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Cell suspension cultures of *Populus tremula* × *P. tremuloides* exhibit a high level of cellulose synthase gene expression that coincides with increased in vitro cellulose synthase activity

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Summary. Compared to wood, cell suspension cultures provide convenient model systems to study many different cellular processes in plants. Here we have established cell suspension cultures of *Populus tremula* L. × *P. tremuloides* Michx. and characterized them by determining the enzymatic activities and/or mRNA expression levels of selected cell wall-specific proteins at the different stages of growth. While enzymes and proteins typically associated with primary cell wall synthesis and expansion were detected in the exponential growth phase of the cultures, the late stationary phase showed high expression of the secondary-cell-wall-associated cellulose synthase genes. Interestingly, detergent extracts of membranes from aging cell suspension cultures exhibited high levels of in vitro cellulose synthesis. The estimated ratio of cellulose to callose was as high as 50 : 50, as opposed to the ratio of 30 : 70 so far achieved with membrane preparations extracted from other systems. The increased cellulose synthase activity was also evidenced by higher levels of Calcofluor white binding in the cell material from the stationary-phase cultures. The ease of handling cell suspension cultures and the improved capacity for in vitro cellulose synthesis suggest that these cultures offer a new basis for studying the mechanism of cellulose biosynthesis.

Keywords: Calcofluor white binding; Cellulose synthase; In vitro cellulose synthesis; *Populus tremula* × *P. tremuloides*; Reverse transcription PCR; Suspension culture.

Abbreviation: XET xyloglucan endotransglycosylase.

Introduction

Cellulose is the most abundant carbohydrate polymer on Earth, and its biosynthesis and biodegradation are thus essential for efficient carbon recycling in nature. Cellulose is also an important raw material for many current and future industries. In spite of the vast biological and practical significance of cellulose, as well as intensive research efforts over many years, the mechanism of cellulose biosynthesis has remained poorly understood.

Cellulose biosynthesis has so far been mostly studied in the model plant *Arabidopsis thaliana* and in cotton. Cellulose is synthesized by large membrane-bound protein complexes, rosettes, located at the plasma membrane (reviewed by Doblin et al. 2002). The catalytic subunits of the cellulose-synthesizing complexes are encoded by a family of genes designated *CesA*. According to the current hypothesis, three different CesA isoenzymes are required for the formation of a functional cellulose-synthesizing complex (reviewed by Williamson et al. 2002). Genome and expressed sequence tags sequencing have so far revealed up to 10 different *CesA* genes in *Arabidopsis thaliana*, rice, and barley (Richmond and Somerville 2000, Tanaka et al. 2003, Burton et al. 2004). Wood formation in trees represents an intensive period of cellulose synthesis, during which most of the glucose from carbohydrate metabolism is channeled to cellulose in the secondary walls. Perhaps reflecting this enormous capacity for cellulose synthesis, 18 different *CesA* genes have been recently identified in the genome of the

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first tree to be sequenced, *Populus trichocarpa* (Djerbi et al. 2005). Studies using different model systems have revealed that secondary-cell-wall synthesis in plants is characterized by the activation of three additional copies of the *CesA* genes, presumably to provide a higher concentration of active rosettes (reviewed by Williamson et al. 2002). In the hybrid aspen, *Populus tremula* × *P. tremuloides*, we have recently shown that four *CesA* genes, *PttCesA1*, *PttCesA3-1*, *PttCesA3-2*, and *PttCesA9*, are activated during xylogenesis and tension wood formation (Djerbi et al. 2004). These genes also have orthologs in *P. tremuloides*, where they appear to be involved in xylogenesis (Wu et al. 2000, Joshi et al. 2004).

In spite of considerable efforts, the other components required for the formation of fully functional rosettes have not been identified, although many candidates have been implicated (reviewed by Doblin et al. 2002). This is mainly due to the difficulty of isolating active protein complexes that are membrane bound. Since wood tissues are a particularly challenging starting material, different model systems have been used instead to study xylogenesis. These include callus tissue from various plant species (Aloni 1980), hypocotyls with secondary xylem development from *Arabidopsis thaliana* (Chaffey et al. 2002) and wound-induced mesophyll cells from *Zinnia elegans* (reviewed by Roberts and McCann 2000). A significant advantage of the so-called zinnia system is that a considerable portion of the cells differentiate into tracheary elements in relatively good synchrony, which allows in vitro study of the general process of secondary-cell-wall biosynthesis (see Fukuda 1997, Milioni et al. 2001, Demura et al. 2002, Pesquet et al. 2003, Mourelatou et al. 2004), including aspects of cellulose biosynthesis (Babb and Haigler 2001, Roberts et al. 2004). Plant cell cultures in liquid medium have been used as a rapid and controlled model system to study many different aspects of plant physiology, biochemistry, and molecular biology (Kuboi and Yamada 1978, Tsutsumi and Sakai 1994, Blee et al. 2001, Kärkönen et al. 2002). In particular, detergent extracts of microsomal fractions from suspension-cultured *Rubus fruticosus* (blackberry) cells have been used successfully for in vitro synthesis of sizable quantities of cellulose from UDP-glucose (Lai Kee Him et al. 2002). Poplar cell cultures have previously been used to study the physiology and activities of selected cell wall enzymes (Bilisics et al. 1982; Tsutsumi and Sakai 1994; Ohmiya et al. 1995, 2000; Takeda et al. 1996). However, such studies have generally focused on analyzing the cultures only until their late exponential growth phase. Here we have developed cell suspension cultures of the hybrid aspen, *Populus tremula* L. × *P. tremuloides* Michx., and analyzed the specific gene expression and/or enzymatic activity of selected cell wall-associated enzymes until the late stationary phase. Interestingly, we found that some of the secondary-cell-wall-associated *CesA* genes are induced in the late stationary phase of the cultures and that the increased level of *CesA* gene expression coincides with unusually high levels of in vitro cellulose synthesis.

