Characterization of Cellulose synthase-like D (CSLD) family revealed the involvement of \textit{PtrCslD5} in root hair formation in \textit{Populus trichocarpa}

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Cellulose synthase-like D (CSLD) family was characterized for their expression and functions in \textit{Populus trichocarpa}. Ten members, \textit{PtrCslD1-10}, were identified in the \textit{P. trichocarpa} genome, and they belong to 4 clades by phylogenetic tree analysis. qRT-PCR and promoter:GUS assays in Arabidopsis and \textit{P. trichocarpa} displayed divergent expression patterns of these 10 \textit{PtrCSLD} genes in root hairs, root tips, leaves, vascular tissues, xylem and flowers. Among \textit{PtrCslD2}, \textit{PtrCslD4}, \textit{PtrCslD5}, \textit{PtrCslD6}, and \textit{PtrCslD8} that all exhibited expression in root hairs, only \textit{PtrCslD5} could restore the root hairless phenotype of the \textit{atcsld3} mutant, demonstrating that \textit{PtrCslD5} is the functional ortholog of \textit{AtCslD3} for root hair formation. Our results suggest more possible functions for other \textit{PtrCslD} genes in poplar.

Root hairs are integral for anchorage, enlarging surface area for absorption of water and nutrients, symbiosis interface between plants and soil biome, and expanding exploited soil area to avoid soil erosion\textsuperscript{1,2}. Arabidopsis root hairs have been serving as a model to study cellular morphogenesis, such as plant cell growth and tip growth\textsuperscript{3}. Trichoblast cell’s basal ends of root epidermis specialized to give rise to bulges, which serve as primordia and elongate into thin tubular structures called root hairs\textsuperscript{4,5}. Trichoblasts progressively divide and expand specialized elongating cells at the tip-growing pole\textsuperscript{6}. Differentiating morphological stages of root hairs include specification, initiation, elongation and cessation\textsuperscript{1,3}. A number of genes such as \textit{AthA}, \textit{AthB}, CPC, \textit{ROP}, \textit{RSW1}, \textit{RHD}, \textit{COW}, \textit{TIP}, \textit{CEN}, \textit{SCN} and \textit{BST} play key roles in root hair tip growth\textsuperscript{7–10}. For examples, CPC promotes trichoblast cell differentiation, \textit{AtROP2} and \textit{AtROP4} are key factors in bulge initiation\textsuperscript{11,12}, \textit{COW1} (CAN OF WORMS), \textit{TIP1}, \textit{CEN1} (CENTEPEDE), \textit{CEN2}, \textit{CEN3} and \textit{BST1} (BRISTLED) control one root hair per trichoblast\textsuperscript{7,9,13}. Rapid polarized exocytosis by cell division at the root hair tip supports cell wall maintenance by deposition of cellulose, and loss in any cellulose component would lead to rupture and ectopic root hair formation\textsuperscript{1,14}. To elucidate gene functions in root hair formation, genetic analysis are required\textsuperscript{8}.

The cellulose synthase like (CSL) gene superfamily is composed of 30 genes in Arabidopsis, which encode glycosyltransferases for biosynthesis of polysaccharides and have tissue-specific expression patterns\textsuperscript{15–18}. Based on the sequences, the CSL gene family is divided into CSLA to CSLG groups\textsuperscript{19}. CSLA, CSLC and CSLF are involved in mannan, xyl glucan, and (1→3; 1→4)-β-D-glucan biosynthesis, respectively\textsuperscript{15,20–25}. The CSLD family member shares high amino acid similarity with the \textit{CESA} family and is involved in root tip formation\textsuperscript{22,25}. Abnormal flowers, pollen tubes and pollen grains were observed in \textit{atcsld1}, \textit{atcsld4} and \textit{nacsld3} mutants\textsuperscript{26–28}, while \textit{atcsld2}, \textit{atcsld3}, \textit{atcsld5}, \textit{oscsld1} and \textit{oscsld4} mutant seedlings were root hairless\textsuperscript{16,29–31}. \textit{PtrCslD2}, an ortholog of \textit{AtCslD3}, showed its expression level in xylem also\textsuperscript{32}.

