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Research article

Reference gene selection for quantitative real-time PCR in *Solanum lycopersicum* L. inoculated with the mycorrhizal fungus *Rhizophagus irregularis*

Alejandra Fuentes [a, b], Javier Ortiz [a], Nicolás Saavedra [c], Luis A. Salazar [c], Claudio Meneses [d], Cesar Arriagada [a, *]

[a] Laboratorio Biorremediacion, Departamento de Ciencias Forestales, Facultad de Ciencias Agropecuarias y Forestales, Universidad de La Frontera, Temuco 4811230, Chile
[b] Programa de Doctorado en Ciencias Mención Biología Celular y Molecular Aplicada, Universidad de la Frontera, Temuco 4811230, Chile
[c] Center of Molecular Biology and Pharmacogenetics, Scientific and Technological Bioresource Nucleus (BIOREN), Universidad de La Frontera, Temuco 4811230, Chile
[d] Centro de Biotecnología Vegetal. Universidad Andres Bello, Republica 217, Santiago, Chile

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ABSTRACT

The gene expression stability of candidate reference genes in the roots and leaves of *Solanum lycopersicum* inoculated with arbuscular mycorrhizal fungi was investigated. Eight candidate reference genes including elongation factor 1 α (*EF1*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), phosphoglycerate kinase (*PGK*), protein phosphatase 2A (*PP2Acs*), ribosomal protein L2 (*RPL2*), β-tubulin (*TUB*), ubiquitin (*UBI*) and actin (*ACT*) were selected, and their expression stability was assessed to determine the most stable internal reference for quantitative PCR normalization in *S. lycopersicum* inoculated with the arbuscular mycorrhizal fungus *Rhizophagus irregularis*. The stability of each gene was analysed in leaves and roots together and separated using the geNorm and NormFinder algorithms. Differences were detected between leaves and roots, varying among the best-ranked genes depending on the algorithm used and the tissue analysed. *PGK*, *TUB* and *EF1* genes showed higher stability in roots, while *EF1* and *UBI* had higher stability in leaves. Statistical algorithms indicated that the *GAPDH* gene was the least stable under the experimental conditions assayed. Then, we analysed the expression levels of the *LePT4* gene, a phosphate transporter whose expression is induced by fungal colonization in host plant roots. No differences were observed when the most stable genes were used as reference genes. However, when *GAPDH* was used as the reference gene, we observed an overestimation of *LePT4* expression. In summary, our results revealed that candidate reference genes present variable stability in *S. lycopersicum* arbuscular mycorrhizal symbiosis depending on the algorithm and tissue analysed. Thus, reference gene selection is an important issue for obtaining reliable results in gene expression quantification.

1. Introduction

Arbuscular mycorrhizal (AM) fungi belonging to the phylum Glomeromycota (Schüβler et al., 2001) are important components of soil microbial biomass. These fungi are the most extended symbiotic organism related to land plants (Wang and Qiu, 2006) and are involved in the bidirectional transference of nutrients between symbionts (Bago et al., 2003; Smith and Read, 1997). This symbiosis can benefit plant growth, promoting primarily higher uptake of phosphorus, water and minerals (Smith and Read, 1997) through the external mycelium. AM symbiosis benefits plants under stress conditions such as salt (Feng et al., 2002), drought (Ruiz-

Abbreviations: EF1, elongation factor 1 α; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *PGK*, phosphoglycerate kinase; *PP2Acs*, protein phosphatase 2A; *RPL2*, ribosomal protein L2; *TUB*, β-tubulin; *UBI*, ubiquitin; *ACT*, actin; *18S* rRNA, 18S ribosomal RNA; *28S* rRNA, 28S ribosomal RNA; *β2M*, β2-microglobulin; *ALB*, albumin; *L32RPL32*, ribosomal protein; *TBP*, TATA sequence-binding protein; *CYCC*, cyclophilin C; *HPRT*, hypoxanthine phosphoribosyl transferase; *RPII*, RNA polymerase II; AMF, arbuscular mycorrhizal fungi; Ct, cycle threshold.

* Corresponding author. Laboratorio Biorremediación, Departamento de Ciencias Forestales, Facultad de Ciencias Agropecuarias y Forestales, Universidad de La Frontera, Casilla 54-D, Avenida Francisco Salazar 01145, Temuco 4811230, Chile.
E-mail address: cesar.arriagada@ufrontera.cl (C. Arriagada).

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Lozano et al., 1996), pathogens (Azcón-Aguilar and Barea, 1997) and heavy metal contamination (Aloui et al., 2012; Arriagada et al., 2009; Hildebrandt et al., 2007; Miransari, 2011). Numerous studies have examined the biological, chemical and molecular characteristics of arbuscular mycorrhizal symbiosis to achieve a better understanding of its effect on gene expression profiles of mycorrhizal plants in different symbiosis stages using quantitative PCR techniques (León-Morcillo et al., 2012).

