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Molecular cloning and characterization of a brassinosteriod biosynthesis-related gene PtoCYP90D1 from Populus tomentosa

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Abstract： Brassinosteroids (BRs), one of the major classes of phytohormones are essential for various processes of plant growth, development, and adaptations to biotic and abiotic stresses. In Arabidopsis, AtCYP90D1 acts as a bifunctional cytochrome P450 monooxygenase, catalyzing C-23 hydroxylation in the brassinolide biosynthetic pathway. The present study reports the functional characterizations of PtoCYP90D1, one of the AtCYP90D1 homologous genes from Populus tomentosa. The qRT-PCR analysis showed that PtoCYP90D1 was highly expressed in roots and old leaves. Overexpression of PtoCYP90D1 (PtoCYP90D1-OE) in poplar promoted growth and biomass yield, as well as increased xylem area and cell layers. Transgenic plants exhibited a significant increase in plant height and stem diameter as compared to the wild type. In contrast, the CRISPR/Cas9-generated mutation of PtoCYP90D1 (PtoCYP90D1-KO) resulted in significantly decreased biomass production in transgenic plants. Further studies revealed that cell wall components increased significantly in PtoCYP90D1-OE lines but not in PtoCYP90D1-KO lines, as compared to wild-type plants. Overall, the findings indicate a positive role of PtoCYP90D1 in improving growth rate and elevating biomass production in poplar, which will have positive implications for its versatile industrial or agricultural applications.

Keywords: Populus tomentosa, biomass, brassinosteroid, CYP90D1, secondary cell wall.

1.Introduction

Brassinosteroids (BRs) are steroid hormones with great potential for crop improvement. These were first discovered from the pollens of *Brassica napus* [1, 2]. It is well known that BRs play critical roles in plant physiological and developmental processes, such as cell expansion and differentiation, vascular formation [3], crop improvement [4], and tolerance to abiotic and biotic stresses [5, 6]. The plant metabolism and plant biomass can be significantly altered by genetically modulating BR content or enhancing signal transduction [7, 8].

The BR-biosynthetic pathways in higher plants have been revealed during the past few decades. Brassinolide (BL), the most active BR, is synthesized from campesterol (CR) via two parallel pathways, known as the early and late C-6 oxidation pathways [9]. Meanwhile, certain components of the BR signaling pathways have also been identified [10, 11]. 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 [12]. Other BR regulatory proteins,such as BRASSINOSTEROID INSENSITIVE2 (BIN2), BRI1 INHIBITORY PROTEIN BKI1, BR-SIGNALING

KINASE 1 (BSK1), 14-3-3 proteins, BRI1 SUPPRESSOR 1 (BSU1), BRI1-EMS SUPPRESSOR1 (BES1) and BRASSINAZOLE-RESISTANT1 (BZR1), have also been discovered through genetic and biochemical methods. [11, 13-17]. It suggests that the perception of BR signaling occurs through the plasma membrane-localized receptor kinase BRI1, which then transduces signals into the nucleus via these signaling components [12].

BRs are produced from campesterol via a series of enzymatic processes. Numerous genes encoding these pivotal enzymes in the BR-biosynthetic pathway have been identified in various plant species, including *Arabidopsis*, rice (*Oryza sativa*), tomato (*Solanum lycopersicum*), and poplar (*Populus tomentosa*) [18]. In BR biosynthesis, DEETIOLATED2 (DET2) is involved in catalyzing a 5α-reduction reaction of several related sterols [19]. The *deetiolated2* (*det2*) mutant, characterized by a dwarfed phenotype, was initially discovered in *Arabidopsis* [20] and subsequently found in maize [21]. Further investigation of mutants exhibiting severe pleiotropic phenotypes led to the identification of additional key BR-biosynthetic genes, such as CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM (CPD) [22], DWARF4 (DWF4) [23], ROTUNDFOLIA3 (ROT3) [24] and BR-6-OXIDASE1(BR6OX1) [25], in both *Arabidopsis* and rice. Mutants lacking these crucial BR-biosynthetic enzymes manifest various phenotypic changes, including dwarfism, delayed senescence, dark-green leaves, shortened petioles, and reduced hypocotyl length in darkness [23]. For example, the *Arabidopsis dwf1* mutant, which has a defect in BR biosynthesis, exhibits reduced inflorescence length and internode distance[26]. Similarly, the *cpd* mutant shows shortened hypocotyls, obsolete apical hooks, and opening cotyledons [22], while the *cyp90* mutant displays reduced fertility with shorter petals and anthers compared to wild-type plants [27]. These observations underscore the significant role of BRs in plant morphogenesis and phylogenesis.

In the biosynthetic pathway of BRs, the *AtCYP90D1* is involved in C-23 hydroxylation [27]. Two rice genes *OsCYP90D2*/*D3*, homologous to *AtCYP90D1* were cloned, and their mutants exhibited a typical BR-deficient phenotype [28, 29]. Additionally, *OsCYP90D2* can participate in the C-3 dehydrogenation reaction. *CYP90D1* encodes a cytochrome P450 protein which catalyzes the C-23 hydroxylations (22-OH-3-one, 3-epi-6-deoxoTY to 6-deoxo3DT, 6-deoxoTY) during BR biosynthesis [24].

The effects of *CYP90D1* in *Arabidopsis thaliana* and rice have been studied, but the role of *CYP90D1* in poplar growth and development has not been established yet. In this study, the *PtoCYP90D1*, homologous to *Arabidopsis CYP90D1*, was isolated from *Populus tomentosa* Carr. The effect of overexpression of *PtoCYP90D1* in transgenic plants was investigated as compared to the wild type. Similarly, the impact of its absence on the growth rate of poplars was elucidated by knocking out *PtoCYP90D1* via CRISPR/Cas9-mediated mutation of *PtoCYP90D1*. Thus, the role of *PtoCYP90D1* in the growth and biomass regulation of poplar was comprehensively encompassed in the present study.

