Biocatalysis and Agricultural Biotechnology 20 (2019) 101246

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Selection and validation of reference genes for quantitative real-time PCR in *Rosmarinus officinalis* L. in various tissues and under elicitation

Zahra Aminfar[a], Babak Rabiei[a,**,1], Masoud Tohidfar[b], Mohammad Hossein Mirjalili[c,*,1]

[a] Department of Agronomy and Plant Breeding, Faculty of Agricultural Sciences, University of Guilan, Rasht, Iran
[b] Department of Biotechnology, Faculty of Science and Biotechnology, Shahid Beheshti University, G.C, Tehran, Iran
[c] Department of Agriculture, Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, G. C, Tehran, Iran

ARTICLE INFO

Keywords:
Gene expression
geNorm
NormFinder
Reference gene
RefFinder
Rosemary

ABSTRACT

The data normalization is a major subject in studies on the gene expression by quantitative polymerase chain reaction (qPCR) to ensure the reliability of obtained results. The normalization aims to remove variation sources with non-biological origins at the highest possible level. The accurate expression analysis greatly relies on the utilization of stable reference gene as an internal control for data normalization because expression patterns of these genes considerably alter across various kinds of tissue or within individual experimental conditions. However, little information is available on the stable reference genes in *Rosmarinus officinalis* L. The present study selected seven genes from the frequently-used housekeeping genes as candidate reference genes including Elongation factor1-alpha (*EF1-α*), 18S ribosomal RNA (*18S rRNA*), Actin (*ACT*), alpha-Tubulin (*α-TUB*), Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), Cyclophilin (*CYP*), and beta-Tubulin (*β-TUB*). Four widely-used statistical algorithms, called NormFinder, geNorm, RefFinder, and BestKeeper, were utilized to evaluate the stability of expressing candidate reference genes in different types of tissue (callus, leaf, flower, stem, and root) and under the elicitor treatment (salicylic acid and methyl jasmonate). According to comprehensive results, *GAPDH* and *18S rRNA* were suitable and unsuitable candidate genes, respectively. The obtained findings are helpful for future studies on the gene expression in *R. officinalis* so as to attain improved and reliable results.

1. Introduction

Rosemary (*Rosmarinus officinalis* L., Lamiaceae) is a perennial herb with a woody form and various pharmacological effects due to its abundant secondary metabolites such as antioxidant, (Rašković et al., 2014), antibacterial, (Bozin et al., 2007), antimutagenic (Tajehmiri et al., 2014), antidiabetic (Bakırel et al., 2008), anti-inflammatory and antinociceptive (de Melo et al., 2011; Estevez et al., 2007; González-Trujano et al., 2007), hepatoprotective (Abdel-Wahhab et al., 2011), and anticancer (Moore et al., 2016; Ngo et al., 2011). The main chemical elements of rosemary (i.e., triterpenoid acids, carnosic acid, caffeic acid, carnosol, and rosmarinic acid) have high antioxidant activities as the cause of a majority of its pharmacological effects (Ngo et al., 2011). Due to these natural compounds, it has been studied for its therapeutic potential and theses pharmaceutically valuable secondary compounds have attracted much attention in industrial production. Nevertheless, there is a little information about genes and metabolic pathways; hence, the gene expression analysis has been widely considered to investigate transcript levels in specific tissues or different conditions to assess metabolic pathways of secondary metabolites. qPCR is a very sensitive, specific, rapid, and accurate technique to quantitatively measure changes in the gene expression on transient and spatial scales under fluctuating ecological or trial conditions (Valasek and Repa, 2005). Moreover, it is the only system that can recognize a few copies of mRNA. Appropriate normalization methods are necessary to achieve the highest analytical ability because of troubles in different steps of preparation and sample processing (Bustin et al., 2009). The precision of accomplished outcomes by qPCR toughly relies on the normalization against the stable reference gene expression to reduce the impact of technical noise and differences in the sample preparation (Guénin et al., 2009).

Despite the intrinsic ability of reference genes to catch all non-

* Corresponding author.
** Corresponding author.
E-mail addresses: rabiei@guilan.ac.ir (B. Rabiei), m-mirjalili@sbu.ac.ir (M.H. Mirjalili).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.bcab.2019.101246
Received 15 May 2019; Received in revised form 10 July 2019; Accepted 10 July 2019
Available online 12 July 2019
1878-8181/

biological variations, the regulation of reference genes under some circumstances have been proved in studies (Vandesompele et al., 2009); hence, the validation in an experimental condition is a crucial task whether a candidate reference gene is appropriate for normalization. The selection of unsuitable reference genes for qPCR can prompt incorrect and deluding results in the normalizing step. (Bustin et al., 2013, L Gutierrez et al., 2008). It is more important in trying to quantify the gene expression for metabolic engineering applications. The ideal endogenous reference gene should not co-regulate with the target gene and experienced expression with a fixed amount in all tissues, development phases and distinctive experimental conditions in the qPCR analysis (Radonić et al., 2004). Determination of a suitable reference gene is a big challenge as the expression stability should be calculated without utilizing other references (Guénin et al., 2009). Therefore, some statistical algorithms like NormFinder (Vandesompele et al., 2002), BestKeeper (Andersen et al., 2004), and geNorm (Pfaffl et al., 2004) were evolved to assess the strength and distinguish the best reference genes under certain circumstances.

Numerous studies have sought to investigate stable reference genes in a lot of plant specimens such as *Arabidopsis thaliana*, as a model plant, and non-model plants such as *Oryza sativa* (Jain et al., 2006; Ji et al., 2014), *Glycine max* (Nakayama et al., 2014), *Solanum lycopersicum* (Expósito-Rodríguez et al., 2008), *Solanum tuberosum* (Nicot et al., 2005), *Triticum aestivum* (Paolacci et al., 2009), *Zea mays* (Lin et al., 2014; Manoli et al., 2012), *Cichorium intybus* (Maroufi et al., 2010), *Salvia hispanica* (Gopalam et al., 2017). Published reports on internal control genes have mainly focused on HKGs in which protein products are engaged with fundamental routes in cell, for example ribosomal subunit synthesis, the glycolytic pathway, protein folding and parts of cytoskeleton. 18S ribosomal RNA (*18S rRNA*), Ubiquitin (*UBQ*), Actin (*ACT*), α-Tubulin (*α-TUB*), β-Tubulin (*β-TUB*), Elongation factors (*EF*), Cyclophilin (*CYP*), and Glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*), are the most popular validated endogenous genes in plants (Kozera and Rapacz, 2013) that express with an steadily and consistent patterns within various tissue types, developmental stages and different conditions. However, it is found that no single gene can be introduced as a reference gene with the greatest stability and reliability to normalize the derived raw data from the whole trial background and plants. Therefore, the identification and evaluation of appropriate reference genes should be accomplished in different plant species. There has been no report on the assessment of reference genes in *R. officinalis*; hence, it is essential to monitor a preeminent reference gene for the accurate normalization in the analysis of expression of genes by qPCR.

The present study systematically and quantitatively evaluated a panel of seven candidate reference genes to examine stable conditions of expression in different tissues (callus, leaf, flower, stem, and root) and under elicitor treatments including salicylic acid and methyl jasmonate that could simulate various abiotic and biotic stress in *R. officinalis*. Ranking of stable candidate reference gene was performed by NormFinder, BestKeeper, RefFinder, and geNorm. The research outcomes are practical for analyzing the gene expression in *R. officinalis* in the future to achieve reliable results.

2. Materials and methods

2.1. Plant material and elicitor treatment

A cultivated population of *R. officinalis* was chosen in a botanical garden of the Medicinal Plants and Drugs Research Institute (MPDRI), Shahid Beheshti University, Tehran, Iran, as a plant material and explant source. Four tissues, namely leaf, flower, stem and root were collected from plants; and the callus induction was performed in an experimental design. The surface-sterilization process of plant shoot tips (10–15 cm) was done after washing them plenty under tap water for 20 min. The explants were sprayed by 70% ethyl alcohol for a minute followed by the surface sterilization in 1% sodium hypochlorite (NaOCl) for 15 min. The process was followed by three times washing of explants by the autoclaved distilled water. For the callus initiation, Murashige & Skoog medium (MS medium) containing Gamborg B5 vitamin (Duchefa no. M 0231, the Netherlands) with 7 gL^{-1} agar and 3% (w = v) sucrose was used as the basal media that was complemented with various concentrations of NAA (0.5, 1.0, 1.5 and 2.0 mgL^{-1}) combined with BAP (1.0 and 2.0 mgL^{-1}). Before adding agar, the pH was adjusted to 5.8 by 1N HCI or NaOH followed by autoclaving at the temperature of 121 °C and duration of 20 min (1.4 $Kg\,cm^{-2}$). The culture incubation was performed at 23 ± 4 °C in a dark place. The callus induction (%) of calli was evaluated after 40 days as follows:

Rate inducing callus = (total amount of callus generated from explants/total amount of cultured explants) × 100%.

R software was used to analyze statistics. Duncan's multiple range test (to compare the statistically significant mean) and ANOVA (analysis of variance) were used for the analysis. $P \leq 0.05$ was considered as the statistical significance value. Calli were then sub-cultured in fresh medium at a three-week interval. Four-month-old calli were used for the RNA extraction. For the elicitor treatment, salicylic acid (SA) and methyl jasmonate (MeJA) were applied by spraying the foliage of plant in an aqueous solution at concentrations of 1 and 2 mM. Sampling from leaves was done in 0, 8, 12, 24, 48 and 72 h after treatment. Three independent biological replicates were allocated to the experiment. The samples were directly plunged in the liquid nitrogen for snap-freezing and put away in ultra-cold conditions until the further use.

