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Research paper

Molecular cloning and characterization of a brassinosteriod biosynthesis-related gene *PtoDWF4* from *Populus tomentosa*

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Brassinosteroids (BRs) as steroid hormones play an important role in plant growth and development. However, little is known about how BRs affect secondary wall biosynthesis in woody plants. In this study, we cloned and characterized *PtoDWF4*, a homologus gene of Arabidopsis *DWF4* encoding a cytochrome P450 protein, from *Populus tomentosa*. qRT-PCR analysis showed that *PtoDWF4* was highly expressed in stems, especially in xylem. Overexpression of *PtoDWF4* (*PtoDWF4-OE*) in poplar promoted growth rate and biomass yield, increased area and cell layers of xylem. Transgenic plants showed a significant increase in plant height and stem diameter compared with the wild type. In contrast, the CRISPR/Cas9-generated mutation of *PtoDWF4* (*PtoDWF4-KO*) resulted in significantly decreased biomass production in transgenic plants. Further studies revealed that constitutive expression of *PtoDWF4* up-regulated the expression of secondary cell wall (SCW) biosynthesis-related genes, whereas knock-out of *PtoDWF4* down-regulated their expression. Quantitative analysis of cell wall components showed a significant increase in *PtoDWF4-OE* lines but a reduction in *PtoDWF4-KO* lines compared with wild-type plants. Taken together, our results indicate that *PtoDWF4* plays a positive role in improving growth rate and elevating biomass production in poplar.

Keywords: biomass, brassinosteroid, *DWF4*, lignin biosynthesis, poplar, secondary cell wall.

Introduction

Brassinosteroids (BRs) as plant steroid hormones were first discovered from the pollens of *Brassica napus* in 1979 (Grove et al. 1979). It is well known that BRs play essential roles in plant physiological and developmental processes, such as embryonic development, cell expansion and differentiation (Clouse and Sasse 1998), vascular formation (Ibañes et al. 2009), crop improvement (Vriet et al. 2012), in response to abiotic and biotic stresses (Sahni et al. 2016). Mutants defective in BR biosynthesis or signal transduction display stunted growth, including short petioles, rounded and curled leaves, pleiotropic dwarf phenotypes and accelerated senescence (Clouse et al. 1996, Fujioka et al. 1997, Choe et al. 1998).

Over the past decades, the BR-biosynthetic pathways have been illuminated in higher plants. Brassinolide (BL), which represents as the most active BR, is synthesized from campesterol (CR) through two parallel pathways, which are called the early and the late C-6 oxidation pathways (Vriet et al. 2013). Meanwhile, some BR signaling pathway components were also identified (Wang and He 2004). For example, BRASSINOSTEROID INSENSITIVE1 (BRI1), which acts as a leucine-rich membrane-localized protein, can interact with BRI1-ASSOCIATED RECEPTOR KINASE (BAK1) to modulate BR signaling (Kinoshita et al. 2005). Other BR regulatory proteins, including BRASSINOSTEROID INSENSITIVE2 (BIN2), BRI1 inhibitory protein BKI1, BR-signaling kinase 1 (BSK1), 14-3-3 proteins, BRI1 SUPPRESSOR 1 (BSU1), BRI1-EMS SUPPRESSOR1

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(BES1) and BRASSINAZOLE-RESISTANT1 (BZR1), are also identified via various genetic and biochemical approaches (Wang et al. 2004, Mora-Garcia et al. 2004, Wang and Chory 2006, Ryu et al. 2007, Tang et al. 2008, Yu et al. 2011). Evidence to date suggests that BRs signal is perceived by plasma membrane-localized receptor kinase BRI1, and transduced into the nucleus through these signaling components (Kinoshita et al. 2005).

BRs are synthesized from campesterol through a series of enzymatic steps. Several genes encoding these key enzymes in BR-biosynthetic pathway have been isolated from many plant species, such as Arabidopsis, rice (*Orzya sativa*) and tomato (*Solanum lycopersicum*) (Chung et al. 2010, Yokot et al. 1997). DEETIOLATED2 (DET2) is associated with catalyzing a 5α-reduction step of multiple related sterols during BR biosynthesis (Fujioka et al. 1997). The *de-etiolated2* (*det2*) mutant with a dwarfed phenotype was firstly identified from Arabidopsis (Chory et al. 1991) and also obtained from pea (Nomura et al. 2004). Through investigation on the mutants with severely pleiotropic phenotypes, other key BR-biosynthetic genes, including CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM (CPD; Szekeres et al. 1996), DWARF4 (DWF4; Choe et al. 1998), ROTUNDFOLIA3 (ROT3; Kim et al. 1998) and BR-6-oxidase1 (BR6ox1; Bishop et al. 1999), have also been identified in Arabidopsis and rice. These mutants defective in the BR-biosynthetic enzymes motioned above exhibit changed phenotypes such as dwarfism, delayed senescence, dark-green leaves, shorten petioles and short hypocotylsin darkness (Chory et al. 1991, Li et al. 2012, Chory and Li 1997, Choe et al. 1998). For instance, in Arabidopsis, the *dwf1* mutant is defective in the BR biosynthesis, leading to reduced inflorescence length and internode distance (Choe et al. 1999). The *cpd* mutant exhibits short hypocotyls, obsolescent apical hooks and opening cotyledons (Szekeres et al. 1996) and the *cyp90* mutant displays lower fertility with shorter petals and anthers, compared with wild-type plants (Ohnishi et al. 2006). These results show that BRs functionally affect morphogenesis and phylogenesis in plants.

In woody plants, secondary cell wall (SCW) formation is a complex process which is precisely controlled by a regulatory network at the level of transcription for the coordinating regulation of several diverse metabolic pathways (Larson 1994, Zhong and Ye 2014). It is well known that phytohormones participate in SCW formation. For example, auxin polar transport may modulate vascular meristem and gibberellin (GA) plays an important role in controlling tension wood formation (Schrader et al. 2003, Jiang et al. 2008). BRs are considered to be a possible regulator for xylem development and can regulate the formation of vascular bundles together with auxin (Miyashima et al. 2013). Enhanced BR-signaling and/or synthesis led to increased number of vascular bundles in Arabidopsis, indicating the role of BRs in promoting the formation of xylem vascular (Nagata et al. 2001, Ibañes et al. 2009). In addition, Arabidopsis DIMINUTO 1 (DIM1) involved in BR biosynthesis had an influence on lignin composition and SCW formation (Hossain et al. 2012). However, the role of BRs in controlling SCW formation in woody species remains still unclear.

DWF4 encodes a cytochrome P450 protein which catalyzes a rate-limiting step during BR biosynthesis (Choe et al. 1998, 2001). Overexpression of *AtDWF4* in *B. napus* enhanced abiotic and biotic stress tolerance and plant productivity (Sahni et al. 2016). Similarly, transgenic Arabidopsis overexpressing *BdDWF4* from *Brachypodium distachyon* displayed long and slender phenotypes (Corvalánl et al. 2017). Although *DWF4* in herbaceous plants has been well identified, little information about its function is known in woody plants so far. More recently, ectopically expression of *PeDWF4* from *Populus euphratica* in Arabidopsis resulted in an alteration of microstructure of inflorescence stems (Si et al. 2016), but the molecular mechanism remains largely unclear. In this study, we isolated *PtoDWF4* from *Populus tomentosa* Carr., which was homologous to Arabidopsis *DWF4*, and determined its role in SCW formation during wood development. Overexpression of *PtoDWF4* (*PtoDWF4-OE*) in poplar increased stem diameter and xylem cell layers, resulting in higher biomass in transgenic plants compared to the wild type, whereas a significant reduction in growth rate was found in transgenic *PtoDWF4*-knock-out (*PtoDWF4-KO*) poplars. Our results suggest that *PtoDWF4* could be involved in the positive regulation of wood formation in poplar.