2.Materials and methods

2.1Plant materials and growth conditions

Plants of *Populus tomentosa* Carr. were grown in a greenhouse under a 16/8-hour light/dark cycle with supplemental light of 4500 lux at temperatures ranging from 22 to 25°C and 60% relative humidity. Watering was adjusted based on evapotranspiration requirements at various growth stages, and fertilization was performed using ½-strength Hoagland nutrient solutions.

2.2Sequence alignment and phylogenetic analysis

Total RNA was extracted from leaf samples using plant Trizol Reagent (Tiangen, China). The full-length *PtoCYP90D1* cDNA was cloned from *P. tomentosa*, verified by sequencing, and inserted into the plant binary vector pCXSN under the control of the 35S promoter. The PCR reaction was carried out for 34 cycles with DNA polymerase (Takara, Dalian, China) in a total volume of 50 μl. The reaction system contained 25 μl of Primer star Max DNA Polymerase (Takara, Dalian, China), 2 μl of each primer (Forward primer and Reverse primer), 2 μl of cDNA as DNA template, and 19 μl of nuclease-free water. The steps constituted of initialization at 98 °C for 2 min, denaturation at 98 °C for 15 s, annealing at 56 °C for 30 s, and extension at 72 °C for 1 min 30 s, followed by final extension of 72 °C for 10 min. The PCR products were purified and ligated into pSH737 plasmid (Takara, Dalian, China).

2.3Construction of the PtoCYP90D1-OE vector and generation of transgenic poplar plants

The full-length coding sequence (CDS) of *PtoCYP90D1* was digested with *BamHl* restriction enzyme (Takara, Dalian, China), and ligated into the plant binary vector pCXSN [30] via the same restriction site, under the control of *Cauliflower mosaic virus* (CaMV) 35S promoter. The resultant construct *PtoCYP90D1*-OE was introduced into *Agrobacterium tumefaciens* GV3101 by the freeze-thaw method [31]. *P. tomentosa* transformation was carried out using the *Agrobacterium*-mediated method [32]. Briefly, after being infected with *Agrobacterium*, poplar leaf discs were co-cultured in Murashige and Skoog Medium (MS), containing 2.0 mg/L zeatin (ZT), 1.0 mg/L naphthalene acetic acid (NAA) and 100 μM-Acetosyringone (AS), in dark for 48 h. Two days later, the leaf discs were transferred to Woody Plant Medium (WPM) with 2.0 mg/L ZT, 1.0 mg/L NAA, and 9 mg/L Kanamycin (Kana) for callus induction. After one month of subculturing in the dark, these leaf discs with calli were transferred to a selective medium containing 2.0 mg/L ZT, 0.1 mg I-1 NAA, and 9 mg/L Kana. Subsequently, regenerated shoots were transferred to rooting medium with 0.1 mg/L NAA, and 9 mg/L Hygromycin (Hyg). After the regeneration of roots, the seedlings were planted in the greenhouse. Genomic DNA isolated from leaves of transgenic plants was used for PCR analysis, using Kana gene-specific primers. The expression levels in transgenic plants were evaluated by quantitative real-time PCR (qRT-PCR) using total RNA extracted from leaves. Table S1 enlists all primers and is available as supplementary data at Tree Physiology Online.

2.4CRISPR/Cas9-mediated mutation of PtoCYP90D1 in poplar

For mutation of *PtoCYP90D1* in poplar, the pYLCRIPSR/Cas9 vectors for multiplex genome targeting vector system of CRISPR/Cas9 were used[33]. Based on their position and GC percentage, two CRISPR/Cas9 target sites of *PtoCYP90D1* were chosen for the sgRNA sequences. 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, resulting in *PtoCYP90D1*-KO [7].

To identify the CRISPR/Cas9-mediated mutation of *PtoCYP90D1* in transgenic poplar plants, genomic DNA was extracted from stems via the CTAB method. The *PtoCYP90D1* genomic fragment was amplified using the gene-specific primers of *PtoCYP90D1* (see Table S1). The amplified products were cloned into the pSH737 simple vector (Takara, Dalian, China), followed by sequencing. The *PtoCYP90D1*-Cas9 transgenic lines (KO) were chosen for propagation. For further validation, PCR genotyping and DNA sequencing were

confirmed in six regeneration plants of KO transgenic lines.

2.5Quantification of xylem relative area

Xylem relative area was measured by ImageJ software [7]. Three biological replicates were measured for each line.

2.6Quantitative RT-PCR

Total RNA was extracted from various tissues of 3-month-old *P. tomentosa* plants, including roots, the sixth stem internode, and seven leaves, by using the Trizol Reagent (Tiangen, China). The sixth internode of stems was selected for separating the xylem and phloem. DNase treatment was utilized to remove genomic DNA from total RNA. DNA was synthesized by PrimeScript™ RT reagent Kit with a genomic DNA Eraser (Takara, Dalian, China). The qRT-PCR was conducted on a TP700 Real-Time PCR machine (Takara, Japan), employing the SYBR Green PCR master mix (Takara, Dalian, China). The poplar 18S rRNA gene served as the reference gene for the internal standard(Table S1).

2.7Quantitative estimation of BRs

The content of BRs in vivo was measured using stems from 3-month-old *PtoCYP90D1*-OE, *PtoCYP90D1*-KO, and wild-type plants. Following cutting, stem samples were immersed in PBS(phosphate-buffered saline, pH 7.4) and subsequently frozen using liquid nitrogen. BR levels were determined using the Plant Brassinolide ELISA Kit (Beijing Chenglin Biotechnology Company, China).

