








ORIGINAL RESEARCH

Biomass formation and sugar release efficiency of *Populus* modified by altered expression of a NAC transcription factor

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Funding information

This work was supported by the U.S. Department of Energy (DOE) BioEnergy Science Center and Center for Bioenergy Innovation projects. The BioEnergy Science Center and Center for Bioenergy Innovation are a Bioenergy Research Center supported by the Office of Biological and Environmental Research in the DOE Office of Science. This manuscript has been authored by UT-Battelle, LLC under Contract No. DE-AC05-00OR22725 with the U.S. Department of Energy.

Abstract

Woody biomass is an important feedstock for biofuel production. Manipulation of wood properties that enable efficient conversion of biomass to biofuel reduces cost of biofuel production. Wood cell wall composition is regulated at several levels that involve expression of transcription factors such as wood-/secondary cell wall-associated NAC domains (WND or SND). In *Arabidopsis thaliana*, *SND1* regulates cell wall composition through activation of its down-stream targets such as MYBs. The functional aspects of *SND1* homologs in the woody *Populus* have been studied through transgenic manipulation. In this study, we investigated the role of *PdWND1B*, *Populus SND1* sequence ortholog, in wood formation using transgenic manipulation through over-expression or silencing under the control of a vascular-specific 4-coumarate-CoA ligase (*4CL*) promoter. As compared with control plants, *PdWND1B*-RNAi plants were shorter in height, with significantly reduced stem diameter and dry biomass, whereas there were no significant differences in growth and productivity of *PdWND1B* over-expression plants. Conversely, *PdWND1B* over-expression lines showed a significant reduction in cellulose and increase in lignin content, whereas there was no significant impact on lignin content of downregulated lines. Stem carbohydrate composition analysis revealed a decrease in glucose, mannose, arabinose, and galactose, but an increase in xylose in the over-expression lines. Transcriptome analysis revealed upregulation of several downstream transcription factors and secondary cell wall related structural genes in the *PdWND1B* over-expression lines, partly explaining the observed phenotypic changes in cell wall

Abbreviations: 4CL, 4-coumarate: CoA ligase; 4CL, 4-coumarate-CoA ligase; CCR, cinnamoyl-CoA reductase; CesA, Cellulose synthase; COMT, caffeic acid/5-hydroxyconiferaldehyde O-methyltransferase; CTAB, cetyltrimethylammonium bromide; DAPI, 4,6'-diamidino-2-phenylindole; GT43, Glucosyltransferase family 43; HPLC, high performance liquid chromatography; KOR, Korrigan; LPI, leaf plastochron index; MEGA, Molecular Evolutionary Genetics Analysis; NAC, NAM, ATAF1/2, and CUC2; NST, NAC secondary wall thickening promoting factor; PAL, phenylalanine ammonia lyase; PBS, phosphate-buffered saline; PCR, RNAi, RNA interference; qRT-PCR, quantitative reverse transcriptase; S:G, syringyl to guaiacyl ratio; SHF, separate hydrolysis and fermentation; SHN, SHINE; SND, secondary wall associated NAC domains; SUSY, sucrose synthase; VND, vascular related NAC domain; WND, wood associated NAC domain transcription factors.

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chemistry. Relative to the control, glucose release efficiency and ethanol production from stem biomass was significantly reduced in over-expression lines. Our results show that *PdWND1B* is an important factor determining biomass productivity, cell wall chemistry and its conversion to biofuels in *Populus*.

KEYWORDS

biomass, cell wall, cellulose, lignin, MYB, NAC transcription factor, SND, sugar release, WND1B

1 | INTRODUCTION

Woody biomass, harvested as feedstock for the pulp and paper, bioproduct, and biofuel industries, is formed by tightly regulated biological and molecular genetic xylogenesis mechanisms. Primary xylem is formed from procambium while secondary xylem is formed from vascular cambium during secondary growth. The major constituents of secondary cell walls are cellulose, lignin, and hemicellulose (Darvill et al., 1980). Cellulose is the most abundant polymer in plants and is a polymer of glucose synthesized on the plasma membrane by the cellulose synthase (CesA) complex (Doblin et al., 2002). Lignin is the second most abundant polymer and is composed of guaiacyl (G), syringyl (S), and *p*-hydroxyphenyl (H) units derived through the phenylpropanoid pathway (Boerjan et al., 2003). In addition to cell division and expansion that occurs in primary cells, secondary xylem formation includes secondary wall deposition, lignification, and programmed cell death (Plomion et al., 2001). The formation of xylem cell walls is coordinately regulated at multiple layers by dozens of structural genes and transcription factors.

The major transcription factors that regulate secondary cell wall synthesis include SHINE (SHN), NAC (which stands for NAM, ATAF1/2 and CUC2) domain transcription factors, and MYBs (Yamaguchi & Demura, 2010; Zhong et al., 2006). SHN is the master switch that controls the expression of down-stream transcription factors, NAC and MYBs (Ambavaram et al., 2011). Over-expression of *AtSHN* in rice (*Oryza sativa*) increased cellulose and decreased lignin (Ambavaram et al., 2011). The master and downstream transcription factors in the secondary cell wall transcription factor hierarchy include wood or secondary wall associated NAC domains (WND/SND), NAC secondary wall thickening promoting factor (NST), and vascular-related NAC domain (VND) transcription factors (Lin et al., 2017; Yamaguchi & Demura, 2010). The protein structure of NAC domain members is highly conserved in the N-terminal region and is required for nuclear localization and homo- or hetero-dimerization (Olsen et al., 2005). The C-terminal region has two conserved motifs, the LP-box and the WQ-box that regulate transcriptional activation (Ko et al., 2007; Yamaguchi et al., 2008). There is evidence for role of NAC family members in multiple plant processes, and these functional roles can be redundant among sequence homologs (Aida et al., 1997; He et al., 2005; Hibara et al., 2003).

The NAC domain transcription factor is one of the largest families, with ~100 genes in *Arabidopsis* and soybean (*Glycine max*) and ~140 genes in rice (*Oryza sativa*) (Ooka et al., 2003; Pinheiro

et al., 2009). In *Populus*, there are 163 genes clustered in 18 subfamilies (Hu et al., 2010). Among these, few transcription factors have been functionally characterized in model species such as *Arabidopsis* and rice. In *Arabidopsis*, at least three NAC domain members, *NST1*, *NST2*, and *NST3/SND1*, have been shown to have functional roles in regulating secondary cell wall biosynthesis (Mitsuda et al., 2007; Mitsuda & Ohme-Takagi, 2008; Zhong et al., 2006). T-DNA knockout mutants of *AtSND1* showed no difference from wild type suggesting that the other isoforms might have compensated for the loss (Zhong et al., 2006). In contrast, either over-expression or dominant repression of *AtSND1* results in plants with weak stems and drastically reduced interfascicular fiber and xylary fiber wall thickness (Zhong et al., 2006). Over-expression of *AtSND1* resulted in massive deposition of lignified secondary cell walls suggesting that normal levels of *AtSND1* transcripts are necessary for maintaining proper cell wall thickening in secondary stems (Zhong et al., 2006). The defective secondary cell wall formation phenotype observed in *Arabidopsis snd1nst2* double mutants was restored by complementation with WNDs from *Populus*, suggesting that *Populus* WNDs regulate secondary wall biosynthesis (Mitsuda et al., 2007; Zhong et al., 2010, 2007a). The NAC transcription factors bind to SNBE (secondary wall NAC binding elements) in the promoters of its downstream targets and regulate their expression. *PtWND2B* induces expression of several wood associated MYB transcription factors and genes involved in secondary cell wall biosynthesis (McCarthy et al., 2011; Zhong et al., 2011). Over-expression of another NAC transcription factor gene, *Ptr-SND1-B1*, in *Populus* stem-differentiating xylem (SDX) protoplasts was reported to induce 178 differentially expressed genes (DEGs) of which 76 were identified to be its direct targets (Lin et al., 2013). Furthermore, two splice variants from NAC and VND transcription factor families are involved in reciprocal cross-regulation during wood formation (Lin et al., 2017). However, much less is known about the role of these transcription factors in maintaining cell wall composition. Recently, over-expression of a NAC family member, *PdWND3A*, was reported to affect lignin biosynthesis, decrease the rate of sugar release, and reduce biomass (Yang et al., 2019). Given there is redundancy reported among functional roles of some NAC transcription factor family members and the knowledge of upstream master regulators of secondary wall biosynthesis, *AtSND1*, in *Arabidopsis*, here we sought to characterize the role of sequence ortholog, *PdWND1B*, in *Populus deltoides* in the context of biomass formation. To advance our knowledge on the role of additional NAC/WND transcription members in secondary cell wall



biosynthesis, we developed transgenic *Populus deltoides* plants with xylem-specific over-expression or RNAi mediated silencing of *PdWND1B*, Potri.001G448400; *WND1B* has previously been referred to as PNAC017, VNS11, SND1-A2 (Ohtani et al., 2011; Zhong et al., 2010; Li et al., 2012; Hu et al., 2010). RNAi transgenic plants displayed weaker stems and altered cell wall composition as compared with control plants. Over-expression lines showed increased lignin content and significantly reduced ethanol production from stem biomass as compared with control plants. Our results confirm that *WND1B* plays an important role in secondary cell wall biosynthesis.

