Transcription Factor Families Regulate the Anthocyanin Biosynthetic Pathway in *Capsicum annuum*

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ABSTRACT. Anthocyanin structural gene transcription requires the expression of at least one member of each of three transcription factor families: MYC, MYB, and WD40. These transcription factors form a complex that binds to structural gene promoters, thereby modulating gene expression. *Capsicum annuum* L. (pepper) displays a wide spectrum of tissue-specific anthocyanin pigmentation, making it a useful model for the study of anthocyanin accumulation. To determine the genetic basis for tissue-specific pigmentation, we used real-time polymerase chain reaction to evaluate the expression of anthocyanin biosynthetic (*Chs*, *Dfr*, and *Ans*) and regulatory (*Myc*, *Myb_A*, and *Wd*) genes in flower, fruit, and foliar tissue from pigmented and nonpigmented *C. annuum* genotypes. No differences were observed in expression of the *Wd* gene among these tissues. However, in all cases, biosynthetic gene transcript levels were significantly higher in anthocyanin-pigmented tissue than in nonpigmented tissues. *Myb_A* and *Myc* transcript levels were also substantially higher in anthocyanin-pigmented floral and fruit tissues. Our results demonstrate that differential expression of *C. annuum* *Myb_A* as well as *Myc* occurs coincident with anthocyanin accumulation in *C. annuum* flower and fruit tissues. In contrast to the situation in flowers and fruit, differential expression of *Myb_A* and *Myc* was not observed in foliar tissue, suggesting that different mechanisms contribute to the regulation of anthocyanin biosynthesis in different parts of the *C. annuum* plant. Cloning and sequencing of *Myb_A* genomic and cDNA clones revealed two introns of 249 and 441 bp between the R2R3 domains. Whereas the *Myb* R2R3 domains were conserved between *C. annuum* and *Petunia ×hybrida* Vilm., the sequence of the non-R2R3 domains was not conserved, with very little homology in these related Solanaceous species.

Pigmentation of plant vegetative tissue, including leaves, stems, and roots and reproductive tissue in flowers and fruit, is attributed to anthocyanin and carotenoid pigments. These colored compounds are produced by independent metabolic pathways and are responsible for yellow to orange coloration and gradations of red through blue. Anthocyanins arise from the phenylpropanoid pathway and are heterocyclic compounds consisting of a three-ring skeleton with sugar and acyl moieties attached at specific locations. Anthocyanin pigments serve a wide variety of functions in plants that include protection against ultraviolet and oxidative light stress, feeding deterrents, insect pollinator attraction, and seed dispersal. Anthocyanins play a role in consumer preference for flower and fruit quality,

potential food health properties, and related horticultural attributes. As a result, classical breeding, as well as transgene technologies, have been used to enhance or create novel colors in ornamental and food crops.

The biochemistry and genetics of anthocyanin-based pigmentation has been studied extensively since early work by Mendel in the 1800s on pea (*Pisum sativum* L.) flower color. Model systems for study of anthocyanin accumulation include *Zea mays* L., *Petunia ×hybrida*, *Antirrhinum majus* L., and *Arabidopsis thaliana* (L.) Heynh. (Griesbach, 2005; Holton and Cornish, 1995; Winkel-Shirley, 2001). The enzymes in the anthocyanin biosynthetic pathway are well characterized and many of the genes encoding these enzymes have been cloned and share high sequence similarity across species and typically exhibit tissue- or development-specific expression.

