

Discovery, The Student Journal of Dale Bumpers College of Agricultural, Food and Life Sciences

Volume 21

Article 7

Fall 2020

Estimation of additive and dominance effects of a mutant glutathione S-transferase gene on anthocyanin content in muscadine grape (*Vitis rotundifolia*)

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Recommended Citation

Brown, A., Worthington, M., Varanasi, A., Nelson, L., Threlfall, R. T., & Howard, L. R. (2020). Estimation of additive and dominance effects of a mutant glutathione S-transferase gene on anthocyanin content in muscadine grape (*Vitis rotundifolia*). *Discovery, The Student Journal of Dale Bumpers College of Agricultural, Food and Life Sciences*, 21(1), 15-22. Retrieved from <https://scholarworks.uark.edu/discoverymag/vol21/iss1/7>

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Estimation of additive and dominance effects of a mutant glutathione S-transferase gene on anthocyanin content in muscadine grape (*Vitis rotundifolia*)

Cover Page Footnote

Autumn Brown is a senior with a major in Horticulture. Margaret Worthington is the faculty mentor and an Assistant Professor in the Department of Horticulture. Aruna Varanasi was a Postdoctoral Associate in the Department of Horticulture and is now a scientist at Bayer Crop Science. Lacy Nelson is a Program Associate in the Department of Horticulture. Renee T. Threlfall is a Research Scientist in the Department of Food Science. Luke Howard is a Professor in the Department of Food Science.

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Meet the Student-Author



Autumn Brown

Research at a Glance

- VrunGST4 is a dominant gene controlling berry color and anthocyanin content in muscadine grapes.
- Berry skin color is not a good indicator of total anthocyanins in black-fruited muscadine grapes.
- The average anthocyanin content in the two mapping populations was very different. This suggests that other genes may also contribute to variation in total anthocyanin content in muscadine grapes.

I am from Mammoth Spring, Arkansas. I will be graduating with honors from the University of Arkansas in the Fall of 2020 with a degree in Horticulture and minors in History and Crop Biotechnology. While at the University of Arkansas, I have learned about research and horticulture in ways I never imagined. I have been working in the Fruit Breeding Lab, under the direction of Dr. Margaret Worthington, since freshman year, during which time I have learned invaluable skills and made many networking connections. I attended the Southern Region and National American Society for Horticultural Science conferences and won first place in the undergraduate oral research presentation competitions at both meetings. In the summer between sophomore and junior year, I interned at Edward Vinson Ltd. in Faversham, England. This helped spur my interest in study abroad, and since then, I studied in Belize and became a Study Abroad Mentor. I served for two years on the Bumpers Honors Student Board. I want to thank my amazing mentor, Margaret Worthington, for her help in this project and my entire college career. I also want to thank Lacy Nelson, Aruna Varanasi, Cindi Brownmiller, Renee Threlfall, Luke Howard, John Clark, the fruit breeding team, and the Horticulture Department for their countless hours of support. Lastly, I want to thank my mother and father for helping me with everything and always offering me their unwavering support.



Autumn with her mentor Margaret Worthington accepting her first place award for undergraduate student paper at the Southern Regional American Society for Horticultural Science.

Estimation of additive and dominance effects of a mutant glutathione S-transferase gene on anthocyanin content in muscadine grape (*Vitis rotundifolia*)

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Abstract

The skin color of muscadine grapes (*Vitis rotundifolia*) is typically classified as black or bronze. A glutathione S-transferase, *VrunGST4*, has been identified as a candidate gene for berry skin color in muscadine grapes. A molecular marker was developed within *VrunGST4* to distinguish between muscadine genotypes (cultivars and selections) with bronze (T:T), heterozygote black (C:T), and homozygote black (C:C) berries. The objectives of this study were to determine whether there was a correlation between berry skin color and total anthocyanin content and to calculate additive and dominance effects of *VrunGST4* in determining total anthocyanins in the berries of two biparental F₁ muscadine populations with the intragenic *VrunGST4* marker. No correlation was found between the berry skin color measurements of hue and lightness and anthocyanin content of black-fruited genotypes in either population. However, there was a slight correlation ($r = 0.64$) between anthocyanin content and chroma in one of the populations. There was no difference in total anthocyanin content of homozygote black (C:C) and heterozygote black (C:T) genotypes in either population, indicating that *VrunGST4* had completely dominant gene action. The total anthocyanin content of the berry skins from black-fruited genotypes in one population was approximately four times greater than black-fruited genotypes in our other population. This finding suggests that other genetic loci may contribute to variation in total anthocyanin content in black-fruited muscadine grapes.

