Front. Biol. 2013, 8(6): 577–598
DOI 10.1007/s11515-013-1281-z

REVIEW

A review of target gene specificity of flavonoid R2R3-MYB transcription factors and a discussion of factors contributing to the target gene selectivity

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Abstract **Flavonoid biosynthetic genes are often coordinately regulated in a temporal manner during flower or fruit development, resulting in specific accumulation profiles of flavonoid compounds. R2R3-MYB-type transcription factors (TFs) "recruit" a set of biosynthetic genes to produce flavonoids, and, therefore, R2R3-MYBs are responsible for the coordinated expression of structural genes. Although a wealth of information regarding the identified and functionally characterized R2R3-MYBs that are involved in flavonoid accumulation is available to date, this is the first review on the global regulation of MYB factors in the flavonoid pathway. The data presented in this review demonstrate that anthocyanin, flavone/flavonol/3-deoxyflavonoid (FFD), proanthocyanidin (PA), and isoflavonoid are independently regulated by different subgroups of R2R3-MYBs. Furthermore, FFD-specific R2R3-MYBs have a preference for early biosynthetic genes (EBGs) as their target genes; anthocyanin-specific R2R3-MYBs from dicot species essentially regulate late biosynthetic genes (LBGs); the remaining R2R3-MYBs have a wider range of target gene specificity. To elucidate the nature of the differential target gene specificity between R2R3-MYBs, we analyzed the DNA binding domain (also termed the MYB-domain) of R2R3-MYBs and the distribution of the recognition *cis*-elements. We identified four conserved amino acid residues located in or just before helix-3 of dicot anthocyanin R2R3-MYBs that might account for the different recognition DNA sequence and subsequently the different target gene specificity to the remaining R2R3-MYB TFs.**

Keywords MYB, *cis*-element, DNA-binding domain, flavonoid, transcription factor, target gene specificity

Introduction

Flavonoids comprise one of the most abundant and important groups of secondary metabolites, which are widely distributed in plants (Ferrer et al., 2008). The flavonoid biosynthetic pathway is illustrated in Fig. 1. Chalcone synthase (CHS) is positioned at the entry point in the pathway and introduces the metabolic flux from the general phenylpropanoid pathway. After the step catalyzed by CHS, the flavonoid pathway branches off into seven branch-pathways, leading to anthocyanins, proanthocyanidins (or condensed tannins), 3-deoxyflavonoids (such as phlobaphene), aurones, flavones, flavonols, and isoflavonoids (Winkel-Shirley 2001). Chalcone and flavandiol (or leucoanthocyanidin) are another two common flavonoids, and flavonoid-related stilbenes, formed by CHS-like stilbene synthase (STS) (Schröder et al., 1988), are often mentioned in reviews of flavonoids. There has long been great interest in the flavonoid biosynthetic pathway because flavonoids provide important colors, and thereby beauty, to the plant kingdom, and many human health benefits have been ascribed to these molecules (Mol et al., 1998; Koes et al., 2005). Flavonoids are also closely related to the defense mechanism of plants against biotic and abiotic stress (Winkel-Shirley, 2002). The flavonoid biosynthetic pathway has been extensively studied over the past 30 years and is arguably the best understood pathway of secondary metabolites in higher plants (Quattrocchio et al., 2006).

A common denominator in the regulation of structural flavonoid pathway genes is the R2R3-MYB transcription

Received May 12, 2013; accepted September 10, 2013
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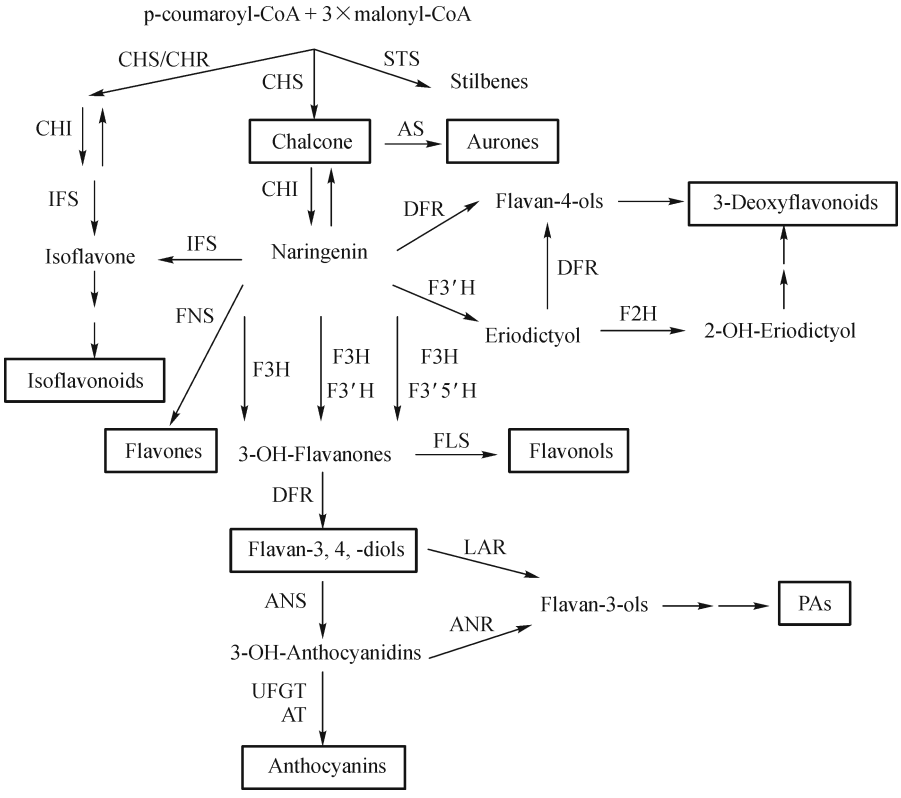

Figure 1 Schematic of the major branch pathways of flavonoid biosynthesis. The major classes of flavonoid products are shown in boxes. CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3′H, flavanone 3′-hydroxylase; F3′5′H, flavanone 3′5′-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanin synthase; GT, glycosyltransferases; AT, acyltransferase; CHR, chalcone reductase; IFS, isoflavone synthase; STS, stilbene synthase; AS, aurone synthase; FLS, flavonol synthase; FSN, flavone synthase; LAR, leucoanthocyanidin reductase; ANR, anthocyanin reductase; F2H, flavanone 2-hydroxylase. 160 × 139mm (200 × 200 DPI).

factor (TF), which is characterized by a structurally conserved DNA binding domain (also termed the MYB-domain) consisting of two imperfect repeats R2 and R3. It is known that MYB proteins regulating the anthocyanin and PA pathways require bHLH (basic Helix Loop Helix) and WD-repeat protein partners to synergistically enhance the expression of structural genes (Hichri et al., 2011a). They form a MYB-bHLH-WD40 (MBW) complex in the transcriptional regulation. In the complex, MYB proteins are the key components to activate discrete subsets of anthocyanin biosynthesis genes and therefore determine target genes selectivity; bHLH proteins may have overlapping regulatory targets; the WD40 regulators are expressed more or less ubiquitously and may not directly be involved in transcriptional activation. Recent studies indicate bHLH can also determine target genes selectivity in some regulation (Hichri et al., 2011b; Kong et al., 2012). For flavone/flavonol/3-deoxyflavonoid (FFD) regulation, however, the two partner factors are not required.

The R2R3-MYB regulatory genes constitute a large family of more than 100 members in higher plant species, including *Arabidopsis* (Stracke et al., 2001), maize (Du et al., 2012a), soybean (Du et al., 2012b), and *Camellia sinensis* (Zhao et al., 2013). The study of plant R2R3-MYBs began with the identification of maize (*Zea mays*) *C1* (Cone et al., 1986; Paz-Ares et al., 1987) and *PL* (Cone et al., 1993) genes that regulate anthocyanin synthesis in different tissues. Grotewold et al. (1991) determined the sequence of the *P* gene, which controls phlobaphene pigmentation in maize floral organs. Although *P* and *C1* regulate distinct flavonoid branches, these genes show a high homology (70%) in the MYB-domain. In contrast to the wealth of knowledge available for the R2R3-MYB regulation of the anthocyanin branch, a long period elapsed before much was known regarding the regulation of other flavonoid branches. The R2R3-MYB regulation of flavones, flavonols, and PAs has only been elucidated in a number of plant species in recent decade, see Table 1. These studies indicate that anthocyanins, FFDs, and PAs are independently regulated and that this differential regulation is managed via R2R3-MYB TFs. R2R3-MYB factors were previously categorised into dozens of subgroup on the basis of the conserved MYB-domain and amino acid sequence motifs present to the C-terminal side of MYB-domain (Stracke et al., 2001; Jia et al., 2004; Dubos et al., 2010). R2R3-MYB factors within a subgroup are usually conserved in regulating a similar flavonoid branch, but this does not mean they regulate the same set of flavonoid genes. How about the conservation of target gene specificity in each

Table 1 Cloned and identified *R2R3-MYB* genes that regulate the flavonoid biosynthetic pathway.

Species	MYB[1]	Regulation targets[2,3]	Functional description
Anthocyanin-specific R2R3-MYBs in dicots			
Antirrhinum majus	Roseal 1	CHS, CHI, F3H, F3′H, FLS, DFR, ANS, UFGT, AT	Floral pigmentation
	Roseal 2	CHS, CHI, F3H, F3′H, FLS, DFR, ANS, UFGT, AT	Floral pigmentation
	Venosa	CHS, CHI, F3H, F3′H, FLS, DFR, ANS, UFGT, AT	Floral pigmentation
Arabidopsis thaliana	PAP1	PAL1, CHS, F3′H, DFR, ANS, UFGT, GST, GT	Pigments in vegetative organs
	PAP2	PAL1, CHS, F3′H, DFR, ANS, UFGT, GST, GT	Pigments in vegetative organs
	MYB113	PAL1, CHS, F3′H, DFR, ANS, UFGT, GST, GT	Pigments in vegetative organs
	MYB114	PAL1, CHS, F3′H, DFR, ANS, UFGT, GST, GT	Pigments in vegetative organs
Brassica oleracea	MYB2	CHS, CHI, *F3H*, *F3′H*, *DFR*, *ANS*, *TT8*, *EGL3*	Red cabbage, purple kale, cauliflower
Capsicum annuum	A	CHS, DFR, ANS	Pigments in fruits
Citrus sinensis	MYBA		Red flesh of blood orange
Epimedium sagittatum	MYBA1	DFR, ANS	Red leaves
Garcinia mangostana	MYB10	DFR	Fruit colouration
Gentiana triflora	MYB3	CHS, F3′5′H, 5AT	Floral pigmentation
Gerbera hybrida	MYB10	*Strongly induces LBGs*	Floral pigmentation
Ipomoea batatas	MYB1	*CHS*, *CHI*, *F3H*, *DFR*, *ANS*, *GT*	Purple flesh of tuberous roots
Ipomoea nil	MYB1	CHS, CHI, F3H, F3′H, DFR, ANS, GT, GST	Floral pigmentation
Malus×domestica	MYB1[4]	DFR, GT	Skin colouration
	MYBA[4]	ANS	Skin colouration
	MYB10	*CHS*, *CHI*, *F3H*, *DFR*, *ANS*, *UFGT*	Skin and flesh colouration
Medicago truncatula	LAP1	*CHS*, CHI, *F3′H*, *DFR1*, *ANS*, *GT*, *LAR*, *ANR*	Anthocyanin accumulation
Myrica rubra	MYB1	*CHS*, *CHI*, *F3H*, *F3′H*, *DFR*, *ANS*, *UFGT*	Fruit colouration
Nicotiana tabacum	MYBAN2	CHS, CHI, F3H, DFR, ANS	Floral pigmentation
Petunia hybrida	AN2	DFR	Floral pigmentation
	DPL	*Induces flavonoid genes except FLS, 3GT, AT*	Anthocyanin in petal veins
	PHZ	*Induces flavonoid genes except F3′5′H*	Light-induced bud flush
Pyrus communis	MYB10	*PAL*, *CHS*, *CHI*, *F3H*, *DFR*, *ANS*, *UFGT*	Fruit skin pigmentation
Pyrus pyrifolia	MYB10	*PAL*, *CHS*, *CHI*, *F3H*, *DFR*, *ANS*	Fruit skin pigmentation
Prunus persica	MYB10	DFR, UFGT, LAR	Fruit skin pigmentation
Solanum lycopersicum	ANT1	*Induces both EBGs and LBGs*	Anthocyanin accumulation
Solanum tuberosum	D		Tuber skin
Vitis labruscana	MYBA	*PAL*, *CHS*, *CHI*, *F3H*, *DFR*, *ANS*, *UFGT*	Berry skin
Vitis vinifera	MYBA	CHI, FLS1, ANS, ANR, UFGT	Berry skin
Anthocyanin-specific R2R3-MYBs in monocots			
Lilium hybrid	MYB12	CHS, F3H, DFR	Floral pigmentation
Oncidium	MYB1	*Transient expression induces CHI, DFR*	Floral pigmentation
Zea mays	C1	At least FLS1, DFR, ANS, UFGT, GST	Anthocyanin, flavonol
	PL	At least CHS, DFR, UFGT	Vegetative and floral tissues
Flavone-, flavonol-, and 3-deoxyflavonoid-specific R2R3-MYBs			
Gentiana triflora	MYBP3	CHS, F3′H, FNSII, F3′5′H	Flavone in young buds
Solanum ycopersicum	MYB12	*PAL*, *C3H*, *CHS*, *CHI*, *F3H*, *F3′H*, *FLS*, *GT*	Red peel
Arabidopsis thaliana	MYB12	CHS, CHI, F3H, FLS1, F3′H, DFR	Flavonol mainly in roots
	MYB11	CHS, CHI, F3H, FLS1, F3′H, DFR	Flavonol mainly in cotyledons
	MYB11	CHS, CHI, F3H, FLS1, F3′H, DFR	Flavonol mainly in cotyledons
Vitis vinifera	MYBF1	CHI, FLS1, ANS, ANR, UFGT	Flavonol in flowers, skin
Sorghum bicolour	MYBY1	CHS, CHI, DFR	Phlobaphene
Zea mays	P	CHS, CHI, F3′H, FLS1, DFR, F2H1	Flavonol, maysin, phlobaphene

(*Continued*)

Proanthocyanidin-specific R2R3-MYBs			
Arabidopsis thaliana	TT2	CHS, CHI, F3H, F3′H, FLS, DFR, ANS, BAN	PA in seeds
Brassica napus	MYBTT2		PA in seeds
Diospyros kaki	MYB2	CHS, F3′H, F3′5′H, DFR, ANR, LAR	Wound-induced PA
	MYB4	ANR, LAR	PA in fruit; wound-induced PA
Fragaria×ananassa	MYB9	*F3′H*, *DFR*, *ANS*, *3GT*, *ANR*, *LAR*	PA in young fruits
	MYB11	*F3′H*, *DFR*, *ANS*, *3GT*, *ANR*, *LAR*	PA in young fruits
Lotus japonicas	MYBTT2	ANR	PA in roots and stems
Medicago truncatula	PAR	WD40	PA in seed coat
Populus tremuloides	MYB134	*Strongly induces LBGs*	Inducible PA in leaves
Prunus persica	MYBPA1	DFR, LAI, UFGT	PA in fruits
Theobroma cacao	MYBPA	*CHS*, *CHI*, *FLS*, *F3H*, *DFR*, *ANS*, *BAN*, UFGT	PA and anthocyanin
Trifolium arvense	MYB14	*Strongly induces LBGs in T. repens*	PA in leaves
Vaccinium corymbosum	MYBPA1		PA in young berries
Vitis vinifera	MYBPA1	CHI, FLS1, F3′5′H1, ANS, LAR1, LAR2, ANR, UFGT	PA mainly in seeds
	MYBPA2	*DFR*, *ANS*, *LAR1*, *LAR2*, *ANR*, *GT*, *PA1*	PA mainly in berry skin
	MYB5a	CHI, F3′5′H, ANS, ANR, LAR1, UFGT	PA in berries; general flavonoid
	MYB5b	CHI, F3′5′H, ANS, ANR, LAR1, UFGT	PA in berries; general flavonoid
Isoflavonoid and general flavonoids R2R3-MYBs, MYB repressors			
Glycine max	MYB176	CHS8	R1-MYB; isoflavonoid
Antirrhinum majus	MYB305	CHS, CHI, F3H, DFR, AS	Flavonoid in flowers
	MYB340	CHS, CHI, F3H, DFR, AS	Flavonoid in flowers
	MYB308	*Suppresses4CL*, *CHSin tobacco*	Repressor of lignin
	MYB330	*Suppresses4CL*, *CHSin tobacco*	Repressor of lignin
Arabidopsis thaliana	MYB4	*Suppresses CHS*, *C4H*	Phenylpropanoid
	MYBL2	*Suppresses CHS*, *CHI*, *F3H*, *F3′H*, *DFR*, *ANS*, *TT8*	R3-MYB; anthocyanin
Fragaria×ananassa	MYB1	*Suppresses CHS*, *F3H*, *DFR*, *ANS*, *GT*, RT, *GST in tobacco*	Repressor of flavonoid
Malus×domestica	MYB6	*Suppresses all flavonoid genes in Arabidopsis*	Repressor of flavonoid
Perilla frutescens	PfMYBP1	DFR	R3-MYB; anthocyanin
Petunia hybrida	MYB3	CHSJ	Flavonoid in petals
Pisum sativum	MYB26		Phenylpropanoid in flowers

