



Nanoparticles Affect the Expression Stability of Housekeeping Genes in Plant Cells

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Purpose: We report on the expression stability of several housekeeping/reference genes that can be used in the normalization of target gene expression in quantitative real-time PCR (qRT-PCR) analysis of plant cells challenged with metal nanoparticles (NPs).

Materials and Methods: Uniform cell suspension cultures of *Hypericum perforatum* were treated with 25 mg/l silver and gold NPs (14–15 nm in diameter). Cells were collected after 0.5, 4.0, and 12 h. The total RNA isolated from the cells was analyzed for the stability of *ACT2*, *ACT3*, *ACT7*, *EF1- α* , *GAPDH*, *H2A*, *TUB- α* , *TUB- β* , and 18S rRNA genes using qRT-PCR. The cycle threshold (Ct) values of the genes were analyzed using the geNorm, NormFinder, BestKeeper, and RefFinder statistical algorithms to rank gene stability. The stability of the top-ranked genes was validated by normalizing the expression of *HYP1*.

Results: The expression of the tested housekeeping genes varied with treatment duration and NP types. *EF1- α* in gold NP treatment and *TUB- α* and *EF1- α* in silver NP treatment ranked among the top three positions. However, none of the genes retained their top ranking with time and across NP types.

Conclusion: *EF1- α* can be used as a reference for treatment involving both silver and gold NPs in *H. perforatum* cells. *TUB- α* can be used only for silver NP-treated cells. The expression instability of most of the housekeeping genes highlights the importance of systematic standardization of reference genes for NP treatment conditions to draw proper conclusions on the target gene expression.

Keywords: nanoparticle, plant cell, *Hypericum*, gene expression, reference gene, quantitative real-time PCR

Introduction

Metal nanoparticles (NPs) possess better physico-chemical properties than those of their bulk form and are useful in various industrial sectors, including agriculture. Recent developments in NP-based pesticides, fungicides, and fertilizers have significantly increased the possibilities of the direct exposure of plants to engineered NPs. Similarly, the environmental concentration of contaminating NPs is steadily increasing owing to the release of metal NPs as waste products from various consumer industries and products. Thus, plants are directly exposed to NPs present in crop protectants and plant growth enhancers, and indirectly to NPs present in the environment as contaminants. These exposures have spurred recent studies regarding the impact of nanomaterials on plant metabolism.¹

Quantitative real-time PCR (qRT-PCR) analysis of gene expression would provide a clear understanding of the changes in the physiology, biochemistry, metabolism, and other aspects of plants under various physiological and environmental conditions. This

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technique is widely used for the quantification of gene expression, reflecting its accuracy across a relatively broad and dynamic range.² However, the reliability of the results depends on the reference genes used in the normalization step of qRT-PCR. A good reference gene should be consistently expressed under both control and experimental conditions. *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*, *actin (ACT)*, *tubulin alpha (TUB- α)*, *tubulin beta (TUB- β)*, *translation elongation factor*, *ubiquitin-conjugating enzyme (UBC)*, *18S ribosomal RNA (18SrRNA)*, *mitogen-activated protein kinase 6 (MPK6)*, and *25S ribosomal RNA (25SrRNA)* are common reference genes.^{2,3}

The stability of reference genes differs according to the experimental conditions and plant species. *EF1- α* has been found to be the most stable gene under heat stress, whereas the *ADP-ribosylation factor 1 (ADP)* gene is the most stable under salt stress in *Hordeum brevisubulatum*.⁴ In *Calotropis procera* subjected to salt stress, *CYP23* has been found to be the most stable reference gene.⁵ The *GAPDH* gene has been demonstrated to be the most reliable reference gene for cold stress, whereas it is less stable under heat stress in *Hypericum perforatum* plants.⁶ These variations in the expression of the housekeeping genes in different conditions highlight the need to assess gene stability in each experimental condition before using the genes for the normalization of target gene expression in qRT-PCR analysis.

Despite the need to understand plant responses upon NP stress at the transcriptional level, a formal standardization of reference genes for the qRT-PCR analysis of gene expression in NP-treated plant cells has not been reported. Plant culture systems can possibly avoid the interference of the structural complexity of plant tissues and environmental fluctuations, which would aid in understanding the response of plants to NPs at the cellular level more precisely. Plant cell culture systems can also be potentially useful in the exploitation of NPs as elicitors of important secondary metabolites. These reasons prompted the present evaluation of the stability of nine housekeeping genes—*ACT2*, *ACT3*, *ACT7*, *EF1- α* , *GAPDH*, *Histone H2A (H2A)*, *TUB- α* , *TUB- β* , and *18SrRNA*— in plant cells challenged with NPs.

Materials and Methods

Nanoparticle Size, Polydispersity Index, and Zeta Potential

Water dispersions of silver (US7140) and gold (US7805) NPs (14–15 nm) were purchased from US Research

Nanomaterials Inc. (Houston, TX, USA). These NPs were diluted to 25 mg/L in Millipore water. They were characterized by mean size, polydispersity index (PDI), and zeta potential using dynamic light spectroscopy with a Mastersizer 3000 zetasizer (Malvern Instruments, Malvern, UK).

Fate of Nanoparticles in Culture Medium

To understand the changes of the NPs in culture medium, NPs diluted to 25 mg/L in MS medium were spectroscopically characterized using a model UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). The ultraviolet/visible (UV-VIS) spectra were recorded from 190 to 1000 nm with a stepwise increment of 1 nm.

Nanoparticle Treatment of *H. perforatum* Suspension Cultures

Various concentrations of silver NPs (5–50 mg/L) were added to *H. perforatum* (cv Helos) suspension cultures, which were then analyzed for changes in color, secondary metabolic profiles, and alkalization. Based on these preliminary data, the 25 mg/L concentration was selected and used for the subsequent experiments. Final concentrations of 25 mg/L of the silver and gold NPs were added to 50 mL cell suspension cultures containing approximately 150 mg (fresh weight) cells/mL in 250 mL Erlenmeyer flasks in triplicate. Cultures that received a corresponding volume of sterile distilled water served as the control. Samples (10 mL) collected at 0.5, 4, and 12 h were filtered through a PluriStrainer[®] 1 μ m filter (pluriSelect, Leipzig, Germany) to remove the medium. The cells were collected in 50 mL Falcon tubes and frozen in liquid nitrogen.

