Gene xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Validation of reference genes for quantitative real-time PCR during latex regeneration in rubber tree

Xiangyu Long [a,1], Bin He [a,b,1], Xinsheng Gao [a], Yunxia Qin [a], Jianghua Yang [a], Yongjun Fang [a], Jiyan Qi [a], Chaorong Tang [a,*]

[a] Key Laboratory of Biology and Genetic Resources of Rubber Tree, Ministry of Agriculture, Rubber Research Institute, Chinese Academy of Tropical Agricultural Sciences, Danzhou, Hainan 571737, PR China
[b] College of Agronomy, Hainan University, Haikou, Hainan 570228, PR China

ARTICLE INFO

Article history:
Received 12 December 2014
Received in revised form 26 February 2015
Accepted 13 March 2015
Available online xxxx

Keywords:
Hevea brasiliensis
Reference genes
Latex regeneration
Algorithms
Quantitative real-time PCR

ABSTRACT

In rubber tree, latex regeneration is one of the decisive factors influencing the rubber yield, although its molecular regulation is not well known. Quantitative real-time PCR (qPCR) is a popular and powerful tool used to understand the molecular mechanisms of latex regeneration. However, the suitable reference genes required for qPCR are not available to investigate the expressions of target genes during latex regeneration. In this study, 20 candidate reference genes were selected and evaluated for their expression stability across the samples during the process of latex regeneration. All reference genes showed a relatively wide range of the threshold cycle values, and their stability was validated by four different algorithms (comparative delta Ct method, Bestkeeper, NormFinder and GeNorm). Three softwares (comparative delta Ct method, NormFinder and GeNorm) exported similar results that identify *UBC4*, *ADF*, *UBC2a*, *eIF2* and *ADF4* as the top five suitable references, and 18S as the least suitable one. The application of the screened references would improve accuracy and reliability of gene expression analysis in latex regeneration experiments.

© 2015 Elsevier B.V. All rights reserved.

1. Introduction

In biological research, the technology of gene expression is broadly applied to understand the biological roles and interrelation of genes in molecular pathways. At transcriptional level, several methods provide high sensitivity and accuracy in the quantification of gene expression, such as transcriptome sequencing, cDNA microarray, Northern blotting and quantitative real-time PCR (qPCR) technology (Kubista et al., 2006; Josefsen and Nielsen, 2011; Lang et al., 2014; Le et al., 2014). Owing to technical ease, low reagent cost, less hand-on time and high throughput, qPCR is increasingly and widely used to measure the expression of target genes across different samples (Kubista et al., 2006). Unfortunately, there are several factors which can affect the quantitative measurement of gene expression by qPCR, including initial sample amount, RNA recovery, RNA integrity and efficiency of cDNA synthesis. To achieve accurate and stable results, it is essential that one or several reference genes should be used as internal control to normalize variations (Vandesompele et al., 2002; Andersen et al., 2004; Huggett et al., 2005).

Theoretically, an ideal reference gene is stably expressed in various samples across different experimental conditions or treatments. Housekeeping genes (HKGs) have been historically used as reference genes for normalization, relating to basal cell activities and cellular structure components (Thellin et al., 1999; Daud and Scott, 2008; Frericks and Esser, 2008). Several housekeeping genes, including 18S or 26S ribosomal RNA (18S or 26S rRNA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), α or β-actin (ACT), β or γ-tubulin (TUB), ubiquitin C (UBC) and elongation factor-1 alpha (EF-1α) have been validated as suitable reference genes for qPCR analysis in the past (Bustin, 2000; Goidin et al., 2001; Kim and Kim, 2003; Lossos et al., 2003; Mitter et al., 2009). However, no genes are universally stable across different plant species and differing experimental conditions. More than chance, the usage of the reported suitable reference genes in a different species or an altered condition leads to misleading results. Recently, more candidate reference genes are isolated and identified using gene expression profile data in many plants, including *Saccharum* (Ling et al., 2014), *Elaeis guineensis* (Chan et al., 2014), *Tectona grandis* L.f. (Galeano et al.,

