REFEREED MANUSCRIPT

Proc. Fla. State Hort. Soc. 124:7–12. 2011.

Molecular Assessment of Anthocyanin Biosynthesis Pathway in Synchronized In Vitro Red Cell Cultures of American Native Grapes

ANTHONY ANANGA, STOYANKA KRASTANOVA, SAFIRA SUTTON, STEPHEN LEONG, AND VIOLETKA COLOVA (TSOLOVA)*

Viticulture Lab, Center for Viticulture and Small Fruit Research, College of Agriculture and Food Sciences, Technology and Agriculture, Florida A&M University, 6505 Mahan Drive, Tallahassee, FL 32317

ADDITIONAL INDEX WORDS. grape, *Vitis,* muscadine, flavonoids, anthocyanins, cell cultures

Anthocyanins are flavonoid compounds that are responsible for the red color of grapes and wines. Because anthocyanins are the most important colorants in grapes, they have been widely studied for their numerous beneficial effects on human health. The study of the flavonoid boisynthetic pathway genes is critical for anthocyanin biosynthesis in *Vitis vinifera* grape. Little has been done to study the influence of these genes in muscadine grapes. Muscadines are extensively cultivated in Florida and the southeastern United States. Using in vitro syncronized cell cultures obtained from superepidermal cells of red berries of 'Cynthiana' and 'Noble' varieties, we examined the expression of 13 genes involved in the flavonoid biosynthetic pathway. By using gene specific primers in combination with a reliable normalization gene (*Actin*), we compared relative expression of the flavonoid biosynthetic genes between 'Noble' and 'Cynthiana'. Our study revealed that there was no significant difference in the expression levels of these genes in the in vitro red cell cultures of both varieties. Among them, CHS2, ANS, DFR, PAL, GST4, CHI4, LDOX, GST5, CHS3, and UFGT showed more expression in 'Noble' than 'Cynthiana'. This study shows that the genes involved in the flavonoid biosynthetic pathway are expressed in the in vitro red cells as the cells multiply. This makes the study of red cells useful and validates their future use as nutraceuticals. It is important to note that this is the first attempt to elucidate the expression pattern of flavonoid biosynthetic pathway genes in synchronized in vitro red cell cultures of grapes.

For many years, berries of the grapevine (*Vitis vinifera* L.) have received more attention due to their significance as an important edible source of flavonoid compounds with nutrient and health benefits for humans (Havsteen, 2002). Different flavonoid compounds are largely localized in berry skin and play a critical role in the quality of wine by contributing to its astringency and color (Koes et al., 2005; Winkel-Shirley, 2001). Muscadine grapes are also gaining a lot of recognition due to their high antioxidant levels, especially the presence of ellagic acid. There are three major classes of flavonoids in plants: anthocyanins, proanthocyanidins, and flavonols that are synthesized via the branched flavonoid biosynthetic pathway (Holton and Cornish, 1995; Winkel-Shirley, 2001). Flavonoids are involved in several physiological and biochemical processes in plants such as UV protection, insect attraction, herbivore defense and symbiosis (Gould and Lister, 2006; Koes et al., 2005; Peters and Constabel, 2002). The quantity and composition of anthocyanins in the skin of the grape berry contributes to its color when they are synthesized (Boss et al., 1996a, 1996b).

Several researchers have studied the compositions of anthocyanin in grape cultivars (Boss et al., 1996b; Mazza and Miniati, 1993; Shiraishi and Watanabe, 1994). According to the study that was carried out by Shiraishi and Watanabe (1994), it was determined that most red cultivars contain mainly derivatives of the anthocyanidins, cyanidin, and/or peonidin, while black cultivars contain primarily delphinidin, petunidin, and/or malvidin derivatives. The expression of the flavonoid biosynthetic genes is crucial for anthocynin biosynthesis in the berry (Boss et al., 1996a, 1996b). In their experiment, Boss et al. (1996b) used several tissues of 'Shiraz', which is a black cultivar, to examine the expression of seven genes involved in anthocyanin biosynthesis and showed that all genes except *UFGT* were expressed in most tissues. In their research, they used skins of white and red cultivars, and their result indicated that the *UFGT* gene was expressed in all red cultivars and not in white ones (Boss et al., 1996a).

