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Invited Review

Advances in the MYB–bHLH–WD Repeat (MBW) Pigment Regulatory Model: Addition of a WRKY Factor and Co-option of an Anthocyanin MYB for Betalain Regulation

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Flavonoids are secondary metabolites derived from the general phenylpropanoid pathway and are widespread throughout the plant kingdom. The functions of flavonoids are diverse, including defense against phytopathogens, protection against UV light damage and oxidative stress, regulation of auxin transport and allelopathy. One of the most conspicuous functions of flavonoids has long attracted the attention of pollinators and scientist alike: the vivid shades of red, pink, orange, blue and purple on display in the flowers of angiosperms. Thus, flavonoid pigments have perhaps been the most intensely studied phenylpropanoids. From Mendel to McClintock and up to the present, studies centered on flavonoid pigments have resulted in some of the most important scientific discoveries of the last 150 years, including the first examples of transcriptional regulation in plants. Here we focus on the highly conserved MYB–bHLH–WD repeat (MBW) transcriptional complex model for the regulation of the flavonoid pigment pathway. We will survey the history of the MBW model spanning the last three decades, highlighting the major findings that have contributed to our current understanding. In particular, recent discoveries regarding WRKY protein control of the flavonoid pigment pathway and its relationship to the MBW complex will be emphasized. In addition, we will discuss recent findings about the regulation of the beet betalain pigment pathway, and how a MYB member of the MBW complex was co-opted to regulate this chemically unrelated but functionally equivalent pathway.

Keywords: Anthocyanins • Betalains • Flavonoids • MBW • Proanthocyanidins • WRKY.

Abreviations: *AN*, *Anthocyanin*; ANS, anthocyanidin synthase; *B*, *Booster*; *BAN*, *Banyuls*; bHLH, basic helix–loop–helix protein; BiFC, bimolecular fluorescence complementation; *C1*, *Colorless1*; CYP, Cyt P450; DFR, dihydroflavonol 4-reductase; DODA, DOPA 4,5-dioxygenase; *EGL3*, *ENHANCER of GLABRA3*; *GL*, *GLABRA*; MATE, multidrug and toxin extrusion; MBW, MYB–bHLH–WD repeat protein; MYB, myeloblastosis protein; *PAC1*, *Pale aleurone color1*; PA, proanthocyanidin; *PAP*, production of anthocyanin pigment; PH, increased pH of petunia petal homogenates; *Pl*, *Purple Plant*; *R*, in maize or beet = *Red*; *SPL9*, *Squamous promoter binding-protein-like 9*; TCP, protein family named after teosinte branched1, cycloidea, proliferating cell nuclear antigen factor proteins; TT, transparent testa; TTG, transparent testa glabra; WD, protein domain containing a tryptophan–aspartate amino acid motif; WDR, WD repeat; WIP, class of zinc finger domain containing a tryptophan–isoleucine–proline amino acid motif; WRKY, protein domain containing a tryptophan–arginine-lysine–tyrosine amino acid motif; *Y*, *Yellow*; ZIM, ZO-1 interaction motif.

Introduction to MYBs, bHLHs and WD Repeat Proteins

The myeloblastosis protein (MYB) DNA-binding domain defines a class of transcriptional regulators found in all eukaryotic organisms. This domain was first identified in the v-*myb* oncogene of the avian myeloblastosis virus and in its cellular homolog c-*myb*. Animal MYB transcription factors typically contain three imperfect MYB repeats (R1R2R3 repeats) and comprise a small family of two or three proteins with roles in cell proliferation (Lipsick 1996). Each repeat is about 52 amino acids long, containing regularly spaced tryptophan residues and folding into a helix–turn–helix variant related to those of prokaryotic repressors.

The MYB gene family expanded dramatically in higher plants, with Arabidopsis containing approximately 339 MYB genes and rice containing about 230 MYB genes (Feller et al. 2011). Accordingly, MYB proteins function in a diverse array of processes including the regulation of plant form, the cell cycle, cell differentiation, metabolism and stress response (for a general review of plant MYB transcription factors, see Dubos et al. 2010, Feller et al. 2011).

The majority of plant MYB proteins contain two imperfect MYB repeats corresponding to the R2R3 Myb repeats of the three repeat R1R2R3 animal MYB proteins. Sequence-specific DNA binding has been demonstrated for several R2R3 MYB transcription factors, with these Mybs binding to one or

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more DNA sequence types (Romeo et al. 1998, Feller et al. 2011). Additionally, R2R3 plant MYB proteins may contain an acidic transcriptional activation domain. In the context of the MYB–bHLH–WD repeat protein (MBW) complex considered in this review, a notable exception to the R2R3 organization is a small group of single MYB (R3) repeat proteins of Arabidopsis (Schellmann et al. 2002). These proteins have only one MYB domain, lack a transcriptional activation domain and function as negative regulators of transcription.

The basic helix–loop–helix (bHLH) domain was originally identified as a similar region shared among a group of DNA-binding proteins from animals (Murre et al. 1989). At approximately the same time, the *R* locus of *Zea mays* (maize) was shown to encode a bHLH transcriptional regulator of biosynthetic genes necessary for pigment production (Ludwig et al. 1989). A typical bHLH domain contains about 18 hydrophilic and basic amino acids at its N-terminal side followed by two amphipathic α-helices separated by a loop. Functionally, the basic region is important for making DNA contacts while the HLH region allows dimerization between bHLH proteins (Massari and Murre 2000). Animal bHLH proteins have broad functions in regulating cell proliferation and differentiation.

In plants, bHLH proteins comprise the second largest transcription factor class. They have very diverse roles in processes ranging from regulation of secondary metabolism, cellular differentiation and patterning, plant growth and development via the regulation of brassinosteroid and ABA hormone signaling pathways, iron uptake in roots and phytochrome-mediated light signaling. bHLH proteins can regulate transcription by recognizing E-boxes, DNA sequences with the consensus of CANNTG (for a general review on plant bHLH proteins, see Feller et al. 2011).

