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Identification of suitable reference genes for studying gene expression in cucumber plants subjected to abiotic stress and growth regulators

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Abstract It has been shown that genes considered to be valid reference genes using semi-quantitative techniques (e.g. northern blot) appear to be less reliable when highly sensitive real-time PCR (qPCR) or microarrays are used. Therefore, the validation of expression stability of reference genes has become an important component of any study using such types of assay. No reference genes have been validated for expression studies of cucumber genes to date. Since the genome of this widely cultivated crop has been recently sequenced, the availability of suitable reference genes for expression analyses of the new cucumber genes is urgently required. For the purpose of normalization in studying expression of cucumber target genes, the stability of twelve reference genes in different cucumber tissues and under various stresses and growth regulators were determined in this study. These included commonly used cucumber reference genes, such as *actin*, *EF*, *cyclophilin*, *ubiquitin* and *tubulin* and the newly identified candidates for reference genes that encode clathrin adaptor complex subunit (CACS), F-box protein, PPA2 activator (tonoplast intrinsic protein, TIP41), mitosis protein (YSL8), protein phosphatase 2 (PDF2), helicase (HEL) and protein homolog of At4g33380. Analyses of quantitative real-time PCR data by three commonly used Excel-based applets, BestKeeper, geNorm and NormFinder, confirmed that expression stability of reference genes depends on the experimental parameters. In addition, they revealed that, except for *EF*, the most stable cucumber genes included mainly the new reference genes: *CACS*, *F-box* and *TIP41*, whereas the commonly used internal controls demonstrated various (*actin, cyclophilin, ubiquitin*) or much lower stability (*tubulin*). Hence, the authors of this study assume that the novel cucumber reference genes will enable better normalization and quantification of transcript levels in future expression studies on cucumber plants.

Keywords Real-time PCR · Reference gene · qPCR Normalization · Cucumber · Gene expression

Abbreviations

ABA	Abscisic acid
ACT	Actin
CACS	Clathrin adaptor complex subunit
cDNA	Complementary DNA
Cp	Crossing point
CYP	Cyclophilin
2,4-D	2,4-Dichlorophenoxy acetic acid

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DTT	Dithiothreitol
EF	Elongation factor
GA_3	Gibberelic acid
GSH	Gluthatione reduced
GSSH	Gluthatione oxidized
HEL	Helicase
IAA	Indole-3-acetic acid
NF	Normalization factor
PDF	Protein phosphatase 2
PEG	Polyethylene glycol
qPCR	Quantitative PCR
TIP41	PPA2 Activator (tonoplast intrinsic protein)
TUA	Tubulin
UBQ	Ubiquitin
VBA	Visual basic applet
YSL8	Mitosis protein

Introduction

The Cucurbitaceae family includes crops such as cucumbers, squashes, luffas, melons and watermelons, and is widely cultivated and distributed around the world. However, genomic resources for all cucurbits are still scarce, which makes this family less attractive for genetic study and molecular breeding. Cucumber (*Cucumis sativus* L., 2n = 2x = 14) with the lowest chromosome number in the genus *Cucumis* is one of the most cultivated vegetable crops in the world, so the extensive research on this species has always been beneficial for the agriculture industry (Koo et al. 2004). Biochemical, physiological and cytogenetic studies of this plant have been carried out for several decades (Bhaduri and Bose 1947; Burzyński et al. 2005; Chen et al. 1998; Janicka-Russak et al. 2008; Koo et al. 2002; Migocka and Klobus 2007; Ramachandran and Seshadri 1986), whereas the analysis of cucumber genes, gene transcription and expression has grown only slowly, since most of the genes remain unknown. Now that the entire cucumber genome has been sequenced (Huang et al. 2009) and the partial contigs have been already deposited in the NCBI database (http://www.ncbi.nlm.nih.gov), significant progress in research into the molecular biology of this species should be expected. However, no validation of suitable reference genes for cucumber gene expression analysis has been performed to date. An ideal reference gene, known as an internal control gene, should be expressed at a constant level across various conditions, such as developmental stages or tissue types, and its expression should be unaffected by experimental parameters (Schmittgen and Zakrajsek 2000; Thellin et al. 1999). Moreover, the reference gene and the target gene should have similar ranges of expression in the study samples (Cappeli et al. 2008). Common reference genes for normalization of qPCR data in various animal and plant tissues include *β-actin* (*ACTB*), *tubulin* (*TUA*), *cyclophilin* (*CYP2*), *elongation factor (EF)*, *RNA polymerase II* (*RP II*), *phospholipase A2* (*PLA2*), *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) and *18S* or *28S rRNAs* (Czechowski et al. 2005; de Jonge et al. 2007; Murphy et al. 2003; Timmons et al. 2005; Zhaoguo et al. 2009). A number of new candidate reference genes for *Arabidopsis* have been recently proposed (Czechowski et al. 2005) and their expression stability has been already evaluated under some experimental conditions (Czechowski et al. 2005; Remans et al. 2008). They include *AT5G15710* (F-box protein), *AT2G28390* (SAND family protein) and *AT5G08290* (mitosis protein YLS8) which proved to be highly stable during heavy metal stress.

A number of recently performed analyses have shown that in different experimental systems the expression of the commonly used reference genes *18S rRNA*, *ACTB, TUA* or *GAPDH* often remains unstable (Barber et al. 2005; Bas et al. 2004; Dheda et al. 2004; Jemiolo and Trappe 2004; Nicot et al. 2005; Remans et al. 2008; Yperman et al. 2004; Zhaoguo et al. 2009). As a consequence, the normalization of expression data obtained by employing more advanced methods of gene expression study, such as real-time PCR or microarrays, results in the subsequent misinterpretation of the results (Schmittgen and Zakrajsek 2000; Tricarico et al. 2002). It is now assumed that the correct reference genes should be selected by evaluating data from expression studies with the available statistical algorithms, such as geNorm (Vandesompele et al. 2002), Bestkeeper (Pfaffl et al. 2004) or Normfinder (Andersen et al. 2004). Nevertheless, most of the studies validating reference genes in different experimental settings concern human and animal tissues. In plants, suitable internal controls for gene expression studies have been identified for rice (Ding et al. 2004; Jain et al. 2006; Kim et al. 2003), *Arabidopsis thaliana*

(Czechowski et al. 2005), poplar (Brunner et al. 2004), potato (Nicot et al. 2005), grapevine (Reid et al. 2006), soybean (Jian et al. 2008; Libault et al. 2008), tomato (Exposito-Rodriguez et al. 2008), *Orobanche ramose* (González-Verdejo et al. 2008), coffee (Barsalobres-Cavallari et al. 2009) and peach (Tong et al. 2009).

The aim of this research was to evaluate the stability of twelve cucumber genes for the purpose of normalization in studying cucumber target gene expression. These included commonly used cucumber reference genes, such as *actin (ACT)*, *elongation factor* (*EF*), *cyclophilin (CYP)*, *ubiquitin (UBQ)* and *tubulin (TUA),* and the newly identified candidates for reference genes that encode clathrin adaptor complex subunit (CACS), F-box protein, tonoplast intrinsic protein (TIP41), mitosis protein (YSL8), protein phosphatase 2 (PDF2), helicase (HEL) and protein homolog of At4g33380. The last seven genes were identified by screening of the cucumber genome using the known *Arabidopsis* homologs that have been shown to be stably expressed in different experimental conditions (Czechowski et al. 2005). The stability and suitability of cucumber genes as reliable internal controls were assessed by three different statistical applets: geNorm (Vandesompele et al. 2002), NormFinder (Andersen et al. 2004) and Bestkeeper (Pfaffl et al. 2004). Material for the analysis was collected from a wide range of experimental series including different tissue types/state of development/treatments/time-course of treatments. Therefore, the results obtained in the study could be useful in many further expression analyses of cucumber target genes.