Because of the biological and agricultural importance and favorable health benefits, the genetics and biochemistry of the flavonoid biosynthetic pathway has been intensively studied in several plant species (Boss et al., 1996c; Judo et al., 1998; Meyerhans et al., 1990). These studies indicated that flavonoid composition among plant species and even different tissues of a plant can be remarkably different (Boss et al., 1996c; Dooner et al., 1991; Ho et al., 1989; Holton and Cornish, 1995; Horton et al., 1989). Further details about the flavonoid biosynthetic pathway are available in many publications (Gould and Lister, 2006; Holton and Cornish, 1995; Koes et al., 2005; Winkel-Shirley, 2001). However, the synthesis of flavonoids via the flavonoid biosynthetic pathway requires two classes of genes: structural

Acknowledgments. This work was supported in part by College of Agriculture and Food Science, Florida A&M University, Florida Grape Growers Association, and USDA/NIFA/AFRI Plant Biochemistry Program Grant #2009-03127. We sincerely thank Dr. Etti Or from ARO Volcanic Center, Israel, for her helpful comments and suggestions.

*Corresponding author; phone: (850) 412-5195; email: violetka.colova@famu.edu

genes that encode enzymes for synthesis of anthocyanins and other flavonoids, and the regulatory genes involved in spatial and temporal regulation of these structural genes (Deluc et al., 2008). Although these two classes of genes are present in both red- and white-fruited grapevine cultivars, the color pigments are not expressed in white-fruited cultivars due to multiallelic mutations in the regulatory genes called *MybA1* and *MybA2* (Kobayashi et al., 2004; Walker et al., 2006, 2007). These two genes regulate expression of the UDP-glucose: flavonoid 3-O-glucosyltransferase (UFGT) gene, which mediates the conversion of anthocyanidins to anthocyanins by glycosylation (Boss et al., 1996c; Kobayashi et al., 2001). Thus, the last biosynthetic step of UFGT-mediated anthocyanin synthesis does not occur in white-fruited grapevine cultivars and hence these cultivars do not express red color.

Therefore, the focus of this study was to use gene specific primers in combination with a reliable normalization gene to verify and compare relative expression of the flavonoid biosynthetic genes between in vitro synchronized red cell cultures of the American native grapes 'Cynthiana' and 'Noble'.

Materials and Methods

Plant materials and cell growth. In vitro synchronized red cell cultures (Fig. 1 C and D) established by Colova (2011) (Patent publication no. US 2011/0054195 A1) from super-epidermal cells of red berries (Fig. 1 A and B) of North American native grapes: *Muscadinia* 'Noble' and *Aestivalis* 'Cynthiana' were used in this study. The cells were grown in the growth chamber at 23 °C under a white light (150 $\mu E \cdot m^{-2} \cdot s^{-1}$) with 16 h light/8 h dark cycle. Developed calli produced anthocyanin and they were red in color. Figure 1 A and B show the two varieties that were used to initiate the in vitro cell lines at the physiological maturity stage. They are grown at the experimental vineyard of Florida A&M University.

Maintainance of solid cultures. The cells were maintained in B-5 media (Gollop et al., 2002). They were transferred to fresh media every 30 d or observed and transferred when they became thick and had a good color. If the medium was brown or if there was browning around the cells, that meant that they were becoming necrotic and not ideal to transfer. Small or large petri plates with B-5 culture medium were used to grow the cells (Fig. 1 C and D). To transfer the cells, the light forceps were gently used to scoop off the top layers of the cells. The cells were gently spread onto new culture media in three medium size layers in each plate in order to give the cells enough room to multiply.

RNA isolation and analysis. Total RNA was isolated using RNeasy Plant Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA was quantified using UV absorbance, and the inexactness thereof inspected by formaldyhide agarose gel electrophoresis. Purified RNA was treated with RNase-free DNAse 1. The integrity of RNA was verified by resolving in 1% formaldehyde-agarose gels (Fig. 2) and subsequent ethidium bromide staining. RNA purity was assessed based on absorbance ratio of 1.8 to 2.0 at 260/280 nm using a spectrophotometer.