Unlike the Myb and bHLH domains, it is difficult to define a distinct family of proteins based solely on the presence of a WD repeat motif (WDR; Smith et al. 1999, van Nocker and Ludwig, 2003). The WD was defined as an approximately 40 amino acid structural repeat ending with tryptophan–aspartic acid (W–D). Indeed, there can be uncertainty in defining and identifying WDRs due to large variations in the position and length of structural elements composing WDR motifs found in different proteins. For example, Arabidopsis contains 237 proteins with at least four WDRs (269 total proteins with at least one WDR). These 237 proteins were classified into 143 distinct families with about 113 of these families showing clear homology to WDR proteins from yeast, fly and/or human. Thus, WDRs are found in a diverse array of proteins spanning a broad spectrum of functions (for a review of WDR protein functions in plants, see van Nocker and Ludwig 2003, Miller et al. 2016). The WDRs generally serve as a protein–protein interaction platform for the formation of complexes and as mediators of transient connections between other proteins. The WDR motif was first identified in a Gβ subunit of heterotrimeric G proteins (Fong et al. 1986). The crystal structure of this protein shows that the WDRs adopt a β-propeller fold. The plant WDR proteins considered in this review are small proteins consisting only of four or five WDRs such as Transparent Testa Glabra 1 (TTG1) of Arabidopsis and its homologs controlling plant pigment production in other species (de Vetten et al. 1997, Walker et al. 1999, Miller et al. 2016).

The Research History of the MBW complex

The early years: foundational contributions from maize genetics

Because plants must regulate flavonoid pigment biosynthesis developmentally and in response to various biotic and abiotic stresses, this pathway has historically been an excellent model for the study of transcriptional regulation. Indeed, pioneering work performed 30 years ago in maize led to the discovery of transcription factors in plants. The first plant transcription factor gene cloned, via a transposon tagging approach, was *Colorless1* (*C1*) from maize (Cone et al. 1986, Paz-Ares et al. 1987). *c1* mutants lack anthocyanins in the aleurone layer of maize kernels and are transcriptionally down-regulated for a set of flavonoid biosynthetic genes. *C1* encodes a MYB regulator of the anthocyanin biosynthetic pathway and, coincidentally, was the locus disrupted in Barbara McClintock's breeding experiments that led to the discovery of transposable elements in the 1950s. This discovery represents the earliest milestone in the study of transcriptional regulation in plants and the first clue leading to the now canonical MBW model for the control of the flavonoid pigment pathway in all plants studied to date.

Just 2 years later, Ludwig et al. (1989) reported another landmark discovery in the field of plant transcriptional regulation that ultimately put the 'B' in the MBW complex: these researchers identified in maize the first plant bHLH gene encoded by the *Red* (*R*) locus required for pigment synthesis in the seed. Later in the same year, Chandler et al. (1989) reported the cloning of an additional regulatory locus, *Booster* (*B*), encoding a bHLH protein homologous to *R*. Depending on the allele, the *B* locus controls the anthocyanin pathway only in the plant (*B-I*) or in both the plant and the seed (*B-Peru*). Before the *R* and *B* loci were cloned, genetic and biochemical studies demonstrated the necessity of these loci for the activity of pigment production in the seed and plant, and for activating the enzymatic activity encoded by known pigment biosynthetic genes. Moreover, breeding experiments indicated that a functional *R* or *B* allele was not sufficient to activate the expression of anthocyanin structural genes, and thus pigment synthesis, in the absence of a functional *C1* allele in the kernel (Chandler et al. 1989). Later, Cone et al. (1993) showed that the *Purple Plant* (*Pl*) locus required for anthocyanin pigments in the plant body encodes a MYB protein and close homolog to the C1 transcription factor. Similar to the kernel requiring C1 and R, activation of the anthocyanin pathway in the plant body requires both the Pl MYB and the B bHLH proteins. In addition, transactivation of anthocyanin structural gene promoter–reporter contructs bombarded into maize tissues also demonstrated a requirement for both a MYB and a bHLH protein (Goff et al. 1990). However, Goff et al. (1992) reported a novel biochemical observation that helped clarify the genetic interaction data and defined a cornerstone of the MBW transcriptional model as we understand it today: a physical interaction between *B* and *C1*

Table 1 Composition of foundational MBW complexes and biological functions in the major plant pigment models

Species	M–B–W	Function
Zea mays	C1–R/B–PAC1	Anthocyanins in kernels.
	P1–R/B–PAC1	Anthocyanins in the plant body.
Antirrhinum majus	Ros1/Ros2/Ve–Del–W?	Anthocyanin synthesis and patterning in flowers and the plant body.
Petunia hybrida	AN2–AN1–AN11	Anthocyanins in flowers and the plant body.
	PH4–AN1–AN11	Flower color modification.
Arabidopsis thaliana	PAPs–EGL3/GL3/TT8–TTG1	Anthocyanins in the plant body.
	TT2–TT8–TTG1	PAs in seed coats.

was demonstrated in yeast two-hybrid assays suggesting that these regulators function together in a transcriptional complex.

Other plants join the fray: contributions from *Petunia, Antirrhinum* and Arabidopsis

In addition to the early groundbreaking genetic work in maize, studies of the anthocyanin pathway in other species made important contributions to the emerging MBW model (**Table 1**). Petunia (*Petunia hybrida*) and snapdragon (*Antirrhinum majus*) were also major models for the study of anthocyanin biosynthesis, particularly in floral tissues. *Arabidopsis thaliana* was another key species for studying the synthesis and regulation of flavonoid pigments. Arabidopsis flowers, however, are naturally anthocyaninless but flavonoid-based proanthocyanidin pigments (PA; commonly known as tannins) are produced in the seed coat (testa), giving Arabidopsis seeds their characteristic brownish orange color (**Fig. 1**). Hence, the identification of pigment mutants in Arabidopsis primarily focused on convenient screens for yellow or lighter colored seed, otherwise known as *transparent testa* (*tt*) mutants (Koornneef 1990).

