TECHNICAL FOCUS

Genomic DNA-based absolute quantification of gene expression in *Vitis*

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Many studies in which gene expression is quantified by polymerase chain reaction represent the expression of a gene of interest (GOI) relative to that of a reference gene (RG). Relative expression is founded on the assumptions that RG expression is stable across samples, treatments, organs, etc., and that reaction efficiencies of the GOI and RG are equal; assumptions which are often faulty. The true variability in RG expression and actual reaction efficiencies are seldom determined experimentally. Here we present a rapid and robust method for absolute quantification of expression in *Vitis* where varying concentrations of genomic DNA were used to construct GOI standard curves. This methodology was utilized to absolutely quantify and determine the variability of the previously validated RG ubiquitin (*VvUbi*) across three test studies in three different tissues (roots, leaves and berries). In addition, in each study a GOI was absolutely quantified. Data sets resulting from relative and absolute methods of quantification were compared and the differences were striking. *VvUbi* expression was significantly different in magnitude between test studies and variable among individual samples. Absolute quantification consistently reduced the coefficients of variation of the GOIs by more than half, often resulting in differences in statistical significance and in some cases even changing the fundamental nature of the result. Utilizing genomic DNA-based absolute quantification is fast and efficient. Through eliminating error introduced by assuming RG stability and equal reaction efficiencies between the RG and GOI this methodology produces less variation, increased accuracy and greater statistical power.

Introduction

Contemporary biological investigations of gene expression in plants often rely on quantitative polymerase chain reaction -based (qPCR) methodologies of some kind (reviewed in Gachon et al. 2004). qPCR methodologies vary in chemistry, data analysis, etc., but the majority of studies utilize a comparative cycle threshold (C_T) methodology wherein the level of expression of a gene of interest (GOI) is calculated relative to a reference gene (RG) according to the simple expression: $2^{-\Delta C_T}$ where $\Delta C_T = C_T^{RG} - C_T^{GOI}$. This methodology is founded on the assumptions that RG expression is stable across the experimental units being compared (samples, treatments, organs, etc.) and that the reaction efficiencies of

Abbreviations – AE, absolute expression; C_T, cycle threshold; CTAB, cetyltrimethyl ammonium bromide; CV, coefficients of variation; gDNA, genomic DNA; GOI, gene of interest; NE, normalized expression; PCR, polymerse chain reaction; PVPP, polyvinyl polypyrrolidone; qPCR, quantitative polymerse chain reaction; RDS, re-circulating drip system; RE, relative expression; RG, reference gene.

the GOI and RG are equal. Both of these assumptions are widely recognized as faulty and many works have focused on ways to ameliorate these issues through determining those RGs with the most stable expression (Vandesompele et al. 2002), quantifying reaction efficiencies (Ramakers et al. 2003) and providing guidelines for best practices (Bustin et al. 2009). Nevertheless, the true nature of variability in RG expression and differences in reaction efficiencies are seldom determined experimentally (Ramakers et al. 2003, Gutierrez et al. 2008b, Gutierrez et al. 2008a, Guenin et al. 2009). For example in *Vitis*, studies have attempted to assess the stability of widely used RGs through microarray data or simple C_T comparisons (Terrier et al. 2005, Reid et al. 2006), but none have done so through the use of absolute quantification.

A much less commonly utilized alternative is absolute quantification of expression through the construction of a standard curve. This methodology still relies on several assumptions, but benefits from the significant advantage that it is not biased by variation in RG expression and reaction efficiencies are determined empirically. The current scarcity of absolute quantification in qPCR studies likely stems from the historically laborious and time-consuming nature of these methods. In order to obtain a series of known gene copy number from which a standard curve could be produced requires having a standard of known molecular weight and concentration, and typically this was obtained through subcloning the GOI. Today, numerous plant genomes are available which make genomic DNA (gDNA) a feasible standard (Yun et al. 2006). One can determine sequence specificity of the target gene and copy number can be readily calculated for a given gDNA concentration from the genome molecular weight. Nevertheless, absolute quantification sees very little use. It is difficult to find any previous study that directly compared relative and absolute methods of quantification in the same experiment, but Palovaara and Hakman (2008) found significant differences between the two methodologies while assessing conifer WOX-related gene expression.

Ubiquitin (*VvUbi*) is a commonly used RG across organisms, and in *Vitis*. In previous studies, *VvUbi* expression has been found to be stable in grape berries (Reid et al. 2006), and in our own analyses of microarray data across various *Vitis* tissues (unpublished data). In this study, we present a rapid and robust gDNA-based method for absolute quantification of gene expression, use this method to absolutely quantify the variability of the *VvUbi* across test studies in different grapevine tissues (roots, leaves and berries), and compare data sets for two GOIs resulting from relative and absolute methods of quantification.

