Journal of Experimental Botany, Vol. 62, No. 8, pp. 2465–2483, 2011
doi:10.1093/jxb/erq442 Advance Access publication 28 January, 2011

Journal of Experimental Botany
www.jxb.oxfordjournals.org

REVIEW PAPER

Recent advances in the transcriptional regulation of the flavonoid biosynthetic pathway

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Received 11 November 2010; Revised 2 December 2010; Accepted 10 December 2010

Abstract

Flavonoids are secondary metabolites involved in several aspects of plant development and defence. They colour fruits and flowers, favouring seed and pollen dispersal, and contribute to plant adaptation to environmental conditions such as cold or UV stresses, and pathogen attacks. Because they affect the quality of flowers (for horticulture), fruits and vegetables, and their derivatives (colour, aroma, stringency, etc.), flavonoids have a high economic value. Furthermore, these compounds possess pharmaceutical properties extremely attractive for human health. Thanks to easily detectable mutant phenotypes, such as modification of petal pigmentation and seeds exhibiting transparent testa, the enzymes involved in the flavonoid biosynthetic pathway have been characterized in several plant species. Conserved features as well as specific differences have been described. Regulation of structural gene expression appears tightly organized in a spatial and temporal way during plant development, and is orchestrated by a ternary complex involving transcription factors from the R2R3-MYB, basic helix–loop–helix (bHLH), and WD40 classes. This MYB–bHLH–WD40 (MBW) complex regulates the genes that encode enzymes specifically involved in the late steps of the pathway leading to the biosynthesis of anthocyanins and condensed tannins. Although several genes encoding transcription factors from these three families have been identified, many gaps remain in our understanding of the regulation of this biosynthetic pathway, especially about the respective roles of bHLH and WD40 proteins. A better knowledge of the regulatory mechanisms of the flavonoid pathway is likely to favour the development of new biotechnological tools for the generation of value-added plants with optimized flavonoid content.

Key words: bHLH, flavonoids, MYB, transcription factors, WD40.

Introduction

Flavonoid compounds are secondary metabolites widely accumulated in vascular plants and to a lesser extent in mosses. They accumulate in all organs and tissues, at different stages of development, and depending on the environmental conditions. Beside their multiple roles in plant development and adaptation to the environment, these molecules are of major interest for human nutrition and health. Indeed, they contribute to the organoleptic quality of plant-derived products (colour, taste, flavour, etc.), and, in addition, they have been shown to be beneficial to human health and in prevention of cell ageing. In grape (*Vitis vinifera* L.) berries for instance, the flavonoid composition is essential for wine quality and conservation. Moreover, the regular consumption of red wine is thought to explain the 'French paradox', whereby the French population suffers a relatively low incidence of coronary

heart disease in spite of a diet rich in saturated fat (Renaud and Gueguen, 1998). The mechanisms involved have long been related to the presence of flavonoids and stilbenes in red wine.

Work achieved on model plants pinpointed the tight regulation of the flavonoid biosynthetic pathway during plant development. It is now established that the transcriptional regulation of the structural genes is controlled by MYB and basic helix–loop–helix (bHLH) transcription factors, together with WD40 proteins. Special attention has hitherto been devoted to MYB, as demonstrated by the reported publications. Herein, the recent advances in the knowledge of the transcriptional regulation of the flavonoid pathway are discussed, with a particular focus on bHLH transcription factors.

Flavonoids are key molecules for plant development and fitness

Flavonoids belong to the large family of phenolic compounds also known as polyphenols. During evolution, phenolic compounds played a key role by contributing to the adaptation of plants to life on land. These molecules are derived from phenylalanine via the general phenylpropanoid pathway (Fig. 1), so called because of the C6–C3 scaffold resulting from the first step of biosynthesis. The general phenylpropanoid pathway provides precursors for several branches leading to the elaboration of thousands of compounds. Among them, lignins are structural polymers that impart strength and stiffness to the secondary cell wall, and are essential components in waterproofing vascular cells (Vanholme *et al.*, 2010).

The flavonoid family encompasses at least 6000 molecules, chiefly divided into phlobaphenes, aurones, isoflavonoids, flavones, flavonols, flavanols, and anthocyanins (Fig. 1). Unlike the other classes of flavonoid compounds, phlobaphenes and isoflavonoids are synthesized almost exclusively by some maize varieties and leguminous plants, respectively. All flavonoids display a C6–C3–C6 skeleton structure, except for the aurones (C6–C2–C6) (Harborne and Williams, 2000; Marais *et al.*, 2006). Their classification is based upon the oxidation level of the central C heterocycle (Fig. 1), the presence of hydroxyl and methyl substitutions on the A and B rings, and also on supplemental modifications such as glycosylation (glucose, galactose, arabinose, rhamnose, and, to a lesser extent, disaccharides), acylation (notably coumaric and caffeic acids), and polymerization (Kong *et al.*, 2003; Macheix *et al.*, 2005; Aron and Kennedy, 2008). Among flavonoids, flavanols represent the largest class of monomeric compounds, and exist as non-glycosylated monomers, dimers, and polymers [proanthocyanidins (PAs) or condensed tannins]. Flavanols, and mainly the stereoisomers 2-3-*trans*(+)-catechine and 2-3-*cis*(–)-epicatechine, are the most abundant flavonoids in the

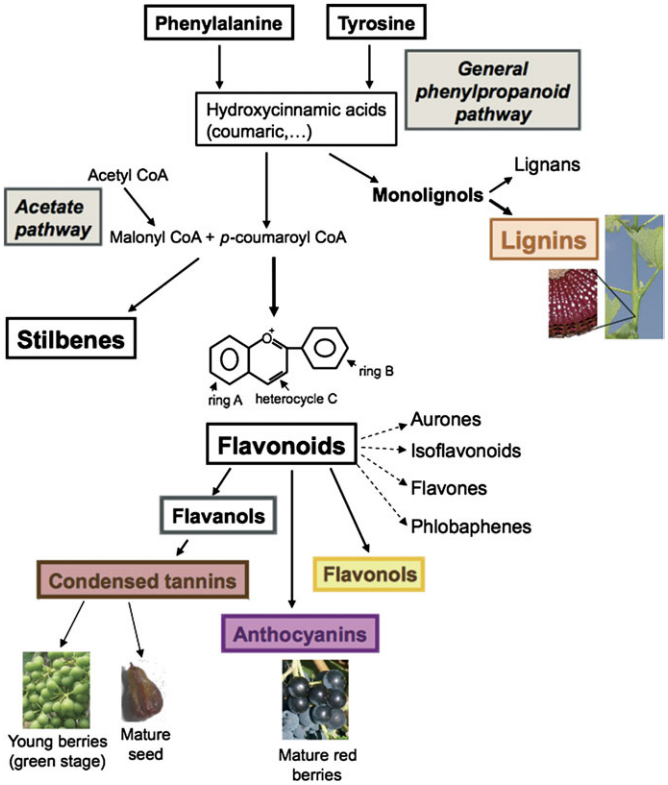

Fig. 1. The general biosynthetic pathway of the phenolic compounds leading to the main subgroups, and including flavonoids and lignins. Accumulation of flavonoid compounds such as anthocyanins and condensed tannins, as well as lignins, is illustrated in grape berries, seeds, and stems, respectively. Red staining indicates the presence of lignified tissues on a grapevine stem cross-section (phloroglucinol-HCl staining). The structure of the flavylium cation (2-phenylbenzopyrylium), which is the backbone of the flavonoid molecules, is indicated.

grape berry for instance. They are located in skin and seeds, and play an important role in the taste and conservation of wine (Waterhouse, 2002; Bogs *et al.*, 2005; Dixon *et al.*, 2005; Lepiniec *et al.*, 2006). Anthocyanin pigments are the glycosylated form of anthocyanidin precursors, derived from the flavylium cation (2-phenylbenzopyrylium; Fig. 1). This subgroup includes at least 400 molecules and exhibits colours ranging from orange-red to purple, depending on pH, co-pigmentation, available metal cations, and modifications undergone by the backbone (Grotewold, 2006; Tanaka *et al.*, 2009). In fruits accumulating anthocyanins such as bilberry (*Vaccinium myrtillus*), apple (*Malus domestica*), or grape, these pigments accumulate in the skin and more rarely in the flesh of the coloured cultivars during the ripening process. In addition, each species exhibits a different fruit anthocyanin profile (Jaakola *et al.*, 2002; Espley *et al.*, 2007; Boss and Davies, 2009). Flavonoids are synthesized in the cytosol and are mainly transported to the vacuole for storage. They can also be found in cell walls, the nucleus, chloroplasts, and even in the extracellular space, depending on the plant species, the tissue, or the stage of development (Hutzler *et al.*, 1998; Kuras *et al.*, 1999; Feucht *et al.*, 2004; Gagné *et al.*, 2006; Zhao and Dixon, 2010).

