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Stable reference genes for studies of gene expression in *Prunus persica* under water stress

Leticia Neutzling Rickes*, Elsa Kuhn Klumb, Letícia Carvalho Benitez, Eugenia Jacira Bolacel Braga, Valmor João Bianchi

Institute of Biology, Departament of Botany, University Federal of Pelotas, Campus Universitário, s/n C.P. 345, Capão do Leão, RS, Brazil

***Corresponding author: leticiarickes@hotmail.com**

Abstract

Strategies which allow the unequivocal identification of genes expressed in response to different treatments and/or stress conditions are important to obtain reliable results in plant physiology studies. However, in order to analyze the expression levels of target genes through RT-qPCR technique, it is essential to use model genes with uniform expression levels under specific experimental conditions. The present study aimed at investigating the stability of reference genes in peach leaves cultivar scion Chimarrita grafted onto 'Aldrighi 1' and 'Tsukuba 2' rootstock, subjected to water deficit for a 9-day period. The experiment was conducted in a completely randomized design with four treatments that correspond the evaluations period: zero (control), 4th, 7th, and 9th stress day of water deficit. For each treatment three biological replicates was used. Eight reference genes were analyzed, among them: *ACT*, *CYP2*, *Ef-1α*, *GAPDH*, *TUA*, *TUB*, *UBQ10* and *18SrRNA*. For the normalization and validation of RT-qPCR data were used the four major software programs currently available: geNorm, NormFinder, BestKeeper, and Comparative ΔC_T. The results showed that there was no influence of different plant grafting combinations in the expression of all reference genes evaluated, yet genes *TUA* and *CYP2* had the most stable expression in the leaves of the peach plants under water deficit for 9-d, whereas genes *Ef-1α* and *ACT* were the least stable ones for the same stress conditions.

Keywords: cyclophilin 2; rootstock; RT-qPCR; water stress, α-Tubulin.
Abbreviations: *ACT*_actin; *αTUA*_alfa-tubulin; CV_coefficient of variation; C_T_Cycle Threshold; *CYP2*_Cyclophilin 2; *Ef-1α*_Elongation factor 1-α; *GAPDH*_Glyceraldehyde-3-phosphate dehydrogenase; RT-qPCR_Reverse transcription quantitative PCR; SD_standard deviation; *UBQ10*_Ubiquitin10; *18SrRNA* _18S ribosomal RNA.

Introduction

Peach (*Prunus persica* L. Batsch) is one of the most important fruit species of temperate climate worldwide (Luo et al., 2014). However, among abiotic factors, water stress is a common phenomenon that seriously affects the yield of this crop at some phenological stages, such as flowering which can lead to the abortion of flowers and fruits, in the intense cell division period of fruits, as well as at the stage preceding fruit ripening (Dichio et al., 2006; Timm et al., 2007; Mercier et al., 2009; Wang and Gartung, 2010). Water deficit inhibits the growth and development of plants, directly affecting the photosynthetic process lead to the reduction of stomatal conductance, transpiration, osmotic potential and leaf area, speeding up the senescence and leaf abscission (Chaves et al., 2009), resulting in physiological limitations that may cause severe decreases in plant yield (Nakashima et al., 2014).

Plants have evolved under different environmental conditions and, thus, developed extremely complex molecular and genetic processes important for their survival in such conditions (Le et al., 2012). Many of the primary processes that plants use in face of adverse conditions are not constitutively active all the time, but rather induced when they are present, triggering signal transduction cascades which, therefore, activate/suppress several genes related to stress response, leading to biochemical and physiological changes (Nakashima et al., 2014). There are several studies on *Prunus* using strategies that allow the identification of genes expressed in response to different treatments and/or stress conditions (Almada et al., 2013; Jiménez et al., 2013; Niu et al., 2014; Arismendi et al., 2015), however, it is necessary that in such trials a few genes are selected as candidate genes, which must be validated in more specific analyses in order to quantify their expression in response to stress. One technique widely used for the determination of gene expression is RT-qPCR (quantitative RT-PCR) based on the procedure of reverse transcription (RT) followed by DNA-Polymerase Chain Reaction (PCR). The outcomes are monitored at each reaction cycle, enabling faster and more accurate detection of amplified cDNA with high sensitivity and specificity in the analysis of transcripts (Gachon et al., 2004). The reliability of RT-qPCR technique depends on specific experimental strategies which seek to minimize variations in quality, stability, and integrity of RNA, as well as on the efficiency of cDNA synthesis (Derveaux et al., 2010). Among such strategies, the selection of reference genes suitable to normalize the data is essential to provide accurate results and reliable interpretation. A suitable reference gene must be expressed at a constant level among samples and its expression cannot be affected by different experimental conditions (Bustin, 2002). The use of unsuitable reference genes may result in quantifications errors and, subsequently, expression data may be misinterpreted (Jain et al., 2006; Amil-Ruiz et al., 2013).