2.8Chemical analysis of secondary cell wall (SCW) components

The sixth internode of 3-month-old poplars was used for chemical analysis of SCW components. The absorbance was measured by a Bio UV-visible spectrophotometer (Shimadzu UV-2401 PC UV-VIS). The concentration of acetyl bromide (AcBr)-soluble lignin was determined by an extinction coefficient of 20.01 $g^{-1} \cdot cm^{-1}$. The content of cellulose and lignin was determined by the Van Soest method [34]. All experiments were conducted in triplicates.

2.9Statistical analysis

Statistical analysis of qRT-PCR data among the different tissue types, in response to exogenous hormones, and decapitation was performed in Excel 2010. The Statistical Program for Social Science 18 (SPSS, Chicago, IL, USA) was used for ANOVA and Student's t-tests (two tail distribution and two samples with unequal variances). The statistical significance was considered at $*0.01<P<0.05$ and $**P<0.01$.

3Results

3.1Isolation and characterization of PtoCYP90D1 from P. tomentosa

The sequencing analysis revealed that *PtoCYP90D1*, which contained a full-length CDS of 1,497 bp, encoded a putative protein of 493 amino acid residues with a predicted molecular weight of 56.7 kDa and isoelectric point (pI) of 8.97. The predicted amino acid sequence of PtoCYP90D1 contained three conserved domains, including the Proline-rich domain (pxgxxgwpxxget), oxygen-binding domain (mipxxk, exxr, and pxrx), steroid-binding domain (ftqxvitetlrxgn), and heme-binding domain[35]. Multiple sequence alignment revealed that PtoCYP90D1 shared a high identity with PtCYP90D1 (76.4%) in *Populus trichocarpa*, and AtCYP90D1 (74%) in *Arabidopsis* (Figure 1B).

Previous studies have reported that *CYP90D1* belongs to the cytochrome P450 superfamily D [24]. phylogenetic (Figure 1A) analysis revealed that *PtoCYP90D1* was grouped into Class I together with *AtCYP90D1*, *PtCYP90D1*, and *OsCYP90D2/3*, which have been identified to participate in BR biosynthesis and promote the growth and development of plants [28, 29].

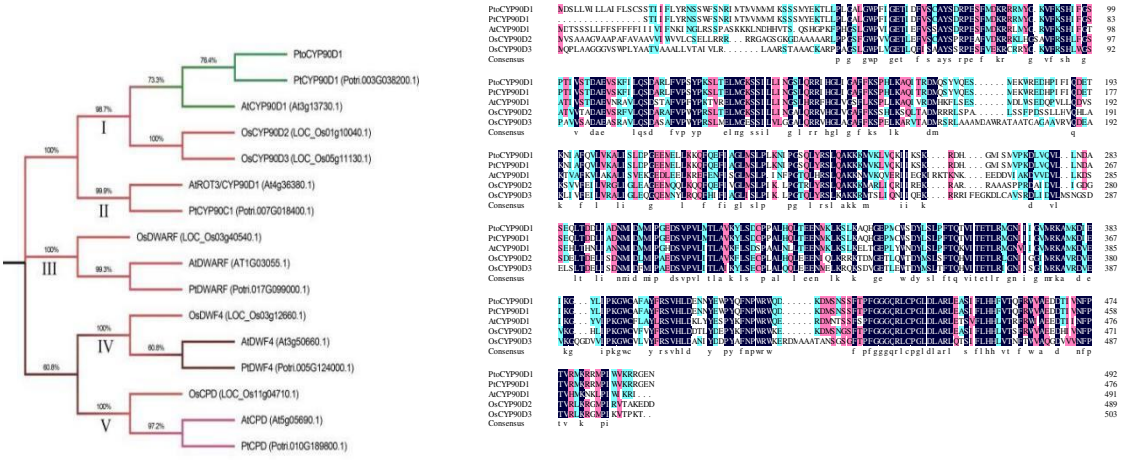


Figure 1 Bioinformatics analysis. (A): Phylogenetic tree of BR's gene of *P. tomentosa* with closest components in *Populus trichocarpa*, *Arabidopsis thaliana,* and *Oryza sativa.* M: methionine start codon, pxgxxgwpxxget: proline-rich domain. Mipxxk, exxr, and pxrx: dioxygen binding domain. Ftqxvitetlrxgn: steroid binding domain. Fgggrlcpgldlarl:hemo binding domain. (B): Multiple sequence alignment of CYP90D1 amino acid sequence. PtCYP90D1: Potri.003G038200.1. AtCYP90D1: AT3g13730.1. OsCYP90D2: LOC_Os01g10040.1. OsCYP90D3: LOC_Os05g11130.1.

3.2Expression patterns of PtoCYP90D1 in poplar

To determine the expression profiles of PtoCYP90D1, total RNA was extracted from different tissues of 3-month-old P. tomentosa. The findings of qRT-PCR showed that the expression level of the PtoCYP90D1 gene in the root and old leaf was significantly higher than in shoot, bud, juvenile leaf, and internode (Figure 2), suggesting that PtoCYP90D1 is involved in the development and maturation of leaf.

