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ORIGINAL PAPER

Identification and characterization of a wood-associated NAC domain transcription factor PtoVNS11 from *Populus tomentosa* Carr.

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Abstract

Key message **Here, we isolated a NAC transcription factor PtoVNS11 from *Populus tomentosa* Carr. Molecular characterization showed that PtoVNS11 was involved in the regulation of secondary cell wall formation in poplar.**

Abstract NAC domain transcription factors are important regulators that activate the secondary wall biosynthesis in wood formation. In this study, we isolated a NAC domain transcription factor PtoVNS11 from *Populus tomentosa* Carr. PtoVNS11 shares high identity with SND1 of *Arabidopsis thaliana* (59.6 %) and PtrWND1B (97.9 %) of *P. trichocarpa*. Gene expression analyses showed that *PtoVNS11* gene was mainly accumulated in xylem and stem of poplar. Further, GUS expression driven by the *PtoVNS11* promoter was observed in vascular tissues of vegetative and reproductive organs in transgenic *Arabidopsis*. Promoter deletion analysis revealed that the fragment (−283 to −171) with the secondary wall NAC-binding element was required for tissue-specific expression of the *PtoVNS11* gene. Subcellular localization analysis showed that *PtoVNS11:GFP* fusion protein was localized in the nucleus. Transgenic poplar plants carrying *35S:PtoVNS11* exhibited dwarf phenotypes with shorter internode as compared to wild-type plants. *PtoVNS11* overexpression resulted in massive deposition of lignin and increased thickening of secondary walls in transgenic poplar. Transcription analysis showed that the expression levels of many wood-associated genes were up-regulated in transgenic plants overexpressing *PtoVNS11*. Taken together, our results indicate that PtoVNS11 may be involved in the regulation of the secondary wall biosynthesis during wood formation in poplar.

Keywords *Populus tomentosa* carr · Lignin · NAC transcription factor · Secondary cell wall · *Arabidopsis thaliana*

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L. Yang and Y. Hou contributed equally to this work.

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Introduction

Wood is mainly composed of cellulose, hemicellulose, lignin, and extractives. It is the major product from forests and constitutes the primary raw material in a number of industrial production chains, including construction, pulping, paper making, direct burning for energy, and so on. Wood formation involves a series of complex developmental processes, including cambial cell division and

differentiation into xylem cells, cell elongation, secondary wall deposition, and programmed cell death (Plomion et al. 2001). In the last several decades, considerable studies have identified a number of genes involved in cambial cell activity, xylem cell differentiation, and secondary wall biosynthesis in tree species, but our understanding of the molecular control of wood formation is still in infancy, and only a small number of wood-associated genes have been isolated from tree species (Zhao and Dixon 2011). Therefore, it is very important to elucidate the molecular mechanisms underlying the transcriptional regulation of secondary wall biosynthesis during wood formation in plants.

Wood formation requires fine temporal and spatial regulation and many transcription regulators are expected to precisely coordinate the expression of hundreds of genes involved in this process (Zhong and Ye 2007). A number of transcription factors belonging to the MYB and NAC families have been demonstrated to be involved in wood formation (Patzlaff et al. 2003; Schrader et al. 2004; Karpinska et al. 2004; Prassinos et al. 2005; Goicoechea et al. 2005; Andersson-Gunneras et al. 2006; Bedon et al. 2007; Lee et al. 2011). For example, overexpression of *PtMYB4* from pine and *EgMYB2* from *Eucalyptus* in *Nicotiana tabacum* resulted in ectopic deposition of lignin and increased thickness of secondary walls in xylem, respectively (Goicoechea et al. 2005; Patzlaff et al. 2003). In poplar, two MYB transcription factors, PtrMYB3 and PtrMYB20 have previously been shown to be involved in the regulation of secondary wall biosynthesis (McCarthy et al. 2010). To date, it has not been reported that wood-associated transcription factors act as master switches activating the entire biosynthetic pathway of wood formation in a tree species.

In *Arabidopsis*, a group of NAC domain proteins, including NAC SECONDARY WALL THICKENING PROMOTING FACTOR1 (NST1), NST2, SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN1 (SND1, also known as NST3), VASCULAR-RELATED NAC-DOMAIN6 (VND6) and VND7, have been demonstrated to regulate the expression of a bunch of secondary wall-associated transcription factors (Kubo et al. 2005; Mitsuda et al. 2005; Zhong et al. 2006; Mitsuda et al. 2007; Zhong and Ye 2007; Zhong et al. 2008). When overexpressed in *Arabidopsis*, these wood-associated NACs, including SND1, NST1/2, and VND6/7, were able to induce the expression of secondary wall-associated transcription factors and result in a concomitant ectopic deposition of secondary walls (Yamaguchi et al. 2008; Zhong et al. 2006; Zhong and Ye 2007). In addition, there are conserved gene structure and NAC domain motifs in multiple species, suggesting that the lignin biosynthesis is likely analogical in angiosperms (Ooka et al. 2003; Shen et al. 2009; Hu et al. 2010; Hussey et al. 2014).

Recently, 16 wood-associated NAC domain proteins PtrWNDs (also called PtVNSs), which are putative functional orthologs of *Arabidopsis* SND1, NST1/2, and VND6/7, have been found in the genome of *P. trichocarpa* (Zhong et al. 2011; Ohtani et al. 2011). Constitutive expression of *PtVNS/PtrWND* genes resulted in ectopic secondary wall thickening in poplar and in *Arabidopsis* with different levels of induction efficiency depending on the specific gene (Zhong et al. 2010; Ohtani et al. 2011). PtrWNDs have also been shown to activate the expression of a number of other wood-associated transcription factors, including PtrMYB3 and PtrMYB20 (McCarthy et al. 2010). PtrWND1B, homolog to *Arabidopsis* SND1, has been reported to undergo alternative splicing (AS) to produce a splice variant PtrSND1-A2^{IR} in developing xylem tissue (Li et al. 2012) and *PtrWND1B* AS functions as a means to regulate and maintain homeostasis of PtrVNS/PtrWNDs for safeguarding steady fiber wall thickening in *Populus* (Zhao et al. 2014). These results suggest that wood formation in poplar is regulated by cooperative functions of the NAC domain proteins. However, there are only very limited reports on the functional characterization of NAC transcription factors in poplar. Here, we isolated a NAC transcription factor, named *PtoVNS11*, from Chinese white poplar (*P. tomentosa* Carr.). We found that *PtoVNS11* is preferentially expressed not only in vascular tissues of developing xylem, but also in developing anthers, sepals, stigmas, and siliques. Overexpression of *PtoVNS11* resulted in massive deposition of lignin and increased secondary cell wall thickening in transgenic poplar plants. Our findings suggest that *PtoVNS11* is involved in the regulation of wood formation in poplar.

