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ORIGINAL ARTICLE

PdMYB118, isolated from a red leaf mutant of *Populus deltoids*, is a new transcription factor regulating anthocyanin biosynthesis in poplar

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Abstract

Key message **A new anthocyanin biosynthesis transcription factor PdMYB118, which could be used for the genetic engineering of colorful tree species, was indentified from a red leaf mutant of *Populus deltoids*.**

Abstract In higher plants, the biosynthesis of anthocyanins is regulated by several classes of transcription factors (TFs), including R2R3-MYB, bHLH and WD-repeat proteins. In this work, we isolated an MYB gene regulating anthocyanin biosynthesis from a red leaf mutant of *Populus deltoids,* which accumulated more anthocyanins in the leaves and showed higher expression levels of anthocyanin biosynthesis genes than did the wild type. Gene expression analyses of all TFs regulating anthocyanin biosynthesis demonstrated that only a *MYB118* homologous gene, *PdMYB118,* was up-regulated in the mutant compared with the wide type. Subcellular localization analyses in poplar leaf mesophyll protoplasts showed that PdMYB118-YFP fusion protein was specifically located in nucleus. When transiently expressed in poplar leaf protoplasts, PdMYB118 specifically promoted the expression of anthocyanidin biosynthesis genes. Dual-luciferase assays revealed that PdMYB118 can directly activate the promoters of these genes. When overexpressed in Shanxin Yang (*P. davidiana* × *P. bolleana*), a hybrid clone commercially grown for landscaping in the northern part of China, transgenic plants overexpressing *PdMYB118* produced more anthocyanins in the leaves and turned their color into redness when grown in both greenhouse and field. Consistently, transcripts of some important anthocyanidin biosynthesis genes were significantly increased in the leaves of transgenic plants. All these results indicate that *PdMYB118* functions as an essential transcription factor regulating anthocyanin biosynthesis in poplar and could be used for the genetic engineering of colorful tree species.

Keywords Anthocyanins · PdMYB118 · Poplar · Red leaf · Transcription factor

Introduction

Anthocyanins, which play multiple roles in plant development and adaptation to environmental stresses, are the major pigments for red, purple, and blue colors. In addition

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to providing fruits and flowers with beautiful colors to improve their organoleptic qualities and attract pollinators and seed dispersers (Winkel-Shirley 2001), protecting plants from insect attacks and pathogen infections (Lorenc-Kukuła et al. 2005), and enhancing the resistance of plants to abiotic stresses (i.e. low temperature, UV, oxidation, drought), anthocyanins also provide trees with beautiful colors for their ornamental values (Christie et al. 1994; Sarma and Sharma 1999; Steyn et al. 2002; Nakabayashi et al. 2014).

The biosynthesis pathway of anthocyanins has been well elucidated as a specific branch of the flavonoid pathway, which is almost ubiquitous in plants (Winkel-Shirley 2001; Espley et al. 2007; Hancock et al. 2012). According to the summarized flavonoid pathway in poplar (Tsai et al. 2006; Mellway et al. 2009), anthocyanin biosynthesis starts from chalcone synthase (CHS), which synthesizes chalcone from 4-coumaroyl CoA and malonyl CoA. Following isomerization, naringenin chalcone is converted into flavanone by chalcone isomerase (CHI). Then, flavanone is hydroxylated by flavanone 3-hydroxylase (F3H) and subsequently hydroxylated at the B-ring by flavanone 3′-hydroxylase (F3′H) and flavonoid 3′5′-hydroxylase (F3′5′H) to form dihydroflavonols. By dihydroflavonol 4-reductase (DFR), dihydroflavonols are reduced to form leucoanthocyanidins. A part of leucoanthocyanidins are used to produce anthocyanidins (cyanidin and delphinidin) by anthocyanidin synthase (ANS); meanwhile, the other part of leucoanthocyanidins are catalyzed by leucoanthocyanidin reductase (LAR) to form transflavan-3-ols (catechin and gallocatechin), the monomeric PA (proanthocyanins) units. The second kind of monomeric PA units, cis-flavan-3-ols (epicatechins and epigallocatechin), is produced by anthocyanidin reductase (ANR), using anthocyanins as substrates.

The temporal and spatial expressions of these anthocyanin biosynthetic genes are subtly regulated by the ternary complexes named MBW, including R2R3-MYB TFs, basic helix-loop-helix (bHLH) TFs, and WD40-repeat proteins (Hichri et al. 2011; Petroni and Tonelli 2011; Xu et al. 2015). In *Arabidopsis*, the multiple MBW complexes activating anthocyanin synthesis are formed with the R2R3-MYB factors (PAP1, PAP2, MYB113, MYB114), the bHLH factors (TT8, GL3, EGL3), and a WD40 factor TTG1 (reviewed by Petroni and Tonelli 2011). Similar regulatory systems in other species are also identified and are involved in anthocyanin biosynthesis in flowers (Elomaa et al. 2003; Nakatsuka et al. 2008), fleshy fruits (Espley et al. 2007; Matus et al. 2010), and seeds (Grotewold et al. 2000; Sweeney et al. 2006).

