Screening and Validation of Internal Reference Genes for Quan-Titative Real-Time PCR Analysis of Leaf Color Mutants in Den-Drobium Officinale

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Keywords: Dendrobium officinale; leaf color; qRT-PCR; reference gene; leaf color mutant

Article

Screening and Validation of Internal Reference Genes for Quantitative Real-Time PCR Analysis of Leaf Color Mutants in *Dendrobium officinale*

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Abstract: Leaf color mutants (LCMs) are important resources for studying diverse metabolic processes such as chloroplast biogenesis and differentiation, pigments' biosynthesis and accumulation, and photosynthesis. However, in *Dendrobium officinale*, LCMs are yet to be fully studied and exploited due to the unavailability of reliable RGs (reference genes) for qRT-PCR (quantitative real-time reverse transcription PCR) normalization. Hence, this study took advantage of previously released transcriptome data to select and evaluate the suitability of ten candidate RGs, including Actin (*Actin*), polyubiquitin (*UBQ*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), elongation factor 1-α (*EF1α*), β-tubulin (*β-TUB*), α-tubulin (*α-TUB*), 60S ribosomal protein L13-1 (*RPL13AD*), aquaporin PIP1-2 (*PIP1-2*), Intima protein (*ALB3*) and Cyclin (*CYCB1-2*) for normalizing leaf color-related genes' expression levels via qRT-PCR. Stability rankings analysis via common software Best-Keeper, GeNorm, and NormFinder disclosed that both ten genes met the requirements of RGs. Of them, *EF1α* exhibited the highest stability and was selected as the most reliable. The reliability and accuracy of *EF1α* were confirmed through qRT-PCR analysis of fifteen chlorophyll pathway-related genes. The expression patterns of these genes via *EF1α* normalization were consistent with the results by RNA-Seq. Our results will pave the way for molecular dissection of leaf color mutations in *D. officinale*.

Keywords: *Dendrobium officinale*; leaf color; qRT-PCR; reference gene; leaf color mutant

1. Introduction

Leaf color mutations are common and easily detectable morphological phenotype variations in higher plants [1,2]. They represent essential resources for insight into plant physiology and metabolism as they mostly affect plants' photosynthetic efficiency, which results in poor growth and development performances and considerable economic losses [1,2]. Accordingly, LCMs have become ideal materials for investigating pigments' metabolism, chloroplast formation and differentiation, photosynthesis, biotic and abiotic stress responses, etc. [1,2]. In rice, studies on LCMs have significantly contributed to the crop improvement [1].

D. officinale (also called *D. catenatum*) is an Orchid with high medicinal and ornamental values [3,4]. Therefore, understanding leaf color mutation mechanisms will contribute to developing diverse attractive *D. offficinale* genotypes and deepen our knowledge of plant physiology and metabolism. In previous studies, we combined physiological and comparative transcriptomics analysis of LCMs of *D. officinale* and unveiled that variation in leaf colors is associated with significantly reducing the number of chloroplasts and chlorophyll and carotenoid contents [5]. We found that the photosynthetic efficiency of LCMs is greatly influenced by light intensity [6]. Importantly, we have

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identified key DEGs (differentially regulated genes) related to variation in leaf color [5]. Unfortunately, we could not validate these DEGs as potential candidate genes and perform functional investigations due to the unavailability of a reliable RG for qRT-PCR normalization.

Currently, qRT-PCR analysis is the widely used method to verify the reliability of transcriptome data due to its high sensitivity, accuracy, specificity, and reproducibility [7–11]. But, its accuracy relies on various factors, including the integrity of initial samples, quality of RNA, primers specificity, reliability of RGs, and the efficiency of the reverse transcription and amplification [12]. Of them, the suitability of RGs is very critical. The selection of inappropriate RG(s) will cause erroneous results by qRT-PCR analysis, resulting in wrong conclusions [13]. Therefore, using one or more stable RGs as the calibration standard(s) is recommended [11,14]. In many plants, reliable RGs are often selected from housekeeping genes with stable expressions, such as actin (*ACT*), polyubiquitin (*UBQ*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), elongation factor (*EF*), 18S ribosomal RNA (*18S*), Tubulin (*TUB*), etc. [14–17]. For instance, *actin2* and *18SRNA* are used to calibrate the expression levels of leaf color-related genes in *Lilium regale* and wheat, respectively. Notably, a reliable RG should display stable expressions at different developmental stages or under diverse experimental conditions in all plant organs [9,18,19]. But, other studies in many species have revealed that RGs do not always exhibit stable expression levels, specifically under changing environments [19–21]. Therefore, it is essential to identify stable and reliable RG(s) for specific traits and experimental conditions in each plant species [9,22,23].

