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Screening of Reference Genes for RT-qPCR in *Euphorbia maculata* Postprint

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Abstract

One of the prerequisites for real-time quantitative PCR (RT-qPCR) is having suitable reference genes. To screen suitable RT-qPCR reference genes for different tissues at different growth stages of *Euphorbia maculata* L., gene fragments of GAPDH, EF-1α, act, UBQ, TUB-α, eIF-4A, and CYP were cloned from *E. maculata* using homology-based cloning. RT-qPCR was used to detect the expression of seven candidate reference genes in roots, stems, leaves, and fruits of *E. maculata* at different growth stages, and software tools such as geNorm, NormFinder, and BestKeeper were used to evaluate the expression stability of each candidate gene. The results showed that the cloned gene fragments of GAPDH, EF-1α, act, UBQ, TUB-α, eIF-4A, and CYP were 729, 808, 753, 422, 233, 656, and 313 bp, encoding 242, 269, 250, 140, 77, 218, and 103 amino acids, respectively, with the highest homology to corresponding amino acid sequences from other plants being above 85%. Based on comprehensive evaluation of expression stability in different tissues at each growth stage by the three software programs, the ranking of expression stability was UBQ>EF-1α>TUB-α>eIF-4A>GAPDH>CYP>act. Therefore, UBQ can be selected as the reference gene for RT-qPCR analysis in *E. maculata* for studying tissue-specific gene expression at different growth stages.

Full Text

Screening of Reference Genes for RT-qPCR in *Euphorbia maculata*

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Abstract

The availability of suitable reference genes is a prerequisite for reliable real-time quantitative PCR (RT-qPCR) analysis. To identify appropriate reference genes for RT-qPCR in different tissues of *Euphorbia maculata* L. across various growth stages, we cloned partial sequences of seven candidate reference genes—GAPDH, EF-1α, actin, UBQ, TUB-α, eIF-4A, and CYP—using homologous cloning. RT-qPCR was then employed to examine the expression patterns of these genes in roots, stems, leaves, and fruits at different developmental phases. Expression stability was evaluated using geNorm, NormFinder, and BestKeeper software. The cloned fragments spanned 729, 808, 753, 422, 233, 656, and 313 bp, encoding 242, 269, 250, 140, 77, 218, and 103 amino acids, respectively. All deduced amino acid sequences exhibited greater than 85% identity with corresponding sequences from other plant species. Comprehensive analysis across the three software platforms revealed the following stability ranking: UBQ > EF-1α > TUB-α > eIF-4A > GAPDH > CYP > actin. Therefore, UBQ can be selected as a reference gene for RT-qPCR normalization in *E. maculata* during studies of tissue-specific gene expression across different growth stages.

Keywords: *Euphorbia maculata*; gene cloning; reference genes; RT-qPCR

Introduction

Euphorbia maculata and *Euphorbia humifusa* are annual herbs belonging to the Euphorbiaceae family, commonly known as "dijincao" in traditional Chinese medicine. Distributed widely across Jiangsu, Jiangxi, Zhejiang, Hubei, Henan, Hebei, Xinjiang, and Inner Mongolia, this medicinal material is used in Traditional Chinese Medicine, Uyghur Medicine, and Mongolian Medicine for clearing heat, detoxifying, cooling blood, stopping bleeding, and promoting diuresis (Delectis Florae Reipublicae Popularis Sinicae Agendae Academiae Sinicae Edita, 1997; Chinese Pharmacopoeia Commission, 2015). The plant contains flavonoids, terpenoids, phenolic acids, and alkaloids, exhibiting antioxidant, anti-inflammatory, antibacterial, hemostatic, and immunomodulatory activities (Liu et al., 2001; An et al., 2008). Current research has primarily focused on quality control, pharmacological effects, and chemical constituent identification (Tian et al., 2019; Hu et al., 2018), with limited molecular biology studies reported to date.

