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Research

Characterization of cellulose synthase complexes in *Populus* xylem differentiation

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Summary

- It is generally hypothesized that the synthesis of cellulose in higher plants is mediated by cellulose synthase complexes (CSCs) localized on the plasma membrane. However, CSCs have not been investigated thoroughly through their isolation. The availability of ample *Populus* tissue allowed *Populus* CSCs to be isolated and characterized in association with xylem differentiation.
- The methods used here included co-immunoprecipitation, proteomic analysis, laser microdissection, immunolocalization and others.
- Western blot analysis of the immunoprecipitated CSCs led to the identification of at least two types of CSC in the membrane protein of *Populus* xylem tissue. Proteomic analysis further revealed that the two types of CSC were assembled from different cellulose synthase proteins. Immunolocalization confirmed that both types of CSC were involved in secondary cell wall formation. In addition, a number of noncellulose synthase proteins were also identified in association with CSC precipitation.
- The results indicate that two types of CSC participate in secondary wall formation in *Populus*, suggesting a new mechanism of cellulose formation involved in the thickening of wood cell walls. This study also suggests that the CSC machinery may be aided by other proteins in addition to cellulose synthase proteins.

Introduction

Cellulose is the most abundant biopolymer on earth and a major component of plant cell walls. In perennial trees, stem growth is derived from the vascular cambium, which divides and differentiates inwards into the secondary xylem and outwards into the secondary phloem. The secondary xylem cells then develop very thick secondary cell walls which contain 42–50% cellulose (Sjöström, 1993). The secondary wall is further divided into three layers: S1, S2 and S3. These layers differ from one another with respect to their cellulose content, degree of polymerization (DP), and crystallites and microfibril angles (Sjöström, 1993; Buchanan *et al.*, 2000; Washusen & Evans, 2001; Barnett & Bonham, 2004; Müller *et al.*, 2006; Mellerowicz & Sundberg, 2008). Although the characteristics of the three layers are known, the mechanisms regulating cellulose synthesis, which results in the formation of different cellulose microfibrils in the layers of the secondary wall, remain unclear.

The synthesis of cellulose in higher plants is generally believed to be catalyzed by cellulose synthase (CesA), which is organized into cellulose synthase complexes (CSCs) localized on the plasma membrane. In freeze–fracture studies, CSC has been visualized as a six-lobed rosette structure (Kimura *et al.*, 1999). Each rosette is believed to be composed of six subunits, with each subunit being composed of six CesA proteins (Delmer, 1999; Doblin *et al.*, 2002; Somerville, 2006; Mutwil *et al.*, 2008). Under this hypothesis, each rosette would contain 36 CesA proteins which can simultaneously catalyze the elongation of 36 β(1–4) glucan chains to form the microfibril in cell walls (Doblin *et al.*, 2002; Saxena & Brown, 2005; Somerville, 2006). In Arabidopsis, different CSCs are believed to mediate cellulose synthesis in primary vs secondary walls (Desprez *et al.*, 2007; Atanassov *et al.*, 2009).

A family of 10 *CesA* genes has been identified in the *Arabidopsis* genome (Richmond & Somerville, 2000). Biochemical interactions of multiple CesA proteins in both primary and secondary cell walls have been reported in the

results of co-immunoprecipitation studies (Taylor *et al.*, 2003; Desprez *et al.*, 2007; Wang *et al.*, 2008; Atanassov *et al.*, 2009). In primary cell walls, the CesA proteins CesA1, CesA3 and CesA6 assemble to form a large (840 kDa) complex which contain six CesA protein subunits (Wang *et al.*, 2008). However, whether each of the three different CesA proteins contributes two subunits to the hexameric complex remains unknown. During secondary cell wall formation, Arabidopsis xylem tissue was identified to contain oligomers of CesA4, CesA7 and CesA8 arranged in dimeric, tetrameric or hexameric complexes. The pattern suggests that the assembly of CSCs may involve a sequential oligomerization process mediated by the formation of disulfide bonds and stable noncovalent interactions (Atanassov *et al.*, 2009).

Meanwhile, 18 *CesA* gene loci (17 protein sequences) have been identified in *Populus trichocarpa* (Djerbi *et al.*, 2005; Suzuki *et al.*, 2006; Kumar *et al.*, 2009). In order to establish a uniform nomenclature, *Populus CesA* genes were recently renamed in a format consistent with the *Arabidopsis CesA* numbering system (Kumar *et al.*, 2009). The new naming system is adopted in this article. Although three *CesA*s are specifically expressed in the primary xylem and involved in cellulose synthesis in Arabidopsis, more than three *CesA* genes are expressed in developing xylem tissue undergoing secondary wall thickening in *Populus* (Suzuki *et al.*, 2006). Based on sequence analysis, *Populus* CesA proteins PtiCesA4, PtiCesA7-A and -B, and PtiCesA8-A and -B were found to be homologous to AtCesA4, AtCesA7 and AtCesA8, respectively, which are expressed in the xylem of Arabidopsis.

So far, only CesA proteins have been identified in CSCs as catalytic units for glucan β(1–4) linkage formation (Pear *et al.*, 1996; Ding & Himmel, 2006; Atanassov *et al.*, 2009; Kumar *et al.*, 2009). However, genetic evidence suggests that a number of other genes, such as the korrigan gene and sucrose synthase gene (*SuSy*), may also be related to cellulose biosynthesis, although it has not been verified whether they are actually involved in the formation of the CSC machinery (Nicol *et al.*, 1998; Sato *et al.*, 2001; Ruan *et al.*, 2003; Szyjanowicz *et al.*, 2004; Coleman *et al.*, 2009). The korrigan (KOR) protein, which encodes a membrane-localized β-1,4-glucanase, has been hypothesized to play a role in relieving the tensional stress generated during the assembly of multiple glucan chains into microfibrils (Somerville, 2006). Mutations in the korrigan gene lead to defects in cellulose accumulation (Nicol *et al.*, 1998; Sato *et al.*, 2001; Szyjanowicz *et al.*, 2004). UDP-glucose, a substrate of CesA in the synthesis of cellulose glucan in plants, can be made from sucrose through a reaction catalyzed by sucrose synthase (SUSY) (Amor *et al.*, 1995; Haigler *et al.*, 2001). The downregulation of the *GhSUSY1* gene in cotton plants can repress the initiation and elongation of cotton fiber cells (Ruan *et al.*, 2003), suggesting that SUSY proteins may channel UDP-glucose to CSCs for cellulose glucan formation. Overexpression of the *Gossypium hirsutum SuSy* gene in poplar lead to increased cellulose formation (Coleman *et al.*, 2009). COBRA, an Arabidopsis extracellular glycosyl-phosphatidylinositol-anchored protein (GPI), has also been linked with cellulose deposition and regulation of cell wall biosynthesis (Gillmor *et al.*, 2005; Roudier *et al.*, 2005). Nonetheless, overt links between these proteins and CSC have yet to be established, and the association of CSCs with nonCesA proteins remains poorly understood.

In this study, we report CSC characterization in *Populus* differentiating xylem tissue. Although the literature widely suggests that different *CesA* genes are responsible for cellulose synthesis in primary and secondary cell walls (Turner & Somerville, 1997; Arioli *et al.*, 1998; Richmond & Somerville, 2000; Mutwil *et al.*, 2008), this study describes the discovery that two types of CSC are involved in the process of secondary wall formation of xylem cells in *Populus*. Furthermore, we provide new evidence supporting the possibility that CSCs may be composed of other proteins in addition to CesAs.