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Introduction

Muscadine grapes (*Vitis rotundifolia*) have been cultivated since the mid-18th century and are native to the Southeastern United States, where winter temperatures do not drop below -12 °C (Conner, 2009). They have a very distinctive fruit, thick skin, large seeds, and a unique fruity and musky aroma. Muscadines are valuable for their fresh-market fruits, wine, and juice production. Most commercial muscadine production goes into juice or winemaking (Morris and Brady, 2007).

Muscadines contain many different phytochemicals, most of which are found in their skins and seeds (King and Young, 1999). Anthocyanins are phenolic pigments responsible for giving many different plants their blue, purple, or reddish color (Wrolstad, 2006). Anthocyanin content in bunch grapes and black muscadine skins vary widely, ranging from 1000 µg.g⁻¹ to over 5000 µg.g⁻¹ fresh weight (Conner and MacLean, 2013).

The total amount of anthocyanins in the berry and the relative proportion of the individual anthocyanins affect muscadine juice color quality and stability (Conner and MacLean, 2013). Delphinidin is the most prominent type of anthocyanin in muscadine grapes, with malvidin found in very small amounts, partly responsible for the poor color stability of muscadine juice and wines (Conner and MacLean, 2013). Muscadines form diglucosidic anthocyanins, which have a decreased ability to form polymeric pigments, making them more prone to oxidation and browning (King and Young, 1999).

In general, muscadine grapes have two main skin colors: black and bronze. Nearly all wild muscadines produce a dark purple, almost black-colored berry. Bronze (light green-brown) berries are present in a much lower amount in the wild, though many bronze cultivars have been developed for fresh market and processing. Our research group recently constructed the first saturated genetic linkage maps of muscadine in two F₁ biparental populations segregating for berry color, 'Black Beauty' × 'Nesbitt' and 'Supreme' × 'Nesbitt' (Lewter et al., 2019). All three parents were heterozygous for black color, and the progeny in both populations segregated at an expected 3:1 ratio for black and bronze berry color (Fig. 1). The muscadine berry color locus mapped to a region on linkage group (LG) 4 aligning to 11.09–11.88 Mbp on *V. vinifera* chromosome 4 (Lewter et al., 2019). There were 21 predicted genes in this interval, including *VviGST4*. Glutathione S-transferases (GST) are required for transporting anthocyanins from the cytosol into the vacuole, where they are sequestered.

Our team sequenced the *VrunGST4* gene in several bronze and black muscadine cultivars and found a non-synonymous single nucleotide polymorphism (CCG/CTG), resulting in proline to leucine substitution in bronze muscadines. We also developed an intragenic Kompetitive Allele Specific PCR (KASP) marker that can distinguish between homozygote black (C:C), heterozygote black (C:T), and bronze (T:T) genotypes (cultivars and selections) and used it to genotype the mapping population and progeny (Varanasi et al., 2020; Fig. 2). It is unknown whether homozygote black genotypes have significantly higher an-