2 | METHODS

2.1 | Phylogenetic analysis

Protein sequences of *Populus* WND isoforms were retrieved from [Phytozome v9.1: *Populus trichocarpa* v3.0](#) (Tuskan et al., 2006), and those corresponding to other plant species were obtained from NCBI. Phylogenetic analysis was performed in MEGA (Molecular Evolutionary Genetics Analysis) using the Neighbor-Joining method (Tamura et al., 2011). Bootstrap values were calculated from 500 independent bootstrap runs. Protein sequence alignment was performed using ClustalW, and shading and percent similarity were predicted by GeneDoc (Nicholas et al., 1997).

2.2 | GFP localization

The full length coding regions of *PdWND1A* (Potri.011G153300) and *PdWND1B* (Potri.001G448400) were amplified from a *P. deltoides* xylem cDNA library (primers listed in Supplemental File 1) using Q5 High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA) and cloned in a pENTR vector (Invitrogen, Carlsbad, CA, USA). After sequence confirmation, the coding region fragment was recombined into a Gateway binary vector pGWB405 (Tsuyoshi et al., 2009) using LR clonase (Invitrogen). Plasmid from a positive clone was transformed to *Agrobacterium tumefaciens* strain GV3101. Tobacco infiltration and protein localization were performed as described previously (DePaoli et al., 2011; Sparkes et al., 2006). *Agrobacterium* harboring the binary constructs *PdWND1A* or *PdWND1B* were cultured overnight in LB media. Following brief centrifugation, the supernatant was discarded, and the pellet was dissolved in 10 mM $MgCl_2$. The culture was infiltrated into 4-week-old tobacco leaves. After 48 h, roughly 4 mm² leaf sections were cut and fixed in 3.7% formaldehyde, 50 mM NaH_2PO_4 , and 0.2% Triton X-100 for 30 min, rinsed with phosphate-buffered saline (PBS), and stained in DAPI (4,6'-diamidino-2-phenylindole, 1.5 $\mu g ml^{-1}$ in PBS) for 30 min. GFP visualization and imaging was performed on a Zeiss LSM710 confocal laser scanning microscope (Carl Zeiss Microscopy, Thornwood, NY) equipped with a Plan-Apochromat 63x/1.40 oil immersion objective.

2.3 | Plant materials

The over-expression construct was developed by amplifying and ligating the 1235 bp coding region fragment of *PdWND1B* (gene model: Potri.001G448400, primers presented in Supplemental File) under the control of a vasculature specific *4-coumarate CoA-ligase* (*4CL*) promoter. The RNAi construct, targeting both splice variants of *PdWND1B* gene, was generated by amplifying a 300 bp coding region fragment (from 800 to 1100 bp) and ligating in sense and antisense orientation to form a hairpin with the chalcone synthase intron, under the control of *4CL* promoter. The binary constructs were transformed into wild-type *P. deltoides* "WV94" using an *Agrobacterium* method (Ma et al., 2004). Transgenic plants and empty vector transformed control plants that were roughly 10 cm tall were moved from tissue culture to small tubes with soil. After 2 months, plants were moved to bigger pots (6 L) and were propagated in a greenhouse maintained at 25°C with a 16 h day length. At the time of harvest (6-month-old plants), plant height was measured from shoot tip to stem base, and diameter was measured 2 in. from the base of the stem. The bottom 10 cm stem portion was harvested, air-dried, and used for carbohydrate composition, cellulose, lignin, S:G ratio, and sugar and ethanol release analysis. Initial phenotyping studies were performed using six transgenic lines for each of the overexpression and RNAi constructs corresponding to six independent transformation events per construct and additional in depth characterization studies were performed on two to four selected lines. Data presented here are from two representative lines. Biological replicates for each of the transgenic plant line (control, RNAi, or overexpression) were generated by growing, vegetatively propagated replicate cuttings plants under identical growth conditions. Biological replicates were generated, grown, and harvested at the same time, in the same location (greenhouse) and for the same duration. To capture effects of gene modification and not genome position effects of modification, two independent lines representing two independent genetic transformation events were assessed for each of the RNAi and overexpression constructs in our study. At the time of harvest, young leaf (leaf plastochron index, LPI-0 and 1), mature leaf (LPI-6⁺), and stem (internode portion between LPI 6 and 8) were collected, frozen in liquid nitrogen, and stored at -80°C until they were processed further.

2.4 | RNA extraction and gene expression studies

RNA from frozen ground stem samples was extracted using a Plant Total RNA extraction kit (Sigma, St Louis, MO) with modifications to the kit protocol. Briefly, 100 mg of frozen ground tissue was incubated at 65°C in 850 μl of a 2% CTAB + 1% βme buffer for 5 min, followed by the addition of 600 μl of chloroform:isoamylalcohol (24:1 v/v). The mixture was spun at full speed in a centrifuge for 8 min after which the supernatant in the top layer was carefully removed and passed through a filtration column included in the kit. The filtered elutant was diluted with 500 μl of 100% EtOH and passed through a binding column. This was repeated until all of the filtered

elutant/EtOH mixture were passed through the binding column. Further steps including on-column DNase digestion (DNase70, Sigma), filter washes, and total RNA elution were followed as per the manufacturer's protocol. cDNA was synthesized from 1.5 μ g total RNA using random primers and RevertAid Reverse Transcriptase (Thermofisher). Quantitative reverse transcriptase PCR (qRT-PCR) was performed in a 384-well plate using cDNA (3 ng), gene specific primers (250 nM, list provided in Supplemental File), and iTaq Universal SYBR Green Supermix (1X, Bio Rad). Relative gene expression was calculated using the threshold cycle (Ct) values of the target gene and the housekeeping gene (18S ribosomal RNA) using Δ Ct or $\Delta\Delta$ Ct methods method (Livak & Schmittgen, 2001). Gene accession numbers and primer sequence information are presented in Supplemental File 1.

