

Article

Screening and Validation of qRT-PCR Reference Genes in Different Tissues and Autumn Leaf Coloration Period of *Euonymus maackii*

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Abstract

Euonymus maackii is an important ornamental tree species valued for its autumn foliage in northern China. To precisely elucidate the molecular mechanisms underlying the distinct leaf coloration types in *E. maackii* during autumn, this study aimed to identify the optimal reference genes for qRT-PCR normalization across different tissues, developmental stages, and autumn leaf coloration types. Using 10 different tissues of *E. maackii* as experimental materials, 10 candidate reference genes were comprehensively evaluated for expression stability using three software tools, including geNorm, and the RefFinder online platform, with structural genes of the pigment biosynthesis pathway employed for validation. The comprehensive evaluation revealed that *TIP41* was the most stable reference gene across different tissues, developmental stages, and the full sample set; *EF-1 α* exhibited the highest stability among samples representing different autumn leaf coloration types; and *GAPDH* was the least stable in all sample groups. Quantitative validation of target genes demonstrated that when *EF-1 α* and *TIP41* were used in combination for normalization, the expression patterns of pigment biosynthesis structural genes were highly consistent with the phenotypic changes observed in autumn leaf coloration. In summary, this study recommends *TIP41* as a universal reference gene for expression analysis across different tissues and developmental stages of *E. maackii*; for studies involving different autumn leaf coloration types, a dual-reference normalization approach combining *EF-1 α* and *TIP41* is recommended.

Keywords: anthocyanin biosynthesis; internal control; transcript normalization; autumn leaf color; *EF-1 α* ; *TIP41*



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1. Introduction

Euonymus maackii is a deciduous small tree belonging to the genus *Euonymus* in the family Celastraceae. It is an excellent autumn foliage plant in northern China [1]. Autumn-colored tree species play a significant role in beautifying the environment, enriching garden landscapes, and bridging the gap in visual appeal between spring and autumn; they are indispensable ornamental plants in landscape design [2]. Plants of the genus *Euonymus* generally possess excellent characteristics such as vigorous growth, tolerance to poor soils, pruning tolerance, and strong stress resistance, making them widely used in landscape construction [3]. Among species in this genus, the *E. maackii* is at its most ornamental in

autumn, when its leaves turn yellow or red and its fruits turn pink; upon splitting, the fruits reveal orange–red arils. The interplay of colorful foliage and pink fruits creates a striking visual effect, and this exceptional ornamental value makes it an indispensable autumn-colored tree species in landscape design across northern China [4]. In recent years, green-leaved plants with monotonous coloration have failed to meet public demands for urban landscapes [5]. Therefore, a thorough analysis of the molecular regulatory mechanisms underlying the autumn leaf coloration in *E. maackii* is of great significance for developing superior cultivars with longer ornamental periods and more vibrant colors through molecular breeding techniques.

In elucidating the mechanisms underlying autumn leaf coloration in *E. maackii*, accurately determining the spatiotemporal expression patterns of key structural genes and transcription factors is a critical step. Quantitative real-time PCR (qRT-PCR), with its high sensitivity, strong specificity, and good reproducibility, has become the technique of choice for detecting the transcriptional levels of such genes [6]. However, the accuracy of qRT-PCR results depends heavily on the standardization of data; that is, an internal reference gene with constant expression levels must be included to eliminate systematic experimental errors such as variations in RNA quality, reverse transcription efficiency, and sample volume [7]. Ideal internal reference genes are typically involved in fundamental cellular biochemical metabolic processes. Common candidate genes include those encoding transcription elongation factors (elongation factor 1- α , *EF-1 α*), structural proteins such as actin (*ACT*) and tubulins (α -tubulin, *TUA*; β -tubulin, *TUB*), and metabolic enzymes like glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), cyclophilin (*CYP*), and phosphoglycerate kinase (*PGK*). Additionally, genes encoding ubiquitin-conjugating enzymes (*UBC*), protein phosphatase 2C (*PP2C*), and the phosphatase activator TIP41 (*TIP41*) are frequently utilized for normalization [8–11]. In theory, internal reference genes should not be affected by experimental conditions; however, numerous studies have shown that there is no single internal reference gene that is universal across species and conditions. A large number of previous studies have shown that the optimal types of reference genes can undergo drastic changes, whether in quantitative analysis of the anthocyanin synthesis pathway [12,13] or under different developmental stages and stress treatments [14,15]. This heterogeneity of expression profiles is not only widely present among plants of different families and genera but also shows significant differences among closely related species within the same genus [16] and different physiological phenotypes of the same species (such as different flower color genotypes) [17]. Therefore, directly applying empirical reference genes in a specific experimental system carries a high risk, and conducting independent reference gene assessment for specific species and treatment conditions is a prerequisite for ensuring data reliability.

For the woody plant *E. maackii*, the selection of reference genes poses a significant challenge. First, *E. maackii* comprises a wide range of tissue types, from highly lignified old roots and stem phloem to tender leaf buds and flowers, with significant differences in intracellular metabolic components. Second, autumn leaf coloration is accompanied by chlorophyll degradation and the massive synthesis of carotenoids and anthocyanin glycosides; these physiological changes are highly likely to cause fluctuations in the expression of traditional housekeeping genes. Although there has been considerable research on *E. maackii* regarding its landscape applications and physiological characteristics, current investigations into the molecular mechanisms underlying its leaf color formation remain largely confined to the stages of cultivation technique improvement and preliminary physiological indicator monitoring [2,3]. The molecular regulatory networks underlying its tissue differentiation and the various autumn leaf color types remain unclear, particularly given the lack of systematic and accurate screening and evaluation of qRT-PCR internal reference genes for

this species across different developmental stages and physiological coloration periods. In light of this, this study used *E. maackii* as the experimental material and constructed 10 representative sample. Ten candidate internal reference genes, including *ACT7*, *EF1- α* , and *TUB*, were selected, and their stability was comprehensively evaluated using three algorithms—geNorm [7], NormFinder [18], and BestKeeper [19]—along with their online platform RefFinder [20]. Finally, the selected optimal internal reference was used to validate key structural genes involved in the autumn coloration pathway. This study provides a reference framework for qRT-PCR normalization in *E. maackii* and related species.

2. Materials and Methods

2.1. Plant Materials

To comprehensively evaluate reference gene stability in *E. maackii*, ten representative sample types were collected (Figure 1). For spatial and developmental analyses, seven sample types (new and old roots, new stems, lignified stem phloem, flowers, leaf buds, and young leaves) were harvested from healthy clonal cuttings grown under controlled greenhouse conditions at Beijing Forestry University. To investigate natural autumn coloration, mature green leaves were collected prior to the autumn color transition. Subsequently, red and yellow leaves were sampled from two adjacent adult trees in Wenyu River Park, Beijing. All samples were collected on clear mornings in three biological replicates. For the adult trees, these replicates were defined as intra-individual replicates, systematically collected from different canopy positions to account for spatial variance (Figure S1). All tissues were immediately flash-frozen in liquid nitrogen and stored at -80°C .

