Reference Gene Validation for Quantitative RT-PCR during Biotic and Abiotic Stresses in *Vitis vinifera*

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Abstract

Grapevine is one of the most cultivated fruit crop worldwide with *Vitis vinifera* being the species with the highest economical importance. Being highly susceptible to fungal pathogens and increasingly affected by environmental factors, it has become an important agricultural research area, where gene expression analysis plays a fundamental role. Quantitative reverse transcription polymerase chain reaction (*q*RT-PCR) is currently amongst the most powerful techniques to perform gene expression studies. Nevertheless, accurate gene expression quantification strongly relies on appropriate reference gene selection for sample normalization. Concerning *V. vinifera*, limited information still exists as for which genes are the most suitable to be used as reference under particular experimental conditions. In this work, seven candidate genes were investigated for their stability in grapevine samples referring to four distinct stresses (*Erysiphe necator*, wounding and UV-C irradiation in leaves and *Phaeomoniella chlamydospora* colonization in wood). The expression stability was evaluated using geNorm, NormFinder and BestKeeper. In all cases, full agreement was not observed for the three methods. To provide comprehensive rankings integrating the three different programs, for each treatment, a consensus ranking was created using a non-weighted unsupervised rank aggregation method. According to the last, the three most suitable reference genes to be used in grapevine leaves, regardless of the stress, are *UBC*, *VAG* and *PEP*. For the *P. chlamydospora* treatment, *EF1*, *CYP* and *UBC* were the best scoring genes. Acquaintance of the most suitable reference genes to be used in grapevine samples can contribute for accurate gene expression quantification in forthcoming studies.

Introduction

Grapevine is one of the most cultivated fruit crop worldwide with *Vitis vinifera* being the species with the highest economical importance due to the high quality standards of its berries. Nonetheless, it is also the most susceptible *Vitis* species to fungal pathogens which can have devastating consequences in the vineyards [1,2]. The associated crop losses, together with the elevated financial and environmental costs to control these diseases, have made *Vitis vinifera* and its pathogens an increasingly important research area. Common approaches to study the corresponding pathosystems often involve, at least at one point, transcriptomic analysis and gene expression quantification [3–5]. Among the several existing techniques to analyze mRNA levels, *q*RT-PCR is currently the most widely used due to its high sensitivity and reproducibility [6]. However, accurate gene expression quantification strongly relies on appropriate reference gene selection for sample normalization [7–9]. Though this requirement has always been an important criterion for gene expression quantification studies, during the early stages of *q*PCR expansion and data analysis development, reference gene selection was rather based on assumptions more than evidence on expression stability. As consequence, several studies might have been conducted using unsuitable or unvalidated reference genes [10,11]. Recent awareness regarding this matter has lead to an increasing number of studies whose main objective is to evaluate the expression stability of candidate genes for normalization in *q*PCR analysis [12–15]. Under the most diverse experimental conditions, including a variety of organisms or tissues and a multitude of biotic and abiotic stimulus, such analysis can provide a valuable tool for accurate gene expression quantification in forthcoming studies. To assess the gene expression stability of potential reference genes, several programs and statistical algorithms have been developed, facilitating the analysis and selection of suitable reference genes for the desired experimental condition. geNorm, NormFinder and BestKeeper are among the most widely used algorithms [7,16,17]. With respect to *V. vinifera*, a non-model organism, some studies have already been conducted in order to evaluate and validate *q*RT-PCR reference genes to be used under distinct physiological and pathological conditions. Those comprise specific aspects of *V. vinifera* such as genotypes,

organs, developmental stages, abiotic stresses and biotic stresses: (i) berries at different stages of development [18]; (ii) leaves subjected to two different abiotic stresses: drought and temperature [19]; (iii) berries from different genotypes at distinct phenological stages, treated or not with gibberellic acid [20]; (iv) leaves at late stages of infection with *Plasmopara viticola* and berries at late stages of infection with *Botrytis cinerea* [11]; (v) two genotypes of *V. vinifera* leaves exhibiting differential sensitivity to *Plasmopara viticola* at the early stages of infection with the oomycete [21]; (vi) leaves infected with *Plasmopara viticola* at late stages of infection [22]. Also, a previous work performed in our laboratory evaluates reference genes to be used in leaf samples at late stages of infection with *Erysiphe necator* [23].

