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Characterization of underlying transcription factors that regulate betalain pigment formation in beets

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Characterization of underlying transcription factors that regulate betalain pigment formation in beets

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Dedication

Dedicated to my family, my grandmother, Robab Azad, my husband, Hossein Mousavi, my siblings, Vahid and Mina, and the memory of my mom and dad, Vajiha and Mostafa Akhavan.

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Finally, I must also thank the members of the ICMB core facility, Protein facility, Texas Advanced Computing Center, and Plant Biology coordinator for providing me resources and supplies to work with.

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Characterization of underlying transcription factors that regulate betalain color formation in beets

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Abstract: The plant kingdom is a colorful place with most vascular plants producing phenylalanine-based red/violet anthocyanin pigment. Only a single order of flowering plants, the Caryophyllales, is known to produce an unusual pigment known as Betalain. Betalains encompasses an entire range of colors between yellow to red, are nitrogen-containing water-soluble compounds derived from tyrosine, and are acidic in nature due to the presence of several carboxyl groups. The betalain and the anthocyanin pigments are mutually exclusive. The pathway and enzymes for betalain biosynthesis, from tyrosine to the end products, red/violet betacyanins and yellow betaxanthins, has largely been determined. Little however is known about the regulation of the biosnynthetic genes. The strong biological correlation between the anthocyanin and the betalains prompted the suggestion that the molecular regulation of betalains and anthocyanins uses the same MYB and bHLH and WD-repeat regulators, the MBW complex. The work described here strives to understand the regulatory mechanisms controlling betalain pigmentation in the Caryophyllales and how they can be controlled

and influenced. To understand the pathway, there was a pressing need for analysis at the biochemical, molecular, and genetic levels. Before the work reported here, two pigment biosynthetic genes were identified. The gene/ enzyme responsible for step two was identified as DOPA 4, 5-dioxygenase (DODA) functioning to produce betalamic acid (BA) from LDOPA (Christinet et al., 2004), and later we showed that a novel cytochrome P450, CYP76AD1, is absolutely required for the red pigment in beets by catalyzing the step producing cyclo-DOPA from the LDOPA substrate (Hatlestad et al., 2012). Through this project I have: (1) discovered and characterized Beet MYB1 (BvMYB1), a MYB evolved from the anthocyanin regulating MYBs, and analyzed how BvMYB1 regulates betalain production by its interaction with DNA and other proteins; (2) determined overlapping functional redundancies of BvMYB1 with two other R2R3 BvMYBs, BvMYB2 and BvMYB3; (3) identified a beet bHLH protein, BvbHLH1, and a beet WD-repeat protein, BvTTG1, that function similar to the *Arabidopsis thaliana* proteins; and finally (4) I worked towards characterizing a novel BvMYB1 Response Element (MRE) that BvMYB1 directly binds to activate betalain biosynthetic genes. By identifying members of the regulatory complex for the betalain pathway I hope to contribute toward understanding the evolutionary replacement of anthocyanins by betalains within a single flowering order, and fill a lack of knowledge about producing, controlling, and influencing this valuable natural pigment.

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CHAPTER 1: General Introduction

Pigments

Plants are the principle producers of natural pigments. These pigments can be found in leaves, fruits, roots, and flowers. Their varied structures, colors, and tastes have long fascinated scientists, who have examined their chemical and physical properties, their mode of synthesis, and their physiological and ecological roles. Their importance in our everyday lives has attracted researchers to thoroughly understand the biosynthetic pathway and regulation of these pigments (Tanaka et al., 2008). Most of the pigments produced by plants are characterized into four major groups: chlorophylls, carotenoids, anthocyanins, and betalains (Delgado-Vargas et al., 2000). While chlorophylls, anthocyanins and carotenoids are almost universal among the higher plants, little is known about betalain biosynthetic genes and even less is known about their regulation. In my work and this dissertation I have focused on understanding the regulation mechanism of betalain pigments and how they can be controlled and influenced.

Roles and Phylogeny

Betalains, producing a range between yellow to red colors, are nitrogen-containing water-soluble compounds derived from tyrosine and are acidic in nature due to the presence of several carboxyl groups. Betalain pigments are found only in a limited number of plant lineages figure 1-1. Betalains are restricted to families in a single angiosperm order, the Caryophyllales. It is known that 9 out of 11 families of the Caryophyllales order (based on the phylogeny of Cuénoud et al., 2002) contain betalains. The remaining two families in the order of Caryophyllales, the Molluginaceae and

Caryophyllaceae, are anthocyanin producing plants instead of betalains. There is a mutually exclusive relationship between anthocyanins and betalains.

The two main types of betalains, the red-purple betacyanins and the yellow betaxanthins, can be found among important staple crops grown worldwide in many agricultural systems, including beets, spinach, amaranthus, quinoa, and prickly pear cactus (Harris et al., 2012). Therefore, betalain pigments can play important roles in nutrition in our everyday diet.

Betalains assume the roles of anthocyanin pigments in all biological senses (Fig.1-2). These roles include attracting pollinators such as insects, birds, and animals, and mediating seed dispersal. Betalain also functions as a protective pigment against damages caused by stresses such as ultraviolet and visible light, senescence, and adverse climate (Delgado-Vargas et al., 2000). Due to betalain's extensive biological roles and its presence in everyday products and diets, it is vital to learn more about this pigment.

Economic Uses

Betalain pigments are an important and well-known dietary source of antioxidant and radical scavenger activity (Uttara et al., 2009). In fact, purified forms are used in food industries that use betalains as natural red and yellow food coloring. But betalain pigment are not only consumed as part of people's diets, recently this pigment has found its way into other industries (Kanner et al., 2001). Some of the uses of betalains are in natural cosmetic industry that uses the deep red color of betacyanins to produce lipsticks (Delgado-Vargas et al., 2000). They are also used in power plants that use the solar energy conversion efficiency of betalains as a coating to produce very sensitive solar

panels (Zhang et al., 2008). These are a few of the economical applications of betalains in various industries.

Biosynthetic genes

The chemical precursors, intermediates, and products of the betalain ring structure biosynthetic pathway have been determined. Unlike the unrelated phenylalanine based anthocyanin pigments, betalains are synthesized from tyrosine. The synthesis of the betalain ring structure is proposed to require three enzyme-mediated steps (Fig. 1-3) (Gandía-Herrero et al., 2005) to reach the red pigment backbone from tyrosine (as opposed to 8 steps from phenylalanine to anthocyanin).

The enzyme responsible for steps 1 and 3 in figure 3 had been proposed to be polyphenol oxidase (PPO) (Steiner et al., 1999), mainly because this enzyme will do these steps *in vitro*, converting tyrosine to L-DOPA and L-DOPA to cyclo-DOPA. But this had never been demonstrated *in planta* (Mayer 2006). Recently, the Lloyd lab identified a cytochrome P450 in beet that performs step 3 (Hatlestad et al., 2012). This finding is consistent with the genetic fact that it is relatively easy to obtain yellow mutants, i.e. mutant plants missing step 3 but maintaining step 1.

Our lab has further shown that CYP76AD1 can perform both steps 1 and 3, while two related enzymes, CYP76AD5 and CYP76AD6, can perform step 1 but not step 3. So all three can perform step 1, and beets appear to be redundant for step 1, while step 3 is uniquely performed only by CYP76AD1 (Sunnadeniya 2014).

In step 2, the 4, 5 dopa-dioxygenase (DODA) cleaves the LDOPA ring to produce 4, 5-seco-DOPA, which spontaneously re-circularizes to form betalamic acid. The gene

encoding this enzyme has been reported from Portulaca, a betalain producing taxa and was recently cloned from beets (Christinet et al., 2004).

Lastly, Betalamic acid condenses with the product of step 2, cyclo-DOPA, to form the red betacyanin pigment, or condenses with other amine groups to form the yellow betaxanthin pigments (Schliemann et al., 1999). The ring structure is often decorated on the cyclo-DOPA moiety and genes encoding UDP glucosyltransferases (UGT) with *in vitro* activity on the 5 and 6 positions of betanidin (the un-glycosylated betacyanin) have been cloned from *Dorotheanthus* (Vogt et al., 1999) and beet (Isayenkova et al., 2006).

As a final point, the steps marked "S" all occur spontaneously *in vitro* and most likely *in vivo*. As an intermediate, betalamic acid produces both yellow and red pigments depending on what condenses with it.

Relative to the detailed understanding of anthocyanin biosynthetic pathway, a lot of work is still needs to be done to better understand the biosynthetic genes of betalain synthesis.

Regulation

Transcription is the first step in the flow of biological information from genome to proteome and its tight regulation is a crucial checkpoint in most biological processes occurring in all living organisms (Dubos et al., 2010). A great place to study transcriptional regulation in eukaryotes is in plant pigment production. One reason for this is because plants can be easily manipulated and screened for pigment differences. Anthocyanin pigments, which are derived from the amino acid phenylalanine, are produced by the majority of plants, from corn and rice to apples and broccoli. Much is

known about the biosynthesis and regulation of anthocyanins in model genetic plants (Gonzalez et al., 2008; Zimmermann et al., 2004). However, unlike the anthocyanin pigments, little is known about transcriptional regulatory complexes regulating betalains, pigments that are derived from tyrosine.

Transcriptional regulation of the anthocyanin pigment pathway is well studied, and it is known to be regulated by a complex of transcription factors that includes MYB, bHLH and WD-repeat proteins. Despite the wealth of knowledge regarding the WD/bHLH/MYB regulatory model governing the anthocyanin/ phenylpropanoid pathway in many species including *Arabidopsis* (Dubos et al., 2010; Stracke et al., 2001) nothing was known about transcriptional regulatory complexes in betalain regulation until our work (Hatlestad et al., 2015). Recently three novel beet (*Beta vulgaris*) MYBs were identified in our lab. Phylogenetic analysis clearly indicates that these MYBs are derived from anthocyanin regulatory MYBs. BvMYB1 and BvMYB2 have been tested and they positively regulate the betalain biosynthetic pathway. Unlike the anthocyanin MYBs that regulate the late biosynthetic genes, BvMYB1 will not regulate the anthocyanin genes in *Arabidopsis*, nor will it interact with anthocyanin bHLH proteins. Through this dissertation I have analyzed the transcriptional regulation of the betalain pathway by a series of experiments in table beet, yeast, and *Arabidopsis*.

Betalain pigments replace anthocyanin pigments

The basic biosynthetic pathway and molecular regulation of anthocyanins have been known for many years, but a full enzymatic pathway and a complete set of regulators for betalain production remain elusive. Additional research is necessary to determine the genetics and molecular biology of both the structural genes and the

transcriptional regulation in betalain production. It is thought that both the betalain and anthocyanin pathways occurred in a common ancestor. Through evolution one or the other of these pathways was lost within the Caryophyllales, thus today plants have either the betalain or anthocyanin pathway but not both (Brockington et al., 2011; Clement and Mabry, 1996). Hypotheses about the phylogeny of these two secondary pigment pathways cannot be tested without further molecular advances in the betalain model. My project aims to understand how pigment-related properties are generated in the betalain accumulating plants through several systems level approaches.

Similar biological regulation and tissue distribution among anthocyanin and betalain plants

Anthocyanins and betalains seem to have more or less the same regulation and tissue distribution at the biological level. They are both water-soluble, synthesized in the cytosol, and localized in vacuoles (Tanaka et al., 2008). Their colors can be altered due to various modifications, such as hydroxyl, methyl, glycosyl and acyl groups, which functionally and structurally change the ring structure. pH can affect both betalain and anthocyanin structure transformations, modifications and stability. Therefore pH is also really important in determining different color hues (Kugler et al., 2004). Finally, similar to anthocyanins, betalains generally accumulate in external tissues exposed to direct light, such as the upper epidermis (Harris et al., 2012; McClure, 1975). These conservation of pigment tissue specificity may suggest similar functional roles among anthocyanin and the betalains.

Furthermore the evolutionary convergence for the ability to accumulate red pigments with either red anthocyanins or betalains in different plant species suggests that

these pigments fulfil a similar function (Brockington et al., 2011). Therefore our first hypothesis was that they might be regulated the same way at the molecular level. Thus we initiated a search for MBW complex members similar to the anthocyanin regulators that might regulate betalain accumulation.

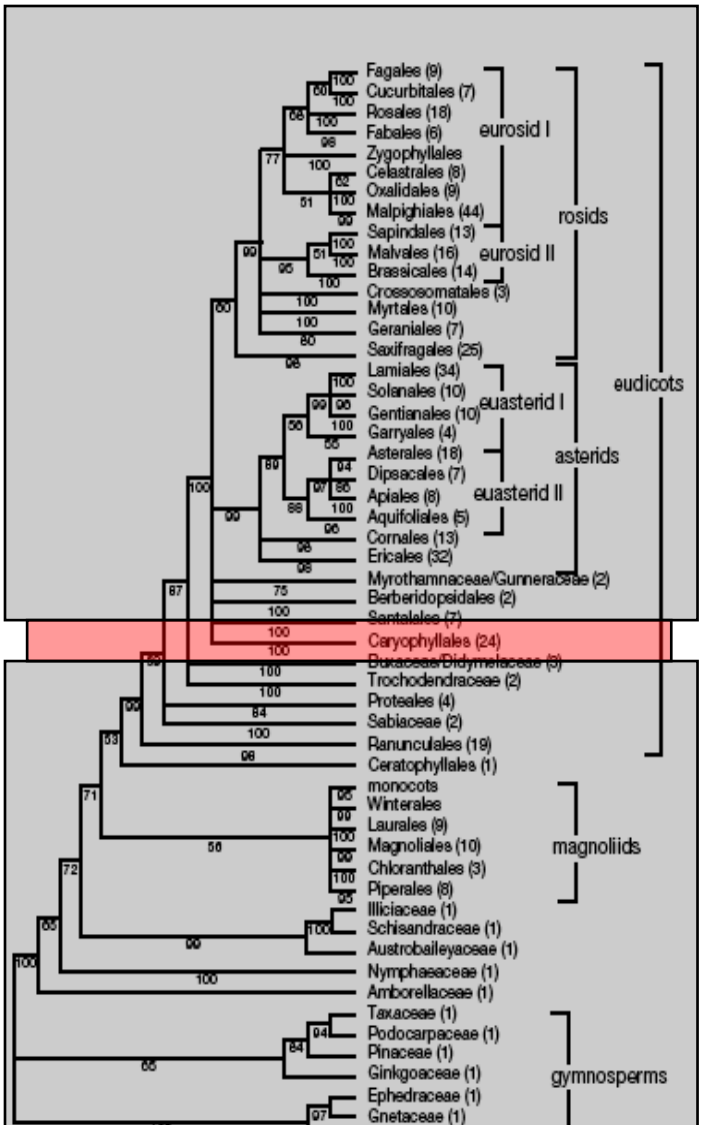

Figure 1-1: Betalains are found in a single order of flowering plants

Betalain pigments are restricted to one order of flowering plants, the Caryophyllales. All other angiosperms produce anthocyanin pigments.

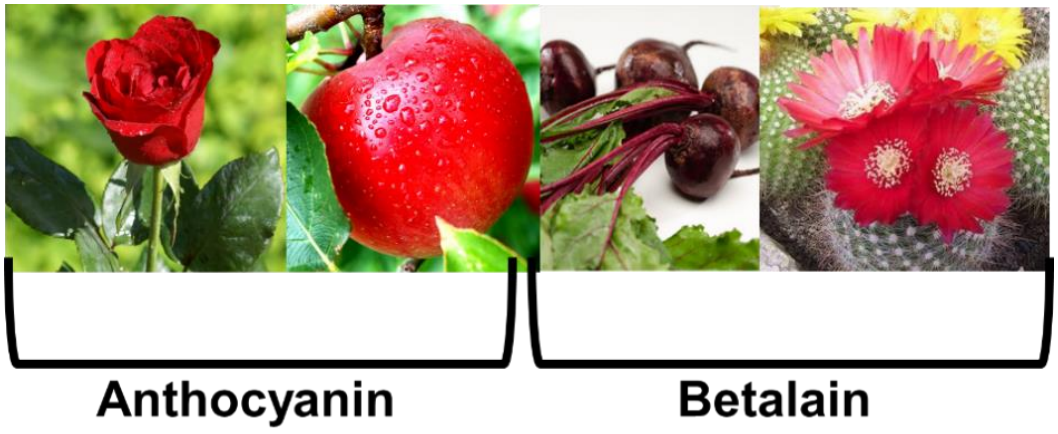

Figure 1-2: Anthocyanins vs. Betalains.

Betalains, a minor class of red plant pigment, replace a major flowering plant pigment known as the anthocyanins in one order of flowering plants.

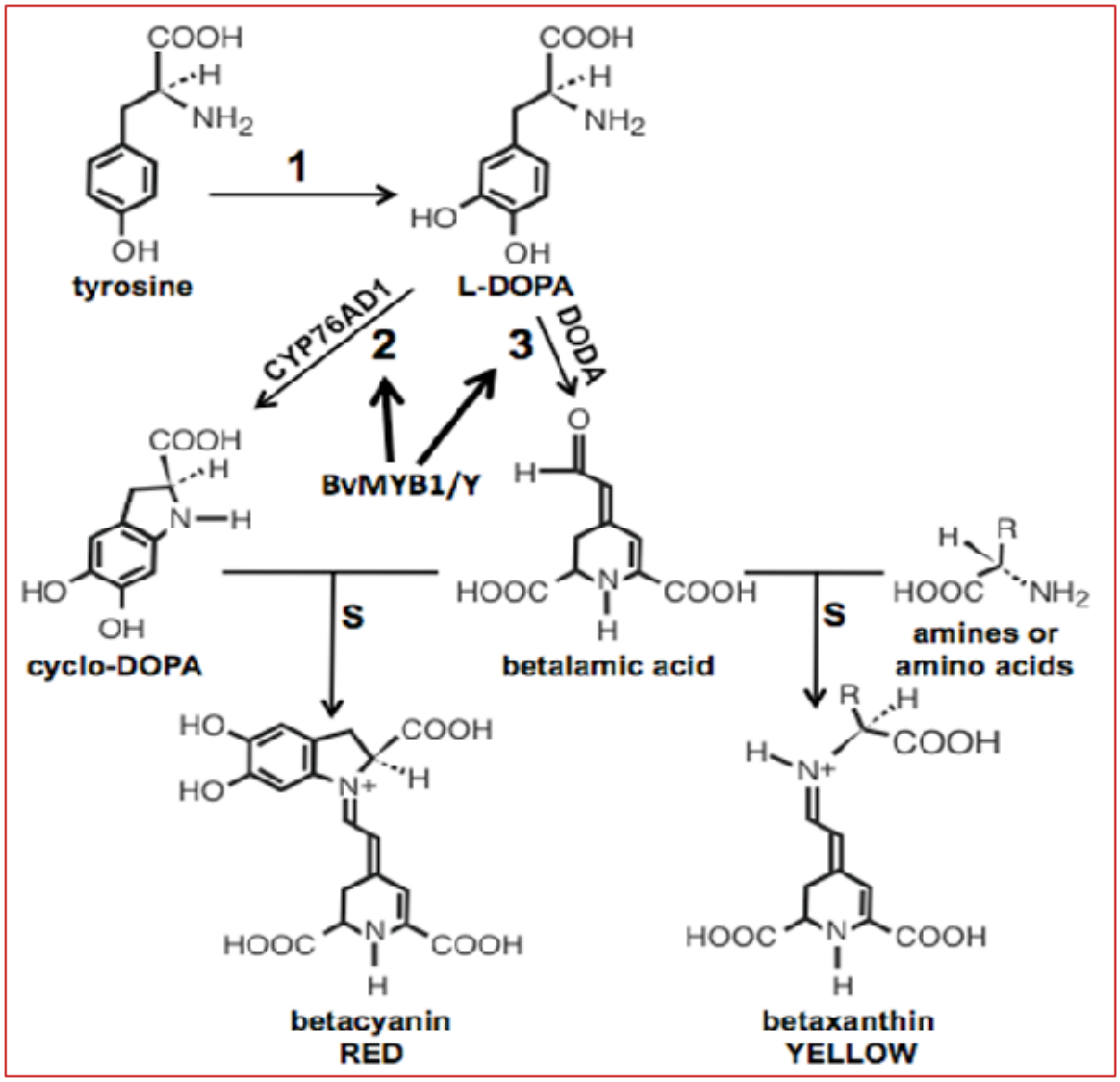

Figure 1-3: Proposed betalain biosynthetic pathway

This is redrawn from Hatlestad et al. 2012. Steps 1, 2, and 3 are proposed to be enzyme mediated, whereas steps marked S are proposed to be spontaneous.

CHAPTER 2: The beet Y locus encodes an anthocyanin MYB-like protein that activates the betalain red pigment Pathway[1]

SUMMARY

Nearly all flowering plants produce red/violet anthocyanin pigments. Caryophyllales is the only order containing families that replace anthocyanins with unrelated red and yellow betalain pigments (Brockington et al., 2011; Clement and Mabry, 1996). Close biological correlation of pigmentation patterns suggested that betalains might be regulated by a conserved anthocyanin-regulating transcription factor complex consisting of a MYB, a bHLH and a WD repeat–containing protein (the MBW complex) (Feller et al., 2011). Here we show that a previously uncharacterized anthocyanin MYB-like protein, *Beta vulgaris* MYB1 (BvMYB1), regulates the betalain

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[1]The data presented here was previously published in: The beet Y locus encodes an anthocyanin MYB-like protein that activates the betalain red pigment pathway.
By Gregory J Hatlestad[2], Neda A Akhavan[2], Rasika M Sunnadeniya, Antonio Gonzalez, Irwin Goldman, J Mitchell McGrath & Alan M Lloyd, Nature Genetics (2015) 47, 92-96.
[2]These authors contributed equally to this work. Neda Akhavan was co-first author on this publication and contributed over half of the data.
Neda was the main scientist in the following experiments: yeast two-hybrid to determine protein-protein interactions; yeast one-hybrid to determine protein-DNA interactions; promoter analysis of the y (white) and Y (red) plants and how the Y locus is transcriptionally regulated; expression analysis of BvMYB1 and GUS in both *Arabidopsis* and beet plants; site-direct mutagenesis to identify residues responsible for MYB-bHLH interaction; glucocorticoid receptor induction to show that the biosynthetic genes are regulated by BvMYB1; qPCR 2nd and 3rd biological replicate of all silencing and overexpression in beets of BvMYB1; analysis of betalain gene expression among varieties of beet plants: Bull's Blood red table beets, Albina vereduna white table beets and C869 white sugar beets; analysis of anthocyanin gene homolog expression in Albina vereduna beet roots. Neda also contributed to developing two novel protocols used in this paper: first, producing 35S:BvMYB1-GR transgenic beet roots on plates where they are very easy to propagate; second Virus Induced Gene Silencing (VIGS) plant transformation using *Agrobacterium rhizogenes*.

pathway in beets. Silencing BvMYB1 downregulates betalain biosynthetic genes and pigmentation, and overexpressing BvMYB1 upregulates them. However, unlike anthocyanin MYBs, BvMYB1 will not interact with bHLH members of heterologous anthocyanin MBW complexes because of identified nonconserved residues. *BvMYB1* resides at the historic beet pigment-patterning locus, *Y*, required for red-fleshed beets (Keller, 1936). We show that *Y* and *y* express different levels of *BvMYB1* transcripts. The co-option of a transcription factor regulating anthocyanin biosynthesis would be an important evolutionary event allowing betalains to largely functionally replace anthocyanins.

INTRODUCTION

The Caryophyllales order alone contains families that produce only anthocyanins or only betalains; no known species make both, and betalains may have arisen once or twice within these taxa (Brockington et al., 2011; Clement and Mabry, 1996). Betalain-accumulating families encompass crops such as beets, *Amaranthus*, spinach and prickly pear cactus. The pathway for betalain biosynthesis, from tyrosine to the end products red/violet betacyanins and yellow betaxanthins, has largely been determined (Fig. 2-1a). Where they occur, betalains assume many of the roles of anthocyanins, coloring flowers and fruits and responding to stress signals. This strong biological correlation prompted the suggestion that the molecular regulation of betalains and anthocyanins uses the same MYB and bHLH regulators, the MBW complex (Brockington et al., 2011; Stafford, 1994).

Red beets produce high concentrations of betalains from the epidermis to the central core, whereas white beets have white flesh with betalains restricted to outer cell layers. Red flesh (*Y*) is dominant over white (*y*), and red beets are a betalain overproducer selected for during domestication (Keller, 1936) (Fig. 2-5). We used red beets as a tissue likely to highly express betalain pathway genes.

BLAST searches of a red beet RNA-sequencing (RNA-seq) cDNA database (Hatlestad et al., 2012) with anthocyanin MYBs identified a highly expressed beet *MYB* gene. To obtain a full-length clone, we identified and sequenced a sugar beet BAC containing this gene. Based on the BAC sequence, *BvMYB1*cDNAs encoding identical proteins were amplified and cloned from red table and white sugar beets, both *B. vulgaris* (*BvMYB1*).

BvMYB1 is a typical plant R2R3-MYB containing the R2 and R3 MYB domains and a C-terminal activation domain (Fig. 2-6). Clustal analysis using the R2R3 domains showed that BvMYB1 forms a monophyletic clade with dicot anthocyanin MYBs (subgroup 6) (Dubos et al., 2010; Stracke et al., 2001), indicating a possible common evolutionary origin (Fig. 2-1b and Fig. 2-7). When we performed a BLAST search of *Arabidopsis thaliana*, the most common matches for BvMYB1 were anthocyanin MYBs, specifically AtMYB113. BvMYB1 is also linked with subgroup 6 when using the resources at IT3F (An Interspecies Transcription Factor Function Finder for Plants). Although it is clear that BvMYB1 is most closely related to subgroup 6, it does not contain the conserved C-terminal motif of the subgroup, [R/K]Px[P/A/R]xx[F/Y] (Stracke et al., 2001; Lin-Wang, 2010), and it contains an INDV motif in the R3 domain where dicot members of the subgroup contain the (A/S/G)NDV motif (Lin-Wang, 2010).

MATERIALS AND METHODS

RNA-seq and beet lines.

RNA-seq and contig assembly were performed on 7-d-old W357B hypocotyls as described (Hatlestad et al., 2012). Bull's Blood red beet was used in most subsequent analyses because it is commercially available and is darker red than W357B. C869 is a white sugar beet breeding line (McGrath et al., 2007), and Albina vereduna is a commercially available white table beet.

Phylogenetic analysis.

The R2 and R3 domains of BvMYB1, nine other beet MYBs, six dicot and one monocot anthocyanin MYBs, and 29 other *Arabidopsis* MYBs representing the broad spectrum of MYB diversity (Table 2-3) were subjected to Clustal alignment and neighbor-joining tree analysis (1,000 bootstrap replicates) using MEGA5 software (Tamura, 2011). BvMYB1 clustered with the dicot anthocyanin MYBs (Fig. 2-7). A representative sample using a subset of these MYBs is shown in Figure 2-1b.

Quantitative RT-PCR gene expression analysis.

Tissues were harvested from beet hypocotyls, induced hairy roots, and leaf sections of VIGS-treated plants or whole *Arabidopsis* seedlings, as appropriate. Total RNA was extracted using RNeasy kits (Qiagen). Quantitative RT-PCR was performed as described (Gonzalez et al., 2008; Hatlestad et al., 2012). Five reactions per target were performed using an actin control with 400 nM of the appropriate primers from the following list: RTPCRBvACTF and RTPCRBvACTR; RTPCRMYB1F and RTPCRMYB1R; RTPCRDODA1F and RTPCRDODA1R; RTPCR76AD1F and RTPCR76AD1R; GUSF and GUSR; BvDFRRTF and BvDFRRTR; BvLDOXRTF and

BvLDOXRTR (Table 2-4). Results were analyzed using the comparative cycle threshold method (User Bulletin 2, ABI PRISM Sequence Detection System). Expression experiments were performed three or more times for each gene with consistent results. Representative experiments are presented in the main figures. Data analysis for three biological replicates are shown in figures and in spreadsheet form in the supporting data. For hairy root samples, each independent biological replicate was composed of hairy roots from a single unique individual beet seedling. Semiquantitative RT-PCR of *AtMYB114* shown in Figure 2-15a was performed using primers AtMYB114F and AtMYB114R.

Hairy root inoculation.

The *BvMYB1* cDNA from the start to stop codons was amplified from W357B beet with primers BvMYB1start and BvMYB1stop (Table 2-4). The *BvMYB1ΔAc* fragment was amplified using primers BvMYB1start and BvMYB1ΔAcR. Gateway recombination sequences were included on cloning primers but are not shown. The products were recombined into*pDONR222* (Invitrogen), sequenced and recombined into pB7WG2 plasmid (Karimi et al., 2002). This created*p35S::BvMYB1* and *p35S::BvMYB1ΔAc* for overexpression of full-length and truncated *BvMYB1*in plants. The *35S::AtMYB114* and *35S::AtMYB114ΔAc* constructs have been described (Gonzalez et al., 2008).*AtMYB114* is the full-length allele and *AtMYB114ΔAc* is a natural truncation allele. *pB7WG2* containing the *GUS* gene was used as a control.

