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Regulation of secondary cell wall biosynthesis by poplar R2R3 MYB transcription factor PtrMYB152 in *Arabidopsis*

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Poplar has 192 annotated R2R3 MYB genes, of which only three have been shown to play a role in the regulation of secondary cell wall formation. Here we report the characterization of PtrMYB152, a poplar homolog of the Arabidopsis R2R3 MYB transcription factor AtMYB43, in the regulation of secondary cell wall biosynthesis. The expression of *PtrMYB152* in secondary xylem is about 18 times of that in phloem. When expressed in Arabidopsis under the control of either *35S* or *PtrCesA8* promoters, PtrMYB152 increased secondary cell wall thickness, which is likely caused by increased lignification. Accordingly, elevated expression of genes encoding sets of enzymes in secondary wall biosynthesis were observed in transgenic plants expressing *PtrMYB152*. Arabidopsis protoplast transfection assays suggested that PtrMYB152 functions as a transcriptional activator. Taken together, our results suggest that *PtrMYB152* may be part of a regulatory network activating expression of discrete sets of secondary cell wall biosynthesis genes.

In *Arabidopsis thaliana* (Arabidopsis), there are nearly 200 genes encoding MYB transcription factors[1], which are classified according to the number of N-terminal DNA binding domain repeats. R2R3 MYB proteins containing two N-terminal DNA binding domain repeats are the largest MYB transcription factor subfamily with 126 members[1,2]. R2R3 MYB transcription factors control several aspects of plant growth and development. For example, GLABRA1 (GL1) and WEREWOLF (WER) are involved in determining cell fate during trichome and root hair cell differentiation, respectively[3,4], while AtMYB77 regulates lateral root formation[5], and ASYMMETRIC LEAVES1 (AS1) regulates shoot morphogenesis and leaf patterning[6]. Recently, several R2R3 MYB transcription factors have been found to regulate secondary cell wall biosynthesis in Arabidopsis[7–9]. Similar findings were observed in *Populus* spp. (poplar) and *Eucalyptus* spp. (Eucalyptus)[10–12].

Unlike primary cell walls, which are synthesized at the cell plate when cells divide and during cell expansion in growing cells, secondary cell walls are deposited in defined cell types such as tracheary elements and fibers after cell expansion has ceased. The massive deposition of lignin, cellulose and hemicelluloses inside primary walls gives secondary walls their characteristic thickness and strength[13]. Genetic analyses using the Arabidopsis inflorescence stems, roots, and *in vitro* secondary cell walls induced in cell culture, have identified a transcription factor network that regulates secondary cell wall biosynthesis[7,14,15]. In the network, several closely related NAC domain transcription factors, including SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN1 (SND1), NAC SECONDARY WALL THICKENING PROMOTING FACTOR1 (NST1), NST2, VASCULAR-RELATED NAC DOMAIN6 (VDN6) and VND7 have been identified as master regulators that are capable of modulating the entire biosynthetic pathways of the secondary wall components cellulose, xylan and lignin[7,14,16–18]. These NAC domain transcription factors can directly activate the expression of secondary wall specific biosynthetic genes[7,19,20] and activate the expression of several downstream transcription factor genes that also directly regulate secondary wall component biosynthetic genes[7,20].

Among the identified downstream transcription factor genes, two encode NAC domain transcription factors (SND2 and SND3), and one encodes the KNOTTED ARABIDOPSIS THALIANA7 (KNAT7) KNOTTED1-like

homeodomain (KNOX) transcription factor, which has recently been shown to negatively regulate secondary cell wall biosynthesis via interaction with OVATE FAMILY PROTEIN4 (OFP4)[20], a transcription factor from a newly identified transcription repressor family[21]. All other downstream transcription factors identified thus far encode for R2R3 MYB transcription factors, including AtMYB46, AtMYB52, AtMYB54, AtMYB58, AtMYB63, AtMYB85 and AtMYB103[7,16,19]. Among these, *AtMYB46*, *AtMYB58*, *AtMYB63*, *AtMYB83*, and *AtMYB103* have all been shown to be the direct targets of the master regulators SND1/VND6/VND7[7,18,19,22]. *AtMYB20*, *AtMYB42* and *AtMYB43* have also been shown to be regulated by SND1[7], but their functions in secondary cell wall biosynthesis have not been characterized. Ectopic expression of *AtMYB46* alone has been shown to be sufficient to induce the entire secondary cell wall biosynthesis program, while AtMYB58 and AtMYB63 specifically activate lignin biosynthesis genes during secondary cell wall formation. AtMYB75 has also been shown to regulate secondary cell wall biosynthesis by interacting with KNAT7[8,9], though it is unclear if its expression is regulated by NAC domain master switch transcription factors.

