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Two R2R3-MYB proteins are broad repressors of flavonoid and phenylpropanoid metabolism in poplar

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SUMMARY

The phenylpropanoid pathway leads to the production of many important plant secondary metabolites including lignin, chlorogenic acids, flavonoids, and phenolic glycosides. Early studies have demonstrated that flavonoid biosynthesis is transcriptionally regulated, often by a MYB, bHLH, and WDR transcription factor complex. In poplar, several R2R3 MYB transcription factors are known to be involved in flavonoid biosynthesis. Previous work determined that poplar *MYB134* and *MYB115* are major activators of the proanthocyanidin pathway, and also induce the expression of repressor-like MYB transcription factors. Here we characterize two new repressor MYBs, poplar *MYB165* and *MYB194*, paralogs which comprise a subgroup of R2R3-MYBs distinct from previously reported poplar repressors. Both *MYB165* and *MYB194* repressed the activation of flavonoid promoters by *MYB134* in transient activation assays, and both interacted with a co-expressed bHLH transcription factor, bHLH131, in yeast two-hybrid assays. Overexpression of *MYB165* and *MYB194* in hybrid poplar resulted in greatly reduced accumulation of several phenylpropanoids including anthocyanins, proanthocyanidins, phenolic glycosides, and hydroxycinnamic acid esters. Transcriptome analysis of *MYB165*- and *MYB194*-overexpressing poplars confirmed repression of many phenylpropanoid enzyme genes. In addition, other MYB genes as well as several shikimate pathway enzyme genes were downregulated by *MYB165*-overexpression. By contrast, leaf aromatic amino acid concentrations were greater in *MYB165*-overexpressing poplars. Our findings indicate that *MYB165* is a major repressor of the flavonoid and phenylpropanoid pathway in poplar, and may also affect the shikimate pathway. The coordinated action of repressor and activator MYBs could be important for the fine tuning of proanthocyanidin biosynthesis during development or following stress.

Keywords: transcriptional repressor, gene regulatory network, plant secondary metabolism, phenolic metabolism, *Populus tremula* × *tremuloides*.

INTRODUCTION

Flavonoids are important plant secondary metabolites belonging to the polyphenol group of natural products. Common flavonoids are anthocyanins, flavonols, flavones, and proanthocyanidins (PAs), many of which are widely studied and generally considered to function in defense against biotic and abiotic stress. For example, flavonols protect plants from UV damage and anthocyanins function as signals for insects, while PAs act as herbivore deterrents and antimicrobial compounds (Gould, 2004; Treutter, 2006; Barbehenn and Constabel, 2011). Many flavonoids also have *in vitro* antioxidant activity and, when consumed in the diet, are beneficial to human health (Chung *et al.*, 1998; Ververidis *et al.*, 2007).

Flavonoids are derived from the general phenylpropanoid and flavonoid pathways, first elucidated in model species including Arabidopsis, petunia, and maize (Dixon *et al.*, 2002). Phenylpropanoid metabolism starts with the deamination of phenylalanine by phenylalanine ammonia lyase (PAL) to yield *trans*-cinnamic acid, which is further converted by cinnamate 4-hydroxylase (C4H) and 4-coumarate: coenzyme A (CoA) ligase (4CL) to *p*-coumaroyl CoA. This central intermediate is involved in the biosynthesis of many secondary metabolites, including lignin, phenolic acids, phenolic glycosides, and flavonoids (Figure S1). Flavonoids are synthesized from *p*-coumaroyl-CoA and three molecules of malonyl-CoA by chalcone synthase

(CHS) and chalcone isomerase (CHI), followed by further reduction and hydroxylation. Most enzymes in flavonoid biosynthesis are encoded by single genes in Arabidopsis, but by multiple genes in poplar, grape, and apple (Sparvoli *et al.*, 1994; Kim *et al.*, 2003; Tsai *et al.*, 2006). The PAs share many pathway intermediates and enzymes with anthocyanins. However, there are two enzymes in the pathway specific for PA biosynthesis: leucoanthocyanidin reductase (LAR) and anthocyanin reductase (ANR), which lead to catechin and epicatechin, respectively (Dixon *et al.*, 2005). LAR also regulates the polymerization and extension of PAs (Liu *et al.*, 2016).

The biosynthesis of anthocyanins and PAs is each regulated by distinct transcription factor complexes. Both are composed of a MYB, a basic helix–loop–helix (bHLH) and a WD-repeat (WDR) protein. MYB and bHLH transcription factors are encoded by large gene families in plants (Xu *et al.*, 2015). The MYB transcription factors interact with both bHLH and WDR proteins, but only the MYB and bHLH bind to DNA to recruit transcription machinery (Xu *et al.*, 2015). Specificity is determined by the MYB factor. In addition to the R2R3-MYBs, the R3-MYBs are sometimes involved in flavonoid biosynthesis. R2R3-MYBs contain two conserved MYB DNA-binding domains while R3-MYBs contain one (Feller *et al.*, 2011).

Many R2R3-MYB activators of flavonoid biosynthesis have been characterized, and they can be grouped in a phylogeny that reflects functional specialization (Dubos *et al.*, 2010; Yoshida *et al.*, 2015). The R2R3-MYB flavonoid activators generally fall into three subgroups that are important for anthocyanin, PA, or flavonol regulation. In Arabidopsis, *PAP1*/*MYB75* specifically regulates anthocyanin accumulation, while *TT2*/*MYB123* regulates PA synthesis, which here occurs only in the seed coat (Borevitz *et al.*, 2000; Nesi *et al.*, 2001). By contrast, Arabidopsis *MYB11*, *MYB12* and *MYB111* all regulate flavonol synthesis (Mehrtens *et al.*, 2005). These functionally specialized types of flavonoid R2R3-MYBs are also found in other species. In grape, the *PAP1*-like *VvMYBA1* specifically regulates anthocyanin biosynthesis and transport (Cutanda-Perez *et al.*, 2009), while PA biosynthesis is regulated by *VvMYBPA1*, *VvMYBPA2,* and *VvMYBPAR* (Bogs *et al.*, 2007; Terrier *et al.*, 2008; Koyama *et al.*, 2014). *VvMYBF1* regulates flavonol biosynthesis (Czemmel *et al.*, 2009). In *Medicago*, *LAP1* was identified as a *PAP1*-type anthocyanin regulator, while *MtPAR* regulates PA synthesis (Peel *et al.*, 2009; Verdier *et al.*, 2012). Similarly, in apple *PAP1* homologs *MYB1* and *MYB10* genes regulate anthocyanin biosynthesis in flesh, and *MYB3* in flowers (Takos *et al.*, 2006; Kortstee *et al.*, 2011; Vimolmangkang *et al.*, 2013), but *MsMYB12* and *MsMYB22* encode distinct transcription factors specialized for PA and flavonol biosynthesis, respectively (Wang *et al.*, 2017). In petunia, *PH4* and *AN2* are the R2R3-MYBs that control anthocyanin biosynthesis (Quattrocchio *et al.*, 2006). Despite this conserved specialization for some MYBs, however, other R2R3-MYBs regulate more than one terminal product and have more general impact on flavonoid biosynthesis, such as *VvMYB5a* and *VvMYB5b* in grape, which are involved in both anthocyanin and PA biosynthesis (Deluc *et al.*, 2006, 2008). Interestingly, anthocyanin and PA MYBs usually require interaction with bHLH co-factors to activate flavonoid gene transcription, while flavonol MYBs do not have this requirement.

In addition to the large number of R2R3-MYB activators, more recently the R2R3-MYB repressors have been recognized as significant regulators of flavonoid biosynthesis in plants. These MYB repressors typically fall into the C4 clade of the MYB transcription factor family and have characteristic repression domains in the C-terminal region. Thus all R2R3-MYB repressors contain a conserved LxLxL sequence within the C-terminal region (Kranz *et al.*, 1998). Unlike most flavonoid MYB activators, R2R3-MYB repressors appear to repress more than one end product. All flavonoid MYB repressors characterized to date also bind to the bHLH co-factor. For example, in *Medicago MYB2* represses both PA and anthocyanin biosynthesis, and physically interacts with the Arabidopsis bHLH TT8. *Myb2* mutant plants have enhanced accumulation of anthocyanin in hypocotyls and flowers, and increased PA accumulation in the seed coat. By contrast, overexpression of *MYB2* in transgenic hairy roots led to reduced anthocyanin and PA accumulation (Jun *et al.*, 2015). In petunia, *MYB27* was identified as a key anthocyanin repressor, binding to bHLH factors AN1 and JAF13. Overexpression of *MYB27* in petunia showed reduced anthocyanin pigmentation in flowers and stems and a small reduction of PA in the seed coat, while *MYB27* RNAi lines showed increased anthocyanin accumulation (Albert *et al.*, 2014).

The grapevine genome has seen an expansion of the flavonoid MYB C4 repressor group. Three flavonoid repressors MYBs, *MYBC2-L1*, *MYBC2-L2,* and *MYBC2-L3* were identified, but only *MYBC2-L1* is expressed highly in berries and flowers. MYBC2-L1 and MYBC2-L3 were shown to physically interact with PhAN1 and VvMYC1 from petunia and grape, respectively, and heterologous expression of *MYBC2-L1* and *MYBC2-L3* in transgenic petunia led to reduced anthocyanin and PA accumulation in petals and seeds (Cavallini *et al.*, 2015). Full characterization of MYB repressors in other species is still rare due to the limitations of plant transformation for many plants. For example, heterologous expression of MYB repressors in petunia or tobacco may not accurately reveal the *in planta* function of MYB repressors.

