ANTHOCYANIN CONTENT AND DIVERSITY IN CRUCIFER VEGETABLES

A Thesis

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Masters of Science

by

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ABSTRACT

The Brassicaceae family is one of the most diverse groups of economically important crops. Brassicaceae plants are known for the production of health promoting phytochemicals including glucosinolates, carotenoids, and anthocyanins. The red and purple colors of Brassicaceae plants is primarily associated with anthocyanins, which are a diverse group of water-soluble polyphenolic pigments produced across many plant species and organs. Anthocyanins are water-soluble vacuolar pigments that may appear red, purple, or blue depending on pH, which has led to interest in their use by the food industry as natural colors.

B. oleracea (cabbage and kale) and *R. sativus* (radish) were initially assessed for the distribution of anthocyanins within plant tissues through cytological methods. Anthocyanins were found in the epithelial and outer mesophyll cells of *B. oleracea* stems and leaves. *R sativus* produced anthocyanins within internal tissues and roots in addition to the outer cells of stems and leaves, indicating the ability to produce high concentrations and total quantities of anthocyanins.

The species were also assessed for anthocyanin pigment quality. High quality anthocyanins have high levels of acylation which results in molecular stability and a ‘blue-green’ color. Radish, cabbage, kale, and Brussels sprouts samples were extracted in buffers ranging from pH 1 through 12. Images of these extracts were used for RGB measurements. Results indicated that *B. oleracea* morphotypes produced strong ‘red’ pigmentation at low pH values, ‘purple’ pigmentation at slightly acidic conditions, ‘blue-green’ at neutral conditions and ‘yellow’ at basic conditions. Radish contained ‘orange’

and ‘brown’ color profiles throughout the pH range due to pelargonidin, indicating that this source may be less desirable for the food industry.

When comparing juvenile types of *B. oleracea* morphotypes (cabbage, kale and Brussels sprouts) and *B. rapa* and *B. juncea* mustard types, kale juvenile types produced the highest levels of anthocyanins (3.49 mg/g dry weight Cy-3,5-DiG Equiv.). The mustards produced unique chromatograms and higher levels of diacylation (84.5%) with the lowest concentrations of anthocyanins (1.15 mg/g Cy-3,5-DiG Equiv.) when compared to their *B. oleracea* counterparts.

B. oleracea genotypes were identified as having preferable natural colors with red cabbage mature morphotypes being typically high in anthocyanins. Screening of germplasm from four repositories was initiated comprising a total of 173 red cabbage accessions. These were evaluated using high performance liquid chromatography (HPLC) methods to assess anthocyanin content. The results showed strong phenotypic diversity both in total anthocyanin content and in percent diacylation of anthocyanins. Several accessions were noted for their high levels of anthocyanins and/or levels of diacylation: HRI 002657A (4.29 mg/g Cy-3,5-DiG Equiv. and 74.4% diacylated), BRA 770 (14.24 mg/g Cy-3,5-DiG Equiv. and 68% diacylated), and CGN 07090 (12.07 mg/g Cy-3,5-DiG Equiv. and 49.1% diacylated). These results indicate that genetic diversity is available for selection of high levels of anthocyanins in *Brassica* vegetables through plant breeding.

A subset of accessions were treated with cold temperature to assess the effect of post-harvest cooling on anthocyanin content of mature cabbage types. Cooling significantly increased both total anthocyanin concentrations and the relative percentage of diacylation (an increase from 9.14 mg/g Cy-3,5-DiG Equiv. (32.7% diacylated) to

11.91mg/g Cy-3,5-DiG Equiv. (39.5% diacylated)). These results indicate that post-harvest storage and cooling may be a beneficial process for industry purposes prior to extraction of anthocyanins.

Plant stresses often reduce biomass yet increase anthocyanin concentration. A study was initiated to evaluate whether biomass directly correlated with anthocyanin concentration. *B. juncea* cultivar 'Crimson Red' was selected for evaluation due to foliar anthocyanin concentration less dependent upon environment variation when compared to *B. oleracea* genotypes. HPLC data indicated a negative correlation (-0.44) between biomass and anthocyanin concentration. A negative correlation (18 cell Styrofoam tray density, -0.25; 32 cell, -0.72; and 72 cell, -0.60) was also observed with the biomass and percentage of diacylated anthocyanins. This suggests that growing plants to a desired biomass prior to inducing stress would be the most pragmatic approach to increasing anthocyanin concentration.

This research has identified genotypes of interest for anthocyanin production and contributes to understanding the effects of post-harvest environmental stresses on anthocyanin quantity and type within Brassicaceae. Research into radish anthocyanin profiles would contribute further to a better understanding of anthocyanin diversity within Brassicaceae Future research using light-emitting diode (LED) lighting for controlled production of desirable genotypes could enable precise production of anthocyanin concentration and type. Pairing this with bioclimatic chambers and vertical farming would allow for increased environmental control of anthocyanin production capitalizing on controlled production of natural colors from plant tissues.

BIOGRAPHICAL SKETCH

Alexandra Bennett is from Eagle Bay, New York located in the Adirondack Park. She completed her undergraduate studies at Le Moyne College in biological education in 2012. Later, she began her graduate work to obtain a Master of Science in horticulture at Cornell University in 2013. Her studies were located at the New York State Agricultural Experiment Station (NYSAES) in Geneva, New York under the supervision of Dr. Phillip D. Griffiths.

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ANTHOCYANINS IN BRASSICACEAE

1.1 ECONOMIC IMPORTANCE OF BRASSICACEAE

1.1.1 Brassicaceae family

The Brassicaceae or crucifer family is a diverse group comprising several agriculturally important crop species. Species domesticated into vegetable crops include *Raphanus sativus* (radish), *Brassica oleracea* (cabbage, broccoli, cauliflower, Brussels sprouts, kale, and kohlrabi), *Brassica rapa* (turnip, pak choi, bok choi), *Brassica napus* (rutabaga), *Eruca sativus* (arugula) and *Brassica carinata* (Ethiopian mustard). The Brassica genus comprises important crops economically among the species: *Brassica oleracea, B. rapa, B. nigra, B. juncea, B. napus, and B. carinata* (Al-Shehbaz, 2001). The phenotypic diversity among and within these species has led to crops with harvestable and consumable stems, leaves, floral structures, hypocotyls, and seeds.

Described as cruciferous or ‘cross-bearing’, the flowers of the Brassicaceae family are uniform in structure, with four petals that resemble a cross, four free saccate sepals, and six stamens. Four of the stamens are more elongated and protrude just above the two short fused carpels that make up the lobed pistil. The fruit of this family form long capsules that are referred to as a siliques (Linnaeus, 1755). Economically important traits within this family are as diverse as the Brassicaceae family itself, which consists of 310 genera (Al-Shehbaz, 2001).

1.1.2 *Brassica oleracea* subspecies and morphotypes

Wild *B. oleracea* are typically biennial plants, having vegetative growth within the first year and flowering for reproduction after a period of cooling, or vernalization. This is an important trait for production of storage vegetables that has been selected in several domesticated species (Pelofske & Baggett, 1979).

Wild *Brassica* species are prevalent in the coastal cliff environments of the Mediterranean basin, north along the Atlantic coast of Europe, and into the United Kingdom (Gates, 1950). Wild species were domesticated into leafy non-heading greens by the Greeks and Romans, with further domestication along the Atlantic coast in Portugal, Spain, France and United Kingdom to diversify leafy kale (*B. oleracea* var. *acephala*) (Mitchell, 1976). The Celts also had an important role in both domestication and development of *Brassica* crops, which derive their name from the Celtic word "Bresic" (Dixon, 2007). During the domestication and cultivation of the *Brassica* species, there was extensive introgression from nearby wild types, which contributed to the large diversity within the genus (Quiros & Farnham, 2011). Early European movement of *Brassica oleracea* into China also led to the development of Chinese kale (kailaan) as a market class.

Non-leafy *Brassica* market classes were also developed as harvestable crops. Progenitors of broccoli (*B. oleracea* var. *italica*) and cauliflower (*B. oleracea* var. *botrytis*) began developing in the west Mediterranean basin at least by 300-500 BCE, with broccoli first described in Etruscan communities by 400–600 BCE (Quiros & Farnham, 2011). Evidence indicates cauliflower arose from broccoli, as cauliflower has greatly reduced fitness due to abortion of floral meristem, leading to low rates of flower

maturation (Gray, 1982). There was likely genetic introgression between the two morphotypes. There is evidence that broccoli was re-introduced in the 17th century, possibly as a mutation of cauliflower, based on the narrow genetic pool of broccoli compared to cauliflower. Brussels sprouts (*B. oleracea* var. gemmifera) were developed in Brussels, Belgium as early as the fourteenth century and well described by the seventeenth century (Gomez-Campo, 1999; John Gerard, 1636).

B. oleracea has the capacity for sporophytic self-incompatibility to ensure outcrossing, this trait is of agronomic importance as it can be utilized to prevent self-pollination of the seed parent during hybrid seed production (Nasrallah & Nasrallah, 1993).The self-incompatibility genes of *B. oleracea* can be dominant, co-dominant, mutual weakening, and intermediate (Dickson & Wallace, 1986).

1.1.3 Domesticated *Brassica* species and the triangle of U

There are six major *Brassica* species within the 'triangle of U' or 'Brassica triangle' first described by U Nagaharu (1935). The ancestral diploid species are made up of the AA genome *Brassica rapa* (2n=20), BB genome *B. nigra* (2n=16), and CC genome *B. oleracea* (2n=18) that all share a common ancestor. While genetically distinct, these three progenitor species have crossed naturally to produce amphidiploid offspring. This has resulted in species with the AABB genome of *B. juncea* (2n=36), the AACC genome *B. napus* (2n=38), and the BBCC genome *B. carinata* (2n=34) (Figure 1.1) (U, 1935).

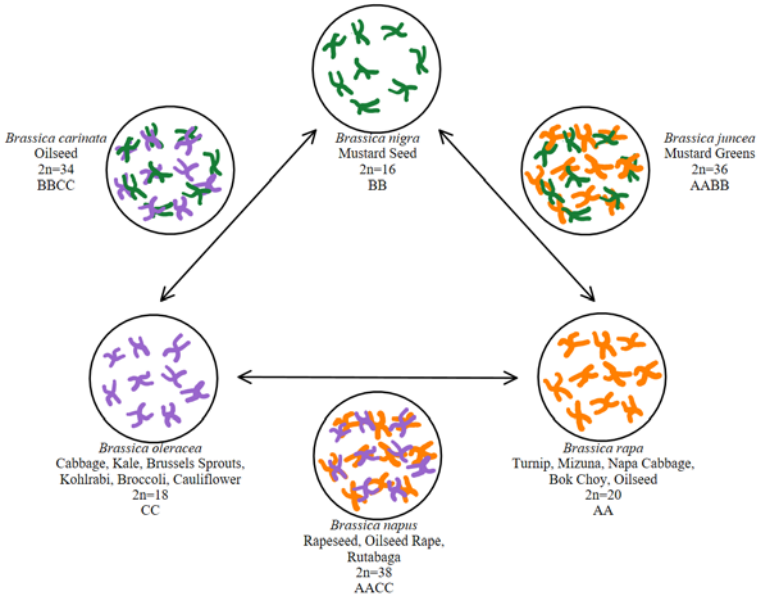


Figure 1.1 Triangle of U depicting interrelations among different *Brassica* species as originally described by U (1935)

The most economically important *B. oleracea* morphotypes, or market classes, that make up the majority of products consumed by humans are cabbage (var. *capitata*), Brussels sprouts (var. *gemmifera*), kale/collard (var. *acephala*), cauliflower (var. *botrytis*), broccoli (var. *italica*), and kohlrabi (var. *gongylodes*) (Figure 1.2). Each of these domesticated subspecies has been developed through direct and indirect selection of plants for different harvestable portions of the plant for consumption. All of these morphotypes readily cross with one another even though they have very different harvestable tissues (Kianian & Quiros, 1992).

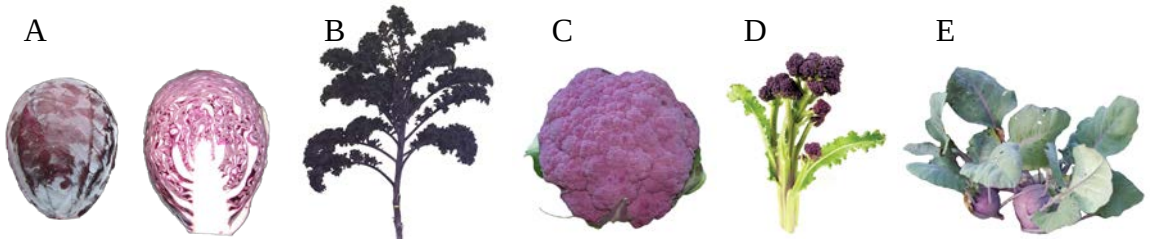


Figure 1.2 Pigmented high anthocyanin morphotypes of *Brassica oleracea* A) cabbage B) kale C) cauliflower D) purple sprouting broccoli, and E) kohlrabi

1.1.4 *Brassica* species and phytochemicals

Brassica vegetables, including kale and cabbage, are relatively low in oxalic acid, a toxic compound associated with plant defense against insects. Oxalic acid is high in other dark leafy greens vegetables including spinach, beet leaves, and parsley (Noonan & Savage, 1999). Low oxalic acid levels are desirable for human consumption as these compounds can precipitate in the body and cause kidney stones and kidney failure (Zarembski & Hodgkinson, 1966).

Brassica species are also rich in a wide variety of health promoting phytochemicals including glucosinolates, carotenoids and anthocyanins. Glucosinolates, which are secondary metabolites, are a group of organic anionic water-soluble compounds. They are found in a variety of plant species and are associated with plant defense mechanisms against herbivory from both specialist insect pests (including *Psylliodes chrysocephala* and *Pieris rapae*) and generalist pests (including pigeons and slugs)(Giamoustaris & Mithen, 1995, 1996; Martin & Müller, 2007). These compounds can be found in almost all plants within the Brassicaceae family (Fahey, Zalcmann, & Talalay, 2001). There are approximately 132 different known natural glucosinolates (Agerbirk & Olsen, 2012). These compounds are derived from a glucose and an amino acid, and contain sulfur and nitrogen with a central carbon atom (Figure 1.3). This central carbon atom may have a variety of side groups attached, which can affect biological activity (Prestera et al., 1996). Both genetics and environment have an effect on glucosinolate content in *B. oleracea* (Farnham, Wilson, Stephenson, & Fahey, 2004). Screening of *B. oleracea* germplasm has identified diversity in content of these

compounds (Stansell, Cory, Couillard, & Farnham, 2015). Glucosinolates have been under investigation for their role in human health.

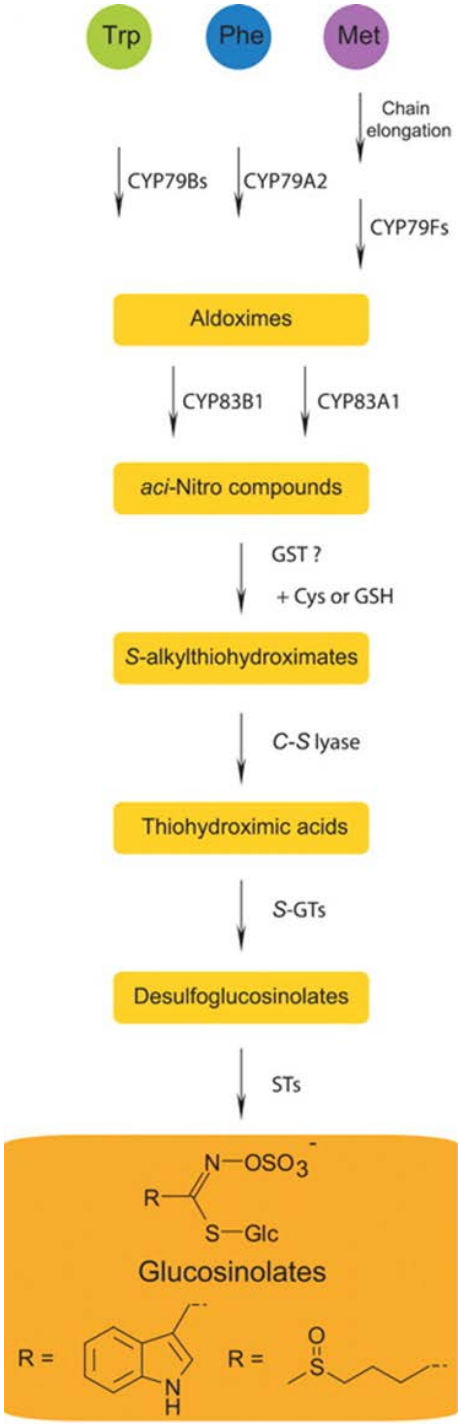


Figure 1.3 General glucosinolate pathways as depicted by Bednarek et al. (2009)

Carotenoids, or tetraterpenoids, are naturally occurring lipid soluble pigments located within the chloroplasts and chromoplasts of plants that increase the photosynthetic spectrum and protect chlorophyll from photo-damage (Beddard, Davidson, & Trethewey, 1977). These pigments may appear yellow, orange, or red, and absorb wavelengths ranging from 400-550 nm. These compounds are called tetraterpenoids as they are made from 8 isoprene molecules, yielding a 40 carbon molecule (Figure 1.4) (Charlton, Treharne, & Goodwin, 1967). There are over 600 known carotenoids, that are broken into two classes: xanthophylls (which contain oxygen) and carotenes (pure hydrocarbons)(T. Goodwin, 2012). Many of these carotenoids are antioxidants and have vitamin A activity, meaning they can serve as precursors to retinol, retinal, and/or retinoic acid (Goodman & Huang, 1965; Napoli & Race, 1988). Retinal is important for eye health, as it forms part of the retina (Fukuda & Stone, 1974). Several carotenoids, including lutein and zeaxanthin, act directly within the eyes by absorbing damaging wavelengths to protect the retina (Sommerburg et al., 1999). Retinoic acid is important to embryonic development (Niederreither, Subbarayan, Dollé, & Chambon, 1999). Most animals are unable to synthesize carotenoids or vitamin A on their own. They must consume carotenoid compounds and convert that to vitamin A or consume vitamin A directly.

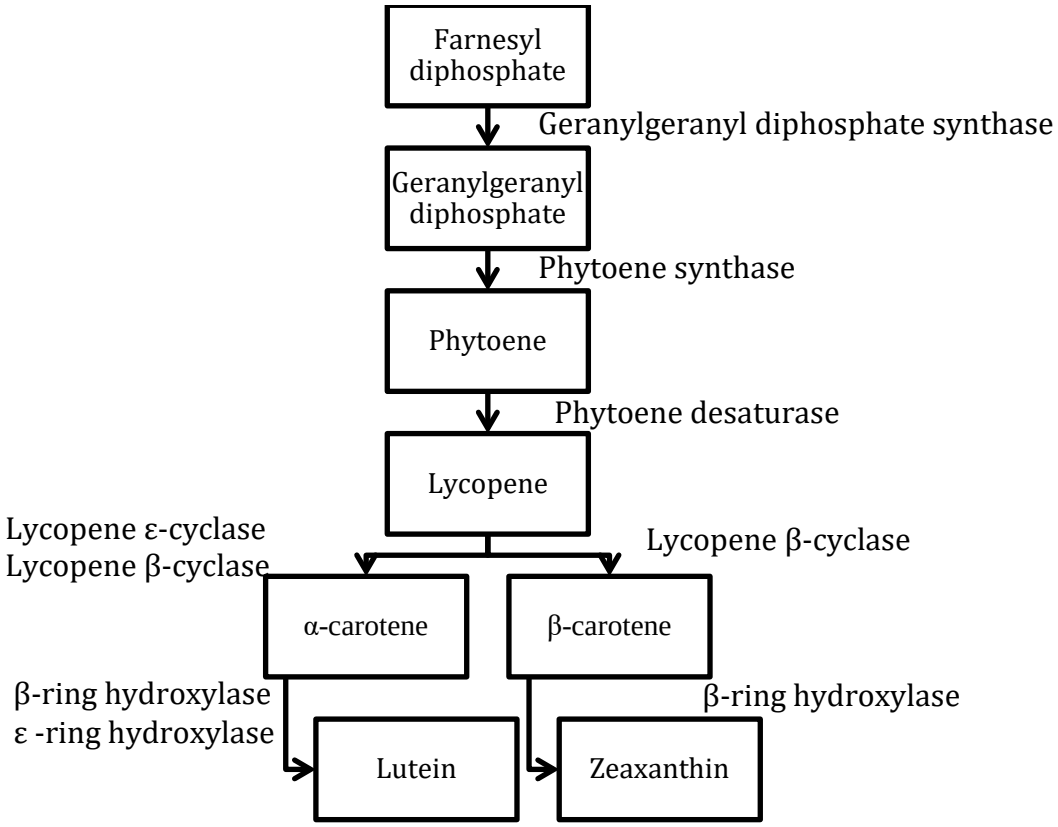


Figure 1.4 General carotenoid pathway

There are a variety of widely available dietary sources of carotenoids. Among these are leafy *Brassica* vegetables. Kale has been reported to have an abundance of lutein, with a range of 4.8 to 13.4 mg/100g fresh weight depending upon environment and genotype. β-carotene (vitamin A) has the ability to form two retinol molecules per molecule, and is the second most available carotenoid in kale, with levels ranging from 3.8 to 10.0 mg/100 g FW (Kopsell, Kopsell, Lefsrud, Curran-Celentano, & Dukach, 2004).

Anthocyanins are another class of naturally occurring phytochemicals abundant within pigmented *Brassica* genotypes. They belong to a parent class of polyphenolic molecules called flavonoids synthesized via the phenylpropanoid pathway. Anthocyanins

are derived from anthocyanidins through sugar addition (Cheng et al., 2014; Nakayama, Suzuki, & Nishino, 2003) and are present in photosynthetic tissues to protect cells from high-light damage by absorbing blue-green (Krol et al., 1995) and ultraviolet light (Giusti, Rodríguez-Saona, & Wrolstad, 1999). They also act as antioxidants with free radical scavenging capabilities, which can confer many health benefits to consumers (Andres-Lacueva et al., 2005; Kalt et al., 2008; Lee et al., 2005; Stull, Cash, Johnson, Champagne, & Cefalu, 2010; H. Wang et al., 1999; L.-S. Wang & Stoner, 2008; Youdim, McDonald, Kalt, & Joseph, 2002).

1.2 ANTHOCYANINS

1.2.1 Role as Phytochemicals

Anthocyanins are a diverse group of water-soluble pigments produced across many plant species and organs. ‘Anthocyanin’ is a word derived from Greek ‘anthos’ (flower) and ‘kyanos’ (blue). They are vacuolar pigments that may appear red, purple, or blue depending on the pH (Brouillard, Delaporte, & Dubois, 1978; T. W. Goodwin & Mercer, 1972). Anthocyanins occur in all tissues of higher plants, including leaves, stems, roots, flowers, and fruits. They are polyphenolics, share a basic flavilyum skeleton (Figure 1.6), and can have a wide variety of hydroxyl and methoxy attachments. Among the anthocyanidins, the most common forms are cyanidin, pelargonidin, delphinidin, malvidin, petunidin, and peonidin. Once glycosylated, anthocyanidins are considered anthocyanins. Anthocyanins have greater variability than anthocyanidins due to added variation in glycosylation and acylation (Gould, Davies, & Winefield, 2008; Holton & Cornish, 1995).

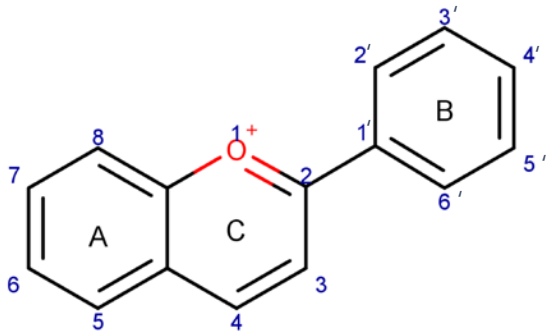


Figure 1.5 Flavylium skeleton molecule of anthocyanins and the two phenyl rings (A and B) and heterocyclic ring (C) that comprise all flavonoid based molecules

Anthocyanin compounds range in purpose, as they can act as pollinator attractants in floral tissue, signal herbivores in fruit ripening, dissuade other herbivores from consuming important leaf tissue, aid in sun protection in leaf tissue, and act as an electron sink/antioxidant in root and internal stem tissues (Gould et al., 2008; Hatier & Gould, 2009; Neill & Gould, 2003).

1.2.2 Biochemistry and genetics

There are two categories of genes responsible for the synthesis of anthocyanins: structural genes and regulatory genes. The structural genes are important for producing enzymes involved in the biochemical reactions that create the aglycone anthocyanidin structure. These pathways, which produce a variety of anthocyanidins, are well known and conserved across plant species. Anthocyanins originally derive from the shikimate pathway, which funnels into the phenylpropanoid pathway. The first committed stop to anthocyanin production is initiated by chalcone synthase (CHS) to produce chalcone (Figure 1.6) (Dooner, Robbins, & Jorgensen, 1991).

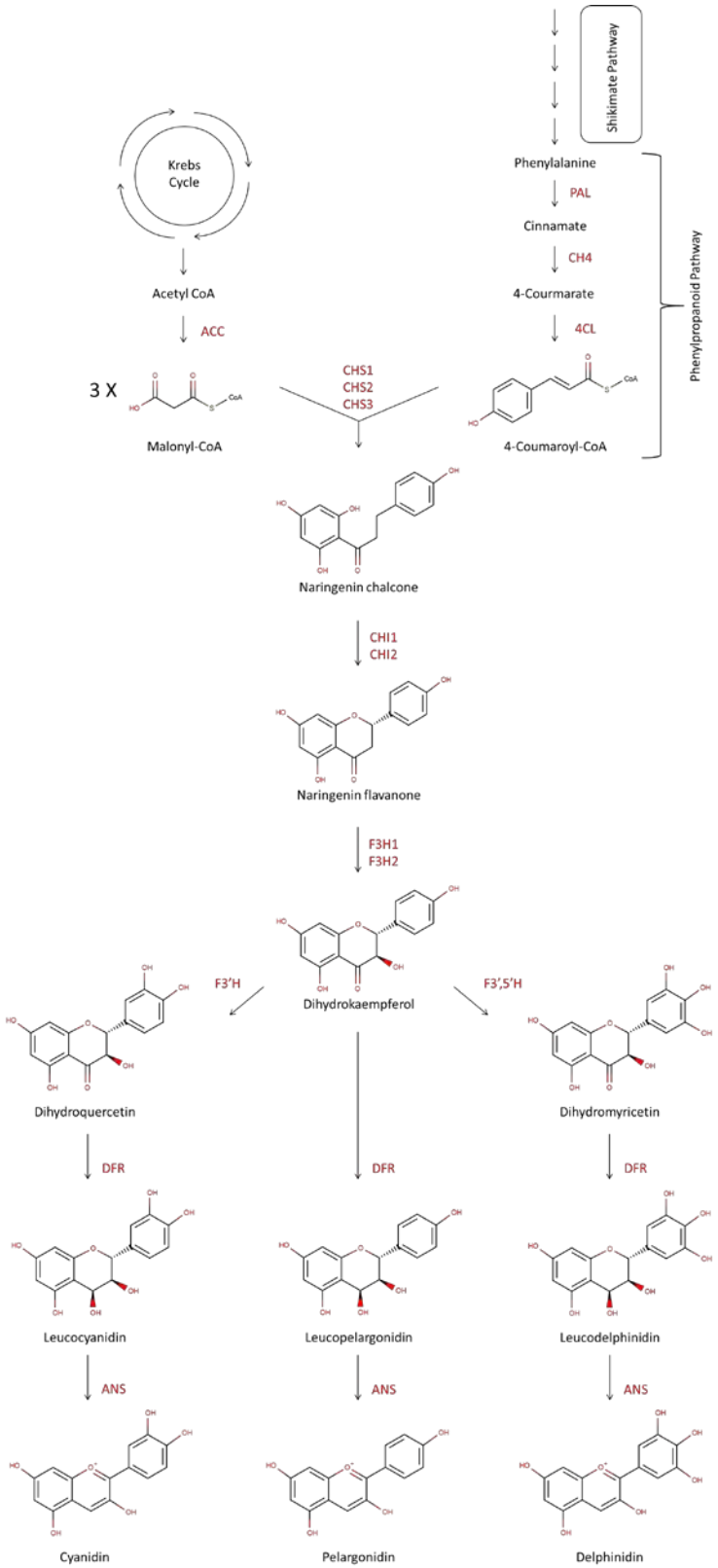


Figure 1.6 General pathway for 3 major anthocyanidin molecules: cyanidin, pelargonidin, and delphinidin.

Further structural genes, including glycosyltransferase/glycotransferase (GT) and anthocyanin acyltransferase (AAT), have also been documented (Fukuchi-Mizutani et al., 2003; Milkowski, Baumert, Schmidt, Nehlin, & Strack, 2004; Nakayama et al., 2003). The GT genes are responsible for transferring activated carbohydrate moieties from donor UDP molecules to anthocyanidins to produce glycosylated anthocyanins. Glycosylation most often occurs at the C-3 position of the flavylium ion skeleton, with the C-5 position being an alternate location for attachment (Andersen & Jordheim, 2001). Anthocyanins with glycosyl groups (i.e., glucose, galactose, xylose, glucuronic acid, and arabinose) attached can be further glycosylated and/or acylated. Anthocyanin GTs, while specific in which sugars they donate can also act as generalists when it comes to sugar acceptors (i.e. flavonoids) (Ford, Boss, & Høj, 1998; Fukuchi-Mizutani et al., 2003).

