PdWND3A, a wood-associated NAC domain-containing protein, affects lignin biosynthesis and composition in *Populus*

Yongil Yang1, Chang Geun Yoo1,2,3, William Rottmann4, Kimberly A. Winkeler4, Cassandra M. Collins4, Lee E. Gunter1,2, Sara S. Jawdy1,2, Xiaohan Yang1,2, Yunqiao Pu1,2,3, Arthur J. Ragauskas1,2,3,5, Gerald A. Tuskan1,2 and Jin-Gui Chen1,2*

Abstract

**Background:** Plant secondary cell wall is a renewable feedstock for biofuels and biomaterials production. *Arabidopsis* VASCULAR-RELATED NAC DOMAIN (VND) has been demonstrated to be a key transcription factor regulating secondary cell wall biosynthesis. However, less is known about its role in the woody species.

**Results:** Here we report the functional characterization of *Populus deltoides* WOOD-ASSOCIATED NAC DOMAIN protein 3 (PdWND3A), a sequence homolog of *Arabidopsis* VND4 and VND5 that are members of transcription factor networks regulating secondary cell wall biosynthesis. PdWND3A was expressed at higher level in the xylem than in other tissues. The stem tissues of transgenic *P. deltoides* overexpressing PdWND3A (OXPdWND3A) contained more vessel cells than that of wild-type plants. Furthermore, lignin content and lignin monomer syringyl and guaiacyl (S/G) ratio were higher in OXPdWND3A transgenic plants than in wild-type plants. Consistent with these observations, the expression of *FERULATE 5-HYDROXYLASE1* (F5H1), encoding an enzyme involved in the biosynthesis of sinapyl alcohol (S unit monolignol), was elevated in OXPdWND3A transgenic plants. Saccharification analysis indicated that the rate of sugar release was reduced in the transgenic plants. In addition, OXPdWND3A transgenic plants produced lower amounts of biomass than wild-type plants.

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Background
Plant cell walls define cellular space and protect internal cellular component against extracellular biotic and abiotic stimuli. In addition to the structural roles, plant cell walls have become an attractive target for conversion into biofuels and biomaterials due to their abundance, alternate chemical composition properties, and renewability. Plant cell walls are generally composed of two types of walls, i.e., the primary cell wall and secondary cell wall. The primary cell wall typically consists of cellulose, hemicellulose and pectin whereas the secondary cell wall contains a larger proportion of lignin [22, 56]. Of these secondary cell wall components, cellulose and hemicellulose are polysaccharides and are being considered as substrates for conversion into biofuels [4, 10, 26]. Lignin as a polyphenolic biopolymer contributes to cell rigidity and protection against pathogens [3]. In addition, lignin facilitates hydrophilic transport by coating the interior of vessels which helps regulate water relations in the plant. However, from the perspective of biofuels production lignin is regarded as a major recalcitrance factor limiting access to cell wall polysaccharides. Therefore, genetic modification of lignin biosynthesis pathway has become an effective approach for reducing recalcitrance and improving biofuel conversion and production.