Materials and methods

Plant materials

Populus tomentosa Carr. (Clone 741) is native in China. Poplar plants were grown in the greenhouse at 25 °C under a 14-/10-h light/dark cycle with supplemental light (4500 lux). Wild-type *Arabidopsis thaliana* (ecotype Columbia) were grown in a greenhouse. The temperature was maintained at 23–25 °C with the 8-/16-h dark/light photoperiod.

Cloning of *PtoVNS11* gene

Total RNA was isolated from the fourth internode stem of *P. tomentosa* Carr. grown in the greenhouse using a RNA RNeasy Plant Mini Kit (Qiagen, Germany) following the manufacturer's instructions. First-strand cDNA was synthesized from 2 μg DNase-treated RNA with RT-AMV transcriptase (TaKaRa, Dalian, China) in a total volume of

20 µl using oligo d (T) at 42 °C for 30 min. The full open reading frame of *PtoVNS11* was amplified with gene-specific primers (Table S1) based on *PtrVNS11* from *Populus trichocarpa* (Joint Genome Institute, http://genome.jgi-psf.org/poplar/poplar.info.html) by PCR with cDNA from stems. The PCR reaction was carried out with Pfu DNA polymerase (TaKaRa) in a total volume of 50 µl with an initial denaturing step at 94 °C for 3 min, 32 cycles of 94 °C for 45 s, 56 °C for 45 s, and 72 °C for 90 s and a final extension step at 72 °C for 10 min. The amplification products were cloned into the plant binary vector pCXSN, which is a zero-background TA cloning system that provides high-efficiency direct cloning of PCR-amplified DNA fragments (Chen et al. 2009). These plant transformation vectors were transformed into *Agrobacterium tumefaciens* EHA105 by the freeze–thaw method (Hofgen and Willmitzer 1988).

Construction of the *PtoVNS11* promoter deletion vectors

Genomic DNA was isolated from young stem of poplar plants using cetyltrimethylammonium bromide method (Sambrook and Russell 2001). Based on phylogenetic analysis, an 2 k-bp 5′ upstream sequence of *PtrWND1B* (http://phytozome.jgi.doe.gov/pz/portal.html#!gene?search=1&detail=1&method=3252&searchText=transcriptid:27041362) was used as templates to amplify promoter deletion series of *PtoVNS11* from *P. trichocarpa* genomic DNA. A series of PCR reactions were carried out with five pairs of primers (Table S2), respectively. After verification by DNA sequence analysis in both directions, each of the fragments was inserted into plant binary vector pCXGUS-P (Chen et al. 2009).

Transformation of *P. tomentosa* Carr. plants

Transgenic poplar plants were generated by *Agrobacterium*-mediated transformation as described previously (Jia et al. 2010). Recombinant *Agrobacterium* was used to infect poplar leaf discs and putative transgenic plants were selected on woody plant medium (WPM) (Lloyd and McCown 1980) supplemented with 9 mg l^{-1} hygromycin. Rooted plantlets were acclimatized in pots at 25 °C in a 14-/10-h light/dark cycle and then transferred to the greenhouse for further studies.

DNA extraction and PCR analysis

Genomic DNA was extracted from leaves (300 mg) of untransformed control and hygromcyin-resistant plants using the modified cetyltrimethylammonium bromide (CTAB) extraction method as previously described (Jia et al. 2010). To determine the presence of transgenes, putative transgenic plants were screened preliminarily by PCR analysis (Luo et al. 2006). The gene-specific primers designed for *Hpt* are listed in Table S1. The PCR condition was an initial denaturing step at 94 °C for 3 min and 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min. The amplification products were electrophoresed on a 1 % (w/v) agarose gel and visualized by ethidium bromide staining.

Semi-quantitative RT-PCR and quantitative real-time PCR analysis

Total RNA was extracted from leaves, roots, stems, and petioles of poplar plants and treated with DNase I (TaKaRa) according to the manufacturer's instructions. To isolate the xylem from *Populus tomentosa*, the fifth and seventh internodes were cut into about 1-cm segments, with the bark and pith removed by blade under a stereomicroscope. About 10 main stems from individual plants were used for isolation of xylem, which were pooled and frozen in liquid nitrogen for RNA extractions. All RNA was purified and first-strand cDNA was synthesized as described above. The reverse-transcribed cDNA samples were used for quantitative real-time PCR, which was performed on a TaKaRa real-time-PCR detection system. *18S rRNA* was used as an internal control. The RT-PCR reactions were carried out with an initial denaturation step at 94 °C for 3 min, 25 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min, and an extension step at 72 °C for 10 min. The amplification products were electrophoresed on a 1 % (w/v) agarose gel. The electropherograms of DNA were visualized with ethidium bromide under UV light. Quantitative real-time PCR analysis was performed as described by Tsai et al. (2006) in a 20-µl reaction volume containing 10 µl of SYBR Green master mix reagent (TaKaRa). The primers were designed using Primer 5.0 software and are listed in Table S1. Each reaction was performed in duplicate and with three biological replicates along with no-template controls. The gene quantification method was based on the relative expression of the target gene versus the reference gene (*18S*) (Li et al. 2012).

