

Review

Anthocyanin biosynthesis in fruit tree crops: Genes and their regulation

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The anthocyanin biosynthesis pathway is a little complex with branches responsible for the synthesis of a variety of metabolites. In fruit tree crops, during the past decade, many structural genes encoding enzymes in the anthocyanin biosynthetic pathway and various regulatory genes encoding transcription factors that regulate the expression of structural genes have been cloned and then functionally characterized in detail. In general, the structural genes involved in anthocyanin synthesis were coordinately expressed and their levels of expression were positively related to the degree of anthocyanin concentration; while, the coordinated expression pattern is striking a diverse among fruit crop species. Regulatory genes regulate spatiotemporally the structural genes and then form complicated metabolic network. Anthocyanin biosynthesis can be affected by external and internal factors, such as light, UV-B, low temperature and ABA through changes in expression of structural and regulatory genes.

Key words: Anthocyanin, regulatory genes, structural genes, fruit tree crops, factors.

INTRODUCTION

Fruits, such as apple, pear, peach, citrus, Chinese bayberry, play an important role in nutrient and health benefits for its rich sources of vitamin C, flavonoids, carotenoids (provitamin A), and other nutraceutical compounds. Of these healthful materials, anthocyanins belonging to the flavonoids compound family are water solvable pigments and have been suggested to reduce certain cancers, coronary heart diseases, oxidative stress and other age-related diseases (Ross and Kasum, 2002). Therefore, they are usually components of human diet and are not only considered exclusively as food products but also as therapeutic agents (Piero et al., 2005). A large number of fruits contain substantial anthocyanins, making their flesh and/or skin pink, red or purple as well as blue. Color is an important quality trait for fruit because consumers generally prefer fruits with red, blue or purple skin and flesh, and then obviously increase their marketability. For these reasons, anthocyanin biosynthesis in fruits becomes one aspect of currently active research

area nowadays, which is helpful to understand the mechanism better and develop novel fruit cultivars with higher anthocyanin content.

In fruit tree crops, during the past decade, many structural genes encoding enzymes in the anthocyanin biosynthetic pathway and various regulatory genes encoding transcription factors that regulate the expression of structural genes have been cloned and then functionally characterized in detail (Boss et al., 1996; Honda et al., 2002; Cultrone et al., 2010; Borsani et al., 2010). In apple and grape, having a better knowledge of anthocyanin biosynthetic mechanisms compared to other fruit tree species, whereas many questions are yet to be determined (Matus et al., 2008), because some genes implicated in anthocyanin biosynthesis have pleiotropy and are affected by many internal and external factors. More recently, many new regulatory genes have been continually identified in grape, apple and other fruit trees (Huguene et al., 2009; Cultrone et al., 2010; Feng et al., 2010). Some of these genes are sensitive to environmental stimuli, for example temperature, light, nutrition and hormones (Jeong et al., 2004; Piero et al., 2005; Ubi et al., 2006; Guo et al., 2008; Bureau et al., 2009; Moreno

