Elevated Gene Expression in *Chalcone Synthase* Enzyme Suggests an Increased Production of Flavonoids in Skin and Synchronized Red Cell Cultures of North American Native Grape Berries

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Anthocyanins are antioxidants and are among the natural products synthesized via the flavonoid biosynthesis pathway. Anthocyanins have been recommended for dietary intake in the prevention of cardiovascular diseases, cancer, and age-related conditions such as Alzheimer's disease or dementia. With an increasingly aging population in many parts of the world, strategies for the commercial production of *in vitro* synchronized red cell cultures as natural antioxidants will be a significant contribution to human medicine. Red pigmented fruits such as grapes (*Vitis* sp.) are a major source of bioavailable anthocyanins and other polyphenols. Since the level of antioxidants varies among cultivars, this study is the first one that phytochemically and genetically characterizes native grape cultivars of North America to determine the optimal cultivar and berry cells for the production of anthocyanins as antioxidants. Using real-time PCR and bioinformatics approaches, we tested for the transcript expression of the chalcone synthase *(CHS)* gene, an enzyme involved in the flavonoid and anthocyanin biosynthesis pathway, in different parts of physiologically mature grape berries and *in vitro* synchronized red cells. A low level of expression was recorded in berry flesh, compared with an elevated expression in berry skins and *in vitro* synchronized red cells, suggesting increased production of flavonoids in skin and cell cultures. This preliminary study demonstrates the potential of functional genomics in natural products research as well as in systematic studies of North American native grapes, specifically in muscadine (*Vitis rotundifolia*).

Introduction

FLAVONOIDS ARE ANTIOXIDANTS that form various natural products such as anthocyanin, proanthocyadin, and phylobaphene pigments. They are involved in many aspects of plant growth and development, such as pathogen resistance, pigment production, UV light protection, pollen growth, and seed coat development (Harbone, 1986). Red pigmented fruits such as grapes are a major source of anthocyanins, as the latter is responsible for the red pigment. The pigments of anthocyanin dictate the degree of redness of the berry skin in different cultivars of grapevine, and this applies to wines fermented in the presence of red skins. The red color is crucial to the wine industry, and wine redness has valuable commercial and economic relevance—premium red wine is considered to be of high quality, which is also a primary factor in visual characterization. Red grapes and

wines are particularly rich in bioavailable anthocyanins that are rapidly absorbed as intact molecules (Bitsch *et al.*, 2004) and delivered into the brain within minutes of ingestion (Passamonti *et al.*, 2005).

Due to their high antioxidant capacity, there is increasing evidence which suggests that flavonoids are health-protecting components in the human diet (Sugihara *et al.*, 1999; Dugas *et al.*, 2000). Anthocyanins have been used in therapy against cardiovascular diseases, cancer (Trevisanato and Kim, 2000), and age-related conditions such as Alzheimer's disease or dementia (Commenges *et al.*, 2000). Due to increasing health problems in many parts of the world, ways for the commercial production of antioxidants in functional foods will be a significant contribution to human health.

Native grapes of North America (*Muscadine or Vitis rotundifolia*) are considered the most important cultivated *Vitis* species in the Southeastern part of the United States

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due to their possession of a high level of antioxidants. However, the lack of genomic studies and the phytochemistry of muscadine has limited the rapid discovery of genes that influence specific traits within the species. Available information indicates that muscadine grape genotypes contain several unique polyphenolic compounds that are beneficial in the human diet. Apart from being the only cultivar producing ellagic acid, muscadine contains important genetic resources that are not utilized in grape breeding (Samuelian *et al.*, 2009b).