Six enzymes are generally involved in anthocyanin biosynthesis. Chalcone synthase (CHS) is the first enzymatic step of the biosynthetic pathway. Twelve different CHS genes (*ChsA*, *B*, *C*, *D*, *E*, *F*, *G*, *H*, *I*, *J*, *K*, and *L*) have been identified in *P. ×hybrida* (Koes et al., 1989) and two (*c2* and *whp*) in *Z. mays* (Coe et al., 1981; Dooner, 1983). Chalcone is isomerized

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by chalcone isomerase (CHI) to naringenin. Two CHI genes (*chiA* and *chiB*) have been identified in *P. ×hybrida* (van Tunen et al., 1988, 1989) and three CHI homologues are present in some *Z. mays* genotypes (Grotewold and Peterson, 1994). Flavanone 3-hydroxylase (F3H) converts naringenin into dihydrokaempferol. The dihydroflavonols are subsequently converted to anthocyanins by the action of three enzymes. Dihydroflavonol is first converted to a colorless leucoanthocyanidin by dihydroflavonol 4-reductase (DFR). Three different DFR genes (*dfrA*, *dfrB*, and *dfrC*) have been described in *P. ×hybrida* (Huitts et al., 1994). The *dfrA* gene corresponds to the *P. ×hybrida* *An6* locus. Leucoanthocyanidins are subsequently converted to colored anthocyanidins by anthocyanidin synthase (ANS). Finally, UDP-glucose-flavonoid 3-O-glucosyltransferase (UFGT) creates the anthocyanin-3-glucoside. Two UFGT gene copies have been identified in *P. ×hybrida* (Yamazaki et al., 2002). CHS is the first and key regulatory enzyme of flavonoid biosynthesis, and DFR is the first committed enzyme of anthocyanin biosynthesis in the flavonoid pathway (Griesbach, 2005).

Tissue- or developmental-specific expression exhibited by anthocyanin structural genes is controlled by regulatory genes. A common set of proteins, comprised of MYB and bHLH MYC proteins plus WD40 repeat proteins, interact to form a regulatory complex that controls transcription of anthocyanin structural genes (Ramsay and Glover, 2005). Most plant MYB proteins are characterized by two (R2 and R3) imperfect repeats of the conserved MYB DNA-binding motif (Martin and Paz-Ares, 1997). Each repeat encodes three α -helices, with the second and third helices forming a helix-turn-helix (HTH) structure when bound to DNA. Anthocyanin-related R2R3-MYB proteins were first characterized in *Z. mays* where anthocyanin accumulation requires the R2R3-MYB proteins C1 for seed pigmentation and P1 for pigmentation in other plant tissues (Cone et al., 1986, 1993). The MYC class of proteins is defined by a conserved basic helix-loop-helix domain consisting of a presumed DNA-binding basic region and a protein-protein dimerization motif. Plant MYC proteins were also first identified in *Z. mays*, where R-like MYC proteins determine the timing, amount, and distribution of anthocyanin pigmentation. The *R* gene family consists of the *R* locus, which also includes *S*, *Lc*, and *Sn*, and the *B* locus (Dooner et al., 1991). R-like MYC proteins were shown to physically interact with C1 MYB proteins to coactivate anthocyanin synthesis by directly binding to the promoters of the biosynthetic genes.

The third component of the anthocyanin regulatory protein complex is represented by a family of WD40 repeat (WDR) proteins (deVetten et al., 1997; Smith, 1999). They comprise a 40-residue WD motif core region that contains the glycine-histidine (GH) dipeptide at the N-terminus and the tryptophan-aspartate (WD) dipeptide at the C-terminus. The WD motif is tandemly repeated four to 16 times and forms the WDR protein, a conserved β -propeller structure that acts as a stable surface to facilitate protein-protein interactions. Plant anthocyanin-related WD40 proteins include the *Z. mays* PAC1 and *P. ×hybrida* AN11 proteins (Carey et al., 2004; deVetten et al., 1997). In a functional MYB-MYC-WD complex, the MYB factor is hypothesized to bind directly to the cis-element of the structural gene, while R-like MYC might bind indirectly via a hypothetical R interaction protein (RIP) (Ramsay and Glover, 2005). R-like MYC is centered in the complex that interacts with a MYB factor with WD proteins on its sides. Together, they activate the entire set of anthocyanin biosynthesis genes.

