Plant Cell Rep (2011) 30:63–74
DOI 10.1007/s00299-010-0944-3

ORIGINAL PAPER

Comparison of predictive methods and biological validation for qPCR reference genes in sunflower leaf senescence transcript analysis

Paula Fernandez · Julio A. Di Rienzo · Sebastián Moschen · Guillermo A. A. Dosio · Luis A. N. Aguirrezábal · H. Esteban Hopp · Norma Paniego · Ruth A. Heinz

Received: 10 August 2010 / Revised: 21 October 2010 / Accepted: 22 October 2010 / Published online: 13 November 2010

Abstract The selection and validation of reference genes constitute a key point for gene expression analysis based on qPCR, requiring efficient normalization approaches. In this work, the expression profiles of eight genes were evaluated to identify novel reference genes for transcriptional studies associated to the senescence process in sunflower. Three alternative strategies were applied for the evaluation of gene expression stability in leaves of different ages and exposed to different treatments affecting the senescence process: algorithms implemented in geNorm, BestKeeper software, and the fitting of a statistical linear mixed model (LMModel). The results show that geNorm suggested the use of all combined genes, although identifying *α-TUB1* as the most stable expressing gene. BestKeeper revealed *α-TUB* and *β-TUB* as stable genes, scoring *β-TUB* as the most stable one. The statistical LMModel identified *α-TUB*, *actin*, *PEP*, and *EF-1α* as stable genes in this order. The model-based approximation allows not only the estimation of systematic changes in gene expression, but also the identification of sources of random variation through the estimation of variance components, considering the experimental design applied. Validation of *α-TUB* and *EF-1α* as reference genes for expression studies of three sunflower senescence associated genes showed that the first one was more stable for the assayed conditions. We conclude that, when biological replicates are available, LMModel allows a more reliable selection under the assayed conditions. This study represents the first analysis of identification and validation of genuine reference genes for use as internal control in qPCR expression studies in sunflower, experimentally validated throughout six different controlled leaf senescence conditions.

Keywords Sunflower · Reference genes · qPCR · Senescence

Communicated by M. Jordan.

Electronic supplementary material The online version of this article (doi:10.1007/s00299-010-0944-3) contains supplementary material, which is available to authorized users.

P. Fernandez · S. Moschen · H. E. Hopp · N. Paniego · R. A. Heinz (✉)
Instituto de Biotecnología, CICVyA, INTA Castelar, Las Cabañas y Los Reseros, (1686) Hurlingham, Buenos Aires, Argentina
e-mail: rheinz@cnia.inta.gov.ar

J. A. Di Rienzo
Cátedra de Estadística y Biometría, Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba, Córdoba, Argentina

S. Moschen · G. A. A. Dosio · L. A. N. Aguirrezábal
Laboratorio de Fisiología Vegetal, Unidad Integrada (Facultad de Ciencias Agrarias UNMdP/EEA INTA) Balcarce, C.C. 276, 7620 Balcarce, Argentina

H. E. Hopp · R. A. Heinz
Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina

P. Fernandez (✉)
Escuela de Ciencia y Tecnología, Universidad Nacional de San Martín, Buenos Aires, Argentina
e-mail: pfernandez@cnia.inta.gov.ar

Introduction

Gene expression analysis requires sensitive, precise, and reproducible measurements against reference genes (RGs). RGs, usually miscalled as housekeeping genes (Bustin et al. 2009), have been used to standardize the relative amount of

mRNA in a sample and used as controls for sample loading, blot transfer and probe hybridization under traditional RNA analysis methods (Szabo et al. 2004). Advanced gene expression techniques such as qPCR require accurate normalization approaches to be informative (Bustin 2002; Czechowski et al. 2004; Quackenbush 2002). In this context, RGs must fulfill two main requirements: to be stably expressed in target tissues and cells, and to have an expression level above background (Andersen et al. 2004). However, multiple reports demonstrate that genes extensively used as RGs were not stable enough along different experimental conditions (Barsalobres-Cavallari et al. 2009; Brunner et al. 2004; Coker and Davies 2003; Dombrovski and Martin 2008; Paolacci et al. 2009) and consequently, normalization with multiple genes is becoming a standard strategy (Guenin et al. 2009; Remans et al. 2008). Thus, it has been recommended to use at least three different RGs for wide gene expression analysis (Vandesompele et al. 2002). Highly expressed genes having fundamental roles in the cell are commonly selected as RGs (Spanakis 1993; Suzuki et al. 2000). Even though *actin*, *tubulin*, and *ubiquitin* have been reported as highly variant among treatments in different plant species (Czechowski et al. 2005; Jian et al. 2008; Kim et al. 2003; Martin et al. 2008; Nicot et al. 2005; Wan et al. 2009), they are still widely used as RGs in condition-specific experiments (Guenin et al. 2009), including functional studies in response to abiotic stresses in sunflower (Fernandez et al. 2008; Hewezi et al. 2006). Till now, several studies have reported evaluations of candidate RGs in plants such as potato (Nicot et al. 2005), rice (Jain 2009; Kim et al. 2003; Narsai et al. 2010), *Arabidopsis thaliana* (Czechowski et al. 2005), soybean (Jian et al. 2008; Libault et al. 2008), perennial ryegrass (Martin et al. 2008), poplar (Brunner et al. 2004), tomato (Exposito-Rodriguez et al. 2008; Lovdal and Lillo 2009), grape berries (Reid et al. 2006), the parasitic plant *Orobanche ramosa* (broomrape) (Gonzalez-Verdejo et al. 2008), wheat (Paolacci et al. 2009), coffee (Barsalobres-Cavallari et al. 2009), Darnel ryegrass (Dombrovski and Martin 2008), forage grass *Brachiaria brizantha* (Duarte Silveira et al. 2009), maize (Scholdberg et al. 2009), peach (Tong et al. 2009), cucumber (Wan et al. 2009), chicory (Maroufi et al. 2010), longan tree (Lin and Lai 2010), garden Petunia (Mallona et al. 2010), tobacco (Schmidt and Delaney 2010), *Salvia miltiorrhiza* (Yang et al. 2010), cotton (Artico et al. 2010), faba bean (Gutierrez et al. 2010), flax (Huis et al. 2010), and water lily (Luo et al. 2010). In spite of all these preceding antecedents, specific RGs have not been identified in *Asteraceae* and more specifically in sunflower, yet.

Sunflower (*Helianthus annuus L.*) is the third most important source of edible vegetable oil worldwide and the second one in Argentina. It is also considered as an important source of biodiesel (Sunflower Statistics NSA 2007–2009, USA) (Fernandez et al. 2008). Biotic and abiotic stresses represent serious limitation in sunflower production and both stresses induce senescence (Buchanan-Wollaston et al. 2003; Gan 2003). Leaf senescence occurs during the last stage of leaf development. It is characterized by dramatic changes in cellular metabolism and sequential degeneration of cellular structures. Despite the fact that senescence is a genetically programmed process, its progress could be modulated by either environmental or plant endogenous conditions. Environmental factors such as light (Borrás et al. 2003; Weaver and Amasino 2001), extreme temperatures, nutrient limitations (Borrás et al. 2003; Pommel et al. 2006), drought (Lechner et al. 2008; Pic et al. 2002), level of CO_2, pathogens and plant endogenous effects such as hormonal levels and reproductive development stages (Sadras et al. 2000) might be involved in leaf senescence progression. Several genes regulating the whole process have been identified in *Arabidopsis* (Lim et al. 2003), including genes for hormonal and proteosomal regulation. These changes are accompanied by, or perhaps driven by changes in gene expression (Gan and Amasino 1997; Lim et al. 2007). Identifying suitable RGs is a prerequisite when investigating changes in gene expression profiles for genes potentially involved in leaf senescence.

