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Reference gene validation for qRT-PCR based gene expression studies in different developmental stages and under biotic stress in apple

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ABSTRACT

Apple is one of the most important fruit crops and the availability of recently sequenced genome facilitates research on gene–trait relationship. Quantitative real-time PCR (qRT-PCR) is a sensitive technique to study gene expression, but its accuracy largely depends on stability of reference gene used for data normalization. Therefore, present study was aimed to identify and validate suitable reference gene in apple. Expression stabilities of 10 housekeeping genes, which are commonly used as reference genes in qRT-PCR studies were evaluated in samples of different developmental stages, various tissue types and under diverse biotic stress conditions in apple. The PCR efficiency of all the genes was found to be ranging between 94–107.6%. Analysis using geNorm, NormFinder and Bestkeeper programs demonstrated that their expression stabilities vary among sample sets. However, protein phosphatase 2A (*PP2A*), ribosomal protein L2 (*RPL2*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were found to be suitable reference genes across all the tissue types and under different biotic stress conditions, while 18S ribosomal RNA (*18S*), β-tubulin (*TUB*) and ubiquitin (*UBQ*) were found to be the least stable genes. Moreover, a combination of different reference genes was suggested for different sample sets. These, results suggest that selection of suitable reference gene depends on the tissue type and development stage or disease condition. Further, use of more than one reference genes in respective tissue types of apple is suggested to accurately normalize qRT-PCR data, in future.

1. Introduction

Gene expression analysis is often used to understand the role of genes in various processes and pathways in living organisms. Among the commonly used methods to study gene expression, quantitative real-time polymerase chain reaction (qRT-PCR) is most accurate, efficient, sensitive and cost effective (Udvardi et al., 2008). Therefore, qRT-PCR has become the method of choice for accurate measurement of target gene expression. Although, qRT-PCR is widely used, the accuracy of its results is dependent on several factors, such as variability in efficiencies of reverse transcription and PCR reaction etc. Thus, normalization according to the total amount of mRNA present in the sample is essential to account for variation in the amount of starting material between samples. The most commonly used approach to normalize qRT-PCR data is to use an internal reference gene, which is stably expressed under relevant experimental conditions and whose abundance is strongly correlated with the amount of total mRNA present in each sample (Gutierrez et al., 2008). However, it is reported that there is no universally applicable reference gene (Nolan et al., 2006; Gutierrez et al., 2008; Chandna et al., 2012), thus validation of a suitable reference gene should be performed in the same organism and sample under the same conditions as those used to study the expression of the target gene(s).

Owing to the importance of reference genes in qRT-PCR data normalization, a number of reference genes have been evaluated to identify the most appropriate reference gene under various experimental conditions in various plants such as, *Arabidopsis* (Czechowski et al., 2005; Remans et al., 2008), tobacco (Schmidt and Delaney, 2010), brassica (Wang et al., 2014), rice (Kim et al., 2003; Jain et al., 2006), poplar (Brunner et al., 2004; Basa et al., 2009; Pettengill et al., 2012), peach (Tong et al., 2009), strawberry

Abbreviations: *18S*, 18S ribosomal RNA; *ACT*, actin; *APRT*, adenine phosphoribosyl transferases; CT, cycle threshold; *CYP*, cyclophilin; *EF-1α*, elongation factor-1α; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *PP2A*, protein phosphatase 2A; *qRT-PCR*, quantitative real-time polymerase chain reaction; *RPL2*, ribosomal protein L2; *TUB*, β-tubulin; UBQ, ubiquitin.

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(Clancy et al., 2013), banana (Chen et al., 2011a,b) and papaya (Zhu et al., 2012) etc.

Apple (*Malus domestica* Borkh.) is one of the most widely cultivated fruit trees, known for its highly nutritious fruits. The phenological events that occur during dormancy induction to fruit ripening are very complex and are sensitive to variation in climatic conditions. Thus, it appears that a complex interplay of endogenous and environmental factors govern the change in the transcriptional activity of the determinant genes. Among environmental factors, low temperature plays a crucial role in setting and development of apple fruit. Thus, understanding the expression pattern of some key genes involved in dormancy establishment and dormancy release and various stages of floral and fruit development will provide insight into the mechanisms involved in these processes. To date, most of the gene expression studies in apple using qRT-PCR have used either *Actin* (Varkonyi-Gasic et al., 2010; Zhu et al., 2011; Zhang et al., 2013) or elongation factor *EF-1α* (Malnoy et al., 2007; Baldo et al., 2010) or ubiquitin *UBQ* (Botton et al., 2009; Kanchiswamy et al., 2013) as reference gene. However, validation of these genes for their suitability as reference genes was not done before use in either of these studies. Recent studies on stability of reference genes for qRT-PCR data normalization in various tissues and developmental stages of apple have been carried out (Perini et al., 2014; Bowen et al., 2014). However, expression stability of reference genes under various biotic stresses in apple has not been investigated, yet. Therefore, present study was aimed to identify the suitable reference gene for qRT-PCR experiments in apple at different temporal developmental stages from dormancy induction to fruit ripening, in addition to leaf and root samples under different biotic stresses. We have evaluated the expression stability of 10 commonly used reference genes *viz,* 18S ribosomal RNA (*18S*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), adenine phosphoribosyl transferases (*APRT*), ribosomal protein L2 (*RPL2*), elongation factor-1α (EF-1α), β-tubulin (*TUB*), ubiquitin (*UBQ*), protein phosphatase 2A (*PP2A*), actin (*ACT*) and cyclophilin (*CYP*), in total 27 samples including 4 leaf and 1 root samples under different biotic stress along with corresponding uninfected healthy samples and, 20 samples of different developmental stages of apple covering the whole phenological year, under natural field conditions.