2.5 | Micro chromatin immunoprecipitation (μ ChIP) assay from protoplasts

Transcription factor PdWND1B was cloned in-frame with 10X Myc tag and used to transfect protoplasts derived from *Populus* 717-1B4 tissue culture grown plants (Guo et al., 2012). ChIP assays were performed using the modified protocol from Dahl and Collas (2008) and Adli and Bernstein (2011). Briefly, transfected protoplasts were resuspended in W5 solution (154 mM NaCl, 125 mM CaCl₂, 5 mM KCl, 2 mM MES [pH 5.7]), crosslinked by adding 1% (v/v) formaldehyde and gently rotating the tubes for 8 min. To stop crosslinking, glycine was added to a final concentration of 0.125 M and gently rotated at room temperature for 5 min. The crosslinked protoplasts were washed once with W5 solution and lysed by mixing with SDS Lysis Buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 10 mM EDTA [pH 8.0], 1% SDS, 1 mM PMSF, protease inhibitor) followed by incubation on ice for 10 min with intermittent and brief vortexing. The lysate was supplemented with RIPA ChIP Buffer (10 mM Tris-HCl [pH 7.5], 140 mM NaCl, 1 mM EDTA [pH 8.0], 1% Triton X-100, 0.1% Na-deoxycholate, 1 mM PMSF, protease inhibitor) and sonicated for 150 s with 0.7 s "On" and 1.3 s "Off" pulses at 20% power amplitude on ice using Branson 450 Digital sonifier to generate 150- to 600-bp chromatin fragments. Additional ice-cold RIPA ChIP buffer was added to aliquot the sample into three separate tubes—500 μ l Antibody (Ab) sample, 500 μ l No-Antibody (NAb) sample, and 75 μ l input chromatin. To the Ab sample, 0.75–1 μ g anti-c-Myc antibody (Sigma-Aldrich #C3956) was added and gently rotated overnight at 4°C. Protein A Mag Sepharose (Sigma-Aldrich #28-9440-06) beads were washed with RIPA buffer (10 mM Tris-HCl [pH 7.5], 140 mM NaCl, 1 mM EDTA [pH 8.0], 1% Triton X-100, 0.1% SDS, 0.1% Na-deoxycholate), added to Ab and NAb samples and gently rotated at 4°C for 120 min. The beads were then collected and washed twice with low-salt wash buffer (150 mM NaCl, 0.1% SDS, 20 mM Tris-HCl [pH 8.0], 2 mM EDTA [pH 8.0], 1% Triton X-100), twice with LiCl buffer (0.25 M LiCl, 1% Na-deoxycholate, 10 mM Tris-HCl [pH 8.0], 1% NP-40, 1 mM EDTA [pH 8.0]), and twice with TE Buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]). The beads were subjected

to reverse crosslinking by adding Complete Elution Buffer (20 mM Tris-HCl [pH 8.0], 5 mM EDTA [pH 8.0], 50 mM NaCl, 1% SDS, 50 μ g/ml Proteinase K) and incubating for 120 min on thermomixer at 68°C and 1300 rpm to elute protein-DNA complexes. Input samples were added with elution buffer (20 mM Tris-HCl [pH 8.0], 5 mM EDTA [pH 8.0], 50 mM NaCl) and 50 μ g/ml proteinase K before placing on thermomixer. After incubation, supernatants were collected, and the ChIP DNA was purified using MinElute PCR Purification Kit (Qiagen #28004). Real-time PCR was performed for the ChIPed DNA by promoter specific primers (Supplemental File 1), and the obtained Ct values were used to calculate the signal intensity by Percent Input Method. At least three biological replicates (with two technical replicates each) representing independent protoplast transfections were used. The ChIPed DNA was also used for PCR reactions by promoter specific primers to analyze the products on agarose gel.

2.6 | Transcriptional activator assay

The coding sequence (CDS) of *WND1B* was in-frame cloned in a Gal4 binding domain (GD) effector vector (Wang et al., 2007). For the trans-activator assays, the GD-fusion constructs were co-transfected with Gal4:GUS reporter construct into *Populus* 717-1B4 protoplasts (Guo et al., 2012). For the trans-repressor assays, GD-fusion constructs were co-transfected with LexA binding-domain fused VP16 (LD-VP) and LexA:Gal4:GUS reporter (Wang et al., 2007). An empty GD effector vector was co-transfected with reporter vectors for the control experiments. The transfected protoplasts were incubated in dark for 16–20 h and GUS activity was quantitatively measured. All the protoplast transfections were included with equal amounts of 35S:Luciferase reporter and Luciferase activity was used for normalization of GUS activity.

2.7 | Cellulose and lignin estimation

Cellulose was estimated on debarked, ground, and air-dried stem tissue using the anthrone method (Updegraff, 1969). Stem sample (25 mg) was first digested with 500 μ l of acetic-nitric acid reagent (100 ml of 80% acetic acid mixed with 10 ml of nitric acid) at 98°C for 30 min. After cooling, the sample was centrifuged, the supernatant was discarded, and the remainder was washed with water. After brief centrifugation, water was discarded, and the pellet was digested with 67% (v/v) sulfuric acid for 1 h at room temperature. An aliquot of the mix was diluted (1:10) with water. In a PCR tube, 10 μ l of diluted reaction mix, 40 μ l of water, and 100 μ l of freshly prepared anthrone reagent (0.5 mg anthrone ml⁻¹ of cold concentrated sulfuric acid) were added and heated for 10 min at 96°C. Samples were cooled, and absorbance (A₆₃₀) was measured. Cellulose was then estimated based on the absorbance of glucose standards. Lignin and its monomer composition were analyzed using pyrolysis molecular beam mass spectrometry at the National Renewable Energy Laboratory as described previously (Mielenz et al., 2009).

2.8 | Stem carbohydrate composition analysis

Roughly 25 mg of air-dried stem sample was weighed in a 2 ml tube and extracted twice at 85°C with a total of 2 ml of 80% ethanol. The supernatant was collected in a new 2 ml tube and was re-extracted with 50 mg activated charcoal (Sigma) to eliminate pigments that interfere with sugar analysis. A 1 ml aliquot of the pigment free extract was incubated overnight in a heating block maintained at 50°C, and the resulting pellet was dissolved in 120 µl of water. A 10 µl aliquot was used for estimation of sucrose and glucose using assay kits (Sigma). Starch from the pellet was digested using 1 U of

α -amylase (from *Aspergillus oryzae*, Sigma) and amyloglucosidase (from *Aspergillus niger*, Sigma). After starch removal, the pellet was dried overnight at 95 °C and used for estimating structural sugars. Roughly, 5 mg of sample was weighed in a 2 ml tube and digested with 50 µl of 75% v/v H₂SO₄ for 60 min. The reaction was diluted by adding 1.4 ml of water, and tubes were sealed using lid-locks and autoclaved for 60 min in a liquid cycle. After cooling, the sample was neutralized with CaCO₃ and sugar composition was estimated using high performance liquid chromatography (HPLC, LaChrom Elite® system, Hitachi High Technologies America, Inc.) as described previously (Fu et al., 2011; Yee et al., 2012).

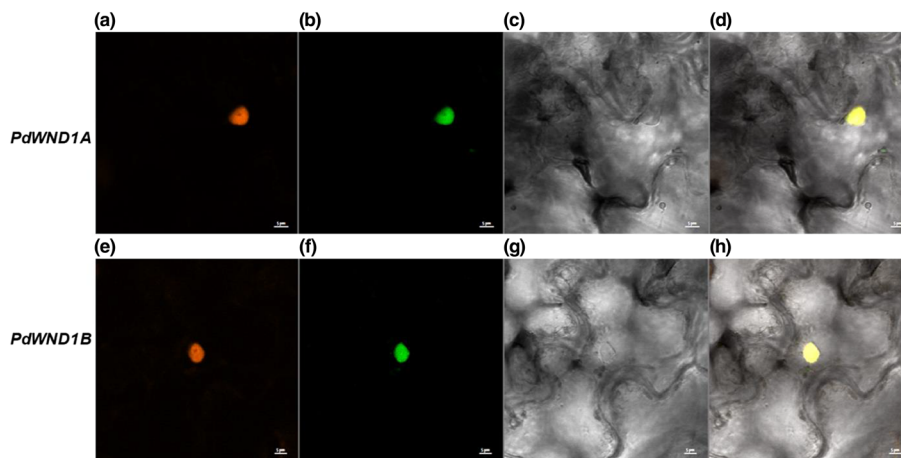
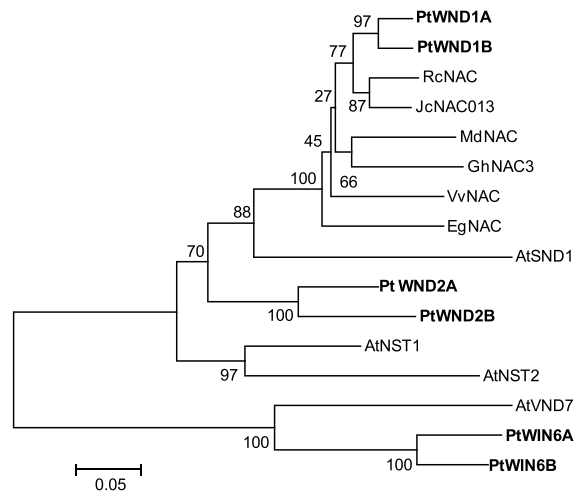