2.2. Total RNA isolation and cDNA synthesis

Total RNA extraction was done with Trizol (TRI reagent SIGMA-ALDRICH, USA) based on the company guidelines. The isolated RNAs were separately diluted at a ratio of 1/50 in treated water with DEPC, and were then visually assessed by measuring optical density at 260, 280 and 230 nm in the UV spectrophotometer; and A260/A280 and A260/A230 ratios were obtained. RNA samples with A260/280 ratios ranging from 1.8 to 2.2 and A260/230 > 1.8 were only considered for the cDNA synthesis. The synthesis of the full-length first strand cDNA was done from 2 μg of RNA templates via RevertAid™ H Minus M-MuLV RT First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Fermentas); and the diluted cDNA (1:5) was applied as the qPCR template.

2.3. Primer design and analysis of qRT-PCR

Seven candidate reference genes were chosen for reference genes as reported in the previous literature and plant-based studies (Table 1). Since most of the sequences were not deposited in GenBank for *R. officinalis*, assembled transcripts from high throughput sequencing (http://medicinalplantgenomics.msu.edu) were used to get sequences and primer designs. The local blast (TBLASTN) was performed to obtain predicted sequences encoding protein products of reference genes through BlastStation2 Version 2.71 software (https://www.blaststation.com/intl/en/blaststation2.php). In the similarity search, amino acid sequences of Arabidopsis homologs of reference genes were retrieved via TAIR database (https://www.arabidopsis.org/) and applied as inquiries for searching TBLASTN versus the assembled transcript of *R. officinalis*. The hit sequence with the highest bit scores and the lowest E-value reflected the best hit for each gene. According to sequences of such unigenes, particular primers were developed and checked for their properties by Oligo Calc (http://biotools.nubic.northwestern.edu/OligoCalc.html) and OligoAnalyzer (https://eu.idtdna.com/calc/analyzer). Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used for checking the primer pair specificity. Table 2 shows all sequences of primers. The qPCR analysis was performed on the Rotor-Gene® 6000 (Qiagen, Germany). The reaction mixture contained 2 μL diluted cDNA, 300 nM of each primer, 5 μl qPCR GreenMaster (Jena Bioscience, Germany) and Milli-Q H2O in a final

Table 1
Candidate reference genes in *R.officinalis*.

Gene abbreviations	Gene names	Arabidopsis homolog locus	Arabidopsis TBLASTN E-value	Identity (%) to *A. thaliana*
ACT	Actin	AT3G12110	0.0	90%
α-TUB	Alpha tubulin	AT5G19780	0.0	98%
β-TUB	Beta tubulin	AT4G20890	0.0	96%
CYP	Cyclophilin	AT5G35100	7E-77	64%
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	AT1G13440	1.1E-158	89%
18SrRNA	18S ribosomal RNA	AT3G41768	4E-65	88%
EF1-α	Elongation factor-1alpha	AT5G60390	0.0	73%

volume of 10 μL. All reactions were implemented by a no-template negative control (NTC) and 3 technical and biological replicates. Thermal cycling was performed as follows: 2 min at 95 °C accompanied by 40 cycles of 95 °C for 20 s, 54–57 °C for 20 s, and 72 °C for 20 s. Results were confirmed by the melt curve and agarose gel analysis. Correlation coefficient (R^2) and amplification efficiency (E) were calculated for all primer pairs using 10 fold serial dilutions of cDNA and a relative standard curve by qBaseplus V3.2 software (Hellemans et al., 2007). PCR efficiency was measured according to the standard curve slope and the following formula:

$$E = (10^{-1/\text{slope}} - 1) \times 100$$

2.4. Data analysis and assessment of expression stability

Data analysis was performed by 3 statistical software, namely geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004) and BestKeeper (Pfaffl et al., 2004) to evaluate the expression stability of candidate internal reference gene over each experimental subset. The whole statistical software included Microsoft Excel-based algorithms. GeNorm calculated M value (a measure of gene expression stability) for candidate reference genes. The genes with the minimum M values were expected to be stable at an expression level, and thus they could be considered as the finest references. NormFinder detected the reference gene with the greatest stability using an ANOVA-based approach that calculated intragroup and intergroup differences. The genes with the greatest stability represented the least variation. BestKeeper program determined the stability rank of reference gene via two factors, namely the coefficient of variance (CV) (the closeness to 1 was better) and the standard deviation (SD) (the bigger was the worse) of average Ct values. Moreover, the algorithm calculated pair-wise correlations and the probability p value. The genes with significant higher correlation coefficients were accepted as the most stable one. To obtain a consensus, Reffinder (Chen et al., 2011a), a web-browser program, was utilized to consolidate aftereffects of three techniques and produce a complete rank based on geometric means of 3 algorithms that corresponded to ranks.

2.5. Validation of reference gene

In order to justify the selection of a reliable reference gene, an expression pattern of the targeted gene, Squalene synthase (*SQS*), which participated in the isoprenoid biosynthetic pathway, was estimated by normalization by two genes with the greatest and lowest detected stability. *SQS* sequence was obtained according to data of *R. officinalis* transcriptome. The amplification efficiency was calculated by qBaseplus software. qPCR amplification was performed as described above. The amount of expression of target gene was estimated in five different tissues under the MeJA treatment with forward primer, 5′-GTCATGTTCTCCGGACTTG-3′, and reverse primer, 5′-TGGGAATAC CTGGGGAATTG-3′. The approximate amounts of the gene expression were calculated via the $E^{-\Delta\Delta CT}$ method (Wong and Medrano, 2005).

3. Results

3.1. Callus induction

Different NAA concentrations combined with BAP were used in the MS medium and applied to culture the primarily nodal sections for callusing. Visible calli were produced at two ends of shoot explants after three weeks (Fig. 1). Different percentages of callus formation were observed based on the applied treatments (Table 3). No calli was induced in the media without any hormone indicating that plant growth regulators were necessary for the callus induction. Other reports also emphasized the importance of exogenous plant growth regulators for the callus formation in other Lamiaceae members including *Salvia fruticose* (Karam et al., 2003), *Satureja khuzistanica* (Sahraroo et al., 2014) and *Ocimum basilicum* (Wongsen et al., 2015).

In the present study, the highest percentage of callus induction (96.67%) was obtained from the MS medium containing 2 mgL^{-1} BAP and 0.5 mgL^{-1} NAA (Table 3). Previous studies indicated the callogenesis from explants of *R. officinalis*. For instance, Dong et al. (2012)

Table 2
Comprehensive details of genes and primer sets used for qRT-PCR.

Gene symbol	Primers (F/R) 5′→3′	Amplicon length (bp)	Amplicon Tm (°C)	PCR efficiency (E%)	(R2)
ACT	ATGATCGGAATGGAAGCTGC CCACCACCTTAATCTTCATGC	196	85.6	98.6	0.999
α-TUB	GAGAGCGTTTGTTCACTGGTA CACCTTCTGCACCAACTTC	116	82	102.1	0.999
β-TUB	ATGATGCTCACTTTCTCTGT GCTTCATTGTCCAACACCAT	131	83	92.4	0.994
CYP	TCGGCTCGTGTCGTATTTCA ACATCAGCAACTTGGGCGAC	170	82.7	89.9	0.996
GAPDH	GCTCTAAACGGAAAGTTGAC ACACTCAAGAAACTGAATACTC	173	82.7	107.8	0.999
18SrRNA	ACCATAAACGATGCCGACC AGCTCTCAGTCTGTCAATCC	228	86.5	106.4	0.992
EF1-α	GCTGAGATGAACAAGAGGT TGAAATCACGATGTCCAGG	157	83	102.7	0.995

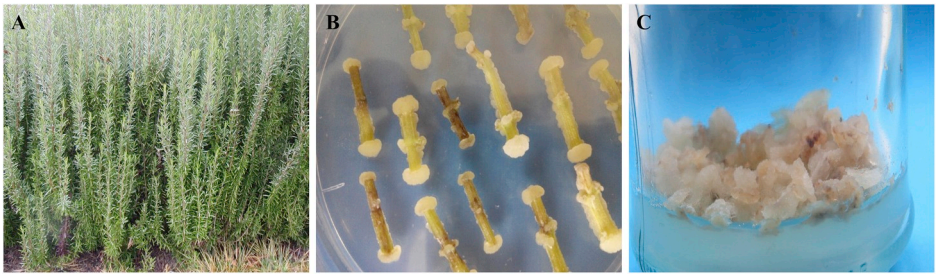

Fig. 1. (**A**) Explant source of *R. officinalis* plant (**B**) Callus induction at two ends of shoot explants after three weeks (**C**) Four-months-old calli.

obtained an induction rate of 88.8% using the MS medium that was complemented with 0.5 mgL^{-1} NAA, 50 gL^{-1} sucrose, and 0.5 mgL^{-1} BAP (Dong et al., 2012), whereas MS medium, which was supplemented by 1 mgL^{-1} NAA, was considered as the best medium for the biomass production by Yesil-Celiktas et al. (2007). These results indicated that the callus induction and growth of *R. officinalis* mostly depended on the nature and types explants as well as concentration and combination of growth regulators, especially cytokinin and auxin.

3.2. Verification of amplified product and Ct value analysis

A total of seven widely-used candidate reference genes including *ACT*, *α-TUB*, *β-TUB*, *CYP*, *EF1-α*, *GAPDH* and *18S rRNA* were assessed in different Rosemary tissues under the elicitor treatment of salicylic acid and methyl jasmonate. Amplification specificity for each gene was verified by a peak in melt curves (Fig. S1) and a band of target product size in the agarose gel electrophoresis analysis (Fig. S2). The results revealed that there was no non-specific amplification or primer-dimers and no fluorescent signal in the no template control (NTC) indicating the specificity of each primer pair and no genomic DNA contamination. The standard curves were designed for candidate genes via serial dilution series to calculate amplification efficiency (E) and correlation coefficient (R^2). The PCR efficiency (E) of primers varied from 89.9% for *CYP* to 107.8% for *GAPDH*; and correlation coefficients were all above 0.99 (Table 2). Expressing level of candidate reference gene were measured using qPCR assay crosswise over 3 subsets of samples consisting of various tissues, SA and MeJA treatments. Seven candidates exhibited great variations in Ct from 14.77 (*18S rRNA*) to 30.45 (*ACT*) with the highest falling in the range of 22–26.