The anthocyanin pigment found in *Capsicum annuum* leaves, flowers, and immature fruit is delphinidin-3-p-coumaroyl-rutinoside-5-glucoside (Lightbourn et al., 2008). Anthocyanin accumulation in *C. annuum* is reportedly influenced by an incompletely dominant gene *A* and a second modifying gene *MoA* (Deshpande, 1933; Peterson, 1959). The *A* locus encodes a *Myb* transcription factor (*Myb_A*) that is absent in genotypes that do not accumulate anthocyanin (Borovsky et al., 2004). Consistent with these reports, we previously demonstrated that anthocyanin accumulation in immature *C. annuum* fruit was simply inherited with modifying gene action (Stommel and Griesbach, 2008). In contrast with fruit color, inheritance of leaf color in anthocyanin accumulating foliar mutants was complex, involving the action of multiple genes (Lightbourn et al., 2007; Stommel and Griesbach, 2008). To further characterize the tissue-specific regulation of the anthocyanin biosynthetic pathway in *C. annuum*, we compared the expression of the anthocyanin regulatory gene complex (*Myb_A*, *Myc*, and *Wd*) and three structural genes (*Chs*, *Dfr*, and *Ans*) between genotypes with anthocyanin-free and anthocyanin-containing flowers, fruit, and leaves.

Materials and Methods

PLANT MATERIAL AND GROWING CONDITIONS. *C. annuum*, plants of true-breeding U.S. Department of Agriculture (USDA) breeding lines divergent for anthocyanin accumulation in flower, fruit, and leaf tissues were produced from seed and grown under standard greenhouse conditions for *C. annuum* (Stommel and Bosland, 2006). Line 06C19-2 produced white flowers, green immature fruit, and green foliage. Line 06C59 produced purple flowers, black immature fruit, and black foliage. These inbred lines share a common pedigree but are divergent for anthocyanin pigmentation.

FLAVONOID ANALYSIS. Analytical high-performance liquid chromatography (HPLC) (Griesbach et al., 1991) was used to quantify the anthocyanin pigments in black foliage and immature black *C. annuum* fruit. Anthocyanins from flower, fruit, and leaf tissue were extracted in acidified methanol (1% HCl), evaporated to dryness, washed with acetone, and resolubilized in acidified methanol. HPLC characterization of anthocyanidins was performed on a 7.8 $\times$ 300-mm column of 5- μ m Bondapak C18 (Waters, Milford, MA) using a 30-min linear gradient of 0% to 10% (v/v) acetonitrile in aqueous 1.5% (v/v) phosphoric acid and 15% (v/v) acetic acid followed by a 10-min linear increase to 20% (v/v) acetonitrile and held at 20% (v/v) acetonitrile for an additional 20 min. The flow rate was 1.0 mL/min and detection was by absorption at 540 nm. Anthocyanins were identified as previously described (Lightbourn et al., 2008). The amount of anthocyanin was determined by measuring the peak area of three replicate samples using the Maxima software (Waters). The relative amount of pigment was calculated as the mean of the area of absorption per gram of fresh weight of three replicates.

GENE EXPRESSION. Flowers, immature fruit, and leaves were harvested from actively growing plants, frozen in liquid nitrogen, and stored at -80°C . Total RNA was isolated from ≈ 100 mg frozen tissue using the RNeasy Plant Mini Kit with the optional DNase digest (Qiagen, Valencia, CA). RNA quantity was recorded using a spectrophotometer (ND1000; NanoDrop Technologies, Wilmington, DE) and integrity and quantification were confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