In plants, flavonoids exhibit a wide range of biological functions. Pigments absorbing visible light such as anthocyanins and aurones (yellow) colour pollen, flower, and fruits, and are thus at the origin of pollinator attraction and seed dispersal (Winkel-Shirley, 2000; Lepiniec *et al.*, 2006). Flavonoids also play a role in the interaction between plants and animals, as exemplified in leaves, where the concentration and nature of PAs determine the bitter taste and thus prevent feeding by herbivores (Harborne and Williams, 2000; Aron and Kennedy, 2008). In seeds, PAs are major determinants of seed coat-imposed dormancy (Debeaujon *et al.*, 2001, 2003). In addition, flavonoids control pollen fertility, and modulate auxin transport (Brown *et al.*, 2001; Peer and Murphy, 2007; Thompson *et al.*, 2010). As well as controlling physiological traits of plant development, flavonoids play a protective role against an array of abiotic stresses. Flavones, flavonols, and anthocyanins accumulate in leaf epidermal cells, waxes, and trichomes, where they act as UV-B filters, but can also complex with DNA and protect it from oxidative damage (Sarma and Sharma, 1999; Harborne and Williams, 2000; Dixon, 2005; Dixon *et al.*, 2005; Aron and Kennedy, 2008; Albert *et al.*, 2009). Likewise, cold stress induces anthocyanin accumulation in maize (*Zea mays*) and *Arabidopsis thaliana* seedlings (Christie *et al.*, 1994; Leyva *et al.*, 1995). Flavonoids, and more generally phenolic compounds, also contribute to defence against biotic stresses (Bhattacharya *et al.*, 2010). They may either be constitutively synthesized or accumulate in response to microbial invasion, since most of these compounds exhibit antimicrobial and pesticide properties, by acting as a repellent, and inhibiting growth and development of pests (Dixon *et al.*, 2002; Chong *et al*, 2009). The major function of PAs for instance is to provide protection against microbial pathogens, insect pests, and herbivores (Dixon *et al.*, 2005). Stilbenes have been shown *in vitro* to have antifungal activity and were thus identified as phytoalexins. Overexpression of stilbene synthase in different species led in most cases to an increased disease resistance against pathogenic fungi (Richter *et al.*, 2006, and references therein). The synthesis, release, and accumulation of phenolics, such as salicylic acid, are central to many defence strategies employed by plants against microbial invaders (Lu, 2009).

Besides their numerous functions in plants, flavonoids present a plethora of medicinal, pharmaceutical, and nutritional properties, and are thus termed 'nutraceutical' compounds (Lin and Weng, 2006). These metabolites represent a source of interest for prevention of several diseases including cancer. They induce apoptosis, stimulate DNA repair, and protect it against oxidative stress, and inhibit the division of cancer cells (Khan *et al.*, 2010). In addition, polyphenols have been shown to possess cardioprotective effects. Initially, this effect was thought to be driven by the postulated major action of polyphenols in inhibiting low-density lipoprotein oxidation and the aggregation of platelets, thereby reducing the risk of atherosclerosis (Zern and Fernandez, 2005; Aron and Kennedy, 2008; Brown *et al.*, 2009; Paredes-Lopez *et al.*, 2010). However, recent work demonstrated that red wine polyphenols, especially delphinidin, exert their endothelial benefits via activation of the oestrogen receptor α (Chalopin *et al.*, 2010). Flavonoids also have demonstrated neuroprotective, anti-inflammatory, analgesic, bactericidal, fungicidal, and spasmolytic properties (Harborne and Williams, 2000; Sun *et al.*, 2002).

Because of these multiple biological activities, flavonoids have attracted the attention of both researchers and consumers. Understanding the different steps of the flavonoid biosynthetic pathway and their regulation is important to generate and select fruits and vegetables enriched in these compounds, with desirable dietary and medicinal properties.

What are basic helix–loop–helix transcription factors?

Together with MYB and WD40, bHLH proteins, also known as MYC, are the main transcriptional regulators of the flavonoid biosynthetic pathway genes. bHLH proteins, which are named thus with respect to their conserved domain, constitute a widespread family of ubiquitous transcription factors, ranging from yeast to human, and widely distributed in plants (Massari and Murre, 2000; Pires and Dolan, 2010). The first bHLH transcription factors were identified in the early 1990s as regulators of cellular proliferation and differentiation, myogenesis, or neurogenesis, but they are also involved in a broad array of additional developmental processes in mammals (Massari and Murre, 2000). In plants, bHLH proteins belong to multigenic families, encompassing 162 members in *Arabidopsis* and 167 in rice (*Oryza sativa*) (Bailey *et al.*, 2003; Heim *et al.*, 2003; Toledo-Ortiz *et al.*, 2003; Li *et al.*, 2006). In grapevine, the bHLH family includes at least 119 members

according to its genome sequence, making it currently the second most important family after the MYB-like proteins (Fig. 2) (Jaillon *et al.*, 2007; Velasco *et al.*, 2007). Plant bHLH proteins are classified into 12 (sub)groups according to Heim *et al.* (2003). Transcription factors belonging to the same subgroups show a comparable number of amino acids, a conserved position of the bHLH domain, as well as the presence of specific regions outside the bHLH domain. To date, more than 40 specific regions found in at least three proteins have been described. Likewise, genes encoding these transcription factors show a similar structure, with an analogous number and position of introns (Buck and Atchley, 2003; Heim *et al.*, 2003; Toledo-Ortiz *et al.*, 2003; Li *et al.*, 2006).

The bHLH domain is constituted of nearly 60 amino acids, and is characterized by the presence of 19 conserved amino acids, five in the basic region, five in the first helix, one in the loop, and finally eight amino acids in the second helix (Fig. 3) (Toledo-Ortiz *et al.*, 2003). The basic region, consisting of 15–17 amino acids, is essential for DNA binding thanks to the basic residues (5.8 on average). bHLH proteins lacking this basic domain represent around