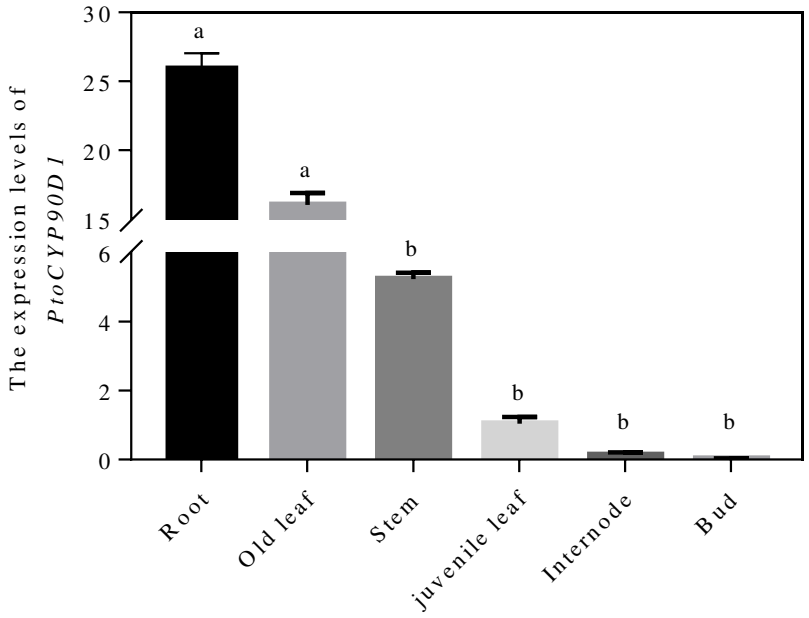
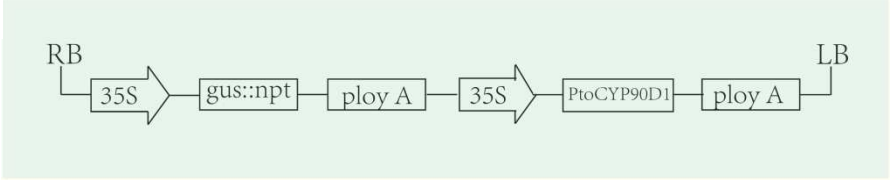


Figure 2 Expression analysis of *PtoCYP90D1* in different tissues (root, old leaf, bud, stem, internode, and juvenile leaf) of *P. tomentosa*. Significant difference analysis using Duncan's test, n=3. Error bars represent the SDs, and the different lowercase letters indicate statistically significant differences at $P<0.01$.

3.3Construction and identification of transgenic poplars

To explore *PtoCYP90D1*'s role in poplar growth and development regulation, the coding sequence of *PtoCYP90D1* was isolated and fused with the CaMV 35S promoter to generate a plant binary expression vector pSH737-35S-*PtoCYP90D1* (Figure 3A). After transformation, putative transgenic plants were selected for confirming the integration of the transgene by PCR analysis using the primers of the *Kana* gene (TableS 2). Two transgenic lines *PtoCYP90D1*-OE and *PtoCYP90D1*-KO were propagated and used for further studies (Figure 3B).

Furthermore, the *PtoCYP90D1* mutant lines were generated using the CRISPR/Cas9-based genome editing system. Two 20 bp sequences followed by a trinucleotide (5'-NGG-3') protospacer adjacent motif (PAM) located in the first three exons of *PtoCYP90D1* were assigned (Figure 3D). The *Agrobactium*-mediated leaf disc method was used to introduce *PtoCYP90D1*-knock-out construct into *P. tomentosa*. More than 2 putative transgenic plants were generated and PCR analysis showed that the *PtoCYP90D1* fragments containing two sgRNA-targeted sites were amplified from all transgenic lines (Figure S2B available as supplementary data at Tree Physiology Online). 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 one transgenic line (*PtoCYP90D1*-KO). In *PtoCYP90D1*-KO, small deletions (1 bp) were detected at the two target sites, suggesting the efficiency of mutagenesis. These deletions detected in the *PtoCYP90D1*-KO lines resulted in translational frame-shift or premature termination of *PtoCYP90D1* (Figure 3C), indicating the successful generation of *PtoCYP90D1* loss-of-function mutants using CRISPR/Cas9-based genome editing.



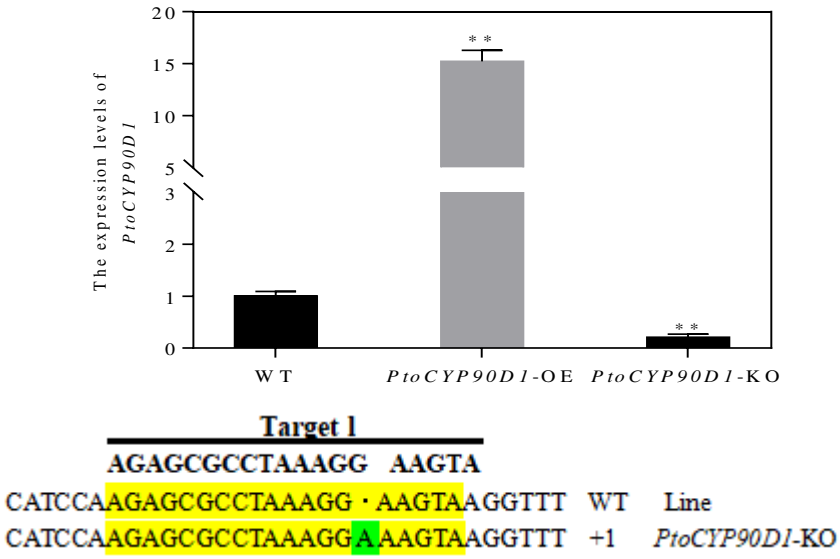


Figure 3 Generation of transgenic poplars. (A) Schematic representation of the *PtoCYP90D1*-OE vector. (B) The expression levels of *PtoCYP90D1* in the transgenic and wild-type plants. Error bars represent the SD (n=3). Statistical analyses were performed using Student's *t*-test as ** $P<0.01$ and * $0.01<P<0.05$. (C) Determination of the mutations in the coding region of *PtoCYP90D1* generated by the CRISPR/Cas9 system. The text on the right summarizes mutation details in the independent CRISPR/Cas9-generated lines.

3.4Overexpression of PtoCYP90D1 promotes the growth and development of poplar

All transgenic and wild-type poplar plants were grown under the same environmental conditions. The 3-month-old transgenic *PtoCYP90D1*-OE plants exhibited rapid growth and development, as compared to the wild type. Inversely, transgenic *PtoCYP90D1*-KO lines exhibited delayed morphological changes, including retarded growth, and reduced plant weight (Figure 4).