Phenylpropanoids, derived from phenylalanine, are the pivotal metabolic precursors to monolignol biosynthesize [16, 29, 45]. The general phenylpropanoid pathway includes three initial steps that are catalyzed by L-phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H) and 4-coumarate-CoA ligase (4CL) [15, 35, 37]. 4-coumaryl-CoA is the final product of general phenylpropanoid pathway and is the precursor chemical for synthesizing three different chemical families, i.e., flavonoids, monolignols, and phenolic acids. The lignin biosynthetic pathway has been well characterized and most biosynthetic enzymes have been identified [2, 29, 44]. Lignin is composed of three monomers known as hydroxymethyl (H), guaiacyl (G) and syringyl (S) that are derived from p-coumaryl, coniferyl and sinapyl alcohols, respectively, and whose productions are regulated by caffeoyl-CoA O-methyltransferase (CCoAOMT), ferulate 5-hydroxylase (F5H), cinnamoyl-CoA reductase (CCR), and cinnamyl alcohol dehydrogenase (CAD) [12, 14, 25, 29, 31, 52]. The regulation and expression of lignin biosynthetic genes is associated with several transcription factors, including NAC (No Apical Meristem (NAM), Arabidopsis Transcriptional Activation Factor (ATAF1/2), Cup-shaped Cotyledon (CUC2)), and V-myb myeloblastosis viral oncogene homolog (MYB) [11, 16, 56]. Of these transcription factors, NAC family proteins function as the master switch regulator of secondary cell wall formation. Kubo et al. [13] suggested that the NAC transcription factors of VASCULAR-RELATED NAC-DOMAIN (VND) 1–7 subfamily act as master regulators of meta and proto xylem vessel formation in Arabidopsis root. NAC SECONDARY WALL THICKENING PROMOTING FACTOR1 (NST1) and NST3/SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN1 (SND1) have also been shown to act as master transcriptional regulators of secondary cell wall formation and fiber cell differentiation [13, 20, 21, 23, 41, 47]. SND1 has been reported to bind directly to the promoter of MYB6 [53]. SND1 also acts as a switch to regulate the expression of many downstream genes related to the secondary cell wall biosynthesis including cellulose and lignin biosynthesis. In Arabidopsis, the intricate network of transcriptional regulation of secondary cell wall biosynthesis has been summarized in several recent review articles [16, 22, 49, 55, 56].

As NAC family members, Arabidopsis VND 1–7 (AtVND1–7) were initially identified in the early stage of xylem vessel cell trans-differentiation using Arabidopsis suspension cultures [5]. The transgenic Arabidopsis overexpressing AtVND1–7 resulted in ectopic formation of xylem vessel element [6, 13, 39, 58]. The comparative transcriptome analysis of inducible expression of AtVND6 and AtSND1 in transgenic Arabidopsis system showed that the upregulated genes by AtVND6 were overlapped with those genes by AtSND1 [23]. However, there were also genes that were preferentially regulated by AtVND6 or AtSND1 [23]. Furthermore, a total of 63 genes encoding a broad range of proteins, including both transcription factors and non-transcription factors involved in the programmed cell death were identified as target genes of AtVND7 in an overexpression study [40]. Therefore, AtVNDs share with AtSND a common set of downstream target genes but also regulate the expression of target genes that are distinct from those regulated by AtSND. Electrophoretic mobility shift assay of AtVND1–7 and transactivation analysis of AtVND6 and AtVND7 showed that AtVNDs bind to the 19-bp consensus DNA sequence of secondary wall NAC binding element (SNBE) and the 11-bp tracheary-element-
regulating cis-elements (TERE) in the promoter region of a group of genes involved in the secondary cell wall biosynthesis, cell wall modification, and programmed cell death [6, 23, 48]. Both TERE and SNBE were also found in the promoter sequences of some direct target genes of AtSND1 [23, 28, 48, 50, 51].

In the woody perennial species *Populus trichocarpa*, a total of eight genes among 16 *Populus* NAC domain protein genes were sub-grouped as *Populus* VND (PtrWND/PtrVNS) [22, 24, 48]. Dominant repression of PtrWND2B/PtrVNS10 and PtrWND6B/PtrVNS08 using EAR-induced dominant repression approach in hybrid *Populus* (*P. tremula × P. alba*) resulted in reduction of wall thickness of xylary fibers [51], whereas ectopic secondary wall thickening phenotype was observed in transgenic *Populus* plants over-expressing all PtrWND/PtrVNS genes driven by cauliflower mosaic virus 35S promoter [24]. Moreover, ectopic deposition of lignin, cellulose, and hemicellulose was observed in transgenic *Arabidopsis* and *Populus* overexpressing PtrWND6B (an AtVND7 homolog) [48, 51]. Therefore, *Populus* VND-related proteins (PtrVND) appeared to function similarly as AtVND in the regulation of vascular vessel formation and secondary cell wall biosynthesis [24, 48, 51]. This was further supported by the observation that heterologous expression of PtrWND3A/PtrVNS05 and PtrWND3B/PtrVNS06 (AtVND4 and 5 homologs) in *Arabidopsis* resulted in ectopic secondary wall deposition in leaf [24].