The Ct value is a sign of transcription level; and a high Ct value reflects a low transcription level. Narrower CT range values indicate more stable gene expression. Among samples, *18S rRNA* showed the highest expression abundance with the lowest mean Ct value of 17.35 ± 1.60 (mean ± SD) accompanied by *GAPDH* (22.33 ± 1.16), *EF1-α* (22.88 ± 1.54), *α-TUB* (23.71 ± 1.24), *β-TUB* (24.50 ± 1.61), *CYP* (25.99 ± 1.34) and *ACT* (26.92 ± 1.53) (Fig. 2). Genes with high standard variations were considered as an unreliable reference gene because of higher variable expressions. The data was seemed to suggest that none of genes could be considered as the stable reference gene; hence, it was essential to assay reference genes for normalization in qPCR for *R. officinalis*.

3.3. Data analysis for assessment of expression stability of reference genes

The stable expression of seven candidate reference genes was assessed by three most common algorithms, namely geNorm, Normfinder and BestKeeper.

3.3.1. Analysis of geNorm

GeNorm operates based on an algorithm to determine the stable expression of the reference gene by which the mean stability of expression (M-value) could be calculated for each gene based on the non-normalized expression level. In this method, the accepted cut-off M-value of 0.50 was usually proposed for stability of reference genes (Hellemans et al., 2007). Lower M-values indicated the higher stability. In the present analysis, Fig. 3 exhibited stable expression ranks of seven candidate reference genes in various tissues and under SA and MeJA treatments and in all samples. *GAPDH* and *α-TUB* indicated the smallest M value, and were thus considered as the stable expressions in both sets of tissues and SA treatment (Fig. 3A and B). Based on the M-value, *18S rRNA* (1.25) in tissue group and *EF1-α* (1.45) in SA treatment group were the least stable genes. In the MeJA treatment group, *α-TUB* and *β-TUB* were the top ranked candidates (0.49 and 0.55 respectively) that were characterized by the greatest stability, accompanied by *GAPDH* (0.6) and *EF1-α* (0.7). *18S rRNA* (1.07) and *ACT* (0.89) were the least stable genes in this group (Fig. 3C). When all samples were mixed and tested, no gene exhibited M-values of less than 0.5, but *GAPDH* (0.82), *CYP* (0.88) and *α-TUB* (0.92) were more stable among seven tested genes (Fig. 3D).

This algorithm also indicated how many reference genes were necessary for precise normalization via calculating a pairwise variation (Vn/n + 1) between sequential ranked genes in different samples. The value of Vn/n + 1 ≤ 0.15 meant that it was not essential for the further reference gene (Vandesompele et al., 2002). The histogram of Fig. 4 indicated that pairwise variation values were all above 0.15 in groups of SA and MeJA treatments and also in all samples. Therefore, no optimized number of reference genes could be specified because of relatively high values of V. The evaluation of further candidate reference genes is probably helpful. If this is impossible, It is recommended using 5 or 6 reference genes with the lowest M values since the normalization with multiple reference genes (non-optimal in this case) can lead to more accurate outcomes than the normalization with a single non-validated reference gene. In contrast, the reported V2/3 value was less

Table 3
Effect of different concentrations of BAP and NAA on callus induction (%) of *Rosmarinus officinalis*.

Callus induction (%)				
BAP (mgL^{-1})	NAA (mgL^{-1})			
	0.5	1.0	1.5	2.0
1.0	36 ± 6.38^{f}	58.33 ± 13.74^{cd}	48.33 ± 11.39^{def}	41.67 ± 13.74^{ef}
2.0	**96.67 ± 3.85^{a}**	68.33 ± 8.39^{bc}	53.33 ± 12.17^{de}	80.0 ± 5.44^{b}

Means ± SD with different letters are significant according to the Duncan's multiple range test (P ≤ 0.05).

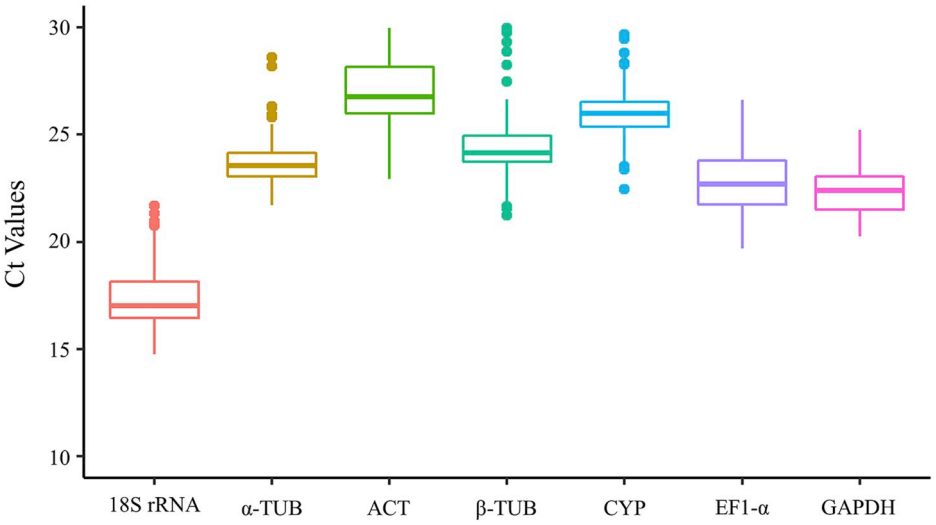

Fig. 2. Distribution of cycle threshold (Ct) values of seven candidate reference genes across all of the samples in *R. officinalis*. The horizontal line within the box indicates the median, boundaries of the box indicate the 25th and 75th percentile, and the whiskers indicate the highest and lowest Ct values. The circles depict potential outliers.

than 0.15 (0.14) for tissue group reflecting two optimal reference genes for this group (Fig. 4). In this regard, the geometric mean of *α-TUB* and *GAPDH* genes can be considered as the optimal normalization factor.

3.3.2. Normfinder analysis

The algorithm of NormFinder used a strategy for immediate determination of the expression variation in order to assess the systematic error during the normalization process. The algorithm assigned a lower value of stability for higher stable genes. NormFinder calculated Normalization factors (NF) by intra- and inter-group variations in order to estimate stability values of all reference genes. According to NormFinder ranking, the gene expression of three candidate reference genes, *α-TUB* (0.44), *CYP* (0.48) and *GAPDH* (0.75), had lower stability values in five tissue samples (Fig. 5A). *GAPDH* (0.42) and *α-TUB* (0.27) for SA and MeJA treatments were top ranks respectively (Fig. 5B and C). According to the combination of the expression data in all groups, the results revealed that *GAPDH* (0.58) and *CYP* (0.76) had more stable expression among candidate reference genes (Fig. 5D). In all samples or each group, the highest and lowest stable genes, which were detected by both geNorm and NormFinder methods, were consistent, but ranking the NormFinder scenario was a little dissimilar to generated findings by geNorm.

