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Evaluation of RNA Extraction Methods and Identification of Putative Reference Genes for Real-Time Quantitative Polymerase Chain Reaction Expression Studies on Olive (*Olea europaea* L.) Fruits

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Supporting Information

ABSTRACT: Genome wide transcriptomic surveys together with targeted molecular studies are uncovering an ever increasing number of differentially expressed genes in relation to agriculturally relevant processes in olive (*Olea europaea* L). These data need to be supported by quantitative approaches enabling the precise estimation of transcript abundance. qPCR being the most widely adopted technique for mRNA quantification, preliminary work needs to be done to set up robust methods for extraction of fully functional RNA and for the identification of the best reference genes to obtain reliable quantification of transcripts. In this work, we have assessed different methods for their suitability for RNA extraction from olive fruits and leaves and we have evaluated thirteen potential candidate reference genes on 21 RNA samples belonging to fruit developmental/ripening series and to leaves subjected to wounding. By using two different algorithms, GAPDH2 and PP2A1 were identified as the best reference genes for olive fruit development and ripening, and their effectiveness for normalization of expression of two ripening marker genes was demonstrated.

KEYWORDS: *gene expression, reference gene, qPCR, normalization, fruit development*

INTRODUCTION

Olive (*Olea europaea* L.) is a crop tree widely cultivated in the Mediterranean area for its economical importance as a source of fruits for table consumption and oil extraction. For its many economical and social implications, research on *O. europaea* represents an important field of study for agricultural and food sciences, plant physiology, biology, nutrition, medical sciences, and pharmacology.

An increasing amount of data is becoming available dealing with the characterization of molecular aspects of olive fruit development and ripening and olive tree responses to environmental stresses. Untargeted systematic approaches,[1,2] together with targeted studies on molecular aspects of specific processes,[3−5] have recently risen and pinpointed a significantly high number of differentially expressed genes in a range of developmental and physiological situations in olive fruits, roots, and leaves. The availability of such a large body of sequence data is significantly fostering targeted gene expression studies.[4,5] Systematic/untargeted and targeted transcriptional studies both require the availability of tools for the precise and reliable quantification of transcripts. In fact, systematic approaches, while providing a global overview of transcriptional regulatory networks, suffer from their intrinsic wide experimental variability of data and thus need validation of differential transcript accumulation of selected genes of interest by more precise quantitative approaches. The method of choice to assess the regulation of transcript abundance of a given gene, either in terms of its relative or absolute changes, is qPCR. For qPCR to be performed reliably and provide reproducible results, extraction of RNA of optimal quality together with the careful choice of reference genes displaying stable expression values over the widest range of experimental situations are absolute requirements. The identification and choice of the best reference genes is essential for the outcome and reliability of qPCR,[6] by providing internal controls to ensure minimization of nonbiological variation. Ideally, to be useful as a reference, a transcript should be equally abundant among all tissues and experimental conditions under investigation. In the past, genes involved in basic cellular processes were considered to fulfill these requirements since they were assumed to be stably expressed in all conditions. For this reason they were defined as "housekeeping genes". In the past few years, with the rapid spreading of qPCR technology through laboratories, this assumption has been questioned, and it has become evident that no gene is transcriptionally stable in all situations, leading to the conclusion that strictly speaking housekeeping genes do not exist.[7] Thus, the concept of "housekeeping genes" has been substituted with that of "reference genes", underlying the fact that the latter ones must be validated in each experimental set prior to be used. These concepts have been highlighted in recent years, with the drafting of the comprehensive rules for minimum information for publication of qPCR data (MIQE),[8,9] described in order to help standardization of experiments between different laboratories. Bustin et al.[9] strongly encouraged submission of the MIQE workflow checklist as a supplemental file along with submitted manuscripts. In this list,

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Table 1. Description and Accession Number of Candidate Reference Genes, Primer Sequences, Product Size, and PRaTo Score[28]

gene name (in this study)	OLEAEST TC identifier or GenBank accession no.	gene description	primer sequences (forward/reverse)	product size	PRaTo ALL score
14-3-3	OLEEUCl002244|Contig1	14-3-3 protein	TCCTGGGCTGATTTGTAAGC	134	−4
			TCCAGTGGTGATTCCAAGGT		
ACT7a	OLEEUCl025648|Contig2	actin-7	AACGGAATCTCTCAGCTCCA	123	−5
			TTGCTTACGTGGCACTTGAC		
ACT7b	OLEEUCl004175|Contig3	actin-7	GTGCTGAGGGATGCAAGAAT	142	−10
			CCATGTTCCCAGGTATTGCT		
EF1a	OLEEUCl014934|Contig1	elongation factor 1-alpha	CCTCTTGGACGATTTGCTGT	86	−1
			CCTGTTGGCTCCTTCTTGTC		
EF1b	OLEEUCl018061|Contig5	elongation factor 1-alpha	CCAAAGGTGACGACCATACC	109	−3
			CTCTCCGTCTCCCACTTCAG		
GAPDH1	OLEEUCl022518|Contig2	glyceraldehyde-3-phosphate dehydrogenase	CAGCTCTTCCACCTCTCCAG	131	−4
			TCCATTGGCAAAGGTTCTTC		
GAPDH2	OLEEUCl004899|Contig2	glyceraldehyde-3-phosphate dehydrogenase	CCTTCCGTGTGCCTACTGTT	92	−2
			GATGGCTGCCTTGATTTCAT		
PP2A1	OLEEUCl021848|Contig2	serine/threonine-protein phosphatase 2A	TGCAGTGGCTACAGGACAAG	83	−5
			TGGACCAAATTCTTCAGCAA		
PP2A2	OLEEUCl021775|Contig2	serine/threonine-protein phosphatase 2A	GGATGCCATATTCCCACAAC	75	−5
			TGGTCCCATGAACAAAAGGT		
UBC1	OLEEUCl010470|Contig3	ubiquitin-conjugating enzyme	GCCCTTATGCTGGAGGTGTA	100	−4
			GGATGGAAAACCTTGGTCCT		
UBC2	OLEEUCl004061|Contig1	ubiquitin-conjugating enzyme	TTGCAGAAAGACCCTCCTGT	92	−8
			CTGTCCGTAGGTCCCATGAT		
UBQ	OLEEUCl002233|Contig1	ubiquitin	GGTGGAATGCCCTCCTTATC	86	−2
			GGGAAAACCATTACCCTTGAG		
OUB2	AF429430.1	polyubiquitin	GCTGGAGGATGGAAGGACTC	191	−1
			CCACGACTCAACAGAGACGA		

several details have to be disclosed including those regarding RNA quality and data normalization.