In this study, based on the previously released RNA-seq data [5], we selected ten candidate RGs and investigated their expression stability using common statistical algorithms (BestKeeper, GeNorm, and NormFinder). As a result, we identified and validated the most suitable RG for qRT-PCR normalization of leaf color-related genes in *D. officinale*. Our findings will promote genomics studies on LCMs *D. officinale*.

2. Materials and Methods

2.1. Plant Materials and Sampling

The wild-type (green leaf, CK) and a leaf color-mutant (yellow) *D. officinale* tissue culture materials were used in this study (Figure 1). When the tissue cultures were at the seedlings stage, leaves were sampled from ten plants of each genotype, mixed, and immediately frozen in liquid nitrogen. Subsequently, the collected samples were stored at – 80 °C in a refrigerator until further experimentations. Three biological and experimental repeats were achieved for each analysis.

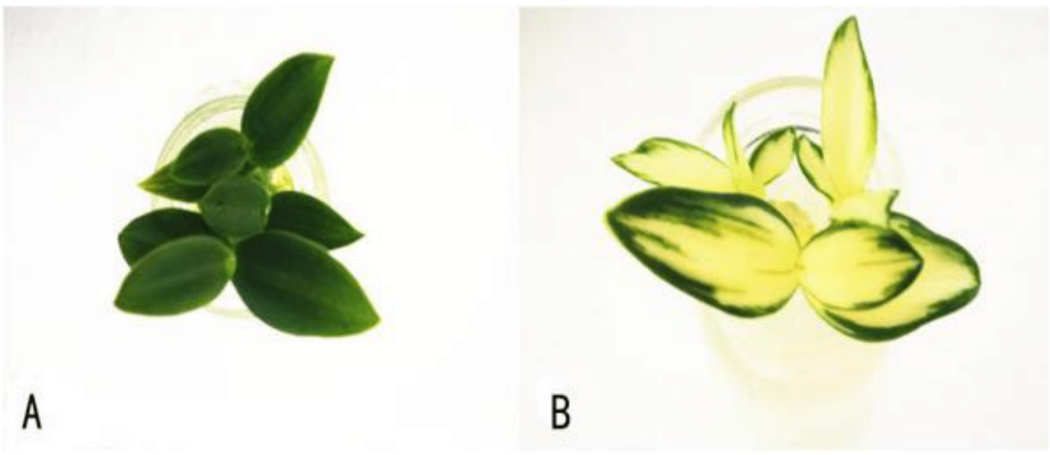

Figure 1. Phenotypic characteristics of *D. officinale* (**A**) and its yellow leaf mutant (**B**).

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2.2. Selection of Candidate Internal RGs and Chlorophyll Pathway-Related Genes

We previously analyzed the transcriptome of wild-type and leaf color-mutant of *D. officinale* [5]. Based on the relative stability of the expression patterns of genes (FPKM values) in all groups based, ten commonly used internal RGs, including *Actin* (Actin 7), *UBQ* (polyubiquitin), *GAPDH* (3-Glyceraldehyde phosphate dehydrogenase), *EF1α* (elongation factor α), *β-TUB* (β-Tubulin), *α-TUB*, *RPL13AD* (60S ribosomal protein l13a-4), *PIP1-2* (water protein channel pip1-2), *ALB3* (intimal protein ppf-1), and *CYCB1-2* (Cyclin B1-2) were screened out as potential RGs for analyzing the relative expression of leaf color-related genes. The expression patterns and FPKM values of these genes are shown in Figure 2A and Table S1, respectively. In addition, based on the functional annotation of DEGs (differentially expressed genes) from the transcriptomics analysis [5], we selected fifteen genes assigned to pigment synthesis (chlorophyll synthesis pathway) for validation of the most reliable RG (Figure 2B, Table S2). Primer Premier 5.0 software was used to design specific primers for each gene. Primers of the ten potential RG and fifteen genes are presented in Table 1 and Table S3, respectively.