Populus trichocarpa is a good model system for studying wood development, perenniality, phenology and ecological interactions, processes that cannot be truly evaluated in annual model plants systems, such as Arabidopsis[23,24]. With growing interests in the use of lignocellulose as a source of biomass for bioenergy, insight into the control and regulation of secondary cell wall biosynthesis will help guide genetic improvement strategies for energy crops, such as *Populus*[25].

In *Populus*, there are 192 annotated genes encoding R2R3 MYB transcription factors[26]. To date, several lines of evidence support the involvement of R2R3 MYB transcription factors in the regulation of secondary cell walls biosynthesis in poplar. First, several poplar *MYB* genes have been shown to be highly expressed during secondary vascular tissue formation[27,28]. Second, PttMYB21, an ortholog of Arabidopsis AtMYB46 has been shown to be expressed primarily in xylem tissues[12]. Third, *Populus trichocarpa* PtrMYB03 and PtrMYB20, PtrMYB21 paralogs, were also shown to be orthologous to Arabidopsis AtMYB46 and its paralog AtMYB83 that can activate the biosynthetic pathways for cellulose, xylan and lignin when overexpressed in Arabidopsis[11]. Finally, overexpression of *PtrWND2B*, a NAC domain master switch transcription factor in poplar induced the expression of several poplar R2R3 *MYB* genes including *PtrMYB03*, *PtrMYB018*, *PtrMYB20*, *PtrMYB21*, *PtrMYB028* and *PtrMYB192*[29]. However, the expression of *PtrMYB152* was not affected in transgenic poplar overexpressing *PtrWND2B*. Since PtrMYB152 and its paralog PtrMYB018 are orthologous to Arabidopsis AtMYB43 and its paralog AtMYB20[29], it would be of great interest to determine if PtrMYB152 is involved in the regulation of secondary cell wall biosynthesis.

Results

PtrMYB152 is a poplar homologue of Arabidopsis R2R3 MYB transcription factor AtMYB43. The entire amino acid sequences of each of the five Arabidopsis R2R3 MYB proteins AtMYB20, AtMYB43, AtMYB46, AtMYB58 and AtMYB63 were used in BLAST searches of the poplar protein database (www.phytozome.net), to identify the most closely related poplar MYBs for each of them. Based on their predicted amino acid sequence similarities to their Arabidopsis homologues, poplar genes encoding 5 R2R3 MYBs including *PtrMYB018*, *PtrMYB021*, *PtrMYB028*, *PtrMYB152* and *PtrMYB192* were identified. PtrMYB018 was identified as the most closely related poplar MYB to AtMYB20, however, when using the amino acid sequence of the identified poplar MYBs to do BLAST, we found AtMYB43 is the Arabidopsis MYB with highest amino acid sequence similarities to PtrMYB018, similar to the results obtained by Wilkins et al[26], thus AtMYB20 was excluded from phylogenetic analysis. As shown in Figure 1A, PtrMYB018 and PtrMYB152 are paralogs and related to AtMYB43; PtrMYB028 and PtrMYB192 are paralogs closely related to AtMYB58 and AtMYB63, and PtrMYB021 is closely related to AtMYB46[11,12,26].

***PtrMYB152* is highly expressed in xylem and encodes a transcriptional activator.** Among the 5 poplar R2R3 MYB genes, all but *PtrMYB152* have been shown to be induced by NAC domain master switch transcription factor PtrWND2B in poplar[29]. So we decided to further investigate if PtrMYB152 is involved in secondary cell wall formation. We first compared its expression in poplar cells undergoing secondary wall development and in developing phloem by quantitative RT-PCR (qRT-PCR). The results showed that *PtrMYB152* is highly expressed in secondary xylem, i.e., about 18 times higher than in phloem (Figure 1B).

To determine if PtrMYB152 functions as transcriptional activator or repressor, we employed a protoplast transfection system. PtrMYB152 was fused to an N-terminal *GAL4* DNA binding domain (GD), and plasmids harboring this fusion or the GD domain alone as a control were co-transfected with a plasmid containing the *GAL4-GUS* reporter gene into protoplasts. Co-transfection with *GD* alone had little effect on the expression of the reporter gene, while co-transfection with the *GD-PtrMYB152* fusion resulted in the activation of the reporter gene (Figure 2). This implies that PtrMYB152 functions as a transcriptional activator.

Tissue specific expression of the *PtrCesA8* promoter in Arabidopsis. The finding that the PtrMYB152 functions as a transcription activator (Figure 2) and is highly expressed in poplar xylem (Figure 1B) prompted us to further investigate its potential functions in secondary cell wall biosynthesis by heterologously expressing *PtrMYB152* gene in Arabidopsis wild-type plants. We employed the commonly used *35S* promoter to ectopically express the genes at high levels. In addition, we employed the poplar *CesA8* promoter[30], from the poplar homolog of the Arabidopsis *CesA8* gene associated with secondary cell wall biosynthesis[31] for its ability to specifically direct expression to developing xylem of secondary walls in Arabidopsis. Since PtrCesA8 enzyme function is required for the biosynthesis of cellulose in secondary walls and it is specifically expressed in cells undergoing secondary wall thickening in poplar, expression of *PtrMYB152* under the control of *PtrCesA8* will be activated in cells undergoing secondary wall thickening and may work in a feedback mechanism to constitutively active secondary cell wall biosynthesis genes.

