Research Article

Reference Gene Selection for Quantitative Real-Time RT-PCR Normalization in Iris. lactea var. chinensis Roots under Cadmium, Lead, and Salt Stress Conditions

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Quantitative real time PCR (RT-qPCR) has emerged as an accurate and sensitive method to measure the gene expression. However, obtaining reliable result depends on the selection of reference genes which normalize differences among samples. In this study, we assessed the expression stability of seven reference genes, namely, ubiquitin-protein ligase UBC9 (UBC), tubulin alpha-5 (TUBLIN), eukaryotic translation initiation factor (EIF-5A), translation elongation factor EF1α, translation elongation factor EF1B (EF1b), actin11 (ACTIN), and histone H3 (HIS), in Iris. lactea var. chinensis (I. lactea var. chinensis) root when the plants were subjected to cadmium (Cd), lead (Pb), and salt stress conditions. All seven reference genes showed a relatively wide range of threshold cycles (Ct) values in different samples. GeNorm and NormFinder algorithms were used to assess the suitable reference genes. The results from the two software units showed that EIF-5A and UBC were the most stable reference genes across all of the tested samples, while TUBLIN was unsuitable as internal controls. I. lactea var. chinensis is tolerant to Cd, Pb, and salt. Our results will benefit future research on gene expression in response to the three abiotic stresses.

1. Introduction

Quantitative real time PCR (RT-qPCR) is a powerful technique to evaluate the quantification of target gene expression. It has advantages of high sensitivity, outstanding accuracy, and broad dynamic range compared with Northern blotting and reverse transcription PCR (RT-PCR) [1]. Nevertheless, it is necessary to use reliable reference gene(s) to normalize the relative expression of target genes. However, the expression of reference gene(s) was not stable under many conditions, which may lead to erroneous normalization [2–4]. As far as is known, many studies have been carried out to select stable reference genes in plants [5, 6].

Cd and Pb are two important heavy metal pollutants which have high toxicity to living beings [7, 8], and salinity is one of the major abiotic stresses which limit the yield of major crops [9]. A number of attempts for reference gene validation have been reported under heavy metal stress in Arabidopsis thaliana [10], soybean [11], cucumber [12], citrus [2], and poplar [13] and under salt stress in potato [4], rice [14], tobacco [15], cucumber [16], and Brachypodium distachyon [17]. I. lactea var. chinensis is a perennial ornamental plant, having potential application in phytoremediation of Cd and Pb [7, 18]. Moreover it is a promising halophyte for the improvement of saline land [9]. To further elucidate the excellent characteristic, more studies are needed to analyze the expression of functional genes and transcription factors under these three abiotic stresses. However, previously studies showed that no single reference gene can be used under various experiment stresses [4, 19, 20]. Thus, it is necessary to identify a set of stable reference genes in I. lactea var. chinensis under these three stress conditions.
Table 1: Description of *Iris lactea* var. *chinensis* reference genes for RT-qPCR.

| Gene  | NCBI accession number | Arabidopsis ortholog locus | Arabidopsis locus description                        |
|-------|-----------------------|----------------------------|-----------------------------------------------------|
| UBC   | EX953716              | AT4g27960                  | Ubiquitin-protein ligase UBC9                       |
| TUBLIN| EX954248              | AT5G19780                  | Tubulin alpha-5                                      |
| EIF-5A| EX954588              | AT1G69410                  | Eukaryotic translation initiation factor            |
| EF1α  | EX950257              | AT5G60390                  | Translation elongation factor EF1A                  |
| EF1b  | AB907790              | AT2G18110                  | Translation elongation factor EF1B                  |
| ACTIN | EX952640              | AT3G12110                  | ACT11                                               |
| HIS   | FD387291              | AT4G04040                  | Histone H3                                           |

*a* All genes were named on the basis of similarity to Arabidopsis proteins determined via BLASTX.

*b* Closest Arabidopsis homolog identified using TAIRBLAST (http://www.arabidopsis.org/Blast/index.jsp).