Since extraction of high quality RNA and identification of reference genes are among the most important factors for reliable qPCR results, in this paper we have compared three different RNA extraction methods, one of which was optimized to yield high quality RNA from olive fruit mesocarp and stressed leaves.

Furthermore, the stability of 13 putative reference genes was evaluated on 21 samples collected from a series of developmental and ripening stages of olive fruits and from leaf tissues subjected to wounding, aiming for the identification of a panel of reference genes useful for future qPCR studies. A number of similar studies already exist for many crop species,[10−14] but no such study is currently available for *Olea europaea*. Two independent algorithms (genorm^{PLUS} and NormFinder) were used to evaluate the 13 olive candidate genes for their performance as reference genes to assess and quantify stability of transcript abundance. Since the output ranking can be slightly different from one software package to another,[15−17] most authors use more than one application to analyze data. geNorm[7] and NormFinder[18] are the two most widely used algorithms. The first one has been recently improved, renamed genorm^{PLUS} and embedded in the qbase^{PLUS}[19] software dedicated to qPCR data analysis. genorm^{PLUS} has many advantages, since analysis is fast, results are reported automatically, no data handling is needed, and the identification of the single best reference gene is possible (instead of the "best two" combination) (http://medgen.ugent.be/~jvdesomp/genorm/#WhatIs). Similarly to the previous version, genorm^{PLUS} provides also the optimal number of reference genes to be used in the considered experimental set.

Since stability of reference genes should be assessed carefully for each experimental condition,[20] each tissue (fruit and leaf) was considered independently, giving particular emphasis to the fruit development series, an aspect of outstanding importance for its economical implications. To provide further evidence on the importance of having validated reference genes and to show the validity of the identified ones for gene expression studies on olive fruits, the normalization of expression data of two target transcripts was also reported.

MATERIALS AND METHODS

Plant Material. Olive fruits (*Olea europaea* L., cv. Frantoio) were harvested during the 2009 season in an orchard in Verona province (Italy, 45° 28′ 52.85″ N, 11° 8′ 32.97″ E) at ten consecutive sampling dates (ca. one week intervals) at 90, 102, 109, 116, 123, 130, 137, 144, 151, 158 DAF. The véraison stage occurred at 123 DAF, and olives picked in the last sampling date were overripe. For wounding experiments, lateral branches were collected from olive (*Olea europaea* L., cv. Frantoio) trees in an orchard in Padova province (Italy, 45° 17′ 8.02″ N, 11° 43′ 42.52″ E) during the 2010 season and wounding was exerted by pressing the leaves with a blunt metal point. The branches bearing wounded leaves were separated into the following groups according to the different treatments: (1) branches treated with 500 ppm propylene (ethylene analogue); (2) branches treated with 1 ppm of 1-methylcyclopropene (1-MCP; ethylene inhibitor); (3) untreated branches kept in air. A negative control (branches with unwounded leaves) was included. For all treatments branches were kept in large volume sealed vessels through which air was flushed. Leaf samples were collected after 4 and 20 h of treatment for detection of early and late responses. For treatments on olives, fruits were picked 109 DAF in Padova province (Italy, 45° 17′ 8.02″ N, 11° 43′ 42.52″ E) during the

2009 season and sorted into three groups in three sealed vessels flushed with air, kept in 1 ppm 1-MCP, or flushed with 500 ppm propylene, respectively. Treatments were performed for 24 h.

Olives mesocarp and leaves were frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

RNA Extraction, Evaluation of RNA Quality, and cDNA Synthesis. Total RNA was extracted from frozen olive fruit mesocarp and olive leaves using the following three different protocols: (1) an RNA extraction procedure by means of illustra RNAspin Mini Isolation Kit (GE Healthcare, Little Chalfont, UK) based on affinity purification on silica columns performed following the manufacturer's instructions; (2) a protocol developed for RNA extraction[21] from grapevine with some modifications;[22] (3) a hot borate method, developed for RNA extraction from recalcitrant species as cotton[23] and previously used on olives,[24] which was finally optimized with the following modifications. Three hundred milligrams of frozen tissue was ground in a mortar with liquid nitrogen, and the finely ground powder was resuspended in preheated borate buffer.[23] All extraction steps were substantially carried out as described by Wan and Wilkins[23] until LiCl precipitation. After LiCl precipitation and centrifugation, the RNA pellet was resuspended in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) and samples were extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). After centrifugation, the aqueous phase was recovered and the RNA was reprecipitated by adding sodium acetate (final concentration of 0.3 M, pH 4.8) and 1 volume of cold isopropyl alcohol. The precipitated RNA was pelleted, washed in 80% ethanol, resuspended in water, and finally subjected to DNase treatment (10U of RQ1 RNase-free DNase; Promega, Milan, Italy) and affinity column purification (RNeasy MinElute cleanup kit; Qiagen, Milan, Italy) as described in ref 25. An aliquot of RNA obtained from each extraction protocol was quantified spectrophotometrically using a Nanodrop 2000 (Scientific, Nanodrop Products, Wilmington, DE, USA), and 260/280 and 260/230 nm absorbance ratios were calculated. RIN numbers were obtained using a Bioanalyzer 2100 (Agilent Technologies Italia, Cernusco sul Naviglio, Italy), and finally 500 ng of RNA was separated on a 1% agarose gel to further check integrity.

For first strand cDNA synthesis, 500 ng of total RNA and 1 μL of Oligo dT (10 μM) were denatured in a total volume of 12.7 μL by incubation at 70 °C for 5 min followed by 5 min at 37 °C. cDNA synthesis was performed by adding 100 U of M-MLV reverse transcriptase (Promega, Milano, Italy), 25 U of RNase Inhibitor (Affymetrix, High Wycombe, U.K.), and dNTPs (10 μM each), in a final volume of 20 μL and by keeping the mixture at 37 °C for 85 min, followed by 5 min at 94 °C. Two independent reactions were pooled to minimize cDNA synthesis variation.

