MYB transcription factor 161 mediates feedback regulation of *Secondary wall-associated NAC-Domain 1* family genes for wood formation

Short title: TF-Mediated Feedback Regulation for Wood Formation

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One-Sentence Summary: A MYB transcription factor mediates feedback mechanisms in a hierarchical transcriptional regulatory network for wood formation in *Populus*. 
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ABSTRACT

Wood formation is a complex process that involves cell differentiation, cell expansion, secondary wall deposition, and programmed cell death. We constructed a 4-layer wood formation transcriptional regulatory network (TRN) in black cottonwood (*Populus trichocarpa*) that has four Secondary wall-associated NAC-Domain 1 (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-PtrMYB161s*) led to reduced wood, altered cell type proportions, and inhibited growth. Integrative analysis of wood cell-based chromatin binding assays with *OE-PtrMYB161s* 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 co-factors. PtrMYB161 protein does not contain any known negative regulatory domains. CRISPR-based mutants of *PtrMYB161* in *P. trichocarpa* exhibited phenotypes similar to the wildtype, suggesting 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.
INTRODUCTION

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 Sperry, 1989).

Wood formation is a complex developmental process involving 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; Ohtani et al., 2011; Zhong et al., 2011; Li et al., 2012; 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; Ohtani et al., 2011; Zhong et al., 2011; Li et al., 2012; Lin et al., 2013; Lin et al., 2017; Chen et al., 2019). Overexpression of *P.*
trichocarpa SND1-B2 (or WND2B) and SND1-A2 (or WND1B) 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 SND1 genes affected the transcript levels of many other TFs, such as MYBs (Zhong et al., 2011; Zhao et al., 2014). Knockout mutation of four \textit{P. tremula} × \textit{P. tremuloides} SND1s (SND1-A1, -A2, -B1, and -B2) 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 SNDs are higher level TFs in a regulatory network, and revealed what other network TFs are regulated by SNDs, 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 \textit{P. trichocarpa}, 266 MYB genes (\textit{PtrMYBs}) have been identified (Wilkins et al., 2009; Tian et al., 2019). Many of the \textit{PtrMYBs} or their homologs in other \textit{Populus} spp. have been studied for functions in regulating the SCW component genes for wood formation, using both genetic and molecular approaches. These MYBs include \textit{PtrMYB002}, \textit{PtrMYB003}, \textit{PdMYB10}, \textit{PtrMYB020}, \textit{PtrMYB021}, \textit{PtrMYB074}, \textit{PtrMYB90}, \textit{PtoMYB092}, \textit{PtrMYB152}, \textit{PtoMYB156}, \textit{PtoMYB170}, \textit{PtrMYB189}, \textit{PtMYB194}, \textit{PtoMYB216}, and \textit{PdMYB221} (also called LTF1) (McCarthy et al., 2010; Tian et al., 2013; Zhong et al., 2013; Chai et al., 2014; Li et al., 2014; Wang et al., 2014; Li et al., 2015; Tang et al., 2015; Wang et al., 2016; Xu et al., 2017; Yang et al., 2017; Wang et al., 2018; 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* MYBs, PtoMYB156 (Yang et al., 2017), PtrMYB189 (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* MYBs, PtMYB165 (Huang et al., 2014; James et al., 2017), PtreM MyB182 (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 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 these subgroup 4 members and the other MYB repressors discussed above, except for PtrMYB189, 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., 2017; Ma and Constabel, 2019). For PtrMYB189, the repressor function was demonstrated by site-directed deletion and mutagenesis of the MYB’s C-terminal 13 aa (277–289, GDDYGNHGMIKKE), 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-seq and ChIP-seq/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 4-layered TRN mediated by PtrSND1-B1 for wood formation in *P. trichocarpa* (Figure 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 (S)-specific monolignol genes (*PtrCald5H1* and *PtrCald5H2*) (Osakabe et al., 1999; Wang et al., 2014; Wang et al., 2018), as well as two key secondary cell-wall 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 Ludwig, 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 the current 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 and one second-layer regulators, 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 *Populus trichocarpa*

*PtrMYB161* expresses more specifically in stem differentiating xylem (SDX) (Figure 2A; Lin et al., 2017; Yeh et al., 2019), and the transcript expression becomes apparent starting from the 4th internode (Figure 2B), where stem secondary growth begins and the S-lignin-rich fiber cells
emerge and differentiate (Li et al., 2001). *In situ* RNA localization suggested that *PtrMYB161* expresses more abundantly in S-lignin-rich fiber cells than in G-lignin-rich vessel elements (Fergus and Goring, 1970; Musha and Goring, 1975; Li et al., 2001), and accumulates its transcripts in differentiating fiber cells at the late stage of SCW formation (Figure 2C). Laser capture microdissection (LCM) based cell-type RNA-seq analysis confirmed this fiber preferential expression (Figure 2D; Lin et al., 2017; Yeh et al., 2019). These tissue- and cell-specific expression patterns suggested that *PtrMYB161*’s functions are associated with wood formation. To determine these functions and *PtrMYB161*’s roles in the wood formation TRN *in planta*, we overexpressed and generated loss-of-function CRISPR-edited *PtrMYB161* in *P. trichocarpa* for characterizations.

**Overexpression of *PtrMYB161* in Transgenic *P. trichocarpa* Resulted in Stunted Phenotypes**

We overexpressed *PtrMYB161* in *P. trichocarpa* under the control of a CaMV 35S promoter. Three independent transgenic lines (*OE-PtrMYB161-L5, OE-PtrMYB161-L8* and *OE-PtrMYB161-L9*) were generated, and all had increased *PtrMYB161* transcript abundances (Figure 3A). These three transgenic lines all showed stunted growth relative to wildtype (Figures 3B and 3C and Supplemental Figure 1). These transgenic lines had significant growth retardation in height, internode length, stem diameter, and leaf size but had similar numbers of internodes compared to wildtype (Figures 3C to 3H). These growth effects were sustained for the period of the study (Figure 3B and Supplemental Figure 1).

