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Selection of suitable inner reference genes for normalisation of microRNA expression response to abiotic stresses by RT-qPCR in leaves, flowers and young stems of peach

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ABSTRACT

RT-qPCR is currently the most accurate, sensitive and widely used technique to investigate gene expression. However, the selection of a suitable internal reference gene is a crucial factor for an accurate and precise result. To date, numerous studies have detected miRNA expression in different experimental conditions to facilitate studies of their biological function, whereas only few studies on reference genes have been performed in plants and none in peach (*Prunus persica* L.). Therefore, the present study aimed to identify suitable reference gene(s) for the normalisation of miRNA expression in peach. In this study, two previously reported housekeeping genes and 15 ppe-miRNAs (*Prunus persica* miRNAs) were selected as reference candidates and their expression stability was investigated in different peach tissues as well as following drought stress, salinity stress, and waterlogging stress treatments. After analysis and validation, the pair of *miR5059* and *miR5072* genes was found to be the best reference genes, which might aid further studies on miRNA function in response to abiotic stresses and other development processes in peach.

1. Introduction

The peach tree (*Prunus persica* L.) is one of the most common and economically important species in the world. However, drought, waterlogging and salt stress are common phenomena that seriously affect peach production yields. Peach trees are highly sensitive to drought stress at particular phenological stages, such as flowering and during stem extension and fruit growth (Berman and DeJong, 1996; Dichio et al., 2006). Due to its high root respiration and large oxygen consumption, peach is also the one of the most intolerant fruit trees to flooding (Ma et al., 2006). It has been reported that peach is relatively sensitive to soil salinity compared with other crop plants and Na^+ accumulation, rather than Cl^- accumulation, has been associated with the familiar marginal and interveinal scorch symptoms observed in salt-stressed peach leaves (Karakas and Rieger, 2000; Rieger, 2000).

Advances in sequencing technology have provided high-throughput gene expression analysis and have contributed to the identification of new genes, including microRNAs (miRNAs). miRNAs act as post-transcriptional modulators of gene expression, acting on several biological functions (Mestdagh et al., 2009; Rubio-Somoza and Weigel, 2011). RT-qPCR is a highly sensitive and widely applied method to analyse miRNA expression in different stress conditions. Within the last two years, both conserved and novel miRNAs have been identified in peach by computational approaches and high-throughput sequencing methods (Barakat et al., 2012; Eldem et al., 2012; Gao et al., 2012a; Zhang et al., 2012a,b; Zhu et al., 2012). RT-qPCR has been routinely used to investigate the expression of miRNAs to better understand their function. To quantify miRNAs and other small RNA molecules, the poly(A)-tailed RT-qPCR approach has also been widely used to amplify mature miRNAs as well as the stem-loop RT-qPCR approach (Chen et al., 2005; Shi and Chiang, 2005). To accurately quantify miRNA expression, many experimental conditions should be considered, such as different sample materials, the quality of RNA extraction and primer amplification efficiency. Thus, for the acquisition of biologically meaningful data, the selection of an appropriate normalisation strategy is crucially important (Tong et al., 2009).

An ideal reference gene should be expressed at a constant level across various conditions and its expression should be unaffected by experimental parameters. Most previous studies in plants, used *5.8S* rRNA (Gao et al., 2012b; Song et al., 2010; Yu et al., 2011) and *U6* snRNA as reference genes for miRNA RT-qPCR analyses, rather

Abbreviations: miRNA, microRNA; ppe-miRNA(s), *Prunus persica* miRNAs; RT-qPCR, quantitative reverse transcription polymerase chain reaction.

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than a few commonly used internal reference genes, such as *18S* ribosomal RNA (*18S* rRNA), *beta-tubulin* (*TUB*), *actin* (*ACT*) or *RNA polymeraseII* (*RPII*). To date, three studies have reported the use of systematically selected miRNAs as RT-qPCR reference genes in soybean (Kulcheski et al., 2010), wheat (Feng et al., 2012) and citrus (Kou et al., 2012), respectively, whereas no such no report exists for peach.

To normalise peach miRNA expression in this study, 17 candidate reference genes were evaluated by RT-qPCR in different biotic stress treatments (drought, waterlogging and salt). Statistical methods implemented in geNorm (Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004) were used.

2. Materials and methods

2.1. Plant materials and treatments

Two-year-old peach trees (*P. persica* L.) of the cultivar 'Zaofengwang', a melting flesh peach genotype, were sampled from the garden of Nanjing Agricultural University, China. Twenty peach trees showing robust and consistent growth were selected and divided into four groups: control, drought, salinity and waterlogging. The abiotic treatments were as follows: (1) for drought stress, the water supply was withheld for seven days (Berman and DeJong, 1996; Eldem et al., 2012); (2) for salinity stress, five thousands of sodium chloride was provided for 24 h (Rieger, 2000); (3) for waterlogging stress, the trees were immersed in water for seven days (Ma et al., 2006). Following treatments, samples were collected from young roots, stems and leaves and were immediately frozen in liquid nitrogen and stored at −70 °C until miRNA extraction. Three biological replicates were performed for each sample.