Primer Design. Primers were designed with Primer3 (http://frodo.wi.mit.edu/primer3/[26]) and checked with OligoCalc (http://www.basic.northwestern.edu/biotools/oligocalc.html[27]). Best resulting pairs were ranked by means of PRaTo (http://prato.daapv.unipd.it[28]), and the best hit was selected. *Olea*ESTdb BLAST (http://140.164.45.140/oleaestdb/blast.php[1]) against TCs database was performed to avoid multiple amplifications. Oligonucleotides were ordered deprotected and desalted (Sigma-Aldrich, Milano, Italy). Gene name, accession number, description, primer sequences, and product size of the reference genes used in this study are given in Table 1. For the two target genes, namely, PG (Polygalacturonase) and FPS (Farnesyl Pyrophosphate Synthase), the following primers were used: PG forward 5′-CATGGGAGTTCAGCATCAGA-3′; PG reverse 5′-GACAAGCAGCTATTTGGCTCA-3′; FPS forward 5′-GGGATCCTGAGGTGATTGGT-3′; FPS reverse 5′-TTTTCGCTACACAAGCAGGA-3′.

qPCR Amplifications. Reactions were set up manually by mixing 5 μL of Fast SYBR Green PCR Master Mix (Applied Biosystems, Monza, Italy), 0.2 μL of each primer (200 nM final), and 2.5 μL of cDNA (corresponding to 2.5 ng of total RNA) in a final volume of 10 μL. Each sample was run in triplicate. Reactions were set in 96-well fast plates sealed with optical foils and loaded into a StepOnePlus (Applied Biosystems, Monza, Italy) platform. A first denaturation step at 95 °C for 20 s was followed by 40 cycles each including a denaturation at 95 °C for 1 s and a combined annealing and extension step at 60 °C for 15 s. A melting curve analysis protocol was executed in the temperature range from 60 to 95 °C, using steps of 0.3 °C and fluorescence reading on hold. For each sample a no-RT control was run and NTC was included in each plate and for each primer pair. A calibration curve was built for each target using 5 cDNA concentrations (equivalent to 62.5, 12.5, 2.5, 0.5, 0.1 ng of total RNA). The StepOne software (ver. 2.1; Applied Biosystems, Monza, Italy) was used for data processing, including the identification of outliers, settings of thresholds, and C_q exporting. MIQE précis guidelines[9] were followed (details are provided in Supplementary File 1 in the Supporting Information).

Determination of Transcript Stability of Putative Reference Genes and Quantification of Target Gene Expression. Two algorithms were used to assess expression stability: genorm^{PLUS} (included in the qbase^{PLUS} package; trial version; Biogazelle, Zwijnaarde, Belgium[7,19]) and NormFinder (ver. 0.953;[18]). Amplification cycles (C_q values) were exported using the StepOne software, converted into correct input files, and uploaded into software applications. qbase^{PLUS} was also used to calculate the expression levels of PG and FPS for the validation of the selected reference genes.

RESULTS

Comparison of RNA Extraction Methods from Olive Fruits and Leaves. Extracting total RNA of good quality and quantifying it correctly are mandatory requirements for qPCR to be performed reliably. To this end, we have preliminarily evaluated three methods for RNA extraction from olive fruits and leaves for their performance in providing RNA of good quality, based respectively on affinity purification of RNA on silica columns from commercial kits and already successfully used on olive fruits and shoots,[1,29,30] on the use of a CTAB extraction buffer (previously employed for grapes[22]), or on the "hot borate" procedure developed for RNA extraction from recalcitrant plant tissues[23] and already used, with no modifications, on olive fruits.[24] We have further improved this latter method to obtain RNA of high quality also from olive leaves by applying some modifications, including a final column affinity purification step (as described in Materials and Methods). Total RNA yield (μg/g of fresh weight), absorbance ratios (260/230 and 260/280), and RIN[31] values were calculated from at least three independent replicas for each tissue to compare the quality of RNAs extracted with these methods and are shown in Table 2. High quality RNA was reproducibly obtained from both fruit and leaf tissues by means of the column affinity purification (commercial kit) and of the hot borate methods, which gave higher yields and showed 260/280 ratios and RIN values always higher than 2 and 7, respectively. 260/230 ratios resulted to be higher than 2 for RNA extracted by the hot borate and CTAB methods and

Table 2. Comparison of Three RNA Extraction Methods from Olive Tissues[a]

	yield (μg of total RNA/g of frozen tissue)	260/280 ratio	260/230 ratio	RIN range
hot borate	132 ± 57	2.13 ± 0.03	2.22 ± 0.14	7.2−7.5
commercial kit	159 ± 71	2.12 ± 0.01	1.65 ± 0.69	8.2−8.6
CTAB	58 ± 21	2.04 ± 0.03	2.20 ± 0.38	6.7−7.2

[a]Yield and absorbance ratios (260/280 nm and 260/230 nm) are average values of at least six replicates (three for fruit and three for leaf tissues) with standard deviation. The range of RIN[31] shows minimum and maximum values obtained by the different extraction methods.

slightly lower than 2 for RNA extracted by affinity purification on silica columns. A lower yield and RIN values lower than 7 were obtained with the CTAB extraction method (Table 2). Total RNA extracted by the hot borate method was used for all subsequent experiments, due to its higher 260/230 absorbance ratios and satisfactory RIN values (higher than 7), even though the highest RIN values (>8) were obtained for RNA extracted by using silica columns from commercial kits.

Selection of Candidate Reference Genes and Evaluation of Primer Pair Performance. For the selection of candidate reference genes, we could not adopt a data mining approach on publicly available microarray data repositories for genes that may display stable expression throughout different tissues, conditions, and developmental stages, since this kind of data are still unavailable for *Olea europaea*. Therefore, previously validated reference genes from other species were retrieved and orthologous genes were searched for in the available olive *Olea*ESTdb (http://140.164.45.140/oleaestdb/[1]). For this purpose, an in-depth bibliographical search was performed to gather all studies regarding validations of reference genes, with particular regard to those concerning horticultural species. Genes that were frequently ranked as the best reference genes for a relatively high number of species[11−14,17,32−45] were selected as potential candidates (Table 3). Among these genes, the transcript of elongation factor 1-α was reported to be equally abundant in at least 13 different species. Additional genes that were selected for their uniformity of transcript abundance on the basis of literature data were actin (3 species), glyceraldehyde 3-phosphate dehydrogenase (4 species), serine/threonine protein phosphatase 2A (4 species), and ubiquitin (3 species) (Table 3). 14-3-3 protein was also considered, although it had been ranked as one of the best only in coffee. Polyubiquitin was tested not only for the good stability of its transcript levels in blueberry, cotton, and poplar but also because it had been already adopted in a previous work regarding olive fruit development and ripening.[5] Similarly, a ubiquitin-conjugating enzyme encoding gene was found to be a useful reference in peach,[46] *Arabidopsis*, and blueberry, and defined as one of the most stable genes in a maize atlas obtained with large scale microarray analyses.[47] Tubulin was not studied since controversial data have been reported on its reliability as a reference gene, being considered one of the best in certain species and among the worst ones, according to the ranking order, in others.[39,41,42]

To retrieve olive expressed sequences, we referred to the *Olea*ESTdb (http://140.164.45.140/oleaestdb/[1]), selecting contigs made up of a high number of ESTs. Primer pairs were designed on these sequences (Table 1), each one producing an amplicon ranging between 75 and 142 bp in length (excluding the already designed pair for polyubiquitin producing an amplicon of 191 bp, as described in ref 5). Since the selected candidate transcripts belonged to multigene families, two genes were chosen and tested for each gene family (shown in Table 1). For polyubiquitin, only the mRNA previously published in ref 5 was kept for analysis.