Material and methods

Plant material

Hybrid aspen (*Populus tremula* L. × *P. tremuloides* Michx.) (Ptt) cultures were established from a sterile plantlet obtained from the Umeå Plant Science Center, Sweden. Pieces cut from the stem were placed on agar or in liquid medium of different compositions to achieve callus growth. The cultures were grown at 23 °C at a photoperiod of 12 h light and 12 h dark (granular culture) or in darkness (fine suspension culture) in a modified MS medium (Garve et al. 1980) containing sucrose (3%), 2,4-dichlorophenoxyacetic acid (1 mg/liter), and kinetin (0.02 mg/liter). The fine suspension culture was subcultivated weekly and the granular culture every second week.

Growth and harvest of cultures

Growth curves were determined by subcultivation of the culture at the phase of maximal growth into a number of parallel flasks containing 50 ml of culture medium. Sampling was made by harvesting whole flasks in duplicate at the time points indicated. The cultures were vacuum filtrated, the pH in the culture medium was measured, and aliquots of cell material and culture medium were frozen until analysis (−80 °C for RNA extraction and −20 °C for enzyme activity measurements). Cellulose and callose synthase activities were systematically measured from freshly harvested cells.

Microscopy

Cell material was treated with a mixture of 10% HCl and 10% CrO_3 (1 : 1) overnight, washed with deionized water, and homogenized with a syringe and needle (Bolwell 1985). The cells were examined with an Olympus BX 51 microscope, using an UplanF1 objective.

Gene expression studies

RNA extractions were performed using the RNeasy Plant Mini Kit (Qiagen). Gene expression analyses were made using the Titanium One-Step RT-PCR Kit (Clontech) according to the manufacturer's recommendations with the following modifications. The reaction volume was reduced by 50% with a total amount of 200 ng of total RNA for each reaction. For analysis of the *PttCesA* genes and ubiquitin protein-ligase I (housekeeping gene), the target transcripts were reverse transcribed without the oligo(dT) primer. The amplification was performed using 35 PCR cycles with annealing temperatures and elongation times optimized for each primer mix and PCR product. The housekeeping gene and the target genes were amplified in separate reactions. For analysis of *PttExpα1*, each reaction was performed with two sets of primer pairs (ratio, 1:1) for *PttExpα1* and *S23-40S* (housekeeping gene). The amplification was performed using 30 PCR cycles with an annealing temperature of 60 °C. The following specific primers were designed and synthesized on the basis of the cDNA sequences encoding the CesA isoenzymes (Djerbi et al. 2004) and PttExpα1 (Gray-Mitsumune et al. 2004). Prim-CesA1F, 5′-GCGA TTGCTGGCCTTCAT-3′; prim-CesA1R, 5′-TCTTTGCCCTTTCTTCTT

AGACTTTT-3′; prim-CesA2F, 5′-CCACCTTTACCCGTTCCTCA-3′; prim-CesA2R, 5′-CAGAAGCTAGAAGGATCGACCATAC-3′; prim-Ces A3-1F, 5′-GGCTGGCATTGATACGAACTTT-3′; prim-CesA3-1R, 5′-TTGACCATATAGAGCTCCCCGA-3′; prim-CesA3-2F, 5′-GAATTG GAATATGATCCTCGTTGTAG-3′; prim-CesA3-2R, 5′-GAGAACAAT CATCTTGCAGTCCATT-3′; prim-CesA4F, 5′-GGAACTGTGTGGCTT GAACTGTGATTAGG-3′; prim-CesA4R, 5′-GAAGAACATAACCCT CTAGAACCCACAACAAAT-3′; prim-CesA9-1F, 5′-TTAAATGTGTGC TTGTTTTCTAGTTGATTTGT-3′; prim-CesA9-1R, 5′-GTATTCCCATA GAAGCAATTATGTACATGTATATTCTAG-3′; prim-CesA9-2F, 5′-TAA AATGTGTGCCACGGTGACCTAG-3′; prim-CesA9-2R, 5′-CACTTGT ATACGCAATATGTACACTGTATATTCTC-3′; prim-PttExpα1F, 5′-AGA ATTCCTGGTGTTTACTCCGGGG-3′; prim-PttExpα1R, 5′-TTAAAC CCTGAAATTCTTGCCGGTAAAAG-3′; prim-ubiquitin protein-ligase IF, 5′-GCCCTCCTCGCGGATCTTTGATAGA-3′; prim-ubiquitin protein-ligase IR, 5′-TGGGTTTGGATCACAGAGCAGGAC-3′; prim-S23-40SF, 5′-CTGGTCGCAAGCTCAAGTCCCAC-3′; prim-S23-40SR, 5′-GAC CTTGGCTTCTCCTTCTTCTCTTTG-3′.

