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Expression of stable reference genes and *SPINDLY* gene in response to gibberellic acid application at different stages of grapevine development

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Abstract

Gibberellic acid (GA_3) is widely used at different stages of berry development, and to understand the molecular mechanism of its action requires identification of stable reference genes. We sprayed grapevine (*Vitis vinifera* L.) cv. Thompson Seedless with GA_3 at rachis stage for rachis elongation, at flower cluster stage for flower thinning, and at 3 - 4 mm berry stage for berry elongation. Tissue samples were collected at different time points after GA_3 application. The expression of 10 candidate reference genes was analyzed using 4 different algorithms to assess their suitability for real time-PCR data normalization. Based on the overall ranking, *PP2A*, *Sutra*, and *SAND* were identified as the most stably expressed genes across all samples. With regard to different stages, *tubulin*, *EF1α*, and *UBC* were the most stable genes during rachis elongation; *PP2A*, *SAND*, and *Sutra* were the most suitable at the flower cluster and berry stages. The expression of GA signaling gene *SPINDLY* (*VvSpy*) was analyzed to validate the stable reference genes. After the GA_3 application, the expression of *VvSpy* was reduced at the rachis stage but did not change at the flower cluster and berry stages. The expression profile of *VvSpy* was comparable when two or three reference genes were used for data normalization.

Additional key words: developmental stages, *Vitis vinifera.*

Introduction

Grapevine is one of the most widely cultivated commercial fruit crops in the world. In India, cv. Thompson Seedless and its clonal selections occupy more than 90 % of the area and are grown mainly for fresh consumption. Morphology of bunches and berry size are two economically important traits in table grape production and are usually controlled by the application of gibberellins and cytokinins (Dookoozlian and Peacock 2001). In cv. Thompson Seedless, the production of loose clusters with bold berries requires mechanical and chemical thinning through the application of GA_3 at different stages of cluster development. A spray with a very low concentration of GA_3 immediately after cluster emergence results in rachis elongation, whereas the second spray at the full bloom stage is important for flower thinning. The third application of GA_3 at the 3 - 4 mm and 8 - 10 mm berry stages results in berry elongation (Roper and Williams 1989, Ramteke and Somkuwar 2006). The use of GA_3 increases the berry size of cv. Emperatriz Seedless grapes depending on the phenological stages and concentrations applied (Casanova *et al.* 2009). Similarly, treating cv. Flame Seedless grapes with 20 mg dm^{-3} GA_3 at the pre-bloom, post-bloom, and pre-veraison stages increases masses of clusters and berries and also increases transportability of berries (Dimovska *et al.* 2014). Although GA_3 is extensively used, there is lack of knowledge about molecular mechanisms of GA_3 response in grape.

Gene expression analysis by qPCR is a powerful technique to understand changes occurring at molecular level in response to different conditions. Accuracy of qPCR is affected by several factors including technical and experimental variations. Stability of an internal reference gene used for data normalization is considered to be the most important factor which might cause a significant variation in results. Doubtful results of gene expression arise when target genes are normalized against a single reference gene without adequate justification and

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Abbreviations: cDNA - complementary DNA; CV – coefficient of variance; dNTP - deoxyriboso-nucleotide triphosphate; GA_3 - gibberellic acid; NCBI - National Center for Biotechnology Information; qPCR - quantitative polymerase chain reaction.
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when non-validated reference genes are used (Bustin *et al.* 2009, Derveaux *et al.* 2010). A reference gene is considered to be good if its expression remains stable across different tissues/organs under experimental conditions. The most commonly used reference genes across plant species are *β-actin*, *GAPDH*, *18sRNA*, *25sRNA*, *ubiquitin conjugating enzymes (UBC)*, *elongation factor 1α (EF1α)*, *tubulin*, *etc*. However, an unstable expression under different sets of experimental conditions has been reported for many of these genes (Gutierrez *et al.* 2008a). The need to validate reference genes for each experimental system has also been demonstrated (Dheda *et al.* 2005, Gutierrez *et al.* 2008b). In recent years, increased awareness about the selection of appropriate gene for data normalization has resulted in numerous studies on reference gene validation in a wide spectrum of plant species including grapevine (Reid *et al.* 2006, Gutha *et al.* 2010), sugarcane (Iskandar *et al.* 2004), poplar (Xu *et al.* 2011), banana (Chen *et al.* 2011), litchi (Zhong *et al.* 2011), citrus (Mafra *et al.* 2012), papaya (Zhu *et al.* 2012), apple (Perini *et al.* 2014), and African oil palm (Xia *et al.* 2014) under different experimental conditions.

