In the Solanaceae, a hierarchy of bHLHs confer distinct target specificity to the anthocyanin regulatory complex

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Abstract

The anthocyanin biosynthetic pathway is regulated by a transcription factor complex consisting of an R2R3 MYB, a bHLH, and a WD40. Although R2R3 MYBs belonging to the anthocyanin-activating class have been identified in many plants, and their role well elucidated, the subgroups of bHLH implicated in anthocyanin regulation seem to be more complex. It is not clear whether these potential bHLH partners are biologically interchangeable with redundant functions, or even if heterodimers are involved. In this study, AcMYB110, an R2R3 MYB isolated from kiwifruit (Actinidia sp.) showing a strong activation of the anthocyanin pathway in tobacco (Nicotiana tabacum) was used to examine the function of interacting endogenous bHLH partners. Constitutive expression of AcMYB110, an R2R3 MYB isolated from kiwifruit (Actinidia sp.) showing a strong activation of the anthocyanin pathway in tobacco (Nicotiana tabacum) was used to examine the function of interacting endogenous bHLH partners. Constitutive expression of AcMYB110 in tobacco leaves revealed different roles for two bHLHs, NtAN1 and NtJAF13. A hierarchical mechanism is shown to control the regulation of transcription factors and consequently of the anthocyanin biosynthetic pathway. Here, a model is proposed for the regulation of the anthocyanin pathway in Solanaceous plants in which AN1 is directly involved in the activation of the biosynthetic genes, whereas JAF13 is involved in the regulation of AN1 transcription.

Key words: bHLH, MYB, Solanaceae, tobacco, transcriptional control, transcription factors.

Introduction

Anthocyanins are the major pigmented group of flavonoid compounds responsible for a range of colours varying from pink to blue, through to red and violet in flowers, fruits, or leaves. The anthocyanin biosynthetic pathway is one branch of flavonoid metabolism (Winkel-Shirley, 2001) and its regulatory system has been extensively studied. The anthocyanin biosynthetic genes are transcriptionally regulated by a MYB–bHLH–WD40 (MBW) complex that contains R2R3 MYB and basic helix-loop-helix (bHLH) transcription factors and a WD40 protein (Feller et al., 2011). The expression patterns of the MBW components thus define the variety of pigmentation patterns of plant tissues (Davies et al., 2012).

The R2R3 MYBs are numerically the largest class of transcription factors in plants. The C1 MYB (Zea mays) was the first transcription factor identified to regulate anthocyanin biosynthesis (Paz-Ares et al., 1987); subsequently a large number of MYBs controlling anthocyanin colour in flowers (Quattrocchio et al., 1999; Schwinn et al., 2006; Albert et al., 2011) and fruit (Kobayashi et al., 2004; Takos et al., 2011).
Basic helix-loop-helix proteins are the second largest class of transcription factor. Generally, the first 200 amino acids of the protein are involved in the interaction with the MYB partner, whereas the following 200 amino acids interact with the WD40 protein. The bHLH domains are involved in the formation of homo- or heterodimers with other bHLH proteins, which often is a prerequisite for DNA recognition and contributes to DNA-binding specificity (Feller et al., 2011; Hichri et al., 2011). The bHLH group of transcription factors have been divided into 26 subgroups (Pires and Dolan, 2010), but if ‘atypical’ bHLHs are included this is extended to 32 subgroups (Carretero-Paulet et al., 2010). Flavonoid related bHLHs have been grouped into subgroup III.

The first bHLHs regulating the flavonoid pathway were identified in maize, Boosterl (B) and Redl (R) (Chandler et al., 1989). Subsequently a number of additional bHLH transcription factors able to regulate the flavonoid pathway have been identified: these include, AN1 and JAF13 in Petunia (Petunia hybridra) (Quattrocchio et al., 1998; Spelt et al., 2000), Delila and Mutabilis in snapdragon (Antirrhinum majus) (Martin et al., 1991), TT8 and GLABRA3 in Arabidopsis (Nesi et al., 2000; Feyissa et al., 2009), VvMYC1 and VvMYCA1 in grape (Vitis vinifera) (Hichri et al., 2010) and A in pea (Pisum sativum) (Hellens et al., 2010). Many of these have been shown to regulate different physiological or morphological events including different branches of the flavonoid pathway, vacuole acidification, and epidermal cell fate (Hichri et al., 2011).