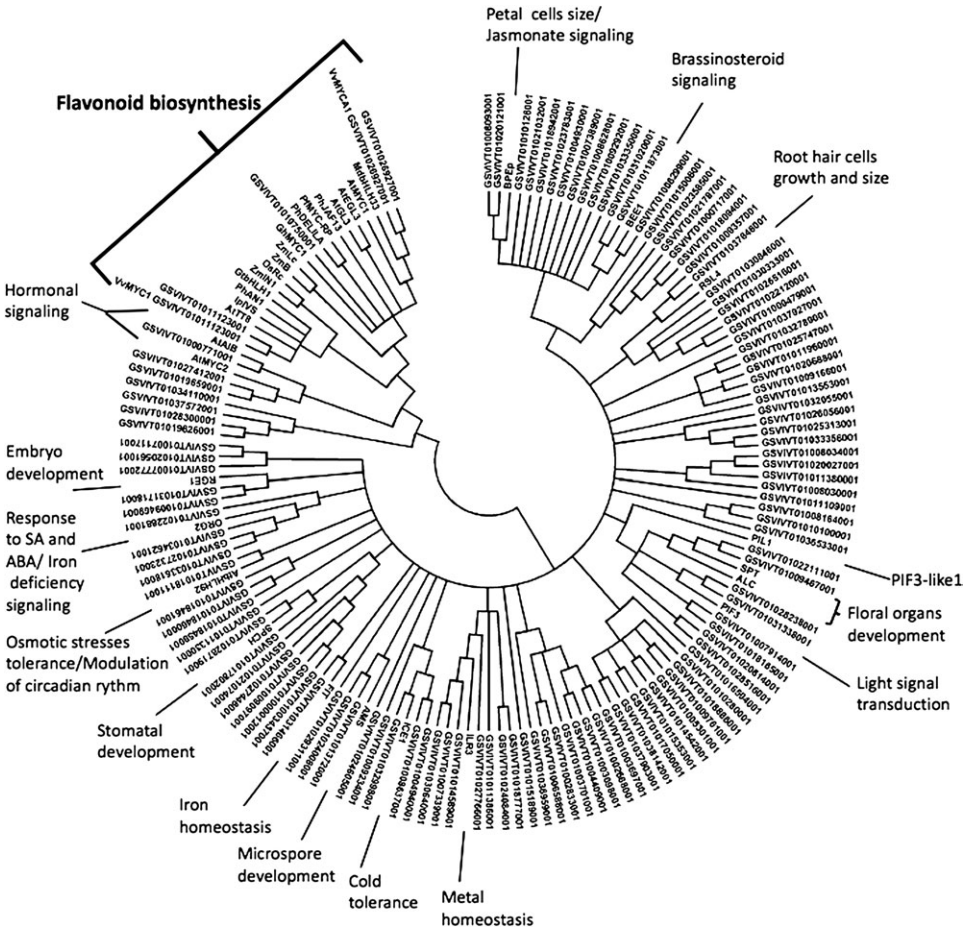

Fig. 2. Evolutionary relationships of 154 bHLH proteins from *Vitis vinifera* [including VvMYC1 (EU447172) and VvMYCA1 (EF193002)], *Arabidopsis thaliana* TT8 (AtbHLH42), GL3 (AtbHLH01), EGL3 (AtbHLH02), MYC1 (AtbHLH12), MYC2 (AtbHLH06), bHLH92, AIB (ABA-Inducible bHLH-Type Transcription Factor; AtbHLH17), SPT (Spatula; AtbHLH24), ALC (Alcatraz; AtbHLH73), AMS (Aborted Microspores; AtbHLH21), ILR3 (IAA-Leucine Resistant 3; AtbHLH105), ICE1 (Inducer of CBF Expression 1; AtbHLH116), ORG2 (OBP3-Responsive Gene 2; AtbHLH38), PIF3 (Phytochrome Interacting Factor 3; AtbHLH08), PIL1 (Phytochrome Interacting Factor 3-Like 1; AtbHLH124), BEE1 (Brassinosteroids Enhanced Expression 1; AtbHLH44), RSL4 (Root Hair Defective 6-Like 4; AtbHLH54), RGE1 (Retarded Growth of Embryo 1; AtbHLH95), FIT (Fe-Deficiency Induced Factor 1; AtbHLH29), BPEp (Big Petal; AtbHLH31), SPCH (Speechless; AtbHLH98), *Antirrhinum majus* DELILA (AAA32663), *Oryza sativa* Rc (BAF42667), *Petunia hybrida* AN1 (AAG25928) and JAF13 (AAC39455), *Zea mays* B (CAA40544), Lc (AAA33504), and IN1 (AAB03841), *Malus domestica* bHLH33 (ABB84474), *Gerbera hybrida* MYC1 (CAA07614), *Perilla frutescens* MYC-RP (BAA75513), *Ipomoea purpurea* Ivory Seed (BAD18982), and *Gentiana triflora* GtbHLH1 (BAH03387). Phylogenetic analyses were conducted using MEGA4 (Tamura *et al.*, 2007). Full-length protein sequences were aligned with Muscle (Edgar, 2004), and the phylogenetic tree was constructed according to the Neighbor–Joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 2000 replicates is taken to represent the evolutionary history of the proteins (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). There were a total of 1379 positions in the final data set. ABA, abscisic acid; SA, salicylic acid.

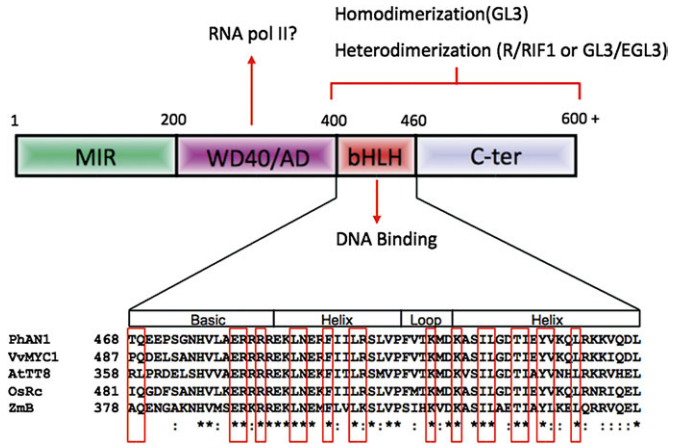

Fig. 3. General structure of the bHLH transcription factors regulating flavonoid biosynthesis. MIR, MYB-interacting region; WD40/AD, domain of interaction with WD40 and/or with the RNA polymerase II through the acidic domain (AD); bHLH, basic helix–loop–helix domain; C-ter, carboxy-terminal end. The length in amino acids of the different regions of the protein is approximate. 600+ indicates that the protein length can exceed 600 amino acids. Sequence alignment of the bHLH domain of four plant bHLH proteins involved in the regulation of flavonoid biosynthesis is indicated (accession numbers of the sequences are indicated in the legend of Fig. 2). Numbers indicate the position of the first amino acid of the domain within the corresponding full-length protein sequence. *, identical amino acids; :, similar amino acids. The 19 conserved residues characteristic of the bHLH domain (Toledo-Ortiz *et al.*, 2003) are highlighted using red boxes.

20% of the *Arabidopsis* bHLH transcription factors, and can act as repressors by forming heterodimers unable to bind DNA (Toledo-Ortiz *et al.*, 2003).

bHLH proteins can bind the canonical *cis*-element E-box (5′-CANNTG-3′), but the variant G-box (5′-CACGTG-3′) is the most commonly recognized sequence and the target of 81% of the bHLH proteins predicted to bind DNA (Toledo-Ortiz *et al.*, 2003; Li *et al.*, 2006). 3′-Flanking nucleotides may also play a role in target *cis*-element recognition (Shimizu *et al.*, 1997). In the classification of animal bHLH proteins (from A to F), G-box binders belong to group B (Atchley and Fitch, 1997; Morgenstern and Atchley, 1999; Ledent and Vervoort, 2001). In *Arabidopsis*, the DNA binding property is conferred by two particular amino acids of the basic region, Glu13 and Arg16, forming the E-box recognition motif (Ellenberger *et al.*, 1994). Indeed, Glu13 directly contacts the CA bases of the E-box, while Arg16 seems to help Glu13 binding and stabilization. Specific binding to the G-box is conferred by His/Lys9, Glu13, and Arg17 (HER motif). Arg17 interacts with the inner G base, while His/Lys9 interacts with the last G residue of the *cis*-element (Massari and Murre, 2000; Toledo-Ortiz *et al.*, 2003; Li *et al.*, 2006).

The α-helices, formed by the hydrophobic isoleucine, leucine, and valine residues, are involved in homo- and heterodimerization. For instance, the homodimer of the human bHLH Max involves the residues Leu23 (27 according to Toledo-Ortiz *et al.*, 2003) of the first protein and Leu23 of the second one (Ferré-d'Amaré *et al.*, 1993; Brownlie *et al.*, 1997). In *Arabidopsis*, this residue is conserved in all bHLH proteins, confirming its importance for interaction. During dimerization of two bHLH transcription factors, the basic region of both proteins is necessary for binding to DNA, each subunit binding to one half of the target *cis*-element (Ellenberger *et al.*, 1994; Shimizu *et al.*, 1997; Massari and Murre, 2000; Heim *et al.*, 2003; Toledo-Ortiz *et al.*, 2003). The second helix, similarly to the basic region and the loop, can also be involved in DNA binding by a direct contact with the E-box (Ellenberger *et al.*, 1994).

The loop separating the two α-helices exhibits a minimal size of 5 bp, and is variable in sequence. This loop is mainly responsible of the three-dimensional arrangement and stabilization of the α-helices, and residues of the helix1–loop junction are involved in the association between bHLH proteins (Ellenberger *et al.*, 1994). Its deletion can decrease the DNA binding capacity of bHLH transcription factors. Indeed, the loop can increase DNA recognition specificity, by recognizing and binding to nucleotides surrounding the G-box (Ferré-d'Amaré *et al.*, 1993; Toledo-Ortiz *et al.*, 2003; Li *et al.*, 2006).

It is also noteworthy that binding of bHLH proteins to DNA can be fostered by dimerization. In yeast, the PHO4 bHLH protein is involved in the regulation of phosphate uptake, and binding of PHO4 to a specific *cis*-element is cooperatively enhanced by binding of the homedomain protein PHO2. The PHO4–PHO2 interaction allows transactivation to occur by abolishing a PHO4 internal interaction with the repressive domain (Barbaric *et al.*, 1998).