Table 2: Primer sequences and amplicon characteristics for each of the seven reference genes.

| Name | Primer sequence (forward/reverse primer) | Size (bp) | T<sub>m</sub> (°C) | E (%) | R<sup>2</sup> |
|------|----------------------------------------|-----------|----------------|-------|-------------|
| UBC  | 5'-TCTCGGCTTTCGGGTTTGTG-3' 5'-ACCTTGGGTTCGGTGAATGG-3' | 224 | 88.0 | 1.965 | 0.9993 |
| TUBLIN | 5'-TTACCGTCACTATTCCACCA-3' 5'-CAGCCAAGAACCCAAAACAGAT-3' | 214 | 87 | 1.905 | 0.9991 |
| EIF-5A | 5'-GGATGAGGAGCACCTTCG-3' 5'-GGCGGTTCTTGATGACGATG-3' | 110 | 90.5 | 1.912 | 0.9997 |
| EF1α | 5'-CCATTTCTGGATTGAGGGTG-3' 5'-AGTCGAAGAGGCTTGTCGGTAG-3' | 133 | 86.5 | 2.016 | 0.9958 |
| EF1b | 5'-ATCTTCTGACGGAGGTATCC-3' 5'-TACCTCTGACCCAGGATAC-3' | 115 | 83.3 | 2.006 | 0.9997 |
| ACTIN | 5'-CTCAACCCGAAGGCAACAGAG-3' 5'-CGAAGGTCCAGGAGGAATA-3' | 216 | 87.0 | 1.966 | 0.9994 |
| HIS  | 5'-GGGCTCGTACCAAGGGAAG-3' 5'-TTCCAGACGGTAAAGCAGTA-3' | 134 | 89.4 | 1.929 | 0.9993 |

In this study, we used RT-qPCR to examine expression variations of seven candidate reference genes. Then, we compared their stabilities across a large set of *I. lactea* var. *chinensis* samples representing Cd, Pb, and salt stress treatments using GeNorm and NormFinder software units. This work will benefit future gene expression analysis in *I. lactea* var. *chinensis*.

2. Materials and Methods

2.1. Plant Materials and Treatments. 10 cm height *I. lactea* var. *chinensis* plants grown in the 1/2 Hoagland nutrient solution at *Iris* Resource Collection Garden of Institute of Botany, Nanjing Sun Yat-Sen Memorial Botanical Garden, were selected and transferred into 500 mL plastic pots for hydroponic cultivation [7]. After two weeks, the uniform and healthy seedings were used to examine the gene expression patterns under different treatments. For Cd, Pb, or salt treatment, the plants were transferred to pots containing nutrient solution added with 80 mg/L CdCl<sub>2</sub> [18], or 10 mM Pb(NO<sub>3</sub>)<sub>2</sub> [7], or 100 mM NaCl stress [9], kept in the same growth pots for designated time (0, 1, 3, 6, 12, and 24 h). The roots were harvested after three treatments. After harvesting, the roots were frozen immediately in liquid nitrogen and stored at -80°C until use for RNA extraction.

2.2. RNA Extraction and cDNA Synthesis. Frozen roots were ground in liquid nitrogen using a mortar and a pestle. Total RNA was extracted using the RNAiso reagent (TaKaRa) according to the manufacturer's instructions. Potentially contaminating DNA was eliminated from total RNA with RNase-free DNaseI (TaKaRa). Only RNA samples with an optical density absorption ratio A260/A280 of 1.8–2.0 and an A260/A230 ratio > 2.0 were used for subsequent analysis [21]. RNA purity was assessed on a BioPhotometer D30 (Eppendorf) [22]. First-strand cDNA was synthesized with the M-MLV (RNase H<sup>-</sup>) (TaKaRa, Japan) and oligo-dT primers.

2.3. Primer Design. The first important step in RT-qPCR reference gene selection is to select an initial set of candidate reference genes. Seven genes that were commonly used as stable reference genes in abiotic stresses were chosen [2, 19, 23] (Table 1). Primers were designed using Primer Premier v5.0 software (Premier Biosoft International) with melting temperatures (T<sub>m</sub>) of 83.3–90.5°C, primer lengths of 20–22 bp, and amplicon lengths of approximately 110–224 bp.
ranges for total samples. Median, squares represent the means, and whiskers indicate the genes. Boxes indicate the 25th/75th percentiles, the line marks the EF1b, ACTIN show in Table 2, the correlation coefficients (peak in melt curve indicated the expected amplicons. As candidates. A single band in gel electrophoresis and a single by qPCR. The expected size of the primer amplicons was further verified by agarase gel electrophoresis. Amplicon purity was assumed where a single melting peak was produced.