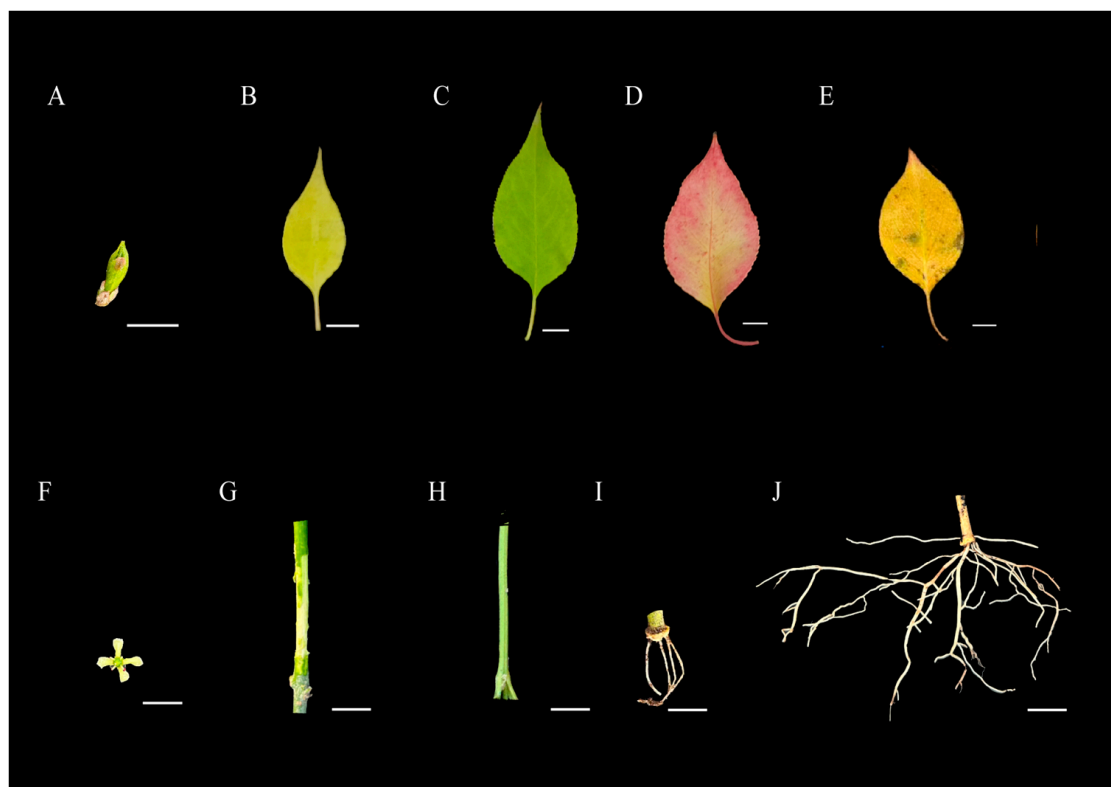


Figure 1. Different samples of *E. maackii*, including (A) Leaf buds, (B) Young leaves, (C) Mature green leaves, (D) Red leaves (E) Yellow leaves, (F) Flowers, (G) Phloem, (H) New stems, (I) New roots, (J) Old roots. Scale 1 cm. Grouping includes different tissues: new roots, new stems, flowers, leaf buds, mature green leaves; different developmental stages: new roots, old roots, new stems, phloem of lignified stems, leaf buds, young leaves, mature green leaves; different autumn leaf coloration types: mature green leaves, yellow leaves, red leaves; and all samples.

2.2. Total RNA Extraction and cDNA Synthesis of Different *E. maackii* Samples

For each of the 10 different samples, 100 mg of tissue was frozen in liquid nitrogen and ground into a fine powder. Total RNA was extracted using the FastPure Universal Plant Total RNA Isolation Kit (Vazyme, Nanjing, China). RNA concentration and purity were determined, and RNA quality was assessed using 1% agarose gel electrophoresis (Figure S2). One microgram (1 µg) of total RNA was reverse transcribed into cDNA according to the manufacturer's instructions for the HiScript III 1st Strand cDNA Synthesis Kit (+gDNA wiper) (Vazyme, Nanjing, China). Total RNA was stored at -80°C , and cDNA was stored at -20°C for later use.

2.3. Selection of Candidate Reference Genes and Primer Design

Based on the FPKM data from the different autumn leaf coloration type transcriptome of *E. maackii* and commonly reported reference genes, ten reference genes were selected for validation: *ACT7*, *TUA*, *TUB*, *PP2C*, *TIP41*, *UBC*, *EF-1 α* , *GAPDH*, *CYP*, and *PGK* [8–11]. Based on the coding sequences (CDS) obtained from the *E. maackii* transcriptome database, we redesigned the quantitative primers for all candidate reference genes and pigment-related structural genes. Primers were designed using the Primer 3 online tool (<https://primer3.ut.ee/> (accessed on 9 March 2026)), with an amplification length set to 180–200 base pairs, GC content to 40–60%, and a melting temperature (T_m) of $(55 \pm 5)^{\circ}\text{C}$. To validate the reliability and accuracy of the reference genes, pigment-related structural genes were chosen from the transcriptome as target genes for verification. Mainly two screening principles were followed, mapping to the flavonoid biosynthesis (ko00941), anthocyanin biosynthesis (ko00942), and carotenoid biosynthesis (ko00906) pathways in KEGG and having highly significantly differentially expressed genes (DEGs) with different autumn leaf color phenotypes ($|\log_2\text{FC}| > 1.0$ and $\text{FDR} < 0.05$). The primers used are listed in Table 1. All designed primers are fully analyzed before synthesis to detect potential secondary structures to ensure that the formation of primer dimers, secondary structures, homodimers, heterodimers, and hairpin structures is strictly controlled within the standard acceptable range. The *E. maackii* leaf transcriptome database utilized for candidate reference gene screening and target gene identification was retrieved from an unpublished in-house database previously established by our research group.

Table 1. Information on primers for structural genes of pigment biosynthesis pathways.

Gene	Full Name	Primer Sequence (5'-3')
<i>CHS</i>	<i>Chalcone synthase</i>	F: CAAACAGCGAGCACAAGACC R: TTGGGACCTCAACGACCAC
<i>CHI</i>	<i>Chalcone isomerase</i>	F: ACCTGGAAACGAACCTCGCAA R: AGGCTTCCTCTTCCTCTTCCT
<i>F3H</i>	<i>Flavanone 3-hydroxylase</i>	F: CGTCTCCAGTCATCTCCAGG R: ATTGCCTCTGACAGCACCTC
<i>F3'5'H</i>	<i>Flavonoid 3',5'-hydroxylase</i>	F: CCAGAGAGATTGATGAGTGGCA R: TGGTCAACCCCATAGGCAG
<i>DFR</i>	<i>Dihydroflavonol 4-reductase</i>	F: TGTTTGAGAATCCACACGCC R: TGAACCGAAACCCCATGTCC
<i>ANR</i>	<i>Anthocyanidin reductase</i>	F: GTTTTCCATGTCGCAACCCC R: CAACAACCAAACCTGTGCCA
<i>LAR</i>	<i>Leucoanthocyanidin reductase</i>	F: TCCGAGGTTATTCCACCGTTG R: TTCTTTCCCAACAAAGCGGC
<i>ANS</i>	<i>Anthocyanidin synthase</i>	F: ATTGACGCCGAGGACAAAGT R: TCCCTGAAGCCTGGTCATTG
<i>UFGT</i>	<i>UDP-Glucose Flavonoid Glucosyltransferase</i>	F: TTAGCTTCGGGACTGTGGTG R: CAAAACCTGTGTTTGGGGCG
<i>PSY</i>	<i>Phytoene synthase</i>	F: AACTGGTTGCGGAAATCCCA R: CTTCGCCACACCTCTGTAG

2.4. RT-PCR Amplification and Primer Specificity Detection of Candidate Reference Genes

Conventional PCR amplification was performed for the primers of each candidate reference gene. RT-PCR reactions were conducted following the instructions of the 2× Phanta Flash Master Mix (Dye Plus) (Vazyme, Nanjing, China). The PCR amplification products were then analyzed by 2.0% agarose gel electrophoresis (Figure S3).

