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***Review*:**

Transcriptional regulation of secondary metabolism

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Abstract. Plants produce secondary metabolites during development and in response to environmental stimuli such as light or pathogen attack. Transcriptional regulation provides the most important control point for the secondary metabolic pathways studied to date. In this article we review the data on the transcription factors that modulate this regulation. For the phenylpropanoid pathway, much is understood about both the specific sequences in the target genes (*cis*-elements) that are involved in responses to environmental and developmental stimuli, and the transcription factors involved. Most information is available for the light induction of the genes for hydroxycinnamic acid production, the production of anthocyanins in leaves and floral tissues, and the production of proanthocyanidins in seeds. Some of the functional interactions between the different types of transcription factor are now being elucidated, and upstream regulators of the genes encoding the transcription factors identified. For other secondary metabolic pathways much less is known, although good progress has been made on identifying transcription factors involved in controlling terpenoid indole alkaloid production. The identification of defined transcription factor genes provides tools for modulating both the amount and distribution of secondary metabolites in plants, and the validity of this approach has been well established by transgenic plants with modified flavonoid accumulation patterns.

Introduction

The plant kingdom produces a huge array of secondary metabolites during development and in response to developmental, environmental, pathogen and symbiont signals. Usually changes in the transcription rate of secondary metabolite biosynthetic genes precede this production, although translational and post-translational regulation may also be important in specific cases. This emphasis on transcriptional regulation may be due to lack of appropriate studies to identify post-transcriptional control, as illustrated by the recent use of genomics to show rhythmic expression of many *Arabidopsis* genes, including those of the phenylpropanoid and carotenoid pathways, with the potential involvement of changing RNA stability in generating the rhythms (Staiger 2002). Nevertheless, transcriptional regulation is the major controlling step for secondary metabolite pathways studied in detail to date (reviewed in Memelink *et al.* 2000, 2001; Martin *et al.* 2001). In this article we review the role of transcription factors (TFs) in control of plant secondary metabolism. Typically, TFs are proteins that regulate gene activity by binding to motifs within genes (usually in gene promoters) in a sequence-specific manner, and modulate the rate of transcriptional initiation by the basal transcription machinery that includes RNA polymerase II (Ranish and Hahn 1996; Pugh 1997; Sawadogo and Szentirmay 1997). TFs may be activators or repressors depending on whether they increase or decrease the rate of initiation (HannaRose and Hansen 1996; Schwechheimer and Bevan 1998). The activity of such TFs may be dependent on direct interaction with other proteins, which themselves do not bind directly to DNA (co-activators and co-repressors). They may also be influenced in their activity by competition with other TFs, or by reversible, post-transcriptional modifications to the TF itself. All these types of regulatory protein have been described for secondary metabolism. Owing to space limitations, few details are given of the structure or properties of the different TFs discussed; rather our focus is on their role in the regulatory process. Details of plant TF structure and function can be found in the reviews of Ellenberger (1994), Martin and

Abbreviations used: ACE, ACGT-containing element; ACO, acyl-CoA oxidase; bHLH, basic helix-loop-helix; bZIP, basic region/leucine zipper; CHI, chalcone isomerase; CHS, chalcone synthase; CIP, COP1-interacting protein; COP, constitutive photomorphogenic; CPRF, common plant regulatory factor; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; DET, de-etiolated; DFR, dihydroflavonol 4-reductase; EBG, early biosynthetic gene; FLS, flavonol synthase; FNS, flavone synthase; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′5′-hydroxylase; GST, glutathione-S-transferase; HCA, hydroxycinnamic acid; LBG, late biosynthetic gene; LRU, light-responsive unit; MRE, MYB recognition element; PAL, phenylalanine ammonia lyase; Str, strictosidine synthase; TF, transcription factor; TIA, terpenoid indole alkaloid; TT, TRANSPARENT TESTA; TTG, TRANSPARENT TESTA GLABROUS; Vp1, Viviparous 1.

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Paz-Ares (1997), Schwechheimer and Bevan (1998), Eulgem *et al.* (2000), Stracke *et al.* (2001), Jakoby *et al.* (2002) and Vom Endt *et al.* (2002). When evaluating the role of transcriptional regulation in secondary metabolism, one pathway, the phenylpropanoid pathway, has been the leading model for studies on plant gene regulation but little is known for any of the other major metabolic pathways. Thus, by necessity, much of the review focuses on the phenylpropanoid pathway.

Regulation of phenylpropanoid biosynthesis

The phenylpropanoid pathway produces a wide range of compounds derived from phenylalanine, including lignins, lignans, stilbenes, and flavonoids such as anthocyanins, flavonols, proanthocyanidins (condensed tannins) and isoflavonoids (Fig. 1; reviewed by Bohm 1998; Petersen *et al.* 1999; Davies 2000; Winkel-Shirley 2001). These compounds have diverse functions in plants, as structural components and in interactions with the environment and other organisms. The initial products of phenylpropanoid biosynthesis, hydroxycinnamic acids (HCAs), are formed from phenylalanine by phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H) and 4-coumarate:CoA ligase (4CL). All the other types of phenylpropanoid are derived from the HCAs, with the first of the flavonoids being formed by chalcone synthase (CHS). Key phenylpropanoid biosynthetic genes were identified early on in molecular studies, and some have been extensively studied for promoter *cis*-regulatory regions that are candidate TF binding sites. In particular the genes encoding the initial steps in general phenylpropanoid metabolism have been studied in detail for their response to environmental stimuli, such as light and biotic stresses. The regulation of the genes of the later stages of the flavonoid pathway, producing the anthocyanin and proanthocyanidin pigments, have been at the forefront of all plant transcriptional regulation studies, in no small part due to the ease of identification of genetic mutants. There are now data from a number of different plant species concerning the TFs regulating the anthocyanin pathway, and general trends across species are emerging.

Early biosynthetic steps

Given the range of functions of HCA-derived compounds, it is no surprise that the genes for the biosynthetic enzymes have been found to respond to many environmental and developmental stimuli. A key focus has been on induction in response to stresses such as UV-light and pathogen-related elicitors, and the cross-talk between the different stress-signaling pathways. Unlike anthocyanin and proanthocyanidin biosynthesis, there is a lack of mutants for early stages of phenylpropanoid metabolism, and most data are from sequence analysis of promoters, *in vitro* biochemical studies and transgenic plants. *Cis*-regulatory elements and interacting TFs have been identified for the promoters of *PAL*, *C4H*, *4CL* and *CHS* genes of several species, and some commonalities are obvious. The UV-light response acts through a light-responsive unit (LRU) that contains a MYB recognition element (MRE) and an ACGT-containing element (ACE). The ACE is recognized and bound by a variety of basic region/leucine zipper (bZIP) proteins. The ACEs have also been referred to as 'G-box-like sequences' in some promoter studies, but include A-box (TACGTA)-, C-box (GACGTC)-, G-box (CAGCTG)- and T-box (AACGTT)- type elements (named after the last nucleotide in the recognition motif). Some of these *cis*-elements may also be involved in pathogen response, along with one or more copies of a TGAC (W-box) sequence that interacts specifically with a WRKY TF (Eulgem *et al.* 1999). There are various *cis*-elements related in sequence to the MRE and

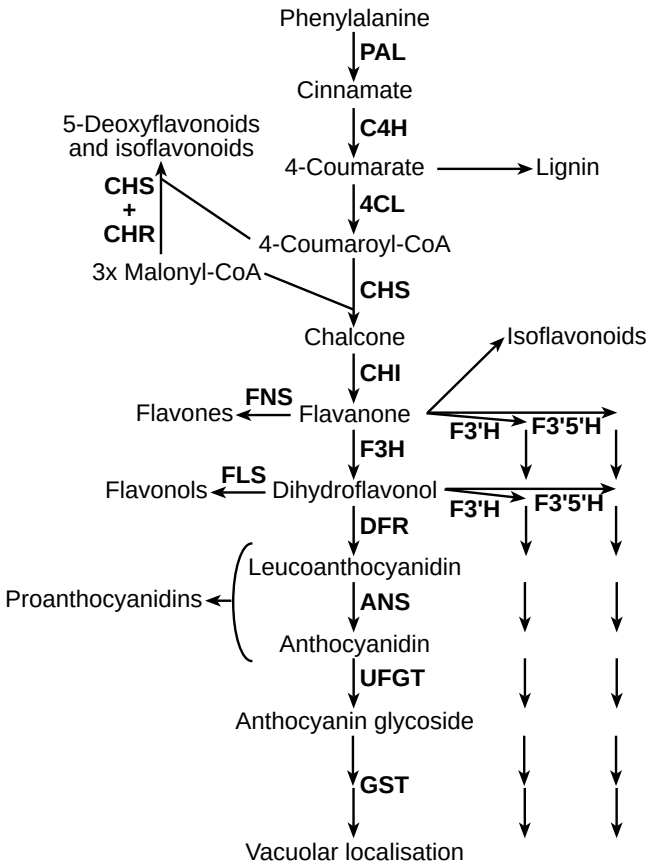

Fig. 1. Diagrammatic representation of a section of the phenylpropanoid pathway leading to lignin, isoflavonoids and the major flavonoid types. Enzyme abbreviations are as given in the text, except for ANS (anthocyanidin synthase) and CHR (chalcone reductase). The arrows on the right represent the action of the enzymes flavonoid 3′-hydroxylase (F3′H) and flavonoid 3′5′-hydroxylase (F3′5′H), that add hydroxyl groups to the 3′ and 5′ carbons of the B-ring of flavonoids, in particular at the flavanone and dihydroflavonol level. The differently hydroxylated substrates progress through the same subsequent enzyme reactions to produce the common anthocyanins that are glycosides of pelargonidin (4′-OH), cyanidin (3′4′-OH) and delphinidin (3′4′5′-OH).