Several methods employing different algorithms have been used for estimating the expression stability of reference genes. The *geNorm* (Vandesompele *et al.* 2002) and *NormFinder* (Andersen *et al.* 2004) are the most frequently used methods. The *Bestkeeper* (Pfaffl *et al.* 2004) and *delta Ct* (Silver *et al.* 2006) methods are also used to determine the expression stability of reference genes. These four algorithms have been integrated into web based program *RefFinder* (Xie *et al.* 2012). Based on rankings from each program, it assigns an appropriate weight to an individual gene and calculates the geometric (geo) mean of their weights for the overall final ranking.

In this study, 10 candidate reference genes were evaluated to identify the most stable reference genes after GA_3 application to grapevine. Samples were collected at different stages of berry development at different time points after the application of GA_3. The most stable reference genes suitable for normalization of qPCR data were identified for different stages. Expression data of gibberellin signaling gene *VvSpy* were analyzed to validate the stable reference genes.

Materials and methods

Plants and treatments: The experiments were conducted on 13-year-old grapevine (*Vitis vinifera* L.) cv. Thompson Seedless at the ICAR National Research Centre for Grapes, Pune, during October - November 2012. The vines were planted at a spacing of 3.3 m between rows and 1.8 m between vines and were trained to a flat roof gable system. Vines with a uniform growth were selected based on pruning biomass accumulated during the previous pruning season.

The details of hormone application and sampling are given in Table 1. Control vines were sprayed/dipped with water at each stage. Six vines were used for sampling at each time point. Samples from a single vine were pooled and considered as one biological replicate. Collected samples were snap frozen in liquid nitrogen and stored at -80 °C until use.

Table 1. Details of hormone treatment and sampling.

Treatment	GA_3 [mg dm^{-3}]	Stage and mode of application	Sampling time points	Sampled tissue
T1	10	rachis at pre bloom stage (spray)	6 and 24 h after spray	rachis
T2	25	flower cluster at full bloom stage (spray)	6, 24, and 48 h after spray	flower cluster
T3	25	berries at 3-4 mm size stage (dipping)	6, 24, and 48 h after dip	berries

RNA extraction and first strand cDNA synthesis: Total RNA was extracted from 1 g of the deep frozen tissue using a modified sodium perchlorate method (Davies and Robinson 1996) followed by purification using a *Spectrum* plant total RNA extraction kit (*Sigma-Aldrich*, St. Louis, USA). On-column DNase digestion was performed before RNA elution from the column. The RNA was extracted from three biological replicates for each sample. The RNA concentrations and quality were evaluated by measuring the ratios of absorbances A_{260}/A_{280} and A_{260}/A_{230} using a nano-spectrophotometer (*Implen*, Munich, Germany). The integrity of the total RNA was determined by electrophoresis on 1 % (m/v) agarose gels using Tris/borate/EDTA (TBE) as electrophoresis buffer. Total RNA (1 μg) was reverse transcribed in 20 mm^3 of a reaction mixture containing 2.5 μM oligo-dT primer, 500 μM dNTPs, 1× reaction buffer, and 4 units of *Superscript III* reverse transcriptase (*Invitrogen*, Carlsbad, USA) according to the manufacturer's instructions. After the first strand synthesis, all cDNA samples were diluted 10-fold with RNase-free water and used as templates in qPCR analysis.

Candidate and target gene selection and primer design: A total of 10 candidate reference genes reported in earlier studies were evaluated for their stability under our experimental conditions. The selected genes were *actin*, *AqPo* (coding aquaporin protein), *EF1α* (elongation factor 1α), *GAPDH* (glyceraldehyde-3-phosphate

dehydrogenase), *PP2A* (protein phosphatase), *SAND* (SAND family protein), *Sutra* (sucrose transporter), *α-tubulin*, *UBC* (ubiquitin conjugating enzyme) reported by Reid *et al.* (2006) and *UBI* (ubiquitine) (Fujita *et al.* 2005). Primer sequences for these genes were taken from these published reports.