In *Petunia*, *Antirrhinum* and Arabidopsis, genes encoding MYB and bHLH proteins were identified as regulators of anthocyanin structural genes as in maize, demonstrating broad conservation of this regulatory mechanism in the plant kingdom. *Delila*, encoding a bHLH factor homologous to the product of *R* that controls pigment accumulation in *Antirrhinum* flowers, was the first dicot anthocyanin regulatory locus cloned (Goodrich et al. 1992). Later, it was shown that three *Antirrhinum* MYB genes, *Rosea1*, *Rosea2* and *Venosa*, homologous to maize *C1*, differentially regulate structural genes of the anthocyanin pathway (Schwinn et al. 2006). In *Petunia*, *Anthocyanin 1* and *2* (*AN1* and *AN2*) were shown to encode a MYB and a bHLH transcription factor, respectively, necessary for pigmentation of floral organs (Quattrocchio et al. 1999, Spelt et al. 2000). Interestingly, the first anthocyanin regulatory locus cloned from *Petunia*, *ANTHOCYANIN 11* (*AN11*), encoded neither a MYB nor a bHLH but a novel regulator protein containing five WDRs (de Vetten et al. 1997), a motif originally noted in a bovine G protein β subunit (Fong et al. 1986). Almost 20 years after its discovery (Koornneef 1981), the *TTG1* locus of Arabidopsis was also shown to encode a WDR protein (Walker et al. 1999) homologous to that encoded by *AN11*. However, the relationship between the WDR protein and the MYB/bHLH transcriptional regulatory model was not immediately clear.

An epidermal cell fate digression in the development of the anthocyanin MBW model

Ironically, the now well-established placement of the WDR protein in the MBW complex did not come from anthocyanin studies in maize or the *Petunia* or *Antirrhinum* floral pigment model species, but instead from trichome cell fate studies in Arabidopsis. Besides lacking both anthocyanins and proanthocyanidin (PA) pigments, the *transparent testa glabra1* (*ttg1*) mutant of Arabidopsis is deficient for trichome (plant hairs) initiation, root hair patterning and differentiation of the mucilage-producing outer seed coat cells. Although it had been known for some time that the maize *R* bHLH gene could rescue the entire suite of *ttg1* mutant phenotypes (Lloyd et al. 1992), the first MBW complex bHLH genes identified in Arabidopsis were the PA pigment regulator *TT8* (Nesi et al. 2000) and the trichome regulator *GLABRA 3* (*GL3*). In yeast two-hybrid analysis, Payne et al. (2000) reported that GL3 could physically interact (via different domains) with both TTG1 and the Arabidopsis GL1 trichome regulator, a homolog of MYB anthocyanin regulators (Oppenheimer et al. 1991). Moreover, GL1 and TTG1 were not capable of direct physical interaction but could presumably form a complex together with GL3. Thus, the now canonical MBW transcriptional complex that regulates anthocyanin production throughout the plant kingdom (**Table 1**; **Fig. 1**) was first proposed in the context of Arabidopsis trichome production (Payne et al. 2000). A specific combination of MYB and bHLH proteins in Arabidopsis has since been identified for the regulation of specific TTG1-dependent epidermal cell fate pathways (Nesi et al. 2001, Bernhardt et al. 2003, Zhang et al. 2003, Bernhardt et al. 2005, Gonzalez et al. 2008, Gonzalez et al. 2009). Also, WDR proteins orthologous to AN11 and TTG1 have been identified as anthocyanin regulators in other species, including Pale Aleurone Color1 (PAC1) of maize (Carey et al. 2004).

In the case of anthocyanin pigment regulation in Arabidopsis, clear identification of the MBW complex was complicated by the redundancy of bHLH and MYB loci, and by the fact that Arabidopsis evolved different MBW complexes for anthocyanin and PA pigment regulation (**Table 1**; **Fig. 1**). The *tt* mutant screen approach described earlier yielded a

