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ORIGINAL PAPER

RNA-Seq Analysis of Spatiotemporal Gene Expression Patterns During Fruit Development Revealed Reference Genes for Transcript Normalization in Plums

Ho-Youn Kim · Prasenjit Saha · Macarena Farcuh · Bosheng Li · Avi Sadka · Eduardo Blumwald

Abstract Transcriptional analysis that uncovers fruit ripening-related gene regulatory networks is increasingly important to maximize quality and minimize losses of economically important fruits such as plums. RNA sequencing (RNA-Seq) and quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) are important tools to perform high-throughput transcriptomics. The success of transcriptomics depends on the high-quality transcripts from polyphenolic- and polysaccharide-enriched plum fruits, whereas reliability of quantification data relies on accurate normalization using suitable reference gene(s). We optimized a procedure for high-quality RNA isolation from vegetative and reproductive tissues of climacteric and non-climacteric plum cultivars and conducted high-throughput transcriptomics. We identified 20 candidate reference genes from significantly non-differentially expressed transcripts of RNA-Seq data and verified their expression stability using qRT-PCR on a total of 141 plum samples which included flesh, peel, and leaf tissues of several cultivars collected from three locations over a 3-year period. Stability analyses of threshold cycle (C_T) values using BestKeeper, delta (Δ) C_T, NormFinder, geNorm, and RefFinder software revealed *SAND protein-related trafficking protein* (*MON*), *elongation factor 1 alpha* (*EF1*α), and *initiation factor 5A* (*IF5A*) as the best reference genes for precise transcript normalization across different tissue samples. We monitored spatiotemporal expression patterns of differentially expressed transcripts during the developmental process after accurate normalization of qRT-PCR data using combination of two best reference genes. This study also offers a guideline to select best reference genes for future gene expression studies in other plum cultivars.

Keywords Fruit development · Gene expression · Plum · Quantitative real-time reverse transcription PCR · Reference gene(s)

Ho-Youn Kim and Prasenjit Saha contributed equally to this work.

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H.-Y. Kim · P. Saha · M. Farcuh · B. Li · E. Blumwald (✉)
Department of Plant Sciences, University of California Davis, Mail Stop 5, One Shields Ave, Davis, CA 95616, USA
e-mail: eblumwald@ucdavis.edu

H.-Y. Kim
e-mail: jsakim@ucdavis.edu

P. Saha
e-mail: psaha@ucdavis.edu

M. Farcuh
e-mail: mfarcuh@ucdavis.edu

B. Li
e-mail: bosli@ucdavis.edu

A. Sadka
Department of Fruit Tree Sciences, Institute of Plant Sciences, A.R.O. Volcani Center, PO Box 6, Bet Dagan 50250, Israel
e-mail: vhasadka@volcani.agri.gov.il

Introduction

The complex process of fruit ripening includes a series of biochemical, metabolic, and physiological changes that lead to the alteration in quality characteristics, such as aroma, color, taste, and texture (Giovannoni 2004). These changes are typically associated with hormonal regulation, cell wall modifications, enzymes synthesis/degradation, and sugar alterations which are controlled by transcriptional regulation of a group of ripening-related genes in a well-defined manner (Giovannoni 2004; Prasanna et al. 2007; Osorio and Fernie 2013). Uncovering such fruit ripening-related gene regulatory

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networks would be critical to increase quality and decrease losses of economically important fruits.

Plum (*Prunus salicina* Lindl.) belongs to the family Rosaceae and is an economically important fruit commercialized around the world for several nutritional benefits to human diet (Okie and Ramming 1999). Plum cultivars present a high variability due to differences among their peel/skin color, harvesting dates, as well as ripening behavior (Singh and Khan 2010). Although plums have been classified as climacteric fruits (i.e., they display an increase in respiration and ethylene production rates during ripening), it has also been reported that some cultivars exhibit a suppressed climacteric ripening and others behave as non-climacteric fruits (Abdi et al. 1997; Abdi et al. 1998; Kim et al. 2015). These differences among cultivars are due to differences in ethylene biosynthesis, perception, and signal transduction processes. Understanding transcriptional changes associated with plum fruit development can shed light on the regulation of the complex ripening process.

Transcriptomics using RNA sequencing (RNA-Seq) and quantitative real-time PCR (qRT-PCR) are common platforms to conduct gene expression profiling during plant developmental processes (Czechowski et al. 2005; Gonzalez-Aguero et al. 2013). Additionally, qRT-PCR is the most common method to conduct validation of transcriptomics data due to its sensitivity, specificity, and broad quantification range for high-throughput and accurate expression profiling (Wong and Medrano 2005; Gonzalez-Aguero et al. 2013). However, high-throughput transcriptomics studies require good quality RNA transcripts (Die and Roman 2012). Several RNA isolation protocols are available, and many have been tested on fruits to isolate good quality RNA for gene expression studies (Chomczynski and Sacchi 1987; Chang et al. 1993; Reid et al. 2006). Specifically, the presence of abundant polysaccharides and polyphenolics in the plum fruits requires its own examination for an optimized RNA extraction protocol to conduct efficient gene expression studies. Moreover, the applicability of qRT-PCR methodology for accurate transcript quantification during gene expression studies relies on the normalization process of expression levels (Guenin et al. 2009). It has been demonstrated that the "reference gene" also known as "internal control gene" or "housekeeping gene" is the preferred choice for the quantification of gene expression using qRT-PCR (Thellin et al. 1999; Kozera and Rapacz 2013). The reliability of qRT-PCR gene expression patterns depends on the expression stability of reference gene(s) among experiments (Thellin et al. 1999). It is assumed that the expression of the reference gene(s) should remain unaltered irrespective of developmental and experimental conditions, because the expression fluctuation of reference gene leads to erroneous gene expression profiles (Thellin et al. 1999). However, the lack of systematic validation of reference genes is one of the major limitations for gene expression studies using qRT-PCR in plants (Gutierrez et al. 2008), including fruits such as plums. No single gene has been found to show stable expression across all tissue samples (Thellin et al. 1999); therefore, it is recommended that the selection of reference gene(s) should be validated in the given set of experimental conditions or tissue samples (Gutierrez et al. 2008). This suggests that the best reference gene(s) in one species may not be suitable for normalization of gene expression in another species or even in different experiments for the same organism.

Considering the enormous importance of reference gene(s) for normalization of gene expression data using qRT-PCR, several studies have been conducted in fruits, for example, in banana (Chen et al. 2011b), citrus (Mafra et al. 2012), grape (Reid et al. 2006), papaya (Zhu et al. 2012), pear (Imai et al. 2014), peach (Tong et al. 2009), and tomato (Exposito-Rodriguez et al. 2008) among others, to identify suitable reference gene(s). One of the straightforward approaches to identify the best reference gene(s) in new species is the search for orthologous sequences of common reference genes reported in model species and assess their expression stability into the desired species (Gimeno et al. 2014; Saha and Blumwald 2014). In this respect, several algorithms, namely delta (Δ) C_T (Silver et al. 2006), BestKeeper (Pfaffl et al. 2004), geNorm (Vandesompele et al. 2002), NormFinder (Andersen et al. 2004), and RefFinder (Chen et al. 2011a), have been developed to validate the stability ranking of the commonly used reference genes. However, Czechowski et al. (2005) demonstrated a new approach for the identification of reference gene(s) from the *Arabidopsis* ATH1 microarray data. Subsequently, similar strategies have been implemented in many fruit species to identify the best candidate reference gene(s) (Coito et al. 2012; Gonzalez-Aguero et al. 2013), although a limited number of qRT-PCR experiments have been performed in plums, and most of them have been carried out using non-validated reference gene(s) (El-Sharkawy et al. 2007; El-Sharkawy et al. 2008; El-Sharkawy et al. 2009). No methodical analysis for the selection of suitable reference gene(s) for qRT-PCR analysis in plums has been reported.

In this study, we conducted an RNA-Seq transcriptomics analysis of climacteric and non-climacteric plums during different fruit development stages using RNA transcripts isolated following an in-house developed and optimized protocol. We categorized significantly non-differentially expressed (SNDE) genes and identified 20 candidate reference genes from our own SNDE gene dataset. We further verified their expression pattern in a large set of plum samples comprised of several tissues including different developmental stages, cultivars, geographical locations, and years. We examined the stability of these genes using five statistical algorithms to characterize the best reference gene(s) for accurate normalization of qRT-PCR under several experimental conditions. Finally, we validated expression patterns of selected significantly differentially expressed (SDE) genes obtained from RNA-Seq analysis

after normalization with the best ranked reference gene(s) using qRT-PCR. We monitored the spatiotemporal expression patterns of SDE genes during fruit developmental processes of plum cultivars after accurate normalization using the best reference gene(s) identified in this study. In summary, the resources developed in this study provide a validated set of reference genes for qRT-PCR transcript normalization in plums, which will ensure more accurate and reliable gene expression results in this species.

