The transcription factor MYB115 contributes to the regulation of proanthocyanidin biosynthesis and enhances fungal resistance in poplar

Lijun Wang1*, Lingyu Ran1*, Yisu Hou1*, Qiaoyan Tian1, Chaofeng Li2, Rui Liu1, Di Fan1 and Keming Luo1

1Key Laboratory of Eco-environments of Three Gorges Reservoir Region, Ministry of Education, Institute of Resources Botany, School of Life Sciences, Southwest University, Chongqing 400715, China; 2Key Laboratory of Adaptation and Evolution of Plateau Biota, Northwest Institute of Plateau Biology, Chinese Academy of Sciences, 810008 Xining, China

Summary

- Proanthocyanidins (PAs) are major defense phenolic compounds in the leaves of poplar (Populus spp.) in response to abiotic and biotic stresses. Transcriptional regulation of PA biosynthetic genes by the MYB-basic helix–loop–helix (bHLH)-WD40 complexes in poplar is not still fully understood.
- Here, an Arabidopsis TT2-like gene MYB115 was isolated from Populus tomentosa and characterized by various molecular, genetic and biochemical approaches.
- MYB115 restored PA productions in the seed coat of the Arabidopsis tt2 mutant. Overexpression of MYB115 in poplar activated expression of PA biosynthetic genes, resulting in a significant increase in PA concentrations. By contrast, the CRISPR/Cas9-generated myb115 mutant exhibited reduced PA content and decreased expression of PA biosynthetic genes. MYB115 directly promoted the expression of PA-specific structural genes. MYB115 interacted with poplar TT8. Coexpression of MYB115, TT8 and poplar TTG1 significantly enhanced the expression of ANR1 and LAR3. Additionally, transgenic plants overexpressing MYB115 had increased resistance to the fungal pathogen Dothiorella gregaria, whereas myb115 mutant exhibited greater sensitivity compared with wild-type plants.
- Our data provide insight into the regulatory mechanisms controlling PA biosynthesis by MYB115 in poplar, which could be effectively employed for metabolic engineering of PAs to improve resistance to fungal pathogens.

Introduction

Flavonoids are plant secondary metabolites that constitute a wide variety of compounds including flavones, flavonols, anthocyanins and proanthocyanidins (PAs, also called as condensed tannins) (Winkel-Shirley, 2001; Dixon et al., 2005; Koes et al., 2005; Lepiniec et al., 2006). PAs are oligomeric and polymeric end-products of the flavonoid biosynthetic pathway and can accumulate in high concentrations in seed coats, leaves, stems, bark and roots (Dixon et al., 2005; Paolocci et al., 2007). PA compounds play important roles in plant defense, such as in protection against UV light damage, mechanical wounding, insect infestation, fungal infection and environmental stresses (Shirley et al., 1995; Weisshaar & Jenkins, 1998; Harborne & Williams, 2000; Pietta, 2000; Winkel-Shirley, 2001; Barbehenn & Peter Constabel, 2011). In alfalfa (Medicago sativa), the increase of PAs could reduce pasture bloat and improve protein utilization in ruminant animals (Dixon et al., 1996; McMahon et al., 2000). PAs, which are oxidized to form brown pigments in seed coats, also are present in many fruits, teas and wines (Wrangham et al., 1998; Forkner et al., 2004). In addition, PAs act as potential dietary antioxidants with beneficial effects for human health (Bagchi et al., 2000; Middleton et al., 2000; Cos et al., 2004). For these reasons, there is an increasing interest in understanding the mechanisms of PAs biosynthesis in plants.

To date, the flavonoid biosynthetic pathway is one of the most extensively studied pathways of plant secondary metabolites (Koes et al., 2005; Grotewold, 2006). The biosynthesis of anthocyanins and PAs share most steps in the flavonoid pathway and leucoanthocyanidins are the first branch point between these two biosynthesis pathways. The main structural genes of PA biosynthetic pathway have been isolated from many species, including Arabidopsis, maize, petunia, snapdragon, sweet potato, alfalfa, apple and grape (Beld et al., 1989; Holton & Cornish, 1995; Boss et al., 1996; Honda et al., 2002; Abrahams et al., 2003; Pang et al., 2009). Leucoanthocyanidin reductase (LAR) and anthocyanidin reductase (ANR), which function at the branching points of the PA pathway, leading to catechin and epicatechin synthesis, respectively, have been reported in Desmodium uncinatum (Tanner et al., 2003), Medicago truncatula (Xie et al.,...
352 Research

The PA biosynthetic pathway is transcriptionally regulated by many transcription factors, such as R2R3 MYB, basic helix-loop-helix (bHLH) and WD40 repeat proteins (Hichri et al., 2011; Huang et al., 2013). The Arabidopsis TRANSPARENT TESTA2 (TT2) gene, which encodes a MYB protein, has been identified as a regulator controlling the transcription of ANR, DFR (encoding dihydroflavonol 4-reductase) (Nesi et al., 2001; Sharma & Dixon, 2005). In grapevine (Vitis vinifera), four MYB-like members – VvMYBPA1, VvMYBPA2, VvMYBPAR and VvMYBC2-L1 – have been demonstrated as specific regulators of the PA pathway (Bogs et al., 2007; Terrier et al., 2009; Huang et al., 2014; Koyama et al., 2014). TT2-like MYB transcription factors were also identified in Brassica napus (Wei et al., 2007) and Lotus japonicus (Yoshida et al., 2008). In persimmon, DkMYB2 and DkMYB4 act as regulators for PA biosynthesis in developing fruits (Akagi et al., 2009, 2010). Recently, a MYBPA1-like protein, activated the DFR and LAR promoters to modulate PA biosynthesis in Prunus persica (Ravaglia et al., 2013).