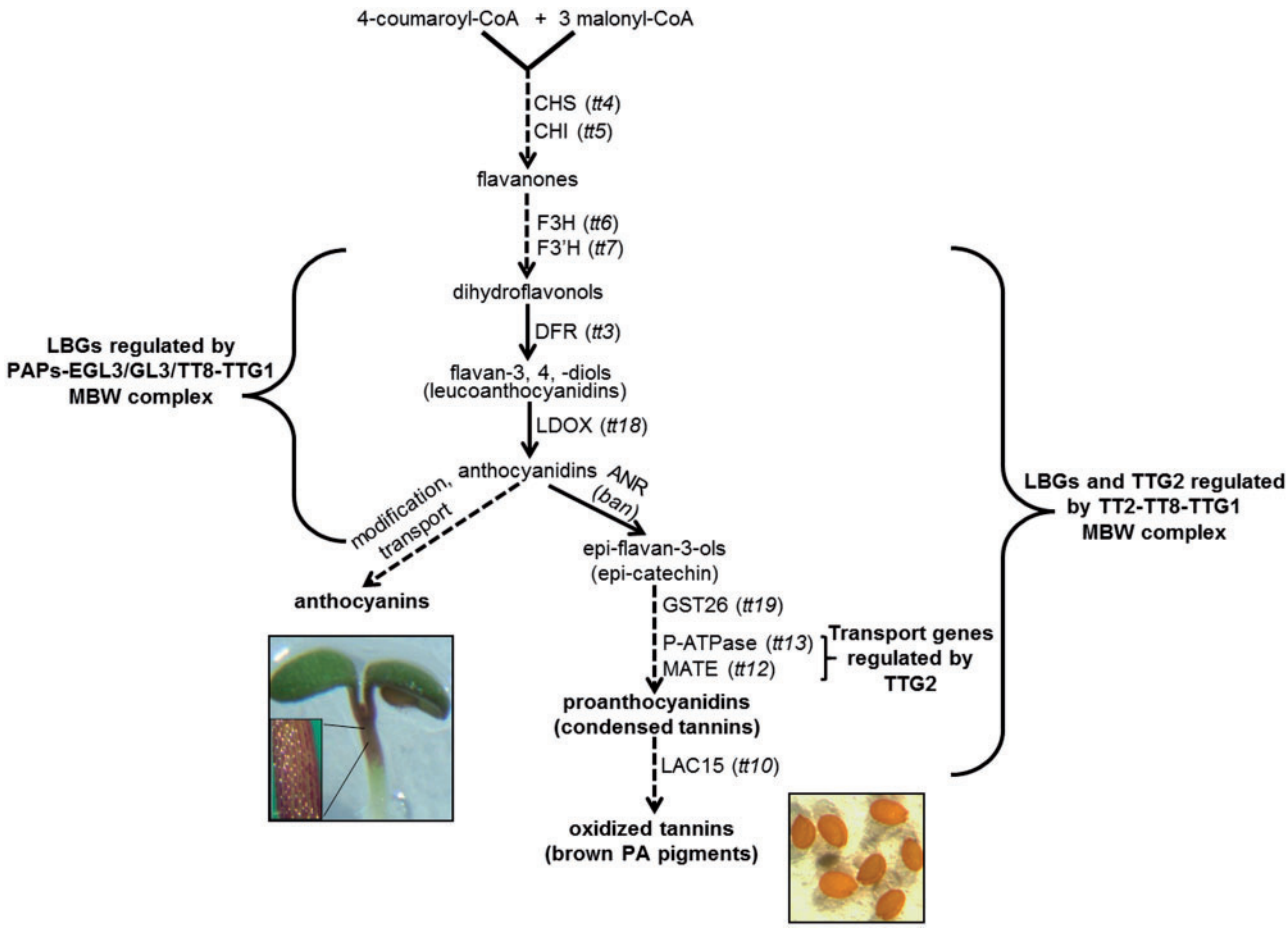

Fig. 1 The Arabidopsis flavonoid pathway for the synthesis of anthocyanins and oxidized tannins, or proanthocyanidins (PAs). The large brackets indicate the late biosynthetic genes (LBGs) directly regulated by the MBW complex: on the left is the anthocyanin-specific MBW complex; on the right is the PA-specific MBW complex. The small bracket on the right indicates the narrow subset of LBGs encoding the transport steps regulated by TTG2. Dashed lines indicate multiple steps. Depicted on the left is a wild-type seedling expressing purplish red anthocyanins in the hypocotyl and parts of the cotyledons early in Arabidopsis development. Depicted on the right are Arabidopsis wild-type dry seed showing the brownish orange PA pigments expressed in the seed coat.

pair of TTG1-dependent regulators, *TT8* and *TT2* (Nesi et al. 2000, Nesi et al. 2001). *TT8* and *TT2* encode a bHLH and MYB protein, respectively, that together with TTG1 regulate the biosynthesis of PA pigments in the Arabidopsis seed coat (Baudry et al. 2004). However, *tt8* and *tt2* mutants show no other *ttg1*-like mutant phenotypes, even displaying normal pigmentation in young Arabidopsis seedlings where anthocyanins are developmentally produced and *TT8* is also expressed. This suggested a distinct, as yet unknown, set of TTG1-dependent MYB and bHLH regulators controlling anthocyanin biosynthesis in the plant body. As hypothesized, the characterization of a third bHLH regulatory gene, *ENHANCER of GLABRA 3* (*EGL3*), uncovered the partially redundant roles of the three Arabidopsis bHLH proteins in regulating anthocyanin biosynthesis (Zhang et al. 2003). In addition, ectopic gene expression and RNA interference (RNAi) approaches helped identify four partially redundant Arabidopsis MYB genes (*PAP1*, *PAP2*, *MYB113* and *MYB114*, collectively known as PAP MYBs for production of anthocyanin pigment) dedicated to the regulation of the anthocyanin pathway in the shoot (Borevitz et al. 2000, Gonzalez et al. 2008). Thus, in retrospect, it is clear why the Arabidopsis anthocyanin-specific MBW complex long evaded detection in screens focused on *tt* phenotypes.

Distillation of universal features of MBW regulation

After 30 years of investigating the regulation of anthocyanin biosynthesis via the MBW complex, some general trends may be noted across the numerous plant species studied to date. Based in part on the timing of structural gene expression and by the subset of structural genes regulated by the MBW complex, the flavonoid biosynthetic pathway is subdivided into 'early' steps and 'late' steps. The MBW complex is predominantly required for the regulation of late flavonoid biosynthetic genes over early genes (**Fig. 1**). However, the particular pathway steps comprising the late and early sets and the degree of regulation of the sets differ between species and even tissues (Taylor and Briggs 1991, Martin et al. 1991, Quattrocchio et al. 1993, Deboo et al. 1995, Winkel-Shirley et al. 1995, Pelletier and Winkel Shirley 1996, Pelletier et al. 1997, Zhang et al. 2003, Morita et al. 2006, Gonzalez et al. 2008). Moreover, the MBW complex is necessary and sufficient for the direct transcriptional

regulation of late flavonoid biosynthetic genes (Baudry et al. 2004, Gonzalez et al. 2008, Xu et al. 2014). In addition, just as different MBW combinations can discriminate between different TTG1-dependent epidermal pathways in Arabidopsis, so too can the exact composition of pigment MBW complexes influence which type of flavonoid pigment is produced, which biosynthetic pathway genes are activated and the strength of target gene expression (**Table 1**; **Fig. 1**; Zhang et al. 2003, Baudry et al. 2004, Schwinn et al. 2006, Lea et al. 2007, Feyissa et al. 2009). Thus, different MYB and bHLH members of pigment MBW complexes within and across species have functionally diversified for more subtle regulation of the flavonoid pathway, with the MYBs contributing more of the fine-tuning of pigment production in terms of pigment type and patterns in the plant (see Xu et al. 2015 for a thorough review on the structure, function and regulation of the flavonoid MBW complex; see Davies et al. 2012 for a review on how diversification of MBW complex members accomplish fine control of the flavonoid pigment pathway leading to pattern formation).

It is also worth noting that several other transcription factor proteins can directly interact with the flavonoid pigment MBW complex either to repress or to promote pigment production. For example, hormone-regulated anthocyanin biosynthesis in Arabidopsis is repressed by jasmonate ZO-1 interaction motif (ZIM) domain proteins interacting with bHLH regulators of the pigment pathway (Qi et al. 2011). Similarly, Squamous Promoter Binding Protein-Like 9 (SPL9) represses pigment accumulation by directly binding to Arabidopsis PAP MYBs, thus disrupting the MBW complex (Gou et al. 2011). In contrast, the TCP3 bHLH protein enhances the transcriptional activity of the flavonoid pigment MBW complex in Arabidopsis by direct interaction with the R2R3 MYBs, thus promoting pigment biosynthesis (Li and Zachgo 2013). Similarly, TT1 (a WIP type zinc finger transcription factor) can interact with the TT2 MYB member of the PA MBW complex and enhance its transcriptional activity (Appelhagen et al. 2011). (For a more thorough review on the post-translational regulation of the flavonoid pigment MBW complex, see Xu et al. 2015.)