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Yin et al. developed atcsld2/csld3, atcsld2/csld5, atcsld3/csld3 and atcsld2/csld3/csld5 double and triple Arabidopsis knockout mutants. All mutants were dwarf and displayed severe necrosis, indicating the collaborative effects among AtCslD2, AtCslD3 and AtCslD5. The expression of AtCslD2 is AtCslD3-dependent, and the defects in the atcsld3 mutant were partially compensated for by AtCslD2 overexpression. Two other CSLD genes, PdCslD5 and PdCslD6, were complementation remedies for defects and abnormalities of atcsld3 mutants, which proved that the aforementioned genes are functional orthologs of the AtCslD3.

AtCslD1 and AtCslD4 are responsible for cellulose deposition in cell walls to avoid ectopic pollen tubes and pollen grains. AtCslD3 is crucial for the tensile strength of root hair tip cells by deposition of cellulose, and atcsld3 mutants were unable to maintain homeostasis, and terminated bulge elongation at early stage was observed. Stunted root and shoot growth, a decreased concentration of homogalacturonan and xylans, and an elevated concentration of the cellulose synthase inhibitor isoaxaben were observed in the atcsld5 mutants. The rice oscsld1 mutant had normal root hair initiation, but displayed stunted root hair growth, swelling and kinking, showing that OsCslD1 is a functional ortholog of AtCslD3/KOJAK/RHD7 and functioning in root hair elongation. Retarded growth and arrested cell division due to lack of cellulose deposition in culm and root tips of rice nd1 mutants (OsCslD4) was observed.

Root and root hair growth have already been explored in maize, rice, cotton and Arabidopsis. In this study, we identified 10 CSLD genes (PtrCslD1-10) in P. trichocarpa and investigated their possible functions. We studied their expression pattern by qRT-PCR and promoter::GUS staining, and their involvement in root hair formation was investigated by complementation in the Arabidopsis atcsld3 mutant. We demonstrate the functions of PtrCslD5 in root hair formation and provide preliminary evidence of the involvement of CSLD members in xylem formation.

**Results**

**Characterization of the CSLD family in Populus trichocarpa.** We used Arabidopsis CSLD gene sequences to BLASTN (E-value < 1.0) P. trichocarpa genome and obtained 10 homologous gene models. We named these genes PtrCslD1 (Potri.002G200300), PtrCslD2 (Potri.014G125100), PtrCslD3 (Potri.003G097100), PtrCslD4 (Potri.001G136200), PtrCslD5 (Potri.019G046700), PtrCslD6 (Potri.013G082200), PtrCslD7 (Potri.004G208800), PtrCslD8 (Potri.009G170000), PtrCslD9 (Potri.003G177800), and PtrCslD10 (Potri.001G050200). To understand dynamic topological evolution, a neighbor joining phylogenetic tree was constructed by MEGA 7.0 using CSLD genes, including the above P. trichocarpa CSLD genes and the CSLD gene in Arabidopsis. On phyletic lineage, gene pair PtrCslD1 and PtrCslD2 shared the same clade I with ZmCslD4, OsCslD4, AtCslD5, GsCslD5, GsCslD5, and GrCslD5 (Fig. 1). In this clade, functions of OsCslD4 and AtCslD5 were studied, and both mutants displayed defective root hairs. In clade II, PtrCslD3 and PtrCslD4 shared the same lineage with AtCslD6, GrCslD6, GaCslD6, GhCslD6; none of those were identified for their functions. PtrCslD5 and PtrCslD6 belong to gene pair, sharing high similarity with AtCslD2, AtCslD3, OsCslD1, OsCslD2, GrCslD2, GaCslD2, GhCslD2, NaCslD3, ZmCslD1 and ZmCslD2 in clade III. Among these 9 genes, AtCslD2, AtCslD3, OsCslD1 are required for root hair morphogenesis, and ZmCslD1 is essential for cell division of rapidly growing tissues. Gene pair PtrCslD7 and PtrCslD8 and gene pair PtrCslD9 and PtrCslD10 belong to clade IV. PtrCslD7 and PtrCslD8 are closely related to AtCslD4, and PtrCslD9 and PtrCslD10 are closely related to AtCslD1. Mutation of both AtCslD1 and AtCslD4 caused abnormal flowers, pollen tubes, and pollen grains. Based on the phylogenetic tree analysis, PtrCslD1, PtrCslD2, PtrCslD5 and PtrCslD6 may function in root hair formation, and PtrCslD7, PtrCslD8, PtrCslD9 and PtrCslD10 may participate in flower and pollen tube development.