Materials and Methods

Plant Materials

Vegetative tissues (VT, leaves) and reproductive tissues (RT, fruits) of plums (*P. salicina* cv. Lindl.) were collected from three different commercial orchards located in California, USA. RT from development stage 2 (S2, pit hardening), stage 3 (S3, second exponential growth phase), and stage 4 (S4, ripe stage), as well as VT from cultivars Burbank (BB), Burgundy (BG), Dolly (DL), Elephant Heart (EH), Methley (MT), Simka (SK), Santa Rosa (SR), Sweet Miriam (SM), Queen Ann (QA), were harvested. These samples were harvested from three orchards located at Parlier (N 36° 35′ 49.292″, W 119° 30′ 27.759″), Reedley (N 36° 37′ 49.504″, W 119° 28′ 16.477″), and Davis (N 38° 32′ 18.982″, W 121° 47′ 36.793″) over the collection periods of 2011, 2012, and 2014. Fruit growth stages were defined based on fruit size, skin color, changes, and firmness as indicators (Trainotti et al. 2003; El-Sharkawy et al. 2007). Samples were collected in three biological replicates from the same side of three individual trees, and each bio-replicate included six fruits and a pool of leaves from each tree. After harvesting, all the samples were quickly transported to the nearby laboratory located either at the Kearney Agricultural Research and Extension Center (Parlier) or Plant Reproductive Biology Building (University of California, Davis). Descriptions of all samples mentioned above are summarized in Fig. S1 and Table S1. In the laboratory, VT and RT were washed with deionized water and air dried under paper towels. Peel (pericarp) was separated from flesh (mesocarp) for each cleaned fruit, and flesh was cut into small pieces. Tissues from each bio-replicate were pooled together and immediately frozen in liquid nitrogen and stored at −80 °C until further use.

RNA Isolation

Frozen tissues were homogenized to a fine powder in liquid nitrogen using an A11 basic analytical mill grinder (IKA Works, Inc. Wilmington, NC, USA). For high-quality RNA isolation from plum fruit tissue rich in polysaccharide and polyphenolics, we tested phenol/chloroform (López-Gómez and Gómez-Lim 1992), RNeasy Plant Mini Kit (Qiagen) (Chomczynski and Sacchi 1987), TRIzol-Qiagen hybrid (Rio et al. 2010), and cetyl trimethylammonium bromide (CTAB)/NaCl (Chang et al. 1993) methods. Homogenized tissues (100 mg) were extracted in 800 μl of CTAB/NaCl buffer (2 % CTAB, 2 % polyvinylpyrrolidone (PVP), 25 mM EDTA, 2.0 M NaCl, 100 mM Tris–HCl, and 2 % β-mercaptoethanol, pH 8.0) in 2-ml tubes. Homogenates were incubated at 65 °C for 10 min and extracted twice with equal volumes of chloroform/isoamylalcohol (24:1, *v*/*v*) at 12,000×*g* for 10 min, and RNA was selectively precipitated with 0.25 volume of 10 M LiCl overnight at 4 °C. RNA was collected by centrifugation at 12,000×*g* for 20 min, and the pellet was dissolved in 500 μl of SSTE buffer (1.0 M NaCl, 0.5 % SDS, 1 mM Tris-EDTA pH 8.0, and 10 mM Tris–HCl, pH 8.0). RNA was extracted with an equal volume of chloroform/isoamylalcohol (24:1) again and precipitated with two volumes of ethanol at −20 °C for 2 h. After spun down at 12,000×*g* for 20 min, the pure RNA was resuspended in 30 μl of

Table 1 Quantity and quality of RNA obtained from plum tissues using various RNA extraction methods

Extraction method	Tissue	Yield (μg/g tissue)	260/280	260/230	RIN
Phenol/chloroform[a]	flesh	3.72±0.90	1.37±0.01	0.6±0.08	ND
RNeasy Plant Mini Kit[b]	flesh	3.70±1.42	1.5±0.15	1.2±0.64	ND
TRIzol-Qiagen hybrid[c]	flesh	4.70±0.10	1.6±0.04	0.7±0.11	ND
CTAB/NaCl[d]	Leaf	97.0±56.93	2.14±0.01	2.10±0.08	ND
	Peel	64.8±15.80	2.13±0.02	2.10±0.04	ND
	flesh	38.4±16.61	2.10±0.02	2.00±0.11	8.1±0.26

Data represent mean±SD. For each RNA extraction method, $n=15$ samples were used

RIN RNA integrity number, *ND* not determined

[a] López-Gómez and Gómez-Lim (1992)

[b] Chomczynski and Sacchi (1987)

[c] Rio et al. (2010)

[d] Chang et al. (1993)

RNAse-free water. The quality of the RNA samples was also assessed on 1.0 % agarose gel electrophoresis, and RNA concentration and purity were determined using a NanoDrop spectrophotometer ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). The integrity of the RNA samples was also assessed using the Agilent 2100 Bioanalyzer with a 6000 nanochip. The extraction method which gave RNA 260/280 absorption ratio between 1.9 to 2.1 and 260/230 absorption ratio higher than 2.0 with high yield was used for subsequent analyses (Table 1).

RNA-Seq Analysis

Total RNA samples of cultivars SM and SR at developmental stages S2 and S4 in three biological replicates were submitted to UC Davis genomic core facility for high-throughput sequencing. Barcoded Illumina RNA-Seq libraries were created using the TruSeq RNA Sample Preparation Kit V2 following the manufacturer's instructions (Illumina, San Diego, CA).

Libraries were analyzed with a Bioanalyzer 2100 instrument (Agilent, Santa Clara, CA), quantified by Qubit fluorometer (Life Technologies, Carlsbad, CA), and pooled in equimolar ratios according to the fluorometric measurements. The pooled library was quantified by qPCR with a Kapa Library Quant kit (Kapa, Cape Town, South Africa) and sequenced with paired-end 100 bp reads on two lanes of an Illumina HighSeq 2500 instrument. The raw reads generated after sequencing were analyzed by the UC Davis bioinformatics core facility using the in-house developed tool "qrqc" (http://www.bioconductor.org/packages/release/bioc/html/qrqc.html) for quality assessment and improvement while Scythe and Sickle (https://github.com/ucdavis-bioinformatics) for adapter and quality trimming. The closest well-annotated peach (*Prunus persica*) genome obtained from Phytozome (http://www.phytozome.net/peach.php) was used as the primary reference, and the transcriptome fasta was derived from the genome and annotation (gff3 file) using the cufflinks utility gffread. High-quality trimmed reads were aligned to the transcriptome fasta using Burrows-Wheeler Aligner (BWA) v.0.6.2 (http://biobwa.sourceforge.net/) (Li and Durbin 2009). Raw counts per gene were generated from the alignments using sam2counts.py (https://github.com/ucdavis-bioinformatics). TheTopHat/Cufflinks (http://cbcb.umd.edu/software) package was used to derive potential novel transcripts (Trapnell et al. 2012). The trimmed reads were aligned with BWA v.0.6.2 to a new transcriptome fasta which include both the existing peach and novel transcripts generated by gffread to generate raw gene counts table using sam2counts.py. The tables of raw counts per gene were separately used as input to edgeR (http://bioconductor.org/packages/release/bioc/html/edgeR.html) (McCarthy et al. 2012) and (http://www.bioconductor.org/packages/release/bioc/html/limma.html) for differential ($P<0.05$) and non-differential ($P>0.05$) expression patterns with a false discovery rate (FDR)=0.05. The RNA-Seq data used in this study is available at the Sequence Read Archive (SRA, http://www.ncbi.nlm.nih.gov/sra) in the National Center for Biotechnology Information (NCBI) with an accession number SRX 863786.