Materials and methods

gDNA preparation, primer design and standard curves

gDNA was prepared from grapevine tissues using a modified cetyltrimethylammonium bromide (CTAB) extraction (Lodhi et al. 1994, Steenkamp et al. 1994, Di Gaspero and Cipriani 2002). In short, tissue was homogenized under liquid nitrogen with 2% polyvinyl polypyrrolidone (PVPP) and then 200–300 mg of tissue was resuspended in 2 ml of pre-heated CTAB extraction buffer (1 *M* Tris–HCl pH = 8.0, 20 m*M* ethylenediaminetetraacetic acid, 1.4 *M* NaCl and 2.5% w/v CTAB); β-mercaptoethanol was added to 0.2% (v/v). The suspension was incubated at 60°C for 30 min mixing frequently. Suspension was centrifuged at maximum speed for 5 min and the supernatant extracted with equal volume of chloroform:isoamyl alcohol, 24:1 (v/v). The aqueous phase was collected, DNA precipitated with isopropanol and then washed with 70% ethanol. DNA was resuspended in 300 µl TE (10 mM Tris-HCl pH = 8.0, 1mM EDTA) with RNase (10 µg ml^{-1} final concentration) and incubated at 37°C for 30 min. DNA was precipitated with NH_4Ac and ethanol and resuspended in water. Preparations were then quantified using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Asheville, NC).

All primer pairs were determined using the FastPCR software's unique primer design. The actual primer pair was chosen manually from those produced by FastPCR based on the following criteria making it most suitable for qPCR; the product is approximately 100 bp in length and represents the most 3′ sequence possible. Primer pairs used for *VvUbi* were forward 5′-GAGGCCAAAGAAGAAGATCAAGT-3′ and reverse 5′-AAAACAAAAGCATCGTTTGTGACC-3′. Primers for *VvPIP1-1* have been used in a previous study (Choat et al. 2009a). The primer pairs used to amplify *VvAOS1* were: forward 5′-CTCATTGTTCGCCCGATATT-3′ and reverse 5′-CCGATGAAAGGGAGACCATA-3′. All primers were validated by confirming a single product via agarose gel electrophoresis followed by gel isolation and direct sequencing of that product.

The concentrations of the gDNA preparations described above were converted to target copies per liter using the following calculations. The molecule weight of a *Vitis vinifera* haploid genome (MW_{Vv}) was approximated as follows:

$$MW_{Vv} = (n_{Vv}) \left(660 \, \text{Da basepair}^{-1} \right) \qquad (1)$$

where n_{Vv} is the number of base pairs in the haploid genome $= 487$ Mb (Jaillon et al. 2007). Therefore:

$$MW_{Vv} = \left(487 \times 10^6 \text{basepairs}\right)\left(660 \text{ Da basepair}^{-1}\right)$$
$$= 3.21 \times 10^{11} \text{g mol}^{-1} \quad (2)$$

This is approximate molecular weight of a *V. vinifera* haploid genome which would contain one copy of the target gene. Thus the concentration of gDNA can be expressed as the equivalent number of target copies per liter as follows:

$$\text{Copy number } l^{-1} = \left[\left(\text{gDNA conc. g } l^{-1}\right)\right.$$
$$\left./ \left(3.21 \times 10^{11} \text{g mol}^{-1}\right)(N_A)\right] \quad (3)$$

where N_A is the Avogadro's number. In order to produce the standard curve, the gDNA was diluted to obtain a series at 1 $\log_{10}$ intervals.

qPCR was performed in a total reaction volume of 12 μl reaction. Reactions included template DNA, 6 μl of Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA), 1 μ*M* forward primer, 1 μ*M* reverse primer and sterile molecular biology grade water to a total volume of 12 μl. All PCR reactions were performed in 96-well plates and the exact reaction cycling conditions were as follows: 95°C for 10 min, 40 cycles of 95°C for 10 s and 60°C for 1 min. Amplification and data analysis were carried out on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). All primers and probe concentrations were optimized. All reactions were carried out in triplicate and reproducibility of the standard curves was assessed by repeating the reactions on four or more occasions.

Calculations of expression values

Relative expression (RE) was calculated according to the expression: $2^{-\Delta C_T}$ where $\Delta C_T = C_T^{RG} - C_T^{GOI}$. Absolute expression (AE) normalized to the absolute level of *VvUbi* expression [normalized expression (NE)] was calculated by first determining the absolute level of *VvUbi* expression from the standard curve (Fig. 1) for each sample, where:

$$C_T = -3.11\left(\log_{10} \text{copies}\right) + 36.8, \quad \text{so } \log_{10} \text{copies}$$
$$= (C_T - 36.8)/-3.11 \quad (4)$$

Then the RE of each sample was multiplied by the corresponding absolute level of *VvUbi* expression. For example, in sample 1 relative *VvPIP1-1* expression was 1.5. Absolute level of *VvUbi* expression was 100 copies ng^{-1} RNA. Therefore, NE $= 1.5 \times 100$ copies ng^{-1} RNA $= 150$ copies ng^{-1} RNA.

AE was calculated for each sample directly from each GOI's corresponding standard curve.

A

$C_T = -3.11(\text{Log}_{10} \text{ copies}) + 36.8$

$r^2 = 0.999$

Cycle threshold (C_T)

Log_{10} copies *VvUbi*

B
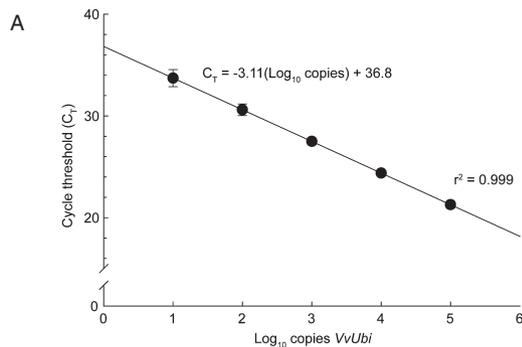
$\log_{10}$ copies	Cycle threshold (C_T)						
	Prep 1	Prep 2	Prep 3	Prep 4	Mean	SD	CV (%)
5	21.0	21.0	21.7	21.3	21.3	0.34	1.6
4	24.5	24.3	24.6	24.2	24.4	0.18	0.7
3	27.9	27.5	27.4	27.1	27.5	0.32	1.2
2	31.4	30.7	30.3	30.1	30.6	0.57	1.9
1	34.8	33.9	33.1	33.0	33.7	0.84	2.5