Real-time polymerase chain reaction (PCR) was used to compare flavonoid gene expression (*Chs*, *Dfr*, *Ans*, *Myb_A*, *Myc*, and *Wd*) between anthocyanin-pigmented and nonpigmented tissues collected from contrasting genotypes. *C. annuum* specific primers were used for *Myb_A* identification (Borovsky et al., 2004). Degenerate primer sets from *P. ×hybrida* (Griesbach and Beck, 2005) were used to generate PCR products from all other genes. PCR products amplified using degenerate primers were cloned and sequenced to develop PCR primers for amplification of gene-specific products from *C. annuum*. Primer sequences, with the exception of *Myb_A* (forward 5′–AGA TTG CCG GGA AGA ACA GCA AAC–3′; reverse 5′–TTG CAC TTG ATG AGA AGG TCC GAG–3′), were previously described (Lightbourn et al., 2007). For real-time PCR, cDNA synthesis was performed with 1 µg of RNA using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Real-time PCR amplification of the cDNA was carried out as described by Lightbourn et al. (2007) using the iCycler 1Q™ Multicolor Real-Time Detection System (Bio-Rad). Results were normalized to the expression of tubulin (*Tub*) where degenerate primers were designed based on gene sequences reported in GenBank (forward 5′–TGG CTA CCA TCA AGA CTA AGC GCA–3′; reverse 5′–AGA CCT CAG CAA CAC TGG TTG AGT–3′). Normalization to *Tub* was substantiated by comparison with normalization to expression of the 18S ribosomal gene (QuantumRNA™ 18S Internal Standards; Ambion, Austin, TX).

Standard calibrations were computed and the efficiency of reaction kinetics and R values were tabulated and found to fall within the range of 95% to 105% (efficiency) and 0.98 to 1.0 (R value). Each genotype was represented by four replicates. Threshold values [Ct (iQ™ 5 v 2.0; Bio-Rad)] were manually checked for $SD > 0.5$ within triplicate reactions. Normalized relative fold expression values were derived using the Pfaffl method (Pfaffl, 2001), and pairwise comparisons between means were made using T-tests.

MYB_A SEQUENCING. A full-length *Myb_A* genomic clone was generated by PCR using PfuUltra II Fusion HS DNA polymerase (Stratagene, La Jolla, CA) and the *Myb_A* primers (forward 5′–GAT ATC ATG AAT ACT GCT ATT–3′; reverse 5′–CTT ACA TTG AAG ATG CGT GGA–3′) described by Borovsky et al. (2004). PCR products of expected size, obtained after 35 cycles of 95 °C for 20 s, 57 °C for 20 s, and 72 °C for 60 s, were purified from the gel and cloned using the StrataClone Blunt PCR Cloning kit (Stratagene) and the associated TA cloning kits when post-PCR 3′–A addition by Taq DNA polymerase was performed. Plasmid DNA was purified using Genelute Plasmid Mini-Prep Kit (Sigma, St. Louis) and was commercially sequenced (Macrogen, Rockville, MD) to identify the correct clones. All the sequence data were edited manually based on the chromatogram output files before being blastx searched against the GenBank nr database.

The 5′ untranslated region (UTR) region was analyzed following the GeneRacer Kit protocol (Invitrogen, Carlsbad, CA). Purple flowers were used to generate a 5′–RACE ready cDNA PCR template. Using forward and forward-nested primers provided in the GeneRacer kit and reverse and reverse-nested primers designed from the proximal 5′ region of the *Myb_A* ORF, PCR products representing the 5′ UTR were formed. The reverse primer was 5′–CTC CAG AGG ACT TGG CAA TAA TAG CAG–3′, and the nested reverse primer was 5′–AGA GGA CTT GGC AAT AAT AGC AGT ATT C–3′.

The amplified 5′ products were cloned using the StrataClone Blunt PCR Cloning kit (Stratagene). Cloned products were sequenced and forward primers designed to confirm clone identity. The primers were 5′–CAA AGA GCA AGG GAC ACA GCT–3′. Resulting PCR products were cloned and sequenced as described above.