Candidate Reference Gene Identification and Primer Design

Identification of putative reference genes was conducted following the same bioinformatics pipeline shown earlier (Gonzalez-Aguero et al. 2013). From RNA-Seq analysis, SNDE genes were identified based on non-significant ($P>0.05$) difference in gene expression patterns between the two genotypes. Further, the mean of read counts (>500) and the coefficient of variation (CV=SD/mean) for each gene among 12 samples were used as criteria to narrow down the number of candidate genes, and a cutoff (CV<40 %) was considered to generate a set of putative candidate genes. Stepwise parameters for identification of candidate reference genes from RNA-Seq analysis are given in Table 2. A list of potential candidate reference genes was generated from previous investigations on fruit plants (Reid et al. 2006; Exposito-Rodriguez et al. 2008; Tong et al. 2009; Wan et al. 2010; Chen et al. 2011b; Zhong et al. 2011; Mafra et al. 2012; Zhu et al. 2012; Gonzalez-Aguero et al. 2013; Wang et al. 2013; Imai et al. 2014). Orthologous locus identifiers (IDs) of potential candidate reference genes from peach, a close relative to plum, were identified using locus search from Phytozome (Table 3). Using the peach locus IDs, 20 plum SNDE genes were selected from RNA-Seq data (Fig. S2) as candidate reference genes (Tables 2 and 3), and their sequences were pulled out (Table S2). Wherever possible, primers were designed to anneal near the 3′ end or at the 3′ UTR of most of the genes using PrimerQuest online tools (http://www.idtdna.com/primerquest/Home/Index) considering the following parameters: length 20±3 mer; product size range 50–200 base pair; melting temperature 60±3 °C; guanine-cytosine (GC) content ~50 %, with no or weak hairpin structures and self-complementation or dimers at the 3′ end by choosing the design qPCR 2primers Intercalating Dyes. Sequences with detailed criteria for all primer pairs are given in Table S3.

cDNA Synthesis and qRT-PCR

First strand cDNA synthesis was carried out according to the protocol described before (Saha and Blumwald 2014) using the QuantiTect reverse transcription kit (Qiagen). A gDNA wipeout reaction was carried out on 150 ng total RNA to eliminate genomic DNA contamination following the manufacturer's instructions. Reverse transcription was performed using a combination of both oligo-dT and random primers in

Table 2 Parameters and criteria of RNA-Seq analysis for the selection of putative reference genes from non-differentially expressed genes

Parameters	Descriptions
Number of samples	12 from two developmental stages (S2 and S4) of plum cultivars SR (climacteric) and SM (non-climacteric)
Number of runs/lanes	Two HiSeq lanes to produce approximately 25 million 100×100 nt paired-end reads per sample
Reads before trimming	526 million raw reads (263 million pairs)
Reads after trimming	511 million reads
Number of reads normalized read counts	42 million reads
Number of SNDE genes ($P>0.05$, average read>500, CV<40 %)	4,967 (17.8 million reads)
Top candidate genes from various range of read counts (500~1,000, 1,000~5,000, 5,000~10,000, >10,000)	218 genes (2.4 million reads)
Search for potential reference genes	20

P values were determined using Student's *t* test between cultivars SR and SM

SDE significantly differentially expressed, *SNDE* significantly non-differentially expressed, *CV* coefficient of variance

a 20 μl reaction mixture. The total volume of cDNA was adjusted to 400 μl (20 times dilution) for qRT-PCR.

qRT-PCR was carried out as described elsewhere (Saha et al. 2013) in an optical 96-well plate (Applied Biosystems, Foster City, CA) using a StepOnePlus™ Real-Time PCR System (Applied Biosystems) equipment. The qRT-PCR reaction mixture contained 2.5 μl of 2× Fast SYBR Green PCR MasterMix (Applied Biosystems), 1 μl of 1 μM each of forward and reverse primers, 1 μl of diluted cDNA, and 0.5 μl of RNAse-free water in 5 μl total volume per well. The qRT-PCR thermal cycles included 50 °C for 2 min and 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. After 40 cycles, the melting curves were generated by heating from 60 to 95 °C with a ramp speed of 1.9 °C min^{-1}. Each

Table 3 Characteristics of candidate reference genes

Genes	Description	Peach locus IDs[a]	Average read[b]	CVr/q[c]	% of SNP[d]
ACT	Actin 2/7	ppa007242m.g	81,307.8	0.10/0.08	0.7
CC55	Coiled-coil domain-containing protein 55	ppa008496m.g	1,175.3	0.12/0.05	1.5
CYC	Cyclophilin	ppa009354m.g	770.9	0.10/0.05	0.8
EF1α	Elongation factor 1 alpha	ppa005702m.g	59,947.3	0.15/0.06	1.6
EREB	Ethylene-responsive element binding factor	ppa006366m.g	65,213.6	0.32/0.09	1.9
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	ppa008227m.g	47,695.9	0.10/0.07	1.8
IF5A	Initiation factor 5A	ppa012654m.g	37,575.1	0.10/0.06	1.8
MADS	MADS box transcription factor	ppa010308m.g	29,097.9	0.36/0.12	1.3
MON	SAND-related trafficking protein MON	ppa003026m.g	2,134.6	0.13/0.05	0.6
PEPCK	Phosphoenolpyruvate carboxykinase	ppa002490m.g	243,129.6	0.15/0.18	1.8
PP2A	Protein phosphatase 2A	ppa009114m.g	4,628.8	0.05/0.05	0.9
RAN	GTP-binding nuclear protein Ran	ppa008371m.g	28,941.3	0.16/0.07	0.4
RCA	Rubisco activase	ppa005184m.g	4,242.3	0.23/0.09	0.9
SAMDc	S-adenosyl methionine decarboxylase	ppa007294m.g	94,551.4	0.32/0.05	0.8
TUA	Tubulin-α	ppa005642m.g	9,665.8	0.24/0.09	0.7
TIP41	TIP41-like family protein	ppa009483m.g	1,498.3	0.14/0.09	1.0
TUB	Tubulin-β	ppa004884m.g	46,894.5	0.10/0.08	1.1
UBC	Ubiquitin-conjugating enzyme	ppa012730m.g	2,323.7	0.22/0.10	1.5
UBQ	Ubiquitin 10	ppa005503m.g	135,612.3	0.27/0.07	1.3
UNK	Transmembrane protein	ppa009826m.g	1,675.3	0.15/0.06	0.7

[a] Homologous locus identifiers (IDs) of selected candidate reference genes from closely related species peach

[b] Average number of reads derived from RNA-Seq analysis

[c] Coefficient of variance, *r*/*q*, RNA-Seq reads ($n=12$)/qPCR C_T values ($n=141$)

[d] Percentage of single nucleotide polymorphisms of each candidate genes between plum and peach

qRT-PCR included water instead of cDNA as a negative no-template control (NTC) reaction. The baseline-subtracted plot of the logarithmic increase in fluorescence signal (ΔRn) data, threshold cycle (C_T) values, and the resulting melt curves was analyzed using StepOne Software (v 2.3). The PCR efficiencies (E) including the linear regression analysis (R^2) of each amplification curve were calculated using the LinRegPCR software (Ramakers et al. 2003). The mean C_T values from three biological replicates with SD and CV for all genes and experimental sets were calculated (Table 3).

Reference Gene Expression Stability Analysis

Computer-based algorithms such as BestKeeper (Pfaffl et al. 2004), ΔC_T (Silver et al. 2006), geNorm (Vandesompele et al. 2002), NormFinder (Andersen et al. 2004), and RefFinder (Chen et al. 2011a) were used to judge the expression stability of each reference gene. For each program, data were processed in a similar way as previously described (Saha and Blumwald 2014). The geomean ranking values of the best recommended comprehensive reference genes were obtained using the stability values (M) calculated by BestKeeper, ΔC_T, geNorm, and NormFinder analyses in RefFinder. The best combinations of reference genes required to obtain accurate normalization were determined by pairwise variation (Vn/Vn+1) between two sequential normalization factors (NFn and NFn+1) using the geNorm-based software in R (Kohl 2007).

Accurate Normalization of Gene Expression

Five SDE genes were selected from RNA-Seq data (Table S2) to verify their expression pattern after accurate normalization using the optimum number of reference genes either singly or in combination across experimental sets (Table S4). Sequences of these genes were obtained from RNA-Seq data (Table S2), and primer sequences and parameters are given in Table S4. qRT-PCR analysis was carried out in the similar manner as described earlier (Saha and Blumwald 2014), and the relative expressions of each gene were determined following the calculation described previously (Czechowski et al. 2004). After normalization with the best reference gene(s), the mean expression level from three biological replicates with standard error (SE) is presented.

Statistical Analysis

All calculations were done using Microsoft Excel (v 2010), while graphs and figures were created using either Microsoft Excel (v 2010) or SigmaPlot (v 10.0). Significant differences between SDE and SNDE genes were tested using t test in Microsoft Excel (v 2010). Tukey's range test was performed for multiple comparisons to evaluate significant differences in gene expression patterns among tissue samples and genotypes, using JMP (v 7.0.2) for significant differences at $P<0.05$. All figures were exported to Adobe Photoshop CS5 (v 12.0) and improved for publication.