RT-qPCR is a powerful technique for gene expression analysis that incorporates fluorescent reporters to monitor amplification in real time, offering high

specificity, sensitivity, and reproducibility (Wang et al., 2019; Zhang et al., 2014). However, obtaining reliable quantitative results requires appropriate reference genes for normalization (Shakeel et al., 2018). Variations in RNA extraction, cDNA synthesis, and PCR amplification efficiency can introduce significant errors, which are typically corrected using stably expressed reference genes (Bustin, 2002). Recent studies demonstrate that no universal reference gene exists; using an unvalidated gene under arbitrary conditions can compromise accuracy or produce erroneous results (Zhu et al., 2019). Moreover, reliance on a single traditional reference gene may affect precision, prompting the adoption of novel reference genes or gene combinations to minimize expression errors (Nguyen et al., 2018; Yuan et al., 2012). Transcriptome databases and microarray data also facilitate identification of reliable reference genes (Liang et al., 2018). Consequently, screening and evaluating candidate reference genes prior to target gene analysis is essential, with geNorm, NormFinder, and BestKeeper being among the most widely used evaluation tools (Kiarash et al., 2018; Zhong et al., 2019).

Commonly used reference genes include actin (act), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), EF-1α (elongation factor-1 alpha), UBQ (ubiquitin), TUB-α (tubulin alpha), eIF-4A (eukaryotic translation initiation factor 4A), CYP (cyclophilin), and 18S rRNA (Haller et al., 2004; Kozera & Rapacz, 2013). This study cloned partial sequences of seven candidate reference genes from *E. maculata*—GAPDH, EF-1α, actin, UBQ, TUB-α, eIF-4A, and CYP—and examined their expression in roots, stems, leaves, and fruits at the seedling, flowering, and fruiting stages using RT-qPCR. Expression stability was assessed using geNorm, NormFinder, and BestKeeper to establish a foundation for tissue-specific gene expression studies in *E. maculata* across different developmental stages.

Materials and Methods

1.1 Plant Materials

Euphorbia maculata seeds were kindly provided by Jiangxi Tianshikang Ecological Chinese Herbal Medicine Planting Co., Ltd. and cultivated in the experimental field of Fuzhou Medical College, Nanchang University. Plant material was authenticated by the Department of Traditional Chinese Medicine at Jiangxi College of Traditional Chinese Medicine. Samples were collected at three developmental stages: seedling stage (70 days post-planting, pre-flowering), flowering stage (with more than three open flowers and no fruits), and fruiting stage (with more than three fruits). For each stage, roots, stems, and leaves were harvested; fruits were additionally collected during the fruiting stage. All samples comprised pooled tissues from at least three individual plants and were immediately stored at -80°C until use.

1.2 Reagents and Instruments

Total RNA extraction and gel recovery kits (Spin Column Plant Total RNA Purification Kit and SanPrep Column DNA Gel Extraction Kit), primer synthesis, and sequencing services were obtained from Sangon Biotech (Shanghai). Reverse transcription and qPCR reagents (PrimeScript™ RT Reagent Kit with gDNA Eraser and PrimeScript™ RT Master Mix) were purchased from TaKaRa (Dalian). Pfu polymerase (TransStart® FastPfu DNA Polymerase), Taq polymerase, and T-vector (pEASY®-Blunt Simple Cloning Kit) were from TransGen Biotech (Beijing). PCR amplification was performed using a T100™ Thermal Cycler and CFX96 Real-Time PCR System (Bio-Rad, USA). RNA concentration and quality were assessed with a NanoDrop One spectrophotometer (Thermo Scientific, USA).

1.3 Experimental Procedures

1.3.1 Total RNA Extraction and cDNA Synthesis Total RNA was extracted from roots, stems, leaves, and fruits at different growth stages using the Spin Column Plant Total RNA Purification Kit according to the manufacturer' s instructions. RNA integrity was verified by agarose gel electrophoresis, and concentration was determined using a NanoDrop One spectrophotometer to ensure suitability for downstream applications.

1.3.2 Primer Design Conserved sequences of GAPDH, EF-1α, actin, UBQ, TUB-α, eIF-4A, and CYP were identified in GenBank, particularly from related Euphorbiaceae species including *Ricinus communis*, *Jatropha curcas*, and *Hevea brasiliensis*. Degenerate primers were designed based on these conserved regions for initial cloning. Following sequencing of the cloned fragments, RT-qPCR primers were designed using Primer Premier 5.0. All primers used in this study are listed in Table 1 .

