Studies on Apple Peel Color Regulation

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Dedication

This dissertation is dedicated to my parents, Moisés and Rebeca, for their continuous encouragement and support.

Abstract

One of the most important factors determining apple [*Malus pumila* P. Mill.] market acceptance is peel color. Most apple cultivars (e.g. 'Royal Gala') produce fruit with a defined fruit pigment pattern, but in the case of 'Honeycrisp' apple, trees can produce fruits of two different kinds: striped and blushed. The causes of this phenomenon are unknown. We compared 'Honeycrisp' fruit from trees that were propagated from buds occurring on branches carrying only blushed or only striped fruit and concluded that blushed trees tend to produce a higher percentage of blushed fruit than striped trees, indicating a mechanism conserved through cell division. The percentage of blushed fruit on any given tree changed from year to year. Blushed and striped fruit occurred together on the same branch, and even on the same spur, with fruits located in the outer canopy being more likely to be striped. Higher crop loads were associated with a lower percentage of blushed fruit on the tree. Blushed and striped fruit do not consistently differ in their maximum pigment accumulation before ripening. The comparison of average hue angle for the whole peel at harvest indicates that blushed fruit are redder on average. We have also shown that striped areas of 'Honeycrisp' and 'Royal Gala' are due to sectorial increases in anthocyanin concentration. Transcript levels of the major biosynthetic genes and *MdMYB10*, a transcription factor that upregulates apple anthocyanin production, correlated with increased anthocyanin concentration in stripes. However, changes in the promoter and coding sequence of *MdMYB10* do not correlate with skin pattern in 'Honeycrisp' and other cultivars differing in peel pigmentation patterns. A survey of methylation levels throughout the coding region of *MdMYB10* and a 2.5 kb region 5' of the ATG translation start site indicated that an area 900 bp long, starting 1400 bp upstream of the translation start site, is highly methylated. Comparisons of methylation levels of red and green stripes indicated that the degree of methylation of the *MdMYB10* promoter is likely to be associated with the presence of stripes in these cultivars, with red stripes having lower methylation levels. Methylation may be associated with the presence of a TRIM retrotransposon within the promoter region, but the presence of the TRIM element alone cannot explain the phenotypic variability observed in 'Honeycrisp'. We suggest that methylation in the *MdMYB10* promoter is more variable in 'Honeycrisp' than in 'Royal Gala', leading to more variable color patterns in the peel of this cultivar.

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1 Literature review

1.1 Introduction

Apple production is of great importance in the United States, with 4.5 billion kilograms of traded production in 2008 and value equivalent to 2.6 billion U.S. dollars. A key quality attribute of apple fruit is its peel color, which not only affects consumer preferences, but is also associated with the fruit's nutritional value. Pigment accumulation in apple fruit can be affected by environmental conditions and fruit production practices. In recent years, significant progress has been made in understanding the genetic regulation of anthocyanin accumulation in apple. Here we summarize the current state of knowledge regarding the regulation of anthocyanin accumulation in apple peels.

1.2 Apple peel color

1.2.1 Marketing importance

Apple peel color is one of the most important factors determining apple market acceptance. In general, red cultivars are the most preferred, and within a cultivar, better colored fruits are in higher demand (Saure, 1990). However, consumer preferences vary from country to country and region to region (Cliff et al., 2002). New Zealand consumers prefer striped apples; consumers from Nova Scotia, Canada favor blushed apples, while consumers in British Columbia, Canada were more accepting of a range of apple types. Panelists in Lleida, Spain, on the contrary, did not show a preference for peel appearance when presented with eight 'Gala' strains with varying pigmentation (Iglesias et al., 2008). In addition, peel color is one of the main traits enabling cultivar discrimination, and there is increasing interest in breeding materials with altered color. In New Zealand, for instance, researchers are working towards the development of a red fleshed apple cultivar (Chagne et al., 2007).

1.2.2 Apple flavonoids and their beneficial health effects

Flavonoids, including anthocyanins flavones and flavonols, are polyphenolic plant secondary metabolites. Anthocyanins, the main red pigments in apple peels, are vitally important in attracting animals for pollination and seed dispersal. Flavones and flavonols, although not visible to the human eye, may attract insects which see farther into the ultraviolet (UV) range of the spectrum and can also protect cells

from excessive UV-B radiation. Additional functions of flavonoids include a role in nitrogen fixation, auxin transport and plant defense (Taiz and Zeiger, 2002).

Total polyphenolic content and levels of individual compounds were shown to vary significantly among eight apple cultivars grown in Ontario, Canada (Tsao et al., 2003). Peels have, in general, a much higher concentration of polyphenols than flesh. The predominant groups of polyphenolics in apple peel and flesh are the colorless procyanidin, followed by the colorless or yellow quercetin glycosides in the peel and hydroxycinnamic acid esters in the flesh (Tsao et al., 2003). The main anthocyanin identified in apple skin is cyanidin 3-galactoside, while the cyanidin 3-glucoside level is very low (Ben-Yehuda et al., 2005; Honda et al., 2002; Kondo et al., 2002; Tsao et al., 2003).

Apples have been associated with lowered risks of cancer and cardiovascular diseases, which are thought to be caused by oxidative processes. Polyphenolics are the major source of antioxidants in apple and this fruit is a very important source of flavonoids in the U.S. and European diets. In the U.S., 22% of the phenolics consumed from fruit come from apples, making them the largest source of these compounds. Several studies have linked apple consumption with reduced risk for cancer, especially lung cancer, cardiovascular disease, asthma, pulmonary problems, diabetes and obesity. These effects are due to the fruit's antioxidant activity, anti-proliferative activity, inhibition of lipid oxidation and cholesterol-lowering (Boyer and Liu, 2004). Antioxidants are mainly localized in the apple peel, but cultivars exhibit a wide variation in the distribution pattern (Eberhardt et al., 2000; Lata, 2007). Flavan-3-ols and procyanidins are the most important contributors to the in vitro antioxidant activity of apples, while procyanidin B2 and epicatechin are the most important individual antioxidants in apple. Also, hydroxycinnamic acids may have a significant antioxidant role in the flesh (Tsao et al., 2005).

Anthocyanins, and co-pigment flavonols, produce most of the pink, red, mauve and blue color found in higher plants (Lancaster, 1992). In apple fruits, the red color is produced by anthocyanins, which accumulate as granules in the inner side of vacuoles (Bae and Kim, 2006), while the background color is determined by the green and yellow chlorophylls and carotenoids in plastids (Lancaster et al., 1994). Co-pigmentation is a solution phenomenon in which pigments and other noncolored organic components form molecular associations, resulting generally in an enhancement in light absorbance and in some cases, a shift in the wavelength of the maximum absorbance of the pigment (Boulton, 2001). Evidence suggests that co-pigmentation does not explain differences in shade of red observed in the peel of different apple genotypes. Instead, this variation might be associated with the visual blending of chlorophyll, carotenoids and anthocyanins (Lancaster et al., 1994).

1.2.3 Anthocyanin accumulation in apple peels

Apple fruit color is determined primarily by the ground color of the skin and secondly by the superimposed anthocyanin pigmentation, if present (Janick et al., 1996). When red pigmentation is present, there are two peaks of coloration during fruit development. The first occurs during the phase of intense cell

division in the fruit and the second coincides with ripening of red cultivars (Saure, 1990). Red pigmentation can adopt different patterns, from small red flecks to bold stripes, and from a weak blush to solid red. The expression of these characters can be affected by environmental, nutritional and orchard management factors, the stage of maturity of the fruit, and by the microenvironment within the canopy. Normally when blushed apples are intercrossed, the offspring will be either blushed or non-pigmented, but not striped, suggesting an additional role for genetic regulation (Janick et al., 1996).

In general, apple skin coloring most often takes the form of striping, but blushes may also occur. The blush may be pronounced but in some cases is ephemeral, being extremely light-sensitive. For instance, quite a number of normally yellow apples will, if exposed to full sun, accumulate red pigments in part of their skin (Janick et al., 1996). There are very few apple cultivars that are truly blushed (David Bedford, University of Minnesota, pers. comm.), with blushed being defined as lacking obvious peel stripes (Fig. 1a). Most fruits are "solidly striped", an appearance that is hard to distinguish from truly blushed in fruit grown under good light conditions. But unlike truly blushed fruit, solidly striped fruit display a range of obviously striped phenotypes (Fig. 1b) when grown under poor coloring conditions such as shading. The classic example of a blushed fruit is 'Connell Red', which is a blushed sport of 'Fireside' (striped). These two cultivars showed no differences when molecular marker profiles were compared (Cabe et al., 2005; MacKay pers. comm.) indicating that the differences observed might be caused by very few genome changes, or even a single mutation. In 'Honeycrisp', fruit pigmentation can adopt two basic patterns: blushed or striped, where blushed fruits are defined as those that never show stripes, even in less colored areas of the peel. These two types of fruit appear together on the same tree, and even on the same spur, a very unusual phenomenon in apple. In contrast to what is observed in 'Honeycrisp', neither 'Connell Red' nor 'Fireside' seems to deviate from their respective patterns. This stable behavior has also been observed for blushed sports of other cultivars (Janick et al., 1996). The striped pattern might be detrimental for fruit marketing, especially in poor coloring conditions, since striped fruits are less red on average than blushed fruits (Telias et al., 2008).

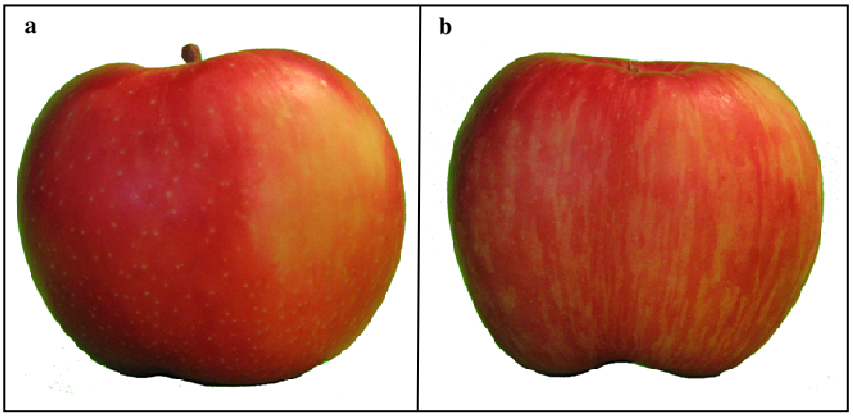

Figure 1. The two different types of fruit peel pigment patterns in 'Honeycrisp' apple. Distribution of anthocyanin in apple peels of blushed a) and striped b) fruits of 'Honeycrisp'.

Anthocyanin is found in cells of the epidermis and sub-epidermal layers, but not all cells are pigmented. Some apples have no pigment in the epidermis, and the color intensity depends on the proportion of cells in each subepidermal layer that contain pigment. The peel of both 'Delicious' and its bud sport 'Starking' contains many stripes, but since the epidermal layer of these cultivars is colorless, the difference in color between a stripe and the adjacent area involves only the hypodermal layers. In 'Northern Spy', which has a small proportion of pigmented cells in the epidermis, red stripes have a greater proportion of pigmented epidermal cells than adjacent areas, as well as more intense color (Dayton, 1959). Striped patterns in 'Royal Gala' and 'Honeycrisp' apples are caused by higher anthocyanin accumulation in the epidermis and hypodermal layers in red stripes as compared to green stripes (Chapter 3 of this dissertation). Light and electron microscopy studies in 'Fuji' apple revealed that redder skins have more layers of epidermal cells containing anthocyanins (Bae and Kim, 2006). The density of anthocyanin is higher in cells of the outer layer of the fruit skins than in inner layers.

1.2.4 Color measurements

Color measurements in plants are performed using a variety of methods. Color assessment by visual comparison to color charts is the most economic, but also the most tedious and subjective. Colorimeters provide, at a higher cost, an objective measurement, but usually of a localized area. Software packages have been implemented to perform evaluations of the fruit surface (e.g. detection of fruit blemishes, see Du and Sun (2004) and references therein), but none of these methods can perform color measurements of "large" surface areas automatically, on a large set of images. More recently, new image processing tools to automatically measure color and other fruit quality variables have been developed and made freely available (Darrigues et al., 2008; Telias et al., 2008).

Multiple color spaces can be used to define color. The CIE *L*a*b** (CIELAB) is a color space specified by the International Commission on Illumination. The three coordinates of CIELAB represent the lightness of the color (*L**) ranging from 0.0 for black and 100.0 for diffuse white, its position between red/magenta and green (*a**) and between yellow and blue (*b**). Hue is defined as the attribute of a visual sensation to which an area appears to be similar to one of the perceived colors such as "red", "yellow", etc. and can be computed from the *a** and *b**values (Fairchild, 2005).

Hue angle and the a*/b* ratio can be used as predictors of anthocyanin concentration in red raspberry fruit (Moore, 1997). Greer (2005) compared different methods to measure color change in apples throughout development, concluding that hue angle is the best indicator, as compared to chlorophyll florescence, chroma or lightness coefficient. Studies of the diffuse reflectance spectra of whole apple fruit in the 400-800 nm range with a spectrophotometer allows for the construction of an index linearly related to anthocyanin concentration (Merzlyak et al., 2003).

1.3 Genetic control of anthocyanin accumulation

1.3.1 Introduction

Two categories of genes affect anthocyanin biosynthesis. The first category encodes enzymes required for pigment biosynthesis (structural or biosynthetic genes), which have been widely studied in apple (Ben-Yehuda et al., 2005; Honda et al., 2002; Kondo et al., 2002; Takos et al., 2006b). The second category is comprised of transcription factors, which are regulatory genes that influence the intensity and pattern of anthocyanin accumulation and generally control expression of many different biosynthetic genes (Goodrich et al., 1992).

1.3.2 Biosynthetic genes

Flavonoids are synthesized by the phenylpropanoid metabolic pathway in which the amino acid phenylalanine is used to produce 4-coumaroyl-CoA (Fig. 2). This compound can in turn be combined with malonyl-CoA to yield the backbone of all flavonoids, a group of compounds called chalcones, which contain two phenyl rings. The conjugate ring-closure of chalcones results in the familiar form of flavonoids, the three-ringed structure of a flavone. The metabolic pathway continues through a series of enzymatic modifications to yield flavanones, dihydroflavonols and anthocyanins. Along this pathway, many products can be formed, including flavonols, flavan-3-ols and proanthocyanidins or tannins (Ververidis et al., 2007).

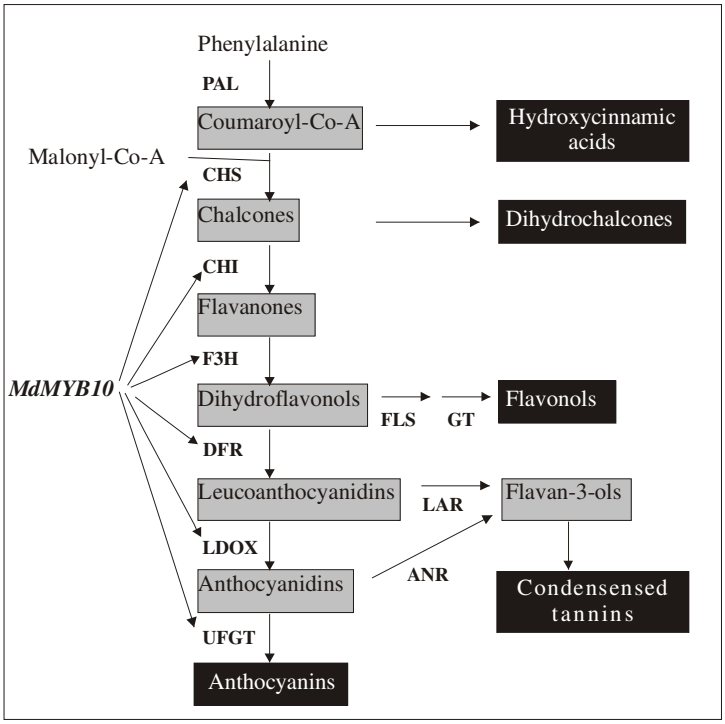

Figure 2. Schematic representation of the flavonoid biosynthetic pathway in apple regulated by *MdMYB10*. Flavonoid intermediates (gray boxes) and end products (black boxes) are indicated. Enzymes required for each step are shown in bold uppercase letters (PAL, phenylalanine ammonia lyase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone-3 b-hydroxylase; FLS, flavonol synthase; GT, unidentified enzyme encoding a glycosyl transferase for flavonol glycone synthesis; DFR, dihydroflavonol-4-reductase; LAR, leucoanthocyanidin reductase; LDOX, leucoanthocyanidin dioxygenase; ANR, anthocyanidin reductase; UFGT, UDP-glycose:flavonoid-3-O-glycosyltransferase (adapted from Takos et al. 2006b).

Five genes encoding the anthocyanin biosynthesis enzymes, chalcone synthase (*CHS*), flavonone 3-hydroxylase (*F3H*), dihydroflavonol 4-reductase (*DFR*), leucoanthocyanidin dioxygenase (*LDOX*), and UDP glucose: flavonoid 3-*O*-glucosyltransferase (*UFGluT*), are coordinately expressed during apple fruit development and their transcription levels are positively correlated with anthocyanin concentration (Ben-Yehuda et al., 2005; Honda et al., 2002; Ju et al., 1999b). Expression of these genes in different cultivars may be controlled by regulatory genes and environmental factors might affect anthocyanin synthesis through these regulatory genes (Ben-Yehuda et al., 2005; Honda et al., 2002; Ju et al., 1999b). However, low levels of expression of the biosynthetic genes do not induce anthocyanin accumulation in ripening fruits of 'Orin' at 168 days after full bloom, but give rise to its accumulation in immature fruits of 'Jonathan' at 142 days after full bloom, suggesting that there are additional mechanisms controlling red coloration during fruit ripening in addition to the regulation of gene expression (Honda et al., 2002).