Poplar is commonly used as a woody plant model system. Because the genus is rich in phenolic phytochemicals including PAs, flavonoids, salicinoids, and hydroxycinnamic acid esters, it can be used to address important questions in chemical ecology. Additionally, poplar PAs and

other phytochemicals are induced by herbivory, wounding, pathogen attack, UV-B exposure, and high light stress (Mellway *et al.*, 2009), making this a powerful system for studies of phenolic metabolism and its regulation. In previous work, we characterized poplar *MYB134* and *MYB115*, both specific activators of PA accumulation. These transcription factors physically interact with poplar bHLH131, and their overexpression transgenic poplar leads to hyperaccumulation of PAs (Mellway *et al.*, 2009; James *et al.*, 2017). Anthocyanin accumulation in poplar is controlled by two *PAP1*-like activators, *MYB117* and *MYB119* (Yoshida *et al.*, 2015; Cho *et al.*, 2016). As in grapevine, the poplar genome also contains an expanded suite of flavonoid MYB repressors. We recently identified four poplar flavonoid R2R3-MYB repressor-like genes, *MYB181*, *MYB182*, *MYB165,* and *MYB194*. Functional studies indicated that *MYB182* regulates flavonoid gene expression and that its overexpression represses both PA and anthocyanin accumulation (Yoshida *et al.*, 2015). In this study, we characterized poplar *MYB165* and *MYB194*, which belong to a distinct subgroup of R2R3-MYB repressors. We show that these genes repress not only flavonoid genes and anthocyanin and PA biosynthesis, but also suppress salicinoid and hydroxycinnamic acid ester accumulation. Importantly, aromatic amino acid biosynthesis was affected by the overexpression of *MYB165*, suggesting a broad effect on metabolism.

RESULTS

MYB165 and *MYB194* are paralogous R2R3-MYB transcription factors with C-terminal repression motifs and help define a separate flavonoid MYB repressor subgroup

MYB165 and *MYB194* were first identified by being upregulated in *MYB134*-overexpressing transgenic poplars, with overexpression ratios of 3.55- and 7.97-fold, respectively (James *et al.*, 2017). *MYB165* and *MYB194* are paralogs from a whole genome duplication (Chai *et al.*, 2014), with 87% DNA sequence similarity and 79% protein sequence similarity between them. Both genes fall into R2R3-MYB clade four and both contain an N-terminal conserved [D/E]Lx2[K/R]x3Lx6Lx3R amino acid domain, the bHLH factor binding site (Zimmermann *et al.*, 2004). Multiple sequence alignments showed that MYB165 and MYB194 have three C-terminal conserved motifs: C1 (LIxxxGIDPxxHRL), C2 (pdLNLDLxxS) (Kranz *et al.*, 1998), and TLLLRF motifs (Matsui *et al.*, 2008) (Figure 1a). The C1 motif is a conserved sequence found in MYB repressors, however it also has putative activator activity (Matsui *et al.*, 2008). The C2 motif, also called the ethylene response factor-associated amphiphilic repression (EAR) motif, has an *in vivo* repressor function when fused to transcription factors and expressed in Arabidopsis (Hiratsu *et al.*, 2003; Matsui *et al.*, 2004).

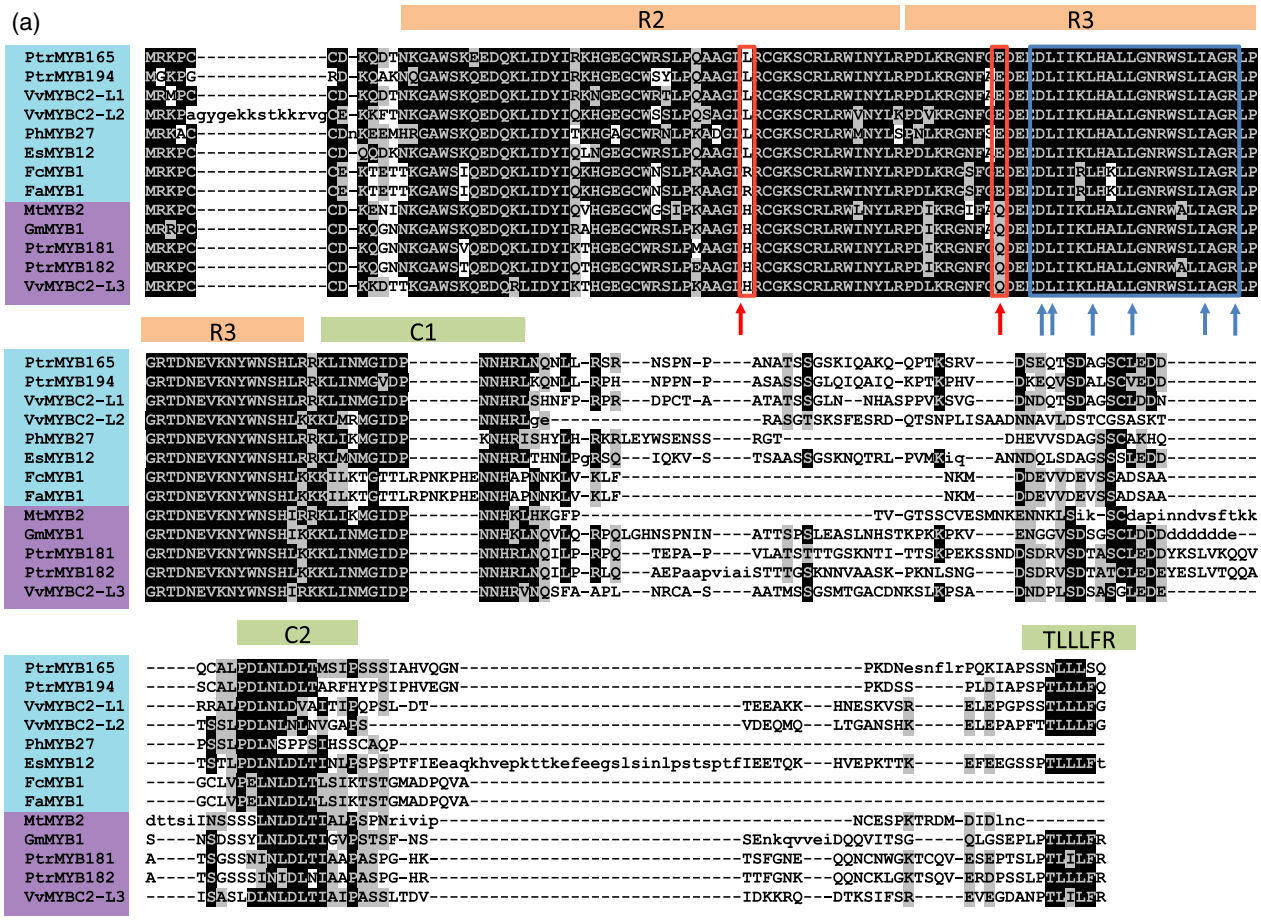
A phylogenetic analysis of characterized and recently discovered poplar clade four R2R3-MYBs showed two distinct subclades, and confirmed that *MYB182*, *MYB165,* and *MYB194* belonged to the group regulating flavonoid accumulation (Figure 1b). *MYB165* and *MYB194* clustered with grape *MYBC2-L1* and petunia *MYB27*, while *MYB182* fell into a separate group with grape *MYBC2-L3* and *Medicago MYB2*. Closer inspection of the sequence alignments of anthocyanin and PA MYB repressors identified two conserved different amino acids within the DNA-binding sites that were restricted to this subgroup. Specifically, at position 62, the MYB165- and MYB194-containing subgroup had a Leu residue, whereas the MYB182- containing subgroup had His at this position; at position 87, MYB165 and MYB194 contained a Glu residue while MYB182 had Gln (Figure 1b). This observation gave rise to the idea that these subgroups could have slightly different specificities and functions.

The expression profiles of *MYB165* and *MYB194*, as assayed by RT-qPCR, indicated that *MYB165* transcript levels were about 10–20-fold higher than those of *MYB194* (Figure S2). MYB165 showed higher expression in young leaves than in older leaves, stems, and roots. Transcript levels were not significantly different between plants grown in the greenhouse and natural sunlight, a treatment previously shown to induce PA and anthocyanin synthesis (James *et al.*, 2017).

MYB165, MYB194, and MYB182 proteins repress activation of PA biosynthesis promoters and physically interact with a bHLH factor

To confirm the repressor function of *MYB165* and *MYB194* on flavonoid gene expression, promoters of the poplar flavonoid and PA enzyme genes *DFR2*, *ANR1*, *LAR1*, and *ANS1* were cloned upstream of the firefly luciferase reporter gene. The constructs also contained the Renilla luciferase gene, controlled by the CaMV35S promoter as a normalizer. The ratio of firefly:Renilla luciferase therefore indicates relative promoter activation. Plasmids encoding MYB134 and the bHLH131 co-activator, MYB165 and MYB194 repressors controlled by CaMV35S promoter, and the promoter–luciferase reporter constructs were co-transformed into poplar suspension cells in different combinations. MYB182 was included for comparison and as a positive control. In the presence of bHLH131, MYB134 strongly activated *DFR2*, *ANR1*, and *LAR1* promoters as previously shown (Figure 2a; James *et al.*, 2017). When MYB165, MYB194, or MYB182 expression constructs were added, the activation was repressed (Figure 2a). This confirmed that MYB165, MYB194, and MYB182 are functional MYB repressors, and that they act on flavonoid promoters. By contrast, MYB134 activation of the *ANS1* promoter was weak, and not strongly affected by the MYB repressors in our assay; this could suggest a different activator, and

(a)



(b)

