* **2000**, *63*, 79–85. [\[CrossRef\]](#)
6. Zhang, R.; Zeng, B.; Chen, T.; Hu, B. Genotype–Environment Interaction and Horizontal and Vertical Distributions of Heartwood for *Acacia melanoxylon* R.Br. *Genes* **2023**, *14*, 1299. [\[CrossRef\]](#) [\[PubMed\]](#)
7. Zotz, G.; Wilhelm, K.; Becker, A. Heteroblasty—A Review. *Bot. Rev.* **2011**, *77*, 109–151. [\[CrossRef\]](#)
8. Forster, M.A.; Bonser, S.P. Heteroblastic Development and the Optimal Partitioning of Traits among Contrasting Environments in *Acacia implexa*. *Ann. Bot.* **2009**, *103*, 95–105. [\[CrossRef\]](#)
9. Forster, M.A.; Bonser, S.P. Heteroblastic Development and Shade-Avoidance in Response to Blue and Red Light Signals in *Acacia implexa*. *Photochem. Photobiol.* **2009**, *85*, 1375–1383. [\[CrossRef\]](#)
10. Winn, A.A. The Functional Significance and Fitness Consequences of Heterophylly. *Int. J. Plant Sci.* **1999**, *160*, S113–S121. [\[CrossRef\]](#)
11. Pinkard, E.A.; Beadle, C.L. Blackwood (*Acacia melanoxylon* R. Br.) Plantation Silviculture: A Review. *Aust. For.* **2002**, *65*, 7–13. [\[CrossRef\]](#)
12. Bustin, S.A.; Benes, V.; Garson, J.; Hellemans, J.; Huggett, J.; Kubista, M.; Mueller, R.; Nolan, T.; Pfaffl, M.W.; Shipley, G.; et al. The Need for Transparency and Good Practices in the qPCR Literature. *Nat. Methods* **2013**, *10*, 1063–1067. [\[CrossRef\]](#) [\[PubMed\]](#)
13. VanGuilder, H.D.; Vrana, K.E.; Freeman, W.M. Twenty-Five Years of Quantitative PCR for Gene Expression Analysis. *BioTechniques* **2008**, *44*, 619–626. [\[CrossRef\]](#) [\[PubMed\]](#)
14. Radonić, A.; Thulke, S.; Mackay, I.M.; Landt, O.; Siebert, W.; Nitsche, A. Guideline to Reference Gene Selection for Quantitative Real-Time PCR. *Biochem. Bioph. Res. Commun.* **2004**, *313*, 856–862. [\[CrossRef\]](#) [\[PubMed\]](#)
15. Kurkela, S.; Brown, D.W.G. Molecular Diagnostic Techniques. *Medicine* **2009**, *37*, 535–540. [\[CrossRef\]](#) [\[PubMed\]](#)
16. Huggett, J.; Dheda, K.; Bustin, S.; Zumla, A. Real-Time RT-PCR Normalisation; Strategies and Considerations. *Genes. Immun.* **2005**, *6*, 279–284. [\[CrossRef\]](#) [\[PubMed\]](#)
17. Joseph, J.T.; Poolakkalody, N.J.; Shah, J.M. Plant Reference Genes for Development and Stress Response Studies. *J. Biosci.* **2018**, *43*, 173–187. [\[CrossRef\]](#) [\[PubMed\]](#)
18. Zhao, F.; Maren, N.A.; Kosentka, P.Z.; Liao, Y.-Y.; Lu, H.; Duduit, J.R.; Huang, D.; Ashrafi, H.; Zhao, T.; Huerta, A.I.; et al. An Optimized Protocol for Stepwise Optimization of Real-Time RT-PCR Analysis. *Hortic. Res.* **2021**, *8*, 179. [\[CrossRef\]](#)
19. Sang, J.; Wang, Z.; Li, M.; Cao, J.; Niu, G.; Xia, L.; Zou, D.; Wang, F.; Xu, X.; Han, X.; et al. ICG: A Wiki-Driven Knowledgebase of Internal Control Genes for RT-qPCR Normalization. *Nucleic Acids Res.* **2018**, *46*, D121–D126. [\[CrossRef\]](#)
20. Wang, X.; Wu, Z.; Bao, W.; Hu, H.; Chen, M.; Chai, T.; Wang, H. Identification and Evaluation of Reference Genes for Quantitative Real-Time PCR Analysis in *Polygonum cuspidatum* Based on Transcriptome Data. *BMC Plant Biol.* **2019**, *19*, 498. [\[CrossRef\]](#)