Here we report the functional characterization of PdWND3A, an AtVND4/5 sequence homolog, using *Populus* transgenics. The transgenic *Populus* overexpressing PdWND3A displayed increased vessel formation in the stem. Both lignin content and lignin S/G ratio were increased in the transgenic plants. Interestingly, RT-PCR analysis indicated that among tested secondary cell wall biosynthesis-related genes, the expression of FSH1 was predominantly up-regulated in the transgenic plants, suggesting that PdWND3A may affect lignin biosynthesis and composition by regulating FSH1 expression.

**Results**

**Phylogenetic analysis of Populus NAC domain-containing proteins**

In a previous studies, Zhong and Ye [54] used AtSND1 (AT1G32770) as a template to search for *Populus* homologs and defined their nomenclatures. In another study, Ohtani et al. identified 16 NAC domain protein genes in the *Populus* genome based on the protein homology analysis with *Arabidopsis* VND/NST/SND protein [24]. With the availability of the latest *P. trichocarpa* genome annotation (v3.1), we used AtSND1 as a template to search of all possible AtSND1 sequence homologs in Phytozome (https://phytozome.jgi.doe.gov) [8] and identified a total of 21 *Populus* loci with a cutoff of amino acid sequence identity >30% (Additional file 2). Among these proteins, a group of eight *Populus* proteins showing high amino acid sequence identity with respective AtVND proteins were selected for further study (Additional file 3). Two clades, including four *Populus* proteins (Potri.012G126500, Potri.015G127400, Potri.001G120000 and Potri.003G113000), shared a cluster with AtVND4 and AtVND5. On the basis of these results, we selected Potri.015G127400, which was previously designated as PtrWND3A [54] for further characterization. PtrWND3B (Potri.012G126500), in the same clade with PtrWND3A, shared 95.3% similarity with PtrWND3A at the amino acid level (Additional file 3). A DNA fragment of 24 bp is absent in the middle of coding sequence of PtrWND3A; therefore, we were able to use gene-specific primer for this region to distinguish PtrWND3A from PtrWND3B (Additional file 4). Similar to *Arabidopsis* VND proteins, NAC domain at the N-terminus of PtrWND is the only predictable domain (Additional file 3).

**Expression pattern of PdWND3A**

To functionally characterize PdWND3A, we first examined PdWND3A transcript abundance in various tissues and organs by using quantitative RT-PCR. PdWND3A transcript was detected in all tested tissues and organs with the highest abundance in the xylem, suggestive of a role in secondary cell wall biosynthesis (Fig. 1).

**Transgenic Populus plants overexpressing PdWND3A**

Subsequently, we generated transgenic *P. deltoides* plants overexpressing PdWND3A (Fig. 2a). A total of 14 independent transgenic lines were generated and six of them were confirmed to overexpress PdWND3A (Additional file 5). We selected two independent lines for further analyses. These two transgenic lines were designated as OXPdWND3A-1 and OXPdWND3A-2 and were confirmed by quantitative RT-PCR to be PdWND3A overexpressers (Fig. 2b).

AtVND family proteins are viewed as master switch transcription factors regulating vessel formation in xylem tissue [22]. To examine whether such a function is conserved in *Populus*, we examined vessel formation in OXPdWND3A transgenic plants. Cytological analysis with cross-section specimen of mature stem revealed a dense vessel formation in the stem of OXPdWND3A (Fig. 2c), with the number of xylem vessel significantly higher in OXPdWND3A transgenic plants compared to wild-type WV94 (Fig. 2d). These results support the view that the regulation of vessel formation is a common function of VND proteins in both *Arabidopsis* and *Populus*.

**Chemical composition analysis of secondary cell wall components in OXPdWND3A transgenic plants**

To examine possible changes in the contents of secondary cell wall components in the stem tissue of OXPdWND3A,
**Fig. 1** Expression of *PdWND3A* in various tissues and organs. Different tissues and organs were collected between 12:00 PM and 2:00 PM from three WV94 plants. Total RNAs were extracted from root, young leaf, mature leaf, young stem (internodes 1 to 3), mature stem (internodes 6 to 8), petiole of mature leaf, inner bark (bark of mature stem) and cambium layer (scraped stem under bark of mature stem). The *PdWND3A* transcript level was measured by qRT-PCR. Shown are mean values of three biological replicates ± S.D.