AAT proteins work similarly by transferring acyl groups from a donor molecule to attached glycosyl moieties on anthocyanins. There are two types of acyl groups that can further separate anthocyanins: aliphatic types (malonyl, acetyl, succinyl, malyl, oxalyl, and tartaryl groups) and aromatic types (which are usually hydroxycinnamoyl) (p-coumaryl, caffeyl, feruryl, and sinapyl groups) (Figure 1.7). Both groups typically attach at the 6-positions of glucosy groups. Two major types of enzymes catalzye the attachment of acyl groups to anthcyanins: the serine carboxypeptidase-like (SCPL) group (acyl donors are acyl-activated sugars) (Milkowski et al., 2004) and the BAHD superfamily of enzymes (acyl donor is acyl coenzyme A thioesters (acyl-CoA)) (Nakayama et al., 2003). Intramolecular association and copigmentation begins to occur

as the molecule grows larger (Malien-Aubert, Dangles, & Amiot, 2000) from addiction of glycosyl and acyl groups, leading to higher molecular stability and quality.

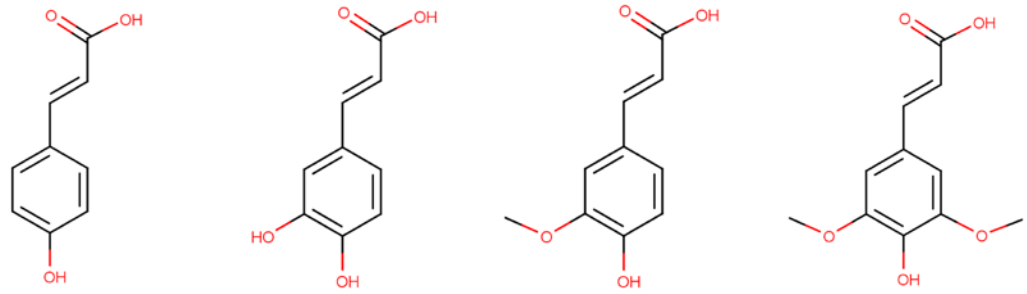


Figure 1.7 Common molecules that acylate anthocyanins: sinnapic acid, caffeic acid, ferulic acid, and para-coumaric acid.

Regulatory genes control the quantity of each anthocyanin produced from the structural pathway. Anthocyanins are primarily controlled at the transcriptional level by MYB proteins, which form a complex with MYC proteins (a type of basic Helix-Loop-Helix (bHLH) protein) and a WD40 protein (Jaakola, 2013). The MYB proteins are more important and specific to anthocyanin synthesis than the rest of the complex (Jaakola, 2013; Ma, Pooler, & Griesbach, 2008). The transcription factors can promote anthocyanin production by serving as activators, or can prevent anthocyanin production by blocking RNA polymerase through repression. MYBL2, for example, is a repressor in *Arabidopsis thaliana* that is typically expressed in older tissue and inhibits production of anthocyanins (Dubos et al., 2008; Matsui, Umemura, & Ohme-Takagi, 2008). Higher expression of PAP1 (a MYB protein) through an ELA1 mutation caused a two fold increase of anthocyanins when compared to wild types of *Arabidopsis thaliana* (Choi et al., 2009). The diversity of these regulatory proteins is complex, with several different

proteins involved in the regulation within a single species. While many of the genes for anthocyanin regulation have been reported, there are still many transcription genes and complexes yet to be discovered.

Structural and regulatory genes must be associated with certain genes in order to be expressed within different organs and developmental stages of a plant. A plant may produce different classes of anthocyanins (or no anthocyanins at all) in different organs depending upon association of promoter genes with different structural genes. Environmental factors also interplay with the production and expression of these transcriptional regulatory complexes. (Figure 1.8) (Jaakola, 2013). Different ratios and concentrations of anthocyanins may also be produced depending upon the association of the promoter with the regulatory gene. This knowledge has been used to generate transgenic plants, which are able to express anthocyanins in specific plant tissues, including ones where anthocyanins are typically not found. For example, a purple-fleshed tomato with high anthocyanin content has been produced through transformation with anthocyanin genes from snapdragon (Butelli et al., 2008)

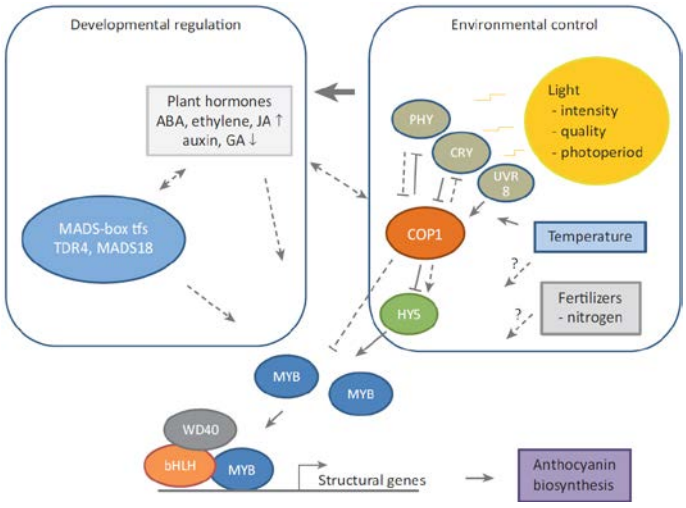


Figure 1.8 developmental and environmental effects on anthocyanin regulatory genes (Jaakola, 2013)

1.2.3 Presence in plants

The role of anthocyanins within plants has been well documented. They are generated for a variety of reasons depending on the tissue in which they are produced (Gould et al., 2008). Anthocyanins that are present in floral tissues are known to attract pollinators, as anthocyanins absorb and reflect certain UVA (315-400 nm) and UVB (280-315 nm) wavelengths that are visible to certain insect and avian pollinators (Grayer & Harborne, 1994). Anthocyanin content coincides with flower fertility, and these pigments may begin to degrade post pollination (Böhm, Boeing, Hempel, Raab, & Kroke, 1998; Farzad, Griesbach, & Weiss, 2002; Weiss et al., 1995). Anthocyanins also signal fruit ripening to attract herbivores to fruit for seed dispersal (Willson & Whelan, 1990).

Light stress on anthocyanin accumulation is well documented, and often induces production in leaf and stem tissue (Chalker-Scott, 1999). This indicates that the compounds act as a visible light and UV light protectant. Anthocyanins are typically red to purple in these tissues, as they reflect visible purple and red while absorbing green and yellow light wavelengths that cannot be easily used by chloroplasts. This reduces damage to plant tissues, serving as an alternative or complimentary process to photoinhibition. Anthocyanins, and more specifically acylated anthocyanins, also absorb UV-B light (Figure 1.9), reducing the penetration of UV-B light into tissue and lessening damage to DNA (Hatier & Gould, 2009).

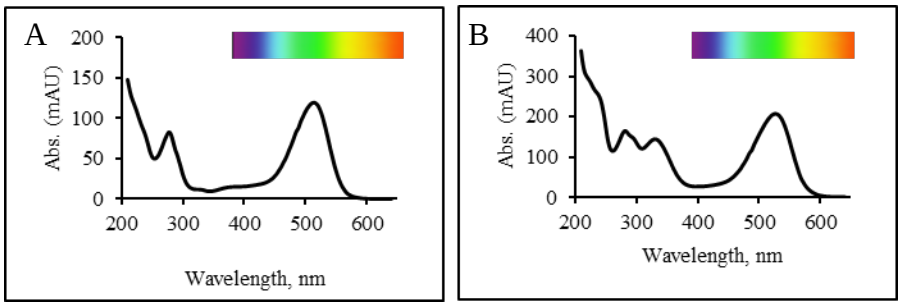


Figure 1.9 Absorbance spectrum of A) non-acylated cyanidin based anthocyanins and B) acylated cyanidin based anthocyanins

Production of anthocyanins can occur in interior tissues and non-epithelial or outer mesophyll tissues. Nutrient deficiency and water stress can induce anthocyanin production, indicating that anthocyanins may be beneficial for other reasons (Hatier & Gould, 2009). Anthocyanins are controversially considered to be free-radical scavengers and metal-chelators (Gould, McKelvie, & Markham, 2002). While anthocyanins have been shown to have strong antioxidant properties *in vitro,* with the ability to be electron or proton donors, there is some controversy on their *in vivo* bioavailability and effectiveness. In most species these compounds are constrained to the vacuole, although some studies have shown *in vivo* effectiveness of anthocyanins within vegetative systems (Agati, Meyer, Matteini, & Cerovic, 2007). The effectiveness of anthocyanins as antioxidants appears to vary among plant species. In sugar maples (*Acer saccharum*), as the concentration of anthocyanins increased with leaf senescence, antioxidant capabilities also increased (Neill & Gould, 2003).

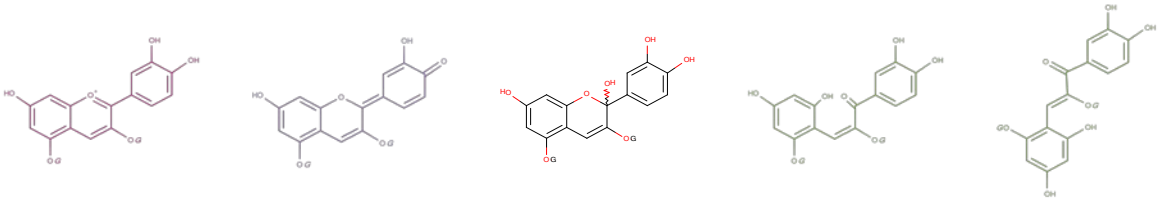
1.2.4 Role in health

While plants produce anthocyanins for their own benefit, some of their beneficial properties, such as antioxidant capabilities, can be transferred over to an animal system

upon consumption (Gabrielska, Oszmiański, Komorowska, & Langner, 2014). The breakdown, distribution, and bioavailability within animal systems are not clear, studies have found anthocyanins within liver, eye, and brain tissues after consumption of a diet high in anthocyanins (Kalt et al., 2008). This indicates that anthocyanins are not completely broken down and are distributed within animals' systems well enough to cross the blood-brain barrier. Other benefits including neuro-protective, visual improvement, lowered weight gain, increased insulin sensitivity, cardiovascular protection, cancer prevention, and anti-ulcer properties have also been explored in recent years with similar controversy (Magistretti, Conti, & Cristoni, 1988; Tsuda, 2012; L.-S. Wang & Stoner, 2008).

1.2.5 Use of anthocyanins as a natural color

Anthocyanins can be used as a natural colorant due to the different colors they produce. Depending upon pH, anthocyanins can be perceived as 'red,' 'purple,' 'blue,' 'green,' or 'yellow' (Brouillard, 1983; Mazza & Brouillard, 1987). The base molecule of anthocyanins is reported to be a flavylium ion (AH^+), as this is the most stable form for anthocyanins. However, the base molecule may shift into a quinoidal base (A), a carbinol pseudobase (B), or deteriorate into a *cis*-chalcone (Cc) that isomerizes into a trans-chalcone (Ct). This is important, as all of these different forms have different absorption spectra. The AH+ forms appear 'red,' the A forms appear 'blue', and the B forms appear colorless, and both the Cc and Ct forms appear light 'yellow.' (Figure 1.10) (Andersen & Jordheim, 2001; Markakis, 1982)



AH^- A B Cc Ct

Figure 1.10 Possible structures of the anthocyanin base molecule. AH^- shows the flavylium ion form, 'A' depicts the quinoidal from, 'B' the carbinol pseudobase, 'Cc' the cis-chalcone, and 'Ct' displays the trans-chalcone.

It is well documented that anthocyanins are affected by pH (Brouillard, 1983; Mazza & Brouillard, 1987). At pH 1-3, anthocyanins are predominantly in the AH^+, or most stable, form. From pH 4-5 they are predominantly in the B form for non-acylated anthocyanins. At pH 4-6, depending upon temperature, deprotonation of hydroxyl groups attached to the flavylium cation begins to occur, producing different A forms (Brouillard et al., 1978). If three or more hydroxyls are present on the original AH^- structure, further deprotonation can occur to create an anionic quinoidal. These anion forms are most stable, and dominate at neutral pH ranges of 7-8 where they appear 'blue' in color. When a solution becomes alkaline, the anionic A forms can even covert into an unstable dianion (A^{2-}). The B form, at room temperature, tends to be 43 times more abundant than any A structure in nonacylated anthocyanins. In acylated anthocyanins the equilibrium distributions are different, and the A forms are in higher concentration than the B forms, resulting in solutions that do not appear colorless at any pH. In acidic environments, the B form is unable to become ionized into the B^-. For anthocyanidins such as cyanidin that have a 3-subsituent in the C ring, it is harder for the conversion from B to B^- to occur, preventing eventual breakdown of anthocyanins into the C form. As pH increases to 8,

the B^- form becomes more stable and is then able to convert into the C^- form. Below pH 8, B^- typically converts back to the B form. The C^- form attracts free hydrogens and rapidly converts to the C form. (Markakis, 1982). The mixture of these different forms can cause a single species of anthocyanin to produce colors 'red,' 'purple,' 'blue,' 'green,' and 'yellow' depending upon pH (Figure 1.11).

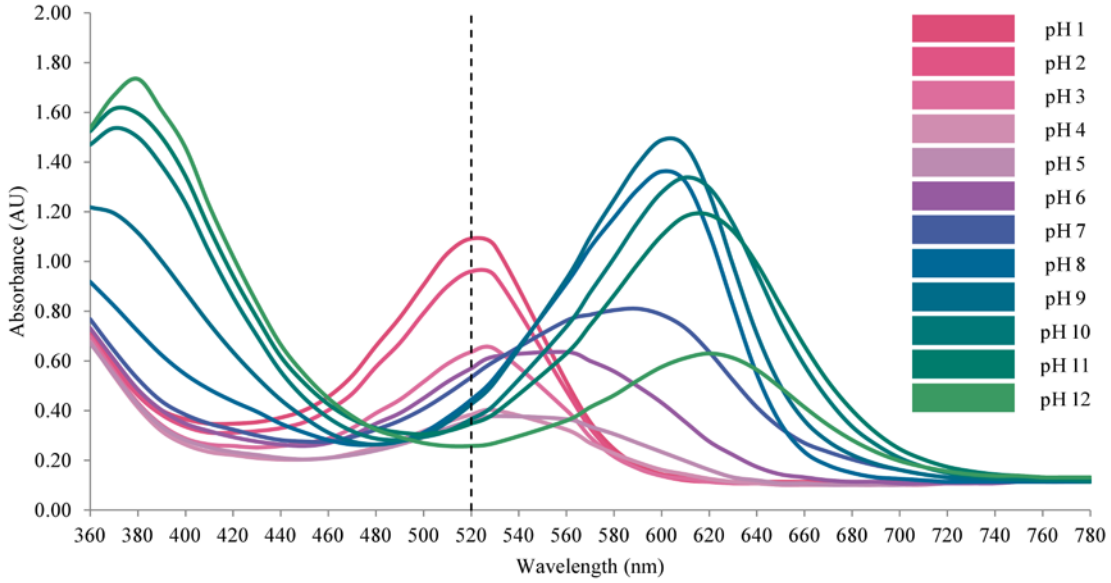


Figure 1.11 Absorption spectrum of anthocyanins from cabbage extract at pH values ranging from 1 to 12

1.3 ANTHOCYANINS IN CRUCIFER SPECIES

B. oleracea mostly contains the agylcone cyanidin as a base molecule. While the gene(s) to produce pelargonidin are present in *B. oleracea*, these gene(s) are either not expressed or the genes for the cyanidin pathway are highly active. Only cyanidin is produced in sufficient quantities to affect the phenotype in an important way. In a study on *B. juncea* red mustard types, only cyanidin based species were found; however,

research on anthocyanins in *B. juncea* is currently limited, with only one well defined study documented (Lin, Sun, Chen, & Harnly, 2011a). Among the related Brassicaceae species, there is a wider variation of aglycone base molecules. For example, *R. sativus* has genes for pelargonidin that are expressed in higher quantities (Giusti et al., 1998).

Anthocyanins are most commonly found in the leaf tissues of *Brassica* species. While anthocyanins can be produced in epithelial and outer mesophyll cells, they are not typically produced within the inner mesophyll tissue. They can act as a natural sun protectant for cells involved in photosynthesis, resulting in their presence in the epithelial tissues of the stems (Gould, Dudle, & Neufeld, 2010).

Anthocyanins can also be found in floral petal tissue (Tatsuzawa et al., 2008) in some crucifer market classes including radish. *B. oleracea* has yellow or white flowers but can have floral meristems with high concentrations of anthocyanins in cauliflower curds (Chiu et al., 2010; Chiu & Li, 2012). Broccoli can also have purple sprouting or normal morphotype purple forms (Porter, 2012; Rodríguez-Hernández, Moreno, Carvajal, García-Viguera, & Martínez-Ballesta, 2012). Plant breeding has been a major factor driving the high accumulation of anthocyanins in these genotypes.

R. sativus can produce anthocyanins within root tissue and internally within the hypocotyl (Tatsuzawa et al., 2008). The reason for this is not clear, as it does not act as a photo protectant or as an attractant. This could be due to artificial selection of the anthocyanins, or potentially this tissue could act as an electron sink (Hatier & Gould, 2009).

1.4 GERMPLASM AND BIODIVERSITY IN COLLECTIONS

Germplasm (‘germ’ = life, ‘plasm’ = mold) refers to living material that can be used to regenerate complete new living organisms. Germplasm repositories maintain genetic diversity of crop plants, and can act as a source of new traits for introgression into crop species. Germplasm repositories are an important resource for plant research and breeding, as they maintain tissues and seed that can be used to generate new whole plants through seeding, cloning, or tissue culture. These tissues are stored and maintained in seed banks, as cryogenically frozen sections of tissue, or as trees growing in nurseries. These tissues and seeds can be well-developed into elite inbred, hybrid, or genetically stable open-pollinated plants, or even wild genotypes.

Germplasm curators manage specific collections of plant species. The primary *B. oleracea* germplasm collections are held by: the United States Department of Agriculture (USDA) in Geneva NY, USA; the Horticulture Research International (HRI), University of Warwick, in Coventry, England; the Institute of Plant Genetics and Crop Plant Research (Leibniz-Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK)) in Gatersleben, Germany; and the Centre for Genetic Resources (CGN) in Wageningen, Netherlands.

The National Plant Germplasm System (NPGS) is a collaborative effort between private organizations and the United States government designed to preserve genetic diversity in horticultural, agricultural and economically important plant species. Plant accessions/germplasm enter the system through donations from other germplasm collections or governments and are given Plant Introduction number (PI number) identifiers. The USDA works with the NPGS by maintaining, evaluating, and distributing

their accessions at several local and regional repositories across the United States. One such regional repository is North East 9 (NE-9) located in Geneva, New York where the *B. oleracea* and *R. sativus* collections are maintained. The oilseed and mustard species including *B. rapa, B. nigra, B. napus, B. carinata and B. juncea* are held at the North Central 6 (NC-6) regional repository in Ames, Iowa.

HRI was previously a horticultural research and development institution located in Wellesbourne, UK responsible for maintaining and developing germplasm. It was primarily used as a tool for industry in the UK. It arose in May 1990 from the AFRC Institute of Horticultural Research Stations. As of November 2009, Warwick University merged this with the University's Department of Biological Sciences into a new School of Life Sciences. The Institute of Plant Genetics and Crop Plant Research (Leibniz-Institut für Pflanzengenetik und Kulturpflanzenforschung) IPK, Gatersleben, is a nonprofit German institution that provides services, data, and information on crop genetics and molecular biology alongside maintaining their germplasm repository. IPK is part of the Leibniz Association. This association consists of 88 independent research institutions that perform knowledge-driven experiments with an emphasis on applied sciences and research. Wageningen is a university in the Netherlands with a core focus in life sciences and agriculture. It has a strong history in agriculture as it was originally established in 1876 as the Rijkslandbouwschool (National Agricultural College) in Wageningen. It houses the Center for Genetic Resources (CGN) that contains their germplasm repository.

Germplasm has been assessed and screened within *B. oleracea* genotypes to assess lutein, β-carotene and chlorophyll concentrations (Kopsell et al., 2004), carotenoid, tocopherol and ascorbate content (Kurilich et al., 1999) and diversity of

collard glucoraphanin and other glucosinolates (Stansell et al., 2015). Anthocyanin content is a trait that has been screened within several different species. Grains such as barley and corn (Harakotr, Suriharn, Scott, & Lertrat, 2014; M.-J. Kim et al., 2007), grape, blueberry, and cranberry fruits (Liang et al., 2008) (Stevenson & Scalzo, 2012; Vorsa, Polashock, Cunningham, & Roderick, 2003), and vegetables such as sweet potato (Todd, Truong, Pecota, & Yencho, 2015) have been assessed for anthocyanin content. Anthocyanins have also been investigated in *B. oleracea* cultivars (Ahmadiani, Robbins, Collins, & Giusti, 2014; Arapitsas, Sjöberg, & Turner, 2008); however, no one has examined anthocyanin concentrations, content, and diversity within *B. oleracea* among the germplasm repositories where the complete diversity of anthocyanins among *B. oleracea* genotypes is unclear.

1.5 MASS SPECTROMETRY AND HIGH PERFORMACE LIQUID CHROMTRAGRAPHY

Quantification of anthocyanins can be achieved using high performance liquid chromatography (HPLC) methods (Wu & Prior, 2005). An HPLC pumps solvents, also referred to as mobile phases, into a sample loop to which the analyte is added using an injector. At this point, the analyte is run through a column where its chemical components are separated based upon their polarity or hydophobicity. These separated components are then sent to the detector individually where they are measured. Many HPLC systems rely on a UV-visible diode array detector (DAD), which is able to read the concentration of each compound based upon their absorbance. This analysis is non-

destructive and results in peaks at different retention times representing each molecule within the analyte on a chromatogram (Wrolstad, 2005).

Speciation of anthocyanins is more accurate using liquid chromatography mass spectrometry (LC-MS) (Arapitsas et al., 2008). Identifications measured with HPLC alone are more tentative than with LC-MS, as retention time is the only way to identify a compound using HPLC, and this shifts between protocols and sample runs due to minor fluctuations in the HPLC environment or sample preparation. MS is able to determine what an analyte is, based upon its mass-to-charge ratio. After being separated by the HPLC, analytes in the sample are sent individually to the MS system. There, the analyte is initially ionized, using an electrospray ionization (ESI) technique that nebulizes the analyte by spraying it through a thin needle with a high voltage charge applied to it. This causes the analyte to evaporate into an ionized gas. The sample is then sent to a mass analyzer (quadrupole), which selects and separates the compounds based upon their mass-to-charge ratio. Mass spectrometers can be single quadrupole or triple quadrupole (also known as tandem mass spectrometry). Within a single quadrupole system, samples are sent directly from the first mass analyzer to the detector. Multiple mass analyzers can be used in tandem where two quadrupole mass analyzers flank a radio frequency-only (RF) quadrupole. This is called tandem mass spectrometry. The Q1, or first mass analyzer and quadrupole, selects out analytes based upon m/Z and sends it to the next quadrupole. This allows for fragmentation to occur within q2 RF, or second, quadrupole. The Q3, or third, quadrupole then separates these fragments by m/Z. After being separated, the fragmented and ionized samples are then sent to a detector(Burlingame, Boyd, & Gaskell, 1996; Gross, 2011; Hoffmann, 2007).

CHAPTER 2

ANTHOCYANIN LOCALIZATION IN PIGMENTED BRASSICACEAE GENOTYPES

2.1 INTRODUCTION

2.1.1 Anthocyanin localization and diversity

2.1.1.1 Anthocyanin accumulation in different plant organs and species

Anthocyanins are polyphenolic phytochemical pigments that accumulate within plant tissues for a wide variety of reasons. Anthocyanins accumulate across many species and plant structures such as leaves, stems, flowers, fruits, seeds, and grains (Tatsuzawa et al., 2008; Wu & Prior, 2005). Many of these species and structures are not useable for industrial scale production as they don't produce large quantities of anthocyanins. For example, many berries produce anthocyanins to signal fruit ripeness (S. Y. Wang & Lin, 2000; Willson & Whelan, 1990). These tissue types can produce concentrations of anthocyanins much higher than in other tissues (S. Y. Wang & Lin, 2000; Welch, Wu, & Simon, 2008) but do not comprise most of the plant biomass and so overall yields of anthocyanins are low.

Plant biomass is comprised primarily of stem and foliar tissues. Within these tissues, anthocyanins act as an aid in preventing and dealing with over-excitation of photosystems, and are associated with cells performing photosynthesis (Gould et al., 2010). It would be of interest to assess the ability of different species, morphotypes, and genotypes to produce anthocyanins within internal cells, or investigate those types that

have a high density of cells that produce high levels of anthocyanins within vegetative tissues.

Brassicaceae was selected as a family of interest for its ability to produce anthocyanins both within root tissue and internally in *Raphanus sativus* (radish) (Tatsuzawa et al., 2008), and for the ability of *Brassica oleracea* morphotypes cabbage and kale to produce large quantities of pigmented leaves (Ahmadiani et al., 2014; Arapitsas et al., 2008; Socquet-Juglard et al., 2016).

2.1.1.2 Cytological assessment of cellular anthocyanin accumulation

On a cellular level, anthocyanins are generated on the cytosolic side of endoplasmic reticulum. They accumulate within the cytoplasm in structures that resemble vesicles called prevacuolar compartments (PVCs). They then travel to the tonoplast and on to the central vacuole, where they aggregate in anthocyanic vacuolar inclusions (AVI) (Markham et al., 2000; Nozzolillo & Ishikura, 1988; Pecket & Small, 1980; Small & Pecket, 1982).

While general anthocyanin distribution is visible to the naked eye, these microscopic structure are not. Cytology paired with microscopy enables the visual study of cells in terms of structure, function and chemistry. With these methods it is possible to determine the localization of anthocyanin-containing vacuoles within plant tissues. This is a quick way to assess types of cells accumulating anthocyanins, density of cells accumulating anthocyanins, and intensity of vacuoles. Such assessment can indirectly and loosely give information on anthocyanin concentration possibilities within different species, morphotypes, and genotypes.

2.1.2 Influence of pH on anthocyanin absorption spectra

Anthocyanins are a highly diverse group of phytochemicals, with cyanidin, pelargonidin, delphinidin and malvidin being the most common forms (Wu et al., 2006). All differ in the number and location of hydroxyl and methoxy groups attached, and have different absorption spectra (Francis & Markakis, 1989). *B. oleracea* morphotypes and *B. juncea* (red mustard) mostly contain the aglycone cyanidin as a base molecule (Chiu & Li, 2012; Lin et al., 2011a; Socquet-Juglard et al., 2016). Brassicaceae species, however, have a wider variety of aglycone base molecules. For example, *R. sativus* has genes for cyanidin and pelargonidin (M. Monica Giusti et al., 1998).

Further molecular alteration through glycosylation and acylation can influence pigmentation. The change in structure can intrinsically change absorption spectra, as intramolecular copigmentation begins to occur as the molecule grows larger (Malien-Aubert et al., 2000). The copigmentation causes a hyperchromic and bathochromic shift. A hyperchromic shift causes an increase in the absorbance (optical density) of a material. As anthocyanins begin intermolecular copigmentation, self-association causes the pigments to increase their absorbance and color intensity. A bathochromic shift results in a shift in the wavelengths actually being absorbed (Parisa, Reza, Elham, & Rashid, 2007). Bathochromic shifts within cyanidin-type anthocyanins cause a shift 5 to 20 nm higher, causing anthocyanins that may normally appear red to appear purple or blue (Boulton, 2001)

The base molecule of anthocyanins is reported as a flavylium ion (AH^+), as this is the most stable form for anthocyanins. Anthocyanins are reported to be found in this form

at low pH of 1-3. Depending upon the change in pH and the glycosyl and acyl attached to the anthocyanin, the base molecule may shift into a quinoidal base (A), a carbinol pseudobase (B), or deteriorate into a *cis*-chalcone (Cc) that isomerizes into a *trans*-chalcone (Ct). These different forms have different absorption spectra, causing the AH^+ forms to appear 'red,' the A form to appear 'blue', and the B form to be colorless. Both Cc and Ct forms are a light 'yellow'(Andersen & Jordheim, 2001; Markakis, 1982). The mixture of these different forms can cause a single species of anthocyanin to produce colors 'red,' 'purple,' 'blue,' 'green,' and 'yellow' depending upon pH.

The first major objectives of this study were to assess species, morphotypes, and genotypes within Brassicaceae for their ability to produce large quantities of highly concentrated anthocyanins. The second was to assess anthocyanin pigment quality, as indicated by desired pigment as stability from acylation, produced by these plant types.

2.2 METHODS AND MATERIALS

2.2.1 Plant materials and growth conditions

Seed of pigmented genotypes of *B. oleracea* var. *capitata* were obtained from two germplasm repositories: the United States Department of Agriculture (USDA) collection in Geneva, NY, USA (NE-9), and the Horticultural Research International (HRI) University of Warwick, Coventry in the United Kingdom (UK) (Appendix 3). Eight red cabbage cultivars and two green cabbage cultivars were used as controls (Table 2.1). *B. oleracea* morphotype diversity samples were also obtained (Table 2.2). Internally pigmented red radish breeding lines developed from watermelon radish types 'Red Meat' (Johnny's Selected Seeds, Maine, USA) and daikon types were also assessed. These were

used to generate interspecific seed with kohrabi cultivar ‘White Kossak’ (Johnny's Selected Seeds, Maine, USA) through bud pollination.

Table 2.1 Green and red *Brassica oleracea* var. *capitata* cultivars grown in Geneva, NY in 2013 for cytology and pH analysis.