1.3.3 Cloning and Analysis of Candidate Reference Gene Fragments PCR amplification was performed in a 25 µL reaction containing 5 µL 5×GC Buffer, 2.5 µL dNTPs (2.5 mmol $\cdot$ L^{-1} each), 1 µL each of forward and reverse degenerate primers (10 µmol$\cdot$L^{-1}), 1 µL cDNA template, 0.3 µL Pfu polymerase (5 U$\cdot$µL^{-1}), and 14.2 µL sterile ddH$_2$O. Cycling conditions were: 94°C for 5 min; 30 cycles of 94°C for 30 s, annealing at the appropriate temperature for 30 s, and 72°C for 60 s; followed by 72°C for 5 min. PCR products were separated on 1.5% agarose gels, and target fragments were purified using the gel extraction kit. Purified DNA was ligated into the pEASY®-Blunt Simple Cloning Kit T-vector and transformed into *E. coli* DH5α. Positive clones were identified by colony PCR and sequenced. Resulting sequences were subjected to BLAST analysis in GenBank.

1.3.4 RT-qPCR Primer Validation and Melting Curve Analysis To verify primer specificity and exclude interference from primer dimers or non-

specific amplification, conventional PCR was performed using qF/qR primers, followed by RT-qPCR and melting curve analysis. For melting curve analysis, RT-qPCR products were heated from 65°C to 95°C, holding at 0.5°C increments for 5 s while monitoring fluorescence. RT-qPCR reactions (20 µL) contained 10 µL 2×SYBR Green qPCR Master Mix, 0.4 µL each of forward and reverse primers (10 µmol·L^{-1}), 2 µL cDNA template, and 7.2 µL sterile ddH_2O. Cycling parameters were: 95°C for 5 min; 40 cycles of 95°C for 30 s, annealing at the appropriate temperature for 30 s, and 72°C for 30 s.

1.3.5 Screening of Candidate Reference Genes cDNA synthesized from total RNA of each sample served as template for RT-qPCR amplification. Expression stability of the seven candidate reference genes was evaluated using geNorm, NormFinder, and BestKeeper. Ct values were converted to relative expression quantities using the formula Q = 2^(Ct_{min} - Ct_{sample}) for geNorm and NormFinder analysis (Wu et al., 2017), while Ct values were directly input into BestKeeper. A comprehensive ranking was generated to identify the most suitable reference gene for tissue-specific expression studies in *E. maculata* across different growth stages.

Results

2.1 Cloning and Analysis of Candidate Reference Gene Fragments

Using cDNA synthesized from total RNA as template and corresponding degenerate primers, PCR amplification yielded products of expected sizes (Figure 1 [Figure 1: see original paper]). Sequencing confirmed that the cloned fragments of GAPDH, EF-1α, actin, UBQ, TUB-α, eIF-4A, and CYP measured 729, 808, 753, 422, 233, 656, and 313 bp, encoding 242, 269, 250, 140, 77, 218, and 103 amino acids, respectively. BLASTp analysis in GenBank revealed highest homologies of 95%, 99%, 100%, 99%, 99%, 87%, and 86% with corresponding sequences from lettuce GAPDH (XP023733724), castor bean EF-1α (XP002518073), litchi actin (ADV17460), *Actinidia rufa* UBQ (GFZ13847.1), barley TUB-α (CAB76917.1), maize eIF-4A (U17979.1), and tung tree CYP (ARV78452.1). The nucleotide sequences were deposited in GenBank under accession numbers EmGAPDH (MT044466), EmEF-1α (MT044465), Emactin (MT044464), EmUBQ (MW815120), EmeIF-4A (MW815119), EmTUB-α (MW815118), and EmCYP (MW815117).

2.2 RT-qPCR Primer Validation and Melting Curve Analysis

Conventional PCR using qF/qR primers produced amplicons of expected lengths (Figure 2 [Figure 2: see original paper]). Melting curve analysis of RT-qPCR products revealed single peaks for all genes (Figure 3 [Figure 3: see original paper]), confirming the absence of primer dimers and non-specific amplification.

These results demonstrate that the designed primers are suitable for subsequent RT-qPCR analysis.