**Fig. 2** *Populus* transgenic plants overexpressing *PdWND3A* (OXPdWND3A). a The gene construct used to generate transgenic plants in *P. deltoides* WV94 background overexpressing *PdWND3A*. b Quantitative RT-PCR analysis of *PdWND3A* transcript level in the transgenic lines. Leaf tissues were used for RNA extraction. c Phloroglucinol-HCl staining image of stem cross-section of 6-month-old OXPdWND3A transgenic plants. d Vessel number. The vessel number was counted in 1 cm² area in the microscopic image. Shown are the mean values of three biological replicates ± S.D. The asterisk marks the statistical significance against WV94 (*p* < 0.01, *n* = 3)
we performed chemical composition analysis. We found that OXPdWND3A lines had significantly higher lignin content than WV94 ($p < 0.01$; Fig. 3). As a predictable monosaccharide from cellulose, the glucose content was reduced in OXPdWND3A lines while no significant difference was observed in xylose content (Fig. 3). The contents of other chemical components such as arabinose and mannose were also not altered in OXPdWND3A compared to WV94 (Fig. 3).

**Lignin physicochemical characterization**

Because lignin content was increased in the OXPdWND3A transgenic plants (Fig. 3), we sought further evidence supporting a role of PdWND3A in lignin biosynthesis. We performed Klason lignin (acid insoluble lignin) analysis with both leaf and stem tissue of the same plants. The Klason lignin content in stem tissue of OXPdWND3A-1 and OxPdWND3A-2 were 12.71 and 11.89% higher than wild-type WV94, respectively (Fig. 4a). OXPdWND3A-1 and OXPdWND3A-2 also contained 16.42 and 13.36% more lignin in leaf tissue compared to WV94, respectively (Fig. 4a).

To further examine the structural characteristics of lignin, the 2D $^1$H-$^1$C NMR analysis was conducted with stem tissue. The OXPdWND3A had relatively high S content and low G content compared to those of WV94, resulting in higher S/G ratio (Table 1). The chemical linkage between lignin subunit was altered in the stem of OXPdWND3A. The relative content of the carbon-carbon bond such as resinol ($\beta$-$\beta$) linkages was significantly increased in OXPdWND3A compared to WV94 (Table 1).

**Gene expression analysis**

To determine whether PdWND3A impacts expression of genes involved in lignin biosynthesis or monomer composition, we performed quantification analysis of the expression of lignin biosynthetic genes. We measured the relative transcript abundance of eight representative lignin biosynthetic genes, including PAL1 and PAL4, CAD1 and 4CL for general phenylprophenoid pathway, and CCoAOMT1, F5H1, COMT3 and COMT4 for monolignol biosynthesis. Among these eight tested genes, F5H1, a gene involved in S unit lignin monomer biosynthesis, was upregulated in both leaf and young stem tissues in the transgenic lines compared to the wild-type control (Fig. 4b). Although COMT and CAD had been reported to regulate S unit lignin monomer biosynthesis [27], no significant difference in their transcript level was observed between OXPdWND3A transgenic plants and the wild-type WV94. The transcript level of CCoAOMT1, another key enzyme involved in the monolignol biosynthesis of G- and S-type lignin [18, 19], in leaf tissue was also higher in the transgenic plants than WV94. Other tested genes were not altered in either tissues between the transgenic lines and WV94 (Fig. 4b). Collectively, these results support that PdWND3A has a role in regulating the expression of genes involved in lignin biosynthesis and lignin monomer composition.

**Saccharification efficiency of OXPdWND3A**

The saccharification efficiency is an important indicator of the usefulness of genetically modified plant biomass for biofuel production. It is generally recognized that high lignin content negatively impacts saccharification efficiency [34]. In contrast, in hardwood species, higher...
S-to-G (S/G) ratio is often considered to be a factor positively influencing saccharification efficiency [34, 43]. *OX*Pd*WND3A* transgenic lines have higher lignin content and higher S/G ratio compared to the control. Therefore, we wanted to examine how these two contrasting factors (i.e., high lignin content and high S/G ratio) impact saccharification efficiency. We measured glucose release, without pretreatment, by enzyme treatment for 48 h. The *OX*Pd*WND3A* transgenic plants displayed lower glucose release compared to the control (Fig. 5). Therefore, in *OX*Pd*WND3A* transgenic lines high lignin content appeared to dominate over high S/G ratio in the process of saccharification.