In plants, bHLH transcription factors regulate many cellular processes such as development of floral organs (Heisler *et al.*, 2001; Sorensen *et al.*, 2003), photomorphogenesis (Leivar *et al.*, 2008), fate of epidermal cells such as trichomes, root hair, and stomata (Bernhardt *et al.*, 2003; Zhang *et al.*, 2003; Morohashi *et al.*, 2007; Serna 2007), hormonal response (Abe *et al.*, 2003; Lorenzo *et al.*, 2004; Li *et al.*, 2007; Bou-Torrent *et al.*, 2008), and metal homeostasis (Rampey *et al.*, 2006; Séguéla *et al.*, 2008; Long *et al.*, 2010), to name a few (Fig. 2). Among these diverse functions, bHLH transcription factors also regulate the biosynthetic pathway of flavonoids in several plant species.

The bHLH transcription factors regulating the flavonoid pathway

The first bHLH transcription factors regulating the flavonoid pathway were identified in maize in 1989, and included B (Booster 1) and R (Red 1), members of the B/R family, before the identification later on of Lc, Sn, and R-ch Hopi (Chandler *et al.*, 1989; Goff *et al.*, 1990; Petroni *et al.*, 2000). bHLH proteins involved in the regulation of the flavonoid pathway share several common features. In *Arabidopsis*, they belong to the subgroup IIIf of the classification established by Heim *et al.* (2003). The first

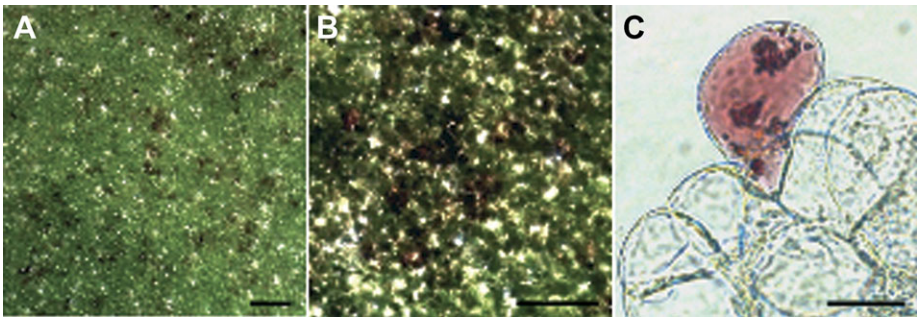

Fig. 4. Cooperative interaction of VvMYC1 and VvMYBA1 to induce anthocyanin accumulation in agro-infiltrated tobacco leaves (A and B) and in grape suspension cells after particle bombardment (C). Tobacco leaves and grape cells were observed 8 d and 4 d, respectively, after transformation. Cells transformed with VvMYC1 or VvMYBA1 alone do not synthesize anthocyanins. Scale bars indicate 0.5 mm in A and B, and 20 μm in C.

200 amino acids on the N-terminal side are involved in the interaction with MYB transcription factors (Fig. 3). The following 200 amino acids often include a negatively charged region necessary for interaction with WD40 proteins and/or the RNApolII complex. Finally, the bHLH domain itself and the C-terminal region are known to participate in homodimer (such as GL3) or heterodimer [such as R/RIF1 (see below) or GL3/EGL3] formation (Fig. 3) (Goff *et al.*, 1992; Ferré-d'Amaré *et al.*, 1994; Payne *et al.*, 2000; Buck and Atchley, 2003; Zhang *et al.*, 2003; Pattanaik *et al.*, 2008; Hichri *et al.*, 2010). These bHLH proteins can bind a G-box on their own, as already described for CrMYC1 (*Catharanthus roseus* MYC1) or perilla (*Perilla frutescens*) MYC-RP and snapdragon (*Antirrhinum majus*) Delila using the yeast one-hybrid technique (Gong *et al.*, 1999; Chatel *et al.*, 2003). However, the binding characteristics seem different according to the transcription factor and the target gene. Indeed, co-expression of the petunia MYB/bHLH pair AN2/JAF13 or *Arabidopsis* TT2/TT8 is necessary for the binding of the resulting dimers to the carophylla *Spinacia oleracea DFR* promoter in yeast. However, the JAF13 and TT8 proteins can also individually bind the *SoANS* and *AtDFR* promoters (Shimada *et al.*, 2006). Together, these results indicate that the bHLH proteins can bind DNA either alone or as a dimer with MYB, depending on the target promoter.

Like *Arabidopsis* bHLH proteins regulating the flavonoid biosynthesis pathway, those involved in trichome/root hair formation or development also belong to the subgroup IIIf (Heim *et al.*, 2003). ZmLc regulates only anthocyanin accumulation in maize, but its overexpression in *Arabidopsis* induces an elevated number of trichomes, together with an ectopic biosynthesis of anthocyanins (Llyod *et al.*, 1992). Similarly, AtGL3 (Glabra3) and AtEGL3 (Enhancer of Glabra3), which share 74% identity, overlap for the control of trichome initiation and development in leaves, and of atrichoblast determination in roots (Bernhardt *et al.*, 2003; Zhang *et al.*, 2003; Morohashi *et al.*, 2007). Transient overexpression of *GL3* in white petals of *Matthiola incana* induces anthocyanin accumulation (Ramsay *et al.*, 2003). In the morning glories *Ipomoea purpurea* and *Ipomoea tricolor*, a mutant phenotype indicates that bHLH2 and IVS (Ivory Seed), respectively, regulate anthocyanin biosynthesis in the corolla and accumulation of PAs in seed. In addition, bHLH2 controls seed trichome formation (Park *et al.*, 2007). Taken together, these results indicate that some bHLH proteins are involved in different physiological events such as the regulation of flavonoid biosynthesis and the determination of epidermal cell fate, but the underlying mechanisms of these different specializations remain unknown.

The number of plant bHLH transcription factors known to regulate the anthocyanin biosynthesis pathway is increasing steadily and they include maize B, R, Lc, and Sn (Chandler *et al.*, 1989; Goff *et al.*, 1990, 1992; Consonni *et al.*, 1993), snapdragon Delila (Gong *et al.*, 1999), perilla MYC-RP (Gong *et al.*, 1999), petunia PhAN1 and PhJAF13 (Quattrocchio *et al.*, 1998; Spelt *et al.*, 2000, 2002), apple MdbHLH3 and MdbHLH33 (Espley *et al.*, 2007), and gentian GtbHLH1 (Nakatsuka *et al.*, 2008) (Table 1 describes some of the bHLH proteins identified in different species and their functions). Only ZmIn1 shows repressive properties, by inhibiting the *CHS White pollen1* and *UFGT Bronze1* expression in maize aleurone (Burr *et al.*, 1996). Among these regulators, the rice Rc/Rd specifically governs PA synthesis in rice grain pericarp (Sweeney *et al.*, 2006; Furukawa *et al.*, 2007). Other bHLH proteins can control both anthocyanin and PA pathways, such as *Arabidopsis* TT8 (Nesi *et al.*, 2000) and morning glory bHLH2 and IVS (Park *et al.*, 2004, 2007). More recently, two grape bHLH proteins, VvMYC1 and VvMYCA1, have been identified (Fig. 3). While VvMYC1 was clearly demonstrated to promote anthocyanin accumulation in transiently transformed grape and tobacco cells (Fig. 4), a possible involvement of VvMYC1 and VvMYCA1 in PA synthesis remains to be investigated (Hichri *et al.*, 2010; Matus *et al.*, 2010).

Compilation of these data definitely indicates that bHLH transcription factors can regulate, sometimes in an overlapping way, one or more branches of the flavonoid pathway, and additional physiological events such as epidermal cell fates. Regulation of these phenomena appears highly dependent on the available partners present in the cells at a given developmental stage.

Table 1. bHLH, MYB, and WD40 proteins involved in the regulation of the flavonoid biosynthetic pathway identified in some major species

Their indicated functions have been determined using mutants or ectopic expression in heterologous and/or homologous species.