It has been demonstrated that PA accumulation is often induced by insect herbivory and fungal infection, mechanical wounding and high-intensity light, suggesting its involvement in defense against biotic and abiotic stresses (Hemming & Lindroth, 1999; Peters & Constabel, 2002; Stevens & Lindroth, 2005; Miranda et al., 2007; Mellway et al., 2009). The toxic effects of PAs on fungi were estimated by measurement of the reduction in the in vitro growth of mycelium (Yuan et al., 2012). In trees, PAs also preserve heartwood from fungal decay and inhibit extracellular hydrolases from invading pathogens, thus preventing their rapid deployment in plants (Scalbert, 1991). Dothiorella gregaria is a fungal species that causes a bark necrosis on the trunk and branches of poplar, resulting in massive production losses worldwide (Yang et al., 1980). Rooted plantlets were grown in the greenhouse at 25°C under a 14 h:10 h, light:dark cycle with supplemental light (4500 lux). The Arabidopsis tt2 mutants (CS83) were obtained from the Arabidopsis Biological Resource Center (ABRC). Seeds of Arabidopsis thaliana and tobacco (Nicotiana benthamiana) were surface-sterilized with 10% bleach and germinated on plates with ½-strength Murashige and Skoog (½MS) medium (Murashige & Skoog, 1962). Seedlings were grown in a glasshouse (16 h:8 h, light:dark) at 22°C and 80% relative humidity.

The leaves of 3-month-old poplar plants were used for different treatments. Fungal inoculations were performed as described previously (Yuan et al., 2012). Marssonina brunnea f. sp. multiagrumtubi and Dothiorella gregaria Sacc. were used in our study. For wounding treatments, poplar young leaves were harvested after being punctured with sterile needles and placed at 20°C for 2 h. For high light (HL) exposure experiments, poplar plants in the glasshouse (355 mol m⁻²s⁻¹) were used as controls and then transferred into full natural sunlight intensity conditions (1756 mol m⁻²s⁻¹).

In order to determine the resistance of transgenic poplar against fungal infections, the in vivo test was performed with...
**Research 353**

**D. gregaria** as described previously (Huang et al., 2012). Adobe Photoshop was used to calculate lesion area.

**RNA extraction and quantitative RT-PCR**

Total RNA was extracted from plant tissues by Plant RNeasy Mini Kit (Qiagen) and reverse transcribed into cDNA using PrimeScript™ RT reagent Kit with gDNA Eraser according to the manufacturer’s instructions (Takara, Dalian, China). Quantitative real time polymerase chain reaction (qRT-PCR) analysis was performed in a 25-µl reaction volume containing 12.5 µl of SYBR Premix Ex Taq™ (Takara), 2 µl cDNA (20 ng), 0.4 µM of each primer. The amplification was monitored on a Thermal Cycler Dice Real Time System TP800 (Takara). Differences in gene expression, expressed as fold change relative to controls, were calculated using the 2−ΔΔCt method (Livak & Schmittgen, 2001). The primers for qRT-PCR were designed according to Tsai et al. (2006) and Shi et al. (2010).

**Sequence comparisons and phylogenetic analysis**

Full-length amino acid sequences were retrieved from JGI (https://phytozome.jgi.doe.gov/pz/portal.html). Amino acid sequence alignments of MYB proteins were performed using DNAMAN v.7 (Lynnon Biosoft, Quebec, Canada). A neighbor-joining phylogenetic tree of R2R3-MYB proteins was constructed using MEGA v.4.1 (Tamura et al., 2007) with 1000 bootstrap replicates. The GenBank accession numbers for genes used in this study are listed in Supporting Information Methods S1.

**Cloning of MYB115**

The open reading frame (ORF) of **MYB115** was amplified from cDNAs from leaves of *P. tomentosa* using gene-specific primers (Table S1) designed according to the sequence of **MYB115** (Potri.002G173900). The PCR products were amplified using PrimeSTAR HS DNA Polymerase (Takara), followed by the A-addition procedure using Ex Taq Polymerase (Takara). The PCR products were cloned into the vector pCXSN-FLAG (Chen et al., 2009) digested with *Xcm* (New England Biolabs, Beverly, MA, USA) to generate the 355:MYB115 construct and confirmed by sequencing.

**Plant transformation**

Poplar transformation was performed using *Agrobacterium*-mediated methods described previously (Jia et al., 2010). The poplar leaf discs were infected by recombinant *Agrobacterium* and putative transgenic plants were selected on WPM supplemented with 9 mg l−1 hygromycin. *Agrobacterium tumefaciens* harboring the 355:MYB115 construct was transformed into the Arabidopsis *tt2* mutants by the floral dip method (Clough & Bent, 1998). Selection of transformants was performed on ½MS medium supplied with 50 mg l−1 hygromycin. Seed phenotypes were observed in progeny from T2 transformants with a single copy of the transgene and further screened for homozygotes after germination.