Materials and methods

Plant materials and growth conditions

Populus tomentosa Carr. (Clone 741) plants were grown in a greenhouse the conditions of 16/8 h light/dark cycle with the 4500 lux supplementary light at 22–25 °C and relative humidity ~60%. Poplar plants were watered according to the evapotranspiration demands during different growth stages and fertilized with 1/2 strength Hoagland nutrient solutions.

Sequence alignment and phylogenetic analysis

The amino acid sequences of DWF4 proteins and related cytochrome P450s used in our experiments were obtained from the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al. 1990), using DWF4 proteins of *Arabidopsis thaliana* and rice (*O. sativa*) as a query. Multiple sequence alignments were performed with DNAMAN7.0 (Lynnon Corporation, USA) and the phylogenetic analysis of CYP proteins were performed with the software MEGA6 (Tamura et al. 2013).

Cloning of PtoDWF4

Total RNA was extracted from leaf samples using plant Trizol Reagent (Tiangen, China) The full-length cDNA fragment (1467 bp) of *PtoDWF4* was amplified with gene-specific primers (see Table S1 available as Supplementary Data at *Tree Physiology* Online) based on *PtrDWF4* (Potri.007G026500.1) by polymerase chain reaction (PCR). The PCR reaction was carried out with Pfu DNA polymerase (Takara, Dalian, China) in a total volume of

50 μl at 98 °C for 2 min, 34 cycles of 98 °C for 15 s, 56 °C for 30 s, 72 °C for 1 min 30 s, followed by final extension of 72 °C for 10 min. The reaction system contained 25 μl of Primer star Max (TAKARA), 2 μl of each primer (Forward primer and Reverse primer), 2 μl of cDNA as DNA template, 19 μl of nuclease-free water. PCR products were purified and ligated into pMD19-T plasmid (Takara, Dalian, China).

Construction of the PtoDWF4-OE vector and generation of transgenic poplar plants

The full-length coding sequence (CDS) of *PtoDWF4* was digested with *Bam*HI restriction enzyme (Takara, Dalian, China), and ligated into plant binary vector pCXSN (Chen et al. 2009) via the same restriction site, under the control of Cauliflower mosaic virus (CaMV) 35 S promoter. The resultant construct *PtoDWF4-OE* was introduced into *Agrobacterium tumefaciens* strain GV3101 by the freeze–thaw method (Hofgen and Willmitzer 1988).

Populus tomentosa transformation was carried out using *A. tumefaciens*-mediated leaf disc method (Jia et al. 2010). In brief, leaf discs of poplar were co-cultured in Woody Plant Medium (WPM), containing 2.0 mg l^{-1} zeatin (ZT), 1.0 mg l^{-1} 1-naphthalene acetic acid (NAA) and 100 μM^{-1} acetosyringone (AS), in dark for 48 h after infected with *Agrobacterium*. Two days later, the leaf discs were transferred to WPM medium with 2.0 mg l^{-1} ZT, 1.0 mg l^{-1} NAA, 400 mg l^{-1} cefotaxime (Cef), 9 mg l^{-1} hygromycin (Hyg) for callus induction. After one month of subculture in the dark, these leaf discs with calli were transferred to selective medium containing 2.0 mg l^{-1} ZT, 0.1 mg l^{-1} NAA, 400 mg l^{-1} Cef, 9 mg l^{-1} Hyg. Subsequently, regenerated shoots were transferred to rooting medium with 0.1 mg l^{-1} NAA, 400 mg l^{-1} Cef, and 9 mg l^{-1} Hyg. After regeneration of roots, the seedlings were planted in the greenhouse. Genomic DNA extracted from leaves of transgenic plants was used for PCR analysis with gene-specific primers of *Hyg* gene. Expression levels of the transgenes in transgenic plants were determined by quantitative real-time PCR (qRT-PCR) using total RNA extracted from leaves. All primers are listed in Table S1 available as Supplementary Data at *Tree Physiology* Online.

CRISPR/Cas9-mediated mutation of PtoDWF4 in poplar

The pYLCRIPSR/Cas9 vectors for multiplex genome targeting vector system of CRISPR/Cas9 were used for mutation of *PtoDWF4* in poplar (Ma et al. 2015). The coding region of *PtoDWF4* was monitored via ZiFiT Targeter (http://zifit.partners.org; Version 4.2) (Sander et al. 2010). Three CRISPR/Cas9 target sites of *PtoDWF4* were chosen for the sgRNA sequences based on their location and GC percentage. These sgRNA cassettes were driven by Arabidopsis AtU3b, AtU6-1 and AtU6-29 promoters, respectively. The entire construction process was followed by Golden Gate Cloning, generating the resultant construct *PtoDWF4-OE* (Fan et al. 2015). Transgenic plants harboring *PtoDWF4-KO* were produced by *Agrobacterium*-mediated transformation as described previously (Jia et al. 2010).

To identify CRISPR/Cas9-mediated mutation of *PtoDWF4* in transgenic poplar plants, genomic DNA was extracted from stems *via* the CTAB method. The *PtoDWF4* genomic fragment was amplified using the gene-specific primers of *PtoDWF4* (see Table S1 available as Supplementary Data at *Tree Physiology* Online). The amplified products were cloned into the pMD19-T Simple vector (Takara, Dalian, China) and then sequenced. The *PtoDWF4*-Cas9 transgenic lines (L15 and L17) were chosen for propagation. PCR genotyping and DNA sequencing was further confirmed in six regeneration plants of transgenic lines L15 and L17. At least 20 clones for each line were randomly selected for DNA sequencing.

Quantification of xylem relative area and SCW thickness

Xylem relative area and SCW thickness were measured by Image J software (Long et al. 2013). Three biological replicates were measured for each line.

Quantitative RT-PCR

Total RNA was extracted from different tissues of 5-month-old *P. tomentosa* plants including roots, the sixth internode of stems, the second young leaves, the 32nd mature leaves, by using the Trizol Reagent (Tiangen, China). The sixth internode of stems was selected for separating xylem and phloem. Genomic DNA was removed from total RNA by DNase. cDNA was synthesized by PrimeScript™ RT reagent Kit with genome DNA Eraser (Takara, Dalian, China). qRT-PCR was performed on a TP700 Real-Time PCR machine (Takara, Japan) using the SYBR Green PCR master mix (Takara, Dalian, China). The poplar *18S rRNA* gene was used as the reference gene for internal standard. The primers used for qRT-PCR are listed in Table S1 available as Supplementary Data at *Tree Physiology* Online. Three biological and three technical replicates were carried out for each gene.

Measurement of BRs content

Stems of 6-month-old *PtoDWF4-OE*, *PtoDWF4-KO* and wild-type plants were used to measure the content of BRs in vivo. After cutting samples, add PBS (phosphate-buffered saline, pH 7.4) and then frozen with liquid nitrogen. BR levels were assayed by Plant Brassinolide (BR) ELISA Kit (Beijing Chenglin Biotechnology Company, China).

