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References genes for qRT-PCR in guaraná (*Paullina cupana* var. *sorbilis*)

Flávia Camila Schimpl[1] · Adilson Pereira Domingues Júnior[1] · José Francisco de Carvalho Gonçalves[2] · José Ferreira da Silva[3] · Paulo Mazzafera[1]

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Abstract Gene expression has been extensively studied in plant science research, mainly for the assessment of plant stress responses. Real-time-quantitative polymerase chain reaction (RT-qPCR) is an important tool for obtaining this information because it is a quick and easy technique to acquire a large amount of molecular data for both model and non-model plants. For a successful RT-qPCR analysis, gene expression should be carefully normalised. Genes involved in essential biological processes that exhibit constitutive expression are commonly selected as internal standards to normalise RT-qPCR experiments. In this study, the transcription profiles of 13 candidate reference genes for RT-qPCR were evaluated in three guarana cultivars (BRS-Amazonas, BRS-Maués and BRS-Luzéia) using different tissues (vegetative and fruit) in varying developmental stages. Two different algorithms, NormFinder and GeNorm, were utilised to assess gene stability. In general, the two algorithms did not select the same pairs of genes for all analysed conditions. For the largest group (the fruits of all cultivars), NormFinder selected the pair *EF1A*/UBQ, whereas GeNorm chose *ACT*/*GAPDH* as the best normalising genes. Thus, we recommend the use of at least four reference genes for the normalisation of gene expression in guarana plant studies.

Keywords Amazon · Caffeine · Gene expression · Real-time PCR · Sapindaceae

✉ Paulo Mazzafera
pmazza@unicamp.br

[1] Departamento de Biologia Vegetal-IB, CP 6109, Universidade Estadual de Campinas, Campinas, São Paulo 13083-970, Brazil

[2] Instituto Nacional de Pesquisas da Amazônia, Manaus, Amazonas, Brazil

[3] Departamento de Produção Animal e Vegetal, Faculdade de Ciências Agrárias, Universidade Federal do Amazonas, Manaus, Amazonas, Brazil

Introduction

Gene expression analysis is an important tool to study complex regulatory networks in biological systems (Chandna et al. 2012). In plants, gene expression analysis has been used extensively to investigate development and growth as well as the metabolic responses induced by environmental changes and responses to insects and pathogens (Remans et al. 2008; Borges et al. 2012; Han et al. 2012; Moura et al. 2012; Fernández-Aparicio et al. 2013; Wei et al. 2013).

Among the different techniques used to analyse genes, including macro- and microarrays, Northern blot, RNA-seq and semi-quantitative RT-PCR, quantitative RT-PCR (qRT-PCR) is the primary technique used. Its main advantages include its relatively low cost; rapid implementation; and high sensitivity, reproducibility and accuracy (Rajeevan et al. 2001; Aerts et al. 2004; Czechowski et al. 2005; Oliveira et al. 2012). Real-time-quantitative polymerase chain reaction (RT-qPCR) can be used to study any type of living organisms, including both model and non-model organisms (Thellin et al. 1999; Gachon et al. 2004). This technique allows for the rapid generation of quantitative data for in-depth analysis of the expression of several target genes (Chandna et al. 2012). However, for a correct interpretation of results, data must be normalised to minimise experimental errors introduced at various stages throughout the process, which may arise from the use of

different individuals, RNA isolation and storage techniques, the use of different amounts of RNA in each assay (pipetting errors), and the efficiency of cDNA synthesis (Guénin et al. 2009; Podevin et al. 2012; Borges et al. 2012). Thus, the reliability of the technique and the accurate quantification of the expression of target genes depend on the experimental conditions and the use of appropriate internal controls (Borges et al. 2012; Han et al. 2012).

Genes involved in essential biological processes that exhibit constitutive expression are commonly selected as internal standards to normalise qRT-PCR experiments (Gachon et al. 2004; Podevin et al. 2012). Ideal reference genes (or normalising genes) must maintain a constant level of expression under different experimental conditions, in different tissues, and at different developmental stages (Thellin et al. 1999; Dheda et al. 2004; Guénin et al. 2009). However, even these genes exhibit variations depending on the living conditions and the organism studied. Thus, a universal reference gene does not exist, and even those described as suitable for some species and/or experimental conditions are not necessarily suitable for the same species grown under varying conditions or for different species grown under the same conditions (Thellin et al. 1999; Guénin et al. 2009; Wei et al. 2013).

