

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

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13

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26

27 **Abstract**

28 Woody biomass is an important feedstock for biofuel production. Manipulation of wood properties
29 that enable efficient conversion of biomass to biofuel reduces cost of biofuel production. Wood
30 cell wall composition is regulated at several levels that involve expression of transcription factors
31 such as wood-/secondary cell wall- associated NAC domains (WND or SND). In *Arabidopsis*
32 *thaliana*, *SND1* regulates cell wall composition through activation of its down-stream targets such
33 as MYBs. The functional aspects of *SND1* homologs in the woody *Populus* have been studied
34 through transgenic manipulation. In this study, we investigated the role of *PdWND1B*, *Populus*
35 *SND1* sequence ortholog, in wood formation using transgenic manipulation through over-
36 expression or silencing under the control of a vascular-specific 4-coumarate-*CoA* ligase (*4CL*)
37 promoter. As compared to control plants, *PdWND1B*-RNAi plants were shorter in height, with
38 significantly reduced stem diameter and dry biomass, whereas there were no significant differences
39 in growth and productivity of *PdWND1B* over-expression plants. Conversely, *PdWND1B* over-
40 expression lines showed a significant reduction in cellulose and increase in lignin content, whereas
41 there was no significant impact on lignin content of down-regulated lines. Stem carbohydrate
42 composition analysis revealed a decrease in glucose, mannose, arabinose, and galactose, but an
43 increase in xylose in the over-expression lines. Transcriptome analysis revealed upregulation of
44 several downstream transcription factors and secondary cell wall related structural genes in the
45 *PdWND1B* over-expression lines that corresponded to significant phenotypic changes in cell wall
46 chemistry observed in *PdWND1B* overexpression lines. Relative to the control, glucose release
47 and ethanol production from stem biomass was significantly reduced in over-expression lines but
48 appeared enhanced in the RNAi lines. Our results show that *PdWND1B* is an important factor
49 determining biomass productivity, cell wall chemistry and its conversion to biofuels in *Populus*.
50

51 **Introduction**

52 Woody biomass, harvested as feedstock for the pulp and paper, bioproduct and biofuel industries,
53 is formed by tightly regulated biological and molecular genetic xylogenesis mechanisms. Primary
54 xylem is formed from procambium while secondary xylem is formed from vascular cambium
55 during secondary growth. The major constituents of secondary cell walls are cellulose, lignin, and
56 hemicellulose (Darvill *et al.*, 1980). Cellulose is the most abundant polymer in plants and is a

57 polymer of glucose synthesized on the plasma membrane by the cellulose synthase (CesA)
58 complex (Doblin *et al.*, 2002). Lignin is the second most abundant polymer and is composed of
59 guaiacyl (G), syringyl (S), and p-hydroxyphenyl (H) units derived through the phenylpropanoid
60 pathway (Boerjan *et al.*, 2003). In addition to cell division and expansion that occurs in primary
61 cells, secondary xylem formation includes secondary wall deposition, lignification, and
62 programmed cell death (Plomion *et al.*, 2001). The formation of xylem cell walls is coordinately
63 regulated at multiple layers by dozens of structural genes and transcription factors.

64

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

80

81 The NAC domain transcription factor is one of the largest families, with ~ 100 genes in
82 *Arabidopsis* and soybean (*Glycine max*) and ~ 140 genes in rice (*Oryza sativa*) (Ooka *et al.*, 2003;
83 Pinheiro *et al.*, 2009). In *Populus*, there are 163 genes clustered in 18 subfamilies (Hu *et al.*, 2010).
84 Among these, a few candidate transcription factors have been functionally characterized in model
85 species such as *Arabidopsis* and rice. In *Arabidopsis*, at least three NAC domain members, *NST1*,
86 *NST2*, and *NST3/SND1*, have been shown to have functional roles in regulating secondary cell wall
87 biosynthesis (Mitsuda *et al.*, 2007; Mitsuda and Ohme-Takagi, 2008; Zhong *et al.*, 2006). T-DNA

88 knockout mutants of *AtSND1* showed no difference from wildtype suggesting that the other
89 isoforms might have compensated for the loss (Zhong *et al.*, 2006). In contrast, either over-
90 expression or dominant repression of *AtSND1* results in plants with weak stems and drastically
91 reduced interfascicular fiber and xylary fiber wall thickness (Zhong *et al.*, 2006). Over-expression
92 of *AtSND1* resulted in massive deposition of lignified secondary cell walls suggesting that normal
93 levels of *AtSND1* transcripts are necessary for maintaining proper cell wall thickening in secondary
94 stems (Zhong *et al.*, 2006). The defective secondary cell wall formation phenotype observed in
95 *Arabidopsis* *snd1inst2* double mutants was restored by complementation with WNDs from
96 *Populus*, suggesting that *Populus* WNDs regulate secondary wall biosynthesis (Mitsuda *et al.*,
97 2007; Zhong *et al.*, 2010; Zhong *et al.*, 2007a). The NAC transcription factors bind to SNBE
98 (secondary wall NAC binding elopements) in the promoters of its downstream targets and regulate
99 their expression. *PtWND2B* induces expression of several wood associated MYB transcription
100 factors and genes involved in secondary cell wall biosynthesis (Zhong *et al.*, 2011; Mc Carthy *et*
101 *al.* 2011). Over-expression of another NAC transcription factor gene, *Ptr-SND1-B1*, in *Populus*
102 stem-differentiating xylem (SDX) protoplasts was reported to induce 178 differentially expressed
103 genes (DEGs) of which 76 were identified to be its direct targets (Lin *et al.*, 2013). Furthermore,
104 two splice variants from NAC and VND transcription factor families are involved in reciprocal
105 cross-regulation during wood formation (Lin *et al.*, 2017). However, much less is known about the
106 role of these transcription factors in maintaining cell wall composition. Recently, over-expression
107 of a NAC family member, *PdWND3A*, was reported to affect lignin biosynthesis, decrease the rate
108 of sugar release and reduce biomass (Yang *et al.*, 2019). Given there is redundancy reported among
109 functional roles of some NAC transcription factor family members and the knowledge of upstream
110 master regulators of secondary wall biosynthesis, *AtSND1*, in *Arabidopsis*, here we sought to
111 characterize the role of sequence ortholog, *PdWND1B*, in *Populus deltoides* in the context of
112 biomass formation. To advance our knowledge on the role of additional NAC/WND transcription
113 members in secondary cell wall biosynthesis, we developed transgenic *Populus deltoides* plants
114 with xylem-specific over-expression or RNAi mediated silencing of *PdWND1B*,
115 Potri.001G448400; *WND1B* has previously been referred to as PNAC017, VNS11, SND1-A2
116 (Ohtani *et al.* 2011; Zhong *et al.* 2010; Li *et al.* 2012; Hu *et al.* 2010; Takata *et al.* 2019). RNAi
117 transgenic plants displayed weaker stems and altered cell wall composition as compared to control
118 plants. Over-expression lines showed increased lignin content and significantly reduced ethanol

119 production from stem biomass as compared to control plants. Our results confirm that *WND1B*
120 plays an important role in secondary cell wall biosynthesis.

121

122 **Methods**

123 **Phylogenetic analysis**

124 Protein sequences of *Populus* WND isoforms were retrieved from *Phytozome v9.1: Populus*
125 *trichocarpa v3.0* (Tuskan *et al.*, 2006) and those corresponding to other plant species were
126 obtained from NCBI. Phylogenetic analysis was performed in MEGA (Molecular Evolutionary
127 Genetics Analysis) using the Neighbor-Joining method (Tamura *et al.*, 2011). Bootstrap values
128 were calculated from 500 independent bootstrap runs. Protein sequence alignment was performed
129 using ClustalW and shading and percent similarity were predicted by GeneDoc (Nicholas *et al.*,
130 1997).

131

132 **GFP localization**

133 The full length coding regions of *PdWND1A* (Potri.011G153300) and *PdWND1B*
134 (Potri.001G448400) were amplified from a *P. deltoides* xylem cDNA library (primers listed in
135 Supplemental file 1) using Q5 High-Fidelity DNA polymerase (New England Biolabs, Ipswich,
136 MA) and cloned in a pENTR vector (Invitrogen, Carlsbad, CA, USA). After sequence
137 confirmation, the coding region fragment was recombined into a Gateway binary vector pGWB405
138 (Tsuyoshi *et al.*, 2009) using LR clonase (Invitrogen). Plasmid from a positive clone was
139 transformed to *Agrobacterium tumefaciens* strain GV3101. Tobacco infiltration and protein
140 localization were performed as described previously (DePaoli *et al.*, 2011; Sparkes *et al.*, 2006).
141 *Agrobacterium* harboring the binary constructs *PdWND1A* or *PdWND1B* were cultured overnight
142 in LB media. After brief centrifugation, supernatant was removed and the pellet was dissolved in
143 10 mM MgCl₂. The culture was infiltrated into four-week-old tobacco leaves. After 48 h, roughly
144 4 mm² leaf sections were cut and fixed in 3.7% formaldehyde, 50 mM NaH₂PO₄, and 0.2% Triton
145 X-100 for 30 min, rinsed with phosphate-buffered saline (PBS), and stained in DAPI (4,6'-
146 diaminino-2-phenylindole, 1.5 µg ml⁻¹ in PBS) for 30 min. GFP visualization and imaging was
147 performed on a Zeiss LSM710 confocal laser scanning microscope (Carl Zeiss Microscopy,
148 Thornwood, NY) equipped with a Plan-Apochromat 63x/1.40 oil immersion objective.

149

150 **Plant materials**

151 The over-expression construct was developed by amplifying and ligating the 1235 bp coding
152 region fragment of *PdWND1B* (gene model: Potri.001G448400, primers presented in
153 Supplemental file) under the control of a vasculature specific *4-coumarate CoA-ligase (4CL)*
154 promoter. The RNAi construct for the same gene was developed by amplifying a 300 bp coding
155 region fragment (from 800 to 1100 bp) and ligated in sense and antisense orientation to form a
156 hairpin with the chalcone synthase intron, under the control of *4CL* promoter. The binary
157 constructs were transformed into wild-type *P. deltoides* 'WV94' using an *Agrobacterium* method
158 (Caiping *et al.*, 2004). Transgenic plants and empty vector transformed control plants that were
159 roughly 10 cm tall were moved from tissue culture to small tubes with soil. After 2-months, plants
160 were moved to bigger pots (6 liter) and were propagated in a greenhouse maintained at 25°C with
161 a 16 h day length. At the time of harvest (six-month-old plants), plant height was measured from
162 shoot tip to stem base, and diameter was measured two inches from the base of the stem. The
163 bottom 10 cm stem portion was harvested, air-dried, and used for carbohydrate composition,
164 cellulose, lignin, S:G ratio, and sugar and ethanol release analysis. Initial studies were performed
165 on 10 transgenic lines for each construct and additional studies were performed on two to four
166 selected lines. Data presented here is from two representative lines. Young leaf (leaf plastochron
167 index, LPI-0 and 1), mature leaf (LPI-6⁺), and stem (internode portion between LPI 6 and 8) were
168 collected, frozen in liquid nitrogen, and stored at -80°C until they were processed further.