Quantitative real-time PCR (qPCR) is used extensively for the quantification of mRNA levels in different tissues, cell types and experimental conditions. qPCR is a rapid, sensitive and specific technique (Bustin, 2002; Valasek and Repa, 2005). However, to obtain reliable and reproducible results, qPCR requires an accurate normalization method to avoid some errors that could affect its results such as differences in RNA input amounts, variability introduced by co-purification of inhibitors during RNA isolation and differences in reverse transcription and PCR efficiencies (Huggett et al., 2005). The normalization of target messenger RNA (mRNA) levels using an internal reference gene is a widely accepted procedure. An ideal reference mRNA must be unaffected by experimental conditions including treatments and growth conditions. Thus, the reference mRNA must be expressed at constant levels among different tissues of an organism and at all stages of development (Løvdal and Lillo, 2009). Traditionally, the so-called housekeeping genes, which are essential for cell metabolism and consequently expressed at constant levels in all tissues, have been used for these purposes (Thellin et al., 1999). The use of these candidate reference genes has been broadly accepted for normalization of semi-quantitative and qualitative techniques such as Northern blot, RNase protection assay and conventional reverse transcription PCR, which are used to evaluate changes at the mRNA level (Huggett et al., 2005). Reference genes used for semi-quantitative techniques include *18S* rRNA (18S ribosomal RNA), *28S* rRNA (28S ribosomal RNA), *TUB A* (α-tubulin), *ACT B* (β-actin), *β2M* (β2-microglobulin), *ALB* (albumin), *RPL32* (ribosomal protein L32), *TBP* (TATA sequence-binding protein), *CYCC* (cyclophilin C), *EF1A* (elongation factor 1α), *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), *HPRT* (hypoxanthine phosphoribosyl transferase) and *RPII* (RNA polymerase II) (Kozera and Rapacz, 2013). However, qPCR sensitivity has allowed the detection of varied expression levels of some candidate reference genes under specific experimental conditions (Dheda et al., 2004; Thellin et al., 1999); therefore, the use of these genes for qPCR normalization can lead to inaccurate results (Czechowski et al., 2005; Nicot et al., 2005). Previous reports have shown that AM colonization induces changes in gene expression that promote the coding of proteins and enzymes in the host plant (Ouziad et al., 2005; Pallara et al., 2013; Rivera-Becerril et al., 2005). However, previous reports have also demonstrated variations in the expression stability of candidate reference genes in plants under stressors such as drought, higher salinity, pathogens and heavy metals (Borowski et al., 2014; Sang et al., 2013; Wang et al., 2012). Moreover, no studies have assessed the expression stability of candidate reference genes in mycorrhizal plants.

Thus, the aim of this study was to rank eight candidate reference genes based on their expression stability in the roots and leaves of *S. lycopersicum* plants inoculated with AM fungi.

2. Material and methods

2.1. Arbuscular mycorrhizal (AM) fungi

The AM fungus *Rhizophagus irregularis* (Schüßler et al., 2001) was obtained from the fungal culture collection maintained in the Bioremediation Laboratory, Universidad de La Frontera, Temuco, Chile. The AM fungal inoculum was a root-and-soil inoculum consisting of 8 g of rhizosphere soil containing spores and colonized root fragments of *Medicago sativa* L. (approximately 100 spores in 100 g of soil). The uninoculated plants with AM fungus were given a filtrate (passed through Whatman no. 1 paper) of the inocula containing common soil microflora (principally soil bacteria) but free of AM fungal propagules.

2.2. Plant growth and fungal inoculation

S. lycopersicum seeds were sterilized with NaClO (0.05% v/v) for 15 min and then rinsed with sterile distilled water. Plants were inoculated at the time of germination in each cell of seedling trays containing sterile vermiculite as the substrate; the seed was deposited on the inoculum and covered with sterile vermiculite. Germination was performed in a bioclimatic chamber with artificial light provided by Sylvania incandescent, cool white lamps (400 μmol m^{-2} s^{-1}, 400–700 nm) with a 16/8 h day/night cycle at 24/18 °C and 60% relative humidity. After 24 days, uniform seedlings from control and AM-inoculated groups were transferred to 500 mL pots (one per pot) containing an Entisol soil with phosphorus (40.3 mg kg^{-1}) and a moderately acidic pH (pH 5.44). The plants were supplemented every week with 10 mL of a nutrient solution plus 50 mg^{-1} phosphorus (Hewitt, 1952), which did not affect the mycorrhizal plant roots (Arriagada et al., 2009). The plants grew for 2 months with 100 mL of distilled water irrigation every 48 h in a climatic chamber. Then, root and shoot samples were frozen in liquid nitrogen and stored at −80 °C until use. Five replicate pots were included in both AM-uninoculated and AM-inoculated plant groups.

