Biosynthesis and Metabolic Engineering of Anthocyanins in Arabidopsis thaliana

Ming-Zhu Shi and De-Yu Xie*

Department of Plant and Microbial Biology, North Carolina State University, Raleigh, NC 27695, USA

Received: September 21, 2012 Revised: October 24, 2012 Accepted: November 6, 2012

Abstract: Arabidopsis thaliana is the first model plant, the genome of which has been sequenced. In general, intensive studies on this model plant over the past nearly 30 years have led to many new revolutionary understandings in every single aspect of plant biology. Here, we review the current understanding of anthocyanin biosynthesis in this model plant. Although the investigation of anthocyanin structures in this model plant was not performed until 2002, numerous studies over the past three decades have been conducted to understand the biosynthesis of anthocyanins. To date, it appears that all pathway genes of anthocyanins have been molecularly, genetically and biochemically characterized in this plant. These fundamental accomplishments have made Arabidopsis an ideal model to understand the regulatory mechanisms of anthocyanin pathway. Several studies have revealed that the biosynthesis of anthocyanins is controlled by WD40-bHLH-MYB (WBM) transcription factor complexes under lighting conditions. However, how different regulatory complexes coordinately and specifically regulate the pathway genes of anthocyanins remains unclear. In this review, we discuss current progresses and findings including structural diversity, regulatory properties and metabolic engineering of anthocyanins in Arabidopsis thaliana.

Keywords: Anthocyanins, Arabidopsis thaliana, biosynthetic pathway, structural diversity, transcriptional regulation.

INTRODUCTION

Anthocyanins are a group of colorful and bioactive natural pigments with numerous important physiological and ecological functions in plants. In general, anthocyanins attract pollinators and seed dispersers, protect plants from high light irradiation and scavenge free radicals produced in cells under stress conditions [1-6]. In addition, anthocyanins have many promising benefits for human health. Numerous studies have demonstrated that anthocyanins have antioxidative, anti-inflammatory, anti-carcinogenic and anti-microbial activities, and can prevent against cardiovascular diseases and diabetes and improve vision [7-12]. A recent study showed that feeding mice with a diet supplemented with transgenic tomatoes rich in anthocyanins resulted in an extension of life span [13].

Arabidopsis thaliana is the first model plant, the genome of which has been sequenced. Over the past nearly three decades, intensive studies on this model plant have greatly updated our understandings in plant biology including the biosynthesis and functions of anthocyanins and other metabolites. In this report, we review and discuss the structural diversity, biosynthesis and metabolic engineering of anthocyanins in this model plant.

STRUCTURAL FEATURES OF ANTHOCYANINS

The study of anthocyanin biosynthesis has become one of the main focuses of the study of flavonoids in Arabidopsis since late 1980s. However, the structural properties of anthocyanins in Arabidopsis were unknown until 2002 when two anthocyanins were identified from leaf tissues [14]. Since then, new anthocyanin molecules have been continuously characterized, most of which were identified from pap1-D (production of anthocyanin pigment 1-Dominant) and 35S:PAP1 transgenic plants as well as red pap1-D callus cultures [15-18]. PAP1 encodes a transcription factor that has been demonstrated to be a master regulator activating anthocyanin biosynthesis in Arabidopsis. The pap1-D and 35S:PAP1 plants are featured by an enhanced accumulation of anthocyanins resulting from the overexpression of PAP1 [19]. To date, more than twenty-nine anthocyanin molecules including trans- and cis- isomers have been identified from Arabidopsis (Table 1; Fig. (1)), which are grown in different growth conditions such as high light intensities [16] and low temperature combined with high light [15].

Cyanidin has been identified as the predominant anthocyanin aglycone in Arabidopsis. To date, all identified Arabidopsis anthocyanin molecules are derived from cyanidin through different modifications such as glycosylation, acylation and methylation, Fig. (1). These anthocyanin molecules are numerated as A1, A2, A3 and so on, in which “A” means “Anthocyanin” (Fig. (1); Table 1). Anthocyanin profiles seem to differ in distinct tissues. For example, A11 appears to be the most abundant anthocyanin molecule in leaf tissues [14-16,18], while A5 is the most abundant one detected from roots [18]. Interestingly, anthocyanin molecules with a sinapoyl moiety (A4, A7, A9, A10 and A11) were not detected in roots [18] as well as in pap1-D callus cultures [17]. Several methylated anthocyanins (A14, A15, A16, A17, and A19) have been identified, but for most of
them the methylation site in the structure has not been determined yet [16,17]. In addition, seedlings treated with anthocyanin precursors have been reported to form new anthocyanin molecules. Seedlings of both Col and Ler ecotypes treated with naringenin were able to synthesize cyanidin 3-O-glucosides (C3Gs) (449 m/z) and three unknown anthocyanin molecules featured by a mass spectrum of 611 m/z [23]. These four anthocyanin molecules are not detectable in plants in untreated conditions. This study indicates that the number and types of anthocyanins that can be produced by this model plant are likely more complicated than our current understanding. As more experiments are being continued, resulting data will enhance our understanding of the structural diversity of anthocyanin molecules in Arabidopsis.

AGLYCONE STRUCTURE MODIFICATIONS

To date, all identified anthocyanin molecules of Arabidopsis are derived from side group modifications of cyanidin through mechanisms of glycosylation, acylation and/or methylation. These modifications have been reported to increase the stability of anthocyanins in aqueous solution and may likely alter their light absorption properties [24,25]. Eight genes have been isolated and biochemically characterized to be associated with these different modifications as described below (Table 2).

Glycosylation is one of the main biochemical mechanisms leading to diverse anthocyanin molecules in Arabidopsis. All anthocyanins identified in Arabidopsis contain at least one sugar group. The hydroxyl groups at C3 and C5 positions of cyanidin have been reported to be the two commonest targets of glucosylation [18,23,25]. These two glucosylation reactions have been characterized to be catalyzed by two major glucosyltransferases, UGT78D2 and UGT75C1, which are encoded by At5g17050 and At4g14090, respectively [18]. UGT78D2 has been reported to glucosylate the hydroxyl group at C3 to form cyanidin 3-O-glucoside. In addition, this enzyme has been reported to catalyze the glycosylation of the hydroxyl group at C3 of flavonols and thus is called a flavonoid 3-O-glycosyltransferase. UGT75C1 has been reported to glucosylate the hydroxyl group at C5 to form cyanidin 5-O-glucosides. In cyanidin 3,5-O-glucosides, the glycosylation of the hydroxyl group at C3 has been reported to occur prior to that at C5 [18]. The formation of cyanidin 3-O-glucoside, cyanidin 5-O-glucoside and cyanidin 3,5-O-glucoside most likely are the beginning steps of glycosylation. Subsequent glycosylations lead to more diverse and
Table 1. Major anthocyanin molecules identified from *Arabidopsis thaliana*.

| Anthocyanin | ESI-MS | Reference about NMR Data | Detected Distribution in Tissues |
|-------------|--------|--------------------------|---------------------------------|
| A1          | 743    | NA                       | Leaves and roots                |
| A2          | 829    | NA                       | Leaves, roots and callus cultures |
| A3          | 889    | [20] b                   | Leaves, roots and callus cultures |
| A4          | 949    | NA                       | Leaves                          |
| A5          | 975    | [20] b                   | Leaves, roots and callus cultures |
| A6          | 1051   | [20] b                   | Leaves                          |
| A7          | 1095   | NA                       | Leaves                          |
| A8          | 1137   | [21]                     | Leaves and roots                |
| A9          | 1118   | [22] c                   | Leaves                          |
| A10         | 1257   | [21]                     | Leaves                          |
| A11         | 1343   | [14]                     | Leaves                          |
| A12         | 1373   | NA                       | Leaves                          |
| A13         | 1357   | [14]                     | Leaves                          |
| A14         | 1395   | NA                       | Leaves                          |
| A15         | 1005   | NA                       | Leaves                          |
| A16         | 1195   | NA                       | Leaves                          |
| A17         | 1115   | NA                       | Leaves                          |
| A18         | 1035   | NA                       | Leaves                          |
| A19         | 843    | NA                       | Callus cultures                 |

*both trans and cis isomers were detected. "NMR data of the same molecule identified in the garden plants of Cruciferae. "NMR data of the same molecule identified in *Matthiola Incana*. NA: not available.