The *PtrCesA8* promoter (~3000 bp *PtrCesA8* promoter fragment) was first shown to be fully active by fusing it to the *GUS*

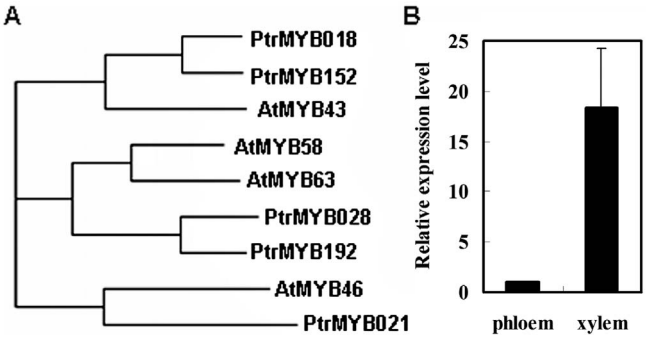

Figure 1 | PtrMYB152 is a homolog of Arabidopsis R2R3 MYB transfection factor AtMYB43. (A) Phylogenetic analysis of poplar homologues of Arabidopsis R2R3 MYB transcription factors. All bootstrap values were >80 (out of 100 replicates) (B) Relative expression levels of *PtrMYB152* in poplar xylem and phloem by real time RT-PCR analysis. Expression of *C672* was used as a reference to normalize the expression of poplar genes, and expression level of *PtrMYB152* in phloem was set as 1.

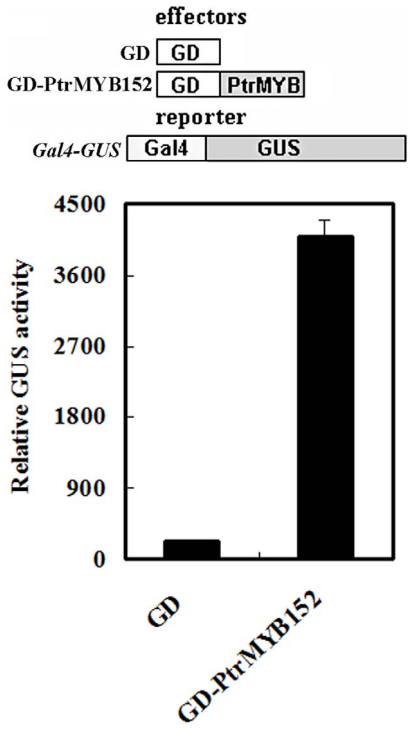

Figure 2 | PtrMYB152 is transcriptional activator. A protoplast transfection system was used to assay the ability of PtrMYB152 to activate the GUS reporter gene. Effectors and reporter (diagrammed on the top of the figure) plasmids were co-transfected into protoplasts isolated from Arabidopsis rosette leaves. GUS activity was assayed after protoplasts had been incubated in darkness for 20–22 h.

reporter gene to generate *PtrCesA8prom:GUS*, and GUS activity was examined histochemically in transgenic Arabidopsis plants. Expression patterns were similar among five individual transgenic lines examined. At the seedling stage, GUS activity was mainly detected in the veins of cotyledons, rosette leaves, and hypocotyls, and in the stele of roots (Figure 3A–E). In the inflorescence stem, GUS activity was primarily observed in the vascular bundles and interfascicular fibers (Figure 3F, G), suggesting that the promoter could be used to direct *PtrMYB152* expression preferentially to developing xylem.

Heterologous expression of *PtrMYB152* affects secondary cell wall development. Constructs of PtrMYB52 with an HA tag at its N-terminus under the control of the *35S* and *PtrCesA8* promoters were made and transformed into Arabidopsis. Multiple lines for each construct were obtained and four lines analyzed in the T2 to T4 generations for each construct. Phenotypes observed were consistent in the four independent lines.