FIGURE 1 (Top figure) Phylogenetic analysis of selected secondary cell wall associated NAC transcription factors from *Populus* and other plant species. Transcription factors from *Populus* are in bold. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. Accessions are provided below. AtSND1: At1g32770 (*Arabidopsis thaliana*); AtNST1: At2g46770; AtNST2: At3g61910; AtVND7: AT1G71930; RcNAC: XP_002518924 (*Ricinus communis*); VvNAC: XP_002279545 (*Vitis vinifera*); JcNAC013: AGL39669 (*Jatropha curcas*); MdNAC: NP_001280877 (*Malus domestica*); GhNAC3: ADN39415 (*Gossypium hirsutum*); EgNAC: KCW72583 (*Eucalyptus grandis*). PtWND1A (Potri.011G153300), PtWND1B (Potri.001G448400), PtWND2A (Potri.014G104800), WND2B (Potri.002G178700), PtWND6A (Potri.013G113100) and PtWND6B (Potri.019G083600). (Bottom figure) Localization of the PdWND1A and PdWND1B in tobacco epidermal cells. Nuclear targeting of GFP: PdWND1A (a to d) and PdWND1B (e to h) in *Nicotiana benthamiana* mesophyll cells after agroinfiltration. Panels (a) and (e) are cells stained with DAPI to show nuclei (color channel set to orange), (b) and (f) are GFP localization (color channel set to green), (c) and (g) are colocalization of DAPI and GFP (yellow color), and (d) and (h) are bright field images. Scale bar represents 5 µm.

2.9 | Glucose release and ethanol conversion

Separate hydrolysis and fermentation (SHF) was used to evaluate digestibility of biomass samples as described previously (Fu et al., 2011; Yee et al., 2012). Extract free biomass was autoclaved for sterilization purposes and the hydrolysis and fermentations were performed in biological triplicate at 5.0% (w/v) biomass loading in a total volume of 20 ml at a pH of 4.8 with a final concentration of 50 mM citrate buffer and 0.063 mg ml⁻¹ streptomycin. The hydrolysis was performed using commercial hydrolytic enzyme blends (Novozymes, Wilmington, DE, USA). Cellic[®]-Ctec2 was loaded at 20 mg protein gram⁻¹ dry biomass, and Novozyme 188 and Cellic[®] Htec2 were loaded at 25% and 20% (v/v) of Ctec2, respectively. The biomass and enzymes were incubated at 50°C and 120 rpm for 5 days. The hydrolysate was then fermented with *Saccharomyces cerevisiae* D5A (ATCC 200062) at 35°C and 150 rpm with a final concentration of 0.5% (w/v) yeast extract. Hydrolysate and fermentation broth samples were analyzed for glucose and ethanol using HPLC equipped with a refractive index detector (model L-2490). The products were separated on an Aminex[®] HPX-87H column (Bio-Rad Laboratories, Inc.) at a flow rate of 0.5 ml min⁻¹ of 5.0 mM sulfuric acid and a column temperature of 60°C and were quantified as described previously (Fu et al., 2011; Yee et al., 2012).

3 | RESULTS AND DISCUSSION

3.1 | Phylogenetic analysis, gene expression, and localization

In *Arabidopsis*, at least three NAC transcription factors, *SND1*, *NST1*, and *VND7* have a proposed role in regulation of secondary cell wall formation. To retrieve their sequence orthologs from *Populus trichocarpa*, protein sequences of the three genes were blasted in *Phytozome* (v 9.1) and the two best hits were retrieved for each sequence resulting in a total of six sequences. These were designated as *PtrWND1A* (Potri.011G153300), *PtrWND1B* (Potri.001G448400), *PtrWND2A* (Potri.014G104800), *WND2B* (Potri.002G178700), *PtrWND6A* (Potri.013G113100) and *PtrWND6B* (Potri.019G083600). The nomenclature used in this study was based on Zhong et al. (2010). All six genes have alternate names; *WND1B* has also been named *SND1A2* or *VNS11* in previously reports (Li et al., 2012; Ohtani et al., 2011). In the phylogenetic tree developed using protein sequences, *PtrWND1A* and *PtrWND1B* were clustered together and share 94% similarity at the protein level (Figure 1; Supplemental File 2), suggesting they originated from a recent genome duplication (Tuskan et al., 2006). They share only approximately 50% similarity with *AtSND1* and *AtNST1*, approximately 56% with *PtrWND2A* and *PtrWND2B*, and approximately 41% with *PtrWND6A* and *PtrWND6B*, but more than 83% with *RcNAC* (*Ricinus communis*) and *JcNAC013* (*Jatropha curcas*). *PtrWND2A* and *PtrWND2B* are clustered together and share 88% similarity, while *PtrWND6A* and *PtrWND6B* share 92% similarity. Protein sequence alignment revealed they are highly

conserved in the NAC domain located in the N terminal region. Conversely, they are highly diverse in the C terminal region, which has putative activation domains (Olsen et al., 2005; Xie et al., 2000). At least 163 NAC domain transcription factors have been reported in *Populus*. Based on phylogenetic analysis, these are classified into 18 groups (Hu et al., 2010). *PtrWND1A* and *B* and *PtrWND2A* and *B* are closely clustered in the NAC-B subgroup, while *PtrWND6A* and *B* are clustered in the NAC-O subgroup.

The expression of the above six NAC transcription factors was studied in eight different tissues of *Populus deltoides* including YL (young leaf), ML (mature leaf), YS (young stem), MS (mature stem), PH (phloem), XY (xylem), RT (root), and PT (petiole). In *Populus*, *WND1B* undergoes alternate splicing, resulting in two variants designated as the small and large variants. The large variant retains intron 2 (Li et al., 2012; Zhao et al., 2014). In this study, to account for both splice variants, primers were designed in the region common to both variants. In general expression of all the genes was much higher in xylem than in other tissues (Supplemental File 3). Within xylem, expression of *PdWND1A* and *PdWND1B* was much higher relative to the other genes. Among other tissues, expression of *PdWND1A* was much higher relative to the other genes except in phloem, where *PdWIN2B* was strongly expressed. Expression of *PdWIN6A* and *PdWIN6B* was weaker in all tissues relative to the other genes. In *Populus*, these transcription factors are most abundantly expressed in stems, which is

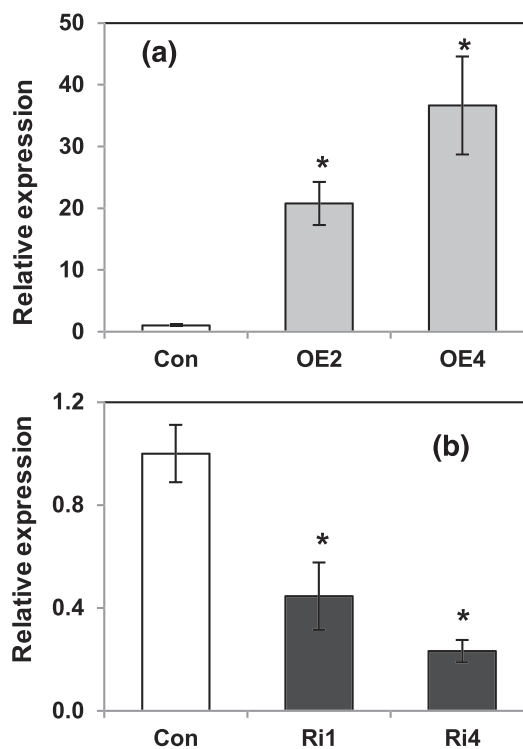


FIGURE 2 Relative expression of *PdWND1B* in control and transgenic lines. Gene expression (arbitrary units) in control (Con), over-expression (OE) lines (a), and RNAi suppressed (Ri) lines (b) was relative to the housekeeping *18S rRNA* gene. The data represent means \pm SE ($n \geq 3$ biological replicates). * indicates statistical significance based on Student's *t* test ($p \leq .05$).

likely regulated differentially in a xylem cell type manner as shown for two isoforms previously (Ohtani et al., 2011). Finally, to confirm the nuclear localization of the two abundantly xylem expressed genes, *PdWND1A* and *PdWND1B*, tobacco leaf infiltrations with GFP:*PdWND1A* and GFP:*WND1B* and DAPI staining was undertaken, which showed that *PdWND1A* and *PdWND1B* are targeted to the nucleus (Figure 1) as has been reported previously for *AtSND1* and *PtWND1B* supporting their potential function as transcription factors (Li et al., 2012; Zhong et al., 2006).