In addition to PAP1/PAP2/MYB113/MYB114 in *Arabidopsis*, PL1 in *Zea mays*, AN2/AN4 in *Petunia hybrida*, VvMYB5a/5b in *Vitis vinifera*, and MdMYB10 in *Malus domestica* (reviewed by Petroni and Tonelli 2011), a number of anthocyanin-associated R2R3-MYB TFs were also characterized in horticultural plants: more than 30 in ornamental plants, 24 in fruit crops, and 19 in vegetables (Naing and Kim 2018). Although a large amount of R2R3-MYB members (up to 192 MYBs) have been found in the poplar genome (Wilkins et al. 2009), the biological functions of anthocyanin-associated MYBs and their regulatory mechanisms in anthocyanin biosynthesis are still not fully illustrated. In poplar, MYB134 specifically regulated the biosynthesis of proanthocyanidins, and overexpression of *MYB134* led to increased accumulation of proanthocyanidins in transgenic poplar, but did not affect the levels of anthocyanins (Mellway et al. 2009). MYB115 was identified as the second positive MYB regulator of proanthocyanidins, which regulated a set of common genes involved in proanthocyanidin biosynthesis (James et al. 2017). But the transcriptional activation of MYB134 was disrupted by PtMYB182, a suppressor of flavonoids which down-regulated the biosynthesis of proanthocyanidin and anthocyanin in poplar (Yoshida et al. 2015). PtrMYB57 was another repressor of proanthocyanidin and anthocyanin by suppressing the transcriptional activation of flavonoid structural genes (Wan et al. 2017). However, only one R2R3-MYB transcription factor PtrMYB119 was identified as a positive regulator in anthocyanin biosynthesis in poplar (Cho et al. 2016). Overexpression of *PtrMYB119* increased the content of total flavonoids and cyaniding-3-*O*-glucoside.

Since most colorful tree species from the southern part of China cannot survive the cold winter, poplar has been used as a major landscaping tree species in the northern part of China due to its hardiness to cold weather. Therefore, breeding colorful trees which can survive in the northern regions has been in great demand. In this work, we isolated a red leaf poplar mutant from *Populus deltoids*, a hybrid clone widely grown in the southern part of China, and identified a poplar MYB transcription factor PdMYB118 from the red leaf mutant. We found that PdMYB118 works as an essential regulator for anthocyanin biosynthesis in poplar. Overexpression of *PdMYB118* in Shanxin Yang, a hybrid clone commercially grown in the northern part of China as a landscaping tree, turned it into a red color tree species.

Materials and methods

Plant materials and growth conditions

Populus deltoids, its red leaf mutant, and a commercial hybrid clone Shanxin Yang (*P. davidiana*×*P. bolleana*) were used in this study. Poplar shoots were grown in greenhouse at 25 °C (day)/18 °C (night) in a 12 h light/12 h dark photoperiod as described previously (Wang et al. 2011).

Plasmid, vector, and plant transformation

To construct the plant expression vector, the full-length CDS of *PdMYB118* was cloned from the red leaf mutant using the gene-specific primers PdMYB118-OF and PdMYB118-OR (Table S1). After sequence confirmation, *PdMYB118* CDS was cloned into the modified pCAMBIA-2301 vector (Huang et al. 2009). Then, the resultant construct was introduced into Shanxin Yang by *Agrobacterium*-mediated transformation as described previously (Wang et al. 2011). Transgenic shoots were propagated on MS medium, transferred to soil, and grown in greenhouse for further study (Jin et al. 2017).

Quantitative real-time RT-PCR

For the expression analysis of anthocyanin biosynthesis genes and transcription factor genes of poplar, total RNA was extracted from the leaves using the RNAiso Reagent (Takara, Japan), as described before (Yang et al. 2015). A total amount of 2 μg RNA was subjected to reverse transcription reaction using the HiScript® II Q RT SuperMix for qPCR (+gDNA wiper) (Vazyme, China). qRT-PCR was performed using a AceQ qPCR SYBR Green Master Mix (Vazyme, China) and a CFX Connect Real-Time System (Bio-Rad, USA). The elongation factor gene *PtrEF1β* was employed as an internal control. The relative expression of each target gene was normalized using *PtrEF1β* (Wang et al. 2013a, b). Gene-specific primers used in this study are listed in Table S1.

Anthocyanin measurement

Anthocyanin content in the leaves of WT, mutant and transgenic plants was measured as described previously (Yoshida et al. 2015). After being ground in liquid N_2, 0.1 g of each sample was extracted with 2 mL of 0.1% HCl/methanol for 12 h at 4 °C. The samples were centrifuged and the extraction was repeated once. Then the supernatants were pooled and diluted with an equal volume of water and chloroform to remove chlorophylls. Finally, the absorption of the aqueous phase was measured at 530 nm. To show the quantity of anthocyanin, A_{530} per gram fresh weight (FW) was used. All experiments were repeated at least three times.

Transient expression of PdMYB118 in poplar mesophyll protoplasts

To analyze the transcription function of PdMYB118, transient expression analyses were performed. The effector was constructed by inserting *PdMYB118* into the *Bam*HI and *Sal*I sites of pGreenII62-SK driven by the CaMV 35S promoter (Hellens et al. 2005). Protoplasts were extracted from the leaves of Shanxin Yang, and then the effector was transformed into the protoplasts as described previously (Tang, et al. 2010; Wang et al. 2013b). The empty vector was used as a negative control. After cultured in dark for 16 h, the transfected protoplasts were collected for RNA extraction and qRT-PCR analyses of anthocyanin biosynthetic genes as described above.

Subcellular localization of PdMYB118-YFP fusion protein

To determine the subcellular localization of PdMYB118 protein, the full-length coding sequences of *PdMYB118* without the stop codon was fused in-frame to the N-terminal of yellow fluorescent protein (YFP) in the pA7-YFP vector. The final plasmid, pA7-YFP or pA7-PdMYB118-YFP, was transfected into Shanxin yang leaf mesophyll protoplasts, essentially as described previously (Sheen 2001; Yoo et al. 2007). After incubated at 23 °C for 18 h, the protoplasts were analyzed using a confocal microscope at 514 nm wavelength (Zeiss LSM 510 META).