Transgenic lines expressing *PtrMYB152* were assessed for variation in secondary cell wall thickening and chemical content. Cross sections were taken from the bases of the inflorescence stems from transgenic plants and control wild-type (Col-0) plants at principal growth stage 6.10[32] when the inflorescence stems of the plants were at similar height of ~12 cm with two to three fully expanded siliques. Preliminary observation of secondary cell wall thickness and morphology using cross-sections stained with toluidine blue showed that heterologous expression of *PtrMYB152* under the control of either the *35S* promoter or the *PtrCesA8* promoter had interfascicular fibers with significantly thicker cell walls than wild-type Col-0 (data not shown). A more detailed view of the effects of *PtrMYB152* on secondary cell wall biosynthesis was obtained by examining sections using transmission electron microscopy (TEM). This analysis

Figure 3 | Expression of *PtrCesA8prom:GUS* in Arabidopsis transgenic plants. 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) was used as a substrate for histochemical staining for GUS activity. (A) whole seedling, (B) close-up views of shoot, (C) cotyledon, (D) hypocotyls, (E) root tip, (F) upper stem section, and (G) lower stem section.

revealed that all vessel, interfascicular fiber and xylary fiber secondary cell walls in the transgenic plants were thicker than those of wild type (Figure 4, Table 1).

Cross-sections were also stained with phloroglucinol to visualize lignin deposition. As shown in Figure 5, more intense phloroglucinol staining was observed in sections from the transgenic lines relative to wild-type, suggesting an increase in lignin content in the transgenic plants.

Cell wall chemistry of *PtrMYB152* expressing lines. Since observations from phloroglucinol-stained stem cross sections suggested an increase in lignin content in transgenic plants expressing *PtrMYB152*, cell wall chemistry was assessed in inflorescence stems of transgenic plants relative to wild-type plants. Because chemical

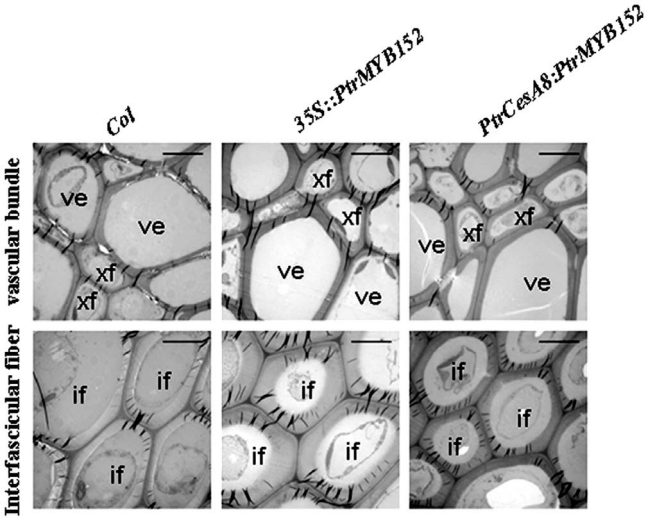

Figure 4 | Secondary cell wall thickening in Arabidopsis transgenic plants expressing *PtrMYB152*. Cross-sections from basal inflorescence stems of wild type and transgenic plants at principal growth stage 6.10.If, interfascicular fiber; ve vessel; xf, xylary fiber; p, phloem. Bars, 10 μm.

Table 1 | Wall thickness of vessels and fibers in the stems of wild type (Col-0) and *PtrMYB152* transgenic plants. Data represent mean ± SD from at least 50 cells measured from TEM images taken from the bases of primary inflorescence stems

	Cell type		
Genotype	Interfascicular fibers (μm)	Vessels (μm)	Xylary fibers (μm)
Col-0	1.66 ± 0.46	1.36 ± 0.41	1.03 ± 0.30
35S:PtrMYB152	4.03 ± 0.90*	2.00 ± 0.47*	1.47 ± 0.51*
PtrCesA8:PtrMYB152	2.71 ± 0.67*	1.81 ± 0.47*	1.48 ± 0.47*

*: The differences to Col-0 were shown to be statistically significant ($p < 0.0001$).

analysis requires relative large amounts of dry samples, pools of stems from three independent homozygous lines expressing each construct were used for chemistry analysis.

As shown in Table 2, total lignin content in the transgenic plants was elevated, with an 24% and 18% increase for *35:PtrMYB152* and *PtrCesA8:PtrMYB152* relative to wild-type respectively. We further examined the relative amounts of syringyl (S) and guaiacyl (G) monomers in the inflorescence stem cell walls, which showed that the S/G monomer ratio was higher in the transgenic plants than wild-type plants (Table 3). The significantly increased interfascicular fiber cells wall thicknesses of the transgenic lines may explain this alteration, since such cells develop the bulk of secondary walls in the Arabidopsis inflorescence stem, and fiber cells are enriched in S-lignin[33].