Results

Quality and Quantity of RNA from Fruit Tissue

In this study, we evaluated four RNA extraction methods for high-quality and quantity RNA isolation from polysaccharide- and polyphenol-enriched flesh (mesocarp) tissue of plum fruits. Results showed that with exception of the CTAB/NaCl method, the other three methods yielded low quantity of RNA in the range of 3.70±1.4 to 4.70±0.1 μg/g tissues (Table 1). We found 260/280 nm absorption ratios of 1.37±0.01 to 1.61±0.04 and 260/230 nm absorption ratios of 0.6±0.08 to 1.2±0.64, suggesting poor RNA quality using phenol/chloroform, RNeasy Plant Mini Kit, and TRIzol-Qiagen hybrid methods for downstream applications. We obtained relatively high amounts (38.4±16.6) of total RNA from flesh tissue with a 260/280 absorption ratio of 2.10±0.02 and 260/230 absorption ratio of 2.00±0.11 for RNA integrity using CTAB/NaCl. The RNA integrity number (RIN) of 8.1±0.26 revealed an acceptable quality of total RNA for future RNA-Seq and qRT-PCR analysis (Table 1). In order to verify the reliability of CTAB/NaCl method, we applied this method to leaf and peel tissues for total RNA preparations. This resulted in 97.0±56.9 and 64.8±15.8 μg/g of total RNA including an appropriate range of RNA quality (260/280 ratio>2 and 260/230 ratio>2) from leaf and peel tissues, respectively (Table 1). Using the CTAB/NaCl method, we further extracted total RNA from the rest of the 126 test samples used in this study (Table S1).

Selection of Potential Reference Genes from Non-Differentially Expressed Genes

To assess the gene expression changes during plum fruit developmental processes, we conducted high-throughput RNA-Seq analyses on two developmentally distinct stages (S2 and S4) of two plum cultivars (SR and SM) that differ remarkably in their fruit ripening behavior (Fig. S1). Sequencing of 12 paired-end libraries in two HiSeq lanes generated a total of 526 million raw reads (263 million pairs). After quality processing and trimming, a total of 511 million reads (98.41 % of raw reads) were further processed. An average 82.2 % (varying between 81.0 and 83.9 % by sample) of trimmed reads were aligned to the peach transcriptome database. The total RNA-Seq reads were separated into 42 million SDE reads (unpublished) and 17.8 million SNDE reads based on significant differences ($P>0.05$) in expression patterns between SR

and SM (Table 2). Further, we categorized all SNDE reads into 4,967 genes with an average reads >500 and CV<40 % (Supplementary data 1). We searched the orthologous locus IDs of previously published putative candidate reference genes tested in fruit and tree species and identified them in SNDE potential candidate genes (Supplementary data 1) following the criteria given in Table 2. We selected a total of 20 SNDE genes as candidate reference genes for transcript normalization during plum fruit developmental process using qRT-PCR (Table 3 and Fig. S2). These candidate reference genes included *actin* (*ACT*), *coiled-coil domain-containing protein 55* (*CC55*), *cyclophilin* (*CYC*), *elongation factor 1 alpha* (*EF1α*), *ethylene-responsive element binding factor* (*EREB*), *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), *initiation factor 5A* (*IF5A*), *MADS box transcription factor* (*MADS*), *SAND protein-related trafficking protein* (*MON*), *phosphoenolpyruvate carboxykinase* (*PEPCK*), *protein phosphatase 2A* (*PP2A*), *GTP-binding nuclear protein* (*RAN*), *rubisco activase* (*RCA*), *S-adenosyl methionine decarboxylase* (*SAMDc*), *tubulin-α* (*TUA*), *tonoplast intrinsic protein* (*TIP41*), *tubulin-β* (*TUB*), *ubiquitin-conjugating enzyme* (*UBC*), *ubiquitin* (*UBQ*), and *transmembrane protein 56* (*UNK*) (Table 3). We observed a wide range of expression levels of these genes from RNA-Seq read data (Tables 3 and Fig. S2). The highest expression level was measured in *PEPCK* (average read 243,129.6), while *CYC* (average read 770.9) showed the lowest expression levels in RNA-Seq analyses (Table 3). The CV value of each gene calculated from RNA-Seq read data identified *PP2A* (CV 0.05) as the most stable SNDE genes among all 20 candidate reference genes (Table 3). In addition, we also noticed relatively low nucleotide polymorphisms compared to peach (Table 3). The highest and lowest nucleotide polymorphisms in sequences generated through RNA-Seq were found in *EREB* (1.9 %) and *RAN* (0.4 %), respectively, among all 20 genes compared to peach orthologs (Tables 3 and S2).

Accurate Amplification of Candidate Reference Genes

We designed gene-specific qRT-PCR primers for all 20 reference genes from sequences obtained from RNA-Seq data (Table S2). The detailed parameters with the amplification characteristics of each primer pair are given in Table S3. We followed a two-step qRT-PCR protocol for cDNA synthesis and amplification in successive steps to reduce the undesired primer dimer formation using SYBR Green. After qRT-PCR, dissociation melt curve analysis of each primer pair revealed a single dominant peak for specific amplification of individual gene (Fig. S3). In addition, we did not see any amplification or nonspecific products when water was used as template instead of cDNA in the no-template control (NTC) reactions (Fig. S3). Furthermore, presence of one amplicon of expected size in agarose gel electrophoresis of qRT-PCR amplified product confirmed the gene-specific amplification of each primer (Fig. S4). Our assessment of amplification efficiency of primer pair using LinRegPCR software showed that all the primer pairs had $E>1.8$ with $R^2>0.97$ in this study (Table S3).

Determination of Non-Differential Expression Patterns of Candidate Reference Genes

We performed qRT-PCR of these 20 reference genes to monitor the non-differential expression patterns (stability) on 141 samples. These samples included VT and RT at S2, S3, and S4 from and nine cultivars collected from three separate locations (Davis, Parlier, and Reedley in California) over three different seasons (2011, 2012, and 2014). We determined the expression levels of all the candidate reference genes by checking the C_T values from all qRT-PCR reactions. The candidate reference genes showed a range of distribution of C_T values from minimum of 20.5±1.8 (mean C_T±SD) in *ACT* to maximum of 31.3±3.1 in *RCA* in all tissue samples studied (Fig. 1). We also noticed the expression stability of these genes from SD and CV of C_T values for each candidate gene. The CV values of *ACT* and *RCA* were 0.09 and 0.10, respectively. The lowest SD values of 1.2 and 1.3 with a CV of 0.05 were found in *PP2A* and *MON* (a *SAND*-related protein gene), respectively, while highest SD (4.4) and CV (0.19) were recorded for *PEPCK* (Fig. 1). We grouped all the C_T values with SD and CV values as fruit developmental stages (S4, S3, and S4), tissue (VT and RT), cultivars (Cv), collection season, and collection location according to the experimental sets described in Table S1 for each gene and provided in the Supplementary data 1. In these experimental sets, *PP2A*, *IF5A*, and *MON* were the most stable (CV>0.05), while *PEPCK* (CV 0.19) and *EREB* (0.09) were the most variable among all 20 genes tested (Supplementary data 2).

Analysis of Expression Stability

We implemented BestKeeper, ΔC_T, NormFinder, geNorm, and RefFinder programs to assess the stability ranking of each candidate reference gene using C_T values from different experimental sets. For this purpose, we categorized our samples into six experimental sets as follows: (i) total (n=141), (ii) S2 (n=30), (iii) S4 (n=60), (iv) VT (n=39), (v) RT (n=102), and (vi) Cv (n=69), and the results are presented in Fig. 2.

In this study, BestKeeper analysis identified *MON* and *PP2A* with CV of 3.3 and 3.6 and SD of 0.9 and 0.9 as the top two most stable genes, while *PEPCK* with CV of 16.0 and SD of 3.7 was the least stable in the total experimental set. In the S2, S4, VT, RT, and Cv experimental sets, *CC55* (3.1±0.9, CV±SD), *CC55* (2.6±0.7), *ACT* (2.4±0.5), *IF5A* (3.5±0.8), and *PP2A* (3.6±0.9) genes showed best stability among all the candidate genes tested by BestKeeper analyses (Fig. 2).

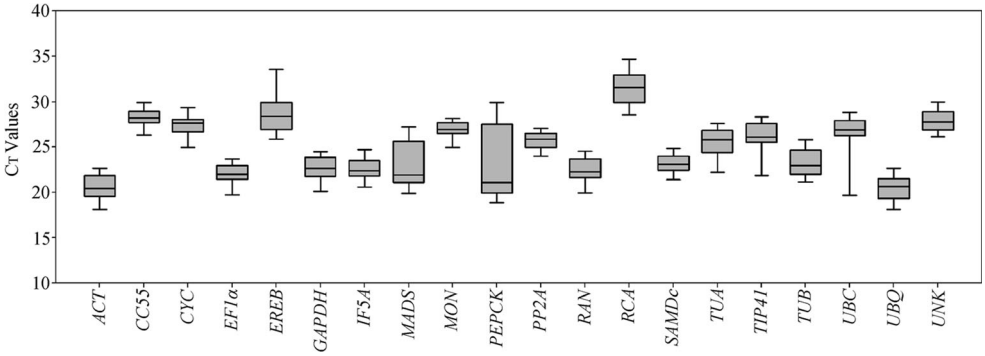

Fig. 1 Distribution of expression levels (C_T values) of 20 candidate reference genes in all plum samples tested. The *box* depicts the 25th and 75th percentiles of data. A *line across the box* represents the median. *Whiskers* show the maximum and minimum values

The ΔC_T method recognized *EF1α* and *MON* with average STDEV values of 1.5 and 1.6 as the two optimum reference genes for normalization in the total experimental set. The candidate reference genes *MON* (1.5), *IF5A* (1.5), *EF1α* (1.1), *MON* (1.3), and *RAN* (1.7) were ranked superior in the S2, S4, VT, RT, and Cv experimental sets, respectively (Fig. 2). The stability values of the 20 candidate reference genes estimated by NormFinder analysis showed that *EF1α* (0.4), *MON* (0.5), *IF5A* (0.8), *EF1α* (0.3), *MON* (0.4), and *RAN* (0.6) were the most stable reference genes in each of the six (i to vi) experimental sets tested (Fig. 2).

