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Selection and validation of reference genes for quantitative RT-PCR analysis in peach fruit under different experimental conditions

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ABSTRACT

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) is a powerful tool for the detection and quantification of target gene expression levels. The accuracy of qRT-PCR data largely depends on the stability of the reference genes used for data normalization. Thus, the selection of suitable reference genes for qRT-PCR is necessary for accurate gene expression, especially under specific experimental conditions. In this study, stably expressed genes in peach fruit stored at various postharvest temperatures were screened based on RNA-seq data. Four newly identified stable genes and 12 traditional reference genes were selected as candidates. Their expression stability was examined in the peel and flesh of peach stored at five different temperatures (5 °C, 15 °C, 25 °C, ambient temperature, and 35 °C). Gene expression was further characterized in different plant tissues (root, stem, leaf, flower, and fruit) and fruit developmental stages. The overall performance of each candidate in all sample sets was also evaluated. As a result, the expression of *PpeIF-1A* was the most stable across the set of all samples, peel and flesh samples at different storage temperatures, and fruit developmental stages. *PpGAPDH* stability ranked highest in plant tissues. In general, *PpeIF-1A* and *PpMUB6* were the most appropriate reference genes for all five experiments whereas *PpRPS28*, *PpRPT-5A,* and *PpAKT3* were unsuitable control genes with variable expression patterns. The relative expression of the phytoene synthase gene *PpPSY1* was assessed to confirm the utility of the selected reference genes in this study. Taken together, this study identified the reference genes suitable for the accurate and reliable normalization of peach gene expression data under different experimental conditions. The novel reference gene *PpMUB6* exhibited stable expression compared to most of the traditional reference genes. These results provide guidance for selecting reliable internal control genes and will benefit future gene expression analysis in a wide variety of conditions in peach.

1. Introduction

Quantitative reverse transcription-PCR (qRT-PCR) has emerged as an essential and widely used technique for quantification of mRNA expression levels due to its simple operationhigh sensitivity reproducibility specificity and high-throughput (Huggett et al., 2005). Its accuracy however is strongly affected by many experimental factors such as the amount of starting material purity and integrity of RNA extraction and the efficiency of reverse transcription and PCR reactions. Therefore a normalization step is a necessary prerequisite. The most effective way to normalize qRT-PCR data is to use reliable reference genes that correct for experimental inaccuracies (Vandesompele et al., 2002; Maroufi et al., 2010). Statistical algorithms such as geNorm (Vandesompele et al., 2002) NormFinder (Andersen et al., 2004) and BestKeeper (Pfaffl et al., 2004) were proposed for the expression stability evaluation of reference gene and selection of the most suitable reference gene in given experimental conditions. With these procedures reference genes in many plant species such as Arabidopsis (Czechowski et al., 2005) chicory (Maroufi et al., 2010) rice (Jain et al., 2006; Li et al., 2010) potato (Nicot et al., 2005) wheat (Long et al., 2010) Brassica napus (Yang et al., 2014) cucumber (Migocka and Papierniak, 2011) longan (Lin and Lai, 2010) citrus (Liu et al., 2013) peach (Tong et al., 2009) have been identified.

An ideal internal gene is one that is stably expressed in different tissues and plant developmental stages, and its expression level must not be affected by environmental conditions (Wan et al., 2010; Radonic

Abbreviations: qRTPCR, quantitative reverse transcription-polymerase chain reaction; Ct, cycle threshold

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et al., 2004). The best known and frequently used reference genes for qRT-PCR assays in plants and animals were mostly housekeeping genes including those of actin (*ACT*), glyceraldehyde-3-phosphate (*GAPDH*), elongation factor 1-α (*EF-1α*), tubulin (*TUB*), ubiquitin (*UBQ*), and 18S ribosomal RNA (*18SrRNA*). Nevertheless, it is increasingly recognized that their transcript levels are not always stable across different species and experimental conditions. Unstable internal control genes for data normalization may result in inappropriate quantitative target gene data and wrong conclusions (Czechowski et al., 2005; Jain et al., 2006; Yan et al., 2012). Therefore, reference genes for qRT-PCR analysis must be selected carefully.

The recent advent of very large data sets like gene chips, expressed sequence tags, and transcriptome sequences have helped identify novel reference genes for transcript normalization under specific experimental treatments. This strategy has already been successfully applied in many plants and has identified several reference genes that outperform the traditional ones (Czechowski et al., 2005; Expósito-Rodríguez et al., 2008; Paolacci et al., 2009). Based on high-throughput technology, 15 potential inner reference genes were selected for peach mRNA normalization and some of them were proved to have higher expression stability than commonly used reference genes (Luo et al., 2014). Similarly, several novel reference genes isolated from EST libraries were confirmed to be more stable than the classical housekeeping genes used for qRT-PCR normalization in wheat (Paolacci et al., 2009) and tomato (Expósito-Rodríguez et al., 2008). Plant transcriptome data are invaluable as a source of internal control genes for qRT-PCR analysis and can be used for the rapid screening of novel stably expressed reference genes.