We used a 1-ml syringe with 30-gauge needle to puncture 2- to 3-mm longitudinal wounds in beet or *Amaranthus hypochondriacus* hypocotyls and inject droplets of

the *Agrobacterium rhizogenes* cultures (Quandt et al., 1993). 'Hairy roots' emerged from hypocotyl wounds usually within 2 weeks.

Multiple roots from a single wound site were used in each biological replicate for gene expression analysis. Three biological replicates were performed for each experiment. Representative samples were tested for antibiotic resistance on MS plates (Fig. 2-18).

Virus-induced gene silencing (VIGS).

A PCR fragment of *BvMYB1* cDNA (448 bp; primers VIGSBvMYB1F and VIGSBvMYB1R; Table 2-4) was cloned into *pDONRSpec* (Invitrogen) and sequenced. This fragment, and control fragments containing the *GUS* gene, a beet laccase-like gene and a beet phytoene synthase gene were recombined into *pTRV2-Gateway* (Liu et al., 2002) and transformed into *Agrobacterium tumefaciens* (Koncz and Schell, 1986).

Seven-day-old beet seedlings were transformed by immersion-vacuum infiltration in the culture for 1 to 2 min as described (Liu et al., 2002). Infiltrated seedlings were maintained at 100% humidity for 24 h and then grown under 24-h fluorescent light. Changes in pigmentation could typically be observed 3 to 4 weeks after infiltration. VIGS of the three control genes never produced any changes in betalain pigmentation.

HPLC/mass spectrometry analysis of betalain pigment.

Pigments were analyzed as described (Kugler et al., 2004). For pigment analysis of silenced *BvMYB1* in Bull's Blood, pigments were extracted from green (non-red) silenced sectors and from non-silenced (red) sectors and leaves. For hairy root pigment analysis, pigments were extracted from roots overexpressing the MYB or MYB fragment and from control roots. A 0.1% ascorbic acid extraction buffer was used to inhibit

betalain oxidation, and the extract was processed through a 0.2-μm filter. The pigment extracts were analyzed by HPLC/mass spectrometry exactly as described (Hatlestad et al., 2012). Careful measurements of inputs (5 mg/ml) were performed to allow for reliable relative quantification of pigments analyzed. Complete HPLC data for Fig. 2-1 e, f for the wavelength of betanin (530 nm) are shown in Fig. 2-19.

Analysis of *Arabidopsis* relative anthocyanin content.

Seedlings from five independent transformed lines (five biological replicates) for each construct, as well as five biological replicates of wild type, were measured for anthocyanin content. For each sample, ten 5-d-old *Arabidopsis* seedlings, grown on MS medium with 3% sucrose, were extracted overnight at 4 °C with 150 μl methanol acidified with 1% HCl. 100 μl of water was added to each tube, and then 250 μl of chloroform was added to extract chlorophylls. The absorbance of the methanol/water fraction was measured at 530 nm.

Analysis of relative betalain content in transgenic beet roots.

Roots from four independent seedlings transformed with *35S::BvMYB1* (four biological replicates) and three independent *35S::GUS* lines were measured for betalain content. Three technical replicates for each transformed line were analyzed. For each sample, 5 mg of transgenic root tissue was extracted overnight at 4 °C in 150 μl water with 0.1% ascorbic acid to suppress pigment oxidation. The absorbance of the extract was measured at 530 nm.

Yeast one-hybrid analysis.

Fragments upstream of the translational start of *BvDODA1* (2,218 bp; primers BvDODA1upstrF and BvDODA1startR), *CYP76AD1* (494 bp; primers 76AD1upstrF and 76AD1startR) and *BvDFR* (527 bp; primers BvDFRupstrF and BvDFRstartR) were amplified from Albina vereduna beet cultivar, recombined into *pDONRP4-P1R* (Kugler et al., 2004), and sequenced. These fragments were recombined into DNAbait::HIS3 reporter vector (pMW2) (Deplancke et al., 2004). These vectors were digested with AflII and transformed into yeast strain YM4271, where they integrate into the genome. These strains were then transformed with *BvMYB1* in the activation domain vector (*pBvMYB1ΔAcAD*) or *pACT* empty vector. Selection for transformants containing both constructs was on –His –Leu medium. Interaction was assayed on –His –Leu with 25 mM 3-amino-1, 2, 4-triazole (3AT).

Glucocorticoid receptor induction.

The full-length *BvMYB1* coding region without a stop codon was amplified using primers BvMYB1start and BvMYB1nostop, recombined into pDONR/zeo and sequenced. This was recombined into *pR1R2ΔGR* (Baudry, 2004) in frame with the hormone binding domain of the rat glucocorticoid receptor (GR) under the control of CAMV35S promoter to create *35S::BvMYB1-GR*.

Albina vereduna seeds were sterilized and sown on MS media with 3% sucrose in Petri dishes. Plates were kept in darkness for 4 days at 25 °C. They were then grown under continuous fluorescent light for 7 days. These seedlings were transformed with *Agrobacterium rhizogenes* containing *35S:BvMYB1-GR* by syringe injection of hypocotyls, and after 2 d they were moved to new MS media with 3% sucrose and 200

mg/l Timentin. The transformed roots were propagated and used for different treatments. The treatments used were H2O (mock), 20 μM DEX and/or 100 μM CHX for 4 h. Total RNA extraction and qRT-PCR were performed as previously described.

Yeast two-hybrid analysis.

The *BvMYB1* full-length and truncated fragments described above were recombined into the Gal4 DNA binding vector (*pGBT9-RFB*) and activation vector, (*pACTGW*-attR) (Nakayama et al., 2002) to create *BvMYB1BD,BvMYB1ΔAcBD*, *BvMYB1AD* and *BvMYB1ΔAcAD*.

The *EGL3*, *R* and *PAP2ΔAc* 2-hybrid constructs have been described (Gonzalez et al., 2008). Constructs were transformed into yeast strain Y190 in various combinations. Selection was on –Leu, –Trp medium, and interaction was assayed on –Leu, –Trp, –His medium with 25 mM 3-amino-1, 2, 4-triazole. All hybrid bait proteins were tested for self-activation.

Mapping.

Fifty-four F6- generation, C869 × W357B RILs(McGrath et al., 2007) were screened for a set of polymorphisms (A/T SNP and 3-bp insertion) approximately 19 kb from *BvMYB1* by sequencing PCR fragments generated using MYB1MAPF and MYB1MAPR primers (Table 2-4). These 54 RILs were also screened for polymorphisms (C/T and T/C SNPs) within *BvCYP76AD1* using Bv76AD1MAPF and Bv76AD1MAPR (Table 2-4). Results of these mapping experiments are presented in Table 2-1. Map distances were calculated as described (Haldane and Waddington, 1931).

We screened 54 F2 Sea beet × W357B for a 3-bp insertion using MYB1MAPF and MYB1MAPR primers. Results of these mapping experiments are presented in Table 2-2.

The Sea beet × W357B and RIL samples were selected to be an adequate sample size for Pearson's χ^2 analysis, and no samples were excluded from the analysis.

Analysis of *Y* and *y* regulatory regions.

Sequence flanking the *BvMYB1* translational start site (GenBank JF432079) was obtained from BAC sequencing and used to design primers for amplification of white beet (*y*) upstream regulatory region. These primers would not amplify a fragment from any red beet variety we tested. We performed SOLiD mate-paired genomic DNA sequencing on the red (*Y*) W357B variety and obtained pairs of reads with one member within the first exon of *BvMYB1*. A primer was designed to the opposite pair-member, and the DNA between the pair was amplified and sequenced. A new mate pair was identified with a member within this new sequence, and the DNA between the pairs was amplified and sequenced. In this iterative manner, we walked upstream of the start for approximately 1,600 bp. We sequenced this region from two white (C869 and Albina vereduna) and two red (W357B and Bull's Blood) beet varieties and found that they were identical within their group. Primer pairs (GWC869-460F and GWC869startR; GW357-460F and GW357startR) were used to amplify 460 bp upstream of *BvMYB1* from the C869 and W357B varieties, respectively. These fragments were cloned into *pDONR222*, sequenced and then cloned into *pKGWFS7* (Karimi et al., 2002) to create plant vectors with *GUS* driven by the *y* and *Y* regulatory regions. These vectors were placed in *A. tumefaciens* for *Arabidopsis* and *Agrobacterium rhizogenes* for beet transformation.

Accession Codes

Sequences are available in GenBank for *B. vulgaris BvDODA1* (HQ656027); *B. vulgaris CYP76AD1* (HQ656023),

Beet *MYB* gene, GenBank JF432079; *BvMYB1*, GenBank JF432080 coding sequence.

RESULTS

A novel BvMYB, BvMYB1 is highly expressed in red beet seedlings

We used quantitative RT-PCR to test the potential of BvMYB1 to regulate betalains. Compared with the level of expression in red Bull's Blood beet (*Y*), *BvMYB1* is expressed at levels at least tenfold lower in sugar beet and Albina vereduna (white, y) (Keller, 1936). Genes encoding the two known biosynthetic enzymes (Christinet et al., 2004; Hatlestad et al., 2012), BvDODA1 and BvCYP76AD1 (Fig. 2-1), were also expressed at low levels in white beets and high levels in red beets (Fig. 2-8). This correlation indicated that BvMYB1 is a good candidate to regulate betalains.

BvMYB1 activates betalain biosynthetic genes

If BvMYB1 activates betalain biosynthetic genes and works like many anthocyanin MYBs (Borevitz et al., 2000; Gonzalez et al., 2008), its overexpression should upregulate the betalain pathway. We used a *35S::BvMYB1*overexpression construct in *Agrobacterium rhizogenes* to infect white beet hypocotyls to produce transgenic fibrous 'hairy' roots. Transformation of white beets using *35S::GUS* resulted in the emergence of white roots only, matching normal white beet fibrous roots (Fig. 2-

1c and Fig. 2-5d). Transformation with *35S::BvMYB1* resulted in the emergence of red fibrous roots that were similar to fibrous roots from red beets (Fig. 2-1d and Fig. 2-5d), indicating that BvMYB1 can upregulate the betalain pathway in white beets.

HPLC–mass spectrometry analysis showed that relative betanin pigment (the major beet red betalain) concentration was greatly increased in *35S::BvMYB1*-transformed over control*35S::GUS*-transformed roots (Fig. 2-1 e, f). Pigment absorbance at 530 nm indicated a 30- to 88-fold increase (Fig. 2-9). Quantitative RT-PCR verified that *BvMYB1* was highly overexpressed in *35S::BvMYB1*-transformed roots and that both *BvDODA1* and *BvCYP76AD1*were upregulated over controls (Fig. 2-1 g and Fig. 2-10).

Silencing BvMYB1results white phenotype in red beets

If BvMYB1 regulates betalain genes, silencing *BvMYB1* should lead to reduced betalain pigmentation and gene expression. Virus-induced gene silencing(Hatlestad et al., 2012; Liu et al., 2002) of *BvMYB1* in Bull's Blood beets produced plants with green, betalain-free sectors on normally dark-red leaves (Fig. 2-2 a). Mass spectrometry analysis showed that silenced sectors contained far less of the pigment than nonsilenced sectors (Fig. 2-2 b,c). *BvMYB1* expression in silenced tissues was downregulated at least fivefold, whereas expression of both *BvDODA1* and *BvCYP76AD1* was at less than 1% of that observed in nonsilenced tissues (Fig. 2-2d and Fig. 2-11), indicating that *BvDODA1* and *BvCYP76AD1* are regulated by BvMYB1.

Truncated BvMYB1 protein deactivated BvMYB1 function

We tested whether a truncated BvMYB1 protein without the transcriptional activation domain (*35S::BvMYB1ΔAc*) would have a dominant-negative activity like that

of similarly truncated anthocyanin MYBs (Gonzalez et al., 2008) (Fig. 2-6). Transformation with *35S::BvMYB1ΔAc* resulted in the emergence of white roots from white beets (data not shown). *35S::GUS* in red beets produced red roots with white tips (Fig. 2-2e). *35S::BvMYB1ΔAc* roots emerging from red beets were white from base to tip, indicating that truncated BvMYB1 represses the betalain pathway (Fig. 2-2f). We verified that *35S::BvMYB1ΔAc* was expressed at high levels and that *BvDODA1* and*BvCYP76AD1* were downregulated 20-fold or more in dominant-negative tissues compared with controls (Fig. 2-2g and Fig. 2-12).

BvMYB1 directly regulates betalain biosynthetic genes

To examine the possibility that BvMYB1 directly regulates *BvDODA1* and *BvCYP76AD1*, we carried out two types of analyses: yeast one-hybrid analysis and expression of a dexamethasone-inducible *35S::BvMYB1* construct fused to the glucocorticoid receptor (*35S::BvMYB1-GR*). Yeast one-hybrid analysis showed that BvMYB1 was able to activate a reporter fused to upstream regulatory regions of each gene (Fig. 2-13), indicating that BvMYB1 can bind to upstream *cis* sequences in both genes in yeast without a partner. *35S::BvMYB1-GR* also seemed to directly regulate *BvDODA1* in beets, as induction with dexamethasone caused upregulation of*BvDODA1* both with and without cycloheximide (Fig. 2-14). However, induced *35S::BvMYB1-GR* did not upregulate *BvCYP76AD1* within the 4-h time frame of this experiment, indicating indirect regulation or slower response.

Next, we tested whether BvMYB1 would function in related betalain species by transforming *Amaranthus hypochondriacus* with *35S::BvMYB1*. *35S::BvMYB1* induced

red *A. hypochondriacus* roots, and control constructs induced white roots (Fig. 2-2 h, i), suggesting that a similar MYB functions in *A. hypochondriacus*.

BvMYB1 does not function in anthocyanin pathway

We then determined whether betalain and anthocyanin MYBs were interchangeable. BvMYB1 did not regulate the anthocyanin pathway in *Arabidopsis*, and *Arabidopsis* MYBs did not regulate the betalain pathway (Fig. 2-3 a–d and Fig. 2-15). *Arabidopsis* AtMYB114 was able to activate the expression of the beet homolog of the anthocyanin pathway gene *leucoanthocyanidin dioxygenase (LDOX)* approximately fourfold and seemed to weakly suppress *dihydroflavonol-4-reductase (DFR)* homolog expression about twofold (Fig. 2-16), but it had no effect on pigment accumulation in beets. This may indicate that there is an unidentified bHLH in beet, as anthocyanin MYBs are dependent on interaction with anthocyanin bHLHs in their native and heterologous species (Zimmermann et al., 2004), but we do not have data to address this in beet. However, we found that BvMYB1 would not interact with anthocyanin bHLHs in yeast two-hybrid studies (Fig. 2-3 e). Examination of BvMYB1 showed that it did not match five of seven conserved amino acids previously determined to be important for bHLH interaction (Grotewold, 2000; Zimmermann et al., 2004) (Fig. 2-3 f). We changed four and then all five of these residues to match anthocyanin MYBs, but this did not result in BvMYB1-bHLH interaction. We identified a sixth conserved residue within the bHLH-interacting MYBs that BvMYB1 did not share. We altered this residue along with the other five, resulting in a BvMYB1 version that interacted strongly with the bHLH (Fig. 2-3e). Changing this residue in the anthocyanin MYB to match the betalain MYB sequence resulted in a large reduction in interaction (data not shown).

BvMYB1 is the Y locus

In 1936, Keller (Keller, 1936) described two betalain loci in beet, *R* and *Y*, linked at approximately 7 cM. We recently identified *R* as *BvCYP76AD1* (Hatlestad et al., 2012) (Fig. 2-1a). Alleles at the *Y* locus dictate whether betalains (red or yellow) are produced in the inner flesh of beets. Red flesh (*Y*) is dominant over white (*y*). We used F6-generation recombinant inbred lines (RILs;) (McGrath et al., 2007) between W357B red beet and C869 white beet to map *BvMYB1* alleles relative to red-flesh and white-flesh segregation (Table 2-1). We found that 28 of 28 red lines were homozygous for the W357B allele (*Y*) and 26 of 26 white lines were homozygous for the C869 allele (*y*). Pearson's χ^2 analysis showed significant linkage ($P < 1.74 \times 10^{-14}$).

To verify, we scored *BvMYB1* alleles versus red or white phenotype in an F2 population between W357B (*Y*) and sea beet (*y*; accession PI562601, US Department of Agriculture Germplasm Resources Information Network; Table 2-2). Of 28 red F2 beets, 6 were homozygous for W357B (*YY*) and 22 were heterozygous for the W357B/PI562601 alleles (*Yy*). Of 26 white F2 beets, all were homozygous for the PI562601 allele (*yy*). Pearson's χ^2 analysis showed significant linkage here also ($P < 2.75 \times 10^{-21}$).

Using polymorphisms in the RIL population, we mapped the distance between the *BvMYB1* and *BvCYP76AD1* (*R*) genes and found linkage of 7.6 cM. This closely agrees with Keller's distance between *Y* and *R* and more recent data (Goldman and Austin, 2000; Keller, 1936). On the basis of functional and genetic evidence reported here, *BvMYB1* is identified as lying at the *Y* locus.

Because the functional difference between the *Y* and *y* alleles seemed to be at the level of mRNA abundance and not coding region polymorphisms (*yy* plants still can

make pigment but never make as much of it or have it in the same locations as *YY* or *Yy* plants), we tested potential upstream regulatory regions of both. We sequenced fragments of ~1,600 bp for each allele. The alleles were identical for 25 bp upstream of the start site, at which point the sequences diverged completely for their entire length (Fig. 2-17). The *Y* upstream sequence from W357 was not found in the published incomplete beet genome, nor did it contain any significant similarity to known sequences, and thus it seems to be unique. We cloned 460 bp of each allele adjacent to the start into a GUS expression construct and transformed it into *Arabidopsis* and beet. In both beet and *Arabidopsis,* GUS histochemical assays and quantitative RT-PCR of the *GUS* transcript showed that the red beet regulatory region (*Y*) results in a far more highly expressed *GUS* transgene than the white region (*y*; Fig. 2-4 and Fig. 2-17). This region may contain 5′ UTR elements that affect mRNA stability or translation, and it must contain transcriptional activation elements causing expression of the *GUS* transgene.

DISCUSSION

In summary, we have discovered a MYB transcription factor in beet that positively regulates betalain pathway genes and maps to the historic *Y* locus. Red flesh in beets is due to a dominant overexpression allele of *Y* explained by the genome sequence immediately upstream of the coding region. Phylogenetic analyses indicate that *BvMYB1* and anthocyanin MYBs probably derive from a common ancestor, consistent with the hypothesis that betalain and anthocyanin biosynthetic genes are regulated by the same type of transcription factors. Although it seems that BvMYB1

evolved from anthocyanin MYBs, it has evolved new characteristics; most notably, it does not interact with heterologous bHLH members of the MYB-bHLH-WD complex. However, it is possible that beets contain BvMYB1-interacting bHLHs with compensatory sequence changes allowing such interactions.

Gene cooption is usually thought of as the use of an existing gene for a new purpose. In the case of the BvMYB1 transcription factor, it seems that an existing MYB somehow acquired new transcriptional targets, allowing it to regulate betalain biosynthetic genes. This regulation of structural genes may be direct, as it seems to be for BvMYB1, or it may be indirect through the regulation of a regulatory intermediate. If true, this specific case of coopting an anthocyanin MYB is particularly noteworthy because it would imply that a novel pigment pathway has coopted an ancestral pigment pathway regulator. Anthocyanin MYB cooption could have led to new and old pigments with the same expression pattern. Two redundant pathways would allow for the loss of either pathway, and the fact that anthocyanins and betalains are mutually exclusive in modern species implies that they provide largely the same function.

In this case, several functions are largely the same: pigmentation of flowers, fruits and epidermis. However, we note that betalains can be yellow and anthocyanins can be purple, so the spectrum of colors and thus function is not strictly identical. The functions and interactions of the anthocyanin MYB-bHLH-WD complex are conserved across widely divergent taxonomic boundaries (Feller et al., 2011; Gonzalez et al., 2008; Grotewold, 2000; Quattrocchio et al., 1993; Schwinn, 2006). Our evidence shows that BvMYB1 has probably diverged from the anthocyanin MYBs, losing the ability to regulate anthocyanins or interact with the MBW complex, at least in the same way. A

possibility is that the coopted MYB or complex only needed to regulate one or two betalain-encoding genes when first coopted (and even now), relieving it from severe evolutionary constraints on the MBW complex, which regulates more than ten genes coordinately to produce anthocyanins.

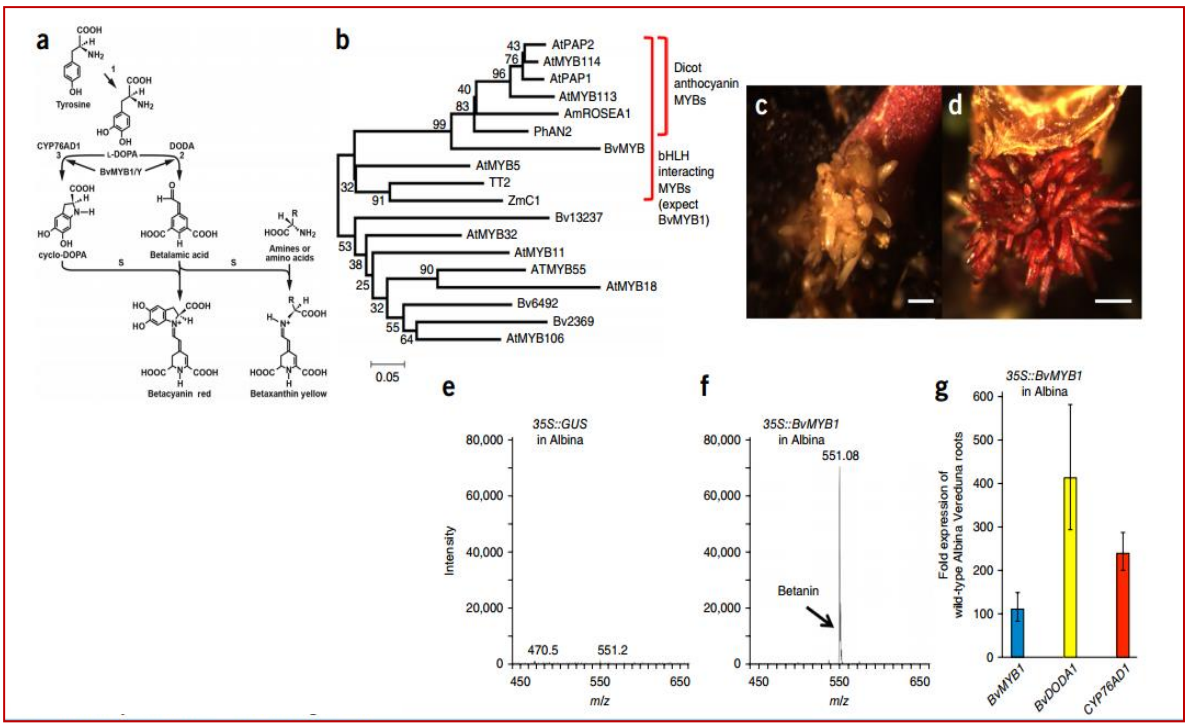


Figure 2-1: Betalain biosynthetic pathway and analysis of BvMYB1 overexpression in beets

(a) Steps 1, 2 and 3 of the biosynthetic pathway are proposed to be enzyme mediated; steps marked S are spontaneous. BvMYB1 controls the expression of the key *BvDODA* and *BvCYP76AD1* genes. (b) Neighbor-joining tree of R2R3 MYB domains of BvMYB1 and other MYBs. Scale represents amino acid changes per position. BvMYB1 clusters with dicot anthocyanin MYBs within the larger bHLH-interacting cluster. *Beta vulgaris* is a dicot. (c, d) Overexpression of GUS (c) and BvMYB1 (d) in white Albina vereduna beets. Scale bars, 1 mm. (e, f) HPLC–mass spectrometry analysis of red betanidin pigment in the roots of white Albina vereduna transformed with *35S::GUS* (e) versus *35S::BvMYB1* (f). (g) Quantitative RT-PCR showing relative *BvMYB1*, *BvDODA1* and *BvCYP76AD1* expression in*35S::BvMYB1* roots versus *35S::GUS* controls. Bars are average of five technical replicates. Error bars are s.d. Every experiment was replicated three times.

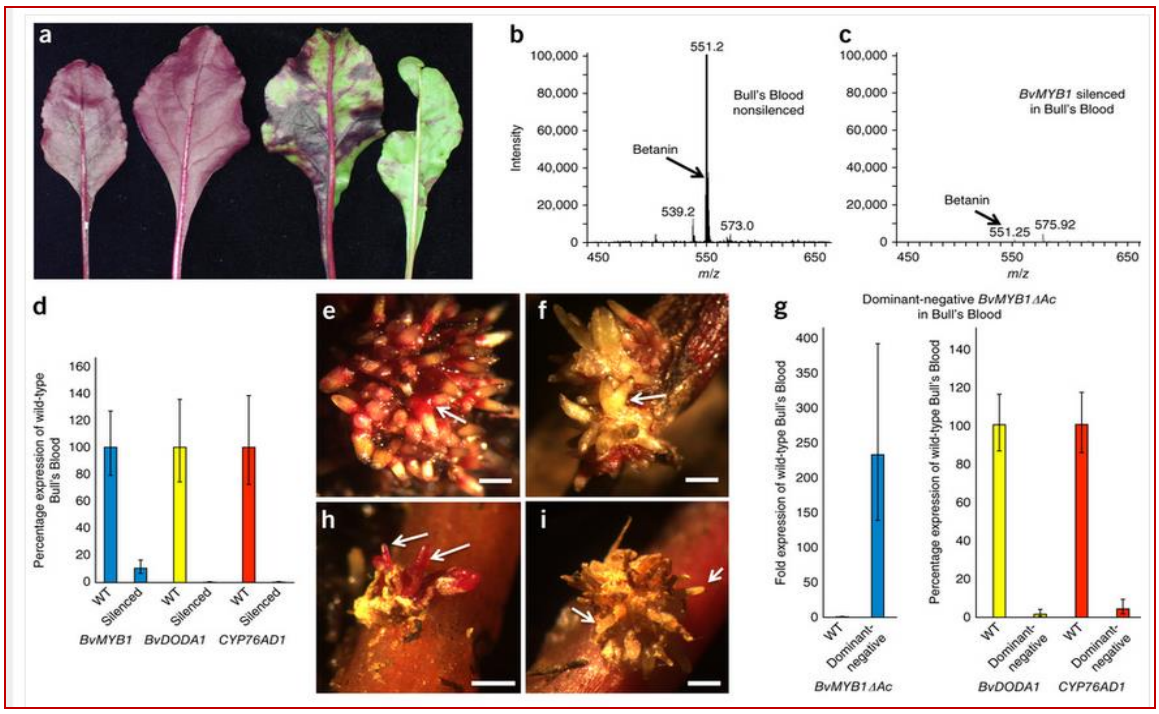


Figure 2-2: Reverse genetic analysis of BvMYB1

(a) Virus-induced gene silencing of *BvMYB1* in Bull's Blood leaves (wild type on left and silenced on right). (b, c) HPLC and mass spectrometry analysis of betanin pigment in nonsilenced (b) versus silenced (c) tissues. (d) Quantitative RT-PCR showing relative gene expression in these tissues. WT, wild type. (e, f) *35S::GUS* (e) and dominant-negative *35S::BvMYB1ΔAc* (f) in Bull's Blood. Scale bars, 1 mm. Arrows point to roots displaying the phenotype. (g) Quantitative RT-PCR showing gene expression in control versus dominant-negative Bull's Blood lines. The *35S::BvMYB1ΔAc* bar is measuring overexpression of the dominant-negative truncation fragment. Every quantitative RT-PCR experiment was replicated three times; results from a single biological replicate are shown. Bars are average of five technical replicates; error bars are s.d. (h, i) *35S::BvMYB1* (full length) (h) and *35S::GUS* (i) expressed in *A. hypochondriacus* cv. Plainsman. Scale bars, 1 mm. Arrows point to roots displaying the phenotype.

