MYB Transcription Factor161 Mediates Feedback Regulation of Secondary wall-associated NAC-Domain1 Family Genes for Wood Formation

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Wood formation is a complex process that involves cell differentiation, cell expansion, secondary wall deposition, and programmed cell death. We constructed a four-layer wood formation transcriptional regulatory network (TRN) in Populus trichocarpa (black cottonwood) that has four Secondary wall-associated NAC-Domain1 (PtrSND1) transcription factor (TF) family members as the top-layer regulators. We characterized the function of a MYB (PtrMYB161) TF in this PtrSND1-TRN, using transgenic P. trichocarpa cells and whole plants. PtrMYB161 is a third-layer regulator that directly transactivates five wood formation genes. Overexpression of PtrMYB161 in P. trichocarpa (OE-PtrMYB161) led to reduced wood, altered cell type proportions, and inhibited growth. Integrative analysis of wood cell-based chromatin-binding assays with OE-PtrMYB161 transcriptomics revealed a feedback regulation system in the PtrSND1-TRN, where PtrMYB161 represses all four top-layer regulators and one second-layer regulator, PtrMYB021, possibly affecting many downstream TFs in, and likely beyond, the TRN, to generate the observed phenotypic changes. Our data also suggested that the PtrMYB161’s repressor function operates through interaction of the base PtrMYB161 target-binding system with gene-silencing cofactors. PtrMYB161 protein does not contain any known negative regulatory domains. CRISPR-based mutants of PtrMYB161 in P. trichocarpa exhibited phenotypes similar to the wild type, suggesting that PtrMYB161’s activator functions are redundant among many TFs. Our work demonstrated that PtrMYB161 binds to multiple sets of target genes, a feature that allows it to function as an activator as well as a repressor. The balance of the two functions may be important to the establishment of regulatory homeostasis for normal growth and development.

Wood is one of the world’s most important raw materials for timber, pulp, and energy (Sarkanen, 1976). Wood in angiosperms is derived from the vascular cambium, which differentiates into three types of cells, fiber, vessel, and ray, with the biosynthesis and deposition of three major cell wall components, lignin, cellulose, and hemicelluloses (Esau, 1965; Sarkanen and Ludwig, 1971; Evert, 2006). Fibers and vessel elements are the two major cell types, with fibers accounting for approximately 60% to 80% of the wood volume (Chen and Evans, 2005; Ohshima et al., 2011). Fibers provide mechanical support, and vessels mediate water and nutrient conduction (Wardrop, 1981; Tyree and Serrry, 1989).

Wood formation is a complex developmental process involving the differentiation of vascular cambium into secondary xylem mother cells followed by cell expansion, secondary wall deposition, and programmed cell death (Evert, 2006). Wood formation is controlled by a regulatory network consisting of transcription factors (TFs) and secondary cell wall (SCW) component genes (Zhong et al., 2010, 2011; Ohtani et al., 2011; Li et al., 2012a; Lin et al., 2013; Lu et al., 2013; Chen et al., 2019; Yeh et al., 2019). A group of NAC (for NAM, ATAF1/2, and CUC2) TFs, including Secondary wall-associated NAC-Domains (SNDs) and Vascular-related NAC-Domains (VNDs), were suggested as master regulators controlling the expression of downstream TFs, which ultimately regulates the expression of SCW genes for wood formation (Kubo et al., 2005; Zhong et al., 2006, 2011; Ohtani et al., 2011; Li et al., 2012b; Lin et al., 2013, 2017; Chen et al., 2019). Overexpression of Populus trichocarpa SND1-B2 (orWND2B) and SND1-A2 (orWND1B) in transgenic hybrid poplar altered the deposition patterns of cell wall components and increased the thickness of fiber cell walls, while suppression of the
two genes reduced the thickness of fiber cell walls (Zhong et al., 2011; Zhao et al., 2014). Altered expression of these two SN Де genes affected the transcript levels of many other TFs, such as MYBs (Zhong et al., 2011; Zhao et al., 2014). Knockout mutation of four Populus tremula × Populus tremuloides SN Де genes (SN Де-А1, SN Де-А2, SN Де-Б1, and SN Де-Б2) led to a severe reduction of SCW in xylem and phloem fibers and xylem ray parenchyma cells (Takata et al., 2019). These results support the view that SN Деs are higher level TFs in a regulatory network and revealed what other network TFs are regulated by SN Деs, leading to the transregulation of SCW genes for wood formation. However, the specificity (direct or indirect interactions) of these regulations is still in an early stage of identification and verification.

MYBs have been known as SND’s downstream TFs in a regulatory network for wood formation (McCarthy et al., 2010; Lin et al., 2013; Nakano et al., 2015; Tang et al., 2015; Jiao et al., 2019; Ohtani and Demura, 2019). In P. trichocarpa, 266 MYB genes (PtrMYBs) have been identified (Wilkins et al., 2009; Tian et al., 2019). Many of the PtrMYBs or their homologs in other Populus species have been studied for functions in regulating the SCW component genes for wood formation, using both genetic and molecular approaches. These MYBs include PtrMYB002, PtrMYB003, PdMYB10, PtrMYB020, PtrMYB021, PtrMYB074, PtrMYB090, PtoMYB092, PtoMYB152, PtoMYB156, PtoMYB189, PtoMYB194, PtoMYB216, and PdMYB221 (also called LTF1; McCarthy et al., 2010; Tian et al., 2013; Zhong et al., 2013; Chai et al., 2014; Li et al., 2014, 2015, Wang et al., 2014b, 2016; Tang et al., 2015; Xu et al., 2017; Yang et al., 2017; Chen et al., 2019; Gui et al., 2019; Jiao et al., 2019). These MYB proteins are all of the R2R3 type, and most of them were demonstrated as transcriptional activators in homologous or heterologous hosts, activating, in general, cell wall component biosynthesis and wall thickening in xylem cells.

Of the studied Populus species MYBs, PtoMYB156 (Yang et al., 2017), PtoMYB189 (Jiao et al., 2019), and PdMYB221 (Tang et al., 2015; Wang et al., 2018; Gui et al., 2019) have been identified as regulatory repressors that negatively affect cell wall properties and wood formation. Three other Populus species MYBs, PтMYB165 (Huang et al., 2014; James et al., 2017), PtremMYB182 (Yoshida et al., 2015), and PtMYB194 (James et al., 2017), were found as repressors in regulating flavonoid biosynthesis. The first MYB repressors were found ~20 years ago, and they are snapdragon (Antirrhinum majus) AmMYB308 and Arabidopsis (Arabidopsis thaliana) AtMYB3, AtMYB4, AtMYB7, and AtMYB32 (Tamagnone et al., 1998; Jin et al., 2000; Preston et al., 2004; Dubos et al., 2010); all are members of the MYB subgroup 4 gene family (Kranz et al., 1998). They negatively regulate enzyme-encoding genes in the general phenylpropanoid biosynthetic pathways, including monolignol and flavonoid biosynthesis (Jin et al., 2000; Dubos et al., 2010; Ma and Constabel, 2019). All of these subgroup 4 members and the other MYB repressors discussed above, except for PтMYB189, have a C-terminal conserved EAR (ethylene-responsive element binding factor-associated amphiphilic repression) motif, demonstrated in vitro and in vivo to be essential for repressing gene expression (Jin et al., 2000; Zhou et al., 2015; Li et al., 2017b; Ma and Constabel, 2019). For PтMYB189, the repressor function was demonstrated by site-directed deletion and mutagenesis of the MYB’s C-terminal 13 amino acids (277–289, GDDYGNHCMKKE), suggesting the essential role of this region for target repression (Jiao et al., 2019).