The quantitative measures showed that the plant height and stem diameter were significantly increased in *PtoCYP90D1*-OE plants, but not dramatically reduced in *PtoCYP90D1*-KO lines in comparison to the wild-type control (Figure 4B and C). Fresh weight increased by 46.94% in transgenic *PtoCYP90D1*-OE lines and reduced by 42.54% in *PtoCYP90D1*-KO lines, as compared to the wild type (Figure 4D). These phenotypical alterations are in line with previous studies, which reported that BRs-related genes could enhance cell elongation and promote plant growth[36, 37]. The findings of the present study also indicate the crucial role *PtoCYP90D1* plays in regulating poplar growth and development.

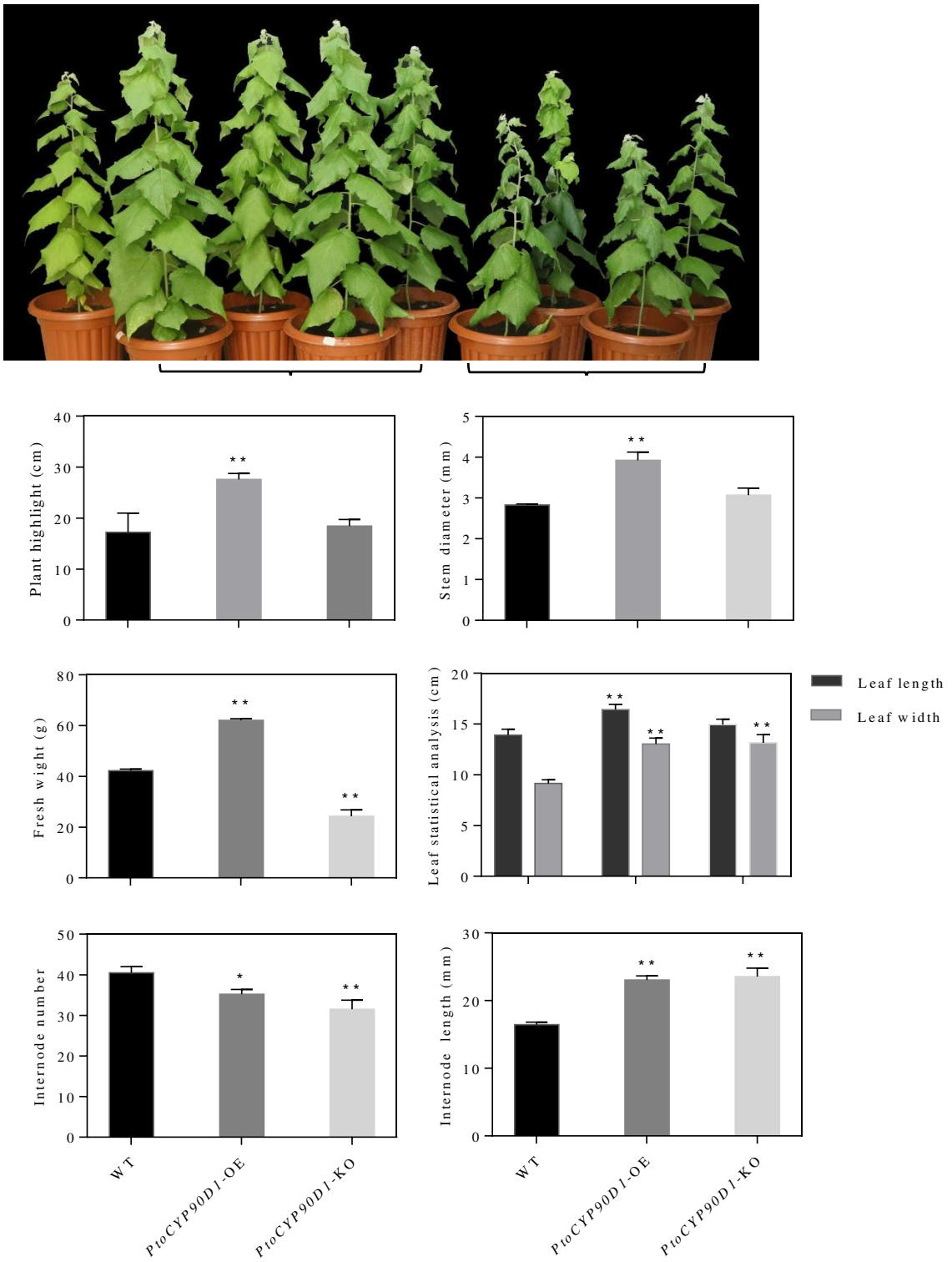


Figure 4 Growth comparisons of wild-type and transgenic plants. (A) Plants after growing for 3 months. (B) Plant height. (C) Stem diameter. (D) Fresh weight. (E) The leaf length and width at the seventh node of wild-type and transgenic plants. (F-G) Internode number and length. Statistical analysis using Student's *t*-test, n≥3: *, significant difference, 0.01<*P*<0.05, **, extremely significant difference, *P*<0.01.

3.5PtoCYP90D1 is involved in the positive regulation of wood formation in poplar

To determine the potential role of *PtoCYP90D1* in wood formation, SCW development was analyzed in the same internode of 3-month-old wild-type and transgenic plants. The paraffin section analysis showed that transgenic *PtoCYP90D1*-OE exhibited a larger stem diameter compared to the wild-type control (Figure 5).

To further verify the effect of *PtoCYP90D1* on xylem development, the content of the chemical components, including lignin and cellulose, was examined in transgenic lines. The findings revealed that lignin contents in *PtoCYP90D1*-OE lines were significantly increased by over 8 % and Less than 8% increase 8.79 % in *PtoCYP90D1*-KO lines, compared to wild-type plants (Figure 6). These results indicated that *PtoCYP90D1* is a positive regulator for improving xylem development in poplar.