Further, analyses based on geNorm normalization (*M*) value revealed significantly high stability of several reference genes at the suggested cutoff range (1.5) of *M* value (Fig. 2). In the total, VT, and RT experimental sets, the best combinations of two reference genes ranked by geNorm were *MON EF1α*, *EF1α GAPDH*, and *MON IF5A* with the lowest *M* values of 0.59, 0.57, and 0.64, respectively (Fig. 2 and Table 4).

In addition, we estimated the geomean of ranking values obtained from BestKeeper, ΔC_T, NormFinder, and geNorm programs using RefFinder software. This allowed us to

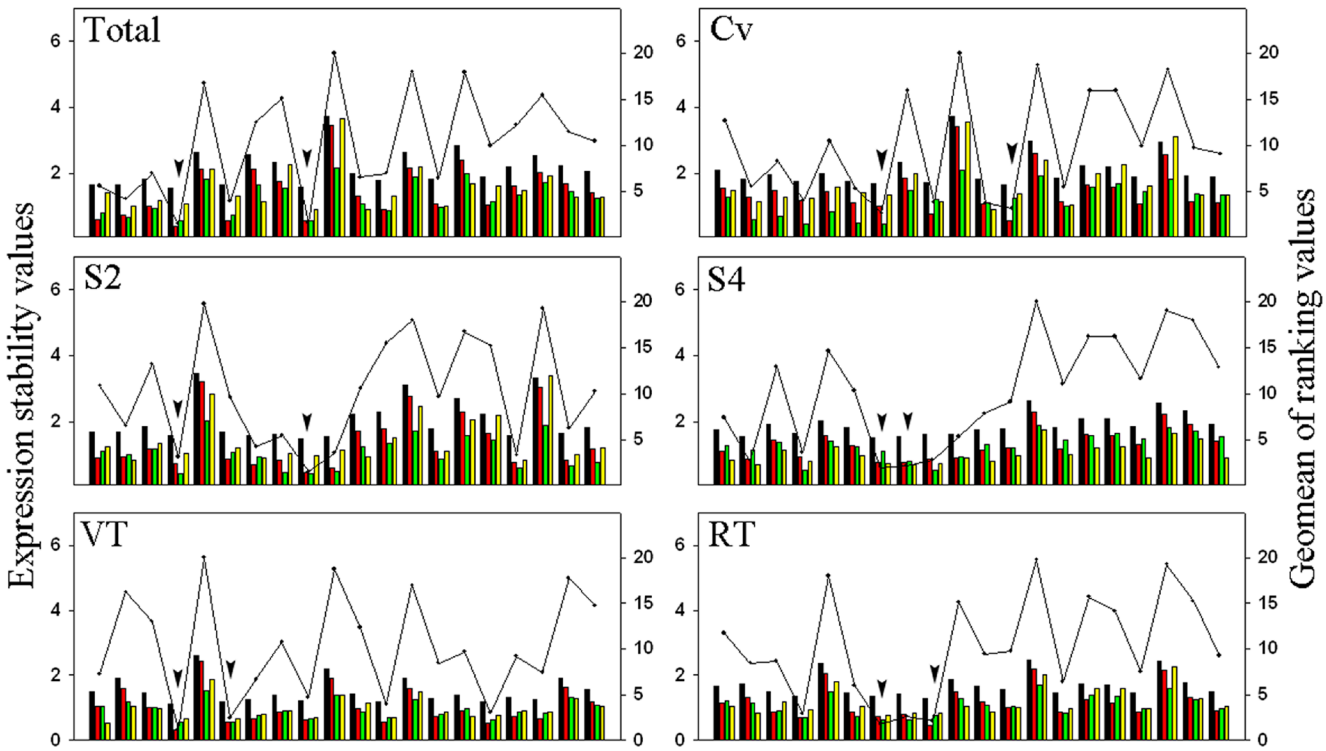

Fig. 2 Ranking of candidate reference genes based on their expression stability (*M*) and geomean of ranking values calculated for each experimental set using different algorithms. Experimental sets presented are *Total*, across all samples; *Cv*, cultivar (SR + SM); *S2*, stage 2 (pit hardening); *S4*, stage 4 (ripe stage); *VT*, vegetative tissue (leaf); and *RT*, reproductive tissue (flesh + peel). *Bars* on the primary *y*-axis represent expression stability based on STDEV (*black*), stability (*S*, *red*), normalization (*M*, *green*), and SD (*yellow*) values estimated by ΔC_T, NormFinder, geNorm, and BestKeeper, respectively, while *line* on the secondary *y*-axis shows geomean of ranking values calculated by RefFinder. *Arrowheads* illustrate the two best ranked genes based on geNorm, NormFinder, and RefFinder analyses (see Table 4 for additional information)

Table 4 Summary of the stability ranking of reference genes

Experimental sets	Best combination (*M* value)	Third ranked gene (*M* value)	V2/3 value	Least stable gene (*M* value)
Total	*MON*I*EF1α* (0.59)	*GAPDH* (0.66)	0.013	*PEPCK* (2.17)
Stage 2 (S2)	*MON*I*EF1α* (0.43)	*MADS* (0.48)	0.010	*EREB* (2.05)
Stage 4 (S4)	*IF5A*I*MADS* (0.56)	*CC55* (0.81)	0.019	*RCA* (1.91)
Vegetative tissue (VT, leaf)	*EF1α*I*GAPDH* (0.57)	*TIP41* (0.62)	0.010	*EREB* (1.51)
Reproductive stage (RT, flesh + peel)	*MON*I*IF5A* (0.64)	*MADS* (0.69)	0.014	*RCA* (1.69)
Cultivars (Cv)	*IF5A*I*RAN* (0.47)	*PP2A* (0.51)	0.011	*PEPCK* (2.12)

V2/3 value represents minimum optimal number of reference genes required for qRT-PCR data normalization at the suggested cutoff range of 0.15 as determined by pairwise variation analyses using gNorm software (Vandesompele et al. 2002)

M value geNorm stability value

generate a recommended comprehensive ranking of reference genes for accurate transcript normalization in each experimental set (Fig. 2). However, in most cases, we found that the RefFinder program recommended the best combination of genes selected by geNorm analyses. A summary of geNorm and RefFinder stability ranking of reference genes for each experimental set is given in Table 4.

We further employed geNorm software to determine the pairwise variation (*V*) values for the best combination of reference gene(s). In this analysis, the estimated pairwise variation (Vn/Vn+1) values between normalization factors NFn and NFn+1 revealed that the V2/3 values were significantly lower than the cutoff range of 0.15 for minimum numbers of reference genes required to normalize transcripts in different experimental sets (Fig. 3).

