Journal of Plant Physiology 170 (2013) 1267–1276

Contents lists available at SciVerse ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Molecular Biology

Two poplar cellulose synthase-like D genes, *PdCSLD5* and *PdCSLD6*, are functionally conserved with *Arabidopsis CSLD3*

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ARTICLE INFO

Article history:
Received 30 November 2012
Received in revised form 1 April 2013
Accepted 7 April 2013
Available online 5 June 2013

Keywords:
Cellulose synthase-like D
Crystalline cellulose
Poplar
Root hair

ABSTRACT

Root hairs are tip-growing long tubular outgrowths of specialized epidermal cells, and are important for nutrient and water uptake and interaction with the soil microflora. Here we characterized two poplar *cellulose synthase-like D* (*CSLD*) genes, *PdCSLD5* and *PdCSLD6*, the most probable orthologs to the *Arabidopsis AtCSLD3*/*KOJAK* gene. Both *PdCSLD5* and *PdCSLD6* are strongly expressed in roots, including in the root hairs. Subcellular localization experiments showed that these two proteins are located not only in the polarized plasma membrane of root hair tips, but also in Golgi apparatus of the root hair and non-hair-forming cells. Overexpression of these two poplar genes in the *atcsld3* mutant was able to rescue most of the defects caused by disruption of *AtCSLD3*, including root hair morphological changes, altered cell wall monosaccharide composition, increased non-crystalline β-1,4-glucan and decreased crystalline cellulose contents. Taken together, our results provide evidence indicating that *PdCSLD5* and *PdCSLD6* are functionally conserved with *AtCSLD3* and support a role for *PdCSLD5* and *PdCSL6* specifically in crystalline cellulose production in poplar root hair tips. The results presented here also suggest that at least part of the mechanism of root hair formation is conserved between herbaceous and woody plants.

Introduction

Root hairs are polarized, tip-growing and tubular outgrowths of specialized root epidermal cells called trichoblasts (Dolan et al., 1994), which are important for the uptake of water and nutrients, and also serve as the interface between the plant and soil microbe (Peterson, 1992). Root hair development was divided into three structural phases: cell specification, initiation and elongation. In this process, rapid cell wall assembly at the growing tip is required for the root hair development since growing hairs can be deformed or ruptured at the very tips when any cell wall components is missing or disrupted (Favery et al., 2001; Foreman and Dolan, 2001; Carol and Dolan, 2002; Galway, 2006). The cell wall at root hair tips mainly comprises primary cell wall, with similar polysaccharide composition to other cells, consisting typically of cellulose, hemicelluloses and pectins (Somerville et al., 2004; Galway, 2006; Lerouxel et al., 2006).

Understanding the synthesis of cell walls has attracted considerable interests, not only because of their biological functions, but also due to their extensive applied uses as food ingredients, fiber and biofuel feedstocks. Cellulose is one of the main components of cell walls and is thought to be synthesized by cellulose synthase (CESA) family (Somerville et al., 2004; Lerouxel et al., 2006). In *Arabidopsis*, there are ten CESAs (Richmond and Somerville, 2000), and their roles in cell wall synthesis have been extensively characterized (Taylor et al., 2003; Desprez et al., 2007; Persson et al., 2007). These proteins reside in plasma membrane-embedded complexes termed rosettes for cellulose synthesis (Somerville et al., 2004). Previous research showed that *Arabidopsis* root hairs rupture at their tips when treated with 2,6-dichlorobenzonitrile (DCB), an inhibitor of cellulose synthesis, indicating that cellulose is required for the integrity of cell walls at the hair tips, and mutations of the CESAs at the root hair tips are expected to result in the rupture of root hairs (Favery et al., 2001; Carol and Dolan, 2002). However, so far, there is no report concerning the essential roles of CESA proteins for cellulose synthesis in tip-growing root hairs (Caño-Delgado et al., 2003; Singh et al., 2008), suggesting that cellulose at root hair tips is synthesized by

Abbreviations: CSL, CESA-like; GUS, β-glucuronidase; qRT-PCR, quantitative real-time RT-PCR; GFP, green fluorescent protein.