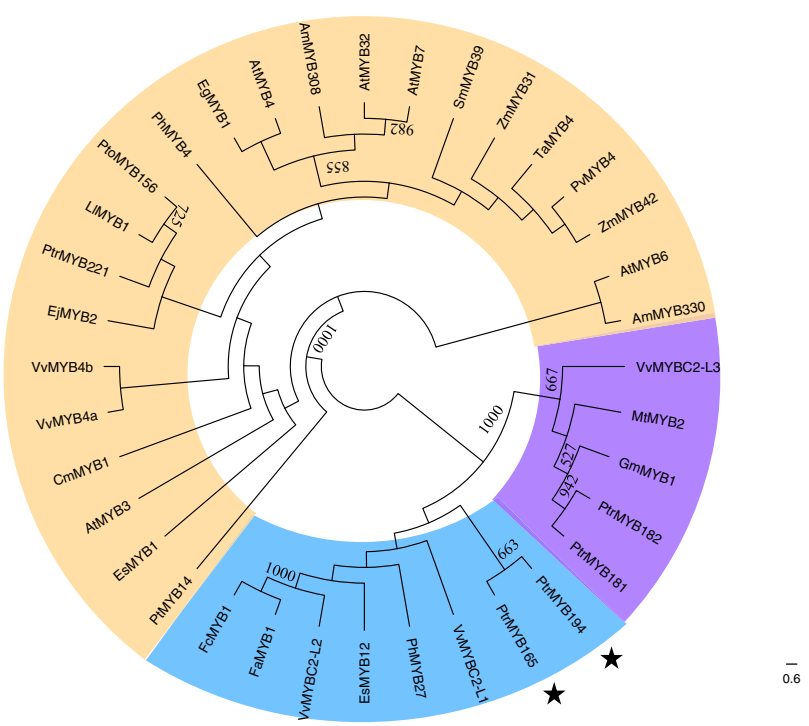


Figure 1. Sequence analysis of repressor R2R3-MYBs. (a) Alignment of amino acid sequences of new poplar and previously characterized R2R3-MYB repressors regulating anthocyanin and PA biosynthesis. White letters on a black background represent amino acids conserved in at least 50% of all sequences. Orange bars indicate R2 and R3 MYB DNA-binding domains, and green bars indicate C1, C2, and TLLLFR motifs. Blue frame and arrows represent the bHLH-binding domain and conserved residues. Names of genes are colored blue and purple to indicate two subgroups, with red arrows indicating conserved amino acids within each group.
(b) Phylogeny of R2R3-MYB subgroup 4 repressors constructed using the maximum-likelihood method. Bootstrap values over 500 are shown (1000 replicates). Orange background indicates lignin repressors, while blue and purple indicates two subgroups of anthocyanin and PA repressors. Asterisks indicate new MYB repressors studied here. Accession numbers for sequence data for phylogeny are AtMYB4, NP_195574; AtMYB32, NP_195225; ZmMYB31, CAJ42202; ZmMYB42, CAJ42204; PvMYB4, AEM17348; PtoMYB156, AMY62793; PtrMYB221, ACN97176; PtrMYB182, AJI76863; PtrMYB181, XP_006372586; VvMYBC2-L1, NP_001268133; VvMYBC2-L2, NP_001268180; VvMYBC2-L3, AIP98385; PhMYB27, AHX24372; FaMYB1, AAK84064; AmMYB308, P81393; AmMYB330, P81395; EgMYB1, CAE09058; AtMYB3, NP_564176; AtMYB7, NP_179263; AtMYB6, NP_192684; CmMYB1, AEO27497; PhMYB4, ADX33331; PtMYB14, ABD60279; EsMYB1, AFH03053; EsMYB12, AFH03064; LIMYB1, ADY38393; SmMYB39, AGS48990; FaMYB1, AAK84064; GmMYB1, ACM62749; TaMYB4, AEG64799; EjMYB2, AID56314.

repressor mechanism for this promoter. We note that ANS1 is also required for anthocyanin biosynthesis, which is known to be regulated independently of the PAs.

Both MYB134 and MYB115 were previously shown to physically interact with bHLH131 as part of the MBW complex (James *et al.*, 2017). As all three MYB repressors contain the bHLH-binding site, we tested whether these also bind to the bHLH131 co-factor in yeast two-hybrid assays. MYB repressors and bHLH131 were fused to the *Gal4* binding domain and the *Gal4* activation domain respectively. On selective medium, only cells expressing both MYB repressors and the bHLH131 factor showed growth (Figure 2b). This situation indicated a direct interaction of the three MYB repressors with bHLH131, which presumably contributes to their activity as repressors. We also plated serial dilutions (1:5) of yeast cells with the same MYB repressor and bHLH vector combinations on selective medium, together with the MYB134 and MYB115 activators for comparison (Figure 2c). Based on colony growth, in this assay we detected no significant difference in the strength of the interaction between the MYB repressors and activators with bHLH131.

Overexpressing *MYB165* and *MYB194* in transgenic poplar leads to reduced accumulation of anthocyanin in leaves, reduced PA concentrations in roots, and downregulation of phenylpropanoid pathway genes

To further characterize the function of *MYB165* and *MYB194 in planta*, they were constitutively overexpressed in hybrid poplar (*P. tremula* × *P. tremuloides*). Four successfully transformed lines for each gene were chosen for further study. In their respective transgenic lines, the *MYB165* and *MYB194* transgenes were each upregulated 50- to 100-fold compared with wild-type poplars (Figure 3a). The transgenics showed no obvious physical abnormalities, however we observed that the roots of tissue culture transgenics showed less coloration and that the mean mature leaf length of both greenhouse-grown and sunlight-exposed MYB repressor overexpressors was approximately 15–20% smaller than that of wild-type poplar (Figure S3).

In greenhouse-grown poplars, the concentration of PA in both wild-type and MYB repressor–overexpressor leaves was below the limit of detection of the butanol–HCl (Bu–HCl) assay. We therefore assayed PAs in roots, which accumulate more PAs than leaves of greenhouse-grown poplar. Treatment with the PA stain 4-dimethylaminocinnamaldehyde (DMACA) resulted in much weaker staining in transgenic roots compared with wild-type roots (Figure S3). When we quantified PAs in methanolic root extracts we found that, in *MYB165*-overexpressor roots, PA concentrations were reduced by 90% (Figure 3b). In *MYB194*-overexpressor roots, the effect was less pronounced, but the PA concentration in the roots of three of the four transgenic lines was nonetheless significantly lower than that in the wild-type.

To examine if *MYB165* and *MYB194* also repress anthocyanin accumulation, we exposed the plants to sunlight for 1 week to induce anthocyanin synthesis. The sunlight-exposed leaves of wild-type poplars turned red and accumulated anthocyanin, particularly in young leaves. By contrast, the MYB repressor–overexpressors were only slightly reddish and accumulated much less anthocyanin than controls (Figures 3c and S3b). Based on a spectrophotometric assay, we observed a reduction of anthocyanin of approximately 80% in both young and medium-aged leaves of *MYB165*-overexpressors. Likewise, the *MYB194*-overexpressors showed a similar pattern but less pronounced: two of the four *MYB194*-overexpressor lines had a 20% reduction in anthocyanin concentration (Figure 3d).

We next used RT-qPCR to measure transcript levels of selected phenylpropanoid and flavonoid genes, including those genes that we previously found to be most affected by *MYB134*-overexpression (James *et al.*, 2017). *PAL1* was downregulated in both *MYB165*- and *MYB194*-overexpressors. *DFR2*, *ANS1*, and *F3′5′H1* also showed strong downregulation in both transgenic plants, in particular in *MYB165*-overexpressors (Figure 4). This pattern is consistent with the more pronounced PA and anthocyanin phenotype in *MYB165*- relative to *MYB194*-overexpressors.

MYB165- and *MYB194*-overexpressor leaves show a strong reduction in salicinoid and hydroxycinnamic ester acid content

To determine if other phenolics were affected by *MYB165*- and *MYB194*-overexpression, methanolic leaf extracts were analyzed using high performance liquid chromatography coupled to UV detection (HPLC-UV). We tested extracts from sunlight-exposed leaves, as natural light stimulates

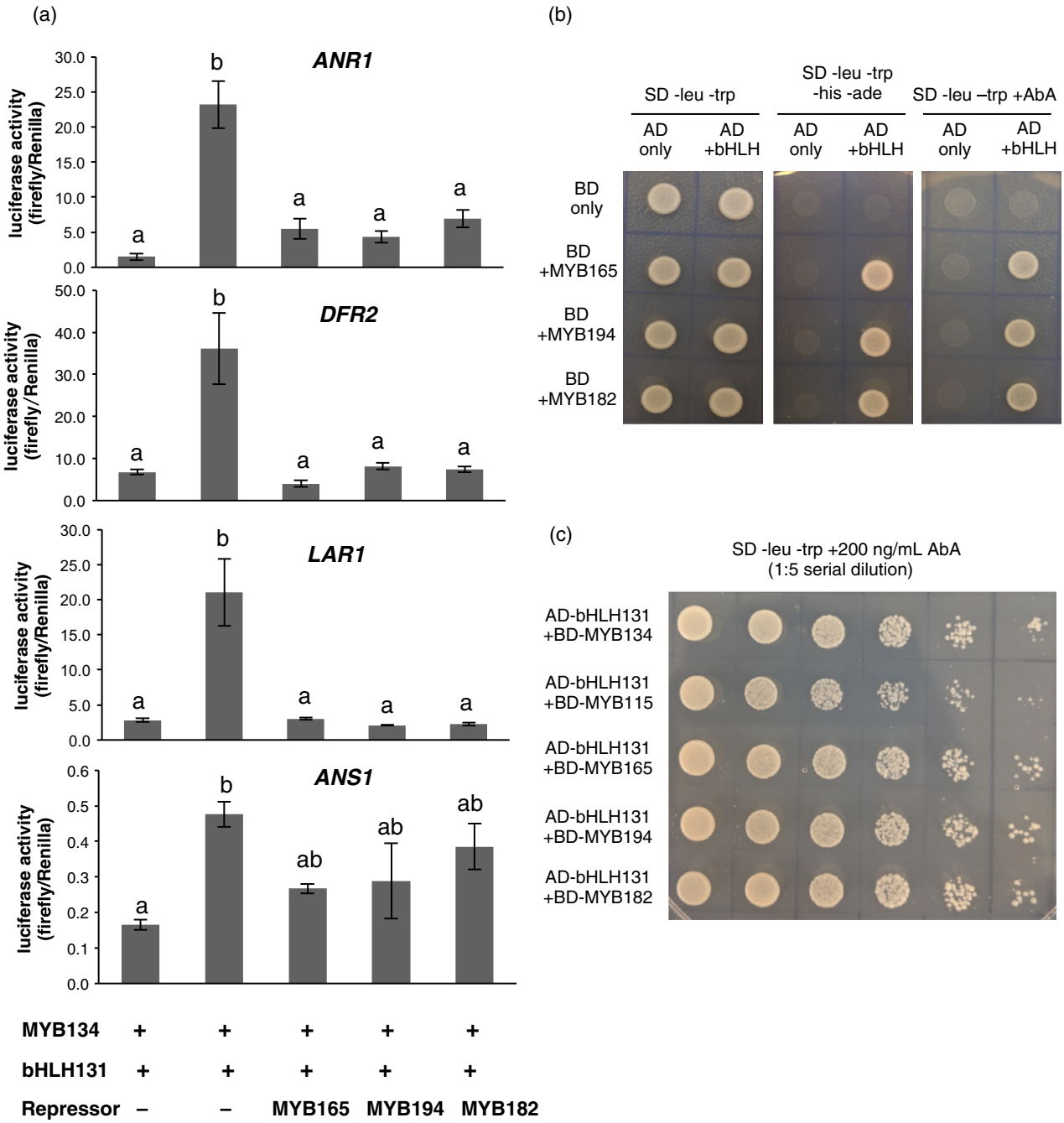


Figure 2. MYB165, MYB194, and MYB182 repress flavonoid gene promoters and interact with poplar bHLH.
(a) Promoter activation assays were carried in transiently transformed poplar cells on *ANR1*, *ANS1*, *DFR2,* and *LAR1* promoters fused to the luciferase reporter gene. MYB134 and bHLH131 constructs were co-expressed with MYB165, MYB194, and MYB182 repressor construct, as indicated at the bottom. Bars indicate promoter activation as normalized by Renilla activity. Different letters on the bar indicate significant differences according to Tukey's HSD test (*P*-value <0.05).
(b) Yeast two-hybrid assays to show interaction of MYB repressors with a bHLH co-activator. MYB165, MYB194, and MYB182 were fused to the DNA-binding domain (BD) and bHLH to the activation domain (AD) in pGBKT7 and pGADT7 vectors respectively, and co-transformed into Y2H Gold strain cells. Cells were grown without selection (left panel) and on selection medium without his or adenine (middle panel) or media with aureobasidin A (AbA) selection (right panel).
(c) Yeast two-hybrid assay serial dilutions (1:5) carried out with MYB- and bHLH-containing strains using AbA selection media. MYB134- and MYB115-containng vectors were from James *et al.* (2017). [Colour figure can be viewed at wileyonlinelibrary.com].