Materials and Methods

Differentiating xylem collection

Samples were collected from the same clone of 3-yr-old *Populus* trees (*P. deltoides* × *trichocarpa*) grown in an experimental field in Jiangsu Province during the growing season. After the bark had been removed, the developing xylem tissues were scraped with a razor blade and immediately frozen in liquid nitrogen. A total of 2 kg of developing xylem tissue was harvested.

Antibody production

A hypervariable region (HVR) was amplified from *PtiCesA1-A*, *PtiCesA3-C*, *PtiCesA7-A* and *PtiCesA8-B* by PCR with the following primers: *PtiCesA1-A*: 5′-*CCATGG*GCCAGTGGCAGGGTGATGATATAGAAC-3′ and 5′-*AAGCTT*CGAACCAGTACCTTCCATGTCCC-3′; *PtiCesA3-C*: 5′-*CCATGG*GCAATTACTCTTCAGAAAA TCAAAACCAG-3′ and 5′-*AAGCTT*TGGTGAACCAAA CTCCCTTACTG-3′; *PtiCesA7-A*: 5′-*CCATGG*GCATC ATTGAAGATGAGCAAGACAAG-3′ and 5′-*AAGCTT*C CCTCCCTCAGGTTCAGAAAC-3; *PtiCesA8-B*: 5′-*CCATGG*GCCCAGAGAACTTGCTGGATGATGTAG-3′ and 5′-*AAGCTT*CGGTTTATCTTCCATCTGCTGTTCC-3′. The PCR products were sequenced and then cloned into the *Nco*I and *Hin*dIII sites of pET28b (Novagen, Madison, WI, USA), respectively. Recombinant peptides were induced in *Escherichia coli BL21(DE3)* by 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and purified with Ni-NTA

Superflow (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The eluates were re-purified and concentrated with centrifugal filter devices. Each pure peptide was dialyzed against phosphate-buffered saline (PBS) to remove imidazole and then used to raise antibodies in rabbits (Shanghai Immune Biotech, Shanghai, China). Two rabbits were injected for each peptide. Crude antisera and preimmunosera were purified using protein-A Sepharose Cl-4B for further use. Each of the antibodies was applied to Western blot hybridization with the four peptide antigens to examine the antibody specificity.

Microsomal fraction preparation

The microsomal fraction was prepared according to the method from previous studies with slight modifications (Osakabe *et al.*, 1999; Suzuki *et al.*, 2006). The tissue sample was ground to a fine powder in liquid nitrogen together with 1% polyvinylpolypyrrolidone (PVPP) and homogenized at 4°C in extraction buffer (1/10, w/v) containing 0.5 M Tris-HCl, pH 8.5, 0.5 M sucrose, 0.1 M KCl, 50 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM leupeptin and 1 mM pepstatin. The homogenate was filtered with Miracloth and centrifuged at 10 000 ***g*** for 10 min at 4°C. The supernatant was centrifuged at 150 000 ***g*** for 30 min to collect the microsomal fraction.

Immunoprecipitation and Western blot analysis

The microsomal pellet (*c.* 5 mg protein) was resuspended in 900 μl of ice-cold IP buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2% v/v Triton X-100, 2% w/v dodecyl-β-D-maltoside (DDM), 1 mM PMSF, 1 mM leupeptin and 1 mM pepstatin) and clarified by centrifugation at 20 000 ***g*** for 30 min to remove insoluble debris. The supernatant was added with 5 μg of antibodies and incubated for 1 h; 100 μl of protein A Sepharose was then added to the mixture, together with Cl-4B, and gently shaken for 2 h at 4°C with end-over-end rotation. After centrifugation for 30 s at 2000 ***g***, the harvested pellet was washed three times with ice-cold IP buffer and twice with ice-cold washing buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM PMSF, 1 mM leupeptin and 1 mM pepstatin). The pellet was boiled in 100 μl of 2 × sampling buffer. A 20 μl sample was loaded onto a 9% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. After electrophoresis separation, proteins were electrotransferred onto polyvinylidene difluoride (PVDF) membrane. The membranes were then blocked with TBS buffer (10 mM Tris, pH 7.5, 0.1% NaCl) with additional 5% nonfat dry milk for 30 min. Afterwards, the membranes were rinsed with TBST buffer (10 mM Tris, pH 7.5, 0.1% NaCl, 0.05% Tween 20, 1% nonfat dry milk) for 5 min. After the rinse had been repeated three times, the membranes were incubated with anti-PtiCesA antibodies (1 μg μl^{-1}) diluted at 1 : 1000 in TBST buffer for 1 h. They were then rinsed in TBST buffer for 5 min. This was repeated three times. The membranes were reacted with the secondary antibodies (donkey anti-rabbit antibodies linked to alkaline phosphatase) at a dilution of 1 : 5000 for 1 h. After rinsing in TBST and alkaline phosphatase buffer (100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$), the membranes were stained with 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitroblue tetrazolium chloride (NBT). Color was developed at room temperature in 5–15 min.

Protein digestion and nanoflow liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

The immunoprecipitated CSC isolates prepared as above were denatured and separated by SDS-PAGE. After staining with Coomassie Blue R-250, the protein bands were cut into 1 mm widths and destained with 100 μl of a solution containing 50% v/v acetonitrile (ACN) (McVety *et al.*, 2006) and 25 mM ammonium bicarbonate for 1 h with three repeats. The gel blocks were then dehydrated with 100 μl of 100% ACN for 40 min and dried in a SpeedVac concentrator for 30 min. The gels were incubated with a 10 ng μl^{-1} trypsin solution in 25 mM ammonium bicarbonate at 37°C for 12 h for hydrolysis. The hydrolyzed peptide mixtures were extracted twice with 8 μl of 50% v/v ACN, 0.5% v/v formic acid (FA). The extracts were dried under the protection of N_2 and resuspended with 5% ACN in 0.1% FA for LC-MS/MS analysis.

The analysis was performed on an LC-20AD system (Shimadzu, Tokyo, Japan) connected to an LTQ Orbitrap mass spectrometer (ThermoFisher, San Jose, CA, USA), as described previously with some modifications (Cao *et al.*, 2009). The extracts were injected onto a CAPTRAP column at a flow rate of 20 μl min^{-1} and subsequently separated by a C18 reverse-phase column at a flow rate of 500 nl min^{-1}. The sample was eluted with a linear gradient of 5–45% with mobile phases (phase A, 5% ACN in 0.1% FA; phase B, 95% ACN with 0.1% FA) over 90 min. The separated sample was then injected into the mass spectrometer and the spray voltage was set at 1.8 kV. The mass range was set from 400 to *c.* 2000 Da with a resolution power of 60 000 using the Orbitrap section, followed by MS/MS experiments for the eight strongest peaks using the LTQ section with a dynamic exclusion duration of 1 min.

Tandem mass spectra were extracted by BioWorks version 3.3.1 sp1 (ThermoFisher). All MS/MS samples were analyzed using Sequest (ThermoFisher, version 28). The parameters for Sequest were: peptide tolerance, 50 ppm; MS/MS tolerance, 1.0 Da. Peptide identifications were accepted if they could be established at > 95.0%

probability as specified by the Peptide Prophet algorithm (Keller *et al.*, 2002). Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii *et al.*, 2003).