Despite the valuable contribution of the previous works, other stress conditions of great importance to plant studies, such as the abiotic stresses caused by wounding or UV-C irradiation, remain to be addressed for this species [24]. Due to their sessile nature, plants are permanently exposed to a wide range of structurally damaging agents which include environmental stresses such as wind, rain or hail, and herbivore attack. Wound occurrence is inevitable and, besides compromising the physical structure of the plant, it constitutes a potential infection site for pathogens [24,25]. To cope with this dual threat, plants might have evolved to integrate both wounding and pathogen response. In fact, some studies have already demonstrated that both stresses can share common signaling pathways and, moreover, regulate the same stress responsive genes. For this reason, wound response stands as an important area of interest in plant studies [26,27]. As for the UV-C stimulus, though such short wavelength radiation is not likely to reach the ground, it has been shown, in several species, that UV-C irradiation can enhance host resistance to pathogens

[28]. Also, in the particular case of *V. vinifera*, UV-C was shown to induce the accumulation of several phenolic defense-related compounds, including resveratrol and other stilbene derivatives. Thus, UV-C irradiation constitutes a practical experimental model to study plant defense responses [29,30].

Concerning the biotic stresses affecting grapevine, besides *P. viticola* and *E. necator*, also *Phaeomoniella chlamydospora* stands amongst the most concerning pathogens for viticulture. While the former oomycetes infect the leaves and berries of the host, the second is a wood colonizing fungus known to participate in the esca disease complex. Taken together, these diseases account for huge economical losses and, therefore, represent priority research areas [21,31–33]. Yet, to our knowledge, no reference gene stability studies have been performed for the host during *P. chlamydospora* infection.

Given the relevance of gene expression studies and the imperative need to use suitable reference genes for sample normalization in *q*PCR analyses, the previous considerations prompted us to determine the most stable reference genes, among seven candidates, during three distinct stresses: *P. chlamydospora* trunk infection, leaf wounding and UV-C irradiation. Moreover, we complemented our previous stability analysis concerning *V. vinifera*-*E. necator* interaction for which only geNorm software had been used [23]. In view of the different stresses known to occur in plants, the selection of the stimuli addressed in the present work aims at increasing the existing information concerning suitable grapevine reference genes to be used in future gene expression studies.
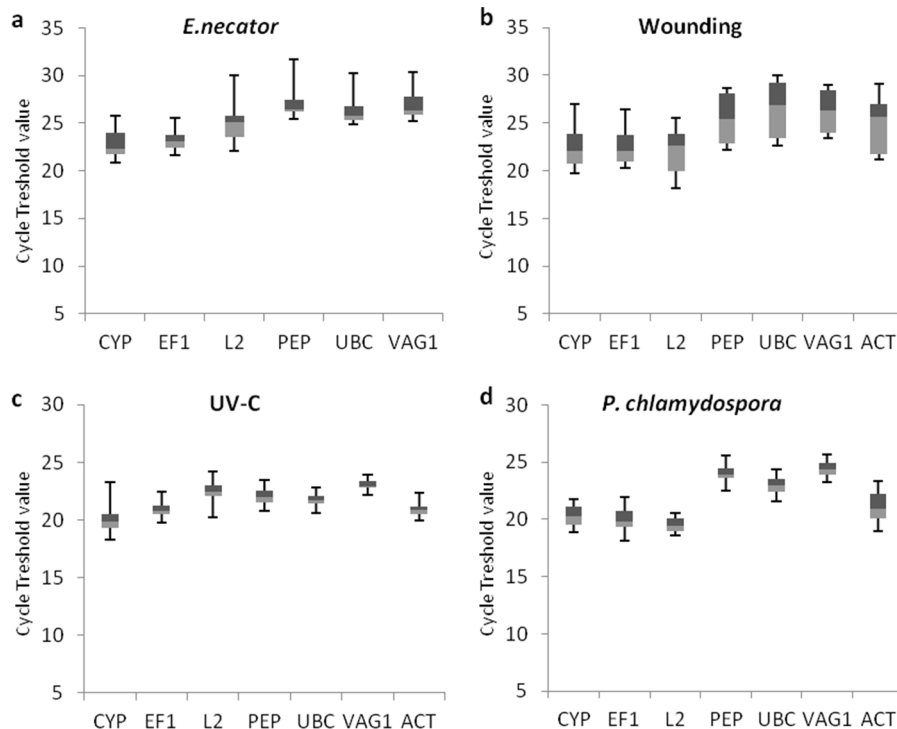
Figure 1. Expression profile of candidate normalization genes in grapevine samples during (a) leaf infection with *E. necator*, (b) leaf wounding, (c) leaf irradiation with UV-C and (d) wood infection with *P. chlamydospora*. Absolute *Ct* values for each treatment and the corresponding controls were combined. Each sample group comprises 5 to 7 biological replicates. The boxes indicate the 25th and 75th percentiles. Lines within the boxes represent the median. Maximum and minimum values are represented by wiskers.
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Table 1. Candidate genes and primer pairs for qRT-PCR normalization in grapevine samples.