Elucidation of WRKY Factor Control Mechanisms Reveals a New Twist on the MBW Pigment Regulatory Model

For about the last 15 years, a wrinkle in the flavonoid pigment MBW regulatory paradigm existed in the form of the *TTG2* locus of Arabidopsis. In 2002, Smyth and co-workers (Johnson et al. 2002) reported that *TTG2* encoded a WRKY class transcription factor (containing the WRKY amino acid motif) that is necessary for trichome and seed coat development. As the '*tt*' designation implies, among the phenotypes in the *ttg2-1* mutant is a lack of PA flavonoid pigments in the seed coat. In addition, it was shown that the *TTG2* gene is regulated by the MBW complex in the context of trichome and seed coat development (Johnson et al. 2002, Lepiniec et al. 2006, Ishida et al. 2007, Gonzalez et al. 2008, Zhao et al, 2008). Unlike other TT regulators such as TT8 and TT2, TTG2 is not required for the expression of *Banyuls* (*BAN*), the first committed step in the PA branch of the flavonoid pigment pathway (Debeaujon et al. 2003). However, like *tt2* and *tt8* mutants, the *ttg2* mutant pigment phenotype is restricted to the seed coat; no lack of anthocyanins in the plant body has been observed in *ttg2* mutants (Johnson et al. 2002, Lepiniec et al. 2006, Appelhagen et al. 2014, Gonzalez et al. 2016). These observations raised some interesting questions: if the MBW complex (specifically TT2/TT8/TTG1) is necessary and sufficient for the direct regulation of pigment biosynthetic genes (especially of *BAN* and other PA pathway-specific genes), how exactly does TTG2 regulate the pathway? Aside from being a direct transcriptional target, what exactly is the nature of the relationship, particularly at the protein level, between TTG2 and the MBW complex?

Another interesting question relates to how taxonomically widespread the WRKY strategy is for the control of the pigment pathway in plants. Among all of the flavonoid pigment model species studied, it appeared that the evolution of a WRKY protein specifically and narrowly controlling the PA branch of the pigment pathway may be unique to Arabidopsis, or unique to instances of PA pathway regulation and perhaps not anthocyanin regulation. Again, considering that Arabidopsis pigment mutant screens focused on *tt* phenotypes while screens in other plant models focused on anthocyanin phenotypes in floral organs, it is not surprising (perhaps even expected) that genes and regulatory mechanisms unique to PA biosynthesis would be uncovered.

However, a recent discovery revealed the conservation of the WRKY-based regulatory mechanism operating beyond the Arabidopsis PA pathway and in the anthocyanin pathway in *Petunia* (Verweij et al. 2016). This and other findings reported in three recent publications have uncovered some fascinating insights regarding the novel mechanisms by which WRKY factors together with the MBW complex regulate the anthocyanin and PA pigment pathways, and the trichome pathway in Arabidopsis (Pesch et al. 2014, Gonzalez et al. 2016, Verweij et al. 2016).

Some recent answers to long-standing questions: reconciliation between the WRKY and MBW control mechanisms suggests new regulatory models.

Pesch et al. (2014) provided a wealth of novel insights into regulatory mechanism of TTG2 and its relationship to the MBW complex in the context of trichome development. These authors showed that TTG2 primarily directly regulates the trichome patterning R3 MYB gene *TRIPTYCHON* (*TRY*). In addition, this regulation is dependent on the MBW complex, although TTG2 enhances the MBW activation of *TRY*. Consistent with these observations, TTG2 was shown to interact physically with TTG1. Moreover, yeast three-hybrid experiments showed that the TTG2 protein and the GL3 bHLH trichome regulator interact via TTG1 as a mediator. Yeast four-hybrid experiments indicate that the trichome R2R3 MYB regulator GLABRA 1 (GL1) does not interrupt the GL3–TTG1–TTG2 interaction. Based on these findings, Pesch et al. (2014) present a model in which, after activation of the *TTG2*

gene by the trichome MBW complex, the TTG2 protein joins the complex via interaction with TTG1 to enhance the direct regulation of *TRY*.

Verweij et al. (2016) recently reported the cloning of the *PH3* locus in *Petunia*, which encodes a WRKY transcription factor homologous to *TTG2*. *PH* mutants of *Petunia* all show similar phenotypes consisting of bluish-hued flower petals and increased pH of petal homogenates (de Vlaming et al. 1983, van Houwelingen et al. 1998). Interestingly, *ph6* was shown to be an allele of the *AN1* bHLH gene that had retained the ability to activate anthocyanin biosynthesis but not the ability to acidify the vacuole (Spelt et al. 2002). Moreover, a distinct *Petunia* MYB regulator, PH4, can form a complex with AN1 and AN11 to activate a separate pathway that alters flower color (**Table 1**). This is accomplished by the PH4–AN1–AN11 complex targeting the expression of two genes, *PH1* and *PH5*, both encoding tonoplast-localized P-ATPase transmembrane transporters. Besides vacuolar acidification, PH4 also regulates volatile floral emissions (Cna'ani et al. 2015). Thus, differential activation of distinct anthocyanin or vacuole acidification/volatile emission pathways is achieved by altering the MYB regulator (AN2 or PH4) or by altering the function of the AN1 bHLH factor (encoded by the *AN1* or *ph6* alleles). The PH3 WRKY factor was also shown to regulate *PH1* and *PH5*. In addition, the PH4–AN1–AN11 MBW complex regulates *PH3*. Lastly, Verweij et al. (2016) showed that the PH3 WRKY factor physically interacts with the AN11 WDR in yeast two-hybrid analysis. Based on these findings, Verweij et al. (2016) present a model in which the *Petunia* MBW complex activates the expression of *PH3*. Then, the PH3 WRKY factor joins the MBW complex to target the expression of *PH1* and *PH5*, ultimately resulting in the acidification of the vacuole as a means of flower color modification (**Fig. 2A**). The authors also speculate that a similar mechanism may be operating in Arabidopsis to acidify the vacuole of inner seed coat cells that produce PA pigments: the TT2–TT8–TTG1 MBW complex directly targets the expression of *TTG2* and then a TT2–TT8–TTG1–TTG2 complex activates the expression of a tonoplast-localized P-ATPase encoded by *TT13/AHA10*. However, recent developments reported in Gonzalez et al. (2016) provide a more comprehensive mechanism of TTG2 control of the PA pathway through the specific regulation of genes involved in vacuolar transport of PA precursors (**Fig. 1**).