Different algorithms, such as GeNorm (Vandesompele et al. 2002), NormFinder (Andersen et al. 2004) and Bestkeeper (Pfaffl et al. 2004), have been used as statistical tools to assist in selecting the best normalising genes for a given set of samples, and these algorithms are very useful when a large number of genes are examined. The selection of suitable reference genes for different experimental conditions or plant species has been evaluated in a series of qRT-PCR studies of several plant species, including model plants, such as *Arabidopsis thaliana* (Czechowski et al. 2005), *Petunia hybrida* (Mallona et al. 2010), tobacco (Schmidt and Delaney 2010), and poplar (Brunner et al. 2004), and crops of economic importance, such as rice (Ding et al. 2004; Jain et al. 2006), soybean (Jian et al. 2008), coffee (Barsalobres-Cavallari et al. 2009; Cruz et al. 2009), sugarcane (Iskandar et al. 2004), tomato (Expósito-Rodríguez et al. 2008), citrus (Yan et al. 2012), and eucalyptus (Marta et al. 2010; Moura et al. 2012; Oliveira et al. 2012).

The Amazonian plant guarana (*Paullinia cupana* var. *sorbilis*) produces seeds with a high caffeine content that are used to produce the soft drink of the same name, Guaraná (Schimpl et al. 2013). Guarana plants growing in the wild have been used in breeding programmes to improve pathogen and insect tolerance, productivity, and caffeine content (Heard et al. 2006; Atroch 2009; Nascimento Filho et al. 2009; Bentes and Costa Neto 2011; Albertino et al. 2012). As a result, an EST database was generated (Ângelo et al. 2008) and used to study a variety of topics, including the biosynthesis of caffeine (Schimpl et al. 2014). Thus, this study aimed to assess 13 candidate reference genes for qRT-PCR assays of guarana. We used ten different testing conditions, combining different cultivars and tissues at different developmental stages.

Materials and methods

Plant materials

The plant materials were collected from guarana pants growing at the farm of Agropecuaria Jayoro Ltda. located at Presidente Figueiredo, Amazonas State, Brazil. Fruits were collected from adult plants of the cultivars BRS-Amazonas, BRS-Maués and BRS-Luzéia at different stages of development/maturation, including green, intermediate and mature fruits. The fruits were then separated into seed and pericarp. Samples were collected from three biological replicates. Additionally, three young plants of the BRS-Amazonas cultivar were used for the collection of vegetative tissues, such as stem (apical portion) and leaves (young and mature). The collected materials were immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

Extraction of total RNA and cDNA synthesis

RNA extraction was performed using the sodium perchlorate method (Rezaian and Krake 1987) as followed by (Schimpl et al. 2014). After extraction, the RNA was treated with DNase "Turbo DNA-free" (Ambion, Inc.) and quantified in a spectrophotometer at 260 nm. The determination of RNA concentration in μg μL^{-1}, was obtained from the equation: RNA = (Abs260 × dilution)/25. The quality of RNA was checked by electrophoresis in agarose gel 1 % with ethidium bromide, with visualization under UV light fotodocumentador (Gel Doc 2000, Bio RAD). Two μg of total RNA was used to synthesize first-strand cDNA with the kit SuperScript III First-Strand (Invitrogen) according to the manufacturer's instructions.