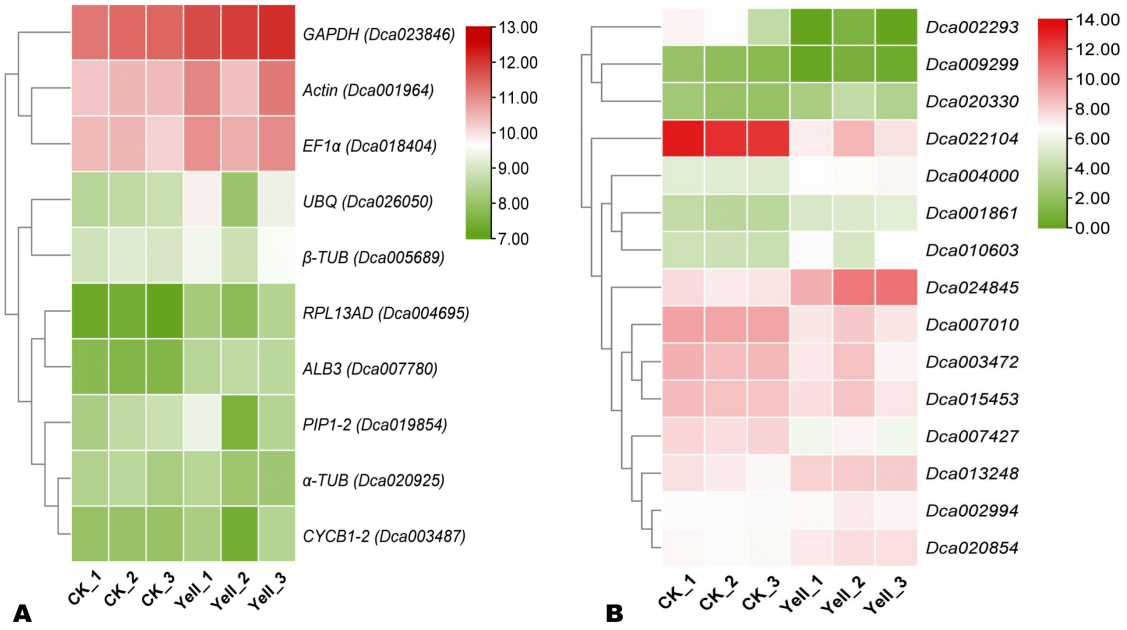

Figure 2. Expression patterns of candidate RGs (**A**) and selected chlorophyll pathway-related genes (**B**) based on Log2FPKM.

Table 1. Primers of the candidate reference genes for qRT-PCR analysis.

No	Genes	Primers (5′-3′)	Product Length (bp)	Tm Value/°C
1	*Actin*	F: CCCTTTATGCTAGTGGTCGAA; R: CCTGAGAATCGCATGTGGTA	110	60
2	*UBQ*	F: GCCGACTACAACATCCAGAA; R: TGATGGTCTTGCCTGTGAG	100	60
3	*GAPDH*	F: ATTTCTTGGGTGACAGCAG; R: TGTCATACCAAGCCACGA	93	60
4	*EF1α*	F: GCTGTGAAGGATCTAAAGCG; R: TGGGAGGTAAAGTTGGCG	83	60
5	*β-TUB*	F: GGCAAGATGAGCACCAAAG; R: GGAATCCACTCCACGAAG	83	60
6	*α-TUB*	F: GAGAGGTTGTCCGTGGACTA; R: CTACTGCTGTGGAAACCTG	82	60
7	*RPL13AD*	F: CGGGCAAAGGTTGCATAC;	82	60

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8	*PIP1-2*	R: CGAGTTTCTCTTCAGCTGTT F: TTGGCGCTGAGATCATCG; R: TGGAACATGAGAGTCCCTG	92	60
9	*ALB3*	F: GTTGCTAGGGTTCGGATGA; R: AAGTAATTCCGCCCAAGTC	82	60
10	*CYCB1-2*	F: TACCAAGATGCCCTTCGC; R: GTCTCCCGCAGATATTCCAG	83	60

2.3. Total RNA Extraction and cDNA Synthesis

Total RNA from all samples was extracted using the RNAprep pure polysaccharide polyphenol plant total RNA extraction kit according to the manufacturer's instructions. The extracted RNA concentration and quality (OD_{260}/ OD_{280} value) were assessed with Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Finally, the integrity of total RNA was confirmed through electrophoresis (1% agarose gel).

The GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA, USA) was used for cDNA construction. The reverse transcription was achieved using Hiscript II QRT super mix according to the manufacturer's instructions for users. The reaction conditions were 15 min at 42 °C and 5 s at 85 °C. Each synthesized cDNA was ten-time diluted (with nuclease-free water)and stored in the refrigerator at - 20 °C.