The reference genes used are generally those involved in basic cellular processes, such as primary metabolism and maintenance of cell structure (Expósito-Rodríguez et al., 2008). Those most commonly tested in RT-qPCR studies in plants include Actin (*ACT*), glyceraldehyde-3-phosphate dehydrogenase (*GADPH*), Elongation factor-1α (*Ef-1α*), Tubulin (*α-TUA* and *β-TUA*), ubiquitin (*UBQ5*, *UBQ10*), ribosomal RNA (*18SrRNA*, *40S*), and cyclophilin (*CYP*) (Jain et al., 2006; Tong et al., 2009; Le et al., 2012; Huang et al., 2014; Hu et al., 2014; Niu et al., 2014; Moraes et al., 2015; Ye et al., 2015; Galli et al., 2015). Nevertheless, studies have shown that the expression of many of these reference genes may have different expression between genotypes, tissues, organs, and developmental stages (Tong et al., 2009; Le et al., 2012; Hu et al., 2014; Niu et al., 2014; Galli et al., 2015; Moraes et al., 2015; Ye et al., 2015). Thus, the selection of suitable reference genes for the normalization of each crop and experimental condition is crucial.

In the past decades, several mathematical methods and software programs were developed to analyze the variability of reference gene expression, such as NormFinder, which evaluates the total variation in gene expression through the sum of the variance (Andersen et al., 2004). BestKeeper, in turn, uses values of standard deviation and coefficient of variation to calculate the intrinsic variance of expression (Pfaffl et al., 2004). The algorithm geNorm selects the two most stable genes or the multiple combination of several stable genes by calculating the mean stability of expression (M) through the arithmetic mean of each normalizing gene compared pairwise (Vandesompele et al., 2002). The ΔC_T method calculates the relative expression of pairs of genes within each treatment by measuring the gene stability through mean standard deviation values comparing a normalizing gene and other candidate genes (Silver et al., 2006).

Considering the *Prunus* plants can be affected by various abiotic stress, in different seasons of the year around the world, the identification of more stable expressed genes in each condition of stress is important to obtain unequivocal gene expression data interpretation. Thereby, the present study aimed at investigating the expression of eight reference genes through RT-qPCR technique in order to identify proper normalizing genes for studies in peach trees under water deficit.

Results

Specificity and efficiency of standard curve of reference genes

Primers amplified specific products which was confirmed by the presence of a single peak of dissociation curves (melting curve), demonstrating the specificity of the PCR product (Fig 1. and Fig 2). By analyzing the curve at the end of the reaction, it is possible to evaluate the fluorescence of the samples in relation to the increasing temperature because each amplicon has a specific fusion temperature, which enables the differentiation of products resulting from PCR (Pfaffl, 2001).

The efficiency of primers amplification in each one graft combinations 'Chimarrita'/rootstock peach plant was based on values obtained from the Logarithm (log) of cDNA dilutions (1:1, 1:5, 1:25 and 1:125). The efficiency of amplification varied between 1.89 and 2.20, indicating that at the end of each cycle, the target transcript was duplicated (Fig 1. and Fig 2). However, there was an exception for the reference gene *18SrRNA* tested in the graft combination 'Chimarrita/Aldrighi 1', which showed an efficiency over the threshold usually acceptable (E = 2.39). The same has not been found for the combination 'Chimarrita/Tsukuba 2' with values of E = 1.92.

For the most gene tested, the coefficient of determination (R^2) were above the minimum value acceptable of 0.7 or 70%, except for the genes *Ef-1α* and *18SrRNA* in the combination 'Chimarrita/Aldrighi 1', which had R^2 bellow the acceptable (R^2 = 0.673 and 0.594, respectively) (Fig 1.), as proposed by Pfaffl (2001).

Standard deviation and coefficient of variation of reference genes

With respect to the standard deviation (SD) and coefficient of variation (CV%), *TUA* and *CYP2* were those genes with the lowest SD (0.40 and 0.36, respectively) and CV% (11.01 and 11.22, respectively) values for the graft combination 'Chimarrita/Aldrighi 1' (Table 2). The same trend has been found for 'Chimarrita/Tsukuba 2' where *TUA* had the lowest SD (0.20) and CV% (8.42) values, indicating higher stability of expression of this gene for both combinations. On the other hand, the *ACT* gene had the highest SD values, between 2.36 ('Chimarrita/Aldrighi 1') and 1.25 ('Chimarrita/Tsukuba 2') and CV% values between 45.71 ('Chimarrita/Aldrighi 1') and 63.44 ('Chimarrita/Tsukuba 2') in comparison to the other genes tested.

Analysis by RefFinder of reference genes

The results obtained with RefFinder were similar in the subset of both graft combinations of 'Chimarrita'/rootstocks of peach trees in the present study. The most unstable gene was *ACT* followed by *Ef-1α* (Fig 3 A-E and Fig 4 A-E), suggesting that these classic reference genes are unsuitable to normalize RT-qPCR data under the experimental conditions investigated in the present study.

According to ΔC_T comparative method, the most stable reference gene under the experimental condition tested (water deficit) was the gene *TUA* with the lowest M values for 'Chimarrita/Aldrighi 1' (1.19) and 'Chimarrita/Tsukuba 2' (0.79), followed by the second most stable gene, *CYP2* (Fig 3A and Fig 4A). The M value is defined by Chen et al. (2011) as the mean variation of a given gene in relation to all the other tested genes.