Materials and methods

Plant material

Plants were grown under a 16-h photoperiod (180 mmol m^{-2} s^{-1}) at 24°C during the day and 22°C during the night for 6–7 days or 2 weeks on standard nutrient solution (Migocka and Klobus 2007), pH 5.0 (with metals) or 6.0 (with other supplements). All growth solutions were filter-sterilized. For the assays including short-term metal treatment, 6 day-old plants were transferred on nutrient solutions supplemented with 20 μM metals (Cd, Pb, Cu, Zn, Mn, Ni) for 4, 12 or 36 h (Experiment 1). The solutions containing metals were exchanged every 12 h to ensure a steady metal concentration was accessible to plant roots. In the experiments with long-term metal stress, the germinated seeds were planted at once on nutrient solution containing metals and grown for 2 weeks (Experiment 2). During that time the solutions with metals were exchanged twice a week. In this experimental system, Pb was applied at 20 μM concentration while the level of other metals was decreased to 10 μM (Cd, Mn, Zn, Ni) or 5 μM (Cu), as those metals in higher concentrations considerably affected plant growth. For the assays including short-term treatment of plants with plant growth regulators or the agents inducing salt, osmotic or oxidative stress (Experiment 3), 7 day-old seedlings were transferred on the nutrient solutions containing NaCl (50 or 100 mM), mannitol (100 or 200 mM), PEG (5 or 10%), GSH, GSSH, DTT or H_2O_2 (all to the final 5 mM concentration), ascorbic acid (10 mM), ABA, IAA, 2,4-D, kinetin, salicylic acid or GA_3 (all to the final 5 μM concentration). For each treatment, four root samples, four leaf samples or four shoot samples of 100 mg from four different plants were taken for RNA extraction and immediately frozen in liquid nitrogen before storage at −80°C.

Total RNA isolation and cDNA synthesis

Frozen cucumber tissues were disrupted under frozen conditions using mortar and pestle. Approximately 50 mg of the ground powdered tissues were resuspended in 500 μl of cold Tri-Reagent (Sigma). The following total RNA extraction was performed according to the manufacturer's protocol. The RNA pellet was washed with ice-cold 75% ethanol, air-dried and dissolved in 30 μl of DEPC-water. To avoid any DNA contamination, samples were treated with RNAse-free DNAse I (Fermentas) at 37°C for 30 min. The reaction was stopped by addition of 2.5 mM EDTA to the samples immediately before 10 min incubation at 65°C. RNA concentration and purity was determined before and after DNA digestion using a NanoDrop spectrophotometer ND-1000 (Thermo Scientific) and the 260/280-nm ratio showed expected values between 1.8 and 2.0. The RNA integrity of randomly chosen samples was also confirmed by electrophoresis in denaturing formaldehyde agarose

gel. Before cDNA synthesis, RNA samples were also screened for genomic contamination by PCR using the primer pairs designed for real-time PCR analysis according to the following scheme: 2 min at 94°C, followed by 40 cycles of 30 s at 94°C, 30 s at 55°C and 1 min at 72°C, with final extension for 10 min at 72°C. Finally, 1 μg of total RNA was used for cDNA synthesis with 2.5 μM oligonucleotide dT primers using the First Strand Synthesis Kit (Applied Biosystem) according to the manufacturer's instructions.

Selection of cucumber reference genes and primer design

Twelve genes were selected for investigation to identify the most stable reference genes for qPCR expression analyses of target cucumber genes. These included the five genes already identified and commonly used as internal controls (*ACT*, *CYP*, *TUA*, *UBQ* and *EF*) as well as the newly identified homologs of *Arabidopsis thaliana* stable reference genes: *clathrin adaptor complex subunit* (*CACS; At5g46630*), *expressed protein At33380*, *TIP41*(*At54g34270*), *helicase* (*HEL; At1g58050*), *phospholipase 2* (*PDF2; Atg13320*), *mitosis protein* (*YSL8; At5g08290*) and *F-box* (*At5g15710*). The cucumber whole-genome shotgun reads were queried with *Arabidopsis* nucleotide sequences using BLASTn to select cucumber homologs. The selected cucumber sequences were then input into the programs identifying the full cDNA sequences (comprising the 5′ and 3′ ends): GeneMark (http://exon.gatech.edu/eukhmm.cgi) and FGENESH (http://www.softberry.ru/berry.phtml). The primer pairs for qPCR amplification of the newly identified expressed sequence tags as well as for the already identified cucumber reference genes were designed using Lightcycler Probe Design Software 2 (Roche). The primers were further employed in a standard RT-PCR reaction using the Titan One Step RT-PCR System (Roche) to check for size specificity of the amplicon size. The amplicons of seven newly identified reference genes were sequenced to confirm specificity of the PCR products.

Quantitative real-time PCR and data analysis

Real time PCR was performed in 20-μl capillaries (Roche) with a LightCycler 2.0 (Roche). Reaction mixtures contained 1 μl of cDNA, 5 μl of RealTime 2 × PCRMaster Mix SYBR® (A&A Biotechnology) and 1 μl of each of 10 μM primers in a total volume of 10 μl. Primer sequences for the reference genes are listed in Table 1. The following amplification conditions were applied: 30 s at 95°C; 45 cycles of 10 s at 95°C, 10 s at 55°C and 15 s at 72°C, with final melting for 15 s at 65°C. The general quality assessment of the PCR results was based on the amplification and melting curve profile of the samples in relation to the assay controls (non-template controls). Melting curve analysis was performed to confirm the specific amplicon and to identify putative unspecific PCR products (e.g. primer dimers, reaction mix contamination). Successive dilutions of the sample with the lowest crossing point (Cp) were used as a standard curve. Amplification efficiency was around 2. For each RNA extraction, measurements of gene expression were obtained in triplicate, and the mean of these values was used for further analysis. The data obtained were converted into correct input files, according to the requirements of the software, and analyzed using three different VBA (Visual Basic Applet) applets: geNorm (version 3.4), NormFinder (version 0.953) and BestKeeper (version 1.0).

