Tree Physiology 36, 1162–1176
doi:10.1093/treephys/tpw046

Research paper

Overexpression of *PtrMYB119*, a R2R3-MYB transcription factor from *Populus trichocarpa*, promotes anthocyanin production in hybrid poplar

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Received October 13, 2015; accepted April 26, 2016; published online June 3, 2016; handling Editor Chung-Jui Tsai

Anthocyanins are a group of colorful and bioactive natural pigments with important physiological and ecological functions in plants. We found an MYB transcription factor (PtrMYB119) from *Populus trichocarpa* that positively regulates anthocyanin production when expressed under the control of the CaMV 35S promoter in transgenic *Arabidopsis*. Amino acid sequence analysis revealed that PtrMYB119 is highly homologous to *Arabidopsis* PAP1 (PRODUCTION OF ANTHOCYANIN PIGMENT1), a well-known transcriptional activator of anthocyanin biosynthesis. Independently produced transgenic poplars overexpressing *PtrMYB119* or *PtrMYB120* (a paralogous gene to *PtrMYB119*) (i.e., 35S::PtrMYB119 and 35S::PtrMYB120, respectively) showed elevated accumulation of anthocyanins in the whole plants, including leaf, stem and even root tissues. Using a reverse-phase high-performance liquid chromatography, we confirmed that the majority of the accumulated anthocyanin in our transgenic poplar is cyanidin-3-*O*-glucoside. Gene expression analyses revealed that most of the genes involved in the anthocyanin biosynthetic pathway were highly upregulated in 35S::PtrMYB119 poplars compared with the nontransformed control poplar. Among these genes, expression of *PtrCHS1* (*Chalcone Synthase1*) and *PtrANS2* (*Anthocyanin Synthase2*), which catalyze the initial and last steps of anthocyanin biosynthesis, respectively, was upregulated by up to 350-fold. Subsequent transient activation assays confirmed that PtrMYB119 activated the transcription of both *PtrCHS1* and *PtrANS2*. Interestingly, expression of *MYB182*, a repressor of both anthocyanin and proanthocyanidin (PA) biosynthesis, was largely suppressed in 35S::PtrMYB119 poplars, while expression of *MYB134*, an activator of PA biosynthesis, was not changed significantly. More interestingly, high-level accumulation of anthocyanins in 35S::PtrMYB119 poplars did not have an adverse effect on plant growth. Taken together, our results demonstrate that PtrMYB119 and PtrMYB120 function as transcriptional activators of anthocyanin accumulation in both *Arabidopsis* and poplar.

Keywords: pigment, transcriptional activator, transgenic poplar.

Introduction

Anthocyanins are a group of colorful and bioactive natural pigments with important physiological and ecological functions in plants, including attraction of pollinators and seed dispersers, protection of plants from high light irradiation and scavenging of free radicals produced in cells under stress conditions (Gould et al. 2002, Steyn et al. 2002, Grotewold 2006, Zhang et al. 2011).

Anthocyanin biosynthesis is one of the most comprehensively studied secondary metabolic pathways in plants and shares a particular branch of the flavonoid pathway (Grotewold

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2006, Petroni and Tonelli 2011). In the initial steps of the flavonoid pathway, phenylalanine ammonia-lyase (PAL), cinnamate-4-hydroxylase (C4H) and 4-coumaroyl-CoA: ligase (4CL) enzymes catalyze sequential reactions to produce coumaroyl-CoA from phenylalanine (Ohl et al. 1990, Lee et al. 1995, Bell-Lelong et al. 1997). Then, chalcone synthase (CHS), the first enzyme in the flavonoid biosynthetic pathway, converts coumaroyl-CoA to naringenin chalcone, which is further isomerized to flavanones by chalcone isomerase (CHI) (Feinbaum and Ausubel 1988, Shirley et al. 1992). Chalcone isomerase is encoded by a single gene in both *Arabidopsis* and *Populus*. After this step, the pathway branches off to make different classes of flavonoids. Flavanones of the anthocyanin biosynthetic branch are hydroxylated to produce dihydroflavonols by flavanone 3-hydroxylase (F3H) (Pelletier and Shirley 1996). Dihydroflavonols are further reduced to flavan-3,4-diols (leucoanthocyanins) by dihydroflavonol reductase (DFR) (Shirley et al. 1992). Anthocyanidin synthase (ANS) catalyzes the last step in the biosynthesis of anthocyanins. UDP glucose: flavonoid-3-*O*-glucosyltransferase (UFGT) functions to transfer the glucosyl moiety from UDP glucose to the 3-hydroxyl group of anthocyanidins and had been shown to be key for anthocyanidin stability and water solubility (Ford et al. 1998). Anthocyanidin reductase (ANR) and leucoanthocyanidin reductase (LAR) are specific to the proanthocyanidin (PA) branch of the pathway (Abrahams et al. 2003, Xie et al. 2003).

The regulatory mechanism of anthocyanin biosynthesis has also been studied extensively. Three types of transcription factors (TFs), namely R2R3-MYB factors, basic helix-loop-helix (bHLH) proteins and WD40 repeat proteins, interact physically to form the MYB–bHLH–WD40 (MBW) TF complex that activates transcription of target genes by interacting directly with the promoter DNA via multiple *cis*-elements (Koes et al. 2005, Ramsay and Glover 2005, Feller et al. 2011, Lai et al. 2013, Xu et al. 2014). MYB TF recognizes the 'Myb-response element' and 'AC elements', while bHLH proteins interact with the 'E-box' or 'bHLH-binding motif'. WDR proteins are thought to function by interacting with MYB and bHLH proteins without direct binding to DNA (Baudry et al. 2004, Feller et al. 2011, Lai et al. 2013, Xu et al. 2014). In *Arabidopsis*, the direct interaction of R2R3-MYB TFs, PRODUCTION OF ANTHOCYANIN PIGMENT1 (PAP1), PAP2, MYB113 and MYB114 and the WD40 repeat protein TTG1 with bHLH TFs GL3, EGL3 and TT8 was demonstrated by yeast two-hybrid assays (Zhang et al. 2003, Zimmermann et al. 2004); these TFs can form multiple MBW complexes to regulate the expression of anthocyanin biosynthesis genes.

Tree species in the genus *Populus* spp., commonly known as poplars, aspens and cottonwoods, are widespread in the northern hemisphere and are important both economically and scientifically. Economically, poplars are used as lumber for construction, pulp for the paper industry and feedstock for biofuel production. Scientifically, poplars serve as a model genetic system for research into tree-specific perennial growth and development, primarily because of the availability of a wide range of genetic resources and the relative ease of genetic transformation. *Populus* spp. contain eight major classes of flavonoids, including chalcones, dihydrochalcones, flavanones, flavones, dihydroflavonols, flavonols, anthocyanins and PAs (Tsai et al. 2006). The occurrence and concentration of flavonoids vary among *Populus* species and clones (Greenaway et al. 1991, 1992, Donaldson et al. 2006). Proanthocyanidins are major flavonoids that accumulate in the leaves of poplars in response to stresses such as wounding, pathogen attack, nitrogen deficiency and UV light (Osier and Lindroth 2004, Miranda et al. 2007, Mellway et al. 2009). Recently, several poplar MYB TFs involved in the PA biosynthesis were functionally characterized. PtMYB134 has been identified as an activator of PA biosynthesis (Zifkin et al. 2012, Gesell et al. 2014). Transgenic poplars overexpressing PtMYB134 accumulated 50-fold higher levels of PAs in their leaves than a nontransformed control, while no significant changes in anthocyanin levels were found, suggesting that PtMYB134 is specific to PA biosynthesis (Mellway et al. 2009). In addition, MYB182 was found to function as a negative regulator of the flavonoid pathway (Yoshida et al. 2015). Overexpression of MYB182 in poplar plants led to reduced PA and anthocyanin levels with a reduction in the expression of key flavonoid genes, suggesting that MYB182 plays an important role in fine-tuning PtMYB134-mediated flavonoid metabolism (Yoshida et al. 2015).

In poplars, anthocyanin accumulates mainly in male catkins, which have characteristic deep red-colored anthers. Wilkins et al. (2009) identified a group of PAP1-like MYB TFs from poplar that are highly expressed in male catkins, suggesting their functional implications in anthocyanin biosynthesis. Very recently, Yoshida et al. (2015) showed that poplar *MYB117* elevated anthocyanin level in *Nicotiana benthamiana* leaves by the agroinfiltration method.

In this study, we investigated five poplar R2R3-MYB TFs (i.e., *PtrMYB116*, *PtrMYB117*, *PtrMYB118*, *PtrMYB119* and *PtrMYB120*), which are probably paralogous to each other. Overexpression of *PtrMYB119* in both *Arabidopsis* and poplar resulted in elevated accumulation of anthocyanins in whole plants. Subsequent gene expression analyses revealed that PtrMYB119 activates most of the genes involved in the anthocyanin biosynthetic pathway, especially *PtrCHS1* and *PtrANS2*, which catalyze the initial and last steps of anthocyanin biosynthesis, respectively. In addition, expression of *MYB182*, a repressor of both anthocyanin and PA biosynthesis, was largely suppressed in 35S::PtrMYB119 poplars, while expression of *MYB134*, an activator of PA biosynthesis, was not changed significantly. Interestingly, 35S::PtrMYB119 poplars exhibited no detrimental growth defects, despite accumulation of high levels of anthocyanins.

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Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana, ecotype Columbia (Col-0), was used in both wild-type and transgenic plant experiments. *Arabidopsis* were grown in soil in a growth room (14 h light; light intensity, 150 μmol m^{-2} s^{-1}) at 23 °C or on half-strength Murashige and Skoog (MS) medium (Sigma-Aldrich Co., St Louis, MO, USA) containing 2% sucrose with appropriate antibiotics for screening. Hybrid poplars (*Populus alba* × *P. tremula* var. *glandulosa*, clone BH) were used as both the nontransformed control plants and transgenic plants in this study. Plants were acclimated in soil and grown in controlled conditions in a growth room (16 h light; light intensity, 150 μmol m^{-2} s^{-1}; 24 °C).

Vector construction and production of transgenic poplars

Full-length cDNAs encoding PtrMYB119 or PtrMYB120 were amplified by polymerase chain reaction (PCR) and inserted downstream of the 35S promoter in the pK2GW7 vector (Karimi et al. 2002) using the Gateway cloning system to produce 35S::PtrMYB119 and 35S::PtrMYB120 constructs. The resulting constructs were verified by DNA sequencing, and the primers used in this study are listed in Table S1 available as Supplementary Data at *Tree Physiology* Online. Vector constructs were introduced into *Agrobacterium tumefaciens* strain C58, which was used to transform *Arabidopsis* and poplar by the floral-dip method (Clough and Bent 1998) and leaf disk transformation-regeneration method (Horsch et al. 1985, Choi et al. 2005), respectively. Transformed cells from poplar were selected on MS medium containing 1.0 mg l^{-1} 2,4-dichlorophenoxyacetic acid, 0.01 mg l^{-1} benzylaminopurine, 0.1 mg l^{-1} 1-naphthylacetic acid (NAA), 500 mg l^{-1} cefotaxime and 50 mg l^{-1} kanamycin. Shoots were regenerated from calli by transferring them to woody plant medium containing 1.0 mg l^{-1} zeatin, 0.1 mg l^{-1} benzyladenine and 0.01 mg l^{-1} NAA. Throughout the experiments, cultures were maintained in a culture room at 25 ± 2 °C and were provided with cool white fluorescent light (150 μmol m^{-2} s^{-1}, 16 h photoperiod).