Transient transcription dual-luciferase assays

For dual-luciferase assays, the LUC reporter constructs were generated by cloning the promoter of *PtrCHS1*, *PtrDRF2* or *PtrANS1* into the *Bam*HI and *Sal*I sites of pGreenII0800-LUC (Hellens et al. 2005). The resultant pGreenII62-SK-PdMYB118 was used as the effector construct as described above. To detect the transcription activity of PdMYB118, the PdMYB118 effector was co-expressed with each reporter construct in the poplar leaf protoplasts, respectively. After 16 h, the transfected cells were collected and homogenized in 300 μL of passive lysis buffer. The crude extract (20 μL) was mixed with 40 μL of luciferase assay buffer and the firefly luciferase (LUC) activity was measured using a GLOMAX 20/20 luminometer (Promega, Wisconsin, USA). Stop and Glow Buffer (40 μL) was then added to the reaction solution and the renillia luciferase (REN) activity was measured. The LUC/REN ratio was used to represent the relative activity of the transcription factors (Bao et al. 2014).

Results

Anthocyanin biosynthesis genes are up-regulated in the red leaf mutant

The leaves of normal poplar trees like *P. deltoids* are green in color. We identified a red leaf mutant of *P. deltoids,* which accumulated more anthocyanins than did the wild type (Fig. 1a, b). Since anthocyanins are synthesized by a series of genes step by step as shown in Fig. S1 (Li

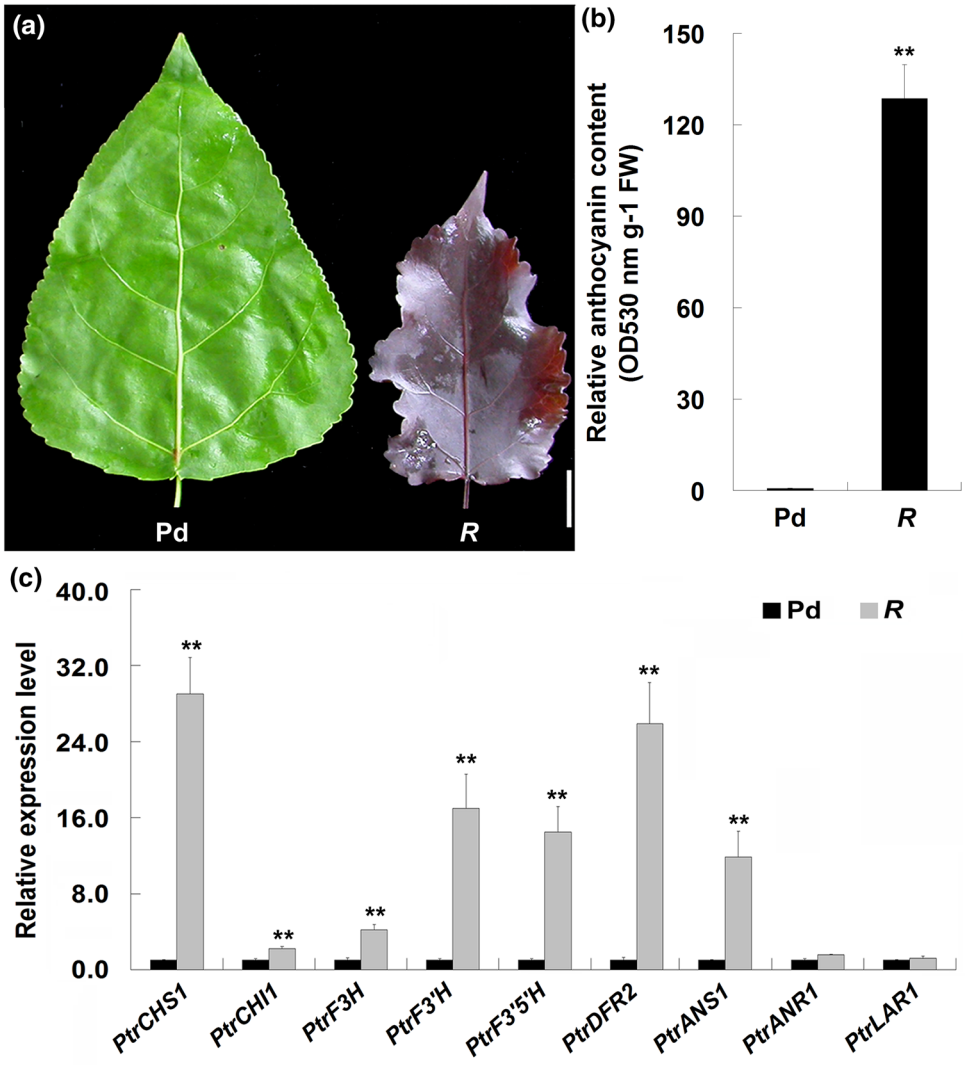


Fig. 1 Expression analyses of anthocyanin biosynthetic genes in the leaves of red leaf mutant. **a** Leaf color of the red leaf mutant. Scale bar 1 cm. **b** Anthocyanin content in the leaves of wild type and red leaf mutant. **c** qRT-PCR analyses of poplar anthocyanin- and proanthocyanin-biosynthesis genes in the red leaf mutant. Total RNA was isolated from the leaves of wild type and red leaf mutant. Expression in the leaves of *Populus deltoids* was set to 1. Error bars represent the SDs from three biological replicates Statistical differences were determined by a Student's *t* test (**$P < 0.01$). *PtrCHS1*, *PtrCHI1*, *PtrF3H*, *PtrF3'H*, *PtrF3'5'H*, *PtrDFR2*, *PtrANS1*, *PtrANR1,* and *PtrLAR1,* respectively, encode chalcone synthase, chalcone isomerase, flavanone 3-hydroxylase, flavanone 3′-hydroxylase, flavonoid 3′5′-hydroxylase, dihydroflavonol 4-reductase, anthocyanidin synthase, anthocyanidin reductase, and leucoanthocyanidin reductase in *Populus*. Pd, *Populus deltoids*; *R*, red leaf mutant of *Populus deltoids*

2014), we searched the putative anthocyanin biosynthesis genes in the poplar genome database and examined their transcriptional levels in the mutant's leaves by qRT-PCR analyses. We observed that anthocyanin biosynthetic genes: *PtrCHS1*, *PtrCHI1*, *PtrF3H*, *PtrF3'H*, *PtrF3'5'H*, *PtrDFR2,* and *PtrANS1*, were all up-regulated in the mutant compared with the wide type. However, the expressions of *PtrANR1* and *PtrLAR1* for no-color pro-anthocyanidin biosynthesis were not changed (Fig. 1c). These results indicate that anthocyanidin accumulation in the red leaf mutant could be a result of the up-regulated expression of anthocyanidin biosynthesis genes.