169

170 **RNA extraction and gene expression studies**

171 RNA from frozen ground stem samples was extracted using a Plant Total RNA extraction kit
172 (Sigma, St Louis, MO) with modifications to the kit protocol. Briefly, 100 mg of frozen ground
173 tissue was incubated at 65°C in 850 μ l of a 2% CTAB + 1% β me buffer for 5 min, followed by the
174 addition of 600 μ l of chloroform:isoamylalcohol (24:1 v/v). The mixture was spun at full speed in
175 a centrifuge for 8 min after which the supernatant in the top layer was carefully removed and
176 passed through a filtration column included in the kit. The filtered elutant was diluted with 500 μ l
177 of 100% EtOH and passed through a binding column. This was repeated until all of the filtered
178 elutant/EtOH mixture was passed through the binding column. Further steps including on-column
179 DNase digestion (DNase70, Sigma), filter washes, and total RNA elution were followed as per the
180 manufacturer's protocol. cDNA was synthesized from 1.5 μ g total RNA using oilgodT primers

181 and RevertAid Reverse Transcriptase (Thermofisher). Quantitative reverse transcriptase PCR
182 (qRT-PCR) was performed in a 384-well plate using cDNA (3 ng), gene specific primers (250 nM,
183 list provided in Supplemental file), and iTaq Universal SYBR Green Supermix (1X, Bio Rad).
184 Relative gene expression was calculated using the delta CT or delta-delta CT method (Livak and
185 Schmittgen, 2001). Template normalization was done using two housekeeping genes, 18S
186 ribosomal RNA and Ubiquitin-conjugating enzyme E2. Gene accession numbers and primer
187 sequence information are presented in Supplemental file 1.

188

189 **Micro Chromatin Immunoprecipitation (μChIP) assay from protoplasts:**

190 Transcription factor PdWND1B was cloned in-frame with 10X Myc tag and used to transfect
191 protoplasts derived from *Populus* 717-1B4 tissue culture grown plants (Guo et al, 2012). ChIP
192 assays were performed using the modified protocol from Dahl and Collas (2008) and Adli and
193 Bernstein (2011). Briefly, transfected protoplasts were resuspended in W5 solution (154mM NaCl,
194 125 mM CaCl₂, 5 mM KCl, 2 mM MES (pH 5.7)), crosslinked by adding 1% (v/v) formaldehyde
195 and gently rotating the tubes for 8 min. To stop crosslinking, Glycine was added to a final
196 concentration of 0.125 M and gently rotated at room temperature for 5 min. The crosslinked
197 protoplasts were washed once with W5 solution and lysed by mixing with SDS Lysis Buffer (50
198 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM EDTA (pH 8.0), 1% SDS, 1 mM PMSF, protease
199 inhibitor) followed by incubation on ice for 10 min with intermittent and brief vortexing. The
200 lysate was supplemented with RIPA ChIP Buffer (10mM Tris-HCl (pH 7.5), 140 mM NaCl, 1 mM
201 EDTA (pH 8.0), 1% Triton X-100, 0.1% Na-deoxycholate, 1 mM PMSF, protease inhibitor) and
202 sonicated for 150 s with 0.7 s ‘On’ and 1.3 s ‘Off’ pulses at 20% power amplitude on ice using
203 Branson 450 Digital sonifier to generate 150- to 600-bp chromatin fragments. Additional ice-cold
204 RIPA ChIP buffer was added to aliquot the sample into three separate tubes – 500 µl Antibody
205 (Ab) sample, 500 µl No-Antibody (NAb) sample and 75µl input chromatin. To the Ab sample,
206 0.75-1 µg anti-c-Myc antibody (Sigma-Aldrich #C3956) was added and gently rotated overnight
207 at 4°C. Protein A Mag Sepharose (Sigma-Aldrich #28-9440-06) beads were washed with RIPA
208 buffer (10 mM Tris-HCl (pH 7.5), 140 mM NaCl, 1 mM EDTA (pH 8.0), 1% Triton X-100, 0.1%
209 SDS, 0.1% Na-deoxycholate), added to Ab and NAb samples and gently rotated at 4°C for 120
210 min. The beads were then collected, washed twice with low-salt wash buffer (150 mM NaCl, 0.1%
211 SDS, 20 mM Tris-HCl (pH 8.0), 2 mM EDTA (pH 8.0), 1% Triton X-100), twice with LiCl buffer

212 (0.25 M LiCl, 1% Na-deoxycholate, 10 mM Tris-HCl (pH 8.0), 1% NP-40, 1 mM EDTA (pH 8.0))
213 and twice with TE Buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)). The beads were
214 subjected to reverse crosslinking by adding Complete Elution Buffer (20 mM Tris-HCl (pH 8.0),
215 5 mM EDTA (pH 8.0), 50 mM NaCl, 1% SDS, 50 µg/ml Proteinase K) and incubating for 120
216 min on thermomixer at 68°C and 1300 rpm to elute protein-DNA complexes. Input samples were
217 added with elution buffer (20 mM Tris-HCl (pH 8.0), 5 mM EDTA (pH 8.0), 50 mM NaCl) and
218 50 µg/ml Proteinase K before placing on thermomixer. After incubation, supernatants were
219 collected and the ChIP DNA was purified using MinElute PCR Purification Kit (Qiagen #28004).
220 Real-time PCR was performed for the ChIPed DNA by promoter specific primers (Supplemental
221 File 1) and the obtained Ct values were used to calculate the signal intensity by Percent Input
222 Method. At least three biological replicates (with two technical replicates each) representing
223 independent protoplast transfactions were used. The ChIPed DNA was also used for PCR reactions
224 by promoter specific primers to analyze the products on agarose gel.

225

226 **Transcriptional activator assay**

227 The coding sequence (CDS) of *WND1B* was in-frame cloned in a Gal4 binding domain (GD)
228 effector vector (Wang et al, 2007). For the trans-activator assays, the GD-fusion constructs were
229 co-transfected with Gal4:GUS reporter construct into *Populus* 717-1B4 protoplasts (Guo et al,
230 2012). For the trans-repressor assays, GD-fusion constructs were co-transfected with LexA
231 binding-domain fused VP16 (LD-VP) and LexA:Gal4:GUS reporter (Wang et al, 2007). An empty
232 GD effector vector was co-transfected with reporter vectors for the control experiments. The
233 transfected protoplasts were incubated in dark for 16-20 h and GUS activity was quantitatively
234 measured. All the protoplast transfactions were included with equal amounts of 35S:Luciferase
235 reporter and Luciferase activity was used for normalization of GUS activity.

236

237 **Cellulose and lignin estimation**

238 Cellulose was estimated on debarked, ground, and air-dried stem tissue using the anthrone method
239 (Updegraff, 1969). Stem sample (25 mg) was first digested with 500 µl of acetic - nitric acid
240 reagent (100 ml of 80% acetic acid mixed with 10 ml of nitric acid) at 98°C for 30 min. After
241 cooling, the sample was centrifuged, the supernatant was discarded, and the remainder was washed
242 with water. After brief centrifugation, water was discarded, and the pellet was digested with 67%

243 (v/v) sulfuric acid for 1 h at room temperature. An aliquot of the mix was diluted (1:10) with water.
244 In a PCR tube, 10 μ l of diluted reaction mix, 40 μ l of water, and 100 μ l of freshly prepared anthrone
245 reagent (0.5 mg anthrone ml^{-1} of cold concentrated sulfuric acid) was added and heated for 10 min
246 at 96°C. Samples were cooled and absorbance (A_{630}) was measured. Cellulose was then estimated
247 based on the absorbance of glucose standards. Lignin and its monomer composition was analyzed
248 using pyrolysis molecular beam mass spectrometry at the National Renewable Energy Laboratory
249 as described previously (Mielenz *et al.*, 2009).

250

251 **Stem carbohydrate composition analysis**

252 Roughly 25 mg of air-dried stem sample was weighed in a 2-ml tube and extracted twice at 85°C
253 with a total of 2 ml of 80% ethanol. The supernatant was collected in a new 2-ml tube and was re-
254 extracted with 50 mg activated charcoal (Sigma) to eliminate pigments that interfere with sugar
255 analysis. A 1 ml aliquot of the pigment free extract was incubated overnight in a heating block
256 maintained at 50°C and the resulting pellet was dissolved in 120 μ l of water. A 10 μ l aliquot was
257 used for estimation of sucrose and glucose using assay kits (Sigma). Starch from the pellet was
258 digested using 1U of α -amylase (from *Aspergillus oryzae*, Sigma) and amyloglucosidase (from
259 *Aspergillus niger*, Sigma). After starch removal, the pellet was dried overnight at 95°C and used
260 for estimating structural sugars. Roughly, 5 mg of sample was weighed in a 2-ml tube and digested
261 with 50 μ l of 75% v/v H_2SO_4 for 60 min. The reaction was diluted by adding 1.4ml of water, tubes
262 were sealed using lid-locks, and autoclaved for 60 min in a liquid cycle. After cooling, the sample
263 was neutralized with CaCO_3 and sugar composition was estimated using high performance liquid
264 chromatography (HPLC, LaChrom Elite® system, Hitachi High Technologies America, Inc.) as
265 described previously (Fu *et al.*, 2011; Yee *et al.*, 2012).

266

267 **Glucose release and ethanol conversion**

268 Separate hydrolysis and fermentation (SHF) was used to evaluate digestibility of biomass samples
269 as described previously (Fu *et al.*, 2011; Yee *et al.*, 2012). Extract free biomass was autoclaved
270 for sterilization purposes and the hydrolysis and fermentations were performed in biological
271 triplicate at 5.0% (w/v) biomass loading in a total volume of 20 ml at a pH of 4.8 with a final
272 concentration of 50 mM citrate buffer and 0.063 mg ml^{-1} streptomycin. The hydrolysis was
273 performed using commercial hydrolytic enzyme blends (Novozymes, Wilmington, DE, USA).

274 Cellic®-Ctec2 was loaded at 20 mg protein gram⁻¹ dry biomass, and Novozyme 188 and Cellic®
275 Htec2 were loaded at 25% and 20% (v/v) of Ctec2, respectively. The biomass and enzymes were
276 incubated at 50°C and 120rpm for 5 days. The hydrolysate was then fermented with
277 *Saccharomyces cerevisiae* D5A (ATCC 200062) at 35°C and 150 rpm with a final concentration
278 of 0.5% (w/v) yeast extract. Hydrolysate and fermentation broth samples were analyzed for
279 glucose and ethanol using HPLC equipped with a refractive index detector (model L-2490). The
280 products were separated on an Aminex® HPX-87H column (Bio-Rad Laboratories, Inc.) at a flow
281 rate of 0.5 ml min⁻¹ of 5.0 mM sulfuric acid and a column temperature of 60°C and were quantified
282 as described previously (Fu *et al.*, 2011; Yee *et al.*, 2012).

283

284 **Results and Discussion**

285 **Phylogenetic analysis, gene expression, and localization**

286 In *Arabidopsis*, at least three NAC transcription factors, *SND1*, *NST1*, and *VND7* have a proposed
287 role in regulation of secondary cell wall formation. To retrieve their sequence orthologs from
288 *Populus trichocarpa*, protein sequences of the three genes were blasted in *Phytozome* (v 9.1) and
289 the two best hits were retrieved for each sequence resulting in a total of six sequences. These were
290 designated as *PtrWND1A* (Potri.011G153300), *PtrWND1B* (Potri.001G448400), *PtrWND2A*
291 (Potri.014G104800), *WND2B* (Potri.002G178700), *PtrWND6A* (Potri.013G113100) and
292 *PtrWND6B* (Potri.019G083600). The nomenclature used in this study was based on Zhong *et al.*,
293 (2010). All six genes have alternate names; *WND1B* has also been named *SND1A2* or *VNS11* in
294 previously reports (Li *et al.*, 2012; Ohtani *et al.*, 2011). In the phylogenetic tree developed using
295 protein sequences, *PtrWND1A* and *PtrWND1B* were clustered together and share 94% similarity
296 at the protein level (Figure 1; Supplemental file 2), suggesting they originated from a recent
297 genome duplication (Tuskan *et al.*, 2006). They share only approximately 50% similarity with
298 *AtSND1* and *AtNST1*, approximately 56% with *PtrWND2A* and *PtrWND2B*, and approximately
299 41% with *PtrWND6A* and *PtrWND6B*, but more than 83% with *RcNAC* (*Ricinus communis*) and
300 *JcNAC013* (*Jatropha curcas*). *PtrWND2A* and *PtrWND2B* are clustered together and share 88%
301 similarity, while *PtrWND6A* and *PtrWND6B* share 92% similarity. Protein sequence alignment
302 revealed they are highly conserved in the NAC domain located in the N terminal region.
303 Conversely, they are highly diverse in the C terminal region, which has putative activation domains
304 (Olsen *et al.*, 2005; Xie *et al.*, 2000). At least 163 NAC domain transcription factors have been

305 reported in *Populus*. Based on phylogenetic analysis, these are classified into 18 groups (Hu *et al.*,
306 2010). *PtrWND1A* and *B* and *PtrWND2A* and *B* are closely clustered in the NAC-B subgroup,
307 while *PtrWND6A* and *B* are clustered in the NAC-O subgroup.