2.3. Estimation of mycorrhizal colonization

After the harvest, root samples were taken from the entire root system at random, cleared in KOH and stained with trypan blue in lactic acid (Phillips and Hayman, 1970). The results are expressed as percentages of root colonization (*M*), frequency (*F*) and abundance of arbuscules in the colonized portion (*a*) according to Trouvelot et al. (1986).

2.4. Isolation of DNA-free total RNA

Total RNA was obtained from 100 mg of leaf or root tissues after pulverization using a mortar and liquid nitrogen. Total RNA was isolated using RNA-Solv reagent (E.Z.N.A) and then re-suspended in 50 μL of RNase-free water. RNA integrity was verified by denaturing gel electrophoresis. Moreover, RNA concentration and purity were measured by UV spectrophotometry at 260/280 nm (MaestroNano spectrophotometer, Maestrogen®). To avoid sample contamination with genomic DNA and false positives in the RT-qPCR, RNA samples (10 μg) were treated with an RNase-free DNase I Set (E.Z.N.A). Finally, RNA was purified using clean-up Hi-Bind RNA Mini Columns from a Total RNA Kit I (E.Z.N.A) and subjected to denaturing gel electrophoresis to confirm RNA integrity.

2.5. cDNA synthesis and RT-qPCR

cDNA synthesis was performed using an AffinityScript qRT-PCR commercial kit (Stratagene, Cedar Creek, TX, USA) according to the manufacturer's recommendations. First, 1 μg of total RNA was added to a mixture containing 170 ng of oligo (dT) primer and 30 ng of random primers in a final volume of 20 μL. The mixture was incubated for 5 min at 25 °C, 45 min at 42 °C and 5 min at 95 °C. For RT-qPCR, cDNA samples were diluted ten-fold with RNase-free water. The selection of reference genes for quantitative real-time

PCR was performed according Løvdal and Lillo (2009) and Goupil et al. (2009), considering eight classic reference genes: elongation factor 1 α (*EF1*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), phosphoglycerate kinase (*PGK*), protein phosphatase 2A (*PP2Acs*), ribosomal protein L2 (*RPL2*), β-tubulin (*TUB*), ubiquitin (*UBI*) and actin (*ACT*) (Table 1). The product size and specificity for each pair of primers were verified by RT-PCR and visualized using a 2% agarose gel. The qPCR reaction was performed at a final volume of 20 μL containing 2 μL of the diluted cDNA, a mixture of 0.3 μM forward and reverse primers, 10 μL of 2× Fast SYBR Green Master Mix (Applied Biosystems®) and 7.5 μL of nuclease-free water. Controls lacking template and containing 2 μL of nuclease free-water were also included. PCR quantification was performed using a StepOnePlus Real-Time PCR System (Applied Biosystems®) with the following thermal cycling conditions: 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 15 s at 60 °C, and 15 s at 72 °C. Melting curve analysis was performed after 40 cycles to verify the reaction specificity by the amplification of a single product for each gene. To validate the reference gene selection, the expression of the *LePT4* gene, a transporter phosphate in *S. lycopersicum* induced by mycorrhization (Nagy et al., 2005), was analysed using the selected reference genes. The induced changes in relative expression levels were calculated using the $2^{\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The analysis of gene expression using real-time PCR was performed using two technical replicates for each biological replicate.

2.6. Statistical analysis

geNorm™ (Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004) algorithms were used to evaluate the stability of candidate reference gene expression using the Ct values transformed to relative quantities as input. Gene expression data were compared by analysis of variance followed by Tukey's multiple range test. Statistical analyses were conducted using SPSS software version 11.0 (SPSS Inc., 1989–2001). Statistical significance was determined at $p < 0.05$.

3. Results

Eight candidate reference genes, *EF1*, *GAPDH*, *PGK*, *PP2Acs*, *RPL2*, *TUB*, *UBI* and *ACT*, were evaluated by RT-qPCR to quantify their mRNA levels in the leaves and root of mycorrhizal plants inoculated with *R. irregularis*.

3.1. Mycorrhizal colonization

The intensity of root colonization and the percentage of arbuscles on the colonized roots were assessed. The percentage of colonized roots (M) with AM fungal structures was 51%; its frequency (F), 37.6%; and arbuscle abundance (a), 35.7%. Non-mycorrhizal plants also showed root colonization but at a reduced percentage and frequency (M%, 0.1; F%, 2.1), and no arbuscles were detected.

3.2. Primer specificity and expression profiles of commonly used reference genes

The melting curve analysis of the amplification products showed a single amplified product for all genes, confirming the specificity of the primer pairs. The amplicons were also examined by 2% agarose gel electrophoresis using GelRed nucleic acid stain (Biotium, Hayward, CA), and a single band of the expected size for each primer pair was observed (data not shown). The average cycle threshold (Ct) values were calculated to reveal mRNA levels among the reference genes across all samples. Candidate reference genes showed differences in their expression levels between the experimental conditions. The *EF1* gene had the lowest Ct value (mean Ct = 18.47), while *GAPDH* showed the highest Ct value (mean Ct = 27.46). The transcription levels of *PGK*, *PP2Acs*, *RPL2*, *TUB*, *UBI* and *ACT* were moderately abundant, with Ct values ranging from 19 to 28 (Fig. 1).