**Expression patterns of CSLD members in P. trichocarpa plants.** Understanding the gene expression pattern can give some clues to their possible functions. We used quantitatively RT-PCR to examine the expression patterns of these CSLD genes in P. trichocarpa. Their absolute transcript abundance in young roots, mature roots, young stem, leaves, xylem, phloem, and apex were determined. Considering the high nucleotide similarity between gene pairs, we designed specific primers to distinguish the gene pairs.

PtrCslD1 and PtrCslD2 displayed similar expression patterns, with high expression levels in young roots, mature roots, young stems, and phloem, with relatively low levels in xylem and leaf, and the lowest level in leaves. However, PtrCslD1 and PtrCslD2 displayed inverse expression levels between young and mature roots (Fig. 2A,B). A comparatively higher transcript abundance of PtrCslD3, 4, 5 and 6 was detected in roots than in young stems, xylem and phloem, and apex had low transcript abundance (Fig. 2C-F). A high expression level in roots was observed for PtrCslD7 and PtrCslD8. PtrCslD7 was highly expressed in both young and mature roots, while PtrCslD8 was detected only for its expression in young roots (Fig. 2G,H). PtrCslD9 and PtrCslD10 were expressed in all tissues, but absolute transcript abundance of PtrCslD10 was very low in these tissues (Fig. 2J). Although similar expression patterns between each gene pair were generally observed, in some tissues the gene pair exhibited variable expression levels. For example, high transcript abundance was detected in mature roots for PtrCslD7 but not for PtrCslD8.

**Expression analysis by promoter::GUS staining.** We used the β-glucuronidase (GUS) gene driven by these 10 CSLD gene promoters to provide more detailed information about their gene expression patterns. About 2.3–3.4 kb of the promoter regions upstream start codon were amplified and the promoter::GUS was transformed into Arabidopsis. GUS signals were stained in roots, leaves, xylem and flowers (petal and style) in both PtrCslD1::GUS and PtrCslD2::GUS transgenic Arabidopsis (Fig. 3A,B). Strong signals were observed in root tips.
and leaf vascular tissues in both transgenics, but GUS signals were observed only in root hairs of pPtrCslD2:GUS transgenics (Fig. 3A,B). PtrCslD4 promoter-driven GUS signals were detected in various tissues, including root hairs, vascular tissues of leaves and petals, vascular bundles of stems, and pollen grains (Fig. 3D). Compared to the PtrCslD4 promoter, the PtrCslD3 promoter only gave weak GUS signals in phloem and vascular tissue of leaves (Fig. 3C). GUS staining showed both PtrCslD5 and PtrCslD8 promoters were activated in root hairs (Fig. 3E,F), and PtrCslD5 promoter-driven GUS expression was also detected in pollen sac (Fig. 3E). The observed GUS signals in Arabidopsis root hairs were consistent with the high transcript abundance determined by qRT-PCR (Fig. 2H). In pPtrCslD9:GUS transgenic Arabidopsis, GUS signals were detected only in pollen grains (Fig. 3G). We did not observe any GUS signals in the transgenic Arabidopsis of pPtrCslD6:GUS and pPtrCslD7:GUS.