Primer design

Sequences of *Arabidopsis thaliana* were used as baits for a search of homologous sequences in Realgene, which is a guarana EST database (Ângelo et al. 2008). The selected genes were among those known to be constitutively expressed or involved in basal metabolism in different cell types (Czechowski et al. 2005; Yan et al. 2012), including 18S ribosomal RNA (*18S*), actin 7 (*ACT*), alcohol dehydrogenase (*ADH*), tubulin alpha 6 (*TUBa*), tubulin beta chain 3 (*TUBb*), cyclophilin-ROC3 (*CYP*) translation

Table 1 Identification of reference genes and sequence of primers used in RT-qPCR analysis

Gene name	Gene description	RealGene Bank	*A. thaliana* homologue	Primer forward/reverse (5′–3′)	Amplicon (bp)	Amplification efficiency (%)*
18S	18S Ribossomal RNA	Contig2420	At3g41768	TGACGGAGAATTAGGGTTCG CTTGGATGTGGTAGCCGTTT	71	99.0
ACT	Actin 7	Contig1017	At5g09810	AGATGACCCAGATTATGTTTGAGACCTTC ACCATCACCAGAATCCAACACAATACC	122	99.4
ADH	Alcohol dehydrogenase	Contig193	AT1G77120	GTTGCAGCATCAGGTCAAGA ACGTCCATTTTCAGCACACA	248	95.2
TUBa	Tubulin alpha 6	Contig1517	At4g14960	CACACAGCACGCTGTACCTT AAGGACCGTTCAATTCGTTG	92	90.0
TUBb	Tubulin beta chain 3	Contig2535	At5g62700	TCCCAACAACGTGAAGTCAA CTTTCGGCCTCAGTGAACTC	197	110.0
CYP	Cyclophilin (ROC3)	Contig12	At2g16600	CGGACCTGGTATTCTCTCCA TGGAGGTCCTTCCAGAGCTA	182	98.3
EF1A	Translation elongation factor A	Contig2542	At1g18070	AAGGAGGCAGCTGAGATGAA AGGGGCATCAATAACAGTGC	150	102
EF1B	Translation elongation factor B	Contig499	At5g19510	ACAGATCTCCACACCGAAGC CAGCAGCTTTACCAGGGAAG	208	99.7
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase C2	Contig644	At1g13440	CTCATTTGAAGGGTGGTGCT GTCTTTTGGGTGGCTGTGAT	232	99.8
ICDH	$NADP^+$ isocitrate dehydrogenase	Contig496	At1g65930	AAAGGTGGCCGCTTTATCTT GCTAGGAAATCGCTCTGCAC	238	104.4
S24	Ribosomal protein S24e	Contig2137	At3g04920	TCATCCTGGAAGGCCTAATG CAACCTTGGTGTCAAGTCCA	221	106
UBQ	Polyubiquitin 11	Contig1935	At4g05050	TAAGGAAGGCATTCCACCTG TAATCCGCTCAATGGTGTCA	212	95.1
UBC	Ubiquitin-conjugating enzyme 10	Contig140	At5g53300	GCGGATCTTGAAGGAGTTGA TGGGTGGCTTGAAAGGATAG	185	90.3

* $E = [10^{(-1/\text{slope})}]^{-1}$ (Rasmussen 2001)

elongation factor A (*EF1A*), translation elongation factor B (*EF1B*), glyceraldehyde-3-phosphate dehydrogenase C2 (*GAPDH*), $NADP^+$-isocitrate dehydrogenase (*ICDH*), ribosomal protein S24e (*S24*), polyubiquitin 11 (*UBQ*) and ubiquitin-conjugating enzyme 10 (*UBC*) (Table 1). Primers were designed using the online tool Primer3 (http://frodo.wi.mit.edu/primer3/) with the following parameters: an approximately 60 °C melting temperature, amplification product between 70 and 250 base pair in length, and 45–55 % GC content.

Quantitative RT-PCR

RT-qPCR (qPCR) was performed using cDNA samples (diluted 50x) synthesised from 2 µg of total RNA. The reactions were processed in triplicate and prepared with a SYBR® Green QuantiFast™ PCR Kit (Qiagen). PCR was performed with an iCycler iQTM5 Multicolour Real-Time PCR Detection System (Bio-Rad) under the following conditions: 95 °C for 3 min followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. Confirmation of the specificity of the amplicon was based on a dissociation curve and performed at the end of each assay. An amplification efficiency curve was generated for each pair of primers, which revealed efficiencies of between >90 % and <110 % for all primers (Table 1).

Selection of the best reference genes

The best combination of reference genes was determined by the quantification of the relative expression value for each gene using two different algorithms, GeNorm (Vandesompele et al. 2002) and NormFinder (Andersen et al. 2004).

