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Identification of the most suitable reference genes for nanoparticle stress response in *Salvia rosmarinus* (rosemary) produced under *in vitro* conditions

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Article

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Abstract

Salvia rosmarinus L. (rosemary) is known to have a wide range of pharmacological effects including antidiabetic, anticarcinogenic, and antitumorigenic properties owing to its secondary metabolites. Studies aiming for the elevation of these metabolites have utilized various elicitors and stresses under *in vitro* conditions, although underlying molecular mechanisms remain unexplored. Gene expression studies using qRT-PCR might provide valuable information regarding how plant and plant cells interact and perceive various treatments and elicitors. Although, despite being able to calculate accurate fold changes, the accuracy of the qRT-PCR data highly depends on the expression of housekeeping (H.K.) genes. To the best of our knowledge, there is no information available on the stable H.K. genes in rosemary under *in vitro* conditions. Thus, in this paper, we assessed the stability of seven commonly used H.K. genes with qRT-PCR. Thereafter, the five most commonly used software (comparative ΔCt, BestKeeper, NormFinder, geNorm, and RefFinder) were used to rank the candidates based on their expression stabilities. We recommend using a combination of *F1-ATPase* and *18S rRNA* to normalize the gene expression experiments in rosemary under *in vitro* conditions. The selected H.K. genes were verified using *4-coumarate-CoA ligase* i.e., a pharmacologically important gene, whose expression might alter under nanoparticle treatment. Additionally, reference genes for several plant tissues, elicitors, and stresses are also proposed. The conclusions obtained from this current study will accelerate the future molecular work in *S. rosmarinus* and other related species.

1. Introduction

Rosemary (*Salvia rosmarinus* L.), belonging to the lamiaceae family, is an aromatic plant with well-known pharmacological effects. The wide range of pharmacological effects of this perennial herb is mainly due to its ample secondary metabolites which are known to have antioxidant, antibacterial, anti-inflammatory, antidiabetic, anticarcinogenic, and antitumorigenic activities[1, 2]. In addition to these, recent studies have also shown that carnosic acid, carnosol, and rosmanol (phytochemicals present in rosemary) have inhibitory effects against the coronavirus's main protease (SARS-CoV-2 M^{pro})[3, 4]. SARS-CoV-2 M^{pro} contains important catalytic site residues, which play an essential role in virus proliferation. Consequently, interest has been raised in increasing these pharmacologically important secondary metabolites of this species among the industries. In this scenario, plant tissue culture technology can be an excellent option for large-scale disease-free plant and tissue production with elevated quantities of secondary metabolites from rosemary and several other medicinal plants, irrespective of their growing seasons. Furthermore, treatment of various elicitors such as nanoparticles and others in culture systems can also boost or change the secondary metabolite level[5]. Even though studies aiming to increase the secondary metabolites through plant tissue culture in rosemary are constantly in process, many more will be needed in the future to accomplish its demands in industrial production. In order to effectively manipulate the synthesis of these metabolites under *in vitro* conditions, underlying molecular mechanisms needs to be explored. Currently, based on the previous literature studies, there is limited information available about the genes and metabolic pathways in rosemary[6]. Hence gene expression analysis using quantitative real-time PCR (qRT-PCR) can be an essential tool to get insights into the metabolic pathways of secondary metabolites in specific tissues or under different conditions.

Owing to its high specificity, rapidity, and sensitivity, qRT-PCR has gained an edge over the traditional polymerase chain reaction (PCR) for comparative expression studies. Although qRT-PCR can calculate accurate fold changes, its accuracy is extremely reliant on the expression of a suitable housekeeping gene[7]. The steps of qRT-PCR are prone to technical noises and variations in the sample preparation. Hence, to nullify these variations, appropriate normalization methods are necessary[8]. The target gene transcription levels must be normalized with suitable reference gene transcription levels. Any inaccuracies in selecting a suitable reference gene may lead to deceptive results[9, 10]. The most commonly used references genes in plant gene expression studies include *ubiquitin (UBQ)*, *eukaryotic elongation factor (eEF)*, *α-tubulin (α-TUB)*, *β-tubulin (β-TUB)*, *actin (ACT)*, *ribosomal RNA genes (rRNA)*, *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*, Acetyl *CoA Carboxylase (ACCase)*, etc.[8]. Although it can be assumed that these genes will have a stable expression in any given condition, however, numerous studies have provided evidence of their variability in their expression level between species of plants or different stress conditions or developmental stages[7, 11] (Joseph *et al*., 2018; Czechowski *et al*., 2005).

Interest in plant tissue culture or *in vitro* plant culture has grown mainly due to its promising ability to produce improved crop varieties and high yield of crucial secondary metabolites. Several efforts have been made to enhance the production of important

secondary metabolites using different biotic and abiotic factors. Currently, the addition of elicitors such as nanoparticles has gained worldwide interest owing to their success in enhancing secondary metabolites in many species. Soltanabad *et al*., 2020 showed that silver nanoparticle treatment could boost the carnosic acid content in *Rosmarinus officinalis* L.[12] The rising interest in using nanoparticles in commercially important medicinal plants (such as rosemary) will increase the need for gene expression studies in these species. To the best of our knowledge, there are no reports on the identification of the most suitable reference genes for gene expression studies under nanoparticle stress in rosemary produced under *in vitro* conditions. Hence, this study aims to identify a suitable reference gene for gene expression studies in *in vitro S. rosmarinus*. We had selected and identified seven common candidate reference genes (*18S rRNA, 25S rRNA, 28S rRNA, ACCase, GAPDH, ATP-synthase*, and *F1-ATPase*) in *S. rosmarinus* and assessed their gene expression stability in three different plant tissues/organs (callus, stem, and leaf), temperature stress (heat stress and cold stress), two elicitor stress (casein hydrolysate and jasmonic acid), osmotic stress (sorbitol) and salt stress (NaCl). Five different widely used statistical software for reference gene analysis (comparative ΔCt[13], BestKeeper[14], NormFinder[15], geNorm[16], and RefFinder[17]) were used to identify the best candidate. Thereafter, the most suitable reference gene was used to validate using the *4-coumarate-CoA ligase (4CL)* gene under a nanoparticle (NP)-stress experiment. *4CL* catalyzes the enzymatic reactions in the general phenylpropanoid pathway and participates in the synthesis of flavonoids. All flavonoids are produced from one molecule of p-coumaroyl CoA and three molecules of malonyl CoA. Flavonoids are pharmacologically important compounds with numerous probable medicinal properties, including anticancer, antioxidant, anti-inflammatory, and anti-inflammatory activities[18]. Additionally, suitable candidates under each mentioned experimental condition were also identified. Our study will provide a basis for current and future gene expression studies in *S. rosmarinus* and its related species and will assist in forthcoming molecular studies aiming for a better understanding of the metabolic pathways associated with secondary metabolites.

2. Materials And Methods

2.1 Plant material acquisition, surface sterilization, preparation of culture medium and growth conditions

Explants of rosemary were taken from the well-maintained botanical garden of the Faculty of Tropical AgriSciences (FTZ), Czech University of Life Sciences, Prague. No specific permissions were necessary to obtain the plant samples. All experiments conducted in the current study, are in compliance with relevant institutional, national, and international guidelines and legislation. Thereafter, the explants were rinsed with distilled water for 10 minutes. Following the rinsing, the explants were treated with 70% ethanol (v/v) for 2 min, then 1% solution of sodium hypochlorite (v/v) (NaOCl, commercial bleach-SAVO) containing two drops of tween 20 for 10 min. Finally, explants were rinsed with autoclaved distilled water for three times, then explants were transferred to Murashige and Skoog's (1962) (MS) basal medium under a laminar flow hood for initiation. The pH of the medium was adjusted to 5.7 ± 1, solidified with 8 g/L agar, and sterilized in an autoclave (Nuve 0T-032 model) at 121℃ for 20 mins. Inoculated cultures were maintained at 24/20 ± 1℃ (except for treatments: heat and cold), 16/8h (light/dark) photoperiod, and relative humidity of 60–70%.