**Generation of the myb115 mutant by CRISPR/Cas9**

The full-length DNA sequence of **MYB115** was screened in the online tool ZiFiT TARGETER v.4.2 (http://zifit.partners.org/ZiFiT/Introduction.aspx) (Sander et al., 2010). Three putative target sites located at the first exon of the **MYB115** coding sequence were selected for designing the sgRNA sequences based on their GC abundance. Three pairs of oligos (Table S1) were designed to specifically target **MYB115** and sgRNA cassettes were assembled into binary pYLCRIPSR/Cas9 vector (Ma et al., 2015) based on Golden Gate Cloning (Fan et al., 2015). The genomic DNA was isolated from the **myb115** mutant using a CTAB method, followed by PCR amplification using gene-specific primers (Table S1). The PCR product was cloned into the pMD19-T vector (Takara) and at least 30 clones for each transgenic line were randomly selected for sequencing.

**Yeast two-hybrid (Y2H) assays**

Y2H assays were performed according to the manufacturer’s instructions (Clontech, Palo Alto, CA, USA). The AD and BD fusion constructs were co-transformed into yeast strain Gold2 using the PEG/LiAC method as described by Gietz & Schiestl (2007). The transformants were then screened on SD medium lacking Trp, Leu, histidine (His) and adenine (Ade) (SD/-Trp/-Leu/-His/-Ade) in the presence of X-α-galto identify the interaction of **MYB115** with other transcription factors.

**Bimolecular fluorescent complementation (BiFC)**

For BiFC assays, expression vectors pG-RN159 and pG-RC160, harboring the N- and C-terminal halves of red fluorescent protein (RFP), respectively, were used as described by Fan et al. (2008). The ORF of **MYB115** was cloned into pG-RN159 to produce the **MYB115-nRFP** construct. The full-length coding sequences of **TT8** and **TTG1** were cloned into pG-RC160 to generate **TT8-cRFP** and **TTG1-cRFP**, respectively. The resulting constructs were bombarded into onion epidermal cells using Gene Gun GJ-1000 (S cientz, Ningbo, Zhejiang, China) for transient assays. Transfected cells were imaged using a confocal microscope (Leica TCS SP5, Wetzlar, Germany).

**Coimmunoprecipitation (CoIP) analysis**

The ORFs of **TT8** and **TTG1** were cloned into pCXSN-FLAG (Chen et al., 2009) to generate the constructs **TT8-FLAG** and **TTG1-FLAG**, respectively. *Agrobacterium* cells harboring **TT8-FLAG** or **TTG1-FLAG** were co-transformed with 355:MYB115: GFP into tobacco leaves (Yang et al., 2000). Soluble proteins were extracted with extraction buffer (50 mM Tris-HCl, 500 mM sucrose, 1 mM MgCl2, 10% glycerol, 10 mM EDTA, 0.1% Triton X-100, 0.2% Nonidet P-40, 5 mM DTT, 1 mM PMSE and complete protease inhibitor cocktail tablet (Boehringer Mannheim)). After centrifugation at 18 000 g for 15 min. The precleared extracts were incubated with 10 µl of Protein A sepharose (GE Healthcare, Piscataway, NJ, USA) at
4°C for 1 h, followed by centrifugation at 800 g at 4°C for 5 min. The supernatant was incubated with 5 μl of anti-FLAG antibody (Sigma-Aldrich) at 4°C for 2 h. The centrifuged (800 g) supernatant was pre-cleared with 30 μl of pre-washed protein A sepharose beads at 4°C for 1 h. After removing the supernatant, the beads were washed three times in CoIP buffer at 4°C. Bound proteins were released by adding 5× protein loading buffer and boiled for 2 min at 95°C. The fusion proteins were detected by immunoblotting using monoclonal anti-GFP antibody (Sigma-Aldrich) and monoclonal anti-FLAG antibody (Sigma-Aldrich).

**Results**

**Identification of stress-responsive R2R3 MYB genes involved in the PA biosynthetic pathway in poplar**

In order to identify candidate regulators of stress-induced PA metabolism in poplar, we analyzed expression profiles of *Populus*R2R3-MYB genes of clades C25, C26, C27 and C32 (Wilkins et al., 2009) in the leaves after treatments with *M. brunnea* and mechanical wounding. Transcriptome analysis revealed that transcript abundances of *MYB50*, *MYB61* and *MYB115* were dramatically upregulated in response to the treatments (Fig. S1; Methods S1). Among them, MYB115 is classified as the MYBPA1-type positive PA regulator (Yoshida et al., 2015). We isolated MYB115 from a leaf cDNA library of *P. tomentosa* by qRT-PCR using gene-specific primers (Table S1). The ORF encodes a protein of 285 amino acid residues with a predicted mass of 32 kD and a calculated pI of 9.2. Analyses of the deduced amino acid sequence reveals that MYB115 contains an N-terminal R2R3 repeat with a [DE]Lx2[RK]x3Lx6Lx3R motif which specifically interacts with bHLH proteins (Fig. 1a) (Zimmermann et al., 2004). Compared with other MYB proteins, MYB115 showed the highest identity with VvMYBPA1 (64.7%) which was identified to regulate PA synthesis in the early stage of grape berry development (Bogs et al., 2007).

Phylogenetic tree reveals that these MYB proteins associated with the regulation of flavonoid pathway are classified into two major groups (Fig. 1b). MYB115 belongs to the same subclade as VvMYBPA1, DkMYB4 and PrtMYB123 (Bogs et al., 2007; Akagi et al., 2009), and is clearly distinct from regulators of the anthocyanin pathway, such as AtPAP1 (Bhargava et al., 2010), AtPAP2 (Borevitz et al., 2000), VvMYBA1 (Kobayashi et al., 2004), VvMYBA2 (Walker et al., 2007), PhAN2 (Quattrocchio et al., 1999) and LeANT1 (Mathews et al., 2003) (Fig. 1b). PrtMYB134, DkMYB2 and VvMYBPA2 appear to be TT2-like R2R3 MYB factors (Fig. 1b), which are involved in the specific regulation of PA metabolism (Mellway et al., 2009; Terrier et al., 2009; Akagi et al., 2010).