ACE motifs, such as the MRE-related H-box and P-box, that are part of the response to developmental, tissue specific and environmental stimuli. These *cis*-elements and interacting TFs are discussed in more detail in the following sections.

The LRU of the *CHS* genes of *Petroselinum crispum* (parsley) and *Arabidopsis thaliana* (*Arabidopsis*) have been shown to be necessary and sufficient for induction of gene expression in response to UV-A/blue light or UV-B in transgenic plant tissues (Schulze-Lefert *et al.* 1989; Weisshaar *et al.* 1991; Feldbrügge *et al.* 1997; Hartmann *et al.* 1998). Many different bZIP proteins have been shown to interact with ACE elements present in a range of promoters (reviewed by Menkens *et al.* 1995; Jakoby *et al.* 2002). For phenylpropanoid gene regulation, attention has focused on the common plant regulatory factor (CPRF) type of bZIP protein. In parsley, the LRU ACE specifically interacts with at least seven CPRFs that bind the ACE with varying affinities and that can form both homo- and heterodimers (reviewed by Jakoby *et al.* 2002). Heterodimerization, along with phosphorylation, may be important to the subcellular localization and function of bZIP proteins (Terzaghi *et al.* 1997; Wellmer *et al.* 2001). *PcCPRF1* transcripts increase in abundance in parsley in response to UV-light, and precede the increase in *CHS* transcript abundance, suggesting it may be part of the UV-induction process. The *PcCPRF1* promoter itself contains ACEs (Feldbrügge *et al.* 1994). In *Arabidopsis*, HY5 is a bZIP protein that interacts directly with ACE-containing, light-responsive promoters (Chattopadhyay *et al.* 1998) and regulates a number of pathways, including the phenylpropanoid pathway (Ma *et al.* 2002)

A number of MYB proteins have been shown to interact with the LRU MRE and with related MREs present in the promoters of phenylpropanoid genes of several species. The product of the *Antirrhinum majus* (*Antirrhinum*) *AmMYB305* gene, and the equivalent protein from *Nicotiana* (tobacco), has been shown to bind and activate the P-box MRE that is associated with petal-enhanced expression (Sablowski *et al.* 1994). AmMYB305 also activates the promoters of *chalcone isomerase* (*CHI*) and *flavanone 3-hydroxylase* (*F3H*), suggesting it may play a role in regulation of flavonol biosynthesis (Moyano *et al.* 1996). The structurally related AmMYB340 protein also binds to the P-box and regulates *CHI*, and may serve a related function to AmMYB305 (Moyano *et al.* 1996). In yeast and plant protoplast studies with target promoters AmMYB340 had stronger transactivation strength but weaker promoter-binding affinity than AmMYB305. This difference in binding affinity was associated with phosphorylation of AmMYB340 but not AmMYB305. As both genes show only slight variation in temporal and spatial expression, AmMYB305 could potentially out-compete the stronger activator for promoter sites, providing for fine gearing of levels of gene expression. Both proteins were able to bind the promoters in the absence of any basic Helix-Loop-Helix (bHLH) TF partner. The MRE of the parsley *CHS* promoter is also recognized by AmMYB305 and an unusual MYB from parsley, PcMYB1, that has only one repeat of the DNA binding domain rather than the more common two-repeat structure of plant MYBs (Feldbrügge *et al.* 1997).

There is considerable data, particularly from *Arabidopsis*, on light regulation of gene expression as part of general photomorphogenesis, some of which includes signaling processes to the LRUs of phenylpropanoid genes. The *constitutive photomorphogenic* (COP) system appears to control the ubiquitin-mediated, light-dependent degradation of downstream signaling components of many photoreceptors (reviewed by Hardtke and Deng 2000; Schwehheimer and Deng 2001). It is composed of the COP9 signalosome, made up of the products of several *COP*, *De-etiolated* (*DET*) and *Fusca* genes, and at least four related proteins COP1, COP10, COP1 INTERACTING PROTEIN (CIP) and DET1 that act outside the COP9 complex. COP1 is a putative E3 ubiquitin ligase that interacts directly with several TFs, and COP10 and CIP probably encode other components of the ubiquitin degradation pathway (Hardtke *et al.* 2002; Suzuki *et al.* 2002). Affected genes include *AtMYB21*, that regulates *PAL* gene activity when overexpressed in *Arabidopsis* (Shin *et al.* 2002), *HY5* (Osterlund *et al.* 2000), and a putative TF gene, *CIP7*, that is required for full light regulation of the *Arabidopsis CHS* gene (Yamamoto *et al.* 1998).

MYB proteins also act as light-responsive repressors of early phenylpropanoid biosynthesis. Overexpression of *AmMYB308* or *AmMYB330* caused dramatic reductions in the levels of lignin and HCA derivatives in transgenic tobacco (Tamagnone *et al.* 1998). An indication of a physiological function for this activity was obtained by studying knockout mutants for the *Arabidopsis* orthologue of *AmMYB308*, *AtMYB4*. Analysis of the *atmyb4* mutant showed increased *C4H* transcript levels, elevated levels of sinapate esters (HCA derivatives) and enhanced tolerance to UV-B exposure (Jin *et al.* 2000). While light was required for *AtMyb4* gene expression, transcript levels fell markedly within 24 h of exposure to UV-B. In *35S*::*AtMyb4* plants, additional genes to *C4H* were down-regulated, including *CHS* and *4CL*.

Repression of transcription may be achieved through two mechanisms: 'passive' repression through competition with activators for target promoter binding sites, and 'active' repression through direct interaction with specific TFs or the basal transcription machinery (HannaRose and Hansen 1996; Schwechheimer and Bevan 1998). The first type of repression has been suggested for a truncated, mutant form of the C1 MYB protein of maize that is normally an activator of anthocyanin biosynthesis. C1-I is a dominant negative allele that lacks the *C*-terminal activation domain normally present, and represses the synthesis of anthocyanin (Paz-Ares *et al.* 1990). It may also occur with *Intensifier1* of

maize, which encodes a bHLH protein similar to a positive regulator of anthocyanin biosynthesis, R, of maize (Burr *et al.* 1996). The *Intensifier1* gene generates a majority of transcripts that encode truncated versions of the bHLH protein that may act as competitive inhibitors. For AtMYB4, deletion analysis and creation of fusion proteins indicate a role for the *C*-terminal domain of the protein in the repression function, perhaps through a direct effect on the basal transcription machinery (Jin *et al.* 2000).

Pathogen elicitation can also down-regulate gene transcription. For parsley it has been shown that pathogen elicitation leads to reduced transcript levels for *CHS* and *acyl-CoA oxidase* (*ACO*), the enzyme that produces acetyl-CoA in the precursor pathway for flavonoids. Both genes are induced by UV-light, and signal transduction operates through the ACE and MRE motifs described earlier. When the plant is placed under both pathogen and UV-light stresses, the pathogen repression signal overrides the UV-light induction signal. For the *ACO* gene, both signals converge on two very similar ACEs (Logemann and Hahlbrock 2002). The switch from activation to repression might be achieved through replacement of one or more of the activating CPRF bZIP or MYB proteins with a repressor protein. *PcCPRF1* transcripts are normally induced by UV-light treatment, but this is prevented by elicitor treatment. In contrast, *PcCPRF2* transcript abundance is repressed by UV-light and induced by elicitation. Thus, these provide candidate proteins for such pathogen-induced responses.

H-boxes are present in the promoters of *PAL*, *4CL* and *CHS* genes of a number of species, and have been associated with stress induction and tissue specificity. The multiple H-box sequence of the *Phaseolus vulgaris* (French bean) *CHS15* gene promoter has been shown to be required for tissue-specific expression in transgenic tobacco (Faktor *et al.* 1997*a*, *b*) and to be involved, together with the neighboring G-box/ACE, in induction in response to pathogen elicitor (Faktor *et al.* 1997*b*). The sequence of the H-box (CCTACC) closely resembles MREs such as the P-box (e.g. CCA[C/A]C[A/T]AC[C/T]CC). However, two DNA-binding proteins, KAP-1 and KAP-2, which have been shown to interact specifically with this sequence, are not MYB proteins. KAP-2 was purified from French bean and protein sequence obtained (Lindsay *et al.* 2002). This was used to isolate a *KAP-2* cDNA from French bean and *Medicago truncatula* (Barrel medic). The KAP-2 protein can promote activity of H-box-containing promoters in *in vitro* systems, and has sequence similarity to the mammalian Ku autoantigen protein involved in control of DNA recombination and transcription. *KAP-2* transcript is constitutively present in French bean tissues, suggesting post-transcriptional control of its activity. This is supported by evidence that elicitor-induced phosphorylation may be important for binding activity and movement of the protein from the cytoplasm to the nucleus (Yu *et al.* 1993).