Enzyme activities

Xyloglucan endotransglycosylase (XET) (E.C. 2.4.1.207) and cellulase (E.C. 3.2.1.4) were extracted for activity measurements by homogenization of 1 g of frozen cell material with a mortar and pestle in 1 ml of extraction buffer (350 mM sodium citrate, pH 5.5, containing 10 mM $CaCl_2$). The homogenates were centrifuged at 4 °C at 10,000 **g** for 10 min and the supernatants (crude extracts) were used for enzyme assays. Enzyme activities were also determined in the culture medium.

XET activity was assayed essentially as described by Sulová et al. (1995). The reaction mixture contained 0.15 mg of xyloglucan, 0.15 mg of xyloglucan oligomers (XGOs), and 50 µl of crude extract in 50 mM citrate-phosphate buffer (pH 5.5). The mixtures were incubated at 37 °C overnight and the reaction was stopped by the addition of 0.1 ml of 1 M HCl and 0.8 ml of 20% Na_2SO_4. Following the addition of 0.2 ml of color reagent (0.5% [w/v] I_2 and 1.0% KI [w/v]), the absorbance was measured at 620 nm. Parallel assays were performed without xyloglucan oligomers to distinguish XET activity from endoglucanase activities, such as endocellulase activities, that are present in crude extracts. The transglycosylating activity of XET was calculated by subtracting the activity measured in the absence of xyloglucan oligomers from that measured in their presence, as described by Sulová et al. (1995).

Cellulase activity was determined by measuring the reducing sugars released from carboxymethyl cellulose, essentially as described by Wood and Bhat (1988). A 50 µl sample of crude extract was mixed with 0.45 ml of 1% CMC (C-5678; Sigma Chemical Co.) in 50 mM sodium acetate buffer (pH 5.0), and incubated for 30 min at 40 °C. Following the addition of 0.75 ml of 3,5-dinitrosalicylic acid, the mixture was kept at 100 °C for 5 min. After cooling to room temperature, the absorbance was measured at 640 nm.

Preparation and assay of β-glucan synthases

Hybrid aspen cells were harvested at the exponential and stationary phases of the granular and fine suspension cultures by vacuum filtration onto filter paper and washed with an excess of distilled water. The cells were disrupted as previously described (Colombani et al. 2004) and microsomal fractions were isolated by differential centrifugation (Lai Kee Him et al. 2001). The protein content in microsomal fractions resuspended in the minimal volume of extraction buffer (100 mM 3-(N-morpholino)-propanesulfonic acid [MOPS]–NaOH buffer, pH 7.0, containing 2 mM EDTA, 2 mM EGTA, and 10% [v/v] glycerol) was determined by the Bradford dye-binding assay (Bradford 1976). The membrane suspensions were diluted to obtain a final protein concentration of 4 mg/ml. Detergent extraction of proteins was performed for 30 min under continuous stirring in the presence of digitonin at a final concentration of 1%. The preparation was centrifuged at 150,000 **g** for 1 h and the supernatant was used for glucan synthase assays.

Conditions that favor cellulose synthesis have been described previously (Colombani et al. 2004). Briefly, the assays were performed in a final volume of 200 µl, using 50 µl of digitonin extract as a source of enzymes. The assay mixture consisted of 100 mM MOPS–NaOH buffer (pH 7.0), 20 mM cellobiose, 1 mM $CaCl_2$, 8 mM $MgCl_2$, 1 mM UDP-glucose and 0.16 µM UDP-D-[U-^{14}C]glucose (Perkin-Elmer; 11,318 MBq/mmol) (final concentrations). The reactions were stopped after 1 h incubation at 25 °C by the addition of 400 µl of ethanol. The ethanol-insoluble polysaccharides were recovered by filtration on glass-fiber filters (GF/C Whatman), which were successively washed with 4 ml of water and 4 ml of 66% ethanol. The radioactivity retained on the filters was measured in 4 ml of liquid scintillation cocktail (Perkin-Elmer), using a Wallac liquid scintillation counter.