Accession number for sequences used in this study

The genes mentioned in the semi-quantitative RT-PCR and quantitative real-time PCR analysis referred to genes as follows: PtrPAL1, Potri.006G126800.1; PtrC4H2, Potri.019G130700.1; Ptr4CL5, Potri.003G188500.2; PtrHCT1, Potri.001G042900.2; PtrC3H3, Potri.006G033300.2; PtrCCoAOMT1, Potri.009G099800.4; PtrCCR, Potri.003G181400.2; PtrCAld

5H, Potri.007G016400.1; PtrCOMT, Potri.012G006400.2; and PtrCAD, Potri.009G095800.2.

Sequence alignment and phylogenetic analysis

The deduced amino acid sequences were aligned with the program DNAMAN7.0 (Lynnon Corporation, USA). The phylogenetic relationships of NAC proteins were analyzed with the neighbor-joining method using MAGE 5.0. The sequences for NACs are as follows: PtrWND1B, POPTR_0001s45250.1; AtSND1, AT1G32770.1; PtrWND 1A, POPTR_0011s15640.1; OsSWN2, AEO53047; PtrSN D1-L-1, POPTR_0010s18420.1; PtrSND1-L-2, POPTR_00 08s07950.1; AtNST1, AT2G46770.1; MtNST, GU144511; PtrWND2A, HQ215849; PtrWND2B, POPTR_0002s179 50.1; AtNST2, AT3G61910.1; and BdSWN5, JQ693426.

Arabidopsis transformation

The plant transformation vectors were transformed into *A. tumefaciens* EHA105 by the freeze–thaw method (Hofgen and Willmitzer 1988). *A. tumefaciens* strain EHA105 containing the *PtoVNS11* promoter::GUS construct was used to transform *A. thaliana* (Col) plants via the floral dip method (Zhang et al. 2006). Transgenic lines were selected on MS media containing 50 $mg.l^{-1}$ hygromycin and grown in a growth chamber set at 25 °C under long-day conditions (the 8-/16-h dark/light photoperiod).

GUS activity assays

Histochemical staining of GUS activity was performed according to the method described previously (Jefferson et al. 1987). Transgenic *Arabidopsis* samples were immersed into GUS reaction buffer [1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-d-glucuronide), 100 mM phosphate buffer pH 7.0, 0.1 % Triton X-100, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 10 mM EDTA, and 20 % methanol]. After overnight incubation in dark at 37 °C, stained samples were bleached with 70 % (v/v) ethanol and then photographed.

For the fluorometric assay, various tissues of transgenic or control plants were homogenized in GUS extraction buffer (50 mM phosphate buffer, pH 7.0, 10 mM EDTA, 0.1 % Triton X-100, 0.1 % sodium lauryl sarcosine, and 10 mM b-mercaptoethanol). The homogenate was then centrifuged for 10 min at 12,000*g* at 4 °C, and the GUS activity of the supernatant was assessed according to the method described by Jefferson (1987). Three replicates were performed for each sample.

Subcellular localization

The *PtoVNS11*–GFP construct was created by ligating the PCR-amplified cDNA of *PtoVNS11* in the pCX-DG (Chen et al. 2009). The expression vectors were introduced into onion epidermal cells by Gene Gun (GJ-1000, SCIENTZ, China). The onion skin was stained with DAPI, and then photographed by confocal microscopy (Leica TCS SP5).

Transcriptional activation assay of PtoVNS11

Transcriptional activity assays were performed essentially as described previously (Tian et al. 2013). The *PtoVNS11* full-length gene sequence was fused in pGBKT7 (Clontech) and introduced into the yeast strain *Saccharomyces cerevisiae* Gold2. X-α-gal was used to identify the transcription activation activity of PtoVNS11.

Microscopic experiments

The basal parts of the fifth and seventh internodes from 3-month-old independent transgenic plants were dissected transversely with blades and then stained for lignin with phloroglucinol–HCl and observed under a Zeiss Axio Lab.A1 light microscope. (Tian et al. 2013).

The 4th internode of stems was fixed in formaldehyde–acetic acid solution [formaldehyde: glacial acetic acid: ethanol (1:1:18)] and embedded in paraffin. 10 μm sections were cut using a Thermo Scientific Finesse325 rotary microtome. After the removal of paraffin, the stems were stained with toluidine blue 0.05 % (w/v) and observed with a Zeiss Axio Lab.A1 light microscope. Transmission electron microscopy was viewed under SEM (Phenom™ Pure FEI, USA) following the manual's recommendations and the images were captured digitally.

Statistical analysis

The Student's *t* test program (http://www.graphpad.com/quickcalcs/ttest1.cfm) was used for statistical analysis of the data from plant height, internode length, cell wall width, lignin content, and quantitative RT-PCR.

Results

Isolation and characterization of *PtoVNS11* from *P. tomentosa* Carr.

A full-length cDNA of *PtoVNS11*, was isolated from Chinese white poplar (*Populus tomentosa* Carr.) based on the sequence in the Joint Genome Institutive poplar database (JGI, *P. trichocarpa* genome portal v1.1, http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html) by reverse transcription (RT)-PCR. An open reading frame (ORF) of 1257 bp encoded 419 amino acids (Fig. S1). Analysis of the deduced amino acid sequence indicates that PtoVNS11

contains a highly conserved N-terminal NAC domain, divided into five subdomains (A–E) and a highly divergent C-terminal transcriptional activation region (Fig. 1a). The alignment analysis and domain comparison showed that PtoVNS11 shares high identity with secondary cell wall-associated NAC proteins from *P. trichocarpa* (PtrWND1B, 97.90 %) and *A. thaliana* (AtSND1, 59.60 %) (Fig. 1a).