Species	Protein	Function(s)	References
bHLH			
Zea mays (maize)	ZmB, ZmR, ZmLc	Regulation of the anthocyanin pathway	Chandler *et al.* (1989); Ludwig *et al.* (1989); Goff *et al.* (1990)
	ZmIn1	Repression of flavonoid gene expression in maize aleurone	Burr *et al.* (1996)
Arabidopsis thaliana	AtTT8	Control of proanthocyanidins and anthocyanins in seeds and seedlings	Nesi *et al.* (2000); Zhang *et al.* (2003)
	GL3/EGL3	Epidermal cell fate determination and induction of anthocyanin accumulation	Bernhardt *et al.* (2003); Ramsay *et al.* (2003)
Vitis vinifera (grape)	VvMYC1	Promotion of anthocyanin accumulation in grape cells	Hichri *et al.* (2010)
Antirrhinum majus (snapdragon)	Delila	Control of anthocyanin biosynthesis in flowers	Martin *et al.* (1991)
Petunia hybrida	PhAN1	Control of anthocyanin pathway in flowers	Quattrocchio *et al.* (1993)
	PhJAF13		Quattrocchio *et al.* (1998)
Perilla frutescens	MYC-RP	Regulation of anthocyanin synthesis in leaves and stem	Gong *et al.* (1999)
MYB			
Zea mays (maize)	ZmC1, ZmPl1, ZmP1	Control of flavonoid biosynthesis in kernels	Paz-Arès *et al.* (1997); Chandler *et al.* (1989); Goff *et al.* (1990); Petroni *et al.* (1990)
Arabidopsis thaliana	AtTT2	Regulation of proanthocyanidin synthesis in seed coat	Nesi *et al.* (2001)
	AtMYBL2	Repression of anthocyanin biosynthesis	Dubos *et al.* (2008); Matsui *et al.* (2008)
	AtCPC	Regulation of epidermal cell fates and inhibition of anthocyanin accumulation	Schellmann *et al.* (2002); Zhang *et al.* (2009); Zhu *et al.* (2009)
Vitis vinifera (grape)	VvMYBPA1	Induction of proanthocyanidin synthesis	Bogs *et al.* (2007)
	VvMYBPA2		Terrier *et al.* (2009)
	VvMYB5a, VvMYB5b	Regulation of phenylpropanoid accumulation	Deluc *et al.* (2006, 2008)
Antirrhinum majus (snapdragon)	Rosea1, Rosea2, Venosa	Regulation of floral pigmentation intensity and patterning	Schwinn *et al.* (2006)
	AmMYB308, AmMYB330	Control of phenylpropanoid biosynthesis	Tamagnone *et al.* (1998)
Petunia hybrida	AN2	Regulation of anthocyanin biosynthesis in flowers	Quattrocchio *et al.* (1999)
WD40			
Zea mays (maize)	ZmPAC1	Regulation of anthocyanin pathway in seed aleurone and scutellum	Carey *et al.* (2004)
Arabidopsis thaliana	AtTTG1	Control of flavonoid pathway, trichomes and roots hair determination, seed mucilage production	Walker *et al.* (1999)
Vitis vinifera (grape)	WDR1	Contributes to the accumulation of anthocyanins	Matus *et al.* (2010)
Petunia hybrida	AN11	Regulation of anthocyanin production and vacuolar pH in flowers	de Vetten *et al.* (1997)
Perilla frutescens	PFWD	Induction of anthocyanin synthesis, trichome formation, and reduction of root hair	Sompornpailin *et al.* (2002)

The MYB transcription factors

The first MYB transcription factors regulating the flavonoid pathway were identified in 1987 in maize, and comprised C1 (Colorless 1) and Pl1 (Purple leaf 1), in addition to P1 (Paz-Arès *et al.*, 1987; Chandler *et al.*, 1989; Goff *et al.*, 1990; Petroni *et al.*, 2000). At that time, identification of C1 indicated that plant transcription factors were closely related to those of mammals, constituting a milestone in plant molecular biology. Indeed, C1 showed a significant homology with the vertebrate c-MYB proto-oncogene, derived from avian myeloblastosis virus and known to control cell proliferation and differentiation (Lipsick, 1996). MYB transcription factors are characterized by the so-called N-terminal MYB domain, consisting of 1 to 3 imperfect repeats of almost 52 amino acids (R1, R2, and R3). While the MYB domain is involved in DNA binding and dimerization, the C-terminal region regulates target gene expression (i.e. activation or repression). Plant MYB transcription factors bind different *cis*-elements, called

MYB-binding sites (MBSs), and some MYB transcription factors show a certain flexibility of recognition (Romero *et al.*, 1998; Jin and Martin, 1999). However, MYB transcription factors belonging to different species and regulating the same pathway, such as PA biosynthesis for instance, seem to bind the same motif (Akagi *et al.*, 2009).

MYB transcription factors regulating the flavonoid pathway have been widely investigated and identified in crop, ornamental, and model plants (Table 1). Most of them present two R repeats (R2R3 MYB proteins), and belong to subgroups 1–7 of the classification of Stracke *et al.* (2001). Regulators of the PA and anthocyanin pathways display the [D/E]Lx$_2$[R/K]x$_3$Lx$_6$Lx$_3$R motif necessary for interaction with bHLH transcription factors in their R3 repeat (Grotewold *et al.*, 2000; Zimmermann *et al.*, 2004), while MYB transcription factors governing flavonol biosynthesis exhibit the SG7 [K/R][R/x][R/K]xGRT[S/x][R/G]xx[M/x]K and the SG7-2 ([W/x][L/x]LS) motifs in their C-terminal end (Stracke *et al.*, 2001; Czemmel *et al.*, 2009). Nevertheless all regulators of the flavonoid pathway do not fit this classification perfectly. In potato, a single domain MYB protein, similar to soybean MYB73, is 44 times more expressed in purple flesh compared with white flesh, suggesting a role in the control of anthocyanin biosynthesis (Stushnoff *et al.*, 2010).

Most of the MYB transcription factors characterized to date control only one branch of the flavonoid pathway. Specific regulators of the anthocyanin pathway have been identified in petunia (Quattrochio *et al.*, 1999, 2006), *Arabidopsis* (Borevitz *et al.*, 2000; Gonzalez *et al.*, 2008), strawberry (Aharoni *et al.*, 2001), grapevine (Kobayashi *et al.*, 2002; Deluc *et al.*, 2006, 2008; Walker *et al.*, 2007; Cutanda-Perez *et al.*, 2009), tomato (Mathews *et al.*, 2003; Ballester *et al.*, 2010), gerbera (Elomaa *et al.*, 2003), apple (Takos *et al.*, 2006; Ban *et al.*, 2007; Espley *et al.*, 2007), potato (Mano *et al.*, 2007; Jung *et al.*, 2009), tobacco (Pattanaik *et al.*, 2010), and pear (Feng *et al.*, 2010), to name a few. Among them, the R3 AtMYBL2 is an anthocyanin repressor (Dubos *et al.*, 2008; Matsui *et al.*, 2008), and the R2R3 AtMYB60 inhibits anthocyanin synthesis in lettuce (Park *et al.*, 2008). Extensive protein sequence alignments of 134 MYB transcription factors regulating the anthocyanin pathway revealed conserved residues in the R3 repeat (arginine, valine, and alanine) of dicots, as well as a short conserved motif ANDV (Lin-Wang *et al.*, 2010). In addition, the [R/K]Px[P/A/R]xx[F/Y] motif has been identified in the C-terminal region of these anthocyanin-regulating MYBs (Lin-Wang *et al.*, 2010).

Regulators of PA biosynthesis have been identified in *Arabidopsis* (Nesi *et al.*, 2001), grapevine (Bogs *et al.*, 2007; Terrier *et al.*, 2009), leguminous plants (Yoshida *et al.*, 2008), persimmon (Akagi *et al.*, 2009), and poplar (Mellway *et al.*, 2009). More recently, MYBs regulating the flavonol branch have also been identified in *Arabidopsis* and grapevine (Mehrtens *et al.*, 2005; Stracke *et al.*, 2007; Czemmel *et al.*, 2009). As already mentioned above, MYBs generally regulate only one branch of the flavonoid pathway. In grapevine for instance, overexpression of *VlMYBA1-2* in hairy roots induced only expression of structural genes related to anthocyanin biosynthesis and transport (Cutanda-Perez *et al.*, 2009). Likewise, ectopic expression of *VvMYBPA1* and *VvMYBPA2* in grapevine hairy roots exclusively activated genes encoding enzymes of the PA pathway such as anthocyanidin reductase and leucoanthocyanidin reductase (Bogs *et al.*, 2007; Terrier *et al.*, 2009). Despite this highly specific function, some MYB transcription factors may play different roles. Overexpression of VvMYB5b in tomato affected both phenylpropanoid and carotenoid metabolism (Mahjoub *et al.*, 2009). The single R3 repeat CAPRICE (CPC) is known to regulate epidermal cell fates such as trichome and root hair formation in *Arabidopsis* (Schellmann *et al.*, 2002). Furthermore, CPC inhibits anthocyanin accumulation in homologous and heterologous hosts, by competing with R2R3 MYB transcription factors regulating the flavonoid pathway. Since CPC does not bind to DNA, it is likely that this transcription factor interferes by interacting with bHLH partners, as demonstrated by yeast two-hybrid assays (Zhang *et al.*, 2009; Zhu *et al.*, 2009).