Gene	Accession number	Primer	Sequence 5'->3'	Amplicon lenght (bp)	PCR amplification Efficiency (E %)			
					PM[a]	Wound	UV-C	Pch[c]
CYP	ES880796	Fw	ACAGCCAAGACCTCGTG	138	78.8	91.2	91.1	78.3
		Rv	GCCTTCACTGACCACAAC					
EF1	GU585871.1	Fw	GAACTGGGTGCTTGATAGGC	164	86.7	93.1	91.4	77.8
		Rv	AACCAAAATATCCGGAGTAAAAGA					
L2	AJ441290.2	Fw	TCTACTTCAACCGATATGC	199	92.3	93.7	96.3	83.3
		Rv	CCACCTGTCCGACTG					
PEP	AF236126.1	Fw	CCTCCTCCTCCAGATTGC	198	89.6	94.0	97.8	82.9
		Rv	GGCTTGCTTGATTCCATTATC					
UBC	EE253706	Fw	CATAAGGGCTATCAGGAGGAC	161	87.2	92.7	94.8	83.4
		Rv	TGGCGGTCGGGAGTTAGG					
VAG	XM_002281110.1	Fw	TTGCCTGTGTCTCTTGTTC	174	91.8	92.3	99.1	84.0
		Rv	TCAATGCTGCCAGAAGTG					
ACT	XM_002282480	Fw	GACTACCTACAAACTCCATCAT	113	b	94.2	92.6	82.5
		Rv	TCATTCTGTCAGCAATACCA					
PAL[d]	XM_002268220	Fw	TTCCGAACCGAATCAAGG	193	b	90.2	91.5	b
		Rv	GGAGCACCGTTCCAAGC					

[a]Powdery mildew (Erysiphe necator).
[b]Not determined.
[c]Phaeomoniella chlamydospora.
[d]Responsive gene used for differential expression quantification.
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Table 2. Grapevine candidate reference gene stability rankings during different treatments according to geNorm, NormFinder and BestKeeper.

Rank	Program					
	geNorm		NormFinder		BestKeeper	
	Gene	M	Gene	SV	Gene	CC
E. necator						
1	UBC	0.238	VAG	0.219	PEP	0.953
2	VAG	0.238	CYP	0.231	VAG	0.911
3	PEP	0.290	EF1	0.237	CYP	0.885
4	CYP	0.331	UBC	0.251	UBC	0.878
5	EF1	0.402	PEP	0.265	EF1	0.856
6	L2	0.757	L2	0.875	L2	0.382
Wounding						
1	PEP	0.287	PEP	0.786	UBC	0.997
2	UBC	0.287	L2	0.939	VAG	0.996
3	ACT	0.366	CYP	0.952	ACT	0.995
4	VAG	0.471	VAG	0.961	CYP	0.985
5	L2	0.531	EF1	1.060	L2	0.978
6	CYP	0.692	UBC	1.170	PEP	0.977
7	EF1	0.776	ACT	4.918	EF1	0.945
UV-C						
1	UBC	0.152	VAG	0.111	L2	0.897
2	VAG	0.152	UBC	0.151	UBC	0.821
3	PEP	0.267	PEP	0.184	PEP	0.800
4	L2	0.400	ACT	0.352	VAG	0.747
5	ACT	0.505	EF1	0.640	ACT	0.519
6	CYP	0.661	CYP	0.684	CYP	0.133
7	EF1	0.746	L2	0.948	EF1	0.050
P. chlamydospora						
1	EF1	0.282	EF1	0.062	ACT	0.986
2	CYP	0.282	CYP	0.068	EF1	0.965
3	VAG	0.335	PEP	0.081	PEP	0.955
4	UBC	0.362	UBC	0.082	CYP	0.955
5	ACT	0.396	VAG	0.090	UBC	0.942
6	PEP	0.410	L2	0.091	VAG	0.933
7	L2	0.421	ACT	0.096	L2	0.922

SV, stability value; CC, Pearson coefficient of correlation.
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Materials and Methods

Plant material and growth conditions

Grapevine (*Vitis vinifera* L., cultivar Touriga Nacional) cuttings used in the experiment were collected from Centro Experimental de Pegões, Portugal and subjected to heartwood disease screening through microbiology assays. Sample collection was gently and duly authorized by Dr. Antero Martins, Associação Portuguesa para a Diversidade da Videira (PORVID), Portugal. The microbiologic screening was performed using the bottom of the cuttings. Thin wood slices were removed from each cutting, surface-sterilized (ethanol, flame and sodium hypochlorite) and then placed in 0.03% (w/v) chloramphenicol-containing PDA medium (five slices per cutting). The plates were incubated at room temperature for a maximum period of one month, during which morphological identification of the microorganisms present in the wood was performed. Diseased cuttings were discarded.

Healthy *V. vinifera* cuttings, with three buds each, were rooted in water and then transferred to soil (1 L pot per plant). Plants were maintained in a growth chamber at 25°C with a photoperiod of 16 h (480 μmol.m^{-2}.s^{-1}). After one month of acclimatization period, whole plants or detached leaves were subjected to the different treatments.