308

309 The expression of the above six NAC transcription factors was studied in eight different tissues of
310 *Populus deltoides* including YL (young leaf), ML (mature leaf), YS (young stem), MS (mature
311 stem), PH (phloem), XY (xylem), RT (root), and PT (petiole). In *Populus*, *WND1B* undergoes
312 alternate splicing, resulting in two variants designated as the small and large variants. The large
313 variant retains intron 2 (Li *et al.*, 2012; Zhao *et al.*, 2014). In this study, to account for both splice
314 variants, primers were designed in the region common to both variants. In general expression of
315 all the genes was much higher in xylem than in other tissues (Supplemental file 3). Within xylem,
316 expression of *PdWND1A* and *PdWND1B* was much higher relative to the other genes. Among
317 other tissues, expression of *PdWND1A* was much higher relative to the other genes except in
318 phloem, where *PdWIN2B* was strongly expressed. Expression of *PdWIN6A* and *PdWIN6B* was
319 weaker in all tissues relative to the other genes. In *Populus*, these transcription factors are most
320 abundantly expressed in stems. *In situ* localization studies suggested both *PtrWND1B* and
321 *PtWND6A* are expressed in xylem vessels and fibers and in phloem fibers after secondary growth,
322 whereas in primary xylem vessels only *PtrWND6A* expression was observed, suggesting
323 developmental regulation (Ohtani *et al.*, 2011). The two abundantly expressed genes, *PdWND1A*
324 and *PdWND1B*, were selected for localization studies using tobacco infiltration. GFP:*PdWND1A*
325 and GFP:*WND1B* were colocalized with DAPI stain confirming both *PdWND1A* and *PdWND1B*
326 are targeted to the nucleus (Supplemental file 4). *AtSND1* and *PtWND1B* have previously been
327 localized to the nucleus supporting their function as transcription factors (Li *et al.*, 2012; Zhong *et*
328 *al.*, 2006).

329

330 **Plant morphology and growth**

331 In the present study, we focused on studying the functional aspects of *PdWND1B* through over-
332 expression and RNAi-mediated suppression using a xylem specific *4CL* promoter. In order to
333 selectively down-regulate *PdWND1B*, sequence in the 3` region that has distinct differences with
334 *PdWND1A* was selected for RNAi construct development. In our preliminary study, six
335 independent over-expression (OE) and six independent RNAi lines were propagated in the

336 greenhouse. Plant height of over-expression lines was not different as compared to controls, but
337 RNAi lines were shorter (Supplemental file 5A). Lignin content was significantly higher in all
338 over-expression lines but showed a slight decreasing trend in RNAi lines (Supplemental file 5B).
339 In-depth characterization was performed on two to four selected lines and data presented in this
340 study is representative of two over-expression lines (designated as OE2 to OE4) and two RNAi
341 suppression lines (Ri1 and Ri4). The extent of alteration in *PdWND1B* expression in transgenic
342 lines was measured using qRT-PCR. As compared to control lines, *PdWND1B* expression was
343 increased by 40-fold in OE4 and by 23-fold in OE2 (Figure 2). In RNAi lines, *PdWND1B*
344 expression was reduced by 73% in Ri4 and by 65% in Ri1.

345

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

354

355 Evidence suggests that *SND/WND* are required for normal plant development (Zhao *et al.*, 2014;
356 Zhong *et al.*, 2010). Over-expression of the full-length coding region of *AtSND1* in *Arabidopsis*
357 and *PtWIN2B* or *PtWIN6B* in *Populus tremula x alba*, under the control of a CaMV 35S promoter,
358 resulted in plants with weaker stems, small leaves, and stunted growth. This strongly supports the
359 hypothesis that the WNDs play a significant role in maintenance of growth and development
360 (Zhong *et al.*, 2006; Zhong *et al.*, 2011). In contrast, over-expression of the *PtWND1B* whole gene
361 (including exons and introns) in *Populus x euramericana*, under the control of a CaMV 35S
362 promoter, did not affect plant growth, but only reduced leaf size (Zhao *et al.*, 2014). Our study
363 included overexpression of the shorter variant of *PdWND1B* under the control of a xylem-specific
364 promoter and the observation of no apparent growth impact in overexpression lines. Previous study
365 reported that over-expression of the *PtWND1B* longer splice variant in *Populus x euramericana*,
366 under the control of its own promoter, affected plant development, but the same effect was not

367 observed when the small variant of *PtWND1B* was over-expressed (Zhao *et al.*, 2014). Zhong *et*
368 *al.*, (2006) also report that an *Atsnd1* mutation did not affect plant development. However,
369 consistent with our study in *Populus*, down-regulation of *PtWND1B*, controlled by its own
370 promoter, resulted in plants with weak stems that did not grow straight (Zhao *et al.*, 2014).
371 Therefore, it appeared that *WND* genes may have species-specific effect on plant growth and
372 development. It is also possible that the differences in promoters used (i.e., native promoter (Zhao
373 *et al.*, 2014) and tissue-specific promoter (in the present study) may contribute to differences in
374 phenotypic observations between *Arabidopsis* and *Populus*.

375

376 **Structural polymers**

377 *WND* transcription factors have a proposed function in secondary cell wall biosynthesis.
378 Therefore, the effect of altered *PdWND1B* expression on secondary cell wall composition were
379 studied in stems. Stem secondary cell walls are composed predominantly of cellulose, lignin, and
380 hemicellulose (Bailey, 1938; Darvill *et al.*, 1980). Cellulose, estimated by the anthrone method,
381 was significantly reduced by 9 to 13% in OE lines, but was increased by 6% in RNAi lines (Figure
382 4A). Lignin content was significantly increased in OE lines but decreased in RNAi4 (Figure 4B).
383 To understand changes in other sugars, stem cell walls were digested and sugars were quantified
384 using HPLC. Glucose and xylose were the predominant sugars in control plant stem material, at
385 45% and 15%, respectively (Figure 5). However, while glucose was reduced in OE lines, xylose,
386 representing the hemicellulose fraction, was significantly increased (Figure 5). Levels of minor
387 sugars including galactose, arabinose, and mannose were also significantly reduced in OE lines.
388 Trace compounds, 5-(Hydroxymethyl) furfural was reduced (up to four fold) in RNAi lines, while
389 2-furfural was significantly reduced by 60 to 75% in RNAi lines.

390

391 Over-expression of *AtSND1* induced ectopic deposition of lignified secondary cell walls in leaf
392 and stem epidermal and mesophyll cells that normally do not undergo lignification (Zhong *et al.*,
393 2006). In addition, cellulose and hemicellulose were also deposited. A similar response was
394 observed in *Populus*, where *PtWND2B* and *PtWND6B* were over-expressed under the control of a
395 CaMV 35S promoter (Zhong *et al.*, 2011). To address the biomass chemistry context of the present
396 study, over-expression of *PdWND1B* in our study was driven by a xylem-specific promoter to
397 avoid confounding growth effects arising from ectopic lignification. In the context of stem cell

398 wall phenotype, our results indicate an increase in lignin and xylose in stems of OE lines while
399 cellulose levels were reduced. A negative relationship has been proposed between levels of
400 cellulose and lignin (Hu *et al.*, 1999). We observed an increase in lignin and a concomitant
401 decrease in cellulose of overexpression lines relative to the control. In *Arabidopsis*, silencing of
402 *AtSND1* and *AtNST1* simultaneously reduced lignin, cellulose, and hemicellulose (Zhong *et al.*,
403 2007a). In the present study, significant differences were not observed in levels of lignin or other
404 sugars in RNAi lines, suggesting that the reduction in expression and function of *PdWND1B*
405 potentially is partly compensated by other members of the NAC family (i.e., PdWND1A)
406 members. In future studies, it would be interesting to generate and characterize double
407 knockout/knockdown plants of *PdWND1A* and *PdWND1B*, and similarly, for other closely related
408 paralogs, which can address the potential functional redundancy and reveal their more precise
409 functions in secondary cell wall biosynthesis.

410

411 **Sugar release and ethanol conversion**

412 The effect of altered cell wall composition on sugar release and ethanol conversion was studied in
413 OE and RNAi lines. Glucose release was significantly reduced by 65 to 70% in OE plants
414 compared to that of control plants (Figure 6A). This is consistent with a significant reduction in
415 ethanol production from biomass. In contrast, glucose release was increased by 15% and 20% in
416 RNAi1 and RNAi4 lines, respectively; however, ethanol production was increased (30%) only in
417 RNAi4, the lines with greater downregulation (Figure 6B).

418

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

424

425 **Gene expression changes**

426 In *Arabidopsis* and *Populus*, over-expression of *AtSND1* and *PtrWND2B* induced expression of a
427 cascade of other transcription factors and structural genes involved in lignin, cellulose, and
428 hemicellulose formation (Zhong *et al.*, 2006; Zhong *et al.*, 2011). A set of 26 *Populus* transcription

429 factors homologous to *Arabidopsis* secondary cell wall associated transcription factors induced by
430 *AtSND1* over-expression were studied here. The expression of all 26 transcription factors was
431 examined in xylem cDNA libraries obtained from two OE lines and two RNAi lines. Over-
432 expression of *PdWND1B* significantly increased expression of several MYBs. Among these, the
433 most prominent were *NAC154*, *NAC156*, *MYB18*, *MYB75*, *MYB199*, *MYB167*, *MYB175*, *MYB28*,
434 *MYB31* and *MYB189*, where the expression was increased by 3 to 9-fold (Figure 7A). However,
435 the expression of two genes, *WIN2A* and *MYB165* was decreased by up to 65% in the same OE
436 lines. In *PdWND1B* RNAi lines, expression of *WIN2A*, *MYB18*, *MYB152*, and *MYB175* were
437 increased by 2- to 3-fold while that of *WIN2B*, *MYB2*, and *MYB161* were reduced by 60 to 80%
438 compared to controls (Figure 7B).