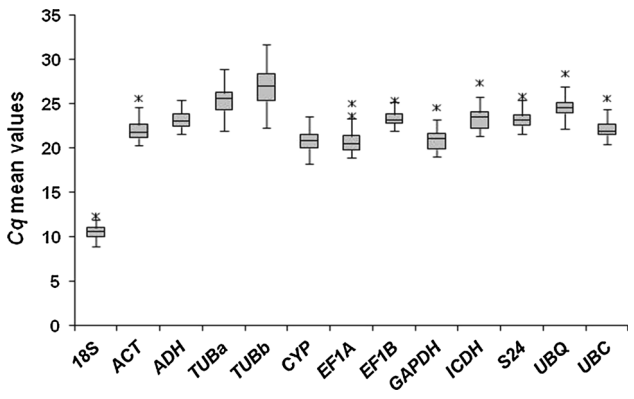

Fig. 1 *Cq values* (cycle threshold) from qRT-PCR analysis of each reference gene for all experimental conditions. The *box* indicates the 25th and 75th percentiles, *vertical bars* represent the minimum and maximum values, and the *bar* within the *box* represents the median. The *asterisks* represent the outliers

Results

The transcriptional profiles of 13 candidate reference genes for qRT-PCR were evaluated in three guarana cultivars using various vegetative and fruit tissues at different developmental stages. Gene names and descriptions, sequence accession numbers, primers used, lengths of the amplified fragments and amplification efficiencies are listed in Table 1.

The average value of the quantification cycle (*Cq*) for all genes in the tissues and stages examined was 22.1. The *18S* gene exhibited the lowest average *Cq* value (10.6), suggesting that this gene was highly expressed in the tissues analysed (Fig. 1). For the other candidate genes, the average *Cq* value was approximately 23.1. *CYP*, *EF1A* and *GAPDH* exhibited the lowest *Cq* values (higher expression), whereas *TUBb*, *TUBa* and *ACT* exhibited the highest average *Cq* values (lower expression). The gene encoding *TUBb* showed the greatest variation in expression, indicating high instability (Fig. 1).

EF1B, *UBQ* and S*24* displayed narrow *Cq* value ranges under the various experimental conditions analysed in this study and exhibited the lowest coefficients of variation (0.15, 0.17 and 0.18 %, respectively). Although it had the lowest *Cq* value, the *18S* gene exhibited the highest coefficient of variation ($CV = 0.73$ %).

To obtain more detailed information for the 13 genes, the expression data were divided into eight groups according to tissue type and stage of development. Then, the stability of gene expression was evaluated using two different algorithms, NormFinder (Table 2) and GeNorm (Table 3). NormFinder calculates a normalisation factor from comparisons of pairs, and this factor is based on inter- and intra-group variations. When the best pair of genes is indicated, this software also calculates a stability value for the combination (Andersen et al. 2004). GeNorm compares the stability of the average expression (*M*) of each gene, choosing those with more stable expression (Vandesompele et al. 2002). Thus, the best pair of genes may vary depending on the algorithm used, based on the classification (ranking) generated by each one.

None of the sample conditions resulted in the selection of the same pair of reference genes by both algorithms. Moreover, only three possible pairs contained at least one gene similarly suggested by GeNorm and NormFinder. NormFinder more frequently suggested *UBQ* and *GAPDH* as stable genes for the different combinations, whereas GeNorm selected *S24* and *ADH* as the most stable genes, which were chosen as the best pair for five tissues (Tables 2, 3).

GeNorm software also determines the required number of reference genes (n) for normalisation between two

Table 2 Stability of the expression of candidate reference genes calculated by the Norm Finder algorithm in the eight conditions analysed