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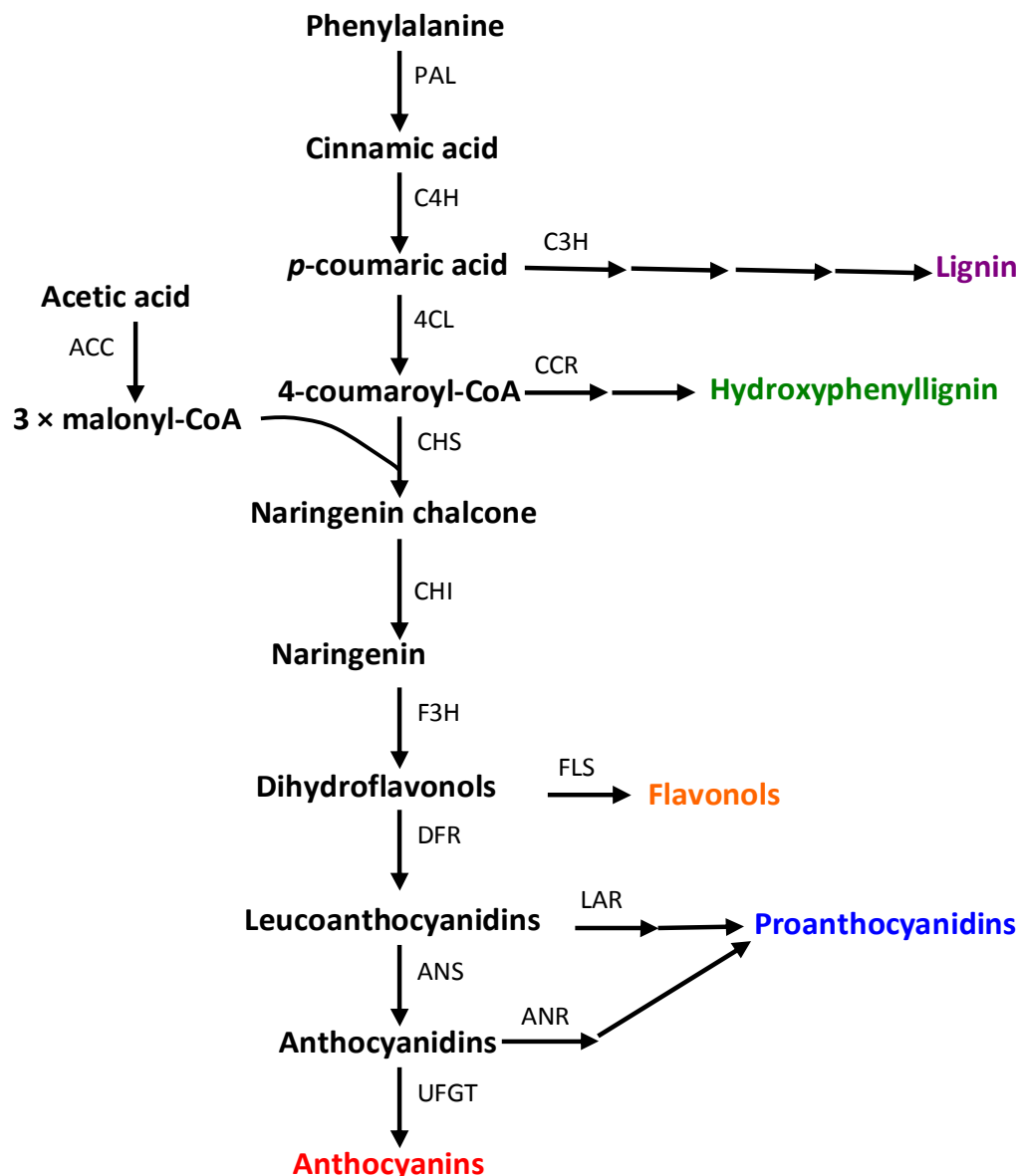


Figure 1. Simplified schematic of anthocyanin biosynthesis pathway and its branches. PAL, Phe ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate-CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; UFGT, UDPG-flavonoid-3-O-glucosyltransferase; ANR, anthocyanidin reductase; LAR, leucoanthocyanidin reductase; FLS, flavonol synthase; ACC, acetyl CoA carboxylase; CCR, cinnamyl-CoA reductase; C3H, 4-coumarate 3-hydroxylase.

et al., 2010; Crifó et al., 2011), which generally can lead to change in accumulation of anthocyanins in fruit. In practice, some cultural methods could be taken to increase anthocyanins content to satisfy the consumers.

Previous reviews in detail, described different aspects of anthocyanins biosynthesis, regulation, and their functions in plants and humans (Liu et al., 2006; Guo et al., 2008; Li et al., 2010). Herein, we will confer an overview of anthocyanins research, with a major focus on updating the recent progress on genes involved in anthocyanins biosynthetic pathway in fruit tree crops.

