Running head:

A negative MYB regulator of flavonoids in poplar

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The MYB182 protein downregulates proanthocyanidin and anthocyanin biosynthesis in poplar by repressing both structural and regulatory flavonoid genes

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One-sentence Summary:

The poplar MYB182 repressor protein negatively regulates flavonoid and proanthocyanidin pathway genes by interacting with other transcription factors
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Abstract

Trees in the genus *Populus* contain phenolic secondary metabolites including the proanthocyanidins (PAs) which help to adapt these widespread trees to diverse environments. The transcriptional activation of proanthocyanidin (PA) biosynthesis in response to herbivory and UV light stress has been documented in poplar leaves, and a regulator of this process, the R2R3-MYB transcription factor MYB134, has been identified. MYB134 overexpressing transgenic plants show a strong high-PA phenotype. Analysis of these transgenic plants suggested the involvement of additional MYB transcription factors including repressor-like MYB factors. Here, MYB182, a subgroup 4 MYB factor, was found to act as a negative regulator of the flavonoid pathway. Overexpression of MYB182 in hairy root culture and whole poplar plants led to reduced PA and anthocyanin levels, as well as a reduction in expression of key flavonoid genes. Similarly, a reduced accumulation of transcripts of a MYB PA activator and a bHLH co-factor was observed in *MYB182* overexpressing hairy roots. Transient promoter activation assays in poplar cell culture demonstrated that MYB182 can disrupt transcriptional activation by MYB134, and that the bHLH-binding motif of MYB182 was essential for repression. Microarray analysis of transgenic plants demonstrated that downregulated targets of MYB182 also include shikimate pathway genes. This work shows that MYB182 plays an important role in the fine-tuning of MYB134-mediated flavonoid metabolism.
Introduction

Flavonoids are widely distributed secondary plant metabolites that include the anthocyanins, flavonols, flavones, and proanthocyanidins (Koes et al., 2005). Collectively, these compounds play many roles in the interaction of plants with their environment, such as protection against ultraviolet (UV) and visible light stress, resistance to herbivores and pathogens, attraction of pollinators and seed dispersers, and modulation of physiological and developmental signals (Mol et al., 1998). The proanthocyanidins (PAs), also known as condensed tannins, are polymers of flavan-3-ols, typically consisting of 2-50 subunits. PAs are present in many plants but are especially abundant in trees, where they are found in vegetative tissues such as leaves, bark, and roots (Barbehenn and Constabel 2011). They are also present in seeds and many types of fruit, where they are thought to discourage premature consumption by frugivores or prevent spoilage by fungi (Cipollini and Stiles, 1993).

In humans, a diet high in PAs has been linked to reduced risk of chronic cardiovascular diseases (Rasmussen et al., 2005; Scalbert et al., 2005). PAs are thought to prevent atherosclerosis by inhibiting the oxidation of low-density lipoproteins (LDLs), based on their electron-donating properties and ability to scavenge reactive oxygen species (Scalbert et al., 2005). Berry fruits, whole grains, wine and beer are considered good sources of PAs (Prior and Gu, 2005). In the diets of ruminants such as sheep and cattle, PAs can be important modulators of excess rumen bacterial activity, preventing bloat and improving protein use efficiency (Min et al., 2003). This effect makes these compounds important components of silage and forage crops, and targets for manipulation via agricultural biotechnology.

In nature, the PAs are associated with diverse ecological functions. They typically possess broad antimicrobial properties, and by inhibiting bacterial activity in soils they affect nutrient cycling. As a result, the PA content of litter from keystone trees species such as poplar correlates strongly with community structure and ecosystem dynamics (Schweitzer et al., 2008). Many studies have attempted to show that PAs are important in defense against insect herbivores. The results of such experiments are mixed, however, as PAs do not consistently show strong effects against major tree pests (Barbehenn and Constabel, 2011; Boeckler et al., 2014). By contrast, they can adversely affect performance and reproductive success in mammals due to their ability to bind dietary protein in mammalian digestive systems (Barbehenn and Constabel, 2011). In planta, PAs may also function to inhibit pathogenic microorganisms (Constabel et al., 2014). Tannins are also often found in the roots of trees, and though their roles here are poorly defined.

The flavonoids, including PAs, are derived from phenylpropanoids and malonyl-CoA, which serve as substrates for chalcone synthase (CHS), the first enzyme in the flavonoid biosynthetic pathway. Following isomerization and hydroxylation by flavanone 3-hydroxylase (F3H),
intermediates are reduced by dihydroflavonol 4-reductase (DFR) (Marles et al., 2003). Anthocyanin synthase (ANS) then catalyses the last common step in the biosynthesis of anthocyanins and PAs. Anthocyanidin reductase (ANR) and leucoanthocyanidin reductase (LAR) are specific to the PA branch of the pathway and produce flavan-3-ols, typically epicatechin and catechin, respectively (Abrahams et al., 2003; Xie et al., 2003). These are polymerized via an unknown mechanism and stored in the vacuole. Genetic studies in Arabidopsis revealed that MATE efflux family proteins TT12, glutathione S-transferase TT19 and Autoinhibited H1-ATPase isoform 10 are required for transport of PAs to the vacuole.

The biosynthesis of anthocyanins and PAs is regulated by three types of transcription factors: R2R3-MYB factors, basic helix–loop–helix (bHLH) proteins, and conserved WD40 repeat (WDR) proteins (Ramsay and Glover, 2005; Koes et al., 2005). The MYB, bHLH and WDR proteins physically interact to form the MBW complex that activates transcription, and which has best been described using genetic tools in Arabidopsis. The MBW complex interacts directly with promoter DNA via multiple cis-elements. Known target sequences include the MRE (Myb-response element) and AC-elements for R2R3-MYB proteins, and the E-box or bHLH-binding motif which is bound by the bHLH proteins (Feller et al., 2011; Lai et al., 2013, Xu et al., 2014). In contrast, WDR proteins have not been shown to bind DNA, but are thought to function by interacting with MYB and bHLH proteins (Baudry et al., 2004). It is the specific combination of interacting R2R3-MYB, bHLH and WDR factors within the complex that determines which target genes are expressed in a given cell (Baudry et al., 2004; Broun, 2005; Koes et al., 2005; Ramsay and Glover 2005).

The bHLH and WDR cofactors are adaptable and can be involved in multiple processes. For example, the bHLH factor TT8 is required for PA synthesis in Arabidopsis when it interacts with the PA-specific R2R3 MYB TT2, but is also involved in the synthesis of anthocyanins and seed coat mucilage when combined with other co-factors (Nesi et al., 2000). A second Arabidopsis bHLH protein, GL3, belongs to a distinct bHLH subgroup and has several non-overlapping functions in determining epidermal cell fate (Zhang et al., 2003. TT8 and GL3 are partially redundant, however, and can compensate for each other in knock-out mutants (Broun, 2005). In petunia, both subgroups of bHLH cofactors are represented by AN1 and JAF13, respectively (Koes et al., 2005). Both TT8 and GL3 have been shown to directly interact with the WDR protein TTG1 (Zhang et al., 2003). TTG1 has roles in the regulation of trichome and root hair formation in addition to its function in the synthesis of Arabidopsis seed coat PAs (Ramsay and Glover, 2005). By contrast, the MYBs are functionally divergent and appear to be the major determinants of whether a gene or pathway is expressed in a given cell type (Koes et al., 2005). In Arabidopsis, the R2R3-MYB factor WER functions exclusively in root hair patterning (Lee and Schiefelbein,
1999), while TT2 is specific to PA biosynthesis and PAP1 regulates only anthocyanin biosynthesis (Nesi et al., 2001; Teng et al., 2005). Nevertheless, these MYBs can all interact with the same bHLH protein such as TT8 to then activate distinct target promoters (Nesi et al., 2000; Gonzalez et al., 2008). In addition, a given MYB can interact with several different bHLHs, providing multiple functional combinations (Zimmermann et al., 2004).

Homologs of Arabidopsis TT2 and PAP1 are found in many species of plants, where they have conserved roles as PA and anthocyanin regulators, respectively. TT2-type PA regulators have been characterized in fruit, including DkMYB2 from persimmon, VvMYBPA2 from grapevine, and FaMYB9 in strawberry (Akagi et al., 2010; Terrier et al., 2009; Schaart et al., 2013). MYBs from the TT2 group are also active in regulating PAs in vegetative tissues, such as LjTT2 from Japanese lotus, TaMYB14 from clover, and MYB134 from poplar (Yoshida et al., 2008; Hancock et al., 2012; Mellway et al., 2009). These MYB transcription factors act as part of the MBW complex that promotes flavonoid gene expression. For MYB134, we demonstrated direct binding to AC-like elements that are found in phenylpropanoid gene promoters (Mellway et al., 2009). A second type of PA MYB regulator was defined by the discovery of VvMYBPA1 from grapevine (Bogs et al., 2009). Orthologs of this MYB were subsequently described in other species (Akagi et al., 2009). These PA MYBs appear to act in parallel as well as in tandem with the TT2-type PA MYBs; in grapevine, both PA MYBs regulate flavonoid promoters directly, but VvMYPA2 overexpression also induces transcripts of VvMYBPA1 (Terrier et al., 2009). Most species examined have both types of PA MYBs; Arabidopsis is a notable exception and has only the TT2-type. A further subgroup of MYB factors are the PAP1-like anthocyanin regulators. In Arabidopsis, PAP1 regulates stress-induced anthocyanins (Teng et al., 2005), and PAP1 orthologs are responsible for coloration in a variety of fruit (Lin-Wang et al., 2010). The floral color regulators AN2 from petunia and ROSEA1/2 from snapdragon are also part of this MYB clade (Koes et al., 2005; Ramsay and Glover, 2005). In poplar, Wilkins et al. (2009) identified a group of PAP1-like MYBs that are highly expressed in pigmented tissues, suggesting conserved functions in anthocyanin synthesis, but none have been characterized to date.