### Discussion

Woody biomass is mainly composed of cellulose, hemicellulose and lignin. The development of applicable method to convert biomass to biofuel has been regarded as a pivotal research for cost-effective biofuel production. In the last decade, molecular and genetic studies of woody plants suggested that transcription factors are critical for regulating secondary cell wall biosynthesis. Of these transcription factors, NAC family proteins are viewed as master switches [22, 56]. We provide evidence here that Pd*WND3A*, a member of NAC family, negatively affects biomass production.
domain-containing protein family, is involved in the regulation of lignin biosynthesis and composition.

PdWND3A and lignin biosynthesis and composition

The physicochemical analysis of OXPdWND3A transgenic lines suggest that overexpression of PdWND3A affects lignin biosynthesis (Fig. 4). Consistent with the physicochemical analysis, the histochemical image showed more xylem vessel formation in OXPdWND3A than WV94 (Fig. 2c and d). In earlier reports, overexpression of PtrWND6B, a homolog of AtVND7 protein, induced ectopic deposition of lignin in leaf epidermal and mesophyll cells [48, 51]. In addition, inducible expression of AtVND6 or AtVND7 in Populus resulted in ectopic lignin deposition [39]. More specifically, overexpression of PtrWND3A was shown to induce ectopic secondary cell wall deposition in poplar leaves [24]. Although a microscopic examination of ectopic secondary

![Fig. 5 Saccharification efficiency of OXPdWND3A transgenic plants. Debarked and dried Populus stem was treated with cellulase enzyme for 48 h. The rate of glucose release was calculated from the detected data by the ion chromatography system. Shown are the averages of two biological replicates ± S.D.](image)

![Fig. 6 Biomass production of OXPdWND3A transgenic plants. a The whole plant picture of 3-month-old plants grown under greenhouse conditions. Scale bar, 25 cm. b The stem volume of OXPdWND3A transgenic plants compared to WV94. Shown are the average of estimated stem volume ± S.D. (n = 5) by using the \( \pi r^2h \) equation with height and diameter of primary stem. The asterisk marks the statistical significance against WV94 (\( p < 0.01, n = 5 \)).](image)
cell wall deposition in the leaves of transgenic plants overexpressing PdWND3A was not conducted in the present study, we provided physicochemical analysis which confirms that the lignin is accumulated in both the leaf and the stem of OXPdWND3A transgenic plants (Fig. 4a). In addition, monolignol composition between S and G unit was altered by overexpression of PdWND3A (Table 1). The structural properties in lignin were also altered in OXPdWND3A. We observed significant increase of resinol (β-β) linkages (Table 1). It was reported that β-β linages are primarily associated with S unit whereas phenylcoumaran is associated with G unit [33]. Therefore, the observed increased lignin resinol abundance is consistent with the observed increased lignin S/G ratio in OXPdWND3A transgenic lines. Collectively, these results suggest that PdWND3A is involved in the regulation of both lignin biosynthesis and lignin monomer composition. It remains unclear whether PdWND3A preferentially regulates lignin biosynthesis or composition and how PdWND3A achieves it. Because PdWND3A functions as a transcription factor, it may do so through the regulation of specific lignin pathway genes.

