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Selection of appropriate reference genes for gene expression studies by quantitative real-time polymerase chain reaction in cucumber

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ABSTRACT

Quantitative real-time polymerase chain reaction (QRT-PCR) has become one of the most widely used methods for gene expression analysis. However, the expression profile of a target gene may be misinterpreted due to unstable expression of the reference genes under different experimental conditions. Thus, a systematic evaluation of these reference genes is necessary before experiments are performed. In this study, 10 putative reference genes were chosen for identifying expression stability using geNorm, NormFinder, and BestKeeper statistical algorithms in 12 different cucumber sample pools, including those from different plant tissues and from plants treated with hormones and abiotic stresses. *EF1α* and *UBI-ep* exhibited the most stable expression across all of the tested cucumber samples. In different tissues, in addition to expression of *EF1α* and *UBI-ep,* the expression of *TUA* was also stable and was considered as an appropriate reference gene. Evaluation of samples treated with different hormones revealed that *TUA* and *UBI-ep* were the most stably expressed genes. However, for abiotic stress treatments, only *EF1α* showed a relatively stable expression level. In conclusion, *TUA, UBI-ep,* and *EF1α* will be particularly helpful for reliable QRT-PCR data normalization in these types of samples. This study also provides guidelines for selecting different reference genes under different conditions.

Introduction

Gene expression analysis has become extremely important in many fields of biological research. Compared with conventional methods for transcript analysis, quantitative real-time polymerase chain reaction (QRT-PCR)[2] is a new quantitative nucleic acid technique and has become the most reliable method of choice for gene expression analysis [1]. QRT-PCR has many distinct characteristics, including a large dynamic range [2], tremendous sensitivity, high sequence specificity, no postamplification processing [3], and amenability to increased sample throughput [4]. Consequently, it is widely applied in a number of biological areas, including biotechnology, microbiology, the diagnosis of infectious disease, and human genetic testing [5].

However, when conducting gene expression analyses by QRT-PCR, many variables must be considered, including the amount of starting material, the quality of RNA, amplification efficiencies, and the selection of endogenous reference genes. Among these, the use of suitable reference genes for the normalization of gene expression is an elementary prerequisite for reliable results in any QRT-PCR analysis [6]. A suitable reference gene for QRT-PCR analysis can be defined as a gene (i) that is stably expressed among different analyzed samples and is unaffected by any experimental treatment; (ii) that is not associated with any pseudogenes so as to avoid genomic DNA amplification; (iii) whose amplification would reflect variations in RNA quality, quantity, and/or complementary DNA (cDNA) synthesis efficiency; (iv) whose stability should be equivalent to that of the target gene transcript(s) or whose impaired amplification should be accompanied by a corresponding reduction in the quantity of target gene transcript(s); and (v) whose expression should not be very low (threshold cycle [C_t] > 30) or very high (C_t < 15) [7].

Previously, a number of reference genes, such as β-actin (*ACTB*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), α- or β-tubulin, cyclophilin, ribosomal units (*18S* or *28S rRNA* [ribosomal RNA]), and ubiquitin (*UBQ*), have been widely used to normalize data of the measured gene expression in plants. However, recent studies have shown that genes commonly used as references might not be stably expressed under different experimental conditions [8]. Use of nonvalidated references could greatly affect the quantification of expression levels of a target gene. For example, a previous study had reported that up to 100-fold variation found in the expression of a target gene could actually be attributed only to variations in the expression of the reference genes. Consequently,

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[2] *Abbreviations used:* QRT-PCR, quantitative real-time polymerase chain reaction; cDNA, complementary DNA; C_t, cycle threshold; ACTB, β-actin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; rRNA, ribosomal RNA; UBQ, ubiquitin; SA, salicylic acid; MeJA, methyl jasmonic acid; ABA, absciscic acid; H_2O_2, hydrogen peroxide; MMLV, Maloney marine leukemia virus; T_m, melting temperature; SD, standard deviation; *r*, coefficient of correlation; CV, percentage covariance.