The interaction between the MYB and bHLH proteins has often been used to study combinatorial gene regulation in plants. In maize the presence of an amino acid motif in the R3 region of the MYB, identified as (DE)Lx4(RK)x4Lx4Lx4R, determines the binding specificity of the MYB to the bHLH partner and is responsible for the different ability of ZmCl and a related R2R3 MYB, ZmPl, to interact with the bHLH R. The ability of the MYB to interact with the bHLH triggers the activation of the biosynthetic gene ZmB1, with R playing a key role in the target specificity of the MBW complex (Grotewold et al., 2000; Zimmermann et al., 2004).

In Petunia, the MBW complex AN2–AN1–AN11 (MYB–bHLH–WD40, respectively) regulates anthocyanin biosynthesis in the flower petals (deVetten et al., 1997; Quattrocchio et al., 1999; Spelt et al., 2000). AN1 and AN11 are also involved in the control of other physiological events such as acidification of the vacuole when associated in a MBW complex that involves the MYB Ph4 (Quattrocchio et al., 2006). In Petunia, two bHLHs have been identified: JAF13 and AN1. Both bHLHs have been shown to regulate anthocyanin biosynthesis in Petunia flowers (Quattrocchio et al., 1998; Spelt et al., 2002) and they have often been used interchangeably in functional assays. However, their role in anthocyanin regulation has not been fully resolved, and they have been shown to have functional differences. In mutant lines, such as the anl mutant (Spelt et al., 2000) and in over-expression lines, JAF13 is not able to complement the lack of AN1.

It seems likely that the MBW complex includes different classes of MYBs and bHLHs with specific functions in regulating the transcription of the flavonoid pathway. Alternatively, different bHLHs may be involved as heterodimers in the MBW complex to regulate the anthocyanin pathway. This specificity of functions amongst similar transcription factors, or involvement of multiple bHLH classes, would allow for more subtle regulation of the transcriptional mechanism through different combinations of transcription factors within the MBW complex. It is therefore necessary to understand how apparently similar MYB or bHLH transcription factors within the MBW complex can have differing regulatory activities.

Here it is shown that AcMYB110, an R2R3 MYB from kiwifruit (Fraser et al., 2013), elicits strong induction of anthocyanin when transiently expressed in the model plant tobacco (Nicotiana tabacum). This response enables us to further define the specific roles of the bHLH proteins NtAN1 and NtJAF13 and to present a model that explains the hierarchical interactions of the MYB and bHLH partners within the MBW complex.

Materials and methods

Phylogenetic analysis

Protein alignment used ClustalW within the software package Geneious 6.1.7 (Biombatters Ltd., Auckland, New Zealand), with manual refinement. A phylogenetic tree was formed from the alignment within the Geneious package using PhyML v2.2 and the maximum likelihood method (Guindon and Gascuel, 2003), with the default settings (NNIs tree topology search, BioNJ initial tree, LG model for amino acids substitutions, and 1000 bootstrap replicates).

Transient assay

Transient assays were performed as described previously (Hellens et al., 2005) using tobacco (Nicotiana tabacum) ‘Samsun’ and Nicotiana benthamiana plants grown in a glasshouse, using natural light with daylight extended to 16h. Transient dual-luciferase and colour assay were executed infiltrating approximately 300 µl of Agrobacterium tumefaciens GV3101 (MP90) mixture into young leaves. Agrobacterium were cultured on Lennox agar plates supplemented with selection antibiotics and incubated at 28 °C. The freshly grown bacteria were re-suspended in infiltration media and incubated at room temperature for at least 2 h (Hellens et al., 2005) before infiltration.