PdWND3A and F5H1 expression
Gene expression analysis of lignin biosynthetic genes in OXPdWND3A transgenic plants indicated that among all tested genes, PdWND3A overexpression primarily affected the expression of F5H1 in both stem and leaf. This was in contrast to previous studies in Arabidopsis in which overexpression of every AtVND gene (AtVND1 to AtVND7) was shown to induce the expression of PAL1, CCoAOMT1 and 4CL of lignin biosynthetic genes but not F5H1 [23, 40, 58]. Zhou et al., [58] demonstrated that the promoters of CCoAOMT1 and 4CL are directly activated by AtVND proteins (AtVND1 to AtVND5). In another study, Populus transgenic plants expressing AtVND7 showed increased expression of genes encoding cationic peroxidase, laccase, CCR, and phenylcoumaran benzylc ether reductase related to lignin biosynthesis [24]. Arabidopsis transgenic plants expressing PtrWND6B, a Populus homolog of AtVND6, also showed increased expression of 4CL1 and CCoAMT1 [54]. The transactivation assay using PtrWND6B as the effector construct identified laccase, CCoAMT1 and COMT1 as direct target genes of PtrWND6B [24, 48]. Therefore, the regulation of gene expression of lignin biosynthetic genes by VND homologs appeared to be conserved between Arabidopsis and Populus. However, no report had shown the relationship between F5H1 expression and VND in previous studies. Our study showed that the expression of F5H1 is upregulated by PdWND3A, implying that there may be regulatory specificity among members of VND/WND transcription factor family regarding their downstream direct or indirect target genes.

F5H mediates the chemical conversion from coniferaldehyde to 5-OH coniferaldehyde in the S monolignol biosynthesis pathway [33]. Overexpression of F5H from Liquidambar styraciflua in Pinus radiata produced more sinapyl alcohol in the lignin polymer [36]. Accumulative evidence suggested that the regulation of F5H1 gene expression may be distinct from the common regulation of other lignin biosynthetic genes. For example, overexpression of AtMYB58 and AtMYB63 activated lignin biosynthetic genes except F5H1 [45, 57]. This result is consistent with the observation that the AC cis-acting element, a binding site for AtMYB58, is absent in the promoter of F5H1 [46, 57]. In Arabidopsis, AtSND1 has been reported to regulate F5H1 gene expression [46]. In the present study, we showed that the expression of F5H1 is up regulated by PdWND3A overexpression (Fig. 4b). Because AtVNDs (AtVND1 to AtVND7) have been shown to bind to the consensus DNA sequence of secondary wall NAC binding element (SNBE) in the promoter region of a group of genes associated with cell wall biosynthesis [6, 23, 48] and the SNBE consensus is present in the F5H1 promoter [50], it is plausible that F5H1 may serve as a direct target PdWND3A. The biochemical determination of F5H1 as a potential PdWND3A target gene (i.e., via protein-DNA binding assays) deserves further investigation.

PdWND3A and sugar release
Previous studies using P. trichocarpa natural variants showed that both lignin content and S/G ratio affect saccharification efficiency [34, 43]. Glucose release was significantly correlated with both lignin content and S/G ratio [34, 43]. However, the glucose release depended on lignin content but not on S/G ratio when sugar release was measured without pretreatment [34]. In the present study, OXPdWND3A transgenic lines showed both higher lignin content and higher S/G ratio (Fig. 3, Fig. 4, Table 1) with lower saccharification efficiency measured without pretreatment (Fig. 5), which is consistent with the observation in P. trichocarpa natural variants [34]. Therefore, lignin content seems to play a more dominant role than S/G ratio in the process of saccharification without pretreatment. Collectively, PdWND3A, when overexpressed, negatively impacts saccharification efficiency. As a future study, creating and characterizing Populus PdWND3A knockdown or knockout transgenic plants may complement and potentially strengthen the conclusion on the role of PdWND3A in lignin biosynthesis and sugar release drawn from overexpression study.

Conclusions
Our results indicate that PdWND3A, a member of NAC domain-containing protein family, impacts both lignin biosynthesis and lignin monomer composition. Specifically,
PdWND3A regulates the expression of F5H gene. Overexpression of PdWND3A negatively impacts saccharification efficiency and biomass production.

Methods

Plant materials
The full-length open reading frame of PdWND3A was amplified from Populus deltoides genotype WV94 and cloned into the pAGW560 binary vector for transformation into WV94. We followed the same procedure for growing and maintaining transgenic plants in the greenhouses as reported in a previous publication [38]. The growth conditions were set with constant 25 °C with 16 h/8 h photoperiod.

