Plant Physiology and Biochemistry 136 (2019) 178–187

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Review

Advance of the negative regulation of anthocyanin biosynthesis by MYB transcription factors

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ARTICLE INFO

Keywords:
Anthocyanin
MYB
Repressor
MBW
Transcription factor

ABSTRACT

Anthocyanins are secondary metabolites derived from the specific branch of the flavonoid pathway, responsible for red, purple and blue coloration display in the flowers and fruits. The functions of anthocyanins are diverse, including acting as visual signals to pollinators, defense against biotic and abiotic stresses. Thus, anthocyanins have been the most intensely studied secondary metabolite pathway. From model plants to horticultural crops, numerous studies have resulted in the discovery of highly conserved MYB–bHLH–WDR (MBW) transcriptional complex for the regulation of anthocyanin biosynthesis in plants. Recent discoveries have revealed that the anthocyanin biosynthesis pathway is also controlled by MYB repressors. Here we focus on the research progress into the role of MYB repressors in anthocyanin biosynthesis. In particular, we will discuss their functions and relationship to the MBW complex in the control of anthocyanin accumulation. In addition, an integrated regulatory network of anthocyanin biosynthesis controlled by MYB repressors and MBW activation complex is built based on the significant progress.

1. Introduction

Anthocyanins are water-soluble flavonoids that responsible for red, purple and blue coloration of flowers and fruits. They act as visual signals to pollinators as well as seed distributors (Davies et al., 2012). In vegetative tissues, anthocyanins are often induced by various biotic and abiotic stresses, such as pathogen attack, high light, low temperature, and phosphate deficiency (Dixon and Paiva, 1995). This induction suggests anthocyanins may serve as protectant due to their antioxidant activities (Albert et al., 2009). Anthocyanins are potent antioxidants efficiently scavenging free radicals and reactive oxygen species (ROS) during environmental stresses (Gould, 2004). Moreover, there are of growing interest in dietary anthocyanin-rich foods for human health benefits (Tsuda, 2012). Therefore, a better understanding of anthocyanin biosynthesis and its regulation is of both scientific and economic importance.

The anthocyanin biosynthesis pathway is conserved in higher plants (Koes et al., 2005; Hichri et al., 2011; Lloyd et al., 2017; Naing and Kim, 2018). Several enzyme-coding structural genes, including phenylalanine-ammonia lyase (*PAL*), 4-coumaryl:CoA ligase (*4CL*), chalcone synthase (*CHS*), chalcone isomerase (*CHI*), flavonoid-3′-hydroxylase (*F3′H*), flavonoid-3′,5′-hydroxylase (*F3′5′H*), flavanone 3-hydroxylase (*F3H*), dihydroflavonol 4-reductase (*DFR*), anthocyanidin synthase (*ANS*), and UDP-glucose: flavonoid 3-*O*-glucosyltransferase (*UFGT*) are involved in anthocyanins biosynthesis (Saito et al., 2013). The expression of these structural genes is regulated at transcriptional level by transcription factors (TFs). A transcriptional activation complex composed of R2R3-MYB, basic-helix-loop-helix (bHLH), and WD40 proteins (named MBW complex) has been shown to control the expression of anthocyanin structural genes (Koes et al., 2005; Xu et al., 2015). In addition, several TFs have also been reported to regulate anthocyanin biosynthesis via interaction with the MBW complex, such as COP1, JAZ, NAC, SPL, and WRKY (Gou et al., 2011; Qi et al., 2011; Maier et al., 2013; Zhou et al., 2015; Gonzalez et al., 2016; Verweij et al., 2016). Moreover, microRNA 156 and microRNA 858 negatively regulate anthocyanin biosynthesis by repressing the expression of their target genes, *MYB* genes and *SPL9*, respectively (Gou et al., 2011; Jia et al., 2015; Liu et al., 2017).

MYB TFs have been identified to be the major determinant regulators in the MBW complex in anthocyanin biosynthesis (Lai et al., 2014; Jin et al., 2016). Their temporal and spatial expression patterns determine the patterning and localization of anthocyanins. *AtPAP1* (*AtMYB75*) and its homologs have been shown to positively regulate the production of anthocyanins via activating the expression of structural genes (Cutanda-Perez et al., 2009; Rowan et al., 2009; Chagne et al., 2012). In addition to being activators in anthocyanin biosynthesis, two

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https://doi.org/10.1016/j.plaphy.2019.01.024
Received 10 September 2018; Received in revised form 8 January 2019; Accepted 19 January 2019
Available online 22 January 2019
0981-9428/

distinct groups of MYBs have been identified to repress anthocyanin production: R2R3-MYB and R3-MYB repressors, which contain one or two repeats of the MYB domain, respectively (Albert et al., 2014). In this review, we aim to give an overview of the research progress into the role of MYB repressors in anthocyanin biosynthesis and the further research to understand their complex regulatory network.

2. MYB transcription factors in plants

MYB proteins are a class of TFs with diversity of functions found in all eukaryotic organisms (Lipsick, 1996). They are characterized with one to four imperfect MYB repeats (R) conferring their ability to bind DNA. Each MYB repeat is about 52 amino acids long, forming three a–helices (Dubos et al., 2010; Feller et al., 2011). In plants, the largest group of MYB proteins is R2R3-MYBs, containing two MYB repeats that are most similar to R2 and R3 from c-MYB (Feller et al., 2011). R2R3-MYB TFs have a modular structure, with conserved N terminal MYB domains and variable C-termini usually containing an activation or repression domain. They have been divided into 22 subgroups on the basis of amino acid motifs in their C-termini (Stracke et al., 2001; Dubos et al., 2010).

The large size of MYB family in plants indicates their importance in the control of plant specific processes. In Arabidopsis, mutants have been extensively used to analyze the functions of R2R3-MYBs, leading to the discovery of their important roles in development, cell fate and identity, primary and secondary metabolism, and plant hormone- and pathogen-mediated stress responses (Stracke et al., 2001; Dubos et al., 2010; Feller et al., 2011; Ambawat et al., 2013). Recently, several single MYB (R3-MYB) proteins that appear to be functionally divergent from R2R3-MYB proteins have been described (Feller et al., 2011).

The phenylpropanoid pathway gives rise to a variety of compounds, such as stilbenes, lignins, and flavonoids (flavonols, anthocyanidins, and proanthocyanidins). Increasing evidences indicate that these phenylpropanoid pathways are finely tuned and tightly controlled by MYBs (Koes et al., 2005; Liu et al., 2016). In Arabidopsis, AtMYB75 (PAP1, At1g56650), AtMYB90 (PAP2, At1g66390), AtMYB113 (At1g66370), and AtMYB114 (At1g66380) of subgroup 6 promote anthocyanin biosynthesis in vegetative tissues by transcriptionally up-regulating the expression of the structural genes (Dubos et al., 2010). Three of these MYBs occur in order on chromosome 1, suggesting they may have arisen by tandem duplications of AtMYB75 (Lin-Wang et al., 2010). By examining gene families of MYBs of subgroup 6 in different angiosperms, they have generally retained the ability to activate anthocyanin biosynthesis through divergence in expression patterns and activation strength (Davies et al., 2012; Liu et al., 2015). Based on sequence homology, Lin-Wang et al. (2010) identified anthocyanin-promoting *MYB10* genes in rosaceous crops. However, some MYBs that repress the formation of anthocyanin have also been documented in some plant species (Liu et al., 2015). MYB proteins can act as transcriptional activators as well as repressors, providing the means for both reinforcement and feedback regulation. MYBs involved in anthocyanin formation or suppression in plants could be identified by phylogenetic analysis (Naing and Kim, 2018). The repression of anthocyanin by MYBs regarding transcriptional control of the structural genes is discussed in detail in this review.