Table 2. List of anthocyanin modification genes identified in *Arabidopsis thaliana*.

| AGI No. | Gene Name | Annotation | Reference |
|---------|-----------|------------|-----------|
| At5g17050 | UGT78D2 | Flavonoid 3-O-glucosyltransferase | [18] |
| At4g14090 | UGT75C1 | Anthocyanin 5-O-glucosyltransferase | |
| At5g54060 | UGT79B1 | Anthocyanin 3-O-glucoside: 2''-O-xylosyltransferase | [26] |
| At3g21560 | UGT84A2 | Sinapic acid: UDP-glucosyltransferase | |
| At3g29590 | A5G6 MaT | Anthocyanin 5-O-glucoside: 6''-O-malonyltransferase | [24] |
| At1g03940 | A3G6 p-ConT | Anthocyanin 3-O-glucoside: 6''-O-p-coumaroyltransferase | |
| At1g03495 | A3G6 p-ConT | Anthocyanin 3-O-glucoside: 6''-O-p-coumaroyltransferase | |
| At2g23000 | SCPL10 | Sinapoylglucose:anthocyanin acyltransferase | [27] |

complex cyanin molecules in this plant. Two other glycosyltransferases encoded by *UGT79B1* and *UGT84A2* respectively were recently identified to be involved in subsequent glycosylation of cyanidin 3-O-glucosides [26]. *UGT79B1* is a cyanidin 3-O-glucoside: 2''-O-xylosyltransferase that adds a xylosyl group to the hydroxyl group at C2''. *UGT84A2* is a sinapic acid: UDP-glucosyltransferase that catalyzes the formation of 1-O-sinapoylglucose by adding glucose to
sinapic acid. The knockout mutation of UGT84A2 lead to the reduction of the levels of A11, a dominant sinapoylated cyanin in wild-type (WT) Arabidopsis leaves [26]. This result suggests that 1-O-sinapoylglucose serve as a donor of sinapoyl moieties to form sinapoylated cyanins. The biochemical mechanism by which the glucose group is attached to the p-coumaroyl moiety on the anthocyanin structures remains to be elucidated.

Acylation is another main biochemical mechanism leading to diverse anthocyanin molecules in Arabidopsis [25,28]. To date, several enzymes have been characterized to catalyze these acylation reactions. At3g29590, At1g03940 and At1g03495 have been identified to encode three BAHD types of anthocyanin acyltransferases (AATs) that use malonyl-CoA or p-coumaroyl-CoA as substrates to transfer the malonyl or p-coumaroyl groups to cyanin structures [24]. In addition, At2g23000 has been characterized to encode a serine carboxypeptidase-like (SCPL) type of AAT. This enzyme has been shown to use sinapoylglucoses as substrates to transfer sinapoyl groups to cyanins to form sinapoylated cyanins [27].

Methylated forms of cyanin molecules have been detected from Arabidopsis [14,16,17]. Although, to date, genes encoding anthocyanin methyltransferases have not been characterized in Arabidopsis, several of them have been identified from other species such as petunia and grape [25]. S-adenosyl-L-methionine (SAM) dependent O-methyltransferases (OMTs) have been reported to be responsible for catalyzing the methylation of various natural products [25,29]. The methylation process of anthocyanins in Arabidopsis is unclear and whether there exist OMTs in Arabidopsis responsible for the formation of methylated anthocyanins remains to be elucidated.

**BIOSYNTHETIC PATHWAY**

The anthocyanin biosynthetic pathway is a major branch of the general phenylpropanoid pathway that starts with phenylalanine, Fig. (2). In general, from phenylalanine to anthocyanins, the biosynthetic pathway can be divided into three phases: beginning steps of the general phenylpropanoid pathway, early steps of the flavonoid pathway and late steps of the anthocyanin specific pathway.

The beginning steps of the phenylpropanoid pathway include three consecutive steps from phenylalanine through cinnamic acid and coumaric acid to 4-coumaroyl CoA, which are catalyzed by phenylalanine ammonia-lyase (PAL), cinnamate-4-hydroxylase (C4H) and 4-coumaroyl: CoA-ligase (4CL), respectively. In addition to flavonoid biosynthesis, these three steps of the phenylpropanoid pathway also lead to the production of hydroxycinnamic acid derivatives such as sinapate esters and monolignols. Genes encoding PAL, C4H and 4CL have been cloned and characterized Fig. (2).

![Fig. (2). Anthocyanin biosynthetic pathway in Arabidopsis. Enzymes catalyzing corresponding steps are indicated. Related branches of the pathway leading to the production of other phenylpropanoid compounds are also indicated. PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumaroyl: CoA-ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3’H, flavonoid 3’-hydroxylase; DFR, dihydroflavonol reductase; ANS, anthocyanin synthase; FLS, flavonol synthase; ANR, anthocyanidin reductase; AGT, anthocyanin glycosyltransferase; AAT, anthocyanin acyltransferase; AMT, anthocyanin methyltransferase; TT19, Transparent Testa 19; TT12, Transparent Testa 12.](image_url)
from Arabidopsis. Four genes have been identified to encode isomers of PAL. Knockout mutant analyses and gene expression experiments under nitrogen depletion and low temperature conditions have shown that two isomers, PAL1 and PAL2, are preferably involved in the flavonoid pathway [30-32]. A small gene family has been identified to encode 4CL in Arabidopsis. Studies of gene expression pattern and enzyme properties have revealed that 4CL3 appears to be preferably associated with the flavonoid pathway, while 4CL1 and 4CL2 are most likely involved in the formation of hydroxycinnamic acid derivatives [33]. In contrast to PAL and 4CL, only one gene in the Arabidopsis genome has been identified to encode C4H.

The early steps of the flavonoid pathway are from 4-coumaroyl CoA through chalcone and naringenin to dihydroflavonol. These three reaction steps are catalyzed by chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3'H), respectively, and as a result, dihydrokaempferol characterized by a hydroxyl group at C4' in the B-ring is produced. The subsequent hydroxylation of dihydrokaempferol at C3' catalyzed by the flavonoid 3'-hydroxylase (F3'3'H) leads to the synthesis of dihydroquercetin. To date, dihydrokaempferol and dihydroquercetin are the only two dihydroflavonol molecules identified in Arabidopsis. Genes encoding these pathway enzymes have been biochemically and genetically characterized in Arabidopsis. Knockout mutations of these genes lead to the lack of production of both anthocyanins and proanthocyanidins in seeds resulting in transparent testa [34-36].

The late steps of the anthocyanin pathway include steps from dihydroflavonols through leucoanthocyanidins to anthocyanidins. These three reaction steps are catalyzed by leucoanthocyanidin dioxygenase (ANS, also called leucoanthocyanidin dioxygenase, LDOX), hydroflavonol reductase (DFR) and anthocyanidin synthase (ANS, also called leucoanthocyanidin dioxygenase, LDOX). These two enzymes are encoded by a single gene respectively. The knockout mutants of either of these two genes lead to transparent testa phenotypes in seeds [34,35]. In addition, as described above, modifications including glycosylation and acylation convert anthocyanidins to diverse anthocyanin molecules.

**METABOLIC CHANNELING IN VIVO**

Successive enzymes of the phenylpropanoid pathway are proposed to be grouped together and associated with the membrane of the endoplasmic reticulum (ER) to form protein complexes that direct the channeling of the intermediate precursors in the complex without diffusing to the cytosol [34,37,38]. Evidence for the channeling of intermediates and the co-localization of pathway enzymes has been reported [39]. In addition, direct in vitro studies have shown that PAL and C4H were co-localized on ER membranes of tobacco cells [40]. It has been hypothesized that the membrane-anchored C4H and F3'H, two members of the cytochrome P450 family proteins, might act as nucleation sites for the binding of other soluble enzymes to the complex [34,40]. Although evidence is limited, this hypothesis is considered as a favorable model for the synthesis and channeling of anthocyanins and other flavonoids.

**TRANSPORT AND COMPARTMENTATION**

Anthocyanins are stored in the central vacuole of cells. As described above, the biosynthesis of anthocyanins takes place in the cytosol. Anthocyanins need to be transported from the cytosol to the vacuole. Transporter-mediated and vesicle-mediated transport are two major hypotheses proposed for the transport of anthocyanins to the vacuole [23,41,42].