Sample fixation and sectioning

The stem internodes (fifth to seventh internodes) of glasshouse-grown *Populus* trees were sampled and fixed according to the acetone-fixation paraffin-embedding protocol with some modifications (Tang *et al.*, 2006). Briefly, the internodes were cut into *c.* 2 mm lengths and fixed by ice-cold pure acetone with vacuum infiltration, and acetone was changed every hour three times. Fixation was further accelerated by microwave treatment at 37°C for 15 min, followed by vacuum infiltration for 15 min, and this treatment was repeated three times. The acetone was then sequentially replaced with acetone : xylene (1 : 1) at 67°C microwaved for 1 min 15 s, with pure xylene at 67°C microwaved for 1 min 15 s, with xylene : paraplast-X (1 : 1) at 60°C microwaved for 10 min, and with fresh paraplast-X at 70°C microwaved for 10 min; the last replacement was repeated five times. The samples were then embedded following standard methods (Tang *et al.*, 2006). The embedded samples were stored at 4°C before sectioning. Sections, 10 μm thick, were cut with a Leica RM 2126 microtome. The sections were mounted on PSA-1X adhesive-coated slides (Instrumedics, St Louis, MO, USA) and dried at 42°C. Dry slides were deparaffinized twice for 10 min each in pure xylene and air dried, and were then ready for microscopic observation and laser microdissection.

Laser microdissection

We used a Veritas Microdissection System (Arcturus Bioscience, Mountain View, CA, USA) to isolate cells from the prepared tissue sections. Tissues were visualized under bright field illumination through a video camera. Cambium cells were cut along the cambium zone and xylem cells were collected from the xylem differentiating zone. The target cells were captured onto a Capsure HS cap (Arcturus Bioscience) and then transferred to a 0.5-ml RNase-free reaction tube containing extraction buffer (PicoPure RNA isolation kit; Arcturus Bioscience). Approximately 500 cells were collected in each sample and the collection time was controlled to < 30 min. After collection, the cells were incubated at 42°C for 30 min and then spun down for RNA extraction.

RNA extraction, RNA amplification and quantitative PCR measurement

We used a PicoPure RNA isolation kit (Arcturus Bioscience) for RNA extraction, according to the manufacturer's protocol. The quality and concentration of total RNA were examined using an RNA 6000 Pico Assay kit coupled with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), according to the manufacturer's instructions.

A TargetAmp two-round aRNA amplification kit (Epicentre Biotechnologies, Madison, WI, USA) was employed to amplify the RNAs. For amplification, *c.* 1 ng of total RNA (in a volume of 1 μl) was used as starting material and, typically, 15 μg of cDNA was produced.

The cDNA was used to measure *Pd×tCesA* expression in different cells. Primers were designed to amplify a specific fragment (100–200 bp in length) from the *Pd×tCesA* genes detected in the isolated CSCs. The primers produced were examined and screened for their specificity and amplification efficiency. Using 10 ng of cDNA as template, quantitative real-time PCR was performed using SYBR Green® Real Time PCR Master Mix QPK212 (TOYOBO, Osaka, Japan) performed on a CFX96™ Real-Time PCR Detection System (Bio-Rad, Winston-Salem, NC, USA). The PCR thermal cycle conditions were as follows: one cycle of 95°C for 2 min, followed by 45 cycles of 95°C for 15 s, 55°C for 15 s and 72°C for 20 s. Following amplification, the PCR product was examined for reaction specificity, and the relative abundance of the gene transcripts was normalized against *Pd×tActin2* expression.

Immunolocalization

Stem tissue fixed and embedded as described above was sliced into sections 10 μm thick. The sections were deparaffinized in xylene and rehydrated by passing through graded alcohols and rinsed in PBS. Blocking solution (PBS with 0.5% normal goat serum and 1% BSA) was applied to the sections for 1.5 h. The first antibody diluted at 1 : 500 in blocking solution was added to the sections in a moist chamber for 1 h. After rinsing for 10 min in PBS and repeating three times, the sections were reacted with the secondary antibodies (donkey anti-rabbit antibody linked to alkaline phosphatase) at a dilution of 1 : 5000 for 2 h in a moist chamber. After rinsing three times for 10 min in PBS and twice for 5 min in alkaline phosphatase buffer (100 mM Tris-Cl, pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween-20, 5 mM levamisole), the sections were stained with BCIP/NBT at room temperature. After color development, the sections were then dehydrated with graded alcohols, cleared with xylene and mounted with aqueous mounting medium.

Electron microscopic observation of cell walls

The second stem internodes were cut into *c.* 2 mm lengths, which were then fixed by 3% paraformaldehyde and 0.5%

glutaraldehyde in PBS (0.1 M, pH 7.4) with vacuum infiltration, and stored at 4°C overnight. The tissues were then washed with 0.1 M PBS and fixed by 0.5% osmic acid for 2 h. After three washes with 0.1 M PBS, the tissues were dehydrated in an ethanol series (final concentration of 80% ethanol) and embedded in LR white resin (Polysciences, Warrington, PA, USA), which was polymerized at 50°C. Ultrathin sections were mounted on gold mesh grids and dried at room temperature. After staining with uranyl acetate for 5 min and lead citrate for 5 min, the grids were observed under an electron microscope (H-7650; HITACHI, Kyoto, Japan) at 80 kV.

Results

Identification of CesA proteins in the differentiating xylem tissue of *Populus* trees

Studies have reported that multiple *CesA* genes at the transcript level are expressed during secondary xylem differentiation in trees (Djerbi *et al.*, 2005; Paux *et al.*, 2005; Ranik & Myburg, 2006; Suzuki *et al.*, 2006; Lu *et al.*, 2008). However the number of CesA proteins actively involved in cellulose synthesis in the secondary xylem is unknown. We prepared specific antibodies against various CesA proteins to determine which CesA proteins are expressed in the differentiating xylem tissue of *Populus* trees. Previous studies have shown that four *CesA* genes (*PtiCesA1-A*, *PtiCesA3-C*, *PtiCesA7-A* and *PtiCesA8-B*) are highly expressed in the differentiating xylem tissue of *P. trichocarpa* (Suzuki *et al.*, 2006). An HVR peptide in each of the four CesA proteins was selected to produce antibodies to recognize specific CesA proteins. The alignment of the amino acid sequences indicated that the four HVRs share few similarities with one another (Fig. 1a).