In Arabidopsis, a quantitative PCR approach comparing the expression of a broad set of pigment biosynthetic genes between wild-type and *ttg2* developing seeds revealed only two obvious targets of regulation: *TT12* and *TT13/AHA10* (Gonzalez et al. 2016; **Fig. 1**). This is in contrast to the much broader regulation by the MBW complex of the late biosynthetic gene subset (which includes *TT12* and *TT13/AHA10*). *TT12* encodes a MATE (multidrug and toxin extrusion)-type vacuolar transporter necessary for the proton gradient-dependent pumping of epicatechin-3′-*O*-glucoside (E3′OG), a glycosylated PA pigment precursor (Marinova et al. 2007). *TT13/AHA10* encodes a tonoplast-localized P_{3A}-ATPase (Baxter et al. 2005, Appelhagen et al. 2015) that presumably establishes the H^+ gradient necessary to power TT12. In addition, TTG2 is capable of binding a 1 kb fragment of DNA containing several putative WRKY boxes immediately upstream of the *TT12* gene, suggesting direct regulation. A similar mechanism has been described in *Medicago* involving an epicatechin-specific glucosyltransferase (yet to be identified in Arabidopsis) and a MATE-type pump for the transport of E3′OG PA precursors into the vacuole of seed coat cells (Zhao and Dixon 2009, Pang et al. 2013). Similar to findings in *Petunia* and in the trichome pathway in Arabidopsis, yeast two-hybrid and bimolecular fluorescence complementation (BiFC) experiments demonstrated an interaction between TTG2 and TTG1, but not between TTG2 and other MBW complex members such as TT2 and TT8 (Gonzalez et al. 2016). Moreover, overexpressing *TT12* in *ttg2* mutants restored the seed coat color phenotype to almost wild-type levels, consistent with the findings that TTG2 has a narrow range of targets consisting of one or two genes involved specifically in vacuolar transport. These findings are consistent with at least two regulatory models presented in Gonzalez et al. (2016) and in **Fig. 2**. In one model, the TT2–TT8–TTG1 MBW complex directly activates *TTG2*. Similar to the models presented in Pesch et al. (2014) and Verweij et al. (2016), TTG2 then interacts with TTG1 to form a TT2–TT8–TTG1–TTG2 quartet (**Fig. 2A**). The consequence of this 'MBWW quartet' model is to narrow greatly the target range of the MBW complex from all the late flavonoid pigment biosynthetic genes to just two vacuolar transport genes (*TT12* and *TT13/AHA10*) near the end of PA pathway (**Fig. 1**). Alternatively, the observations made thus far do not rule out the possibility of a second model, the 'WW duo' model, in which a transcriptional complex centered on the TTG1 WDR protein and the TTG2 WRKY factor, but omitting the TT2 MYB and TT8 bHLH factors, regulates only the vacuolar transport steps in the PA pathway (**Fig. 2B**). In this model, the TTG1–TTG2 complex would reinforce the MBW complex in the regulation of *TT12*. It is also possible that these two TTG2 regulatory models are not mutually exclusive, with multiple co-existing transcriptional complexes in plants providing various means for fine-tuned control of the flavonoid pigment pathway.

Evidence for multiple TTG2 regulatory models

Indeed, extensive genetic data available from studies in the PA pathway may reveal additional, subtle insights into the WRKY and MBW regulatory mechanisms. It is noteworthy that there seems to be a qualitative difference between the degree of regulation of target genes by TTG2 and TTG1 compared with TT2 and TT8. For example, in *ttg2* developing seed, the expression of *TT12* is completely repressed. Similarly, the expression of *TT12* is undetectable in *ttg1* seed coats (Xu et al. 2014, Gonzalez et al. 2016). Although greatly reduced in expression, *tt2* and *tt8* seed coats still show detectable residual expression of *TT12*, in contrast to *ttg1* and *ttg2* (Xu et al. 2014, Gonzalez et al. 2016). As previously mentioned, TTG2 physically interacts with TTG1 but not with MYB or bHLH MBW complex members, including TT2 and TT8 (Pesch et al. 2014, Gonzalez et al. 2016). Additionally, TTG2's control of *TT12* is dependent upon TTG1.

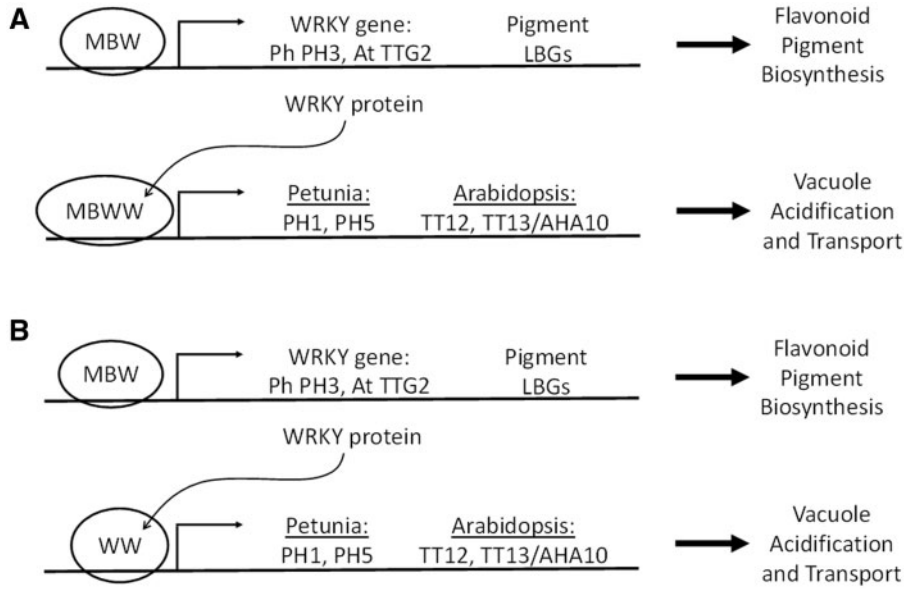

Fig. 2 Possible MBW and WRKY transcriptional models for the regulation of the flavonoid pigment pathway. In (A), the MBW complex controls the expression of the LBGs and the WRKY gene (PH3 in *Petunia*, TTG2 in Arabidopsis). The WRKY protein then possibly enters the MBW complex, via physical interaction with the WDR protein (AN11 in *Petunia* and TTG1 in Arabidopsis), and narrows the range of LBG targets to the vacuole acidification and transport genes: *PH1* and *PH5* in *Petunia*, *TT12* and *TT13/AHA10* in Arabidopsis. In (B), the MBW complex regulates the LBGs and the WRKY gene but then the WRKY and WDR proteins physically interact possibly to form a separate transcriptional complex, without the MYB and bHLH proteins, that narrowly targets the vacuole acidification and transport genes. Ph: *Petunia hybrida*; At: *Arabidopsis thaliana*; LBGs: late biosynthetic genes of the flavonoid pigment pathway.