Abbreviations: qRT-PCR, Quantitative real-time PCR; Ct, threshold cycle (previously); Cq, quantification cycle; HKGs, Housekeeping genes; SD, standard deviation; CV, coefficient of variation; 18S, 18s ribosomal RNA; ACT7a, Actin (ACTIN7); ACT7b, Actin (ACTIN7); ADF, Actin depolymerizing factor; ADF4, Actin depolymerizing factor 4; eIF1Aa, Eukaryotic translation initiation factor 1A; eIF1Ab, Eukaryotic translation initiation factor 1A; eIF2, Eukaryotic translation initiation factor; eIF3, Eukaryotic translation initiation factor; FP, F-box family protein; PTP, Trosine phosphatase; RH2a, DEAD box RNA helicase,RH2; RH2b, DEAD box RNA helicase,RH2; ROC3, Cytosolic cyclophilin (ROC3); UBC1, Ubiquitin-protein ligase; UBC2a, Ubiquitin-protein ligase (ATUBC2); UBC2b, Ubiquitin-protein ligase (ATUBC2); UBC3, Ubiquitin-protein ligase; UBC4, Ubiquitin-protein ligase; YLS8, Mitosis protein YLS8.

* Corresponding author.
E-mail address: chaorongtang@126.com (C. Tang).

[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.gene.2015.03.026
0378-1119/© 2015 Elsevier B.V. All rights reserved.

ARTICLE IN PRESS

2014), *Iris lactea* var. *chinensis* (Gu et al., 2014), *Triticum aestivum* L. (Long et al., 2010). To improve the results accuracy, before performing qPCR analyses in a new experimental system, it is vital to evaluate multiple reference genes and utilize the most suitable one(s) to quantify gene expression. Four systematic and statistical algorithms, comparative delta Ct method (Silver et al., 2006), Bestkeeper (Pfaffl et al., 2004), NormFinder (Andersen et al., 2004) and GeNorm (Vandesompele et al., 2002) have been developed to assess and identify the most suitable reference genes for qPCR data normalization.

Natural rubber, cis-polyisoprene, is an essential industrial substance and strategic material, and is mainly acquired from *Hevea brasiliensis*, the Pará rubber tree. Natural rubber biosynthesis occurs in the cytoplasm (latex) of laticifers, which are a strong sink and highly specialized cells in the phloem (Metcalfe, 1967; Lewinsohn, 1991). In production, huge amount of latex (30–40% of its dry matter as natural rubber) is harvested by tapping for every two or three days. So, latex regeneration between two consecutive tappings is one of the major limiting factors determining rubber yield (Kongsawadworakul et al., 2009). And the earlier studies found that physiologically, latex regeneration mainly depends on the availability and metabolism of sugar and nitrogen compounds (Tupy, 1973; Pujade-Renaud et al., 1994). At present, the development of molecular biology makes the studies of gene expression and regulation related to natural rubber biosynthesis become a hotspot in undressing the mechanisms of latex regeneration. For instance, several researchers have focused on the genes involved in the supply and utility of sucrose in the latex, using gene expression profile especially qPCR analysis (Dusotoit-Coucaud et al., 2010; Tang et al., 2010; Liu et al., 2015). To reveal the underlying molecular mechanism and regulatory network of latex regeneration, it is necessary and crucial to screen the suitable reference genes for normalization in relevant gene expression analysis. Although we previously characterized a total of 22 candidate genes for their suitability as reference genes in several experimental conditions in rubber tree (Li et al., 2011), the screening of the suitable references for studying latex regeneration has not been touched. In this study, 20 common reference genes were tested for their expression stability across the samples during the process of latex regeneration. Of the four softwares used for evaluating the suitability of reference genes, three (comparative delta Ct method, NormFinder and GeNorm) exported similar results showing UBC4, ADF, UBC2a, eIF2 and ADF4 as the suitable reference genes for normalization in latex regeneration samples. Evaluation and application of them would improve accuracy and reliability of gene expression analysis in latex regeneration experiments.

2. Materials and methods

2.1. Plant materials

In this study, two clones of rubber tree, RRIM600 and CATAS628 were applied as experimental materials with different types of metabolism. Two types of tree were growing for 13 years at the experimental plantation of the Rubber Research Institute of the Chinese Academy of Tropical Agricultural Sciences (CATAS) (Danzhou, Hainan, China), and which had been regularly tapped for 6 years in a half spiral pattern, every three days (S/2, d/3).