21. Sankar, K.; Yoon, H.J.; Lee, Y.B.; Lee, K.Y. Evaluation of Reference Genes for Real-Time Quantitative PCR Analysis in Tissues from Bumble Bees (*Bombus terrestris*) of Different Lines. *Int. J. Mol. Sci.* **2022**, *23*, 14371. [\[CrossRef\]](#) [\[PubMed\]](#)
22. Hu, A.; Yang, X.; Zhu, J.; Wang, X.; Liu, J.; Wang, J.; Wu, H.; Zhang, H.; Zhang, H. Selection and Validation of Appropriate Reference Genes for RT-qPCR Analysis of *Nitraria sibirica* under Various Abiotic Stresses. *BMC Plant Biol.* **2022**, *22*, 592. [\[CrossRef\]](#) [\[PubMed\]](#)
23. Gutierrez, L.; Mauriat, M.; Gunin, S.; Pelloux, J.; Lefebvre, J.-F.; Louvet, R.; Rusterucci, C.; Moritz, T.; Guérineau, F.; Bellini, C.; et al. The Lack of a Systematic Validation of Reference Genes: A Serious Pitfall Undervalued in Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis in Plants. *Plant Biotechnol. J.* **2008**, *6*, 609–618. [\[CrossRef\]](#)
24. Tang, F.; Chu, L.; Shu, W.; He, X.; Wang, L.; Lu, M. Selection and Validation of Reference Genes for Quantitative Expression Analysis of miRNAs and mRNAs in Poplar. *Plant Methods* **2019**, *15*, 35. [\[CrossRef\]](#) [\[PubMed\]](#)
25. Silver, N.; Best, S.; Jiang, J.; Thein, S.L. Selection of Housekeeping Genes for Gene Expression Studies in Human Reticulocytes Using Real-Time PCR. *BMC Mol. Biol.* **2006**, *7*, 33. [\[CrossRef\]](#) [\[PubMed\]](#)
26. Vandesompele, J.; De Preter, K.; Pattyn, F.; Poppe, B.; Van Roy, N.; De Paepe, A.; Speleman, F. Accurate Normalization of Real-Time Quantitative RT-PCR Data by Geometric Averaging of Multiple Internal Control Genes. *Genome Biol.* **2002**, *3*, research0034.1. [\[CrossRef\]](#) [\[PubMed\]](#)
27. Andersen, C.L.; Jensen, J.L.; Ørntoft, T.F. Normalization of Real-Time Quantitative Reverse Transcription-PCR Data: A Model-Based Variance Estimation Approach to Identify Genes Suited for Normalization, Applied to Bladder and Colon Cancer Data Sets. *Cancer Res.* **2004**, *64*, 5245–5250. [\[CrossRef\]](#) [\[PubMed\]](#)

28. Pfaffl, M.W.; Tichopad, A.; Prgomet, C.; Neuvians, T.P. Determination of Stable Housekeeping Genes, Differentially Regulated Target Genes and Sample Integrity: BestKeeper—Excel-Based Tool Using Pair-Wise Correlations. *Biotechnol. Lett.* **2004**, *26*, 509–515. [\[CrossRef\]](#)
29. Pihur, V.; Datta, S.; Datta, S. RankAggreg, an R Package for Weighted Rank Aggregation. *BMC Bioinform.* **2009**, *10*, 62. [\[CrossRef\]](#)
30. Gao, M.; Liu, Y.; Ma, X.; Shuai, Q.; Gai, J.; Li, Y. Evaluation of Reference Genes for Normalization of Gene Expression Using Quantitative RT-PCR under Aluminum, Cadmium, and Heat Stresses in Soybean. *PLoS ONE* **2017**, *12*, e0168965. [\[CrossRef\]](#)
31. Han, B.; Yang, Z.; Samma, M.K.; Wang, R.; Shen, W. Systematic Validation of Candidate Reference Genes for qRT-PCR Normalization under Iron Deficiency in Arabidopsis. *Biometals* **2013**, *26*, 403–413. [\[CrossRef\]](#) [\[PubMed\]](#)