The HVRs were expressed in *E. coli* and the expressed polypeptides were purified and used to raise antibodies in rabbit. The four antibodies (anti-CesA7-A, anti-CesA8-B, anti-CesA1-A and anti-CesA3-C) were examined for their specificity through cross-reactions with various expressed polypeptides (Supporting Information Fig. S1a). The results demonstrated that the antibodies reacted only with their corresponding antigens without cross-hybridization with other CesA peptides. Using the specific antibodies, the presence of specific CesA proteins in differentiating xylem tissue was investigated by Western blot analysis. To examine the localization of CesA, proteins (5 μg) from crude microsomal and soluble fractions were separated by SDS-PAGE (Fig. S1b) for CesA identification. The blot hybridization results clearly showed that the four antibodies were only able to detect their corresponding CesA proteins in the microsomal protein of the differentiating xylem (Fig. 1b). The detected Pd×tCesA protein bands also showed distinct molecular weights, which corresponded to the relative sizes

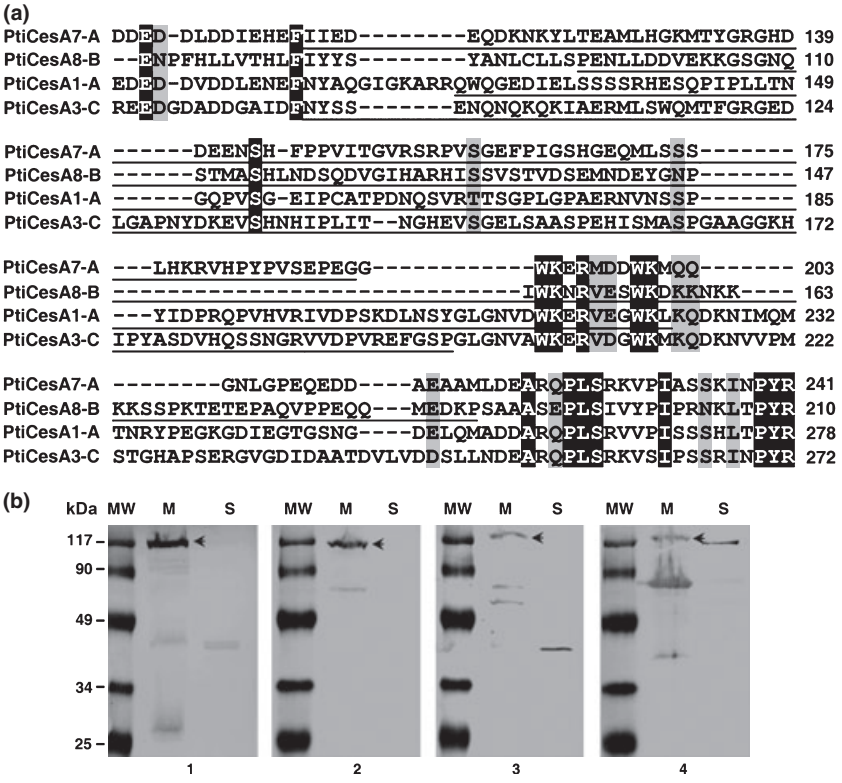

Fig. 1 Selected peptide sequences for antibody production and Western blot identification of PtiCesA proteins in the microsomal fraction. (a) Alignment of a hypervariable region (HVR) of the four cellulose synthase genes in poplar. The peptides selected for antibody production are underlined. The four peptides are PtiCesA7-A, I^{112} to G^{190}; PtiCesA8-B, P^{95} to P^{187}; PtiCesA1-A, Q^{125} to L^{223}; and PtiCesA3-C3, N^{96} to P^{197}. (b) Four Pd×tCesA proteins were specifically identified by their corresponding antibodies. Microsomal (M) and soluble (S) proteins were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then immunoblotted with anti-CesA7-A, anti-CesA8-B, anti-CesA1-A and anti-CesA3-C, respectively. The full sizes of the detected Pd×tCesAs (arrows) were consistent with the predicted molecular weights of the PtiCesA proteins. 1, PtiCesA7-A (116.3 kDa); 2, PtiCesA8-B (114.5 kDa); 3, PtiCesA1-A (122.0 kDa); 4, PtiCesA3-C (120.6 kDa).

of the predicted PtiCesAs [PtiCesA1-A (122.0 kDa) > PtiCesA3-C (120.6 kDa) > PtiCesA7-A (116.3 kDa) > PtiCesA8-B (114.5 kDa)]. In addition to bands corresponding to the full size of the CesA proteins, weaker bands of smaller molecular sizes were also detected. These bands could represent the products of degraded CesA proteins which have also been observed in other studies (Taylor *et al.*, 2003; Atanassov *et al.*, 2009).

Immunoprecipitation of CSC from secondary differentiating xylem

To characterize CSCs from the microsomal fraction of the secondary xylem, we used the aforementioned antibodies for specific CesAs to pull down CSCs through co-immunoprecipitation. After precipitation, the CesA proteins were solubilized with both Triton and DDM according to our modified protocol (see details in Materials and Methods). The CesA protein components of the precipitated CSCs were examined through Western blot analysis (Fig. 2). The CSC that was pulled down with the anti-CesA7-A antibody was able to react with both anti-CesA7-A and anti-CesA8-B antibodies, indicating that this CSC contained at least Pd×tCesA7-A and Pd×tCesA8-B proteins. Similarly, CSC precipitated with the anti-CesA8-B antibody was found to contain both Pd×tCesA8-B and Pd×tCesA7-A proteins.

Anti-CesA3-C and anti-CesA1-A antibodies were also used to harvest CSCs. CSC precipitated with the anti-CesA3-C antibody was found to include Pd×tCesA3-C and Pd×tCesA1-A proteins. Conversely, both Pd×tCesA1-A and Pd×tCesA3-C proteins were identified in CSCs pulled down with anti-CesA1-A antibodies. However, the CSCs harvested using the anti-CesA7-A or anti-CesA8-B antibodies did not contain either Pd×tCesA1-A or Pd×tCesA3-C proteins, and vice-versa. Pull down with a preimmune IgG, which was used as a negative control, failed to precipitate any CesA proteins (Fig. 2).

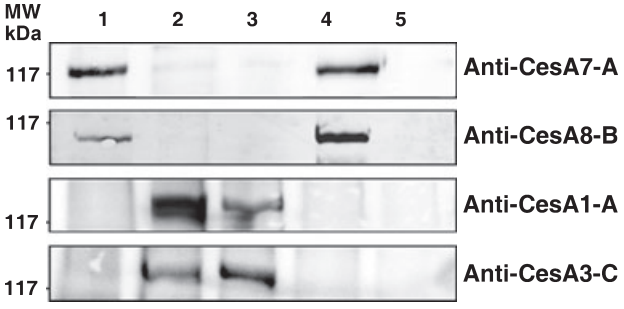

Fig. 2 Detection of two types of cellulose synthase complex (CSC) in the differentiating xylem of poplar. Four antibodies were used for the co-immunoprecipitation of CSCs from the microsomal fraction of protein in poplar differentiating xylem: 1, precipitant by anti-CesA7-A; 2, precipitant by anti-CesA1-A; 3, precipitant by anti-CesA3-C; 4, precipitant by anti-CesA8-B; 5, precipitant by preimmune IgG. The precipitated CSCs were reacted with each of the four antibodies (labeled on the right). Two types of CSC were identified. Pd×tCesA1-A co-immunoprecipitated with Pd×tCesA3-C, but not with Pd×tCesA7-A or Pd×tCesA8-B. Pd×tCesA7-A co-immunoprecipitated with Pd×tCesA8-B, but not with Pd×tCesA1-A or Pd×tCesA3-C. No Pd×tCesAs co-immunoprecipitated with preimmune IgG.

Taken together, these results indicate that two different types of CSC were identified in the differentiating secondary xylem of *Populus*. One type contains Pd×tCesA7-A and Pd×tCesA8-B proteins and the other includes Pd×tCesA1-A and Pd×tCesA3-C proteins.