Deduced amino acid sequences of the genes encoding F3H, DFR, LDOX and UFGluT (prepared from the skin tissues of 'Fuji' apple) showed high homology to corresponding protein sequences from other plants and indicate that each protein is encoded by a multigene family (Kim et al., 2003). The mRNAs of anthocyanin biosynthetic genes are detected preferentially in the skin tissue and transcription of the genes was coordinately induced by light (Kim et al., 2003). The transcripts were detected abundantly in the skin of red cultivars, but rarely in that of a cultivar bearing non-red fruit, confirming that these genes have major roles in determination of apple skin color (Kim et al., 2003). Southern hybridization using fragments of anthocyanin structural genes as probes revealed little polymorphism between green ('Mutsu') and red cultivars ('Fuji' and 'Jonathan'), demonstrating that both green and red cultivars carry anthocyanin structural genes. This further indicates that the expression of the anthocyanin genes in green cultivars is somehow affected, leading the authors to suggest that transcription factors might be altered in the green cultivar (Kim et al., 2003).

According to Saure (1990), the enzyme phenylalanine ammonia-lyase (PAL) is critical in the regulation of flavonoid synthesis in apple, and is a key enzyme in anthocyanin synthesis. Later reports conclude that, while PAL catalyzes a reaction to produce precursors of anthocyanin synthesis, changes in anthocyanin accumulation can occur independently of changes in PAL activity under conditions of sufficient precursors (Ju et al., 1995b). Further support is provided by Ju et al. (1999b), who found that red-striped areas contained more anthocyanin and higher UDP galactose:flavonoid-3-o-glucosyltransferase (UFGalT) activity than their adjacent areas, but no differences in PAL and CHS activities were found.

The higher abundance of cyanidin 3-galactoside as compared to cyanidin 3-glucoside in apple skin suggests that the functional enzyme is an UFGalT, which is responsible for the transfer of galactose to the 3-o position of flavonoids. However, researchers could not determine whether the apple UFGluT functions as UFGalT by transferring the galactosyl moiety to cyanidin (Honda et al., 2002). In experiments performed by Kondo et al. (2002) *UFGluT* transcripts were not detected when anthocyanin did not appear later in development. However, *MdCHS*, *MdF3H*, *MdDFR*, *MdLDOX* and *MdUFGluT* transcripts are all detected at 20 days after full bloom in both dark grown fruit without anthocyanin accumulation and light grown fruit with anthocyanin accumulation. These results, in agreement with those of Ju et al. (1995a), suggest that the role of UFGluT may change during fruit development and that anthocyanin formation depends on cyanidin presence rather than on UFGalT activity.

Correlation between DFR activity and anthocyanin accumulation in apple fruit was not significant. It appears that DFR is necessary, but not a limiting point, for anthocyanin synthesis in apples (Ju et al., 1997). Finally, UDOP-glucose 4 epimerase (UGE), an enzyme that catalyses the reversible epimerization of UDP-glucose to UDP-galactose (the major sugar donor for cyanidin-glycoside in apple), was more active in apple skins that accumulated anthocyanin, indicating its possible contribution to red coloration (Ban et al., 2007a).

1.3.3 Regulatory genes

Regulatory genes, controlling the expression of genes of the anthocyanin pathway, have been identified in many plant species. These genes influence the intensity and pattern of anthocyanin biosynthesis and generally control expression of many different structural genes (Espley et al., 2007; Gonzalez et al., 2008; Holton and Cornish, 1995; Mol et al., 1998; Spelt et al., 2002). The R/B gene family of transcription factors determines the timing, distribution and amount of anthocyanin pigmentation in maize. This family encodes proteins with homology with the basic helix-loop-helix (bHLH) motif in the MYC transcription activator (Chandler et al., 1989). Accumulation of anthocyanins in competent tissues of the maize plants requires that members of the R/B family interact with either C1 (in the seed) or Pl (in the plant tissue). C1 and Pl share sequence similarity with the DNA binding domains of the MYB oncogenes. *Pl* and *C1* encode proteins with 90% or more amino acid identity in domains important for the regulatory function of the C1 protein (Cone et al., 1988). Pigment production in any particular part of the maize plant requires the interaction of a member of the R/B family and a member of the C1/Pl family (Cocciolone and Cone, 1993). Experiments with petunia indicated that floral anthocyanin production requires a WD repeat-containing protein (AN11), a MYB (AN2) and bHLH (AN1) transcription factor. These genes not only control synthesis of anthocyanins, but also acidification of vacuoles in petal cells and the size and morphology of cells in the seed coat epidermis (Spelt et al., 2002).

In apple, three groups have independently identified an R2R3 MYB transcription factor responsible for anthocyanin accumulation (Ban et al., 2007b; Espley et al., 2007; Takos et al., 2006a). *MdMYB10* transcript levels strongly correlate with peel anthocyanin levels, and this gene is able to induce anthocyanin accumulation in heterologous and homologous systems (Espley et al., 2007). In addition, *MdMYB10* co-segregates with the *Rni* locus, a major genetic determinant of red foliage and red color in the flesh of apple fruit (Chagne et al., 2007). *MdMYB1* transcription also correlates with anthocyanin synthesis and is higher in red fruit peel sectors (more exposed to light) and in red peel cultivars (including 'Cripps' Red', 'Gala' and 'Galaxy') than in green peel sectors or non-red cultivars (including 'Golden Delicious', 'Granny Smith' and 'Grandspur'). Transcription of *MdMYB1* increased in dark grown apples once exposed to light, providing additional evidence of its role as an anthocyanin regulator (Takos et al., 2006a). The expression of several anthocyanin pathway genes was found to be regulated by both MdMYB10 and MdMYB1 (Espley et al., 2007; Takos et al., 2006a) (Fig. 2). *MdMYBA* is also more highly expressed in redder peels and the redder cultivars such as 'Jonathan', as compared to paler cultivars like 'Tsugaru', and its transcription is induced by UV-B light and low temperature (Ban et al., 2007b). The coding region of *MdMYB1* is 100 and 98% identical to *MYBA* and *MYB10,* respectively (Ban et al., 2007b). In addition, *MdMYB10* and *MdMYBA* have been mapped to the same region on linkage group 9 (Ban et al., 2007b; Chagne et al., 2007), suggesting that these three genes differ minimally and are probably alleles of each other.

The striking phenotype of some apple varieties producing red fleshed fruit and red foliage is caused by the occurrence of an autoregulatory mechanism associated with MdMYB10. In these genotypes, five direct tandem repeats of a 23 bp sequence in the promoter of *MdMYB10*, 275 bp upstream of the ATG translation start codon, are the target of the MdMYB10 protein itself, and the number of repeat units correlates with an increase in transcription activation caused by the MdMYB10 protein (Espley et al., 2009). In contrast, no sequence differences were detected when comparing promoter and coding regions of *MdMYB10* among 'Fireside', 'Connell Red', and blushed and striped 'Honeycrisp', using RNA and DNA extracted from peel tissue (Chapter 3 of this dissertation), implying that different pigment patterns in these cultivars are not caused by changes at the primary DNA sequence level. A dCAPs PCR marker derived from a SNP in the promoter of *MdMYB1* is able to discriminate between most red and green apple cultivars (Takos et al., 2006a).

Transcript levels of *MdbHLH3* (similar to Arabidopsis *TT8),* and *MdbHLH33* (similar to snapdragon *Delila)*, remain more constant throughout apple fruit development and do not follow the same pattern as the biosynthetic genes or *MdMYB10* (Espley et al., 2007). Results from reporter assays suggest that in cultivars carrying the 23 bp sequence repeats and the *MdMYB10* autoregulatory system, the influence of *MdbHLH3* on *MdMYB10* transcription is reduced (Espley et al., 2009).

Repressors of anthocyanin production have also been identified within the MYB class of transcription factors. These include *FaMYB1* in strawberry (Aharoni et al., 2001) and *AtMYBL2* in Arabidopsis (Dubos et al., 2008; Matsui et al., 2008). *FaMYB1* is up-regulated jointly with late anthocyanin pathway genes, and it has been suggested that its role is to balance anthocyanin levels produced at later states of strawberry maturation (Aharoni et al., 2001). A gene in apple, *MdMYB17*, shows high homology to *AtMYB4*, a repressor of sinapate esters production in Arabidopsis. *AtMYB4* expression is downregulated by exposure to UV-B light, indicating that derepression is an important mechanism for acclimation to UV-B in this species (Jin et al., 2000). *AtMYB4* has been found to repress its own expression in a negative autoregulatory loop (Zhao et al., 2007b).

Both *MdMYB10* and *MdMYB17* transcript levels correlate with anthocyanin concentration in stripes of both 'Honeycrisp' and 'Royal Gala', with higher mRNA levels in red stripes as compared to green stripes. Transcript levels of structural genes follow the same pattern as those of *MdMYB10* and *MdMYB17*, suggesting that the presence of stripes is correlated with differential transcript accumulation of *MdMYB10* and *MdMYB17* in the differentially pigmented stripes, which in turn regulates transcription of structural genes. Levels of *MdbHLH3* and *MdbHLH33* transcripts do not differ in green and red stripes, and therefore correlate poorly with anthocyanin concentration (Chapter 3 of this dissertation).

1.4 Factors affecting anthocyanin accumulation in apple

1.4.1 Light

Elevated sunlight promotes ripening associated pigment changes in apple, including more profound breakdown of chlorophyll, induction of carotenoid synthesis and specific changes in carotenoid patterns (Solovchenko et al., 2006). Anthocyanin production in apples has a tight dependence on light; not only the intensity but also the quality of light influences anthocyanin formation, with blue-violet and UV light being most effective, and far-red being least effective or even inhibitory (Saure, 1990). Both phytochrome and specific UV-B photoreceptors appear to be involved in a synergistic activation of anthocyanin synthesis (Arakawa, 1988). Studies of the effect of fruit position on the tree on flavonoid and chlorogenic acid contents showed that both anthocyanin and quercetin-3-glycoside concentration are tightly linked to light levels. There is a critical far red/red light ratio of approximately 1, below which no anthocyanin and only minimal quercetin 3-glycosides are formed. In addition, photosynthesis is necessary for the full expression of the response (Awad et al., 2001). Postharvest irradiation is effective in increasing red color only when applied to apples at commercial harvest, and not earlier, indicating that light responses are developmentally controlled (Marais et al., 2001)

When fruits are unbagged and exposed to light, *MdCHI* transcription increases 240 fold, followed by *MdCHS* and *MdLDOX* at 80- and 60-fold, respectively (Takos et al., 2006b), in agreement with results obtained by Ben Yehudah et al. (2005). *MdMYB1* was identified as the light-responsive regulatory factor controlling transcription of apple flavonoid genes (Takos et al., 2006a). In different studies, transcription of *MdMYBA*, *MdCHS*, *MdDFR*, *MdLDOX* and *MdUFGluT* in apple peels was also found to be regulated by light, particularly UV-B radiation (Ban et al., 2007b; Ju et al., 1997; Ubi et al., 2006). Condensed tannin production, on the other hand, was not light induced (Takos et al., 2006b).

Flavonols accumulate in apple skin during acclimation to strong sunlight. They can serve as an efficient UV-B screen, playing an important role in the resistance of the photosynthetic apparatus to the UV-B component of solar radiation. Anthocyanins do not exhibit a detectable synergistic effect in UV-B protection, and seemingly serve for protection from damage only by radiation in the blue-green part of the visible spectrum (Solovchenko and Schimtz-Elberger, 2003). Reay and Lancaster (2001) studied the potential of detached fruit to accumulate anthocyanins and quercetin glycosides and found that the shaded side of the fruit has a much greater potential than the exposed side. They concluded that the fruit skin's previous exposure to light is a modifying factor in the potential for accumulation of anthocyanins by 'Gala' and 'Royal Gala'. A study of the effect of light irradiation on the accumulation of phenolic compounds in slices of apple flesh indicated that phenolic acids, anthocyanin and flavonols are the phenolics that increase rapidly by irradiation whereas flavanols, procyanidins and dihydrochalcones levels remain unchanged (Bakhshi and Arakawa, 2007).

The position of the fruit on the tree can affect the pattern of anthocyanin deposition in 'Honeycrisp' apple. More striped fruit are produced on SW facing branches (most sun exposed in the northern hemisphere); these fruits were additionally more strongly striped than those on the least sun exposed NE

branches. These results suggest that in this cultivar, higher light incidence or temperature on the bud or the fruit correlates with an increase in the occurrence and strength of stripes (Telias et al., 2008).

1.4.2 Temperature

Low temperatures promote, and high temperatures in the fall inhibit anthocyanin synthesis in apple (Marais et al., 2001; Saure, 1990). The effect of low temperatures can be explained, at least in part, by a promotion of *MdCHS*, *MdLDOX* and *MdUFGluT* transcription (Ubi et al., 2006). The amount of pigment in the fruit is more closely correlated to average night temperature than to the average day temperature. The negative effect of higher temperature on color formation in 'McIntosh' apples is more pronounced in attached fruit (no color formation) than in detached fruit (slight color formation) suggesting that factors necessary for the formation of red color in apple may not be the same for detached and attached fruit (Uota, 1952).

Curry (1997), working with skin tissue discs, found that the optimal temperature for anthocyanin accumulation in pre-climacteric fruit tissue is 25°C. When fruit has entered the climacteric phase of ripening it shows a greatly reduced capacity for anthocyanin formation, indicating that delaying harvest in order to increase anthocyanin accumulation under advanced ripening conditions might be ineffective. The study showed that a period of cold temperature stimulates anthocyanin production; two or three nights with temperatures in the range of 2-5°C followed by warm sunny days promote red color development. Bakhshi and Arakawa (2007), on the other hand, found that synthesis of phenolic acids, anthocyanins and flavonols in slices of apple flesh is maximum at 24°C regardless of maturity stage and cultivar.

In the same orchard and at the same declining temperature, in trees with high crop load as compared with trees with lower crop loads, fruit ripening starts later and anthocyanin formation is postponed. This suggests that the increase in anthocyanin in autumn appears to be more closely related to ripening, as indicated by the rise in ethylene, than to a fall in temperature (Faragher, 1983).

1.4.3 Mineral nutrition

Generally, surplus nitrogen fertilization is associated with a reduction in the percentage of well-colored fruits at harvest time, although the total yield of well-colored fruit may be higher (Beattie, 1954). Reay and Lancaster (2001) found that the application of urea increases the chlorophyll and carotenoid concentrations in the fruit skin, and reduces anthocyanin concentrations in the blush side of the fruit at maturity.

Awad and de Jager (2002b) obtained negative correlations between nitrogen concentration in the fruit and anthocyanin and total flavonoid concentration at maturity. Nitrogen may inhibit flavonoid synthesis by enhancing the channeling of L-phenylalanine towards protein synthesis, or alternatively, it might negatively influence the enzyme system involved in the biosynthesis of phenolics. Strissel et al. (2005) studied the

effect of nitrogen on the activities of anthocyanin biosynthetic enzymes and found that PAL activity seems to be downregulated by high nitrogen levels. A high potassium supply supplements the positive effect of low nitrogen on anthocyanin formation. Further evidence was obtained by Bongue-Bartelsmann and Phillips (1995), who found that in tomato nitrogen deficiency increases anthocyanins and flavonol concentration two to three-fold in leaves and produces an increase in the steady-state mRNA levels of *CHS* and *DFR*, while chalcone isomerase (*CHI*) levels decreased. Results in Arabidopsis indicate that anthocyanin accumulation is an early senescence response to nitrogen deficiency and is controlled by the *NLA* gene (Peng et al., 2008).

The effects of other mineral nutrients on apple peel color are not so well documented. Several authors found a positive effect of potassium application on anthocyanin formation in apple, as reviewed by Saure (1990), and a recent study by Funke and Blanke (2006) found an increase in fruit anthocyanins with monophosphate application in 'Elstar' apple.

1.4.4 Carbohydrate availability

In Arabidopsis, PAP1, a MYB transcription factor controlling anthocyanin biosynthesis, upregulates anthocyanin production in response to high sucrose levels (Teng et al., 2005). In addition, *PAP1* expression is induced by light, nitrogen and phosphorus deficiencies, and is therefore considered to be a key mediator in environmental regulation of anthocyanin biosynthesis (reviewed by Rowan et al. 2009). In apples evidence for sugar involvement in anthocyanin accumulation comes from results indicating lower sugar concentration in fruits that do not develop satisfactory color, as well as from experiments with skin discs. Sugars may also be involved in a reduction of anthocyanidin degradation; an increase in galactose prior to harvest could enable anthocyanidin to accumulate by forming the glycoside (anthocyanin). Several authors nevertheless have found no correlation between sugar content and color development at the time of fruit maturity. Some treatments that may promote anthocyanin formation can even reduce sugar content (reviewed by Saure, 1990).