32. De Almeida, M.R.; Ruedell, C.M.; Ricachenevsky, F.K.; Sperotto, R.A.; Pasquali, G.; Fett-Neto, A.G. Reference Gene Selection for Quantitative Reverse Transcription-Polymerase Chain Reaction Normalization during In Vitro Adventitious Rooting in *Eucalyptus globulus* Labill. *BMC Mol. Biol.* **2010**, *11*, 73. [\[CrossRef\]](#) [\[PubMed\]](#)
33. Yan, H.; Zhang, Y.; Xiong, Y.; Chen, Q.; Liang, H.; Niu, M.; Guo, B.; Li, M.; Zhang, X.; Li, Y.; et al. Selection and Validation of Novel RT-qPCR Reference Genes under Hormonal Stimuli and in Different Tissues of *Santalum album*. *Sci. Rep.* **2018**, *8*, 17511. [\[CrossRef\]](#)
34. Derveaux, S.; Vandesompele, J.; Hellemans, J. How to Do Successful Gene Expression Analysis Using Real-Time PCR. *Methods* **2010**, *50*, 227–230. [\[CrossRef\]](#) [\[PubMed\]](#)
35. Lü, J.; Yang, C.; Zhang, Y.; Pan, H. Selection of Reference Genes for the Normalization of RT-qPCR Data in Gene Expression Studies in Insects: A Systematic Review. *Front. Physiol.* **2018**, *9*, 1560. [\[CrossRef\]](#) [\[PubMed\]](#)
36. Wu, Y.; Zhang, C.; Yang, H.; Lyu, L.; Li, W.; Wu, W. Selection and Validation of Candidate Reference Genes for Gene Expression Analysis by RT-qPCR in *Rubus*. *Int. J. Mol. Sci.* **2021**, *22*, 10533. [\[CrossRef\]](#) [\[PubMed\]](#)
37. Song, H.; Mao, W.; Duan, Z.; Que, Q.; Zhou, W.; Chen, X.; Li, P. Selection and Validation of Reference Genes for Measuring Gene Expression in *Toona ciliata* under Different Experimental Conditions by Quantitative Real-Time PCR Analysis. *BMC Plant Biol.* **2020**, *20*, 450. [\[CrossRef\]](#) [\[PubMed\]](#)
38. Bustin, S.A.; Beaulieu, J.-F.; Huggett, J.; Jaggi, R.; Kibenge, F.S.; Olsvik, P.A.; Penning, L.C.; Toegel, S. MIQE Précis: Practical Implementation of Minimum Standard Guidelines for Fluorescence-Based Quantitative Real-Time PCR Experiments. *BMC Mol. Biol.* **2010**, *11*, 74. [\[CrossRef\]](#)
39. Bustin, S.A.; Benes, V.; Garson, J.A.; Hellemans, J.; Huggett, J.; Kubista, M.; Mueller, R.; Nolan, T.; Pfaffl, M.W.; Shipley, G.L.; et al. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clin. Chem.* **2009**, *55*, 611–622. [\[CrossRef\]](#)
40. Hou, S.; Zhao, T.; Yang, D.; Li, Q.; Liang, L.; Wang, G.; Ma, Q. Selection and Validation of Reference Genes for Quantitative RT-PCR Analysis in *Corylus heterophylla* Fisch. × *Corylus avellana* L. *Plants* **2021**, *10*, 159. [\[CrossRef\]](#)
41. Sun, H.; Jiang, X.; Sun, M.; Cong, H.; Qiao, F. Evaluation of Reference Genes for Normalizing RT-qPCR in Leaves and Suspension Cells of *Cephalotaxus hainanensis* under Various Stimuli. *Plant Methods* **2019**, *15*, 31. [\[CrossRef\]](#) [\[PubMed\]](#)
42. Horiguchi, G.; Van Lijsebettens, M.; Candela, H.; Micol, J.L.; Tsukaya, H. Ribosomes and Translation in Plant Developmental Control. *Plant Sci.* **2012**, *191–192*, 24–34. [\[CrossRef\]](#) [\[PubMed\]](#)
43. Dai, F.; Zhao, X.; Tang, C.; Wang, Z.; Kuang, Z.; Li, Z.; Huang, J.; Luo, G. Identification and Validation of Reference Genes for qRT-PCR Analysis in Mulberry (*Morus alba* L.). *PLoS ONE* **2018**, *13*, e0194129. [\[CrossRef\]](#) [\[PubMed\]](#)