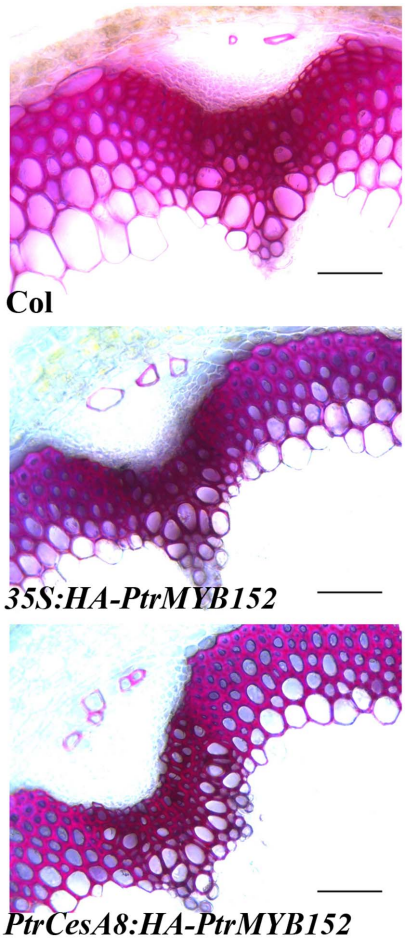

Figure 5 | Secondary cell wall lignification in Arabidopsis transgenic plants expressing *PtrMYB152*. Phloroglucinol stained sections from basal inflorescence stems of wild type and transgenic plants at principal growth stage 6.10. Bars, 50 μm.

PtrMYB152 regulates secondary cell wall biosynthetic gene expression. To test the effect of heterologous expression of *PtrMYB152* on potential target genes, we examined the expression of a suite of secondary cell wall biosynthetic genes in Arabidopsis *PtrMYB152* expressing lines that exhibited altered cell wall properties. The analyses were carried out using seedlings, pooled from three independent lines for each construct, to ensure tissue of uniform age for each genotype. Using seeding rather the stems also allowed us to identify genes that may be directly activated by PtrMYB152. As shown in Figure 6, the transgenic lines had increased expression of several lignin biosynthetic genes including *4CL1* (At1g51680), *HCT* (At5g48930), *C3H1* (At2g40890), *CCoAOMT1* (4g34050) and *CAD6* (Atg37970). This suggests that PtrMYB152 regulate secondary cell wall biosynthesis by activating a subset of secondary cell wall biosynthetic genes.

Discussion

In Arabidopsis, secondary cell wall biosynthesis is controlled by a transcription factor network. Several closely related NAC domain transcription factors, including SND1, NST1, NST2, VDN6 and VND7 work together to activate several downstream transcription factor genes, and the downstream transcription factors directly activate secondary cell wall components biosynthesis genes[7,14,15,17]. Most of the downstream transcription factors identified so far are R2R3 MYB transcription factors, which play important roles in regulating secondary cell wall biosynthesis[7,15]. In poplar, several R2R3 MYB transcription factors have also been shown to be involved in the regulation of secondary cell wall biosynthesis[11,12,27–29].

In this report, we provide evidence showing that PtrMYB152 is involved in the regulation of secondary cell wall biosynthesis. In poplar, there are a total of 192 genes encoding R2R3 MYB transcription factors[26], however, their functions in plant growth and development remain largely unknown. By searching for poplar homologues of Arabidopsis R2R3 MYBs that are known to be involved in the regulation of secondary cell wall biosynthesis, we identified five poplar MYBs including PtrMYB018, PtrMYB021, PtrMYB028, PtrMYB152 and PtrMYB192 (Figure 1A). Among them, PtrMYB018, PtrMYB021, PtrMYB028, and PtrMYB192 have been shown to be regulated by PtrWND2B and involved in the regulation of secondary cell wall biosynthesis, however, the expression of *PtrMYB152* was not affected

Table 2 | Lignin content (μg/mg DW) in the stems of the wild type (Col-0) and *PtrMYB152* transgenic plants. Data indicate two (I and II) independent assays

Genotype		Acid insoluble lignin	Acid soluble lignin	Total lignin
Col-0	I	81.31	53.85	135.16
	II	87.63	53.48	141.11
35S:PtrMYB152	I	134.90	40.30	175.20
	II	124.42	41.96	166.38
PtrCesA8:PtrMYB152	I	128.24	48.23	176.47
	II	96.63	53.51	150.14

Table 3 | Lignin monomer composition in inflorescence stems of the wild type (Col-0) and *PtrMYB152* transgenic plants. S, syringyl units; G, guaiacyl units. Data indicate two (I and II) independent assays

		Lignin Monomer Composition	
Genotype		%S	S:G
Col-0	I	23.50	0.31
	II	23.74	0.31
35S:PtrMYB152	I	27.84	0.39
	II	26.17	0.37
PtrCesA8:PtrMYB152	I	29.32	0.41
	II	29.43	0.42

in *PtrWND2B* transgenic poplar[28]. Real-time RT-PCR results showed that *PtrMYB152* is differentially expressed in developing xylem and phloem in poplar, and its expression level in secondary xylem is about 18-fold great than in phloem (Figure 1B), indicating PtrMYB152 may be involved in the regulation of secondary cell wall formation. Consistent with this hypothesis, phenotypic analysis showed that overexpression of *PtrMYB152* increased secondary cell wall thickness of vessel and fiber cells in Arabidopsis inflorescence stems (Figure 4, Table 1). Chemical analysis suggested an increase in lignification and other changes in cell wall composition in the stems of transgenic plants (Figure 5, Table 2, Table 3). These results demonstrate that PtrMYB152 regulates secondary cell wall biosynthesis in Arabidopsis.