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there was huge potential scope for misinterpretation of the results [9]. Therefore, there is an urgent need to perform systematic validation of reference genes as an essential component of real-time reverse transcription–PCR analysis so as to improve the reliability of published results [10–12].

Recently, a number of statistical analysis methods have been proposed for evaluation of expression stability of reference genes and selection of the most suitable reference gene under different experimental conditions [10,13–16]. Several analytical programs can be downloaded free of charge (e.g., geNorm [http://medgen.ugent.be/~jvdesomp/genorm] [2], BestKeeper [http://www.gene-quantification.de/bestkeeper.html] [6], NormFinder [http://www.mdl.dk/publicationsnormfinder.htm]) [17].

Cucumber (*Cucumis sativus* L.) is one of the most important vegetables worldwide. With the complete genome sequence of cucumber now available, functional genomics, based heavily on gene expression analysis, will become the future research focus. Up to date, very few studies were carried out through QRT-PCR analysis in cucumber, and no article was found on study of appropriate reference gene. In the current study, we have evaluated a number of reference genes as potential candidates for use in normalizing QRT-PCR data from cucumber tissues and plants exposed to various stress or hormonal treatments.

Materials and methods

Plant material

5211S, an introgression line of *Cucumis hystrix*/*C. sativus* that is highly resistant to downy mildew, was studied. In the spring of 2009, seeds of 5211S were germinated and grown in growth chambers for 12 h light at 25 °C and 12 h dark at 18 °C. The relative humidity was kept at 65% to 75%.

Biotic stress treatments

For biotic stress, seedlings at the second true leaf stage were infected with *Pseudoperonospora cubensis* in a greenhouse. The second true leaf from each seedling was inoculated with a single drop (~0.01 ml) of inoculum containing 1.2×10^5 sporangia per milliliter. The seedlings were placed inside plastic boxes and incubated at 20 °C at approximately 100% relative humidity in darkness for 24 h and were then placed in a chamber (24–30 °C) with a 16-h photoperiod.

Abiotic stress treatments

For salt and drought stress treatments, seedlings at the second true leaf were transferred to 300 mM NaCl or 400 mM mannitol for 5 h.

For cold and heat shock treatments, the seedlings were kept at 4 ± 1 and 42 ± 1 °C, respectively, for 3 h. Seedlings kept in water for the same duration at 25 ± 1 °C served as the control.

Hormone treatments

For hormone treatments, seedlings at the second true leaf stage were sprayed with solutions of salicylic acid (SA, 100 μM), methyl jasmonic acid (MeJA, 100 μM), or absciscic acid (ABA, 100 μM) and were sampled 3 h later. For the hydrogen peroxide (H_2O_2) treatment, seedlings were sprayed with H_2O_2 (10 μM) in sterile water. Control plants were sprayed with sterile water only.

RNA isolation and cDNA preparation

Total RNA was isolated from the leaves, stems, and roots using TRIzol reagent (invitrogen) and was treated with DNase I (Promega) to remove any traces of genomic DNA according to the manufacturer's instructions. Successful removal of DNA contamination was confirmed by the presence of PCR amplification product using a primer pair (5′-GTCAAAATACTGGGAAGATC-3′ and 5′- TTTGAGGTAGG AAGTGTAGT-3′) designed to amplify an intron sequence of a gene encoding the HSP70 gene (GenBank accession no. EF208125, unpublished). The first-strand cDNA synthesis was performed using oligo$(dT)_{15}$ primers (Promega) and 200 U of Maloney marine leukemia virus (MMLV) reverse transcriptase (Promega) for 1 h at 42 °C. RNA extraction and cDNA synthesis from all of the different samples were performed for two biological replicates.