Control of gene expression by the MBW complex can be further modulated by the involvement of repressor MYB proteins. The first such negative MYB regulators to be characterized include *Antirrhinum* MYB308 and MYB330, which negatively affect phenolic acid and lignin biosynthesis (Tamagnone et al., 1998), and Arabidopsis MYB4 and MYB32, which regulate sinapate esters and lignin biosynthesis (Jin et al., 2000; Preston et al., 2004). These MYB repressors defined subgroup 4 within the MYB gene family, which to date includes only negative regulators. Recently, Arabidopsis MYB7, the downstream target of MYB4, was identified as a repressor of flavonol biosynthesis and demonstrated to directly target DFR and UGT genes.
(Fornalé et al., 2014). Chrysanthemum MYB1, the repressor of lignin biosynthesis, also downregulates the flavonoid pathway (Zhu et al., 2013).

Repressor MYBs of the anthocyanin pathway have also been identified. These include strawberry FaMYB1 and FcMYB1, and petunia PhMYB27 (Aharoni et al., 2001; Salvatierra et al., 2013; Albert et al., 2014). Overexpression of FaMYB1 causes the suppression of anthocyanin synthesis in tobacco, and suppression of FcMYB1 by transient RNAi in strawberry fruit increased the accumulation of anthocyanin. Likewise, RNAi-suppression of PhMYB27 increases the accumulation of anthocyanin in Petunia flowers and vegetative tissues (Albert et al., 2014). In phylogenies, these flavonoid R2R3 repressor MYBs all cluster together within MYB subgroup 4 (Stracke et al., 2001), and all share the C2-motif [pfLNLD/ELxiG/S] with a core consensus sequence of LxLxL or DLNxxP (Dubos et al., 2010; Aharoni et al., 2001). These consensus sequences are the signature patterns of the EAR (ERF-associated amphiphilic repression) motif, the predominant form of transcriptional repressor motif identified in plants (Kagale and Rozwadowski, 2011). Elegant work with PhMYB27 has begun to illuminate the mechanism of action of the subgroup 4 R2R3 repressor MYBs. In yeast two-hybrid assays, PhMYB27 interacts directly with bHLHs of both the GL3 and TT8 subgroups, though with different affinities (Albert et al., 2014). Furthermore, deletion of the DLNxxP-type EAR motif reduced PhMYB27 repressor activity; the function of this motif was not defined here but may involve chromatin remodeling (Kagale and Rozwadowski, 2011). Recently, the first suppressor of the PA pathway, VvMYBC2-L1, was identified in grapevine as a new locus co-located with eQTLs for PA-related genes (Huang et al., 2014). This subgroup 4 repressor MYB downregulates PAs and flavonoid biosynthetic genes when overexpressed in hairy roots. Little is known about its mechanism of action, but it contains a partial LxLxL-type EAR motif.

In addition to subgroup 4 R2R3 MYB repressors, the Arabidopsis single repeat R3 MYB repressors CPC and ETC1 were shown to be involved in the downregulation of anthocyanin biosynthesis (Zhu et al., 2009, Nemie-Feyissa et al., 2014). In petunia, the R3 MYB factor MYBx acts as negative regulator of anthocyanin accumulation in parallel to PhMYB27 (Albert et al., 2014). These R3 MYB proteins do not contain the repressor motif, but act as passive repressors by binding bHLH proteins required for formation of MBW complexes (Albert et al., 2014). A distinct type of repressor from Arabidopsis, AtMYBL2, also contains only a single repeat, but is otherwise more closely related to the R2R3 MYBs than other characterized R3 MYBs (Dubos et al., 2008; Matsui et al., 2008). This protein has a unique C-terminal TLLLFR motif that is required for repressor activity, and so the mechanism appears to be distinct from the small R3 MYB repressors.

In general, the flavonoid MYB repressors are associated with specific flavonoids, but may
also exert effects on related pathways. For example, FaMYB1 suppresses anthocyanin biosynthetic genes when overexpressed in tobacco, including ANS and GT (Aharoni et al., 2001), and MYBL2 targets CHS, CHI, F3H, DFR, ANS, and TT8 (Dubos et al., 2008; Matsui et al., 2008). However, when overexpressed in *Lotus corniculatus*, FaMYB1 also reduced PA biosynthesis (Paolocci et al., 2011), and *AtMYBL2* suppresses ANR expression in the seed coat (Dubos et al., 2008). Likewise, the *VvMYBC2-L1*-overexpressing grapevine hairy roots have a reduction in stilbene content in addition to reduced PAs and PA precursors (Huang et al., 2014). Therefore, the determinants of target specificity and details about the mechanism of repression still remain to be elucidated, especially in perennial plants that accumulate significant concentrations of PAs.

The genus *Populus* consists of widespread trees of the northern hemisphere that are commonly known as poplars, aspens and cottonwoods. *Populus* typically contain substantial amounts of phenolic metabolites, including hydroxycinnamate esters, salicinoids, and PAs, which in *P. tremuloides* can accumulate to 25% DW of leaves (Donaldson et al., 2006). Furthermore, in *Populus* PA biosynthesis and accumulation can be rapidly induced by stresses including wounding, herbivore damage, pathogen attack, nitrogen deficiency, and UV light (Osier and Lindroth, 2004, Miranda et al., 2007; Mellway et al., 2009), suggesting a complex system of regulators. Stress induction of PAs in poplar involves the upregulation of flavonoid biosynthesis genes. This response is mediated by MYB134, a TT2 type R2R3 MYB transcription factor (Mellway et al., 2009).

In the poplar genome, 192 genes encode R2R3 MYB transcription factors (Wilkins et al., 2009), but very few poplar MYBs with roles in flavonoid regulation are functionally characterized. MYB134 is an activator of PA biosynthesis and stimulates the poplar ANR1 promoter in transient expression assays in Arabidopsis (Zifkin et al., 2012; Gesell et al., 2014). Transgenic poplar plants overexpressing *MYB134* accumulate up to 50 times more PAs in their leaves, yet they have normal anthocyanin levels and only slightly elevated flavonol contents, suggesting MYB134 regulates PAs specifically (Mellway et al., 2009). Concurrently, microarray analysis revealed that all known early and late structural genes for PA biosynthesis were upregulated in these plants (Mellway, 2009). In addition, several MYB genes predicted to encode both positive and negative regulators were expressed at elevated levels in the MYB134 overexpressors. One such regulator is MYB115, which belongs to the MYBPA1 subgroup of R2R3 MYB activators (Terrier et al., 2009). We also identified several genes encoding R2R3 repressor MYBs of subgroup 4, and one single repeat R3 MYB. Here, we characterize the *MYB182* R2R3 MYB repressor-like gene in detail. We demonstrate that, when MYB182 is overexpressed in poplar hairy roots and transgenic poplar plants, PA accumulation is reduced, as
is expression of flavonoid biosynthetic genes. Using transient expression assays, we further show that MYB182 represses gene expression induced by the activator MYBs, and that this repression requires interaction with a bHLH co-factor. In addition, MYB182 may repress other regulatory genes, as well as other enzyme-encoding genes important for phenolic metabolism in poplar.
Results

*The poplar repressor-like MYB165, MYB182 and MYB194 belong to a separate subclade within R2R3 MYB subgroup 4*

The protein sequences of the poplar repressor-like MYB genes MYB165, MYB182 and MYB194, which were identified by their enhanced expression in MYB134 overexpressor plants, were aligned and compared to characterized R2R3 MYBs from other species (Fig. 1). MYB181 was included in the phylogenetic tree as it showed high identity (86%) to MYB182, although unlike the other three MYBs this gene was not upregulated in MYB134 overexpressors. The alignment shows that the N-terminal R2R3 domain is highly conserved and includes a bHLH binding domain, while the C-terminal domain is very divergent (Fig. 1a). However, the latter region contains two conserved protein motifs, the C1 motif (LlsrGIDPxT/SHRxI/L; Shen *et al*., 2011) and the C2 motif (pdLNLD/ELxiG/S), which is diagnostic for subgroup 4 MYB proteins and contains the EAR repressor domain (Jin *et al*., 2000; Kagale and Rozwadowski, 2011). The new poplar repressor MYBs share the LxLxL type EAR motif with the lignin/phenylpropanoid MYBs, although in MYB181 and MYB182 this motif contains conservative substitutions. Thus, whereas LNLDL is most common, MYB 181 and MYB182 C2 domains contain INLDL and INIDL, respectively. The poplar repressors also diverge in the xiG/S portion of the C2 motif (Fig 1a). In the lignin or phenylpropanoid repressor MYBs such as AtMYB4 and EgMYB1 (Jin *et al*., 2000; Legay *et al*., 2010), a zinc-finger motif (CX1–2CX7–12CX2C) and a C4 motif (FLGLx4–7V/LLDG/FYRSx1LEMK) were present. These motifs were not found in FaMYB1, VvMYBC2-L1, PhMYB27, and the new poplar MYBs. In its place these MYB repressor proteins all contained a complete (MYB165 and MYB194) or partial (MYB181 and MYB182) TLLLFR repressor motif (Matsui *et al*., 2008).

To better define the structure of the subgroup 4 and to identify closely related R2R3 MYB factors, a phylogenetic analysis of the repressor MYBs and known anthocyanidin and PA regulators was performed (Fig. 1b, Supplemental Fig. S1). This phylogeny resolved a number of clades according to function. As expected, clades for known anthocyanin, flavonol, and the two types of PA activators (TT2- and MYBPA1-types) were clearly defined with strong bootstrap support. The new poplar repressor MYBs grouped together with the previously characterized anthocyanin repressors FaMYB1 andPhMYB27 from strawberry and petunia, and the PA repressor VvMYBC2-L1 from grapevine (Huang *et al*., 2014; Aharoni *et al*., 2001; Albert *et al*., 2014). This subclade was separated from the second major group of R2R3 repressor MYBs that comprises the general phenylpropanoid and lignin repressors from a variety of plants including monocots. Together with the presence of different motifs in the C-terminus, this analysis confirms
that the R2R3 MYB repressors of flavonoid metabolism are distinct from other types of repressors.

