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

Autumn Brown

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Autumn Brown, Honors Student, Horticulture Department, University of Arkansas

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 and proanthocyanidin in the seeds of two biparental F_1 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 higher 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. We also tested total proanthocyanidin content in the seeds of

homozygous black, heterozygote black, and homozygous bronze genotypes and found there was no significant difference between the three genotype classes in either population.

Introduction

Muscadine grapes

Table and wine grapes (*Vitis vinifera*) are important sources of nutrients, antioxidants, and other phenolic compounds. Unfortunately, *V. vinifera* is not adapted to all production regions and is very susceptible to diseases in humid regions like Arkansas. 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). There they are most abundant in the coastal plains along the Atlantic ocean and the gulf of Mexico (Olien 1990). Muscadines were the first cultivated grape in North America and play an important part in American history (Conner, 2009). The earliest Western Explorers wrote about and were enthralled with this grape. There are records that date that Spanish Missionaries in Florida were making wine from muscadines as early as 1565 (Olien, 1990).

Both muscadines and *V. vinifera* belong to the *Vitis* genus, but *V. vinifera* and other bunch grapes are in the subgenus *Euvitis*, while muscadines are in subgenus *Muscadinia*. Muscadines are the only cultivated member of the *Muscadinia* subgenus (Conner and MacLean, 2013). There are many key differences between muscadines and bunch grapes. Both are diploid organisms, but bunch grapes have 38 chromosomes while muscadines have 40 chromosomes. Muscadines have a very distinctive fruit, thick skin, large seeds, and a unique fruity and musky aroma. *V. vinifera* is susceptible to many pathogens, such as downy mildew (*Peronospora sparsa*), powdery mildew (*Uncinula necator*) and Pierce's disease (*Xylella fastidiosa*) whereas muscadines are resistant. 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).

Anthocyanins in grapes

Muscadines contain many different phytochemicals, most of which are found in their skins and seeds (King and Young, 1999). Phenolic compounds, the largest category of phytochemicals, have been the subject of numerous studies as they potentially have health-related impacts such as reduction of heart disease and cancer (Wrolstad, 2006). The skins and seeds of muscadines are rich in antioxidant activity and have major phenolics (Conner and MacLean, 2013). Anthocyanins are phenolic color pigments responsible for giving many different plants their blue, purple or reddish color (Wrolstad, 2006). Anthocyanin content in bunch grapes and black muscadines skins vary widely, ranging from 1000 $\mu g.g^{-1}$ to over 5000 $\mu g.g^{-1}$ 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). Bunch grapes and muscadines differ in their anthocyanin content. Bunch grapes tend to contain mostly malvidin and peonidin, with a small amount of delphinidin (Conner and MacLean, 2013). Malvidin is the reddest individual anthocyanins, which gives *V. vinifera* wines their characteristic dark red color. Malvidin and peonidin are also the most stable individual anthocyanins, which allows *V. vinifera* wines to retain their color during long periods of storage. The common anthocyanins found in grapes in order of decreasing stability are malvidin, peonidin, pelargonidin, petunidin, cyanidin, and delphinidin (King and Young, 1999). 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). The good color stability in *V. vinifera* wines is also due to the fact that *V. vinifera* grapes do not contain the dominant allele for production of diglucosidic anthocyanins, which causes grapes to only produce monoglucosides. In contrast, muscadines can form diglucosidic anthocyanins. Diglucosidic anthocyanins have a decreased ability to form polymeric pigments, making them more prone to oxidation and browning (King and Young, 1999).

Proanthocyanidins in grapes

Proanthocyanidins are the second most abundant of the phenolic compounds (Hümmer and Schreier, 2008). They are found in fruits, bark, leaves, and seeds of many plants, and they provide protection against predation. These colorless flavonoid polymers are responsible for the flavor and astringency in many teas, wines, and fruit juices (Dixon et al., 2005). When muscadines are processed for wine, or juice, both their skin and seeds are discarded. These by-products are called pomace and are often thrown out and not utilized. Due to the high amounts of phenolic compounds that reside in the pomace, much research has been done on transforming these by-products into a nutraceutical product (García-Lomillo and González-SanJosé, 2017).