1.4.5 Growth regulators

Ethylene is a key factor in the regulation of anthocyanin biosynthesis and color development in apples, with positive correlations being found between ethylene and total anthocyanin, but not with other flavonoid compounds (Whale and Singh, 2007). According to Faragher and Brohier (1984), ethylene initiates rapid anthocyanin accumulation during apple ripening by increasing the level of PAL in the skin. A transgenic line of 'Royal Gala' apple that produces no detectable levels of ethylene was produced to study ethylene response in this cultivar. Seventeen genes in the phenylpropanoid biosynthetic pathway were upregulated in response to ethylene application, including PAL (Schaffer et al., 2007).

Awad and de Jager (2002a) found that the accumulation of anthocyanin is clearly stimulated by ethephon (an ethylene releasing agent) and delayed by ABG-3168 (a likely inhibitor of ethylene production), whereas maturity related fruit characteristics are not significantly affected by the different treatments. From this they concluded that anthocyanin formation in apple skin is not simply a ripening-related phenomenon; anthocyanin formation is apparently regulated by both the developmental signals and ethylene signaling. Whale et al. (2008) reported that the best fruit color and firmness are obtained with an application of the ethylene synthesis inhibitor AVG five weeks before harvest, followed by an ethephon application two weeks later.

The application of ethephon and a seniphos-like substance (a phosphorus-calcium mixture) produces an enhancement of red skin color and an increase in concentration of flavonoid compounds (Li et al., 2002). In these experiments PAL and UFGalT activities are not closely related to increased ethylene concentration, while the increase in CHI activity may possibly be caused by an increase in ethylene. No involvement of ethylene is found in the coloration responses due to sunlight (Arakawa et al., 1985), temperature (Blankenship, 1987) or bagging (Kubo et al., 1988).

Gibberellic acid reduces anthocyanin accumulation, without influencing fruit maturation (Awad and de Jager, 2002a). However, application of the gibberellin inhibitors cycocel and prohexadione-calcium does not significantly influence the formation of anthocyanin or fruit maturation. In another study, Mata et al. (2006) found that prohexadione-calcium applications increase red color in 'Fuji', but not in 'Royal Gala' apples.

Jasmonic acid increases anthocyanin concentration in apples (Kondo, 2006), and synergistic or additive responses were found between ethylene and methyl jasmonate in apple peel pigment synthesis pathways (Rudell and Mattheis, 2007). Abscisic acid, auxins and cytokinins have previously been implicated in anthocyanin regulation, but a clear role for these growth regulators has not yet been established (Saure, 1990).

1.4.6 Orchard management practices

Bagging is widely applied in Japan as an effective practice for inducing some color formation even in cultivars that do not usually show any red color upon ripening. Bagging, by covering apples with bags made of paper or other materials, is done about one month after full bloom. Once fruits are bagged, anthocyanin accumulation is inhibited, consistent with the requirement of light for anthocyanin accumulation. Following removal of the bags some months later, fruit rapidly develop red color, and after several days the coloration exceeds that of control fruits in most cultivars. However, differences at harvest may be negligible especially if a decreasing temperature promotes color formation in untreated fruits of red cultivars (Saure, 1990). Arakawa (1988) noted that fruits initially produce more anthocyanin at immature and mature stages after bagging than control fruits when exposed to white and UV-B light. However, anthocyanin synthesis decreases rapidly towards harvest, whereas in the control fruit it increases further for

varying periods of time. Bagging is also a useful model for studying anthocyanin synthesis and gene expression in apples. The potential of bagged fruit to synthesize anthocyanin when exposed to light remains constant during five months of cold storage (Ju, 1998).

Another practice that has proven to increase anthocyanin concentration is to cover the orchard floor with reflecting films. These films stimulate internal ethylene synthesis and elevate UFGalT activity (Ju et al., 1999a). The use of a white polypropylene ground cover (Extenday^{TM}), an aluminized plastic film, and a reflective foil all increase apple peel red color (Funke and Blanke, 2005; Glenn and Puterka, 2007; Jakopic et al., 2007).

Fruit thinning can increase total red color in 'Honeycrisp' fruit peels, as demonstrated in experiments by Delong (2006) and Wright et al. (2006). Crop loads can additionally affect the pattern of pigment accumulation in this cultivar, with higher crop loads being associated with a lower percentage of blushed fruit (Telias et al., 2008).

Storage conditions used after harvest can have an effect on the flavonoid levels in the fruit. Antioxidant status is maintained more efficiently in controlled atmosphere than in common cold storage if fruit are stored for 90 days (Lata, 2008).

1.5 Mechanisms that can affect the pattern of anthocyanin accumulation in apple peels

1.5.1 Chimeras

A plant is said to be a chimera when cells of more than one genotype are found growing adjacent in the tissues of that plant. Chimeras arise when mutations occur in cells in apical meristems, giving origin to regions of the bud that are affected in one or more characteristics. In apple production, new materials with a chimeral origin are known as "sports" and are selected for changes in fruit or tree characteristics. Many apple sports have been identified and selected for changes in fruit color. Some cultivars such as 'Cox's Orange Pippin', 'Delicious' and 'Elstar' are prone to produce mutants with increased amounts of anthocyanin in the outer cell layers of the fruit skin, while other widely grown cultivars are stable and seem seldom to mutate (Janick et al., 1996).

Red sports of a single cultivar can differ in the area of the fruit affected and in the intensity of the pigmentation in the outer cell layers (Dayton, 1959). Very often the mutation is limited to one cell layer in the apical meristem; therefore, the plant is likely to be a periclinal chimera. The mutation is usually not heritable unless the second layer (L-2) which gives rise to gametes, is involved (Pratt, 1983). Pratt et al. (1975) suggested that blushed fruited sports of 'Northern Spy', a striped apple, have a mutation for this color pattern in the L-1 and L-2 layers of the apical meristem. According to Dayton (1969), many striped strains of 'McIntosh' are the result of undesirable mutations from the color pattern of the original

'McIntosh'. Mutations in L-1 probably account for this altered pigmentation, and results show that this cultivar may also be quite heterogeneous in its internal layers. Dark stripes on the fruits of the pear clone P1571, that have a layer pattern of L-1/green, L-2/green, L-3/red, indicate areas where the L-3 contribution to the flesh color is close enough to the epidermis to receive light and develop anthocyanins (Chevreau et al., 1989).

McMeans et al. (1998) were not able to obtain tissue culture plants of 'Gala' and 'Royal Gala' that produced pure red or pure green fruits, suggesting that the source of color patterning in these striped cultivars is not chimeral. Trees in that experiment were obtained from leaf tissue culture and were classified as red or green depending on the pigments present in leaves and stems. However, the distribution of anthocyanin in the tree depends on so many factors, and there are so many exceptions to correlations between red fruit color and anthocyanin pigmentation of other parts of the trees, as to make this of no value as a method for predetermining fruit color (Janick et al., 1996).

'Honeycrisp' trees were propagated from buds occurring on branches with only striped ("striped trees") or only blushed ("blushed trees") fruits in order to study the stability of the blushed and striped traits. Striped trees not only produce a significantly higher percentage of striped fruit, but they also produce fruit with a higher intensity of striping when compared to blushed trees. The percentage of blushed fruit on any given tree or branch changes from year to year, indicating that in the case of 'Honeycrisp' the variation in pigment pattern is not chimeral in origin (Telias et al., 2008).

1.5.2 Cytosine methylation

The addition of methyl groups to cytosines in DNA is a chemical modification that can be inherited and subsequently removed without changing the original DNA sequence. DNA methylation is part of the epigenetic code and is the best characterized epigenetic mechanism. In plants, cytosines are methylated both symmetrically (at CG dinucleotides or CHG trinucleotides) and asymmetrically (at CHH), where H represents any nucleotide but guanine. In eukaryotes, DNA methylation functions as a gene-silencing mechanism that regulates endogenous genes and protects the genome by inactivating selfish DNA elements (Chan et al., 2005). Environmental factors can affect the methylation state of DNA: Steward et al. (2002) observed that exposure to cold stress resulted in genome wide demethylation in maize seedlings roots.

Methylation in Arabidopsis is the result of three overlapping systems: 1) CG methylation controlled primarily by methyltransferase 1, 2) CHG methylation, maintained by chromomethylase 3 interacting with Histone 3 lysine 9 dimethylation pathway and 3) CHH methylation, maintained by domains rearranged methylase 1 and 2 and requiring the active targeting of small interfering RNAs (Zhang, 2008). Demethylation by DNA glycosylases of the demeter family can also control the resulting methylation landscape (Penterman et al., 2007).

Microarray experiments indicate that 20% of the Arabidopsis genome is methylated (Zhang, 2008). Methylated regions consist mostly of transposable elements and other repeats, while gene promoters are

rarely methylated, probably because methylation in these regions can interfere with transcription. Unexpected results indicated that methylation in transcribed regions is present in one third of Arabidopsis genes; this type of methylation is enriched in the 3' half of the transcribed regions and occurs primarily in CG sites (Zhang, 2008). In rice, DNA methylation of the transcribed region is enriched for and associated with a larger suppressive effect than DNA methylation of the promoter region (Li et al., 2008).

Cocciolone and Cone (1993) reported that differential DNA methylation in the 3' untranslated region of *Pl-Bh*, a MYB transcription factor allelic to *Pl* regulating anthocyanin accumulation in maize, can cause striped patterns in all plant tissues. The level of anthocyanin production within the stripes is not uniform; rather, pigmentation levels vary from cell-to-cell. The common pattern for all blotchy organs is that not every cell within a pigmented sector is pigmented to the same degree. Additionally, pigmented cells frequently occur in clusters, resembling clonal sectors. Patterns of cell division appear to dictate the spatial arrangement of pigmented cells within any organ. The authors hypothesized that early during development, the *Pl-Bh* gene would be differentially methylated and this methylation would be more or less maintained through subsequent cell divisions, producing clonal sectors in plant tissues of predominantly pigmented cells (unmethylated) and sectors of predominantly unpigmented cell (methylated). This hypothesized mechanism for the variability of the blotched phenotype can account for the activation of anthocyanin synthesis by Pl-Bh within some cells early in development, thereby yielding clonal sectors of pigmentation. It is also capable of explaining the cell-to-cell variability of gene expression, where not all cells attain the fully activated state. This regulatory mechanism is also heritable and can explain the stable transmission of the *Pl-Bh* phenotype to the progeny from generation to generation (Cocciolone and Cone, 1993).

Methylation plays a role in the regulation of additional genes controlling pigment production. Sekhon and Chopra (2009) identified a gene called *Ufo1* which controls methylation levels in *P1*, a gene that regulates phlobaphene (brick red flavonoids) biosynthesis in maize kernel pericarp and cob glumes, and whose activity may also produce variegation in maize pericarp. Ectopic expression of *P1-wr* correlated with hypomethylation of an enhancer region, 5 kb upstream of the transcription start site. The molecular nature of *Ufo1* is still unknown.

Cytosine methylation was found to be involved in the reduced expression of endogenous duplications. The duplicated *R* and *Sn* genes regulate the maize anthocyanin biosynthetic pathway and encode tissue specific products that are homologous to bHLH transcriptional activators. As a consequence of their coupling in the genome, *Sn* is partially silenced. Researchers found that differences in pigmentation are inversely correlated with differences in the methylation of the *Sn* promoter. Accordingly, treatment with 5-azacytidine (AZA), a demethylation agent, restores a strong pigmentation pattern that is transmitted to the progeny and that is correlated with differential expression of the *Sn* transcript (Ronchi et al., 1995).

In apple a region of the *MdMYB10* promoter, encompassing the region between nucleotide positions -1411 and -555 relative to the translation start site, is highly methylated in 'Royal Gala' and 'Honeycrisp' apple (Chapter 3 of this dissertation). Green stripes of 'Honeycrisp' show higher methylation levels for *MdMYB10* than red stripes, with the largest differences being found within the highly methylated region;

methylation levels therefore correlate with transcript levels of *MdMYB10*. Although differences between red and green striped are consistent, overall methylation levels vary between years. Similar trends are observed in 'Royal Gala', except that the differences between red and green stripes are smaller and significant only for a portion of the highly methylated region. Overall, higher methylation levels are observed for 'Royal Gala' than 'Honeycrisp'. Comparisons between red and green regions of the peel of blushed 'Honeycrisp' apples indicated no methylation differences, and interestingly, in two out of the three promoter regions studied, red stripes have methylation levels comparable to those in the peels of blushed apples, while green stripes have methylation levels higher than those of red stripes or red and green regions of blushed apples. As suggested for maize (Cocciolone and Cone, 1993), one hypothesis is that early in apple fruit development, differences in *MdMYB10* methylation are present among individual cells. Throughout fruit growth, these differentially methylated cells would give origin to sectors of peel varying in their ability to accumulate anthocyanins.

Methylation of the *MdMYB10* promoter may affect transcription through interference with the RNA-polymerase transcription complex, or by preventing binding of additional factors required for transcription. It is known that within the most methylated region of the *MdMYB10* promoter are five putative E-box motifs (Espley et al., 2009), a bHLH-related cis-acting element (CACATG) (Atchley and Fitch, 1997; Heim et al., 2003). The region also has a motif needed for *MdMYB10* transactivation by the MdMYB10 protein itself (Espley et al., 2009). The occurrence of DNA methylation might therefore interfere with these regulatory mechanisms.

Different methylation levels early in apple fruit development could be mitotically maintained from those in the meristematic cells that gave origin to the fruit, or could result from demethylation or *de novo* methylation. Results in 'Honeycrisp' suggest that there is at least some mitotic maintenance of methylation states, given that trees clonally propagated from buds on branches with exclusively blushed fruits, tend to produce a higher percentage of blushed fruit (Telias et al., 2008). Nonetheless, results from the same study indicate that additional factors can influence the pattern in the peel, namely position of the fruit on the tree and crop load. An assessment of the exact location and context of *MdMYB10* DNA methylation, as well as other types of epigenetics marks such as histone methylation, can shed light on the mechanism involved in *MdMYB10* regulation.

1.5.3 Transposable elements

The presence of transposable elements can affect gene expression both at the transcriptional (e.g. through the introduction of an alternative transcription start site) and at the post-transcriptional level (Feschotte, 2008). Evidence linking transposable elements to pigment variegation in plants was first found by Barbara McClintock during the 1940s in her studies of the *Dissociator* (*Ds*) and *Activator* (*Ac*) loci in maize (McClintock, 1949). She later worked on the *Spm* family of transposable elements, which comprises fully functional autonomous transposable elements and a variety of moderately to severely disabled

relatives in maize. Her studies focused on defective *Spm* (*dSpm*) insertions into the *A* and *A2* loci, both of which encode enzymes in the anthocyanin biosynthetic pathway. In *Spm*-suppressible plants, the transposon-disrupted gene continued to be expressed, but only in the absence of the autonomous *Spm* element. When *Spm* was present in the same plant, this type of transposon-disrupted gene was not expressed. In *Spm*-dependent plants, the gene with the transposon insertion was generally expressed only in the presence of a fully functional *Spm*. *Spm* codes for a trans-acting protein that binds to the transposon sequence inserted into or near the gene. Binding of these proteins either increases or decreases expression of the transposon-disrupted gene. In some cells, these proteins, probably acting together with other cellular proteins, excise the transposon from the gene and move it to a different location (Fedoroff, 1996).

Expression and transposition of the *Spm* transposon of maize is controlled by interacting epigenetic and autoregulatory mechanisms. Methylation of critical transposable element sequences prevents both transcription and transposition in maize, heritably inactivating the *Spm* element. The promoter of the transposable element and a highly GC-rich downstream sequence are the methylation target sequences. The transposable element encodes two proteins necessary for transposition, *TnpA* and *TnpD*. In addition to its role in transposition, *TnpA* is both a positive and a negative regulator of transcription. *TnpA* represses the promoter of the transposable element when it is not methylated. When the transposable element is inactive and its promoter is methylated, *TnpA* activates the methylated promoter and facilitates both its transient and heritable demethylation (Fedoroff, 1996).

Pooma et al. (2002) investigated the effects of *Mutator*, another type of transposable element, and *Spm* insertions on the expression of maize *a1*, encoding for the enzyme dihydroflavonol-4-reductase, independently regulated by the transcription factors C1 and P. The *a1-mum2* and *a1-m2* alleles carry *Mu1* and *Spm* insertions respectively in the anthocyanin regulatory element (ARE), a conserved motif present in several flavonoid biosynthetic gene promoters where MYB transcription factors bind and activates transcription. *a1-m2* belongs to the *Spm*-dependent class of alleles in which the expression of the *a1* gene happens only in the presence of trans-active, nondefective *Spm* elements. The *a1-mum2* allele carries the nonautonomous *Mu1* transposable element inserted in the ARE element. It belongs to the *Mu*-suppressible mutant class, in which the mutant phenotype (no anthocyanin pigmentation) is suppressed, resulting in pigmentation in the absence of the autonomous *MuDR* element. In the presence of *MuDR*, excision of *Mu1* in the aleurone results in the formation of frequent revertant sectors on a colorless background, because of the inhibitory effect of *MuDR* on the expression of *a1*. The presence of *MuDR* is associated with an extensive hypomethylation of *Mu1* and may interfere with the activation of a regulatory element present in *Mu1*. The unexpected pigmentation patterns provided by the *Mu1* and *Spm* insertions are a consequence of the disruption by the transposons of cis-regulatory elements important for the regulation of *a1*.

Studies in grape indicated that the presence of a retrotransposon named *Gret1* in the promoter of MYB transcription factors controlling anthocyanin production in this species, leads to the white fruited phenotype (This et al., 2007; Walker et al., 2007). Subsequent studies by Cadle-Davidson and Owens (2008) further demonstrated that *Gret1* copy number is significantly higher in white fruited accessions.