Overexpression of *PtrMYB161* disrupted the normal stem vascular meristematic activities (wood differentiation), leading to a reduced secondary xylem (wood) area (Figure 4A). Stem transverse
and tangential longitudinal sections of the differentiating vascular cambium show the presence of fusiform initials (FIs, including their derivatives, the dividing and enlarging cells) and ray initials (RIs) in the vascular cambium of OE-PtrMYB161 transgenics (Supplemental Figures 2A and 2B). These initials and their derivative 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 (Figure 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 (Figure 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 (Figures 4A to 4D). The number of fiber cells differentiated per unit area was reduced (Figure 4E), and the number of vessel elements was increased by 2-fold compared to wildtype (Figure 4E). These transgenic vessels were significantly smaller in diameter, as revealed by their lumen areas (Figure 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 (16 to 26) (Supplemental Figure 2C).

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 to wildtype (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 0.50 compared to 1.94 in wildtype) (Table 2). Significant reductions were also observed for total wood glucose (ranging from ~45 to ~58% reductions) and xylose (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 Secondary Cell-Wall Component Genes**

We performed RNA-seq on stem differentiation xylem (SDX) tissues of *OE-PtrMYB161* transgenics (Figure 5 and Supplemental Dataset 1). When compared with the wildtype transcriptome, the expression of all known monolignol biosynthetic pathway genes in *OE-PtrMYB161* exhibited a greater than 2-fold reduction (Figure 5A). *PtrCAld5H1*, a direct target of *PtrMYB161* (Chen et al., 2019) and a key regulator of S-monomolignol biosynthesis (Wang et al., 2014), was the most highly repressed gene (98% repression) (Figure 5A, Supplemental Dataset 1). All the secondary cell-wall 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 (Figure 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 (Figure 5B, Supplemental Dataset 1). Transcript levels of all genes known to be associated with the biosynthesis of either the backbone or side-chain structures in the two key hemicelluloses, xylan (17 genes) and glucomannan (2 genes; *PtrCslA1* and *PtrCslA2*) (Shi et al., 2017; Kumar et al., 2019), were drastically reduced (Figure 5C, Supplemental Dataset 1). The drastic suppression of these cell-wall component genes was consistent with the severely altered wood lignin and polysaccharide properties (Tables 1 and 2).

The *PtrMYB161*-mediated effects on plant growth, gene suppression, and wood cell-wall morphological and chemical alterations were all similar to transgenics overexpressing the functionally known MYB repressors, AmMYB308, PtoMYB156, PtrMYB189, 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* function as a strong activator and verified its TF-DNA transactivation effects *in vivo* (Chen et al., 2019). Furthermore, *PtrMYB161*’s C-terminal 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 Suggested a Feedback Regulation Point at PtrMYB161 in the
PtrSND1-Mediated TRN

In the PtrSND1-B1 TRN, PtrMYB161 directly activates the expression of 3 monolignol pathway
genes (PtrCAlld5H1, PtrCAlld5H2 and PtrCCoAOMT2) and 2 secondary cell-wall cellulose
biosynthetic genes (PtrCesA4 and PtrCesA18) (Figure 1; Chen et al., 2019). But here,
overexpression of PtrMYB161 in transgenics repressed not only these 5 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.

In *P. trichocarpa* there are four SND1 members, PtrSND1-A1, -A2, -B1 and -B2 (Li et al.,
2012; Lin et al., 2017), that are higher level TFs regulating plant secondary cell-wall formation
(Ohtani et al., 2011; Zhong et al., 2011; Li et al., 2012; Lin et al., 2013; Chen et al., 2019).
Before assessing the suggested feedback regulations, we first tested whether all these four SND1
TFs are constituents of the PtrSND1-B1-mediated TRN (Figure 1; Chen et al., 2019).

PtrSND1-B1 TRN is Part of PtrSND1 Family-Mediated TRN Where Four PtrSND1
Members Directly Activate the Expression of *PtrMYB021* and *PtrMYB074*