Fig. 1. *Vitis* ubiquitin (*VvUbi*) standard curve and its reproducibility. (A) *VvUbi* standard curve with linear regression equation and regression coefficient (r^2) presented. Data points represent means $\pm$ SD bars (n = 4). (B) Cycle thresholds for given *VvUbi* copy number across four independent genomic DNA preparations; the mean, SD, and CV are presented for each $\log_{10}$ copy level. Each 'Prep' value represents the mean of three technical replicates.

Test experiment 1

Rootstock material was acquired from plants grown hydroponically in a continuous re-circulating drip system (RDS). The plants were fed a modified Hoagland's solution which consisted of 2000 μ*M* Ca(NO$_3$)$_2$, 3000 μ*M* KNO$_3$, 1250 μ*M* KH$_2$PO$_4$, 1500 μ*M* MgSO$_4$, 100 μ*M* Na$_2$SiO$_3$, 40 μ*M* H$_3$BO$_3$, 9 μ*M* MnSO$_4$, 4 μ*M* ZnSO$_4$, 4 μ*M* CuSO$_4$, 0.10 μ*M* H$_2$MoO$_4$ and 100 μ*M* NaFeDTPA. pH was adjusted to approximately 5.8. The Hoagland's solution was changed in all RDS systems as needed based upon total volume losses due to transpiration and evaporation. Roots were collected from individual plants for each replicate, immediately frozen on dry ice and then transferred to −80°C. Total RNA was isolated from each root and *VvUbi* and *VvPIP1-1* expression was quantified as in previous studies in our laboratory (Choat et al. 2009a, Gambetta et al. 2010).

Test experiment 2

Leaves were obtained from a commercial vineyard of Chardonnay grapevines (Beringer Vineyards, Yountville, CA). Plants were identified that exhibited all of the hallmarks of *Xylella fastidiosa* infection (a.k.a. Pierce's disease), including shriveled fruit, leaf-scorch symptoms, petiole matchsticks and green islands. Ten plants were tagged, basal leaves collected, total DNA isolated and the presence of *X. fastidiosa* confirmed by qPCR

according to Gambetta et al. (2007). Leaves that exhibited various levels of leaf scorch were then collected, photographed, immediately frozen on dry ice and then transferred to -80°C. The image collected for each leaf was used to quantify the proportion of leaf scorch relative to green area using IMAGEJ software as in Choat et al. (2009b). Total RNA was isolated from each leaf and *VvUbi* and *VvAOS1* expression was quantified as in previous studies in our laboratory (Choat et al. 2009a, Gambetta et al. 2010).

Test experiment 3

Field-grown Chardonnay berries were obtained from UC Davis experimental vineyards (Davis, CA). The berries were harvested at approximately 60 days after anthesis which represents the onset of ripening. Berries were immediately frozen on dry ice and then transferred to -80°C. Berries were dissected frozen and total RNA was isolated from each of seven parts: pedicel, proximal skin, proximal flesh, distal skin, distal flesh, whole skins, whole flesh and *VvUbi* and *VvPIP1-1* expression was quantified as in previous studies in our laboratory (Choat et al. 2009a, Gambetta et al. 2010).

Data analysis

All expression data was log transformed (Willems et al. 2008, Rieu and Powers 2009) prior to statistical analysis. Reaction specific efficiencies were determined using LINREGPCR software (Ramakers et al. 2003). For treatment effects, means at each sample date were compared by Tukey's HSD. All analyses were carried out using SAS software (SAS Institute Inc., Cary, NC).

Results

Standard curves

A standard curve for the commonly used RG *ubiquitin* (*VvUbi*) was established utilizing gDNA standards (Fig. 1A). The relationship between gene copy number and cycle threshold (C_T) was log linear over five orders of magnitude ranging from 10^1 to 10^5 copies and the reaction efficiency calculated from the curve was 105%. The reproducibility of the gDNA standards was validated by carrying out four independent gDNA isolations and quantifications, and standard curves were established for each preparation (Fig. 1B). This methodology yielded highly consistent standards. Coefficients of variation (CVs) in the C_Ts for individual gene copy numbers varied from 0.7–2.5% between the preparations.