Histochemical staining of lignin

The sixth internode of the 5-month-old poplar stems were fixed in FAA buffer (formaldehyde: glacialacetic acid: 50% ethanol, 1:1:18). After embedding in paraffin, the stems were cross-sectioned by using an Ultra-Thin Semiautomatic Microtome (FINESSE 325, Thermo) and stained with 0.05% (w/v) toluidine blue O and then observed under Zeiss optical microscope (Zeiss, Oberkochen, Germany).

For histochemical staining, stem sections of poplar were hand-cut with a razor blade and an Ultra-Thin Semiautomatic Microtome

(FINESSE 325, Thermo). The micro-sections were stained for 15 s with 1.0% (w/v) phloroglucinol after dissociation for 60 s by 40% (v/v) HCl, and then observed under an Olympus BX53 microscope. After the digital images were captured with a diagnostic instrument, the software Adobe Photoshop CC was used to process them.

Chemical analysis of SCW components

The sixth internode of 5-month-old poplars was used for chemical analysis of SCW components. Total lignin content was measured by classical Klason, acid-soluble and acetyl bromide-soluble (AcBr) lignin methods as previously described by Li et al. (2014). The absorbance was measured by a Bio UV-visible spectrophotometer (Shimadzu UV-2401 PC UV-VIS). The concentration of AcBr-soluble lignin was determined by an extinction coefficient of 20.0 l g^{-1} cm^{-1}. The content of cellulose and xylan was examined by the Van Soest method (Van Soest and Wine 1967). Each experiment repeated three times.

Scanning electron microscope analysis

To perform scanning electron microscopy (SEM), cross sections were obtained by dissecting transversely with razor blade by hand and the samples were attached using double-sided stick tapes. The samples were observed by SEM (Phenom^{tm}Pure FEI, USA) following the manual's recommendations and images were captured digitally. In addition, the software Revolution 1.6.1 was used to measure vessel cell wall thickness (μm).

Statistical analyses

The quantitative data were evaluated for statistical significance (*P* value) using Student's *t*-test. All the tests were two-tailed. The data was normalized and all samples were normally distributed with homogeneity of variance.

Results

Isolation and characterization of PtoDWF4 from *P. tomentosa*

The full-length coding sequence of *PtoDWF4* (Potri.007G026500.1) was isolated from *P. tomentosa* Carr. according to the genome sequence data (http://genome.jgi-psf-org/Poptrl_l/Poptrl_l.home.html) using gene-specific primers (see Table S1 available as Supplementary Data at *Tree Physiology* Online) by reverse transcription PCR (RT-PCR). Sequence analysis results showed that *PtoDWF4*, which contained a full-length CDS with a length of 1467 bp, encoded a putative protein of 488 amino acid residues with a predicted molecular weight of ~55.2 kDa and a calculated isoelectric point (pI) of 9.08. The predicted amino acid sequence of PtoDWF4 contained three conserved domains, including the heme binding domain A, the steroid binding domain B and domain C (Nebert and Gonzalez 1987). Multiple sequence alignment revealed that PtoDWF4 shared high identity with PtrDWF4 (98.57%) in *P. trichocarpa* (Torr. & Gray), AtDWF4 (74.08%) in Arabidopsis, and relative lower identity with OsDWF4 (65.82%) in rice and ZmDWF4 (63.13%) in maize (Figure 1A).

Previous studies have reported that DWF4 belongs to the cytochrome P450 superfamily (CYP90B1) (Choe et al. 1998, 2001). As shown in Figure 1B, phylogenetic analysis revealed that PtoDWF4 was grouped into Class I together with AtDWF4, ZmDWF4 and OsDWF4, which have been identified to participate in BR biosynthesis and promote the growth and development of plants (Choe et al. 1998, Hong et al. 2003, Liu et al. 2007).

Expression patterns of PtoDWF4 in poplar

We predicted the expression pattern of *PtoDWF4* in an associated web resource (http://aspwood.popgenie.org) (Sundell et al. 2017). The result showed that *PtoDWF4* was highly expressed in wood-forming zone (see Figure S1 available as Supplementary Data at *Tree Physiology* Online). To further determine the expression profiles of *PtoDWF4*, total RNA was extracted from different tissues of 5-month-old *P. tomentosa*. qRT-PCR analysis showed that *PtoDWF4* was detected in all tissues tested, but highly expressed in stems, especially in xylem (Figure 2). By contrast, relatively low expression level was detected in roots.

Construction and identification of transgenic poplars

To investigate the function of *PtoDWF4* in the regulation of the growth and development of poplar, we isolated the coding sequence of *PtoDWF4* and fused it with the *CaMV 35S* promoter to generate a plant binary expression vector *PtoDWF4-OE* (Figure 3A). After transformation, putative transgenic plants were selected for confirming the integration of the transgene by PCR analysis using the primers of the hygromycin phosphotransferase gene (*Hyg*) (see Figure S2A available as Supplementary Data at *Tree Physiology* Online). Ten independent transgenic lines harboring *PtoDWF4* were chosen for qRT-PCR analysis. Two transgenic lines (L2 and L4) with high transcript levels of *PtoDWF4* were propagated and used for further studies (Figure 3B).

Meanwhile, we also generated the *PtoDWF4* mutant lines using the CRISPR/Cas9-based genome editing system. Three 20-bp sequences followed by a trinucleotide (5'-NGG-3') protospacer adjacent motif (PAM) located in the first three exon of *PtoDWF4* were assigned (Figure 3C). The *PtoDWF4*-knock-out construct (*PtoDWF4-KO*) was introduced into *P. tomentosa* by *Agrobactium*-mediated leaf disc method. More than 20 putative transgenic plants were generated and PCR analysis showed that the *PtoDWF4* fragments containing three sgRNA-targeted sites were amplified from all transgenic lines (see Figure S2B available as Supplementary Data at *Tree Physiology* Online). PCR products from lines L2, L8, L15 and L17 were amplified and cloned into PMD19 vector. A total of 20 PCR clones for each mutant were randomly chosen for DNA sequencing. The results revealed that deletions (–), insertions (+) and substitutions of one or a few nucleotides were detected at the three sgRNA-targeted sites in two transgenic lines (L15 and L17) In line L15, small deletions

