Intron-Mediated Alternative Splicing of WOOD-ASSOCIATED NAC TRANSCRIPTION FACTOR1B Regulates Cell Wall Thickening during Fiber Development in *Populus* Species[1][W]

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Alternative splicing is an important mechanism involved in regulating the development of multicellular organisms. Although many genes in plants undergo alternative splicing, little is understood of its significance in regulating plant growth and development. In this study, alternative splicing of black cottonwood (*Populus trichocarpa*) wood-associated NAC domain transcription factor (PtrWNDs), *PtrWND1B*, is shown to occur exclusively in secondary xylem fiber cells. *PtrWND1B* is expressed with a normal short-transcript *PtrWND1B-s* as well as its alternative long-transcript *PtrWND1B-l*. The intron 2 structure of the *PtrWND1B* gene was identified as a critical sequence that causes *PtrWND1B* alternative splicing. Suppression of *PtrWND1B* expression specifically inhibited fiber cell wall thickening. The two PtrWND1B isoforms play antagonistic roles in regulating cell wall thickening during fiber cell differentiation in *Populus* spp. *PtrWND1B-s* overexpression enhanced fiber cell wall thickening, while overexpression of *PtrWND1B-l* repressed fiber cell wall thickening. Alternative splicing may enable more specific regulation of processes such as fiber cell wall thickening during wood formation.

Wood is an important renewable resource for the production of pulp, chemicals, timber products, and energy. Wood formation in tree species is the result of a highly ordered and finely regulated process of cell differentiation and secondary cell wall thickening, initiated from the vascular cambium and resulting in thick-walled xylem cells. Wood formation is regulated at the genomic and molecular levels, but the mechanism underlying the regulation has largely not been elucidated.

Alternative splicing (AS) is an important regulatory mechanism in the development of multicellular organisms (Irimia et al., 2007). In plants, an estimated 60% or more of intron-containing genes undergo AS (Syed et al., 2012). AS occurs in many circadian clock genes in Arabidopsis (*Arabidopsis thaliana*), suggesting that it controls the plant circadian clock through modulating the level of productive and nonproductive mRNA isoforms (James et al., 2012). A large number of variations in AS have been identified in different Arabidopsis genotypes (Gan et al., 2011), while organ-specific and abiotic stress-responsive AS patterns have been detected in natural and synthetic polyploids of *Brassica napus* (Zhou et al., 2011). Combined evidence suggests that AS may be a mechanism employed by plants to achieve phenotypic plasticity to adapt to different environments. AS often results in changes in protein sequences, likely affecting protein domain structure and function (Severing et al., 2012). However, knowledge of the functional significance of AS in plants is limited due to the lack of characterization of AS variants. Even less is known about the role of AS in wood formation, a complex process involving many AS events (Bao et al., 2013).

A number of transcription factor (TF) genes are involved in the regulation of wood formation. Among them, No Apical Meristem/Arabidopsis Transcription Activation Factor/Cup-shaped Cotyledon (NAC) family genes play critical roles in regulating xylem cell differentiation and cell wall thickening (Zhong et al., 2010a). These NAC TFs in Arabidopsis include NAC SECONDARY WALL THICKENING PROMOTING FACTORs (NSTs; Mitsuda et al., 2005, 2007; Mitsuda and Ohme-Takagi, 2008), SECONDARY WALL-ASSOCIATED NAC DOMAIN proteins (SNDs; Zhong et al., 2006, 2008), and VASCULAR-RELATED NAC DOMAIN proteins (VNDs; Kubo et al., 2005; Yamaguchi et al., 2008). Their homologs in tree species are named WNDs (for wood-associated NAC domain transcription factors; Zhong et al., 2010b) or VNSs (for VND-, NST/SND-, SOMBRERO-related proteins; Ohtani et al., 2011). NAC genes play a role in regulating the expression of other downstream secondary wall-

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associated TF genes and wall synthesis genes in Arabidopsis (Zhong et al., 2007, 2008; Zhou et al., 2009; Yamaguchi et al., 2011; Zhong and Ye, 2012). NAC TF genes are seen as the key players regulating the complex transcriptional network leading to wall-thickening cell differentiation. Despite their importance, much is still unknown regarding how these NAC TF proteins interact to form transcriptional cascades. In addition, wood formation derived from vascular cambial activity in tree species exhibits distinct properties, such as perennial secondary growth, cambium dormancy, longer lifespan, heavily thickened walls, juvenile and mature wood, heartwood formation, and other characteristics, which differ from xylem development in herbaceous Arabidopsis (Taylor, 2002). Wood tissue is structured with vessel elements, ray parenchyma cells, and fibers in woody dicots. The formation of wood tissue involves a well-programmed, coordinated process of cell division, cell differentiation, cell wall thickening, and cell death (Mellerowicz et al., 2001; Plomion et al., 2001). Compared with herbaceous plants such as Arabidopsis, woody plants need to synthesize the secondary cell wall in a large and stable quantity against the forces of gravity and other environmental factors. To elaborate, wood cell walls are composed of the primary cell wall and multiple layers of secondary cell walls, which can be divided into three different layers, S1, S2, and S3 (Plomion et al., 2001). Each of these layers displays different thicknesses, cellulose microfibril angles, lignin and hemicellulose contents, and compositions (Plomion et al., 2001). The formation of these wood cell wall structures involves many yet to be elucidated molecular mechanisms. Therefore, the regulation of secondary cell wall formation in woody plants is conceivably different compared with herbaceous plants.

A group of NAC domain TFs, WNDs, constitutes the transcriptional regulatory networks, which activate the secondary wall biosynthetic program in *Populus* spp. Among them, black cottonwood (*Populus trichocarpa*) wood-associated NAC domain transcription factors PtrWND2B and PtrWND6B are considered functional orthologs of Arabidopsis SND1 and are involved in the regulation of wood formation (Zhong et al., 2010b). *PtrSND1-A2* (also named *PtrWND1B/PtrVNS11*, homolog to Arabidopsis *AtSND1*) was previously reported by Li et al. (2012) to undergo AS to produce the splice variant *PtrSND1-A2*^{IR} in developing xylem tissue. The *PtrSND1-A2*^{IR} complementary DNA (cDNA) encodes a NAC domain protein that has no activation domain but has a protein dimerization domain. Further subcellular protein colocalization and translocation, yeast two-hybridization, and bimolecular fluorescence complementation evidence confirmed that PtrSND1-A2 and its splice variant PtrSND1-A2^{IR} can form heterodimers or homodimers with other *PtrSND1* members or themselves. Using a stem-differentiating protoplast system, transient transcriptional perturbation and transactivation were conducted. Integrating with results of