3.3. Expression stability of commonly used reference genes

Two different algorithms were used to evaluate the expression stability of the eight selected reference genes: geNorm (Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004). For these analyses and selection of the most stably expressed genes, Ct values were transformed to relative quantities using the delta-Ct method.

3.3.1. geNorm analysis

The geNorm algorithm classifies the stability of gene expression according to the *M* value, establishing a cut-off of M = 1.5, in which an *M* value less than 1.5 is considered a stably expressed gene. Conversely, an *M* value over 1.5 indicates lower expression stability (Vandesompele et al., 2002). The stability of these genes was evaluated using roots, leaves and both tissues together. In the analysis including root and leaves from both mycorrhizal and non-mycorrhizal plants, *PP2Acs* and *TUB* showed higher stability, followed by *EF1* and *RPL2*, while *GAPDH* presented the lesser stability (Fig. 2A). Then, the differences in the stability of the reference genes between both tissues was analysed, and *EF1* and *UBI* showed the most stable expression in leaves (Fig. 2B). In contrast, *PGK* and *TUB* had the lowest *M* values in roots (Fig. 2C). The data suggest a tissue-specific utility of these genes for qPCR normalization. Similarly, as described in the analysis using both roots and leaves together, *GAPDH* had the least stable expression in both leaves and roots.

geNorm calculates the pairwise variation (*V*) to determine the optimal number of reference genes required for accurate normalization. The pairwise variation (V_n/V_{n-1}) between two sequential

Table 1
Primer sequences of candidate reference genes.

Name gene	Accession Number	Primer sequence (Forward primer 5′–3′)	Forward primers (Reverse primer 5′–3′)	Amplicon length (Bp)	Reference
TUB	DQ205342	AACCTCCATTCAGGAGATGTTT	TCTGCTGTAGCATCCTGGTATT	180	Løvdal and Lillo (2009)
EF1	X14449	GGAACTTGAGAAGGAGCCTAAG	CAACACCAACAGCAACAGTCT	158	Løvdal and Lillo (2009)
GAPDH	TC198136	CTGCTCTCTCAGTAGCCAACAC	CTTCCTCCAATAGCAGAGGTTT	157	Løvdal and Lillo (2009)
PGK	TC203809	CTTCCTCCTTAAAACTCCTCTCC	CTAAGGTCTCCAACGCTCTTCT	162	Løvdal and Lillo (2009)
PP2Acs	AY325817	CGATGTGTGATCTCCTATGGTC	AAGCTGATGGGCTCTAGAAATC	149	Løvdal and Lillo (2009)
RPL2	X64562	GTCATCCTTTCAGGTACAAGCA	CGTTACAAACAACAGCTCCTTC	156	Løvdal and Lillo (2009)
UBI	TC193502	GGACGGACGTACTCTAGCTGAT	AGCTTTCGACCTCAAGGGTA	134	Løvdal and Lillo (2009)
ACT	EU884309	GGGATGGAGAAGTTTGGTGGTGG	CTTCGACCAAGGGATGGTGTAGC	150	Goupil et al. (2009)
LePT4	AY885652	GAAGGGGAGCCATTTAATGTGG	CCATCTTGTGTGTATTGTTGTATC	182	Nagy et al. (2005)

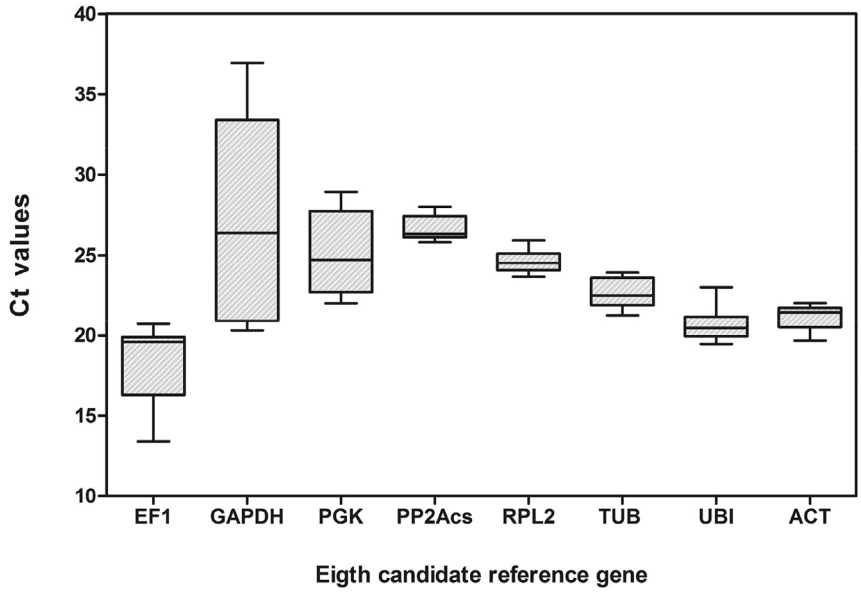

Fig. 1. Variations in cycle threshold (Ct) values of each candidate reference genes.