In general, the hypothesis of transporter-mediated transport is supported by the identification of flavonoid transporters involved in the vacuolar transport of specific types of anthocyanins and proanthocyanidin precursors in different plant species [43-46]. In Arabidopsis, three genes, TT12, TT19 and AHA10, have been functionally characterized to be associated with the transport of anthocyanins. TT12 encodes a multidrug and toxic efflux (MATE) antipporter that has been demonstrated to be responsible for the vacuolar uptake of glycosylated flavan-3-ols and possibly glycosylated anthocyanidins in the endothelial cells of seeds [43,47]. The tt12 mutants lack the formation of proanthocyanidins in seeds and show a transparent testa phenotype. Also, the endothelial cells of tt12 mutants form multiple vesicles instead of a large central vacuole. AHA10 encodes a plasma membrane H+-ATPase that has been reported to likely function in endosomal or vacuolar compartments [48]. The aha10 mutants form multiple vesicles instead of a large central vacuole; instead, produce numerous vesicles filled with epicatechin molecules that are precursors of proanthocyanidins. Experiments have shown that AHA10 is essential for the acidification of the central vacuole and the formation of the proton gradient necessary for the function of TT12 in the seed endothelial cells. Given that TT12 and AHA10 are primarily expressed in developing seeds, these two genes likely co-ordinate the subcellular transport and compartmentation of anthocyanins and proanthocyanidins in the seed coat. The mechanism of the vacuolar uptake of anthocyanins in vegetative tissues remains unclear. It has been hypothesized that homologs of TT12 likely function in vegetative tissues to mediate the transport of anthocyanins [43]. In addition, homologs of the multidrug resistance-associated protein (MRP) type of ABC transporters similar to the ZmMRP3 in maize [44] are also potential candidates involved in anthocyanin transport from the cytosol to the large central vacuoles in vegetative tissues. TT19 encodes a glutathione S-transferase (GST) that has been demonstrated to be involved in the vacuolar uptake of both anthocyanins and proanthocyanidin precursors [49]. The tt19 mutants lack the production of proanthocyanidins in the seed coat and show transparent testa phenotypes. TT19 was proposed to function as a carrier protein to ‘escort’ anthocyanins or proanthocyanidin precursors to the vacuole [34,42,49-51]. In vitro biochemical analysis has shown that TT19 has a very weak GST activity, and no anthocyanin-glutathione conjugates have been detected in Arabidopsis [34,49,51,52]. A recent study demonstrated that TT19 can bind to not only cyanidin but also to cyanidin 3-O-glucoside, although the affinity to the latter is lower than to the former [53]. Based on the cytosolic localization of TT19, the binding of TT19 to cyanidin most likely occurs near the cytosolic surface of ER. TT19 might function in protecting cyanidin from degrada-
tion during the transport process. Furthermore, given that recently the TT19 fusion protein was observed to be localized in the tonoplast as well [53], it likely has additional functions that needs further characterization.

The evidence for vesicle-mediated transport results from the observation of cytoplasmic vesicle-like structures filled with anthocyanins and the anthocyanic vacuolar inclusions (AVIs) that exist in the large central vacuole [23,41]. This with anthocyanins and the anthocyanic vacuolar inclusions the observation of cytoplasmic vesicle-like structures filled with anthocyanins and the anthocyanic vacuolar inclusions is strong evidence for vesicle-mediated transport and vesicle-mediated mechanisms may act in concert to direct the transport of anthocyanins.

**METABOLIC ENGINEERING OF ANTHOCYANINS IN VITRO**

The isolation of anthocyanin-producing cells in vitro from Arabidopsis has not been reported until recently. We established anthocyanin-producing cell lines through tissue culture from rosette leaves of pap1-D plants [17,54]. On a modified MS medium (without NH4NO3 and with half-strength KNO3) supplemented with 0.1 mg L\(^{-1}\) 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.25 mg L\(^{-1}\) kinetin, red calli were selected and maintained. During in vitro selection, metabolic differentiation occurred in cultured cells. As a result, several red cell lines with different anthocyanin levels were developed. In addition, anthocyanin-free cells from pap1-D plants were also established. Microarray and RT-PCR analysis showed up-regulation of the expression of most late pathway genes as well as transcription factors including PAP1, TTS and GL3 in red pap1-D cells. LC-MS based profiling identified seven cyanin molecules from red pap1-D cells. The anthocyanin-producing pap1-D cells provide an appropriate model system to understand the mechanisms of how other factors control the activities of the WBM complexes discussed below.

**TRANSCRIPTIONAL REGULATION OF PATHWAY GENES**

Over the past two decades, the regulation of the anthocyanin biosynthetic pathway has gained intensive investigations in Arabidopsis [55,56]. Pathway genes of flavonoid biosynthesis were shown to be co-regulated [15,18,55,57]. Particularly, studies of mutants, gene expression profiling, protein-DNA and protein-protein interactions have shown that the expression of late biosynthetic genes of anthocyanins is regulated by a ternary WD40-bHLH-MYB (WBM) complex composed of MYB, bHLH and WD40 transcription factors.

Four MYB transcription factors, PAP1/ MYB75, PAP2/ MYB90, MYB113 and MYB114 with relatively high sequence similarities, have been identified to control anthocyanin biosynthesis in vegetative tissues. All these four genes are R2R3-MYB proteins that contain two imperfect repeats in the MYB domain [58,59]. PAP1 (Production of Anthocyanin Pigmentation 1) was identified by T-DNA activation tagging [19]. The overexpression of PAP1 in pap1-D activation tagging lines and 35S:PAP1 transgenic plants leads to high accumulation of anthocyanins in leaves, stems, flowers and roots [15,18,19]. In addition, the overexpression of PAP2, MYB113 and MYB114 also leads to an increase in anthocyanin production [19,60]. In contrast, the pap1 knockout mutants and the knockdown plants of PAP1, PAP2, MYB113 and MYB114 by RNAi lack anthocyanins in leaves and seedlings [60]. Gene expression analysis has shown that the expression of DFR and ANS is highly activated in plants overexpressing these genes [15,16,18,19,60], but reduced or inactivated in pap1 knockout mutants and PAP1 RNAi knockdown plants [60]. Among the four MYB transcription factors, it appears that PAP1 is a master regulator of anthocyanin biosynthesis. PAP1 is expressed at the highest level in comparison with its homologs. The metabolic engineering of red pap1-D cells has demonstrated that the overexpression of PAP1 alone can activate the anthocyanin pathway especially the expression of late pathway genes [17,54,61]. In addition, the overexpression of PAP1 in several other plant species has resulted in obvious increases in anthocyanin levels [62-66]. These data show that PAP1 is a key regulator controlling the biosynthesis of anthocyanins. It is hypothesized that PAP2, MYB113 and MYB114 might be specialized in regulating anthocyanin biosynthesis under certain conditions or at specific developmental stages of plants.

Three members of the bHLH transcription factor family, GL3 (Glabra 3), EGL3 (Enhancer of Glabra 3) and TT8 (Transparent testa 8), have been identified to positively regulate anthocyanin biosynthesis. Based on the classification of the bHLH protein family, these three members belong to the subgroup III [67-70]. These three homologs are not simply functionally redundant. In contrast, they have overlapping but distinct functions in regulating several physiological and developmental processes, such as trichome initiation, non-root hair cell fate determination, seed coat muclilage formation, anthocyanin and proanthocyanidin biosynthesis [60,71-75]. GL3 and EGL3 were identified from the phenotypes of their knockout mutants. In Arabidopsis, gene expression and biochemical analysis have shown that GL3 and EGL3 were essentially associated with trichome development, pavement cell fate determination and cell patterning. In particular, promoter activity analyses have shown that the expression of these two genes spatially occurs in mature embryos, expanding cotyledons, root tips, leaf primordium and young seedlings [55,60,74,75]. The function of GL3 in regulating anthocyanin biosynthesis was first observed in a transient expression experiment, in which the co-expression of GL3 and MYC-146 led to the formation of anthocyanins in white flower mutants of Matthiola incana [76]. The involvement of GL3 in anthocyanin biosynthesis subsequently was supported by mutant analysis and gene expression studies. The pigmentation of anthocyanins in the cotyledon and hypocotyl of seedlings was phenotypically lower in egl3, gl3 and egl3 gl3 mutants than in wild-type plants. The egl3 gl3 mutants lost the most reddish pigmentation, followed by egl3 and then gl3 mutants [75]. In addition, the overexpression of EGL3 in the ttgl1 mutant background resulted in more anthocyanin pigmentation than the overexpression of GL3 in the same mutant background [75]. These two observations were supported by results from inducible gene expression experiments. In brief, the expression of the recombinant GL3 induced by dexamethasone in gl3 and gl3 egl3 mutant backgrounds revealed that when EGL3 was present, the gene ex-
expression levels of DFR and ANS were similar no matter whether GL3 was present or not [60]. These observations suggested that EGL3 had a stronger regulatory activity on anthocyanin biosynthesis than GL3 in seedlings [60]. However, the regulatory function of GL3 in anthocyanin biosynthesis was also shown by experiments testing the effects of nitrogen depletion. This study revealed the involvement of GL3 but not EGL3 in the formation of anthocyanins in rosette leaves under nitrogen deficient conditions [77].