Results

Expression profiles of reference genes

The raw expression data analyses obtained from the qPCR assay revealed differences between the twelve reference genes (Electronic Supplementary Material Table). Based on the Cp value, the traditional reference genes encoding EF, cyclophilin, actin and tubulin were expressed at the highest level, reaching threshold fluorescence after 13–19, 15–20, 16–21 or 17–21 cycles, respectively. The transcript of *CACS* was slightly less abundant with the Cp in the range of 18–22 cycles. In addition, it was observed that the Cp values for *GW881873* and *TIP41* were not significantly higher (20–23 cycles), whereas in the case of *F-box*, *YSL8* and *HEL*, these values reached 20–24 cycles. The genes with the least abundant transcripts included *UBQ* and *PDF2* with Cp values in the range of 22–26 cycles. The expression of each gene varied across all RNA samples studied, with higher

Table 1 Cucumber reference genes and their primer sequences

Accession number	Annotation	Forward primer	Reverse primer
AB010922	*Actin* (*ACT*)	CCGTTCTGTCCCTCTACGCTAGTG	GGAACTGCTCTTTGCACTCTCGAG
EF446145	*Elongation factor 1-alpha* (*EF1*)	ACTTTATCAAGAACATGATTAC	TTCCTTCACAATTTCATCG
AY942800	*Cyclophilin* (*M2*)	GGAAATGGTACAGGAGGTG	CATACCCTCAACGACTTGAC
AJ715498	*Alpha-tubulin* (*TUA*)	CATTCTCTCTTGGAACACACTGA	TCAAACTGGCAGTTAAAGATGAAA
AF104391	*Ubiquitin-like protein* (*UBI-1*)	CCTTATTGACCAACCAGTAGT	GGACAATGTTGATTTCCTCG
GW881874	*Clathrin adaptor complex subunit* (*CACS*)	TGGGAAGATTCTTATGAAGTGC	CTCGTCAAATTTACACATTGGT
GW881870	*F-box protein*	GGTTCATCTGGTGGTCTT	CTTTAAACGAACGGTCAGTCC
GW881871	*TIP41-like* (*PP2A phosphatase activator*)	CAACAGGTGATATTGGATTATGATTATAC	GCCAGCTCATCCTCATATAAG
GW881872	*YSL8* (*mitosis protein*)	CCTTGTGGATATCACAGAAGTT	CTTGTTTATCCTTGAGTGCC
GW881868	*Protein phosphatase 2* (*PDF 2*)	GTAGGACCTGAACCAACTA	CTTCACGCAGGGAAGA
GW881869	*Helicase*	TTCTCGAAGATTTAGTGATTCATGTG	CAATGGACGAATGCAAAGG
GW881873	*Expressed protein* (*At4g33380 homolog*)	GGGTCTAACTCATCATAAAGAAAGCG	CAGCATTCCATATGCTTATGTTCGT

expression variations observed for *TUA*, *ACT, EF* and *CYP* (5 cycles) and lower expression variations for the remaining genes (below 5 cycles). When samples of one experiment were considered, the expression variations were lower for all genes except *ACT*. The discrepancy in the expression of the twelve genes in different cucumber tissue samples implied that a selection of reliable reference genes to normalize gene expression under various experimental conditions should be the prerequisite. In this experiment, the relative expression values were used for each cDNA sample as inputs for the geNorm and the NormFinder algorithms. In addition, amplification efficiencies and Cp values obtained during expression measurements of reference genes were used for the BestKeeper applet.

geNorm analysis

For each reference gene, the geNorm algorithm calculated the gene expression stability measure *M* as the average pairwise variation *V* for that gene with the other tested reference genes. The lower value of *M* reflected more stable gene expression and was the basis for ranking the genes by expression stability. Vandesompele et al. (2002) recommend the use of at least three reference genes for robust normalization of expression stability. To evaluate the impact of differential treatment/organ combinations on the expression stability of the reference genes, the geNorm input was separated to consider only short-term metal treatment (Experiment 1), long-term metal treatment (Experiment 2) or short-term treatment of plants with plant growth regulators or agents inducing salt, osmotic or oxidative stress (Experiment 3). According to the results obtained for Experiment 1 (Fig. 1, top), the average expression stability values *M* for the traditional genes were significantly (*TUA*, *UBQ*, *CYP*) or slightly (*EF*, *ACT*) higher than for the novel cucumber reference genes identified for this assay. Thus, the expression of the novel reference genes was significantly more stable under short-term metal stress, with the three genes *TIP41*, *GW881873* and *CACS* ranked in the top positions. In addition, *CACS* and *GW881873* together with *F-box* also proved to be the most stable genes in Experiment 2 (Fig. 1, middle); however, the other novel reference genes, such as *HEL*, *YSL8* and *PDF,* were ranked among the five least stable genes during the 2 weeks-long heavy metal stress. In this experiment, the traditional reference gene, *EF,* was also stably expressed (fourth position according to geNorm),

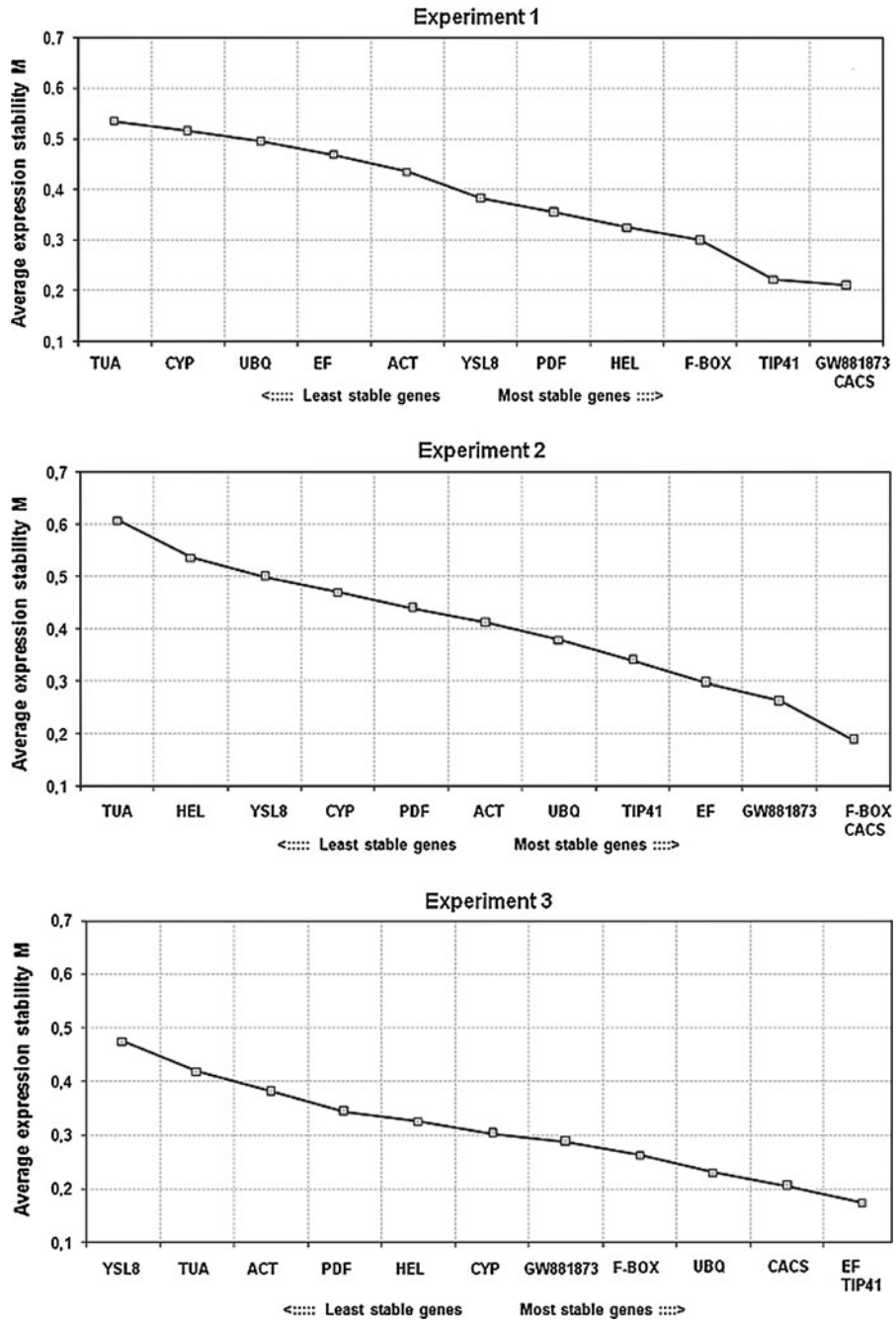

Fig. 1 Ranking of the candidate reference genes according to geNorm analysis. The *M* value reflecting gene expression stability was calculated separately for short-term metal treatment (*Experiment 1*) and long-term metal treatment (*Experiment 2*) as well as for treatment concerning salt, osmotic and oxidative stresses and plant growth regulators (*Experiment 3*)