Genetic control of berry color in grapes and muscadines

In general, muscadines 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 F_1 biparental populations segregating for berry color, ‘Black Beauty’ x ‘Nesbitt’ and ‘Supreme’ x ‘Nesbitt’ (Lewter et al., 2019). While the MYB transcription factor genes controlling fruit color in *V. vinifera* are located on

chromosome 2, 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. *VviGST4* is a homolog of bronze II (*bz2*) and *AN9*, which are GSTs that cause color variations in maize (*Zea mays*) and petunia (*Petunia* × *atkinsiana*) respectively (Nash et al., 1990 ; Harjes et al., 2008). Proanthocyanidins are also sequestered in the vacuole and transported by GSTs in a similar fashion to anthocyanins.

Our team sequenced the *VrunGST4* gene in several bronze and black muscadine cultivars and found a nonsynonymous single nucleotide polymorphism (CCG/CTG) resulting in a shift from proline to leucine in bronze muscadines. We also developed an intragenic Kompetitive Allele Specific Primer (KASP) marker that can distinguish between homozygote black (C:C), heterozygote black (C:T), and bronze (T:T) genotypes 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 anthocyanin 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 heterozygous 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 and proanthocyanidin content in seeds. If homozygote black progeny have significantly higher

anthocyanin or proanthocyanidin 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 three main objectives for this research: (1) assess whether individual anthocyanin content varies between homozygote and heterozygote black muscadines, (2) determine if there is a correlation between berry skin color and total anthocyanin content in muscadines, and (3) determine if the total proanthocyanidin content of the muscadine seeds varies between homozygous, heterozygous black muscadines and homozygous bronze muscadine

Material and Methods

Mapping populations

This research was conducted using two mapping populations segregating for berry color, 'Black Beauty' x 'Nesbitt' and 'Supreme' x 'Nesbitt'. Each of these populations consisted of 172 individuals. 'Black Beauty' and 'Supreme' are both pistillate cultivars, while 'Nesbitt' is a perfect-flowered cultivar. All three parents are black-fruited, but the bronze, pistillate cultivar 'Fry' is prominent in each pedigree (Clark 1997). 'Fry' is the female parent of both 'Nesbitt' and 'Black Beauty' and is also represented in the maternal and paternal lineage of 'Supreme' (Goldy and Nesbitt 1985; Clark 1997; Conner 2013).

Both crosses were made in 2007 at the University of Arkansas Fruit Research Station (FRS) in Clarksville, AR. Seedlings were planted at FRS in May 2008 and trained to a single-wire trellis with a 0.6 m cordon established for each vine. Vines received routine cultural care including annual dormant pruning to three or four bud spurs. Applications of 43 kg.ha-1 of N

fertilizer were made each year and trickle irrigation was provided as needed. No fungicides or insecticides were applied to the vines. Chi-Square goodness-of-fit tests were performed to test whether the progeny fit the 1:1 segregation ratio of female and perfect-flowered vines expected in each population. The correct flower sex of two vines from each population with conflicting sex phenotypes recorded in 2011 and 2012 was verified in June 2017. Berry color was scored as a qualitative trait (black or bronze) and Chi Square tests were performed to test whether the progeny fit the 3:1 ratio of black- and bronze-fruited vines expected for a cross between two heterozygote black parents. The progeny in both populations segregated at an expected 3:1 ratio for black and bronze berry color (Fig. 1)

Harvest

Fruit from selected progeny in the 'Black Beauty' x 'Nesbitt' and 'Supreme' x 'Nesbitt' mapping populations were harvested from vines grown at the University of Arkansas Fruit Research Station in Clarksville on September 13, 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 University of Arkansas 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 individual berry using a CR 400 colorimeter (Konica Minolta, Ramsey, NJ). 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 $h° = \tan–1(b^*/a^*)$

following (Mcguire, 1992). After color measurements were completed, the flesh was removed from the slipskin fruit and seeds where extracted. Both the skins and seeds were frozen (-20 °C) for anthocyanin and proanthocyanidin analysis, respectively.