Regulation of anthocyanin and proanthocyanidin biosynthetic genes

Biosynthesis of anthocyanins, proanthocyanidins and other flavonoids has now been studied in detail in a number of species, allowing for some general conclusions to be made. Firstly, as mentioned earlier, control of transcription seems to be the key point for regulation amongst the steps leading to protein production. Secondly, the biosynthetic genes are regulated as groups. The specific groupings vary between species. In studies of flower petals of several dicotyledonous species, a pattern allowing separation of early biosynthetic genes (EBGs) from late biosynthetic genes (LBGs) has emerged. The point of division is at flavanone 3-hydroxylase (F3H) or dihydroflavonol 4-reductase (DFR) depending on whether there is a predominant co-production with anthocyanins of flavones or flavonols, respectively. In seed and vegetative tissues of *Zea mays* (maize) and leaves of *Perilla frutescens* the biosynthetic genes for anthocyanin production from CHS down to those involved in transport to the vacuole are regulated by common TFs (Dooner 1983; Dooner *et al.* 1991; Saito and Yamazaki 2002). In *Vitis vinifera* (grape) berries the UDP-glu:flavonoid 3-*O*-glucosyltransferase (UFGT) is regulated separately from the other genes, and seems to be the key step for triggering anthocyanin production during berry ripening (Boss *et al.* 1996; Kobayashi *et al.* 2002). A third commonality emerging is that branches from the pathway to anthocyanins, and some secondary modification steps in the pathway, are regulated separately from the core anthocyanin biosynthetic genes. Thus, TFs regulating anthocyanin production do not seem to regulate the flavonol synthase (FLS), flavone synthase (FNS), flavonoid 3′-hydroxylase (F3′H) or F3′5′H, although limited data are available as yet. In terms of the TFs involved in regulating anthocyanin and proanthocyanidin biosynthesis, the major common feature is the key role of interacting MYB and bHLH type TFs as activators of the biosynthetic genes, and the involvement of WD40 proteins in assisting the process. Table 1 summarizes the isolation of *MYB*, *bHLH* and *WD40* genes, for both vegetative and floral systems, for which a role in regulation of anthocyanin or proanthocyanidin production has been confirmed through genetic mutants or transgenic studies. In the next sections we look at the TFs known to be involved in more detail.

The first plant system for which the involvement of MYB and bHLH proteins was demonstrated was maize. Expression of one member of a MYB gene family (the *C1*/*Pl* family, Cone *et al.* 1986; Paz-Ares *et al.* 1986; Cone *et al.* 1993) and a bHLH gene family (the *R*/*B* family, Chandler *et al.* 1989; Ludwig *et al.* 1989; Perrot and Cone 1989; Tonelli *et al.* 1991) is necessary and sufficient to induce expression of most of the anthocyanin biosynthetic genes, from *CHS* through to *Bronze2*, a gene that encodes a glutathione-S-transferase (GST) enzyme involved in

vacuolar transport. Different members of the families encode functionally related proteins, but differences in the temporal and spatial control of their expression allow for varying anthocyanin and flavonoid production in response to environmental and developmental signals.

A similarly complex regulatory system of MYB and bHLH proteins has been defined for anthocyanin biosynthesis in petals of *Antirrhinum* and *Petunia*, with the LBGs being the prime regulatory targets. In *Antirrhinum*, *Delila* (Goodrich *et al.* 1992) and *Mutabilis* (Martin *et al.* 2001) encode the bHLH factors and the anthocyanin MYB gene family consists of *Rosea1*, *Rosea2* and *Venosa* (Martin *et al.* 2001). The combined action of these genes provides for complex control of the spatial and temporal production of anthocyanins in the petals. *Delila* is active in both petal lobes and tube, while *Mutabilis* is active only in the lobes. *Rosea1* gives strong pigment production, *Rosea2* weaker pigmentation and *Venosa*, pigmentation only in epidermal cells overlying veins, producing a striking venation pattern (Martin *et al.* 2001). This diversity of action is partly the result of specificity of expression of the *MYB* genes.

Several genes are also involved in controlling anthocyanin biosynthesis in floral tissues in *Petunia*. *Anthocyanin1* (*An1*) and *Jaf13* encode bHLH factors and *An2*, and probably *An4*, encode MYB proteins (Quattrocchio *et al.* 1998, 1999; Spelt *et al.* 2000, 2002). All are required for expression of the LBGs. JAF13 and AN1 diverge in their amino acid sequence, and may also differ in function within the regulatory cascade. AN1 is structurally most similar to INTENSIFIER1 of maize, and transgenic *Petunia* with AN1–glucocorticoid receptor constructs have demonstrated it is a direct activator of the LBGs and also a *myb* gene of unknown function (*Pmyb27*). JAF13 is more similar to DELILA of *Antirrhinum* and R of maize, and *Jaf13* expression does not compensate for loss of *An1* activity in *Petunia* petals. Furthermore, *An1* gene expression, but not *Jaf13* gene expression, is dependent on the activity of *An2*. This data has lead to the suggestion that hierarchical control may be operating between the MYBs and bHLHs controlling anthocyanin biosynthesis in *Petunia* (Spelt *et al.* 2000).

The regulation of production of proanthocyanidins, and of the closely related phlobaphenes (3-deoxyflavonoid compounds) in seed, has been extensively studied in *Arabidopsis* and maize, respectively. MYB and bHLH proteins are again critical as direct regulators. In maize phlobaphene production is controlled by a MYB protein, P, that up-regulates *CHS* and *DFR*, and, presumably, the flavan-3-ol biosynthetic enzyme, but does not up-regulate *F3H* (Lechelt *et al.* 1989;

Table 1. Plant MYB, bHLH and WD40 proteins whose activity in regulating anthocyanin or proanthocyanidin production has been confirmed by genetic mutant or transgenic plant studies

Species	Metabolite	Protein type	Name	Reference
Antirrhinum majus	anthocyanins	MYB	ROSEA1	Martin *et al.* 2001
		MYB	ROSEA2	Martin *et al.* 2001
		MYB	VENOSA	Martin *et al.* 2001
		bHLH	DELILA	Goodrich *et al.* 1992
		bHLH	MUTABILIS	Martin *et al.* 2001
Arabidopsis thaliana	anthocyanins and proanthocyanidins	MYB	PAP1 (AtMYB75)	Borevitz *et al.* 2000
		MYB	PAP2 (AtMYB90)	Borevitz *et al.* 2000
		MYB	TT2	Nesi *et al.* 2001
		bHLH	TT8	Nesi *et al.* 2000
		WD40	TTG1	Walker *et al.* 1999
Fragaria × *ananasa*	anthocyanins	MYB	FaMYB1 (repressive?)	Aharoni *et al.* 2001
Gerbera hybrida	anthocyanins	bHLH	GMYC1	Elomaa *et al.* 1998
Perilla frutescens	anthocyanins	MYB	MYB-P1	Gong *et al*. 1999*a*
		bHLH	MYC-RP/GP	Gong *et al.* 1999*b*
		WD40	PFWD	Sompornpailin *et al.* 2002
Petunia	anthocyanins	MYB	AN2, AN4	Quattrocchio *et al.* 1998, 1999
		bHLH	AN1, JAF13	Spelt *et al.* 2000
		WD40	AN11	de Vetten *et al.* 1997
Sorghum bicolor	phlobaphenes	MYB	Y	Chopra *et al.* 1999
Vitis vinifera	anthocyanins	MYB	MYBA	Kobayashi *et al.* 2002
Zea mays	anthocyanins and phlobaphenes	MYB	C1	Cone *et al.* 1986; Paz-Ares *et al.* 1986
		MYB	Pl	Cone *et al.* 1993
		MYB	P	Lechelt *et al.* 1989; Grotewold *et al.* 1991
		bHLH	B	Chandler *et al.* 1989
		bHLH	LC	Ludwig *et al.* 1989
		bHLH	R	Perrot and Cone 1989
		bHLH	SN	Tonelli *et al.* 1991
		bHLH	IN (repressive)	Burr *et al.* 1996

Grotewold *et al.* 1994). Unlike C1 of maize, the regulatory activity of P does not require a companion bHLH protein, even though P and C1 recognize the same promoter elements of the *DFR* gene. By comparing P to C1, Grotewold *et al.* (2000) were able to define which amino acid residues in the C1 protein defined the interaction with the maize bHLH protein.