ANTHOCYANINS BIOSYNTHESIS PATHWAY

The anthocyanin biosynthesis pathway (Figure 1) is a little complex with branches responsible for the synthesis of a variety of metabolites and has been, nowadays, almost completely elucidated (Boss et al., 1996). There are a wide range of constructive genes, such as phenylalanine ammonia lyase (PAL), chalcone synthase (CHS), anthocyanidin synthase (ANS) involved in anthocyanin biosynthesis. Phenylalanine is a direct precursor for the synthesis of anthocyanidins. The conversion from

phenylalanine to anthocyanines requires a series of enzyme-catalyzed reaction. First of all, phenylalanine transformed to trans-cinnamic acid through trans-elimination of ammonia catalyzed by PAL. After that trans-cinnamic acid mediated by C4H and 4CL changes into 4-coumaroyl-CoA. One molecular of 4-coumaroyl-CoA together with three molecules of malonyl CoA were catalyzed by CHS form naringenin chalcone which is rapidly and stereospecifically isomerized to Naringenin by CHI. After hydroxylated at the 3-position by F3H, naringenin converts to dihydroflavonols which, subsequently, is reduced to leucoanthocyanidin by DFR. ANS catalyzes the synthesis of corresponding colored anthocyanidins using leucoanthocyanidin as substrate. At last, the hydroxyl group at C3 of anthocyanidins is glycosylated by glycosyl moieties from UDP-activated sugar donor molecule by the action of UFGT to yielded corresponding anthocyanins stored finally in the vacuole, a subcellular compartment with an acidic environment. Anthocyanidins are modified by glycosyl moieties in various ways in a family- or species-specific manner, and their color greatly depends on the number of hydroxyl groups on the B-ring; the larger the number of groups, the darker the blue color (Tanaka et al., 2008).

STRUCTURAL GENES

Structural genes encoding the enzymes of the anthocyanin biosynthetic pathway are conserved in plant kingdom (Holton and Cornish, 1995). In fruit crops, most of them have been isolated and well characterized (Boss et al., 1996; Piero et al., 2005; Ubi et al., 2006; Borsani et al., 2010).

A large number of studies pointed out that the structural genes involve in anthocyanin synthesis were coordinately expressed and their levels of expression were positively related to the degree of anthocyanin concentration (Honda et al., 2002; Piero et al., 2005; Borsani et al., 2010; Crifó et al., 2011), but there is some variability in the specific steps involved. In apple, as an example, coordinated expression of *MdCHS*, *MdF3H*, *MdANS*, *pDFR* and *pUFGluT* together was correlated well with anthocyanin synthesis (Honda et al., 2002). While, the transcript levels of *UFGT* were higher in red fruits compared to white in grape (Kobayashi et al., 2001) and *CHS*, *ANS* and *UFGT* in citrus (Cotroneo et al., 2006). From the aforementioned information, the coordinated expression pattern of structural genes related to improving anthocyanin synthesis is dependent on fruit crop species.

Among structural genes, PAL is the first gene involved in anthocyanin synthesis, while its role has been controversial. Some previous works (Zhao et al., 1994; Wang et al., 2000) supposed that PAL activity was closely related to anthocyanin synthesis; while others (Ju et al., 1995; Lo Piero et al., 2005; Feng et al., 2008) point to that PAL

may not be a key enzyme for anthocyanin development.

These seemingly contradictory results can be elucidated by the hypothesis supposed by Ju et al. (1995) that only when anthocyanin was synthesized beginning with the deamination of Phenylalanine would PAL be critical for anthocyanin synthesis; on the other hand, when precursors were sufficient, anthocyanin synthesis would not depend on PAL activity. PAL, not a tissue-specific gene, could express in flower, leaf, roots and seeds (Boss et al., 1996; Salvatierra et al., 2010), and was enhanced by light (Feng et al., 2010; Niu et al., 2010), low temperature (Lo Piero et al., 2005; Crifó et al., 2011) and abscisic acid (ABA) (Jeong et al., 2004), while suppressed by 1-methylcyclopropene (Jiang et al., 2001), *a*-naphthaleneacetic acid (NAA) and shading (Jeong et al., 2004). Interestingly, Feng et al. (2008) found that the effect of bagging treatment toward PAL activity was not obvious, indicating PAL was likely to be regulated by other genes rather than light directly. In grapevine, PAL has been shown to be encoded by multigene families (Sparvoli et al., 1994), but no one member of these multigene families, to date, is identified in fruit tree crops. In this case, PAL maybe has no other members indeed, just more likely post-translational processing results in PAL functional diversity.