3. Anthocyanin-related R2R3-MYB repressors

In plants, R2R3-MYBs play a vital role in regulation of anthocyanins biosynthesis. Among them there are positive and negative regulators of the structural genes of the pathway, which allows maintaining the balance of anthocyanin levels in different plant organs (Pérez-Díaz et al., 2016). R2R3-MYB repressors act to reduce anthocyanin production. The first such negative MYB regulator, AmMYB308, was characterized from *Antirrhinum majus* (Tamagnone et al., 1998). Subsequently, R2R3-MYB repressors have been identified in few plant species (Table 1), including strawberry FaMYB1 (for *Fragaria* × *ananassa*) (Aharoni et al., 2001) and FcMYB1 (for *Fragaria chiloensis*) (Salvatierra et al., 2013), petunia PhMYB27 (Albert et al., 2014), grapevine VvMYBC2-L1/3 and VvMYB4-like (Cavallini et al., 2015; Pérez-Díaz et al., 2016), poplar PtrMYB182 and PtrMYB57 (Yoshida et al., 2015; Wan et al., 2017), peach PpMYB17-20 (Zhou et al., 2016), apple MdMYB16 and MdMYB15L (Xu et al., 2017, 2018), Chinese narcissus NtMYB2 (Anwar et al., 2018). Interestingly, all these repressors belong to the subgroup 4 R2R3-MYBs, which to date includes only negative regulators involving in the regulation of the phenylpropanoid metabolism (Jin et al., 2000). Ectopic expression of these repressors in tobacco leads to the loss of pigmentation in flowers due to the repression in anthocyanin biosynthesis (Tamagnone et al., 1998; Aharoni et al., 2001; Cavallini et al., 2015; Pérez-Díaz et al., 2016; Anwar et al., 2018). Likewise, overexpression of PtrMYB57 repressed anthocyanin accumulation in poplar. By contrast, anthocyanin content was increased in *Ptrmyb57* mutants generated by the CRISPR/Cas9 system (Wan et al., 2017). Therefore, genetic, biochemical, and cell biological evidences suggest that these repressors function as part of a multidimensional regulatory network to regulate anthocyanin biosynthesis linked to environmental stimuli or developmental processes (Jun et al., 2015).

3.1. Structure of R2R3-MYB repressors

R2R3-MYB repressors contain conserved R2R3 domain at the N-terminal end (Fig. 1 and S1). In addition, they have the conserved motif of [D/E]Lx2[R/K]x3Lx6Lx3R within the R3 domain for interaction with the bHLH proteins (Zimmermann et al., 2004). Yeast two-hybrid experiments confirmed that PhMYB27, VvMYBC2-L1/3, and MdMYB16 could bind the bHLH proteins PhJAF13, PhAN1, MdbHLH33, respectively (Albert et al., 2014; Cavallini et al., 2015; Xu et al., 2017). In apple, MdMYB15L could not interact with MdbHLH33 when the bHLH binding sequence was knocked out (Xu et al., 2018). Furthermore, PtrMYB182 without a functional bHLH-binding site lost its repressor activity (Yoshida et al., 2015). Therefore, specific bHLH cofactors are needed for R2R3-MYB repressors to regulate anthocyanin biosynthesis, as well as plant MYB activators (Espley et al., 2007).

Within the C-terminal, the protein sequences are divergent both in sequence and in length (Figure S1). Two conserved motifs, namely, C1 motif (LIsrGIDPxT/SHRxI/L) and C2 motif (pdLNLD/ELxiG/S), are found in most of the R2R3-MYB repressors. The C2 motif has been proposed to be involved in the transcriptional repression of flavonoids and lignin biosynthesis pathway, containing the core sequence LxLxL or DLNxxP, which is part of the EAR repressor motif (Jin et al., 2000). EAR motif functions as transcriptional repressors, which is present in several repressor proteins, such as ethylene-responsive element binding factors (ERFs), SUPERMAN (SUP), and AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) proteins (Kagale et al., 2010). It has the ability to convert a transcriptional activator into a repressor by chimeric protein fusion experiments (Matsui et al., 2004; Kasajima and Sasaki, 2016). In contrast, deletion or mutation of the EAR motif results in a reduction or loss of suppression. For example, PhMYB27, PtrMYB57, and MdMYB16, require the EAR motif to repress the expression of target genes, as deletion of this motif reduce their repressor activity (Albert et al., 2014; Wan et al., 2017; Xu et al., 2017). MtMYB2 has a semi-conserved EAR sequence (MDIDL) at the C-terminal end in addition to the canonical EAR motif (LNLDL) in the C2 domain (Jun et al., 2015). Mutation of C2 motif (three Leu residues were changed to Ala) in MtMYB2 only partially compromised its repression, but additional mutation of the semi-conserved EAR sequence at the C-terminal end completely abolished its repressive activity (Jun et al., 2015). The amplification of the EAR motif in R2R3-MYB repressors may strengthen the mechanism of repression although the biological and evolutionary significance has not been systematically analyzed. Moreover, the function of EAR motif is not defined but may involve in chromatin remodeling (Kagale and Rozwadowski, 2011).

Table 1
MYB repressors involved in regulation of anthocyanins biosynthesis in plants.