**MYB115 is induced by wounding, high light and fungal inoculation**

In order to further determine whether MYB115 is a regulator of stress-activated PA synthesis, the promoter fragment of *MYB115* was isolated from *P. tomentosa* and predicted by PLACE and
PlantCARE databases (Higo et al., 1999; Lescot et al., 2002). A number of elements responsible for different stresses, including light-responsive motifs, and wounding and pathogen-responsive elements, are found in the MYB115 promoter (Fig. S2; Methods S1). Furthermore, we determined the transcript level of MYB115 by qRT-PCR at different times after wounding. As expected, mechanical wounding of leaf margins induced MYB115 expression, reaching a peak at 24 h after wounding, and beginning to decrease at 48 h (Fig. 2a). Consistently, PA concentrations elevated four times in the damaged leaves after wounding for 24 h (Fig. 2d).

In order to determine the expression level of MYB115 under HL, we transferred poplar plants from the glasshouse into full natural sunlight as described by Mellway et al. (2009). qRT-PCR analysis showed a strong activation of MYB115 by 6 h in the HL-exposed leaves, and the highest transcript level at 48 h (Fig. 2b). The analyses of extracts from the leaves after 48 h of HL exposure revealed a significant increase in total PA accumulation (Fig. 2e).

Transcript levels of MYB115 were also induced after inoculation with a hemibiotrophic fungus D. gregaria, and the highest level was found at 72 h after infection (Fig. 2c). PA concentration was determined at 96 h after infection and showed a five-fold increase (Fig. 2f).

MYB115 seems to act as a transcriptional activator

Sequence analysis showed that the MYB115 protein contains the predicted nuclear localization signals (NLS) in the N-terminal domain (Fig. 1a). To determine the subcellular localization of MYB115, we transiently expressed 35S:MYB115:GFP in the onion epidermal cells and observed the tissue by confocal microscopy. The 35S:MYB115:GFP in the onion epidermal cells and observed the tissue by confocal microscopy. The 35S:MYB115:GFP fusion protein exhibited its activation property in yeast cells, indicating that MYB115 acts as a potential transcriptional activator.
Expression profiles of MYB115 in poplar

In order to determine expression patterns of MYB115, the expression levels of MYB115 in various tissues of *P. tomentosa* were evaluated by qRT-PCR. MYB115 was expressed in all tissues tested with the highest mRNA level in roots (Fig. 3a). To investigate the relationship between MYB115 expression and PA accumulation in poplar leaves, we examined transcript levels of MYB115 at different developmental stages of poplar leaves. MYB115 was highly expressed in young leaves (leaf plastochron index, LPI-1, -2 and -3), but a sharp decrease was detected in mature leaves (LPI-4 and -5) (Fig. 3b).

**MYB115 complements the Arabidopsis tt2 mutant phenotype**

In order to confirm the function of MYB115 as a TT2-like regulator of PA synthesis, the 35S:MYB115 construct was introduced into the Arabidopsis *tt2* mutant (Nesi *et al.*, 2001). Three independent transgenic lines with high transcript levels of *MYB115* were selected for further analysis (Fig. 4a). Overexpression of MYB115 in the *tt2* mutant rescued the *tt2* phenotype (bright yellow seed color), producing the brown colored mature seeds typical of WT (Fig. 4b). The Arabidopsis seeds were further stained with DMACA, and these results showed that MYB115 could complement the PA-deficient phenotype of the *tt2* mutant (Fig. 4c). Quantification of PAs demonstrated that the *tt2* mutant seeds complemented with 35S:MYB115 restored the production of both soluble and insoluble PAs up to the contents found in WT (Fig. 4d).

Overexpression of MYB115 induces PA accumulation in transgenic poplar

In order to investigate the function of MYB115, the 35S:MYB115 construct was introduced into Chinese white poplar by *A. tumefaciens*-mediated transformation. A total of 16 transformants were obtained and grown in the glasshouse, and three independent lines (OE-1, -9 and -13) with high transcript levels of MYB115 were chosen for further analysis.

In order to determine PA localization in poplar, different tissues were stained with DMACA. As expected, histochemical
staining showed that PAs were present at much greater concentrations in the MYB115-OE tissues compared with these of the control (Fig. 5a,b). In petioles, blue staining was observed only in the epidermal cells of the controls, whereas stronger staining was observed in the epidermal, phloem and xylem cells of MYB115-OE plants. Quantitative measurement showed that there was a significant increase in the concentrations of both soluble and insoluble PAs in MYB115-OE plants compared with WT (Fig. 5d). These results indicate that MYB115 positively regulates the expression of these structure genes involved in PA pathway and also affects lignin biosynthesis.