The result seems to be controversial to the previous observations about the relative contribution of GL3 and EGL3 on anthocyanin biosynthesis, but this difference might be explained by different experimental materials and/or treatments used in the studies. Taken together, all these experiments indicated that the involvement of EGL3 in the regulation of anthocyanin biosynthesis is likely conditional; GL3 and EGL3 might have functional specificity under different developmental stages and/or environmental conditions. From mutant analysis, the locus TT8 was first identified to encode a transcription factor [35]. The seeds of this mutant lack the brownish pigmentation produced by oxidation of proanthocyanidins, but anthocyanin biosynthesis was only moderately affected in young seedlings and leaves. The subsequent gene cloning and characterization demonstrated that TT8 encoded a bHLH protein regulating the expression of DFR, ANS and BAN (ANR) in the endothelial layer of seed coat [73]. Its expression was detected in seedlings, buds, flowers, and developing siliques, but barely detectable in rosette leaves, stems and roots [73]. Promoter analysis also revealed the pattern of expression of TT8 in developing siliques and young seedlings [78] as well as in the main veins of rosette leaves [79]. We recently isolated red cells from tissue culture of pap1-D rosette leaves overexpressing PAP1. Comparative qRT-PCR and microarray analyses showed a strong up-regulation of TT8 in red pap1-D cells [17]. All data have suggested that TT8 not only regulates anthocyanin production towards the synthesis of proanthocyanidins in seeds, but is also involved in the regulation of anthocyanin biosynthesis in vegetative tissues and cell cultures. In addition, EGL3 and TT8 have been identified to have a shared role in regulating seed coat mucilage production [75]. Moreover, studies have shown that TT8 expression can be controlled by several MYB and bHLH transcription factors. The expression of TT8 is increased in transgenic plants overexpressing PAP1 or TT2 [78]. In the gl3 egl3 mutant background, TT8 promoter has been shown to have a lower activity than in wild-type plants, indicating the necessity of GL3 and EGL3 in controlling the expression of TT8 [78]. Also, TT8 has been shown to be able to regulate its own expression [78]. Although most of the investigations have not reported the involvement of TT8 in epidermal cell fate determination during normal growth of plants, a recent report showed that TT8 was involved in the development of marginal trichomes of rosette leaves treated withjasmonic acid (JA), 6-benzylaminopurine (BAP) and gibberellic acid (GA) [80].

TTG1 is the only WD40 protein member currently determined to regulate anthocyanin biosynthesis in Arabidopsis. Mutation in the TTG1 locus results in pleiotropic impacts on plant development and metabolism, including the deficiency of anthocyanin production in vegetative tissues, the deficiency of proanthocyanidins in seed coat and defects in trichome initiation, non-root hair cell fate determination and seed mucilage production [35,81,82]. Multiple experiments have demonstrated that TTG1 is constitutively expressed in all tissues throughout the entire development of plants; in addition, its expression does not respond to alteration of environmental conditions tested [83-85]. All current data have shown that TTG1 has a central role in the WBM regulatory complexes to regulate epidermal cell fate and metabolic specificity leading to the production of anthocyanins and proanthocyanidins.

**THE WD40/BHLH/MYB REGULATORY COMPLEXES**

It has been shown that the activation of anthocyanin biosynthetic pathway, especially late biosynthetic steps in Arabidopsis, is controlled by a ternary complex formed by WD40, bHLH and MYB transcription factors, including TTG1, GL3, EGL3, TT8, PAP1, PAP2, MYB113 and MYB114 described above. The WD40/bHLH/MYB (WBM) complexes controlling anthocyanin biosynthesis have been identified from other plant species as well such as maize and petunia [55,56]. To date, TTG1 has been demonstrated to play a central role in the regulatory network in all WBM complexes potentially identified. The function of TTG1 in the WBM complex has been suggested to stabilize the protein-protein interactions [56,86]. The WD motifs in TTG1 are normally the sites responsible for interacting with other proteins. TTG1 has been found to be required for the normal distribution of GL3 in the nucleus. The loss of TTG1 caused the GL3-YFP protein to be distributed abnormally in the nucleus resulting in ‘speckles’ [74]. Also, a recent study demonstrated that nuclear-localized GL3 can recruit TTG1 to the nucleus by interacting with the TTG1 protein [87].

Protein-protein interactions among bHLHs, MYBs and TTG1 have been demonstrated by different experiments. Yeast two-hybrid and pull down assays have provided evidence that GL3, EGL3 and TT8 interact with TTG1, MYB family proteins PAP1/PAP2 and bHLH proteins themselves [71,75,88]. In addition, TT8 has been demonstrated to interact with TT2 and TTG1 to regulate proanthocyanidin biosynthesis [71]. GL3 and EGL3 also interact with GL1 and WER, which are involved in the regulation of trichome initiation and non-root hair cell fate determination, respectively [75,88]. Sequence analysis revealed a conserved motif consisting of [DE]Lx2[RK]x3Lx2Lx2R in the R3 repeat of MYB proteins interacting with bHLHs. Site mutation studies confirmed that this motif is responsible for the interaction with bHLH proteins [88]. Those R2R3-MYBs such as MYB11, MYB12 and MYB111 that do not contain this motif have been demonstrated to be unable to interact with bHLHs. Two transient expression experiments have also indicated that MYB proteins interact with bHLH proteins to activate the transcription of late biosynthetic genes [88,89].

The C-terminal region of bHLH proteins is suggested to be required for regulating anthocyanin biosynthesis. Sequence analysis has identified that the N-terminal of bHLH proteins contains a region interacting with MYB and an acidic region while the C-terminal includes a bHLH domain likely involved in the formation of homodimer and heterodimer [56,90]. A recent study showed that the ectopic expression of the N-terminal region of the maize R protein, a
homolog of GL3 and EGL3, can regulate leaf trichome and root hair differentiation in Arabidopsis, but for activating anthocyanin biosynthesis, the full length R is required [91]. These data suggest that the dimerization of the bHLH proteins is likely a prerequisite for activating anthocyanin biosynthesis. It is also possible that other co-factors might be recruited to the C-terminal regions of bHLH proteins and are required for the activation of target genes. In maize, an EMSY-related factor involved in the histone modification has been found to specifically interact with the bHLH region of R and is necessary for the activation of the expression of anthocyanin biosynthetic genes [92].

The component properties of different WBM complexes remain to be further elucidated. Although biochemical and genetic studies have shown that TTG1 (WD40), GL3/EGL3/TT8 (bHLH) and PAP1/PAP2/MYB113/MYB114 (MYB) are components of potential WBM complexes [54,60]. The understanding of how many bHLH and MYB members are necessary to form a functional complex to activate the biosynthesis of anthocyanins at different developmental stages and environmental conditions is limited. To date, it appears that only the WBM complex in endothelial layers of seed coats has been determined to consist of TTG1, TT8 and TT2 [71]. In comparison, the components of bHLHs and MYBs in leaves are more complicated. Gene expression studies and protein profiles in single cells or in metabolically and morphologically identical cells in leaves might be helpful. We recently isolated red cells from rosette leaves of pap1-D plants and cultured them in vitro. Although these red cells were characterized by heterogeneity of pigmentation, no specific morphological differentiations, such as the formation of pavement cells and trichomes, occurred in the culture conditions [17]. Genome-wide gene expression analysis identified the up-regulation of PAP1 and TT8 in red cells. In addition, qRT-PCR analysis showed the up-regulation of the expression of GL3 in red cells. Our experiments suggest that TTG1, GL3/TT8 and PAP1 likely form the only WBM complex that activates the high production of anthocyanins in engineered pap1-D cells.

In Arabidopsis, in addition to regulating anthocyanin biosynthesis, WBM complexes are involved in the control of other physiological and developmental processes including trichome initiation, non-root hair cell fate determination and mucilage production in the seed coat. This is in contrast to the function of WBM complexes in maize which seems to only control anthocyanin biosynthesis [55,56,71,74]. The involvement of WBM complexes in such broad aspects of cellular events in Arabidopsis likely results from the overlapping but distinct functions of multiple members of bHLH and MYB proteins as discussed above.

REGULATION OF THE ACTIVITIES OF WBM COMPLEXES BY FACTORS IN PLANTA

The activities of WBM complexes can be affected by factors in planta. As described above, TTG1, bHLHs (GL3/EGL3/TT8) and MYBs (PAP1/PAP2/MYB113/MYB114) can form different WBM complexes to activate anthocyanin biosynthesis. However, other proteins, such as CPC and MYBL2 which are 1R-MYB members, have been demonstrated to negatively control the activities of WBM complexes resulting in the decrease in the biosynthesis of anthocyanins shown in Fig. (3). Results from transient expression and protein interaction studies have suggested that CPC and MYBL2 compete with positive regulators PAP1/PAP2 to bind bHLH proteins and interfere with the formation of active WBM complexes thus negatively regulating the expression of pathway genes [79,88,89]. In addition to anthocyanin biosynthesis, CPC was identified to negatively regulate trichome initiation and non-root hair cell fate determination [93,94]. The overexpression of MYBL2 has been shown to repress trichome development [95]. The C-terminal of MYBL2 contains a repression domain composed of TLLLFR that has been shown to have a strong repressive activity [79]. A recent study has shed some light on the mechanisms in determining epidermal cell fate. The results revealed that the cell fate of root epidermal cells is determined by the quantitative competition between the levels of the positive R2R3-MYB regulator WER and the negative 1R-MYB protein CPC [96]. We propose that a similar regulatory mechanism might also control the production of anthocyanins in Arabidopsis cells. The quantitative competi-

Fig. (3). Regulation of anthocyanin production in Arabidopsis cells by quantitative competition between active WBM complexes and inactive or repressive WBM complexes.
tion between positive regulators PAP1/PAP2/MYB113/MYB114 and negative regulators CPC/MYB1L2 may determine the activation/repression of the expression of pathway genes, Fig. (3).