normalization factors contains an increasing number of genes. A *V* value of 0.15 was proposed as a cut-off value. Below that point, the inclusion of an additional control gene is not required (Vandesompele et al., 2002). As shown in Fig. 3, the pairwise variation of $V_{2/3}$ was lower than 0.15. These results showed that two reference genes would be optimal for normalizing the gene expression in all *S. lycopersicum* samples.

3.3.2. NormFinder analysis

The NormFinder algorithm was also used to analyse expression stability. This software performed a separate analysis of sample subgroups, estimating intra- and intergroup expression variations and calculating gene stability values (Andersen et al., 2004). Similar to geNorm, the most stable genes are ranked according to their stability values: the lowest stability value corresponds to the most stably expressed gene. Moreover, this algorithm suggests the best pair of genes among the candidate reference genes analysed using the intra- and intergroup variance. The stability values for all reference genes determined by the NormFinder algorithm are shown in Table 2. According to the stability ranking of the eight candidate genes, slight deviations from the geNorm results were observed. The analysis of leaves and roots together showed higher stability of *EF1* and *PGK* genes. In contrast, *UBI* and *EF1* were ranked as the best reference genes for leaves and *EF1* and *TUB* for roots when analysed separately. *EF1/PP2Acs, PGK/TUB and EF1/TUB* corresponded to the best gene pairs suggested for normalization. In this analysis, *GAPDH* was also identified as the most variable gene between the samples, reaching a maximum stability value of 1.061 in roots. Thus, *GAPDH* is the least recommended gene for qPCR normalization under the examined experimental conditions.

3.4. Validation of the reference genes

To evaluate the effect of reference gene selection on the assessment of relative gene expression, we quantified the *S. lycopersicum PT4* gene (*LePT4*), a phosphate transporter induced by mycorrhization and commonly used to detect the development and symbiosis functionality of roots (Fiorilli et al., 2009). *LePT4* expression levels were quantified using those candidates suggested from each algorithm as reference genes. First, according to geNorm pairwise variation (V) that indicated two genes as adequate for optimal normalization, we selected a combination of two reference genes to normalize *LePT4* expression. Thus, we normalized *LePT4* expression using the best two reference genes according to geNorm (*PGK/TUB*) and the best pair identified by NormFinder (*EF1/TUB*). Moreover, we evaluated *LePT4* expression following normalization with the two best-ranked individual reference genes from each algorithm, *PGK* and *TUB* from geNorm and *EF1* and *TUB* from NormFinder. The least stable gene (*GAPDH*) identified in these analyses was also used. All expression values obtained by normalizing *LePT4* expression with the selected reference genes showed significant differences between the roots from mycorrhizal and non-mycorrhizal plants. When the best pairs were used as reference genes, relative *LePT4* expression values in roots were 20.6 ± 1.6 and 22.1 ± 0.8 for *PGK/TUB* and *EF1/TUB*, respectively. Then, we used *PGK*, *EF1* and *TUB* individually, obtaining expression rates of 22.7 ± 5.9, 23.6 ± 1.0 and 20.7 ± 0.8, respectively. No significant differences in the relative expression of the *LePT4* gene were observed using either individual reference genes or the above-described pairs. However, relative *LePT4* gene expression showed a marked increase when using *GAPDH* as reference gene, reaching an expression rate of 260.8 ± 27.4 (Fig. 4).

4. Discussion

Real-time quantitative PCR provides reliable data with high specificity and sensibility for the detection and quantification of mRNA target sequences (Gachon et al., 2004). However, quantification of gene expression is affected by several factors (Maroufi et al., 2010); therefore, an accurate normalization method is required. The expression level of a target gene is normalized using reference genes (Bustin et al., 2009). These genes that are used as internal controls are commonly called housekeeping or reference genes (Huggett et al., 2005) and should be steadily expressed and correlated with the total amounts of mRNA (Bustin et al., 2009). However, other studies have reported that many candidate reference genes are not suitable for normalizing gene expression due to variations in expression depending on the experimental conditions (Dheda et al., 2004). Our results suggest reference genes obtained from an experimental validation of qPCR normalization in the roots,