Monitoring spatiotemporal gene expression patterns during fruit developmental process after accurate normalization of differentially expressed genes

We monitored spatiotemporal gene expression patterns of SDE genes from our RNA-Seq data during the fruit ripening process. To authenticate the expression patterns of RNA-Seq data using qRT-PCR, we applied the best reference gene(s) that we identified in this study for accurate transcript normalization. For this purpose, we selected five SDE genes, of which *ACS3*, *PAL*, and *PG* encode key enzymes, while *EXP2* and *ST* regulate cellular and metabolic processes, during fruit ripening. The details of each of the five target genes including primer sequences are presented in Table S4. The relative transcript levels of these genes were calculated after normalizing using either single two top ranked genes or best combination of reference genes as recommended for RT and VT by geNorm and RefFinder (Tables 4 and 5 and S5). We monitored spatiotemporal expression patterns of these genes in RT tissues at S2 and S4 developmental stages of SR and SM cultivars in samples collected during the 2011 harvesting season (Fig. 4a). We validated similar expression patterns of these genes in the same RT samples after transcript quantification using *MON* + *IF5A* reference genes in combination (Fig. 4b and Table 5) or *MON* and *IF5A* singly of qRT-PCR data (Table S5). We further extended qRT-PCR analysis and confirmed spatiotemporal expression patterns of these genes in S2, S3 of RT (which included both flesh and peel), and VT

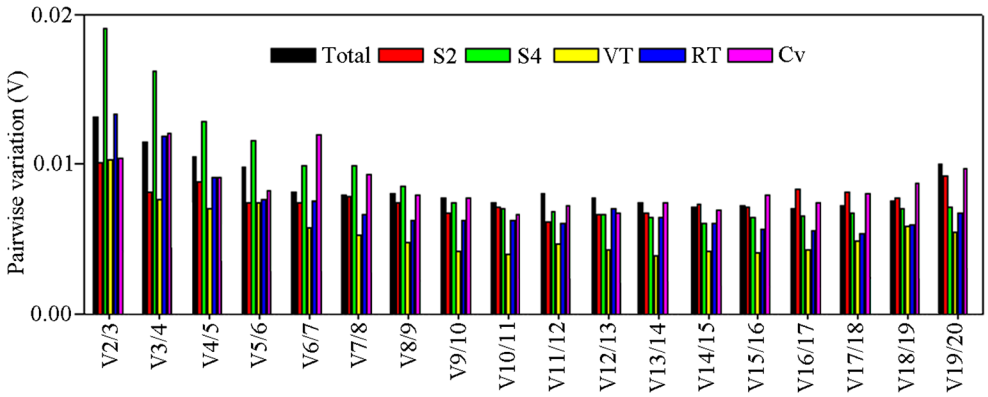

Fig. 3 Determination of pairwise variation (*V*) values for best combination of reference genes. The pairwise variation (Vn/Vn+1) was estimated between normalization factors NFn and NFn+1 by the geNorm software. *Bars* illustrate respective *V* values for *Total*, across all samples; *Cv*, cultivar (SR+SM); *S2*, stage 2 (pit hardening); *S4*, stage 4 (ripe stage); *VT*, vegetative tissue (leaf); and *RT*, reproductive tissue (flesh + peel). Minimum numbers of reference genes required for accurate normalization at different experimental sets are represented by V2/3 values at the cutoff range of 0.15 (Vandesompele et al. 2002)

Table 5 Comparison of expression fold changes (log2) of five differentially expressed target genes after normalization with the best combination of reference genes

Cultivar	Tissue	Stage	Season	Location	*ACS3*	*PAL*	*PG*	*EXP2*	*ST*
SR	RT	S2	2012	R	ND	-5.7^{e}	ND	-5.6^{bc}	-6.7^{ab}
SM	(flesh)		2012		ND	-6.5^{e}	ND	-5.9^{c}	-7.5^{bcde}
SM			2012		ND	-5.8^{e}	ND	-6.7^{c}	-6.2^{a}
EH			2014	P	ND	-6.9^{e}	ND	-6.2^{d}	-8.6^{cde}
SR		S3	2011	R	-9.4^{b}	-8.3^{e}	ND	-5.8^{c}	ND
SM			2011		-9.5^{b}	-10.2^{e}	ND	-9.0^{c}	ND
EH			2014	D	-12.0^{b}	-11.1^{e}	ND	-7.1^{c}	ND
SR		S4	2012	R	-3.2^{a}	-1.4d^{e}	-1.7^{a}	-3.4^{a}	ND
SR			2014	D	-5.9^{ab}	-3.1^{e}	-10.0^{a}	-6.3^{c}	ND
SR			2014	R	-5.0^{ab}	-4.3^{e}	-4.6^{a}	-5.9^{c}	ND
EH	RT	S3	2014	P	ND	-4.1^{e}	ND	-9.5^{c}	-6.9^{abc}
EH	(peel)		2014	D	ND	-0.8^{bc}	ND	-8.1^{c}	-7.3^{bcd}
SR		S4	2012	R	ND	0.9^{b}	ND	-4.0^{ab}	-7.6^{bcde}
SR			2014		ND	-1.7^{de}	ND	-5.6^{bc}	-5.9^{a}
SR			2014	D	ND	0.5^{bc}	ND	-5.8^{c}	-9.1^{de}
SR	VT		2012	R	ND	-2.5^{e}	ND	-8.4^{c}	ND
SM			2014		ND	-3.2^{e}	ND	-8.7^{c}	ND
EH			2014	P	ND	-2.8^{e}	ND	-10.7^{c}	ND
SM			2014	R	ND	1.5^{a}	ND	-12.2^{c}	ND
EH			2014	D	ND	-2.8^{e}	ND	-9.5^{c}	ND
SR			2014	R	ND	-0.3^{cd}	ND	-11.8^{c}	ND
SR			2014	D	ND	-2.0^{de}	ND	-7.8^{c}	ND

Data showed log-transformed values of mean relative expression levels from three biological replicates. For target genes name, see Table S4. Best combinations of reference genes *MONIIF5A* and *EF1αIGAPDH* were used for qRT-PCR data normalization for RT and VT samples, respectively (see Table 4). Green-yellow-red color scale depicts low-medium-high expression levels of each gene. Different superscript letters indicate significant differences at the $P \leq 0.05$ level

SR Santa Rosa, *SM* Sweet Miriam, *EH* Elephant Heart, *RT*, reproductive tissue, *VT* vegetative tissue, *S2* stage 2, *S3* stage 3, *S4* stage 4, *R* Reedley, *P* Parlier, *D* Davis (see Table S1 and Fig. S1), *ND* not detected transcripts

from several Cvs (SR, SM and EH) collected from different locations (Reedley, Parlier, and Davis) over three harvesting seasons (2011, 2012, and 2014) (Tables 5 and S5). We used reference genes *EF1α* and *GAPDH* separately or in combination (*EF1α* + *GAPDH*) to normalize qRT-PCR data (Tables 5 and S5) as suggested for VT (Table S4). Transcript normalization using a single reference gene demonstrated a strong bias in the relative expression pattern with variation in relative expression levels (Table S5), due to statistically significant differences among the expression results obtained by using each reference gene separately, whereas incorporation of two reference genes together revealed more accurate and reliable expression profiles of these genes across tissues (Table 5). Finally, when we validated the expression profiles of five fruit ripening-related genes using the best combination of reference genes obtained in this study, we noticed that *ACS3* expressed only in flesh at S3 and S4, neither in peel at S3 and S4 nor in VT at S2. Similarly, *PG* expression was specific to S4, while

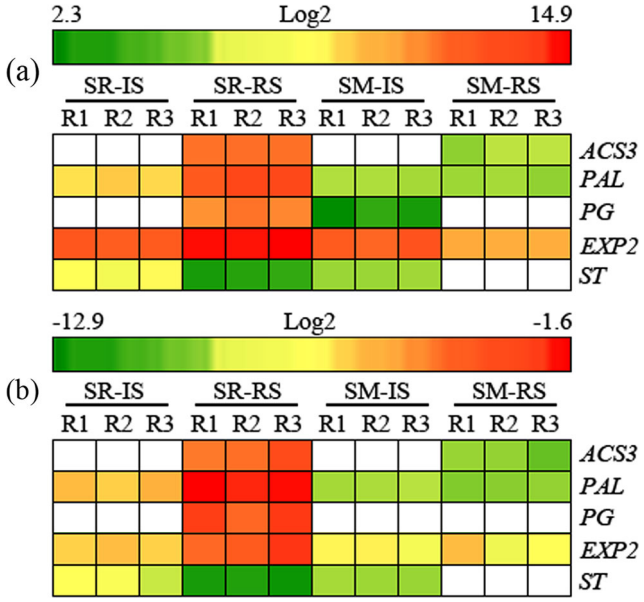

Fig. 4 Expression patterns of five differentially expressed genes during plum fruit development. **a** Fold change in expression patterns of each gene as determined by RNA-Seq analysis. **b** qRT-PCR validation of expression profile of each gene. Data represent log2-transformed values of total reads of RNA-Seq data or mean relative expression values after normalization with best combinations of reference genes (*MON* + *IF5A*) for RT of qRT-PCR data. Data demonstrated comparable differential expression profiles of five genes in immature stage (IS, S2) and ripe stage (RS, S4) of SR and SM from three biological replicates (R1, R2, and R3) collected during 2011 using RNA-Seq and qRT-PCR. *Green-yellow-red* color scale depicts low-medium-high expression levels of each gene. Undetected transcripts were shown in *white* color. See Fig. S1 and Tables 4 and S3 for detail fruit developmental stages, target, and reference genes, respectively

ST expression was observed in flesh at S2 and peel at S3 and S4 (Fig. 4 and Tables 5 and S5). *PAL* and *EXP2* expressions were detected in varying levels in all the tissues studied. However, *PAL* transcript levels were significantly higher ($P<0.05$) in peel compared to flesh at S3 and S4 of RT (Tables 5 and S5).

