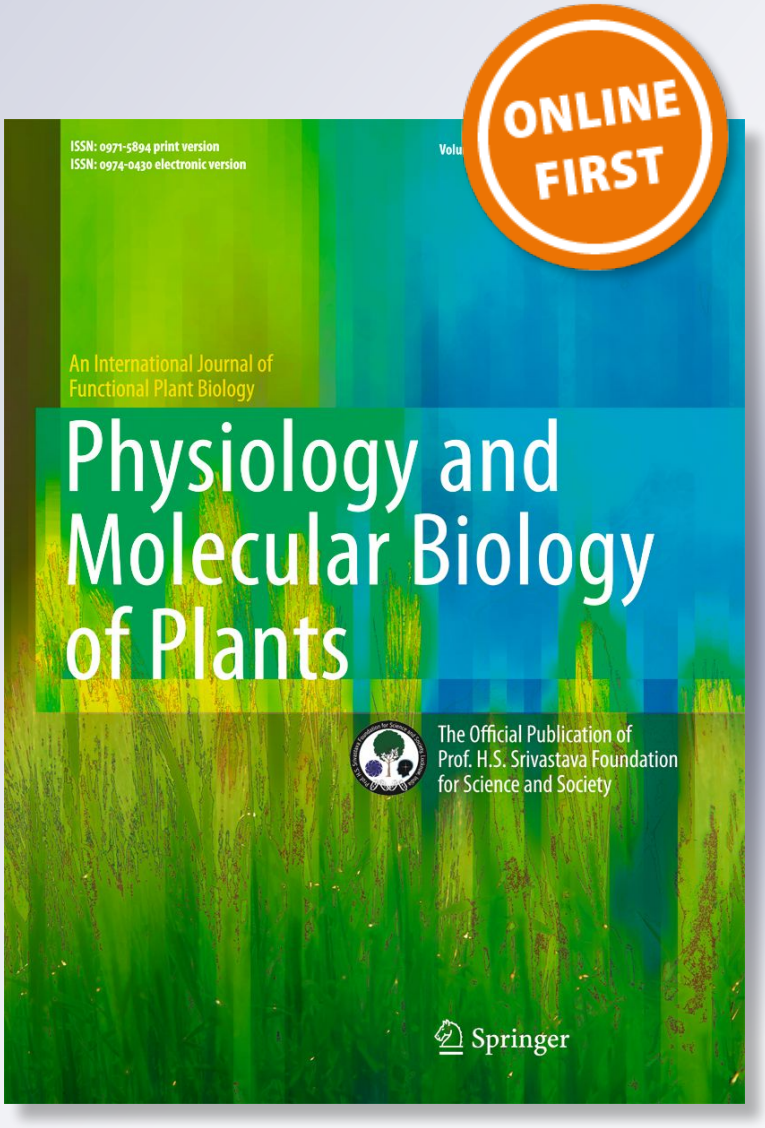
Selection of reference genes for reverse transcription-qPCR analysis in the biomonitor macrophyte Bidens laevis *L.*

Germán Lukaszewicz, María Valeria Amé & Mirta Luján Menone

Physiology and Molecular Biology of Plants
An International Journal of Functional Plant Biology

ISSN 0971-5894

Physiol Mol Biol Plants
DOI 10.1007/s12298-018-0534-3



Springer

Physiol Mol Biol Plants
https://doi.org/10.1007/s12298-018-0534-3

RESEARCH ARTICLE

Selection of reference genes for reverse transcription-qPCR analysis in the biomonitor macrophyte *Bidens laevis* L.

Germán Lukaszewicz[1,2] · María Valeria Amé[2] · Mirta Luján Menone[1,2]

Received: 6 November 2017 / Revised: 27 February 2018 / Accepted: 9 April 2018

Abstract The RT-qPCR has been the method used to analyze gene expression in plants but its benefits have not been completely exploited in the field of plants ecotoxicology when used as molecular biomarkers. The correct use of RT-qPCR demands to establish a certain number of reference genes (RG) which are expected to be invariable in their expression although it does not always happen. The main goals of this work were to: (1) analyze the stability of six potential RG, (2) establish the optimum number of RG, (3) select the most suitable RG to be applied in *Bidens laevis* under different test conditions and tissues and (4) confirm its convenience by normalizing the expression of one gene of interest under three different challenges. When all data were pooled together, the geNorm algorithm pointed out beta-actin and beta-tubulin (TUB) as the optimal RG pair while NormFinder algorithm selected nicotinamide adenine dinucleotide dehydrogenase (NADHD) and histone 3 (H3) as possessing the most invariable levels of expression. On the other hand, when data were grouped by tissues, ANOVA test selected H3 and TUB, while data grouped by conditions indicated that H3 and NADHD were the most stable RG under this analysis. Therefore, for a general-purpose set of RG, the overall analysis showed that a set of three RG would be optimum, and H3, TUB and NADHD were the selected ones. On the other hand, as RG can vary depending on the tissues or conditions, results achieved with ANOVA would be more reliable. Thus, appropriate normalization process would clearly need more than one RG.

Keywords Molecular biomarkers · RT-qPCR · Reference genes · Aquatic macrophyte · Ecotoxicology · Xenobiotics

Introduction

Most ecotoxicological studies make use of different species to determine the status of an environment by using a battery of biomarkers in order to assess the effect due to a given xenobiotic or condition on the environment. Not every species can be used as a model species to carry out this kind of studies. Living organisms selected as alternative test species should fulfill certain conditions concerning their availability, manipulation, short life cycles and most important, they should show sensitivity to xenobiotics and be representative of their environment (Ferrat et al. 2003; den Besten et al. 2007). The study of macrophytes is relevant to understand the effect that an environmental pollutant could have on the whole ecosystem because they play an important role in the aquatic environment acting not only as primary producers and source of food, but also as shelter and support for other organisms (Mohan and Hosetti 1998).

To the best of our knowledge, there are few aquatic plant model species that have been used in ecotoxicological studies, even fewer for analyzing biomarkers. Compared to animals, the subject of biomarkers in plants is

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12298-018-0534-3) contains supplementary material, which is available to authorized users.

✉ María Valeria Amé
vame@fcq.unc.edu.ar

[1] Instituto de Investigaciones Marinas y Costeras (IIMyC) UNMDP, CONICET, Mar del Plata, Argentina

[2] Dto. Bioquímica Clínica—CIBICI, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba - CONICET, Haya de la Torre esq. Medina Allende, 5000 Córdoba, Argentina

Published online: 26 June 2018

comparatively less explored with limited examples in the literature (Brain and Cedergreen 2009). In previous studies, Perez et al. (2011) proposed the use of *Bidens laevis* L. as a model species in genotoxicity studies, fulfilling the general conditions of a bioindicator. Moreover, other studies have shown its high sensitivity to different chemicals, including potent genotoxic compounds and environmental contaminants, measured by biochemical and genetic biomarkers (Pérez et al. 2011, 2014). To understand the underlying mechanisms of these responses, reverse transcription-real time polymerase chain reaction (RT-qPCR) would be the chosen method, due to its proved sensitivity and accuracy to determine the expression of biomarker genes involved. This method has been used several times in the last decade to analyze gene expression in molecular medicine, biotechnology, microbiology, diagnosis (Nolan et al. 2006) and, in plants, mainly in crops and species of economic concern. However, its benefits have not been completely exploited in the field of plant ecotoxicology.