Primer design and QRT-PCR and data analysis

Ten pairs of specific primers for reference genes were designed using Beacon Designer 2.06 (Premier Biosoft International). Real-time PCR reactions were carried out in a total volume of 25 μl containing 12.5 μl of 2× SYBR Green PCR Master Mix (Applied Biosystems), 1 μl (10 pmol) of each primer, 25 ng of template (15× diluted cDNA from samples), and 9.5 μl of sterile distilled water. The thermal conditions for real-time PCR were 95 °C for 10 min (denaturation) followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. All reactions were performed in triplicate in 96-well reaction plates using the iQ5 machine (Bio-Rad). Quantification analysis was performed by the comparative C_T method, which mathematically (standard curve) transforms the threshold cycle into the relative expression level of genes (PerkinElmer User Bulletin). Primer efficiencies and standard deviations were calculated using qBase software (version 1.3.5) [18] on a standard curve generated using a 5-fold dilution series of one sample over at least six dilution points measured in triplicate. Relative expression levels of these genes were imported to BestKeeper (version 1) [6], geNorm (version 3.4) [12], and NormFinder [17] analysis tools, which were used as described in their respective manuals. Data for two biological replicates were assayed in triplicate for each reference gene.

Results and discussion

High variation in expression levels of cucumber putative reference genes under different experimental conditions

Based on SYBR Green detection, the QRT-PCR analysis method was used to evaluate the stability of expression of 10 putative reference genes in different experimental conditions (Table 1). Melting curve analysis of amplification products confirmed that, at the expected melting temperature (T_m), the primers amplified a single product (see supplementary material). For each assay, a standard curve was generated using 5-fold serial dilutions of pooled cDNAs (not shown). All of the PCR assays were run in triplicate to increase reliability of the results. The C_t value of each reference gene under different experimental conditions was used to compare the various degrees of expression. Fig. 1 shows a relatively wide range of C_t values for all 10 putative reference genes. The highest C_t value was 36.50 (*ACT1*), and the lowest was 15.61 (*18S rRNA*). Most of the remaining C_t values were distributed between 22 and 28. In addition, each individual reference gene had different C_t values in all of the applied conditions. These results clearly indicate that none of the 10 reference genes had a constant expression level. Therefore, it is critical to select a reliable reference gene(s) for cucumber gene expression analysis under the conditions to be tested.

Evaluation of expression stability of putative reference genes

Expression profiles for each reference gene were analyzed using three different methods with BestKeeper [6], geNorm [12], and NormFinder [17] software packages.

Table 1
Primer sequences of candidate reference genes for normalization, amplification length, and melting temperature of the amplified product in cucumber QRT-PCR assays.

Name	Accession number	Gene description	Forward primer (5′–3′)	Reverse primer (5′–3′)	Amplicon length (bp)	PCR efficiency (%)	T_m (°C)
ACT	AB010922	Actin	TTCTGGTGATGGTGTGAGTC	GGCAGTGGTGGTGAACATG	149	97.2	60
ACT1	DQ115881	Actin 1	GTGGTGGTGAATGAGTAGCC	TTGGATTCTGGTGATGGTGTC	150	100.1	62
ACT2	DQ115882	Actin 2	GAAGGAATAACCACGCTCAG	ACACAGTTCCCATCTACGAG	117	99.3	60
ACT3	DQ115883	Actin 3	GGCAGTGGTGGTGAACATG	TTCTGGTGATGGTGTGAGTC	149	96.7	60
18S rRNA	AF206894	18S Ribosomal RNA	TCTGCCCGTTGCTCTGATG	TCACCCGTCACCACCATAG	130	97.5	60
EF1α	EF446145	Elongation factor 1-α	ACTGTGCTGTCCTCATTATTG	AGGGTGAAAGCAAGAAGAGC	98	103.2	60
CYP	AY942800	Cyclophilin	CGTTGAGGGTATGAATGTGG	CCACAATCGGCAATGACAAC	88	96.8	60
TUA	AJ715498	α-Tubulin	ACGCTGTTGGTGGTGGTAC	GAGAGGGGTAAACAGTGAATC	106	97.1	60/62
UBI-1	AF104391	Ubiquitin-like protein	CCAAAGCACAAGCAAGAGAC	AGTAGGTTGTCTTATGGCGC	143	99.1	60
UBI-ep	AY372537	Ubiquitin extension protein	CACCAAGCCCAAGAAGATC	TAAACCTAATCACCACCAGC	220	97.6	58