To generate overexpression vectors the open reading frames of AcMYB110 and NtAN1 were amplified using specific primers (Supplementary Table S1) and cloned into pENTR-TOPO and subsequently a Gateway reaction was performed with destination expression vector pHEX2 (http://www.lifetechnologies.com). PhAN1 expression vector, as described in Albert et al. (2011), was provided by Nick Albert (Plant and Food Research, Palmerston North, NZ).

To generate the RNA interference constructs, small fragments were amplified of NtAN1 and NtJAF13 (respectively 383 bp and 197 bp long) with specific primers (Supplementary Table S1) and the cloned products were put into pENTR-TOPO, followed by a Gateway reaction with RNAi destination vector pTKO2 (http://www.lifetechnologies.com).

For the colour assay, a mixture of Agrobacterium transformed with constructs constitutively expressing transcription factors fused to the 35S promoter in pHEX2 and silencing the endogenous bHLHs NtAN1 and NtJAF13 by RNAi using a pTKO2 vector was used. Final colour development was assayed at four days and seven days after infiltration.
Tobacco AN1, CHS, and DFR promoter sequences were obtained by a genome walking strategy; approximately 1 kb of target promoter was amplified using specific primers (Supplementary Table S1) and inserted into pGreen 0800-LUC. Promoter activation assays were performed in tobacco leaves. Agrobacterium containing the promoter–LUC fusions were mixed with Agrobacterium containing the transcription factor to be tested (ratio 1:3) and used to infiltrate tobacco leaves (Hellens et al., 2005). The resulting luminescence was measured four days after infiltration.

Pigment analysis

Five independent replicates were collected for each infiltration. Leaf samples were freeze-dried and ground. Anthocyanins were extracted in acidified MeOH (0.1% HCl) and centrifuged at 3000 g. Aliquots were dried down and re-suspended in 20% v/v aqueous MeOH (0.2 ml). The re-dissolved extracts were analysed by reversed phase HPLC using a method previously described (Andre et al., 2012). Anthocyanin peaks were identified by comparison of retention times with authentic standards and absorption at 520 nm. Total anthocyanin content was calculated using standard solutions of cyanidin-3-galactoside (Extrasynthese, Genay, France) and expressed as cyanidin galactoside equivalents ng mg⁻¹ of fresh weight.

Real time qPCR analysis

Gene expression analysis was performed on infiltrated leaves, 4 d after infiltration. RNA was isolated using TRIzol® (http://www.lifetechnologies.com) and DNase-treated with DNA-free:Ambion (http://www.ambion.com/). Reverse transcription was performed using oligo(dT) according to the manufacturer’s protocol (Transcriptor: Roche Diagnostics, http://www.roche.com/). Quantitative real-time PCR was carried out using the LightCycler® 480 System, using 480 SYBR® Green I Master (Roche Diagnostics). Reactions were performed in quadruplicate and a non-template control was included in each run. The data were analysed using the LightCycler® 480 software 1.5 normalised to tobacco actin (GQ281246) and calibrated to the control (empty vector) infiltration (primer sequences in Supplementary Table S1).

Yeast one-hybrid and yeast two-hybrid assays

Yeast one-hybrid experiments were conducted using the Matchmaker Gold Y1H library Screening System (Clontech). 490 bp and 562 bp of the NiANI and NiDFR promoters, respectively, were cloned in the pAbAi vector and transformed into Y1HGold strain to generate the bait reporter strains. NiANI and NiJAF13 full-length coding sequence were cloned into pDEST22 (Life Technologies) and transfected into yeast as a fusion to the GAL4 activating domain (AD). MYB110 was cloned using Gateway recombination technology (Life Technologies) in pGW6, a Gateway-adapted version of pTFT1 vector (Egea-Cortines et al., 1999) which allows expression of an untagged protein in yeast. The different pairwise combinations of MYB and bHLHs and corresponding empty vector controls were transfected in the PJ69-4A and PJ69-4alpha yeast strains (James et al., 1996). Pairwise combinations of MYBs and bHLHs, expressed as fusion proteins to the GAL4 activating (AD) and binding (BD) domains respectively, were obtained by mating and screened for growth after up to 3 d at 30 °C on SD media lacking tryptophan (T), leucine (L) and histidine (H) and supplemented by increasing amount of 3-amino-1,2,4-triazole (3AT) (10, 25, 50, 75, and 100 mM) to increase the stringency of the histidine selection (Supplementary Fig. S1).