while *TUA* displayed the lowest stability of all the tested genes. Additionally, *CACS* was again highly ranked in Experiment 3, though *EF* and *TIP41* were the most stable genes under different abiotic stresses and the growth regulators (Fig. 1, bottom). Another traditional reference gene, *UBQ*, also remained highly stable in this experiment, since it was ranked by the applet in the fourth position. On the contrary, *YSL8*, *TUA* and *ACT* were among the three least stable reference genes, demonstrating the highest *M* values. Based on the results obtained for the three different experimental series, it may be concluded that genes encoding CACS and TUA displayed the most and the least stable expression, respectively

(Fig. 1). Considering the whole geNorm curves, the *M* values of the reference genes were the most diverse in the experiment, including the samples from roots, shoots and leaves of plants treated with metals for 2 weeks. Hence, the expression of some reference genes seems to be less stable when comparing different tissues and organs or during the long-term stresses than during the short-term changes in the environment. It has been recently shown that the use of the geometric mean of several internal control genes as a normalization factor is more accurate than the use of a single reference gene. However, the inclusion of unstable genes might in turn negatively influence the normalization factor. The optimal number of control genes for normalization was determined with geNorm by means of the pairwise variations ($V_{n/n+1}$) between the sequential normalization factors (NF_n and NF_{n+1}) after successive inclusion of less stable reference genes (Fig. 2). According to Vandesompele et al. (2002), a variation of <0.15 indicates no significant contribution of an additional control gene to the normalization factor, which means that the optimal number of reference genes is achieved and the inclusion of an additional reference gene is not required. In all three experiments, $V_{2/3}$ was already below this cut-off, implying that the employment of the two best reference genes is likely to result in reliable gene expression normalization of target genes (Fig. 2).

NormFinder analysis

Similar to geNorm, the NormFinder program is another Visual Basic application tool for Microsoft Excel used for the determination of expression stability of reference genes. This program allows the estimation not only of the overall expression variation of the candidate genes but also of the variation between sample subgroups of one sample, e.g. roots, shoots and leaves. The results of the NormFinder analysis are presented in Table 2. Samples from three experiments were input separately and analyzed separately as one group or as three subgroups of one group. This separation resulted from a previous observation that the separation and definition of sample subgroups could have a significant effect on NormFinder's output (Tong et al. 2009).

The group of samples from short-term metal stress was divided into three subgroups according to the length of metal treatment: 4, 12 and 36 h. The seven subgroups of the samples separated according to the metal treatment (six metals plus control) were also analyzed but the output was not significantly affected (data not shown). The set of samples from long-term metal stress was divided into three subgroups based on the tissue type (roots, shoots and leaves). In addition, analysis of this set was performed including seven subgroups separated according to the type of metal. However, this separation did not influence the output, and therefore the data were not included in Table 2. Three subgroups in the third set of experiments were separated as follows: osmotic and salt stress (1), oxidative stress (2) and growth regulator treatment (3).

The NormFinder outputs with different sample subgroups and without any subgroups demonstrated common features. In the experiments concerning heavy metal stress, the best reference genes assigned by the applet included *CACS* ranked in the first position and *EF, TIP41, GW881873* and *F-box,* ranked among the top positions in different order depending on the time-course of the stress. On the contrary, *TUA* displayed the least stable expression under heavy metal stresses. More striking differences between experiments 1 and 2 were related to the middle positions of the ranking: *CYP* and *UBQ* were expressed the least stably during short-term metal stress while their stability during permanent metal stress was ranked higher. Similar to the geNorm analyses, the least stable genes in Experiment 1 included mostly traditional reference genes: *TUA*, *CYP* and *UBQ*. However, NormFinder assigned *EF* as the most stable reference gene during temporary metal treatment, whereas geNorm ranked it in one of the last positions (Fig. 1, top). In Experiment 2, the results obtained from both geNorm and NormFinder calculations were consistent, indicating *CACS*, *F-box* and *GW881873* as the best internal controls and *TUA*, *HEL* and *YSL8* as the least stable genes. The NormFinder analyses of the results from Experiment 3 were also in accordance with the geNorm output. As was shown by the two applets, under the short-term courses of growth regulator treatment and abiotic stresses other than heavy metal pollution, the most reliable genes included *EF*, *TIP41*, *F-box*, *CACS* and *UBQ*, whereas the expressions of *YSL8*, *TUA* and *ACT* were the least stable. As for the geNorm results, the NormFinder output indicated that