2.4. qRT-PCR Analysis and Validation of the Most Reliable RG

The qRT-PCR was conducted on Lightcycle®480 type II fluorescent quantitative PCR (Roche, Switzerland) using the quantifast®SYBR®green PCR kit and following instructions by the manufacturer. The PCR reaction was a mixture of cDNA 1 μL; Upstream and downstream primers (10μMol · L^{-1}) 0.2 μL for each; 2× QuantiFast®SYBR®Green PCR Master Mix 5 μL; and Nuclease-free H_2O 3.6 μL. The reaction procedure was as follows: pre-denaturation at 95 °C for 5 min; denaturation at 95 °C for 10 s (40 cycles); and annealing at 60 °C for 30 s. Three technical and biological repetitions were set for each gene. At the end of the cycle, the melting curve was used to detect the product specificity, and each gene's Ct (cycle threshold) values were computed automatically [15,24].

The reliability of the most reliable RG was confirmed through qRT-PCR analysis of the relative expression of the fifteen selected chlorophyll pathway-related genes, followed by expression level calculation via the $2^{-\Delta\Delta Ct}$ method [25]. The internal control was the most stable RG.

2.5. Stability and Statistical Analyses

The Ct values were considered as relative quantities to perform gene expression stability analysis [15,24]. Three commonly used software, including NormFinder [7,9], BestKeeper [26], and geNorm [22]. Finally, we applied the GM (geometric mean) method to fit the results from the three software and generate a comprehensive stability ranking for the candidate RG [27]. Data Processing System, GraphPad Prism v9.0.0121 (GraphPad 159 Software Inc., La Jolla, CA, USA), and Microsoft Excel 2016 were used for data analysis and graphing [27,28]. Statistical differences were obtained via an independent t-test at $P < 0.05$. Finally, we used TBtools software to construct the heatmap of genes [29].

3. Results

3.1. Primers Specificity and Expression Profiles Analyses of Candidate RGs

To uncover a suitable RG for normalizing the relative expression levels of leaf color-related genes in *D. officinale* via qRT-PCR, we selected ten candidate RGs from transcriptome data and subjected them to qRT-PCR analysis. The melting curves showed that the primers of each potential RG were highly specific (Figure 3). All ten genes exhibited a single peak with no primer dimer, and

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all amplicons showed good repeatability, confirming the accuracy, reliability, and specificity of primers.