3.2 | Plant morphology and growth

In the present study, we focused on studying the functional aspects of *PdWND1B* through over-expression and RNAi-mediated suppression using a xylem specific 4CL promoter. While transgenic plant production was attempted with the other paralog gene, *PdWND1A*, was attempted but sufficient independent transformants were not successful in tissue culture. Sufficient independent events were available for overexpression and downregulation lines for an in-depth thorough study of *PdWND1B*. In order to selectively downregulate *PdWND1B*, a sequence in exon region at the 3' end that has distinct differences with *PdWND1A* was selected for RNAi construct development, targeting both the splice variants reported previously for the gene (Zhao et al., 2014). In our preliminary study, six independent over-expression (OE) and six independent RNAi lines were propagated in the greenhouse. Plant height of over-expression lines was not different as compared with controls, but RNAi lines were shorter (Supplemental File 5A). Lignin content was significantly higher in all over-expression lines but showed a slight decreasing trend in RNAi lines (Supplemental File 5B). In-depth characterization was performed

on two to four selected lines and data presented in this study is representative of two over-expression lines (designated as OE2 to OE4) and two RNAi suppression lines (Ri1 and Ri4). The extent of alteration in *PdWND1B* expression in transgenic lines was measured using qRT-PCR. As compared with control lines, *PdWND1B* expression was increased by 40-fold in OE4 and by 23-fold in OE2 (Figure 2). In RNAi lines, *PdWND1B* expression was reduced by 73% in Ri4 and by 65% in Ri1.

At the time of harvest (~6 months of growth), control plants reached an average of 130 cm (Figure 3a). The OE plants were similar in height with that of controls. However, Ri lines were significantly shorter by 40 to 50% and reached an average of 66 to 78 cm (Figure 3a). A similar trend was also observed in stem diameter. As compared with controls, stem diameter in OE expression lines was not significantly altered but was reduced by 40% in Ri1 and Ri4 (Figure 3b). The combined effect of reduced plant height and stem diameter resulted in a roughly 75% reduction in total stem dry weight in Ri1 and Ri4 lines (Figure 3c). RNAi lines also developed smaller leaves and thus had a roughly 70% reduction in leaf weight (Figure 3d).

Evidence suggests that *SND/WND* are required for normal plant development (Zhao et al., 2014; Zhong et al., 2010). Over-expression of the full-length coding region of *AtSND1* in *Arabidopsis* and *PtWIN2B* or *PtWIN6B* in *Populus tremula x alba*, under the control of a CaMV 35S promoter, resulted in plants with weaker stems, small leaves, and stunted growth. This strongly supports the hypothesis that the WNDs play a significant role in maintenance of growth and development (Zhong et al., 2006, 2011). In contrast, over-expression of the *PtWND1B* whole gene (including exons and introns) in *Populus x euramericana*, under the control of a CaMV 35S promoter, did not affect plant growth, but only reduced leaf size (Zhao et al., 2014). Our

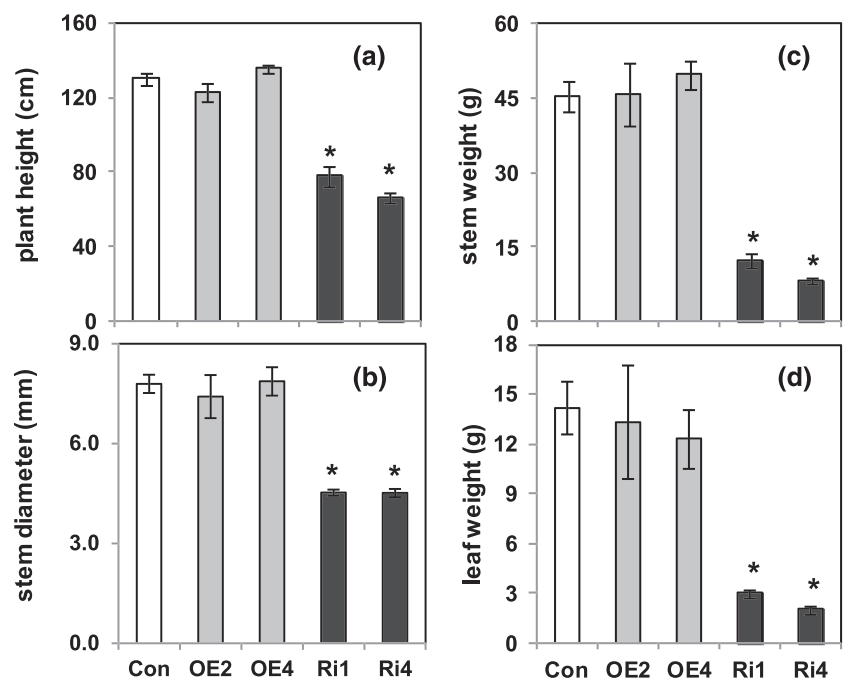


FIGURE 3 Growth and biomass productivity in control and *PdWND1B* transgenic lines. Plant height (a), stem diameter (b), stem weight (c), leaf weight (d) of empty vector transformed control (Con), and *PdWND1B* over-expression (OE) and RNAi suppressed (Ri) lines. The data represent means \pm SE ($n \geq 3$ biological replicates). * indicates statistical significance based on Student's *t* test ($p \leq .05$).

study included overexpression of the shorter variant of *PdWND1B* under the control of a xylem-specific promoter and the observation of no apparent growth impact in overexpression lines. Previous study reported that over-expression of the *PtWND1B* longer splice variant in *Populus x euramericana*, under the control of its own promoter, affected plant development, but the same effect was not observed when the small variant of *PtWND1B* was over-expressed (Zhao et al., 2014). Zhong et al. (2006) also report that an *Atsnd1* mutation did not affect plant development. However, consistent with our study in *Populus*, downregulation of *PtWND1B*, controlled by its own promoter, resulted in plants with weak stems that did not grow straight (Zhao et al., 2014). Therefore, it appeared that *WND* genes may have species-specific effect on plant growth and development. It is also possible that the differences in promoters used (i.e., native promoter (Zhao et al., 2014) and tissue-specific promoter (in the present study) may contribute to differences in phenotypic observations between *Arabidopsis* and *Populus*.

3.3 | Structural polymers

WND transcription factors have a proposed function in secondary cell wall biosynthesis. Therefore, the effect of altered *PdWND1B* expression on secondary cell wall composition was studied in stems. Stem secondary cell walls are composed predominantly of cellulose, lignin, and hemicellulose (Bailey, 1938; Darvill et al., 1980). Cellulose, estimated by the anthrone method, was significantly reduced by 9 to 13% in OE lines, but was increased by 6% in RNAi lines (Figure 4a). Lignin content was significantly increased in OE lines but no significant effect was observed in the RNAi lines (Figure 4b). Relative to the control, lignin S:G ratio appeared lower as a result of manipulating expression of *PdWND1B* gene either via overexpression or downregulation, as a significant phenotypic change was observed in both OE lines and in one RNAi line (Figure 4c). To understand changes in other sugars, sugars were quantified post cell wall digestion using HPLC. Glucose and xylose were the predominant sugars in control plant stem material, at 45% and 15%, respectively (Figure 5). However, while glucose was reduced in OE lines, xylose, representing the hemicellulose fraction, was significantly increased (Figure 5). Levels of minor sugars including galactose, arabinose, and mannose were also significantly reduced in OE lines. Trace compounds, 5-(hydroxymethyl) furfural, were reduced (up to fourfold) in RNAi lines, while 2-furfural was significantly reduced by 60 to 75% in RNAi lines.