Stability value								
Gene	Fruit—all cultivars	Seed—all cultivars	Pericarp—all cultivars	Fruit BRS-Maués	Fruit BRS-Luzéia	Fruit BRS-Amazonas	Leaves/shoots BRS-Amazonas	All tissues BRS-Amazonas
18S	0.168	0.197	0.112	0.164	0.171	0.14	0.347	0.116
ACT	0.078	0.078	0.064	0.063	0.063	0.098	0.384	0.120
ADH	0.105	0.117	0.075	0.082	0.101	0.100	0.488	0.103
TUBa	0.122	0.127	0.101	0.110	0.146	0.103	0.684	0.109
TUBb	0.114	0.124	0.093	0.131	0.098	0.104	1.375	0.135
CYP	0.087	0.099	0.066	0.088	0.089	0.067	0.419	0.074
EF1A	0.124	0.111	0.099	0.118	0.082	0.102	0.506	0.107
EF1B	0.128	0.154	0.082	0.076	0.107	0.133	0.412	0.127
GAPDH	0.113	0.117	0.055	0.097	0.126	0.108	0.180	0.084
ICDH	0.091	0.129	0.045	0.095	0.084	0.092	0.348	0.084
S24	0.096	0.133	0.051	0.102	0.106	0.064	0.313	0.071
UBQ	0.095	0.111	0.071	0.087	0.062	0.075	0.371	0.102
UBC	0.131	0.122	0.096	0.093	0.127	0.125	0.441	0.133
Best pairs	*ACT* *UBQ*	*EF1A* *UBQ*	*GAPDH* *S24*	*ICDH* *UBC*	*ACT* *UBQ*	*CYP* *S24*	*GAPDH* *ICDH*	*CYP* *S24*
Stability value	0.054	0.052	0.033	0.044	0.046	0.039	0.131	0.049

Fruits include samples of seeds and pericarp together

Table 3 Stability of the expression of candidate reference genes calculated by the GeNorm algorithm after the gradual exclusion of the least stable reference gene the eight conditions analysed

Stability value (*M*)								
Gene	Fruit—all cultivars	Seed—all cultivars	Pericarp—all cultivars	Fruit BRS-Maués	Fruit BRS-Luzéia	Fruit BRS-Amazonas	Leaves/shoots BRS-Amazonas	All tissues BRS-Amazonas
18S	0.754	0.792	0.554	0.657	0.669	0.734	0.829	1.117
ACT	0.593	0.296	0.397	0.540	0.462	0.550	0.834	0.971
ADH	0.434	0.490	0.359	0.265	0.419	0.340	0.841	0.850
TUBa	0.841	0.879	0.663	0.776	0.702	0.650	1.337	1.150
TUBb	0.997	1.093	0.763	0.964	0.971	0.905	2.606	1.883
CYP	0.550	0.570	0.493	0.471	0.577	0.564	0.979	0.880
EF1A	0.647	0.380	0.523	0.605	0.537	0.538	0.967	0.992
EF1B	0.698	0.715	0.430	0.314	0.312	0.820	0.794	1.246
GAPDH	0.565	0.296	0.477	0.433	0.610	0.477	0.758	0.839
ICDH	0.576	0.538	0.411	0.559	0.512	0.495	0.925	0.945
S24	0.434	0.618	0.359	0.265	0.312	0.340	0.730	0.821
UBQ	0.506	0.594	0.457	0.363	0.491	0.449	0.773	0.978
UBC	0.626	0.453	0.390	0.517	0.554	0.578	0.844	0.903
Best pairs	*ADH* *S24*	*ACT* *GAPDH*	*ADH* *S24*	*ADH* *S24*	*EF1B* *S24*	*ADH* *S24*	*ADH* *EF1B*	*ADH* *S24*

Fruits include samples of seeds and pericarp together

sequential normalisation factors (V_n/V_{n+1}) based on pair-wise variation (V). According to Vandesompele et al. (2002), 0.150 is the cut-off value for V. Below this value, the addition of another reference gene for normalisation is unnecessary. As expected, different sample sets exhibited a distinct number of "*n*" values (Fig. 2).