Results

Phylogenetic analysis of anthocyanin-related bHLHs

Phylogenetic analysis, performed on the deduced amino acid sequences of more than 30 flavonoid-related bHLHs belonging to the subgroup III, clearly separates them in two clades (Fig. 1): clade A and clade B, as described in Davies et al. (2012). Clade A included JAF13 (Petunia), R (maize), Delila (snapdragon), and GL3/EGL3 (Arabidopsis), whereas clade B contained AN1 (Petunia), Intensifier (maize), Mutabilis (snapdragon), and TT8 (Arabidopsis). Members of the same subgroups are often involved in the same biological process, although it is not always clear if their functions overlap. For example; GL3, EGL3, and TT8 are partially redundant in the control of anthocyanin biosynthesis in Arabidopsis. However,
each of these have also more specific functions such as PA biosynthesis (TT8), trichome formation (GL3/EGL3), and production of seed coat mucilage (TT8/EGL3) (Feller et al., 2011). Moreover, in Petunia the lack of AN1 (clade B) cannot be complemented by JAF13 (clade A), despite both being able to regulate anthocyanin biosynthesis (Spelt et al., 2000). On the other hand, in snapdragon Delila and Mutabilis, respectively from clade A and clade B, seem to be functionally redundant and able to complement for the loss of the other factor (Schwinn et al., 2006).

Ectopic expression of AcMYB110 reveals different abilities to promote red pigmentation depending on the availability of endogenous bHLHs

When anthocyanin-related MYBs are ectopically expressed transiently in leaves of tobacco (Nicotiana tabacum) an anthocyanic patch can be generated (Espley et al., 2007). In the current study, kiwifruit AcMYB110 (Fraser et al., 2013) was used because of its strong ability to promote anthocyanin biosynthesis in tobacco leaves without the need to provide ectopic expression of a bHLH partner. This ability of AcMYB110 to activate the anthocyanin pathway of tobacco when transient expressed on its own is different from many other anthocyanin-related R2R3 MYBs (Lin-Wang et al., 2010). Four days after infiltration red pigmentation was evident, and this developed into a dark red patch after 7 days (Fig. 2).

This ability of AcMYB110 to induce anthocyanin biosynthesis, without co-infiltration of a bHLH, could be due to an efficient interaction with the endogenous tobacco bHLH and WD40 proteins. To investigate these interactions, RNA interference (RNAi) was used to inhibit the activity of each of the endogenous tobacco bHLHs known to be involved in anthocyanin biosynthesis: NtAN1a (HQ589208), NtAN1b (HQ589209), and NtJAF13b (KF298397). When NtAN1 was silenced (the same RNAi construct was able to target both NtAN1a and NtAN1b) no red colour occurred, despite

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**Fig. 2.** (A) Red colour development in Nicotiana tabacum leaves 7 days after ectopic expression of AcMYB110 in combination with bHLH RNAi constructs targeting tobacco NtAN1 and NtJAF13, and constructs constitutively expressing Petunia hybrida PhJAF13. (B) Anthocyanin content in infiltrated patches 4 and 7 days after infiltration. Anthocyanin content is expressed as cyanidin galactoside equivalents ng mg⁻¹ of fresh weight. Error bars are SE for five replicates.
constitutive expression of *AcMYB110* (Fig. 2A, B). In contrast, constitutive expression of *AcMYB110* and *NtJAF13* RNAi still resulted in a red patch, although knock-down of *NtJAF13b* had the effect of delaying the development of the pigmentation (Fig. 2A, B). Four days after infiltration the anthocyanic patches where *AcMYB110* and *NtJAF13* RNAi were co-infiltrated were less intense compared with the darker pigmentation of the leaves infiltrated with *AcMYB110* only, and even after 7 days they did not reach the same anthocyanin content of *AcMYB110* only (Fig. 2A, B).