One of the most substantial findings of these MYB repressors is their pleiotropic effects, which affect traits beyond phenylpropanoid biosynthesis. Overexpression of these repressors induces complex modifications in cell wall component synthesis and plant development, causing severe growth inhibition in transgenic hosts (Tamagnone et al., 1998; Jin et al., 2000; Wang et al., 2011; Tang et al., 2015; Yang et al., 2017; Gui et al., 2019; Jiao et al., 2019). These results suggest a transcriptional regulatory network (TRN) where activators and repressors function coordinately to establish transcriptional homeostasis for normal growth and development. The results also suggest that MYB repressors or those regulating diverse phenotypes are potential key TFs for establishing and maintaining the regulatory homeostasis. However, we do not know how these MYB repressors are arranged in a TRN and how they transduce regulations to maintain proper growth and development. Knowledge of the functions of individual MYB activators and repressors in wood formation is extensive. What is needed now is knowledge about the TRN regulatory system (i.e. the identity, hierarchy, and interactions of key TFs that coordinate the regulatory homeostasis).

We have begun to uncover the hierarchical TRN for wood formation by establishing a portion of the
hierarchy directed by PtrSND1-B1. We previously developed a wood-forming cell system that coupled RNA sequencing (RNA-seq) and chromatin immunoprecipitation (ChIP) sequencing/PCR for studying early and transient transcriptome responses induced by TF transregulation (Lin et al., 2013; Chen et al., 2019; Li et al., 2019). Using this system, we constructed a four-layered TRN mediated by PtrSND1-B1 for wood formation in *P. trichocarpa* (Fig. 1; Chen et al., 2019). In this TRN, many TFs are either lignin pathway- or cellulose pathway-specific transregulators, except PtrMYB161 (Potri.007G134500). PtrMYB161, a third-layer TF, can directly regulate genes in both lignin and cellulose biosynthetic pathways. It directly transregulates the two syringyl-specific monolignol genes (*PtrCAla5H1* and *PtrCAla5H2*; Osakabe et al., 1999; Wang et al., 2014a, 2018) as well as two key SCW cellulose synthase genes, *PtrCesA4* and *PtrCesA18* (*PtrCesA8-B*; Suzuki et al., 2006; Kumar et al., 2009). Syringyl-lignin is a unique and major part of the total lignin in angiosperm tree species (Towers and Gibbs, 1953; Sarkanen and Ludvig, 1971; Higuchi, 1997). It evolved for mechanical support and pathogen defense functions, a product of speciation and adaptation (Scurfield, 1973; Vance et al., 1980; Evert, 2006). Therefore, PtrMYB161 may have diverse regulatory functions associated with wood cell wall formation, growth, and adaptation, making it a potential regulator that can affect the regulatory homeostasis for normal growth and development. We then continued to characterize the TRN work by focusing on PtrMYB161.

In this study, we combined transient and stable transgenesis to demonstrate that the previously constructed PtrSND1-B1-directed TRN is controlled by all PtrSND1 family members as the top-layer regulators. In this wood formation TRN, PtrMYB161 functions as an activator as well as a repressor. Under normal growth conditions, PtrMYB161 transactivates specific cell wall component genes. At high expression levels, PtrMYB161 then acts as a feedback repressor that silences four top-layer regulators and one second-layer regulator, which results in a diverse range of phenotypic changes including growth inhibition. The discovered TRN and its feedback regulation systems may provide a useful source of knowledge on the complex regulations in wood formation and growth.

**RESULTS**

**Tissue- and Cell Type-Specific Expression Patterns of *PtrMYB161* in *P. trichocarpa***

*PtrMYB161* expresses more specifically in stem differentiating xylem (SDX; Fig. 2A; Lin et al., 2017; Yeh et al., 2019), and the transcript expression becomes apparent starting from the fourth internode (Fig. 2B), where stem secondary growth begins and the syringyl-lignin-rich fiber cells emerge and differentiate (Li et al., 2001). In situ RNA localization suggested that *PtrMYB161* expresses more abundantly in syringyl-lignin-rich fiber cells than in guaiacyl-lignin-rich vessel

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**Figure 1.** PtrSND1-B1-directed regulatory network. The arrows indicate the protein-DNA regulatory interaction with activation ability, and the blunted line indicates negative regulation. CW, Cell wall. The figure was modified from Chen et al. (2019).
Overexpression of PtrMYB161 in Transgenic *P. trichocarpa* Resulted in Stunted Phenotypes

We overexpressed *PtrMYB161* in *P. trichocarpa* under the control of a cauliflower mosaic virus 3SS promoter. Three independent transgenic lines (OE-*PtrMYB161*-L5, OE-*PtrMYB161*-L8, and OE-*PtrMYB161*-L9) were generated, and all had increased *PtrMYB161* transcript abundances (Fig. 3A). These three transgenic lines all showed stunted growth relative to the wild type (Fig. 3, B and C; Supplemental Fig. S1). These transgenic lines had significant growth retardation in height, internode length, stem diameter, and leaf size but had similar numbers of internodes compared with the wild type (Fig. 3, C–H). These growth effects were sustained for the period of the study (Fig. 3B; Supplemental Fig. S1).

Overexpression of *PtrMYB161* disrupted the normal stem vascular meristematic activities (wood differentiation), leading to a reduced secondary xylem (wood) area (Fig. 4A). Stem transverse and tangential longitudinal sections of the differentiating vascular cambium show the presence of fusiform initials (including their derivatives, the dividing and enlarging cells) and ray initials in the vascular cambium of OE-*PtrMYB161* transgenics (Supplemental Fig. S2, A and B). These initials and their derivatives indicate lateral meristematic activities for vascular cambium differentiation in OE-*PtrMYB161* transgenics. Magnified images of stem cross sections of the xylem clearly show the presence of fiber cells, vessels, and ray parenchyma cells in the xylem of OE-*PtrMYB161* transgenics (Fig. 4B). Moreover, it is observed that SCW formation was severely suppressed in the xylem fibers of OE-*PtrMYB161* transgenics. Additionally, tangential longitudinal sections show that the xylem fibers of OE-*PtrMYB161* transgenics are narrow and exhibited elongated cells with tapered ends (Fig. 4C). The fiber cells exhibited a feature of intrusive apical growth, which is responsible for the elongation of fibers. Stem transverse and tangential longitudinal sections, together with scanning electron micrographs, show that elevated *PtrMYB161* expression resulted in thinner fiber walls, but the effect was not obvious for vessel cells (Fig. 4, A–D). The number of fiber cells differentiated per unit of area was...
reduced (Fig. 4E), and the number of vessel elements was increased by twofold compared with the wild type (Fig. 4E). These transgenic vessels were significantly smaller in diameter, as revealed by their lumen areas (Fig. 4F). In addition, OE-PtrMYB161-L8 seemed to exhibit a cessation of secondary growth (lateral thickening), as stem wood areas and stem diameters remained essentially unchanged in the older internodes (internodes 16 to 26; Supplemental Fig. S2C).

Figure 3. Effects of overexpressing PtrMYB161 on P. trichocarpa growth. A, Expression levels of the PtrMYB161 transgene in SDX tissues of three PtrMYB161 transgenic lines (OE-PtrMYB161-L5, -L8, and -L9). The error bars represent SD values from three biological replicates. Asterisks indicate significant differences between each line of the transgenics and wild-type (WT) plants by Student’s t test (*P < 0.05). B, Growth phenotypes of 4-month-old OE-PtrMYB161 (L5, L8, and L9) and wild-type plants. Bars = 10 cm. C to H, Statistical analysis of height (C), internode number (D), internode length (E), stem diameter (F), leaf length (G), and leaf width (H) of OE-PtrMYB161 (L5, L8, and L9) and wild-type plants. Error bars represent SD values of three independent experiments with five P. trichocarpa plants for each genotype in each replicate. Asterisks indicate significant differences between each line of the transgenics and wild-type plants by Student’s t test (**)P < 0.01.