In order to carry out our test studies, standard curves for two GOIs, the aquaporin *VvPIP1-1* and the jasmonic
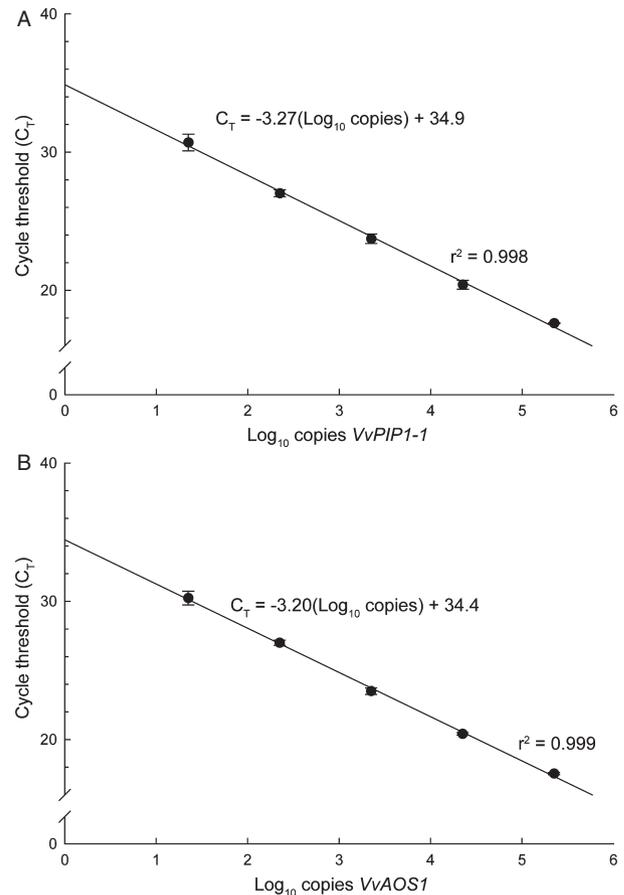
Fig. 2. GOI standard curves used in this study. (A) *Vitis* aquaporin (*VvPIP1-1*) standard curve. (B) *Vitis* jasmonic acid biosynthetic gene (*VvAOS1*) standard curve. Linear regression equation and regression coefficient (r^2) presented. Data points represent means $\pm$ SD bars ($n = 4$).

acid biosynthetic gene *VvAOS1* were also established (Fig. 2). For both *VvPIP1-1* and *VvAOS1* the relationship between gene copy number and C_T was log linear over five orders of magnitude ranging from 10^1 to 10^5 copies and their reaction efficiencies were 101 and 103%, respectively.

RG expression

Absolute level of expression of the commonly used RG *ubiquitin* (*VvUbi*) was quantified across three test studies in three different tissues (roots, leaves and berries). Mean *VvUbi* expression differed by as much as 200-fold between experiments (Fig. 3A). *VvUbi* expression varied widely and the CVs differed greatly between the test studies. In roots, *VvUbi* expression varied over 16-fold and in berries expression varied over 64-fold (Fig. 3B). These experiments contrast with *VvUbi* expression in leaves that varied only fourfold

A Ubiquitin expression in test case experiments

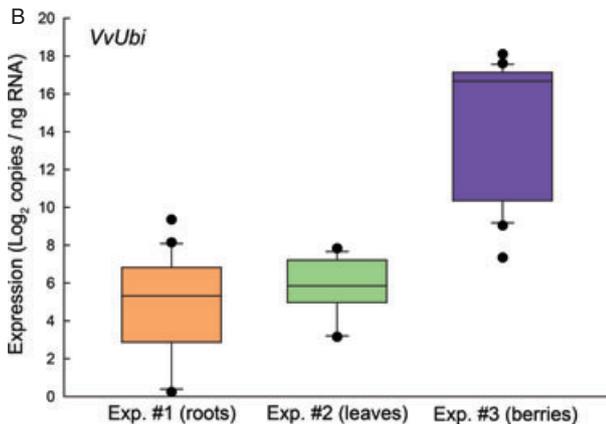| Experiment #	Expression (log$_2$ copies / ng RNA)		
	Mean*	SD	CV (%)
1 (roots)	4.9 a	2.7	55.3
2 (leaves)	5.8 a	1.5	24.9
3 (berries)	14 b	3.6	24.3

* Different letters represent significant differences (P<0.001, Tukey's HSD)

Fig. 3. *VvUbi* expression in the three test experiments. (A) table including mean *VvUbi* expression, SD, and CV for each experiment. (B) Box plot illustrating the variation of *VvUbi* expression in each experiment. Boxes represent the 25–75th percentile, horizontal line within the box the median, whiskers the 10–90th percentile and individual points the outliers. Experiment #1 (n = 20), experiment #2 (n = 16) and experiment #3 (n = 21).

(Fig. 3B). Even considering 10–90th percentiles and outliers, *VvUbi* expression was much less variable in leaves when compared with roots and berries.

Test experimentation

GOIs were quantified in our test studies and the expression of each GOI was represented in three ways: (1) as RE utilizing the $2^{-\Delta C_T}$ method, (2) as AE normalized to the absolute level of *VvUbi* expression (NE) and (3) as AE calculated from each genes' standard curve (AE). RE represents the $2^{-\Delta C_T}$ method, NE accounts for the variability in *VvUbi* expression but still assumes equal reaction efficiencies, and AE quantifies each GOIs' expression absolutely without either assumption.