Phylogenetic analysis of PtoVNS11

The NAC proteins in plants form a large family and can be classified into two groups and 18 subgroups by sequence similarity of NAC domains (Ooka et al. 2003). In this study, the full-length amino acid sequences were used for phylogenetic analysis and a neighbor-joining phylogenetic tree was constructed by with the MEGA 5.0 program (Wang et al. 2013b). The results show that PtoVNS11 has a close relationship with AtSND1, PtrWND1A, and PtrWND1B (Fig. 1b), indicating that PtoVNS11 could be a potential transcriptional regulator in secondary wall biosynthesis.

Expression profiling of *PtoVNS11* in poplar

The expression profiles of *PtoVNS11* in various tissues of Chinese white poplar were determined by quantitative real-time PCR and semi-quantitative RT-PCR. Transcript accumulation was detected in all tissues analyzed, including roots, stems, young leaves, barks, and petioles, except for old leaves, and the highest mRNA level of *PtoVNS11* was found in xylems (Fig. 2a, b), consistent with the expression patterns of *PtrWND1B* from *P. trichocarpa* in different organs and tissues (Li et al. 2012; Zhao et al. 2014).

Promoter analysis of *PtoVNS11* in *Arabidopsis*

To further investigate the spatial and temporal expression pattern of *PtoVNS11*, a 2069-bp promoter fragment was isolated from the genome of *P. tomentosa* Carr. by PCR. Transgenic *Arabidopsis* plants expressing β-glucuronidase (GUS) gene under the control of the *PtoVNS11* promoter were produced and analyzed histochemically. In 2-week-old *Arabidopsis* seedlings, GUS activity was mainly confined to cotyledons, stems, and veins (Fig. 3a). *PtoVNS11*::*GUS* expression was also detected in the vascular region of two-week-old roots and tip region (Fig. 3b, c). Further, GUS staining was also detected during the development of the reproductive organs. In flowers, high GUS activity was predominantly observed in the pistils, stigma, stamens, anther, and petals (Fig. 3d). In siliques, GUS activity was present in the adhesion zones (Fig. 3e). Moreover, GUS activity was also observed in the stems and leaves of *Arabidopsis*, especially strong in the stem (Fig. 3f, g). Consistent with real-time PCR data above, these results indicate that *PtoVNS11* is expressed in all the vegetative tissues including young leaves, roots, and stems as well as reproductive organs.

Since *PtoVNS11* is highly expressed in developing xylem, we examined the *PtoVNS11* 5′-flanking sequence for the presence of *cis*-elements using the PLACE database (http://www.dna.affrc.go.jp/PLACE/) (Table S2). In a 1150-bp promoter fragment of the *PtoVNS11* gene, at least

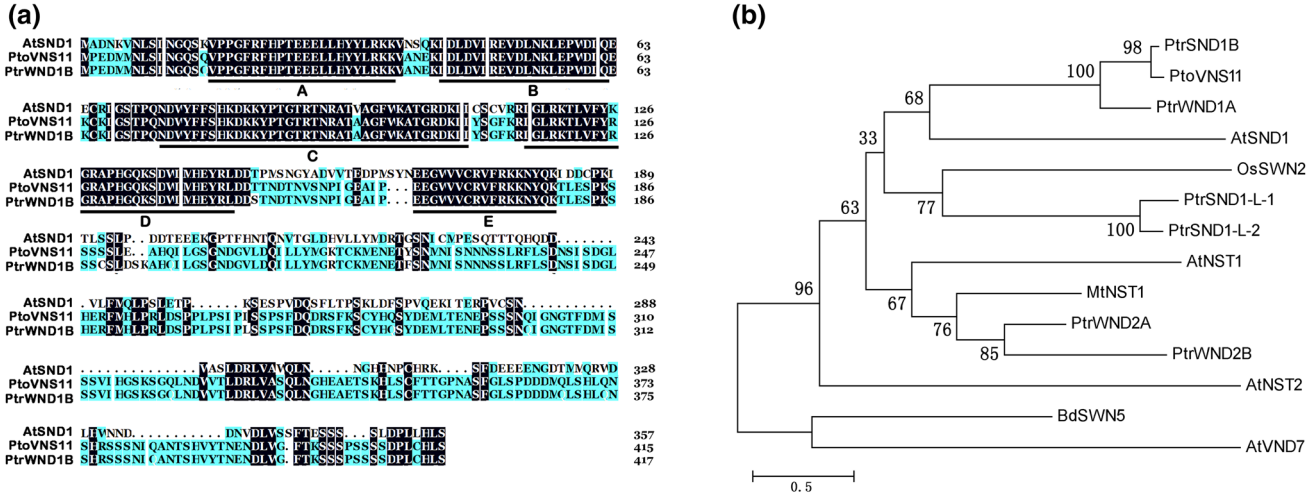

Fig. 1 Phylogenetic analyses and comparison of the putative amino acid sequence of PtoVNS11 with that of the other NAC domain proteins. **a** Multiple sequence alignment of PtoVNS11 and the other NAC domain proteins. Identical amino acids are *shaded* in *gray*. The locations of the five highly conserved amino acid motifs (*A–E*) are underlined. **b** Poplar NAC homologs of *Arabidopsis* SND1 were analyzed for their phylogenetic relationship with SND1, PtrWND1B using the DNAMAN7.0 with 1000 replicates of the neighbor-joining algorithm, and the phylogenetic tree was displayed using the MEGA5.0 (Wang et al. 2013b)