Further magnification of histochemical staining revealed that overexpression of *PtoCYP90D1* improved secondary xylem development (Figure 5E), leading to an increased number of cell layers in xylem (9 layers), phloem (7 layers), cortex (4 layers), and cortical areas (4 layers) (Figure 6), compared to wild-type plants (Figure 5D). In contrast, the knock-out of *PtoCYP90D1* resulted in reduced vessel development (Figure 6K). Similar results were also observed in the fifth and seventh internodes between transgenic lines and wild-type plants (Figure 7).

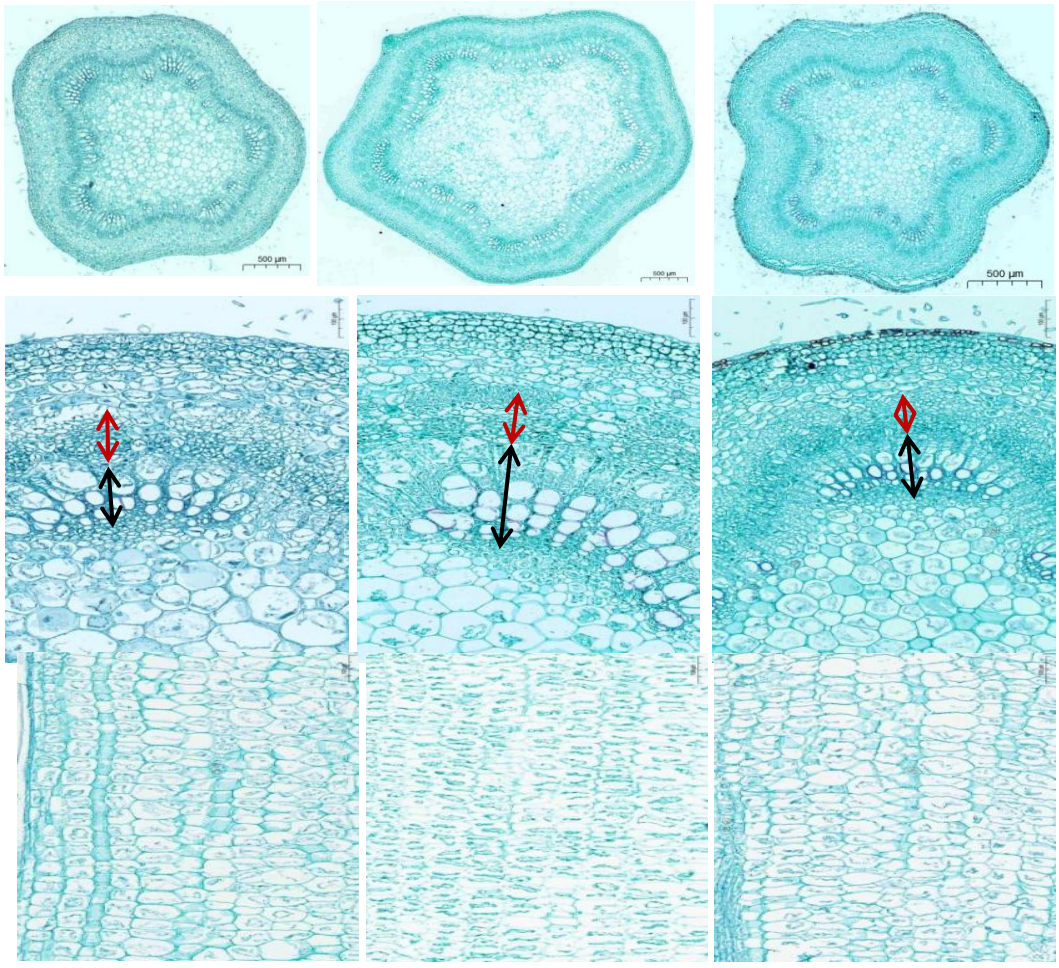


Figure 5 Cross sections of the stem in the 8th internode showing xylem and phloem area, Longitudinal cutting of the pith. (A, D, G) wild-type, (B, E, H) *PtoCYP90D1*-OE and (C, F, L) *PtoCYP90D1*-KO. P: phloem, X: xylem.