Chalcone synthases (*CHS*) is the first committed enzyme in the flavonoid pathway that facilitates the stepwise synthesis of chalcone. The function of *CHS* is believed to be a regulatory focal point of substrate flow between the flavonoid pathway and the other competing directions of the phenylpropanoid pathway. This leads to the diversity of phenolic compounds such as lignins and phytoalexins (Winkel-Shirley, 2001; Szalma *et al.*, 2002). Genes encoding *CHS* constitute a multigene family in which the copy number varies among the plant species, and functional divergence appears to have repeatedly occurred (Tuteja *et al.*, 2004; Yang *et al.*, 2004). The expression of the *CHS* gene in fruit tissue is developmentally regulated and associated with fruit coloring (Aharoni and O'Connell, 2002; Kumar and Ellis, 2003). *CHS* is encoded by a single gene in *Arabidopsis thaliana* and by a large multiple gene family in other plants such as soybean and petunia (Koes *et al.*, 1994). In soybean, there are nine members of the *CHS* gene families sharing high nucleotide sequence identity within their coding regions (Tuteja and Vodkin, 2008). Many genes for the anthocyanin biosynthesis pathway have been isolated and characterized in *Vitis vinifera* grapes. However, only one member of the *CHS* gene family (*CHS1*) has been reported in muscadine (Samuelian *et al.*, 2009a). It is not clear whether there are other *CHS* gene families available in *M. rotundifolia* genome.

With an increasingly aging population in many parts of the world, strategies for the commercial production of natural antioxidants from *in vitro* synchronized red cells of muscadine will be a significant contribution to human medicine. The purpose of this study was to investigate the expression patterns of the muscadine *CHS* gene in the physiologically mature berries and *in vitro* synchronized red cell cultures of North American native grapes. This is because our goal is to phytochemically and genetically characterize the native grape cultivars of North America and to determine the tissues that express high anthocyanin levels as potential candidates for the commercial production of anthocyanins as antioxidants.

Materials and Methods

Plant materials and cell growth

In vitro synchronized red cell cultures (Fig. 1A, B) established from super-epidermal cells of red berries (Fig. 2A, B) of North American native grapes: muscadinia "Noble" var. and aestivalis "Cynthiana" var. by Colova, (2011) (patent publication no. US 2011/054195 A1) were used in this study. The cells were grown in a growth chamber at 23°C under a white light ($150 \mu E\ m^{-2}S^{-1}$) with a 16 h light/8 h dark cycle. The callus developed produces anthocyanin, and it is red in color. Figure 2A and B shows 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 the Florida A&M University in Tallahassee, Florida.

Maintenance of cell cultures

The cells were maintained in B-5 media in petri plates, as described by Gollop *et al.* (2002). The cultures were transferred to a fresh medium every 30 days or when thickness and the required color were attained. To transfer the cells, light forceps were gently used to scoop off the top layers of the cells. The cells were gently spread onto new culture media in 3 medium-sized layers in each plate in order to give them enough room to multiply.

Protein three-dimensional structure prediction

The Muscadine CHS Structural model was obtained from its amino-acid sequence by using the SWISS MODEL (http://swissmodel.expasy.org) (Kiefer *et al.*, 2009) and Protein Homology/analogy Recognition Engine (PHYRE) (www.sbg.bio.ic.ac.uk/phyre) prediction servers (Altschul *et al.*, 1997, Bennett-Lovsey *et al.*, 2008). The model obtained was classified according to identity percentage.

RNA isolation and gel electrophoresis

Samples were prepared from different tissues of Noble and Cynthiana grape varieties (*In vitro* synchronized red cell cultures, physiologically mature berry skins, and flesh). Total RNA was isolated using the RNeasy Plant Mini Kit according to the manufacturer's protocol. RNA was quantified