Discussion

Transcriptomics studies are increasingly important to understand signaling and metabolic pathways underlying molecular and cellular processes during fruit ripening (Coito et al. 2012; Gonzalez-Aguero et al. 2013). While performing transcriptomics during fruit ripening, the success depends on the quality of transcripts obtained from polysaccharide- and polyphenolic-enriched fruit tissues (Reid et al. 2006; Die and Roman 2012). Presence of these compounds made it challenging to extract high-quality RNA from plum fruits for downstream applications such as RNA-Seq and qRT-PCR analyses (Die and Roman 2012). Therefore, prior to gene expression profiling, it is advisable to develop a robust isolation protocol which yields high-quality RNA devoid of polyphenolic and polysaccharide compounds from plum tissues. For this purpose, we evaluated four major RNA extraction protocols which have been tested in fruits and woody species (Chomczynski and Sacchi 1987; Chang et al. 1993). The phenol/chloroform method is known to be suitable for mango fruit (López-Gómez and Gómez-Lim 1992); a commonly used commercialized Qiagen mini kit shown to be ideal for many crop plants (Chomczynski and Sacchi 1987); and the guanidinium thiocyanate-based TRIzol method (Rio et al. 2010) works well with peaches (Meisel et al. 2005). However, in our study, all three methods resulted in poor quality and quantity of total RNA from plum flesh tissue (Table 1). The low 260/230 and 260/280 absorption ratios indicated that total RNA contained polysaccharides, polyphenolic compounds, and proteins (Table 1). We adapted a CTAB/NaCl-based RNA isolation protocol from a previously described procedure developed for pine tree (Chang et al. 1993) and modified it according to our needs. Several studies have reported that the CTAB/NaCl method is a suitable protocol for tissues with abundant polysaccharides and polyphenol contents (Chang et al. 1993; Reid et al. 2006). However, as each fruit is unique in composition and secondary metabolites, a dedicated protocol for quality and quantity of RNA depleted of polysaccharides, polyphenols, and proteins would be useful for plums. Unlike earlier studies (Chang et al. 1993; Reid et al. 2006), here we scaled down the whole protocol to reduce the starting material and reagent volume from all stages in order to handle the large number of samples in this study (Table S1). Our optimization also included the application of high concentration of NaCl, PVP, three chloroform/isoamylalcohol extractions, and overnight precipitation with LiCl at 4 °C. Using this protocol, we obtained total RNA of 38.4±16.61 μg/g tissue with 260/230 and 260/280 ratios of >2 and RIN >8 from plum flesh tissues (Table 1), suggesting its acceptable quality and quantity for downstream high-throughput studies (Bustin et al. 2009).

Analyses of microarray-based expression profiles in crop plants allowed the identification of potential reference genes during development and different environmental conditions (Czechowski et al. 2005; Coito et al. 2012). Unfortunately, the information obtained from crop plants cannot be directly relevant to the transcriptional abundance during unique fruit developmental processes. In addition, the limited genomic information and non-availability of microarray chips drove us to use RNA-Seq analysis in order to understand the transcriptional changes occurring during climacteric and non-climacteric plum fruit ripening. Our results of high-throughput transcriptome sequencing of 12 samples revealed 511 million reads after trimming from two HiSeq lanes, which was quite comparable to the 477 million trimmed reads obtained from 47 grapevine samples in a full Genome Illumina Analyzer II run (Gonzalez-Aguero et al. 2013). However, in their analysis, 91 % of the trimmed

reads were located in the reference grape genome, that is ~10 % lower (82.2 % of trimmed reads were aligned to peach transcriptome database) in our case which could be attributed to the difference between peach and plum genomes. Furthermore, identification of candidate genes from either microarray or RNA-Seq data in fruits has been described recently (Coito et al. 2012; Gonzalez-Aguero et al. 2013). Based on the parameters given in Table 2, we chose 20 SNDE ($P>0.05$) genes that had CV<40 % as putative reference genes (Table 3) which represent potential candidate reference genes for fruits in many previous studies (Chen et al. 2011b; Mafra et al. 2012; Zhu et al. 2012). Moreover, Gonzalez-Aguero et al. (2013) reported CV>40 % of candidate reference genes selected from RNA-Seq non-differentially expressed genes.

We evaluated the expression stability of 20 candidate reference genes which included both commonly used reference genes as well as new reference genes from RNA-Seq SNDE genes. The expression stability of candidate genes was tested in 141 diverse plum tissues using qRT-PCR (Table 1 and Fig. S1). These genes showed non-differential expression patterns in RNA-Seq (Fig. S2) and relatively low (% of SNP<2) nucleotide variation compared to orthologous genes from peach (Tables 3 and S2). Previous studies have reported more than 90 % identity of orthologous reference genes from eucalyptus (de Oliveira et al. 2012) and tomato (Exposito-Rodriguez et al. 2008). It has been suggested that primer or amplification specificity must be validated empirically with direct experimental evidence such as melting profile, electrophoresis gel, amplicon size, and sequence (Bustin et al. 2009). In our study, we documented single peak melting curve with no amplification peak in the NTC for specific amplification of reference genes with their respective primer pairs and confirmed the absence of primer dimers or non-specific products during the dissociation curve analysis (Fig. S3). We also provided the gene sequences including primer sequences with expected amplicon size (Tables S2 and S3) and agarose gel electrophoresis profile to support the specific amplification of each primer pair from qRT-PCR (Fig. S4). Furthermore, our LinRegPCR-based calculation of PCR efficiency of each primer pair revealed mean efficiency values over 1.8 for the candidate reference genes (Table S3), suggesting specific genes being amplified at least at 80 % efficiency per cycle during qRT-PCR. A similar range of PCR efficiencies were reported for many orthologous candidate reference genes during grapevine berry development using LinRegPCR (Reid et al. 2006).

One of the direct ways to examine the expression stability of reference gene(s) was to assess the mean C_T values with range of SD and calculate the CV among all the studied samples. As summarized in Fig. 1, the distribution of C_T values for all genes varied from 20.4±1.7 in the most abundant *ACT* to 31.2±2.9 in the least abundant *RCA*, while for the majority of genes, the C_T ranged from 22.0±1.4 to 28.5±2.7 in the total experimental set. Furthermore, we observed the least variation of SD and CV for most stable reference gene(s). The minimum SD (1.2) and CV (0.05) values of *MON* and *PP2A* suggest a narrow variation, whereas *PEPCK* with SD and CV of 4.1 and 0.18 showed the highest variation in expression stability among all candidate genes studied. However, we also saw a wide distribution range of C_T values and similar pattern of poor performance of *PEPCK* in each experimental set. Supporting this analysis the poor performance of *PEPCK* was reported previously (Saha and Blumwald 2014). Therefore, the data showed that these genes are potential candidates for accurate normalization of qRT-PCR data in plum after proper validation for individual experimental set. In agreement with our study, CV<50 % of C_T values of reference genes were reported in earlier investigations (Reid et al. 2006; Saha and Blumwald 2014).

In this study, we compared four different statistical approaches, BestKeeper, ΔC_T, geNorm, and NormFinder, to reduce the bias in identifying the most stably expressed reference gene(s) and evaluated their stability ranking. The BestKeeper program calculates the stability of reference gene(s) based on SD of C_T values, and a gene with SD<1 is considered the most stable (Pfaffl et al. 2004). On the other hand, ΔC_T method follows a relatively simple approach to calculate stability based on ΔC_T for all pairwise gene combinations (Silver et al. 2006). Both programs were reported to be useful for stability ranking of the best reference genes in fruit plants (Tong et al. 2009; Amil-Ruiz et al. 2013; Imai et al. 2014). The NormFinder algorithm employs a model-based approach by considering intra- and inter-group variations for calculation of a normalization factor (Andersen et al. 2004), whereas geNorm algorithm evaluates gene expression stability based on log2-transformed expression ratios for all pairwise gene combinations and calculates their SD as *V* value which represents an indicator of stability expression *M* value (Vandesompele et al. 2002). A stepwise elimination of the least stable genes is carried out until only two genes are left, these being the most stably expressed genes at $M<1.5$ as suggested by geNorm (Vandesompele et al. 2002). One of the most prominent outcomes of these analyses is that each software selected a different set of top ranked reference genes (Table 4). This is expected because each program uses different algorithms and analytical procedures for stability ranking of candidate reference genes. Earlier reports on banana (Chen et al. 2011b), papaya (Zhu et al. 2012), and peach (Tong et al. 2009) showed that these computational programs did not place the top ranked genes in identical order. In conjunction with our study, discrepancies between NormFinder and geNorm have also been reported previously (Exposito-Rodriguez et al. 2008; Zhong et al. 2011; Saha and Blumwald 2014). The reason behind the apparent divergence between these two programs is that NormFinder does not examine systematic errors during sample preparation and only accounts for variation within and between sample groups,

whereas the geNorm software is sensitive to the co-regulation and apparently tends to select those genes that have highest degree of similarity in their expression profile (Vandesompele et al. 2002). However, a comparison of different algorithms using RefFinder for selection of reference gene allows a better evaluation of the most reliable controls and reduces the risk of artificial selection of co-regulated genes (Chen et al. 2011a). In our analysis, all programs very consistently excluded genes that showed unstable expression patterns as least stable (Table 4). Therefore, we generated a non-biased recommended comprehensive ranking of reference genes by combining the results of all four programs in RefFinder (Chen et al. 2011a) during the plum fruit developmental process. Previously, RefFinder proved to be a useful program for the comprehensive ranking of superior reference genes in many studies (Zhu et al. 2012; Saha and Blumwald 2014).