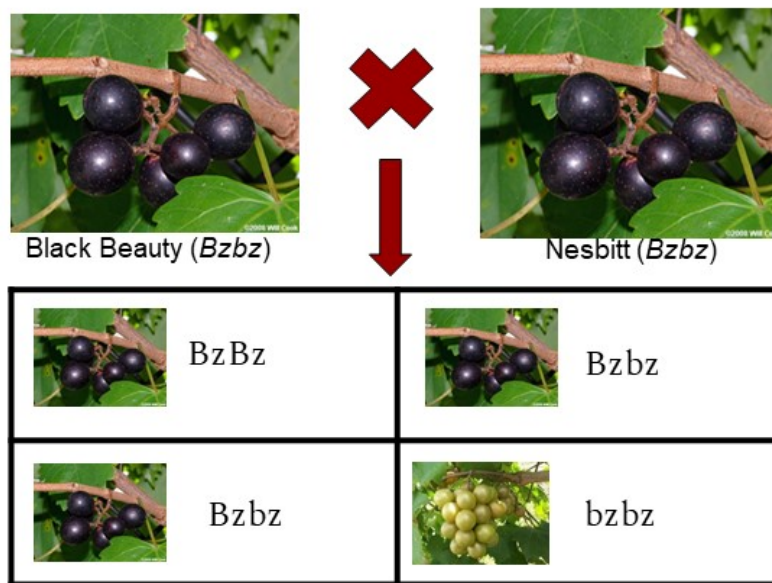


Fig. 1. The expected 3:1 segregation ratio of black (BzBz and Bzbz) and bronze (bzbz) progeny in the 'Black Beauty' × 'Nesbitt' muscadine grape mapping population.

thocyanin content than heterozygote black genotypes. Both genotype classes appear black, but color is not always a good predictor of nutraceutical content. Allele dosage plays a major role in determining anthocyanin content in *V. vinifera*. Most phenotypic variation in grape anthocyanin content has been attributed to additive effects, with dominance playing a minor role (Fournier-Level et al., 2009).

Now that we can accurately discern which progeny are heterozygote black and homozygote black, it is possible to determine whether allele dosage (additive genetic variation) at *VrunGST4* plays a significant role in determining anthocyanin content in muscadine skins. If homozygote black progeny have significantly higher anthocyanin content, breeders can use the intragenic *VrunGST4* KASP marker to select progeny with high anthocyanin production for processing and nutraceutical industries (Varanasi et al., 2020).

There were two main objectives for this research: (1) to assess whether individual anthocyanin content varies between homozygote and heterozygote black muscadines, and (2) to determine if there is a correlation between berry skin color and total anthocyanin content in muscadines.

Materials and Methods

Harvest

Fruit from selected progeny in the ‘Black Beauty’ × ‘Nesbitt’ and ‘Supreme’ × ‘Nesbitt’ mapping populations was harvested from vines grown at the University of Arkansas System Division of Agriculture Fruit Research Station in Clarksville on 13 September 2018. Forty-eight progeny, 16 from each genotype class (C:C, C:T, T:T), with sufficient fully colored mature fruit on the date of harvest were selected from each population. Harvested plants were selected by walking both populations and taking notes on which vines had adequate amounts of ripe fruit. Sixteen plants from each genotype class were randomly selected from the list of plants with adequate ripe fruit.

Color Analysis

Five berries were collected from each progeny vine, transported back to the Department of Food Science, Fayetteville in coolers, and stored in a cold room (2 °C). The next day, skin color was measured at the equator of each in-