2.4. RT-qPCR. The RT-qPCR reactions were run on a Mastercycler ep realplex real-time PCR system (Eppendorf, http://www.eppendorf.com/) with SYBR Premix Ex Taq II (Perfect Real Time) (TAKARA). Each reaction was performed in 20 μL mix containing 50 ng of each cDNA, 200 nM of each primer, and 10 μL SYBR Premix. The following amplification program was used: initial denaturation 95°C for 120 s, then 40 cycles of 95°C at 15 seconds, 55°C at 15 seconds, and 72°C at 20 seconds. Melting curves were recorded after cycle 40 by heating from 60 to 95°C at a rate of 0.5°C s⁻¹. Each RT-qPCR was run in triplicate, and mean Ct values were calculated. Reverse transcription negative control was also included for each primer pair.

2.5. Statistical Analysis. PCR efficiency was calculated from amplification plots using the LinRegPCR program [24]. The quantification cycle values were converted into relative quantities via the delta-Cq method [25]. Two statistical approaches were used to determine the stability of the candidate samples: GeNorm software [26] and NormFinder software[27].

3. Results

3.1. Performance of the Primers and Ct Value Analysis. A total of seven genes, including UBC, TUBLIN, EIF-5A, EFla, EIFb, ACTIN, and HIS, were selected as reference gene candidates. A single band in gel electrophoresis and a single peak in melt curve indicated the expected amplicons. As shown in Table 2, the correlation coefficients (R²) ranged in value between 0.9958 and 0.9997, and PCR amplification efficiencies between 1.905 and 2.016. The two results were from the LinRegPCR program [24].

In our study, Ct values of the seven reference genes showed a relatively wide range from 18.01 to 28.67 in tested samples (Figure 1). The least abundant transcripts were HIS and EF1b with Ct values of 28.67 and 27.46, respectively. However, EIF-5A presented the highest transcriptional level and the lowest Ct value of 18.01. The average Ct value of the selected genes was about 23.82. The coefficient of variation of EFla was smallest (6.24), while the coefficient of variation of TUBLIN was the largest (7.64).

3.2. The Stability of Reference Genes. In our study, two methods were selected to analyze the stability of seven reference genes. GeNorm calculates M (average expression stability) for the identification of the most suitable reference gene(s) and V (average pairwise variation) to define the optimal number of genes that should be used. On the basis of M, a lower Μ value indicates more stable genes. Genes which had M values more than 1.5 indicated the need for additional reference gene(s) [22]. The ranking order according to the M value was showed in Figure 2. The M values for all genes were below 1.5. For total samples, UBC and HIS were the most stably expressed genes with an M value of 0.524. ACTIN and TUBLIN were the least stable genes (Figure 2(a)). In different samples across Cd treatment, UBC and EF1b performed well with an M value of 0.149, while EFla and TUBLIN have relative high M value (Figure 2(b)). For NaCl treatment, UBC and HIS were the most highly ranked with an M value of 0.278. EFla and TUBLIN were the least stable genes like under NaCl treatment (Figure 2(c)). For Pb treatment, UBC and HIS showed the lowest M value of 0.506 and ACTIN was the highest with an M value of 0.894 (Figure 2(d)). Vandesompele defined V (the pairwise variation Vrst/Vn+1) to choose the optimal number of reference genes [26]. As showed in Figure 3, three groups of samples, that is, total samples, Cd stress treatment samples, and Pb stress treatment samples, showed higher V2/3 value more than 0.15 (Figure 3). Thus, three reference genes in Cd stress and four reference genes in total and Pb stress were necessary to obtain accurate results in gene expression normalization.

GeNorm and NormFinder were developed based on a different strategy. Each one has its own advantages and disadvantages [19]. To further confirm the result obtained by the GeNorm software, we further analyzed using NormFinder software, an algorithm which depends on a statistical and mathematical model that estimates the overall expression variation of a set of candidates to identify the optimal normalization gene [21, 27]. Results indicated that the most unstable gene in total, Cd stress, NaCl stress, and Pb stress, was consistent with the GeNorm analysis (Table 3). EIF-5A ranked as the most stable gene in total (stability value = 0.295) and Cd stress (stability value = 0.084). His was optimal with a stability value of 0.194 the NaCl treatment. During the Pb stress, UBC ranked in the top in NormFinder analysis.