The standard stability of expression found with the BestKeeper algorithm differed from the other algorithms, evidencing that *18SrRNA* genes in the graft combination 'Chimarrita/Aldrighi 1' and *UBQ10* in the combination 'Chimarrita/Tsukuba 2' were the most stable genes, meanwhile *ACT* followed by *EF-1α* were the least stable ones for both 'Chimarrita/rootstock' combinations (Fig 3B and Fig 4B).

According to the algorithm of the program NormFinder, the reference gene with the lowest M value was *GAPDH* (M = 0.58 – 'Chimarrita/Aldrighi 1'), followed by *TUA* (M = 0.65) (Fig 3C). For the graft combination 'Chimarrita/Tsukuba 2', in turn, the gene *TUA* was the most stable with the lowest M value (0.46) followed by *TUB* (M = 0.53) and *UBQ10* (M = 0.555). In this graft combination, the gene *GAPDH* was highly unstable, ranking in the seventh position (M = 0.74) (Fig 4C). The same has been found with the algorithms of ΔC_T comparative method (M = 0.97) (Fig 4A) and geNorm (M = 0.81) (Fig 4D).

Based on M values calculated for eight genes tested, it was found that *TUA/CYP2* (M = 0.43; M = 0.35) (Fig 3D and Fig 4D, respectively) were the most stable for both graft combinations cultivar-scion/rootstocks in the present study,

Table 1. Description of eight reference genes tested in RT-qPCR reactions in peach trees under water deficit.

Gene symbol	Local and number access	TºMelting (°C)	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Reference
ACT	Peach EST TC1223	57.5	GTTATTCTTCATCGGCGT CTTCG	CTTCACCATTCCAGTTCCA TTGTC	Tong et al. (2009)
Ef-1α	GeneBank DW355834	56.2	AATTGCCTTTGTTCCCAT CTCTG	TGGGCTCCTTCTAATCTCC TTA	Xu et al. (2008)
CYP2	Peach EST TC1916	56.3	ACTCCAAAGCGTGTTAG AAAAGG	GTCTCTTCCACCATAACGA TAGG	Tong et al. (2009)
GADPH	Peach EST TC3113	57.0	ATTTGGAATCGTTGAGG GTCTTATG	AATGATGTTGAAGGAAGC AGCAC	Tong et al. (2009)
TUA	Peach EST TC2873	56.9	TTCTCTCTACTCATTCCC TCCTTG	GATTGGTGTATGTTGGTCT CTCG	Tong et al. (2009)
TUB	Peach EST TC3624	59.5	CCGAGAATTGTGACTGC CTT CAAG	AGCATCATCCTGTCTGGGT ATTCC	Tong et al. (2009)
UBQ10	Peach EST TC2782	57.0	AAGGCTAAGATCCAAGA CAAAGAG	CCACGAAGACGAAGCACT AAG	Tong et al. (2009)
18SrRNA	Peach EST TC1229	59.0	TAGTTGGTGGAGCGATT TGTCTG	CTAAGCGGCATAGTCCCTC TAAG	Tong et al. (2009)

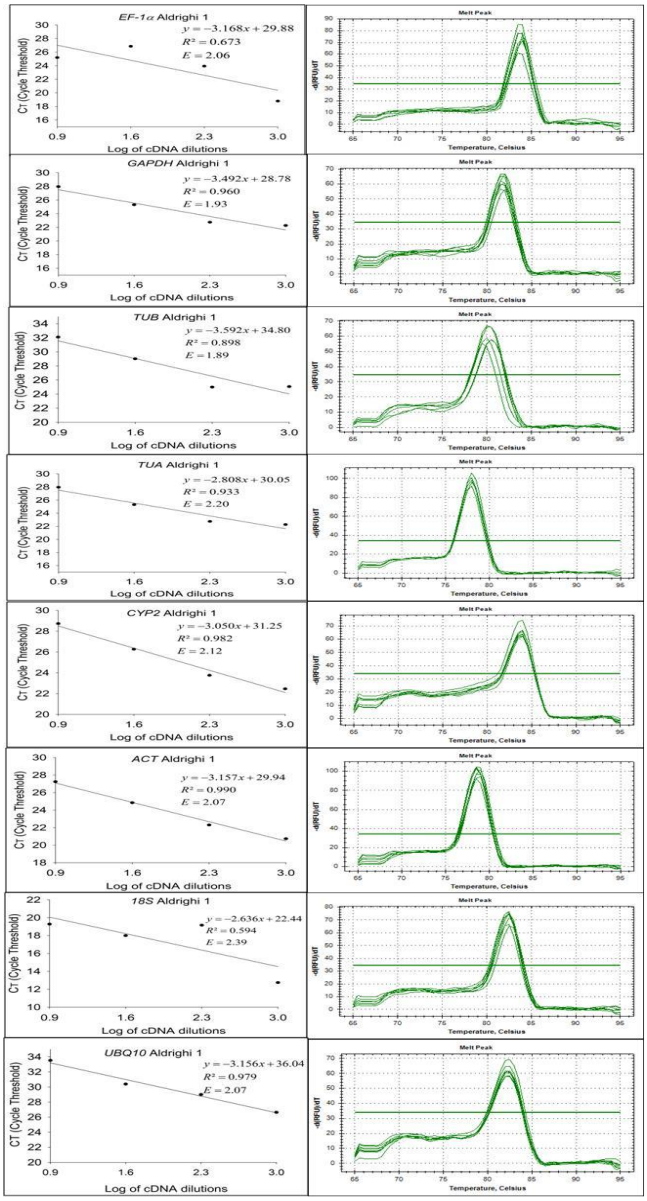

Fig 1. Efficiency and specificity of eight reference genes in leaf samples of peach tree cultivar Chimarrita grafted onto 'Aldrighi 1' rootstock, subjected to water deficit for different periods.