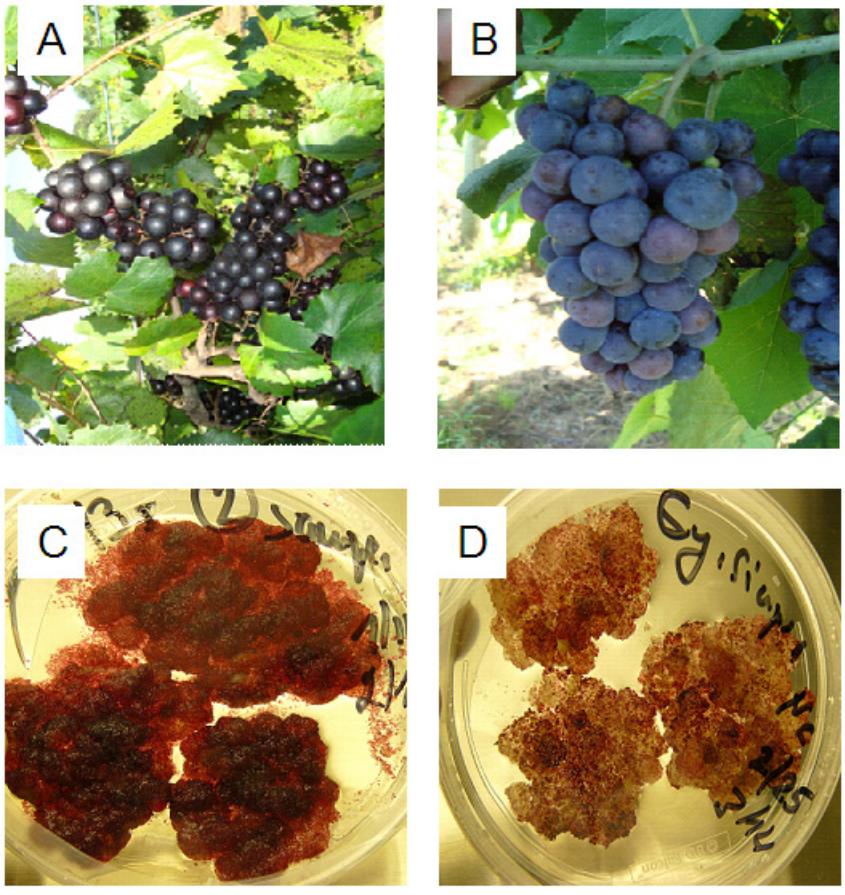

Fig. 1. Plant materials used in this study: (**A**) fruits from 'Noble', (**B**) fruits from 'Cynthiana', (**C**) synchronized red cells established from 'Noble', and (**D**) synchronized red cells from 'Cynthiana'.

Table 1. Genes targeted and the primers used to amplify their sequences.

Targeted gene	Forward primer 5´–3´	Reverse primer 5´–3´
Actin	TAGAAGCACTTCCTGTGGAC	GGAAATCACTGCACTTGCTC
CHS2	ACGTCCCAGGGTTGATTTCC	TGCAGTCCTCACCAGTCTC
ANS	ATCTGGCCTAAGACACCAAGC	ATGTTGGGAAAAGGTGCGAGG
Vexp-1	CTCCTACTTCAACCTAGTCC	AAGGCCACATTGACAAATGC
DFR	AGCTATTAAAGGCTGCACCG	ACACCTTTGAACTCTGTGGG
PAL	TCTTGAAGCTCATGTCTACC	TAGCAGATTGGGAGAGGTG
GST4	ATGGTGATGAAGGTGTATGG	ACTCAGATCAGCGAGAGTGA
CHI4	TCGAGAACGTCCTATTTCC	ATCTTTGGAGAAGCTCATCG
LDOX	AAATGAATGCAGGGAACTGG	AGAAGTAGGCTAGTGAGACG
GST5	TTGGGGTTCCAAACTAGAAC	ACCAAACCTCTTTCTCATCC
ANR	TGCGAATTTGGAAGTGAGGGC	TGTGCCTGGCAAACATCTTCC
F3H	CTCTCTCTCATACTTCTTCG	AAACCATAGAGCCTGCAAG
CHS3	TGCTGACTACCAACTCACC	TACTCGCTCAAGACGTGTCG
UFGT	ATAAACTCCTTCGAGGAGC	TCCCATTGAGCCTTTGGTC