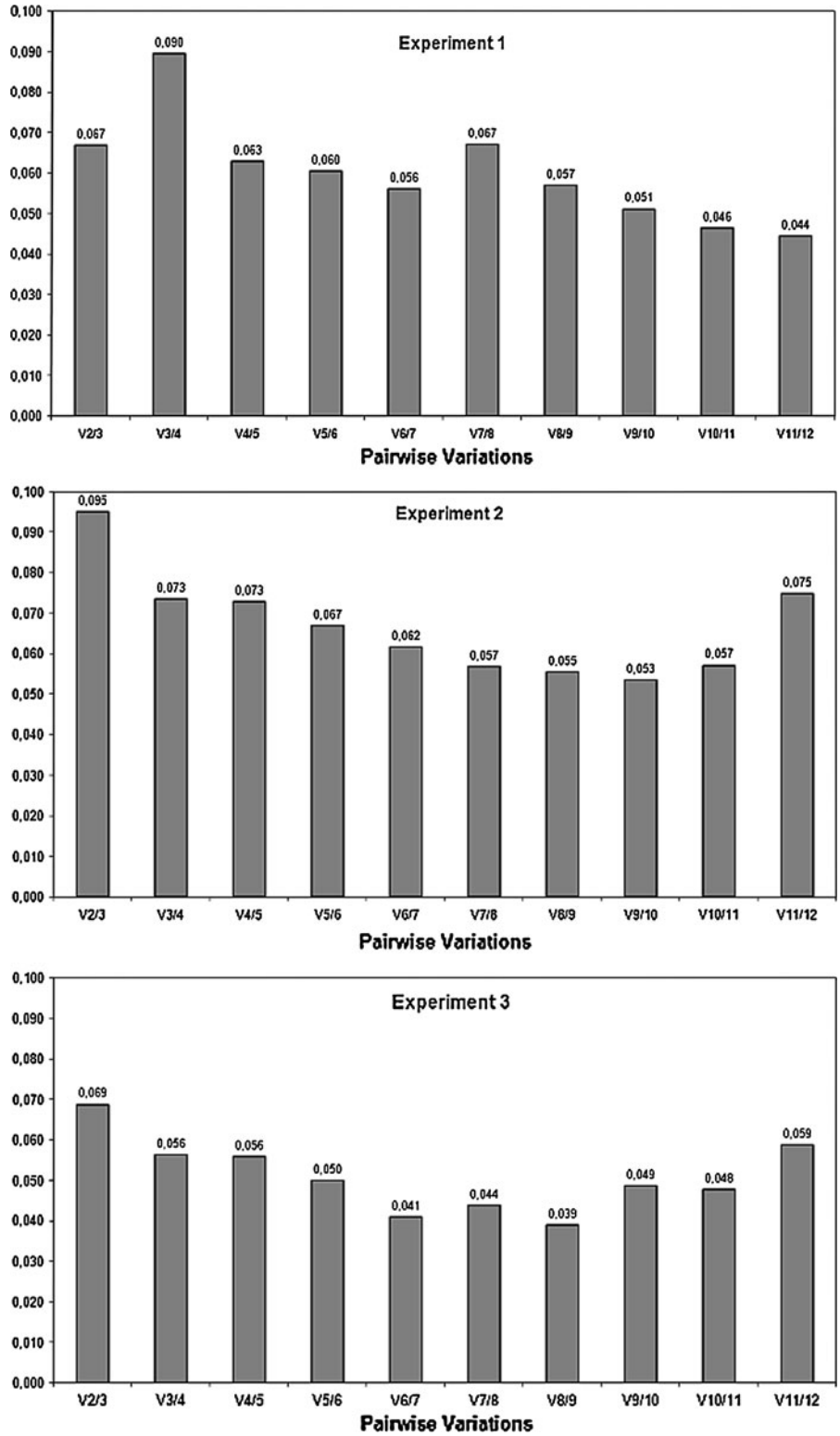

Fig. 2 Determination of the optimal number of control genes for normalization. A variation <0.15 indicates no significant contribution of an additional control gene to the normalization factor, and hence the use of two best reference genes should result in reliable gene expression normalization in all three experiments

Table 2 Ranking of candidate genes and their expression stability values calculated by NormFinder

Ranking order	Experiment 1[a]		Experiment 2[b]		Experiment 3[c]	
	No subgroups	3 subgroups	No subgroups	3 subgroups	No subgroups	3 subgroups
1	CACS	CACS	CACS	CACS	EF	EF
	0.116	0.063	0.086	0.068	0.078	0.050
2	EF	TIP41	F-box	F-box	TIP41	TIP41
	0.154	0.071	0.158	0.106	0.092	0.066
3	TIP41	EF	GW881873	GW881873	F-box	F-box
	0.161	0.072	0.182	0.157	0.135	0.092
4	GW881873	GW881873	TIP41	TIP41	CACS	CACS
	0.193	0.086	0.216	0.190	0.140	0.093
5	F-box	F-box	EF	Ubiquitin	Ubiquitin	Ubiquitin
	0.197	0.088	0.241	0.192	0.168	0.107
6	Helicase	Helicase	Ubiquitin	Actin	Helicase	Helicase
	0.217	0.090	0.243	0.197	0.171	0.109
7	YSL8	YSL8	Actin	EF	PDF	PDF
	0.220	0.094	0.262	0.229	0.223	0.143
8	Actin	Actin	Cyclophilin	Cyclophilin	GW881873	GW881873
	0.235	0.095	0.307	0.254	0.260	0.151
9	PDF	PDF	YSL8	Helicase	Cyclophilin	Cyclophilin
	0.245	0.097	0.354	0.273	0.360	0.155
10	Ubiquitin	Cyclophilin	PDF	PDF	Actin	Actin
	0.308	0.243	0.361	0.280	0.363	0.167
11	Cyclophilin	Ubiquitin	Helicase	YSL8	Tubulin	Tubulin
	0.318	0.280	0.430	0.287	0.377	0.188
12	Tubulin	Tubulin	Tubulin	Tubulin	YSL8	YSL8
	0.504	0.304	0.619	0.396	0.484	0.229

[a] Samples from roots of 7 day-old seedling subjected to 4, 12 or 36 h of metal treatment

[b] Samples from roots, shoots and leaves of 2 week-old seedling subjected to permanent metal treatment

[c] Samples from roots of 7 day-old seedling subjected to salt, osmotic or oxidative stress and plant growth regulator treatment

the novel cucumber reference genes might be suitable candidates for reliable internal controls useful for normalization assays. According to geNorm and NormFinder calculations, *CACS* should be the most reliable gene for the study of the expression of cucumber target genes in different tissues and under heavy metal stress, whereas *EF* showed remarkable stability of expression level under a broad range of various temporary treatments of cucumber plants.

BestKeeper analysis

Based on the results obtained in the geNorm and NormFinder analyses, we excluded the two most unstable genes in each experiment, namely *TUA* and *CYP*, *TUA* and *HEL* or *YSL8* and *TUA* in experiments 1, 2 and 3, respectively. The expression of the ten remaining reference genes was further analyzed using the Bestkeeper applet. The third Excel-based spreadsheet software application designed to test gene expression stability estimates inter-gene relationships of possible reference gene pairs by the analysis of the numerous pairwise correlations of raw Cp values of each gene. Moreover, since the previously presented geNorm and NormFinder software was restricted to reference gene expression analyses, the BestKeeper software could analyze the expression levels of the target genes together with the reference genes (Pfaffl et al. 2004). For each pairwise correlation, the Pearson correlation coefficient (r) and the probability

(*P*) value were calculated. Four different target genes were used for the evaluation of reference gene stability, using this applet. Similar to the previous analyses, the samples were analyzed as three separate inputs according to the category of experimental conditions.

The results are presented in Table 3. Reference genes used for the test demonstrated significant correlation with each other and strong correlation with the BestKeeper index ($r > 0.9$ in the vast majority of cases). The strongest inter-gene correlation was observed for *CACS* and *TIP41* in the short-term metal stress, *F-box* and *CACS* in the long-term metal stress and *TIP41* and *EF* during short-term treatment of plants with oxidative, osmotic or salt stress or growth regulators ($r > 0.93$). The highest Pearson correlation coefficient indicates that these pairs of genes showed the most similar expression

Table 3 The expression stability values for cucumber reference genes calculated by the BestKeeper applet