CRISPR/Cas9-mediated mutations in MYB115 result in a reduction of PA accumulation in poplar

In order to further examine the role of MYB115 in regulation of PA biosynthesis, we generated targeted mutagenesis of MYB115 via the CRISPR/Cas9 system according to our previous report (Fan et al., 2015). Three 20-bp sequences followed by a 5'-NGG-3' protospacer adjacent motif (PAM) located in the first exon region of MYB115 were selected as sgRNA complementary sites (Fig. 6a). To confirm the targeted mutagenesis of MYB115, the genomic DNA from 11 transgenic lines harboring the Cas9-SgRNAs construct was extracted for PCR amplification (Fig. S4a). The PCR-amplified products were detected by sequencing analysis of randomly selected clones from individual transgenic plants (Fig. S4b). The results revealed that there were insertions (+) or deletions (−) at the desired target sites caused by the CRISPR/Cas9 system, introducing indels into the MYB115 gene via the nonhomologous end-joining (NHEJ) repair pathway. Among them, sequence rearrangements in the first exon were detected in transgenic lines L3, L5, L8 and L10, leading to translational frameshift of MYB115 (Fig. S4b). To investigate the stability of heritable targeted mutagenesis in the progenies, lots of somaclones were regenerated from the myb115 mutants. The PCR products were amplified using genomic DNA extracted from the clonal progenies of the myb115 mutants (L5 and L10), and transformed into the A/T cloning vector, respectively. A total of 30 PCR clones for each mutant were randomly selected for sequencing (Fig. 6a). In the myb115 mutant L5, small deletions (2 bp or 19 bp) were detected at the desired target sites T1 & T2 in all of the progenies tested, suggesting the 100% efficiency of mutagenesis. No DNA edition was found in the T3 site. Similar results were obtained in the progenies of the myb115 mutant L10, in which a high efficiency (93.33%) of mutagenesis also appeared in sites T1 & T2 of the MYB115 gene (Fig. 6a). These results indicated that Cas9-sgRNA successfully generated heritable mutations in the MYB115 gene in poplar.

Quantification of PAs revealed that both soluble and insoluble PA contents were significantly reduced in the MYB115 mutant lines (L5, L10) compared with WT (Fig. 6b). Furthermore, the contents of total phenolics, phenolic acids, flavonol glycosides, flavonoids, anthocyanins and lignins in WT, MYB115-OE and myb115 mutant plants were examined (Methods S1). Varying degree increases in contents of total phenolics and flavonoids only were detected in MYB115-OE plants, but obvious decreases were detected in myb115 mutants in comparison to WT (Fig. S5a,b). By contrast, there were no significant differences in accumulation of phenolic acids, anthocyanins, flavonol glycosides and acid-insoluble lignins between MYB115-OE, myb115 mutants and WT (Fig. S5d–f).

qRT-PCR analysis showed that the expression levels of some structural genes of the PA biosynthetic pathway, including PAL5, CHS4, CHI1, DFR1, ANR1, LAR1 and LAR3 were consistently upregulated in the MYB115-OE plants compared with WT (Fig. 5d), whereas the transcript levels of PAL5, F3H, FLS1 and UFGT1 were not different in transgenic MYB115-OE plants and WT (Fig. 5d). However, lignin biosynthetic genes including CCoAOMT1, CAD1 and CCR2 were significantly downregulated in transgenic MYB115-OE plants as compared with WT (Fig. 5d). These results indicate that MYB115 positively regulates the expression of these structure genes involved in PA pathway and also affects lignin biosynthesis.
loss-of-function of MYB115 and reduced PA accumulation in poplar, indicating that MYB115 acts as a specific regulator of the PA pathway.

MYB115 interacts with TT8 protein to form a regulatory complex

In order to examine whether MYB115 is capable of forming regulatory complexes with TTG1- and TT8-like proteins to regulate the PA pathway, the putative sequences of poplar TT8 (bHLH131, Potri.005G208600) and TTG1 were obtained by searching the JGI P. trichocarpa v.12 database (https://phytozome.jgi.doe.gov/pz/portal.html) using BLAST with the protein sequences of TTG1 (AT5G24520) and TT8 (AT4G09820) from A. thaliana as the query sequences. MYB115, TTG1 and TT8 were cloned into the vectors pGADT7 and pGBKT7, respectively. Y2H assays showed that MYB115 could interact with TT8 to activate the His, Ade and Mel (encoding a-galactosidase) reporter genes in yeast cells (Fig. 7a).

The specific interactions of MYB115, TTG1 and TT8 were also analyzed in plant cells by BiFC assays. When MYB115-nRFP and TT8-cRFP were co-expressed in onion epidermal cells, the RFP fluorescence was reconstituted, but fluorescence was absent in these cells coexpressing MYB115-nRFP and TTG1-cRFP, indicating the occurrence of a physical interaction between MYB115 and TT8 (Fig. 7b).
Fig. 5 Accumulation of proanthocyanidins (PAs) and expression of phenylpropanoid biosynthetic genes in MYB115-OE poplar. (a, b) PAs were localized by staining different tissues of wild-type (WT) (a) and MYB115-OE (b) plants with DMACA staining. Bars: 1 cm, leaves; 2 mm, roots; 200 μm, stems and petioles. (c) Quantification of soluble and insoluble PAs in different transgenic lines (OE-1, OE-9 and OE-13). (d) Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of MYB115 and phenylpropanoid biosynthetic genes in MYB115-OE plants. Error bars represent mean ± SD of three biological replicates. Significant differences using Student’s t-test: *, P < 0.05; **, P < 0.01.
Fig. 6 Characterization of targeted mutagenesis of MYB115 in poplar. (a) Targeted mutagenesis of MYB115 in poplar is identified by sequencing two representative transgenic plants. The CRISPR/Cas9 targeted sequences (T1, T2 and T3) in the region of exon 1 of MYB115 are indicated in the red box. The targeted sequences (Target 1, 2, 3) are shaded in gray and the protospacer adjacent motif (PAM) sequence is in bold type. The deleted nucleotide is replaced by short red lines, the inserted nucleotide is written in red uppercase letters, and the substituted nucleotide is written in red lowercase letters. The numbers in the left-most column preceded by ‘/C0’ and ‘/+’ show how many nucleotides are deleted or inserted, and those in parentheses represent the frequency of detected clones with such mutant allele. (b) Quantification of soluble and insoluble proanthocyanidins (PAs) in the wild-type (WT) and myb115 mutant lines (L5, L10). (c) Quantitative RT-PCR analysis of PA biosynthetic genes in myb115 mutant plants. Error bars represent ± SD of three biological replicates. Significant differences using Student’s t-test: **, P < 0.01.
Furthermore, we performed a CoIP experiment to confirm this interaction. TT8-FLAG or TTG1-FLAG were co-transformed with 35S:MYB115:GFP into tobacco leaves. Proteins were extracted and immunoprecipitated with anti-FLAG antibody, and the IP fraction was analyzed in a protein blot with anti-GFP antibody. MYB115-GFP proteins were detected to interact with TT8, confirming that the physical interaction exists only between TT8 and MYB115 (Fig. 7c).