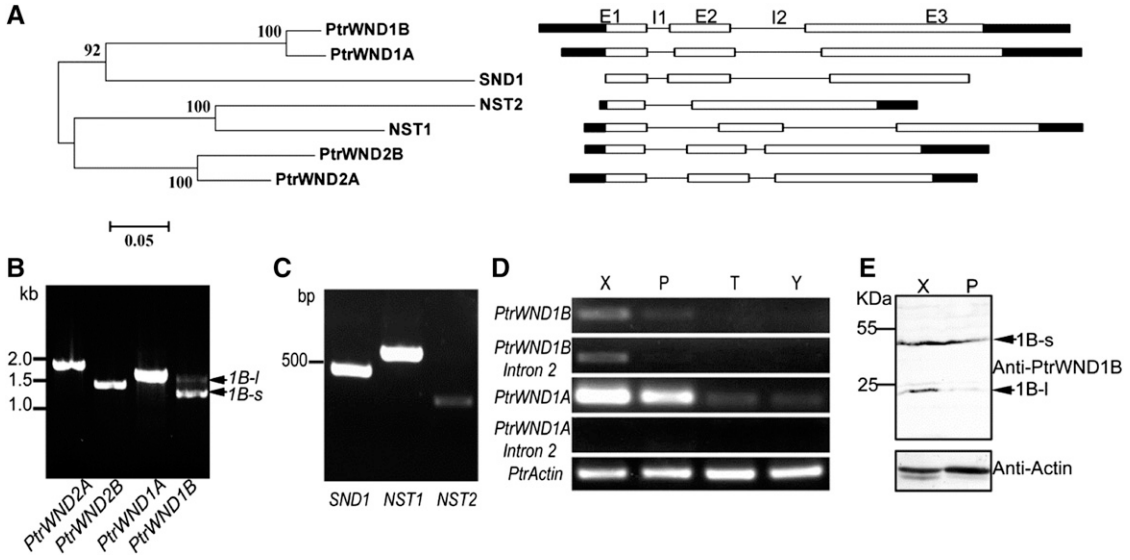

Figure 1. AS of *PtrWND1B* in black cottonwood. A, Phylogenetic relationships of the NST group genes and their exon-intron structures in black cottonwood and Arabidopsis. In exons (E1, E2, and E3), untranslated regions are shown in black boxes and coding sequences in white boxes. Introns (I1 and I2) are shown as lines. B, RT-PCR amplification of *PtrWND1A*, *PtrWND1B*, *PtrWND2A*, and *PtrWND2B* from cottonwood xylem. Two alternative *PtrWND1B* transcripts, *WND1B-l* and *WND1B-s*, are indicated by arrows. C, RT-PCR amplification of *SND1*, *NST1*, and *NST2* from Arabidopsis inflorescence stem. Specific forward and reverse primers were designed to flank the sequence between the first and last exons. D, Tissue-specific expression of *PtrWND1B*, *PtrWND1B* intron 2, *PtrWND1A*, and *PtrWND1A* intron 2. *PtrACTIN2* was used as a reference gene. X, Xylem; P, phloem; T, tip; Y, young stem of the second internode. E, Western blot of cottonwood xylem (X) and phloem (P) proteins. Two isoforms (indicated by arrows) were identified by anti-PtrWND1B. ACTIN was used as a reference protein.

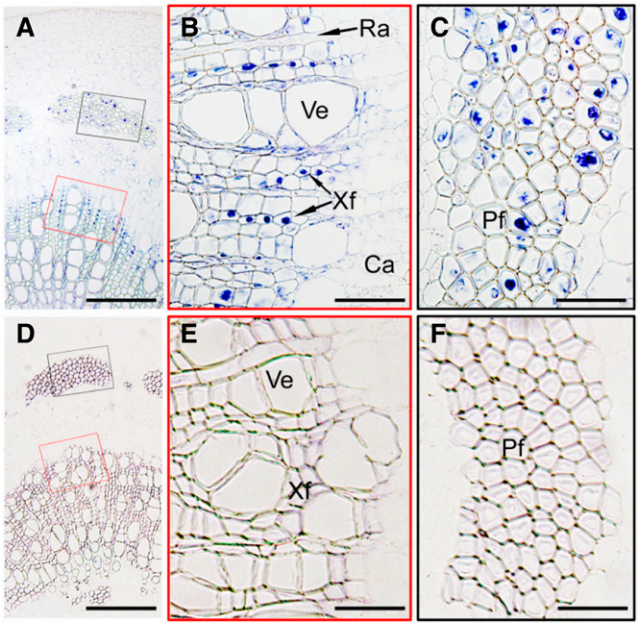

Figure 2. Immunolocalization of PtrWND1B in *Populus* spp. stems. A and D, Cross sections of the stem at the 10th internode were hybridized with anti-PtrWND1B antibodies (A) or preimmune IgG (D). B and E, High magnification of the red frames in A and D, respectively. C and F, High magnification of the black frames in A and D, respectively. Ca, Cambium cell; Pf, Phloem fiber cell; Ra, ray cell; Ve, vessel cell; Xf, xylem fiber cell. Bars = 200 μm (A and D) and 50 μm (B, C, E, and F).

electrophoretic mobility shift assays, the authors provided strong evidence for the regulatory function of *PtrSND1-A2*^{IR} in vitro: *PtrSND1-A2*^{IR} can dominant-negatively regulate the autoregulation of its family number as well as the activation of their common target gene *PtrMYB021* (Li et al., 2012). In this independent study, we also found that the *Populus* spp. ortholog of *SND1*, *PtrWND1B*, undergoes AS in woody species but *SND1* does not undergo AS in herbaceous Arabidopsis. That AS is specifically associated with the wood formation process in *Populus* spp. can be attributed to the sequence structure of *WND1B*. Evidence from reverse genetics demonstrated that *WND1B* AS is a mechanism to regulate fiber cell wall thickening in woody dicots that, to our knowledge, had not been described previously. Demonstrating the linkage between AS and the dominant-negative regulation of NAC TF genes in planta offers important insight into the significance of this mechanism during wood formation.

RESULTS

Analysis of NAC TF Gene AS in Arabidopsis and *Populus* spp.

NAC TF genes involved in the regulation of secondary xylem differentiation and cell wall biosynthesis were analyzed in studies of wood formation in cottonwood (Zhong et al., 2010b; Ohtani et al., 2011). In this study, those transcription factors were named WNDs according to the nomenclature used in previous studies (Zhong et al., 2010b). Among the WNDs studied, four share a high sequence similarity with each other in the cottonwood genome: *PtrWND1A* (Potri.011G153300; also named *PtVNS12* [Ohtani et al., 2011] or *PtrSND1-A1* [Li et al., 2012]), *PtrWND1B* (Potri.001G448400; also named *PtVNS11* [Ohtani et al., 2011] or *PtrSND1-A2* [Li et al., 2012]), *PtrWND2A* (Ptri.014G104800; also named *PtVNS09* [Ohtani et al., 2011] or *PtrSND1-B1* [Li et al., 2012]), and *PtrWND2B* (Potri.002G178700; also named *PtVNS10* [Ohtani et al., 2011] or *PtrSND1-B2* [Li et al., 2012]). These *WNDs* are homologous to the Arabidopsis genes *AtSND1/AtNST3*, *AtNST1*, and *AtNST2*, respectively. *PtrWND1A* and *PtrWND1B* form a duplicated pair and have a similar structure to Arabidopsis *SND1* in that they contain three exons and two introns (Fig. 1A). These NAC TF genes were all found to be expressed in cottonwood developing xylem or Arabidopsis inflorescence stem.

Interestingly, two transcripts from *PtrWND1B* AS were detected uniquely in *Populus* spp. (Fig. 1, B and C),

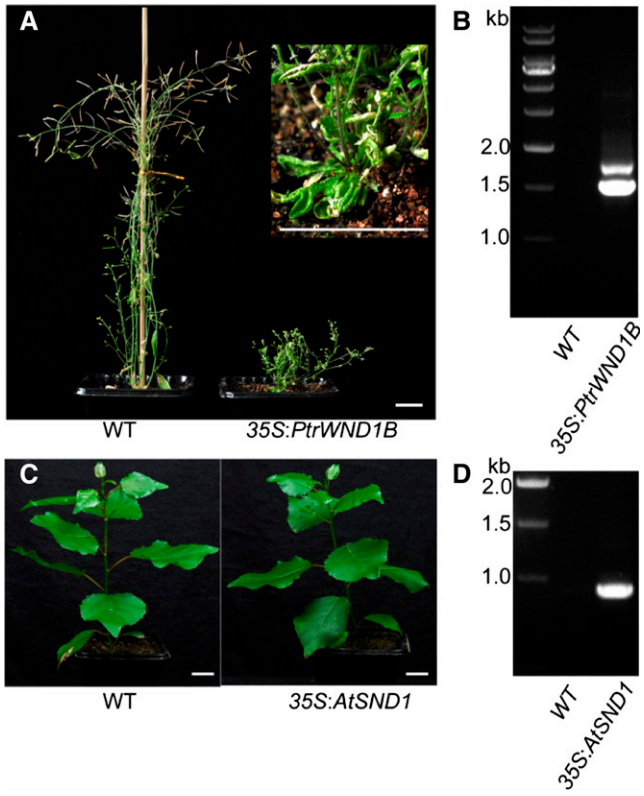

Figure 3. AS is specific for the *PtrWND1B* gene. A, Two-month-old seedlings of wild-type (WT) and 35S:*PtrWND1B* transgenic Arabidopsis. Curling leaves are shown in the enlarged inset image. B, RT-PCR amplification of PtrWND1B from wild-type or 35S:*PtrWND1B* transgenic Arabidopsis. C, Three-month-old seedlings of wild-type and 35S:*AtSND1* transgenic *Populus* spp. D, RT-PCR amplification of *SND1* from wild-type or 35S:*AtSND1* transgenic *Populus* spp. Bars = 2 cm (A and C).