pPtrCslD2:GUS in Arabidopsis had GUS signals in root hairs, but pPtrCslD2:GUS did not, indicating PtrCslD2 might express in root hairs of P. trichocarpa. To confirm the expression of PtrCslD2 in root hairs of P. trichocarpa, we transformed pPtrCslD2:GUS into P. trichocarpa. GUS staining in P. trichocarpa showed strong signals in developing xylem, root hairs, and root tips (Fig. 4A–C), consistent with the GUS staining in Arabidopsis. Both PtrCslD6 and PtrCslD7 promoters did not give GUS signals in Arabidopsis. We selected the PtrCslD6 promoter to test its ability in P. trichocarpa. GUS was stained in xylem, root hairs and root tips in pPtrCslD6:GUS transgenic poplar (Fig. 4D–F).

Complementation to the atcsld3 mutant. In the above promoter:GUS experiments, we observed GUS signals in root hairs in several transgenics. The promoters of PtrCslD2, PtrCslD4, and PtrCslD8 could drive GUS expression in Arabidopsis (Fig. 3). Transformation of PtrCslD2 and PtrCslD6 promoter-driven GUS in P. trichocarpa gave GUS signals in root hairs. To identify the functions of these five CSLD genes in root hairs, we overexpressed these genes in the root hair mutant atcsld3. Absolute transcript abundance of transgenes in the complementation Arabidopsis was determined by qRT-PCR to confirm their expression (Fig. 5). Numerous root hairs were observed in wildtype Arabidopsis (Fig. 6A). In the atcsld3 mutant, root hairs were hardly seen (Fig. 6B). Complementation of atcsld3 with PtrCslD2, PtrCslD4 and PtrCslD8 gave only a few short root hairs (Fig. 6C–E). Compared with PtrCslD2, PtrCslD4 and PtrCslD8, overexpression of PtrCslD6 in atcsld3 produced more and longer root hairs, but the root hair number was much fewer and root hairs were shorter than in wildtype (Fig. 6F).
PtrCslD5 complemented mutants appeared with bunches of root hairs (Fig. 6G), and the root hair length was the same as in the wildtype, indicating that \textit{PtrCslD5} is the functional ortholog of \textit{AtCslD3}.

\textbf{Discussion}

Root hairs, tubular appendages of trichoblast cells of rhizodermis are integral for plant growth, soil anchorage, water and mineral adsorption, symbiotic interface for mychorhizae and nitrogen-fixing bacteria. \textit{Arabidopsis thaliana} root epidermis cells are being used as a model to study cell growth and function\textsuperscript{1,2,14,48–50}. Rapid axillary mitotic division of root hairs requires proper cellulose deposition\textsuperscript{1,14}. \textit{Cellulose synthase A} (\textit{CesA}) family members are responsible for cellulose biosynthesis, while \textit{cellulose synthesis like D} (\textit{CSLD}) family members are involved in cellulose deposition in both primary and secondary cell walls\textsuperscript{5}. Disruption of cellulose deposition would affect root hair formation. In Arabidopsis, several \textit{CSLD} members, including \textit{AtCslD2}, \textit{AtCslD3} and \textit{AtCslD5}, have been characterized for their functions in root hair formation\textsuperscript{33}. The root hairless phenotype was observed in the \textit{atcsld3} mutant\textsuperscript{31}, and mutant characterization showed that \textit{AtCslD3} is functioning in the initiation of root hair formation\textsuperscript{33}. \textit{AtCslD2} is functioning at a later stage of root hair development, and the \textit{atcsld2} mutant had abnormal root hairs, with many rupturing late in development\textsuperscript{43}. \textit{AtCslD5} has functions redundant with \textit{AtCslD2} and \textit{AtCslD3}\textsuperscript{33}. In our studies on \textit{CSLD} members in \textit{P. trichocarpa}, only \textit{PtrCslD5} could restore the root hairless phenotype of \textit{atcsld3}, indicating that \textit{PtrCslD5} is the functional ortholog of \textit{AtCslD3}. However, \textit{PtrCslD6} shared 96% amino acid sequence identity with \textit{PtrCslD5} but had very little complementation with \textit{atcsld3}. In another study, both \textit{PdCslD5} and \textit{PdCslD6} from \textit{P. deltoids} could rescue the root hairless phenotype in the \textit{atcsld3} mutation\textsuperscript{34}. The difference on the complementation to \textit{atcsld3} between \textit{PtrCslD6} and the other three \textit{CSLD} genes (\textit{PtrCslD5},