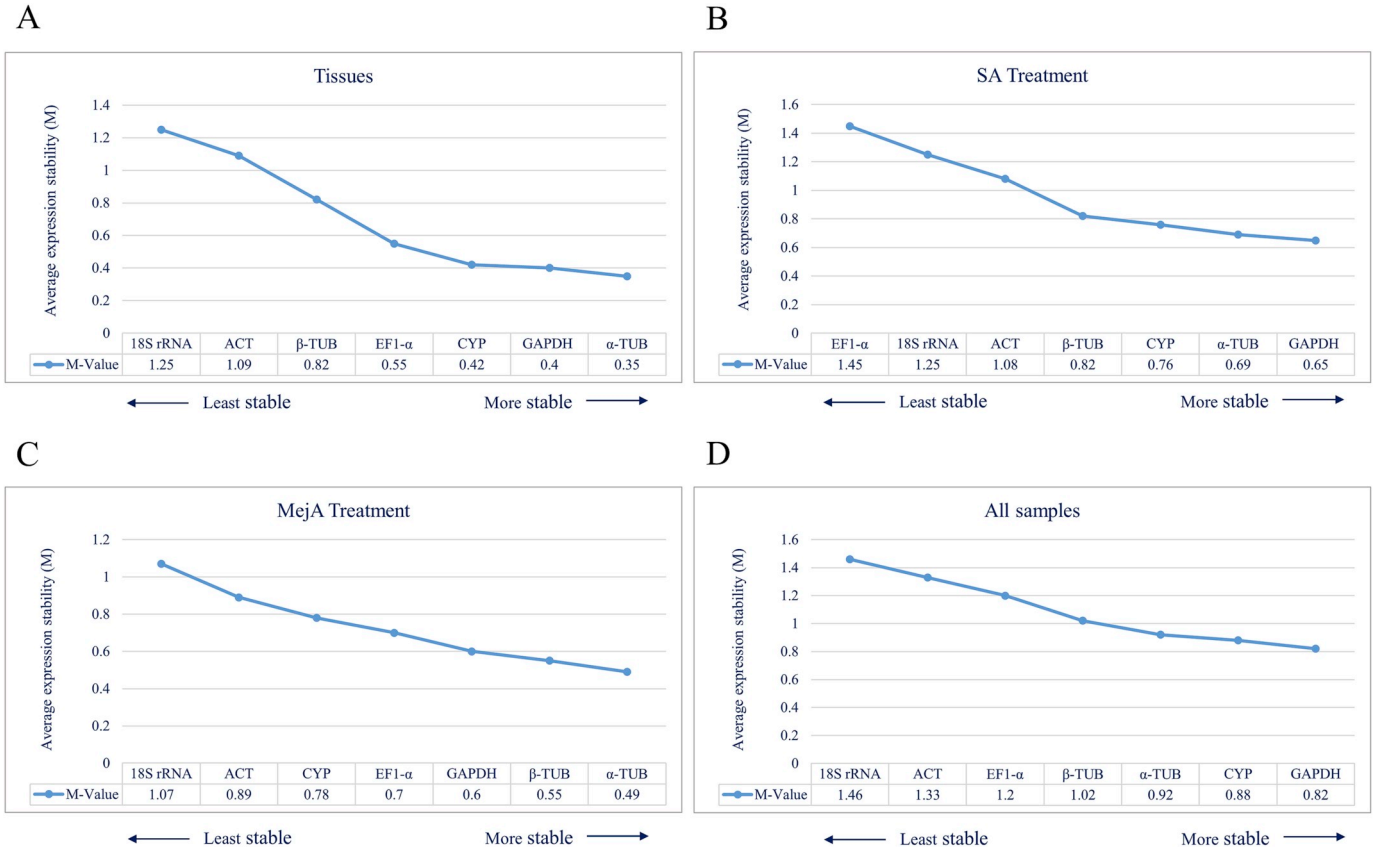

Fig. 3. Average expression stability values (M) of seven candidate reference genes calculated by geNorm. (**A**) Tissue; (**B**) SA treatment; (**C**) MeJA treatment; (**D**) all of the samples in our given conditions.

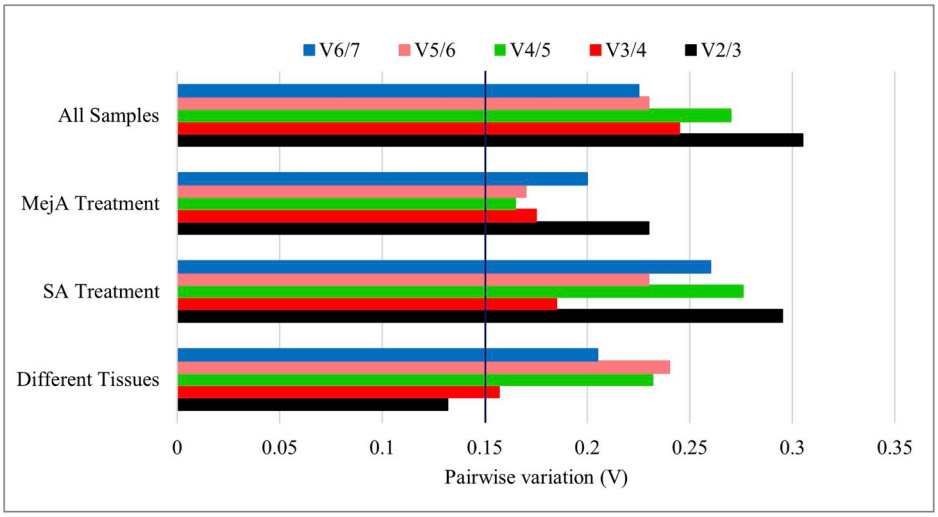

Fig. 4. The optimal number of reference genes for normalization in the tested experimental conditions. Pairwise variation (Vn/Vn + 1) values were calculated by geNorm. The vertical bold line indicates a cut-off value of 0.15, below which additional reference genes are not essential for normalization.

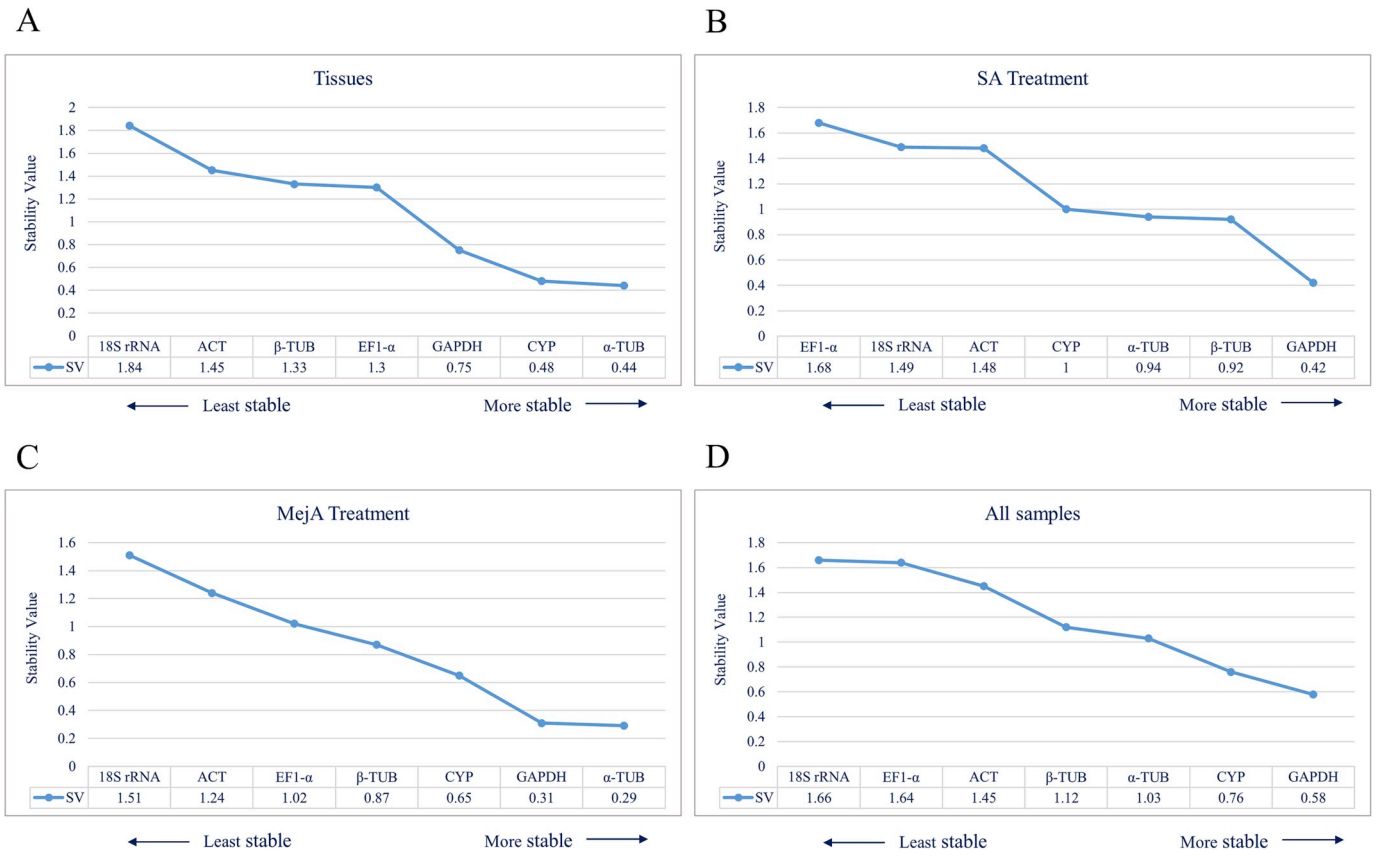

Fig. 5. Stability values of seven candidate reference genes calculated by NormFinder. (**A**) Tissue; (**B**) SA treatment; (**C**) MeJA treatment; (**D**) all of the samples in our given conditions.

3.3.3. *BestKeeper analysis*

Raw Ct values are inputs of BestKeeper software. The values of CV ± SD determine the stability of expression. In this workflow, the more stable genes possess the lower CV ± SD values. SD greater than 1 were unacceptable (less stable) for genes and these genes were introduced as unstable genes that should be ignored (Ma et al., 2016; Pfaffl et al., 2004). According to the Bestkeeper evaluation, the CV ± SD ranking of the candidate reference gene increased like in geNorm and Normfinder suggesting the gradual decrease in the stability (Fig. 6). For instance, CYP showed a CV ± SD value of 1.91 ± 0.51 and identified as the best reference gene for normalization in the tissue group, while *18S rRNA* was the worse with a CV ± SD of 6.66 ± 1.27. In the SA-treated subset, CV ± SD values of all genes were in the same range; and only *EF1-α* had SD < 1. In MeJA-treated subset and all sample subsets, *α-TUB* and *CYP* exhibited the lowest CV ± SD values of 2.69 ± 0.62 and 3.66 ± 0.95; hence, they were considered as genes with the highest stability. In this scenario, the least stable reference gene with a high SD value reported *18S rRNA* in all groups. BestKeeper