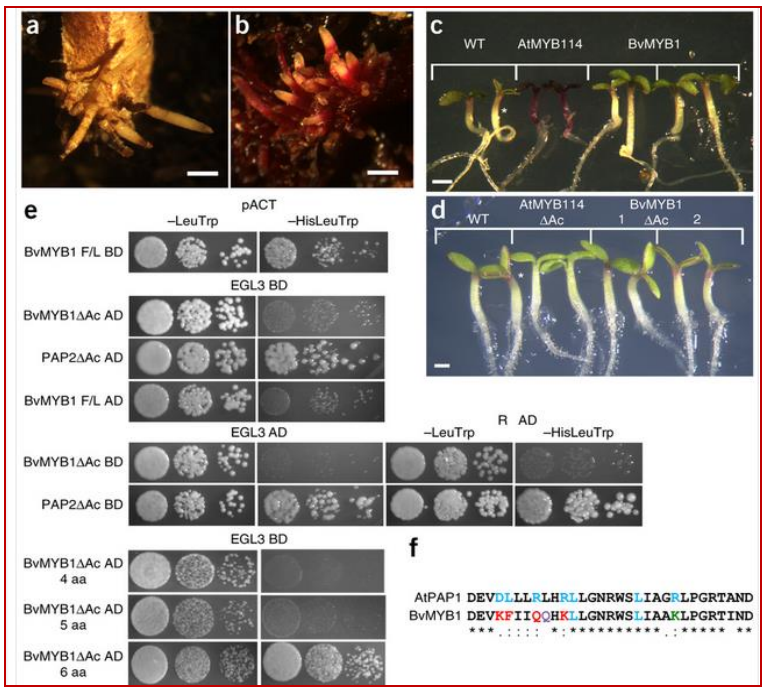


Figure 2-3: Analysis of BvMYB1 versus *Arabidopsis* anthocyanin MYBs

(a) Overexpression of the *Arabidopsis* anthocyanin MYB, AtMYB114, in white beet does not activate the betalain pathway. (b) Overexpression of AtMYB114ΔAc in red beet does not act as a dominant negative. Scale bars, 1 mm in a and b. (c, d) Overexpression of AtMYB114 and BvMYB1 (c) and of AtMYB114ΔAc and BvMYB1ΔAc (d) in 3-day-old *Arabidopsis* seedlings. Anthocyanin pigmentation is produced on the *Arabidopsis* hypocotyl just below the cotyledons marked by asterisks. 1 and 2 are independent transgenic lines. AtMYB114 activates anthocyanins, whereas AtMYB114ΔAc acts as a dominant negative. BvMYB1 and BvMYB1ΔAc do not affect anthocyanin production. Scale bars, 1 mm. (e) Yeast two-hybrid analysis of possible MYB-bHLH interaction. Top, BvMYB1 in the DNA-binding-domain vector activates transcription in yeast. Middle, PAP2, but not BvMYB1, interacts with *Arabidopsis* EGL3 and maize R bHLH proteins. Bottom, changing six amino acids in BvMYB1 to match conserved residues in anthocyanin MYBs allows BvMYB1 to interact with EGL3. F/L, full length; AD, Gal4 activation domain fusion; BD, Gal4 DNA-binding-domain fusion. (f) Analysis of the bHLH interaction region of PAP1 (PAP1, PAP2, AtMYB113 and AtMYB114 are identical in this region) and BvMYB1. Seven identified residues required for bHLH binding in PAP1 are highlighted (Nakayama et al., 2002; Schwinn, 2006). The four changed residues are red in BvMYB1, the fifth is green and the sixth is purple. PAP1, PAP2, AtMYB113 and AtMYB114 all behave the same in assays like those in c–e and are 87% identical in the MYB domains. All experiments were repeated at least three times.

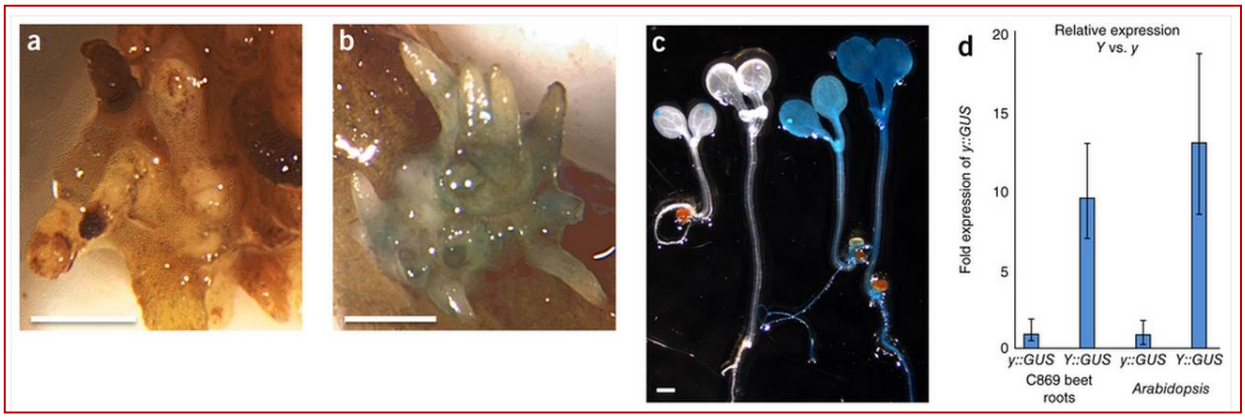


Figure 2-4: Analysis of *BvMYB1 y* versus *Y* allele regulatory regions

(a, b) Albina vereduna white beet transformed with y::GUS (a) or Y::GUS (b) and stained for GUS activity with X-Gluc. Scale bars, 1 mm. (c) *Arabidopsis* transformed with *y::GUS* (left) or *Y::GUS* (right) and stained for GUS activity. Scale bar, 1 mm. (d) Quantitative RT-PCR showing relative *GUS* gene expression of *y* versus *Y* regulatory regions in white beet tissue and *Arabidopsis*. Every quantitative RT-PCR experiment was replicated three times; results from a single biological replicate are shown. Bars are average of five technical replicates; error bars are s.d. All experiments were repeated at least three times.

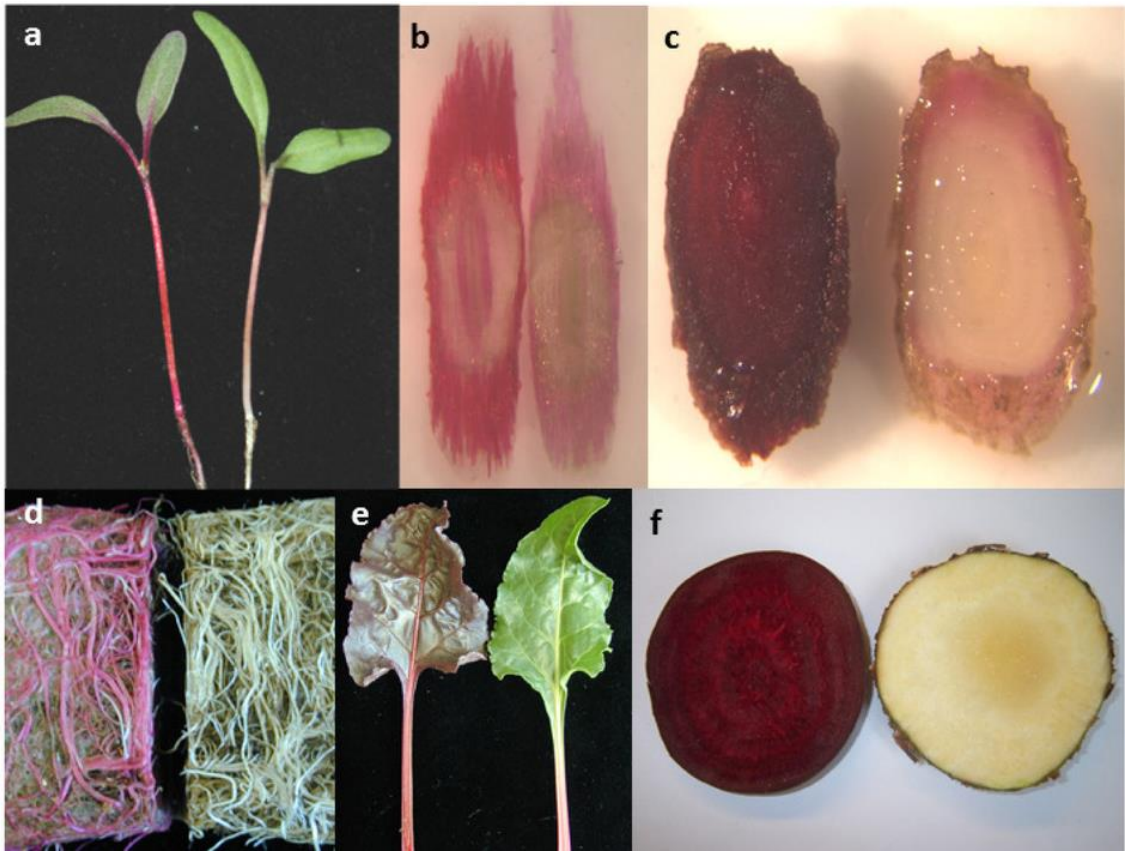


Figure 2-5: Bull's Blood red table beet (YY genotype) is on the left and Albina vereduna white table beet (yy genotype) is on the right in each panel

(a) Seven-day-old seedlings. (b) Oblique hand section through 7-day-old hypocotyl. (c) Sections through same position several weeks later. (d) Fibrous roots on pot-bound plants. (e) Mature true leaves. (f) Mature beets.

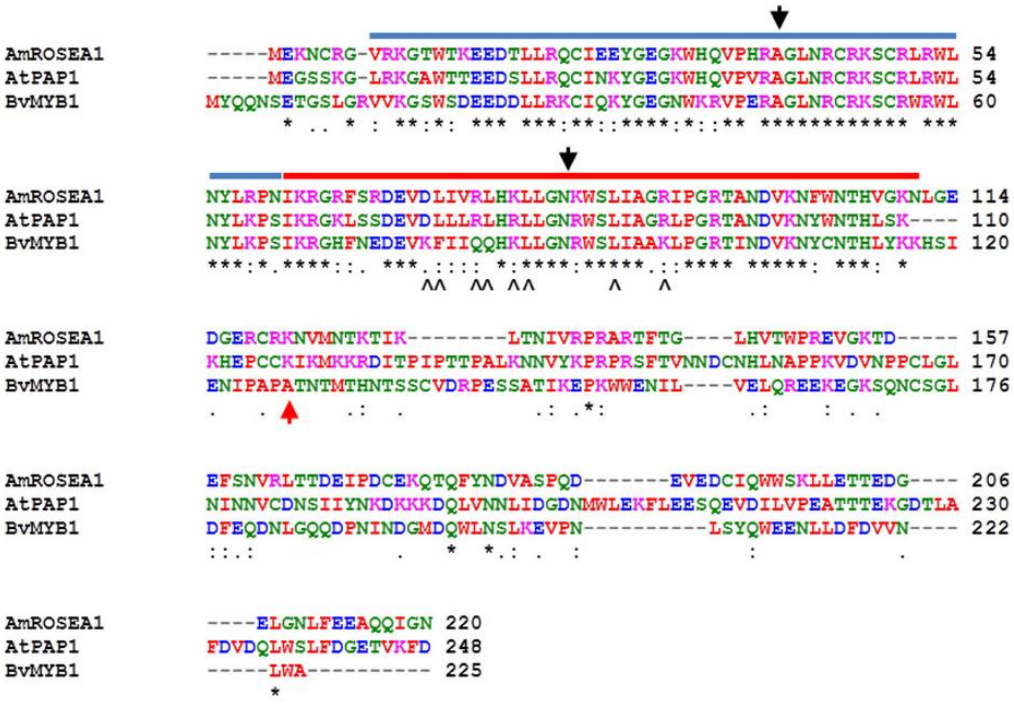


Figure 2-6: Alignment of BvMYB1 with two anthocyanin regulatory MYBs, AmROSEA1 from *Antirrhinum majus* and AtPAP1 from *Arabidopsis thaliana*

Blue and red bars designate the R2 and R3 MYB domains, respectively. Black arrowheads indicate the position of introns in all three genes. A red arrowhead indicates the last amino acid in the truncated BvMYB1ΔAc. Carats indicate the amino acids involved in bHLH interaction discussed in the text. The alignment illustrates the high degree of identity throughout the MYB domains but lack of homology throughout the activation domain approximately after amino acid positions 110 to 115. The alignment was created with ClustalW2.

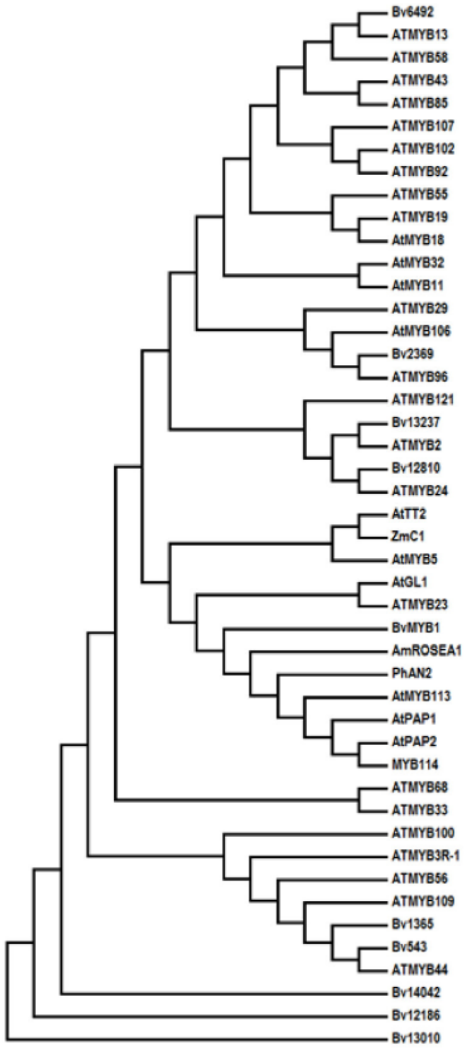


Figure 2-7: Neighbor-joining tree of the R2R3 MYB domains of BvMYB1 and a representative selection of R2R3 MYBs across the gene family

This tree includes all the genes from Figure 2-1b and at least one member from most of the subgroups previously defined.

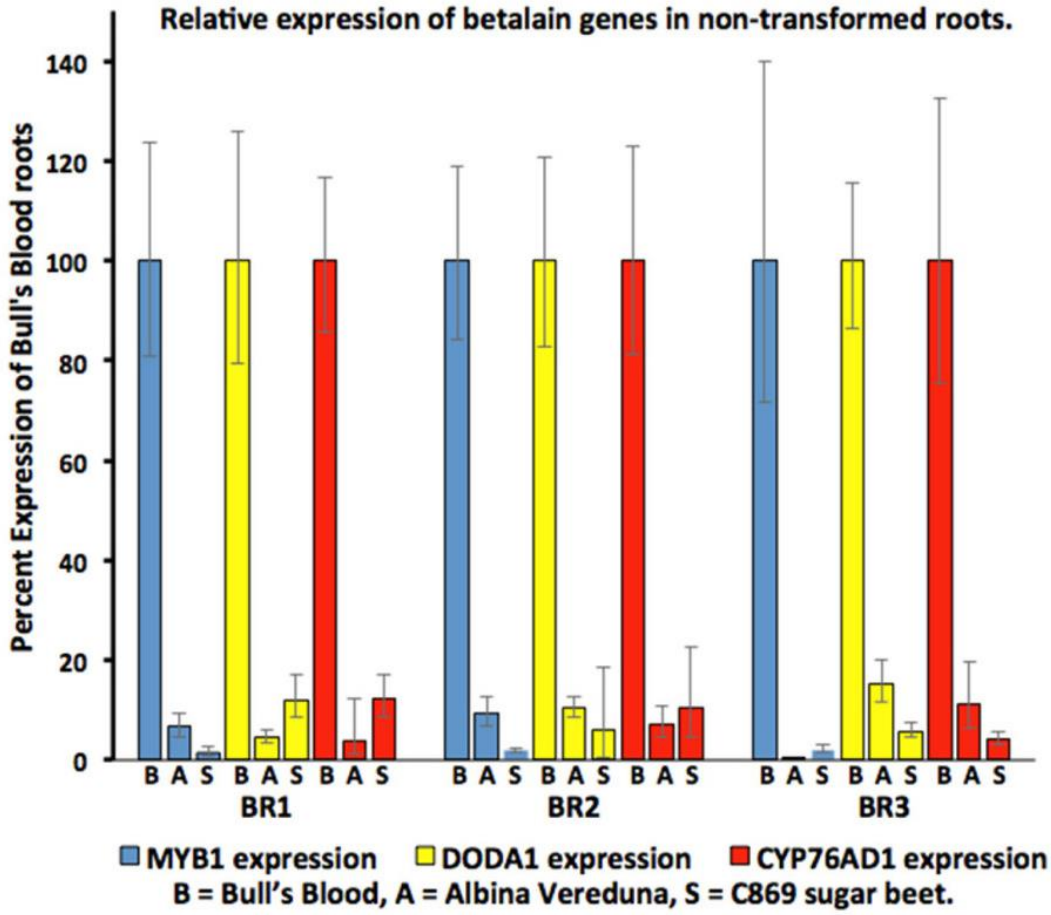


Figure 2-8: Betalain gene expression is upregulated in Bull's Blood red table beets (Y) versus Albina vereduna white table beets (y) and C869 white sugar beets (y)

Every qRT-PCR experiment was replicated three times, and all three biological replicates (BR1, 2, and 3) are shown. Bars represent the average of five technical replicates; error bars, s.d.

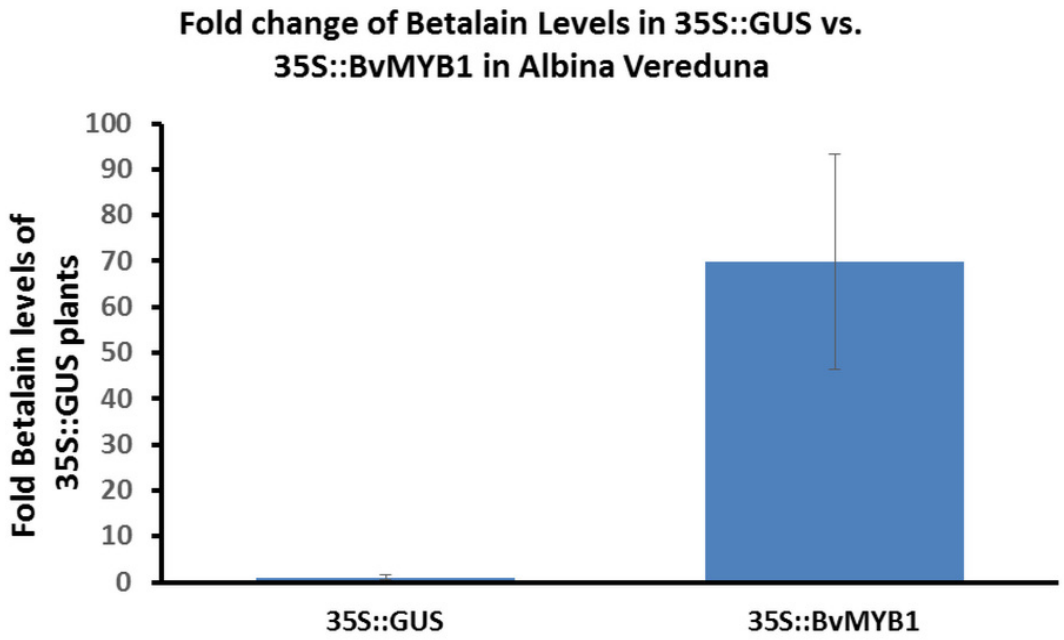


Figure 2-9: Betalain pigment is increased in 35S::BvMYB1 compared to 35S::GUS in Albina vereduna

Extracted pigment was quantified by measuring absorbance at 530 nm. Data for 35S::GUS are the average of three biological replicates with three technical replicates each. Data for 35S::BvMYB1 are from four independent biological replicates (A, B, C and D). Bars represent the average of three technical replicates each; error bars, s.d.

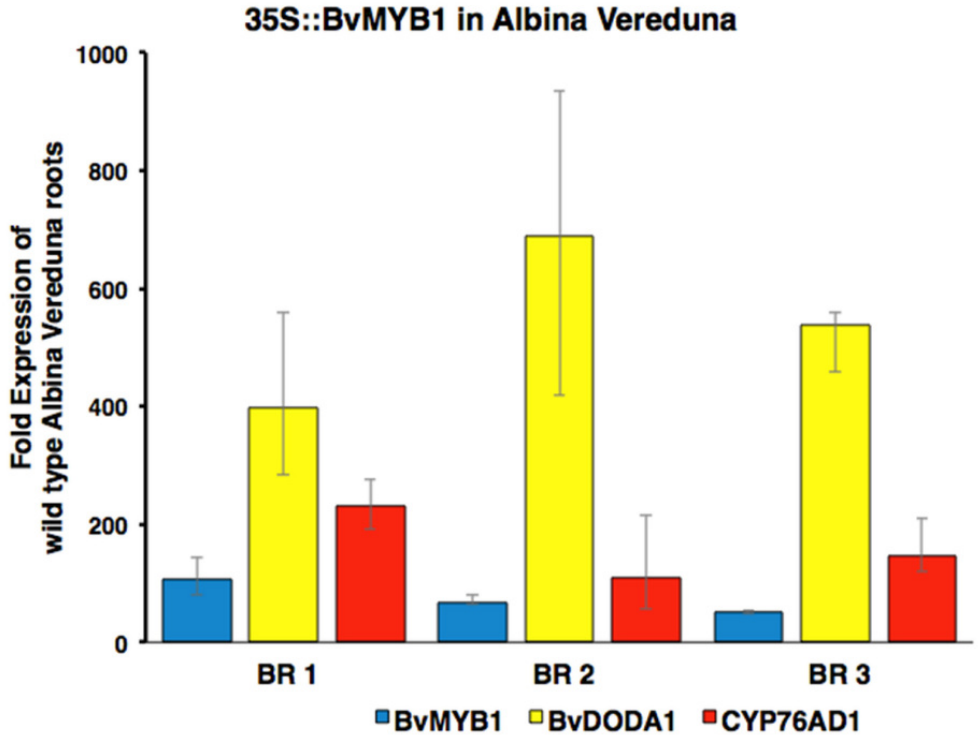


Figure 2-10: BvMYB1 overexpression induces expression of BvDODA1 and BvCYP76AD1

Every qRT-PCR experiment was replicated three times, and all three biological replicates (BR) are shown. Bars represent the average of five technical replicates; error bars, s.d.

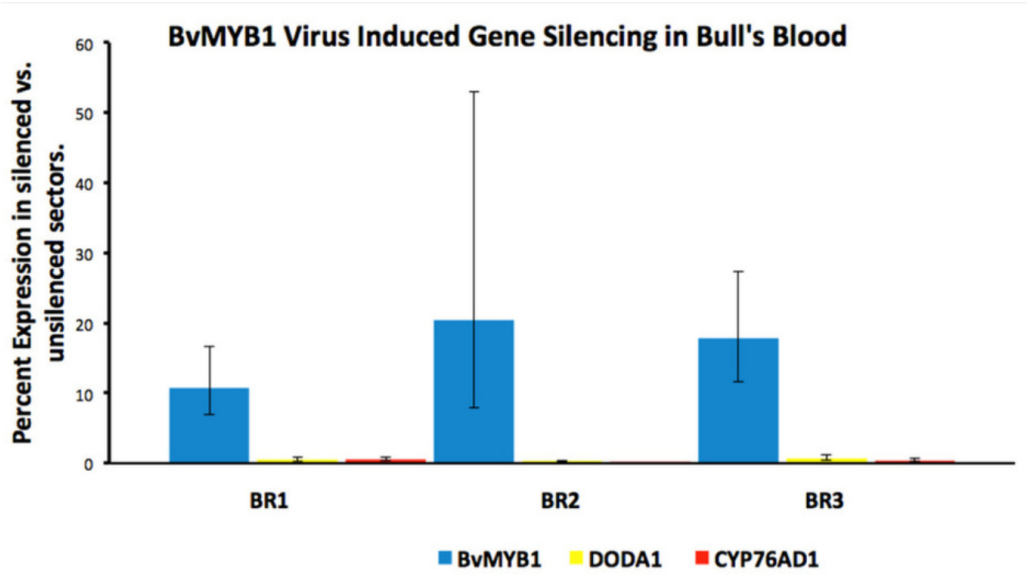


Figure 2-11: Virus-induced gene silencing of BvMYB1 in Bull's Blood results in downregulation of BvDODA1 and BvCYP76AD1

Every qRT-PCR experiment was replicated three times, and all three biological replicates (BR) are shown. Bars represent the average of five technical replicates; error bars, s.d.

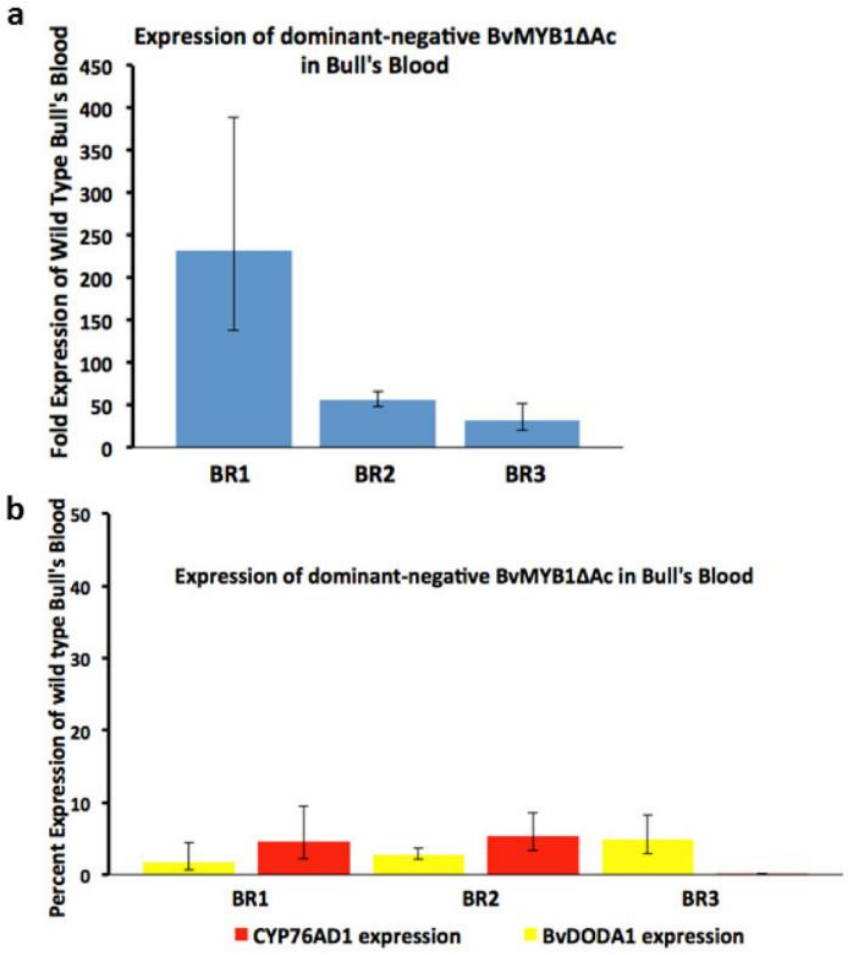


Figure 2-12: Dominant-negative 35S::BvMYB1ΔAc results in downregulation of BvDODA1 and BvCYP76AD1

(a, b) qRT-PCR expression analysis of *BvMYB1ΔAc* (a), *BvDODA1* (b) and *BvCYP76AD1* (b) in 35S::BvMYB1ΔAc Bull's Blood beet hairy roots. Every qRT-PCR experiment was replicated three times, and all three biological replicates are shown. Bars represent the average of five technical replicates; error bars, s.d.

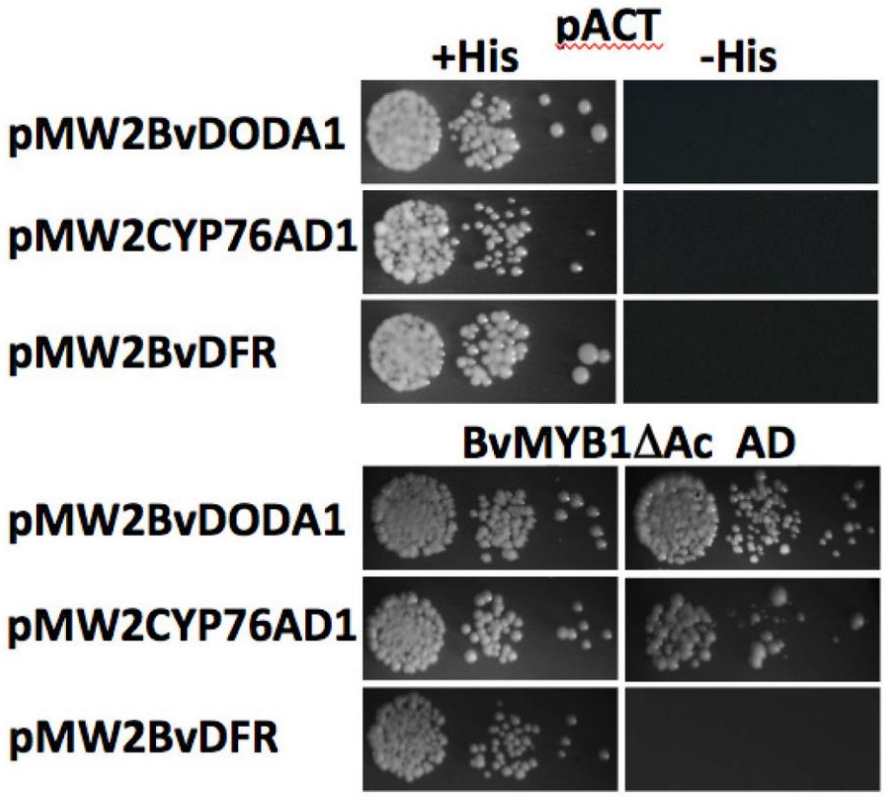


Figure 2-13: Yeast one-hybrid analysis indicates that BvMYB1 can bind to upstream regulatory regions of BvDODA1 and BvCYP76AD1

Sequence upstream of the translation start codons for two betalain structural genes, *BvDODA1* and *BvCYP76AD1*, and a putative phenylpropanoid gene, *BvDFR*, were fused upstream of the *HIS3* reporter gene. The upper panels show that these upstream regions do not activate the reporter in the absence of BvMYB1 expression. The lower panels show that BvMYB1 is able to activate *BvDODA1* and *BvCYP76AD1* but not *BvDFR*.