439
440 In a previous study, over-expression of *PtrWND2B* induced expression of *PtrWND1A* and *B*,
441 *PtrWND2A*, and *PtrWND6A* and *B* (Zhong *et al.*, 2011). However, over-expression of *PdWND1B*
442 induced only *PdWND6A* in our study. Also, *PtrWND2B* induced expression of all transcription
443 factors except *PtrMYB152* (Wang *et al.*, 2014). In contrast, several transcription factors were not
444 induced in our study, suggesting that *WND1B* and *WND2B* may have distinct targets with some
445 overlap. Alternatively, in the previous study, gene expression was quantified in leaf tissue where
446 secondary wall formation is uncommon, while our study employed developing xylem tissue where
447 secondary cell wall biosynthesis-related genes are viewed to be more specifically regulated by
448 those TFs. The increase in *PdWND2A* in the *PdWND1B* suppression lines indicates the existence
449 of a compensatory mechanism. Although induction of *PdWND2A* or, more likely, other MYBs
450 compensated for cell wall composition, they did not compensate and maintain normal growth in
451 RNAi lines. In herbaceous plants such as *Arabidopsis*, *snd1* or *nst1* single mutants had no obvious
452 growth defects, but *snd1 nst1* double mutants had severely affected stem strength suggesting that
453 either one is sufficient for proper growth (Zhong *et al.*, 2007a). In *Arabidopsis*, over-expression
454 of *AtSND1* induced expression of *AtMYB46* (Zhong *et al.*, 2007b), but over-expression of
455 *PdWND1B* did not induce expression of *PdMYB002* and *PdMYB021*, the homologs of *AtMYB46*,
456 implying the existence of potential species-specific regulation. Over-expression of *AtSND1* and
457 *AtNST1* induced expression of *AtMYB58*. However, only *AtNST1* induced *AtMYB63* (Zhou *et al.*,
458 2009). Our results were consistent with *Arabidopsis* in that over-expression of *PdWND1B* induced
459 expression of *PdMYB28*, the closest homolog of *AtMYB58* but not *PdMYB192*, the closest

460 homolog of *AtMYB63*, suggesting that WND/NAC master regulators have both redundant and
461 distinct gene targets, and exhibit species-specificity in downstream regulation. *AtMYB58* and
462 *AtMYB63* induced lignin formation but not cellulose and hemicellulose formation, suggesting that
463 individual MYBs are specific to each pathway (Zhou *et al.*, 2009). Relative to *PdWND1B* RNAi
464 lines, the observed greater impact of *PdWND1B* overexpression on expression of cell wall
465 transcription factor genes was also observed on expression of secondary cell wall (Shi *et al.* 2021)
466 and sugar metabolism related genes (Figure 8).

467

468 **Promoter binding and transcriptional activation**

469 *PdWND1B* has been previously reported as a transcription activator and is found to bind to
470 promoters of *MYB002* (Lin *et al.* 2013), as well as the newly reported cell wall transcriptional
471 regulators, *HB3* (Badmi *et al.* 2018) and *EPSP* (Xie *et al.* 2018), in *Populus*. Transactivation assays
472 confirmed that *PdWND1B* acts as a transcriptional activator and not as a transcriptional repressor
473 (Supplemental File 5). *In vivo* DNA binding assay using micro-chromatin immunoprecipitation
474 (μ ChIP) confirmed the binding of *PdWND1B* on the promoter of *PdMYB002*, a known target of
475 *Ptr-SND1-B1* (Lin *et al.*, 2013) (Supplemental File 6), pointing to the overlapping functions of
476 two poplar NAC homologs. Overexpression of *PdWND1B* induces the expression of a gene
477 encoding 5-Enolpyruvylshikimate 3-Phosphate Synthase (EPSP), an enzyme that has been
478 demonstrated activity as a transcriptional repressor and is involved in lignin biosynthesis (Xie *et*
479 *al.*, 2018). ChIP and transactivation assays suggest that *PdWND1B* binds to the promoters of the
480 two *Populus EPSP* homologs, *EPSP1* and *EPSP2*, and activates their transcription *in vivo*
481 (Supplemental File 7). These results indicate that *PdWND1B* is the upstream regulator of EPSP in
482 lignin biosynthesis. The HD-ZIP III family of transcription factors have known roles in stem
483 development (Robischon *et al.*, 2011; Zhu *et al.*, 2013). *PdWND1B* binds to the two homologs of
484 the HD-ZIP III family of transcription factors, *PtHB3* and *PtHB4* and activates their transcription
485 *in vivo* (Supplemental File 8). It has also previously been reported that *PdWND1B* binds to the
486 promoter of a calmodulin binding protein *PdIQD10*, which is also involved in secondary cell wall
487 biosynthesis (Badmi *et al.*, 2018). Our results provide molecular evidence to further substantiate
488 the role of *PdWND1B* as a master regulator of secondary cell wall biosynthesis during woody stem
489 development in *P. deltoides*.

490

491 **Conclusion**

492 Secondary cell wall composition depends on expression of *WND* transcription factors. The
493 functional role of *WND1B* in *Populus* was studied by over-expression and down-regulation under
494 the control of a xylem specific promoter. Over-expression of *PdWND1B* induced a cascade of
495 transcription factors and structural genes involved in secondary cell wall biosynthesis. Phenotypic
496 changes were aligned with molecular changes, specifically, over-expression of *PdWND1B* resulted
497 in increased lignin and xylose content, but decreased glucose resulting in a significant reduction in
498 ethanol conversion. Down-regulation of *PdWND1B*, on the other hand, did not consistently alter
499 lignin and cellulose content in stems but did impact other wall components and resulted in stunted
500 growth. It is plausible that a functional compensation, as has been reported before, by other NAC
501 members including *WND2A* and MYBs such as *MYB18*, *MYB152* and *MYB175*, in part explains
502 the lack of significant impact on cell wall chemistry as a result of down-regulation of *PdWND1B*.
503 Taken in total, our results suggest that *PdWND1B* does play a functional role in secondary cell
504 wall biosynthesis through coordination with transcription factors and structural genes, which is
505 further supported by the molecular evidence of its function to activate the transcription of several
506 secondary cell wall pathway genes reported in the literature. In the future, studies designed to
507 dissect the redundant and non-redundant functions of *PdWND1B*, its other homologs, and
508 downstream transcription factors in stem, as well as root tissues, are needed to shed important and
509 timely light on the redundant, conserved, and divergent mechanisms of plant biomass chemistry
510 and productivity. Such fundamental understanding is critical to developing biodesign-based
511 approaches to co-optimize aboveground performance for bio-derived fuels and products and soil
512 health belowground.

513

514 **Figures and Tables**

515 Figure 1. Phylogenetic analysis of selected secondary cell wall associated NAC transcription
516 factors from *Populus* and other plant species

517 Figure 2. Relative expression of *PdWND1B* in control and transgenic lines

518

519 Figure 3. Growth and biomass productivity in control and *PdWND1B* transgenic lines

520

521 Figure 4. Stem cell wall composition of control and *PdWND1B* transgenic lines
522
523 Figure 5. Sugar composition in stem cell walls of control and *PdWND1B* transgenic lines
524
525 Figure 6. Glucose release and ethanol conversion efficiency from stems of control and *PdWND1B*
526 transgenic lines
527
528 Figure 7. Expression of secondary cell wall related transcription factors in control and *PdWND1B*
529 transgenic lines
530
531 Figure 8. Expression of secondary cell wall related and sugar metabolism related genes in control
532 and *PdWND1B* transgenic lines
533
534 **Supplemental Files**
535 Supplemental File 1. List of gene models and their primer sequence information.
536
537 Supplemental File 2. Percentage protein similarity matrix of selected secondary wall associated
538 transcription factors from *Populus* and other species.
539 Supplemental File 3. Expression of the six *NAC* genes in different tissues of *Populus*.
540 Supplemental File 4. Localization of the *PdWND1A* and *PdWND1B* in tobacco epidermal cells.
541 Supplemental File 5. Plant height (A) and lignin content (B) in control (Con) and *PdWND1B* over-
542 expression (OE) and RNAi suppression (Ri) lines.
543
544 Supplemental File 6. *PdWND1B* has transcriptional activator activity.
545 Supplemental File 7. *PdWND1B* binds to promoter of *MYB002* secondary cell wall transcription
546 factor gene.
547 Supplemental File 8. *PdWND1B* regulates the expression of *EPSP* genes *in vivo*.
548 Supplemental File 9. *PdWND1B* regulates the expression of *HB3-like* genes *in vivo*.

549

550 **Abbreviations:**

551 4CL, 4-coumarate-*CoA* ligase; DAPI, 4,6'-diamidino-2-phenylindole; CTAB,
552 cetyltrimethylammonium bromide; HPLC, high performance liquid chromatography; LPI, leaf
553 plastochron index; MEGA, Molecular Evolutionary Genetics Analysis; NAC, NAM, ATAF1/2
554 and CUC2; NST, NAC secondary wall thickening promoting factor; PBS, phosphate-buffered
555 saline; qRT-PCR, quantitative reverse transcriptase; *PAL*, phenylalanine ammonia lyase; PCR;
556 RNAi, RNA interference; S:G, syringyl to guaiacyl ratio; SHF, separate hydrolysis and
557 fermentation; SHN, SHINE; SND, secondary wall associated NAC domains; VND, vascular
558 related NAC domain; WND, wood associated NAC domain transcription factors. *4CL*, 4-
559 coumarate:CoA ligase; *COMT*, caffeic acid/5-hydroxyconiferaldehyde O-methyltransferase; *CCR*,
560 cinnamoyl-CoA reductase; *CesA*, Cellulose synthase; *KOR*, Korriagan; *GT43*, Glucosyltransferase
561 family 43; *SUSY*, sucrose synthase.

562

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580

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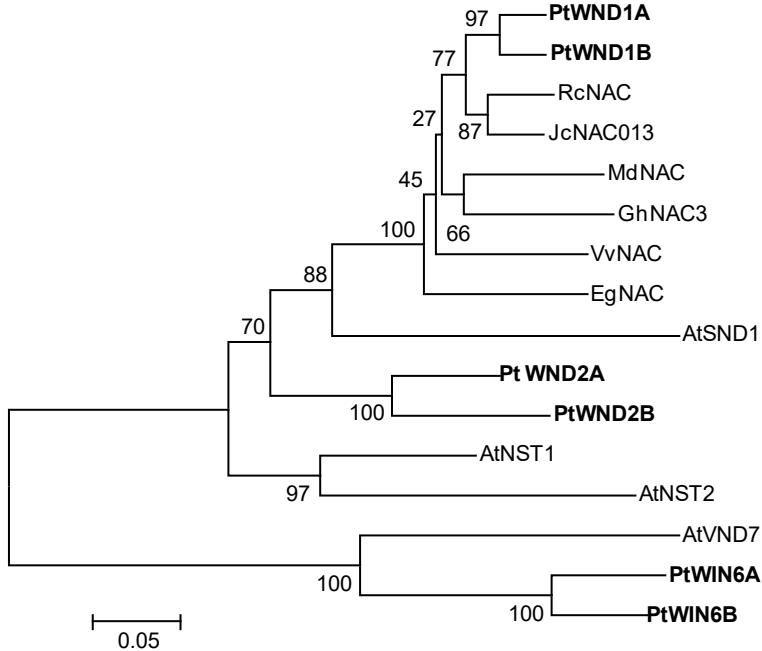
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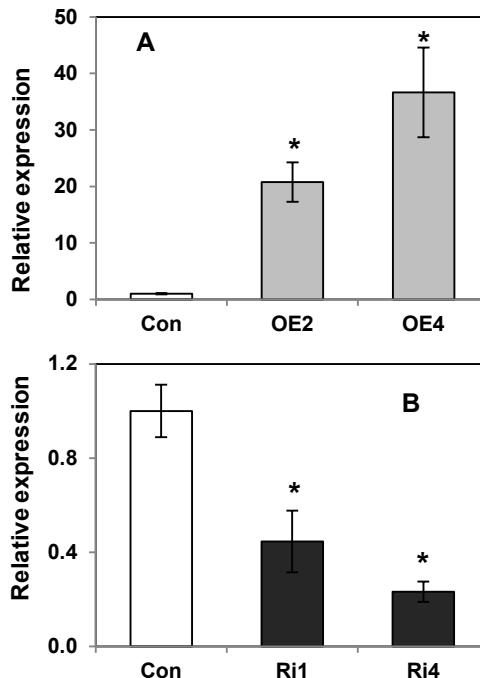
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760 **Figure 1. Phylogenetic analysis of selected secondary cell wall associated NAC transcription**
761 **factors from *Populus* and other plant species.** Transcription factors from *Populus* are in bold.
762 The percentage of replicate trees in which the associated taxa clustered together in the bootstrap
763 test (500 replicates) are shown next to the branches. Accessions are provided below. AtSND1:
764 At1g32770 (*Arabidopsis thaliana*); AtNST1: At2g46770; AtNST2: At3g61910; AtVND7:
765 AT1G71930; RcNAC: XP_002518924 (*Ricinus communis*); VvNAC: XP_002279545 (*Vitis*
766 *vinifera*); JcNAC013: AGL39669 (*Jatropha curcas*); MdNAC: NP_001280877 (*Malus*
767 *domestica*), GhNAC3: ADN39415 (*Gossypium hirsutum*); EgNAC: KCW72583 (*Eucalyptus*
768 *grandis*). PtWND1A (Potri.011G153300), PtWND1B (Potri.001G448400), PtWND2A
769 (Potri.014G104800), WND2B (Potri.002G178700), PtWND6A (Potri.013G113100) and
770 PtWND6B (Potri.019G083600).