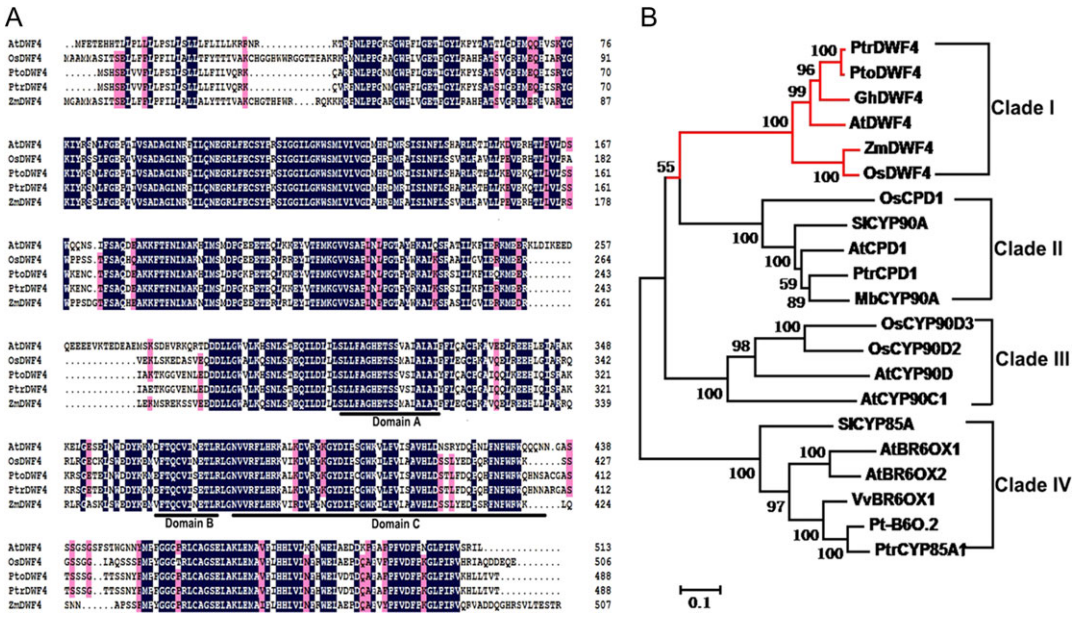

Figure 1. Multiple sequence alignment and phylogenetic analysis of amino acid sequences of PtoDWF4 with other cytochrome P450s from different species. (A) Sequence alignments of *PtoDWF4* with other CYTOCHROME P450s. Amino acid sequences were aligned with the software of DNAMAN 8. The conserved P450s domains are underlined. Identical and similar amino acid residues are shaded with black and gray, respectively. (B) The phylogenetic relationship of *PtoDWF4* with other CYP450 proteins was constructed by the Neighbor-Joining method in MEGA6 software. The bootstrap values are shown as percentages at the nodes. Bar = 0.05 substitutions per site. The GenBank accession numbers of P450s as follows: *AtDWF4* (*Arabidopsis*, AF044216), *ZmDWF4*, (*Zea mays*, CYP90b2), *OsDWF4* (*Oryza sativa*, AB206579), *PtrDWF4* (*P. trichocarpa*, Potri.007G026500.1), *GhDWF4* (*Gossypium hirsutum* L, DQ996567), *AtCPD* (*Arabidopsis*, X87367), *OsCPD1* (*O. sativa*, AK060257), *SlCYP90A* (*Solanum lycopersicum*, BT014380), *MbCYP90A2* (*Vigna radiata*, AF279252), *AtCYP90C1* (*Arabidopsis thaliana*, AB008097), *AtCYP90D1* (*A. thaliana*, AB066286), *OsCYP90D2* (*O. sativa*, AP003244), *OsCYP90D3* (*O. sativa*, AC130732), *AtBR6OX1* (*A. thaliana*, AB035868), *AtBR6OX2* (*A. thaliana*, AB087801), *VvBR6OX1* (*Vitis vinifera*, DQ235273), *PtrCYP85A1* (*P. trichocarpa*, Potri.004G117700.1), *Pt-B6O.2* (*P. trichocarpa*, Potri.017G099000.1), *SlCYP85A* (*S. lycopersi*, U54770).

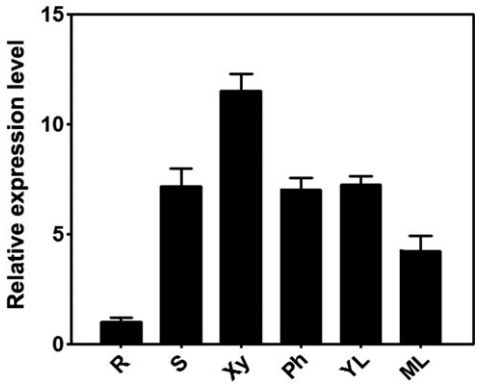

Figure 2. Expression patterns of *PtoDWF4* in poplar. qRT-PCR was performed to analyze the transcript levels of *PtoDWF4* in different tissues of poplar. The poplar *18S* gene was used as an internal control. Error bars represent ± SD from three biological repeats. R: roots; S: stems; Xy: xylem; Ph: phloem; YL: young leaves; ML: mature leaves.

(1 bp or 2 bp) were detected at the three target sites, and small insertions (1 bp) happened at target sites T1 and T3, suggesting the efficiency of mutagenesis. Similar results were obtained in line L17 (Figure 3D). These insertions and deletions detected in the *PtoDWF4-KO* lines resulted in translational frame-shift or premature termination of *PtoDWF4* (see Figure S3 available as Supplementary Data at *Tree Physiology* Online), indicating the successful generation of *PtoDWF4* loss-of-function mutants using CRISPR/Cas9-based genome editing approach. No mutations were found in other lines (L2 and L8) (data not shown). Considered that *PtoDWF4* shared high identity (91.43%) with a close paralog (Potri.005G124000.1), we also detected whether the mutations occurred in the targets of Potri.005G124000.1 in transgenic lines L15 and L17. The sequencing results showed that no mutations were found in these target sites of Potri.005G124000.1 in transgenic lines (see Figure S4 available as Supplementary Data at *Tree Physiology* Online).

Overexpression of PtoDWF4 promotes growth and development of poplar

All transgenic and wild-type poplar plants were grown in the greenhouse under the same environmental conditions. Compared with the wild type, 5-month-old transgenic *PtoDWF4-OE* plants exhibited rapid growth and development. Inversely, transgenic *PtoDWF4-KO* lines delayed morphological changes, including retarded growth, reduced internode number and smaller leaf size, but no significant alteration in leaf shape (Figure 4A). Quantitative measure showed that the stem diameter and plant height were