Cultivar	Source	Cultivar Group
Bartolo	Bejo, Netherlands	Green
Bronco	Bejo, Netherlands	Green
Futurima	Bejo, Netherlands	Red
Red Express	Johnny's Selected Seeds, Maine, USA	Red
Red Jewel Cabbage	Stokes Seed, Ontario, Canada	Red
Rio Grande Red	Orsetti Seed Company, California, USA	Red
Rondale Red	Stokes Seed, Ontario, Canada	Red
Ruby's Perfection	Johnny's Selected Seeds, Maine, USA	Red
Super Red 80	Sakata Seed, Yokohama, Japan	Red
Super Red 90	Sakata Seed, Yokohama, Japan	Red

Table 2.2 *Brassica oleracea* genotype diversity samples grown along red cabbage types in Geneva, NY in 2013 for cytology and pH analysis.

Accession/ Cultivar	Source	Morphology	Cultivar Group
FalStaff	Park Seed, South Carolina, USA	Brussels Sprouts	Red
G 29761	(USDA) Geneva, NY, USA	Brussels Sprouts	Red
G 30871	(USDA) Geneva, NY, USA	Brussels Sprouts	Green
HRI 001912	(HRI) Warwick, Coventry,UK	Brussels Sprouts	Red
HRI 001945	(HRI) Warwick, Coventry,UK	Brussels Sprouts	Red
HRI 002147	(HRI) Warwick, Coventry,UK	Brussels Sprouts	Red
Red Ball	Gurney's Seed and Nursery Co., Indiana, USA	Brussels Sprouts	Red
Redbor	Bejo, Netherlands	Kale	Red

Seeds of each genotype were sown in 72-cell styrofoam trays (Speedling, Sun City, Fla.) filled with 'Cornell Mix' (Boodley and Sheldrake, 1982), with one seed per cell, on June 6th of 2013. They were grown in a controlled environment greenhouse with 70°C/65°C night/day temperatures and a 14 hour photoperiod. After five weeks, materials were transplanted to a field in Geneva, NY onto raised plastic beds with 180 cm centers and 45 cm spacing. They were irrigated and fertigated as needed through a drip irrigation system. Interspecific plants were not transplanted to fields and instead

maintained within the greenhouse. Plant tissue was harvested after 90 days and cabbage types were stored in cold chambers at 8 °C until processing for evaluation; other types were processed immediately.

Juvenile types were additionally assessed within greenhouse conditions for HPLC analysis. Three red cabbage (‘Futurima’, ‘G30902’ (USDA, Geneva, NY, USA), and ‘Red Express’), three kale (‘Redbor’, ‘Red Russian’ (Johnny's Selected Seeds, Maine, USA), and ‘Scarlet’ (Johnny's Selected Seeds, Maine, USA)), three Brussels’ sprout (‘Rubine’ (Park Seed, South Carolina, USA), ‘Red Ball’, and ‘Falstaff’) and three mustard (‘Red Muzuna’ (Kitazawa Seed Co, California, USA), ‘Scarlet Frills’ (Johnny's Selected Seeds, Maine, USA), and ‘Red Giant’ (Park Seed, South Carolina, USA)) genotypes were selected for evaluation of Brassica market class. These were seeded in triplicate with two repetitions in 72-cell styrofoam trays in 'Cornell Mix' (Boodley and Sheldrake, 1982) with one seed per cell (Speedling, Sun City, Fla.). They were grown in a controlled environment greenhouse with 70°C/65°C night/day temperatures and a 14 hour photoperiod. Samples were harvested five weeks post seeding.

2.2.2 Cytology

2.2.2.1 Sectioning

Leaves were unwrapped from the head of the cabbage and a leaf interior to the head was selected for cytological evaluation. For kale genotypes samples were taken from the mid-section of the plant. Samples were sectioned using double edge stainless chrome steel razor blades (Personna, Israel). A rectangular section 3mm long by 1.5 mm wide, between major veins two thirds of the way from the leaf midrib to the leaf edge,

was selected and sectioned. From this section, smaller cross sections were made transversely from the end of the section, beginning the cut at 100 µm thick and gradually tapering to 10 µm. Sections were made at 25 µm. This generated a section of the sample approximately one to two cells thick for imaging. An optical works 422 radial microtome (Erma, Tokyo, Japan) with Personna blade clamped with a microtome blade holder (Spencer, Buffalo, NY, USA) was used for later samples.

Cryotome sections 1mm x 3mm were placed in a Tissue-Tek standard 25 mm x 20 mm x 5 mm cryomold (Sakura Americas, Torrance, CA, USA) containing Tissue-Tek optimal cutting temperature (OCT) compound (Sakura Americas, Torrance, CA, USA) compound. 2-Methylbutane (isopentane) (Sigma Aldrich, St. Louis, MO, USA) was cooled in liquid nitrogen. OCT imbedded samples were placed in 2-Methylbutane for approximately two minutes until completely frozen. Sectioning was set to 10 µm on a cryotome (Thermo Scientific, Waltham, MA, USA) in order to obtain thickness of one to two cells.

Pigmented root sections were taken from interspecific plants. The plant was displaced from the soil and roots were washed using deionized water. Roots were sectioned both along and across the root using double edge stainless chrome steel razor blades (Personna, Israel).

2.2.2.2 Microscopy

After sectioning, samples were immediately placed on a slide (Fisher Scientific, Waltham, MA, USA) in deionized water and a coverslip (Fisher Scientific, Waltham, MA, USA) was positioned. Images were taken within 1 minute of sectioning in order to

avoid pigment bleeding. For cryotome imaging, samples were placed on a slide (Fisher Scientific, Waltham, MA, USA) and kept frozen within cryotome to avoid destruction of cells and vacuoles. A coverslip was placed and images were taken within 30 seconds to avoid thawing and cellular breakdown.

Slides were placed under a BX60 microscope (Olympus, Shinjuku, Tokyo, Japan). Images were taken at 10 X magnification and 20 X magnification using a MicroPublisher camera (QImaging Scientific, Surrey, BC V3S 6K3, Canada). Sections were positioned relative to the camera with xylem on the uppermost side of the image.

Root samples from interspecific plants were magnified using a M5 stereo microscope (Wild, Heerbrugg, Switzerland) to 5 X magnification in the absence of cover slip and slide. Images were again taken with a MicroPublisher camera.

2.2.3 pH gradient analysis

2.2.3.1 Buffer Solutions

pH 1 and 2 buffers were made up of 50 mM KCL (3.728g/L) (Fisher Scientific, Waltham, MA, USA). pH 3, 4, and 5 buffers contained 200 mM sodium citrate (J.T. Baker, Center Valley, PA, USA) (58.82g/L). pH 6 and 7 solutions were created using 200 mM sodium phosphate (23.992 g/L) (Sigma Aldrich, St. Louis, MO, USA). Buffer solutions for pH 8 and 9 contained 200 mM Tris (24.228 g/L) (OmniPur, Billerica, MA, USA). pH 1-9 buffers were adjusted with 1M HCl (LabChem Inc, Zelienople, PA, USA) and an Orion 3 Star pH Benchtop Meter (Thermo Scientific, Waltham, MA,USA). pH 10, 11, and 12 buffers used 200 mM N-cyclohexyl-3-aminopropanesulfonic acid (CAPS)

(44.262 g/L) (J.T. Baker, Center Valley, PA, USA) and were adjusted with 1M NaOH (Fisher Scientific, Waltham, MA, USA).

2.2.3.2. Sample Preparation

After harvest, samples were divided into representative sections. Cabbage samples were divided into four quarters representing the whole cabbage head. The core of the samples were then removed. Radish samples were sectioned by taking representative wedges from the hypocotyl. Multiple Brussels sprouts axillary buds (>10) were selected for representation of the harvestable plant product. Kale was sampled by removing multiple (> 7) representative healthy leaves from both new and old growth.

After sampling, specimens were frozen at -20°C and lyophilized over a 48 hr period using an MX53 Magnum series freeze-drying unit (Millrock Technology, Kingston, NY, USA). Each sample was then ground to a fine powder using a mortar and pestle and stored at 24°C while being shielded from light until further analysis. 5 mL of buffer solutions were transferred to a 10 mL beaker. This was repeated for pH buffer solutions 1-12. Fifty mg of dried and ground samples were added to each beaker. Samples were given a minute to allow for maximum extraction of sample.

2.2.3.3 Digital Imaging

Samples images were made with a white background for contrast and in the absence of natural sunlight. Samples were aligned from pH 1 to 12 within the 5 mL beakers used for extraction. Images were taken on normal camera setting (COOLPIX S5300, Nikon, Tokyo, Japan) within 5 minutes of sample extraction in buffer solutions.

2.2.3.4 RGB analysis

RGB values were assessed using GNU Image Manipulation Program (GMP) software (open source) on images taken. RGB values were measured at pH values 1-12. These mean values for pigmented *B. oleracea*, control *B. oleracea*, and *R. sativus* types were calculated.

2.2.4 High performance liquid chromatography (HPLC)

2.2.4.1 Reagents and Standards

Ultrapure water used in chemical extraction and processing was generated by filtering deionized water through a 0.22 µm filter in a Milli-Q integral water purification system (Millipore Corporation, Bedford, MA, USA). Acids and organic solvents were HPLC grade and purchased from Fisher Scientific (Pittsburgh, PA, USA). cyanidin-3,5-diglucoside reference standard was obtained from Extrasynthese (Genay, France).

2.2.4.2 Sample preparation

Bulked samples were frozen at -20°C post-harvest and over a 48 hour period of time were lyophilized with a MX53 Magnum series freeze-drying unit (Millrock Technology, Kingston, NY, USA). Dry samples were ground with a mortar and pestle until particles were fine enough to fit through a 40mm mesh screen. Samples were placed in opaque 50mL centrifuge tubes and stored at room temperature.

Samples were weighed into 50 mg portions, placed into 15 ml Eppendorf centrifuge tubes (Eppendorf, Hauppauge, NY, USA), and triple extracted in 1ml 0.01M

hydrochloric acid (HCl) in methanol (MeOH). After each extraction, the samples were sonicated for 1 minute, centrifuged for 5 minutes at 4°C using and 3000 x g with an Eppendorf 5810R centrifuge (Eppendorf, NA, Hauppauge, NY, USA). Each of the three supernatant aliquot portions were combined in a glass Pyrex test tube (Corning Incorporated, Corning, NY, U.S.), placed in a 35°C water bath, and dried with a stream of nitrogen gas generated by a Parker Balston model N2-04 nitrogen generator (Parker Hannifin Corp, Haverhill, MA, USA). Dried samples were resuspended in 1 ml of 0.01M HCl in water, filtered throught a 0.22 µm polyethersulfone (PES) membrane (Krackeler Scientific, Inc., Albany, NY, USA) and injected into an Agilent 1260 Infinity series high performance liquid chromatography (HPLC) system (Agilent Technologies, Santa Clara, CA, US).

2.2.4.3 Anthocyanin speciation via high performance liquid chromatography (HPLC)

The Agilent 1260 Infinity series HPLC system was fitted with a Kinetex® core-shell pentafluorophenyl (PFP) column (100 mm x 2.1 mm, 2.6 µm diameter particle size, 100 Å pore size) proceeded by an inline Krudkatcher® column filter (Phenomenex, Torrance, CA, USA). Five µl of sample was injected into the HPLC with a flow rate of 0.2 ml/min and heated to 35°C. Mobile phase A consisted of 0.5% (v/v) phosphoric acid (H_3PO_4) in water and mobile phase B consisted of 0.5% (v/v) H_3PO_4 in MeOH. Mobile phase concentrations and run time are shown in Table 4.1. After each run was completed, a ten minute post run of 85% A and 15% B ensured a return to chemical equilibrium. Samples were assessed by a diode array detector, and a Max-Light cartridge flow cell (1 µl volume and 10 mm path) was set to monitor at 520 nm using a 630 nm reference.

Table 2.3 Concentration of mobile phase A (0.5% (v/v) phosphoric acid (H_3PO_4) in water) and mobile phase B (0.5% (v/v) H_3PO_4 in methanol (MeOH)) shown in percentages at each time point during a sample run of 40 minutes

Time (Minutes)	% mobile phase A	% mobile phase B
0	85%	15%
10	70%	30%
15	65%	35%
38	50%	50%
40	85%	15%

A standard curve ranging from 0.01 ppm to 250 ppm was made in triplicate (r^2 < 0.9999) using a cyanidin-3,5-diglucoside standard that resulted in a percent relative standard deviation (%RSD) of less than 0.5. The computer system was monitored and data analyzed using Agilent Chemstation software version B.04.03 and service pack 2 with spectral software module (Agilent Technologies, Santa Clara, CA, US). Speciation between nonacylated, monoacylated and diacylated anthocyanins was originally tentatively judged based on elution time, absorbance spectrum, and previously published literature (Arapitsas et al., 2008; Harborne, 1958; Lin et al., 2011a).

2.2.4 Statistical Analysis

Statistical analysis was undertaken using Excel software (Microsoft, New Mexico, USA). Means were calculated to produce figures for pH analysis. JMP (SAS Institute, Cary, NC, USA) was used to calculate Student's t test and generate connected letter reports.

2.3 RESULTS AND DISCUSSION

2.3.1 Cytology

Anthocyanin accumulation differed between green and red cabbage types. The green cabbage control cultivar ‘Bartolo’ had little or no anthocyanins, as shown through lack of visible color with microscopy methods (Figure 2.1.A). Red cabbage accessions and cultivars generated anthocyanins in outer mesophyll cells, of leaves both external and internal. External leaves were exposed to natural light in the field while the internal leaves were not. This indicates relatively low environmental control by light of anthocyanin production of inner leaves. Interior leaves of red cabbage genotypes also lacked chlorophyll pigmentation.

Anthocyanins did accumulate in the epidermal cells of some cultivars and accessions, such as in ‘Futuruima’ (Figure 2.1.B). However, accumulation did not always occur in epidermal cells, as was the case with ‘PI 275004’ (Figure 2.1.B). All red cabbage cultivars and accessions had anthocyanin accumulation in the outer mesophyll cells, penetrated two to four cells deep. No anthocyanin production within interior mesophyll cells of *B. oleracea* samples was observed. Anthocyanins aggregated within vacuoles as observed in prior studies (Pourcel et al., 2010).

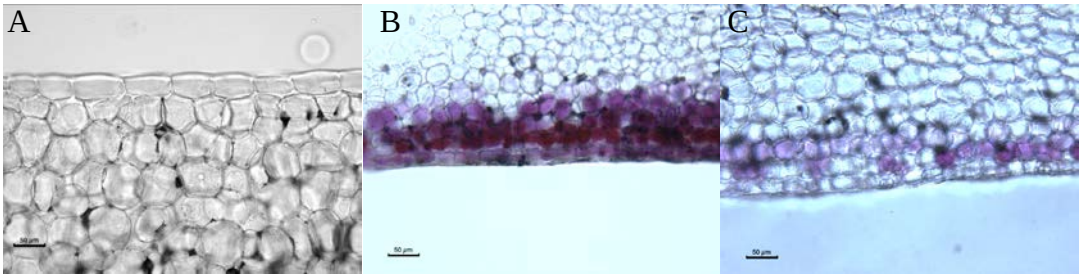


Figure 2.1 Anthocyanin accumulation in epidermal and outer mesophyll cells in A) ‘Bartolo’, B) ‘Futurima’, and C) ‘PI 275004’ cabbage genotypes at 20 X magnification.

Kale cultivar ‘Redbor’ had strong anthocyanin pigmentation in the adaxial side of leaves exposed to sunlight and lower accumulation on the abaxial side of the leaves (Figure 2.2.B). This suggests light regulation of anthocyanin production in these tissues. Interference from chlorophyll made in situ anthocyanin visual quantification unreliable, as the underlying chlorophyll in cells located below the adaxial epidermis causes a darkening of the pigments when visualized in the open field that is not necessarily indicative of high leaf anthocyanin concentrations. Because of this, visual selection in the field is not the most effective way of selecting high anthocyanin genotypes. Cabbage had thicker leaves than kale (Figure 2.2.).

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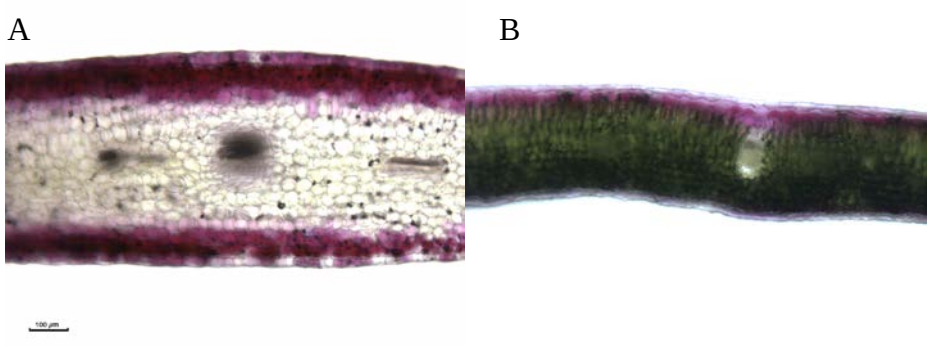


Figure 2.2 A) Thickness and anthocyanin localization in internal leaf of ‘Futurima’ cabbage with an absence of chlorophyll compared to B) anthocyanin and chlorophyll accumulation in a ‘Redbor’ kale leaf in upper mesophyll and epidermal tissue compared to lower epidermal and mesophyll at 10X magnification.

R. sativus has the ability to produce anthocyanins both internally within hypocotyl and in root tissues (data not shown). This trait was heritable and segregating in interspecific breeding lines developed and used for evaluation (Figure 2.4). This ability to produce pigmented callus tissue under tissue culture methods correlated with interior pigmented plants.

Figure 2.3 Internal anthocyanin pigmentation of interspecific plants generated from crossing internally pigmented radish (*Raphanus sativus*) breeding lines (developed from a cross of daikon radish types and red-hearted radish genotypes) with 'White Kossak' kohlrabi. A) A section was made along the root with B) other sections being made across the root. Images were taken at 5X magnification.

Radish was found to be good candidate for high anthocyanin production because it can produce anthocyanins internally within the hypocotyl, particularly in genotypes where the anthocyanin quantity is high in the absence of environmental stress. Radish genotypes are also able to produce anthocyanins within root tissue, a trait not observed in the cabbage and kale genotypes studied. Expression in multiple tissues can produce higher amounts of anthocyanin for a given biomass.

Cabbage and kale have different harvestable plant tissues resulting in challenges unique to each morphotype. Kale has thinner leaves than cabbage, and consequently have more exposure to light and the environment. They also have high levels of chlorophyll underlying the leaf-based anthocyanins, a feature only observed in the outer wrapper leaves of cabbage.

While thinner leaves in kale could lead to a higher percentage of cells that produce anthocyanins within foliar tissues, these leaves are more exposed to and influenced by changes in light and temperature. Cabbage produces a dense number of internal leaves in which anthocyanin accumulation was high in many genotypes and appeared to be less influenced by light. Figure 2.3 illustrates the low anthocyanin accumulation on the abaxial side of the leaf, while leaves internal to the cabbage head can

produce anthocyanins in high concentrations on both sides of the leaf that have little or no chlorophyll. As the internal leaves make up the majority of the cabbage biomass, this makes red cabbage amenable to both storage and high production of anthocyanins. High levels of phenotypic diversity were also observed among red cabbage genotypes. Genotypes such as 'Futurima' produced anthocyanins deeper in mesophyll tissue and at higher vacuolar concentrations when compared to kale genotypes such as 'Redbor.' Genotypic diversity among red kale genotypes is very limited. Investigations focused on 'Redbor' as the primary red kale cultivar grown as it is one of the most deeply pigmented kale genotypes. Further investigation or genetic improvement through breeding could identify genotypes that have higher cellular concentrations and deeper tissue penetration within the kale market class.

2.3.2 pH gradient pigmentation of different species and cultivar groups

Results from the anthocyanin extraction and pH imaging revealed three patterns when comparing green cabbage, pigmented *B. oleracea* and *R. sativus*. Green cabbage cultivar controls 'Bartolo' and 'Bronco' had, on average, equal red (R), green (G) and blue (B) values from pH 1-7, indicating equal reflection of the three colors. Their extracts appeared clear against a white background (Figure 2.4 A). From pH 7 to 11, B values dropped from 229 to 176. R value decreased from 231 to 211 from pH 7 to 11. G values remained above the R a B values. This was visualized as a slight 'green-yellow' pigment at high pH values (pH 9-12) (Figure 2.4). The pigmentation at higher values (pH 9-12) could be due to production of chalcone molecules from non-anthocyanin flavonoid molecules (Dall'Acqua, Miolo, Innocenti, & Caffieri, 2012; D.-O. Kim, Padilla-Zakour,

& Griffiths, 2004). Red cabbage genotypes that are high in other flavonoid molecules could have interference and artificially stronger or ‘green-yellow’ skewed pigmentation at pH values 8 to 11 that cannot be attributed to anthocyanin degradation into chalcone alone. The sample preparation used did not result in pure anthocyanin. Using better extraction methods could avoid these complications.

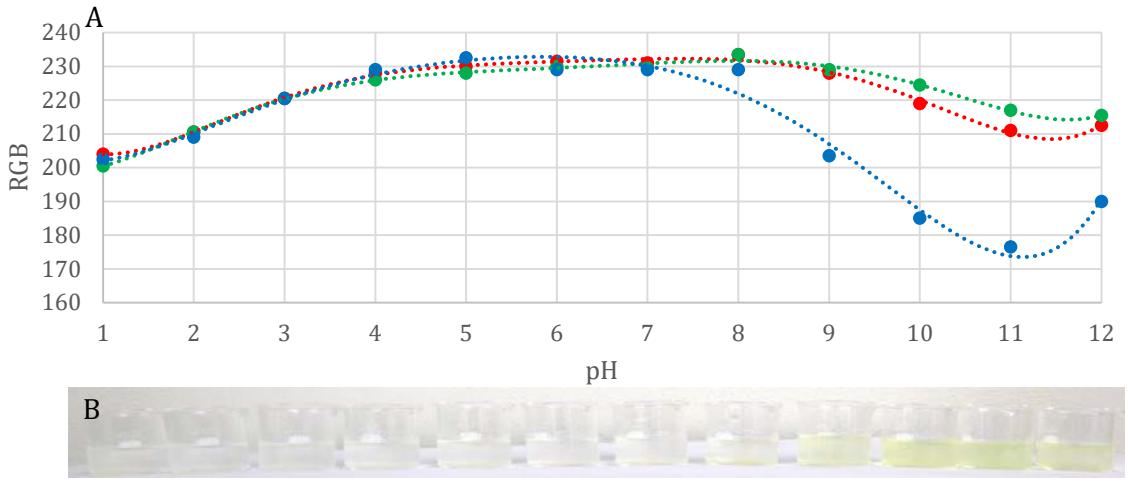


Figure 2.4 A) average RGB values of control cabbage cultivar extractions at pH values 1-12 with B) showing extraction image for control cultivar ‘Bartolo’ in buffer solutions ranging from pH 1-12.

All *B. oleracea* samples had consistent RGB profiles across the pH range. The mean R values remained at 205 from pH 1-3 (Figure 2.5 A). These high R values could be attributed to anthocyanins in the flavylium ion form. R values decreased to 30 between pH values 3 through 8 as the flavylium ions begin to convert into quinoidal forms. Between pH 8 to 11, R values had a mean of 29. At pH 12, R values increased to 46. G values remained the lowest, keeping levels at or below 60 until pH 12 where G values peak at 109. At pH 2 and pH 10, B values had a mean of 60. These B values peaked at

130 in buffer solutions of pH 5. B values were higher than R and G values from pH 6 to 11. Coupled with relatively high G values and low R values, the extracts have a strong 'cyan' pigmentation in the pH 8-10 range (Figure 2.5). This can be attributed to the fact that *B. oleracea* produces predominantly cyanidin based upon previous literature (Arapitsas et al., 2008).

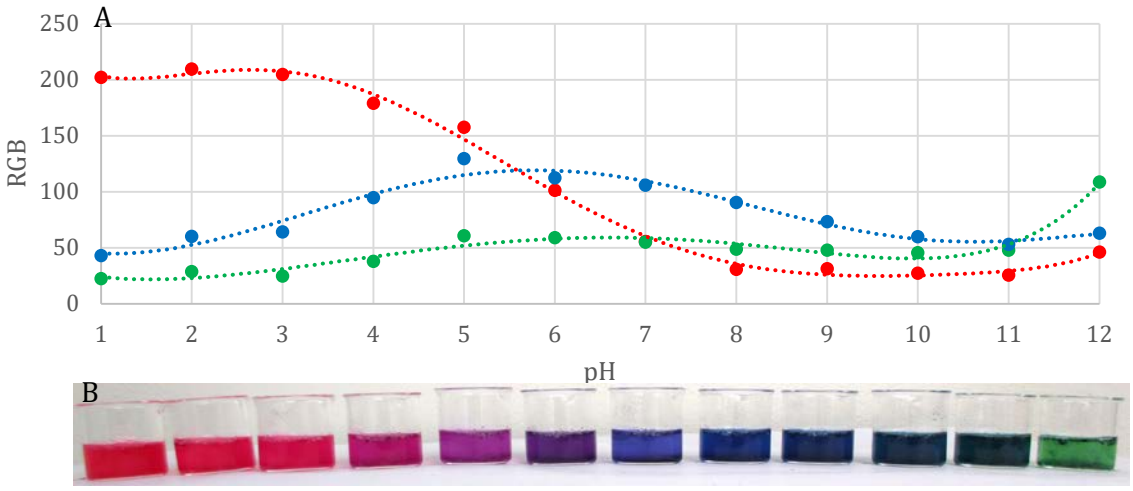


Figure 2.5 A) mean RGB values of red cabbage cultivars and accession extractions at pH values 1-12 with B) showing extraction image for red cabbage control check 'Futurima' in buffer solutions ranging from pH 1-12.

Prior studies have found that anthocyanins are in the chalcone form in the pH 4 to 5 range. These chalcone molecules are colorless or light yellow. While there is some increase in G values beginning around pH 4, samples did not become more translucent around pH 4 as reported in prior studies (Markakis, 1982). This could be attributed to the larger and more stable molecular structure of anthocyanins commonly found in red cabbage. Stability is derived from glycosylation and acylation in anthocyanins. High levels of acylation cause intramolecular self-association, which could prevent anthocyanins from entering the unstable chalcone form as easily at acidic pH values

(Malien-Aubert et al., 2000). Instead, more flavylium cation forms divert directly into the quinoidal form. This could explain why intramolecular copigmentation causes bathochromic and hyperchromic shifts (Parisa et al., 2007).

Radish genotypes had similar trends in B and G values compared to cabbage genotypes with lower values at all pH levels (Figure 2.6 A). B values peaked at pH 5 at 100. G values were highest (73) in pH 5 buffer solutions. With lower B and G values, R values were proportionally higher when compared to cabbage types, averaging 207 at pH 1-5. After pH 5, R values dropped to 21 at pH 11. The relatively high R values and low G and B values resulted in extracts that looked more ‘orange’ at low pH values and ‘brown’ at higher pH values (Figure 2.6). This is likely due to higher levels of pelargonidin found in radish, as pelargonidin is a more ‘orange’ anthocyanin (Francis & Markakis, 1989).

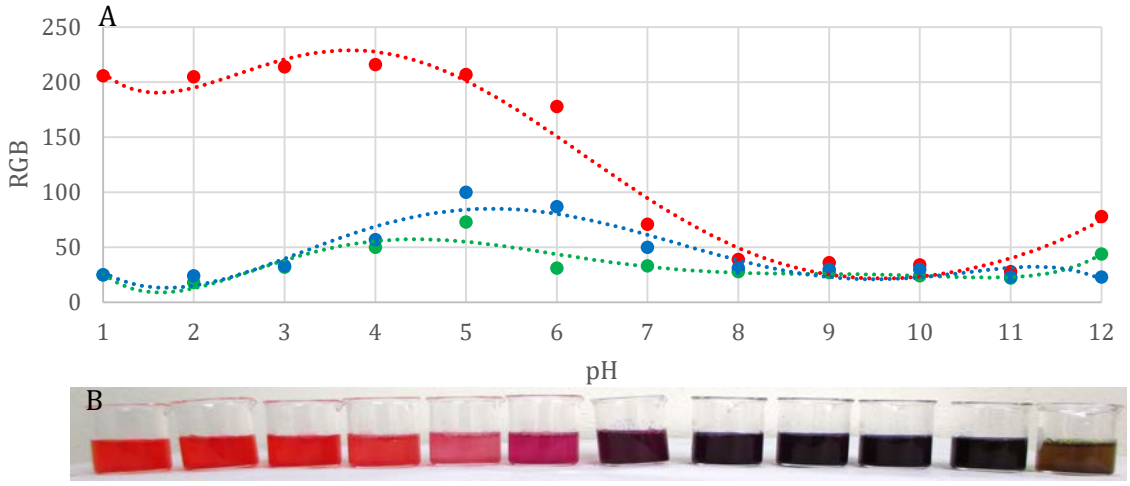


Figure 2.6 A) average RGB values of radish extractions at pH values 1-12 with B) showing extraction image for internally colored breeding line ‘11RD18’ in buffer solutions ranging from pH 1-12.

Improved extraction procedures need to be implemented in future studies to ensure less interference from contaminating particles and molecules such as chalcone.

Methodologies such as colorimetry could also be utilized to distinguish differences between harvestable types and tissues and the exact bathochromic shifts occurring. High performance liquid chromatography (HPLC) and mass spectrometry (MS) more accurately measure anthocyanin quantities, but localization in plant tissues is important for studying both the environmental stresses and targeting genotypes for crop improvement. Evaluating localization and color variation using cytology and pH provide an excellent first step for determining appropriate genotype groups and understanding the likely interactions and issues that may be encountered working with harvested plants from a physiological and chemical standpoint.

2.3.3 Identification of pigments

Nineteen different anthocyanins were identified for *Brassica* genotypes and analyzed for this study based upon absorption spectra analysis paired with previous literature (Figure 2.7 and Table 2.4). Peaks were classified as either nonacylated, monoacylated or diacylated.