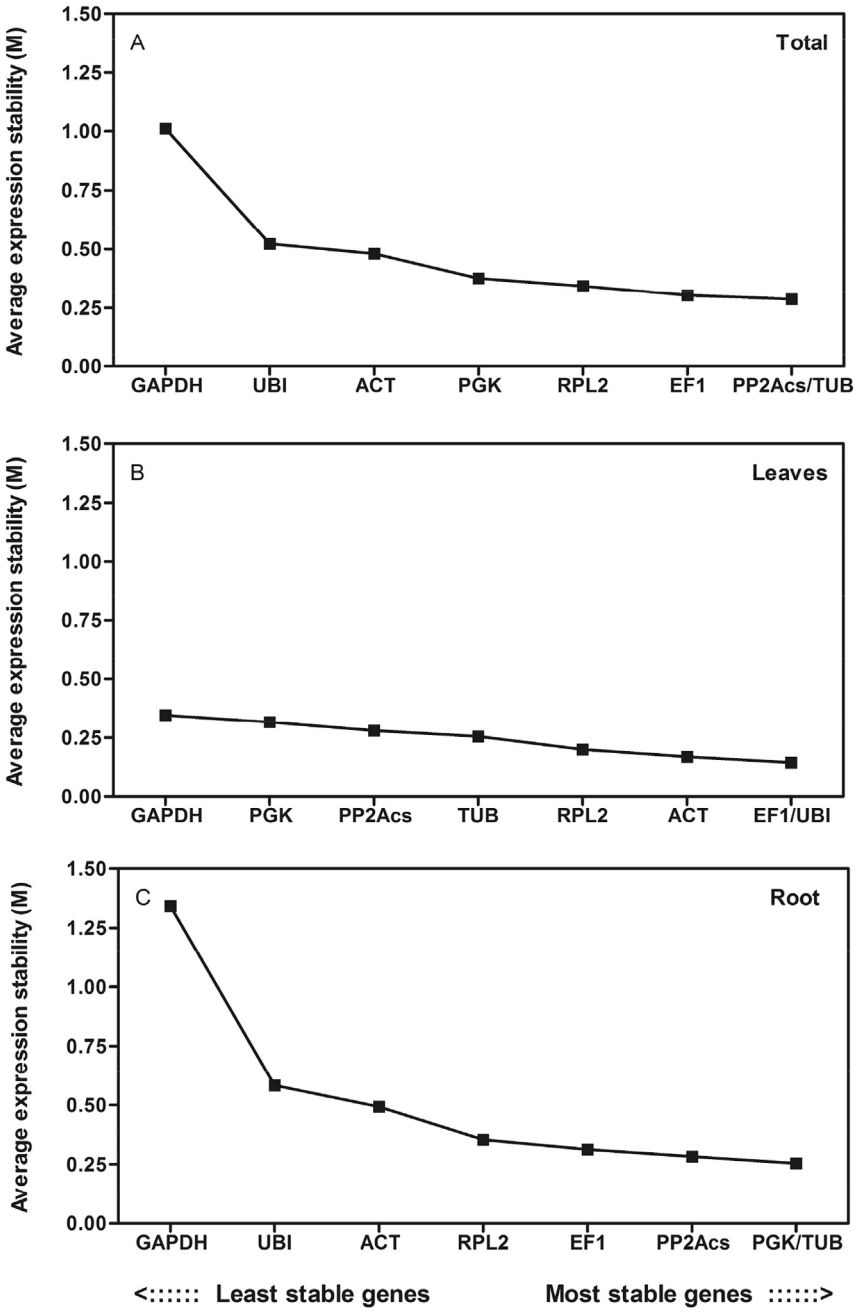

Fig. 2. Gene expression stability of eight reference genes ranked according to geNorm analysis. Ranking of gene expression stability was performed using tissues from leaves and root together (A) and leaves (B) or roots (C) separately. Mean expression stability (M) was calculated following stepwise exclusion of the least stable gene across all experimental groups.

leaves and total tissue of *S. lycopersicum* under AM symbiosis. AM colonization of plants has been studied because this colonization confers increased in plant growth and enhanced phosphorus, water and mineral nutrient uptake (Pearson and Jakobsen, 1993) and increase plant stress tolerance exposed to biotic and abiotic stress (Barea et al., 2005). At the molecular level AM fungi induces gene expression in plants, among them some nitrogen and phosphate transporters in roots (Burleigh, 2001; Rosewarne et al., 1999). Moreover, the mycorrhizal plants exhibit differential expression in roots and leaves (Taylor and Harrier, 2003). In the shoots, genes that are differentially regulated are involved in the primary and secondary metabolism, in stress or defense responses (Liu et al., 2007; López-Ráez et al., 2010; Mandal et al., 2014). Furthermore, other authors indicate that mycorrhizal inoculation could modulate the changes in gene expression in response to abiotic stress (Cicatelli et al., 2012; Ouziad et al., 2005; Pallara et al., 2013; Rivera-Becerril et al., 2005). However, no studies have been conducted regarding the stability of reference genes in AM colonization necessary for accurate and reliable normalization of RT-qPCR. In this work, we selected and validated reference genes for gene expression analysis in mycorrhizal plants inoculated with *R. irregularis*.