FIG. 1. *In vitro* synchronized red cell cultures established from super-epidermal cells of red berries of North American native grapes: muscadinia Noble var. **(A)** and aestivalis Cynthiana var. **(B)** (patent publication no. US 2011/0054195A1). Color images available online at www.liebertonline.com/dna
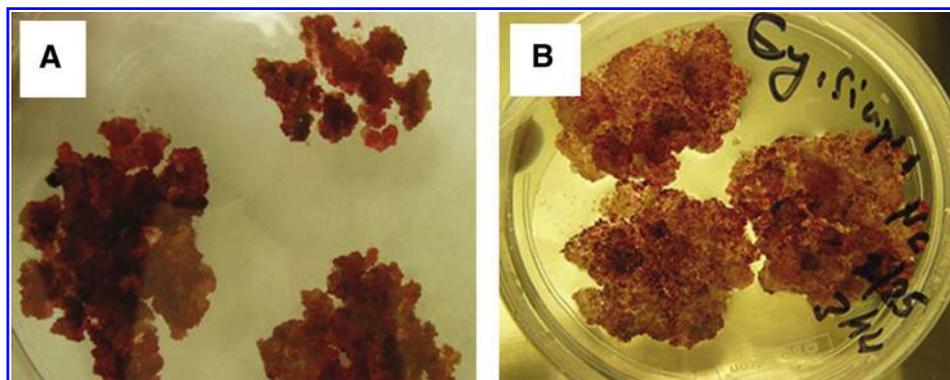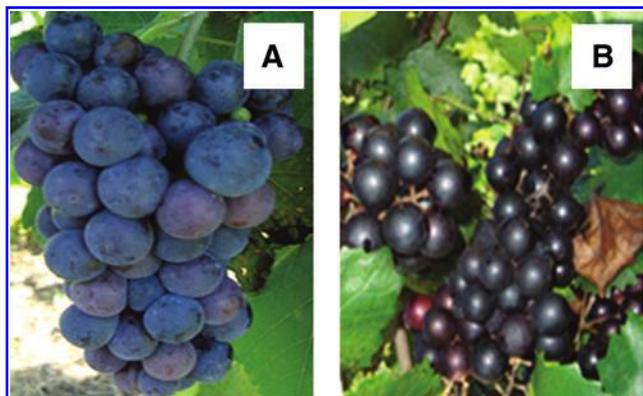
FIG. 2. Physiologically mature berries from Cynthiana var. **(A)** and Noble var. **(B)** used to analyze *CHS* expression profile. *CHS*, chalcone synthase. Color images available online at www.liebertonline.com/dna

using UV absorbance, and the inactivity was inspected by formaldehyde agarose gel electrophoresis. Purified RNA was treated with RNase-free DNAse 1, and immediately frozen to −20°C. Formaldehyde gel electrophoresis (1.2% agarose) was used to evaluate the RNA quality. The gel apparatus (including the gel tray and comb) was treated with RNase Away and rinsed with distilled water.

cDNA synthesis

Total RNA was used in primary gene expression profiling. The SuperScript First-strand Synthesis System for RT-PCR (Invitrogen) 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 MgCl2, 10 mM dithiothreitol, 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 the 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 1μL 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 it was ready for use.

Relative-quantitative real-time PCR using SYBR green assay

Relative-quantitative real-time PCR reactions were performed in a 96-well plate with an iCycler iQ Multicolor Real-Time PCR Detection system (Bio-Rad; Vandesompele *et al.*, 2002), using an iQ SYBR Green Supermix (Bio-Rad) to monitor cDNA amplification, according to the manufacturer's protocol. As a control, parallel amplification reactions of *Actin* (a housekeeping gene) were also performed. Each primer set was designed based on the 3′-end cDNA sequence of the corresponding gene. The specific primers used for real-time PCR were as follows: for *CHS*, 5′-TACTCGCTCAA GACGTGTCG-3′ (Forward) and 5′-CCTACCCACACA CAACTTCG-3′ (Reverse), giving a product of 245 bp; for *Actin* 5′-TAGAAGCACTTCCTGTGGAC-3′ (Forward) and

5′-GGAAATCACTGCACTTGCTC-3′ (Reverse), giving a product of 184 bp. RT-PCR was performed using the iQ SYBR Green Supermix (Bio-Rad) on an 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 72°C). PCR amplification was done in a 25 μL total volume, containing 3 μL of diluted cDNA (duplicate), 0.5 μM of each primer, and 11 μL of 2 × iQ SYBR Green Supermix. In each experiment, the target and the control were amplified in the same plate with a series of diluted cDNA (10^{-5}–10^{-9} ng) to generate calibration-specific curves. Three independent experiments were performed for each sample. After the real-time PCR had been performed, the absence of unwanted by-products was confirmed by an automated melting curve analysis and agarose gel electrophoresis of the PCR products.