Results

Two related genotypes of common pedigree with contrasting anthocyanin pigmentation were selected for gene expression studies. Line 06C59 produced purple-colored flowers and black fruit and foliage, while line 06C19–2 flowers were white in color, and fruit and foliage were green. Even though line 06C19–2 lacked anthocyanin in the flowers, fruit, and foliage, it did contain anthocyanin pigment in stem internodes. Therefore, functional copies of the anthocyanin genes must be present and the lack of anthocyanin in the flowers, fruit, and foliage must be the result of differential tissue-specific gene expression.

In agreement with previous analysis of independent genotypes (Lightbourn et al., 2008), HPLC analyses demonstrated that anthocyanin-pigmented tissues in line 06C59 contained a single major anthocyanin compound, delphinidin-3-p-coumaroyl-rutinoside-5-glucoside. Black pigmentation, in contrast to purple pigmentation, is attributed to high concentrations of chlorophyll in combination with anthocyanin in *C. annuum* fruit and foliar tissues (Lightbourn et al., 2008). Flower, fruit, and leaf tissues of line 06C19–2 contained little or no anthocyanin compared with line 06C59 (Table 1).

To determine the genetic basis for tissue-specific pigmentation, we evaluated the expression of anthocyanin biosynthetic (*Chs*, *Dfr*, and *Ans*) and regulatory (*Myc*, *Myb_A*, and *Wd*) genes in flower, fruit, and foliar tissue from anthocyanin-pigmented (06C59) and nonpigmented (06C19–2) genotypes. In all cases, biosynthetic gene transcript levels were significantly higher in anthocyanin-pigmented tissues in comparison with nonanthocyanin-pigmented tissues. Levels of *Chs*, *Dfr*, and *Ans* transcript were 16-, 240-, and 56-fold higher, respectively, in anthocyanin-pigmented flowers of 06C59 in comparison with white flowers of line 06C19–2 (Fig. 1A). Similar to flowers, *Chs*, *Dfr*, and *Ans* displayed the greatest fold difference in transcript levels between fruit of anthocyanin-pigmented versus green-colored fruit (20-, 95-, and 195-fold greater for *Chs*, *Dfr*, and *Ans*, respectively) (Fig. 1B). In foliar tissue, *Ans* exhibited the greatest fold difference in transcript level between anthocyanin-pigmented versus green-colored leaves relative to that observed for *Chs* and *Dfr* (13-, 13-, and 42-fold greater for *Chs*, *Dfr*, and *Ans*, respectively) (Fig. 1C).

Table 1. Anthocyanin content in flowers, fruit, and leaves of *Capsicum annuum* genotypes 06C19–2 (nonanthocyanin pigmented: white flowers, green fruit, green foliage) and 06C59 (anthocyanin pigmented: purple flowers, black fruit, black leaves).

Tissue	Genotype	
	06C19–2	06C59
	<i>(peak area/g fresh wt tissue) × 10³</i>	
Flower	0	42,682 ± 4,246
Fruit	4.7 ± 1.1	1,600 ± 439
Leaf	16.8 ± 4.6	27,148 ± 5,487

Fig. 1. Flavonoid structural (*Chs*, *Dfr*, and *Ans*) and regulatory (*Myb_A*, *Myc*, and *Wd*) gene expression in (A) flower, (B) fruit, and (C) leaf tissue of *Capsicum annuum* line 06C19-2 (nonanthocyanin pigmented: white flowers, green fruit, green foliage) and line 06C59 (anthocyanin pigmented: purple flowers, black fruit, black leaves). Gene expression was measured using real-time PCR and normalized to tubulin gene expression. Data are presented as the mean of three replicates. Error bars represent SE $\pm$ mean. Letters denote Fisher's least significant difference multiple comparison test ($P \leq 0.001$); means followed by the same letter are not significantly different.