Elevated Levels of PtrMYB161 Transcripts Drastically Affect Lignin Structure and Polysaccharide Contents in Stem Wood

We next characterized and quantified the three components in wood cell walls. In the stem wood of OE-PtrMYB161 transgenics, lignin contents were slightly reduced compared with the wild type (by ~1% to 11%; Table 1). Lignin composition was significantly altered in the transgenics, with up to ~57% reduction in
S-monomers and ∼115% increase in G-monomers (S:G ratios ranging from 0.38 to 0.5 compared with 1.94 in the wild type; Table 2). Significant reductions were also observed for total wood Glc (ranging from ∼45% to ∼58% reductions) and Xyl (ranging from ∼35% to ∼57% reductions; Table 1), suggesting that the biosynthesis of cellulose and xylan was strongly inhibited in the presence of a high level of PtrMYB161 transcripts. We then examined the effects of PtrMYB161 overexpression on the transcript levels of cell wall component genes.

Overexpression of PtrMYB161 Strongly Represses the Expression of SCW Component Genes

We performed RNA-seq on SDX tissues of OE-PtMYB161 transgenics (Fig. 5; Supplemental Data Set S1). When compared with the wild-type transcriptome, the expression of all known monolignol biosynthetic pathway genes in OE-PtMYB161 exhibited a greater than twofold reduction (Fig. 5A). PtcCald5H1, a direct target of PtMYB161 (Chen et al., 2019) and a key regulator of S-monolignol biosynthesis (Wang et al., 2014a), was the most highly repressed gene (98% repression; Fig. 5A; Supplemental Data Set S1). All the SCW cellulose biosynthetic genes, PtrCesA4, PtrCesA7 (PtrCesA7-A), PtrCesA17 (PtrCesA7-B), PtrCesA8 (PtrCesA8-A), and PtrCesA18 (PtrCesA8-B; Suzuki et al., 2006; Kumar et al., 2009; Shi et al., 2017), were nearly completely repressed (Fig. 5B). However, the remaining CesA homologs, mainly those for primary cell wall cellulose biosynthesis (Suzuki et al., 2006; Kumar et al., 2009; Shi et al., 2017), were generally not affected (Fig. 5B; Supplemental Data Set S1). Transcript levels of SCW cellulose biosynthetic genes were strongly repressed in OE-PtMYB161 transgenics.
that were not obvious in the PtrSND1-B1 TRN. Regulatory mechanisms associated with PtrMYB161 most likely, the expression of known MYB repressors. Most likely, the PtrMYB161 may be a regulator with transcriptional silencing functions that are distinct from those of known MYB repressors. Most likely, the results from the stable transgenics suggest in planta regulatory mechanisms associated with PtrMYB161 that were not obvious in the PtrSND1-B1 TRN derived from transient transcriptome responses (Chen et al., 2019).

OE-PtrMYB161 Transgenic Suggested a Feedback Regulation Point at PtrMYB161 in the PtrSND1-Mediated TRN

In the PtrSND1-B1 TRN, PtrMYB161 directly activates the expression of three monolignol pathway genes (PtrCAlDH, PtrCAlDH2, and PtrCCoAOMT2) and two SCW cellulose biosynthetic genes (PtrCesA4 and PtrCesA18; Fig. 1; Chen et al., 2019). But here, overexpression of PtrMYB161 in transgenics repressed not only these five genes but also most of the other cell wall component genes. Together, the results from transgenic protoplasts and whole plants suggest a possible feedback regulation in the TRN where PtrMYB161 may negatively regulate key higher level TFs, such as PtrSND1-B1 or PtrMYB021/074, to directly or indirectly attenuate the expression of many downstream TFs, including PtrMYB161, leading to a suppression of all major cell wall component genes.

P. trichocarpa, there are four SND1 members, PtrSND1-A1, -A2, -B1, and -B2 (Li et al., 2012b; Lin et al., 2017), that are higher level TFs regulating plant SCW formation (Ohtani et al., 2011; Zhong et al., 2011; Li et al., 2017), which play a role in plant growth, gene suppression, and wood cell wall morphological and chemical alterations were all similar to those of transgenics overexpressing the functionally known MYB repressors, AmMYB308, PtoMYB156, PtoMYB189, and PdMYB221 (Tamagnone et al., 1998; Tang et al., 2015; Yang et al., 2017; Jiao et al., 2019). Therefore, PtrMYB161 may also be a repressor. However, we have demonstrated that PtrMYB161 functions as a strong activator and verified its TF-DNA transactivation effects in vivo (Chen et al., 2019). Furthermore, PtrMYB161’s C-terminal end has neither EAR nor GDDYGNHGMIKKE conserved motifs that are necessary for gene repression functions (Jin et al., 2000; Zhou et al., 2015; Jiao et al., 2019). We argue that PtrMYB161 may be a regulator with transcriptional silencing functions that are distinct from those of known MYB repressors. Most likely, the results from the stable transgenics suggest in planta regulatory mechanisms associated with PtrMYB161 that were not obvious in the PtrSND1-B1 TRN derived from transient transcriptome responses (Chen et al., 2019).

OE-PtrMYB161 transgenics were tested. Three biological replicates from independent pools of P. trichocarpa stems were carried out. Data are means of three independent assays. Asterisks indicate significant differences between each line of the transgenics and wild-type plants by Student’s t test (*P < 0.05 and **P < 0.01). Units are g per 100 g of dry extractive-free wood.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild Type</th>
<th>OE-PtrMYB161-L5</th>
<th>OE-PtrMYB161-L8</th>
<th>OE-PtrMYB161-L9</th>
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<tr>
<td>Acid-insoluble lignin</td>
<td>17.19 ± 0.60</td>
<td>18.59 ± 0.77</td>
<td>15.53 ± 0.30</td>
<td>17.71 ± 0.69</td>
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<tr>
<td>Acid-soluble lignin</td>
<td>4.27 ± 0.18</td>
<td>2.60 ± 0.31**</td>
<td>3.44 ± 0.03**</td>
<td>3.04 ± 0.16**</td>
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<tr>
<td>Total lignin</td>
<td>21.43 ± 0.63</td>
<td>21.43 ± 0.63</td>
<td>18.97 ± 0.28*</td>
<td>20.75 ± 0.83</td>
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<tr>
<td>Glc</td>
<td>53.33 ± 0.50</td>
<td>26.79 ± 1.94**</td>
<td>29.1 ± 2.98**</td>
<td>22.1 ± 2.88**</td>
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<tr>
<td>Xyl</td>
<td>12.1 ± 0.51</td>
<td>6.18 ± 0.43**</td>
<td>7.9 ± 0.36**</td>
<td>5.2 ± 0.93**</td>
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<tr>
<td>Gal</td>
<td>1.07 ± 0.09</td>
<td>6.86 ± 1.01**</td>
<td>3.63 ± 0.03**</td>
<td>7.11 ± 0.22**</td>
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<td>Ara</td>
<td>2.4 ± 0.15</td>
<td>2.18 ± 0.23</td>
<td>0.43 ± 0.03**</td>
<td>1.38 ± 0.50</td>
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<td>Total carbohydrate</td>
<td>68.9 ± 0.06</td>
<td>42.01 ± 1.23**</td>
<td>41.13 ± 2.68**</td>
<td>35.79 ± 3.28**</td>
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<tr>
<td>Carbohydrate:lignin ratio</td>
<td>3.22 ± 0.10</td>
<td>1.99 ± 0.13*</td>
<td>2.17 ± 0.18**</td>
<td>1.74 ± 0.23**</td>
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PtrSND1-B1 TRN Is Part of the PtrSND1 Family-Mediated TRN, Where Four PtrSND1 Members Directly Activate the Expression of PtrMYB021 and PtrMYB074

In the PtrSND1-B1 TRN, PtrSND1-B1 directly activates the expression of three monolignol pathway genes (PtrCAlDH1, PtrCAlDH2, and PtrCCoAOMT2) and two SCW cellulose biosynthetic genes (PtrCesA4 and PtrCesA18; Fig. 1; Chen et al., 2019). But here, overexpression of PtrMYB161 in transgenics repressed not only these five genes but also most of the other cell wall component genes. Together, the results from transgenic protoplasts and whole plants suggest a possible feedback regulation in the TRN where PtrMYB161 may negatively regulate key higher level TFs, such asPtrSND1-B1 or PtrMYB021/074, to directly or indirectly attenuate the expression of many downstream TFs, including PtrMYB161, leading to a suppression of all major cell wall component genes.