[1] The corresponding references are: AmRoseal1, AmRoeal2 and AmVenosa (Schwinn et al., 2006); AtPAP1 and AtPAP2 (Borevitz et al., 2000; Gonzalez et al., 2008); AtMYB113 and AtMYB114 (Borevitz et al., 2000; Gonzalez et al., 2008); BoMYB2 (Chiu et al., 2010; Chiu and Li 2012; Zhang et al., 2012); CaMYBA (Borovsky et al., 2004); CsMYBA (Li et al., 2012b); EsMYBA1 (Huang et al., 2013); GmMYB10 (Palapol et al., 2009); GtMYB3 (Nakatsuka et al., 2008); GhMYB10 (Elomaa et al., 2003; Laitinen et al., 2008); IbMYB1 (Mano et al., 2007); InMYB1 (Morita et al., 2006); MdMYB1 (Takos et al., 2006); MdMYBA (Ban et al., 2007); MdMYB10 (Espley et al., 2007); MtLAP1 (Peel et al., 2009); MrMYB1 (Niu et al., 2010); NtMYBAN2 (Pattanaik et al., 2010); PhAN2 (Quattrocchio et al., 1998; Quattrocchio et al., 1999; Spelt et al., 2000); PhDPL and PhPHZ (Albert et al., 2011); PcMYB10 (Li et al., 2012a); *Pyrus pyrifolia* PpMYB10 (Feng et al., 2010); *Prunus persica* MYB10 (Ravaglia et al., 2013); SlANT1 (Mathews et al., 2003); StD (Jung et al., 2009); VlMYBA (Kobayashi et al., 2002); VvMYBA (Czemmel et al., 2009); LhMYB12 (Yamagishi et al., 2010; Lai et al., 2012); OMYB1 (Chiou and Yeh, 2008); ZmC1 (Cone et al., 1986; Goff et al., 1992; Sainz et al., 1997); ZmPL (Cone et al., 1993); GtMYBP3 (Nakatsuka et al., 2012); SlMYB12 (Ballester et al., 2010); AtMYB12 (Mehrtens et al., 2005; Stracke et al., 2007; Stracke et al., 2010); AtMYB11 and AtMYB111 (Stracke et al., 2007; Stracke et al., 2010); VvMYBF1 (Deluc et al., 2006; Czemmel et al., 2009; Matus et al., 2009); SbMYBY1 (Boddu et al., 2005, 2006); ZmP (Grotewold et al., 1994; Falcone Ferreyra et al., 2010; Sharma et al., 2012); AtTT2 (Nesi et al., 2001; Baudry et al., 2004); BnMYBTT2 (Wei et al., 2007); DkMYB2 (Akagi et al., 2010); DkMYB4 (Akagi et al., 2009); FaMYB9 and FaMYB11(Schaart et al., 2013); LjMYBTT2 (Yoshida et al., 2008); MtPAR (Verdier et al., 2012); PpMYBPA1 (Ravaglia et al., 2013); PtMYB134 (Mellway et al., 2009); TcMYBPA (Liu, 2010); TaMYB14 (Hancock et al., 2012); VcMYBPA1 (Zifkin et al., 2012); VvMYBPA1 (Bogs et al., 2007; Czemmel et al., 2009); VvMYBPA2 (Terrier et al., 2009); VvMYB5a (Deluc et al. 2006); VvMYB5b (Deluc et al., 2008); GmMYB176 (Yi et al., 2010; Dhaubhadel and Li, 2010); AmMYB305 (Jackson et al., 1991; Sablowski et al., 1994; Moyano et al., 1996); AmMYB340 (Jackson et al., 1991; Moyano et al., 1996); AmMYB308 and AmMYB330 (Tamagnone et al., 1998); AtMYB4 (Jin et al., 2000); AtMYBL2 (Dubos et al., 2008; Matsui et al., 2008); FaMYB1 (Aharoni et al., 2001); MdMYB6 (Gao et al., 2011); PfMYBP1 (Gong et al., 1999); PhMYB3 (Solano et al., 1995); PsMYB26 (Uimari and Strommer, 1997).

[2]The genes underlined are the target genes.

[3]Those in regular font indicate results based on direct evidence such as promoter assays, mRNA quantity analyses in loss-of-function plants, and in vitro DNA binding. Those in italic font indicate results based on indirect evidence from correlation analyses of expression patterns and overexpression evaluation in a heterologous or homologous system.

[4]MdMYB1and MdMYBA are identical in sequence.

R2R3-MYB subgroup? Can MYB domain explain target gene specificity? To address these questions, we present a deep review on the target gene specificity of the flavonoid R2R3-MYBs identified and characterized to date. We analyzed the DNA binding domain of R2R3-MYBs and the *cis*-element distribution in promoters of flavonoid structural

genes. This review provides information for the understanding of how R2R3-MYBs regulate flavonoid accumulation by controlling a set of structural genes and how R2R3-MYB regulation determines the accumulation pattern of flavonoid metabolites.

R2R3-MYB regulation of flavonoid branches

Flavones, flavonols, PAs, and anthocyanins are widely distributed among higher plant species. Isoflavonoids are only found in legumes and a small number of non-legume plants, and aurones are found in a few species of a range of plant genera, particularly in Scrophulariaceae, Plumbaginaceae, and Compositae (Falcone Ferreyra et al., 2012). A few species, including sorghum, maize, and Gloxinia, produce 3-deoxyflavonoids, and a number of unrelated plant species, including peanut, grape, and pine, produce stilbene (Falcone Ferreyra et al., 2012). The flavonoid metabolites that accumulate in a limited number of plant species might have evolved multiple times or might have been lost from specific plant lineages over the course of evolution (Winkel-Shirley, 2001).

R2R3-MYBs that regulate the biosynthesis of flavones, flavonols, anthocyanins, and PAs have been isolated and characterized from a range of plant species, whereas those regulating 3-deoxyflavonoids and isoflavonoids have been characterized only from Gramineae plants and soybean, respectively, see Table 1. No R2R3-MYBs that regulate biosynthesis pathway of aurone and stilbene have yet been reported. Chalcones and flavan-3,4-diols are the two major detected intermediate flavonoid metabolites in plants. Their regulation systems are not likely to be independent from those of flavonoid end products. In addition to R2R3-MYBs that are specific to a single flavonoid branch, there are some contributing to multiple flavonoid braches, essentially regulating the general flavonoid pathway.

Anthocyanin-specific R2R3-MYBs

There have been several important reviews on the molecular structure and biosynthetic pathway of anthocyanin (Springob et al., 2003; Grotewold, 2006). After characterizing the anthocyanin structural genes, researchers began identifying the regulatory factors, and it was found that R2R3-MYB TFs play a central role in anthocyanin regulation (Allan et al., 2008). Such knowledge is the foundation for creating novel flower colors through genetic engineering (Tanaka et al., 2009; Nishihara and Nakatsuka, 2011).

Under normal conditions, anthocyanins are typically produced in flowers and fruits during late developmental stage, a time when these reproductive organs undergo large physiologic changes. For flowers, it is the stage when the petals rapidly grow due to cell expansion (Martin and Gerats, 1993); for small berries, it is the stage called veraison, which represents the transition from berry growth to berry ripening. The investigation of the transcription profiles of anthocyanin structural genes in various plant species suggests that the genes usually can be divided into two groups. For example, in gentian (*Gentiana triflora*) for which the major flower pigment is delphinidin-based anthocyanin, *F3H*, *F3'5'H*, *DFR*, *ANS*, *UFGT*, and *5AT* have a transcription profile during petal development paralleling with anthocyanin biosynthesis, whereas *CHS*, *CHI*, and *F3'H* do not (Nakatsuka et al., 2005). This difference in transcription profile is consistent with the positions of the enzyme coding genes in flavonoid pathway (Fig. 1). *CHS* and *CHI* are upstream of *F3H*, *F3'5'H*, *DFR*, *ANS*, *UFGT*, and *5AT* in the pathway. In eustoma, *F3H* is grouped with *CHS* and *CHI* instead of with *F3'5'H*, *DFR*, and *ANS* based on their expression profiles (Noda et al., 2004). *F3H* classification is different between gentian and eustoma. This is because *F3H* is required for flavonol in addition to anthocyanin biosynthesis in eustoma. In fact, studies in major dicot flowers, fruits and small berries reveal similar patterns of coordinated gene expression, as found in snapdragon (Schwinn et al., 2006), pear (Li et al.,, 2012a) and bayberry (Niu et al., 2010). Grape (*Vitis vinifera*) is an exception, as only *UFGT* shows an expression profile consistent with anthocyanin accumulation (Boss et al., 1996). Monocot flowers, however, exhibit a different coordinated expression pattern of anthocyanin genes from dicot species. Orchids (*Oncidium*) and lilies (*Lilium* spp.) essentially show a pigmentation-correlated expression profiles of *CHS* genes (Chiou and Yeh, 2008; Lai et al., 2012). In this review, we divided the enzyme coding genes for anthocyanin biosynthesis (or central flavonoid pathway genes) into two groups: early biosynthetic genes (EBGs) that includes *CHS* and *CHI* and late biosynthetic genes (LBGs) that includes *DFR*, *ANS*, *UFGT* and *AT*. Genes *F3H*, *F3'H*, and *F3'5'H* belong to either EBGs or LBGs depending on their expression pattern. This classification method is different to the traditional method as shown in gentian and eustoma which is totally based on expression pattern.

The differential expression pattern of EBGs and LBGs in gentian flowers is due to R2R3-MYB regulation. Anthocyanin-specific GtMYB3 activates the promoters of *GtF3'5'H* and *Gt5AT* but not *GtCHS* in a transient expression assay, indicating that EBGs are not the target genes (Nakatsuka et al., 2008). A later study found that EBGs and *F3'H* are under the control of GtMYBP3, a flavone-specific R2R3-MYB TF (Nakatsuka et al., 2012). It is interesting that the primary target genes of dicot anthocyanin-specific R2R3-MYBs appear to be LBGs, at least not EBGs. Anthocyanin-specific VvMYBA can activate the *VvUFGT* promoter but not those of any other genes, including *VvANS* (Kobayashi et al., 2002; Czemmel et al., 2009). Some studies indicate that EBG expression can be closely correlated with *R2R3-MYB* regulator genes and R2R3-MYBs can induce EBGs in transgenic plants, but there is no evidence demonstrating that anthocyanin R2R3-MYBs of dicot species directly

activate EBGs (Table 1). Overexpression evaluation method is unreliable for the determination of the primary target genes of an R2R3-MYB regulator. For example, Borevitz et al. (2000) identified AtPAP1, an anthocyanin activator in *Arabidopsis* seedlings, by activation tagging methods and found that entire phenylpropanoid pathway was upregulated by AtPAP1. However, plants harbouring an RNAi construct targeting *AtPAP1* and similar sequences of *AtPAP2*, *AtMYB113*, and *AtMYB114* exhibited the downregulation of only LBGs (Gonzalez et al., 2008). This result clarifies that the target genes of AtPAP1 are restricted to only LBGs. Therefore, we emphasize the information of target gene specificity based on direct evidences as defined in Table 1. In contrast to those of dicot species, anthocyanin-specific R2R3-MYBs of monocots seem to regulate both EBGs and LBGs, as demonstrated in *Oncidium*, lily, and maize (Table 1).

To date, 34 anthocyanin-specific R2R3-MYBs from species in 21 genera have been functionally characterized (Table 1), with apparent diversity in target gene specificity between monocot and dicot species. Specifically, anthocyanin-specific R2R3-MYB TFs in dicot species have not been reported to activate EBGs such as *CHS*, whereas those in monocot species have a wide range of target genes. It is interesting that, based on the MYB-domain and conserved motif in the carboxy terminus, dicot R2R3-MYB TFs (except GtMYB3) belong to subgroup 6 (SG6), whereas monocot R2R3-MYB TFs (except LhMYB12) belong to SG5. The structural differences between the SG5 and SG6 proteins may account for their different target gene specificity. As the MYB-domain is known to contribute to the binding selectivity of an R2R3-MYB TF, we performed a phylogenetic tree analyses based only on the MYB-domain (Fig. 2). Basically, utilizing only the MYB-domain or the full amino acid (AA) sequence does not make a large difference to the R2R3-MYBs group based on sequence similarity. More details will be discussed in the section "DNA binding site of MYBs".

Proanthocyanidin-specific R2R3-MYBs

PAs are oligomeric and polymeric flavan-3-ols (Fig. 1). The biosynthetic pathway leading to PAs has been reviewed previously (Dixon et al., 2005; Xie and Dixon, 2005). LAR and ANR are the two key enzymes introducing the metabolic flow into the biosynthesis of catechin and epicatechin, respectively, two flavan-3-ols PA monomers. Pollination constant and non-astringent (PCNA)-type persimmons (*Diospyros kaki*) produce PAs in the fruit flesh within 9 weeks after blooming, whereas astringent (A)-type persimmons produce PAs throughout fruit development (Ikegami et al., 2007). This differentiated PA accumulation pattern is due to DkMYB4, a protein that regulates PA biosynthesis in the fruit flesh (Akagi et al., 2009). DkMYB4 is able to activate the *DkANR* promoter but not the *DkLAR* promoter in a dual-luciferase assay (Akagi et al., 2010). Moreover, a DkMYB4 recombinant protein bind to the promoters of *DkF3'5'H* and *DkANS* in an electrophoretic mobility shift assay (EMSA), indicating that these genes are also putative primary targets (Akagi et al., 2009). Another PA regulator, DkMYB2, is able to significantly activate the promoters of *DkANR* and *DkLAR*, though it is less expressed than DkMYB4 and its expression profile appears not to be correlated with structural gene expression under normal conditions (Akagi et al., 2010). During the fruit development of blueberry (*Vaccinium corymbosum*), *LAR* and *ANR* show PA-specific expression profile, while the remaining flavonoid genes that are also required for anthocyanin biosynthesis do not (Zifkin et al., 2012). VcMYBPA1 is able to activate the poplar *PtANR* promoter in a dual-luciferase assay, indicating its role in PA regulation, though the primary target genes of VcMYBPA1 need to be identified. VcMYBPA1 has a transcription profile that parallels PA and flavonol accumulation during the early stage and anthocyanin accumulation during the late stage, indicating this R2R3-MYB may also control flavonol and anthocyanin. However, no R2R3-MYB TFs playing a central role in multiple flavonoid branches have been identified thus far, except for maize P and C1. Grape produces PAs mainly in the berry skin during the early stages and in seeds at the veraison stage. Two major PA-specific R2R3-MYB TFs in grape have been identified, VvMYBPA1 (Bogs et al., 2007) and VvMYBPA2 (Terrier et al., 2009), and their tissue-specific expression patterns indicate they may be responsible for regulation in seeds and berry skin, respectively. VvMYBPA2 may upregulate VvMYBPA1 *in vivo* because plants overexpressing MYBPA2 have enhanced transcription level of MYBPA1 (Terrier et al., 2009). It is notable that neither the overexpression of VvMYBPA1 nor that of VvMYBPA2 enhances the transcription level of *LAR2*. Another two R2R3-MYB TFs, VvMYB5a (Deluc et al., 2006) and VvMYB5b (Deluc et al., 2008), are reported to regulate PA syntheses in grape. In contrast with other PA-specific R2R3-MYBs, VvMYB5a and VvMYB5b are suggested to participate in the regulation of multiple flavonoid products since these two genes induce strong accumulation of other flavonoids such as anthocyanins in tobacco. Here, since there are no solid evidences showing VvMYB5a and VvMYB5b control other branches and these two factors in fact are not capable of activating *VvUFGT* promoter, we group them with PA-specific MYBs. In strawberry (*Fragaria* × *ananassa*), FaMYB9 and FaMYB11 have recently been reported to regulate PA syntheses during the small green fruit stage (Schaart et al., 2013). The studies in fruits or berries mentioned above indicate the entire flavonoid pathway genes that are necessary for PAs syntheses are regulated by PA-specific R2R3-MYBs; especially, the study in grape has provided direct evidence to support this.