RNA Isolation and cDNA Synthesis

Approximately 100 mg of the frozen cells from NP treated and control cultures were ground into fine powder using liquid nitrogen and a sterile, pre-chilled mortar and pestle. Total RNA was isolated using a spectrum RNA extraction kit (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's information. Column DNase treatment (Sigma-Aldrich) was performed to remove contaminating DNA. RNA quantity was measured using a NanoDrop[™] One^C (Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity (RIN) was measured using the 2100 Bioanalyzer System (Agilent Technologies, Santa Clara, CA, USA). Approximately 1 μ g of RNA with a RIN value >7 was converted to cDNA using the M-MLV Reverse Transcriptase kit

(Promega, Madison, WI, USA) following the manufacturer's instructions.

Genes, Primers, and qRT-PCR Conditions

Nine housekeeping genes (*ACT2*, *ACT3*, *ACT7*, *EF1- α* , *GAPDH*, *H2A*, *TUB- α* , *TUB- β* , and *18SrRNA*) were tested as candidate reference genes (Table 1). The forward and reverse primer sequences used in the amplification of these genes were derived from previous reports.^{2,6} Primers were synthesized and their specificity was tested using PCR and qRT-PCR based on melting curve analysis. Amplification of specific fragments of the genes using qRT-PCR was carried out in a Lightcycler 480 real-time PCR system (Roche, Basel, Switzerland) using the SYBR Green-based PCR assay. The final volume for each reaction was 10 μ L with the following components: 4.2 μ L diluted cDNA template (0.1 μ g), 5 μ L SensiFAST SYBR No-ROX (Bioline, London, UK), 0.4 μ L forward primer (10 μ M), and 0.4 μ L reverse primer (10 μ M). The reaction was conducted in 96-well plates (Brooks Life Sciences, Manchester, UK) at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 15 s, and extension at 72°C for 25 s. The melting curve was obtained by heating the amplicon from 65°C to 97°C in the continuous acquisition mode and five acquisitions at each temperature. qRT-PCR analysis was performed with three technical replicates for each biological replicate. The standard curve for each set of primers was generated with 10-fold serial dilution of the pooled cDNA of all treatment conditions. Average cycle threshold (Ct) values for each dilution were used to calculate the amplification efficiency (E%) of each primer pair using the equation: $E (\%) = (10^{(-1/\text{slope})} - 1) \times 100\%$.^{7,8} The E% of all primer sets was 90% to 110% (Table 1).

Ranking of Genes Based on Ct Values

To test the stability of the selected genes in the various NP treatment conditions, the Ct values obtained in qRT-PCR analyses were analyzed using four statistical algorithms: geNorm, NormFinder, BestKeeper, and RefFinder. The mean pairwise variation (V) between the Ct values of the tested genes was used to calculate the expression stability value (M) of each reference gene in the geNorm analysis.⁹ An ANOVA-based model was applied to determine inter- and intra-group variations of the Ct values of the reference genes. The findings were used to calculate the expression stability value in NormFinder.¹⁰ The standard deviation and coefficient of variance of raw Ct values were used in the calculation of expression stability of the candidate reference genes in BestKeeper.¹¹ All the statistical data were used to generate a comprehensive ranking of genes in RefFinder.¹²

Validation of Housekeeping Genes for Stability Upon Nanoparticle Treatment

Phenolic oxidative coupling protein (HYP1), a gene that is up-regulated by various biotic and abiotic stress conditions in *H. perforatum*, was used as the target gene to confirm the stability of the reference genes predicted by the statistical analyses. The relative expression of *HYP1* after NP treatment at different times was calculated by normalizing with the most and least stable reference genes by $2^{-\Delta\Delta C_t}$ method.⁷

Results and Discussion

Characteristics of Nanoparticles

The mean particle size of both silver and gold NPs was higher than the actual sizes reported by the manufacturer. The mean size of silver and gold NPs was 38.57 (nm

Table 1 Details of the Housekeeping Genes Tested for Their Stability After Gold and Silver NP Treatment

Gene	Accession Number	Primer Sequence	Amplification Efficiency (E%)
<i>ACT2</i>	MK054303	Fw- AGGAGTCCCTCCACGACCAC Rv- GCCGTTGTGTACCGGGTAGG	106.04
<i>ACT3</i>	MK106364	Fw- ATCCTTCCCACGGTGTTGC Rv- CAATCGCCTCGTCGCCTACA	103.09
<i>ACT7</i>	MK106365	Fw- GAGCAGCAGCAGGTCGACAA Rv- ACCCACTCGAGCCCAGTGTA	109.42
<i>EF1-α</i>	MK106356	Fw- TGGAGGCTCTCCCTGGTGAC Rv- AAGTTGGCAGCCTCCTTGGC	99.72
<i>TUB-α</i>	MK106362	Fw- TGCTGCGGTTGCCACCATT Rv- CGCTGCACCTTTGCAAGATCG	98.71
<i>TUB-β</i>	MK106361	Fw- CGACGGGAGTGACAGCCTTG Rv- CGACCACATCGCTCGTCTCC	105.76
<i>GAPDH</i>	EU301783	Fw- GGTCGACTTCAGGTGCAGTGA Rv- CACCATGTCGTCTCCCATCA	99.64
<i>H2A</i>	EU034009	Fw- CCGGTTGGGAGGGTTCA Rv-TGCACCGACCCTTCCATT	100.64
<i>18SrRNA</i>	AF206934	Fw- CGTCCCTGCCCTTTGTACAC Rv- CGAACACTTCACCGGACCAT	92.34

Table 2 Mean Particle Size, Polydispersity Index and Zeta Potential of the NPs

	Mean Particle Size (d.nm)	Polydispersity Index (Pdl)	Zeta Potential (mV)
Silver	38.57	0.289	-9.56
Gold	84.05	0.178	-13.4

diameter) and 84.05 (nm diameter), respectively (Table 2). The size distribution of silver and gold NPs is shown in Figure 1. The PDI of silver and gold NP suspensions was

0.289 and 0.178, respectively. The zeta potential of silver and gold NPs was -9.56 and -13.4, respectively. The UV-VIS spectra of the NPs remained more or less similar in the MS medium after different time-points (Figure 2).