The sequence of target gene *SPINDLY* (*VvSpy*, coding for O-linked N-acetylglucosamine transferase) was identified by using the grape genome sequence from the *NCBI* database using the *Arabidopsis* homologue (*NCBI* gene ID 820327) as query. Specific primers for the target gene were designed with *Primer3Plus v. 2.3.6* (Untergasser *et al.* 2007). The following parameters were used for designing the primers: the amplicon length between 150 - 220 bp, the primer size of 20 ± 2 bp, the melting temperature (Tm) between 55 and 60 °C and the GC content of 45 - 50 %. The forward and reverse primers purposely lay on adjoining exons which were separated by an intron. A slightly higher amplicon length of 150 - 220 bp was used since the software did not predict a primer in adjoining exons when a lower amplicon length was used. The specificity of the primer set was confirmed by the presence of a single symmetrical amplicon peak in the melting curve analysis and no primer-dimer peaks in no-template-control (NTC) reactions.

qPCR analysis: An optimal primer concentration was estimated by assay of a few samples with varying concentrations (50, 100, 200, and 300 nM) of the primer and the primer concentration of 200 nM was found to be optimal. The 20 mm^3 of a qPCR cocktail contained 3 mm^3 of diluted template cDNA, 200 nM of each primer, and 10 mm^3 of *Absolute blue qPCR SYBR Green Master Mix* (2×) (*Thermo Scientific*, Waltham, USA). Aliquots from the same cDNA sample were used with all primer sets. A robotic liquid handling system *QIAgility 2000* (*Qiagen*, Hilden, Germany) was used for setting up qPCR reactions. The qPCR was performed on *Rotor-Gene Q* (*Qiagen*) and the following thermal cycling program was used: an initial denaturation and enzyme activation at 95 °C for 10 min, 40 cycles at 95 °C for 20 s, at 60 °C for 20 s, and at 72 °C for 20 s, followed by a final extension at 72 °C for 5 min. The primer specificity was monitored by melting curve analysis consisting of slow heating at 50 °C with a rate of 1 °C per 5 s up to 99 °C with a continuous measurement of fluorescence. No template controls were included in each PCR run for each primer pair. Each sample was amplified in two technical replicates and the mean was used for data analysis.

Data analysis: *Rotor-Gene Q* software *v. 2.3.1* was used for data acquisition and analysis. The amplification plots were analyzed with a default threshold value and amplification cycles (Cq) were obtained. PCR efficiency and correlation efficiency (R^2) for all primer pairs were estimated using a five-point standard curve based on serial dilutions of one of the cDNA templates. The PCR efficiency was calculated based on the slope of the standard curve using the following formula: reaction efficiency = $[10^{(-1/M)}] - 1$; where M is the slope of the standard curve.

The Cq values were used to evaluate the stability of reference genes under our experimental conditions. Four analysis methods which use different algorithms were used: *BestKeeper* (Pfaffl *et al.* 2004), *NormFinder* (Andersen *et al.* 2004), *geNorm* (Vandesompele *et al.* 2002), and comparative *delta Ct* (Silver *et al.* 2006). The web-based tool *RefFinder* (http://www.leonxie.com/referencegene.php; Xie *et al.* 2012), which integrates these four methods, was used to evaluate and screen the reference genes.

Validation of reference genes: Gibberellin signaling gene *SPINDLY* (*VvSpy*) was used as target gene to confirm the stability of reference genes under our experimental conditions. The software *REST2009* (Pfaffl *et al.* 2002) was used to calculate the relative expression of the target gene. The *REST2009* software applies a mathematic model that takes into account different PCR efficiencies of the gene of interest and reference genes. It also includes integrated randomization and bootstrapping to test the statistical significance of calculated expression ratios.

Results and discussion

The amplification specificity of the 10 candidate reference genes and the target gene was evaluated by melting curve analysis of PCR products. The presence of a single peak with no visible primer-dimer formation confirmed the specific amplification for each gene. Also, no signals were detected in the no-template controls. Amplification efficiency (E), which indicates the amplicon doubling rate of a specific primer pair, ranged from 86 % (*SAND*) to 110 % (*GAPDH*). The correlation coefficients (R^2) of linear standard curves varied between 0.980 - 0.998 indicating good fits of the standard curves to plotted data points and strong correlations between detected Cq values and amounts of templates in the reactions (Table 1 Suppl.). These results confirm the specificity and high-efficiency RT-qPCR systems for the selected genes.