In all the experiments, three replicates for each RNA sample were included; averages were calculated, and differences in the threshold cycle (Ct) were evaluated. The comparative Ct method was used, which mathematically transforms the Ct data into the relative transcription-level genes. When comparing the expression of *CHS* in different tissues, the relative quantification of *CHS* expression was achieved by calibrating its transcription level to that of the reference gene, *Actin*. The expression level calculated by the formula $2^{-\Delta\Delta Ct}$ represents the x-fold difference from the calibrator.

Search for muscadine CHS-related sequences

Putative CHS sequence was retrieved through Basic Local Alignment Tool (BLAST), homology, and domain searches in public domains, namely GenBank (www.ncbi.ncbi.nlm.nih .gov). The GenBank CHS protein sequence of muscadine (Accession no. ACN30003; Samuelian *et al.*, 2009a) was used for BLAST and homology searches against other plants.

Multiple sequence alignments and phylogenetic tree construction

Multiple alignment of the putative amino-acid sequence of muscadine CHS was performed using the T-Coffee program (Notredame *et al.*, 2000). The alignment of 14 CHS proteins was summarized using the Plotcon sequence similarity graph (http://bioweb2.pasteur.fr/docs/EMBOSS/plotcon .html), which represents the similarity along the set of aligned sequences. The molecular phylogenetic tree for CHS was built with Neighbor Joining, using p-distance as a substitution model, and Maximum Parsimony methods in MEGA Version 4.0, with 5000 iterations for calculating boostrap confidence levels (Tamura *et al.*, 2007). The phylogenetic tree construction included the sequences for the muscadine CHS protein and/or putative CHS proteins reported in the NCBI database for 13 plants (Table 1).

Results

RT-PCR primers used were specific to the target gene

The *CHS* fragment showed a sharp peak for the PCR product of the quantitative standard sample, Cynthiana, and Noble at 88°C (Fig. 3). A single melting peak at the same melting temperature was produced for the PCR product of

TABLE 1. PERCENT SIMILARITY OF MUSCADINE CHALCONE
SYNTHASE AMINO-ACID SEQUENCE WITH CHALCONE
SYNTHASE AMINO SEQUENCE FROM OTHER PLANT SPECIES

	Plant species	NCBI accession no.	Sequence similarity (%)
1	*Vitis rotundifolia*	ACN30003	100
2	*Vitis vinifera*	BAA31259	100
3	*Camellia japonica*	BAI66465	98
4	*Gossypium hirsutum*	AEO96985	98
5	*Camellia chekiangoleosa*	ADW11243	98
6	*Camellia sinensis*	AAO13091	98
7	*Nelumbo nucifera*	ADD74168	98
8	*Abelmoschus manihot*	ACE60221	98
9	*Rhododendron simsii*	CAC88858	98
10	*Pyrus communis*	ABI79305	98
11	*Humulus lupulus*	CAK19317	98
12	*Citrus maxima*	ACX37403	99
13	*Glycine max*	XP_003553986	98
14	*Nicotiana alata*	ACS12837	98

the cDNA samples. Every PCR product also generated prominent bands with the expected sizes in the gel electrophoresis. These results indicated that nonspecific PCR products were not detected in the analyzed temperature range. The fidelity of the real-time PCR assay is also demonstrated by the lack of specific signals for the no-RT and H_2O controls and by the Ct determined for each gene.

