Validation of reference genes for normalization of qPCR gene expression data from Coffea spp. hypocotyls inoculated with Colletotrichum kahawae

Andrelia Figueiredo1†, Andreia Loureiro2†, Dora Batista2, Filipa Monteiro1, Vítor Várzea2, Maria Salomé Pais1, Elijah K Gichuru3 and Maria Céu Silva2

Abstract

Background: Coffee production in Africa represents a significant share of the total export revenues and influences the lives of millions of people, yet severe socio-economic repercussions are annually felt in result of the overall losses caused by the coffee berry disease (CBD). This quarantine disease is caused by the fungus Colletotrichum kahawae Waller and Bridge, which remains one of the most devastating threats to Coffea arabica production in Africa at high altitude, and its dispersal to Latin America and Asia represents a serious concern. Understanding the molecular genetic basis of coffee resistance to this disease is of high priority to support breeding strategies. Selection and validation of suitable reference genes presenting stable expression in the system studied is the first step to engage studies of gene expression profiling.

Results: In this study, a set of ten genes (S24, 14-3-3, RPL7, GAPDH, UBQ9, VATP16, SAND, UQCC, IDE and β-Tub9) was evaluated to identify reference genes during the first hours of interaction (12, 48 and 72 hpi) between resistant and susceptible coffee genotypes and C. kahawae. Three analyses were done for the selection of these genes considering the entire dataset and the two genotypes (resistant and susceptible), separately. The three statistical methods applied GeNorm, NormFinder, and BestKeeper, allowed identifying IDE as one of the most stable genes for all datasets analysed, and in contrast GAPDH and UBQ9 as the least stable ones. In addition, the expression of two defense-related transcripts, encoding for a receptor like kinase and a pathogenesis related protein 10, were used to validate the reference genes selected.

Conclusion: Taken together, our results provide guidelines for reference gene(s) selection towards a more accurate and widespread use of qPCR to study the interaction between Coffea spp. and C. kahawae.

Background

Coffea arabica L. production in Africa can be seriously affected by coffee berry disease (CBD), caused by the hemibiotrophic fungus Colletotrichum kahawae JM [1]. Despite the fact that several plant organs can be affected, maximum production losses occur when infection takes place in expanding green berries, leading to their premature dropping and mummification [2,3]. Crop damages due to CBD, along with chemical control costs, accounts annually for a loss of US$ 300–500 millions in Arabica coffee production [4].

Since its first report in 1922 in Kenya [5], CBD rapid outbreak throughout Eastern Africa urged the development of breeding programmes in several countries (such as Kenya, Ethiopia and Tanzania) giving rise to the release of several resistant coffee varieties for coffee growers [2,4]. In Kenya, the most relevant example is the hybrid commercial variety Ruiru 11, which was bred for resistance to CBD and coffee leaf rust (Hemileia vastatrix) using lines of the coffee cultivar Catimor as
resistance sources. Breeding programmes remain the most economical and environmentally sound method for a sustainable coffee production. Presently, with the advances in genomic and transcriptomic resources, breeding for resistance can be supported and thus improved by molecular based-information.

Molecular research on the coffee - *C. kahawae* pathosystem has been lagging behind other plant-*Colletotrichum* spp. interactions although advances on the mechanisms of pathogen infection and host resistance at cellular level were achieved [2,3,6] Deepening the knowledge on the molecular basis governing coffee resistance to *C. kahawae* is thus fundamental to get some insights on the distinctive processes underlying plant resistance response. Monitoring gene differential expression and validating high-throughput RNA sequencing (RNA-seq) data is ideally achieved through quantitative real-time PCR (qPCR) analysis. Regardless of being an extremely powerful technique relative to sensitivity, specificity and broad quantification range, accurate data normalization with a reference gene(s) is an absolute requirement for qPCR correct measurement of gene expression. In this study, we have tested 10 candidate genes for qPCR normalization of gene expression during the first hours of interaction (12, 48 and 72 hpi) with *C. kahawae*. Two coffee genotypes, resistant and susceptible to *C. kahawae* were used. The best combination of reference genes determined for each dataset was used to further assess the expression of a pathogenesis-related protein 10 (*PR10*) and a receptor like kinase (*RLK*) known to be induced during coffee – leaf rust interactions [7,8]. Here we provide, for the first time, a set of reference genes suitable for gene expression studies in both resistant and susceptible coffee genotypes to *C. kahawae*.

**Results and discussion**

Normalization is one of the key factors affecting the accuracy and reliability of quantitative gene expression analysis. Here, we describe an assessment of ten candidate reference genes (RGs) for their use as internal controls in gene expression studies of coffee defence responses against *C. kahawae* in two different genotypes, Caturra as susceptible and Catimor 88 as resistant. Data were analyzed considering the entire dataset and each of the coffee genotypes separately.