Species	Gene	Type	Accession	Reference
Apple (*Malus* × *domestica*)	*MdMYB6*	R2R3	AAZ20429	Gao et al. (2011)
	MdMYB16	R2R3	HM122617	Xu et al. (2017)
	MdMYB15L	R2R3		Xu et al. (2018)
Arabidopsis	*AtMYB60*	R2R3	AF062895	Park et al. (2008)
	CPC	R3	BAA21917	Zhu et al. (2009)
	AtMYBL2	R3	AEE35154	Dubos et al. (2008); Matsui et al. (2008)
Brassica oleracea	*BoMYBL2–1*	R3		Song et al. (2018)
Brassica rapa	*BrMYB4*	R2R3	HQ317143	Zhang et al. (2014)
Chinese narcissus (*Narcissus tazetta*)	*NtMYB2*	R2R3	KY860527.1	Anwar et al. (2018)
Erythranthe cardinalis	*ROI1*	R3	AGC66792	Yuan et al. (2013)
Gentian (*Gentiana triflora*)	*GtMYB1R1*	R3	AB779612	Nakatsuka et al. (2013)
	GtMYB1R9	R3	AB779613	Nakatsuka et al. (2013)
Ginkgo biloba	*GbMYBF2*	R2R3	JQ068807	Xu et al. (2014)
Grapevine (*Vitis vinifera*)	*VvMYB4-like*	R2R3	XP_002273328	Pérez-Díaz et al. (2016)
	VvMYBC2-L1	R2R3	ABW34393	Cavallini et al. (2015)
	VvMYBC2-L3	R2R3	KM046932	Cavallini et al. (2015)
Iochroma loxense	*IlMYBL1*	R3	KY658469	Gates et al. (2018)
Medicago truncatula	*MtMYB2*	R2R3	XM_003616340	Jun et al., 2015
Peach (*Prunus persica*)	*PpMYB17*	R2R3	KT159233	Zhou et al. (2016)
	PpMYB18	R2R3	KT159234	Zhou et al. (2016)
	PpMYB19	R2R3	KT159235	Zhou et al. (2016)
	PpMYB20	R2R3	KT159236	Zhou et al. (2016)
Petunia (*Petunia hybrida*)	*PhMYB27*	R2R3	KF985023	Albert et al. (2014)
	PhMYBx	R3	KF985022	Albert et al. (2014)
Poplar (*Populus trichocarpa*)	*PtrMYB182*	R2R3	AJI76863	Yoshida et al. (2015)
	PtrMYB57	R2R3		Wan et al. (2017)
	PtrRML1	R3		Hu et al. (2016)
Snapdragon (*Antirrhinum majus*)	*AmMYB308*	R2R3	P81393	Tamagnone et al. (1998)
Strawberry (*Fragaria* × *ananassa*)	*FaMYB1*	R2R3	AF401220	Aharoni et al. (2001)
Strawberry (*Fragaria chiloensis*)	*FcMYB1*	R2R3	GQ867222	Salvatierra et al. (2013)
Tomato (*Solanum cheesmaniae*)	*SlTRY*	R3	MF197521	Tominaga-Wada et al. (2013)
	SlMYBATV	R3	MF197509	Cao et al. (2017)
Trifolium repens	*TrMYB133*	R2R3	KT699108	Albert (2015)

Phylogenetic analyses together with protein motif discovery using the Multiple EM for Motif Elicitation (MEME) bioinformatic tool divide the R2R3-MYB repressors into two clades such as AtMYB4-like and FaMYB1-like (Fig. 2a). GbMYBF2 (Xu et al., 2014) and BrMYB4 (Zhang et al., 2014) are closely related to AmMYB308 and AtMYB60, whereas TrMYB133 (Albert, 2015) and MYBC2 are more related to the strawberry FaMYB1 and petunia PhMYB27 anthocyanin repressors (Fig. 2a). In addition to the C1 and C2 motifs, some AtMYB4-like repressors possess C3 or zinc-finger (ZnF) motif (CX1-2CX7-12CX2C) and C4 motif (FLGLx4-7V/LLD/GF/YR/Sx1LEMK), which are absent in FaMYB1-like repressors (Fig. 2b and S1). Interestingly, a second repressor motif named TLLLFR (C5) was found at the C-terminus of FaMYB1-like repressors, such as VvMYBC2, VvMYB4-like, and PtrMYB182. TLLLFR-type repressor motif was originally identified in the flavonoid repressor AtMYBL2 (Dubos et al., 2008; Matsui et al., 2008). In Populus, PtrMYB182 had the TLLLFR-type repressor motif to function efficiently instead of the EAR repressor domain (Yoshida et al., 2015).

R2R3 domain is responsible for DNA binding of specific target and interaction with other cofactors. The two clades of R2R3-MYB repressors showed differences in the conserved element termed A2 box or element 3 (Cavallini et al., 2015). In AtMYB4-lke clade, the sequence of this element is DNEI, however, the sequence is DNEV in FaMYB1-like clade (Fig. 1). The importance of this element in R2R3-MYB repressors is worth further research in the future. The C-terminal of R2R3-MYBs is divergent and believed to be responsible for different regulatory functions. The presence of different motifs in the C-terminus of AtMYB4-like and FaMYB1-like repressors suggests potentially distinct mechanisms of action.

3.2. Expression patterns of R2R3-MYB repressors

R2R3-MYB repressors are constitutively expressed in both vegetative and reproductive plant tissues. In *Ginkgo biloba*, *GbMYBF2* was constitutively expressed in leaves, fruits, stems, and roots, with the relative higher expression in the roots (Xu et al., 2014). In grapevine, expression of *VvMYB4-like* was detected in roots, leaves, flowers, and skin, except in seed, where it was no longer detected (Pérez-Díaz et al., 2016). The level of transcription of *NtMYB2* gene in petals and corona was significantly higher than that in other tissues and the transcription level at the bud stage was highest (Anwar et al., 2018). Organ-specific expression analysis revealed lower levels of *FcMYB1* mRNA in flower, leaf, runner and roots of *F. chiloensis* compared to fruit (Salvatierra et al., 2013). To investigate the spatial and temporal expression patterns of *PtrMYB57*, Wan et al. (2017) transformed the plant binary vector containing a β-glucuronidase (*GUS*) reporter gene driven by the promoter of *PtrMYB57* into poplar, and histochemical GUS staining analysis showed that GUS activity was detected in all tissues except for roots (Wan et al., 2017).

Considering the relationship between R2R3-MYB repressors expression and anthocyanin accumulation, two expression pattern behaviors are observed reflecting different roles in response to environmental or developmental signals. The expression patterns of *MdMYB16* showed to be opposite to anthocyanins biosynthesis and the positive regulator (Xu et al., 2017). Such negative correlations in developmental or tissue-specific expression have been observed with VvMYB4-like, VvMYBC2-L3, PtrMYB182, GbMYBF2, and PhMYB27 repressors (Albert et al., 2011; Xu et al., 2014; Cavallini et al., 2015; Yoshida et al., 2015; Pérez-Díaz et al., 2016). These R2R3-MYB repressors are expressed in tissues to prevent ectopic accumulation of anthocyanins. On the other hand, *FaMYB1*, *PtrMYB57*, and *Tr-MYB133* expression correlated with the accumulation of anthocyanins, with high transcript abundance detected in high-anthocyanin tissues or development stages (Aharoni et al., 2001; Albert et al., 2011; Wan et al., 2017). These R2R3-MYB repressors are expressed when anthocyanins are being biosynthesized, and provide feedback regulation. The difference in the expression patterns of R2R3-MYB repressors raises questions about the prevention of