2.2. Selection of candidate reference genes and primer design

In this study, two pairs of primers (*5S* and *U6*), the most commonly used genes as internal controls for miRNA, were obtained from Gao et al. (2012a,b) and Kou et al. (2012) and (15) peach miRNAs were sequenced by high-throughput technology (unpublished data). During the analyses of 15 miRNAs for RT-qPCR analysis in peach, we observed that *miR156*, *miR159*, *miR162*, *miR164*, *miR167*, *miR168*, *miR171*, *miR172*, *miR396*, *miR827*, *miR894*, *miR1511*, *miR3630*, *miR5059* and *miR5072* showed uniform stability in different tissues. The miRNA primers were designed using Primer 5 (http://frodo.wi.mit.edu/primer5/) with the following parameters: a Tm of about 60 °C, an amplicon length of 59–126 bp, and a GC content of 45–60% to ensure optimal hybridisation efficiency (Table 1).

2.3. Small RNA extraction and cDNA synthesis

The extraction of small RNAs was carried out independently using the CTAB reagent as previously described (Wang et al., 2010). The quality was evaluated by electrophoresis on a 2.0% agarose gel and measured by a UV-1800 spectrophotometer (Eppendorf, Germany) at 260 and 280 nm. The small RNAs were polyadenylated using a poly(A) polymerase (NEB, USA) and then recovered by phenol/chloroform extraction followed by ethanol precipitation. The cDNAs were synthesised from 1 μg poly(A)-tailed RNA using the PrimeScript RT reagent Kit with gDNA Eraser (Takara, Japan) and the RT primer 5′-CCAGTAGCGTATGATGAGCACAGAGTCTGAGATCACTCGTAGCGAGG-d(T)$_{33}$-V(A/C/G)N(A/C/G/T)-3′ (Shi and Chiang, 2005; Shi et al., 2012).

2.4. Quantitative real-time PCR

To monitor the amplification efficiency, the cDNA templates were diluted 5, 10, 15, 20 or 25 times. The amplification efficiency was determined in a RT-qPCR assay using triplicates of each dilution and miRNA-specific primers. A universal reverse primer 5′-CCAGTAGCGTATGATGAGCA-3′ was used for real-time quantitative PCR (Table 1). The RT-qPCR reaction volume was 20 μL and included 10 μL SYBR Premix ExTaq^{TM} (Takara), 1 μL cDNA, 0.2 μM of each primer and 8.2 μL sterile water. All quantitative PCRs were performed in an ABI 7300 Real-Time PCR System (Bio-Rad) and the amplification conditions were 95 °C for 30 s, 40 cycles of 95 °C for 30 s and 60 °C for 34 s and the fluorescence signal was measured once every 1 °C. Negative PCR controls (no cDNA template) were prepared, to detect possible contamination. The specificity of the primer amplicons was tested by the analysis of a melting curve. Each sample was amplified in three technical replicates and the mean was used for RT-qPCR analysis.

2.5. Data analysis

Primer efficiency (E) and correlation coefficients (R^2) were calculated using Microsoft Office Excel (2007), based on a standard curve generated using a series of concentration dilutions. The PCR efficiency was calculated using the formula: $E = [10^{-1/\text{slope}} - 1] \times 100\%$. Box plots of raw Ct values were analysed with a threshold fluorescence value of 0.1, from which the expression stability of the candidate reference genes can intuitively be derived. For more in-depth analysis, the software programs geNorm v3.5 (Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004) were used for evaluation of the stability of candidate reference miRNA expression of the three sample groups (all samples group, different tissues samples and the different abiotic stresses group). For both programs, raw Ct values were transformed to relative quantities via the comparative Ct method.

2.6. Reference gene validation

Ppe-miR163 and *ppe-miR1077* were used as target genes to confirm the stability of the reference gene(s) between drought-stressed and control subgroups. The primers for *miR163* and *miR1077* are shown in Table 1. To calculate the relative expression of the two target genes, the $2^{-\Delta\Delta Ct}$ method was used (Schmittgen and Livak, 2008). Data analyses were conducted using SPSS version 17.0 statistical software.

3. Results

3.1. PCR efficiency and amplification specificity

A total of 17 candidate genes, including 15 miRNAs and two small RNAs, were selected for normalisation of gene expression in different samples by RT-qPCR. The amplification efficiency (E) and correlation coefficient (R^2) for each pair of primers indicates the amplicon doubling rate of a specific primer pair during PCR amplicifacion and the quality of the fit of the standard curve to the plotted data points, respectively. Regression analysis of the primer sets showed that the E values of the 17 candidate reference genes ranged from 81% to 121% and the R^2 values were between 0.934 and 0.998 (Table 1), demonstrating the high quality and specificity of the PCR reaction for these candidate genes.

3.2. Expression profiles of the candidate reference genes

A total of 17 candidate reference genes from the 12 samples had a wide expression range, with Cq values between 11.95 and 29.28