**Amplification specificity and efficiency**

To examine the expression stability of the candidate RGs selected, transcript levels of the ten candidates were measured by qPCR using gene-specific primer pairs (Table 1). Melting curves of the genes tested were analysed to detect the absence/presence of primer dimer or non-specific PCR products (Additional file 1). For V-Type proton ATPase (*VATP16*), SAND family protein (*SAND*) and Ubiquinol-cytochrome c reductase complex chaperone (*UQCC*), melting curves profiles revealed non-specific amplification and primer dimer formation on the amplicon region (Additional file 1), rendering these genes as unsuitable for further analysis (Table 1). For all remaining genes, melting curve analysis showed no amplification peak in the no-template controls (NTCs) (Additional file 1).

PCR efficiency of each primer pair was calculated through the standard curve method using a pool of all cDNA samples in a ten-fold serial dilution. The amplification efficiency (E) of the reactions ranged from 1.886 (89%) to 2.033 (103%) with the correlation coefficients 

\[ R^2 \]

varying from 0.98 to 0.99 (Table 1). To assure that any variation between biological replicates was not related to the treatments but intrinsic to the gene itself, data from the biological replicates was analysed separately by statistical algorithms [9,10].

**Analysis of gene expression stability data**

The expression stability of the selected RGs was addressed by three different statistical applets: GeNorm, NormFinder and BestKeeper. The results were analyzed by dividing the data into three different datasets: all samples in the assay (entire dataset), biotic stress applied to the coffee cultivars Catimor 88 and Caturra, separately.

**GeNorm**

The geNorm algorithm calculates the gene expression stability measure (M value) for each gene based on its average pairwise expression ratio relative to each of the other genes in the analysis. A gene displaying a high M value presents a high variance in its expression. After, GeNorm estimates the normalization factor (NF) using the geometric mean of expression levels of n best reference genes, using a pairwise variation (V) with a cut-off value of 0.15 as threshold [11].

The gene encoding for an Insuline Degrading Enzyme (*IDE*) presented high stability in all datasets analysed (lower M value), however, different combinations of genes showed low M values when samples were analyzed only considering one coffee genotype. Therefore, the gene pairs indicated for expression normalization were *IDE/14-3-3* protein (*14-3-3*) (Table 2) for the entire dataset, *IDE/Tubulin beta-9 (*β-tub9*) (Table 3) for the susceptible cultivar Caturra during inoculation time-course and *IDE/60S ribosomal protein L7 (*RPL7*) (Table 3) for the resistant variety Catimor 88 during inoculation time-course. The orthologous gene *IDE* was defined as a reference gene for common bean hypocotyls inoculated with *Colletotrichum lindemuthianum* [12]. In other studies, this same gene was also determined as the most stable and suitable for validation of subtractive libraries of common bean during compatible and incompatible interactions with the rust fungus *Uromyces appendiculatus* [13]. *IDE* gene encodes for a
| Gene (coffee source gene) | Reference | Primer sequence | Amplicon length (bp) | Ta (°C) | Tm (°C) | PCR efficiency (%) | Regression coefficient (R²) | Average Cq value |
|---------------------------|-----------|-----------------|----------------------|---------|---------|-------------------|-----------------------------|------------------|
| VATP16 (GT00136.1)*      | Gamm et al. [29] | Fw: ATAGAGAGAGAGCCCAATTC<br>Rev: CCGAGGAAGGCAAAAAAG | 150 | 60 | 78.63 | | | |
| UQCC (isotig09120)       | Discarded (unspecific amplification, primer dimer) | | | | | | | |
| SAND (isotig05620)       | Selim et al. [30] | Fw: GATTGGTCAGAAGGCTGTT<br>Rev: GGACCACGCACACCAACAC | 116 | 60 | 78.70 | | | |
| UBQ9 (AF29708)*          | Ramiro et al. [7] | Fw: AACATTGGGAGGTGTTCTG<br>Rev: GCAGAAAACCAACTAAGACCAAA | 79 | 60 | 76.16 | 92 | 0.987 | 24.5 ± 0.95 |
| S24 (SGN-U349723)*      | Cruz et al. [28] | Fw: GCCCAAATACGCGCTATCA<br>Rev: TCTCTTGGGCGCTGTTGTC | 92 | 60 | 76.60 | 91 | 0.996 | 20.61 ± 1.04 |
| IDE (isotig10635)        | Borges et al. [12] | Fw: GCCTGCCACGAACTGATGA<br>Rev: AGATGCCACTGCTGATGAT | 91 | 55 | 76.28 | 92 | 0.994 | 24.46 ± 1.30 |
| β- Tub9 (isotig08544)    | | Fw: ACCCTCCAGCAAATCTGAAG<br>Rev: TCAGGTGCGATGATT | 100 | 55 | 77.27 | 96 | 0.996 | 19.40 ± 0.82 |
| 14-3-3 (SGN-U356404)*    | Barsalobres-Cavallari et al. [18] | Fw: CTCCAAGCACATATGTGTTAAC<br>Rev: AACATGGGTGCACTGCTGCT | 75 | 60 | 73.33 | 103 | 0.999 | 22.76 ± 1.47 |
| GADPH (SGN-U347734)*     | Barsalobres-Cavallari et al. [18] | Fw: TGGAGGCGCTGCGAA<br>Rev: AACAAGGTGCACTGCTGCT | 59 | 60 | 75.73 | 96 | 0.998 | 21.42 ± 1.96 |
| RPL7 (SGN-U351477)*      | Barsalobres-Cavallari et al. [18] | Fw: CATTGGCTATCAGATGCTATG<br>Rev: TGTCGTCGAGGCAAGCT | 66 | 55 | 76.48 | 89 | 0.999 | 23.76 ± 1.28 |