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<th>OE-PtrMYB161-L8</th>
<th>OE-PtrMYB161-L9</th>
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<td>S-Lignin</td>
<td>62.02% ± 0.50%</td>
<td>31.64% ± 0.77%%</td>
<td>26.45% ± 0.80%%</td>
<td>26.64% ± 2.33%%</td>
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<td>G-Lignin</td>
<td>31.99% ± 0.93%</td>
<td>63.19% ± 0.84%%</td>
<td>68.81% ± 0.53%%</td>
<td>67.40% ± 2.49%%</td>
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<td>H-Lignin</td>
<td>5.99% ± 0.60%</td>
<td>5.17% ± 0.81%</td>
<td>4.74% ± 0.28%</td>
<td>5.93% ± 0.21%</td>
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<td>S:G ratio</td>
<td>1.94 ± 0.07</td>
<td>0.50 ± 0.02**</td>
<td>0.38 ± 0.01**</td>
<td>0.40 ± 0.05**</td>
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previously demonstrated that the other three PtrSND1 members can activate *PtrMYB021* (Li et al., 2012b), but whether these are direct or indirect activations remained undetermined in vivo. While these three members can activate *PtrMYB021*, we do not know whether they can also activate *PtrMYB074* or whether the activation is by direct TF-DNA interactions.

First, we determined whether *PtrSND1*-A1, *PtrSND1*-A2, and *PtrSND1*-B2 can directly transactivate *PtrMYB074*. To that, we transfected SDX protoplasts to overexpress each of these *PtrSND1* members (*pUC19-35S-PtrSND1*-sGFP) and analyzed the transcript abundance of *PtrMYB074* by RT-qPCR after 12 h of protoplast incubation. Protoplasts transfected with a *pUC19-35S-sGFP* plasmid were used as an empty-vector control. The results showed that each of the three *PtrSND1* member genes could activate the expression of *PtrMYB074* (Fig. 6A). Therefore, all four *PtrSND1* family members, *PtrSND1*-A1, *PtrSND1*-A2, *PtrSND1*-B1, and *PtrSND1*-B2, can activate the expression of both *PtrMYB021* and *PtrMYB074*.

Next, we analyzed whether *PtrSND1*-A1, *PtrSND1*-A2, and *PtrSND1*-B2 can directly transactivate *PtrMYB021* and *PtrMYB074* by testing if each of the *PtrSND1* members can bind to the promoter of *PtrMYB021* and *PtrMYB074*, using anti-GFP antibody ChIP analysis in SDX protoplasts (Chen et al., 2019; Li et al., 2019; Yeh et al., 2019). We isolated SDX protoplasts and transfected a portion of the protoplasts with a plasmid DNA (*pUC19-35S-PtrSND1 member-sGFP*) for overexpressing *PtrSND1* member-sGFP. Another portion of the protoplasts was transfected with a *pUC19-35S-sGFP* plasmid as a mock control. Following cross-linking and anti-GFP antibody purification, qPCR was performed for four fragments of the ~2,000-bp chromatin DNA fragments (promoter sequences) upstream of the coding region of each tested *PtrMYB* gene, with expected qPCR products ranging from 80 to 200 bp (Fig. 6B). RT-qPCR analysis of specific chromatin enrichments in protoplasts overexpressing the *PtrSND1* member-sGFP fusion indicated that each of these three *PtrSND1* members could bind to the ~2,000-bp promoter in at least one location (Fig. 6C; Supplemental Fig. S3). Previously, we had shown that GFP fusion did not affect their native transactivation functions (Chen et al., 2019). Therefore, all four *PtrSND1* members can directly transactivate *PtrMYB021* and *PtrMYB074*, extending the previously established *PtrSND1*-B1 TRN into a *PtrSND1* family-mediated TRN (Fig. 6D).

**PtrMYB161 Mediated Direct Feedback Repression of Four *PtrSND1* Member Genes and *PtrMYB021* But Not *PtrMYB074***

As suggested above, *PtrMYB161* in *OE-PtrMYB161* transgenic lines may negatively regulate higher level TFs, such as *PtrSND1*s, initiating a cascade of feedback regulation for a broad suppression of cell wall component genes (Fig. 5; Supplemental Data Set S1). We then examined RNA-seq results and conducted RT-qPCR analysis of transcripts in SDX tissues of *OE-PtrMYB161-L8* and the wild type. These analyses revealed that overexpression of *PtrMYB161* repressed the expression of the four *PtrSND1* members as well as *PtrMYB021* and *PtrMYB074* (see Fig. 7A and Supplemental Fig. S4 for RNA-seq data). The results support our hypothesis that *PtrMYB161* acts as a feedback repressor in the *PtrSND1* family-mediated TRN for wood formation. We next tested whether these repressions are direct or indirect regulatory effects.

We performed anti-GFP antibody ChIP, as we did above, using SDX protoplasts constitutively expressing a *PtrMYB161*-sGFP fusion (*pUC19-35S-PtrMYB161*-sGFP). Protoplasts transfected with a *pUC19-35S-sGFP*
plasmid were used as the control. Four chromatin fragments in the ~2-kb promoter of each PtrSND1 member (P1–P4 in Fig. 7B) and PtrMYB021 and PtrMYB074 (P1–P4 in Fig. 6B) were amplified (by RT-qPCR) from the transfected protoplasts following ChIP. We detected twofold to 12-fold enrichment of at least one sequence fragment within the ~2-kb promoter of each PtrSND1 member gene and PtrMYB021 (Fig. 7C), indicating that PtrMYB161 can bind to these upstream cell wall TF genes for a direct feedback repression in the wood formation TRN (Fig. 7D). The ChIP analysis suggests that PtrMYB161 cannot directly repress PtrMYB074 (Supplemental Fig. S4B). The repressed transcript abundance of PtrMYB074 observed in OE-PtrMYB161 lines (Fig. 7A) was most likely an indirect effect through the repression of PtrSND1 members by PtrMYB161.

There is growing evidence that transcriptional repression events may result from histone deacetylation (Shahbazian and Grunstein, 2007; Zentner and Henikoff, 2013; Liu et al., 2014; Gao et al., 2015; Han et al., 2016; Li et al., 2017a; Zhang et al., 2018; Park et al., 2019; Ueda and Seki, 2020; Zeng et al., 2020). We hypothesized that the repression mechanism of PtrMYB161 in P. trichocarpa might involve corepressors such as histone deacetylase to effect transcriptional repression via histone deacetylation. To test this hypothesis, we analyzed the expression of all known P. trichocarpa histone deacetylase genes in OE-PtrMYB161 transgenics using the RNA-seq of SDX tissues. In all the OE-PtrMYB161 transgenic lines, the three epigenetic regulators in the PtrHDT3 family (also known as the HD2C family; Dangl et al., 2001; Pandey et al., 2002) were drastically up-regulated (Fig. 8; Supplemental Data Set S2), suggesting that these three PtrHDT3 members (PtrHDT3-A, PtrHDT3-B1, and PtrHDT3-B2) may be key components in PtrMYB161-mediated feedback regulation.