In silico expression profiles using the *P. trichocarpa* expression database and eFP browser were generated for the new repressor MYBs in different poplar tissues (Supplemental Fig. S2). For comparison, the genes encoding the poplar PA regulator MYB134 and ANR1, a key enzyme for PA biosynthesis, were also profiled. Both genes are strongly expressed in young leaves, roots and seedlings, all tissues with high PA synthesis. In etiolated seedlings, transcripts of MYB134 are also slightly induced by light. Except for MYB182, all of the repressors are mainly expressed in young leaves. By contrast, the expression profile for MYB182 was the opposite of MYB134, the positive PA regulator. This is consistent with the idea that MYB182 could have a negative effect on PA accumulation. Such negative correlations in developmental expression have been observed with AtMYBL2, PhMYB27, and VvMYBC2-L1 repressors (Dubos et al., 2008; Albert et al., 2011; Huang et al., 2014). We next used RT-qPCR to confirm that MYB182 expression is upregulated in MYB134-overexpressing poplars, as this is how it was first identified (Supplemental Fig. S3). The MYB182 repressor was also induced by wound stress, similar to MYB134 and the PA biosynthetic genes. Based on these results, we focused on MYB182 for more detailed analyses on the role of this gene in PA and flavonoid metabolism.

**Poplar hairy roots overexpressing MYB182 show reduced proanthocyanidin levels and flavonoid gene expression**

Since poplar roots accumulate high levels of PAs and express both positive and negative PA MYB regulators, we first used a hairy root expression system to directly test the function of MYB182. Induction of hairy roots by *Agrobacterium rhizogenes* is a relatively fast and efficient way of obtaining transgenic tissues (Giri and Narasu, 2000). Transgenic hairy roots have been used successfully for analysis of transcriptional regulation of secondary metabolism in alfalfa, grape and soybean (Pang et al., 2013; Terrier et al., 2009; Huang et al., 2014). The MYB182 coding sequence was cloned into a binary vector harboring GFP marker gene under the control of a root-specific promoter. Two weeks following co-cultivation with *Agrobacterium rhizogenes*, leaf explants began to develop hairy roots. GFP-expressing roots were subcultured and analyzed further. Susceptibility to *Agrobacterium* is known to vary among *Populus* species and genotypes; we obtained the highest frequency of hairy root formation with *Populus tremula x P. alba* clone 717-1-B4 and the Arqual A. *rhizogenes* strain (Fig. 2a). Only GFP-expressing roots were analyzed further. All GFP-positive lines that expressed MYB182 showed dramatically reduced PA levels compared to empty vector control hairy roots (Fig. 2c). RT-qPCR confirmed the overexpression of MYB182 in all three lines examined (Fig. 2b).
To test the prediction that MYB182 overexpression leads to reduced PA levels by downregulating the flavonoid pathway, expression of flavonoid structural genes was examined by RT-qPCR. Reduced transcript levels were observed for the ANR1 and CHS1 genes (Fig. 2d). This suggested an effect on both early and late flavonoid biosynthesis genes. DFR1 showed a similar pattern but its overall expression was too low to observe a consistent reduction in transcripts. By contrast, the flavonol synthase 2 (FLS2) gene, which does not participate in PA or anthocyanin synthesis, was not affected significantly by MYB182 overexpression (Fig. 2d). These experiments suggested that MYB182 specifically impacts PA and core flavonoid transcripts. We also assayed expression of the positive PA regulator, MYB134, and other regulatory genes we previously observed to be upregulated in MYB134-overexpressing poplar. MYB134 and a GL3-like bHLH cofactor (bHLH079, see below) were not affected by MYB182 overexpression. However, MYB115, the MYBPA1-type positive PA regulator, and bHLH131, a PA synthesis co-factor of the TT8/DEL/JAF13 group (Gesell et al., 2014), were affected in the MYB182 overexpressing hairy roots, and both showed reduced expression (Fig. 2d).

**MYB182 overexpression in transgenic poplar plants leads to reduced anthocyanin and proanthocyanidin synthesis and accumulation**

To investigate the role of MYB182 in whole poplar plants, it was overexpressed in transgenic poplar plants under the control of the double cauliflower mosaic virus 35S promoter. Multiple independently transformed lines were generated, propagated, and grown in the greenhouse together with wild type poplar plants (see Materials and Methods). Overexpression of MYB182 in poplar did not lead to any visible abnormalities when plants were grown under normal greenhouse conditions (Supplemental Fig. S4).

Previous work in our laboratory had shown that in the greenhouse, poplar produces relatively low concentrations of PAs and other phenolics, but that these accumulate to much higher levels when plants are exposed to direct sunlight (Mellway et al., 2009). Therefore, we exposed plants to natural summer sunlight (up to 2080 µmol s⁻¹ m⁻²) outside the greenhouse in July 2013. Within one week, the effect of the increased light exposure was apparent, observed in the reddening of the young leaves of both transgenic and control lines. However, this light-induced accumulation of red anthocyanin pigment was much less marked in the transgenic lines than in the controls (Fig. 3a-e). Direct measurement of anthocyanins confirmed significant differences between transgenic and control leaf extracts (Fig. 3f). Likewise, the BuHCl assay revealed a significant decrease in PA concentration in leaves of the MYB182 overexpressors compared to control lines (Fig. 3g). The direct effect of MYB182 on the flavonoid pathway was confirmed by RT-qPCR analysis: while
*MYB182* is overexpressed in each transgenic line, the structural genes *DFR1*, *ANS1* and *ANR1* genes were repressed relative to controls (Fig. 3h). This downregulation is consistent with the observed decrease in both anthocyanin and PA levels, as DFR and ANS are common to both flavonoid branches.

HPLC analysis of methanolic extracts of *MYB182* overexpressor and control leaves was carried out to determine if *MYB182* expression altered the abundance of other phenolics in the transgenic plants. The HPLC method separates the major poplar phenolic constituents, including salicinoids, hydroxycinnamate esters, and flavonol glycosides (Supplemental Fig. S5). We could detect no significant differences between control and *MYB182* overexpressor extracts, suggesting that the other phenolic pathways were not significantly affected by MYB182.

*Transcriptome analysis of MYB182 overexpressor plants reveals additional downregulated genes and potential targets of MYB182*

To identify additional genes downregulated by MYB182, the GeneChip® Poplar Genome Array was used to compare leaf transcriptomes of three wild type and three *MYB182* overexpressor plants. In order to reduce environmentally-induced variability in expression levels, the array was probed with cDNA isolated from leaves of greenhouse-grown plants. We defined a fold-change threshold of \(<0.5\) and \(>2\), with a *P*-value \(<0.05\) and a *Q*-value of \(<0.1\). Using these criteria, 318 and 153 probes were classified as downregulated and upregulated, respectively. This supports the idea that MYB182 has an overall repressive effect. To validate the microarray data, RT-qPCR for *MYB182* and a set of downregulated genes was carried out for the *MYB182* overexpressors (Supplemental Fig. S6). A strong correlation of deregulated genes seen in both microarray and RT-qPCR confirmed the robustness of the array data.

Significantly downregulated genes were annotated by Blast2GO (Conesa *et al*., 2005) and categorized by predicted function. In total, 189 of the 318 downregulated gene probes were classified by gene ontology ID as being involved in 'metabolic process'; this is consistent with a role of MYB182 in secondary metabolism (Fig. 4b). All annotations for genes with flavonoid or phenylpropanoid functions were verified by manual BLAST searches of the GenBank database. A number of core flavonoid enzymes were significantly downregulated, including chalcone synthase (CHS2), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR2), and chalcone isomerase (CHI2) (Table 1). In poplar, F3H and CHI are both a single-copy genes, while DFR exists as two isoforms; these genes are all wound-stress induced and contribute to PA synthesis (Tsai *et al*., 2006). While we did not detect significant downregulation of all the isoforms for the flavonoid enzymes using our cut-offs, the analysis confirms that when
overexpressed, MYB182 has repressive effects on the flavonoid pathway.

In addition to the core flavonoid genes, two genes annotated as UGT-flavonoid glycosyltransferases were also downregulated, as well as two core phenylpropanoid genes: phenylalanine ammonia lyase 3 (PAL3) and 4-coumarate-CoA ligase 4 (4CL4). The downregulation of a suite of uncharacterized poplar genes annotated as flavonoid 3'-monooxygenase-like genes was intriguing; these are being investigated further but their connection to flavonoid metabolism and their specific biochemical functions are unknown. Interestingly, transcripts encoding five shikimate pathway genes were identified among the significantly downregulated genes. These include phospho-2-dehydro-3-deoxyheptonate aldolase/3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHPS), 5-enolpyruvylshikimate-3-phosphate synthase (ESPS), chorismate mutase (CM), and arogenate dehydratase (ADT)-like genes (Table 1). The shikimate pathway is well known, and is required by plants to provide aromatic amino acids for proteins as well as phenylpropanoids. Thus, it is possible that MYB182 not only affects PA and anthocyanin metabolism, but also has the potential to downregulate the shikimic acid pathway at the level of gene expression (Fig. 4b).

**MYB182 inhibits the transactivation of a PA promoter by positive MYB regulators**

To determine if MYB182 can directly repress PA synthesis, we performed promoter activation assays in transiently transformed poplar suspension cells using promoter-luciferase fusion constructs. We had previously shown that the poplar ANR1 promoter is activated by MYB134 when co-transformed with the poplar bHLH131 co-factor into a transient expression system (Zifkin et al., 2012; Gesell et al., 2014). To show negative regulation by MYB182, a positive regulator such as MYB134 was used in combination with the MYB182 repressor construct, and the reduction in activation measured. We first tested the activator and repressor constructs at different ratios, with up to a five-fold excess of activator. Even at this reduced ratio (repressor to activator of 1:5), MYB182 was able to repress activation of poplar ANR1 (Fig. 5a). Based on these results, for subsequent experiments a repressor to activator ratio of 1:4 was used. For comparison, we also tested constructs encoding the single repeat R3 MYB, MYB179, since this gene is also upregulated by MYB134 and is similar to CPC and MYBx, single repeat R3 MYB repressors that downregulate anthocyanin biosynthesis in Arabidopsis and petunia (Wester et al., 2009; Zhu et al., 2009; Albert et al, 2014). In our experiments, MYB179 only repressed ANR1 activation at a repressor:activator ratio of 1:1. When the relative amount of MYB179 was reduced, it was no longer active (Fig. 5a). This difference with MYB182 suggested potentially distinct mechanisms of action for MYB179 and MYB182.