MYB115 contributes to the regulation of the PA biosynthesis pathway

In order to identify structural genes of PA biosynthesis activated by MYB115, promoter trans-activation assays were conducted in tobacco leaves by transient expression experiments. The promoters of PA biosynthetic genes were fused with GUS reporter gene to generate the reporter constructs. The 35S:MYB115-GFP, 35S:TTG1 and 35S:TT8 constructs were used as effectors (Fig. 8a). As expected, MYB115 activated the promoter of the genes PAL1, CHS, DFR, ANR1 and LAR3 12-, 8.5-, 9-, 6.6- and 7.2-fold, compared with the corresponding control, respectively, whereas the promoter of FLS1 was not activated by MYB115 (Fig. 8b).

Furthermore, the promoter activities of poplar ANR1 and LAR3 activated by MYB115, TT8 and TTG1 were analyzed by transient expression assays. We found that TT8 and TTG1 were not able to activate these promoter expressions, whereas coexpression of MYB115, TTG1 and TT8 activated the ANR1 and LAR3 promoters by 12- and 22-fold, respectively (Fig. 8c, d). These results suggest that MYB115 seems to interact with TT8 and TTG1 to form a regulatory complex to activate the PA biosynthetic pathway.

MYB115 overexpression enhances resistance to D. gregaria in transgenic poplar

In order to determine the resistance of the MYB115-OE and myb115 mutant plants against fungi, excised leaves of transgenic and WT plants were inoculated with agar plugs containing hyphae of hemibiotrophic fungus D. gregaria. Compared with the severe disease symptoms appeared on the control leaves at 4 d post inoculation (dpi), only slight necrotic lesions were on the
leaves of the MYBI15-OE lines tested (Fig. 9a). Although the leaves of the myb115 mutants showed larger lesion areas than WT (Fig. 9b). Quantification assays showed that lesion areas in the MYBI15-OE and myb115 mutant lines were c. 50% (Fig. 9c) and 137.5% (Fig. 9d) of WT, respectively. To detect the antifungal activity in vitro, D. gregaria was inoculated on PDA medium containing the crude extracts from the poplar leaves. As shown in Fig. 9(e), mycelial growth of the pathogens was inhibited on the media with the extracts from WT and transgenic plants. Among them, the most obvious inhibitory effect was detected in the MYBI15-OE lines but this was relatively weak in the myb115 mutants compared with WT. No inhibition zone was found on the medium without extracts (Fig. 9e). Quantitative measurement revealed that colony diameter of D. gregaria grown on the medium with the extracts from the MYBI15-OE plants was reduced by 36% compared with that of the control without extracts (Fig. 9g).

In order to examine whether the fungal resistance in transgenic plants was correlated with the expression levels of resistance genes, we detected the expression of pathogenesis-related gene PR5, which is a marker gene of the SA signaling pathway, MYB44 (a transcription activator of the SA signaling pathway), NPR1 (nonexpressor of PR1, a key node of the SA pathway) and JAZ10 (belonging to the jasmonate-zim-domain protein family) (Spoel et al., 2003; Loake & Grant, 2007; Thines et al., 2007; Shim et al., 2013). No significant differences in the expression levels of these resistance-related genes were found in WT and transgenic plants (Fig. 6), indicating that the sensitivity of transgenic plants to pathogens might be caused mainly by PA accumulation. Furthermore, the inhibition against D. gregaria by PAs was also detected microscopically. The abnormal hyphal growth of D. gregaria, such as short hyphae, swollen tips and fewer hyphal branches, was observed when supplied with the MYBI15-OE extracts (Fig. 9f), consistent with PA toxicity to filamentous fungi (Scalbert, 1991). These results indicate that MYBI15 acts as a positive regulator of basal resistance to infection of hemibiotrophic fungal pathogens in poplar.

Discussion

MYBI15 is a stress-induced regulator of PA biosynthesis in poplar

Proanthocyanidin (PA) compounds are important secondary metabolites for plant adaptation to the environmental conditions, such as mechanical wounding, pathogen attacks and UV stresses. Previous studies have demonstrated that PA accumulation begins in the youngest leaves of poplar and reaches a peak in mature leaves (Salminen et al., 2004; Wang et al., 2013). PAs as antimicrobial agents are rich in poplar roots (Cushnie & Lamb, 2005; Yuan et al., 2012), and are able to affect microbial activity in the soil (Schimmel et al., 1996). Damage to poplar leaves by fungal infection or insect herbivory will induce the expression of genes involved in the PA biosynthetic pathway and cause rapid PA accumulation (Peters & Constabel, 2002; Mellow et al., 2009). It is well established that the transcriptional regulation of these PA structural genes is controlled by MYB proteins as shown by previous studies (Terrier et al., 2009; Hichri et al., 2011; Huang et al., 2012; Koyama et al., 2014).