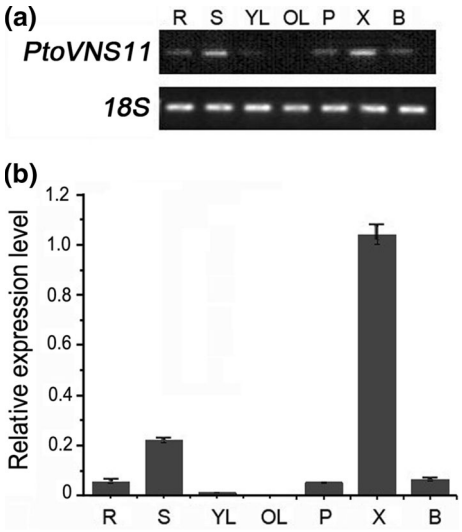

Fig. 2 Expression analysis of *PtoVNS11* using semi-quantitative RT-PCR and quantitative real-time PCR. **a** Semi-quantitative RT-PCR analysis of *PtoVNS11* expression in various tissues of *P. tomentosa* Carr. **b** Quantitative real-time PCR analysis of *PtoVNS11* transcript levels in various tissues of *P. tomentosa* Carr. The relative mRNA abundance of *PtoVNS11* was normalized with respect to reference gene *18 s rRNA* in different tissues. *R* root apices, *S* stem, *YL* young leaf (1–2 internodes), *OL* old leaf (5–6 internodes), *P* petiole, *X* xylem, *B* bark. The *bars* are standard deviations (SD) of three technical repeats

two secondary wall NAC-binding elements (SNBEs) (Fig. 3f). A *cis*-acting regulatory element involved in auxin responsiveness (AUXRR) was also found at position-95 (sequence GGTCCAT). In addition, some biotic and abiotic stress-related *cis*-elements, such as ABA-responsive elements (ABRE), W-Box (binding site for the WRKY transcription factors) and PASR (pathogen- and salt-induced expression) were located at the promoter region between −1090 and −360 (Fig. 3f). We also determined the effect of promoter length of *PtoVNS11* on GUS expression in transgenic *Arabidopsis* plants. A series of fragments of the *PtoVNS11* promoter with decreasing lengths were amplified and inserted into the pCXGUS-P vector. The GUS activity detected in transgenic plants carrying each promoter deletion category is shown in Fig. 3i. The fluorometric measurement revealed that the highest GUS activity was detected in transgenic plants containing the *PtoVNS11*-1150 promoter. GUS activity was markedly decreased when the promoter was shortened to −652. But transgenic plants carrying the −283 promoter showed relatively high expression level. The shortest promoter, −171, could still confer GUS expression in transgenic plants; however, GUS activity was only 5 % of the activity of the −1150 promoter. These results suggested that the *PtoVNS11*-1150 promoter region from −283 to

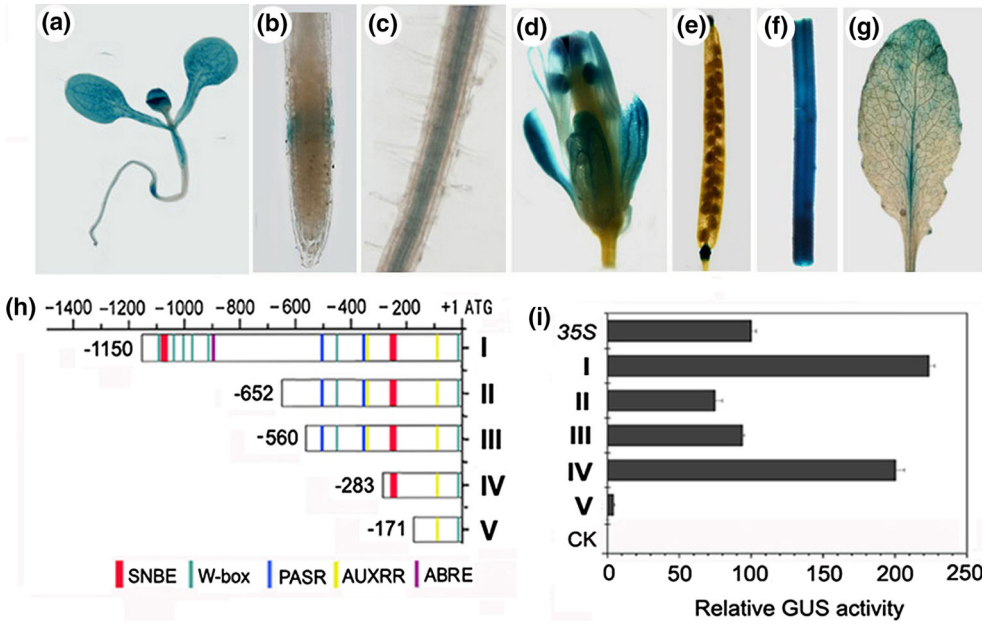

Fig. 3 GUS activity of transgenic *Arabidopsis* carrying the pro*PtoVNS11:GUS* vector. GUS activity was detected in 2-week-old seedings (**a**) and root tip region (**b**) and the vascular region (**c**) of roots. GUS expression patterns in the flower (**d**), silique (**e**), stem (**f**), and mature leave (**g**) during the development of the reproductive organs. **h** Schematic representation of constructs carrying different *PtoVNS11* promoters. The promoter fragments with different lengths were inserted into pCXGUS-P vector (Chen et al. 2009). Plants were also transformed with the promoterless GUS gene (CK, as a negative control) or the GUS gene under the control of the CaMV 35S promoter (35S, as a positive control). **i** GUS activity in transgenic plants was measured by fluorometric analysis. *Error bars* represent standard error ($n = 3$)

−171 might be required for regulating the activity of tissue-specific expression.

Subcellular localization and transcriptional activation activity of PtoVNS11

Sequence analysis showed that the PtoVNS11 protein possesses the typical domain organization of known plant NAC transcription factors, including the NAC domain located at the N-terminus and the putative activation domain at the C terminus (Olsen et al. 2005). To determine the subcellular localization of PtoVNS11 protein in poplar, we fused the green fluorescent protein (GFP) to the N-terminus of PtoVNS11 under the control of CaMV 35S promoter. The *PtoVNS11*:*GFP* and GFP control were transformed into onion epidermal cells by particle bombardment. We found that the fusion protein accumulated in the nucleus (Fig. 4a), consistent with the predicted function of PtoVNS11 as transcription factors. Meanwhile, we assayed the GAL4 DNA-binding domain for transactivation analysis in yeast. It was shown that PtoVNS11 was able to induce the expression of the *LacZ* reporter gene (Fig. 4b), indicating that it is a transcriptional activator.