Table 2. Average ($\bar{X}$), coefficient of variation (CV%) and standard deviation (SD) of eight reference genes in leaf samples of peach tree cultivar Chimarrita grafted onto 'Aldrighi 1' and 'Tsukuba 2' rootstocks, subjected to water deficit.

ALDRIGHI 1	*TUA*	*CYP2*	*UBQ10*	*TUB*	*18SrRNA*	*GADPH*	*Ef-1α*	*ACT*
Average $\bar{X}$	3.69	3.26	3.84	2.98	3.11	3.98	5.91	5.17
CV%	11.01	11.22	18.30	39.90	25.12	24.04	25.10	45.71
SD	0.40	0.36	0.70	1.18	0.78	0.95	1.48	2.36
TSUKUBA 2								
Average $\bar{X}$	2.45	2.10	1.40	1.56	1.47	1.96	2.31	1.97
CV%	8.42	22.40	47.01	28.91	32.96	38.53	17.66	63.44
SD	0.20	0.47	0.66	0.45	0.48	0.75	0.40	1.25

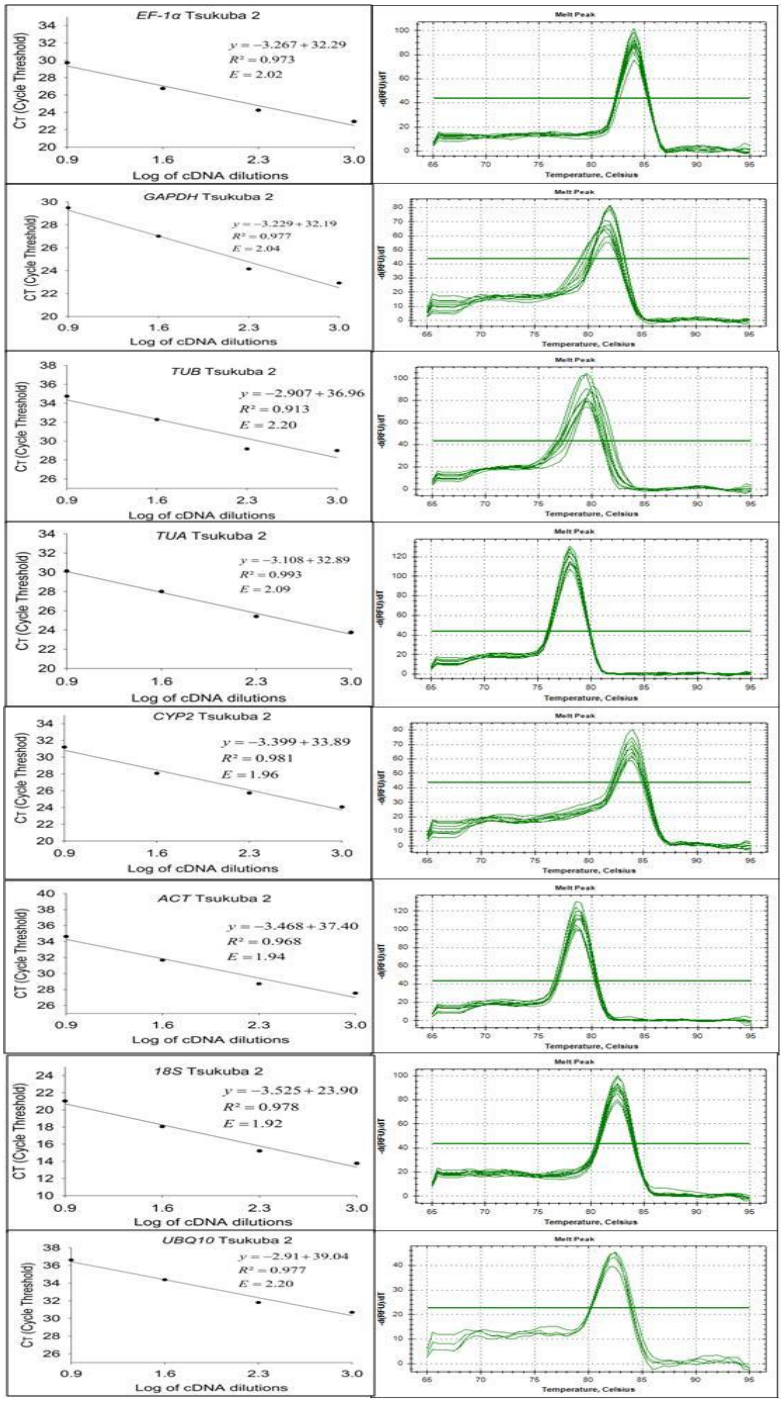

Fig 2. Efficiency and specificity of eight reference genes in leaf samples of peach tree cultivar Chimarrita grafted onto 'Tsukuba 2' rootstock, subjected to water deficit for different periods.