So, what is providing the residual *TT12* gene expression in *tt2* and *tt8* mutant seed coats that is otherwise completely absent in *ttg1* and *ttg2* seed coats (Xu et al. 2014)? Could it be TTG1 in a complex with TTG2 protein, produced from residual *TTG2* gene expression, in *tt2* and *tt8* mutants? If so, this would suggest that something akin to the WW duo model (**Fig. 2B**) is operating in *tt2* and *tt8* mutants to yield detectable *TT12* expression. Ectopically expressing TTG2 in *ttg1* mutants did not restore *TT12* expression at all (Gonzalez et al. 2016), although this result would be predicted given either of the two proposed TTG2 regulatory models. However, it would be interesting to express *TTG2 ectopically* in *tt2* and *tt8* mutant seed coats. If *TT12* expression is restored in these mutants, this would indicate that TTG2 could function independently of the MYB and bHLH factors, providing evidence that the TTG1–TTG2 model is feasible.

Co-option of Phenylpropanoid-Regulating MBW Complex Members to Regulate Unrelated Pathways

The MBW complex regulates epidermal developmental processes in the Arabidopsis lineage

While the MBW complex has been most extensively studied in the context of the anthocyanin and PA pathways, the complete complex, or complex members, have been co-opted to regulate unrelated pathways in certain plant taxa. As discussed above, in Arabidopsis, in addition to the anthocyanin and PA pathways, the complex is well known to regulate various epidermal cell fate pathways including trichome initiation, root hair/non-root hair cell fate and outer seed coat differentiation. The taxonomic extent of this co-opted regulation extends to at least the closely related Malvaceae family where it regulates the economically important cotton fiber initiation process, another seed coat differentiation process (Wan et al. 2014). In Arabidopsis, this MBW regulation includes both activation of trichome cell fate by the complex and near-neighbor repression of the trichome fate by truncated MYBs descended from full-length activator MYBs (see Feller et al. 2011 for a review).

Co-option of an anthocyanin-regulating MYB to regulate the betalain pigment pathway

Betalains are red and yellow pigments that are produced in some families of the Caryophyllales order. Where they occur, they are mutually exclusive with the anthocyanins and they appear to provide all of the functions of anthocyanins, pigmenting flowers and fruits and responding to the same environmental stimuli as the anthocyanin pathway (**Fig. 3**). Betalains and anthocyanins are chemically unrelated, with the betalains being derived from tyrosine while anthocyanins are derived from phenylalanine. Their biological functions as pigments are so similar that betalains were known as 'nitrogenous anthocyanins' before the structure was determined by Mabry (Mabry et al. 1962, Mabry 1964). Mabry coined the term betalain naming these pigments after the beet genus, *Beta*.

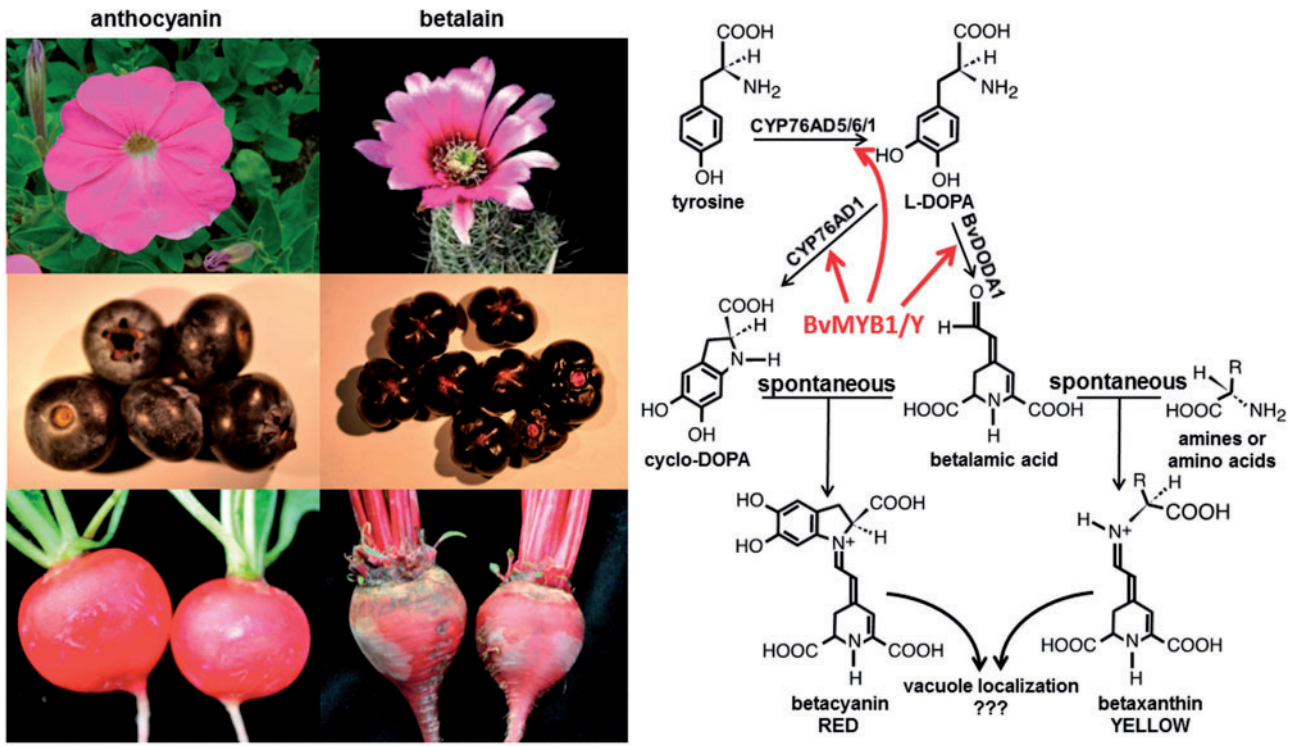

Fig. 3 Betalain pathway with comparision of analogous structures pigmented with anthocyanin or betalain. The six left panels are representative flowers, fruits and roots, with the far left panels pigmented with anthocyanins and the right with betalains. Upper left, *Petunia* flower; upper right, cactus flower; middle left, blueberries; middle right, malabar spinach berries; lower left, radish roots; lower right, beet roots. The betalain pathway is shown on the right, with the enzymatic steps regulated by BvMYB1 indicated with arrows. The condensation of betalamic acid with cyclo-DOPA to produce red betalains, or amine compounds to produce yellow betalains is spontaneous. Betalains are trafficked to the vacuole by unknown means. Note that betacyanins are usually modified, often by glycosylation on the cyclo-DOPA moiety (not shown).