Plant treatments

For the powdery mildew (PM) treatment, all plants within the same growth chamber were simultaneously inoculated with *E. necator* by direct contact with naturally infected grapevine leaves. The primary inoculum was collected from a vineyard in Instituto Superior de Agronomia, Lisbon, Portugal and passed to a set of
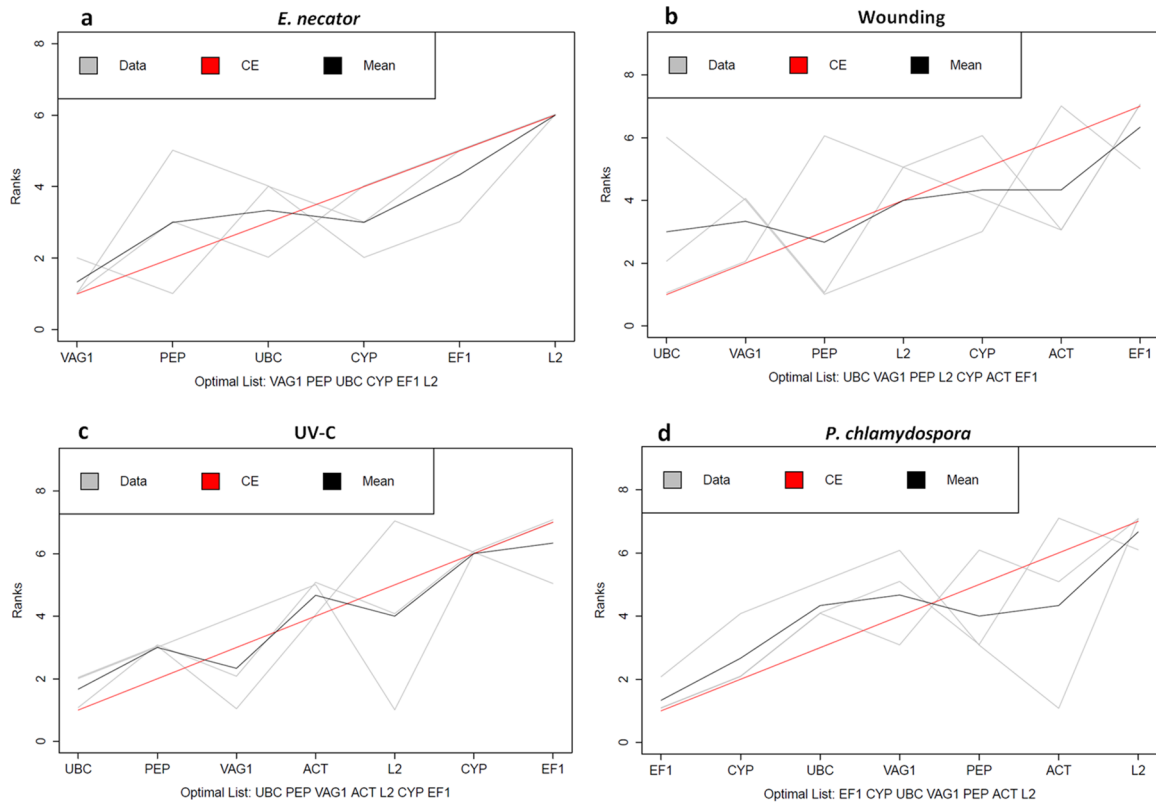
Figure 2. Consensus stability rankings generated by Monte Carlo algorithm for (a) leaf infection with *E. necator*, **(b) leaf wounding, (c) leaf irradiation with UV-C, and (d) wood infection with** *P. chlamydospora*. RankAggreg (v. 0.4–3) package for R was used to compute Monte Carlo algorithm with the Spearman footrule distances on the rank lists generated by each applet. Individual stability measurements (geNorm, NormFinder or BestKeeper) are shown in grey, average rank positions in black and the computed Monte Carlo model in red.
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grapevines in greenhouse which provided the experimental inoculum source. Plants were allowed to grow with generalized powdery mildew infection for 30 days prior to sample collection.

