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Selection of appropriate reference genes in eggplant for quantitative gene expression studies under different experimental conditions

Xiaohui Zhou, Jun Liu, Yong Zhuang*

Institute of Vegetable Crops, Jiangsu Academy of Agricultural Sciences, Nanjing 210014, China

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ABSTRACT

Quantitative real-time polymerase chain reaction (RT-qPCR) has become one of the most widely used methods for gene expression analysis, and the evaluation of appropriate reference genes should be the first step to consider in experiments based on RT-qPCR. In this work, nine candidate reference genes, including *18sRNA*, *CYP*, *UBQ*, *GAPDH*, *TUB*, *TUA*, *EF1*, *ACTIN* and *RPOB*, were investigated for their expression stability in five different eggplant sample pools using three algorithms: geNorm, NormFinder and RefFinder. The samples were collected under different experimental conditions, including those from different plant tissues, hormone treatments, biotic and abiotic stresses. Our results demonstrated that the expression stability varied from different experimental conditions between reference genes. In general, *GAPDH* and *18sRNA* had a good performance under most experimental conditions, whereas *TUB* and *RPOB* exhibited poor stability. This study should provide guidelines for the selection of reference genes for gene expression studies in eggplant.

1. Introduction

Gene expression profiling is an important step in elucidating the complex regulatory networks of the genetic, signaling and metabolic pathway mechanisms that underlie the developmental, biological and cellular processes (Yeap et al., 2014; Hu et al., 2009; Wan et al., 2010). RT-qPCR is the most commonly used method for monitoring gene expression profiles, it being superior in speed, reproducibility, sensitivity and specificity (Bustin, 2002; Nolan et al., 2006; VanGuilder et al., 2008). Nonetheless, the reliability of RT-qPCR is influenced by variations caused in various steps of the experimental procedure (Bustin and Nolan, 2004; Goulao et al., 2012). Thus, the selection of an appropriate normalization is essential to avoid these variations and the employment of internal reference genes has been considered the most common method of normalization (Goulao et al., 2012; Bustin et al., 2005; Gao et al., 2012).

Suitable reference genes should be expressed at a constant level across various samples and must be unaffected by the experimental conditions and treatments introduced (Wan et al., 2010; Schmittgen and Zakrajsek, 2000). The common choices of reference genes are housekeeping genes, which are required for basic cellular processes, e.g., actin, tubulin and glyceraldehyde-3-phosphate dehydrogenase (Wan et al., 2010; Nicot et al., 2005; Zhu et al., 2012). However, increasing evidence shows that the mRNA level of commonly used reference genes is often not constant among individuals, tissues or experimental conditions (Hu et al., 2009; Gao et al., 2012). Thus, the evaluation of the stability of potential reference genes in every individual experimental setup is required and necessary (Goulao et al., 2012; Zhu et al., 2012).

The eggplant (*Solanum melongena* L.) belongs to the Solanaceae family, is an important vegetable crop and is of substantial economic importance in Asia, Africa and the subtropics (Zhuang et al., 2012; Collonnier et al., 2001). However, eggplant production is severely threatened by numerous abiotic or biotic stresses, and there is an urgent need to explore the useful genes of interest for eggplant improvement (Zhuang et al., 2012; Toppino et al., 2008; Tamura et al., 2002). With the development of genomic technologies, gene expression analysis could provide a better understanding of the molecular mechanisms associated with abiotic and biotic stress tolerance and, in turn, improve the resistance to different stresses in eggplant. Despite the economic importance of the eggplant, gene expression analysis is rather limited, compared to those of the other cultivated solanaceous crops, such as the tomato, pepper and potato. Thus, the identification and evaluation of the suitability and stability of reference genes could be the first step to promoting gene expression analysis in eggplant.

Currently, the most common housekeeping genes studied for the normalization of expression signals in solanceous crops include

* Corresponding author. Fax: +86 25 84391752.
E-mail address: jaaszy@163.com (Y. Zhuang).

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actin, glyceraldehyde-3-phosphate dehydrogenase, cyclophilin, elongation factor, tubulin, *18sRNA* and ubiqutin (Lopez-Pardo et al., 2013; Exposito-Rodriguez et al., 2008; Gantasala et al., 2013; Nicot et al., 2005). However, some of the most commonly used reference genes were reported not to be suitable for most of the experimental conditions (Zhu et al., 2012; Exposito-Rodriguez et al., 2008; Tong et al., 2009). Gantasala et al. (2013) evaluated the stability in the expression of six candidate reference genes (*18sRNA*, *APRT*, *GAPDH*, *Cyclophilin*, *Actin* and *RuBP*) of eggplant, but only in a set of tissues representing six developmental stages, and there have been no reports of the suitability of the reference genes under different experimental conditions in eggplant.

In this study, nine classical reference genes, including 18s ribosomal RNA (*18sRNA*), Cyclophilin (*CYP*), ubiquitin (*UBQ*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), beta-tubulin (*TUB*), alpha-tubulin (*TUA*), elongation factor 1-alpha (*EF1*), actin (*ACTIN*) and RNA polymerase beta subunit (*RPOB*) were selected based on the previous studies of solanaceous crops. The gene's suitability and stability as internal controls under different stresses or treatments (salt, drought, cold, heat and *Verticillium dahliae* infection), three hormone treatments (salicylic acid, abscisic acid and) methyl jasmonate and three different eggplant tissues (root, stem and leaf) were evaluated by three different statistical programs that have been well developed: geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004) and RefFinder. The results obtained in the study could provide useful information and enhance further expression research of eggplant target genes.