The R2R3 repressor-like MYBs contain a bHLH binding region (Fig. 1) and are thus
predicted to interact directly with bHLH co-factors. To probe this interaction, we assayed repressor and activator constructs together with two poplar bHLHs belonging to different subclades, poplar bHLH131 which is part of the TT8 subclade (Gesell et al., 2014), and poplar bHLH079 which belongs to the JAF13/GL3/subclade (Supplemental Fig. S7). For these experiments, we also included poplar MYB179. In Arabidopsis, GL3 interacts with the PA or anthocyanin activator MYBs to form a MBW complex and activate the DFR promoter, while the single R3 repeat MYB CPC disrupts this interaction (Zimmermann et al., 2004; Wester et al., 2009). Our luciferase assays indicated that MYB182 was less effective as a repressor when co-bombarded with bHLH079 than with bHLH131 (Fig. 5b). For MYB179, however, we found the opposite pattern: greater repression with bHLH079 than with bHLH131 (Fig. 5c). This dependency on the type of bHLH cofactor suggested that the bHLH interaction is important for the mechanism of action of MYB repressors. We also tested MYB182 and MYB179 repressor activity with the second PA MYB activator, MYB115; the pattern of repression here was generally similar to that seen using MYB134 as the activator (Fig. 5d, e).

Since the transgenic poplar overexpressing MYB182 showed reduced anthocyanin accumulation (Fig. 3), we designed experiments to test whether MYB182 could be directly involved in the negative regulation of anthocyanin biosynthesis (Fig. 6). Based on the many characterized anthocyanin regulators of the PAP1-type (Fig. 1), we chose MYB117 as a likely ortholog for functional studies. In bombardment experiments carried out as previously, MYB117 activated the DFR promoter from Arabidopsis with both bHLH co-factors (Fig. 6). However, unlike the previous activation experiments using MYB134 or MYB115, MYB117 activation of DFR was repressed by MYB182 only when co-transformed with bHLH131. As before, MYB179 showed the opposite pattern, repressing MYB117 only if bHLH079 was used as a cofactor. The repression of anthocyanin biosynthesis was confirmed using transient transformation in agroinfiltrated Nicotiana benthamiana leaves. In this experiment, MYB117 induced visible anthocyanin accumulation when infiltrated with the bHLH131 cofactor (Fig. 6, c). Co-infiltration of MYB182 with the above activators, however, prevented anthocyanin synthesis and red leaf coloration.

Together, the transient expression experiments demonstrated that MYB182 repressed promoter activation driven by both anthocyanin and PA activator MYBs. With the PA regulators MYB134 and MYB115, the repressor activity of MYB182 was clear with either bHLH cofactor. With the MYB117 anthocyanin activator, however, repression by MYB182 was detected only in combination with bHLH131.
**MYB182 repressor activity requires interaction with the bHLH co-factor**

The alignment of MYB182 and other repressor-like MYBs had revealed the two common motifs found in all R2R3 repressor-like MYBs: the bHLH-binding motif in the R3 domain and a C2 region with a partial EAR motif on the C-terminal region (Fig. 1a; Fig. 7a). To probe the functions of these conserved regions, we created mutant versions of MYB182 for promoter activation and repression constructs, in which one or both of the motifs were altered. For the bHLH mutation, we altered the first and last L residues to I and T, respectively, whereas to generate a C2 mutant we replaced IDLNI with VDTNI (Fig. 7a). MYB182 without a functional bHLH binding site (mutant 1) lost its ability to repress MYB134 activation of the ANR promoter (Fig. 7b). In contrast, mutation of the C2 motif (mutant 2) did not result in a detectable reduction of repressor function. A construct with both motifs altered (double mutant) showed the same results as mutant 1. These results corroborate the idea that the primary mechanism of action for MYB182 involves an interaction with the bHLH co-factors. In addition, based on the presence of the TLLLFR motif in the C-terminal region, we created mutant constructs to test whether it this motif might be required for repressor activity of MYB182. Neither a complete deletion or a partial modification of this sequence prevented MYB182 from repressing promoter activation (Fig. 7c).
Discussion

The proanthocyanidin pathway in poplar responds to multiple developmental and environmental signals, reflecting the action of both positive and negative regulators. Here, we characterize a novel poplar repressor-like R2R3 MYB, MYB182, and demonstrate that it can downregulate PA as well as anthocyanin metabolism. MYB182 is the second MYB repressor of PA metabolism described to date. Our analysis shows that MYB182 acts on flavonoid gene promoters and may also affect other regulatory genes. Our data further indicate that MYB182 can repress activation by both anthocyanin- and PA-specific MYB activators, and that interaction with a bHLH cofactor is required for this repression. These conclusions are based on two lines of evidence. First, transgenic poplars and hairy root cultures that overexpress MYB182 have diminished PA concentrations, and the transgenic leaves also show lower levels of anthocyanins. The reduction in PAs in transgenic tissues correlates with reduced transcript levels of key flavonoid pathway genes. Second, transient expression and promoter activation assays directly demonstrate the repressive effects of MYB182 on relevant promoters activated with both PA and anthocyanin activator MYBs.

MYB182 is a repressor that reduces PA and anthocyanin accumulation and represses flavonoid gene expression when overexpressed in transgenic poplar

Our data from MYB182-overexpressing hairy roots clearly indicate that PA accumulation is decreased and transcripts of relevant flavonoid genes are reduced in abundance in transgenic hairy roots (Fig. 2). Effects on anthocyanin accumulation could not be studied in this system as hairy roots do not make these pigments. In parallel, our whole-plant transformations with MYB182 also showed reduced PA levels and decreased anthocyanin accumulation in leaves (Fig. 3). In contrast to plants overexpressing other repressor-like MYBs such as AmMYB308, AtMYB4 and PvMYB4 (Tamagnone et al., 1998; Jin et al., 2000; Shen et al., 2012), in MYB182-overexpressors no phenotypic abnormalities were detected.

The MYB182-overexpressing plants showed only mild effects of the transgene in the greenhouse-grown plants, but developed a clearer phenotype when they were moved into natural sunlight to induce flavonoid biosynthesis (Fig. 3a-e). While the light levels in such an experimental setup are difficult to control, the intensity of the light was not excessive, and we have previously used this treatment to reliably stimulate flavonoid metabolism (Mellway et al., 2009; Gesell et al., 2014). However, the transition from greenhouse conditions with no UV-B penetration and lower intensity visible light to natural sunlight likely constitutes a mild stress treatment (see Materials and Methods). Nevertheless, the reduction of PA metabolites and gene
expression in transgenic plants was less marked than that in the hairy root culture system. This was perhaps due to a difference between the relative expression of the PA pathway in leaves and roots. Whereas leaves accumulate PAs together with large amounts of other phenolics, including salicinoids, flavonols, and hydroxycinnamic acid esters (Constabel and Lindroth, 2009), poplar roots have a much simpler phenolic profile that is dominated by the PAs (C. P. Constabel, unpublished data). Hairy roots are thus more sensitive to manipulations of the PA pathway, which will make them an ideal system for testing PA repressor activity. In grapevine, hairy root transformation has been used to validate the function of the VvMYBC2-L1 gene (Huang et al., 2014).

The microarray analysis and RT-qPCR of the transgenic plants demonstrated the repressor function of MYB182, showing downregulation of genes encoding flavonoid enzymes (Fig. 3, Table 1), and is similar to the RT-qPCR analysis of hairy root flavonoid gene expression. An effect of MYB182 on PA, anthocyanin, and core flavonoid genes is consistent with several features of the MYB182 protein sequence, which contains amino acid motifs shared by other flavonoid regulators. A recent study by Heppel et al. (2013) identified two regions of conserved amino acid residues in the R2 and R3 domain of activator MYB transcription factors and proposed that these residues are important for the specific regulation of the PA and anthocyanin pathway. The MYB182 sequence contains a Gly at position 49 in the R2 domain, a conserved feature in all PA regulators and in the other R2R3 MYB repressors (Fig. 1a). At positions 90-93 of the C-terminal end of the R3 repeat, a short stretch of amino acid residues was observed by Heppel et al. (2013) to be different in the PA MYBs (Asp–Asn–Glu–Ile) from the anthocyanin MYBs (Ala–Asn–Asp–Val). In MYB182, this region has residues characteristic of both the PA and anthocyanin regulators (Asp–Asn–Glu–Val), which could reflect MYB182’s capacity to repress both pathways. However, other regions must also be important, since the (Asp–Asn–Glu–Ile) motif is found in some subgroup 4 lignin and phenylpropanoid repressor MYBs as well.

In addition to downregulation of core flavonoid genes, in the MYB182 overexpressors we also noted the reduced transcript abundance of other genes with annotations suggesting peripheral roles in flavonoid synthesis. For example, two UDP-glucose dependent glucosyltransferases (UGTs) are repressed by MYB182, including UGT78M1, an enzyme that we had found was induced in Melampsora medusae-infected poplar leaves (Miranda et al., 2007). However, the recombinant UGT78M1 protein was not active with any anthocyanin, flavonol, or flavan-3-ol substrate that we tested, so its biochemical function remains undefined (Veljanovski and Constabel, 2013). The functions of the other UGT are also unknown; however, it is expressed in herbivore-stressed poplars (Ralph et al., 2006). A conspicuous group of downregulated genes is annotated as encoding flavonoid 3’- monooxygenase-like enzymes, (i.e., flavonoid
3'-hydroxylase-like), which belong to the cytochrome P450 gene family. A phylogenetic analysis of the most related P450s indicated that the genes identified in our experiment are not highly similar to any characterized genes (Supplemental Fig. S8). They are closest to GhDDWF1 and NtCYP71D20 (approximately 69-79% protein identity), which are involved in the biosynthesis of brassinosteroids and capsidiol, respectively (Kang et al., 2001; Ralston et al., 2001). Until other genes in this subgroup are functionally characterized, the significance of the downregulation of the poplar flavonoid 3'-monooxygenase-like genes remains unclear.