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773 **Figure 2. Relative expression of *PdWND1B* in control and transgenic lines.** Gene expression
774 (arbitrary units) in control (Con), over-expression (OE) lines (A), and RNAi suppressed (Ri) lines
775 (B) was relative to the housekeeping genes *Ubiquitin conjugating enzyme E2* and *18S RNA*. The
776 data represents means \pm SE ($n = 3$). * Indicates statistical significance based on Student's *t*-test (p
777 ≤ 0.05).

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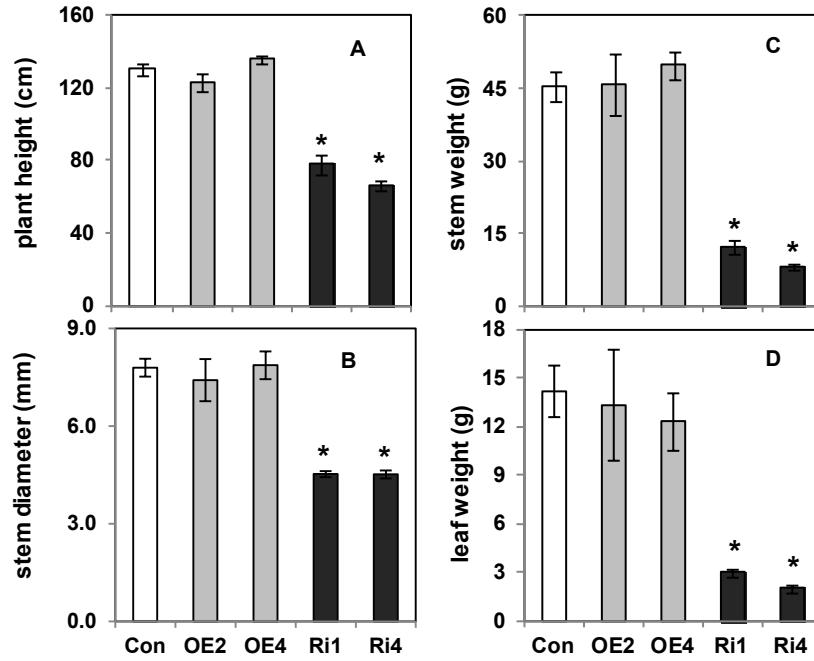
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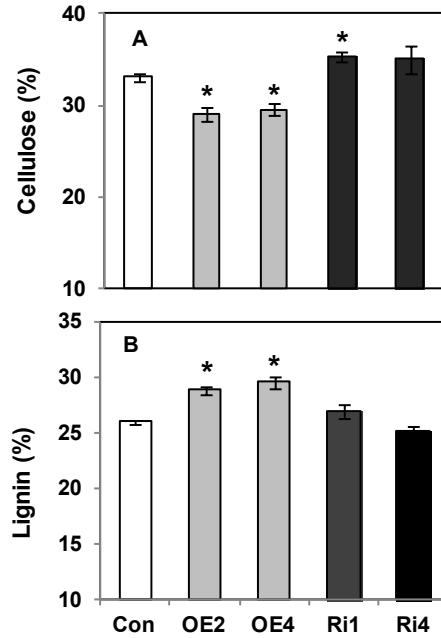
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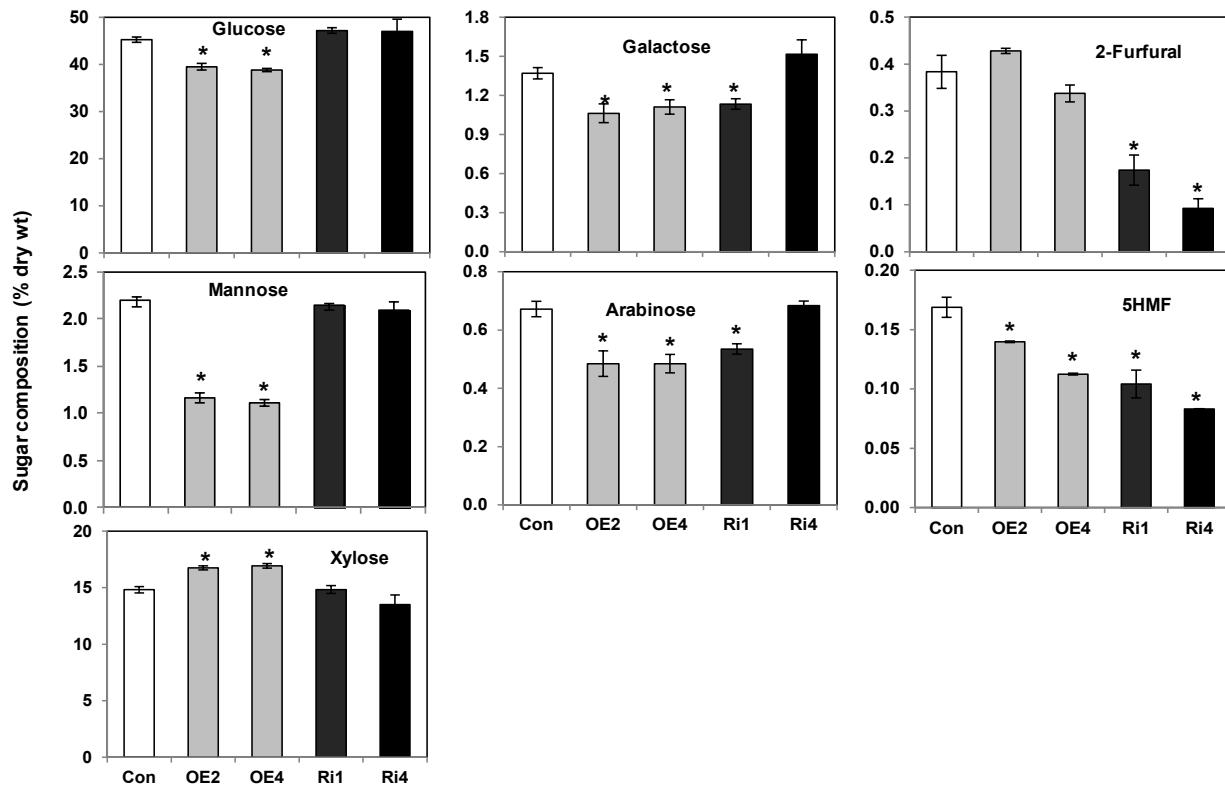
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791 **Figure 3. Growth and biomass productivity in control and *PdWND1B* transgenic lines.** Plant
792 height (A), stem diameter (B), stem weight (C), leaf weight (D) of empty vector transformed
793 control (Con), and *PdWND1B* over-expression (OE) and RNAi suppressed (Ri) lines. The data
794 represents means \pm SE ($n = 3$). * Indicates statistical significance based on Student's *t*-test ($p \leq$
795 0.05).

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805 **Figure 4. Stem cell wall composition of control and *PdWND1B* transgenic lines.** Levels of
806 cellulose (A) and lignin (B) in empty vector transformed control (Con), *PdWND1B* over-
807 expression (OE), and RNAi suppressed (Ri) lines. The data represents means \pm SE (n = 3 to 5). *
808 Indicates statistical significance based on Student's *t*-test (p \leq 0.05).
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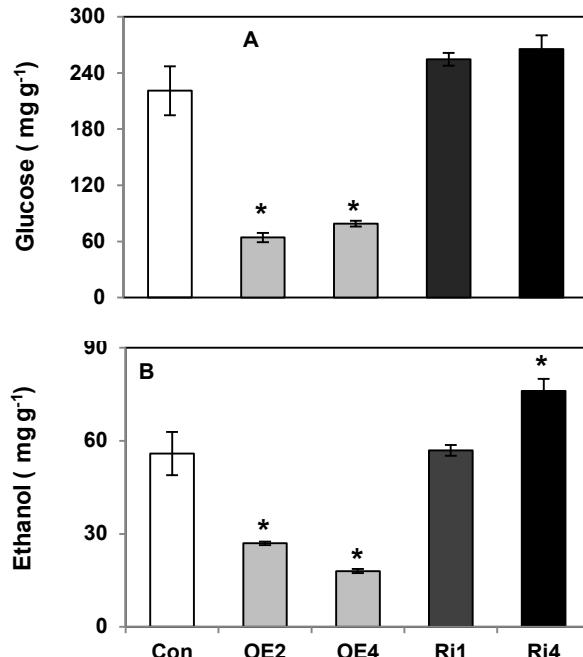
813 **Figure 5. Sugar composition in stem cell walls of control and *PdWND1B* transgenic lines.**

814 Levels of different sugars in empty vector transformed control (Con), *PdWND1B* over-expression

815 (OE), and RNAi suppressed (Ri) lines. The data represents means ± SE (n = 3). * Indicates

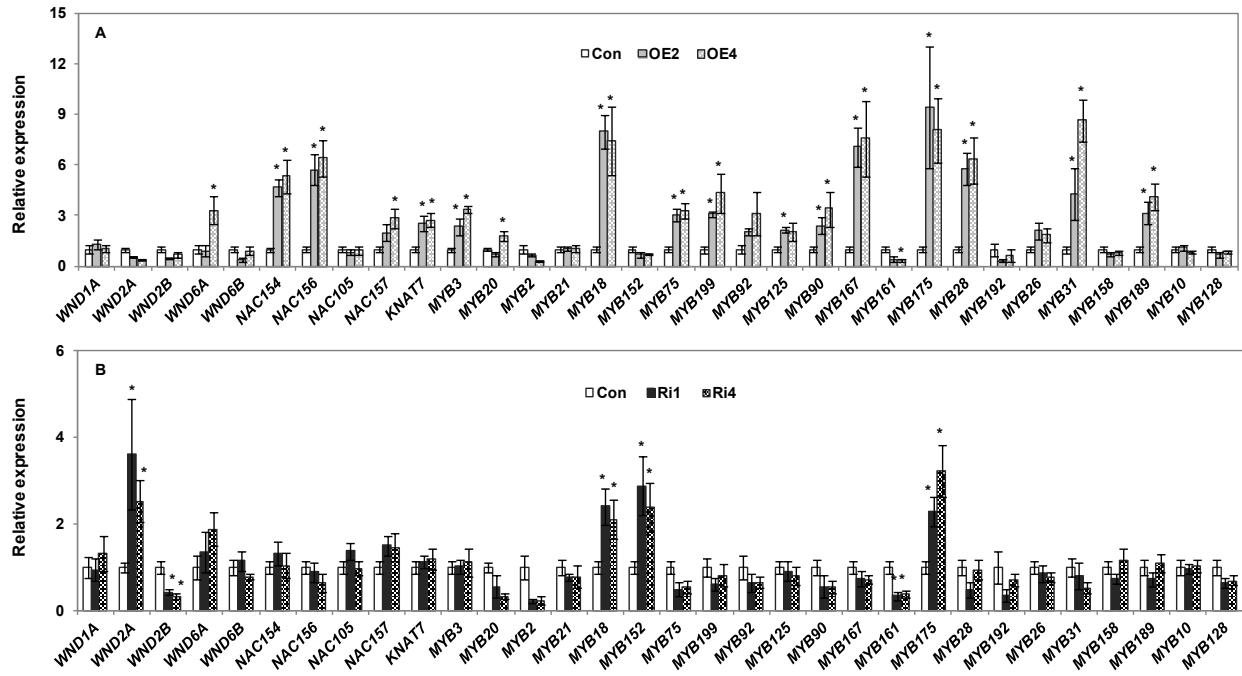
816 statistical significance based on Student's *t*-test (p ≤ 0.05).