2.2 Callus induction

Surface sterilized leaf explants were utilized for the callus induction. Different combinations of 6-benzylaminopurine (BAP), 2,4-Dichlorophenoxyacetic acid (2,4-D), and Kinetin (Kn) (Table 1) were used to test optimum concentration and combination to induce callus.

Table 1
Combinations of phytohormones utilized for callus induction in *Salvia rosmarinus*.

Combinations	BAP (mg/L)	2,4-D (mg/L)	Kinetin (mg/L)
C1	1.5	0.5	-
C2	1.0	1.0	-
C3	0.5	1.5	-
C4	-	1.5	0.5
C5	-	1.0	1.0
C6	-	0.5	1.5

2.3 Stress, elicitor, and nanoparticle treatment

The *in vitro* grown plants and callus of *S. rosmarinus* were subjected to different stresses and elicitors supplemented with the basic MS. The treatments included: Osmotic stress (Sorbitol, 200mM), Salt stress (NaCl, 100mM), Heat stress (30℃), Cold stress (4℃), Jasmonic acid (1 mg/L), Casein hydrolysate (1 mg/L). Additionally, ZnO (Zinc Oxide) NPs at a concentration of 10 mg/L were used for nanoparticle stress. ZnO NPs nanopowder, < 50nm particle size (BET), > 97% SIGMA- ALDRICH (677450-5G) were obtained from Sigma-Aldrich (Burlington, MA, USA). To disrupt and dissolve the ZnO NPs, they were dispersed in sterile distilled water for 60 min using a sonicator (BANDALIN SONOPLUS). After that, the solution was further diluted to prepare desired concentrations. All the treatments had five replications and were maintained for 24 hours except for nanoparticle treatment which was maintained for 7 days. Plant samples from leaves and stems and callus were collected from each treatment to be stored at -80°C (until further use).

2.4 qRT-PCR experiment

The plant RNA was extracted from the fresh tissues (± 60–80 mg per sample) using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The RNA sample integrity was confirmed by running the samples on 1.2% agarose gel electrophoresis. 1µg (per reaction) of quality-checked gDNA-free RNA template was used as a template for cDNA synthesis. For this purpose, TURBO DNA-free™ (Invitrogen, US) Kit was used, according to the manufacturer's instructions. Degenerate primers were designed for seven common candidate reference genes (*18S rRNA, 25S rRNA, 28S rRNA, ACCase, GAPDH, ATP-synthase* and *F1-ATPase*). The candidate genes were chosen based on previous literature studies and the primers were designed based on their homologous sequences in other plants species using Primer3 software (https://bioinfo.ut.ee/primer3-0.4.0/). All the designed primers were tested by general PCR (C1000 thermocycler, Bio-Rad, Hercules, CA, USA) and verified in the 1.8% agarose gel electrophoresis. The qRT-PCR assay was done in StepOne™ Real-Time PCR System (Applied Biosystems™, USA) using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems™, USA). The reaction mixture used was as follows: 5 µL of SYBR Green Master Mix, 1 µL of primer mix (10 mM each), and 4 µL of cDNA (2.5 ng/µL). The primer efficiency (E) and correlation coefficient (R^2) calculation was done according to Sen *et al*., 2021[8]. The qRT-PCR thermocycler was programmed at an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 57 to 62°C (based on the annealing temperature of the primer pairs). For melting curve analysis, stepwise heating was performed from 60°C to 95°C. 5 biological replicates were used for all the qRT-PCR experiments.

2.5 Reference gene analysis

Gene expression stabilities of the candidate genes were examined by comparative ΔCt, RefFinder, geNorm, NormFinder and BestKeeper, according to Sen *et al*., 2021. Before and after nanoparticle stress treatment, the relative *4CL* gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method[19]. NormFinder software ranks the gene based on the stability values and the gene with minimal variation is ranked as the best by the software. geNorm program estimates the ranking of the candidate genes based on

expression stability value (M). The M value is inversely proportional to the ranking of the gene. BestKeeper relies on the standard deviation values of cycle threshold (Ct) or crossing point values (CP) and coefficient of correlation (r) values. RefFinder integrates all the softwares for reference gene screening (geNorm, NormFinder, BestKeeper, and the comparative Delta-Ct method) and give the complete ranking based on the geometric mean of ranking values[20].

3. Results

3.1 Callus Induction and Plant Regeneration

Nodal segments of *S. rosmarinus* subjected to basic MS without any phytohormones were sufficient to establish and propagate under *in vitro* conditions. For callus induction, all the studied combinations of phytohormones were able to induce callus using leaf segment as explant after 2 weeks (Table 2 & Fig. 1). Despite all combinations being successful in inducing callus, healthy calli with a faster proliferation rate were generated on media C1 when compared to other combinations. The generated calli on C1 were light green to dark green in colour, structurally friable, and loosely packed (Fig. 1). Hence were selected for further treatments. The calli were sub-cultured every 30 days.

Table 2
Effect of different concentrations of phytohormones on callus induction (%) of *Salvia rosmarinus*.

Treatment	Callus induction % (± standard deviation)	Growth status of callus
C1	100 ± 0.0	Proliferated fast, Light green to dark green in colour and structurally friable
C2	100 ± 0.0	Proliferated fast, light green in colour and structurally friable
C3	100 ± 0.0	Proliferated slowly, light green to brown in colour and structurally friable
C4	100 ± 0.0	Proliferated slowly, brown colour and structurally friable
C5	100 ± 0.0	Proliferated slowly, brown in colour and structurally friable
C6	100 ± 0.0	Proliferated slowly, brown in colour and structurally friable

3.2 Primer efficiency and candidate genes expression

The quality and the quantity of the extracted RNA was checked using 1.2% agarose gel electrophoresis and nanodrop spectrophotometer (Thermo Scientific™, US), respectively. A260/A280 values ranged from 1.90 to 1.96 for all the samples. 1000ng of each RNA sample was further used to synthesize the cDNA. Thereafter, the designed primers were tested in general PCR using diluted cDNA samples. All the amplicons produced from the primers showed a single band in 1.8% agarose gel (supplementary Fig. 1). In all the qRT-PCR experiments, a single peak was obtained for all the primers (Fig. 2). Their efficiency values and correlation coefficient values ranged between 93.87–104.69% and 0.9823 to 0.9997, respectively (Table 3). The expression profile of the eight nominees under distinct experimental conditions is shown in Fig. 3. Among the seven candidate genes, *18S rRNA* (mean Ct value 14.78 ± 3.59) is the most abundantly expressed gene (i.e., lowest average Ct value), whereas *GAPDH* (mean Ct value 31.43 ± 4.02) showed the lowest expression (i.e., highest average Ct value).

Table 3
Primer efficiency and correlation coefficient values of the candidate reference genes.

Gene	Designed based on	Sequence	Annealing temperature (°C)	Amplicon length (bp)	Primer efficiency (%)	R^2 value
18S rRNA_Fwd	X16077.1, AH001709.2, XR_004642731.1	TCTGCCCTATCAACTTTCGATGGTA	62	168	93.90	0.9823
18S rRNA_Rev		AATTTGCGCGCCTGCTGCCTTCCTT				
28S rRNA_Fwd	XR_006991982.1, AH001710.2	CCTGATCTTCTGTGAAGGGTTCGA	60	95	93.93	0.9904
28S rRNA_Rev		GGTTCGATTAGTCTTTCGCCCCTA				
25S rRNA_Fwd	XR_006995432.1, XR_006614521.1	CGTCCCTTAGGATCGGCTTAC	57	182	104.69	0.9956
25S rRNA_Rev		AAGGCCGAAGAGGAGAAAGGT				
ATP-Synthase_Fwd	NM_114510.3, OM691673.1, NC_061230.1	GGCTTGAACGAAACGGAAGA	59.5	115	101.13	0.9874
ATP-Synthase_Rev		AGAGTTGGTTTGACTGCCCT				
F1-ATPase_Fwd	D88375.1	TATCTGTCAGTCGTGTCGGG	59.1	110	98.84	0.9948
F1-ATPase_Rev		AAAGGCGGCTACTTCTCGAT				
GAPDH_Fwd	KX086568.1	GGACTGGAGAGGTGGAAGAG	60.1	135	100.92	0.9973
GAPDH_Rev		GGAACCCTGAATGACATGCC				
ACCase_Fwd	E09394.1	GCTGCTATTGCCAGTGCTTA	57	53	93.87	0.9997
ACCase_Rev		AAGCTTGTTCAGGGCAGAAA				

3.3 Gene expression stability using comparative ΔCt and BestKeeper

The method ΔCt evaluates the stability of the candidate reference gene expression based on the standard deviation values whereas the BestKeeper software determines the consistency of the reference genes based on the crossing point (CP) and standard deviation (SD) values. In the case of expression studies with the plant organs (callus, stem and root), both ΔCt and BestKeeper identified *18S rRNA* as the best candidate for reference gene (Fig. 4 and Fig. 5). Under all the other experimental conditions, ACCase was observed to be the most stable reference gene, according to BestKeeper (Fig. 5). However, ΔCt suggested *ATP-synthase* as the best reference gene under salt stress and *F1-ATPase* under osmotic stress, elicitor stress, temperature stress and also under combined conditions (Fig. 4).