It must be stressed that the genes affected by MYB182 in overexpression experiments remain tentative targets of this repressor, as overexpression experiments in themselves are not sufficient to unequivocally demonstrate function. Analysis of transgenic and wildtype plants under stress conditions may also help illuminate the role of MYB182 in poplar adaptation. Ultimate proof of the function of MYB182 and validation of its targets will require the creation of MYB182 knock-out plants.

**A bHLH-interacting site in the R3 domain of MYB182 is critical for repression**

Analysis of amino acid sequences revealed that the MYBs in the PA/anthocyanin clade of subgroup 4 repressors harbor neither the zinc-finger nor the C4 motif, but do contain the conserved C1 and C2 motifs as well as the bHLH binding region (Fig. 1). Previous work had demonstrated direct protein-protein interactions of bHLH proteins with MYB repressors, including FaMYB1, AtMYB4 and PhMYB27 (Aharoni et al., 2001; Zimmermann et al., 2004; Albert et al., 2014). *In vitro* mutagenesis of our MYB182 constructs allowed us to test the functions of the putative motifs directly. Disruption of the bHLH-binding site clearly abolished the repressor activity of MYB182 (Fig. 7), indicating that its activity depends on interaction with bHLH factors. This is consistent with our observation that the pattern of repression is altered depending on which type of bHLH factor is used as a cofactor. MYB182 showed repression with either bHLH079 (GL3-type) or bHLH131 (TT8-like) cofactor using the PA activators, although to varying degrees. When activation was driven by the PAP1-like anthocyanin regulator MYB117, however, MYB182 could repress promoter activation only if the bHLH079 was co-expressed (Fig. 6). These data corroborate the importance of the bHLH co-factor for repressor function, so that MYB182 could be acting in part by competing for bHLH with the activator MYBs. Furthermore, it may also act as part of a MBW complex, as suggested by experiments on PhMYB27 in petunia (Albert et al. (2014). These authors suggest that in this case specificity may depend on the activator, and that the R2R3 repressor MYBs switches the complex from activation to repression. In any event, the strength of the MYB repressor- bHLH interaction is likely to be a critical element. The correlation between the strength of protein-protein interactions and repressor
function has not yet been examined for the R2R3 subgroup 4 MYBs. For the R3 single repeat MYBs including Arabidopsis CPC, gentian MYBR1, and petunia MYBx, protein binding affinities do differ among these R3-MYB factors and this can affect their function (Kirik et al., 2004; Wester et al., 2009; Nakatsuka et al., 2013). Quantitative analysis of protein-protein interactions may reveal the interaction and affinity of MYB182, MYB179 and MYB117 with bHLH131 and bHLH079.

A key question that still needs to be answered directly is if MYB182 itself can bind to flavonoid promoters and thus directly repress flavonoid gene expression. While we could not test this possibility, mutating the R3 domain of Arabidopsis AtMYB4 impeded its ability to bind DNA and abolished its ability to repress transcription. This would suggest that DNA binding is also essential for its repressor function (Jin et al., 2000). Other subgroup 4 MYB factors are reported to directly bind target gene promoters (Jin et al., 2000; Fornalé et al., 2010; Shen et al., 2012; Bomal et al., 2014). We cannot rule out that mutation of the MYB182 bHLH binding site might also have affected its DNA binding ability, since Baudry et al. (2004) had shown that the Arabidopsis MYB TT2 can only bind to its target gene promoter in yeast in the presence of a bHLH or WDR factor. Currently our data does not allow us to distinguish this possibility from a mechanism based on competition for bHLH co-factors, although we note that these are not mutually exclusive mechanisms. Protein-protein interaction experiments and yeast one-hybrid assays should be able to distinguish between these mechanisms.

Surprisingly, mutation of the C2 motif in MYB182 did not diminish its repressor activity, despite the documented role of this motif in MYB subgroup 4 lignin and phenylpropanoid repressors and the anthocyanin repressor PhMYB27. It is possible that the differences in the motif in MYB182, though conservative (INIDL vs LNLDL), reduce the motif's functionality. However, even the previous studies showed that repressor activity could not be completely eliminated by disruption or removal of the C2 motif (Jin et al., 2000; Shen et al., 2012; Albert et al., 2014). This suggests that additional mechanisms are important. Nevertheless, the C2 region with the core EAR motif is considered to be the predominant transcriptional repression motif in plants (Ohta et al., 2001; Hiratsu et al., 2003; Tiwari et al., 2004). In tobacco ERF3 (NtERF3) where the EAR motif was first characterized, deletion mutations within the EAR motif eliminated its ability to repress transcription (Ohta et al., 2001). Although the mechanism of repression is still unclear, some reports show that the EAR motif is important for interaction with histone modification proteins (Kagale and Rozwadowski, 2011). In Arabidopsis, the IAA12/BDL auxin transcriptional repressor, and NINJA (Novel Interactor of JAZ), which functions in transcriptional repression of JA signaling, have been shown to interact with the TPL (TOPLESS) co-repressor (Szemenyei et al., 2008; Pauwels et al., 2010). The genetic interaction between TPL or TPL-related 1 (TPR1)
and histone deacetylase AtHDA19 supports a model for EAR motif-mediated repression via the recruitment and action of chromatin modifiers (Kagale and Rozwadowski, 2011). Such a model could explain why deletion of the C2 motif did not impact MYB182 in our transient expression assays, since the target promoter is transferred into the cell as a plasmid and is not present as chromatin. An alternate explanation would be that the effects of C2 motif deletion were obscured by the action of the bHLH binding site, since binding to a bHLH co-factor seems to be sufficient to repress transcriptional activation (Fig. 7, mut.1). This could explain why Ohta et al. (2001) saw effects of the EAR motifs in transient assays using plasmids while we did not, since the repressor used by these authors does not require such co-factors. Disruption of the TLLLFR motif likewise did not inhibit repressor activity of MYB182, unlike what was reported for AtMYBL2 (Matsui et al., 2008). The discrepancy could be due to different assay systems, as Matsui et al. (2008) used the yeast Gal4 binding domain, or the dominant effect of the bHLH interaction. In order to better understand the mechanism(s) by which MYB182 acts, as well as the potential role of the different motifs within MYB182, the ratios of activator, co-factor, and mutant repressor will likely need to be examined carefully. However, overall our experimental system indicates a greater importance of the bHLH-binding site of MYB182 relative to the conserved C2 motif, at least against the MYB134/bHLH131 activator complex.

**MYB182 is part of a complex network of positive and negative regulators for flavonoid metabolism in poplar**

MYB182 was first identified due to its enhanced expression in transgenic plants that overexpress *MYB134*, and was hypothesized to help modulate PA induction. Our data have corroborated this idea, and core flavonoid genes are indeed targets of the MYB182 repressor. However, our work has uncovered additional connections to other parts of the flavonoid and PA regulatory network in poplar. Poplar hairy roots overexpressing MYB182 showed reduced expression of MYB115 and bHLH131, both positive regulators which stimulate PA synthesis (Fig. 2; Gesell et al., 2014). An inhibitory effect of MYB182 on these and other positive transcriptional regulators still needs to be confirmed, but would provide another level of control with additional feedback loops for downregulating the PA pathway. Poplar MYB165 and MYB194 also need to be studied in more detail. Their roles are unknown to date, but could provide additional features that will contribute to the development of a regulatory network model for stress-induced PA regulation in poplar.

The recent characterization of the anthocyanin repressor PhMYB27 in petunia provides a model for such multi-level regulation: this protein acts to repress thebHLH co-factor AN1, MYBx, and its own expression, in addition to downregulating the structural genes (Albert et al., 2014).
Such negative regulation via repression of the MYB PA activators was also suggested by work on the regulation of grapevine PA biosynthesis (Terrier et al., 2009; Huang et al., 2014), where expression levels of positive regulators VvMYBPA1 and VvMYBPA2 in VvMYBC2-L1-overexpressing hairy roots were reduced. Work in these distinct experimental systems all suggest there are common regulatory mechanisms among subgroup 4 R2R3 MYB factors regulating late flavonoid genes in higher plants.

Overall, our data suggest that MYB182 can inhibit flavonoid biosynthesis at three levels. First, it acts to repress transcription of genes encoding flavonoid enzymes that are required for PA synthesis. Second, it counteracts the effects of the positive regulators MYB134 and MYB115 by repressing the expression of components of the PA-activating MBW complexes themselves. In their work on petunia PhMYB27 and anthocyanin regulation, Albert et al. (2014) refer to this two-level repression as a “double lock-down” mechanism: direct downregulation of flavonoid structural genes, and the parallel downregulation of positive flavonoid regulators. Third, based on our microarray analysis of MYB182 overexpressor plants, we speculate that the availability of shikimate phenylpropanoid precursors may also be reduced, although this will need to be validated directly.

Conclusions

Our work has used both transient and stable transformation of poplar to illuminate the role of MYB182 as a repressor of the flavonoid pathway leading to anthocyanins and PAs. Its repressor activity in this pathway is consistent with its position in the phylogeny of subgroup 4 MYB factors, and helps to define a subclade of flavonoid R2R3 MYB repressor proteins. While we could not demonstrate a role for the C2 motif or TLLLFR motif of MYB182, its bHLH-binding regions is clearly essential for repression. The mechanism by which MYB182 downregulates flavonoid genes appears to be similar to recently characterized anthocyanin repressors in petunia. MYB 182 is part of a complex network of positive and negative transcriptional regulators for PAs that now includes several activator MYBs and bHLHs as well as three additional MYB repressors. These remain to be characterized in future work.
MATERIALS AND METHODS

Plant growth conditions and stress treatments

_Populus tremula x P. tremuloides_ (clone INRA 353-38) and _P. tremula x P. alba_ (clone INRA 717-1-B4) were micropropagated _in vitro_ on solid Lloyd & McCown's Woody Plant Medium (WPM; Caisson Laboratories, North Logan, UT) supplemented with 0.5 µg/mL indole butyric acid (IBA) in growth chambers under long-day condition (16 h light/8 h dark, 25°C). These plants were the source of material for stable transformation experiments. For greenhouse experiments, rooted _in vitro_ plantlets were transplanted to soil and acclimated in a mist chamber for 3 weeks prior to being moved into the greenhouse.