Experiment 1	UBQ	EF	ACT	GW881873	F-box	YSL8	TIP41	CACS	HEL	PDF
UBQ	0.888	–	–	–	–	–	–	–	–	–
EF	0.886	0.925	–	–	–	–	–	–	–	–
ACT	0.870	0.904	0.821	–	–	–	–	–	–	–
33380	0.852	0.893	0.921	0.858	–	–	–	–	–	–
F-box	0.552	0.594	0.570	0.734	0.622	–	–	–	–	–
TIP41	0.895	0.951	0.872	0.957	0.884	0.743	-	–	–	–
CLAT	0.932	0.946	0.906	0.959	0.869	0.616	0.950	–	–	–
HEL	0.860	0.891	0.916	0.883	0.965	0.634	0.905	0.899	–	–
PDF	0.901	0.962	0.908	0.938	0.859	0.730	0.972	0.964	0.879	–
BK	0.912	0.953	0.931	0.956	0.930	0.765	0.979	**0.980**	0.976	0.971
Experiment 2	CYP	UBQ	EF	ACT	GW881873	PDF	YSL8	TIP41	CACS	F-box
UBQ	0.898	–	–	–	–	–	–	–	–	–
EF	0.900	0.943	–	–	–	–	–	–	–	–
ACT	0.951	0.887	0.941	–	–	–	–	–	–	–
33380	0.856	0.903	0.966	0.908	–	–	–	–	–	–
F-box	0.714	0.753	0.819	0.792	0.906	–	–	–	–	–
TIP41	0.803	0.863	0.877	0.851	0.866	0.888	–	–	–	–
CLAT	0.896	0.945	0.953	0.928	0.927	0.852	0.937	–	–	–
HEL	0.913	0.936	0.975	0.959	0.968	0.843	0.861	0.958	–	–
PDF	0.928	0.950	0.979	0.956	0.952	0.824	0.889	0.977	0.983	-
BK	0.930	0.949	0.982	0.965	0.970	0.873	0.918	0.980	0.986	**0.990**
Experiment 3	CYP	UBQ	EF	ACT	GW881873	F-box	TIP41	CACS	HEL	PDF
UBQ	0.918	–	–	–	–	–	–	–	–	–
EF	0.882	0.928	–	–	–	–	–	–	–	–
ACT	0.754	0.784	0.876	–	–	–	–	–	–	–
33380	0.896	0.871	0.834	0.625	–	–	–	–	–	–
F-box	0.887	0.901	0.938	0.910	0.859	–	–	–	–	–
TIP41	0.885	0.950	0.973	0.834	0.880	0.945	–	–	–	–
CLAT	0.901	0.922	0.928	0.751	0.963	0.922	0.958	–	–	–
HEL	0.782	0.851	0.932	0.921	0.745	0.916	0.936	0.866	–	–
PDF	0.749	0.833	0.908	0.778	0.824	0.872	0.900	0.898	0.855	–
BK	0.920	0.949	0.980	0.884	0.897	0.974	**0.983**	0.964	0.939	0.915

The highest Person coefficient values representing the most stable genes are marked in bold

patterns in the conditions of our experiments. *CACS* demonstrated the strongest correlation with the BestKeeper index in Experiment 1 ($r = 0.98$), while *F-box* ($r = 0.99$) and *TIP41* ($r = 0.983$) were the most reliable reference genes in experiments 2 and 3, respectively. However, the differences in the Pearson correlation coefficients between the first and the second reference gene in the three rankings were minute. The Bestkeeper selection of the best reference genes was in agreement with the geNorm and NormFinder analyses. According to the calculations based on the raw Cp values in relation to Bestkeeper index, *YSL8* ($r = 0.765$), *PDF* ($r = 0.873$) and *ACT* ($r = 0.884$) were the least stable genes in experiments 1, 2 and 3, respectively. All three applets ranked *PDF* and *ACT* poorly in experiments 2 and 3, respectively, whereas *YSL8* held a higher position in the geNorm and NormFinder rankings than in the Bestkeeper calculations. Nevertheless, the three different statistics produced highly comparable results in selecting the most reliable cucumber reference gene for normalization of target gene expression in different tissues and under heavy metal stress (*CACS*) or during the exposure of plants to other environmental stresses or growth regulators (*EF*).

Discussion

The invention of quantitative PCR (qPCR) has significantly improved the detection and quantification of expression profiles of selected genes in distinct biological samples. The main advantages of this technique are high sensitivity, high specificity and broad quantification range (Bustin et al. 2005; Bustin and Nolan 2004; Gahon et al. 2004; Ginzinger 2002). However, in recent years it has been well proven that accurate data normalization is an absolute requirement for correct measurement of gene expression using the qPCR method (Andersen et al. 2004). Until recently, it has often been assumed that the best normalization was achieved using an internal reference gene, since this takes into account variation in the amount of starting material between samples caused, e.g., by sample-to-sample variations, variations in RNA integrity, RT efficiency differences and cDNA sample loading variations. Commonly used housekeeping genes, e.g. *GAPDH*, *albumin*, *actins*, *tubulins*, *cyclophilin*, *micro-globulins*, *18S rRNA* or *28S rRNA* seemed to be suitable, since they are present in all nucleated cell types, being necessary for basic cell survival. Hence, their expression has been considered to be stable in various tissues. Nevertheless, numerous treatments and studies have already shown that the expression of these genes might also be regulated and vary under experimental conditions (Thellin et al. 1999). Therefore, normalization with multiple reference genes is becoming the standard and the reports that identify such genes in plant research have been increasing in number now that algorithms measuring gene expression stability are available (Vandesompele et al. 2002; Andersen et al. 2004; Pfaffl et al. 2004). The present study reports the first validation of housekeeping genes in cucumber allowing the identification of the most suitable reference gene(s) for normalization of qPCR data. The detailed analysis included a broad spectrum of samples obtained from cucumber plants: the experiments considered the analyses of gene expression in different plant tissues (roots, shoots, leaves), under different external factors (stresses or growth regulators) and under different time-courses for the presence of external factors (metals).

Several approaches have been proposed to measure the stability of reference gene expression and it is assumed that a comparison of different algorithms allows better evaluation (Tong et al. 2009). Therefore, we used three statistical applets, geNorm, NormFinder and BestKeeper, to validate five cucumber reference genes in different experimental conditions. Since the number of potential candidate genes identified in cucumber has been limited to date, we have selected and identified seven novel reference genes based on the available data from the genome-wide study and testing of *Arabidopsis thaliana* reference genes (Czechowski et al. 2005). The novel cucumber reference genes included *clathrin adaptor complex subunit* (*CACS*), *homolog of At4g33380*, *RNA helicase*, *PDF2-like protein*, *YSL8-like mitosis protein*, *TIP41-like protein* and *F-box protein*. Their homologs in *A. thaliana* displayed a remarkably stable expression pattern, as calculated by geNorm software on a variety of samples including different developmental stages, organs, tissues, genotypes and various cultivation conditions (Czechowski et al. 2005). This report has clearly shown that among the 21 analyzed *Arabidopsis* reference genes, the majority of novel genes were expressed considerably more

stably than the traditional and commonly used *ACT*, *UBQ* or *EF*. In our research, the novel cucumber reference genes were also mostly ranked higher than traditional genes, except *EF* which was assigned as the best internal control in Experiment 3.

Comparing the outputs obtained from three different applets, it may be concluded that *CACS* should be the most reliable reference gene for studies on the effect of temporary and permanent heavy metal stress on target gene expression in cucumber roots, shoots and leaves. This gene also ranked very high in the geNorm analyses of *Arabidopsis* samples from different developmental series and different nutrient-related stresses (Czechowski et al. 2005). Thus *CACS* may be considered as a reliable internal control for organ and tissue expression analyses as well as during heavy metal stress or deprivation and/or readdition of different nutrients (N, C, P, S). Other suitable novel genes considered as reliable genes during heavy metal stress included cucumber *F-box* and *At4g33380 homolog*, as well as *TIP41-like protein*. Their *Arabidopsis* homologs ranked high in the complex analyses of Czechowski et al. (2005); although *TIP41* was ranked as the weakest candidate of ten reference genes tested by Remans et al. (2008) in samples from roots and leaves of plants treated with Cu and Cd. Due to problems with primers specific for the genes, Remans et al. (2008) could not verify the expression of *Arabidopsis At4g33380* and *CACS* under Cd and Cu stress. Comparing the expression of some novel and traditional (*ACT*, *EF*, *UBQ*) reference genes, Remans et al. (2008) selected *F-box*, *YSL8* and *SAND family protein* as the most stable genes when considering the effect of Cu and Cd treatment on *Arabidopsis* roots and leaves. In our analyses concerning heavy metal stress and different cucumber organs, the stability of *YSL8* expression was lower than the stability of some other novel reference genes. This might be a result of the differences in the range of samples that were used for the expression assay. These samples included not only roots and leaves but also shoots collected from plants and the treatment included six (Cd, Pb, Cu, Zn, Mn, Ni) and not two different heavy metals.