When recruited to the promoter region of the *Gal4-GUS* reporter gene via a fused GD domain in a protoplast transient express system, PtrMYB152 activated *GUS* expression, indicating that it is a transcription activator (Figure 2). Consistent with this observation, real-time RT-PCR showed that some genes required for the biosynthesis of major secondary cell wall components cellulose, xylan and lignin are activated by overexpression of *PtrMYB152* (Figure 6), indicating that PtrMYB152 controls secondary cell biosynthesis by inducing the expression of discrete sets of secondary cell wall biosynthesis genes.

Secondary cell walls are formed by deposition of cell wall polymers mainly lignin, cellulose and xylan after cell expansion has ceased[13,34], and this process is regulated by a transcription factor regulatory network[7,15]. Because spatially and temporally expression of related transcription factor genes may be important for their proper functions, in addition to the *35S* promoter, we also used a tissue specific *PtrCesA8* promoter (Figure 3) to drive the expression of *PtrMYB152*. We found that overexpression of *PtrMYB152* by either the *PtrCesA8* promoter or the *35S* promoter resulted in similar but slightly different effects on secondary cell wall thickening in Arabidopsis (Figure 4). These data suggest that spatially and temporally regulated expression may play a role in the proposed functions of PtrMYB152.

Our data on the poplar MYB transcription factor PtrMYB152, combined with other recent studies of poplar genes encoding orthologs of Arabidopsis MYB, NAC domain, and homeodomain proteins in the secondary cell wall regulatory network support the idea that the transcription regulatory network governing secondary cell wall biosynthesis is largely conserved in poplar and Arabidopsis. Figure 7 summarizes these data, which indicate that for each of the known Arabidopsis transcriptional regulators, poplar orthologs with the similar functions have been identified. These transcription factors provide new tools for understanding and manipulating wood formation in poplar and other tree species. It should be noted that by examining four in-house secondary cell wall formation related transcriptomic datasets, Cassan-Wang et al[38] identified new potential transcription factor genes that are regulated by MYB transcription factors, and are involved in cell wall biosynthesis[38], so it is likely that PtrMYB152 may also regulate genes encoding other transcription factors, which in turn activate some of the secondary cell well biosynthesis genes.

In summary, our study provides evidence that PtrMYB152 regulates secondary cell wall biosynthesis in way similar to that of Arabidopsis MYBs. Information gained from this study may be used to guide to genetic modification of poplars to make it an improved feedstock for biofuels.

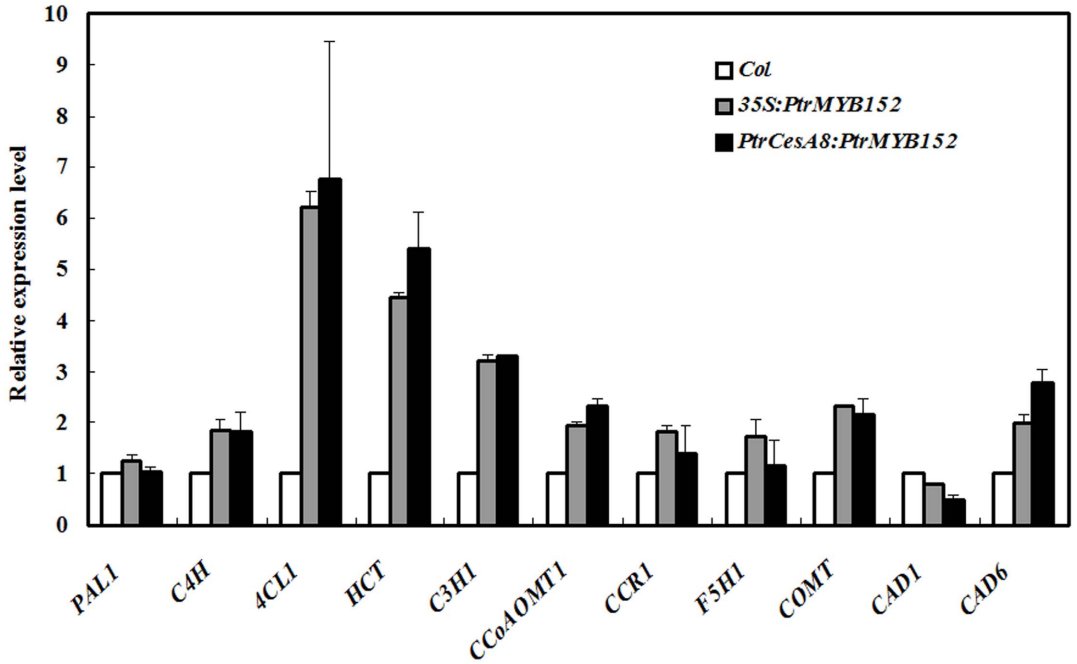

Figure 6 | Lignin biosynthetic genes expression in transgenic plants. RNA was isolated from 7-day old seedlings of transgenic and wild-type plants. qRT-PCR was used to detect the expression of related genes. Expression of *ACTIN2* was used as a control reference gene. Expression of each gene in wild type was set as 1.