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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 represents means \pm SE ($n = 3$). * Indicates statistical significance based on Student's *t*-test ($p \leq 0.05$).



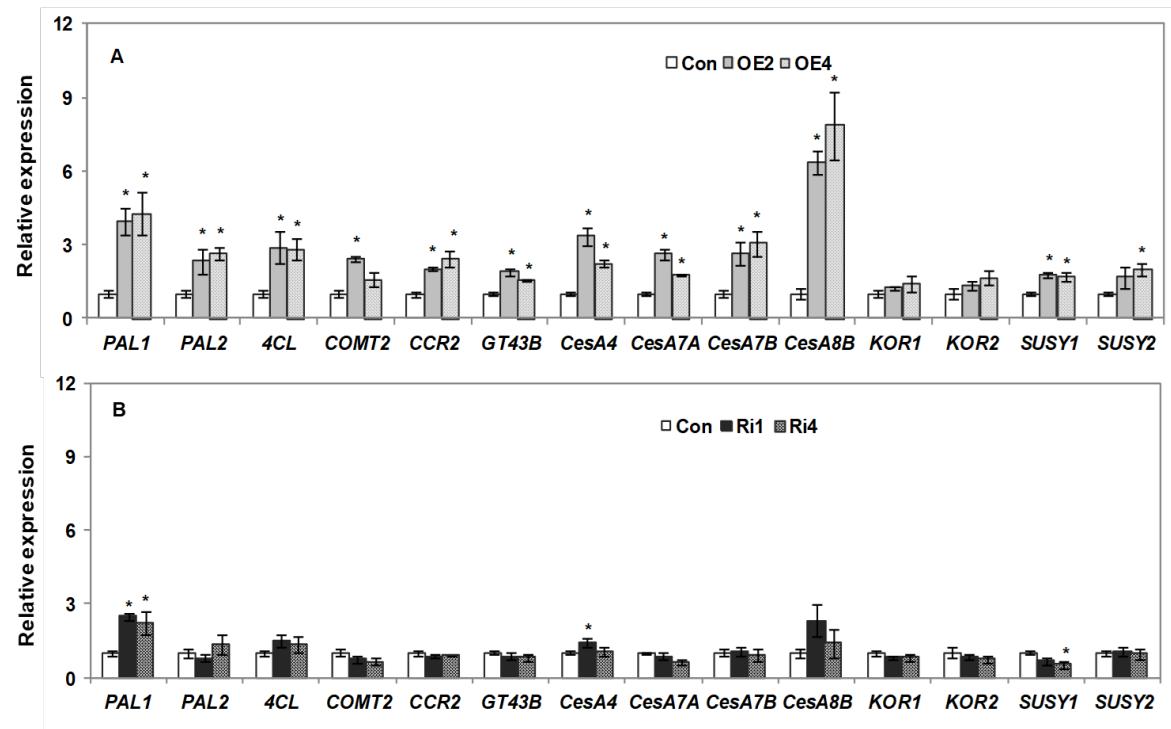
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828 **Figure 7. Expression of secondary cell wall related transcription factors in control and**
829 ***PdWND1B* transgenic lines.** Relative gene expression (arbitrary units) in control (Con), over-
830 expression (OE) lines (A), and RNAi suppressed (Ri) lines (B) was calculated based on the
831 expression of target genes relative to house-keeping genes, *Ubiquitin conjugating enzyme E2* and
832 *18S RNA*, and then normalized to control. The data represents means \pm SE ($n = 3$). * indicates
833 statistically significant, $p \leq 0.05$ based on Student's *t*-tests.

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838 **Figure 8. Expression of secondary cell wall related and sugar metabolism related genes in**
839 **control and *PdWND1B* transgenic lines.** Relative gene expression (arbitrary units) in control
840 (Con), over-expression (OE) lines (A), and RNAi suppressed (Ri) lines (B) was calculated based
841 on the expression of target genes relative to house-keeping genes, *Ubiquitin conjugating enzyme*
842 *E2* and *18S RNA*, and then normalized to control. *PAL*, phenylalanine ammonia lyase; *4CL*, 4-
843 coumarate:CoA ligase; *COMT*, caffeic acid/5-hydroxyconiferaldehyde O-methyltransferase; *CCR*,
844 cinnamoyl-CoA reductase; *GT43*, Glucosyltransferase family 43; *CesA*, Cellulose synthase; *KOR*,
845 Korrigan; *SUSY*, sucrose synthase. The data represents means \pm SE (n = 3). * indicates statistically
846 significant, $p \leq 0.05$ based on Student's *t*-tests.
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853 **Supplemental File 1. List of gene models and their primer sequence information.** OE and
854 RNAi primers were used to design over-expression and RNAi-knockout construct respectively and
855 the rest for qRT-PCR. F and R indicate forward and reverse primers, respectively.
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Name	Primer sequence	Gene Model/Accession
WND1B-F-OE	CACCCCCGGGATGCCTGAGGATATGATGAA	Potri.001G448400
WND1B-R-OE	ACCGCGTTGTTACCGATAAGTGGCAT	
WND1B-F- RNAi	CACCCCCGGGCCTCTATTCCACTAAGCAG	
WND1B-R- RNAi	TCTAGACATCATCAGGAGAAAGACCA	
WND1B-F	GGAAGGACTTGCAAGATGGAGAA	Potri.001G448400
WND1B-R	CCCATCACTGATGCTATTGTCTGAG	
WND1A-F2	TGACCTCATCCTCAATGACTGA	Potri.011G153300
WND1A-R2	CCATGAGAATGCTGTTGTGTG	
WND2A-F	GGACGATAGCACCAGTGATACC	Potri.014G104800
WND2A-R	GCCGCCTCCTCTTCCATAAC	
WND2B-F	CCCACCATGACTTATTGTACCC	Potri.002G178700
WND2B-R	CCAGAGGTCGATTCACTGTTAT	
WIN6A-F	AGCCCTAGTACACTTCAACAA	Potri.013G113100
WIN6A-R	GATGCAAGTAAGGTGTCCAAAC	
WIN6B-F	GCCCTAGTACACTCTCAACAAG	Potri.019G083600
WIN6B-R	GACGCTAGTAAGGTGTCAAAGT	
NAC154-F	GGTACAAAGTAGTGGGCATGA	Potri.017G016700
NAC154-R	AAATGGGAGAGTAGCTGTTGAG	
NAC156-F	GGTACAAAGTAGTGGACATGAGG	Potri.007G135300
NAC156-R	GTGAAAATGGGAAGTAGTTG	
NAC105-F	CCCACCTCAATTCTCCCTAAT	Potri.011G058400
NAC105-R	TGCTTTCTCTCATCACCAT	
NAC157-F	CAGGCCTCCTGATCAGTTCTA	Potri.004G049300
NAC157-R	GCTTTCTGATCACATCACTGC	
KNAT7-F	ACTACCGGGTGACACTACTT	Potri.001G112200

KNAT7-R	TGCCAGTCCTCTCCTTG		
MYB3-20-F	GTGGGTATGGGAGATGGATT	Potri.001G267300	
MYB3-R	CAGGTACTATCAAAGTGGTGG		
MYB20-R	GCAAGTATTATCAAAGCAGTTG	Potri.009G061500	
MYB2-F	GGGATCTTGGAGGTGTTAATG	Potri.001G258700	
MYB2-R	GTAAAGACTACTCACGTAGCT		
MYB21-F	GCTCAAGTTGGAGTCCTAACAA	Potri.009G053900	
MYB21-R	CCATGCAGCTTACACTCTCTAA		
MYB18-F	CAACCTCAACCTCTCATTAACATC	Potri.004G086300	
MYB18-R	TCCCAACTACTCAAGTCATCATC		
MYB152-F	TCGTCATCGACATCATCTTCTTC	Potri.017G130300	
MYB152-R	CACCGACAGCATCACTGATTA		
MYB75-F	CCAAGCCACGAGAGAAGATTAC	Potri.015G129100	
MYB75-199-R	TTGCCACCCATGTCTAGGATAC		
MYB199-F	CAAGCCAGTAGAGGAGGAGATT	Potri.012G127700	
MYB199-R	TTGCCACCCATGTCTAGGATAC		
MYB92-F	TTACACATGGTTATCGGACTG	Potri.001G118800	
MYB92-R	AAATCTTCTCATCATCGCTCTA		
MYB125-F	AACTACACAGGGTTATCGGATTG	Potri.003G114100	
MYB125-R	ACCGTATAATCTAGCGATTGAG		
MYB90-F	TCGGCCCATTGAGTTCTAC	Potri.015G033600	
MYB90-R	AGCCTTGCTCTGATGTTCC		
MYB167-F	AGCAGGAAGCCTGGAAA	Potri.012G039400	
MYB167-R	TCGTTGACACACCACCA		
MYB161-F	GATGATGTCGAGGTGGATCAG	Potri.007G134500	
MYB161-R	TCAAGACCTACAATCCACTAAC		
MYB175-F	CCCTCGACAATGCTAGAAGAG	Potri.017G017600	
MYB175-R	GTGAAGGGAACCCGCTAAT		
MYB28-F	CGTTGAAGCATGCCAAATCTC	Potri.005G096600	
MYB28-R	GTGTCTCGGCAGCATTCTT		
MYB192-F	TTGAAGCTGGCCAGAGCTCA	Potri.007G067600	
MYB192-R	CTCTCCGCAGCATTCTCGATAA		
MYB26-31-F	GGTGATGGTTATGGAAGCAATAAA	Potri.005G063200	
MYB26-R	CCTCCATGATCTCCTTGCTCTT		