PdMYB118 is the key regulator leading to the red leaf phenotype of mutant

The increased expressions of anthocyanin biosynthetic genes may be regulated by the MBW complex composed of R2R3 MYB TFs, bHLH TFs and WD40 proteins (Fig. S1; Li 2014). We searched these kinds of TF genes in the poplar genome database and examined their expression levels in the leaves of red leaf mutant. We found that only a MYB transcription factor gene *PtrMYB118* (the homolog of *MdMYB10/AtPAP1*) was remarkably up-regulated in the red leaf mutant, with an increase of more than 200 times compared with the wide type; other kinds of transcription factor genes *PtrMYB134* (the homolog of *AtTT2*), *PtrTTG1.2* (the homolog of *AtTTG1*), as well as the bHLH protein genes *PtrLC1* (the homolog of *MdbHLH33/GL3/AtEGL3*) and *PtrTT8* (the homology of *MdbHLH3/AtTT8*), were all down-regulated (Fig. 2).

We then cloned the MYB transcription factor from the red leaf mutant and compared it with the putative poplar MYB factors involved in anthocyanin and pro-anthocyanin biosynthesis. By amino acid sequence alignment, we found that the MYB protein shared very high identity with PtrMYB118 and then named it as PdMYB118 (Fig. 3a, b). In *Populous*, the PdMYB118 subfamily includes three

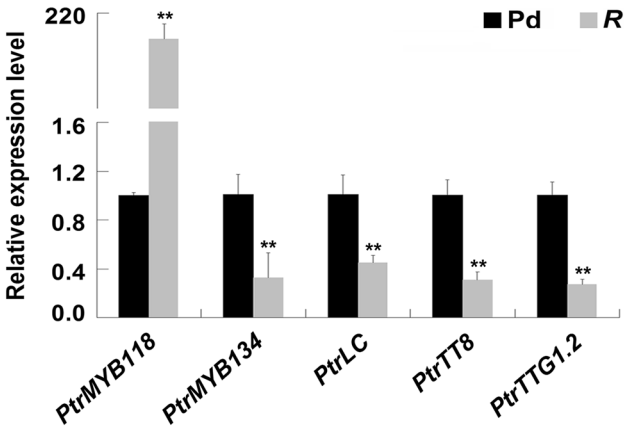


Fig. 2 qRT-PCR analysis of MBW TFs genes in the leaves of red leaf mutant. Putative MBW TFs genes in *Populus,* including two MYB transcription factor genes (*PtrMYB118* and *PtrMYB134*), two bHLH transcription factor genes (*PtrLC1* and *PtrTT8*), and a WD40 protein gene (*PtrTTG1.2*), were chosen for the study. Total RNA was isolated from the leaves of wild-type and red leaf mutant. Expression in the leaves of *Populus deltoids* was set to 1. Error bars represent the SDs from three biological replicates. Statistical differences were determined by a Student's *t* test (**$P < 0.01$). Pd, *Populus deltoids*; *R*, red leaf mutant of *Populus deltoids* (color figure online)

members, PtrMYB118, PtrMYB119, and PtrMYB120. They all contain an N-terminal R2R3 repeat, a typical domain for anthocyanin- and proanthocyanidin-associated MYB TFs (Fig. 3a). In addition, PdMYB118 and its homologs contain the M1 motif, which is in consensus in the anthocyanin-related MYBs, rather than the M2 motif, which is typical in proanthocyanin-related MYBs. Consistently, PdMYB118 and its homologs clustered with the anthocyanin regulated MYB TFs AtPAP1/2 and MdMYB10 (Fig. 3b). We further examined the expression levels of all these poplar *MYBs* and found that only *PtrMYB118* was up-regulated in the red leaf mutant (Fig. 3c), suggesting that PdMYB118 may play an important role in anthocyanin biosynthesis in poplar, like PtrMYB119, which has been reported as an important regulator anthocyanin biosynthesis (Cho et al. 2016).

PdMYB118 is a transcription factor regulating the expression of anthocyanin biosynthesis genes

To understand the exact functions of PdMYB118 in anthocyanin biosynthesis, we transiently expressed PdMYB118-YFP fusion protein in the mesophyll protoplasts of Shanxin Yang. We observed that, unlike the ubiquitous distribution of free YFP, the fluorescence signal of PdMYB118-YFP fusion protein was specifically localized to the cell nuclear (Fig. 4), indicating that PdMYB118 is indeed a transcription factor working in the nucleus.

To verify the transcriptional activity of PdMYB118 on the expressions of anthocyanin biosynthetic genes, we transiently over-expressed it in the leaf protoplasts of wild-type Shanxin Yang (Fig. 5a). As expected, PdMYB118 up-regulated the expressions of anthocyanidin biosynthesis genes in the protoplasts overexpressing *PdMYB118* (Fig. 5b), although proanthocyanin biosynthesis genes such as *PtrANR* and *PtrLAR* were down-regulated (Fig. S2).