The reliable identification of senescence-associated genes (SAGs) (Buchanan-Wollaston and Ainsworth 1997) involved in leaf senescence depends on an accurate selection of RGs. Therefore, the use of suitable statistical methods seems mandatory for obtaining meaningful results (Pabinger et al. 2009). Many algorithms have been developed to identify RGs, among them geNorm (Vandesompele et al. 2002), followed by BestKeeper (Pfaffl et al. 2004), are the most frequently used software for this purpose. On the other hand, statistical linear models were also applied for selecting RGs from a wide range of candidate stable genes (Andersen et al. 2004; Chervoneva et al. 2010; Reid et al. 2006; Szabo et al. 2004).

The aim of this work was to identify reliable candidate sunflower RGs adequate for leaf senescence expression studies under different experimental conditions. Expression profiles of eight genes of *H. annuus L.* were evaluated using three alternative approaches for selection of genuine RGs: application of the geNorm algorithm (Vandesompele et al. 2002), the BestKeeper algorithm (Pfaffl et al. 2004), and the fitting of a statistical linear-mixed model (LMModel). Three SAGs previously reported for *Arabidopsis thaliana* (Gepstein et al. 2003), a vacuolar processing enzyme involved in maturing and activation of vacuolar proteins (AN At4g32940) (D3 gene); an aleurain protease, belonging to cystein-proteases family (AN At5g60360) (D4 gene), and a third SAG gene with a calcium binding function, acting mainly in signaling regulation (AN At1g18210) (R2

gene) were then validated by qPCR analysis normalized against the two most stable RGs detected.

Materials and methods

Plant materials and experimental conditions

Field experiments were carried out at INTA Balcarce Experimental Station (37°45′*S*, 58°18′*W*) during the 2003/2004 crop season. Sunflower hybrid VDH 481 (Advanta Seeds) was sown at a 7.2 plants/m^2 density and seeds emerged 11 days later. Diseases, weeds, and insects were adequately controlled. Soil fertility assured maximum yields under adequate water conditions. Rainfall was complemented with irrigation when necessary to avoid water deficit, except for the case of water deficit treatment. Volumetric humidity of the soil was measured periodically by the Time Domain Reflectometry method, with a moisture measuring system (Trase System, Model 6050X1, Soilmoisture Equipment Corp., Santa Barbara, CA, USA). Leaves 15 and 25 (numbered from the bottom to the top of the plant) were considered as initiated when their primordia were visible (about 40 μm long) on the apical meristem under a microscope.

The following experimental conditions were applied to either accelerate or delay leaf senescence in comparison with control plants:

Water deficit (D). A mild water deficit was achieved by covering the soil with a 200 μm plastic mesh to avoid rainfall penetration into the soil. The mesh was installed 10 days before flowering, and the volumetric humidity of the soil was reduced about 40%. Controlled irrigation helped to maintain the deficit level from treatment application till the sample harvest day.

Head excision (FE). The head of several plants was cut with a fine scalpel 4 days before flowering to suppress the reproductive sink.

Control (C). Untreated plots were kept as controls.

A total of six treatments were considered to evaluate gene stability: water deficit−leaf 15 (D.L1), water deficit−leaf 25 (D.L2), head excision−leaf 15 (FE.L1), head excision−leaf 25 (FE.L2), control−leaf 15 (C.L1), and control−leaf 25 (C.L2).

The experiment was conducted as a randomized complete block design with three replicates (plant–plots). Each experimental unit was integrated by three randomly selected plants from each plot.

RNA isolation and quality controls

The analysis was performed on healthy green leaf samples from 63-day-old plants to assure RNA integrity. Samples were immediately frozen in liquid nitrogen and conserved at −80°C until their processing. High-quality total RNA was isolated and reverse transcribed. The same cDNA sample was used for qPCR of each of the eight studied genes. RNA was extracted using the RNeasy plant mini kit, including DNase treatment according to manufacturer's instructions (Qiagen, Germany).

RNA concentration was measured using a Nanodrop ND-100 spectrophotometer (NanoDrop Technologies, USA). Purity and integrity of total RNA were determined by 260/280 nm ratio and the integrity was checked by electrophoresis in 1% agarose gel.

Primer design for RGs and SAGs

Eight plant genes commonly used as internal control for expression studies (Brunner et al. 2004; Czechowski et al. 2005; Jain et al. 2006; Kim et al. 2003; Nicot et al. 2005) were selected for RG evaluation in sunflower; *α-tubulin* (*α-TUB*), *β-tubulin* (*β-TUB*), *18S rRNA*, *26S rRNA*, *actin*, *ubiquitin* (*UBQ13*), *plastid-encoded RNA polymerase* (*PEP*) and *elongation factor 1-α* (*EF-1α*).

Sunflower EST Gene Index Project was queried to detect sunflower homologous of genes commonly used as internal control for gene expression analysis. The evaluated sunflower RGs with the corresponding accession numbers and gene function are listed in Table 1.

Specific primer pair sets for qPCR were designed based on selected sequences using Primer Express 2.0 software (PE Applied Biosystems, USA) under default parameters (Table 1). Expression stabilities of these genes were assessed by qPCR for 18 tissue samples including three experimental conditions, two leaf ages and three biological replicates. The same cDNA sample was used for real-time PCR of each of the eight tested genes. In order to validate suitable RGs, three sunflower genes homologous to *A. thaliana* SAGs genes (AN At4g32940, At5g60360, At1g18210) (D3, D4 and R2 genes) were identified (Gepstein et al. 2003). Specific primer sets for qPCR were designed as described before and are listed in Table 2.

qPCR analysis with SYBR green

Two-step qPCR was performed. cDNA was synthesized from 500 ng DNase treated RNA using Superscript first-strand synthesis system for reverse transcription (Invitrogen, USA) with random hexamer primers according to manufacturer's instructions. cDNAs were diluted 1:5 with nuclease-free water, except for the *18* and *26S rRNA* reactions for which the samples were diluted 1:10. The reaction was carried out in a 25-μl reaction mix containing 200 nM of each RG primer, 1 μ of cDNA sample and 1× QuantiTect SYBR Green PCR master mix (Qiagen,

Table 1 Primer sequences of eight RGs, product size, and melting temperature of the amplified product

Gene name[a]	*H. annuus* public EST AN[b]	Homolog GenBank AN[b]	Gene description	Organism	Primer sequence 5′–3′	Primer sequence 3′–5′	Product size (bp)/ efficiency (%)[c]	Temperature (°C)
Actin	DY915068	AF282624.1	Actin	*H. annuus*	AGGGCGGTCTTTCCAAGTAT	ACATACATGGCGGGAACATT	122/100	67
EF-1α	DY910258	AF308938.1	Elongation factor	*S. lycopersicum*	ACCGAGTGGTGGGTATTCAG	TCAACGCTTGATCACACC	113/92	64
18S rRNA	BQ976519	AF107577.1	18S ribosomal RNA gene	*H. annuus*	TTTTAGGCCACGGAAGTTTG	GTACAAAGGGCAGGGACGTA	107/95	64
26S rRNA	BQ969069	AF479183.1	26S ribosomal RNA gene	*H. annuus*	GCTTCCGATTCACGGAATAA	AGAATCAGCGGGGAAAGAAG	151/90	66
α-TUB	GE504647	AF401481.1	α-tubulin gene	*H. annuus*	TGAAGGTGGAGAGACCAACC	ACTGGTGCCAATGAGGAAAG	175/92	68
β-TUB	DY921120	AF276945.1	β-tubulin gene	*A. rosea*	AAGCAATTGCCTCCGTAAGA	TGTTGCTCAACAAGGCTACG	153/92	68
UBQ13	DY914108	L05401	Polyubiquitin gene sequence	*A. thaliana*	AAACCTTGACAGGCAAGACA	GCTAAAGTCCTACCATCCTCCA	105/88	67
PEP	DY912398	Q1KXX0	Plastid-encoded RNA polymerase subunit beta	*H. annuus*	TTTGTGGTGCACAATTTCGT	ACACGCCGAGCATAAATACC	126/85	66