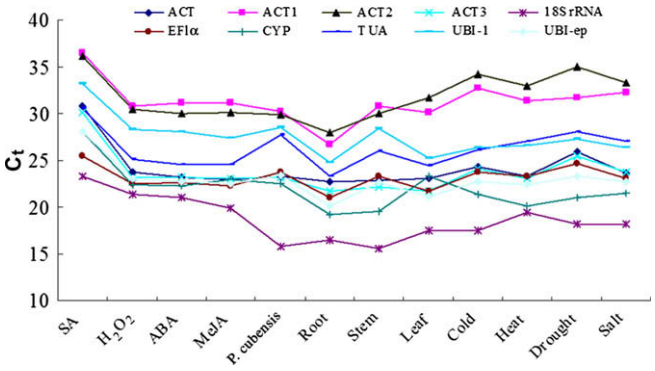

Fig. 1. RNA transcription levels of the 10 candidate reference genes tested in cucumber, presented as C_t mean values in all 12 sample pools. Each C_t value is the mean of three replicates.

geNorm analysis

geNorm software was used to rank the tested reference genes based on their expression stability value *M*. The most stable reference gene has the lowest *M* value, whereas the least stable one has the highest *M* value. The *M* value is the average pairwise variation of a particular gene with all other reference genes. Table 2 shows that the *M* values for the 10 putative reference genes under different experimental conditions were less than 1.5 [12], indicating that these genes have stable expression levels. However, the most stable reference gene was not identical among individual cucumber samples. For treatments with cold and heat stress, expression levels of the *ACT3* and *CYP* genes were the most stable, whereas *ACT2, ACT1,* and *ACT* had nearly constant expression stability. *ACT2* and *EF1α* exhibited the most stable expression levels in all 10 reference genes under salt and drought stress treatments. *TUA* and *UBI-ep* were the most stable reference genes under the growth hormone and H_2O_2 treatments and in different plant tissue samples. In addition, for all tested sample pools, *ACT* and *ACT3* ranked highly and may be useful for multiple experimental purposes. On the other hand, *18S rRNA* was the least stable among the reference genes examined.

Expression levels of these 10 genes are inconsistent under different experimental conditions. For example, the *CYP* gene was most stable under cold and heat stress treatments, whereas it was least stable in different plant tissues (Table 2). This stresses the importance of choosing an appropriate reference gene(s) before performing experiments so as to obtain reliable target gene results.

To determine the optimal number of reference genes in each experimental condition, pairwise variation (V_n/V_{n+1}) was calculated using geNorm. Vandesompele and coworkers [12] usually used 0.15 as a cutoff value to determine the optimal number of reference genes, below which the inclusion of additional reference genes is not required. However, 0.15 is not an absolute cutoff value but rather an ideal value. Whether 0.15 is used as a cutoff value will depend on the data. Fig. 2 shows the pairwise variation values for each experimental condition. Analysis of the pairwise variation in salt and drought treatments revealed that the $V_{2/3}$ value is 0.06 (significantly < 1.5), indicating that the two reference genes, *ACT2* and *EF1α*, would be sufficient for normalizing gene expression. Similarly, in cold- and heat-treated samples, *ACT3* and *CYP* were the optimal normalization factors for gene expression analysis. In addition, evaluation of different hormones and H_2O_2-treated samples and various tissues revealed that *TUA* and *UBI-ep* were the best reference genes and would be appropriate for normalizing gene expression data. A decrease in the pairwise variation was seen on the inclusion of a seventh gene. The $V_{6/7}$ value was 0.145, which is below the cutoff value of 0.15, indicating that at least six reference genes should be included for gene expression studies.

Table 2
Average expression stability values (*M*) of 10 candidate reference genes as calculated by geNorm.

