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Selection of reliable reference genes for gene expression studies using quantitative real-time PCR in navel orange fruit development and pummelo floral organs

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ABSTRACT

Quantitative real-time PCR (qRT-PCR) has become a widely used method of analyzing gene expression levels. The stability of selected reference gene(s) is very important for the accuracy of qRT-PCR. In the present study, eight candidate reference genes including *CsActin*, *CsGAPDH*, *CsTUB1*, *CsUBQI*, *CsUBL5*, *CsIF4α*, *CsUBQ4* and *CsTUA3* were chosen, and their expression levels were analyzed by qRT-PCR in the fruit pulp of navel orange (*Citrus sinensis* L. Osbeck) at five different ripening stages and pummelo (*Citrus grandis* L. Osbeck) with six different floral organs (ovary, style, anther, filament, flower stalk and petal). GeNorm, NormFinder and BestKeeper programs were used to assess the stability of the candidate reference genes. The three outputs were merged by RefFinder, a web-based comprehensive tool for the final ranking. The results showed that *CsGAPDH* and *CsUBL5* determined by geNorm were the best combination for all the three experimental sets. The expressions of *CsGAPDH* and *CsTUA3* were most stable at different fruit ripening stages or different floral organs. For all samples, *CsUBL5* and *CsGAPDH* were the most stable genes while *CsTUA3* was the least stable one and *CsTUB1* was the most unsuitable reference gene in many sample sets. The relative quantification of the *CsNCED1* and *CgSRK1* genes varied according to the type and number of reference genes, which further proved the importance of reference gene selection.

1. Introduction

The methods for detecting gene expression levels include semi-quantitative PCR (semi-PCR), Northern blot, RNase protection analysis (RPA), quantitative real-time PCR (qRT-PCR), gene chips and RNA-seq. qRT-PCR is a powerful tool for quantification of gene expression and an effective method for studying low-abundant mRNA expression (Bustin et al., 2005), due to its high sensitivity, specificity and broad quantification range (Bustin, 2005; Bustin and Nolan, 2004; Gachon et al., 2004). Besides, Huggett et al. (2005) found that qRT-PCR was the only method for detecting mRNA levels of low copy number target genes in many situations. However, the data obtained by qRT-PCR can be strongly affected by reference genes (Czechowski et al., 2005; Gutierrez et al., 2008), thus the expression of reference genes should not be affected throughout any biological context; otherwise, it may lead to erroneous results (Jain et al., 2006).

Ideal reference genes are constitutively expressed, exhibiting little variation under various experimental conditions such as tissue types, plant developmental stages and external stimuli (Banda et al., 2008). Some classical housekeeping genes have been used extensively as reference genes for gene expression analysis in citrus without validation of their expression stability under specific experimental conditions, such as actin (*ACT*) (Chai et al., 2011; Liu et al., 2009), 18S ribosomal RNA (*18SrRNA*) (Albrecht and Bowman, 2008), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (Fan et al., 2010) and tubulin (*TUB*) (Tan and Swain, 2007). Until recently, the expression stability of several reference genes has been validated in specific experimental conditions or different tissues/organs of citrus. *18SrRNA*, *ACTB* and *rpII* have been proved to be the most stable ones among six leaf samples of different citrus genotypes (Yan et al., 2012) and Liu et al. (2013) validated the expression stability of 10 potential reference genes in diverse sets of biological citrus samples, including embryonic callus, different plant organs and floral tissues, and concluded that *CitUBQ1* was

Abbreviations: qRT-PCR, quantitative real-time polymerase chain reaction; DAF, days after flowering; Ct, cycle threshold.

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the most stable one across the set of all samples. In addition, there is a growing recognition that the expression of classical reference genes may also vary considerably under different experimental conditions, which may result in low precision or misleading results (Beekman et al., 2011; Czechowski et al., 2005; Gutierrez et al., 2008; Remans et al., 2008). Therefore, it is necessary to perform systematic validation of suitable reference gene(s) for different species according to different experimental and developmental stages prior to their use for qRT-PCR normalization.

Citrus is among the most important and widely grown commodity fruit crops. The ripening stage is important for citrus fruit development. However, the underlying mechanisms explaining this biological process remain unknown. Therefore, it is important to know that the expression patterns of some key genes can be differentially expressed during fruit ripening, which can help to better understand the complex interactions among the regulatory networks. Furthermore, studies of the molecular events associated with various floral tissues and organs help to understand self-incompatible and seedless characteristics of citrus. Thus, the genes expressed in floral tissues and organs need an in-depth study. However, the expression levels of so-called reference genes differ in distinct tissues and experimental conditions. Thus, the selection of reliable reference genes for gene expression studies in citrus fruit ripening stage and different floral tissues and organs is a base for these studies.

In this study, the stability of eight candidate reference genes was validated. Genes including actin-7 (*CsActin*), glyceraldehyde-3-phosphate dehydrogenase 1 (*CsGAPDH*), tubulin beta-1 chain (*CsTUB1*), ubiquitin extension protein 1 (*CsUBQI*), ubiquitin-like protein 5 (*CsUBL5*), translation initiation factor 4α (*CsIF4α*), polyubiquitin 4 (*CsUBQ4*) and tubulin alph-3 (*CsTUA3*) are commonly used as reference genes. In addition, the expression profile of *CsNCED1* gene during citrus fruit ripening and *CgSRK1* gene in different floral organs was also analyzed using the selected reference genes, respectively.