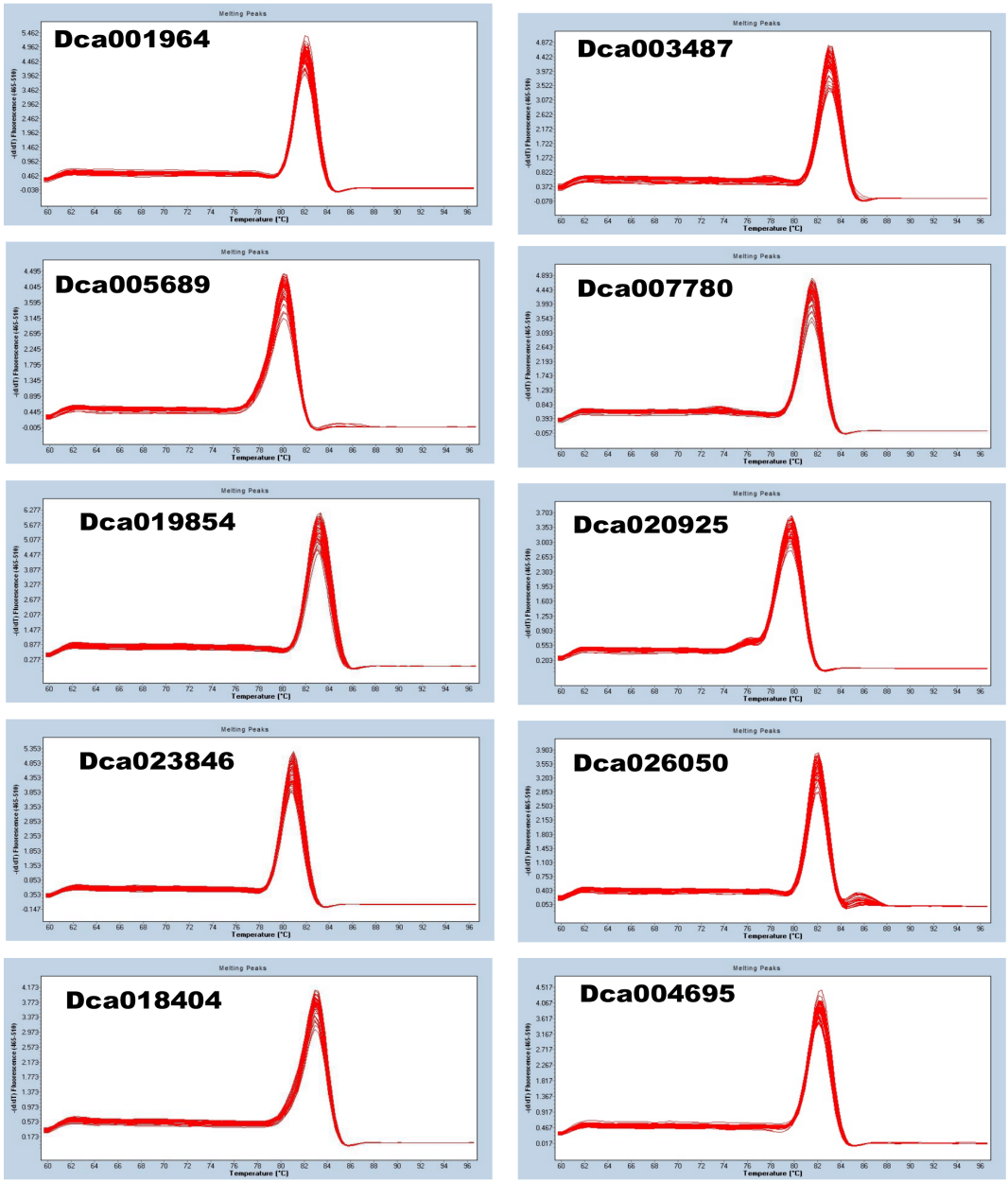

Figure 3. Melting curves of the ten candidate RGs (reference genes).

In order to assess the expression levels of each potential RGs, we automatically computed their respective Ct values. The results showed that the Ct values of the ten genes ranged from 18.858 (*GAPDH*) to 26.915 (*RPL13AD*) on average (Figure 4). The average Ct value of *actin-7*, *UBQ10*, *EF1α*, *β-TUB*, *α-TUB*, *PIP1-2*, *ALB3*, and *CYCB1-2* was 22.241, 21.288, 21.493, 22.678, 23.386, 23.571, 26.069, and 23.230, respectively. *CYCB1-2* exhibited the least expression variation from 22.94 to 23.93, followed by *EF1α* from 20.84 to 22.20 (Figure 4). The summary of the variation in expression levels (Ct values) of all candidate RGs is presented in Table S4.

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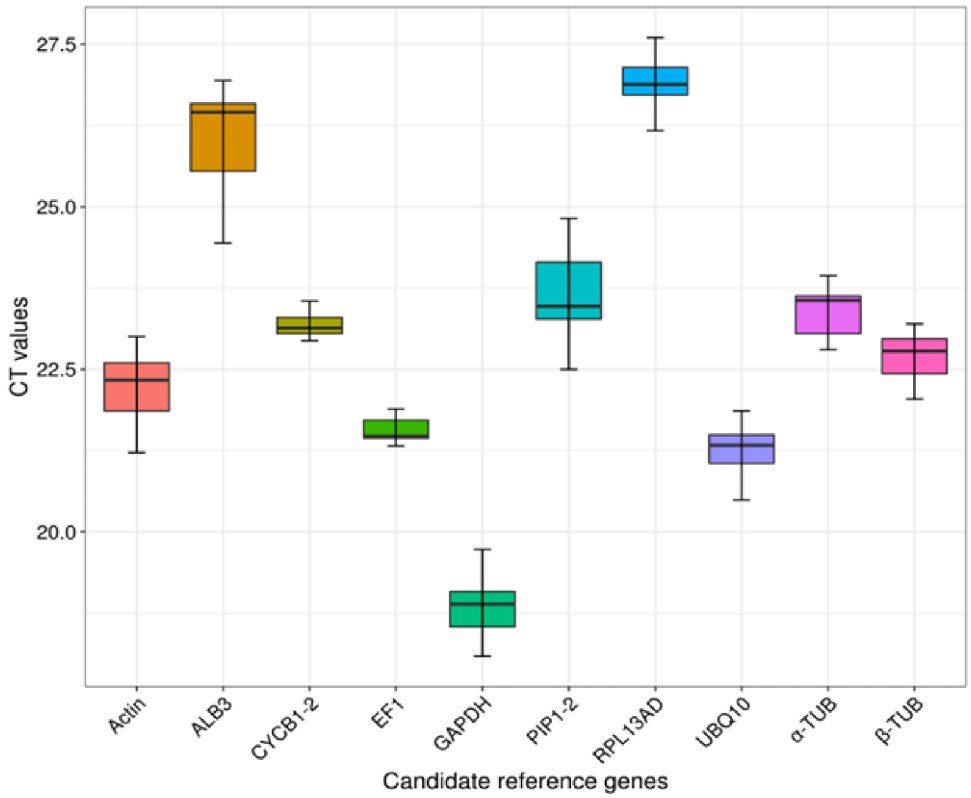

Figure 4. qRT-PCR Ct values for all candidate reference genes in all samples.