3.4 Gene expression stability using NormFinder

NormFinder software assesses the intra- and intergroup variation, which are then combined into stability values. Finally, the candidate gene with the least variation is ranked as the best by the software. Additionally, this software also recommends the best combination of genes using a pairwise comparison approach. In this study, *ATP-synthase* has been considered to be the most stable candidate under osmotic stress, salt stress and combined condition, whereas *25S rRNA* has been observed to be the best candidate for the expression studies with plant organs and under elicitor and temperature stress. *GAPDH* has been considered to the least stable under all the conditions. NormFinder algorithm also identifies the best pair of genes. Table 4 shows the most stable reference gene as well as the best combination in rosemary, as evaluated by NormFinder.

Table 4
Evaluation of expression stability by NormFinder.

Rank	Plant organ	Osmotic stress	Salt stress
1	*25S rRNA*	*ATP-synthase*	*ATP-synthase*
2	*28S rRNA*	*25S rRNA*	*F1-ATPase*
3	*18S rRNA*	*ACCase*	*18S rRNA*
4	*ATP-synthase*	*F1-ATPase*	*25S rRNA*
5	*F1-ATPase*	*18S rRNA*	*ACCase*
6	*ACCase*	*28S rRNA*	*28S rRNA*
7	*GAPDH*	*GAPDH*	*GAPDH*
Best pair	*25S rRNA/28S rRNA*	*ATP-synthase/25S rRNA*	*ATP-synthase/F1-ATPase*
Rank	**Elicitor stress**	**Temperature stress**	**All combined**
1	*25S rRNA*	*25S rRNA*	*ATP-synthase*
2	*F1-ATPase*	*18S rRNA*	*25S rRNA*
3	*18S rRNA*	*F1-ATPase*	*F1-ATPase*
4	*ATP-synthase*	*ATP-synthase*	*18S rRNA*
5	*ACCase*	*ACCase*	*ACCase*
6	*28S rRNA*	*28S rRNA*	*28S rRNA*
7	*GAPDH*	*GAPDH*	*GAPDH*
Best pair	*F1-ATPase/25S rRNA*	*F1-ATPase/25S rRNA*	*F1-ATPase/18S rRNA*

3.5 Gene expression stability using geNorm

geNorm software algorithm assesses the stability of the reference genes based on the geometric means values or “M” value. This software considers 1.5 as the threshold value. The genes with an M value < 1.5, are deemed to be the most stable reference gene candidate. In addition to the reference gene expression stability, geNorm is also used to calculate the pairwise variation, based on which the optimal number of reference gene/s required for a particular experimental condition is/are calculated. The results for the gene expression stability analysis with geNorm are shown in Fig. 6. In our study, geNorm identified *F1-ATPase/18S rRNA* as the best combination for studies with the plant organs and under temperature stress. The gene pairs, *ATP-synthase/F1-ATPase* and *18S rRNA/25S rRNA* were the recommended genes under salt and elicitor stress, respectively. geNorm ranked *25S rRNA/ACCase* as the best reference gene combination under osmotic stress as well as under combined conditions. Irrespective of the experimental conditions, *GAPDH* is not recommended for gene expression normalization by geNorm software. Based on the pairwise analysis the number using a combination of two reference genes is suggested for studies with the plant organs and studies under osmotic and temperature stress (Fig. 7). Using the combination of three and six reference genes is recommended for the gene expression studies under salt and elicitor stress, respectively (Fig. 7). Though, when all the experimental conditions were combined together, our pairwise analysis suggested use of one gene is adequate, however, we recommend using a combination of two reference genes.

3.6 Gene expression stability using RefFinder

To remove the heterogeneity in ranking order (provide by all the mentioned software), RefFinder is used to produce a comprehensive ranking of the candidate reference genes (Table 5). This software integrates the results obtained from geNorm, NormFinder, BestKeeper, and the comparative ΔCt method to produce an overall grading of the most stable reference genes. In this

study, for gene experiments with plant organs, RefFinder recommends using *18S rRNA* as the most stable reference gene. *GAPDH* is considered the least stable gene. *ATP-synthase* and *25S rRNA* is considered the best and the worst reference gene (respectively), under the salt and temperature stress conditions. Under osmotic stress, *ACCase* and *25S rRNA* are observed as the most and least stable candidates, respectively. Under elicitor stress and the combined condition, RefFinder recommends *F1-ATPase*, as the most stable reference gene.

Table 5
Evaluation of expression stability by RefFinder.

Rank	Plant organ	Osmotic stress	Salt stress
1	*18S rRNA*	*ACCase*	*ATP-synthase*
2	*ACCase*	*F1-ATPase*	*F1-ATPase*
3	*25S rRNA*	*18S rRNA*	*GAPDH*
4	*28S rRNA*	*ATP-synthase*	*ACCase*
5	*ATP-synthase*	*28S rRNA*	*18S rRNA*
6	*F1-ATPase*	*GAPDH*	*28S rRNA*
7	*GAPDH*	*25S rRNA*	*25S rRNA*
Rank	**Elicitor stress**	**Temperature stress**	**All combined**
1	*F1-ATPase*	*ATP-synthase*	*F1-ATPase*
2	*ATP-synthase*	*F1-ATPase*	*ATP-synthase*
3	*18S rRNA*	*ACCase*	*ACCase*
4	*ACCase*	*GAPDH*	*GAPDH*
5	*28S rRNA*	*18S rRNA*	*18S rRNA*
6	*GAPDH*	*28S rRNA*	*25S rRNA*
7	*25S rRNA*	*25S rRNA*	*28S rRNA*

3.8 Choice of the best reference gene

Based on the pairwise variation results, when all the conditions were combined, even though the results suggested that one reference gene is enough, however, we recommend using a combination of genes to avoid any biasness. Although NormFinder and GeNorm suggested two different combinations, we had taken into consideration the results obtained from other softwares. Finally, we suggest using the combination of *F1-ATPase* and *18S rRNA* for normalization of the gene expression studies in rosemary produced by plant tissue culture methods. Moreover, we also endorse not using *GAPDH* for qRT-PCR studies with *S. rosmarinus*.

3.9 Validation of the best reference gene under nanoparticle stress

Based on the reference gene analysis, *F1-ATPase* and a combination of *F1-ATPase* and *18S rRNA* were identified as the most suitable candidate genes, whereas *GAPDH* was the most unstable gene. To substantiate the trustworthiness of the candidate genes, relative expression of the *4CL* under nanoparticle stress was assessed using the best and the least stable candidate genes. The normalized relative expression of *4CL* (by the best and the worst gene) in *S. rosmarinus* plants is shown in Fig. 8. In all the cases the relative expression of the *4CL* is higher following the nanoparticle treatment, however, while normalization using *GAPDH*, no significant difference was detected. Even though validation under nanoparticle stress indicated that both *F1-ATPase* and a combination of *F1-ATPase* and *18S rRNA* could be suitable, however, to avoid any biasness, we recommend using a combination of *F1-ATPase* and *18S rRNA* to normalize the gene expression studies in *in vitro* produced rosemary under nanoparticle stress.