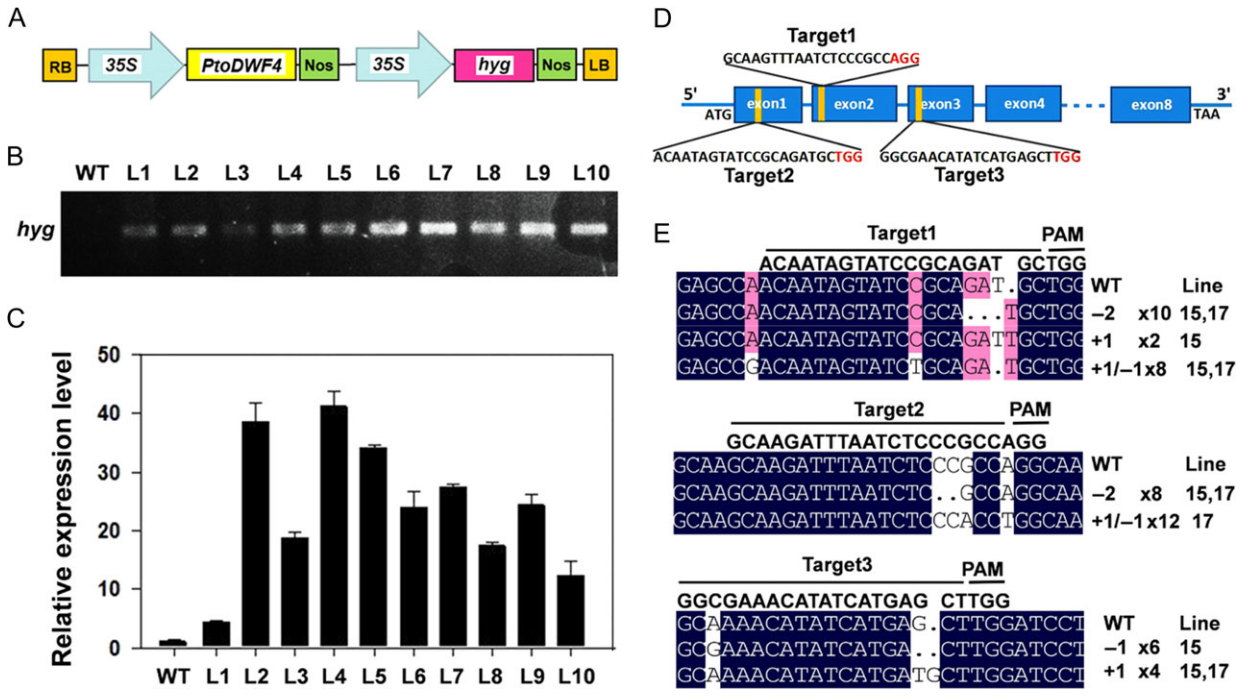

Figure 3. Generation of transgenic poplars. (A) Diagram of the *PtoDWF4-OE* vector. (B) The expression levels of *PtoDWF4* in the *PtoDWF4-OE* lines. The poplar *18S* gene was used as an internal control. Error bars represent ± SD from three biological repeats. (C) Diagram of three CRISPR/Cas9 target sites of *PtoDWF4*. T1, T2 and T3 indicate the positions of sgRNA-targeted sites. (D) Determination of the mutations in the coding region of *PtoDWF4* generated by the CRISPR/Cas9 system. The text on the right summarizes mutation details in two independent CRISPR/Cas9-generated lines (L15 and L17).

significantly increased in *PtoDWF4-OE* plants but dramatically reduced in *PtoDWF4-KO* lines in comparison to the wild-type control (Figure 4B and C). Shoot dry weight increased 33–46% in transgenic *PtoDWF4-OE* lines and reduced 23–43% in *PtoDWF4-KO* lines compared to the wild type (Figure 4D). These phenotypical alterations coincide with previous studies, which showed that BRs-related genes could enhance cell elongation and promote plant growth (Mora-Garcia et al. 2004, He et al. 2007, Wang et al. 2013). Taken together, our results suggested that *PtoDWF4* might play an important role in regulating poplar growth and development.

PtoDWF4 is involved in the positive regulation of wood formation in poplar

In order to determine the potential role of PtoDWF4 in wood formation, SCW development was analyzed in the sixth internode of 5-month-old wild-type and transgenic plants. Toluidine blue staining analysis showed that transgenic *PtoDWF4-OE* lines exhibited larger stem diameter compared to the wild-type control, while *PtoDWF4-KO* lines had a significantly smaller the size of stem diameter (Figure 5A–C). Further magnification of histochemical staining revealed that overexpression of *PtoDWF4* improved secondary xylem development (Figure 5E), leading to expanded xylem area (Figure 5J) and increased number of xylem cell layers (28 layers) (Figure 5K), compared to wild-type plants (Figure 5D). In contrast, knock-out of *PtoDWF4* resulted in reduced xylem development (Figure 5F and I) and only 16 layers were observed in *PtoDWF4-KO* plants (Figure 5K). Similar results were also observed in the fifth and seventh internode between transgenic lines and wild-type plants (data not shown).

Additionally, the lignin deposition in the vascular tissues of transgenic *PtoDWF4-OE* plants and *PtoDWF4-KO* mutants were determined by Phloroglucinol/HCl staining. The lignin-specific histochemical staining showed that no obvious changes in lignin deposition were detected in the stem xylem of the *PtoDWF4-OE* and *PtoDWF4-KO* plants compared to the wild-type (see Figure S5A–C available as Supplementary Data at *Tree Physiology* Online). Scanning electron microscope (SEM) also revealed that there was no significant difference in the thickness of the vessel cell walls between transgenic *PtoDWF4-OE* and *PtoDWF4-KO* lines as well as the wild-type control (see Figure S5D–G available as Supplementary Data at *Tree Physiology* Online). These results suggested that *PtoDWF4* mainly improved the xylem development during wood formation, but did not affect the cell wall thickness of stem xylem in poplar.

To further verify the effect of *PtoDWF4* on xylem development, the content of the chemical components, including lignin, cellulose and xylan, was examined in transgenic lines. Glucan is the most important component of cellulose, which may reflect the content of cellulose. The results showed that lignin contents in PtoDWF4-*OE*

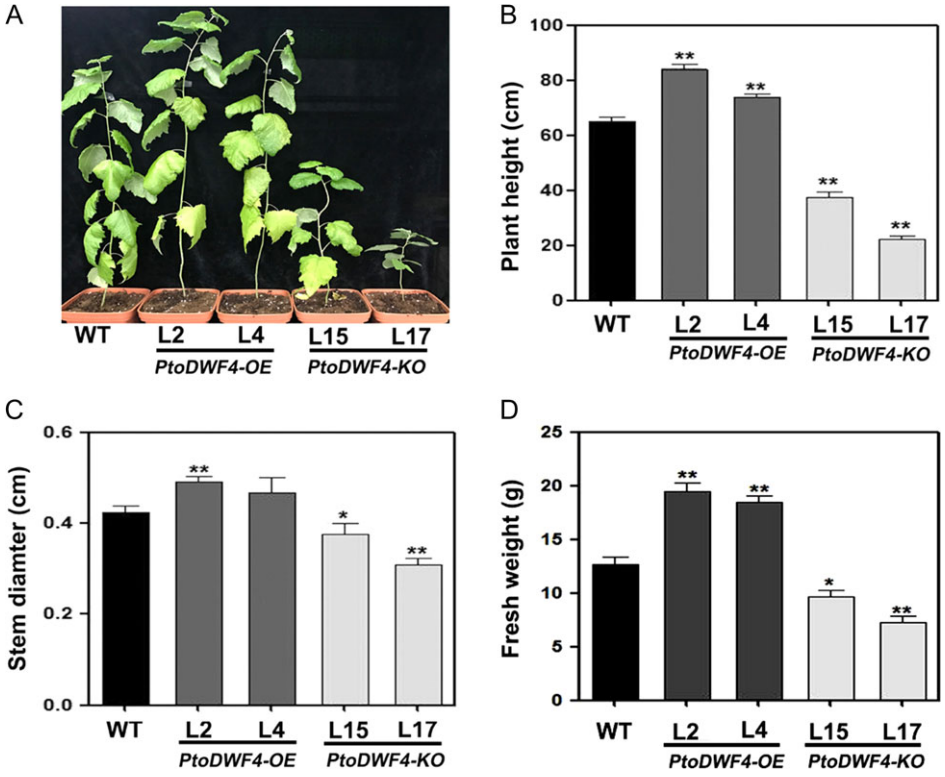

Figure 4. Morphological phenotypes of transgenic *PtoDWF4-OE* and *PtoDWF4-KO* poplar plants. (A) Phenotypes of the 5-month-old wild-type and transgenic plants. Measurement of plant height (B), stem diameter of the sixth internode (C) and shoot dry weight (D) in the wild-type and transgenic lines. Scale bars = 10 cm (A). Error bars: ± SD. *$P < 0.05$; **$P < 0.01$.

lines were significantly increased by 10.64–13.09% and decreased by 8.79–11.67% in *PtoDWF4-KO* lines, compared to wild-type plants (Table 1). Similar changes in the xylan and cellulose contents were obtained from these transgenic plants (Table 1). These results indicated that *PtoDWF4* is a positive regulator for improving xylem development in poplar.