These results demonstrated that, in the tobacco system, *AcMYB110* requires *NtAN1* to activate anthocyanin biosynthesis, whereas the lack of *NtJAF13b* results in a slower development of pigmentation (Fig. 2A, B). A related phenotype is seen in colour development of flowers of stable RNAi tobacco lines targeting endogenous *NtAN1* (*a* and *b*) and *NtJAF13b*. *NtAN1* RNAi lines developed white flowers, whereas the *NtJAF13* RNAi lines developed less intense pink flowers compared with the wild type (Supplementary Fig. S2). To further characterize the functional interaction of the two bHLHs, the down-regulated *NtAN1* (*NtAN1* RNAi) was complemented by constitutively expressing the *Petunia hybrida JAF13* (*PhJAF13*; AF020545). Using the *Petunia PhJAF13* to complement the reduction in expression of the tobacco bHLH allows the monitoring by qPCR of expression of the endogenous *NtbHLH* as there is sufficient sequence difference between the genes from the two species. Constitutive expression of *PhJAF13* was not able to complement the lack of tobacco *NtAN1* (RNAi) and did not restore the ability of *AcMYB110* to drive red pigmentation (Fig. 2). This demonstrates distinct functional properties for the two bHLHs and suggests differing roles in the anthocyanin regulatory system.

**Ectopic expression of AcMYB110 up-regulates tobacco NtAN1 and the biosynthetic genes of the anthocyanin pathway in distinct ways**

*AcMYB110* is able to form a complex with either *NtAN1* or *NtJAF13*, as confirmed by yeast two-hybrid assay (Fig. 3A). *NtJAF13* and *NtAN1* may target the MBW complex to different promoters within this pathway. The gene expression of chalcone synthase (*NtCHS*; AB213651) and dihydroflavonol 4-reductase (*NtDFR*; EF421429) were therefore examined, as representative of early and late stages of anthocyanin biosynthesis; as well as the expression of the endogenous *NtJAF13b* and *NtAN1* genes. Ectopic expression of *AcMYB110* in the tobacco leaf up-regulated the transcript level of the endogenous *NtAN1* and the biosynthetic genes *NtCHS* and *NtDFR* (Fig. 3B). This is consistent with the development of a red pigmentation of the infiltrated leaf patches. In contrast, *AcMYB110* did not have a significant effect on the expression of tobacco *NtJAF13*.

As expected, co-infiltration of the *NtAN1* RNAi with *AcMYB110* strongly reduced the transcript level of the endogenous *NtAN1* and consequently the expression of the biosynthetic genes of the pathway and the colour of the leaf patch. On the contrary, *NtJAF13* expression was slightly elevated when RNAi was directed at *NtAN1*. *NtJAF13* RNAi did not completely silence the endogenous *NtJAF13* transcript, but reduced its expression by approximately half. The reduced expression of *NtJAF13* and ectopic expression of *AcMYB110* resulted in a reduced up-regulation of *NtAN1* and consequently of *NtCHS* and *NtDFR* (Fig. 3B).