CRISPR-Based Editing of PtrMYB161 Did Not Significantly Affect the Plant Phenotype, Wood Structure, and Wood Cell Wall Composition

We demonstrated, through transgenic plant overexpression, that PtrMYB161 is a strong negative regulator of the wood formation gene PtrMYB074. As a control, we used PtrMYB021, which is known to positively regulate cell wall biosynthesis genes (Wu et al., 2015; Sun et al., 2017; Xue et al., 2017; Zhang et al., 2018; Liu et al., 2019; Zhang et al., 2019). The results showed that the expression of PtrMYB074 in the OE-PtrMYB161 lines was significantly reduced compared to the control and the OE-PtrMYB021 lines. The repression effect was further confirmed by ChIP-qPCR analysis, where the enrichment of TF-GFP was significantly lower in the OE-PtrMYB161 lines than in the control and OE-PtrMYB021 lines. These findings suggest that PtrMYB161 plays a crucial role in the regulation of cell wall biosynthesis genes.
Figure 7. PtrMYB161 mediates the feedback regulation of PtrSND1-directed regulatory network. A, The transcript abundances of PtrSND1-A1, PtrSND1-A2, PtrSND1-B1, PtrSND1-B2, PtrMYB021, and PtrMYB074 were detected using RT-qPCR in xylem tissues of 4-month-old wild-type (WT) and OE-PtrMYB161-L8 transgenic plants. Error bars indicate SE values of three biological replicates from independent pools of P. trichocarpa xylem tissues. B, Locations of the promoter fragments amplified by qPCR following the ChIP assays. P1 to P4 show the approximate locations of the promoter fragments amplified by qPCR following ChIP assays. C, ChIP-qPCR showed that PtrMYB161 directly binds to the promoters of PtrSND1-A1, PtrSND1-A2, PtrSND1-B1, and PtrMYB021, indicating the enriched promoter segments of PtrSND1-A1, PtrSND1-A2, PtrSND1-B1, and PtrMYB021. The unenriched promoter segments are shown in Supplemental Figure S4. Enrichment of DNA was calculated as the ratio between PtrMYB161-GFP and GFP (control), normalized to that of the PtrACTIN gene. Control values were set as 1. Error bars indicate SE values of three biological replicates (three independent batches of SDX protoplast transfections). Statistical significance in A and C was estimated using Student t test (*P < 0.05 and **P < 0.01). D, Diagram showing that PtrMYB161 feedback regulates the PtrSND1-directed regulatory network. The light gray and black arrows indicate the protein-DNA regulatory interaction with activation ability, and the blunted lines indicate negative regulation. CW, Cell wall.
that affects plant growth, cell wall biosynthesis, and wood formation (Figs. 3–5; Tables 1 and 2). To further test PtrMYB161’s regulatory roles, we generated loss-of-function mutants of PtrMYB161 in P. trichocarpa using CRISPR-based genome editing with Streptococcus pyogenes Cas9 (Deltcheva et al., 2011; Jiang et al., 2013; Heler et al., 2015). A single guide RNA (sgRNA) was designed to target single-nucleotide polymorphism-free regions in PtrMYB161’s exon II (see “Materials and Methods”; Fig. 9A). The sgRNA was cloned into the pEGP237-2A-GFP vector (Osakabe et al., 2016; Ueta et al., 2017) for CRISPR editing using our improved P. trichocarpa transformation protocol (Song et al., 2006; Li et al., 2017c, 2019). We generated two independent biallelic mutants, ptrmyb161-2 and ptrmyb161-3, with each containing heterozygous edits for PtrMYB161 (Fig. 9B). These mutants were clonally propagated for further analysis.

The ptrmyb161 lines had slightly improved growth in height, internode number, and stem diameter but had similar internode lengths compared with the wild type (Fig. 9C). These growth effects were sustained for the period (~4 months) of the study (Fig. 9D; Supplemental Fig. S5). The morphology, size, and wall thickness of the three main stem xylem cells (fibers, vessels, and rays) in the ptrmyb161 lines were similar to those in wild-type plants (Supplemental Fig. S6). Loss of function of PtrMYB161 in P. trichocarpa also did not significantly alter the lignin quantity, lignin SG ratio, and neutral sugar composition and contents in wood (Supplemental Tables S1 and S2).

CRISPR-Edited Mutations in PtrMYB161 Did Not Disturb Overall Effects of PtrSND1 Family-Mediated Transregulation in P. trichocarpa

We performed RNA-seq and RT-qPCR to examine SDX gene expression in ptrmyb161-2 and ptrmyb161-3 lines. We found that mutation of PtrMYB161 in ptrmyb161-2 did not cause a substantial change in the transcript abundances of the PtrSND1 members, PtrMYB021 and PtrMYB074, the higher level transregulators in the PtrSND1 family-mediated TRN (Supplemental Fig. S7A). The overall effect of PtrMYB161 mutation on the PtrSND1-mediated wood formation TRN appeared to be minor in both ptrmyb161-2 and ptrmyb161-3 because the RNA-seq results showed that transcript levels of monolignol, cellulose, and hemicellulose biosynthetic pathway genes in these mutants were similar to those in the wild type (Supplemental Fig. S7, B–D; Supplemental Data Set S3). RT-qPCR analysis of a few such genes confirmed the RNA-seq results (Supplemental Fig. S7, E–G). These in-substantial changes in the expression of cell wall component genes in ptrmyb161s are consistent with the similar wood properties between the mutants and the wild type (Supplemental Tables S1 and S2). Minor gene transregulation alterations in a key wood formation TRN may also be an important reason for normal growth and development in these ptrmyb161 mutants.

DISCUSSION

In this study, we continued to explore the PtrSND1-B1-mediated TRN for wood formation in P. trichocarpa. We revealed that the PtrSND1-B1 TRN in P. trichocarpa is mediated not only by PtrSND1-B1 but also by all PtrSND1 family members (Fig. 6). The PtrSND1 members-mediated transregulation (of PtrMYB021 and PtrMYB074) was established in a P. trichocarpa SDX protoplast system, and the regulatory effect (direct transactivation) was validated by PtrSND1 TF-sGFP fusion proteins in the system using anti-GFP antibody ChIP (Fig. 6). In our previous studies, we used the same transregulation system to discover TF-DNA interactions for wood formation and histone acetylation-mediated drought tolerance responses in P. trichocarpa (Lin et al., 2013; Chen et al., 2019; Li et al., 2019; Yeh et al., 2019). In those studies, we demonstrated that GFP-tagged TFs retain their native transactivation function and tested 82 protoplast-inferred TF-DNA interactions in 68 genotypes of transgenic and CRISPR-edited P. trichocarpa, and we verified that ~93% (76 of 82) of the tested interactions function in planta. Therefore, this work established an extended wood formation TRN, where four PtrSND1 members (PtrSND1-A1, PtrSND1-A2, PtrSND1-B1, and PtrSND1-B2) are top-layer TFs regulating directly both PtrMYB021 and PtrMYB074, which then guide 55 TF-DNA interactions to transactivate 26 cell wall genes for wood formation (Fig. 7D). We further established that the transactivation may be restrained by a feedback regulation network mediated by a TRN member, PtrMYB161.

The feedback network was discovered based on genetic analysis of regulatory effects in transgenic whole plants in combination with the protoplast system (Fig. 7). Overexpressing PtrMYB161 in P. trichocarpa repressed the expression of four PtrSND1 members, PtrMYB021, and PtrMYB074, the six regulators of the
top two layers of the TRN (Fig. 7D). The transgenesis combined with ChIP assays using the protoplast system suggested that five of these six regulators were directly repressed by PtrMYB161 (Fig. 7). The expression of the sixth regulator, PtrMYB074, may be indirectly repressed by PtrMYB161. It is also possible that the repression is directly mediated by PtrMYB161 through binding to PtrMYB074’s promoter in regions other than those tested.