Total anthocyanins

Anthocyanins were extracted by homogenizing the grape skins (3.5 g) and the extraction solution, methanol/water/formic acid (60:37:3 v/v/v), with a Euro Turrax T18 Tissuemizer (Tekmar-Dohrman Corp, Mason, OH, USA) for approximately 1 min. The samples were then centrifuged for 5 min at 10,000 rpm, and filtered through miracloth into a 100 mL or 200 mL volumetric flask. This process was repeated again with 25 mL of extraction solution containing acetone/water/acetic acid. The entire process was repeated until all color was removed from the supernatant. Total anthocyanins were then measured using the pH differential method quantified as cyanidin-3-glucoside equivalents following Lawless et al. (2012). 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, NY) and then resuspended in 1 mL of 5% formic acid. The samples were passed through 0.45-mm polytetrafluoroethylene (PTFE) syringe filters (Varian Inc, Palo Alto, CA) before High-Performance Liquid Chromatography (HPLC) analysis. Anthocyanin analysis by HPLC was performed following a procedure from Mi 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 × 250 mm Symmetry® C18 column (Waters Corp, Milford, MA) preceded by a 3.9 mm × 20 mm

Symmetry® C18 guard column. The mobile phase was a linear gradient of 5% formic acid and methanol from 2% to 60% for 60 min at 1 $mL.min^{-1}$. 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.

Total proanthocyanidins

The seeds of the same berries from the subset of the mapping population used to assess total anthocyanins were ground into a fine powder for analysis. All seeds were first lyophilized overnight. Four to six seeds per sample were ground in a 2.0 mL centrifuge tube with one or two 5/32" grinding balls (OPS Diagnostics, Lebanon, NJ) in a ball mill (Mixer Mill MM 400, Retsch, Haan, Germany) for 10 min at 30 Hz. Then the samples subsequently were ground by hand in a mortar and pestle until they became a coarse powder. The samples were placed back into the ball mill for an additional 10 min until a fine powder was formed.

Samples were weighed to fit in the range of 40-60 mg and placed in 50 mL centrifuge tubes. The PAC extraction solution (20 mL) was added to the samples. The samples were vortexed for 30 sec followed by sonication at room temperature for 30 min. Samples were placed on an orbital shaker for 1 h and subsequently centrifuged at 1000 × *g* at 12 ºC for 5 min. The supernatant was collected for analysis.

Total proanthocyanidins present in the phenolic extract were measured using the 4-dimethylaminocinnamaldehyde (DMAC) assay following the methods of Prior et al. (2010). A solution of 3 mL HCl in 27 mL ethanol was prepared and 0.03 g of DMAC was added to the solution. Aliquots (50 mL) of blanks, standards, and extracts were prepared. Two hundred and fifty mL of DMAC solution was added to all prepared blanks, standards, and extracts. The plate

was read immediately at 640 nm. A2 proanthocyanidin was used as the standard (50 ppm, 25 ppm, 12.5 ppm and 6.25 ppm) with results expressed as mg total proanthocyanidins /100g seeds

Statistical analysis

Statistical analyses were performed in SAS 9.4 (Cary, NC). 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 ANOVA to test whether total anthocyanins and total proanthocyanidins 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 significant correlation between total anthocyanins and lightness, hue, or chroma in the 'Supreme' x 'Nesbitt' population (Fig. 3). In the 'Black Beauty' x 'Nesbitt' mapping population, there was no significant correlation between total anthocyanins and lightness or hue. However, total anthocyanins were negatively correlated with chroma in the 'Black Beauty' x 'Nesbitt' mapping population (r = -0.64, $P < 0.001$). Other researchers have also found that color is not a good indication for nutraceutical content. A 2014 study determined that color was not 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 significant difference between the C:C and C:T genotypes in either population (Fig. 4). In the 'Supreme' x 'Nesbitt' mapping population, C:C genotypes had an average of 263.8 mg/100g total