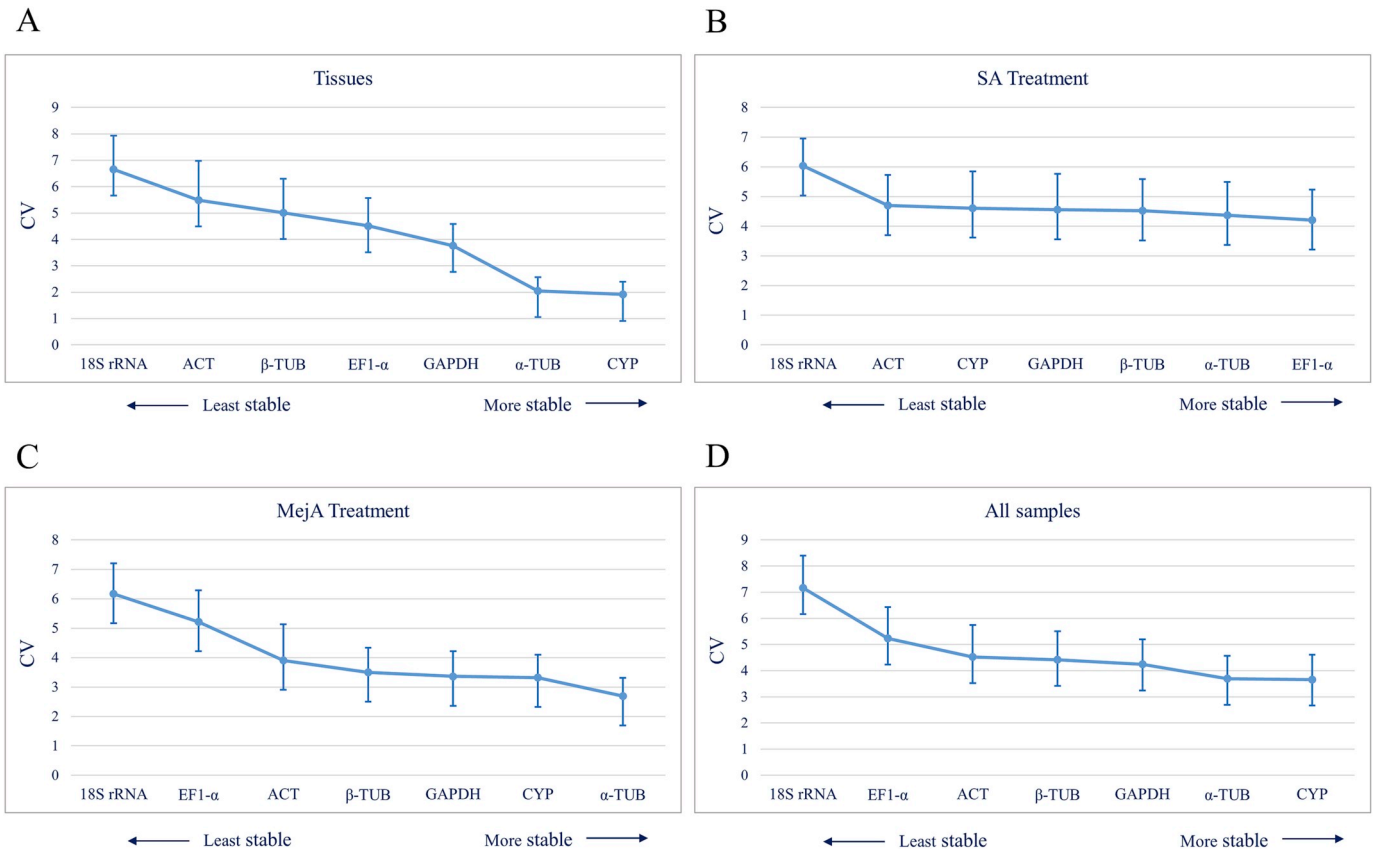

Fig. 6. Expression stability of seven reference genes calculated by BestKepper. The CVs and SDs of the candidate reference genes were used to evaluate the stability of the candidate reference genes. The gene with the lowest CV and SD was considered the most stably expressed, which is on the right, while the least stable gene is on the left. (**A**) Tissue; (**B**) SA treatment; (**C**) MeJA treatment; (**D**) all of the samples in our given conditions.

Table 4
Pearson correlation coefficient of Reference Genes with Bestkeeper index.

Bestkeeper coefficient of correlation (Bestkeeper index vs Reference Genes)							
group	18S rRNA	ACT	α-TUB	β-TUB	CYP	EF1-α	GAPDH
Tissue	0.140	0.743*	0.613*	0.762*	0.608*	0.553*	0.633*
SA treatment	0.518*	0.498*	0.736*	0.913*	0.853*	0.001	0.893*
MeJA treatment	0.347*	0.461*	0.815*	0.633*	0.792*	0.839*	0.891*
All samples	0.481*	0.481*	0.587*	0.796*	0.806*	0.369*	0.783*

*Indicates significance at p-value $<$ 0.05 level.

software also calculates an index called the BestKeeper Index which is obtained from correlation coefficients of Ct value of each gene with geometric mean of all their Ct values. The Pearson correlation coefficient between Bestkeeper index and genes were calculated in the next step. The probability p-value indicated a statistically significant correlation. Table 4 present computed correlation coefficients of studied reference genes in this survey. According to this data, it seems that there was some conflict. Therefore, correlation coefficients and related p-values, which were calculated by BestKeeper, were obtained only from groups with homogeneous variance between transcript levels. The observed significant variances may be because of differences in expression values (Ct) of selected internal controls (Neerukonda et al., 2016). In the present research, Ct values were ranging from ~17 (*18S rRNA*) to ~27 (*ACT*). In the tissue subset, both in SA and MeJA-treated subsets and all samples group, *β-TUB*, *GAPDH* and *CYP* were significantly correlated with one another and the BestKeeper index and were considered as genes with higher stability.

3.3.4. Comprehensive stability analysis of reference genes

Ranking the reference genes stability via the above-mentioned three algorithms were slightly different, and thus it was essential to get a comprehensive rank. RefFinder is a comparative algorithm that produces overall ranking and subsequently a consensus result. The comprehensive rank for studied reference genes in each subset was obtained from RefFinder as presented in Table 5. According to the ranking order by RefFinder, *GAPDH* was comprehensively ranked first in all samples and groups. The results was the same as the list by geNorm and NormFinder for all combined data supporting that the same result with these two different algorithms and the proposed best gene by multiple strategies were the same. *ACT* was in the lowest rank for groups of tissue and SA treatment; and *18S rRNA* was the last rank for the MeJA treatment and all tested samples indicating that the genes were unstably expressed in tested groups.

Table 5
Comprehensive ranking of seven candidate reference genes integrated by RefFinder.

Ranking	Tissue	SA treatment	MeJA treatment	Total
1	GAPDH	GAPDH	GAPDH	GAPDH
2	CYP	β-TUB	α-TUB	α-TUB
3	β-TUB	CYP	CYP	CYP
4	α-TUB	α-TUB	β-TUB	β-TUB
5	EF1-α	EF1-α	EF1-α	ACT
6	18SrRNA	18SrRNA	ACT	EF1-α
7	ACT	ACT	18SrRNA	18SrRNA

3.4. Validating reference genes

Approximate amounts of expression of a targeted gene were tracked in various tissues under MeJA treatment in *R. officinalis* using qRT-PCR in order to validate the best reference genes. The expression was quantified and underwent the normalization via two stable reference genes, individually and jointly, and a reference gene with the lowest stability according to comprehensive ranking in each group. Squalene synthase (*SQS*), involved in isoprenoid and sterol biosynthesis pathway, considered as a target gene. The enzyme catalyzed the final branch point of both biosynthetic pathways and the formation of squalene as a two-step reaction. In different plant species, sterol and triterpenic compounds were enhanced by the overexpression of *SQS* (Grover et al., 2013, OT Kim et al., 2010, Y-K Kim et al., 2014; Seo et al., 2005). In different tissues, the relative expression level of *SQS* was higher in leaves and flower when it underwent the normalization with two reference genes (*GAPDH* or *CYP*) with the highest stability individually or in combination state. In contrast, when it was normalized with an unstable endogenous control *18S rRNA*, the highest expression of *SQS* was found in stem (Fig. 7A). Under the MeJA treatment, the transcript abundance increased at 12 h and gradually decreased till 72 h, whereas the relative expression level of *SQS* was overestimated at 12 h and 48 h while using the least stable reference gene (Fig. 7B). In general, the same pattern of gene expression was seen in application of two stable reference genes (*GAPDH* or *α-TUB*) individually and in combination for normalization; and the expression model was entirely altered when the normalization was done by the least reported stable gene, *18S rRNA*. The results highlighted the importance of selecting housekeeping genes that could lead to a significant effect on the final normalized results.

4. Discussion

Undoubtedly, qPCR is a sensitive accurate and powerful technique to detect low levels of RNA (Kozera and Rapacz, 2013), but the major challenge of this technique is the normalization step with the validated reference genes in order to avoid the influence of nonbiological variation(s) such as errors of operator, sample preparation, performance of reverse transcription and platform differences. (Gadkar and Filion, 2015; Huggett et al., 2005). The reliable reference genes have a number of standard features: involved in the basic necessary processes for the cell survival (Housekeeping Genes), minimal variability in their expression between different tissues and under various experimental conditions, unaffected by experimental factors, stably expressed and non-regulated continuous level (Chervoneva et al., 2010; Kozera and Rapacz, 2013). Therefore, the selection of inappropriate internal genes can bring to erroneous normalization and subsequent false interpreted results (Dekkers et al., 2012; Dheda et al., 2004). In a research, a series of candidate reference genes for normalization was evaluated in *R. officinalis* as the first systematic survey. A systematic survey was conducted on the expression stability of a set of candidate reference genes for normalization in *R. officinalis* in different experimental situations (different tissues, SA treatment and MeJA treatment).

