Vitis **55**, 157–160 (2016) DOI: 10.5073/vitis.2016.55.157-160

Reference gene validation for gene expression studies using quantitative RT-PCR during berry development of 'Aki Queen' grapes

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Summary

In order to understand the gene regulation during berry development and examine the effect of abscisic acid (ABA) on gene expression related to berry maturation, we evaluated the validity of four housekeeping genes, *elongation factor 1-α* (*EF1-α*), *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), *ubiquitin-conjugating enzyme* (*UBC*), *and 60S ribosomal protein L40-1 (VvUbiquitin1*), as references by using 'Aki Queen' grapes with and without ABA treatment. The main contribution of this study is that a gene expression analysis using *GAPDH* as a reference gene will help to elucidate the berry development process and the physiological effects of ABA on berry maturation of 'Aki Queen' grapes.

Key words: abscisic acid; berry maturation; quantitative RT-PCR; reference gene.

Introduction

Grape berries undergo a double-sigmoidal pattern of growth, where two rapid growth phases of cellular expansion are separated by a lag phase (Coombe and Hale 1973). The onset of the second growth phase, called véraison, is characterised by increases in sugar and anthocyanin contents, decreases in acidity and rapid decrease in fruit firmness. This maturation process is possibly triggered by phytohormone abscisic acid (ABA) (Kuhn *et al*. 2014). ABA is a good candidate as plant growth regulator to control grape berry maturation; however, the physiological effects of ABA on berry maturation are not fully understood.

Quantitative RT-PCR (qRT-PCR) is a powerful tool for analysing gene expression, and it has recently been used to monitor gene expression in plants (Gachon *et al*. 2004). To account for sample variations and the efficiency of the quantification process, the results of qRT-PCR should be normalised with the total amounts of mRNA present in the samples. For this purpose, the expression levels of target genes are usually described in terms of the ratios between target and reference mRNA levels. Hence, reference genes should be carefully selected to reflect the total amounts of mRNA (Huggett *et al*. 2005). It is generally assumed that housekeeping genes are expressed uniformly in different tissues and organs; however, some exhibit varied expression in different experimental conditions (Gutierrez *et al*. 2008).

In the present study, to understand the gene regulation during berry development and examine the effect of ABA on gene expression related to berry maturation of 'Aki Queen', a standard red grape cultivar in Japan that frequently suffers from poor or over-coloration on the berry skin, we evaluated the validity of four housekeeping genes that previously served as references, *elongation factor 1-α* (*EF1-α*), *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), *ubiquitin-conjugating enzyme* (*UBC*), *and 60S ribosomal protein L40-1* (*VvUbiquitin1*, Bogs *et al*. 2006), as references, by using 'Aki Queen' grapes with and without ABA treatment immediately before the véraison.

Material and Methods

Plant materials and ABA treatment: We used 25-year-old 'Aki Queen' (*Vitis vinifera* × *V. labruscana*) plants grown at the Experimental Farm of Ishikawa Prefectural University in 2009. About 6 mL of 400 ppm ABA solution (BAL Planning, Japan) was sprayed onto the clusters at 39 d after full-bloom (DAF). The véraison occurred around 40 DAF and clusters were harvested at 83 DAF. The untreated clusters were sprayed with the solvent that dissolved ABA. Each treatment was performed in 8 replications.

Gene expression analysis: Total RNA was extracted from berry skins using the hot borate method (Wan and Wilkins 1994). After treatment with DNase I (Thermo, USA), single-strand cDNAs were synthesised from 500 ng of total RNA using SuperScript III Reverse Transcriptase (Invitrogen, USA) in the presence of an RNasin Ribonuclease Inhibitor (Promega, USA) and oligo-dT primer. qRT-PCR was performed with a StepOnePlus Real-Time PCR System (Applied Biosystems, USA) and SYBR Pre-mix Ex-Taq II (Takara-Bio, Japan). The standard amplification protocol consisted of an initial denaturing step at 95 °C for 30 s, followed by 45 cycles at 95 °C for 5 s, 60 °C for 30 s. Primer sequences were listed in the Table. Three independent RNA extractions were used for the analysis. All PCR reactions were replicated twice with the same cDNA samples and Tukey's test was used to determine significant differences.