Overexpression of *PtoVNS11* results in morphological changes in poplar

To investigate whether PtoVNS11 is involved in the regulation of secondary wall biosynthesis, the open reading frame of *PtoVNS11* under the control of the CaMV 35S promoter was introduced into Chinese white poplar by *A. tumefaciens*-mediated transformation. More than 20 putative transformants with hygromycin resistance were produced and grown in the greenhouse. PCR analysis revealed that an expected amplification product specific for hygromycin phosphotransferase (HPT) gene was obtained from all putative transgenic lines tested but not from the non-transformed plants (Fig. S2), confirming the integration of the transgene into the poplar genome. The expression level of the *PtoVNS11* gene in transgenic and control plants was detected by real-time PCR analyses. Expression of *PtoVNS11* was significantly higher in the transgenic lines compared with the control plants (Fig. 5c), further confirming that *PtoVNS11* had been transferred into the poplar genome and was highly expressed in the transgenic seedlings.

The transgenic lines overexpressing *PtoVNS11* showed dwarf phenotypes with obviously shorter internodes (Fig. 5a). For further analysis, three transgenic lines (T3, T5 and T8) and wild-type plants were grown under the same conditions, and plant height was determined after 3 months. Compared to the control which was approximately 23.5 cm in height, transgenic lines only reached about 11–19 cm (Fig. 5b). Reduced internode length was observed in the transgenic lines compared to the wild-type controls (Fig. 5d), with the strongest effect on the sixth internode, which was shortened by a mean of 51.58 % (Fig. 5d). These results suggested that PtoVNS11 plays an important role in the stem development of poplar.

Overexpression of *PtoVNS11* affects secondary cell wall formation

To determine lignin deposition site in transgenic *35S:PtoVNS11* poplar plants, stem sections were stained with toluidine blue and phloroglucinol–HCl. As shown in

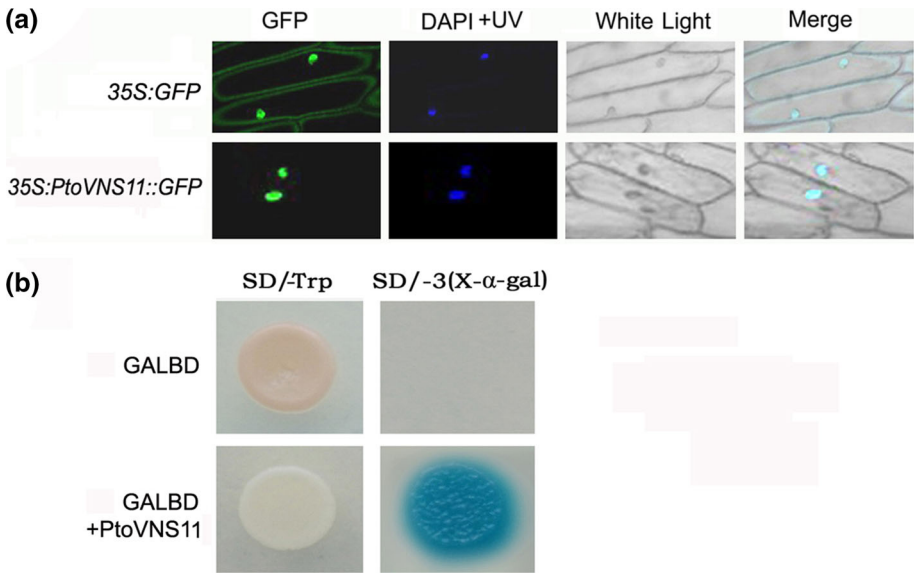

Fig. 4 Transcriptional activity and nuclear localization of PtoVNS11. **a** Onion epidermis was transformed with *35S:PtoVNS11:GFP* and *35S:GFP* constructs by particle bombardment. GFP fluorescent images were examined with confocal microscope at 20 h after bombardment. The position of nuclei was confirmed by DAPI staining. **b** Transcriptional activation analysis of *PtoVNS11* ORF fused with the GAL4 DNA-binding domain (GAL4BD) showing its ability to activate the expression of the Trp and α-Gal reporter genes in yeast