[a] All sunflower ESTs were named based on similarity to protein sequences by using BLAST

[b] Accession number

[c] qPCR efficiency was determined according to previously reported work (Radonic et al. 2004)

Table 2 Oligonucleotides of three sunflower genes homologous to *A. thaliana* SAGs previously reported (Gepstein et al. 2003)

R2F 5′ GTTCATTTTCCTGTGTTTAGTGT 3′
R2R 5′ CTCCAAATTGTTCTAGGTTCATA 3′
D3F 5′ TCTCCTCATAGTATTTAGGTCCA 3′
D3R 5′ TATCTAATATACAAACGCGGGTA 3′
D4F 5′ ATGAATTGAAGCATGCAGTAG 3′
D4R 5′ ATGAGTTCTTTATCAGCCAGTAT 3′

Germany). Control PCRs with no template and minus RT–PCR were performed for each primer pair. qPCRs were performed using a 96-well plate machine (ABI Prism 7000 Sequence Detection System and software, PE Applied Biosystems, USA). The thermal profile was set to 60°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s, and 60°C for 1 min. Specificity of amplicons was verified by melting curve analysis (60 to 95°C) after 40 PCR cycles and by agarose gel electrophoresis. Three biological replicates for each treatment and two technical replicates for each biological replicate were used for qPCR analysis. qPCR efficiency was determined for each gene and each stress treatment with the slope of a linear regression model (Pfaffl 2001). For this test, each cDNA sample was bulked and then used as the PCR template in a range of 50, 25, 10, 5 and 2 ng. The corresponding qPCR efficiencies were calculated according to (Radonic et al. 2004): $E = (10^{[-1/\text{slope}]} - 1) \times 100$. All PCRs displayed efficiencies between 85 and 100% according to this formula.

Measurement of gene expression stability

Gene-stability was evaluated by geNorm (Vandesompele et al. 2002), BestKeeper software (Pfaffl et al. 2004), and by application of LMModel.

Normalized Cq values of the biological replicates were the input data for geNorm (Bustin et al. 2009), to assess stability among treatments and replicates. Cq values were previously transformed to relative quantities using the comparative Cq method (Livak and Schmittgen 2001; Supplementary Table 1). The highest relative quantity within each gene, across treatments and replicates, was set to 1. Software default values were applied.

In BestKeeper, the input data to evaluate gene stability was the biological replicate average value of each treatment.

Regarding the analysis by LMModel, Cq values of every gene in each condition were considered as input data. The model included the stress factor (D, FE, and C), the leaf-age factor (L1, L2), the interaction between them and the blocks and plots-within-blocks random effects. The last factor was included considering a possible correlation

between the observations of leaves 15 and 25 within plots. Subsequently, using the Akaike Information Criterion (Akaike 1974), the model was reduced eliminating the plots-within-blocks random effect. The effects of fixed terms of the model were summarized by their associated *P* values. A *P* value that is lower than a given significance level is indicative of the presence of systematic changes in gene expression among treatments. Considering that the purpose of the assay was to identify candidate RGs, a significance level of 0.10 was set, instead of the standard of 0.05. The reason for using a higher significance level was to tighten up the criterion to declare a gene as a RG.

The uncontrolled variability of Cq (not explained by the fixed terms of the model) was reported as CV value calculated as follows: $\mathrm{CV} = \sqrt{\sigma_b^2 + \sigma_\varepsilon^2} \Big/ \bar{C}q$ where σ_b^2 is the between blocks variance, σ_ε^2 is the residual variance, and $\bar{C}q$ is the overall mean.

Results

Identification of sunflower candidate RGs

BLAST algorithm (Altschul et al. 1990) was performed against the Sunflower EST Gene Index Project to detect sunflower homologous to RG genes that are commonly used as internal control for gene expression analysis in the literature. Gene name, accession number, gene description, primer sequences, and amplicon length are provided in Table 1. All sunflower unigenes were named based on similarity to known proteins or nucleotide sequences using BLAST with a score value higher than 80 and *E* value lower than 0.01.

In order to verify that the selected genes were suitable for sunflower gene expression studies, a qPCR assay based on SYBR green detection was designed for transcript profiling. Considering that normalization with multiple references is now becoming a standard procedure (Lovdal and Lillo 2009), we applied two of the most widely used softwares for RG selection (geNorm and BestKeeper) and a statistical analysis using LMModel in order to rank the potential RG genes according to their inner stability along treatments.

geNorm results

Gene stability was estimated by the *M* value using geNorm. This software calculates *M* (a gene stability value) and the pairwise variation *V* between two sequential normalization factors containing an increasing number of genes (Vandesompele et al. 2002). *M* value is defined as the average pairwise log2-transformed expression ratios of a certain gene respect to all other candidates RGs, whereas *V* is used to select the minimum number of RGs needed for an accurate normalization.

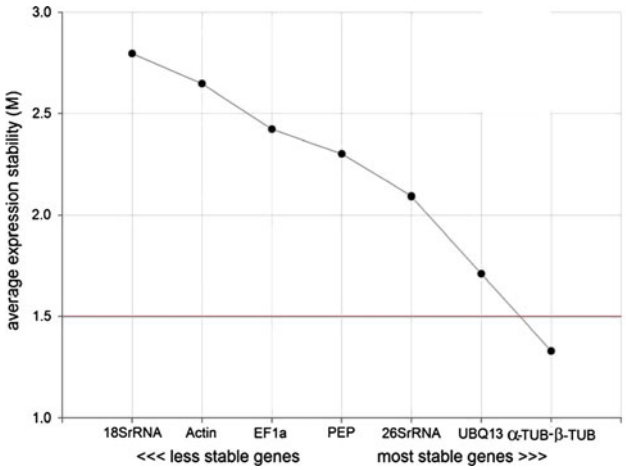

Fig. 1 Expression stability and ranking of RGs as calculated by geNorm in all 18 cDNAs samples series The lower value of average expression stability (*M* values) indicates more stable expression

Data were transformed to relative quantities of Cq values for each gene and their calculated *M* value is presented, showing the output results by geNorm (Fig. 1; Supplementary Table 1). Besides, to evaluate the optimal number of genes required for accurate normalization, pairwise variations (V_n/V_{n+1}) between consecutively ranked normalization genes were calculated (Fig. 2). Hence, according to the geNorm stepwise elimination procedure, the most stable genes among the tested ones were *α-TUB* and *β-TUB*, both with a final *M* value below 1.5 (default geNorm cut-off value). However, according to the 0.15 cut-off for the *V* value proposed by geNorm in the pairwise variation analysis (Vandesompele et al. 2002), the eight genes should be included as RGs along the experiment ($V_{7/8} = 0.229$) for normalizing gene expression (Fig. 2).

BestKeeper results

BestKeeper estimates gene expression stability for all individual reference genes by performing pairwise correlation analyses using raw Cq values of each gene. RGs are combined into an index that is (BestKeeper index, BKI) used to rank the best control genes according to their correlations with BKI (Pfaffl et al. 2004).