In the PtrSND1-B1 TRN, PtrSND1-B1 directly transactivates *PtrMYB021* and *PtrMYB074*
(Figure 1). We also previously demonstrated that the other three PtrSND1 members can activate
*PtrMYB021* (Li et al., 2012), 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 activate *PtrMYB074*. To do that, we transfected SDX protoplasts to overexpress each of these *PtrSND1* members (*pUC19*-35S-*PtrSND1*-35S-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* (Figure 6A). Therefore, all four *PtrSND1* family members, *PtrSND1*-A1, -A2, -B1 and -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 SND1 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 crosslinking 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* genes, with expected qPCR products ranging from 80 to 200 bp (Figure 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 (Figure 6C and Supplemental Figure 3). 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 (Figure 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 *PtrSND1s*, initiating a cascade of feedback regulation for a broad suppression of cell-wall component genes (Figure 5 and Supplemental Dataset 1). 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* (Figure 7A and Supplemental Figure 4A 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 to P4 in Figure 7B) and *PtrMYB021* and *PtrMYB074*...
(P1 to P4 in Figure 6B) were amplified (by RT-qPCR) from the transfected protoplasts following ChIP. We detected 2- to 12-fold enrichment of at least one sequence fragment within the ~2-kb promoter of each *PtrSND1* member genes and *PtrMYB021* (Figure 7C), indicating that *PtrMYB161* can bind to these upstream cell-wall TF genes for a direct feedback repression in the wood formation TRN (Figure 7D). The ChIP analysis suggests that *PtrMYB161* can not directly repress *PtrMYB074* (Supplemental Figure 4B). The repressed transcript abundance of *PtrMYB074* observed in *OE-PtrMYB161* lines (Figure 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., 2017; 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 (Figure 8 and Supplemental Dataset 2), 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 that affects plant growth, cell-wall biosynthesis, and wood formation (Figures 3, 4, 5 and Tables 1, 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). An sgRNA was designed to target SNP-free regions in *PtrMYB161*’s exon II (see Methods; Figure 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., 2017; 2019). We generated two independent biallelic mutants, *ptrmyb161*-2 and *ptrmyb161*-3, with each containing heterozygous edits for *PtrMYB161* (Figure 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 to wildtype (Figure 9C). These growth effects were sustained for the period (~4 months) of the study (Figure 9D, Supplemental Figure 5). 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 wildtype plants (Supplemental Figure 6). Loss-of-function of *PtrMYB161* in *P. trichocarpa* also did not significantly alter the lignin quantity, lignin S/G ratio, and neutral sugar composition and contents in wood (Supplemental Tables 1 and 2).
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 Figure 7A). 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 wildtype (Supplemental Figures 7B-D, Supplemental Dataset 3). RT-qPCR analysis of a few such genes confirmed the RNA-seq results (Supplemental Figures 7E-G). These insubstantial changes in the expression of cell-wall component genes in *ptrmyb161s* are consistent with the similar wood properties between the mutants and wildtype (Supplemental Tables 1 and 2). 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 (Figure 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 (Figure 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 these 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 verified that ~93% (76/82) of the tested interactions function *in planta*. Therefore, the current work established an extended wood formation TRN, where four PtrSND1 members (PtrSND1-A1, -A2, -B1, and -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 (Figure 7D). We further established that the transactivation may be restrained by a feedback regulation network mediated by a TRN’s member, PtrMYB161.

The feedback network was discovered based on genetic analysis of regulatory effects in transgenic whole plants in combination with the protoplast system (Figure 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 (Figure 7D). The transgenesis combined with ChIP assays using the protoplast system suggested that five of these six regulators were directly repressed by PtrMYB161 (Figure 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 regulate 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 regulations are further supported by transgenesis of SND1 in several Populus species. Dominant repression of the P. trichocarpa PtrSND1-B2 functions (named PtrWND2B in 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). RNAi of SND1 members in transgenic P. euramericana using sequence fragments of P. trichocarpa PtrSND1-A2 (named PtrWND1B in 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 current work revealed that SND1 members exert redundant functions in regulating plant growth (Figure 3), xylem fiber frequency and cell-wall thickness (Figure 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; Ohtani et al., 2011; Zhong et al., 2011; Li et al., 2012; Li et al., 2012; Lin et al., 2013; Zhao et al., 2014; Hussey et al., 2015; Nakano et al., 2015; Ye and Zhong, 2015; Sakamoto et al., 2016; Lin et al., 2017; 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 transcriptional regulatory networks (TRNs), revealing specifically and quantitatively how regulations are transduced from SND1s through pair-wise 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 SNDs regulations affect the quantities of the network’s end-products—the three cell-wall components and lignin structures. Our current work demonstrated that lowering the transcript levels of PtrSND1 members, particularly those of PtrSND1-A1 and PtrSND1-A2 together (Figure 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 (Figure 5), supporting a SND1s-guided TRN involving PtrMYB161-mediated multi-layer, feedback repression paths (Figure 7).

We analyzed the expression of key cambium identity genes in wild-type and OE-PtrMYB161 transgenic plants (Supplemental Figure 8). These genes include homologs of WUSCHEL-RELATED HOMEOBOX4 (WOX4), ARBORKNOXI (ARK1), ARK2, and popREVOLUTA (PRE),
which are well-known to act as positive regulators of cambium identity in *Populus* (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-PtrMYB161* transgenics (Supplemental Figure 8), which is in agreement with the detection of fusiform and ray initials, key features that are characteristic of vascular cambium development (Supplemental Figures 2A and 2B). Moreover, high expression levels of the cambium identity genes such as *ARK1* (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*, which are consistent with reduced stem secondary growth of transgenic *P. trichocarpa* overexpressing *PtrMYB161*.

The *PtrMYB161*-mediated repression paths affect plant development and growth (Figures 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 (HATs), whereas differential gene repression is accompanied by hypoacetylated histones mediated by histone deacetylases (HDACs) (Allfrey et al., 1964; Shahbazian and Grunstein, 2007; Li et al., 2011; Zhou et al., 2011; Zentner and Henikoff, 2013; Li et al., 2019; Ueda and Seki, 2020). Our RNA-seq result reveals that of all the currently known *P. trichocarpa* histone deacetylase genes, only three *PtrHDT3* members (*PtrHDT3-A, PtrHDT3-B1*, and *PtrHDT3-B2*) were drastically up-regulated in the *OE-PtrMYB161s* (Figure 8 and Supplemental Dataset 2). These up-regulated genes are preferentially expressed in the vascular...
cambium based on the high spatial resolution RNA-seq analyses (Sundell et al., 2017). Overexpression of *PtrMYB161* decreased the cambial activities (wood differentiation), leading to a reduced secondary xylem (wood) area (Figure 4A and Supplemental Figure 2C). Therefore, *PtrMYB161* overexpression-induced high *PtrHDT3* levels may repress wood differentiation genes through histone deacetylation in the chromatin of such genes, causing reduced stem secondary growth. High expression levels of epigenetic regulators *HD2* (putative orthologs of *HDT3*) are known to provoke developmental abnormalities in Arabidopsis (Zhou et al., 2004).