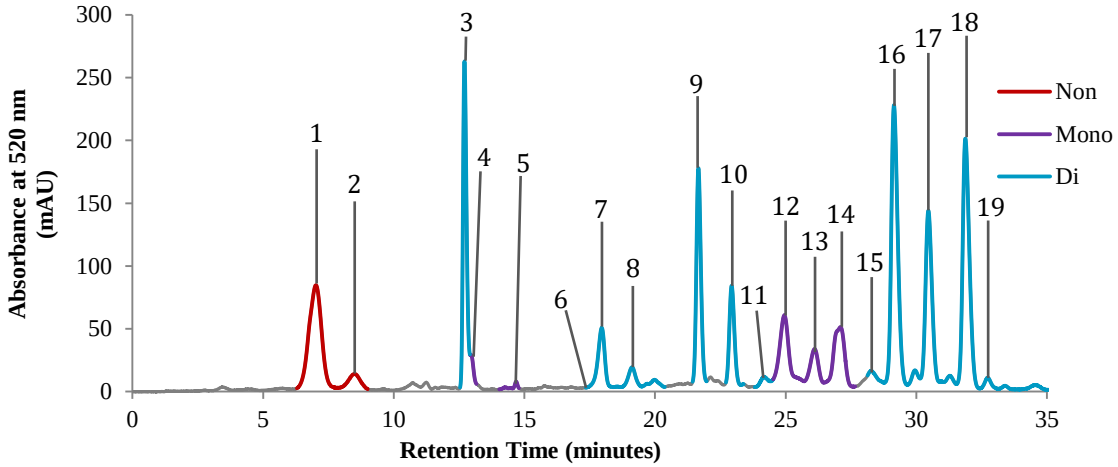


Figure 2.7 Chromatogram indicating the position and retention time of the 19 peaks identified through LCMS methods.

Table 2.4 The 19 peaks identified through LCMS. Rentention time, maximum absorption (Λmax (nm)), mass to charge ratio ([M]+ (m/z)) and assignment of non (N), mono (M), or Di-acylated (D) given along with tennative chemical identification.

Peak	RT	Λmax (nm)	[M]+ (m/z)	Assignment	Tentative Identification
1	6.358	514	733	N	Cyanidin-3-diglucoside-5-glucoside
2	7.952	514	611	N	Cyanidin 3,5-diglucoside
3	12.417	528	1141	D	Cyanidin-3-(glycopyranosyl-sinapoyl)-diglucoside-5-glucoside
4	12.669	528	979	M	Cyanidin-3-(sinapoyl)-diglucoside-5-glucoside
5	13.831	520	979	M	Cyanidin-3-(sinapoyl)-diglucoside-5-glucoside
6	17.433	522	1081	D	Cyanidin-3-(caffeoyl)(p-coumaroyl)-diglucoside-5-glucoside
7	18.566	524	1111	D	Cyanidin-3-(glycopyranosyl-feruloyl)-diglucoside-5-glucoside
8	19.363	524	1141	D	Cyanidin-3-(glycopyranosyl-sinapoyl)-diglucoside-5-glucoside
9	21.135	534	1287	D	Cyanidin-3-(feruloyl)(feruloyl)-triglucoside-5-glucoside
10	22.393	534	1317	D	Cyanidin-3-(sinapoyl)(feruloyl)-triglucoside-5-glucoside
11	23.712	532	1347	D	Cyanidin-3-(sinapoyl)(sinapoyl)-triglucoside-5-glucoside
12	24.078	522	919	M	Cyanidin-3-(p-coumaroyl)-diglucoside-5-glucoside
13	25.259	522	949	M	Cyanidin-3-(feruloyl)-diglucoside-5-glucoside
14	26.292	524	979	M	Cyanidin-3-(sinapoyl)-diglucoside-5-glucoside
15	27.505	522	1111	D	Cyanidin-3-(glycopyranosyl-feruloyl)-diglucoside-5-glucoside
16	28.487	534	1125	D	Cyanidin-3-(sinapoyl)(p-coumaroyl)-diglucoside-5-glucoside
17	29.793	536	1155	D	Cyanidin-3-(sinapoyl)(feruloyl)-diglucoside-5-glucoside
18	31.19	536	1185	D	Cyanidin-3-(sinapoyl)(sinapoyl)-diglucoside-5-glucoside
19	31.761	534	1155	D	Cyanidin-3-(sinapoyl)(feruloyl)-diglucoside-5-glucoside

2.3.3 Chromatographic differences among market classes

Of the market classes and species observed, kale produced the highest anthocyanins on average, at 3.49 mg/g dry weight in cyanidin-3,5-diglucoside equivalents (Cy-3,5-DiG Equiv.) compared to cabbage (2.25 mg/g Cy-3,5-DiG Equiv.), Brussels sprouts (2.86 mg/g Cy-3,5-DiG Equiv.), and mustards (1.15 mg/g Cy-3,5-DiG Equiv.) (Figure 2.8.A). However, mustards produced the highest percentage of diacylated anthocyanins on average (84.5%), compared to kale (70.7%), Brussels sprouts (64.1%), and cabbage (53.5%). All market classes show promise for a breeding program due to differing benefits such as high concentrations, high acylation, or different combinations of the two (Figure 2.8.B).

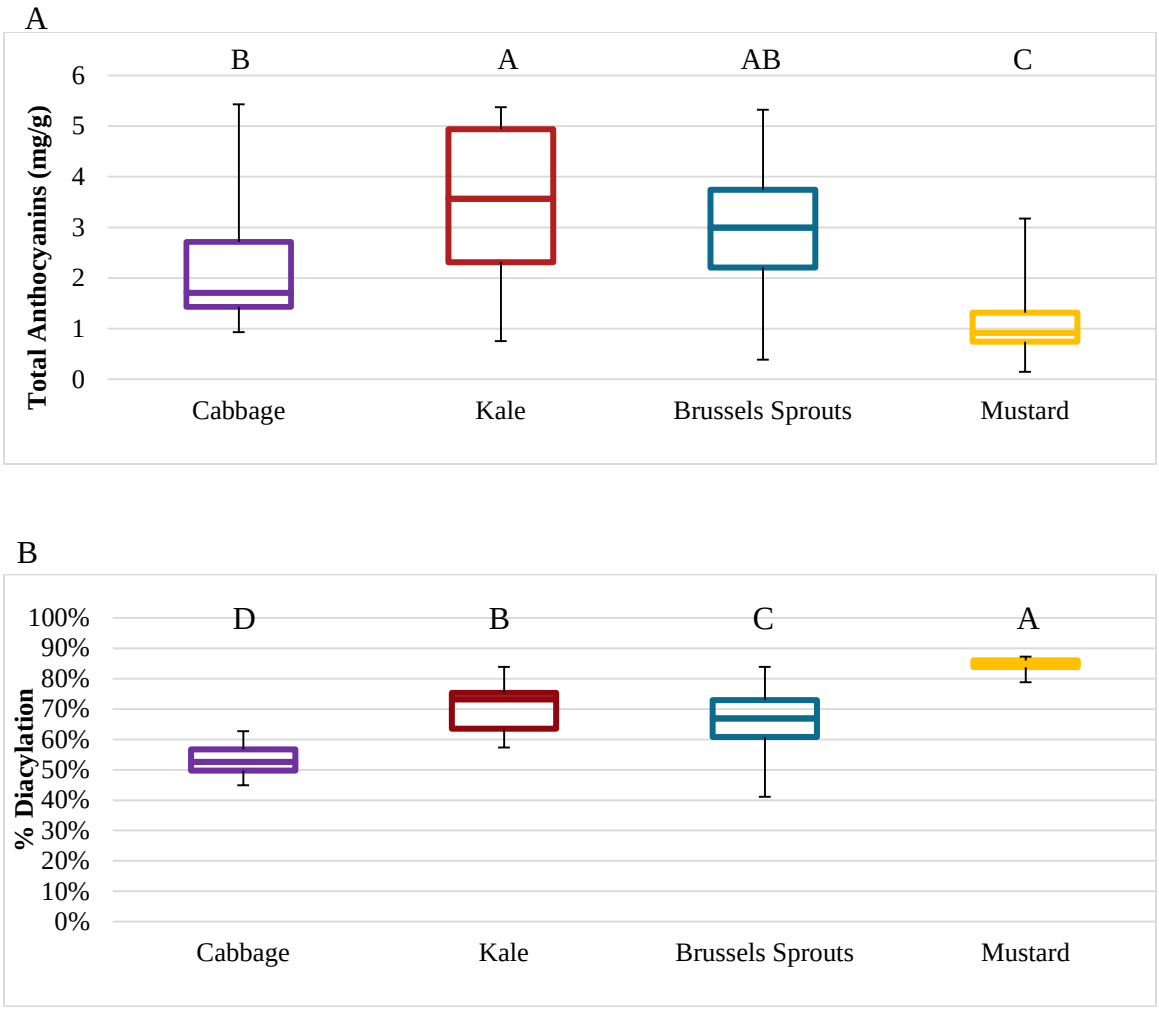


Figure 2.8 A) Anthocyanin leaf concentration (mg/g dry weight cyanidin 3,5-diglucoside equivalent) variation between three cultivars for each market classes (cabbage, kale, Brussels sprouts (*B. oleracea*), and mustard (*B. rapa* and *B. juncea*)). B) diacylated anthocyanin percentage variation between market classes.

Red mustard types (*B. rapa* and *B. juncea*) had anthocyanin profiles with later peak retention times compared to *B. oleracea* genotypes (Figure 2.9). Higher diacylation in anthocyanins results in later elution. These genotypes could be of interest for vertical farming as juveniles for their ability to provide unique, highly acylated anthocyanins.

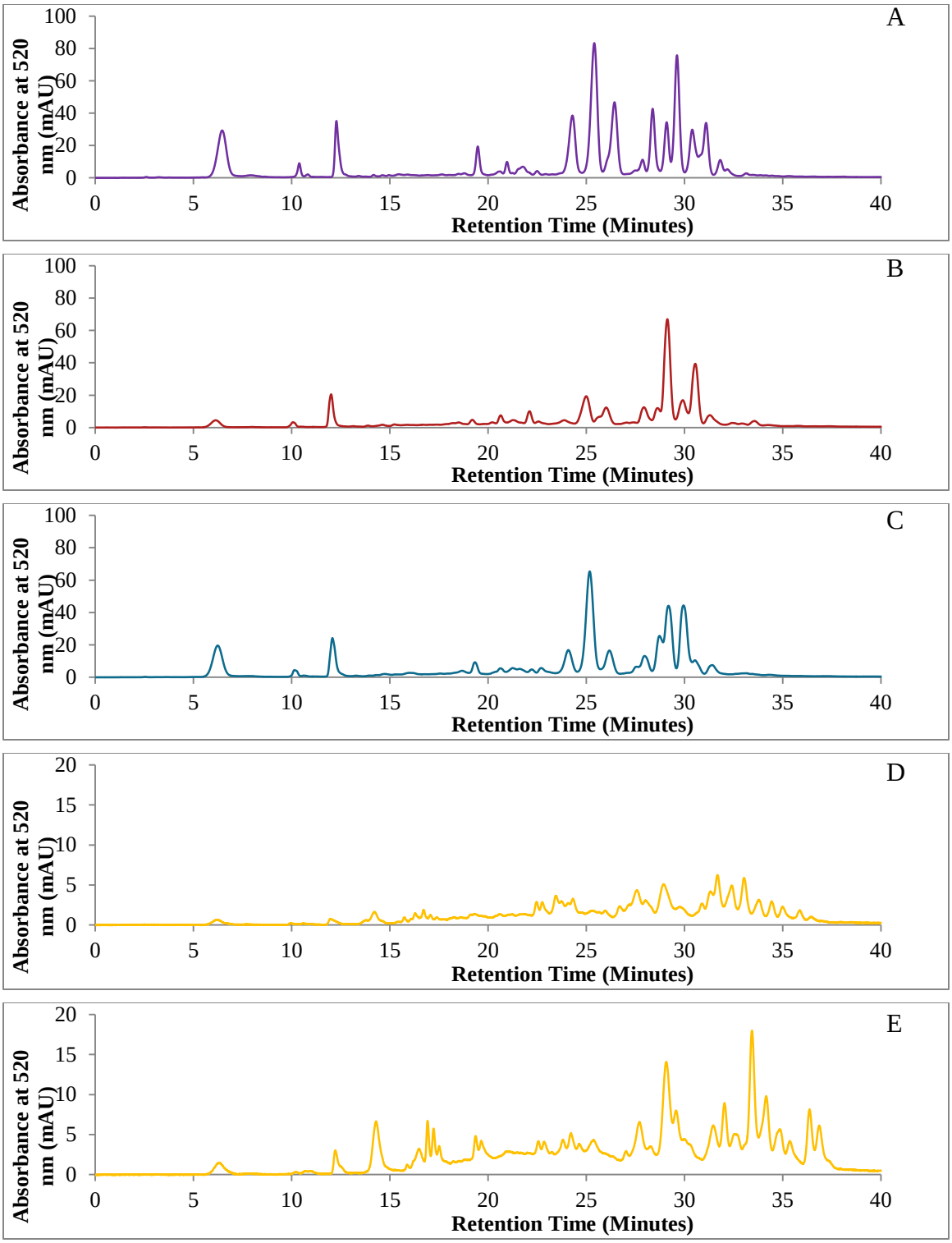


Figure 2.9 Representative HPLC chromatograms of leaves from the different species and market classes. *B. oleracea* (**A.** cabbage (Red Express) **B.** Kale (Redbor) and **C.** Brussels sprouts (Rubine)), *B. juncea* (**D.** mustard (Scarlet Frills)), and *B. rapa* (**E.** mustard (Red Mizuna))

2.3.4 Implications for future work

While internally pigmented radish genotypes are good candidates for producing high quantities of anthocyanins when compared to cabbage and kale, they also contain more pelargonidin than *B. oleracea* morphotypes. While this may be beneficial is some scenarios, pelargonidin produces more ‘orange’ and ‘brown’ colors rather than the ‘blue’ and ‘cyan’ pigmentation of cyanidin in *B. oleracea*. The colors produced by cyanidin are less common. This makes cabbage and kale overall better candidates for further research. Storage of the harvested product is also important, as it enables processing over an extended period of time if there is not a significant change in the quality and quantity of anthocyanins over time. Radish and cabbage are more practical market classes for storage as they are biennial crops that can maintain quality over extended periods in cold storage.

Additionally, when looking juvenile types, mustards are of interest for their unique and highly diacylated anthocyanin profiles. This could make them of interest for future work if a genotype with higher anthocyanin concentration is available, especially as the mustard cultivars assessed within this study appeared less affected by environment than *B. oleracea* juvenile types. These could be of interest for vertical farming production.

CHAPTER 3

SCREENING OF ANTHOCYANIN CONTENT AND ASSESSMENT OF POSTHARVEST COOLING IN MATURE B*rassica oleracea* VAR. *capitata* PIGMENTED GERMPLASM

3.1 INTRODUCTION

3.1.1 Anthocyanins

Anthocyanins are polyphenolic water-soluble pigments can have a wide variety of hydroxyl, methoxyl, sugar, and acyl groups attached to their flavylium cation base molecule. Cyanidin, pelargonidin, delphinidin, malvidin, petunidin, and peonidin are the most common and well documented anthocyanins. These are well conserved in the plant kingdom and can be found across many species and organs such as leaves, shoots, roots, seed, and grains (Wu & Prior, 2005).

Anthocyanins are produced within foliar and stem epidermal tissues in response to cold, light, drought, nutrient deficiency, and biotic stressors (Leyva, Jarillo, Salinas, & Martinez-Zapater, 1995; Mark Hodges & Nozzolillo, 1995; Socquet-Juglard et al., 2016). They act both to protect photosystems from over excitation and they reduce free radicals by acting as antioxidants (Hatier & Gould, 2009; Neill & Gould, 2003; Steyn, Wand, Holcroft, & Jacobs, 2002). These antioxidant benefits contribute to reduced cardiovascular problems, act in neuroprotection, and reduce the risk of cancer both *in vivo* and *in vitro* (Kalt et al., 2008; Lau, Shukitt-Hale, & Joseph, 2005; Prior et al., 2010; Tsuda, Horio, Uchida, Aoki, & Osawa, 2003; L.-S. Wang & Stoner, 2008).

3.1.2 Germplasm screening of anthocyanins

Anthocyanin content has been screened in barley and corn grains (Harakotr et al., 2014; M.-J. Kim et al., 2007), grape skin (Liang et al., 2008), small fruits such as blueberries and cranberries (Stevenson & Scalzo, 2012; Vorsa et al., 2003) and within vegetables such as sweet potato (Todd et al., 2015). These studies found that anthocyanin content within all of the different species and plant tissues assessed has significant variation, which can be a genetic base for crop improvement. While research has been undertaken on anthocyanin content in *B. oleracea* cultivars (Ahmadiani et al., 2014; Arapitsas et al., 2008; Chiu & Li, 2012), screening germplasm of different collections has not been documented. Determining the diversity of anthocyanin quantity and quality in genotypes from collections could form the base for selection of plants with desirable type and anthocyanin production.

3.1.3 Anthocyanins in *B. oleracea* var. *capitata*

Cabbage (*B. oleracea* var. *capitata*) has diverse pigmented genotypes available within germplasm repositories. Currently, the available materials include 83 red cabbage accessions listed in the United States Department of Agriculture (USDA) NE-9 germplasm repository, 34 in the Horticultural Research International (HRI) repository, 97 in the Institute of Plant Genetics and Crop Plant Research (Leibniz-Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK)) repository, and 32 in the Centre for Genetic Resources (CGN) repository.

Anthocyanin pigmentation can occur within underdeveloped floral meristem (curd) tissues such as in pigmented broccoli (*B. oleracea* var. *italaca*) and cauliflower

(*B. oleracea* var. *botrytis*) (Chiu & Li, 2012). Pigmented cauliflower genotypes have the potential to produce higher anthocyanin concentration within curd tissues than does red cabbage. Pigmentation in cauliflower is thought to have arisen from a single spontaneous mutation in the 1980's that activated a MYB transcription factor (Chiu et al., 2010), which explains the low level of diversity available in germplasm repositories for the pigmented trait in these market classes.

B. oleracea morphotypes mostly contain the aglycone cyanidin as a base molecule for anthocyanins. The diversity of *B. oleracea* cyanidin anthocyanins is abundant, with at least 39 different types of anthocyanins currently identified; the majority of these are at least monoacylated and many are diacylated (Arapitsas et al., 2008; Chiu et al., 2010; Socquet-Juglard et al., 2016). Among the genotypes studied, many express anthocyanins at low levels in internal tissues. The highest concentrations localized in the epithelial and outer mesophyll cells of leaves and stems.

The objectives of this study included the assessment of genetic diversity with respect to anthocyanin quantity and quality in *B. oleracea* var. *capitata*. The effect of post-harvest cooling on anthocyanin content was also investigated relative to anthocyanin concentration and acylation among genotypes.

3.2 METHODS AND MATERIALS

3.2.1 Plant materials and growth conditions

3.2.1.1 2012 Season

Seed of pigmented *B. oleracea* var. *capitata* genotypes were obtained from two germplasm repositories: 43 accessions from the United States Department of Agriculture

(USDA) NE-9 collection in Geneva, NY, USA (Appendix 3.1) and 24 accessions from the Horticultural Research International (HRI), University of Warwick, Coventry in the United Kingdom (UK) (Appendix 3.2). Eight red cabbage cultivars were included as checks for variation among commercial cultivars and two green cabbage cultivars were included as non-pigmented checks (Table 3.1).

Table 3.1 Green and red *B. oleracea* var. *capitata* cultivars grown in Geneva, NY in 2012 for

Cultivar	Source	Cultivar Group
Bartolo	Bejo, Netherlands	Green
Bronco	Bejo, Netherlands	Green
Futurima	Bejo, Netherlands	Red
Integro	Johnny's Selected Seeds, Maine, USA	Red
Mammoth Red Rock	Johnny's Selected Seeds, Maine, USA	Red
Red Express	Johnny's Selected Seeds, Maine, USA	Red
Red Jewel Cabbage	Sakata Seed, Yokohama, Japan	Red
Rio Grande Red	Orsetti Seed Company, California, USA	Red
Rondale Red	Stokes Seed, Ontario, Canada	Red
Ruby's Perfection	Johnny's Selected Seeds, Maine, USA	Red
Super Red 115	Sakata Seed, Yokohama, Japan	Red
Super Red 80	Sakata Seed, Yokohama, Japan	Red
Super Red 90	Sakata Seed, Yokohama, Japan	Red

Seeds of each genotype were sown in 72-cell styrofoam trays in 'Cornell Mix' (Boodley and Sheldrake, 1982) with one seed per cell (Speedling, Sun City, Fla.) on May 10th 2012. Materials were transplanted to a field in Geneva, NY after 35 days on raised plastic beds with 180 cm centers and 45 cm spacing. They were irrigated and fertigated as needed through a drip irrigation system. Plants were harvested in three sets dependent on maturity starting in mid-September.

3.2.1.2 2014 Season

Seeds from 73 accessions collected from the Institute of Plant Genetics and Crop Plant Research (Leibniz-Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK)) in Gatersleben, Germany (Appendix 3.4), were sown in 72-cell styrofoam trays in 'Cornell Mix' (Boodley and Sheldrake, 1982) with one seed sown per cell (Speedling, Sun City, Fla.) on June 9th, 2014. Three red cabbage cultivar checks were seeded along with these accessions: ‘Futurima’ (Bejo, Netherlands), ‘Integro’ (Johnny's Selected Seeds, Maine, USA), and ‘Ruby’s Perfection’ (Johnny's Selected Seeds, Maine, USA). Seedlings were transplanted to a field in Freeville, NY after five weeks where they were irrigated and fertilized. Mature cabbage heads were harvested from the field on October 13th, 2014.

3.2.1.3 2015 Season

Seed from 32 accessions was collected from the Centre for Genetic Resources (CGN) in Wageningen, Netherlands in 2015 (Appendix 3.5). Seed were sown one seed per cell in a 72-cell styrofoam trays in 'Cornell Mix' (Boodley and Sheldrake, 1982) with ‘Futurima’ used as a control cultivar. Seed for the first replication was sown in May 7th, 2015 while seed for the second replication was sown on May 12th, 2015. Five week old seedlings were transplanted to two different fields in Freeville, NY. They were irrigated and fertilized in the field until the first replication was harvested on September 28th, 2015 and the second replication was harvested on October 14th, 2015. A sample from each replication was placed in cold storage at 4° C for five months. Samples were then lyophilized and analyzed for comparison with samples analyzed direct from the field.

3.2.2 Sample preparation

Harvested cabbage heads were sectioned into four quarters longitudinally to be most representative of the anthocyanin distribution throughout the head relative to the core. The core was removed from two of these quarters and samples were frozen at -20°C. Once completely frozen, samples were lyophilized for a total of 48 hours with a MX53 Magnum series freeze-drying unit (Millrock Technology, Kingston, NY, USA). Dried samples were ground with a mortar and pestle until particles could fit through a 40mm mesh screen. Samples were then stored at room temperature in opaque 50 mL centrifuge tubes.

A 50 mg portion of each sample was extracted three times in three separate 5 ml aliquots of 0.01M hydrochloric acid (HCl) in methanol (MeOH). After each extraction, samples were sonicated for 1 minute and then centrifuged at 3000 x g with an Eppendorf 5810R centrifuge (Eppendorf, NA, Hauppauge, NY, USA). Supernatant from each extraction was pooled into a glass Pyrex test tube (Corning Incorporated, Corning, NY, U.S.). Combined extracts were placed in a 35°C water bath and evaporated under a stream of nitrogen gas generated by a Parker Balston model N2-04 nitrogen generator (Parker Hannifin Corp, Haverhill, MA, USA). Dried samples were reconstituted in 1 ml of 0.01M HCl in water and filtered through a 0.22 µm polyethersulfone (PES) membrane (Krackeler Scientific, Inc., Albany, NY, USA). Samples were immediately analyzed via HPLC.

3.2.3 Reagents and Standards

Cyanidin-3,5-diglucoside was used as a primary reference standard for this study and was sourced from Extrasynthese (Genay, France). All pH modifiers and organic solvents were HPLC grade and retrieved from Fisher Scientific (Pittsburgh, PA, USA). All sources of ultrapure water were generated by processing deionized water through a 0.22 µm filter with a Milli-Q integral water purification system (Millipore Corporation, Bedford , MA, USA).

3.2.4 Anthocyanin speciation via high performance liquid chromatography (HPLC)

An Agilent 1260 Infinity series HPLC system was used in combination with a Kinetex® core-shell pentafluorophenyl (PFP) column (100 mm x 2.1 mm, 2.6 µm diameter particle size, 100 Å pore size) along with an inline Krudkatcher® column filter (Phenomenex, Torrance, CA, USA) to determine anthocyanin content and concentration. Five µl of sample was injected into the HPLC system at a flow rate of 0.2 ml/min and heated to 35°C. Mobile phase A was made up of 0.5% (v/v) phosphoric acid (H_3PO_4) in water while mobile phase B consisted of 0.5% (v/v) H_3PO_4 in MeOH. The sample ran through the column in reverse phase over the course of 40 minutes with a mobile phase A concentration of: 0 min, 85%; 10 min, 70%; 15 min, 65%; 3 min, 50%; 40 min, 85%.

After this 40 minute period, a ten minute post run of 85% A and 15% B ensued in order to return the column to chemical equilibrium. The sample was measured with a diode array detector with a Max-Light cartridge flow cell (1 µl volume and 10 mm path) that monitored the sample at 520 nm using a 630 nm reference. Agilent Chemstation

software version B.04.03 with service pack 2 and spectral software module (Agilent Technologies, Santa Clara, CA, US) monitored the system and recorded data.

A standard curve was generated in triplicate ($r^2 > 0.9999$) using a cyanidin-3,5-diglucoside standard and ranged from 0.01 ppm to 250 ppm. This resulted in a percent relative standard deviation (%RSD) of less than 0.5. Speciation between non-acylated, mono-acylated and di-acylated anthocyanins was initially judged based on elution time and absorbance spectrum, and then supported by previously published literature (Arapitsas et al., 2008; Charron et al., 2008) and our previous mass spectrometry work (Socquet-Juglard et al., 2016).

4.2.5 Statistical analysis

JMP Pro 12 (SAS Institute, Cary, NC, USA) software was used to do all statistical analysis within this study. Significance was determined by ANOVA with *P* values < 0.05. Pearson correlation paired with ANOVA analysis assessed relationships between total anthocyanins and percent diacylation. JMP was also used to determine distribution of anthocyanins and percent diacylation within populations.

3.3 RESULTS AND DISCUSSION

3.3.1 Identification of pigments

Based upon absorption spectra analysis paired with previous literature, 19 different anthocyanins were identified and analyzed for this study (Figure 3.1 and Table 3.2). Peaks were classified as either nonacylated, monoacylated or diacylated. Of the 19 peaks, only 2 peaks were nonacylated, 5 were monoacylated, and 12 were found to be

diacylated. ‘Futurima’ was used for the representative chromatogram as it contained all 19 of the peaks identified. Not all samples contained all 19 peaks, especially samples with low anthocyanin concentrations. Certain samples, such as BRA793, contained peaks not previously identified.

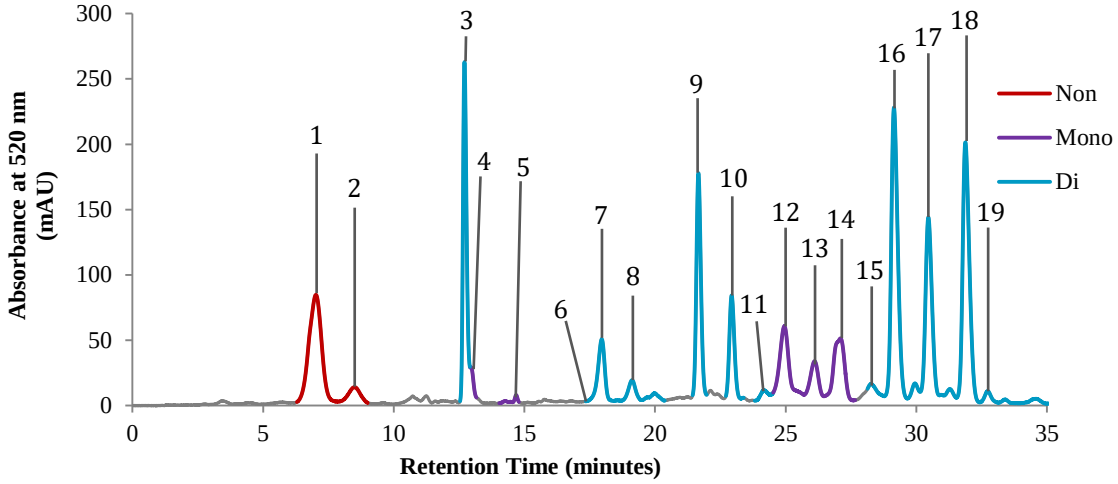


Figure 3.1 Chromatogram indicating the position and retention time of the 19 peaks identified through LCMS methods. Chromatogram produced from a samples of ‘Futurima’ grown in 2014.

Table 3.2 The 19 peaks identified through LCMS. Rentention time, maximum absorption (Λmax (nm)), mass to charge ratio ([M]+ (m/z)) and assignment of non (N), mono (M), or Di-acylated (D) given along with tennative chemical identification.