The correct use of RT-qPCR demands to establish a certain number of Reference Genes (RG) that are ideally expected to be invariable in their expression and therefore correlate strongly with the total amount of mRNA present in a sample (Hruz et al. 2011). If RG are not properly selected, incorrect and unreliable results are very likely to be obtained. Hence, a suitable set of RG should be determined prior to any RT-qPCR analysis. In the past, most RG were developed mainly in yeast, animals and bacteria and were extrapolated to plant studies without proper validation (Gutierrez et al. 2008). Only recently, plant ecotoxicologists are making efforts to overcome this issue in order to improve qRT-PCR as a sensitive tool (Brulle et al. 2014; Lee et al. 2015; Peng et al. 2015; Chaâbene et al. 2017).

Therefore, taking this into account, the main goals of this work were to analyze the stability of six potential RG under different test conditions (control, plant size, xenobiotic exposure and temperature) and in different tissues (root, stem and leaf), establish the optimum number of RG, and normalize the expression of one GOI (BlGSTphi) under three different treatments using the chosen combination of RG.

Materials and methods

Plant material

Seeds of *B. laevis* were collected from La Brava lake (37°53′S, 57°59′W), Argentina. Seeds were sterilized in a 10% solution of commercial bleach (DEM Argentina, 5.5 g/L) for 10 min, rinsed several times in distilled water, and placed in Petri dishes with moist filter paper for imbibition. Germination was synchronized by scarifying the seeds in the same day. Seedlings were transferred to vermiculite-containing pots and grown for 60 days in a growing chamber until exposure. A photoperiod of 12 h light/12 h darkness and a room temperature of 22 °C were set up. Seedling tissues were carefully rinsed in distilled water before the exposure. Different plant sizes due to genotypes variability were addressed as small and large plants.

Exposure conditions

Individual plants were exposed in a 330 mL flask keeping the same photoperiod, light intensity and humidity setting with 3 replicates tested for each condition. Seedlings of *B. laevis* were exposed to four conditions: *1-control* Large plants (> 1500 mg), temperature 22 °C, media: Hoagland solution; *2-plant size* Small plants (< 250 mg), temperature 22 °C, media: Hoagland solution; *3-temperature* Large plants, temperature 5 °C, media: Hoagland solution; *4-xenobiotic exposure* Large plants, temperature 22 °C, media: Hoagland solution + endosulfan 10 µg/L. This concentration of endosulfan was selected, taking into account the sublethal effects that it exerts, such as genotoxicity and oxidative stress in *B. laevis* (Pérez et al. 2011, 2014). After 24 h, seedlings were dissected into three different tissues (root, stem and leaf), shocked in liquid nitrogen and stored at − 80 °C until further processing.

RNA extraction and cDNA synthesis

Frozen samples were ground to a fine powder in liquid nitrogen with a pestle and mortar. About 30 mg were resuspended in 900 µL RNA extraction solution and further extracted according to the acid guanidinium thiocyanate–phenol–chloroform method described by Chomczynski and Sacchi (1987). The obtained RNA pellet was resuspended in 100 µL nuclease-free water (Biodynamics S.R.L, Buenos Aires, Argentina). The purity of RNA was determined by measuring absorbance at 260 and 280 nm with a Take3 Micro-Volume Plate in a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Winooski, USA) and by running an electrophoretic 1% agarose gel. In this way, RNA was quantified, and its purity and integrity was confirmed. First strand cDNA was synthesized from 1 µg of total RNA using MMLV-Reverse Transcriptase (Invitrogen, Carlsbad, USA) and OligodT_{15} (Biodynamics S.R.L, Buenos Aires, Argentina) primers in a final reaction volume of 20 µL.

Primers selection and product identity

The expression of six candidate genes was analyzed: Elongation Factor 1-alfa (EF1a), Beta-Actin (BACT), Histone 3 (H3), Nicotinamide Adenine Dinucleotide Dehydrogenase (NADHD), Glyceraldehyde-3-phosphate Dehydrogenase (GADPH) and Beta-Tubulin (TUB).

All the genes analyzed in this work were previously tested and used as reference genes by Maroufi et al. (2010) and Maroniche et al. (2011). Selected primers were purchased from Sigma-Aldrich (Saint Louis, USA) and are described in Table 1 together with their sources. All the amplicons obtained in *B. laevis* by q-PCR with the previously mentioned primers were subsequently sequenced (Macrogen Inc., Korea) and analyzed with Basic Local Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi) in order to confirm their identity and similarity to the sequences obtained using these primers in the other species (Table 1).

q-PCR protocol

For mRNA relative expression levels assessment, real-time PCR was performed on a Bio-Rad iQ5 cycler (Bio-Rad, Hercules, USA) with 25 ng cDNA, QuantiTect SYBR Green PCR Master Mix (Qiagen, Foster City, USA) spiked with fluorescein 10 nM and 10 μM primer mix in a final volume of 20 μL. PCR cycling conditions consisted of: 1× 95 °C, 15 m; 45× [94.0 °C, 15 s, 61.2 °C, 30 s and 72.0 °C, 30 s].

Real-time PCR reactions were run in duplicate for each cDNA sample. The occurrence of a specific and sole product for each set of primers was tested by running a melting curve from 52 to 96 °C and a 2% agarose electrophoresis ethidium bromide-stained gel for each q-PCR reaction. A 50 bp ladder DNA (Productos Bio-lógicos, Bernal, Argentina) was used to estimate and verify the amplicon length.

Amplification products were quantified by comparison of experimental Ct (threshold cycle, defined as the PCR cycle where an increase in fluorescence over background levels first occurred) number. Consequently, the greater the quantity of target cDNA in the starting material, the faster a significant increase in fluorescent signal, yielding a lower Ct. Therefore, RG expression, number and selection were established through the Ct values from the qPCR reactions.

Data analysis

PCR efficiency calculation

It is mandatory to calculate the efficiency of the PCR reaction in order to determine the presence of inhibitors or contaminants in the samples. The method designed by

Table 1 Primer sequences of the genes used their corresponding annealing temperature and product size and qPCR efficiencies

Gene name	Sequence	Reference	Tm (°C)	Amplicon size (bp)	E value	qPCR efficiency (%)
NADHD	F: 5′-TGCAGCAAAGGCTTGTCAAA-3′	Maroufi et al. (2010)	66.8	102	2e−21	77.60
	R: 5′-TCGAAACTTCCCGTTATCCAA-3′		65.3			
H3	F: 5′-ACAGCTCGCAAATCAACCG-3′	Maroufi et al. 2010	66.3	100	3e−20	95.48
	R: 5′-GCGGCTTCTTCACTCCACC-3′		67.3			
EF-1a	F: 5′-TTGAGGCTGGTATCTCGAAGAAC-3′	Maroniche et al. 2011	56.4	111	8e−46	89.70
	R: 5′-GCTCGGTGGAGTCCATCTTG-3′		57.9			
BACT	F: 5′-AAAGCCAACAGGGAGAAGATGAC-3′	JX843817[a]	60.6	85	–	86.96
	R: 5′-GCCTGGATGGCAACGTACA-3′		60.0			
GADPH	F: 5′-AGGGCGGTGCTAAGAAAGTCA-3′	Maroufi et al. 2010	66.9	91	n.a.	86.63
	R: 5′-TCTGGCTTGTATTCCTTCTCATT-3′		62.7			
TUB	F: 5′-GCACGGCATTGATGTGACC-3′	Maroufi et al. 2010	67.8	101	4e−7	97.31
	R: 5′-GAACAAACCTCCCGCCACT-3′		66.3			
GSTF4	F: 5′-CCCGATCTCTCACTCTCTCG-3′	Cummins et al. (2003)	61.4	193	0.01	92.1
	R: 5′-GAGATGCTCAGGGCTCTTGT-3′		59.4			

NADHD, nicotinamide adenine dinucleotide dehydrogenase; H3, histone 3; EF-1a, elongation factor 1 alpha; BACT, beta-actin; GADPH, glyceraldehyde-3-phosphate dehydrogenase; TUB, beta-tubulin; GSTF4, glutathione S-transferase phi; n.a., not analyzed

[a]Genbank accession number of the sequence in *Bidens laevis*

Zhao and Fernald, which is free and available online (http://www.miner.ewindup.info), was used to calculate PCR efficiency from the raw fluorescence data. Although the most common approach is to design a standard curve with serial dilutions of a plasmid or pooled samples of cDNA, it is time-consuming and requires production of repeatable and reliable standards (Pfaffl 2001 in Zhao and Fernald 2005) which are not easily achieved. The method chosen in this work is independent of any assumptions or subjective judgments and therefore, more objective than the traditional ones.