In summary, many recent studies, together with the analysis of new plant genomes, suggest that primary protein structures and biological functions are correlated within MYB subgroups that are conserved between divergent species. This is especially true for MYB transcription factors regulating the flavonoid pathway, where specific motifs and conserved residues have been identified in anthocyanin (Lin-Wang *et al.*, 2010) and flavonol (Czemmel *et al.*, 2009) regulators. However, the biological functions of the consensus motifs present in the C-terminus of the proteins are just beginning to be investigated. It would be of great interest to determine if these specific motifs can provide the specificity for a MYB transcription factor to regulate a given branch of the flavonoid pathway, by modulating interactions with DNA and/or with protein partners such as bHLH and/or WD40 proteins.

The WD40 proteins

WD40 or WDR (WD repeat) proteins are involved in many eukaryotic cellular processes including cell division, vesicle formation and trafficking, signal transduction, RNA processing, and regulation of transcription (Van Nocker and Ludwig, 2003). They notably participate in chromatin remodelling, through modifications of the histone proteins, and can thus influence transcription (Couture *et al.*, 2006; Suganuma *et al.*, 2008; Zhu *et al.*, 2008).

WD40 proteins are characterized by a peptide motif of 44–60 amino acids, typically delimited by the GH dipeptide on the N-terminal side (11–24 residues from the N-terminus) and the WD dipeptide on the C-terminus (Smith *et al.*, 1999). This motif can be tandemly repeated 4–16 times within a protein, with a large majority of *Arabidopsis* WD40 proteins exhibiting 4 or more WD repeats (Van Nocker and Ludwig, 2003). WD40 proteins are not thought to have any catalytic activity (DNA binding or regulation of expression of a target gene), but rather seem to be a docking platform,

as they can interact with several proteins simultaneously (Van Nocker and Ludwig, 2003). Only *Arabidopsis* TTG1 (Transparent Testa Glabra 1) was clearly demonstrated, using chromatin immunoprecipitation, to bind the promoter of *AtTTG2*, a gene encoding a WRKY transcription factor mainly involved in trichome patterning (Zhao *et al.*, 2008).

A small number of WD40 proteins involved in the regulation of the flavonoid pathway have been identified so far (Table 1), and include petunia AN11 (Anthocyanin 11; de Vetten *et al.*, 1997), *Arabidopsis* TTG1 (Walker *et al.*, 1999), perilla PFWD (Sompornpailin *et al.*, 2002), maize ZmPAC1 (Pale Aleurone Color1; Carey *et al.*, 2004), *Medicago trunculata* MtWD40-1 (Pang *et al.*, 2009), and grapevine WDR1 and WDR2 (Matus *et al.*, 2010). These WD40 proteins appear to be highly conserved among species. Indeed, PFWD and PhAN11 show 81.3% identity, whereas PFWD and AtTTG1 share 77.8% identity (Walker *et al.*, 1999). The WD40 protein family seems to be less expanded than the the MYB or bHLH families, since *MtWD40-1*, *AN11*, and *PAC1*, are single-copy genes (de Vetten *et al.*, 1997; Carey *et al.*, 2004; Pang *et al.*, 2009).

WD40 proteins, regulating the flavonoid pathway, such as TTG1, can control many other physiological processes, such as trichome and root hair determination and seed mucilage production, and are accordingly expressed in tissues both accumulating and not accumulating flavonoids (Walker *et al.*, 1999). In petunia, *an11* mutants show a reduced anthocyanin content in the corolla. Disturbance of petal coloration is attributed both to a reduction in the expression of flavonoid structural genes and to a modification of the vacuolar pH, indicating that AN11 is involved at least in the regulation of these two metabolic events (de Vetten *et al.*, 1997). In *Medicago truncatula*, *MtWD40-1* mutants are deficient in accumulation of mucilage, and the synthesis of PAs, flavonols, anthocyanins, and benzoic acid in seeds, but only in PA synthesis in flowers, and finally they show no modification of epidermal cell fate. *MtWD40-1* mutants show a strong reduction of the expression of flavonoid structural genes, whereas overexpression of *MtWD40-1* in *M. truncatula* hairy root does not induce PA accumulation (Pang *et al.*, 2009).

Altogether, these data clearly indicate that WD40 proteins can be involved in various physiological and metabolic events, but also point to the fact that the underlying regulatory mechanisms of these events require the presence of additional partners.

The MYB–bHLH–WD40 (MBW) complex

Although flavonoid subgroups are derived from the same biosynthetic pathway, they accumulate differentially in plant organs and tissues, depending on the developmental stage and the environmental conditions, since they fulfil different biological functions. Thus, their distribution implies an accurate spatial and temporal regulation of the flavonoid biosynthetic pathway, requiring a specific combination of transcription factors. The involvement of a ternary complex formed by proteins from the bHLH, MYB, and WD40 families, the MBW complex, has been clearly demonstrated in *Arabidopsis* and petunia.

The MBW complex is highly organized, and each subunit fulfils a specific function such as binding to DNA, activation of expression of a target gene, or stabilization of the transcription factor complex. The interaction between members of the MBW complex may determine the subcellular localization of the complex itself. For instance, bHLH–WD40 interaction seems to be necessary for translocation of the WD40 proteins into the nucleus. Indeed, the PFWD–green fluorescent protein (GFP) fusion protein is localized in the cytosol when expressed alone, and co-expression of *PFWD* and *MYC-RP* in onion cells allows PFWD transport to the nucleus (Payne *et al.*, 2000; Sompornpailin *et al.*, 2002). Similar results have been described in petunia, where AN11 has also been localized in the cytosol (de Vetten *et al.*, 1997). Likewise, in tobacco leaves infiltrated with the grapevine *WDR1*, the encoded protein is localized either in the cytosol or in the cytosol and nucleus depending on the observed cell, while VvMYCA1 is localized in both cellular compartments (Matus *et al.*, 2010). Moreover, within the nucleus, members of the MBW complex can influence each other's accumulation. In *Arabidopsis ttg1* and *gl1* mutants, GL3–yellow fluorescent protein (YFP) is partitioned to the nucleus, but is unevenly distributed into speckles, indicating that TTG1 and GL1 transcription factors are required for the proper subnuclear distribution of GL3 (Zhao *et al.*, 2008).

Using knockout mutants and overexpression experiments, two MBW complexes have been clearly identified so far and described in *Arabidopsis* and petunia, namely TT2/TT8/TTG1 (Transparent Testa 2/Transparent Testa 8/Transparent Testa Glabra 1) and AN2/AN1/AN11 (Anthocyanin 2/1/11), respectively.

In *Arabidopsis*, the MBW complex TT2/TT8/TTG1 regulates PA accumulation in the seed coat (Debeaujon *et al.*, 2003; Baudry *et al.*, 2004), whereas the GL1/GL3–EGL3–TT8/TTG1 (Glabrous 1/Glabra 3–Enhancer of Glabra 3–Transparent Testa 8/Transparent Testa Glabra 1) complex controls trichome initiation and formation (Payne *et al.*, 2000; Zhang *et al.*, 2003; Maes *et al.*, 2008). A physical interaction between TT8 and TT2, as well as between TT8 and TTG1, has been demonstrated using yeast two-hybrid experiments (Baudry *et al.*, 2004). In addition, TTG1 can also directly interact with TT2 or the trichome regulator GL1, but without showing any obvious catalytic activity. Thus, it has been proposed that TTG1 may act as a bridge to stabilize the MBW complex (Baudry *et al.*, 2004; Zhao *et al.*, 2008). As described above, the *ttg1* mutant phenotype indicates that TTG1 is involved in several physiological responses. bHLH proteins TT8, GL3, and EGL3 also show partially overlapping functions (Zhang *et al.*, 2003). Consequently, the target gene specificity of the MBW complex seems to be conferred by the MYB protein. Indeed, PAP1/PAP2 (Production of Anthocyanin Pigment 1/2), TT2, GL1, WER (WEREWOLF), and AtMYB61 regulate anthocyanin accumulation in seedlings, PA biosynthesis in seed teguments, trichome formation, root hair

initiation, and mucilage production in seed teguments, respectively (Zhang *et al.*, 2003; Baudry *et al.*, 2004). Except for TT2, none of its closest homologues (PAP1, PAP2, WER, and AtMYB111) could activate the *AtBAN* promoter (*BAN* encodes an anthocyanidin reductase). In contrast, TT2 could interact either with TT8, EGL3, or GL3 to increase *BAN* activity significantly (Baudry *et al.*, 2004).