However, PA-specific R2R3-MYBs in other plant species show a different target gene range. AtTT2 controls PAs syntheses in *Arabidopsis* seed coat. PA accumulation begins to occur approximately 5 days after flowering (Routaboul et

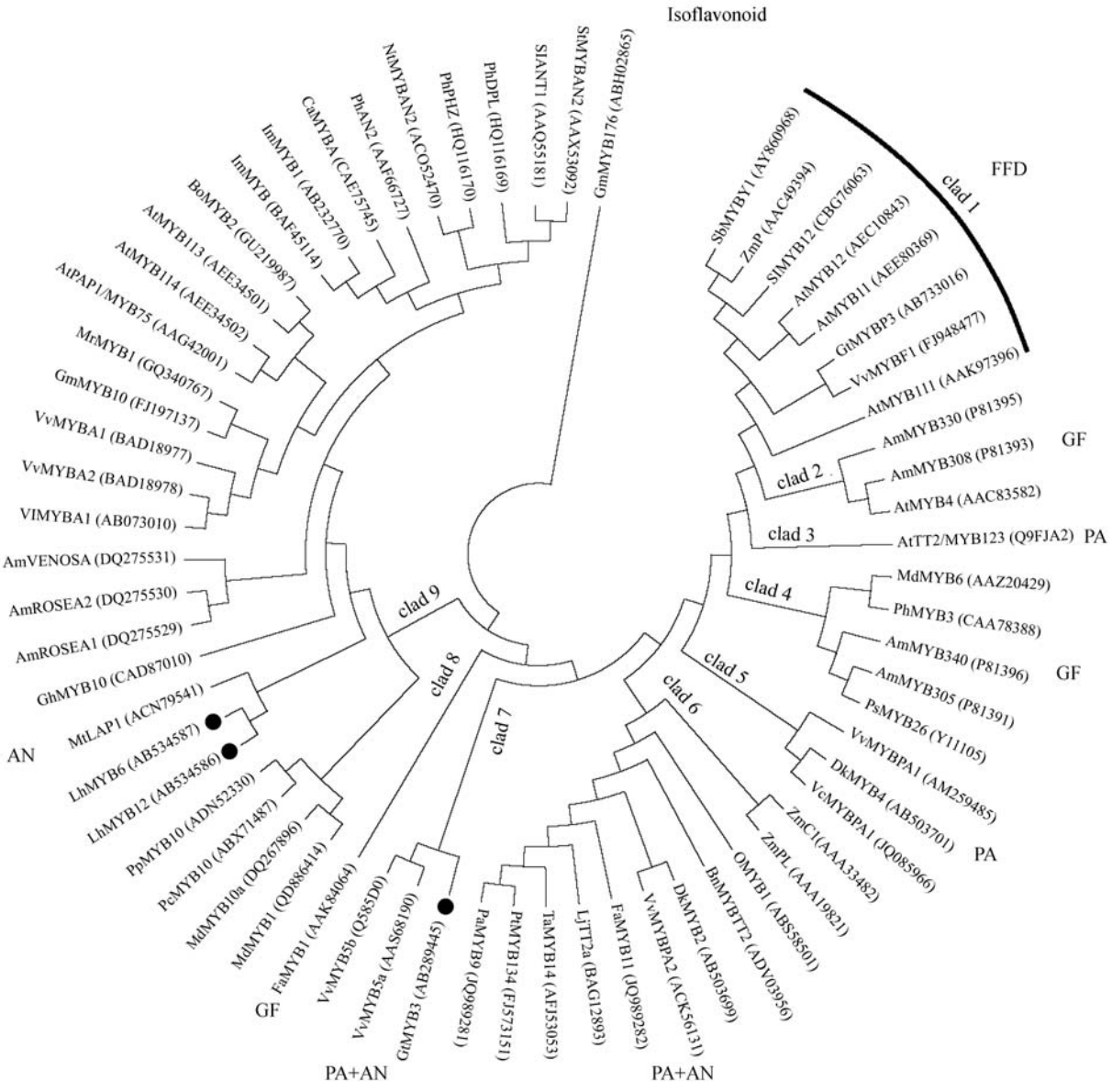

Figure 2 Phylogenetic tree based on DNA binding domains showing that flavonoid-regulating R2R3-MYBs form nine clades. The tree was constructed using MEGA 4.0 (neighbor joining) and displays only topology. The amino acid sequences were retrieved from the DDBJ/EMBL/GenBank databases with the accession numbers shown in brackets. LhMYB12 and LhMYB6 (black dot) are from the monocot lily but cluster with dicot species; gentian GtMYB3 (black dot) clusters with VvMYB5a and VvMYB5b that regulate PA synthesis. Flavonoid branches that each clade is related are given in the outer ring. FFD, flavone/flavonol/3-deoxyflavonoid; GF, general flavonoids; PA, proanthocyanidin; AN, anthocyanin. 166 × 160mm (200 × 200 DPI).

al., 2006). Transcripts of *CHS*, *CHI*, *DFR*, *ANS*, and *ANR* are detected in the seed coats, but these genes showed different expression profiles prior to the torpedo stage and, therefore, can be divided into two subsets (Devic et al., 1999). On the one hand, the transcription levels of *CHS*, *CHI*, *F3H*, *F3'H*, and *FLS1* are high from the bud stage onward. On the other hand, the *DFR*, *ANS*, and *BAN* transcription levels peak at the globular stage, consistent with TT2 (Nesi et al., 2000). The *tt2* mutation results confirm that TT2 does not regulate EBGs (Nesi et al., 2001). A recent study suggests that TaMYB14 may control PA synthesis in the young leaves of *Trifolium arvense* (Hancock et al., 2012). The overexpression of *TaMYB14* in *Trifolium repens*, which does not produce PAs, results in the consistent upregulation of LBGs and PA-specific genes. Poplar (*Populus tremuloides*) constitutively produces a large amount of PAs in the leaves, but PA biosynthesis is also often upregulated by environmental stimuli, including herbivore attack or wounding (Peters and Constabel, 2002), fungal infection (Miranda et al., 2007), and abiotic stresses of high light levels and nutrient limitation

(Osier and Lindroth 2001). Poplar PtMYB134 is reported to regulate inducible PA accumulation in the leaves. The genes induced by high light stimulation can be divided into two groups according to their induced expression pattern (Mellway et al., 2009). The genes after *F3H* are coordinately induced with PtMYB134, indicating that PtMYB134 targets LBGs in light induction. In summary, AtTT2, TaMYB14 and PtMYB134 are likely to primarily target LBGs in addition to *LAR* and *ANR in planta*.

In conclusion, the target gene specificities of PA-specific R2R3-MYBs vary and appear to depend on plant species. Based on our phylogenetic tree analyses, PA-specific R2R3-MYBs form four clades: clade 3, clade 5, clade 6, and clade 7 (Fig. 2). DkMYB2 and PtMYB134 cluster together in clade 6 and recognize AC-elements (Mellway et al., 2009; Akagi et al., 2010). In contrast, clade 2 members seem to recognize a *cis*-element called MYBCORE (5′-CNGTTR-3′) that is similar to animal MYB recognition site. DkMYB4 binds to MYBCORE but not any other MYB binding *cis*-element (Akagi et al., 2009). VvMYBPA1 is suggested to recognize the same MYBCORE element and, thus, regulate a range of structural genes (Bogs et al., 2007). However, there seems no difference regarding target gene specificity between clade 2 and clade 6. This may be due to a wide distribution of *cis*-elements recognized by clade 2 and clade 6 members among flavonoid genes. Analyses of the binding ability and target gene specificity of more PA-specific R2R3-MYBs are necessary to obtain further insight.

Flavone- and flavonol-specific MYBs

The biosynthetic pathway of flavones and flavonols have been previously reviewed (Martens and Mithöfer, 2005; Martens et al., 2010). Many flowers produce flavones or flavonols to protect the reproductive organs against UV damage. Gentian (Fujiwara et al., 1998; Nakatsuka et al., 2005) and snapdragon (Martin et al., 1991) produce flavones at the early flower stages when anthocyanin biosynthesis has not yet begun. The same accumulation pattern of flavonols is found in petunia (Saito et al., 2006) and Eustoma (Noda et al., 2004). *FNS* and *FLS* are specific to flavones and flavonols, respectively, and are often coordinately expressed with EBGs, as found in most flowers. GtMYBP3 and GtMYBP4 are reported to control flavone synthesis in gentian flowers (Nakatsuka et al., 2012). Both genes can activate the promoter of *GtF3'H*, in addition to *GtCHS* and *GtFNS*, but not *GtF3'5'H*. *F3'H* is most likely essential for flavone formation after the step catalyzed by FNS.

In lily, flowers produce a single flavonol and anthocyanin at the same time during the late stages, with the flavonol concentration being much lower than that of the anthocyanin (Lai et al., 2012). The R2R3-MYB TFs regulating flavones or flavonols in monocot flowers remain unknown; furthermore, it is not clear whether other monocot flowers have the same accumulation profiles of flavonols or flavones.

Flavonols accumulate in the peel of ripening tomato (*Solanum lycopersicum*) fruits. In red-fruited tomatoes, *CHS*, *F3H*, and *FLS* transcription levels increase during ripening, peak at the turning stage, and then decrease slightly at the red stage; the transcription level of *CHI* remains low and even decreases upon ripening (Bovy et al., 2002). Therefore, *CHI* is thought to be a rate-determining step in flavonol accumulation. Grape VvMYBF1 specifically activates the promoters of the flavonoid pathway genes involved in flavonol synthesis (Czemmel et al., 2009). Surprisingly, the promoter of the *VvDFR* gene that is involved in the synthesis of anthocyanins and PAs is moderately activated by VvMYBF1. DFR may participate in an unknown alternative route to produce flavonols, similar to the role of F3′H in gentian flowers.

The regulation system of flavonol accumulation has been systematically revealed in *Arabidopsis*. AtMYB12, AtMYB11, and AtMYB111 control flavonol biosynthesis in *Arabidopsis* (Mehrtens et al., 2005; Stracke et al., 2007). These three TFs show differential spatial regulation, but they bind to the same targets in a promoter assay. MYB12 mainly controls flavonol biosynthesis in roots, whereas MYB111 predominantly regulates biosynthesis in cotyledons. These three factors are required for flavonol accumulation because mutant *MYB11-12-111* seedlings cannot produce flavonol compounds. The targets of the three TFs are EBGs and *FLS*. In general, AtMYB12 has stronger *trans*-activation capacity for the four target genes than AtMYB111, which, in turn, has higher activity than AtMYB11. Although *F3'H* is a potential target of these regulatory factors, a promoter assay study did not prove this; an unknown additional co-factor might be required to active the *F3'H* promoter. *F3'H* is a special structural gene that it is necessary for quercetin flavonols but not for kaempferol flavonols. *Arabidopsis* leaves and stems predominantly accumulate kaempferol flavonols, whereas inflorescences and siliques show a broad spectrum of flavonoids (Stracke et al., 2010). Therefore, plants need more regulatory factors to regulate *F3'H* when and where necessary the synthesis of quercetin flavonols is required. In the seed coats, the predominant flavonol is quercetin-3-*O*-rhamnoside (Q3R); quercetin-3-*O*-rhamnoside-7-*O*-glucoside (Q3R7G) accumulates in even higher concentrations than Q3R but disappears during seed maturation (Routaboul et al., 2006). Q3R7G accumulation begins approximately 5 days after flowering and drastically decreases in quantity at approximately 15 days after flowering, a time when the large accumulation of Q3R begins. The accumulation of Q3R7G and Q3R during the early stages appears to be mainly influenced by AtMYB12 and during the late stages by AtMYB11 and AtMYB12 (Stracke et al., 2010).

In conclusion, flavone- and flavonol-specific R2R3-MYBs are extremely conserved with regard to their target gene specificity. This is likely related to their extremely conserved

MYB-domain structure since all of these regulatory proteins are clustered in clade 1 in our phylogenetic tree analyses (Fig. 2).

3-Deoxyflavonoid-specific MYBs

The 3-deoxyflavonoids include phlobaphene, 3-deoxyanthocyanidins, and *C*-glycosyl flavones. Phlobaphenes and 3-deoxyanthocyanidins are derived from the flavan-4-ols luteoforol or apiforol. *C*-glycosyl flavones consist of apimaysin and maysin and are synthesized through a different pathway than FNS, with flavanone 2-hydroxylase (F2H) catalyzing the first step (Fig. 1). Perhaps the most well-studied plant system with regard to phlobaphenes is maize; phlobaphene pigments accumulate most conspicuously in the floral organs, including the cob glumes and pericarp, though it is also found in the tassel glumes and husk (Sharma et al., 2012); *C*-glycosyl flavones and 3-deoxyanthocyanidins primarily accumulate in maize silks and act as defense compounds (Waiss et al., 1979).

Two tightly linked MYB homologous genes, *P1* and *P2*, control 3-deoxyflavonoid synthesis (Zhang et al., 2000). *P1* is primarily expressed in the pericarp, cob glumes, and silks, whereas the expression of *P2* is restricted to the silks and anther wall. Despite the differential spatial expression patterns, these two TFs show high sequence similarity and are identical in function. In maize silks, both *P1* and *P2* regulate maysin synthesis because maysin cannot be detected in plants in which these genes have been deleted; maize cells transformed with either *P1* or *P2* synthesize phlobaphene and *C*-glycosyl flavones (Zhang et al., 2003). Transformation of maize callus and plants with the *P1* gene provides further evidence for its function in regulating phlobaphenes and maysin (Grotewold et al., 1998; Cocciolone et al., 2005). In an initial study analyzing *P1* mutant plants, *CHS*, *CHI*, and *DFR* were identified as the target genes of P1 (Grotewold et al., 1994), and sorghum Y1 controls the same set of genes and regulates phlobaphene in the pericarp (Boddu et al., 2006). *ZmFLS1* (Falcone Ferreyra et al., 2010) and *ZmF3'H* (Sharma et al., 2012) are the most recently reported regulation targets of P1. *ZmF3'H* is known to be required for anthocyanin biosynthesis and also participates in the synthesis of 3-deoxyflavonoids; *ZmF3'H* adds a hydroxyl group to the 3′-position of apiforol to form luteoforol.