Dissection of the CSCs in *Populus* by LC-MS/MS analysis

We analyzed the proteomic composition of the harvested CSCs to further confirm which CesA proteins are actually present in the two different types of CSC, and to determine whether other proteins in addition to CesAs are associated with the CSCs. The harvested CSC isolates were separated through SDS-PAGE partition to remove the added antibody proteins. From each separation, protein gel bands, except those in the range 45–60 kDa (which correspond to the size of the antibodies), were cut successively into 15 sections that were recovered and subjected to LC-MS/MS analysis. The analysis yielded a large number of peptide sequences corresponding to CesA proteins for each of the two types of isolated CSC (Table S1). The corresponding CesA proteins were identified from a search of the *Populus* genome database and are summarized in Table 1.

The results in Table 1 further revealed that different CesA proteins were detected in the two types of isolated CSC. The first type of CSC ('type I'), pulled down by anti-PtiCesA7-A antibodies, contained five different CesA proteins, including Pd×tCesA4, Pd×tCesA7-A, Pd×tCesA7-B, Pd×tCesA8-A and Pd×tCesA8-B. In comparison, the second type of CSC ('type II'), pulled down by anti-CesA3-C antibodies, contained another six CesA proteins: Pd×tCesA1-A, Pd×tCesA1-B, Pd×tCesA3-C, Pd×tCesA3-D, Pd×tCesA6-E and Pd×tCesA6-F. Significantly, these CesA constituents of the two types of CSC were highly reproducible without crossover in our repeated analyses (Tables S1, S2). Interestingly, the sequences of all five CesA proteins detected in type I CSCs shared high homology with the CesAs found in the secondary cell wall in Arabidopsis, whereas type II CesAs were homologous to the CesAs found in the Arabidopsis primary cell wall.

Localization of the CSCs in the xylem cells of *Populus*

Secondary xylem tissue of tree stem is composed of several cell types, including fiber cells, vessels and ray parenchyma cells. During secondary xylem differentiation, the formation

Table 1 Cellulose synthase (CesA) protein constitution in the two types of cellulose synthase complex (CSC)

CSC type	Protein name	Total peptides detected		Gene-specific peptides detected		Corresponding gene model
		Expt 1	Expt 2	Expt 1	Expt 2	
Type I	Pd×tCesA4	10	23	7	16	eugene3.00002636
	Pd×tCesA7-A	52	80	23	41	estExt_Genewise1_v1.C_LG_VI2188
	Pd×tCesA7-B	44	66	14	27	gw1.XVIII.3152.1
	Pd×tCesA8-A	12	11	3	3	gw1.XI.3218.1
	Pd×tCesA8-B	13	20	4	10	eugene3.00040363
Type II	Pd×tCesA1-A	10	9	2	3	estExt_fgenesh4_pm.C_LG_XVIII0125
	Pd×tCesA1-B	13	7	3	3	fgenesh4_pg.C_LG_VI001789
	Pd×tCesA3-C	17	26	8	8	estExt_fgenesh4_pg.C_LG_IX0979
	Pd×tCesA3-D	14	30	9	10	estExt_Genewise1_v1.C_LG_I1792
	Pd×tCesA6-E	7	3	2	1	fgenesh4_pm.C_LG_XIII000084
	Pd×tCesA6-F	8	2	2	0	fgenesh4_pg.C_scaffold_133000012

of secondary cell walls is a major activity in fiber cells and vessels. Although the two types of CSC were identified from differentiating xylem tissue, a second goal was to determine the type of cell in which the CSCs were located. Thus, the same antibodies were used to localize the two types of CSC in cross-sections of *Populus* stem. As examined above, anti-CesA7-A and anti-CesA8-2 antibodies are able to recognize type I CSCs, whereas anti-CesA1-A and anti-CesA3-C antibodies are particularly reactive with type II CSCs.

Both anti-CesA7-A and anti-CesA8-2 antibodies showed the same localization signal in differentiating fiber cells, vessel cells and phloem fiber cells, whereas anti-CesA1-A and anti-CesA3-C antibodies were reactive with cambium cells, differentiating xylem cells and phloem fibers. Fig. 3 shows that Pd×tCesA7-A was localized in xylem fiber cells, vessel cells and phloem fiber cells (Fig. 3a–c), but was hardly detectable in vascular cambium cells (Fig. 3b). By contrast, signals corresponding to anti-CesA1-A antibodies were detected in cambium cells, parenchyma cells, xylem fiber cells, phloem fiber cells and vessel cells (Fig. 3d–f). Electron microscopy was used to image newly divided cambium cells that were undergoing primary cell wall formation (Fig. 4a,b). In differentiating fiber cells (Fig. 4c,d), secondary cell walls were observed to be undergoing strong thickening adjacent to the plasma membrane, whereas the primary wall, an outer layer far from the plasma membrane, appeared to have completed synthesis earlier. This observation, together with the results of immunolocalization, indicates that the signal of anti-CesA7-A antibodies, which detect Pd×tCesA7-A found in type I CSCs, was mainly localized in cells undergoing secondary cell wall formation, whereas the anti-CesA1-A signal, indicative of Pd×tCesA1-A contained in type II CSCs, was localized in the cells undergoing primary wall formation, as well as cells going through secondary wall thickening.

The distinct localization pattern of the two types of CSC was further reinforced by gene expression characterization. Using laser microdissection, cells of different types were collected from the cross-sections of the same *Populus* stem as used for immunolocalization. The collected cells included cambium zone cells that were mainly undergoing primary cell wall synthesis and differentiating xylem cells that were strongly undergoing secondary cell wall formation (Fig. 5a). Based on the detected CesA proteins, the designed primers were examined and screened for their specificity and amplification efficiency to ensure the accuracy of the real-time PCR analysis (see Table S3 for the primer sequences used). The corresponding gene expression of the 11 CesA proteins detected in the two types of CSC was characterized in the collected cells. Except for Pd×tCesA6-E, for which the expression was below the detectable level, the other 10 genes were detected as being fairly well expressed. As indicated in Fig. 5b, all of the type I CesA genes exhibited a similar expression pattern – highly expressed in differentiating xylem cells, but weakly expressed in cambium zone cells – which is closely related to the formation of the secondary cell wall. On the other hand, type II CesA genes exhibited a different expression pattern (Fig. 5c). They were expressed in both cambium zone cells and differentiating xylem cells, with a higher expression level in cambium zone cells than in xylem cells, suggesting that type II CesA genes are involved in both primary and secondary cell wall synthesis.

It was noted that the CesA proteins were detected in different abundances. Interestingly, different *CesA* genes were expressed at different levels even when they were constituents of the same CSC type. *Pd×tCesA8-B* was the most strongly expressed of the type I *CesA*s by a relative difference of > 10-fold over the least expressed protein. *Pd×tCesA3-D* was the most highly expressed of the type II *CesA*s. It is unclear whether different levels of *CesA* gene expression translated into differences in the relative representations of the proteins within CSC.