In *Arabidopsis*, knockout and gain of function experiments have demonstrated TRANSPARENT TESTA 2 (TT2) up-regulates several proanthocyanidin biosynthetic genes in the seed coat, including *BANYULS* (Nesi *et al.* 2001), encoding the first committed step in flavan-3-ol production. TT2 requires an interacting bHLH factor, encoded by *TT8* (Nesi *et al.* 2000). *TT2* expression patterns show much greater spatial and temporal variation than those of *TT8*, suggesting it provides the controlling step in tannin gene regulation. TT2 and TT8 are required for transcription only of the LBGs, as EBG transcript levels are not reduced in developing seeds of *tt2*, *tt8* and *ttg* mutants (Nesi *et al.* 2000). *TT2* is also expressed during seedling development, where it is required for full anthocyanin production (Nesi *et al.* 2000).

Some of the anthocyanin regulatory genes also impact on other processes. *Rosea1* of *Antirrhinum* (K. Schwinn, unpublished data) and *An1*, *An2* and *An11* of *Petunia* (Spelt *et al.* 2002) also regulate vacuolar pH, and additionally, *An1* influences seed coat epidermal cell development. The best model for studies of such overlapping pathways is *Arabidopsis*, in which the regulatory pathways for anthocyanin and proanthocyanidin production share components with those controlling developmental processes such as trichome formation, root epidermal cell development and seed mucilage production.

Interacting with, or controlling production of, the MYB and bHLH proteins are other types of factor, some of which are common to some of the various pathways. These may act upstream or downstream in the signal cascade, or interact directly with the MYB/bHLH complex. As yet, there are few candidates for direct regulators of the *MYB* and *bHLH* genes. Many of the genes identified as upstream in the signaling pathways seem likely to be involved in general development or environmental response pathways, rather than specifically with phenylpropanoid production.

Mutant plants lacking activity of WD-repeat proteins in *Arabidopsis* (*TRANSPARENT TESTA GLABROUS1*, *TTG1*) and *Petunia* (*anthocyanin 11*, *An11*), lose the ability to produce anthocyanins (in *Petunia*, de Vetten *et al.* 1997) or anthocyanins, proanthocyanidins, seed mucilage and trichomes (in *Arabidopsis*, Walker *et al.* 1999; Western *et al.* 2001). Also, a WD40 (PFWD) that promotes anthocyanin production in transgenic *Arabidopsis* has been isolated from *Perilla* (Sompornpailin *et al.* 2002). Introduction of TFs active in the phenylpropanoid and trichome pathways can complement the *ttg1* phenotype of *Arabidopsis*, completely with the maize bHLH *R* (Lloyd *et al.* 1992) and partially with the *Arabidopsis* bHLH *GLABROUS3* (Payne *et al.* 2000). However, the WD-repeat proteins are not working through transcriptional control of the *MYB* and *bHLH* genes, as mRNA levels for the MYB *An2* are not reduced in the *Petunia an11* mutant (de Vetten *et al.* 1997). Thus, they may work by promoting activity of the MYB and/or bHLH proteins, perhaps by stabilizing the MYB:bHLH transcription complexes. This is supported by the physical interaction shown between WD40 and the bHLH proteins in yeast two-hybrid assays (Payne *et al.* 2000; Sompornpailin *et al.* 2002).

TRANSPARENT TESTA GLABROUS2 (*TTG2*) is required for proanthocyanidin production in *Arabidopsis*, and has recently been shown to encode a member of the plant-specific WRKY group of TFs (Johnson *et al.* 2002). The *ttg2* mutant has greatly reduced production of seed coat proanthocyanidins and mucilage and has fewer, less branched trichomes, but is wild type for anthocyanin production in vegetative tissues. TTG2 probably functions downstream of TTG1, and it has been suggested that it may regulate the activity of the proanthocyanidin specific biosynthetic genes, starting with the first committed step to flavan-3-ol production in *Arabidopsis*, *BANYULS*. *TRANSPARENT TESTA* (*TT*) *16* activity is also required for proanthocyanidin production and expression of *Banyuls*, and encodes a MADS domain protein (identical to ARABIDOPSIS BSISTER) that may be involved in endothelium development. *TT1* is also required for endothelium development, as the *tt1* mutant causes loss of proanthocyanidin production and altered seed coat morphology. It encodes a member of a new group of zinc finger proteins, termed the WIP subfamily (Sagasser *et al.* 2002). The *ANTHOCYANINLESS2* (*ANL2*) gene of *Arabidopsis* encodes a homeobox protein, of the HD-GLABRA2 group, that is required for full anthocyanin production, particularly in the subepidermal cells (Kubo *et al.* 1999). Unlike the *tt* mutants, the *anl2* mutant has no effect on the pigmentation of the seed coat, but affects anthocyanin production in the seedling and other vegetative tissues. Homeodomain proteins are generally involved in cell specification and pattern formation, and the *anl2* mutant shows aberrant cellular organization in the roots. Whether any of these genes of *Arabidopsis* encode direct regulators of the flavonoid-related *MYB* and *bHLH* genes active in vegetative tissues is not known.

The best characterized gene for an upstream regulator of the anthocyanin TF genes is *Viviparous 1* (*Vp1*). *Vp1* encodes a unique type of TF that, along with ABA-regulated TFs, is required for seed maturation. Originally identified in maize (McCarty *et al.* 1991; Hattori *et al.* 1992), the equivalent gene has now been identified in a number of species, including *Arabidopsis* (*ABI3*, Giraudet *et al.* 1992). It has multiple regulatory roles in maize seed development, including both up- and down-regulation of gene expression,

and is required for production of anthocyanins in the aleurone. VP1 up-regulates the *C1* gene directly, through the *Sph cis*-element of the promoter. *Sph* recognition occurs through the B3 domain of the VP1 protein, a domain distinct from those involved in regulation of other seed development genes that contain G-box *cis*-elements (Suzuki *et al.* 1997). VP1 seems to function as part of a complex of TFs that may include 14-3-3 proteins (Schultz *et al.* 1998).

Regulation of tryptophan production

In addition to its requirement for protein synthesis, the tryptophan biosynthetic pathway provides the precursors for a number of plant secondary metabolites, including anthranilate- and indole-derived alkaloids, glucosinolates and indolic phytoalexins (Radwanski and Last 1995). The first committed enzyme in tryptophan biosynthesis, anthranilate synthase, provides a key control point for the pathway. The control of levels of active enzyme is under both transcriptional and non-transcriptional control. In *Arabidopsis* the *ASA1* gene, one of two genes encoding the α-subunit of the enzyme, is transcriptionally induced by several environmental signals, including pathogen infection and amino acid starvation. Transcription of the three *Arabidopsis* genes encoding the β-subunit, *ASB* genes, is also induced by the environmental signals that up-regulate *ASA1*. The identification of *Arabidopsis* mutants with altered tryptophan regulation (*atr*) has enabled significant progress to be made in understanding the transcriptional regulation of tryptophan biosynthesis. Two dominant mutants have been obtained with elevated tryptophan levels and they have been shown to correspond to mutations in TF genes. The *atr1D* mutation corresponds to an overexpression allele of a MYB gene, named *ATR1* (Bender and Fink 1998), which causes increased transcript abundance from *ASA1* (Bender and Fink 1998) and other tryptophan biosynthetic genes (Smolen and Bender 2002). The *atr2D* mutation corresponds to an allele of a bHLH gene, named *ATR2*, with a single amino acid change in the encoded ATR2 protein (Smolen *et al.* 2002). Overexpression of the *atr2D* gene, but not the wild-type *ATR2* gene, produces pleiotropic phenotypes in transgenic *Arabidopsis*, including dark pigmentation and sterility, and it has been suggested that ATR2 may not normally be directly involved in regulating tryptophan biosynthetic genes (Smolen *et al.* 2002). The *atr1D atr2D* double mutant displays an additive phenotype, indicating the MYB and bHLH may act as part of independent pathways. This is supported by a lack of interaction between them in yeast two-hybrid assays (Smolen *et al.* 2002).