Recently, Morohashi et al. (2012) introduced a functional *P1-rr* allele into a A619 inbred line that harbours a recessive null *P1-ww* allele and then identified the primary target genes of P1 through a combination of chromatin immunoprecipitation and high-throughput sequencing (ChIP-Seq). These advanced techniques allow the comprehensive identification of P1-regualted genes, identifying *CHS* (*C2*), *CHI1*, and *DFR* (*A1*), in agreement with previous studies. New target genes were also identified: a second *CHS WHP1*, a second *DFR A1**, and *ZmF2H1*, which catalyzes the key branch point in the pathway leading to 3-deoxyflavonoid. The promoters of *WHP1*, *A1**, and *CHS* were activated by P1 in a luciferase reporter assay; interestingly the activation of *WHP1* and *A1** by the C1/R complex were not efficient. In addition to target genes in the flavonoid pathway, 1500 putative targets of P1 were identified, including phenylpropanoid genes, translation-associated genes, and TF genes, in maize pericarp and silks. P1 has many more target genes than expected and plays a role in various plant physiologic activities. This finding is in agreement with the previous finding that P1-expressing maize cells also accumulate ferulic acid (Grotewold et al., 1998) and that P1 is one of the two QTLs that exerts a major effect on chlorogenic acid accumulation (Bushman et al., 2002). In summary, 3-deoxyflavonoid-specific MYBs regulate *DFR*, *F3'H*, *F2H*, as well as EBGs in flavonoid pathway.

Isoflavonoid-specific MYBs

Isoflavonoids are abundant in soybeans and other leguminous plants, but some non-legume plant species also produce isoflavonoids (Mackova et al., 2006). Isoflavonoid biosynthesis has been reviewed (Du et al., 2010). Wang (2011) recently provided a review on the structural studies of the key enzymes involved in isoflavonoid biosynthesis. In soybean (*Glycine max*), isoflavonoids are detected throughout the plants and show the greatest amount in embryo tissues at late stages, the mature seeds, and the leaves (Dhaubhadel et al., 2003). Changes in *IFS2* transcript abundance are consistent with isoflavonoid accumulation in embryo tissues, whereas *CHR*, *CHI*, and *IFS1* show steady-state constitutive transcription, indicating that these genes are activated for processes other than isoflavonoid syntheses. The *GmMYB176* gene encoding an R1-MYB was identified to regulate isoflavonoid biosynthesis, and GmMYB176 is capable of binding a TAGT element *in vitro* and of activating the *GmCHS8* promoter in *Arabidopsis* protoplasts (Yi et al., 2010; Dhaubhadel and Li 2013). The study of the GmMYB176 in vivo function showed that GmMYB176 is essential but insufficient to regulate isoflavonoid biosynthesis in soybean.

General flavonoid pathway MYBs and negative regulators

Snapdragon AmMYB305 and AmMYB340 are capable of activating the promoters of *AmCHI* and *AmF3H* and bean *GmPAL2* but not *AmDFR* and *AmAS* (Moyano et al., 1996). AmMYB305 and AmMYB340 are expressed only in flowers, and their temporal expression mimics exactly that of *AmF3H* during flower development (Jackson et al., 1991). This result indicates their roles in anthocyanin biosynthesis, but further investigation using in situ hybridization suggests that their flower tissue-specific expression patterns are correlated to sites of flavonol accumulation (Moyano et al., 1996). AmMYB305 and AmMYB340 are not highly expressed in

the epidermal tissues of petals that synthesize anthocyanins. Additional evidence, particularly with regard to the activation of the *FSL* promoter, is needed to identify the function of AmMYB340 in flavonol biosynthesis. Because AmMYB305 and AmMYB340 do not cluster with flavonol-related clade 1 (Fig. 2), we postulate these factors participate in the regulation of general flavonoid genes for multiple purposes.

In addition to flavonoid activators, some repressor MYB regulators play an important role in balancing flavonoid biosynthesis. There are two types of MYB repressors: single R repeat MYBs and R2R3-MYBs. *Arabidopsis* AtMYBL2 is an R3-MYB that inhibits the flavonoid pathway by competing with R2R3-MYB for interaction with a necessary bHLH co-factor, thereby negatively regulating flavonoid synthesis (Dubos et al., 2008; Matsui et al., 2008). However, it is unlikely that this MYB repressor selects its target genes by directly interacting with the gene promoters, even though it is reported that an R3-MYB can still bind to *DFR* promoter (Gong et al., 1999). R2R3-MYB repressors fulfil their functions via conserved repression domains at the carboxy terminus. *Arabidopsis* AtMYB4 is such a repressor (Jin et al., 2000); although AtMYB4 mutant plants enhance the transcription of *C4H*, other genes, such as *PAL* and *CHS*, are not affected. FaMYB1 from strawberry is related to fruit ripening and contains a repression domain similar to that of AtMYB4 (Aharoni et al., 2001). The overexpression of *FaMYB1* in tobacco results in a reduction of anthocyanins and flavonols in flowers and a corresponding reduction of *ANS* and *DFR* expression but not *CHS* and *F3H*. More recently, Gao et al. (2011) reported that apple MdMYB6 affects structural gene expression and inhibits *Arabidopsis* anthocyanin biosynthesis. These studies indicate that R2R3-MYB repressors involved in flavonoid pathway select their target genes in a manner similar to R2R3-MYB activators, though the mechanism is not clear, and there is no direct evidence showing their interaction with promoters.

Factors accounting for differential target gene specificity

To control a particular flavonoid branch, a R2R3-MYB activator targets a set of flavonoid structural genes. The selectivity of target genes relies on the two components of *cis-trans* regulation: a MYB-domain that directly recognizes a DNA sequence and the presence of related DNA sequences in the promoters of flavonoid genes. In addition, the interaction with co-factors is also involved in the DNA specific recognition. Here, we tentatively explain the diversity of target gene specificity of R2R3-MYB TFs by closely examining these three factors.

DNA binding site of MYBs

MYB TFs recognize the corresponding *cis*-elements through their MYB-domain. The MYB-domain of R2R3-MYB consists of two imperfect repeats (R2 and R3), and each is approximately 50-53 amino acids and forms three α-helix (Ogata et al., 1992). The second and third helices form a helix-turn-helix (HTH) structure that intercalates in the major groove of DNA (Rabinowicz et al., 1999; Tahirov et al., 2002). Maize C1 and P are both capable of activating the *ZmDFR* (*A1*) promoter, though only C1 targets the *ZmUFGT* (*Bz1*) promoter. However, replacement of the MYB region of C1 with that of P results in the inability to activate *ZmUFGT* promoter but not the *ZmDFR* promoter (Grotewold et al., 1994). This result clearly indicates that the MYB-domain is sufficient for specific DNA binding. In the existing literatures about R2R3-MYBs, phylogenic analyses using MYB domain is usually done to predicate their function and study their relationship. For example, Lin-Wang et al. (2010) studied the phylogenetic relationship between anthocyanin-related MYBs of rosaceous and other species. Only a few reports discussed the relation between MYB domain and DNA binding selectivity by phylogenic analyses. Agaki et al. (2010) divided PA-specific R2R3-MYBs into three groups based on MYB domain structure and discussed their subfunctionalization.

We analyzed the MYB-domains of all the available flavonoid-regulating R2R3-MYBs by constructing a phylogenetic tree (Fig. 2.). The flavonoid R2R3-MYBs form 9 clades, essentially the same result as the analyses using the full amino acid (AA) sequence of each protein. When using the full AA sequences, clade 3 is placed between clade 4 and clade 5, and clade 7 is placed between clade 5 and clade 6 (data not shown). The members within clades 1 and 9 share a similar set of flavonoid target genes. Clade 1 members are those that regulate FFD synthesis and are characterized by EBG target genes. In contrast, all clade 9 members (except for lily LhMYB12) primarily target LBGs. Clade 9 consists of all the anthocyanin-specific R2R3-MYBs from dicot species, with the exception of gentian GtMYB3, which clusters with grape PA-regulating MYBs. It is noteworthy that the anthocyanin-specific R2R3-MYBs from maize and *Oncidium* cluster with the PA-specific R2R3-MYBs in clade 6, suggesting that the monocot anthocyanin regulators are closer to the PA regulators than to the dicot anthocyanin regulators. All the R2R3-MYB repressors cluster together in clade 2, with the exception of apple MdMYB6 and strawberry FaMYB1. Clade 4 members are chiefly activators regulating the general flavonoid pathway. The PA-regulating R2R3-MYBs span four clades: clade 3, 5, 6, and 7. In summary, the MYB-domain feature of FFD-specific R2R3-MYBs and dicot anthocyanin-specific R2R3-MYBs show a significant correlation with the target gene selectivity.

To obtain a further understanding of the relation between MYB-domain feature and the differential target gene specificity, we generated a multiple sequence alignment of R2R3-MYB proteins (Fig. 3), including nearly all the identified flavonoid R2R3-MYBs. If a residue actually contributes to the differential target gene specificity between

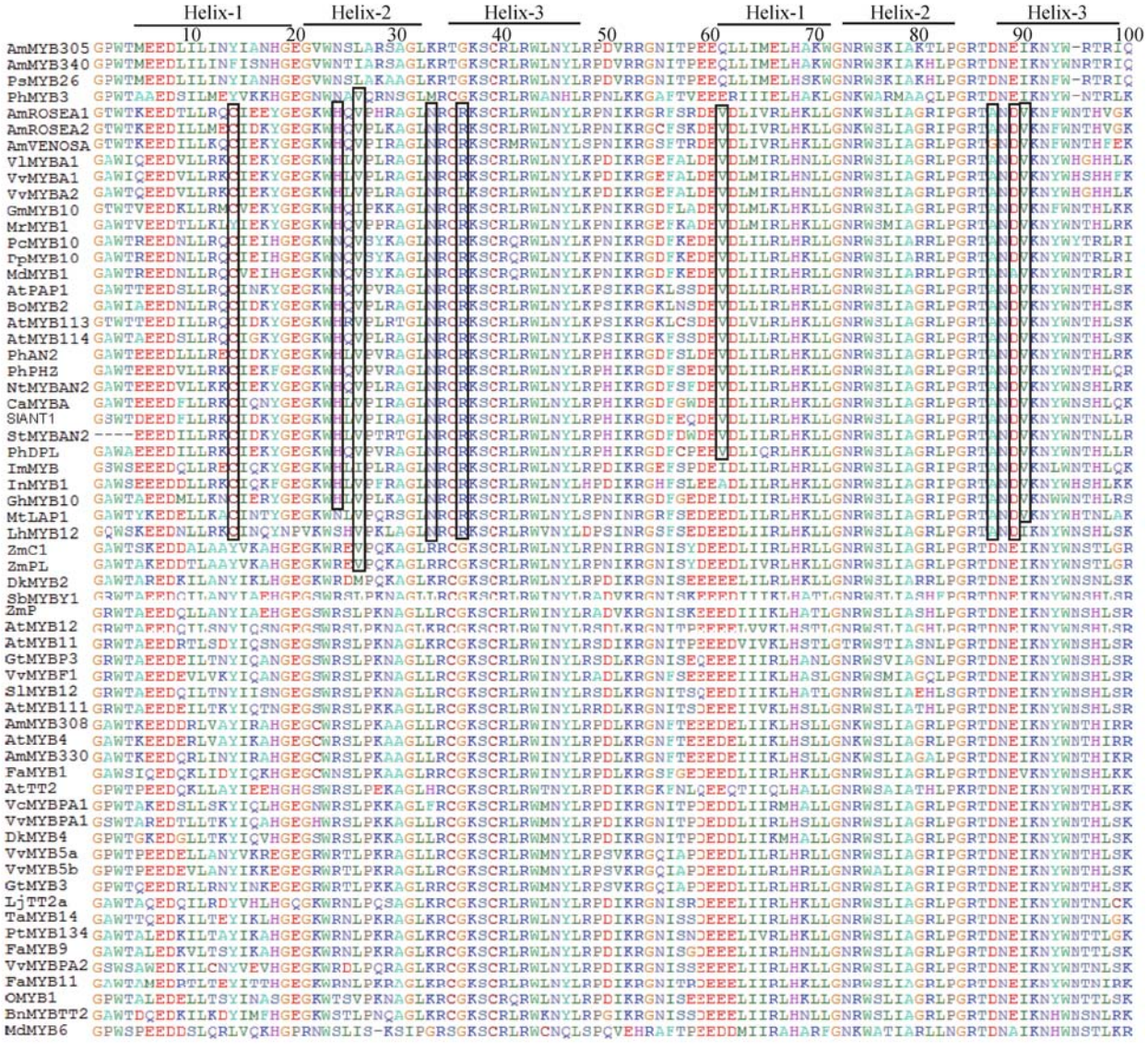

Figure 3 Alignment of the MYB-domain of known flavonoid R2R3-MYB TFs using the ClustalW2 program. The boxed residues are those that are conserved within clade 9 but different from the remaining R2R3-MYBs. 185 × 172mm (136 × 136 DPI).

the different R2R3-MYB clades, it should be conserved within that clade but be different to that of other clades. We identified nine such residues that may account for the limited target range of dicot anthocyanin-specific R2R3-MYBs (Fig. 3), with residues 36, 87, 89, and 90 showing unique patterns among these MYB sequences. These amino acids are 36R, 87A, 89D, and 90V in the clade 9 R2R3-MYBs, whereas they are 36G, 87D, 89E, and 90I in the remaining R2R3-MYBs. There are four exceptions for the clade 9 MYBs, including 36L of VvMYBA2, 87G of AmVENOSA, 89A of MdMYB1, and 90I of LhMYB12, and two exceptions for the remaining MYBs, including 89A of MdMYB6 and 90V of FaMYB1. Regardless, the number of exceptions is very small when considering the large number of R2R3-MYBs analyzed, and it is rare to find such a distribution of other residues among these proteins. We must note that residues 36, 89, and 90 are positioned in helix-3 of R2 or R3 and that residue 87 is positioned just before helix-3 of R3. Experimental evidences have indicated that helix-3 plays a central role in recognizing specific base. Mutations occurring in helix-3 often abolish specific DNA binding and result in the loss of function (Saikumar et al., 1990; Frampton et al., 1991; Gabrielsen et al., 1991; Ogata et al., 1994; Sasaki et al., 2000). The difference in residues 36, 87, 89, and 90 between clade 9 and the remaining proteins may account for the different target gene specificity.

The importance of residue 89 has been demonstrated to date. When mutation of 89D (101D in the original paper) to 89E occurred, ZmC1 became defective in DNA binding because its ability to activate *UFGT* and *DFR* promoter was reduced (Goff et al., 1991; Sainz et al., 1997). A similar DNA binding defect is caused by the same mutation in PhMYB3

(Solano et al., 1995). Most recently, Heppel et al. (2013) reported identification of key amino acids for the differential in target specificity between PA-specific R2R3-MYBs and dicot anthocyanin-specific R2R3-MYBs. Their experimental results are highly consistent with our *in silico* predictions. Exchange of residues 36, 87, 89, and 90 between TT2 and PAP1-4 can swap the pathway selection of TT2 and PAP1-4 (Heppel et al., 2013). We tentatively propose that clade 9 members recognize different DNA sequence to others, see Table 2, due to their distinctive residue 36, 87, 89, and 90, and therefore are not capable of activating EBGs like most of the remaining flavonoid R2R3-MYBs. On the surface, the features of the MYB-domain cannot fully explain the observed differential target gene specificity because lily LhMYB12 contains 36R, 87A, and 89D, similar to dicot anthocyanin R2R3-MYBs, but is capable of activating *CHS* (Lai et al., 2012). However, we must remind that the *LhCHS* promoter fragment activated by LhMYB12/LhbHLH2 contains a perfect G-box but not any typical anthocyanin elements. Therefore, LhbHLH2 rather than LhMYB12 likely determine DNA binding via ACT domain OFF model, see the section "Roles of bHLH co-factors".

In addition to the key residues described above, several other residues have been experimentally identified to determine the differential in target specificity between AmMYB305 and vertebrate c-MYB. AmMYB305 and vertebrate c-MYB bind to two different *cis*-element MBSII and MBSI, respectively; PhMYB3 binds to both of these elements (Table 2). Three residues were first identified to be the key residues in the MBSI recognition by protein mutant experiment (Ogata et al., 1994); their counterparts in our multiple sequence alignment are residue 37, 91 and 92. After that, Solano et al. (1997) identified five new important residues; their counterparts in our multiple sequence alignment are residue 41, 45, 88, 95 and 96. The eight residues in total were suggested be responsible for the differential target DNA sequence between c-MYB and AmMYB305.

cis-elements for MYB binding

The regulation of coordinated expression of flavonoid structural genes is achieved through the interaction of the MYB-domain with conserved *cis*-elements that are present in target genes. A limited number of such *cis*-regulatory motifs for flavonoid MYB binding have been well characterized and reported (Table 2). According to their sequence and cognate MYB, these motifs can basically be divided into two classes: AC-rich *cis*-motifs and vertebrate c-MYB sites. Several critical DNA sequences for anthocyanin regulation have been identified and they are very different to the known AC-rich motifs and vertebrate c-MYB sites (Table 2).