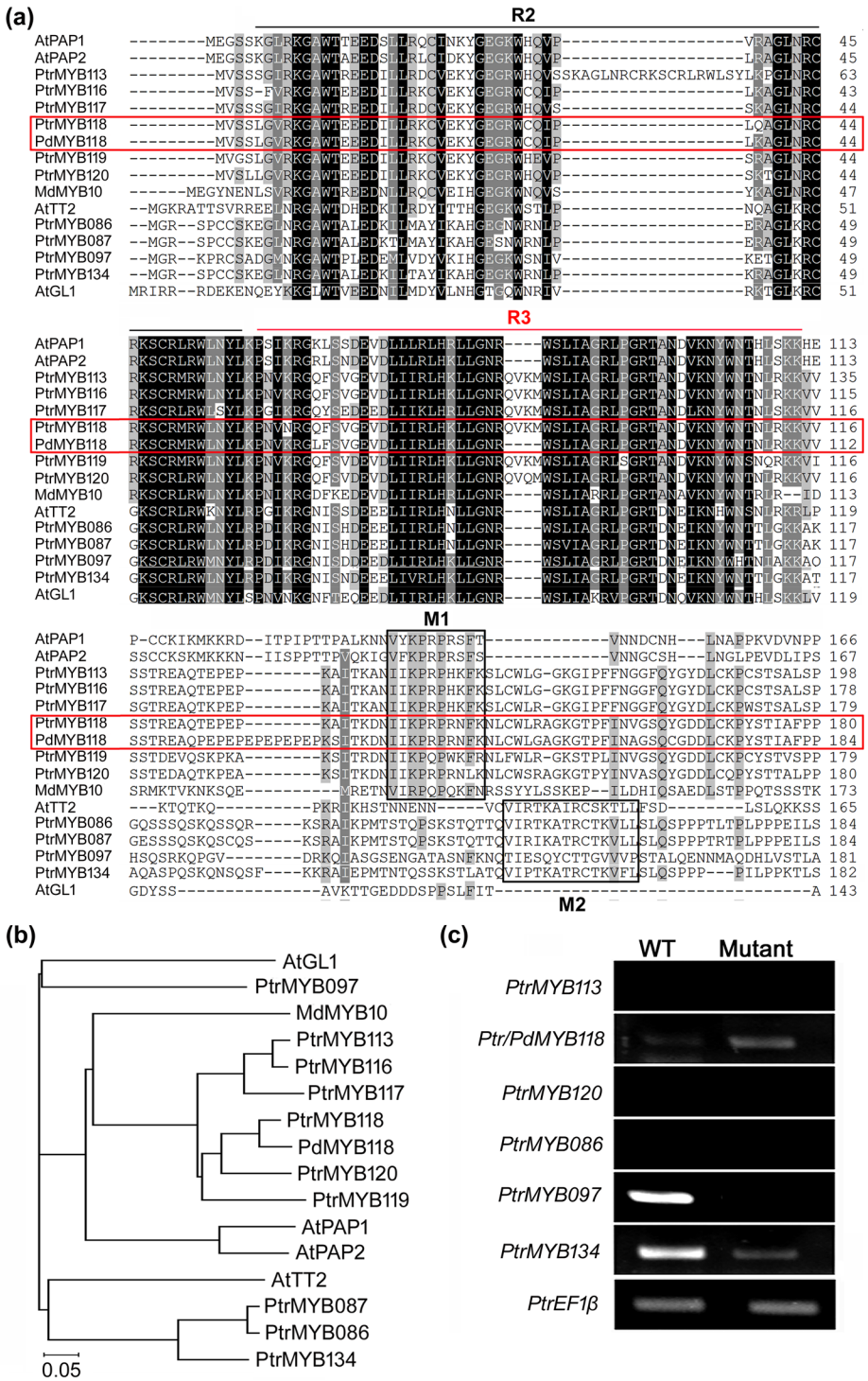
We further confirmed whether PdMYB118 directly activates the pomoters of anthocyanin biosynthetic genes by dual-luciferase assays. Promoters of three anthocyanin biosynthetic genes, *PtrCHS1*, *PtrDFR2,* and *PtrANS1,* were cloned and assessed. We found that PdMYB118 directly activated the promoters of *PtrCHS1*, *PtrDFR2,* and *PtrANS1*, showing a higher LUC/REN ratio than the control (Fig. 6). These results suggest that PdMYB118 is a transcription factor regulating the anthocyanin biosynthesis in poplar.

Overexpression of *PdMYB118* in Shanxin Yang leads to red leaf phenotype of transgenic plants

Since high expression of *PdMYB118* caused the red leaf phenotype by activating the transcriptions of anthocyanidin biosynthesis genes rather than the proanthocyanin biosynthesis genes, leading to enhanced production of anthocyanins in the red leaf mutant, we postulated that PdMYB118 could have a great potential to be used for the engineering of red leaf poplar trees. To further investigate this speculation, we generated an overexpression vector of *PdMYB118* driven by 35S promoter and introduced it into Shanxin Yang, a hybrid clone commercially grown in north China. PCR and qRT-PCR analyses confirmed the successful integration and high expression of *PdMYB118* in all tested independently regenerated lines (Fig. S3). The tissue culture plantlets of transgenic poplar were transferred into soil and grown in greenhouse. The leaves of transgenic plants overexpressing *PdMYB118* gradually turned into red and accumulated more anthocyanins than did the wide type (Fig. 7a–e). When plants were grown in field at Yantai (Shandong province, China), the leaves of transgenic plants also showed red color and successfully survived the cold winter (Fig. 7f). All these results indicate that *PdMYB118* could be used as a valuable candidate gene for the engineering of colorful tree species suitable for landscaping use in the vast areas of northern China.