2. Materials and methods

2.1. Plant materials

Fruit samples were harvested from 'Fengjie 72-1' navel orange (*Citrus sinensis* L. Osbeck) which was cultivated in an orchard at Fengjie, Chongqing city, China. Fruit samples were harvested at 150, 170, 190, 210 and 240 DAF (days after flowering) from 3 different trees and 12 fruit samples were harvested from each tree. After separating the pulp from the peel, the pulp was sliced. With all the sliced pulp mixed, the samples were frozen rapidly in liquid nitrogen and kept at −80 °C. The floral organs (including ovary, style, anther, filament, flower stalk and petal) and leaf were sampled from 'Shatian' pummelo (*Citrus grandis* L. Osbeck) at its full-bloom stage from five trees, which were cultivated in Guilin, Guangxi, China. The floral organs and leaf samples were also frozen in liquid nitrogen immediately after being harvested. All the samples were transported to the laboratory and stored at −80 °C until total RNA was isolated.

2.2. Total RNA isolation and cDNA synthesis

Total RNAs were extracted from all samples according to Liu et al. (2006). The total RNA of fruit samples of each development stage was extracted from the mixture of 12 fruits pulps from 3 different trees. The total RNA of each floral organ was extracted from the mixture of more than 50 flowers from 5 trees. The total RNA quality was controlled by a series of methods according to Liu et al. (2013). A total of 1 μg RNA was reversely transcribed for first-strand cDNA synthesis using the RevertAid™ First Strand cDNA synthesis kit (Fermentas, Lithuania) according to the instructions of the manufacturer.

2.3. Primer design

The eight chosen candidate reference genes were *CsActin*, *CsGAPDH*, *CsTUB1*, *CsUBQ1*, *CsUBL5*, *CsIF4α*, *CsUBQ4* and *CsTUA3*, and their sequences were obtained from the *Citrus sinensis* Annotation Project (CAP) database (http://citrus.hzau.edu.cn/) (Table 1). The ortholog genes of *Arabidopsis thaliana* were obtained by BLASTP in the Arabidopsis Information Resource (TAIR) (http://www.arabidopsis.org/). Eight pairs of specific primers of eight candidate reference genes and the primers of target gene *CgSRK1* were designed according to their sequences with the Primer Express 3.0 (Applied Biosystems, Foster City, CA, USA) (Table 1, Supplementary Table S1). For *CsNCED1*, primers were previously designed by Wu et al. (2014). The primers were further confirmed with a melting curve analysis after amplification of eight reference genes (Supplementary Fig. S1). To estimate PCR efficiencies, standard curves of a 10-fold dilution series from pooled cDNA were made to calculate the gene-specific PCR efficiency and regression coefficient (R^2) for each gene (Table 1).

2.4. Real-time quantitative PCR

qRT-PCR was performed in ABI 7900HT Fast Real-time system (PE Applied Biosystems, Foster City, CA, USA) with optical 384-well plates. The SYBR Green PCR Master Mix (PE Applied Biosystems) was used in reactions. Ten microliters of the reaction mixture was added to each well. The thermal cycling program was set at 50 °C for 2 min, 95 °C for 1 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The real-time PCR was performed with four replicates for each sample, and data are indicated as the means ± standard error (SE) ($n = 3$).

2.5. Gene sequencing

In order to verify the correct sequences of the reference genes, all the amplicons of eight candidate genes were sequenced. The PCR products were separated by 2.0% agarose gel electrophoresis, and purified using a GeneJET Gel Extraction Kit (Thermo scientific). The purified products were cloned into the T&A™ Cloning Vector (Yeastern Biotech, Taipei, Taiwan) and the independent clones were sequenced in Beijing Genomics Institute (BGI).

2.6. Data analysis

Software packages for data analysis included geNorm (version 3.5), NormFinder (version 0.953), BestKeeper (version 1) and RefFinder (http://www.leonxie.com/referencegene.php?type=reference). The PCR efficiency shown in Table 1 was calculated according to Rasmussen (2001).

2.7. Normalization of CsNCED1 and CgSRK1

9-cis-epoxycarotenoid dioxygenase gene (*NCED1*) is a key gene for ABA biosynthesis and the transcripts of *NCED1* accumulate in fruit pulp during the fruit development and ripening stage (Rodrigo et al., 2006). S-receptor kinase-like protein 1 (*CgSRK1*) is a *S*-locus receptor kinase, which is a key gene for self-incompatibility, displaying different expression profiles in different floral organs (Dwyer et al., 2013). The sequence of *CsNCED1* was obtained from the *Citrus sinensis* Annotation Project (CAP) database and the sequence of *CgSRK1* was obtained from a *Citrus grandis* flower-derived cDNA library (Biswas et al., 2012). *CsNCED1* and *CgSRK1*