Histological analysis

Cross sections of poplar stem or petioles were prepared by hand-cutting and observed for anthocyanin pigment accumulation without staining. Proanthocyanidins were detected by staining sections for 10 min with dimethylaminocinnamaldehyde (DMACA) (1% [w/v] in ethanol : 6 N HCl, 1 : 1 [v/v]). Images were captured using a microscope (CHB-213, Olympus, Tokyo, Japan) and camera (DCM900, Oplenic, Hangzhou, China).

RNA extraction and qRT-PCR

Total RNAs were extracted using the cetyl trimethylammonium bromide (CTAB) method with slight modification (Logemann et al. 1987). In brief, plant tissues were ground into a fine powder using liquid nitrogen and mixed with CTAB buffer followed by phenol : chloroform : isoamyl alcohol (25 : 24 : 1) extraction. Isopropanol was added to the mixture to isolate RNA. One microgram of total RNA was reverse-transcribed to produce first-strand cDNA using the PrimeScript™ RT reagent kit (Takara, Otsu, Japan) following the manufacturer's instructions. A semi-quantitative real-time PCR (RT-PCR) was performed as described (Lee et al. 2014). Quantitative real-time PCR (qRT-PCR) was performed using the CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with iQ™ SYBR® Supermix (Bio-Rad). Poplar *ACTIN2* gene was used as the internal quantitative control (Kim et al. 2011), and relative expression level was calculated by the $2^{-\Delta\Delta C_t}$ method (Pfaffl 2001). All primer sequences were designed using Primer3 software (http://fokker.wi.mit.edu). Sequences are provided in Table S1 available as Supplementary Data at *Tree Physiology* Online.

Transient TAA

Preparation of *Arabidopsis* leaf protoplasts and transient transfection of reporter and effector constructs were performed as described previously (Ko et al. 2009, 2012). For the effector constructs, full-length cDNA of PtrMYB119 was ligated between the CaMV 35S promoter and the nopaline synthase terminator after removing GUS from the pTrGUS vector. Reporter constructs were created by placing promoter fragments (*Pro_PtrANS1*, *Pro_PtrANS2*, *Pro_PtrCHS1* and *Pro_AtCesA4*) in front of the GUS reporter gene after removing the 35S promoter from the pTrGUS vector. The primers used for PCR amplification of full-length genes and promoters are listed in Table S1 available as Supplementary Data at *Tree Physiology* Online. Plasmid DNA was prepared using a Plasmid Plus Maxi kit (QIAGEN, Valencia, CA, USA), and 7 μg of reporter and 7 μg of effector plasmids were used for transfections. For internal control for GUS activity normalization, 1 μg of PtrNAN plasmid (Kirby and Kavanagh 2002) was added. Then, 15 μl of plasmid mixture (15 μg) and 200 μl of protoplasts were transferred to 2 ml microcentrifuge tubes following the procedure described by Yoo et al. (2007). β-Glucuronidase and NAN enzyme assays were performed according to Kirby and Kavanagh (2002). NAN and GUS activities were measured using MUN (Sigma-Aldrich Co.) and MUG (Sigma-Aldrich Co.) as substrates, respectively, against MU standards on a Hoefer TK 100 fluorometer (excitation: 355 nm, emission: 460). The ratio of GUS and NAN activities is represented as relative GUS/NAN units. Three biological replicates were used in the experiments.

Sample preparation for biochemical analysis

Freeze-dried samples (leaf tissues of both transgenic and non-transgenic poplar plants) were ground with mortar and pestle. Ground samples (0.8 g) were extracted by 320 ml of methanol/water/acetic acid (79.6 : 19.9 : 0.5, v/v) for 3 h in shaking incubator (25 °C, 150 r.p.m.). The solvent was filtered through Whatman No. 1 filter paper (Whatman International Ltd,

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Maidstone, UK). Extraction procedures were repeated under the same conditions. The solvent was evaporated using a vacuum rotary evaporator (Eyela Co., Tokyo, Japan) at 40 °C.

Quantification of total phenolics and flavonoids

Contents of total phenolic compounds were determined by the method reported by Eom et al. (2009) with some modification. Extract and Folin–Ciocalteu's phenol reagent (20 µl) were added to 2.6 ml of distilled water. After 6 min, 2.0 ml of 7% Na_2CO_3 was added. After 90 min, the absorbance was measured at 750 nm using a spectrophotometer (S-4100; Scinco, Seoul, Korea). The content of total phenolics was determined using a calibration curve for gallic acid (Sigma-Aldrich Co.) as a standard and expressed as mg gallic acid equivalents (GAE) g^{-1} DW.

Contents of total flavonoids were determined by the method described by Zhishin et al. (1999) with some modification. Extract (0.5 ml) was mixed with 3.2 ml of distilled water. Then, 5% $NaNO_2$ (0.15 ml), 10% $AlCl_3$ (0.15 ml) and 1 M NaOH (1.0 ml) were added to the mixture. The absorbance of the mixture was measured at 510 nm using a spectrophotometer (S-4100; Scinco). The content of total flavonoids was determined using a calibration curve for quercetin (Sigma-Aldrich Co.) as the standard and expressed as mg quercetin equivalents (QE) g^{-1} dry weight (DW).

Reverse-phase HPLC analysis for anthocyanins

The extracts were analyzed by reverse-phase high-performance liquid chromatography (HPLC) (Waters 2695 Alliance HPLC, Waters Inc., Milford, MA, USA) using an octadecylsilane column (Prontosil 120-5-C18-ace-EPS, Bischoff, Leonberg, Germany). The flow rate of mobile phase was 1.0 ml min^{-1}. The mobile phases were (i) 10% aqueous formic acid and (ii) 10% methanolic formic acid. Gradient elution was performed as follows: 12–25% of solvent B for 32 min and 25–60% of solvent B for 32–48 min. The injection volume of samples (3 mg ml^{-1} of methanol) was 20 µl. Quantifications of cyanidin-3-*O*-glucoside in transgenic poplars were performed by using cyanidin-3-*O*-glucoside chloride (Chengdu SinoStandards Bio-Tech Co., Chengdu, China) as a standard. Peaks were monitored at 517 nm with a Waters 996 photodiode array detector (Waters Inc.). High-performance liquid chromatography grade solvents were used for mobile phases.

Measurement of chlorophyll fluorescence

To analyze the photosynthetic capacity of transgenic poplars, chlorophyll fluorescence (F_v/F_m) was measured using a Pocket PEA chlorophyll fluorometer (Hansatech, Reutlingen, Germany). The fifth to seventh leaves from poplars grown for 3 months in a greenhouse were placed in the dark for 15 min and then exposed to a strong flash beam. The ratio of variable to maximal fluorescence (F_v/F_m), which approximates the maximum efficiency of photosystem II, was then calculated from the measured normal yield of chlorophyll fluorescence (Bilger et al. 1995, Gao and Peng 2006).

Measurement of growth parameters of poplar

Overall growth parameters of poplars grown for 2 months at living modified organism (LMO) sites (latitude 37.2N, longitude 126.9E) after transplanting 4-month-old greenhouse grown poplars were measured. Parameters assessed were stem height (measured height from top to bottom), diameter (measured stem thickness at 10 cm above the soil level using slide calipers), number of internodes (counted from top to bottom) and leaf area (measured on the 10–12th leaves from the top using a LI-3100 area meter, LI-COR Biosciences, Lincoln, NE, USA).

Results

Identification of poplar MYB TFs that positively regulate anthocyanin biosynthesis

We found a MYB TF (PtrMYB119) that positively regulates anthocyanin production when expressed under the control of the CaMV 35S promoter in transgenic *Arabidopsis* (see Figure S1a and b available as Supplementary Data at *Tree Physiology* Online). Nucleotide sequence analysis in the poplar genome database (Phytozome; http://phytozome.jgi.doe.gov/pz/portal.html) revealed that the *PtrMYB119* gene is located in tandem on chromosome 17 with four more genes (*PtrMYB116*, *PtrMYB117*, *PtrMYB118* and *PtrMYB120*), and all of these genes are homologous to each other. Further analysis revealed that PtrMYB119 is highly homologous to *Arabidopsis* PAP1, a well-known transcriptional activator of anthocyanin biosynthesis (Figure 1).

Phylogenetic analysis showed that all five poplar MYB genes (*PtrMYB116*, *PtrMYB117*, *PtrMYB118*, *PtrMYB119* and *PtrMYB120*) are in the same clade (denoted 'AN' in Figure 1a) as dicot anthocyanin-producing MYBs, including *Petunia* hybrida Anthocyanin2 (PhAN2), tomato Anthocyanin1 (LeANT1), sweet potato MYB1 (IbMYB1), grape VvMYBA1 and *Arabidopsis* AtPAP1. The 'AN' clade is clearly separated from the 'PA' clade, which includes PA-producing MYBs such as AtTT2, VvMYBPA1 and PtrMYB134 (Figure 1a).

In a subsequent amino acid sequence alignment analysis, we found that all five poplar MYBs have three conserved motifs identified in anthocyanin-producing R2R3-MYB TFs in plants (e.g., *Arabidopsis*, sweet potato and grape), apart from the very well-conserved DNA-binding domain, referred to as the R2R3 domain (Figure 1b). The first motif, [D/E]Lx2[R/K]x3Lx6Lx3R (also known as the ID domain, Grotewold et al. 2000, Stracke et al. 2001, Zimmermann et al. 2004), is involved in the interaction with bHLH proteins, and it is present in the R3 domain of PtrMYBs. The second conserved motif, ANDV, is also found in the R3 domain of PtrMYBs. The ANDV motif was identified by Lin-Wang et al. (2010) in their comparative sequence analysis of anthocyanin-promoting MYBs of dicot plants. The [R/K]Px[P/A/R]xx[F/Y] motif, which has been found in the C-terminal region of anthocyanin-regulating MYBs

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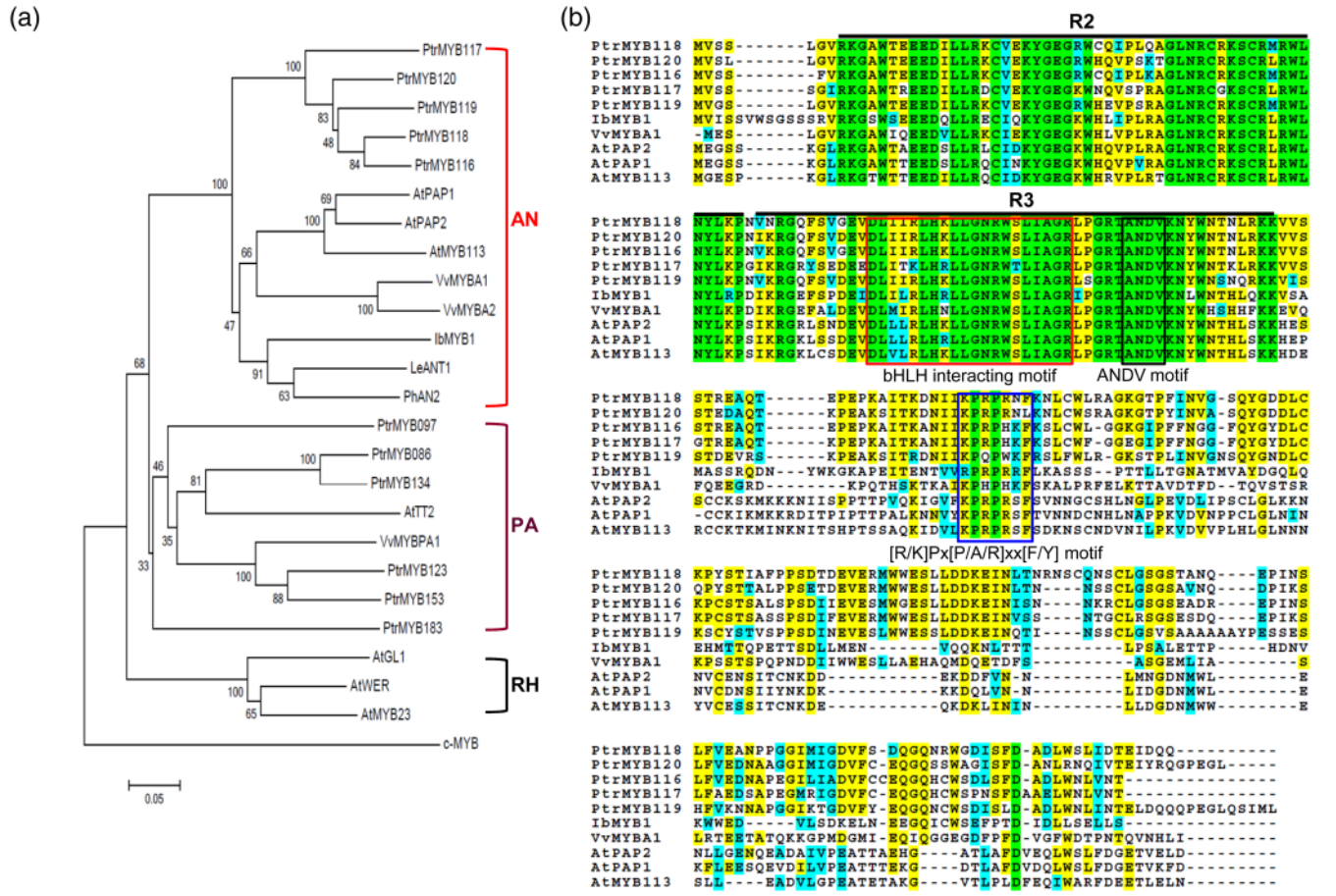