Overexpression of *PdMYB118* promotes the expression of anthocyanin biosynthesis genes in red leaves of transgenic poplar

Based on the observation that up-regulated expression of *PdMYB118* in the *red leaf* mutant prompted the transcriptions of anthocyanin biosynthetic genes, we speculated that overexpression of *PdMYB118* in transgenic poplar



plants would have also activated the expressions of anthocyanin biosynthetic genes. Indeed, expression of most of anthocyanin biosynthetic genes such as *PtrCHS1*, *PtrF3H*, *PtrF3'H*, *PtrDFR2,* and *PtrANS1* was obviously up-regulated in the red leaves of transgenic plants (Fig. 8). Although *PtrLAR* was down-regulated and *PtrANR* expression was not changed in the transgenic plants, these observations are consistent with the previous reports that anthocyanin MYBs, such as PAP1 in *Arabidopsis*, VvMYBPA1 in grape, MdMYB9/11 in apple, and PtrMYB119 in poplar, regulated the expressions of anthocyanin biosynthesis genes (Borevitz et al. 2000; Nesi et al. 2001; Bogs et al. 2007; Cho et al. 2016). All these results imply that the increased anthocyanin accumulation in red leaves of transgenic plants is a result of

◂**Fig. 3** Poplar *MYBs* involved in anthocyanin and proanthocyanin biosynthesis. **a** Amino acid alignment of poplar MYBs with known R2R3-MYB regulators regulating anthocyanin and proanthocyanin biosynthesis. The alignment was generated by ClustalX (1.83) (http://www.clustal.org/clustal2/). R2 and R3, the consensus motifs of anthocyanin- and proanthocyanin-regulated MYBs; M1, the KPRPRS/T motif of anthocyanin-related MYBs. M2, the typical motif V[V/I]xT[K/R]Ax[K/R]C[S/T]K in proanthocyanin-related MYBs (Wilkins et al. 2009). The red box indicates the high indentity between PdMYB118 and PtrMYB118. **b** Phylogenetic tree of anthocyanin- and proanthocyanin-related MYBs from poplar and other species. The scale bar measures substitutions per site. The length of a branch is the average number of substitutions that occurred at each site of the sequence between the two ends of the branch. GenBank numbers or gene model names: AtGL1, NM_113708; AtPAP1, CAB09230; AtPAP2, NP176813; AtTT2, Q9FJA2; MdMYB10, DQ267896; PtrMYB086, Potri.018G049600; PtrMYB087, Potri.018G049200; PtrMYB097, Potri.006G275900; PtrMYB113, Potri.017G125600; PtrMYB116, Potri.017G125900; PtrMYB117, Potri.017G126000; PtrMYB118, Potri.017G125800; PtrMYB119, Potri.017G125600; PtrMYB120, Potri.017G125700; PtrMYB134, Potri.006G221800. **c** RT-PCR analysis of poplar MYBs in the leaves of red leaf mutant. WT, *Populus deltoids*; Mutant, red leaf mutant (color figure online)

the up-regulated expressions of anthocyanidin biosynthesis genes caused by *PdMYB118* overexpression.

Discussion

As a classic green-leaf-tree species, infrequent red leaf mutation has limited the study on red color formation in poplar. To date, only a PA-specific R2R3-MYB MYB134 regulating the colorless PA synthesis and a R2R3-MYB transcription factor PtrMYB119 from *Populus trichocarpa* promoting anthocyanin production in hybrid poplar were characterized (Mellway et al. 2009; Cho et al. 2016). We obtained a poplar mutant with red leaf from *P. deltoids* and identified a key transcription factor PdMYB118, which is the main cause of the red pigmentation of mutant, based on the observations that only *PdMYB118* was up-regulated and other putative poplar transcription factor genes including *PtrMYB134*, *PtrLC1*, *PtrTT81,* and *PtrTTG1.2* were all down-regulated (Fig. 2). In vitro assays confirmed that PdMYB118 was a transcription factor regulating the expressions of anthocyanin synthesis genes by activating their promotes (Figs. 4, 5, 6). Combining the finding that expressions of anthocyanin synthesis genes were up-regulated in the mutant (Fig. 1c), we speculate that the enhanced production of anthocyanins in the mutant was caused by the up-regulated transcriptions of anthocyanidin biosynthesis genes.

The amino sequences of PdMYB118 and their homologies all contain a typical M1 motif for anthocyanin biosynthesis related MYBs, which is obviously distinguished from the M2 motif typically for proanthocyanin biosynthesis related MYBs (Fig. 3a). In addition, high expression of PdMYB118 in the RED-LEAF mutant, as well as in the poplar leaf protoplast, specifically up-regulated the expression of anthocyanin biosynthesis genes (Figs. 1c, 5). This is consistent with previous reports that anthocyanin MYBs, such as PAP1 in *Arabidopsis*, VvMYBPA1 in grape, MdMYB9/11 in apple, and PtrMYB119 in poplar, regulated the expressions of anthocyanin biosynthesis genes (Borevitz et al. 2000; Nesi et al. 2001; Bogs et al. 2007; Cho et al. 2016).

Similar to the findings in the RED-LEAF mutant, and transgenic plants overexpressing PtrMYB119 (Cho et al. 2016), overexpression of PdMYB118 obviously activated the expressions of anthocyanin biosynthetic genes, leading to red colored phenotype in the leaves of transgenic plants (Figs. 7, 8). However, PdMYB118 functioned as a specific regulator in anthocyanindis biosynthesis other than colorless proanthocyanin biosynthesis. Only anthocyanindis biosynthesis genes were up-regulated in the mutant, the proanthocyanin biosynthesis genes PtrANR and PtrLAR were down-regulated (Fig. 1c), which is consistent with the gene expression results in the protoplasts transiently expressing *PdMYB118* and in the leaves of transgenic poplar plants overexpressing *PdMYB118* (Figs. 5, 8, S2). However, PtrMYB119 generally regulated the expression of anthocyanin biosynthetic genes and proanthocyanin biosynthesis genes (Cho et al. 2016). ANS (anthocyanidin synthase) and LAR (leucoanthocyanidin reductase) share the same substrate leucoanthocyanidins: ANS could produce the colorful anthocyanidins, while LAR generates colorless *trans*-flavan-3-ols (Fig. S1). In addition, ANR (anthocyanidin reductase) transfers anthocyanidins into colorless *cis*-flavan-3-ols. Down-regulated expression of PtrLAR and PtrANR by PdMYB118 could have increased the substrate of ANS and reduced the consumption of anthocyanidins and therefore benefited the accumulation of colorful anthocyanidins.