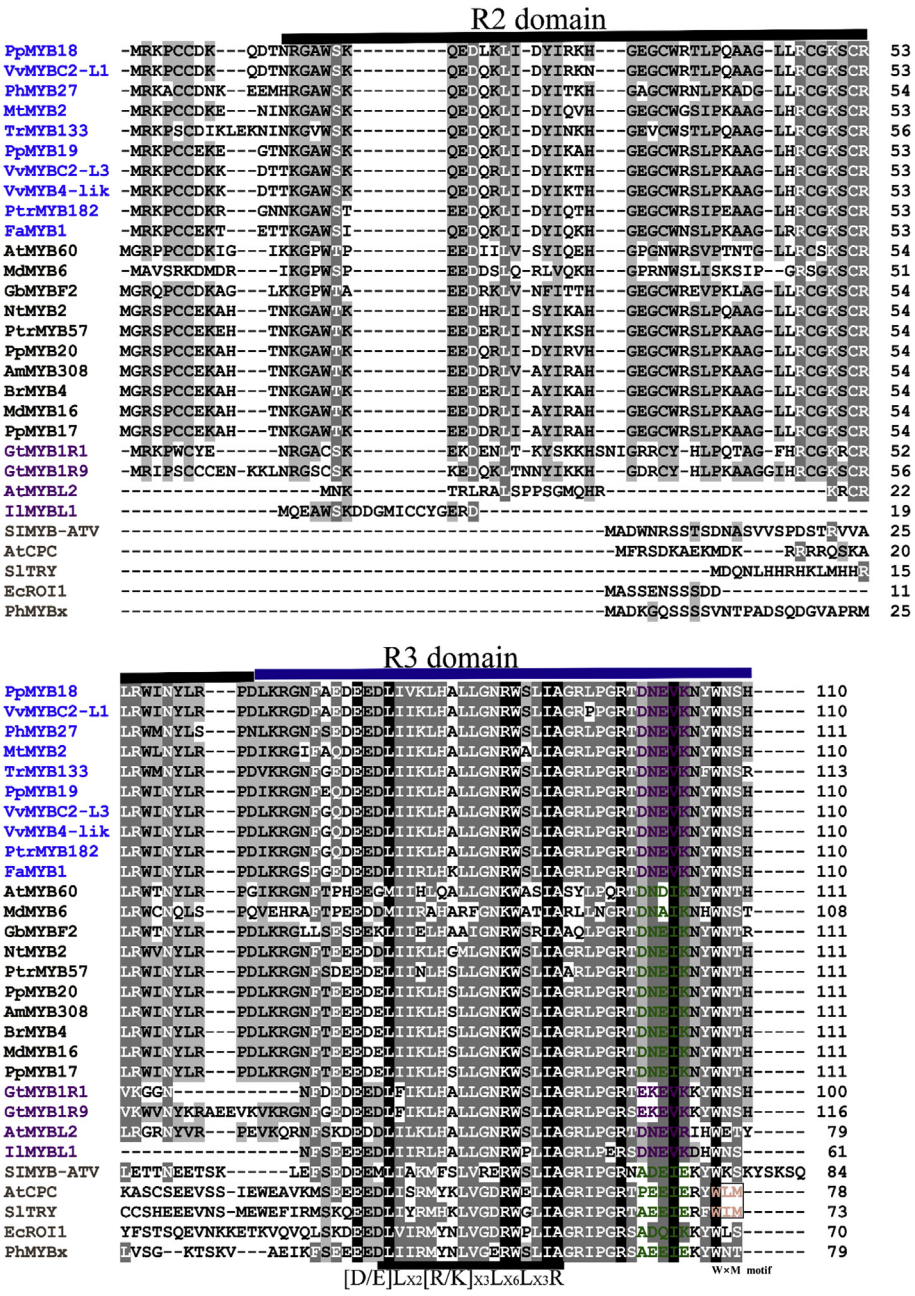

Fig. 1. Protein sequence alignment of R2R3 and R3 domains. Protein sequences of R2R3 domain or R3 domain for MYB repressors were aligned by the ClustalW program with default setting integrated in Geneious Pro5.5.6 software (Biomatters). Sub-clade members are shown with their names in different colors. The R2 and R3 MYB domains are indicated on top. bHLH-interacting residues within the R3 domain are underlined. Sub-clade specific features are highlighted in different colors. Sequences utilized for alignment were obtained from GenBank and the accession numbers are listed in Table 1. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

ectopic accumulation or feedback regulation are performed by same repressive MYB family members, or by other additional TFs. Moreover, the expression of R2R3-MYB repressors determines the anthocyanin levels in different genotypes. Fruit with high anthocyanin levels had low *MdMYB16* expression levels, while fruit with low anthocyanin levels had high *MdMYB16* expression levels (Xu et al., 2017). Likewise, *FcMYB1* showed higher transcript levels in white than in red strawberry fruits (Salvatierra et al., 2013).

The expression of R2R3-MYB repressors has been shown to be altered by different light conditions (Jin et al., 2000; Albert et al., 2011). *PhMYB27* is highly expressed during non-inductive shade conditions and down-regulated by high light (Albert et al., 2011). However, it is not clear how *PhMYB27* expression is repressed under high-light treatment. Overall, the R2R3-MYB repressors, which are either restricted to specific tissues or ubiquitous but differentially regulated by light or developmental signals, reflect different roles.

3.3. R2R3-MYB repressors negatively regulate anthocyanin biosynthesis

To determine R2R3-MYB repressors function in planta to repress anthocyanin biosynthesis, stable transgenic tobacco and Arabidopsis are usually generated. Heterologous expression of R2R3-MYB repressors in tobacco repressed anthocyanin biosynthesis to change the flower to almost white (Tamagnone et al., 1998; Aharoni et al., 2001; Pérez-Díaz et al., 2016; Anwar et al., 2018; Anwar et al., 2018, 2018). Notably, the expression level of these repressors in tobacco correlated with severity in loss of pigmentation in petals (Pérez-Díaz et al., 2016). Similarly, transgenic Arabidopsis plants that overexpress R2R3-MYB repressors accumulate less anthocyanin than wild type plants (Gao et al., 2011; Xu et al., 2014; Pérez-Díaz et al., 2016). However, the evidence obtained from heterologous systems is limited, because the need for considering the genetic background used in expression experiments (Salvatierra et al., 2013). Recently, overexpression of *PtrMYB182* in Poplar plants reduced anthocyanin biosynthesis and accumulation (Yoshida et al., 2015). In petunia, representative *PhMYB27*