3.2. Stability Analysis of Candidate Internal RGs

To determine the stability rankings of the ten potential RGs, three independent software (BestKeeper, GeNorm, and NormFinde) were used, and the results were integrated via the GM method to obtain the comprehensive stability ranking. Firstly, we used geNorm to compute the expression stability measured (M) of each potential RG based on average pairwise expression ratios. A gene can be selected as an internal RG if its M value is below 1.5, and the lower the M value, the more the gene is stable [15]. The analysis by geNorm showed that the M values of all ten genes were > 0.9, indicating they both met the basic requirements for an internal RG (Figure 5a). *EF1α* was the most stable gene, with an M value of 0.395, followed by *CYCB1-2* (M value of 0.439) (Figure 5a). Usually, a single RG is insufficient to achieve high accuracy. Accordingly, sometimes using two or more RGs for precise and reliable normalization is recommended. The optimal number of RGs is determined by pairwise variation value (Vn/n + 1, V value). If Vn/n + 1 ≥ 0.15, the adequate number of RGs equals n + 1. In contrast, if Vn/n + 1 < 0.15, the number is n [14,22]. The V value of all pairwise comparisons was less than 0.15 (Figure 5D), indicating two RGs might be required. Thus, the M values indicate *EF-1α* + *CYCB1-2* is the best combination for accurate normalization of leaf color-related genes' expression levels in *D. officinale*.

NormFinder serves to compute the SV (stability value) of RGs and unveil the optimal number of RGs for precise normalization through analysis of intra- and inter-group variations [15]. The most stable gene is one that exhibits a lower expression level than the average SVs. The stability ranking by NormFinder and GeNorm analyses were somewhat similar, with a little variation in the classification of the top four genes (Figure 5B). Interestingly, *EF1α* (SV = 0.064) occupied the first rank, confirming it was the most stable RG. β-*TUB* (0.115), *RPL13AD* (0.117), and *CYCB1-2* (0.143) ranked second, third, and fourth in terms of stability by NormFinder, respectively (Figure 5B).

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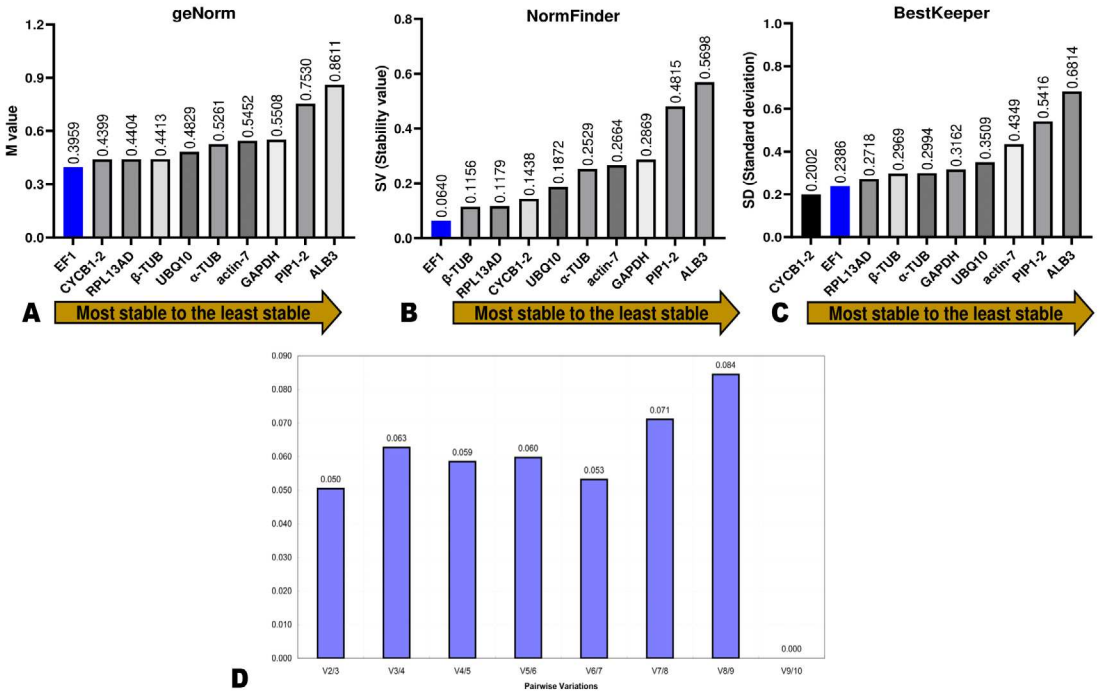

Figure 5. Expression stability of candidate RGs (reference genes) by geNorm analysis (**A**), NormFinder (**B**), and BestKeeper (**C**). (**D**) The pairwise variation values (Vn/Vn + 1, V) of candidate RGs.