Interestingly, only the novel reference genes were among the best five reference genes assigned by the software through the analyses of samples from Experiment 1, whereas the analyses of samples from Experiment 2 revealed that of the five traditional cucumber reference genes tested, *EF* and *UBQ* could be considered as stable internal controls. Arabidopsis *EF1α* and *UBQ10* were also highly ranked in the Czechowski et al. (2005) analyses of 79 samples from different developmental series. Nutrient-related stresses significantly affected *EF1α* but not *UBQ* expression patterns (Czechowski et al. 2005). Thus, it is suggested that *EF* and *UBQ* should be used as internal controls in organ/tissue/developmental expression analyses rather than during various cultivation conditions.

The analyses of reference gene stability in the third experiment also revealed similarities in geNorm, NormFinder and BestKeeper outputs: all algorithms proved that *EF* and *TIP41* were the most reliable reference genes for the normalization of gene expression results obtained from studies using cucumber roots treated with various stresses (oxidative, osmotic or salt stress) and growth regulators. According to geNorm and NormFinder, *TUA* and *YSL8* were the least stable reference genes in Experiment 3, so they were excluded from further Bestkeeper analysis. On the other hand, Bestkeeper selected *GW881873* and *ACT* as the least reliable internal controls and *ACT* was also ranked poorly by geNorm and NormFinder.

Hence, the completion of the three analyses has shown that although all three softwares produced slightly different rankings of reference gene stability, the set of most reliable genes was similar in their outputs. Rankings for the three diverse experiments were different, particularly in the middle positions. The inconsistency between geNorm, NormFinder and BestKeeper results has already been observed and is generally expected since all three applets are based on distinct statistical algorithms (Cruz et al. 2009). It has been recently proposed that the geometric mean of several internal control genes as a normalization factor is more accurate than the use of a single reference gene. The rankings presented in this study provide a basis for the further selection of genes that could be combined together to normalize qPCR analysis of cucumber gene expression. The results also confirm that the study of reference gene expression stability should be frequently performed before the normalization of target gene expression data. In general, the analyses revealed that novel cucumber reference genes *CACS*, *TIP41*, *F-box protein*, *GW881873* and traditional *EF* were among the best

reference genes for accurate data normalization in the conditions of our experiments. In contrast, the traditional reference genes (*UBQ*, *CYP*, *TUA* and *ACT*) were ranked lower in the stability rankings. Previous data regarding traditional reference genes as internal controls are inconsistent. It was previously proved that *UBQ10* expression was unstable in different tissues at various developmental stages in rice, soybean and peach (Coker and Davies 2003; Jain et al. 2006; Tong et al. 2009). However, the gene displayed high stability in *Arabidopsis* throughout the development and under a wide range of environmental conditions including biotic and abiotic stress as well as growth regulators (Czechowski et al. 2005). *UBQ* was also assigned as a reliable internal control in studies on tomato subjected to nitrogen, cold and light stress (Løvdal and Lillo 2009). Some recently published reports have also proved that *ACT* might not fulfill the requirements of reference gene definition (Gutierrez et al. 2008; Nicot et al. 2005; Tong et al. 2009). This might be of great importance, since this gene is one of the most frequently used reference genes in studies on gene expression in many organisms, including plants. Among the tested cucumber reference genes, both *CYP* and *TBA* were ranked poorly as reliable internal controls when considering overall analyses. It had been previously proved that the expression of *CYP* varied in different tissue samples of peach, in tomato subjected to different biotic and abiotic stress, and in grapevine throughout berry development (Nicot et al. 2005; Reid et al. 2006; Tong et al. 2009). *CYP* mRNA synthesis was also shown to be variable at different developmental stages of maize and stimulated in maize and bean by mercuric chloride treatment and other abiotic stresses, including heat-shock, wounding, salt stress and low temperature (Marivet et al. 1992, 1995). In addition to this, different drugs significantly induced *CYP* transcription in human tissues (Pérez et al. 2003). Therefore, particular caution should be taken when *CYP* is considered as a reliable reference gene. A similar conclusion has been proposed for *TUA*, which displayed highly variable expression patterns in different peach tissues samples (Tong et al. 2009). On the other hand, the expression of *TUA* and *UBQ* remained quite stable in various poplar tissues collected at different developmental stages, and at different times of the year (Brunner et al. 2004).

Taken together, the data obtained during previous studies and our study confirmed the need for the evaluation of reference genes under different experimental conditions, proving that a reference gene that is stable in one experiment may not be a reliable internal control in other conditions. Our results also confirm the data from previous studies indicating that the novel reference genes should be selected and evaluated alongside the traditional reference genes, as they are very often more stable in various experimental conditions than the commonly used internal controls. This first evaluation of the expression stability of cucumber reference genes in a wide range of experimental samples should be useful in further molecular research in this species. Furthermore, the novel cucumber reference genes that displayed a remarkably stable expression pattern in our experimental samples can be successfully used for the accurate quantification of cucumber target gene expression using real-time PCR assay.

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References

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

Barber RD, Harmer DW, Coleman RA, Clark BJ (2005) GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues. Physiol Genomics 21:389–395

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

Bas A, Forsberg G, Hammarstrom S, Hammarstrom ML (2004) Utility of the housekeeping genes 18S rRNA, beta-actin and glyceraldehyde-3-phosphate-dehydrogenase for normalization in real-time quantitative reverse transcriptase-polymerase chain reaction analysis of gene expression in human T lymphocytes. Scand J Immunol 59:566–573

Bhaduri PN, Bose PC (1947) Cytogenetical investigations in some common cucurbits, with special reference to fragmentation of chromosomes as physical basis of speciation. J Genet 48:237–256

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

Burzyński M, Migocka M, Kłobus G (2005) Cu and Cd efflux from cucumber root plasma membranes. Plant Sci 168:1609–1614

Bustin SA, Nolan T (2004) Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. J Biomol Tech 15:155–166

Bustin SA, Benes V, Nolan T, Pfaffl MW (2005) Quantitative real-time RT-PCR–a perspective. J Mol Endocrinol 34:597–601

Cappelli K, Felicetti M, Capomaccio S, Spinsanti G, Silvestrelli M, Supplizi AV (2008) Exercise induced stress in horses: selection of the most stable reference genes for quantitative RT-PCR normalization. BMC Mol Biol 9:49

Chen JF, Staub JE, Jiang J (1998) A reevaluation of karyotype in cucumber (*Cucumis sativus* L.). Genet Resour Crop Evol 45:301–305