MYB31-R	GATGATAAAACTGAAGCTTGG	Potri.007G106100
MYB158-F	TGAAGAAAGGGTGAGGAAAGG	Potri.005G156600
MYB158-R	GCTTCCATGGCTAACATTGC	
MYB189-F	AGGGTTGTTCCAAGTCCATTAG	Potri.002G073500
MYB189-R	GGTTACTCGTCGCTCTCATATT	
MYB10-F	GAGTGCTTACAGAGGCAAGAG	Potri.001G099800
MYB10-R	CAGCTCCATGTTAGATGAATTG	
MYB128-F	TGGTGCCTATTGAGATGCAATCC	Potri.003G132000
MYB128-R	CTTCTCCACCAAGTGGTCCTTC	
PAL1-F	ACAACTTCTTAGTGGCACTCTGC	Potri.006G126800
PAL1-R	GCTCCTCAAGTTCTCCTCCAAATG	
PAL2-F	ACTCCTTGGGCTTGATTCTGC	Potri.008G038200
PAL2-R	ACCAACCAGGTGGTAGACATGAG	
4CL1-1-F	CGAAGCTTGTACTAGCCCATCC	Potri.001G036900
4CL1-1-R	TCCTGCATCCTCATCTTCATTCC	
COMT2-F	AGCTGTCGTTAACACCATCGTC	Potri.012G006400
COMT2-R	ACATGCTCCACACCAGGATAAG	
CCR2-F	TGGAGAGGTGGTGGAAATCCTTG	Potri.003G181400
CCR2-R	CTTCTCATCTGAGCACTGGTAGG	
GT43B-F	GTCGCCCTCTTCAGTCCAGCA	Potri.016G086400
GT43B-R	ACAGTCCTCTGGTGGGATTCCCT	
CesA4-F	AGCATCCAGGACTTGTGGCGTAAT	Potri.002G257900
CesA4-R	TGAGGAGGGTGGTCCATTGAAGA	
CesA7A-F	AGCTCTCTTGCCCTCTGGGTGA	Potri.006G181900
CesA7A-R	TGAKTCCACATTGCTTGGTGTCA	
CesA7B-F	GTCCGGATTGATCCATTGT	Potri.018G103900
CesA7B-R	CCCTTAGAACAGCAGGATGCAC	
CesA8B-F	GCTGTTGGCCTCTGTCTTCT	Potri.004G059600
CesA8B-R	CGCAACCAAGGTGTTATCAA	
KOR1-F	CCATGAGATGCCACAGTTGA	Poptri.003G151700
KOR1-R	TCCCAAGATGTTCCAAGTCC	
KOR2-F	CCTTGGAGACCATGAGATGC	Poptri.001G078900
KOR2-R	CCGTGGAGTCGCATTATCTT	
SUSY1-F	GAACCTTGATCGTCTTGAGAGYCG	Potri.018G063500

SUSY1-R	GGTTCTGTCTCCMAACYGAAACCA		
SUSY2-F	CAACCTYGATCAYCGTGAGAGCCG	Potri.006G136700	
SUSY2-R	ACCATTATTCTGGACCCGGAACCC		
18S-F	AATTGTTGGTCTTCAACGAGGAA	AF206999	
18S-R	AAAGGGCAGGGACGTAGTCAA		
UBCc-F	CTGAAGAAGGAGATGACARCMCCA	Potri.006G205700	
UBCc-R	GCATCCCTTCAACACAGTTCAMG		
ProMYB002-F	ACCTCTCTCATTTCCCCTGC	Potri.001G258700	
ProMYB002-R	TCCCTGTCACTAGAAAGGTGATCT		
ProHB3-F	GCCTGCCTCTCATTATTCTCTAC	Potri.011G098300	
ProHB3-R	CACCTAAAGAAAGAACTAAAAC TTG		
ProHB4-F	TCTCGATGTCTTGATGATTG	Potri.001G372300	
ProHB4-R	TCAACAAAAACACCTAATAAAAG		
ProEPSP1-F	TCTTCACGTCCTCTCACCAACCC	Potri.002G146400	
ProEPSP1-R	GGCTTCACTCTGTTCTCTCC		
ProEPSP2-F	CACGAAGAAAACACAGTGTGGG	Potri.014G068300	
ProEPSP2-R	CTGAATGACAGATGAAAACAAG		
SND1clo-F	CACCATGCCTGAGGATATGATGAATC	Potri.001G448400	
SND1-Rstop	TTATAACCGATAAGTGGCATAATGG		

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866	PtWND1B	AtSND1	PtWND2A	PtWND2B	AtNST1	AtNST2	RcNAC	JcNAC013	MdNAC	GhNAC3	VvNAC	EgNAC	PtWND6A	PtWND6B	AtVND7	
867	PtWND1A	94%	52%	57%	56%	52%	48%	86%	83%	72%	74%	79%	75%	40%	41%	40%
	PtWND1B		53%	57%	56%	53%	49%	85%	83%	72%	74%	80%	75%	42%	42%	40%
	AtSND1			56%	55%	58%	55%	53%	54%	54%	56%	56%	53%	46%	46%	45%
	PtWND2A				88%	65%	58%	56%	58%	55%	57%	57%	57%	45%	46%	45%
	PtWND2B					64%	57%	55%	57%	54%	56%	57%	57%	46%	47%	43%
	AtNST1						72%	51%	53%	51%	52%	53%	53%	46%	46%	44%
	AtNST2							47%	47%	48%	49%	49%	49%	45%	45%	42%
868	RcNAC								87%	69%	72%	78%	72%	41%	41%	40%
	JcNAC013									71%	70%	79%	73%	42%	43%	39%
869	MdNAC										77%	69%	65%	40%	41%	39%
	GhNAC3											70%	69%	41%	41%	40%
	VvNAC											71%	42%	43%	42%	
870	EgNAC												43%	44%	42%	
	PtWND6A													92%	69%	
	PtWND6B														67%	

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873 **Supplemental File 2. Percentage protein similarity matrix of selected secondary wall**
874 **associated transcription factors from *Populus* and other species.** Accessions are provided
875 below. AtSND1: At1g32770 (*Arabidopsis thaliana*); AtNST1: At2g46770; AtNST2: At3g61910;
876 AtVND7: AT1G71930; RcNAC: XP_002518924 (*Ricinus communis*); VvNAC: XP_002279545
877 (*Vitis vinifera*); JcNAC013: AGL39669 (*Jatropha curcas*); MdNAC: NP_001280877 (*Malus*
878 *domestica*), GhNAC3: ADN39415 (*Gossypium hirsutum*); EgNAC: KCW72583 (*Eucalyptus*
879 *grandis*). PtWND1A (Potri.011G153300), PtWND1B (Potri.001G448400), PtWND2A
880 (Potri.014G104800), WND2B (Potri.002G178700), PtWND6A (Potri.013G113100) and
881 PtWND6B (Potri.019G083600).

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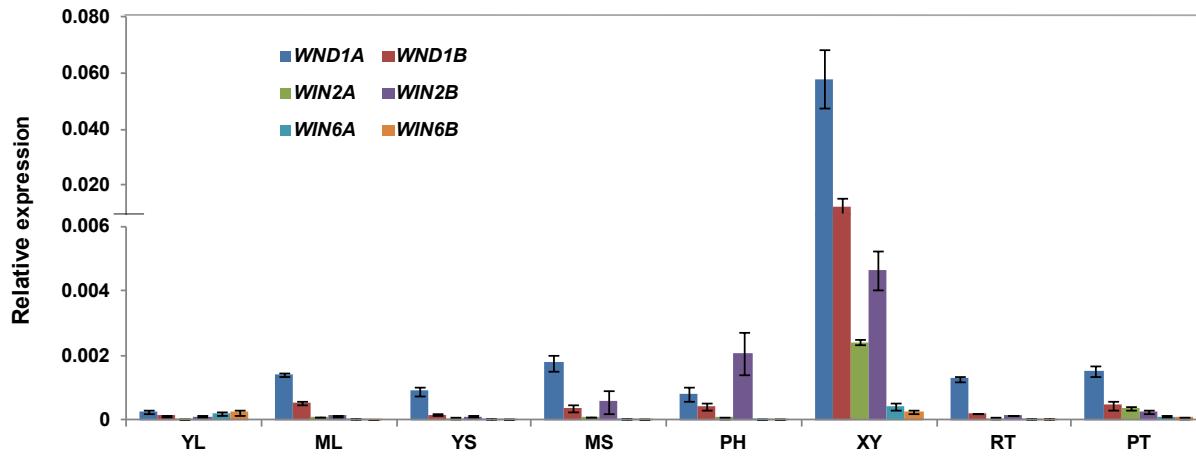
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892 **Supplemental File 3. Expression of the six NAC genes in different tissues of *Populus*.** YL
893 (young leaf), ML (mature leaf), YS (young stem), MS (mature stem), PH (phloem), XY (xylem),
894 RT (root), PT (petiole). Relative expression (arbitrary units) was calculated based on the
895 expression of target genes relative to house-keeping genes, *Ubiquitin conjugating enzyme E2* and
896 <18S RNA. A break in the Y-axis represents discontinuous scale. The data represents mean values
897 of three biological replicates \pm SE.

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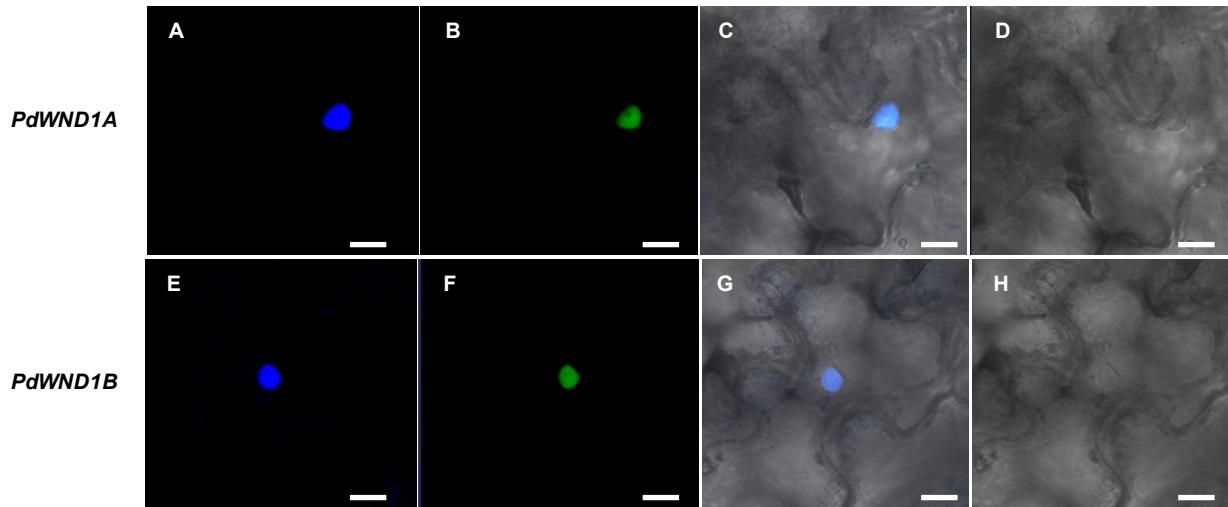
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911 **Supplemental File 4. Localization of the PdWND1A and PdWND1B in tobacco epidermal**
912 **cells.** Nuclear targeting of GFP: **PdWND1A** (A to D) and **PdWND1B** (E to H) in *Nicotiana*
913 *benthamiana* mesophyll cells after agro infiltration. Panels A and E are cells stained with DAPI to
914 show nuclei (blue stain), B and F are GFP localization, C and G are colocalization of DAPI and
915 GFP, and D and H, no fluorescence control. Scale bar represents 10 μ M.