In poplar, a large number of R2R3 MYB family members have been predicted (Wilkins et al., 2009), but only a few MYB
proteins have been identified to specifically regulate PA biosynthesis (Mellway et al., 2009; Yoshida et al., 2015). In this study, we describe the identification and characterization of MYB115 from poplar to regulate PA synthesis. Expression of MYB115 was induced by different stresses including mechanical wounding, HL and fungal pathogens in poplar leaves (Fig. 1). Overexpression of MYB115 in Arabidopsis tt2 mutant can restore PA accumulation in seed coats of tt2 mutants (Fig. 4), suggesting that MYB115 specifically regulates PA synthesis. Constitutive expression of MYB115 in transgenic poplar activated the PA biosynthetic pathway, including general phenylpropanoid genes, both early and late flavonoid genes, resulting in a significant accumulation of PAs (Fig. 5). By contrast, mutation of MYB115 in transgenic poplar led to downregulation of many structural genes.

Fig. 9 Resistance of transgenic poplar plants to fungal infections. (a) Disease symptoms of the third leaf from wild-type (WT) and MYB115-OE plants after 4 d of Dothiorella gregaria infection. (b) Disease symptoms of the sixth leaf from WT and myb115 plants after 4 d of D. gregaria infection. (c) Ratio of the lesion area in the infected leaves from WT and MYB115-OE plants (OE-13). (d) Ratio of the lesion area in the infected leaves from WT and myb115 plants (L10). Values are means of three replications. Error bars indicate ± SD. Significant differences using Student’s t-test: **, P < 0.01. (e, f) In vitro antifungal activity of proanthocyanidins (PAs). (e) Dothiorella gregaria was inoculated on PDA medium supplied with crude leaf extracts from MYB115-OE, WT and myb115 mutant plants. NA, a negative control without crude leaf extracts. (f) Microscopic observation of hyphal growth of D. gregaria. Pictures were taken after 72 h of incubation of D. gregaria. Arrows indicate the position of hyphal branches. (g) Quantitative measurement of the diameters of these colonies on the medium after 72 h of incubation with D. gregaria. Error bars represent ± SD of three biological replicates.
of the flavonoid pathway (Fig. 6c), indicating that MYB115 acts as a direct or indirect regulator of these genes. Previous studies have shown that poplar ANR1 and LAR3 are two PA-specific genes, and overexpression of these genes in transgenic plants led to an increase in PA accumulation (Yuan et al., 2012; Wang et al., 2013). Similar results were obtained in grapevine (Vitis vinifera), in which the expression of VvMYBPA1, a MYB homolog of MYB115, activated the PA-specific genes VvLAR1 and VvANR (Bogs et al., 2007).

**MYB115 encodes a VvMYBPA1-like transcription factor**

MYB-related transcription factors are well known to regulate various branches of the flavonoid pathway in plants and are believed to play wide roles in the regulation of phenylpropanoid metabolism in general (Tamagnone et al., 1998; Deluc et al., 2006). Many Transparent Testa 2 (TT2)-like MYB proteins that regulate PA biosynthesis in different species have been characterized. As shown in Fig. 2(b), these PA regulators except for PtrMYB182 fall within two subclades, PA clades 1 and 2. Likewise, the formation of two separate subgroups of PA regulators was reported by Hancock et al. (2012) and Koyama et al. (2014). Obviously, PA clade 1 included VvMYBPA1, PtrMYB123, MYB115 and DkMYB4 (Bogs et al., 2007; Akagi et al., 2009). Sequence alignment analysis showed that these proteins are very similar (Fig. 2a). MYB115 exhibits the highest sequence similarity to VvMYBPA1, which is specific to regulation of PA biosynthesis in grapes (Fig. 1). By contrast, PA clade 2 included FaMYB9/FaMYB111 from Strawberry (Fra-garia × ananassa) (Schaart et al., 2013), MtMYB14 from M. truncatula (Liu et al., 2014), Arabidopsis TT2 (Nesi et al., 2001), DkMYB2 from Diospyros kaki (Akagi et al., 2010) and PrMYB134 from P. trichocarpa (Mellway et al., 2009). In a previous study, ectopic expression of VvMYBPA1 in Arabidopsis can activate both general pathway genes and those on the PA-specific branch and induce high concentrations of PAs, resulting in the death of transgenic plants at the early stage of development (Bogs et al., 2007). In the present study, however, no phenotypic alteration except for color of seed coats was found in transgenic Arabidopsis tt2 mutants harboring 35S:MYB115 (Fig. 4).

Like VvMYBPA1 and VvMYBPA2 in grapevine (Bogs et al., 2007; Terrier et al., 2009), transgenic plants overexpressing PrtMYB134 and MYB115 exhibited similar phenotypes, indicating that these two proteins have apparent redundancy as activators of PA biosynthesis in poplar. Most species examined except for Arabidopsis, which has only the TT2 type, have both types of PA MYBs. These PA-specific MYBs appear to act in parallel as well as in tandem with the TT2-type PA MYBs. In grapevine, both VvMYBPA1 and VvMYBPA2 regulate the flavonoid structural genes, but VvMYBPA2 overexpression also activates expression of VvMYBPA1 (Terrier et al., 2009), suggesting that VvMYBPA2 acts upstream of VvMYBPA1. Despite the phenotypic similarities of transgenic plants, the differences of regulatory functions in PA biosynthesis between MYB134 and MYB115 remain unclear.