BestKeeper helps to evaluate the stability index of RGs by calculating mainly the SD (standard deviation) [26]. A gene is stable if its SD is lower than 1.0 [15,26]. The results by BestKeeper indicated that the SD of all ten genes was lower than 0.7, supporting that they are suitable for use as internal RGs Figure 5C). *CYCB1-2* (SV = 0.2002) and *EF1α* (SV = 0.2386) ranked first and second in terms of stability, respectively (Figure 5C). Both three software revealed that *PIP1-2* and *ALB3* were the least stable genes.

We applied the GM method to integrate the results from the three software and determine the comprehensive stability ranking of the ten RGs. As presented in Table 2, GM values confirmed that *EF1α* was the most suitable and reliable RG, followed by *CYCB1-2*, *RPL13AD*, *β-TUB*, *UBQ10*, *α-TUB*, *GAPDH*, *Actin*, *PIP1-2*, and *ALB3*.

Table 2. The comprehensive ranking of candidate RGs (reference genes) stability by GM method.

Ranking	Reference Genes	Geomean of Ranking Values
1	*EF1α*	1.260
2	*CYCB1-2*	2.000
3	*RPL13AD*	3.000
4	*β-TUB*	3.175
5	*UBQ10*	5.593
6	*α-TUB*	5.646
7	*GAPDH*	7.268
8	*Actin*	7.319
9	*PIP1-2*	9.000
10	*ALB3*	10.000

3.3. Validation of EF1α as the Most Reliable and Stable Internal RG

To confirm the reliability and stability of *EF1α* for accurate normalization of relative expression levels of leaf color-related genes, we investigated the expression of fifteen selected genes from the

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chlorophyll biosynthesis pathway (Figure 2B, Table S2) via qRT-PCR. As shown in Figure 6, the expression patterns of the targeted genes via qRT-PCR and RNA-Seq were consistent with identical regulation patterns.

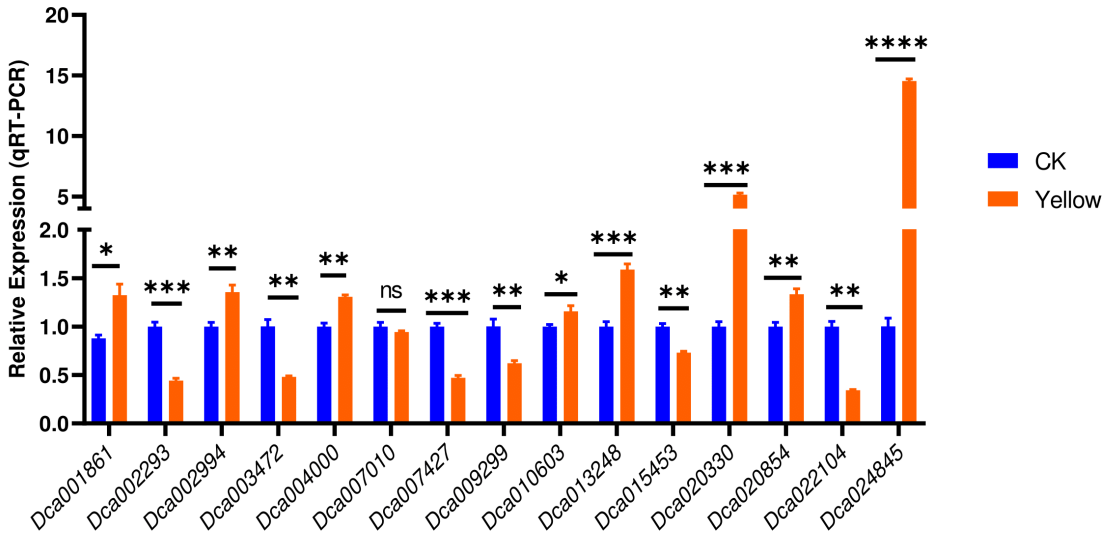

Figure 6. The relative expression levels of fifteen chlorophyll pathway-related genes via normalization with the most suitable RG (reference gene) *EF1α*. *, **, ***, **** indicate statistical differences at $P < 0.05$, 0.01, 0.001, and 0.0001, respectively.