anthocyanins while the C:T genotypes averaged 265.4 mg/100g total anthocyanins. The T:T genotypes averaged 9.43 mg/100g total anthocyanins. In the ‘Black Beauty’ x ‘Nesbitt” population the C:C genotypes averaged 890.2 mg/100g total anthocyanins and the C:T population had 883.1 mg/100g total anthocyanins, the T:T genotypes averaged 18.6 mg/100g 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’ x ‘Nesbitt’ mapping population than the ‘Supreme’ x ‘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/100g to over 500 mg/100g (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 were able to determine the percent of each individual anthocyanin in three genotype classes (C:C, C:T, and T:T) of both mapping populations. Conner and MacLean (2013) previously reported that delphinidin was the predominant type of 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%-5.00%).

Proanthocyanidins

In the 'Supreme' x 'Nesbitt' population C:C genotypes averaged 1343.23 mg total proanthocyanidins /100g seeds, the C:T genotype class genotypes had 1103.31mg total proanthocyanidins /100g seeds, and the T:T genotypes had 1048.05 mg total proanthocyanidins /100g berry seeds (Fig. 6). In the 'Black Beauty' x 'Nesbitt' population the C:C genotype class had 1122.02 mg total proanthocyanidins /100g seeds, the C:T genotype class had 1169.99 mg total proanthocyanidins /100g seeds, and the T:T genotype class had 1117.84 mg total proanthocyanidins /100g seeds. There was no significant difference in total proanthocyanidin content among the three genotype classes in either mapping population. Our results are similar to those of Pastrana-Bonilla et al. (2003), with both bronze and black berries having similar proanthocyanidin amounts. *VviGST3* was more highly expressed in seeds, whereas *VviGST4* was more highly expressed in the berry skins in *V. vinifera* (Pérez-Díaz et al., 2016). Our results suggest that the mutated *VrunGST4* gene is highly expressed in muscadine berry skins but that other GSTs, such as *VrunGST3*, may play a larger role in proanthocyanidin transport in the seeds.

Conclusions

For both total and individuals anthocyanins we found a dominant gene action regarding the *VrunGST4* gene, with no significant 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. Seeds from homozygous black (C:C), heterozygous black (C:T), and homozygous bronze (T:T) muscadines

did not differ significantly in total proanthocyanidin content. 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 and proanthocyanidin content in muscadines.

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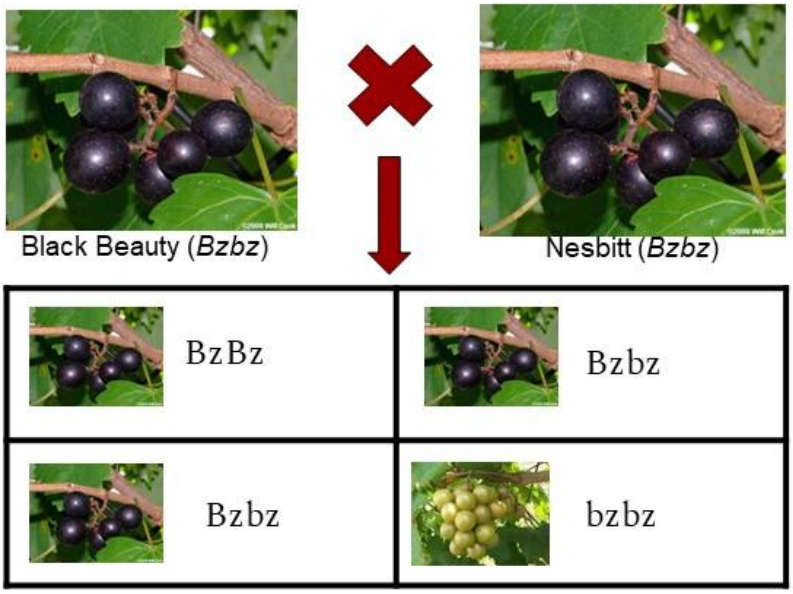