Regulation of terpenoid indole alkaloid production

Terpenoid indole alkaloids (TIAs) are derived from products of two separate biosynthetic pathways that generate the tryptophan and terpenoid precursors. Although they are produced in a taxonomically limited group of species, their powerful bioactive properties have made them the focus of much research. In particular, the production of medicinal compounds in *Catharanthus roseus* (Madagascar periwinkle) has become a model system for deciphering TIA biosynthesis. More than twenty biosynthetic steps are involved in producing the major bioactive TIAs, vinblastine and vincristine, from geranoil and tryptophan (reviewed by Roberts and Strack 1999; Memelink *et al.* 2000, 2001). Some steps are plastid-located, some occur on the endoplasmic reticulum and some are associated with the vacuole, and there is also evidence of transport of intermediates between specialized cell types. Thus, regulation of the pathway is likely to be complex. TIA production is induced by jasmonates [jasmonic acid and its volatile derivative methyl jasmonate (MeJA)] and fungal elicitors. As with phenylpropanoids, the primary control point for enzyme activity appears to be through transcriptional regulation of the biosynthetic genes (Memelink *et al.* 2000, 2001). Both induction pathways may intersect, as elicitors can induce jasmonic acid production. A promoter element involved in both the jasmonate and the elicitor response (jasmonate and elicitor responsive element, JERE) was identified in the promoter region of the *Strictosidine synthase* (*Str*) gene, close to the TATA box. It consists of a 24-bp sequence with a GCC core (Menke *et al.* 1999; Memelink *et al.* 2000). Also present was a G-box sequence (CACGTG) that is common to many abiotic and biotic stress induced genes, and an upstream 'BA region' containing quantitative enhancer elements (Memelink *et al.* 2000). Proteins interacting with all three regions have now been identified, with those binding to the JERE being important for the induction of transcription by stress, via jasmonates. Yeast one-hybrid screens identified two JERE-interacting proteins that belong to the AP2/ERF-domain family of TFs, and these were named Octadecanoid Responsive *C. roseus* AP2 (ORCA)1 and ORCA2 (Menke *et al.* 1999). ORCA2, but not ORCA1, activity is induced by MeJA and elicitors, through increased transcript abundance. ORCA2 was shown to activate a *Str*::*GUS* construct in transiently co-transformed *C. roseus* cells (Menke *et al.* 1999). A third member of the family, ORCA3, was isolated by T-DNA tagging (van der Fits and Memelink 2000). ORCA3 bound to the JERE promoter region of *Str* and other TIA biosynthetic gene promoters, and promoted their transcription in transient assays and stably transformed cell cultures. Yeast one-hybrid screens also identified a MYB-like protein (CrBPF-1) that interacts with the BA-region and whose transcription is induced by fungal elicitors, but not jasmonate, by a protein kinase-dependent process (van der Fits *et al.* 2000). Thus, elicitors may induce TIA gene expression by jasmonate-dependent and jasmonate-independent pathways. Two G-box binding proteins have also been identified for the *Str* gene but do not seem to be part of the stress induction process (Siberil *et al.* 2001), a finding supported by results in transgenic tobacco,

showing deletion of the G-box does not reduce elicitor responsiveness (Pasquali *et al.* 1999).

Regulation of carotenoid production

Carotenoids are ubiquitous plant pigments that protect the chloroplast against photo-oxidation, and that are also commonly used to provide bright colors in flowers and fruits. Surprisingly, no TFs involved in regulating carotenoid biosynthetic gene transcription have been reported to date. However, some elements of the regulatory pathways have been determined, in particular, the ethylene-mediated regulation of carotenoid production during fruit ripening (Wang *et al.* 2002; White 2002) and the light-induced accumulation of carotenoids during photomorphogenesis of seedlings (Fankhauser and Chory 1997).

The *Ripening-inhibitor* (*Rin*) locus of *Lycopersicon esculentum* (tomato) has been found to encode a MADs box protein essential for various aspects of fruit ripening, including carotenoid production (LeMADS-RIN, Vrebalov *et al.* 2002). Production of LeMADS-RIN is induced in fruit during ripening, and it probably lies early in the ripening signaling pathway, possibly preceding the ethylene climacteric response. However, no direct link to carotenoid gene regulation has been demonstrated. Many components of the ethylene signal transduction pathway of tomato fruit have also been determined (Wang *et al.* 2002), but again these may lie well upstream of the direct carotenoid gene regulation. Similarly, much is known of the processes regulating gene expression during photomorphogenesis, but there are few studies on how the accumulation of carotenoids is controlled at the level of gene regulation. In seedlings of *Sinapis alba* (white mustard) and *Arabidopsis*, phytochrome has been shown to mediate the increase in transcript abundance for the key carotenoid biosynthetic enzyme phytoene synthase (von Lintig *et al.* 1997). However, the steps linking the phytochrome signal to changes in *phytoene synthase* gene expression were not determined.

Isolation of TF genes

Transcription factors with defined functions in regulation of secondary metabolism were originally identified through two main routes; cloning of genes associated with mutations or screening for proteins binding to defined promoter *cis*-elements. More recently, however, more general approaches to identifying and assigning function to plant TFs have emerged. One approach is to identify sequences of a group of putative TFs of unknown function and then systematically characterize them. Early examples include the isolation of six *myb* cDNAs from *Antirrhinum* petals (Jackson *et al.* 1991), and the subsequent functional studies of gene expression patterns, promoter binding activities and phenotypes resulting from their expression in transgenic plants (Sablowski *et al.* 1994; Moyano *et al.* 1996; Tamagnone *et al.* 1998), and 14 *myb* sequences from tomato (Lin *et al.* 1996). With the completion of the *Arabidopsis* genome, such studies can be conducted on a much larger scale, which may result in identification of many TFs for secondary metabolism. Two European Union collaborative projects across many laboratories have aimed to characterize all the TF genes of *Arabidopsis*, starting with the *myb* genes (Martin *et al.* 2001; Stracke *et al.* 2001). General outputs from the MYB project are already available, such as the phylogeny analysis, transcript abundance data and the generation of a gene knockout collection (Kranz *et al.* 1998; Meissner *et al.* 1999). Similar programs in other species may assist in defining regulatory activities for secondary metabolites not produced by *Arabidopsis*, for example for phytoalexins in the legume *M. truncatula* (May 2002).

Biotechnology applications of TF genes

Most current strategies for metabolic engineering in plants have involved altering the production of a single enzyme. For secondary metabolism this may be targeted at reducing flow through a pathway, adding a new biosynthetic step or steps, or attempting to raise production levels of a group of compounds. However, unless a rate-limiting step can be identified, it can be difficult to raise pathway output significantly by altering activity of a single enzyme. Indeed, introducing whole pathways may be required, for example to produce anthocyanins, proanthocyanidins or health-related flavonoids in tissues that lack them.

As the activity of the biosynthetic genes of secondary metabolism (at least of those studied to date) appears determined primarily by the expression patterns of the regulatory genes, altering the patterns of regulatory gene expression may allow the temporal and spatial modification of secondary metabolite production. This prospect has been tested by placement of *MYB* or *bHLH* cDNAs, in particular *C1* and *Lc* from maize, under the control of the *CaMV 35S* promoter and introduction of these constructs into a range of species. The power of this approach is demonstrated by the successful up-regulation of anthocyanin production in *Trifolium repens* (white clover, de Majnik *et al.* 2000), *Petunia* (Bradley *et al.* 1998), tobacco and tomato (Lloyd *et al.* 1992; Mooney *et al.* 1995), of flavonol production in tomato (Bovy *et al.* 2002), and of levels of various flavonoids and other metabolites in maize cell lines (Grotewold *et al.* 1998). These experiments have also illustrated the ability to apply the gene technology in heterologous species. There are also examples of using TFs to inhibit branches of the phenylpropanoid pathway (Tamagnone *et al.* 1998; Jin *et al.* 2000; Aharoni *et al.* 2001).

Evidently, the availability of defined flavonoid regulatory genes provides tools for modulating both the amount and distribution of anthocyanins in floral and vegetative tissues. However, the results to date also highlight problems with the available technology. Experiments with the closely related bHLH TFs LC and DELILA have shown that, firstly, the

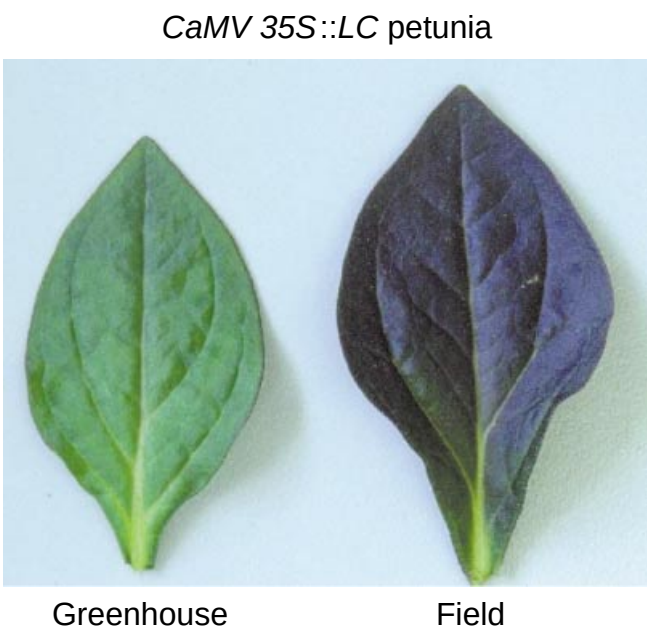

Fig. 2. Leaves from *CaMV 35S*::*Lc Petunia* grown in a plastic greenhouse (left) or an open field (right). Greenhouse grown plants have a weak pigmentation phenotype while field grown plants have deeply pigmented foliage. Non-transgenic plants (not shown) have green foliage under either growth conditions.

same gene constructs may have markedly different effects in transgenics of different species, and secondly, the similar TFs produce quite different transgenic phenotypes (Mooney *et al.* 1995; Bradley *et al.* 1999; de Majnik *et al.* 2000). This variation may reflect differences in both the promoter sequences of the endogenous flavonoid biosynthetic genes, and the presence or absence of interacting endogenous TFs. Furthermore, regulatory proteins with very similar amino acid sequences may differ in their target sequences, their DNA binding strength, and in how they interact with other TFs. The influence of endogenous factors on the transgenic phenotypes is illustrated by our own results with *CaMV 35S*::*Lc Petunia* transgenics. Homozygous plants of the same transgenic line and same age have dramatically different phenotypes when grown in the field or in a plastic greenhouse. Field-grown plants show the strong vegetative pigmentation seen in the original transgenics (Bradley *et al.* 1998), while greenhouse plants have weak pigmentation, barely greater than that of non-transgenic plants (Fig. 2). Controlled environment experiments subsequently confirmed that this was due to high light levels in the field, presumably changing activities of endogenous TFs (the authors' and coworkers' unpublished data).