Software description

Different available computational programs have been specifically designed in order to evaluate the stability of a set of candidate RG under different conditions and to determine the optimum number of RG to be used. As suggested by Jacob et al. (2013), more than two algorithms should be applied for the selection of RG. In this study, two software [geNorm (Vandesompele et al. 2002) and NormFinder (Andersen et al. 2004)] included in the Genex package (Multid Analyses AB) and the ANOVA calculations were used to analyze the gene stability. In addition, to establish the optimal number of RG, a specific function of NormFinder was used. Finally, once the stability and the optimal number of RG were defined, we were able to establish a general set of recommended RG as well as a specific set for each tissue or condition assessed.

The raw Ct values for each candidate RG were corrected by PCR efficiency (E), and then transformed into relative expression ratios (R) using the formula $R = E^{-Ct\ sample}/E^{Ct\ calibrator}$, where the calibrator is the sample with the lowest Ct value (higher expression) within samples of the corresponding candidate RG. This step was performed for the three methods used to examine the candidate RG.

geNorm

GeNorm is an algorithm that selects an optimal pair of reference genes from a great variety of candidate genes. For every control gene tested, the pairwise variation with all other control genes was determined as the standard deviation of the logarithmically transformed expression ratios, and the internal control gene-stability measure (M-value) was defined as the average pairwise variation of a particular gene with respect to all other control genes. Afterwards, a stepwise elimination of the genes with the highest M-value (least stable) results in the two most stably-expressed genes among those tested (Vandesompele et al. 2002). This model assumes that each candidate gene is not co-regulated, which means that there wouldn't be two genes varying their expression in a similar way under a given treatment, although this cannot be verified by this software. The formulas on which this software is based are further described in Vandesompele et al. (2002).

NormFinder

NormFinder is an algorithm that determines an optimum reference gene (or more than one) within a group of candidates. Unlike geNorm, it considers the variation from grouped samples (e.g. 1-Control/2-Plant size/3-Temperature/4-Xenobiotic exposure, or 1-Root/2-Stem/3-Leaf). It estimates both intergroup and intragroup variation through a model-based approximation and, by combining these sources of error, an individual stability value is provided (Andersen et al. 2004), using analysis of variance (ANOVA) on logarithmically transformed expression values (Mehdi Khanlou and Van Bockstaele 2012). The statistical formulas are described in the original paper by Andersen et al. (2004). This software can also determine the optimum number of RG by calculating the accumulated standard deviation when more than one reference gene is added.

ANOVA based stability index

The algorithms previously described assume that two candidate RG do not vary their expression profile systematically in the same way across all the samples considered. However, most of the publications failed to comply with this assumption in practice. Mehdi Khanlou and Van Bockstaele (2012) suggested the use of a stability index based on the analysis of variance (ANOVA) model which is free from the assumption made by the algorithms. Therefore, we included the use of ANOVA test as described by these authors, which is not included in any software package. Data of the relative expression ratios (obtained as previously described) were transformed using a natural log function to obtain X value, and followed by a one-way ANOVA. Intragroup variance (among tissues or conditions) was represented by the mean square errors while intergroup variance was represented by mean square of groups. Then, both variances were divided by $-1 = |X$, where $|X$ is the average of X, to obtain the intergroup variation index (V_B) and intragroup variation index (V_W) of each RG. Finally, V_B and V_W were multiplied to obtain a stability index for each gene.

Glutathione S-transferase expression

In order to confirm the suitability of the chosen RG, we measured the expression of one Glutathione S-transferase enzyme (BlGSTphi) in roots of *B. laevis* using the most convenient combinations of RG obtained by the use of the

three methods. This Glutathione S-transferase phi is a very abundant enzyme associated with the detoxification of several xenobiotics and selective herbicides (Jain et al. 2010). To achieve this aim, plants of *B. laevis* (n = 3) were exposed to three xenobiotics which are well known GST expression regulators: CDNB (10 mg/L) (Choi et al. 2013), 2,4-D (5 mg/L) (Flury et al. 1995; Watahiki et al. 1995; Coskun and Zihnioglu 2002) and metolachlor (567 mg/L) (DeRidder et al. 2002; Mezzari et al. 2005) for 24 h. The exposure and PCR conditions were the same as mentioned above for RG validation.

Results

RT-qPCR specificity, efficiency and expression profile

Table 1 shows the candidate reference genes tested as well as the E-values calculated by comparing the sequences obtained for *B. laevis* in the qPCR to those from the available databases. Since BACT has been partially sequenced for *B. laevis* (GenBank nucleotide sequence database accession number: JX843817), this calculation was not necessary for this gene. The qPCR efficiency varied from 77.60 to 97.31%.

All the candidate genes had the expected amplicon size. However, the identity of GADPH could not be confirmed by its sequence, and consequently this gene was excluded from any further analysis. In all the other cases, similarity to the original sequences was confirmed (Table 1). Melting curves showed a single peak corresponding to a single amplification product for every candidate gene (Fig. 1). Thirty-six individual samples per gene were analyzed, corresponding to three replicates of the three tissues and four conditions tested. In all cases, two technical duplicates were run for each sample, giving a total of 72 qPCR reactions. For a better visualization, ten of them are shown in Fig. 1. Agarose gel electrophoresis also confirmed a sole PCR product for each pair of primers (data not shown).

Figure 2 shows the average Ct values as indicators of the expression of each candidate gene. Most of the candidate RG showed an average expression level ranging 24–32 Ct.

Software analysis

geNorm

geNorm calculations pointed out BACT and TUB as the optimal RG pair when all data were pooled together, getting an M-value of 1.35 (Fig. 3), while EF-1a resulted as the least stable RG.

NormFinder

Firstly, in order to obtain a general-purpose set of RG, the grouping of data according to different tissues or conditions has not been taken into consideration. The most stable candidate gene was NADHD with the lowest Standard Deviation (SD) of 0.64, followed by H3 and BACT (Fig. 4a, b). However, the accumulated SD slightly decreases when considering the first four genes of the ranking, and increases again when adding the fifth one (Fig. 4).

Secondly, the analysis was performed grouping the samples according to different tissues or conditions, deriving in coincident results with respect to general-purpose set of RG, which are shown below.

Grouping by tissues The gene expression variability was analyzed in three different tissues (group 1: Root, group 2: Stem, group 3: Leaf). The most stable gene obtained was NADHD (SD 0.1971), and the addition of the second most stable gene (H3) decreased the accumulated SD to 0.1813 (data provided by Normfinder function in Genex software).