\begin{figure}
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\caption{Expression analyses of \textit{PtrCSLD} genes in \textit{P. trichocarpa}. Absolute transcript abundance of \textit{PtrCslD1} (A), \textit{PtrCslD2} (B), \textit{PtrCslD3} (C), \textit{PtrCslD4} (D), \textit{PtrCslD5} (E), \textit{PtrCslD6} (F), \textit{PtrCslD7} (G), \textit{PtrCslD8} (H), \textit{PtrCslD9} (I), and \textit{PtrCslD10} (J), were examined in young roots (root 1), mature roots (root 2), stems of internodes 1–3 (stem 1–3), leaves, xylem, phloem, and shoot apex. The plasmid containing the gene was used as a standard for establishing a quantitative correlation between the copy number of the target gene transcript molecules and the \textit{C}_\text{t} values. Error bars represent standard errors of triplicate assay.}
\end{figure}
*PdCslD5* and *PdCslD6* indicates that some key amino acids may be changed in *PtrCslD6*, reducing its function in root hair formation. *PtrCslD6* and *PdCslD6* share a 99% amino acid identity, with two amino acid difference (valine versus isoleucine, and glycine versus serine). It will be interesting to further investigate the roles of these two amino acids in root hair formation. Based on the GUS staining in promoter:GUS transgenic Arabidopsis and poplar, *PtrCslD2*, *PtrCslD4*, *PtrCslD6*, and *PtrCslD8* may also function in root hair formation. It is interesting that the GUS expression driven by *PtrCslD5* and *PtrCslD8* promoters were at the same places, root hair and epidermis of root hair zone, but *PtrCslD8* did not restore the phenotype of *atcsld3*. GUS staining shows that *PtrCslD2* promoter activity is induced at an early stage of root hair development, while *PtrCslD4* promoter activity is induced at a later stage of root hair development, suggesting *PtrCslD2* and *PtrCslD4* may function at different stages of root hair development. The functions of *PtrCslD2*, *PtrCslD4*, *PtrCslD6*, and *PtrCslD8* genes in root hair formation and whether they are functioning cooperatively with *PtrCslD5* need further studies, such as complementation to *atcsld2* and *atcsld5* mutants or knockout poplar mutant generation.

In Arabidopsis and other species, CSLD members function not only in root hair formation but also in other tissues, such as vascular tissues and pollen. Strong GUS staining of *PtrCslD1* and *PtrCslD2* promoters was observed in vascular tissue of leaves and vascular bundles of stems (Fig. 3A,B), and comparatively light GUS signals in vascular tissue were detected for *PtrCslD3* and *PtrCslD4* promoters (Fig. 3C,D). Phylogenetic tree analysis shows *PtrCslD1* and *PtrCslD2* are in the clade with *AtCslD5* and *OsCslD4*, and *PtrCslD3* and *PtrCslD4* are in the same clade with *AtCslD6*. Triple mutant *csld2/csld3/csld5* had asymmetric loops and discontinuous