**Genes of interest (GOIs)**

| PR10 (CF589103)*         | Ramiro et al. [7] | Fw: GCCACCATCCCTGAAGAGAA<br>Rev: CAACTCTCTCTGCGGCTGCT | 151 | 55 | 80.17 | 99 | 0.999 | 19.35 ± 2.28 |
| RLK (CF589181)*          | | Fw: ATGGGAGAAGGAGATGCGCAGAG<br>Rev: GCCCAATTACAGTTTGGAACAC | 189 | 55 | 81.15 | 91 | 0.998 | 24.81 ± 2.02 |

*Unigene accession number according to the SOL Genomics Network.
Ta, annealing temperature; Tm, melting temperature; SD, standard deviation. * NCBI accession number or TC TIGR number.
peptidase that could be related to the cleavage control of proline-rich signalling proteins [14]. We may point out that due to its important role in basic cell processes, this gene could be a good reference gene. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Ubiquitin (UBQ9) were the least stable genes in both genotypes.

The analysis carried out by geNorm also enables the determination of the optimal number of reference genes, through the calculation of pairwise variation (\(V_{n}/V_{n+1}\)) between two sequential candidate genes. High values indicate the need for the inclusion of another gene to obtain a reliable normalization factor, which should contain at least two internal controls. Thus, extra reference genes can be included until the \(V_{n}/V_{n+1}\) value is smaller than a threshold of 0.15 as recommended by Vandesompele et al. [11]. Based on this parameter, the use of three reference genes were set for the entire dataset (V3/4 = 0.148), four reference genes for Catimor 88 (V4/5 = 0.145) and two or three reference genes (V2/3 = 0.148, V3/4 = 0.109) for Catimor hypocotyls inoculated with C. kahawae (Figure 1). For culti-var Caturra during inoculation time-course \(β\)-Tub9 and S24 were considered the most stable with SD values of 0.46 and 0.48, respectively, with the best correlations \(r = 0.882\) and 0.787, respectively) and a \(p\) value of 0.005.

### Table 2 Reference genes ranking for the entire dataset calculated by the GeNorm, NormFinder and BestKeeper

| Gene | GeNorm | Normfinder | BestKeeper |
|------|--------|------------|------------|
| IDE  | 0.578(1/2) | 0.402(1) | 2.54 ± 0.62(2) | 0.801* |
| 14-3-3 | 0.578(1/2) | 0.624(4) | 2.61 ± 0.59(4) | 0.723* |
| RPL7 | 0.604(3) | 0.542(3) | 1.98 ± 0.47(1) | 0.701* |
| \(β\)-Tub9 | 0.684(4) | 0.478(2) | 2.44 ± 0.47(3) | 0.720* |
| S24  | 0.731(1/2) | 0.610(5) | 2.79 ± 0.57(5) | 0.660* |
| UBQ9 | 0.785(6) | 0.699(6) | 2.23 ± 0.54(6) | 0.560 |
| GAPDH | 1.019(7) | 1.597(7) | n.a | n.a |

SV: stability value; CV, coefficient of variance; SD, standard deviation of Cq value; \(r\), Pearson coefficient of correlation; * \(p < 0.001\), \(p\)-value associated with the Pearson coefficient of correlation. Ranking order is presented in parenthesis, n.a., not assigned.

### Table 3 Reference genes ranking for Catimor 88 and Caturra inoculated with C. kahawae

| Biotic stress | Catimor 88 (resistant) | Caturra (susceptible) |
|--------------|------------------------|-----------------------|
| Gene | GeNorm | NormFinder | BestKeeper | r | GeNorm | NormFinder | BestKeeper | r |
| IDE | 0.640(1) | 0.505(2) | 2.82 ± 0.69(4) | 0.790* | 0.392(1/2) | 0.358(3) | 2.09 ± 0.50(2) | 0.786* |
| 14-3-3 | 0.669(2) | 0.759(4) | 2.59 ± 0.58(3) | 0.689* | 0.518(5) | 0.517(5) | 2.71 ± 0.61(5) | 0.778* |
| RPL7 | 0.640(3) | 0.626(3) | 2.09 ± 0.49(1) | 0.619* | 0.502(4) | 0.455(4) | 1.86 ± 0.44(1) | 0.684* |
| \(β\)-Tub9 | 0.795(4) | 0.479(1) | 2.34 ± 0.45(2) | 0.726* | 0.392(1/2) | 0.322(1) | 2.37 ± 0.46(4) | 0.822* |
| S24 | 0.914(5) | 0.807(6) | 2.87 ± 0.59(5) | 0.543 | 0.476(3) | 0.341(2) | 2.34 ± 0.48(3) | 0.787* |
| UBQ9 | 0.870(6) | 0.735(5) | 1.75 ± 0.43(6) | 0.303 | 0.648(7) | 0.649(7) | 2.61 ± 0.63(6) | 0.642* |
| GAPDH | 1.169(7) | 1.779(7) | n.a | n.a | 0.600(6) | 0.580(6) | 2.85 ± 0.58(7) | 0.778* |