Small regulatory RNAs were recently uncovered to control anthocyanin biosynthesis through a mechanism of regulating the expression of the members of the WBM complexes. TAS4-siR81(-), which is derived from TAS4 and miR828, is a trans-acting siRNA. TAS4-siR81(-) and miR828 were shown to target PAP1/PAP2/MYB113 [97,98]. In phosphate deficient conditions, the expression level of PAP1 is increased in tissues. PAP1 has been demonstrated to activate the expression of TAS4 and miR828, which may further function in a feedback manner to target PAP1 and its homologs to reduce their expression [97]. These results have revealed a potential autoregulatory mechanism of PAP1 expression through TAS4-siR81(-) and miR828. In addition, transgene silencing of PAP2 has been observed in homozygous transgenic tobacco plants and was suggested to be caused by small regulatory RNAs similar to TAS4-siR81(-) and miR828 in Arabidopsis [99]. Another example of small RNA involved in the regulation of anthocyanin biosynthesis is miR156. The SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) transcription factor targeted by miR156 has been demonstrated to negatively regulate the acropetal accumulation of anthocyanins in the inflorescent stem [100]. SPL9 was observed to be able to interact with PAP1 and can directly bind to the promoter of DFR. SPL9 was suggested to negatively control the expression of anthocyanin pathway genes by competing with bHLH proteins for binding with PAP1. The high expression of miR156 indirectly positively regulates the expression of anthocyanin pathway genes.

REGULATION OF ANTHOCYANIN BIOSYNTHESIS BY ABIOTIC FACTORS AND PHYTOHORMONES

Anthocyanin biosynthesis can be induced by various abiotic factors such as high light, low temperature, sucrose, nutrient depletion and phytohormones [15,77,84,101-105]. Numerous significant advances have been made in elucidating the molecular mechanisms of anthocyanin biosynthesis in response to these factors, several of which are summarized below.

Light

Light is one of the most important environmental factors affecting biosynthesis of anthocyanins. Strong light conditions can increase the production of anthocyanins [15,16,83]. In contrast, dark conditions can lead to the decrease of anthocyanins. Although the mechanism of light regulation on anthocyanin biosynthesis remains to be completely elucidated, many studies have demonstrated that the expression of pathway and regulatory genes involved in anthocyanin biosynthesis is controlled by different light conditions. As multiple experiments have shown, all pathway genes are expressed in seedlings and rosette leaves of Arabidopsis plants in strong light conditions [15,16,83]. Also, it has been shown that the activation of these pathway genes in light conditions is likely through controlling the expression of the members of the WBM complexes [15,16,83]. For example, the expression of PAP1, PAP2 and bHLH genes GL3, EGL3 and TT8 were all induced by various light spectra [83]. As described above, PAP1 is a master regulator of anthocyanin biosynthesis. Nevertheless, several studies showed that PAP1 overexpression alone was not sufficient for the activation of anthocyanin biosynthesis in the dark or under low light conditions [15,16,83], which suggests that the accumulation of other factors such as bHLH or HY5 proteins in response to light is needed to activate anthocyanin pathway gene expression.

In addition, light signaling components have been demonstrated to play important roles in controlling anthocyanin biosynthesis. HY5, a bZIP protein, is a positive regulator of photomorphogenesis and can be degraded by COP1 in dark-grown seedlings [106]. In far-red light conditions, HY5 and PIF3 (a phytochrome interacting factor) collaboratively regulate anthocyanin biosynthesis in germinating seedlings. HY5 and PIF3 can simultaneously bind to different sequence elements in the promoters of several anthocyanin pathway genes and positively regulate their expression [107]. In addition, HY5 has been demonstrated to be a key effector in the UV light signaling pathway that was mediated by UV RESISTANCE LOCUS8 (UVR8) [108] and also in the cryptochrome photoreceptor-mediated blue light response [109]. The light-regulated zinc finger protein 1 (LZP1), which functions in the downstream of HY5, has also been identified to act as a positive regulator in de-etiolation. LZP1 has been shown to positively regulate anthocyanin biosynthesis through a mechanism of directly or indirectly controlling the expression of PAP1 [110]. Furthermore, light regulatory units (LRUs) sufficient for light responsiveness have been identified in the promoters of the CHS, CHI, F3H and FLS genes in studies conducted under UV-containing white light. The LRUs have been characterized to include a MYB-recognition element (MRE) and an ACGT-containing element (ACE), the latter of which is recognized by bZIP proteins such as HY5 [111].

Sucrose

Sucrose has been demonstrated to regulate anthocyanin biosynthesis in plants and cell cultures. In general, treating Arabidopsis seedlings with increased levels of sucrose can enhance the production of anthocyanins [104]. A time course study of gene expression has shown that most pathway genes are induced in seedlings treated with sucrose [103]. The increase of pathway gene expression most likely results from the induction of PAP1. A QTL analysis has shown that the expression of PAP1 is responsible for sucrose-induced anthocyanin accumulation [104]. In addition, a microarray study on seedlings treated with sucrose versus controls has revealed a strong up-regulation of PAP1 but not PAP2 [103].

Sucrose transporters appear to play a role in sucrose-induced anthocyanin biosynthesis. The mutants of SUC1 (SUCROSE TRANSPORTER) showed less anthocyanin accumulation in response to sucrose [112]. In addition, SUC2, a homolog of SUC1, has been shown to be involved in anthocyanin production in conditions of phosphate deficiency. The expression of SUC2 is highly up-regulated in the hypersensitive to phosphate starvation1 (hps1) mutant, which has an enhanced sensitivity to phosphate starvation [113]. Con-
sequently, in this mutant, the levels of sucrose are much higher than in wild-type plants. As a result, the seedlings of hps1 mutants have enhanced production of anthocyanins.

In addition, a crosstalk between sucrose and plant growth regulators has been shown to regulate anthocyanin biosynthesis. Jasmonate and cytokinin are known to induce anthocyanin production in plants; however, in the absence of sucrose, the regulatory functions of these plant hormones are not obvious [105,114]. Ethylene has been observed to suppress the sucrose-induced anthocyanin biosynthesis. One mechanism is that ethylene treatments lead to the down-regulation of the expression of GL3, TT8, and PAP1 [115,116]. In addition, ethylene treatments cause the down-regulation of SUC1 in roots [115].

**Nitrogen**

Nitrogen sources can strongly control the biosynthesis of anthocyanins in Arabidopsis. A general trend is that seedlings produce low levels of anthocyanins in high concentrations of total nitrogen, in contrast, high levels of anthocyanins in low concentrations of nitrogen. Under nitrogen deficient conditions, seedlings have been reported to accumulate high levels of both anthocyanins and flavonols [84,85]. Pathway genes and regulatory genes have been shown to be regulated in response to nitrogen treatment. Transcriptional analyses have revealed that nitrogen depletion conditions induced the expression levels of PAP1 and PAP2 [84,117]. In comparison, PAP2 was shown to have a stronger response to nitrogen limitation than PAP1. This observation was supported by another experiment, in which the expression of PAP2 was strongly induced in senescing leaves treated by high sugar/nitrogen ratios [118]. For three bHLH genes, GL3 but not EGL3 was highly up-regulated in rosette leaves of wild-type plants under nitrogen depletion [84]. In contrast, the GL3 mutants accumulate much lower amounts of anthocyanins in rosette leaves under nitrogen depletion conditions compared with WT and egl3 mutants. A recent study suggested that the FRUITFULL (FUL) gene is also likely involved in the regulation of anthocyanin biosynthesis in response to nitrogen. The FUL gene regulates cell differentiation during fruit and leaf development in Arabidopsis [119]. Its homolog VmTDR4 has been identified to be an important regulatory gene in regulating anthocyanin accumulation during the ripening of bilberry fruits [120]. Gene expression analysis revealed that FUL is necessary for the expression of PAP2 under nitrogen depletion conditions [120]. Moreover, three LATERAL ORGAN BOUNDARY DOMAIN (LBD) family proteins, LBD37, LBD38 and LBD39, were recently identified to negatively regulate anthocyanin biosynthesis under nitrogen sufficient conditions [121]. The overexpression of these genes strongly suppressed anthocyanin production in plants grown under a nitrogen depletion condition. In contrast, the knockout mutants of these three genes accumulated high levels of anthocyanins even though grown under a nitrogen sufficient condition. Transcriptional analysis has revealed that these three regulators repress anthocyanin biosynthesis through suppressing the expression of PAP1 and PAP2 [121].