2.5. RT-qPCR Amplification of Candidate Reference Genes

cDNA templates were serially diluted to five concentrations (1 , 5^{-1} , 5^{-2} , 5^{-3} , 5^{-4}). Standard curves were generated using qRT-PCR reactions. Amplification efficiency (E , %) was calculated using the formula $E = (5^{-1/\text{slope}} - 1) \times 100$. The standard curve slope and the linear correlation coefficient R^2 were also determined [21] to assess the reliability of the standard curve regression equation. qRT-PCR reactions were performed using a Bio-Rad CFX Duet real-time quantitative polymerase chain reaction system (Bio-Rad, Hercules, CA, USA). A 20 µL reaction system was employed, consisting of 10 µL of 2× HQ SYBR qPCR Mix, 2 µL of diluted cDNA, 0.4 µL each of forward and reverse primers ($10 \mu\text{mol}\cdot\text{L}^{-1}$), and ddH₂O added to a final volume of 20 µL. The amplification program included an initial denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 10 s and annealing at 60 °C for 30 s. Product specificity was confirmed by melting curve analysis, with conditions set at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. Three technical replicates were performed for each sample, and the Ct value was calculated as the average of the three results. qRT-PCR reactions were conducted on 10 different samples using the most suitable dilution factor.

2.6. Candidate Reference Gene Stability Analysis

The expression stability of ten candidate reference genes was analyzed using geNorm, NormFinder, and BestKeeper algorithms. A comprehensive evaluation was performed based on the online website RefFinder (<http://blooge.cn/RefFinder/> (accessed on 15 April 2026)) to screen for the most ideal reference genes. The screened reference genes were then used to analyze and validate the expression patterns of key structural genes involved in different autumn leaf coloration types, with three technical replicates for each sample, and the Ct value was calculated as the average of the three results. The relative expression levels of target genes were calculated using the $2^{-\Delta\Delta\text{CT}}$ method [22].

2.7. Data Processing

All statistical analyses and data visualization were performed using GraphPad Prism software (version 11.0.0). The statistical significance of the relative expression differences of the target genes between the red and yellow leaf phenotypes was determined using an unpaired two-tailed Student's *t*-test. Prior to the analysis, the data were assessed to ensure they met the underlying assumptions of normality and homogeneity of variances (*F*-test, $p > 0.05$). Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Screening of Candidate Reference Genes and Primers Specificity Analysis

Ten candidate reference genes with FPKM values greater than 10 were identified from the transcriptome, namely *EF1-α*, *ACT7*, *TUA*, *TUB*, *GAPDH*, *CYP*, *PGK*, *UBC*, *PP2C*, and *TIP41*. Among these 10 candidate reference genes, *ACT7* showed the highest FPKM value and expression level, while *TIP41* and *TUB* exhibited lower expression levels. However, the FPKM values of all 10 candidate reference genes were generally stable across different leaf coloration types in autumn (Figure 2A).

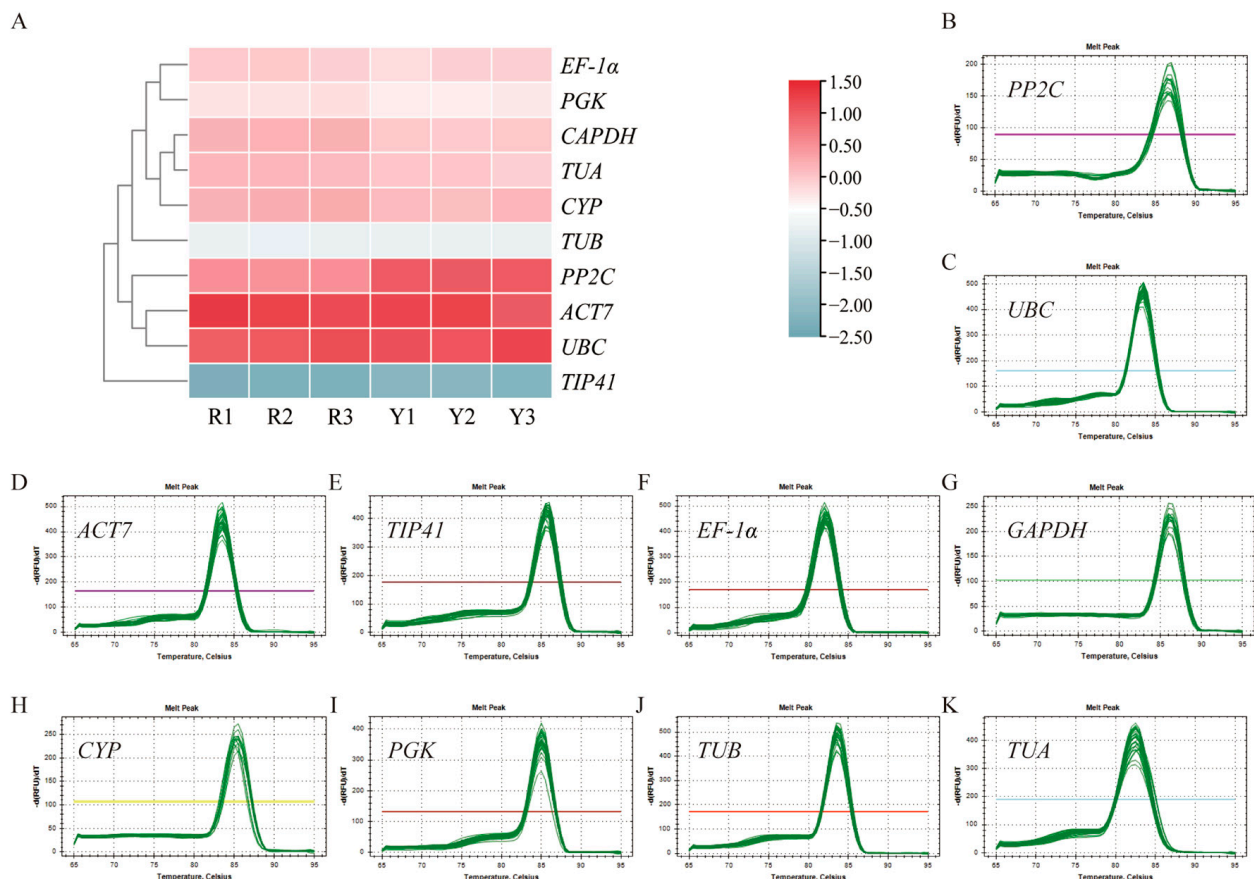


Figure 2. Expression of candidate reference genes, including (A) Heatmap of expression for 10 candidate endogenous reference genes in *E. maackii*. R1–R3 and Y1–Y3 display the three intra-individual replicates for the red leaf and yellow leaf samples respectively. The color scale represents the row-scaled Z-scores of log-transformed expression values, where red indicates relatively higher expression levels and blue indicates lower expression levels across the samples. (B–K) melting curves of 10 candidate internal reference genes. Abbreviations: elongation factor 1- α (*EF-1 α*), actin 7 (*ACT7*), α -tubulin (*TUA*), β -tubulin (*TUB*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), cyclophilin (*CYP*), phosphoglycerate kinase (*PGK*), ubiquitin-conjugating enzymes (*UBC*), protein phosphatase 2C (*PP2C*), and the phosphatase activator TIP41 (*TIP41*).