Transposon activation and suppression may be responsible for some of the genetic variation that occurs in color or spur habit in pome fruits (Brown, 2003). Retrotransposons have been identified in apple, including TRIM retrotransposons (Antonius-Klemola et al., 2006), copia-like retrotransposons (Tignon et al., 2001; Zhao et al., 2007a) and the *dem1* retroelement (Yao et al., 2001b). Transposable elements identified in apple include *Ars1* and *Ars2*, two short repeat sequences present in high number in apple genome. *Ars1* is thought to represent miniature inverted-repeat transposable elements (MITEs), while the identity of *Ars2*, though it is undoubtedly a transposable element, remains undetermined (Hadanou et al., 2003). Finally, Han and Korban (2007) reported the presence of Spring transposons in apple. Despite ample evidence of the presence of multiple kinds of transposable elements, there is no evidence associating transposable elements with fruit peel variegation in apple.

Tignon et al. (2001) looked for cDNA fragments that might be differentially expressed in relation with apple fruit color, by comparing mRNA populations extracted from the fruit skin of 'Jonagold' and some of its mutants. A partial reverse transcriptase gene of several copia-like retrotransposons was isolated from expressed messenger RNAs from only one of the two color mutants of 'Jonagold' studied, suggesting that vegetative multiplication of apple could induce retrotransposon activation and lead to mutations linked to the interruption of genes by retrotransposons.

A simple genetic basis for the red/yellow skin color polymorphism in apple was verified using RAPD markers. The A^1 marker co-segregates with red color, while a^1 and a^2 are associated with yellow skin color (Cheng et al., 1996). Results are consistent with the hypothesis that the red/yellow dimorphism is controlled by a monogenic system with the presence of the red anthocyanin pigmentation being dominant. There is no indication that other modifier genes could reverse the effect of the locus linked to the markers. The marker does not correlate with the presence or absence of stripes on red fruit or with the intensity of the anthocyanin pigmentation. Subsequent studies performed by Wakasa et al. (2003) demonstrated that the sequence information of the a^1 and a^2 fragments were virtually identical to A^1 except for their respective insertions. Sequence data revealed that the 76 bp a^1 insertion is an inverted repeat, while the 163 bp insertion in a^2 contains a duplication of a 10-bp target site. The 163 bp insertion of a^2 is called *Majin*, and is characterized as a mobile element. There are approximately 6000 copies of *Majin* per haploid apple genome.

Sequence-specific amplified polymorphisms (S-SAP), which allow the identification of dominant markers for the detection of variation in the DNA flanking retrotransposon insertion sites, were used to distinguish several clones of the cultivars 'Gala' and 'Braeburn'. Bud mutations, which have generated new patented varieties of 'Gala' and 'Braeburn', appear to derive from retrotransposon insertions (Venturi et al., 2005)

A sequence highly homologous to *TRIM2*, a terminal-repeat retrotransposon in miniature (Antonius-Klemola et al., 2006) is present 2.5 kb upstream of the *MdMYB10* translation start site in several cultivars differing in the total levels and pattern of anthocyanin accumulation, indicating that the presence of the transposon alone does not explain pigment differences between cultivars. This transposon does not seem to

insert new transcription start sites, since no transcription of the downstream *MdMYB10* gene from the transposon was observed in the cultivars tested (Chapter 3 of this dissertation).

1.5.4 Other mechanisms

Other mechanisms, described previously in other plant species, might be responsible for color variability in apple fruit, including paramutation, recurrent somatic mutations and the role of specific genes in controlling pigment patterns.

Paramutation is a phenomenon in which the interaction between two alleles leads to a directed alteration in transcription that is both mitotically and meiotically heritable. It is a widespread epigenetic phenomenon extensively studied in maize. Two models have been proposed to explain the trans-communication that occurs during paramutation. The first suggests that epigenetic states are altered by direct interactions between chromatin complexes, whereas the other invokes the participation of RNA-mediated chromatin changes. In maize, paramutation has been described for four genes (*R1*, *B1*, *Pl1* and *P1*), and regulatory sequences required for paramutation have been identified for *B1* and *P1* (Chandler et al., 1996).

Two alleles are involved in *B1* paramutation: *B'* (light purple plant) and *B-I* (dark purple plant). The paramutagenic *B'* state arises spontaneously from the unstable, highly expressed paramutable *B-I* state. Unlike *B-I*, *B'* is extremely stable. A 6 kb region, located 100 kb upstream of the *B1* transcription start site, that was required for paramutation and *B-I* enhancer activity was identified. In this region, *B-I* and *B'* have seven tandem repeats. DNA sequences are identical in *B-I* and *B'*, but the tandem repeats are differentially methylated and have greater *DNAseI* hypersensitivity in *B-I* relative to *B'*, indicating that epigenetic mechanisms mediate the stable transcriptional silencing that is associated with *B1* paramutation (Chandler and Stam, 2004).

In crosses that bring together the *P1-rr* allele (red cob and pericarp) and a transgene carrying elements that have been identified as necessary for paramutation, a subset of the transgenic plants displayed patterned pigment or colorless pericarps. *P1* paramutation requires a mutation in the mediator of paramutation 1 (*mop1*) gene, which encodes a RNA-dependent RNA polymerase, indicating that RNA-mediated gene silencing is involved in this type of paramutation (Sidorenko and Chandler, 2008).

In *Ipomoea tricolor* cv. Blue star, variegations of flowers and seeds were proven to be caused by recurrent somatic mutation. This mutation results from a tandem duplication within a gene encoding a bHLH transcriptional regulator functionally equivalent to Petunia *AN1* and Arabidopsis *TT8*. The truncated peptide produced lacks the bHLH motif (Park et al., 2004)

The presence of spots in the tepals (constituting the outer whorl in flowers where calyx and corolla are phenotypically indistinguishable) of Asiatic hybrid lily is controlled by several genes that determine the number of spots per square centimeter (Abe et al., 2002). The authors identified two putative QTLs for spot formation in tepals, and found that their dominant alleles might suppress spot formation. A single dominant

gene controls the presence or absence of anthocyanin in the tepals. These results indicate that the traits of anthocyanin pigmentation in tepals and of spot formation are under distinct regulation, although both tepals and tepal spots contain the same anthocyanin pigment.

The phenomena previously described have not been implicated in regulation of apple anthocyanins, but the conservation of anthocyanin pathways across plant species leads to the possibility that similar mechanisms control accumulation in this fruit crop. Further research is needed to establish the role of additional mechanisms in regulating anthocyanin accumulation in apple.

1.6 Conclusions

Recent advances in the field of genetics and epigenetics are broadening our understanding of the complex regulation of anthocyanin accumulation in apple. This new knowledge, coupled with greater understanding of the effects of light, temperature and other environmental factors, management practices, and the role of tissue organization, will undoubtedly provide new tools for achieving outstanding fruit quality, both in terms of visual appeal, and nutritional value.

2 Plant and environmental factors influencing the pattern of pigment accumulation in 'Honeycrisp' apple peels, using a novel color analyzer software tool.

2.1 Abstract

One of the most important factors determining apple [*Malus pumila* P. Mill.] market acceptance is peel color. Coloring of 'Honeycrisp' fruits can adopt two patterns: blushed or striped. This is an unusual phenomenon in apple. The objective of this study was to compare 'Honeycrisp' fruit from trees that were propagated from buds occurring on branches carrying only blushed or striped fruit. We concluded that blushed trees tend to produce a higher percentage of blushed fruit than striped trees, indicating a mechanism conserved through cell division. The percentage of blushed fruit on any given tree changed from year to year. Blushed and striped fruit occurred together on the same branch, and even on the same spur, with fruits located in the outer canopy being more likely to be striped. Higher crop loads were associated with a lower percentage of blushed fruit on the tree. Blushed and striped fruit do not consistently differ in their maximum pigment accumulation before ripening. The comparison of average hue angle for the whole peel at harvest indicates that blushed fruit are redder on average. Stripes were caused by reduced anthocyanin accumulation in certain portions of the peel, and not by a deeper localization. We speculate that an epigenetic mechanism regulates the pattern of anthocyanin accumulation in 'Honeycrisp' apple. Increased production of blushed, redder apples, may be achieved through clonal selection and crop load regulation. A software tool for efficient relative color evaluations was developed and is freely available to the community.

2.2 Introduction

‘Honeycrisp’ is a cultivar developed by the University of Minnesota and released in 1991 (Luby and Bedford, 1992). Many of its characteristics have attracted growers and consumers, resulting in a major increase in production. Coloring of ‘Honeycrisp’ fruits can adopt two basic patterns: “blushed” or “striped” (Fig. 1). For the purposes of this study, fruits are blushed when the surface is partly covered with a red tinge that is not broken, and striped when the color is in alternating lines in some or all portions of the peel (Zielinski, 1977) and the two categories are mutually exclusive. The presence of both kinds of fruit on the same tree as observed in ‘Honeycrisp’ has not been described in other cultivars to our knowledge. The causes of this phenomenon are unknown.

Peel color variability is important because color is one of the most important factors determining apple market acceptance. In general, red cultivars are the most preferred, and within a cultivar, redder fruits are

more demanded (Saure, 1990). Consumer preferences differ among regions: New Zealand consumers prefer striped apples; Nova Scotia consumers prefer blushed apples; in British Columbia consumers were more accepting of a range of apple types (Cliff et al., 2002). In recent years, consumers have become increasingly interested in quality attributes with beneficial health effects. Apples are an important source of antioxidants, including anthocyanins, the primary red pigments in apple peel. Consequently, apple consumption has been associated with lowered risks of cancer, cardiovascular disease and other chronic diseases (Boyer and Liu, 2004; Eberhardt et al., 2000; Knekt et al., 1996; Le Marchand et al., 2000).

Apple peel color is determined first by the ground color of the peel and second by the superimposed red anthocyanin pigmentation (if present). Anthocyanin can accumulate following different patterns: flecks; stripes; blush, all of which can range widely in their intensity. These characters can be affected by environmental, nutritional and cultural factors, the stage of maturity of the fruit, and by the microenvironment within the canopy (Janick et al., 1996). There are two peaks of anthocyanin accumulation in apple, the first occurring during the phase of intense cell division in the fruit and the second coinciding with ripening of red cultivars (Saure, 1990).

Anthocyanin can be found in the epidermis and subepidermal layers of the peel, but not all cells within a layer are pigmented. Some apples have no pigment in the epidermis, and the color intensity depends on the proportion of cells in each subepidermal layer that contains pigment. In the striped cultivars 'Delicious' and 'Northern Spy' the difference in color between a stripe and the adjacent area is due to the presence of more pigmented cells per unit area in red stripes. Color sports can differ from the cultivar from which they originated in the proportion of pigmented cells, the amount of anthocyanin accumulation in the cells, and the tissue layers in which anthocyanins accumulate (Dayton, 1959).

Color measurements in plants are performed using a variety of methods. Color assessment by comparison to color charts is the most economical, but also the most tedious and subjective. Colorimeters provide, at a higher cost, an objective measurement but usually of a localized area. To facilitate this work we developed image processing software to make relative color measurements of "large" surface areas in an unlimited number of photos. Other software packages have been implemented to perform similar functions (e.g. detection of fruit blemishes, see Du and Sun (2004) and references therein), but to our knowledge none of them can perform this function automatically, on a large set of images and at the same time being freely available. A high throughput image processing method, that is fully automatic, and that is made freely available to the academic community can be very valuable.

In this study, 'Honeycrisp' trees were propagated from buds occurring on branches with exclusively striped or blushed fruits to study the stability of the blushed and striped traits following asexual propagation. Contrary to the stable behavior of many apple color sports, preliminary observations indicated that 'Honeycrisp' trees propagated in this way did not consistently produce blushed or striped fruit over time. Additional objectives of this study were to determine whether blushed and striped fruit are distributed on the tree following a consistent pattern that could be explained by environmental effects or plant

conditions; to assess the effect of crop load on the percentage of blushed fruit on each tree and finally to compare the timing, intensity and location of anthocyanin accumulation in blushed and striped fruits.

2.3 Materials and Methods

Experiments were conducted during the 2005, 2006 and 2007 growing seasons at the University of Minnesota Horticultural Research Center in Chanhassen, MN (45°N). Preliminary data were collected in 2003 and 2004. 'Honeycrisp' trees used in this study were planted in 1999, on B.9 rootstock, at a 1.8 m spacing within rows and 4.9 m between rows, in a randomized fashion. A total of 73 of these trees were propagated from buds on 23 selected branches with exclusively blushed fruit (here referred to as "blushed trees") and 99 were propagated from 27 branches with exclusively striped fruit (here referred to as "striped trees"). Fruit was thinned each year after June drop on each tree to homogenize crop load, leaving approximately 5 fruit/cm^2 of trunk cross sectional area. Leaf and soil analyses were performed in August 2005.

For the 2005 color evaluations, 16 blushed trees that produced only blushed fruit and 16 striped trees that produced only striped fruit in 2004 were selected. The most intense red portion of the petals was measured on 10 tagged flowers per tree on Apr. 29 (pink blossom) using the Royal Horticultural Society color charts (1995) . Using the same trees, fruit color measurements at the most intense red portion of the peel were taken on June 1 with the color chart. We measured fruit color in 10 fruits per tree in early, mid and late August with a colorimeter (Minolta chroma meter CR-200, Minolta Camera Co., Ltd, Japan) using the CIE *L*, a*, b** system. Hue angle was estimated using the formulas provided by McGuire (1992) and reported in degrees (0° = red-purple, 90° = yellow and 180° = bluish-green). Two measurements per fruit were taken on the reddest side of the fruit.

In 2006, 30 flowers were tagged, when available, only on the 16 blushed trees selected in 2005. This change in the way of selecting fruit was justified by the very low incidence of blushed fruit observed in 2005 on the selected blushed trees, as well as on the block as a whole. Petal color measurements were taken on tagged flowers on Apr. 27 (pink blossom) using the color chart. The early June fruit color evaluation was not possible in 2006 due to absence of red coloration. Fruit color measurements were started on June 15, and performed every 18 days on average, using the colorimeter. One measurement per fruit was taken.

Four branches per tree, carrying up to 15 fruits each, were selected on 32 trees that produced both kinds of fruits in 2004. Each year we recorded the number of blushed and striped fruit on each branch a week before harvest. All fruit was harvested each year and classified as striped or blushed for each tree individually and the percentage of blushed fruit was then calculated for each tree. In addition, in 2007 the intensity of the stripes was visually evaluated and fruits were scored as either weakly or strongly striped.

Each year we took 2 (2005) or 4 (2006 and 2007) photos per individual blushed and striped fruit after harvest; fruits were rotated 180 (when 2 pictures were taken) or 90 degrees (when 4 pictures were taken) between pictures so that all the surface of each fruit appears in at least one picture (except for small areas at

the top and bottom of the fruit). When 4 pictures were taken, every point on the fruit equator is seen exactly twice (once close to frontally), reducing the bias introduced by the orientation of the fruit chosen. Pictures were taken in the lab, under constant light conditions from fluorescent tubes, using a Canon digital camera model A75 at 2048 x 1536 (2005 and 2006) or 2816 x 2112 (2007) resolution. Translucent paper was placed between the source light and the fruit to avoid highlights. The images were then processed using the color analyzer software we developed. A total of 168, 210 and 537 fruits were evaluated in 2005, 2006 and 2007 respectively. Color was also measured with a colorimeter in the reddest portion of the peel, except in 2005 because a high proportion of the fruit had been damaged by hail, and reddest areas were usually associated with this damage.

The color analyzer is a software tool developed in Matlab (Version 7.1, The Mathworks) to measure statistics (e.g. mean color, color variance, etc.) of areas of interest in a set of pictures. The measurements are assumed to be made under unknown but constant illumination conditions. Therefore the resulting color estimates are relative to these illumination conditions and can only be used for comparisons of pictures taken under the same conditions, and not for absolute measurements. In an initial stage the software must be "trained" (using this term as understood in the machine learning community) to segment these areas. The user trains the program by selecting and labeling points in the picture as belonging to the class of interest (foreground) or to the rest of the picture (background). The training is interactive: the user selects some points and is shown the result of the segmentation, adds more points if necessary to correct the result, until it is satisfactory. This training usually takes less than 15 minutes and is performed only once at the beginning of the photo analysis process. Given the automatic nature of the process, no noticeable differences that can be attributed to variability among individual trainers of the program are observed. After the training is finished, the program automatically processes one picture at a time, by first segmenting the area of interest and then computing the color statistics of this area (Fig. 3). To select the area of interest, each point in the image is classified as foreground or background using a nearest-neighbor classifier (Duda et al., 2001). The nearest-neighbor classifier assigns to each pixel the label of the closest (according to Euclidian distance in RGB space) training pixel. To remove small interspersed regions, two morphological operations (Gonzalez and Woods, 1987) are performed in sequence on the result of the classification: first the closure operation and then the opening of the complement. The statistic computed for the selected region was its mean color, calculated in RGB coordinates and expressed in the HSV (hue, saturation and value) color space. Note that in this output hues are also expressed in degrees. The output is automatically exported to Excel. The code can be obtained from the authors by request and can be used within Matlab or as a standalone application. Running the program within Matlab has the advantage that it can then be easily modified to return any other statistic of the segmented region (e.g., shape, area, visual appearance) of the crop of interest.