**AcMYB110 activates the promoters of NtAN1 and the biosynthetic genes NtCHS and NtDFR**

Using a transient dual luciferase assay (Hellens et al., 2005), the ability of *AcMYB110* to regulate the tobacco *NtCHS*, *NtDFR*, and *NtAN1* promoters was tested. Different combinations of *AcMYB110* and RNAi knock-down constructs directed at the endogenous bHLHs were co-infiltrated in tobacco leaves together with the pGREEN-LUC constructs, which contained the target promoters (tobacco *CHS, DFR* or *AN1*) driving the expression of the luciferase reporter gene (LUC). The results are presented relative to the level of the Renilla (REN) reporter gene, which is under control of a 35S
promoter. As expected, AcMYB110 was able to activate the promoters of the biosynthetic genes, *NtCHS* and *NtDFR* (Fig. 4). However, when *NtANI* was down-regulated there was a significant drop in the activation of these promoters. Co-infiltration of *NtJAF13* RNAi did not significantly affect AcMYB110 activation of the *NtCHS* and *NtDFR* promoters. Moreover, AcMYB110 was able to activate the *NtANI* promoter (Fig. 5A), and addition of the *NtANI* RNAi construct had no effect on this activation. In contrast, activation of the *NtANI* promoter was reduced in the presence of the *NtJAF13* RNAi construct (Fig. 5A). Ectopic expression of PhJAF13 enhanced activation of the *NtANI* promoter, whereas overexpression of *NtANI* did not improve the activation of the *NtANI* promoter (Fig. 5B).

Direct binding to the *NtANI* promoter by AcMYB110 was assayed by yeast one-hybrid (Fig. 5C). AcMYB110 was shown to bind the *NtANI* promoter only in presence of the NtJAF13, whereas no binding was demonstrated in the presence of NtANI (Fig. 5D). This result confirms the transient luciferase assay results where NtJAF13 seems to be involved in the regulation of *NtANI*.

### Comparative in silico analysis of the promoter regions of AN1, CHS, and DFR

The promoter sequences of *N. tabacum* NtANI, NtCHS, and NtDFR were analysed using the PLACE database SignalScan program (Higo et al., 1999) and several potential MYB (Dare et al., 2008) and bHLH binding sites were identified (Fig. 6 and Supplementary Fig. S3). Nine different variants of the canonical CANNTG bHLH binding motif were identified. When focusing on the 650 bp region upstream of the ATG, it was found that the CACGTG variant was only present in the promoters of the two biosynthetic genes whereas two other motifs, CATCTG and its reverse complement CAGATG, were only found in the *NtANI* promoter. The promoter analysis of *AN1*, *CHS*, and *DFR* was extended to three other species of the Solanaceae family (*Petunia*, potato, tomato) as well as to *Arabidopsis*. The results confirmed that the CACGTG motif was specific to the CHS and DFR biosynthetic genes and that either, or both, CATCTG and CAGATG motifs were present in the *ANI-like* promoters in the Solanaceae. However, this did not occur in the *Arabidopsis* promoters as the CATCTG motif was present in the *AtCHS* promoter and none of the two *ANI*-specific motifs were present in the *TT8* promoter. Although the *ANI-like* promoters of the four Solanaceae share a low pairwise identity (48–55% with one another and 81% between tomato and potato AN1), the common CACGTG motif sits in a highly conserved stretch of 27 bp which also contains conserved putative MYB binding sites. This suggests that this region is under high selective pressure and so may have an important regulatory function.

### Discussion

The transcriprional regulation of anthocyanin biosynthesis has been well described in plants and involves an MBW complex consisting of an R2R3 MYB, a bHLH, and a WD40 protein. MYBs and bHLHs that regulate the anthocyanin biosynthetic pathway have been extensively described in several plants of the Solanaceae family and are often found as small multi-gene families e.g. *AN1*, *JAF13*, *AN2*, *DPL*, and *PHZ* in *Petunia* (Quattrocchio et al., 1998; Quattrocchio et al., 1999; Spelt et al., 2000; Albert et al., 2011).