The proportion of callose and cellulose recovered in the assay mixtures were measured with specific glycoside hydrolases (*exo*-(1→3)-β-glucanase from *Trichoderma* sp. or a mixture of the cellobiohydrolase II from Megazyme and the Novozyme 188 cellobiase, which was a generous gift from the late Dr. Schülein, Novo Nordisk), exactly as described by Colombani et al. (2004). The extent of hydrolysis of the radioactive polysaccharides synthesized in the presence of UDP-D-[U-^{14}C]glucose under the conditions described above was determined by comparison with controls in which no hydrolytic enzyme was used.

Estimation of the amount of cellulose in cell walls

The amount of cellulose present in the walls of cells from the granular and fine suspension cultures, harvested after different periods of growth, was estimated by a fluorescence test based on the affinity of cellulose for the dye Calcofluor white M2R (Sigma-Aldrich). Frozen cells were homogenized with a mortar and pestle, and 50 mg of the homogenates were mixed with 1 ml of Calcofluor white solution (5 µg/ml in 20 µM aqueous NaOH). After 30 min in the dark at room temperature, the mixture was centrifuged for 10 min at 10,000 **g** and the fluorescence of the supernatant was measured with a spectrofluorimeter (FLUOstar Optima; BMG Labtechnologies) at excitation and emission wavelengths of 340 and 460 nm, respectively. The percentage of Calcofluor white bound to the cell walls was determined by measuring the decrease in fluorescence in the supernatant, compared to the fluorescence of the Calcofluor white solution alone.

Results and discussion

Growth of the hybrid aspen cell suspension cultures

Cell suspension cultures were developed in order to investigate the function of enzymes involved in cell wall biosynthesis in the hybrid aspen, *P. tremula* × *P. tremuloides*. The cultures were initiated from a sterile grown plantlet and grown as described in the Material and methods section. A modified MS-based medium (Garve et al. 1980) supplemented with 2,4-dichlorophenoxyacetic acid (1 mg/liter) and kinetin (0.02 mg/liter) was used for maintaining the cultures. Two different cultures were selected for the present study: an undifferentiated fine suspension culture (grown in darkness) and a nongreen granular culture (grown in light). The fine suspension culture consisted of small, pale, slightly yellowish and relatively homogeneous aggre-

gates, while the granular culture contained yellowish aggregates of different sizes, up to 10 mm in diameter. The granular culture was established from a fine suspension culture by selection of slowly growing aggregates.

The activity and gene expression of selected cell wall-specific enzymes were analyzed up to the late stationary phase, i.e., up to 30 and 25 days for the granular and fine suspension cultures, respectively (Fig. 1). Both cultures had an initial lag phase of 3–4 days followed by an exponential phase with maximal growth rate between days 5 and 15 for the granular culture and between days 5 and 9 for the fine suspension culture. The cell mass (wet weight) increased to approximately three and six times the inoculum in the granular and fine suspension cultures, respectively. The growth curve of the present hybrid aspen fine suspension culture was similar to that previously observed for *Populus alba* (Ohmiya et al. 1995, Takeda et al. 1997), in terms of the length of the rapid-growth period and the occurrence of different phases of growth. In order to characterize the cultures, the activities or the amount of mRNA transcripts specific for certain enzymes connected to cell wall synthesis were determined in samples from different time points during growth. All experiments were carried out using at least two independently grown cultures.