C4H, 4CL sequentially convert cinnamic acid to phenolic precursors for anthocyanin biosynthesis. In strawberry, these two structural genes do not seem to have a determining role in the differences of fruit pigmentation, but had a closely related to synthesis of lignin monomers (Salvatierra et al., 2010). Compared to other structural genes, the information about C4H, 4CL enhancing anthocyanin synthesis is relatively poor. Consequently, further investigations are necessary for the role of these two genes in anthocyanin synthesis.

CHS (Chalcone synthases), the well-known representatives of the type III polyketide synthase (PKS) superfamily, catalyze the condensation of 4-hydroxycinnamoyl-CoA and three malonyl-CoA molecules to form the chalcone derivative, naringenin chalcone, which is the first committed step in the phenylpropanoid pathway of plants, leading to the biosynthesis of flavonoids, isoflavonoids, and anthocyanins (Ferrer et al., 1999). The genes encoding CHS constitute a multigene family. In grape, three *CHS* members, to date have been identified referred to as *CHS1*, *CHS2*, *CHS3*, respectively (Jeong et al., 2008). On the amino acid level, the homology between *CHS1* and *CHS2* was 96%, and between *CHS3* and *CHS1*, and *CHS3* and *CHS2* both were 89%, indicating very high homologies among CHSs. *CHS3* was predominantly expressed in the berry skin of red cultivars during coloration, while *CHS1* and *CHS2* mainly in the leaves and berry skin of both white and red cultivars (Yamamoto et al., 2002) implicating that *CHS3* was mainly responsible for anthocyanin synthesis, and *CHS1* and *CHS2* for other metabolites. More recently, it has been demonstrated that the expression of *CHS1* and

CHS2 coincided with flavonol; and CHS2 and CHS3 with anthocyanin biosynthesis (Jeong et al., 2008). From these two studies, CHS2 can be considered as a multi-function CHS member. In citrus, two CHS cDNAs were obtained (Moriguchi et al., 1999; Lu et al., 2009), and shared 86.6% identity each other at the amino-acid level, but their detailed function remains unknown.

The CHS members in other fruit tree species are necessary to be identified. Numerous studies, not including Boss et al. (1996), have demonstrated that the expression of the *CHS* in fruit tissue was passively associated with fruit coloring as shown in apple (Honda et al., 2002), citrus (Cotroneo et al., 2006; Bernardi et al., 2010; Wang et al., 2010), pear (Zhang et al., 2011), grape (Yamamoto et al., 2002), bilberry (Jaakola et al., 2002), etc; and was up-regulated by light (Feng et al., 2010), low temperature (Lo piero et al. 2005; Crifó et al., 2011), and UV-B (Ubi et al., 2006), and was inhibited or unchanged by the ethanol treatment (Kereamy et al., 2002). Thus, the *CHSs* in fruit tree crops seemed to be under different transcription controls, respectively.

Like CHS genes, *CHIs* also constitute a multigene family. Thus far, two CHI members (CHI1 and CHI2) were isolated and exhaustively analyzed (Jeong et al., 2008), indicating that CHI1 strongly associated with anthocyanin synthesis (Jeong et al., 2004), while CHI2 with flavan-3-ol (Jeong et al., 2008). In apple, two sequences similar to MdCHI in the apple genome have not been isolated as yet (Tacos et al., 2006a, b). Some studies suggested expression of CHI was related to anthocyanin synthesis (Feng et al., 2008, 2010), others showed not (Niu et al., 2010). Thus far, incongruence can be reasonably explained through leaning from PAL as earlier discussed. The level of transcript of CHS gene can be affected by environmental stimuli, such as light.