AC-rich *cis*-motifs are widely distributed and recognized by R2R3-MYBs that regulate anthocyanin, 3-deoxyflavonoid, and PA biosynthesis. The P binding site 5′-CCWACC-3′ (−64 bp to −59 bp, relative to the transcriptional start site) was first identified in the proximal *ZmDFR* (*A1*) promoter using a DNase I footprinting approach (Grotewold et al., 1994). When this region was mutated, *trans*-activation by P was decreased by approximately 50%. Later, in 1997, Sainz et al. found an additional P binding site, 5′-AACTACCGG-3′ (−116 bp to −124 bp), within the distal region of the *ZmDFR* promoter. However, P only bound to a large degree to the distal site when the proximal site was mutated due to the lower affinity of the distal site. These two P binding sites in the *ZmDFR* promoter are also bound by C1, though the proximal site is bound with a lower affinity by C1 relative to

Table 2 *Cis*-elements interacting with flavonoid R2R3-MYBs.

MYB TF	*cis*-element			Reference
	Description[1]	Sequence[2]	Promoter	
ZmP/C1	P/C1-site1	CCWACC	*DFR*	Grotewold et al.,, 1994
PcMYB1	MRE	ACCTACC	*CHS*	Feldbrügge et al.,, 1997
PtMYB134	AC-element	ACCTACC	*PAL1*, *DFR1*, *ANR2*	Mellway et al., 2009
DkMYB2	AC-element	MACCWAMC	*ANR*, *LAR*	Akagi et al., 2010
AmMYB305	Box-P	GAACCTAACT	*PAL2*[3]	Sablowski et al., 1994
PhMYB3	MBSII	TAAC-TAACT[4]	*CHSJ*	Solano et al., 1995
ZmP/C1	P/C1-site2	AAC-TACCGG	*DFR*	Sainz et al., 1997
ZmC1	MYB site	TAAC-TG[4]	*UFGT*	Roth et al., 1991
DkMYB4	MYBCORE	YAAC-NG[4]	*F3′5′H*, *ANS*, *ANR*	Akagi et al., 2009
PhMYB3	MBSI	TAAC-SG[4]	*CHSJ*	Solano et al., 1995
ZmC1	C1-site3	GGTGGTTG	*ANS*	Lesnick and Chandler, 1998
ZmC1	ARE	TTGACTGGNGGNTGCG	*ANS*	Lesnick and Chandler, 1998
GhMYB10	ARE	AGTTGAATGGGGG-TGCA	*UFGT*	Elomaa et al., 2003
ZmC1	ARE	CGACTGGCNGGTGC	*DFR*	Tuerck and Fromm, 1994
AtPAP1	PCE	MSYGTGG-NGR[4]	*CHS*, *F3H*, *DFR*, *ANS*, *UFGT*, *GST*	Dare et al., 2008

[1] Description from the original paper or related papers.
[2] R = A/G, M = A/C, W = A/T, S = G/C, K = G/T, Y = C/T, *N* = A/G/C/T.
[3] *Phaseolus vulgaris PAL2*; the remaining promoters are from the same plant species with the corresponding MYB TFs.
[4] The reverse complementary sequence of the original reported sequence.

P. When a random pool of oligonucleotides was prepared, P primarily selected sequences with an 5′-ACCWACC-3′ motif, whereas C1 selected 26 diverse sequences, with only some of the fragments having a site similar to the two binding sites in the *ZmDFR* prompter (Sainz et al., 1997). This result suggests that C1 has a broader DNA binding specificity than P. Indeed, ZmC1 is even capable of activating *Arabidopsis AtANR* (Baudry et al., 2004). The MYB-recognition element (MRE), first identified in the *PcCHS* promoter, is required for light induction (Feldbrügge et al., 1997), and the MRE sequence 5′-ACCTACC-3′ is very similar to P/C1-site 1. MREs are distributed among the promoters of the co-activated *Arabidopsis* flavonol synthesis genes *AtCHS*, *AtCHI*, *AtF3H*, and *AtFLS1* (Hartmann et al., 2005), and MREs are recognized by AmMYB305 (Feldbrügge et al., 1997), PcMYB1 (Feldbrügge et al., 1997), ZmC1 (Hartmann et al., 2005), and AtMYB12 (Mehrtens et al., 2005). The principle of the 'one *cis*-element versus many factors' type of regulation was previously proposed to be a common feature of the large TF families found in plants. PtMYB134 and DkMYB2 that control PA biosynthesis in poplar and persimmon, respectively, was found to directly bind to AC-rich *cis*-motifs in an EMSA experiment (Mellway et al., 2009; Akagi et al., 2010).

The consensus sequence of 5′-CNGTTR-3′ was proven to be the recognition site of vertebrate c-MYB (Lüscher and Eisenman 1990), and some studies suggest plant R2R3-MYBs also recognize these sequences. The region from −47 to −78 bp of *ZmUFGT*(*Bz1*) is sufficient to respond to C1/R-specific regulation in transient assay under the help of CaMV35 minimal promoter (Roth et al., 1991). Inspection of this region reveals that one of the two critical *cis*-elements is homologous to the consensus sequence 5′-CNGTTR-3′; the other one is the bHLH biding site 5′-CANNTG-3′. DkMYB4 directly targets MYBCORE motifs that are similar to the vertebrate c-MYB site but not other representative MYB sites, including AC-rich *cis*-motifs (Akagi et al., 2009). MYBCORE motifs are not present in the promoters of *DkLAR* and *DkF3'H*, in agreement with the observation that the expression of *DkLAR* and *DkF3'H* is not correlated with DkMYB4 in persimmon fruits. Petunia PhMYB3 recognizes two DNA sequences: MBSI and MBSII (Solano et al., 1995). MBSI resembles the vertebrate c-MYB site; MBSII is similar to Box-P and can be recognized by AmMYB305 but not by vertebrate c-MYB.

DNA sequences crucial for the activation of anthocyanin-specific R2R3-MYBs are conserved within the promoter regions of anthocyanin structural genes and, therefore, have been assigned the name of an anthocyanin regulatory element (ARE). By investigating the C1/B (MYB/bHLH) activation of deleted and mutated variants of the *ZmDFR* (A1) promoter, Tuerck and Fromm (1994) identified that the most important region for C1/B *trans*-activation was between −98 to −88 bp, a region overlapping ARE^{ZmDFR} (−101 to −88 bp); mutation in this region reduced C1/B activation severely to below 8% of normal. It is notable that ARE^{ZmDFR} is located between the two P/C1 binding sites which both contribute to C1/B activation (Sainz et al., 1997). The two binding sites most likely increase the local concentration of the C1 protein on DNA and, thereby, help the ARE to recruit C1. AREs appears to be specific for anthocyanin regulation because a promoter fragment containing this motif does not confer efficient P activation of *ZmUFGT* (Grotewold et al., 1994). Inspection of the *ZmCHS* (*C2*), *ZmANS* (*A2*), *ZmUFGT* (*Bz1*), and *ZmGST* (*Bz2*) promoters reveals homology of the ARE motif in these promoters (Tuerck and Fromm 1994), with a consensus sequence of 5′-CGACTGGCNGGTGC-3′. Because these DNA sequences have high similarity and a similar distance to the transcription start site, they are likely conserved with regard to anthocyanin regulation in maize. Lesnick and Chandler (1998) have since proven that ARE^{ZmANS} (−92 to −107 bp) is also crucial for *ZmANS* transcription activated by C1/B, though the motif is not the motif that Tuerck and Fromm predicted by sequence comparison. Moreover, ARE^{ZmANS} overlaps the third binding site for C1 (5′-GGTGGTTG-3′). In gerbera, an ARE^{GhDFR2} in the proximal promoter region is critical for GhMYB10 regulation (Elomaa et al., 2003). Dare et al., (2008) identified the PAP1 *cis*-regulatory element (PCE) as 5′-YCNC-CACRWK-3′ by scanning the eight promoters that were activated by AtPAP1 in *N. benthamiana* leaves. Using microarray analyses, the eight genes were selected from 33 genes that showed significant alteration in transcription level due to the overexpression of the *AtPAP1* gene. Deleting and mutating the PCE sequence in promoters of *AtUFGT* and *AtGST* resulted in reduced activation by PAP1. Since PCE shows a low similarity to the previously identified ARE, there is a discrepancy in recognition site between AtPAP1 and GhMYB10. GhMYB10 shows monocot type of recognition site. ARE and PCE were identified by specific mutation to the DNA sequences and therefore in our review it is still far to know how these sequence is "crucial" to regulation of anthocyanin-specific R2R3-MYBs. It is possible that other factors that are necessary for anthocyanin regulation may interact with C1 through AREs. Thus, it is too early to draw a conclusion whether anthocyanin-specific R2R3-MYBs of dicots have the same recognition site to those of monocots or not. Nonetheless, AtPAP1 and GhMYB10, the two clade 9 members with known recognition sites, do not appear to select their target genes through interacting with AC-rich elements and animal MYB sites *in vivo* that are widely distributed among flavonoid promoters (Table 2).

Despite the advances in identifying the DNA binding sequence or functionally crucial DNA sequence, the distribution of *cis*-elements alone cannot fully explain the observed target gene specificity. For example, gentian GtMYBP3 targets *GtCHS*, *GtF3'H*, and *GtFNS* and regulates flavone production, whereas GtMYB3 targets *GtF3'5'H* and *GtAT* and regulates anthocyanin synthesis (Nakatsuka et al., 2008; Nakatsuka et al., 2012). The *GtCHS*, *GtF3'H*, *GtF3'5'H*, and *GtFNS* promoters present P binding sites and MYBCORE

binding sites, and the *GtAT* promoter lacks a P binding site. Such a distribution pattern of *cis*-elements in flavonoid genes alone cannot fully explain the target gene specificity of GtMYBP3 or GtMYB3. Indeed, the presence of *cis*-elements appears insufficient to confer R2R3-MYB target specificity to a structural gene *in vivo*.

Roles of bHLH co-factors

Kong et al. (2012) recently proposed a combinatorial gene regulatory framework that placed bHLH TFs in an important role in the coordinated regulation of genes lacking obviously conserved *cis*-regulatory elements. Maize R has two models to activate anthocyanin genes that depend on the homo-dimerization of the C-terminal ACT domain. When the ACT domain is in the dimer form (ON model), the monomeric conformation of the bHLH region is preserved. In this case, R is tethered to DNA through its interaction with C1, indicating that C1 determines DNA binding. In contrast, when the ACT domain is the monomeric form (OFF model), the bHLH region of R forms homodimers that bind the G-box motif; although C1 is still required to form a complex for activation, R determines DNA binding. It is interesting that R and C1 regulate *ZmDFR* via the ACT domain ON model, whereas they regulate *ZmUFGT* via the ACT domain OFF model. This new finding can explain some cases where genes lacking obviously conserved *cis*-elements are regulated coordinately. Hichri et al. (2011b) provided one another evidence that the target gene selectivity of VvMYB5b depends on the interaction with bHLH co-factors. The single residue mutated protein VvMYB5b^{L} lost its ability to correctly interact with VvMYC1, and thereby resulted in severely reduced *trans*-activation activity in tobacco stamens. However, VvMYB5b^{L} overexpression appeared to retain *trans*-activation activity in tobacco corolla and target *NtCHS*, *NtDFR* and *NtANS*. The tissue-dependent *trans*-activation activity of VvMYB5b^{L} indicates that bHLH or unknown co-factors affect the target gene selectivity of R2R3-MYBs.

R2R3-MYB regulation in developing flowers and fruits

The major flavonoid compounds in flowers are flavones/flavonols and anthocyanins, and in fruits and seeds they are flavones/flavonols, anthocyanins, and PAs. The flavonoids in these organs show spatiotemporal accumulation patterns, and different classes of flavonoids usually do not occur simultaneously. The spatiotemporal regulation of flavonoid branches is essential to direct the biosynthesis of different flavonoids. Metabolic channelling by R2R3-MYBs in plant flavonoid metabolism enables plants to effectively synthesize the appropriate metabolites and avoid metabolic interference. Below, we demonstrate how R2R3-MYBs regulate their target genes of necessity for flavonoid biosynthesis during development of flowers and berry skins.

Flower development

Flower development can be divided in to the early stage and the late stage as demonstrated in gentian (Nakatsuka et al., 2005) and eustoma (Noda et al., 2004). The R2R3-MYB TFs of clade 1 induce the coordinated expression of EBGs and *FSN* or *FLS* to produce flavones or flavonols during the early stage (Fig. 4A). The transcription levels of clade 1 members then drastically decrease before pigmentation, resulting in decreases in *FSN* or *FLS* but not EBGs. At the late stage, the R2R3-MYB TFs of clade 9 induce the coordinated expression of LBGs in dicot species with the help of their bHLH co-factor. However, it remains unclear which factors are responsible for the activation of EBGs because clade 9 R2R3-MYBs in dicot species never regulate these genes, and, moreover, transcripts of the clade 1 R2R3-MYBs are absent at the late stage. Some other regulatory proteins may contribute to activate EBGs at the late stage. Another likely mechanism for the expression of early genes might be light activation during photomorphogenesis. Light-independent *CHS* expression is mediated by regulators, such as HY5, which can bind to a minimal light-responsive region of the *CHS* promoter (Ang et al., 1998; Lee et al., 2007). R2R3-MYB regulation in monocot flower development remains to be further clarified. Lily synthesizes flavonol during the late stage, and LhMYB12 appears to contribute to the activation of the entire anthocyanin pathway (Lai et al., 2012).

Unlike flowers of big size mentioned above, grape flowers synthesizes flavonols and PAs during the late flower stage. VvMYBPA1 and VvMYB5b show a maximum of expression one week prior to anthesis, preceding maximum PA accumulation at flowering (Bogs et al., 2007; Deluc et al., 2008). The highest transcription level of VvMYBF1 also occurs at anthesis (Czemmel et al., 2009). However, it is noteworthy that entire grape buds or flowers must be used to analyze the accumulation of flavonoids and gene transcripts because these organs are small. Indeed, this observation could result in different findings from studies of large flowers that examine only flower petals.

Fruit development

Grape berry development can be divided into four phases: fruit set stage (0–2 weeks after flowering, WAF), young fruit stage (3–6 WAF), veraison stage (7–8 WAF), and post-veraison stage ($>$ 8 WAF). Grape is an important plant for the study of the biosynthesis and regulation of flavonoids because the berries are able to produce three common flavonoids: anthocyanins, flavonols, and PAs (Fig. 4B), and the temporal control of PA, anthocyanin, and flavonol synthesis needs to be coordinated during berry development. Czemmel et al. (2012) recently provided a deep review on R2R3-MYBs that regulate flavonoids in grapevine.