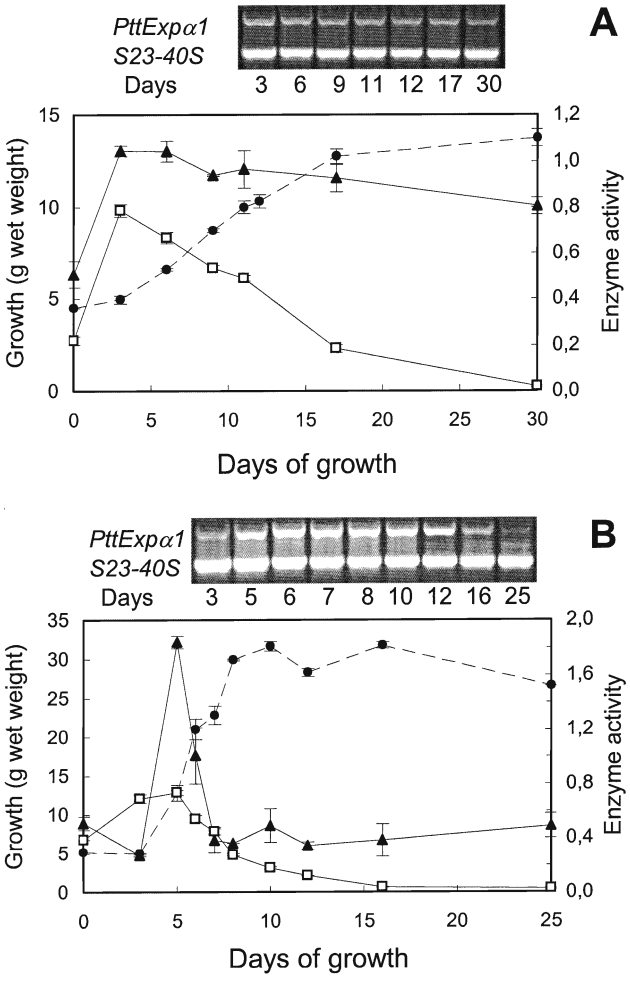

Fig. 1 A, B. Expansin expression profiles and cellulase and XET enzyme activities in *P. tremula* × *P. tremuloides* cell cultures during growth. Growth (●) is expressed as grams of wet weight per flask (50 ml of culture medium per flask). The expression of *PttExpα1* was analyzed by reverse transcription PCR with *S23-40S* as a reference gene. Cellulase activity (□) is expressed as ΔA_{640}/min per g of wet weight. XET activity (▲) is expressed as ΔA_{620}/h per g of wet weight. Samples from duplicate flasks were analyzed and means with standard errors are presented. **A** Granular culture. **B** Fine suspension culture

Cellulase, expansin, and XET

Cellulase, XET, and expansin can be used as markers for primary-cell-wall formation (Rose and Bennett 1999, Darley et al. 2001). The transglycosylating activity of XET was determined by calculating the difference between the activity measured in the presence and absence of xyloglucan oligomers. Thus, XET could be specifically assayed, even in the presence of endoglucanases that use xyloglucan as a substrate, as demonstrated by Sulová et al. (1995). Cellulase and XET activities measured in the cell extracts are shown in Fig. 1. In the absence of a simple assay for expansin, the specific mRNA levels of the *PttExpα1* gene (Gray-Mitsumune et al. 2004) were determined using reverse transcription PCR (Fig. 1). The highest cellulase and XET activities were observed in the early stages of growth in both cultures. While the cellulase activity decreased in both cultures towards the stationary phase, the XET activity remained at a relatively high level in the granular culture. Likewise, a relatively high expression of the expansin gene, *PttExpα1*, was maintained throughout the growth cycle of the granular culture, while its transcription peaked during the period of maximal growth (days 5 to 12) in the fine suspension culture.

According to the present hypothesis, XET activity contributes to cell expansion by catalyzing transient weakening followed by reestablishment of the cellulose-xyloglucan networks in the primary cell walls (reviewed by Rose and Bennett 1999). Previous studies of cell suspension cultures of white poplar (Takeda et al. 1996) and carrot (Hetherington and Fry 1993) have generally associated XET activity with rapid growth of the cells. Expansins constitute a large and ancient family of proteins associated with cell wall expansion, although their mode of action is not known (Cosgrove 1999, Cosgrove et al. 2002, Li et al. 2002). Expression profiling during xylem development in poplar indicated up-regulation of the *PttExpα1* gene during cell expansion

(Hertzberg et al. 2001). This is analogous with the relatively high expression of *PttExpα1* during the exponential phase of the present fine suspension cell culture. Randomly acting endolytic cellulases are thought to be involved in cell wall expansion and growth (Nicol and Höfte 1998, Hertzberg et al. 2001), which is consistent with the high cellulase activity observed during the early exponential growth phase of the present cultures. In summary, the phase of rapid growth in the present cultures coincided with the activity or increased expression of enzymes generally linked to primary-cell-wall synthesis and cell expansion.

Cellulose synthase gene expression

Inspection of the cell suspension cultures by optical microscopy revealed the occurrence of tracheary elements in their stationary phases, particularly in the granular culture (Fig. 2). This prompted us to determine the expression patterns of seven different *CesA* genes as a function of growth in both cultures (Fig. 3). Among these, *PttCesA2* and *PttCesA4* have been previously associated with primary cell wall synthesis in poplar (Hertzberg et al. 2001, Djerbi et al. 2004). In turn, *PttCesA1*, *PttCesA3-1*, *PttCesA3-2*, and *PttCesA9* exhibit highest levels of expression in xylem and tension wood tissues, both undergoing intensive cellulose synthesis (Hertzberg et al. 2001, Djerbi et al. 2004). These genes fall into the same phylogenetic groups as the secondary-cell-wall-associated *CesA* genes in other plants (Djerbi et al. 2005). Furthermore, we have recently discovered that *PttCesA9* occurs as two paralogs, *PttCesA9-1* and *PttCesA9-2*, in hybrid aspen (S. Djerbi unpubl. data).