Fully expanded leaves (fourth and fifth positions from the tip of each shoot) with and without *E. necator* infection symptoms (visible mycelia on the upper leaf surface) were harvested.
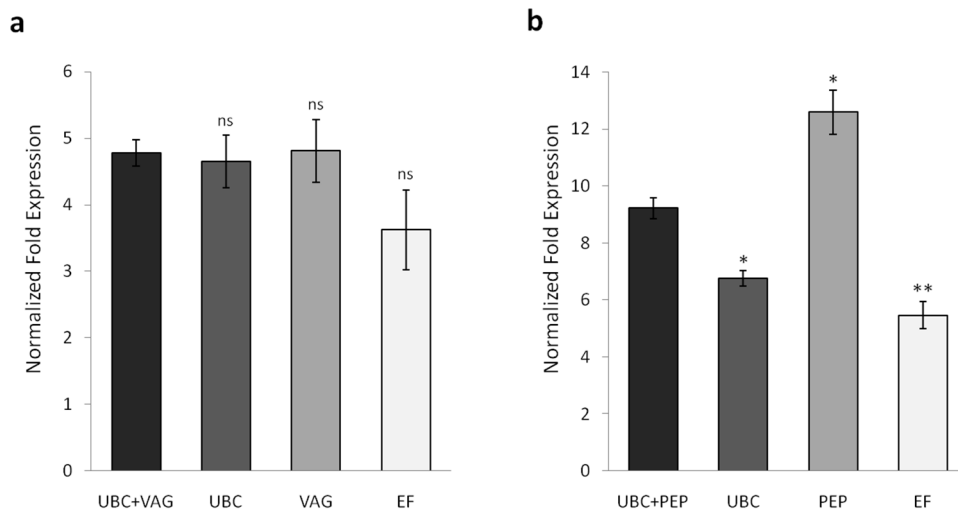
Figure 3. Differential gene expression of *PAL* **in grapevine leaves induced by (a) wounding and (b) UV-C irradiation.** Relative gene expression quantification was performed using four different normalization factors derived from: the combination of the two top ranked genes, the best ranked gene, the second most stable gene and the worst ranked gene. All values are mean±SD (n=4). Statistical differences (one-way ANOVA followed by Dunnett's multiple comparison test) to UBC+VAG – wounding or UBC+PEP – UV-C irradiation are marked: ns – not significant ($P>0.05$); * – significant ($0.01<P<0.05$); ** – very significant ($0.001<P<0.01$).
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For the wounding treatment, fully expanded leaves were cut using a sterile razor blade. Each leaf was subjected to six 1 cm-long cuts and collected 24 h after the treatment. Control samples were left untreated and maintained under the same conditions.

For the UV irradiation treatment, leaves were detached and their undersides were exposed to UV-C radiation (Philips TUV 30 W, 92 µW cm-2 at 253 and 7 nm) at a distance of 15 cm from the source during 10 min. Following irradiation, treated and control samples were incubated in a dark wet chamber at room temperature for 48 h.

For the *Phaeomoniella chlamydospora* treatment, a pure fungal isolate was obtained from CBS (CBS 239.74) and propagated in PDA medium at 23°C in dark. Inoculation was performed at the base of the primary shoot by removing a small section of the bark with a scalpel and placing a 5 mm inoculation plug (cut from the actively growing margin of the colony) into the wound (mycelium side down). Each wound was then be covered with moist cotton wool and sealed with parafilm. The same procedure was followed for negative control plants using non inoculated PDA plugs. Plants were maintained under the above described conditions for one week prior to sample collection.

Following the mentioned incubation periods for individual treatments, all samples were harvested and immediately frozen in liquid nitrogen. Biological replicates for all treatments and corresponding controls were created by pooling either four leaves or two 5 cm-long stem sections per sample.

RNA extraction and cDNA synthesis

Total RNA extraction was performed using the Rapid CTAB (hexadecyltrimethyl ammonium bromide) method, especially suited for high phenolic content material, adapted as follows [34]. Biological samples were ground in liquid nitrogen, homogenized at approximately 150 mg per mL in extraction buffer (2% (w/v) CTAB, 2.5% (w/v) polyvinylpoly-pyrrolidone, 2 M NaCl, 100 mM Tris-HCl pH 8.0, 25 mM ethylenediaminetetraacetic acid (EDTA), 2% (v/v) β-mercaptoethanol) and incubated at 65°C for 10 min. Samples were extracted twice with one volume of chloroform:isoamylic alcohol (24:1, v/v) and centrifuged at 12,000 g during 10 min at 4°C. The recovered aqueous phase was supplemented with ¼ volume of 10 M LiCl and incubated during 30 min at 4°C. RNA was collected by centrifugation at 21,000 g, 4°C during 20 min, and resupended in 500 µL of pre-warmed (65°C) SSTE buffer (0.5% w/v sodium dodecyl sulfate (SDS), 1 M NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA). Samples were again extracted with one volume of chloroform:i-soamylic alcohol (24:1, v/v) followed by centrifugation at 12,000 g during 10 min. The recovered supernatant was supplemented with 0.7 volumes of cold isopropanol and immediately centrifuged at 21,000 g, 4°C during 15 min. RNA *pellet* was washed with 70% (v/v) ethanol and resuspended in water. Prior to reverse transcription, samples were treated with RQ1 RNase-Free Dnase (Promega) according to the manufacturer's protocol.

All samples were reverse transcribed using ThermoScript RT-PCR System (Invitrogen) as described by the manufacturer. cDNA was synthesized from 1.5 µg of total RNA and oligo(dT)20 primers. RT reactions were carried at 55°C for 60 min.