Expression of genes was determined based on Cq value. The Cq data indicate that the transcripts of these genes showed varying abundance. The mean Cq value of the 10 reference genes varied between 14.82 and 24.61. Among these candidate reference genes, *EF1α* was the most abundantly expressed gene in all the samples as indicated by its low Cq values (14.82 ± 0.96) followed by *GAPDH* (15.39 ± 1.00), whereas *SAND* was the least abundant gene (24.61 ± 1.21). The calculated coefficient of variance (CV) of the Cq values of the reference genes

across all the samples ranged from 0.92 to 2.50 (Table 2 Suppl.). The *EF1α* and *UBC* had the lowest CV of 0.92 and 0.94, respectively, thus showing the least variation in expression. *Actin* (2.50) and *AqPo* (2.29) had large variances in their expression. The CV values of *GAPDH*, *α-tubulin*, *PP2A*, and *Sutra* were 1.12, 1.23, 1.31, and 1.27, respectively.

We also analyzed stage wise variation in expression of the candidate reference genes (Table 2 Suppl.). At rachis elongation stage, *SAND* (CV = 0.54) was the least variable gene followed by *GAPDH* (CV of 0.57), whereas *AqPo*, *UBI*, and *actin* had large variations in their expression with CV of 1.94, 1.48, and 1.39, respectively. At flower cluster stage, *UBC* was the least variable gene (CV of 0.30) followed by *AqPo* and *Sutra* (CV of 0.58 and 0.62 respectively). *UBI* showed the most variable expression profile at cluster stage. *GAPDH*, *EF1α*, and *SAND* were the least variable in berry stage, whereas *actin* showed the highest variation. A significant variation in the expression of *actin* was revealed in all the treatments and tissues.

The expression of an ideal reference gene should remain stable under experimental conditions. Four different approaches were used to analyze the expression stability of the candidate genes in different data sets: *1*) all experimental samples, *2*) samples of rachis, *3*) samples of flower cluster, and *4*) samples of berry stage.

The Cq values of all the samples which included control and GA_3 treated samples at three stages and different time points were integrated and analyzed by the above mentioned four algorithms (Table 2). The *PP2A* and *Sutra* were identified as the best reference genes by

Table 2. The stage wise ranking of 10 candidate genes determined by different methods.