Primers, RT-PCR, and cDNA synthesis. Primers used in this study were the same as those designed by Samuelian et al. (2009). They were used to amplify partial gene-specific sequences. A list of primer pairs and genes targeted are provided in Table 1. Total RNA was used in primary gene expression profiling. The SuperScript First Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) was used to synthesize cDNA in a 20 µL reaction containing 1 µg of DNase I-treated total RNA, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM dithiothreitol (DDT), 0.5 µg oligo (dT), 0.5 mM each of dATP, dGTP, dCTP, and dTTP, and 200 U SuperScript II Reverse Transcriptase. RNA, dNTPs, and oligo (dT) were mixed first, heated to 65 °C for 5 min, and placed on ice until addition of the remaining reaction components. The reaction was incubated at 50 °C for 50 min, and terminated by heat inactivation at 85 °C for 5 min. The cDNA product was treated with 1 µL of Rnase H (Invitrogen) for 20 min at 37 °C. An identical reaction without the reverse transcriptase was performed to verify the absence of genomic DNA (no-RT control). The cDNA was stored at –20 °C until ready to use. Purification of cDNA prior to real-time PCR was not necessary.

Real-time PCR using SYBR green assay. Real-time PCR (RT-PCR) reactions were performed in 96-well plates with iCycler iQ Multicolor Real-Time PCR Detection system (Bio-Rad, Hercules, CA; Vandesompele et al., 2002) using iQ SYBR Green Supermix (Bio-Rad) as described in the manufacturer's manual. All RT-PCR assays were performed with proper controls according to Minimum Information for Publication of Real-Time PCR Experiments (MIQE) guidelines. Each reaction was carried out in 25 µL reaction mixture containing 3 µL of cDNA, 0.5 µM each of gene-specific forward and reverse primer (Table 1), and 11 µL of 2× iQ SYBR Green Supermix (Bio-Rad). RT-PCR was performed using iQ SYBR Green Supermix (Bio-Rad) on a iQ-Cycler (Bio-Rad) under the following conditions: an initial denaturating cycle (2 min at 95 °C), followed by 40 cycles constituted by three steps of denaturation, annealing, and polymerization (20 s at 95 °C, 30 s at 60°, and 30 s at 72 °C). All assays included no-RT and no-template controls to verify non-specific amplification. At the end of each RT-PCR, a melting curve analysis was performed over the range 65 °C to 97 °C to determine the specificity of amplicons (Fig. 3). The amplicons were also resolved in 1% agarose gels, stained with ethidium bromide and visualized under UV light (Fig. 4). cDNA from three biological replicates were used for RT-PCR analysis, and three technical replicates were analyzed for each biological replicate. Aliquots from the same cDNA were used in all technical replications. Differences in gene expression among red cells of 'Noble' and 'Cynthiana' varieties were calculated according to the delta-delta C_t method (Pfaffl, 2001).