Consistent with elevated levels of structural gene transcript in anthocyanin-pigmented tissues, anthocyanin-specific *Myb_A* and *Myc* transcript levels were significantly higher in anthocyanin-pigmented fruit and floral tissues (Fig. 1, A and B). *Myb_A* transcript levels were ≈ 20 -fold greater in anthocyanin-pigmented flowers and fruit (06C59) in comparison with white flower and green fruit tissue (06C19-2). *Myc* showed an ≈ 40 -fold increase in pigmented versus unpigmented flowers and fruit. In contrast to *Myb_A* and *Myc*, *Wd* transcript levels in pigmented flowers and fruit (06C59) was very low and did not differ significantly from unpigmented tissue (06C19-2; Fig. 1, A and B).

In leaf tissue, there was no significant difference in *Myb_A*, *Myc*, and *Wd* transcript levels between anthocyanin-pigmented (06C59) and unpigmented (06C19-2) lines (Fig. 1C). The *Myb_A* and *Myc* transcript levels in anthocyanin-pigmented leaf tissue of 06C59 were significantly lower than those found in anthocyanin-pigmented flower and fruit tissue of this genotype. About equivalent levels of *Myb_A* and *Myc* transcript were noted in nonanthocyanin-pigmented flower, fruit, and leaf tissue of 06C19-2. The *Wd* transcript level was about the same in flower, fruit, and leaf tissue.

Analysis of the full-length *Myb_A* genomic clone revealed a 1652-bp sequence containing two introns of 249 and 441 bp (Fig. 2). 5'-RACE was used to derive an UTR sequence upstream of the coding region in *Myb_A*.

Discussion

C. annuum displays a wide spectrum of tissue-specific anthocyanin pigmentation, making it a useful model for the study of anthocyanin accumulation. Differential expression of *Chs*, *Dfr*, and *Ans* structural genes was observed in anthocyanin-pigmented versus nonanthocyanin-pigmented *C. annuum* flower, fruit, and leaf tissues. In anthocyanin-pigmented tissues, all three structural genes were markedly upregulated. Borovsky et al. (2004) reported that the *Chs* structural gene was expressed in leaves, flowers, and fruit of an anthocyanin-containing *C. annuum* genotype (5226), as well as in an anthocyanin-free *C. chinense* Jacq. (PI 159234) accession. Differential expression of *Chs* was not observed between these two genotypes with contrasting anthocyanin pigmentation.

In the anthocyanin-containing genotype (5226), they found that *Dfr* and *Ans* were upregulated in immature fruit before accumulation of visible anthocyanins. No transcript was evident in purple-pigmented fruit, flowers, or leaves of 5226. Because *Dfr* and *Ans* transcript are required for anthocyanin production, we suggest that *Dfr* and *Ans* transcript in anthocyanin-pigmented fruit may be more transient in 5226. *Dfr* and *Ans* were not expressed in leaves, flowers, and fruit of the anthocyanin-free PI 159234. In contrast, we demonstrated that in the anthocyanin-containing genotype (06C59) we studied, *Chs*, *Dfr*, and *Ans* were all upregulated in anthocyanin-pigmented flower, fruit, and leaf tissues. Differential regulation

Fig. 2. Nucleotide sequence alignment of the full-length *Myb_A* genomic and cDNA clones from anthocyanin-pigmented *Capsicum annuum* genotype 06C59 (Myb59). Comparative *Myb* cDNA sequence data from GenBank for *C. annuum* [Ca A (GenBank accession AJ608992)] and *Petunia ×hybrida* [Ph An2 (GenBank accession AF146702)] cDNA are shown. The R2R3 *Myb* domain is underlined. Start and stop codons are highlighted in bold. Dissimilarities in nucleotide sequence between R2R3 regions in respective *Myb* clones are highlighted in gray.

of all three of these biosynthetic pathway genes was evident between our genotypes with contrasting anthocyanin pigmentation. Therefore, it appears that different genotypes have different anthocyanin gene expression patterns. This is not unexpected because tissue-specific anthocyanin pigmentation in *C. annuum* has independently evolved (Stommel and Bosland, 2006).