In the present study, the expression stability of seven nominee reference genes was assessed by four statistical procedures, NormFinder, geNorm, BestKeeper and RefFinder. In the research, the top-ranked genes, which were generated by geNorm and Normfinder algorithms, were identical across different conditions. Moreover, the bottom position of ranking was the same in each subset. Nevertheless, the stability rank of two algorithms were partially dissimilar because of different strategy of each algorithms to measure the stability. The gene ranking of geNorm was based on the low pairwise variation (the expression stability value, M) (Vandesompele et al., 2002), while the stability was determined via NormFinder according to the combination of intra- and inter-group variations (Andersen et al., 2004). In addition, PCR efficiencies largely affected the output of geNorm according to NormFinder. In contrast, NormFinder was more sensitive to small sample sizes (De Spiegelaere et al., 2015). *α-TUB* was the gene with the greatest stability in tissue group and MeJA treatment; and *GAPDH* was ranked as the best gene in the SA treatment and all samples group, so they were two reference genes with the greatest recommended stability by both geNorm and Normfinder in studied experimental conditions. *18S rRNA* and *EF1-α* were considered as unstable reference genes in the SA treatment and other three groups respectively by geNorm and Normfinder. BestKeeper provides SD of Ct values, and Pearson correlation of

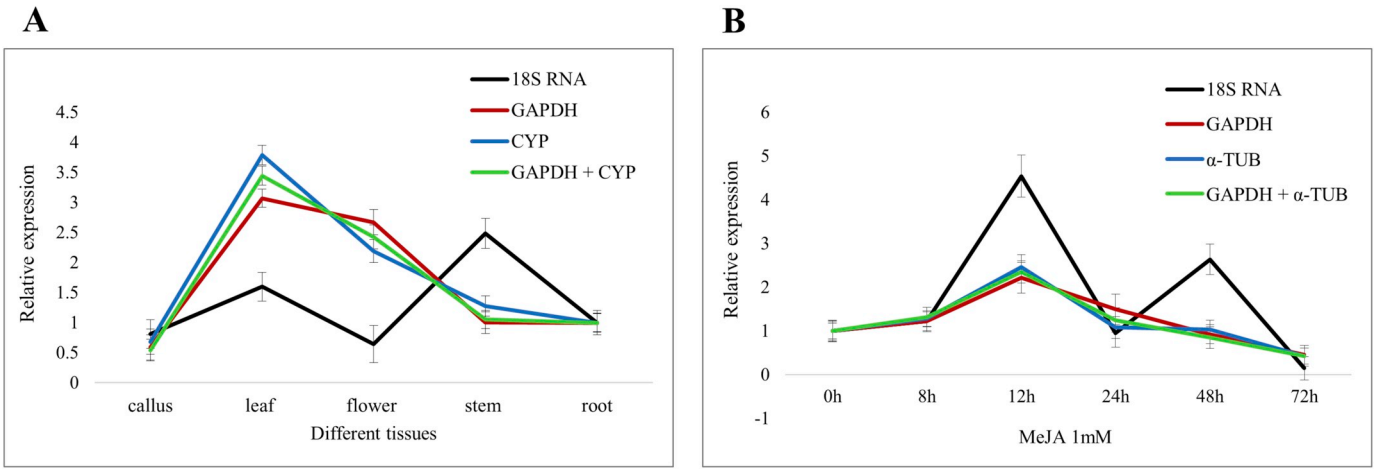

Fig. 7. Validation of the reference genes. **(A)** Expression level of *SQS* in different tissues. **(B)** Expression level of *SQS* under MeJA treatment. The most stable reference genes (*GAPDH*, CYP, *α-TUB*) individually and in combination, and the least stable reference gene (*18S rRNA*) were used. The error bars represent the mean of three biological replicates ± SD.

every gene by a BestKeeper Index (Pfaffl et al., 2004). This algorithm is less sensitive to the co-regulation like NormFinder, whereas geNorm ranking can be affected by co-regulated genes that appear to be more stable (Peng et al., 2018; Wu et al., 2016). Ranking BestKeeper was not similar to geNorm and Normfinder in the present study. For instance, BestKeeper could not recommend the most stable reference genes based on the standard deviation in the SA treatment. Therefore, results of three algorithms were integrated by RefFinder. This web-based tool represented the comprehensive ranking by calculating geometric means of the weight of each gene generating by programs (Chen et al., 2011a). This algorithm has been widely used to explore the proper reference gene in particular conditions (Duan et al., 2017, Galli et al., 2015, L Huang et al., 2014, T Huang et al., 2018, Kiarash et al., 2018, Zhu et al., 2012). According to the comprehensive ranking (Table 5), we found that *GAPDH* and *α-TUB* have been the most appropriate genes when each sample pooled. The most stable expression based on all three given conditions was related to *GAPDH*. This gene was the highest three ranked reference gene in each group by all three programs. Based on a literature survey, there are contradictory reports on the expression stability of *GAPDH* in different trials and species of plants. The present study indicated that it was a gene with the highest stability in various tissues, SA and MeJA treatment in *R. officinalis*. According to the same way, *GAPDH* seemed to have a stable expression gene in *Salvia hispanica* in various abiotic stresses (Gopalam et al., 2017), *Narcissus pseudonarcissus* in different organs and different cultivar (X Li et al., 2018), *Cicer arietinum* under stress conditions (Garg et al., 2010), *Cajanus cajan* under heat and salinity stress (Sinha et al., 2015), *Panax ginseng* in seedlings treated with heat (Wang and Lu, 2016), *Paeonia suffruticosa* in different cultivars (J Li et al., 2016), *Linum usitatissimum* in various tissues and development phases (Huis et al., 2010), *Brachypodium distachyon* through cold and heat stress (Hong et al., 2008), *Coffea Arabica* in different tissues and organs (Barsalobres-Cavallari et al., 2009) and cereal plants (*Triticum aestivum*, *Hordeum vulgare* and *Avena sativa*) under viral infections (Jarošová and Kundu, 2010). In contrast, other reports considered *GAPDH* as an inappropriate reference gene for normalization, for instance, in strawberry under drought and salt stress condition and different cultivars (Galli et al., 2015), *Zea mays* under flowering and water deficit stress (Oneto et al., 2016), *Nicotiana tabacum* across developmental stages and abiotic stress (Schmidt and Delaney, 2010), *Cichorium intybus* In cell cultures grown under various conditions (Delporte et al., 2015) and *Carica papaya* at different storage temperatures, modified atmosphere packaging, hot water treatment and biotic stress (Zhu et al., 2012).

The second top stable reference gene was different in each group in comprehensive ranking. *CYP*, *β-TUB* and *α-TUB* were ranked second in various tissues, SA and MeJA treatment respectively. Similar to the present study, *CYP* also exhibited uniform expression pattern in *Panicum virgatum* under different environmental stresses and different plant tissues (Gimeno et al., 2014), *Panax ginseng* in different organs and different growth stages (Liu et al., 2014), *Salvia hispanica* in the different abiotic stress treatments (Gopalam et al., 2017), Peanut (Reddy et al., 2013) and Sorghum under abiotic stress (Sudhakar Reddy et al., 2016), Soybean in various tissues (Jian et al., 2008) and in developing and germinating seed (Q Li et al., 2012), *Vicia faba* in different tissues and genotypes (N Gutierrez et al., 2011) and *Solanum tuberosusm* in salt stress (Nicot et al., 2005) and banana under different conditions (Chen et al., 2011b); however, it was recognized as an inappropriate reference gene in the grapevine berry development (Reid et al., 2006), and *Cicer arietinum* under various experimental conditions (Reddy et al., 2016). The microtubules are parts of the cytoskeleton of eukaryotic cells that are involved in a variety of cellular processes. They participate in the cell division (segregation of chromosomes during the mitosis and meiosis), positioning of organelles and the intracellular transport (Rebouças et al., 2013). Therefore, they were traditionally used as reference genes in several species.

In the present specific research, they had a uniform expression pattern after *GAPDH* in SA and MeJA treatment; hence, they could be considered as stable genes for normalization. According to the studies, the genes had stable expression In *Buglossoides arvensis* under different experimental conditions and in different tissues (Gadkar and Filion, 2015), durum wheat in drought stress (Kiarash et al., 2018), *Orobanche ramose* (González-Verdejo et al., 2008), *Cucumis sativus* (Wan et al., 2010) and *Helianthus annuus* (Fernandez et al., 2011) during development, chrysanthemum in aphid infestation (Gu et al., 2011) and *Salvia hispanica* in vegetative stages (Gopalam et al., 2017). On the other hand, they were less constant under different stress treatments in maize (Sekhon et al., 2011). In the present study, *ACT* and *18S rRNA* exhibited variations in the expression, and thus they should be prudently considered as reference genes. They are widely used as internal controls in qPCR among a number of different plant species and experiments. Nevertheless, some studies have suggested that they may be unsuitable to compare expression among different experimental conditions because of the variability in the expression in different conditions. In this case, the normalization can lead to wrong evaluations. These results were consistent with studies by Gadkar and Filion (2015) who considered *18S rRNA* and *ACT* as genes with the greatest instability of expression for selected experimental conditions in the assay (Gadkar and Filion, 2015).

5. Conclusion

It was the first study on the validation of reference genes in *R. officinalis* for the qPCR normalization. The experimental condition to assess reference genes included five different tissues and SA and MeJA treatments at six-time intervals. In conclusion, *GAPDH* and *CYP* in different tissues, *GAPDH* and *β-TUB* in SA treatment, and *GAPDH* and *α-TUB* in MeJA treatment were considered as appropriate internal controls for the qPCR-based normalization in *R. officinalis*. For reliable application of reference genes, it is recommended to validate more genes in more conditions because most of reference genes are not always constant in different conditions. Therefore, the amount of expression of reference genes should be measured under the same conditions as those that are used for related genes.

Conflicts of interest

No potential conflict of interest was reported by the authors.

Acknowledgements

The authors thank the Shahid Beheshti University Research Council, the University of Guilan, Iran for their financial support of this project.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101246.

References

Abdel-Wahhab, K.G.E.-D., El-Shamy, K.A., El-Beih, N.A.E.-Z., Morcy, F.A., Mannaa, F.A.E., 2011. Protective effect of a natural herb *(Rosmarinus officinalis)* against hepatotoxicity in male albino rats. Comun. Sci. 2, 9–17.

Andersen, C.L., Jensen, J.L., Ørntoft, T.F., 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.

Bakırel, T., Bakırel, U., Keleş, O.Ü., Ülgen, S.G., Yardibi, H., 2008. In vivo assessment of antidiabetic and antioxidant activities of rosemary (*Rosmarinus officinalis*) in alloxan-diabetic rabbits. J. Ethnopharmacol. 116, 64–73.