4. Discussion

The pharmacological properties of rosemary are mainly attributed due to the secondary metabolites produced by the plant[21]. Several attempts have been made to increase the production of these metabolites under *in vitro* conditions[22]. The *in vitro* systems offer several advantages over field conditions like extraction of metabolites is independent of environmental factors and growing seasons; any plant or plant cells can be multiplied targeting specific metabolites; Elicitors and hormones can be efficiently employed; organic substances could be extracted which cannot be achieved by chemical synthesis. To achieve these, researchers have utilized several approaches like callus culture and bioreactor culture along with several biotic and abiotic stresses, elicitor, and nanoparticle treatment[23, 24, 25]. However, the underlying molecular mechanisms behind this secondary metabolite production are yet to be explored and need to be studied extensively, which will require qRT-PCR experiments.

qRT-PCR has the ability to detect very low-level expression of genes providing it an edge over other traditional methods. However, the accuracy of this method is highly dependent on the selection of stable reference gene/s for the normalization process. Earlier, housekeeping genes were believed to be stable in all given conditions and were utilized for the normalization process however, recent findings have reported that the expression of these genes could vary considerably, even the classical housekeeping genes[20, 26]. Given that, identification of reference gene/s for normalization in any given experimental condition is very crucial, if not optimized properly might lead to ambiguous outcomes.

Contemplating at industrial importance of rosemary, any validated reference gene has not been reported for *in vitro* produced *S. rosmarinus*. Hence, in this study, we used qRT-PCR to evaluate the expression stability of seven candidate reference genes under various experimental conditions. The minimum number of required reference genes for each experimental condition was also identified. Additionally, the most stable reference gene/s for each experimental condition were recommended, individually. Based on the pairwise analysis, we suggest using a combination of two reference genes for the studies with the plant organs (*18S rRNA* and *ACCase*) and studies under osmotic (*ATP-synthase* and *ACCase*) and temperature stress (*F1-ATPase* and *25S rRNA*). For the gene expression studies under salt stress, pairwise analysis suggests three reference genes (*ATP-synthase, F1-ATPase*, and *18S rRNA*) and under elicitor stress, using a combination of six reference genes (*ACCase, F1-ATPase, 18S rRNA, ATP-synthase, GAPDH*, and *28S rRNA*) is suggested. However, considering all the experimental conditions, we recommend using a combination of *F1-ATPase* and *18S rRNA* for normalization of the gene expression studies in rosemary produced under plant tissue culture. Even though in a previous study with *Rosmarinus officinalis* L., the authors recommend using *GAPDH* for normalization of the qRT-PCR data[27], the current study suggested not using this gene for expression studies with plant tissue culture-produced rosemary. This difference might be attributed to plant tissue selection and experimental treatments carried out under *in vitro* conditions.

Understanding how plant and plant cells interact and perceive various treatments and elicitors will provide valuable insights to further enhance metabolite production in industrially important plants such as rosemary. Rapid progress in molecular biology methods in plant biotechnology has raised the demand for the identification of reference genes. Hence, the reference genes recommended and validated in our study will aid in the studies related to the elucidation of elicitor stresses and their regulatory mechanisms.

Declarations

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Author contributions

Rohit Bharati and Madhab Kumar Sen designed the experiment. Rohit Bharati, Madhab Kumar Sen, Ram Kumar, Aayushi Gupta, and Ingrid Melnikovová conducted experiments. Rohit Bharati, Madhab Kumar Sen, and Vishma Pratap Sur analyzed and interpreted the results. The manuscript draft was prepared by Rohit Bharati, Madhab Kumar Sen, and Aayushi Gupta. Eloy

Fernández Cusimamani was responsible for funding acquisition, project administration, and overall supervision of the study. All authors contributed to the proofreading of the final version of the draft.

Competing interests

The authors declare no competing interests.

Ethics statement

This article does not contain any studies with human or animal subjects.

Ethical approval

No permissions were necessary to obtain the plant samples. All experiments conducted in the current study, including the collection of plant material, are in compliance with relevant institutional, national, and international guidelines and legislation.

Data availability

The datasets generated and/or analysed during the current study are available in the NCBI repository (https://www.ncbi.nlm.nih.gov/).

References

1. Kompelly, A., Kompelly, S., Vasudha, B. & Narender, B. Rosmarinus officinalis L.: an update review of its phytochemistry and biological activity. Journal of Drug Delivery and Therapeutics **9**, 323–330 (2019). https://doi.org/10.22270/jddt.v9i1.2218
2. Moore, J., Yousef, M. & Tsiani, E. Anticancer Effects of Rosemary (Rosmarinus officinalis L.) Extract and Rosemary Extract Polyphenols. Nutrients **8**, 731 (2016). https://doi.org/10.3390/nu8110731
3. Villegas-Sánchez, E. et al. In Vitro Culture of Rosmarinus officinalis L. in a Temporary Immersion System: Influence of Two Phytohormones on Plant Growth and Carnosol Production. Pharmaceuticals **14**, 747 (2021). https://doi.org/10.3390/ph14080747
4. Cui, W., Yang, K. & Yang, H. Recent Progress in the Drug Development Targeting SARS-CoV-2 Main Protease as Treatment for COVID-19. Frontiers in Molecular Biosciences **7**, 398 (2020). https://doi.org/10.3389/fmolb.2020.616341
5. Rivero-Montejo, S. de J., Vargas-Hernandez, M. & Torres-Pacheco, I. Nanoparticles as Novel Elicitors to Improve Bioactive Compounds in Plants. Agriculture **11**, 134 (2021). https://doi.org/10.3390/agriculture11020134
6. Yao, D., Zhang, Z., Chen, Y., Lin, Y., Xu, X. & Lai, Z. Transcriptome Analysis Reveals Differentially Expressed Genes That Regulate Biosynthesis of the Active Compounds with Methyl Jasmonate in Rosemary Suspension Cells. Genes **13**, 67 (2022). https://doi.org/10.3390/genes13010067
7. Joseph, J. T., Poolakkalody, N. J. & Shah, J. M. Plant reference genes for development and stress response studies. J. Biosci. **43**, 173–187 (2018). https://doi.org/10.1007/s12038-017-9728-z
8. Sen, M. K., Hamouzová, K., Košnarová, P., Roy, A. & Soukup, J. Identification of the most suitable reference gene for gene expression studies with development and abiotic stress response in *Bromus sterilis*. Sci. Rep. **11**, 13393 (2021). https://doi.org/10.1038/s41598-021-92780-1
9. Chapman, J. R. & Waldenström, J. With Reference to Reference Genes: A Systematic Review of Endogenous Controls in Gene Expression Studies. PLOS ONE **10**, e0141853 (2015). https://doi.org/10.1371/journal.pone.0141853
10. Bustin, S. A. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. Journal of Molecular Endocrinology **29**, 23–39 (2002). https://doi.org/10.1677/jme.0.0290023
11. Czechowski, T., Stitt, M., Altmann, T., Udvardi, M. K. & Scheible, W.-R. Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. Plant Physiology **139**, 5–17 (2005). https://doi.org/10.1104/pp.105.063743
12. Hadi Soltanabad, M., Bagherieh-Najjar, M. B. & Mianabadi, M. Carnosic Acid Content Increased by Silver Nanoparticle Treatment in Rosemary (Rosmarinus officinalis L.). Appl Biochem Biotechnol **191**, 482–495 (2020).