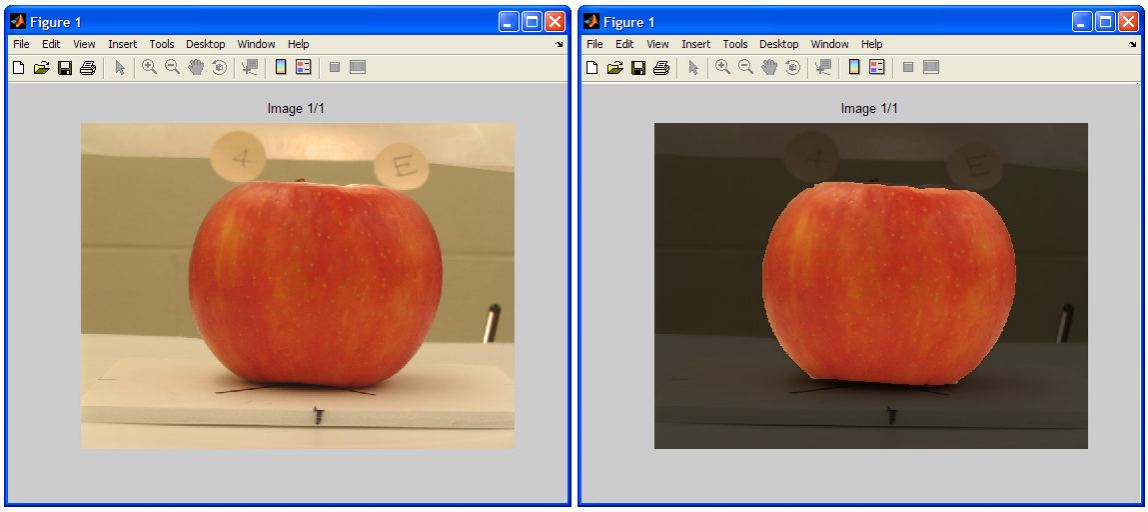

Figure 3. The implemented color analyzer image processing tool. Two screenshots of the implemented color analyzer software tool showing the original image (left) and the segmented result (right).

To study the distribution of blushed and striped fruit on individual trees and the effect of light interception, we used nine 30-year-old 'Honeycrisp' trees on M26 planted on the same farm at a 2.4 m spacing within rows and 4.9 m between rows. An upper (upper third of the canopy) and a lower (lower third of the canopy) scaffold branch were selected both on the SW and NE sides of the tree. Each branch was divided into an inner portion, extending one meter from the trunk, and an outer portion, extending from one meter out from the trunk to the branch tip. At the time of commercial harvest we recorded the number of blushed, weakly striped and strongly striped fruit on each branch and branch sector, and noted whether they were single fruits or part of fruit clusters.

We observed multiple peel cross sections to learn about the histological localization of anthocyanin containing cells in both kinds of fruits. In striped fruits we compared red and green stripes. Fruit peels were free-hand sectioned from mature blushed and striped fruit using a razor blade. They were then mounted on slide glasses with a drop of distilled water and examined using an optical microscope (Leitz Wetzlar, Germany).

The incidence of blushed fruit and the effect of crop load on the incidence of blushed fruit were analyzed by binomial regression analysis in the program ARC (Cook and Weisberg, 1999). The effect of fruit position on the tree and frequencies of blushed, weakly and strongly striped fruit were analyzed using Chi-square tests and contrasts in SAS system for Windows, release 9.1 (SAS Institute Inc, Cary, NC). Color measurements for blushed and striped fruit were compared within each date using the procedure GLM of the SAS. The mean separation test used was least significance difference.

2.4 Results and Discussion

Blushed trees produced a significantly ($p<0.01$) higher percentage of blushed fruit than striped trees in 2005 (23.0 vs. 5.0%), 2006 (8.4 vs. 2.2%) and 2007 (1.7 vs. 0.2%). Striped trees additionally produced fruit with a higher intensity of striping when compared to blushed trees, with proportionally more fruit falling under the category of strongly striped (96.6 vs. 88.6%) and less under weakly striped (2.9 vs. 8.1%) ($p<0.01$).

The percentage of blushed fruit on any given tree or branch changed from year to year. On trees carrying blushed fruit, higher crop loads were associated with lower percentage of blushed fruit (Fig. 4). The binomial regression models constructed using data from trees that produced at least one blushed fruit indicate a significant effect of the number of fruit per unit of trunk cross sectional area on the incidence of blushed fruit on individual trees for the three years ($p<0.01$). Models were statistically different among years. The increase in average crop load from 1.2 fruit/cm^2 in 2005 to 5.1 fruit/cm^2 in 2007 for the block as a whole may partially explain the decreasing percentages of blushed fruit observed. Additional factors that were not measured in this study, such as climate variables, higher pest pressure, or others, could have also influenced the behavior of the plants.

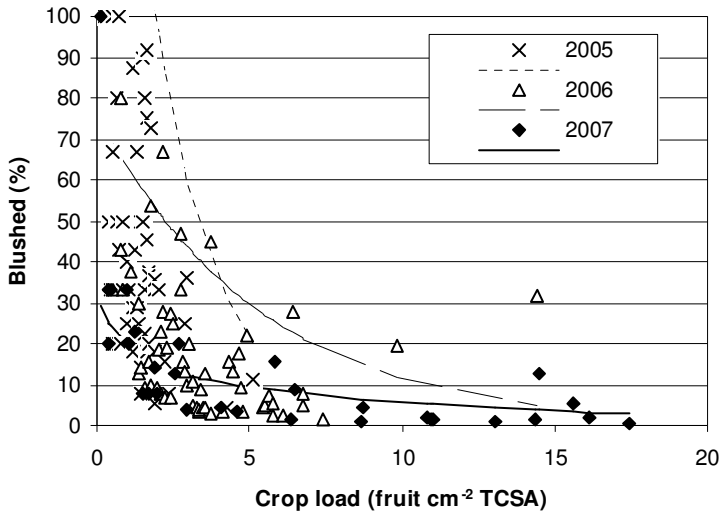

Figure 4. Crop load effect on the percentage of blushed fruit. Crop load (expressed as number of fruits per trunk cross sectional area) and the percentage of blushed fruit for individual trees that produced at least one blushed fruit (2005, 2006 and 2007). The equations for the models fitted are: Blushed 2005=$\exp^{(0.31-0.51*F)}$/1+$\exp^{(0.31-0.51*F)}$, Blushed 2006=$\exp^{(-0.96-0.19*F)}$/1+$\exp^{(-0.96-0.19*F)}$ and Blushed 2007=$\exp^{(-1.87-0.099*F)}$/1+$\exp^{(-1.87-0.099*F)}$ where F is the number of fruits per cm^2 of trunk cross sectional area.

Observations performed on older 'Honeycrisp' trees indicated that the position of the fruit on the tree is related to the pattern of anthocyanin deposition. Thirteen percent of the fruit on NE facing branches was blushed, as compared to only 6.0% on SW facing branches ($p<0.05$). Lower branches and inner sections of

the branches tended to produce more blushed fruit, but these differences were not statistically significant. Similar results were obtained when considering the intensity of stripes, with SW branches, which in addition to producing a higher percentage of striped fruit, also showed a significantly higher proportion (76.4 vs. 68.8%) of strongly striped fruit than the NE branches ($p<0.0001$). These results suggest that higher light incidence on the bud or the fruit correlates with an increase in the occurrence and strength of stripes.

Fruits within clusters were more likely ($p<0.01$) to be all of the same kind (blushed, weakly or strongly striped). This analysis took into account the relative probabilities of each type of apple on the tree. Although spurs carrying fruits of different kinds were observed, fruits in closer proximity were more likely to have similar phenotypes, suggesting the existence of a localized mechanism of regulation of the pattern of anthocyanin accumulation.

We did not find significant differences in petal color when comparing blushed and striped fruit. In 2005, blushed fruit measured early in development (June 1) were darker than striped fruit. This early fruit evaluation could not be performed in 2006 due to absence of red coloration at this stage. This was probably a consequence of weather conditions more conducive to faster pigment degradation than in the previous year. Blushed fruits in 2005 were significantly redder (color measured at reddest point) than striped fruit on August 23 ($p<0.05$), with a similar trend observed in earlier dates (Fig. 5). Differences in color between blushed and striped fruit were not observed in 2006 (Fig. 4) or 2007. Collectively, these data indicated no consistent differences in maximum pigment accumulation during fruit development between fruit that would ultimately be classified as striped or blushed.

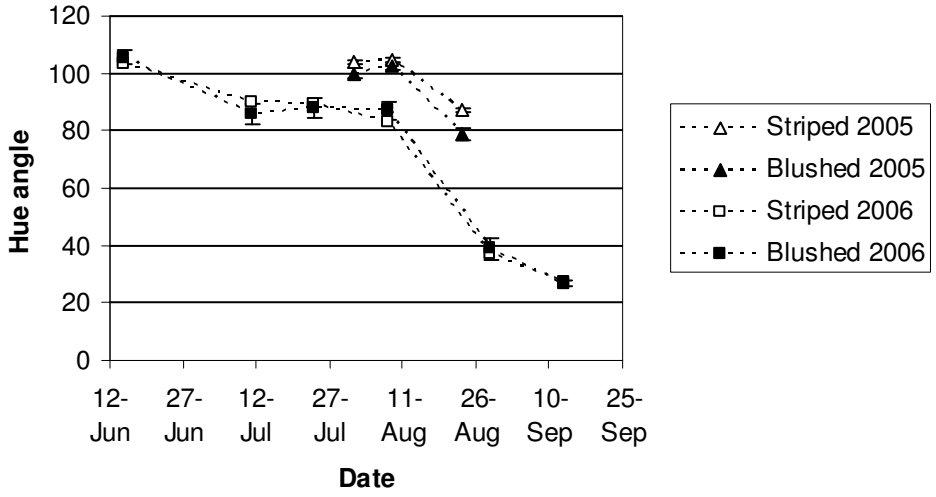

Figure 5. Blushed and striped fruit color throughout the growing season. Fruit peel hue angle measured at the reddest point of the fruit surface. Data points represent the average for all blushed or striped fruits measured on each date. Bars represent standard errors.

The comparison of average hue angle for the whole peel at harvest from photographic data indicated that blushed fruit were significantly redder ($p<0.01$) in all years (14.0 vs. 22.3, 10.8 vs. 14.0 and 28.1 vs. 31.7° for 2005, 2006 and 2007 respectively).

The lack of consistent color differences throughout development in the reddest point of the fruit peel indicated that differences between blushed and striped fruit are only consistently detectable when measuring color for the whole peel. We cannot rule out that measurements of color in the whole peel like those performed from photos at harvest might give different results in earlier dates, but such measurements are impractical before harvest since they require controlled light conditions. Given that blushed fruit are redder than striped ones, the goal should be to increase blushed fruit production when target markets prefer redder fruit.

Blushed and striped fruits did not accumulate anthocyanins in different layers of the peel (Fig. 6A and 6B). Both kinds of fruits showed anthocyanin accumulation in the epidermis and hypodermis; color intensity was related to the proportion of cells accumulating anthocyanins. In striped fruit, the difference between stripes was only due to lack of anthocyanin accumulation in the paler stripes, and not due to a deeper localization of the anthocyanin-containing cells (Fig 5C and 5D) as proposed by McMeans et al. (1998) for the case of 'Gala' apple.

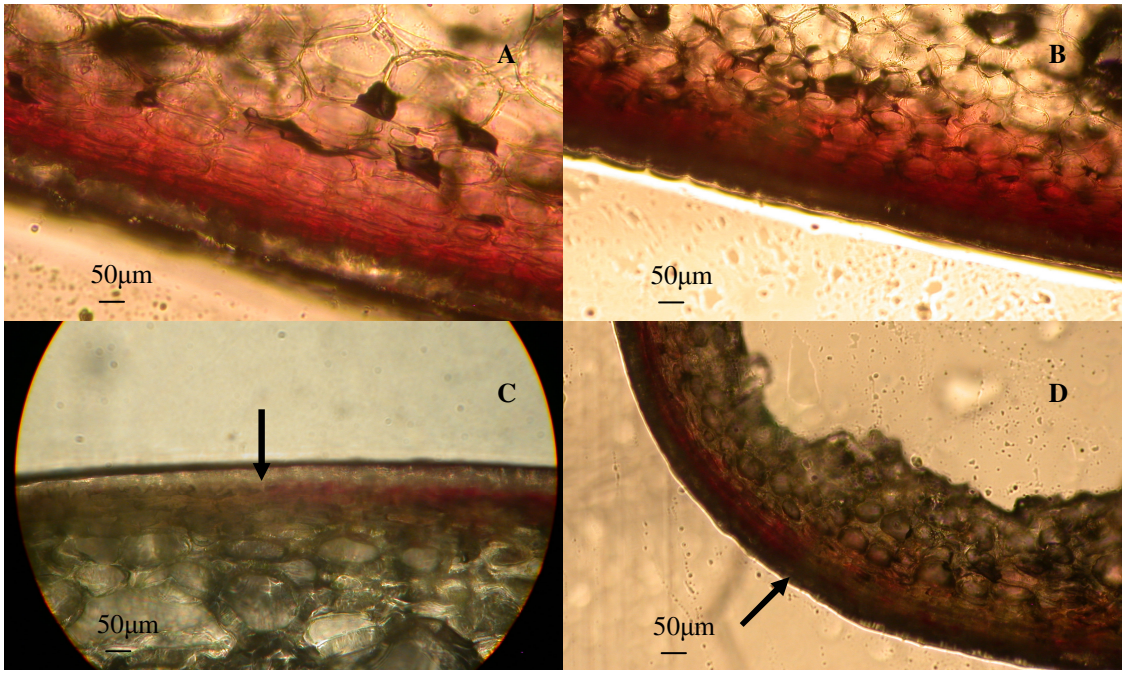

Figure 6. Anthocyanin tissue localization in blushed and striped fruit. Peel cross sections of A) a striped fruit, B) a blushed fruit, C and D) striped fruit, showing the intersection of a red and a green stripe (indicated with an arrow).

Among the trees that produced the highest percentages of blushed fruit throughout this study all but one were originally blushed, but in no case was the percentage higher than 50% across all years. The

absence of trees with consistently high percentage of blushed fruit highlights the importance of regulating crop load if the objective is to increase blushed fruit production. Even then, reaching consistently high percentages of blushed fruit might be challenging under our conditions.

The tendency of blushed trees to produce a higher percentage of blushed fruit indicated that the blushed/striped trait in 'Honeycrisp' apple is partially conserved after asexual propagation. Given the significant environmental effects and that single plants and even single spurs produced both kinds of fruits, a strictly genetic control of the trait can be ruled out. These results also indicated that the blushed/striped phenomenon is probably not chimeral.

We speculate that an epigenetic mechanism (i.e. involving changes in gene function that do not involve changes in DNA sequence) controls the pattern of anthocyanin deposition in 'Honeycrisp' apple peels. Crop load and environmental effects can be some of the factors involved in the regulatory mechanism. Many examples of epigenetic mechanisms controlling pigment accumulation in plants have been described. In corn (*Zea mays* L.), variegated leaf phenotypes are due to the accumulation of anthocyanin in some clonal sectors but not in others. According to Cocciolone and Cone (1993) differential DNA methylation in a gene controlling anthocyanin accumulation (*Pl-Bh*) can account for the variation observed. Plants carrying the *Pl-Bh* allele show sectors of pigmented and unpigmented cells. The authors propose that the *Pl-Bh* gene is silenced through DNA methylation early in development in a portion of the cells. Cells subsequently divide resulting in sectors of predominantly pigmented cells (where *Pl-Bh* in unmethylated) and cells predominantly lacking pigment (methylated). Transposons are another likely explanation for the phenomenon observed; like differential methylation, transposon activity could be both maintained through cell division and environmentally regulated. Different kinds of transposons have been identified in apple to date (Antonius-Klemola et al., 2006; Han and Korban, 2007; Tignon et al., 2001; Venturi et al., 2005; Yao et al., 2001a).

In summary, the blushed/striped phenomenon in 'Honeycrisp' apple is controlled both at the genetic and the environmental level, leading us to suggest an epigenetic mechanism of control. Increasing blushed fruit production, both through clonal selection and crop load regulation, should be the goal if apples with redder peels are desired. However, our results suggest that obtaining clones that produce exclusively striped or blushed fruit may be difficult. A tool for efficient relative color evaluations (or any other statistics of the segmented area that the user inputs) was developed and is freely available to the community.

3 Apple skin patterning is associated with methylation changes in the promoter of *MdMYB10*

3.1 Abstract

Some apple (*Malus pumila* P. Mill) varieties have attractive striping patterns, a quality attribute that is important for determining apple fruit market acceptance. Most apple cultivars (e.g. 'Royal Gala') produce fruit with a defined fruit pigment pattern, but in the case of 'Honeycrisp' apple, trees can produce fruits of two different kinds: striped and blushed. The causes of this phenomenon are unknown. Here we show that striped areas of 'Honeycrisp' and 'Royal Gala' are due to sectorial increases in anthocyanin concentration. Transcript levels of the major biosynthetic genes and *MdMYB10*, a transcription factor that upregulates apple anthocyanin production, correlated with increased anthocyanin concentration in stripes. However, changes in the promoter and coding sequence of *MdMYB10* do not correlate with skin pattern in 'Honeycrisp' and other cultivars differing in peel pigmentation patterns. A survey of methylation levels throughout the coding region of *MdMYB10* and a 2.5 kb region 5' of the ATG translation start site indicated that an area 900 bp long, starting 1400 bp upstream of the translation start site, is highly methylated. Comparisons of methylation levels of red and green stripes indicated that the degree of methylation of the *MdMYB10* promoter is likely to be associated with the presence of stripes in these cultivars, with red stripes having lower methylation levels. Differences in anthocyanin levels between red and green stripes can be explained by differential transcript accumulation of *MdMYB10*. Different transcript levels of *MdMYB10* in red versus green stripes are inversely associated with methylation levels in its promoter, especially in a 900 bp region upstream of the translation start site. Methylation may be associated with the presence of a TRIM retrotransposon within the promoter region, but the presence of the TRIM element alone cannot explain the phenotypic variability observed in 'Honeycrisp'. We suggest that methylation in the *MdMYB10* promoter is more variable in 'Honeycrisp' than in 'Royal Gala', leading to more variable color patterns in the peel of this cultivar.