Inter- and intra-group variation explains the total variation of a set of samples. Intergroup variation is a measure of variability among different groups, which shows the deviation of each group from the average, being NADHD, the gene with the highest intergroup variation. This bias represented in Table S1, showed that each RG has a particular variation depending on the tissue analyzed. In this sense, NADHD in root showed a deviation value of 0.68 while in leaf it is only 0.19. On the other hand, EF-1a presented a value close to the average (0.01) in root while in the other two tissues, values were more distant (0.34 and − 0.36).

Intragroup variation is a measure of the SD within each group. In this case, contrary to the results observed in the intergroup variation, NADHD showed the lowest values within each tissue whereas the most variable gene turned out to be EF-1a (Table S1), particularly in root and leaf. Similar to NADHD, the other RG analyzed showed low values, ranging 0.21–3.4 in contrast with EF-1a that reached values as high as 14.05.

Grouping by conditions

The gene expression variability was analyzed under four conditions (group 1: Control, group 2: Small plants, group 3: Endosulfan, group 4: Cold). The candidate gene with the lowest SD was NADHD (0.72), but in this case, the addition of a second candidate gene (H3) did not decrease the accumulated SD as expected, instead, it increased this value up to 0.92 (data provided by Normfinder function in Genex software).

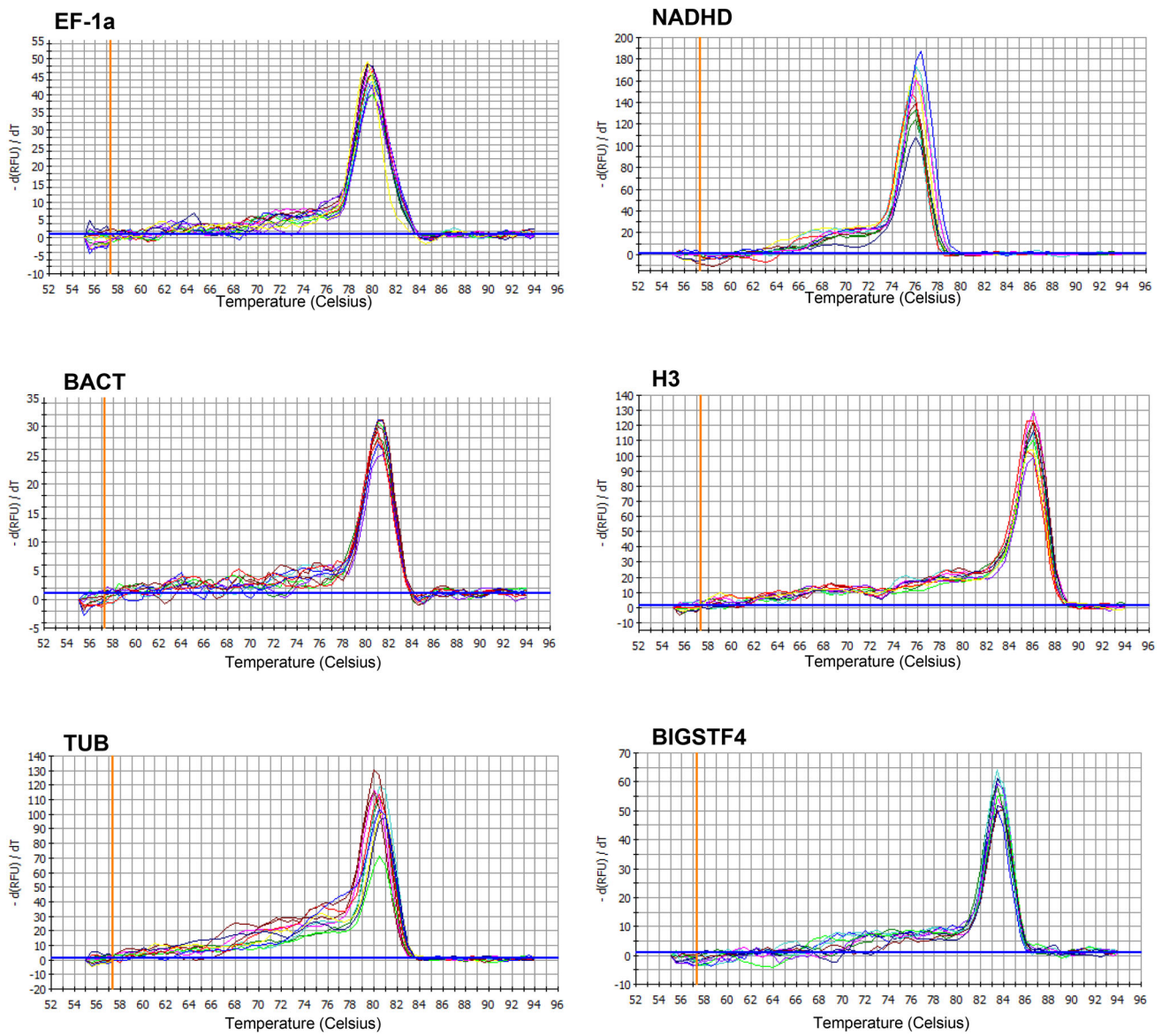


Fig. 1 Melting curve of the five candidate genes and one gene of interest. **EF-1a** Elongation Factor 1 alpha. **NADHD** Nicotinamide adenine dinucleotide dehydrogenase, **BACT** beta-actin, **H3** histone 3, **TUB** beta-tubulin, **BIGSTF4** glutathione S-transferase Phi

The analysis of variation pointed out EF-1a as possessing the highest intergroup value and the highest intragroup value (Table S2). On the other hand, the most stable candidate RG, showing the lowest inter- and intragroup variation values were NADHD and TUB, respectively (Table S2). As we can observe in the same table, the exposure to the xenobiotic endosulfan exerted the highest intergroup variation mainly in EF-1a, BACT and TUB.

ANOVA based stability index

Grouping by tissues The results of the stability index achieved by ANOVA revealed that H3 and TUB were the most stable RG when all data were grouped by tissues (Table 2).

In order to assess the variation across tissues, data corresponding to each particular condition (e.g. Control, Small, Endosulfan or Cold) were analyzed grouped by tissue. Thus, none of the resulting genes for each condition showed complete coincidence with the others. For example, using the subset corresponding to Control plants, EF-1a and TUB were the least variable RG (Fig. 5a), while NADHD and H3 resulted as the selected pair for Endosulfan-treated plants (Fig. 5c).

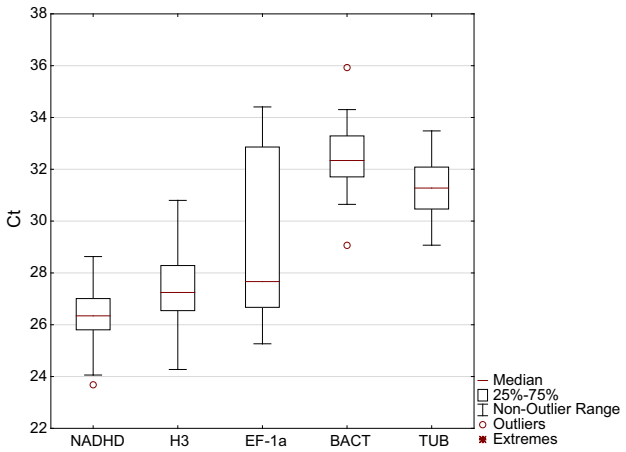


Fig. 2 The range of expression levels of five reference genes expressed by cycle threshold values. The boxes and whiskers indicate 25–75% and non-outlier range, respectively. The inner lines in boxes represent the median value. NADHD: nicotinamide adenine dinucleotide dehydrogenase, H3: histone 3, EF-1a: elongation factor 1 alpha, BACT: beta-actin, TUB: beta-tubulin

Grouping by conditions

The results of the stability index achieved by ANOVA revealed that H3 and NADHD were the most stable RG when all data were grouped by condition (Table 3).