SV: stability value; CV, coefficient of variance; SD, standard deviation of Cq value; \(r\), Pearson coefficient of correlation; * \(p < 0.05\), \(p\)-value associated with the Pearson coefficient of correlation. Ranking order is presented in parenthesis, n.a., not assigned.
For Catimor 88, IDE was selected as the most stable with an SD value of 0.69, high correlation \( r = 0.790 \) and a p value of 0.005 (Table 3).

Interestingly, despite belonging to important classes of cellular functioning, GAPDH and UBQ9 were indicated by BestKeeper analysis as the least stable expressed genes for the entire dataset and for Catimor 88 under biotic stress, which was consistent with the results obtained from geNorm and NormFinder. GAPDH has been widely used as a reference gene in many experimental conditions [17] and is one of the best RGs for measuring gene expression in different \( C. arabica \) tissues/organs, namely root, stem, leaf, flower and fruit [18]. However previous examples of this gene leading to wrong results have also been reported due to its lack of stability under specific experimental conditions [17,19]. In the present analysis, GAPDH was not among the best RGs between experimental groups. One of the possible reasons for those discrepancies may be that GAPDH not only acts as a component of the glycolytic pathway but takes part in other processes as well, thus the expression profile of GAPDH might fluctuate according to the corresponding experimental conditions. UBQ9 was also pointed out as a good RG for \( C. arabica \) leaves inoculated with \( Hemileia vastatrix \) [7]. Yet, for coffee hypocotyls inoculated with \( C. kahawae \) it was considered one of the least stable genes, which is in accordance with Borges et al. [12] whom pointed out Ubiquitin as one of the least stable genes for common bean inoculated with \( C. lindemuthianum \) in several tissues including hypocotyls. These variations in the expression profiles of genes normally used as internal controls confirm the need for validation of RGs under each specific condition of interest.

**A comprehensive ranking analysis considering the three applets (Additional file 2) revealed that, in the specific pathosystem coffee hypocotyls-\( C. kahawae \), the two most stable genes, were IDE and \( \beta \)-Tub9 for the resistant genotype and the combination IDE + \( \beta \)-Tub9 or S24+ \( \beta \)-Tub9 for the susceptible genotype.**

**Expression analysis of PR10 and RLK**

The coffee RLK gene is predicted to encode a receptor-like kinase [20], being some members of this class of proteins involved in resistance signalling pathways [21], while PR10 seems to be related with the jasmonic acid (JA)-dependent resistance pathway [22] and to accumulate in host cells in incompatible interactions [23,24]. Both PR10 and RLK genes have been described to be induced during coffee infection with \( Hemileia vastatrix \) [8,20].

The expression of both PR10 and RLK was studied during both coffee compatible and incompatible interactions with \( C. kahawae \). Three normalization strategies were followed in order to validate the results obtained by GeNorm, Normfinder and Bestkeeper: 1) using the two best RGs given by the comprehensive ranking, (2) selecting the optimal number of RGs based on GeNorm pairwise variation value (the 0.15 cut-off value was followed) and (3) using the two least stable RGs. Thus, PR10 and RLK expression was normalized in Catimor 88 with a normalization factor (NF) calculated based on the expression of (1) IDE and \( \beta \)-Tub9, (2) RPL7, IDE, 14-3-3 and \( \beta \)-Tub9 and (3) GAPDH and UBQ9. For Caturra, the NF was calculated based on the expression of (1) IDE and \( \beta \)-Tub9 or S24 and \( \beta \)-Tub9, (2) IDE, S24 and \( \beta \)-Tub9 and (3) GAPDH and UBQ9.