For natural sunlight exposure, 2-month old potted poplar plants were moved from the greenhouse (mean maximum PAR, 392 µmol s⁻¹ m⁻²; UV-B irradiance, 0.003 mW cm⁻²; temperature 17 - 28°C) to full natural sunlight during July in Victoria, British Columbia, Canada (mean maximum PAR, 2080 µmol s⁻¹ m⁻²; UV-B irradiance, 0.2 mW cm⁻²; temperature 12 - 32°C). For wounding experiments, leaves of greenhouse-grown, 3-month old wild-type 353-38 plants (Leaf Plastochron Index, LPI 10-12) were crushed along their margins with pliers. Leaves were rewounded 1 h following initial wounding and harvested at 24 h.

Suspension cell cultures of _Populus trichocarpa x P. deltoides_ (H11-11) were maintained in 40 mL of Murashige-Skoog liquid medium (MS, Sigma) supplemented with 3% sucrose and maintained in 250 mL flasks on a rotary shaker (130 rpm) in the dark at 22°C. Seven mL of suspension culture was sub-cultured into 40 mL of fresh MS medium every 7 d, and four individual 7-d-old cultures were used for each bombardment.

_Nicotiana benthamiana_ plants for agroinfiltration were grown in a growth chamber under long-day conditions (16 h light/8 h dark, at 22°C) and used when four weeks old.

Phylogenetic and sequence analysis

Full-length amino acid sequences were retrieved from public databases. For phylogenetic tree construction of R2R3 MYB proteins, multiple sequence alignments were performed using Dialign (Al Ait _et al._, 2013; http://dialign.gobics.de). Only sequence regions with at least 10% diagonal similarity were retained, which restricted the alignment to the conserved DNA-binding N-terminal region. After first using ProtTest (Abascal _et al._, 2005; http://darwin.uvigo.es/software/protttest2_server.html) which determined that the most suitable model for Maximum Likelihood analysis was JTT+I+G, we generated the phylogenetic tree using a local PhyML (Guindon _et al._, 2013) via JTT model with manual modification of gamma distribution and proportion of invariable sites. Bootstrapping was carried out with 1000 replicates.
using the same evolution model for the original tree using local PhyML. Trees were displayed using Figtree (http://tree.bio.ed.ac.uk/software/figtree/) and rooted at the mid-point. For phylogenetic tree construction of flavonoid 3’-monooxygenase-like and bHLH genes, multiple alignments of full-length protein sequences were performed with the ClustalW algorithm implemented in MEGA package version 5.2 (Tamura et al., 2011) with default parameters. The phylogenetic tree was constructed using the means of maximum likelihood method with bootstrapping (500 replicates).

Cloning of poplar MYB factors and plasmid construction
Total RNA was isolated from young leaves of *P. tremula x P. tremuloides* INRA 353-38 plants using the method outlined below. Reverse transcription was carried out using SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. The coding regions of *MYB117, MYB179, MYB182* and *bHLH079* were amplified with Phusion High Fidelity DNA Polymerase (New England Biolabs) using the gene specific primers designed based on the coding sequences for *P. trichocarpa* in the Phytozome database (http://www.phytozome.net/) (Table S1). Each PCR product was digested with SpeI and KpnI, then ligated behind the double 35S promoter of the pMDC32 overexpression vector (Curtis and Grossniklaus, 2003). For hairy root constructs, the region containing a double 35S promoter, the *MYB182* coding sequence, and the nopaline synthase terminator were amplified with Phusion High Fidelity DNA Polymerase from pMDC32-PtMYB182 with the primers containing the attB site for Gateway cloning (Table S1). This PCR product was transferred into the Gateway binary vector pKGWD,0 (Karimi et al., 2002) through LR recombination (LR clonase II; Invitrogen) to yield the *MYB182* overexpressor construct with a GFP marker. Mutations for pMDC32-MYB182 mutant plasmids were performed by site-directed mutagenesis via overlap extension using PCR with mutated primers (Table S1). Activator and cofactor plasmids (pMDC32-bHLH131, pMDC32-MYB134, pMDC32-MYB115), and reporter plasmids pGREEN800LUC-ANR1 promoter constructs are described previously (Zifkin et al., 2012; Gesell et al., 2014;). The Arabidopsis *DFR* promoter (1.0 kb upstream region) was amplified from *Arabidopsis thaliana* (ecotype Columbia) leaf genomic DNA extracted using the DNeasy Plant Mini Kit (Qiagen). The PCR products were digested with SpeI and KpnI, and then ligated upstream of the firefly luciferase gene in the same reporter vector.

Generation of transgenic poplar hairy roots and whole plants
For hairy root generation, leaves of *in vitro* grown *P. tremula x P. alba* clone INRA 717-1-B4 were excised and placed on pre-culture solid medium [0.25 g MES, 0.1 g myo-inositol, 30 g sucrose, 4.33 g MS (Caisson) salts in 1L, pH5.7] for 16 h. These leaves were wounded by cutting the
leaf-veins gently before transformation with *Agrobacterium*. pKGWD,0-MYB182 and pKGWD,0 (empty vector) were moved into *Agrobacterium rhizogenes* strain ARqua1 by electroporation. Transformed colonies were grown on MG/L-agar medium at 28°C with spectinomycin and biotin for vector selection. Liquid cultures were grown in MG/L medium for 16h before spinning down. The bacterial pellet was resuspended in induction broth [0.25 g MES, 0.1 g myo-inositol, 30 g sucrose, 4.33 g MS (Caisson) salts in 1 L] with 200 µM acetosyringone to an OD of 0.6-0.8 and used to inoculate excised leaves. Hairy roots arising from transformed leaves were excised and maintained in the dark at 25°C in petri dishes on solid antibiotic-containing medium (preculture medium with carbenicillin, cefotaxime, and timentin). After hairy roots appeared on the leaves, infected leaves were moved to solid WPM supplemented with 0.25 mg/L indole-3-butyric acid and 0.25 mg/L 1-naphthaleneacetic acid. Individual hairy root clones were subcultured every 21 d. Screening for positive transformants was performed by GFP detection under UV light using an Olympus SZX7 Zoom Stereomicroscope. Hairy roots were harvested and flash-frozen in liquid nitrogen for nucleic acid extraction and PA analysis.

For generating transgenic plants, the binary vector pMDC32-MYB182 was transferred to the *Agrobacterium tumefaciens* strain GV3101 ::pMP90. *P. tremula x tremuloides* clone 353-38 leaf explants were transformed as described previously (Mellway *et al*., 2009). Positive independently transformed lines were identified by selection of plantlets on hygromycin-containing shooting and rooting medium, and subsequently confirmed by PCR. Transgenics were maintained and propagated on solid WPM medium as described above prior to acclimation to the greenhouse for further analysis.

**RNA extraction and real-time quantitative PCR**

Total RNA was extracted from frozen poplar hairy roots or ground leaf powder using the modified cetyl trimethyl ammonium bromide (CTAB) method for polyphenol-rich plant tissue described by Muoki *et al.* (2012). The RNA preparations were checked with agarose electrophoresis and analysis on a Nanodrop 2000 (ThermoScientific, Wilmington, NC, USA). Prior to qPCR analysis, total RNA (2.5 µg) was treated with amplification grade DNase I (Invitrogen) according to the manufacturer’s instructions. DNase I-treated RNA (250 ng) was used for reverse transcription by SuperScript II reverse transcriptase (Invitrogen). For real-time qPCR, two technical replicates were analyzed using a Stratagene Mx4000 thermal cycler. Each qPCR reaction (15 µl total volume) consisted of the QuantiTect SYBRGreen mix (Qiagen) with 0.67 µM gene-specific primers and 2 µl of cDNA template diluted into 1:20 from original reverse transcription. The amplification protocol was 95°C for 3 min followed by 40 cycles of 94°C for 30 s, 58-64°C for 30 s, and 72°C for 30 s. Annealing temperatures for each primer were determined based on tests to
determine their efficiency (see Table S1). Gene expression levels were quantified by normalization to the mean of elongation factor 1 beta (EF1b, alpha subunit) and ubiquitin/ribosomal protein 27a expression (GenBank Accession XM_002299613 and XM_002320914.2) and calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

**Microarray analysis**

DNaseI-treated RNA from wild type and MYB182 overexpressor transgenic plants (leaves 10-12) was further purified with the NucleoSpin RNA II clean-up kit (Clontech, Mountain View, CA, USA). These RNA preparations were used for Affymetrix GeneChip® Poplar Genome Array microarray hybridizations conducted at the Genome Quebec Innovation Centre at McGill University (Montreal, QC, Canada). Data was normalized with FlexArray using a GC Robust Multi-array Average normalization and an Empirical Bayes (Rocke) significance test. Genes were considered differentially expressed in the transgenics if they showed a fold change of $>2$ or $<0.5$ with a p-value $< 0.05$ and q-value $<0.1$. Q-value was calculated using the R package QVALUE (Storey, 2002). Annotations of differentially expressed genes were performed with Blast2Go (Conesa et al., 2005, http://blast2go.com/b2ghome) and Phytozome (http://www.phytozome.net/), and were manually checked via BLAST database searches (NCBI) and literature searches. The categorization of gene ontology was performed with Blast2Go.