In the first test experiment, the *Vitis* aquaporin *VvPIP1-1* was quantified in six different rootstocks (Fig. 4). RE was highly variable with an average CV among biological replicates of 45% (Table 1). NE and AE both resulted in marked decreases in the average CV among biological replicates to 8 and 9%, respectively. RE overestimated the actual magnitude of *VvPIP1-1* expression, which was 15-fold greater than *VvUbi*
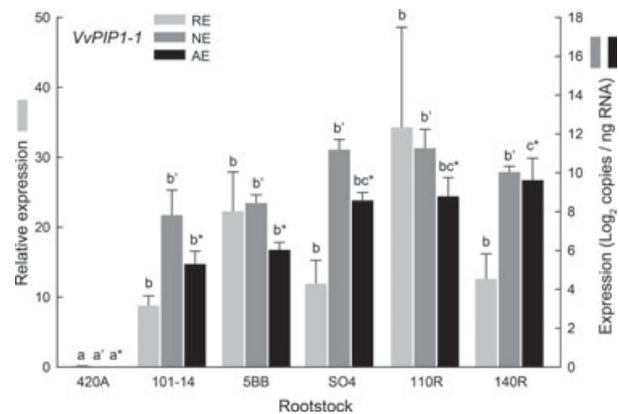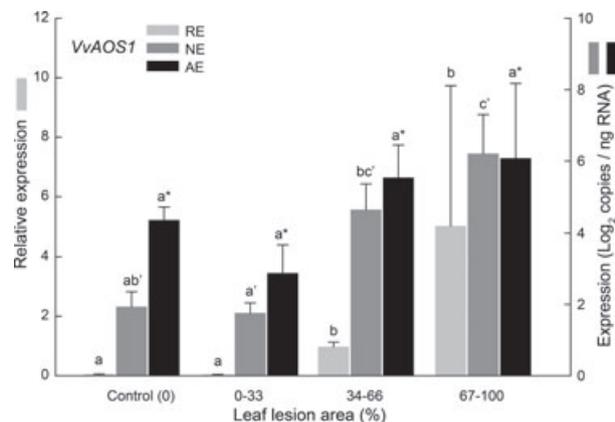

Fig. 4. *VvPIP1-1* expression in test case experiment #1 represented as RE (light gray), NE (gray), and AE (black). Bars represent standard error. Different letters represent significant differences within RE (letter only), NE (') or AE (*), across rootstocks ($P < 0.05$; Tukey's HSD, n = 4). Note, axis scales differ for RE (left) and NE, AE (right).

Table 1. GOI variability for each method of quantification. [a]Represents the mean of all CV calculated across biological replicates.

Experiment #	Gene	Average CV (%)[a]		
		RE	NE	AE
1 (roots)	*VvPIP1-1*	44.7	7.8	9.3
2 (leaves)	*VvAOS1*	64.4	33.9	39.0
3 (berries)	*VvPIP1-1*	70.3	22.4	24.0

when averaged across all rootstocks (Fig. 4, Table 2). However, AE was only 3.4-fold greater than *VvUbi* levels (Table 2). With regards to the different rootstocks, RE resulted in few statistically significant differences in expression despite large quantitative differences (Fig. 4, light gray bars). Rootstock 420A exhibited extremely low levels of expression, while 101–14, SO4 and 140R levels were intermediate. 5BB and 110R had the highest levels of expression, approximately 34 and 22 times *VvUbi*, respectively. NE yielded a different result; 420A still exhibited the lowest level of expression, but 110R and SO4 exhibited the highest levels of expression, while 101–14, 5BB and 140R exhibited intermediate levels (Fig. 4, gray bars). Again, there were few statistically significant differences in expression. Finally, AE resulted in the greatest number of statistically significant differences and the nature of the result was different than both RE and NE. 420A still exhibited the lowest levels of expression, but 140R had the highest level of expression (Fig. 4, black bars).

In the second test experiment, the jasmonic acid biosynthetic gene *VvAOS1* was quantified in leaves of grapevines responding to the pathogen *X. fastidiosa* (Fig. 5). RE was again extremely variable with an average

Table 2. Average GOI expression relative to *VvUbi*. [a]Represents the mean of all expression values calculated across biological replicates.

		Average expression relative to *VvUbi* (fold change)[a]	
Experiment #	Gene	RE	AE
1 (roots)	*VvPIP1-1*	15	3.4
2 (leaves)	*VvAOS1*	1.5	1.0
3 (berries)	*VvPIP1-1*	0.20	0.11

Fig. 5. *VvAOS1* expression in test case experiment #2 represented as RE (light gray), NE (gray), and AE (black). Bars represent standard error. Different letters represent significant differences within RE, NE, or AE, across leaf lesion area ($P < 0.05$; Tukey's HSD, n = 3). Note, axis scales differ for RE (left) and NE, AE (right).

CV among biological replicates of 64% (Table 1). NE and AE both resulted in decreases in the average CV among biological replicates to 34 and 39%, respectively. As in test experiment 1, RE overestimated the actual magnitude of *VvAOS1* expression, but to a much lesser extent being only 1.5-fold greater than *VvUbi* when compared with *VvAOS1* and *VvUbi* having equivalent expression values using AE (Table 2). With regards to changes in *VvAOS1* expression, all methods demonstrated that *VvAOS1* expression increased with increasing leaf lesion area, but differed in magnitude and statistical significance. RE and NE both resulted in significant differences, but both tended to grossly underestimate *VvAOS1* expression at lower expression levels when compared with AE (Fig. 5). As a result, AE resulted in smaller, non-significant differences in expression (Fig. 5, black bars).