3.2 Introduction

Apple peel color is one of the most important factors determining apple market acceptance. In general, red cultivars are the most preferred, and within a cultivar, better colored fruits are in higher demand (Saure, 1990). Consumer preferences vary from country to country and region to region: New Zealand consumers prefer striped apples, consumers in Nova Scotia, Canada prefer blushed apples, while consumers in British Columbia, Canada are more accepting of a range of apple types (Cliff et al., 2002). Peel pigments not only affect visual appeal, they also contribute to the fruit's nutritional value. Apples have been associated with

lowered risks of cancer and cardiovascular diseases, which are thought to be caused by oxidative processes. Polyphenolics, including anthocyanins, the red pigments in apple peels, have been found to be the major source of antioxidants in apple (Boyer and Liu, 2004). Antioxidants are mainly localized in the apple peel, but cultivars exhibit a wide variation in the distribution pattern (Eberhardt et al., 2000; Lata, 2007). Anthocyanin accumulation in apple peels can be affected by genetic, environmental, nutritional and cultural factors, the stage of maturity of the fruit, and by the microenvironment within the canopy (Janick et al., 1996).

The main anthocyanin identified in apple skin is cyanidin 3-galactoside, while cyanidin 3-glucoside levels are very low (Ben-Yehuda et al., 2005; Honda et al., 2002; Kondo et al., 2002). Two categories of genes affect the biosynthesis of anthocyanin. The first category encodes enzymes required for pigment biosynthesis (structural or biosynthetic genes), which have been widely studied in apple (Ben-Yehuda et al., 2005; Honda et al., 2002; Kondo et al., 2002; Takos et al., 2006b) (Fig. 2). The second category is comprised of transcription factors, regulatory genes that influence the intensity and pattern of anthocyanin accumulation and generally control transcription of many different biosynthetic genes. At least three families, MYB, bHLH and WDR, have been found to be involved in the regulation of anthocyanin synthesis, but the specific classes and genes involved vary depending on the species (Espley et al., 2006; Gonzalez et al., 2008; Spelt et al., 2002).

Flavonoid intermediates (gray boxes) and end products (black boxes) are indicated. Enzymes required for each step are shown in bold uppercase letters (PAL, phenylalanine ammonia lyase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone-3 b-hydroxylase; FLS, flavonol synthase; GT, unidentified enzyme encoding a glycosyl transferase for flavonol glycone synthesis; DFR, dihydroflavonol-4-reductase; LAR, leucoanthocyanidin reductase; LDOX, leucoanthocyanidin dioxygenase; ANR, anthocyanidin reductase; UFGT, UDP-glycose:flavonoid-3-O-glycosyltransferase (adapted from Takos et al. 2006b).

In apple, three research groups have independently identified an R2R3 MYB transcription factor responsible for anthocyanin accumulation in peels, and the loci have been named *MdMYB10*, *MdMYB1* and *MdMYBA* (Allan et al., 2008; Ban et al., 2007b; Espley et al., 2007; Takos et al., 2006a). The MYB transcription factors discovered in these experiments likely originate from linked loci or are allelic. *MdMYB10* transcription strongly correlates with peel anthocyanin levels and this gene is able to induce anthocyanin accumulation in heterologous and homologous systems (Espley et al., 2007). In addition, *MdMYB10* co-segregates with the *Rni* locus, a major genetic determinant of red foliage and red color in the core of apple fruit (Chagne et al., 2007). Transcript levels of *MdMYB1*, identified by Takos et al. (2006a) also correlate with anthocyanin accumulation and are higher in red fruit peel sectors (more exposed to light) and in red peel cultivars than in green peel sectors or cultivars. Transcript levels of *MdMYB1* increased in dark-grown apples once exposed to light, providing additional evidence of its role as an anthocyanin regulator. Three alleles of *MdMYB1* have been described based on SNPs present in the promoter and coding sequence, and only one of them, *MdMYB1-1,* co-segregates with red skin color (Takos

et al., 2006a). The expression of several anthocyanin pathway genes was found to be regulated by both MdMYB10 and MdMYB1 (Espley et al., 2007; Takos et al., 2006a) (Fig. 2). *MdMYBA* is also more highly transcribed in redder peels, and its transcription is induced by UV-B light and low temperature (Ban et al., 2007b). The coding region of *MdMYBA* is 100 and 98% identical to *MdMYB1* and *MdMYB10*, respectively (Ban et al., 2007b). In addition, *MdMYB10* and *MdMYBA* have been mapped to the same region on linkage group 9 (Ban et al., 2007b; Chagne et al., 2007), supporting the idea that these three genes differ minimally or may be alleles of each other. In apple, two candidate bHLH transcription cofactors (bHLH3 and bHLH33*)* are also needed for activating promoters of anthocyanin structural genes and *MdMYB10* (Espley et al., 2009; Espley et al., 2007).

Repressors of anthocyanin production were also identified within the MYB class of transcription factors, including *FaMYB1* in strawberry (Aharoni et al., 2001) and *AtMYBL2* in Arabidopsis (Dubos et al., 2008; Matsui et al., 2008). *FaMYB1* is up-regulated jointly with late anthocyanin pathway genes, and it was suggested that its role is to balance anthocyanin levels produced at later stages of strawberry maturation (Aharoni et al., 2001).

'Honeycrisp', an increasingly important apple cultivar developed at the University of Minnesota (Luby and Bedford, 1990), produces fruits that can adopt two basic peel color patterns: blushed or striped (Fig. 1). For the purposes of this study, fruits are defined as striped when the color alternates between vertically elongated regions in some or all portions of the peel. Fruits are termed blushed when the surface is partly covered with a red tinge that is not broken. These two phenotypic categories are mutually exclusive. In 'Honeycrisp' both kinds of fruit may be present on the same tree, a characteristic that has not been described in other cultivars. The molecular basis of this phenomenon is unknown.

Different mechanisms can cause variegation in plants, including chimeras (Dayton, 1969), transposable element activity (Pooma et al., 2002), cytosine methylation (Cocciolone and Cone, 1993) and paramutation (Chandler and Stam, 2004). Previous results do not provide evidence for a chimeral source of variegation in the case of 'Honeycrisp', since the phenotype is not stable after propagation (Telias et al., 2008) as would be expected if changes were caused by a periclinal chimera. Microscopic observations indicated that the difference between stripes is due to a reduction in pigment accumulation in the paler stripes, both in the epidermis and in the first hypodermal layers (Telias et al., 2008).

Activation and suppression of transposable elements may be responsible for some of the genetic variation that occurs in peel color in pome fruits (Brown, 2003). Transposable elements have been identified in apple (Antonius-Klemola et al., 2006; Hadanou et al., 2003; Han and Korban, 2007; Tignon et al., 2001; Venturi et al., 2005; Wakasa et al., 2003; Yao et al., 2001a; Zhao et al., 2007a) but to date there is no evidence associating transposable elements with fruit peel variegation. The presence of transposable elements can affect gene expression both at the transcriptional (e.g. through the introduction of an alternative transcription start site), and at the post-transcriptional level (Feschotte, 2008).

Cocciolone and Cone (1993) reported that striped patterns of anthocyanin accumulation in maize were due to differential DNA methylation in the 3' untranslated region of *Pl-Bh*, a MYB transcription factor

regulating anthocyanin accumulation. Methylation was found to be inversely correlated with *Pl-Bh* mRNA levels in variegated plant tissues. The authors hypothesized that early during development, the *Pl-Bh* gene would be differentially methylated and this methylation would be more or less maintained through subsequent cell divisions, producing clonal sectors in plant tissues of predominantly pigmented cells (unmethylated) and sectors of predominantly unpigmented cells (methylated). It is not known whether methylation is responsible for color differences in apple.

We therefore sought to understand the molecular mechanism responsible for 'Honeycrisp' color pattern regulation and instability. We also included in this study two stably striped cultivars ('Royal Gala' and 'Fireside'), a stably blushed cultivar ('Connell Red', a sport of 'Fireside') and other cultivars differing in the degree of peel pigmentation. Our results showed that variation in pigment accumulation between red and green stripes correlates with anthocyanin levels, and the steady state mRNA levels of both the anthocyanin biosynthetic genes and the transcription factor *MdMYB10*. Sequence variation in the *MdMYB10* promoter and coding region does not explain the observed phenotypes. The upstream and coding regions of *MdMYB10* were examined in red and green stripes for DNA methylation levels and a 900 bp region, starting 1400 bp upstream of the predicted translation start site, was found to be highly methylated in both 'Honeycrisp' and 'Royal Gala'. Red stripes were associated with lower methylation of this region in both cultivars, but no differences were found between blushed 'Honeycrisp' green and red peel regions.

3.3 Materials and Methods

3.3.1 Plant material

Leaf samples of 'Honeycrisp', 'Connell Red', 'Fireside', '1807', 'Honeygold' and 'Geneva' apple were collected in early spring of 2005 and fruits of the same cultivars were collected at maturity during the 2005, 2006, 2007 and 2008 growing seasons from trees at the Horticultural Research Center in Chanhassen, Minnesota. In February 2008, 'Royal Gala' fruits were harvested at Plant and Food Research orchards (Nelson, New Zealand). 'Royal Gala' apples grown in Chile were purchased in a Minnesota grocery store in April 2008 to be used for methylation experiments. For the *MdMYB10* characterization experiments, whole fruit peels were used. For anthocyanin quantification, transcript analyses and methylation studies, red and green stripes were carefully separated using a razor blade. Since stripes cannot be absolutely classified as green or red, samples are more accurately described as "red stripe enriched" or "green stripe enriched". Both green and red stripes were obtained from the same region of the peel at each time, preventing the possibility that the "red stripe enriched" sample would also be enriched for fruit peel regions more exposed to light and vice versa. For comparisons between different blushed fruit regions, light-exposed (redder) and -unexposed (greener) peel regions were separated. For both blushed and striped fruit

regions, peel tissue from at least two apples was pooled. In all cases, leaves and peels were immediately frozen in liquid nitrogen and placed at -80ºC before anthocyanins, DNA or RNA was extracted.

3.3.2 Identification and quantification of anthocyanins

Apple peel samples were finely ground in liquid nitrogen and then resuspended in 1 ml methanol and 0.1% HCl. Samples were sonicated for 4 min, stored at room temperature in the dark for 3 h and then centrifuged at 3,000 x g. Aliquots of 1.0 ml were dried down to completion in a Labconco Centrivap Concentrator (Labconco, Kansas City, MO, USA). Samples were resuspended in 20% methanol (250 ul). Anthocyanins were identified by LC-MS analysis as described previously (Stevenson et al., 2006). Identification was based both on masses (M^+) of molecular ions and characteristic fragments/neutral losses and comparison of retention times and fragmentation with authentic standards of cyanidin-3-O-glucoside and cyanidin-3-O-galactoside. M^+ fragments observed were 303 Da (delphinidin), 287 Da (cyanidin) and 271 Da (pelargonidin). Neutral losses (i.e. mass differences between fragments) observed were 162 Da (hexoside sugar, e.g. galactose), 146 Da (deoxyhexoside sugar, e.g. xylose) and 132 Da (pentoside sugar, e.g. arabinose). MS cannot distinguish between sugars of the same mass (e.g. glucose/galactose). Anthocyanins and other phenolic compounds were quantified by HPLC as described previously (Stevenson et al., 2006). Quantification was achieved by reference to standards of anthocyanins and other phenolic compounds, using LC-MS data to confirm identification of peaks.

3.3.3 Real-time transcript analysis

Mature 'Honeycrisp' fruit peels were very finely ground in liquid nitrogen and RNA was extracted using the Lopez-Gomez and Gomez-Lim extraction method (1992) as modified by Mann et al. (2008). Briefly, after precipitation with 3M LiCl, RNA was collected by centrifugation at 12,000 x g for 30 min, and second day LiCl washes were eliminated. RNA pellets were resuspended in 400 µl RNAse free sterile water, potassium acetate was added to a final concentration of 0.3M, and the RNA was precipitated with two volumes of absolute ethanol. After incubation for at least 1 hour at 20°C, RNA was pelleted by centrifugation (20,000 x g for 30 min) and resuspended in RNAse free sterile water. RNA was treated with RQ1 RNAse-free DNAse (Promega Corp., Madison, WI) and then purified using the RNeasy RNA clean-up procedure (Qiagen, Valencia, CA). RNA quantification was performed using a Qubit™ fluorometer (Invitrogen Corp., Carlsbad, CA). Total RNA was reverse-transcribed into cDNA using the Super-Script III (Invitrogen Corp.) reverse transcriptase kit.

Real-time PCR amplification and analysis were carried out using an Applied Biosystems 7500 real-time PCR system (Applied Biosystems, Foster City, CA). Reactions were performed in triplicate using 10 ul 2X iTaq SYBR Green Supermix with ROX (Bio-Rad, Hercules, CA) Master Mix, 1 µl 10 mM of each

primer, 1 µl template and nuclease-free water (Qiagen) to a final volume of 20 µl. Primers were designed to amplify *MdMYB10, MdCHS, MdCHI, MdF3H, MdDFR1, MdLDOX, MdUFGT, MdbHLH3, MdbHLH3* and *MdActin* (Appendix I). Primers were also designed to a gene sequence termed *MdMYB17,* which shows highest homology, by BLAST match, to Arabidopsis *AtMYB4* (Jin et al., 2000) and strawberry *FaMYB1* (Aharoni et al., 2001), repressors of anthocyanin biosynthesis. A negative nuclease-free water control was included in each run. PCRs used an initial denaturation step at 95°C for 3 min, followed by 40 cycles of denaturation for 15 s at 95°C and annealing and elongation for 60 s at 60°C. Fluorescence was measured at the end of each annealing step at 60 °C. Amplification was followed by a melting curve evaluation. The data were analyzed with the Applied Biosystems Sequence Detection Software, version 1.4.0.25 (Applied Biosystems), and transcript levels were normalized to *Malus x domestica* actin (*MdActin*, Genbank accession number CN938023) to minimize variation in cDNA template levels. *MdActin* was selected for normalization due to its consistent transcript levels throughout leaf and fruit tissues, with crossing threshold (Ct) values changing by less than 2. For each gene, a standard curve was generated using a cDNA serial dilution, and the resultant PCR efficiency calculations (ranging between 1.839 and 1.945) were used for relative transcript level analysis. Error bars shown in real-time PCR data are biological and technical replicates, representing the means ± SE of three biological samples and three technical replicates. Analysis of variance (ANOVA) on real-time PCR data was performed using JMP® 7.0 statistical software (SAS Institute Inc, Cary, NC). Student's t-test was used to establish significant differences in transcript accumulation between biological replicates.

Mature 'Royal Gala' peel RNA was isolated by a method adapted from that described by Chang et al. (1993), quantified in a NanoDrop nd-100 spectrophotometer running software version 3.0.1 (NanoDrop Technologies, Wilmington, DE) and treated with DNA-free DNAse (Ambion, Austin, TX). For real time-PCR analysis, total RNA was reverse-transcribed into cDNA using the Super-Script III (Invitrogen Corp.) reverse transcriptase kit. Real-time PCR amplification and analysis were carried out using the Roche 480 LightCycler System (Roche Diagnostics, Mannheim, Germany). All reactions were performed using the LightCycler 480 SYBR Green I Master Mix (Roche Diagnostics) following manufacturer instructions. Reactions were performed in triplicate using 5 µl 5 x Master Mix, 1.0 µM each primer and 3 µl diluted cDNA. A negative nuclease-free water (Roche Diagnostics) control was included in each run. Primers used are the same as described above. PCRs used an initial denaturation step at 95°C for 5 min, followed by 50 cycles of denaturation for 10 s at 95°C, annealing for 10 s at 60°C and elongation for 20 s at 72°C. Fluorescence was measured at the end of each annealing step at 72 °C. Amplification was followed by a melting curve analysis with continual fluorescence data acquisition during the 65–95ºC melt curve. The raw data were analyzed with the LightCycler software, (LightCycler 480, Software 1.5) and transcript levels were normalized to *MdActin* to minimize variation in cDNA template levels. For each gene, a standard curve was generated using a cDNA serial dilution, and the resultant PCR efficiency calculations (ranging between 1.81 and 2.0) were imported into relative transcript level analysis. Error bars shown in

real-time PCR data are technical replicates, representing the means ± SE of three replicate real-time PCR reactions. ANOVA on real-time PCR data was performed as described above.