Genotype 5226 was derived from round-fruited *C. annuum* germplasm, while genotype 06C59 was derived from elongated Tabasco type *C. annuum* germplasm. The unpigmented genotype PI 159234 was derived from *C. chinense*, and the unpigmented genotype 06C19-2 was derived from *C. annuum* germplasm. Unlike previous studies, we used genetically related lines to contrast anthocyanin and anthocyanin-free gene expression.

Our *Myb_A* cDNA sequence was identical to the sequence reported by Borovsky et al. (2004). Analysis of the genomic sequence revealed two introns of 249 and 441 bp, both of which were between the R2R3 domains (Fig. 2). As expected, the R2R3 domains were conserved between the two Solanaceous genera, *Petunia* and *Capsicum*. The sequence of the non-R2R3 domains was not conserved, with very little homology in these related genera. Comparisons are restricted to coding regions because a full-length *P. ×hybrida* genomic *Myb* is not available. In unrelated species, *Malus ×domestica* Borkh. (apple) and *Ipomoea batatas* (L.) Lam. var. *batatas* (sweetpotato), the *Myb* controlling anthocyanin production had two introns between the R2 and R3 domains. In *M. ×domestica*, the first intron between R2 domains was 323 bp, while the second intron between R3 domains was 3000 bp (Takos et al., 2006). In *I. batatas*, the first intron between the R2 domains was 131 bp, while the second intron between R3 domains was 720 bp (Mano et al., 2007). Interestingly, the *Myb* controlling trichome development in *Gossypium hirsutum* L. (cotton) also contains two introns between the R2 and R3 domains (Wang et al., 2004). The first intron acts as an enhancer in trichome development and a repressor in the development of other cell types. This is similar to what was found for the *cMyb* in mouse (*Mus musculus* L.) (Seib et al., 1994). In the mouse *cMYB*, there are 10 introns; however, only intron 4 was critical for expression.

It is widely accepted that the anthocyanin biosynthesis pathway is regulated largely by the MYB-MYC-WD regulatory protein complex (Koes et al., 2005). These transcription factors form a complex that binds to structural gene promoters, thereby modulating gene expression. WDs serve as a platform for protein-protein interactions with MYCs, which in turn recruit MYBs to form a transcription complex. The working hypothesis is that the ubiquitous WD interacts with multiple MYCs, and each MYC enlists different MYBs that eventually bind to the unique target gene to initiate gene expression.

We demonstrate that differential expression of *Myb_A* as well as *Myc* is required for anthocyanin accumulation in *C. annuum*. Prior pepper investigations were confined to the study of *Myb_A* regulatory gene expression (Borovsky et al., 2004). Consistent with our results, *Myb_A* expression in the anthocyanin-pigmented genotype 5226 was differentially expressed in anthocyanin-pigmented and unpigmented tissues.

There was no difference in the expression of *Wd* in pigmented versus unpigmented tissue. This lack of differential *WD* expression in pigmented and unpigmented tissue was also observed in *Phalaenopsis* Blume by Ma et al. (2009). This

observation was explained by the network regulation model as discussed above because MYB and MYC together appeared to be sufficient to dictate the specificity of the regulatory complex.

Structural gene regulation is defined by the diversity among the anthocyanin *Myc* and *Myb* alleles, each of which regulates expression in a different manner. For example, in *P. ×hybrida*, the combination *Myc_{An1}/Myb_{An2}* induces anthocyanin pigmentation in the flower, whereas the *Myc_{An1}/Myb_{Ph4}* combination induces vacuolar acidification (Quattrocchio et al., 2006). In addition to controlling structural gene expression, these regulatory genes can also control their own expression. In *A. thaliana* seed, a MYC, MYB, WD40 complex controls flavonoid structural gene transcription. This same complex also controls the transcription of one its members (*Myc*) (Baudry et al., 2006).