When comparing the resistant genotype Catimor 88 after inoculation with \( C. kawahae \) with control samples, RLK expression increased to reach a maximum at 48hpi and decreased thereafter, while PR10 expression increased during inoculation time-course (Figure 2). Considering the normalization strategies tested, the expression level of both RLK and PR10 did not differ significantly when using the two best RGs from the three applets or the four best RGs given by GeNorm V = 4/5 (Figure 1). Using the two best RGs, the expression levels were higher than 11.3 –fold for RLK and 5.5-fold for PR10, decreasing slightly to 8.5-fold for RLK and 4.0-fold for PR10, when data were normalized with the four best RGs from geNorm V = 4/5. In contrast, data normalization with the two more unstable RGs, GAPDH and UBQ9, led to significant variable expression levels for both genes analysed, presenting also higher SD values (Figure 2A, Additional file 3), which corroborates the indications from the expression stability analyses that these genes should not be used as RGs for Catimor 88 hypocotyls inoculated with \( C. kahawae \).
For Caturra inoculated with C. kahawae, β-Tub9 appeared as the most stable, followed by both IDE and S24. Thus, normalization with the two best RGs was tested using either a combination of IDE + β-Tub9 or S24+ β-Tub9. When analysing RLK and PR10 expression in comparison to control samples, no significant expression differences were obtained at 12 hpi with the three normalization strategies tested (Figure 2B, Additional file 3). Also, at 48 hpi and 72 hpi, using the two best RGs for normalization (two best genes selected by the statistical applets and also by GeNorm V2/3 value), expression levels and SD values did not vary significantly either with IDE + β-Tub9 or S24+ β-Tub9 (Figure 1). However, when using the combination S24+ β-Tub9, lower SD values were obtained and PR10 and RLK expression levels were very similar to those obtained with the three best RGs given by GeNorm V3/4 value (Additional file 3). It may be considered that despite IDE and S24 shared the second position in the comprehensive ranking of the three statistical applets; S24 is more stable than IDE and thus S24+ β-Tub9 combination should be used for normalization. Interestingly, expression of both genes was not significantly altered when normalizing with the two most unstable genes (GADPH and UBQ9). On the contrary, PR10 and RLK expression levels were slightly smaller or presented higher SD values when normalizing with the three best RGs given by geNorm V = 3/4 (Figure 1). For the susceptible coffee cultivar Caturra

![Figure 2](link)
inoculated with C. kahawae, despite being considered the most unstable RGs by GeNorm, NormFinder and BestKeeper, GADPH and UBQ9 presented a more stable expression than in the resistant genotype Catimor 88 (Figure 2). These results reflect the need to not only validate reference genes for specific experimental conditions, but also for different genotypes.

A non-parametric test was used to account for any significant differences in the expression of PR10 and RLK calculated with the different normalization strategies (Additional file 4). As no statistically differences were found, we may assume that for an accurate normalization, in the present experimental system, the two most stable RGs suggested by the three applets may be used, i.e. IDE + β-Tub9 for Catimor 88 and S24+ β-Tub9 for Caturra.

Conclusions
In the present study, we have evaluated the expression stability of 10 candidate reference genes along C. kahawae inoculation time-course of two coffee genotypes showing a susceptible vs resistant response towards this pathogen, aiming to identify a set of stable reference gene(s) for data normalization of gene expression studies. Analysis of expression stability using GeNorm, NormFinder and Bestkeeper revealed that the expression of β-Tub9 and IDE is most stable across all datasets tested. In addition, several normalization strategies were tested under an additional validation step using RLK and PR10, and statistical analysis revealed that for an accurate normalization the two most stable RGs suggested by the three applets should be used and that a different combination of reference genes should be applied for susceptible and resistant genotypes inoculated with C. kahawae. Thus IDE + β-Tub9 should be used for transcript normalization of Catimor 88 hypocotyls inoculated with C. kahawae and S24+ β-Tub9 for transcript normalization of Caturra hypocotyls inoculated with C. kahawae.

Since, up to our knowledge, no reference genes have yet been established for inoculation studies of coffee hypocotyls, we believe that the information provided by our study will greatly facilitate future coffee research and enable sensitive and accurate quantification of gene expression in coffee genotypes showing different degrees of resistance towards C. kahawae.

Methods
Inoculation of coffee hypocotyls
Hypocotyls were used as a model material to study CBD in controlled conditions since previous studies have shown a correlation between the pre-selection test on hypocotyls and mature plant resistance in the field \( r = 0.73–0.80 \) [25]. Coffee seeds were sown in greenhouse conditions at temperatures between 16°C and 28°C (average minimum and maximum temperatures respectively), during 8 weeks. Conidia of C. kahawae isolate Que2 (from Kenya) were produced after 7 days at 22°C on extract malt agar [26]. Hypocotyls of cultivars Catimor 88 (from Kenya) and Caturra (CIFC 19/1), resistant and susceptible to the C. kahawae isolate used, were then inoculated according to the technique described [25] with slight modifications. Briefly, hypocotyls were vertically placed on plastic trays containing a wet nylon sponge and sprayed with a conidia suspension \( (2 \times 10^6/\text{ml}) \) or with water (mock-inoculated hypocotyls – control samples). After inoculation, trays were covered with plastic bags and kept in a Phytotron 750 E at 22°C in the dark for 24 h, and then under a photoperiod of 12 hours during the infection time-course.

Sample collection
According to previous microscopic analysis [27], hypocotyl tissues were sampled at times corresponding to different stages of pathogenesis: i) Conidia germination and appressoria differentiation (in both coffee genotypes) at 12 hpi; ii) fungal penetration and establishment of biotrophic phase (susceptible genotype) or beginning of hypersensitive cell death (HR) and accumulation of phenols (resistant genotype) at 48 hpi; iii) switch to the necrotrophic phase (susceptible genotype) or display of HR and phenols deposition in more that 50% of infection sites (resistant genotype) at 72 hpi. Hypocotyls were thus harvested at 12, 48 and 72 hours post inoculation (hpi). Two independent experiments were conducted and 40 hypocotyls were collected for each coffee genotype (Catimor 88 and Caturra, control and inoculated) at each of the three time-points. Plant material was immediately frozen in liquid nitrogen and stored at 80°C.

