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

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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 ∆Ct, 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

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 *B1*-like protein (*B1*) 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 ∆Ct [25], geNorm [26], NormFinder [27], BestKeeper [28], and RankAggreg [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.cnccb.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 R² 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>
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<th>Gene Symbol</th>
<th>Gene ID</th>
<th>Gene Description</th>
<th>Forward/Reverse Primer (5′-3′)</th>
<th>Amplicon Length (bp)</th>
<th>Primers TM (°C)</th>
<th>E (%)</th>
<th>R²</th>
</tr>
</thead>
</table>
| ACT7        | evm.model.Chr8.816 | actin 7          | F:AGATTCCGCTACCCAGAAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA...
2.3. Expression Stability of the Candidate Reference Genes

This paper employed four different statistical algorithms (comparative ΔCt, 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. ΔCt Algorithm

Figure 2a and Table S2 show that PP2a exhibited the highest stability in the DCV, DTO, ST, GA, ET, ETHs, 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 ETHs groups.

![Figure 2](image)

Figure 2. The heat map presents the stability of the ten candidate reference genes calculated by comparative ΔCt and NormFinder. (a) Comparative ΔCt; (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 value = 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.
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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.
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 ± 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.

![Figure 4](image)

**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.

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<th>SD ± CV</th>
<th>LDGS</th>
<th>SD ± CV</th>
<th>PEG</th>
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<td>OTUD6B</td>
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<td>PP2a</td>
<td>0.80 ± 3.80</td>
<td>TIP41</td>
<td>0.88 ± 3.85</td>
<td>UBI11</td>
<td>1.92 ± 10.30</td>
</tr>
<tr>
<td>7</td>
<td>PP2a</td>
<td>0.83 ± 4.04</td>
<td>EF1a</td>
<td>0.83 ± 3.70</td>
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<td>0.92 ± 4.36</td>
<td>PAT10</td>
<td>0.88 ± 3.90</td>
</tr>
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<td>8</td>
<td>EF1a</td>
<td>0.97 ± 4.29</td>
<td>UBI11</td>
<td>0.98 ± 5.67</td>
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<td>1.88 ± 8.70</td>
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<tr>
<td>9</td>
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<td>0.78 ± 2.89</td>
<td>UBI3</td>
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<td>1.23 ± 4.66</td>
<td>ACT7</td>
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<td>1.36 ± 5.91</td>
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<td>0.65 ± 3.40</td>
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<td>2</td>
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</tr>
<tr>
<td>5</td>
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<td>UBI3</td>
<td>0.83 ± 4.38</td>
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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, \textit{PAT10} and \textit{TIP41} were identified as the most stable genes in DCV, LDGS, ST, and IAA sets; \textit{PP2a} and \textit{RPL4} were optimal combinations in DTO and All groups; \textit{PP2a} and \textit{PAT10} exhibited stable expression in GA and ASs groups; \textit{PP2a} and \textit{UBI11} were identified as the two most stable genes in PEG treatment; \textit{UBI3} combined with \textit{OTUB6B}, \textit{RPL4} or \textit{PP2a} were the two most stable genes in HT, ET, or ETHs, respectively.

![Figure 5](image-url)

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 ΔCt 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 *TUB82*. 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.](image-url)
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 ∆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 ∆Ct, geNorm, and NormFinder results. Previous studies conducted on N. sibirica [22], Rubus [36], and Toona ciliate [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,
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 RNAprep Pure Plant Plus Kit (Tiangen, 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 PrimeScript™ 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 TaqTM 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 TaqTM 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 (R²) 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 ΔCt, geNorm, NormFinder, and BestKeeper) and a comprehensive sorting tool (RankAggreg) in different experimental conditions.

The mean standard deviation (mSD) was calculated using the comparative ΔCt 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 ΔCt, 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 ‘convln’ argument with 50.
4.6. Validation of Reference Genes

To validate the dependability of selected reference genes, the expression patterns of \textit{AmWRKY33} (Primer F:5’-CTTCTCTCCCAATCTCG-3’; Primer R: 5’-CATCATCCCCCATCGATA-3’) and \textit{AmWRKY6} (Primer F:5’-CCGCTCTCCGCCGAAGATT-3’; Primer R: 5’-AGCCACAAGTGTGCGAGT-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 \textit{AmWRKY33} and \textit{AmWRKY6} with the two most stable (alone and in combination) and the least stable reference genes were calculated using the $2^{-\Delta\Delta\text{Ct}}$ 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 \textit{A. melanoxylon}. Our results show that \textit{PAT10} and \textit{TIP41} emerged as the most stable reference genes across the DCV, LDGS, ST, and IAA groups; \textit{PP2a} and \textit{PAT10} were the top two choices for GA and ASs groups; \textit{PP2a} and \textit{RPL4} maintained stable expression in DTO and All samples; in PEG treatment, both \textit{PP2a} and \textit{UBI11} exhibited stable expression; \textit{UBI3} and \textit{OTUD6B} were identified as stable reference genes in HT treatment; and \textit{UBI3} combined with \textit{RPL4} or \textit{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 \textit{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 (Ct) value range of the 10 candidate reference genes in 12 set samples. Figure S4: Morphological of different developmental stages of \textit{Acacia melanoxylon} leaves. Table S1: The raw Ct values of ten genes across all samples. Table S2: Stability of ten candidate reference genes by comparative $\Delta\text{Ct}$ 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.

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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; \textit{ACT7}: actin 7; \textit{OTUD6B}: deubiquitinase \textit{OTUD6B}; \textit{EF1a}: elongation factor1-alpha; \textit{PAT10}: protein S-acyltransferase 10; \textit{PP2a}: protein phosphatase 2a; \textit{RPL4}: 60S ribosomal protein L4-like; \textit{TIP41}: tonoplast intrinsic protein; \textit{TUB2}: beta-tubulin; \textit{UBI3}: ubiquitin 3; \textit{UBI11}: ubiquitin 11.
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