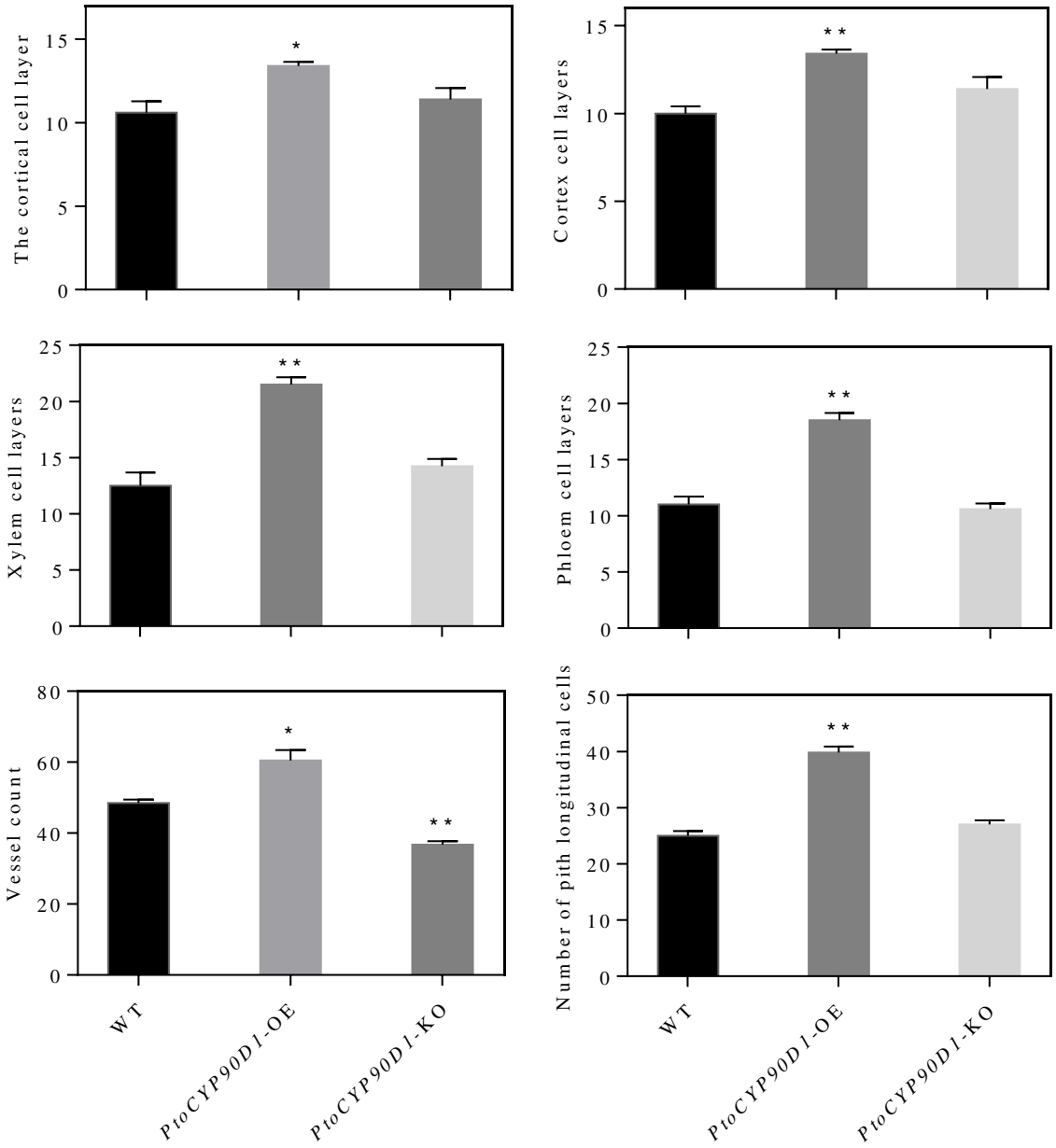


Figure 6 Comparison of stem microstructures between transgenic and wild-type poplar plants. The cell layers of (G) cortex (H) cortical (R) xylem and (J) phloem in wild-type and transgenic plants. (K) The vessel counts. (L) The number of piths. * indicates differences in comparison with WT at $0.01<P<0.05$, **indicates significant differences in comparison with WT at $P< 0.01$ (Student's *t*-test).