44. Máthé, C.; M-Hamvas, M.; Freytag, C.; Garda, T. The Protein Phosphatase PP2A Plays Multiple Roles in Plant Development by Regulation of Vesicle Traffic—Facts and Questions. *Int. J. Mol. Sci.* **2021**, *22*, 975. [\[CrossRef\]](#) [\[PubMed\]](#)
45. Li, C.; Xu, J.; Deng, Y.; Sun, H.; Li, Y. Selection of Reference Genes for Normalization of Cranberry (*Vaccinium macrocarpon* Ait.) Gene Expression under Different Experimental Conditions. *PLoS ONE* **2019**, *14*, e0224798. [\[CrossRef\]](#)
46. Zhang, J.-R.; Feng, Y.-Y.; Yang, M.-J.; Xiao, Y.; Liu, Y.-S.; Yuan, Y.; Li, Z.; Zhang, Y.; Zhuo, M.; Zhang, J.; et al. Systematic Screening and Validation of Reliable Reference Genes for qRT-PCR Analysis in Okra (*Abelmoschus esculentus* L.). *Sci. Rep.* **2022**, *12*, 12913. [\[CrossRef\]](#) [\[PubMed\]](#)
47. Guo, R.; Guo, H.; Zhang, Q.; Guo, M.; Xu, Y.; Zeng, M.; Lv, P.; Chen, X.; Yang, M. Evaluation of Reference Genes for RT-qPCR Analysis in Wild and Cultivated cannabis. *Biosci. Biotechnol. Biochem.* **2018**, *82*, 1902–1910. [\[CrossRef\]](#) [\[PubMed\]](#)
48. Tian, Y.; Chu, Z.; Wang, H.; Wang, G.; Wu, S.; Yang, Y. Selection and Validation of Reference Genes for Quantitative Real-Time PCR in *Cymbidium sinense*. *BioTechniques* **2022**, *72*, 51–59. [\[CrossRef\]](#)
49. Dash, P.K.; Rai, R.; Pradhan, S.K.; Shivaraj, S.M.; Deshmukh, R.; Sreevathsa, R.; Singh, N.K. Drought and Oxidative Stress in Flax (*Linum usitatissimum* L.) Entails Harnessing Non-Canonical Reference Gene for Precise Quantification of qRT-PCR Gene Expression. *Antioxidants* **2023**, *12*, 950. [\[CrossRef\]](#)
50. Sudhakaran, S.; Thakral, V.; Padalkar, G.; Rajora, N.; Dhiman, P.; Raturi, G.; Sharma, Y.; Tripathi, D.K.; Deshmukh, R.; Sharma, T.R.; et al. Significance of Solute Specificity, Expression, and Gating Mechanism of Tonoplast Intrinsic Protein during Development and Stress Response in Plants. *Physiol. Plant.* **2021**, *172*, 258–274. [\[CrossRef\]](#)
51. Reddy, D.S.; Bhatnagar-Mathur, P.; Reddy, P.S.; Cindhuri, K.S.; Ganesh, A.S.; Sharma, K.K. Identification and Validation of Reference Genes and Their Impact on Normalized Gene Expression Studies across Cultivated and Wild Cicer Species. *PLoS ONE* **2016**, *11*, e0148451. [\[CrossRef\]](#) [\[PubMed\]](#)

52. Chen, Y.; Tan, Z.; Hu, B.; Yang, Z.; Xu, B.; Zhuang, L.; Huang, B. Selection and Validation of Reference Genes for Target Gene Analysis with Quantitative RT-PCR in Leaves and Roots of Bermudagrass under Four Different Abiotic Stresses. *Physiol. Plant.* **2015**, *155*, 138–148. [[CrossRef](#)] [[PubMed](#)]
53. Qu, R.; Miao, Y.; Cui, Y.; Cao, Y.; Zhou, Y.; Tang, X.; Yang, J.; Wang, F. Selection of Reference Genes for the Quantitative Real-Time PCR Normalization of Gene Expression in *Isatis indigotica* Fortune. *BMC Mol. Biol.* **2019**, *20*, 9. [[CrossRef](#)] [[PubMed](#)]