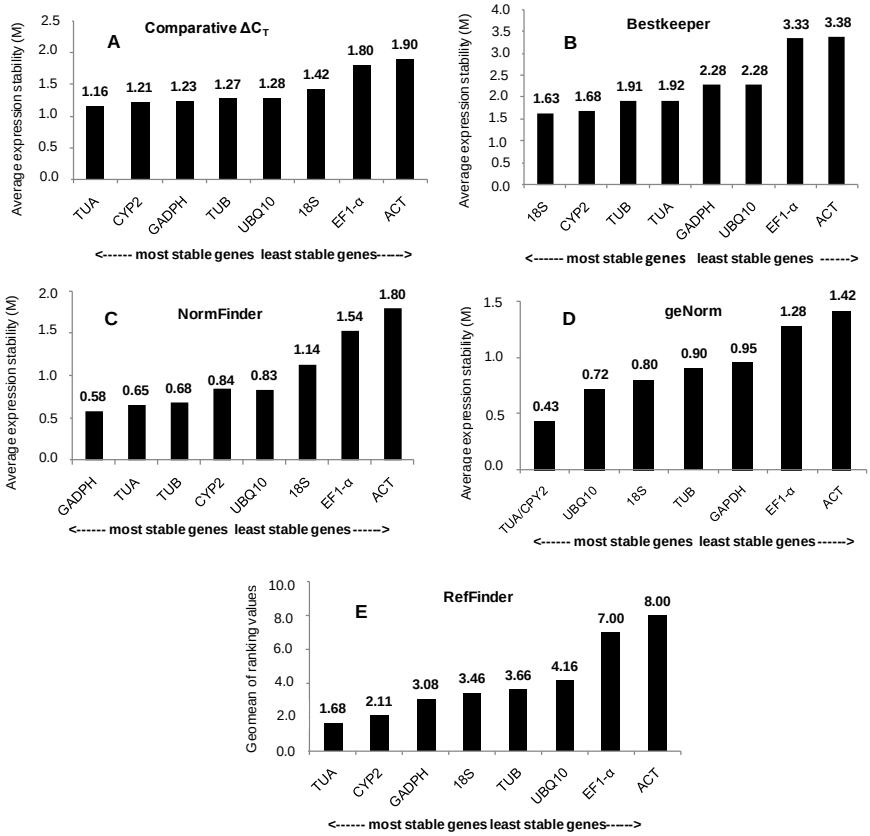

Fig 3. Average stability of expression of eight reference genes according to the algorithms (A) ΔC_T comparative method, (B) Bestkeeper, (C) NormFinder, (D) geNorm, and (E) RefFinder-Ranking in leaf samples of peach trees cultivar scion 'Chimarrita' grafted onto 'Aldrighi 1' rootstock, subjected to water deficit.

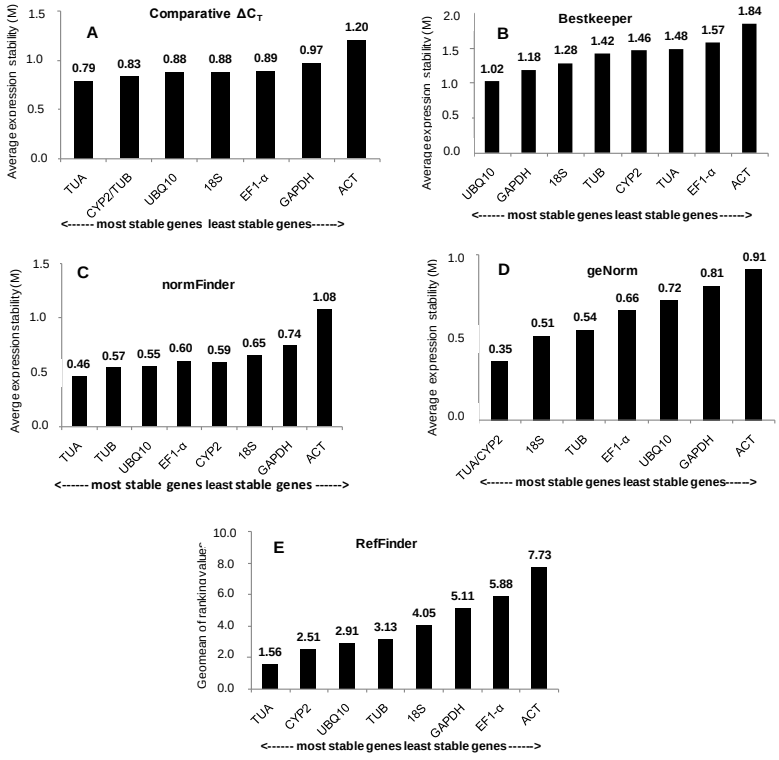

Fig 4. Average stability of expression of eight reference genes according to the algorithms (A) ΔC_T comparative method, (B) Bestkeeper, (C) NormFinder, (D) geNorm, and (E) RefFinder-Ranking in leaf samples of peach trees cultivar scion 'Chimarrita' grafted onto 'Tsukuba 2' rootstock, subjected to water deficit.

whereas *ACT* (M = 1.42 and M = 0.91) was the most variable/unstable (Fig 3D and Fig 4D, respectively).