A wide body of studies suggested that F3H was related to anthocyanin synthesis (Honda et al., 2002; Castellarin et al., 2007a, b, c; Palapol et al. 2009; Feng et al., 2010; Niu et al. 2010); and enhanced by light (Kim et al., 2003), exogenous ethylene (Ashraf et al., 2003), UV-B (Ubi et al. 2006), water deficit (Castellarin et al., 2007b) and low temperature (Ban et al., 2007), etc., but not affected by ethanol (Kereamy et al., 2002). Sparvoli et al. (1994) pointed out that F3H has been shown to be encoded by multi-gene families. In grape, two F3H members, to date were isolated and subsequently analyzed (Jeong et al., 2004, 2008), indicating that the transcription of CHS1, CHS2, F3H1, and F3H 2 coincided with flavonol; and CHS2, CHS3, and F3H2 with anthocyanin biosynthesis. While, in other fruit tree species, no one F3H members has been reported as yet.

DFR and ANS reported previously were enhanced by light (Kim et al., 2003; Feng et al., 2010), low temperature (Piero et al., 2005), UV-B (Ubi et al., 2006), but unaffected by ethanol (Kereamy et al., 2002) and 2-chloroethylphosphonic acid (Kereamy et al., 2003) and had a major role in enhancing anthocyanin synthesis

(Honda et al., 2002; Cultrone et al., 2010; Jaalola et al., 2010; Niu et al., 2010; Zhang et al., 2011).

Of these two genes, only two *DFR* members have been isolated and analyzed in apple, referred to as MdDFR1 and DdDFR2, and in Chinese bayberry, referred to as MrDFR1 and MrDFR2, respectively (Espley et al., 2007; Niu et al., 2010). In apple, MdDFR1 were shown to be up-regulated in pigmented fruit tissue but not to any great extent in leaf. In contrast, MdDFR2 showed little or no activity in fruit but was highly elevated in the leaves of 'Red Field'.

In Chinese bayberry, MrDFR1 was clear-cut associated with anthocyanin content, but not MrDFR2, indicating that DFR members each are generally responsible for different function in fruit tree crops.

UFGT, last step gene of anthocyanin synthesis, could be a key enzyme in coloration during anthocyanin accumulation in grape (Kobayashi et al., 2001), apple (Ubi et al., 2006), mangosteen (Palapol et al. 2009), strawberry (Salvatierra et al., 2010) and Chinese bayberry (Niu et al., 2010) and so forth. However, this gene was expressed in all of the citrus leaves tested, including the non-pigmented and thus was not related to anthocyanin synthesis, but *ANS* or *DFR* could be (Lo Piero et al., 2005, 2006; Cultrone et al., 2010).

Cultrone et al. (2010) believed that UFGT is a family of enzymes that exhibits broad substrate specificity for flavonoids and anthocyanidins with 3-hydroxyl groups. Based on this hypothesis, it is reasonable that some of *UFGT* members were not related to anthocyanin synthesis. To our knowledge, this case is true to apple, in which had two *UFGT* members, referred to as UFGluT and UFGalT, respectively, and UFGluT expression is strong related to anthocyanin synthesis but UFGalT not (Honda et al., 2002). UFGT could be enhanced by environmental stimuli, such as ethanol (Kereamy et al., 2002), UV-B, low temperature (Ubi et al., 2006; Lo Piero et al., 2005), ethylene (Umphon et al., 2007), water deficiency (Castellarin et al., 2007b).

Collectively, the structural genes involved in anthocyanin synthesis are coordinately expressed and regulated by a wide variety of environmental factors, which control the anthocyanin synthesis.

The multigene family phenomenon make anthocyanin synthesis pathway more complex. To date, for grape and apple, the mechanism of anthocyanin synthesis is relatively clear. However, in other fruit tree species, such pear, Chinese bayberry and Citrus, etc; more investigations are required to elucidate the mechanism of anthocyanin synthesis.