geNorm and NormFinder algorithms were used to evaluate the stability of eight candidate reference genes in plants under AM colonization. The geNorm and NormFinder analyses showed some differences in the ranking of genes depending on the analysed

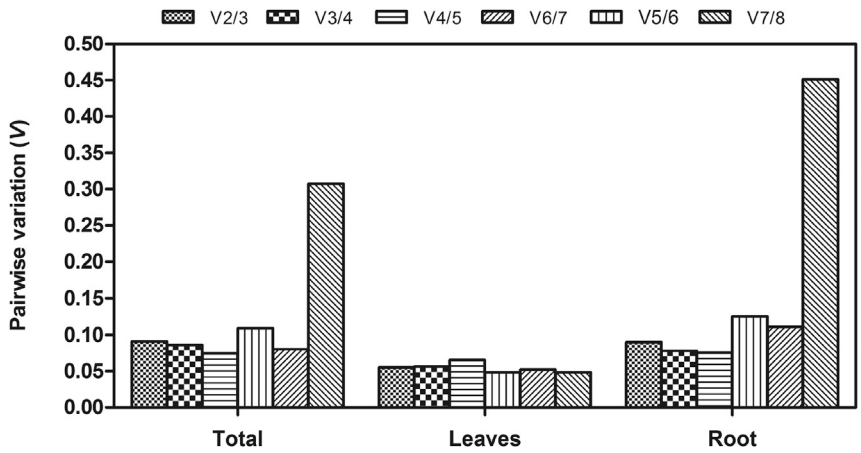

Fig. 3. Average pairwise variation analysis to determine the optimal number of reference gene suggested for gene expression normalization in root, leaves and total tissues from S. lycopersicum under arbuscular mycorrhizal colonization.

Table 2
Expression stability of the reference gene in leaves and roots of S. lycopersicum inoculated or not with R. irregularis analysed using NormFinder algorithm.

Rank	Total		Leaves		Root	
	Gene	Stability	Gene	Stability	Gene	Stability
1	*EF1*	0.058	*UBI*	0.073	*EF1*	0.075
2	*PGK*	0.137	*EF1*	0.079	*TUB*	0.123
3	*PP2Acs*	0.164	*LeAct*	0.144	*PP2Acs*	0.252
4	*TUB*	0.164	*RPL2*	0.171	*PGK*	0.297
5	*RPL2*	0.208	*PP2Acs*	0.191	*RPL2*	0.410
6	*LeAct*	0.453	*TUB*	0.200	*LeAct*	0.619
7	*UBI*	0.506	*PGK*	0.209	*UBI*	0.823
8	*GAPDH*	1061	*GAPDH*	0.229	*GAPDH*	2020
Best pair	*EF1/PP2Acs*	0.073	*PGK/TUB*	0.055	*EF1/TUB*	0.057

tissue. When analysing leaves and roots together (total), *PP2Acs* and *TUB* were ranked at the top position according to geNorm, while *EF1* was the best reference gene according to NormFinder. When the references genes were evaluated in leaves and roots separately, the ranking order between genes varied depending on the analysed tissue. When we analysed leaves, *EF1* and *UBI* were the best-ranked genes using either geNorm or NormFinder, while in roots, the most stably expressed genes were *PGK* and *TUB* according to geNorm and *EF1* using NormFinder. The best reference gene pairs were also estimated by NormFinder, which identified *EF1/PP2Acs, PGK/TUB* and *EF1/TUB* as better pairs for normalization in total tissue, leaves and roots, respectively. As shown, the two better-ranked reference genes did not necessarily match he best pair proposed by the algorithm because the resulting pair might have compensating expression among them. Therefore, if a reference gene is slightly overexpressed in one group, then the other chosen gene will be correspondingly underexpressed in the same group. Variations in gene expression stability rankings for the same reference genes have also been reported by other studies; these variations are likely related to the computational method used (Li et al., 2014; Løvdal and Lillo, 2009; Wang et al., 2012).

GAPDH was defined by the two analysis methods as least stably expressed gene for all analysed tissues. Recent studies regarding the selection of reference genes have shown that *GAPDH* is unstable under different experimental conditions and varies between different tissues. Moreover, a previous report (Løvdal and Lillo, 2009) showed a loss of stable *GAPDH* expression in tomato under cold stress. Other authors also showed unstable expression of this gene in leaves and roots under drought stress in cotton and *Cichorium intybus* (Maroufi et al., 2010; Wang et al., 2012).