Figure 1. The expected 3:1 segregation ratio of black and bronze progeny in the ‘Black Beauty’ x ‘Nesbitt’ mapping population

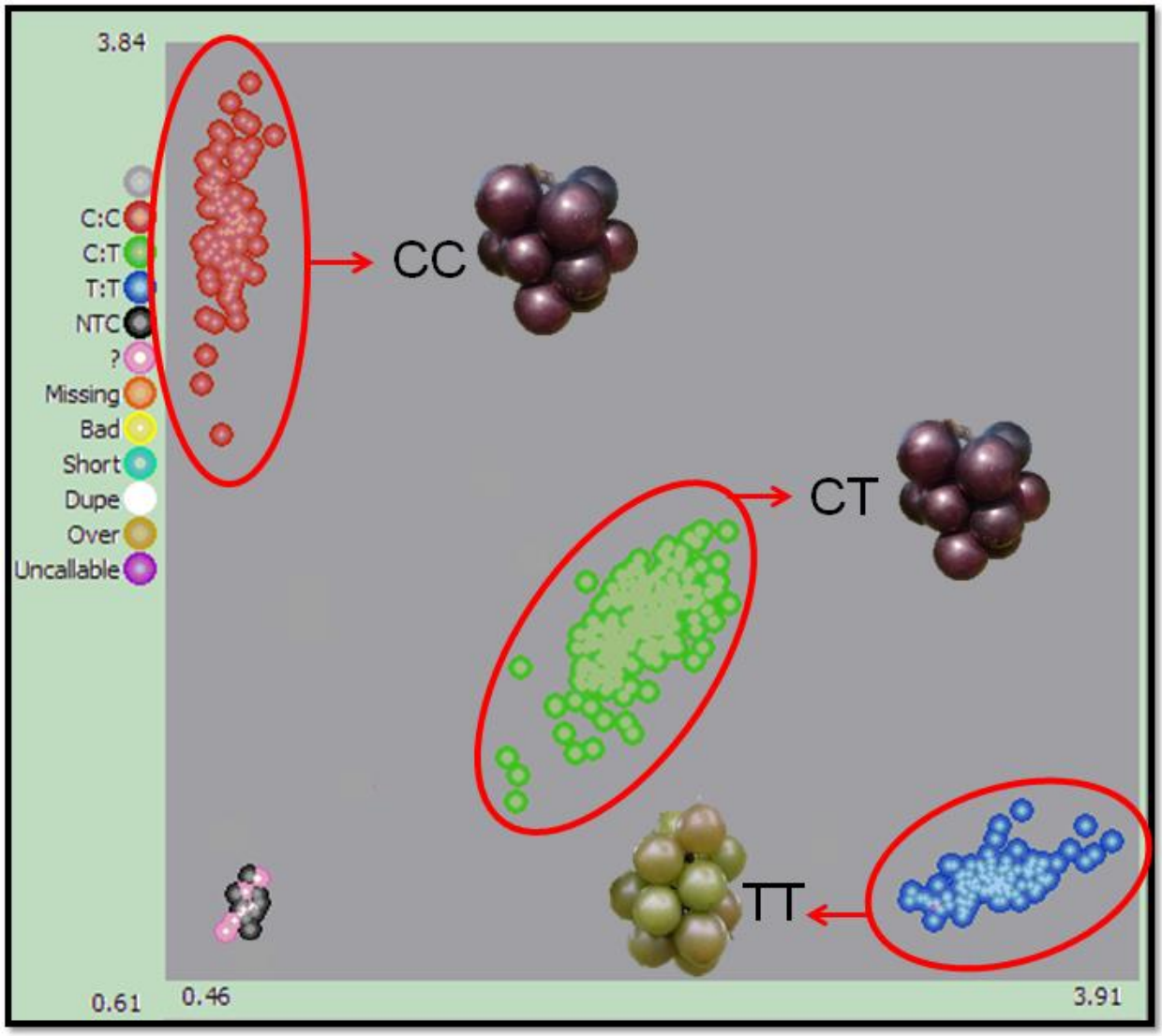

Figure 2. KASP (Kompetitive Allele-Specific PCR) cluster plot showing clusters of bronze (T:T), heterozygote black (C:T), and homozygote black (C:C) progeny from the 