Specific primers were designed for the 10 candidate reference genes of *E. maackii*. qRT-PCR validation was conducted using serially diluted cDNA from the mixed sample as a template. Melting curve analysis revealed that all 10 candidate reference genes exhibited a single signal peak (Figure 2B–K).

3.2. Amplification Efficiency Analysis of Candidate Reference Gene Primers

The amplification efficiency values for all primers ranged from 90.40% (*EF1- α*) to 115.24% (*ACT7*), with R^2 values consistently ≥ 0.99 (Table 2). This demonstrates that the reference gene primers used were both specific and effective, and the resulting experimental data are suitable for subsequent analysis. Based on these standard curves, a uniform 5-fold dilution of the cDNA templates was confirmed to be perfectly within the optimal linear range for all evaluated genes, yielding highly reliable Ct values ranging from 15 to 30. To maintain strictly consistent initial template inputs across all reactions, this 5-fold dilution factor was adopted for all subsequent qRT-PCR validation experiments.

Table 2. Information on primers for candidate housekeeping genes.

Gene	Primer Sequence (5'-3')	Product Length/bp	Slope	Amplification Efficiency (E)%	Linear Correlation Coefficient R ²
<i>ACT7</i>	F: AAGATTCCGTGCCAGAGG R: CCTTGCTCATACGGTCTGC	188	−2.1951	115.2387	0.9901
<i>TUA</i>	F: CGAGTGAGGAAGTTGGCTGA R: ACTGCTGTTGAGACCTGTGG	182	−2.1205	113.6132	0.99384
<i>TUB</i>	F: CATGATGTGTGCTGCTGACC R: GCCATCCTCAAGCCCTTAGG	198	−2.1146	114.0657	0.9971
<i>PP2C</i>	F: GATTCGAACGCTGTTGAGGC R: CAACGTGGCCATCGAAAAC	182	−2.1940	108.2461	0.9982
<i>TIP41</i>	F: AGGTGGACGACAAGGACTA R: ACAACTGTCCCCAAACACC	189	−2.2345	105.4966	0.9869
<i>UBC</i>	F: CCCAAACATCAACACAATGGT R: CTCGTACTTGCTCCGGTCAG	181	−2.2431	104.9336	0.9989
<i>EF-1α</i>	F: TGTTGAGATGCACCACGAGG R: AGCCATTTCCAATCTGCCCCA	197	−2.4994	90.3956	0.9936
<i>GAPDH</i>	F: ATGCCTTACCTGCTGTCACC R: TCCTTCCGTGTTCCAACCTGT	183	−2.2103	107.1223	0.9956
<i>CYP</i>	F: CGCCGATGAGAACTTCCAGA R: ATCCCGATCCGACCTTCTCA	197	−2.2002	107.8181	0.9973
<i>PGK</i>	F: AACTGGTTGCGGAAATCCCA R: CTTCCGCCACACCCTCTGTAG	182	−2.2265	106.0283	0.9964

3.3. Expression Abundance of Candidate Reference Genes

The comparison of average Ct values showed that *EF1-α* and *CYP* exhibited higher expression levels, while *PGK* and *TIP41* showed lower expression levels. The comparison of Δ Ct values indicated that *TIP41* had the smallest fluctuations among different tissues, developmental stages, and all samples. In the autumn leaf coloration type, *EF1-α* was the most stable, followed by *TIP41*. In contrast, *GAPDH* showed the greatest variability across all groups (Table 3).

Table 3. Ten candidate reference gene Δ CT value ranking.

Groups	Method	Ranking									
		1	2	3	4	5	6	7	8	9	10
Different tissues	Mean Ct	<i>EF1-α</i>	<i>CYP</i>	<i>ACT7</i>	<i>UBC</i>	<i>GAPDH</i>	<i>TUA</i>	<i>TUB</i>	<i>PP2C</i>	<i>PGK</i>	<i>TIP41</i>
	Δ Ct	<i>TIP41</i>	<i>TUA</i>	<i>TUB</i>	<i>ACT7</i>	<i>EF1-α</i>	<i>UBC</i>	<i>PP2C</i>	<i>PGK</i>	<i>CYP</i>	<i>GAPDH</i>
Different stages of development	Mean Ct	<i>CYP</i>	<i>EF1-α</i>	<i>ACT7</i>	<i>UBC</i>	<i>TUA</i>	<i>GAPDH</i>	<i>TUB</i>	<i>PP2C</i>	<i>PGK</i>	<i>TIP41</i>
	Δ Ct	<i>TIP41</i>	<i>ACT7</i>	<i>TUA</i>	<i>UBC</i>	<i>TUB</i>	<i>EF1-α</i>	<i>PGK</i>	<i>PP2C</i>	<i>CYP</i>	<i>GAPDH</i>
Different types of leaf coloration	Mean Ct	<i>CYP</i>	<i>UBC</i>	<i>EF1-α</i>	<i>ACT7</i>	<i>PP2C</i>	<i>TUA</i>	<i>TUB</i>	<i>GAPDH</i>	<i>TIP41</i>	<i>PGK</i>
	Δ Ct	<i>EF1-α</i>	<i>TIP41</i>	<i>TUB</i>	<i>TUA</i>	<i>ACT7</i>	<i>UBC</i>	<i>CYP</i>	<i>PP2C</i>	<i>PGK</i>	<i>GAPDH</i>
All samples	Mean Ct	<i>CYP</i>	<i>EF1-α</i>	<i>UBC</i>	<i>ACT7</i>	<i>TUA</i>	<i>GAPDH</i>	<i>TUB</i>	<i>PP2C</i>	<i>PGK</i>	<i>TIP41</i>
	Δ Ct	<i>TIP41</i>	<i>ACT7</i>	<i>TUA</i>	<i>TUB</i>	<i>UBC</i>	<i>EF1-α</i>	<i>CYP</i>	<i>PP2C</i>	<i>PGK</i>	<i>GAPDH</i>

3.4. Stability Analysis of Candidate Reference Genes

3.4.1. geNorm Analysis

The geNorm software evaluates gene stability based on the expression stability (M value), where a lower M value indicates greater stability of the candidate reference gene (Figure 3). *TUA* and *TUB* showed the most stable expression in different tissues; *ACT7* and *TIP41* were the most stable across different developmental stages; *TIP41* and *EF1-α* exhibited the most stable expression in different leaf coloration types; and *ACT7* and *TIP41* were the most stable across all samples. *GAPDH* was the most unstable across all four groups. The combination of three reference genes determined by the geNorm-based pairwise variation analysis ($V_n/V_{n+1} < 0.15$) is optimal for different tissues, developmental

stages, and all sample groups, while the combination of two reference genes is optimal for different color variant types (Figure 4).