The protein localization and gene expression experiments consistently suggested that, although both types of CSC are

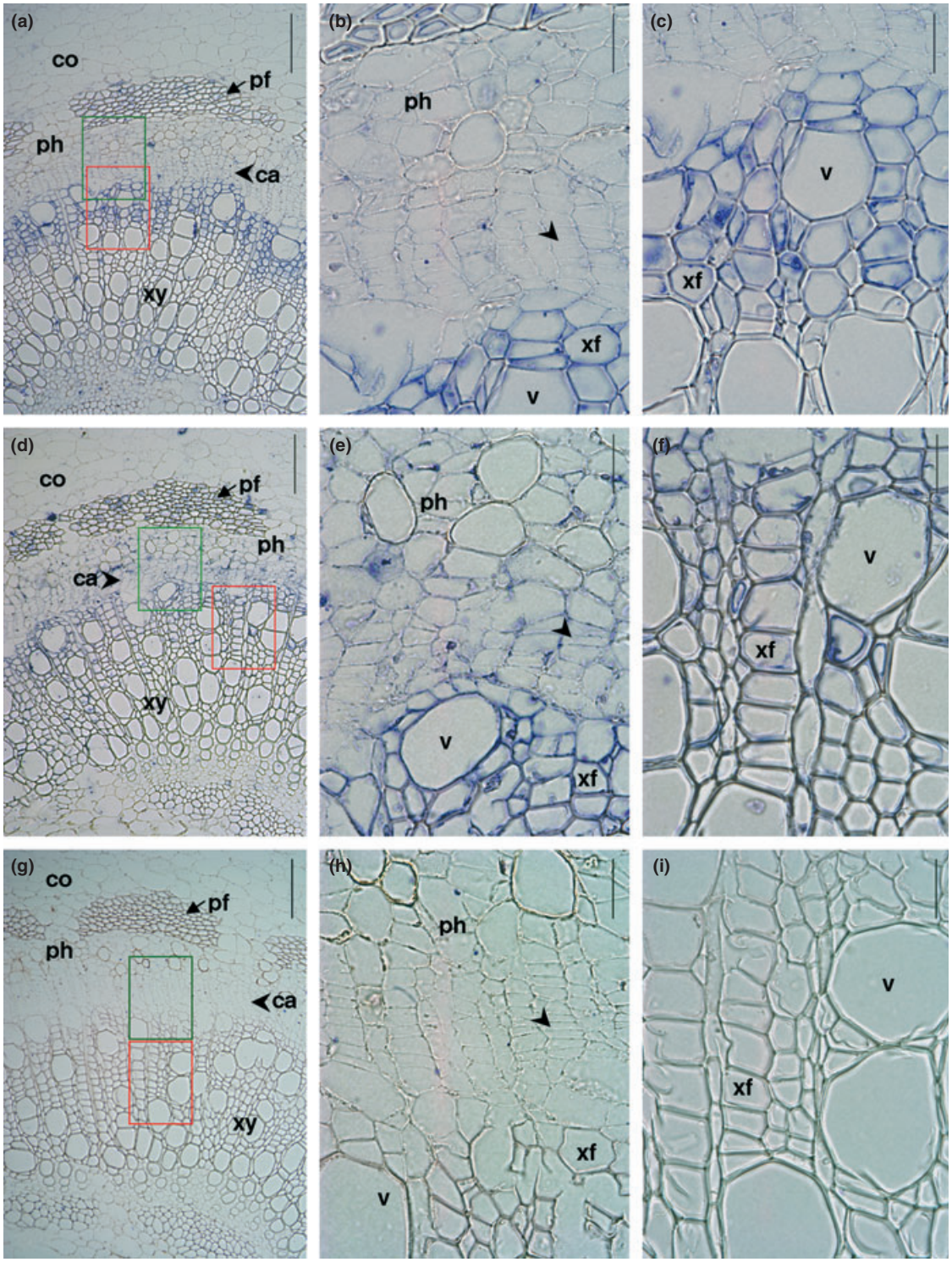

Fig. 3 Immunolocalization of cellulose synthase complexes (CSCs) in differentiating xylem cells. Cross-sections of *Populus* stem were taken at the sixth internode. The sections were hybridized with anti-CesA7-A antibodies for the recognition of type I CSC (a–c), with anti-CesA1-A antibodies for recognition of type II CSC (d–f), and preimmune IgG (g–i). Type I CSCs were localized in vessel and xylem fiber cells, but hardly present in cambium cells. (b, c) High magnification of the green- and red-framed zones in (a). Type II CSCs were localized in both cambium cells and xylem cells. (e, f) High magnification of the green- and red-framed zones in (d). No hybridization signal was detected with the preimmune IgG. (h, i) High magnification of the green- and red-framed zones in (g). Arrowheads show cambium cells; arrows show phloem fiber cells. ca, cambium; co, cortex; pf, phloem fiber; ph, phloem; v, vessel; xf, xylem fiber; xy, xylem. Bars: (a, d, g) 100 μm; (b, c, e, f, h, i) 20 μm.

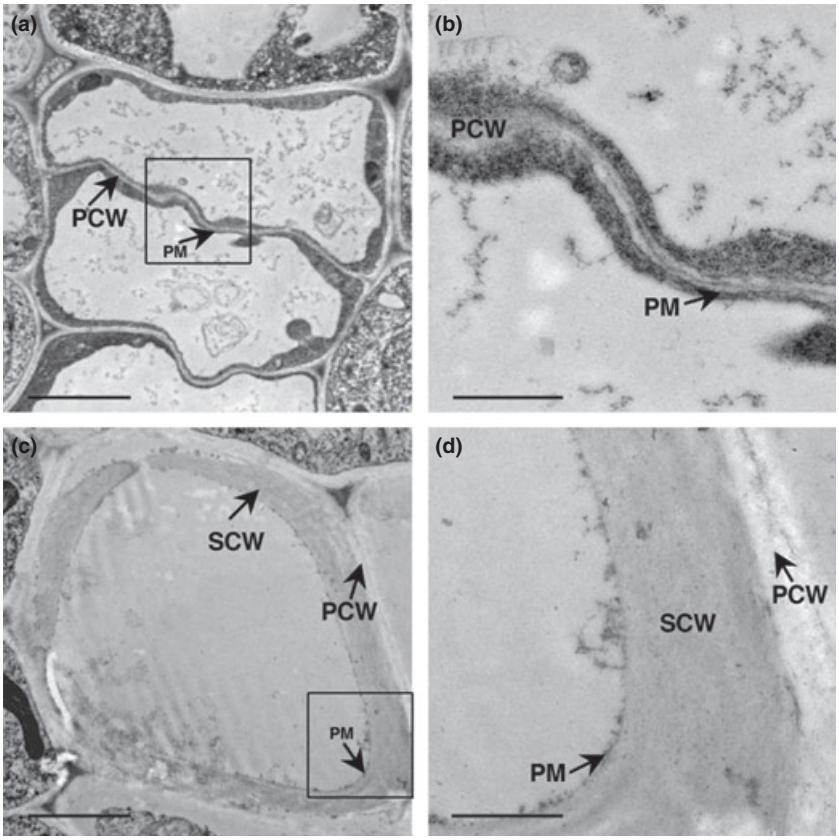

Fig. 4 Cell walls in *Populus* cambium and fiber cells. Newly divided cambium cells and differentiating fiber cells were observed by electron microscopy. In newly divided cambium cells (a), only the primary cell wall was synthesized as indicated by the arrow. The primary cell wall from the framed zone in (a) was observed under high magnification (b). In differentiating fiber cells, secondary cell walls were synthesized inside the primary cell wall (c). Thickening secondary cell walls from the framed zone in (c) were observed under high magnification (d). PM, plasma membrane; PCW, primary cell wall; PM, plasma membrane; SCW, secondary cell wall. Bars: (a, c) 2 μm; (b, d) 500 nm.

required for xylem cell wall formation, type I CSCs are specifically involved in the formation of secondary cell walls, whereas type II CSCs contribute to both primary and secondary cell wall synthesis.