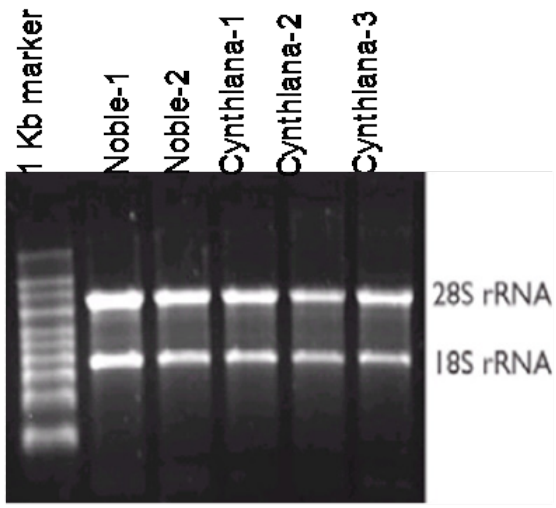

Fig. 2. Gel electrophoretic analysis of total RNA product from the synchronized red cells of 'Noble' and 'Cynthiana' clearly showing 18S and 28S. The extraction was repeated more than two times as indicated above.

Data acquisition. Expression levels were determined as the number of cycles needed for the amplification to reach a threshold fixed in the exponential phase of PCR reaction (C_t). The C_t values were transformed into quantities using PCR efficiencies according to Vandesompele et al. (2002)

Statistical analysis. C_t values from the iCycler iQ Multicolor Real-Time PCR Detection system (Bio-Rad) were analyzed. The statistical significance of experimental variation was calculated using ANOVA at confidence levels of 95% ($P < 0.05$). A P value superior to 0.05 indicated that no difference of variation of expression could be deduced.

Results and Discussion

The amplification efficiency (data not shown) of each gene-specific primer indicated that primer pairs were suitable for amplification and quantification of target genes. Melting curve analysis for each amplicon showed a single peak (Fig. 3), further

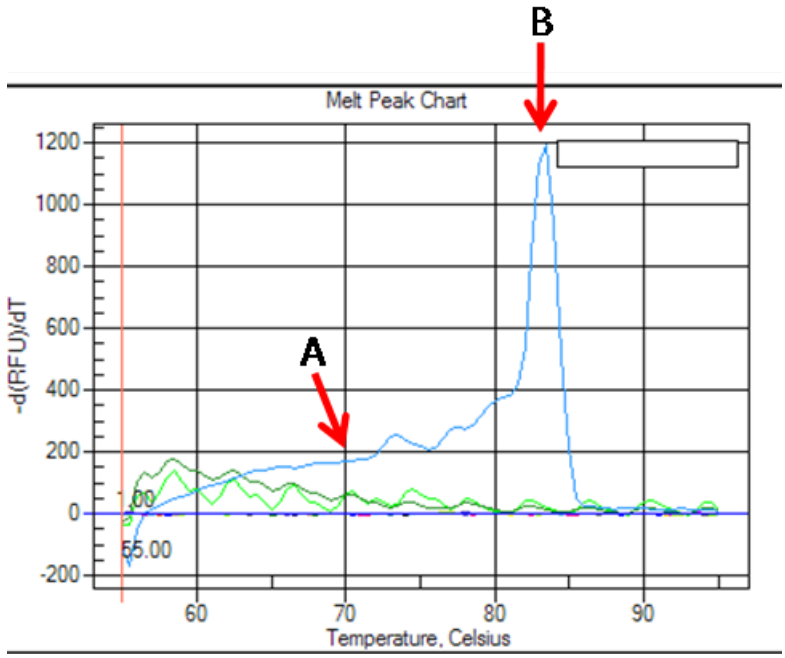

Fig. 3. Confirmation of PCR amplification specificities of the primers used in this study. Melting peaks were examined with cDNA templates and the experiment was replicated 3 times. The melting temperature was 84 °C. (**A**) Indicates the melting curve with no peaks for water and no-RT controls; (**B**) indicates the melting temperature of specific product in these reactions.

confirming the specificity of amplicons produced in RT-PCR for all targeted genes. Agarose gel electrophoretic separation of each amplicon showed DNA fragments of the expected size with no visible primer-dimer products (Fig. 4). All these results indicated that the total RNA and the derived cDNA template were free of contaminating genomic DNA, demonstrating high quality of nucleic acid preparations obtained for gene expression level analyses by RT-PCR.