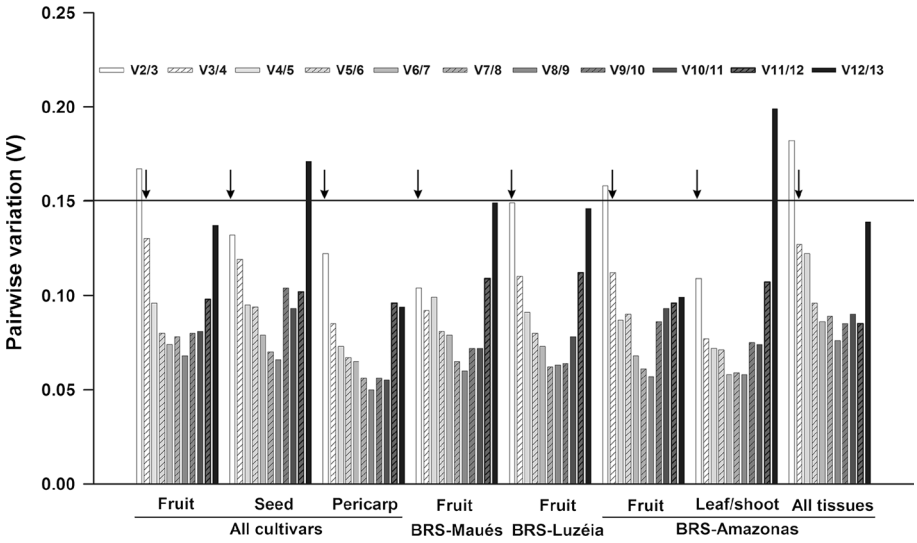

Fig. 2 Pairwise variation (V) and the determination of the optimal number of reference genes that should be used for normalisation according to each condition analysed. The *arrows* indicate the ideal numbers of reference genes for the normalisation of gene expression

Discussion

The study of gene expression is important and has been increasingly used in plant science research to better understand metabolic process (Conrath 2011; Nakashima et al. 2014). Additionally, an increasing interest in plant species containing secondary metabolites of physiological and pharmacological importance has resulted in the generation of transcriptome databases for several medicinal species (MedPlants—http://medplants.ncgr.org/; and MPGR—http://medicinalplantgenomics.msu.edu/) (Hao et al. 2012). A recent example is the guarana plant, *Paullinia cupana*, which is a native species from the Amazon region with a high caffeine content (Ângelo et al. 2008). This plant's transcriptome has been used to investigate the biosynthesis of alkaloids (Schimpl et al. 2014).

In this study, we evaluated the stability of 13 genes in different tissues from three cultivars of guarana using two algorithms, GeNorm and NormFinder. NormFinder analysis showed that each sample group had a unique pair of highly stable genes. Exceptions included the BRS-Amazonas-fruit and BRS-Amazonas-all tissues together sets, for which *CYP* and *S24* were identified as a highly stable pair. In this case, because the proportion of fruit samples in this group (the pericarps and seeds of the three maturation stages) was greater than that of the vegetative tissues (the young and mature leaves and stems), it is possible that the final result could be affected by the fruit/vegetative tissue ratio, resulting in the identification of the same pair in both cases (Table 2). For the set BRS-Amazonas-leaves/shoots, the best pair included *GAPDH* and *ICDH*. Consistently, the GeNorm algorithm suggested the pairs *ADH* and *S24* for the same sets of BRS-Amazonas-fruit and all tissues mixed, whereas *ADH* and *EF1B* was determined to be the best pair for the set BRS-Amazonas-leaves/shoots (Table 3).

Based on the differences between algorithm output and the sample group characteristics (cultivars and tissue), it was not possible to select one single set of genes for the normalisation of qPCR studies of guarana tissues. Even for seeds, which contain the highest caffeine content in the guarana plant (Schimpl et al. 2014), the two algorithms did not identify the same pairs of genes; NormFinder chose the pair *EF1A*/*UBQ,* whereas GeNorm selected *ACT*/*GAPDH*. This variation might be associated with the distinct nature of the samples analysed, which included vegetative and reproductive tissues at various maturation stages, representing different cellular events involving cell division and differentiation. Even the tissues from the same organ, such as the pericarp and seed, showed different pairs depending on the algorithm used. Therefore, the choice of the best reference genes depends on the nature of the tissue analysed, and the transition between the vegetative and reproductive phases must be carefully observed.

Overall, we conclude that more than four reference genes are required for the normalisation of gene expression in expression studies of guarana. We do not exclude the possibility that the use of other cultivars may result in the detection of new pairs of reference genes using the GeNorm and NormFinder algorithms.

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