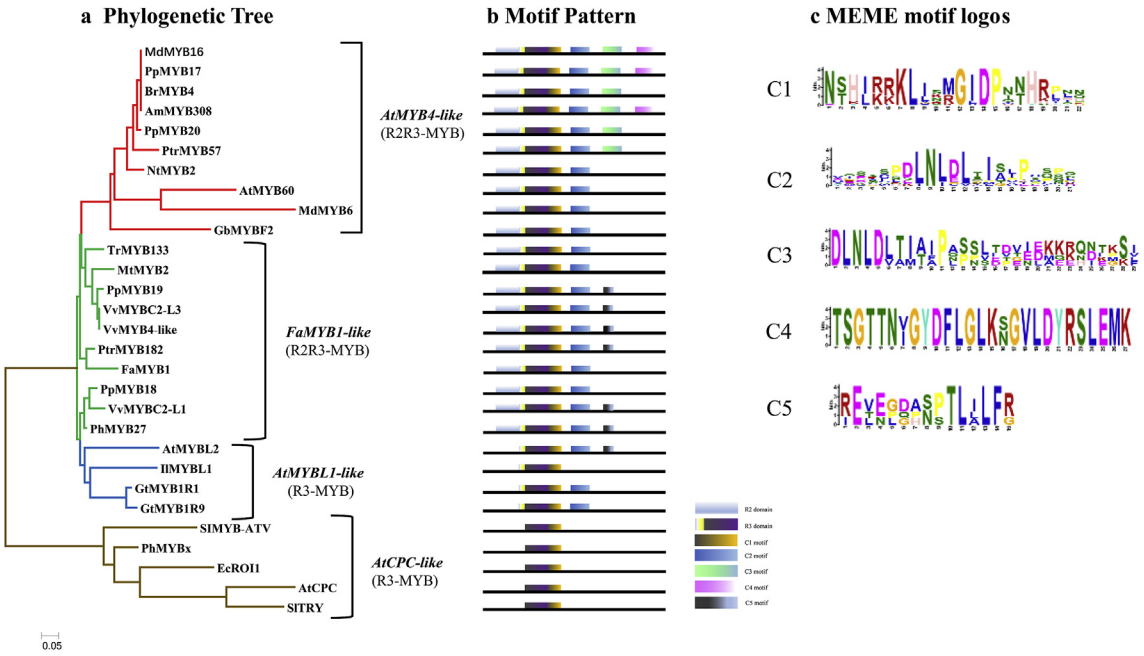

Fig. 2. Phylogenetic relationships and architecture of conserved protein motifs in R2R3-and R3-MYB repressors related to anthocyanin biosynthesis. a The phylogenetic tree was constructed based on the entire protein sequences using MEGA 5 software. Accession numbers are listed in Table 1. b The motif composition on every branch of the phylogenetic tree are represented by colored boxes. c The sequence for motifs C1-5 identified by MEME.

RNAi lines had increased anthocyanin accumulation, while overexpression lines had reduced anthocyanin level, indicating PhMYB27 as a repressor of anthocyanin biosynthesis (Albert et al., 2014). However, studies on gene function in plant species with either long life cycles or difficulties in transformation and regeneration impede the research process. Transient genetic transformation in crops might overcome this obstacle. Xu et al. (2017) overexpressed *MdMYB16* in the red-fleshed callus resulted in a change in callus coloration from red to yellow, a decrease in the anthocyanin content.

The repression of anthocyanin biosynthesis was also confirmed using transient assay in tobacco leaves. Visible anthocyanin accumulation was observed with syringe-infiltrated MYB activators. Co-infiltration of R2R3-MYB repressors with the above activators, however, prevented anthocyanin accumulation (Lin-Wang et al., 2011; Yoshida et al., 2015; Anwar et al., 2018). Overexpression of *MdbHLH33* in callus overexpressing *MdMYB16* weakened the inhibitory effect of *MdMYB16* on anthocyanin biosynthesis (Xu et al., 2017). Together, these results suggested that the anthocyanin biosynthetic pathway is controlled by a complex regulatory loop between R2R3-MYB repressors and MBW complexes (Albert et al., 2014).

3.4. Molecular mechanism of R2R3-MYB repressors in relation to anthocyanin biosynthesis

R2R3-MYB repressors play important roles in the regulation of anthocyanin biosynthesis. However, the molecular mechanism of the R2R3-MYB repressors in relation to anthocyanin biosynthesis still remains to be elucidated. Based on current research for Eudicots (particularly Arabidopsis and petunia), R2R3-MYB repressors may have two types, FaMYB1-like acted upon MBW complexes such as PhMYB27 (Albert et al., 2014) and AtMYB4-like directly binds on the promoter of target genes such as MdMYB16 (Xu et al., 2017).

3.4.1. FaMYB1-like type

In petunia, PhMYB27 was found to act upon MBW complexes to repress anthocyanin biosynthesis (Albert et al., 2014) and similar modes have also been shown for FaMYB1, VvMYBC2, MdMYB15L, PtrMYB182, and MtMYB2 (Paolocci et al., 2011; Cavallini et al., 2015; Jun et al., 2015; Yoshida et al., 2015; Xu et al., 2018). Based on these findings, a hierarchical and feedback gene regulatory network for anthocyanin biosynthesis has been suggested in eudicots, where MYB repressors are able to interfere with the proper assembly of the MBW activation complex (Albert et al., 2014).

In this model, FaMYB1-like type repressor cannot directly bind to the promoter of target genes and it is, therefore, unable to repress the transcription by itself. Instead, it acts as a corepressor that is incorporated into or binds MBW complexes to repress genes normally targeted by the MBW activation complex. The recruitment of FaMYB1-like type repressor to MBW complexes is bridged through dimerized bHLH factors present within the MBW complex (Albert et al., 2014). This is supported by the fact that they contain a bHLH interaction signature (Fig. 1) and directly interact with bHLH proteins (AtTT8 and PhAN1/PhJAF13) (Aharoni et al., 2001; Albert et al., 2014; Cavallini et al., 2015). Therefore, repression by FaMYB1-like type repressor requires the formation of the MBW complex, which specify the target genes, and the repressive domains such as EAR or TLLLFR motif, which perform transcriptional repression (active repression) (Fig. 3a).

FaMYB1-like type repressors target the same genes as those targeted by the anthocyanin MBW activation complex, suggesting that the recruitment of FaMYB1-like type repressors to MBW complexes change the complex activity from activation to repression (Albert et al., 2014). The characterization of FaMYB1-like type repressors in petunia, grapevine, and poplar suggest that they can inhibit anthocyanin biosynthesis at multilevels (Albert et al., 2014; Cavallini et al., 2015; Yoshida et al., 2015). First, they act to repress the transcription of late anthocyanin structural genes, such *ANS*, *DFR*, and *UFGT*. In addition to repression of structural genes, FaMYB1-like type repressors also restrain the abundance of bHLH and MYB activators. Albert et al. (2014) refer to this two-level repression as a “double lock-down” mechanism: direct down-regulation of flavonoid structural genes and the parallel down-regulation of the MBW transcriptional activation complex.

3.4.2. AtMYB4-like type

AtMYB4-like type repressors act as direct repressors by binding to the MYB motifs found in the promoters of many structural genes in the anthocyanin pathway, leading to the downregulation of this pathway