3.3.4 *MdMYB10* characterization

To study sequence diversity in the *MdMYB10* coding region, fruit peel RNA and cDNA were obtained using the modified Lopez-Gomez and Gomez-Lim extraction method as described above. *MdMYB10* coding region was amplified using PfuUltra™ Hotstart DNA Polymerase (Stratagene, La Jolla, CA) using *MdMYB1* primers (Appendix I) and DNA template. Since *MdMYB10* and *MdMYB1* differ minimally within the coding region, primers used to study this region are able to amplify both targets. However, melting curves from PCR suggested one product that, after sequence, was 100% identical to *MdMYB1-1*. Reactions were performed in a 50 µl total volume (15 ng template, 100 ng/µl each primer, 25 mM each dNTP, 2.5 units AmpliTaq™ (Applied Biosystems), 10 x buffer provided by manufacturer and 25 mM $MgCl_2$). PCRs used 35 cycles of 94°C 30 s, 50°C 30 s, 72°C 120 s (Gene Amp PCR system 9700, Applied Biosystems). Fragments were then poly A-tailed by incubating 3 ul PCR product with 1 ul AmpliTaq™ (Applied Biosystems), 1 ul buffer provided by manufacturer, 1 ul 2 mM dATP, and 1 ul sterile water for 24 minutes at 70°C. Fragments were then desalted through a MicroSpin™ S-200 HR column (Amersham Biosciences, Piscataway, NJ) according to manufacturer's recommendations. Desalted fragments were cloned into the pGEM®-T Easy Vector (Promega Corp.), also according to manufacturer's instructions. Plasmids were purified from 3 ml overnight cultures using the Wizard *Plus* SV Minipreps DNA Purification system (Promega Corp.). To verify insert size, 3 µl of plasmid DNA were digested in 1 x manufacturer supplied buffer by 10 units *Eco*RI (Invitrogen) in a 10 µl total volume at 37°C for 1 h. The entire reaction was loaded and separated on 1% agarose gels in TBE buffer, stained with ethidium bromide, and photographed under UV light. Inserts were compared to DNA standards of known size. Subsequently, undigested plasmids were sequenced using 3.2 pM of M13 forward or reverse primer. All nucleotide sequences were determined by Applied Biosystems BigDye Terminator version 3.1 cycle sequencing on an Applied Biosystems 3130xl or 3730xl automatic sequencer (Applied Biosystems) at the University of Minnesota DNA Biomedical Genomics Center's sequencing and analysis facility. Sequences were analyzed, assembled into contigs, and compared to known sequences using SeqMan™ II (Windows 32 vs. 5.08; DNASTAR Inc, Madison, WI).

For characterization of the *MdMYB10* region upstream of the translation start site (referred to as "promoter" for simplification), leaf tissues or fruit peels were very finely ground in liquid nitrogen, and DNA was isolated using the Haymes' method (Haymes, 1996) or using the DNeasy Plant mini Kit (Qiagen). Three promoter regions were amplified using PfuUltra ™ Hotstart DNA Polymerase (Stratagene) using *MdMYB10* primer pairs -2029/-1229, -1411/-678 and -677/47 (Appendix II). Reactions were performed as described above, but without additional $MgCl_2$. PCR fragments were then desalted through a MicroSpin™ S-300 HR column (Amersham Biosciences) according to manufacturer's

recommendations and fragments from three independent replicate reactions per sample were sequenced directly using 3.2 pM of the corresponding forward and reverse primers, as detailed above.

Within the Plant & Food *Malus* gene database (Newcomb et al., 2006) was a DNA sequence identical to Genbank accession EU518249, the promoter of *MdMYB10*. However, between positions -3038 and -2420 upstream of the ATG translation start site of *MdMYB10* (EU518249, 'Royal Gala') was sequence with 85% identity to *TRIM2,* a terminal-repeat retrotransposon in miniature (Antonius-Klemola et al., 2006). To amplify the TRIM element in the cultivars studied, standard PCRs were performed using AmpliTaq™ (Applied Biosystems) in a 50 µl total volume (15 ng genomic DNA as template, 1 µM each TRIM primer (Appendix I), 200 µM each dNTP, 1.25 units Taq, 10 x buffer provided by manufacturer). PCRs used 35 cycles of 94°C 30 s, 55°C 30 s, 72°C 60 s. These same thermocycling conditions were used to study whether the TRIM element is transcribed in the cultivars studied. The template in transcription studies consisted of cDNA obtained as described above, and TRIM forward and reverse primers (Appendix I) or TRIM forward combined with *MdMYB10* -1873 were used (Appendix 2).

3.3.5 Methylation studies

Peel genomic DNA (less than 1 µg) from red or green stripes, or from red and green areas of blushed apples, was digested with McrBC (New England Biolabs, Beverly, MA) in 100 µl total volume including 1 x NEB2 buffer, 0.1 mg/mL bovine serum albumin, 1 mM GTP and 40 U McrBC or 50% glycerol (mock digested reactions). Digestions were incubated overnight at 37°C to ensure complete digestion and then incubated at 65°C for 20 min to halt enzyme activity. Real-time experiments were performed on McrBC and mock digested template as described above for 'Honeycrisp' and primers used are presented in Appendix 2. For each experiment, real-time PCR runs, including a control (mock digested DNA) and a McrBC digested sample, were performed in triplicate, and two or three independent digestions were used. Percent methylation for individual samples was calculated as a function of the delta CT between control and McrBC treated DNA, using the formula $\%\, methylation = 100 - \frac{100}{efficiency^{\Delta CT}}$. Student's t-test was used to establish significant differences in template amounts between biological replicates and subsequently calculate sample size. The estimated sample size was used when determining whether significant differences occurred between red and green peel regions.

3.4 Results

3.4.1 Anthocyanin quantification and transcript levels of biosynthetic genes

Red stripes of 'Royal Gala' and 'Honeycrisp' contained approximately eight and four times as much anthocyanin as green stripes (83 vs. 10 and 38 vs. 10 µg/g of anthocyanin monoglycoside equivalent for 'Royal Gala' and 'Honeycrisp', respectively). In all cases, the major anthocyanin detected was cyanidin-3-galactoside (Fig. 7).

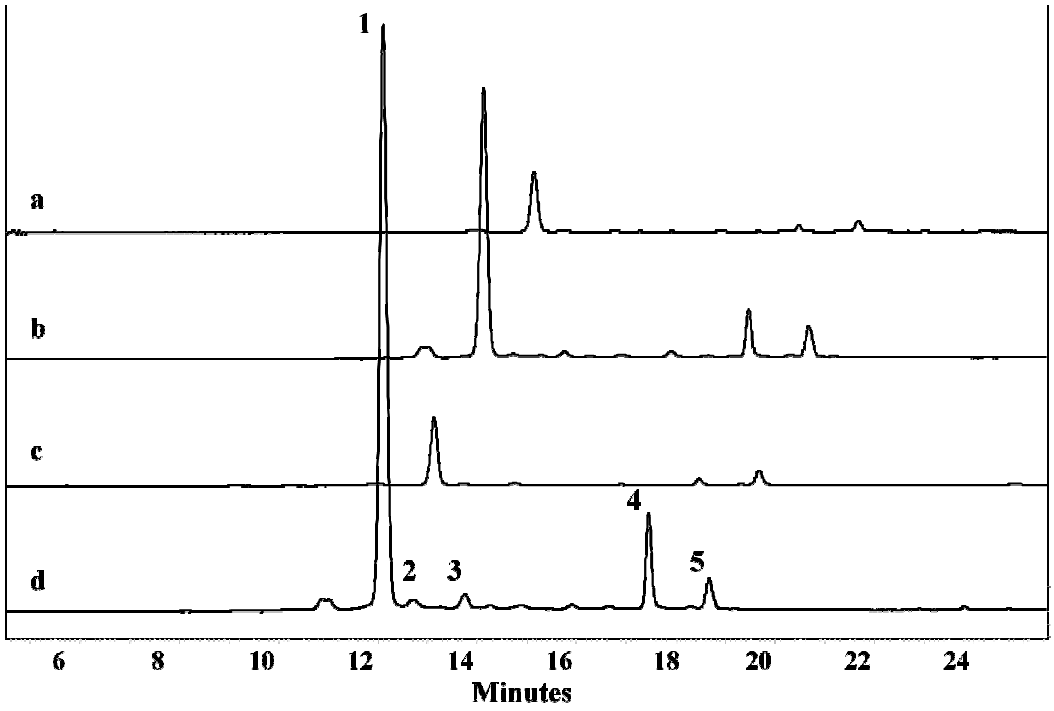

Figure 7. Levels of cyanidin-3-galactoside differ in red and green stripes of 'Honeycrisp' and 'Royal Gala'. HPLC traces at 520nm of a) green and b) red stripes of 'Honeycrisp' and c) green and d) red stripes 'Royal Gala'. Peak identification (observed molecular ion/major fragment, masses in Da):

1 - Cyanidin-3-galactoside (M^+ = 449, 287);

2 - Cyanidin-3-glucoside (M^+ = 449, 287);

3 - Cyanidin pentoside (M^+ = 419, 287 most likely the arabinoside);

4 and 5 - Tentatively identified (ions were low intensity) as pelargonidin derivatives (M^+ = 557, 395, 271 Da, implies presence of pelargonidin, hexoside sugar and an unidentified species; mass 124).

We subsequently compared the transcript levels of regulatory genes *MdMYB10*, *MdMYB17*, *MdbHLH3* and *MdbHLH33* and biosynthetic genes *MdCHS, MdCHI, MdF3H, MdDFR1, MdLDOX, MdUFGT,* in RNA isolated from red and green stripes of 'Royal Gala' and 'Honeycrisp' (Fig. 8). *MdMYB10* and *MdMYB17* transcript levels correlated with anthocyanin concentration in both 'Honeycrisp' and 'Royal Gala', with higher mRNA levels in red stripes as compared to green stripes (ratios significantly larger than 1, $p \leq 0.05$). Transcript levels of structural genes followed the same pattern as those of *MdMYB10* and *MdMYB17*. Levels of the two bHLH transcription factors did not differ in green and red stripes ($p \leq 0.05$), and therefore correlated poorly with anthocyanin concentration. These results suggest that the presence of

stripes is correlated with differential transcript accumulation of *MdMYB10* and *MdMYB17* in the differentially pigmented stripes, which in turn results in a corresponding modulation of transcript levels of structural genes. *MdMYB10* is a known activator of the apple anthocyanin pathway (Espley et al., 2007) and *MdMYB17* is a likely repressor as it is highly homologous to *AtMYB4*, a repressor of the phenylpropanoid pathway (Jin et al., 2000; Zhao et al., 2007b). We decided to further characterize *MdMYB10* coding and upstream regions in order to determine whether sequence polymorphisms can explain different pigmentation patterns.

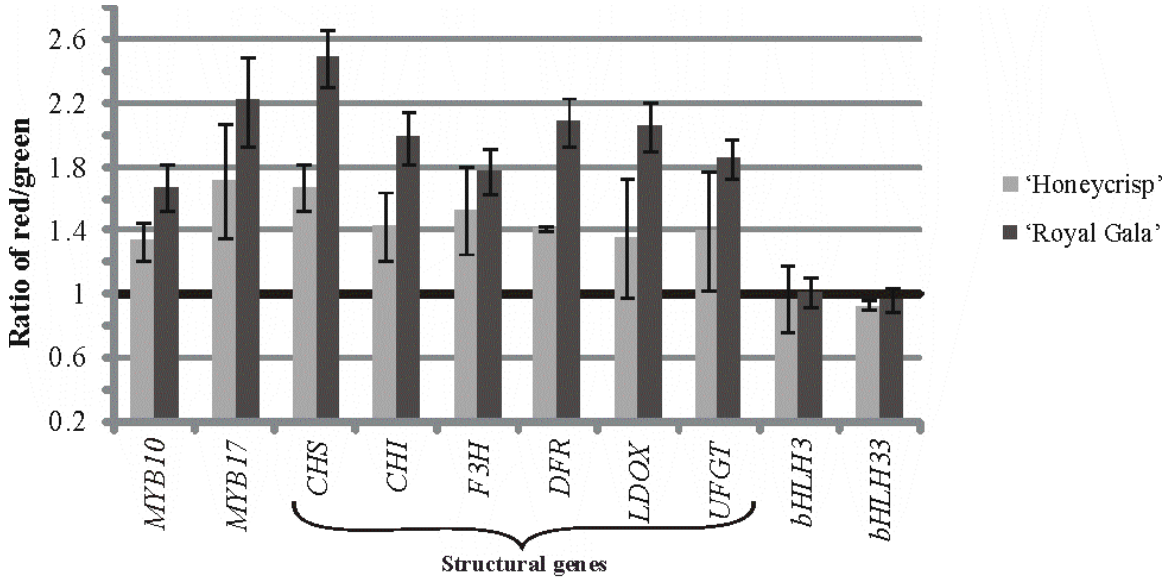

Figure 8. Transcript levels of apple anthocyanin genes determined by real-time PCR. Values indicate the ratio between the normalized transcript levels (relative to actin) in red and green stripes of 'Honeycrisp' and 'Royal Gala' as indicated. Error bars are SE.

3.4.2 *MdMYB10* sequence characterization in 'Honeycrisp', 'Connell Red' and 'Fireside'

3.4.2.1 The *MdMYB10* coding region

To study the possibility that sequence differences are the cause of differential color patterns in the peel, we sequenced a total of 94 cDNA clones of the 'Honeycrisp' *MdMYB10* coding region, 47 from a phenotypically uncharacterized 'Honeycrisp' fruit (harvested in late August when pigment pattern could not yet be conclusively determined), 24 from a mature striped and 23 from a mature blushed fruit. Ninety-two percent of the sequences obtained were 100% identical to *MdMYB1-1* (Takos et al., 2006a). We found three single nucleotide polymorphisms (SNP) that produce changes in protein sequence, but since each one appeared only once in our dataset, and in phenotypically different apples, they most likely represent

copying or sequencing errors. These results indicate low levels of sequence diversity in *MdMYB10* coding region in 'Honeycrisp', with no evidence suggesting that the blushed/striped phenomenon is associated with modifications at the primary DNA sequence level within the coding region. We then sequenced the coding regions of *MdMYB10* in 24 clones of the striped cultivar 'Fireside' and 23 clones of 'Connell Red', a stably blushed sport of 'Fireside'. *MdMYB10* coding region in these two cultivars is identical to that of the most abundant version found in 'Honeycrisp' and the previously published *MdMYB1-1* sequence, again suggesting that no differences in primary DNA sequence are the source of differential patterns of pigment accumulation in these two cultivars.

3.4.2.2 The *MdMYB10* promoter region

We amplified three regions (-2029 to -1229, -1411 to -678, and -677 to 47) (nucleotide positions on the Genbank accession EU518249 relative to translation start site) spanning 2 kb of the *MdMYB10* promoter. PCR results did not indicate any fragment size differences among 'Honeycrisp' blushed and striped, 'Connell Red' and 'Fireside' DNA, therefore no large insertion or deletions were evident. We sequenced the PCR products of each of these fragments from three independent reactions, and found no differences between blushed and striped 'Honeycrisp', or between 'Connell Red' and 'Fireside', but there were 14 SNPs between 'Honeycrisp' and the other two cultivars.

3.4.3 TRIM Transposon

Following our discovery of the TRIM transposable element in the promoter of MdMYB10 in 'Royal Gala', we checked for its presence in 'Honeycrisp' (blushed and striped), 'Connell Red', 'Fireside', '1807' (green selection) and 'Geneva' (ultra red cultivar) via PCR, combining a primer designed from the TRIM element (TRIM forward primer) with one designed from the promoter region of MdMYB10 (primer -1873). Results confirmed the presence of the TRIM element in each of these cultivars in the expected location (Fig. 9). We subsequently cloned and sequenced three of these PCR products from 'Honeycrisp' (blushed and striped), 'Connell Red' and 'Fireside'. Half of the fragments yielded sequences showing 99% or more identity to the upstream region of *MdMYB1/MdMYB10* previously published (Takos et al., 2006, Espley et al., 2009; DQ886414 and EU518249 respectively). The other sequences were probably amplifications from insertions of similar TRIM elements located elsewhere in the genome, with percent identities to *TRIM2* ranging from 40 to 56.5%.

We tested for TRIM transcript presence in blushed and striped 'Honeycrisp', 'Connell Red', 'Fireside', 'Geneva' (ultra red cultivar) and 'Honeygold' (green cultivar), and found it to be transcribed in all cases. However, a fragment spanning a portion of the TRIM element and extending 500 bp into the promoter of *MdMYB10* did not amplify from total RNA, indicating that transcription from the TRIM element did not

extend into *MdMYB10* in these cultivars. Overall, results indicated that neither the presence of the TRIM element in the *MdMYB10* promoter region nor its transcription explained the differences in peel pigment accumulation among the cultivars studied.

3.4.4 Methylation levels in *MdMYB10*

DNA samples from green and red stripes of 'Honeycrisp' (2007 samples) and 'Royal Gala' were treated with the methylation sensitive restriction enzyme McrBC to ascertain whether the observed differences in transcript accumulation were associated with methylation differences in the promoter or coding region of *MdMYB10* (Fig. 9). Eighteen fragments spanning the three exons of *MdMYB10* and 2.5 Kb of the promoter, starting at the transposon insertion were evaluated. McrBC treated and mock-digested templates were compared using real-time PCR, and percent methylation was calculated. Results indicated that a region of the *MdMYB10* promoter, encompassing the fragments between nucleotide positions -1411 and -555 is highly methylated (above 60%) in both cultivars. 'Connell Red' and 'Fireside' had low methylation (20-40%) in the -2254 to -2098 fragment and high methylation (95%) in the -846 to -651 fragment, indicating a similar pattern of *MdMYB10* methylation in these cultivars to those observed in 'Royal Gala' and 'Honeycrisp'.