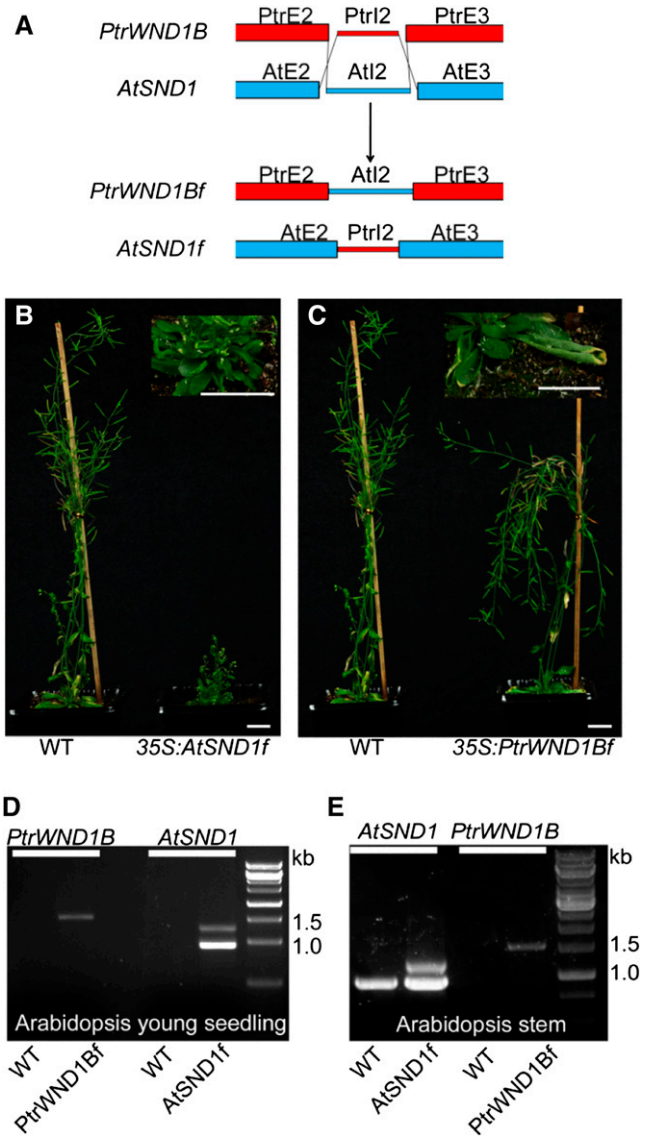

Figure 4. The AS of *PtrWND1B* is mediated by its intron 2. A, Schematic diagram of the intron 2 swap between *PtrWND1B* and *AtSND1*. PtrE2, PtrE3, AtE2, and AtE3 indicate partial exon 2 or 3 in *P. trichocarpa* and Arabidopsis, respectively. PtrI2 and AtI2 indicate full-length intron 2 in *P. trichocarpa* and Arabidopsis, respectively. B, Wild-type (WT) and 35S:*SND1f* transgenic Arabidopsis at the age of 7 weeks. Curling leaves are shown in the enlarged inset image. C, Wild-type and 35S:*PtrWND1Bf* transgenic Arabidopsis at the age of 7 weeks. Curling leaves are shown in the enlarged inset image. D and E, RT-PCR amplification of intron-swap genes *PtrWND1Bf* and *AtSND1f* from young seedlings (D) and inflorescence stem (E) of 35S:*PtrWND1Bf* or 35S:*AtSND1f* transgenic Arabidopsis. Specific forward and reverse primers were designed to flank the sequence between the first and last exons. Bars = 2 cm (B and C).

which was also reported by Li et al. (2012). Here, the longer of the two splice variants was named *WND1B-l* and the shorter splice variant was named *WND1B-s*. Sequencing of the two transcripts indicated that they differed with respect to the retention of intron 2; intron 2 is retained in *WND1B-l* but not in *WND1B-s*. As in *Populus* spp., two *SND1* homologs (Eucgr.E01053 and Eucgr.D01671) were identified in the differentiating xylem tissue of *Eucalyptus grandis* (Supplemental Fig. S1A). RNA sequencing of the xylem transcriptome of *Eucalyptus grandis* identified mRNA from the two splicing variants of Eucgr E01053 (a likely *WND1B* ortholog), including a form with retention of intron 2 (Supplemental Fig. S1B). Given that AS was also not detected with the orthologs of *WND1B* in Arabidopsis, AS regulation of these types of transcriptional factor genes may be different in woody plants and herbaceous plants.

Tissue Specificity of *PtrWND1B* Expression in Cottonwood

Although *PtrWND1A* and *PtrWND1B* are a duplicated pair, they exhibited different expression patterns in differentiating xylem, differentiating phloem, shoot tips, and young stem internode (the second internode; Fig. 1D). Transcripts of *PtrWND1A* were detected in all four tissues, while transcripts of *PtrWND1B* and its alternative splice variant were only detected in differentiating xylem and differentiating phloem. The expression of *PtrWND1B* and its splice variants may be specific to differentiating secondary vascular tissue. The *PtrWND1B-s* transcript is predicted to encode a protein of 418 amino acids, while the *PtrWND1B-l* transcript is predicted to encode a protein of only 166 amino acids due to the presence of a premature termination codon in the retained intron 2 (Li et al., 2012). Specific peptides were selected as immunogens to generate antibodies (Supplemental Fig. S2A) that can recognize PtrWND1B-s and PtrWND1B-l isoforms but not PtrWND3B (Supplemental Fig. S2, B and C). Two bands were detected with molecular sizes of 47.2 and 19.5 kD (Fig. 1E), corresponding to the protein sizes encoded by *PtrWND1B-s* and *PtrWND1B-l*, respectively. The relative abundance of the two isoforms in different tissues appeared consistent with their transcript levels (Fig. 1, D and E). Detection of the two forms of the PtrWND1B protein demonstrates that both alternative splice transcripts are translated into their corresponding proteins in secondary vascular tissue. Immunolocalization was performed to determine the cellular location of PtrWND1B. PtrWND1B protein (potentially including both protein isoforms, PtrWND1B-s and PtrWND1B-l) was found to be predominantly located in thick-walled fiber cells in xylem and phloem tissues (Fig. 2). No such signals were observed in vessel or cambium cells, which suggests that expression of the proteins is associated with secondary wall thickening in both xylem and phloem fibers.

PtrWND1B AS Is Determined by Its Intron 2

To investigate what causes AS of *PtrWND1B* in *Populus* spp., we transformed Arabidopsis with a construct of the *PtrWND1B* genomic fragment under the control of the cauliflower mosaic virus (CaMV) 35S promoter

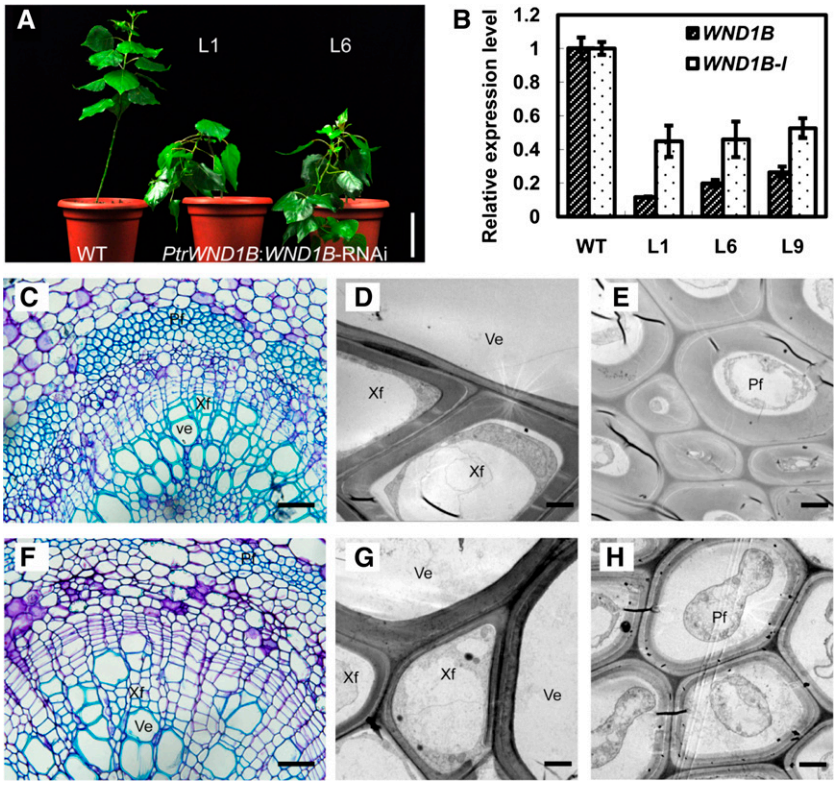

Figure 5. Effects of *PtrWND1B* suppression on the secondary wall thickening in xylem cells of *Populus* spp. A, A wild-type plant (WT) and transgenic *PtrWND1B*:*WND1B-RNAi* plants (L1 and L6) at the age of 3 months. B, *WND1B* and *WND1B-l* expression in the stems of transgenic lines (L1, L6, and L9). Error bars represent SE of three independent biological replicates. *WND1B* includes *WND1B-s* and *WND1B-l*, but mainly *WND1B-s*. C and F, Cross sections of the eighth internode stem showing the xylem fibers (Xf) and phloem fibers (Pf) in the wild type and *PtrWND1B*:*WND1B-RNAi* transgenic line L1, showing thinner fiber cell walls developed in the transgenic lines (F) compared with those in the wild type (C). D and G, Transmission electron micrographs of xylem fiber and vessel (Ve) walls in the wild type (D) and *PtrWND1B*:*WND1B-RNAi* plant L1 (G). E and H, Transmission electron micrographs of phloem fiber walls in the wild type (E) and *PtrWND1B*:*WND1B-RNAi* plant L1 (H). Similar phenotypes were observed with other lines. Bars = 10 cm (A), 50 μm (C and F), and 2 μm (D, E, G, and H).