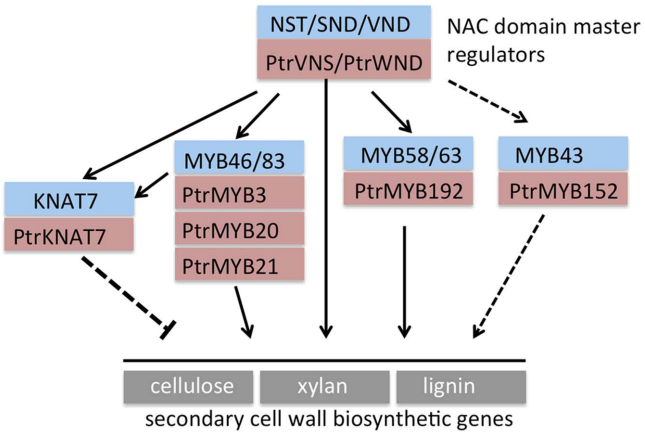

Figure 7 | Poplar orthologs of Arabidopsis transcription factors in the cell wall transcription regulatory network. Arabidopsis transcription factors are indicated in blue, poplar transcription factors in pink. Arabidopsis NAC domain transcription factors (e.g., NST1, SND1, VND6, VND7) act as master regulators and directly activate the *MYB* and *KNAT7* targets shown, as well as biosynthetic genes, and Arabidopsis *MYB* genes work in feed-forward mechanism to activate biosynthetic target genes (see Demura and Ye[25], Zhao and Dixon[35], and Zhong *et al.*[22] for recent reviews). Arabidopsis KNAT7 is transcriptional repressor that directly or indirectly down regulates biosynthetic target genes[20]. AtMYB43 has been implicated in the Arabidopsis network, but is not known to be a direct target of NAC domain master regulators, and its target genes have not been identified. Poplar NAC domain orthologs of Arabidopsis master regulators have been identified by Zhong *et al.*[19] (*PtrWND* genes) and Ohtani *et al.*[36] (*PtVNS* genes). The poplar *KNAT7* ortholog was described by Li *et al.*[37]. Poplar *MYB* genes are named according to Wilkins *et al.*[26], and are described in this study, McCarthy *et al.*[11], and Winzell *et al.*[12]. Solid lines indicate known direct targets in Arabidopsis; dashed lines indicate regulator relationships but unknown direct targets. Arrows, positive regulation; line with block, negative regulation.

Methods

Identification of poplar homologues of Arabidopsis R2R3 MYB transcription factors. To identify poplar homologues of Arabidopsis R2R3 MYB transcription factors that are known to be involved in regulating secondary cell wall biosynthesis, the entire amino acid sequences of Arabidopsis AtMYB20 (At1g66230), AtMYB43 (At5g16600), AtMYB46 (At5g12870), AtMYB58 (At1g16490) and AtMYB63 (At1g79180) were used in BLAST searches of the *Populus trichocarpa* proteome (www.phytozome.net). Full-length amino acid sequences of the selected poplar and Arabidopsis MYBs were subjected to phylogenetic analysis using AliBee-Multiple Alignment software, release 2.0 (http://www.genebee.msu.su/services/malign_reduced.html), and the most closely related homologs, based on this analysis, were chosen for in-depth characterization. We used poplar *MYB* gene names according to the nomenclature of Wilkins *et al.*[26], corresponding gene names are as follows: *PtrMYB018*, POPTR_0004s08480; *PtrMYB021*, POPTR_0009s05860; *PtrMYB028*, POPTR_0005s09930; *PtrMYB152*, POPTR_0017s02850; *PtrMYB192*; POPTR_0007s08190.

Plant materials and growth conditions. *Populus trichocarpa* tissue was collected from about 15-year-old field-grown poplar trees in July, and used for *PtrMYB152* gene cloning and gene expression assays.

Arabidopsis thaliana (Arabidopsis) ecotype Columbia (Col-0) was used for plant transformation. For RNA isolation from Arabidopsis seedlings, seeds were sterilized and sown on ½ strength Murashige & Skoog (MS) basal medium with vitamins (PlantMedia, http://www.plantmedia.com). For plant transformation or phenotypic analysis, seeds were directly sown into soil, and grown at 22°C, with a 14/10 hour photoperiod at approximately 120 μmol $m^{-2}s^{-1}$.