Barsalobres-Cavallari, C.F., Severino, F.E., Maluf, M.P., Maia, I.G., 2009. Identification of suitable internal control genes for expression studies in *Coffea arabica* under different experimental conditions. BMC Mol. Biol. 10, 1.

Bozin, B., Mimica-Dukic, N., Samojlik, I., Jovin, E., 2007. Antimicrobial and antioxidant properties of rosemary and sage (*Rosmarinus officinalis* L. and *Salvia officinalis* L.,

Lamiaceae) essential oils. J. Agric. Food Chem. 55, 7879–7885.
Bustin, S.A., Benes, V., Garson, J., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G., 2013. The need for transparency and good practices in the qPCR literature. Nat. Methods 10, 1063.
Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55, 611–622.
Chen, D., Pan, X., Xiao, P., Farwell, M.A., Zhang, B., 2011a. Evaluation and identification of reliable reference genes for pharmacogenomics, toxicogenomics, and small RNA expression analysis. J. Cell. Physiol. 226, 2469–2477.
Chen, L., Zhong, H.-y., Kuang, J.-f., Li, J.-g., Lu, W.-j., Chen, J.-y, 2011b. Validation of reference genes for RT-qPCR studies of gene expression in banana fruit under different experimental conditions. Planta 234, 377.
Chervoneva, I., Li, Y., Schulz, S., Croker, S., Wilson, C., Waldman, S.A., Hyslop, T., 2010. Selection of optimal reference genes for normalization in quantitative RT-PCR. BMC Bioinf. 11, 253.
de Melo, G.A.N., Grespan, R., Fonseca, J.P., Farinha, T.O., Silva, E.L., Romero, A.L., Bersani-Amado, C.A., Cuman, R.K.N., 2011. *Rosmarinus officinalis* L. essential oil inhibits in vivo and in vitro leukocyte migration. J. Med. Food 14, 944–946.
De Spiegelaere, W., Dern-Wieloch, J., Weigel, R., Schumacher, V., Schorle, H., Nettersheim, D., Bergmann, M., Brehm, R., Kliesch, S., Vandekerckhove, L., 2015. Reference gene validation for RT-qPCR, a note on different available software packages. PLoS One 10 e0122515.
Dekkers, B.J., Willems, L., Bassel, G.W., van Bolderen-Veldkamp, R., Ligterink, W., Hilhorst, H.W., Bentsink, L., 2012. Identification of reference genes for RT–qPCR expression analysis in Arabidopsis and tomato seeds. Plant Cell Physiol. 53, 28–37.
Delporte, M., Legrand, G., Hilbert, J.-L., Gagneul, D., 2015. Selection and validation of reference genes for quantitative real-time PCR analysis of gene expression in *Cichorium intybus*. Front. Plant Sci. 6, 651.
Dheda, K., Huggett, J.F., Bustin, S.A., Johnson, M.A., Rook, G., Zumla, A., 2004. Validation of housekeeping genes for normalizing RNA expression in real-time PCR. Biotechniques 37, 112–119.
Dong, Y., Wang, R., Li, Z., Qi, C., Liu, B., Duan, R., Liu, Y., 2012. Callus induction and plant regeneration from rosemary leaves. Biosci. Methods 3.
Duan, M., Wang, J., Zhang, X., Yang, H., Wang, H., Qiu, Y., Song, J., Guo, Y., Li, X., 2017. Identification of optimal reference genes for expression analysis in Radish (*Raphanus sativus* L.) and its relatives based on expression stability. Front. Plant Sci. 8, 1605.
Estevez, M., Ramirez, R., Ventanas, S., Cava, R., 2007. Sage and rosemary essential oils versus BHT for the inhibition of lipid oxidative reactions in liver pâté. Lebensm. Wiss. Technol. 40, 58–65.
Expósito-Rodríguez, M., Borges, A.A., Borges-Pérez, A., Pérez, J.A., 2008. Selection of internal control genes for quantitative real-time RT-PCR studies during tomato development process. BMC Plant Biol. 8, 131.
Fernandez, P., Di Rienzo, J.A., Moschen, S., Dosio, G.A., Aguirrezábal, L.A., Hopp, H.E., Paniego, N., Heinz, R.A., 2011. Comparison of predictive methods and biological validation for qPCR reference genes in sunflower leaf senescence transcript analysis. Plant Cell Rep. 30, 63–74.
Gadkar, V.J., Filion, M., 2015. Validation of endogenous reference genes in *Buglossoides arvensis* for normalizing RT-qPCR-based gene expression data. SpringerPlus 4, 178.
Galli, V., Borowski, J.M., Perin, E.C., da Silva Messias, R., Labonde, J., dos Santos Pereira, I., dos Anjos Silva, S.D., Rombaldi, C.V., 2015. Validation of reference genes for accurate normalization of gene expression for real time-quantitative PCR in strawberry fruits using different cultivars and osmotic stresses. Gene 554, 205–214.
Garg, R., Sahoo, A., Tyagi, A.K., Jain, M., 2010. Validation of internal control genes for quantitative gene expression studies in chickpea (*Cicer arietinum* L.). Biochem. Biophys. Res. Commun. 396, 283–288.
Gimeno, J., Eattock, N., Van Deynze, A., Blumwald, E., 2014. Selection and validation of reference genes for gene expression analysis in switchgrass (*Panicum virgatum*) using quantitative real-time RT-PCR. PLoS One 9 e91474.
González-Trujano, M., Peña, E., Martínez, A., Moreno, J., Guevara-Fefer, P., Deciga-Campos, M., López-Muñoz, F., 2007. Evaluation of the antinociceptive effect of *Rosmarinus officinalis* L. using three different experimental models in rodents. J. Ethnopharmacol. 111, 476–482.
González-Verdejo, C., Die, J., Nadal, S., Jiménez-Marín, A., Moreno, M., 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, 176–181.
Gopalam, R., Rupwate, S.D., Tumaney, A.W., 2017. Selection and validation of appropriate reference genes for quantitative real-time PCR analysis in *Salvia hispanica*. PLoS One 12 e0186978.
Grover, A., Samuel, G., Bisaria, V.S., Sundar, D., 2013. Enhanced withanolide production by overexpression of squalene synthase in *Withania somnifera*. J. Biosci. Bioeng. 115, 680–685.
Gu, C., Chen, S., Liu, Z., Shan, H., Luo, H., Guan, Z., Chen, F., 2011. Reference gene selection for quantitative real-time PCR in Chrysanthemum subjected to biotic and abiotic stress. Mol. Biotechnol. 49, 192.
Guénin, 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, L., Mauriat, M., Guénin, S., Pelloux, J., Lefebvre, J.F., Louvet, R., Rusterucci, C., Moritz, T., Guerineau, F., Bellini, C., 2008. The lack of a systematic validation of reference genes: a serious pitfall undervalued in reverse transcription-polymerase chain reaction (RT-PCR) analysis in plants. Plant Biotechnol. J. 6, 609–618.
Gutierrez, N., Giménez, M.J., Palomino, C., Avila, C.M., 2011. Assessment of candidate reference genes for expression studies in *Vicia faba* L. by real-time quantitative PCR. Mol. Breed. 28, 13–24.
Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., Vandesompele, J., 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol. 8, R19.
Hong, S.-Y., Seo, P.J., Yang, M.-S., Xiang, F., Park, C.-M., 2008. Exploring valid reference genes for gene expression studies in *Brachypodium distachyon* by real-time PCR. BMC Plant Biol. 8, 112.
Huang, L., Yan, H., Jiang, X., Zhang, Y., Zhang, X., Ji, Y., Zeng, B., Xu, B., Yin, G., Lee, S., 2014. Reference gene selection for quantitative real-time reverse-transcriptase PCR in orchardgrass subjected to various abiotic stresses. Gene 553, 158–165.
Huang, T., Long, J., Liu, S.-W., Yang, Z.-W., Zhu, Q.-J., Zhao, X.-L., Peng, C., 2018. Selection and validation of reference genes for mRNA expression by quantitative real-time PCR analysis in *Neolamarckia cadamba*. Sci. Rep. 8, 9311.
Huggett, J., Dheda, K., Bustin, S., Zumla, A., 2005. Real-time RT-PCR normalisation; strategies and considerations. Genes Immun. 6, 279.
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, 71.
Jain, M., Nijhawan, A., Tyagi, A.K., Khurana, J.P., 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.
Jarošová, J., Kundu, J.K., 2010. Validation of reference genes as internal control for studying viral infections in cereals by quantitative real-time RT-PCR. BMC Plant Biol. 10, 146.
Ji, Y., Tu, P., Wang, K., Gao, F., Yang, W., Zhu, Y., Li, S., 2014. Defining reference genes for quantitative real-time PCR analysis of anther development in rice. Acta Biochim. Biophys. Sin. 46, 305–312.
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.
Karam, N.S., Jawad, F.M., Arikat, N.A., Shibl, R.A., 2003. Growth and rosmarinic acid accumulation in callus, cell suspension, and root cultures of wild *Salvia fruticosa*. Plant Cell Tissue Organ Cult. 73, 117–121.
Kiarash, J.G., Wilde, H.D., Amirmahani, F., Moemeni, M.M., Zaboli, M., Nazari, M., Moosavi, S.S., Jamalvandi, M., 2018. Selection and validation of reference genes for normalization of qRT-PCR gene expression in wheat (*Triticum durum* L.) under drought and salt stresses. J. Genet. 1–12.
Kim, O.T., Kim, S.H., Ohyama, K., Muranaka, T., Choi, Y.E., Lee, H.Y., Kim, M.Y., Hwang, B., 2010. Upregulation of phytosterol and triterpene biosynthesis in *Centella asiatica* hairy roots overexpressed ginseng farnesyl diphosphate synthase. Plant Cell Rep. 29, 403–411.
Kim, Y.-K., Kim, Y.B., Uddin, M.R., Lee, S., Kim, S.-U., Park, S.U., 2014. Enhanced triterpene accumulation in *Panax ginseng* hairy roots overexpressing mevalonate-5-pyrophosphate decarboxylase and farnesyl pyrophosphate synthase. ACS Synth. Biol. 3, 773–779.
Kozera, B., Rapacz, M., 2013. Reference genes in real-time PCR. J. Appl. Genet. 54, 391–406.
Li, J., Han, J., Hu, Y., Yang, J., 2016. Selection of reference genes for quantitative real-time PCR during flower development in tree peony (*Paeonia suffruticosa* Andr.). Front. Plant Sci. 7, 516.
Li, Q., Fan, C.-M., Zhang, X.-M., Fu, Y.-F., 2012. Validation of reference genes for real-time quantitative PCR normalization in soybean developmental and germinating seeds. Plant Cell Rep. 31, 1789–1798.
Li, X., Tang, D., Shi, Y., 2018. Selection of reference genes for quantitative real-time PCR normalization in *Narcissus pseudonarcissus* in different cultivars and different organs. Heliyon 4 e00686.
Lin, F., Jiang, L., Liu, Y., Lv, Y., Dai, H., Zhao, H., 2014. Genome-wide identification of housekeeping genes in maize. Plant Mol. Biol. 86, 543–554.
Liu, J., Wang, Q., Sun, M., Zhu, L., Yang, M., Zhao, Y., 2014. Selection of reference genes for quantitative real-time PCR normalization in *Panax ginseng* at different stages of growth and in different organs. PLoS One 9 e112177.
Ma, R., Xu, S., Zhao, Y., Xia, B., Wang, R., 2016. Selection and validation of appropriate reference genes for quantitative real-time PCR analysis of gene expression in *Lycoris aurea*. Front. Plant Sci. 7, 536.
Manoli, A., Sturaro, A., Trevisan, S., Quaggiotti, S., Nonis, A., 2012. Evaluation of candidate reference genes for qPCR in maize. J. Plant Physiol. 169, 807–815.
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.
Moore, J., Yousef, M., Tsiani, E., 2016. Anticancer effects of rosemary (*Rosmarinus officinalis* L.) extract and rosemary extract polyphenols. Nutrients 8, 731.
Nakayama, T., Rodrigues, F., Neumaier, N., Marcelino-Guimarães, F., Farias, J., De Oliveira, M., Borém, A., de Oliveira, A., Emygdio, B., Nepomuceno, A., 2014. Reference genes for quantitative real-time polymerase chain reaction studies in soybean plants under hypoxic conditions. Genet. Mol. Res. 13, 860–871.
Neerukonda, S.N., Katneni, U.K., Golovan, S., Parcells, M.S., 2016. Evaluation and validation of reference gene stability during Marek's disease virus (MDV) infection. J. Virol. Methods 236, 111–116.
Ngo, S.N., Williams, D.B., Head, R.J., 2011. Rosemary and cancer prevention: preclinical perspectives. Crit. Rev. Food Sci. Nutr. 51, 946–954.
Nicot, N., Hausman, J.-F., Hoffmann, L., Evers, D., 2005. Housekeeping gene selection for real-time RT-PCR normalization in potato during biotic and abiotic stress. J. Exp. Bot. 56, 2907–2914.
Oneto, C.D., Bossio, E., Faccio, P., Beznec, A., Blumwald, E., Lewi, D., 2016. Validation of housekeeping genes for qPCR in maize during water deficit stress conditions at flowering time. Maydica 62, M13.