Peak	RT	Λmax (nm)	[M]+ (m/z)	Assignment	Tentative Identification
1	6.358	514	733	N	Cyanidin-3-diglucoside-5-glucoside
2	7.952	514	611	N	Cyanidin 3,5-diglucoside
3	12.417	528	1141	D	Cyanidin-3-(glycopyranosyl-sinapoyl)-diglucoside-5-glucoside
4	12.669	528	979	M	Cyanidin-3-(sinapoyl)-diglucoside-5-glucoside
5	13.831	520	979	M	Cyanidin-3-(sinapoyl)-diglucoside-5-glucoside
6	17.433	522	1081	D	Cyanidin-3-(caffeoyl)(p-coumaroyl)-diglucoside-5-glucoside
7	18.566	524	1111	D	Cyanidin-3-(glycopyranosyl-feruloyl)-diglucoside-5-glucoside
8	19.363	524	1141	D	Cyanidin-3-(glycopyranosyl-sinapoyl)-diglucoside-5-glucoside
9	21.135	534	1287	D	Cyanidin-3-(feruloyl)(feruloyl)-triglucoside-5-glucoside
10	22.393	534	1317	D	Cyanidin- 3-(sinapoyl)(feruloyl)-triglucoside-5-glucoside
11	23.712	532	1347	D	Cyanidin-3-(sinapoyl)(sinapoyl)-triglucoside-5-glucoside
12	24.078	522	919	M	Cyanidin-3-(p-coumaroyl)-diglucoside-5-glucoside
13	25.259	522	949	M	Cyanidin-3-(feruloyl)-diglucoside-5-glucoside
14	26.292	524	979	M	Cyanidin-3-(sinapoyl)-diglucoside-5-glucoside
15	27.505	522	1111	D	Cyanidin-3-(glycopyranosyl-feruloyl)-diglucoside-5-glucoside
16	28.487	534	1125	D	Cyanidin-3-(sinapoyl)(p-coumaroyl)-diglucoside-5-glucoside
17	29.793	536	1155	D	Cyanidin-3-(sinapoyl)(feruloyl)-diglucoside-5-glucoside
18	31.19	536	1185	D	Cyanidin-3-(sinapoyl)(sinapoyl)-diglucoside-5-glucoside
19	31.761	534	1155	D	Cyanidin-3-(sinapoyl)(feruloyl)-diglucoside-5-glucoside

3.3.2 USDA population diversity

There were 43 cabbage accessions screened in the USDA collection. The total anthocyanin concentration ranged from 9.60 mg/g Cy-3,5-DiG Equiv. in PI 246109 to 0.06 mg/g Cy-3,5-DiG Equiv. in PI 329197. These had an average concentration of 3.04 mg/g cabbage, dry weight, cyanidin-3,5-diglucoside equivalent (Cy-3,5-DiG Equiv Figure 3.2 shows the sample distribution and variation range of total anthocyanin content.

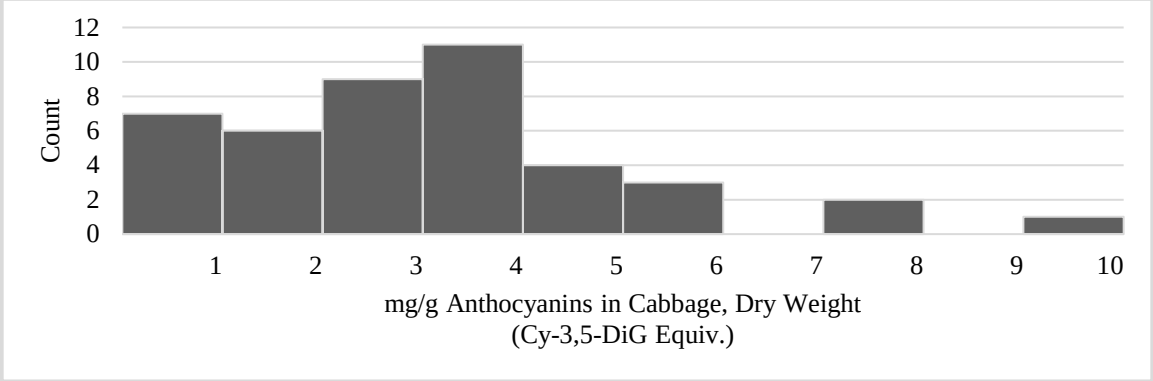


Figure 3.2 Distribution of total anthocyanin concentration (mg/g cabbage, dry weight, cyanidin-3,5-diglucoside equivalent (Cy-3,5-DiG Equiv.)) of 43 USDA accessions grown in summer of 2012 in Geneva, NY, USA

Within the 43 accessions, 3 contained anthocyanin concentrations above 7 mg/g Cy-3,5-DiG Equiv.: PI 246109, PI 662648, and G 30902. These all outperformed the cultivar controls used for anthocyanin concentration comparison. Seven accessions had concentrations below 1 mg/g Cy-3,5-DiG Equiv.: PI 296133, PI 370358, G 29936, G 29941, PI 357387, PI 291998, and PI 329197 (Appendix 3.6). The mean anthocyanin content for the USDA cabbage accessions was 46.9% diacylated. Accessions ranged from 19.9% diacylated to 74.2% diacylated. Percent diacylation followed a normal distribution in the 43 USDA accessions (Figure 3.3).

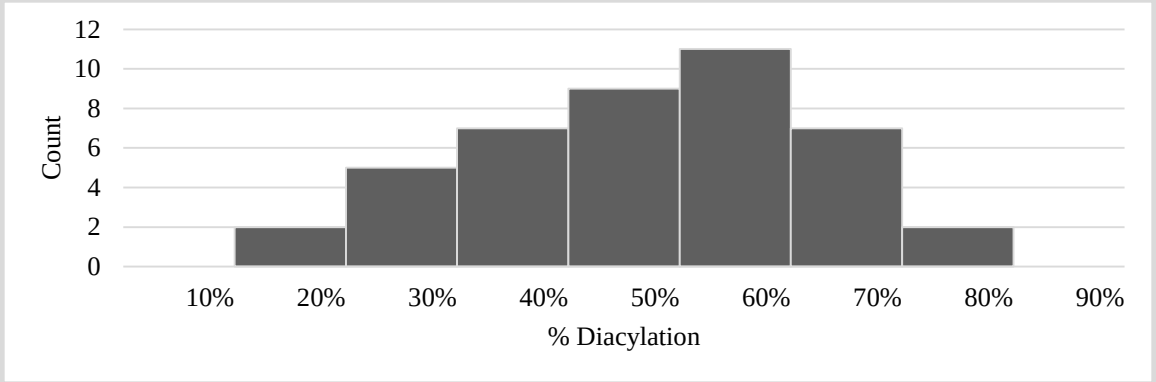


Figure 3.3 Percent diacylation distribution of 43 USDA accessions grown in summer of 2012 in Geneva, NY, USA

Of the 19 peaks identified, only peaks 1, 12, 13, 14, 16, 17, and 18 made up a significant portion (77.5%) of anthocyanins produced within the USDA red cabbage germplasm at 10.2%, 15.9%, 9.8%, 13.9%, 8.2%, 7.4% and 12.2% respectively on average (Table 3.3).

Table 3.3 Anthocyanin composition of the 43 USDA accessions. Peak number and non, mono, and diacylation identification given. Numbers expressed as mg/g cabbage, dry weight, cyanidin-3,5-diglucoside equivalent (Cy-3,5-DiG Equiv.). Data summarized by mean, standard deviation (SD), minimum (min), maximum (max) of anthocyanin peaks and their ratios (%).

Peak #	Class	Mean mg/g	SD mg/g	Min mg/g	Max mg/g	Mean %	SD %	Min %	Max %
1	N	0.33	0.27	0.01	1.14	10.2	4.0	4.0	22.92
2	N	0.04	0.03	0.00	0.13	1.6	1.6	0.5	10.09
3	D	0.08	0.06	0.00	0.25	2.6	1.3	0.8	8.20
4	M	0.03	0.02	0.00	0.13	1.3	0.7	0.4	3.59
5	M	0.01	0.01	0.00	0.05	0.7	1.1	0.1	5.92
6	D	0.08	0.05	0.00	0.25	3.0	1.4	0.9	6.65
7	D	0.06	0.05	0.00	0.19	2.3	1.1	0.5	5.28
8	D	0.05	0.03	0.00	0.14	1.8	1.0	0.4	7.32
9	D	0.08	0.08	0.00	0.35	3.0	2.6	0.8	17.38
10	D	0.07	0.06	0.00	0.27	2.7	1.8	0.5	9.31
11	D	0.03	0.02	0.00	0.07	1.2	0.9	0.3	5.71
12	M	0.57	0.54	0.01	2.25	15.9	9.8	3.1	41.58
13	M	0.34	0.36	0.00	2.10	9.8	4.5	2.3	23.22
14	M	0.47	0.40	0.01	1.57	13.9	5.6	2.4	23.54
15	D	0.04	0.06	0.00	0.26	1.4	2.2	0.1	12.58
16	D	0.25	0.19	0.00	0.63	8.2	5.2	2.1	28.67
17	D	0.20	0.16	0.00	0.74	7.4	5.9	1.1	36.20
18	D	0.31	0.24	0.00	1.14	12.2	8.0	0.3	32.60
19	D	0.02	0.02	0.00	0.14	0.8	0.8	0.2	4.97

PI 244991 was noted within the USDA germplasm for high levels of acylation when compared to other accessions (63.1% diacylation) paired with above average levels of anthocyanins (3.28 mg/g Cy-3,5-DiG Equiv.) (Figure 3.4). Breeding this accession with other notable genotypes with concentrated anthocyanins would allow for the ability to generate new genotypes with improved anthocyanin content.

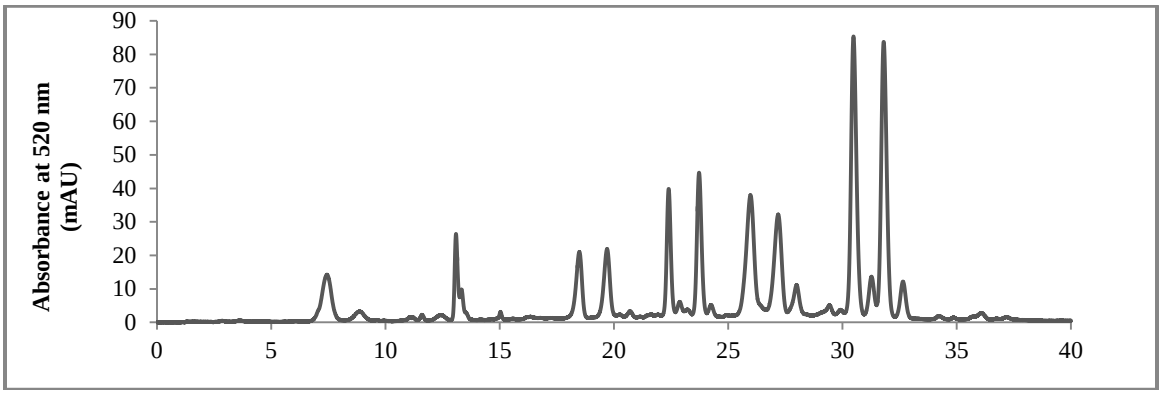


Figure 3.4 Chromatogram of anthocyanin peaks for USDA accession PI 24499

3.3.3 HRI population diversity

The 24 accessions screened from the HRI germplasm red cabbage collection contained a mean of 4.97 mg/g Cy-3,5-DiG Equiv. anthocyanins. Anthocyanin concentrations ranged from 1.65 mg/g Cy-3,5-DiG Equiv. to 10.95 mg/g Cy-3,5-DiG Equiv. As shown in Figure 3.5, the variation range and sample distribution of total anthocyanin content generally formed a normal distribution.

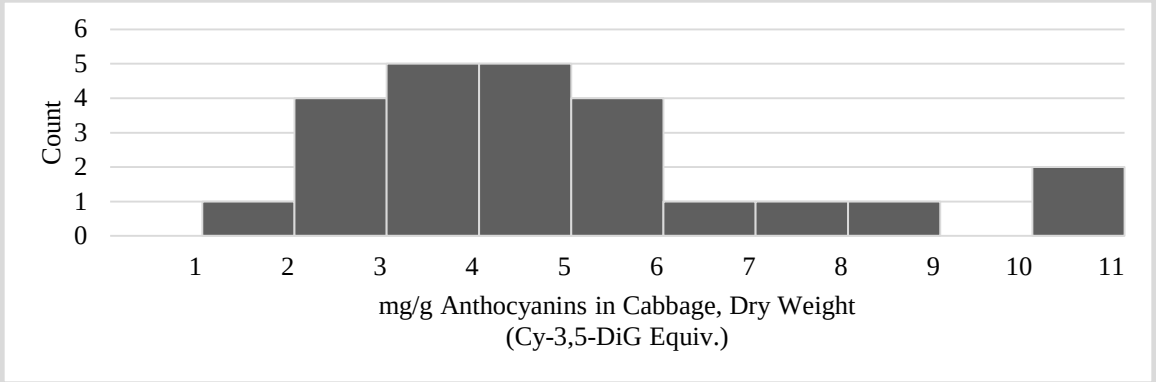


Figure 3.5 Distribution of total anthocyanin concentration (mg/g cabbage, dry weight, cyanidin-3,5-diglucoside equivalent (Cy-3,5-DiG Equiv.)) of 24 HRI accessions grown in summer of 2012 in Geneva, NY, USA

Among 24 HRI accessions, there were 4 accessions that contained anthocyanins greater than 7 mg/g Cy-3,5-DiG Equiv.: HRI 005887, HRI 003586, HRI 012994, HRI 007852A. These all outperformed the cultivar standard used for comparison. All HRI accessions contained anthocyanins above1 mg/g Cy-3,5-DiG Equiv. and only one, HRI 007858A, contained fewer than 2 mg/g Cy-3,5-DiG Equiv. (Appendix 3.8). The HRI repository had significantly higher anthocyanin concentrations overall when compared to the USDA repository (*P* value < 0.05), even though entries were all from the same replicated fields. HRI germplasm had better plant quality and uniformity overall.

The 24 HRI accessions had a mean 43.7% of diacylated anthocyanins and ranged from 28.1% to 74.4% in percent diacylation in the samples evaluated. As shown in Figure 3.6, percent diacylation formed a relatively normal distribution. While the HRI repository had larger concentrations of anthocyanins overall when compared to the USDA collection, the percent diacylation distribution was not significantly different between HRI and USDA.

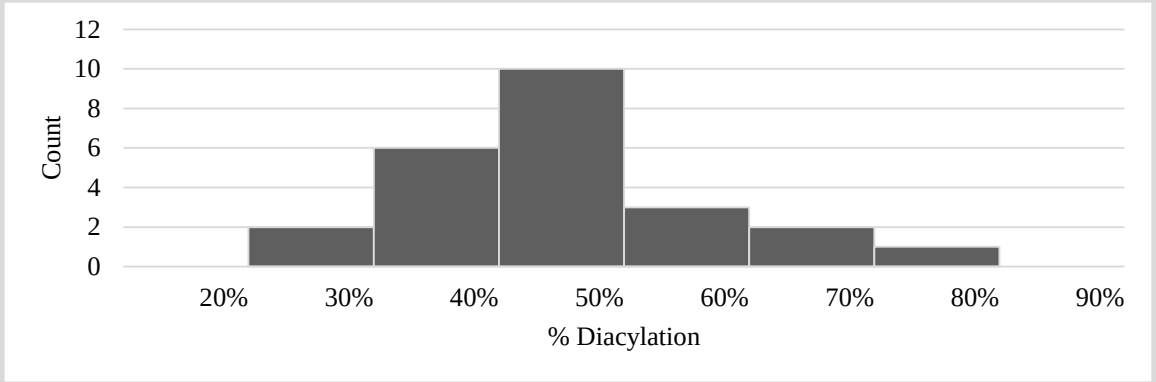


Figure 3.6 Percent diacylation distribution of 24 HRI accessions grown in summer of 2012 in Geneva, NY, USA

Seven peaks were found to make up 81.0% of the anthocyanin content on average for the HRI germplasm repository: 1 (10.8%), 12 (17.2%), 13 (10.8%), 14 (15.1%), 16 (9.0%), 17 (6.9%), and 18 (11.2%) (Table 3.4).

Table 3.4 Anthocyanin composition of the 24 HRI accessions. Peak number and non, mono, and diacylation identification given. Numbers expressed as mg/g cabbage, dry weight, cyanidin-3,5-diglucoside equivalent (Cy-3,5-DiG Equiv.). Data summarized by mean, standard deviation (SD), minimum (min), maximum (max) of anthocyanin peaks and their ratios (%).

Peak #	Class	Mean mg/g	SD mg/g	Min mg/g	Max mg/g	Mean %	SD %	Min %	Max %
1	N	0.55	0.37	0.14	1.48	10.8	3.7	5.4	20.49
2	N	0.04	0.02	0.02	0.09	1.0	0.5	0.4	2.57
3	D	0.14	0.10	0.03	0.38	2.7	1.2	1.0	5.18
4	M	0.06	0.04	0.02	0.15	1.2	0.5	0.5	2.12
5	M	0.01	0.01	0.01	0.03	0.3	0.2	0.1	0.76
6	D	0.13	0.05	0.06	0.27	2.8	0.9	1.1	4.76
7	D	0.09	0.05	0.04	0.32	2.0	0.5	1.0	2.90
8	D	0.06	0.02	0.03	0.12	1.4	0.4	0.8	2.19
9	D	0.14	0.09	0.04	0.40	2.9	1.5	0.8	7.97
10	D	0.12	0.10	0.03	0.52	2.4	1.2	0.8	5.66
11	D	0.04	0.02	0.02	0.09	0.9	0.2	0.5	1.37
12	M	0.79	0.43	0.21	1.61	17.2	8.4	4.8	35.49
13	M	0.55	0.39	0.12	1.59	10.8	3.9	4.0	20.29
14	M	0.76	0.50	0.22	2.14	15.1	4.5	8.2	23.38
15	D	0.04	0.03	0.01	0.13	0.7	0.7	0.2	2.60
16	D	0.43	0.24	0.12	1.00	9.0	3.4	2.6	16.14
17	D	0.36	0.26	0.07	1.00	6.9	2.9	2.5	12.50
18	D	0.55	0.41	0.13	1.86	11.2	6.1	4.0	25.62
19	D	0.03	0.02	0.01	0.05	0.6	0.3	0.4	1.59

HRI 005887 was noted for its ability to produce high amounts of anthocyanins. It produced the highest concentration of anthocyanins from the HRI collection at 10.05 total mg/g Cy-3,5-DiG Equiv. Additionally, it had above average diacylation at 47.5% diacylated content (Figure 3.7).

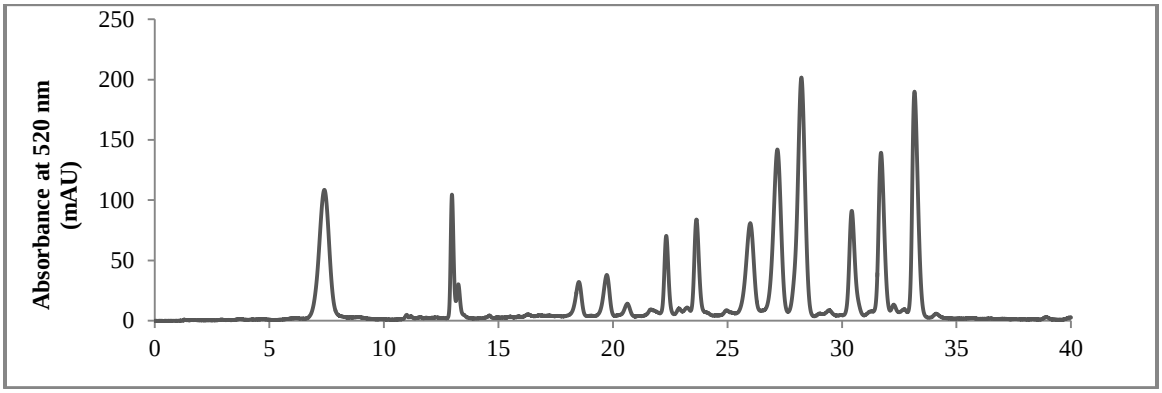


Figure 3.7 Chromatogram of anthocyanin peaks for HRI accession HRI 005887

HRI 002657A was of note for its high percent diacylation at 74.4% diacylation. It also produced anthocyanin concentrations at the upper end of the distribution for this germplasm repository at 4.29 mg/g Cy-3,5-DiG Equiv. It had a notable profile due to large amount of peak 16, 17, and 18, all of which are diacylated, and low levels of peak 12, 13, and 14, all of which are monoacylated (Figure 3.8). These two accessions would be good to use for breeding as they have the combined possibility to produce genotypes with increased levels of anthocyanins and high levels of acylation.

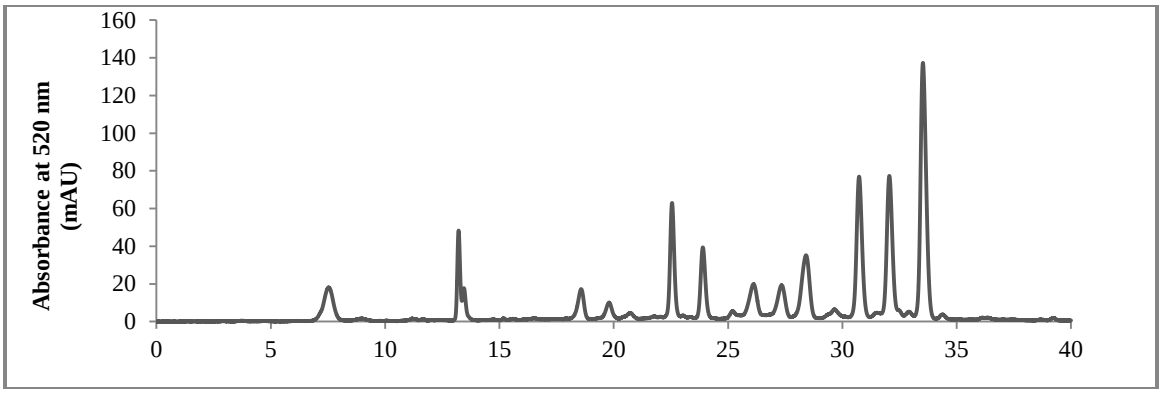


Figure 3.8 Chromatogram of anthocyanin peaks for HRI accession HRI 002657A

3.3.4 IPK population diversity

The 73 accessions screened from the IPK germplasm in 2014 contained a mean of 8.81 mg/g Cy-3,5-DiG Equiv. anthocyanins. The concentration of anthocyanins from this repository ranged from 0.14 mg/g to 21.64 mg/g Cy-3,5-DiG Equiv. The anthocyanin concentration from the IPK accessions generally followed a normal distribution (Figure 3.9).

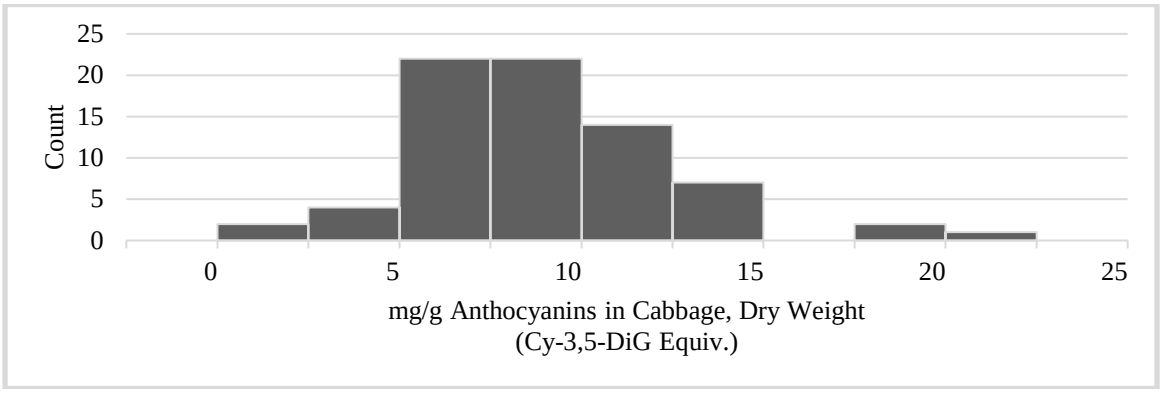


Figure 3.9 Distribution of total anthocyanin concentration (mg/g cabbage, dry weight, cyanidin-3,5-diglucoside equivalent (Cy-3,5-DiG Equiv.)) of 73 IPK accessions grown in summer of 2014 in Freeville, NY, USA.

Within the IPK germplasm repository, three red cabbage accessions had total anthocyanin concentrations greater than 17 mg/g Cy-3,5-DiG Equiv.: BRA 789, BRA

762, and BRA778. Six accessions contained concentrations less than 5 mg/g Cy-3,5-DiG Equiv.: BRA400, BRA2200, BRA752, BRA792, BRA793 (Appendix 3.10). Cultivars and accessions replicated from previous years did not remain consistent in ranking due to environmental differences affecting anthocyanin concentrations in different seasons. This agrees with the previous literature (Wiczkowski, Topolska, & Honke, 2014).

Anthocyanin content from the IPK repository had a mean of 47.9% diacylated and ranged from 16.7% to 73.8% in diacylation. BRA770 stood out from other accessions for having both high anthocyanin concentrations (14.24 mg/g Cy-3,5-DiG Equiv.) and high levels of acylation (68.0% diacylated, 9.69 mg/g Cy-3,5-DiG Equiv. diacylated anthocyanins total on average). Percent diacylation formed a normal distribution (Figure 3.10). Anthocyanin concentration was higher in accessions from the IPK repository that had the most uniform and highest quality accessions; however, percent diacylation and distribution was not significantly different in the IPK accessions.

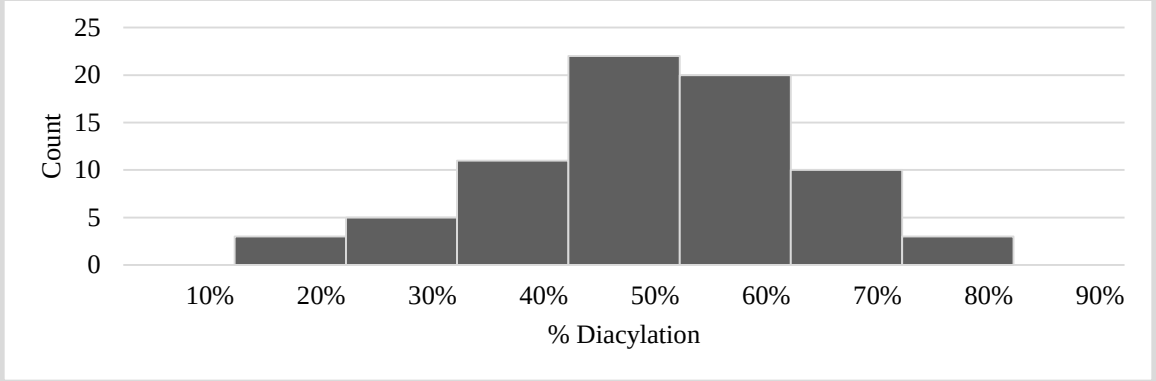


Figure 3.10 Percent diacylation of 73 IPK accessions grown 2014 in Freeville, NY, USA.

The same 7 peaks made up the majority of anthocyanins within the IPK repository that was consistent with results from the USDA and HRI repositories. These 7 peaks

made up 89.7% of the anthocyanin content: 1 (11.0%), 12 (12.4%), 13 (9.3%), 14 (16.4%), 16 (9.5%), 17 (8.4%), and 18 (13.8%) (Table 3.5).

Table 3.5 Anthocyanin composition of the 73 IPK accessions. Peak number and non, mono, and diacylation identification given. Numbers expressed as mg/g cabbage, dry weight, cyanidin-3,5-diglucoside equivalent (Cy-3,5-DiG Equiv.). Data summarized by mean, standard deviation (SD), minimum (min), maximum (max) of anthocyanin peaks and their ratios (%).

Peak #	Class	Mean mg/g	SD mg/g	Min mg/g	Max mg/g	Mean %	SD %	Min %	Max %
1	N	0.78	0.50	0.01	3.13	11.0	4.6	4.5	26.2
2	N	0.06	0.06	0.00	0.35	0.9	0.7	0.1	3.5
3	D	0.26	0.15	0.00	0.82	3.6	1.3	1.3	8.6
4	M	0.07	0.04	0.00	0.20	1.0	0.3	0.3	1.9
5	M	0.01	0.01	0.00	0.02	0.1	0.1	0.0	0.7
6	D	0.12	0.07	0.00	0.34	1.8	1.0	0.2	5.1
7	D	0.08	0.05	0.00	0.25	1.2	0.7	0.1	4.5
8	D	0.04	0.03	0.00	0.14	0.6	0.3	0.1	2.6
9	D	0.16	0.10	0.00	0.48	2.3	1.5	0.2	7.8
10	D	0.15	0.09	0.01	0.49	2.3	1.3	0.4	8.3
11	D	0.05	0.03	0.00	0.19	0.7	0.3	0.1	1.4
12	M	0.89	0.53	0.01	2.50	12.4	5.4	0.8	30.6
13	M	0.68	0.50	0.00	3.23	9.3	3.5	1.9	23.4
14	M	1.13	0.61	0.01	3.46	16.4	6.0	6.1	31.6
15	D	0.14	0.10	0.00	0.59	1.9	1.0	0.3	4.4
16	D	0.69	0.46	0.00	2.13	9.5	4.7	1.2	22.4
17	D	0.59	0.39	0.01	1.77	8.4	4.2	1.2	21.4
18	D	0.90	0.46	0.03	2.56	13.8	5.9	3.8	33.8
19	D	0.03	0.03	0.00	0.13	0.5	0.3	0.0	2.3

BRA793 (Figure 3.11) was an interesting accession as it had few chromatographic peaks previously identified for being common within *B. oleracea*. Its profile was different from the rest of the accessions screened, with six anthocyanins that eluted at later retention times. It had the lowest concentrations of anthocyanins (0.14 mg/g Cy-3,5-DiG Equiv.) compared to other accessions within this repository, though it is still of value for breeding efforts due to its ability to contribute a unique anthocyanin profile.