Supplementary Table 2 summarizes the descriptive statistics given by BestKeeper for the eight candidate RGs. The table shows mean deviations of six RGs in bold letters. According to this software, genes having mean deviations of Cqs over 1 are inconsistent and consequently they should not be chosen as RGs. Then, *α-TUB* and *β-TUB* were evaluated in a subsequent step of BestKeeper

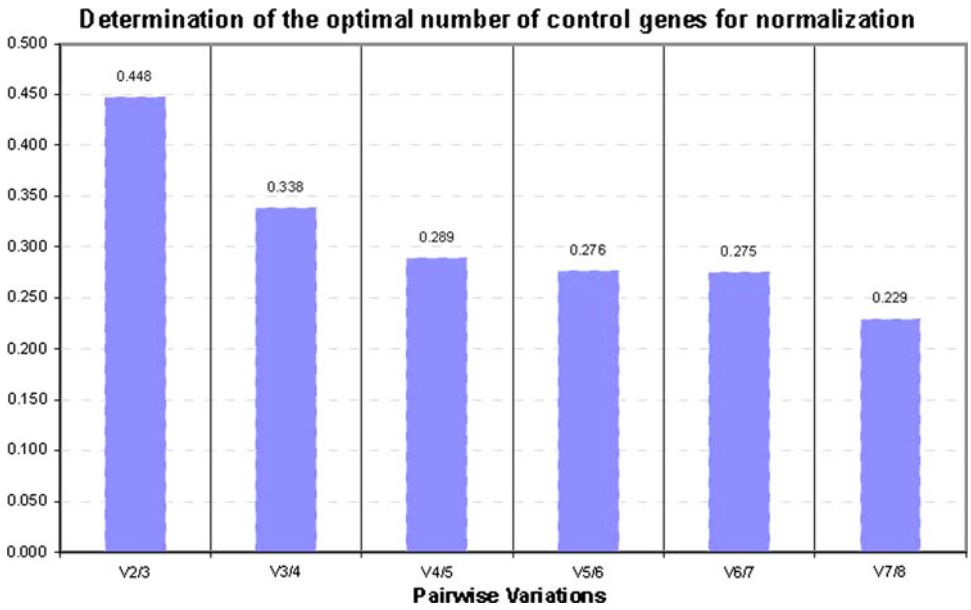

Fig. 2 Determination of the optimal number of RGs pairwise variation analysis (*V*) of selected RGs. *Each bar* represents change in normalization accuracy when stepwise adding more RGs according to the ranking in Fig. 1

algorithm. This step consists in calculating the BKI and its correlation with selected genes. The correlation between BKI and *α-TUB* was 0.745 ($P = 0.001$) while BKI and *β-TUB* was 0.773 ($P = 0.001$). Thus, according to BestKeeper, *β-TUB* should be selected considering that it showed the highest correlation to BKI.

LMModel analysis

Analyzing candidate RGs expression stability by LMModel, we found that results were quite different to geNorm or BestKeeper output scorings, except for *α-TUB* (Table 3). The *P* values for *26S rRNA*, *UBQ13*, and *β-TUB* were indicative of systematic variations among treatments, making them not appropriate for RGs. Considering the remaining genes, *α-TUB* showed the higher stability according to CV value. Under the same criteria, *actin*, *PEP* and *EF-1α* transcripts may also be used as RGs. It has to be pointed out that the CV values for *actin*, *PEP* and *EF-1α* transcripts are larger than the CV for *α-TUB*. *18S rRNA* showed the highest CV value and a border-line *P* value. Figure 3 illustrates the changes in gene expression (Cq) among treatments, confirming that *α-TUB*, *actin*, *PEP* and *EF-1α* were the best candidate RGs, in this order.

The scoring of each gene according to the three RG selection methods (geNorm, BestKeeper and LMModel) is shown in Table 4. A score of 1 implies more stability in gene expression. For each selection method, the score was calculated leaving one replicate out at a time ("$-r$" implies that replication "*r*" was omitted from calculations). It is possible to visualize the stability of the scoring system provided by each of the methods and easily compare their results. In general, scoring was quite stable within methods and *α-TUB* was top-positioned across all methods. However, there are important discrepancies regarding *β-TUB*, *UBQ13* and *actin*. For LMModel, *β-TUB* should not be recommended; meanwhile geNorm and BestKeeper suggest the opposite. In the same way, a discussion may be held regarding the performance of the other genes.

Considering the contrasting results among scoring systems, we decided to further validate two potential RGs for experimental validation: one scored by the three methods and the other one scored only by the LMModel.

Validation of selected RGs for SAGs expression studies in sunflower

Considering that normalization with a single reference gene can lead to significant errors (Vandesompele et al. 2002), two RGs, *α-TUB*, and *EF-1α*, showing different stability scores (Tables 3, 4), were selected for SAGs expression studies normalization.

The expression of three sunflower putative genes homologous to *A. thaliana* SAGs (Gepstein et al. 2003) are as follows: a vacuolar processing enzyme (AN At5g60360) (D3 gene); an aleurain protease, belonging to cystein-proteases family (AN At1g18210) (D4 gene); and a calcium binding function (AN At4g32940) (R2 gene) was analyzed by qPCR using *α-TUB* and *EF-1α* as RGs.

Average Cq of both selected RGs versus target SAGs are listed in Table 5. Cq values for *α-TUB* showed less variation than *EF-1α*. Consistently with this observation when *α-TUB* was used as RG, no significant difference was observed among treatments whereas a slight difference was detected between D4 expression data and D3 or R2 values

Table 3 qPCR statistics for the evaluated RGs by LMModel

Gene name	Mean Cq	CV	Fixed effects *P* value
α-TUB	33.82	3.2	0.9227
Actin	33.41	7.7	0.4179
EF-1α	28.10	10.2	0.2582
PEP	29.14	7.7	0.2608
18S rRNA	19.29	16.1	0.1102
β-TUB	35.82	2.3	0.0426
UBQ13	31.51	4.3	0.0190
26S rRNA	18.94	8.2	0.0035

when *EF-1α* was used as RG, reemphasizing the better reliability of using multiple reference genes as normalization factor (Figs. 4, 5).

Discussion

Gene expression analysis using qPCR requires efficient normalization approaches to be informative. Thus, selection of RGs should be carefully evaluated for differential expression assays. When data from large scale transcriptional studies are available, such as in the case of *Arabidopsis* (Czechowski et al. 2005) and rice (Narsai et al. 2010), RGs can be directly chosen from those data sets. Alternatively, the species for which these tools are not available, selection of candidate genes relies on the evaluation of genes widely described as more stably expressed in different systems. For the present study, eight traditionally considered RGs were evaluated in sunflower for identification of true RGs for senescence transcriptomic profiling assays. Selection of RGs by geNorm and