Primer design and qPCR

PCR primers were designed with Beacon Designer software (Premier Biosoft International) to target amplicons between 80 and 300 bps. Amplification specificity was first assessed though Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) using *V. vinifera* database as template. qPCR was performed with iQ SYBR Green supermix (Bio-Rad) using iCycler equipment (Bio-

Rad). Prior to use, cDNA samples were diluted to 50 ng/µL. Reaction mixtures (20 µL) were prepared according to the following: 5 µL of the diluted template, 1 µL primer mix (10 µM each), 10 µL iQ SYBR Green supermix (Bio-Rad), 4 µL H_2O. Thermal cycling was composed of an initial denaturation step for 3 min at 95°C, 40 cycles at 95°C for 10 s, 55°C for 30 s and 72°C for 30 s. All reactions were performed in triplicate and amplification specificity was confirmed through melting curve analysis.

Data analysis

Raw data (i.e. not baseline-corrected) belonging to each individual amplification curve was imported from iQ5 into LinRegPCR software (version 11.0) for baseline and PCR efficiency estimation. Log-linear phases were automatically determined containing four to six points with the highest correlation coefficient. According to the obtained linear regressions, individual PCR amplification efficiencies were calculated. Student's t-test was used to compare amplification efficiencies of each amplicon between treated samples and corresponding controls of the same treatment ($P<0.05$). Since no differences were observed, mean efficiencies for each amplicon within each treatment were used for subsequent analysis. Ct values were retrieved using a fluorescence threshold defined within a common window-of-linearity (WoL) for each dataset.

To evaluate the expression stability of the selected candidate genes for the different stimulus, three different Visual Basic Application (VBA) applets for Microsoft Excel were used: geNorm v.3.5 [7], NormFinder v. 0953 [16] and BestKeeper [17]. Input file creation and subsequent data analysis was performed according to the corresponding manuals. For both GeNorm and NormFinder software, Ct values were transformed into relative quantities (amplification efficiency corrected) using the lowest Ct sample as calibrator. For BestKeeper analysis, raw Ct values as well as PCR amplification efficiencies were directly inserted into the software.

Consensus ranks, integrating the results of the different algorithms, were generated using a non-weighted unsupervised rank aggregation method. Data analysis was carried out using the RankAggreg v. 0.4–3 package [35] for R. RankAggreg input was a matrix of rank-ordered genes according to the different algorithms used. Comprehensive ranks were obtained from the calculated Spearman footrule distances and the Cross-Entropy Monte Carlo algorithm.

Results and Discussion

Expression profile of candidate reference genes

Reference gene validation for qRT-PCR expression studies has become a fundamental requisite for reliable quantification results. To provide information regarding potential reference genes for future use in qRT-PCR studies involving *V. vinifera*, we decided to evaluate the expression stability of a set of commonly used housekeeping genes during four distinct stimuli comprising biotic and abiotic stresses [36–38]. These include powdery mildew infection, mechanical wounding and UV-C irradiation in leaves and xylem colonization with *P. chlamydospora* in woody tissues. Treatment selection was based on its potential application for future gene expression studies in grapevine. Candidate genes were selected taking into consideration either its frequent use as sample normalizers or their repeated participation in similar studies. To avoid co-regulation events, candidate genes were also required to belong to distinct metabolic pathways. Accordingly, the selected candidates for this study were cyclophilin (*CYP*), elongation factor

1 α (*EF1*), ribosomal protein L2 (*L2*), phosphoenolpiruvate carboxylase (*PEP*), ubiquitin conjugating enzyme (*UBC*), vacuolar ATPase subunit G (*VAG*) and actin (*ACT*) [36–42]. Following PCR amplification, a general overview of the expression profile and relative abundance of each candidate gene was obtained by plotting the *Ct* values obtained for all samples (control and treatment) under the different conditions studied (Figure 1). *ACT* expression stability upon *E. necator* infection (Figure 1a) was not assessed as it was not considered as a candidate in our previous study [23].

All genes displayed moderate expression levels with mean *Ct* values ranging from 19.7 to 27.2 for *L2* and *PEP* during *P. chlamydospora* and *E. necator* interaction, respectively. Minimum *Ct* values, meaning higher abundance, were observed for *EF1* (18.1) during *P. chlamydospora* interaction, whilst *PEP* displayed the highest *Ct* (31.6) for *E. necator* treatment. Overall, gene expression variation across samples within each treatment ranged from 1.9 to 7.9 *Ct*s with the highest expression fluctuations being observed for the wounding experiment. Though preliminary information can be obtained through absolute *Ct* analysis, to correctly assess the expression stability of candidate genes, raw amplification data must be first linearized. This was carried out by converting the *Ct* values into relative quantities which were normalized to the sample with the lowest *Ct*.