Several investigations regarding plants in symbiotic association with AM fungi that used qPCR experiments did not examine gene expression stability, and *UBI*, *EF1*, *TUB* and *18S* were frequently used as reference genes (León-Morcillo et al., 2012; Mandal et al., 2015; Ouziad et al., 2006; Pallara et al., 2013; Pérez-Tienda et al., 2014). Genes such as *UBI* have been used for gene expression normalization in root and leaves (Fiorilli et al., 2009; Pallara et al., 2013), which, according to our results, is less stable in roots, possibly leading to erroneous results in these experiments.

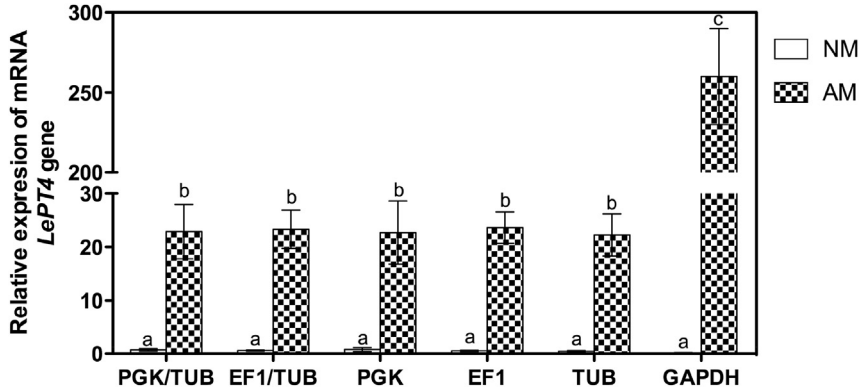

Fig. 4. Variations on LePT4 relative gene expression depending on the reference gene selected for normalization in root of S. lycopersicum under arbuscular mycorrhizal colonization. The data are the means ± standard error of means. Bars with the same letters are not significantly different ($p < 0.05$) according to Tukey's multiple range test.

The reliability of reference gene selection was evaluated by determining root transcript levels of *LePT4* normalized using as references the best-ranked individual (*PGK*, *EF1* and *TUB*) and paired genes (*PGK/TUB* and *EF1/TUB*, Fig. 4) for root tissue obtained from the analysis using geNorm and NormFinder. No differences in gene expression were observed using these selected genes as references. However, the relative quantification of *LePT4* increased when *GAPDH* was used as the reference gene. Therefore, the use of that gene in expression normalization by qPCR can induce an overestimation for relative expression of *LePT4*. Regarding the induction of *LePT4* by mycorrhizal colonization, we observed an overexpression of *LePT4* approximately 20-fold in AM plants compared to NM ones, including lower *LePT4* mRNA amounts in NM. However, relative *LePT4* expression was previously reported to increase and then decrease depending on the time from inoculation (Grabherr et al., 2011). Moreover, and in contrast to our results, several studies showed no *LePT4* expression in the absence of mycorrhization. However, these studies used conventional RT-PCR for quantification (Bray, 1997), which is a less sensitive technique than qPCR, or instead, they used real-time PCR but without evaluating the stability of the reference genes used for normalization (Robinson et al., 2010).

According to stability analyses for genes in mycorrhizal plants, results that are more accurate for relative quantification could be achieved by considering the plant tissues separately due to the observed slight variations in the stability of reference genes in the leaves and roots (Fig. 2 and Table 2). geNorm and NormFinder selected *GAPDH* as the least stable gene, and that instability was greater in the roots than in the leaves or total tissue. This finding is particularly important for the evaluation of symbiosis functionality using the root expression of *LePT4* by qPCR. These data confirm that the selection of reference genes must be analysed separately according to the tissue under study, as reported previously by other authors (Li et al., 2014; Wang et al., 2012; Warzybok and Migocka, 2013). Moreover, the selection of reference genes must be analysed under each particular experimental condition. Our data suggest that *TUB* was one of the most suitable reference genes when using a model in which *LePT4* expression was assessed 60 days after mycorrhizal inoculation. However, published data have demonstrated that tubulin is involved in cytoskeletal rearrangement in mycorrhizal roots that occurs during the early stages of colonization (Bonfante et al., 1996); thus, the *TUB* can be used only to evaluations of gene expression when the mucorrhizal colonization is already established.

Our data suggest that the selection of reference genes for *S. lycopersicum* can be performed in either geNorm or NormFinder, considering the results collected from all biological replicates and experimental conditions as input data. Finally, our results provide a list of potential reference genes for use in the normalization of RT-qPCR experiments in *S. lycopersicum* under symbiotic association with AM fungi, and this study design can be used as a guide for the evaluation of appropriate reference genes for expression analyses in other plants, tissues or experimental conditions.

Authors contributions

A, Fuentes carried out the experimental design. J, Ortiz collected samples and helped on harvest. A, Fuentes; N, Saavedra; L, Salazar; C, Meneses and C, Arriagada performed analysis and writing the manuscript.

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