Samples	Overall ranking	Geometric mean	*geNorm*	Stability value	*NormFinder*	Stability value	*delta Ct*	Average SD	*BestKeeper*	SD
All samples	*PP2A*	1.63	*PP2A*	0.33	*PP2A*	0.32	*PP2A*	0.65	*EF1α*	1.65
	Sutra	2.99	*Sutra*	0.33	*SAND*	0.33	*SAND*	0.66	*UBC*	1.65
	SAND	3.13	*SAND*	0.38	*tubulin*	0.37	*tubulin*	0.69	*GAPDH*	1.73
	UBC	3.34	*tubulin*	0.47	*Sutra*	0.43	*Sutra*	0.70	*UBI*	1.82
	tubulin	3.83	*UBC*	0.54	*UBC*	0.55	*UBC*	0.79	*Sutra*	1.83
	EF1α	4.56	*EF1α*	0.60	*EF1α*	0.57	*EF1α*	0.80	*tubulin*	1.85
	GAPDH	6.05	*GAPDH*	0.65	*UBI*	0.61	*UBI*	0.83	*PP2A*	1.86
	UBI	6.29	*UBI*	0.68	*GAPDH*	0.62	*GAPDH*	0.83	*SAND*	2.00
	AqPo	9.00	*AqPo*	0.72	*AqPo*	0.74	*AqPo*	0.90	*AqPo*	2.21
	actin	10.00	*actin*	0.79	*actin*	0.96	*actin*	1.09	*actin*	2.39
Rachis	*tubulin*	2.11	*PP2A*	0.34	*tubulin*	0.09	*tubulin*	0.62	*GAPDH*	0.53
	EF1α	2.91	*Sutra*	0.34	*EF1α*	0.25	*EF1α*	0.65	*UBC*	0.60
	UBC	3.22	*EF1α*	0.46	*UBC*	0.34	*UBC*	0.67	*SAND*	0.61
	Sutra	3.64	*tubulin*	0.49	*SAND*	0.38	*SAND*	0.69	*UBI*	0.64
	SAND	3.94	*SAND*	0.51	*Sutra*	0.46	*Sutra*	0.71	*tubulin*	0.68
	PP2A	4.12	*UBC*	0.52	*PP2A*	0.47	*PP2A*	0.72	*EF1α*	0.73
	GAPDH	5.20	*UBI*	0.54	*UBI*	0.51	*UBI*	0.75	*Sutra*	0.75
	UBI	6.09	*AqPo*	0.62	*AqPo*	0.83	*AqPo*	0.98	*PP2A*	0.81
	AqPo	8.24	*GAPDH*	0.70	*GAPDH*	0.83	*GAPDH*	0.99	*AqPo*	1.06
	actin	10.00	*actin*	0.80	*actin*	1.12	*actin*	1.22	*actin*	1.14
Flower cluster	*PP2A*	1.41	*PP2A*	0.21	*PP2A*	0.16	*PP2A*	0.49	*UBC*	0.39
	SAND	2.30	*SAND*	0.21	*SAND*	0.22	*SAND*	0.51	*Sutra*	0.62
	Sutra	2.91	*Sutra*	0.31	*EF1α*	0.39	*Sutra*	0.59	*AqPo*	0.67
	EF1α	4.16	*AqPo*	0.36	*Sutra*	0.42	*EF1α*	0.60	*PP2A*	0.69
	AqPo	4.56	*EF1α*	0.44	*tTubulin*	0.46	*tubulin*	0.64	*EF1α*	0.71
	UBC	4.74	*tubulin*	0.48	*AqPo*	0.49	*AqPo*	0.64	*tubulin*	0.74
	tubulin	5.48	*UBC*	0.52	*GAPDH*	0.51	*GAPDH*	0.67	*SAND*	0.77
	GAPDH	7.71	*GAPDH*	0.57	*UBC*	0.57	*actin*	0.71	*actin*	0.77
	actin	8.49	*actin*	0.60	*actin*	0.58	*UBC*	0.71	*GAPDH*	0.82
	UBI	10.00	*UBI*	0.63	*UBI*	0.63	*UBI*	0.76	*UBI*	0.96
Berry	*SAND*	1.63	*SAND*	0.24	*SAND*	0.27	*SAND*	0.56	*UBC*	0.43
	PP2A	3.08	*Sutra*	0.24	*GAPDH*	0.32	*PP2A*	0.59	*UBI*	0.47
	Sutra	3.31	*PP2A*	0.29	*PP2A*	0.33	*Sutra*	0.61	*GAPDH*	0.47
	GAPDH	3.81	*EF1α*	0.36	*EF1α*	0.35	*EF1α*	0.62	*EF1α*	0.49
	EF1α	4.00	*AqPo*	0.42	*Sutra*	0.38	*GAPDH*	0.63	*PP2A*	0.51
	UBC	4.76	*tubulin*	0.47	*tubulin*	0.43	*tubulin*	0.68	*tubulin*	0.59
	tubulin	6.00	*GAPDH*	0.50	*AqPo*	0.51	*AqPo*	0.71	*SAND*	0.59
	UBI	6.18	*UBC*	0.54	*UBC*	0.55	*UBC*	0.74	*Sutra*	0.62
	AqPo	6.85	*UBI*	0.60	*UBI*	0.67	*UBI*	0.84	*AqPo*	0.74
	actin	10.00	*actin*	0.72	*aActin*	1.11	*actin*	1.19	*actin*	0.96

geNorm with M values of 0.33 followed by *SAND* (M of 0.38). The *NormFinder* and *delta Ct* methods showed *PP2A* and *SAND* to be the most stably expressed genes. However, the results of *BestKeeper* were very different; *EF1α* and *UBC* were the best reference genes by this algorithm. In overall ranking, *PP2A*, *Sutra*, and *SAND* with geometric (geo) means of 1.63, 2.99, and 3.13 were found to be the most stable genes under the GA_3 application. *Actin* and *AqPo* were considered to be the least stable genes by all the four methods as well as in overall ranking.

We also evaluated the stability of candidate reference genes at individual stages of sampling. In rachis, *geNorm* considered *PP2A* and *Sutra* (M of 0.34) and *EF1α* (M of 0.46) as the best reference genes. *Tubulin* and *EF1α* were considered to be the best reference genes by the *NormFinder* and *delta Ct* methods, whereas *GAPDH* (standard deviation, SD, of 0.53) and *UBC* (SD of 0.60) were identified as the two best genes by *BestKeeper*. In overall ranking, *Tubulin* and *EF1α* (geo means of 2.11 and 2.91, respectively) were found to be the most stably expressed genes. *Actin* was the least stable gene in rachis by all the algorithms.

In the flower cluster, *PP2A*, *SAND*, and *Sutra* with stability values of 0.21, 0.21, and 0.31 were considered the most stable genes by *geNorm*. Both the *NormFinder* and *delta Ct* algorithms suggested *PP2A* and *SAND* as the best reference genes, whereas *UBC* and *Sutra* were ranked as the most stable genes by *BestKeeper*. In overall ranking, *PP2A* and *SAND* were the two most stable reference genes. All the methods considered *UBI* to be the least stable reference gene in the flower cluster.