cDNA encoding muscadine CHS amplified

The results showed that the *CHS* transcript accumulated in the skin, flesh, and *in vitro* synchronized cell cultures of Noble
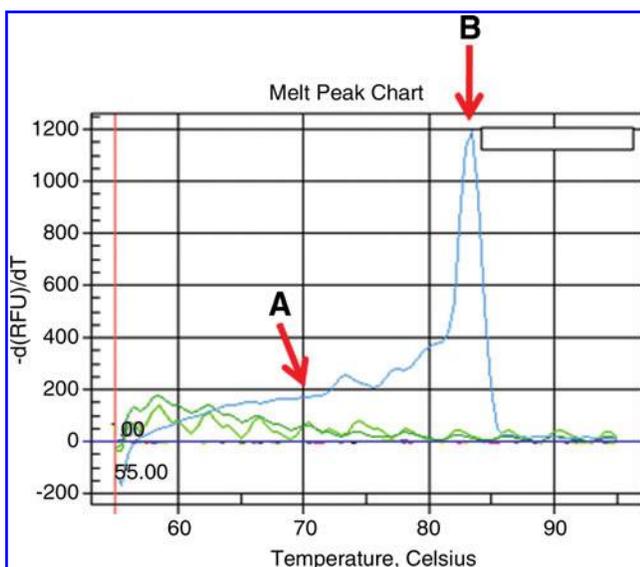

FIG. 3. Melting curve indicating temperature verses fluorescence [-d(RFU)/dT] for the *CHS* gene. All reactions with the cDNA template showed one sharp and fully overlapping melting peak, indicating the specificity of primers designed from a partial gene sequence. **(A)** indicates the melting curve with no peaks for water and no RT controls. **(B)** indicates the melting temperature of specific products in these reactions. Color images available online at www.liebertonline.com/dna
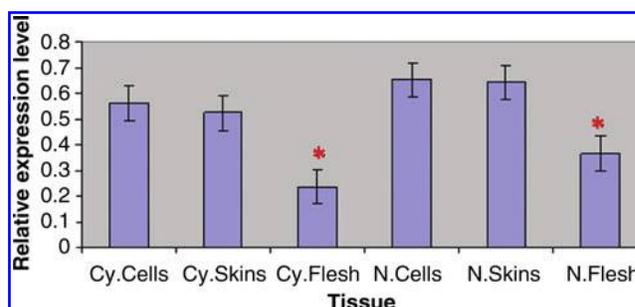

FIG. 4. Expression profile of *CHS* in different tissues collected from the muscadine and hybrid berries. A real-time PCR analysis was performed using total RNA from tissues of Cynthiana var. such as red cells (Cy. Cells), skins of physiologically mature berries (Cy. skins), and flesh of physiologically mature berries (Cy. Flesh). Real-time PCR analysis was also performed using total RNA from tissues of Noble var. such as red cells (N. Cells), skins of physiologically mature berries (N. Skins), and flesh of physiologically mature berries (N. Flesh). Actin and ubiquitin were used as an internal control. Data are mean values ± SDs from the relative expression of *CHS* in cells, skins, and flesh analyzed from two independent varieties. *Significant difference between flesh and other tissues ($p < 0.05$). Color images available online at www.liebertonline.com/dna

and Cynthiana. However, the relative expression levels varied significantly (Fig. 4). *CHS* showed the highest transcript levels in the skins, and *in vitro* red cell cultures, and low levels in the flesh of physiologically mature berries. Our study confirms, for the first time, the expression of *CHS* in *in vitro* synchronized red cell cultures of Noble and Cynthiana varieties after the cells have gone through the tissue culture process, which involves different hormone (Auxins and Cytokines) stimuli. The RT-PCR analysis of total RNA from the *in vitro* synchronized red cells of Noble and Cynthiana varieties indicated that there was a slight difference in the expression of the *CHS* gene transcripts in both varieties.

