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Identification of internal control genes for gene expression studies in olive mesocarp tissue during fruit ripening

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ABSTRACT

Olive (*Olea europaea* L.) is one of the most important fruit trees in the Mediterranean zone. For olive gene expression studies, reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) is the most used and chosen technique in addition to the use of appropriate reference genes. So in this context, in our research, we analyzed six different genes of control in order to select the most stable one during olive fruit development. We used ten samples from five periods of fruit maturity of two different olive cultivars "Chemlali Sfax" and "Chétoui" during the year 2016. The profiles of expression of studied genes were assessed in three replicates. To analyze the results, we tried to use two Excel-based software: GeNorm and Bestkeeper. It results that software packages selected Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and Elongation factor-2alpha (*EF-2α*) as stable gene in Chétoui and across cultivars. *GAPDH* and *ACT1* were found as the steadiest ones in Chemlali Sfax. Our study suggests the use of both *GAPDH* and *EF-2α* as two reference genes for the normalizing RT-qPCR data in future researches of olive fruit tissue development.

1. Introduction

Olea europaea L. is a species of fruitful tree, known and cultivated in different countries of the Mediterranean basin. Olive oil is considered to be an economically important product, especially for the producing countries, such as Tunisia, where it plays a very important role in the economy of the country. An increasing attention is being paid for its health benefits (Pérez-Jiménez et al., 2007). These characteristics are strongly related to the composition of olive oil (particularly its high monounsaturated fatty acids) and to numerous active bio-molecules synthesized during fruit development process.

Researches in the topic of the characterization of molecular aspects of olive fruit development provide a large and rising quantity of information that becomes accessible (Nonis et al., 2012; Ray and Johnson, 2014). Last years, researchers explored the transcriptome of olive drupes during different developmental stages and tissues using Next generation sequencing (NGS) technologies (Alagna et al., 2009). Data resulting following these sequencing experiments are an important source for gene detection and characterization in olive. But, in the case of gene expression studies in olive tree, it becomes important to determine a reference gene that should be stable and proves a reliable expression in olive samples for particular experimental conditions and different type of tissues.

Real time quantitative polymerase chain reaction (RT-qPCR) is widely used for applications ranging from genotyping, gene expression analysis, miRNA and non-coding RNA analysis, and many other purposes. It is an effective technique to study the variation in levels of gene expression. It is a commonly used method for gene expression due to its high precision, but we should take in consideration that gene expression can be affected by diverse factors. So, it is necessary to use reference genes as an internal control for the reaction to raise the precision of gene quantification and to reduce the effect of such factors. Endogenous gene of control should not vary under diverse experimental conditions (Gachon et al., 2004).

Frequently, genes used as control are considered to have a standardized expression level under a series of diverse conditions, and they are involved in basic cellular processes (Resetic et al., 2013). The most used reference genes above the last few years in both plants and animals consist of *EF-1α*, *GAPDH*, *ACT1*, *18S* or *26S RNA*, *α-tub*, Ubiquitin carrier protein, TATA-Box binding protein and β-tubulin (Czechowski et al., 2005; Jarošová and Kundu, 2010; Gamm et al., 2011; Long et al., 2011).

In recent years, three research works were carried out in order to select steady housekeeping genes in olives through fruit maturity.

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Thirteen housekeeping genes have been evaluated by Nonis et al. (2012) on olive fruits and leaves samples (from the variety Frantoio) above various maturity periods. In this study, they demonstrated that *PP2A1*and *GAPDH2* were the best housekeeping genes for olive fruit maturity and development in cited cultivar. A similar study have been carried out by Resetic et al. (2013) using 29 genes in Istrska Belica variety and identified *TATA* binding protein (*TBP*) and TIP41-like family protein (*TIP41*) genes as the best ones for RT-qPCR analysis in olive fruit development, whereas some classical reference genes showed not to be appropriate. Ray and Johnson (2014) conducted a combined analysis using 8 housekeeping genes on three different olive cultivars to evaluate the appropriateness of traditional reference genes and discover the most relevant ones for different developmental stage of maturation. They concluded that *EF-1α*, *GAPDH*, *60S RBP L18-3*, *OUB2* and *PP2A* were the most stables genes across the 3 studied cultivars. Taken together, the data from the last three reports indicate that the stability of housekeeping genes have to be confirmed for every experimental set and especially for each cultivar.

We believe that this is the first work that aims on the validation of housekeeping genes for RT-qPCR analysis on Tunisian olive cultivars. Therefore, six commonly used housekeeping genes were analyzed in order to select the steadiest ones in olive fruit development. We used different samples from five periods of olive fruit maturity of "Chemlali Sfax" and "Chétoui" cultivars. Analysis of RT-qPCR results were achieved using two Excel-based software: GeNorm and Bestkeeper.