Influence of Nanoparticle Treatment on *H. perforatum*

Gradual darkening of *H. perforatum* suspension cultures was observed by time after challenging them with the gold (Figure 3D-F) and silver (Figure 3G-I) NPs. However, no color change was observed in the control (Figure 3A-C).

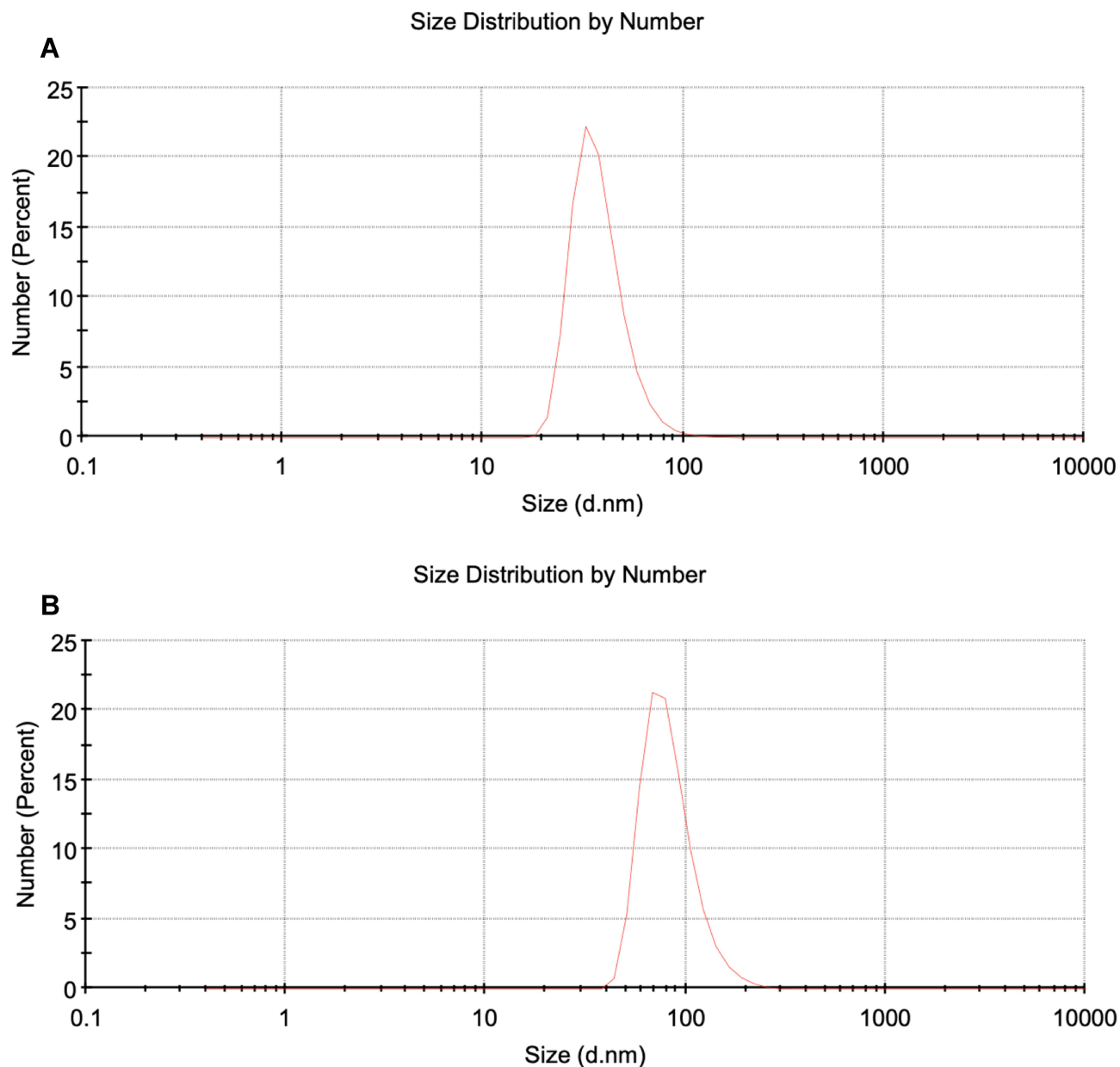


Figure 1 Size distribution of silver (A) and gold (B) NPs used in the present study.

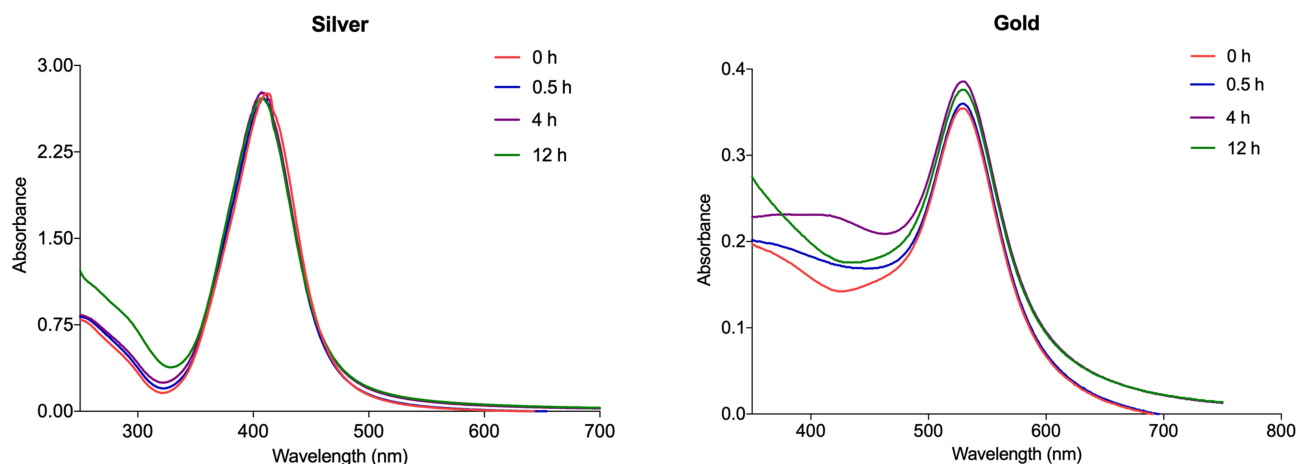


Figure 2 UV-VIS spectra of NPs diluted in MS medium.