and transformed *Populus* spp. with a construct of CaMV 35S:*AtSND1*. The transgenic Arabidopsis lines exhibited small rosette sizes and stunted leaves with severely curled blades (Fig. 3A), a phenotype similar to that of Arabidopsis overexpressing *AtSND1* (Zhong et al., 2006). We then examined whether *PtrWND1B* underwent AS in the heterologous system. Both splice transcript variants, *PtrWND1B-s* and *PtrWND1B-l*, were detected in Arabidopsis with a similar ratio of abundance as in cottonwood (Fig. 3B). The occurrence of *Populus* spp. *PtrWND1B* AS in Arabidopsis suggests that the AS machinery is conserved in the two plant systems and that the AS event may be attributed to the sequence of *PtrWND1B*. When *AtSND1* was expressed in *Populus* spp., the transferred *AtSND1* gene produced only one form of the transcript, as it did in Arabidopsis. No AS was detected (Fig. 3, C and D), which again suggests that the AS of *PtrWND1B* relies on its genomic sequence. No phenotypic changes were observed in the transgenic plants. When the expression of *WND1A* and *WND1B* was examined in the transgenic plants, the inherent level of *PtrWND1B* expression was suppressed and the ratio of *PtrWND1B-l* abundance was increased compared with the wild type (Supplemental Fig. S3).

Next, we compared the genomic sequences of the *AtSND1*, *PtrWND1A*, and *PtrWND1B* genes. The genes contain similar exon and intron structures, but the intron 2 sequence in *PtrWND1B* differed from that in *AtSND1* and *PtrWND1A* (Supplemental Fig. S4). The intron 2 fragment of *AtSND1* and *PtrWND1B* was then swapped to examine how the structure of intron 2 contributes to intron retention during AS. The intron 2 of *AtSND1* was exchanged with the intron 2 of *PtrWND1B* and named *AtSND1f*. The intron 2 of *PtrWND1B* was replaced with the intron 2 from *AtSND1* and named *PtrWND1Bf* (Fig. 4A). Then, *AtSND1f* and *PtrWND1Bf* were placed under the control of a CaMV 35S promoter and transferred into Arabidopsis. More than 30 independent transgenic lines were generated. Both the *AtSND1f* and *PtrWND1Bf* transgenic lines exhibited curled leaves and growth retardation, with the *AtSND1f*

Table I. *Cell wall thickness of fibers and vessels in the stems of wild-type and transgenic plants*

Cell wall thickness was measured from transmission electron micrographs of fibers and vessels in the 3-month-old stem. Data are means (μm) ± SE from at least 30 cells in three different plants. Asterisks represent $P < 0.01$ in Student's *t* test.

Sample	Wild Type	*PtrWND1B*:*WND1B-RNAi*	*35S*:*PtrWND1B*	*PtrWND1B*:*PtrWND1B-l*	*PtrWND1B*:*PtrWND1B-s*
Phloem fibers	2.11 ± 0.50	1.06 ± 0.20**	2.27 ± 0.41	1.20 ± 0.28**	2.90 ± 0.53**
Xylem fibers	1.21 ± 0.21	0.79 ± 0.19**	1.38 ± 0.31	0.83 ± 0.21**	1.46 ± 0.31**
Vessels	1.00 ± 0.22	2.21 ± 0.45**	0.95 ± 0.12	1.91 ± 0.37**	0.96 ± 0.21

lines exhibiting the more severe phenotypes (Fig. 4, B and C). Expression of the transferred genes was examined in young seedlings and mature stems of the transgenic plants. *AtSND1* was highly expressed, while *PtrWND1B* expression was relatively lower. AS occurred in *AtSND1f* lines, and two transcript bands were detected (Fig. 4, D and E). The sequence corresponding to the smaller band did not contain the intron sequence, while sequencing of the transcripts verified that *PtrWND1B* intron 2 was retained in the larger band. In contrast, only one transcript band without intron retention was detected in the *PtrWND1Bf* lines, indicating the absence of the AS event. These results verify that the AS event is mediated by intron 2 of the *PtrWND1B* gene.

Regulation of *PtrWND1B* Expression Affects Secondary Wall Thickening of Fiber Cells

To further examine the function of *PtrWND1B* in wood formation, *PtrWND1B* expression was down-regulated through RNA interference suppression. A *PtrWND1B*-specific RNA interference fragment under the control of its own promoter (*PtrWND1B*:*WND1B-RNAi*) was constructed and transferred into *Populus* spp. The resulting transgenic plants were unable to grow straight (Fig. 5A). The transcript abundance of *PtrWND1B* and *PtrWND1B-l* was significantly suppressed in those plants (Fig. 5B). The eighth internode of the stem was sectioned to examine the wood morphology. Compared with the wild type (Fig. 5, C–E), secondary cell wall thickness in fiber cells was reduced in both xylem and phloem tissues (Fig. 5, F–H). The thickness of phloem fiber cell walls and xylem fiber cell walls was reduced by 50% and 35% (Table I), respectively. However, the thickness of vessel cell walls increased by about 120% (Table I; Fig. 5G). Suppression of *PtrWND1B* expression led to specific inhibition of fiber secondary wall thickening in xylem and phloem tissues, resulting in corresponding stem phenotypes.

To investigate how *PtrWND1B* suppression affects the transcription process leading to cell wall thickening in the transgenic plants, we examined the expression of cell wall thickening-associated genes, including fiber-associated *NST* transcription factor genes such as *WND1A* and *WND2A*, vessel-associated *VND* transcription factor genes such as *WND3A* and *WND6A*, their downstream transcription factor genes such as *MYB3*, *MYB20*, and *MYB28*, and typical secondary cell wall biosynthesis genes such as cellulose synthesis-related *Cellulose synthase8* (*CesA8*), xylan-related *Glycosyltransferase family43B* (*GT43B*), and lignin-related *4-Coumarate-CoA Ligase1* (*4CL1*). Transcript levels of the NST group and secondary wall biosynthesis genes were reduced, while the abundance of the VND group transcripts increased slightly (Fig. 6). These results are consistent with suppressed wall thickening in fiber cells and enhanced wall thickening in vessel cells.

Up-regulation of *PtrWND1B* transcripts was carried out by transferring a genomic fragment of *PtrWND1B* into *Populus* spp. under the control of the CaMV 35S promoter (35S:*PtrWND1B*). Three lines were selected for further analysis, and these transgenic plants exhibited a phenotype of small leaves that were curled upward or downward (Fig. 7, A and B). No obvious physical changes were observed in the stem when dissected, and the wall thickness of xylem and phloem fiber cells was not changed. No changes were observed in the vessel walls (Fig. 7, E and G; Table I). Both *PtrWND1B* and *PtrWND1B-l* transcripts were increased in the stems of the transgenic plants (Fig. 7C). *WND1A* transcripts also increased, but the relative abundance of the transcripts for other transcription factors and secondary cell wall biosynthesis genes was not affected (Supplemental Fig. S5A). Introduction of an additional copy of *PtrWND1B* resulted in the concurrent increase of both splice variants but did not affect secondary wall thickening in stem fiber cells. However, secondary cell wall biosynthesis was significantly up-regulated in the curled leaves of the transgenic plants (Supplemental Fig. S5B). This could be due to the ectopic expression of *PtrWND1B* in leaf cells in which the transcript abundance for cell wall formation genes was modified.