2.3 Ct Value Analysis of Candidate Reference Genes

RT-qPCR amplification of cDNA from various tissues (roots, stems, leaves, and fruits) at different growth stages yielded Ct values that inversely correlate with expression level. The expression abundance ranking was: EF-1α > TUB-α > eIF-4A > UBQ > CYP > GAPDH > actin, with Ct ranges of 17.04–19.55, 18.52–21.81, 18.58–22.04, 20.19–22.90, 20.63–24.99, 21.85–24.95, and 24.44–29.26, respectively (Figure 4 [Figure 4: see original paper]). Technical replicates showed minimal variation, though expression levels were not constant across all samples.

2.4 Expression Stability Analysis of Candidate Reference Genes

2.4.1 geNorm Analysis geNorm calculates stability values (M) where lower M values indicate greater stability. All candidate genes except actin exhibited M values below 1.5 across different tissues, with stability ranking: UBQ > TUB-α > EF-1α > eIF-4A > GAPDH > CYP > actin (Table 2).

2.4.2 NormFinder Analysis Similar to geNorm, NormFinder assesses stability based on variance-derived M values. Analysis revealed differential stability among the seven genes, with ranking: UBQ > TUB-α > EF-1α > eIF-4A > GAPDH > CYP > actin (Table 3). UBQ displayed the lowest M value, indicating highest overall stability.

2.4.3 BestKeeper Analysis BestKeeper evaluates stability using standard deviation (SD) and coefficient of variation (CV) calculated directly from Ct values, where stable genes exhibit lower SD and CV values. The resulting ranking was: EF-1α > UBQ > eIF-4A > GAPDH > TUB-α > CYP > actin (Table 4). EF-1α, UBQ, and eIF-4A showed comparable SD and CV values, indicating relatively stable expression.

2.4.4 Comprehensive Analysis Given the slight variations in rankings across the three software platforms, geometric mean values were calculated for comprehensive evaluation. Lower geometric mean values correspond to higher stability. The final integrated ranking across different tissues and growth stages was: UBQ > EF-1α > TUB-α > eIF-4A > GAPDH > CYP > actin (Table 5).

Discussion and Conclusion

Euphorbia maculata is a commonly used medicinal herb in multiple traditional medicine systems, containing flavonoids, terpenoids, phenolic acids, and alka-

loids, with quercetin content serving as a quality standard. Despite its pharmacological importance, molecular biology research on this species remains limited. This study marks the first cloning of seven traditional reference gene fragments from *E. maculata* and their evaluation as candidate normalization genes across three developmental stages (seedling, flowering, and fruiting) in roots, stems, leaves, and fruits using RT-qPCR.

All candidate genes except actin showed relatively high expression abundance, with Ct values below 25, meeting the requirements for reliable quantification. As different algorithms employed by geNorm, NormFinder, and BestKeeper often yield divergent results, comprehensive analysis is necessary to identify optimal reference genes (Kiarash et al., 2018; Zhong et al., 2019). geNorm and NormFinder produced consistent results, with UBQ, TUB-α, EF-1α, eIF-4A, and GAPDH showing M values <1, indicating stable expression, with UBQ ranked as the best single reference gene. BestKeeper analysis differed slightly, identifying EF-1α as the top-ranked gene, though UBQ and eIF-4A also demonstrated stable expression with SD values <1, showing no significant difference from EF-1α.

UBQ encodes ubiquitin, a protein involved in protein regulation and cellular metabolism that serves as a commonly used reference gene in many plants, such as in studies of gene expression during petal development in herbaceous peony (*Paeonia lactiflora*) (Li, 2017). EF-1α, the alpha subunit of elongation factor-1, participates in transcriptional control, apoptosis, and signal transduction in eukaryotes and represents another widely used reference gene that shows stable expression across different tissues in *Hippeastrum rutilum* (Liu et al., 2018). GAPDH, actin, TUB-α, eIF-4A, and CYP were not identified as suitable reference genes for *E. maculata* across different tissues and growth stages, though they may be stably expressed in other plant species, underscoring the necessity of reference gene validation for specific experimental conditions (Kozera & Rapacz, 2013).

Therefore, UBQ is recommended as a reference gene for studying tissue-specific gene expression in *E. maculata* across different growth stages. For applications requiring reference gene combinations, UBQ and EF-1α represent an appropriate pair. These findings provide a valuable foundation for future molecular biology research on *E. maculata*. As gene discovery and expression studies in this species advance, additional reference genes with even greater stability may be identified.

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