https://doi.org/10.1007/s12010-019-03193-w

13. Silver, N., Best, S., Jiang, J. & Thein, S. L. Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. BMC Molecular Biology **7**, 33 (2006). https://doi.org/10.1186/1471-2199-7-33
14. Pfaffl, M. W., Tichopad, A., Prgomet, C. & Neuvians, T. P. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper – Excel-based tool using pair-wise correlations. Biotechnology Letters **26**, 509–515 (2004). https://doi.org/10.1023/B:BILE.0000019559.84305.47
15. Andersen, C. L., Jensen, J. L. & Ørntoft, T. F. 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 Research **64**, 5245–5250 (2004). https://doi.org/10.1158/0008-5472.CAN-04-0496
16. Vandesompele, J. et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* **3**, research0034.1 (2002). https://doi.org/10.1186/gb-2002-3-7-research0034
17. Xie, F., Xiao, P., Chen, D., Xu, L. & Zhang, B. miRDeepFinder: a miRNA analysis tool for deep sequencing of plant small RNAs. Plant Mol Biol **80**, 75–84 (2012). https://doi.org/10.1007/s11103-012-9885-2
18. Ullah, A. et al. Important Flavonoids and Their Role as a Therapeutic Agent. Molecules **25**, 5243 (2020). https://doi.org/10.3390/molecules25225243
19. Roy, A. & Palli, S. R. Epigenetic modifications acetylation and deacetylation play important roles in juvenile hormone action. BMC Genomics **19**, 934 (2018). https://doi.org/10.1186/s12864-018-5323-4
20. Ashrafi, M., Azimi Moqadam, M. R., Moradi, P., Mohsenifard, E. & Shekari, F. Evaluation and validation of housekeeping genes in two contrast species of thyme plant to drought stress using real-time PCR. Plant Physiology and Biochemistry **132**, 54–60 (2018). https://doi.org/10.1016/j.plaphy.2018.08.007
21. Alvi, S. S., Ahmad, P., Ishrat, M., Iqbal, D. & Khan, M. S. Secondary Metabolites from Rosemary (Rosmarinus officinalis L.): Structure, Biochemistry and Therapeutic Implications Against Neurodegenerative Diseases. in *Natural Bio-active Compounds: Volume 2: Chemistry, Pharmacology and Health Care Practices* (eds. Swamy, M. K. & Akhtar, M. S.) 1–24 (Springer, 2019). doi:10.1007/978-981-13-7205-6_1.
22. Coskun, Y., Duran, R. E. & Kilic, S. Striking effects of melatonin on secondary metabolites produced by callus culture of rosemary (*Rosmarinus officinalis* L.). Plant Cell Tiss Organ Cult **138**, 89–95 (2019). https://doi.org/10.1007/s11240-019-01605-7
23. Mosavat, N., Golkar, P., Yousefifard, M. & Javed, R. Modulation of callus growth and secondary metabolites in different Thymus species and *Zataria multiflora* micropropagated under ZnO nanoparticles stress. Biotechnology and Applied Biochemistry **66**, 316–322 (2019). https://doi.org/10.1002/bab.1727
24. Ali, A. et al. Silver nanoparticles elicited in vitro callus cultures for accumulation of biomass and secondary metabolites in Caralluma tuberculata. Artificial Cells, Nanomedicine, and Biotechnology **47**, 715–724 (2019). https://doi.org/10.1080/21691401.2019.1577884
25. Singh, A. & Dwivedi, P. Methyl-jasmonate and salicylic acid as potent elicitors for secondary metabolite production in medicinal plants: A review. J Pharmacogn Phytochem **7**, 750–757 (2018).
26. Ling, H., Wu, Q., Guo, J., Xu, L. & Que, Y. Comprehensive Selection of Reference Genes for Gene Expression Normalization in Sugarcane by Real Time Quantitative RT-PCR. PLOS ONE **9**, e97469 (2014). https://doi.org/10.1371/journal.pone.0097469
27. Aminfar, Z., Rabiei, B., Tohidfar, M. & Mirjalili, M. H. Selection and validation of reference genes for quantitative real-time PCR in Rosmarinus officinalis L. in various tissues and under elicitation. Biocatalysis and Agricultural Biotechnology **20**, 101246 (2019). https://doi.org/10.1016/j.bcab.2019.101246

Figures

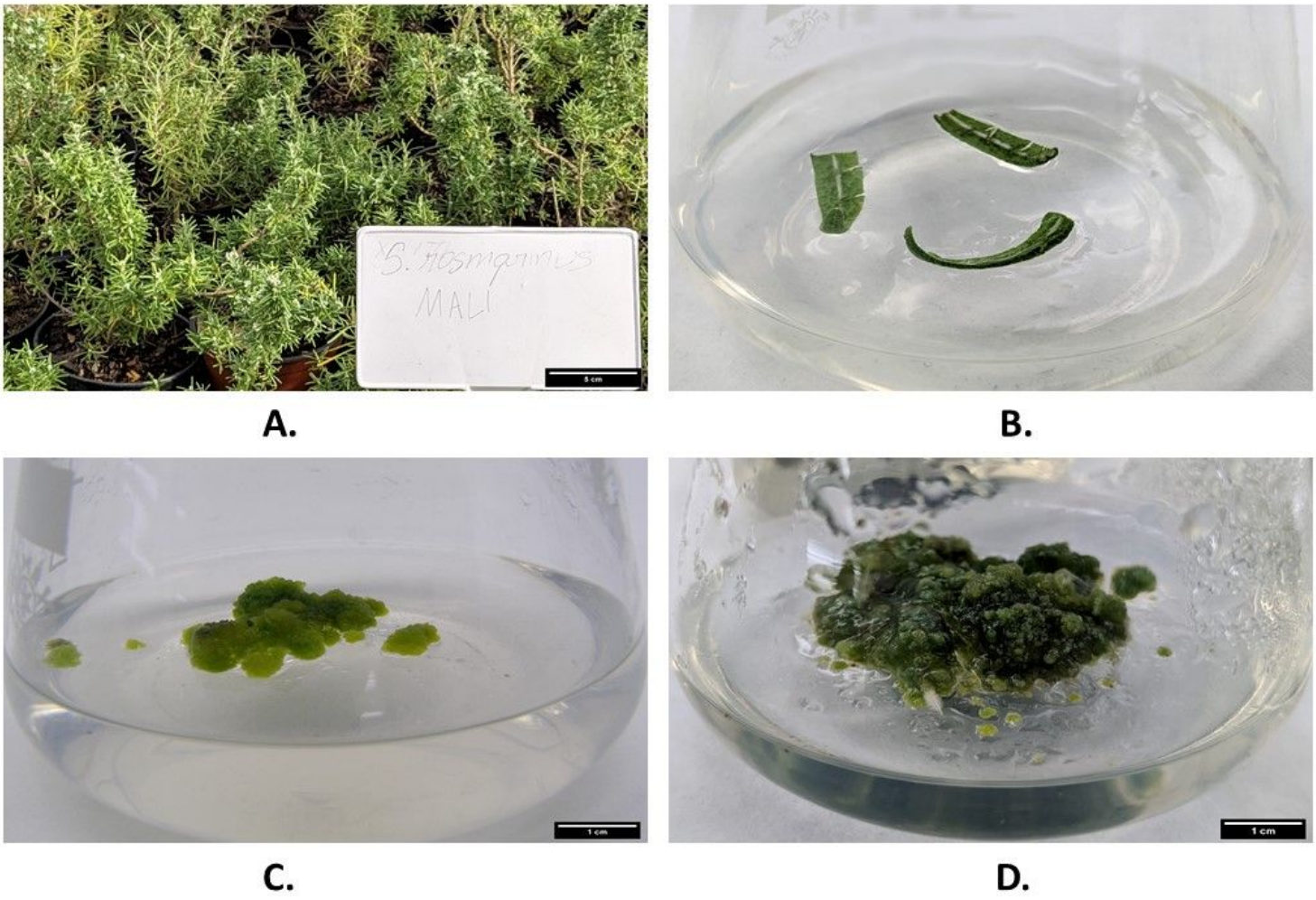

Figure 1

In vitro **culture of** ***Salvia rosmarinus*** **L. A:** Source of the explant. The explants were collected from the well-maintained botanical garden of the Faculty of Tropical AgriSciences, Czech University of Life Sciences, Prague, **B:** The leaves were used as explants and transferred to Murashige and Skoog's (1962) basal medium, **C:** Callus after 10 days and **D:** Callus after 20 days.