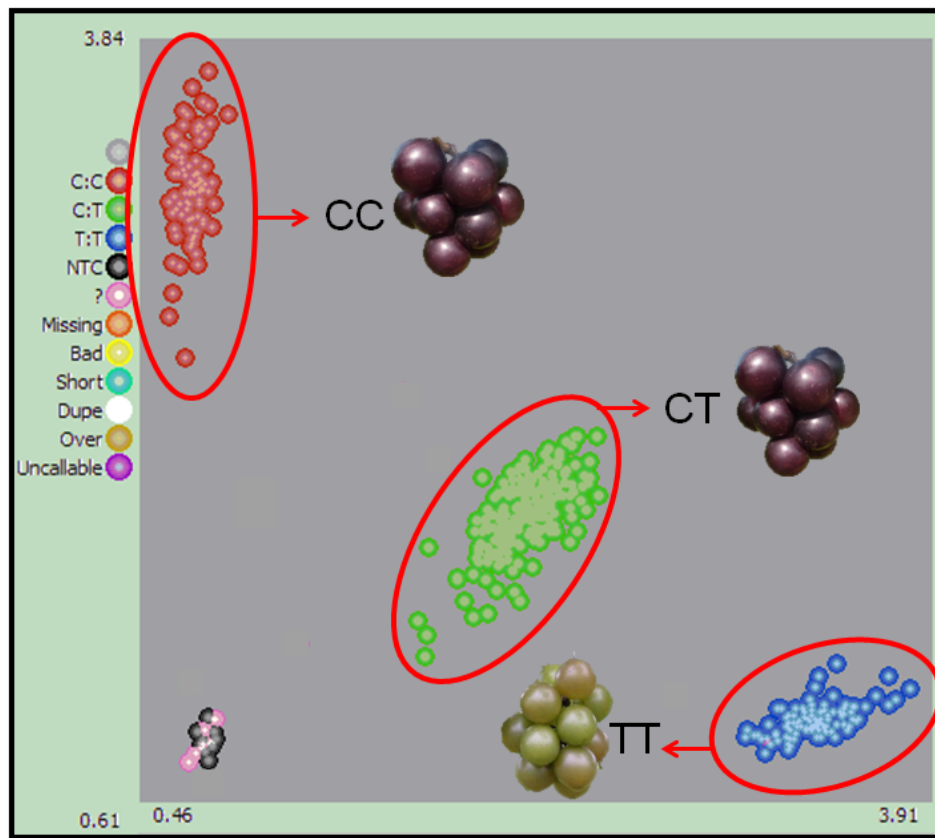


Fig. 2. Competitive Allele-Specific (KASP) PCR cluster plot showing clusters of bronze (T:T), heterozygote black (C:T), and homozygote black (C:C) progeny from the ‘Supreme’ x ‘Nesbitt’ and ‘Black Beauty’ x ‘Nesbitt’ muscadine grape mapping populations.

dividual berry using a CR 400 colorimeter (Konica Minolta, Ramsey, New Jersey). Color was measured as L^* (lightness), a^* (green-red), and b^* (yellow-blue) coordinates. Coordinates a^* and b^* were transformed into chroma (C^*) and hue angle (h°) using the equations: $C^* = (a^{*2} + b^{*2})^{1/2}$ and $h^\circ = \tan^{-1}(b^*/a^*)$ following (McGuire, 1992). After color measurements were completed, the flesh was removed from the slipskin fruit (pulp releases easily from the skin), and seeds were extracted. The skins were frozen (-20°C) for anthocyanin analysis.

Total Anthocyanins

Anthocyanins were extracted by homogenizing the grape skins (3.5 g) in the extraction solution, methanol/water/formic acid (60:37:3 v/v/v), with a Euro Turrax T18 Tissuemixer (Tekmar-Dohrman Corp, Mason, Ohio) for approximately 1 min. The samples were then centrifuged for 5 min at 10,000 rcf, and filtered through miracloth into a 100-mL or 200-mL volumetric flask. This process was repeated with 25 mL of extraction solution containing acetone/water/acetic acid. The entire process was repeated until all color was removed from the residue. Total anthocyanins were then

measured using the pH differential method and quantified as cyanidin-3-glucoside equivalents following Giusti and Wrolstad (2001). Absorbance was measured spectrophotometrically at 510 and 700 nm and at pH 1 and pH 4.5.

Individual Anthocyanins

Aliquots (7.5 mL) of five extracts (chosen randomly from each genotype class and mapping population) were dried using a Speed Vac concentrator (ThermoSavant, Holbrook, New York) and then re-suspended in 1 mL of 5% formic acid. The samples were passed through 0.45-mm polytetrafluoroethylene (PTFE) syringe filters (Varian Inc, Palo Alto, California) before High-Performance Liquid Chromatography (HPLC) analysis. Anthocyanin analysis by HPLC was performed following a procedure from Cho et al. (2004). All samples were analyzed using a Waters HPLC system equipped with a model 600 pump, a model 717 Plus autosampler, and a model 996 photodiode array detector. Separation was carried out using a 4.6 mm \times 250 mm Symmetry[®] C₁₈ column (Waters Corp, Milford, Massachusetts) preceded by a 3.9 mm \times 20 mm Symmetry[®] C₁₈ guard column. The mobile phase was a linear gradient of 5% formic acid and methanol