REGULATED GENES

In fruit tree crops just as other plant species, the structural genes of the anthocyanin synthesis were largely regulated by a complex of MYB transcription

Table1. Regulatory genes identified in fruit tree crops and its targeting structural genes.

Fruit species	MYB	bHLH	Structural genes	Fruit tissue	Reference
Apple	MdMYB1	MdHHLH3,	ANS	peel	Takos et al., 2006a
	MdMYBA	HLH33		peel	Ban et al., 2007
	MdMYB10			Peel/flesh/leaf	Espley et al., 2009
Grape	VvMYB5b	MYCA1	VvLAR and VvANR	Peel	Deluc et al., 2008
	VvMYB5a		UFGT, C4H, ANS, F3H,	Peel	Deluc et al., 2006
	VvMYBA1		CHI, CHS, 4CL, DFR	Peel	Walker et al., 2007
	VvMYBA2		UFGT UFGT ANR, UFGT		Walker et al., 2007
Pear	PyMYB10,		DFR	Peel	Feng et al., 2010
	PcMYB10			Peel	Pierantoni et al., 2010
	PpyMYB10				Wang et al., 2010a
	PbMYB10				Wang et al., 2010a
mangosteen	GmMYB10		GmDFR, GmUFGT, GmLDOX	pericarp	Palapol et al., 2009
strawberry	FaMYB1		ANS, UFGT	flesh	Aharoni et al., 2001
	FaMYB10		DFR		Wang et al., 2010a
	FvMYB10		DFR		Wang et al., 2010a
biberry	VmTDR4		VmMYB2	Peel/flesh	Jaakola et al., 2010
Chinese bayberry	MrMYB1		AtDFR	flesh	Niu et al., 2010
Loquat	EjMYB10			Unknown	Wang et al., 2010a
Medlar	MgMYB10			Unknown	Wang et al., 2010a
Quince	CoMYB10			Unknown	Wang et al., 2010a
Apricot	ParMYB10		DFR	Unknown	Wang et al., 2010a
Plum	PiMYB10			Unknown	Wang et al., 2010a
	PdmMYB10			Unknown	Wang et al., 2010a
	PsMYB10			Unknown	Wang et al., 2010a
Cherry	PavMYB10		DFR	Unknown	Wang et al., 2010a
	PcrMYB10		DFR	Unknown	Wang et al., 2010a
	PcfMYB10			Unknown	Wang et al., 2010a
Almond	PdMYB10		DFR	Unknown	Wang et al., 2010a
Peach	PprMYB10		DFR	Unknown	Wang et al., 2010a
Red raspberry	RiMYB10		DFR	Unknown	Wang et al., 2010a

factors (TF), basic helix-loop-helix (bHLH) TFs and WD-repeat proteins (MYB-bHLH-WD40: MBW) at the level of transcription. Apple and grape were the best studied species in terms of regulation of anthocyanin synthesis by TF, extending our knowledge about anthocyanin

synthesis metabolism. Many IFs have been isolated and analyzed (Table 1), because TF was highly conserved in fruit tree species.

In grapevine, the VvMYBA1 and VvMYBA2 regulate specifically the expression of the UFGT gene and thereby