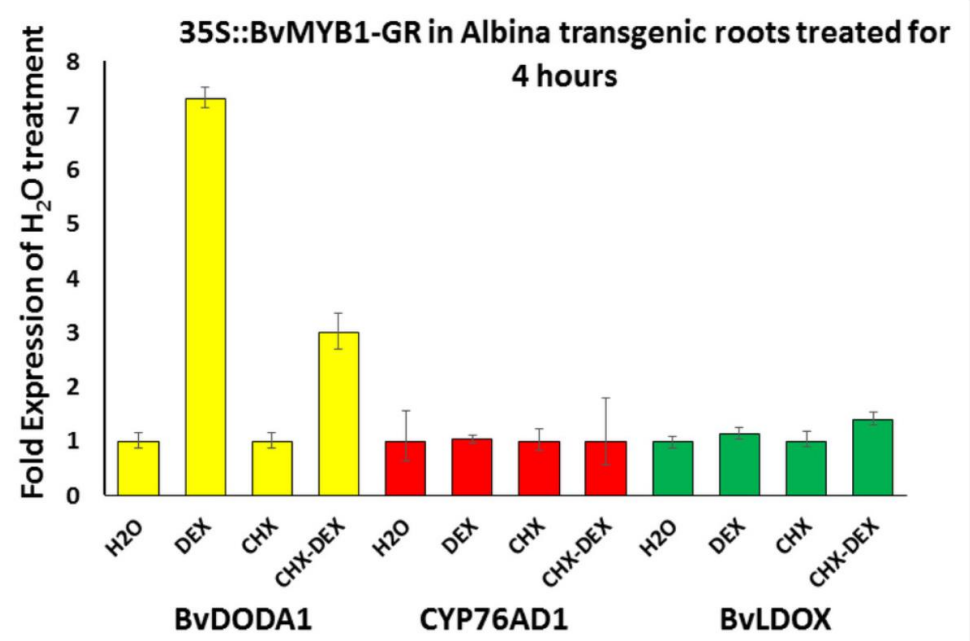


Figure 2-14: Analysis of 35S::BvMYB1-GR in Albina vereduna hairy roots

qRT-PCR after 4 hours of treatment for transgenic roots. Results shown as relative gene expression compared to expression in the mock (H20) sample. BvDODA1 and CYP76AD1 are our biosynthetic genes, and BvLDOX is our negative control. Bars represent the average of five technical replicates; error bars, s.d. Every experiment was replicated three times.

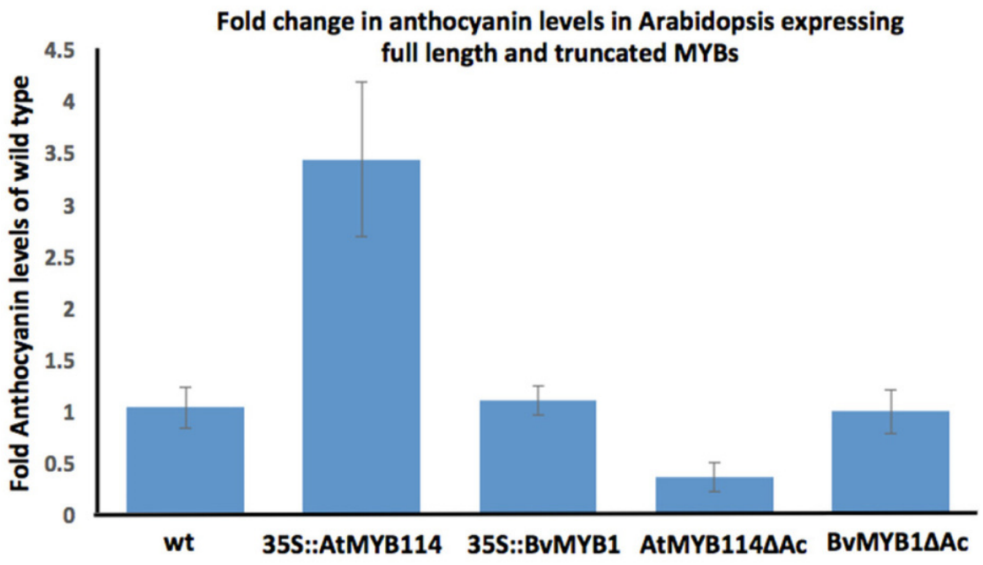


Figure 2-15: Relative anthocyanin content of *Arabidopsis thaliana* expressing MYB constructs versus wild type

Extracted pigment was quantified by measuring the absorbance at 530 nm. Each bar represents the average of five biological replicates (independent transformants); error bars, s.d.

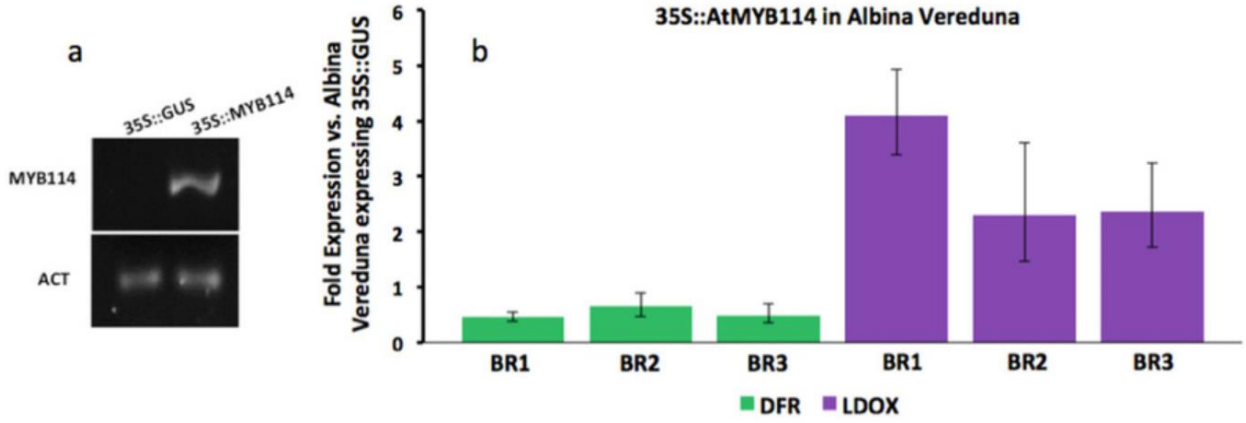


Figure 2-16: Analysis of anthocyanin gene homolog expression in Albina vereduna beet roots expressing 35S::AtMYB114

(a) Semiquantitative RT-PCR of *AtMYB114* in roots expressing 35S::GUS versus 35S::AtMYB114. (b) qRT-PCR of *BvDFR* and *BvLDOX* in these same lines. Results are shown as relative gene expression compared to expression in the 35S::GUS sample. Every qRT-PCR experiment was replicated three times, and all three biological replicates are shown. Bars represent the average of five technical replicates; error bars, s.d.

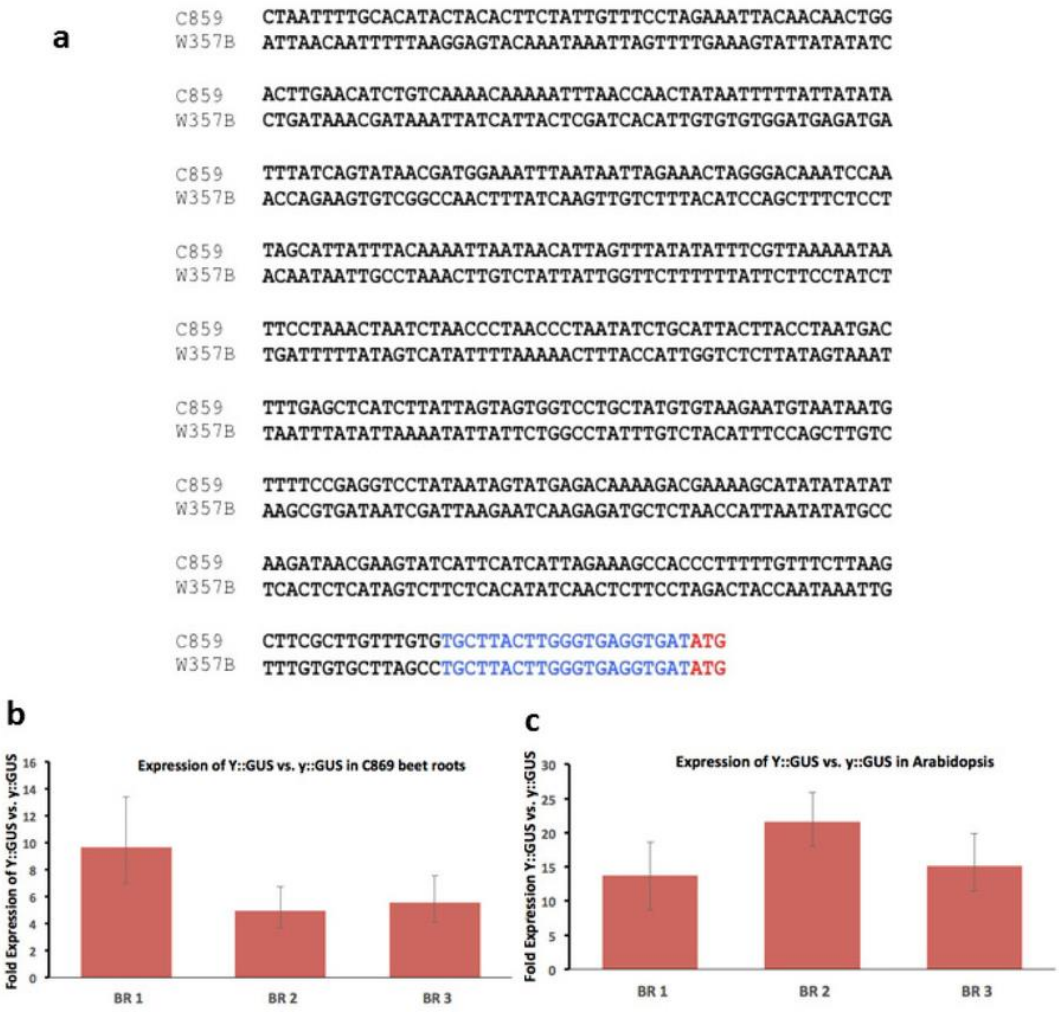


Figure 2-17: Y regulatory sequence more strongly induces GUS expression than y in both C869 beet roots and *Arabidopsis*

(a) The sequences of the white (*y*; upper) and red (*Y*; lower) *BvMYB1* regulatory regions used for the analysis in Figure 2-4 are shown. The sequences are identical for 21 bp upstream of the start codon (in red and blue). At that point, homology ends. Both sequences have approximately 70% AT content. (b, c) GUS expression in comparison of Y::GUS to y::GUS in C869 roots (b) and *Arabidopsis* (c). Every qRT-PCR experiment was replicated three times, and all three biological replicates are shown. Bars represent the average of five technical replicates; error bars, s.d.

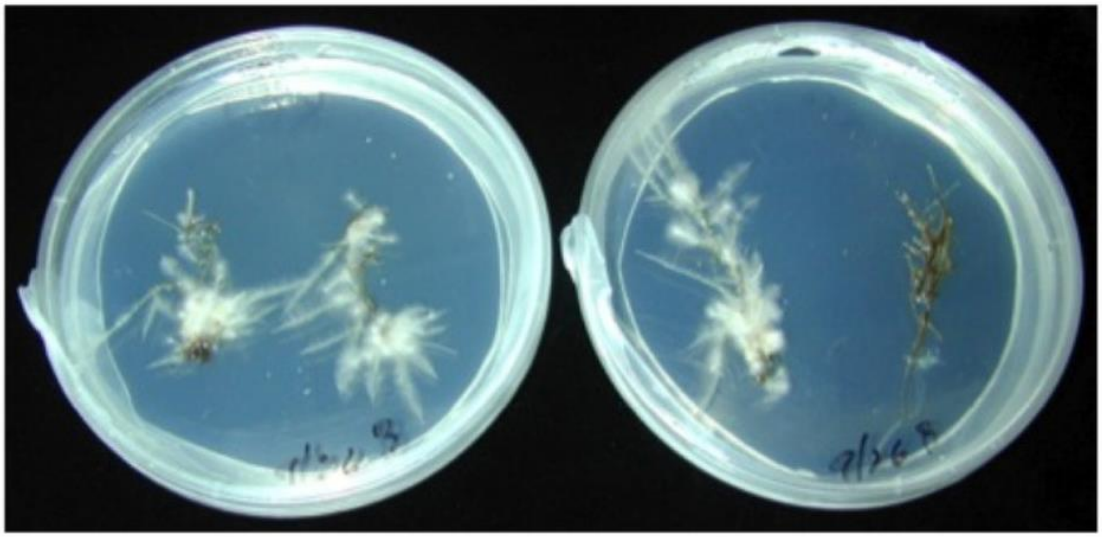


Figure 2-18: Representative example of antibiotic selection of transformed hairy roots

Each plate contains kanamycin-resistant T-DNA–transformed roots on the left and no-plasmid control hairy root culture on the right. The left plate contains no antibiotic, whereas the plate on the right contains 50 mg/l kanamycin.

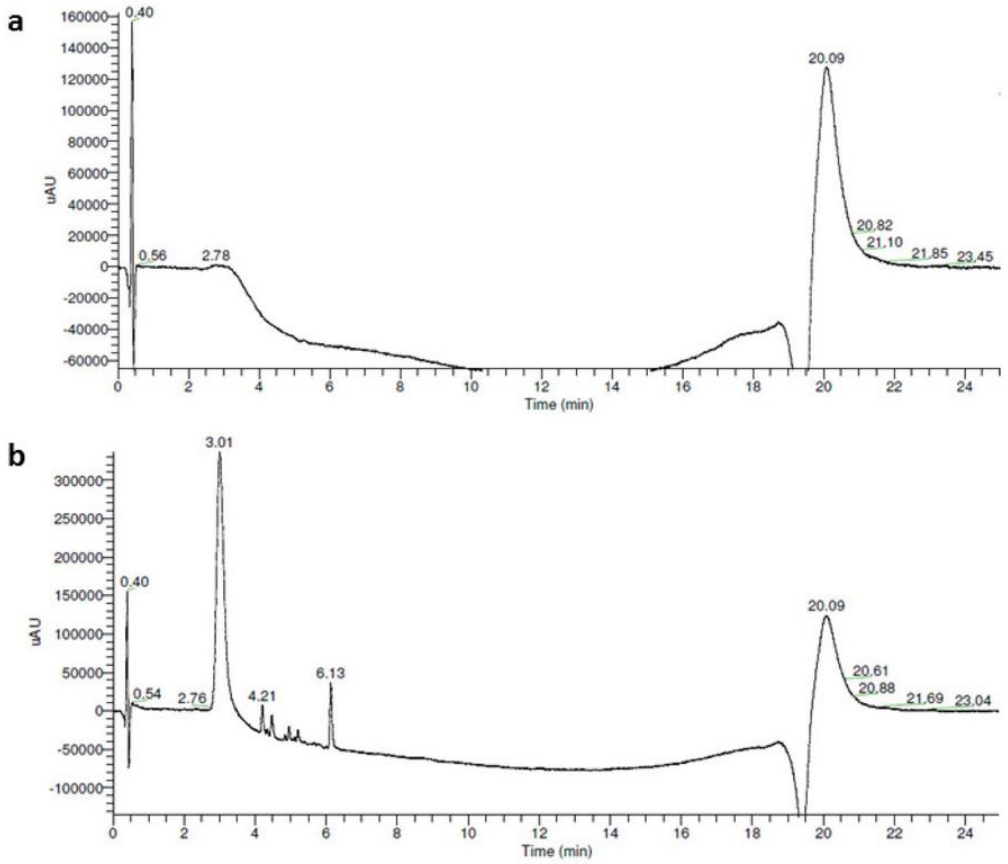


Figure 2-19: Complete HPLC data at 530 nm, the wavelength for detecting betanin

(a) 35S::GUS in Albina vereduna roots. (b) 35S::BvMYB1 in Albina vereduna roots. These HPLC data correspond to the mass spectrometry data shown in Figure 2-1 e, f. The betanin peak is at minute 3.01.

Table 2-1: RIL Mapping Results, BvMYB1 allele vs. CYP76AD1 (R) allele.

All c869 MYB allele RILs were also y phenotype and all w357 MYB allele were Y phenotype

sample	MYB Allele	R allele	Genotype	sample	MYB Allele	R allele	Genotype
203-02	c869	c869	cc	025646	w357	w357	ww
303-01	c869	w357	cw	024045	w357	w357	ww
201-02	c869	c869	cc	025697	w357	w357	ww
217-01	c869	c869	cc	024142	w357	w357	ww
215-02	c869	c869	cc	025409	w357	c869	wc
310-01	c869	c869	cc	025539	w357	c869	wc
310-02	c869	c869	cc	025302	w357	w357	ww
203-01	w357	w357	ww	025909	w357	w357	ww
235-01	w357	w357	ww	024236	w357	w357	ww
167-01	w357	w357	ww	025376	w357	c869	wc
167-02	w357	w357	ww	023975	w357	w357	ww
215-04	w357	w357	ww	023912	w357	c869	wc
271-01	w357	w357	ww	024946	c869	c869	cc
271-02	w357	w357	ww	024043	c869	c869	cc
025677	c869	c869	cc	025191	c869	c869	cc
024117	c869	c869	cc	024179	c869	c869	cc
025628	c869	c869	cc	024080	c869	c869	cc
025789	c869	c869	cc	024197	c869	c869	cc
023967	c869	c869	cc	023951	c869	c869	cc
024147	c869	c869	cc	023969	c869	c869	cc
024239	c869	c869	cc	025448	w357	w357	ww
023421	c869	c869	cc	025356	w357	w357	ww
025186	c869	c869	cc	024144	w357	c869	wc
025638	c869	c869	cc	025505	w357	w357	ww
023431	c869	c869	cc	025537	w357	w357	ww
023460	w357	w357	ww	023923	w357	c869	wc
213-01	w357	w357	ww	025367	w357	w357	ww

Table 2-2: Sea Beet Mapping Results, phenotype (red or white beet) vs. BvMYB1 allele

Sample	Phenotype	MYB Allele	Sample	Phenotype	MYB Allele	Sample	Phenotype	MYB Allele
1	Y (red)	w357	19	Y (red)	het	37	y (white)	sea
2	Y (red)	het	20	y (white)	sea	38	Y (red)	het
3	Y (red)	het	21	y (white)	sea	39	Y (red)	het
4	Y (red)	w357	22	Y (red)	het	40	y (white)	sea
5	Y (red)	het	23	y (white)	sea	41	y (white)	sea
6	y (white)	sea	24	y (white)	sea	42	Y (red)	het
7	y (white)	sea	25	Y (red)	het	43	y (white)	sea
8	y (white)	sea	26	Y (red)	w357	44	Y (red)	het
9	y (white)	sea	27	Y (red)	het	45	y (white)	sea
10	y (white)	sea	28	Y (red)	het	46	y (white)	sea
11	Y (red)	w357	29	Y (red)	het	47	y (white)	sea
12	y (white)	sea	30	y (white)	sea	51	Y (red)	het
13	Y (red)	het	31	Y (red)	w357	52	Y (red)	w357
14	Y (red)	het	32	Y (red)	het	53	Y (red)	het
15	Y (red)	het	33	y (white)	sea	55	y (white)	sea
16	Y (red)	het	34	y (white)	sea	56	y (white)	sea
17	Y (red)	het	35	y (white)	sea	57	y (white)	sea
18	Y (red)	het	36	y (white)	sea	58	y (white)	sea

Table 2-3: MYB genes included in Figure 2-2 neighbor-joining tree

	Genbank	KEGG entry*	TAIR locus	Species
BvMYB1	JF432079			*Beta vulgaris*
Bv2369		T20031:2369		*Beta vulgaris*
Bv6492		T20031:6492		*Beta vulgaris*
Bv13237		T20031:13237		*Beta vulgaris*
AtMYB106	AEE73615.1		AT3G01140	*Arabidopsis thaliana*
AtMYB32	NM_119665		AT4G34990	*Arabidopsis thaliana*
AtMYB113	NM_105308		AT1G66370	*Arabidopsis thaliana*
AtPAP1	NM_104541		AT1G56650	*Arabidopsis thaliana*
AtPAP2	NM_105310		AT1G66390	*Arabidopsis thaliana*
AtMYB114	NM_105309		AT1G66380	*Arabidopsis thaliana*
AtMYB55	NM_116398		AT4G01680	*Arabidopsis thaliana*
AtMYB11	NM_116126		AT3G62610	*Arabidopsis thaliana*
AtMYB18	NM_118688		AT4G25560	*Arabidopsis thaliana*
PhAN2	EF423868.1			*Petunia hybrida*
ZmC1	P10290.1			*Zea mays*
AtTT2	NM_122946		AT5G35550	*Arabidopsis thaliana*
AmROSEA1	ABB83826.1			*Antirrhinum majus*
AtGL1	NM_113708		AT3G27920	*Arabidopsis thaliana*
AtMYB23	NM_123397		AT5G40330	*Arabidopsis thaliana*
AtMYB5	NM_112200		AT3G13540	*Arabidopsis thaliana*
AtMYB13	NM_100499		AT1G06180	*Arabidopsis thaliana*
AtMYB58	NM_101514		AT1G16490	*Arabidopsis thaliana*
AtMYB43	NM_121666		AT5G16600	*Arabidopsis thaliana*
AtMYB85	NM_118394		AT4G22680	*Arabidopsis thaliana*
AtMYB107	NM_111164		AT3G02940	*Arabidopsis thaliana*
AtMYB102	NM_118264		AT4G21440	*Arabidopsis thaliana*
AtMYB92	NM_121066		AT5G10280	*Arabidopsis thaliana*
AtMYB29	NM_120851		AT5G07690	*Arabidopsis thaliana*
AtMYB96	NM_125641		AT5G62470	*Arabidopsis thaliana*
AtMYB121	NM_113920		AT3G30210	*Arabidopsis thaliana*
AtMYB2	NM_130287		AT2G47190	*Arabidopsis thaliana*
AtMYB24	NM_123399		AT5G40350	*Arabidopsis thaliana*
AtMYB68	NM_125976		AT5G65790	*Arabidopsis thaliana*
AtMYB56	NM_121786		AT5G17800	*Arabidopsis thaliana*
AtMYB100	NM_128080		AT2G25230	*Arabidopsis thaliana*
AtMYB33	NM_120692		AT5G06100	*Arabidopsis thaliana*
AtMYB19	NM_124605		AT5G52260	*Arabidopsis thaliana*
AtMYB3R-1	NM_119426		AT4G32730	*Arabidopsis thaliana*
AtMYB44	NM_126130		AT5G67300	*Arabidopsis thaliana*
AtMYB109	NM_115431		AT3G55730	*Arabidopsis thaliana*
Bv12810		T20031:12810		*Beta vulgaris*
Bv1365		T20031:1365		*Beta vulgaris*
Bv543		T20031:543		*Beta vulgaris*
Bv14042		T20031:14042		*Beta vulgaris*
Bv12186		T20031:12186		*Beta vulgaris*
Bv13010		T20031:13010		*Beta vulgaris*

* http://www.genome.jp/kegg-bin/show_organism?org=T20031

Table 2-4: Primers used as indicated in the text and methods of chapter 2

Primer Name	Primer sequence 5' to 3'
RTPCRBvACTF	TCTATCCTTGCATCTCTCAG
RTPCRBvACTR	ATCATACTCGCCCTTGGAGA
RTPCRMYB1F	GCCGACGATTCTGGCC
RTPCRMYB1R	GATGGTCTTTGATAGCAGC
RTPCRDODA1F	CATTGGTTCAGGAAGTGCAA
RTPCRDODA1R	ACGAAGCCATGAATCAAAGG
RTPCR76AD1F	CTTTTCAGTGGAATTAGCCCACC
RTPCR76AD1R	CCCAATATCTTCCATAATGTTCCA
BvMYB1start	TGATGTACCAGCAGAATAGTGAAAC
BvMYB1stop	GCTATGCCCACAAGTTCACAAC
BvMYB1□AcR	TCAAGCTGGTGCGGGAATGTTTTC
BvMYB1nostop	GTGCCCACAAGTTCACAAC
VIGSBvMYB1F	GCCGACGATTCTGGCCTGTCC
VIGSBvMYB1R	GTGAGGTGATATGTACCAGCAG
BvMYB1MAPF	AGGTACAAGTATACATATAGTTAC
BvMYB1MAPR	AATGATTGTATAATATGGATGGTG
Bv76AD1MAPF	GCTAATCTTGCTAAAATTCACGG
Bv76AD1MAPR	TTATGGTGGGCTAATTCCACTG
GWC869-460F	CTAATTTTGCACATACTACAC
GWC869startR	ACCTCACCCAAGTAAGCAC
GW357-460F	ATTAACAATTTTTAAGGAGTAC
GW357startR	ACCTCACCCAAGTAAGCAGG
GUSF	CATGAAGATGCGGACTTACG
GUSR	GCCCAGTCGAGCATCTCTTC
BvDFRRTF	AGTGCAGCATTTGTTGG
BvDFRRTR	GTGGCAACATGAAAGAC
BvLDOXRTF	GAGAAAGAAGTAGGAGGCATAG
BvLDOXRTR	GTTGTGTAGGATGAAAGTTAAG
AtMYB114F	CTGAGTAAGAAGCATGAACCGTG
AtMYB114R	TTGGGCCGGTGTATTAGGAG
BvDODA1upstrF	CCTATTCCGACTGCTACC
BvDODA1startR	CTCTTTTTTTTTTGTTTGCTTTC
76AD1upstrF	GTTGGACCTCTGAGTGAGTGG
76AD1startR	GATGATCTCACAAATAACACG
BvDFRupstrF	TTTTCATTTAAGCTTTTCCAAG
BvDFRstartR	TTTTGTGGTTATATGATAGATTG

CHAPTER 3: The beet genome contains three similar MYBs, BvMYB1, 2, and 3, with betalain pigment regulatory activities

SUMMARY

Betalains are yellow and red-violet plant pigments that are restricted to several families in the order Caryophyllales, where the betacyanins (red betalains) play biological roles analogous to anthocyanins. Recently we identified novel cytochrome P450 enzymes that function in the betalain network to produce LDOPA and cyclo-DOPA from tyrosine, and a MYB-type transcription factor that activates the genes encoding these enzymes. Other than the identification of BvMYB1, the regulation of betalain pigmentation still remains under-studied. Two other similar beet MYBs, BvMYB2 and BvMYB3, were recently identified through BAC and genomic DNA sequencing. These two MYBs function very similarly and are in close proximity in the beet genome to BvMYB1. Overexpressing any of the 3 MYBs up-regulates the betalain biosynthetic genes and pigments, and silencing any of the 3 BvMYBs down-regulates them. Due to sequence similarities between the three, there is a chance however that silencing one will silence all three. So the effect may just be due to silencing MYB1. In this chapter I have characterized the three MYBs through sequence analysis, functional experiments, and protein-protein interactions. These discoveries will contribute to our ability to understand the regulatory system of the nutritionally valuable betalain pigments.

INTRODUCTION

The MYB family of transcriptional regulators was first identified and named after the avian MYeloBlastosis (MYB) protooncogene (Chen et al., 2007). In plants the *R2R3-MYB* genes comprise the largest MYB gene group and one of the largest transcription factor gene families, playing roles in metabolism, development, and defense responses. Recently, the sequence of most *R2R3-MYB* genes of *Beta vulgaris* (sugar beet) in the order Caryophyllales has been recognized (Stracke et al., 2014). Where a total of 75 *R2R3-MYB* gene sequences, protein structures, and chromosomal locations were identified in beet. By conducting RNA sequencing of different organs of the white sugar beet, organ specific expression patterns of several R2R3-MYBs were determined (Stracke et al., 2014). These results can help us characterize the functionality of many of the R2R3-MYBs by comparing these mRNA and genomic sequences to characterized R2R3-MYBs among other flowering plant species.

Structural redundancy between R2R3-MYBs is common and has been found in many flowering plant species including *Arabidopsis thaliana*. A prominent example of redundancy is the two highly similar MYB factors GLABROUS1 (GL1/AtMYB0), and WEREWOLF (WER/AtMYB66), which form a monophyletic clade with **subgroup 15** of the R2R3-MYB family (Kranz et al., 1998; Stracke et al., 2001). WER/AtMYB66 and GL1/AtMYB0 are functionally equivalent proteins (Lee and Schiefelbein, 2001) that display their different biological functions in root hair and trichome formation, respectively. The only difference is their different spatial expression patterns and cis-regulatory sequences. Other well-known examples of redundant R2R3-MYB function are the RAX1/MYB37, RAX2/MYB38, and RAX3/MYB84 proteins that make up subgroup

14 (SG14) (Müller et al., 2006). *RAX* genes are to some extent redundant in function and allow a fine-tuning of secondary axis formation in *Arabidopsis*. Together the *RAX* genes control a very early step in axillary meristem initiation. Another example of redundancy is between MYB11, MYB12, and MYB111, which are all part of subgroup 7 (SG7) of the R2R3-MYBs. These proteins encode three functionally redundant MYBs regulating the expression of several early acting flavonoid biosynthetic genes (Gou et al., 2011). Furthermore, the proliferation and divergence of MYB genes during plant evolution coincided with the acquisition of new cellular functions and morphological forms (Martin and Paz-Ares, 1997). These works provide new insights into the evolution of redundant MYB gene family members in *Arabidopsis* and other flowering plants, and more generally, they demonstrate novel ways that new developmental gene functions can arise, such as the modification of cis-regulatory sequences. These findings prompt suggestions that MYB gene divergence and variation played an important role in the diversification of plant developmental and metabolic mechanisms.