RNA extraction and cDNA synthesis
Total RNA was isolated from hypocotyls with the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, USA) according to the manufacturer’s instructions. Residual genomic DNA was digested with DNase I (On-Column DNase I Digestion Set, Sigma-Aldrich, USA). RNA purity and concentration were measured at 260/280 nm and 260/230 nm using a spectrophotometer (NanoDrop-1000, Thermo Scientific), while RNA integrity was verified by agarose gel electrophoresis. Genomic DNA (gDNA) contamination was checked by qPCR analysis of a target on the crude RNA [11]. Complementary DNA (cDNA) was synthesized from 2.5 μg of total RNA using RevertAid™H Minus Reverse Transcriptase (Fermentas, Ontario, Canada) anchored with Oligo (dT)\(_{18}\) primer (Fermentas, Ontario, Canada), according to manufacturer’s instructions.
Candidate gene selection and primer design

Ten candidate genes were selected based on their previous reports of suitable qPCR reference genes associated either to Coffea arabica or to biotic stress [7,12,18,28-30]. Five of these genes were previously described as reference genes for C. arabica: S24, 14-3-3, RPL, GAPDH and UBQ9. The other genes were retrieved from coffee (HDT 832/2) database (unpublished data) as being homologous to grapevine V ATP16, SAND, UQCC and homologous to common bean IDE and β-Tub9.

Specific primers (Table 1) were designed with Primer Express software version 3.0 (Applied Biosystems, Sourceforge, USA) using the following parameters: amplicon length between 75 and 250 bp; size: 20 ± 2 bp; annealing temperature (Ta) between 55°C and 60°C; GC content: ± 50%.

Quantitative real time PCR

Quantitative RT-PCR (qPCR) experiments were carried out using Maxima™ SYBR Green qPCR Master Mix (2×) kit (Fermentas, Ontario, Canada) in a StepOne™ Real-Time PCR system (Applied Biosystems, Sourceforge, USA). A final concentration of 2.5 mM MgCl2 and 0.2 μM of each primer were used in 25 μL volume reactions, together with cDNA as template. The amplification efficiency of each candidate/target gene was determined using a pool of identical volumes of all cDNA samples. The pool was diluted and used to generate a five-point standard curve based on a ten-fold dilution series. Each standard curve was amplified in two independent qPCR runs and each dilution was run in triplicate. Amplification efficiency (E) was calculated from the slope of the standard curve (E = 10(-1/a) -1) where a is the slope of the linear regression model (y = a log(x) + b) fitted over log-transformed data of the input cDNA concentration (y) plotted against Cq values (x).

To investigate candidate reference gene stability, cDNA samples were 10-fold diluted. Thermal cycling for all genes started with a denaturation step at 95°C for 10 min followed by 45 cycles of denaturation at 95°C for 15 s and annealing temperatures (Table 1) for 30 s. Each set of reactions included a negative control with no template. Dissociation curves and agarose gel electrophoresis were used to analyze non-specific PCR products. Two biological replicates and three technical replicates were used for each sample.

Determination of gene expression stability

The expression stability of each candidate reference gene and the best combination of reference genes were obtained using a pairwise method by GeNorm [11], a model-based method by NormFinder [15] software and the BestKeeper tool [16]. The analysis was performed considering three groups: resistant hypocotyls (Catimor 88) compared to control (mock-inoculated) samples dataset, susceptible hypocotyls (Caturra) compared to control (mock-inoculated) samples dataset and entire dataset. The definition of the optimal number of genes required for normalization was achieved by GeNorm pairwise variation analysis [31]. A comprehensive ranking was established by calculating the arithmetic mean ranking value of each gene using the three applets [32], and each gene was ranked from 1 (most stable) to 7 (least stable).

Finally, RefFinder (http://www.leonxie.com/referencegene.php) was used as a verification tool of our results (Additional file 5). RefFinder is a comprehensive tool that integrates the currently available major computational programs (GeNorm, NormFinder, BestKeeper, and the comparative ΔCt method) and based on the rankings from each program assigns an appropriate weight to an individual gene, and calculates the geometric mean of their weights for the overall final ranking [33].

PR10 and RLK gene expression

The expression of two defense-related genes, a receptor like kinase (RLK, CF589181) and a pathogenesis-related protein 10 (PR10, CF589103), previously described as being differentially expressed in coffee leaves inoculated with Hemileia vastatrix [7,8] was studied in the coffee-C. kahawae interaction. Three normalization strategies were tested: 1) using the two top genes given by a comprehensive ranking considering the three methods (GeNorm, NormFinder and BestKeeper); 2) using the optimal number of reference genes selected by the GeNorm V value (pairwise variation analysis, Figure 1) for each condition studied, and 3) using the two most unstable genes considering the comprehensive ranking of the three methods (GAPDH and UBQ9).