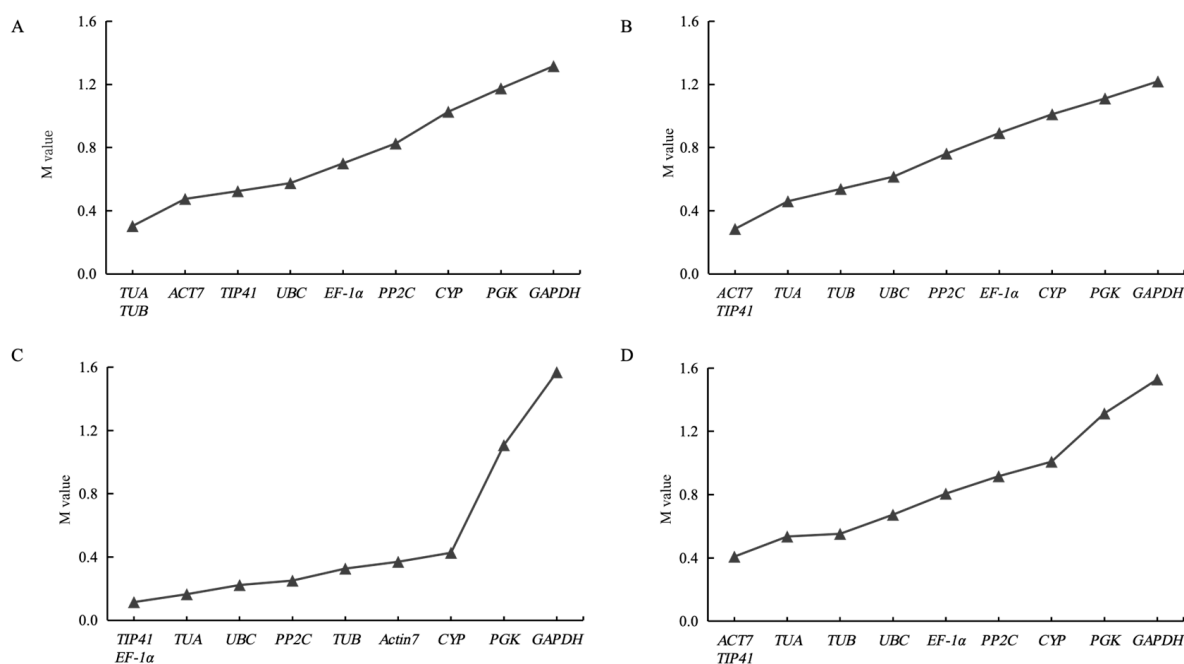


Figure 3. geNorm analysis of expression stability values for candidate internal reference genes, including (A) Different tissues, (B) Different developmental stages, (C) Different leaf coloration types, (D) All samples. Abbreviations: elongation factor 1- α (EF-1 α), actin 7 (ACT7), α -tubulin (TUA), β -tubulin (TUB), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), cyclophilin (CYP), phosphoglycerate kinase (PGK), ubiquitin-conjugating enzymes (UBC), protein phosphatase 2C (PP2C), and the phosphatase activator TIP41 (TIP41).

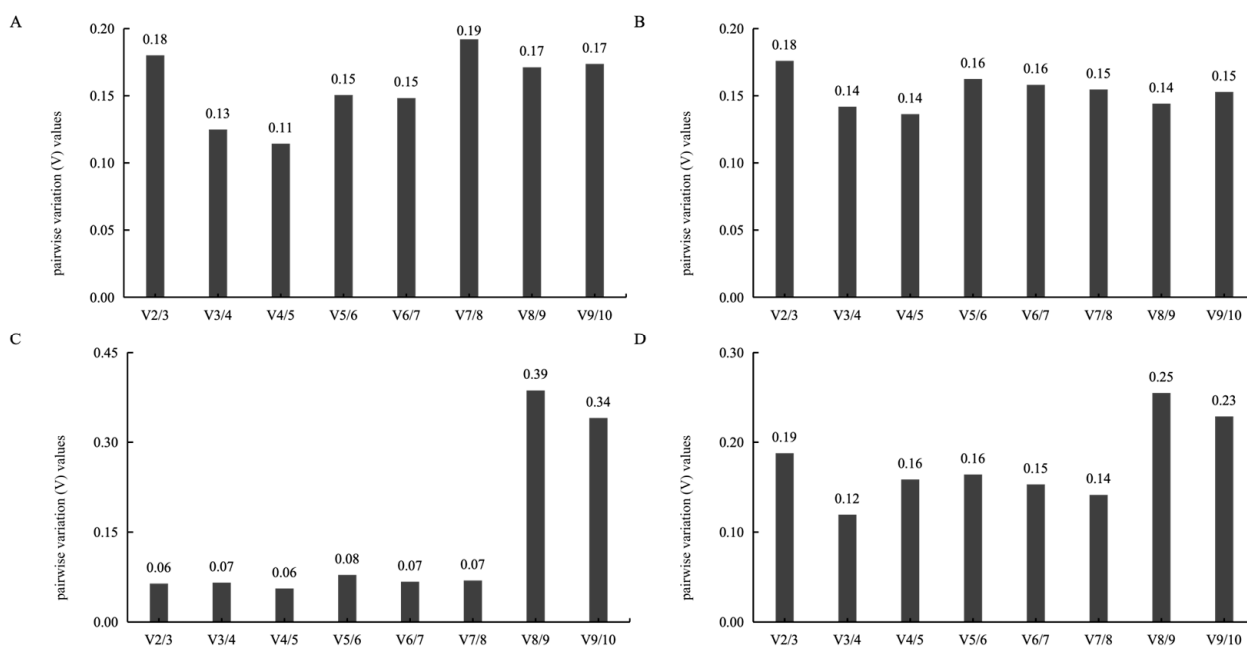


Figure 4. Pairwise variation (V) of the 10 candidate reference genes, including (A) Different tissues, (B) Different developmental stages, (C) Different leaf coloration types, (D) All samples.

3.4.2. NormFinder Analysis

The NormFinder software assesses the expression stability of candidate reference genes based on stability values (S values), where a lower S-value indicates higher stability (Figure 5). *TIP41* showed the most stable expression in different tissues, different developmental stages and all samples; *CYP* exhibited the most stable expression in different leaf coloration types. *GAPDH* was the most unstable across all four groups.