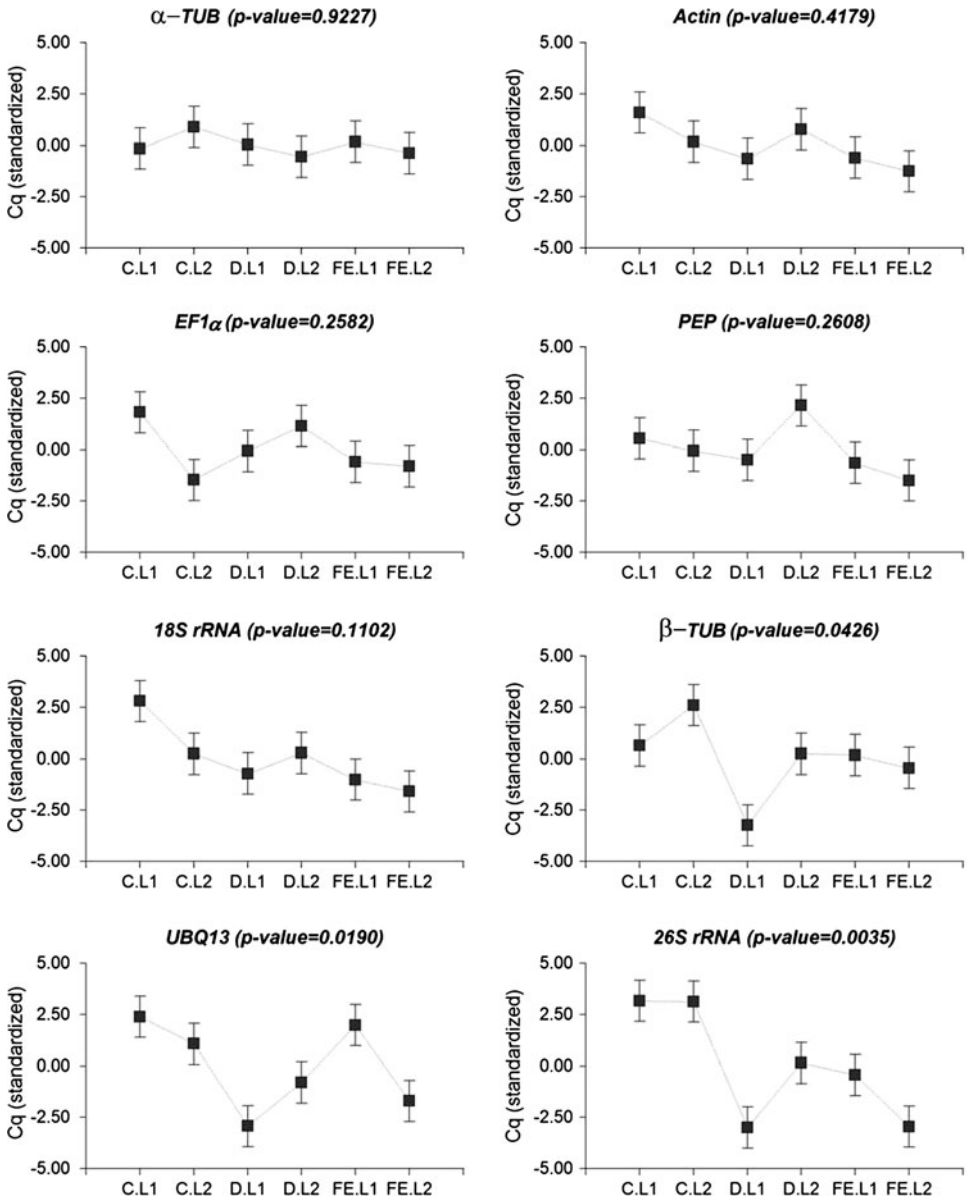

Fig. 3 RNA transcription levels of RGs tested, presented as Cq mean value in the different samples. Each Cq value is the mean of three replicates. *C.L1* Control leaf 15, *C.L2* Control leaf 25; *D.L1* Water deficit leaf 15, *D.L2* Water deficit leaf 25, *FE.L1* Head excision leaf 15, *FE.L2* Head excision leaf 25

Table 4 Gene scoring according to RG-selection methods: geNorm, BestKeeper and LMModel

	α-TUB	*Actin*	*EF1α*	*PEP*	*18S rRNA*	*β-TUB*	*UBQ13*	*26S rRNA*
GeNorm-1	2	5	7	6	8	1	3	4
GeNorm-2	2	7	8	1	6	3	4	5
GeNorm-3	2	5	7	6	8	1	3	4
GeNorm average	2.0	5.7	7.3	4.3	7.3	1.7	3.3	4.3
BestKeeper-1	1	5	7	4	8	2	3	6
BestKeeper-2	1	5	7	2	8	3	4	6
BestKeeper-3	1	5	7	4	8	2	3	6
BestKeeper average	1.0	5.0	7.0	3.3	8.0	2.3	3.3	6.0
LMModel-1	2	3	1	4	5	6	8	7
LMModel-2	1	2	7	3	4	5	6	8
LMModel-3	1	3	4	2	6	5	7	8
LMModel average	1.3	2.7	4.0	3.0	5.0	5.3	7.0	7.7

The table shows the scoring of each gene according to the three RG-selection methods: GeNorm, BestKeeper and LMModel being a score of one ("1") the best score. For each method the score was calculated leaving aside one of the three replicates at a time ("$-r$" indicates that replication "r" was omitted from calculations). The average of score for each method is shown for each gene as a summary

Table 5 Average Cq and CV value for R2, D3 and D4 genes and the two best ranked RGs for three biological replicates per treatment

Treatment	Samples	SAGs genes (Gepstein et al. 2003)						RGs genes			
		R2 (AN At4g32940)		D3 (AN At5g60360)		D4 (AN At1g18210)		*α-TUB* (AN GE504647)		*EF-1α* (AN DY910258)	
		Cq	CV	Cq	CV	Cq	CV	Cq	CV	Cq	CV
C.L1	3	30.49	2.5	31.06	5.0	28.74	3.9	33.69	1.7	30.08	2.6
C.L2	3	30.00	0.8	30.19	3.8	27.42	4.5	34.20	1.9	25.57	7.2
FE.L1	3	30.46	3.9	28.96	4.3	26.42	6.6	33.52	2.9	27.16	1.7
FE.L2	3	29.28	0.6	29.13	1.1	24.84	2.7	33.32	2.6	26.73	12.9
D.L1	3	30.45	2.1	30.31	1.7	26.10	5.1	33.67	0.2	27.80	6.7
D.L2	3	29.75	2.0	29.12	5.5	24.98	1.5	33.38	4.5	30.07	5.7

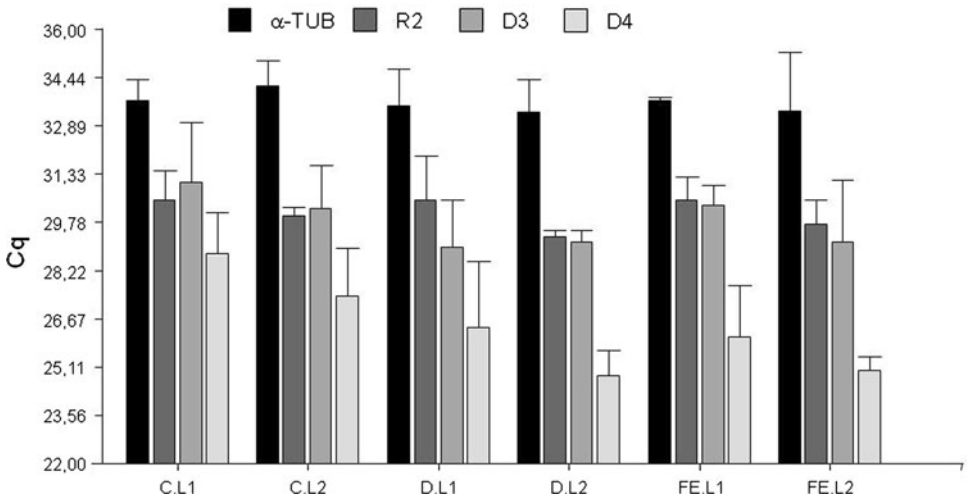

Fig. 4 Average Cq of analyzed SAGs genes normalizing against *α-TUB* as RG. *Error bars* show standard deviation

BestKeeper algorithms is commonly used in relative gene expression studies (Duarte Silveira et al. 2009; Guenin et al. 2009; Tong et al. 2009). NormFinder is another widely used method, but it requires a minimum of eight samples per group to obtain reliable results (Andersen et al. 2004). Both, geNorm and BestKeeper methods are often