2. Material and methods

2.1. Olive samples collection and origin areas

The selection of variety "Chemlali Sfax" plants was performed in the region of Sfax (Center of Tunisia), and "Chétoui" was collected from the North of Tunisia (Beni Khalled region, Governorate of Nabeul). The olive fruits were taken during different periods of mesocarp ripening. Sampling was done once a month from July until the end of December 2016. Immediately after harvesting, fruits were congealed at −80 °C.

2.2. Isolation of RNA and synthesis of cDNA

We isolated total RNA from the mesocarp of olive fruit tissues from olive cultivars Chemlali Sfax and Chétoui using RNA Extraction Kit 'PureLink RNA Mini Kit' (Thermo Fisher Scientific) using protocol given by the manufacturer. The quality of RNA was checked by the integrity of RNA bands on 1% agarose gel electrophoresis, which showed intact and intense RNA bands (18S and 28S). Concentrations of RNA and A260/A280 ratios were measured by spectrophotometer, and samples were stored at −80 °C. One microgram of every RNA sample was transformed by reverse transcription to cDNA using cDNA Synthesis Kit (Invitrogen SuperScript® III First-Strand Synthesis System) using random hexamer primers. Obtained cDNA samples after reverse transcription were stored at −20 °C.

2.3. Selection of reference gene

In the objective of selection and validation of the most stable housekeeping genes for RT-qPCR expression analyses in olive tissues, we used primers for six genes designed and available in our laboratory, Alpha-tubulin (*α-tub*), Factor elongation 2-alpha (*EF-2α*), Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), Factor elongation 1-alpha (*EF-1α*), Actin (*ACT1*) and Factor elongation 1-A (*EF-1A*). Those genes have been previously identified and have been used in previous researches as reference for RT-qPCR quantification (Libault et al., 2008; Kozera and Rapacz, 2013; Monteiro et al., 2013; Jiang et al., 2014) (Table 1).

We proved the specificity of the amplicons by detecting a single band with expected size after electrophoresis in 2% agarose gel, and also this was confirmed by the presence of a single peak in the qPCR melting curve.

2.4. Reverse transcription-quantitative PCR assay

We carried out RT-qPCR reactions in 20 μl of solution in a micro plate (48-well) on MJ Mini Opticon Bio Rad thermo cycler (Bio-Rad). Reaction mixes contained the following: SYBR Green Mix (10 μl), an appropriate cDNA dilution (a volume of 2 μl), a final primer concentration of 100 nM and, we complete to 20 with, RNase free water.

The amplification conditions for RT-qPCR were as follows: 95 °C for 30 s; then 45 cycles at 95 °C for 5 s; and 60 °C for 20 s, and finally, we used the melting curve from 65 °C to 95 °C to verify the specificity of primers. A standard curve was done using 5-fold series of dilution points. Efficiencies of reactions were calculated using the following equation: $E = (10^{[-1/\text{slope}]} - 1) - 100$ (Pfaffl et al., 2004).

2.5. Data analysis

In order to classify the expression stability, we used two different types of software: GeNorm (Vandesompele et al., 2002) and BestKeeper (Pfaffl et al., 2004). For BestKeeper analysis, we used data directly for stability calculations, and in the case of the GeNorm analysis, we converted into relative quantities using the formula 2-ΔCq. (ΔCq = the corresponding Cq value − minimum Cq). The raw data are listed in Table 2. We can consider a gene as the most stable when it has the lowest M value (Vandesompele et al., 2002).

In addition, we used a useful tool using algorithms of four programs (GeNorm, Normfinder, BestKeeper and ΔCq) and the RefFinder (Xie et al., 2011), which gives a proper weight to each gene and counts the geometric mean of these weights for a definitive ranking.

3. Results and discussion

3.1. Evaluation of RNA quality and affirmation of PCR products

The RT-qPCR is the most used technique for gene expression analysis. So as to get convincing results and valid analysis of RT-qPCR, it is

Table 1
List of primers referred to used reference genes.

Gene Symbol	Gene name	Primer	Amplicon size	Efficiency (%)	R^2
EF-1α	Elongation factor 1-alpha (contig1)	F: CCCCTTCGTCCCCATCTCTGGT R: ACACGACCCACTGGCACCGT	201 pb	100	0.991
EF-2α	Elongation factor 1-alpha (contig2)	F: CCCCTTCGTCCCCATCTCTGGT R: GGAGGGGCTTGTCTGAAGGCC	140 pb	98	0.995
EF-1A	Elongation factor alpha	F: GCACGGTCATTGATGCCCCTGGA R: TACCCCAACCTTCCTCCAAGTAGG	293 pb	90	0.892
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	F: GGAGCTGCCAAGGCTGTCGG R: AGCTTGTCCTCAGATTCCTCCTTGA	176 pb	91	0.973
α-tub	Alpha tubilin	F: ACAACTTCGCCCGTGGGCAC R: GCTGCGTTGACGTGCTTGGG	691 pb	94	0.870
ACT1	Actin	F: AGCACCTGAAGAGCACCCGGT R: GCACAATACCAGTACGCCAC	170 pb	91	0.990