Multiple alignments and phylogenetic analysis of the CHS protein

The BLAST search for *CHS*-related sequences in the NCBI Genebank database showed that homologous protein sequences are present in other plants, as indicated in Table 1. The alignment of the deduced amino-acid sequences indicated that muscadine *CHS* shares the most identity with *V. vinifera CHS*, showing 100% identity. Furthermore, the alignment of muscadine *CHS* with other plants revealed that there are conserved amino-acid substitutions between *CHS* from muscadine and *CHS* from other plants mentioned in Table 1. The same conservation pattern was observed when the corresponding nucleotide sequences were aligned (>99% identity). Similarity percentages were found in the multiple amino-acid alignment of other plant species within the genus such as *Citrus maxima* (99%), *Camellia japonica* (98%), *Pyrus communis* (98%), and *Glycine max* (98%), showing that CHS is highly conserved at the genus level.

The three-dimensional (3D) structure of muscadine *CHS* (Fig. 5) shared an 84.3% similarity with the template, which further facilitated the positive identification of muscadine *CHS*. The cluster tree of the alignment of *CHS* proteins and
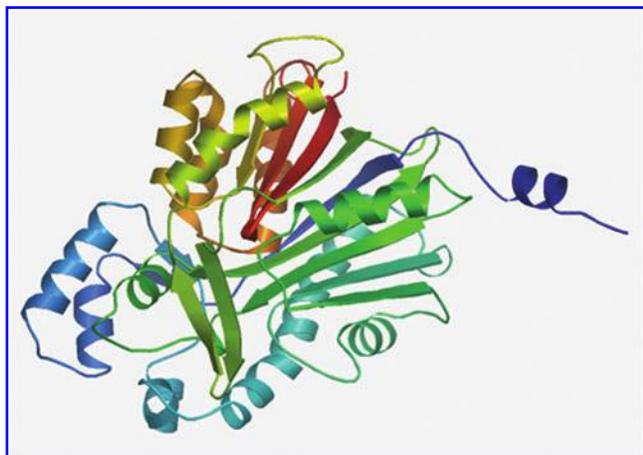
FIG. 5. The computational modeled three-dimensional structure of muscadine CHS. Color images available online at www.liebertonline.com/dna

the cladogram confirmed the close relationship between muscadine *CHS* and *V. vinifera* CHS (Fig. 6I). The phylogenetic tree was grouped into three distinct clades (Fig. 6I–III). Muscadine *CHS* was located in cluster I Fig. 6, while cluster II was further divided into several subgroups based on the
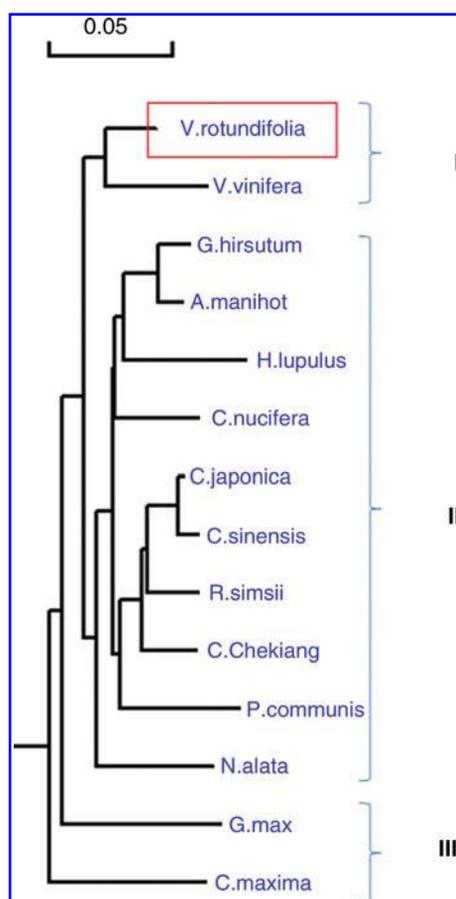