In the final test experiment, *VvPIP1-1* was quantified in different parts of field-grown Chardonnay berries (Fig. 6). RE was again extremely variable with an average CV among biological replicates of 70% (Table 1). NE and AE both decreased the average CV among biological replicates to 22 and 24%, respectively. Again, RE overestimated the actual magnitude of *VvPIP1-1*
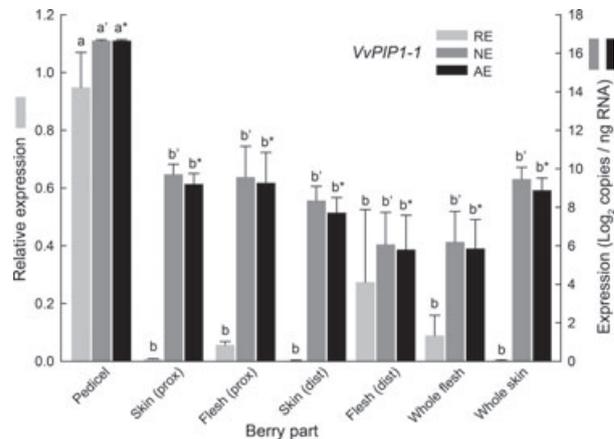

Fig. 6. *VvPIP1-1* expression in test case experiment #3 represented as RE (light gray), NE (gray), and AE (black). Bars represent standard error. Different letters represent significant differences within RE, NE or AE, across berry parts ($P < 0.05$; Tukey's HSD, n = 3). Note, axis scales differ for RE (left) and NE, AE (right).

Table 3. Comparison of *VvPIP1-1* expression in experiment #3. [a]There were no significant differences between methods of representing AE ($P < 0.05$, Tukey's HSD). [b]AE with reaction efficiencies determined from the standard curve. [c]AE with reaction efficiencies determined by LinRegPCR.

	VvPIP1-1 expression (Log_2 copies ng^{-1} RNA)[a]			
Berry part	NE	AE[b]	AE[c]	CV (%)
Pedicel	16.7	16.7	16	2.3
Skin (proximal)	9.7	9.2	10.3	5.6
Flesh (proximal)	9.6	9.3	10.1	4.1
Skin (distal)	8.4	7.7	7.3	6.6
Flesh (distal)	6.1	5.8	5.0	10
Whole skin	6.2	5.9	5.8	3.6
Whole flesh	9.5	8.9	9.5	3.9

expression (Table 2). With regards to changes in expression, all methods demonstrated that *VvPIP1-1* expression was greatest in the berry pedicel (Fig. 6). In this test experiment, reaction efficiencies were determined for all individual amplifications by using LINREGPCR (Ramakers et al. 2003). The value of *VvPIP1-1* expression determined by NE or AE did not differ significantly when reaction specific efficiencies were determined (Table 3).

Discussion

Assumption and error

The assumptions of RG stability and equal reaction efficiencies implicit in RE introduce significant error; however the nature of these errors is quite different.

The error introduced by variation in RG expression acts independently on each sample. The CVs of *VvUbi* expression determined in this study (25 and 55%) are equivalent to those determined by Reid et al. (2006). Although, Reid et al. (2006) did not employ absolute quantification; they found CVs of cycle thresholds of about 1–5% (which would correspond to CVs of approximately 25–100% in expression) across a range of candidate RGs. In both Arabidopsis and Poplar, variation in the expression of several RGs was also found to be quite high (Gutierrez et al. 2008b, Gutierrez et al. 2008a). RGs, even when properly validated, are not always stable across samples within or between experiments. The degree to which RG variations bias results is dependent on the magnitude of RG variation. This is illustrated in this study where in test experiment #1 *VvUbi* expression was the most variable (CV > 50%) and RE resulted in more pronounced error when compared with absolute methods. Methods that reduce the error due to RG variability include the use of multiple validated RGs (Vandesompele et al. 2002), and depending on the exact experimental design, additional statistical corrections (Willems et al. 2008).

While variation in RG expression acts independently on each sample, differences in reaction efficiencies produce systematic errors that affect all data in a C_T-dependent fashion. In this study, RG reaction efficiency (105%) was found to be greater than GOI efficiency for both GOIs (101 and 103%), which produces a situation where RE tends to overestimate expression. This is reflected in the test experiments where RE overestimates GOI expression by as much as fourfold when compared with AE (Table 2). Reactions efficiencies, even when determined for a given primer pair/template combination via the construction of a standard curve, can vary from reaction to reaction (Ramakers et al. 2003). This error is complicated by the fact that determination of fluorescent baseline values can influence the calculation of reaction efficiencies as well (Ruijter et al. 2009). Both of these issues can be resolved by obtaining reaction specific efficiencies directly from individual amplification plots using the program LinRegPCR (Ramakers et al. 2003). Determining reaction specific efficiencies did not substantially change the results in this study (Table 3). Furthermore, even NE, which still relies on the RG reaction efficiency, did not result in a significantly different measure of expression level when compared with AE. These results suggest that the majority of error is due to variability in RG expression. This is important because NE can be used as a proxy for AE, obviating the need for producing individual standard curves for every GOI in cases where the size of the experiment makes this unfeasible.

Absolute quantification does control for multiple sources of error, but it is not assumption free. Some of these assumptions are present in both relative and absolute quantification. For example, variability in RNA extraction (i.e. high abundance transcripts being extracted with higher efficiency than low abundance transcripts) is not controlled for in either methodology. Other sources of variation are controlled for in RE, but not in absolute. One of these possible sources of error is variability in the reverse transcription during the production of cDNA (Stahlberg et al. 2004a, 2004b). However, Stahlberg et al. (2004a, 2004b) found that this variability is well controlled by utilizing the same reaction conditions and template concentration across all samples, as was done in this study. When using gDNA there is the possibility of copy number variations, which could confound results (assuming every gene exists as diploid). However, when using a sequenced genome this potential pitfall is easily identifiable.