control fruit coloration. The skin color mutation from black to white/green arise from a retrotransposon insertion inside the promoter sequence of MYBA1 and several nonconservative substitutions in coding sequence of MYBA2 (Kobayashi et al., 2002; Walker et al., 2007). These two regulated genes locate on a single bacterial artificial chromosome and either of them can regulate colour in the grape berry, so mutational events taking place in two these adjacent regulated genes were essential for the genesis of the white grapes, but did not know which one first (Walker et al., 2007). Other MYBs (VvMYB5a and VvMYB5b) are involved in the control of different branches of the phenylpropanoid pathway, including anthocyanin synthesis pathway. In tobacco transformed with VvMYB5a, the biosynthesis of condensed tannins was enhanced, and lignin metabolism altered (Deluc et al., 2006). Like VvMYB5a, VvMYB5b regulate particularly the anthocyanin and the proanthocyanidin pathways (Deluc et al., 2008). In grapevine, to date, two WD32 (VvWDR1, VvWDR2) and two bHLH proteins (VvMYCA1, VvMYC1) have been identified, respectively. However, only VvMYCA1 and VvWDR1 are closely related to anthocyanin synthesis. VvMYCA1 may regulate UFGT and ANR through combining with similar elements in promoters of these two structure genes, whereas VvWDR1 may be part of a MYB/bHLH complex, making them both affect positively the synthesis of anthocyanins. *CsMYB8* and *CsMYC2* identified from *Citrus Sinensis* were not related to anthocyanin biosynthesis, while *CsMYC2* may be involved in the regulation of the flavonoid biosynthetic pathway (Cultrone et al., 2010).

In apple, three MYB genes, that is, MdMYB1, MdMYBA, MdMYB10, have been identified to belong to the R2R3 class and are responsible for anthocyanin accumulation (Takos et al., 2006a; Espley et al., 2007). At the deduced amino acid level, MdMYB1 and MdMYBA are identical and differ from MdMYB10 in three amino acids (Ban et al., 2007). Wang et al. (2010) believed that these three MYB genes were likely alleles of each other. MdMYB1 and MdMYBA expression are correlated with anthocyanin synthesis in the fruit skin (Takos et al. 2006a) and MdMYB10 controlled anthocyanin production in fruit (peel and flesh) and leaves (Espley et al., 2007). The methylation within MdMYB10 promoter seemingly decides apple skin patterning (blushed and striped) (Telias et al., 2011). Interestingly, MdMYB10 can activate its own promoter via an upstream MYB-binding motif for its own protein product (Espley et al., 2009). From previous studies, MYBs needs a bHLH partner to enhance anthocyanin accumulation. Thus far, two bHLH genes, that is, MdbHLH3 and MdbHLH33, have been identified in apple and MdbHLH3 is more suitable to MdMYB10 than MdbHLH33.

Besides apple and grapevine, MYBs has been studied intensively in other fruit tree crops, such as pear (Feng et al., 2010), mangosteen (Palapol et al., 2009), Chinese bayberry (Niu et al., 2010) and strawberry (Aharoni et al.,

2001). In general, MYBs are helpful in enhancing anthocyanin accumulation, but some repressing anthocyanin production were also identified, including MdMYB17 in apple (Wang et al., 2011), and FaMYB1 in strawberry (Aharoni et al., 2001). External and internal factors, such as light (Feng et al., 2010; Niu et al., 2010), UV-B, low temperature (Ban et al., 2007) and ABA (Jeong et al., 2004) affect IF expression, and then regulate spatio-temporally the structural genes. More recently, an IF, VmTDR4, regulating MYBs related to anthocyanin synthesis has been identified from Bilberry (Jaakola et al., 2010), indicating that a complex regulating network is present in fruit tree crops regulating the anthocyanin biosynthesis pathway. Furthermore, it were these suchlike IFs that, perhaps, make anthocyanin biosynthesis pathway organically linked with other metabolic pathways such soluble sugar, organic acid, and hormone.

FUTURE DEVELOPMENT

Anthocyanins biosynthesis in fruit tree crops is one aspect of currently active research area not only due to visual appeal but nutritional value. To develop an anthocyanin-rich cultivar, a better understanding of the regulatory network controlling anthocyanin biosynthesis in fruit tree crops is a prerequisite. However, transcription factors identified in fruit tree crops are limited, and how to cooperatively respond to internal and external factors, and then regulate structural genes needs to further investigate. Furthermore, according to mutated gene controlling anthocyanin biosynthesis, we can develop functional molecular markers to carry out marker-assisted selection in fruit tree crops. This promising work needs numerous explorations in future. Another challenging aspect would be to understand fully the effects of internal and external factors on anthocyanin accumulation in different fruit tree crops. In this case, we can use some strategies to regulate anthocyanin accumulation in practice.

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