Again, to assess the variation across conditions, data corresponding to one particular tissue at a time (root, stem or leaf) were analyzed grouped by conditions. Thus, taking into account the first two optimal genes selected by ANOVA, NADHD was always chosen (Fig. 6a–c). In addition, BACT was selected only for root and leaf (Fig. 6a, c) while TUB only for stem (Fig. 6b).

Overall comparative rankings of 6 candidate reference genes

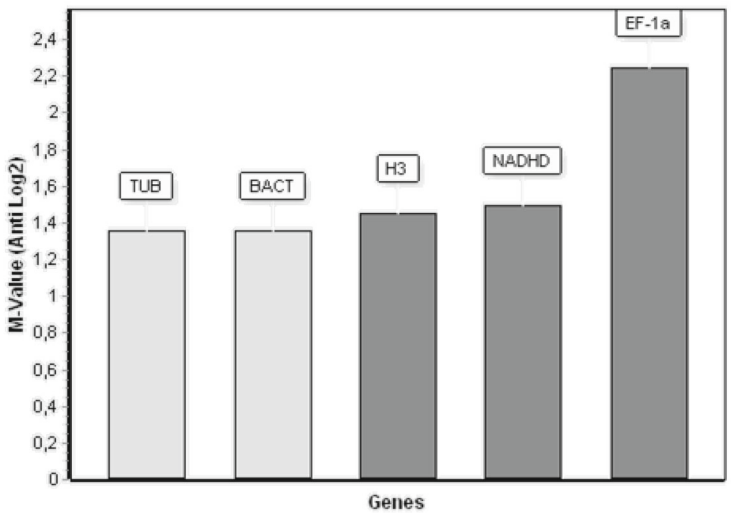
Table 4 shows a general-purpose ranking of RG obtained using geNorm and NormFinder algorithms, while ANOVA values are specific for tissues or conditions.

In general terms, BACT ranked within the first two more stable genes and NADHD within the first three genes. On the other hand, EF-1a always ranked last (Table 4).

Analysis of the expression of BlGSTphi

The average Ct value obtained for BlGSTF4 was 31.5. Taking into account the results obtained in roots by ANOVA calculations (Fig. 5a), geNorm (Fig. S6) and NormFinder (Fig. S8), three different combinations of RG pairs (BACT/TUB, BACT/NADHD and TUB/NADHD), as well as the combination of the three of them, were used as internal controls (Fig. 7).

In spite of the higher expression levels shown, metolachlor treatment presented the highest variability with all the combinations of RG. Therefore, these results were not used in the selection of the most convenient set of RG. The 2,4-D treatment did not show a noticeable difference in the variability using the different combinations. However, CDNB data allowed us to select BACT/TUB combination as the most stable (SD 0.27), only slightly over TUB/NADHD (SD 0.29) and NADHD/BACT/TUB (SD 0.30) (Fig. 7).



Gene Name	M-Value
EF-1a	2.24
NADHD	1.49
H3	1.44
BACT	1.35
TUB	1.35

Fig. 3 Expression stability values (M-value) calculated with geNorm algorithm and ranking of the candidate reference genes. TUB: Beta-tubulin, BACT: beta-actin, H3: histone 3, NADHD: nicotinamide adenine dinucleotide dehydrogenase, EF-1a: elongation factor 1 alpha

A

Gene Name	SD	Acc. SD
NADHD	0.64	0.64
H3	1.04	0.61
BACT	1.20	0.57
TUB	1.22	0.52
EF-1a	3.37	0.79

B

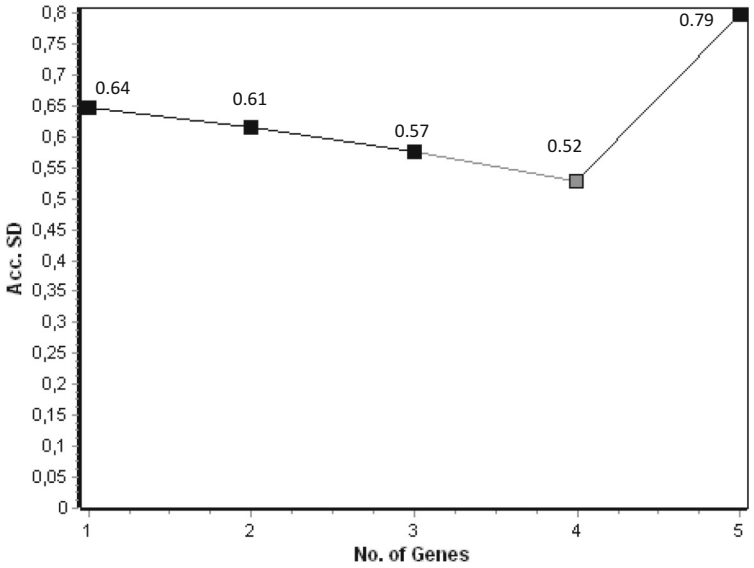


Fig. 4 **a** Standard deviation (SD) obtained with NormFinder algorithm, ranked from the most stable (lower SD) to the least stable (higher SD). **b** Accumulated SD when using additional candidate reference genes to the most stable. NADHD, nicotinamide adenine dinucleotide dehydrogenase; H3, histone 3; BACT, beta-actin; TUB, beta-tubulin; EF-1a, elongation factor 1 alpha

Table 2 Intergroup and intragroup variances, their components, and the Stability Index calculated with ANOVA method, when data were grouped by tissues (root, stem and leaf)

Genes	Source of variation	MS (variance) components			Total MS (variance)	− 1/lX	V_B	V_W	Stability index
		Roots	Stems	Leaves					
NADHD	Between groups	1.73	1.68	0.00	3.41	0.56	6.06	0.90	5.45
	Within groups	0.10	0.25	0.16	0.51				
H3	Between groups	0.01	0.08	0.15	0.24	0.46	0.52	2.15	1.11
	Within groups	0.32	0.27	0.40	1.00				
EF-1a	Between groups	0.04	0.01	0.09	0.14	0.33	0.42	16.54	6.87
	Within groups	2.19	1.70	1.57	5.46				
BACT	Between groups	0.05	1.01	0.62	1.68	0.43	3.94	1.74	6.86
	Within groups	0.31	0.34	0.09	0.74				
TUB	Between groups	0.55	0.35	0.02	0.92	0.65	1.42	0.93	1.32
	Within groups	0.22	0.13	0.24	0.60				

V_B, intergroup variation index; V_W, intragroup variation index; lX, average of the natural log of the relative expression ratios for each gene; NADHD, nicotinamide adenine dinucleotide dehydrogenase; H3, histone 3; EF-1a, elongation factor 1 alpha; BACT, beta-actin; TUB, beta-tubulin

Discussion

RT-qPCR specificity, efficiency and expression profile

The range of the expression level observed for the candidate RG was similar to that reported by Nicot et al. (2005), Maroufi et al. (2010) and Die et al.(2010) in *Solanum tuberosum*, *Cichorium intybus* and *Pisum sativum*, respectively. In addition, the level of expression of the candidate genes tested was mostly in a similar range to that of many target genes found in the literature. According to Hruz et al. (2011), an ideal reference gene must have an abundance of transcripts similar to that of the target genes under investigation. For example, Maroufi et al.(2010) obtained a mean Ct of 31 for its target gene fructan 1-exohydrolase IIa