To assess gene expression, relative quantities (RQ) were calculated for both RGs and genes of interest (GOIs) by the formula RQ = 2ΔCq, where E represents the amplification efficiency (E) for each gene and ΔCq the difference in the Cq from each target sample and calibrator (ΔCq = Cqcalibrator – Cqtarget) [16,34]. A normalization factor calculated as the geometric mean of the relative expression of the RGs selected for each normalization strategy was used to obtain the normalized relative quantities (NRQ) [35].

The statistical significance (p < 0.05) between the three normalization strategies used was determined by the Kruskall–Wallis test using IBM® SPSS® Statistics version 20.0.0 (SPSS Inc., USA) software.

Additional files

Additional file 1: Figure S1. Primer specificity test through dissociation curve analysis collected from StepOne™ software ver. 2.2.2 (Applied Biosystems). 14-3-3 (A), IDE (B), RPL7 (C), S24 (D), β-Tub9 (E), GADPH (F).
Acknowledgements
This work was funded by Portuguese national funds through Fundação para a Ciência e a Tecnologia (project PTD/AGR-GPL/112217/2009, grants SFRH/BPD/47008/2008 and SFRH/BPD/63641/2009) and through BIOFISQ PEST-OE/BIA/UI4046/2011. We also appreciate the technical support provided by Paula Leandro and Sandra Sousa.

Author details
1 Plant Systems Biology Lab, Center of Biodiversity, Functional & Integrative Genomics (BioFIG), Science Faculty of Lisbon University, Lisbon 1749-016, Portugal. 2 CFC-Biotrop/ICT-Instituto de Investigação Científica Tropical, Quinta do Marquês, Ceasar 2784-505, Portugal. 3 Coffee Research Foundation (Crf), P.O. Box 4-00232, Ruini, Kenya.

Received: 21 May 2013 Accepted: 25 September 2013 Published: 28 September 2013

References
1. Waller J, Bridge P, Black R, Haskia G. Characterization of the coffee berry disease pathogen, Colletotrichum kahawae sp. Mycol Res 1993, 97:989-994.
2. Silva MC, Vazera V, Guerra-Guimarães L, Azinheira HG, Fernandez D, Petiot A-S, Bertrand B, Lashermes P, Nicole M. Coffee resistance to the main diseases: leaf rust and coffee berry disease. Braz J Plant Physiol 2006, 18:119-147.
3. Loureiro A, Nicole M, Vazera V, Moncada P, Bertrand B, Silva M. Coffee resistance to Colletotrichum kahawae is associated with lignification, accumulation of phenols and cell death at infection sites. Physiol Mol Plant Pathol 2012, 77(1):23-32.
4. Van Der Vossen H, Waluyo D. Additional evidence for oligogenic inheritance of durable host resistance to coffee berry disease (Colletotrichum kahawae) in arabica coffee (Coffee arabica L.). Euphytica 2009, 165(1):105-111.
5. McDonald J. A preliminary account of a disease of green coffee berries in Kenya Colony. 1926, 11:145-154.
6. Chen Z, Nunes M, Silva M, Rodrigues C. Appressorium turgor pressure of Colletotrichum kahawae might have a role in coffee cuticle penetration. Mycologia 2004, 96(6):1199-1208.
7. Ramiro D, Escoute J, Petiot A, Nicole M, Maluf M, Fernandez D. Biphasic haustorial differentiation of coffee rust (Hemileia vastatrix race 8) associated with defence responses in resistant and susceptible coffee cultivars. Plant Pathol 2009, 58(5):944-955.
8. Diniz I, Talhinhas P, Azinheira H, Varzea V, Medeira C, Maia I, Petiot A, Nicole M, Fernandez D, Silva M. Cellular and molecular analyses of coffee resistance to Hemileia vastatrix and nonhost resistance to Uromyces vignae in the resistance-donor genotype HD9832/2. Eur J Plant Pathol 2012, 133(1):141-157.
9. Remans T, Smeets K, Opdenakker K, Mathijssen D, Vangronsveld J, Cuypers A. Normalisation of real-time RT-PCR gene expression measurements in Arabidopsis thaliana exposed to increased metal concentrations. Plant Bio 2008, 227(6):1435-1439.
10. Castro P, Roman B, Rubio I, De J. Selection of reference genes for expression studies in Cicer arietinum L: analysis of cy3/5E3 gene expression against Ascochyta rabiei. Mol Breed 2012, 29(1):261-274.
11. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalisation of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002, 3(7).
12. Borges A, Tsai S, Caldas D. Validation of reference genes for RT-qPCR normalization in common bean during biotic and abiotic stresses. Plant Cell Rep 2012, 31(5):827-838.
13. Thibivilliers S, Joshi T, Campbell K, Scheffler B, Xu D, Cooper B, Nguyen H, Stacey G. Generation of Phaeolus vulgaris ESTs and investigation of their regulation upon Uromyces appendiculatus infection. BMC Plant Biol 2009, 9:46.
14. Cunningham D, O’Connor B. Proline specific peptidases. Biochem Et Biophys Acta-Protein Struct Mol Enzymol 1997, 1334(2):160-186.
15. Andersen C, Jensen J, Omtont T. 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 2004, 64(15):5245-5250.
16. Pfaffl M, Tichopad A, Prgomet C, Neuvians T. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper - Excel-based tool using pair-wise correlations. Biotechnol Lett 2004, 26(6):509-515.
17. Suzuki T, Higgins P, Crawford D. Control selection for RNA quantitation. Biotechniques 2000, 29(1):32-41.
18. Bansalbhes-Cavallari C, Severino F, Maluf M, Maia I. Identification of suitable internal control genes for expression studies in Coffea arabica under different experimental conditions. BMC Mol Biol 2009, 10:1.
19. Schmidt G, Delaney S. Stable internal reference genes for normalization of real-time RT-PCR in tobacco (Nicotiana tabacum) during development and abiotic stress. Mol Gen Genomics 2010, 283(3):233-241.
20. Fernandez D, Santos P, Agostini C, Bon M, Petitot A, Silva M, Guerra Guimaraes L, Ribeiro A, Argout X, Nicole M. Coffee (Coffee arabica L) genes early expressed during infection by the rust fungus (Hemileia vastatrix), Mol Plant Pathol 2004, 5(6):527-536.
21. Morris E, Walker J. Receptor-like protein kinases: the keys to response. Curr Opin Plant Biol 2003, 6(4):339-342.
22. van Loon L, Rep M, Pieterse C. Significance of inducible defense-related proteinases in infected plants. Annu Rev Phytopathol 2006, 44:35-52.
23. Figueiredo A, Almeida P, Fortes A, Bonow-Rex M, Zyprian E, Sousa L, Pais M. Cultivar-specific kinetics of gene induction during downy mildew early infection in grapevine. Funct Integr Genomics 2012, 12(2):379-386.
24. Söh H, Park A, Park S, Back K, Yoon J, Park H, Kim Y. Comparative analysis of pathogenesis-related protein 10 (PR10) genes between fungal resistant and susceptible peppers. Eur J Plant Pathol 2012, 132(1):37-48.
25. van der Vossen H, Cook R, Marakar G. Breeding for resistance to coffee berry disease caused by Colletotrichum coffeae in Doi Inthanon (sensa Hindorf) in Coffee arabica L. Herb pre-selec res Euphytica 1976, 25:733-745.
26. Rodrigues C, Varzea V, Hindorf H, Medeiros E. Strains of Colletotrichum-Coffeae noac causing coffee berry disease in Angola and Malawi with characteristics different to the Kenya strain. J Phytopathol-Phytopathol Zeitsschrift 1991, 131(3):205-209.
27. Loureiro A, Figueiredo A, Batista D, Baraldi T, Várzea V, Azinheira HG, Talhinhas P, Pais MS, Gichuru EK, Silva MC: New cytological and molecular data on coffee – Colletotrichum kahawae interactions. Proceed 24th Int Conf Coffee Sci (ASIC) 2012:593–598.