Over-expression of *AtSND1* induced ectopic deposition of lignified secondary cell walls in leaf and stem epidermal and mesophyll cells that normally do not undergo lignification (Zhong et al., 2006). In addition, cellulose and hemicellulose were also deposited. A similar response was observed in *Populus*, where *PtWND2B* and *PtWND6B* were over-expressed under the control of a CaMV 35S promoter (Zhong et al., 2011). To address the biomass chemistry context of the present study, over-expression of *PdWND1B* in our study was driven by a xylem-specific promoter to avoid confounding growth effects arising from ectopic lignification. In the context of stem cell wall

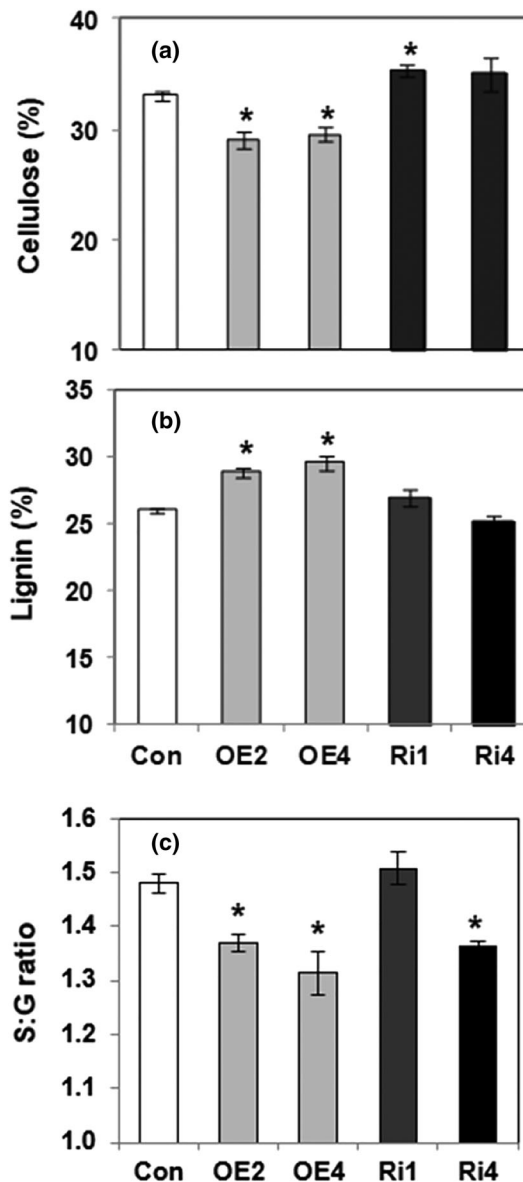


FIGURE 4 Stem cell wall composition of control and *PdWND1B* transgenic lines. Cellulose (a) and lignin (b) content and (c) lignin S:G ratio measured dried debarked stems of empty vector transformed control (Con), and *PdWND1B* over-expression (OE), and RNAi suppressed (Ri) lines. The data represent means \pm SE ($n \geq 3$). * indicates statistical significance based on Student's *t* test ($p \leq .05$).

phenotype, our results indicate an increase in lignin and xylose in stems of OE lines while cellulose levels were reduced. A negative relationship has been proposed between levels of cellulose and lignin (Hu et al., 1999). We observed an increase in lignin and a concomitant decrease in cellulose of overexpression lines relative to the control. In *Arabidopsis*, silencing of *AtSND1* and *AtNST1* simultaneously reduced lignin, cellulose, and hemicellulose (Zhong et al., 2007a). In the present study, significant differences were not observed in levels of lignin or other sugars in RNAi lines, suggesting that the reduction in expression and function of *PdWND1B* potentially is partly compensated by other

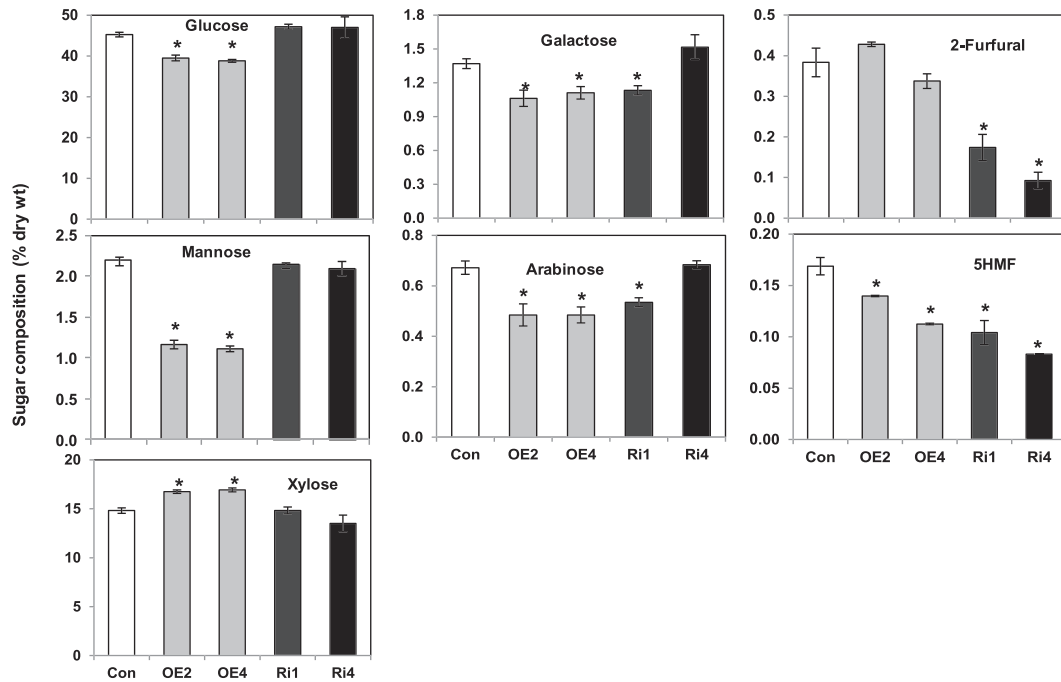


FIGURE 5 Sugar composition in stem cell walls of control and *PdWND1B* transgenic lines. Levels of different sugars in empty vector transformed control (Con), *PdWND1B* over-expression (OE), and RNAi suppressed (Ri) lines. The data represent means \pm SE ($n \geq 3$). * indicates statistical significance based on Student's *t* test ($p \leq .05$).

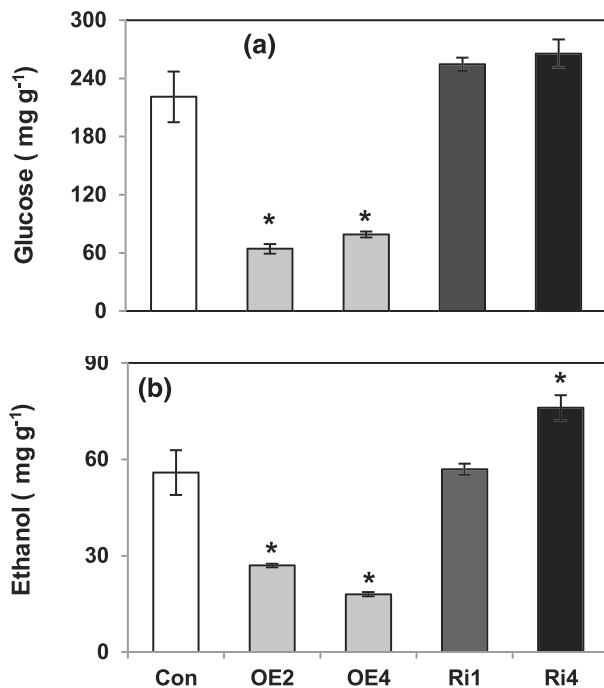


FIGURE 6 Glucose release and ethanol conversion efficiency from stems of control and *PdWND1B* transgenic lines. Levels of glucose (a) and ethanol (b) in empty vector transformed control (Con), *PdWND1B* over-expression (OE), and RNAi suppressed (Ri) lines. The data represent means \pm SE ($n \geq 3$). * indicates statistical significance based on Student's *t* test ($p \leq .05$).

members of the NAC family (i.e., *PdWND1A*) members. In future studies, it would be interesting to generate and characterize double knockout/knockdown plants of *PdWND1A* and *PdWND1B*, and similarly, for other closely related paralogs, which can address the potential functional redundancy and reveal their more precise functions in secondary cell wall biosynthesis.