Figure 1. PtrMYB119 and PtrMYB120 belong to the R2R3-MYB family of TFs involved in anthocyanin biosynthesis. (a) Phylogenetic analysis of the five poplar R2R3-MYB proteins identified in this study with selected R2R3-MYB proteins. Complete amino acid sequences were aligned using ClustalW and the rooted phylogenetic tree was constructed using MEGA 6.0 (Tamura et al. 2013) with the minimum evolution method (1000 bootstrap replicates) and p-distance model. Anthocyanin (AN) and proanthocyanidin (PA) biosynthetic subgroups are indicated. Human c-Myb and the root hair regulating subgroup (RH) were included as outgroups. GenBank accession numbers or locus IDs are as follows: PtrMYB116 (*Populus trichocarpa*, Potri.017G125900), PtrMYB117 (*P. trichocarpa*, Potri.017G126000), PtrMYB118 (*P. trichocarpa*, Potri.017G125800), PtrMYB119 (*P. trichocarpa*, Potri.017G125600), PtrMYB120 (*P. trichocarpa*, Potri.017G125700), AtPAP1 (*Arabidopsis thaliana*, AAG42001), AtPAP2 (*A. thaliana*, NP_176813), AtMYB113 (*A. thaliana*, NP_176811), VvMYBA1 (*Vitis vinifera*, BAD18977), VvMYBA2 (*V. vinifera*, BAD18978), IbMYB1 (*Ipomoea batatas*, BAG68211), LeANT1 (*Lycopersicon esculentum*, AAQ55181), PhAN2 (*Petunia × hybrida*, AAF66727), PtrMYB086 (*P. trichocarpa*, Potri.018G049600), PtrMYB097 (*P. trichocarpa*, Potri.006G275900), PtrMYB123 (*P. trichocarpa*, Potri.003G144200), PtrMYB134 (*P. trichocarpa*, Potri.006G221800), PtrMYB153 (*P. trichocarpa*, Potri.003G144300), PtrMYB183 (*P. trichocarpa*, Potri.006G221500), AtTT2 (*A. thaliana*, Q9FJA2), VvMYBPA1 (*V. vinifera*, AM259485), AtGL1 (*A. thaliana*, P27900), AtMYB23 (*A. thaliana*, NP_198849), AtWER (*A. thaliana*, NP_196979) and c-Myb (*Homo sapiens*, AAB49039). (b) Amino acid sequence alignment of the five poplar R2R3-MYB proteins with *Arabidopsis* (AtPAP1, AtPAP2 and AtMYB113), grape (VvMYBA1) and sweet potato (IbMYB1) R2R3-MYB proteins. The black bars indicate the R2 and R3 repeats of the MYB domain. The [D/E]Lx2[R/K]x3 Lx6Lx3R motif necessary for interaction with bHLH TFs is indicated by the box in the R3 repeat. ANDV and [R/K]Px[P/A/R]xx[F/Y] motifs are indicated by the boxes.

(Lin-Wang et al. 2010), was conserved in all the aligned sequences (Figure 1b).

Taken together, our sequence analyses suggested that PtrMYB119 belong to the R2R3-MYB family of TFs involved in anthocyanin biosynthesis, which is consistent with the result of Wilkins et al. (2009).

PtrMYB119 is highly expressed in male catkin tissues

Tissue-specific expression of all five PtrMYBs was evaluated using previously generated poplar tissue-specific transcriptome data (Wilkins et al. 2009, Ko et al. 2012). There was no significant expression of any of the five PtrMYB genes in any of the tissues tested (e.g., shoot apical, leaf primordia, mature leaf and stem tissues) with the exception of male and female catkins (see Figure S2 available as Supplementary Data at *Tree Physiology* Online). All PtrMYBs were expressed at high levels in male catkins. Because male catkins have anthers with a strong red pigmentation (Wilkins et al. 2009), our expression data suggested that PtrMYB119 may have a functional role in anthocyanin accumulation.

35S::PtrMYB119 transgenic hybrid poplars exhibit high-level accumulation of anthocyanins

To further characterize the molecular function of putative anthocyanin-producing MYB TFs of poplar, we produced transgenic

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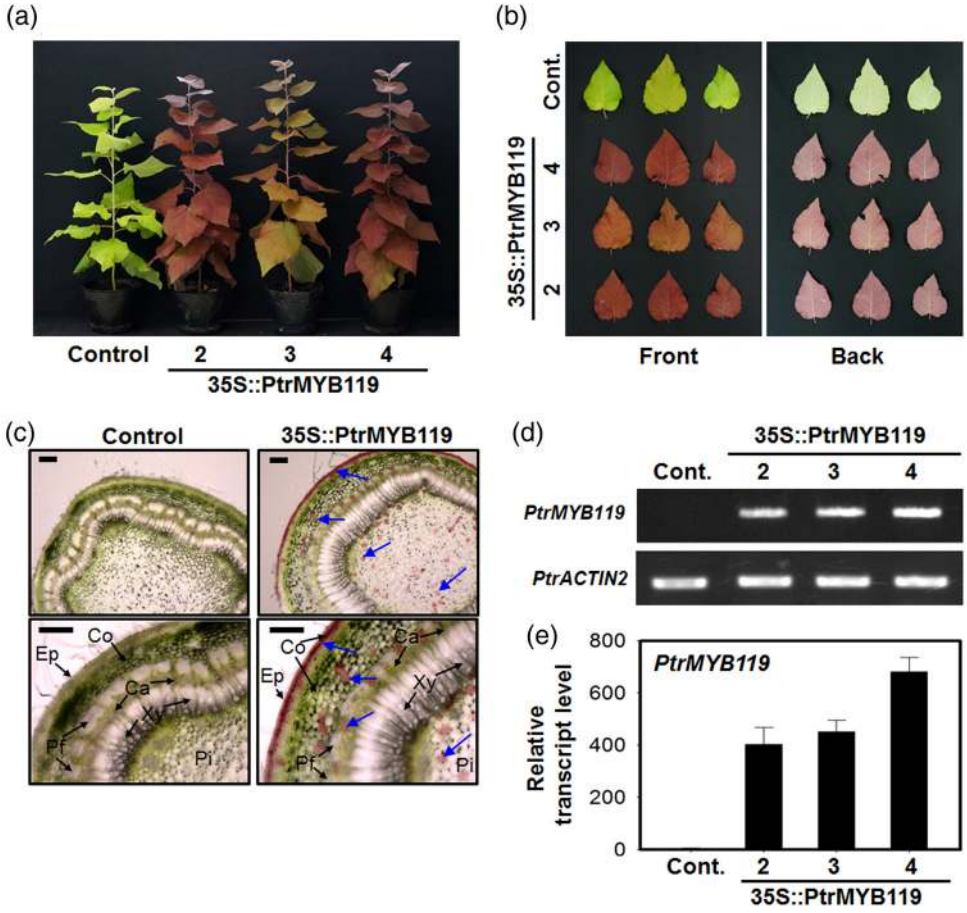

Figure 2. Overexpression of PtrMYB119 in a transgenic hybrid poplar resulted in high-level accumulation of red pigmentation. (a) Transgenic poplars overexpressing PtrMYB119 (35S::PtrMYB119) had strong red-color pigmentation in whole plant relative to nontransformed control plants (control). Three independent transgenic poplar lines (e.g., #2, 3 and 4) and the control are shown. (b) Red-colored pigmentation in the leaves of transgenic poplars. The 10–12th leaves from the top of transgenic poplars were arranged for comparison with control. (c) Observation of red-colored pigmentation at the cellular level in stem cross sections of transgenic poplars and the control. Gray-colored arrows indicate red-colored cells. Ep, epidermis; Co, cortex; Ca, cambium; Pf, phloem fiber; Xy, xylem; Pi, pith. Scale bars represent 100 μm. (d, e) Expression of the *PtrMYB119* gene in the independent transgenic poplar lines compared with the control. First-strand cDNAs were synthesized from total RNA extracted from leaf tissues and used as template in semi-quantitative RT-PCR (d) or qRT-PCR (e) experiments. Relative transcript levels were determined using the *PtrACTIN2* gene as a quantitative control. Four-month-old poplars grown in a pot were used in these experiments. Error bars indicate standard deviations of three independent experiments. Note that red-colored pigmentations are shown in a darker tone in the black and white image.

hybrid poplar lines overexpressing *PtrMYB119* and *PtrMYB120*, respectively (i.e., 35S::PtrMYB119 and 35S::PtrMYB120). As expected, significant changes in pigmentation were observed in all parts of both 35S::PtrMYB119 and 35S::PtrMYB120 transgenic hybrid poplars. In this report, we focused on characterizing 35S::PtrMYB119 poplar lines, because no significant differences were observed between this transgenic line and 35S::PtrMYB120 lines (see Figure S1c available as Supplementary Data at *Tree Physiology* Online). For further comparative phenotypic analysis, we selected two strong (i.e., #2 and 4) and one mild (i.e., #3) transgenic 35S::PtrMYB119 poplar lines in terms of red-color pigmentation in stem and leaf tissues (Figure 2a and b). To visualize the accumulation of red pigments at the cellular level, we prepared stem cross sections and observed the tissue without any staining. Compared with nontransformed control poplar cross sections, numerous red pigmented cells were found in the pith, cortex, phloem, ray cells and even cambium layers of 35S::PtrMYB119 poplars (Figure 2c, see Figure S4 available as Supplementary Data at *Tree Physiology* Online). In particular, red pigments were concentrated in the outer layer of subepidermal cells, which explained the intense redness of the stem and leaves of 35S::PtrMYB119 poplar lines. Expression level of the *PtrMYB119* gene in three selected lines (i.e., #2, 3 and 4) was analyzed by semi-quantitative RT-PCR as well as qRT-PCR and compared with the nontransformed control poplar (Figure 2d and e). *PtrMYB119* was upregulated by as much as 400-fold in the selected lines relative to nontransformed control poplar (Figure 2e). However, the expression level of the *PtrMYB119* gene in each line was not linearly correlated to the red phenotype.