In Arabidopsis, AtSND1 directly regulates AtMYB46 (the homolog of PtrMYB021; Zhong et al., 2007; Chen et al., 2019), which directly regulates AtMYB4, AtMYB7, and AtMYB32, three MYB repressors (Ko et al., 2009; Chen et al., 2019). In this regulatory network, the three MYBs were also demonstrated for functions as negative feedback regulators of AtSND1 (Wang et al., 2011). In addition, overexpression of AtMYB32 (the PtrMYB161 close homolog) resulted in reduced expression of AtSND1 as well as retarded growth (Wang et al., 2011). These results are consistent with the PtrMYB161-mediated feedback regulation system. Our findings of the PtrMYB161-mediated feedback regulation are further supported by transgenesis of SND1 in several Populus species. Dominant repression of the P. trichocarpa PtrSND1-A2 functions (named PtrWND2B by Zhong et al. [2010]) in transgenic P. alba × P. tremula resulted in retarded growth and reduced thickness of stem xylem secondary cell walls (Zhong et al., 2011). RNA interference of SND1 members in transgenic Populus euramerica using sequence fragments of P. trichocarpa PtrSND1-A2 (named PtrWND1B by Zhao et al. [2014]) also resulted in stunted growth and a thinner wall in secondary xylem cells (Zhao et al., 2014). These heterologous transgene manipulations would likely affect multiple endogenous SND1 members with unknown specificity, leading to the observed phenotypes. More specific manipulation of SND1 functions was reported recently, where CRISPR-Cas9 was applied to edit the four PtrSND1 members’ putative orthologs in P. tremula × P. tremuloides (Takata et al., 2019). The resulting quadruple mutants also exhibited stunted growth and thin-walled stem secondary xylem cells (Takata et al., 2019). All these previous studies and our work here revealed that SND1 members exert redundant functions in regulating plant growth (Fig. 3), xylem fiber frequency and cell wall thickness (Fig. 4), and xylem cell wall composition (Tables 1 and 2).

Studies on genetic functions of SND1s and related SND genes in tree species are extensive and have suggested that they are master TFs mediating regulatory networks for wood formation (Hu et al., 2010; Zhong et al., 2010, 2011; Ohtani et al., 2011; Li et al., 2012a, 2012b; Lin et al., 2013, 2017; Zhao et al., 2014; Hussey
et al., 2015; Nakano et al., 2015; Ye and Zhong, 2015; Sakamoto et al., 2016; Chen et al., 2019; Takata et al., 2019). Many of these networks have been loosely defined as connections among SNDs, their possible downstream TFs, and the three major wood cell wall components, based on gene expression analyses (Zhong et al., 2011; Nakano et al., 2015; Ye and Zhong, 2015). More structured TRNs, revealing specifically and quantitatively how regulations are transduced from SND1s through pairwise TF-DNA interactions to activate wood cell wall genes, have also been established (Lin et al., 2013; Chen et al., 2019). However, despite the previous extensive studies, it is still unknown how SND regulations affect the quantities of the network’s end products: the three cell wall components and lignin structures. Our work here demonstrated that lowering the transcript levels of PtrlSND1 members, particularly those of PtrlSND1-A1 and PtrlSND1-A2 together (Fig. 7A), inhibits the biosynthesis of cellulose and xylan (the two major cell wall polysaccharides) by nearly 60% and alters lignin structures (reduces lignin S/G ratios by 70% to 80%; Table 2). These cell wall component changes are consistent with the reduction in the abundance of component gene transcripts (Fig. 5), supporting an SND1-guided TRN involving PtrlMYB161-mediated multilayer feedback repression paths (Fig. 7).

We analyzed the expression of key cambium identity genes in wild-type and OE-PtrlMYB161 transgenic plants (Supplemental Fig. S8). These genes include homologs of WUSCHEL-RELATED HOMEOBOX4, ARBORKNOX1 (ARK1), ARK2, and popREVOLUTA, which are well known to act as positive regulators of cambium identity in Populus species (Schrader et al., 2004; Groover et al., 2006; Du et al., 2009; Robischon et al., 2011; Kucukoglu et al., 2017). These cambium identity genes were readily detected and upregulated in OE-PtrlMYB161 transgenics (Supplemental Fig. S8), which is in agreement with the detection of fusiform and ray initials, key features that are characteristic of vascular cambium development (Supplemental Fig. S2, A and B). Moreover, high expression levels of the cambium identity genes such as ARKI (Groover et al., 2006) and ARK2 (Du et al., 2009) are known to inhibit the onset and differentiation of secondary vascular tissues, leading to thin stems in Populus species, which is consistent with reduced stem secondary growth of transgenic P. trichocarpa overexpressing PtrlMYB161.

The PtrlMYB161-mediated repression paths affect plant development and growth (Figs. 3 and 4). Complex processes like growth and development are regulated by subsets of differentially activated or repressed TFs through signal transduction (Jaenisch and Bird, 2003; Kouzarides, 2007; Nakashima et al., 2014; Song et al., 2016). Epigenetic modifications are key underlying mechanisms for such differential transregulations. Differential activation of genes is associated with histone hyperacetylation in the chromatin of the genes catalyzed by histone acetyltransferases, whereas differential gene repression is accompanied by hypo-acetylated histones mediated by histone deacetylases (Allfrey et al., 1964; Shahbazian and Grunstein, 2007; Li et al., 2011, 2019; Zhou et al., 2011; Zentner and Henikoff, 2013; Ueda and Seki, 2020). Our RNA-seq result reveals that of all of the currently known P. trichocarpa histone deacetylase genes, only three PtrlHDT3 members (PtrHDT3-A, PtrlHDT3-B1, and PtrlHDT3-B2) were drastically up-regulated in the OE-PtrlMYB161s (Fig. 8; Supplemental Data Set S2). These upregulated genes are preferentially expressed in the vascular cambium based on the high-spatial-resolution RNA-seq analyses (Sundell et al., 2017). Overexpression of PtrlMYB161 decreased the cambial activities (wood differentiation), leading to a reduced secondary xylem (wood) area (Fig. 4A; Supplemental Fig. S2C). Therefore, PtrlMYB161 overexpression-induced high PtrlHDT3 levels may repress wood differentiation genes through histone deacetylation in the chromatin of such genes, causing reduced stem secondary growth. High expression levels of the epigenetic regulators HD2s (putative orthologs of HDT3s) are known to provoke developmental abnormalities in Arabidopsis (Zhou et al., 2004).

In addition to the possible involvement in growth-related regulation, PtrlHDT3s may also be key components in PtrlMYB161-mediated feedback regulation. The induced PtrlHDT3s in OE-PtrlMYB161 lines could be recruited by PtrlMYB161 to its targets, the PtrlSND1 members, for repression. Similar regulatory mechanisms have been demonstrated in our and other previous studies that TF or TF-cofactor modules that bind to target genes recruit histone deacetylases or histone acetyltransferases to specific chromatin sites for target gene repression or activation in response to plant development and adaptation (Kornet and Scheres, 2009; Liu et al., 2014; Zhou et al., 2017; Cheng et al., 2018; Lee and Seo, 2019; Li et al., 2019; Ueda and Seki, 2020). The possibility of the involvement of epigenetic marks as cofactors for PtrlMYB161 to act as a repressor is appealing, particularly because PtrlMYB161 protein does not have the known functional repressor domains, such as the EAR or GDDYGNHGMKKEE motif (Jin et al., 2000; Zhou et al., 2015; Jiao et al., 2019). Such epigenetic regulatory mechanisms will provide insights into network regulation and should be explored in future studies.

Our study suggested that high levels of PtrlMYB161 may act as signals triggering a cascade of regulations affecting adversely wood cell wall formation and plant growth (Supplemental Data Set S4). However, deletion of PtrlMYB161 using CRISPR-Cas9 could not enhance these developmental traits. In fact, the edited ptrmyb161-2 and ptrmyb161-3 mutants were similar to the wild type in all aspects examined, such as cell wall structure, morphology and chemical composition, and other growth developmental features (Fig. 9; Supplemental Figs. S5 and S6; Supplemental Tables S1 and S2). The phenotypic similarity between the mutants and the wild type comports with the full transcriptome analysis showing that deleting PtrlMYB161 had little effect on the transcriptome. Only 1.7% to 4.2% of the
expressed genes were altered for their transcript levels in Ptrmyb161-2 and Ptrlmyb161-3, respectively (Supplemental Table S3; Supplemental Data Set S5). Likely, normal growth and development in P. trichocarpa do not require PtrMYB161 functions. The essentially unaltered transcriptomes and phenotypes suggest that the deleted PtrMYB161 functions, such as the direct transregulation of the specific cell wall genes (Supplemental Fig. S7), may be compensated for by other TFs or combinations of these TFs.