In addition to the possible involvement in growth-related regulation, *PtrHDT3* may also be key components in *PtrMYB161*-mediated feedback regulation. The induced *PtrHDT3* in *OE-PtrMYB161* lines could be recruited by *PtrMYB161* to its targets, the *PtrSND1* 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 HDACs or HATs 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 co-factors for *PtrMYB161* to act as a repressor is appealing, particularly because *PtrMYB161* protein does not have the known functional repressor domains, such as the EAR or GDDYGNHGMIKKE motifs (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 *PtrMYB161* may act as signals triggering a cascade of regulations affecting adversely wood cell-wall formation and plant growth (Supplemental Dataset 4). However, deletion of *PtrMYB161* using CRISPR-Cas9 could not enhance these
developmental traits. In fact, the edited *ptrmyb161-2* and *ptrmyb161-3* mutants were similar to the wildtype in all aspects examined, such as cell-wall structure, morphology and chemical composition, and other growth developmental features (Figure 9, Supplemental Figures 5, 6, and Supplemental Tables 1, 2). The phenotypic similarity between the mutants and the wildtype comports with the full transcriptome analysis showing that deleting *PtrMYB161* 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 *ptrmyb161-3*, respectively (Supplemental Table 3 and Supplemental Dataset 5). 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 Figure 7), may be compensated for by other TFs or combinations of these TFs.

In the 4-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 and one second-layer regulators (Figure 7), which results in a diverse range of phenotypic changes including increased number of xylem vessels and reduced growth (Figures 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 downregulated 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 (Figure 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 co-factors
(Ma, 2004). It is possible that co-repressors (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 (Figures 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 co-factors. Future studies of the co-factors for
PtrMYB161 will improve our understanding of the dual activator-repressor functions.

CONCLUSION

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 on 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 co-factors, 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-PtrMYB161* 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 *P. trichocarpa* genotype Nisqually-1. Wildtype, transgenic plants and knockout mutants 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 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 µg of total RNA was used for reverse transcription to generate cDNA using 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% ethanol, 5% acetic acid, 3.7% formaldehyde) (v/v). 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. 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 probes in hybridization solution. The hybridized signals were detected by incubating with alkaline phosphatase-conjugated antibodies against digoxigenin according to 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 Dataset 6.

**Generation of *P. trichocarpa* Transgenic and Knockout Mutants**

The coding region of *PtrMYB161* was amplified from *P. trichocarpa* plants and cloned into the pBI121 vector to generate overexpression construct pBI121-35S-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/CRI SPR2/CRI SPR). The *PtrMYB161* sgRNA sequences were synthesized and inserted into pEgP237-2A-GFP vector as described (Ueta et al., 2017). The constructs were transformed into *P. trichocarpa* plants according to a rapid genetic transformation method mediated by *Agrobacterium tumefaciens* (Song et al., 2006; Li et al., 2017; 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 were listed in Supplemental Dataset 6.

**Histological Analysis**
Stem internodes of *P. trichocarpa* were cut into 2-mm fragments and fixed with FAA solution (50% ethanol, 5% acetic acid, 3.7% formaldehyde) (v/v). 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 ethanol/xylene solution 75:25, 50:50, 25:75 (v/v) and 100% xylene sequentially at room temperate. 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 to 12-µm sections using a rotary microtome (Leica RM2245) and stained with safranin O and 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 (Au) 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 plants and knockout mutants. The RNA-seq libraries of overexpression transgenics (*OE-PtrMYB161-L5, OE-PtrMYB161-L8*, and *OE-PtrMYB161-L9*) and wildtype plants were generated with 3 biological replicates per sample using 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 (*ptrmyb161-2, ptrmyb161-3*) and wildtype plants were
generated with 3 biological replicates per sample using MGIEasy RNA Library preparation reagent kit V3.0 (BGI). A total of 9 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 *P. trichocarpa* genome v.3.0 (Phytozome; www.phytozome.com) using Bowtie2 (Langmead and Salzberg, 2012). The raw counts were determined and normalized following our established analysis pipeline (Lin et al., 2013). DEGs were identified by DESeq2 (Love et al., 2014) and defined as a threshold (FDR < 0.05; transcript abundance ratio > 2 [overexpression transgenic plants vs. wildtype or knockout mutants vs. wildtype]).

**Wood Chemistry**

Fresh stem segments of 4-month-old *P. trichocarpa* plants were cut and immersed in 90% (v/v) acetone at room temperature for 2 days. The stem segments were transferred into 100% acetone at room temperature for 14 days, with fresh acetone replaced every 2 days. The acetone 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, sugars) and lignin composition (S-lignin, G-lignin, H-lignin) following established procedures (Abraham et al., 2013; Wang et al., 2018).

**SDX Protoplasts Isolation and DNA Transfection**

The *P. trichocarpa* SDX protoplast isolation and transfection were carried out as described (Lin et al., 2013; 2014) with minor modifications. The debarked stem segments of 6-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 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 500 g for 3 min at room temperature. The pelleted protoplasts were resuspended in the MMG solution and the cell density was adjusted to $5 \times 10^5$ cells/mL. For gene expression analysis, 2 mL of protoplasts were used for transfection with 0.2 mL plasmid DNA (2 mg/mL) and 2.2 mL of PEG solution. For ChIP assay, 20 mL protoplasts were used for transfection with 2 mL plasmid DNA (2 mg/mL) and 22 mL of PEG solution. The transfection detail procedure was described as our previous studies (Lin et al., 2013; 2014).

**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 TF-sGFP fusion. The pUC19-35S-sGFP vector was used as 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 M MES pH 5.7, 0.8 M mannitol, 2 M KCl) were cross-linked with 1% 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 anti-GFP 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 Dataset 6.
Gene Expression Analysis in SDX Protoplasts

The full coding sequences of \( \text{PtrSND1-A1}, \text{PtrSND1-A2}, \) and \( \text{PtrSND1-B2} \) were constructed to the pUC19-35S-RfA-35S-sGFP vector as described (Li et al., 2012) and the pUC19-35S-sGFP was used as the control. The plasmids were prepared using a CsCl gradient and transfected into SDX protoplasts as described (Lin et al., 2014). After culturing for 12 h, protoplasts were collected for RNA extraction and RT-qPCR analysis.