**Jasmonate**

Jasmonate (JA) is an elicitor and signal molecule that mediates plant responses to pathogen infection, UV radiation and other abiotic stresses [122]. JA can strongly increase anthocyanin biosynthesis in Arabidopsis. A recent study showed that the F-box protein COI1 was required for the expression of late anthocyanin biosynthetic genes as well as the regulatory genes PAP1, PAP2 and GL3 in response to JA [105]. It has been demonstrated that the COI1 protein interacts with ASK1/ASK2, Cullin1, and Rbx1 to form the SCF<sup>COI1</sup> complex, which mediates the degradation of JA ZIM-domain (JAZ) proteins [123]. JAZ proteins have been shown to repress diverse JA responses including anthocyanin biosynthesis [124]. The potential mechanism is that JAZ proteins can interact with the C-terminal regions of both bHLH (TT8, GL3 and EGL3) and MYB (PAP1 and GL1) transcription factors to interfere the formation of active WBM complexes [125]. These results provide an appealing model for the molecular mechanism of JA-induced anthocyanin production, in which JA induces the degradation of JAZ proteins through the SCF<sup>COI1</sup> complex, thus allowing the formation of the functional WBM complexes and leading to the production of anthocyanins.

**CURRENT AND FUTURE DEVELOPMENTS**

To date, numerous accomplishments of studies in understanding anthocyanin biosynthesis and in metabolic engineering have led to the development of several patents. Major relevant patents regarding the regulation and manipulation of anthocyanin production in plants.

| Patent #  | Title                                                                 | Year of Patent |
|-----------|----------------------------------------------------------------------|---------------|
| US 6573432-B1 | Regulation of anthocyanin pigment production [126]               | 2003          |
| US 7973216-B2 | Compositions and methods for modulating pigment production in plants [127] | 2011          |
| US 20100319091-A1 | Methods of modulating production of phenylpropanoid compounds in plants [128] | 2010          |
| US 20090100545-A1 | Means and methods to modulate flavonoid biosynthesis in plants and plant cells [129] | 2009          |
| US 8008543-B2 | Modification of flavonoid biosynthesis in plants by PAP1 [130]            | 2011          |
| US 7960608-B2 | Modification of flavonoid biosynthesis in plants [131]              | 2011          |
| US 20100186114-A1 | Modification of plant flavonoid metabolism [132]                | 2010          |
Anthocyanin biosynthesis in Arabidopsis

Anthocyanin production in plants are listed in (Table 3). These technologies will likely enhance the further engineering of novel anthocyanin molecules with high nutritional values in plants to benefit human health.

In spite of the numerous progresses made in elucidating the biosynthetic and regulatory processes of anthocyanin biosynthesis, many questions still remain unanswered in this research area. For example, how do WBM complexes respond to different environmental factors? Are there functional specificities for different WBM complexes? How do negative transcription factors interact with WBM complexes? What occurs in the crossstalk between different regulatory complexes? Also, enzymes involved in the synthesis of different anthocyanin molecules remain to be comprehensively elucidated: regulatory mechanisms of tissue specific profiles of anthocyanin molecules need to be further investigated. Most of the current studies have been completed on young seedlings after seed germination. To comprehensively understand anthocyanin biosynthesis, plants grown at different growth stages and under various environmental conditions need to be investigated. To accurately understand the regulatory mechanisms, single cells or metabolically and morphologically identical cells such as anthocyanin-producing pap1-D cells as reported by Shi and Xie (2011) are helpful systems to elucidate the mechanism of environmental regulation of anthocyanin biosynthesis and the functional specificity of WBM complexes.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

ACKNOWLEDGEMENTS

We thank Dr. Rebecca S. Boston from the Department of Plant and Microbial Biology at North Carolina State University for her critical suggestions for the draft of this manuscript.

ABBREVIATIONS

| Abbreviation | Description |
|--------------|-------------|
| 4CL          | 4-coumaroyl CoA-ligase |
| ANS          | Anthocyanidin synthase |
| bHLH         | Basic helix-loop-helix |
| C4H          | Cinnamate 4-hydroxylase |
| CHI          | Chalcone isomerase |
| CHS          | Chalcone synthase |
| COI1         | Coronatine insensitive 1 |
| COP1         | Constitutive photomorphogenic 1 |
| CPC          | Caprice |
| DFR          | Diaprenol reductase |
| EGL3         | Enhancer of glabra 3 |
| ESI-MS       | Electrospray ionization mass spectrometry |
| F3H          | Flavanone 3-hydroxylase |
| F3’H         | Flavonoid 3’-hydroxylase |
| GL3          | Glabra 3 |
| MYBL2        | MYB-like 2 |
| NMR          | Nuclear magnetic resonance |
| PAL          | Phenylalanine ammonia lyase |
| PAP1         | Production of anthocyanin pigment 1 |
| TTG1         | Transparent testa, glabra 1 |
| WBM          | WD40-bHLH-MYB |
| TT8          | Transparent testa 8 |

REFERENCES

[1] Chalker-Scott L. Environmental significance of anthocyanins in plant stress responses. Photochem Photobiol Sci 1999; 70 (7): 1-9.
[2] Gould KS, McKelvie J, Markham KR. Do anthocyanins function as antioxidants in leaves? Imaging of H2O2 in red and green leaves after mechanical injury. Plant Cell Environ 2002; 25: 1261-9.
[3] Neill SO, Gould KS. Anthocyanins in leaves: light attenuators or antioxidants. Funct Plant Biol 2003; 30: 865-73.
[4] Hattier J-HB, Gould KS. Anthocyanin function in vegetative organs. In: Gould K, Davies K, Winfield C, Eds. Anthocyanins: biosynthesis, functions, and applications. New York: Springer 2009: pp. 1-20.
[5] Steyn WJ, Wand SJE, Holcroft DM, Jacobs G. Anthocyanins in vegetative tissues: a proposed unified function in photoprotection. New Phytol 2002; 155: 349-61.
[6] Zhang Y, Zheng S, Liu Z, Wang L, Bi Y. Both HY5 and HYH are necessary regulators for low temperature-induced anthocyanin accumulation in Arabidopsis seedlings. J Plant Physiol 2011; 168 (4): 367-74.
[7] He J, Giusti MM. Anthocyanins: natural colorants with health-promoting properties. Annu Rev Food Sci Technol 2010; 1: 163-87.
[8] Pascal-Teresa DS, Moreno DA, Garcia-Viguera C. Flavanols and anthocyanins in cardiovascular health: a review of current evidence. Int J Mol Sci 2010; 11 (4): 1679-703.
[9] Toufektsian MC, De Lorgeril M, Nagy N, et al. Chronic dietary intake of plant-derived anthocyanins protects the rat heart against ischemia-reperfusion injury. J Nutr 2008; 138 (4): 747-52.
[10] Jing P, Bomser JA, Schwartz SJ, He J, Magnuson BA, Giusti MM. Structure-function relationships of anthocyanins from various anthocyanin-rich extracts on the inhibition of colon cancer cell growth. J Agric Food Chem 2008; 56 (20): 9391-8.
[11] Speciale A, Canali R, Chirafisi J, Saja A, Virgili F, Cimino F, Cyanidin 3-O-glucoside protection against TNF-alpha-induced endothelial dysfunction: involvement of nuclear factor-kappaB signaling. J Agric Food Chem 2010; 58 (22): 12048-54.
[12] Ghosh D, Konishi T. Anthocyanins and anthocyanin-rich extracts: role in diabetes and eye function. Asia Pac J Clin Nutr 2007; 16 (4): 218.
[13] Butelli E, Titta L, Giorgio M, et al. Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors. Nat Biotechnol 2008; 26 (11): 1301-8.
[14] Bloom SJ, Abraham S. The structure of the major anthocyanin in Arabidopsis thaliana. Phytochemistry 2002; 59 (3): 343-6.
[15] Rowan DD, Cao M, Lin-Wang K, et al. Environmental regulation of leaf colour in red 355: PAP1 Arabidopsis thaliana. New Phytol 2009; 182 (1): 102-15.
[16] Shi MZ, Xie DY. Features of anthocyanin biosynthesis in pap1-D and wild-type Arabidopsis plants grown in different light intensity and culture media conditions. Planta 2010; 231 (6): 1385-400.
[17] Shi MZ, Xie DY. Engineering of red cells of Arabidopsis thaliana and comparative genome-wide gene expression analysis of red cells versus wild-type cells. Planta 2011; 235 (4): 787-805.
[18] Tohge T, Nishiyama Y, Hirai MY, et al. Functional genomics by integrated analysis of metabolome and transcriptome of Arabidopsis plants over-expressing an MYB transcription factor. Plant J 2005; 42 (2): 218-35.
[19] Borevitz JO, Xia Y, Blount J, Dixon RA, Lamb C. Activation tagging identifies a conserved MYB regulator of phenylpropanoid biosynthesis. Plant Cell 2000; 12 (12): 2383-94.
Tatsuzawa F, Saito N, Shinoda K, Shigihara A, Honda T. Acetylated cyanidin 3-sambubioside-5-glucocones in three garden plants of the Cruciferae. Phytochemistry 2006; 67 (12): 1287-95.