In berries, *SAND* and *Sutra* (M of 0.24) followed by *PP2A* (M of 0.29) were the three best reference genes by the *geNorm* algorithm. *NormFinder* considered *SAND* and *GAPDH* as the best reference genes. *SAND* (SD of 0.56) and *PP2A* (SD of 0.59) were the most stable genes by the *delta Ct* method, whereas *BestKeeper* identified *UBC* and *UBI* as the most suitable reference genes. In overall ranking, *SAND*, *PP2A*, and *Sutra* with geo mean values of 1.63, 3.08, and 3.31 were considered as the best reference genes for expression analysis in berries.

As expected, variation in gene ranking was observed by the different methods as different algorithms are used by these methods. The *geNorm* is the most widely used statistical algorithm to analyze the stability of reference genes. It calculates gene expression stability measure M for a reference gene as the average pairwise variation V for that gene with all other tested reference genes (Vandesompele *et al.* 2002). For a good internal reference gene, M value should be less than 1.5. The M values for all the studied genes were below the threshold limit in all the data sets (Table 2). The ranking of genes in four different data sets varied. According to *geNorm*, the best three stable genes were *PP2A*, *Sutra*, and *SAND* for the samples of berry and flower clusters; though the stability values for these genes varied marginally in different data sets. In rachis, *PP2A* and *Sutra* were top ranked with M of 0.34 followed by *EF1α* (M of 0.46). *SAND*, which was ranked among the top three genes in other sets, was ranked fifth although its stability value M (0.51) was not significantly higher than the third ranked gene, *EF1α* (M of 0.46).

NormFinder ranks the set of candidate genes according to their expression stability in a given sample set and given experimental design (Andersen *et al.* 2004) and the genes with the lowest stability values are top ranked. The *PP2A* and *SAND*, top-ranked by *geNorm*, were also top-ranked by the *NormFinde*r and *delta Ct* methods for the flower cluster and berry but not for the rachis samples. *BestKeeper* calculates SD and CV based on Cq values of candidate reference genes (Pfaffl *et al.* 2004). The most stable genes are identified as those which exhibit the lowest CVs and SDs. In our study, contrasting results were obtained with *BestKeeper* which identified *EF1α* and *UBC* as the most stable genes and placed *PP2A*, *SAND*, and *Sutra* at the middle rank.

The comprehensive ranking, which involves assigning an appropriate weight to an individual gene based on the ranking by different methods and calculates the geo mean of their weights for the overall final ranking (Xie *et al.* 2012), identified *PP2A*, *Sutra*, and *SAND* as the most stable reference genes in all the samples including the flower cluster and berry samples, which corroborated well with the ranking given by the *geNorm*, *NormFinder*, and *delta Ct* methods. The stable reference genes based on the comprehensive ranking were considered for validation and data normalization of the target gene, *VvSpy*.

Based on the homology search using *Arabidopsis* sequences (*NCBI* gene ID 820327) for *SPINDLY*, the homologous grape genome sequence (GenBank accession - AM436403.1) was used for gene prediction using *FGENESH* (Solovyev *et al.* 2006). A protein containing 7 exons and 457 amino acids was identified. Conserved domains *Glyco_transf_41* and *Spy* were also detected in the protein sequence. Protein *BLAST* showed a high homology to *SPINDLY* or *SPINDLY*-like proteins in other plant species (data not shown), thus confirming that the selected target gene coded for *SPINDLY* protein in grape. We renamed this gene as *VvSpy* and used it for the validation of selected reference genes under GA_3 application.

We analyzed the relative expression of *VvSpy* across all the treatments, time points, and tissues (rachis, flower, cluster, and berry) after the GA_3 treatment. Single (*PP2A*), two (*PP2A* and *Sutra*), and three (*PP2A*, *Sutra*, and *SAND*) most stable reference genes, and the least stable gene (*actin*) based on the comprehensive ranking were used for data normalization. The results (Fig. 1) indicate that the expression of *VvSpy* in the rachis decreased at 6 and 24 h after the GA_3 treatment when recommended reference genes based on all sample data were used for data normalization. The expression profile was similar when two or three genes were used, *i.e.*, a relative expression at 24 h was less than at 6 h. However, when the stage specific reference genes were used for data normalization, the expression profile was reversed.