The WD-bHLH-MYB complex in the anthocyanin pathway provides an excellent model for defining the molecular mechanisms underlying the divergence of gene function during the evolution of multicellular organisms. Work done in various species, most importantly maize and *Arabidopsis*, led to the emergence of the canonical MYB/bHLH/WD-repeat (MBW) combinatorial transcription factor complex model for the regulation of the anthocyanin pathway (Goff et al., 1992; Gonzalez et al., 2008; Zhang et al., 2008). Due to relative conservation between betalain and anthocyanin biological functions, our first hypothesis was that the betalain pathway is regulated by the same MBW complex as are the anthocyanins. From our earlier work however, we show

that changes in several amino acids in a betalain regulator, the BvMYB1 protein, were critical in determining the MYB-bHLH interactions (Hatlestad et al., 2015). These crucial amino acid changes question the presence and contribution of a complete MYB/bHLH/WD complex for the regulation of betalain pathway.

Secondary metabolic pigment biosynthesis, including the betalain pathway, is a great place to study redundancy between R2R3MYB genes. Due to the absence of recent genome duplication events in *B. vulgaris,* there is a relatively low number of redundant genes that function similarly. Only 75 R2R3MYBs were detected in the *Beta vulgaris* unlike the 125 R2R3MYBs detected in the *Arabidopsis thaliana* (Stracke et al., 2001).

It is difficult to study the function of closely related redundant genes and proteins using reverse genetics, due to sequence similarities. In white beets 5% of the R2R3 MYBs were found to be involved in tandem duplication. The three analyzed BvMYBs in this dissertation are very similar in sequence, function and are presumed to be redundant.

I note that the first plant R2R3-*MYB* gene identified was *C1* from *Zea mays* (Paz-Ares et al., 1987). C1 is required for the synthesis of anthocyanins, the plant pigment pathway that functions very similarly to the betalain pigment pathway, in the aleurone of *Z. mays* kernels. Since then, the known range of functions of *MYB* genes in plants has increased widely. *MYB* genes have been shown to regulate other branches of phenylpropanoid metabolism, they influence the cell's shape and differentiation, and some are activated during hormone responses or during plant defense reactions.

Only one beet R2R3-*MYB* that controls betalain pigment has been characterized (Hatlestad et al., 2015), whereas at least 8 *R2R3-MYBs* that positively or negatively control anthocyanin pigment have been well characterized in *A. thaliana* (Borevitz et al.,

2000; Dubos et al., 2010; Gonzalez et al., 2008; Mehrtens et al., 2005; Stracke et al., 2007; Zhu et al., 2009). Recently, I identified two other novel anthocyanin-like beet MYBs in addition to BvMYB1. Phylogenetic analysis clearly indicates that these MYBs are derived from anthocyanin regulatory MYBs. Here I have analyzed the transcriptional regulation of the betalain pathway by BvMYB1, BvMYB2, and BvMYB3 with a series of experiments in table beet, yeast, and *Arabidopsis*. BvMYB2 and BvMYB3 positively regulate the betalain biosynthetic pathway in beet, similar to BvMYB1. Unlike the anthocyanin MYBs that regulate the late biosynthetic genes, neither BvMYB1, BvMYB2, nor BvMYB3 will regulate the anthocyanin genes in *Arabidopsis*, nor will they interact with anthocyanin bHLH proteins.

MATERIALS AND METHODS

Hairy root inoculation

A genomic version of the *BvMYB2* gene from the start codon to stop codon was amplified by PCR from BAC library using the GWBvMYB2F and GWBvMYB2R primers, and a genomic version of *BvMYB3* gene from the start to stop codons was amplified by PCR from sugar beet genomic DNA from the C869 beet variety, using the GWBvMYB3F and GWBvMYB3R primers (Table 3-1). Gateway recombination sequences were also included in primers but are not shown in the table. The products were cloned into pDONR222 (Invitrogen), sequenced and recombined into pB7WG2 (Karimi et al., 2002). This created the *35S::BvMYB2 and 35S::BvMYB3* constructs for overexpression of BvMYB2 and BvMYB3 in plants. pB7WG2 vector containing

the *GUS* gene was used as a control construct. These plasmids were transformed into *Agrobacterium rhizogenes* ARqua1 (Quandt et al., 1993).

Overnight LB cultures of *Agrobacterium rhizogenes* strains were placed into a 1-ml tuberculin syringe fitted with a 30-gauge needle. The needle tip was used to make wounds of 2–3 mm in length in the hypocotyls of beet. During the wounding process, droplets of the *Agrobacterium rhizogenes* culture were injected into the wounds. Induced pigment changes could typically be seen around the wound site within 5 to 10 days. Hairy roots typically emerged from hypocotyl wounds within 2 weeks. Roots were never observed to emerge from inoculated leaf tissues.

RNA was isolated from the transgenic root tissues. To obtain a cDNA version of the genes, RNA was reverse transcribed. cDNA versions of BvMYB2 and BvMYB3 were used in VIGS and Y2H constructs.

Virus Induced Gene silencing (VIGS)

Fragments of BvMYB2 (383 bp) and BvMYB3 (410 bp) cDNA were amplified using the following primers (VIGSBvMYB2F and VIGSBvMYB2R and; VIGSBvMYB2F and VIGSBvMYB3R; Table 3-1). These were cloned into *pDONRZeo* (Invitrogen) and sequenced. These fragments, and control fragments containing the *GUS* gene, a beet laccase-like gene and a beet phytoene synthase gene were recombined into *pTRV2-Gateway* (Liu et al., 2002) and transformed into *Agrobacterium tumefaciens* GV3101 (Koncz and Schell, 1986). This creates VIGS constructs for potential silencing of each of these genes.

For VIGS, seven day old beet seedlings were transformed by inoculation of TRV1 and TRV2 in *Agrobacterium tumefaciens* GV3101. Infiltrated seedlings were maintained

at 100% humidity for 24h and then grown under 24h fluorescent light. Changes in pigmentation could typically be observed 3 to 4 weeks after transformation. VIGS of the three control genes never produced any changes in betalain pigmentation.

Yeast two-hybrid analysis

The BvMYB2 and BvMYB3 cDNA full-length and truncated fragments described above were recombined into the Gal4 DNA binding vector (*pGBT9-RFB*) and activation vector, (*pACTGW*-attR) (Nakayama et al., 2002) to create *BvMYB2BD and BvMYB3BD*, *BvMYB2ΔAcBD and BvMYB3ΔAcBD*, *BvMYB2AD and BvMYB3AD*, and *BvMYB2ΔAcAD and BvMYB3ΔAcAD*.

The *EGL3*, *R* and *PAP2ΔAc* 2-hybrid constructs have been described (Gonzalez et al., 2008). Constructs were transformed into yeast strain Y190 in various combinations. Selection was on –Leu, –Trp medium, and interaction was assayed on –Leu, –Trp, –His medium with 25 mM 3-amino-1, 2, 4-triazole. All hybrid bait proteins were tested for self-activation.

RESULTS

The betalain regulatory complex, identification of BvMYB2 and BvMYB3

Our lab has characterized BvMYB1 in detail as a betalain regulator, as I have described in chapter two (Hatlestad et al., 2015). Recently, I have found two new MYB genes that are very similar to BvMYB1, named BvMYB2 and BvMYB3. When we performed a BLAST search of cDNA sequence on the *Arabidopsis thaliana* database, the most common matches for BvMYB1, BvMYB2, and BvMYB3 were anthocyanin MYBs,

specifically AtMYB113 as the closest relative for all three, as shown in the phylogenetic tree (Fig. 3-1 a and b). The three BvMYBs fit in MYB subgroup 6 when using the resources at IT3F (An Interspecies Transcription Factor Function Finder for Plants). Although it is clear that neither contain the conserved C-terminal motif of the subgroup, [R/K]Px[P/A/R]xx[F/Y] (Stracke et al., 2001; Lin-Wang, 2010), and they all contain an INDV motif in the R3 domain where dicot members of the subgroup contain the (A/S/G)NDV motif (Lin-Wang, 2010).

Just looking at an R2R3 domain alignment (Fig. 3-1c), BvMYB1 and BvMYB2 are more similar to each other than they are to BvMYB3. Out of the 106 amino acids within the R2R3 domain, BvMYB1 and BvMYB2 were different at 7 amino acids, BvMYB2 and BvMYB3 were different in 19 amino acids, and BvMYB1 and BvMYB3 were different in 19 amino acids.

The number of amino acid differences between the genes can help us identify phylogenetic distances between the three BvMYB genes. BvMYB1 and BvMYB2 have much fewer changes in the R2R3 domain to each other than with BvMYB3. This is consistent with the gene organization in the genome, where BvMYB1 and BvMYB2 are very close in distance, and BvMYB3 is farther down on the same chromosome.

Genomic organization of betalain pathway genes

To determine the location of the three BvMYBs and compare them to the organization of the betalain biosynthesis genes in beets, a BLAST search was performed on the published sugar beet genomic sequence database (Dohm et al., 2013) with BvDODA1, BvCYP76AD1, BvMYB1 and the two newly identified BvMYBs, BvMYB2

and BvMYB3. The biosynthetic and regulatory genes all lay on chromosome 2 in very close proximity (Fig. 3-2).

In the previous publications from our lab, segregation analysis of CYP76AD1 polymorphisms in a sugar beet population segregating for red and yellow phenotypes (R vs. r genotype) showed that CYP76AD1 lies at the R locus described more than 70 years ago (Hatlestad et al., 2012; Keller, 1936). Interestingly, in screening a sugar beet BAC library for CYP76AD1 and the highly expressed DODA1, both genes were found on the same BAC, 36 kb apart (Genbank HQ656021).

Remarkably the BvMYBs also lay in close proximity to each other. The discovery of a second and third homologous BvMYB in beet plants was not necessarily surprising. Numerous examples of duplicated MYB genes exist. In *Arabidopsis*, all four genes in the PAP1 anthocyanin regulating MYB family are located on chromosome 1 (Du et al., 2012). In beets we also see that BvMYB1 and BvMYB2 are closely linked (~18 kb apart) and BvMYB3 is also found on the same chromosome (chromosome 2).

BvMYB1 and BvMYB2 were found in the same sugar beet BAC clone, and BvMYB3 was found on the same chromosome by blasting against the sequenced genome (Dohm et al., 2014). The distance between the regulatory MYB genes is: 18,757 bp between BvMYB1 and BvMYB2, and 3,721,384 bp between BvMYB1 and BvMYB3, and 3,740,401 bp between BvMYB2 and BvMYB3. The close proximity of BvMYB1 and BvMYB2 probably indicates a more recent duplication event and explains why there is so much conservation in the R2R3 domains of these two.

Furthermore by examining the sequenced genome we observe that BvDODA1 and CYP76AD1 also lie on chromosome two between the three regulatory MYB genes. BvMYB3 is closer to the two biosynthetic genes than BvMYB1.

BvMYB2 and BvMYB3 genomic and cDNA comparison

BvMYB2 was discovered through sequencing a sugar beet BAC clone that contained BvMYB1, while BvMYB3 was identified in our nextgen genome sequence data of the red table beet W357 variety. Due to low and undetectable gene expression of BvMYB2 and BvMYB3 respectively in wild type beet seedlings, we overexpressed genomic clones of each to obtain cDNA versions of these genes. cDNA versions of both of these genes were obtained from hairy root tissue produced from overexpression of genomic versions of BvMYB2 and BvMYB3 using 35S::BvMYB2 and 35S::BvMYB3 clones in *Agrobacterium rhizogenes* transformation. RNA was extracted from these tissues and the BvMYB2 and BvMYB3 cDNA was synthesized using gene specific primers and reverse transcriptase.

BvMYB2 and BvMYB3 have open reading frames (ORF) of 678 bp and 714 bp respectively and BvMYB1 is 678 bp. The nucleotide identity from start to stop of BvMYB2 and BvMYB3 compared to BvMYB1 are 91.7 % and 72.6%. The amino acid identity between BvMYB2 and BvMYB1 is 87.5% while the identity between BvMYB3 and BvMYB1 is 58.2%.

Overexpression of BvMYB2 and BvMYB3 in beets upregulates the betalain pathway

In order to characterize how BvMYB2 and BvMYB3 might regulate the betalain pathway, we overexpressed these genes through *Agrobacterium rhizogenes* mediated

transformation in white beet roots. Pigment phenotypes were detected in transformed hairy roots. Overexpression of all the three R2R3MYBs, BvMYB1, BvMYB2 and BvMYB3 in Albina vereduna white beets resulted in red pigmentation of the white plant compared to roots from 35S::GUS in which are used as a control (Fig. 3-3 and Fig. 3-4). qRT-PCR was performed to verify that the BvMYB2 (Fig. 3-3) and BvMYB3 (not shown) were overexpressed in the hairy root cultures in white. Both BvMYB2 and BvMYB3 greatly increase betalain pigmentation when overexpressed and therefore they appear to control the same sets of structural genes (Fig. 3-3 and Fig. 3-4).

Because BvMYB2 and BvMYB3 are expressed at very low levels in young seedlings, we tested whether they might be regulated by abiotic stresses and thus control betalain pigmentation under certain environmental conditions. We were however, unable to detect higher levels of these genes present in wild type after high light stresses or cold treatment. Of course there are many more types of environmental stimuli and/or developmental stages that might result in the upregulation of these two transcriptional regulators.

BvMYB2 and BvMYB3 like BvMYB1 do not function in the anthocyanin pathway

Like BvMYB1, BvMYB2 and BvMYB3 did not interact with anthocyanin bHLHs or our newly discovered BvbHLH1 in yeast two-hybrid studies (figure not shown). Examination of BvMYB2, BvMYB3, and BvMYB1 amino acids showed that they did not match 4 of 8 (BvMYB2) , 5 of 8 (BvMYB3), and 6 of 8 (BvMYB1) from the conserved amino acids previously determined to be important for bHLH interaction (Grotewold, 2000; Zimmermann et al., 2004; Hatlestad et al., 2015; Fig. 3-5). BvMYB1 is more different than BvMYB2 and BvMYB3 from the conserved site, this might

suggest that BvMYB2 and BvMYB3 diverged later from AtPAP1 than BvMYB1, thus losing a lot more of the functions.

Conservative and nonconservative changes

The most vital changes in the BvMYBs compared to AtMYBs occur in 3 sets of amino acids (aa). The first being negative charged aspartic acid (D) in PAP1 to positive-charged lysine (K) in BvMB1 and BvMYB3; the second being positive charge arginine (R) in PAP1 to polar uncharged glutamine (Q) in all three of the BvMYBs, and the third being the novel change from hydrophobic amino acid of leucine (L) in PAP1 to polar uncharged glutamine (Q) in BvMYBs. Changing these three sites in betalain producing BvMYBs might recreate anthocyanin producing AtMYBs.

Unlike BvMYB1, BvMYB2 and BvMYB3 are not upregulated in red beets

To confirm that the reason why BvMYB2 and BvMYB3 were not detected in hypocotyl transcriptome data of red beet was due to expression levels, qRT-PCR was done. The CT values were very high (they are inversely proportional to gene expression) or were undetectable, therefore we concluded that the RNAseq was correct and BvMYB2 and BvMYB3 mRNAs were absent or at very low levels in the hypocotyls of the wild-type red or white beets under our laboratory conditions. There however remains a possibility that BvMYB2 and BvMYB3 are expressed at different stages of development, like in the seed coat, or under different environmental conditions.

DISCUSSION

New traits are thought to arise during evolution via gene duplication and gene divergence. The divergence of gene function may result either from changes in regulatory sequences and/or by alterations in protein-coding regions (Carroll, 2000; Doebley and Lukens, 1998).

Here I have discovered and characterized two beet MYB transcription factors, BvMYB2 and BvMYB3, that positively regulate betalain pathway genes. These BvMYBs appear to be the result of duplication and alteration in protein-coding regions of a MYB that eventually gave rise to BvMYB1, 2, and 3. All of these MYBs have a functional coding regions and because they have been maintained through evolution, they appear to be important functionally. Phylogenetic analyses indicate that *BvMYBs* and anthocyanin MYBs probably derive from a common ancestor, consistent with the hypothesis that betalain and anthocyanin biosynthetic genes are regulated by the same type of transcription factors. Interestingly, BvMYB2 and BvMYB3 function very similarly to BvMYB1 in overexpression. All three MYB genes are on chromosome 2, where BvMYB1 and BvMYB2 are very closely linked.

Although it appears that the BvMYBs evolved from anthocyanin MYBs, they have evolved new characteristics; most notably, they do not interact with heterologous bHLH members of the MYB-bHLH-WD complex. Changes among several amino acid appears to be the reason for the lack of MYB-bHLH interaction among the betalain MYBs. The BvMYB that looks closest to the anthocyanin MYBs is BvMYB2 with the fewest changes in the amino acids responsible for MYB-bHLH interaction. Pronounced redundancy in the *Beta vulgaris* genome is very likely. BvMYB1, 2 and 3 is just one of

the duplication events detected in protein-coding regions scattered over the genome (Stracke et al., 2014).

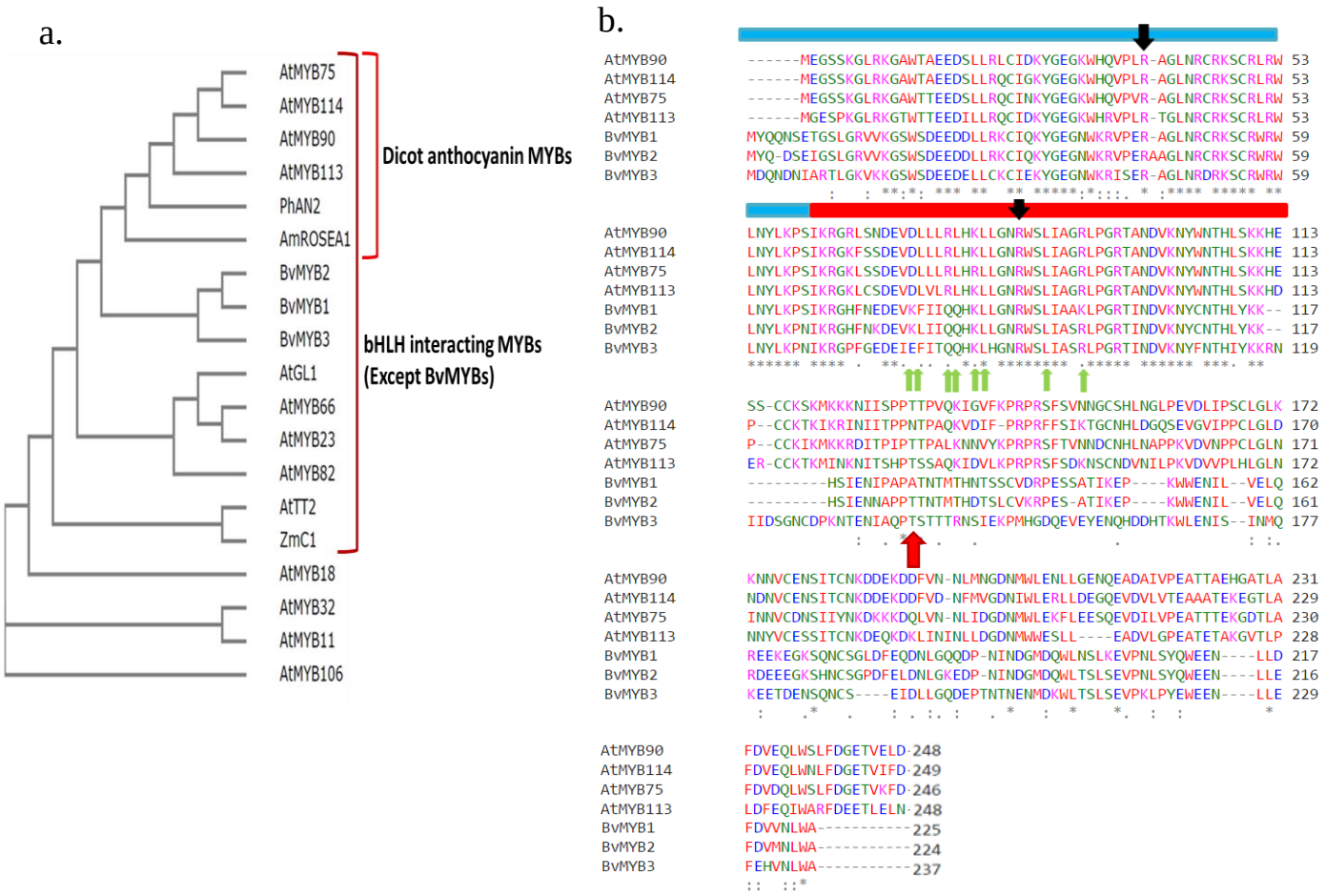


Figure 3-1: Identification of BvMYB2 and BvMYB3

(a) Neighbor-joining tree of several AtMYBs and the BvMYBs that regulate anthocyanin and betalains. The table with the genbank numbers of these genes can be found in chapter 2 (b) Alignment of BvMYBs with four anthocyanin regulatory MYBs. AtMYB75 (PAP1), AtMYB114 you need to put the rest of 114 in the alignment, AtMYB90 (PAP2), and AtMYB113 from *Arabidopsis thaliana.* Blue and red bars designate the R2 and R3 MYB domains, respectively. Black arrowheads indicate the position of introns in all six genes. A red arrowhead indicates the last amino acid in the truncated BvMYB2Δac and BvMYB3 Δac. The green arrowheads indicate the amino acids involved in bHLH interaction discussed in the text. The alignment illustrates the high degree of identity throughout the MYB domains but lack of homology throughout the activation domain approximately after amino acid positions 110 to 115. The alignment was created with ClustalW2.

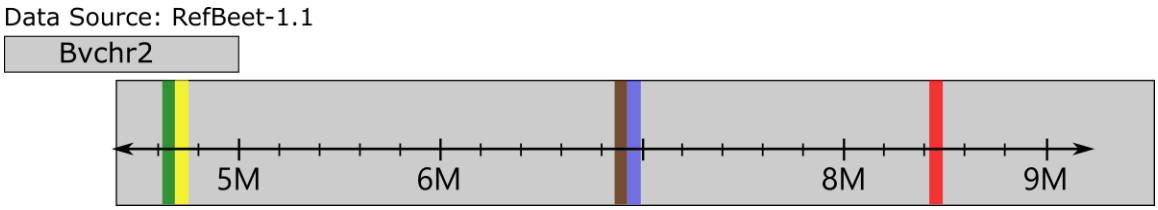
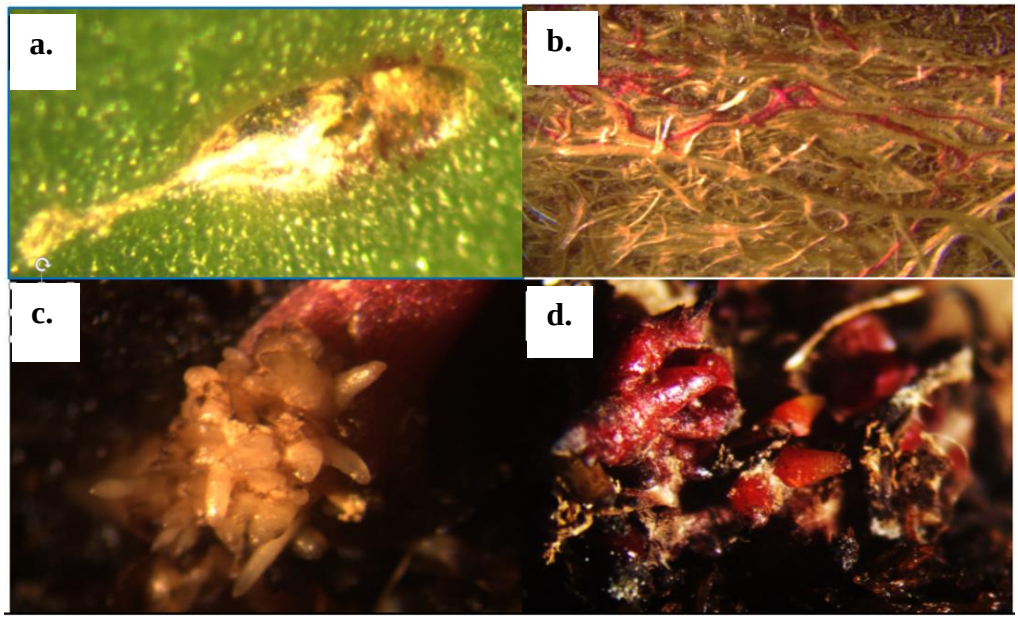


Figure 3-2: Location of betalain biosynthetic genes and the BvMYBs on chromosome 2 of *Beta vulgaris*

The vertical band from left to right who are also color coded indicate:
BvMYB2 (green), BvMYB1 (yellow), BvDODA1 (purple), BvCYP76AD1 (blue), and BvMYB3 (bright red). All these 5 genes are located in close proximity on chromosome 2.



e.

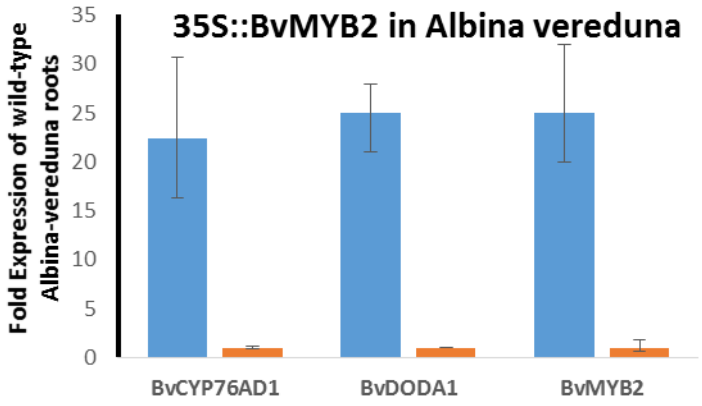


Figure 3-3: Complementing white beets by overexpressing BvMYB2

All this needs more explaining. (a**)** Overexpression of BvMYB2 in leaves, (b) overexpression of BvMYB2 in roots, (c) overexpression of 35S::GUS, (d) overexpression of 35S::BvMYB2 in stem using A*grobacterium rhizogenes*, (e) Quantitative RT-PCR showing relative *BvMYB2*, *BvDODA1* and *BvCYP76AD1* expression in *35S::BvMYB2* roots versus *35S::GUS* controls. Bars are average of five technical replicates. Error bars are s.d. Every experiment was replicated three times.

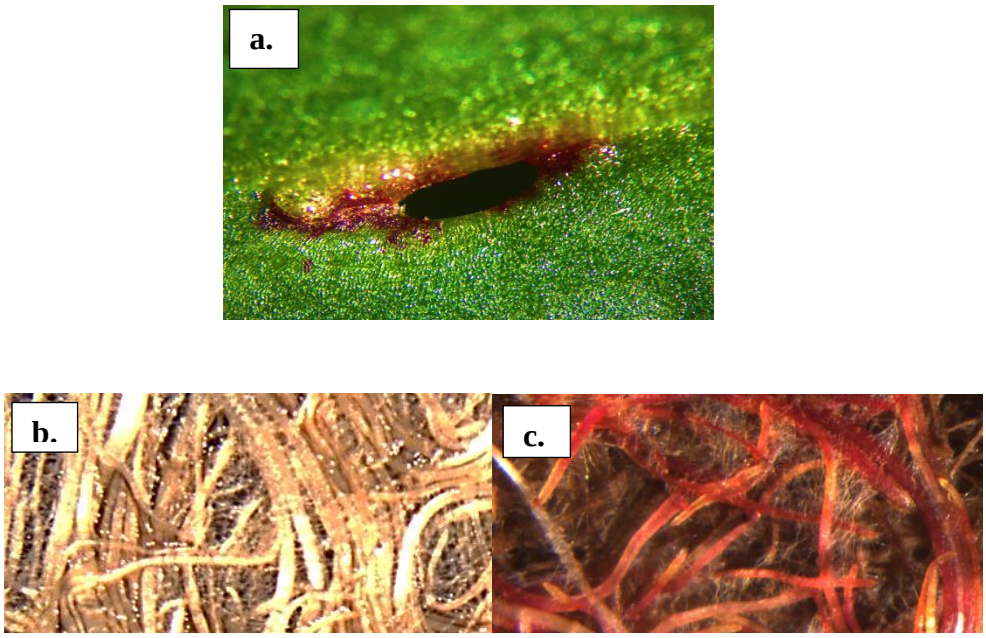


Figure 3-4: Overexpression of BvMYB3 leads to betalain pigment biosynthesis in Albina vereduna, white beets

(a) Overexpression of BvMYB3 in leaves, (b) overexpression of 35S:: GUS, (c) overexpression of 35S:: BvMYB3 in roots using A*grobacterium rhizogenes*.