Overexpression of *PtrWND1B-l* Inhibits Fiber Formation

The full coding sequences of *PtrWND1B-s* and *PtrWND1B-l* cDNAs were overexpressed in *Populus* spp. under the control of the *PtrWND1B* promoter

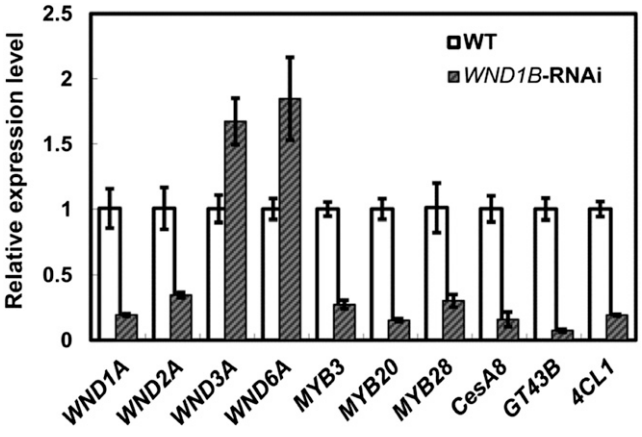

Figure 6. Expression of secondary wall-associated genes in stem of transgenic *WND1B* knockdown (*WND1B-RNAi*) plants. The secondary wall NAC regulators *PtrWNDs* can be divided into two groups, the NST group and the VND group, which may regulate fiber and vessel cell wall thickening, respectively (Ohtani et al., 2011). *WND1A* and *WND2A* belong to the NST group, and *WND3A* and *WND6A* belong to the VND group. Several *MYB* transcriptional factors, including *MYB3*, *MYB20*, and *MYB28*, are considered as common targets of the *PtrWNDs* in the transcriptional networks of secondary cell wall formation (Zhong et al., 2010a). *CesA8*, *GT43B*, and *4CL1* are involved in the biosynthesis of the secondary cell wall components cellulose, hemicellulose, and lignin biosynthesis, respectively. Error bars represent SE of three independent biological replicates. WT, Wild type.

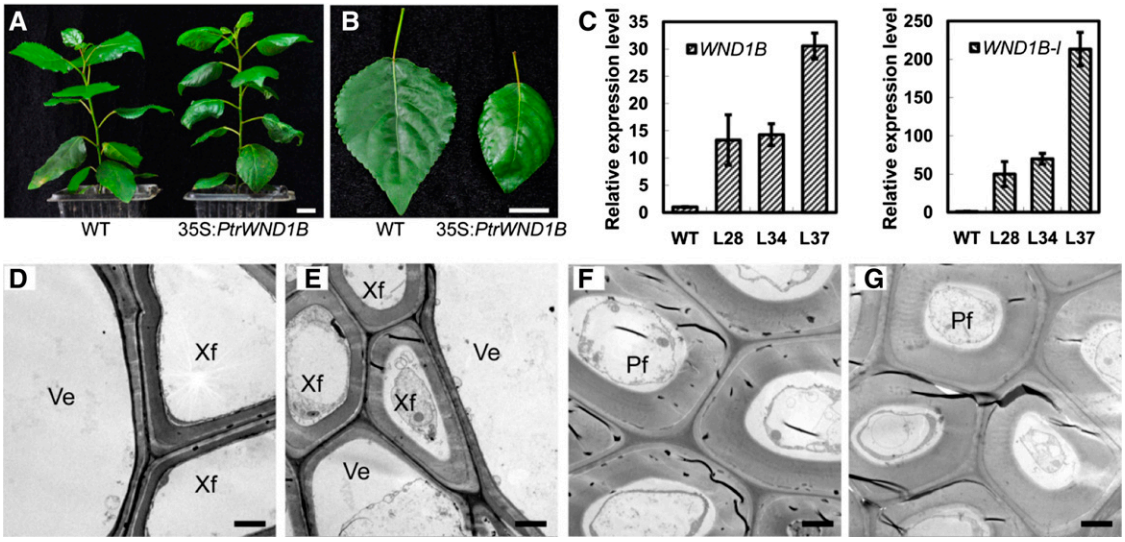

Figure 7. Effects of *PtrWND1B* overexpression on plant growth. A, A wild-type plant (WT) and transgenic *Populus* spp. overexpressing *PtrWND1B* (35S:*PtrWND1B*). B, Representative leaves of wild-type and 35S:*PtrWND1B* plants (L34; similar phenotypes were observed with other lines). C, Quantitative RT-PCR analysis of *PtrWND1B-s* and *PtrWND1B-l* transcripts in 35S:*PtrWND1B* transgenic lines (L28, L34, and L37). D and E, Transmission electron micrographs of xylem fiber and vessel walls in cross sections of the eighth internode stem of the wild type (D) and 35S:*PtrWND1B* transgenic line L34 (E). F and G, Transmission electron micrographs of phloem fiber walls in cross sections of the eighth internode stem of the wild type (F) and 35S:*PtrWND1B* transgenic line L34 (G). Similar phenotypes were observed with other lines. Pf, Phloem fiber cell; Ve, vessel cell; Xf, xylem fiber cell. Bars = 2 cm (A and B) and 2 μm (D–G).

(*PtrWND1B*:*PtrWND1B-s* and *PtrWND1B*:*PtrWND1B-l*) to determine the roles of the two splice variants of *PtrWND1B* in planta. The *PtrWND1B*:*PtrWND1B-s* plants grew straight and appeared similar to the wild type (Fig. 8A). In contrast, *PtrWND1B*:*PtrWND1B-l* lines could not grow upright (Fig. 8B) and appeared similar to the *PtrWND1B-RNAi* lines (Fig. 5A). Expression of the transferred *PtrWND1B-s* and *PtrWND1B-l* genes was verified in the transgenic trees (Fig. 8C). Cross sections of the eighth internode of the stems showed that the secondary cell wall thickening of fiber cells was suppressed in *PtrWND1B*:*PtrWND1B-l* lines but increased in *PtrWND1B*:*PtrWND1B-s* lines (Supplemental Fig. S6). The wall thickness of xylem and phloem fiber cells in *PtrWND1B*:*PtrWND1B-s* plants was increased by 37% and 17%, respectively, but reduced by 43% and 31% in *PtrWND1B*:*PtrWND1B-l* plants compared with the wild type (Fig. 8, D–I; Table I). Meanwhile, the wall thickness of vessel cells in *PtrWND1B*:*PtrWND1B-s* plants was not changed but was increased by 91% in *PtrWND1B*:*PtrWND1B-l* plants (Fig. 8, D–I; Table I). Transcripts of the NST group transcription factor and secondary cell wall biosynthesis genes were increased in *PtrWND1B*:*PtrWND1B-s* plants (Fig. 9A) but reduced in *PtrWND1B*:*PtrWND1B-l* plants (Fig. 9B). However, transcripts of the VND group genes were little changed in *PtrWND1B*:*PtrWND1B-s* plants (Fig. 9A) but increased in *PtrWND1B*:*PtrWND1B-l* plants (Fig. 9B). Together, these results demonstrate that *PtrWND1B-s* and *PtrWND1B-l* play antagonistic roles in fiber secondary wall formation. *PtrWND1B-s* plays a positive role, while *PtrWND1B-l* plays a negative role, in the secondary cell wall thickening of both xylem and phloem fibers.

DISCUSSION

AS of *PtrWND1B* in Trees

Cell wall thickening is a major component of wood formation. However, mechanistic understanding of this process in trees is limited. In herbaceous Arabidopsis, cell wall thickening involves the activation of distinct transcriptional regulation networks in the different cell types of vascular tissue. For example, the NAC TF *VND1-7* preferentially regulates vessel formation (Kubo et al., 2005; Yamaguchi et al., 2008), while two other NAC TFs, *SND1* and *NST1*, preferentially regulate fiber formation (Zhong et al., 2006, 2007; Mitsuda et al., 2007). The TFs switch on the same group of downstream TFs, including *MYB46*, *MYB83*, *MYB58*, and *MYB63*, which results in the regulated biosynthesis of secondary wall components (Zhong et al., 2008; Caño-Delgado et al., 2010; Demura and Ye, 2010). This transcriptional regulation network is believed to be conserved in multiple higher plants (Zhong et al., 2010a). In cottonwood, *PtrWNDs* are considered functional orthologs of Arabidopsis *NSTs* or *VNDs* (Zhong et al., 2010b; Ohtani et al., 2011). However, because wood formation in trees displays very different characteristics from vascular development in herbaceous plants, it is important to determine the similarities and differences in the