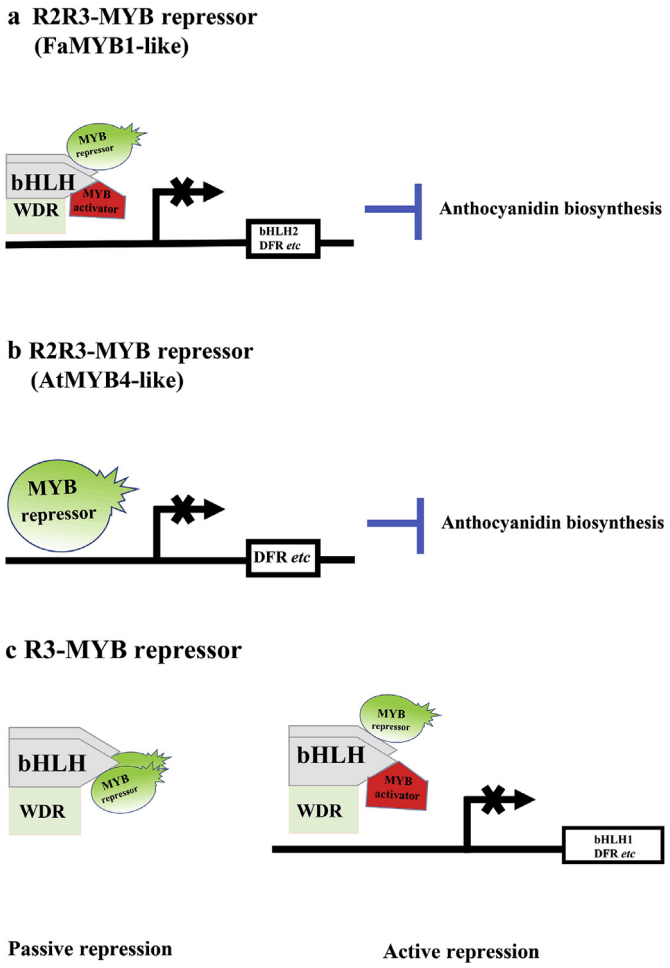

Fig. 3. Putative molecular mechanisms of MYB repressors in the regulation of expression of target gens. Schematic representation of the putative functional involvement of FaMYB1-like type R2R3-MYB repressor (a), AtMYB4-like type R2R3-MYB repressor (b), and R3-MYB repressor in anthocyanin biosynthesis (c). R2R3-MYB repressors could inhibit anthocyanin biosynthesis by incorporating into MBW activation complex as a corepressor or by binding on the promoter of target genes directly. R3-MYB repressors could block anthocyanin accumulation by titrating bHLH as passive repressors or by partnering with MBW activation complex and triggering active repression.

(Fig. 3b). They actively repress transcription through motifs in its C-terminal domains (Jin et al., 2000). Compared with FaMYB1-like repressor, some AtMYB4-like repressors possess C3 or C4 motif in the C-terminus (Fig. 2b and S1). The precise molecular mechanism of these motif mediated gene repression remains to be achieved.

In apple, MdMYB16 was found to form homodimers and directly inhibit the expression of *MdUFGT* and *MdANS* via its C-terminal EAR repressor (Xu et al., 2017). However, overexpression of *MdbHLH33* known as an activator of anthocyanin biosynthesis in callus overexpressing *MdMYB16* weakened the inhibitory effect of MdMYB16 on anthocyanin biosynthesis. The interaction between MdbHLH33 and MdMYB16 might influence the inhibitory effect of *MdMYB16* on anthocyanin biosynthesis (Xu et al., 2017). However, this regulatory model requires further validation.

Suppression of *ANS*, *DFR*, and *UFGT* has been a common feature observed in all transgenic tobacco and other plants heterologous expression of AtMYB4-like repressor (Pérez-Díaz et al., 2016; Xu et al., 2017; Anwar et al., 2018). In *AtMYB60*-overexpressing lettuce, AtMYB60 inhibited the anthocyanin biosynthesis by down regulating the expression of *DFR* gene (Park et al., 2008). The transcripts level of *UFGT* was significantly reduced in all transgenic tobacco overexpressing *NtMYB2* gene (Anwar et al., 2018). This is likely the result that AtMYB4-like repressor primarily exerts its repressive action on the end steps of anthocyanin biosynthesis. Recently, Xu et al. (2017) showed that apple MdMYB16 repressor directly bound the promoter of *ANS* and *UFGT* genes and inhibited their expression.

4. Anthocyanin-related R3-MYB repressors

In addition to R2R3-MYB repressors, single-repeat R3-MYB factors, such as Arabidopsis AtMYBL2 and AtCPC, petunia PhMYBx, gentian (*Gentiana triflora*) GtMYB1R1 and GtMYB1R9, poplar PtrRML1, tomato SlMYBATV and SlTRY have also been identified as negative regulators of anthocyanin production (Table 1) (Dubos et al., 2008; Matsui et al., 2008; Zhu et al., 2009; Nakatsuka et al., 2013; Tominaga-Wada et al., 2013; Albert et al., 2014; Hu et al., 2016; Cao et al., 2017). In *Mimulus*, ROSE INTENSITY1 (ROI1), an R3-MYB protein, has been identified as a major QTL for petal anthocyanin concentration (Yuan et al., 2013). A novel R3-MYB transcriptional repressor, IlMYBL1, was recently reported to be involved in the loss of floral pigmentation in *Iochroma* (Gates et al., 2018). In contrast, Song et al. (2018) reported that the purple *Brassica oleracea var. capitata F. rubra* is due to the loss of *BoMYBL2–1* expression, caused by either the promoter substitution or deletion of the gene. All these evidences suggest that R3-MYB repressors negatively regulate the biosynthesis of anthocyanin in parallel to R2R3-MYB repressors.

4.1. Structure of R3-MYB repressors

For the small R3-MYB repressors, including Arabidopsis CPC, petunia MYBx, and tomato SlMYBATV, they contain only an R3 domain and no repressive motif, but importantly, they have retained the motif responsible for binding to a bHLH partner (Figs. 1 and 2). Besides these small R3-MYB repressors, Arabidopsis AtMYBL2, a factor with a R3 domain and part of a R2 domain is also a negative regulator of anthocyanin biosynthesis (Dubos et al., 2008; Matsui et al., 2008). GtMYB1R1, GtMYB1R9, and IlMYBL1 showed high similarities to AtMYBL2 (Nakatsuka et al., 2013; Gates et al., 2018). Phylogenetic analyses divide the R3-MYB repressors into two clades and reveal the evolutionary derivation from each other (Fig. 2a). CPC-like type R3-MYB repressors fall into a single clade that is distantly related to MYBL2-like R3-MYB repressors and R2R3-MYB repressors. However, MYBL2-like R3-MYB repressors are closely related to R2R3-MYB repressors (Fig. 2a), raising the possibility that MYBL2-like R3-MYB repressors might be originated via tandem duplication followed by large deletions in the R2 domain. Like CPC, the MYBL2 clade has also retained the bHLH interaction motif in the R3 domain (Fig. 1). Additionally, AtMYBL2 has a repression motif (TLLLFR) in its C termini to actively repress transcription, and so the mechanism appears to be distinct from the CPC clade. Moreover, some CPC-like R3-MYB repressors contain the WxM motif that is required for cell-to-cell movement (Fig. 1), whereas the MYBL2 clade does not (Kurata et al., 2005).

4.2. Expression patterns of R3-MYB repressors

Like R2R3-MYB repressors, the expression pattern of R3-MYB repressors is either inversely correlated to anthocyanin biosynthesis to restrict the region of anthocyanin pigmentation or up-regulated when anthocyanin begin to accumulate to provide feedback inhibition. The expression of *AtMYBL2* appeared to be the opposite of anthocyanin biosynthesis in rosette leaves and stems (Dubos et al., 2008; Matsui et al., 2008). BoMYBL2–1, whose expression is inversely correlated to anthocyanin biosynthesis, is not detected in purple cabbages (Song et al., 2018). In contrast, *PhMYBx* was expressed in petals and anthocyanin-induced vegetative tissue (Albert et al., 2011). Likewise, the expression profiles of *GtMYB1R1* and *GtMYB1R9* gene were similar to those of the MYB activator for anthocyanin biosynthesis (Nakatsuka

et al., 2013). Moreover, the expression of R2R3-MYB repressors was sensitive to abiotic stress, such as strong light, or nitrogen depletion. Transcript levels for *PhMYBx* increased with high-light, while AtMYBL2 was negatively regulated by high light (Dubos et al., 2008; Nakatsuka et al., 2013). Interestingly, *CPC* expression was significantly increased in response to nitrogen depletion in Arabidopsis, exerting a negative feedback on anthocyanin accumulation during nitrogen starvation in rosette leaves (Nemie-Feyissa et al., 2014).