Figure 3-5: BvMYB1, 2, and 3 have altered amino acids at conserved bHLH binding positions

Conserved amino acids important for bHLH binding are highlighted in red in AtPAP1.

Table 3-1: Primers used as indicated in the text and methods of chapter 3

Primer Name	Primer sequence 5' to 3'
RTPCRBvACTF	CTATCCTTGCATCTCTCAG
RTPCRBvACTR	ATCATACTCGCCCTTGGAGA
VIGSBvMYB2F	ATGTACCAGGATAGTGAAAT
VIGSBvMYB2R	AGTTGGTGGGGCATTGTTTTC
VIGSBvMYB3F	ATGGACCAAAATGATAATATTG
VIGSBvMYB3R	TGTTGGCTGTGCAATGTTTTC
RTPCRMYB2F	ATGTACCAGGATAGTGAAATTG
RTPCRMYB2R	GACTTACTTCGCAAGTGCATTC
RTPCRMYB3F	ATGGACCAAAATGATAATATTG
RTPCRMYB3R	GAACTCCTTTGCAAGTGCATTG
RTPCRDODA1F	CATTGGTTCAGGAAGTGCAA
RTPCRDODA1R	ACGAAGCCATGAATCAAAGG
RTPCR76AD1F	CTTTTCAGTGGAATTAGCCCACC
RTPCR76AD1R	CCCAATATCTTCCATAATGTTCCA
GWMYB2F	ATGTACCAGGATAGTGAAAT

CHAPTER 4: Beet contains bHLH and WD-repeat orthologs of the canonical anthocyanin-regulating MYB-bHLH-WD transcription factor complex

SUMMARY

Anthocyanin production is regulated by a complex of transcription factors (TF) that include MYB, bHLH and WD-repeat proteins, however betalain production appears to be regulated by only MYB TF members and does not require the whole complex. Here we demonstrate that overexpression of BvbHLH1 and BvTTG1 results in what appears proanthocyanin. Furthermore, BvTTG1 completely complements the *ttg1* null mutant in *Arabidopsis* and BvbHLH1 overexpression dominantly suppressed tannin production in *Arabidopsis* seeds. Yeast two-hybrid studies show that BvbHLH1, like AtGL3 and AtEGL3 (*Arabidopsis* anthocyanin regulating bHLHs), interacts with AtTTG1 and the anthocyanin MYB protein, PAP2, but not the beet betalain MYBs. In yeast two-hybrid analysis we also observe that BvTTG1 interacts with the BvbHLH1 and AtbHLH. These results indicate that unlike BvMYB1, the other anthocyanin-like regulatory members, BvTTG1 and BvbHLH1, likely form complexes with each other in beets.

INTRODUCTION

Transcriptional regulation of the anthocyanin pigment pathway is well studied. It is known to be regulated by a complex of transcription factors that include MYB, bHLH and WD-repeat proteins. Despite the wealth of knowledge regarding the MYB/bHLH/WD regulatory model governing the anthocyanin and tannin pathways in many species, and also various cell fate pathways in *Arabidopsis,* little is known about

transcriptional regulatory complex in betalain regulation, or whether betalain producing species contain a MBW complex.

Tyrosine-based betalain pigments in plants provide an excellent model for the study of transcriptional regulation. Similar to anthocyanins, betalain pigments appear to be regulated developmentally. In anthocyanin regulation a combination of transcription factors, MYB, bHLH and WD-repeat family members, can specify the class of flavonoid pigment produced, where it will be produced, production in response to a particular stimulus, and whether transcriptional regulation of structural genes is positive or negative (Gonzalez et al., 2008; Hartmann et al., 2005; Hatlestad et al., 2015; Jouquet et al., 2011; Lepiniec et al., 2006; Solfanelli et al., 2006). Our original hypothesis was that a MYB-bHLH-WD complex regulated the betalain pigment pathway.

Unlike the betalains, anthocyanins have been extensively studied during the past 50 plus years. The maize R protein is the first bHLH transcription factor identified in plants. R and its orthologs and paralogs, regulate the anthocyanin biosynthetic genes in parallel with MYB proteins (Chandler et al., 1989; Goff et al., 1992). Subsequent to the discovery of the bHLH and MYB regulators, a general MYB/bHLH/WD-repeat model for the regulation of the anthocyanin biosynthetic pathway was found to operate in most plant species studied including *Arabidopsis thaliana seen in* Fig. 4-1a (Schwinn, 2006; Zhang et al., 2008; Zhao et al., 2008), morning glory (*Ipomoea nil*) (Morita et al., 2006; Park et al., 2007), snapdragon (*Antirrhinum majus*) (Goodrich et al., 1992; Schwinn, 2006), maize (*Zea mays*) (Chandler et al., 1989; Lloyd et al., 1992), and petunia (*Petunia hybrida*) (Davies, 2009). Betalains and anthocyanins seem to have more or less the same biological regulation and tissue distribution, therefore our first hypothesis was that they

are regulated the same way at the molecular level. Thus we started the search for members of the MYB/bHLH/WD complex in *Beta vulgaris*, a betalain producing species. Our discovery of MYB-class betalain regulators is described in previous chapters. This chapter describes our work towards identification of a BvbHLH and a BvWD, which might work directly with BvMYB1 to regulate the betalain biosynthetic members.

The WD repeat transcription factor proteins are defined by the presence of four or more recurring units containing a conserved core of approximately 40 amino acids that usually end with tryptophan-aspartic acid (WD) (Li and Roberts, 2001). AN11 from *Petunia* was the first WD-repeat protein identified which can regulate genes in the anthocyanin pathway (Vetten et al., 1997). A few years later a gene known as, Transparent Testa Glabra1 (TTG1) of *Arabidopsis* was identified as a WD repeat protein, encoding gene with a role in several developmental and biochemical pathways including production of seed mucilage and anthocyanin pigments (Walker et al., 1999). *ttg1* mutants in *Arabidopsis* not only completely lack anthocyanins in the epi- and subepidermal layers of leaves and stems, but TTG1 also pleiotropically controls several epidermal developmental pathways including: outer seed coat cell differentiation into the mucilage producing cells; leaf and stem trichome cell-fate initiation; and root hair-nonhair cell-fate. Finding a TTG1-like complex member that functions in betalain pathway regulation like TTG1 does in the anthocyanin pathway would argue for our initial hypothesis.

A network of putative transcriptional regulators, which include the paralogous bHLH proteins GLABRA3 (GL3) and ENHANCER OF GLABRA3 (EGL3), is known to influence the differentiation of many cell types. bHLH proteins in general are a

superfamily of transcription factors that are characterized as containing the basic-Helix-Loop-Helix domain. This domain contains two functionally distinct regions. At the N-terminal end is the basic region, which is involved in DNA binding and consists of approximately 15 amino acids. At the C-terminal end is the hydrophobic HLH region, which functions as a dimerization domain (Ferré-D'Amaré et al., 1994; Murre et al., 1989). Many bHLH proteins form complexes such as homodimers, heterodimers to closely related members of the family, or heterodimers with one or several different partners (Toledo-Ortiz et al., 2003). Usually bHLHs bind as dimers to specific DNA target sites to properly regulate anthocyanin biosynthesis (Gonzalez et al., 2008), trichome (plant hair cell) initiation and patterning (Payne et al., 2000; Zhang et al., 2003), stomata formation in hypocotyls (Berger et al., 1998), and seed coat mucilage production (Zhang et al., 2003). For all these transcription factor combinations, the bHLH proteins require the physical interactions of MYBs and the WD protein (Baudry, 2004; Payne et al., 2000; Zhang et al., 2003).

Our original general hypothesis was that the betalain pathway would be regulated at the molecular level in the same way as the anthocyanin pathway—that anthocyanin regulators had been co-opted to regulate betalains. In order to test the hypothesis, we set about to identify orthologous members of the canonical MYB/bHLH/WD transcription factor complex that regulates anthocyanins. Our identification and characterization of co-opted anthocyanin-like MYB regulators that now regulate betalain pathway genes is described in previous chapters. Here I describe my identification and characterization of anthocyanin-like bHLH (BvbHLH1) and WD (BvTTG1) orthologs from beet.

MATERIALS AND METHODS

Finding BvTTG1 and BvbHLH1 in the database (s)

BLAST searches of a red beet RNA-sequencing (RNA-seq) cDNA database (Hatlestad et al., 2012) with anthocyanin TTG1 identified a beet *WD-40* gene. To obtain a full-length clone, we identified and sequenced a sugar beet BAC containing this gene (McGrath et al., 2007). Based on the BAC sequence, a *BvTTG1* cDNA was amplified and cloned from red table beet.

AtbHLHs, EGL3 and GL3, did not provide good BLAST results (low e-value) after our search in red beet RNA-seq data. To obtain a clone of BvbHLH1 we therefore searched the published genomic sequences to find a candidate bHLH (Dohm et al., 2014). To obtain the full-length genomic clone of BvbHLH1, we identified and sequenced a sugar beet BAC containing this gene. Based on the BAC sequence, *BvbHLH1* cDNAs encoding identical proteins were amplified and cloned from white sugar beets.

Phylogenetic analysis

The bHLH domain of BvbHLH1 was compared to bHLH domains of the 147 members of the AtbHLH protein family (Toledo-Ortiz et al., 2003). The dicot anthocyanin bHLHs that were the top BLAST search hits for BvbHLH1 were aligned using clustalW and neighbor-joining tree analysis was performed using MEGA5 software (Tamura, 2011).

In the TTG1 phylogenetic analysis the full-length (start to stop) protein-coding sequence of BvTTG1 was compared to the top ten BLAST search hits from *Arabidopsis* thaliana database. ClustalW was used for alignment and neighbor-joining tree analysis

was performed very similar to pervious analysis of bHLH using MEGA5 software (Tamura, 2011).

Quantitative RT-PCR gene expression analysis

Tissues were harvested from beet hypocotyls (if the tissue type was not mentioned), induced hairy roots, or leaf sections of VIGS-treated plants, as appropriate. Total RNA was extracted using RNeasy Kits (Qiagen). Quantitative RT-PCR was performed as described (Gonzalez et al., 2008; Hatlestad et al., 2012, 2015). Five reactions per target were performed using an actin control with 400 nM of the appropriate primers from the following list: RTPCRBvACTF, RTPCRBvACTR, RTPCRBvbHLH1F and RTPCRBvbHLH1R; RTPCRBvTTG1F and RTPCRBvTTG1R; (Table 1). Results were analyzed using the comparative cycle threshold method (User Bulletin 2, ABI PRISM Sequence Detection System). Expression experiments were performed three times for each gene with consistent results. The data shown is a representative of one experiment from the 3 biological repetitions. Data analyses for three biological replicates are shown in figures. For hairy root samples, each independent biological replicate was composed of hairy roots from a single unique individual beet seedling.

Hairy root inoculation

The *BvbHLH1* genomic and cDNA fragments from the start to stop codons were amplified from Sugar beet BAC library clones (McGrath et al., 2007) with primers BvbHLH1start and BvbHLH1stop (Table 4-1). Gateway recombination sequences were included on primers but are not shown. The products were recombined into pDONR222 (Invitrogen), sequenced and recombined into pB7WG2 plasmid (Karimi et al., 2002). This created p35S::BvbHLH1cDNA and *p35S::BvbHLH1genomic* for

overexpression of full-length cDNA and genomic versions. The *35S::BvMYB1* and *35S::GUS* constructs have been described (Hatlestad et al., 2015; Chapter 2). pB7WG2 containing the *GUS* gene was used as a control.

We used a 1 ml syringe with 30-gauge needle to puncture 2 to 3 mm longitudinal wounds in beet and inject droplets of the *Agrobacterium rhizogenes* cultures (Quandt et al., 1993). Hairy roots emerged from hypocotyl wounds usually within 2 weeks.

Arabidopsis thaliana L*er*, Col, and *ttg1* mutant plants were transformed with *Agrobacterium tumefaciens* GV3101 cultures of 35S::BvbHLH1 and 35S::BvTTG1 using the floral dip method (Clough and Bent, 1998). Primary transformants were selected on MS plates with 50 mg/L kanamycin or after basta spraying treatments done in soil (0.6 µg/µL).

Virus-induced gene silencing (VIGS)

A PCR-amplified fragment of *BvbHLH1* cDNA (primers VIGSBvbHLHF and VIGSBvbHLHR; Table 1) was cloned into *pDONRSpec* (Invitrogen) and sequenced. This fragment, and control fragments containing the *GUS* gene and a beet phytoene synthase gene were recombined into *pTRV2-Gateway* (Liu et al., 2002) and transformed into *Agrobacterium tumefaciens* (Koncz and Schell, 1986).

Seven-day-old beet seedlings were transformed by immersion-vacuum infiltration in the *A. tumefaciens* culture for 1 to 2 min as described (Liu et al., 2002). Infiltrated seedlings were maintained at 100% humidity for 24 h and then grown under 24 h fluorescent light. If the VIGS resulted in phenotypic changes, these could typically be observed 3 to 4 weeks after infiltration. VIGS of the three control genes never produced any changes in betalain pigmentation.

Yeast two-hybrid analysis

The full cDNA version of BvbHLH1 and BvTTG1 were also cloned into donor vector and then recombined into the Gal4 DNA binding vector (*pGBT9-RFB*) and activation vector, (*pACTGW*-attR) (Nakayama et al., 2002) to create *BvbHLHBD*, *BvbHLHAD, BvTTG1BD,* and *BvTTG1AD.*

Constructs were transformed into yeast strain Y190 in various combinations. Selection was on –Leu, –Trp medium, and interaction was assayed on –Leu, –Trp, –His medium with 25 mM 3-amino-1, 2, 4-triazole. All hybrid bait proteins were tested for self-activation.

The *EGL3, R, PAP2ΔAc,* and BvMYB1 2-hybrid constructs have been described (Gonzalez et al., 2008; Hatlestad et al., 2015). Constructs were transformed into yeast strain Y190 in various combinations. Selection was on –Leu, –Trp medium, and interaction was assayed on –Leu, –Trp, –His medium with 25 mM 3-amino-1, 2, 4-triazole. All hybrid bait proteins were tested for self-activation.

RESULTS

A novel bHLH gene from beet, BvbHLH1, is identified and categorized in the same subfamily as AtEGL3, an anthocyanin-regulating bHLH

BvbHLH1 was identified in BLAST searches of genomic *Beta vulgaris* sequence using EGL3 cDNA sequence as a query. Alignments and phylogenetic analysis of BvbHLH1 and *Arabidopsis thaliana* bHLH proteins indicates that BvbHLH1 is most related to the anthocyanin bHLHs, specifically AtEGL3 and AtTT8, and to other bHLH plant pigment regulators (Fig. 4-1b).

To score the conserved amino acids of BvbHLH1 compared to AtbHLHs, we conducted multiple protein sequence alignments using ClustalW (Fig 4-1c and 4-2). Previously 19 amino acid elements were predicted to be the essential conserved motifs within the bHLH family of genes which are also conserved in the anthocyanin bHLHs. The prediction of this site was based on relative variability at sites from basic and helix components. 18 of these amino acids were from the basic and helix regions and 1 from the loop (Atchley et al., 1999). This site was also essential as the bHLH motif for anthocyanin bHLHs (Atchley et al., 1999; Toledo-Ortiz et al., 2003). 9 from the 19 amino acids were identical between BvbHLH1 and this bHLH motif site. These changes can indicate a significant divergence of the consensus motif between the *Arabidopsis thaliana* anthocyanin regulating bHLH's and their related *Beta vulgaris* bHLH's (Fig. 4-1b). Furthermore, BvbHLH1 is missing all the important residues required for the DNA binding motif in *Arabidopsis thaliana,* and the changes consist of position 9, 13, 16, and 17. These four amino acids are sites that recognize E-box and G-box DNA-protein binding sites. In EGL3 and tt8, positions 9, 13, 16, and 17 consist of His, Glu, Arg, Arg vs. in BvbHLH1 which is Ala, Leu, Trp, Asp consecutively (Fig. 4-1b).

BvbHLH1 encodes a protein with a predicted molecular mass of ~62 kD and a calculated pI value of 4.53 (Compute pI/MW tool at http://www.expasy.ch/tools/pi_tool.html). BvbHLH1 encodes a putative protein of 548 amino acids, while AtEGL3 is 596 and AtTT8 is 518 amino acids. The length difference is mainly in the central region of the coding sequence. If you compare the bHLH motif, BvbHLH1 is similar to AtEGL3 at 76% of the amino acids, and similar to AtTT8 at 69% of the amino acids in the bHLH motif.

A novel beet WD-repeat gene, BvTTG1, is identified and categorized in the same subfamily as AtTTG1, an anthocyanin regulating WD-40 protein

BvTTG1 was identified in through a BLAST search of our red beet RNAseq database using *AtTTG1* as a query. Clustal alignments and phylogenetic analysis of BvTTG1 to *Arabidopsis thaliana* WD-repeat proteins, indicates that it is closely related to the anthocyanin WD-40s AtTTG1 (Transparent Testa Glabra1), AtLWD1 (Light-Regulated WD), and AtAN11 (Anthocyanin 11) (Fig. 4-3a).

BvTTG1 encodes a putative protein of 350 amino acids, 9 amino acid longer than AtTTG1 according to Fig 4-3a. The BvTTG1 protein is approximately 72.6% identical to AtTTG1. An alignment of BvTTG1 with *Arabidopsis thaliana* anthocyanin WD-40 proteins are presented in (Fig. 4-3b). The ribbon model shown in Fig. 4-3D illustrates how similar the predicted tertiary structures of AtTTG1 and BvTTG1 are.

Functional analysis of BvbHLH1 and BvTTG1 in *Beta vulgaris* and *Arabidopsis thaliana* for possible roles in betalain and anthocyanin regulation

To identify the location of BvbHLH1 expression I quantified BvbHLH1 mRNA in different organ types of sugar beet. Compared to young leaves, floral buds expressed significantly more (21-fold) BvbHLH1 than young leaves and other tissues (Fig 4-1 a).

To examine BvbHLH1 and BvTTG1 as potential regulators of the anthocyanin and betalain pathway in *Beta vulgaris* and *Arabidopsis thaliana*, we identified and sequenced sugar beet BAC clones containing these genes. These genes were subsequently identified in the Sugar beet published genome database (Dohm et al., 2014). BvbHLH1 is located on chromosome 1 and BvTTG1 is located on chromosome 3.

The genomic versions of BvbHLH1 and BvTTG1, from start to stop codons, were amplified from the BAC clones and placed under the control of the strong CaMV35S

promoter. *Beta vulgaris* plants were transformed to produce hairy root tissue, which we used to make RNA and subsequently cDNA versions of these genes.

Smaller PCR-amplified fragments of BvbHLH1 and BvTTG1 (cDNA) were cloned in pDONR Zeo, and after confirming the clones, LR reactions were performed to place these fragments into the TRV2 vector, and these were transformed into *Agrobacterium tumefaciens*. Results of the silencing and overexpression of BvbHLH1 and BvTTG1 are discussed below.

In beets, silencing and overexpression of BvbHLH1 did not change the plant phenotype. However in *Arabidopsis thaliana* we see a phenotype change in seed coat color of plants overexpressing BvbHLH1. BvbHLH1 silencing in red and white beet plants resulted in no obvious phenotype (Fig. 4-4b). Take note that we were not able to observe beet seeds under our conditions due to not observing blooming and seeding. BvbHLH1 overexpression in wild-type white beet hairy roots resulted in no pigment production, the same as overexpressing the control GUS gene (Fig 4-4c and 4-4d). Overexpression of BvMYB1 (Fig 4-4e) in white beets resulted in large amounts of betalain production as presented in earlier chapters. We quantified the 35S::BvbHLH1 transgene expression and, (Fig 4-4 f), as expected, BvbHLH1 was highly expressed in transformed tissues compared to wild-type. Overexpression of BvbHLH1 in the L*er* background of *Arabidopsis thaliana* resulted in lighter seed coat color than the wild type (Figure. 4-4g). This could be due to its interference with tannin producing genes in *Arabidopsis*. Tannin production in *Arabidopsis* is controlled by the BvbHLH1 orthologue, AtTT8, and we are testing whether BvbHLH1 can complement the *Arabidopsis tt8* mutant.

Like BvbHLH1 overexpression, BvTTG1 overexpression in wild-type white beet plants resulted in no change in betalain pigment production (figure not shown). We overexpressed BvTTG1 in the *Arabidopsis ttg1* mutant and found that it fully complemented (Gonzalez and Akhavan unpublished data), bringing back seed coat color, anthocyanin pigmentation, trichomes and seed coat mucilage production, which are all deficient in the *ttg1* mutant.

From the above data we conclude that the BvbHLH1 and BvTTG1 may be responsible for tannin or proanthocyanin production in beets. The tannin pathway is closely related to the anthocyanin pathway in that they have many intermediate compounds in common until the very last biosynthetic steps and they are both regulated by versions of the combinatorial MYB/bHLH/WD complex although the specific MYB and bHLH members of the complex are not the same. Betalain accumulating plants do make tannins and beets restrict tannins to their seed coat (Bittrich and Amaral, 1991), just like *Arabidopsis* (Debeaujon et al., 2003). Previously we showed that the Betalain pigment pathway appears to be regulated by only a MYB protein and does not require the whole complex. In betalain producing plants it could be possible that a MYB/bHLH/WD complex regulates the tannin pathway in the seed coat.

BvbHLH1 interacts with anthocyanin and betalain TTG1 proteins and anthocyanin MYB proteins

We measured protein-protein interactions through yeast 2-hybrid assays using the galactosidase reporter activity (Fig. 4-6). We tested suspected protein interactions with known *Arabidopsis* anthocyanin regulatory complex members. These partners include bHLH, MYB, and WD repeat proteins. We previously reported that there were no interactions between BvMYB1 and other complex member proteins including the

bHLHs, AtEGL3 and AtGL3, and the WD-repeat proteins, AtTTG1 and BvTTG1, using the yeast two-hybrid system.

In this study we show that the novel BvbHLH1 does interact with many complex members including the anthocyanin regulatory AtMYB90 (PAP2) and the WD-repeat proteins (AtTTG1 and BvTTG1). However, BvbHLH1 did not interact with other bHLHs (AtEGL3 or AtGL3), or itself. Thus, unlike the anthocyanin bHLHs, BvbHLH1 does not appear to form homo or heterodimers. This could be the lack of the second Leucine (L) responsible for interaction within the HLH domain (Pogenberg et al., 2012).

DISCUSSION

Earlier we showed that a MYB transcription factor, co-opted from the anthocyanin MYB/bHLH/WD (MBW) regulatory complex, regulates DODA and cytochrome P450 betalain biosynthetic genes in beet. In this chapter I have shown the presence of two other beet transcription factors, an AtEGL3-like BvbHLH1 and an AtTTG1-like BvTTG1. Both of these have many similarities to MYB/bHLH/WD anthocyanin-regulating complex members.

My analysis makes us conclude that BvbHLH1 and BvTTG1 do not function in the betalain pathway. Both BvbHLH1 and BvTTG1 do not interact with BvMYB1 in yeast-2-hybrid analysis, therefore they do not appear to be assisting BvMYB1 in the regulation of betalains. Furthermore their overexpression and silencing does not affect betalain production in beets.

In spite of this, BvbHLH1 and BvTTG1 do interact with other anthocyanin-regulating complex members in yeast 2-hybrid. The BvbHLH1 protein interacts with the WD proteins AtTTG1 and BvTTG1, and the *Arabidopsis* MYB AtMYB90 (PAP2).

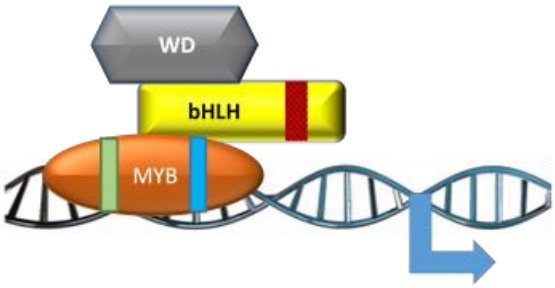
However, BvbHLH1 does not appear to homo- or heterodimerize, possibly because of a lack of leucine amino acid in its first helix domain. Similarly, BvTTG1 does interact with the anthocyanin-regulating bHLHs, AtEGL3 and AtGL3 as well as the beet bHLH, BvbHLH1.

Based on the branch of the phenylpropanoid pathway yielding anthocyanins in plants, we observe that the same combinatorial MYB/bHLH/WD complex controls both anthocyanin and condensed tannin biosynthesis. While beets do not make anthocyanins, they do make condensed tannins (Bittrich and Amaral, 1991). Therefore, although the BvMYB1 appears to regulate betalains on its own, other combinations MYB/bHLH/WD transcription factors including BvbHLH1 and BvTTG1 are likely to regulate other pathways in *Beta vulgaris*, as illustrated in the model in Fig.4-6 (Bittrich and Amaral, 1991). Our work in *Arabidopsis* shows that BvbHLH1 can take a role and intervene with the tannin production in seeds appearing to act as a dominant negative. This may indicate the BvbHLH1 acts very similar to the *Arabidopsis thaliana* MYB/bHLH/WD complex bHLH member, AtTT8, that regulates seed coat pigment (Gonzalez et al., 2009). I note that overexpression of some complex members in *Arabidopsis* leads to hypomorphic phenotypes. For example overexpression of the MYB, GL1, leads to a loss of trichome cell initiation. I would like to use VIGS to silence BvbHLH1 in beet seed coats to test this hypothesis. However, due to need for several months' cold storage in order to induce flowering, I have not been able to complete this experiment.

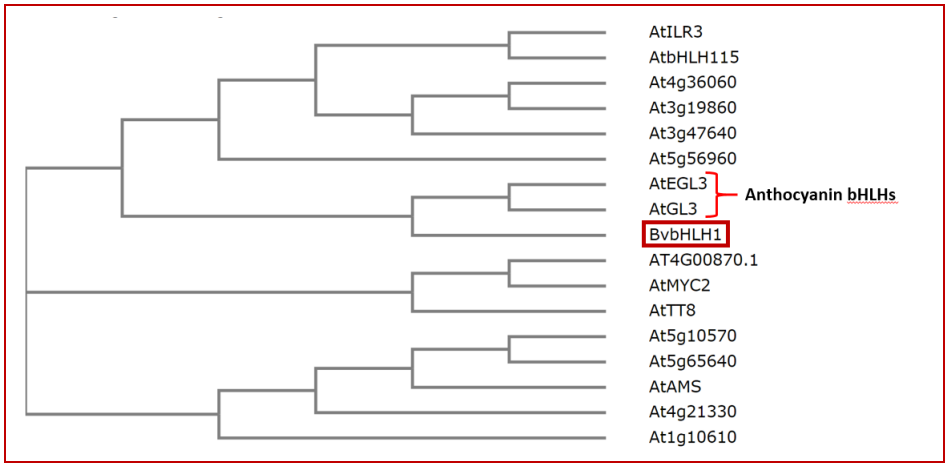
There still remain many gaps in our knowledge about the exact role of these transcription factors in beet. While we have identified a BvTTG1 and a BvbHLH1 that

can regulate tannins when heterologously expressed in *Arabidopsis*, we have not been able to confirm their roles in beets.

a.



b.



c.

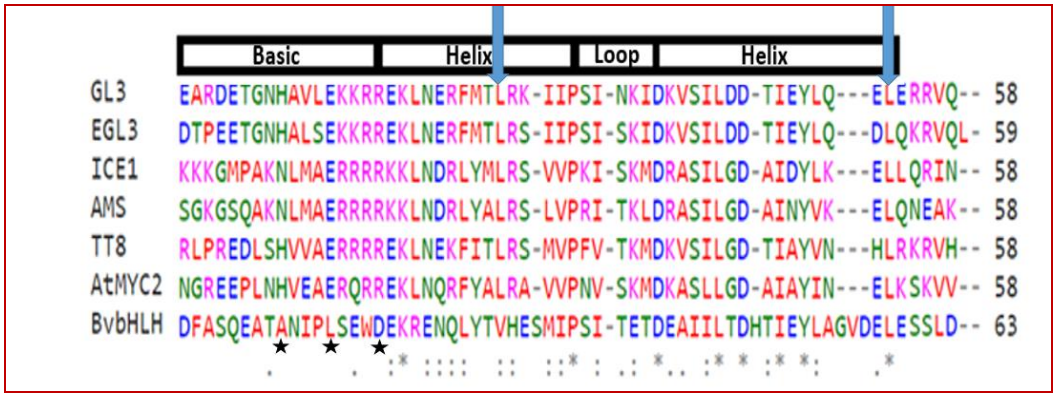


Figure 4-1: Identification of BvbHLH1

(a) Anthocyanin producing plants regulate pigment through a complex of WD-bHLH-MYB proteins (b) Neighbor-joining tree of several different bHLH (c) ClustalW, Alignment of BvbHLH1 with four anthocyanin regulatory bHLHs based on their bHLH domains. Amino acids important for DNA binding is indicated by stars, and the amino acids important for dimerization of the helix-loop-helix domain are indicated by arrows.

