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 3S 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 factors1, 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 members1,2. 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, respectively14, while AtMYB77 regulates lateral root formation3, and ASYMMETrIC LEAVES1 (AS1) regulates shoot morphogenesis and leaf patterning6. Recently, several R2R3 MYB transcription factors have been found to regulate secondary cell wall biosynthesis in Arabidopsis7,8. 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 strength13. 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 biosynthesis7,8. 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 lignin14,16-18. These NAC domain transcription factors can directly activate the expression of secondary wall specific biosynthetic genes19,20 and activate the expression of several downstream transcription factor genes that also directly regulate secondary wall component biosynthetic genes.21

Among the identified downstream transcription factor genes, two encode NAC domain transcription factors (SN2 and SND3), and one encodes the KNOTTED ARABIDOPSIS THALIANA7 (KNAT7) KNOTTED1-like

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targets of the master regulators SND1/VND6/VND7, regulated by SND17, 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.

*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. 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*.

In *Populus*, there are 192 annotated genes encoding R2R3 MYB transcription factors. 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. Second, PtrMYB21, an ortholog of Arabidopsis AtMYB46 has been shown to be expressed primarily in xylem tissues 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 expression cassette. 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.

**Figure 1** | *PtrMYB152* is a homolog of Arabidopsis R2R3 MYB transcription 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.

**Tissue specific expression of the *PtrCesA8* promoter in Arabidopsis.** The finding that the *PtrMYB152* functions as a transcriptional 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, from the poplar homolog of the Arabidopsis CesA8 gene associated with secondary cell wall biosynthesis 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.

**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 AtMYB43; *PtrMYB028* and *PtrMYB192* are closely related to AtMYB58 and AtMYB63, and *PtrMYB021* is closely related to AtMYB46.
reporter gene to generate \textit{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 \textit{PtrMYB152} expression preferentially to developing xylem.

\textbf{Heterologous expression of \textit{PtrMYB152} affects secondary cell wall development.} Constructs of \textit{PtrMYB52} with an HA tag at its N-terminus under the control of the 35S and \textit{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 \textit{PtrMYB152} were assessed for variation in secondary cell wall thickening and chemical content. Cross sections were taken from the bases of the inflorescence stems of wild-type (Col-0) plants at principal growth stage 6.10If, interfascicular fiber; ve vessel; xf xylary fiber; p, phloem. Bars, 10 µm.

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

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure3.png}
\caption{Expression of \textit{PtrCesA8prom:GUS} in Arabidopsis transgenic plants. 5-bromo-4-chloro-3-indolyl-\(\beta\)-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.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Secondary cell wall thickening in Arabidopsis transgenic plants expressing \textit{PtrMYB152}. Cross-sections from basal inflorescence stems of wild type and transgenic plants at principal growth stage 6.10If, interfascicular fiber; ve vessel; xf xylary fiber; p, phloem. Bars, 10 µm.}
\end{figure}
analysis requires relative large amounts of dry samples, pools of cells 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 35S: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-lignin14.

** 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 seedling 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 genes14,15,17. Most of the downstream transcription factors identified so far are R2R3 MYB transcription factors, which play important roles in regulating secondary cell wall biosynthesis15. In poplar, several R2R3 MYB transcription factors have also been shown to be involved in the regulation of secondary cell wall biosynthesis15,16,27.

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 factors56, 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 |
|---------------------|-----------------------|---------------------|--------------|
| Col0                | 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       |

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.
in *PtrWND2B* transgenic poplar. 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 greater 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 expression 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, and this process is regulated by a transcription factor regulatory network. 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. identified new potential transcription factor genes that are regulated by MYB transcription factors, and are involved in cell wall biosynthesis, so it is likely that *PtrMYB152* may also regulate genes encoding other transcription factors, which in turn activate some of the secondary cell wall 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.

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**Table 3 | Lignin monomer composition in inflorescence stems of the wild type (Col-0) and *PtrMYB152* transgenic plants.**

| Genotype                  | Lignin Monomer Composition | %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 |

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**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.
Two μg of total RNA was used for reverse transcriptase synthesis using the Omniscript RT Kit (Qagen) according to the manufacturer’s instructions. ACTIN2 (ACT2) and poplar elongation factor C672 gene were used as controls for qRT-PCR. Gene primers used for RT-PCR analysis of PtrMYB152 are: 5’-TCCACTAATATCGATCTGGAAC-3’ and 5’-TAGCAGGAACCTTATCATGCA-3’. Primers for RT-PCR analysis of expression of Arabidopsis genes were as described by Li et al.10,11.

**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 (Qagen) 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 CaMV19 or the PtrCesA8 promoter.

For plant transformation, corresponding constructs in pUC19 vector were digested with EcoRI, then subcloned into the binary vector pZP2211. Five-week-old plants were exposed to 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.

**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 (Qagen, Valencia, CA). Protoplasts isolation, transfection and GUS activity assays were performed as described previously17.

**Histochemical staining for GUS activity.** 5-bromo-4-chloro-3-indolyl-β-D-glucuronidase (GUS-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 610 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. Two-weeks, 10 days-, and 1 week- and light transmission-electron microscopy (TEM) have been described previously18. 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 Image 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/quickcalc/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 by Li et al.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 Mansfield19.

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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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