the synthesis of phenolic metabolism (Figure S4). Comparative analysis of HPLC-UV profiles indicated that several compounds in the *MYB165*- and *MYB194*-overexpressor chromatograms were significantly reduced relative to the controls. Consistent with our previous observations on the effects of *MYB165* and *MYB194*, these peaks all showed a

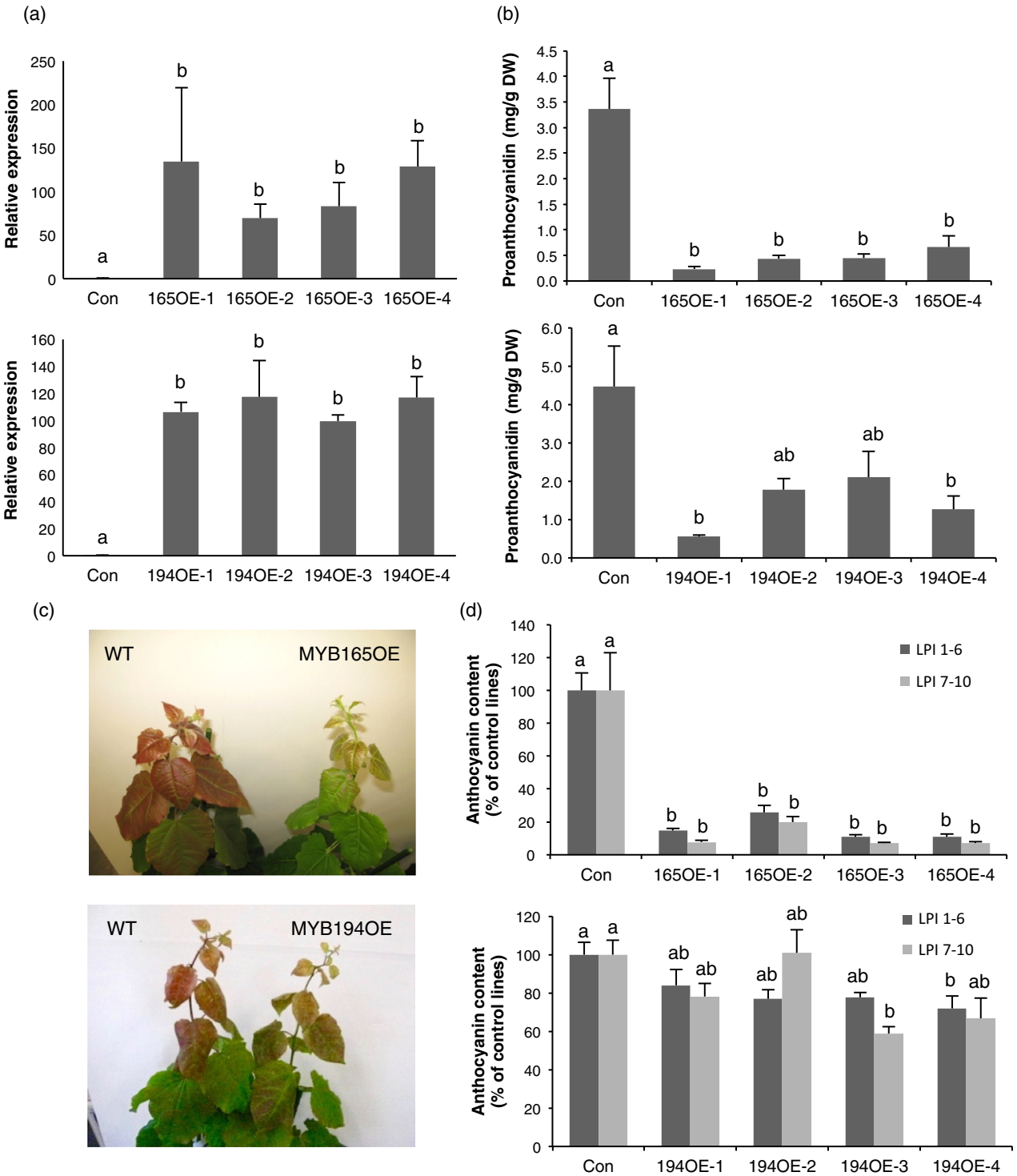


Figure 3. Analysis of *MYB165*- and *MYB194*-overexpressors.
(a) RT-qPCR analysis of *MYB165* and *MYB194* transgenes in four-individually transformed lines of each of *MYB165*- and *MYB194*-overexpressors.
(b) PA concentrations in control and *MYB165*- and *MYB194*-overexpressor roots as assayed by the Bu-HCl assay.
(c) Images of control and MYB-overexpressing transgenic poplars following 1 week of direct sunlight exposure.
(d) Relative anthocyanin content in leaves of sunlight-exposed transgenic poplars normalized to controls. LPI 1–6 refers to leaf plastochron leaves 1–6, the youngest six uncurled leaves; LPI 7–10, leaves in the maturation and expansion zones. Different letters on the bar indicate significant difference according to Tukey's HSD test (*P*-value <0.05). Error bars represent SE. ($n = 3$).

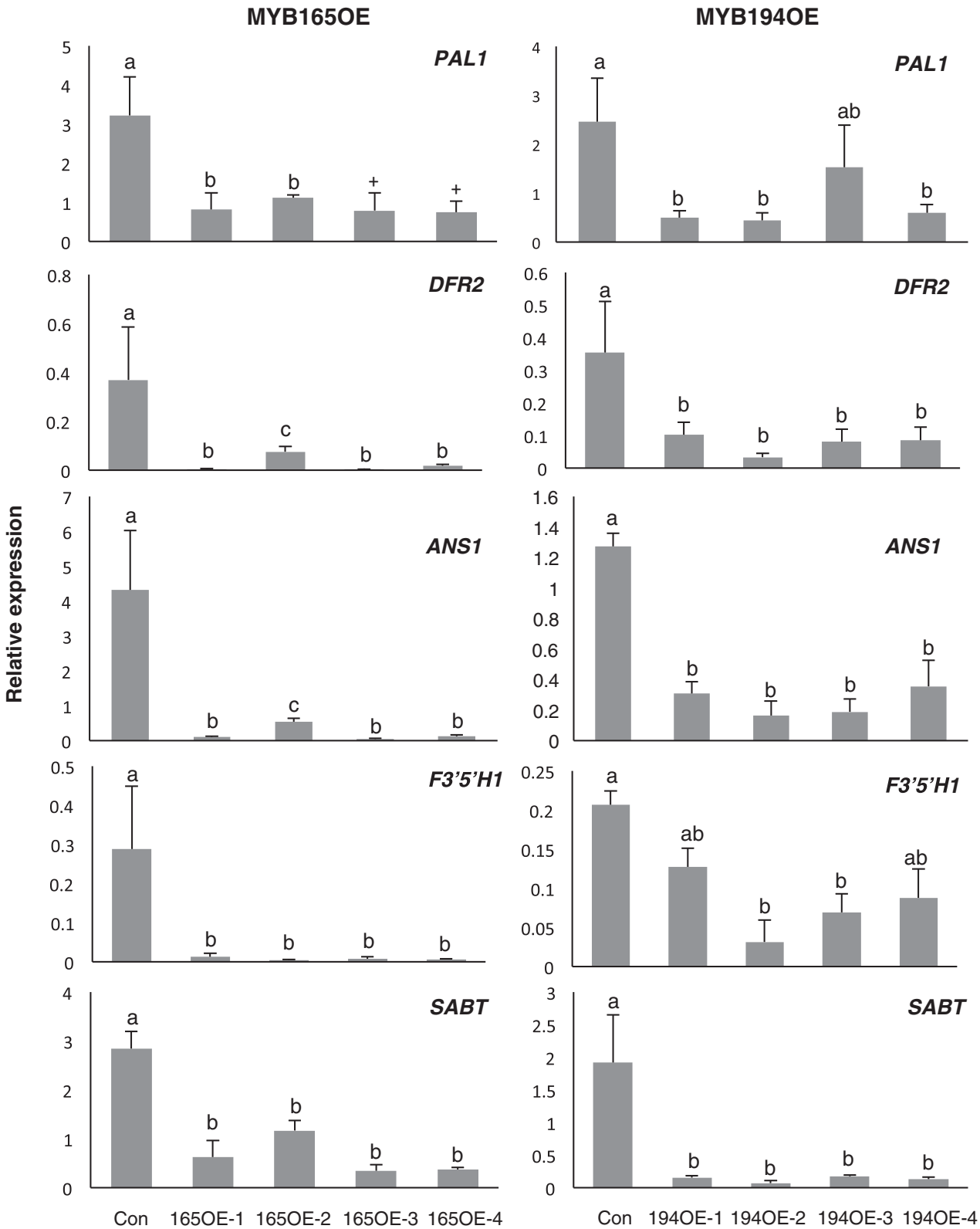


Figure 4. Analysis of differentially expressed flavonoid and phenolic biosynthesis genes in *MYB165*- and *MYB194*-overexpressors.
Transcript levels were quantified in four independent transgenic lines by RT-qPCR as described in Experimental Procedures. *PAL1*, phenylalanine lyase 1; *DFR2*, dihydroflavonoid reductase 2; *ANS1*, anthocyanin synthase 1; *F3′5′H1*, flavonoid 3′, 5′-hydroxylase 1; *SABT*, benzoyl-CoA:salicyl alcohol *O*-benzoyltransferase. Different letters on the bar indicate significant difference of relative expression according to Tukey's HSD test (*P*-value <0.05). Error bars represent SE ($n = 3$).

proportionally greater reduction in *MYB165*- than *MYB194*-overexpressors (Figures S5 and S6).