In the flower cluster, the expression of *VvSpy* gene in the GA_3 treated vines did not change at 6 and 24 h, however, decreased marginally at 48 h. Similarly, the expression of *VvSpy* did not change appreciably in the GA_3 treated berries. The expression varied when one or multiple reference genes were used for data normalization; however, the expression profiles of *VvSpy* in the flower cluster and berries at different time points were similar when one, two, or three reference genes were used for data normalization. Normalization with the least stable

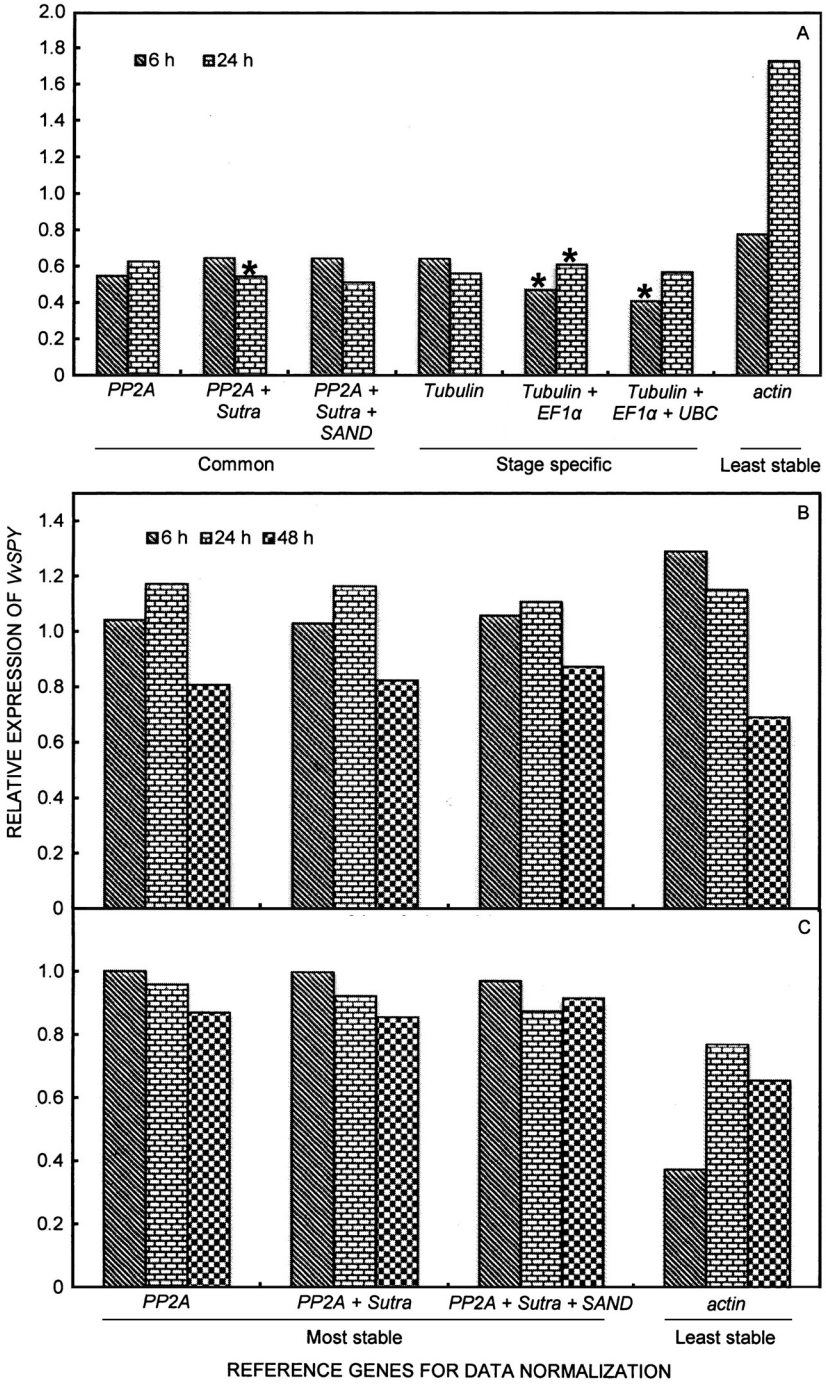

Fig. 1. Relative expression of *VvSpy* in the rachis (*A*), flower cluster (*B*), and 3 - 4 mm berries (*C*) of grapevine cv. Thompson Seedless after GA_3 treatment. Data were normalized with one, two, or three the most stable and least stable reference genes. The *REST2009* software, which takes into account different PCR efficiencies of a gene of interest and reference genes, was used for estimating a relative expression of *VvSpy* in treated samples. The software also includes integrated randomization and bootstrapping to test the statistical significance of calculated expression ratios. Iteration was set at 10 000 during analysis. * - indicates significant difference in the relative expression at $P < 0.01$.

gene *actin* resulted in overestimation of expression in the rachis samples at both the time points, and it was up to two times higher at 24 h. In the flower cluster and berry samples, the expression profiles with *actin* at different time points were different from that obtained with the most stable genes. In the berries, underestimation of expression was recorded at all the time points.