For each rubber clone, 30 trees with similar girth, growth vigor and rubber yield were selected and divided into 6 groups. All groups were tapped simultaneously at the first tapping, and then at 6 h, 12 h, 24 h, 48 h, 72 h and 96 h, respectively, one group for each time was tapped again. After every tapping, latex was collected for RNA extraction according to previous description by Tang (Tang et al., 2007, 2010).

2.2. RNA isolation and cDNA synthesis

Total RNA was extracted by the protocol as described previously by Tang (Tang et al., 2007). RNA samples were treated with DNase I (TaKaRa) to eliminate the trace contaminants of genomic DNA. The integrity of the RNA samples was checked by agarose gel electrophoresis, and the concentration and quality were examined by NanoDrop 2000 (Thermo, USA) at 230 nm, 260 nm and 280 nm. Synthesis of cDNA was performed using the RevertAidTM First Strand cDNA Synthesis Kit (Fermentas, Canada) following the manufacturer's protocol.

2.3. qPCR

A total of 20 candidate reference genes, 18S, ACT7a, ACT7b, ADF, ADF4, eIF2, eIF3, eIF1Aa, eIF1Ab, FP, PTP, RH2a, RH2b, ROC3, UBC1, UBC2a, UBC2b, UBC3, UBC4 and YLS8 were selected from our previous study, and their detail information refers to our previous study including gene annotation, primer sequence, and so on (Table 1S). The real-time PCR was carried out using the SYBR® Premix Ex Taq™ II (Perfect Real Time) (Takara, Dalian, China) and the ABI 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA, USA). The PCR reaction system and procedures were described previously (Tang et al., 2007, 2010; Li et al., 2011). The ABI 7500 Software v2.0.6 was used for visualizing and analyzing the data, including the quantification cycle values, PCR efficiency and correlation coefficients.

2.4. Data analysis

After collecting and converting the quantification cycle data (Cq), Cq average values were calculated to statistical analysis by SPSS 13 (http://www.spss.com/). To obtain reliable results, comparative delta Ct method, Bestkeeper, NormFinder and GeNorm were used to analyze expression stability of reference genes, according to their instructions (RefFinder, http://www.leonxie.com/referencegene.php?type=reference). Pearson correlation coefficients were detected for ranking results from four different algorithms, using Minitab 15 software (http://www.minitab.com/).

3. Results

3.1. Expression profiles of candidate reference genes

A total of 20 candidate reference genes were selected for determining the most stable one during the process of latex regeneration in the rubber varieties of RRIM600 and CATAS628. Amplification of each reference gene in 24 samples (three replicates per sample) produced 72 Cq values, and samples with missing Cq values or inconsistencies between replicates (Cq differences >0.5 cycle) were removed from the analysis. Based on the standard curves using a serial dilution of cDNA samples, the amplification efficiencies of those primers ranged from 94.18% to 102.28%, and the regression coefficient R^2 for all primers varied between 0.997 and 1.000.

Over all samples, the 20 candidate reference genes had a wide range of the Cq values, and the mean Cq values of those gene ranged from 15.60 to 23.07 across all the samples. Among these candidate reference genes, 18S was the most abundantly expressed gene (mean Cq $\pm$ SD $=$ 15.60 $\pm$ 1.07) followed by eIF3 (mean Cq $\pm$ SD $=$ 16.38 $\pm$ 0.72), whereas RH2a was the least abundantly expressed gene (mean Cq $\pm$ SD $=$ 23.07 $\pm$ 1.04). There were small standard deviation and coefficient of variation in all samples. The eIF3 standard deviation (SD) was the lowest (0.72) while PTP presented the largest variation between Cq values (SD $=$ 1.14). The coefficient of variation of UBC4 was the smallest (4.18%), while that of 18S was the largest (6.88%) (Table S1). Additionally, using individual value plot to evaluate and compare all samples, the results showed that those genes had similar distribution or trend except 18S and FP (Fig. 1).

3.2. Expression stability of the twenty candidate reference genes

In order to make a more detailed expression analysis of the candidate reference genes, the 24 samples were divided into three

Please cite this article as: Long, X., et al., Validation of reference genes for quantitative real-time PCR during latex regeneration in rubber tree, Gene (2015), http://dx.doi.org/10.1016/j.gene.2015.03.026

Download English Version:

https://daneshyari.com/en/article/5905469

Download Persian Version:

https://daneshyari.com/article/5905469

Daneshyari.com