Primers were first tested on a pool made of all the olive cDNA samples used in this study. All primer pairs gave a melting curve with a unique peak (Supplementary File 2 in the Supporting Information), except for primer pairs of the second transcript of 14-3-3 and UBQ. These two transcripts were discarded from subsequent analyses. Dilution series of the pooled cDNAs were amplified to calculate the efficiency of primers that ranged between 1.891 and 2.155, with a calculated standard error always lower than 0.007 (Table 4).

Table 3. List of the Candidate Reference Genes Selected for This Study and Corresponding Species for Which They Had Been Validated as Best qPCR Reference Genes

best reference genes	plant species
14-3-3	*Coffea*[32]
actin	chicory[33]
	grapevine[11]
	pea[34]
elongation factor 1-α	chicory[33]
	Chinese cabbage[35]
	chrysanthemum[36]
	fava bean[37]
	flax[38]
	grapevine[11]
	litchi[39]
	poplar[40]
	potato[12]
	rice[13]
	tobacco[41]
	zucchini[42]
glyceraldehyde 3-phosphate dehydrogenase	*Coffea*[32]
	flax[38]
	grapevine[11]
	litchi[39]
polyubiquitin	blueberry[43]
	cotton[44]
	poplar[40]
serine/threonine protein phosphatase 2A	*Arabidopsis*[45]
	chrysanthemum[36]
	pea[34]
	tobacco[41]
ubiquitin	banana[14]
	peach[17]
	rice[13]
ubiquitin-conjugating enzyme	*Arabidopsis*[45]
	blueberry[43]

Expression Stability of Candidate Reference Genes. The selected primer pairs were used to amplify 21 cDNA samples, obtained from leaves and fruits from different developmental stages and treatments, using a sample maximization strategy.[19] In order to have the most complete coverage of the olive fruit developmental stages, mesocarp samples were collected starting from pit hardening until complete ripening (overripe fruits). All data arising from amplifications were used to calculate the expression stability of the candidate reference genes.

A C_q box plot was obtained for each transcript (Figure 1). The 13 considered genes covered a range between 17 and 31 C_qs. The coverage was quite complete except for a small gap around 22 C_q. This analysis enabled a first evaluation of the most stable genes, since a lower span in C_qs corresponds to a higher stability: for example PP2A2 had a limited span, thus a higher uniformity of transcript abundance in comparison with GAPDH1. In order to identify the most stable putative reference genes, two different independent algorithms were used: genorm^{PLUS}[7,19] and NormFinder.[18] genorm^{PLUS} returned M and V parameters. M was defined as the average pairwise variation of a particular gene against all other control genes.[7] Under the assumption that putative reference genes are not

Table 4. Amplicon T_m, C_q of NTC, and PCR Efficiency Calculated from Standard Curve Slope of Selected Candidate Reference Genes

gene	amplicon T_m (°C)	NTC	std curve slope (±SE)	r^2	PCR effic calcd from slope (±SE)
14-3-3	78.32	undetermined	−3.386 ± 0.019	0.996	1.974 ± 0.007
ACT7a	77.27	undetermined	−3.301 ± 0.008	0.999	2.009 ± 0.003
ACT7b	78.31	undetermined	−3.225 ± 0.010	0.998	2.042 ± 0.004
EF1a	76.97	37.21	−3.302 ± 0.008	0.999	2.008 ± 0.003
EF1b	78.32	37.15	−3.409 ± 0.005	0.999	1.965 ± 0.002
GAPDH1	77.12	undetermined	−2.999 ± 0.009	0.998	2.155 ± 0.005
GAPDH2	75.64	undetermined	−3.253 ± 0.012	0.997	2.029 ± 0.005
PP2A1	73.70	undetermined	−3.535 ± 0.011	0.998	1.918 ± 0.004
PP2A2	72.34	undetermined	−3.484 ± 0.016	0.997	1.937 ± 0.006
UBC1	72.80	37.12	−3.236 ± 0.009	0.998	2.037 ± 0.004
UBC2	77.26	undetermined	−3.422 ± 0.015	0.996	1.960 ± 0.006
UBQ	80.85	undetermined	−3.613 ± 0.014	0.998	1.981 ± 0.005
OUB2	77.27	37.06	−3.472 ± 0.009	0.999	1.941 ± 0.004

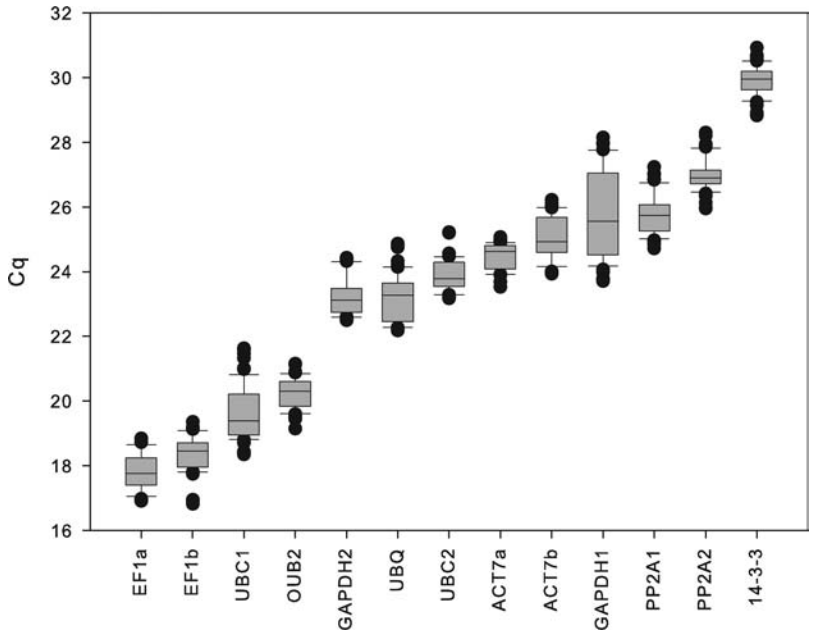

Figure 1. Box plot analysis of C_qs obtained in the whole sample set for each gene. Each box indicates 25/75%iles, with the median line dividing the box in two parts. Whisker caps represent 10/90%iles. Dots indicate outliers.