4.3. R3-MYBs repressors negatively regulate anthocyanin biosynthesis

Increasing evidences have suggested that R3-MYBs repress anthocyanin biosynthesis because loss-of-function mutations in these genes lead to stronger anthocyanin pigmentation (Dubos et al., 2008; Matsui et al., 2008; Zhu et al., 2009; Gates et al., 2018; Song et al., 2018). For example, knockout of the *AtMYBL2* and *CPC* gene in Arabidopsis increase anthocyanin accumulation in seedlings (Matsui et al., 2008). In addition, the negative effect of R3-MYB repressors on anthocyanin accumulation is also confirmed by ectopically expressed these genes. Transgenic tobacco plants expressing *CPC*, *GtMYB1R1*, or *IlMYBL1* gene exhibited significant reductions in floral anthocyanin accumulation, resulting in white-flowered phenotypes (Zhang et al., 2009; Nakatsuka et al., 2013; Gates et al., 2018). Overexpression of *SlTRY* and *SlMYBATV* strongly reduced the leaf anthocyanin content in tomato (Tominaga-Wada et al., 2013; Colanero et al., 2018).

Recently, Albert et al. (2014) demonstrated the non-cell-autonomous activity of PhMYBx in inhibiting anthocyanin biosynthesis using PhAN11 (WDR) complementation assay. Petunia *an11* mutant lacks a functional PhAN11 allele, preventing the formation of the MBW complex and leading to a lack of anthocyanin (de Vetten et al., 1997). Complementation by biolistic transformation with $35S_{pro}$:*PhAN11* resulted in multicellular foci that produced anthocyanins around the single transformed cell. By contrast, transformation with PhAN11 + PhMYBx inhibited the formation of these multicellular colored foci (Albert et al., 2014). Thus it is proposed that the intercellular movement of the WDR protein (PhAN11) and R3-MYB repressor (PhMYBx) might facilitate anthocyanin pigment pattern formation (Albert et al., 2014).

4.4. Molecular mechanism of R3-MYB repressors in regulating anthocyanin biosynthesis

The role of R3-MYB repressors in regulating anthocyanin biosynthesis is less clear than for the R2R3-MYB repressors (Koes et al., 2005; Zhu et al., 2009; Albert et al., 2014). The MYBL2-like R3-MYB repressors contain a repression motif in their C termini to actively repress transcription. They might function as active repressors that act upon MBW complexes to repress anthocyanin biosynthesis, similarly to the FaMYB1-like R2R3-MYB repressors described above. The CPC-like R3-MYB repressors, which contain only an MYB domain and no repressive motif, are thought to function as passive repressors through competition for bHLH partners with R2R3-MYB activators (Fig. 2b).

The CPC-like R3-MYB repressors interact with bHLH proteins in a competitive manner to prevent the formation of the MBW complex and so negatively regulate anthocyanin production. In Arabidopsis, CPC contains the conserved MYB–bHLH interaction motif and physically interacts with GL3/EGL3 (bHLH). CPC competes with the binding of PAP1/2 to GL3/EGL3, thus preventing the PAP1/EGL3 activation of *DFR* expression and negatively controlling anthocyanin biosynthesis (Zhu et al., 2009). Petunia PhMYBx, which is structurally similar to CPC and SlTRY, competes with R2R3-MYB activators for interaction with the bHLH protein PhAN1, leading to PhAN1 in inactive complexes (Albert et al., 2014). Interesting, protein-binding affinities differ between the CPC-like R3-MYB repressors and their bHLH partners, and can affect their competitive function.

5. An integrated regulatory network of anthocyanin biosynthesis controlled by MYB repressors and MBW activation complex

5.1. MYB activators and repressors operate in a hierarchy

Accumulating evidences indicate that anthocyanin regulatory network is organized in a hierarchical manner (Albert et al., 2014). The reciprocal control of expression levels between positive and negative MYB regulators form a complex regulatory loop to control and fine-tune the biosynthesis of anthocyanin. Three levels of action between MYB activators and repressors are proposed based on previous studies: (1) activators induce repressors, (2) repressors repress repressors, and (3) repressors repress activators (Cavallini et al., 2015). Albert et al. (2014) found that the *PhMYB27* and *PhMYBx* repressors were induced after overexpressing the anthocyanin activator *DPL* and *PHZ* in petunia leaves. Similarly, Yoshida et al. (2015) found the induction of PtrMYB182 repressor in poplar hairy roots overexpressing the *PtrMYB134* activator. Remarkably, the action of MYB repressors is subject to autorepression or repression of other members of the MYB repressors. *PhMYBx* and *PhMYB27* itself were identified as target genes of *PhMYB27* in petunia, suggesting they were both activated and repressed by the MBW complex (Albert et al., 2014). A down-regulation of *VvMYBC2-L1* was also observed in the *VvMYBC2-L3* transgenic grapevine hairy roots (Cavallini et al., 2015). This level of action is an indirect regulation or a direct repression remaining to be elucidated. Thus, MYB repressor appears to be transcriptionally activated by the MBW complexes and at the same time under its own repression in a feedback mechanism (Colanero et al., 2018). Concerning the transcriptional hierarchy of repressors on activators, Matsui et al. (2008) found that Arabidopsis AtMYBL2 represses expression of *AtTT8*, in addition to the anthocyanin biosynthetic genes. The repressive action could also be mediated by the down-regulation of MYB activators (Huang et al., 2014). Therefore, MYB repressors might act indirectly to suppress the expression of genes encoding bHLH and MYB activators, thus disrupting the MBW transcriptional activation complex.

5.2. An integrated model for anthocyanin regulation

In recent years, significant progress has been made to identify and characterize the regulatory components for anthocyanin biosynthesis in model and horticultural crops. A model has been built to describe the actions of the activators and repressors of the MBW complexes for anthocyanin biosynthesis (Albert et al., 2014).

Under noninductive conditions, R2R3-MYB activators are not expressed, while MYB repressors are highly expressed and bHLH1 and WDR are constitutively expressed. The MYB repressor may confer a repressive action to the anthocyanin biosynthetic genes, or prevent MBW complex formation through binding free bHLHs, inhibiting anthocyanin biosynthesis (Fig. 4a).