Rather than participating in the specific recognition of a target gene promoter, WD40 proteins are more likely to enhance gene activation. Dissection of the *AtBAN* promoter revealed that a fragment of 86 bp, including an MBS and a G-box at a distance of 36 bp, is sufficient to drive expression of the *uidA* reporter gene specifically in PA-accumulating cells (Debeaujon *et al.*, 2003). If the TT2–TT8 dimer can bind to the *BAN* promoter in yeast and activate it *in planta*, co-expression of *TT2*, *TT8*, and *TTG1* in *Arabidopsis* protoplasts activates the *BAN* promoter almost four times more than the *TT2–TT8* double transformation (Baudry *et al.*, 2004).

In petunia, the AN2/AN1/AN11 complex controls anthocyanin biosynthesis in the corolla, mainly by regulating *DFR* and *CHSJ* expression (Quattrocchio *et al.*, 1993; de Vetten *et al.*, 1997; Spelt *et al.*, 2002). Similarly to AN1, AN11 is involved in the regulation of anthocyanin biosynthesis in the corolla, but also regulates the vacuolar pH in petal limb cells and the morphology of the seed epidermal cells. However, AN2 does not affect these traits and exclusively regulates anthocyanin biosynthesis (Spelt *et al.*, 2002), while a second MYB transcription factor, PH4, controls the vacuolar pH (Quattrocchio *et al.*, 2006). Again, these results are consistent with the specificity of MYB transcription factors. Removal of the AN1 C-terminal end only affects vacuolar pH and morphology of the seed coat cells, indicating that this domain is a domain which interacts with different MYB partners (Spelt *et al.*, 2000, 2002).

Flavonol biosynthesis, at least in *Arabidopsis*, appears to be regulated only by MYB transcription factors that do not exhibit a motif for interaction with bHLH proteins in their R3 repeat. Indeed, AtMYB11, AtMYB12, and AtMYB111 activate on their own the *CHS*, *CHI*, *F3H*, and *FLS* promoters, but neither *DFR* nor *UFGT* (Stracke *et al.*, 2007). In grapevine, VvMYBF1 regulates *VvFLS1* (*Flavonol Synthase 1*) expression without the need for a bHLH partner, and can complement *Arabidopsis myb12* mutants (Czemmel *et al.*, 2009). Surprisingly, co-expression of *ZmC1* and *ZmLc* driven by the fruit-specific *E8* promoter in tomato led to a 60-fold increase in the flavonol kaempferol level in the flesh, while plants transformed with each transcription factor independently showed no significant accumulation of flavonols compared with wild-type plants (Bovy *et al.*, 2002). In maize, *ZmFLS1* expression is controlled by the anthocyanin promoting the MYB–bHLH dimer C1/PL1 + R/B or by the phlobaphene promoting MYB P1 (Ferreyra *et al.*, 2010). These results indicate that, depending on the plant species, regulation of the flavonol pathway may differ, and involves either a MYB transcription factor alone or a MYB–bHLH dimer.

Transcriptional regulation of the regulators

Besides governing the expression of flavonoid structural genes, the members of the MBW complex also regulate their own expression in a complex circuit. TT8, for instance, interacts with TTG1 and MYB transcription factors such as TT2 or PAP1 to regulate its own transcription (Tohge *et al.*, 2005; Baudry *et al.*, 2006). Other MYB–bHLH dimers, such as PAP1/GL3, can regulate *TT8* expression, as shown by yeast one-hybrid experiments and confirmed *in planta* (Baudry *et al.*, 2006). In petunia, the MYB proteins AN2 and AN4 specifically regulate *AN1* expression, without influencing *JAF13* (Quattrocchio *et al.*, 1998; Spelt *et al.*, 2000). In grapevine, VvMYC1 regulates its own expression by interacting with the MYB PA regulator VvMYBPA1 (Hichri *et al.*, 2010). In gentian flower petals, GtMYB3 may control *GtbHLH1* expression as well (Nakatsuka *et al.*, 2008). In addition to bHLH, MYB proteins can also control their own expression. In red-fleshed apples, MYB10 binds to and transactivates its own promoter. Indeed, in these red varieties, a minisatellite located in the promoter region of MdMYB10 constitutes an autoregulatory element, comprising five direct tandem repeats of a 23 bp motif, each one predicted to contain an MBS (Espley *et al.*, 2009).

In these intricate loops, it can also be noted that *ttg1* mutants can be complemented with varying degrees of efficiency by MYB transcription factors such as GL1, or bHLH proteins such as ZmR or GL3, which allow restoration of trichome formation (Lloyd *et al.*, 1992; Larkin *et al.*, 1994; Payne *et al.*, 2000). These results indicate that WD40 proteins act upstream of MYB and bHLH, and are also observed in Japanese morning glory (*Ipomoea nil*) flowers, where *InbHLH2* expression is reduced in *InWDR1* mutants (Morita *et al.*, 2006). In *an11* mutants, DFR activity is restored only by *AN2* and not *AN1* overexpression, indicating that AN11 may act upstream of AN2. However, the *AN2* transcript level is identical in wild-type and *an11* plants, indicating that AN11 could be involved in the post-translational control of AN2 (de Vetten *et al.*, 1997).

The complexity of the regulation of the MYB/bHLH network is also revealed by the transcriptomic analyses of plants from various species overexpressing a MYB transcription factor controlling the flavonoid pathway. In *Gerbera* callus and stamens overexpressing *GMYB10* and strongly pigmented, a MYB transcription factor exhibiting a repressive motif similar to that of the *V. vinifera* C2 MYB protein is in turn overexpressed (Laitinen *et al.*, 2008; Matus *et al.*, 2008). Expression of this *C2* repressor is also induced in grape roots overexpressing the specific anthocyanin regulator *VlMYBA1* (Cutanda-Perez *et al.*, 2009), as well as in roots of grapes overexpressing the specific PA regulator *VvMYBPA2* (Terrier *et al.*, 2009). It is interesting to note that, in both cases, no significant change of *bHLH* or *WD40* gene expression levels has been observed.

To conclude, a tight autoregulation of the MBW network does not appear systematic. In maize, *PAC1* (*WD40*), *R* (*bHLH*), and *C1* (*MYB*) seem to be independently regulated

(Carey *et al.*, 2004). In apple as well, MdMYB10 does not seem to regulate *MdbHLH3* and *MdbHLH33* expression (Espley *et al.*, 2007).

Additional potential regulators of flavonoid biosynthesis

Two-hybrid experiments have allowed the identification of the maize RIF1 (R-Interacting Factor 1) as a partner of the bHLH ZmR. RIF1, an EMSY-related protein localized in the nucleus, contains two peptidic domains necessary for the interaction with R: an ENT domain, which seems to be involved in homodimerization, and an AGENET domain. The RIF1 protein is involved in chromatin remodelling through histone acetylation (H3K9/14) (Hernandez *et al.*, 2007). Albeit that the R–RIF1 interaction is direct and involves the bHLH region of R, C1 is necessary for the *in vivo* formation of the C1/R/RIF1 complex and for tethering this complex to the *A1* (encoding a DFR) promoter. Extinction of *RIF1* expression in maize cells overexpressing *C1* and *R* leads to a reduction of pigmentation of almost 50%, underlining the importance of chromatin structure in the regulatory mechanisms of the flavonoid pathway, as well as the role of the R bHLH transcription factor as an interaction platform (Sainz *et al.*, 1997; Lesnick and Chandler, 1998; Hernandez *et al.*, 2007). A similar type of interaction has been described for the oncogenic c-MYC, which activates transcription through interaction with the chromatin remodelling factors SWI/SNF or co-factors of histone acetylase (Cheng *et al.*, 1999; Massari and Murre, 2000).