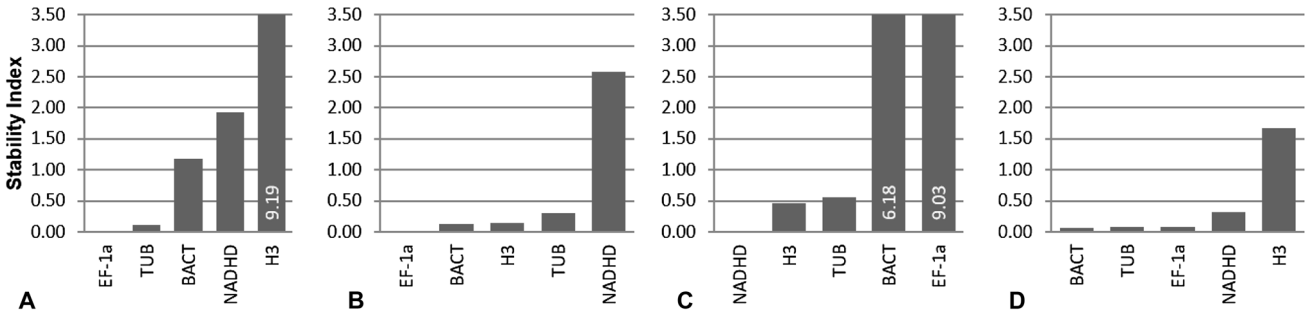


Fig. 5 ANOVA based stability index for different conditions. **a** Control, **b** small, **c** endosulfan, **d** cold, grouping data by tissues. Values higher than 3.5 are indicated in the corresponding bars. NADHD, nicotinamide adenine dinucleotide dehydrogenase; H3, histone 3; BACT, beta-actin; TUB, beta-tubulin; EF-1a, elongation factor 1 alpha

Table 3 Intergroup and intragroup variances, their components, and the Stability Index calculated with ANOVA method, when data were grouped by conditions (control, small, endosulfan and cold)

Genes	Source of variation	MS (variance) components				Total MS (variance)	− 1/x	V_b	V_w	Stability index
		Control	Small	Endosulfan	Cold					
NADHD	Between groups	0.02	0.31	0.29	0.03	0.65	0.56	1.16	1.20	1.39
	Within groups	0.23	0.26	0.05	0.13	0.67				
H3	Between groups	0.00	0.11	0.01	0.13	0.26	0.46	0.55	2.20	1.21
	Within groups	0.56	0.11	0.19	0.16	1.02				
EF-1a	Between groups	15.15	11.81	9.12	18.56	54.64	0.33	165.55	1.56	258.58
	Within groups	0.03	0.06	0.35	0.08	0.52				
BACT	Between groups	0.25	0.00	0.22	0.00	0.47	0.43	1.11	1.94	2.15
	Within groups	0.36	0.13	0.26	0.08	0.83				
TUB	Between groups	1.03	0.31	0.09	0.57	2.01	0.65	3.11	0.75	2.34
	Within groups	0.09	0.15	0.17	0.08	0.49				

V_B, intergroup variation index; V_W, intragroup variation index; lX, average of the natural log of the relative expression ratios for each gene; NADHD, nicotinamide adenine dinucleotide dehydrogenase; H3, histone 3; EF-1a, elongation factor 1 alpha; BACT, beta-actin; TUB, beta-tubulin

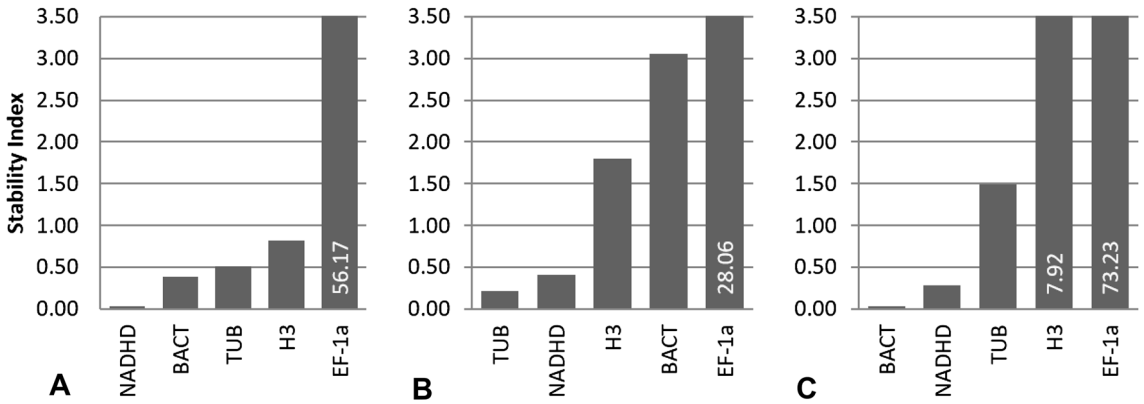


Fig. 6 ANOVA based stability index for different tissues of *Bidens laevis*. **a** Root, **b** stem, **c** leaf, grouping data by conditions. Values higher than 3.5 are indicated in the corresponding bars. NADHD, nicotinamide adenine dinucleotide dehydrogenase; H3, histone 3; BACT, beta-actin; TUB, beta-tubulin; EF-1a, elongation factor 1 alpha

and IIb which was near the range obtained for the RG used (19–29), while Wan et al. (2011) found a Ct ~ 20 for the target gene Cytochrome p450 family 4 which was in a range of Ct ~ 15 to 25 for the RG. In our case, the Ct of the candidate RG ranged between 24 and 32, and the Ct values for BLGSTphi fitted properly in this range.

Table 4 Comparative rankings of candidate Reference Genes in *Bidens laevis* using three different statistical methods

Ranking	geNorm	M-value	NormFinder	SD	ANOVA (tissues)	Stability index	ANOVA (conditions)	Stability index
1°	BACT TUB	1.35	NADHD	0.64	H3	1.11	H3	1.21
2°	H3	1.44	H3	1.04	TUB	1.32	NADHD	1.39
3°	NADHD	1.49	TUB	1.20	NADHD	5.45	BACT	2.15
4°	EF-1a	2.24	BACT	1.22	BACT	6.86	TUB	2.34
5°			EF-1a	3.37	EF-1a	6.87	EF-1a	258.58

NADHD, nicotinamide adenine dinucleotide dehydrogenase; H3, histone 3; EF-1a, elongation factor 1 alpha; BACT, beta-actin; TUB, beta-tubulin