Due to its hardness to cold winter, poplar with green leaves has been used as one of the major landscaping trees in the northern part of China. Along with the urbanization process in the northern cities of China, the demand for landscape tree species with beautiful color is growing. Shanxin Yang is one of the hybrid clones commercially grown in north China. The red-colored phenotype of transgenic Shanxin Yang overexpressing *PdMYB118* showed a great potential of this gene for the breeding of colorful trees (Fig. 7).

In this study, we identified a red leaf poplar mutant with up-regulated *PdMYB118* expression. By directly activating their promoters, PdMYB118 regulated the expression of anthocyanidin biosynthesis genes. Overexpression of *PdMYB118* in Shanxin Yang conferred red leaf phenotype on transgenic plants when they were grown in either green house or field. Therefore, *PdMYB118* could be used as a valuable candidate gene for the breeding of colorful landscaping tree species suitable for the cold winter regions.

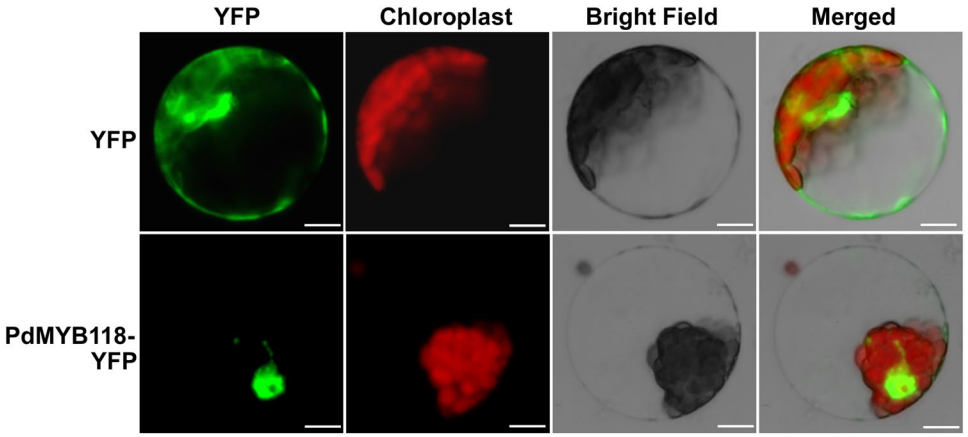


Fig. 4 Subcellular localization of PdMYB118 protein. PdMYB118-YFP or YFP alone was transiently expressed in the leaf protoplasts of Shanxin yang, incubated in dark for 18 h, and observed under a confocal microscope. Bar 10 μm

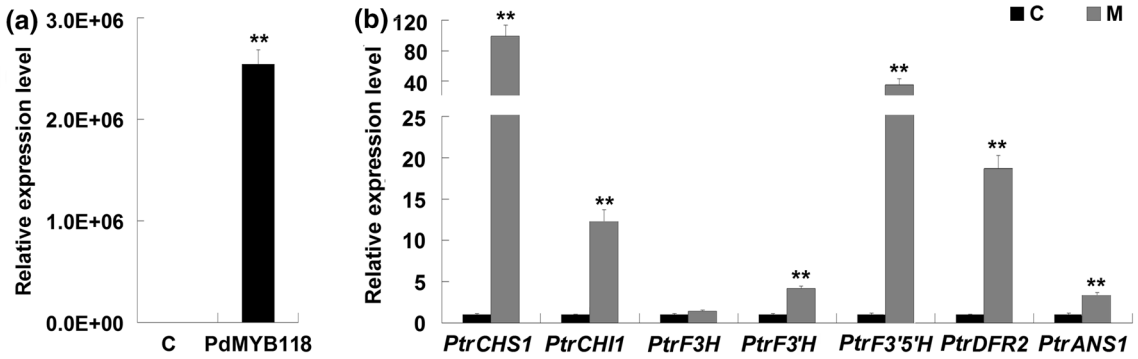


Fig. 5 Transient expression of *PdMYB118* in poplar mesophyll protoplasts. **a** Expression of *PdMYB118* in the transfected protoplasts. **b** Expression levels of poplar anthocyanin biosynthetic genes in the transfected protoplasts. The construct pGreenII62-SK-*PdMYB118* was transfected into the poplar leaf protoplasts. The empty vector was used as a negative control. RNA was extracted from the transfected protoplasts for qRT-PCR analyses of anthocyanin biosynthetic genes and *PdMYB118*. Gene expression level in control was set to 1. Error bars represent the SDs from three biological replicates. Statistical differences were determined by a Student's *t* test (**$P < 0.01$). C protoplasts transfected with pGreenII62-SK (control), M protoplasts transfected with pGreenII62-SK-*PdMYB118*

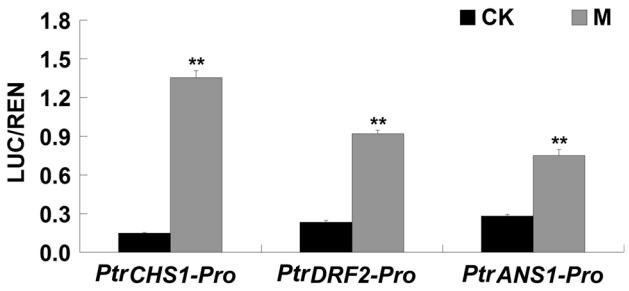


Fig. 6 Transient transcription dual-luciferase assays. The effector of *PdMYB118* (pGreenII62-SK-*PdMYB118*) was co-transfected with the reporters of *PtrCHS1*, *PtrDFR2,* and *PtrANS1* promoters in poplar protoplasts, respectively. The expression level of REN was used as an internal control. The LUC/REN ratios represent relative activities of PdMYB118 on the promoters of *PtrCHS1*, *PtrDFR2,* and *PtrANS1*. Error bars represent the SDs from three biological replicates. Statistical differences were determined by a Student's *t* test (**$P < 0.01$). CK protoplasts transfected with pGreenII62-SK (control), M protoplasts transfected with pGreenII62-SK-*PdMYB118*

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Author contributions statement HW, XW, SW, YJ, CJ and CW performed the experiments and analyzed the data. HW and HZ conceived the study. HW, YB, BL, and HZ wrote the manuscript. All authors read and agreed at the last version of the manuscript.