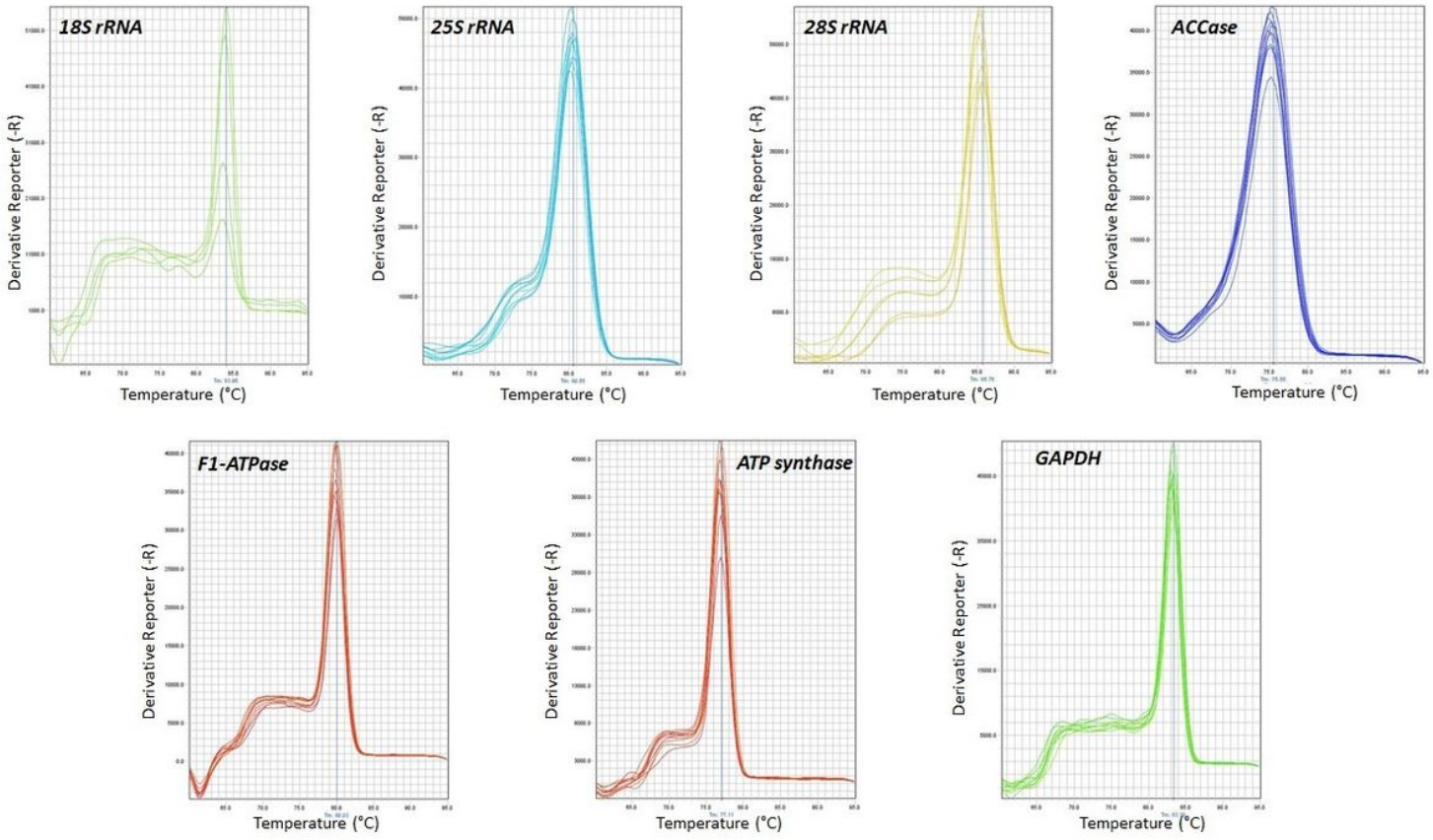

Figure 2

Melting curve analysis of each candidate reference genes.

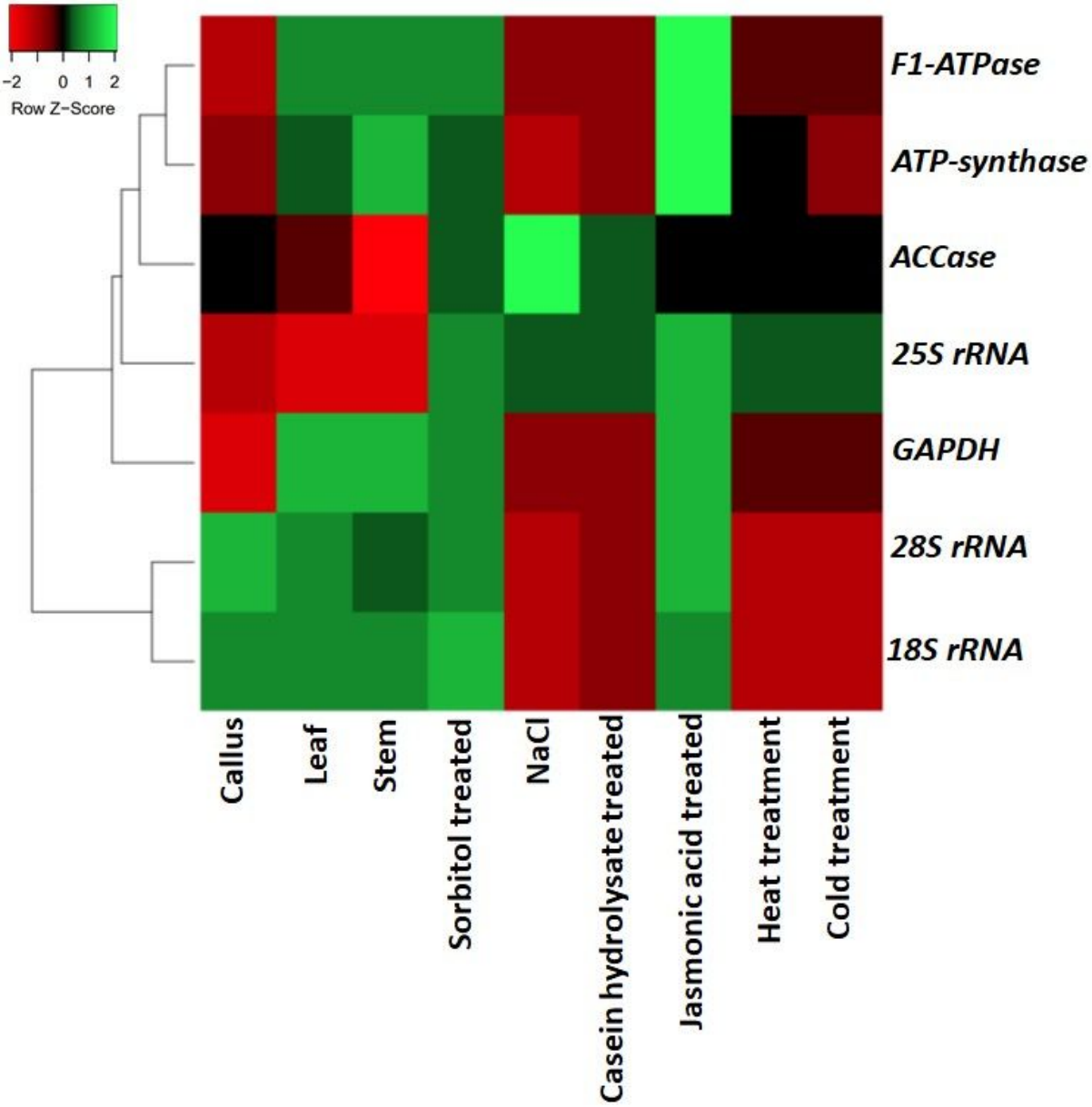

Figure 3

Heat map of candidate reference genes based on the mean Ct values.