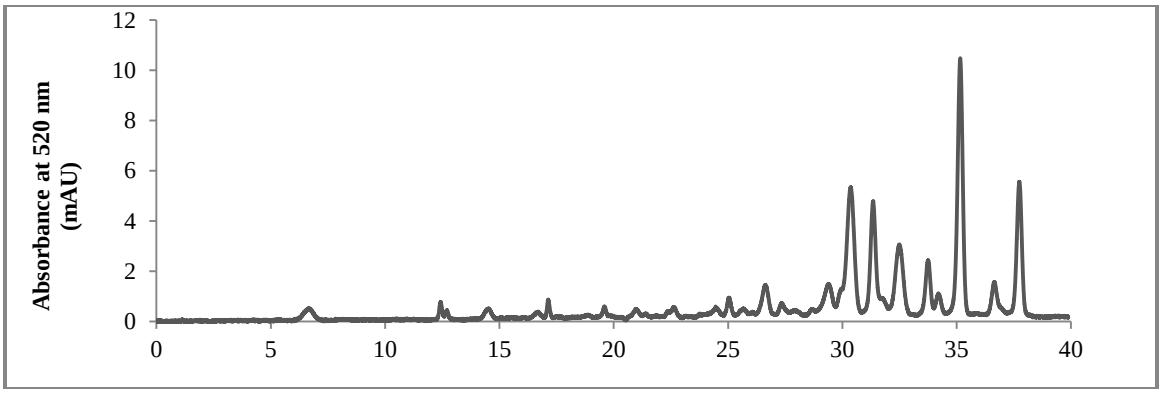


Figure 3.11 Chromatogram of anthocyanin peaks for IPK accession BRA793

The major goal of this screening was to select genotypes able to produce comparably high level of anthocyanin concentrations with high levels of acylation for breeding purposes. BRA 770 was identified for as having high anthocyanin concentrations (14.24 mg/g Cy-3,5-DiG Equiv.) and high levels of acylation (68.0% diacylated, 9.69 mg/g Cy-3,5-DiG Equiv. diacylated anthocyanins total on average) (Figure 3.12). This is an ideal candidate for being the basis of a breeding program.

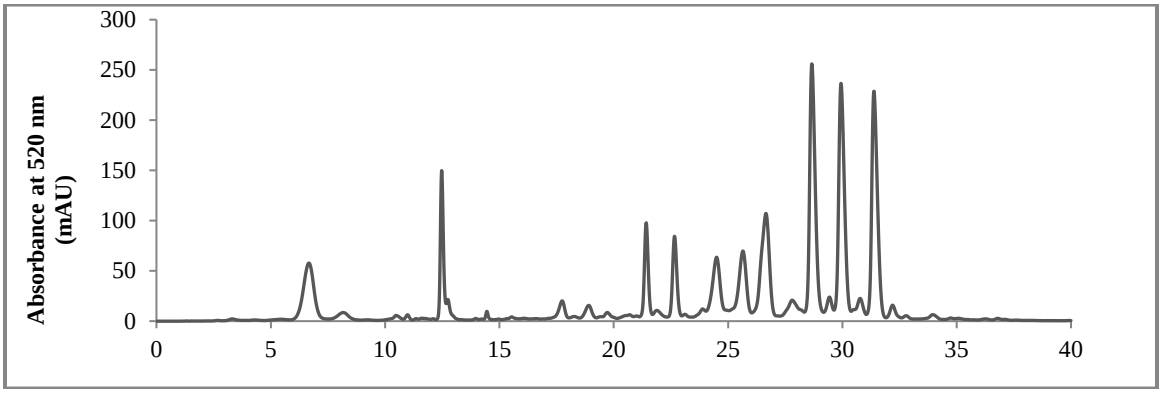


Figure 3.12 Chromatogram of anthocyanin peaks for IPK accession BRA770

3.3.5 CGN population diversity

The 32 red cabbage accessions screened from the CGN germplasm collection produced a mean of 9.10 mg/g Cy-3,5-DiG Equiv. anthocyanins. The total anthocyanin

content ranged from 0.73 mg/g Cy-3,5-DiG Equiv. to 14.92 mg/g Cy-3,5-DiG Equiv. (Figure 3.13). The means and distribution from the CGN repository grown in 2015 are similar to the IPK germplasm accession screened in 2014 and are significantly greater than the USDA and HRI accessions screened in 2012. This could be the result of the season, field location and quality of genotypes from the different repositories.

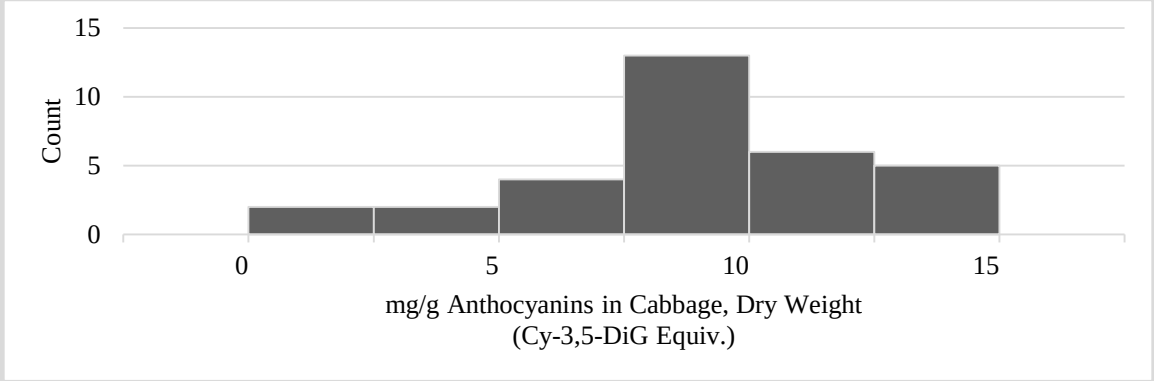


Figure 3.13 Distribution of total anthocyanin concentration (mg/g cabbage, dry weight, cyanidin-3,5-diglucoside equivalent (Cy-3,5-DiG Equiv.)) of 32 CGN accessions grown in summer of 2015 in Freeville, NY, USA.

No accession had a mean total anthocyanin concentration greater than 17 mg/g Cy-3,5-DiG Equiv.. There were 3 accessions with a mean above 14 mg/g Cy-3,5-DiG Equiv.: CGN 07086, CGN 18434, and CGN 07094. Of the 32 accessions, 4 accessions contained less than 5 mg/g Cy-3,5-DiG Equiv. of anthocyanins in total: CGN 07085, CGN 07091, CGN 18435, CGN 15769 (Appendix 3.12). IPK accessions averaged 32.7% diacylated anthocyanins and ranged from 14.9% to 54.4% in percent diacylation. Percent diacylation generally followed a normal distribution (Figure 3.14).

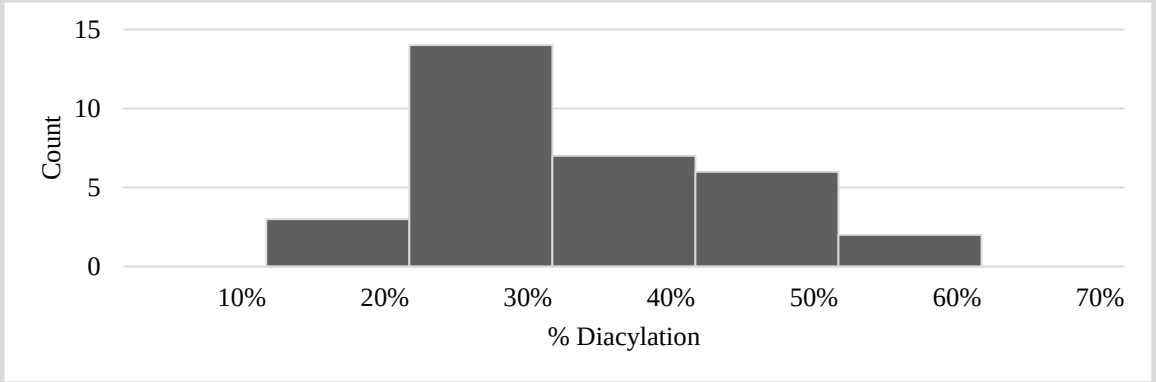


Figure 3.14 Percent diacylation distribution of 32 CGN accessions grown in summer of 2015 in Freeville, NY, USA

Only 6 major peaks contributed to the makeup of the anthocyanins within the CGN repository: peak 1, 12, 13, 14, 16, and 18. These 6 peaks were responsible for 73.5% of the anthocyanin content: 1 (11.8%), 12 (24.0%), 13 (11.9%), 14 (15.5%), 16 (4.4%), and 18 (4.4%) (Table 3.6).

Table 3.6 Anthocyanin composition of the 32 CGN accessions. Peak number and non, mono, and diacylation identification given. Numbers expressed as mg/g cabbage, dry weight, cyanidin-3,5-diglucoside equivalent (Cy-3,5-DiG Equiv.). Data summarized by mean, standard deviation (SD), minimum (min), maximum (max) of anthocyanin peaks and their ratios (%).

Peak #	Class	Mean mg/g	SD mg/g	Min mg/g	Max mg/g	Mean %	SD %	Min %	Max %
1	N	0.82	0.42	0.02	2.01	11.8	3.5	4.8	23.4
2	N	0.11	0.06	0.00	0.36	1.6	0.6	0.3	3.3
3	D	0.09	0.06	0.01	0.28	1.3	0.5	0.4	3.0
4	M	0.10	0.06	0.01	0.32	1.4	0.5	0.3	3.0
5	M	0.07	0.04	0.00	0.22	1.0	0.4	0.3	2.5
6	D	0.25	0.14	0.01	0.69	3.6	1.3	1.5	8.7
7	D	0.18	0.08	0.01	0.40	2.8	1.1	1.1	7.3
8	D	0.14	0.06	0.01	0.32	2.2	0.7	0.8	4.6
9	D	0.13	0.09	0.01	0.48	1.9	0.9	0.7	5.7
10	D	0.14	0.07	0.01	0.36	2.1	0.9	0.9	5.8
11	D	0.10	0.05	0.01	0.21	1.5	0.6	0.4	3.2
12	M	1.77	1.14	0.02	6.37	24.0	9.4	4.1	43.2
13	M	0.84	0.45	0.04	2.00	11.9	3.0	6.6	19.8
14	M	1.14	0.77	0.02	3.60	15.5	5.6	1.2	30.5
15	D	0.20	0.13	0.01	0.70	2.8	1.2	0.7	7.5
16	D	0.33	0.34	0.01	2.70	4.4	2.5	0.9	17.7
17	D	0.22	0.17	0.02	0.89	3.4	2.8	0.4	19.2
18	D	0.40	0.37	0.02	1.91	6.0	4.4	0.5	24.4
19	D	0.05	0.04	0.01	0.18	0.8	0.5	0.1	2.4

CGN 07090 had high diacylation on average (49.1%) with 12.07 mg/g Cy-3,5-DiG Equiv. anthocyanins when compared to the rest of the CGN germplasm. This increased acylation appears to mostly derive from high levels of peak 18 (Figure 3.15).

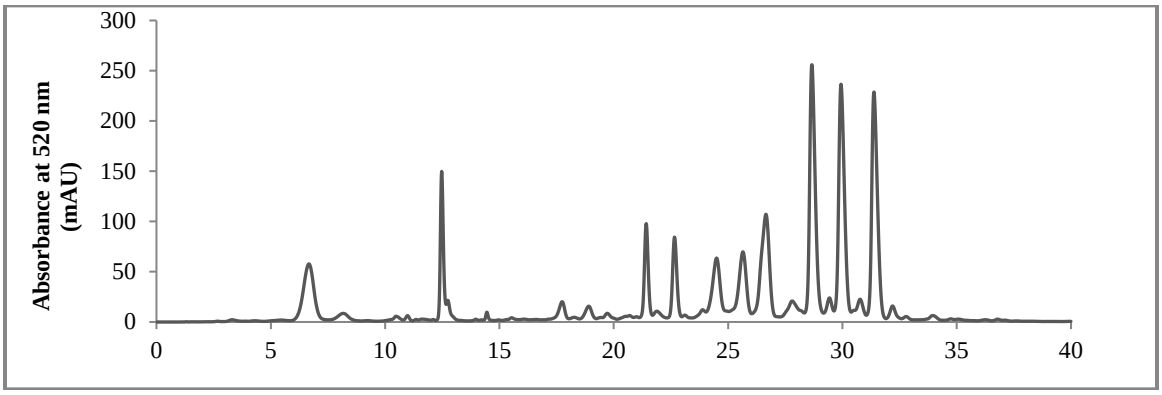


Figure 3.15 Chromatogram of anthocyanin peaks for CGN accession CGN 07090

3.3.6 Implications of genetic diversity

While mean concentrations of anthocyanins varied by year and germplasm repositories, all repositories had mean anthocyanin concentrations that followed a normal distribution. This indicates good genetic diversity in overall anthocyanin concentrations, and that breeding for higher anthocyanin concentrations could be achieved with the genetic materials assessed within this study.

Percent diacylation also followed a normal distribution in all of the germplasm repositories assessed, indicating strong genetic diversity for acylation within red cabbage accessions. This suggests that anthocyanin yields have a strong genetic component that can be selected through crop improvement and genetic selection for percentage of diacylated anthocyanins. Figure 3.16 shows the peak distribution of all of the accessions

and years combined, indicating which peaks (1, 12, 13, 14, 16, 17, and 18) contribute the most to anthocyanin content. These peaks also have the most variation (Table 3.7).

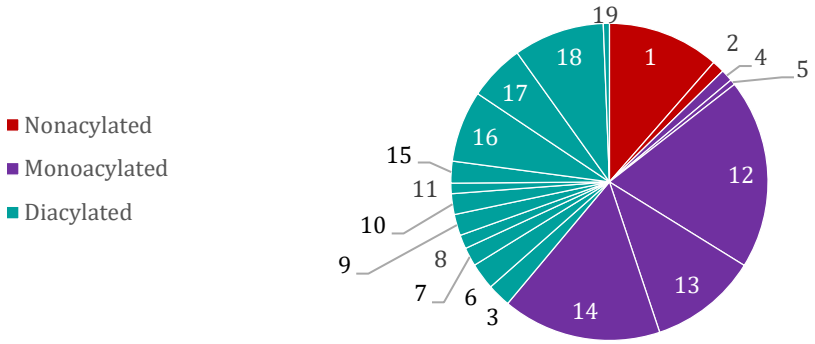


Figure 3.16 Average anthocyanin content composition from 173 total accessions screened showing peak number and non, mono, and diacylation classification.

Table 3.7 Anthocyanin composition of the 173 total accessions screened. Peak number and non, mono, and diacylation identification given. Numbers expressed as mg/g cabbage, dry weight, cyanidin-3,5-diglucoside equivalent (Cy-3,5-DiG Equiv.). Data summarized by mean, standard deviation (SD), minimum (min), maximum (max) of anthocyanin peaks and their ratios (%).

Peak #	Class	Mean mg/g	SD mg/g	Min mg/g	Max mg/g	Mean %	SD %	Min %	Max %
1	N	0.70	0.46	0.01	3.13	11.2	4.0	4.0	26.2
2	N	0.08	0.06	0.00	0.36	1.3	0.9	0.1	10.1
3	D	0.15	0.13	0.00	0.82	2.4	1.5	0.4	8.6
4	M	0.08	0.05	0.00	0.32	1.2	0.5	0.3	3.6
5	M	0.03	0.04	0.00	0.22	0.6	0.7	0.0	5.9
6	D	0.17	0.12	0.00	0.69	2.8	1.4	0.2	8.7
7	D	0.12	0.08	0.00	0.40	2.1	1.2	0.1	7.3
8	D	0.09	0.07	0.00	0.32	1.5	1.0	0.1	7.3
9	D	0.13	0.09	0.00	0.48	2.3	1.6	0.2	17.4
10	D	0.13	0.09	0.00	0.52	2.3	1.3	0.4	9.3
11	D	0.07	0.05	0.00	0.21	1.1	0.7	0.1	5.7
12	M	1.19	0.97	0.01	6.37	18.2	9.7	0.8	43.2
13	M	0.68	0.48	0.00	3.23	10.6	3.7	1.9	23.4
14	M	1.00	0.69	0.01	3.60	15.5	5.7	1.2	31.6
15	D	0.14	0.12	0.00	0.70	2.1	1.5	0.1	12.6
16	D	0.45	0.40	0.00	2.70	7.1	4.6	0.9	28.7
17	D	0.35	0.32	0.00	1.77	6.0	4.5	0.4	36.2
18	D	0.70	0.46	0.01	3.13	10.1	6.7	0.3	33.8
19	D	0.08	0.06	0.00	0.36	0.7	0.5	0.0	5.0

3.3.7 Effect of Cooling on CGN accessions

After post-harvest cooling for five months, accessions from the CGN repository had a total anthocyanin concentration that followed a normal distribution but skewed

toward higher anthocyanin concentrations when compared to their non-cooled counterparts (Figure 3.17). Under normal conditions, mature cabbage heads from the CGN collection had a mean anthocyanin concentration of 9.14 mg/g Cy-3,5-DiG Equiv. When cooled, the mean anthocyanin concentration was increased to 11.91mg/g Cy-3,5-DiG Equiv. (Figure 3.18). This difference was significant ($P < 0.05$).

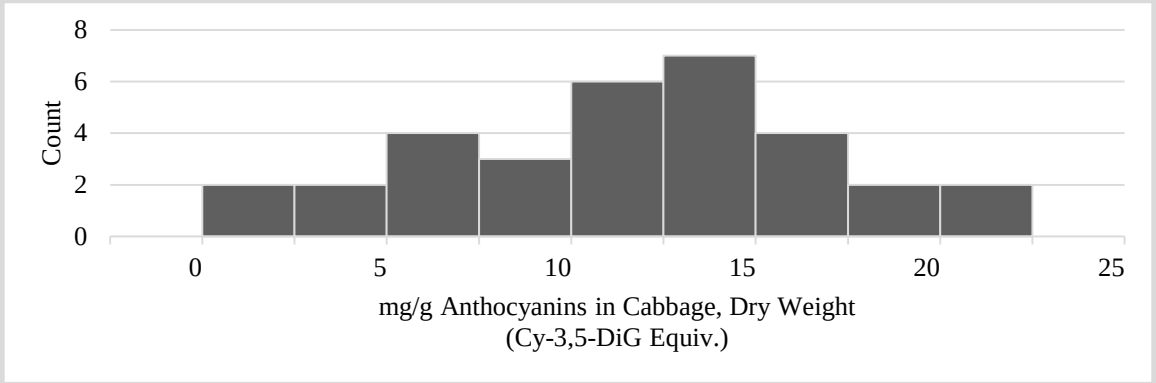


Figure 3.17 Distribution of total anthocyanin concentration (mg/g cabbage, dry weight, cyanidin-3,5-diglucoside equivalent (Cy-3,5-DiG Equiv.)) of 32 CGN accessions grown in summer of 2015 in Freeville, NY, USA and then were subjected to 5 months post-harvest cooling at 4° C.

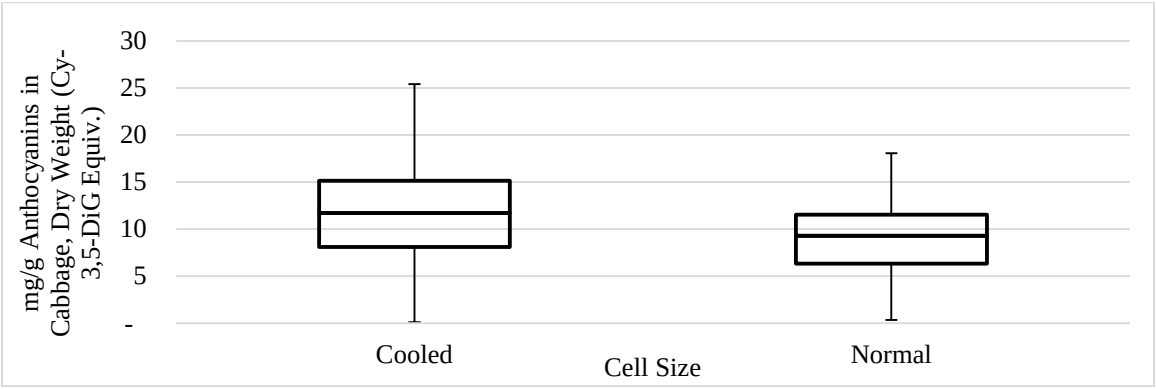


Figure 3.18 differences in total anthocyanin concentrations (mg/g cabbage, dry weight, cyanidin-3,5-diglucoside equivalent (Cy-3,5-DiG Equiv.)) between CGN accessions that had (Cooled) and had not (Normal) undergone 5 months of post-harvest cooling at 4° C. Differences between means assessed by ANOVA was significant ($P < 0.05$)

Percent diacylation was directly influenced by the cold storage process. Prior to cooling, the mean percent diacylation for the CGN accessions was 32.7% that increased to 39.5% after cold storage (Figure 3.19). This difference was significant *(P* < 0.05).

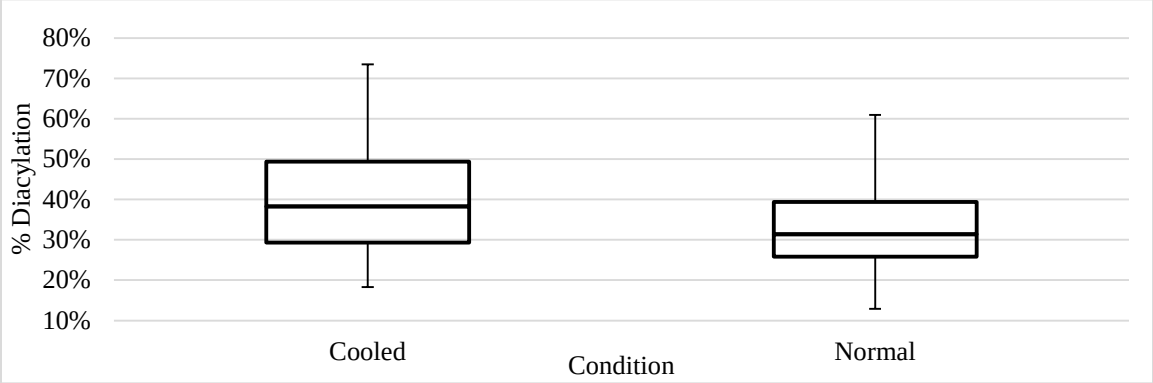


Figure 3.19 Differences in percent diacylation between CGN accessions that had (Cooled) and had not (Normal) undergone 5 months of post-harvest cooling at 4° C as shown by box plots. Differences between means assessed by ANOVA was significant (*P* < 0.05).

The skew towards higher diacylation can be explained by the increase in the percentage of diacylated peaks 16, 17, and 18 and the decrease in the percentage of monoacylated peaks 12 and 13. Peak 16 had a mean increase from 4.4% to 5.8%, peak 17 from 3.4% to 4.2% and peak 18 from 6.0% to 9.3%. All of these changes were significant (*P* values < 0.05*).* A 5.5% increase of these 3 diacylated peaks was observed. Conversely, peak 12 decreased from 24.0% to 17.7% and peak 13 decreased from 11.9% to 9.5% when cooled (Table 3.8). These changes were significant (*P* values < 0.05*).* This resulted in a 8.7% decrease of these two major monoacylated peaks.

Table 3.8 Anthocyanin composition of the 32 CGN accessions that were subjected to 5 months post-harvest cooling at 4° C. Peak number and non, mono, and diacylation identification given. Numbers expressed as mg/g cabbage, dry weight, cyanidin-3,5-diglucoside equivalent (Cy-3,5-DiG Equiv.). Data summarized by mean, standard deviation (SD), minimum (min), maximum (max) of anthocyanin peaks and their ratios (%).

Peak #	Class	Mean mg/g	SD mg/g	Min mg/g	Max mg/g	Mean %	SD %	Min %	Max %
1	N	1.22	0.77	0.01	4.02	13.0	3.6	5.7	25.1
2	N	0.14	0.08	0.00	0.38	1.6	0.6	0.4	3.0
3	D	0.19	0.13	0.00	0.65	2.1	0.9	0.6	6.6
4	M	0.12	0.08	0.00	0.33	1.4	0.7	0.4	3.5
5	M	0.04	0.02	0.00	0.10	0.5	0.2	0.2	1.6
6	D	0.32	0.17	0.00	0.93	3.7	1.4	1.3	9.9
7	D	0.21	0.11	0.01	0.64	2.5	1.1	0.8	7.4
8	D	0.15	0.07	0.00	0.35	1.7	0.4	1.0	3.5
9	D	0.26	0.15	0.00	0.67	3.0	1.3	0.7	7.3
10	D	0.21	0.14	0.01	0.80	2.6	1.7	0.8	11.5
11	D	0.12	0.09	0.00	0.47	1.2	0.5	0.4	2.7
12	M	1.71	1.28	0.00	6.24	17.7	9.0	3.5	39.4
13	M	0.92	0.58	0.01	2.47	9.5	3.0	2.7	17.6
14	M	1.61	1.00	0.01	4.68	16.9	4.9	1.0	30.7
15	D	0.24	0.15	0.00	0.66	2.6	1.1	0.5	6.4
16	D	0.54	0.34	0.00	1.84	5.8	2.7	1.5	18.5
17	D	0.37	0.31	0.01	1.49	4.2	2.9	0.6	15.6
18	D	0.77	0.56	0.01	2.17	9.3	6.2	0.3	30.8
19	D	0.05	0.03	0.00	0.16	0.6	0.3	0.1	2.2

3.3.8 Anthocyanin concentration effects on percent diacylation

Data was pooled across all three different years, all four germplasm repositories and additional accessions to determine possible correlation between total anthocyanin content and percent diacylation. Total anthocyanin had a negative correlation (-0.28) with percent diacylation, as shown in Figure 3.20. The correlation had a *P* value < 0.05, which indicated a significant correlation. There is likely strong genetic and environmental interplay determining percent diacylation and general anthocyanin profile of *B. oleracea*.

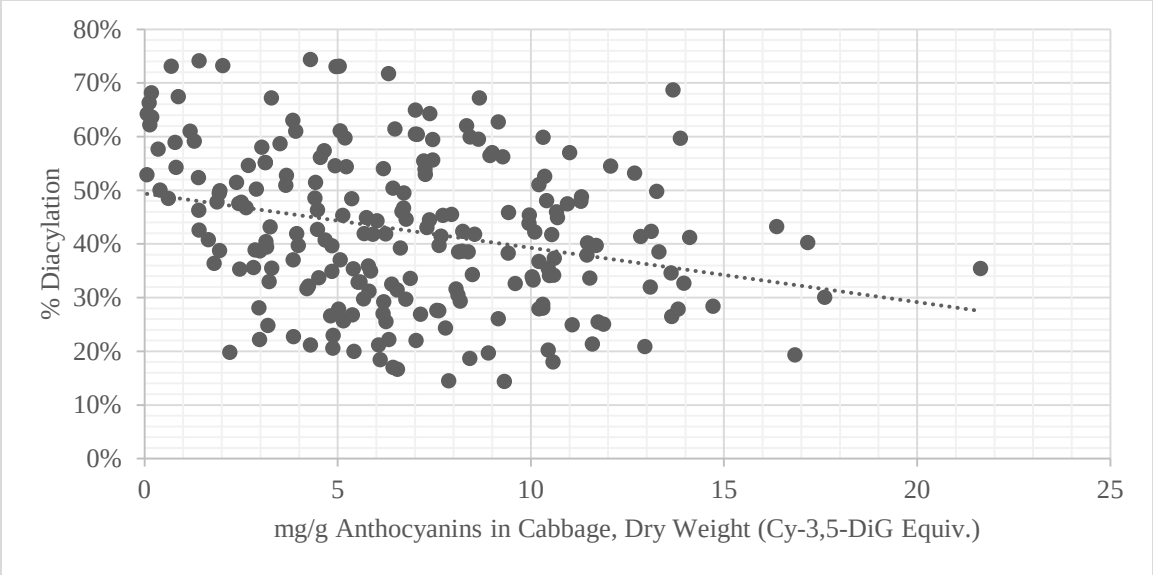


Figure 3.20 Correlation between total anthocyanin concentration (mg/g cabbage, dry weight, cyanidin-3,5-diglucoside equivalent (Cy-3,5-DiG Equiv.)) and percent diacylation across all four germplasm repositories (43 USDA, 24 HRI , 73 IIPK, and 32 CGN accessions) and years screened. Negative Pearson correlation of -0.28 was calculated.

While there is correlation between total anthocyanin concentration and percent diacylation the results indicated that accessions with both high anthocyanin content and high acylation are rare. As noted previously, several genotypes were identified with both high concentrations of anthocyanins and greater than average diacylation, such as BRA770 (Figure 3.12), which can be used for breeding material.

CHAPTER 4

ASSESSMENT OF BIOMASS CORRELATIONS WITH PLANT AGE, PLANT DENSITY AND ANTHOCYANIN CONTENT IN RED MUSTARD (*Brassica juncea)*

4.1 INTRODUCTION

4.1.1 Anthocyanins in *Brassica juncea*

Anthocyanins are polyphenolic phytochemicals produced within diverse plant organs (leaves, stems, flowers, fruits, seeds, and grains) and conserved across many plant species. Cyanidin, pelargonidin, delphinidin, malvidin, petunidin, and peonidin are the most common anthocyanins found within the plant kingdom (Gould et al., 2008). These compounds are of current interest due to their cardiovascular, cancer preventative, and neuroprotective health benefits (Andres-Lacueva et al., 2005; Kalt et al., 2008; Lee et al., 2005; Stull et al., 2010; H. Wang et al., 1999; L.-S. Wang & Stoner, 2008; Youdim et al., 2002) and are relevant for the food industry for their ability to produce a wide variety of colors including red, purple, blue, and green (Markakis, 1982).