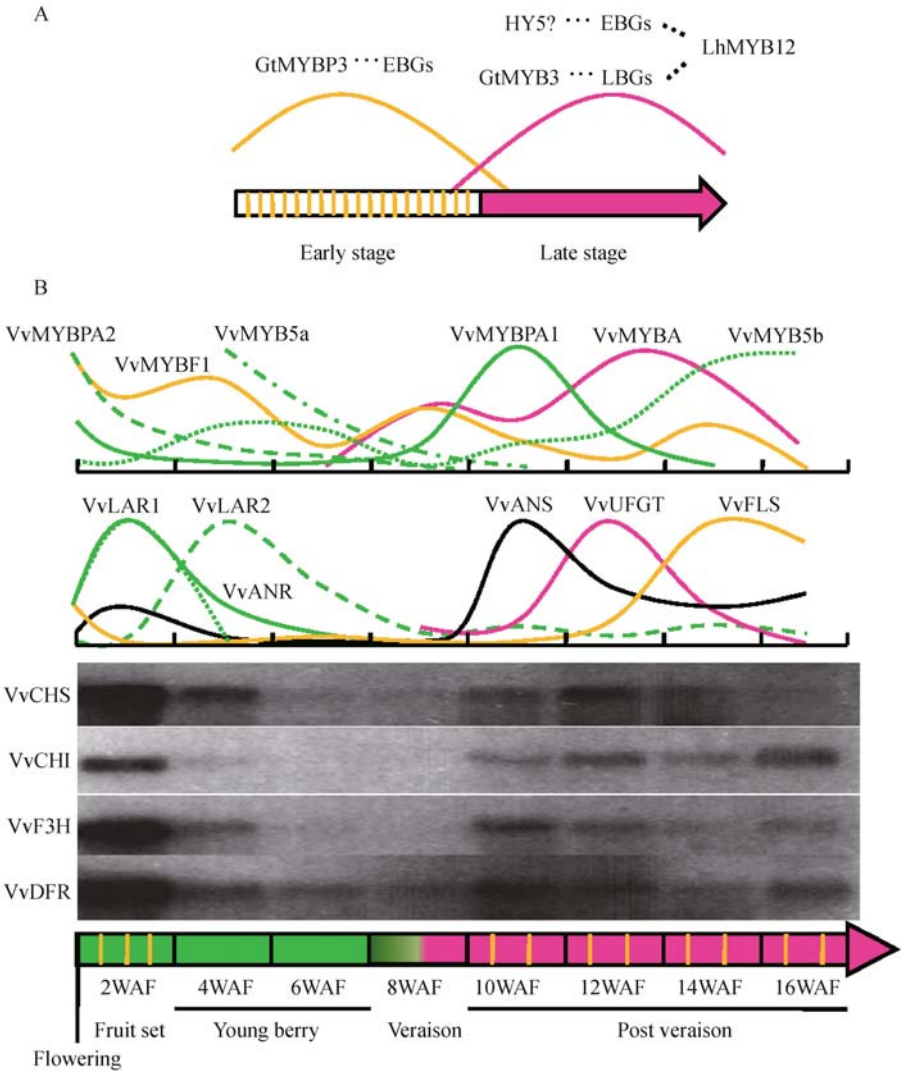

Figure 4 An illustration of the flavonoid regulation mechanism in developing flowers (A) and berry skin of grape 'Shiraz' (B). The pink color indicates anthocyanin pigmentation, the orange color indicates flavone or flavonol accumulation, and the green color indicates PA accumulation. The transcript abundance changes of VvMYBA and VvUFGT (Matus et al., 2010), VvMYBF1 (Czemmel et al., 2009), VvMYBPA1(Bogs et al., 2007), VvMYBPA2 (Terrier et al., 2009), VvMYB5b (Deluc et al., 2008), VvFLS (Downey et al., 2003b), and VvLAR1, VvLAR2, VvANR and VvANS (Bogs et al., 2005) are based on real-time PCR results from the original papers; that of VvMYB5a is roughly quantified from semiquantitative RT-PCR result in the original paper (Deluc et al., 2006); thoes of VvCHS, VvCHI, VvF3H, and VvDFR were investigated through northern blots in the original paper (Boss et al., 1996). The bar indicates a 2-week period during grape berry development. 150 × 176 mm (160 × 160 DPI).

Vitis vinifera 'Shiraz' grape mainly produces quercetin-3-glycoside (Q3G) flavonols, and the deposition tissues are floral organs, tendrils, inflorescences, leaves, and berry skins but not the berry flesh and seeds (Downey et al., 2003b). In reproductive organs, the Q3G concentration is high at flowering and then decreases between flowering and berry set; the total amount of flavonols per berry increases at fruit set stage and then remains the level until post-veraison stage along with the weight and size increase of berry, indicating flavonol syntheses in berries mainly occurs at fruit set stage and post-veraison stage. In strawberry fruit, flavonols largely accumulate in the ripe fruits concomitant with anthocyanin synthesis (Schaart et al., 2013). 'Shiraz' grape also produces PAs and the deposition tissues are flowers, berry skin, seeds, and leaves, and seeds have impressively high concentrations of PAs (Downey et al., 2003a), but the different tissues have PAs of different subunit composition (Bogs et al., 2005). The flowers and berry skins contain mainly catechin, whereas the seeds contain epicatechin. The main accumulation period of PAs in grape berries is from fruit set to 1-2 weeks following veraison (Bogs et al., 2005). Essentially, the biosynthesis of both flavonols and PAs during early fruit developmental stage

is consistent with the finding in blueberry (Zifkin et al., 2012).

VvMYBF1, VvMYBPA1 and VvMYBPA2 show decreasing levels at the fruit set stage; thereafter, VvMYBPA2 remains an undetectable level throughout berry development. Despite the drastic change of transcription levels, these three TFs are highly expressed at fruit set stage. Accordingly, transcription of their target genes *CHS*, *CHI*, *F3H*, *DFR*, *ANS*, *FLS*, *LAR1*, and *ANR* are induced, although *ANS* and *FLS* have moderate transcript abundance. At the young berry stage, the berry skin and seed can be separated, and the following narrative is about berry skins. VvMYBPA1 and VvMYBPA2 are nearly undetectable at this stage; the transcription levels of VvMYB5a and VvMYB5b both dramatically decrease and VvMYB5a transcripts then become undetectable after the veraison stage. This change of R2R3-MYBs transcription levels is consistent with the sharp decrease of all the structural genes. LAR2 is not the target gene of any the R2R3-MYBs mentioned above and therefore shows discrepant change of transcription level. At the stage just before the veraison, all the known flavonoid R2R3-MYBs are inactive except VvMYBF1. This is consistent with the fact that the transcription of all the targeted structural genes become almost undetectable (Boss et al., 1996); *LAR2* shows a remarkable and rapid decrease at the same time (Bogs et al., 2005). There is a discrepancy between FLS and its regulator VvMYBF1 at this stage, which may be due to an additional function of VvMYBF1 or the repression of *VvFLS* by another factor as suggested by the authors (Czemmel et al., 2009). It looks like that the flavonoid biosynthesis in grape berry skins is briefly inhibited around veraison stage; it should be interesting to clarify the possible physiologic or molecular mechanism to explain this phenomenon. After veraison stage, flavonoid synthesis "switches" from PA to anthocyanin synthesis. At 10 WAF, VvMYBPA1 shows the highest transcription level, which is closely correlated with the re-activation of *CHS*, *CHI*, *F3H*, *DFR* and *ANS*; VvMYBA begins to drastically increase at veraison stage which should be responsible for the *UFGT* activation. At 12 WAF, VvMYBPA1 shows a notable decrease in transcript abundance and thereafter remains undetectable; interestingly, VvMYB5b increases its transcription simultaneously and reaches its highest transcription level at 16 WAF. It seems that VvMYBPA1 "passes" the responsibility to activate genes from *CHS* to *ANS* in the pathway onto VvMYB5b that are necessary for anthocyanin biosynthesis. At 14 WAF, the transcription profile of VvMYBF1 has a small peak, which should be responsible for the peak of that of FLS.

Conclusion

Our review demonstrates that dicot anthocyanin-specific R2R3-MYBs and FFD-specific R2R3-MYBs primarily target LBGs and EBGs, respectively, and that PA-specific R2R3-MYBs target both EBGs and LBGs or only LBGs. Our *in silico* analyses of conserved amino acids in MYB domain together with existing experimental data shed light into the understanding of the mechanism of target specificity of R2R3-MYBs. Three factors are involved in the DNA specific recognition of R2R3-MYBs: DNA binding domain, the presence of target *cis*-elements, and co-factors. We identified several unique conserved residues of dicot anthocyanin-specific R2R3-MYBs that may account for the unique recognition DNA sequence and the unique target gene specificity. Our review also demonstrates that the differential target gene specificity of R2R3-MYBs is required to "switch" the metabolic flow between the different flavonoid branches during flower and fruit development.

Acknowledgements

This work is supported by the Department of Education of Sichuan Province, China (Grant-In-Aid for Scientific Research No. 20132A0248).

Compliance with ethics guidelines

Yunsong LAI, Huanxiu LI declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

References

Aharoni A, De Vos C H, Wein M, Sun Z, Greco R, Kroon A, Mol J N, O'Connell A P (2001). The strawberry FaMYB1 transcription factor suppresses anthocyanin and flavonol accumulation in transgenic tobacco. Plant J, 28(3): 319–332

Akagi T, Ikegami A, Tsujimoto T, Kobayashi S, Sato A, Kono A, Yonemori K (2009). DkMyb4 is a Myb transcription factor involved in proanthocyanidin biosynthesis in persimmon fruit. Plant Physiol, 151(4): 2028–2045

Akagi T, Ikegami A, Yonemori K (2010). DkMyb2 wound-induced transcription factor of persimmon (*Diospyros kaki* Thunb.), contributes to proanthocyanidin regulation. Planta, 232(5): 1045–1059

Albert N W, Lewis D H, Zhang H, Schwinn K E, Jameson P E, Davies K M (2011). Members of an R2R3-MYB transcription factor family in Petunia are developmentally and environmentally regulated to control complex floral and vegetative pigmentation patterning. Plant J, 65(5): 771–784

Allan A C, Hellens R P, Laing W A (2008). MYB transcription factors that colour our fruit. Trends Plant Sci, 13(3): 99–102

Ang L H, Chattopadhyay S, Wei N, Oyama T, Okada K, Batschauer A, Deng X W (1998). Molecular interaction between COP1 and HY5 defines a regulatory switch for light control of Arabidopsis development. Mol Cell, 1(2): 213–222

Ballester A R, Molthoff J, de Vos R, Hekkert Bt, Orzaez D, Fernández-Moreno J P, Tripodi P, Grandillo S, Martin C, Heldens J, Ykema M, Granell A, Bovy A (2010). Biochemical and molecular analysis of

pink tomatoes: deregulated expression of the gene encoding transcription factor SlMYB12 leads to pink tomato fruit color. Plant Physiol, 152(1): 71–84

Ban Y, Honda C, Hatsuyama Y, Igarashi M, Bessho H, Moriguchi T (2007). Isolation and functional analysis of a MYB transcription factor gene that is a key regulator for the development of red coloration in apple skin. Plant Cell Physiol, 48(7): 958–970

Baudry A, Heim M A, Dubreucq B, Caboche M, Weisshaar B, Lepiniec L (2004). TT2, TT8, and TTG1 synergistically specify the expression of *BANYULS* and proanthocyanidin biosynthesis in *Arabidopsis thaliana*. Plant J, 39(3): 366–380

Boddu J, Jiang C, Sangar V, Olson T, Peterson T, Chopra S (2006). Comparative structural and functional characterization of sorghum and maize duplications containing orthologous myb transcription regulators of 3-deoxyflavonoid biosynthesis. Plant Mol Biol, 60(2): 185–199

Boddu J, Svabek C, Ibraheem F, Jones A D, Chopra S (2005). Characterization of a deletion allele of a sorghum *Myb* gene *yellow seed1* showing loss of 3-deoxyflavonoids. Plant Sci, 169(3): 542–552

Bogs J, Downey M O, Harvey J S, Ashton A R, Tanner G J, Robinson S P (2005). Proanthocyanidin synthesis and expression of genes encoding leucoanthocyanidin reductase and anthocyanidin reductase in developing grape berries and grapevine leaves. Plant Physiol, 139 (2): 652–663

Bogs J, Jaffé F W, Takos A M, Walker A R, Robinson S P (2007). The grapevine transcription factor VvMYBPA1 regulates proanthocyanidin synthesis during fruit development. Plant Physiol, 143(3): 1347–1361

Borevitz J O, Xia Y, Blount J, Dixon R A, Lamb C (2000). Activation tagging identifies a conserved MYB regulator of phenylpropanoid biosynthesis. Plant Cell, 12(12): 2383–2394

Borovsky Y, Oren-Shamir M, Ovadia R, De Jong W, Paran I (2004). The *A* locus that controls anthocyanin accumulation in pepper encodes a MYB transcription factor homologous to Anthocyanin2 of Petunia. Theor Appl Genet, 109(1): 23–29

Boss P K, Davies C, Robinson S P (1996). Analysis of the expression of anthocyanin pathway genes in developing *Vitis vinifera* L. cv Shiraz grape berries and the implications for pathway regulation. Plant Physiol, 111(4): 1059–1066

Bovy A, de Vos R, Kemper M, Schijlen E, Almenar Pertejo M, Muir S, Collins G, Robinson S, Verhoeyen M, Hughes S, Santos-Buelga C, van Tunen A (2002). High-flavonol tomatoes resulting from the heterologous expression of the maize transcription factor genes *LC* and *C1*. Plant Cell, 14(10): 2509–2526

Bushman B S, Snook M E, Gerke J P, Szalma S J, Berhow M A, Houchins K E, McMullen M D (2002). Two loci exert major effects on chlorogenic acid synthesis in maize silks. Crop Sci, 42(5): 1669–1678

Chiou C Y, Yeh K W (2008). Differential expression of *MYB* gene (*OgMYB1*) determines color patterning in floral tissue of *Oncidium* Gower Ramsey. Plant Mol Biol, 66(4): 379–388

Chiu L W, Li L (2012). Characterization of the regulatory network of *BoMYB2* in controlling anthocyanin biosynthesis in purple cauliflower. Planta, 236(4): 1153–1164

Chiu L W, Zhou X, Burke S, Wu X, Prior R L, Li L (2010). The purple cauliflower arises from activation of a MYB transcription factor. Plant Physiol, 154(3): 1470–1480

Cocciolone S M, Nettleton D, Snook M E, Peterson T (2005). Transformation of maize with the *p1* transcription factor directs production of silk maysin, a corn earworm resistance factor, in concordance with a hierarchy of floral organ pigmentation. Plant Biotechnol J, 3(2): 225–235

Cone K C, Burr F A, Burr B (1986). Molecular analysis of the maize anthocyanin regulatory locus *C1*. Proc Natl Acad Sci U S A, 83(24): 9631–9635

Cone K C, Cocciolone S M, Burr F A, Burr B (1993). Maize anthocyanin regulatory gene *pl* is a duplicate of *c1* that functions in the plant. Plant Cell, 5(12): 1795–1805

Czemmel S, Heppel S C, Bogs J (2012). R2R3 MYB transcription factors: key regulators of the flavonoid biosynthetic pathway in grapevine. Protoplasma, 249(S2 Suppl 2): S109–S118

Czemmel S, Stracke R, Weisshaar B, Cordon N, Harris N N, Walker A R, Robinson S P, Bogs J (2009). The grapevine R2R3-MYB transcription factor VvMYBF1 regulates flavonol synthesis in developing grape berries. Plant Physiol, 151(3): 1513–1530

Dare A P, Schaffer R J, Lin-Wang K, Allan A C, Hellens R P (2008). Identification of a *cis*-regulatory element by transient analysis of co-ordinately regulated genes. Plant Methods, 4(1): 17

Deluc L, Barrieu F, Marchive C, Lauvergeat V, Decendit A, Richard T, Carde J P, Mérillon J M, Hamdi S (2006). Characterization of a grapevine R2R3-MYB transcription factor that regulates the phenylpropanoid pathway. Plant Physiol, 140(2): 499–511

Deluc L, Bogs J, Walker A R, Ferrier T, Decendit A, Merillon J M, Robinson S P, Barrieu F (2008). The transcription factor VvMYB5b contributes to the regulation of anthocyanin and proanthocyanidin biosynthesis in developing grape berries. Plant Physiol, 147(4): 2041–2053

Devic M, Guilleminot J, Debeaujon I, Bechtold N, Bensaude E, Koornneef M, Pelletier G, Delseny M (1999). The *BANYULS* gene encodes a DFR-like protein and is a marker of early seed coat development. Plant J, 19(4): 387–398

Dhaubhadel S, Li X (2010). A new client for 14-3-3 proteins: GmMYB176, an R1 MYB transcription factor. Plant Signal Behav, 5(7): 921–923