2. Materials and methods

2.1. Plant materials and experimental conditions

Plants of the *Solanum melongena* cv. Suzhouniujiao were used in this study. *Solanum melongena* cv. Suzhouniujiao is a local commercial variety, which is a typical Chinese long type and has dark purple skin with a purple calyx. Seeds were germinated and plants were grown in the greenhouse of the Jiangsu Academy of Agricultural Science, Nanjing, China. The different experimental treatments in this study were performed according to Wan et al. (2010, 2011) and Wang et al. (2012).

For salt and drought stress treatments, seedlings at the fourth true leaf stage were transferred to 300 mM NaCl or 400 mM mannitol for 5 h. The seedlings were kept in water for the same duration at $25 \pm 1\,^{\circ}C$ as the control. For heat and cold shock treatments, seedlings were kept for 5 h at 42 ± 1 and $4 \pm 1\,^{\circ}C$, respectively. Leaves were harvested after the prescribed treatment duration.

For biotic stress, seedlings at the fourth true leaf stage were inoculated by dipping the roots in a 1×10^7 spores ml^{-1} suspension of *Verticillium dahliae* for 3 min in the greenhouse, whereas the control plants were immersed in sterile water (Robb et al., 2007; Dervis et al., 2009). Samples including roots, stems and leaves were taken 24 h after treatment.

For hormone treatments, seedlings at the fourth true leaf stage were sprayed with solutions of abscisic acid (ABA, 100 μM), methyl jasmonic (MeJA, 100 μM) and salicylic acid (SA, 100 μM) in sterile water, whereas the control plants were sprayed with sterile water only. The leaves were sampled 5 h after treatment.

For different tissues, the young leaves, stems and roots of *S. melongena* cv. Suzhouniujiao plants were freshly sampled at the fourth true leaf stage. All samples were frozen in liquid nitrogen and stored at $-80\,^{\circ}C$ until use. A total of 13 samples as described above was used in the study. All experiments were performed using three biological triplicates.

2.2. RNA isolation and cDNA preparation

The total RNA was isolated from the leaves, stems and roots using the RNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. The total RNA concentration of each sample was estimated using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). The integrity of the RNA samples was assessed on 1.2% agarose gel electrophoresis. The single-strand cDNA synthesis was performed using the PrimeScript™II 1st Strandc DNA synthesis kit (Takara, Japan) according to the manufacturer's instructions and used for further analysis.

2.3. Selection of reference genes and primer design

Nine candidate reference genes including *18sRNA*, *CYP*, *UBQ*, *GAPDH*, *TUB*, *TUA*, *EF1*, *ACTIN* and *RPOB* were selected for analysis based on previous studies (Exposito-Rodriguez et al., 2008; Gantasala et al., 2013; Wang et al., 2012; Wan et al., 2011). The primer pairs of the nine selected reference genes for RT-qPCR amplification were designed using Primer 3 (version 4.0) software (Rozen and Skaletsky, 2000) and then checked using Oligo 6.0 software. All primer pairs were custom ordered from Invitrogen, Shanghai. The primer specificities were confirmed with 2.5% agarose gel electrophoresis for a single product and the expected size. The specificity of the primer pairs was also checked by the melting-curve after amplification with RT-qPCR analysis.

2.4. Quantitative real-time PCR and data analysis

The RT-qPCR was performed in 96-well plates with a Bio-Rad CFX96 Real-Time PCR system using a SYBR Green-based PCR assay. The reaction containing 10 μl of Fastsmart Universial SYBR Green Master (Rox) (Roche), 5 μl of diluted cDNAs and 0.5 μl (10 pmol) of each primer to a final volume of 20 μl was performed. The PCR conditions were $95\,^{\circ}C$ for 10 min, followed by 40 cycles of $95\,^{\circ}C$ for 15 s, $60\,^{\circ}C$ for 30 s. The mean amplification efficiencies of the primers were estimated by the LinRegPCR program (Ruijter et al., 2009). Expression levels of the reference genes were determined by C_t values, and relative expression levels of these genes were imported to two publicly available software tools, geNorm (version 3.5) and NormFinder, to analyze the gene expression stability according to the manual description. An additional tool, RefFinder (http://www.Leonxie.com/referencegene.php) was used to confirm the reliability of the calculation (Zhu et al., 2012).

3. Results

3.1. Verification of quantitative amplicons and primer specificity

A total of nine commonly used reference genes were selected as candidates for the investigation of expression stability (*18sRNA*, *CYP*, *UBQ*, *GAPDH*, *TUB*, *TUA*, *EF1*, *ACTIN* and *RPOB*). Primer pairs were designed based on the sequence of these candidate reference genes. The specificity of the amplifications was confirmed both in agarose gel electrophoresis and melting curve analysis. Only those showing a single band of expected size for each primer (Fig. 1A) and the single-peak melting curves of the RT-qPCR were selected for further analysis. All nine reference genes were PCR-amplified from the genomic DNA extracted from the eggplant leaves to verify the presence of introns between the primer-binding sites. The size of genomic DNA fragments was the same as that of the cDNA fragments except for the *TUA* gene. The amplicon was larger than the cDNA fragment of the *TUA* gene indicating the presence of an intron. The information for the gene names, primer sequences and amplicon characteristics are listed in Table 1.