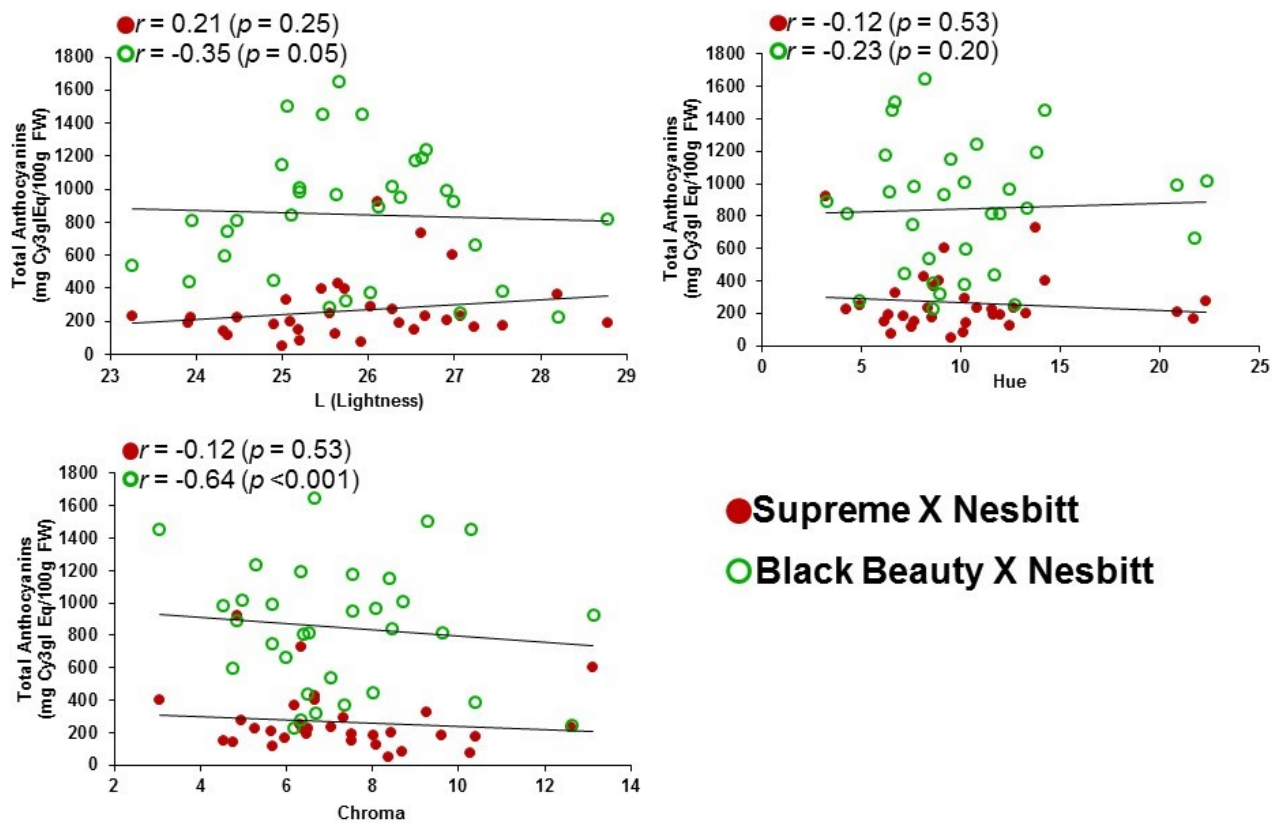


Fig. 3. Correlation between the anthocyanin content in black muscadine grape berry skin and color attributes, (a) lightness, (b) chroma, and (c) hue, in the muscadine mapping populations. The 'Supreme' x 'Nesbitt' and 'Black Beauty' x 'Nesbitt' mapping populations are indicated with black and hollow dots, respectively.

from 2% to 60% for 60 min at 1 mL/min. The system was equilibrated for 20 min at the initial gradient prior to each injection. The anthocyanin peaks were quantified at 510 nm with results expressed as mg cyanidin-3-glucoside equivalents per 100 g fresh fruit weight.