Amino acid sequence alignment and phylogenetic analysis
AtSN1 (AT1G32770) was subjected to Phytozome v12.0 (https://phytozome.jgi.doe.gov) [8] and BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) [1] to identify NAC domain-containing proteins in the Populus (P. trichocarpa) and Arabidopsis (A. thaliana) genomes. The full-length amino acid sequence homologs of AtSN1 from each species were subsequently used to perform reciprocal sequence homolog search with >30% amino acid similarity cutoff (e-value < 0.01). The collected proteins were used as subjects in the Pfam database to predict putative protein domains and functional motifs [7]. The phylogenetic tree was constructed by PhyML (a phylogeny software based on the maximum-likelihood principle) using Jones-Taylor-Thornton (JTT) model matrix of amino acid substitution with 1000 bootstrap replication [9]. Nearest-Neighbor-Interchange (NNI) algorithm was used to perform tree topology search.

Phloroglucinol-HCl staining
To obtain the image of xylem vessel formation from OXPdWND3A transgenic lines, was analyzed and compared with the control (wild-type WV94) by two-step sulfuric acid hydrolysis according to the NREL procedure [32]. Wiley-milled, 6-month-old Populus stems were Soxhlet-extracted using ethanol/toluene (1:2, v/v) for 12 h. For the analysis of leaf tissues, additional 12 h ethanol/toluene extraction and 12 h acetone extraction were conducted. The extractives-free samples were air-dried and hydrolyzed by two-step acid method. Briefly, the biomass was hydrolyzed with 72% H2SO4 at 30 °C for 1 h and 4% H2SO4 at 121 °C for 1 h. The solid residues were filtered and washed with excessive amounts of deionized water and oven-dried at 105 °C for 24 h. Ash content was measured by muffle furnace at 575 °C for 12 h. Klasson lignin content was calculated as below:

\[ \text{Klasson lignin content (\%)} = \frac{\text{Acid insoluble residues (g)} - \text{Ash (g)}}{\text{Extractives free sample (g)}} \times 100 \]

Carbohydrate contents were analyzed using a Dionex ICS-3000 ion chromatography system with external standards.

Lignin S/G ratio analysis
Nuclear magnetic resonance (NMR) analysis was used to measure the lignin S/G ratio. Stem samples were extracted as described above. Cellulolytic enzyme lignin
was isolated from the extractives-free biomass as described in a previous study [42]. The isolated lignin (~30 mg) was dissolved with DMSO-d6 in 5 mm NMR tube. A Bruker Avance III 400 MHz spectroscopy equipped with a 5 mm Broadband Observe probe and Bruker standard pulse sequence (’hsqetgps2’) was used for two-dimensional (2D) 1H,13C heteronuclear single quantum coherence (HSQC) NMR analysis at 300 K. The spectral widths of 11 ppm (1H, 2048 data points) and 190 ppm in F1 (13C, 256 data points) were employed for the 1H and 13C-dimensions, respectively. The number of transients was 64 and the coupling constant (JCH) used was 145 Hz. Bruker Topspin software (v3.5) was used for data processing.

Saccharification efficiency assay

Stem tissues collected at a position 15 cm above the stem base of 6-month-old plants were dried and Wiley-milled to 40-mesh for sugar release measurement. Approximately 250 mg of sample was placed in 50 mM citrate buffer solution (pH 4.8) with 70 mg/g-biomass of Novozymes CTec2 (Novozymes, Franklinton, NC) loading. The enzymatic hydrolysis was conducted at 50°C with 200 rpm in an incubator shaker for 48 h. Enzymes in the hydrolysate were deactivated in the boiling water for 5 min prior to the analysis of released sugars by using the Dionex ICS-3000 ion chromatography system. Each analysis was conducted in duplicates from single plant of each transgenic line.

Statistical analysis

T-test (against WV94) was performed at \( p \leq 0.01 \) by t-test function integrated in the Excel software (Microsoft, Redmond, WA) for all statistical analysis. Asterisk in each figure indicates significant difference from WV94 or control samples \( (p \leq 0.01) \).

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12870-019-2111-5.