Accession Numbers

The RNA-seq data of \( OE-\text{PtrMYB161} \) transgenics and \( \text{ptrmyb161} \) 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 \( \text{Populus trichocarpa} \) v3.0 (Phytozome; www.phytozome.com). All gene ID numbers in this article were listed in Supplemental Dataset 7.

TABLES

Table 1. Wood composition of \( OE-\text{PtrMYB161} \) transgenic and wild-type \( \text{P. trichocarpa} \)

Table 2. Lignin composition of \( OE-\text{PtrMYB161} \) transgenic and wild-type \( \text{P. trichocarpa} \)

SUPPLEMENTAL DATA

Supplemental Figure S1. Effects of overexpressing \( \text{PtrMYB161} \) on \( \text{P. trichocarpa} \) growth.
Supplemental Figure S2. Stem cross and tangential longitudinal sections of four-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 *PtrSND1s* 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 *PtrSND1s, PtrMYB021, PtrMYB074* and cell-wall component genes in xylem tissues of four-month-old wild-type and *PtrMYB161* biallelic mutants.

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 Table S3. Percentage of DEGs in *PtrMYB161* overexpression transgenic plants and mutants.
**Supplemental Dataset S1.** Secondary cell-wall component genes in OE-PtrMYB161 transgenic plants.

**Supplemental Dataset S2.** Histone deacetylase genes in OE-PtrMYB161 transgenic plants.

**Supplemental Dataset S3.** Secondary cell-wall component genes in loss-of-function mutants of PointerMYB161.

**Supplemental Dataset S4.** Differentially expressed genes in OE-PtrMYB161 transgenic plants.

**Supplemental Dataset S5.** Differentially expressed genes in loss-of-function mutants of PointerMYB161.

**Supplemental Dataset S6.** Primer sequences used in this paper.

**Supplemental Dataset S7.** Gene ID of all sequence data from this article.

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Figure legends

**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. TFs, transcription factors; CW, cell wall. The figure was modified from Chen et al., 2019.

**Figure 2. Expression patterns of *PtrMYB161* in *P. trichocarpa.* (A) The transcript abundance of *PtrMYB161* was analyzed by RNA-seq in four *P. trichocarpa* tissues (xylem, phloem, leaf, and shoot). Normalized CPM means normalized transcript abundances as counts-per-million (CPM). (B) The transcript abundance of *PtrMYB161* was detected by RT-qPCR in different stem internodes of *P. trichocarpa*. *PtrACTIN* was used as an internal reference gene. IN, internode. (C) *In situ* mRNA localization of *PtrMYB161* in the 8\(^{th}\) stem internode of *P. trichocarpa*. Cross sections of the stems were hybridized with digoxigenin-labeled antisense RNA probes of *PtrMYB161* (left), or with a digoxigenin-labeled sense *PtrMYB161* RNA probe as a control (right). The hybridization signals are shown in purple. ph, phloem; pf, phloem fiber; ca, cambium; xy, xylem; xf, xylem fiber; v, vessel. Bars=200 μm. (D) The transcript abundance of *PtrMYB161* was analyzed by RNA-seq in two stem differentiating xylem (SDX) cell types (fiber and vessel cells) of *P. trichocarpa*. The error bars in (A), (B), and (D) represent SEs of three biological replicates from independent pools of *P. trichocarpa* tissues or cells.

**Figure 3. Effects of overexpressing *PtrMYB161* on *P. trichocarpa* growth.** (A) The expression levels of the *PtrMYB161* transgene in stem differentiating xylem (SDX) tissues of three *PtrMYB161* transgenic lines (*OE-PtrMYB161*: L5, L8, L9). The error bars represent SEs from three biological replicates. Asterisks indicate significant differences between each line of the transgenics and wild-type plants (*, P < 0.05, Student’s *t* test). (B) The growth phenotypes of four-month-old *OE-PtrMYB161* (L5, L8, 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, L9) and wild-type plants. Error bars represent SEs of three independent experiments with 5 *P. trichocarpa* plants for each genotype in each replicate. Asterisks indicate significant differences between each line of the transgenics and wild-type plants (**, P < 0.01, Student’s *t* test).
Figure 4. Overexpression of *PtrMYB161* affects the number, lignification and cell wall thickness of fiber cells, and the number and lumen area of vessels. (A) Stem cross sections of four-month-old wild-type and *OE-PtrMYB161-L8* transgenic plants with the 10th and 20th internodes (IN). The cross sections were stained with safranin O and fast green. Bars=200 μm. (B) Magnified images of stem cross sections stained with toluidine blue showing the presence of xylem fibers, vessels and ray parenchyma cells. Bars=50 μm. (C) Magnified images of tangential longitudinal sections stained with toluidine blue showing that the xylem fibers of *OE-PtrMYB161* are narrow and elongated cells with tapered ends. Bars=50 μm. F, xylem fiber; V, vessel element; R, xylem ray parenchyma cell. (D) Scanning electron micrographs of wild-type and *OE-PtrMYB161-L8* transgenic plants with the 20th internode imaged at ×500 (left), ×1500 (middle), and ×2000 (right) magnification. Bars=20 μm. (E and F) Statistical analysis the number of fibers and vessels per cross-sectional area (mm²) (E), mean lumen area of individual fibers and vessels (μm²) (F) within the 20th internode. (E and F) Error bars represent SEs of three independent replicates with at least 150 vessel cells and 3000 fiber cells for each genotype in each replicate, and asterisks indicate significant differences between the transgenics and wild-type plants (**, P < 0.01, Student’s *t* test).