Statistical Analysis

Statistical analyses were performed in SAS 9.4 (SAS Institute, Inc., Cary, N.C.). Pearson's correlation coefficient was used to test for the significance of the correlation between color and total anthocyanins in black (C:C and C:T) genotypes. PROC GLM was used to perform an analysis of variance to test whether total anthocyanins differed among C:C, C:T, and T:T genotype classes in the two mapping populations.

Results and Discussion

Berry Skin Color and Total Anthocyanins

There was no correlation between total anthocyanins and the berry skin color attributes (lightness, hue, or chroma) in the 'Supreme' × 'Nesbitt' population (Fig. 3). In the 'Black Beauty' × 'Nesbitt' mapping population, there was no correlation between total anthocyanins and lightness or hue. However, total anthocyanins were negatively correlated with chroma in the 'Black Beauty' × 'Nesbitt' mapping population ($r = -0.64$, $P < 0.001$). Other researchers have also found that color is not a good indication of nutraceutical content. A 2014 study determined that color was not a good indicator for beta carotene in maize (*Zea mays*), with no significant correlation between color and nutraceutical content (Muthusamy et al., 2014).

Total Anthocyanins

The *VrunGST4* gene was determined to have dominant gene action, with no difference between the C:C and C:T genotypes in either population (Fig. 4). In the 'Supreme' × 'Nesbitt' mapping population, C:C genotypes had an average of 263.8 mg/100 g total anthocyanins, while the C:T genotypes averaged 265.4 mg/100 g total anthocyanins. The T:T genotypes averaged 9.43 mg/100 g total anthocyanins. In the 'Black Beauty' × 'Nesbitt' population, the C:C genotypes averaged 890.2 mg/100 g total anthocyanins, the C:T population had 883.1 mg/100 g total anthocyanins, and the T:T genotypes averaged 18.6 mg/100 g total anthocyanins. This finding is in contrast to the *V. vinifera* Myb color genes, which have an additive effect (Fournier-Level et al., 2009).

The greater average anthocyanin content of black-fruited progeny in the 'Black Beauty' × 'Nesbitt' mapping population than in the 'Supreme' × 'Nesbitt' progeny could be attributed to many different factors, including general ripeness when the berries were picked. The large difference between the means of the black-fruited genotypes in the two mapping populations suggests that there may be other loci contributing to total anthocyanin content in addition to *VrunGST4*. Anthocyanin content in the skins of black-fruited muscadines has previously been shown to range from less than 100 mg/100 g to over 500 mg/100 g (Conner and MacLean, 2013). Further investigations are needed to determine which other loci contribute to this large range in total anthocyanin content in black-fruited muscadines.

Individual Anthocyanins

By performing HPLC analysis, we determined the percentage of each individual anthocyanin in three genotype