Rank	Total		Cold/heat		Drought/salt		Different hormone		Different tissue	
	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability
1	*ACT*	0.483	*ACT3*	0.009	*ACT2*	0.020	*TUA*	0.140	*TUA*	0.029
1	*ACT3*	0.483	*CYP*	0.009	*EFlα*	0.020	*UBI-ep*	0.140	*UBI-ep*	0.029
2	*EFlα*	0.756	*ACT2*	0.014	*ACT3*	0.128	*ACT2*	0.152	*EFlα*	0.176
3	*UBI-ep*	0.793	*ACT1*	0.019	*TUA*	0.269	*EFlα*	0.203	*ACT3*	0.565
4	*ACT1*	0.898	*ACT*	0.055	*UBI-1*	0.310	*UBI-1*	0.256	*ACT*	0.733
5	*TUA*	0.965	*EFlα*	0.194	*UBI-ep*	0.382	*CYP*	0.330	*UBI-1*	0.880
6	*UBI-1*	1.037	*UBI-ep*	0.266	*18S rRNA*	0.520	*ACT1*	0.384	*ACT1*	0.990
7	*CYP*	1.165	*UBI-1*	0.392	*ACT1*	0.646	*ACT3*	0.557	*ACT2*	1.136
8	*ACT2*	1.271	*TUA*	0.552	*CYP*	0.704	*ACT*	0.672	*18S rRNA*	1.274
9	*18S rRNA*	1.442	*18S rRNA*	0.790	*ACT*	0.780	*18S rRNA*	0.860	*CYP*	1.455

Note. Higher *M* values indicate genes with low transcriptional stability, whereas lower *M* values indicate genes with high transcriptional stability.

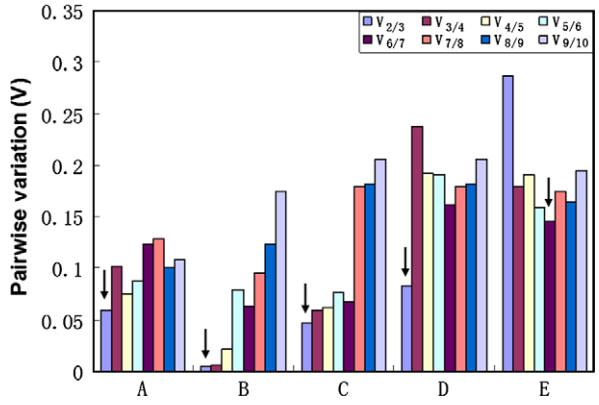

Fig. 2. Pairwise variation (V) analysis of the candidate reference genes. The pairwise variation (V_n/V_{n+1}) was analyzed between the normalization factors NF_n and NF_{n+1} by geNorm software to determine the optimal number of reference genes required for QRT-PCR data normalization in various sample pools: (A) under salt and drought treatments; (B) under cold and heat treatments; (C) under different hormone and H_2O_2 treatments; (D) in different tissues at the same developmental stage; (E) in all 12 tested sample pools. Arrows indicate the best-suited pairwise comparisons.

NormFinder analysis

NormFinder is another algorithm for identifying the optimal normalization gene among a set of candidate reference genes [17]. It ranks the set of candidate normalization genes according to their expression stability in a given sample set and given experimental design. This analysis method identified that *EF1α* was the most stable reference gene in cold- and heat-treated samples and in different tissues. In salt- and drought-treated and different hormone-treated sample pools, *TUA* had the most stable expression and was the ideal reference gene. For all tested samples, *UBI-ep* was the most appropriate for use as a reference gene (Table 3).

The results obtained by NormFinder for hormone treatments differed from those obtained by geNorm. In low- and high-temperature conditions, the geNorm and NormFinder programs showed opposite results for *ACT2*. These differences between the two methods were expected given that the programs are based on distinct statistical algorithms. The geNorm algorithm relies on the principle that the expression ratio of two ideal reference genes is constant in all of the samples, independent of the experimental conditions, whereas the NormFinder algorithm uses a solid statistical framework to estimate both the overall expression variation of the candidate reference genes and the variation between sample subgroups of the sample set. It provides a stability value for each gene that is a direct measure of the estimated expression variation.