'Supreme' x 'Nesbitt' and 'Black Beauty' x 'Nesbitt' mapping populations.

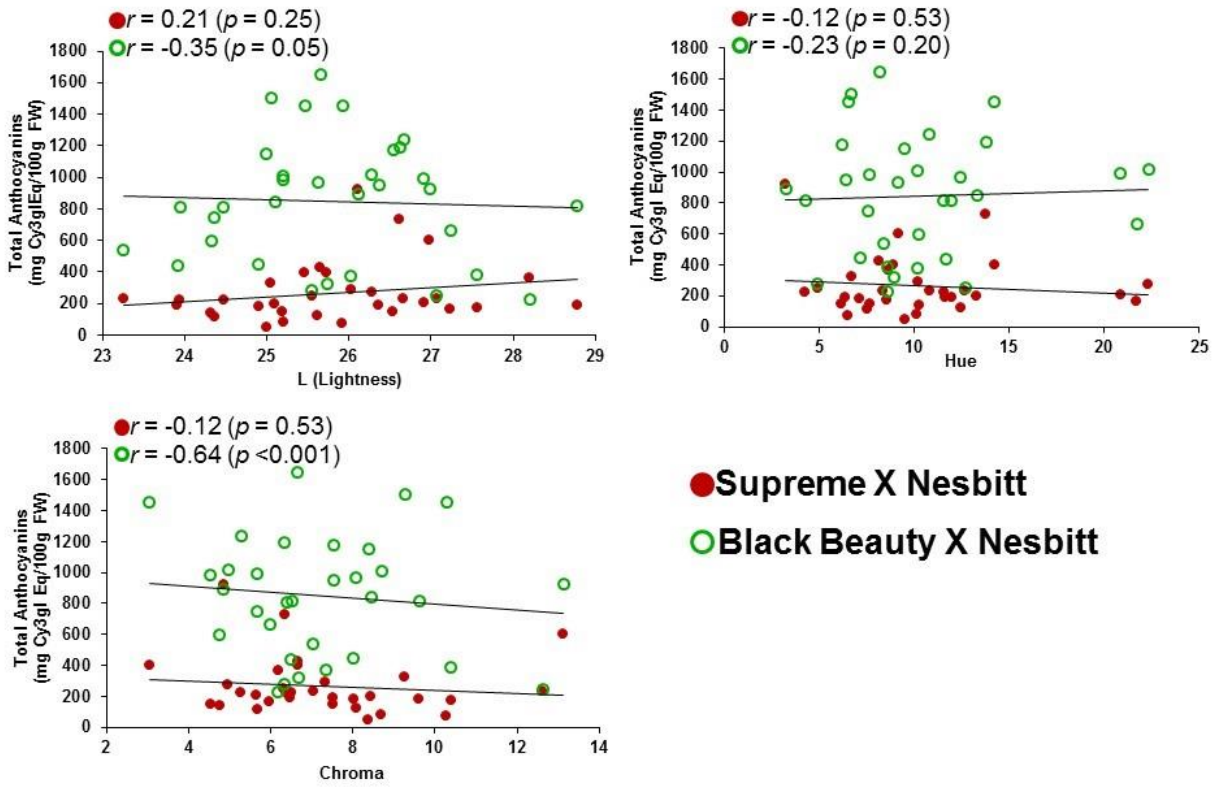

Figure 3. Correlation between the anthocyanin content in black muscadine grape berry skin and color attributes (lightness, chroma and hue) in ‘Supreme’ x ‘Nesbitt’ and ‘Black Beauty’ x ‘Nesbitt’ mapping populations.