FIG. 6. A phylogenetic tree based on the deduced amino-acid sequence of various CHS from other plants. The muscadine CHS protein is boxed in red. Color images available online at www.liebertonline.com/dna

plant species. For example, *CHS* proteins from *Gossypium hirsutum* and *Abelmoschus manihot* (both in *Malvaceae* family) appeared in the same subgroup and those from *C. japonica*, *C. Sinensis*, and *C. chekiangoleosa* (all in the Theaceae family) were nested in another subgroup (Fig. 6). Our results concur with those by Zhou *et al.* (2011), which suggest that CHS is well conserved among plants of different groups and has distinct species specificity.

Discussion

This study amplified a cDNA encoding muscadine *CHS* homolog from physiologically mature berry skins, flesh, and *in vitro* synchronized red cell cultures of North American native grapes and characterized its expression patterns. The expression analysis revealed that the *CHS* gene was highly expressed in the *in vitro* synchronized red cell cultures and the skin of the physiologically mature red berries of North American native grapes. The activation of specific sets of genes depends on plant development and response to environmental stimuli (Ban *et al.*, 2003; Ageorges *et al.*, 2006). Variations in expression can be explained by the fact that *CHS* mRNA and enzyme levels are highly regulated during development associated with tissue and cell-type-specific accumulation of flavonoid pigments, and in response to environmental stimuli for the synthesis of flavonoid or isoflavonoids involved in adaptation (Hahlbrock and Scheel, 1989).

This study has shown that strategies for the commercial production of antioxidants such as bioavailable anthocyanins and other polyphenols should target berry skins and *in vitro* synchronized red cells, which have in this study been shown to harbor increased production of flavonoids. Apart from giving clear solutions in natural products research, this preliminary study demonstrates the potential of functional genomics in the systematic studies of North American native grapes. The high expression of *CHS* in anthocyanin-pigmented tissues (Fig. 4) confirms that *CHS* is responsible for anthocyanin biosynthesis in red berries, as reported earlier in other species: *G. hybrida* (Helariutta *et al.*, 1995), *Asiatic hybrid lily* (Nakatsuka *et al.*, 2003), *Dendodrobium orchid* (Mudalige-Jayawickrama *et al.*, 2005), and *Paeonia suffruticosa* (Zhou *et al.*, 2011).

Sequence analysis and comparison of the novel muscadine *CHS* revealed that the ORF was 1525 bp in length and putatively encoded a polypeptide of 393 amino acids, which had high similarities (98% to 100%) with CHSs from other plant species (Table 1), with a predicted molecular mass of 42.8 kDa and a pI of 6.01. Sequence analysis of the putative amino acids revealed that muscadine CHS contained the family signature and the active amino-acid residues that are highly conserved among all CHS sequences. In addition, the results of the 3D structural modeling and phylogenetic analysis indicated that muscadine *CHS* and *CHS* from *V. vinifera* are closely identical (Fig. 5 and 6). These findings indicate that the muscadine *CHS* characterized in this study is a homolog of the *CHS* gene, and its protein is a typical CHS protein.

Conclusion

In this study, we amplified and characterized muscadine *CHS* and by expression analysis confirmed that *CHS* is

involved in anthocyanin biosynthesis in the *in vitro* synchronized red cell cultures, skins, and flesh of Noble and Cynthiana. To our knowledge, this is the first time that *CHS* has been analyzed in the *in vitro* synchronized red cell cultures of North American native grapes. The amplification, characterization, and expression of other genes related to flavonoid accumulation in North American native grapes may pave the way to elucidate the molecular basis of berry pigmentation and can also facilitate and increase the development of *in vitro* red cell cultures of muscadine with more neutraceuticals compounds by manipulating the flavonoid structural and regulatory genes through functional genomics. The commercial production of *in vitro* synchronized red cell cultures as natural antioxidants will be a significant contribution to human medicine.

Acknowledgments

Disclosure Statement

No competing financial interests exist.

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