RNA isolation, RT-PCR and quantitative RT-PCR (qRT-PCR). Total RNA from poplar tissue was isolated using PureLink Plant RNA Reagent (Invitrogen), cleaned with RNeasy Plant Mini Kit (Qiagen), and treated with RNase-Free DNase (Qiagen) as described previously[39,40]. Total RNA from Arabidopsis seedlings was isolated using RNeasy Plant Mini Kit and treated with RNase-Free DNase set according to the manufacturer's instructions. Three different pools of tissues were generated for each sample, and analyzed independently as biological replicates.

Two μg of total RNA was used for reverse transcriptase synthesis using the Omniscript RT Kit (Qiagen) according to the manufacturer's instructions. *ACTIN2* (*ACT2*) and poplar elongation factor *C672* gene were used as controls for qRT-PCR. Gene primers use for RT-PCR analysis of *PtrMYB152* are: 5′-TCCACTAATATCGTATCTGAAC-3′ and 5′-TAGCGGAACTTCATCTATGCA-3′. Primers for RT-PCR analysis of expression of Arabidopsis genes were as described by Li *et al.*[20,27].

Constructs. To generate the *PtrCesA8prom:GUS* construct, poplar DNA was isolated from the developing xylem collected from field grown poplar tree using DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. A fragment that covers the region −2905 to +1 of the start codon of *PtrCesA8* gene was amplified by PCR using isolated DNA as the template. PCR products were then cloned into *pUC19* vector to drive the expression of the *GUS* reporter gene.

To generate HA or GD tagged constructs for *PtrMYB152*, the full-length open-reading frame (ORF) of *PtrMYB152* was amplified by RT-PCR using RNA isolated from the developing xylem collected from field grown poplar, and the PCR products cloned in frame with an N-terminal HA or GD tag into the *pUC19* vector under the control of either the double *35S* enhancer promoter of *CaMV*[41] or the *PtrCesA8* promoter.

For plant transformation, corresponding constructs in *pUC19* vector were digested with *EcoRI*, then subcloned into the binary vector *pPZP211*. Five-week-old plants with several mature flowers on the main inflorescence were used for transformation with various constructs via *Agrobacterium tumefaciens* (GV3101) using the floral dip method. Phenotypes of transgenic plants were examined in the T1 generation, and confirmed in T2 to T4 generations. For all transgenic plants, at least 4 transgenic lines with similar phenotypes were collected and evaluated. Overexpression of *PtrMYB152* in related lines was confirmed by RT-PCR.

Plasmid DNA isolation, protoplast transfection and β-glucuronidase (GUS) activity assay. All reporter and effector plasmids used in transfection assays were prepared using the EndoFree Plasmid Maxi Kit (Qiagen, Valencia, CA). Protoplasts isolation, transfection and GUS activity assays were performed as described previously[41].

Histochemical staining for GUS activity. 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc; Rose Scientific Ltd) was used as the substrate for the histochemical staining of GUS activity in seven-day old seedlings and stem sections from 6-week old plant.

Microscopy. To study inflorescence stem development, primary stems from soil grown plants at principal growth stage 6.10[32] were collected, and sections ~2 cm away from the bases of the stems were used for phenotypic analysis.

The sections were stained either in aqueous 0.02% toluidine blue O (Sigma) or in phloroglucinol (saturated solution in 2 M HCl) and viewed immediately using an Olympus AX70 light microscope. Tissue embedding and light and transmission electron microscopy (TEM) have been described previously[20]. Photos were taken under an Olympus AX70 light microscope or a Hitachi H7600 PC-TEM (Hitachi Ltd., Tokyo, Japan). Cell wall thickness was measured from TEM micrographs using ImageJ software (http://rsb.info.nih.gov/ij/index.html, Maryland, USA). For each genotype, secondary cell wall measurements were taken from at least 50 separate cells at standardized positions. The measurements were subjected to statistical analysis using the Student's *t* test (http://www.graphpad.com/quickcalcs/ttest1.cfm). The quantitative differences between wild type and transgenic lines in all data sets were shown to be statistically significant.

Chemical analysis. Five-cm stem fragments from the bases of primary inflorescence stems were used as material for chemical analysis. Lignin content was determined by a modified Klason method as described[42].

Ten mg of ground, extract-free oven-dried flour was used for lignin monomer composition analysis, using a downscaled thioacidolysis procedure followed by gas chromatographic analysis as described in details by Robinson and Mansfield[43].

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Author contributions

S.W., J.G.C., S.D.M. and C.J.D. designed the research. S.W. performed homolog identification, gene cloning, transgenic plants generation, protoplast transfection and drafted the manuscript. E.L. performed phenotype analysis and qRT-PCR analysis. I.P. performed chemical analysis. J.G.C., S.D.M. and C.J.D. modified the manuscript.

Additional information

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