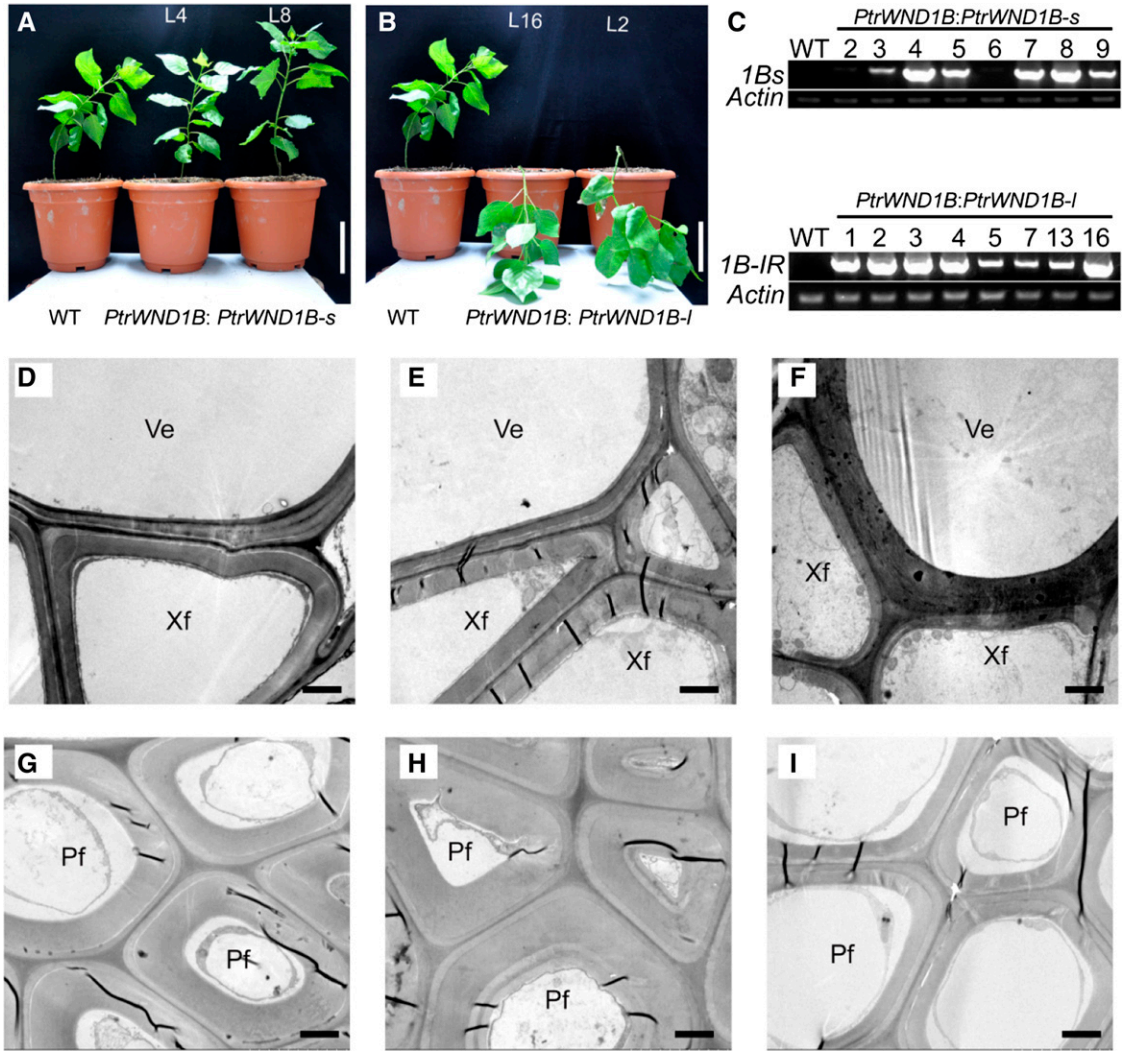

Figure 8. Effects of the AS event of *PtrWND1B* on secondary wall thickening in *Populus* spp. cells. A, The wild type (WT) and *PtrWND1B*:*PtrWND1Bs* transgenic lines L4 and L8. B, The wild type and *PtrWND1B*:*PtrWND1B-l* transgenic lines L16 and L2. C, *PtrWND1B-s* or *PtrWND1B-l* expression in *PtrWND1B*:*PtrWND1B-s* (top) or *PtrWND1B*:*PtrWND1B-l* (bottom) transgenic lines. *PtrACTIN2* was used as a reference. D to F, Transmission electron micrographs of xylem fiber (Xf) and vessel (Ve) walls in cross sections of the eighth internode stem of the wild type (D), *PtrWND1B*:*PtrWND1B-s* L4 (E), and *PtrWND1B*:*PtrWND1B-l* L16 (F). G to I, Transmission electron micrographs of phloem fiber (Pf) walls in cross sections of the eighth internode stem of the wild type (G), *PtrWND1B*:*PtrWND1B-s* L4 (H), and *PtrWND1B*:*PtrWND1B-l* L16 (I). Similar phenotypes were observed with other lines. Bars = 10 cm (A and B) and 2 μm (D–I).

mechanisms regulating secondary cell wall thickening in trees as in Arabidopsis and other herbaceous plants.

PtrWND1B, one of the NST group NAC genes in cottonwood, was found to be subject to AS in this study and a previous study (Li et al., 2012). Two alternative transcripts (*PtrWND1B-s* and *PtrWND1B-l*) were detected specifically in wood-forming tissue. *PtrWND1B-s* encodes a full-length NAC protein with a conserved N-terminal NAC domain and a C-terminal activation domain, and *PtrWND1B-l* encodes a partial NAC domain protein with a protein dimerization domain but without the activation domain, due to the introduction of an early stop codon in the retained intron 2 (Li et al., 2012). AS does not occur in any NST group NAC genes in Arabidopsis but occurs in the homologs of *SND1* in woody plants. In addition to PtrWND1B in *Populus* spp., AS was observed in the SND1 homolog Eucgr.E01053 in *Eucalyptus grandis*. However, the analogous AS event was not observed in monocot rice (*Oryza sativa*; Wang and Brendel, 2006). Thus, the AS event does not occur in all plants but rather appears to be a special event in some woody plants, such as black cottonwood and *Eucalyptus grandis*. The transcripts of both splice variants can be translated into their corresponding proteins, which are located in the fiber cells of secondary vascular tissue. AS may have evolved as a

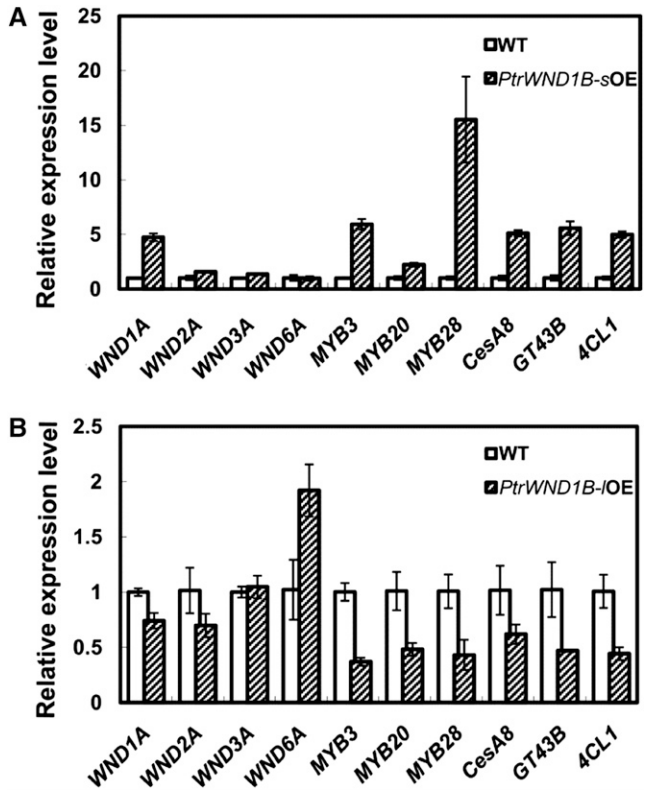

Figure 9. Two alternative *PtrWND1B* transcripts play distinct roles in the regulation of secondary wall formation in *Populus* spp. A and B, Expression of several secondary wall-associated genes was up-regulated in *PtrWND1B*:*PtrWND1B-s* (*PtrWND1B-s*OE) transgenic lines (A) but down-regulated in *PtrWND1B*:*PtrWND1B-l* (*PtrWND1B-l*OE) transgenic lines (B). Error bars represent SE of three independent biological replicates. WT, Wild type.

native mechanism associated with the regulation of wood formation.