**Additional file 1.** The list of primers used for PCR and RT-PCR analyses in this study.

**Additional file 2.** The phylogenetic analysis of *Populus* and *Arabidopsis* VND/NST/SND proteins. (a) PhyML phylogenetic tree analysis. A total of 21 and 22 different loci from *Populus* and *Arabidopsis*, respectively, were identified as AtSN1 homologous proteins. The full-length amino acid sequences were subjected to PhyML, Potri015G127400, a receptor like protein, was used as an outgroup protein sequence in this phylogenetic tree. The AvVND homolog cluster is highlighted by green color and these proteins are used for further analyses shown in Fig. 1. (b) Heatmap illustrating amino acid sequence similarity of 43 VND homologs from *Populus* and *Arabidopsis*.

**Additional file 3.** VND homologs in *Populus* and *Arabidopsis*. (a) Phylogenetic tree of *Populus* NAC domain-containing proteins and *Arabidopsis* NAC proteins known as the master switch transcription factors regulating secondary cell wall biosynthesis. Potri015G127400 shows high amino acid sequence similarity with AtVND4 and VND5; (b) The heatmap illustrating full-length amino acid sequence similarity between VND homologs in *Populus* and *Arabidopsis*. (c) Conserved domain in full-length amino acid sequence of proteins shown in panel (a) and (b). Note that NAC domain is the only conserved region among VND proteins in *Populus* and *Arabidopsis*.

**Additional file 4.** The cDNA sequence alignment between Potri012G126500 (PdWND3A) and Potri015G127400 (PdWND3A). The missing sequence region between these two genes is highlighted with yellow color.

**Additional file 5.** Expression of PdWND3A in the *Populus* transgenic plants. Asterisk marks selected lines for further analysis presented in this study.

**Abbreviations**

4CL: 4-coumarate:CoA ligase; C4H: Cinnamate 4-hydroxylase; CAD: Cinnamoyl alcohol dehydrogenase; CCoAOMT: Caffeoyl-CoA O-methyltransferase; CCR: Cinnamoyl CoA reductase; F5H: Ferulate 5-hydroxylase; F5H1: FERULATE 5-hydroxylase; G: Guaiacyl; H: Hydroxyphenyl; MiR: V-myb myeloblastosis viral oncogene homolog; NAC: No Apical Meristem (NAM); Arabidopsis Transcriptional Activation Factor (ATAF1/2); Cup-shaped Cotyledon (CUC2); NST1: NAC secondary wall thickening promoting factor1; OxPdWND3A: *Populus* detoids transgenic plants overexpressing PdWND3A; PAL: L-phenylalanine ammonia-lyase; PdWND3A: *Populus* detoids wood-associated NAC domain protein 3A; Potri015G127400; PdWND3B: *Populus* detoids wood-associated NAC domain protein 3B; Potri012G126500; S: Syringyl; SNBE: Secondary wall NAC binding element; SND1: Secondary wall-associated NAC domain protein1; TERE: Tracheary-element-regulating cis-elements; VND: Vascular-related NAC domain

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**Authors’ contributions**

YY performed bioinformatics, RT-PCR and qRT-PCR analyses, and microscopic imaging. CGY, YP and AJR performed chemical composition analysis and measured sugar release. WR, KAW and CMC generated *Populus* transgenic lines. LEG and SSJ measured biomass production. XY designed the gene construct for *Populus* transformation. GAT and JGC conceived the study, coordinated research and contributed to experimental design and data interpretation. All authors read and approved the final manuscript.

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**Availability of data and materials**

All data generated or analyzed during this study are included in this published article and its supplementary information files.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

**Author details**

1. 3BioEnergy Science Center and Biosciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831, USA; 2. Center for Bioenergy Innovation, Oak Ridge National Laboratory, Oak Ridge, TN 37831, USA; 3. UT-ORNL Joint
Institute for Biological Science, Oak Ridge National Laboratory, Oak Ridge, TN 37831, USA. ArborGen Inc., Ridgeville, SC 29472, USA. Department of Chemical and Biomolecular Engineering & Department of Forestry, Wildlife, and Fisheries, University of Tennessee, Knoxville, TN 37996, USA.

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