Problems of scale

Typically, AE level is reported as the number of copies of a GOI per amount of total RNA. This leads to two difficulties. First, mRNA makes up a very low percentage of the total RNA in a cell, the majority being rRNAs, and the ratio of mRNA/rRNA is not always equal among cells (Nolan et al. 2006). This is unlikely to cause issues in most studies because it is predominately a problem when comparing tissues proliferating at different rates (Nolan et al. 2006). However, it leads to another question which greatly impacts the presentation and interpretation of the result. Per what amount of total RNA should the number of copies of the GOI be reported: µg, ng, pg? The high sensitivity of qPCR necessitates a discussion of this problem because it allows for detection of so few mRNA copies (<10). In other words, on what scale is the level of gene expression biologically meaningful?

The quantity of RNA in eukaryotic cells varies, but typically ranges on the order of 10–100 pg (Roozemond 1976, Baugh et al. 2001, Alberts 2002). This means that when expression is reported on the order of microgram this represents the number of copies per 10^4–10^5 cells, approximately. For example, in Fig. 8 the high *VvPIP1-1* expression levels in the pedicel, approximately 27 $\log_2$ copies µg^{-1} RNA, correspond to approximately 100–1000 copies per cell. This is reasonable; however, the lower expression levels found throughout other parts of the berry correspond to approximately 1–10 copies cell^{-1} (Fig. 7). A level of expression this low is arguably not biologically meaningful and could represent insignificant basal levels of expression. Alternatively, the expression could be
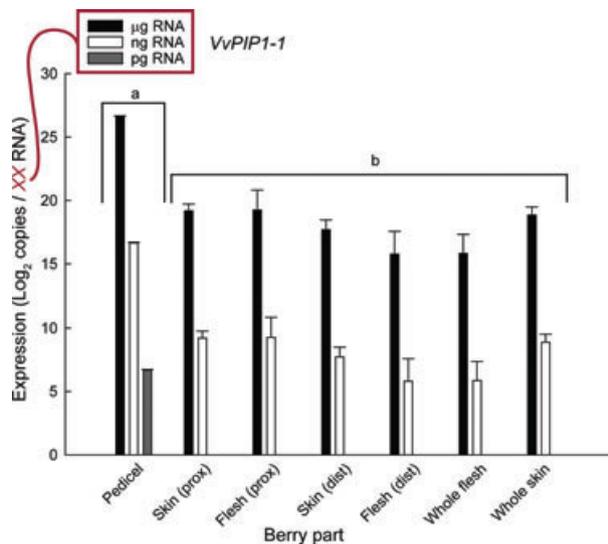
Fig. 7. Differences in the nature of the result due to scaling effects. Data for *VvPIP1-1* expression (from AE in Fig. 6) in experiment #3 is shown in three scales: $\log_2$ copies per microgram (black), nanogram (white), or picogram (gray) of RNA. Bars represent standard error. Scaling does not affect statistical significance ($P < 0.05$; Tukey's HSD, n = 3).

limited to a small fraction of cell types from the sampled tissue. Regardless, reporting the number of copies per microgram of RNA may give a false impression of significant expression levels. Instead, reporting expression as copies per nanogram or picogram of RNA represents a more realistic scale relative to individual cells comprising an organ or tissue (Fig. 7).

Biological and artifactual noise

Gene expression is noisy (reviewed in Raser and O'Shea 2005). Even if it was possible to obtain perfectly reliable measures of gene expression between cells, tissues or across developmental processes the data would be noisy by its nature. This 'biological' noise can arise from a variety of sources but is typically defined as either intrinsic, the noise present even in an idealized homogeneous state due to stochasticity, or extrinsic, the noise produced by differences in cell type, local environment, developmental state, etc. (Elowitz et al. 2002). Further, extrinsic noise can arise from changes that affect the expression of all genes in a sample equally (defined as global) or changes that affect a specific gene or genes (defined as gene-specific) (Raser and O'Shea 2004, Pedraza and van Oudenaarden 2005). This is an important distinction, especially with regard to the method of quantification, because global variation cannot be revealed by relative quantification. RE results in GOI expression appearing constant even while changing because RG expression is changing equally.

With regard to gene-specific variation, RE introduces error through variability in RG expression and reaction efficiencies.

In addition to the biological noise present, there will also be artifactual noise arising from errors discussed throughout this work. This work presents methodologies to minimize the contribution of those errors so to reveal the true biological noise present in the gene expression data. This is important because it is becoming increasingly clear that much can be learned by not just examining expression levels, but also having an understanding of expression variability (reviewed in Raser and O'Shea 2005).

Conclusions

gDNA-based absolute quantification is fast and efficient, requiring little additional time or money when compared with using RE. This methodology produces less variation, increased accuracy and greater statistical power through eliminating error introduced by assuming RG stability and equal reaction efficiencies between the RG and GOI. In addition, this methodology reveals the true biological noise present in the gene expression data by minimizing the artifactual noise.