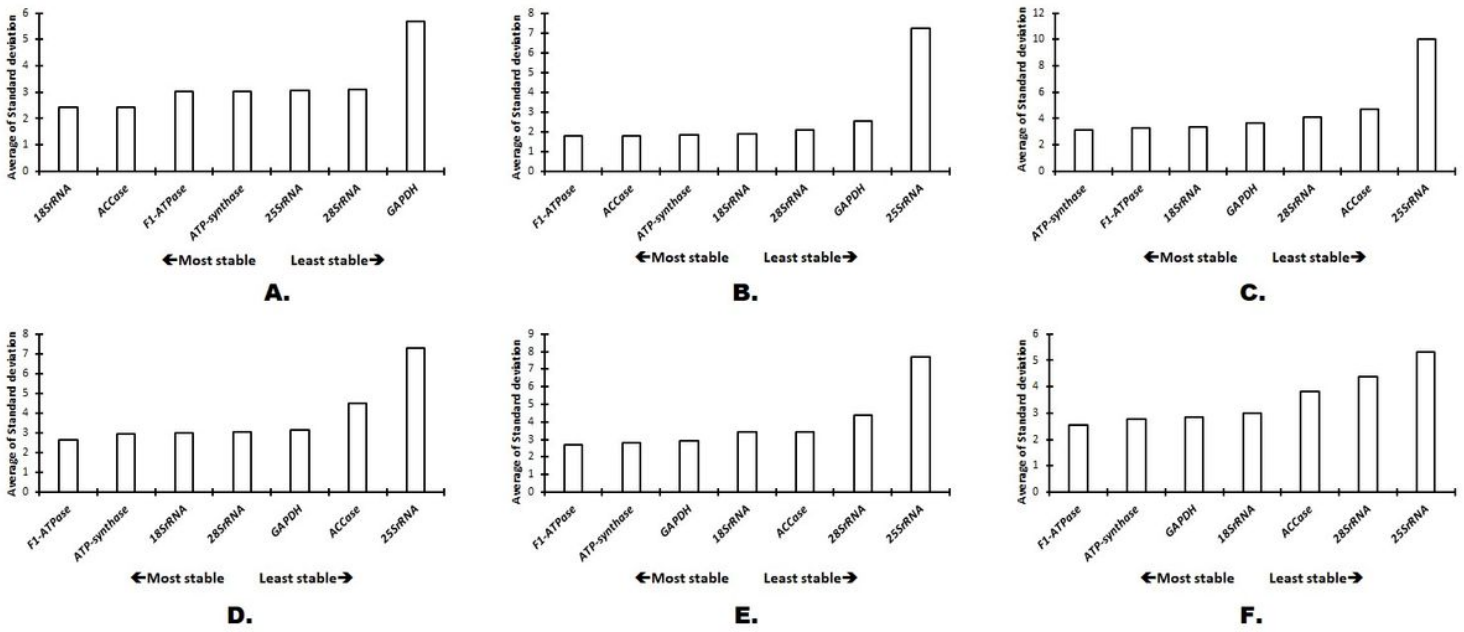

Figure 4

Evaluation of expression stability by ΔCt method. A. Plant organs, **B.** Osmotic stress, **C.** Salt stress, **D.** Elicitor stress, **E.** Temperature stress, **F.** All combined.

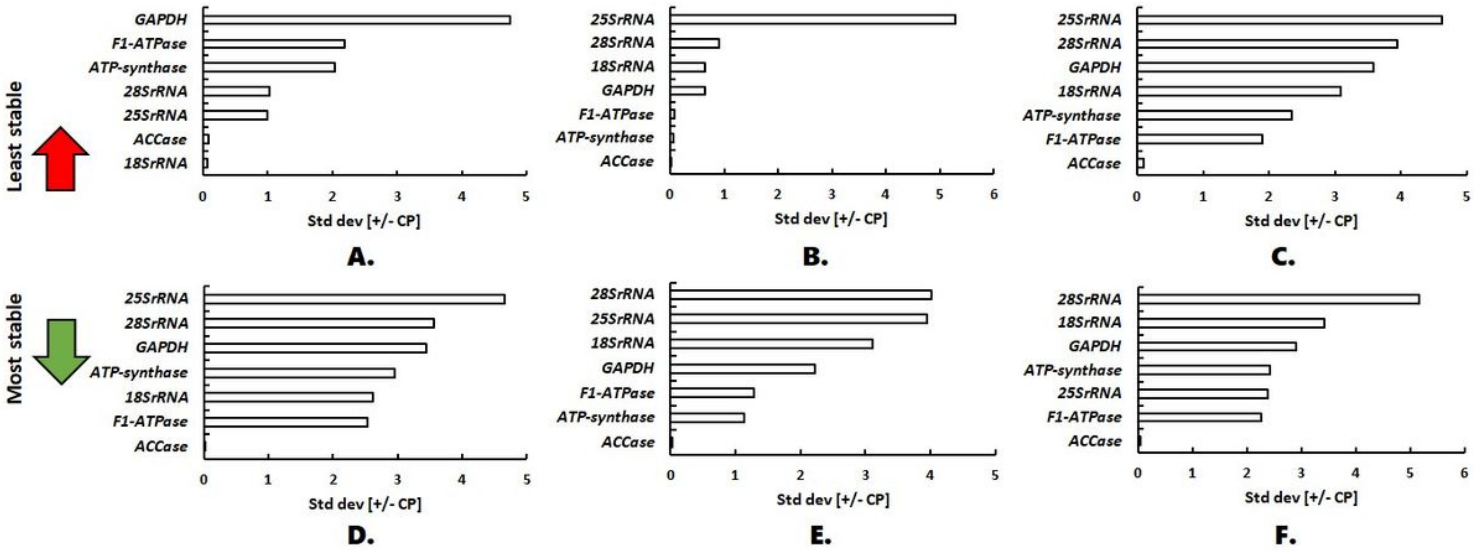

Figure 5

Evaluation of expression stability by BestKeeper. A. Plant organs, **B.** Osmotic stress, **C.** Salt stress, **D.** Elicitor stress, **E.** Temperature stress, **F.** All combined.

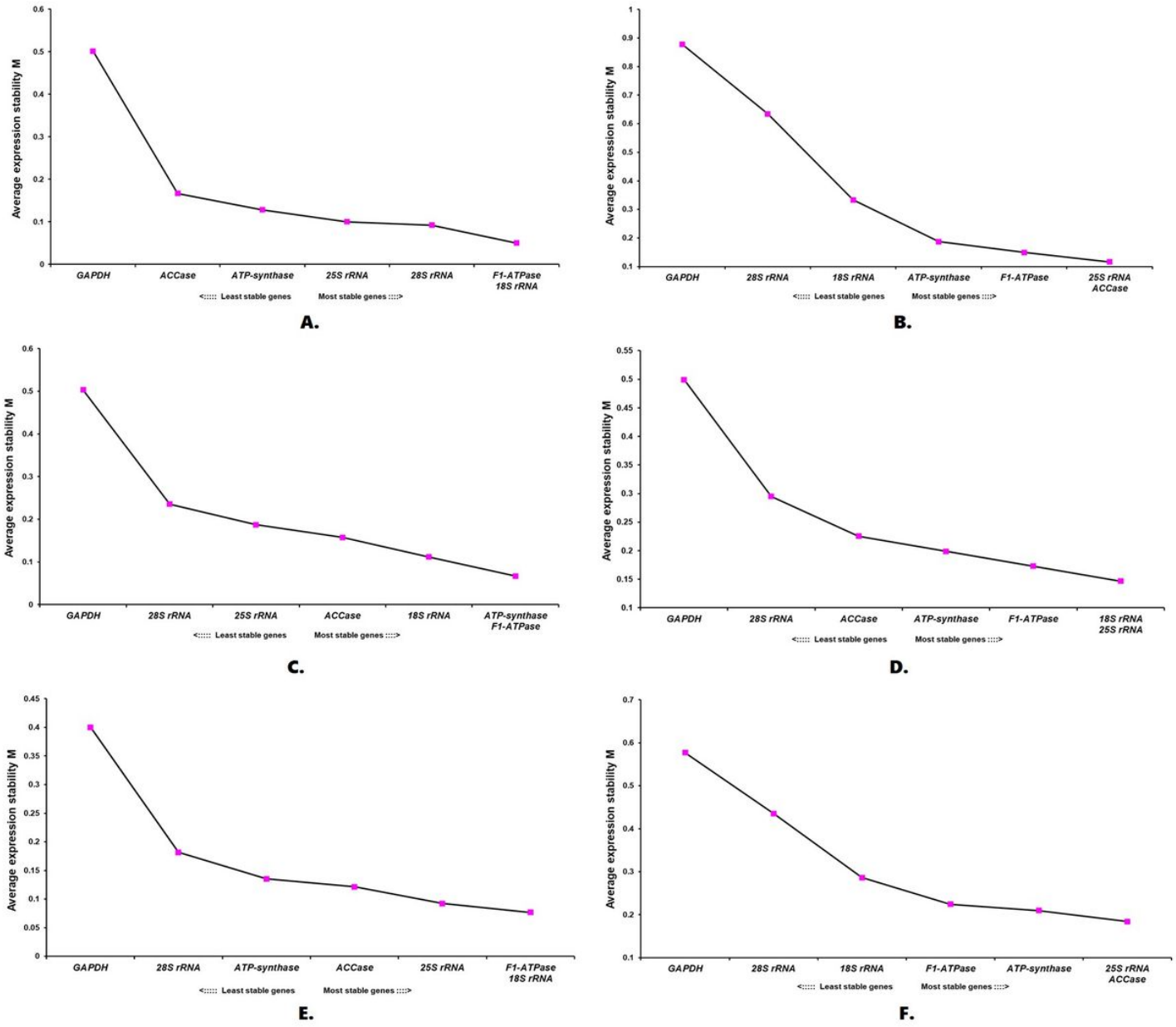

Figure 6

Evaluation of expression stability by geNorm. A. Plant organs, **B.** Osmotic stress, **C.** Salt stress, **D.** Elicitor stress, **E.** Temperature stress, **F.** All combined.