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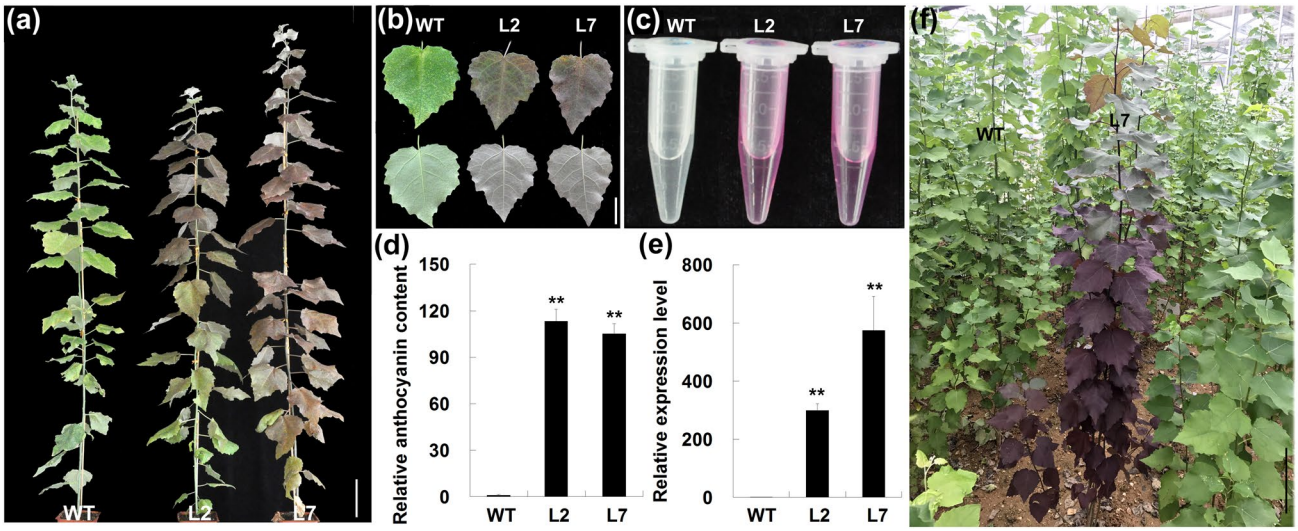

Fig. 7 Red leaf phenotype of transgenic plants overexpressing *PdMYB118*. **a** Red leaves of transgenic plants. Bar 8 cm. **b** Transgenic red leaves were photographed on the adaxial side (up panel) and the abaxial side (down panel), respectively. Bar 4 cm. **c** Anthocyanin solutions extracted from 100 mg leaf of wild-type and transgenic poplar plants. **d** Content of anthocyanins in leaves of transgenic plants. **e** qRT-PCR analysis of *PdMYB118* in the leaves of wild-type and transgenic poplar plants. Gene expression level in wild-type sample was set to 1. **f** Red leaf phenotype of transgenic plants grown in field. Error bars represent the SDs from three biological replicates. Statistical differences were determined by a Student's *t* test (**$P < 0.01$). WT, wild-type poplar (Shanxin Yang); L2 and L7, transgenic plants overexpressing *PdMYB118* (color figure online)

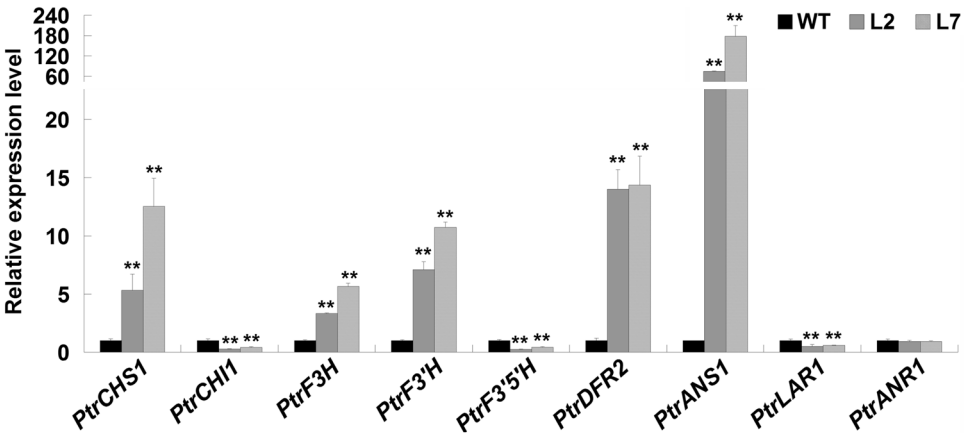

Fig. 8 qRT-PCR analyses of anthocyanin biosynthetic genes in the leaves of wild-type and transgenic poplar plants. Mature leaves were used for analysis. Gene expression level in wide type sample was set to 1. Error bars represent the SDs from three biological replicates. Statistical differences were determined by Student's *t* test (**$P < 0.01$). WT leaves of wild-type poplar plant; L2 and L7 leaves of transgenic plants overexpressing *PdMYB118*

Compliance with ethical standards

Conflict of interest We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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