Besides integrating the four methods abovementioned, the software RefFinder (Fig 3E and Fig 4E) uses C_T values to assign a given value for a single gene and calculates the geometric mean of its values for the final ranking, based on the ratings of each one of the four programs (Chen et al., 2011).

Although RefFinder does not indicate a cutoff value to rank a reference gene as stable or unstable, this software provides a ranking of candidate genes according to their stability. In this case, candidate genes with the lowest ranking were considered to be expressed as stable under the experimental conditions investigated and, therefore, can be selected as ideal reference genes. As it is observed in Fig 3E, the overall ranking of stability in the graft combination 'Chimarrita/Aldrighi 1' was determined as: *TUA* > *CYP2* > *GAPDH* > *18SrRNA* > *TUB* > *UBQ10* > *Ef-1α* > *ACT*, which was a little different compared to the stability of the graft combination 'Chimarrita/Tsukuba 2' whose ranking was: *TUA* > *CYP2* > *UBQ10* > *TUB* > *18SrRNA* > *GAPDH* > *Ef-1α* > *ACT* (Fig 4E). Therefore, the RefFinder was used as a confirmation tool to select the most suitable reference genes, since a few differences in the level of stability of expression were observed among the approaches studied.

Discussion

The analysis of gene expression to better understand certain processes that control biological traits has increased considerably in the past decades and, currently, RT-qPCR is the most common technique used to analyze the expression of transcripts (Mallona et al., 2010). This technique is accurate and reliable, moreover, it is used to validate data obtained by other methods. Undoubtedly, its advantages are the high sensitivity, the real-time detection of the progress of the reaction, velocity of the analysis and accurate measurement of the material examined in the sample (Gachon et al., 2004). Studies of gene expression through RT-qPCR require the knowledge about the reference genes stably expressed for the normalization data of target genes (Bustin et al., 2009; Derveaux et al., 2010). For this purpose, several genes evolved in basic cellular processes are usually known as constitutive and hence extensively used as normalizing genes in RT-qPCR analyses. However, these genes may vary significantly in their expression, therefore being unsuitable for use as internal control of certain experimental conditions because might result in the misinterpretation data (Tong et al., 2009; Hu et al., 2014; Galli et al., 2015).

Glyceraldehyde-3-phosphate dehydrogenase is one of the reference genes commonly used to normalize RT-qPCR data. The use of *GAPDH* in many studies has provided good results (Cruz et al., 2009), otherwise, in other studies it is not recommended due to the variability of expression posed by different experimental conditions (Tong et al., 2009; Hu et al., 2014; Huang et al., 2014; Galli et al., 2015; Ye et al., 2015). In this paper, *GAPDH* has proven to be quite unstable in most of the algorithms used and also had a high CV%, 24.04 for 'Chimarrita/Aldrighi 1' and 38.53 for 'Chimarrita/Tsukuba 2' graft combination. The possible explanation for this high variability may be due to the fact that *GAPDH* plays a role as a compound of the glycolytic pathway by converting glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate along with the reduction of NAD^+ to $NADH_2$, besides taking part in other cell processes, so that, the profile of expression of this gene may have been influenced under the experimental conditions of this study.

Actin is an intracellular protein quite abundant in eukaryotic cells and their highest concentration is in the cell cortex. Genes encoding actin are commonly used as normalizing in many studies of expression (Jain et al., 2006). Among the eight reference genes tested in the present study, *ACT* was considered to be the most unstable in the four algorithms analyzed (Fig 3 A-E and Fig 4 A-E) also having the highest CV% and SD (Table 2). Tong et al. (2009), have also found an increased instability of this gene in peach plants. *ACT* has also demonstrated instability in other studies, such as those carried out by Galli et al. (2015), in *Fragaria* x *ananassa* Duch subjected to water deficit and by Jain et al. (2006) in *Oryza sativa* under different experimental conditions. In contrast, in *Prunus sibirica* L. seeds, *ACT* was one the most stable genes according to the algorithms geNorm and NormFinder (Niu et al., 2014).

In leaves and roots of *Dactylis glomerata* L., the genes *ACT* and *CPY2* had an increased stability in plants subjected to water deficit (Huang et al., 2014). These same genes have also been the most stable based on the algorithms geNorm and NormFinder in flowers and fruits of cherry tree (Ye et al., 2015). In the present study, the gene *CYP2* has also shown high stability as the second most stable gene in the ranking (RefFinder) (Fig 3E and Fig 4E). Cyclophilins are a family of proteins found in very diverse organisms from prokaryotes to humans. Such molecules are related to the function of modulation of membrane permeability in mitochondria and also influence the conformation of proteins in cells (Lewin, 1997), thus, they are often used as reference genes in many studies.