28. Cruz F, Kalaoun S, Nobile P, Colombo C, Almeida J, Barros L, Romano E, Grossi-de-Sa M, Vaslin M, Alves-Ferreira M: Evaluation of coffee reference genes for relative expression studies by quantitative real-time RT-PCR. Mol Breed 2009, 23(4):607–616.

29. Gamm M, Heloir M, Kelloniemi J, Poinssot B, Wendehenne D, Adrian M: Identification of reference genes suitable for qRT-PCR in grapevine and application for the study of the expression of genes involved in pterostilbene synthesis. Mol Genet Genomics 2011, 285(4):273–285.

30. Selim M, Legay S, Berkelmann-Lohnertz B, Langen G, Kogel K, Evers D: Identification of suitable reference genes for real-time RT-PCR normalization in the grapevine-downy mildew pathosystem. Plant Cell Rep 2012, 31(1):205–216.

31. Tunbridge E, Eastwood S, Harrison P: Changed Relative to What? Housekeeping Genes and Normalization Strategies in Human Brain Gene Expression Studies. Biol Psychiatry 2011, 69(2):173–179.

32. Wang Q, Ishikawa T, Michiue T, Zhu B, Guan D, Maeda H: Stability of endogenous reference genes in postmortem human brains for normalization of quantitative real-time PCR data: comprehensive evaluation using geNorm, NormFinder, and BestKeeper. Int J Leg Med 2012, 126(6):943–952.

33. Zhu X, Li X, Chen W, Chen J, Lu W, Chen L, Fu D: Evaluation of new reference genes in papaya for accurate transcript normalization under different experimental conditions. Plos One 2012, 7(8).

34. Livak K, Schmittgen T: Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(−Delta Delta C) method. Methods 2001, 25(4):402–408.

35. Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J: qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol 2007, 8(2).

doi:10.1186/1756-0500-6-388

Cite this article as: Figueiredo et al: Validation of reference genes for normalization of qPCR gene expression data from Coffea spp. hypocotyls inoculated with Colletotrichum kahawae. BMC Research Notes 2013 6:388.