The relative proportions of MYB and MYC can also influence transcription factor activation of anthocyanin structural genes. Using transient expression assays, Ma et al. (2008) demonstrated that altered ratios of *Myb:Myc* transcript produced varying levels of anthocyanin accumulation in normally white *Phalaenopsis* flowers. High levels of *Myb* expression in conjunction with low or high levels of *Myc* expression were required to elicit high levels of anthocyanin accumulation. Neither regulatory gene alone was sufficient to elicit anthocyanin production. We also observed varying *Myb:Myc* transcript ratios in *C. annuum*. *Myb_A:Myc* transcript ratios were ≈4-fold greater in fruit and flower (*Myb_A:Myc* transcript ratio 2.89 and 4.12, respectively) tissue of the anthocyanin-free 06C19-2 in comparison with corresponding anthocyanin-pigmented fruit and flower (*Myb_A:Myc* transcript ratio 0.63 and 1.00, respectively) tissues in 06C59. Results from *Z. mays* (deMajnik et al., 1998) suggested that the threshold requirement for the regulatory genes to activate anthocyanin production is different in different plants. The low *Myb_A* and *Myc* expression levels observed in our nonanthocyanin-pigmented *C. annuum* flower and fruit tissues suggest that differential expression of these regulatory genes is likely the primary determinant of anthocyanin pigmentation in these genotypes.

In contrast to *C. annuum* flowers and fruit, differential expression of *Myb_A* and *Myc* was not observed in foliar tissue, suggesting that a different mechanism contributes to the regulation of anthocyanin biosynthesis in foliage. Likewise, differences in *Myb_A* and *Myc* expression were not responsible for the difference observed in anthocyanin structural gene expression under inductive and noninductive environmental conditions for foliar anthocyanin accumulation (Lightbourn et al., 2007). Inheritance studies also demonstrated that the genetic control of anthocyanin accumulation in *C. annuum* flowers and fruit is different from that in foliage. Whereas anthocyanin-based flower and fruit color can be separated into discreet classes, a continuous range of color scores is evident in breeding populations segregating for foliar pigmentation (Stommel and Griesbach, 2008). High broad sense and narrow sense heritability for foliar anthocyanin pigmentation indicate that inheritance of foliar anthocyanin is more complex and is not limited to the action of *A* and *MoA*. Additive gene action and epistasis contribute to foliar anthocyanin pigmentation.

A possible mechanism that may explain our results involves miRNA. miRNAs control gene expression by targeting specific mRNAs for degradation (Bartel, 2004; Jones-Rhoades et al., 2006; Kidner and Marteinssen, 2005). miRNAs 21 to 22 nucleotides in length are involved in post-transcriptional gene

silencing by targeting mRNAs for degradation via an endonuclease complex (Baulcombe, 2004; Jones-Rhoades et al., 2006).

Preliminary observations suggest that miRNAs could be involved in regulating anthocyanin gene expression in *C. annuum* leaves. One of the symptoms we observed following tobacco etch virus infection of purple-leaved *C. annuum* plants was a reduction in anthocyanin pigmentation (data not shown). *C. annuum* fruit from virus-infected plants can display differential anthocyanin pigmentation (Black et al., 1991). Viral infection is known to modify host gene expression by suppressing RNA silencing. *Glycine max* L. seeds are normally yellow due to the presence of *Chs* miRNAs (Senda et al., 2004). When infected with soybean mosaic potyvirus, there was an increase in *Chs* mRNA that restored anthocyanin seed color. In the *P. ×hybrida* *Star* mutant, virus infection can restore anthocyanin production in white *Star* floral tissue by inhibiting miRNA or siRNA formation responsible for absence of chalcone synthase (Koseki et al., 2005). Further studies are planned to examine anthocyanin biosynthetic and regulatory miRNA genes in *C. annuum*.

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