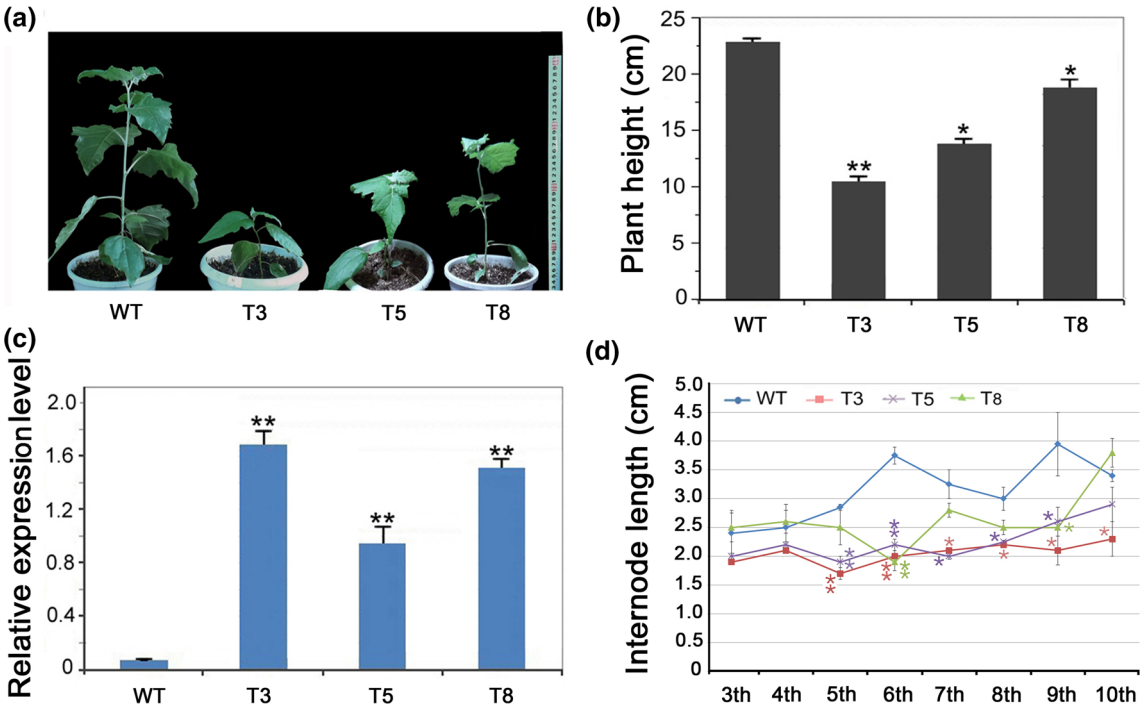

Fig. 5 Overexpression of *PtoVNS11* results in morphological changes in *Populus*. **a** Phenotypes of 12-week-old pro*35S:PtoVNS11* transgenic plants compared with WT. Plant heights (**b**) and stem internode lengths (20-week-old plants) (**d**) of transgenic *35S:PtoVNS11* and wild-type plants. **c** qRT-PCR analysis showing the expression of *PtoVNS11* in the seedlings of three representative transgenic lines (T3, T5 and T8). The expression of the *18S rRNA* gene was used as an internal control. Results are presented as means and standard deviations ($n = 10$). Data are mean ± SE from at least 10 plants in *different lines*. *Double asterisks* indicates significant differences in comparison with WT at $P < 0.01$, respectively (Student's *t* test)

Fig. 6, the stem tissues of the 35*S:PtoVNS11* plants stained more intensely than the 35*S:PtoVNS11* tissues. Compared to the wild-type control (Fig. 6a–c, g), the growth of xylem and phloem fiber was obviously increased from the third internode to the fifth internode in 35*S:PtoVNS11*stems (Fig. 6d–f, h). The xylem zone was significantly widened and the number of radial cell layers in the xylem regions was increased in *PtoVNS11* overexpressors (Fig. 6d–f, h). The transmission electron micrographs of xylem fiber and vessel walls in the *35S:PtoVNS11* plants (Fig. 6j) exhibited denser cell walls than that of in the wild-type plants (Fig. 6i). Quantitative analysis showed that lignin content in stem wood of transgenic lines was increased when compared with the control (Table S3). These results indicated that PtoVNS11 may play an important role during wood formation in poplar.

PtoVNS11 upregulates the expression of secondary cell wall synthesis-associated genes

We investigated the effects of *PtoVNS11* overexpression on the expression of secondary wall biosynthesis genes by qRT-PCR with gene-specific primers. Compared with wild-type plants, the expression levels of *PAL1*, *C4H2*, *4CL5,* and *CCoAOMT1* were significantly increased in transgenic *35:PtoVNS11* lines tested. We also found that no obvious changes in transcript levels of *HCT1*, *C3H3*, *CCR2,* and *LAC40* were detected, but all of the downstream genes including *CAld5H2*, *COMT2*, *CAD1,* and *PO6* were up-regulated in the transgenic plants overexpressing *PtoVNS11* (Fig. 7). These results indicated that PtoVNS11, as a transcriptional activator, is involved in the regulation of secondary cell wall biosynthetic genes in poplar.

Discussion

Wood formation involves complex developmental processes such as cambial cell division, cell expansion, secondary wall thickening, programmed cell death, and finally heartwood formation (Plomion et al. 2001). To genetically modify wood property, it is essential to characterize the regulatory genes that control the various developmental events of wood formation. A number of transcription factors have been identified to be associated with the regulation of lignin biosynthesis in tree species (Patzlaff et al. 2003; Karpinska et al. 2004; Schrader et al. 2004; Prassinos

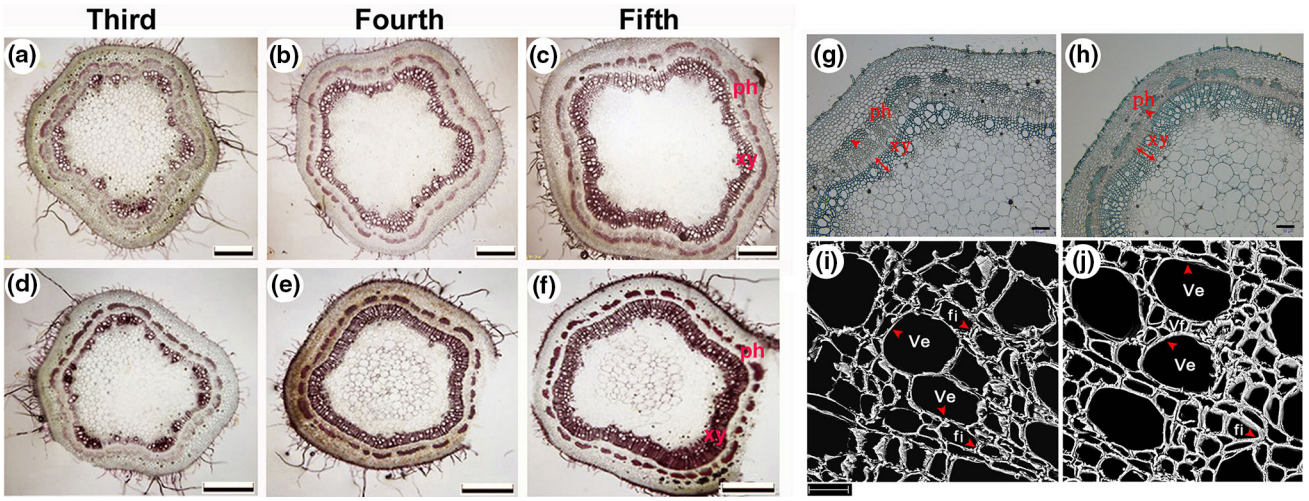

Fig. 6 Constitutive expression of *PtoVNS11* results in an increase of secondary cell wall thickness in transgenic plants. The different internodes in stems of 3-month-old wild-type (**a**–**c**, **g**) and transgenic lines (**d**–**f**, **h**) were sectioned and stained for lignin with phloroglucinol–HCl or Toluidine Blue. Transverse sections were observed by scanning electron microscope (SEM) from the 4th internode of the stems of WT (**i**) and transgenic plants (**j**). *xy* xylem, *ph* phloem, *ve* xylem vessel, *fi* xylem fiber. *Bars* 200 μm in **a**–**f**; 50 μm in **g**, **h**; 17.5 μm in **i**, **j**