The two major salicinoids of the *P. tremula* × *P. tremuloides* hybrid, salicortin and tremulacin, were the mostly dramatically impacted. To quantify these peaks, UV chromatogram peak areas and a purified salicortin standard were used (Figure S9). Both salicortin and tremulacin concentrations were reduced by 80–90% in *MYB165*-overexpressors and approximately by 60% in *MYB194*-overexpressors (Figure 5). A minor salicinoid, salicin, did not elute as a single peak and could not be quantified by HPLC-UV, but was subsequently analyzed by LC–MS (see below). Two large peaks, tentatively identified as *cis*-3-coumaroyl quinate and *trans*-3-coumaroyl quinate isomers based on MS analysis (Figure S7; Clifford *et al.*, 2008), were also found to be reduced by over 50% and 30% in *MYB165*- and *MYB194*-overexpressors, respectively. Our extracts contained at least two caffeoyl quinate (chlorogenic acid) isomers, but these were not readily separated by HPLC-UV. However, preliminary analysis of this mixed peak suggested that caffeoyl quinates were less affected by the MYB repressors than the coumaroyl quinates (Figure 5).

Further phytochemical comparisons were carried out using LC–ESI-iontrap MS, which allowed us to quantify salicin, procyanidin B1, and separate the two isomers of caffeoyl quinate. Salicin and *trans*-3-caffeoyl quinate showed no significant differences between the transgenic poplars and controls (Tables S1 and S2), however the *cis*-3-caffeoyl quinate isomer was reduced by approximately 50% in the transgenics (Table S1). In addition to the salicin and caffeoyl quinates, we found that procyanidin B1 was almost completely absent in the *MYB165*-overexpressors but clearly seen in controls (Table S1; Figure S8). This finding is consistent with the suppression of total PAs in transgenic leaves as determined by the Bu–HCl assay.

The biosynthesis of salicortin, tremulacin, and other salicinoids is poorly understood, although these salicinoids are most likely to be derived from cinnamic acid (Babst *et al.*, 2010). We had previously identified a benzoyl-CoA: salicyl alcohol *O*-benzoyltransferase (SABT) as a candidate enzyme for salicinoid biosynthesis (Chedgy *et al.*, 2015). When we conducted RT-qPCR assays of *SABT* expression, we determined that transcript levels of this gene also showed a significant reduction in both types of overexpressor plants (Figure 4). Together with our phytochemical data, these data support a potential role of SABT in salicinoid biosynthesis.

Transcriptome analysis indicates downregulation of genes encoding phenylpropanoid and flavonoid enzymes, transcription factors, and shikimate enzyme-encoding genes in *MYB165*-overexpressors

As *MYB165*-overexpressors showed a stronger repression phenotype than *MYB194*-overexpressors, we focused additional molecular studies on *MYB165*. We selected one *MYB165*-overexpressor line (165OE-1) for transcriptomic analysis by RNA-seq. cDNA libraries were made from leaves of three biological replicates of the overexpressor and control lines. We sequenced cDNA libraries from both greenhouse-grown and sunlight-exposed plants, in order to study the effects on the *MYB165*-overexpressor under low-stress or high-stress conditions. Comparative analysis of the transcriptomes demonstrated overexpression of *MYB165* reached more than 100-fold in the greenhouse-grown transgenics and more than 50-fold in sunlight-exposed transgenics. At a threshold of a two-fold expression ratio and a q-value <0.05, 514 genes were differentially expressed in the former data set whereas, in the latter, only 236 genes met this criteria. Among the differentially expressed genes in the greenhouse-grown plants, 302 genes were downregulated and 212 genes were upregulated. In sunlight-exposed plants, 141 genes were downregulated and 95 genes were upregulated (Tables S4 and S5).

Among the genes that are differentially expressed, we identified many transcription factors, including six MYB and bHLH genes previously found to be involved in the phenylpropanoid pathway that were downregulated under both low-stress and high-stress conditions (Table 1). Interestingly, the other two poplar flavonoid MYB repressors, *MYB182* and *MYB194*, were in this set. Fourteen phenylpropanoid and flavonoid enzyme genes showed reduced expression in the transgenic poplars; this corresponded to the broad reduction in accumulation of phenolics in these plants. Whereas there was a substantial overlap in the downregulated genes, some genes were found to be significantly downregulated in only one of the two data sets (greenhouse-grown or sunlight-exposed *MYB165*-overexpressor lines), but not in the other. Unexpectedly, 10 shikimate pathway genes were also downregulated in *MYB165*-overexpressors in one or both data sets, including 5′-enolpyruvylshikimate 3-phosphate (*EPSP*) synthase, chorismate synthase, chorismate mutase, and arogenate dehydratase, the last step leading to phenylalanine synthesis. This result clearly suggested that *MYB165* overexpression had directly or indirectly affected genes upstream of the phenylpropanoid pathway.

Increased accumulation of aromatic and branched-chain amino acids in *MYB165*-overexpressors

The shikimate pathway leads to the synthesis of aromatic amino acids including phenylalanine, the precursor of phenylpropanoids. As a number of shikimate pathway genes were downregulated in the *MYB165*-overexpressors, we used an LC–MS/MS method to quantify 17 amino acids for sunlight-exposed poplar leaf extracts (Table S3). For the majority of the transgenic lines, we measured a significantly higher relative concentration of phenylalanine,

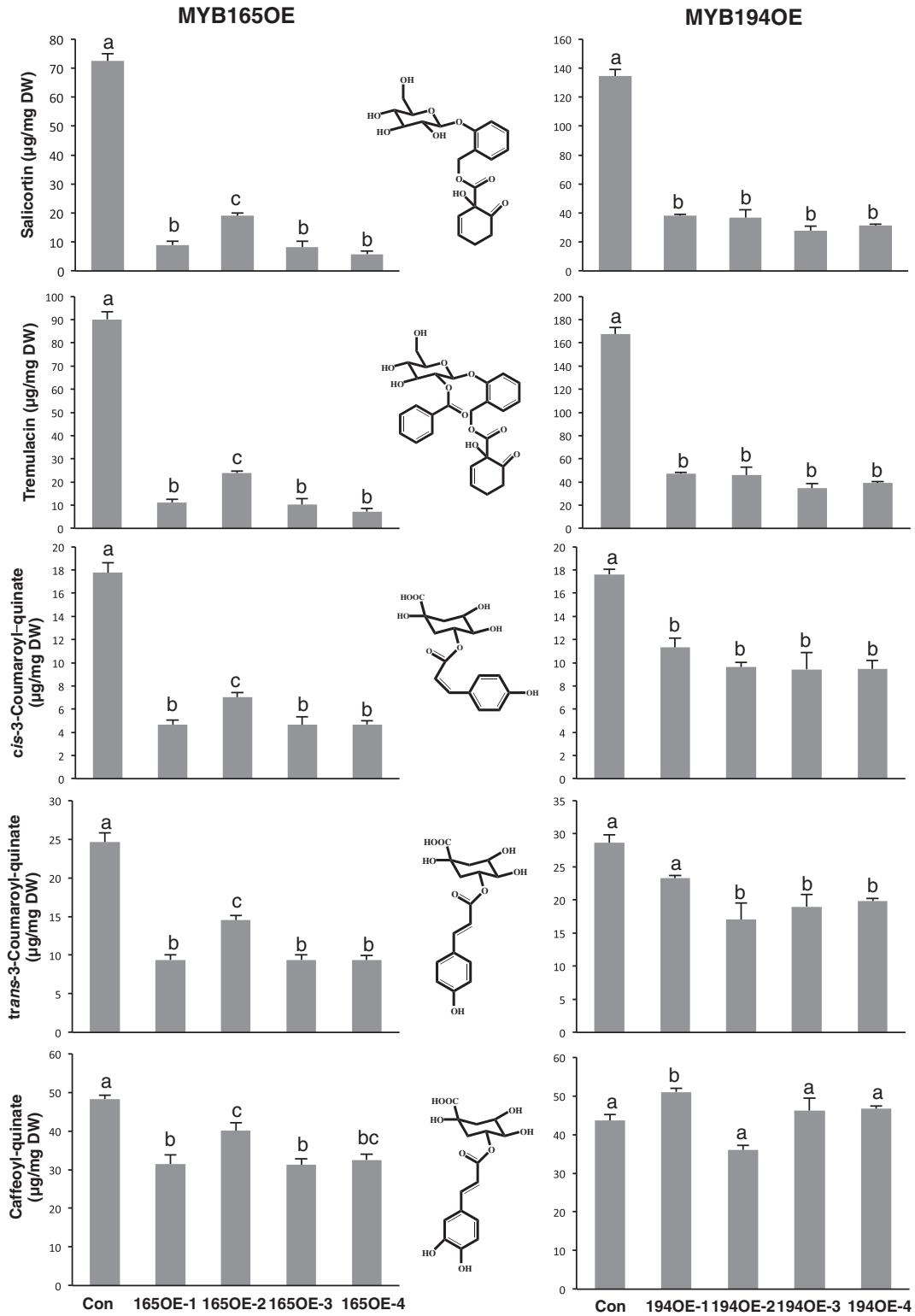


Figure 5. Analysis of salicinoids and hydroxycinnamate esters in *MYB165*- and *MYB194*-overexpressors.
Quantification of control, *MYB165*-, and *MYB194*-overexpressors was carried out by HPLC-UV at 280 nm as described in Experimental Procedures. Different letters on the bar indicate significant difference of concentration of phenolic compounds according to Tukey's HSD test (*P*-value <0.05). Error bars represent SE ($n = 3$).