A total of 13 flavonoid biosynthetic pathway genes and 1 reference gene (Table 1) were evaluated for their expression under our experimental conditions. Since all RT-PCR reactions were performed with cDNA derived from equal quantity of total RNA, transcript abundance of these 13 genes were analyzed by direct comparison of C_t values, assuming equal C_t for equal transcript number. As shown in Fig. 4, all of the 13 analyzed genes were expressed both in 'Cynthiana' and 'Noble' cell lines.

The expression patterns of flavonoid pathway genes (CHS2, ANS, Vexp-1, DFR, PAL, GST4, CH14, LDOX, GST5, ANR, F3H, CHS3, and UFGT) were examined in the red cells of 'Cynthiana' and 'Noble'. The overview of expression levels of these genes (Fig. 4) showed higher transcript levels in the red cells of 'Noble', assuming equal C_t for equal transcript number, since all RT-PCR reactions were performed with equal amount of cDNA derived from equal quantity of total RNA. Using actin (or *ACT*) as a reference gene, we normalized the raw C_t data for each gene from 'Noble' and 'Cynthiana' and their relative expression levels are shown in Fig. 4. Ten flavonoid biosynthetic pathway genes analyzed in this study showed higher expression levels in the red cells of 'Noble' when compared with 'Cynthiana'. However, statistically there was no significant difference in the expression level between the two varieties.

Based on these results, it can be concluded that the expression of some of the flavonoid biosynthetic pathway genes was slightly higher in the red cells of 'Noble' when compared to expression levels of corresponding genes in the red cells of 'Cynthiana'. The isogenes of chalcone synthase (CHS2 and CHS3) that are involved in recruitment of flavonoid precursors to enter the flavonoid biosynthetic pathway also exhibited slightly higher expression levels in 'Noble' compared to 'Cynthiana'. The expression of flavonoid hydroxylases, F3H, which regulates the synthesis of cyanidin-based anthocyanins, was lower in 'Noble' as comapered to 'Cynthiana'.

In grapes, cultivars with red fruits accumulate anthocyanin pigments predominantly in berry skins by displaying various shades of colors ranging from brick red to dark blue and their biosynthesis is developmentally triggered at the onset of véraison via the activation of flavonoid biosynthetic pathway genes (Walker, 2007). In this study we used gene-specific RT-PCR to present the evidence that PAL, an enzyme that commits the flux of primary metabolism into the flavonoid biosynthetic pathway, and both "early" (CHS, CHI, F3´H, F3´5´H, F3H, and FLS) and "late" genes (DFR, LDOX, UFGT, and LAR) of the pathway are expressed in the in vitro synchronized red cell cultures of 'Noble' and 'Cynthiana' (Fig. 5). Our results further strengthen the purpose of the in vitro cultures of red cells and validate their future use as nutraceuticals. This research is crucial as an initial step toward understanding how to change the metabolic flux in specialized in vitro red cells. It is important to note that no studies have been conducted to elucidate the expression pattern of flavonoid biosynthetic pathway genes in synchronized in vitro red cell cultures of grapes.

Literature Cited

Boss, P.K., C. Davies, and S.P. Robinson. 1996a. Expression of anthocyanin biosynthesis pathway genes in red and white grapes. Plant Mol. Biol. 32:565–569.

Boss, P.K., C. Davies, and S.P. Robinson. 1996b. Anthocyanin composition and anthocyanin pathway gene expression in grapevine sports

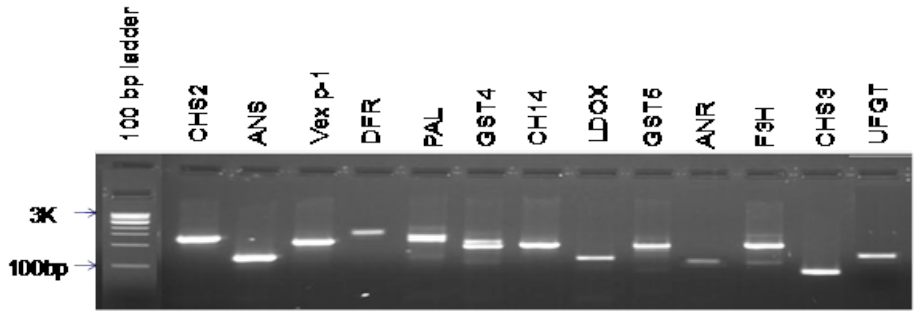

Fig. 4. Expression of flavonoid biosynthetic genes in synchronized red cells of 'Noble'. The amplicons were detected using RT-PCR after cDNA samples were normalized upon *Actin* gene expression.