BestKeeper analysis

BestKeeper evaluates gene expression stability for all individual reference genes based on three variables: standard deviation (SD), coefficient of correlation (r), and percentage covariance (CV) [6]. All reference genes are combined into an index (BestKeeper), and the correlation between each reference gene and the index is calculated based on individual C_t values as the geometric mean of a number of candidate reference genes. Reference genes with SD values greater than 1 are considered as inconsistent and should be excluded. Table 4 shows the results from BestKeeper analysis. *EF1α* had a high r value and low SD and CV values among all of the tested reference genes, indicating that it is the most suitable reference gene. Although *UBI-ep* was excluded according to the principle, it was still considered as a candidate reference gene. BestKeeper and NormFinder produced the same results. However, compared with geNorm, BestKeeper showed a weak difference (*EF1α* and *UBI-ep* ranked as second and third by geNorm, respectively), which may have been caused by the distinct statistical algorithms used by these two methods. geNorm detects the two reference genes

Table 3
The 10 candidate reference genes for normalization and their expression stability values in various sample pools calculated by NormFinder software.

Rank	Total		Cold/heat		Drought/salt		Different hormone		Different tissue	
	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability
1	*UBI-ep*	0.215	*EFlα*	0.018	*TUA*	0.023	*TUA*	0.050	*EFlα*	0.428
2	*EFlα*	0.332	*UBI-ep*	0.018	*UBI-1*	0.023	*UBI-ep*	0.050	*UBI-ep*	0.444
3	*ACT1*	0.455	*UBI-1*	0.230	*UBI-ep*	0.079	*ACT2*	0.052	*TUA*	0.463
4	*ACT3*	0.496	*ACT*	0.300	*EFlα*	0.299	*EFlα*	0.084	*ACT3*	0.523
5	*ACT*	0.534	*ACT1*	0.380	*ACT2*	0.318	*UBI-1*	0.131	*ACT*	0.528
6	*TUA*	0.742	*ACT3*	0.394	*18S rRNA*	0.453	*CYP*	0.285	*ACT1*	0.675
7	*UBI-1*	0.742	*CYP*	0.394	*ACT3*	0.459	*ACT1*	0.358	*ACT2*	0.706
8	*CYP*	0.898	*ACT2*	0.408	*ACT1*	0.680	*ACT3*	0.728	*18S rRNA*	0.984
9	*ACT2*	1.020	*TUA*	0.607	*CYP*	0.689	*ACT*	0.808	*UBI-1*	1.088
10	*18S rRNA*	1.333	*18S rRNA*	1.202	*ACT*	0.738	*18S rRNA*	1.096	*CYP*	1.409

Table 4
Descriptive statistics of stable reference gene expression in cucumber based on the BestKeeper approach [6].

Factor	Reference gene									
	ACT	*ACT1*	*ACT2*	*ACT3*	*18S rRNA*	*EFlα*	*CYP*	*TUA*	*UBI-1*	*UBI-ep*
n	12	12	12	12	12	12	12	12	12	12
GM[C_t]	24.06	31.21	31.70	23.63	18.56	23.29	21.91	26.15	27.47	22.84
AM[C_t]	24.15	31.28	31.79	23.72	18.70	23.36	22.02	26.22	27.55	22.90
Min[C_t]	22.74	26.68	27.95	21.71	15.61	21.01	19.20	23.34	24.75	20.10
Max[C_t]	30.84	36.50	36.19	30.07	23.35	25.51	28.03	30.52	33.17	28.10
SD[±C_t]	1.43	1.34	2.09	1.42	1.92	0.93	1.55	1.54	1.46	1.06
CV[%C_t]	5.91	4.30	6.58	5.98	10.28	5.15	7.04	5.89	5.29	4.61
r	0.936	0.935	0.785	0.967	0.718	0.977	0.827	0.842	0.860	0.788

Note: n, number of cucumber samples; GM[C_t], geometric mean of C_t value; AM[C_t], arithmetic mean of C_t value; Min[C_t] and Max[C_t], extreme values of C_t; SD[±C_t]:, standard deviation of C_t value; CV[%C_t], coefficient of variance expressed as percentage of C_t value; r, coefficient of correlation.

whose expression ratios show least variation from those of the other genes tested, whereas BestKeeper considers the least variation of a single reference gene.