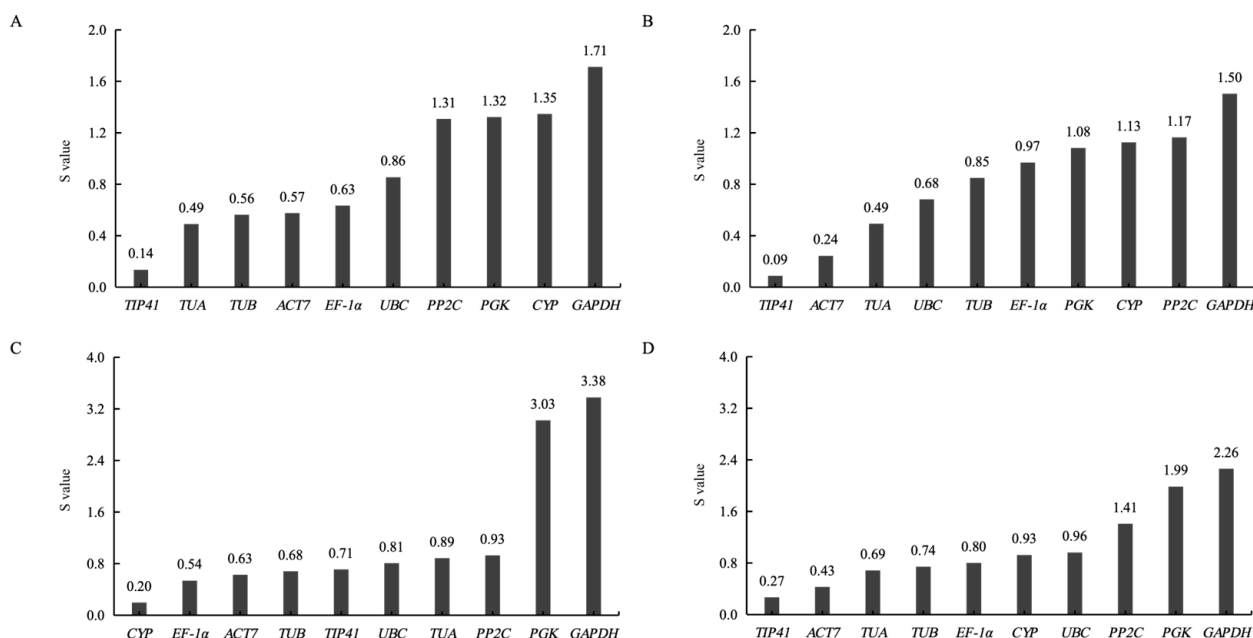


Figure 5. NormFinder analysis of expression stability values for candidate internal reference genes, including (A) Different tissues, (B) Different developmental stages, (C) Different leaf coloration types, (D) All samples. Abbreviations: elongation factor 1- α (*EF-1 α*), actin 7 (*ACT7*), α -tubulin (*TUA*), β -tubulin (*TUB*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), cyclophilin (*CYP*), phosphoglycerate kinase (*PGK*), ubiquitin-conjugating enzymes (*UBC*), protein phosphatase 2C (*PP2C*), and the phosphatase activator *TIP41* (*TIP41*).

3.4.3. BestKeeper Analysis

The BestKeeper software determines the expression stability of candidate reference genes by automatically calculating two variables: standard deviation (SD) and coefficient of variation (CV). A smaller SD value indicates higher stability (Table 4). *TIP41* was identified as the most stably expressed gene in different tissues, *UBC* in different developmental stages, and *ACT7* in both different leaf coloration types and all samples.

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

Gene	Different Tissues		Different Stages of Development		Different Types of Leaf Coloration		All Samples	
	SD	CV/%	SD	CV/%	SD	CV/%	SD	CV/%
<i>ACT7</i>	1.1567	5.0098	0.8139	3.5822	0.1437	0.6359	0.8615	3.7607
<i>TUA</i>	1.3999	5.6635	1.1932	4.8880	0.4755	2.0191	1.2331	5.0625
<i>TUB</i>	1.2494	4.9585	1.3113	5.2413	0.2122	0.8576	1.1758	4.6810
<i>PP2C</i>	1.0826	4.1984	1.1106	4.3387	0.6227	2.6463	1.2383	4.9262
<i>TIP41</i>	0.8793	3.1774	0.8184	2.9858	0.3693	1.3589	0.7920	2.8873
<i>UBC</i>	0.9416	4.0586	0.5722	2.4954	0.5656	2.6326	0.9123	4.0125

Table 4. Cont.

Gene	Different Tissues		Different Stages of Development		Different Types of Leaf Coloration		All Samples	
	SD	CV/%	SD	CV/%	SD	CV/%	SD	CV/%
<i>EF-1α</i>	1.4338	6.3908	1.0653	4.8747	0.3504	1.6197	0.9894	4.5198
<i>GAPDH</i>	2.0203	8.1794	1.5643	6.3715	2.4229	9.1495	1.9412	7.7474
<i>CYP</i>	1.1495	5.2521	1.0797	4.9618	0.2001	0.9373	0.8739	4.0291
<i>PGK</i>	1.4738	5.5590	1.3604	5.1245	2.2413	7.9365	1.6632	6.1903

3.4.4. Comprehensive Stability

To integrate the analysis results from the geNorm, NormFinder, and BestKeeper algorithms, the Ct values of each candidate reference gene across different samples were imported into the online tool RefFinder for a comprehensive evaluation (Table 4). *TIP41* was found to be the most stably expressed gene in different tissues, different developmental stages, and all samples. In contrast, *EF-1 α* was the most stably expressed in different leaf coloration types, followed by *TIP41* (Figure 6).

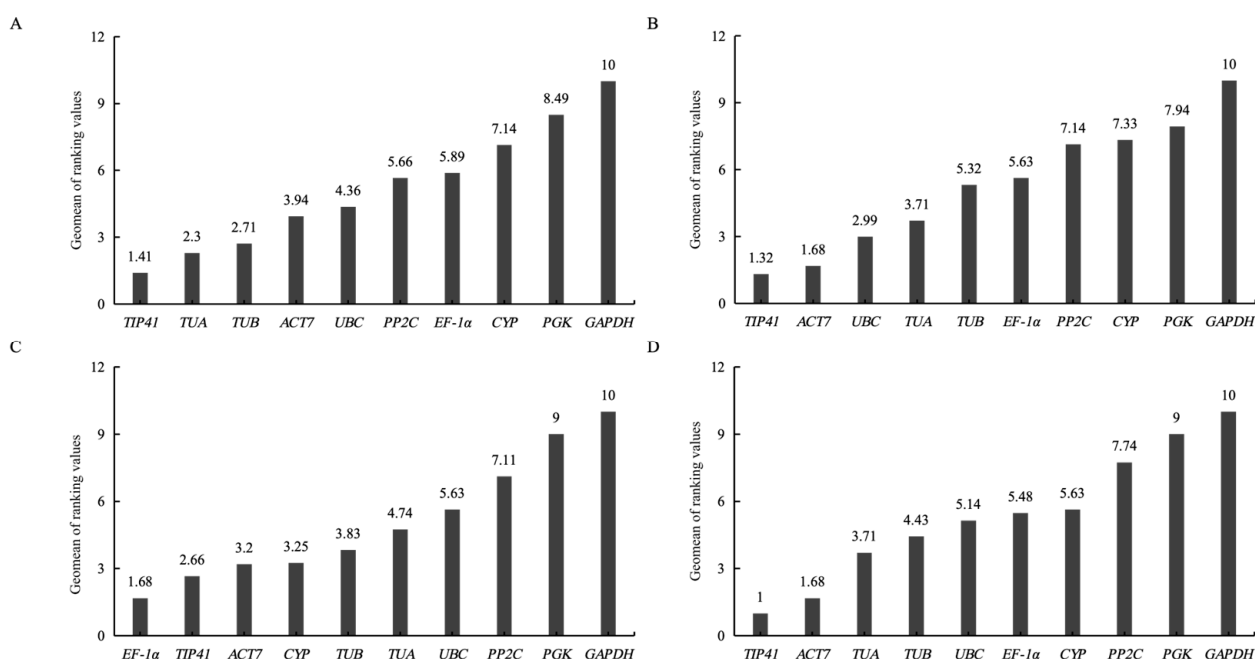


Figure 6. RefFinder analyses the stability of 10 candidate internal reference genes, including (A) Different tissues, (B) Different developmental stages, (C) Different leaf coloration types, (D) All samples. Abbreviations: elongation factor 1- α (*EF-1 α*), actin 7 (*ACT7*), α -tubulin (*TUA*), β -tubulin (*TUB*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), cyclophilin (*CYP*), phosphoglycerate kinase (*PGK*), ubiquitin-conjugating enzymes (*UBC*), protein phosphatase 2C (*PP2C*), and the phosphatase activator *TIP41* (*TIP41*).