PttCesA2 was rather evenly expressed over the different stages of growth in the fine suspension culture, except for a slightly reduced level of expression at around day 16, which we cannot explain. In the granular culture, the same gene had a somewhat higher level of expression during the exponential phase of growth. *PttCesA4* was constitutively expressed in both cultures. In the granular culture, but not the fine suspension, *PttCesA3-2* was up-regulated in two separate periods of growth, first during days 3–5 and later from day 12. It is possible that the earlier up-reg-

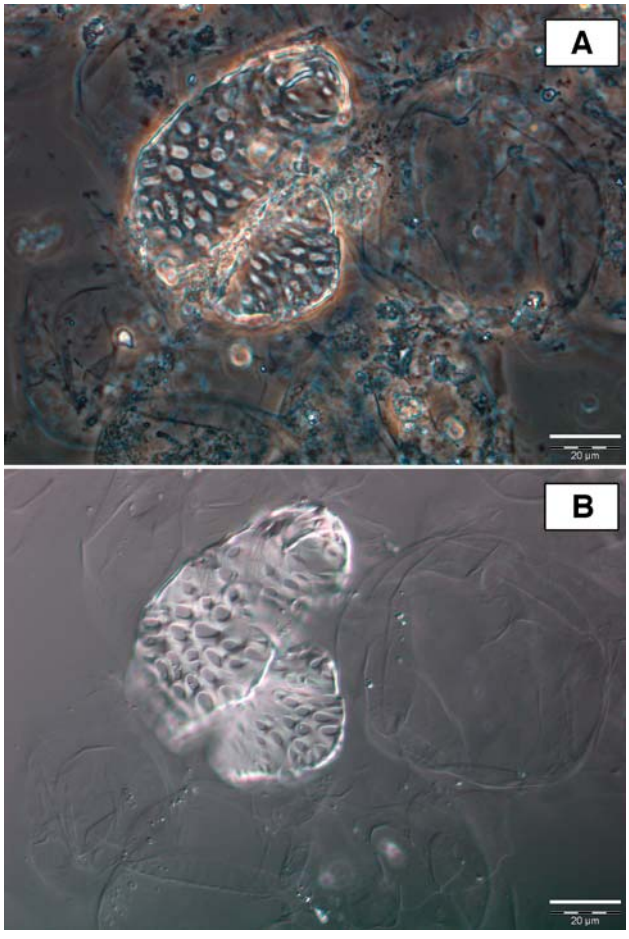

Fig. 2 A, B. Tracheary elements formed in the stationary growth phase of the *P. tremula* × *P. tremuloides* granular cell culture. **A** Phase contrast image. **B** Differential interference contrast image. Bars: 20 µm

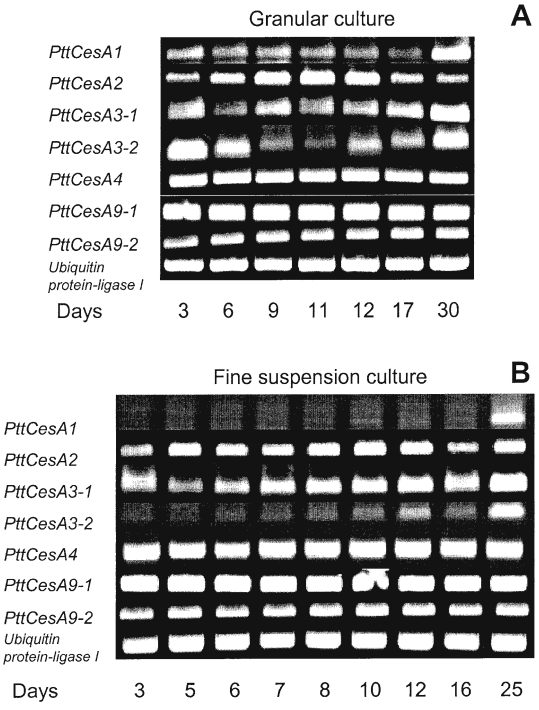

Fig. 3 A, B. Cellulose synthase expression profiles in *P. tremula* × *P. tremuloides* cell cultures. The expression of *PttCesA1*, *PttCesA2*, *PttCesA3-1*, *PttCesA3-2*, *PttCesA4*, *PttCesA9-1*, and *PttCesA9-2* in granular (**A**) and fine suspension (**B**) cell cultures during growth was analyzed by reverse transcription PCR with ubiquitin protein-ligase I as a reference gene

ulation of *PttCesA3-2* was a stress response resulting from the mild mechanical disintegration of the inoculum granules upon subcultivation. *PttCesA3-2* is also up-regulated in tension wood (Djerbi et al. 2004), and its promoter may thus be activated by stress, similar to that of *PtrCesA* from *Populus tremuloides* (Wu et al. 2000).

Among the secondary-cell-wall-associated poplar genes, *PttCesA1*, *PttCesA3-1*, and *PttCesA3-2* were subjected to somewhat different, but clear, up-regulation towards the late stationary phases of both cultures, while both paralogs of *PttCesA9* were constitutively expressed (Fig. 3). This is consistent with the gene expression analyses in poplars, *Arabidopsis thaliana*, barley, and rice, which indicate that three different *CesA* genes are simultaneously expressed at high levels during secondary-cell-wall synthesis (Gardiner et al. 2003; Tanaka et al. 2003; Taylor et al. 2003; Burton et al. 2004; Djerbi et al. 2004, 2005). Our observation that at least four *CesA* genes appear to be highly expressed during xylogenesis in a tree may reflect different physiological states or different cell types, e.g., fibers or vessels in wood. Indeed, the recent discovery of a large number of duplicated *CesA* genes in the genome of *Populus trichocarpa* (Djerbi et al. 2005) indicates that our understanding of the number of genes and proteins involved in cellulose synthesis in trees is by no means complete.