Overexpression of PtoDWF4 improves BR biosynthesis in transgenic poplar

Previous studies have shown that DWF4 was responsible for BR biosynthesis (Choe et al. 1998, 2001). The expression levels of the BRs-related genes in transgenic plants were determined. As shown in Figure 6, qRT-PCR analysis showed that no significant changes occurred in *PtoBZR1* (homology with *AtBZR1*, which functions as a transcriptional repressor with dual roles in BR homeostasis and growth responses) (He et al. 2005), in transgenic and wild-type plants. Notably, the transcriptional levels of *PtoCPD, PtoROT3, PtoCYP85A2* (homology with *AtCPD*, *AtROT*, *AtCYP85a2*, respectively) (Mathur et al. 1998, Shimada et al. 2003, Kim et al. 2005*a*, 2005*b*) were significantly up-regulated in *PtoDWF4-OE* lines, but down-regulated in *PtoDWF4-KO* lines in comparison to the wild type (Figure 6). Furthermore, we examined BR levels in transgenic and wild-type plants. Overexpression of *PtoDWF4* resulted in a significant increase in BR concentrations in transgenic plants. By contrast, in *PtoDWF4-KO* lines (L15 and L17), BR levels were decreased to 89% and 80% of the levels in the wild type, respectively (Figure 7).

PtoDWF4 positively regulated the expression of SCW biosynthesis-related genes in poplar

Given that *PtoDWF4* influenced xylem development in poplar, we examined the expression of SCW biosynthesis-related genes via qRT-PCR. As expected, the expression of lignin biosynthetic genes (*PAL4*, *C4H2*, *F5H2*, *4CL5*, *HCT1*, *C3H3*, *CCOAOMT1*, *CCR2*, *COMT2, CAD1*), a cellulose biosynthetic gene (*CesA2B*) and a xylan biosynthetic gene (*GT43D*) (Joshi et al. 2004, Djerbi et al. 2005, Shi et al. 2010, Lee et al. 2011, Vanholme et al. 2013) were induced in transgenic *PtoDWF4-OE* lines but suppressed in *PtoDWF4-KO* lines (Figure 8A), consistent with the changes of chemical components in stem xylem of transgenic lines (Table 1). Additionally, the expression of SCW-associated transcription factors was also determined in transgenic *PtoDWF4-OE* and *PtoDWF4-KO* plants (Figure 8B). The results revealed that overexpression of *PtoDWF4* induced the expression of 4 *NAC* (*NAC154*, *NAC105*, *WND1B* and *WND2B*), 2 *MYB* (*MYB103* and *MYB092*) and *WRKY12* transcription factor genes (Ye and Zhong 2015), whereas knock-out of *PtoDWF4* resulted in a significant reduction in their expression in transgenic

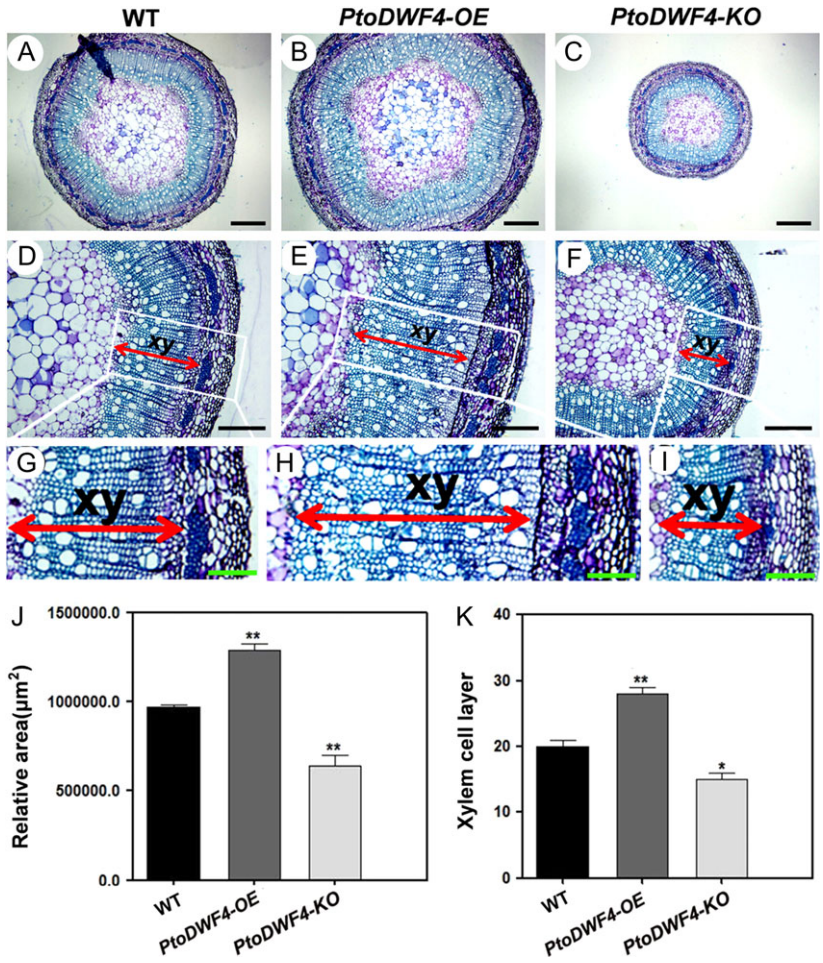

Figure 5. Effects of *PtoDWF4* on xylem development in transgenic lines. (A–I) Cross sections of stems showing increased xylem area in the sixth internode of 5-month-old plants stained with toluidine blue. Stem cross sections from the wild-type (A, D, G), *PtoDWF4-OE* (B, C, E) and *PtoDWF4-KO* (F, H, I) plants. (J) Measurement of relative area of xylem in wild-type and transgenic plants. (K) Numbers of xylem layers in wild-type and transgenic plants. Xy, xylem. Scale bars: (A–C) = 500 μm; (D–F) = 100 μm; (G–I) = 50 μm.

Table 1. Cell wall composition analysis of the stems in wild-type and transgenic plants. Plant materials were prepared from the stems of 6-month-old plants and determined by Klason, AcBr and Van Soest methods. Error bars represent ± SD from three biological repeats. Asterisks indicate significant differences in comparison to WT (Student's *t*-test: **P* < 0.05; ***P* < 0.01). The unit is mg100 mg^{-1}.

Sample	WT	*PtoDWF4-OE*		*PtoDWF4-KO*	
		L2	L4	L2	L4
Lignin					
Acid-insoluble	20.17 ± 0.42	22.83 ± 0.52**	22.03 ± 0.58*	19.53 ± 0.37	18.33 ± 0.49*
Acid-soluble	3.13 ± 0.27	3.52 ± 0.23	3.25 ± 0.25	2.72 ± 0.13	2.25 ± 0.15*
Total	23.30 ± 0.69	26.35 ± 0.75*	25.78 ± 0.83*	21.25 ± 0.50	20.58 ± 0.64*
Polysaccharide					
Xylan	16.34 ± 0.48	18.28 ± 0.51*	17.52 ± 0.45	15.88 ± 0.31	14.52 ± 0.25*
Glucan	42.12 ± 0.75	45.34 ± 1.07*	44.86 ± 0.82*	41.86 ± 1.02	40.34 ± 0.83

plants. These results implied that PtoDWF4 might positively regulate xylem development by activating SCW biosynthetic genes in poplar.