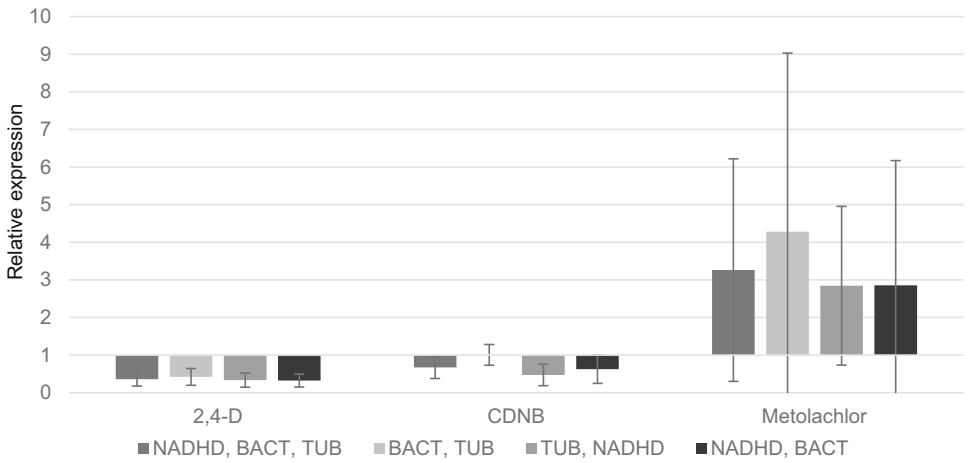


Fig. 7 Expression profile of BlGSTphi in *Bidens laevis* exposed to 2,4-D (5 mg/L), CDNB (10 mg/L), and metolachlor (567 mg/L) determined by qPCR with NADHD/BACT/TUB, BACT/TUB, TUB/NADHD and NADHD/BACT as reference gene combinations. Results are given as the mean of the relative expression ± SD. NADHD, nicotinamide adenine dinucleotide dehydrogenase; H3, histone 3; BACT, beta-actin; TUB, beta-tubulin; EF-1a, elongation factor 1 alpha

Software analysis

As it was pointed out by Jacob et al. (2013), the application of geNorm and NormFinder algorithms delivered identical ranking in only 5 out of the 12 investigated reference genes. This fact presents shortcomings that may lead to false selection of reference genes, recommending that more than two algorithms should be applied for the selection of the reference genes. Therefore, we discuss the comparative results from three different methods.

By means of geNorm software, an M-value lower than 1.5 is recommended for a reliable normalization process (Rivera-Vega et al. 2012). Therefore, the value obtained for the pair BACT-TUB was acceptable. On the contrary, the high M-value observed for EF-1a indicates a lack of stability and consequently, it would be not advisable to use it.

Unlike the results from geNorm, by using NormFinder algorithm, NADHD and H3 showed the most stable expression among all RG tested, supporting the idea that a specific set of RG should be considered for every particular case and that no single gene or combination of them can be used without prior testing. Although the results indicated that the use of 4 RG to normalize data would lead to the most reliable normalization process, the accumulated SD decreases weakly after considering the third and fourth gene (TUB and BACT). This fact would denote only a slight improvement but would also imply an increase in time and costs.

Considering that one of the main purposes of the overall analysis was to find the most suitable set of RG using presence or absence of a pesticide (endosulfan) as a condition, the final part of the present work was to check its applicability when the plants were exposed to other xenobiotics or environmental pollutants, in this case CDNB, 2,4-D and metolachlor. When analyzing the expression of BLGSTphi in a particular tissue (root) with the number and combination of RG required (Fig. 5a, S6 and S8), we could achieve the lowest variation in the expression SD in plants exposed to CDNB. This fact indicates that the chosen set of RG was the most appropriate and highlights the relevance

of using CDNB not only as an in vitro universal substrate for GST activity assay (Nimptsch and Pflugmacher 2005), but also as an in vivo regulator of GST expression.

In the NormFinder analysis, when grouped by tissue, NADHD proved to be the most stable RG and, when adding H3, a marked improvement was noticed. This observation was previously explained by Andersen et al. (2004) as a compensating expression in which one gene is slightly overexpressed in one group but the other gene is correspondingly underexpressed in the same group. On the contrary, when grouping by conditions, the addition of a second gene did not improve the accumulated SD suggesting that it would be enough to use NADHD to normalize data in this kind of studies.

It is noteworthy that NADHD turned out to be the most stable RG across different tissues and conditions as tested with the 3 methods, leading us to conclude that it is a good RG for the aims of this work in *B. laevis*. This is a gene seldom used to normalize qPCR data (Hitchen et al. 2009; Maroufi et al. 2010; Xu et al. 2017) and, therefore, given its high expression stability in *B. laevis*, it would be advisable to include it in future analysis of potential RG in other conditions or in other species.

Interestingly, in spite of having a general very stable expression among tissues under a given condition, EF-1a presented a markedly different expression among the different conditions tested, as seen in its high intragroup variation (Table S1 NormFinder and Fig. 4a, b, d ANOVA test). Contrary to the assumption that EF-1a is an appropriate RG (Weber et al. 2006), in this work, it turned out to be the least stable one. Therefore, the present study shows that no gene could be universally used and also that proper validation should be performed when selecting the optimal number of RG for each condition tested. This fact had been previously pointed out by Gutierrez et al. (2008) who, similarly to the present work, found that the most stably expressed genes varied between different sets of experiments, illustrating their specificity.

Conclusions

By comparing the stability of commonly used candidate genes, we identified the most stable ones for qPCR gene expression analysis using three bioinformatic algorithms. Therefore, for a general-purpose set of RG, the overall analysis showed that a set of three RG will be optimum, being H3, TUB and NADHD, the ones selected. On the other hand, as RG expression can vary depending on the tissues or conditions, an appropriate choice of a set of RG for each of them was necessary, achieving the most applicable results with ANOVA. These results set up the bases for making an appropriate normalization process, where more than one RG is clearly needed, in future qPCR studies.

Furthermore, studies of this kind are the basis of reliability in the use of biomarkers for environmental risk assessment, particularly in the proposal of new species as bioindicators of environmental pollution.

Acknowledgements The authors would like to thank Paula Fernandez from the Instituto de Biotecnología-Instituto Nacional de Tecnología Agropecuaria-INTA-Castelar, Argentina and Khosro Mehdi Khanlou from the Department of Plant Production, Ghent University, Belgium for their selfless contribution to this manuscript.

Funding This work was supported by Grants from National University of Mar del Plata (EXA 608/12), Consejo Nacional de Investigaciones Científicas y Técnicas-CONICET-(PIP 112-200-801-02190) and Fondo para la Investigación Científica y Tecnológica-FONCyT-(PICT 2011 No. 1597).

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. Can Res 64:5245–5250. https://doi.org/10.1158/0008-5472.CAN-04-0496

Brain RA, Cedergreen N (2009) Biomarkers in aquatic plants: selection and utility. Rev Environ Contam Toxicol 198:49–109. https://doi.org/10.1007/978-0-387-09647-6_2

Brulle F, Bernard F, Vandenbulcke F, Cuny D, Dumez S (2014) Identification of suitable qPCR reference genes in leaves of *Brassica oleracea* under abiotic stresses. Ecotoxicology 23:459–471. https://doi.org/10.1007/s10646-014-1209-7

Chaâbene Z et al (2017) Insight into the expression variation of metal-responsive genes in the seedling of date palm (*Phoenix dactylifera*). Chemosphere. https://doi.org/10.1016/j.chemosphere.2017.12.146

Choi Y, Noh E, Kim H, Shim D (2013) Overexpression of poplar GSTU51 confers selective tolerance to both mercury and methyl viologen but not to CDNB or cadmium in transgenic poplars. Plant Biotechnol Rep 7:175–184. https://doi.org/10.1007/s11816-012-0246-z

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal Biochem 162:156–159. https://doi.org/10.1006/abio.1987.9999

Coskun G, Zihnioglu F (2002) Effect of some biocides on glutathione-S-transferase in barley, wheat, lentil and chickpea plants. Turk J Biol 26:89–94