Concluding comments

Transcriptional regulation provides the most important control point for the secondary metabolic pathways studied to date. For the phenylpropanoid pathway, a lot is understood about both the specific *cis*-elements that are involved in responses to environmental and developmental stimuli, and the TFs involved. Most information is available for the light induction process, the production of anthocyanins in leaves and floral tissues, and the production of proanthocyanidins in seeds. Some of the functional interactions between the different types of TF are now being elucidated, and upstream regulators of the genes encoding the TFs identified. In contrast to the wealth of knowledge on regulation of phenylpropanoid biosynthesis, knowledge for other secondary metabolite pathways is poor. To date, some TF genes have been cloned for the tryptophan and TIA biosynthesis pathways, but no genes encoding direct regulators are available for major pathways such as those for glucosinolates, terpenoids, carotenoids or betalains. However, it is anticipated that the major programs arising from the genomics success with species such as *Arabidopsis* and *M. truncatula* will soon lead to identification of TF genes for several of these pathways. The identification of defined TF genes provides tools for modulating both the amount and distribution of secondary metabolites in plants. The validity of this approach has been well established by transgenic plants with modified flavonoid accumulation patterns, and the *atr1D* and *atr2D* mutations affecting tryptophan production illustrate how such gene technology may soon be extended to other secondary metabolism pathways.

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References

Aharoni A, De Vos CH, Wein M, Sun Z, Greco R, Kroon A, Mol JN, O'Connell AP (2001) The strawberry FaMYB1 transcription factor suppresses anthocyanin and flavonol accumulation in transgenic tobacco. *The Plant Journal* **28**, 319–332.

Bender J, Fink GR (1998) A MYB homologue, ATR1, activates tryptophan gene expression in *Arabidopsis*. *Proceedings of the National Academy of Sciences USA* **95**, 5655–5660.

Bohm BA (1998) 'Introduction to flavonoids.' (Harwood Academic Publishers: Amsterdam)

Borevitz JO, Xia Y, Blount J, Dixon RA, Lamb C (2000) Activation tagging identifies a conserved MYB regulator of phenylpropanoid biosynthesis. *The Plant Cell* **12**, 2383–2394.

Boss PK, Davies C, Robinson SP (1996) Analysis of the expression of anthocyanin pathway genes in developing *Vitis vinifera* L. cv. Shiraz grape berries and the implications for pathway regulation. *Plant Physiology* **111**, 1059–1066.

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

Bradley JM, Davies KM, Deroles SC, Bloor SJ, Lewis DH (1998) The maize *Lc* regulatory gene up-regulates the flavonoid biosynthetic pathway of *Petunia*. *The Plant Journal* **13**, 381–392.

Bradley JM, Deroles SC, Boase MR, Bloor S, Swinny E, Davies KM (1999) Variation in the ability of the maize *Lc* regulatory gene to upregulate flavonoid biosynthesis in heterologous systems. *Plant Science* **140**, 31–39.

Burr FA, Burr B, Scheffler BE, Blewitt M, Wienand U, Matz EC (1996) The maize repressor-like gene *intensifier1* shares homology with the *R1*/*B1* multigene family of transcription factors and exhibits missplicing. *The Plant Cell* **8**, 1249–1259.

Chandler VR, Radicella JP, Robbins TP, Chen J, Turks D (1989) Two regulatory genes of the maize anthocyanin pathway are homologous: Isolation of *B* using *R* genomic sequences. *The Plant Cell* **1**, 1175–1183.

Chattopadhyay S, Ang LH, Puente P, Deng XW, Wei N (1998) *Arabidopsis* bZIP protein HY5 directly interacts with light-responsive promoters in mediating light control of gene expression. *The Plant Cell* **10**, 673–683.

Chopra S, Brendel V, Zhang JB, Axtell JD, Peterson T (1999) Molecular characterization of a mutable pigmentation phenotype and isolation of the first active transposable element from *Sorghum bicolor*. *Proceedings of the National Academy of Sciences USA* **96**, 15330–15335.

Cone KC, Burr FA, Burr B (1986) Molecular analysis of the maize anthocyanin regulatory locus *C1*. *Proceedings of the National Academy of Sciences USA* **83**, 9631–9635.

Cone KC, Cocciolone SM, Burr FA, Burr B (1993) Maize anthocyanin regulatory gene *pl* is a duplicate of *c1* that functions in the plant. *The Plant Cell* **5**, 1795–1805.

Davies KM (2000) Plant colour and fragrance. In 'Metabolic engineering of plant secondary metabolism'. (Eds R Verpoorte and W Alfermann) pp. 127–164. (Kluwer Academic Publishers: Dordrecht)

de Majnik J, Weinman JJ, Djordjevic MA, Rolfe BG, Tanner GJ, Joseph RG, Larkin PJ (2000) Anthocyanin regulatory gene expression in transgenic white clover can result in an altered pattern of pigmentation. *Australian Journal of Plant Physiology* **27**, 659–667.

de Vetten N, Quattrocchio F, Mol J, Koes R (1997) The *an11* locus controlling flower pigmentation in *Petunia* encodes a novel WD-repeat protein conserved in yeast, plants, and animals. *Genes & Development* **11**, 1422–1434.

Dooner HK (1983) Co-ordinate genetic regulation of flavonoid biosynthetic enzymes in maize. *Molecular and General Genetics* **189**, 136–141.

Dooner HK, Robbins TP, Jorgensen RA (1991) Genetic and developmental control of anthocyanin biosynthesis. *Annual Review of Genetics* **25**, 173–199.

Ellenberger T (1994) Getting a grip on DNA recognition: structures of the basic region leucine zipper, and the basic region helix-loop-helix DNA-binding domains. *Current Opinion in Structural Biology* **4**, 12–21.

Elomaa P, Mehto M, Kotilainen M, Helariutta Y, Nevalainen L, Teeri TH (1998) A bHLH transcription factor mediates organ, region and flower type specific signals on dihydroflavonol-4-reductase (DFR) gene expression in the inflorescence of *Gerbera hybrida* (Asteraceae). *The Plant Journal* **16**, 93–99.

Eulgem T, Rushton PJ, Schmelzer E, Hahlbrock K, Somssich IE (1999) Early nuclear events in plant defense signalling: rapid gene activation by WRKY transcription factors. *EMBO Journal* **18**, 4689–4699.

Eulgem T, Rushton PJ, Robatzek S, Somssich IE (2000) The WRKY superfamily of plant transcription factors. *Trends in Plant Science* **5**, 199–206.

Faktor O, Kooter JM, Loake GJ, Dixon RA, Lamb CJ (1997*a*) Differential utilization of regulatory *cis*-elements for stress-induced and tissue-specific activity of a French bean chalcone synthase promoter. *Plant Science* **124**, 175–182.

Faktor O, Loake G, Dixon RA, Lamb CJ (1997*b*) The G-box and H-box in a 39-bp region of a French bean chalcone synthase promoter constitute a tissue-specific regulatory element. *The Plant Journal* **11**, 1105–1113.

Fankhauser C, Chory J (1997) Light control of plant development. *Annual Review of Cell and Developmental Biology* **13**, 203–229.

Feldbrügge M, Sprenger M, Dinkelbach M, Yazaki K, Harter K, Weisshaar B (1994) Functional analysis of a light-responsive plant bZIP transcriptional regulator. *The Plant Cell* **6**, 1607–1621.

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

Giraudat J, Hauge BM, Valon C, Smalle J, Parcy F, Goodman HM (1992) Isolation of the *Arabidopsis ABI3* gene by positional cloning. *The Plant Cell* **4**, 1251–1261.

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

Gong ZZ, Yamagishi E, Yamazaki M, Saito K (1999*b*) A constitutively expressed Myc-like gene involved in anthocyanin biosynthesis from *Perilla frutescens*: molecular characterization, heterologous expression in transgenic plants and transactivation in yeast cells. *Plant Molecular Biology* **41**, 33–44.

Goodrich J, Carpenter R, Coen ES (1992) A common gene regulates pigmentation pattern in diverse plant species. *Cell* **68**, 955–964.

Grotewold E, Athma P, Peterson T (1991) Alternatively spliced products of the maize *P* gene encode proteins with homology to the DNA-binding domain of Myb-like transcription factors. *Proceedings of the National Academy of Sciences USA* **88**, 4587–4591.