3.4 | Sugar release and ethanol conversion

The effect of altered cell wall composition on sugar release and ethanol conversion was studied in OE and RNAi lines. Glucose release was significantly reduced by 65 to 70% in OE plants compared with that of control plants (Figure 6a). This is consistent with a significant reduction in ethanol production from biomass. In contrast, in the RNAi lines, no statistically significant difference was observed for glucose release relative to control and a statistically significant increase in ethanol production was observed in only one line, RNAi4 (Figure 6b).

Biomass recalcitrance is determined by many parameters, but predominantly by cellulose and lignin content and composition. Lignin content and S:G ratio have been reported to influence sugar release efficiency in poplar (Studer et al., 2011). An increase in lignin content and decrease in cellulose content had a strong negative impact on sugar release efficiency and ethanol conversion in OE lines in this study.

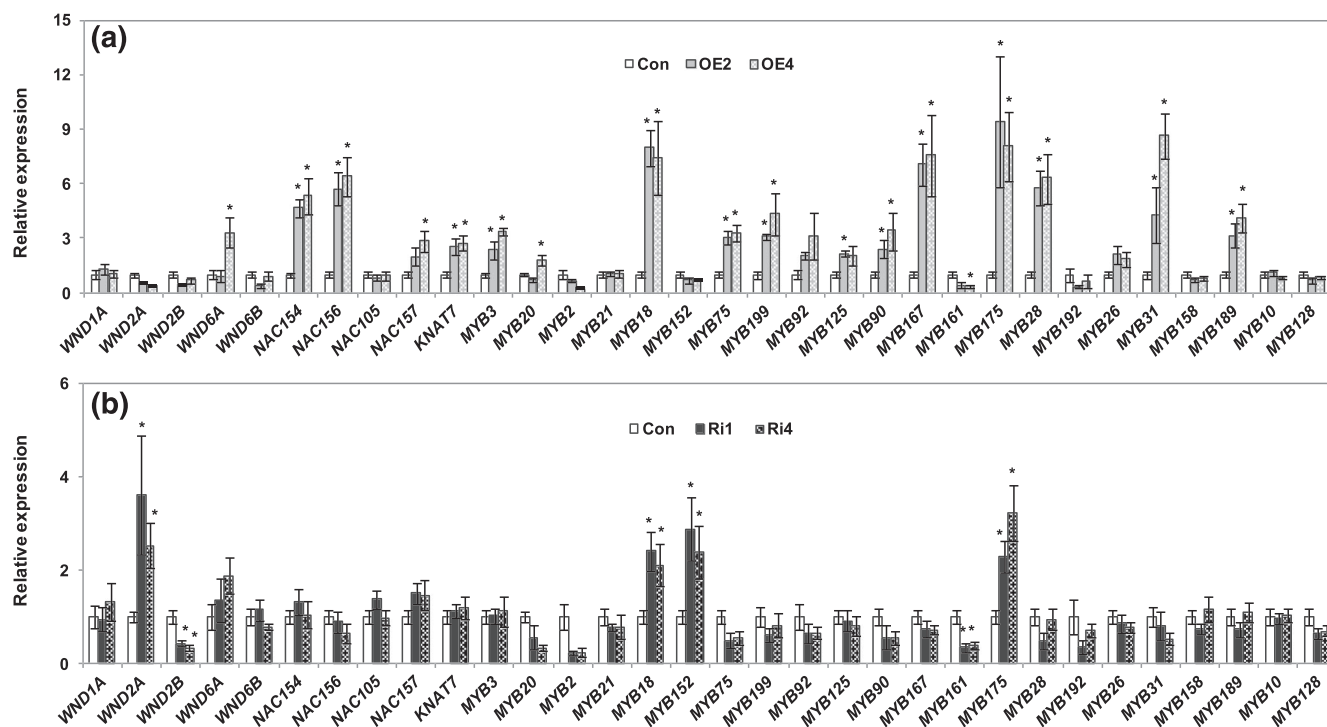


FIGURE 7 Expression of secondary cell wall related transcription factors in control and *PdWND1B* transgenic lines. Relative gene expression (arbitrary units) in control (Con), over-expression (OE) lines (a), and RNAi suppressed (Ri) lines (b) was calculated by normalizing the expression of target genes to the housekeeping gene, *18S rRNA*, and calculating expression change relative to the control line. The data represent means \pm SE ($n = 3$). * indicates statistically significant, $p \leq .05$ based on Student's *t* tests.

3.5 | Gene expression changes

In *Arabidopsis* and *Populus*, over-expression of *AtSND1* and *PtrWND2B* induced expression of a cascade of other transcription factors and structural genes involved in lignin, cellulose, and hemicellulose formation (Zhong et al., 2006, 2011). A set of 26 *Populus* transcription factors homologous to *Arabidopsis* secondary cell wall associated transcription factors induced by *AtSND1* over-expression were studied here. The expression of all 26 transcription factors was examined in xylem cDNA libraries obtained from two OE lines and two RNAi lines. Over-expression of *PdWND1B* significantly increased expression of several MYBs. Among these, the most prominent were *NAC154*, *NAC156*, *MYB18*, *MYB75*, *MYB199*, *MYB167*, *MYB175*, *MYB28*, *MYB31* and *MYB189*, where the expression was increased by 3 to 9-fold (Figure 7a). However, the expression of two genes, *WIN2A* and *MYB165* was decreased by up to 65% in the same OE lines. In *PdWND1B* RNAi lines, expression of *WIN2A*, *MYB18*, *MYB152*, and *MYB175* were increased by 2- to 3-fold while that of *WIN2B*, *MYB2*, and *MYB161* were reduced by 60 to 80% compared with controls (Figure 7b).

In a previous study, over-expression of *PtrWND2B* induced expression of *PtrWND1A* and *B*, *PtrWND2A*, and *PtrWND6A* and *B* (Zhong et al., 2011). However, over-expression of *PdWND1B* induced only *PdWND6A* in our study. Also, *PtrWND2B* induced expression of all transcription factors except *PtrMYB152* (Wang et al., 2014). In

contrast, several transcription factors were not induced in our study, suggesting that *WND1B* and *WND2B* may have distinct targets with some overlap. Alternatively, in the previous study, gene expression was quantified in leaf tissue where secondary wall formation is uncommon, while our study employed developing xylem tissue where secondary cell wall biosynthesis-related genes are viewed to be more specifically regulated by those TFs. The increase in *PdWND2A* in the *PdWND1B* suppression lines indicates the existence of a compensatory mechanism. Although induction of *PdWND2A* or, more likely, other MYBs compensated for cell wall composition, they did not compensate and maintain normal growth in RNAi lines. In herbaceous plants such as *Arabidopsis*, *snd1* or *nst1* single mutants had no obvious growth defects, but *snd1 nst1* double mutants had severely affected stem strength suggesting that either one is sufficient for proper growth (Zhong et al., 2007a). In *Arabidopsis*, over-expression of *AtSND1* induced expression of *AtMYB46* (Zhong et al., 2007b), but over-expression of *PdWND1B* did not induce expression of *PdMYB002* and *PdMYB021*, the homologs of *AtMYB46*, implying the existence of potential species-specific regulation. Over-expression of *AtSND1* and *AtNST1* induced expression of *AtMYB58*. However, only *AtNST1* induced *AtMYB63* (Zhou et al., 2009). Our results were consistent with *Arabidopsis* in that over-expression of *PdWND1B* induced expression of *PdMYB28*, the closest homolog of *AtMYB58* but not *PdMYB192*, the closest homolog of *AtMYB63*, suggesting that WND/NAC master regulators have both redundant and distinct gene