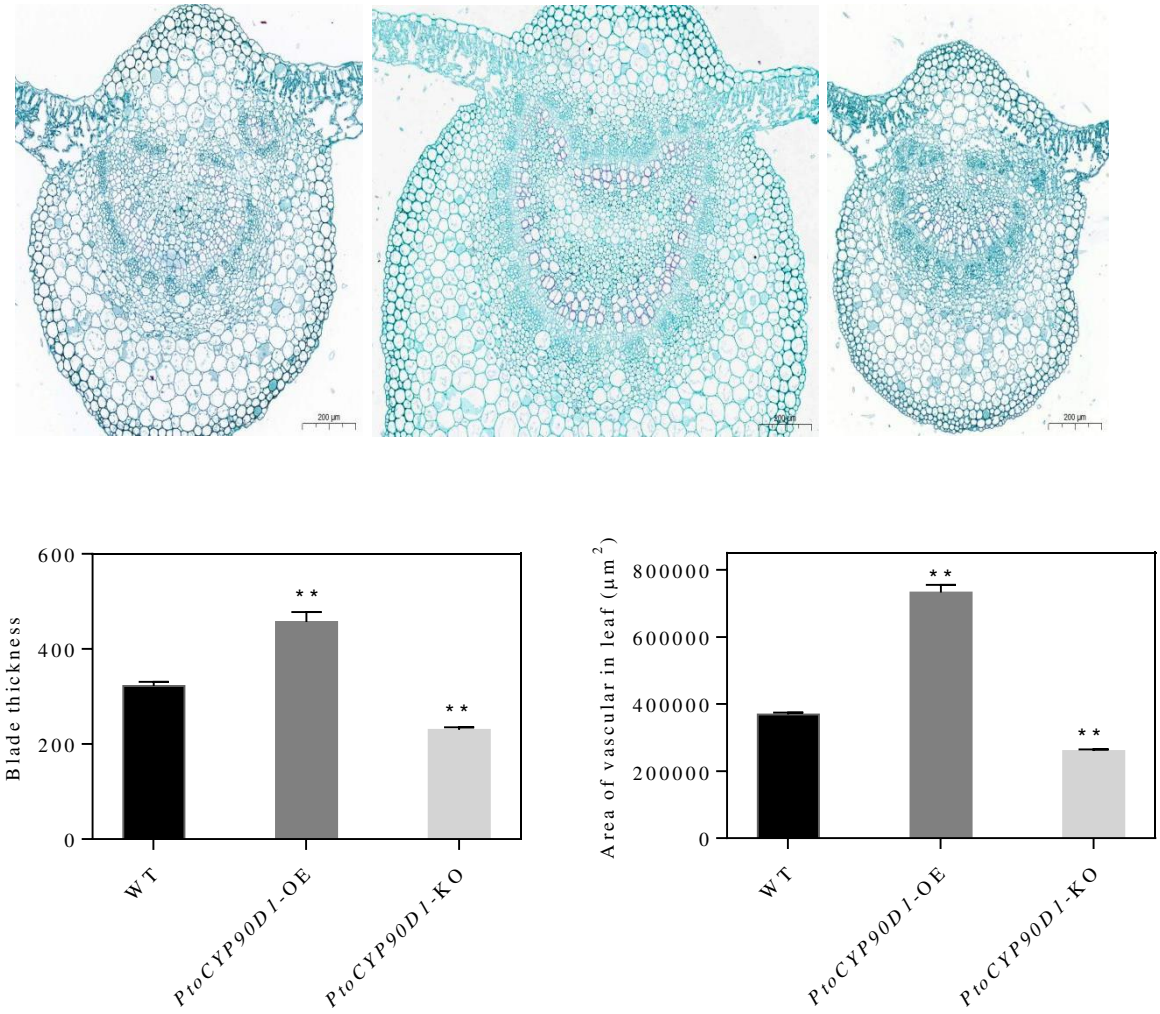


Figure 7 Comparison of leaf vein microstructures between transgenic and wild-type poplar plants. * indicates differences in comparison with WT at 0.01<*P*<0.05, **indicates significant differences in comparison with WT at *P*< 0.01 (Student's *t*-test).

4.Discussion

Previous studies have demonstrated that *AtCYP90D1* encodes C-23 hydroxylation [24], which catalyzes 22-OH-3-one and 3-epi-6-deoxoTY directly into 6-deoxo3DT and 6-deoxoTY in the late C-6 pathway, reducing the two-step synthesis of BR. CYP90D1 is considered one of the main enzymes in BR biosynthesis. To affirm whether Populus *CYP90D1* gene family members have similar functions to *AtCYP90D1*, homologs were identified in the Populus genome. In this study, *PtoCYP90D1* was cloned from *P. tomentosa*. Amino acid sequence analysis showed that PtoCYP90D1 contains one heme-binding domain, the steroid-binding domain, and oxygen-binding domain[35, 38] (Figure 1B), suggesting that it was a conserved cytochrome P450 monooxygenase protein. Phylogenetic analysis showed that *PtoCYP90D1* was grouped with *AtCYP90D1* and *OsCYP90D2/3*, and had the closest relationship with *PtCYP90D1*.

As Populus has been taken as an ideal model tree plant [39], a comparative study on the genetic functions of the *CYP90D1* gene family between *Arabidopsis*

and rice will provide direct evidence in understanding the mechanism of BR-regulated plant growth and development in herbaceous as well as woody plants. The study found that *PtoCYP90D1* was predominantly expressed in roots and old leaves (Figure 2). Therefore, the high expression of *PtoCYP90D1* in old leaves and low expression in young leaves indicated the important role of *PtoCYP90D1* in leaf maturation and development. In a previous study, overexpression of *AtCYP90D1* in transgenic *Arabidopsis* plants increased plant height and internode length [40], while internode cell shortening was reported for*OsCYP90D1* RNA interference mutant [28]. The present study showed that overexpression of *PtoCYP90D1* resulted in an increase in both plant height and stem diameter in poplar, whereas knock-out of *PtoCYP90D1* did not exhibit a significant difference in height or stem diameter as compared to wild type (Figure 4).

BRs play an important role in regulating plant growth and development [41]. It has been verified that genetic modification of BR synthesis promotes plant growth [18]. Plants with BRs metabolic mutants and overexpression exhibited rapid growth, including larger leaves, more height, and increased biomass [7, 18]. These phenotypic alterations are consistent with previous reports on transgenic *Arabidopsis* overexpressing *AtCYP90D1*, which displayed increased inflorescence height and seed and branch counts [40]. Similarly, overexpression of *Populus trichocarpa CYP85A3*, a key enzyme gene for BRs synthesis, in tomatoes increased the height of transgenic plants by 10 ~ 20 cm, which was 2.17 times that of wild type. Overexpression of *PtCYP85A3* in poplar also increased plant height and stem diameter. Overexpression of *PtoDET2* transgenic poplars resulted in faster growth rates and agronomic traits such as the number of internodes, leaves, plant height, and stem diameter increased[7]. The present findings revealed that *PtoCYP90D1*-OE transgenic plants increased plant height and stem diameter relative to wild-type, while knockout ones did not change significantly. At the same time, it was found that in *PtoCYP90D1*-OE transgenic plants, the length and width of leaves, as well as the fresh weight, increased significantly, whereas it decreased significantly in *PtoCYP90D1*-KO plants. These results imply that *PtoCYP90D1* overexpression promotes the growth and development of poplar.

Previous studies have shown that brassinolide plays an important role in xylem formation and vascular development [18]. In BR biosynthesis mutants, the cell wall synthesis genes were down-regulated, while exogenous BRs promoted the differentiation of vessel molecules and xylem formation [42]. Overexpression of *BRI1* in transgenic poplar increased the expression of *CESA*, a gene related to SCW biosynthesis, and promoted wood formation[43]. In this study, the paraffin sections of stem and vein tissues of *PtoCYP90D1* plants showed that the xylem and phloem area of the overexpression plants was greater than that of the wild type, while the knockout plants did not change significantly. Likewise, the number of xylems and phloem cell layers in the stem, as well as the number of pith cells in the overexpressed PtoCYP90D1 transgenic plants, increased significantly, while no substantial alterations occurred in the knockouts. These findings suggest that *PtoCYP90D1* overexpression promotes the growth and development of plant stems by increasing the number of tissue cells.

5. Ethics approval and consent to participate

We ensured that the collection of plant material and experimental research and field studies on plants complied with relevant institutional, national, and international guidelines and legislation.

6. Conflict of Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

7.Supplementary Data

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

8.Contributions

Y.L. and X.Y. designed this study. Y.L., H.T. and S.T. did the main experimental work. All of the authors carried out the field experiments. Y.L. and S.T. wrote the manuscript. X.Y. revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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