Table 1 Selected downregulated genes in greenhouse-grown and sunlight-exposed MYB165-overexpressors with at least two-fold change and *q*-values smaller than 0.05

			Greenhouse		Sunlight	
Potri number	Potra number	Gene	Fold change	*q*-value	Fold change	*q*-value
Transcription factors						
Potri.010G114000	Potra000033g00168	MYB165	104.29	2.14E-72	56.65	4.51E-106
Potri.004G088100	Potra006413g25676	MYB182	0.002	1.80E-41	0.015	6.35E-03
Potri.017G128900	Potra002572g19350	MYB181	–	–	0.181	3.02E-17
Potri.008G128500	Potra002018g15830	MYB194	0.271	5.51E-04	–	–
Potri.006G275900	Potra002457g18619	MYB097	0.046	1.01E-04	–	–
Potri.002G054100	Potra001790g14531	bHLH131	0.045	1.62E-13	–	–
Phenylpropanoid pathway enzyme genes[a]						
Potri.013G074500	Potra003463g21747	SABT	0.037	1.13E-06	0.062	3.12E-05
Potri.005G229500	Potra001823g14702	DFR1	0.043	1.58E-12	0.041	7.34E-03
Potri.006G126800	Potra007760g26019	PAL1	0.064	3.28E-04	0.010	4.00E-02
Potri.019G130700	Potra001148g10006	C4H1	–	–	0.378	1.02E-05
Potri.018G094200	Potra001172g10165	4CL1	0.060	2.48E-12	0.024	7.26E-08
Potri.T071600	Potra177143g27937	4CL4	–	–	0.450	1.54E-02
Potri.014G145100	Potra000539g03787	CHS1	–	–	0.394	8.50E-04
Potri.003G176800	Potra161624g34003	CHS4	–	–	0.005	3.23E-02
Potri.009G069100	Potra001804g14599	F3′5′H1	0.109	3.69E-12	0.018	3.96E-03
Potri.016G091100	Potra000611g04646	PAL3	0.122	1.46E-03	–	–
Potri.005G113700	Potra000959g07878	F3H	0.405	7.85E-04	0.050	3.47E-02
Potri.001G113100	Potra001727g14056	ANS1	0.254	5.98E-04	0.023	2.37E-09
Potri.002G033600	Potra194092g28921	DFR2	0.267	1.23E-02	0.035	7.08E-03
Potri.004G030700	Potra001988g15626	ANR1	0.361	7.19E-05	–	–
Shikimate pathway enzyme genes						
Potri.004G188100	Potra181763g28181	ADT	0.274	7.53E-04	–	–
Potri.010G221600	Potra002082g16231	CS	0.303	8.54E-04	0.274	4.05E-02
Potri.017G088700	Potra005645g25454	CM	0.346	2.22E-02	–	–
Potri.002G146400	Potra000375g01603	EPSP synthase	0.318	1.48E-04	0.308	9.25E-05
Potri.013G029800	Potra003810g22948	SDH	–	–	0.137	4.82E-02
Potri.011G024900	Potra004157g24637	TSβ	2.675	2.67E-03	–	–
Potri.005G079200	Potra000874g07035	PAT	0.488	9.97E-03	0.365	1.30E-03
Potri.002G099200	Potra002057g16045	DAHPS	0.316	2.26E-02	–	–
Potri.005G162800	Potra002402g18269	DAHPS	0.369	4.95E-02	0.276	2.37E-08
Potri.005G110900	Potra003816g22967	DHQS	0.492	5.54E-03	–	–

ADT, arogenate dehydratase; CS, chorismate synthase; CM, chorismate mutase; EPSP synthase, 5′-enolpyruvylshikimate 3-phosphate synthase; SDH, shikimate dehydrogenase; TSβ, tryptophan synthase β chain; PAT, prephenate aminotransferase; DAHPS, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase; DHQS, dehydroquinate synthase.
[a]For abbreviations, see legend to Figure S1.

tryptophan, and tyrosine, all products of the shikimate pathway. We also found that several other amino acids, including His, Val, Leu, and Ile accumulated more in transgenic leaves (Figure 6). While this was unexpected, the branched-chain and aromatic amino acid pathways share the aspartate kinase, chorismate mutase, and TyrA (ACT) regulatory motif, which may explain the observed co-regulation of the branched-chain amino acids (Lang *et al.*, 2014) (see Discussion).

DISCUSSION

The importance of R2R3-MYB repressors in phenylpropanoid and flavonoid metabolism has only recently become apparent with detailed studies on flavonoid regulation in flowers (Albert *et al.*, 2014), fruits (Cavallini *et al.*, 2015), and seeds (Jun *et al.*, 2015). Here, we present *in planta* data on poplar MYB repressors which are implicated in PA metabolism as well as other phenolics. Both MYB165 and MYB194 repressed flavonoid promoters in poplar suspension cells, and reduced PA and anthocyanin synthesis when overexpressed in transgenic poplars. Transcript analysis of *MYB165*- and *MYB194*-overexpressors confirmed the downregulation of genes of the phenylpropanoid and flavonoid pathways. Similar to previous findings of flavonoid R2R3-MYB repressors (Albert *et al.*, 2014; Cavallini *et al.*, 2015; Jun *et al.*, 2015; Yoshida *et al.*, 2015), *MYB165* and *MYB194* can affect both PA and anthocyanin biosynthesis. Our data further suggest that *MYB165* and *MYB194* can also affect the accumulation of other phenolic compounds including the salicinoids and hydroxycinnamate esters, making this a repressor with potentially broad impact on poplar secondary metabolism.

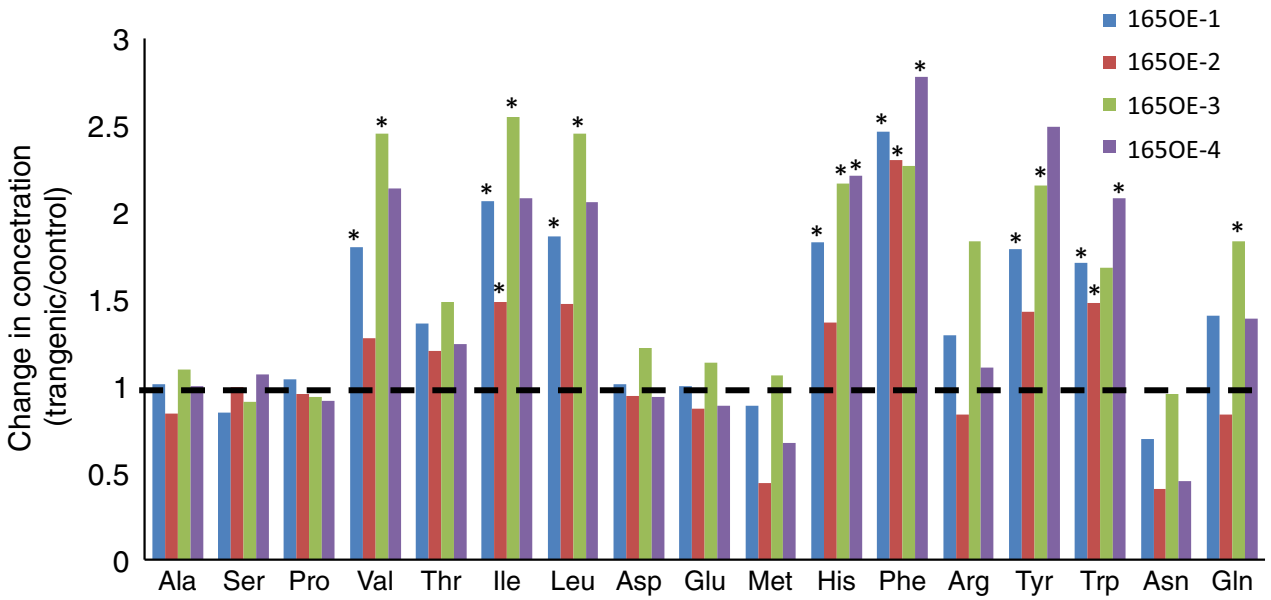


Figure 6. Effect of *MYB165*-overexpression on relative amino acid concentrations. Relative abundance of amino acids in *MYB165*-overexpressors relative to controls. Four independent transgenic lines are shown. A ratio of greater than one indicates enrichment in transgenics; asterisks indicated a significant change (*P*-value <0.05) relative to controls.

MYB165 and *MYB194* help to define a new subgroup of flavonoid MYB repressors

Our previous phylogenetic analysis showed that the C4 clade of MYB repressors separated into two major groups, a lignin or general phenylpropanoid subclade, and a flavonoid subclade (Yoshida *et al.*, 2015). Here we determined that the flavonoid subclade of MYB repressors further separated into two groups. One group comprised poplar MYB165, MYB194, petunia MYB27, and grape MYBC2-L1 and MYBC2-L2, while MYB182, MYB181, *Medicago* MYB2 and grape MYBC2-L3 fall into the second group (Figure 1). Close inspection of their protein sequences identified several conserved different amino acids within their DNA-binding sites that may lead to differential target specificities and repression efficacies. However, such functional differences between these two flavonoid R2R3-MYB repressor subgroups are not yet clear, as only in a few species have both types been studied. In grapevine, the *MYBC2-L3* repressor showed a stronger effect on PAs than *MYBC2-L1* when overexpressed in grape hairy roots. Conversely, when tested in petunia petals, *MYBC2-L3* was a stronger repressor of anthocyanins (Huang *et al.*, 2014; Cavallini *et al.*, 2015). Our analyses were carried out in an homologous system, transgenic poplars, and indicated that *MYB165*, *MYB194,* and *MYB182* showed significant differences in target specificity and repression strength *in planta*, despite similar responses in transient expression or yeast two-hybrid assays. Specifically, transcriptomic analysis showed that at least 10 flavonoid enzyme genes were significantly downregulated in the *MYB165*-overexpressors (Table 1), compared with five in *MYB182*-overexpressors (Yoshida *et al.*, 2015). For genes that were impacted in both *MYB182*- and *MYB165*-overexpressors, such as *PAL3* and *DFR2*, the effect was greater in *MYB165*-overexpressors. These differences align with a greater effect on the end products; in *MYB165*-overexpressors, anthocyanins, and PAs were clearly more severely affected than in *MYB182*-overexpressors, despite similar levels of transgene overexpression.