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

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

Grotewold E, Sainz MB, Tagliani L, Hernandez JM, Bowen B, Chandler VL (2000) Identification of the residues in the Myb domain of maize C1 that specify the interaction with the bHLH cofactor R. *Proceedings of the National Academy of Sciences USA* **97**, 13579–13584.

HannaRose W, Hansen U (1996) Active repression mechanisms of eukaryotic repressors. *Trends in Genetics* **12**, 229–234.

Hardtke CS, Deng X-W (2000) The cell biology of the COP/DET/FUS proteins. Regulating proteolysis in photomorphogenesis and beyond? *Plant Physiology* **124**, 1548–1557.

Hardtke CS, Okamoto H, Stoop-Myer C, Deng XW (2002) Biochemical evidence for ubiquitin ligase activity of the *Arabidopsis* COP1 interacting protein 8 (CIP8). *The Plant Journal* **30**, 385–394.

Hartmann U, Valentine WJ, Christie JM, Hays J, Jenkins GI, Weisshaar B (1998) Identification of UV/blue light-response elements in the *Arabidopsis thaliana* chalcone synthase promoter using a homologous protoplast transient expression system. *Plant Molecular Biology* **36**, 741–754.

Hattori T, Vasil V, Rosenkrans L, Hannah LC, McCarty DR, Vasil IK (1992) The *Viviparous-1* gene and abscisic acid activate the *C1* regulatory gene for anthocyanin biosynthesis during seed maturation in maize. *Genes & Development* **6**, 609–618.

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

Jakoby M, Weisshaar B, Dröge-Laser W, Vicente-Carbajosa J, Tiedemann J, Kroj T, Parcy F (2002) bZIP transcription factors in *Arabidopsis*. *Trends in Plant Science* **7**, 106–111.

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

Johnson CS, Kolevski B, Smyth DR (2002) *TRANSPARENT TESTA GLABRA2*, a trichome and seed coat development gene of *Arabidopsis*, encodes a WRKY transcription factor. *The Plant Cell* **14**, 1359–1375.

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

Kranz HD, Denekamp M, Greco R, Jin H, Leyva A, Meissner RC, Petroni K, Urzainqui A, Bevan M, Martin C, Smeekens S, Tonelli C, Paz-Ares J, Weisshaar B (1998) Towards functional characterisation of the members of the *R2R3–Myb* gene family from *Arabidopsis thaliana*. *The Plant Journal* **16**, 263–276.

Kubo H, Peeters AJM, Aarts MGM, Pereira A, Koornneef M (1999) *ANTHOCYANINLESS2*, a homeobox gene affecting anthocyanin distribution and root development in *Arabidopsis*. *The Plant Cell* **11**, 1217–1226.

Lechelt C, Peterson T, Laird A, Chen J, Dellaporta SL, Dennis E, Peacock WJ, Starlinger P (1989) Isolation and molecular analysis of the maize *P* locus. *Molecular and General Genetics* **219**, 225–234.

Lin Q, Hamilton WDO, Merryweather A (1996) Cloning and initial characterization of 14 *myb*-related cDNAs from tomato (*Lycopersicon esculentum* cv. Ailsa Craig). *Plant Molecular Biology* **30**, 1009–1020.

Lindsay WP, McAlister FM, Zhu Q, He XZ, Dröge-Laser W, Hedrick S, Doerner P, Lamb C, Dixon RA (2002) KAP-2, a protein that binds to the H-box in a bean chalcone synthase promoter, is a novel plant transcription factor with sequence identity to the large subunit of human Ku autoantigen. *Plant Molecular Biology* **49**, 503–514

Lloyd AM, Walbot V, Davis RW (1992) *Arabidopsis* and *Nicotiana* anthocyanin production activated by maize regulators *R* and *C1*. *Science* **258**, 1773–1775.

Logemann E, Hahlbrock K (2002) Crosstalk among stress responses in plants: pathogen defense overrides UV protection through an inversely regulated ACE/ACE type of light-responsive gene promoter unit. *Proceedings of the National Academy of Sciences USA* **99**, 2428–2432.

Ludwig SR, Habera LF, Dellaporta SL, Wessler SR (1989) *Lc*, a member of the maize *R* gene family responsible for tissue-specific anthocyanin production, encodes a protein similar to transcriptional activators and contains the *myc*-homology region. *Proceedings of the National Academy of Sciences USA* **86**, 7092–7096.

Ma L, Gao Y, Qu L, Chen Z, Li J, Zhao H, Deng XW (2002) Genomic evidence for COP1 as a regulator of light-regulated gene expression and development of *Arabidopsis*. *The Plant Cell* **14**, 2383–2398.

Martin C, Paz-Ares J (1997) MYB transcription factors in plants. *Trends in Genetics* **13**, 67–73.

Martin C, Jin H, Schwinn K (2001) Mechanisms and applications of transcriptional control of phenylpropanoid metabolism. In 'Regulation of phytochemicals by molecular techniques'. (Eds JT Romeo, JA Saunders, BF Matthews) pp. 155–170. (Elsevier Science Ltd: Oxford)

May GD (2002) An integrated approach to *Medicago* functional genomics. In 'Phytochemistry in the genomics and post-genomics eras'. (Eds JT Romeo and RA Dixon) pp. 179–195. (Elsevier Science Ltd: Oxford)

McCarty DR, Hattori T, Carson CB, Vasil V, Lazar M, Vasil IK (1991) The *Viviparous-1* developmental gene of maize encodes a novel transcriptional activator. *Cell* **66**, 895–905.

Meissner RC, Jin HL, Cominelli E, Denekamp M, Fuertes A, Greco R, Kranz HD, Penfield S, Petroni K, Urzainqui A, Martin C, Paz-Ares J, Smeekens S, Tonelli C, Weisshaar B, Baumann E, Klimyuk V, Marillonnet S, Patel K, Speulman E, Tissier AF, Bouchez D, Jones JJD, Pereira A, Wisman E, Bevan M (1999) Function search in a large transcription factor gene family in *Arabidopsis*: assessing the potential of reverse genetics to identify insertional mutations in R2R3 MYB genes. *The Plant Cell* **11**, 1827–1840.

Memelink J, Menke FLH, van der Fits L, Kijne JW (2000) Transcriptional regulators to modify secondary metabolism. In 'Metabolic engineering of plant secondary metabolism'. (Eds R Verpoorte and W Alfermann) pp. 111–125. (Kluwer Academic Publishers: Dordrecht)

Memelink J, Verpoorte R, Kijne JW (2001) ORCAnisation of jasmonate-responsive gene expression in alkaloid metabolism, *Trends in Plant Science* **6**, 212–219.

Menke FLH, Champion A, Kijne JW, Memelink J (1999) A novel jasmonate- and elicitor-responsive element in the periwinkle secondary metabolite biosynthetic gene *Str* interacts with a jasmonate- and elicitor-inducible AP2-domain transcription factor, ORCA2. *EMBO Journal* **18**, 4455–4463.

Menkens AE, Schindler U, Cashmore AR (1995) The G-box — a ubiquitous regulatory DNA element in plants bound by the GBF family of bZIP proteins. *Trends in Biochemical Sciences* **20**, 506–510.

Mooney M, Desnos T, Harrison K, Jones J, Carpenter R, Coen E (1995) Altered regulation of tomato and tobacco pigmentation genes caused by the *delila* gene of *Antirrhinum*. *The Plant Journal* **7**, 333–339.

Moyano E, Martinez-Garcia JF, Martin C (1996) Apparent redundancy in *myb* gene function provides gearing for the control of flavonoid biosynthesis in *Antirrhinum* flowers. *The Plant Cell* **8**, 1519–1532.

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

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

Osterlund, MT, Hardtke CS, Wei N, Deng XW (2000) Targeted destabilization of HY5 during light-regulated development of *Arabidopsis*. *Nature* **405**, 462–466.

Pasquali G, Erven ASW, Ouwerkerk PBF, Menke, FLH, Memelink J (1999) The promoter of the strictosidine synthase gene from periwinkle confers elicitor-inducible expression in transgenic tobacco and binds nuclear factors GT-1 and GBF. *Plant Molecular Biology* **39**, 1299–1310.

Payne CT, Zhang F, Lloyd AM (2000) *GL3* encodes a bHLH protein that regulates trichome development in *Arabidopsis* through interaction with GLI and TTG1. *Genetics* **156**, 1349–1362.

Paz-Ares J, Wienand U, Peterson PA, Saedler H (1986) Molecular cloning of the *c* locus of *Zea mays*: a locus regulating the anthocyanin pathway. *EMBO Journal* **5**, 829–833.

Paz-Ares J, Peterson PA, Saedler H (1990) Molecular analysis of the *C1-I* allele from *Zea mays*: a dominant mutant of the regulatory *C1* locus. *EMBO Journal* **9**, 315–321.

Perrot GH, Cone KC (1989) Nucleotide sequence of the maize *R-S* gene. *Nucleic Acids Research* **17**, 8003.