Silver NP treatment reduced cell viability after 12 h (Figure 4C). The cell viability remained almost the same in gold NP-treated (Figure 4B) and control (Figure 4A) cultures. Toxicity mediated by silver NPs involves various mechanisms including the production of reactive oxygen species (ROS).¹³ A lower level of ROS is involved in the regulation of various cellular functions, while cell death is induced by higher levels of ROS.^{14–16} Viability of *Lemna gibba* plant cells reduces after silver NP treatment, which is also significantly correlated with intracellular ROS induction.¹⁶ RNA degradation can be rapidly triggered by cell death.^{17,18} The

proportion of mRNA to total RNA was reduced in *Escherichia coli* treated with silver nitrate compared to untreated *E. coli*.¹⁹

Metal NPs reportedly induce both positive and negative changes in major metabolic processes such as seed germination, growth, shoot/root length, biomass, and physiological or biochemical activities.^{20,21} These effects are largely based on the plant species and the physicochemical properties of the NPs. Magnetic NPs negatively affect the metabolism and induce biosynthesis of protective compounds in suspension cultures of Tobacco BY-2 cells.²² Biomass fresh weight and production of secondary

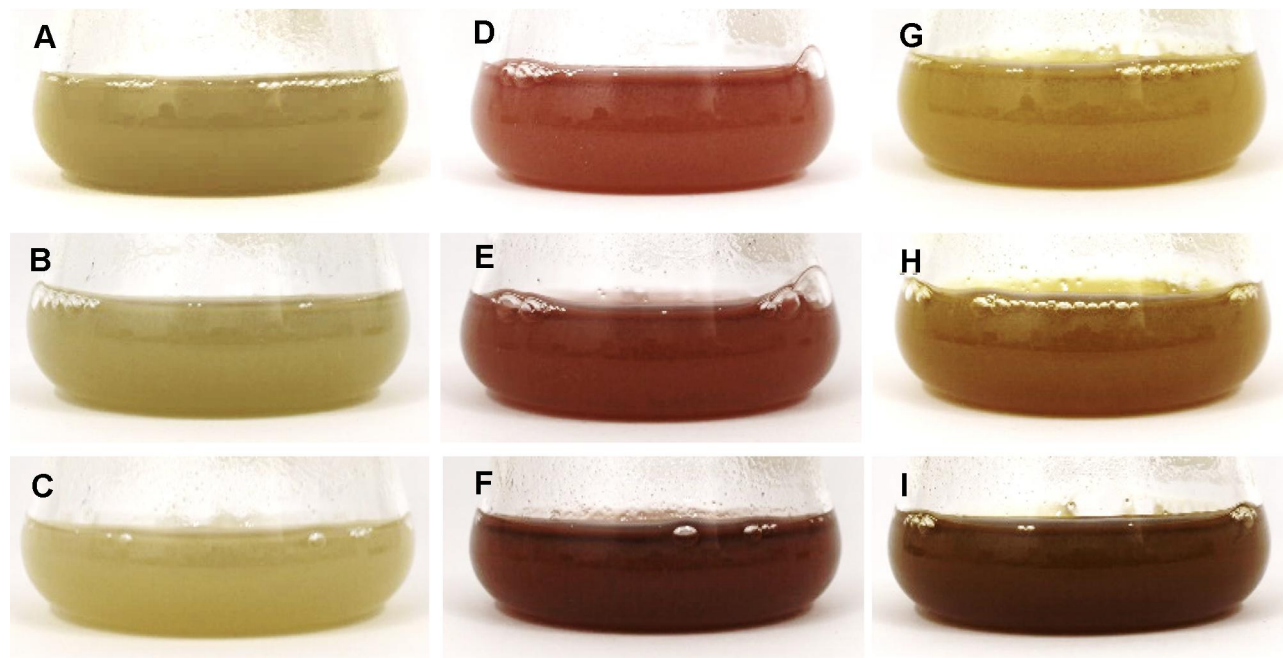


Figure 3 *H. perforatum* cell suspension cultures after NP treatment. Control cultures remained yellowish-green throughout the experimental period ((A) 0.5 h, (B) 4 h (C) and 12 h), whereas cultures gradually darkened with time in the presence of gold ((D) 0.5 h, (E) 4 h, and (F) 12 h), and silver ((G) 0.5 h, (H) 4 h and (I) 12 h), NPs.

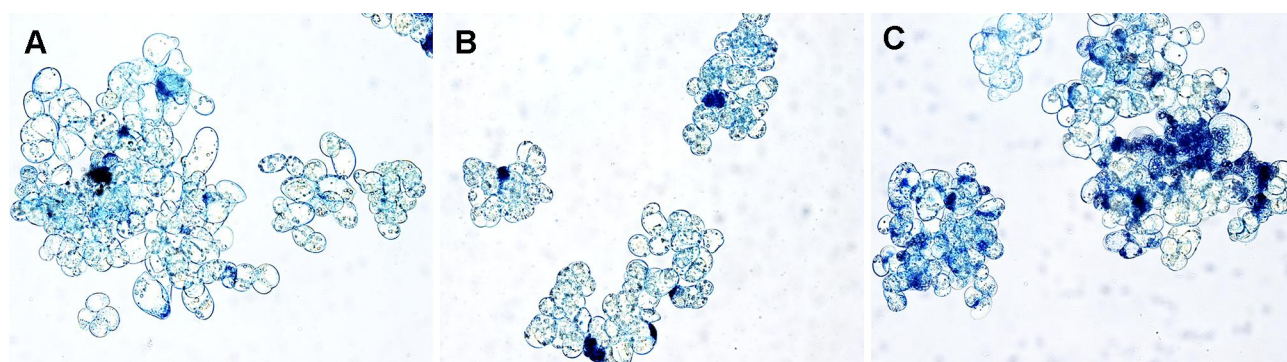


Figure 4 Trypan blue staining of control (A), and cultures treated with gold (B) and silver (C) NPs after 12 h showing cell viability.