Figure 5. RNA-seq analysis of the transcript abundances of cell-wall component genes. (A to C) The transcript abundances of monolignol (A), cellulose (B) and hemicellulose (C) genes were calculated by RNA-seq. The stem differentiating xylem (SDX) of four-month-old wild-type and *OE-PtrMYB161* transgenic plants were used to perform the RNA-seq. Normalized CPM means normalized transcript abundances as counts-per-million (CPM). Error bars indicate SEs of three biological replicates from independent pools of *P. trichocarpa* SDX tissues.

Figure 6. *PtrSND1-A1, PtrSND1-A2* and *PtrSND1-B2* directly activate the expression of *PtrMYB021* and *PtrMYB074*. (A) RT-qPCR was used to detect the transcript abundance of *PtrMYB074* in *P. trichocarpa* stem differentiating xylem (SDX) protoplast overexpressing GFP (control), *PtrSND1-A1, PtrSND1-A2*, or *PtrSND1-B2*. (B) The locations of the amplified promoter fragments. P1 to P4 show the approximate location of the promoter fragments amplified by qPCR following ChIP assays. (C) ChIP-qPCR showing that *PtrSND1-A1, PtrSND1-A2* and *PtrSND1-B2* directly bind to the promoters of *PtrMYB021* and *PtrMYB074*. *P. trichocarpa* SDX.
protoplast system was used to perform the ChIP experiments. P1, P2, P3 indicate the enriched DNA segments in *PtrMYB021* and *PtrMYB074* promoters. The unenriched promoter segments were shown in Supplemental Figure S3. Enrichment of DNA was calculated as the ratio between the TF-GFP fusion (*PtrSND1-A1-GFP, PtrSND1-A2-GFP, PtrSND1-B2-GFP*) and GFP (control), normalized to that of the *PtrACTIN* gene. Control values were set as 1. (A and C) Error bars indicate SEs of three biological replicates (three independent batches of SDX protoplast transfections). Statistical significance was estimated using the Student *t* test (*, P < 0.05; **, P < 0.01). (D) Diagram showing that PtrSND-A1, PtrSND1-A2, PtrSND1-B1, PtrSND1-B2 directly regulate the *PtrMYB021* and *PtrMYB074* genes. The arrows indicate the protein–DNA regulatory interaction with activation ability.

**Figure 7.** *PtrMYB161* mediates feedback regulation of *PtrSND1s* 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 and OE-*PtrMYB161*-L8 transgenic plants. Error bars indicate SEs 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. (C) ChIP-qPCR showed that *PtrMYB161* directly binds to the promoter of *PtrSND1-A1, PtrSND1-A2, PtrSND1-B1, PtrSND1-B2* and *PtrMYB021*. P1 and P2 indicate the enriched promoter segments of *PtrSND1-A1, PtrSND1-A2, PtrSND1-B1, PtrSND1-B2* and *PtrMYB021*. The unenriched promoter segments were 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 SEs of three biological replicates (three independent batches of stem differentiating xylem protoplast transfections). (A and C) Statistical significance was estimated using the Student *t* test (*, P < 0.05; **, P < 0.01). (D) Diagram showing that *PtrMYB161* feedback regulates the *PtrSND1s* directed regulatory network. The light gray and black arrows indicate the protein–DNA regulatory interaction with activation ability, and the blunted line indicates negative regulation. TFs, transcription factors; CW, cell wall.
Figure 8. RNA-seq analysis of the transcript abundances of histone deacetylase genes. The transcript abundances of histone deacetylase genes were calculated by RNA-seq. The stem differentiating xylem (SDX) of four-month-old wild-type and OE-PtrMYB161 transgenic plants were used to perform the RNA-seq. Normalized CPM means normalized transcript abundances as counts-per-million (CPM). Error bars indicate SEs of three biological replicates from independent pools of *P. trichocarpa* SDX tissues. Asterisks indicate significant differences between each line of OE-PtrMYB161 transgenic plants and wild-type plants (*, P < 0.05; **, P < 0.01, Student’s *t* test).

Figure 9. Effects of CRISPR-edited mutations in *PtrMYB161* on *P. trichocarpa*. (A) A simplified gene structure to indicate the locations and sequence of sgRNA. (B) Mutations at the sgRNA target site for *PtrMYB161* biallelic mutants, which were generated using the CRISPR-Cas9 system with nucleotide deletion or insertion. The dash lines indicate the deleted nucleotides. The red letters represent the inserted nucleotides. Number of bp represent the number of deleted or inserted nucleotides. The bottom rows (Reference) denote the native sgRNA target sequence. (C) The growth phenotypes of four-month-old *PtrMYB161* biallelic mutants and wild-type plants. Bars=10 cm. (D) Statistical analysis of height, internode number, internode length, and stem diameter of *PtrMYB161* biallelic mutants and wild-type plants. Error bars represent SEs of three independent experiments with 5 *P. trichocarpa* plants for each genotype in each replicate. Asterisks indicate significant differences between each line of *PtrMYB161* biallelic mutants and wild-type plants (*, P < 0.05; **, P < 0.01, Student’s *t* test).
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Table 1. Wood composition of *OE-PtrMYB161* transgenic and wild-type *P. trichocarpa*