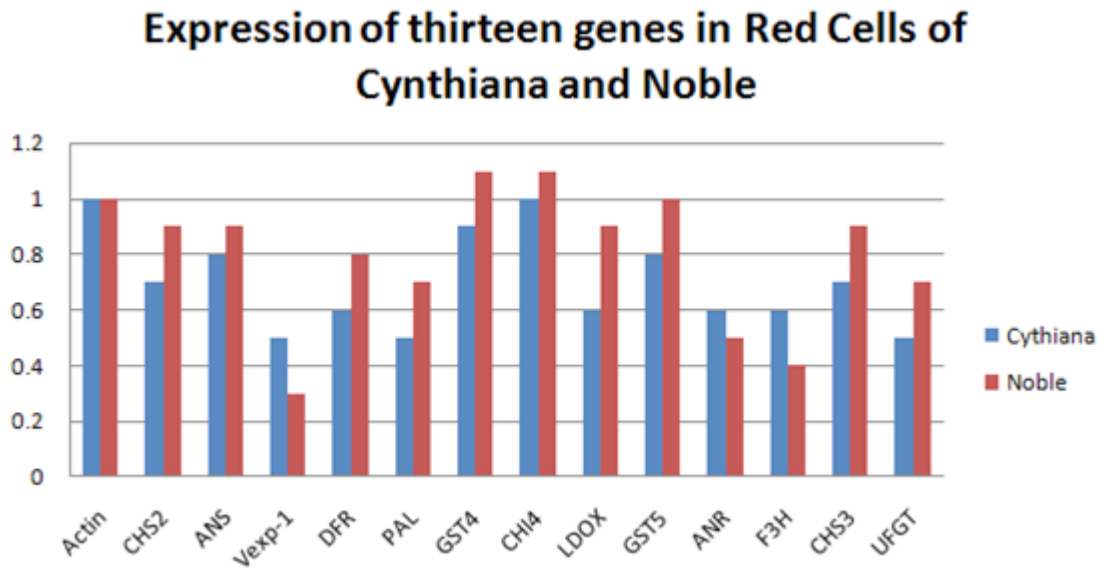

Fig. 5. Expression patterns of flavonoid biosynthetic pathway genes in synchronized red cells of 'Cynthiana' and 'Noble'. The relative expression levels of the flavonoid biosynthetic pathway genes are shown as arbitrary units on the y-axis. The raw Ct values for each gene were normalized using one reference gene (*Actin*). Columns represent mean value from five biological replicates. There were no significant differences in gene expression between 'Noble' and 'Cynthiana'.

differing in berry skin colour, Aust. J. Grape Wine Res. 2:163–170.

Boss, P.K., C. Davies, and S.P. Robinson. 1996c. Analysis of the expression of anthocyanin pathway genes in developing *Vitis vinifera* L. cv. Shiraz grape berries and the implications for pathway regulation, Plant Physiol. 111:1059–1066.

Colova, V. 2011. Synchronized strains of subepidermal cells of muscadine (muscadine sp.) grapevine pericarp for use as a sourse of flavonoids (nutraceuticals). Patent Publication No. US 2011/0054195 A1.

Dooner, H.K., T.P. Robbins, and R.A. Jorgensen. 1991. Genetic and developmental control of anthocyanin biosynthesis, Annu. Rev. Genet. 25:173–199.

Deluc, L., J. Bogs, R.A. Walker, T. Ferrier, A. Decendit, M.J. Merillon, P.S. Robinson, and F. Barrieu. 2008. The transcription factor VvMYB5b contributes to the regulation of anthocyanin and proanthocyanidin biosynthesis in developing grape berries. Plant Physiol. 147(4):2041–2053.