metabolites reportedly increase after treatment with zinc oxide²³ and silver²⁴ NPs in cell suspension cultures of *Linum usitatissimum*. Copper oxide NPs treatment significantly increases the production of gymnemic acid II and phenolic compounds in *Gymnema sylvestre* suspension cell cultures.²⁵ Aluminum oxide NPs were toxic for Tobacco BY-2 cells in suspension, which involved the generation of reactive oxygen and nitrogen species.²⁶

Gold and silver NPs generally behave in an opposite manner to each other across plant species. While gold NPs beneficially influence growth and metabolism in various plant species, silver NPs inhibit growth and metabolism. *Allium cepa* seeds treated with green synthesized gold NPs display enhanced seed germination, emergence, growth, and yield,²⁷ whereas cytotoxic and genotoxic impacts have been observed in *A. cepa* root tip cells treated with silver NPs.²⁸ The application of gold NPs improved the growth and yield of *Brassica juncea*.²⁹ Phytotoxic effects that include lower chlorophyll contents, higher superoxide dismutase activity, and less fruit productivity have been reported in *Lycopersicon esculentum* treated with silver NPs.³⁰ A dose-dependent inhibitory effect of silver NPs has been observed for the germination and subsequent growth and development of *Oryza sativa* seeds.³¹ Similar positive and negative impacts of NPs have also been recorded in plant cell suspension cultures. Silver NPs increase malondialdehyde (MDA) and hydrogen peroxide (H₂O₂) contents in *Momordica charantia* suspension cell cultures,³² while gold NP treatment enhances biomass accumulation in *Prunella vulgaris* suspension cell cultures.³³

Nanoparticles Affect Expression Stability of Housekeeping Genes

The *ACT2*, *ACT3*, *ACT7*, *EF1- α* , *GAPDH*, *H2A*, *TUB- α* , *TUB- β* , and *18SrRNA* housekeeping genes were analyzed

for their stability upon NP treatment. Agarose gel electrophoresis of the amplicon from PCR and melting curve analyses following qRT-PCR confirmed the primer specificity of all the tested genes. The specific fragments of the selected genes could be efficiently amplified using these primers as revealed by agarose gel electrophoresis (Figure S1, Supplementary data). Melting curve analysis showed a single peak, further confirming the primer specificity (Figure S2, Supplementary data).

All the housekeeping genes were previously examined concerning their stability in different abiotic conditions in plants. *ACT2*, *TUB- β* , and *EF1- α* were the most stable reference genes in different tissues, developmental stages, and stress conditions in *H. perforatum*.² Expression of *ACT3* was stable in cucumber upon cold and heat stress.³⁴ *ACT7* was stably expressed in *Corchorus olitorius* during drought and water stress.³⁵ *GAPDH* expression was stable in *Arabidopsis* under cold, heat, and salt stresses.³ Similarly, the expression of *18SrRNA* was stable upon metal stress in *Camellia sinensis*,³⁶ *H2A* expression was stable under heat, salt, drought, and dark stresses in *Reaumuria soongorica*,³⁷ and *TUB- α* was stably expressed in *Hordeum vulgare* subjected to salt and cold stresses.³⁸

Ct represents the number of cycles in which the fluorescent signal crosses the background level (threshold) in qRT-PCR. Ct values were used to determine the expression levels of candidate reference genes by box-plot analysis (Figure 5). The Ct values ranged from 14.71 to 29.44, 14.33 to 29.24, and 15.62 to 29.79 at 0.5 h (Figure 5A), 4 h (Figure 3B), and 12 h (Figure 5C), respectively, in both control and NP treatments. The Ct values were lowest for *18SrRNA* across the NP treatments and times, and highest for *ACT3*. Among the genes analyzed, *TUB- β* showed