Studies show the Brassicaceae family can produce pelargonidin and cyanidin in *Raphanus sativus* (Radish) (Giusti, Ghanadan, & Wrolstad, 1998; Lin, Sun, Chen, & Harnly, 2011b) and cyanidin in *Brassica oleracea* (Ahmadiani et al., 2014; Arapitsas et al., 2008) species. Red mustard genotypes have been selected within *B. juncea* that are reported to be cyanidin based (Lin et al., 2011a). Twenty-seven acylated cyanidin 3-sophoroside-5-diglucosides, 24 acylated cyanidin 3-sophoroside-5-glucosides and 3 acylated cyanidin triglucoside-5-glucosides were identified in a study by Lin et al.

(2011). No nonacylated anthocyanins were present. This study also showed that anthocyanins in *Brassica juncea* are more likely to have sophoroside sugars than their *B. oleracea* relatives. They are also capable of being triacylated according to Lin et al. (2011) that has not been reported within *B. oleracea.*

4.1.2 Plant stress impact on anthocyanin content and plant biomass

Plant stressors, such as light, nutrient deficiency, drought stress, and cold are known to affect anthocyanin concentrations and composition (Chalker-Scott, 1999; Mark Hodges & Nozzolillo, 1995; Socquet-Juglard et al., 2016). These stressors are known to decrease biomass accumulation. Other factors, such as period of time given for growth and development, as well as plant density, are known to affect plant biomass (Momoh & Zhou, 2001).

4.1.3 Absorption spectrum identification of non, mono, and diacylated anthocyanins

Acylated anthocyanins have an absorption peak at 330 nm (E_{acyl}), the maximal absorption of the acyl group. Within the anthocyanin absorption spectrum at pH 2, there is a peak within the visual range at 520 nm (E_{vis}) that is the maximal absorption of cyanidin-based anthocyanins (Figure 4.1). An E_{acyl}/E_{vis} absorptivity ratio of 53–69% indicates monoacylation of anthocyanin species, while E_{acyl}/E_{vis} absorptivity ratio of 98–128% indicated diacylation of anthocyanins (Arapitsas et al., 2008; Harborne, 1958).

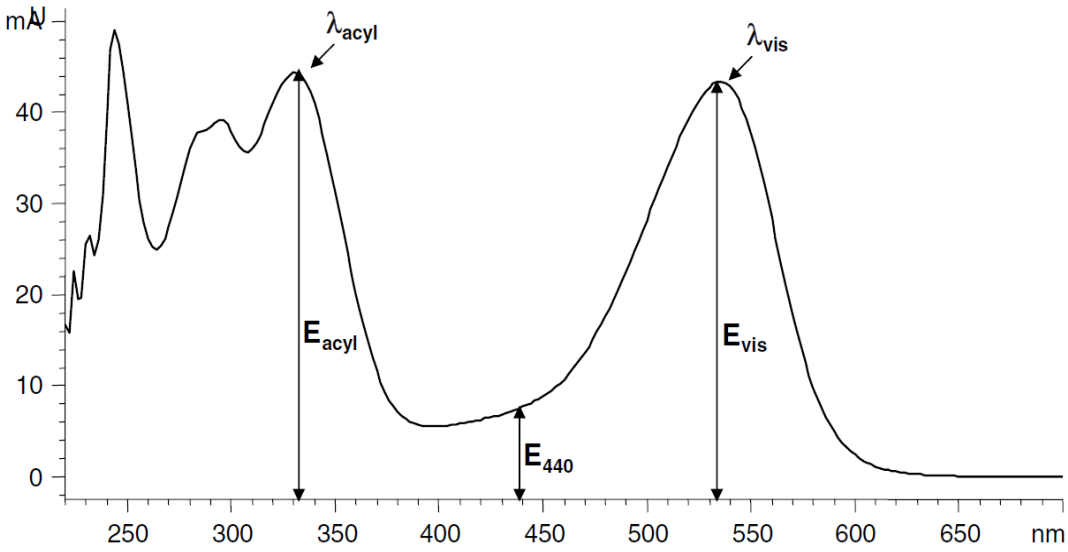


Figure 4.1 Absorption spectrum of cyanidin based anthocyanins at pH 2 indicating Eacyl (the peak at which acyl groups maximally absorb) and Evis (the point where anthocyanins maximally absorb within the visible specrum) (Arapitsas et al., 2008).

The major objectives of this study were to identify non-, mono-, and diacylation of different anthocyanins within *B. juncea;* to assess anthocyanin concentration and profile change with temporal increase of plant biomass; and to evaluate plant density impact on biomass and its influence on anthocyanin content in *B. juncea* red mustards.

4.2 METHODS AND MATERIALS

4.2.1 Plant materials and growth conditions

Three red cabbage (‘Futurima’, ‘G30902’, and ‘Red Express’), three kale (‘Redbor’, ‘Red Russian’, and ‘Scarlet’), three Brussels sprout (‘Rubine’, ‘Red Ball’, and ‘Falstaff’) and three mustard (‘Red Muzuna’, ‘Scarlet Frills’, and ‘Red Giant’) genotypes were selected for evaluation of *Brassica* market class. These were seeded in triplicate with two repetitions in 72-cell styrofoam trays in 'Cornell Mix' (Boodley and Sheldrake,

1982) with one seed per cell (Speedling, Sun City, Fla.). Samples were harvested five weeks post seeding.

Seed of red mustard cultivar ‘Crimson Red’ (Kitazawa Seed Co., Oakland, CA) was chosen for the study based on high expression of anthocyanins in the leaves of juvenile plants. Seeds was sown in 72, 32, and 18- cell styrofoam trays in 'Cornell Mix' (Boodley and Sheldrake, 1982) with one seed per cell (Speedling, Sun City, Fla.). At 29 days post seeding, three plants from each cell size was harvested and bulked. Plants were harvested every other day for 20 days. Three replications of three bulked plants were harvested.

4.2.2 Reagents and Standards

Ultrapure water used in chemical extraction and processing was generated by filtering deionized water through a 0.22 µm filter in a Milli-Q integral water purification system (Millipore Corporation, Bedford, MA, USA). Acids and organic solvents were HPLC grade and purchased from Fisher Scientific (Pittsburgh, PA, USA). Cyanidin-3,5-diglucoside reference standard was obtained from Extrasynthese (Genay, France).

4.2.3 Sample preparation

Bulked samples were frozen at -20°C post-harvest and over a 48 hour period of time were lyophilized with a MX53 Magnum series freeze-drying unit (Millrock Technology, Kingston, NY, USA). Dry samples were ground with a mortar and pestle until particles were fine enough to fit through a 40mm mesh screen. Samples were placed in opaque 50 mL centrifuge tubes and stored at room temperature.

Samples were weighed into 50 mg portions, placed into 15 ml Eppendorf centrifuge tubes (Eppendorf, Hauppauge, NY, USA), and triple extracted in 1ml 0.01M hydrochloric acid (HCl) in methanol (MeOH). After each extraction, the samples were sonicated for 1 minute, centrifuged for 5 minutes at 4°C using and 3000 x g with an Eppendorf 5810R centrifuge (Eppendorf, NA, Hauppauge, NY, USA). Each of the three supernatant aliquot portions were combined into a glass Pyrex test tube (Corning Incorporated, Corning, NY, U.S.), placed in a 35°C water bath, and dried with a stream of nitrogen gas generated by a Parker Balston model N2-04 nitrogen generator (Parker Hannifin Corp, Haverhill, MA, USA). Dried samples were resuspended in 1 ml of 0.01M HCl in water, filtered throught a 0.22 µm polyethersulfone (PES) membrane (Krackeler Scientific, Inc., Albany, NY, USA) and injected into an Agilent 1260 Infinity series high performance liquid chromatography (HPLC) system (Agilent Technologies, Santa Clara, CA, US).

4.2.4 Anthocyanin speciation via high performance liquid chromatography (HPLC)

The Agilent 1260 Infinity series HPLC system was fitted with a Kinetex® core-shell pentafluorophenyl (PFP) column (100 mm x 2.1 mm, 2.6 µm diameter particle size, 100 Å pore size) proceeded by an inline Krudkatcher® column filter (Phenomenex, Torrance, CA, USA). Five µl of sample was injected into the HPLC with a flow rate of 0.2 ml/min and heated to 35°C. Mobile phase A consisted of 0.5% (v/v) phosphoric acid (H_3PO_4) in water and mobile phase B consisted of 0.5% (v/v) H_3PO_4 in MeOH. Mobile phase concentrations and run time are shown in Table 4.1. After each run was completed, a ten minute post run of 85% A and 15% B ensured a return to chemical equilibrium.

Samples were assessed by a diode array detector, and a Max-Light cartridge flow cell (1 µl volume and 10 mm path) was set to monitor at 520 nm using a 630 nm reference.

Table 4.1 Concentration of mobile phase A (0.5% (v/v) phosphoric acid (H_3PO_4) in water) and mobile phase B (0.5% (v/v) H_3PO_4 in methanol (MeOH)) shown in percentages at each time point during a sample run of 40 minutes

Time (Minutes)	% mobile phase A	% mobile phase B
0	85%	15%
10	70%	30%
15	65%	35%
38	50%	50%
40	85%	15%

A standard curve ranging from 0.01 ppm to 250 ppm was made in triplicate ($r^2 <$ 0.9999) using a cyanidin-3,5-diglucoside standard that resulted in a percent relative standard deviation (%RSD) of less than 0.5. The computer system monitored and data analyzed using Agilent Chemstation software version B.04.03 and service pack 2 with spectral software module (Agilent Technologies, Santa Clara, CA, US). Speciation between nonacylated, monoacylated and diacylated anthocyanins was originally tentatively judged based on elution time, absorbance spectrum, and previously published literature (Arapitsas et al., 2008; Harborne, 1958; Lin et al., 2011a).

4.2.5 Statistical analysis

Statistical analysis was undertaken using JMP Pro 12 (SAS Institute, Cary, NC, USA) software. Linear regressions models were preformed to assess trends in anthocyanin content over time and to assess correlation between biomass and anthocyanin content. Significance was determined by ANOVA and student t-tests with *P*

values < 0.05. A Pearson correlation was performed to assess relationships between characteristics.

4.3 RESULTS AND DISCUSSION

4.3.1 Identification of pigments

Twenty-nine peaks were found to have characteristic anthocyanin absorption spectra (Figure 4.2 and Table 4.2). Of these, all had a E_{acyl} peak at 330 nm, indicating acylation of all anthocyanins within *B. juncea* mustard types, in agreement with Lin et al. (2011a). Eleven of these peaks could not be identified as mono, di, or triacylated due to having E_{acyl}/E_{vis} absorption ratios between 70-97% or above 128%. Unidentified peaks were still incuded in total anthcyanin content and maintained as unknowns when calculating percent diacylation.

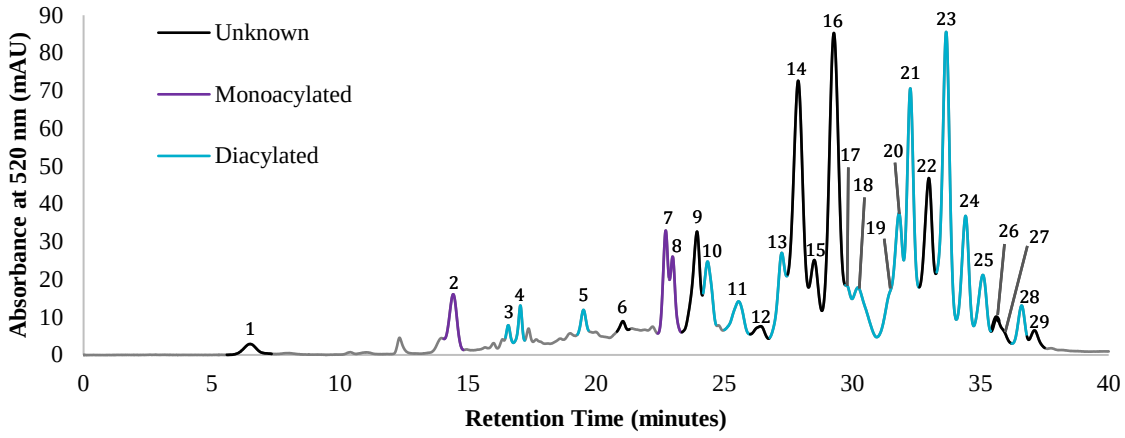


Figure 4.2 Chromatogram indicating the position and retention time of the 29 peaks identified through absorption spectrum E_{acyl}/E_{vis} methods

Table 4.2 Anthocyanin peak characteristics in red mustard (*Brassica juncea*), including retention time, E_{acyl}/E_{vis} percentage for identification, and assignment of unknown (U), non (N), mono (M), or Diacylated (D) given.

#	Time	E_{acyl}/E_{vis}	Assignment
1	6.497	94%	U
2	14.424	66%	M
3	16.57	118%	D
4	17.042	110%	D
5	19.508	101%	D
6	21.046	155%	U
7	22.715	50%	M
8	22.985	58%	M
9	23.935	95%	U
10	24.344	104%	D
11	25.554	120%	D
12	26.427	168%	U
13	27.243	120%	D
14	27.881	88%	U
15	28.507	134%	U
16	29.274	79%	U
17	29.711	114%	D
18	30.226	101%	D
19	31.551	110%	D
20	31.818	108%	D
21	32.259	105%	D
22	32.983	133%	U
23	33.659	108%	D
24	34.417	116%	D
25	35.093	116%	D
26	35.623	129%	U
27	35.8	145%	U
28	36.599	119%	D
29	37.099	133%	U

4.2.2 Biomass increase relative to plant density

Biomass increased over 18 days (Figure 4.3) with a *P* value < 0.05 for 72 (R^2 = 0.814) and 32 (R^2 = 0.8762) cell density and a *P* value < 0.05 for 18 (R^2 = 0.4578) cell density. At day thirty-nine, 32 and 72 cell seedling tray densities began flowering. At day 47, 18 cell density plants began to flower.

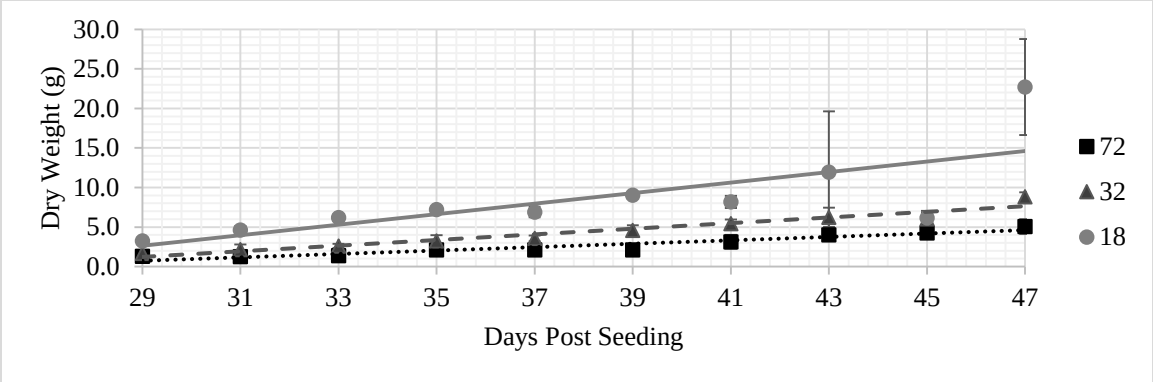


Figure 4.3 Biomass increase of red mustard (*Brassica juncea*) cultivar 'Crimson Red' (Kitazawa Seed Co., Oakland, CA) in dry weight (g) over the course of 18 days. Harvest started at 29 days post seeding and took place asynchronous days until 47 days post seeding. Plants were subjected to three different density conditions: 18 cell, 32 cell, and 72 cell seedling trays. Each data point present three bulked plants. Linear regressions are shown.

Biomass accumulation was affected by plant density, with 18 cell crowding having a mean dry weight of 6.9 g (A), 32 a mean dry weight of 4.4 g (B), and 72 a mean DW of 2.7 g (C) across all harvest days (Figure 4.4). This was significantly different with an ANOVA *P* value < 0.05.

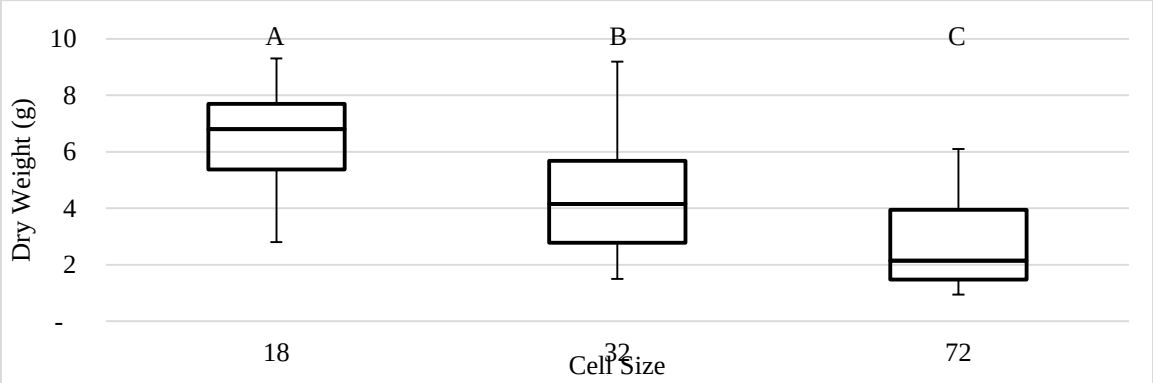


Figure 4.4 Box pots showing biomass differences in dry weight (g) between red mustard (*Brassica juncea*) cultivar 'Crimson Red' (Kitazawa Seed Co., Oakland, CA) plants grown at different plant densities. Plants were seeded in 18, 32, and 72 cell seedling trays. Student's t test statistical analysis produced the connecting letter report given. Non-connecting letters indicate significant differences.

4.3.3 Anthocyanins over time and density effects

Total anthocyanin content was not significantly affected by cell size crowding when assessed by an ANOVA (*P* value > 0.05). Data was combined for further analysis due to this lack of significance. Total anthocyanin content significantly decreased over the course of 18 days (Figure 4.5) (*P* value < 0.05). There was some variation within the data indicated by an $R^2 = 0.4626$ (Figure 4.5). Variation decreased with increased plant age.

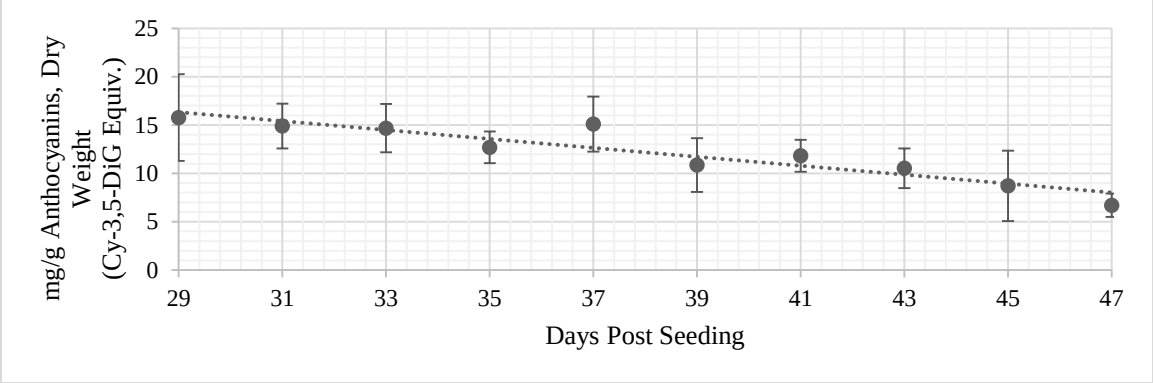


Figure 4.5 Linear regression showing total anthocyanin concentrations (expressed a cyanidin-3,5-diglucoside equivalents (Cy-3,5-DiG Equiv.)) changed with increasing plant age in *Brassica juncea* red mustard cultivar 'Crimson Red' (Kitazawa Seed Co., Oakland, CA). Harvest started 29 days post seeding and continued every two days until 47 days post seeding. All plant density (18, 32, and 72 cell seedling plant density) data was pooled and averaged for each data point. Error bars indicate standard deviation.

Figure 4.6 indicated that percent diacylation decreased over time, and that interaction with cell size also influenced percent diacylation. As styrofoam tray cell size had an effect on percent diacylation, results were analyzed separately. The 72 and 32 cell density plants had higher significance and less variation within the data (*P* value < 0.05 for both with an $R^2 = 0.4744$ for 72 and $R^2 = 0.6167$ for 32) when compared to 18 cell density (*P* value < 0.05 and $R^2 = 0.3775$).

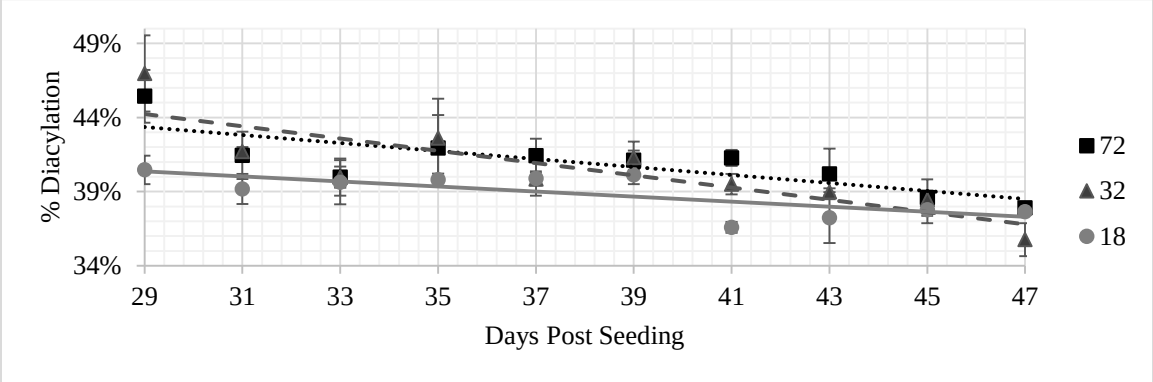


Figure 4.6 Effect of plant age on % diacylation by linear regression in *Brassica juncea* red mustard cultivar 'Crimson Red' (Kitazawa Seed Co., Oakland, CA). Data was separated by plant density. Plants were seeded at 18, 32, and 72 seedling tray densities. Plants were harvested on alternating days between 29 and 27 days post seeding. Each data point represents three bulked plants.

Plant density and resource differences among cell size influenced diacylation of anthocyanins. The mean percent diacylation between 72 cell trays (41.8%) and 32 cell trays (41.7%) were not different. Plants grown in 18 cell trays were different ($P < 0.05$), averaging 39.7% across all repetitions and harvest dates (Figure 4.7). Percent monoacylation increased with increasing cell size: 72 cell averaged 7.6% (B), 32 cell averaged 7.8% (AB), and18 cell averaged 8.1% (A). Unknown anthocyanin content was also higher for 18 cell trays (43.6%) when compared to 32 (41.3%) and 72 (40.7%) cell densities ($P < 0.05$).

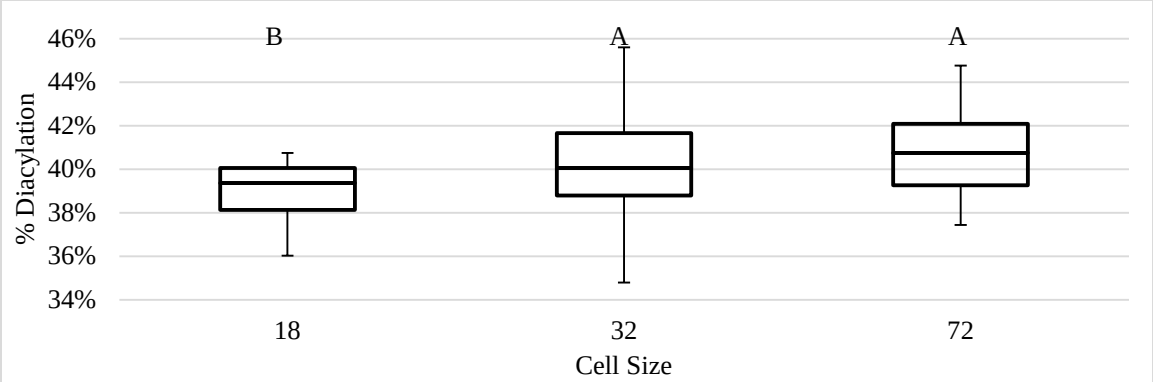


Figure 4.7 Box plot showing percent diacylation differences by plant density. *Brassica juncea* red mustard cultivar 'Crimson Red' (Kitazawa Seed Co., Oakland, CA) was seeded at plant densities of 18, 32, and 72 cell seedling trays. The connected letter report shown was generated using a student's t test and indicated significant differences between 18 cell seedling tray density from the 32 and 72 cell size, but no different between 32 and 72 cell size density.

4.3.4 Effect of biomass on anthocyanin content

Data from all plant ages (29-47 days post seeding) and densities (18, 32, and 72 cell seedling trays) was pooled for assessment of biomass effect on anthocyanin oncetrations. A negative correlation (-0.44, $P < 0.05$) between increasing biomass in dry weight (g) and total anthocyanin concentrations within the red mustards across all harvest dates and density conditions was observed (Figure 4.8). This is consistent with the data reported that biomass increased over time and total anthocyanin concentrations decreased over time. This agrees with previous studies showing that anthocyanins are found in the highest concentrations in certain juvenile types as they act as a photoprotective mechanism before other quenching methods develop (Liakopoulos et al., 2006; Murray, Smith, & Hackett, 1994). Conversely, plant density influenced biomass but did not have a significant effect on total anthocyanin concentrations.

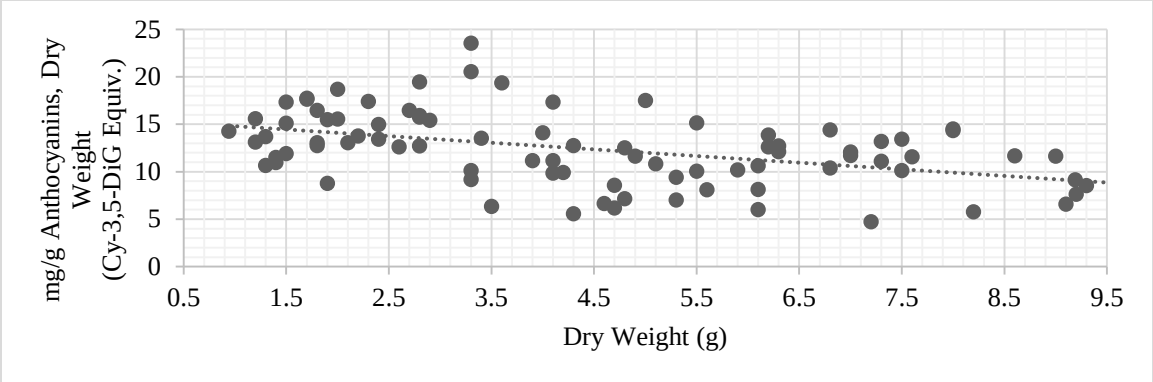


Figure 4.8 *Brassica juncea* cultivar 'Crimson Red' negative Pearson correlation (-0.44) between biomass (dry weight (g)) and anthocyanin concentrations (cyanidin-3,5-diglucoside equivalents (Cy-3,5-DiG Equiv.)). Data from all plant ages (29-47 days post seeding) and densities (18, 32, and 72 cell seedling trays) were pooled. Each data point represents three bulked plants.

There was a strong negative correlation (18 cell, -0.25; 32 cell, -0.72; and 72 cell, -0.60) between total anthocyanins and percent diacylation (*P* value < 0.05 for all cell types) shown in Figure 4.9. Both increased plant age and decreased plant density had a positive correlation with plant biomass and a negative correlation with percent diacylation for all density conditions.

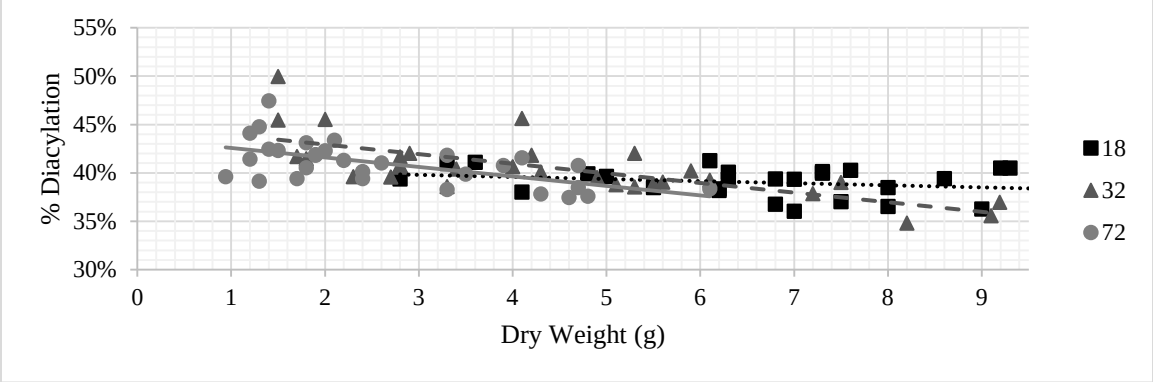


Figure 4.9 Correlation between biomass (dry matter (g)) and percent diacylation of *Brassica juncea* cultivar 'Crimson Red'. Data from all plant ages ranging from 29 to 47 days post seeding was pooled. Cells size effect on diacylation was significant (*P* value > 0.05) and was kept separate with each density condition having a negative Pearson correlation: 18 cell (-0.25), 32 cell (-0.72) and 72 cell (-0.60). Each data point represents three bulked plants.

4.3.5 Conclusions

Plant density had no impact on the concentration of anthocyanins within juvenile pigmented mustard plants. Anthocyanin profile was influenced, however, by increased plant density and decreased plant resources from 18 cell seedling flats to 32 or 72 seedling flats. Environmental stress from different nutrient or water availability may also have contributed to this shift in anthocyanin profile.