Detection of other proteins in addition to CesAs in *Populus* CSCs

To date, CesA proteins are the only verified components of CSCs. We were able to use LC-MS/MS analysis to detect a number of nonCesA proteins together with precipitated CSCs. The CSCs pulled down by anti-CesA7-A and anti-CesA3-C were analyzed by the LC-MS/MS method. Each was repeated with two isolations. All of the detected proteins in the isolated CSCs are listed in Table S2. As a summary (see Table 2), the results indicated that a similar number (*c.* 70) of proteins were identified in both types of isolated CSC, and little difference was detected between the repeated isolations. Among the detected proteins, CesA proteins were detected specifically in association with CSC type as described above. In addition, other different proteins were also detected in the two type CSC precipitates (*c.* 17 proteins in type I and 11–13 proteins in type II), but a majority of the proteins (*c.* 56–58 proteins) were detected in both CSCs. The proteins detected in both CSCs can generally be sorted into three categories. Category I includes proteins that either affect cellulose synthesis or are speculated to associate with CSCs, such as KOR protein, SUSY, COBRA-like protein and others; category II includes proteins related to the cell skeleton and vesicle trafficking, such as actin, tubulin, myosin, kinesin and others; and category III proteins are proteins without a specific classification, such as protein ankyrin-like protein (methyltransferase), protein disulfide isomerase-like protein, receptor-like kinase and others. It is still unclear at this time whether nonCesA proteins detected in the precipitated CSCs are indeed associated with the CSC machinery or appear as a result of impurities in the isolation. However, the presence of category II proteins was not surprising, given that CSCs are assembled in the Golgi and delivered to the plasma membrane in a dynamic process involving the acto-myosin network (Crowell *et al.*, 2009). The CSCs harvested through immuno-pull-down may have included CSCs undergoing assembly and intracellular trafficking, as well as those localized on the plasma membrane. Further investigation is needed, especially for the category III proteins, to determine whether and how these proteins may actually interact with CSCs.

Discussion

The elucidation of the structure and function of CSC has been an ongoing central theme in the understanding of cellulose synthesis in plants. Although a number of reports in

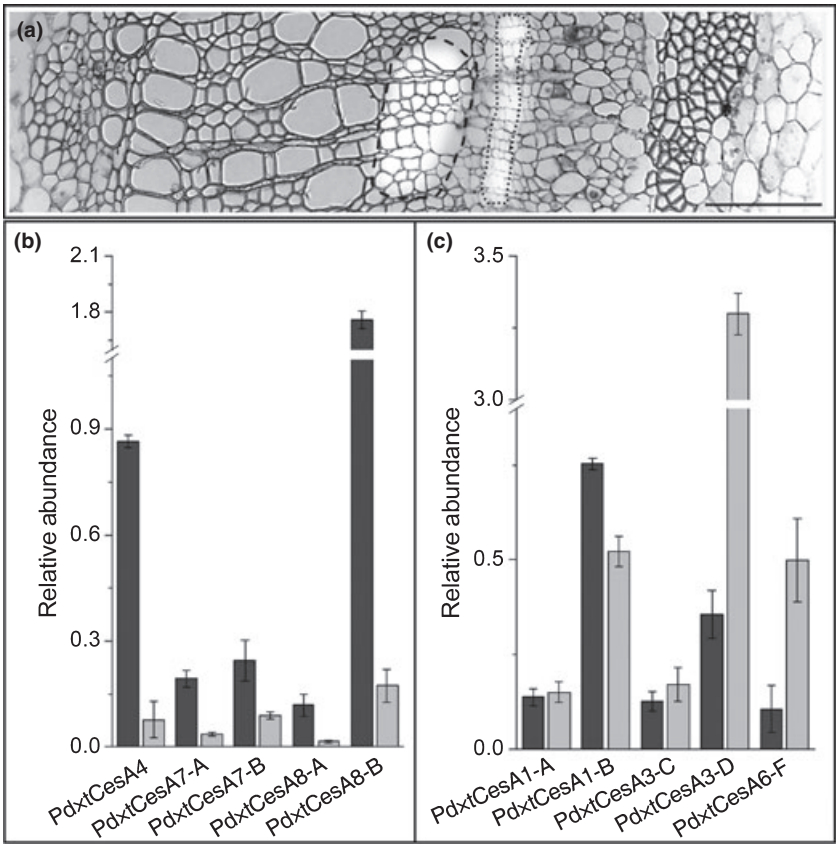

Fig. 5 Expression of *Pd×tCesA* genes in different cells. Two types of cell, representative of primary and secondary cell wall synthesis, were collected using laser microdissection. Expression of *Pd×tCesA* genes in the collected cells was measured using quantitative real-time PCR. (a) Transverse sections of *Populus* stem at the sixth internode. Broken line, xylem cells were collected; dotted line, cambium cells were collected. Bar, 100 μm. (b) Relative abundance of type I *Pd×tCesA* transcripts expressed in the two cell types (xylem, black columns; cambium, gray columns). (c) Relative abundance of the type II *Pd×tCesA* transcripts expressed in the two cell types (xylem, black columns; cambium, gray columns). The values in (b) and (c) are means ± SD.

Table 2 The number of proteins detected in isolated cellulose synthase complexes (CSCs)

	Expt 1		Expt 2	
Isolated CSC samples	Type I CSC	Type II CSC	Type I CSC	Type II CSC
Total number of detected proteins	73	70	75	68
Number of proteins belonging to both CSCs	56	57	58	57
Number of proteins only in type I	17	0	17	0
Number of proteins only in type II	0	13	0	11

the literature have described attempts to isolate CSCs and to assay the CesA activity of CSCs (Lai-Kee-Him *et al.*, 2002; Desprez *et al.*, 2007; Wang *et al.*, 2008; Atanassov *et al.*, 2009; Bessueille *et al.*, 2009), knowledge of how the complex is constituted and how it regulates the formation of cellulose microfibrils on the plasma membrane is still limited.

According to previous studies, only one type of CSC is present in the xylem tissue of Arabidopsis plants (Taylor *et al.*, 2003; Atanassov *et al.*, 2009). In this study, using antibodies against specific CesA proteins, two different types of CSC were isolated from differentiating xylem cells that were undergoing strong secondary cell wall thickening. Proteomic characterization further revealed that more than three different CesA proteins are detected in the isolated CSCs. CesA proteins in type I CSCs include Pd×tCesA4, Pd×tCesA7-A, Pd×tCesA7-B, Pd×tCesA8-A and Pd×tCesA8-B, whereas, in type II CSCs, there are six other CesAs: Pd×tCesA1-A, Pd×tCesA1-B, Pd×tCesA3-C, Pd×tCesA3-D, Pd×tCesA6-E and Pd×tCesA6-F. Based on comparisons of their protein sequences, CesAs from type I CSCs belong to the same clade as those found in the Arabidopsis secondary cell wall, whereas type II CesAs are homologous to the CesAs found in the Arabidopsis primary cell wall.