Although the different members of the bHLH and R2R3 MYB multi-gene families have often been thought of as having very similar roles, there is increasing evidence of specialization of function. Not only is there considerable amino acid sequence variability between the flavonoid-related MYB genes from the Poaceae (such as *ZmC1*) and those of dicots, but different gene family members within a species can vary in function, as seen from markedly different transgenic phenotypes resulting from over-expression of *PhAN2*, *PhDPL*, or *PhPHZ* in *Petunia* (Quattrocchio et al., 1998; Albert et al., 2011). Less information is available for the subfamily IIIF bHLHs belonging to the two different clades; one that includes sequences such as *AN1* (*Petunia*), TT8 (*Arabidopsis*), and Mutabilis (snapdragon), and a second that contains sequences such as *JAF13* (*Petunia*), Delila (snapdragon), and GL3 from *Arabidopsis* (Fig. 1).

Here, the roles of the two anthocyanin-related bHLH clades have been investigated using kiwifruit (*Actinidia*) AcMYB110, previously shown to activate anthocyanin
A hierarchy of bHLHs regulate anthocyanin production. (Fraser et al., 2013), and which is able to promote a red anthocyanic patch when expressed transiently without co-expression of an exogenous bHLH. Because of this strong activating ability, AcMYB110 proved to be a useful tool to study possible functional differences between the two flavonoid-related bHLH clades in tobacco.

The MYB–AN1 complex directly activates anthocyanin biosynthetic genes

Ectopic expression of the MYB NtAN2 in tobacco promotes anthocyanin biosynthesis and results in red pigmentation in both vegetative and floral tissues (Pattanaik et al., 2010). This is achieved by up-regulating the expression of the biosynthetic genes and the NtAN1 bHLH (Bai et al., 2011). Here, similar results were obtained when AcMYB110 was transiently expressed in tobacco leaves: NtAN1 was up-regulated, as were the biosynthetic genes of the pathway, resulting in dark red patches only a few days after infiltration (Fig. 2). However, constitutive expression of AcMYB110 did not affect the expression level of NtJAF13. Co-infiltration of AcMYB110 with the NtAN1 RNAi construct prevented activation of NtCHS and NtDFR and therefore red pigmentation (Figs 2, 3B, and 4), and the constitutive expression of PhJAF13 was not able to complement the lack of NtAN1 (Fig. 2). This is consistent with previous results where AN1 was shown to be necessary to activate the anthocyanin pathway: in Petunia, a white petal an1 mutant is due to the lack of PhAN1 and over-expressing PhJAF13 was not sufficient to restore anthocyanin biosynthesis (Spelt et al., 2000). A similar situation is found in pea, where loss of function of an AN1-like bHLH, corresponding to Mendel’s A gene, results in white flowers (Hellens et al., 2010). However, less specificity is seen in other species than that of Petunia or tobacco, where phenotypic redundancy between the bHLH family members is seen. For example, in snapdragon the bHLH Delila complements for the loss of another anthocyanin-related bHLH, Mutabilis (Schwinn et al., 2006). In contrast, co-infiltration of AcMYB110 with NtJAF13 RNAi resulted in a reduced level of NtJAF13 which did not seem to affect the ability of AcMYB110 to promote anthocyanin accumulation (Figs 2 and 3B).
The MYB–JAF13 complex has an indirect effect on anthocyanin biosynthesis through regulation of AN1 expression

Gene expression data (Fig. 3B) of the infiltrated leaves supported the role of NtJAF13 as an indirect regulator of the pathway by aiding the activation of NtAN1 expression by the MYB. Previous work had suggested that anthocyanin-related MYBs can regulate AN1 expression. In particular, MYB AN2 (or MYB AN4) and MYB75-PAPI were shown to enhance the expression of PhAN1 and TT8 in Petunia and Arabidopsis, respectively (Spelt et al., 2000; Tohge et al., 2005). Moreover, there seems to be some level of functional redundancy amongst the flavonoid-related MYBs in regulating AN1 expression, given that PhAN1 is still expressed in Petunia an2 mutants (Spelt et al., 2000) and that in Arabidopsis TT2 also has been shown to promote TT8 expression (Baudry et al., 2006).