Primer pair amplification efficiencies

To perform data linearization, PCR amplification efficiencies (*E*) must be taken into consideration, preventing significant bias from being introduced in the generated results [43,44]. *E* values were estimated using the absolute fluorescence increase method (Table 1) [44,45]. LinRegPCR software (version 11.0), developed by Ruijter *et al.*, 2009, was used to individually analyze each sample and determine amplification efficiencies based on a proper baseline correction. Considering that *E* value for one primer pair might differ among stimuli and, moreover, between control and treated samples in the same stimulus, we separately analyzed each sample group. Though amplification efficiencies for each gene may vary depending on the treatment, no differences (p<0.05) were observed between control and treated samples of the same stimulus. Thus, mean *E* values (Table 1) were used for subsequent analysis.

Reference gene expression stability

Following baseline estimation and amplification curve analysis for all *q*PCR reactions, the statistical analysis to evaluate the expression stability of the candidate genes was performed using three different programs: geNorm [7], NormFinder [16] and BestKeeper [17]. Though all aim to determine which candidate genes are the most stable under certain conditions, they run under different algorithms and mathematical models. Therefore, the stability ranking of the putative reference genes might differ depending on the program used [46]. GeNorm analysis relies on the intuitive principle that the expression ratio of two ideal reference genes should always remain constant across all samples. Accordingly, it calculates a gene expression stability measure (M) based on the average pairwise expression ratio between each gene and the remaining candidates. Lower M values are indicative of higher stability. The main drawback of geNorm, and consequently one of the most important criteria to be aware of, is that candidate genes must not be co-regulated. This would introduce significant bias as identically regulated genes tend to be top ranked in geNorm even if their expression levels fluctuate considerably among samples [7]. On the other hand, NormFinder analysis, a model-based variance estimation method, displays less sensitivity

to co-regulation events. Expression stability of candidate genes is evaluated according to their overall expression variation among the sample set. For each of the analyzed genes, NormFinder calculates a stability value (SV) according to which a ranking is generated. Similarly to geNorm, a lower SV value is indicative of higher stability [16]. The third and last tool adopted to assess the gene expression stability was BestKeeper software. Unlike the previous methods, input data for this software consists of raw *Ct* values instead of relative quantities. Nevertheless, amplification efficiencies are also considered. The expression variability is assessed through coefficient of variance and standard deviation analysis. The software calculates a "BestKeeper index" referring to each sample and compares the candidate genes based on their pairwise correlation with this index value. Candidates displaying a higher Pearson's correlation coefficient (*r*) correspond to the most stably expressed [17].

Following our gene expression variation analysis over the four particular experimental conditions, the candidate genes were rank ordered according to the stability parameters calculated by each program (Table 2).

Concerning the long term interaction between grapevine and *E. necator*, a previous work conducted in our laboratory was already performed, in which the most suitable *q*PCR reference genes were reported using the geNorm software [23]. Nevertheless, to provide complementary information regarding this matter, in the present study, we decided to re-evaluate the expression stability of the same candidate genes using also NormFinder and BestKeeper. In addition, since a different PCR efficiency determination method was employed, a new geNorm analysis was also performed. Though different M values were obtained for each gene, the stability ranking was not affected.

As expected, regardless of the experimental condition and similarly to other reference gene evaluation studies, the studied genes performed differently depending on the analysis program used. Therefore, in the absence of an ideal or preferred method, it is not possible to determine the precise candidate genes most stable under each condition. However, in certain cases, a simple overview of the three ranks can reveal particular tendencies. For instance, for the *E. necator* treatment, *VAG* was consistently ranked among the two most stable genes. Yet, *UBC*, whose M value was the same as *VAG*, was ranked fourth according to NormFinder and BestKeeper. Full agreement was observed regarding *L2*, which was the worst ranked gene in all three methods. As for the wounding stimulus, a higher discrepancy is observed among the three methods. While *PEP* displayed the best stability performance when evaluated by geNorm and NormFinder, it was classified as one of the worst genes by BestKeeper. A similar situation occurs for UBC. Despite the significant discrepancies occurring among the ranks generated by the three softwares, one must also be aware that, in some of those cases, the ranks were generated based upon small differences in the stability parameters indicating that the genes involved might possess expression variations very close to each other. As for the UV-C irradiation treatment, a reasonable consistency is observed, where for all methods, both *UBC* and *PEP* are among the three best ranked genes. *CYP* on the other hand, was classified as the second worst gene regardless of the analysis type. For the last treatment addressed in this work, *P. chlamydospora* infection in woody tissues, a clear difference is observed when the stability rankings are compared with the previous treatments. Both *EF1* and *CYP*, which were constantly amongst the worst scoring genes, are, in this case, two of the most stable candidates. Though the biotic stress itself can cause significant gene expression variations,

also a considerable effect is expected due to tissue/organ specific metabolism.