Dhaubhadel S, McGarvey B D, Williams R, Gijzen M (2003). Isoflavonoid biosynthesis and accumulation in developing soybean seeds. Plant Mol Biol, 53(6): 733–743

Dixon R A, Xie D Y, Sharma S B (2005). Proanthocyanidins—a final frontier in flavonoid research? New Phytol, 165(1): 9–28

Downey M O, Harvey J S, Robinson S P (2003a). Analysis of tannins in seeds and skins of Shiraz grapes throughout berry development. Aust J Grape Wine Res, 9(1): 15–27

Downey M O, Harvey J S, Robinson S P (2003b). Synthesis of flavonols and expression of flavonol synthase genes in the developing grape berries of Shiraz and Chardonnay (*Vitis vinifera* L.). Aust J Grape Wine Res, 9(2): 110–121

Du H, Feng B R, Yang S S, Huang Y B, Tang Y X (2012a). The R2R3-MYB transcription factor gene family in maize. PLoS One, 7(6): e37463

Du H, Huang Y, Tang Y (2010). Genetic and metabolic engineering of isoflavonoid biosynthesis. Appl Microbiol Biotechnol, 86(5): 1293–1312

Du H, Yang S S, Liang Z, Feng B R, Liu L, Huang Y B, Tang Y X (2012b). Genome-wide analysis of the MYB transcription factor

superfamily in soybean. BMC Plant Biol, 12(1): 106

Dubos C, Le Gourrierec J, Baudry A, Huep G, Lanet E, Debeaujon I, Routaboul J M, Alboresi A, Weisshaar B, Lepiniec L (2008). MYBL2 is a new regulator of flavonoid biosynthesis in *Arabidopsis thaliana*. Plant J, 55(6): 940–953

Dubos C, Stracke R, Grotewold E, Weisshaar B, Martin C, Lepiniec L (2010). MYB transcription factors in *Arabidopsis*. Trends Plant Sci, 15(10): 573–581

Elomaa P, Uimari A, Mehto M, Albert V A, Laitinen R A, Teeri T H (2003). Activation of anthocyanin biosynthesis in *Gerbera hybrida* (Asteraceae) suggests conserved protein-protein and protein-promoter interactions between the anciently diverged monocots and eudicots. Plant Physiol, 133(4): 1831–1842

Espley R V, Hellens R P, Putterill J, Stevenson D E, Kutty-Amma S, Allan A C (2007). Red colouration in apple fruit is due to the activity of the MYB transcription factor, MdMYB10. Plant J, 49(3): 414–427

Falcone Ferreyra M L, Rius S, Emiliani J, Pourcel L, Feller A, Morohashi K, Casati P, Grotewold E (2010). Cloning and characterization of a UV-B-inducible maize flavonol synthase. Plant J, 62(1): 77–91

Falcone Ferreyra M L, Rius S P, Casati P (2012) Flavonoids: biosynthesis, biological functions, and biotechnological applications. Front Plant Sci 3: Article 222

Feldbrügge M, Sprenger M, Hahlbrock K, Weisshaar B (1997). PcMYB1, a novel plant protein containing a DNA-binding domain with one MYB repeat, interacts in vivo with a light-regulatory promoter unit. Plant J, 11(5): 1079–1093

Feng S, Wang Y, Yang S, Xu Y, Chen X (2010). Anthocyanin biosynthesis in pears is regulated by a R2R3-MYB transcription factor PyMYB10. Planta, 232(1): 245–255

Ferrer J L, Austin M B, Stewart C Jr, Noel J P (2008). Structure and function of enzymes involved in the biosynthesis of phenylpropanoids. Plant Physiol Biochem, 46(3): 356–370

Frampton J, Gibson T J, Ness S A, Döderlein G, Graf T (1991). Proposed structure for the DNA-binding domain of the Myb oncoprotein based on model building and mutational analysis. Protein Eng, 4(8): 891–901

Fujiwara H, Tanaka Y, Yonekura-Sakakibara K, Fukuchi-Mizutani M, Nakao M, Fukui Y, Yamaguchi M, Ashikari T, Kusumi T (1998). cDNA cloning, gene expression and subcellular localization of anthocyanin 5-aromatic acyltransferase from *Gentiana triflora*. Plant J, 16(4): 421–431

Gabrielsen O S, Sentenac A, Fromageot P (1991). Specific DNA binding by c-Myb: evidence for a double helix-turn-helix-related motif. Science, 253(5024): 1140–1143

Gao J, Shen X, Zhang Z, Peng R, Xiong A, Xu J, Zhu B, Zheng J, Yao Q (2011). The myb transcription factor MdMYB6 suppresses anthocyanin biosynthesis in transgenic *Arabidopsis*. Plant Cell Tissue Organ Cult, 106(2): 235–242

Goff S A, Cone K C, Chandler V L (1992). Functional analysis of the transcriptional activator encoded by the maize *B* gene: evidence for a direct functional interaction between two classes of regulatory proteins. Genes Dev, 6(5): 864–875

Goff S A, Cone K C, Fromm M E (1991). Identification of functional domains in the maize transcriptional activator C1: comparison of wild-type and dominant inhibitor proteins. Genes Dev, 5(2): 298–309

Gong Z Z, Yamazaki M, Saito K (1999). A light-inducible Myb-like gene that is specifically expressed in red *Perilla frutescens* and presumably acts as a determining factor of the anthocyanin forma. Mol Gen Genet, 262(1): 65–72

Gonzalez A, Zhao M, Leavitt J M, Lloyd A M (2008). Regulation of the anthocyanin biosynthetic pathway by the TTG1/bHLH/Myb transcriptional complex in *Arabidopsis* seedlings. Plant J, 53(5): 814–827

Grotewold E (2006). The genetics and biochemistry of floral pigments. Annu Rev Plant Biol, 57(1): 761–780

Grotewold E, Athma P, Peterson T (1991). Alternatively spliced products of the maize *P* gene encode proteins with homology to the DNA-binding domain of myb-like transcription factors. Proc Natl Acad Sci U S A, 88(11): 4587–4591

Grotewold E, Chamberlin M, Snook M, Siame B, Butler L, Swenson J, Maddock S, St Clair G, Bowen B (1998). Engineering secondary metabolism in maize cells by ectopic expression of transcription factors. Plant Cell, 10(5): 721–740

Grotewold E, Drummond B J, Bowen B, Peterson T (1994). The myb-homologous *P* gene controls phlobaphene pigmentation in maize floral organs by directly activating a flavonoid biosynthetic gene subset. Cell, 76(3): 543–553

Hancock K R, Collette V, Fraser K, Greig M, Xue H, Richardson K, Jones C, Rasmussen S (2012). Expression of the R2R3-MYB transcription factor TaMYB14 from *Trifolium arvense* activates proanthocyanidin biosynthesis in the legumes *Trifolium repens* and *Medicago sativa*. Plant Physiol, 159(3): 1204–1220

Hartmann U, Sagasser M, Mehrtens F, Stracke R, Weisshaar B (2005). Differential combinatorial interactions of *cis*-acting elements recognized by R2R3-MYB, BZIP, and BHLH factors control light-responsive and tissue-specific activation of phenylpropanoid biosynthesis genes. Plant Mol Biol, 57(2): 155–171

Heppel S C, Jaffé F W, Takos A M, Schellmann S, Rausch T, Walker A R, Bogs J (2013). Identification of key amino acids for the evolution of promoter target specificity of anthocyanin and proanthocyanidin regulating MYB factors. Plant Mol Biol, 82(4-5): 457–471

Hichri I, Barrieu F, Bogs J, Kappel C, Delrot S, Lauvergeat V (2011a). Recent advances in the transcriptional regulation of the flavonoid biosynthetic pathway. J Exp Bot, 62(8): 2465–2483

Hichri I, Deluc L, Barrieu F, Bogs J, Mahjoub A, Regad F, Gallois B, Granier T, Trossat-Magnin C, Gomès E, Lauvergeat V (2011b). A single amino acid change within the R2 domain of the VvMYB5b transcription factor modulates affinity for protein partners and target promoters selectivity. BMC Plant Biol, 11(1): 117

Huang W, Sun W, Lv H, Luo M, Zeng S, Pattanaik S, Yuan L, Wang Y (2013). A R2R3-MYB transcription factor from *Epimedium sagittatum* regulates the flavonoid biosynthetic pathway. PLoS One, 8(8): e70778

Ikegami A, Eguchi S, Kitajima A, Inoue K, Yonemori K (2007). Identification of genes involved in proanthocyanidin biosynthesis of persimmon (*Diospyros kaki*) fruit. Plant Sci, 172(5): 1037–1047

Jackson D, Culianez-Macia F, Prescott A G, Roberts K, Martin C (1991). Expression patterns of myb genes from *Antirrhinum* flowers. Plant Cell, 3(2): 115–125

Jia L, Clegg M T, Jiang T (2004). Evolutionary dynamics of the DNA-binding domains in putative R2R3-MYB genes identified from rice subspecies indica and japonica genomes. Plant Physiol, 134(2): 575–585

Jin H, Cominelli E, Bailey P, Parr A, Mehrtens F, Jones J, Tonelli C, Weisshaar B, Martin C (2000). Transcriptional repression by AtMYB4 controls production of UV-protecting sunscreens in Arabidopsis. EMBO J, 19(22): 6150–6161

Jung C S, Griffiths H M, De Jong D M, Cheng S, Bodis M, Kim T S, De Jong W S (2009). The potato developer (*D*) locus encodes an R2R3 MYB transcription factor that regulates expression of multiple anthocyanin structural genes in tuber skin. Theor Appl Genet, 120(1): 45–57

Kobayashi S, Ishimaru M, Hiraoka K, Honda C (2002). Myb-related genes of the Kyoho grape (*Vitis labruscana*) regulate anthocyanin biosynthesis. Planta, 215(6): 924–933

Koes R, Verweij W, Quattrocchio F (2005). Flavonoids: a colorful model for the regulation and evolution of biochemical pathways. Trends Plant Sci, 10(5): 236–242

Kong Q, Pattanaik S, Feller A, Werkman J R, Chai C, Wang Y, Grotewold E, Yuan L (2012). Regulatory switch enforced by basic helix-loop-helix and ACT-domain mediated dimerizations of the maize transcription factor R. Proc Natl Acad Sci U S A, 109(30): E2091–E2097

Lai Y S, Shimoyamada Y, Nakayama M, Yamagishi M (2012). Pigment accumulation and transcription of LhMYB12 and anthocyanin biosynthesis genes during flower development in the Asiatic hybrid lily (*Lilium* spp.). Plant Sci, 193-194: 136–147

Laitinen R A, Ainasoja M, Broholm S K, Teeri T H, Elomaa P (2008). Identification of target genes for a MYB-type anthocyanin regulator in *Gerbera hybrida*. J Exp Bot, 59(13): 3691–3703

Lee J, He K, Stolc V, Lee H, Figueroa P, Gao Y, Tongprasit W, Zhao H, Lee I, Deng X W (2007). Analysis of transcription factor HY5 genomic binding sites revealed its hierarchical role in light regulation of development. Plant Cell, 19(3): 731–749

Lesnick M L, Chandler V L (1998). Activation of the maize anthocyanin gene *a2* is mediated by an element conserved in many anthocyanin promoters. Plant Physiol, 117(2): 437–445

Li L, Ban Z J, Li X H, Wu M Y, Wang A L, Jiang Y Q, Jiang Y H (2012a). Differential expression of anthocyanin biosynthetic genes and transcription factor PcMYB10 in pears (Pyrus communis L.). PLoS One, 7(9): e46070

Li Z T, Gmitter F G, Grosser J W, Chen C, Gray D J (2012b). Isolation and characterization of a novel anthocyanin-promoting MYBA gene family in *Citrus*. Tree Genet Genomes, 8(4): 675–685

Lin-Wang K, Bolitho K, Grafton K, Kortstee A, Karunairetnam S, McGhie T K, Espley R V, Hellens R P, Allan A C (2010). An R2R3 MYB transcription factor associated with regulation of the anthocyanin biosynthetic pathway in Rosaceae. BMC Plant Biol, 10(1): 50

Liu Y (2010) Molecular analysis of genes involved in the synthesis of proanthocyanidins in *Theobroma cacao*. PhD Dissertation (USA: The Pennsylvania State University).

Lüscher B, Eisenman R N (1990). New light on Myc and Myb. Part II. Myb. Genes Dev, 4(12B 12b): 2235–2241

Mackova Z, Koblovska R, Lapcik O (2006). Distribution of isoflavonoids in non-leguminous taxa- an update. Phytochemistry, 67(9): 849–855

Mano H, Ogasawara F, Sato K, Higo H, Minobe Y (2007). Isolation of a regulatory gene of anthocyanin biosynthesis in tuberous roots of purple-fleshed sweet potato. Plant Physiol, 143(3): 1252–1268

Martens S, Mithöfer A (2005). Flavones and flavone synthases. Phytochemistry, 66(20): 2399–2407

Martens S, Preuss A, Matern U (2010). Multifunctional flavonoid dioxygenases: flavonol and anthocyanin biosynthesis in *Arabidopsis thaliana* L. Phytochemistry, 71(10): 1040–1049

Martin C, Gerats T (1993). Control of pigment biosynthesis genes during petal development. Plant Cell, 5(10): 1253–1264

Martin C, Prescott A, Mackay S, Bartlett J, Vrijlandt E (1991). Control of anthocyanin biosynthesis in flowers of *Antirrhinum majus*. Plant J, 1 (1): 37–49

Mathews H, Clendennen S K, Caldwell C G, Liu X L, Connors K, Matheis N, Schuster D K, Menasco D J, Wagoner W, Lightner J, Wagner D R (2003). Activation tagging in tomato identifies a transcriptional regulator of anthocyanin biosynthesis, modification, and transport. Plant Cell, 15(8): 1689–1703

Matsui K, Umemura Y, Ohme-Takagi M (2008). AtMYBL2, a protein with a single MYB domain, acts as a negative regulator of anthocyanin biosynthesis in *Arabidopsis*. Plant J, 55(6): 954–967

Matus J T, Loyola R, Vega A, Peña-Neira A, Bordeu E, Arce-Johnson P, Alcalde J A (2009). Post-veraison sunlight exposure induces MYB-mediated transcriptional regulation of anthocyanin and flavonol synthesis in berry skins of *Vitis vinifera*. J Exp Bot, 60(3): 853–867

Matus J T, Poupin M J, Cañón P, Bordeu E, Alcalde J A, Arce-Johnson P (2010). Isolation of WDR and bHLH genes related to flavonoid synthesis in grapevine (*Vitis vinifera* L.). Plant Mol Biol, 72(6): 607–620

Mehrtens F, Kranz H, Bednarek P, Weisshaar B (2005). The *Arabidopsis* transcription factor MYB12 is a flavonol-specific regulator of phenylpropanoid biosynthesis. Plant Physiol, 138(2): 1083–1096

Mellway R D, Tran L T, Prouse M B, Campbell M M, Constabel C P (2009). The wound-, pathogen-, and ultraviolet B-responsive *MYB134* gene encodes an R2R3 MYB transcription factor that regulates proanthocyanidin synthesis in poplar. Plant Physiol, 150(2): 924–941

Miranda M, Ralph S G, Mellway R, White R, Heath M C, Bohlmann J, Constabel C P (2007). The transcriptional response of hybrid poplar (*Populus trichocarpa* x *P*. deltoides) to infection by *Melampsora medusae* leaf rust involves induction of flavonoid pathway genes leading to the accumulation of proanthocyanidins. Mol Plant Microbe Interact, 20(7): 816–831

Mol J, Grotewold E, Koes R (1998). How genes paint flowers and seeds. Trends Plant Sci, 3(6): 212–217

Morita Y, Saitoh M, Hoshino A, Nitasaka E, Iida S (2006). Isolation of cDNAs for R2R3-MYB, bHLH and WDR transcriptional regulators and identification of *c* and *ca* mutations conferring white flowers in the Japanese morning glory. Plant Cell Physiol, 47(4): 457–470