Nakabayashi R, Kusano M, Kobayashi M, et al. Metabolomics-oriented isolation and structure elucidation of 37 compounds including two anthocyanins from Arabidopsis thaliana. Phytochemistry 2009; 70 (8): 1017-29.

Saito N, Tatsuzawa F, Nishiyama A, Yokoi M, Shigihara A, Honda T. Acetylated cyanidin 3-sambubioside-5-glucocones in Matthiola incana. Phytochemistry 1995; 38 (4): 1027-32.

Pourcel L, Irani NG, La Y, Riedl K, Schwartz S, Grotewold E. The formation of Anthocyanic Vascular Inclusions in Arabidopsis thaliana and implications for the sequestration of anthocyanin pigments. Mol Plant 2010; 3 (1): 78-90.

Luo J, Nishiyama Y, Fuell C, et al. Convergent evolution in the BAHD family of acyl transferases: identification and characterization of anthocyanin acyl transferases from Arabidopsis thaliana. Plant J 2007; 50 (4): 678-95.

Yoneyura-Sakakibara K, Nakayama T, Yamazaki M, Saito K. Modification and stabilization of anthocyanins. In: Gould K, Davies K, Winefield C, Eds. Anthocyanins: Biosynthesis, functions and applications. New York: Springer 2009; pp. 169-85.

Yoneyura-Sakakibara K, Fukushima A, Nakabayashi R, et al. Two glycosyltransferases involved in anthocyanin modification delineated by transcriptome-independent component analysis in Arabidopsis thaliana. Plant J 2012; 69 (1): 154-67.

Fraser CM, Thompson MG, Shirley AM, et al. Related Arabidopsis serine carboxypeptidase-like sinapoylglycerol acyltransferases display distinct but overlapping substrate specificities. Plant Physiol 2007; 144 (4): 1986-99.

Nakayama T, Suzuki H, Nishino T. Anthocyanin acyltransferases: specificities, mechanism, phylogenetics, and applications. J Mol Catal B: Enzym 2003; 23: 117-32.

Joshi CP, Chiang VL. Conserved sequence motifs in plant S-adenosyl-L-methionine-dependent methyltransferases. Plant Mol Biol 1998; 37 (4): 663-74.

Huang J, Gu M, Lai Z, et al. Functional analysis of the Arabidopsis PAL gene family in plant growth, development, and response to environmental stress. Plant Physiol 2010; 153 (4): 1526-38.

Rohde A, Moreel K, Ralph J, et al. Molecular phenotyping of the pal1 and pal2 mutants of Arabidopsis thaliana reveals far-reaching consequences on phenylpropanoid, amino acid, and carbohydrate metabolism. Plant Cell 2004; 16 (10): 2749-71.

Olsen KM, Lea US, Stelmaszt B, Verheul M, Lillo C. Differential expression of four Arabidopsis PAL genes; PAL1 and PAL2 have functional specialization in abiotic environmental responses. Plant Cell 2008; 16 (14): 1491-9.

Ehling J, Buttn L, Wang Q, Douglas CJ, Somssich IE, Krombik E. Three 4-coumarate: CoA ligase in Arabidopsis thaliana represent two evolutionarily divergent classes in angiosperms. Plant J 1999; 19 (1): 9-20.

Lepiniec L, Debeaujon I, Routaboul JM, et al. Genetics and biochemistry of seed flavonoids. Annu Rev Plant Biol 2006; 57: 405-30.

Shirley BW, Kubase KL, Storz G, et al. Analysis of Arabidopsis mutants deficient in flavonoid biosynthesis. Plant J 1995; 8 (5): 659-71.

Winkel-Shirley B. Flavonoid Biosynthesis. A Colorful Model for Genetics, Biochemistry, Cell Biology, and Biotechnology. Plant Physiology 2001; 126: 485-93.

Jorgensen K, Rasmussen AV, Morant M, et al. Metabolon formation and metabolic channeling in the biosynthesis of plant natural products. Curr Opin Plant Biol 2005; 8 (3): 280-91.

Winkel BS. Metabolic channeling in plants. Annu Rev Plant Biol 2004; 55: 85-107.

Winkel-Shirley B. Evidence for enzyme complexes in the phenylpropanoid and flavonoid pathways. Physiologia Plantarum 1999; 107: 142-9.

Achini L, Blancaflor EB, Rasmussen S, Dixon RA. Colocalization of L-phenylalanine ammonia-lyase and cinnaamate 4-hydroxylase for metabolic channeling in phenylpropanoid biosynthesis. Plant Cell 2004; 16 (11): 3098-109.

Gomez C, Conejero G, Torregrosa L, Cheynier V, Terrier N, Ageorges A. In vivo grapevine anthocyanin transport involves vesicle-mediated trafficking and the contribution of anthoMATE transporters and GST. Plant J 2011; 67 (6): 960-70.
Anthocyanin biosynthesis in Arabidopsis

[64] Zvi MM, Shklarman E, Masci T, et al. PAP1 transcription factor enhances production of phenylpropanoid and terpenoid scent compounds in rose flowers. New Phytol 2012; 195 (2): 335-45.

[65] Gatica-Arias A, Farag MA, Stanke M, Matsouk E, Wessjohann L, Weber G. Flavonoid production in transgenic hop (Humulus lupulus) altered by PAP1/MYB75 from Arabidopsis thaliana L. Plant Cell Rep 2012; 31 (1): 111-9.

[66] Zulugua DL, Gonzali S, Loreti E, et al. Arabidopsis thaliana MYB75/PAP1 transcription factor induces anthocyanin production in transgenic tomato plants. Funct Plant Biol 2008 (35): 606-18.

[67] Bailey PC, Martin C, Toledo-Ortiz G, et al. Update on the basic helix-loop-helix transcription factor gene family in Arabidopsis thaliana. Plant Cell 2003; 15 (11): 2497-502.

[68] Heim MA, Jakoby M, Werber M, Martin C, Weisshaar B, Bailey PC. The basic helix-loop-helix transcription factor family in plants: a genome-wide study of protein structure and functional diversity. Mol Biol Evol 2003; 20 (5): 735-47.

[69] Li X, Duan X, Jiang H, et al. Genome-wide analysis of basic/helix-loop-helix transcription factor family in rice and Arabidopsis. Plant Physiol 2006; 141 (4): 1167-84.

[70] Toledo-Ortiz G, Huq E, Quail PH. The Arabidopsis basic/helix-loop-loop transcription factor family. Plant Cell 2003; 15 (8): 1749-70.

[71] Baudry A, Heim MA, Dubreucq B, Coboche M, Weisshaar B, Lepinic L. TT2, TTG1, and TTG1 synergistically specify the expression of BANYULS and proanthocyanin biosynthesis in Arabidopsis thaliana. Plant J 2004; 39 (3): 366-80.

[72] Payne CT, Zhang F, Lloyd AM. GL3 encodes a bHLH protein that regulates trichome development in Arabidopsis in interaction with GL1 and TTG1. Genetics 2000; 156 (3): 1349-62.

[73] Nesi N, Debeaujon I, Jond C, Pelletier G, Coboche M, Lepinic L. The TT8 gene encodes a basic helix-loop-helix domain protein required for expression of DFR and BAN genes in Arabidopsis silique. Plant Cell 2000; 12 (10): 1863-78.

[74] Zhao M, Morohashi K, Hatlestad G, Grotewold E, Lloyd A. The TTG1-bHLH-MYB complex controls trichome cell fate and patterning through direct targeting of regulatory loci. Development 2003; 130 (11): 1991-9.

[75] Zhang F, Gonzalez A, Zhao M, Payne CT, Lloyd A. A network of redundant bHLH proteins functions in all TTG1-dependent pathways of Arabidopsis. Development 2003; 130 (20): 4859-69.

[76] Ramsay NA, Walker AR, Mooney M, Gray JC. Two basic-helix-loop-helix genes (MYC146 and GL3) from Arabidopsis can activate anthocyanin biosynthesis in a white-flowered Matthiola incana mutant. Plant Mol Biol 2003; 52 (3): 679-88.

[77] Feyissa DN, Lovdal T, Olsen KM, Nukumizu Y, Sano R, Wada T. A full-length R-like basic-helix-loop-helix transcription factor is required for anthocyanin upregulation whereas the N-terminal region regulates epidermal hair formation. Plant Sci 2012; 183: 115-22.

[78] Hernandez JM, Feller A, Morohoshi K, Frame K, Grotewold E. The basic helix loop helix domain of maize R links transcriptional regulation and biological function, and is conserved among R/BMyb proteins. Proc Natl Acad Sci USA 2007; 104 (43): 17222-7.