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Table

Genes and primer sets used for quantitative RT-PCR

Gene	Accession	*Arabidopsis* orthologue accession (Description)	Forward and reverse primer sequences	Product size (bp)	Average Ct value ± SD/ Cycle efficiency
EF1-α	XM_002284928.3	AK221176.1 (Elongation factor eEF-1 alpha chain)	5′-CCTTCTTCTCCACGCTCTTG-3′ 5′-TGGTGATGCAGGGTTTGTTA-3′	146	20.8 ± 1.426 / 2.00
GAPDH	XM_002263109.3	AK222046.1 (Glyceraldehyde-3-phosphate dehydrogenase)	5′-CCAAGGCTGGAATAGCACTC-3′ 5′-CCATGTGGACAATCAAGTCG-3′	105	21.9 ± 0.580 / 1.94
UBC	XM_002274299.2	NM_105097.8 (Ubiquitin-conjugating enzyme E2)	5′-CAAGCCACCCAAGGTTTCCT-3′ 5′-AGGGCTCCACTGCTCTTTCA-3′	100	25.8 ± 0.583 / 2.00
VvUbiquitin1	XM_002273532.2	NM_129175.2 (60S ribosomal protein L40-1)	5′-GTGGTATTATTGAGCCATCCTT-3′ 5′-AACCTCCAATCCAGTCATCTAC-3′	182	24.1 ± 0.992 / 2.00

Results and Discussion

The amounts of mRNA of candidate reference genes (*EF1-α*, *GAPDH*, *UBC*, and *VvUbiquitin1*) were determined for the samples obtained from 27 DAF to 68 DAF. All the target genes were specifically amplified with a cycle efficiency of 1.97 to 2.00 (Table). The standard deviation of each Ct value represents the gene expression variance in all the examined samples including ABA-treated and untreated ones. *EF1-α* showed the highest variability of expression with a Ct value of 20.8 ± 1.426, followed by *VvUbiquitin1* with a Ct value of 24.1 ± 0.993. *GAPDH* and *UBC* showed low variance of expression with Ct values of 21.9 ± 0.580 and 25.8 ± 0.583, respectively. These results indicate that expression of *EF1-α* and *VvUbiquitin1* varied considerably during developmental stages, and thus are not suitable as reference genes. Indeed, the relative expression level of *EF1-α* increased between 27 and 42 DAF, and decreased thereafter when the other three genes were used as reference genes (Figure A, B, and C). Accordingly, when *EF1-α* was used as a reference, the apparent expression of *GAPDH*, *UBC*, and *VvUbiquitin1* increased after 54 DAF (Figures 1D, G, and J). Likewise, *VvUbiquitin1* expression decreased around 68 DAF when *GAPDH* and *UBC* were used as references (Figure K and L), and the relative expression of *GAPDH* and *UBC* increased at 68 DAF when *VvUbiquitin1* was used as reference (Figure F and I).

GAPDH and *UBC* were stably expressed during the berry development of 'Aki Queen' grapes, although the relative expression of *GAPDH* decreased slightly after 33 DAF when *UBC* was used as a reference (Figure E), and the relative expression of *UBC* increased slightly when *GAPDH* was used as a reference (Figure H). The standard deviation of the Ct value was the same between *GAPDH* and *UBC* (Table), but *GAPDH* expression showed lower variance than UBC expression when the berries were treated with ABA (data not shown). Indeed, when *UBC* was used as a reference, significant differences were observed in the expression levels of *EF1-α* and *VvUbiquitin*1 between ABA-treated and untreated berries (Figure B and L, 42 DAF). However, no significant differences were detected when *GAPDH* was used as the reference gene (Figure A and K, 42 to 68 DAF). Together, we concluded that *GAPDH* is the most relevant reference for gene expression studies using qRT-PCR during berry development of 'Aki Queen' grapes with and without ABA treatment. Indeed, we have analysed the expression of genes involved in flavonoid biosynthesis with *GAPDH* as reference gene, and confirmed that genes including *UDP-glucose*: f*lavonoid 3-O-glucosyltransferase* and *chitinase 4* was upregulated in response to ABA treatment as previously described (Jeong *et al.* 2004, Koyama *et al.* 2010, Katayama-Ikegami *et al.* 2016). *EF1-α*, *GAPDH*, *UBC*, and *VvUbiquitin1* have been used as reference genes for qRT-PCR analysis in grapes. Reid *et al*. (2006) described that *GAPDH*, *actin*, *EF1-α*, and *SAND* serve as reference genes for developing 'Cabernet Sauvignon' berries. Borges *et al*. (2014) validated *UBC*, *vacuolar ATPase subunit G*, and *phosphoenolpyruvate carboxylase* as the most suitable reference genes for