**Transient expression and dual luciferase promoter activation assays**

For each bombardment assay, aliquots of poplar suspension cells (*P. trichocarpa* x *P. deltoides* H11-11) were collected from four independently grown cultures and placed on four individual filter papers (7 cm diameter) divided into quarters. The filter papers plus cells were placed onto solid MS medium with 0.5 M mannitol in petri dishes and incubated for 1 h prior to bombardment. Aliquots (25 μL) of 0.7 μm diameter tungsten beads (Bio-Rad, 60 mg/ml) were coated with 250 ng of each plasmid (reporter, activator, co-factor, and reporter vectors) under constant vortexing, followed by the addition of 25 μL of 2.5 M CaCl$_2$ and 10 μL of 0.1 M spermidine. Samples were vortexed for an additional 10 minutes at 4°C. The beads were washed first with 200 μL of 70 % ethanol, then with 200 μL of 100 % ethanol, and resuspended in 20 μL of 100% ethanol. The beads were pipetted onto flying disks (Bio-Rad) and allowed to air dry. The bombardments were conducted with a PDS-1000/He Biolistic Particle Delivery System (Bio-Rad) under 900 p.s.i. Filter papers with cells were placed approximately 13 cm from the rupture disc. After bombardment, the cells were incubated in darkness for 48 h at RT prior to the luciferase assay.

Promoter activation was assayed using the Dual-Luciferase® Reporter (DLR™) Assay System (Promega). The cells were scraped from the filter paper, homogenized with a micro-pestle
and mixed by vortexing in 150 μL of Passive Lysis Buffer (Promega DLR™ Assay System). The extract was incubated on ice for 10 min, then centrifuged for 10 min at 13000 rpm. Aliquots (10 μL) were assayed for firefly and Renilla luciferase luminescence, as described by the manufacturer (Promega).

**Nicotiana benthamiana agroinfiltration**
The overexpression vectors pMDC32-bHLH131, pMDC32-MYB117, and pMDC32-MYB182 were transformed into Agrobacterium tumefaciens strain GV3101:MP90. A. tumefaciens cultures harboring these plasmids were grown in liquid LB at 28°C for 16 h. Bacteria were pelleted and resuspended in 10 mM MgCl₂ solution to an OD₆₀₀ of 0.7. Equal amounts of *Agrobacterium* suspensions harboring each construct were infiltrated into the abaxial surface of the oldest two leaves of 4-week old *Nicotiana benthamiana* plants as described by Espley *et al.* (2007). Eight days after infiltration, photographs of infiltrated leaves were taken, and leaf samples were harvested, freeze dried, and analyzed for anthocyanins as described above.

**Phytochemical extraction and assays**
For PA analysis of hairy roots, 50 mg fresh tissue was ground in liquid nitrogen and extracted for 16 h at room temperature in 5 mL of 100% methanol. Extracts were centrifuged to remove solid debris, and an aliquot (0.5 mL) assayed for PAs using the acid butanol assay as described (Porter *et al.*, 1985) using purified *P. tremuloides* PAs as a standard. For analysis of other samples, 25 mg of ground freeze-dried powder was extracted into 1.5 mL of 100% methanol and sonicated for 10 min. Extracts were centrifuged, and extracted two more times with 1.5 mL 100% methanol and sonication. Following a final centrifugation step, the three extracts were pooled and assayed as above. To measure anthocyanins, approximately 50 mg of ground, freeze-dried leaf powder was extracted into 500 μL of 1% (v/v) HCl in methanol overnight at room temperature with shaking. One volume ddH₂O was added to 500 μL extract and mixed. The sample was extracted with 1 mL of chloroform and centrifuged at 13,000 rpm for 5 min (Nemie-Feyissa *et al.*, 2014). Anthocyanins were measured in the aqueous upper phase at 530 nm (modified from Martin *et al.*, 2002).

**Acknowledgements**
We thank Mireille Chabaud and David Barker (INRA) for *Agrobacterium rhizogenes* strain ARqua1, Roger P. Hellens for providing pGREENII0800 plasmid, and Dr. Andreas Gesell for vector constructs for the transient assay. Brad Binges is acknowledged for help with plant care, Tieling Zhang for technical assistance, and Dr. Jürgen Ehlting for help with phylogenetic analysis.
We thank Amy Franklin and Cuong Le for help with microarray data analysis, Dr. John Taylor for access to the stereomicroscope, and the Centre for Forest Biology for growth facilities.
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Figure Legends

**Figure 1.** Sequence analysis of repressor-like R2R3 MYBs relevant to poplar PA regulation. (a) ClustalW alignment of the amino acid sequences of poplar MYB165, MYB181, MYB182, MYB194 and other R2R3-MYB subgroup 4 proteins. The R2 and R3 MYB domains are indicated by black bars. The boxed sequences are the potential functional motifs. White letters on a black background represent residues that are identical in the sequences aligned. White letters on a gray background indicate conservative changes. Boxes indicate conserved elements in the primary sequence: C1 motif, LLsrGIDX(T/S)HRX(L/I); C2 motif, pdLNL(D/E)LXi(G/S); C4 motif, GYDFLG(L/M)X4–7LX(Y/F)(R/S)XLEMK; Zinc finger (Zf) motif, CX1–2CX7–12CX1–2C; and TLLLFR motif. The bHLH binding regions is also marked, with key residues indicated by asterisks. (b) Phylogenetic tree of poplar MYB165, MYB181, MYB182, MYB194 and related functionally characterized R2R3 MYB transcription factors from other plants, constructed from the N-terminal DNA binding domains using the Maximum Likelihood method with all bootstrap values over 500 shown (1000 replicates). Stars indicate the poplar repressor-like MYBs discussed here, the arrow marks MYB182. Clades are indicated in color as follows: in light blue, flavonoid repressors; purple, lignin/phenylpropanoid repressors; green, flavonol activators; light yellow, TT2-type PA activators; dark yellow, MYBPA1-type PA activators; pink, anthocyanidin activators. Genbank accession numbers for all protein sequences are listed in Supplemental Files.

**Figure 2.** Ectopic over-expression of MYB182 in poplar hairy roots. (a) Plasmid construct used for hairy root transformation of poplar. The green fluorescent protein (GFP) marker gene is driven by a rolD promoter and MYB182 by the double cauliflower mosaic virus 35S promoter (shown as 2x35S). The images show GFP expression in hairy roots indicating the presence of the construct. (b) Expression of MYB182 in transgenic hairy root and control lines (c) Proanthocyanidin (PA) content in independent MYB182 over-expression and empty vector control hairy root lines. (d) Relative expression of PA-related genes assayed by reverse transcription quantitative polymerase chain reaction (RT-qPCR). bHLH131 and bHLH079 are poplar members of the TT8/AN1 or GL3/DEL/3AF13 subgroups, respectively (Supplementary Fig. S7). MYB115 is an R2R3 MYB positive PA regulator. Relative gene expression is normalized to the mean of elongation factor 1B and ubiquitin/ribosomal protein 27a expression. All data points are means of three biological replicates for each of three control and three transgenic lines, with error bars indicating standard deviation. Labelled columns not connected by the same letter are significantly different at P < 0.05, based on a Tukey-Kramer Honestly Significant Difference (HSD) test.
**Figure 3.** Effect of *MYB182* overexpression in transgenic poplars grown under natural sunlight. (a) Visual comparison of wild type control and *MYB182* overexpressing (OE) poplar plants after 10 weeks of growth. (b-e) Close-up images of young leaves of *MYB182* OE (b, d) and control (c, e) plants. Panels b and c show top view of plant with youngest leaves of *MYB182* OE and control plants, panels d and e show leaves at LPI 5. (f, g) Anthocyanin and PA content of *MYB182* OE lines and wild type control lines. (h) Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of *MYB182* and *DFR1, ANS1*, and *ANR1* genes in wild-type and three independent *MYB182* OE lines. Relative gene expression is normalized to the mean of elongation factor 1B and ubiquitin/ribosomal protein 27a expression. Data points shown are means from three biological replicates (individual plants) with error bars indicating standard deviations. Asterisks on top of the bars indicate values determined by Student’s t-test to be significantly different from control (P < 0.05).

**Figure 4.** Analysis of target genes based on microarray profiles from *MYB182* overexpressing poplar plants. (a) Pie chart of gene ontology categories corresponding to the 381 downregulated probes classified as “biological process”. Note that any one probe can belong to more than one gene ontology category. (b) Summary of the shikimate, phenylpropanoid and flavonoid pathways leading to PA and anthocyanin synthesis. Abbreviated enzyme names are indicated for each biosynthetic step. Bold-type and underlined names represent genes significantly downregulated in *MYB182* overexpressing plants. DAHPS, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase; DHQS, dehydroquinate synthase, DHQD, dehydroquinate dehydratase, SD, shikimate dehydrogenase, SK, shikimate kinase, EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; CM, chorismate mutase; ADT, arogenate dehydratase; PAL, phenylalanine ammonia lyase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3’H, flavonoid 3’-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; ANR, anthocyanidin reductase; LAR, leucoanthocyanidin reductase; UGT, UDP glycosyl transferase.

**Figure 5.** Transient expression assays to test activation and repression of the poplar *ANR1* promoter by poplar MYB and bHLH regulators in suspension cultured poplar cells. MYB activators and repressor plasmids were tested in a ratio of 4:1 (250: 62.5 ng DNA, which gives
similar ratio of molecular weight) unless otherwise indicated. (a) Transcriptional activation by MYB134 (R2R3 activator MYB) and poplar bHLH131 at different activator to repressor ratios. (b, c) Effects of MYB182 (R2R3-MYB repressor) and MYB179 (R3-MYB) on the activation of the ANR1 promoter by MYB134 in the presence of bHLH131 (TT8/DEL/AN1 homolog) or poplar bHLH079 (GL3/JAF13 homolog). (d, e) Transcriptional activation by MYB115 (R2R3 activator MYB) with bHLH131 or bHLH079, and repression by MYB182 and MYB179. All data points are means of three biological replicates, with error bars indicating standard deviations. Asterisks indicate values determined by Student’s t-test to be significantly different from control (P < 0.05).

Figure 6. Functional analysis and repression of the poplar anthocyanin regulator MYB117 in transiently transformed poplar suspension cells and agroinfiltrated Nicotiana benthamiana leaves. (a, b) Activation of the Arabidopsis DFR promoter by anthocyanin regulators and effects of MYB repressors in poplar cell culture. Assays of MYB117 were carried out with repressors MYB182 or MYB179, and using either bHLH131 (a) or bHLH079 (b) as a cofactor. (c) Anthocyanin content and corresponding image of red leaf coloration at 8 d following agroinfiltration of leaves the presence and absence of MYB182 and MYB117. The ratio of MYB activators to repressors was 4:1. All data points are means of three independent replicates, with error bars indicating standard deviations. Asterisks on top of the bars indicate values determined by Student’s t-test to be significantly different from the control (P < 0.05).