Total flavonoids and total phenolics were quantified using leaf tissues of 2-month-old poplars (Figure 3). The contents of total

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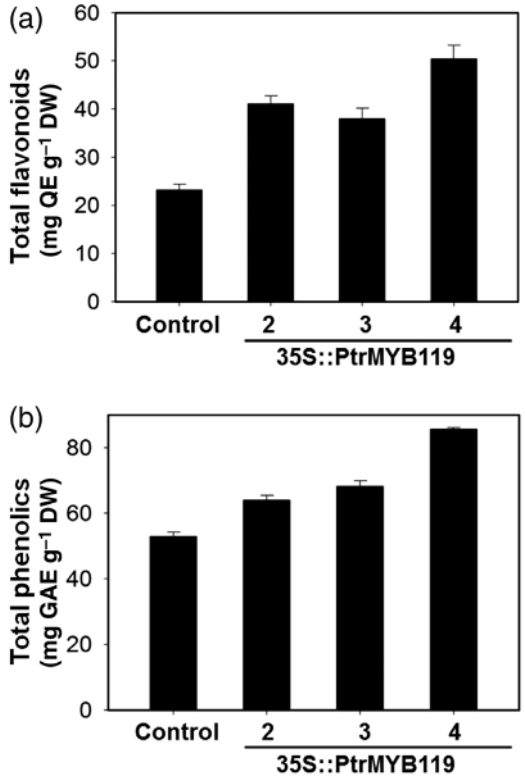

Figure 3. Increased accumulation of total flavonoids and total phenolics in the 35S::PtrMYB119 transgenic poplars. (a) Quantification of total flavonoid contents in the three independent 35S::PtrMYB119 poplar lines with control. Total flavonoids were determined using a calibration curve for quercetin as a standard and expressed as mg QE g^{-1} DW. (b) Quantification of total phenolics in the three independent 35S::PtrMYB119 poplar lines with control. Total phenolics were determined using a calibration curve for gallic acid as a standard and expressed as mg GAE g^{-1} DW. Leaves of 2-month-old poplars grown in pots were used in these experiments. Error bars represent standard deviations of three independent experiments.

flavonoids were increased more than twofold in 35S::PtrMYB119 poplar line (#4) compared with nontransformed control poplars (Figure 3a). Total phenolics were also increased significantly in transgenic lines compared with nontransformed control poplars (Figure 3b).

To characterize the chemical nature of the accumulated anthocyanin in transgenic lines, we performed a reverse-phase HPLC. Based on the UV–visible spectra, showing two peaks at 278.5 and 517 nm (Figure 4), the chemical identity of the accumulated anthocyanin was supposed to be a glucoside derivative of cyanidin as reported by Aguilar and Hernández-Brenes (2015) and Skaar et al. (2014). Using a cyanidin-3-*O*-glucoside as a standard, we confirmed that majority of the accumulated anthocyanin in our transgenic poplar is the cyanidin-3-*O*-glucoside (Figure 4). Our reverse-phase HPLC chromatogram clearly showed a massive accumulation of cyanidin-3-*O*-glucoside in leaf tissues of 35S::PtrMYB119 poplar lines (e.g., 1.17 mg g^{-1} dry weight (DW) of line #4), while no detectable cyanidin-3-*O*-glucoside was found in nontransformed control poplar (Table 1).

These results demonstrated that PtrMYB119 is a positive regulator of anthocyanin biosynthesis in poplar trees and that the red pigmentation of 35S::PtrMYB119 transgenic poplars was caused by accumulation of cyanidin-3-*O*-glucoside.

Expression of genes in the anthocyanin biosynthesis pathway is upregulated in 35S::PtrMYB119 transgenic hybrid poplars

Anthocyanin accumulation has been shown to have a positive correlation with the expression of anthocyanin biosynthetic genes (Paz-Ares et al. 1987, Borevitz et al. 2000, Espley et al. 2007). Because our 35S::PtrMYB119 transgenic poplars exhibited highly enhanced anthocyanin accumulation (Figures 2

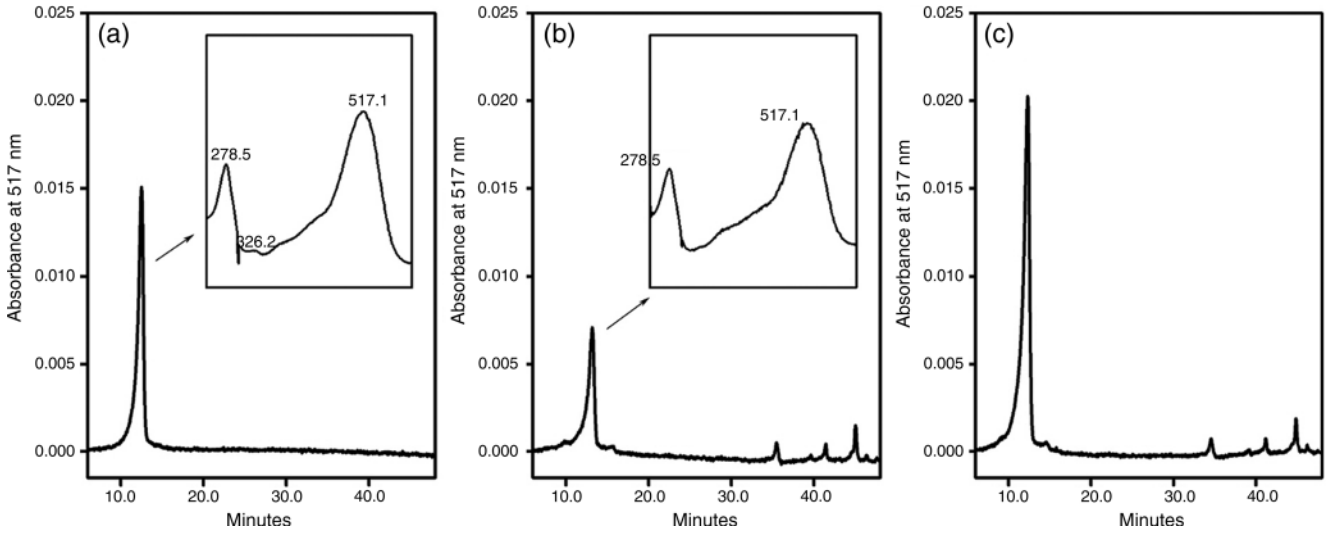

Figure 4. Accumulation of cyanidin-3-*O*-glucoside in 35S::PtrMYB119 transgenic poplars. Reverse-phase HPLC chromatogram monitored at 517 nm and UV–visible spectra of major anthocyanin peak (insets of (a) and (b)). (a) Cyanidin-3-*O*-glucoside as a standard (10 μM). (b) 35S::PtrMYB119 poplar line #2 (1 mg ml^{-1}). (c) 35S::PtrMYB119 poplar line #2 (1 mg ml^{-1}) with cyanidin-3-*O*-glucoside standard (10 μM).

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Table 1. Quantification of cyanidine-3-*O*-glucoside from transgenic poplars. ND, not determined. Data are presented as mean ± standard deviation ($n = 3$).

	Control	35S::PtrMYB119		
		2	3	4
Cyanidin-3-*O*-glucoside (mg g^{-1} DW)	ND	0.85 ± 0.01	0.32 ± 0.10	1.17 ± 0.19

and 3), we quantified the expression of genes involved in the anthocyanin biosynthesis pathway by qRT-PCR in three independent lines and compared this with nontransformed control poplar (Figure 5). Expression of *phenylalanine ammonialyase* (*PtrPAL1*) and *4 coumarate CoA ligase* (*Ptr4CL2*) genes, which are involved in the initial steps of the flavonoid pathway, was upregulated (Figure 5a and b), while no significant changes in *PtrPAL2* and *cinnamic acid 4-hydroxylase* (*PtrC4H1*) expression were observed in 35S::PtrMYB119 transgenic poplars (see Figure S5 available as Supplementary Data at *Tree Physiology* Online). As expected, increased expression of anthocyanin biosynthetic genes such as *chalcone synthase* (*PtrCHS1* and *PtrCHS2*), *chalcone isomerase* (*PtrCHI1*), *flavonoid 3′-hydroxylase* (*PtrF3H1*), *dihydroflavonol reductase* (*PtrDFR1*), *anthocyanidin synthase* (*PtrANS1* and *PtrANS2*) and *UDP glucose: flavonoid-3-O-glucosyltransferase* (*PtrUFGT1*) was observed (Figure 5c–j). Among these genes, expression levels of *PtrCHS1*, which catalyzes the initial step of anthocyanin biosynthesis and *PtrANS2*, which catalyzes the last step of anthocyanin biosynthesis, were upregulated by >300-fold. Taken together, these results suggested that overexpression of *PtrMYB119* induced anthocyanin pigment production by elevating transcript levels of multiple anthocyanin biosynthetic genes.

35S::PtrMYB119 transgenic hybrid poplars accumulate more PAs than nontransformed control poplars

Interestingly, the leucoanthocyanidin reductase (*PtrLAR1*) and anthocyanidin reductase (*PtrANR1*) genes, which are specific to the PA branch of the pathway, were upregulated by up to 12-fold (i.e., *PtrANR1*) in the 35S::PtrMYB119 transgenic poplars. To examine whether the 35S::PtrMYB119 transgenic poplars accumulated more PAs, we performed DMACA staining, which is commonly used for PA detection (Xie et al. 2003). Proanthocyanidins are major flavonoids in poplars (Osier and Lindroth 2004, Miranda et al. 2007, Mellway et al. 2009), and blue coloration, linked to the presence of PA, was observed in the epidermal cell layers of stems and petioles of nontransformed control poplars (Figure 6). The 35S::PtrMYB119 transgenic poplars showed much stronger blue coloration than control poplars not only in epidermal cell layers but also in cortex and pith cells (Figure 6). These results suggested that overexpression of *PtrMYB119* affects PA biosynthesis positively in poplar.

Since poplar MYB134 has been known as a specific and positive transcriptional regulator of PA biosynthesis (Mellway et al. 2009), we quantified expression of *MYB134* in the 35S::PtrMYB119 transgenic poplars compared with nontransformed control. Our result showed no significant changes of *MYB134* expression (Figure 7a). Interestingly, expression of *MYB182*, a repressor of both anthocyanin and PA biosynthesis (Yoshida et al. 2015), was largely suppressed in all the 35S::PtrMYB119 poplar lines (Figure 7b).

PtrMYB119 activates reporter gene expression in transfected protoplasts

To further verify the strong upregulation of *PtrCHS1* and *PtrANS2* in 35S::PtrMYB119 transgenic poplars, we analyzed the ability of PtrMYB119 to activate the promoters of these genes using transient transcriptional activation assays (TAAs) as described previously (Ko et al. 2009). To do this, the promoter regions (~1 kb) of *PtrCHS1* and *PtrANS2* genes were used to drive β-glucuronidase (GUS) reporter gene expression using PtrMYB119 as an effector (Figure 8a). We found that PtrMYB119 strongly activated expression of all promoters tested, with the exception of the *AtCesA4* promoter, which is involved in secondary wall-specific cellulose biosynthesis (Taylor et al. 2003, Ko et al. 2009). We thus used this as an internal negative control in our TAA experiments (Figure 8). Accordingly, when we used AtMYB46 as an effector, a master regulator of secondary wall biosynthesis (Ko et al. 2009), only *AtCesA4* promoter was activated (data not shown). Consistent with the qRT-PCR results (Figure 5), PtrMYB119 activated both *PtrCHS1* and *PtrANS2* promoters by up to 30- and 20-fold more than control levels, respectively, but only upregulated the *PtrANS1* promoter by five-fold (Figure 8b). This result suggested that both *PtrCHS1* and *PtrANS2* may be direct downstream targets of PtrMYB119. To support this result, we used AtMYB46 as an effector, a master regulator of secondary wall biosynthesis, in this experiment and found that AtMYB46 activates only *AtCesA4* promoter but not *PtrCHS1*, *PtrANS1* and *PtrANS2* promoters (data not shown).