Petersen M, Strack D, Matern U (1999) Biosynthesis of phenylpropanoids and related compounds. In 'Biochemistry of plant secondary metabolism'. (Ed. M Wink) pp. 151–221. (Sheffield Academic Press: Sheffield)

Pugh BF (1997) Dynamics of transcription complex assembly. In 'Transcription factors in eukaryotes'. (Ed. AG Papavassiliou) pp. 37–50. (Landes Bioscience: Austin, TX)

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

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

Radwanski ER, Last RL (1995) Tryptophan biosynthesis and metabolism: biochemical and molecular genetics. *The Plant Cell* **7**, 921–934.

Ranish JA, Hahn S (1996) Transcription: basal factors and activation. *Current Opinion in Genes and Development* **6**, 151–158.

Roberts MF, Strack D (1999) Biochemistry and physiology of alkaloids and betalains. In 'Biochemistry of plant secondary metabolism'. (Ed. M Wink) pp. 17–78. (Sheffield Academic Press: Sheffield)

Sablowski RWM, Moyano E, Culianez-Macia FA, Schuch W, Martin C, Bevan M (1994) A flower-specific Myb protein activates transcription of phenylpropanoid biosynthetic genes. *EMBO Journal* **13**, 128–137.

Sagasser M, Lu GH, Hahlbrock K, Weisshaar B (2002) *A. thaliana* TRANSPARENT TESTA 1 is involved in seed coat development and defines the WIP subfamily of plant zinc finger proteins. *Genes & Development* **16**, 138–149.

Saito K, Yamazaki M (2002) Biochemistry and molecular biology of the late-stage of biosynthesis of anthocyanin: lessons from *Perilla frutescens* as a model plant. *New Phytologist* **155**, 9–23.

Sawadogo M, Szentirmay MN (1997) Specific transcription initiation by RNA polymerase II and associated factors. In 'Transcription factors in eukaryotes'. (Ed. AG Papavassiliou) pp. 23–36. (Landes Bioscience: Austin, TX)

Schultz TF, Medina J, Hill A, Quatrano RS (1998) 14-3-3 proteins are part of an abscisic acid–VIVIPAROUS1 (VP1) response complex in the *Em* promoter and interact with VP1 and EmBP1. *The Plant Cell* **10**, 837–847.

Schulze-Lefert P, Dangl JL, Becker-Andre M, Hahlbrock K, Schulz W (1989) Inducible *in vivo* DNA footprints define sequences necessary for UV light activation of the parsley chalcone synthase gene. *EMBO Journal* **8**, 651–656.

Schwechheimer C, Bevan M (1998) The regulation of transcription factor activity in plants. *Trends in Plant Science* **3**, 378–383.

Schwechheimer C, Deng XW (2001) COP9 signalosome revisited: a novel mediator of protein degradation. *Trends in Cell Biology* **11**, 420–426.

Shin B, Choi G, Yi HK, Yang SC, Cho IS, Kim J, Lee S, Paek NC, Kim JH, Song PS (2002) AtMYB21, a gene encoding a flower-specific transcription factor, is regulated by COP1. *The Plant Journal* **30**, 23–32.

Siberil Y, Benhamron S, Memelink J, Giglioli-Guivarc'h N, Thiersault M, Boisson B, Doireau P, Gantet P (2001) *Catharanthus roseus* G-box binding factors 1 and 2 act as repressors of strictosidine synthase gene expression in cell cultures. *Plant Molecular Biology* **45**, 477–488.

Smolen G, Bender J (2002) Arabidopsis cytochrome P450 cyp83B1 mutations activate the tryptophan biosynthetic pathway. *Genetics* **160**, 323–332.

Smolen GA, Pawlowski L, Wilensky SE, Bender J (2002) Dominant alleles of the basic Helix-Loop-Helix transcription factor ATR2 activate stress-responsive genes in *Arabidopsis*. *Genetics* **161**, 1235–1246.

Sompornpailin K, Makita Y, Yamazaki M, Saito K (2002) A WD-repeat-containing putative regulatory protein in anthocyanin biosynthesis in *Perilla frutescens*. *Plant Molecular Biology* **50**, 485–495.

Spelt C, Quattrocchio F, Mol JNM, Koes R (2000) *Anthocyanin1* of *Petunia* encodes a basic helix-loop-helix protein that directly activates transcription of structural anthocyanin genes. *The Plant Cell* **12**, 1619–1631.

Spelt C, Quattrocchio F, Mol J, Koes R (2002) ANTHOCYANIN1 of *Petunia* controls pigment synthesis, vacuolar pH, and seed coat development by genetically distinct mechanisms. *The Plant Cell* **14**, 2121–2135.

Staiger D (2002) Circadian rhythms in *Arabidopsis*: time for nuclear proteins. *Planta* **214**, 334–344.

Stracke R, Werber M, Weisshaar B (2001) The R2R3–MYB gene family in *Arabidopsis thaliana*. *Current Opinion in Plant Biology* **4**, 447–456.

Suzuki M, Kao CY, McCarty DR (1997) The conserved B3 domain of VIVIPAROUS1 has a cooperative DNA binding activity. *The Plant Cell* **9**, 799–807.

Suzuki G, Yanagawa Y, Kwok SF, Matsui M, Deng XW (2002) *Arabidopsis* COP10 is a ubiquitin-conjugating enzyme variant that acts together with COP1 and the COP9 signalosome in repressing photomorphogenesis. *Genes & Development* **16**, 554–559.

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

Terzaghi WB, Bertekap RL, Cashmore AR (1997) Intracellular localization of GBF proteins and blue light-induced import of GBF2 fusion proteins into the nucleus of cultured *Arabidopsis* and soybean cells. *The Plant Journal* **11**, 967–982.

Tonelli C, Consonni G, Faccio Dolfini S, Dellaporta SL, Viotti A, Gavazzi G (1991) Genetic and molecular analysis of *Sn*, a light-inducible, tissue specific regulatory gene in maize. *Molecular and General Genetics* **225**, 401–410.

van der Fits L, Memelink J (2000) ORCA3, a jasmonate-responsive transcriptional regulator of plant primary and secondary metabolism. *Science* **289**, 295–297.

van der Fits L, Zhang H, Menke FLH, Deneka M, Memelink JA (2000) *Catharanthus roseus* BPF-1 homologue interacts with an elicitor-responsive region of the secondary metabolite biosynthetic gene *Str* and is induced by elicitor via a JA-independent signal transduction pathway. *Plant Molecular Biology* **44**, 675–685.

Vom Endt DV, Kijne JW, Memelink J (2002) Transcription factors controlling plant secondary metabolism: what regulates the regulators? *Phytochemistry* **61**, 107–114.

von Lintig J, Welsch R, Bonk M, Giuliano G, Batschauer A, Kleinig H (1997) Light-dependent regulation of carotenoid biosynthesis occurs at the level of phytoene synthase expression and is mediated by phytochrome in *Sinapis alba* and *Arabidopsis thaliana* seedlings. *The Plant Journal* **12**, 625–634.

Vrebalov J, Ruezinsky D, Padmanabhan V, White R, Medrano D, Drake R, Schuch W, Giovannoni J (2002) A MADS-box gene necessary for fruit ripening at the tomato *ripening-inhibitor* (*Rin*) locus. *Science* **296**, 343–346.

Walker AR, Davison PA, Bolognesi-Winfield AC, James CM, Srinivasan N, Blundell TL, Esch JJ, Marks MD, Gray JC (1999) The *TRANSPARENT TESTA GLABRA1* locus, which regulates trichome differentiation and anthocyanin biosynthesis in *Arabidopsis*, encodes a WD40 repeat protein. *The Plant Cell* **11**, 1337–1350.

Wang KLC, Li H, Ecker JR (2002) Ethylene biosynthesis and signaling networks. *The Plant Cell* **14** (Suppl. S), S131–S151.

Weisshaar B, Armstrong GA, Block A, da Costa E, Silva O, Hahlbrock K (1991) Light-inducible and constitutively expressed DNA-binding proteins recognising a plant promoter element with functional relevance in light responsiveness. *EMBO Journal* **10**, 1777–1786.

Wellmer F, Schafer E, Harter K (2001) The DNA binding properties of the parsley bZIP transcription factor CPRF4a are regulated by light. *Journal of Biological Chemistry* **276**, 6274–6279.

Western TL, Burn J, Tan WL, Skinner DJ, Martin-McCaffrey L, Moffatt BA, Haughn GW (2001) Isolation and characterization of mutants defective in seed coat mucilage secretory cell development in *Arabidopsis*. *Plant Physiology* **127**, 998–1011.

White PJ (2002) Recent advances in fruit development and ripening: an overview. *Journal of Experimental Botany* **53**, 1995–2000.

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

Yamamoto YY, Matsui M, Ang LH, Deng XW (1998) Role of a cop1 interactive protein in mediating light-regulated gene expression in *Arabidopsis*. *The Plant Cell* **10**, 1083–1094.

Yu LM, Lamb CJ, Dixon RA (1993) Purification and biochemical characterization of proteins which bind to the H-box *cis*-element implicated in transcriptional activation of plant defense genes. *The Plant Journal* **3**, 805–816.

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