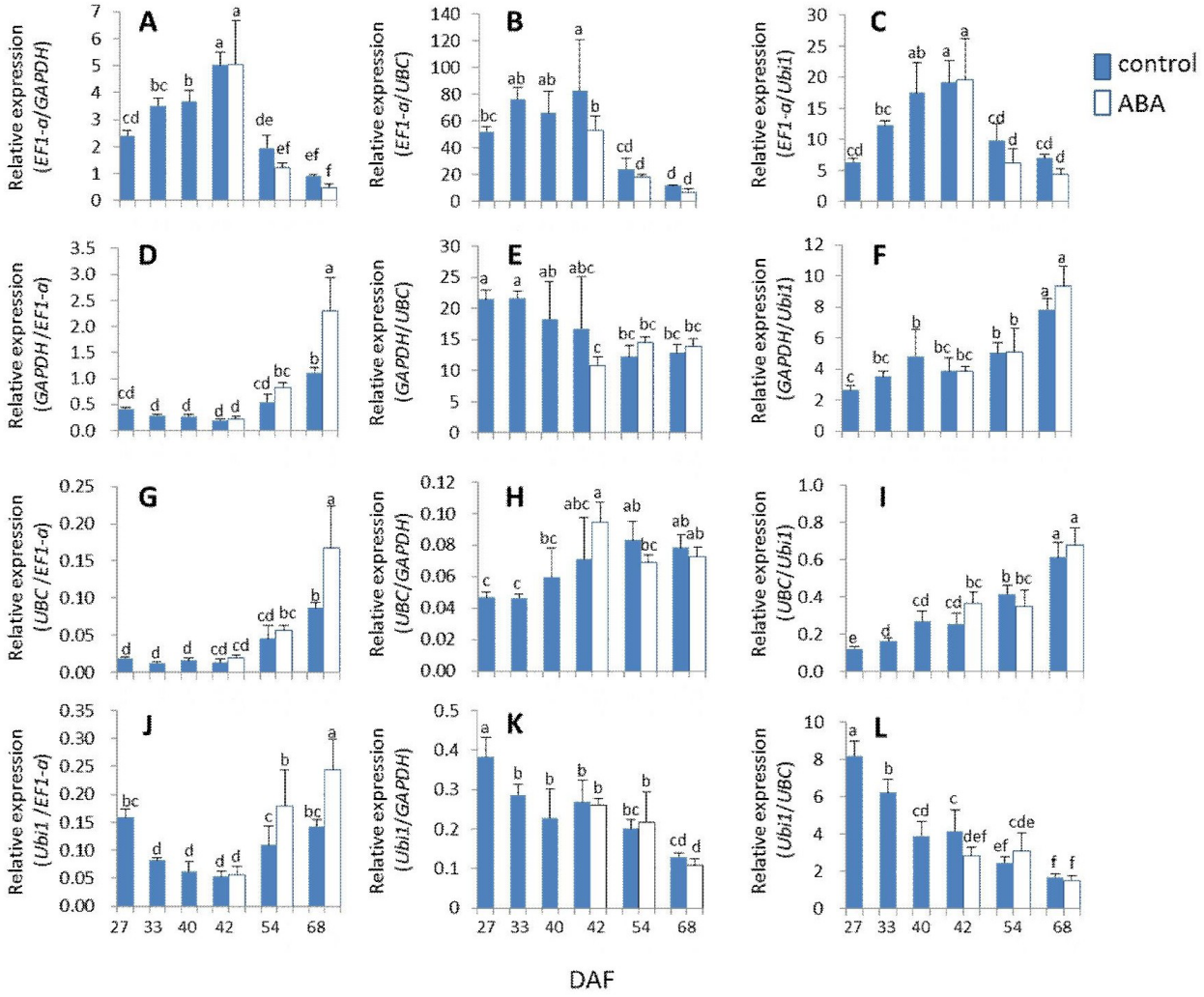

Figure: Relative expression levels of *EF1-α*, *GAPDH*, *UBC*, and *VvUbiquitin1*. *EF1-α* (D, G, J), *GAPDH* (A, H, K), *UBC* (B, E, L), and *VvUbiquitin1* (C, F, I) were used as references, respectively, to compare the expression levels between the four genes. Bars labelled with different letters differ significantly (Tukey's test, $P < 0.05$).

examining the response to biotic and abiotic stress such as *Erysiphe necator*, wounding, and UV-C irradiation in leaves of *V. vinifera* 'Touriga Nacional'. They also showed the relevance of *EF1-α*, *cyclophilin*, and *UBC* for analysing *Phaeomoniella chlamydospora* colonisation of wood. Additionally, *AvrRpt2-induced gene* and *T-complex 1 beta-like protein* were the most stably expressed genes after comparing the complete set of genotypes and phenological stages studied using table grape segregants belonging to a 'Ruby Seedless' and 'Sultanina' crossings (González-Agüero *et al*. 2013). These reports imply that a reference gene should be considered as being valid solely when validated under the same conditions as those used to assess the expression of the target genes.

Conclusion

We envisage that a detailed gene expression analysis using *GAPDH* as a reference gene will help to elucidate the berry development process and the physiological effects of ABA on berry maturation of 'Aki Queen' grapes.

Acknowledgements

This work was supported in part by Grants-in-Aid for Young Scientists (nos. 21880041 and 22780029) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by a Grant for the Promotion of Research from Actree Corporation.

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Received March 18, 2016
Accepted August 4, 2016