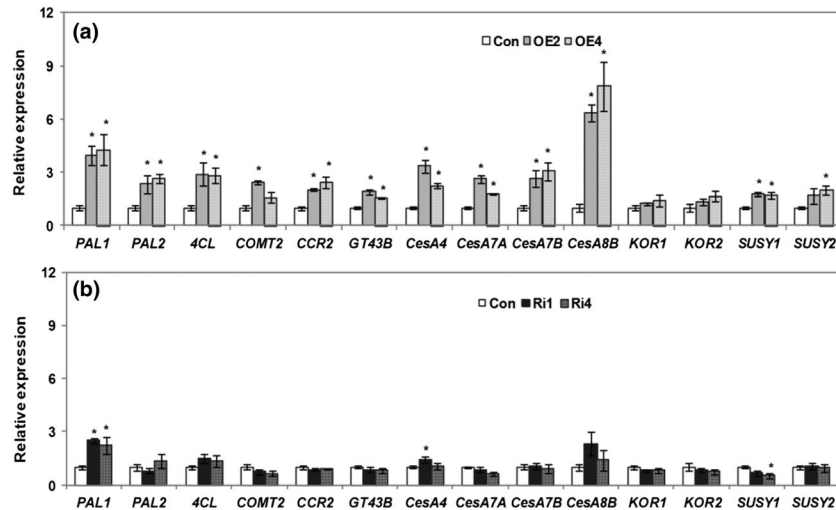


FIGURE 8 Expression of secondary cell wall related and sugar metabolism related genes in control and *PdWND1B* transgenic lines. Relative gene expression (arbitrary units) in control (con), over-expression (OE) lines (a), and RNAi suppressed (Ri) lines (b) was calculated by normalizing the expression of target genes to the housekeeping gene, *18S rRNA*, and calculating expression change relative to the control line. *PAL*, phenylalanine ammonia lyase; *4CL*, 4-coumarate: CoA ligase; *COMT*, caffeic acid/5-hydroxyconiferaldehyde *O*-methyltransferase; *CCR*, cinnamoyl-CoA reductase; *GT43B*, glucosyltransferase family 43; *Cesa*, cellulose synthase; *KOR*, KORRIGAN endoglucanase; *SUSY*, sucrose synthase. The data represent means \pm SE ($n = 3$). * indicates statistically significant, $p \leq .05$ based on Student's *t* tests.

targets, and exhibit species-specificity in downstream regulation. *AtMYB58* and *AtMYB63* induced lignin formation but not cellulose and hemicellulose formation, suggesting that individual MYBs are specific to each pathway (Zhou et al., 2009). Relative to *PdWND1B* RNAi, a greater impact of *PdWND1B* overexpression was observed on expression of cell wall transcription factor genes, secondary cell wall genes (Shi et al., 2010) and sugar metabolism related genes (Figure 8). A significant upregulation of all tested lignin and pathway genes, *PAL1*, *PAL2*, *4CL*, *COMT2*, and *CCR2*, was observed in overexpression lines relative to the control, which corresponded with the observed significant increase in lignin level measured in these overexpression lines (Figure 4b). Among the genes tested in cellulose biosynthesis pathway, *CESA* and *SUSY* genes were upregulated, however, concomitant increases in expression of other key components of cellulose pathway, *KOR1* and *KOR2*, may in part explain the resulting lack of increase in cellulose level in overexpression lines.

3.6 | Promoter binding and transcriptional activation

PdWND1B has been previously reported as a transcription activator and is found to bind to promoters of *MYB002* (Lin et al., 2013), as well as the newly reported cell wall transcriptional regulators, *HB3* (Badmi et al., 2018) and *EPSP* (Xie et al., 2018), in *Populus*. Transactivation assays confirmed that *PdWND1B* acts as a transcriptional activator and not as a transcriptional repressor (Supplemental File 5). In vivo DNA binding assay using micro-chromatin immunoprecipitation (μ ChIP) confirmed the binding of *PdWND1B* on the promoter of *PdMYB002*, a known target of *Ptr-SND1-B1* (Lin et al., 2013)

(Supplemental File 6), pointing to the overlapping functions of two poplar NAC homologs. Overexpression of *PdWND1B* induces the expression of a gene encoding 5-enolpyruvylshikimate 3-phosphate synthase (*EPSP*), an enzyme that has been demonstrated activity as a transcriptional repressor and is involved in lignin biosynthesis (Xie et al., 2018). ChIP and transactivation assays suggest that *PdWND1B* binds to the promoters of the two *Populus EPSP* homologs, *EPSP1* and *EPSP2*, and activates their transcription in vivo (Supplemental File 7). These results indicate that *PdWND1B* is the upstream regulator of *EPSP* in lignin biosynthesis. The HD-ZIP III family of transcription factors has known roles in stem development (Robischon et al., 2011; Zhu et al., 2013). *PdWND1B* binds to the two homologs of the HD-ZIP III family of transcription factors, *PtHB3* and *PtHB4* and activates their transcription in vivo (Supplemental File 8). It has also previously been reported that *PdWND1B* binds to the promoter of a calmodulin binding protein *PdIQD10*, which is also involved in secondary cell wall biosynthesis (Badmi et al., 2018). Our results provide molecular evidence to further substantiate the role of *PdWND1B* as a master regulator of secondary cell wall biosynthesis during woody stem development in *P. deltoides*.

4 | CONCLUSION

Secondary cell wall composition depends on expression of *WND1B* transcription factors. The functional role of *WND1B* in *Populus* was studied by over-expression and downregulation under the control of a xylem specific promoter. Over-expression of *PdWND1B* induced a cascade of transcription factors and structural genes involved in secondary cell wall biosynthesis. Over-expression of *PdWND1B*

resulted increased lignin and xylose content, but decreased glucose resulting in a significant reduction in ethanol conversion. Downregulation of *PdWND1B*, on the other hand, did not consistently alter lignin and cellulose content in stems but did impact other wall components and resulted in stunted growth. It is plausible that a functional compensation, as has been reported before, by other NAC members including *WND2A* and MYBs such as *MYB18*, *MYB152* and *MYB175*, in part explains the lack of significant impact on cell wall chemistry as a result of downregulation of *PdWND1B*. Taken in total, our results suggest that *PdWND1B* does play a functional role in secondary cell wall biosynthesis through coordination with transcription factors and structural genes, which is further supported by the molecular evidence of its function to activate the transcription of several secondary cell wall pathway genes reported in the literature. In the future, studies designed to dissect the redundant and non-redundant functions of *PdWND1B*, its other homologs, and downstream transcription factors in stem, as well as root tissues, are needed to shed important and timely light on the redundant, conserved, and divergent mechanisms of plant biomass chemistry and productivity. Such fundamental understanding is critical to developing biodesign-based approaches to co-optimize aboveground performance for bio-derived fuels and products and soil health belowground.

ACKNOWLEDGMENTS

We thank Brock Carter and Zackary Moore for inventory, propagation, and maintenance of plants in ORNL greenhouses. This manuscript has been authored by UT-Battelle, LLC under Contract No. DE-AC05-00OR22725 with the U.S. Department of Energy. The U.S. Government retains, and the publisher, by accepting the article for publication, acknowledges that the U.S. Government retains a non-exclusive, paid-up, irrevocable, world-wide license to publish or reproduce the published form of this manuscript, or allow others to do so, for U.S. Government purposes. The Department of Energy will provide public access to these results of federally sponsored research in accordance with the DOE Public Access Plan (<http://energy.gov/downloads/doe-public-access-plan>).

CONFLICT OF INTEREST

The Authors did not report any conflict of interest.

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How to cite this article: Payyavula, R. S., Badmi, R., Jawdy, S. S., Rodriguez, M. Jr, Gunter, L., Sykes, R. W., Winkeler, K. A., Collins, C. M., Rottmann, W. H., Chen, J.-G., Yang, X., Tuskan, G. A., & Kalluri, U. C. (2022). Biomass formation and sugar release efficiency of *Populus* modified by altered expression of a NAC transcription factor. *Plant Direct*, 6(8), e419. <https://doi.org/10.1002/pld3.419>