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**Figure 3.** GUS staining of promoter:GUS in Arabidopsis. *PtrCslD1* promoter:GUS (A), *PtrCslD2* promoter:GUS (B), *PtrCslD3* promoter:GUS (C), *PtrCslD4* promoter:GUS (D), *PtrCslD5* promoter:GUS (E), *PtrCslD8* promoter:GUS (F). *PtrCslD9* promoter:GUS (G). *AtCslD2* promoter:GUS (H). Scale bar unit is μm.
vascular elements, showing that AtCslD5, a gene important for root hair formation, is also functioning in vascular tissues. OsCslD4 is expressed in the apex of many organs with rapid growth, and its mutation had many effects, such as inhibited plant growth, thin culms, small grains etc. In clade IV, AtCslD1 and AtCslD4 are closely related to gene pair PtrCslD9/10 and gene pair PtrCslD7/8. Both AtCslD1 and AtCslD4 are important for pollen tube growth. Combining the gene expression patterns and functional characterizations of these 10 PtrCslD genes and other CSLD genes in other species, we found that some CSLD genes are functioning in multiple tissues, such as root hairs, pollen tubes and vascular tissues. For example, the qRT-PCR and promoter:GUS staining experiment (Figs 2 and 4) showed that PtrCslD2 was expressed in various tissues, including root hairs, root tips, and xylem. The expression is in accordance with AtCslD5 expression in Arabidopsis. The expression of PtrCslD6 was observed in root hairs, root tip, and xylem in P. trichocarpa, indicating its roles in the root hairs, root tip and xylem. The expression pattern and predicted function of PtrCslD6 are in accordance with the functions of AtCslD2, AtCslD3 and AtCslD5 in root hairs, xylem and tip growth. Although PtrCslD2 and PtrCslD6 promoters also drove GUS signals in the P. trichocarpa cambium that is lacking in Arabidopsis stems, we assume the expression in cambium is in accordance with the expression of AtCslD2, AtCslD3 and AtCslD5 in tip tissue (Fig. 3H). These results indicate that the CSLD genes share a certain level of conservation between Arabidopsis and poplar, and the CSLD genes in P. trichocarpa may play roles in the same tissues of root hair, vascular tissue and pollen tube, as in Arabidopsis.

We also observed difference between Arabidopsis and poplar related to CSLD gene functions and regulation. The activities of the promoters of PtrCslD1 to PtrCslD9 were studied in Arabidopsis through promoter:GUS experiments. Surprisingly, no GUS staining was observed for PtrCslD6 and PtrCslD7 promoters. However, the PtrCslD6 promoter was active in P. trichocarpa, with staining in root hairs, root tips, cambium, and xylem (Fig. 4). This difference indicates that the upstream regulator(s) of PtrCslD6 between Arabidopsis and poplar may be different. PtrCslD1 and PtrCslD9 transcripts were detected at a very low level in leaves in P. trichocarpa (Fig. 2A,B), but both promoters gave strong signals in Arabidopsis leaves (Fig. 3A,B), indicating that the promoters are activated differently between Arabidopsis and poplar. Besides, the expression level of PtrCslD1 in young roots was higher than that in mature roots, but PtrCslD2 displayed an opposite expression pattern in young roots and mature roots. The occurrence of the different expression patterns between PtrCslD1 and PtrCslD2 in roots may be formed after chromosome duplication.

Methods

Plant Materials and Growth. Populus trichocarpa (Nisqually-1) were obtained from tissue culture and grown on Murashige and Skoog (MS) medium on 16 h/8 h light and dark under aseptic conditions at 25–28 °C as described previously. Seeds of the Arabidopsis thaliana atcsld3 mutant line (AT3G03050) were obtained from Nottingham Arabidopsis Stock Centre (NASC, Nottingham, UK). The seeds were surface-sterilized with sterilizing solution (0.1% Triton and 20% NaClO) for 12 minutes, washed with sterilized distilled water and sown on the solid medium containing MS salts for three days before the seeds were put into an illumination incubator at 22 °C with fluorescent white light at 16/8 h light and dark cycles.