Our promoter activation analysis supports a hierarchical model where NtJAF13 is involved in the transcriptional activation of NtAN1, which in turn regulates the anthocyanin biosynthetic genes (Fig. 7). In tobacco, silencing of NtAN1 by RNAi, or over-expressing NtAN1 did not affect NtAN1 promoter activation, suggesting that NtAN1 is not involved in activating its own promoter, unlike in Arabidopsis, where TT8 in combination with PAP1 or TT2 is able to regulate its own expression (Baudry et al., 2006; Xu et al., 2013). In contrast, the NtJAF13 RNAi had a negative effect on NtAN1 promoter activation (Fig. 5A) and over-expression of NtJAF13 improved NtAN1 promoter activation (Fig. 5B). The role of NtJAF13 as a regulator of NtAN1 expression (in combination with AcMYB110) was further confirmed by yeast one-hybrid assay, in which AcMYB110 was able to bind the NtAN1 promoter only in the presence of the NtJAF13.
(Fig. 5D). These results, in yeast and in planta, establish that NtJAF13 is required by the MYB to activate NtANI expression, whereas NtANI is not involved in its own regulation.

Given that AcMYB110 is able to activate all the promoters tested, our results suggest that the specificity of promoter recognition by the MBW complex is determined by the bHLH partner. The exact role of the bHLH in the MBW complex in different plant species is not yet clear. Studies in maize have suggested roles in reducing the inhibitory effect of single-repeat MYBs and/or in assisting transcription through aiding in chromatin accessibility (Feller et al., 2011; Kong et al., 2012). However, there is less known on the involvement of the bHLH in promoter cis-element recognition, some of which may be explained by the recent identification of further interacting proteins that alter DNA recognition by the bHLH (Kong et al., 2012). Our analysis of the promoter sequences from Arabidopsis and four different Solanaceous species found specific target bHLH cis-elements: a CACGTG bHLH-binding motif was present in the promoters of the two biosynthetic genes and surrounded by several potential MYB binding sites, whereas the CAGATG and/or CATCTG motifs were specific to the ANI promoter (Fig. 6), supporting a model where the AN1–MYB and JAF13–MYB complexes would specifically recognise these motifs in the biosynthetic gene and the ANI promoters, respectively. In Arabidopsis the CACGTG motifs was also found in the biosynthetic gene promoters; consistent with the fact that TT8 is able to regulate its own promoter (Baudry et al., 2006), neither of the two ‘ANI-like’-specific bHLH motifs were present in the TT8 promoter, suggesting that the model of hierarchical regulation is not well conserved outside the Solanaceae family. Future work to confirm this observation is required.

AN1 and JAF13 regulate anthocyanin biosynthesis through a hierarchical mechanism

In the Solanaceae, specificity of the anthocyanin-activating MYBs is defined by the interaction with the bHLH partners JAF13 and AN1. The MYB, to activate anthocyanin biosynthesis, needs to associate specifically to AN1 to induce transcription of the biosynthetic genes, whereas the MYB–JAF13 complex is not able to directly activate the genes of the pathway. The MYBs therefore rely on elevating ANI expression so that the MYB–AN1–WD40 complex can then activate the anthocyanin response. The role of JAF13 is to enhance ANI expression, boosting anthocyanin biosynthesis; this is achieved by forming a complex with the MYB and activating the ANI promoter. JAF13 is suggested to act further upstream in the regulatory cascade in the control of anthocyanin biosynthesis.

Supplementary data

Supplementary data are available at JXB online.

Figure S1. Analysis of MYB110, AN1 and JAF13 protein–protein interactions by yeast two-hybrid.

Figure S2. Colour development in flowers of stable RNAi lines targeting endogenous NtANI and NtJAF13.

Figure S3. (A) Promoter sequence alignment of ANI (A), CHS (B) and DFR (C) genes from tobacco, Petunia, tomato and potato.

Table S1. Oligonucleotide primer sequences.

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