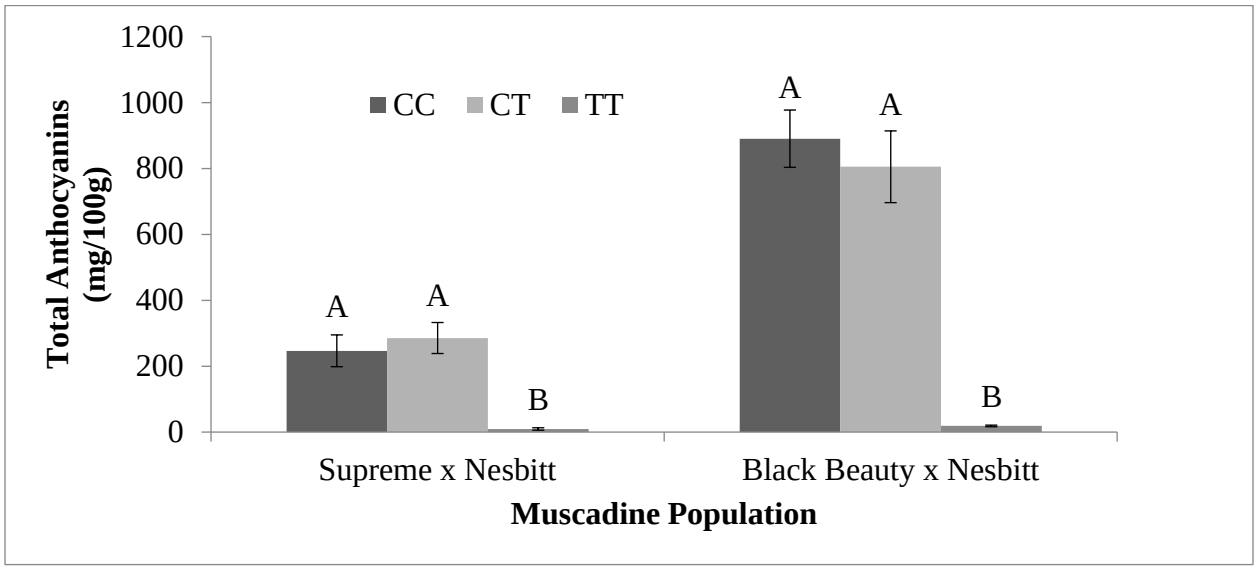

Figure 4. Total anthocyanin content of skin extracts in three different muscadine grape genotype classes; homozygous black (C:C), heterozygous black (C:T) and homozygous bronze (T:T) in the ‘Supreme’ x ‘Nesbitt’ and ‘Black Beauty’ x ‘Nesbitt’ mapping populations.