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http://dx.doi.org/10.1016/j.jplph.2013.04.014

glycosyltransferases (GT) that are not classified within the CESA family.

Cellulose synthase-like (CSL) proteins were identified by the sequence similarity to CESAs. There are 29 CSL genes in *Arabidopsis*, while at least 30 putative CSL genes in poplar (Suzuki et al., 2006). The *Arabidopsis* CSL gene family can be divided into six subfamilies namely *CSLA*, *CSLB*, *CSLC*, *CSLD*, *CSLE* and *CSLG* (Richmond and Somerville, 2000). Two additional subfamilies (*CSLF* and *CSLH*) have been found exclusively in grasses (Lerouxel et al., 2006) and another subfamily (*CSLJ*) is present in grasses and some dicot species (Fincher, 2009). Various CSL proteins have been shown to be involved in synthesizing backbone of hemicelluloses (Dhugga et al., 2004; Liepman et al., 2005; Burton et al., 2006, 2008; Lerouxel et al., 2006; Cocuron et al., 2007; Bernal et al., 2008; Park et al., 2011). Among the *CSL* genes, *CSLD* family has the highest sequence similarity to CESAs, which indicates that CSLDs may also function as cellulose synthases to synthesize β-1,4-linked glucan chains (Doblin et al., 2001; Richmond and Somerville, 2001).

Clues to the biological functions of the CSLs have been sought widely by defining their biochemical activity and/or subcellular localization, but interpretation of this work has not yet been conclusive. There is a growing body of evidence showed that the CSLDs participated in specific polysaccharide synthesis in tip growth and stem development, and several mutants of *Arabidopsis CSLD* family have severe phenotypic defects. Two *Arabidopsis CSLD* genes, *AtCSLD2* and *AtCSLD3/KOJAK*, are specifically involved in root hair formation (Favery et al., 2001; Wang et al., 2001; Bernal et al., 2008; Galway et al., 2011; Park et al., 2011). Root hairs of *atcsld3* mutant rupture during outgrowth, while *atcsld2* mutant hairs rupture later, during tip growth, and the defective root hair phenotype of *atcsld3* could be recovered by overexpression of *AtCSLD2* (Yin et al., 2011), suggesting partial functional redundancy of the two genes. Recent progress has proposed a role for AtCSLD3 with a distinct (1→4)-β-glucan synthase activity in apical plasma membranes during root hair tip growth (Park et al., 2011). In addition, *AtCSLD2* and *AtCSLD3* are also indicated to play a role in female gametophyte development (Yoo et al., 2012). The *atcsld5* mutant has moderately reduced growth due to lower activity of synthesis of xylan and pectin rather than a specific polymer (Bernal et al., 2007), while both *atcsld1* and *atcsld4* mutants are deficient in pollen tube growth (Bernal et al., 2008). Mutation in *OsCSLD1* exhibits similar changes in root hair formation as *atcsld3* (Kim et al., 2007). *NaCSLD1* was shown to be required for the formation of cell wall in developing pollen tubes of tobacco (Doblin et al., 2001). In particular, *ZmCSLD1* reveals a previously unrecognized role for *CSLDs* in plant cell division, especially during early phases of cross-wall formation (Hunter et al., 2012).

In this study, to functionally characterize the root hair-specific *CSLD* genes, two poplar *CSLD* genes, *PdCSLD5* and *PdCSLD6*, were subjected to molecular and genetic identification. We examined their expression and subcellular localization patterns, tested their abilities to complement the defects of *atcsld3* mutant, and gain further insight into functions of CSLDs by cell wall sugar composition analysis. Our results provide evidences showing that PdCSLD5 and PdCSLD6 are functionally conserved with AtCSLD3 and support a role for PdCSLD5 and PdCSL6 specifically in crystalline cellulose synthesis in poplar root hair tips.