coregulated, a stepwise exclusion of the less stable gene was performed and *M* values of the remaining genes were calculated accordingly. The most stable genes were chosen among those displaying the lowest *M* values, considering 0.5 as a good threshold.[19] The *V* parameter represents the pairwise variation (V_n/V_{n+1}) between two consecutively ranked control genes. A minimum *V* cutoff threshold of 0.15 was recommended[7] to determine the optimal number of reference genes. The NormFinder algorithm ranks putative reference genes according to the SV parameter defined as the combination of inter- and intragroup variation.[18] Similarly to genorm^{PLUS}, genes with the lowest SV were defined as the most stable ones. Analyses were first performed on all samples; then, in a second round, fruit and leaf data were considered separately. For what concerns genorm^{PLUS} analysis (Figure 2a), 12 out of the 13 genes displayed an *M* value under the acceptable stability threshold of 0.5 indicated by ref 7. GAPDH1 was the least stable gene with an *M* greater than 0.5 ($M = 0.609$). According to the *V* parameter (Figure 2b), the optimal minimum number of reference genes required to have a pairwise variation under the threshold of 0.15 resulted to be two ($V_{2/3} = 0.088$). Thus the two genes PP2A1 and GAPDH2, that resulted to be the most stable ones by displaying *M* values of 0.216 and 0.244, respectively, would be sufficient to reach a good normalization of data for this experimental set.

The same data processed by the NormFinder algorithm showed consistently similar results (Figure 3a) but with slightly different ranking positions in comparison to genorm^{PLUS}. Stability values (SV) of the GAPDH1 (SV = 0.694) and PP2A1 (SV = 0.065) genes confirmed them as the worst and the best in terms of steadiness of transcript abundance, respectively. PP2A2 (SV = 0.066) was ranked as second in place of GAPDH2 (SV = 0.106), which resulted to be fourth. NormFinder also indicated that SV decreased to 0.053 if the best two reference genes were used in combination in place of the best one alone (PP2A1, SV = 0.065). Since samples could be divided into two subsets, corresponding to leaf and fruit tissues, NormFinder allowed calculation of both intergroup and intragroup variation (Figure 3b). A positive intergroup variation was given when the gene showed systematically higher

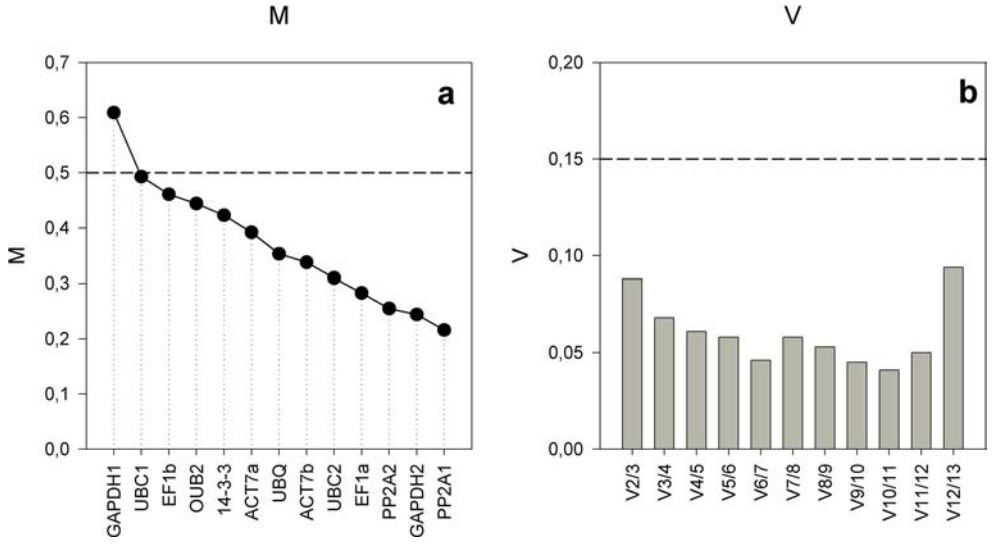

Figure 2. genorm^{PLUS} analysis of candidate reference genes in the fruit and leaf sample set considered as a whole. Genes are ordered by descending *M* values (a), and the minimum number of genes required for normalization is indicated by means of *V* parameter (b). Threshold levels are indicated by dashed lines.

expression in fruits than in leaves, while a negative intergroup variation was given in the opposite case. Intragroup variation is represented by error bars in Figure 3b. From this analysis it was evident that the most stable genes PP2A1, PP2A2, EF1a, GAPDH2, and UBC2 (shown in Figure 3a) displayed intergroup variation close to zero (Figure 3b) by having comparable stability in fruits and leaves.

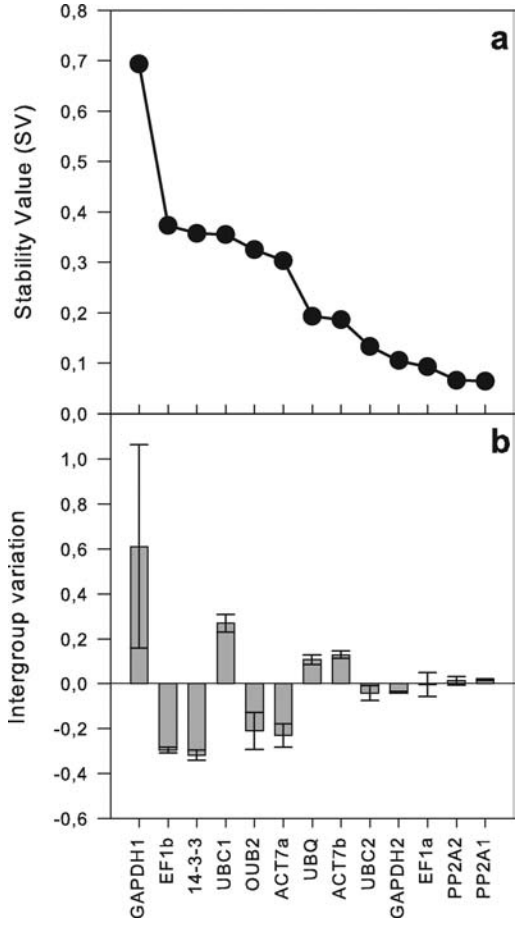

Figure 3. NormFinder analysis of candidate reference genes for the whole sample set. SV (a) and intergroup variation (b) are reported. Error bars (b) represent intragroup variation.