Bioinformatics analysis. The CSLD family genes of A. thaliana were downloaded from the Arabidopsis Tair database (https://www.arabidopsis.org/) and blasted in P. trichocarpa genome via BLASTn search tool with E-value ≤ 1.0. The homologous gene sequences were downloaded from the Phytozome 10.1 plant genomics portal (https://phytozome.jgi.doe.gov/pz/portal.html). We also downloaded CSLD family genes already characterized in different species from the NCBI (https://www.ncbi.nlm.nih.gov/gene) database. The unrooted phylogenetic tree for multiple alignment analysis of protein sequences predicted from cDNA sequences of A. thaliana, G. hirsutum,
G. arboreum, G. raimondii, O. sativa, Z. mays, and P. trichocarpa CSLD genes was constructed with the MEGA 7.0 tool using the Neighbor-Joining (NJ) method through 2000 bootstrap replicates. Each protein encoded by the P. trichocarpa CSLD gene family was assigned a specific name according to Van Erp and Walton. For the PtCslD gene expression pattern analysis, leaves, shoot apices, young stems of 1–3 internodes, xylem, phloem, young roots and mature roots were collected from six-month-old trees and put in liquid nitrogen immediately. Total RNA was extracted using the CTAB method. For the qRT-PCR analysis of PtCslD genes in the atcsld3 mutant, the total RNA was extracted from the roots using an RNeasy Plant Mini Kit (Qiagen). The reverse transcription of RNA to cDNA and quantitative...
polymerase chain reaction (PCR) were carried out as described previously. The primers used in the qRT-PCR are listed in Supplemental Table S1.

Promoter-driven GUS expression in Arabidopsis and P. trichocarpa. The promoter regions of 2.4–3.4 kb upstream start codon were amplified forPtrCslD genes using specific primers (Supplemental Table S1). The sizes of amplified fragments were 3.44 kb (PtrCslD1), 2.34 kb (PtrCslD2), 2.73 kb (PtrCslD3), 2.73 kb (PtrCslD4), 2.5 kb (PtrCslD5), 2.7 kb (PtrCslD6), 2.75 kb (PtrCslD7), 2.78 kb (PtrCslD8), 2.8 kb (PtrCslD9), and 2.67 kb (PtrCslD10). The promoters of PtrCslD1 to PtrCslD9 were successfully amplified. The amplified fragments were cloned into pCR2.1 for sequencing. Further, the promoter fragments were excised from pCR2.1 vectors and inserted into pBI121 by replacing the 35 S promoter, generating pPtrCslD1:GUS, pPtrCslD2:GUS, pPtrCslD3:GUS, pPtrCslD4:GUS, pPtrCslD5:GUS, pPtrCslD6:GUS, pPtrCslD7:GUS, pPtrCslD8:GUS, and pPtrCslD9:GUS. All constructs were introduced into the Agrobacterium tumefaciens strain GV3101. Transformation in Arabidopsis followed the floral dip method. T1 transgenic plants were screened on MS plates with 30 mg/L kanamycin and transferred to MS plates without kanamycin. Agrobacterium-mediated transformation in P. trichocarpa was conducted using 5–8 internode stems as explants following the previous publication. After being verified by PCR using DNA as templates, the transgenic plants were moved into pots and maintained in a greenhouse. GUS staining and observation were conducted as described previously.

Mutant complementation. The total RNA isolated from the xylem for qRT-PCR analysis was reverse-transcribed to cDNA using an Omniscript RT kit (Qiagen). Using the cDNA as templates, the full-length cDNAs of PtrCslD2, PtrCslD4, PtrCslD5, PtrCslD6 and PtrCslD8 were amplified with designed primers (Supplemental Table S1). The PCR fragments were inserted into pBI121 to replace the GUS gene, generating 35 S:PtrCslD2, 35 S:PtrCslD4, 35 S:PtrCslD5, 35 S:PtrCslD6, and 35 S:PtrCslD8. After transformation in the atcsld3 mutant by floral dip method, 6–10 lines were confirmed for transgene expression in roots by qRT-PCR as described above. The root hairs in the wildtype, atcsld3 mutant, and complementation plants were photographed under a Zeiss (Stemi DV4) microscope.

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**Acknowledgements**

This work was supported by a grant from Fundamental Research Funds of Chinese Academy of Forestry (CAFYBB2017MA030).

**Author Contributions**

Q.L. designed the experiments; X.P., H.P., M.A., H.L., X.Y. and X.D. performed the experiments; Y.L. and Q.L. analyzed the data; and M.A., H.P. and Q.L. wrote the manuscript. All authors reviewed the manuscript.

**Additional Information**

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-36529-3.

**Competing Interests:** The authors declare no competing interests.

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