In 1936, Keller described two loci in beet that affect betalain pigmentation, the *R* and *Yellow* (*Y*) genes (Keller 1936). The *R* locus has been shown to encode a Cyt P450, CYP76AD1 (**Fig. 3**; Hatlestad et al. 2012). Mutations at the *R* locus lead to the loss of red pigmentation and the accumulation of yellow. Keller's *Y* locus has been shown to encode a MYB transcriptional regulator, BvMYB1 (Hatlestad et al. 2015). Phylogenetic analysis of BvMYB1 clearly indicates that it is within the anthocyanin MBW complex MYB clade (S6), distinct from the MBW-independent MYB regulators (such as AtMYB11, AtMYB12 and AtMYB111) that regulate early steps of the flavonoid pathway (Liu et al. 2015). However, it has lost the ability to interact with bHLH members of the MBW complex. Furthermore, BvMYB1 cannot up-regulate the anthocyanin pathway in Arabidopsis, and the Arabidopsis PAP MYBs cannot regulate betalains in beet. BvMYB1 has been shown to regulate directly genes in the betalain pigment pathway, *CYP76AD1/R* and *BvDODA1* (Hatlestad et al. 2015; **Fig. 3**). *BvDODA1* encodes the DOPA 4,5-dioxygenase enzyme (DODA) required for all betalain biosynthesis. More recently, CYP76AD5 and CYP76AD6 have been shown to hydroxylate tyrosine to L-DOPA (**Fig. 3**; Polturak et al. 2015, Sunnadeniya et al. 2016) and that these genes are also up-regulated by BvMYB1, but it is not known if this regulation is direct. It was shown that a regulatory mutation at the *Y* locus isolated during beet domestication causes up-regulation of *BvMYB1* and this mutation is responsible for the intense pigment accumulation in the interior of the beet root. It will be interesting to determine if there are also changes in DNA element motifs bound by the beet MYB. In addition, there are two other closely related beet MYBs that can up-regulate the betalain pathway when overexpressed. Neither of these MYBs is able to interact with bHLH proteins (Lloyd lab, unpublished observations). As discussed elsewhere, the duplicated Arabidopsis PAP MYBs differentially regulate the anthocyanin pathway in varied environmental and developmental contexts (Lea et al. 2007, Gonzalez et al. 2008, Shan et al. 2009, Luo et al. 2012, Shin et al. 2013). It may be that the duplicated beet MYBs have similar differential activities.

Mutations leading to the loss of bHLH interaction by the beet myb may have been important for the co-option from anthocyanin regulation, helping to confer betalain specificity. Using site-directed mutagenesis, BvMYB1 was modified so that it interacted with anthocyanin bHLH regulatory proteins. These residue changes all map to a fairly well-characterized region that was previously shown to mediate MYB–bHLH interaction although the analysis expands this region by a single amino acid required for interaction. It was noted that this modified beet MYB is still unable to regulate the anthocyanin pathway when expressed in Arabidopsis. It is likely that the beet MYB no longer binds to the same DNA element, but this has not been verified (Hatlestad et al. 2015).

MBW co-option may partially explain mutual exclusivity of betalains and anthocyanins

Shimada et al. (2005) show that both spinach and pokeweed, two betalain-producing species, encode anthocyanidin synthase (ANS) proteins that are fully functional in producing anthocyanidin, which is required for anthocyanin production. However, they show that the expression of these ANS genes, as well as the requisite dihydroflavonol 4-reductase (DFR) genes, is mostly restricted to the seed, where they may be involved in the production of PA (tannin) pigments. Although it has not been demonstrated, this expression restriction may be the result of co-option of the required anthocyanin MBW MYB to regulate betalain genes and loss of the ability to activate anthocyanin biosynthetic genes in the plant body, flowers or fruit, eliminating the full anthocyanin pathway from these species. It will be interesting to analyze whether there are MBW complex members in betalain-producing species with expression limited to the developing seed coat.

In addition to loss of anthocyanin pathway gene expression, it may be that the latest steps in the anthocyanin pathway, steps such as glycosylation and transport into the vacuole, are simply missing from the betalain species. This is not yet known.

Concluding Remarks

Decades of fruitful research into the regulation of flavonoid plant pigments has taught us a tremendous amount about widely conserved mechanisms of transcriptional control in plants. Much of this work has culminated in the MBW transcriptional complex paradigm regulating not only the flavonoid pigment pathway but also a suite of plant epidermal developmental pathways. Interestingly, recent studies in beets revealed that this regulatory mechanism was partially co-opted to regulate pigment biosynthetic genes in the betalain pathway. In addition, elucidating the TTG2 and PH3 WRKY protein regulatory mechanisms has recently contributed significant, novel modifications to our understanding of the MBW regulatory complex. However, some interesting questions remain regarding the exact molecular make-up, function and conservation of WRKY-containing transcriptional complexes. Do WRKY proteins simply join the MBW complex to modify its transcriptional activity? Alternatively, are there yet to be discovered new complexes centered on TTG2? Besides flower color modification through vacuolar acidification, do WRKY factors such as PH3 in *Petunia* also regulate the genes encoding transporters of pigment precursors into the tonoplast (similar to TTG2 in the Arabidopsis PA pathway)? Like flavonoid pigments, betalain pigments also accumulate in the vacuole. However, to date, there is no information about how betalains are transported to or enter this organelle. Have betalain-producing species co-opted other aspects of flavonoid pigment regulation such as the WRKY strategy for the regulation of pigment transport or acidification of the vacuole? It seems that ongoing studies of pigment pathways will still teach us much about regulatory mechanisms in plants for years to come.

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