MYB115 positively regulates the general flavonoid pathway and PA-specific branch in poplar

Numerous studies have demonstrated that the biosynthesis of PAs is regulated by a MYB-bHLH-WD40 (MBW) complex of three different transcription factors: R2R3 MYB, bHLH and WD40 proteins (Lepiniec et al., 2006; Hichiri et al., 2011). Among them, the bHLH proteins act as bridge between WD40 and MYB proteins (Baudry et al., 2004; Schaart et al., 2013). In the present study, we demonstrated that MYB115 interacted physically with TT8 (Fig. 7), and coexpression of MYB115, TT8 and TTG1 obviously promoted PA biosynthetic gene expression (Fig. 8c,d), indicating that MYB115 may participate in forming of the MBW complex. In transient expression assays, MYB115 was able to induce the expression of the PA pathway genes, but did not affect the expression of the flavonol synthesis gene FLSI (Fig. 8b). Moreover, there were no differences in expression of anthocyanin-biosynthetic gene UFGT1 between myb115 mutants, WT and MYB115-OE lines (Figs 5d, 6c). These results indicated that MYB115 is a PA-specific regulator, consistent with these findings reported in VvMYBPA1 (Bogs et al., 2007).

In grape, VvMYBPA1 is capable of regulating the expression of both early flavonoid biosynthetic genes and PA branch genes (Bogs et al., 2007). In the present study, quantitative analysis by real time polymerase chain reaction revealed that CHI1, CHS4 and DFR1 were also activated in MYB115-OE plants (Fig. 5d) and downregulated in the myb115 mutants (Fig. 6c). Obviously, poplar MYB115, likely VvMYBPA1, is able to activate the general flavonoid pathway. Another poplar VvMYBPA2-type MYB, PtrMYB134, has also been identified to control both early and late flavonoid genes in transgenic poplars and gel shift assays showed thatPtrMYB134 can bind to AC-like elements in ANR, DFR and PAL promoters (Mellway et al., 2009), suggesting that these structural genes are targets ofPtrMYB134. In grape, transcripts of VvMYBPA1 were induced by VvMYBPA2 overexpression (Terrier et al., 2009). It is unclear whether PtrMYB134 also regulates directly MYB115 in poplar.

**Application of MYB115 overexpression in improvement of poplar for resistance to fungal pathogens**

Poplar plants are hosts for a large variety of filamentous fungi, such as Aspergillus niger, Colletotrichum graminicola, Gloeophyllum trabeum, Trichoderma viride and D. gregaria (Scalbert, 1991; Yuan et al., 2012). The transcriptional response of hybrid poplar (P. trichocarpa × P. deltoides) to poplar leaf rust (M. medusae) infection has been analyzed using the Populus cDNA microarray and these PA biosynthetic genes were upregulated dramatically (Miranda et al., 2007). Our data revealed that MYB115 overexpression resulted in PA accumulation in poplar leaves (Fig. 5). In vivo assays showed that, after infection with D. gregaria, there was a significant reduction in disease symptoms in MYB115-OE leaves compared with WT, whereas the myb115 mutant lines displayed more sensitivity to pathogen infection (Fig. 9). These results are in agreement with our previous report...
(Yuan et al., 2012), indicating that MYB115 is involved in the pathogen-defense response in poplar and its overexpression significantly enhanced resistance to fungal pathogens. Our study provides an efficient strategy using genetic engineering to increase PA accumulation in transgenic poplar plants for enhancing fungal resistance.

Acknowledgements

This work was supported by the National Key Research and Development Program (2016YFD0600105), the National Natural Science Foundation of China (31370672, 31300990), the National Key Project for Research on Transgenic Plant (2016ZX08010-003) and the Fundamental Research Funds for the Central Universities (XDJK2014a005, XDJK2013B032).

Author contributions

K.L. and L.W. designed the work; L.W., L.R., Y.H., Q.T., C.L., R.L. and D.F. performed experiments and analyzed data; L.W., L.R. and Y.H. drafted the manuscript; and L.W. and K.L. revised the manuscript.

Reference


Shim JS, Jung C, Lee S, Min K, Lee YW, Choi Y, Lee JS, Song JT, Kim JK, Choi YD. 2013. AMyB44 regulates WRKY70 expression and modulates...
antagonistic interaction between salicylic acid and jasmonic acid signaling. Plant Journal 73: 485–495.


Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Heat map showing transcript levels of PtrMYB genes in response to the fungus (M. brunnea f. sp. multigermutubi) and wounding stresses.

Fig. S2 Plant cis-acting regulatory elements in the MYB115 promoter.

Fig. S3 Nuclear localization and transcriptional activity analysis of MYB115.

Fig. S4 PCR assays to detect CRISPR-induced mutations in 11 representative transgenic plants.

Fig. S5 Quantitation of phenylpropanoid-derived compounds from WT, MYB115-OE and myb115 mutant plants.

Fig. S6 RT-qPCR analysis of resistance genes in MYB115-OE, WT and myb115 mutant plants.

Table S1 Primers used in this study

Methods S1 Supporting Materials and Methods: details of methods used in this study.

Please note: Wiley Blackwell are not responsible for the content or functionality of any Supporting Information supplied by the authors. Any queries (other than missing material) should be directed to the New Phytologist Central Office.