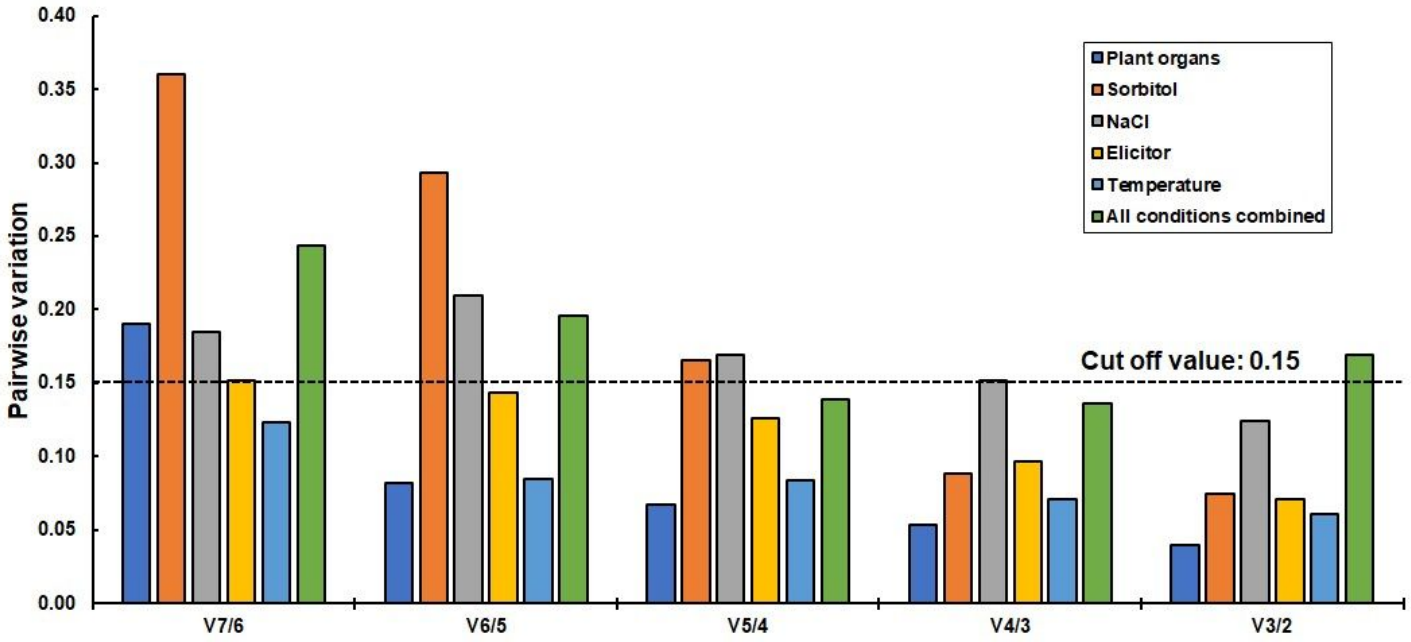

Figure 7

Analysis of the number of optimal genes required for each experimental condition.

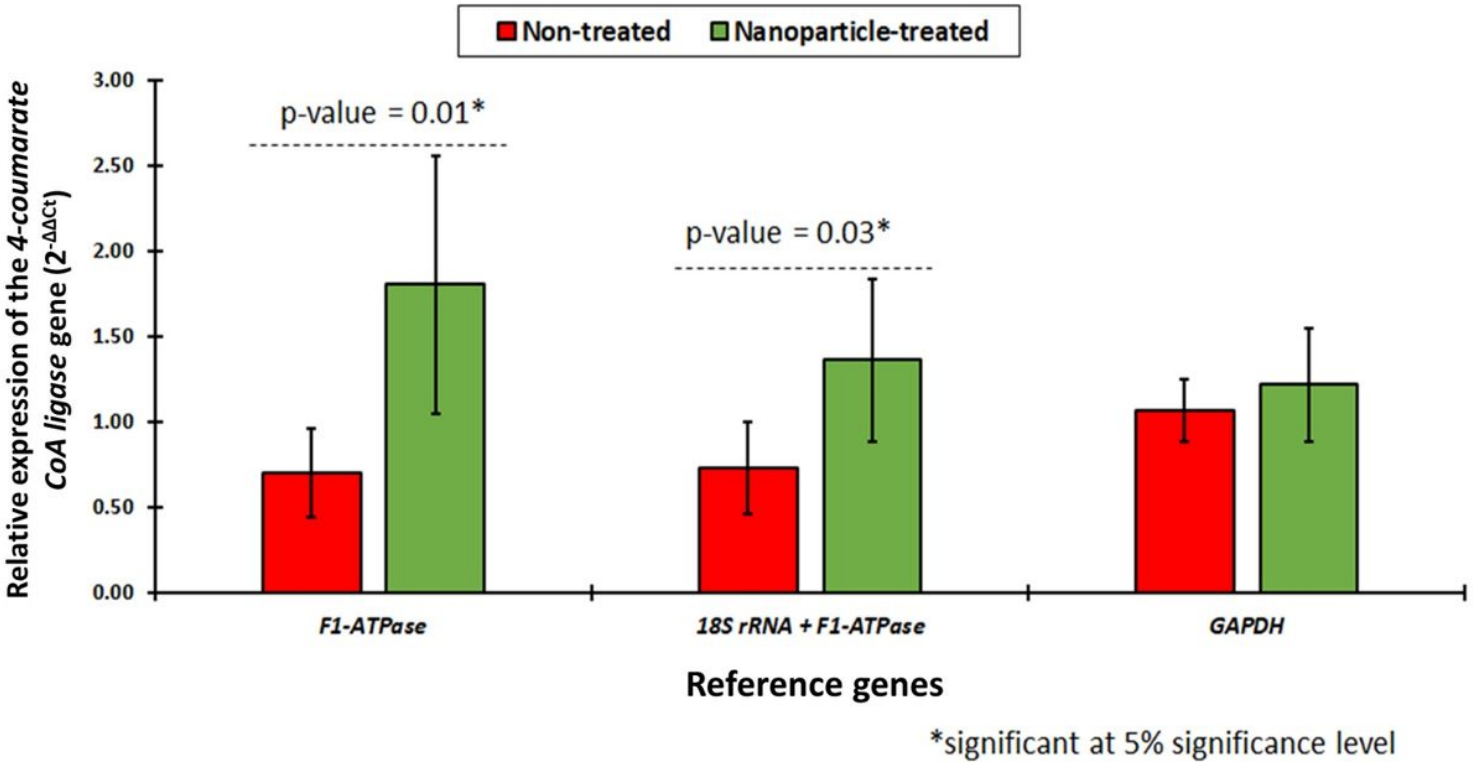

Figure 8

Relative expression of the *4-coumarate CoA ligase* gene under zinc nanoparticle stress. Relative gene expression without any nanoparticle treatment and 7 days after zinc nanoparticle treatment were compared, and normalization was done with *F1-ATPase*, *18S rRNA + F1-ATPase*, and *GAPDH*.

Supplementary Files

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