As procambium and cambium are able to differentiation into xylem (Elo et al. 2009), we also detected the influence of *PtoDWF4* on cambium maker genes. The results were consistent with our expectation. *HB7*, which promoted xylem cell differentiation (Zhu et al. 2013), was elevated in *PtoDWF4-OE* lines (Figure 8C). Meanwhile, the expression levels of cambium genes (*C3H18* and *Ant1*) (Schrader et al. 2004, Chai et al. 2014) were

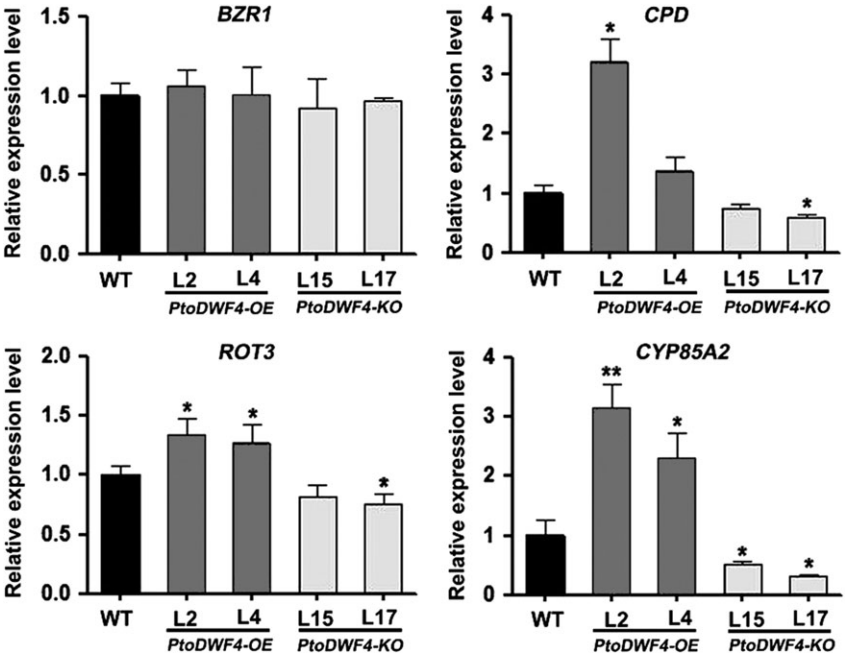

Figure 6. Expression analysis of BR transcriptional regulator *BZR1* and synthetic pathway genes *CPD*, *ROT3* and *CYP85A2* in wild-type, *PtoDWF4-OE* and *PtoDWF4-KO* plants. Primers are listed in Table S1 available as Supplementary Data at *Tree Physiology* Online. The poplar *18S* rRNA gene was used as an internal control. Error bars represent ± SD from three biological repeats. Student's *t*-test: $*P < 0.05$; $**P < 0.01$.

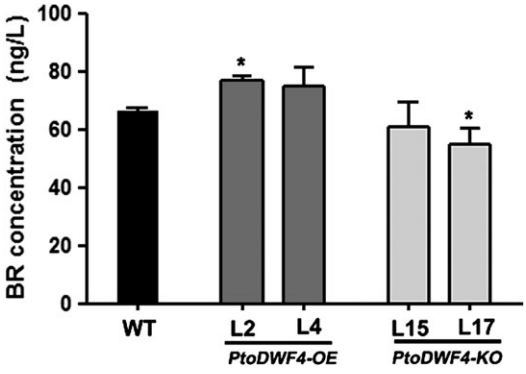

Figure 7. The content of BRs in transgenic plants. Values in the figure represent the means ± SE of three replicates. Student's *t*-test: $*P < 0.05$.

significantly increased in *PtoDWF4-OE* with lower transcriptional levels in *PtoDWF4-KO* lines (Figure 8C). These results indicated that *PtoDWF4* was involved in the regulation of cambium in wood development in poplar.

Discussion

Over the past decades, extensive studies have shown that phytohormones play essential roles in plant growth and development, organ formation. In woody species, it has been demonstrated that hormones such as auxin, cytokinin, GA and ethylene, have important roles in wood formation (Nugroho et al. 2012, Pramod et al. 2012). For instance, auxin plays an important role on embryonic development and procambium patterning (Scarpella et al. 2002, Jenik and Barton 2005), GAs are involved in the regulation of early stages of xylem differentiation during wood formation in aspen (Israelsson et al. 2003). Cytokinins participate in the regulation of cambial development in poplar (Nieminen et al. 2008). Ethylene is also an important natural phytohormone and interacts with other hormones in tension wood formation in poplar (Andersson-Gunneras et al. 2003). Recently, BRs were shown to take part in regulating wood formation. It is well demonstrated that BRs stimulated the tracheary element differentiation, and also had a feedback regulation to ensure homeostasis of cell wall biosynthesis (Yamamoto et al. 1997, Wolf et al. 2012). *DIM1*, a BR biosynthesis gene, participated in controlling cell wall structure and composition in Arabidopsis (Hossain et al. 2012). Although increasing evidence has established the connection between BR and cell wall formation in Arabidopsis, to date, little is known about the roles of BRs in secondary xylem formation of woody species.

Previous studies have demonstrated that *DWF4* (also known as CYP90B1) encodes C-22 hydroxylase (Choe et al. 1998), which plays an important role in the hydroxylation of various C27-29 sterols (Choe et al. 2001, Fujita et al. 2006). Since the ratio of reagents to the direct products of DWF4 was the highest among all the enzymes involved in the BR biosynthesis flux, DWF4 is considered as one of the main enzymes in the BR biosynthesis (Choe et al. 2001, Shimada et al. 2003). In a previous study, *PeDWF4*, homolog of Arabidopsis *DWF4* (*AtDWF4*), was isolated from *P. euphratica* which is a woody species with high tolerances to drought and salt stresses (Si et al. 2016). Overexpression of *PeDWF4* in transgenic Arabidopsis plants resulted in an increase