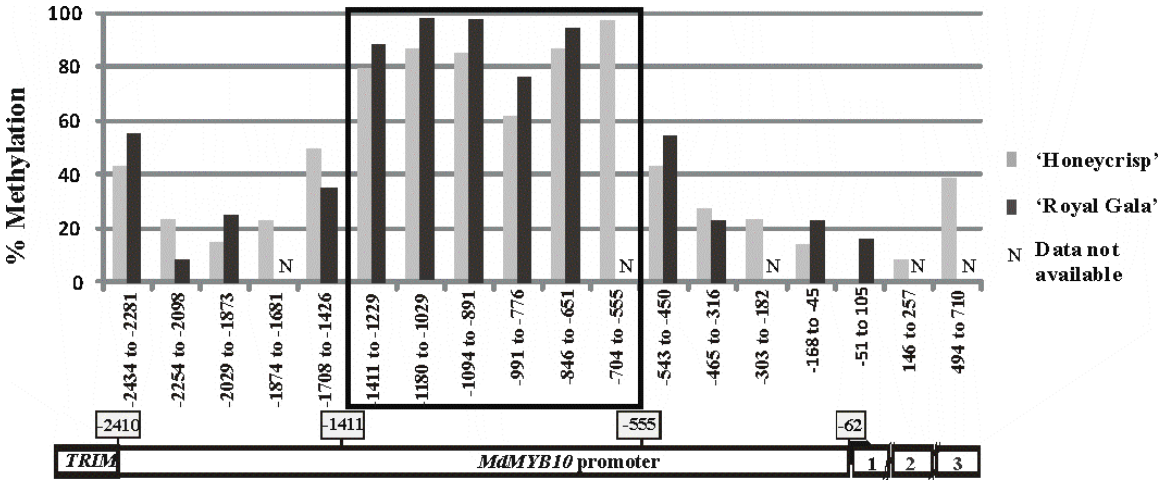

Figure 9. Methylation levels across *MdMYB10* in 'Honeycrisp' and 'Royal Gala'. Percent methylation in 'Honeycrisp' and 'Royal Gala' across the *MdMYB10* locus (Genbank accession EU518249), estimated using an assay combining McrBC digestions and real-time PCR amplification. Values represent the average of green and red stripe samples. Percent methylation indicates the proportion of copies cut by McrBC and is calculated using the following formula: $\%\, methylation = 100 - \frac{100}{efficiency^{\Delta CT}}$. Values on the X axis indicate the location of the primers used relative to the ATG translation start site of *MdMYB10*. The figure at the bottom indicates the relative location of the TRIM element, the *MdMYB10* promoter and three exons;

this figure is not to scale. The calculated % methylation for the -51 to 105 fragment in 'Honeycrisp' was negative, therefore a value of 0 is indicated in the plot. Methylation in the -704 to -555 fragment in 'Royal Gala' could not be estimated given the extremely low template levels in the McrBC treated sample. The -1874 to -1681, -303 to -182, 146 to 257 and 494 to 710 fragments were not evaluated in 'Royal Gala'.

Green stripes of 'Honeycrisp' (2007 samples) showed higher methylation levels overall than red stripes, with the largest differences being found within the highly methylated region (positions -1411 to -555) (Fig. 10A). Comparisons for the -704 to -555 fragment were not reliable since quantification in the McrBC digested samples was highly variable due to extremely low template levels (i.e. this region was so highly methylated that treatment with McrBC resulted in nearly complete digestion of the template DNA). McrBC preferentially cuts DNA containing methylcytosine on one or both strands, between two recognition sites [5'...Pu^{m}C($N_{40\text{-}3000}$)Pu^{m}C...3']. Sequence analysis indicated that differences in predicted methylations levels between regions were not due to difference in the number of potential McrBC recognition sites (data not presented). When 2008 fruits were analyzed, similar results were obtained, but overall methylation levels were higher and differences between red and green stripes were greater, indicating that methylation levels are variable between years (Fig. 10c). Similar trends were observed in 'Royal Gala' for some of the fragments, except that the differences between red and green stripes were smaller and only significant ($p \leq 0.05$) for the -991 to -776 fragment. Overall, higher methylation levels were observed for 'Royal Gala' than 'Honeycrisp' (Fig. 10B).

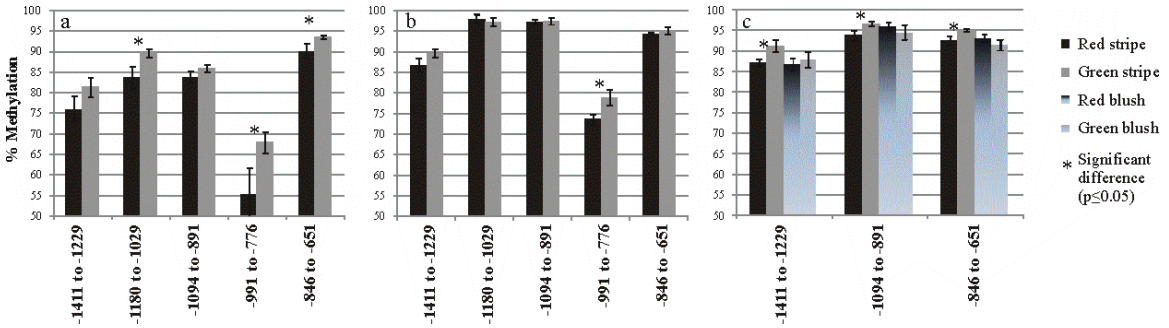

Figure 10. Methylation levels in different fruit peel regions in 'Honeycrisp'. Comparison of percent methylation in the highly methylated region (-1411 to -651) of the *MdMYB10* promoter (GenBank accession EU518249) between: A) red and green stripes of 'Honeycrisp' (2007 fruit samples), B) red and green stripes of 'Royal Gala' (2007 fruit samples), C) red and green stripes and red and green areas of blushed 'Honeycrisp' (2008 fruit samples). Percent methylation was calculated using an assay combining McrBC digestions and real-time PCR. It indicates the proportion of copies cut by McrBC and is calculated using the following formula: $\%\, methylation = 100 - \frac{100}{efficiency^{\Delta CT}}$. The X axis indicates nucleotide positions relative to the ATG translation start site. Error bars are SE and stars indicate significant differences ($p \leq 0.05$).

We hypothesized that color differences between red (exposed to light) and green (unexposed to light) regions of the peel of blushed apples are only due to light effects and not to differences in methylation levels. We therefore compared methylation percentages in red (exposed) and green (unexposed) areas of blushed apples and red and green stripes. Results indicated no significant differences ($p \leq 0.05$) between red and green regions of the peel of blushed apples. Interestingly, in two out of the three regions studied (-1411 to -1229 and -846 to -651), red stripes have methylation levels comparable to those in the peels of blushed apples, while green stripes have methylation levels higher than those of red stripes or red and green regions of blushed apples (Fig. 10C).

3.5 Discussion

3.5.1 Anthocyanin genes transcript levels are associated with the striped patterns observed in some apple cultivars.

Anthocyanin and transcript quantifications in apple peels performed in this study suggested a possible mechanism controlling pigment patterns in apple fruit peels. We have found that green stripes are associated with lower anthocyanin accumulation, which is explained by reduced transcript levels of all the anthocyanin pathway genes evaluated, including the structural genes in the pathway, and *MdMYB10*, a transcription factor which regulates them. An additional gene evaluated in this study, *MdMYB17*, which shows homology to *FaMYB1*, a transcription factor that represses anthocyanin synthesis in strawberry (Aharoni et al., 2001), was found to be transcribed in a similar manner to *MdMYB10*. *MdMYB17* is highly homologous to *AtMYB4*, a repressor of the phenylpropanoid pathway (Jin et al. 2000), and has been found to repress flavonoids when over-expressed in Arabidopsis (Andrew Allan pers. comm.). Given the evidence linking MYB (Sidorenko and Chandler, 2008) and bHLH (Chandler and Stam, 2004) transcription factors to pigment variegation in maize, we considered *MdMYB10*, the main transcription factor regulating the pathway in apple (Ban et al., 2007b; Espley et al., 2007; Takos et al., 2006a), to be the most likely candidate to be involved in peel variegation. We therefore sought to identify a mechanism responsible for *MdMYB10* transcript level differences.

3.5.2 Variegation in apple peels is associated with *MdMYB10* methylation mosaicism

Our results indicate an inverse association between methylation levels in the *MdMYB10* promoter and anthocyanin accumulation in striped apple peels. As previously suggested by Cocciolone and Cone (1993) for maize, we hypothesized that early in apple fruit development, differences in *MdMYB10* methylation are present among individual cells. Throughout fruit growth, these differentially methylated cells would give

origin to sectors of peel varying in their ability to accumulate anthocyanins. Our results indicate that DNA methylation in the promoter of *MdMYB10* is associated with reduced transcript accumulation. DNA methylation may affect transcription through interference with the RNA-polymerase transcription complex or by preventing binding of additional factors required for transcription. Genome wide studies of DNA methylation have indicated that in Arabidopsis, methylation within regulatory regions is rare and probably selected against, as it may interfere with transcription initiation (Zhang et al., 2006). In rice, DNA methylation of the transcribed region is enriched for and is associated with a larger suppressive effect than DNA methylation of the promoter region (Li et al., 2008).

Different methylation levels early in apple fruit development could be mitotically maintained from those in the meristematic cells that gave origin to the fruit, or could result from demethylation or *de novo* methylation. Previous results in 'Honeycrisp' suggest that there is at least some mitotic maintenance of methylation states, given that trees clonally propagated from buds on branches with exclusively blushed fruits, tend to produce a higher percentage of blushed fruit (Telias et al., 2008). Nonetheless, results from the same study indicated that additional factors can influence the pattern in the peel, namely position of the fruit on the tree and crop load. Sekhon and Chopra (2009) identified a gene called *Ufo1* that controls methylation levels in *p1*, a gene that regulates phlobaphene biosynthesis in maize, and whose activity may also produce variegation in the maize pericarp. Ectopic expression of *P1-wr* correlated with hypomethylation of an enhancer region, 5 Kb upstream of the transcription start site. The molecular nature of *Ufo1* is still unknown. An assessment of the exact location and context of *MdMYB10* DNA methylation, as well as other types of epigenetics marks such as histone methylation, can shed light on the mechanism involved in *MdMYB10* regulation. For example, within the most methylated region of the *MdMYB10* promoter in this study (-1411 to -555; Fig. 9) are five putative E-box motifs (Espley et al., 2009), a bHLH-related cis-acting element (CACATG) (Atchley and Fitch, 1997; Heim et al., 2003).

The unstable nature of pigment patterning in 'Honeycrisp' could be a result of a more variable cell to cell methylation pattern than is present in other cultivars producing fruit with consistent pigment patterns, such as 'Royal Gala', 'Fireside' and 'Connell Red'. We speculate that the occurrence of stripes in 'Honeycrisp' is a consequence of the occurrence of higher than normal methylation levels in the green stripes, something that occurs only in some fruit and to varying degrees. In contrast, methylation levels would tend to remain more constant in 'Royal Gala'.

3.5.3 Possible mechanisms controlling *MdMYB10* methylation

The presence of a TRIM transposable element in an upstream region of the promoter might be associated with the changes in methylation observed between different regions of the peel. The TRIM element identified in this study is located 2.5 Kb upstream of the predicted translation start site, and is present in 'Honeycrisp', 'Royal Gala', and five other cultivars of varying peel pigmentation. Lippman et al. (2004) indicated that in Arabidopsis transposable elements can determine epigenetic gene silencing when

inserted within or very near (<500bp) a gene. The effect of a transposable element 2.5 Kb upstream of the coding region is unknown. We did not find any evidence of transposable element sequences within the highly methylated promoter region. Finally, we cannot rule out the possibility of the occurrence of paramutation. This phenomena would occur if an allele of *MdMYB10* controlled the epigenetic state of another allele, a mechanism that has been widely studied in maize (Chandler and Stam, 2004; Sidorenko and Chandler, 2008)

3.6 Conclusions

Differences in anthocyanin levels between red and green stripes can be explained by differential transcript accumulation of *MdMYB10*, a transcription factor that regulates the anthocyanin pathway in apple. Different transcript levels of *MdMYB10* in red versus green stripes are inversely associated with methylation levels in its promoter, especially in a 900 bp region upstream of the translation start site. Methylation might be associated with the presence of a TRIM element within the promoter region, but the presence of the TRIM element alone cannot explain the phenotypic variability observed in 'Honeycrisp'. We suggest that methylation in the *MdMYB10* promoter is more variable in 'Honeycrisp' than in 'Royal Gala', leading to more variable color patterns in the peel of this cultivar.

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Appendix I. Forward and reverse primers used in real-time PCR and RT-PCR analyses

Primers amplified anthocyanin biosynthetic enzymes, candidate transcription factors and TRIM transposable elements.

Gene identifier (Genbank)	Name	Forward primer	Reverse primer
CN938023	*MdActin*	TGACCGAATGAGCAAGGAAATTACT	TACTCAGCTTTGGCAATCCACATC
CN944824	*MdCHS*	GGAGACAACTGGAGAAGGACTGGAA	CGACATTGATACTGGTGTCTTCA
CN946541	*MdCHI*	GGGATAACCTCGCGGCCAAA	GCATCCATGCCGGAAGCTACAA
CN491664	*MdF3H*	TGGAAGCTTGTGAGGACTGGGGT	CTCCTCCGATGGCAAATCAAAGA
AF117268	*MdDFR1*	GATAGGGTTTGAGTTCAAGTA	TCTCCTCAGCAGCCTCAGTTTTCT
AF117269	*MdLDOX*	CCAAGTGAAGCGGGTTGTGCT	CAAAGCAGGCGGACAGGAGTAGC
AF117267	*MdUFGT*	CCACCGCCCTTCCAAACACTCT	CACCCTTATGTTACGCGGCATGT
DQ266451	*MdbHLH33*	ATGTTTTTGCGACGGAGAGAGCA	TAGGCGAGTGAACACCATACATTA AAGG
CN934367	*MdbHLH3*	AGGGTTCCAGAAGACCACGCCT	TTGGATGTGGAGTGCTCGGAGA
CO867070	*MdMYB17*	TGGCTCCAGAAAAGCAAATCA	GGCCGCTTGCAGAATCTGTA
DQ267896	*MdMYB10*	TGCCTGGACTCGAGAGGAAGACA	CCTGTTTCCCAAAAGCCTGTGAA
DQ886414	*MdMYB1*	GCGGTACCGGTAGCAGGCAAAAGAATAGC TAAGC	GCGGATCCCACATTTACAAGCAAG GAAAATA
AY603367	TRIM	CGGGATGTGACAATTTGGTA	GCGATGTGGGATGTTACAAT
EU518249	*MdMYB10* -2029 to -1229	GAAATCGTTCGAAGGTCTAAGG	TTCGTTGGATTCCGTTAAGC
EU518249	*MdMYB10* -1411 to -678	AACCTTCACAAGGGTTGTCG	AATGGATGGAATGGAACGAA
EU518249	*MdMYB10* -677 to 47	TTCGTTCCATTCCATCCATT	AGTCCAGGCACCTTTTCTCA

Appendix II. Forward and reverse primers for apple genes used in McrBC/real-time PCR analysis.

Primers amplified different regions of the *MdMYB10* locus of apple (Genbank accession EU518249). Primer position is indicated relative to the ATG translation start site.

Primer position	Forward primer	Reverse primer
-2434 to -2281	TGTAACAAGATGATGACGACGTGTA	TCTCCGCTCCCCTTCCA
-2254 to -2098	CATTTCCACCGTTCATTTCTAAGTT	CAGTAGAGAGATGAAGAGGCAATGC
-2029 to -1873	GAAATCGTTCGAAGGTCTAAGG	ACAGCAAACACCCAAAATCC
-1874 to -1681	GTTGCCATTTTTGAACACAACA	CCACGTGTTCAGGGTCCTTT
-1708 to -1426	TTTAATAAAAAGGACCCTGAACACG	CGTGATATATGATCTTGATGGTTGA
-1411 to -1229	AACCTTCACAAGGGTTGTCG	TTCGTTGGATTCCGTTAAGC
-1094 to -891	GGTCCCGCAAGACAGATAACC	CACTAAAAAAACACTTAGGCATACGAA
-991 to -776	GGCTGAACCACCTATGAAAATAATG	AGACGCTACACCTAACACATTGCT
-846 to -651	CTCTTGTGAAAGCTTAGTGAGTTGAAG	TGAGAGGAATGGATGGAATGG
-704 to -555	CGGGCTAGGATTTTCTCCTCTT	CTTCTTCATTCCCCTCCTATTTGA
-543 to -450	GGAGAGAATCCTACTCCATAAATTACAAG	CTTTCGCTGCTTTTTCAAGTGTT
-465 to -316	GAAAAAGCAGCGAAAGCATGA	GGAAATCAATCCCAGGGCATA
-303 to -182	GTCGTGCAGAAATGTTAGCTTTTC	CAGAAGCAAACACTGACAAGTTTAAAAC
-168 to -45	TGCACGTCACTGGCCTTGTA	TAAGCTTAGCTATTCTTTTGCCTGCTA
-51 to 105	AGTGGGTAGCAGGCAAAAGA	TCCACTTTCCCTCTCCATGA
146 to 257	GAGCTGCAGACAAAGATGGTTAAA	CCTGTTTCCCAAAAGCCTGTGAAGT
494 to 710	ACCACAAACGTCGTCGTCAAC	CCAAAGGTCCGTGCTAAAGG