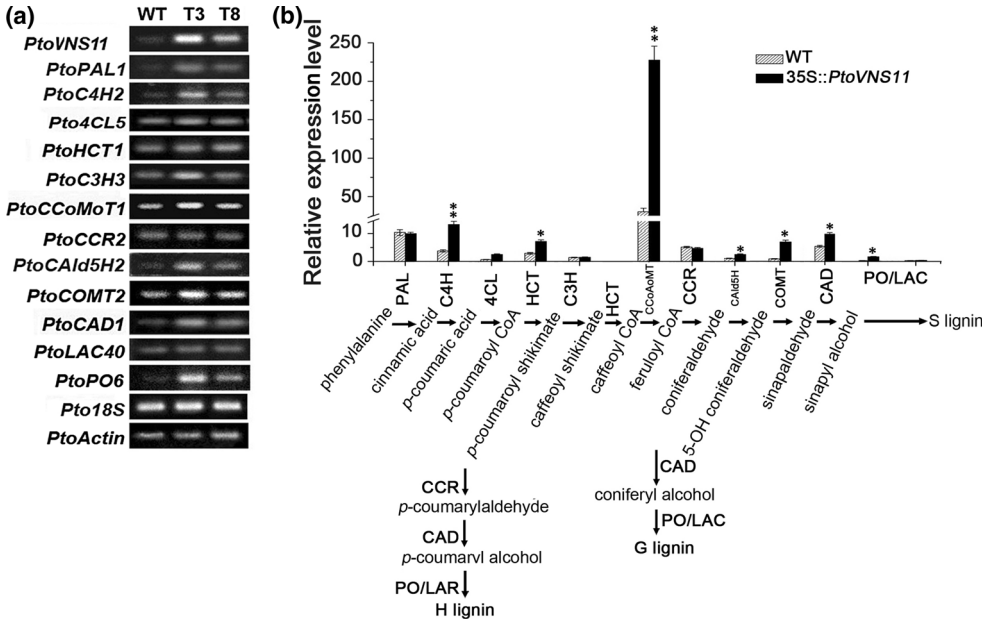

Fig. 7 Secondary cell wall-associated gene expression in *PtoVNS11* overexpressor plants. The expression levels of most of the genes in the lignin pathway were examined by semi-quantitative RT-PCR analysis. PAL (phenylalanine ammonia lyase), C4H (cinnamate-4-hydroxylase), 4CL (4-coumarate:CoA ligase), HCT (p-hydroxycinnamoyl-CoA:quinate shikimate p-hydroxycinnamoyltransferase), C3H (4-coumarate 3-hydroxylase), CCoAOMT (caffeoyl-CoA O-methyltransferase), CCR (cinnamoyl-CoA reductase), CAld5H (coniferyl aldehyde 5-hydroxylase), COMT (caffeic acid/5-hydroxyconiferaldehyde O-methyltransferase). *Error bars* represent SE of three independent biological replicates. *Single asterisk*, *double asterisk* indicate significant differences in comparison with WT at $P < 0.05$, $P < 0.01$, respectively (Student's *t* test)

et al. 2005; Andersson-Gunneras et al. 2006; Bedon et al. 2007; Bomal et al. 2008).

In *Arabidopsis* and poplar, it has been shown that the NAC transcription factors are master switches and their downstream targets are involved in the activation of the secondary wall biosynthetic genes (Wang et al. 2013a; Zhong et al. 2010; Zhong and Ye 2007). In *Arabidopsis*, a group of secondary wall NAC domain transcription factors such as SND1, NSTs and VNDs are important regulators that activate the secondary wall biosynthesis as master

transcriptional switches (Zhong and Ye 2007; Zhong et al. 2011). In poplar, PtrVNSs as SND1 functional orthologs have been demonstrated as activators of secondary cell wall-associated genes during wood formation (Ohtani et al. 2011).

In this study, we found that transcript abundance of *PtoVNS11* was highest in the xylem of poplar stems, the site of high lignin content (Fig. 2a, b). We further found that overexpression of *PtoVNS11* in *P. tomentosa* Carr. significantly reduced the plant height and internode length of stems (Fig. 5d). Similar results were reported by Zhao et al. (2014). That a defining feature of *PtoVNS11* was able to involve in xylem cells occurring during developmental processes, causing the cell wall structure appeared more compact when constitutively expressed in poplar plants (Fig. 6). We also observed that C4H, 4CL, HCT, CCoAOMT, COMT, and CAD, which have been shown to be involved in the biosynthesis of wood components (Ni et al. 1994; Li et al. 1999, 2003; Boerjan et al. 2003; Hisano et al. 2009; Shi et al. 2009), were up-regulated by PtoVNS11 (Fig. 7). These observations are consistent with previous findings in transgenic *Arabidopsis* overexpressing *PtrWND2B* and *PtrWND6B* (Zhong et al. 2010). Up-regulation of these secondary cell wall-associated genes may affect the secondary wall biosynthesis, leading to increased cell wall thickness in the stems of transgenic plants (Fig. 6). Taken together, our studies provided the evidence that PtoVNS11 plays an important role in the development of stem and secondary growth in poplar.

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Conflict of interest The authors declare that they have no conflict of interest.

Author contribution statement Conceived and designed the experiments: LY ST KL. Performed the experiments: LY XZ WL. Analyzed the data: KL WL LY. Contributed reagents/materials/analysis tools: WL LY YF. Wrote the paper: KL LY ST.

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