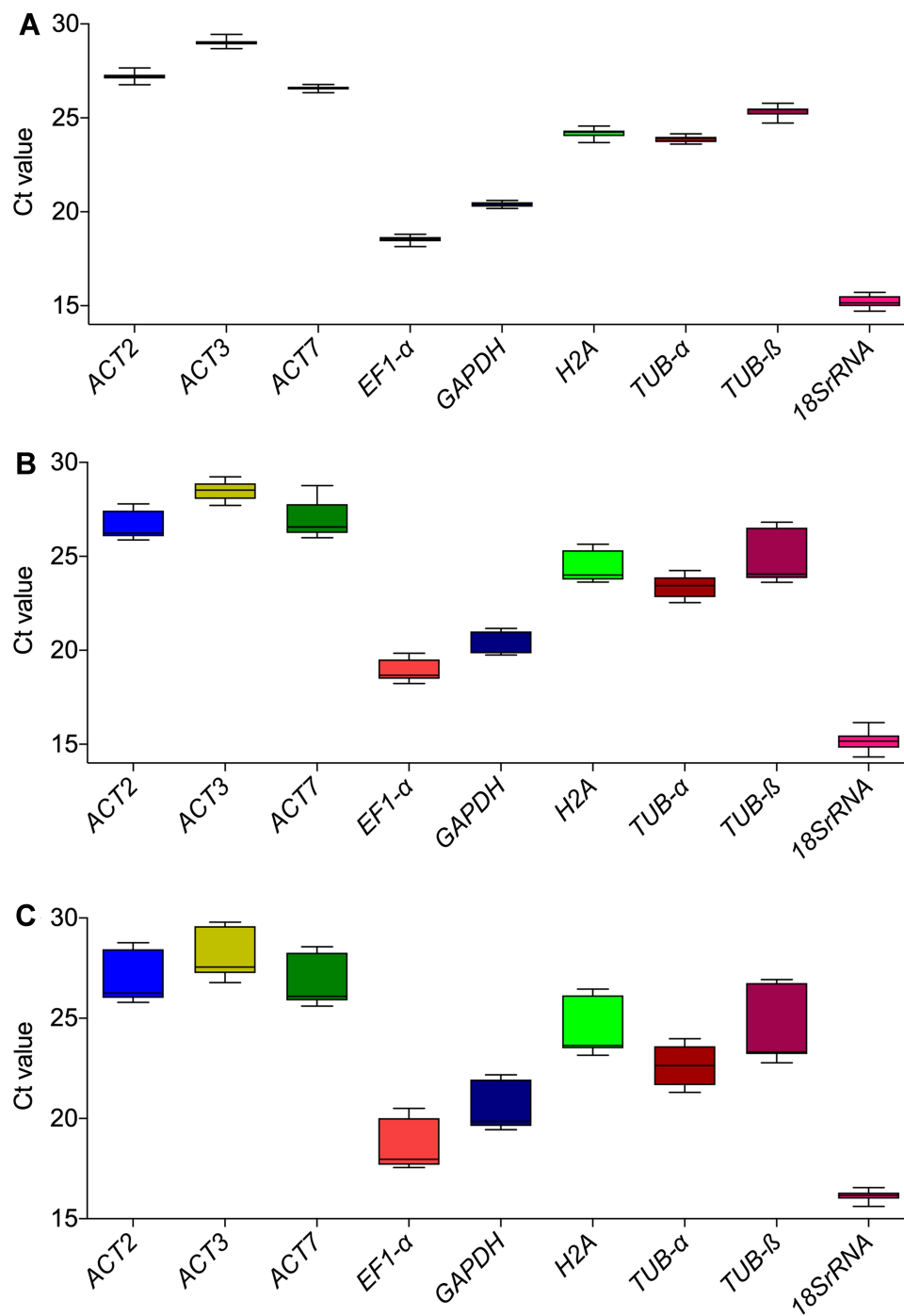


Figure 5 BoxPlot showing Ct values of the housekeeping genes in experimental samples taken at 0.5 h (A), 4 h (B) and 12 h (C) post-treatment time points. The line across the box is the median value, whereas the 25th percentile to the 75th percentile is indicated by the lower and upper boxes. The maximum and minimum values are represented by Whiskers.

a high variation of Ct values in cultures treated with NPs for 12 h. The lowest variation of expression levels was observed in *18SrRNA* at various times.

Ct values of the control and NP treatments were further analyzed by hierarchical clustering to understand the major differences between the expression patterns of

the genes in the different treatment conditions using the ClustVis web tool.³⁹ Heatmap analysis revealed no significant difference compared to the control at 0.5 h of treatment. However, cells treated with silver NPs for longer than 0.5 h showed a greater variation of Ct values than the control and gold NP treatments. Silver

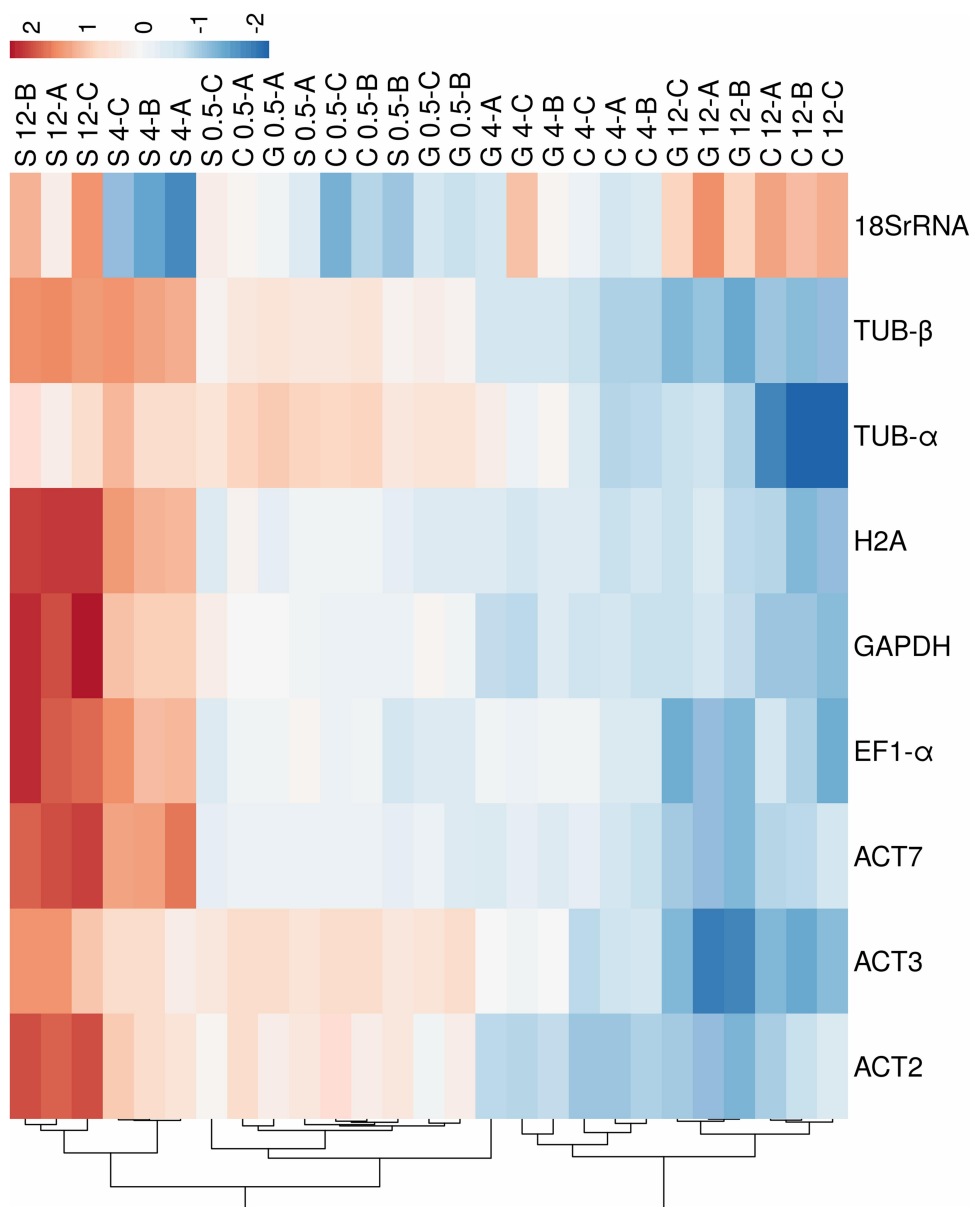


Figure 6 Heatmap constructed by hierarchical clustering analysis (HCA) of Ct values of *ACT2*, *ACT3*, *ACT7*, *EF1- α* , *GAPDH*, *H2A*, *TUB- α* , *TUB- β* and *18SrRNA* genes in control (C), and gold (G) and silver (S) NPs treated cultures at 0.5 h (0.5), 4 h (4) and 12 h (12) post-treatment in three biological replications (A-C). Red color represent positive values or higher Ct values and blue color represents negative values or low Ct values.