Under inductive conditions, anthocyanin biosynthesis is initiated by activating the expression of R2R3-MYB activator. The R2R3-MYB activator interacts with bHLH1 (PhJAF13/AtEGL3 homologs) and WDR to form a MBW activation complex that activates the expression of bHLH2 (PhAN1/AtTT8 homologs). Then the bHLH2 is able to form a core MBW activation complex with the R2R3-MYB activator and WDR, which activates the expression of the anthocyanin biosynthetic genes (e.g., *DFR*) to promote anthocyanin accumulation. The core MBW activation complex also activates the expression of bHLH2 to provide reinforcement, and the expression of R2R3-MYB and R3-MYB repressors to provide feedback inhibition (Fig. 4b). In tomato, the R3-MYB repressor *SlMYBATV* is targeted by the core MBW activation complex and at the same time itself is autoregulated to provide feedback repression (Colanero et al., 2018). A transcriptional regulatory loop is formed by AtMYBL2, AtTT8 and PAP1 that regulate anthocyanin biosynthesis in Arabidopsis. PAP1 activates the positive regulator of AtTT8, which is an activator of AtMYBL2, while AtMYBL2 negatively regulates the

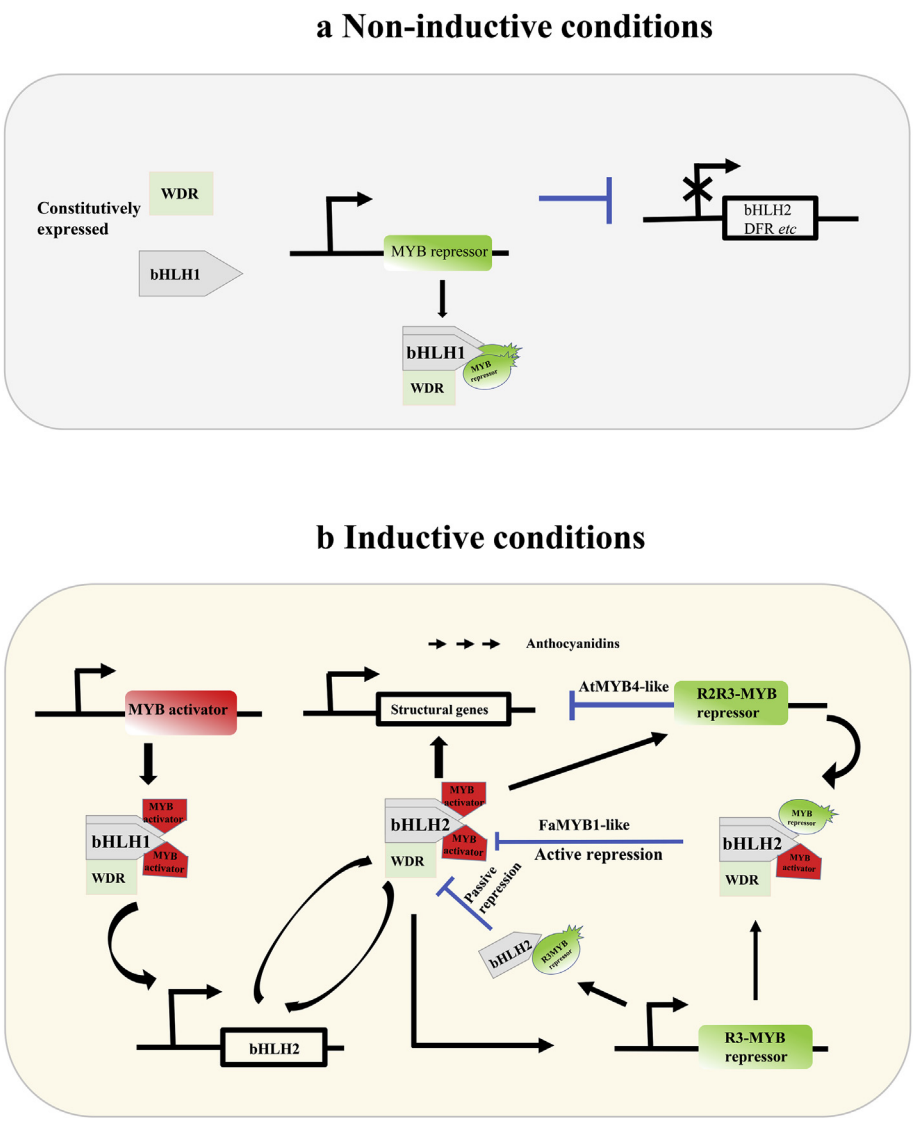

Fig. 4. Model of the anthocyanin regulation network in plant. This model is modified according to Albert et al. [24]. a Under noninductive conditions, R2R3-MYB activators are not expressed, while MYB repressors are expressed at high levels. MYB repressors directly inhibit the expression of target genes through repression motifs or titrate bHLH to prevent the formation of MBW complexes. b Under inductive conditions, R2R3-MYB activators are expressed and then form a MBW activation complex with bHLH1 (PhJAF13/AtEGL3 homologs) and WDR, which activates the expression of bHLH2 (PhAN1/AtTT8 homologs). Then a core MBW activation complex containing bHLH2 forms and activates the expression of the anthocyanin biosynthesis genes, ultimately resulting in anthocyanin accumulation. The core MBW activation complex also activates the expression of bHLH2 (reinforcement) and MYB repressors to provide feedback inhibition.

expression of AtTT8 (Matsui et al., 2008). Thus, antagonistic mechanism incorporating MBW activation complex and multiple negative regulators may facilitate the fine tuning of anthocyanin biosynthesis and protect plants from accumulation of excessive anthocyanins.

6. Conclusions and future prospects

Plants require sophisticated regulatory mechanisms to precisely regulate the biosynthesis of anthocyanins to adapt to myriad developmental and environmental signals. The fruitful research into the regulation of anthocyanin biosynthesis has taught us a widely conserved mechanism of transcriptional control in plants. The gene regulatory network is facilitated by not only the activation of the pathway but also its repression. Moreover, recent studies revealed that the hierarchical and feedback regulation by MYB activators and repressors add the complexity of the regulatory mechanisms. In addition, elucidating the MYB repressors regulatory mechanisms modify our understanding of the MBW regulatory complex. However, some interesting questions remain regarding the exact function and molecular mechanism of MYB repressors. To date, there is no information about the subspecialization of MYB repressors regarding their capacity to affect distinctive points of the phenylpropanoid pathway. It appears that the MYB repressors bind MBW complexes and that the targeting specificity may depend on the activator. Moreover, redirecting the metabolic flux along the phenylpropanoid pathway towards other branches may occur, but it need to be clarified in more diverse species. The correlation between the strength of the MYB repressor-bHLH interaction and the repress ability has not yet been examined. Besides the regulation by light, do other environmental stimuli (i.e. biotic stresses) and nutrient availability (i.e. sugar) also regulate its expression? Indeed, the regulation of MYB repressor expression needs to be elucidated. In addition, how the MYB repressor complex is removed? Is post-translational regulation involved in this process? Further information about the MYB repressors will help us to understand the mechanism for fine-tuning anthocyanin biosynthesis in plants.

Author contributions

L.C., and J.Z., wrote the manuscript and contributed equally. B.H., Y.Q., and G.H., wrote, edited, contributed equally and gave final approval of manuscript.

Acknowledgements

This research was funded by the National Natural Science Fund of China (No. 31872066), the National Key Research and Development Program (2018YFD1000200), the Pearl River S&T Nova Program of Guangzhou (No. 201610010147), China Litchi and Longan Industry

Technology Research System (No. CARS-32-05), the Key Science and Technology Planning Project of Guangzhou (No. 201804020063), YangFan Innovative & Entrepreneurial Research Team Project (No. 2014YT02H013), and the Guangdong Litchi Breeding, Propagation and Extension Union Construction Project.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.01.024.

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