In the current study, *MON*, a SAND-related protein, was ranked as the most stable reference gene in total and reproductive tissue experimental sets. Similarly, SAND was revealed as one of the superior reference genes found for proper normalization in tomato developmental studies (Exposito-Rodriguez et al. 2008) as well as in a set of organs and tissues of pear (Imai et al. 2014). In citrus (Mafra et al. 2012), grapevine (Reid et al. 2006), and tomato (Exposito-Rodriguez et al. 2008), *MON/SAND* was ranked as one of the third most stable reference genes for transcript quantification using qRT-PCR. In addition, our analysis also categorized *MADS*, *PP2A*, *RAN*, and *TIP41* among the top ranked candidate reference genes for accurate data normalization. In banana, both *RAN* and *TIP41* were ranked among the top five most stable genes (Chen et al. 2011b), whereas *PP2A* and *TIP41* were good candidate genes during grape berry development (Reid et al. 2006). Additionally, the housekeeping genes like *EF1α*, *GAPDH*, and *IF5A* were the best ranked reference genes for transcript normalization in plum. These housekeeping genes were commonly used as reference genes for gene expression studies in many plant species (Gimeno et al. 2014; Saha and Blumwald 2014). An *EF1α* has also been identified as a stable reference gene in grape (Reid et al. 2006), litchi (Zhong et al. 2011), and tomato (Exposito-Rodriguez et al. 2008). In accordance to our study, *GAPDH* ranked among the top three genes in both the total and VT tissue sample sets (Table 4). In many areas of research, *GAPDH* has been widely used (Kozera and Rapacz 2013) and reported to be one of the best reference genes for measuring gene expression in fruit tissues (Reid et al. 2006; Zhong et al. 2011; Mafra et al. 2012). Although previous gene expression analysis in plum used *ACT* as an internal control gene (El-Sharkawy et al. 2007; El-Sharkawy et al. 2008), our analysis shows that *ACT* was not the best reliable gene for transcript quantification during fruit ripening. In support of our study, Zhu et al. (2012) also demonstrated that *ACT* did not appear to be the best gene to use as reference gene during the different treatments of papaya fruits. Furthermore, Czechowski et al. (2005) also reported instability of *ACT2* expression pattern in their study which could be due to the fact that *ACT* is one of the major components of cytoplasmic microfilaments in eukaryotic cells, not only supporting cell structure and determining its shape but also participating in other cellular functions. Additionally, few common reference genes, *CYC*, *TUB*, and *UBQ*, also showed poor performance in plum. This is in corroboration with the previously published work by Thellin et al. (1999) where they found high variability in the relative expression of *ACT*, *TUB*, and *UBQ* during various developmental stages in *Arabidopsis*. *TUB* showed an unacceptable variable expression in peach (Tong et al. 2009) and grape berry (Reid et al. 2006). Based on our analysis, *CYC* was not a suitable reference gene for transcript quantification in plum. Similarly, *CYC* was not among the best reference genes in several of the earlier analyses in fruits (Tong et al. 2009; Chen et al. 2011b). This is because *CYP* expression is significantly regulated by development process.

Our analysis indicated the importance of reference gene validation for each experimental condition and exhibited that specific set of reference genes are required during plum fruit developmental process. Overall, these results demonstrated that a reference gene with stable expression in one tissue may not be suitable to normalize gene expression in another tissue. Thus, a single reference gene may not be enough to accurately normalize qRT-PCR data rather a combination of multiple reference genes are preferred. Although most of the earlier work used only one single gene as an internal control for normalization, it has been suggested that the use of two or more reference genes results in a more reliable transcript quantification of qRT-PCR data. Therefore, the number of reference genes to be taken into account must be experimentally determined using pairwise variations Vn/Vn+1 between consecutively ranked normalization factors NFn and NFn+1 as proposed by Vandesompele et al. (2002). The inclusion of an additional reference gene is not required below the proposed cutoff value of 0.15 by geNorm program (Vandesompele et al. 2002). In our present study, V2/3 is lower than this cutoff value (Fig. 3 and Table 4), suggesting that two top reference genes are enough for normalization. Use of two most stable reference genes is a valid normalization strategy in most experimental conditions (Gimeno et al. 2014; Saha and Blumwald 2014), and this work also suggested that only two genes would be sufficient to get more accurate and reliable normalization compared to the use of a single reference gene in most sample sets (Fig. 4 and Table S5). A combination of *MON* + *EF1α* would be appropriate as a reference panel for normalizing gene expression data in the total sample tested, whereas the combination of *MON* + *IF5A* was the most suitable for RT. A combination of *EF1α* and *GAPDH* would be appropriate as a reference panel for normalizing gene expression data in VT.

To demonstrate the usefulness of the validated candidate reference genes to accurately determine the relative expression levels, five ripening-related genes (*ACS3*, *PAL*, *PG*, *EXP*, and *ST*) were investigated in three plum cultivars during different fruit developmental stages using qRT-PCR. It has been shown that *ACS3* (Giovannoni 2004), *PAL* (Faragher and Brohier 1984), and *PG* (Giovannoni 2004; Prasanna et al. 2007) are three crucial genes encoding enzymes essential for metabolic changes, while *EXP* (Giovannoni 2004) and *ST* (Reid et al. 2006) are two important genes critical for cell wall modification and sugar reallocation during fruit ripening. In non-climacteric fruits, the decrease in ethylene production is associated with reduced activity of ACS, which is expected due to the fact that *ACS3* codes for the rate-limiting enzyme in the ethylene biosynthetic pathway (Prasanna et al. 2007). Besides, during the process of fruit ripening, softening is a complex process that has been reported to be strongly dependent on cell wall disassembly (Ruiz-May and Rose 2013) involving the activity of a wide range of cell wall- modifying proteins encoded by specific genes (El-Sharkawy et al. 2007). These involve PGs and EXPs, and some authors have hypothesized that their expression is ethylene regulated (Pech et al. 2008), thus varying among cultivars and developmental stages. Furthermore, the quality characteristics of ripe fruits are also dependent on PAL, a key enzyme of phenylpropanoid metabolism pathway and responsible for anthocyanin accumulation and color changes of fruits (Faragher and Brohier 1984). Finally, one of the essential characteristics of fruit quality is sweetness, which depends on sugar mobilization through the action of sugar transporters during the fruit ripening process (Osorio and Fernie 2013). In this study, we monitored the differential expression patterns of these genes after accurate normalization with the best reference genes either in combination (Table 5) or singly (Table S5). We found the same differential expression profiles of these genes both in RNA-Seq (Fig. 4a) and qRT-PCR (Fig. 4b) after normalization with the combination of *MON* + *IF5A* reference genes in flesh tissue of plum harvested during 2011. Our qRT-PCR data demonstrated spatiotemporal expression profiles for these ripening-related genes throughout plum fruit development, where the expression of *ACS3*, *PG*, and *ST* was present only in RT but not-detected (ND) in VT. Expression of *ACS3* and *PG* was restricted to flesh tissue in S3 and S4, while *ST* expression is limited to flesh of S2 and peel of S3 and S4. Expression of *PAL* was high in VT compared to flesh of RT, while an opposite expression pattern was noticed for *EXP* after normalization with the best combination of reference (*EF1α* + *GAPDH*) genes. In addition, we provided evidence that these sets of reference genes are also useful for transcript quantification in several tissues of different plum cultivars, while incorporation of multiple reference genes provides the most reliable expression pattern after precise normalization.

Conclusions

In this work, RNA-Seq analyses revealed a set of SNDE genes that were tested for their suitability to normalize SDE genes during plum fruit developmental processes using qRT-PCR. Expression analysis and stability ranking of 20 potential candidate genes lead to identify recommended sets of superior reference genes as well as combination of the best reference genes. These results allowed us to obtain a most stable quantification of key fruit ripening-related genes in different tissues of plum cultivars using qRT-PCR. In summary, we show the first in-depth study to validate the optimal reference genes for the quantification of transcript levels during plum development using qRT-PCR. This study also offers a guideline to select the best reference gene(s) for future gene expression studies in different plum cultivars.

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Conflict of Interest The authors declared that they have no conflict of interest.

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