Although three CesAs form the building blocks of the single type of CSC found in the primary xylem of Arabidopsis, 11 CesAs are simultaneously expressed and form two types of CSC in the differentiating xylem of *Populus*. How are the two CSCs associated with different cell types or cell wall formation? To understand the kind of cells in which the CSCs were located, we explored the immunolocalization of CSCs in cross-sections of *Populus* tree stems. Type I CSCs were mainly detected in cells undergoing secondary cell wall formation, whereas type II CSCs were detected in cells undergoing primary cell wall synthesis and in cells undergoing secondary cell wall thickening.

Cellulose in primary and secondary cell walls displays different characteristics, such as different DPs and microfibril crystallization. Usually, cellulose synthesized in primary cell walls contains low levels of microfibril crystallites and low DP, whereas cellulose in the secondary walls has a much higher DP and microfibril crystallites (Blaschek *et al.*, 1982; Sjöström, 1993; Buchanan *et al.*, 2000; Washusen & Evans, 2001; Barnett & Bonham, 2004; Müller *et al.*, 2006; Mellerowicz & Sundberg, 2008). Thick secondary cell walls in woody xylem cells contain three layers: S1, S2 and S3. These layers contain cellulose with high and/or low microfibril crystallites and DP. The detection of one type of CSC in cells undergoing primary cell wall formation and two types of CSC in cells undergoing secondary cell wall formation raises the possibility that the two types of CSC in *Populus* may regulate the synthesis of cellulose with different characteristics. One type of CSC might contribute to the formation of cellulose with low DP and/or low crystallites, whereas the second type may contribute to cellulose with high DP and/or high crystallites.

Cellulose synthesis can be influenced by the growing conditions. In trees, it is known that tension stress affects cellulose synthesis-related gene expression, which leads to changes in cellulose microfibril properties (Paux *et al.*, 2005; Andersson-Gunneras *et al.*, 2006). Expression of secondary wall-specific CesAs, in particular, can change in both directions under tension stress (Joshi, 2003; Andersson-Gunneras *et al.*, 2006), but it is unknown whether the alteration in gene expression is related to CSC modification. Although more direct evidence, such as the characterization of cellulose properties in response to CSC modification, would be needed to verify this proposition, the findings in this study imply that the assembly of CSCs from different CesAs contributes to the production of cellulose with different characteristics, which, in turn, affects the formation of the primary and secondary cell walls.

Compared with the three CesA building blocks in Arabidopsis CSCs, five or six Pd×tCesAs were found in each of the two types of *Populus* CSC. This difference may be explained by two possibilities. One possibility is that it is the result of the duplication of the *Populus* genome which occurred some 65 million yr ago (Tuskan *et al.*, 2006). PtiCesA genes can be grouped into eight sets of clearly defined paralogs (Tuskan *et al.*, 2006). Paralog proteins (Pd×tCesA7-A and -B, Pd×tCesA8-A and -B, etc.) were detected among the five CesAs composing each CSC type. This suggests that both paralog proteins are able to form CSC, but are not necessarily simultaneously present within a single CSC. One CesA may be interchangeable with its paralogous protein among individual CSCs. Another possibility is that the detected CesAs were contributed by both parents. The CSCs isolated in this study were taken from hybrid plant material, and the detected peptide sequences might be a match for CesA homologs from both parents. Although these two possibilities may cause the detection of more than three CesAs in CSC biochemical composition, that is not to say that each individual CSC structure includes all the detected CesAs.

The potential exchange of paralogous CesAs within a CSC and the finding that different combinations of CesAs form different types of CSC emphasize the idea that the assembly of CesA proteins into CSCs is not governed by a random process. Instead, CesAs follow some yet undetermined mechanism to divide into different complex groups. Further studies on the assembly of CesA proteins will lead to greater insights into the functionality of CSCs.

Previous studies have proposed that CSCs in Arabidopsis are composed of three different CesAs with each contributing a pair of proteins to a CSC subunit (Doblin *et al.*, 2002; Somerville, 2006). However, CesAs are usually expressed at different levels in trees (Kalluri & Joshi, 2004; Samuga & Joshi, 2004; Djerbi *et al.*, 2005; Ranik & Myburg, 2006; Suzuki *et al.*, 2006; Lu *et al.*, 2008). The observation that *Populus* CesAs, including those present within the same CSC type, varied a great deal in their expression levels raised the question of whether each of the different CesAs contributes an equal number of proteins in a CSC. Based on peptide mass spectrum signals, we tried to determine whether the abundance of the signals translated quantitatively into the relative composition of different CesA proteins within the CSCs. However, a relationship was difficult to establish because of a number of technical challenges in the experimental procedure from CSC isolation to mass spectrum analysis.

CesA proteins are the only protein components of CSCs confirmed so far; however, cellulose synthesis is believed to involve proteins other than CesAs (Somerville, 2006). CesA complexes of *c.* 840 kDa were detected in our study (data not shown), similar to those reported in Arabidopsis (Wang *et al.*, 2008). These complexes are larger than the predicted size of CSCs assembled purely from six CesAs, raising the possibility that the complexes might include other proteins. Previously, proteins such as SUSY, KOR and COBRA were suspected of being associated with CSCs. In our studies, these proteins were identified as being co-immunoprecipitated with both types of CSC in *Populus*. An earlier report claimed that the KOR protein was not precipitated in co-immunoprecipitation experiments in Arabidopsis (Desprez *et al.*, 2007). Yet, interestingly, during the process of preparing this article, a study was published showing that SUSY is actually an integral component of the CSC according to the observations of immunogold labeling with anti-SUSY antibodies under an electron microscope (Fujii *et al.*, 2010).

CSC formation, delivery and turnover represents a dynamic process (Crowell *et al.*, 2009). It is likely that the CSCs isolated through immuno-pull-down included CSCs undergoing assembly and intracellular trafficking, as well as those localized on the plasma membrane. The proteins

detected together with CesAs could include the proteins that dynamically interact with CSCs at various stages of the process. On the other hand, although immunoprecipitation is able to pull down the CSC and its interacting components, it is technically difficult to harvest the pure CSC structure by this method. The proteomic detection in the immunoprecipitated CSCs may also include some proteins which result from impurities. Here, a number of nonCesA proteins were detected together with the immunoprecipitated CSCs; however, we cannot conclude at this time that they are all actually part of the CSC structure. Nonetheless, these results provide additional clues towards a more sophisticated mechanism of cellulose synthesis involving a network of multiple proteins.

In conclusion, we have isolated two types of CSC from *Populus* xylem. Both may be involved in the formation of secondary cell walls. This finding revisits the idea that the primary or secondary cell wall houses only one type of CSC. Instead, it raises the possibility that different types of CSC may contribute to the synthesis of cellulose microfibrils with specific characteristics, which would in effect result in the partitioning of the cell wall into different layers. Verification of this model would open up new possibilities to engineer cellulose microfibrils, a major component of plant biomass, through CSC modification.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Examination of antibody specificity and sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) protein separation.

Table S1 The peptide sequences of Pd×tCesA proteins detected in the cellulose synthase complexes (CSCs).

Table S2 List of the proteins detected in the immunoprecipitated cellulose synthase complexes (CSCs).

Table S3 Primer sequences for quantitative real-time PCR amplification.

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