3.5. Validation of Candidate Reference Gene Stability

To select the most reliable internal control, we rely on the comprehensive ranking provided by RefFinder and the paired variance analysis by geNorm to determine that the combination of *EF-1 α* and *TIP41* reference genes is optimal for different color variant types. The expression patterns of 10 structural genes in the pigment synthesis pathway were analyzed in autumn red and yellow leaves, using *EF-1 α* and *TIP41* (the most stable), the dual reference gene combination (*EF-1 α* + *TIP41*), and *GAPDH* (the least stable) as calibration references, respectively. The results showed that when using *EF-1 α* or *TIP41* as

single reference genes, or their combined calibration, the expression trends of the target genes were highly consistent. However, when *GAPDH*, the least stable reference gene, was used for standardization, the relative expression patterns of the target genes significantly deviated, and the results of the differential significance analysis among different leaf color samples also showed substantial changes (Figure 7).

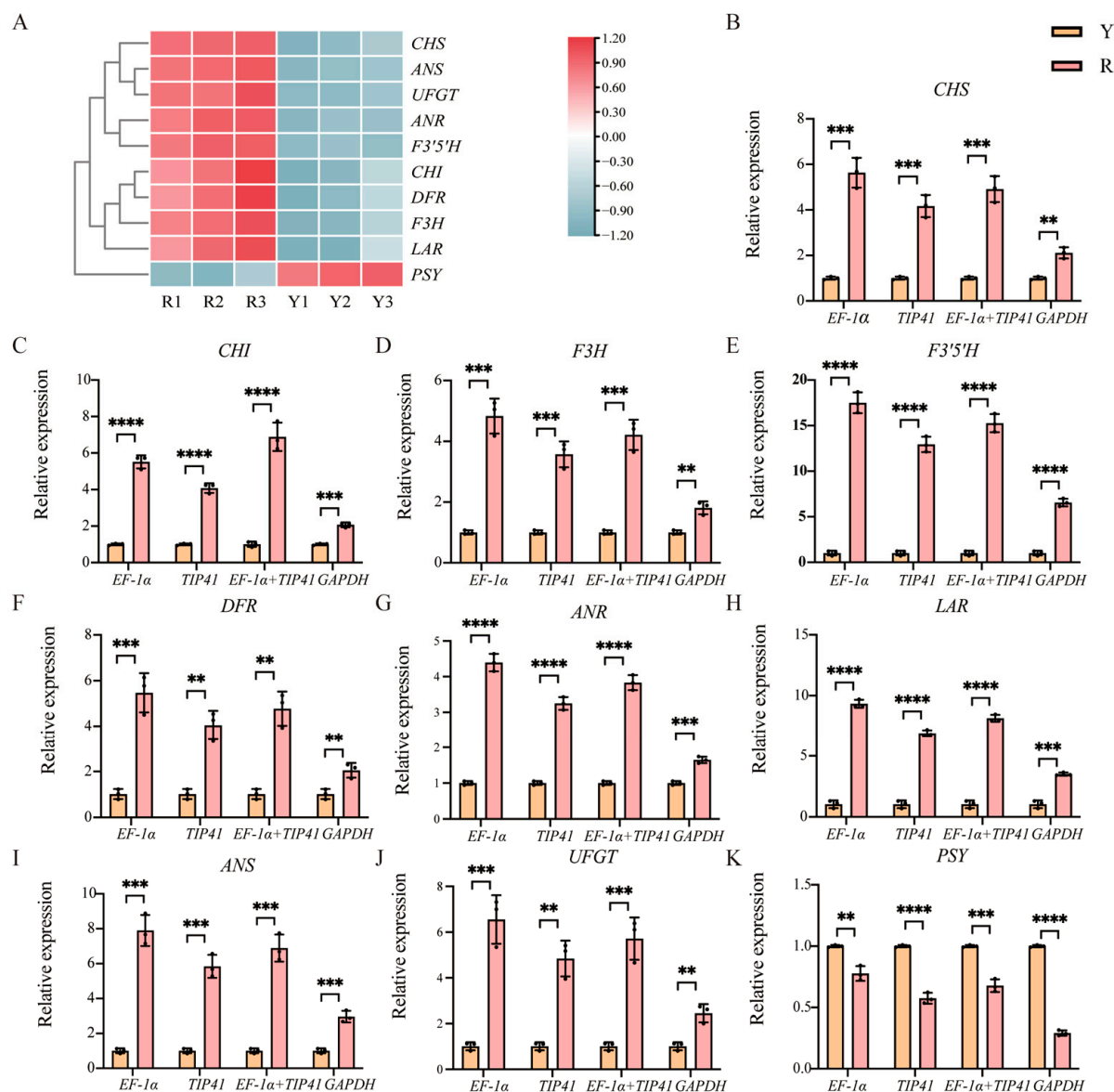


Figure 7. Transcriptomic profiles and qRT-PCR validation of candidate genes. (A) Expression profiles of 10 structural genes of the pigment synthesis pathway in red and yellow leaves. R1–R3 and Y1–Y3 display the three intra-individual replicates for the red leaf and yellow leaf samples respectively. The color scale represents the row-scaled Z-scores of log-transformed expression values, where red indicates relatively higher expression levels and blue indicates lower expression levels across the samples. (B–K) Relative expression levels of structural genes of the pigment synthesis pathway when *EF-1α*, *TIP41*, *EF-1α + TIP41* and *GAPDH* were used as internal reference genes. R and Y denote the red leaf and yellow leaf samples, respectively. Statistical significance between the red and yellow leaves was determined using an unpaired two-tailed Student's *t*-test (* $p < 0.01$, ** $p < 0.001$, **** $p < 0.0001$). Abbreviations: elongation factor 1- α (*EF-1α*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), the phosphatase activator *TIP41* (*TIP41*), chalcone synthase (*CHS*), flavanone 3-hydroxylase (*F3H*), flavonoid 3', 5'-hydroxylase (*F3'5'H*), dihydroflavonol 4-reductase (*DFR*), anthocyanidin reductase (*ANR*), leucoanthocyanidin reductase (*LAR*), anthocyanidin synthase (*ANS*), UDP-Glucose Flavonoid Glucosyltransferase (*UFGT*), and phytoene synthase (*PSY*).

4. Discussion

Selecting appropriate reference genes is a prerequisite for ensuring accurate and reliable qRT-PCR analysis results. However, the expression of reference genes often exhibits strong species, tissue, and developmental stage specificity, with few truly universal reference genes that transcend various physiological conditions [23]. In recent years, the combination of high-throughput sequencing technologies (e.g., RNA-seq) and bioinformatics algorithms has provided strong support for the efficient and standardized screening of reference genes [24]. For instance, candidate reference genes screened using public information databases for *Oryza sativa* have demonstrated excellent stability [25]. In this study, the stability of 10 candidate reference genes in *E. maackii* was evaluated using the geNorm, NormFinder, and BestKeeper algorithms, respectively. As detailed in the Results Section, the three statistical algorithms yielded divergent stability evaluations for the candidate genes. Such discrepancies in evaluation, which vary by algorithm, are quite common in plant reference gene screening studies [26], fundamentally originating from the differences in the underlying statistical models of each software. Previous studies on reference gene screening during fruit development in *Prunus mume* and *Prunus salicina* ‘Qiangcuili’ observed similar phenomena [12,27], specifically that BestKeeper’s ranking results often showed significant deviations from those of the other two software programs. This is primarily attributed to BestKeeper directly performing correlation and standard deviation analysis based on raw Ct values, whereas geNorm and NormFinder assess stability based on relative expression levels. Recognizing the inherent statistical limitations of single algorithms, this study further incorporated the RefFinder online platform. By calculating the geometric mean across multiple algorithms, this platform avoids the limitations of individual models and identifies highly reliable reference genes for *E. maackii*.