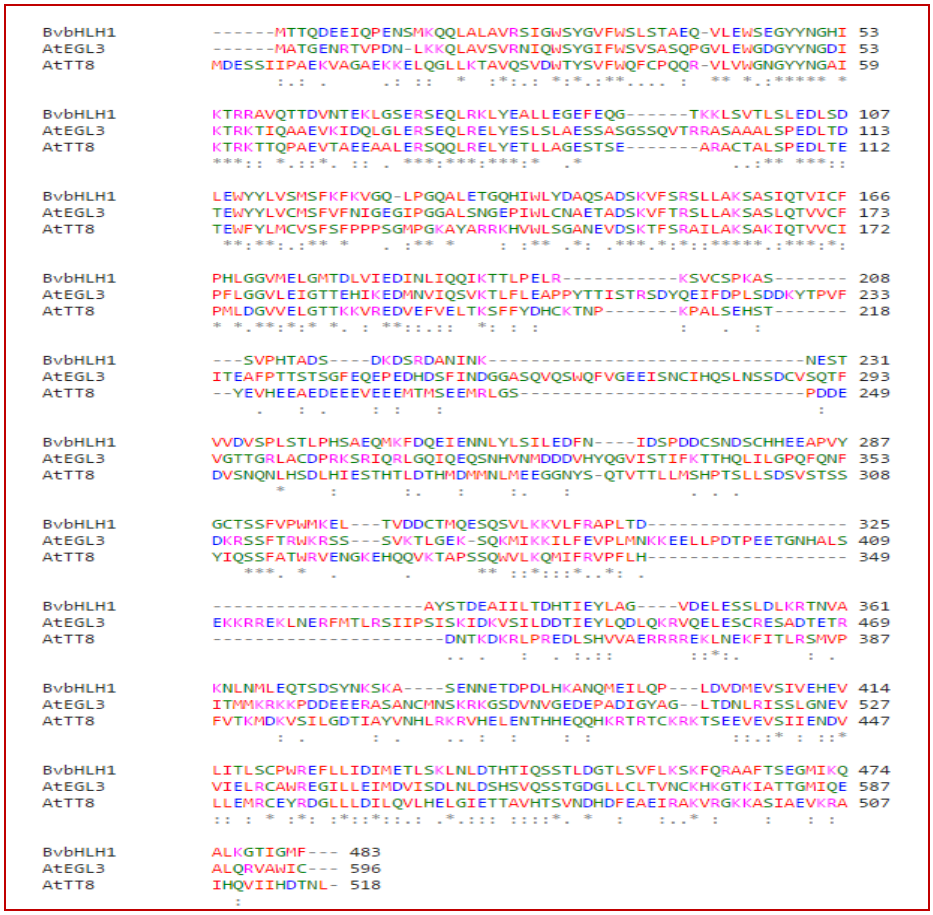
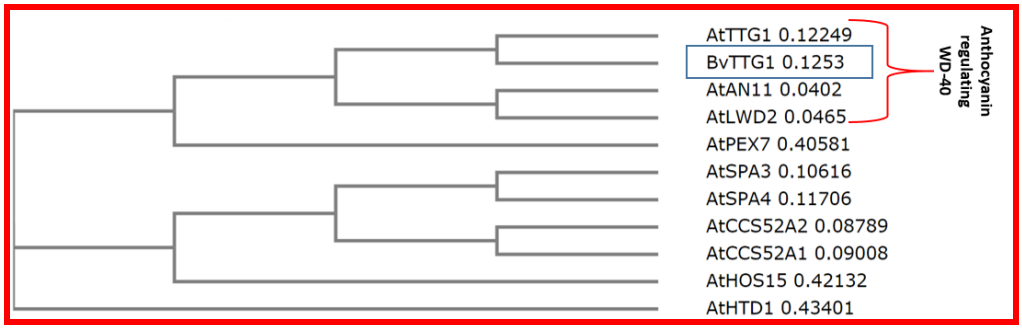


Figure 4-2: Alignment of BvbHLH1, AtTT8, and AtEGL3

a.



b.

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AtAN11          MGTSSDPIQDGSDEQQKRSEIYTYEAPWHIYAMNWSV------RRDKKYRLAITSLLEQY 54
AtLWD2          MVTSSDQIQNGSEEQSKRSEIYTYEAPWQIYAMNWSI------RRDKKYRLAITSLIEQY 54
AtTTG1          MDNSAPDSLSRSE------TAVTYDSPYPLYAMAFSS-----LRSSSGHRIAVGSFLEDY 49
BvTTG1          MENSIEEESHQFSNNNN--NNLVTYDSPYPLYAMSFSLSSAGICRRSSDQIIAVGSFIEEY 58

AtAN11          PNRVEIVQLDESNGEIRSDPNLSFEHPYPPTKTIFIPDK----ECQRPDLLATSSDFLRL 110
AtLWD2          PNRVEIVQLDESNGEIRSDPNLCFEHPYPPTKTSFIPDK----ECQRPDLLATSSDFLRL 110
AtTTG1          NNRIDILSFDSDSMTVKPLPNLSFEHPYPPTKLMFSPPSLRRPSSGD--LLASSGDFLRL 107
BvTTG1          NNRVDLLTFDKETLTLNLTPNLSFSHPYPSTKLMFQPSSSSSSSSSSPPLLASSADYLRL 118

AtAN11          WRIADDHSRVELKSCLNSNKNSEFCGPLTSFDWNEAEPRRIGTSSTDTTCTIWDIEREAV 170
AtLWD2          WRISDDESRVELKSCLSSDKNSEFSGPITSFDWNEAEPRRIGTSSIDTTCTIWDIEREVV 170
AtTTG1          WEINEDSSTVEPISVLNNSKTSEFCAPLTSFDWNDVEPKRLGTCSIDTTCTIWDIEKSVV 167
BvTTG1          YSVKNDS--IELVSLLNNSKSSDFCAPLTSFDWNDVVPQRIGTSSIDTTCTIWDVEKCVV 176

AtAN11          DTQLIAHDKEVFDIAWGGVGVFASVSADGSVRVFDLRDKEHSTIIYESSEPDTPLVRLGW 230
AtLWD2          DTQLIAHDKEVYDIAWGGVGVFASVSEDGSVRVFDLRDKEHSTIIYESGEPSTPLVRLSW 230
AtTTG1          ETQLIAHDKEVHDIAWGEARVFASVSADGSVRIFDLRDKEHSTIIYESPQPDTPLLRLAW 227
BvTTG1          ETQLIAHDKEVYDIAWGEAGVFASVSADGSVRIFDLRDKEHSTIVYESPEPDTSLLRLAW 236

AtAN11          NKQDPRYMATIIMDSAKVVVLDIRFPALPVVELQRHQASVNAIAWAPHSSCHICTAGDDS 290
AtLWD2          NKQDPRYMATVIMGSAKIVVLDIRFPALPVVELQRHQASVNAIAWAPHSSSHICSAGDDS 290
AtTTG1          NKQDLRYMATILMDSNKVVILDIRSPTMPVAELERHQASVNAIAWAPQSCKHICSGGDDT 287
BvTTG1          NKQDLRYMATTLMDSNKVVILDIRSPTMPVAEFERHQACVNAVAWAPQSGRHICSAGDDA 296

AtAN11          QALIWDISSMGQHVEGGLDPILAYTAGAEIEQLQWSSSQPDWVAIAFSTKLQILRV- 346
AtLWD2          QALIWDISSMGQHVEGGLDPILAYTAGAEVEQLQWSSSQPDWVAIAFSNKLQILRV- 346
AtTTG1          QALIWELPTVAG--PNGIDPMSVYSAGSEINQLQWSSSQPDWIGIAFANKMQLLRV- 341
BvTTG1          QALIWELPSMPA--PSGIDPLSVYNATSEINQLQWSAAQPDWIGIAFSNKLQLLRV- 350
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c.

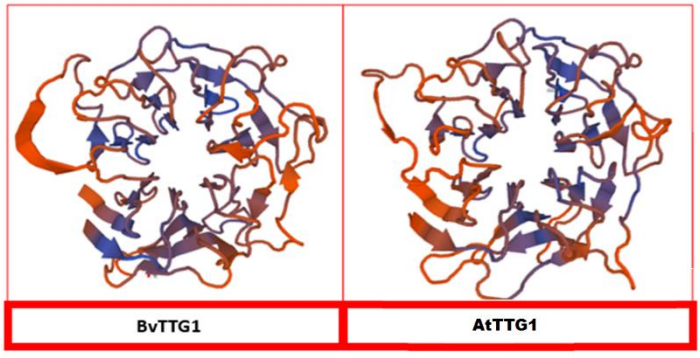


Figure 4-3: ClustalW alignment of BvTTG1 in *Beta vulgaris* genome and 3D structure of BvTTG1

(a) Neighbor-joining tree of WD-40 proteins of *Arabidopsis thaliana* and the selected BvTTG1 protein, phylogeny was made based on the protein sequences (b)Alignment of BvTTG1 from *Beta vulgaris* and AtTTG1 from *Arabidopsis thaliana* (c) Ribbon representations of the TTG1 proteins based on www.swissmodel.expasy.org.

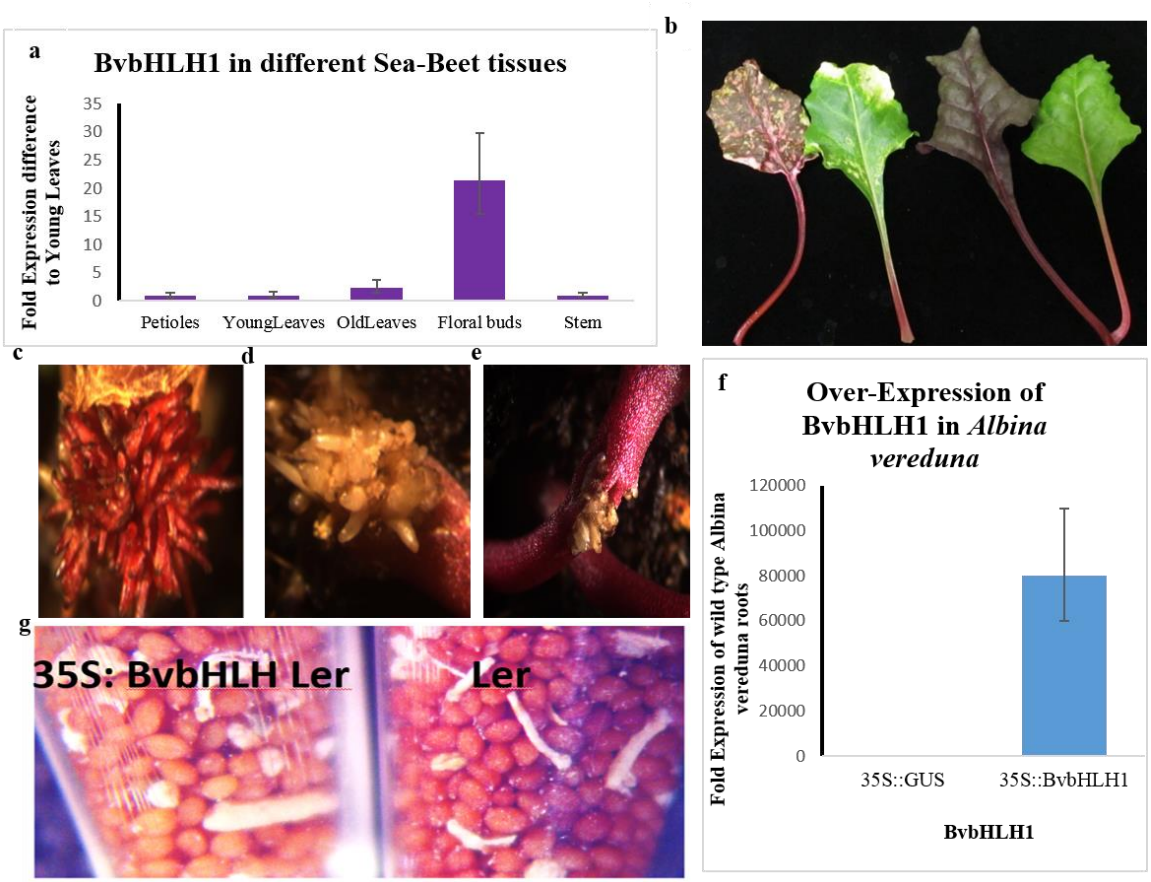


Figure 4-4: Analysis of BvbHLH1 overexpression in *Arabidopsis thaliana*

(a) Expression of BvbHLH1 in different tissue types of Sea Beet (b) Virus-induced gene silencing of *BvbHLH1* (the order of leaves from left to right, silenced BvPSY in Bull's Blood, silenced BvPSY in Albina vereduna, silenced BvbHLH1 in Bull's Blood, silenced BvbHLH1 in Albina vereduna) (c) Overexpression of BvMYB1 (causes red pigment induction) (d) GUS (no pigment induction) (e) and BvbHLH1 (no pigment induction) (f) Quantitative RT-PCR showing relative *BvbHLH1*expression in *35S::BvbHLH1* roots versus *35S::GUS* controls. Bars are average of five technical replicates. (g) Overexpression of BvbHLH1 in Ler (left) and wild-type Ler seedlings (right).

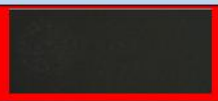
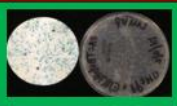
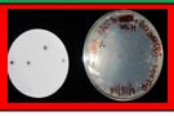
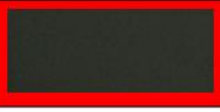
	Interaction	B-gal assay	His	HIS+25mM3AT
pACTBvbHLH1 X EGL3BD	No interaction			
pACTBvbHLH1 X pP2MDB	Positive interaction			
pACTBvbHLH1 X AtTTG1BD	Positive interaction			
pACTBvbHLH1 X BvTTG1BD	Positive interaction			
pACTBvbHLH1 X BvMYB1BD	No interaction			

Figure 4-5: BvbHLH1 interacts with anthocyanin complex members
Yeast two-hybrid analysis of possible MYB-bHLH interaction. The shown figures are BvbHLH1 are in activation domain vector activating transcription in yeast in presence of other complex members. BvbHLH1 acts very similar in binding domain vector.

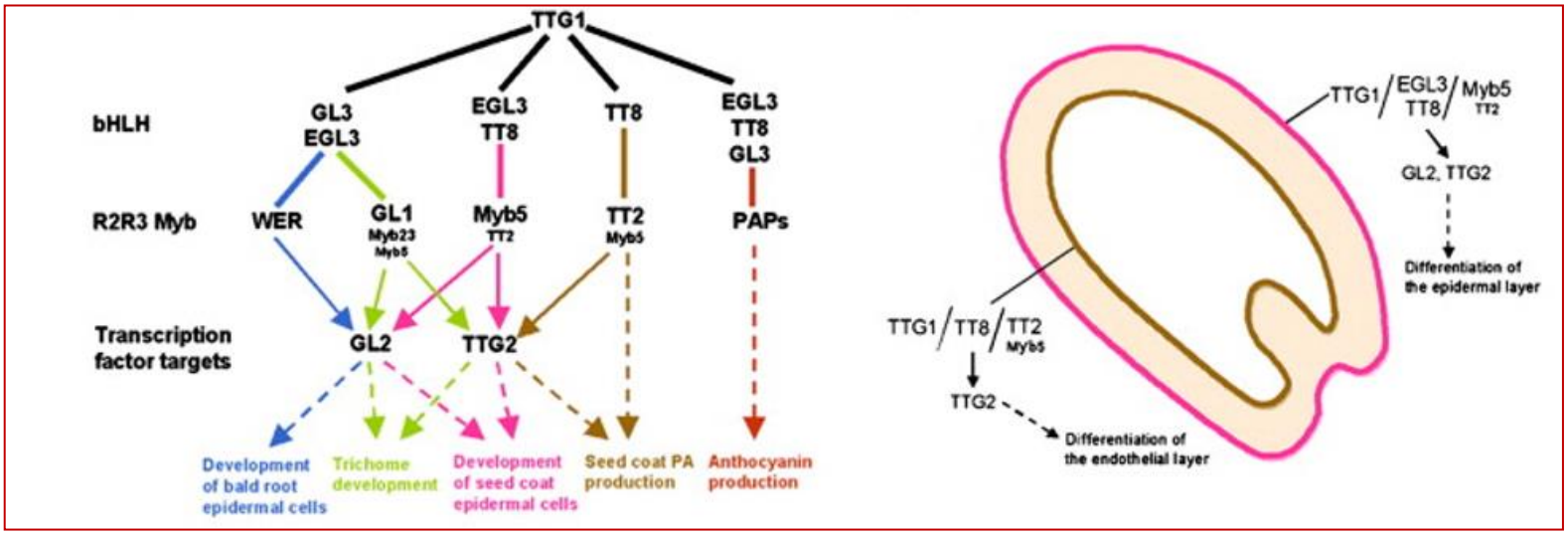


Figure 4-6: Models for the regulation of TTG1-dependent developmental pathways

(A) A regulatory network for the positive control of TTG1-dependent epidermal cell fates. Solid lines indicate interactions between members of a complex. Dashed arrows indicate a multi-step differentiation pathway. Colored lines and arrows indicate specific regulator combinations and the pathway controlled. Text size in the case of the MYBs indicates their relative contributions to cell fate regulation. (B) A regulatory model for the differentiation of the seed coat outer and inner layers specified by specific TTG1-dependent transcriptional complexes. Text size in the case of MYB5 and TT2 MYBs indicates their relative contributions to the development of the outer and inner testa layers (Gonzalez et al., 2009).

Table 4-1: Primers used as indicated in the text and methods of chapter 4

Primer Name	Primer sequence 5' to 3'
RTPCRBvACTF	CTATCCTTGCATCTCTCAG
RTPCRBvACTR	ATCATACTCGCCCTTGGAGA
GWBvbHLHF	ATGACCACCCAAGATGAAG
GWBvbHLHR	TCAAAACATGCCGATAGTTC
VIGSBvbHLHF	CACATTAAGACGAGAAGAGCC
VIGSBvbHLHR	CCAACCTTTGCCAGCAAAGAGC
RTPCRBvbHLH1F	GGGAGGTGTGATGGAGCTAGGG
RTPCRBvbHLH1R	GCTATAAGCATCCGTTAAAGGTG
RTPCRBvTTG1F	CTTTCCTTCCTCCAGAAATCC
RTPCRBvTTG1R	GAGCTTGTTTCTCTTCTCAACAA

CHAPTER 5: BvMYB1 protein directly activates betalain gene expression by binding to a novel BvMYB1 Response Element (MRE)

SUMMARY

Sequence-specific protein-DNA interactions are critical for regulating many cellular processes, including transcription, DNA replication, repair, and rearrangement. We previously characterized an anthocyanin-MYB-like protein, *Beta vulgaris* MYB1 (BvMYB1), which regulates the betalain pathway in beets (Hatlestad et al., 2015). We have identified a novel DNA sequence that is bound by the BvMYB1 transcription factor. Initially, through "promoter bashing" experiments using sequences upstream of BvCYP76AD1 (encoded at the beet R locus; Hatlestad et al.,2012) and testing these sequences with Electrophoretic mobility shift assay (EMSA) we are working towards the identification of a highly active BvMYB1 response element (MRE). Second, we have verified several direct BvMYB1 target genes through yeast 1-hybrid experiments and the *in planta* use of a BvMYB1-glucocorticoid receptor (GR) fusion allele that is inducible with dexamethasone. These targets include BvCYP76AD1, BvDODA1, and BvMRP1. We are now trying to identify the nucleotide sequence of the MRE and how it is different from the anthocyanin MYB cis-regulatory elements. After identifying this element we will analyze it in promoter-reporter experiments. Finally, we have identified two other betalain MYB regulatory transcription factors with high similarity to BvMYB1, BvMYB2 and BvMYB3, which may activate gene expression via the MRE. Through our understanding of interactions between BvMYB1 transcription factors and downstream betalain biosynthetic genes we can better understand how the betalain secondary pigment pathway is regulated.

INTRODUCTION

Protein-DNA interactions are important in many processes including regulation of transcription, translation, DNA replication, repair, recombination, RNA processing, and translocation (Pabo and Sauer, 1984). Overall these interactions allow many cellular processes to occur through different developmental stages. In these complexes the protein functions either as a positive regulator by allowing selective portions of a chromosome to be unraveled so that the DNA is available for transcription or as negative regulator where the protein helps to keep the chromosome tightly packaged so that transcription of the encoded genes is completely silenced (Zhang et al., 2013).

Here we observe Protein-DNA interaction between BvMYB1 and three important betalain biosynthetic genes, BvCYP76AD1, BvDODA1 and BvMRP1. BvMYB1 plays a vital role in regulating betalain pigmentation by directly interacting with critical downstream genes and controlling their transcriptional expression. The BvMYB1 regulates by binding to a cis-regulatory site that appears to be in the enhancer sequence of these three genes. I hope my work here will help us further characterize the activities of the BvMYB1 protein and understand the steps required for betalain regulation in the order of flowering plants known as the Caryophyllales.

MATERIALS AND METHODS

Cloning of BvMYB1 into an *E. coli* expression vector

The BvMYB1 coding region without a stop codon was amplified using primers BvMYB1start and BvMYB1nostop, recombined into pDONR/zeo (Invitrogen) and

sequenced. This was recombined into the *E. coli* expression vector pCOLD (Qing et al., 2004) and placed in BL21 cells (Invitrogen).

Electrophoretic Mobility Shift Assay (EMSA)

BvMYB1-6XHis fusion protein was purified from *Escherichia coli* expression strain BL21 cells (Invitrogen) containing BvMYB1 in the pCOLD protein expression plasmid (Qing et al., 2004). Protein expression was induced in 100-mL expression cultures using 1 mM isopropyl thio-β-d-galactoside (IPTG), and cells were harvested 24 h after induction at 15°C at OD_{600} of 0.5. Cells were sonicated in lysis buffer (20 mM sodium phosphate buffer, pH 7.3, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, and 1 mM phenylmethanesulfonyl fluoride). The supernatant of centrifuged samples were used for purification using a 1-mL Ni-NTA spin column (Sigma-Aldrich; H7787-10Ea, SLBD8198) as directed by the manufacturer. Aliquots of the flow-through fractions were analyzed by SDS-PAGE and Coomassie Brilliant Blue staining.

The DNA probes used in gel-shift experiments were either amplified by PCR or directly annealed by heating the primers to 100°C followed by slow cooling to room temperature. Primers used to amplify DNA fragments used in gel-shift experiments, and mutated fragments are given in Table 1. The binding reaction was performed at room temperature for 20 min in 5X binding buffer (50 mM Tris-HCl (pH 8), 750 mM KCl, 2.5 mM EDTA, 0.5% Triton-X 100, 62.5% glycerol, 1mM DTT; Xie et al., 2009). DNA-protein complexes were separated on 10% native gels (http://www.assay-protocol.com/molecular-biology/electrophoresis/native-page), and ethidium bromide was used to stain the DNA. The DNA bands were visualized and photographed under UV light (Kadonaga and Tjian, 1986).

Chromatin Immunoprecipitation (ChIP) Experiments

ChIP experiments were performed as described previously (Morohashi et al., 2007). Briefly, the tissue used was from Albina vereduna beet hairy roots expressing BvMYB1::GR under control of 35S promoter after induction with dexamethasone (DEX) for 2 days. This construct was created using gateway cloning technology as follows. The full-length BvMYB1 coding region without a stop codon was amplified using primers BvMYB1start and BvMYB1nostop (Table 1), recombined into pDONR/zeo and sequenced. This was recombined into pR1R2ΔGR (Baudry et al., 2004) in frame with the hormone binding domain of the rat glucocorticoid receptor (GR) under the control of CAMV35S promoter to create 35S::BvMYB1-GR.

The tissue was cross-linked using 3% formaldehyde, then ground using mortar and pestle (at -80°C) and sheared on ice using a sonicator. The supernatant was then precleared using salmon sperm DNA/Protein A-agarose beads for 120 min at 4°C. After the preclearing incubation, immunoprecipitations were performed overnight at 4°C with either 1 µg of anti-GR antibody (PA1-516; Affinity BioReagents), or 1 µg of DNA/Protein A-agarose. After incubation, beads were washed two times with LNDET buffer (0.25 m LiCl, 1% NP40, 1% deoxycholate, 1 mm EDTA) and two times with Tris-EDTA buffer. The washed beads and input fraction were resuspended in elution buffer (1% SDS, 0.1 m $NaHCO_3$) with 1 mg/mL proteinase K and incubated overnight at 65°C. Finally, the DNA was purified using phenol:chloroform extraction. Quantitative PCRs were performed using the real-time PCR system (Thermo Fischer Scientific). The primers that were used for qPCR of the ChIP can be found in Table 1.

Yeast one-hybrid analysis

Fragments upstream of the translational start of BvDODA1 (2,218 bp, 500 bp, 200bp); CYP76AD1 (494 bp, 2kb, 1kb, 159 bp, 115bp, 380bp, 200bp) and BvDFR (527 bp; all primers are in Table 5-1) were amplified from Albina vereduna beet cultivar, recombined into pDONRP4-P1R (Kugler et al., 2004), and sequenced. These fragments were recombined into DNAbait::HIS3 reporter vector (pMW2; Deplancke et al., 2004). These vectors were linearized with AflII and transformed into yeast strain YM4271 (Deplancke et al., 2004), where they integrate into the genome creating a bait::His3 reporter that will be activated if the bait is bound by a DNA binding protein with a transcription activation domain, like BvMYB1. These strains were then transformed with BvMYB1 in the activation domain vector (pBvMYB1ΔAcAD) or with the pACT empty vector. Selection for transformants containing both the reporter and the activation domain vector was on –His –Leu medium. Interaction was assayed on –His –Leu with 25 mM 3-amino-1, 2, 4-triazole (3AT).

Overexpression and propagation of beet hairy roots

Seeds of white (Albina vereduna) and red (W357B) beet varieties were sterilized and germinated on 3% MS media on petri plates. They were incubated in the dark for 7 to 10 days to allow for germination. Overnight liquid LB cultures of *Agrobacterium rhizogenes* ARqua1 strains were placed into 1 mL tuberculin syringe. A 30-gauge needle was used to create wounds and inject the culture into the wound. Four days after injection, seedlings were transferred onto a 3% sucrose MS media containing 200 to 400mg/L of Timentin to kill the *Agrobacterium rhizogenes*. Plates were incubated at 25°C under continuous fluorescent light. The “hairy roots” emerged from the wound sites

on the hypocotyl in 2-3 weeks. Roots were excised from the wound site and maintained on 3% sucrose MS media with 200 mg/L Timentin.

RESULTS

The BvMYB1-GR fusion directly activates BvDODA1 in beet roots

The 35S::BvMYB1-GR construct was used to generate beet hairy root cultures. These root cultures were phenotypically and genotypically checked to verify that the BvMYB1-GR did not activate the betalain pathway genes unless the roots were treated with dexamethasone (Fig. 5-1).

In transgenic 35S::BvMYB1::GR roots, we measured the expression of potential MYB-target genes to identify which are activated quickly, after 4 hours DEX treatment (potentially directly regulated), and which take longer to be activated (potentially indirectly regulated). Four hours treatment with DEX activated BvDODA1 but not CYP76AD1, two potential targets (Fig 5-3a). It also did not activate the negative control, BvLDOX.

Treatment with cycloheximide (CHX) inhibits translation. So if a gene is transcriptionally activated by DEX, both without and with CHX, this is considered as evidence for direct activation as opposed to activation after transcription and translation of an intermediate trans-activator (Morohashi et al., 2007)**.** After treatment with DEX and CHX for four hours it appeared BvMYB1 directly regulates BvDODA1 as induction causes upregulation of BvDODA1 both with and without CHX. BvCYP76AD1 and LDOX did not appear to be upregulated within the four-hour time frame of this experiment. This may indicate that the CYP76AD1 potential target is an indirect target,

or that the kinetics of activation are slower/more complex, perhaps requiring other players. It must also be acknowledged that this is a negative experimental result and therefore difficult to interpret.

This experiment was repeated with a 24 hour time frame (Fig 5-3b). In this case BvDODA1 still appears to be a direct target as indicated by activation by DEX and by DEX plus CHX. However, CYP76AD1 now appears to be activated by BvMYB1 even in the presence of CHX, indicating that it is a candidate as a direct target with slower kinetics of activation.

We note that there are real caveats to over-interpretation these types of experiments. For example, treating with DEX plus CHX vs. DEX alone has been reported to both downregulate and upregulate real direct targets (Morohashi et al., 2007).

Yeast 1-hybrid analysis indicates that BvMYB1 binds upstream cis sequences of BvDODA1 and CYP76AD1

Yeast 1-hybrid analysis is used to determine whether a DNA binding protein, like a transcription factor, binds to bait DNA sequences (potential binding sites) in yeast. I used yeast 1-hybrid to examine the possibility that BvMYB1 directly binds to cis sequences upstream of the BvDODA1 and BvCYP76AD1 genes. Before isolating protein in large quantities we conducted mass spectrometry of pcold BvMYB1 vector (Fig. 5-2), to confirm that BvMYB1 protein is produced in large quantities in the cell.