In summary, a comparison of three methods—geNorm, NormFinder, and BestKeeper—suggests that *EF1α* and *UBI-ep* could be considered as suitable reference genes under all of the conditions tested in this study. In different tissues, *EF1α*, *UBI-ep*, and *TUA* are the most appropriate reference genes, and the same three genes show the most stable expression following different hormone and abiotic stress treatments.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2009.12.008.

References

[1] S.A. Bustin, Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays, J. Mol. Endocrinol. 25 (2000) 169–193.
[2] J.A. Garson, P.R. Grant, U. Ayliffe, R.B. Ferns, R.S. Tedder, Real-time PCR quantitation of hepatitis B virus DNA using automated sample preparation and murine cytomegalovirus internal control, J. Virol. Methods 126 (2005) 207–213.
[3] D.G. Ginzinger, Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream, Exp. Hematol. 30 (2002) 503–512.
[4] C.A. Heid, J. Stevens, K.J. Livak, P.M. Williams, Real time quantitative PCR, Genome Res. 6 (1996) 986–994.
[5] S.A. Bustin, S. Dorudi, Molecular assessment of tumour stage and disease recurrence using PCR-based assays, Mol. Med. Today 4 (1998) 389–396.
[6] M.W. Pfaffl, A. Tichopad, C. Prgomet, T.P. Neuvians, Determination of stable housekeeping genes, differentially regulated target genes, and sample integrity: BestKeeper–Excel-based tool using pair-wise correlations, Biotechnol. Lett. 26 (2004) 509–515.
[7] H. Lland, M. Hertzberg, P. Marlton, Myeloid leukenmia, in: S.P. Colgan (Ed.), Methods and Protocols, Humana, Totowa, NJ, 2006, p. 53.
[8] L. Gutierrez, M. Mauriat, S. Guenin, J. Pelloux, J.F. Lefevre, R. Louvet, C. Rusterucci, T. Moritz, F. Guerineau, C. Bellini, O. Van Wuytswinkel, 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 (2008) 609–618.
[9] L. Gutierrez, M. Mauriat, J. Pelloux, C. Bellini, O. Van Wuytswinkel, Towards a systematic validation of references in real-time RT-PCR, Plant Cell 20 (2008) 1734–1735.
[10] N. Nicot, J.F. Hausman, L. Hoffmann, D. Evers, Housekeeping gene selection for real time RT-PCR normalization in potato during biotic and abiotic stress, J. Exp. Bot. 56 (2005) 2907–2914.
[11] T. Suzuki, P.J. Higgins, D.R. Crawford, Control selection for RNA quantitation, BioTechniques 29 (2000) 332–333.
[12] J. Vandesompele, K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, F. Speleman, Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes, Genome Biol. 3 (2002) RESEACH0034.
[13] J. Ding, J. Jia, L. Yang, H. Wen, C. Zhang, W. Liu, D. Zhang, Validation of a rice specific gene, sucrose phosphate synthase, used as quantitative PCR detection of transgenes, J. Agric. Food Chem. 52 (2004) 3372–3377.
[14] M. Jain, A. Nijhawan, A.K. Tyagi, J.P. Khurana, Validation of housekeeping genes as internal control for studying gene expression in rice by quantitative real-time PCR, Biochem. Biophys. Res. Commun. 345 (2006) 646–651.
[15] B.R. Kim, H.Y. Nam, S.U. Kim, S.I. Kim, Y.J. Chang, Normalization of reverse transcription quantitative-PCR with housekeeping genes in rice, Biotechnol. Lett. 25 (2003) 1869–1872.
[16] T. Czechowski, M. Stitt, T. Altmann, M.K. Udvardi, W.R. Scheible, Genome-wide identification and testing of superior reference genes for transcript normalization in *Arabidopsis*, Plant Physiol. 139 (2005) 5–17.
[17] C.L. Andersen, J.L. Jensen, T.F. Orntoft, 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 (2004) 5245–5250.
[18] J. Hellemans, G. Mortier, A. De Paepe, F. Speleman, J. Vandesompele, QBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data, Genome Biol. 8 (2007) R19.