| Plants               | WT      | *OE-PtrMYB161-L5* | *OE-PtrMYB161-L8* | *OE-PtrMYB161-L9* |
|----------------------|---------|-------------------|-------------------|-------------------|
| Acid-Insoluble lignin| 17.19 ± 0.60 | 18.59 ± 0.77      | 15.53 ± 0.30      | 17.71 ± 0.69      |
| Acid-soluble lignin  | 4.27 ± 0.18  | 2.60 ± 0.31**     | 3.44 ± 0.03**     | 3.04 ± 0.16**     |
| Total Lignin         | 21.43 ± 0.63 | 21.20 ± 1.01*     | 18.97 ± 0.28*     | 20.75 ± 0.83      |
| Glucose              | 53.33 ± 0.50 | 26.79 ± 1.94**    | 29.1 ± 2.98**     | 22.1 ± 2.88**     |
| Xylose               | 12.1 ± 0.51  | 6.18 ± 0.43**     | 7.9 ± 0.36**      | 5.2 ± 0.93**      |
| Galactose            | 1.07 ± 0.09  | 6.86 ± 1.01**     | 3.63 ± 0.03**     | 7.11 ± 0.22**     |
| Arabinose            | 2.4 ± 0.15   | 2.18 ± 0.23       | 0.43 ± 0.03**     | 1.38 ± 0.50       |
| Total Carbohydrate   | 68.9 ± 0.06  | 42.01 ± 1.23**    | 41.13 ± 2.68**    | 35.79 ± 3.28**    |
| C:L Ratio            | 3.22 ± 0.10  | 1.99 ± 0.13*      | 2.17 ± 0.18**     | 1.74 ± 0.23**     |

Four-month-old plants were tested. Three biological replicates from independent pools of *P. trichocarpa* stems were carried out. Data are mean of three independent assays. Asterisks indicate significant differences between each line of the transgenics and wild-type plants (*P < 0.05, **P < 0.01, Student’s t test). Units: g/100g of dry extractive-free wood. C:L=Carbohydrate to Lignin Ratio.
Table 2. Lignin composition of *OE-PtrMYB161* transgenic and wild-type *P. trichocarpa*

| Plants | WT     | OE-PtrMYB161-L5 | OE-PtrMYB161-L8 | OE-PtrMYB161-L9 |
|--------|--------|-----------------|-----------------|-----------------|
| S-Lignin | 62.02% ± 0.50% | 31.64% ± 0.77%** | 26.45% ± 0.80%** | 26.64% ± 2.33%** |
| G-Lignin | 31.99% ± 0.93% | 63.19% ± 0.84%** | 68.81% ± 0.53%** | 67.40% ± 2.49%** |
| H-Lignin | 5.99% ± 0.60% | 5.17% ± 0.81% | 4.74% ± 0.28% | 5.95% ± 0.21% |
| S/G ratio | 1.94 ± 0.07 | 0.50 ± 0.02** | 0.38 ± 0.01** | 0.40 ± 0.05** |

Four-month-old plants were tested. Three biological replicates from independent pools of *P. trichocarpa* stems were carried out. Data are mean of three independent assays. Asterisks indicate significant differences between each line of the transgenics and wild-type plants (**P < 0.01, Student’s *t* test). H: H-subunits; G: G-subunits; S: S-subunits; %: percentage weight of total lignin.
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. TFs, transcription factors; CW, cell wall. The figure was modified from Chen et al., 2019.
Figure 2. Expression patterns of *PtrMYB161* in *P. trichocarpa*. (A) The transcript abundance of *PtrMYB161* was analyzed by RNA-seq in four *P. trichocarpa* tissues (xylem, phloem, leaf, and shoot). Normalized CPM means normalized transcript abundances as counts-per-million (CPM). (B) The transcript abundance of *PtrMYB161* was detected by RT-qPCR in different stem internodes of *P. trichocarpa*. *PtrACTIN* was used as an internal reference gene. IN, internode. (C) *In situ* mRNA localization of *PtrMYB161* in the 8th stem internode of *P. trichocarpa*. Cross sections of the stems were hybridized with digoxigenin-labeled antisense RNA probes of *PtrMYB161* (left), or with a digoxigenin-labeled sense *PtrMYB161* RNA probe as a control (right). The hybridization signals are shown in purple. ph, phloem; pf, phloem fiber; ca, cambium; xy, xylem; xf, xylem fiber; v, vessel. Bars=200 μm. (D) The transcript abundance of *PtrMYB161* was analyzed by RNA-seq in two stem differentiating xylem (SDX) cell types (fiber and vessel cells) of *P. trichocarpa*. The error bars in (A), (B), and (D) represent SEs of three biological replicates from independent pools of *P. trichocarpa* tissues or cells.
Figure 3. Effects of overexpressing *PtrMYB161* on *P. trichocarpa* growth. (A) The expression levels of the *PtrMYB161* transgene in stem differentiating xylem (SDX) tissues of three *PtrMYB161* transgenic lines (OE-*PtrMYB161*; L5, L8, L9). The error bars represent SEs from three biological replicates. Asterisks indicate significant differences between each line of the transgenics and wild-type plants (*, *P* < 0.05, Student’s *t* test). (B) The growth phenotypes of four-month-old OE-*PtrMYB161* (L5, L8, 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, L9) and wild-type plants. Error bars represent SEs of three independent experiments with 5 *P. trichocarpa* plants for each genotype in each replicate. Asterisks indicate significant differences between each line of the transgenics and wild-type plants (**, *P* < 0.01, Student’s *t* test).
Figure 4. Overexpression of *PtrMYB161* affects the number, lignification and cell wall thickness of fiber cells, and the number and lumen area of vessels. (A) Stem cross sections of four-month-old wild-type and *OE-PtrMYB161-L8* transgenic plants with the 10th and 20th internodes (IN). The cross sections were stained with safranin O and fast green. Bars=200 μm. (B) Magnified images of stem cross sections stained with toluidine blue showing the presence of xylem fibers, vessels and ray parenchyma cells. Bars=50 μm. (C) Magnified images of tangential longitudinal sections stained with toluidine blue showing that the xylem fibers of *OE-PtrMYB161* are narrow and elongated cells with tapered ends. Bars=50 μm. F, xylem fiber; V, vessel element; R, xylem ray parenchyma cell. (D) Scanning electron micrographs of wild-type and *OE-PtrMYB161-L8* transgenic plants with the 20th internode imaged at ×500 (left), ×1500 (middle), and ×2000 (right) magnification. Bars=20 μm. (E and F) Statistical analysis the number of fibers and vessels per cross-sectional area (mm²) (E), mean lumen area of individual fibers and vessels (μm²) (F) within the 20th internode. (E and F) Error bars represent SEs of three independent replicates with at least 150 vessel cells and 3000 fiber cells for each genotype in each replicate, and asterisks indicate significant differences between the transgenics and wild-type plants (**, P < 0.01, Student’s t test).
Figure 5. RNA-seq analysis of the transcript abundances of cell-wall component genes.