Yeast one-hybrid analysis showed that BvMYB1 was able to activate a reporter fused to 500, 1000, and 2000 bp upstream of the start codon of BvDODA1, and 1000 and 2000 bp upstream of the start codon of CYP76AD1 (Fig. 2-13 and Fig 5-4.). This indicates that BvMYB1 can independently (without a partner from the MBW complex) bind to upstream cis sequences of both genes in yeast. BvDODA1 fragments of 115, 159,

and 380 bp upstream of the start codon did not result in reporter activation. However a 194 bp fragment spanning 175 to 369 bp upstream of the start codon was weakly activated and it is likely to contain a BvMYB1 binding element.

Chromatin Immunoprecipitation (ChIP) analysis indicates that BvMYB1 binds cis sequences of BvDODA1 and CYP76AD1 *in vivo*

Chromatin immunoprecipitation (ChIP) coupled with PCR amplification of sequences at or near a suspected binding element, is used to analyze whether a DNA binding protein is bound at particular locations in the genome. I utilized this method to verify BvMYB1's direct association with specific regulatory regions upstream of the suspected direct targets, BvDODA1 and CYP76AD1, as well as a newly identified potential target, BvMRP1. BvMRP1 is a Multi-drug Resistance Protein-like gene that is highly upregulated by BvMYB1 (Alex Bean and Neda Akhavan, Unpublished result). ChIP experiments were performed on DEX-induced Albina vereduna hairy roots transformed with the 35S::BvMYB1-GR, using antibodies against the glucocorticoid receptor (GR). Immunoprecipitated chromatin was subjected to quantitative polymerase chain reaction (QPCR) using primers upstream of the suspected targets as shown in Figure 5-5. This analysis demonstrated that ChIP of BvMYB1-GR enriches for DNA upstream of each gene indicating that BvDODA1, BvCYP76AD1, and BvMRP1 are all direct targets *in vivo.*

Electrophoretic Mobility Shift Assays (EMSA) identifies DNA fragment bound by BvMYB1

EMSA is used to test for specific DNA binding by DNA binding proteins *in vitro.* Here I performed EMSA with BvMYB1 protein produced in *E. coli* to assay whether BvMYB1 would bind to sequences upstream of CYP76AD1. The goal is to eventually

identify the specific BvMYB1 response element (MRE). Tiled fragments upstream of CYP76AD1 were amplified and the given amplified fragments were confirmed and quantified on agarose gels. BvMYB1 and the potential target binding fragments (probes) were incubated for 20 minutes at Room Temperature and then placed on ice for 30 minutes. The reactions were then loaded and electrophoresed on native polyacrylamide gels (PAGE) to look for mobility shifts indicative of protein-DNA binding. Figure 5-6 a and b shows that BvMYB1 specifically shifts a 194 bp fragment upstream of CYP76AD1 but will not shift a 194 bp fragment from the beet Actin locus. The shifted CYP76AD1 fragment corresponds to the same 194 bp fragment that showed reporter activation in the yeast 1-hybrid assay described above, again indicating that this fragment likely contains a MRE.

Pursuit of the BvMYB1 Response Element (MRE) sequence

Currently I am pursuing three different methods to identify the MRE sequence. The first two are through more refined yeast 1-hybrid and gel-shift. The third method is by selecting for the motif after analyzing what can be cloned from a pull-down using BvMYB1-6XHis to bind fragments in a random-sequence oligonucleotide library. This is the well-documented SELEX system (Blackwell and Weintraub, 1990; Ferré-D'Amaré et al., 1994; Huizenga and Szostak, 1995; Jolma et al., 2013; Oliphant et al., 1989; Wright et al., 1991). It is hoped that identification of the MRE sequence will allow us to manipulate the site by increasing copy number or mutating the site to change the expression of reporter genes or pigment biosynthetic genes. These tests will help us to confirm that the MRE is responsible for BvMYB1 regulation.

DISCUSSION

Sequence-specific protein-DNA interactions are critical for regulating many cellular processes. Understanding interactions between transcription factors and regulatory genomic regions is required to understand cellular responses to different environmental and developmental cues. Here I have used YIH, ChIP, and EMSA in order to identify protein-DNA interactions between our transcription factor BvMYB1 and the betalain biosynthetic genes (BvDODA1, CYP76AD1, and BvMRP1).

I have performed extensive experiments to show that BvMYB1 regulates these downstream genes by direct binding to cis regulatory sequences. However there is still work to be done to identify the MRE sequence.

To fully grasp plant metabolism and development we must first understand the transcriptional networks that control target genes.

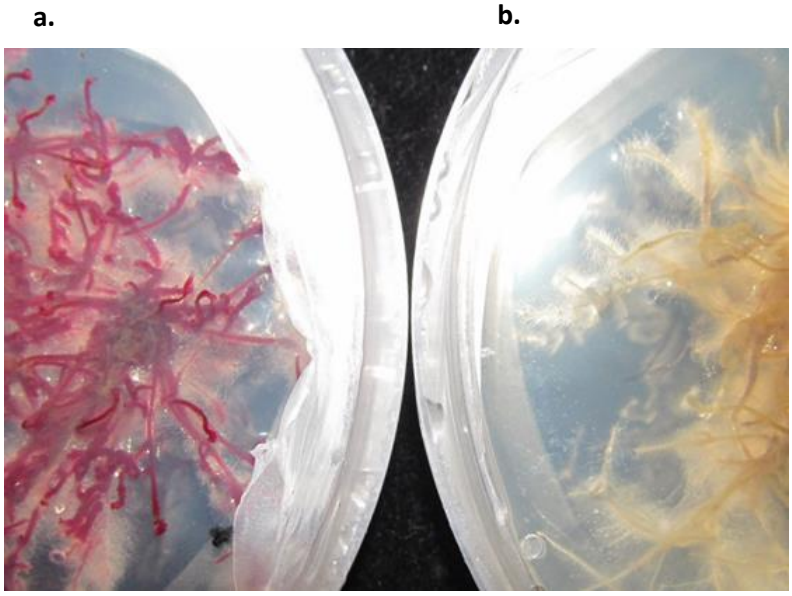


Figure 5-1: Induction of 35S::BvMYB1-GR construct

(a) DEX induced construct (b) No induction of my transformed roots.

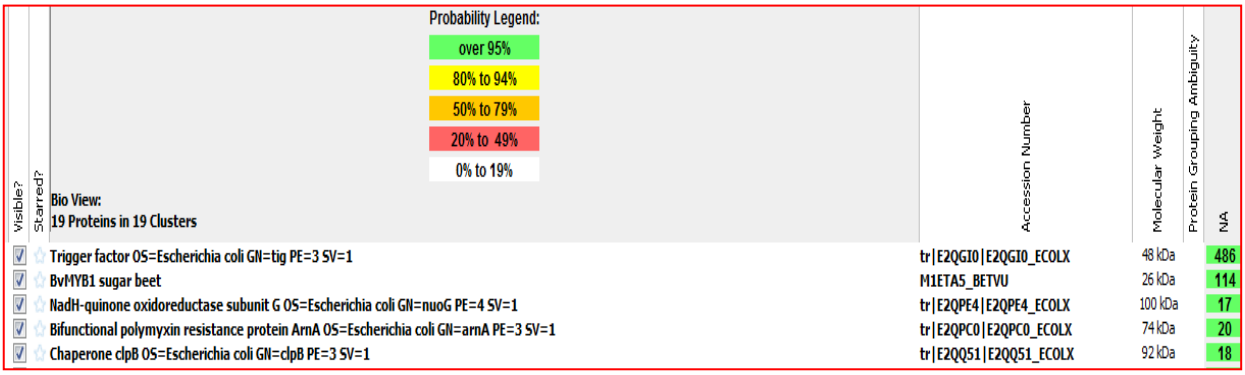
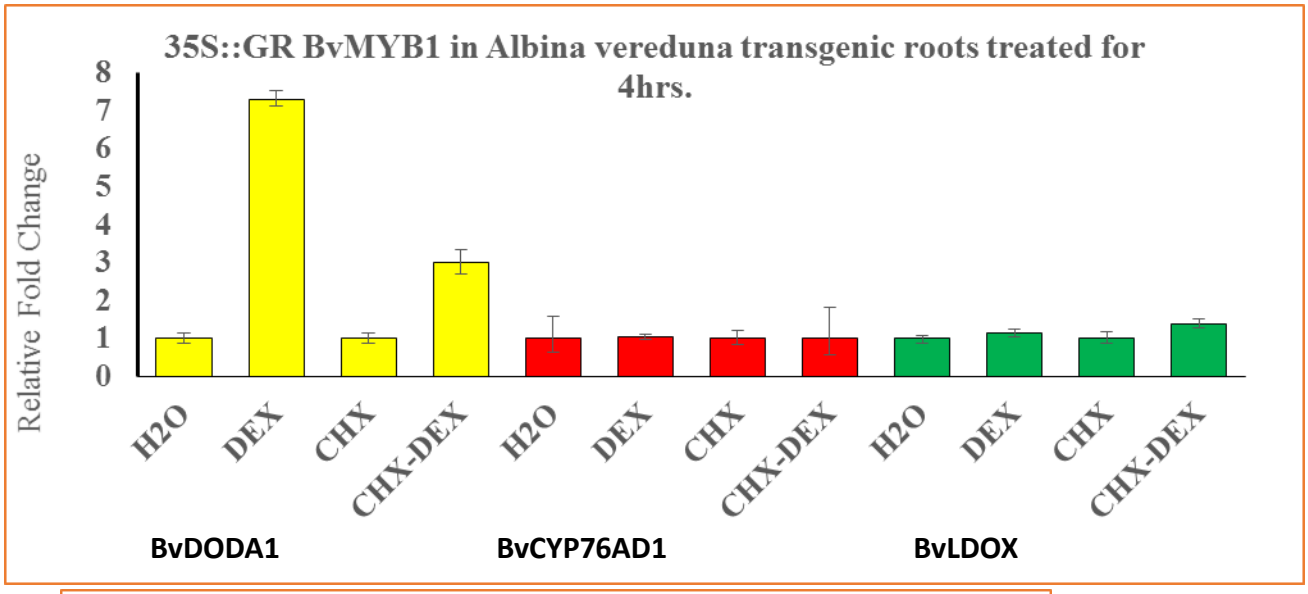


Figure 5-2: Mass spectrometry of BvMYB1 Protein, 35S::BvMYB1-6XHis vector in BL21 *E. coli* bacterial strain

a.



b.

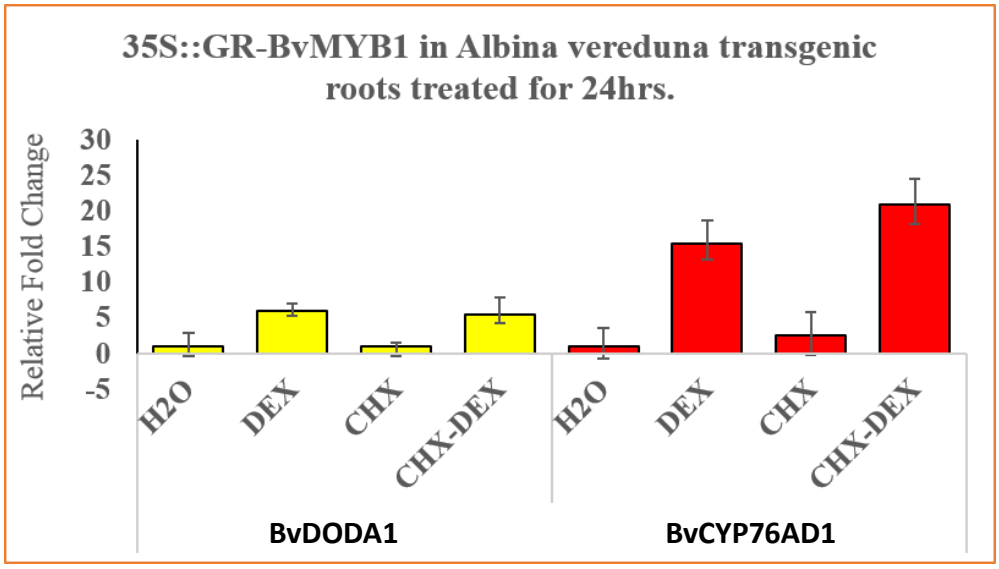


Figure 5-3: Analysis of 35S::BvMYB1-GR in Albina vereduna hairy roots

qRT-PCR after 4 hours (a) and 24 hours (b) of DEX and CHX treatment for transgenic roots. Results shown as relative gene expression compared to expression in the mock (H_20) sample. Bars represent the average of five technical replicates; error bars, s.d. Every experiment was replicated three times.

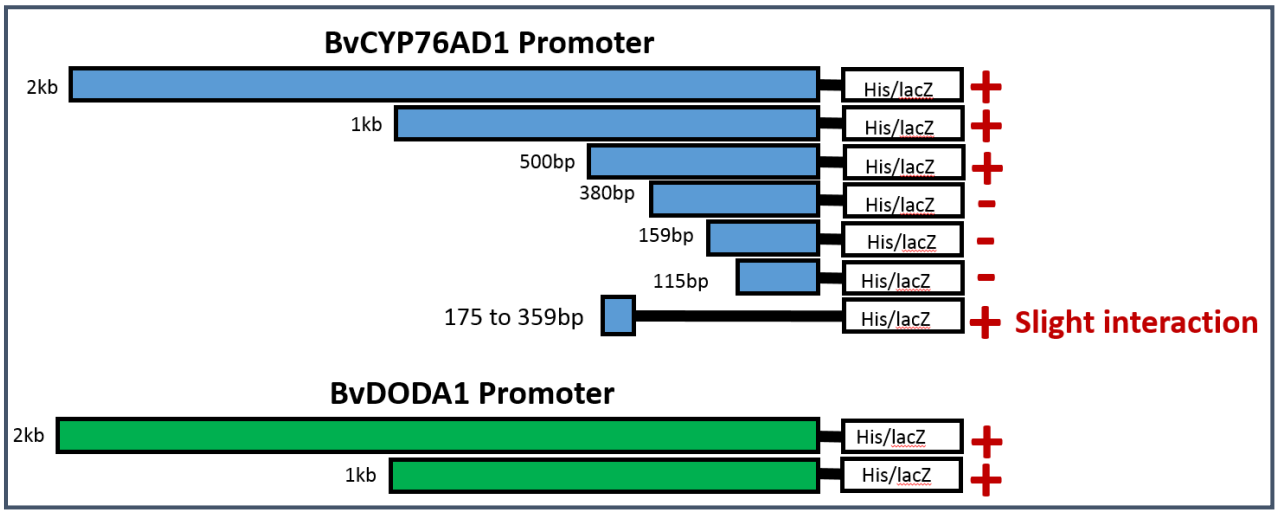


Figure 5-4: Promoter bashing in Y1H to identify the cis-regulatory element

Different constructs made to figure out the BvMYB1 binding site, the (+) indicates positive interactions, and (-) indicates no interaction.

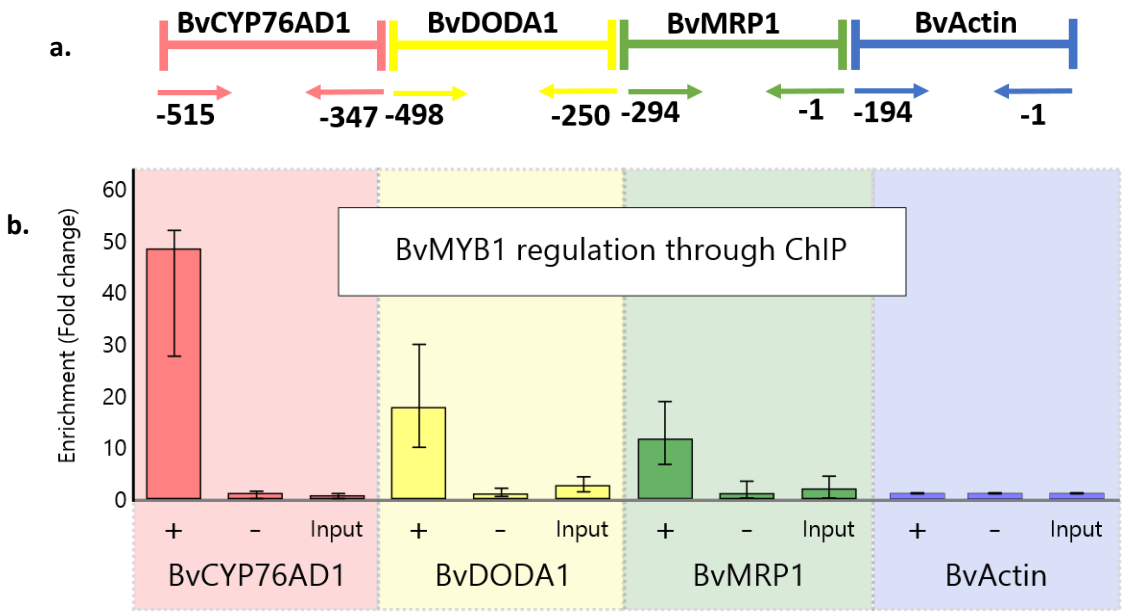


Figure 5-5: Expression of biosynthetic genes in 35S:BvMYB1-GR fusion after ChIP pull-down

BvCYP76AD1, BvDODA1, and BvMRP1 are direct targets of BvMYB1. (a) Distance and location of the primers used relative to the start for ChIP. (b) q-PCR of DNA obtained from red beet tissue of 2-d-induced *35S::BvMYB1-GR* transgenic Albina vereduna plants expressing p35S::GL3-GR treated with DEX for 2 days and ChIP analysis. (+) indicates plus GR antibody, (-) indicates plus salmon sperm DNA/Protein A-agarose beads, (input) was not treated sample, taken out after sonication. The bars indicate the relative expression to actin gene present in sample. Error bars indicate the s.d. of the data obtained from three independent RT-PCR reactions obtained from one induction experiment. All experiments were conducted in at least three biological replicates (three independent inductions), which showed no significant difference with each other.

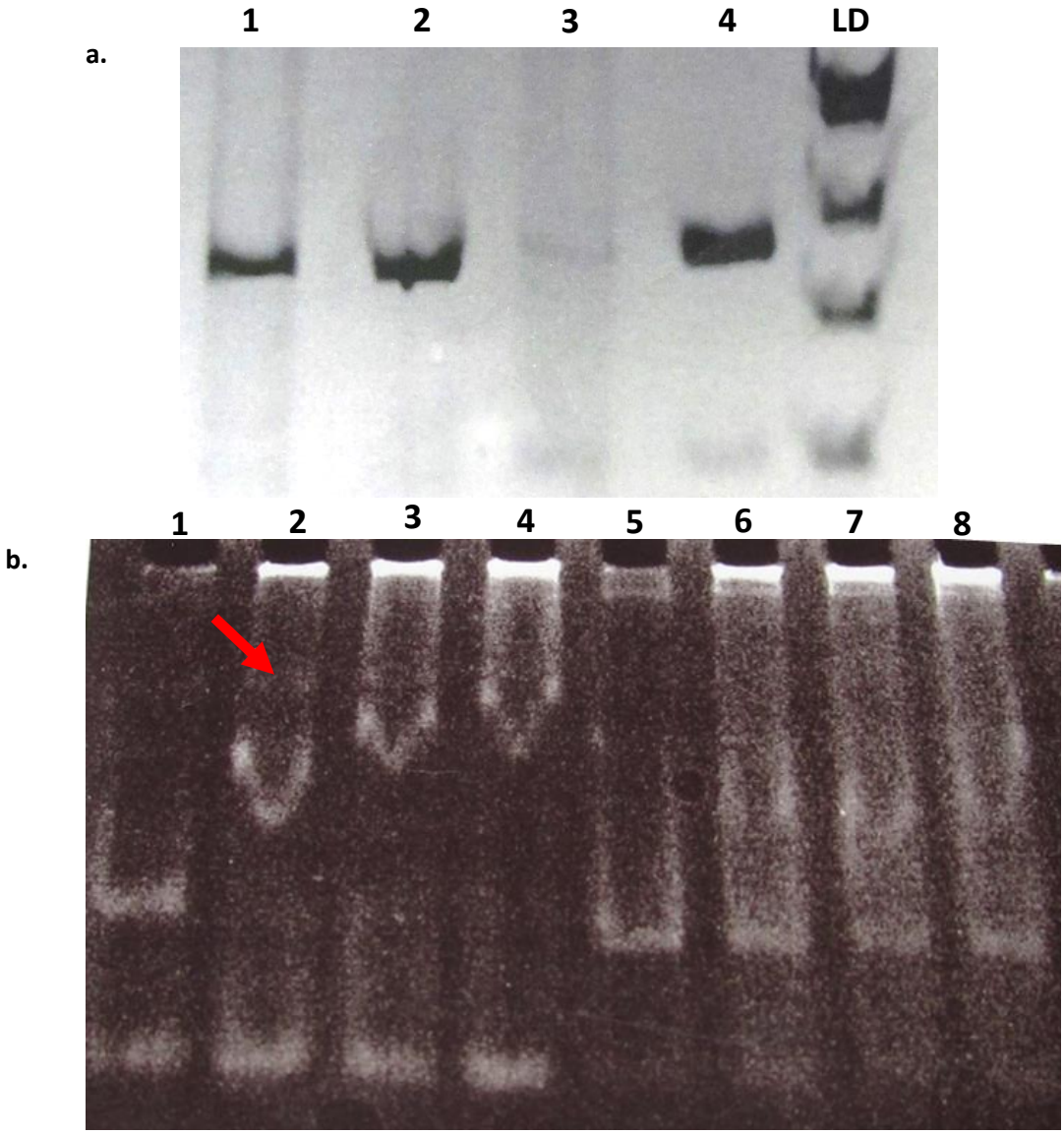


Figure 5-6: BvMYB1 interacts with 194bp of the promoter

(a) 1 and 2 contain actin probe, 3 and 4 contain CYP76AD1 probe. 1 and 3 also include protein aliquot, (b) 1 through 4 contain CYP76AD1 probe, and 5 through 8 contain the actin probe. They are listed in increasing concentration of protein (1-4 and 5-8). Were number 1 and 5 only contain the DNA probe, 4 and 8 contain the probe and the highest concentration of protein. The red arrow indicates the shifted band.

Table 5-1: Primers used as indicated in the text and methods of chapter 5

Primer Name	Primer sequence 5' to 3'
RTPCRBvACTF	CTATCCTTGCATCTCTCAG
RTPCRBvACTR	ATCATACTCGCCCTTGGAGA
BvMYB1start	TGATGTACCAGCAGAATAGTGAAAC
BvMYB1nostop	GTGCCCACAAGTTCACAAC
BvDODA1upstr2kbF	CCTATTCCGACTGCTACC
BvDODA1upstr1kbF	ATCCAAAACAGTACCTCTTTG
BvDODA1startR	CTCTTTTTTTTTTGTTTGCTTTC
76AD1upstr2kbF	GGGTCGCACAAAGTTGACAG
76AD1upstr1kbF	AACAGAACATCTTATCTTATC
76AD1upstr500bpF	GTTGGACCTCTGAGTGAGTGG
76AD1upstr380bpF	CTCAATGGAGAAATAGGAAG
76AD1upstr159bpF	CTTAATACACCATTTAATTCC
76AD1upstr115bpF	CGTAGTATATAATCCTATAC
76AD1startR	GATGATCTCACAAATAACACG
76AD1upstr175bpR	CTGTCCATTCTTTTAAACC
76AD1upstr369bpF	ACCTCAATGGAGAAATAGGAAG
BvDFRupstrF	TTTTCATTTAAGCTTTTCCAAG
BvDFRstartR	TTTTGTGGTTATATGATAGATTG
CYPAD1upst194F	GGGGGGGAGGGGTTGGGGCTTG
CYPAD1upstream194R	CTTCCTATTTCTCCATTGAGGTG
Actin-194F	GCATACGTCGCGCTTGACTTTGAG
Actin-194R	TGGAGTTGTAGGTTGTCTCGTGG
BvMRP1upstrF	CTGGGAAATGGGTCTCATCTGC
BvMRP1startR	CACTGATCACCCTTTTTCTCTC
4Xmotif-76AD1F	AACACCTCAATGGAGAAAACACCTCAATGGAGAAAACACCT CAATGGAGAAAACACCTCAATGGAGAA
4Xmotif-76AD1R	TTCTCCATTGAGGTGTTTTCTCCATTGAGGTGTTTTCTCCATTG AGGTGTTTTCTCCATTGAGGTGTT

CHAPTER 6: Conclusion

In this thesis, I have presented studies on molecular mechanisms for the regulation of the betalain secondary pigment pathway. While there are extensive studies of anthocyanin pigment regulation at the molecular level, nothing was known about betalain pigment regulation until we began these studies. This promising and nutritious pigment has attracted a significant amount of interest in recent years. My project was an endeavor to gain a deeper understanding of regulation of the three major steps in the betalain pathway. Among important findings of my work are (i) characterization of the main betalain regulator in beet, BvMYB1, which encodes an anthocyanin MYB-like protein that activates the betalain red pigment pathway (ii) identification and characterization of two MYBs similar to BvMYB1, BvMYB 2 and 3, which have betalain pigment regulatory activities (iii) identification of bHLH and WD-repeat orthologs in beet of the canonical anthocyanin-regulating MYB-bHLH-WD transcription factor complex members, and (iv) work towards the identification of a novel BvMYB1 Response Element (MRE) that BvMYB1 protein uses to directly activate betalain gene expression.

In Chapter 2, I explored the identification of a MYB gene that positively regulates the three enzymatic steps described earlier in the introduction. This MYB was co-opted from the anthocyanin pathway, supporting one of our primary hypotheses as to how and why the betalain pigments are produced exactly like anthocyanins. This MYB was mapped to the historic Y locus identified by Keller in 1936. Unlike the anthocyanin MYBs that regulate genes late in the biosynthetic pathway in virtually any anthocyanin-

producing plant where they are expressed, BvMYB1 will not regulate anthocyanin genes when expressed in *Arabidopsis*, nor will it interact with anthocyanin bHLH proteins. This work was recently published in Nature Genetics.

In Chapter 3, I studied the functional similarity of BvMYB1, BvMYB2 and BvMYB3. BvMYB2 and BvMYB3 were identified in close proximity in the beet genome to BvMYB1. Overexpression of any of the three MYBs directly correlates with high pigment levels and increased expression of the betalain biosynthetic genes. In this chapter I characterized the three MYBs through sequence analysis, functional experiments, and protein-protein interactions. Further work needs to be done to identify environmental or developmental conditions where these transcriptional regulators are expressed.

In Chapter 4, I explored and characterized other regulatory members of the complex, identifying an EGL3-like gene, BvbHLH1, and a TTG1-like gene, BvTTG1, from *Beta vulgaris*. Unlike the anthocyanin production regulatory complex that includes MYB, bHLH and WD-repeat proteins, betalain production appears to be regulated by only MYB transcription factor members. In this chapter I demonstrate that overexpression of BvbHLH1 and BvTTG1 in *Arabidopsis* appears to regulate proanthocyanidin (tannin) production. In the case of BvTTG1 it can completely complement the *ttg1* null mutation, and in case of BvbHLH1 it can suppress tannin production in wild type *Arabidopsis* seeds. Through yeast two-hybrid analysis, we observe that unlike BvMYB1, which works alone, BvbHLH1 interacts with the AtTTG1, BvTTG1 and AtMYBs. The AtMYB used was AtPAP2 (AtMYB90) which is an anthocyanin regulating MYB. Further work must be done to study the role of these

proteins on proanthocyanidin regulation in *Arabidopsis thaliana* and possibly *Beta vulgaris.*

In Chapter 5, I explored the potential for BvMYB1 binding sites upstream of direct transcriptional targets. Initially, we verified several direct BvMYB1 target genes through yeast 1-hybrid, ChIP, DEX-CHX experiments, and EMSA of sequences upstream of BvCYP76AD1 and BvDODA1. The main genes that are found to be directly regulated include BvCYP76AD1, BvDODA1, and BvMRP1. Through a combination of EMSA, yeast 1-hybrid, and SELEX of sequences upstream of BvCYP76AD1 and BvDODA1, we are working toward identification of a highly active BvMYB1 response element (MRE) sequence. I am also working to show that the two other betalain MYB regulatory transcription factors with close homology to BvMYB1, BvMYB2 and BvMYB3, can activate gene expression via the MRE.

As part of my projects, I performed next-generation sequencing on genomic DNA of red beet (W357B variety) and several beet BAC clones containing betalain pathway gene. The BACs sequenced contain BvDODA1, BvCYP76AD1, BvMYB1, BvTTG1, and BvDODA2. Other BAC clones containing important genes that were identified but not sequenced include BvbHLH1, BvNPR1, and BvMYB3.

To conclude, I hope my findings will be a pillar for the future work of the junior students who will pursue research on this topic. I hope my findings have further enhanced our understanding of the control and regulation of plant secondary metabolic networks in general, and the betalain pathway in particular. Ultimately, this research impacts our ability to create crops that are more nutritious for humans and livestock due to their radical scavenger activity, help the cosmetic industry in producing natural and safe

products, and finally our ability to use betalains in solar energy production due to their high solar energy conversion efficiency.

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Vita

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