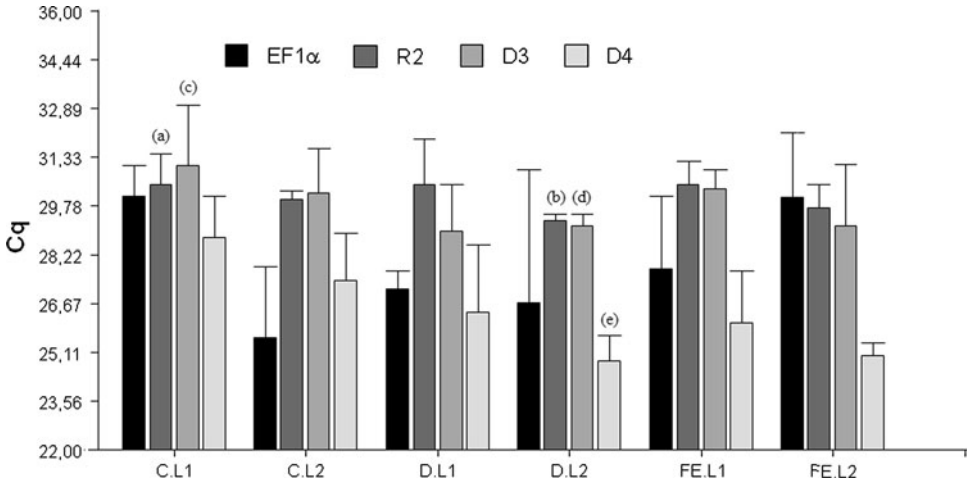

Fig. 5 Average Cq of analyzed SAGs genes normalizing against *EF-1α* as RG. *Error bars* show standard deviation. *a* R2 gene in C.L1 over-expressed respect to C.L2 and a FE.L1, *b* R2 gene in D.L2 over-expressed respect to C.L2, *c* D3 gene in C.L1 over-expressed respect to C.L2, *d* D3 gene in D.L2 over-expressed respect to C.L2, *e* D4 gene in D.L2 over-expressed respect to C.L2 and D.L1

applied even in situations where no biological replicates are available. In contrast, when biological replicates are available, a statistical model can be fitted. LMModel not only allows the estimation of systematic changes in gene expression, but also takes into account the details of experimental design and the sources of random variation through the estimation of variance components. Moreover, LMModel not only measures the stability of relative gene expression but also tests its significance in a well-established theoretical framework. This is also the reasoning behind the application of linear models in other RG's-identification studies (Andersen et al. 2004; Olbrich et al. 2008; Szabo et al. 2004).

When applied to sunflower leaf senescence system, geNorm suggests *α-TUB* and *β-TUB* as the best RGs, although, according to the 0.15 pairwise variation cut-off (*V* value), it encourages to include all eight genes to support gene expression studies. Even though the optimal cut-off *V* number could be around 0.15 (Vandesompele et al. 2002), other works consider a higher *V* value, depending on the dataset tested (De Ketelaere et al. 2006; Kuijk et al. 2007). geNorm software is strongly dependent on the assumption that none of the analyzed genes are co-regulated which would lead to erroneous selection of RGs (Andersen et al. 2004). Hence, in order to revise the effect of gene co-regulation in the geNorm ranking of stable genes, each gene was stepwise eliminated from the panel before running geNorm, resulting in the selection of *α-TUB* or *β-TUB* as the more stable genes, followed by UBQ13. BestKeeper also selects *α-TUB* and *β-TUB* in the first step according to the CV value, and suggests *β-TUB* as the best RG candidate. It is clear that both software-based strategies lead to similar gene scorings. On the other hand, the predictive model-based approach concluded that *α-TUB*, *actin*, *EF-1α* and *PEP* were stably expressed among the different experimental conditions. However, except for *α-TUB* that was selected as RG by all the three methods, there are important differences between model-based approach and software-based approach. LMModel classifies *β-TUB* as a non-stable gene while actin, *EF-1α*, and *PEP* were predicted as appropriate RGs (Fig. 3; Table 3). These contrasting results emphasize the need to standardize and normalize qPCR by a common language and statistical method (Bustin et al. 2009; Pabinger et al. 2009). Considering RGs validation and normalization in other orphan and/or model plants, *ubiquitine* was suggested to be an appropriate internal control for qPCR studies in rice (Jain 2009) but was ranked as unstable in soybean (Jian et al. 2008). However, in *Arabidopsis* (Czechowski et al. 2005) and tomato (Lovdal and Lillo 2009), *ubiquitine* showed highly stable expression levels. In the present study, results from three methodologies showed that this gene underwent variation according to experimental conditions. On the other hand, *tubuline* showed an unacceptable variable expression in peach (Tong et al. 2009), perennial ryegrass (Martin et al. 2008), in the forage grass *Brachiaria brizantha* (Duarte Silveira et al. 2009), wheat (Paolacci et al. 2009), grape berry (Reid et al. 2006), and flax (Huis et al. 2010), but was found to be an appropriate RG in cucumber (Wan et al. 2009), longan tree (Lin and Lai 2010), and in our report on sunflower. Similarly, caution should be taken in the case of *18S* or *28S rRNA* RG selection. The reference gene status of ribosomal proteins lacks a trustable performance because they demonstrated to be more highly expressed in tissues with faster cell division and by showing profound differences between conditions due to expression of tissue-specific isoforms or transcript variants (Thorrez et al. 2008) In spite of this, *28S rRNA* was selected as the most stable RG in rice, being widely used until a recent study based on a *Oryza sativa* microarray reported new reliable RGs (Narsai et al. 2010). In chicory, *actin*, *EF-1α*, and *18S rRNA* were found as the most stable RGs, however, rRNA was excluded as a potential RG due to its high rate level of cellular expression, as previously

discussed (Maroufi et al. 2010). In addition, *EF-1α* kept stable in the apomictic and sexual grass *Brachiaria brizantha* (Duarte Silveira et al. 2009), perennial ryegrass (Martin et al. 2008), grape berry (Reid et al. 2006), potato (Nicot et al. 2005), and also in cucumber (Wan et al. 2009), whereas, interestingly, we reported this gene as variable under salinity conditions in a cDNA microarray analysis for sunflower (Fernandez et al. 2008).

Besides the discrepancies regarding the stability of *β-TUB*, *UBQ13*, and *actin* genes (Tables 3, 4) by means of the three evaluated procedures, other differences emerged regarding the differential performance of remaining genes evaluated. Considering these results, we decided to biologically validate two of the recommended RGs for SAGs expression studies. In this context, we selected *α-TUB* based on its high stability profile under the three statistical methods applied, and *EF-1α* as a second RG which, in contrast, addressed different score values according to the selection approach used. The model-based approach was applied to analyze three sunflower genes homologous to *A. thaliana* SAGs (Gepstein et al. 2003) using *α-TUB* and *EF-1α* for an experimental design that included three treatments, two leaf ages, and three biological replicates. This study confirmed that *α-TUB* was more stable than *EF-1α* within different senescence related treatments, demonstrating that *α-TUB* shows a good hit independently of which RG selection method is applied.

LMModel not only accurately estimates systematic changes in gene expression, but also tests their significance in a well-established theoretical framework (Szabo et al. 2004). It also allows the quantification of sources of random variation and the inclusion into the model of the details of the experimental design. This may be the reasoning behind the application of linear models in other RGs identification studies (Andersen et al. 2004; Olbrich et al. 2008; Szabo et al. 2004). According to these considerations and following the results obtained through the experimental validation of three sunflower putative SAGs genes, the selection of *α-TUB* by the three methods emerge as the most suitable for our experimental conditions. Nevertheless, it is worth noting the importance of the validation of more than one RG out of the list of recommended RGs derived from either selection method. In the present work, even when *α-TUB* and *EF-1α* were both selected as stable by LMModel, the first one proved to be much more stable for SAGs expression studies than the second.