An AS Event Determined by the Intron 2 Sequence of *PtrWND1B*

AS events occur relatively frequently in a large portion of plant genes (Lu et al., 2010; Marquez et al., 2012). However, little is known about how or why AS occurs in plants. In animals, many occurrences of AS are related to intron structure (Reddy, 2007). Research on the AS of plant genes is largely focused on variation in the expression or activity of splicing-related proteins (Sanchez et al., 2010; Sugliani et al., 2010; Reddy et al., 2012). *PtrWND1B* AS occurs naturally in *Populus* spp. and can be induced to occur in Arabidopsis when transformed with the *PtrWND1B* gene. However, Arabidopsis *SND1* does not undergo AS even when it is transferred into *Populus* spp. These results suggest that the AS event of *PtrWND1B* is likely not related to the plant species system but rather is dependent on the gene sequence.

PtrWND1A and *PtrWND1B* were likely generated as the result of a recent duplication of the cottonwood genome (Tuskan et al., 2006). The *PtrWND1A* gene encodes a NAC protein that shares 89% amino acid sequence identity with PtrWND1B. However, AS does not occur in *PtrWND1A* or its Arabidopsis homolog *SND1*. Among the NST group NAC TFs in Arabidopsis and *Populus* spp., *PtrWND1B* was the only gene to undergo AS, which takes place during the process of fiber cell development. The sequence of intron 2 of *PtrWND1B* appears to be a critical determinant of AS. A swap of intron 2 between *SND1* and *PtrWND1B* revealed that the intron 2 sequence of *PtrWND1B* contains a distinct structure that mediates *PtrWND1B* AS, while AS did not occur in WND1A and SND1 as a result of having different intron 2 sequences.

Sequence alignment (Supplemental Fig. S3B) analysis of the three genes indicates that a fragment with TA-rich elements is absent in *PtrWND1B* intron 2 but conserved in *PtrWND1A* and *SND1* intron 2. Generally, the T-rich and TA-rich regions in introns are known to be important for the recognition of splice sites and for the efficient splicing of introns (Reddy, 2007; Reddy et al., 2012). Thus, we hypothesize that *PtrWND1B* may have undergone a deletion mutation and lost part of its intron 2 sequence, possibly containing TA-rich elements. Those elements, which are present in *PtrWND1A* and other paralogs/orthologs, may play a critical role in controlling the intron 2 AS events. Consistent with this hypothesis, the length of the *PtrWND1B* intron 2 sequence (330 bp) is shorter than that of the *PtrWND1A* intron 2 (384 bp) and *SND1* intron 2 (436 bp) sequences. After the duplication of the *SND1* gene in trees, AS may have contributed to the evolution of the role of *PtrWND1B* in regulating wood formation.

PtrWND1B AS May Serve as a Mechanism to Regulate the Secondary Cell Wall Formation of Fiber Cells in *Populus* spp.

Although AS has been detected in many plant genes, little is known about the significance of AS for plant development. Recently, another group (Li et al., 2012) reported the characterization of the alternative *PtrWND1B* splice variant, *PtrSND1-A2*^{IR} (*PtrWND1B-l* in this study), using a protoplast system. Dimerization is required in order for the PtrWND-type transcription factors to perform their transactivating function (Ernst et al., 2004; Li et al., 2012). Overexpression of the splice variant in protoplast repressed the expression of its *PtrSND1* homologs and *PtrMYB021*. The truncated protein encoded by the splice variant, which lacked DNA-binding abilities but retained its dimerization capability, was able to disrupt the function of SND1 homologs, likely through forming nonfunctional heterodimers with normal SND1 proteins (Li et al., 2012). However, no explicit linkage was made between the dominant-negative effect caused by the PtrWND1B AS event and wood formation in vivo.

In this study, PtrWND1B proteins were found to be predominantly localized in xylem fiber cells and phloem

fiber cells. Knockdown of *PtrWND1B* suppressed secondary wall thickening in xylem fiber and phloem fiber cells, while up-regulation of *PtrWND1B-s* expression under the control of its own promoter enhanced the thickening of the fiber secondary wall without affecting vessels. Transcriptional analysis revealed that NST group genes and their downstream transcriptional network genes were correspondingly down-regulated in the *PtrWND1B*-down-regulated trees and upregulated in the *PtrWND1B-s*-overexpressed trees. These results suggest that *PtrWND1B* is primarily associated with fiber secondary wall formation. Meanwhile, knockdown of *PtrWND1B* as well as up-regulation of *PtrWND1B-l* using its own promoter enhanced vessel wall thickness. This indicates that different networks exist to regulate the wall thickening of fiber cells versus vessel cells. Similar phenomena were reported in Arabidopsis, where *SND1* overexpression suppressed fiber secondary wall thickening but promoted vessel secondary wall thickening (Zhong et al., 2006; Ko et al., 2007).

PtrWND1B-s was found to play a positive role, and *PtrWND1B-l* to play a negative role, in fiber secondary wall formation. Up-regulation of *PtrWND1B-s* promoted fiber secondary wall thickening, while up-regulation of *PtrWND1B-l* expression suppressed secondary wall thickening, a similar effect to *PtrWND1B* down-regulation. SND1-type transcript factors are suggested to form dimers to regulate wall thickening in the process of xylem development (Zhong et al., 2010b; Ohtani et al., 2011; Li et al., 2012). The negative role of *PtrWND1B-l* can be attributed to the ability of the truncated PtrWND1B-l protein, which lost its DNA-binding domain and transcription activation domain but retained its dimerization domain, to form heterodimers with NST group PtrWNDs and interfere with their functions (Li et al., 2012). Similar effects have also been investigated in other TFs; for example, artificially truncated $VND7^{1-161}$ was shown to inhibit VND7 function in Arabidopsis (Yamaguchi et al., 2008).

In Arabidopsis, both repression and overexpression of *SND1* caused deficiencies in fiber cell wall formation (Zhong et al., 2006; Ko et al., 2007). In *Populus* spp., AS of the *SND1* homolog *PtrWND1B* produced two forms of *PtrWND1B* transcripts, and individual overexpression of the two *PtrWND1B* splice variants showed different effects on fiber cell wall thickening. *PtrWND1B-s* overexpression manifested in the development of a thicker fiber cell wall, while *PtrWND1B-l* overexpression resulted in thinner fiber cell walls. However, overexpression of *PtrWND1B* had no evident effect on fiber cell wall formation in the stem of *Populus* spp. The two splice variants, which play antagonistic roles, may cancel each other out when *PtrWND1B* is overexpressed. Alternatively, the relative abundance with which the two forms of *PtrWND1B* are expressed could constitute a gradient in regulating the level of fiber cell wall thickening. Consistent with this model, increasing the ratio of *PtrWND1B-l* abundance negated the effect of *SND1*, with no evident impact on the phenotypes of transgenic *Populus* spp. overexpressing Arabidopsis *SND1*. Fiber cell wall thickness is a critical factor affecting wood properties and utilization. The results yielded from this study may have implications for improving wood fiber quality through engineering the AS regulation in order to modify fiber cell wall thickening during wood formation.

The wood formation process is controlled by a complex network of several NAC TF genes (Caño-Delgado et al., 2010; Demura and Ye, 2010; Zhong et al., 2010a). How the various TFs in the network are coordinated remains an area of intense study. AS of *PtrWND1B* may have evolved in trees as a mechanism to regulate fiber cell wall thickening in wood formation. These findings raise outstanding questions, such as what factors control the ratio of the two *PtrWND1B* transcript formats, and more broadly, how are fiber cell wall thickening and the wood formation process coordinated? The expression of two *PtrWND1B* variants could be an example of how AS may serve to help ensure a steady process of wood formation by buffering fluctuations in NAC TF gene expression.

CONCLUSION

PtrWND1B, a key gene in the transcriptional network of secondary cell wall thickening, was shown to undergo AS exclusively in secondary xylem fiber cells in *Populus* spp. The *PtrWND1B* intron 2-mediated AS results in the generation of two isoforms (*PtrWND1B-s* and *PtrWND1B-l*) that play antagonistic roles in regulating cell wall thickening during fiber cell differentiation. *PtrWND1B-s* codes a full-size NAC protein to transactivate the downstream transcriptional network of secondary cell wall thickening, while *PtrWND1B-l* codes a truncated protein to dominantly repress fiber cell wall thickening. Together, the results demonstrated in planta that *PtrWND1B* AS functions as a means to regulate and maintain the homeostasis of PtrWNDs for safeguarding steady fiber wall thickening in woody dicots.