[79] Wada T, Kurata T, Tominaga R, et al. Role of a positive regulator of root hair development, CAPRICE, in Arabidopsis root epidermal cell differentiation. Development 2002; 129 (23): 5409-19.

[80] Wada T, Tachibana T, Shimura Y, Okada K. Epidermal cell differentiation in Arabidopsis determined by a MYB homolog, CPC. Sci. Sci 1997; 277 (5329): 1113-6.

[81] Sawa S. Overexpression of the AtMYL2 gene represses trichome development in Arabidopsis. DNA Res 2002; 9 (2): 31-4.

[82] Song SK, Ryu KH, Kang YH, et al. Cell fate in the Arabidopsis root epidermis is determined by competition between WERE-WOLF and CAPRICE. Plant Physiol 2011; 157 (3): 1106-20.

[83] Hisieh LC, Lin SL, Hsing AC, et al. Uncovering small RNA-mediated responses to phosphate deficiency in Arabidopsis by deep sequenc. Plant Physiol 2009; 151 (4): 2120-32.

[84] Rajagopalan R, Vacheret H, Trejo J, Bartel DP. A diverse and evolutionarily fluid set of microRNAs in Arabidopsis thaliana. Genes Dev 2006; 20 (24): 3407-25.

[85] Velten J, Cakir C, Youn E, Chen J, Cazzonelli CL. Transgene silencing and transgene-derived siRNA production in tobacco plants homozygous for an introduced AtMYB90 construct. PLoS One 2012; 7 (2): e30411.

[86] Gou JY, Felippes FF, Liu CJ, Weigel D, Wang JW. Negative regulation of anthocyanin biosynthesis in Arabidopsis by a miR156-25/78 repeat protein superfamily in plants. Proc Natl Acad Sci USA 2011; 108 (22): 8842-7.

[87] Christie PJ, Alfenito MR, Walbot V. Impact of low-temperature stress on general phenylpropanoid and anthocyanin pathways: Enhancement of transcript abundance and anthocyanin pigmentation in maize seedlings. Planta 1994 (194): 541-9.

[88] Leyva A, Jarillo JA, Salinas J, Martinez-Zapater JM. Low temperature induces the accumulation of phenylalanine ammonia-lyase and chalcone synthase mRNAs of Arabidopsis thaliana in a light-dependent manner. Plant Physiol 1995; 108 (1): 39-46.

[89] Solfancelli C, Poggi A, Loreti E, Alpi A, Perata P. Sucre-specific induction of the anthocyanin biosynthetic pathway in Arabidopsis. Plant Physiol 2006; 140 (2): 637-46.

[90] Teng S, Keurentjes J, Beetsink L, Koornneef M, Smeekens S. Temperature and nitrogen deficiency in Arabidopsis regulates intercellular transport and redistribution of TTG1 protein. Arabidopsis. Mol Plant 2009; 2 (4): 790-802.

[91] Zimmermann IM, Heim MA, Weisshaar B, Uhrig JF. Comprehensive identification of Arabidopsis thaliana MYB transcription factors interacting with R/B-like BHLH proteins. Plant J 2004; 40 (1): 534-44.

[92] Zhu HF, Fitzsimmons K, Khandelwal A, Kranz RG. CPC, a single-repeat R3 MYB, is a negative regulator of anthocyanin biosynthesis in Arabidopsis. Mol Plant 2009; 2 (4): 790-802.

[93] Feller A, Machemer K, Braun EL, Grotewold E. Evolutionary and comparative analysis of MYB and bHLH plant transcription factors. Plant J 2011; 66 (1): 94-116.

[94] Tominaga-Wada R, Iwata M, Nukumizu Y, Sano R, Wada T. A full-length R-like basic-helix-loop-helix transcription factor is required for anthocyanin upregulation whereas the N-terminal region regulates epidermal hair formation. Plant Sci 2012; 183: 115-22.
Brown BA, Headland LR, Jenkins GI. UV-B action spectrum for UVR8-mediated HY5 transcript accumulation in Arabidopsis. Photochem Photobiol 2009; 85 (5): 1147-55.

Vandenbusche F, Habricot Y, Condiffe AS, Maldiney R, Van der Straeten D, Ahmad M. HY5 is a point of convergence between cryptochrome and cytokinin signalling pathways in Arabidopsis thaliana. Plant J 2007; 49 (3): 428-41.

Chang CS, Li YH, Chen LT, et al. LZF1, a HY5-regulated transcriptional factor, functions in Arabidopsis de-etiolation. Plant J 2008; 54 (2): 205-19.

Hartmann U, Sagasser M, Mehr tens F, Stracke R, Weisshaar B. 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 2005; 57 (2): 155-71.

Sivitz AB, Reinders A, Ward JM. Arabidopsis sucrose transporter AtSUC1 is important for pollen germination and sucrose-induced anthocyanin accumulation. Plant Physiol 2008; 147 (1): 92-100.

Lei M, Liu Y, Zhang B, et al. Genetic and genomic evidence that sucrose is a global regulator of plant responses to phosphate starvation in Arabidopsis. Plant Physiol 2011; 156 (3): 1116-30.

Lorette E, Povero G, Novi G, Solfanelli C, Alpi A, Perata P. Giberellins, jasmonate and abscisic acid modulate the sucrose-induced expression of anthocyanin biosynthetic genes in Arabidopsis. New Phytol 2008; 179 (4): 1004-16.

Jeong SW, Das PK, Jeoung SC, et al. Ethylene suppression of sugar-induced anthocyanin pigmentation in Arabidopsis. Plant Physiol 2010; 154 (3): 1514-31.

Kwon Y, Oh JE, Noh H, Hong SW, Bho SH, Lee H. The ethylene signaling pathway has a negative impact on sucrose-induced anthocyanin accumulation in Arabidopsis. J Plant Res 2011; 124 (1): 193-200.

Lillo C, Lea US, Ruoff P. Nutrient depletion as a key factor for manipulating gene expression and product formation in different branches of the flavonoid pathway. Plant Cell Environ 2008; 31 (5): 587-601.

Pourtou N, Jennings R, Pelzer E, Pallas J, Wingler A. Effect of sugar-induced senescence on gene expression and implications for the regulation of senescence in Arabidopsis. Planta 2006; 224 (3): 556-68.

Gu Q, Ferrandiz C, Yanofsky MF, Martienssen R. The FRUIT-FULL MADS-box gene mediates cell differentiation during Arabidopsis fruit development. Development 1998; 125 (8): 1509-17.

Jaakola L, Poole M, Jones MO, et al. A SQUAMOSA MADS box gene involved in the regulation of anthocyanin accumulation in bilberry fruits. Plant Physiol 2010; 153 (4): 1619-29.

Rubin G, Tohge T, Matsuda F, Saito K, Scheible WR. Members of the LBD family of transcription factors repress anthocyanin synthesis and affect additional nitrogen responses in Arabidopsis. Plant Cell 2009; 21 (11): 3567-84.

Kazan K, Manners JM. The interplay between light and jasmonate signalling during defence and development. J Exp Bot 2011; 62 (12): 4087-100.

Xu L, Liu F, Lechner E, et al. The SCF(CO11) ubiquitin-ligase complexes are required for jasmonate response in Arabidopsis. Plant Cell 2002; 14 (8): 1919-35.

Pauwels L, Goossens A. The JAZ proteins: a crucial interface in the jasmonate signaling cascade. Plant Cell 2011; 23 (9): 3089-100.

Qi T, Song S, Ren Q, et al. The Jasmonate-ZIM-domain proteins interact with the WD-Repeat/BHLH/MYB complexes to regulate Jasmonate-mediated anthocyanin accumulation and trichome initiation in Arabidopsis thaliana. Plant Cell 2011; 23 (5): 1795-814.

Borevitz J, Xia YJ, Dixon RA, Lamb CJ. Regulation of anthocyanin pigment production. US 6573432 B1, 2003.

Espey M, Hellens R, Allan AC. Compositions and methods for modulating pigment production in plants. US 7973216-B2, 2011.

Vainstein A, Moyal-Ben-Zvi M, Rimon-Spitzer B. Methods of modulating production of phenylpropanoid compounds in plants. US 20100319091-A1, 2010.

Van Breusegem F, Vanderauwera S. Means and methods to modulate flavonoid biosynthesis in plants and plant cells. US 20090100545-A1, 2009.

Mouradov A, Spangenberg G. Modification of flavonoid biosynthesis in plants by PAP1. US 8008543-B2, 2011.

Mouradov A, Spangenberg G. Modification of flavonoid biosynthesis in plants. US 7960608-B2, 2011.

Spangenberg G, Mouradov A. Modification of plant flavonoid metabolism. US 20100186114-A1, 2010.