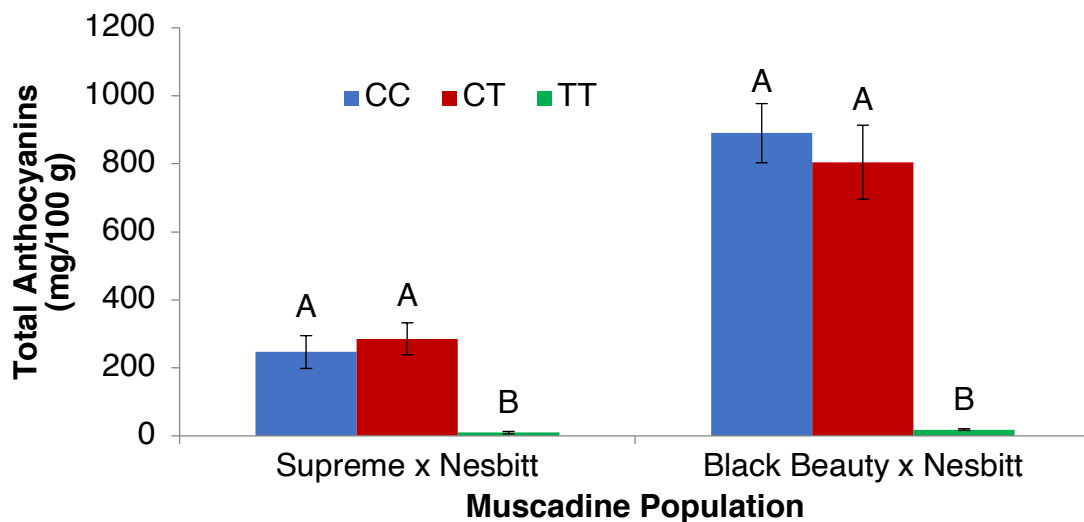


Fig. 4. Total anthocyanin content of skin extracts in three different muscadine grape genotype classes; homozygous black (C:C), heterozygous black (C:T), and homozygous bronze (T:T) in the 'Supreme' × 'Nesbitt' and 'Black Beauty' × 'Nesbitt' mapping populations.

classes (C:C, C:T, and T:T) of both mapping populations. Conner and MacLean (2013) previously reported that delphinidin was the predominant individual anthocyanin in both black and bronze berries from both mapping populations. Our results were similar, with delphinidin as the most abundant anthocyanin making up 22.21% to 37.72% of total anthocyanins in the C:T, C:C, and T:T genotype classes in both mapping populations (Fig. 5). The order of importance of the other individual anthocyanins was petunidin (9.9–12.7%), peonidin (7.08–9.61%), cyanidin (6.4–9.3%), and malvidin (2.68–5%).

Conclusions

For both total and individual anthocyanins, we found a dominant gene action regarding the *VrunGST4* gene, with no difference in anthocyanin content between homozygous black (C:C) and heterozygous black (C:T) muscadines. We also determined that berry skin color is not a good indicator of total anthocyanins in black-fruited muscadine grapes. The *VrunGST4* KASP marker is still predictive for berry color and will be useful for breeding purposes. Further research is needed to determine what other possible genes or loci affect anthocyanin content in muscadines.

Acknowledgments

Funding for this project was provided by the Student Undergraduate Research Fellowship (SURF) program and the Bumpers College Undergraduate Research Grant.

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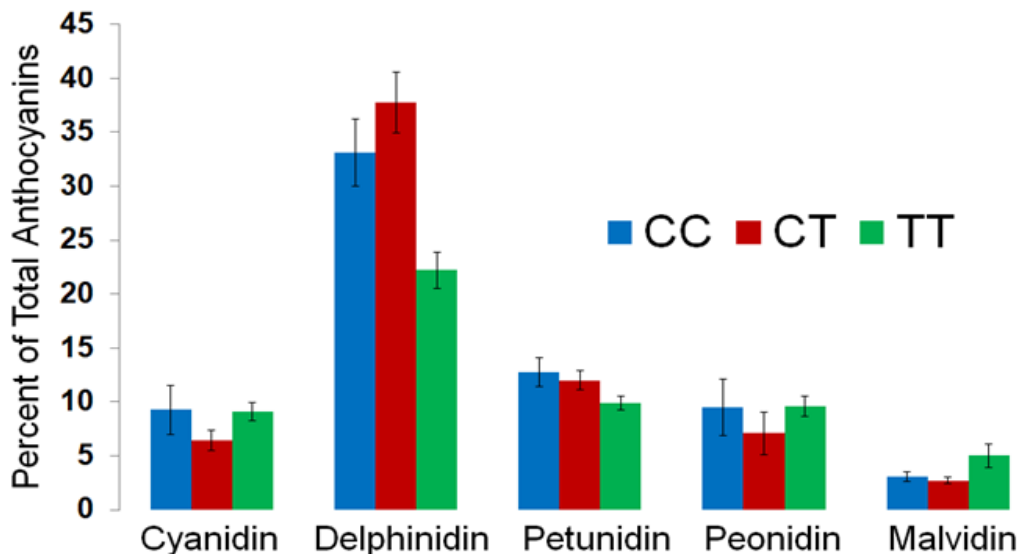


Fig. 5. Individual anthocyanins (percent of total anthocyanins) in homozygous black (C:C), heterozygous black (C:T) and homozygous bronze (T:T) muscadine grape genotype classes in the 'Supreme' x 'Nesbitt' and 'Black Beauty' x 'Nesbitt' mapping populations.

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