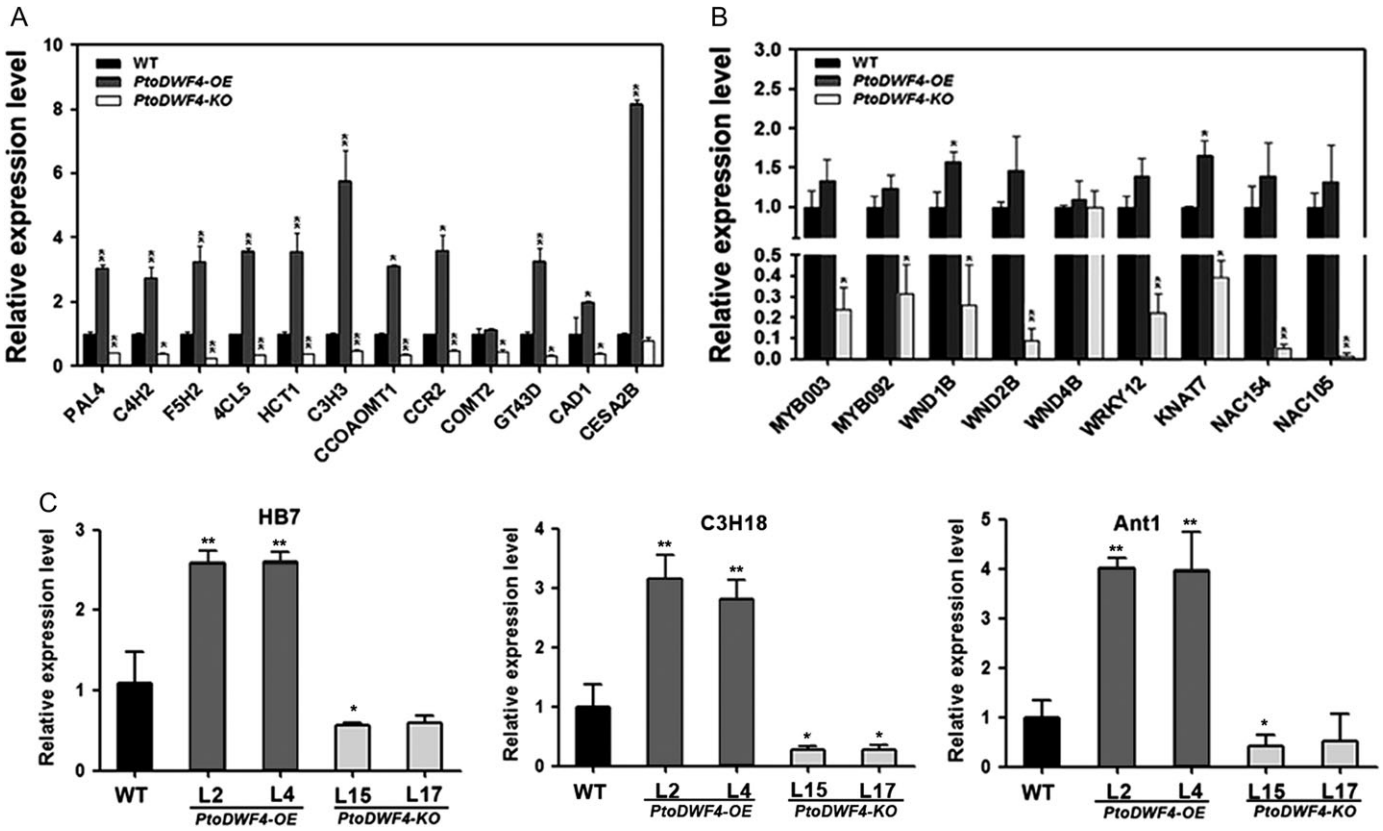

Figure 8. Expression analysis of these genes associated with wood biosynthesis in wild-type and transgenic plants. The poplar *18S* rRNA gene was used as an internal control. Primers are listed in Table S1 available as Supplementary Data at *Tree Physiology* Online. Error bars represent ± SD from three biological repeats. Student's *t*-test: $^{*}P < 0.05$; $^{**}P < 0.01$.

in plant heights, number of siliqua, hypocotyl length and seed yields. In this study, another poplar *DWF4* gene *PtoDWF4* was cloned from *P. tomentosa*. Amino acid sequence analysis showed that *PtoDWF4* contains one heme binding domain A, the steroid binding domain B and domain C (Nebert and Gonzalez 1987) (Figure 1A), suggesting that it was a conserved cytochrome P450 monooxygenase protein. Phylogenetic analysis showed that *PtoDWF4* was grouped together with *AtDWF4*, *ZmDWF4* and *OsDWF4*, but had the closest relationship with*PeDWF4* (Figure 1B). Transgenic plants overexpressing these *DWF4* genes showed a significant increase in vegetative growth, inflorescence stem height and biomass yield, due to an elevation of the endogenous levels of BR biosynthesis (Choe et al. 1998, Hong et al. 2003, Liu et al. 2007, Si et al. 2016). We showed that overexpression of *PtoDWF4* resulted in an increase in both plant height and stem diameter in poplar (Figure 4), whereas knockout of *PtoDWF4* led to a reduction in biomass of transgenic plants (Figure 4D). These phenotypic alterations are consistent with previous reports in transgenic Arabidopsis overexpressing *AtDWF4*, which displayed increased inflorescence height and numbers of both seeds and branches. Similar severe dwarf phenotype was observed in the Arabidopsis *dwf1* mutant (Takahashi et al. 1995, Choe et al. 2001, Liu et al. 2007). These phenotype similarities are probably due to the altered activity of the rate-limiting step enzyme, thus influencing BR biosynthesis in poplar. Our result revealed that *PtoDWF4* had a conserved function in specific hydroxylation steps during BR biosynthesis in poplar.

Extensive evidence has shown that plant hormones play important roles in xylogenesis and vascular development (Zhong and Ye 2014). In BR-biosynthetic mutants, for example, expression of cell wall synthetic genes was down-regulated. Exogenous BRs increased tracheary element differentiation in *Zinnia elegans* (Yamamoto et al. 1997). Moreover, BRs regulate vascular differentiation, promoting xylem formation (Fukuda 2004). A recent study showed that Arabidopsis *DIM1* is involved in BR biosynthesis and its mutant *dim1* displayed a dwarf phenotype with a reduction in lignin and cellulose composition (Hossain et al. 2012). In order to investigate the function of *PtoDWF4* in woody plants, here we generated transgenic plants carrying *PtoDWF4-OE* and *PtoDWF4-KO* cassettes. Histochemical staining of toluidine blue showed that constitutive expression of *PtoDWF4* improved secondary xylem development in transgenic *PtoDWF4-OE* plants (Figure 5), resulting in elevated number of xylem cell layers (Figure 5K), compared with the wild type (Figure 5D). Inversely, the *PtoDWF4-KO* mutants exhibited reduced xylem area (Figure 5F and I) and fewer xylem cell layers (Figure 5K).

Interestingly, Phloroglucinol/HCl staining and SEM analysis showed that no significant difference in the thickness of the vessel cell walls was found between transgenic *PtoDWF4-OE* and *PtoDWF4-KO* lines as well as the wild-type control (see Figure S5 available as Supplementary Data at *Tree Physiology* Online). Taken together, these data implied that *PtoDWF4* is involved in the positively regulation of xylem development during wood formation, but did no change the thickness of SCW in poplar.

Lignin, cellulose and xylan constitute the main components of the SCW in plants and wood formation requires the coordinated regulation of their biosynthesis (Ye and Zhong 2015). qRT-PCR analysis revealed that overexpression of *PtoDWF4* resulted in an elevation in the expression of SCW biosynthetic genes for lignin (*PAL4, C4H2, F5H2, 4CL5, HCT1, C3H3, CCOAOMT1, CCR2, COMT2* and *CAD1*), cellulose (*CesA2B*) and xylan (*GT43D*) (Figure 8A). In contrast, their expression levels were down-regulated in *PtoDWF4-KO* lines (Figure 8A). Meanwhile, *PtoDWF4* activated the expression of NAC and MYB transcription factors, while knock-out of *PtoDWF4* repressed their transcriptional levels (Figure 8B). Similar results were reported in a previous study, in which *PtCYP85A3*, a functional homolog of *AtCYP85A2* worked as a bifunctional cytochrome P450 monooxygenase to catalyze a rate-limiting step in the BR-biosynthetic pathway (Kim et al. 2005*a*, 2005*b*), was isolated from *P. trichocarpa* (Jin et al. 2017). Overexpression of *PtCYP85A3* improved xylem formation without affecting the composition of cellulose and lignin as well as the thickness of SCW in transgenic poplar (Jin et al. 2017).

In addition, as vascular cambium of trees played an important role on producing xylem and phloem cells, we also detected the transcriptional levels of genes involved in vascular cambium development. Overexpression of *PtoDWF4* enhanced the expression of poplar *HB7, C3H18* and *Ant1* genes, while relative lower expression occurred in *PtoDWF4-KO* plants (Figure 8C). In conclusion, our study provides molecular evidence that *PtoDWF4* is involved in the positive regulation of wood development, especially xylem development, in poplar. Although we found that *PtoDWF4* influenced the expression of SCW- and vascular cambium-associated genes, the regulatory mechanism still needs to be further explored.

Supplementary Data

Supplementary Data for this article are available at *Tree Physiology* Online.

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