genorm^{PLUS} and NormFinder analyses were then conducted separately on the two subsets of fruits and leaves samples. As far as fruits are concerned, the two algorithms gave the same results: GAPDH1 was the least stable gene (*M* = 0.438; Figure 4a) (SV = 0.672; Figure 4e) and PP2A1 the most stable one (*M* = 0.173; Figure 4a) (SV = 0.060; Figure 4e). According to the genorm^{PLUS} *V* parameter, the optimal number of reference genes was two (Figure 4c). The second reference gene indicated by genorm^{PLUS} was EF1b (*M* = 0.190; Figure 4a). Differently, GAPDH2 (SV = 0.062; Figure 4e) was ranked by NormFinder at the second place.

In leaves, genorm^{PLUS} indicated that two reference genes were needed as well (*V* = 0.054; Figure 4d). ACT7a (*M* = 0.144) and ACT7b (*M* = 0.155) were the most stable ones (Figure 4b), while OUB2 was the least stable (*M* = 0.375), even if with *M* values below the stability threshold of 0.5 (Figure 4b). Comparably, also in NormFinder OUB2 was the last in the ranking order (SV = 0.295; Figure 4f), while the two most stable genes for leaves resulted to be 14-3-3 (SV = 0.045) and PP2A2 (SV = 0.106) (Figure 4f).

Validation of Reference Genes for Olive Fruit Development and Ripening. In order to test the suitability of the reference genes validated in our sample set, two transcripts were quantified throughout olive fruit development and ripening.

For this aim a putative polygalacturonase (PG; EC 3.2.1.67) and a farnesyl pyrophosphate synthase (FPS; EC 2.5.1.1) were selected. These genes have not been previously studied in olive and were chosen on the basis of their specific pattern of mRNA accumulation during tomato berry development, the model for climacteric fruit ripening. PG is considered a classical marker of fruit ripening and of loss of firmness in fleshy fruits, since its expression increases significantly in the last stages of fruit ripening along with softening in several fruits including tomato.[48,49] FPS was selected for its peculiar biphasic accumulation pattern in tomato berries: high mRNA levels in young fruits followed by a peak at the "late breaker" stage.[50]

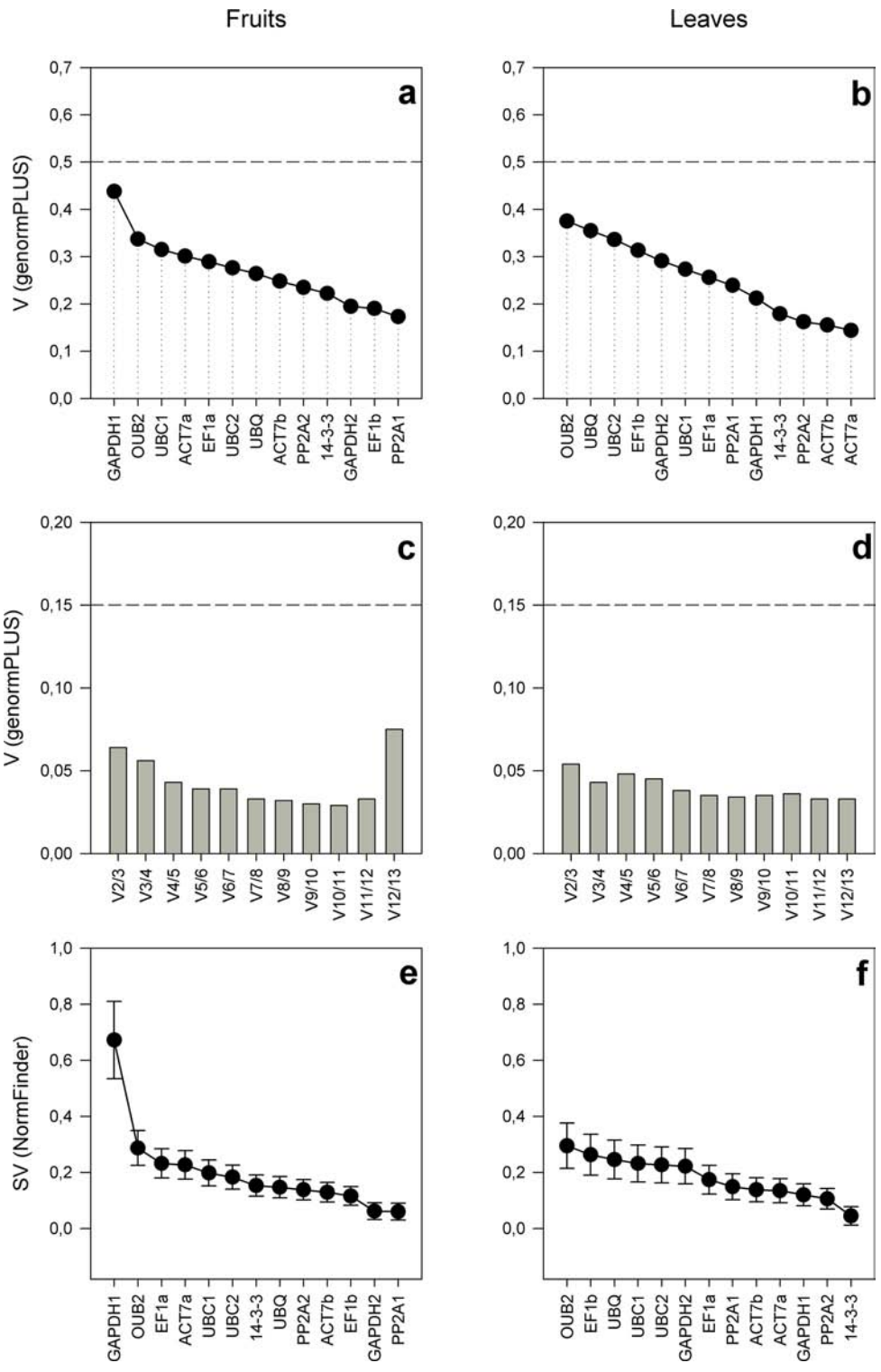

Figure 4. genorm^{PLUS} and NormFinder analysis of candidate reference genes in fruit and leaf samples. For each tested gene genorm^{PLUS} *M* parameter is reported in fruits (a) and leaves (b). The minimum number of reference genes to be used is indicated by genorm^{PLUS} *V* parameter for both fruits (c) and leaves (d). Threshold levels are indicated by dashed lines. NormFinder SV parameter is reported for putative reference genes in fruit (e) and leaf (f) tissues considered separately. Error bars represent SE.