Consensus Stability Rankings

Considering that each of the previous methods has its own limitations and no agreement exists for which software is the most suitable for expression stability analysis, a common approach to perform these studies often involves the use, comparison and integration of all three methods. Several strategies exist to create a comprehensive stability ranking integrating the results of the three applets. In general, each gene is assigned a certain weight corresponding to the rank obtained for each program (*e.g.* 1-most stable to 7- least stable). Subsequent rank aggregation methodologies are then employed, which can, for instance, rely on straightforward arithmetic and geometric mean of the ranks [47–49]. However, in this work a different methodology, suggested by Mallona (2010) and followed by Goulão (2012), was used. The outputs of the different applets were merged by means of a non-weighted unsupervised rank aggregation method using the Cross-Entropy Monte Carlo algorithm. According to the previous method, an optimal stability ranking list for each experimental condition was created (Figure 2).

Overall, the rank aggregation method supports some of our initial observations. When comparing the different optimal lists obtained for each treatment, we can also observe that, for all the three stresses involving grapevine leaves, despite the scoring differences, *UBC*, *VAG* and *PEP* are consistently ranked within the most stable genes. Concerning *P. chlamydospora* treatment, *EF1*, *CYP* and *UBC* are, among all candidates, the most stable reference genes.

For the particular case of *E. necator* infection, an inevitable attempt was made to correlate our results with similar existing studies reporting the most appropriate grapevine normalization genes upon pathogen interaction, namely *Plasmopara viticola*, the causal agent of downy mildew [11,21,22]. Though distinct, both pathogens have similar infection mechanisms, lifestyles and colonize the same tissues. In fact, even though different sets of genes were used in each study, a certain degree of accordance can be observed, at least in one of the cases, when some of the same genes are evaluated. In the work conducted by Selim (2012), in which four of the present genes were also evaluated, *UBC* was ranked as the most stably expressed in *P. viticola* infected leaves. In addition, *EF1*, as well as *CYP*, were the two worst ranked candidates. Conversely, in the work developed by Monteiro (2013), also addressing *P. viticola* leaf infection, *EF1* was, at all times, one of top ranked genes.

In order to further validate the suitability of the top ranked genes identified in this study, we decided to perform the differential expression quantification of a potentially responsive gene, phenylalanine ammonia lyase (*PAL*), for two of the tested conditions (wounding and UV-C irradiation). Given its extensive characterization and general acceptance as a defense related gene whose expression can be induced by a variety of stresses, *PAL*

expression changes caused by the selected stimuli would be predictable to occur [50,51]. In an attempt to evaluate the potential bias arising due to improper reference gene selection, we calculated the fold change expression of *PAL* using both the best and the worst ranked genes for normalization (Figure 3). For each of the treatments, fold change expression values were determined using different normalization factors (NF) derived from: the combination of the two most stable reference genes, the best ranked gene, the second best gene and the worst ranked gene. As expected, regardless of the treatment, an upregulation of *PAL* was observed. For the wounding experiment (Figure 3a), the calculated expression values using the combined and isolated best genes as NFs (*UBC* and *VAG*) were comparable among themselves, with fold change values of 4.78 (*UBC+VAG*), 4.65 (*UBC*) and 4.81 (*VAG*). When using the NF corresponding to the most unstable gene (*EF*), a fold change of 3.62 was obtained. Despite the noticeable difference between the normalization with the worst and best genes, a certain degree of consistency exists within all four quantifications. In fact, the statistical analysis reveals that no significant differences ($P>0.05$) exist when comparing the results obtained from *UBC+VAG* with the remaining. This suggests that the expression of these candidate genes (*UBC*, *VAG* and *EF*), and possibly of all the remaining, was not significantly affected by the experimental wounding stress. Thus, in this case, one could infer that any of the candidates could be used for sample normalization without major bias being introduced. On the other hand, for the UV-C treatment (Figure 3b), larger discrepancies can be observed among the evaluated candidates. *PAL* gene expression normalized to the combination of the two best ranked genes (*UBC* and *PEP*) indicated a 9.22 fold change. However, when normalized for each of the candidates individually, the calculated upregulation was 6.77 (*UBC*), 12.59 (*PEP*) and 5.47 (*EF*). Such accentuated differences highlight not only the importance of selecting the most appropriate reference genes for each experimental conditions, but also the necessity to use multiple genes for sample normalization.

Taken together, besides supporting the already accepted idea that no universal reference genes exist, our results provide information regarding the suitability of potential *q*RT-PCR reference genes to be used in grapevine samples for distinct biotic and abiotic stresses. Such knowledge might prove useful in transcriptomic studies contributing for accurate gene expression quantification.

Author Contributions

Conceived and designed the experiments: SM AML RBF. Performed the experiments: AFB CF. Analyzed the data: AFB CF. Wrote the paper: AFB CF SM AML RBF.

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