NP-treated samples displayed higher Ct values at 4 h and 12 h of treatment, while control and gold NP-treated samples displayed comparatively lower Ct values for the respective genes at these times (Figure 6). The number of mRNA transcripts available for amplification may decrease due to breaks that occur in degraded or partially degraded samples, which may lead to the higher Ct value in the qRT-PCR assay compared to intact RNA.^{40,41} This correlated with the higher Ct values obtained for the silver NP-treated samples with lower RIN (Table S1, Supplementary data).

Statistical Analysis of Gene Stability in Nanoparticle Treated Cells

Ct values from all treatment conditions were analyzed together to identify the stable reference gene suitable for all treatment conditions. The genes were ranked for their stability based on the geNorm, NormFinder, BestKeeper analyses of the Ct values of genes at various times of NP treatment.

Each statistical tool predicted different genes as the top reference gene for the same experimental condition (Table S2-S4, Supplementary data). For example, *18SrRNA* was

Table 3 Comprehensive Stability Ranking of Genes Based on RefFinder Analysis at Various Time Points After NP Treatment of *H. perforatum* Cells

Stability Rank	Gold			Silver		
	0.5 h	4 h	12 h	0.5 h	4 h	12 h
1	<i>EF1-α</i>	<i>EF1-α</i>	<i>GAPDH</i>	<i>TUB-α</i>	<i>EF1-α</i>	<i>TUB-α</i>
2	<i>TUB-α</i>	<i>H2A</i>	<i>H2A</i>	<i>ACT7</i>	<i>TUB-α</i>	<i>GAPDH</i>
3	<i>ACT7</i>	<i>GAPDH</i>	<i>EF1-α</i>	<i>EF1-α</i>	<i>GAPDH</i>	<i>EF1-α</i>
4	<i>TUB-β</i>	<i>ACT2</i>	<i>ACT2</i>	<i>H2A</i>	<i>H2A</i>	<i>ACT7</i>
5	<i>H2A</i>	<i>ACT7</i>	<i>ACT7</i>	<i>ACT3</i>	<i>ACT2</i>	<i>ACT2</i>
6	<i>GAPDH</i>	<i>TUB-α</i>	<i>ACT3</i>	<i>GAPDH</i>	<i>ACT3</i>	<i>18SrRNA</i>
7	<i>ACT3</i>	<i>ACT3</i>	<i>TUB-β</i>	<i>TUB-β</i>	<i>18SrRNA</i>	<i>ACT3</i>
8	<i>ACT2</i>	<i>TUB-β</i>	<i>18SrRNA</i>	<i>ACT2</i>	<i>ACT7</i>	<i>H2A</i>
9	<i>18SrRNA</i>	<i>18SrRNA</i>	<i>TUB-α</i>	<i>18SrRNA</i>	<i>TUB-β</i>	<i>TUB-β</i>

the best reference gene for silver NP-treated cells in the BestKeeper analysis, while geNorm and NormFinder ranked *18SrRNA* as the least reliable gene. Similar differences in the ranking of reference genes between statistical algorithms were reported. Hence, geometrical mean of the results from different algorithms generally ranked by RefFinder was used to precisely select the reference genes.⁴² RefFinder analysis results revealed *TUB- α* , *EF1- α* , and *ACT7* as top three stable genes at 0.5 h treatment with gold and silver NPs (Table 3). After 4 h of treatment, *EF1- α* , *H2A*, and *GAPDH* were ranked as top three stable genes for gold NPs, with *EF1- α* , *TUB- α* , and *GAPDH* being the top three stable genes when silver NPs were used. At 12 h post-treatment, *GAPDH*, *H2A*, and *EF1- α* genes were the top three for gold NPs, and *TUB- α* , *GAPDH*, and *EF1- α* were the top three genes for silver NPs. Interestingly, except for *EF1- α* , none of the tested genes consistently occupied the top three positions for both NPs and their treatment durations.

Although *EF1- α* ranked in the top three positions for both NPs and for the treatment times, its position in the ranking was not consistent in all the variants. *EF1- α* catalyzes the binding of aminoacyl-tRNA to the A-site of the ribosome.^{43,44} The gene exhibits higher expression stability to heat and cold stresses in *Urochloa brizantha*,⁴⁴ cold and salt stress in *Caragana intermedia*,⁴⁵ and cold, heat, drought, and salt stresses in *Achyranthes bidentate*.⁴⁶ Similarly, *TUB- α* ranked in the top two positions across treatment periods for the silver NPs, while it ranked very low in the case of gold NP treatment. Nevertheless, *TUB- α* expression was stable across various abiotic stresses in plants.³⁸ All these findings clearly highlight the necessity

of standardizing reference genes for each parameter when NPs are used.

Validation of Gene Stability

HYP1 gene expression for both NPs and for all treatment durations was normalized with the Ct values of housekeeping genes to validate stability. The fold change in *HYP1* gene expression was approximately equal at any given time of NP treatment when its expression was normalized with the top-ranking housekeeping genes of the corresponding variant. However, when normalized according to the least ranking gene, a difference in the fold change was evident as early as 30 min, with further deviation as the treatment time increased. While normalization using *18SrRNA* for 4 h and 12 h of silver NP treatment indicated down-regulation of *HYP1* expression, normalization with other genes indicated upregulation of the gene for the same variants. Similarly, *TUB- α* showed a higher variation in its expression compared to other housekeeping genes at 12 h of gold NP treatment (Figure 7). Upregulation of *HYP1* was observed at 12 h after treatment with gold and silver NPs. However, no significant change in the expression level was observed at 0.5 h and 4 h.