References

Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P (2002) Molecular Biology of the Cell, 4th Edn. Garland Science, New York

Baugh LR, Hill AA, Brown EL, Hunter CP (2001) Quantitative analysis of mRNA amplification by in vitro transcription. Nucleic Acids Res 29: E29

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55: 611–622

Choat B, Gambetta GA, Shackel KA, Matthews MA (2009a) Vascular function in grape berries across development and its relevance to apparent hydraulic isolation. Plant Physiol 151: 1677–1687

Choat B, Gambetta GA, Wada H, Shackel KA, Matthews MA (2009b) The effects of Pierce's disease on leaf and petiole hydraulic conductance in Vitis vinifera cv Chardonnay. Physiol Plant 136: 384–394

Di Gaspero G, Cipriani G (2002) Resistance gene analogs are candidate markers for disease-resistance genes in grape (Vitis spp.). Theor Appl Genet 106: 163–172

Elowitz MB, Levine AJ, Siggia ED, Swain PS (2002) Stochastic gene expression in a single cell. Science 297: 1183–1186

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

Gambetta GA, Fei J, Rost TL, Matthews MA (2007) Leaf scorch symptoms are not correlated with bacterial populations during Pierce's disease. J Exp Bot 58: 4037–4046

Gambetta GA, Matthews MA, Shaghasi TH, McElrone AJ, Castellarin SD (2010) Sugar and abscisic acid signaling orthologs are activated at the onset of ripening in grape. Planta 232: 219–234

Guenin S, Mauriat M, Pelloux J, Van Wuytswinkel O, Bellini C, Gutierrez L (2009) Normalization of qRT-PCR data: the necessity of adopting a systematic, experimental conditions-specific, validation of references. J Exp Bot 60: 487–493

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

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

Jaillon O, Aury JM, Noel B, Policriti A, Clepet C, Casagre A, Choisne N, Aubourg S, Vitulo N, Jubin C, Vezzi A, Legeai F, Hugueney P, Dasilva C, Horner D, Mica E, Jublot D, Poulain J, Bruyere C, Billault A, Segurens B, Gouyvenoux M, Ugarte E, Cattonaro F, Anthouard V, Vico V, Del Fabbro C, Alaux M, Di Gaspero G, Dumas V, Felice N, Paillard S, Juman I, Moroldo M, Scalabrin S, Canaguier A, Le Clainche I, Malacrida G, Dur E, Pesole G, Laucou V, Chatelet P, Merdinoglu D, Delledonne M, Pezzotti M, Lecharny A, Scarpelli C, Artiguenave F, Pe ME, Valle G, Morgante M, Caboche M, Adam-Blondon AF, Weissenbach J, Quetier F, Wincker P, Public F-I (2007) The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. Nature 449: 463–467

Lodhi M, Ye G-N, Weeden N, Reisch B (1994) A simple and efficient method for DNA extraction from grapevine cultivars and Vitis species. Plant Mol Biol Rep 12: 6–13

Nolan T, Hs RE, Bustin SA (2006) Quantification of mRNA using real-time RT-PCR. Nat Protoc 1: 1559–1582

Palovaara J, Hakman I (2008) Conifer WOX-related homeodomain transcription factors, developmental consideration and expression dynamic of WOX2 during Picea abies somatic embryogenesis. Plant Mol Biol 66: 533–549

Pedraza JM, van Oudenaarden A (2005) Noise propagation in gene networks. Science 307: 1965–1969

Ramakers C, Ruijter JM, Deprez RH, Moorman AF (2003) Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci Lett 339: 62–66

Raser JM, O'Shea EK (2004) Control of stochasticity in eukaryotic gene expression. Science 304: 1811–1814

Raser JM, O'Shea EK (2005) Noise in gene expression: origins, consequences, and control. Science 309: 2010–2013

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

Rieu I, Powers SJ (2009) Real-time quantitative RT-PCR: design, calculations, and statistics. Plant Cell 21: 1031–1033

Roozemond RC (1976) Ultramicrochemical determination of nucleic-acids in individual cells using Zeiss UMSP-1 microspectrophotometer – Application to isolated rat hepatocytes of different ploidy classes. Histochem J 8: 625–638

Ruijter JM, Ramakers C, Hoogaars WM, Karlen Y, Bakker O, van den Hoff MJ, Moorman AF (2009) Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. Nucleic Acids Res 37: e45

Stahlberg A, Kubista M, Pfaffl M (2004a) Comparison of reverse transcriptases in gene expression analysis. Clin Chem 50: 1678–1680

Stahlberg A, Hakansson J, Xian X, Semb H, Kubista M (2004b) Properties of the reverse transcription reaction in mRNA quantification. Clin Chem 50: 509–515

Steenkamp J, Wiid I, Lourens A, Vanhelden P (1994) Improved method for DNA extraction from Vitis vinifera. Am J Enol Vit 45: 102–106

Terrier N, Glissant D, Grimplet J, Barrieu F, Abbal P, Couture C, Ageorges A, Atanassova R, Leon C, Renaudin JP, Dedaldechamp F, Romieu C, Delrot S, Hamdi S (2005) Isogene specific oligo arrays reveal multifaceted changes in gene expression during grape berry (Vitis vinifera L.) development. Planta 222: 832–847

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

Willems E, Leyns L, Vandesompele J (2008) Standardization of real-time PCR gene expression data from independent biological replicates. Anal Biochem 379: 127–129

Yun JJ, Heisler LE, Hwang II, Wilkins O, Lau SK, Hyrcza M, Jayabalasingham B, Jin J, McLaurin J, Tsao MS, Der SD (2006) Genomic DNA functions as a universal external standard in quantitative real-time PCR. Nucleic Acids Res 34: 6718–6718

Edited by V. Hurry