In vitro synthesis of cellulose

Cellulose synthases have been difficult to assay, mainly because of the instability of the enzyme complexes. Another problem is that callose synthase, which uses the same substrate as cellulose synthase, i.e., UDP-glucose, is systematically present in the same preparations, but with a much higher level of activity (Okuda et al. 1993, Doblin et al. 2002). As a consequence, preparations from plant cells usually synthesize callose as a major polysaccharide when UDP-glucose is used as a sugar donor. However, we recently optimized a protocol using digitonin for protein extraction from the plasma membranes of granular hybrid aspen suspension cultures, which enabled us to specifically assay cellulose synthase activity in vitro, even if callose was also synthesized during the assay (Colombani et al. 2004). Cellulose can be quantified by using the Updegraff procedure, which involves a mixture of acetic and nitric acids (Updegraff 1969). The Updegraff reagent is believed to hydrolyze every component in a given preparation, except for crystalline cellulose. However, our earlier experiments have shown that (1→3)-β-glucans can be resistant, especially when their degrees of polymerization and crystallinity are as high as some of those synthesized in vitro by Pelosi et al. (2003). Thus, the Updegraff reagent does

Table 1. β-Glucan and cellulose synthase activities in granular and fine suspension cell cultures recovered during exponential and stationary phases[a]

Culture	Growth phase	Overall β-glucan synthase activities[b]		Cellulose synthase activity[c]		
		Total[d]	Specific[e]	% of overall β-glucan synthase activities	Total[d]	Specific[e]
Granular suspension	exponential (12 days old)	907	9.6	11.0	100	1.06
	stationary (26 days old)	317	11.0	49.6	157	5.47
Fine suspension	exponential (8 days old)	305	4.5	24.0	73	1.09
	stationary (24 days old)	215	4.8	35.0	75	1.68

[a] Values are averages from 3 independent measurements; variations are within the range of ±10%
[b] Callose plus cellulose synthase activities
[c] The level of cellulose synthase activity was determined by measuring the extent of hydrolysis of radioactive polysaccharides synthesized in vitro in the presence of UDP-D-[U-^{14}C]glucose, using specific hydrolytic enzymes as specified in the Material and methods section
[d] Values are given as picomoles of glucose incorporated into glucan per minute per gram of fresh cells
[e] Values are given as nanomoles of glucose incorporated into glucan per minute per milligram of protein

not allow unequivocal discrimination between (1→3)-β-glucans and cellulose. For this reason, cellulose and callose synthesized in vitro were quantified using specific glycoside hydrolases, in the present work, as indicated in the Material and methods section. Such enzymes have been previously successfully used to allow unequivocal distinction between (1→3)-β-glucans and cellulose, as well as quantification of the two polymers (Lai Kee Him et al. 2002, Colombani et al. 2004). Inspired by the high level of the secondary-cell-wall-associated *CesA* gene expression in the aging hybrid aspen cell suspension cultures, this approach was used in the present work to compare the level of cellulose synthase activity in both the granular and fine suspension cultures, at different stages of growth (Table 1).

In order to characterize the cultures, the overall level of β-glucan synthase activity, i.e., the sum of the callose and cellulose synthase activities, was determined per gram of cells (overall activity) and per milligram of total protein (specific activity). The overall β-glucan synthase activity decreased between the exponential and stationary phases, whereas the specific β-glucan synthase activity increased slightly in both cultures, although more so in the granular culture (Table 1). The use of specific glycoside hydrolases to characterize the reaction products suggests that this increase can be attributed to an increase in cellulose synthase, rather than callose synthase activity. While the cellulose synthase activity in the granular cells represented only 11% of the total β-glucan synthase activity at the exponential phase, this value increased to about 50% in the stationary phase. Again, this difference was not as prominent in the fine suspension culture (Table 1).