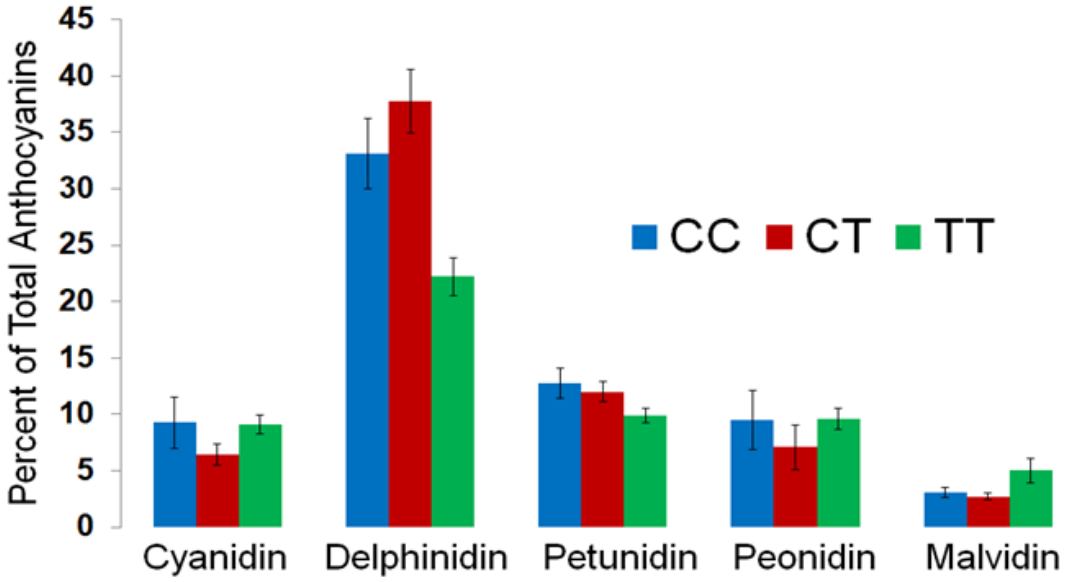

Figure 5. Percent composition of total anthocyanins in homozygous black (C:C), heterozygous black (C:T) and homozygous bronze (T:T) muscadine grape genotype classes in the ‘Supreme’ x ‘Nesbitt’ and ‘Black Beauty’ x ‘Nesbitt’ mapping populations.

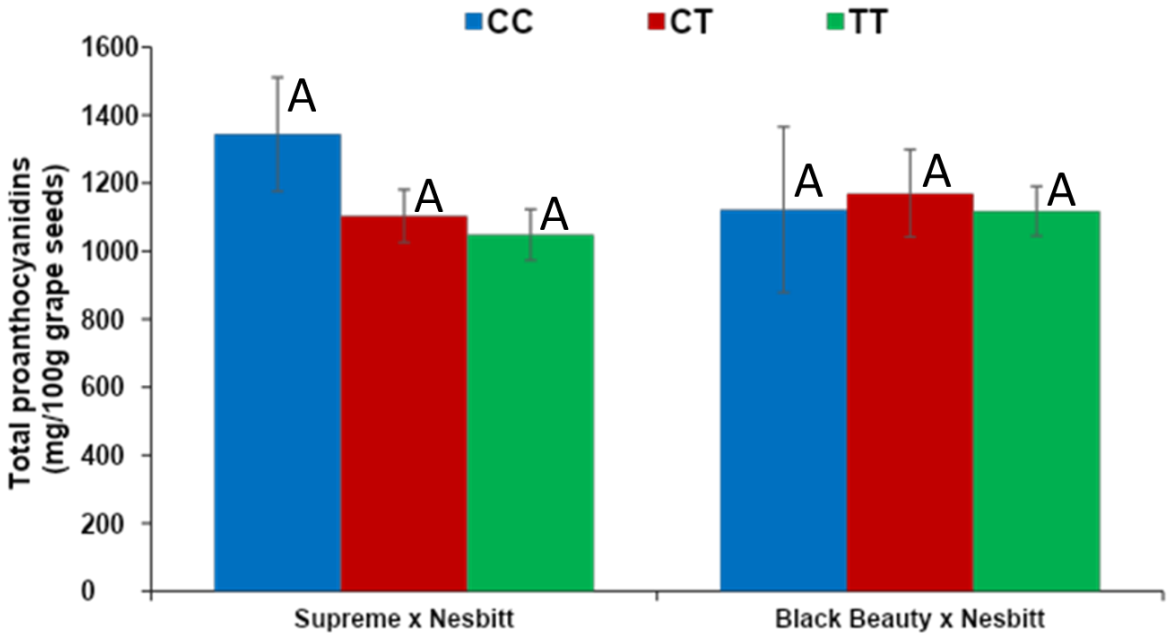

Figure 6. Total proanthocyanidin content in seeds collected from three different muscadine grape genotype classes, homozygous black (C:C), heterozygous black (C:T) and homozygous bronze (T:T), in the 'Supreme' x 'Nesbitt' and 'Black Beauty' x 'Nesbitt' mapping populations