(A to C) The transcript abundances of monolignol (A), cellulose (B) and hemicellulose (C) genes were calculated by RNA-seq. The stem differentiating xylem (SDX) of four-month-old wild-type and OE-PtrMYB161 transgenic plants were used to perform the RNA-seq. Normalized CPM means normalized transcript abundances as counts-per-million (CPM). Error bars indicate SEs of three biological replicates from independent pools of *P. trichocarpa* SDX tissues.
Figure 6. PtrSND1-A1, PtrSND1-A2 and PtrSND1-B2 directly activate the expression of PtrMYB021 and PtrMYB074. (A) RT-qPCR was used to detect the transcript abundance of PtrMYB074 in P. trichocarpa stem differentiating xylem (SDX) protoplast overexpressing GFP (control), PtrSND-A1, PtrSND1-A2, or PtrSND1-B2. (B) The locations of the amplified promoter fragments. P1 to P4 show the approximate location of the promoter fragments amplified by qPCR following ChIP assays. (C) ChIP-qPCR showing that PtrSND1-A1, PtrSND1-A2 and PtrSND1-B2 directly bind to the promoters of PtrMYB021 and PtrMYB074. P. trichocarpa SDX protoplast system was used to perform the ChIP experiments. P1, P2, P3 indicate the enriched DNA segments in PtrMYB021 and PtrMYB074 promoters. The unenriched promoter segments were shown in Supplemental Figure S3. Enrichment of DNA was calculated as the ratio between the TF-GFP fusion (PtrSND1-A1-GFP, PtrSND1-A2-GFP, PtrSND1-B2-GFP) and GFP (control) normalized to that of the PtrACTIN gene. Control values were set as 1. (A and C) Error bars indicate SEs of three biological replicates (three independent batches of SDX protoplast transfections). Statistical significance was estimated using the Student t test (*, P < 0.05; **, P < 0.01). (D) Diagram showing that PtrSND-A1, PtrSND1-A2, PtrSND1-B1, PtrSND1-B2 directly regulate the PtrMYB021 and PtrMYB074 genes. The arrows indicate the protein–DNA regulatory interaction with activation ability.
Figure 7. PtrMYB161 mediates feedback regulation of PtrSND1s 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 and OE-PtrMYB161-L8 transgenic plants. Error bars indicate SEs 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. (C) ChIP-qPCR showed that PtrMYB161 directly binds to the promoter of PtrSND1-A1, PtrSND1-A2, PtrSND1-B1, PtrSND1-B2 and PtrMYB021. P1 and P2 indicate the enriched promoter segments of PtrSND1-A1, PtrSND1-A2, PtrSND1-B1, PtrSND1-B2 and PtrMYB021. The unenriched promoter segments were 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 SEs of three biological replicates (three independent batches of stem differentiating xylem protoplast transfections). (A and C) Statistical significance was estimated using the Student t test (*, P < 0.05; **, P < 0.01). (D) Diagram showing that PtrMYB161 feedback regulates the PtrSND1s directed regulatory network. The light gray and black arrows indicate the protein–DNA regulatory interaction with activation ability, and the blunted line indicates negative regulation. TFs, transcription factors; CW, cell wall.
Figure 8. RNA-seq analysis of the transcript abundances of histone deacetylase genes. The transcript abundances of histone deacetylase genes were calculated by RNA-seq. The stem differentiating xylem (SDX) of four-month-old wild-type and *OE-PtrMYB161* transgenic plants were used to perform the RNA-seq. Normalized CPM means normalized transcript abundances as counts-per-million (CPM). Error bars indicate SEs of three biological replicates from independent pools of *P. trichocarpa* SDX tissues. Asterisks indicate significant differences between each line of *OE-PtrMYB161* transgenic plants and wild-type plants (*, P < 0.05; **, P < 0.01, Student’s *t* test).
Figure 9. Effects of CRISPR-edited mutations in *PtrMYB161* on *P. trichocarpa*. (A) A simplified gene structure to indicate the locations and sequence of sgRNA. (B) Mutations at the sgRNA target site for *PtrMYB161* biallelic mutants, which were generated using the CRISPR-Cas9 system with nucleotide deletion or insertion. The dash lines indicate the deleted nucleotides. The red letters represent the inserted nucleotides. Number of bp represent the number of deleted or inserted nucleotides. The bottom rows (Reference) denote the native sgRNA target sequence. (C) The growth phenotypes of four-month-old *PtrMYB161* biallelic mutants and wild-type plants. Bars=10 cm. (D) Statistical analysis of height, internode number, internode length, and stem diameter of *PtrMYB161* biallelic mutants and wild-type plants. Error bars represent SEs of three independent experiments with 5 *P. trichocarpa* plants for each genotype in each replicate. Asterisks indicate significant differences between each line of *PtrMYB161* biallelic mutants and wild-type plants (*, P < 0.05; **, P < 0.01, Student’s *t* test).
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