Figure 7. Functional analysis of conserved motifs in MYB182. (a) Structure of MYB182 protein showing location of conserved bHLH-binding domain and C2 repressor motif. The design strategy for the mutation of amino acids in the conserved motifs is shown below each site in question. (b) Location of mutations within MYB182 mutant constructs used in the promoter activation/repression assay, and corresponding results showing activation of the poplar ANR1 promoter by MYB134 with bHLH131 and repression by MYB182. Mut.1 and mut.2 refer to mutants 1 and mutant 2 shown above. (c) Schematic and results of TLLLFR mutant tests, where the motif was either mutated or deleted. Data points are means of three biological replicates, with error bars indicating standard deviations. Asterisks on top of the bars indicate values that were determined by Student’s t-test to be significantly different from the control (P < 0.05).
SUPPLEMENTAL FILES:

Supplemental Figure S1. Distance tree representation of the phylogenic analysis shown in Figure 1. The tree highlights poplar MYB165, MYB181, MYB182, MYB194 and key functionally characterized R2R3 MYB transcription factors from other subclades.

Supplemental Figure S2. Tissue-specific pattern of gene expression of positive regulator MYB134, the PA-specific enzyme ANR1, and newly identified MYB-repressor like genes (MYB165, MYB179, MYB182, MYB194).

Supplemental Figure S3. Upregulation of MYB182 transcripts by MYB134 overexpression and stress treatment.

Supplemental Figure S4. Six-week old plants and leaves of wild type and Populus tremula x P. tremuloides overexpressing MYB182.

Supplemental Figure S5. Sample HPLC chromatogram of phenolic profiles comparing wild type and MYB182 overexpressor plants exposed to natural sunlight.

Supplemental Figure S6. Correlation of gene expression between microarray and qPCR data.

Supplemental Figure S7. Phylogenetic analysis of bHLH factors related to poplar bHLH079 and poplar bHLH131.

Supplemental Figure S8. Phylogenetic analysis of flavonoid 3’-monooxygenase-like genes downregulated by MYB182 together with similar and/or functionally characterized cytochrome P450 proteins from diverse species.

Supplemental Table S1. List of Primers used for experiments

Supplemental Table S2. List of accession numbers for phylogenies in Fig. 1, Fig. S7 and Fig. S8.
### Table 1. Lists of selected genes that were down-regulated in MYB182 overexpression plants

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Description is based on Blast2GO annotations (Conesa et al., 2005) unless noted:

<sup>a</sup>described in Veljanovski and Constabel, 2013
<sup>b</sup>described in Tsai et al., 2006
<sup>c</sup>described in Hamberger et al., 2006
**Figure 1.** Sequence analysis of repressor-like R2R3 MYBs relevant to poplar PA regulation. (a) ClustalW alignment of the amino acid sequences of poplar MYB165, MYB181, MYB182, MYB194 and other R2R3-MYB subgroup 4 proteins. The R2 and R3 MYB domains are indicated by black bars. The boxed sequences are the potential functional motifs. White letters on a black background represent residues that are identical in the sequences aligned. White letters on a gray background indicate conservative changes. Boxes indicate conserved elements in the primary sequence: C1 motif, LLsrGIDPX(T/S)HRX(I/L); C2 motif, pdLNL(D/E)LXi(G/S); C4 motif, GYDFLG(L/M)X4–7LX(Y/F)(R/S)XLEMK; Zinc finger (Zf) motif, CX1–2CX7–12CX1–2C; and TLLLFR motif. The bHLH binding regions are also marked, with key residues indicated by asterisks. (b) Phylogenetic tree of poplar MYB165, MYB181, MYB182, MYB194 and related functionally characterized R2R3 MYB transcription factors from other plants, constructed from the N-terminal DNA binding domains using the Maximum Likelihood method with all bootstrap values over 500 shown (1000 replicates). Stars indicate the poplar repressor-like MYBs discussed here, the arrow marks MYB182. Clades are indicated in color as follows: in light blue, flavonoid repressors; purple, lignin/phenylpropanoid repressors; green, flavonol activators; light yellow, TT2-type PA activators; dark yellow, MYBPA1-type PA activators; pink, anthocyanidin activators. Genbank accession numbers for all protein sequences are listed in Supplemental Files.
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(a) Wild type MYB182 OE

(b) (c) (d) (e)

(f) (g) (h)

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Relative expression

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Relative expression

MYB182

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Relative expression

ANR1

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**Figure 3.** Effect of *MYB182* overexpression in transgenic poplars grown under to natural sunlight. (a) Visual comparison of wild type control and *MYB182* overexpressing (OE) poplar plants after 10 weeks of growth. (b-e) Close-up images of young leaves of *MYB182* OE (b, d) and control (c, e) plants. Panels b and c show top view of plant with youngest leaves of *MYB182* OE and control plants, panels d and e show leaves at LPI 5. (f, g) Anthocyanin and PA content of *MYB182* OE lines and wild type control lines. (h) Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of *MYB182* and *DFR1*, *ANS1*, and *ANR1* genes in wild-type and three independent *MYB182* OE lines. Relative gene expression is normalized to the mean of elongation factor 1B and ubiquitin/ribosomal protein 27a expression. Data points shown are means from three biological replicates (individual plants) with error bars indicating standard deviations. Asterisks on top of the bars indicate values determined by Student’s t-test to be significantly different from control (P < 0.05).
(a) Phosphoenolpyruvate (PEP) → Erythrose-4-phosphate (DAHPS)

(b) 3-Deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) → Shikimate 3-phosphate (EPSPS)

Phenylalanine → PAL, CHS, CHI, F3H, F3’H, DFR, UGT

Leucoanthocyanidins → ANS, ANR, LAR

Anthocyanins → Catechin, Epicatechin, Proanthocyanidins
**Figure 4.** Analysis of target genes based on microarray profiles from *MYB182* overexpression poplar plants. (a) Pie chart demonstrating gene ontology categories of the 779 annotations and classifying them by “biological process”. (b) Summary of the shikimate, phenylpropanoid and flavonoid pathways leading to PA and anthocyanin synthesis. Abbreviated enzyme names are indicated for each biosynthetic step. Bold underlined names represent genes significantly downregulated in *MYB182* overexpression plants. DAHPS, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase; DHQS, dehydroquinate synthase, DHQD, dehydroquinate dehydratase; SD, shikimate dehydrogenase, SK, shikimate kinase, EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; CM, chorismate mutase; ADT, arogenate dehydratase; PAL, phenylalanine ammonia lyase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3’H, flavonoid 3’-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; ANR, anthocyanidin reductase; LAR, leucoanthocyanidin reductase; UGT, UDP glucosyl transferase.
Figure 5. Transient expression assays to test activation and repression of the poplar ANR1 promoter by poplar MYB and bHLH regulators in suspension cultured poplar cells. MYB activators and repressor plasmids were tested in a ratio of 4:1 (250: 62.5 ng DNA, which gives similar ratio of molecular weight) unless otherwise indicated. (a) Transcriptional activation by MYB134 (R2R3 activator MYB) and poplar bHLH131 at different activator to repressor ratios. (b, c) Effects of MYB182 (R2R3-MYB repressor) and MYB179 (R3-MYB) on the activation of the ANR1 promoter by MYB134 in the presence of bHLH131 (TT8/DEL/AN1 homolog) or poplar bHLH079 (GL3/JAF13 homolog). (d, e) Transcriptional activation by MYB115 (R2R3 activator MYB) with bHLH131 or bHLH079, and repression by MYB182 and MYB179. All data points are means of three biological replicates, with error bars indicating standard deviations. Asterisks indicate values determined by Student’s t-test to be significantly different from control (P < 0.05).
**Figure 6.** Functional analysis and repression of the poplar anthocyanin regulator MYB117 in transiently transformed poplar suspension cells and agroinfiltrated *Nicotiana benthamiana* leaves. (a, b) Activation of the Arabidopsis DFR promoter by anthocyanin regulators and effects of MYB repressors in poplar cell culture. Assays of MYB117 were carried out with repressors MYB182 or MYB179, and using either bHLH131 (a) or bHLH079 (b) as a cofactor. (c) Anthocyanin content and corresponding image of red leaf coloration at 8 d following agroinfiltration of leaves the presence and absence of MYB182 and MYB117. The ratio of MYB activators to repressors was 4:1. All data points are means of three independent replicates, with error bars indicating standard deviations. Asterisks on top of the bars indicate values determined by Student’s t-test to be significantly different from the control (P < 0.05).
(a) Myb182

R2  R3

bHLH binding site

LXLX-like repressor motif

DLx_2Kx_3Lx_3R \rightarrow DLx_2Kx_3Tx_3R

IDLNI \rightarrow VDTNI

(b) MYB182 mutation 1

MYB182 mutation 2

MYB182 1,2 double mutation

Luciferase activity (Firefly / Renilla)

mut.1

mut.2

double mutation

bHLH131

MYB134 182

mutations

(c) MYB182 TLLLFR mutation

MYB182 ΔTLLLFR

Luciferase activity (Firefly / Renilla)

TLLLFR mut

ΔTLLLFR

bHLH131

MYB134 182

mutations
Figure 7. Functional analysis of conserved motifs in MYB182. (a) Structure of MYB182 protein showing location of conserved bHLH-binding domain and C2 repressor motif. The design strategy for the mutation of amino acids in the conserved motifs is shown below each site in question. (b) Location of mutations within MYB182 mutant constructs used in the promoter activation/repression assay, and corresponding results showing activation of the poplar ANR1 promoter by MYB134 with bHLH131 and repression by MYB182. Mut.1 and mut.2 refer to mutants 1 and mutant 2 shown above. (c) Schematic and corresponding results of TLLLFR mutant tests, where the motif was either mutated or deleted. Data points are means of three biological replicates, with error bars indicating standard deviations. Asterisks on top of the bars indicate values that were determined by Student’s t-test to be significantly different from the control (P < 0.05).