4. Discussion

Many studies have been performed to evaluate and select reliable reference genes in a number of plants, such as in cotton.
subjected to salt and drought stress [19], in *Lycium barbarum* L. under different development stages [28], in *chrysanthemum* during aphid infestation, heat stress, or waterlogging stress [22], in spathe tissue of *Anthurium andraeanum* [29], and in *Coffea arabica* during nitrogen starvation, salt, and heat stress [30]. Here, we have found that seven candidate reference genes performed differently upon three stresses to which *I. lactea* var. *chinensis* plants were subjected.

Two commonly used algorithms (GeNorm and NormFinder) were used to evaluate and identify reference genes. The GeNorm analysis may be biased by the coregulation, since they will show a lower level of pairwise variation than independently regulated genes and occupy closed positions in the ranking coregulated genes [15,26]. However, NormFinder could be more effective in avoiding behavior of gene coregulation because it ranks reference genes according to the intra- and intergroup variation [31, 32]. Our study showed that different gene stability ranking orders were generated by two analysis algorithms. In previous studies, different conclusions were also generated by the two methods such as in *Cineraria* [33], *C. lavandulifolium* [34], radish [32], cucumber [16], and flax [35]. Based on our study, *EIF-5A* and *UBC* exhibited stable expression patterns for accurate normalization when looking at the expression data in all four series. On the other hand, *TUBLIN* performed poorly which indicated that it was not consistently expressed and should not be used as reference genes in our experimental setups. In addition, GeNorm results showed that the choice of the reference gene number depends on the experiment conditions (Figure 3).
**Table 3**: Ranking of seven reference genes in order of their expression stability calculated by NormFinder.

| Ranking order | Gene | Total Stability | Cad Stability | NaCl Stability | Gene | Pb Stability |
|---------------|------|----------------|--------------|---------------|------|-------------|
| 1             | EIF-5A | 0.295       | 0.084       | 0.394       | UBC  | 0.263       |
| 2             | UBC   | 0.308       | 0.296       | 0.225       | EIF-5A | 0.332       |
| 3             | EIFα  | 0.374       | 0.310       | 0.308       | EIFβ  | 0.364       |
| 4             | EIFβ  | 0.379       | 0.366       | 0.342       | HIS   | 0.396       |
| 5             | ACTIN | 0.448       | 0.367       | 0.367       | EIFβ  | 0.446       |
| 6             | HIS   | 0.450       | 0.442       | 0.371       | TUBLIN | 0.599       |
| 7             | TUBLIN | 0.613      | 0.480       | 0.411       | ACTIN | 0.643       |

**Conflict of Interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.

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*EIF-5A* and *UBC* were abundantly and constantly transcribed in all of the samples. Indeed, *EIF-5A* is thought to function in protein synthesis by promoting synthesis of the first peptide bond [36]. *UBC* is known to be ubiquitin conjugating enzyme [37]. So they remained continuously expressed over the different conditions and showed minimal changes in RNA transcription. The most commonly used reference gene, *ACTIN*, was not among the more stable genes in our tests. Previously, *ACTIN* was commonly used as endogenous internal controls to normalize gene expression studies [38]. However, the poor stability of *ACTIN* was found in potato [4], peach [31], and in cucumber [16]. It may be that the total actin content can vary with development, cell culture conditions, and potentially between cells within tissues [38]. *EF1α* and *EF1β* belong to elongation factor-1 gene family. However, *EF1α* was ranked above *EF1β* except for NaCl and Pb stress (Table 3). They were not among the best reference genes in our test just as the earlier analyses [10,32]. This may be that the expression of *EFI* can be modulated in situations involving growth restriction, transformation, ageing, and cell death [39]. Compared with *EF1α* and *EF1β*, *TUBLIN* was the least stably expressed gene found. Surprisingly, *TUBLIN* showed highly stable expression in longan tree [40] and in cucumber [16]. *HIS* was the most stably expressed gene under NaCl stress, but it was not suitable as the best under three analyses (Table 3). These results suggest that we should choose suitable reference genes according to different species and conditions.

**5. Conclusion**

To our knowledge, this study is the first systematic analysis for the selection of superior reference genes for qPCR in *I. lactea* var. *chinensis* roots under different abiotic (Cd, NaCl, and Pb) stress conditions. Analysis using GeNorm and NormFinder algorithms revealed that *EIF-5A* and *UBC* could be considered to be appropriate reference genes for gene expression analysis under different abiotic experiment stress, whereas *TUBLIN* showed relatively low expression stability. This work will enable accurate and reliable gene expression experiments under different abiotic stress conditions in *I. lactea* var. *chinensis* root.
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