Ubiquitin is an essential protein in the regulation of biological processes, directly involved in signaling complexes of other proteins (Sun et al., 1997), besides taking part in the structure and transcription of chromatin, DNA repairing, regulation of endocytosis and transport of proteins (Hernandez-Garcia et al., 2009). When investigating the expression of eleven reference genes in different samples of *Prunus persica*, Tong et al. (2009) found that *UBQ10* had a stable expression as normalizing in this species. In the present study, such behavior has not been proven because *UBQ10* has not been properly stable in all algorithms analyzed (Fig 3 and Fig 4), contrasting with the results found by Galli et al. (2015), where the *UBQ11* gene was the second most stable gene according to the general ranking (RefFinder) in strawberry plants under water deficit.

Ribosomes in eukaryotes have four different rRNA molecules: 5S, 5.8S, 18S, and 28S. These rRNA molecules along with ribosomal proteins make up two ribosomal subunits 40S and 60S. The rRNA molecules play different roles in the synthesis of proteins. Ribosomal subunits, such as 18S, are widely used as reference genes. However, the use of the gene *18S* as a reference is not always suitable because it does not have the poly-A tail that hinders the synthesis of cDNA with oligo-dT primers. Besides, the ribosomal gene *18S* is not always a completely representative of mRNA cell population. In the present study, this gene had varied stability and it was the most stable only in the algorithm BestKeeper for the graft combination 'Chimarrita/Aldrighi 1' (Fig 3B). Galli et al. (2015) did not recommend it as a possible reference gene for strawberry plants under osmotic stress and water deficit. In peach trees, under different experimental conditions and tissues, Tong et al. (2009) have also found an increased variability for this gene.

The elongation factor 1α in eukaryotes is a highly conserved member of the GTPases family, which includes protein factors involved in the initiation, elongation, and end of translation, besides being involved in other cellular

functions, such as interaction with protein of the cytoskeletal, calmodulin as well as with the ubiquitin-dependent proteolytic system (Keeling et al., 2004). As it was found by Niu et al. (2014) in *Prunus sibirica* L., and by Hu et al. (2014) in *Fortunella crassifolia* Swingle under different abiotic stresses, the gene *Ef-1α* had shown variability in the stability expression corroborating our results as it can be seen on the ranking of stability (RefFinder) (Fig 3E and Fig 4E).

Tubulins comprise a small family of globular proteins. The most common members of this family are α-tubulin and β-tubulin, which are the proteins that make up microtubules, playing a crucial role in several basic processes such as cell division, intracellular transport, cell motility, morphogenesis, and maintenance of cell shape (Ludwig et al., 1987; Whittaker and Triplett, 1999). In this paper, we analyzed both tubulin genes (α and β), and the gene *TUB* was more instable than the gene *TUA* in our experimental conditions. *TUA* was the most stable gene in comparison to all the other genes, which is demonstrated in all algorithms analyzed in addition to the lowest SD (0.20) and CV% (8.42) values in the graft combination 'Chimarrita/Tsukuba 2' (Table 2). Moreover, Ye et al. (2015) reported a high stability of this gene in vegetative tissues and organs of cherry trees. In contrast, Tong et al. (2009) reported no stability of this gene in different genotypes, tissues, and developmental stages of fruits in *Prunus persica*. Hu et al. (2014) have also reported no stability of the gene *TUA* in *Fortunella crassifolia* Swingle under water deficit.

The results obtained in this study lead us to suggest that constitutive genes may be regulated differently in different plant species, confirming that in general there is no universal reference gene applicable to all experimental conditions and the reference transcripts commonly used may not always exhibit similar constitutive expression. The contradiction of these, as well as other findings, confirms the need of an adequate selection of the constitutive gene as control for analyses of expression of transcripts through RT-qPCR.

The major characteristic of a reference gene is the stability of expression that should not be affected by the tissue type, development period or physiological conditions of the plant. The use of unsuitable reference genes leads to differences in the profile of relative expression. Then, our results showed the importance of previously validated reference genes for the experimental conditions to be studied. Either erroneous or inconsistent interpretations may be taken forward if a previous validation is not conducted for the desired experimental conditions.

Materials and Methods

Plant material and water deficit conditions

Peach trees cultivar Chimarrita, with 2-year-old, grafted onto two rootstocks of *Prunus persica* ('Aldrighi 1' and 'Tsukuba 2') were growth into pots with 25-liter capacity filled with soil substrate classified as dystrophic yellow-red argisol (Streck et al., 2008). The plants were kept in a greenhouse, and irrigated daily up to field soil capacity until the beginning of the trail on day zero (control), when the irrigation was suspended during 9-day period to induce the stress by water deficit. The 9-d period of plant evaluation was based on morphological and physiological characteristic of the leaves, such as: decrease in stomatal conductance, epinasty, and early leaf abscission, which was also previously referenced by Martinazzo et al. (2011; 2013) in plants of *Prunus* spp. under water stress treatments. In the present study, three plants of each graft combination 'Chimarrita'/rootstock were maintained under regular irrigation supply just to track the physiological characteristics of the leaves in relation to the plants under water deficit.