Cummins I, O'Hagan D, Jablonkai I, Cole DJ, Hehn A, Werck-Reichhart D, Edwards R (2003) Cloning, characterization and regulation of a family of phi class glutathione transferases from wheat. Plant Mol Biol 52:591–603

den Besten PJ, Munawar M, Suter G (2007) Ecotoxicological testing of marine and freshwater ecosystems: emerging techniques, trends and strategies. Integr Environ Assess Manag 3:305–306. https://doi.org/10.1002/ieam.5630030221

DeRidder BP, Dixon DP, Beussman DJ, Edwards R, Goldsbrough PB (2002) Induction of Glutathione-*S*-Transferases in Arabidopsis by herbicide safeners. Plant Physiol 130:1497–1505. https://doi.org/10.1104/pp.010066

Die JV, Roman B, Nadal S, Gonzalez-Verdejo CI (2010) Evaluation of candidate reference genes for expression studies in *Pisum sativum* under different experimental conditions. Planta 232:145–153. https://doi.org/10.1007/s00425-010-1158-1

Ferrat L, Pergent-Martini C, Romeo M (2003) Assessment of the use of biomarkers in aquatic plants for the evaluation of environmental quality: application to seagrasses. Aquat Toxicol 65:187–204. https://doi.org/10.1016/S0166-445X(03)00133-4

Flury T, Adam D, Kreuz K (1995) A 2,4-D-inducible glutathione S-transferase from soybean (*Glycine max*). Purification, characterisation and induction. Physiol Plant 94:312–318. https://doi.org/10.1111/j.1399-3054.1995.tb05317.x

Gutierrez L, Mauriat M, Pelloux J, Bellini C, Van Wuytswinkel O (2008) Towards a systematic validation of references in real-time rt-PCR. Plant Cell 20:1734–1735. https://doi.org/10.1105/tpc.108.059774

Hitchen SJ, Shostak AW, Belosevic M (2009) *Hymenolepis diminuta* (Cestoda) induces changes in expression of select genes of *Tribolium confusum* (Coleoptera). Parasitol Res 105:875. https://doi.org/10.1007/s00436-009-1481-2

Hruz T et al (2011) RefGenes: identification of reliable and condition specific reference genes for RT-qPCR data normalization. BMC Genom 12:156. https://doi.org/10.1186/1471-2164-12-156

Jacob F, Guertler R, Naim S, Nixdorf S, Fedier A, Hacker NF, Heinzelmann-Schwarz V (2013) Careful selection of reference genes is required for reliable performance of RT-qPCR in human normal and cancer cell lines. PLoS ONE 8:e59180. https://doi.org/10.1371/journal.pone.0059180

Jain M, Ghanashyam C, Bhattacharjee A (2010) Comprehensive expression analysis suggests overlapping and specific roles of rice glutathione S-transferase genes during development and stress responses. BMC Genom 11:73. https://doi.org/10.1186/1471-2164-11-73

Lee M-A, Guo R, Ebenezer V, Ki J-S (2015) Evaluation and selection of reference genes for ecotoxicogenomic study of the green alga *Closterium ehrenbergii* using quantitative real-time PCR. Ecotoxicology 24:863–872. https://doi.org/10.1007/s10646-015-1430-z

Maroniche GA, Sagadin M, Mongelli VC, Truol GA, del Vas M (2011) Reference gene selection for gene expression studies using RT-qPCR in virus-infected planthoppers. Virol J 8:308. https://doi.org/10.1186/1743-422X-8-308

Maroufi A, Van Bockstaele E, De Loose M (2010) Validation of reference genes for gene expression analysis in chicory (*Cichorium intybus*) using quantitative real-time PCR. BMC Mol Biol 11:15. https://doi.org/10.1186/1471-2199-11-15

Mehdi Khanlou K, Van Bockstaele E (2012) A critique of widely used normalization software tools and an alternative method to identify reliable reference genes in red clover (*Trifolium pratense* L.). Planta 236:1381–1393. https://doi.org/10.1007/s00425-012-1682-2

Mezzari MP, Walters K, Jelinkova M, Shih MC, Just CL, Schnoor JL (2005) Gene expression and microscopic analysis of Arabidopsis exposed to chloroacetanilide herbicides and explosive compounds. A phytoremediation approach. Plant Physiol 138:858–869. https://doi.org/10.1104/pp.104.056168

Mohan B, Hosetti B (1998) Lead toxicity to *Salvinia natans* grown in macrophyte ponds. J Ecotoxicol Environ Monit 8:03–07. https://doi.org/10.1006/enrs.1999.3960

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. https://doi.org/10.1093/jxb/eri285

Nimptsch J, Pflugmacher S (2005) Substrate specificities of cytosolic Glutathione S-Transferases in five different species of the aquatic macrophyte *Myriophyllum*. J Appl Bot Food Qual 79:94–99

Nolan T, Hands RE, Bustin SA (2006) Quantification of mRNA using real-time RT-PCR. Nat Protoc 1:1559–1582. https://doi.org/10.1038/nprot.2006.236

Peng Y-L, Wang Y-S, Gu J-D (2015) Identification of suitable reference genes in mangrove *Aegiceras corniculatum* under abiotic stresses. Ecotoxicology 24:1714–1721. https://doi.org/10.1007/s10646-015-1487-8

Perez DJ, Lukaszewicz G, Menone ML, Camadro EL (2011) Sensitivity of *Bidens laevis* L. to mutagenic compounds. Use of chromosomal aberrations as biomarkers of genotoxicity. Environ Pollut 159:281–286. https://doi.org/10.1016/j.envpol.2010.08.028

Pérez DJ, Lukaszewicz G, Menone ML, Amé MV, Camadro EL (2014) Genetic and biochemical biomarkers in the macrophyte *Bidens laevis* L. Exposed to a commercial formulation of endosulfan. Environ Toxicol 29:1063–1071. https://doi.org/10.1002/tox.21836

Rivera-Vega L, Mamidala P, Koch JL, Mason ME, Mittapalli O (2012) Evaluation of reference genes for expression studies in ash (*Fraxinus* spp.). Plant Mol Biol Rep 30:4. https://doi.org/10.1007/s11105-011-0340-3

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3:research0034.0001–research0034.0011

Wan Q, Whang I, Choi CY, Lee JS, Lee J (2011) Validation of housekeeping genes as internal controls for studying biomarkers of endocrine-disrupting chemicals in disk abalone by real-time PCR Comparative biochemistry and physiology. Toxicol Pharmacol CBP 153:259–268. https://doi.org/10.1016/j.cbpc.2010.11.009

Watahiki MK, Mori H, Yamamoto KT (1995) Inhibitory effects of auxins and related substances on the activity of an Arabidopsis glutathione S-transferase isozyme expressed in *Escherichia coli*. Physiol Plant 94:566–574. https://doi.org/10.1111/j.1399-3054.1995.tb00969.x

Weber M, Trampczynska A, Clemens S (2006) Comparative transcriptome analysis of toxic metal responses in *Arabidopsis thaliana* and the Cd^{2+}-hypertolerant facultative metallophyte *Arabidopsis halleri*. Plant Cell Environ 29:950–963. https://doi.org/10.1111/j.1365-3040.2005.01479.x

Xu J, Lu M-X, Cui Y-D, Du Y-Z (2017) Selection and evaluation of reference genes for expression analysis using qRT-PCR in *Chilo suppressalis* (Lepidoptera: Pyralidae). J Econ Entomol 110:683–691. https://doi.org/10.1093/jee/tow297

Zhao S, Fernald RD (2005) Comprehensive algorithm for quantitative real-time polymerase chain reaction. J Comput Biol 12:1047–1064. https://doi.org/10.1089/cmb.2005.12.1047