The accumulation of olive PG and FPS transcripts during fruit development was referred to PP2A1 (the best reference gene according to genorm^{PLUS} and NormFinder), to GAPDH1 (the worst one), and to the combination of the best two ones (PP2A1 and GAPDH2) to highlight the effect of the choice of reference genes on expression patterns. PG transcript accumulation was steady until 116 DAFB and then rapidly and progressively increased reaching a maximum at 160 DAFB with an induction that resulted to be 200 or 1600 times, with respect to the level detected at the first sampling date, depending on whether the worst or the best ranked gene was used as reference, respectively (Figure 5a).

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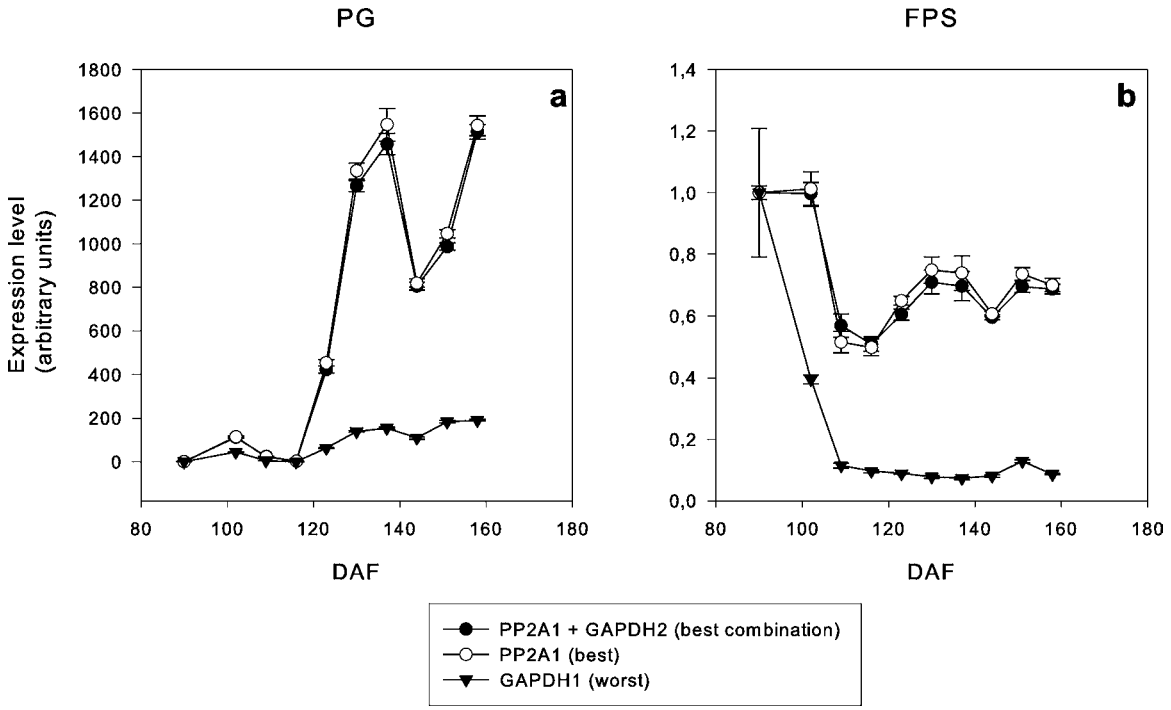

Figure 5. Expression profiles of PG and FPS during fruit development by means of PP2A1 (the best reference gene), GAPDH1 (the worst one), and PP2A1 + GAPDH2 (the best two indicated by genorm^{PLUS}). Expression levels are indicated in arbitrary units, and the sample at 90 DAFB is used as a reference setting the expression value to 1. Errors bars represent SE.

FPS showed even more different profiles if GAPDH1 or PP2A1 were used (Figure 5b). In the first case, FPS transcripts levels decreased until 109 DAFB and then appeared overall stable throughout ripening. On the contrary, when PP2A1 was used as reference, the transcript profile of olive FPS reflected the typical biphasic behavior of tomato FPS, showing high levels of transcript accumulation during the first phase of fruit growth followed by a decline and a transient peak in the late stages of véraison.

For both genes no significant differences could be observed when the combination of the top two reference genes was adopted as reference instead of the best one alone.

DISCUSSION

Commercial interest in olives and olive oil is fostering molecular studies on this plant species. Recently, significant steps ahead have been made in the large scale identification of olive transcripts[1,2] and an increasing number of reports have been published dealing with targeted gene expression analyses on olive developmental and physiological processes. For the reliable fulfillment of such surveys, well-established strategies for mRNA quantification are needed. In recent years, qPCR has become the preferred method for gene expression studies, even if this technique is far from being defined as a "gold standard". Recent reports have set the minimum information needed for publication of qPCR experiments (MIQE[8,9]), but few studies have been performed complying with these rules to date.[51] Many aspects can affect qPCR experiments importantly, and one of these is the quality of the RNA template. As a preliminary step, we have comparatively evaluated the quality of the RNA extracted from olive fruit and leaf tissues by using three procedures based on affinity purification columns from commercial kits, on the use of a CTAB or of a "hot borate" extraction buffer, respectively. In addition, the latter procedure was optimized by modifying the method described in ref 23 to enable extraction of high yield of RNA of satisfactory quality from both olive leaf and fruit tissues. Extraction methods based on the "hot borate" procedure and on affinity purification (commercial kit) enabled the efficient extraction of high quality RNA characterized by RIN values higher than 7, optimal 260/280 nm absorbance ratios (≥2), and 260/230 nm absorbance ratios that resulted satisfactory (≥2) for the "hot borate" method and slightly suboptimal (≤2) for the affinity column (commercial kit) extraction procedure, respectively. On average, the CTAB procedure resulted in lower yields and RIN values.