Materials and methods

Plant materials and growth conditions

One-year-old poplar (*Populus deltoids*) was grown in greenhouse under 16 h light/8 h dark at 25–28 °C. Shoot apices (internodes 1–3 from top, same as below), leaf (from internodes 4–6), developing xylem (from the basal internodes), phloem (from the basal internodes), cortex (from the basal internodes), young and old roots were harvested separately. All samples were immediately frozen in liquid nitrogen and stored at −80 °C until further process.

Arabidopsis thaliana ecotype Columbia (Col-0) was used in this study. The T-DNA insertion line SALK_112105 (*atcsld3*) was obtained from Nottingham *Arabidopsis* Stock Centre (NASC, Nottingham, UK). *Arabidopsis* plants were grown in greenhouse under 16 h light/8 h dark at 22 °C with 65% relative humidity. Seeds were sterilized before sown on half strength MS medium. After stratification at 4 °C for 2 d, *Arabidopsis* seeds were germinated at 22 °C.

Bioinformatic analyses of CSLD protein sequences

Multiple alignment analysis of the full-length protein sequences was performed by Clustal X (version 1.83) program (Thompson et al., 1997). The unrooted phylogenetic tree was constructed with MEGA 4.0 program using the Neighbor-Joining (NJ) method with 1000 bootstrap replicates (Tamura et al., 2007). Synteny information was collected from the Plant Genome Duplication Database (PGDD; http://chibba.agtec.uga.edu/duplication).

Quantitative real-time PCR

Total RNA extraction and quantitative real-time PCR (qRT-PCR) was conducted as described previously (Hu et al., 2010). The expression of the ubiquitin gene (*UBQ10*, BU879229) was used as an internal control. The primers used to amplify the transcripts were as follows: 5′-CGAACACTCCAGAAGAGAAC-3′ and 5′-CTCTGTCACTGGGATTGAGT-3′ for *PdCSLD5*; 5′-CCTATCAACTAGCTCGGATG-3′ and 5′-GATCATCCCTTGAGTAGCTG-3′ for *PdCSLD6*; 5′-GTTGATTTTTGCTGGGAAGC-3′ and 5′-GATCTTGGCCTTCACGTTGT-3′ for *UBQ10*.

In situ hybridization

In situ hybridization was performed as previously described (Zhou et al., 2007). Briefly, poplar roots were fixed in 2.5% formaldehyde and 0.5% glutaraldehyde and embedded in paraffin. Sections (10 μm thick) were cut, mounted on glass slides and hybridized with digoxigenin-labeled *PdCSLD5* and *PdCSLD6* antisense or sense RNA probes. The hybridization signals were detected by incubating with alkaline phosphatase-conjugated antibodies against digoxigenin and subsequent color development with alkaline phosphatase substrates. For the synthesis of antisense and sense probes used for in situ hybridization, a 210 bp fragment of *PdCSLD5* cDNA and a 198 bp fragment of *PdCSLD6* cDNA were PCR amplified with their corresponding primers (5′-GGATTGTGATCATTGTCATGAT-3′ and 5′-GAATCAATTTCAAATATCCG-3′ for *PdCSLD5*; 5′-CCATTTTCAGGCAGATGACT-3′ and 5′-ATTTGAGACAACTTCGGACACG-3′ for *PdCSLD6*). The RNA probes were synthesized with the DIG RNA labeling mix (Roche, Mannheim, Germany) according to the manufacturer's instruction, respectively.

GUS expression assays

To generate *pPdCSLD5*::*GUS* and *pPdCSLD6*::*GUS* transgenic plants, 2015 bp and 2003 bp genomic fragment upstream of the putative ATG start codon of *PdCSLD5* and *PdCSLD6* were PCR amplified using their corresponding primers (5′-CCCCAACAAAACTCCGATTA-3′ and 5′-GAGCTCCGATTGTTCTCTG-3′ for *PdCSLD5*; 5′-GATTGTCACTATATTGATAAACC-3′ and 5′-ATTTTAACACTATAAGAGACTTC-3′ for *PdCSLD6*), and subcloned into the upstream of *GUS* gene in pKGWFS7 vector (Karimi et al., 2002), respectively. The resulting constructs were transformed into wild type (WT) *Arabidopsis* plants, and the T1 transgenic plants were