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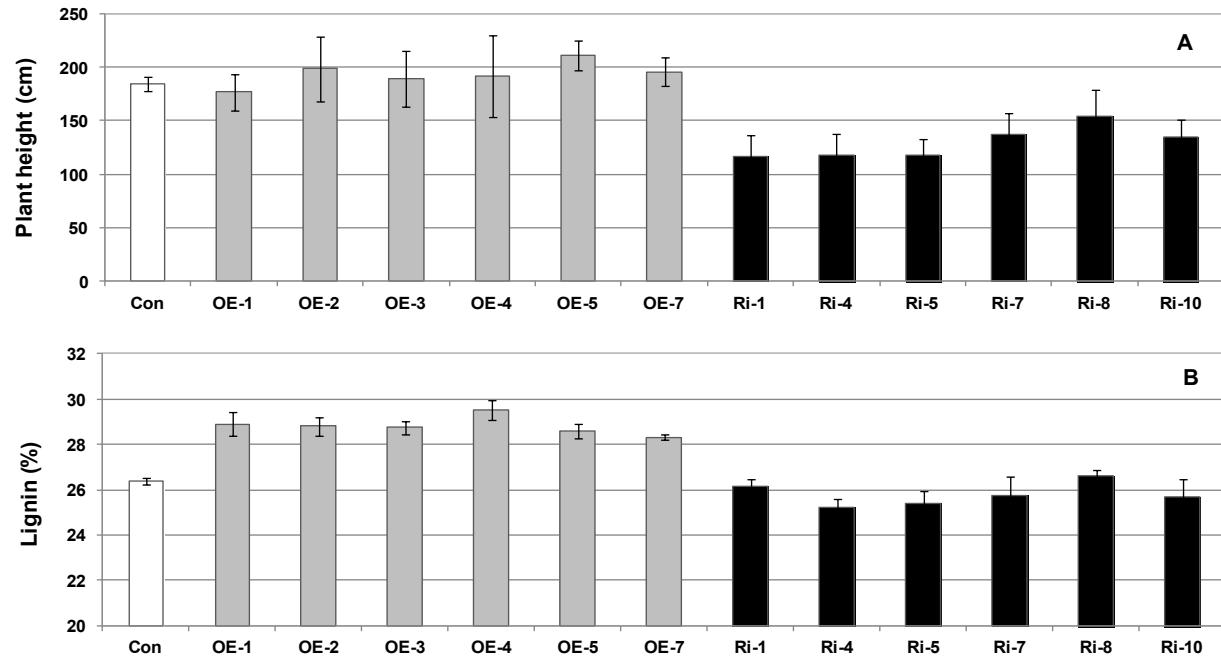
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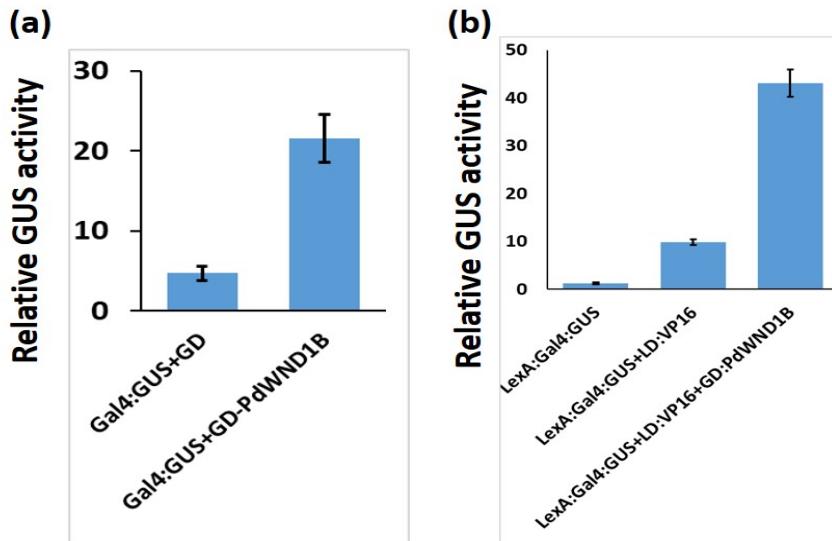
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932 **Supplemental File 5.** Plant height (A) and lignin content (B) in control (Con) and *PdWND1B*
933 over-expression (OE) and RNAi suppression (Ri) lines.
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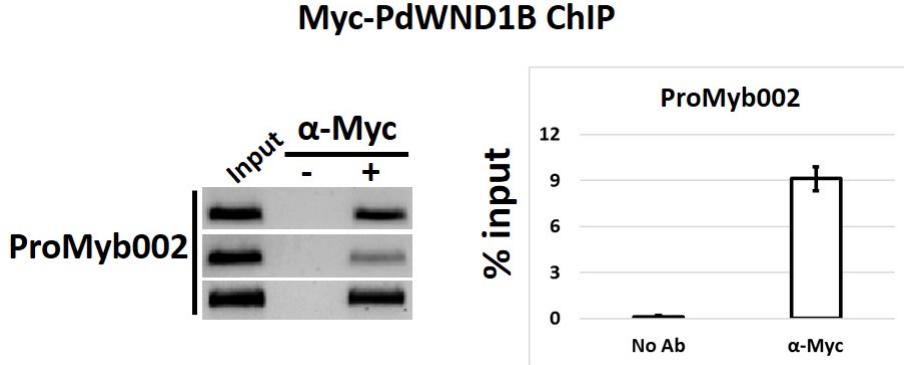


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942 **Supplemental File 6. PdWND1B has transcriptional activator activity.** (a) Protoplasts
943 transfected with Gal4:GUS reporter together with Gal4 binding domain (GD) fused with
944 PdWND1B (GD-PdWND1B) shows increased GUS activity as compared to empty GD vector
945 control. (b) GD-PdWND1B does not repress the expression of GUS reporter when co-transfected
946 with LexA:Gal4:GUS reporter and LexA binding domain (LD) fused transactivator VP16.

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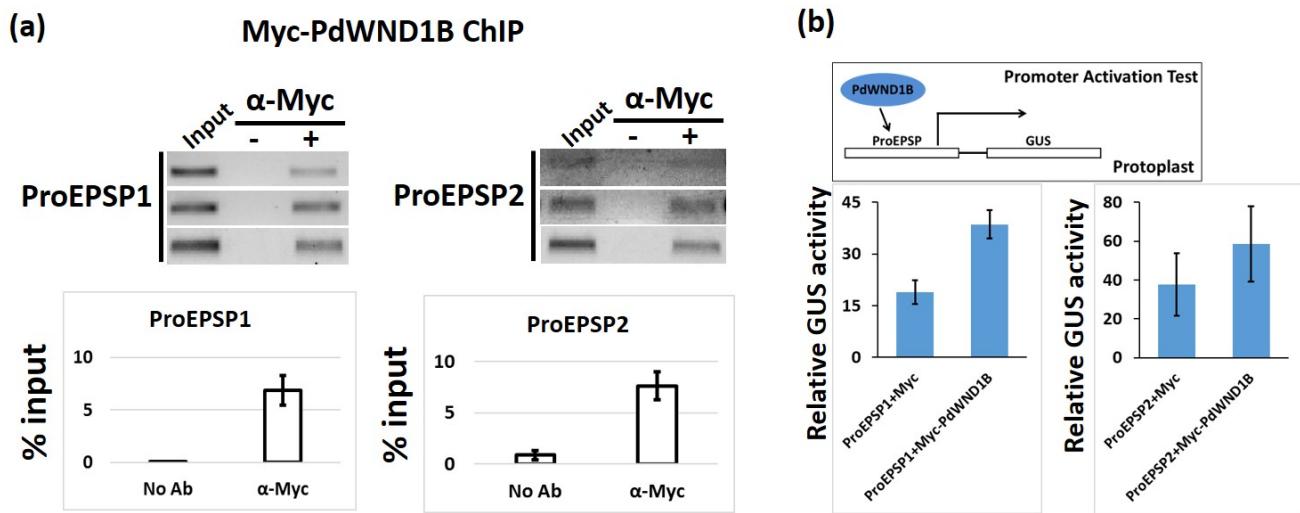
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950 **Supplemental File 7. PdWND1B binds to promoter of *MYB002* secondary cell wall**
951 **transcription factor gene.** Miro Chromatin immunoprecipitation (μ ChIP) from protoplasts
952 transfected with Myc-fused PdWND1B indicates its binding to the promoter region of Myb002
953 gene in vivo. Left panel shows gel bands from three replicates including the input lane, no-antibody
954 negative control and the sample with antibody. ChIP enrichment signal was calculated from
955 quantitative PCR data as percent of input signal.

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961 **Supplemental File 8. PdWND1B regulates the expression of EPSP genes *in vivo*.** (a) μChIP
962 from protoplasts transfected with Myc-fused PdWND1B indicates its binding to the promoter
963 region of EPSP1 and EPSP2 genes *in vivo*. Top panel shows gel bands from three replicates
964 including the input lane, no-antibody negative control and the sample with antibody. Bottom panel
965 shows PCR data for ChIP enrichment signal that was calculated as percent of input. (b) Protoplasts
966 transfected with a EPSP promoter driven GUS reporter together with the Myc-fused PdWND1B
967 show higher GUS activity as compared to empty vector controls.

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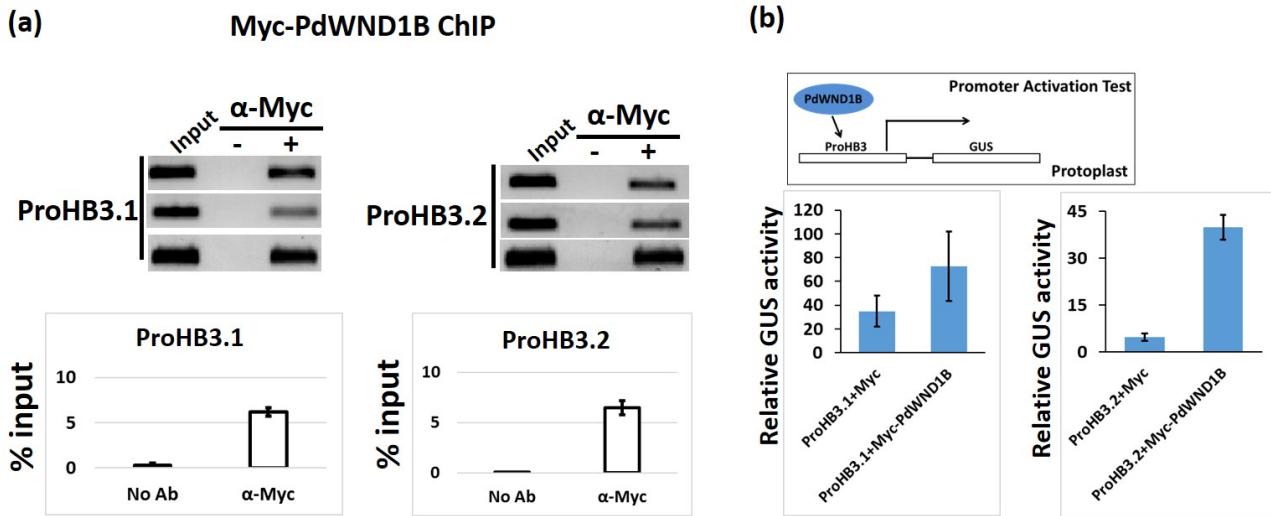
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982 **Supplemental File 9. PdWND1B regulates the expression of *HB3-like* genes *in vivo*.** (a) μChIP
983 from protoplasts transfected with Myc-fused PdWND1B indicates its binding to the promoter
984 region of *PdHB3-like* genes, *PdHB3.1* (*PtHB3*; Potri.011G098300) and *PdHB3.2* (*PtHB4*,
985 Potri.001G372300), *in vivo*. Top panel shows gel bands from three replicates including the input
986 lane, no-antibody negative control and the sample with antibody. Bottom panel shows PCR data
987 for ChIP enrichment signal that was calculated as percent of input. (b) Protoplasts transfected with
988 an HB3 promoter driven GUS reporter together with the Myc-fused PdWND1B show higher GUS
989 activity as compared to empty vector controls.