4. Discussion

Leaf color is a critical agronomic trait, and its variation significantly affects global plant metabolism. Principally, leaf color mutations induce less photosynthetic efficiency, causing poor growth, reduced yield, and important economic losses [1,2]. Accordingly, leaf color mutants have become materials of considerable research interest. They are ideal for studying variations in plant metabolism and physiology, especially the molecular mechanisms governing chloroplast biogenesis and differentiation, pigment synthesis and accumulation, photosynthesis, diverse stress response, etc. [1,2]. In *D. officinale*, previous studies have shown that leaf color mutation is associated with a reduction in plant height, stem diameter, and the number of chloroplasts; low chlorophyll content; and altered chloroplast structure [5,6]. In addition, they have provided insight into the underlying molecular mechanisms through comparative transcriptome analysis and unveiled DEGs [5]. However, the identified potential candidate genes have not been validated, and functional characterizations are yet to be conducted due to the unavailability of a reliable RG for qRT-PCR analysis. Thus, the present study took advantage of the RNA-seq data to comprehensively select candidate RGs, examine their stability, and validate the most stable and reliable to promote genomics studies on leaf color mutants in *D. officinale*. This approach has been widely used in several plants to identify suitable RGs for specific traits and experimental conditions [11,15,30–32].

The efficiency of qRT PCR results depends on the reliability of internal RGs. Only a stable expression of internal RG can guarantee the accuracy of the results. Previous studies have demonstrated that internal RGs are species-specific, and their stability varies according to the traits and experimental conditions [11,15,30–32]. For instance, different RGs have been identified for studying traits-related pigment synthesis in different plant species. In *Catalpa fargesii*, *CfMADH* and *CfEF-1* have been identified as the most reliable RGs to be used individually or in combination for normalizing the expression levels of leaf color-related genes [31]. In *Lagerstroemia indica*, *LiEF1α-2* and *LiEF1α-1* were identified as the most suitable for analyzing the expression levels of leaf color-related genes [33]. In wheat, 18S rRNA is used as the internal RG to calibrate the expression of leaf color-related candidate genes [17]. In *Lilium regale*, *LrActin2* was identified as the best RG for normalizing photosynthesis-related candidate genes' expression levels [16]. Herein, we investigated ten genes,

including *EF1α*, *CYCB1-2*, *RPL13AD*, *β-TUB*, *UBQ10*, *α-TUB*, *GAPDH*, *Actin*, *PIP1-2*, and *ALB3*. Expression stability analysis via GeNorm, NormFinder, and BestKeeper revealed that these ten genes could be used as internal RGs to calibrate leaf color-related genes' expression levels in *D. officinale*. *EF1α* exhibited the highest stability among them, indicating it is the most suitable RG. Using this RG may promote the molecular dissection of the regulatory network of leaf color mutations in *D. officinale*.

To verify the suitability and reliability of *EF1α*, we analyzed fifteen chlorophyll pathway-related DEGs' expression levels via qRT qPCR. The expression patterns of the targeted genes via *EF1α* normalization were consistent with the transcriptome sequencing results. This result further confirms the accuracy of using *EF1α* as the primary RG for qRT-PCR calibration of leaf color-related genes' expression levels in *D. officinale.* Furthermore, of the fifteen analyzed genes, *Dca024845* and *Dca020330* were the most significantly up-regulated in the yellow leaf mutant genotype. Accordingly, these genes may represent key candidate genes for deciphering chlorophyll synthesis-related molecular mechanisms in *D. offinale*. Functional characterization of these genes is required to reveal their roles.

5. Conclusions

In summary, this study investigates ten RGs for qRT-PCR normalization of expression levels of leaf color-related candidate genes in *D. officinale.* Of them, *EF1α* was the most reliable RG, based on stability rankings analysis and confirmatory through the accurate calibration of the expression levels of fifteen chlorophyll pathway-related DEGs. *Dca024845* and *Dca020330* were the most significantly up-regulated genes in the yellow leaf mutant genotype. Therefore, they should be subjected to functional genomics studies to deepen our understanding of the molecular mechanisms involved in leaf color mutations. Our results will enable the identification of candidate genes and functional genomics studies related to leaf color in *D. officinale*. Furthermore, they may provide comprehensive guidelines for uncovering suitable RGs in other plant species.

Supplementary Materials: The following supporting information can be downloaded at: Preprints.org, Table S1: List of the ten candidate reference genes with their FPKM expression values; Table S2: List of the fifteen chlorophyll pathway-related genes with their FPKM expression values; Table S3: Primers of the fifteen chlorophyll pathway-related genes for qRT-PCR analysis; Table S4: Summary of the qRT-PCR Ct value of ten candidate genes.

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