An additional major factor that affects dramatically the accuracy of qPCR results is the selection of reference genes for data normalization to eliminate nonbiological variation. It is widely known that the choice of internal controls may be one of the most critical points, because a wrong choice may bring about misleading results.[6] It has been ascertained that the so-called "housekeeping genes" do not exist,[7] especially when samples coming from very different experimental conditions are compared. Thus, during recent years the number of preliminary screenings on candidate reference genes has grown rapidly. These works have provided the guidelines for careful selection of reference transcripts, allowing a more efficient use of resources and time. In this paper we have selected 13 potential reference genes for *Olea europaea* and we have tested them in 21 olive samples obtained from mesocarp of fruits at 10 successive developmental stages (ranging from 90 DAFB until full ripening, 160 DAFB), of fruits treated with propylene or with the inhibitor of ethylene action 1-MCP and from leaves subjected to abiotic stress (wounding) in combination with treatments with propylene or 1-MCP. Since extensive microarray expression data are not available for olive, the selection of the candidate reference transcripts for this work was made by

choosing olive genes orthologous to the best ranked reference genes from other crops. All the most commonly used reference genes were included in this analysis, except tubulin and 18S rRNA. These two genes were discarded because the stability of their transcript levels appeared to be quite controversial, especially in the case of 18S.[7,52] Overall, this process led to the identification of 13 *Olea europaea* potential reference genes, belonging to 8 gene families.

To evaluate these genes in terms of uniformity of transcript abundance, qPCR experiments were designed to comply at best with MIQE précis guidelines.[9] Due to the lack of genomic data, primer pairs were designed on the available *Olea*ESTdb sequences.[1] The selected genes were first subjected to melting curve analysis to exclude the presence of multiple amplicons. By plotting the C_q distribution for each selected gene, it has been possible to gain hints on which genes had the lowest variation among all samples. GAPDH1 showed the widest variation (roughly a span of 4 C_q) and thus was the least stable gene. This analysis was also useful to demonstrate that the stability of transcript abundance was independent from the average C_q, as more stable genes were not specifically associated with high or low C_qs. Furthermore, the selected genes covered a wide C_q range and thus represented a set of reference genes encompassing a wide range of transcript abundance that may be chosen to study differently abundant targets, since an ideal reference gene should have an expression level comparable to that of the target gene under investigation.[53]

In order to determine the best reference genes in this experimental plot, expression values were analyzed by two algorithms, each based on different calculation principles, namely, genorm^{PLUS}[7,19] and NormFinder.[18] Considering all samples, genorm^{PLUS} classified 12 genes, out of the 13 selected in total, as stable. This percentage of success was quite high if compared to previous works. Probably this could be explained also by the fact that the whole procedure was optimized, starting from a high-quality RNA to the subsequent steps, thus minimizing nonbiological errors. The most stable gene was PP2A1, which was first identified in whole transcriptomic studies carried out on *Arabidopsis*[45] and shown to have high stability also in other species. Also NormFinder analysis, even though not providing a cutoff value, ranked PP2A1 as the most transcriptionally stable and the best one considering the fruit development sample series. The genes that had the best and the worst ranking values were consistently identified by both software applications, thus highlighting the robustness of their transcriptional stability and their value as reference genes. Nonetheless, some minor discrepancies could be observed in the resulting lists: for example GADPH2, which was ranked second according to genorm^{PLUS}, was ranked fourth according to NormFinder. However, this inconsistency is a normal effect due to the different algorithms used, as already observed previously,[15,17] that results on minor differences in the ranking order between genes characterized by similar stability but that does not reflect significant differences in terms of steadiness of transcript abundance.

For what concerns leaves, different ranking orders were obtained by using the two algorithms. It has to be pointed out that all the 13 transcripts were found to be suitable, their genorm^{PLUS} M value being below the acceptable threshold of 0.5. The differences in terms of stability were extremely low, and this could explain why the ranking orders given by the two algorithms did not overlap in leaves. On the other hand, similar divergences were also experienced previously.[16]

As a remark, splitting the two tissue (fruit and leaf) subsets further demonstrated that it is mandatory to validate internal controls for each experimental plot/subset. This notion was also supported by NormFinder analysis of the intergroup variation. For genes having a SV > ∼0.2, intergroup variation was quite far from zero, indicating that those genes showed systematically higher expression levels in one subset compared with the other one.[18]

Regarding the number of reference genes to be used, two genes were shown to be enough for normalization according to geNorm^{PLUS} analyses. This result was valid for the overall data set and also for fruits and leaves considered separately. Notwithstanding multiple reference genes have been considered a gold standard for qPCR data normalization, it was frequently observed that two was the optimal number in several experimental plots.[14,16,42]

To further demonstrate the importance and reliability of the reference genes identified in this work, especially for olive fruit development studies, the expression profiles of two fruit development- and ripening-related genes (PG and FPS) were studied. The expression profiles obtained for olive PG and FPS in fruits were similar to those already described for their orthologues in tomato fruit, when either the best reference gene alone or the best combination (two best reference genes combined) was considered. On the contrary, a significant difference was observed in the resulting pattern when the worst internal control was used instead of the best one, further highlighting the fact that using validated reference genes is mandatory for qPCR studies to be reliable and to avoid pitfalls.

As concluding remarks, in this work two essential steps were studied to enable optimal performance of qPCR experiments in olive. An optimized protocol for RNA extraction from olive leaf and fruit tissues was implemented and compared with currently used procedures. Furthermore a list of validated primers pairs was evaluated for the amplification of putative reference genes for use in future qPCR studies in olive. The effectiveness of these primer pairs was tested with success on a fruit developmental series. To the best of our knowledge, this is the first study providing validated reference genes for gene expression studies in olive (*Olea europaea L.*). Even though reference genes must be selected and validated for each individual experiment, nevertheless those described in this work will be of general use for future qPCR studies by providing a guideline and a source of reference genes to be tested on specific RNA samples and particularly on those dealing with olive fruit development and ripening.

■ ASSOCIATED CONTENT

Supporting Information

Supplementary File 1, MIQE précis checklist consisting of a detailed list of all the parameters involved in qPCR workflow, starting from sample handling to expression data analysis[6], and Supplementary File 2, melting curve analysis of the candidate reference genes considered in the present study and of PG and FPS. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

14-3-3, 14-3-3 protein; 1-MCP, 1-methylcyclopropene; ACT, actin; bp, base pairs; CTAB, hexadecyltrimethylammonium bromide; DAF, days after flowering; EF1, elongation factor 1; EST, expressed sequence tag; FPS, farnesyl pyrophosphate syntase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NTC, no template control; OUB, polyubiquitin; PG, polygalacturonase; PP2A, protein phosphatase 2A; qPCR, quantitative real-time polymerase chain reaction; RT, reverse transcription; SV, stability value (NormFinder); UBC, ubiquitin conjugating enzyme; UBQ, ubiquitin

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