It should be pointed out that information derived from an ongoing large-scale sunflower transcriptomic analysis based on highly dense oligonucleotide microarray analysis designed by our group, which was technically and experimentally validated, may provide identification of other RGs that will be suitable for different tissues and/or growing conditions of this important crop in the near future.

Conclusions

In this work we demonstrate that gene expression of commonly used RGs is highly variable along different treatments affecting leaf senescence in sunflower. Comparisons of three RG selection methods based on qPCR data analyses showed different results, being only *α-TUB* selected as stable RG by either method. According to LMModel, four reference genes were identified: *α-TUB*, *actin*, *PEP* and *EF-1α*, for the tested experimental conditions. Wet lab validation for sunflower putative SAGs expression studies using two of these RGs, *α-TUB* and *EF-1α*, emphasized the need of normalization using multiple genes. Therefore, these results open new insights about carefully selecting organ-specific and stress-specific RGs for functional genomics in this relevant oil crop.

This study represents the first analysis of identification and validation of genuine reference genes to be used as internal control in qPCR expression studies in sunflower, under leaf senescence conditions.

Acknowledgments This research was supported by CONICET PIP 5788, ANPCyT/FONCYT, Préstamo BID PICT 15-32905, INTA-PE AEBIO 241001and 245001, INTA-PE AEBIO 245711, INTA-AEBI0 243532, INTA PN CER 1336, and UNMdP, AGR212, AGR260. Dr. PdCF, Dr. RAH, Dr. NBP, Dr. GAAD, and Dr. LANA are career members of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina), J.A.D.R., M.Sc., is a Professor at the Agricultural College in National University of Cordoba and Dr. HEH is a career member of the Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC) and a Professor at the Facultad de Ciencias Exactas y Naturales, University of Buenos Aires (UBA). We are grateful to Dr. Marisa Farber for critical reading of the manuscript.

References

Akaike H (1974) A new look at the statistical model identification". IEEE Trans Automat Contr 19:716–723

Altschul S, Gish W, Miller W, Myers E, Lipman D (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Andersen C, Jensen J, Orntoft T (2004) Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res 64:5245–5250

Artico S, Nardeli SM, Brilhante O, Grossi-de-Sa MF, Alves-Ferreira M (2010) Identification and evaluation of new reference genes in *Gossypium hirsutum* for accurate normalization of real-time quantitative RT-PCR data. BMC Plant Biology 10:49

Barsalobres-Cavallari C, Severino F, Maluf M, Maia I (2009) Identification of suitable internal control genes for expression studies in *Coffea arabica* under different experimental conditions. BMC Molecular Biology 10(1):1

Borrás M, Madonni G, Otegui M (2003) Leaf senescence in maize hybrids: plant population, row spacing and kernel set effects. Field Crops Res 82:13–26

Brunner AM, Yakovlev IA, Strauss SH (2004) Validating internal controls for quantitative plant gene expression studies. BMC Plant Biology 4:14

Buchanan-Wollaston V, Ainsworth C (1997) Leaf senescence in *Brassica napus*: cloning of senescence-related genes by subtractive hybridization. J Exp Bot 52:1117–1121

Buchanan-Wollaston V, Earl S, Harrison E, Mathas E, Navabpour S, Page T, Pink D (2003) The molecular analysis of leaf senescence—a genomic approach. Plant Biotechnol J 1:3–22

Bustin S (2002) Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J Mol Endocrinol 29:23–39

Bustin S, Benes V, Garson J, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl M, Shipley G, Vandesompele J, Wittwer C (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55:611–622

Chervoneva I, Li Y, Schulz S, Croker S, Wilson C, Waldman SA, Hyslop T (2010) Selection of optimal reference genes for normalization in quantitative RT-PCR. BMC Bioinformatics 11:253

Coker JS, Davies E (2003) Selection of candidate housekeeping controls in tomato plants using EST data. Biotechniques 35:740–748

Czechowski T, Bari R, Stitt M, Scheible W-R, Udvardi M (2004) Real-time RT-PCR profiling of over 1, 400 Arabidopsis transcription factors: unprecedented sensitivity reveals novel root- and shoot-specific genes. Plant J 38:366–379

Czechowski T, Stitt M, Altmann T, Udvardi M, Scheible W-R (2005) Genome-wide identification and testing of superior reference genes for transcript normalization in *Arabidopsis*. Plant Physiol 139:5–17

De Ketelaere A, Goossens K, Peelman L, Burvenich C (2006) Technical note: validation of internal control genes for gene expression analysis in bovine polymorphonuclear leukocytes. J Dairy Sci 89:4066–4069

Dombrovski JE, Martin RC (2008) Evaluation of reference genes for quantitative RT-PCR in *Lolium temulentum* under abiotic stress. Plant Sci 176:390–396

Duarte Silveira E, Alves-Ferreira M, Arrais Guimaraes L, Rodrigues da Silva F, de Tavares Campos Carneiro V (2009) Selection of reference genes for quantitative real-time PCR expresssion studes in the apomictic and sexual grass *Brachiaria brizantha*. BMC Plant Biol 9:84

Exposito-Rodriguez M, Borges A, Borges-Perez A, Perez J (2008) Selection of internal control genes for quantitative real-time RT-PCR studies during tomato development process. BMC Plant Biol 8:31

Fernandez P, Di Rienzo J, Fernandez L, Hopp H, Paniego N, Heinz RA (2008) Transcriptomic identification of candidate genes involved in sunflower responses to chilling and salt stresses based on cDNA microarray analysis. BMC Plant Biol 8:11

Gan S (2003) Mitotic and postmitotic senescence in plants. Science's SAGE KE

Gan S, Amasino RM (1997) Making sense of senescence. Plant Physiol 113:313–319

Gepstein S, Sabehi G, Carp M-J, Hajouj T, Nesher MFO, Yariv I, Dor C, Bassani M (2003) Large-scale identification of leaf senescence-associated genes. Plant J 36:629–642

Gonzalez-Verdejo CI, Die JV, Nadal S, Jimenez-Marín A, Moreno MT, Román B (2008) Selection of housekeeping genes for normalization by real-time RT–PCR: analysis of Or-MYB1 gene expression in *Orobanche ramosa* development. Anal Biochem 379:38–43

Guenin S, Mauriat M, Pelloux J, Van Wuytswinkel O, Bellini C, Gutierrez L (2009) Normalization of qRT-PCR data: the necessity of adopting a systematic, experimental conditions-specific, validation of references. J Exp Bot 60:487–493

Gutierrez N, Giménez MJ, Palomino C, Avila CM (2010) Assessment of candidate reference genes for expression studies in *Vicia faba* L. by real-time quantitative PCR. Mol Breed. doi:10.1007/s11032-010-9456-7

Hewezi T, Leger M, El Kayal W, Gentzbittel L (2006) Transcriptional profiling of sunflower plants growing under low temperatures reveals an extensive down-regulation of gene expression associated with chilling sensitivity. J Exp Bot 57:3109–3122

Huis R, Hawkins S, Neutelings G (2010) Selection of reference genes for quantitative gene expression normalization in flax (*Linum usitatissimum* L.). BMC Plant Biol 10:7

Jain M (2009) Genome-wide identification of novel internal control genes for normalization of gene expression during various stages of development in rice. Plant Sci 176:702–706

Jain M, Nijhawan A, Tyagi AK, Khurana JP (2006) Validation of housekeeping genes as internal control for studying gene expression in rice by quantitative real-time PCR. Biochem Biophys Res Commun 345:646–651