54. Zhou, T.; Yang, X.; Fu, F.; Wang, G.; Cao, F. Selection of Suitable Reference Genes Based on Transcriptomic Data in *Ginkgo biloba* under Different Experimental Conditions. *Forests* **2020**, *11*, 1217. [[CrossRef](#)]
55. Hong, S.-Y.; Seo, P.J.; Yang, M.-S.; Xiang, F.; Park, C.-M. Exploring Valid Reference Genes for Gene Expression Studies in *Brachypodium distachyon* by Real-Time PCR. *BMC Plant Biol.* **2008**, *8*, 112. [[CrossRef](#)] [[PubMed](#)]
56. Wang, W.; Hu, S.; Cao, Y.; Chen, R.; Wang, Z.; Cao, X. Selection and Evaluation of Reference Genes for qRT-PCR of *Scutellaria baicalensis* Georgi under Different Experimental Conditions. *Mol. Biol. Rep.* **2021**, *48*, 1115–1126. [[CrossRef](#)] [[PubMed](#)]
57. Liu, Q.; Qi, X.; Yan, H.; Huang, L.; Nie, G.; Zhang, X. Reference Gene Selection for Quantitative Real-Time Reverse-Transcriptase PCR in Annual Ryegrass (*Lolium multiflorum*) Subjected to Various Abiotic Stresses. *Molecules* **2018**, *23*, 172. [[CrossRef](#)]
58. Guo, Y.; Zhang, S.; Yuan, Q. Deubiquitinating Enzymes and Bone Remodeling. *Stem Cells Int.* **2018**, *2018*, e3712083. [[CrossRef](#)]
59. Radjacomare, R.; Usharani, R.; Kuo, C.-H.; Fu, H. Distinct Phylogenetic Relationships and Biochemical Properties of Arabidopsis Ovarian Tumor-Related Deubiquitinases Support Their Functional Differentiation. *Front. Plant Sci.* **2014**, *5*, 84. [[CrossRef](#)]
60. Greaves, J.; Chamberlain, L.H. DHHC Palmitoyl Transferases: Substrate Interactions and (Patho) Physiology. *Trends Biochem. Sci.* **2011**, *36*, 245–253. [[CrossRef](#)]
61. Wang, Y.; Yang, W. Proteome-Scale Analysis of Protein S-Acylation Comes of Age. *J. Proteome Res.* **2021**, *20*, 14–26. [[CrossRef](#)] [[PubMed](#)]
62. Long, L.; Gu, L.; Wang, S.; Cai, H.; Wu, J.; Wang, J.; Yang, M. Progress in the Understanding of WRKY Transcription Factors in Woody Plants. *Int. J. Biol. Macromol.* **2023**, *242*, 124379. [[CrossRef](#)] [[PubMed](#)]
63. Wani, S.H.; Anand, S.; Singh, B.; Bohra, A.; Joshi, R. WRKY Transcription Factors and Plant Defense Responses: Latest Discoveries and Future Prospects. *Plant Cell Rep.* **2021**, *40*, 1071–1085. [[CrossRef](#)] [[PubMed](#)]
64. Chen, X.; Mao, Y.; Huang, S.; Ni, J.; Lu, W.; Hou, J.; Wang, Y.; Zhao, W.; Li, M.; Wang, Q.; et al. Selection of Suitable Reference Genes for Quantitative Real-Time PCR in *Sapindus sebiferum*. *Front. Plant Sci.* **2017**, *8*, 637. [[CrossRef](#)] [[PubMed](#)]
65. Chen, H.; Yang, Z.; Hu, Y.; Tan, J.; Jia, J.; Xu, H.; Chen, X. Reference Genes Selection for Quantitative Gene Expression Studies in *Pinus massoniana* L. *Trees* **2016**, *30*, 685–696. [[CrossRef](#)]
66. Kubista, M.; Andrade, J.M.; Bengtsson, M.; Forootan, A.; Jonák, J.; Lind, K.; Sindelka, R.; Sjöback, R.; Sjögreen, B.; Strömbom, L.; et al. The Real-Time Polymerase Chain Reaction. *Mol. Asp. Med.* **2006**, *27*, 95–125. [[CrossRef](#)] [[PubMed](#)]
67. Livak, K.J.; Schmittgen, T.D. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. *Methods* **2001**, *25*, 402–408. [[CrossRef](#)]

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.