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

Cruz F, Kalaoun S, Nobile P, Colombo C, Almeida J, Barros LMG, Romano E, Grossi-de-Sá MF, Vaslin M, Alves-Ferreira M (2009) Evaluation of coffee reference genes for relative expression studies by quantitative real-time RT-PCR. Mol Breed 23:607–616

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

de Jonge HJ, Fehrmann RS, de Bont ES, Hofstra RM, Gerbens F, Kamps WA, de Vries EG, Zee AG, de Meerman GJ, der Elst A (2007) Evidence based selection of housekeeping genes. PLoS ONE 2:898

Dheda K, Huggett JF, Bustin SA, Johnson MA, Rook G, Zumla A (2004) Validation of housekeeping genes for normalizing RNA expression in real-time PCR. Biotechniques 37:112–119

Ding J, Jia J, Yang L, Wen H, Zhang C, Liu W, Zhang D (2004) Validation of a rice specific gene, sucrose phosphate synthase, used as the endogenous reference gene for qualitative and real-time quantitative PCR detection of transgenes. J Agric Food Chem 52:3372–3377

Exposito-Rodriguez M, Borges A, Borges-Perez A, Perez J (2008) Selection of internal control genes for quantitative real-time RT-PCR studies during tomato development process. BMC Plant Biol 8:131

Gahon C, Mingam A, Charrier B (2004) Real-time PCR: what relevance to plant studies? J Exp Bot 55:1445–1454

Ginzinger DG (2002) Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. Exp Hematol 30:503–512

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

Gutierrez L, Mauriat M, Guenin S, Pelloux J, Lefebvre JF, Louvet R, Rusterucci C, Moritz T, Guerineau F, Bellini C, van Wuytswinkel O (2008) The lack of a systematic validation of reference genes: a serious pitfall undervaluated in reverse transcription-polymerase chain reaction (RT-PCR) analysis in plants. Plant Biotechnol J 6(6):609–618

Huang S, Li R, Zhang Z, Li L, Gu X, Fan W, Lucas WJ, Wang X, Xie B, Ni P, Ren Y, Zhu H, Li J, Lin K, Jin W, Fei Z, Li G, Staub J, Kilian A, van der Vossen EAG, Wu Y, Guo J, He J, Jia Z, Ren Y, Tian G, Lu Y, Ruan J, Qian W, Wang M, Huang Q, Li B, Xuan Z, Cao J, Asan, Wu Z, Zhang J, Cai Q, Bai Y, Zhao B, Han Y, Li Y, Li X, Wang S, Shi Q, Liu S, Cho WK, Kim J-Y, Xu Y, Heller-Uszynska K, Miao H, Cheng Z, Zhang S, Wu J, Yang Y, Kang H, Li M, Liang H, Ren X, Shi Z, Wen M, Jian M, Yang H, Zhang G, Yang Z, Chen R, Liu S, Li J, Ma L, Liu H, Zhou Y, Zhao J, Fang X, Li G, Fang L, Li Y, Liu D, Zheng H, Zhang Y, Qin N, Li Z, Yang G, Yang S, Bolund L, Kristiansen K, Zheng H, Li S, Zhang X, Yang H, Wang J, Sun R, Zhang B, Jiang S, Wang J, Du Y, Li S (2009) The genome of the cucumber, *Cucumis sativus* L. Nat Genet 41:1275–1281

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

Janicka-Russak M, Kabała K, Burzyński M, Kłobus G (2008) Response of plasma membrane H^+-ATPase to heavy metal stress in *Cucumis sativu*s roots. J Exp Bot 59:3721–3728

Jemiolo B, Trappe S (2004) Single muscle fiber gene expression in human skeletal muscle: validation of internal control with exercise. Biochem Biophys Res Commun 320:1043–1050

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

Kim BR, Nam HY, Kim SU, Kim SI, Chang YJ (2003) Normalization of reverse transcription quantitative-PCR with housekeeping genes in rice. Biotechnol Lett 25: 1869–1872

Koo DH, Hur Y, Jin DC, Bang JW (2002) Karyotype analysis of a Korean cucumber cultivar (*Cucumis sativus* L. cv. Winter Long) using C-banding and bicolor fluorescence in situ hybridization. Mol Cell 13:413–418

Koo D-H, Choi H-W, Cho J, Hur Y, Bang J-W (2004) A high-resolution karyotype of cucumber (*Cucumis sativus* L. 'Winter Long') revealed by C-banding, pachytene analysis, and RAPD-aided fluorescence in situ hybridization. Genome 48:534–540

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

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

Marivet J, Frendo P, Burkard G (1992) Effects of abiotic stresses on cyclophilin gene expression in maize and bean and sequence analysis of bean cyclophilin cDNA. Plant Sci 84:171–178

Marivet J, Frendo P, Burkard G (1995) DNA sequence analysis of a cyclophilin gene from maize: developmental

expression and regulation by salicylic acid. Mol Gen Genet 247(2):222–228

Migocka M, Klobus G (2007) The properties of the Mn, Ni and Pb transport operating at plasma membranes of cucumber roots. Physiol Plant 129:578–587

Murphy RM, Watt KK, Cameron-Smith D, Gibbons CJ, Snow RJ (2003) Effects of creatine supplementation on housekeeping genes in human skeletal muscle using real-time RT-PCR. Physiol Genomics 12:163–174

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

Pérez G, Tabares B, Jover R, Gómez-Lechón M-J, Castell JV (2003) Semi-automatic quantitative RT-PCR to measure CYP induction by drugs in human hepatocytes. Toxicol In Vitro 17:643–649

Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP (2004) Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: Best-Keeper- Excel-based tool using pair-wise correlations. Biotechnol Lett 26:509–515

Ramachandran C, Seshadri VS (1986) Cytological analysis of the genome of cucumber (*Cucumis sativus* L.) and muskmelon (*Cucumis melo* L.). Z Pflanzenzüchtg 96:25–38

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

Remans T, Smeets K, Opdenakker K, Mathijsen D, Vangronsveld J, Cuypers A (2008) Normalization of real-time RT-PCR gene expression measurements in *Arabidopsis thaliana* exposed to increased metal concentrations. Planta 227:1343–1349

Schmittgen TD, Zakrajsek BA (2000) Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. J Biochem Biophys Methods 46:69–81

Thellin O, Zorzi W, Lakaye B, de Borman B, Coumans B, Hennen G, Grisar T, Igout A, Heinen E (1999) Housekeeping genes as internal standards: use and limits. J Biotechnol 75:291–295

Timmons JA, Jansson E, Fischer H, Gustafsson T, Greenhaff PL, Ridden J, Rachman J, Sundberg CJ (2005) Modulation of extracellular matrix genes reflects the magnitude of physiological adaptation to aerobic exercise training in humans. BMC Biol 3:19

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

Tricarico C, Pinzani P, Bianchi S, Paglierani M, Distante V, Pazzagli M, Bustin SA, Orlando C (2002) Quantitative real-time reverse transcription polymerase chain reaction: normalization to rRNA or single housekeeping genes is inappropriate for human tissue biopsies. Anal Biochem 309:293–300

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3:0034.1–0034.11

Yperman J, de Visscher G, Holvoet P, Flameng W (2004) Beta-actin cannot be used as a control for gene expression in ovine interstitial cells derived from heart valves. J Heart Valve Dis 13:848–853