MYB165 and *MYB194* have broad effects on phenolic metabolism in poplar

In addition to PAs and anthocyanins, *MYB165* and *MYB194* also repressed the accumulation of other poplar phenolics. The strong reduction in salicinoid concentrations was unexpected, as this effect was not observed in *MYB182*-overexpressors (Yoshida *et al.*, 2015). By contrast, in our previous work on PA activators, overexpression of *MYB134* and *MYB115* in transgenic plants led to a strong upregulation of PAs together with a downregulation of salicinoids. In the *MYB165*-overexpressors described here, we observed a repression of both pathways by an overexpressed MYB. This situation could suggest a more upstream role for *MYB165*. These data together suggest that the previously observed downregulation of salicinoids in *MYB134*- and *MYB115*-overexpressors could be due to the enhanced expression of *MYB165* and *MYB194* repressors by the activator MYBs that we observed in those plants (Mellway *et al.*, 2009; James *et al.*, 2017). This situation would suggest that *MYB165* and *MYB194* can act as

direct repressors of salicinoid biosynthesis. This interpretation would be consistent with our observed downregulation of the potential salicinoid biosynthetic enzyme gene, *SABT*, in the *MYB165*- and *MYB194*-overexpressors. If *MYB165* can directly downregulate salicinoid synthesis during the induction of PAs, it would suggest a key role in modulating poplar secondary metabolism in response to stress.

We also observed a reduction of hydroxycinnamic acid esters (*cis*-3- and *trans*-3-*p*-coumaroyl quinate, and *cis*-3-caffeoyl quinate) in *MYB165*- and *MYB194*-overexpressors. Hydroxycinnamic acid esters, similar to flavonoids including PAs and anthocyanins, are synthesized from *p*-coumaroyl-CoA (Tsai *et al.*, 2006). As the general phenylpropanoid pathway gene *PAL1* was affected by *MYB165*- and *MYB194*-overexpression, a reduced flux into phenylpropanoid metabolism could explain this broad reduction. The repressive effect was more pronounced on the *p*-coumaroyl quinates than caffeoyl quinates (Figure 5 and Table S1). Therefore it appears that the compounds directly requiring *p*-coumaroyl-CoA are most affected by *MYB165* and *MYB194*. Overall, the phytochemical effects of *MYB165*- and *MYB194*-overexpression are clearly different from our previous observations with *MYB182*, and supports the idea of functional specialization of these repressor subgroups (Yoshida *et al.*, 2015). Furthermore, as *MYB165* and *MYB194* have effects on all major categories of phenolic metabolites, we speculate that they play a central position in the regulation of phenolic metabolism in poplar.

MYB165 may indirectly affect the shikimate pathway and amino acid biosynthesis

MYB165 overexpression caused a minor increase in the accumulation of phenylalanine, tryptophan, and tyrosine content of leaves, but counter-intuitively led to downregulation of at least nine shikimate pathway and aromatic amino acid biosynthesis genes. These trends may reflect multiple levels of regulation and feedbacks, as amino acid biosynthesis is typically controlled via feedback loops. A previous study found that transgenic downregulation of a *PAL* gene in petunia can lead to overaccumulation of Phe, while no change in shikimate gene expression was seen (Lynch *et al.*, 2017). In our *MYB165*-overexpressors, *PAL1* transcripts were also clearly downregulated; we speculate that the increased accumulation of aromatic amino acids was also due to a reduction in *PAL* expression and a general reduction in flux into phenylpropanoids.

Given the many feedbacks that are known to regulate amino acid biosynthesis, the downregulation of shikimate genes in the *MYB165*-overexpressors could be the result of a feedback loop to compensate for the hyperaccumulation of Phe and other aromatic amino acids. Alternatively, there could also be a more direct effect of *MYB165* on shikimate gene expression. Testing this would require identification of R2R3-MYB activators of the shikimate pathway in poplar; such MYB activators are known in other species (Chen *et al.*, 2006), but not in poplar. In petunia, R2R3-MYB *ODO1* regulates both shikimate and phenylpropanoid pathway genes (Verdonk, 2005), and it was previously suggested that ODO1 could interact with bHLH or WDR factors as part of a transcriptional complex (van Schie *et al.*, 2006). As both MYB165 and MYB194 interact with the poplar bHLH in the MBW complex, our poplar repressors may negatively affect shikimate pathway genes by binding to the same bHLH factors required for shikimate pathway regulation.

The enrichment in branched-chain amino acids Val, Leu, and Ile, together with the aromatic amino acids in the *MYB165*-overexpressors was not expected, but is consistent with known regulatory patterns. Biosynthesis of the branched-chain amino acids requires several of the same biosynthetic enzymes, notably acetohydroxyacid synthase (small subunit 2) and isopropyl malate synthase 1, both important for negative feedback regulation (Xing and Last, 2017). These enzymes both contain the ACT motif, a conserved site within the allosteric regions central to their regulatory functions, which is also found in the shikimate pathway enzymes chorismate mutase and prephenate dehydrogenase (Lang *et al.*, 2014; Xing and Last, 2017). This shared regulatory site in both aromatic and branched-chain amino acid pathways is important for coordinate regulation in biosynthetically distant pathways. We speculate that the presence of the ACT motif could explain why both of these pathways were similarly affected by *MYB165*-overexpression. Further research is needed to explore this potential metabolic interaction, however.

MYB165-overexpression identifies other potential phenylpropanoid-related MYB and bHLH factors

In addition to enzyme-encoding genes, multiple transcriptional activators were repressed by *MYB165*-overexpression. For example, in greenhouse plants, *MYB097* and its paralog *MYB101* were downregulated 21-fold and 25-fold, respectively. The function of these two genes is not known, but *MYB097* was previously found to be upregulated in sunlight- and wound-stressed poplars, and was therefore proposed to be a positive regulator of phenylpropanoid and flavonoid biosynthesis (Mellway *et al.*, 2009). Likewise, poplar *MYB006* and its paralog *MYB126*, were downregulated by 2.7-fold and 3.3-fold, respectively, in the greenhouse-grown *MYB165*-overexpressors, and *MYB006* expression was reduced in the sunlight-exposed plants as well. These MYBs belong to the same clade as Arabidopsis *AtMYB5*, grape *VvMYB5a* and *VvMYB5b*. Overexpression of *VvMYB5a* and *VvMYB5b* in tobacco led to overaccumulation of both anthocyanin and PA (Deluc *et al.*, 2006, 2008), suggesting that poplar *MYB006* and *MYB126* also help regulate flavonoid metabolism.

Besides affecting the MYB genes, transcripts of two *TT8*-like bHLH factors were also found to be reduced in *MYB165*-overexpressors. One of these downregulated bHLHs, *bHLH131*, is a *TT8*-homolog, and co-factor of both *MYB134* and *MYB115* during regulation of PA synthesis (James *et al.*, 2017). It is also a co-factor for poplar *MYB117*, the *PAP1*-like anthocyanin regulator in poplar (Yoshida *et al.*, 2015). A second bHLH gene was downregulated 53-fold and 35-fold under greenhouse-grown and sunlight-exposed conditions, respectively, and may encode a new flavonoid regulator in poplar.

Interestingly, the *MYB182* repressor and its paralog *MYB181*, as well as the *MYB194* repressor characterized here, were also downregulated in *MYB165*-overexpressors. Our analyses had indicated that *MYB194* is very similar to *MYB165*. Both genes share 79% protein sequence identity and contain the characteristic C2 and TLLLFR MYB repressor motifs, showed similar repression efficacy on flavonoid promoters in the transient activation assays, and had similar strength of binding to bHLH factor in the yeast two-hybrid assays. This redundancy implies that the downregulation of *MYB194* in *MYB165*-overexpressors may be part of a feedback loop, which maintains stable concentration of phenylpropanoids and flavonoids in poplar. However, we note that our constitutive overexpression approach is fairly non-specific, and we do not know which effects are direct and which are secondary responses. The observation that so many putative flavonoid regulators were downregulated by *MYB165*-overexpression, however, suggests there is a hierarchy of transcription factors to regulate phenylpropanoid and flavonoid metabolism, and that *MYB165* plays a prominent role in this hierarchy.

In conclusion, our results showed that the two poplar MYB repressors, *MYB165* and *MYB194*, repressed flavonoid promoter activity and reduced PA, anthocyanin, salicinoid, and hydroxycinnamic acid ester accumulation in poplar. Although *MYB165* and *MYB194* are similar in sequence, *MYB165* was generally more highly expressed, in different tissues and throughout development, and showed a stronger phenotype in transgenics. Our functional analysis of *MYB165* and *MYB194* helped to define a new flavonoid R2R3-MYB repressor subgroup, with different repression efficacy and target specificity from *MYB182*. *MYB165* also regulated several other MYBs and bHLH factors, and directly or indirectly downregulated the expression of shikimate enzyme genes. In future work, the exact DNA-binding targets of these MYB repressors should be identified, which will help to further elucidate their functions. As poplar is rich in phenolic compounds and reflects an expansion of MYB repressor gene family, identifying specific targets of these repressors should provide the evolutionary rationale for this diversity of function.

EXPERIMENTAL PROCEDURES

Multiple alignments and phylogenetic analysis

Sequences of MYB repressors were taken from NCBI (see accession numbers below). Multiple sequence alignments were performed using Dialign (Ait *et al.*, 2013; https://bibiserv.cebitec.uni-bielefeld.de/dialign/). The most suitable model for maximum-likelihood analysis was determined to be JTT+I using Protest (Abascal *et al.*, 2005; http://darwin.uvigo.es/software/prottest2_server.html). The phylogenetic tree was generated using a local PhyML (Guindon *et al.*, 2010) via JTT model with manual modification of γ-distribution. Bootstrapping was carried out with 1000 replicates. The phylogeny figure was displayed using Figtree (http://tree.bio.ed.ac.uk/software/figtree/).

Plant growth treatments and transformation

Tissue-cultured *P. tremula* × *P. tremuloides* (clone INRA 353–38) were grown in Lloyd & McCown's Woody Plant medium (Caisson Labs, Smithfield, UT, USA) with 1.25 mM indole butyric acid for 2 months under long day conditions (16-h light, 25°C). These plants were used as materials for transformation or further growth in greenhouse. Two-month-old tissue culture plantlets were transplanted to soil and kept in a mist chamber for 4 weeks, then grown in a greenhouse for 2 months before harvesting as previously described (Yoshida *et al.*, 2015). Sunlight-exposed poplars were moved outside in June or August 2014 and exposed to natural sunlight for 1 week.