Among additional potential regulators of the flavonoid pathway, the *Arabidopsis* WRKY transcription factor TTG2 has been reported to be involved in trichome development, but also in condensed tannins and mucilage production in the seed coat, in a TTG1-dependent way (Jonhson *et al.*, 2002; Ishida *et al.*, 2007). In addition, in *Arabidopsis* plants ectopically expressing the anthocyanin regulator *PAP1*, *TTG2* expression is up-regulated, suggesting a possible involvement in anthocyanin regulation (Tohge *et al.*, 2005). In the epidermal cell fate determination circuit, TTG2 acts downstream of TTG1 and other MYB and bHLH regulators, as indicated by the repression of *TTG2* expression in leaves of the *egl3*, *gl3 egl3*, and *ttg1* mutants (Johnson *et al.*, 2002; Western *et al.*, 2004). In root epidermal cells, WER positively regulates *TTG2* expression, while CPC and TRY down-regulate it by forming unproductive complexes with the GL3 and EGL3 bHLH proteins (Ishida *et al.*, 2007).

MADS-box transcription factors belonging to the SQUAMOSA subgroup, mainly known to control the identity of floral meristem and floral/fruit development (Immink *et al.*, 2010), also appear to be implicated in the regulation of anthocyanin biosynthesis. In bilberry, *VmTDR4* is orthologous to the tomato SQUA MADS-box *TDR4* and the *Arabidopsis FRUITFULL* genes. In the white colour mutant of bilberry, the *VmTDR4* transcript level is reduced compared with wild-type plants. In addition, ectopic expression of *TDR4* in *Arabidopsis* siliques induces anthocyanin biosynthesis, while its extinction in bilberry by VIGS (virus-induced gene silencing) results in a substantial decrease in anthocyanin content (Jaakola *et al.*, 2010). This loss of pigmentation correlates with a reduced expression of the flavonoid structural genes, as well as with a suppression of expression of regulatory genes such as *VmMYB2*, which encodes a protein sharing 62% and 86% identity with AtPAP1 and VvMYBPA1, respectively. Together, these results indicate that VmTDR4 can affect anthocyanin biosynthesis during ripening via a monitoring of *MYB* gene expression (Jaakola *et al.*, 2010). Similar results have been described for sweet potato, where IbMADS10 (belonging to the SQUA subfamily) is almost exclusively expressed in pigmented tissues and promotes anthocyanin accumulation in potato calli (Lalusin *et al.*, 2006). In *Arabidopsis*, mutation of the gene encoding the BSISTER (ABS) MADS-box protein TT16 abolishes PA synthesis in the seed endothelium and leads to a transparent testa phenotype. TT16 is necessary for the expression of *BANYULS* and acts upstream of TT2 in the seed PA biosynthesis pathway. It also plays a role in the differentiation of PA-accumulating endothelial cells (Nesi *et al.*, 2002; Debeaujon *et al.*, 2003).

Finally, the bZIP transcription factors must also be mentioned, as they mediate the light-dependent regulation of flavonoid biosynthesis. bZIP proteins bind the ACGT-containing element (ACE) which, together with the MYB recognition element (MRE), constitutes the light response unit (LRU). LRUs have been identified in *Arabidopsis CHS*, *F3H*, and *FLS*, and grapevine *VvFLS1* promoters, and are necessary for light responsiveness, indicating a possible cooperation between MYB and bZIP transcription factors to ensure this function (Hartmann *et al.*, 2005; Czemmel *et al.*, 2009).

Flavonoids and biotechnology applications

Understanding the intricate regulation of the flavonoid biosynthesis pathway has obvious purposes, such as generation of flowers with original colours and fruit varieties presenting attractive visual and/or agronomic properties, thus boosting the natural selection which has occurred since the beginning of time. Flowers displaying a range of pigmentation caused by a mutation in the coding sequence of one or several members of the MBW complex have been described, for instance in petunia with the *an1* (bHLH) or *an2* (MYB) mutants, in morning glory *Ipivs* (bHLH), *c* (InMYB1), and *ca* (InWDR1) mutants, or in gentian *GtMYB3*, to name a few (Quattrocchio *et al.*, 1999; Morita *et al.*, 2006; Park *et al.*, 2007; Nakatsuka *et al.*, 2008). Absence of pigmentation (i.e. of anthocyanin production) in fruits such as grape berry and Chinese bayberry (*Myrica rubra*) is caused by a mutation in the coding sequence of the MYB genes *VvMYBA2* and *MrMYB1*, respectively, together with a transposon insertion in the promoter of the *MYBA1* gene (Kobayashi *et al.*, 2004; Walker *et al.*, 2007; Niu *et al.*, 2010).

Manipulating flavonoid biosynthesis to engineer fruit and vegetables enriched in antioxidant and nutritional compounds, deserving more than ever to be called 'nutraceuticals', would be of great interest for human health. In tomato, ectopic expression of the MYB-encoding gene *LeANT1* induced anthocyanin accumulation in skin and subepidermal cell layers (Mathews *et al.*, 2003). Similarly, co-expression of the *bHLH Delila* and *MYB Rosea 1* genes under the control of the fruit-specific promoter *E8* induced a dramatic increase of anthocyanin pigments in the flesh and skin, leading to dark purple fruits (Butelli *et al.*, 2008). Cancer-susceptible *p53* knockout mice fed with these transgenic tomatoes exhibited a significantly extended longevity (Butelli *et al.*, 2008). This work represents the first example of generation of fruits enriched in flavonoid bioactive compounds that could be part of a healthy daily diet. In apple (*M. domestica*) and in sweet potato (*Ipomoea batatas*), MdMYB10 and IbMYB1 specifically control anthocyanin accumulation in the flesh (Espley *et al.*, 2007; Mano *et al.*, 2007). Modification of their expression could be used to enhance flavonoid contents in these highly consumed products. However, a plant transformation approach can also be considered with bHLH transcription factors, as constitutive expression of *ZmLc*, *Delila*, and *MYC-RP/GP* in tomato led to anthocyanin accumulation in aerial tissues (including fruits) and roots (Mooney *et al.*, 1995; Goldsbrough *et al.*, 1996; Gong *et al.*, 1999).

Finally, strategies to enhance PA contents in forage crops (mainly alfalfa and clover) could help to prevent pasture bloat in ruminant animals, by slowing down the fermentation in the rumen. Overexpression of *ZmLc* in alfalfa (*Medicago sativa*) could induce PA accumulation in leaves (McMahon *et al.*, 2000; Ray *et al.*, 2003). Similarly, overexpression of *ZmSn* in birdsfoot trefoil (*Lotus corniculatus*) increased PA biosynthesis, with subtle extra accumulation of anthocyanin restricted to specific areas in the leaf (Robbins *et al.*, 2003).

However, constitutive expression of a transgene (*MYB* or *bHLH*) in a heterelogous system is often not sufficient to induce flavonoid accumulation automatically, and an environmental stress such as cold and high light is advocated (Ray *et al.*, 2003; Paolocci *et al.*, 2005; Cominelli *et al.*, 2008; Albert *et al.*, 2009; Rowan *et al.*, 2009). Inadequate growth conditions of the *Arabidopsis 35S::PAP1* plants for instance (i.e. high temperature and low light) led to a down-regulation of expression of the positive regulators, in parallel with an up-regulation of expression of the potential transcriptional repressors *AtMYB3*, *AtMYB6*, and *AtMYBL2* (Rowan *et al.*, 2009).

Conclusions

Given the particular attention devoted to health and disease prevention through a balanced diet including natural products, flavonoids appear as possible nutraceuticals widely distributed in vegetables and fruits. In this context, an important research effort is currently underway to understand the biosynthetic pathway and the regulatory mechanisms of flavonoid biosynthesis in various plant species. If the pathway itself is now quite well understood, its regulation appears to be under a hierarchy of complex events, which are slowly being deciphered. The identification of new transcription factors involved in flavonoid biosynthesis should be conducted together with the investigation of the parameters controlling their expression. Modulating the expression of target transcription factors through cultural practices or adequate environmental conditions in order to modify flavonoid contents in plants may provide a good opportunity to avoid genetic engineering. Likewise, determining the endogenous factors which trigger the expression of the regulatory genes can be another path to follow. Finally, investigating the allelic variability between cultivars of the same plant species is likely to allow the use of these transcription factors as molecular markers of the fruit/vegetable quality.

Acknowledgements

We wish to thank Sabine Guillaumie for pictures of grape seeds and the stem cross-section of Figure 1.

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