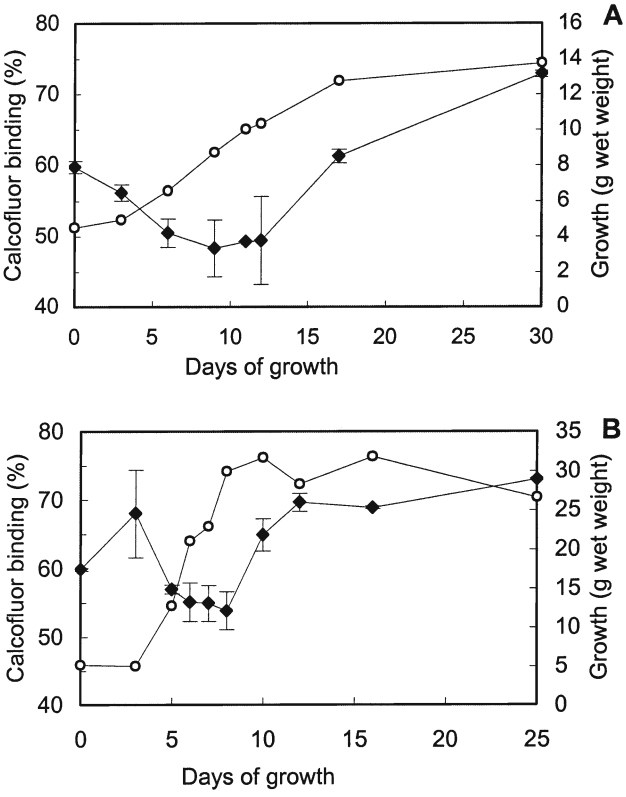

Fig. 4 A, B. Calcofluor white M2R binding to cell homogenates from *P. tremula* × *P. tremuloides* cultures. Growth (○) is expressed as grams of wet weight per flask (50 ml of culture medium per flask). Calcofluor white binding (◆) is expressed as the decrease in fluorescence compared with the original Calcofluor white solution added to the sample. Samples from duplicate flasks were analyzed and means with standard errors are presented. **A** Granular culture. **B** Fine suspension culture

Calcofluor white binding

In order to determine whether the higher levels of cellulose synthase activity during the stationary phases of the cell cultures correlate with a higher amount of cellulose in the corresponding cell walls, we measured the binding of Calcofluor white M2R to cell walls. Calcofluor white M2R is a stilbene-type dye, which has been used previously for microfluorimetric quantification of cellulose in differentiating protoplasts or intact cells (Galbraith 1981). We adapted this method for fluorescence measurement in a spectrofluorometer, recording the decrease in fluorescence caused by adding cell material to the Calcofluor white solution.

Figure 4 shows that Calcofluor white binding followed the same trend for both the granular and fine suspension cultures. The amount of Calcofluor white bound to the cell walls decreased in both cultures in their early exponential growth phases, compared with the initial growth phases. Thereafter, the percentage of dye bound to the cell walls increased steadily during the exponential phases of growth in both cultures. In addition to cellulose, Calcofluor white is known to interact with other polysaccharides containing stretches of (1→4)-linked glucosyl residues (Wood 1980). Some low background can thus be expected due to an interaction with xyloglucan in the primary cell walls, since Calcofluor white preferentially binds strictly linear (1→4)-β-glucans. Even callose binds to Calcofluor white, but with a much lower affinity than (1→4)-β-glucans (Wood 1980). It has been shown before in suspension-cultured wheat that the mitotic index during the lag phase correlates with a peak of callose content, which thereafter disappears (Zabotin et al. 2002). Similarly, the binding of Calcofluor white at the beginning of the growth curves of the present cultures could be partially due to the presence of callose, which is required for cell plate formation and primary-cell-wall deposition (Stone and Clarke 1992). However, the

overall amount of callose in plant cell cultures never exceeds a few percent of the total cell wall components. Another explanation may be that the cellulose content in the newly subcultured cells is diluted in relation to other cell components during cell expansion. The increasing Calcofluor white binding during later stages of growth can be interpreted as increased accumulation of cell wall material, in which cellulose most likely predominates. Thus, together with the observation of a smaller proportion of callose synthase activity in the stationary-phase cultures (Table 1), none or little of the fluorescent-dye binding is expected to be due to the presence of callose. Therefore, the obtained signal must reflect the presence of an increased amount of cellulose in the cell walls of the aging cell cultures. The increased level of Calcofluor white binding, especially in the granular culture, is consistent with the higher level of expression of a subset of *CesA* genes and the higher ratio of cellulose over callose synthesis in the stationary-phase culture.

Conclusions

Here we have shown that poplar cell suspension cultures in their late stationary phase exhibit a high level of transcription of the secondary-cell-wall-associated *CesA* genes. We have also shown that detergent extracts of membrane preparations from the stationary-phase cells are able to synthesize cellulose in vitro at a rate exceeding that observed in other experimental systems. Even though the cultured poplar cells are not synchronized like the zinnia system, they apparently contain, on average, a high concentration of the cellulose-synthesizing protein complexes. As compared with woody tissues, and even with the *Zinnia elegans* mesophyll cells, which need to be mechanically disintegrated from leaf tissues, the cell cultures described here are significantly easier to handle, to grow in relatively large volumes, and to use as a starting material for membrane protein extraction. We are therefore confident that, while the poplar cell culture system at its present state of development cannot compete with the zinnia system in terms of studying the process of xylogenesis, it does offer an interesting new model system for future proteomic and molecular studies of cellulose biosynthesis.

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