The *SPINDLY* (*Spy*) encodes O-linked N-acetylglucosamine transferase (Thornton *et al.* 1999) and has been shown as negative regulator of GA_3 signaling in *Arabidopsis* (Jacobsen and Olszewski 1993). In *Arabidopsis,* mutations in *Spy* gene resulted in suppression of all of the phenotypes caused by GA_3 deficiency to varying levels. Cytosolic *Spy* activity was reported to promote cytokinin responses and repress GA_3 signaling (Maymon *et al.* 2009). In grapes, though GA_3 is extensively used, no information is available about the role of this gene in GA_3 signaling at different stages of berry development. The GA_3 spray at rachis stage resulted in rachis elongation and the effect was markedly visible within 24 h after the application. The expression of *VvSpy* was reduced at rachis stage indicating its role in GA_3 signaling. The reduced expression of *VvSpy* promoted cell elongation in the rachis. These results are in accordance with reports on rice (Shimada *et al.* 2006) where an enhanced elongation of lower internodes is correlated with a decreased *OsSPY* expression in knockdown plants. At flowering stage, the inhibition of pollen tube growth in pistils after GA_3 treatment due to biosynthesis of pollen tube inhibitors has been reported in grapevine cvs. Muscat Bailey (Kimura *et al.* 1996) and Delaware (Okamoto and Miura 2005). In addition to a pollenicidal effect and ABA mediated abscission in the presence of GA_3, an increased ethylene content at the time of flowering also contributes to flower abscission in cv. Chardonnay (Hilt and Bessis 2003). Berry elongation is a result of increased cell expansion. A higher content of GA_3 is known to increase cell expansion through an increased hydrolysis of starch, fructans, and sucrose into glucose and fructose (Glasziou 1969). In the flower cluster and berry, the expression of *VvSpy* in the GA_3 treated vines did not change significantly suggesting that different mechanisms of GA_3 response and/or signaling played roles at these stages.

Precise and consistent estimation of gene expression requires data normalization with an appropriate reference gene. Successful quantification of gene expression requires data normalization with a validated reference gene showing stable expression across all experimental material and conditions. Data normalization with validated reference genes controls variations in RNA extraction yield, reverse transcription yield, and amplification efficiency, allowing comparison of mRNA content across different samples (Bustin *et al.* 2009). In grapevine, Reid *et al.* (2006) evaluated a large number of genes for their stability during berry development across two seasons and recommended the use of combinations of several genes for normalization in grape berry development studies. They also suggested *GAPDH* and *actin* as two the most suitable reference genes for data normalization. Gutha *et al.* (2010) also identified *actin* and *NAD5* as the stable reference genes for studying gene expression in grapevine leaf in response to virus infection. These reports are in contrast to our analysis wherein the expression of *actin* was found to be varying after the GA_3 application. The GA_3 application in grape resulted in rapid changes in the rachis, flower cluster, and berry leading to elongation, thinning, and elongation, respectively. Several changes in the cell shape are linked to actin cytoskeleton (Smith 2003). This may be the reason for a varying expression of *actin* under GA_3 application. Gamm *et al.* (2011) identified *VATP16* and *60SRP* as stable reference genes for studying expression in grapevine leaves and berries infected with *Plasmopara viticola* and *Botrytis cinerea*, respectively. However, several publications on gene expression studies in grape have reported the use of a single reference gene, mainly *VvUbi*, *EF1α*, or *actin* for data normalization without a proper validation under specific experimental conditions. The importance of validation of reference gene/s for experiment sets has been emphasized by several workers (Dheda *et al.* 2005, Gutierrez *et al.* 2008b, Guénin *et al.* 2009). Our results on expression of *VvSpy* with different reference genes also indicate that data normalization with two or three reference genes gave a stable expression profile, and the over- and/or under-estimation of a target gene was obtained if unstable reference genes were used for data normalization. Thus, determination of an appropriate reference gene for different stages, and the use of combination of reference genes for data normalization should be considered for qPCR based gene expression analyses.

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