Gollop, R., S. Even, V. Tsolova, and A. Perl. 2002. Expression of the grape dihydroflavonol reductase gene and analysis of its promoter region. J. Expt. Bot. 53:1397–1409.

Gould, K.S. and C. Lister. 2006. Flavonoid function in plants, p. 397–441. In: M. Andersen and K.R. Markham (eds.). Flavonoids: Chemistry, biochemistry and applications. CRC Press, Boca Raton, FL.

Havsteen, B.H. 2002. The biochemistry and medical significance of the flavonoids. Pharmacol. Ther. 96(2–3):67–202.

Holton, T.A. and C.E. Cornish. 1995. Genetics and biochemistry of anthocyanin biosynthesis. Plant Cell 7(7):1071–1083.

Ho, S.N., H.D. Hunt, R.M. Horton, J.K. Pullen, and L.R. Pease. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene 77:51–60.

Horton, R.M., H.D. Hunt, S.N. Ho, J.K. Pullen, and L.R. Pease. 1989. Engineering hybrid genes without the use of restriction enzymes gene splicing by overlap extension. Gene 77:61–68.

Judo, M.S.B., A.B. Wedel, and C. Wilson. 1998. Stimulation and suppression of PCR-mediated recombination, Nucleic Acids Res. 26:1819–1825.

Kobayashi, S., M. Ishimaru, C.K. Ding, H. Yakushiji, and N. Goto. 2001. Comparison of UDP-glucose : flavonoid 3-O-glucosyltransferase (UFGT) gene sequences between white grapes (*Vitis vinifera*) and their sports with red skin. Plant Sci. 160(3):543–550.

Kobayashi, S., N. Goto-Yamamoto, and H. Hirochika. 2004. Retrotransposon-induced mutations in grape skin color. Science 304:982–989.

Koes, R., W. Verweij, and F. Quattrocchio. 2005. Flavonoids: A colorful model for the regulation and evolution of biochemical pathways. Trends Plant Sci. 10(5):236–242.

Mazza, G. and E. Miniati. 1993. Anthocyanins in fruits, vegetables, and grains, CRC Press, Boca Raton, FL.

Meyerhans, A., J. Vartanian., and S. Wain-Hobson. 1990. DNA recom-

bination during PCR. Nucleic Acids Res. 18:1687–1691.
Peters, D.J. and C.P. Constabe. 2002. Molecular analysis of herbivore-induced condensed tannin synthesis: Cloning and expression of dihydroflavonol reductase from trembling aspen (*Populus tremuloides*). Plant J. 32:701–712.
Pfaffl, M.W. 2001. A new mathematical model for relative quantification in real time RT-PCR. Nucleic Acids Res. 29:2002–2007.
Samuelian, S.K., C. Camps, C. Kappel, E.P. Simova, S. Delrot, and V. Colova. 2009. Differential screening of overexpressed genes involved in flavonoid biosynthesis in North American native grapes: 'Noble' *muscadinia* var. and 'Cynthiana' *astevalis* var. Plant Sci. 177:211–221.
Shiraishi, S. and Y. Watanabe. 1994. Anthocyanin pigments in the grape skins of cultivars (*Vitis* spp.), Sci. Bul. Fac. Agr. Kyushu Univ. 48:255–262.
Vandesompele, J., K. De Preter, P. Poppe, B. Van Roy, N. De Paepe, and A.F. Speleman. 2002. Accurate normalization of real time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3 Res. 0034.
Walker, A.R., E. Lee, and S.P. Robinson. 2006. Two new grape cultivars, bud sports of Cabernet Sauvignon bearing pale-coloured berries, are the result of deletion of two regulatory genes of the berry colour locus. Plant Mol. Biol 62(4–5):623–635.
Walker, A.R., E. Lee, J. Bogs, D.A. McDavid, M.R. Thomas, and S.P. Robinson. 2007. White grapes arose through the mutation of two similar and adjacent regulatory genes. Plant J. 49(5):772–785.
Winkel-Shirley, B. 2001. Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiol. 126(2):485–493.