High-level accumulation of anthocyanin does not have an adverse effect on the growth of hybrid poplars

The bright red colors of the entire plant body of the 35S::PtrMYB119 transgenic poplars were reminiscent of the autumnal tints that signal the end of the growing season. We therefore hypothesized that transgenic poplars with high-level accumulation of anthocyanins would grow less than nontransformed controls. However, the overall growth of transgenic poplars that were grown for 2 months at an LMO field after transplantation of 4-month-old greenhouse grown poplars was comparable to that of nontransformed controls in terms of

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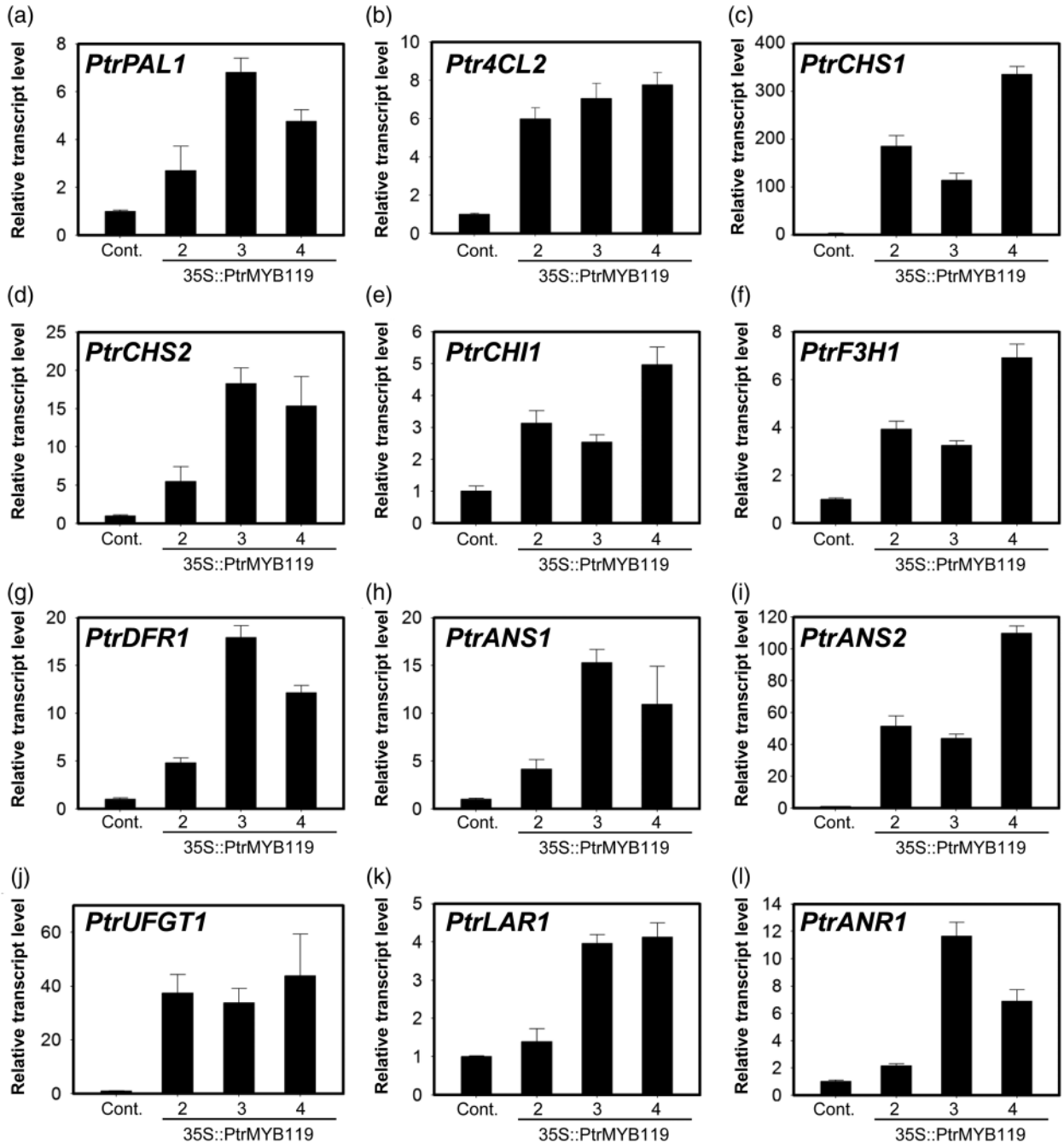

Figure 5. Expressional analysis of anthocyanin biosynthetic genes in 35S::PtrMYB119 transgenic poplars. (a) *PtrPAL1* (phenylalanine ammonia-lyase 1, Potri.006G126800.1), (b) *Ptr4CL2* (4-coumaroyl-CoA: ligase2, Potri.019G049500.2), (c) *PtrCHS1* (chalcone synthase1, Potri.014G145100.2), (d) *PtrCHS2* (chalcone synthase2, Potri.001G051500.1), (e) *PtrCHI1* (chalcone isomerase1, Potri.010G213000.1), (f) *PtrF3H1* (flavanone 3-hydroxylase1, Potri.005G113900.1), (g) *PtrDFR1* (dihydroflavonol reductase1, Potri.002G033600.1), (h) *PtrANS1* (anthocyanidin synthase1, Potri.003G119100.1), (i) *PtrANS2* (anthocyanidin synthase2, Potri.001G113100.1), (j) *PtrUFGT1* (UDP glucose: flavonoid-3-*O*-glucosyltransferase1, Potri.013G118700.1), (k) *PtrLAR1* (leucoanthocyanidin reductase1, Potri.008G116500.1) and (l) *PtrANR1* (anthocyanidin reductase1, Potri.004G030700.1). Quantitative real-time PCRs were performed using first-strand cDNA synthesized from total RNAs that were extracted from leaf tissues of 4-month-old poplars. Relative transcript levels were determined using the *PtrACTIN2* gene as a quantitative control. Error bars indicate standard deviations of three independent experiments.

height, diameter, internode number and leaf area (Figure 9a–d). In addition, we measured chlorophyll fluorescence from leaves to assess the efficiency of photosystem II photochemistry (Bilger et al. 1995, Willits and Peet 2001, Gao and Peng 2006, Kadir 2006). Resulting F_v/F_m ratios were not significantly different between transgenic poplars and nontransformed control poplars (Figure 9f), although there were considerable changes of leaf colors (Figure 9e). These results suggested that

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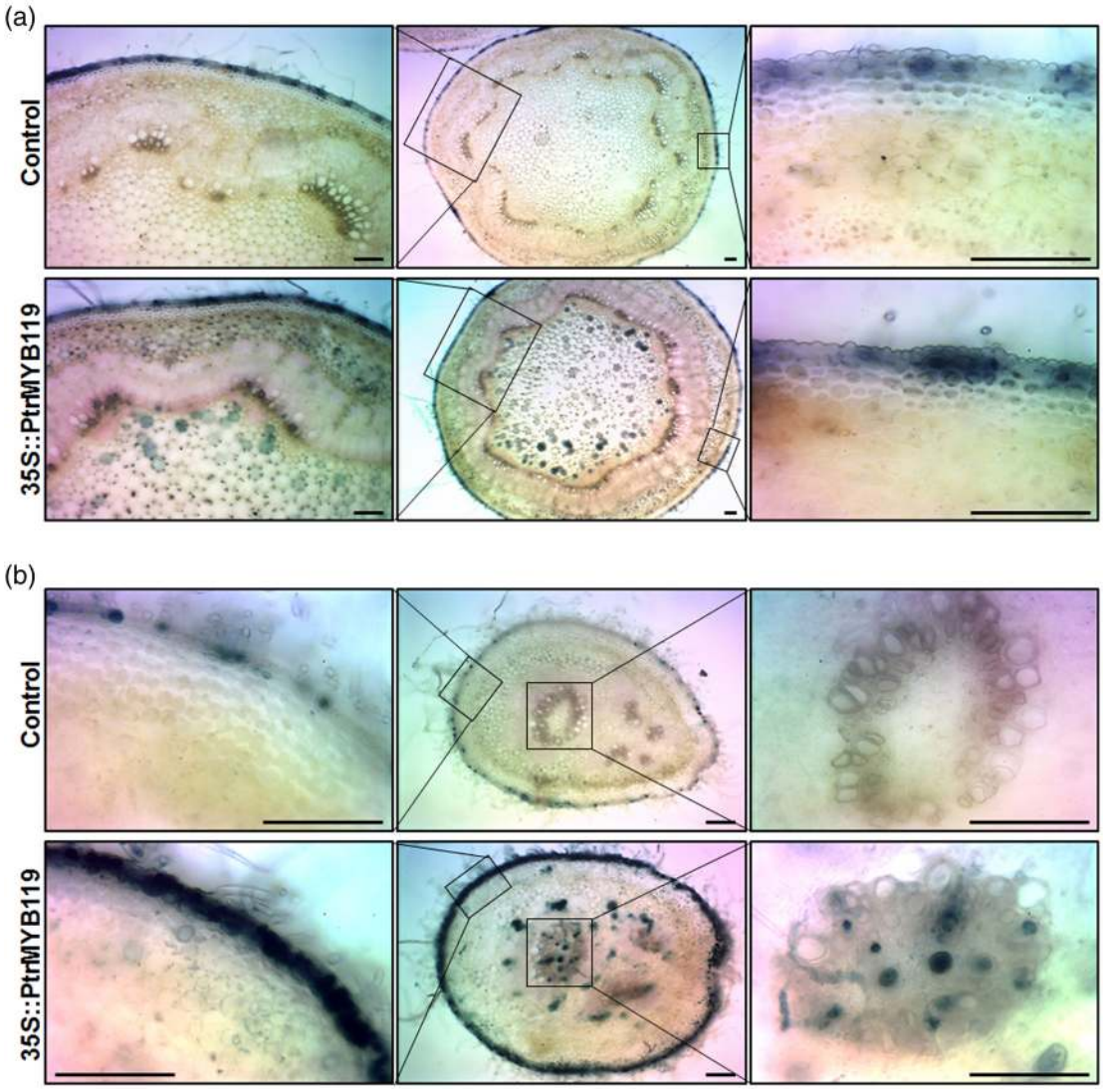

Figure 6. 35S::PtrMYB119 transgenic hybrid poplars accumulate more PAs than nontransformed control poplars. Proanthocyanidin accumulation in transgenic poplar expressing 35S::PtrMYB119. Dimethylaminocinnamaldehyde staining was used to investigate PA accumulation in the seventh internode of the stem (a) and petioles (b) of poplars, which were grown for 2 months at an LMO field after transplanting 4-month-old greenhouse grown poplars. Scale bars represent 100 μm.

elevated accumulation of anthocyanins did not affect the growth of 35S::PtrMYB119 transgenic hybrid poplars adversely, at least within our observation period.