Morohashi K, Casas M I, Falcone Ferreyra M L, Mejía-Guerra M K, Pourcel L, Yilmaz A, Feller A, Carvalho B, Emiliani J, Rodriguez E, Pellegrinet S, McMullen M, Casati P, Grotewold E (2012). A genome-wide regulatory framework identifies maize pericarp color1 controlled genes. Plant Cell, 24(7): 2745–2764

Moyano E, Martínez-Garcia J F, Martin C (1996). Apparent redundancy in *myb* gene function provides gearing for the control of flavonoid biosynthesis in antirrhinum flowers. Plant Cell, 8(9): 1519–1532

Nakatsuka T, Haruta K S, Pitaksutheepong C, Abe Y, Kakizaki Y, Yamamoto K, Shimada N, Yamamura S, Nishihara M (2008). Identification and characterization of R2R3-MYB and bHLH

transcription factors regulating anthocyanin biosynthesis in gentian flowers. Plant Cell Physiol, 49(12): 1818–1829

Nakatsuka T, Nishihara M, Mishiba K, Yamamura S (2005). Temporal expression of flavonoid biosynthesis-related genes regulates flower pigmentation in gentian plants. Plant Sci, 168(5): 1309–1318

Nakatsuka T, Saito M, Yamada E, Fujita K, Kakizaki Y, Nishihara M (2012). Isolation and characterization of GtMYBP3 and GtMYBP4, orthologues of R2R3-MYB transcription factors that regulate early flavonoid biosynthesis, in gentian flowers. J Exp Bot, 63(18): 6505–6517

Nesi N, Debeaujon I, Jond C, Pelletier G, Caboche M, Lepiniec L (2000). The *TT8* gene encodes a basic helix-loop-helix domain protein required for expression of *DFR* and *BAN* genes in *Arabidopsis* siliques. Plant Cell, 12(10): 1863–1878

Nesi N, Jond C, Debeaujon I, Caboche M, Lepiniec L (2001). The *Arabidopsis TT2* gene encodes an R2R3 MYB domain protein that acts as a key determinant for proanthocyanidin accumulation in developing seed. Plant Cell, 13(9): 2099–2114

Nishihara M, Nakatsuka T (2011). Genetic engineering of flavonoid pigments to modify flower color in floricultural plants. Biotechnol Lett, 33(3): 433–441

Niu S S, Xu C J, Zhang W S, Zhang B, Li X, Lin-Wang K, Ferguson I B, Allan A C, Chen K S (2010). Coordinated regulation of anthocyanin biosynthesis in Chinese bayberry (*Myrica rubra*) fruit by a R2R3 MYB transcription factor. Planta, 231(4): 887–899

Noda N, Kanno Y, Kato N, Kazuma K, Suzuki M (2004). Regulation of gene expression involved in flavonol and anthocyanin biosynthesis during petal development in lisianthus (*Eustoma grandiflorum*). Physiol Plant, 122(3): 305–313

Ogata K, Hojo H, Aimoto S, Nakai T, Nakamura H, Sarai A, Ishii S, Nishimura Y (1992). Solution structure of a DNA-binding unit of Myb: a helix-turn-helix-related motif with conserved tryptophans forming a hydrophobic core. Proc Natl Acad Sci U S A, 89(14): 6428–6432

Ogata K, Morikawa S, Nakamura H, Sekikawa A, Inoue T, Kanai H, Sarai A, Ishii S, Nishimura Y (1994). Solution structure of a specific DNA complex of the Myb DNA-binding domain with cooperative recognition helices. Cell, 79(4): 639–648

Osier T L, Lindroth R L (2001). Effects of genotype, nutrient availability, and defoliation on aspen phytochemistry and insect performance. J Chem Ecol, 27(7): 1289–1313

Palapol Y, Ketsa S, Lin-Wang K, Ferguson I B, Allan A C (2009). A MYB transcription factor regulates anthocyanin biosynthesis in mangosteen (*Garcinia mangostana* L.) fruit during ripening. Planta, 229(6): 1323–1334

Pattanaik S, Kong Q, Zaitlin D, Werkman J R, Xie C H, Patra B, Yuan L (2010). Isolation and functional characterization of a floral tissue-specific R2R3 MYB regulator from tobacco. Planta, 231(5): 1061–1076

Paz-Ares J, Ghosal D, Wienand U, Peterson P A, Saedler H (1987). The regulatory *c1* locus of Zea mays encodes a protein with homology to myb proto-oncogene products and with structural similarities to transcriptional activators. EMBO J, 6(12): 3553–3558

Peel G J, Pang Y, Modolo L V, Dixon R A (2009). The LAP1 MYB transcription factor orchestrates anthocyanidin biosynthesis and glycosylation in Medicago. Plant J, 59(1): 136–149

Peters D J, Constabel C P (2002). Molecular analysis of herbivore-induced condensed tannin synthesis: cloning and expression of dihydroflavonol reductase from trembling aspen (*Populus tremuloides*). Plant J, 32(5): 701–712

Quattrocchio F, Verweij W, Kroon A, Spelt C, Mol J, Koes R (2006). PH4 of Petunia is an R2R3 MYB protein that activates vacuolar acidification through interactions with basic-helix-loop-helix transcription factors of the anthocyanin pathway. Plant Cell, 18(5): 1274–1291

Quattrocchio F, Wing J, van der Woude K, Souer E, de Vetten N, Mol J, Koes R (1999). Molecular analysis of the *anthocyanin2* gene of petunia and its role in the evolution of flower color. Plant Cell, 11(8): 1433–1444

Quattrocchio F, Wing J F, van der Woude K, Mol J N, Koes R (1998). Analysis of bHLH and MYB domain proteins: species-specific regulatory differences are caused by divergent evolution of target anthocyanin genes. Plant J, 13(4): 475–488

Rabinowicz P D, Braun E L, Wolfe A D, Bowen B, Grotewold E (1999). Maize R2R3 Myb genes: Sequence analysis reveals amplification in the higher plants. Genetics, 153(1): 427–444

Ravaglia D, Espley R V, Henry-Kirk R A, Andreotti C, Ziosi V, Hellens R P, Costa G, Allan A C (2013). Transcriptional regulation of flavonoid biosynthesis in nectarine (*Prunus persica*) by a set of R2R3 MYB transcription factors. BMC Plant Biol, 13(1): 68

Roth B A, Goff S A, Klein T M, Fromm M E (1991). C1- and R-dependent expression of the maize *Bz1* gene requires sequences with homology to mammalian myb and myc binding sites. Plant Cell, 3 (3): 317–325

Routaboul J M, Kerhoas L, Debeaujon I, Pourcel L, Caboche M, Einhorn J, Lepiniec L (2006). Flavonoid diversity and biosynthesis in seed of *Arabidopsis thaliana*. Planta, 224(1): 96–107

Sablowski R W, Moyano E, Culianez-Macia F A, Schuch W, Martin C, Bevan M (1994). A flower-specific Myb protein activates transcription of phenylpropanoid biosynthetic genes. EMBO J, 13(1): 128–137

Saikumar P, Murali R, Reddy E P (1990). Role of tryptophan repeats and flanking amino acids in Myb-DNA interactions. Proc Natl Acad Sci U S A, 87(21): 8452–8456

Sainz M B, Grotewold E, Chandler V L (1997). Evidence for direct activation of an anthocyanin promoter by the maize C1 protein and comparison of DNA binding by related Myb domain proteins. Plant Cell, 9(4): 611–625

Saito R, Fukuta N, Ohmiya A, Itoh Y, Ozeki Y, Kuchitsu K, Nakayama M (2006). Regulation of anthocyanin biosynthesis involved in the formation of marginal picotee petals in Petunia. Plant Sci, 170(4): 828–834

Sasaki M, Ogata K, Hatanaka H, Nishimura Y (2000). Backbone dynamics of the c-Myb DNA-binding domain complexed with a specific DNA. J Biochem, 127(6): 945–953

Schaart J G, Dubos C, Romero De La Fuente I, van Houwelingen A M, de Vos R C, Jonker H H, Xu W, Routaboul J M, Lepiniec L, Bovy A G (2013). Identification and characterization of MYB-bHLH-WD40 regulatory complexes controlling proanthocyanidin biosynthesis in strawberry (Fragaria × ananassa) fruits. New Phytol, 197(2): 454–467

Schröder G, Brown J W, Schröder J (1988). Molecular analysis of

resveratrol synthase. cDNA, genomic clones and relationship with chalcone synthase. Eur J Biochem, 172(1): 161–169

Schwinn K, Venail J, Shang Y, Mackay S, Alm V, Butelli E, Oyama R, Bailey P, Davies K, Martin C (2006). A small family of MYB-regulatory genes controls floral pigmentation intensity and patterning in the genus *Antirrhinum*. Plant Cell, 18(4): 831–851

Sharma M, Chai C, Morohashi K, Grotewold E, Snook M E, Chopra S (2012). Expression of flavonoid 3′-hydroxylase is controlled by *P1*, the regulator of 3-deoxyflavonoid biosynthesis in maize. BMC Plant Biol, 12(1): 196

Solano R, Fuertes A, Sánchez-Pulido L, Valencia A, Paz-Ares J (1997). A single residue substitution causes a switch from the dual DNA binding specificity of plant transcription factor MYB.Ph3 to the animal c-MYB specificity. J Biol Chem, 272(5): 2889–2895

Solano R, Nieto C, Avila J, Cañas L, Diaz I, Paz-Ares J (1995). Dual DNA binding specificity of a petal epidermis-specific MYB transcription factor (MYB.Ph3) from *Petunia hybrida*. EMBO J, 14 (8): 1773–1784

Spelt C, Quattrocchio F, Mol J N, Koes R (2000). anthocyanin1 of petunia encodes a basic helix-loop-helix protein that directly activates transcription of structural anthocyanin genes. Plant Cell, 12(9): 1619–1632

Springob K, Nakajima J, Yamazaki M, Saito K (2003). Recent advances in the biosynthesis and accumulation of anthocyanins. Nat Prod Rep, 20(3): 288–303

Stracke R, Ishihara H, Huep G, Barsch A, Mehrtens F, Niehaus K, Weisshaar B (2007). Differential regulation of closely related R2R3-MYB transcription factors controls flavonol accumulation in different parts of the *Arabidopsis thaliana* seedling. Plant J, 50(4): 660–677

Stracke R, Jahns O, Keck M, Tohge T, Niehaus K, Fernie A R, Weisshaar B (2010). Analysis of PRODUCTION OF FLAVONOL GLYCOSIDES-dependent flavonol glycoside accumulation in *Arabidopsis thaliana* plants reveals MYB11-, MYB12- and MYB111-independent flavonol glycoside accumulation. New Phytol, 188(4): 985–1000

Stracke R, Werber M, Weisshaar B (2001). The R2R3-MYB gene family in *Arabidopsis thaliana*. Curr Opin Plant Biol, 4(5): 447–456

Tahirov T H, Sato K, Ichikawa-Iwata E, Sasaki M, Inoue-Bungo T, Shiina M, Kimura K, Takata S, Fujikawa A, Morii H, Kumasaka T, Yamamoto M, Ishii S, Ogata K (2002). Mechanism of c-Myb-C/EBP beta cooperation from separated sites on a promoter. Cell, 108(1): 57–70

Takos A M, Jaffé F W, Jacob S R, Bogs J, Robinson S P, Walker A R (2006). Light-induced expression of a MYB gene regulates anthocyanin biosynthesis in red apples. Plant Physiol, 142(3): 1216–1232

Tamagnone L, Merida A, Parr A, Mackay S, Culianez-Macia F A, Roberts K, Martin C (1998). The AmMYB308 and AmMYB330 transcription factors from antirrhinum regulate phenylpropanoid and lignin biosynthesis in transgenic tobacco. Plant Cell, 10(2): 135–154

Tanaka Y, Brugliera F, Chandler S (2009). Recent progress of flower colour modification by biotechnology. Int J Mol Sci, 10(12): 5350–5369

Terrier N, Torregrosa L, Ageorges A, Vialet S, Verriès C, Cheynier V, Romieu C (2009). Ectopic expression of *VvMybPA2* promotes proanthocyanidin biosynthesis in grapevine and suggests additional targets in the pathway. Plant Physiol, 149(2): 1028–1041

Tuerck J A, Fromm M E (1994). Elements of the maize *A1* promoter required for transactivation by the anthocyanin *B*/*C1* or phlobaphene *P* regulatory genes. Plant Cell, 6(11): 1655–1663

Uimari A, Strommer J (1997). Myb26: a MYB-like protein of pea flowers with affinity for promoters of phenylpropanoid genes. Plant J, 12(6): 1273–1284

Verdier J, Zhao J, Torres-Jerez I, Ge S, Liu C, He X, Mysore K S, Dixon R A, Udvardi M K (2012). MtPAR MYB transcription factor acts as an on switch for proanthocyanidin biosynthesis in *Medicago truncatula*. Proc Natl Acad Sci U S A, 109(5): 1766–1771

Waiss A C, Chan B G, Elliger C A, Wiseman B R, McMillian W W, Widstrom N W, Zuber M S, Keaster A J (1979). Maysin, a flavone glycoside from corn silks with antibiotic activity toward corn earworm. J Econ Entomol, 72: 256–258

Wang X (2011). Structure, function, and engineering of enzymes in isoflavonoid biosynthesis. Funct Integr Genomics, 11(1): 13–22

Wei Y L, Li J N, Lu J, Tang Z L, Pu D C, Chai Y R (2007). Molecular cloning of *Brassica napus TRANSPARENT TESTA 2* gene family encoding potential MYB regulatory proteins of proanthocyanidin biosynthesis. Mol Biol Rep, 34(2): 105–120

Winkel-Shirley B (2001). Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiol, 126(2): 485–493

Winkel-Shirley B (2002). Biosynthesis of flavonoids and effects of stress. Curr Opin Plant Biol, 5(3): 218–223

Xie D Y, Dixon R A (2005). Proanthocyanidin biosynthesis—still more questions than answers? Phytochemistry, 66(18): 2127–2144

Yamagishi M, Shimoyamada Y, Nakatsuka T, Masuda K (2010). Two *R2R3-MYB* genes, homologs of Petunia *AN2*, regulate anthocyanin biosyntheses in flower Tepals, tepal spots and leaves of asiatic hybrid lily. Plant Cell Physiol, 51(3): 463–474

Yi J, Derynck M R, Li X, Telmer P, Marsolais F, Dhaubhadel S (2010). A single-repeat MYB transcription factor, GmMYB176, regulates CHS8 gene expression and affects isoflavonoid biosynthesis in soybean. Plant J, 62(6): 1019–1034

Yoshida K, Iwasaka R, Kaneko T, Sato S, Tabata S, Sakuta M (2008). Functional differentiation of *Lotus japonicus* TT2s, R2R3-MYB transcription factors comprising a multigene family. Plant Cell Physiol, 49(2): 157–169

Zhang B, Hu Z, Zhang Y, Li Y, Zhou S, Chen G (2012). A putative functional MYB transcription factor induced by low temperature regulates anthocyanin biosynthesis in purple kale (*Brassica Oleracea* var. acephala f. tricolor). Plant Cell Rep, 31(2): 281–289

Zhang P, Chopra S, Peterson T (2000). A segmental gene duplication generated differentially expressed myb-homologous genes in maize. Plant Cell, 12(12): 2311–2322

Zhang P, Wang Y, Zhang J, Maddock S, Snook M, Peterson T (2003). A maize QTL for silk maysin levels contains duplicated Myb-homologous genes which jointly regulate flavone biosynthesis. Plant Mol Biol, 52(1): 1–15

Zhao L, Gao L, Wang H, Chen X, Wang Y, Yang H, Wei C, Wan X, Xia T (2013). The R2R3-MYB, bHLH, WD40, and related transcription factors in flavonoid biosynthesis. Funct Integr Genomics, 13(1): 75–98

Zifkin M, Jin A, Ozga J A, Zaharia L I, Schernthaner J P, Gesell A, Abrams S R, Kennedy J A, Constabel C P (2012). Gene expression and metabolite profiling of developing highbush blueberry fruit indicates transcriptional regulation of flavonoid metabolism and activation of abscisic acid metabolism. Plant Physiol, 158(1): 200–224