In the four-layer wood formation TRN, PtrMYB161 functions as an activator as well as a repressor. Under normal growth conditions, PtrMYB161 transactivates specific cell wall component genes (Chen et al., 2019). At high expression levels, PtrMYB161 then acts as a feedback repressor that silences four top-layer regulators and one second-layer regulator (Fig. 7), which results in a diverse range of phenotypic changes including increased number of xylem vessels and reduced growth (Figs. 3 and 4). The discovered TRN and its feedback regulation systems may provide a useful source of knowledge on the complex regulations in wood formation and growth.

Previous reporting reveals that PtrSND1-A1 and PtrSND1-A2 genes, which are strongly down-regulated by PtrMYB161, are expressed at the beginning of SCW formation, and then their expression declines (Sundell et al., 2017). Moreover, the expression of PtrMYB21 also peaks at the initiation of SCW formation and then sharply declines (Sundell et al., 2017). Our results from in situ RNA localization indicate late accumulation of PtrMYB161 transcripts in differentiating fiber cells during SCW formation (Fig. 2C). These results suggest the possibility that PtrMYB161 acts to repress PtrSND1-A1, PtrSND1-A2, and PtrMYB21 genes in cells where secondary walls are already being synthesized and where the expression of PtrSND1-A1, PtrSND1-A2, and PtrMYB21 genes are no longer needed for initiating fiber cell differentiation and wall thickening.

Many TFs can work as both transcriptional activators and repressors for different target genes. For instance, the homeodomain TF WUSCHEL (WUS) directly represses the cytokinin-inducible response genes (ARR5, ARR6, ARR7, and ARR15; Leibfried et al., 2005), but it directly activates transcription of the gene for a small signaling peptide, CLAVATA3 (Schoof et al., 2000; Yadav et al., 2011). The two opposite functions of the WUS TF are important for shoot apical meristem regulation. The regulatory activities of TFs often depend on specific cofactors (Ma, 2005). It is possible that corepressors (e.g. histone deacetylases PtrHDT3-A, PtrHDT3-B1, and PtrHDT3-B2) control the activity of PtrMYB161 to repress the expression of PtrSND1 family and PtrMYB21 genes (Figs. 7 and 8). PtrMYB161 may have distinct regulatory activities, activation or repression, at different stages during wood formation, depending upon its combinatorial actions with spatiotemporal-specific cofactors. Future studies of the cofactors for PtrMYB161 will improve our understanding of the dual activator-repressor functions.

Concluding Remarks

We combined two transgenic systems to study wood formation TRN. The transgenic xylem protoplasts coupled with transcriptome and chromatin-binding analyses identified the base network of TF-target gene regulations. The transcriptome information of transgenic whole plants complemented the base TRN with regulations affected by specific developmental or adaptation conditions that are lacking in protoplast cultures. The combination allows more in-depth regulatory information, such as the PtrMYB161-mediated feedback regulation of its upstream regulators, which cannot be readily revealed by using either system alone. Our study suggests that PtrMYB161 has dual functions in regulating different sets of target genes. It binds to specific cell wall component genes and acts as a transactivator to help maintain a normal biosynthesis of the cell wall components. PtrMYB161 also binds to its upstream TFs and acts as a repressor of the TFs, affecting various developmental processes, including increased number of xylem vessels and reduced growth, traits normally adaptive to drought stress. Such repressor activities require high levels of PtrMYB161 and possibly cofactors, such as epigenetic regulators. PtrMYB161 may act as a genetic and epigenetic regulatory switch controlling cell wall component biosynthesis, growth, and adaptation during wood formation. Further study of the OE-PtrMYB161s and ptrmyb161 mutants for their transcriptome and epigenome responses to drought and growth is necessary to test and reveal how PtrMYB161 switches and interacts with other regulatory systems to control wood formation.

Materials and Methods

Plant Materials and Growth Conditions

All experiments were performed with Populus trichocarpa genotype Nisqually-1. Wild-type, transgenic, and knockout mutant plants were grown in a walk-in growth chamber as described (Li et al., 2019). Four-month-old P. trichocarpa plants were harvested for RNA isolation, in situ hybridization, histological analysis, scanning electron micrograph analysis, and wood composition analysis. Stems of 6-month-old wild-type plants were used to isolate SDX protoplasts.

Total RNA Extraction

Total RNA was extracted from SDX or SDX protoplast of P. trichocarpa using the RNeasy plant RNA isolation kit (Qiagen). RNA concentration was determined by NanoDrop 2000 (Thermo Scientific). RNA integrity was examined using a Bioanalyzer 2100 (Agilent). The RNA was used for RT-qPCR and RNA-seq.

RT-qPCR

One microgram of total RNA was used for reverse transcription to generate cDNA using the PrimeScript RT Reagent Kit with gDNA Eraser according to the manufacturer’s instructions (Takara). RT-qPCR was performed with FastStart Universal SYBR Green Master (Roche) on an Agilent Mx3000P Real-Time PCR System.

In Situ RNA Localization

The eighth stem internodes of P. trichocarpa were harvested for fixation with FAA solution (50% [v/v] ethanol, 5% [v/v] acetic acid, and 3.7% [v/v])
formaldehyde). After dehydration, the fixed tissues were embedded in paraffin (Sigma) and sectioned to a thickness of 10 μm using a rotary microtome (Leica RM2245). The 175-bp region of *PtrMYB161* was used as specific probes for in situ hybridization. The antisense and sense probes were generated using T7 and SP6 RNA polymerases, respectively. A digoxigenin RNA labeling kit (Roche) was used for probe labeling. After pretreatment, the sections on slides were hybridized with the digoxigenin-labeled *PtrMYB161* antisense or sense RNA probe in hybridization solution. The hybridized signals were detected by incubating with alkaline phosphatase-conjugated antibodies against digoxigenin according to the digoxigenin nucleic acid detection kit (Roche). The color development was evaluated with alkaline phosphatase substrates. The images were taken with a microscope (Leica DM6B). Primer sequences used for probe amplification are summarized in Supplemental Data Set S6.

**Generation of *P. trichocarpa* Transgenic and Knockout Mutant Plants**

The coding region of *PtrMYB161* was amplified from *P. trichocarpa* plants and cloned into the pBb121 vector to generate overexpression construct pBb121-35S-PtrMYB161. The CRISPR-Cas9 system (Ueta et al., 2017) was used to generate knockout mutants of *PtrMYB161*. The sgRNA sequence of *PtrMYB161* was selected using CRISPR-P 2.0 (http://crispr.hzau.edu.cn/cgi-bin/CRISPR2/CRISPR). The *PtrMYB161* sgRNA sequences were synthesized and inserted into pEGP237-2A-GFP vector as described (Ueta et al., 2017). The constructs were transferred into *P. trichocarpa* plants according to a rapid genetic transformation method mediated by *Agrobacterium tumefaciens* (Song et al., 2006; Li et al., 2017c, 2019). The overexpression transgenic plants were identified by RT-qPCR to determine the expression of *PtrMYB161* in the SDX tissues. Mutation in *PtrMYB161* was detected by PCR amplification to use primers flanking the sgRNA target sequence. The amplified DNA segments were cloned into pMD18-T vector (Takara), and 40 colonies were selected for sequencing. All amplification primers are listed in Supplemental Data Set S6.

**Histological Analysis**

Stem internodes of *P. trichocarpa* were cut into 2-mm fragments and fixed with FAA solution (as above). The fixed stem segments were transferred into a graded ethanol series (50%, 60%, 70%, 85%, and 100%; v/v) at 4°C for dehydration and incubated in xylene:xylene solution (75:25, 50:50, and 25:75; v/v) and 100% xylene sequentially at room temperature. The stem segments were then immersed in 75:25 (v/v) xylene:paraffin solution at 42°C overnight and embedded in 100% paraffin (Sigma). The embedded stem segments were cut into 12-μm sections using a rotary microtome (Leica RM2245) and stained with Safranin O, Fast Green, and Toluidine Blue, respectively. The images were captured using a digital microscope and scanner M8 (Precipoint), and the fiber and vessel cells were measured using LAS v4.8 and LAS X v2.0 software (Leica).