Peach (*Prunus persica* L. Batsch) is an important and widely grown fruit crop. Existing studies on peach fruit focused mainly on the physiological changes in major metabolites like soluble sugar and organic acid, as well as gene expression profiles during fruit development (Lombardo et al., 2011; Desnoues et al., 2014). More recently, an increasing number of studies have been conducted on the effects of postharvest heat treatment (Spadoni et al., 2014) and exogenous phytohormone treatments on peach ripening and quality in postharvest storage (Jiang et al., 2013). Temperature is an important environmental factor affecting postharvest fruit quality (Lurie and Crisosto, 2005; Xi et al., 2012). Nevertheless, the mechanism underlying its impact on postharvest fruit ripening and senescence is poorly understood. The transcriptional profiles of peach fruit were compared at different postharvest storage temperatures by RNA-seq in our earlier work (unpublished data). Further research is required to determine the expression profiles of certain genes expressed at different postharvest stages. The identification of reliable reference genes is a necessary prerequisite and will assist in the subsequent expression analysis. Thus far, only a few studies have been conducted to evaluate the stabilities of reference genes in peach (Tong et al., 2009 Luo et al., 2014). The expressions of selected reference genes, which were limited to the traditional ones published previously in other plants, have proven to vary significantly across different experimental conditions. It is, therefore, necessary to identify novel reference genes and evaluate their expression stabilities over a wide range of experimental conditions.

In the present study, the objective was to select superior reference genes for reliable qRT-PCR normalization in peach. Several novel potential genes were screened based on RNA-seq data. The expression stabilities of four potential novel and 12 traditional reference genes (*PpACT*, *PpCYP2*, *PpTEF2*, *PpGADPH*, *PpPLA2*, *PpRPII*, *PpRPL13*, *PpTUA*, *PpTUB*, *PpUBQ10*, *PpeIF-1A,* and *PpRPS28*) were determined under different experimental conditions. This included peach fruits (peel and flesh) stored at different temperatures (5 °C, 15 °C, 25 °C, ambient temperature and 35 °C), different fruit development stages, and distinct tissues. The expression profile of phytoene synthase *PpPSY1,* which catalyses the initial step of carotenoid synthetic pathways, was also analysed using the selected reference genes. The data indicated several novel potential reference genes from a high-throughput screening and identified reliable combinations guaranteeing the accuracy of gene expression under specific experimental conditions. This work will benefit future studies on gene expression and function research in peach and other higher plants.

2. Materials and methods

2.1. Plant materials

"Jinxiu" yellow-fleshed peach is an important commodity fruit in China. It is very popular due to its bright colour, characteristic aroma, and nutrient density. "Jinxiu" fruits were harvested from an orchard in Yingcheng, Hubei Province. Fruits similar in size, maturity, and free from visible injuries were selected and stored separately at 5 °C, 15 °C, 25 °C, ambient temperature, or 35 °C. Samples were removed from the constant-temperature incubators at the times indicated and divided into peel and flesh. Different organs (roots, shoots, leaves, flowers) and fruit samples at 38, 78, 98, 118 and 138 days after flowering were also collected from adult trees. Fruit samples at 98, 118, and 138 days were divided into peel and flesh then frozen in liquid nitrogen and stored at −80 °C for subsequent analysis. All experiments were repeated three times. Sample data used for reference genes screening are listed in Table 1.

2.2. Selection of stably expressed genes by RNA-Seq analysis

RNA-seq libraries were constructed with NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) using peach flesh samples stored for 1, 2, 3, 5, 6, and 7 days at 15 °C and 25 °C. They were sequenced on an Illumina Hiseq 2000 platform. Unique gene expression levels were estimated using the Fragments Per Kilobase of transcript per Million mapped reads (FPKM) method (Roberts et al., 2011). The mean expression value (x), standard deviation (σ), and the σ/x ratio (coefficient of variance, Vσ) were then calculated. The coefficient of variance is an indicator of gene expression stability. A lower coefficient of variance value indicates a more stable gene expression. The top 100 genes with the lowest Vσ values (the most stably expressed genes at 15 °C and 25 °C) were chosen. Of these, the genes that were most stable at both temperatures were selected as possible normalization genes.

2.3. Total RNA isolation and first strand cDNA synthesis

Total RNA was extracted from all samples and purified using a SK8661 Kit according to the manufacturer's instructions (SK8661, Sangon Biotech). Total RNA purity and concentration were determined using a Nanodrop TM spectrophotometer (NANODROP 1000; Thermo Scientific). Only RNA samples with OD260/280 ratios between 1.8 and 2.2, and OD260/230 ratios higher than 2.0 were used for cDNA synthesis. The integrity of the RNA samples was determined via 1.2% agarose gel electrophoresis. 1 μg RNA was inverse-transcribed to first-

Table 1
Sample sets of "Jinxiu" peach (*Prunus persica* L. Batsch) representing different storage temperatures, fruit development stages and distinct tissues.

Experimental sample sets	Sampling types
Peel and flesh of peach fruit samples stored at different temperatures	5 °C 0 d, 2 d, 4 d, 6 d, 10 d, 14 d, 18 d, 22 d, 26 d 15 °C 2 d, 4 d, 6 d 25 °C 2 d, 4 d, 6 d Room temperature 2 d, 4 d, 6 d 35 °C 2 d, 4 d, 6 d
Fruit developmental stages	38d, 78 d, 98 d, 118 d, 138 d[a] after flowering
Plant tissues	Root, shoot, leaf, flower, fruit[b]

[a] The fruit samples at 98, 118, and 138 days were divided into peel and flesh.
[b] The Ct value of fruit was used for tissue analyses.