Discussion

Many R2R3-MYB TFs responsible for anthocyanin production in fruits, flowers and leaves have been characterized in plant species, e.g., *Arabidopsis* PAP1, maize C1, grapevine VvMYBA1, sweet potato IbMYB1 and *Petunia* PhAN2 (Roth et al. 1991, Quattrocchio et al. 1999, Borevitz et al. 2000, Teng et al. 2005, Lijavetzky et al. 2006, Mano et al. 2007, Gonzalez et al. 2008, Lin-Wang et al. 2010).

In this study, we found a total of five poplar R2R3-MYB TFs (i.e., *PtrMYB116*, *PtrMYB117*, *PtrMYB118*, *PtrMYB119* and *PtrMYB120*) and suggest that these MYB TFs function as positive regulators of anthocyanin biosynthesis for the following reasons. First, all five of the poplar R2R3-MYB TFs are paralogs of one other and exhibit the highest sequence similarity to R2R3-MYB TFs required for anthocyanin biosynthesis, but not PA biosynthesis, in plants (Figure 1a). Second, all the MYB TFs (*PtrMYB116*, *PtrMYB117*, *PtrMYB119* and *PtrMYB120*) are expressed strongly in male catkins, which have anthers with strong red pigmentation (Wilkins et al. 2009, see Figure S2 available as Supplementary Data at *Tree Physiology* Online). Third, overexpression of *PtrMYB119* and/or *PtrMYB120* in both transgenic *Arabidopsis* and poplars driven by the constitutive 35S promoter resulted in marked accumulation of anthocyanins in whole plants (see Figures S1 and S4 available as Supplementary Data at *Tree Physiology* Online, Figures 2–4).

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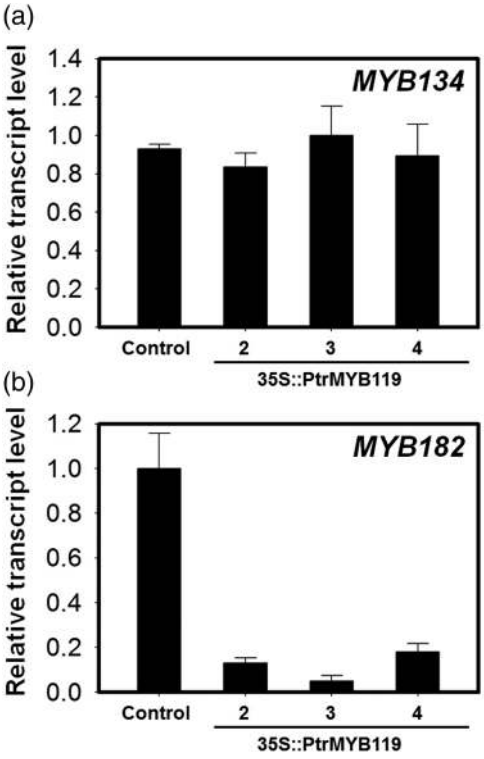

Figure 7. Expression of *MYB134* and *MYB182* in 35S::PtrMYB119 transgenic poplars. (a) *MYB134* (Potri.006G221800.1) and (b) *MYB182* (Potri.004G088100.1). Quantitative real-time PCRs were performed using first-strand cDNA synthesized from total RNAs that were extracted from leaf tissues of 4-month-old poplars. Relative transcript levels were determined using the *PtrACTIN2* gene as a quantitative control. Error bars indicate standard deviations of three independent experiments.

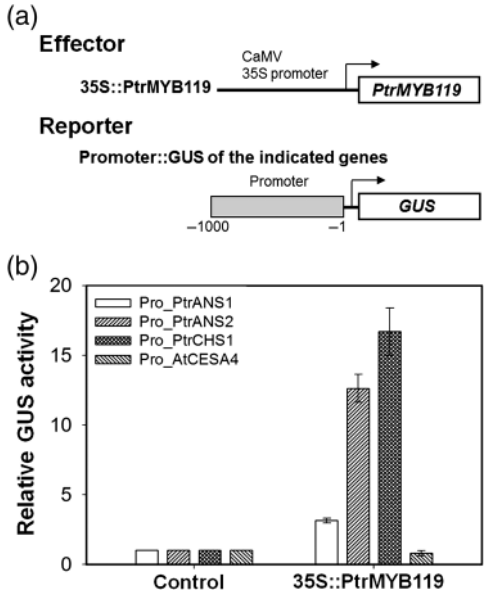

Figure 8. PtrMYB119 activates the expression of genes involved in anthocyanin biosynthesis in vivo. PtrMYB119 was coexpressed in *Arabidopsis* leaf protoplasts with the GUS reporter gene driven by the promoters of the *PtrANS1*, *PtrANS2* and *PtrCHS1* genes, together with PtrNAN for normalization (see Materials and methods). *AtCesA4* promoter was used as a negative control. Activation of the promoter by PtrMYB119 was measured by assaying GUS activity after 16-h incubation. (a) Diagram of the effector and reporter constructs used in this TAA. The effector construct contained the PtrMYB119 gene driven by the CaMV 35S promoter. The reporter constructs consisted of the GUS reporter gene driven by the promoters of the indicated genes. (b) Transcriptional activation assay showing the effects of PtrMYB119 on induction of the promoters of *PtrANS1*, *PtrANS2* and *PtrCHS1*. The expression level of the GUS reporter gene in the protoplast transfected with no effector was used as a control and was set to 1 after normalization. Error bars indicate standard errors of three biological replicates.

Fourth, overexpression of *PtrMYB119* activated transcription of most of the genes involved in the anthocyanin biosynthetic pathway, including *PtrCHS1*, *PtrANS2* and *PtrUFGT1* (Figures 5 and 8).

Wilkins et al. (2009) reported that the five poplar R2R3-MYB TFs have a modified R3 DNA-binding domain, comprising a four amino acid addition (QV[K/Q]M) directly preceding the first conserved Trp in the R3 repeat. They reasoned that the additional four amino acids in the five poplar R2R3-MYB TF proteins likely affected the binding specificity or selectivity of the TFs. However, our sequence analysis revealed no additional four amino sequence in the full-length cDNA clones of either *PtrMYB119* or *PtrMYB120* (Figure 1b). This result indicates that the four amino acid sequence addition comes from an error in splice site prediction during annotation of the poplar genome sequence.

Genes in the anthocyanin biosynthetic pathway are grouped into two subsets in *Arabidopsis*: early biosynthetic genes (EBGs) and late biosynthetic genes (LBGs). The expression of EBGs (e.g., *CHS*, *CHI*, *F3H* and *F3′H*) is regulated by three functionally redundant R2R3-MYB TFs, MYB11, MYB12 and MYB111 (Mehrtens et al. 2005, Stracke et al. 2007, Li 2014), while LBGs (e.g., *DFR*, *ANS* and *UFGT*) are regulated by an MBW transcriptional activator complex (Gonzalez et al. 2008, Petroni and Tonelli 2011, Li 2014, Xu et al. 2014). However, our qRT-PCR and TAA results (Figures 5 and 8) suggested that PtrMYB119 controls the expression of both EBGs (*PtrPAL1*, *Ptr4CL2*, *PtrCHS1*, *PtrCHS2*, *PtrCHI1* and *PtrF3′H1*) and LBGs (*PtrDFR1*, *PtrANS1* and *PtrANS2*) at the transcriptional level. Chu et al. (2013) reported a similar result for sweet potato IbMYB1.

Anthocyanins are inherently unstable under the physiological condition. Thus, immediate modifications (e.g., glycosylation, methylation and acylation) after biosynthesis are required for their stability. Glycosylation is a critical modification for increasing the hydrophilicity and stability of anthocyanins (He and Giusti 2010). UDP glucose: flavonoid-3-*O*-glucosyltransferase has been known to function in this modification and be very important in anthocyanin accumulation in plants (Ford et al. 1998). The expression of *UFGT* was under extremely strict control for its tissue, developmental stages and substrate specificity (Offen et al. 2006). In the 35S::PtrMYB119 transgenic poplars, *PtrUFGT1* was upregulated up to 40-fold compared with control (Figure 5j). This result might explain the high-level and stable

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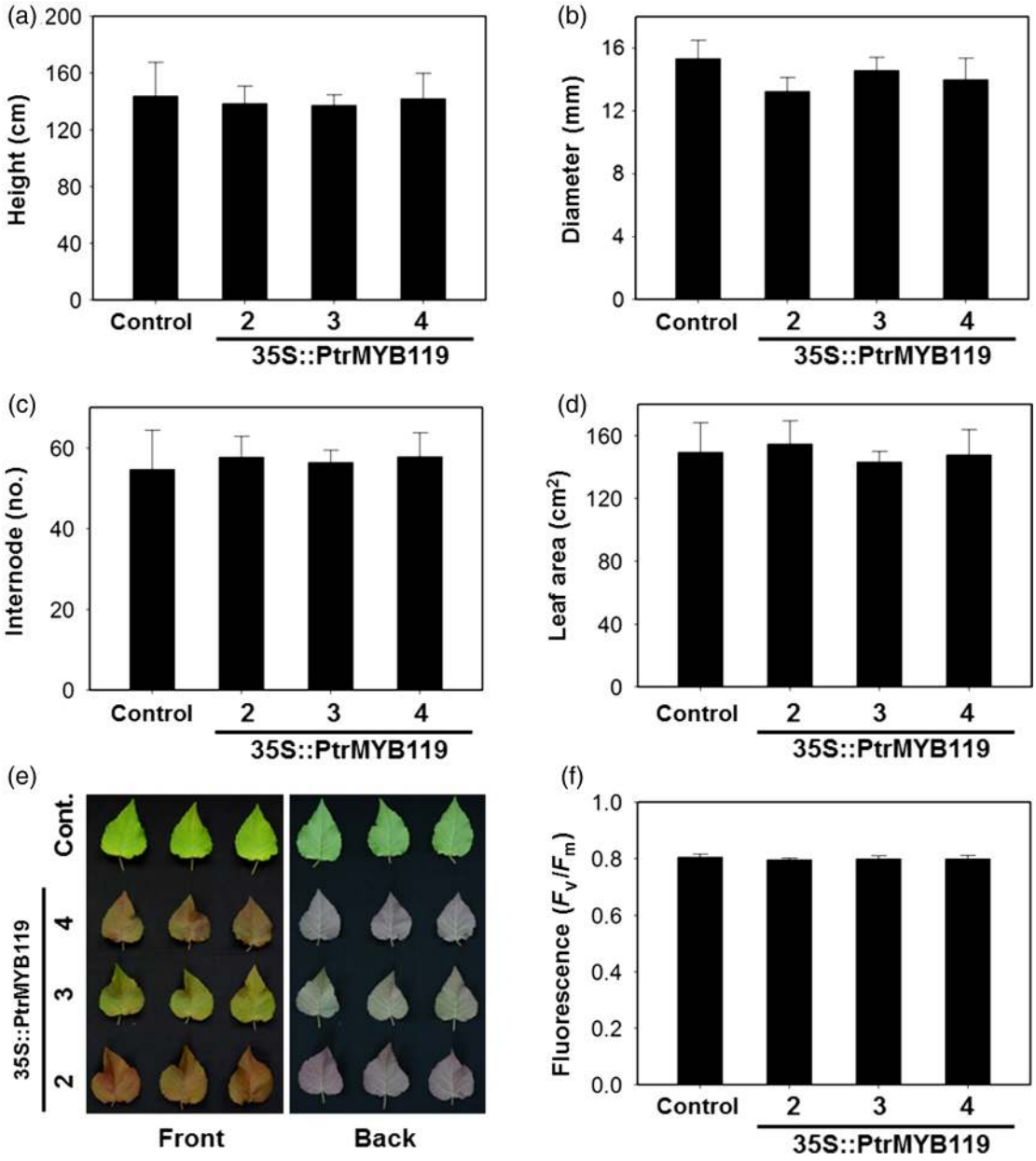

Figure 9. High-level accumulation of anthocyanin does not adversely affect the growth of 35S::PtrMYB119 transgenic hybrid poplars. Overall growth of three independent transgenic poplars grown for 2 months at an LMO field after transplanting 4-month-old greenhouse grown poplars was compared with nontransformed control. (a) Height, (b) diameter, (c) number of internodes, (d) leaf area and (e) shape and colors of leaves. Fifth to seventh leaves from the top are shown. (f) Measurement of fluorescence (F_v/F_m) from poplar leaves grown for 4 months in a greenhouse. Error bars indicate standard deviations of three independent experiments.

accumulation of anthocyanins in the 35S::PtrMYB119 transgenic poplars.