**Scanning Electron Microscopy**

The 20th stem internodes of 4-month-old *P. trichocarpa* were collected and coated with gold at 10 mA for 60 s. The samples were imaged using a scanning electron microscope (Nanotech JCM-5000).

**RNA-Seq Analysis**

RNA-seq was carried out with total RNA of SDX tissues isolated from wild-type, *OE-PtrMYB161* transgenic, and knockout mutant plants. The RNA-seq libraries of overexpression transgenics (OE-*PtrMYB161*-L5, OE-*PtrMYB161*-L8, and OE-*PtrMYB161*-L9) and wild-type plants were generated with three biological replicates per sample using the NEBNext Ultra II RNA library preparation kit. A total of 12 libraries were sequenced using an Illumina Hiseq4000. The RNA-seq libraries of knockout mutants (*ptrmyb161*-1 and *ptrmyb161*-3) and wild-type plants were generated with three biological replicates per sample using the MGIEasy RNA Library Preparation reagent kit V3.0 (BGI). A total of nine libraries were sequenced using a BGISEQ-500. For all libraries, 150-bp average read lengths were obtained from sequencing. After removing the library index sequences from each read, the clean reads were mapped to the *Populus trichocarpa* genome v3.0 (Phytozome) using Bowtie2 (Langmead and Salzberg, 2012). The raw counts were determined and normalized following our established analysis pipeline (Lin et al., 2013). Differentially expressed genes were identified by DESeq2 (Love et al., 2014) and defined as a threshold (false discovery rate < 0.05; transcript abundance ratio > 2 [overexpression transgenic plants versus the wild type or knockout mutants versus the wild type]).

**Wood Chemistry**

Fresh stem segments of 4-month-old *P. trichocarpa* plants were cut and immersed in 90% (v/v) aceton at room temperature for 2 d. The stem segments were transferred into 100% (v/v) aceton at room temperature for 14 d, with fresh aceton replaced every 2 d. The aceton was then discarded, and the stem segments were air-dried. The air-dried stem segments were used to quantify the wood composition (acid-insoluble lignin, acid-soluble lignin, and sugars) and lignin composition (syringyl-lignin, guaiacyl-lignin, and hydroxycinnamyl-lignin) following established procedures (Abraham et al., 2013; Wang et al., 2018).

**SDX Protoplast Isolation and DNA Transfection**

The *trichocarpa* SDX protoplast isolation and transfection were carried out as described (Lin et al., 2013, 2017) with minor modifications. The debarked stem segments of 4-month-old *P. trichocarpa* were immersed in cell wall digestion enzyme solution in a 50-ml centrifuge tube for 3 h at room temperature. The digested debarked stem segments were transferred into the 30-ml MMG solution (4 mM MES pH 5.7, 15 mM MgCl2 and 0.25 mM manganese) in another 50-ml centrifuge tube. The protoplasts were released by gently shaking the 50-ml centrifuge tube for 30 s. The protoplasts were filtered by the 75-μm nylon membrane and centrifuged at 500g for 3 min at room temperature. The pelleted protoplasts were resuspended in the MMG solution, and the cell density was adjusted to 5 × 106 cells ml−1. For gene expression analysis, 2 ml of protoplasts was used for transfection with 0.2 ml of plasmid DNA (2 mg ml−1) and 2.2 ml of polyethylene glycol solution. For ChiP assay, 20 ml of protoplasts was used for transfection with 2 ml of plasmid DNA (2 mg ml−1) and 22 ml of polyethylene glycol solution. The detailed transfection procedure was described in our previous studies (Lin et al., 2013, 2017).

**ChiP Assays**

The full coding sequences of *PtrMYB161*, *PtrSND1-A1*, *PtrSND1-A2*, and *PtrSND1-B2* were constructed to the pUC19-35S-sGFP vector, individually generating the destination vector with the T7-sGFP fusion. The pUC19-35S-sGFP vector was used as the control. The plasmids were prepared and transfected into SDX protoplasts. The ChiP assays were performed in the transfected SDX protoplasts as described (Chen et al., 2019; Li et al., 2019; Yeh et al., 2019). Briefly, the protoplasts in WI buffer (0.2 mM MES pH 5.7, 0.8 mM mannitol, and 2 mM KCl) were cross-linked with 1% (v/v) formaldehyde for 10 min at room temperature. The cross-linked protoplasts were harvested for chromatin extraction and then sonicated using a Bioruptor (Diagenode). The sheared chromatin was immunoprecipitated using 5 μg of anti-CFP antibodies (Abcam; ab290). The purified DNAs were analyzed by ChiP-qPCR as previously described (Li et al., 2014, 2019; Yeh et al., 2019). Primers for ChiP-qPCR are listed in Supplemental Data Set S6.

**Gene Expression Analysis in SDX Protoplasts**

The full coding sequences of *PtrSND1-A1*, *PtrSND1-A2*, and *PtrSND1-B2* were constructed to the pUC19-35S-RfA-35S-sGFP vector as described (Li et al., 2020b), and the pUC19-35S-sGFP vector was used as the control. The plasmids were prepared using a CsCl gradient and transfected into SDX protoplasts as described (Lin et al., 2013). After culturing for 12 h, protoplasts were collected for RNA extraction and RT-qPCR analysis.

**Accession Numbers**

The RNA-seq data of *OE-PtrMYB161* transgenics and *ptrmyb161* mutants have been deposited in the National Center for Biotechnology Information Sequence Read Archive under accession numbers PRJNA630668 and PRJNA630301, respectively. Sequence data from this article can be found in *Populus trichocarpa* genome v3.0 (Phytozome). All gene identifier numbers in this article are listed in Supplemental Data Set S7.
Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Effects of overexpressing PtrMYB161 on P. trichocarpa growth.

Supplemental Figure S2. Stem cross and tangential longitudinal sections of 4-month-old wild-type and OE-PtrMYB161-L8 transgenic plants with different internodes.

Supplemental Figure S3. The unenriched promoter segments in PtrMYB021 and PtrMYB074 promoters.

Supplemental Figure S4. PtrMYB161 mediates feedback regulation of the PtrSNID1-directed regulatory network.

Supplemental Figure S5. Effects of PtrMYB161 biallelic mutants on P. trichocarpa growth.

Supplemental Figure S6. CRISPR-based editing of PtrMYB161 did not substantially affect the development of vascular tissues and cell wall thickness.

Supplemental Figure S7. The transcript abundances of PtrSNID1s, PtrMYB021, PtrMYB074, and cell wall component genes in xylem tissues of 4-month-old wild-type and PtrMYB161 biallelic mutant plants.

Supplemental Figure S8. RNA-seq analysis of the transcript abundances of key cambium identity genes.

Supplemental Table S1. Wood composition of PtrMYB161 mutants and wild-type P. trichocarpa.

Supplemental Table S2. Lignin composition of PtrMYB161 mutants and wild-type P. trichocarpa.

Supplemental Figure S3. Percentage of differentially expressed genes in PtrMYB161 overexpression transgenic plants and mutants.

Supplemental Data Set S1. SCW component genes in OE-PtrMYB161 transgenic plants.

Supplemental Data Set S2. Histone deacetylase genes in OE-PtrMYB161 transgenic plants.

Supplemental Data Set S3. SCW component genes in loss-of-function mutants ofPtrMYB161.

Supplemental Data Set S4. Differentially expressed genes in OE-PtrMYB161 transgenic plants.


Supplemental Data Set S6. Primer sequences used in this article.

Supplemental Data Set S7. Gene identifiers of all sequence data from this article.

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LITERATURE CITED


Feedback Regulation for Wood Formation


Wang et al. 2017


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