Recent advances in the transcriptional regulation of the flavonoid biosynthetic pathway

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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-(+)-catechin and 2-3-cis(−)-epicatechin, 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 flavylum cation (2-phenylbenzopyrylium), which is the backbone of the flavonoid molecules, is indicated.](image-url)
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 cardio-protective 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 spasmyolytic 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

![Flavonoid biosynthesis](image)

**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), *Gerbiera hybridia* MYC1 (CAA07614), *Perilla frutescens* MYC-RP (BA917513), *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.
while His/Lys9 interacts with the last G residue of the Arg17 (HER motif). Arg17 interacts with the inner G base, seems to help Glu13 binding and stabilization. Specific directly contacts the CA bases of the E-box, while Arg16 recognition motif (Ellenberger et al., 2001). 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 transcription 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; Séguela 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 IIIIf of the classification established by Heim et al. (2003). The first
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 RNApolIII 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’Amare 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 III’ (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 (Lloyd et al., 1992). Similarly, AtGL3 (Gabra3) 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 Re/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.

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.
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-Ares 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

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

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<th>Function(s)</th>
<th>References</th>
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<td></td>
<td>seed mucilage production</td>
<td></td>
</tr>
<tr>
<td>Vitis vinifera (grape)</td>
<td>WDR1</td>
<td>Contributes to the accumulation of anthocyanins</td>
<td>Matus et al. (2010)</td>
</tr>
<tr>
<td>Petunia hybrida</td>
<td>AN11</td>
<td>Regulation of anthocyanin production and vacuolar pH in flowers</td>
<td>de Vetten et al. (1997)</td>
</tr>
<tr>
<td>Perilla frutescens</td>
<td>PFWD</td>
<td>Induction of anthocyanin synthesis, trichome formation, and reduction of root</td>
<td>Sompornpailin et al. (2002)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>hair</td>
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</tr>
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</table>
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]Lx2[R/K]x3Lx6Lx3R 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 flavonoid biosynthesis exhibit the SG7 [K/R][R/x][R/K]x2GRT[S/x][R/G]x2[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/P][P/A/R][x3]xF[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 (Mehtrens 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 VvMYBA1-2 in hairy roots induced only expression of structural genes related to anthocyanin biosynthesis and transport (Cutanda-Perez et al., 2009). Likewise, ectopic expression of VvMYBP1 and VvMYBP4 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 Glandula) 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), petulla 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 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 TTG1 is involved at least in the regulation of these two metabolic events (de Vetten et al., 1997). In *Medicago trunculata*, MtWD40-1 mutants are deficient in accumulation of mucilage, and the synthesis of PAs, flavonols, anthocyanins, and benzoic acid (de Vetten et al., 1997; de Vetten et al., 2004). 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 Glandula) 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 Glandula) 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 MBW 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/AN11 complex controls anthocyanin biosynthesis in the corolla, mainly by regulating DFR and CHS1 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 (Ferreya 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 GbHLH1 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 tgt1 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 VmMYBA1 (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
ectopic expression of reduced compared with wild-type plants. In addition, the colour mutant of bilberry, the VmTDR4 is orthologous to the tomato SQUAMADS-box regulation of anthocyanin biosynthesis. In bilberry, productive complexes with the GL3 and EGL3 bHLH while CPC and TRY down-regulate it by forming unproductive complexes with the GL3 and EGL3 bHLH regulators, as indicated by the repression 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). LRU's 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).

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 AGNET 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 over-expressing CI 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 eg3, gl3 eg3, 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).

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 inWDR1 (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; Goldsborough 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 heterologous 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.

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