Jian B, Liu B, Bi Y, Hou W, Wu C, Han T (2008) Validation of internal control for gene expression study in soybean by quantitative real-time PCR. BMC Mol Biol 9:59

Kim B-R, Nam H-Y, Kim S-U, Kim S-I, Chang Y-J (2003) Normalization of reverse transcription quantitative-PCR with housekeeping genes in rice. Biotechnol Lett 25:1869–1872

Kuijk E, du Puy L, TA von Tol H, Haagsman H, Colenbrander P, Roelen B (2007) Validation of reference genes for quantitative RT-PCR studies in porcine oocytes and preimplantation embryos. BMC Dev Biol 7:58

Lechner L, Pereyra Irujo G, Granier C, Aguirrezabal L (2008) Re-watering of plants under water deficit reveals that leaf cells retain their ability to expand after the leaf has apparently reached its final size. Ann Bot 101:1007–1015

Libault M, Thibivilliers D, Bilgin D, Radwan O, Benitez M, Clough S, Stacey G (2008) Identification of four soybean reference genes for gene expression normalization. Plant Genome 1:44–54

Lim PO, Woo HR, Nam HG (2003) Molecular genetics of leaf senescence in *Arabidopsis*. Trends Plant Sci 8:272–278

Lim P, Kim H, Nam H (2007) Leaf senescence. Annu Rev Plant Biol 58:115–136

Lin YL, Lai ZX (2010) Reference gene selection for qPCR analysis during somatic embryogenesis in longan tree. Plant Sci 178:359–365

Livak K, Schmittgen T (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25:402–408

Lovdal T, Lillo C (2009) Reference gene selection for quantitative real-time PCR normalization in tomato subjected to nitrogen, cold, and light stress. Anal Biochem 387:238–242

Luo H, Chen S, Wan H, Chen F, Gu C, Liu Z (2010) Candidate reference genes for gene expression studes in water lily. Anal Biochem. doi:10.1016/j.ab.2010.05.002

Mallona I, Lischewski S, Weiss J, Hause B, Egea-Cortines M (2010) Validation of reference genes for quantitative real-time PCR during leaf and flower development in *Petunia hybrida*. BMC Plant Biol 10:4

Maroufi A, Van Bockstaele E, De Loose M (2010) Validation of reference genes for gene expression analysis in chicory (*Cichorium intybus*) using quantitative real-time PCR. BMC Mol Biol 11:15

Martin R, Hollenbeck V, Dombrowski J (2008) Evaluation of Reference Genes for Quantitative RT-PCR in *Lolium perenne*. Crop Sci 48:1881–1887

Narsai R, Ivanova A, Ng S, Whelan J (2010) Defining reference genes in *Oryza sativa* using organ, development, biotic and abiotic transcriptome datasets. BMC Plant Biol 10:56

National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/)

National Sunflower Association [http://www.sunflowernsa.com/stats/table.asp?contentID=109&htmlID=74&submit170=View&submit.x=57&submit.y=12)]

Nicot N, Hausman J-F, Hoffman L, Evers D (2005) Housekeeping gene selection for real-time PCR normalization in potato during biotic and abiotic stress. J Exp Bot 56:2907–2914

Olbrich M, Gerstner E, Welzl G, Fleischmann F, Obwald W, Bahnweg G, Ernst D (2008) Quantification of mRNAs and housekeeping gene selection for quantitative real-time RT-PCR normalization in European Beech *(Fagus sylvatica L.)* during abiotic and biotic stress. Z Naturforsch C 63:574–582

Pabinger S, Thallinger G, Snajder R, Eichjorn H, Rader R, Trajanoski Z (2009) QPCR: application for real-time PCR data management and analysis. BMC Bioinformatics 10:268

Paolacci A, Tanzarella O, Porceddu E, Ciaffi M (2009) Identification and validation of reference genes for quantitative RT-PCR normalization in wheat. BMC Mol Biol 10:11

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acid Res 29:e45

Pfaffl M, Tichopád A, Prgomet C, Neuvians T (2004) Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper—Excel-based tool using pair-wise correlations. Biotechnol Lett 26:509–515

Pic E, Teyssandier de la Serve B, Tardieu F, Turc O (2002) Leaf senescence induced by mild water deficit follows the same sequence of macroscopic, biochemical, and molecular events as monocarpic senescence in pea. Plant Physiol 128:236–246

Pommel B, Gallais A, Coque M, Quelleré I, Hirel B, Prioul J, Andrieu B, Floriot M (2006) Carbon and nitrogen allocation and grain filling in three maize hybrids differing in leaf senescence. Eur J Agron 24:203–211

Quackenbush J (2002) Microarray data normalization and transformation. Nat Genet 32:496–501

Radonic A, Thulke S, Mackay IM, Landt O, Siegert W, Nitsche A (2004) Guideline to reference gene selection for quantitative real-time PCR. Biochem Biophys Res Commun 313:856–862

Reid KE, Olsson N, Schlosser J, Peng F, Lund ST (2006) An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development. BMC Plant Biol 6:27

Remans T, Smeets K, Opdenakker K, Mathijsen D, Vangronsveld J, Cuypers A (2008) Normalisation of real-time PCR gene expression measurements in *Arabidopsis thaliana* exposed to increased metal concentrations. Planta 227(6):1343–1349. doi:10.1007/s00425-008-0706-4

Sadras V, Echarte L, Andrade F (2000) Profiles of leaf senescence during reproductive growth of sunflower and maize. Ann Bot 85:187–195

Schmidt GW, Delaney SK (2010) Stable internal reference genes for normalization of real-time RT-PCR in tobacco (*Nicotiana tabacum*) during development and abiotic stress. Mol Genet Genomics 283:233–241

Scholdberg TA, Norden TD, Nelson DD, Ronald Jenkins G (2009) Evaluating precision and accuracy when quantifying different endogenous control reference genes in maize using real-time PCR. J Agric Food Chem 57:2903–2911

Spanakis E (1993) Problems related to the interpretation of autoradiographic data on gene expression using common constitutive transcripts as controls. Nucleic Acids Res 21:3809–3819

Suzuki T, Higgins PJ, Crawford DR (2000) Control selection for RNA quantitation. Biotechniques 29:332–337

Szabo A, Perou CM, Karaca M, Perreard L, Quackenbush JF, Bernard PS (2004) Statistical modeling for selecting housekeeper genes. Genome Biology 5:R59

The Dana Farber Cancer Institute Sunflower Gene Index Project (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=sunflower)

Thorrez L, Van Deun K, Tranchevent LC, Van Lommel L, Engelen K, Marchal K, Moreau Y, Van Mechelen I, Schuit F (2008) Using ribosomal protein genes as reference: a tale of caution. PLoS One 3:e1854

Tong Z, Gao Z, Wang F, Zhou J, Zhang Z (2009) Selection of reliable reference genes for gene expression studies in peach using real-time PCR. BMC Mol Biol 10:71

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology 3(7):RESEARCH0034

Wan H, Zhao Z, Qian C, Sui Y, Malik A, Chen J (2009) Selection of appropriate reference genes for gene expression studies by quantitative real-time polymerase chain reaction in cucumber. Anal Biochem. doi:10.1016/j.ab.2009.12.008

Weaver M, Amasino R (2001) Senescence is induced in individually darkened *Arabidopsis* leaves, but inhibited in whole darkened plants. Plant Physiol 127:876–886

Yang Y, Hou S, Cui G, Chen S, Wei J, Huang L (2010) Characterization of reference genes for quantitative real-time PCR analysis in various tissues of *Salvia miltiorrhiza*. Mol Biol Rep 37:507–513