In addition, DMACA staining to analyze the accumulation of PA suggested that PtrMYB119 is a positive regulator of PA biosynthesis (Figure 6). This result is consistent with the findings reported for grape VvMYB5a and VvMYB5b, which promote strong accumulation of both anthocyanins and PAs in flowers (Deluc et al. 2006). In poplar, PtMYB134 has been known as a positive and specific regulator of PA biosynthesis (Mellway et al. 2009). Thus, we performed qRT-PCR to quantify expression of *MYB134* in the 35S::PtrMYB119 transgenic poplars. However, we could not find any significant changes in *MYB134* expression (Figure 7a), suggesting that the PA accumulation observed in the 35S::PtrMYB119 transgenic poplars is probably not caused by MYB134-mediated regulation. To our surprise, expression of *MYB182* was greatly suppressed in all the 35S::PtrMYB119 poplar lines (Figure 7b). This result suggested that high-level accumulation of anthocyandins in the 35S::PtrMYB119 transgenic poplars may be partially attributed to the suppression of a negative regulator, MYB182 (Yoshida et al. 2015).

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The regulatory mechanism of anthocyanin biosynthesis in plants has been studied extensively, and the MBW TF complex has been shown to activate transcription of target genes by interacting directly with the promoter DNA via multiple *cis*-elements (Koes et al. 2005, Ramsay and Glover 2005, Feller et al. 2011, Lai et al. 2013, Xu et al. 2014). We found that PtrMYB119 or PtrMYB120 alone was sufficient in both *Arabidopsis* and poplar plants to promote anthocyanin biosynthesis through transcriptional regulation of anthocyanin biosynthetic genes (see Figure S1 available as Supplementary Data at *Tree Physiology* Online, Figures 2–4). In addition, coexpression of *PtrMYB119* alone in *Arabidopsis* leaf mesophyll protoplasts activated the promoters of the *PtrANS1*, *PtrANS2* and *PtrCHS1* genes (Figure 8). Similar results were reported for sweet potato IbMYB1 (Chu et al. 2013) and *Phalaenopsis* PeMYBs (Hsu et al. 2015). However, we could not exclude the possibility that a bHLH protein involved in anthocyanin biosynthesis was induced in the transgenic *Arabidopsis* and poplars overexpressing either *PtrMYB119* or *PtrMYB120*, because induction of the bHLH/TT8 gene was reported when *IbMYB1* and *PAP1* were overexpressed in *Arabidopsis* (Tohge et al. 2005, Chu et al. 2013). Thus, isolation and characterization of bHLH partners and WD40 coregulators of PtrMYB119 is very important to better understand the transcriptional regulation of anthocyanin biosynthetic genes by the PtrMYB119–bHLH–WD40 complex.

Anthocyanins are a group of colorful and bioactive natural pigments important not only in plants (Gould et al. 2002, Steyn et al. 2002, Zhang et al. 2011) but also in humans because of their many health-promoting properties, such as their antioxidative, anti-inflammatory, anticarcinogenic and antimicrobial activities, and their ability to prevent cardiovascular disease and diabetes (Ghosh and Konishi 2007, Jing et al. 2008, He and Giusti 2010, Pascual-Teresa et al. 2010, Speciale et al. 2010, Cândido et al. 2015). Butelli et al. (2008) reported that feeding mice a diet supplemented with transgenic tomatoes rich in anthocyanins resulted in extension of life span. Thus, producing commercial crop varieties with higher anthocyanin content is highly desirable. In this study, overexpression of a single TF (i.e., PtrMYB119) activated multiple enzymatic steps of the anthocyanin biosynthetic pathway and resulted in accumulation of a considerable amount of anthocyanins in hybrid poplars without affecting their normal growth (Figure 9). We compared anthocyanin quantities in 35S::PtrMYB119 plants with those in various crop plants known for their high anthocyanin content such as tomato, red cabbage, blueberry, cherry and purple sweet potato by using a simple spectroscopy method (Dong et al. 2001). The estimated amount of anthocyanins produced by 35S::PtrMYB119 poplar plants was superior to that in red cabbage, blueberry and cherry (see Figure S6 available as Supplementary Data at *Tree Physiology* Online). These results suggest that molecular breeding for greatly improved anthocyanin content in plant species of interest may be possible by modifying the expression of a single gene that regulates metabolic flux.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

Conflict of interest

None declared.

Funding

This work was funded by a grant to J.-H.K. by the Korea Forest Service (S111213L080110) and by grants from the Basic Science Research Program through the National Research Foundation of Korea (NRF-2011-0008840 and NRF-2015R1D1A1A01060807).

References

Abrahams S, Lee E, Walker AR, Tanner GJ, Larkin PJ, Ashton AR (2003) The *Arabidopsis TDS4* gene encodes leucoanthocyanidin dioxygenase (LDOX) and is essential for proanthocyanidin synthesis and vacuole development. Plant J 35:624–636.

Aguilar O, Hernández-Brenes C (2015) Use of modified phenolic thyme extracts (*Thymus vulgaris*) with reduced polyphenol oxidase substrates as anthocyanin color and stability enhancing agents. Molecules 20:22422–22434.

Baudry A, Heim MA, Dubreucq B, Caboche M, Weisshaar B, Lepiniec L (2004) TT2, TT8, and TTG1 synergistically specify the expression of *BANYULS* and proanthocyanidin biosynthesis in *Arabidopsis thaliana*. Plant J 39:366–380.

Bell-Lelong DA, Cusumano JC, Meyer K, Chapple C (1997) Cinnamate-4-hydroxylase expression in *Arabidopsis* (regulation in response to development and the environment). Plant Physiol 113: 729–738.

Bilger W, Schreiber U, Bock M (1995) Determination of the quantum efficiency of photosystem II and of non-photochemical quenching of chlorophyll fluorescence in the field. Oecologia 102:425–432.

Borevitz JO, Xia Y, Blount J, Dixon RA, Lamb C (2000) Activation tagging identifies a conserved MYB regulator of phenylpropanoid biosynthesis. Plant Cell 12:2383–2393.

Butelli E, Titta L, Giorgio M et al. (2008) Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors. Nat Biotechnol 26:1301–1308.

Cândido TLN, Silva MR, Agostini-Costa TS (2015) Bioactive compounds and antioxidant capacity of buriti (*Mauritia flexuosa* L.f.) from the Cerrado and Amazon biomes. Food Chem 177:313–319.

Choi YI, Noh EW, Lee HS, Han MS, Lee JS, Choi KS (2005) An efficient and novel plant selectable marker based on organomercurial resistance. J Plant Biol 48:351–355.

Chu H, Jeong JC, Kim W-J et al. (2013) Expression of the sweetpotato R2R3-type *IbMYB1a* gene induces anthocyanin accumulation in *Arabidopsis*. Physiol Plant 148:189–199.

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J 16:735–743.

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Deluc L, Barrieu F, Marchive C, Lauvergeat V, Decendit A, Richard T, Carde JP, Merillon JM, Hamdi S (2006) Characterization of a grapevine R_2R_3-MYB transcription factor that regulates the phenylpropanoid pathway. Plant Physiol 140:499–511.

Donaldson JR, Stevens MT, Barnhill HR, Lindroth RL (2006) Age-related shifts in leaf chemistry of clonal aspen (*Populus tremuloides*). J Chem Ecol 32:1415–1429.

Dong X, Braun EL, Grotewold E (2001) Functional conservation of plant secondary metabolic enzymes revealed by complementation of *Arabidopsis* flavonoid mutants with maize genes. Plant Physiol 127:46–57.

Eom SH, Jin CW, Park HJ, Seo DW, Kim WW, Cho DH (2009) Stimulating effects of far-infrared ray radiation on the release of antioxidative phenolics in grape berries. Food Sci Biotechnol 18:362–366.

Espley RV, Hellens RP, Putterill J, Stevenson DE, Kutty-Amma S, Allan AC (2007) Red colouration in apple fruit is due to the activity of the MYB transcription factor, MdMYB10. Plant J 49:414–427.

Feinbaum RL, Ausubel FM (1988) Transcriptional regulation of the *Arabidopsis thaliana* chalcone synthase gene. Mol Cell Biol 8:1985–1992.

Feller A, Machemer K, Braun EL, Grotewold E (2011) Evolutionary and comparative analysis of MYB and bHLH plant transcription factors. Plant J 66:94–116.

Ford CM, Boss PK, Hoj PB (1998) Cloning and characterization of *Vitis vinifera* UDP-glucose:flavonoid 3-*O*-glucosyltransferase, a homologue of the enzyme encoded by the maize *Bronze-1* locus that may primarily serve to glucosylate anthocyanidins *in vivo*. J Biol Chem 273:9224–9233.

Gao J, Peng Z-H (2006) Response of *Populus* × *canescens* (*Populus tremula* × *alba*) to high concentration of NaCl stress. J For Res 17:269–272.

Gesell A, Yoshida K, Tran LT, Constabel CP (2014) Characterization of an apple TT2-type R2R3 MYB transcription factor functionally similar to the poplar proanthocyanidin regulator PtMYB134. Planta 240:497–511.

Ghosh D, Konishi T (2007) Anthocyanins and anthocyanin-rich extracts: role in diabetes and eye function. Asia Pac J Clin Nutr 16:200–208.

Gonzalez A, Zhao M, Leavitt JM, Lloyd AM (2008) Regulation of the anthocyanin biosynthetic pathway by the TTG1/bHLH/Myb transcriptional complex in *Arabidopsis* seedlings. Plant J 53:814–827.

Gould KS, McKelvie J, Markham KR (2002) Do anthocyanins function as antioxidants in leaves? Imaging of H_2O_2 in red and green leaves after mechanical injury. Plant Cell Environ 25:1261–1269.

Greenaway W, Gümüsderea I, Whatley FR (1991) Analysis of phenolics of bud exudate of *Populus euphratica* by GC-MS. Phytochemistry 30:1883–1885.

Greenaway W, English S, Whatley FR (1992) Relationships of *Populus* × *acuminata* and *Populus* × *generosa* with their parental species examined by gas chromatography—mass spectrometry of bud exudates. Can J Bot 70:212–221.

Grotewold E (2006) The genetics and biochemistry of floral pigments. Annu Rev Plant Biol 57:761–780.

Grotewold E, Sainz MB, Tagliani L, Hernandez JM, Bowen B, Chandler VL (2000) Identification of the residues in the Myb domain of maize C1 that specify the interaction with the bHLH cofactor R. Proc Natl Acad Sci USA 97:13579–13584.

He J, Giusti MM (2010) Anthocyanins: natural colorants with health-promoting properties. Annu Rev Food Sci Technol 1:163–187.

Horsch RB, Fry JE, Hoffmann NL, Eichholtz D, Rogers SG, Fraley RT (1985) A simple and general method for transferring genes into plants. Science 227:1229–1231.

Hsu C-C, Chen Y-Y, Tsai W-C, Chen W-H, Chen H-H (2015) Three R_2R_3-MYB transcription factors regulate distinct floral pigmentation patterning in *Phalaenopsis* spp. Plant Physiol 168:175–191.