To generate *MYB194*- and *MYB165*-overexpressors, the binary vector pMDC32 harbouring the *MYB165* or *MYB194* coding sequences were transformed into the *Agrobacterium tumefaciens* strain GV3101::pMP90 by electroporation. *P. tremula* × *P. tremuloides* (clone INRA 353–38) leaves were excised and transformed as described previously (James *et al.*, 2017). Positively transformed lines were selected on shoot-inducing and rooting medium with hygromycin B (Sigma-Aldrich, Oakville, ON, Canada) and the expression of transgene was confirmed by RT-qPCR.

Luciferase transient promoter activation assays

Luciferase promoter activation assay was performed using biolistic transient transformation of hybrid poplar suspension cultured cells as previously described (James *et al.*, 2017). Promoters of flavonoid biosynthesis genes (*ANR1*, *DFR2* and *LAR1*) (1.5 kb upstream region) were cloned from *P. trichocarpa* and inserted into the pGREEN800-LUC vector (Hellens *et al.*, 2005). The *MYB134* coding sequence was cloned from *P. tremuloides*, and *MYB165*, *MYB194*, *MYB182*, and *bHLH131* were amplified from reverse-transcribed *P. tremula* × *P. tremuloides* cDNA and inserted into the pMDC32 vector. Repressor and activator plasmids were added in a molar ratio of 1:4, as previously described (Yoshida *et al.*, 2015). After transient transformation, the cells were incubated on mannitol MS plates for 2 days, then homogenized and luciferase activity assayed with the Promega dual luciferase assay system.

Yeast two-hybrid analysis

Yeast two-hybrid assays were performed using the Clontech Matchmaker Gold Yeast two-hybrid system as previously described (James *et al.*, 2017). MYB repressors were cloned into pGBKT7 and fused to the *Gal4*-binding domain, and *bHLH131* was cloned into pGADT7 and fused to the *Gal4*-activation domain. Different combinations of pGBKT7 and pGADT7 constructs were

transformed into Y2H Gold strain cells following the manufacturer's instruction. Successfully transformed yeast cells were plated on selection medium to examine the physical interaction of MYB repressors and bHLH.

Phytochemical extraction and analysis

Phenolic compounds were extracted from roots using MeOH as described by Yoshida *et al.* (2015). Approximately 50 mg freeze-dried tissue was added to 1.5 mL of 100% MeOH, followed by vortexing and centrifuging at 15 900 ***g*** for 5 min. The tissue was extracted twice more with 1 mL MeOH and the extracts were pooled for further analysis. To measure PA in leaves, 0.5 mL MeOH extract was added to 2 mL of Bu−HCl reagent (butanol:concentrated HCl (95:5 v/v)) with 66.75 μL of iron reagent (2% $NH_4Fe(SO_4)_2$ (w/v) in 2N HCl). In total, 40 μL of the mixture was taken as unheated blank and rest of the mixture was heated in 95°C water bath for 40 min and cooled to room temperature. The unheated and heated mixture were placed in 96-well plates and read at 550 nm on PerkinElmer VICTOR X5 plate reader. A standard curve was generated using purified PA from *P. tremuloides* (Mellway *et al.*, 2009).

To extract anthocyanins, 500 μL of methanol with 1% HCl (v/v) were added to 30–50 mg of freeze-dried poplar leave tissues and shaken at 250 rpm at 20°C overnight. A 500 μL of distilled water was mixed with the extract and vortexed. Next, 1 mL of chloroform was added and the mixture centrifuged at 13 000 rpm for 5 min. Anthocyanin concentration was spectrophotometrically measured at 530 nm in the aqueous layer.

HPLC and LC−MS analysis of phenolic extracts

For HPLC-UV analysis, 3.5 mL of methanol extract was dried by SpeedVac (ThermoSavant SC110A) and resuspended in 100% HPLC-grade methanol to a final concentration of 50 mg tissue/mL. The extract was then diluted to a final concentration of 5 mg/mL in 50% methanol. A System Gold 126 HPLC system with autosampler and System Gold 168 detector (Beckmen Coulter, Missassauga, ON, Canada) equipped with a Kinetics C18 column (150 × 4.6 mm, 2.6 μm; Phenomenex Torrance, CA, USA) were used for phenolic compound analysis. Next, 20 μL of diluted methanol extract was analyzed. Separation of phenolic compounds was performed using the elution gradient previously described (James *et al.*, 2017), with UV detection at 280 nm. A standard curve was made with purified salicortin (a gift from Richard Lindroth) and chlorogenic acid (MP Biomedicals, Santa Ana, CA, USA).

For LC−MS analysis we separated phenolic extracts on a Kinetics C18 column (100 × 4.6 mm, 2.6 μm; Phenomenex) and an Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) using the elution gradient described previously (James *et al.*, 2017). The LC system was coupled to a Bruker Esquire 6000 ion-trap mass spectrometer (Bruker Daltonics, Bremen, Germany) and mass spectra were obtained by applying the method parameters described previously (James *et al.*, 2017). The mass spectrometer was operated in alternating ionization mode in the 60–1000 m/z range.

For the identification of hydroxycinnamic acid esters the mass spectrometer was additionally operated in AutoMS mode to generate MS2 fragmentation spectra in negative mode. MS2 spectra in negative mode were compared with the data given in Clifford *et al.*, 2008. For relative quantification of procyanidin B1, salicin, coumaroyl quinate, and caffeoyl quinate the peak areas of extracted ion chromatogram traces in negative mode at m/z 577, m/z 285, m/z 337, and m/z 353 were used.

For amino acid analysis, methanolic plant extracts were diluted with 1:10 (v/v) in water containing the ^{13}C, ^{15}N labeled algal amino acid mix (Isotec, Miamisburg, OH, USA). Amino acids in the diluted extracts were directly analyzed by LC–MS/MS as described previously (Crocoll *et al.*, 2016) using reversed phase chromatography on a Zorbax Eclipse XDB-C18 column (50 mm × 4.6 mm, 1.8 μm, Agilent Technologies, Germany) coupled to an API5000 mass spectrometer (Applied Biosystems, Foster City, CA, USA) operated in positive mode in multiple reaction monitoring mode.

RNA extraction and RT-qPCR analysis

Total RNA was extracted from poplar leaves as previously described (Yoshida *et al.*, 2015). RNA was then treated with RQ1 DNase (Promega, Madison, WI, USA) to degrade genomic DNA and cDNA was made using Superscript II reverse transcriptase (Invitrogen, Carslbad, CA, USA).

RT-qPCR was performed using Ssofast qPCR mix (Bio-Rad, Mississauga, ON, Canada) on a CFX96 Real Time system and C1000 Thermocycler (Bio-Rad). Transcript abundance data were normalized using the geometric mean of elongation factor *EF1b* and actin expression. Primers are shown in Table S6.

RNA-seq analysis

RNA was extracted following the method described above. MagJET enrichment kit (ThermoScientific, Waltham, MA, USA) was used to purify mRNA from crude RNA. The mRNA was fragmented by heating the samples at 94°C for 5 min. To make cDNA libraries with adaptors NEBNext ultra RNA Library Prep Kit for Illumina (NEB) was used. GeneJET NGS Cleanup Kit (ThermoScientific) was used to remove extra adaptors. Primers for RNA-seq were added to cDNA using NEBNext Multiplex Oligo kit (NEB). MagJET NGS Cleanup and Size Selection kit (ThermoScientific) was used to select DNA fragments around 350 bp. Twelve libraries at the same concentrations (250 ng/μL each) were pooled and sent to the McGill University/GenomeQuébec Innovation Center (http://gqinnovationcenter.com) and sequenced on one HiSeq lane.

The alignment of sequence was performed using Tophat2 and Cufflinks (Trapnell *et al.*, 2013). A *P. tremula* genome sequence was used as reference genome (Sjödin *et al.*, 2009; http://popgenie.org/). Cuffdiff was used to analyze the differentially expressed genes. DESeq package in R (version 3.4.0) was used to generate the final differentially expressed gene table (Anders and Huber, 2010). For gene annotation, an annotated reference transcriptome from *P. tremula* (Sjödin *et al.*, 2009; http://popgenie.org/) was used with modification referencing the annotation from Phytozome.

Statistics analysis

The statistical analysis was done by one-way analysis of variance (ANOVA) using R (version 3.4.0). Tukey's honest significant difference (HSD) test was used to compare the means with cut-off threshold $P = 0.05$.

The RNA-seq data discussed in this paper have been deposited at NCBI's Gene Expression Omnibus (Edgar *et al.*, 2002) and are accessible through GEO accession number GSE118388. Poplar MYB165 and MYB194 sequences are available at NCBI's GenBank Sequence Database under accession numbers MH746926 and MH746927.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. General phenylpropanoid and flavonoid pathway.

Figure S2. Expression profile of *MYB165* and *MYB194* in wild-type poplar (*P. tremula* × *tremuloides*) tissues.

Figure S3. Images comparing leaves and roots of *MYB165*- and *MYB194*-overexpressors.

Figure S4. HPLC analysis of greenhouse-grown and sunlight-exposed wild-type poplar leaf extracts.

Figure S5. Representative HPLC chromatogram of *MYB165*-overexpressor and control poplar leaf extracts.

Figure S6. Representative HPLC chromatogram of *MYB194*-overexpressor and control poplar leaf extracts

Figure S7. LC–MS validation of caffeoyl quinates and *p*-coumaroyl quinates

Figure S8. LC–MS validation of procyanidin B1.

Figure S9. Standard curves for phytochemical analysis.

Table S1. Quantification of procyanidin B1 and two caffeoyl quinate isomers by LC/MS.

Table S2. Quantification of salicin using LC/MS.

Table S3. Quantification of amino acids in *MYB165*-overexpressors.

Table S4. Differentially expressed genes in greenhouse-grown *MYB165*-overexpressors as determined by RNA-seq.

Table S5. Differentially expressed genes in sunlight-exposed *MYB165*-overexpressors as determined by RNA-seq.

Table S6. qPCR primer list.

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