Paolacci, A.R., Tanzarella, O.A., Porceddu, E., Ciaffi, M., 2009. Identification and validation of reference genes for quantitative RT-PCR normalization in wheat. BMC Mol. Biol. 10, 11.
Peng, S., Liu, L., Zhao, H., Wang, H., Li, H., 2018. Selection and validation of reference genes for quantitative real-time PCR normalization under ethanol stress conditions in *Oenococcus oeni* SD-2a. Front. Microbiol. 9, 892.
Pfaffl, M.W., Tichopad, A., Prgomet, C., Neuvians, T.P., 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.
Radonić, A., Thulke, S., Mackay, I.M., Landt, O., Siegert, W., Nitsche, A., 2004. Guideline to reference gene selection for quantitative real-time PCR. Biochem. Biophys. Res. Commun. 313, 856–862.
Rašković, A., Milanović, I., Pavlović, N., Ćebović, T., Vukmirović, S., Mikov, M., 2014. Antioxidant activity of rosemary (*Rosmarinus officinalis* L.) essential oil and its hepatoprotective potential. BMC Complement Altern. Med. 14, 225.
Rebouças, E.d.L., Costa, J.J.d.N., Passos, M.J., Passos, J.R.d.S., Hurk, R.v.d., Silva, J.R.V., 2013. Real time PCR and importance of housekeepings genes for normalization and quantification of mRNA expression in different tissues. Braz. Arch. Biol. Technol. 56, 143–154.
Reddy, D.S., Bhatnagar-Mathur, P., Cindhuri, K.S., Sharma, K.K., 2013. Evaluation and validation of reference genes for normalization of quantitative real-time PCR based gene expression studies in peanut. PLoS One 8 e78555.
Reddy, D.S., Bhatnagar-Mathur, P., Reddy, P.S., Cindhuri, K.S., Ganesh, A.S., Sharma, K.K., 2016. Identification and validation of reference genes and their impact on normalized gene expression studies across cultivated and wild cicer species. PLoS One 11 e0148451.
Reid, K.E., Olsson, N., Schlosser, J., Peng, F., Lund, S.T., 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.
Sahraroo, A., Babalar, M., Mirjalili, M.H., Moghaddam, M.R.F., Ebrahimi, S.N., 2014. In-vitro callus induction and rosmarinic acid quantification in callus culture of *Satureja khuzistanica* Jamzad (Lamiaceae). Iran. J. Pharm. Res. (IJPR) 13, 1447.
Schmidt, G.W., Delaney, S.K., 2010. Stable internal reference genes for normalization of real-time RT-PCR in tobacco (*Nicotiana tabacum*) during development and abiotic stress. Mol. Genet. Genom. 283, 233–241.
Sekhon, R.S., Lin, H., Childs, K.L., Hansey, C.N., Buell, C.R., de Leon, N., Kaeppler, S.M., 2011. Genome-wide atlas of transcription during maize development. Plant J. 66, 553–563.
Seo, J.-W., Jeong, J.-H., Shin, C.-G., Lo, S.-C., Han, S.-S., Yu, K.-W., Harada, E., Han, J.-Y., Choi, Y.-E., 2005. Overexpression of squalene synthase in Eleutherococcus senticosus increases phytosterol and triterpene accumulation. Phytochemistry 66, 869–877.
Sinha, P., Saxena, R.K., Singh, V.K., Krishnamurthy, L., Varshney, R.K., 2015. Selection and validation of housekeeping genes as reference for gene expression studies in pigeonpea (*Cajanus cajan*) under heat and salt stress conditions. Front. Plant Sci. 6, 1071.
Sudhakar Reddy, P., Srinivas Reddy, D., Sivasakthi, K., Bhatnagar-Mathur, P., Vadez, V., Sharma, K.K., 2016. Evaluation of sorghum [*Sorghum bicolor* (L.)] reference genes in various tissues and under abiotic stress conditions for quantitative real-time PCR data normalization. Front. Plant Sci. 7, 529.
Tajehmiri, A., Ghasemi, M., Sabet, F., Doost Chakoosari, M., Abdolahzadegan, N., 2014. Antimutagenic activity of *Rosmarinus officinalis* L. By Ames test. Sci. J. Microbiol. 3, 78–81.
Valasek, M.A., Repa, J.J., 2005. The power of real-time PCR. Adv. Physiol. Educ. 29, 151–159.
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 Biol. 3 research0034. 0031.
Vandesompele, J., Kubista, M., Pfaffl, M.W., 2009. Reference gene validation software for improved normalization. Real-Time PCR: Curr. Technol. Appl. 4, 47–64.
Wan, H., Zhao, Z., Qian, C., Sui, Y., Malik, A.A., Chen, J., 2010. Selection of appropriate reference genes for gene expression studies by quantitative real-time polymerase chain reaction in cucumber. Anal. Biochem. 399, 257–261.
Wang, M., Lu, S., 2016. Validation of suitable reference genes for quantitative gene expression analysis in *Panax ginseng*. Front. Plant Sci. 6, 1259.
Wong, M.L., Medrano, J.F., 2005. Real-time PCR for mRNA quantitation. Biotechniques 39, 75–85.
Wongsen, W., Bodhipadma, K., Noichinda, S., Leung, D., 2015. Influence of different 2, 4-D concentrations on antioxidant contents and activities in sweet basil leaf-derived callus during proliferation. Int. Food Res. J. 22, 638.
Wu, Z.-J., Tian, C., Jiang, Q., Li, X.-H., Zhuang, J., 2016. Selection of suitable reference genes for qRT-PCR normalization during leaf development and hormonal stimuli in tea plant (*Camellia sinensis*). Sci. Rep. 6, 19748.
Yesil-Celiktas, O., Nartop, P., Gurel, A., Bedir, E., Vardar-Sukan, F., 2007. Determination of phenolic content and antioxidant activity of extracts obtained from *Rosmarinus officinalis*' calli. J. Plant Physiol. 164, 1536–1542.
Zhu, X., Li, X., Chen, W., Chen, J., Lu, W., Chen, L., Fu, D., 2012. Evaluation of new reference genes in papaya for accurate transcript normalization under different experimental conditions. PLoS One 7 e44405.