The trial was conducted in a completely randomized design with four treatments that correspond the evaluations period: zero (control), 4^{th}, 7^{th}, and 9^{th} stress day of water deficit. For each treatment three biological replicates was used. In each period of evaluation leaves were sampled from cultivar Chimarrita, immediately frozen in liquid nitrogen and subsequently stored in an ultrafreezer at -80 ºC up to RNA extraction. Leaf samples of each graft combination cultivar Chimarrita onto the two rootstocks were analyzed separately.

Total RNA isolation and cDNA synthesis

The total RNA was extracted from 100 mg of leaf tissue that had been grounded in liquid nitrogen and poured into 2 mL polypropylene tubes, added with 900 µL CTAB buffer 65ºC pre-heated. Samples were homogenized in vortex, incubated in a water bath at 65 ºC for 30 minutes and cooled. Then, the extraction was carried out with successive centrifugation steps, following the protocol proposed by Chang et al. (1993), with small modifications.

The RNA *pellet* was dissolved with 30 µL 0.01% DEPC autoclaved water, homogenized in vortex and stored in ultrafreezer. The concentration and purity of RNA were measured in NanoDrop ND-1000 (260/280nm) whereas the quality and integrity were verified by electrophoresis in 1.0% agarose gel. Single-stranded cDNAs were synthesized by reverse transcription from 1.5 µg total RNA by using the kit GoScript™ Reverse Transcription System (Promega) in a reaction with a final volume of 20 µL with oligo-dT primers according to the manufacturer's instructions.

Selection of candidate reference gene under water deficit

We selected eight reference genes to be tested in this study. They are reported in literature as internal control in RT-qPCR analyses and, supposedly, have no significant variation in different experimental conditions. The genes selected were: *ACT*, *CYP2*, *Ef1-α*, *GAPDH*, *TUA*, *TUB*, *UBQ10* and *18SrRNA* (Table 1).

Reverse transcription quantitative PCR

The total volume of each RT-qPCR reaction was 12 µL, 6.25 µL SYBR Green fluorophore (Invitrogen®), 0.25 µL (10mM) of each primer (forward and reverse), 1 µL cDNA (dilution 1:5, previously determined), 4.15 µL ultrapure water, and 0.1 µL ROX. The amplification was standardized in a CFX-96 Real Time Thermal Cycler (Bio-Rad) equipment, by using the following amplification conditions: 95 ºC for 10 minutes, 40 cycles of 95 ºC for 15 seconds, 60 ºC for 1 minute with the insertion of the melting curve from 65 ºC to 95 ºC, increasing 5 ºC at every fluorescence measurement. Three biological replicates were used.

Specificity and efficiency of standard curve

The specificity of primers was assessed through the dissociation curve (melting curve) and we maintained only primers with specific amplicons that show a single peak of dissociation of strands of PCR products.

The efficiency of PCR (*E*) was obtained from four serial dilutions of cDNA (1:1, 1:5, 1:25, and 1:125) in order to generate the standard curve of each pair of primer tested. The value of *E* was estimated by the equation $E = 10^{-(1/slope)}$

(Rasmussen, 2001), where slope is the curvature of the line obtained from the regression between C_T values (Cycle Threshold) of transcripts and the values from the algorithm of different cDNA dilutions (standard curve of efficiency), considered as acceptable efficiency values ranging from 1.8 to 2.2, which corresponds to a reaction efficiency between 90 – 110% (Fig 1, and Fig 2).

Data analysis

In order to classify and determine the performance of each reference gene, the means of C_T values (Le et al., 2012; Niu et al., 2014) were used for each sample analyzed, obtained from each RT-qPCR reaction cycle. Data was subjected to the analysis of variance through the Statistical Analysis System – WinStat – Version 2.0 (Machado and Conceição, 2003), and the normalizing genes with the lowest standard deviation and coefficient of variation values were considered to be stable.

In parallel with statistical analyses, the stability of normalizing genes for both combinations 'Chimarrita'/rootstocks was assessed with the tool RefFinder, available on the website http://fulxie.0fees.us/?type=reference&ckattempt=1, which integrates the computational algorithms geNorm, NormFinder, BestKeeper, and ΔC_T comparative method to compare and rank the stability of candidate genes to normalizing. C_T values of each gene were used by these algorithms to determine the stability of relative expression. Among these four algorithms, geNorm and NormFinder use relative expression values calculated from C_T values whereas BestKeeper and ΔC_T comparative method use directly C_T values inserted onto RefFinder (Chao et al., 2012). In addition to the isolated analysis of each algorithm, an overall ranking of the best normalizing for the experimental condition investigated was obtained by combining the results of four algorithms. The detailed calculation of each one of the methods is described in Chen et al. (2011).

Conclusion

The analysis of expression stability of eight candidate genes to normalizing for RT-qPCR studies suggests the constitutive genes *TUA* and *CYP2* as the most suitable for studies in peach leaves cultivar Chimarrita grafted onto 'Aldrighi 1' and 'Tsukuba 2' rootstocks under water deficit. The genes *Ef-1α* and *ACT* are the least suited for the experimental conditions tested.

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