In addition to the objective differences in the algorithms themselves, the discrepancies in the stability of candidate genes across different analysis scales deeply reflect their underlying biological functional heterogeneity. In evaluations spanning different tissues (spatial scales) and developmental stages (temporal scales), *TIP41* have shown excellent stability. *TIP41* encodes an intrinsic vacuolar membrane protein involved in regulating fundamental cell signaling and vesicle transport [28]. Maintaining fundamental cell communication and protein translation is essential for survival across all plant organs and developmental stages. Therefore, we hypothesize that genes governing this core homeostasis are better equipped to resist spatial and temporal expression fluctuations, serving as reliable transcriptional landmarks. The extremely high stability of *TIP41* in cross-tissue and cross-developmental stage analyses has been reported in multiple species. For example, it has been shown to maintain stable expression amidst metabolic fluctuations during the development of *Xanthoceras sorbifolium* seeds and the differentiation of various plant organs in the *Brassica* genus [29–32].

In contrast, under the specific physiological scale of autumn leaf color change, reference genes face entirely different transcriptional selection pressures. *GAPDH* encodes a key enzyme involved in glycolysis and the Calvin cycle. When mature green leaves enter the autumn color change stage, photosynthesis rapidly declines, and carbon flow is significantly redistributed to provide precursors for the biosynthesis of secondary metabolites. Therefore, the transcription of primary metabolism genes like *GAPDH* is highly likely to undergo drastic fluctuations, making them extremely unsuitable as reference genes under the specific physiological scale of autumn leaf color change [33,34]. To achieve phenotypic transformation, cells must synthesize substantial amounts of pigment-related structural enzymes. This requires plant cells to maintain high ribosomal activity and translational efficiency during this metabolic transition. As a translation elongation factor, *EF-1 α* is an indispensable core component of protein synthesis [35]. This underlying physiological

logic provides a plausible hypothesis for why *EF-1 α* can still exhibit extremely high stability under the specific physiological scale of autumn leaf color change. Previous studies have widely confirmed the reliability of these genes in plant pigment quantitative analysis, not only in the color changes of reproductive organs such as *Nelumbo nucifera* and *Camellia japonica* [10,34,36], but also in the leaf color differentiation processes of woody and herbaceous plants such as *Liquidambar formosana*, *Dendrobium officinale*, and *Sinobambusa tootsik* [37–40]. This consistency across species and organs further supports the hypothesis that *EF-1 α* can effectively counteract the drastic transcriptional selection pressures during autumn senescence, making it an ideal choice for precise normalization and elucidation of the molecular mechanism of the autumn leaf coloration types.

In traditional qRT-PCR expression analysis, normalization using a single reference gene was once the most common strategy. However, extensive research has confirmed that under extreme developmental transitions or severe environmental fluctuations, even classic housekeeping genes can exhibit significant shifts in expression abundance [41]. Therefore, introducing a multiple reference gene normalization system to eliminate the systematic bias caused by single-gene fluctuations has become a general consensus for improving quantitative accuracy. For example, in complex physiological studies such as the cut flower development of *Paeonia* ‘Bartzella’ [42] and the abiotic stress response of *Salsola ferganica* [43], combinations of two reference genes were employed to ensure data reliability. Based on this, the present study introduced a multiple reference gene joint-normalization model for the autumn leaf coloration types of *E. maackii*. According to the geometric averaging evaluation standard proposed by Vandesompele et al. [7], the core statistical indicator for determining the optimal number of reference genes is the pairwise variation value (V_n/V_{n+1}). The geNorm analysis revealed that in the comprehensive evaluation of all *E. maackii* samples, the V_2/V_3 value was 0.064, which is below the internationally recommended threshold of 0.15. This indicates that in the quantitative study of autumn leaves in *E. maackii*, introducing the two most stable genes, *EF-1 α* and *TIP41*, for combined normalization is sufficient to meet the statistical requirements for accurate quantification.

The physiological essence of autumn leaf coloration is the accelerated degradation of chlorophyll, coupled with the massive synthesis and accumulation of secondary metabolites such as anthocyanins and carotenoids. Among these, the key enzyme genes of the anthocyanin biosynthesis pathway and the core genes of carotenoid biosynthesis directly determine the differentiation of red and yellow leaf phenotypes in plants [44,45]. In quantitative studies of autumn-color tree species such as *Sapium sebiferum* [46] and *Liquidambar formosana* [38], structural genes of pigment biosynthesis pathways, such as *CHS*, were utilized for validation. Therefore, selecting these highly active structural genes of pigment biosynthesis pathways as target genes can accurately reflect the normalization efficacy of reference genes under severe metabolic fluctuations. To validate our findings, we quantified ten pigment-related structural genes. Normalization with *EF-1 α* , *TIP41*, or their combination yielded highly consistent expression profiles across different leaf colors. In contrast, using the unstable *GAPDH* severely distorted these transcriptional trends. These results confirm that *EF-1 α* and *TIP41* represent the optimal reference gene strategy for investigating autumn color transitions in *E. maackii*.

Although this study has successfully identified the reliable reference gene of *E. maackii*, there are certain limitations on the sampling of autumn leaf color samples in the experimental design. Representing red and yellow leaf samples were collected from two adjacent adult trees. Although this can minimize environmental impacts, such as soil composition and light, it is indeed impossible to separate the genotype-specific effect from the phenotype-related expression differences by using only one adult tree for each phenotype. Therefore, the differential expression observed in the verification of the target gene may

partially reflect the genotype variation between the two individuals. However, *EF-1 α* and *TIP41* maintain high stability in samples with different leaf color phenotypes and genetic backgrounds, further highlighting their robust applicability. In order to establish a clearer molecular mechanism for the autumn color of *E. maackii*, future studies should expand the population-level sampling scale or use different leaf color mutation cloning with the same genetic background to completely eliminate genotype interference.

Looking forward, although this study successfully establishes *EF-1 α* and *TIP41* as highly reliable reference genes for studying the autumn leaf color change in *E. maackii*, their potential as universal internal references still require broader exploration. Future research should aim to evaluate the expression stability of these genes across a wider spectrum of developmental stages, diverse abiotic and biotic stress conditions, and alongside a more comprehensive array of functional genes and metabolic pathways. Such extensive validations will not only solidify the universal applicability of *EF-1 α* and *TIP41* for general transcript normalization in *E. maackii* but also provide robust methodological tools for deeper functional genomic and breeding studies in related woody ornamental species.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae12070773/s1>, Figure S1: Environmental consistency and phenotypic divergence of the sampled *Euonymus maackii* individuals; Figure S2: RNA agarose gel electrophoresis of different samples of *E. maackii*; Figure S3: Agarose gel electrophoresis image of PCR products of 10 candidate reference genes.

Author Contributions: Y.H. and J.Y. designed the research framework; J.Y. collected and prepared the materials for the experiment, analyzed the data, and wrote the first draft; Y.H. participated in the manuscript revision. All authors have read and agreed to the published version of the manuscript.

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