2. Materials and methods

2.1. Plant material

Apple (*cultivar* Royal delicious) samples were collected from local orchard of Palchan (32°18′36″N, 77°10′40″E; altitude 2350 m, Manali district, Himachal Pradesh) India throughout the calendar year. These samples include, eight stages of spur buds collected at the interval of 16–18 days (from October 20, 2012 to March 8, 2013), six stages of floral development, namely green tip, tight cluster, initial pink, full pink, full bloom and petal fall collected within 32 days interval (from March 25, 2013 to April 26, 2013) and six stages of fruit development collected within 14–148 days post anthesis (from May 8, 2013 to October 2, 2013) (Supplementary Fig. S1). All the samples of temporal developmental stages can be grouped into three different developmental categories *viz*; bud development, floral development and fruit development. Also, the leaf and root tissues under different biotic stress in natural field conditions were collected. The collected leaf tissues were infected with sap sucking insect, virus and fungal pathogens (*Alternaria* and powdery mildew). The root tissue was infected with root rot. The corresponding healthy sample of respective tissue was taken as control. Thus, all the collected samples contain five tissue types which include leaf, root, bud, flower and fruit tissue. The samples were immediately frozen in liquid nitrogen and stored at −80 °C for further use. The pathogens were identified as described by Arya et al. (2014). Briefly, the sequencing of 16S rRNA and ITS region was used to identify the bacterial and fungal infection, respectively. The virus was identified by using sequencing of conserved region of coat protein encoding gene. The sample sets analyzed in this study are listed in Table 1. All the samples were immediately frozen in liquid nitrogen and stored at −80 °C for further use.

2.2. Total RNA isolation and first strand cDNA synthesis

Total RNA was extracted from 100 mg of plant material using iRIS solution (Ghawana et al., 2011) for samples including dormant buds to petal fall stage and using modified CTAB+iRIS method (Muoki et al., 2012) for samples of fruit development stages. Total RNA sample was treated with RNase-free DNase I (Fermentas Life Sciences, USA) to remove genomic DNA contamination. The RNA concentration and integrity were measured using a NanoDrop UV–vis spectrophotometer (Thermo Scientific) and formaldehyde agarose gel electrophoresis, respectively. First strand cDNA was synthesized in 20 μl reaction with 1 μg of total RNA using superscript-II (Invitrogen) with random hexamers. Each cDNA sample was diluted 10 fold in nuclease free water, prior to qRT-PCR analysis.

2.3. Selection of candidate reference genes and primer designing

Ten candidate reference genes were selected to identify the most stably expressed gene(s) to be used in qRT-PCR studies is apple. These genes include 18S ribosomal RNA (*18S*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), adenine phosphoribosyl transferases (*APRT*), ribosomal protein L2 (*RPL2*), elongation factor-1α (EF-1α), β-tubulin (*TUB*), ubiquitin (*UBQ*), protein phosphatase 2A (*PP2A*), actin (*ACT*) and cyclophilin (*CYP*), which are commonly used reference genes in gene expression studies. Nucleotide sequences of these housekeeping genes of *Arabidopsis* were extracted using TAIR10 (http://www.arabidopsis.org) as described by Czechowski et al. (2005). These nucleotide sequences were then BLAST searched against apple genome database using BLAST tool of AppleGFDB [http://www.applegene.org/] to identify their homologs in apple. Forward and reverse primers were designed using Primer Express software version 3.0.1 (Invitrogen) with default parameters, except amplicon length was set to 90–130 bp. Out of resulting primer pairs, the one with least penalty was selected. The primer specificity was first checked by PCR amplification of target genes followed by gel electrophoresis on 2% agarose gel.

2.4. qRT-PCR conditions and PCR efficiency

The qRT-PCR reactions were performed using StepOnePlus Real-Time PCR system (Applied Biosystems) with universal cycling conditions (initial denaturation at 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 60 s) followed by dissociation curve analysis to determine the specificity of the amplification in each case. Each reaction was performed in 20 μl reaction mixture containing diluted cDNA sample as template and 2x Power SYBR Green PCR master mix (Applied Biosystems, USA), and 200 nM each of forward and reverse gene specific primers. Each experiment was conducted with three technical and three biological replicates for each sample. In order to determine the gene specific PCR amplification efficiency of each primer pair in qRT-PCR experiments, 10 fold dilution series (10–10,000 fold dilution) of cDNA were performed as described (StepOne Plus User's manual; (Bustin et al., 2009). The