All the above observations emphasize that the expression of the housekeeping genes also varies between NPs and their treatment durations. Nevertheless, qRT-PCR studies conducted to date with NP-treated plant samples have used reference genes without formal standardization or validation. The response of *A. thaliana* to treatment with silver NPs was studied using the *mitogen-activated protein kinase 6 (MPK6)* gene as the reference without validation.⁴⁷ In another study, *EF1- α* and *18SrRNA* genes

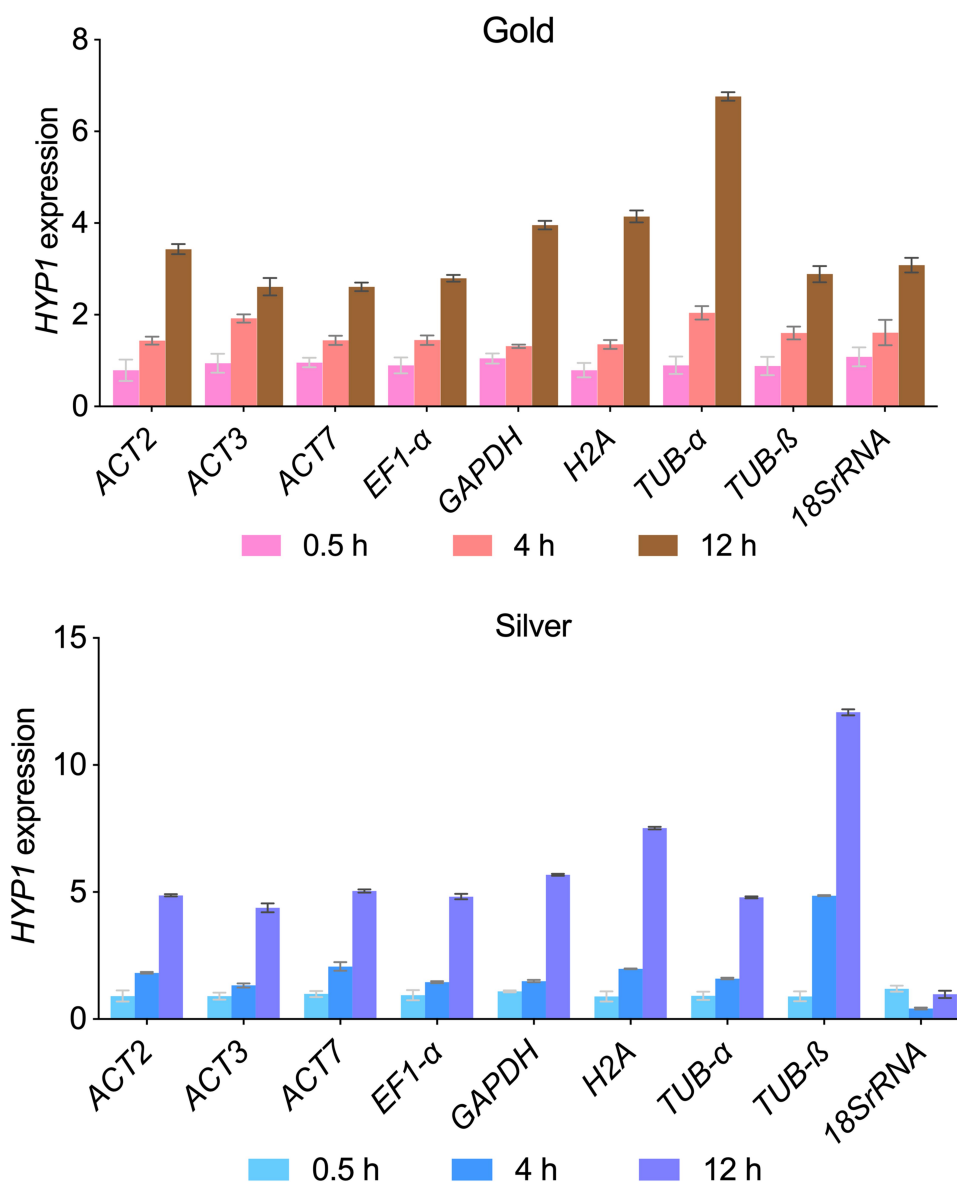


Figure 7 Gold and silver NPs induced *HYP1* gene expression in *H. perforatum* cells after normalization with the expression of housekeeping genes.

were selected as the references based on microarray data in the normalization of target gene expression in qRT-PCR analysis of *A. thaliana* treated with cerium oxide and titanium dioxide NPs.⁴⁸ The *ACT2* gene was used as the reference in the study of the response of *A. thaliana* to cerium and indium oxides,⁴⁹ zinc oxide,⁵⁰ and silver⁵¹ NPs without validation. However, the expression of the *ACT2* gene fluctuated drastically with time during treatment with NPs, even in the uniform cell suspension cultures that were maintained under tightly controlled conditions. Normalization of *HYP1* gene expression with the *ACT2* gene would lead to an erroneous conclusion.

Conclusions

To the best of our knowledge, the present study is the only systematic analysis of the stability of housekeeping genes in plant cells treated with NPs. Results of this study clearly show that the expression of tested housekeeping genes varies in cultures treated with the same NPs based on the treatment duration. The variation between the gold and silver NPs was pronounced. *EF1-α* ranked amongst the top three for both types of NPs and their treatment duration. Thus, the gene can be used as a reliable reference for the normalization of qRT-PCR in studies of gene expression in *H. perforatum* cells treated with gold and silver

NPs. Similarly, *TUB- α* ranked in the top two when silver NPs were used for the treatment. Thus, this gene can be used as a reference for silver NP treatment in *H. perforatum* cells. However, most of the other genes were not consistent in their expression with time for the same NP treatment, clearly emphasizing the need for a formal standardization of reference genes for every condition of NP treatment.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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