A strong negative correlation was identified between overall biomass and both anthocyanin concentration and percent diacylation of anthocyanins in red mustard plants. Biomass production and anthocyanin production are both large carbon sinks for the plant system, and may have had an effect. As the plant diverted more energy to growth and development, anthocyanin content became diluted, which likely resulted in the decrease in diacylation, as the production and attachment of acyl groups takes both time and carbon.

Prior literature suggests that young juvenile plant of certain species, such as grapes and ivy, are more likely to have higher anthocyanin concentrations in foliar tissues than older plants, as they use these compounds as an early stage photoprotector (Liakopoulos et al., 2006; Murray et al., 1994). However, this is not observed to be the case for *B. oleracea* pigmented cabbage and kale types (Socquet-Juglard et al., 2016). It is likely that *B. juncea*, being closely related to *B. oleracea*, does not exhibit the pattern also.

A high correlation was observed between biomass and percent diacylation. This could be an effect of light or resource access for each condition type. The lower density

(18 cell) juvenile plants have more light availability and resources, whereas the more highly populated (72 cell) plants have lower light and resource availability. Studies have linked anthocyanin production to light, water, and nutrient availability (Chalker-Scott, 1999). However, this genotype was selected for low environmental impact on anthocyanin content, so this is likely not a large factor.

In conclusion, biomass had an effect on both anthocyanin concentration and content in the *B. juncea* red mustard cultivar. As biomass was correlated with decreased anthocyanin concentration and percent diacylation, it may be important to grow plant tissue to biomass prior to inducing stressors. This could result in large quantities of high-anthocyanin tissues with increased acylation, and avoid issues with fitness costs. Additional factors that affect biomass, such as nutrient deficiency, cold stress, and light stress, should be further investigated to determine which of the primary resources (e.g. light, water, and nutrient changes), have the highest impact on anthocyanin content and anthocyanin type.

CHAPTER 5

CONCLUSIONS AND DISCUSSION

Anthocyanins are health-promoting in that they reduce cardiovascular issues, reduce cancer rates, and they are neuroprotective (Andres-Lacueva et al., 2005; Kalt et al., 2008; Lee et al., 2005; Stull et al., 2010; H. Wang et al., 1999; L.-S. Wang & Stoner, 2008; Youdim et al., 2002). Additionally, these compounds play a practical role in industry as a natural food color capable of replacing their artificial counterparts

(Bkowska-Barczak, 2005; Francis & Markakis, 1989; He & Giusti, 2010; Markakis, 1982).

The Brassicaceae family is known for producing several health promoting phytochemicals, including anthocyanins. The genes for producing different anthocyanin structures vary within the different species and market classes of this family (Ahmadiani et al., 2014, 2014; Chiu et al., 2010; Giusti et al., 1998; Lin et al., 2011a, 2011b). Environmental factors such as light, cold, and drought stress are known to influence the regulatory genes responsible for controlling concentrations and ratios of different anthocyanins (Chalker-Scott, 1999; Jaakola, 2013).

Germplasm evaluations have been undertaken with *Brassica oleracea* to investigate diversity of collard glucoraphanin and other glucosinolates (Stansell et al., 2015), carotenoid, tocopherol and ascorbate content (Kurilich et al., 1999), with further research investigating lutein, β-carotene and chlorophyll concentrations (Kopsell et al., 2004). Anthocyanins have been researched in some *B. oleracea* cultivars (Ahmadiani et al., 2014; Arapitsas et al., 2008; Wiczkowski, Szawara-Nowak, & Topolska, 2013); however, the diversity of anthocyanin concentrations and content had not been documented within genotypes and the biodiversity available in *B. oleracea* germplasm repositories.

Certain environmental factors and their effect on anthocyanin content have been well researched, such as how stressors including light, cold, and drought may increase or decrease anthocyanin content (Leyva et al., 1995; Mark Hodges & Nozzolillo, 1995; Socquet-Juglard et al., 2016). We examined how cooling may affect diverse genotypes of mature post-harvest cabbage heads for storage consideration. While stress may increase

anthocyanin concentration, these stressors frequently result in reduced biomass in growing plants. This yield trade off, where one beneficial trait is gained at the expense of another, is defined as a fitness cost. It has not been clearly assessed how biomass may correlate with anthocyanin concentrations and content.

Different genetic and environmental influences need to be addressed in order for industry to pragmatically utilize *Brassica* species as a source of anthocyanins. This research forms the base for developing reliable high quality anthocyanin extracts from *Brassica* species.

Characterization of anthocyanin localization can help assess Brassicaceae market classes capable of producing the highest density of anthocyanins. *Raphanus sativus* (radish) genotypes capable of internal pigmentation were evaluated alongside RGB pigmentation analysis of anthocyanins produced from cabbage and kale using a pH range from 1 to 12. This work indicated that radish was capable of producing a higher density of pigmentation when compared to cabbage and kale genotypes, which could only produce anthocyanin in outer mesophyll and epidermal cells; however, the colors produced were more ‘orange’ at all pH values when compared to cabbage and kale genotypes due to the presence of pelargonidin. Red cabbage was chosen as the candidate Brassica most likely to be able to produce both high quality cyanidin based anthocyanins and relatively high concentrations of anthocyanins due to its ability to produce many dense, thin leaves within the head with very low levels of chlorophyll. Cabbage is storable, highly productive, and has a high harvest index, making it a practical crop for low cost production of anthocyanins.

Screening *B. oleracea* var. *capitata* (cabbage) germplasm from four different repositories encompassing 173 accessions was undertaken to assess diversity of anthocyanin content in red cabbage. Within all of the repositories, the large range of anthocyanin concentration and percent diacylation observed suggests genetic diversity within these repositories could be used for crop improvement for higher anthocyanin production. Breeding efforts focused on an increased anthocyanin concentrations and selected for specific anthocyanin profiles could result in new cultivars for use as natural color sources.

Samples from the CGN repository were subjected to cold storage at 10°C, and; this specific post-harvest cooling process on these mature genotypes resulted in an increase in both anthocyanin concentration and percent diacylation. This could be a promising finding for industry use, as it indicates that the practice of growing and harvesting one crop of cabbage per year is a practical method for producing large quantities of anthocyanin without having concerns for anthocyanin degradation during storage. Storage would also enable post-harvest processing over time, improving the processing economics.

Mature and juvenile *B. oleracea* types have anthocyanin concentrations and profiles that are highly influenced by the environment. *Brassica juncea* red mustard genotypes typically show higher anthocyanin expression as juveniles than *B. oleracea*, making ‘Crimson Red’ a good candidate. (for what?). It was of interest to use *B. juncea* to assess correlation between biomass and anthocyanin content with limited environmental interference, as both total anthocyanins and percent diacylation decreased with increased biomass. This has to important practical applications. Thus, harvesting

plant materials at a younger, lower biomass may be important if higher anthocyanin concentrations and levels of acylation are desired. Also, there may be a fitness cost (i.e., lower biomass) for actively growing plants to produce the highest possible anthocyanin concentrations. If this is the case, it might be better to grow plant materials to desired biomass and then induce stressors to slow growth and increase anthocyanin concentrations, as was done with the mature cabbage heads placed in cold storage.

Increasing environmental control may help demonstrate exactly how environment versus biomass affects anthocyanin content. For this purpose, further investigation should be performed in bioclimatic growth chambers paired with hydroponic systems with precise control of temperature, nutrients, and light-emitting diode (LED) lighting intensity and wavelengths. Control of environment could help pinpoint exact genetic versus environmental control of anthocyanin production, as it would enable precise environmental parameters and algorithms that would be most cost effective for producing desired anthocyanin profiles and concentrations.

Radish is only capable of producing pelargonidin, the ‘orange’ anthocyanin, and cyanidin, the ‘cyan’ or ‘blue-green’ anthocyanin (Giusti et al., 1998). Recent studies have shown some purple-hearted cultivars are capable of producing low pelargonidin and high cyanidin anthocyanin extracts (Lin et al., 2011b). Additional screening could highlight genotypes that are able to produce large quantities of anthocyanins high in cyanidin and low in pelargonidin. Further evaluation of controlled environment growth could also enable higher relative production of anthocyanins from this species, especially if combined with breeding efforts.

Cell culture production of cells producing high levels of desirable anthocyanins could also be a workable approach if *R. sativus* callus is capable, for example, of producing anthocyanin. Use of tissue culture or liquid suspension culture has the potential to be more environmentally controlled than bioclimatic growth chambers, eliminating many issues associated with growing plants in the open field where conditions are environmentally inconsistent and vary in soil heavy metal content, nutrient availability (Mark Hodges & Nozzolillo, 1995), and weather patterns (Chalker-Scott, 1999). These conditions can result in inconsistent anthocyanin concentrations and ratios that would not be desirable for industry production; however, it is unlikely that the yields required could be generated efficiently using a cell culture system using current technology.

In conclusion, analysis of different *B. oleracea* morphotypes and related crucifers such as radish (*R. sativus*) and mustard (*B. juncea*) resulted in a focus on assessing anthocyanin levels in cabbage germplasm. The initial Brassicaceae screening also lead to further assessment of 'Crimson Red' mustards, the only *Brassica* crop identified in this thesis with the ability to produce high anthocyanin yields regardless of environment, to better understand how biomass directly effects anthocyanin content in mustard types. This work is the first step for a better understanding of the optimal production of anthocyanins in a controlled environment. This, paired with future work with bioclimatic chambers to assess the perfect growing conditions, can lead to economically efficient vertical farming practices. Pairing select elite genotypes with precise environmental control, as is possible in vertical farming, has potential to change the color industry, and, to a larger extent, the food industry.

APPENDIX

Appendix 3.1 USDA 43 accessions grown and screened in summer of 2012 in Geneva, NY, USA

Accession #	Old Accession #	Other database Names	Country of Origin
G 29739	-	12636	Netherlands
G 29818	-	-	United States
G 29819	-	-	United States
G 29936	-	-	Bulgaria
G 29941	-	-	Bulgaria
G 30902	-	HRI - WSB 3582	United Kingdom
G 30903	-	HRI - WSB 6852	United Kingdom
PI 244987	-	-	United Kingdom
PI 244991	-	-	United Kingdom
PI 244993	-	-	United Kingdom
PI 244994	-	-	United Kingdom
PI 244999			United Kingdom
PI 245000	-	-	United Kingdom
PI 245010	-	-	France
PI 245012	-	-	France
PI 246046	-	-	Netherlands
PI 246047	-	-	Netherlands
PI 246049	-	-	Netherlands
PI 246056	-	-	Netherlands
PI 246057	-	-	Netherlands
PI 246059	-	-	Netherlands
PI 246060	-	-	Netherlands
PI 246085	-	-	Netherlands
PI 246086	-	-	Netherlands
PI 246087	-	-	Netherlands
PI 246106	-	-	Netherlands
PI 246107	-	-	Netherlands
PI 246108	-	-	Netherlands
PI 246109	-	-	Netherlands
PI 261604	-	-	United States
PI 261758	-	-	United States
PI 275004	-	-	United States
PI 291998	-	-	Israel
PI 296133	-	-	United States
PI 329197	-	-	Netherlands
PI 343634	-	-	Russian Federation
PI 357387	-	-	Macedonia
PI 370358	-	-	Macedonia
PI 662582	G 30136	-	United States
PI 662608A	-	-	-
PI 662648	G 30672	-	Bulgaria
PI 662706	G 30904	HRI - WSB 2656	United Kingdom

Appendix 3.2 HRI 34 accessions grown and screened in summer of 2012 in Geneva, NY, USA

Accession #	Country of Origin
HRI 001912	N/A
HRI 001945	N/A
HRI 002147	N/A
HRI 002538A	N/A
HRI 002568	N/A
HRI 002657A	N/A
HRI 002658A	N/A
HRI 003582	N/A
HRI 003583	N/A
HRI 003584	N/A
HRI 003585	N/A
HRI 003586	N/A
HRI 003587	N/A
HRI 003588	N/A
HRI 003589	N/A
HRI 004606	N/A
HRI 004620	N/A
HRI 004621	N/A
HRI 004625	N/A
HRI 005233	N/A
HRI 005697A	N/A
HRI 005887	N/A
HRI 006215	N/A
HRI 006233	N/A
HRI 006852	N/A
HRI 007835	N/A
HRI 007836	N/A
HRI 007851A	N/A
HRI 007852A	N/A
HRI 007854	N/A
HRI 007858A	N/A
HRI 008373	N/A
HRI 012994	N/A

Appendix 3.3 Top USDA and HRI accessions grown, replicated, and assessed in 2013 in Geneva, NY, USA.

Accession #
G 30902
G 30903
G 30904
PI 244993
PI 244999
PI 246047
PI 246085
PI 246109
PI 275004
PI 662605
PI 662648
HRI 002657A
HRI 002658A
HRI 003583
HRI 003586
HRI 003589
HRI 004620
HRI 005887
HRI 007825A
HRI 007835
HRI 007851A
HRI 007852A
HRI 008373
HRI 012994

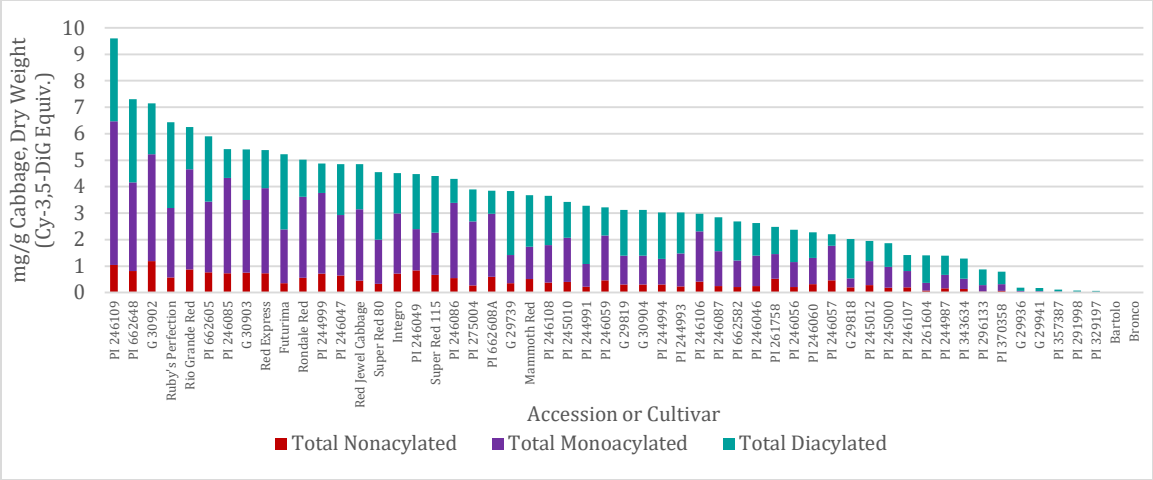
Appendix 3.4 IPK 73 accessions gown and screened at Freeville, NY, USA in 2014.

Accession #	Country of Origin
BRA277	Germany
BRA279	Germany
BRA281	Unknown
BRA282	Unknown
BRA400	United Kingdom
BRA401	Romania
BRA403	Netherlands
BRA404	Netherlands
BRA737	Germany
BRA739	Netherlands
BRA740	Netherlands
BRA741	Denmark
BRA742	Netherlands
BRA751	Hungary
BRA752	-
BRA753	Germany
BRA756	Unknown
BRA758	Czech and Slovak Federal Republic
BRA760	Netherlands
BRA762	Sweden
BRA763	Denmark
BRA764	Netherlands
BRA766	France
BRA770	Sweden
BRA774	Denmark
BRA775	Poland
BRA776	Denmark
BRA778	Unknown
BRA782	Netherlands
BRA783	Netherlands
BRA785	Czech and Slovak Federal Republic
BRA786	United Kingdom
BRA789	Germany
BRA790	Germany
BRA792	Bulgaria
BRA793	Czech and Slovak Federal Republic
BRA794	Czechoslovakia
BRA796	Netherlands
BRA797	USA
BRA798	Denmark
BRA799	France
BRA800	Sweden
BRA802	Netherlands
BRA803	Sweden
BRA805	Austria
BRA902	France
BRA903	Switzerland
BRA1763	Unknown
BRA1764	Unknown
BRA1765	Unknown

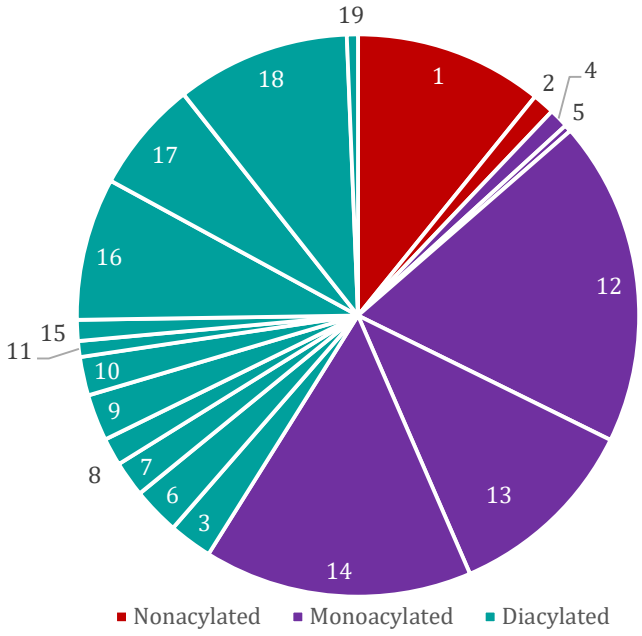
BRA1767	Netherlands
BRA1800	Germany
BRA1801	Unknown
BRA1871	Switzerland
BRA1919	Italy
BRA2112	Germany
BRA2115	Germany
BRA2198	Unknown
BRA2200	Unknown
BRA2418	Germany
BRA2433	Germany
BRA2470	Turkey
BRA2489	Germany
BRA2496	Germany
BRA2536	Germany
BRA2562	Germany
BRA2597	Germany
BRA2609	Germany
BRA2610	Germany
BRA2704	Denmark
BRA2708	Union of Soviet Socialist Republics
BRA2824	Germany
BRA2826	United Kingdom

Appendix 3.5. CGN 32accessions grown and screened in 2015 in Freeville, NY.

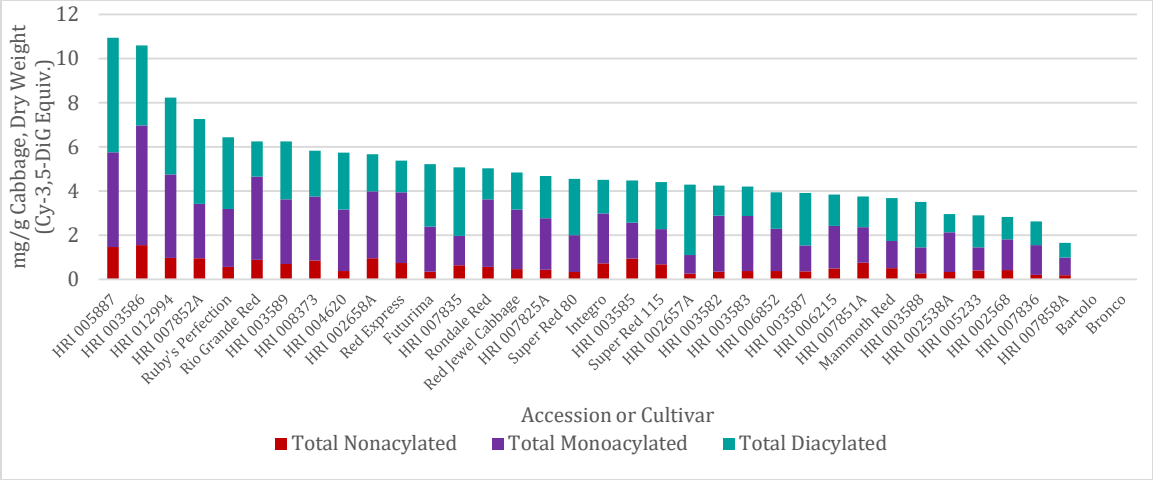
Accession #	Country of Origin
CGN07081	Denmark
CGN07082	Denmark
CGN07083	Denmark
CGN07084	Netherlands
CGN07085	Netherlands
CGN07086	Netherlands
CGN07087	Netherlands
CGN07088	Netherlands
CGN07089	Netherlands
CGN07090	Netherlands
CGN07091	Netherlands
CGN07092	Netherlands
CGN07093	Netherlands
CGN07094	Netherlands
CGN11038	Netherlands
CGN11039	Netherlands
CGN11040	Denmark
CGN11041	Netherlands
CGN11042	Netherlands
CGN11043	Netherlands
CGN11165	Denmark
CGN14019	Netherlands
CGN14063	Netherlands
CGN14064	Denmark
CGN15769	Bulgaria
CGN15771	Netherlands
CGN15772	Netherlands
CGN18434	Switzerland
CGN18435	Czechoslovakia
CGN18436	Germany
CGN18437	Poland
CGN18438	Romania



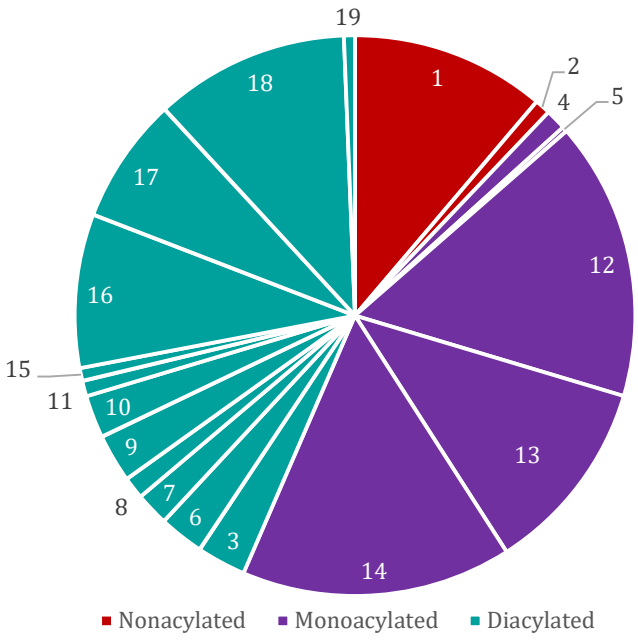
Appendix 3.6 Total anthocyanin content with nonacylation, monoacylation, and diacylation shown for 43 USDA accessions compared to common red cabbage cultivars grown Geneva, NY, USA in summer of 2012



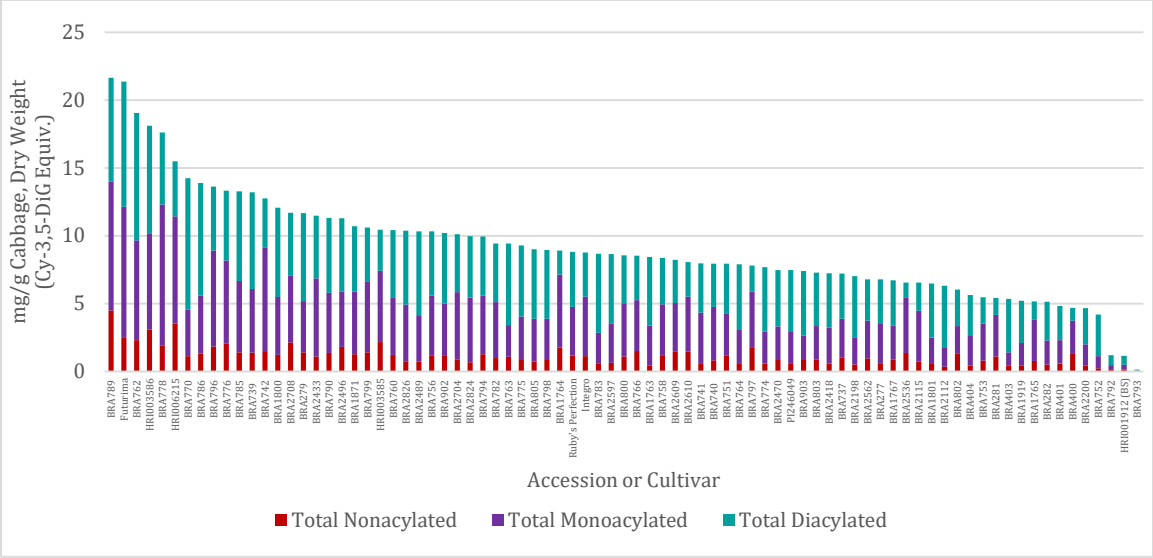
Appendix 3.7 Average anthocyanin content composition from 43 accessions from the USDA germplasm repository showing peak number and by non, mono, and diacylation classification.



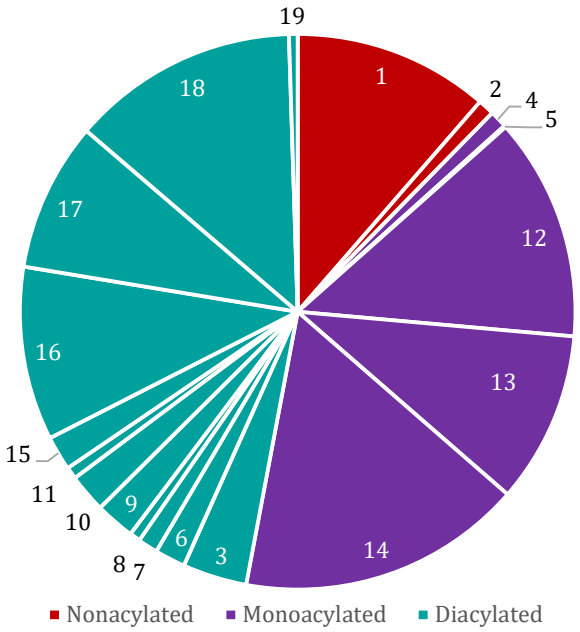
Appendix 3.8 Total anthocyanins, nonacylation, monoacylation, and diacylation of 24 HRI accessions compared to common red cabbage cultivars grown in summer of 2012 in Geneva, NY, USA



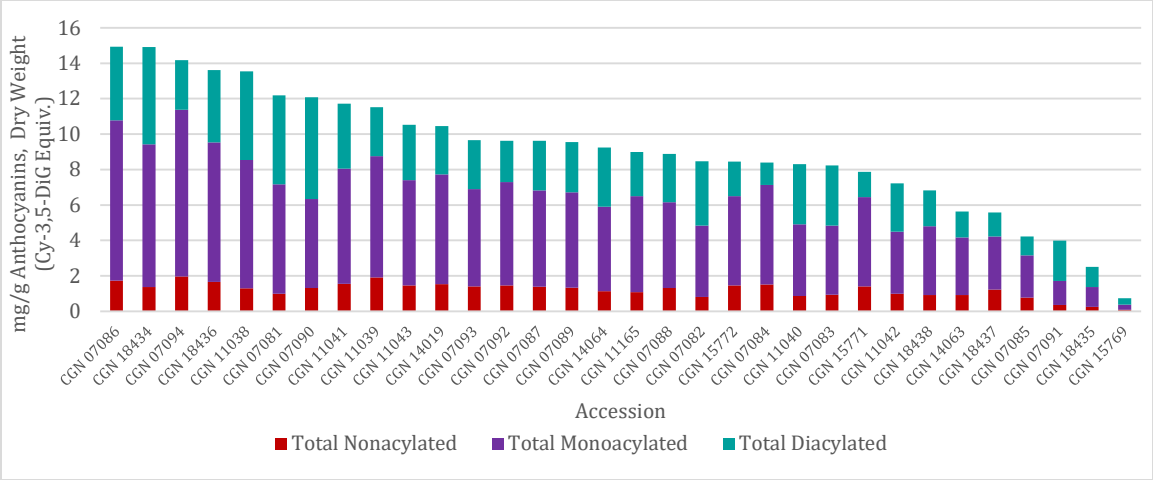
Appendix 3.9 Mean anthocyanin content composition from 24 accessions form the HRI germplasm repository showing peak number and non, mono, and diacylation classification.



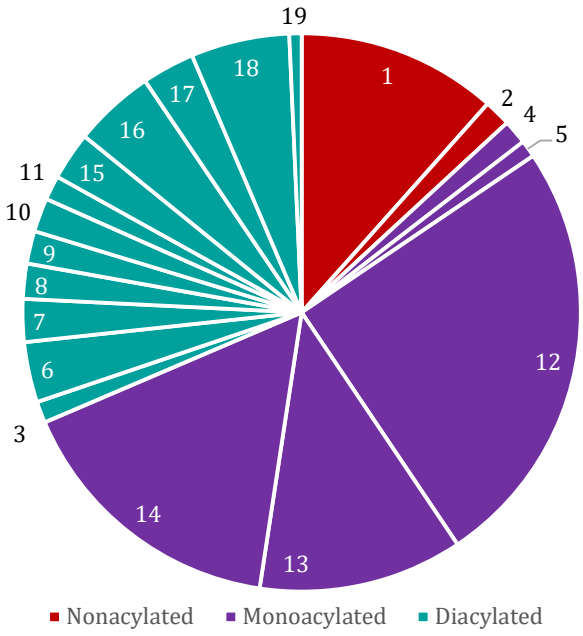
Appendix 3.10 Total anthocyanins content with nonacylation, monoacylation, and diacylation shown of the 73 IPK accessions compared to common red cabbage cultivars grown in Freeville, NY, USA in summer of 2014



Appendix 3.11 Average anthocyanin content composition from 73 accessions form the IPK germplasm repository showing peak number and non, mono, and diacylation classification.



Appendix 3.12 Total anthocyanins content with nonacylation, monoacylation, and diacylation shown of the 32 CGN accessions grown in Freeville, NY, USA in summer of 2015



Appendix 3.13 Average anthocyanin content composition from 32 accessions form the CGN germplasm repository showing peak number and non, mono, and diacylation classification.

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