MATERIALS AND METHODS

Plant Materials

Populus trichocarpa genotype Nisqually-1 was used for gene cloning and expression analysis. *Populus* × *euramericana* 'Nanlin895' was used for genetic transformation, according to Li et al. (2003). Arabidopsis (*Arabidopsis thaliana* Columbia) was used for genetic transformation in accordance with previously described methods (Clough and Bent, 1998). *Eucalyptus grandis* was used for RNA sequencing. *Populus* spp. was maintained in a phytotron or greenhouse as described (Zhao et al., 2013). Arabidopsis was grown in a phytotron with a light and dark cycle of 16 h and 8 h at 22°C.

Sequence Analysis

PtrWND sequences were retrieved from the poplar genome database (http://www.phytozome.net/poplar). *SND1*, *NST1*, and *NST2* sequences were retrieved from The Arabidopsis Information Resource (http://www.arabidopsis.org/). The deduced protein sequences were aligned using the

ClustalW method (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The neighbor-joining tree was constructed using MEGA 5.1 (Tamura et al., 2011). Bootstrap values were calculated from 1,000 trials.

For RNA sequencing analysis of SND1 homologs in *Eucalyptus grandis*, developing xylem tissue of *Eucalyptus grandis* was collected after bark removal. Total RNA was extracted using Trizol reagents (Invitrogen; http://zh.invitrogen.com/). RNA concentration and quality were determined using an Agilent Bioanalyzer 2100. The cDNA libraries were constructed from poly(A)-enriched RNA following Illumina protocols. cDNA fragments of 200 to 300 bp were selected, and nondirectional 100-nucleotide paired-end sequencing was carried out (Illumina HiSEquation 2000). The resulting sequences were mapped to a reference genome (version 1.0; http://www.phytozome.net/) using TopHat (version 2.0.5). The mapped reads of Eucgr.E01053 to the genome were visualized with the Integrative Genomics Viewer (version 2.1.25). Sequencing depths at the nucleotide level were obtained with SAMtools (version 0.1.18) and extracted using PERL scripts.

Molecular Cloning and Expression Analysis

Total RNA was isolated from various tissues and treated with RNase-free DNase I to remove DNA contamination. First-strand cDNA synthesis was performed using the TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix kit (TransGen Biotech; http://english.transgen.com.cn/). Trace genomic DNA was further eliminated by genomic DNA removal (TransGen Biotech) during the first-stand cDNA synthesis. Then, the first-strand cDNA was used as a template for gene cloning and transcript analysis. The cDNAs of the *Populus* spp. *PtrWNDs* and Arabidopsis *SND1*, *NST*, and *NST2* were amplified using specific primer sets (Supplemental Table S1) and cloned into pMD19-T vectors (Takara; http://www.takara.com.cn/) for sequencing. Reverse transcription (RT)-PCR was conducted for variable cycles to determine the logarithmic phase of amplifications for the samples. RT-PCR was repeated three times, and identical results were obtained. The transcript level of the *Populus* spp. *ACTIN2* gene was used as an internal control. For quantitative real-time PCR, primers (Supplemental Table S1) were designed to amplify a specific fragment (100–300 bp in length) from the target genes. Quantitative real-time PCR was performed using iQ SYBR Green Supermix and an iQ5 Real-Time PCR Detection System (Bio-Rad; http://www.bio-rad.com/). Gene expression values were normalized using the *Populus* spp. *ACTIN2* gene as a reference.

Intron Swap of *PtrWND1B* and *SND1*

Intron 2 swap of *PtrWND1B* and *SND1* was performed by PCR-mediated recombination (Fang et al., 1999) and gave *PtrWND1Bf* and *SND1f*. Specific overlapping primers were designed and are listed in Supplemental Table S1. The recombinant chimeric genes were verified by sequencing.

Constructs

For suppression of *PtrWND1B*, a 239-bp fragment in *PtrWND1B* transcriptional activation regions was selected and cloned into a pRNAi vector (Limpens et al., 2004) in both sense and antisense orientations to generate inverted repeat sequences, and a 2,132-bp upstream sequence of the ATG start codon of the *PtrWND1B* gene was cloned into the above vector to replace the CaMV 35S promoter. Then, the fragment including the *PtrWND1B* promoter, inverted repeat sequences, and *Octopine synthase* terminator was subcloned into the binary vector pCAMBIA2300. For overexpression of *PtrWND1B*, a genomic fragment including the full-length coding region and two intron regions was placed under the control of the CaMV 35S promoter in the binary vector pCAMBIA2300.

For individual up-regulation of *PtrWND1B-s* or *PtrWND1B-l*, the full-length coding sequence of *PtrWND1B-s* or *PtrWND1B-l* driven by the 2.1-kb *PtrWND1B* promoter was cloned into the pCAMBIA2300 binary vector. For overexpression of *PtrWND1Bf* and *SND1f*, the recombinant chimeric genes were placed under the control of the CaMV 35S promoter in the binary vector pCAMBIA2300.

Primers used in vector construction are listed in Supplemental Table S1. All constructs above were mobilized into *Agrobacterium tumefaciens* strain GV3101 for transformation of *Populus* spp. or Arabidopsis.

Antibody Production, Western Blot, and Immunolocalization

PtrWND1B-specific peptides PEDMMNLSIN, SLDSKAHQIL, SFDQDRSFKS, SVIHGSKSGQ, SSDPLCHLSV, and QLSHLQNSHR were recombinantly expressed in *Escherichia coli* and used to raise polyclonal antibodies in rabbits (Abmart; http://www.ab-mart.com.cn/). Anti-glutathione *S*-transferase and anti-actin monoclonal antibodies were purchased from Abmart. Western-blot analysis and immunolocalization were performed according to methods detailed in previous studies (Song et al., 2010).

Production of Prokaryotic Recombinant Protein in *Escherichia coli* and Antibody Specificity Test

The full-length cDNAs of *PtrWND1B-s*, *PtrWND1B-l*, and *PtrWND3B* were each cloned into a pET vector and fused in frame with a SUMO tag at the N terminus (Novagen; http://www.merckmillipore.cn). After the sequence was confirmed, the constructs were transformed into *Escherichia coli* BL21 (DE3) strain (Invitrogen; http://zh.invitrogen.com/site/cn/zh/home.html), and recombinant protein expression was induced with 0.5 M isopropylthio-β-galactoside at 37°C for 3 h. The protein samples were loaded onto an SDS-PAGE gel for Commassie blue staining and western-blot analysis.

Microscopy and Wall Thickness Analysis

The eighth internode of stems from 3-month-old *Populus* spp. plants was fixed in formaldehyde-acetic acid solution (formaldehyde:glacial acetic acid:ethanol [1:1:18]) and embedded in Paraplast. Ten-micrometer sections were cut using an RM2235 rotary microtome (Leica; http://www.leica-microsystems.com/products), stained with toluidine blue, and observed with a BX51 light microscope (Olympus; http://www.olympus-global.com/). Transmission electron microscopy was performed according to methods detailed in previous studies (Song et al., 2010). Wall thickness was measured from transmission electron micrographs using ImageJ software (http://rsb.info.nih.gov/ij/). Data from at least 30 cells in each of three plants were collected and analyzed statistically using Student's *t* test.

Sequence data from this article can be found in the GenBank data library under the accession numbers HQ215847, XM_002317023 (*PtrWND1A*), XM_002300464 (*PtrWND1B*), HQ215849, XM_002320861 (*PtrWND2A*), XM_002302636 (*PtrWND2B*), XM_002322362 (*PtrWND3A*), XM_002327206 (*PtrWND6A*), XM_002299908 (*PtrMYB3*), XM_002313267 (*PtrMYB20*), XM_002307154 (*PtrMYB28*), XM_002316779 (*PtrCesA8*), JF518935 (*PtrGT43B*), XM_002297663 (*Ptr4CL1*), and XM_002298674 (*PtrACT2*) and in The Arabidopsis Genome Initiative under locus identifiers At1g32770 (*SND1*), At2g46770 (*NST1*), and At3g61910 (*NST2*).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. *SND1* homolog in *Eucalyptus* spp. also occurs AS.

Supplemental Figure S2. Antibody specificity of anti-PtrWND1B.

Supplemental Figure S3. *AtSND1* overexpression resulted in changes in expression of *WND1B* and *WND1B-l* in *Populus* spp.

Supplemental Figure S4. Sequence alignment of intron 2 of *PtrWND1B*, *PtrWND1A*, and *AtSND1* genes.

Supplemental Figure S5. Expression of secondary wall-associated genes in the 35S: *PtrWND1B* transgenic *Populus* spp.

Supplemental Figure S6. Effects of *PtrWND1B-s* and *PtrWND1B-l* on xylem cell secondary wall thickening.

Supplemental Table S1. List of primers used in this study.

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