Jing P, Bomser JA, Schwartz SJ, He J, Magnuson BA, Giusti MM (2008) Structure–function relationships of anthocyanins from various anthocyanin-rich extracts on the inhibition of colon cancer cell growth. J Agric Food Chem 56:9391–9398.

Kadir S (2006) Thermostability of photosynthesis of *Vitis aestivalis* and *V. vinifera*. J Am Soc Hortic Sci 131:476–483.

Karimi M, Inzé D, Depicker A (2002) GATEWAY™ vectors for agrobacterium-mediated plant transformation. Trends Plant Sci 7:193–195.

Kim Y-H, Kim MD, Choi YI, Park S-C, Yun D-J, Noh EW, Lee H-S, Kwak S-S (2011) Transgenic poplar expressing *Arabidopsis NDPK2* enhances growth as well as oxidative stress tolerance. Plant Biotechnol J 9:334–347.

Kirby J, Kavanagh TA (2002) NAN fusions: a synthetic sialidase reporter gene as a sensitive and versatile partner for GUS. Plant J 32:391–400.

Ko J-H, Kim W-C, Han K-H (2009) Ectopic expression of MYB46 identifies transcriptional regulatory genes involved in secondary wall biosynthesis in *Arabidopsis*. Plant J 60:649–665.

Ko J-H, Kim H-T, Hwang I, Han K-H (2012) Tissue-type-specific transcriptome analysis identifies developing xylem-specific promoters in poplar. Plant Biotechnol J 10:587–596.

Koes R, Verweij W, Quattrocchio F (2005) Flavonoids: a colorful model for the regulation and evolution of biochemical pathways. Trends Plant Sci 10:236–242.

Lai Y, Li H, Yamagishi M (2013) A review of target gene specificity of flavonoid R_2R_3-MYB transcription factors and a discussion of factors contributing to the target gene selectivity. Front Biol 8:577–598.

Lee D, Ellard M, Wanner LA, Davis KR, Douglas CJ (1995) The *Arabidopsis thaliana* 4-coumarate:CoA ligase (*4CL*) gene: stress and developmentally regulated expression and nucleotide sequence of its cDNA. Plant Mol Biol 28:871–884.

Lee DH, Lee IC, Kim KJ et al. (2014) Expression of *gibberellin 2-oxidase 4* from *Arabidopsis* under the control of a senescence-associated promoter results in a dominant semi-dwarf plant with normal flowering. J Plant Biol 57:106–116.

Li S (2014) Transcriptional control of flavonoid biosynthesis: fine-tuning of the MYB-bHLH-WD40 (MBW) complex. Plant Signal Behav 9:e27522. doi:10.4161/psb.27522

Lijavetzky D, Ruiz-garcia L, Cabezas JA et al. (2006) Molecular genetics of berry colour variation in table grape. Mol Genet Genomics 276:427–435.

Lin-Wang K, Bolitho K, Grafton K, Kortstee A, Karunairetnam S, McGhie TK, Espley RV, Hellens RP, Allan AC (2010) An R2R3 MYB transcription factor associated with regulation of the anthocyanin biosynthetic pathway in Rosaceae. BMC Plant Biol 10:50. doi:10.1186/1471-2229-10-50

Logemann J, Schell J, Willmitzer L (1987) Improved method for the isolation of RNA from plant tissues. Anal Biochem 163:16–20.

Mano H, Ogasawara F, Sato K, Higo H, Minobe Y (2007) Isolation of a regulatory gene of anthocyanin biosynthesis in tuberous roots of purple-fleshed sweet potato. Plant Physiol 143:1252–1268.

Mehrtens F, Kranz H, Bednarek P, Weisshaar B (2005) The *Arabidopsis* transcription factor MYB_{12} is a flavonol-specific regulator of phenylpropanoid biosynthesis. Plant Physiol 138:1083–1096.

Mellway RD, Tran LT, Prouse MB, Campbell MM, Constabel CP (2009) The wound-, pathogen-, and ultraviolet B-responsive MYB_{134} gene encodes an R_2R_3 MYB transcription factor that regulates proanthocyanidin synthesis in poplar. Plant Physiol 150:924–941.

Miranda M, Ralph SG, Mellway R, White R, Heath MC, Bohlmann J, Constabel CP (2007) The transcriptional response of hybrid poplar (*Populus trichocarpa* × *P. deltoids*) to infection by *Melampsora medusae* leaf rust involves induction of flavonoid pathway genes leading to the accumulation of proanthocyanidins. Mol Plant Microbe Interact 20:816–831.

Offen W, Martinez-Fleites C, Yang M, Kiat-Lim E, Davis BG, Tarling CA, Ford CM, Bowles DJ, Davies GJ (2006) Structure of a flavonoid glucosyltransferase reveals the basis for plant natural product modification. EMBO J 25:1396–1405.

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Ohl S, Hedrick SA, Chory J, Lamb CJ (1990) Functional properties of a phenylalanine ammonia-lyase promoter from *Arabidopsis*. Plant Cell 2:837–848.

Osier TL, Lindroth RL (2004) Long-term effects of defoliation on quaking aspen in relation to genotype and nutrient availability: plant growth, phytochemistry and insect performance. Oecologia 139:55–65.

Pascual-Teresa DS, Moreno DA, García-Viguera C (2010) Flavanols and anthocyanins in cardiovascular health: a review of current evidence. Int J Mol Sci 11:1679–1703.

Paz-Ares J, Ghosal D, Wienand U, Peterson PA, Saedler H (1987) The regulatory c1 locus of *Zea mays* encodes a protein with homology to myb proto-oncogene products and with structural similarities to transcriptional activators. EMBO J 6:3553–3558.

Pelletier MK, Shirley BW (1996) Analysis of flavanone 3-hydroxylase in *Arabidopsis* seedlings (coordinate regulation with chalcone synthase and chalcone isomerase). Plant Physiol 111:339–345.

Petroni K, Tonelli C (2011) Recent advances on the regulation of anthocyanin synthesis in reproductive organs. Plant Sci 181:219–229.

Pfaffl MW (2001) Development and validation of an externally standardized quantitative insulin like growth factor-1 (IGF-1) RT-PCR using Light Cyclear SYBR Green I technology. In: Meuer S, Witter C, Nakagawara K (eds) Rapid cycle real-time PCR. Springer, Heidelberg, pp 281–291.

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

Ramsay NA, Glover BJ (2005) MYB–bHLH–WD40 protein complex and the evolution of cellular diversity. Trends Plant Sci 10:63–70.

Roth BA, Goff SA, Klein TM, Fromm ME (1991) C1- and R-dependent expression of the maize Bz1 gene requires sequences with homology to mammalian myb and myc binding sites. Plant Cell 3:317–325.

Shirley BW, Hanley S, Goodman HM (1992) Effects of ionizing radiation on a plant genome: analysis of two *Arabidopsis* transparent testa mutations. Plant Cell 4:333–347.

Skaar I, Adaku C, Jordheim M, Byamukama R, Kiremire B, Andersen ØM (2014) Purple anthocyanin colouration on lower (abaxial) leaf surface of *Hemigraphis colorata* (Acanthaceae). Phytochemistry 105:141–146.

Speciale A, Canali R, Chirafisi J, Saija A, Virgili F, Cimino F (2010) Cyanidin-3-*O*-glucoside protection against TNF-α-induced endothelial dysfunction: involvement of nuclear factor-κB signaling. J Agric Food Chem 58:12048–12054.

Steyn WJ, Wand SJE, Holcroft DM, Jacobs G (2002) Anthocyanins in vegetative tissues: a proposed unified function in photoprotection. New Phytol 155:349–361.

Stracke R, Werber M, Weisshaar B (2001) The *R2R3-MYB* gene family in *Arabidopsis thaliana*. Curr Opin Plant Biol 4:447–456.

Stracke R, Ishihara H, Huep G, Barsch A, Mehrtens F, Niehaus K, Weisshaar B (2007) Differential regulation of closely related R2R3-MYB transcription factors controls flavonol accumulation in different parts of the *Arabidopsis thaliana* seedling. Plant J 50:660–677.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30:2725–2729.

Taylor NG, Howells RM, Huttly AK, Vickers K, Turner SR (2003) Interactions among three distinct CesA proteins essential for cellulose synthesis. Proc Natl Acad Sci USA 100:1450–1455.

Teng S, Keurentjes J, Bentsink L, Koornneef M, Smeekens S (2005) Sucrose-specific induction of anthocyanin biosynthesis in *Arabidopsis* requires the MYB_{75}/PAP1 gene. Plant Physiol 139:1840–1852.

Tohge T, Nishiyama Y, Hirai MY et al. (2005) Functional genomics by integrated analysis of metabolome and transcriptome of *Arabidopsis* plants over-expressing an MYB transcription factor. Plant J 42: 218–235.

Tsai CJ, Harding SA, Tschaplinski TJ, Lindroth RL, Yuan Y (2006) Genome-wide analysis of the structural genes regulating defense phenylpropanoid metabolism in *Populus*. New Phytol 172:47–62.

Wilkins O, Nahal H, Foong J, Provart NJ, Campbell MM (2009) Expansion and diversification of the *Populus* R_2R_3-MYB family of transcription factors. Plant Physiol 149:981–993.

Willits DH, Peet MM (2001) Measurement of chlorophyll fluorescence as a heat stress indicator in tomato: laboratory and greenhouse comparisons. J Am Soc Hortic Sci 126:188–194.

Xie D-Y, Sharma SB, Paiva NL, Ferreira D, Dixon RA (2003) Role of anthocyanidin reductase, encoded by *BANYULS* in plant flavonoid biosynthesis. Science 299:396–399.

Xu W, Grain D, Bobet S, Le Gourrierec J, Thévenin J, Kelemen Z, Lepiniec L, Dubos C (2014) Complexity and robustness of the flavonoid transcriptional regulatory network revealed by comprehensive analyses of MYB–bHLH–WDR complexes and their targets in *Arabidopsis* seed. New Phytol 202:132–144.

Yoo S-D, Cho Y-H, Sheen J (2007) *Arabidopsis* mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nat Protoc 2:1565–1572.

Yoshida K, Ma D, Constabel CP (2015) The MYB182 protein downregulates proanthocyanidin and anthocyanin biosynthesis in poplar by repressing both structural and regulatory flavonoid genes. Plant Physiol 167:693–710.

Zhang F, Gonzalez A, Zhao M, Payne CT, Lloyd A (2003) A network of redundant bHLH proteins functions in all TTG1-dependent pathways of *Arabidopsis*. Development 130:4859–4869.

Zhang RF, Zhang FX, Zhang MW, Wei ZC, Yang CY, Zhang Y, Tang XJ, Deng YY, Chi JW (2011) Phenolic composition and antioxidant activity in seed coats of 60 Chinese black soybean (*Glycine max* L. Merr.) varieties. J Agric Food Chem 59:5935–5944.

Zhishin J, Mengcheng T, Jianming W (1999) The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem 64:555–559.

Zifkin M, Jin A, Ozga JA, Zaharia LI, Schernthaner JP, Gesell A, Abrams SR, Kennedy JA, Constabel CP (2012) Gene expression and metabolite profiling of developing highbush blueberry fruit indicates transcriptional regulation of flavonoid metabolism and activation of abscisic acid metabolism. Plant Physiol 158:200–224.

Zimmermann IM, Heim MA, Weisshaar B, Uhrig JF (2004) Comprehensive identification of *Arabidopsis thaliana* MYB transcription factors interacting with R/B-like BHLH proteins. Plant J 40:22–34.

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