Title: What Do We Really Know About Cellulose Biosynthesis in Higher Plants?

Running title: Cellulose Biosynthesis in Higher Plants

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Abstract

Cellulose biosynthesis is one of the most important biochemical processes in plant biology. Despite the considerable progress made during the last decade, numerous fundamental questions related to this key process in plant development are outstanding. Numerous models have been proposed through the years to explain the detailed molecular events of cellulose biosynthesis. Almost all models integrate solid experimental data with hypotheses on several of the steps involved in the process. Speculative models are most useful to stimulate further research investigations and bring new exciting ideas to the field. However, it is important to keep their hypothetical nature in mind and be aware of the risk that some undemonstrated hypotheses may progressively become admitted. In this review, we discuss the different steps required for cellulose formation and crystallization, and highlight the most important specific aspects that are supported by solid experimental data.

Key words: Cellulose biosynthesis, cell wall, higher plants, microfibrils, cellulose polymerization, cellulose crystallization.
Cellulose biosynthesis is a vital biochemical process in higher plants. It is essential to cell
growth and division, tissue formation and differentiation. Owing to its vital function,
cellulose biosynthesis can be the target of specific inhibitors that can be used as herbicides
(Sabba and Vaughn 1999). However, the mode of action of the drugs available remains
largely unknown and the existence of a direct interaction with the cellulose synthase
machinery is not demonstrated in most cases. A typical example of a herbicide affecting
cellulose biosynthesis in an indirect way is 2,6-dichlorobenzonitrile. This drug has been used
for decades to inhibit the growth of undesirable weeds in shrub beds, orchards and berry
fields, but it is only recently that it has been shown to act through an interaction with a
microtubule-associated protein (Rajangam et al. 2008). Most herbicides targeted to cellulose
biosynthesis have in fact been isolated empirically, for instance by screening libraries of
molecules for their effect on plant growth, rather than through rational design. This is
essentially because the process of cellulose biosynthesis remains to be fully understood,
despite the decades of efforts that have been made to decipher the corresponding molecular
events. A number of hypothetical models have been proposed through the years to explain
the polymerization and crystallization mechanisms of cellulose in higher plants (see for
instance Delmer, 1999; Brown and Saxena 2000; Doblin et al. 2002). Even though these
models have been useful in raising important fundamental questions and suggesting possible
answers, their hypothetical nature has sometimes been forgotten with time. As a result, some
molecular events that are still not well demonstrated have become dogmas. The objective of
this review is to summarize the well demonstrated aspects related to cellulose biosynthesis
and revisit a number of arguable concepts that are generally accepted a priori while not being
supported by unequivocal experimental evidence.

In Which Compartment Does the First Step of Cellulose Biosynthesis Occur?

Cellulose is known to be synthesized by membrane-bound complexes that can be visualized by
freeze-fracture techniques (reviewed in Brown 1996). The synthesizing machinery was first
observed at the tip of elongating cellulose microfibrils in the green alga Oocystis and was for this
reason designated as terminal complex (TC) (Brown and Montezinos 1976). As opposed to most
algae and the bacterium Gluconoacetobacter xylinus, whose TCs are organized as linear arrays, the
cellulose-synthesizing machinery of higher plants typically occurs in the form of hexagonal
structures with a six-fold symmetry designated as rosettes (Mueller and Brown 1980; Brown 1996). In addition to the plasma membrane, this form of TCs has also been reported in Golgi cisternae and Golgi-derived vesicles of mesophyll cells of *Zinnia elegans* during differentiation to tracheary elements, suggesting that rosettes are preformed in the Golgi and transported to the plasma membrane by Golgi vesicles (Haigler and Brown 1986). However, the rosette density in these intracellular compartments was low. In addition, as opposed to the rosettes in the plasma membrane, which have been shown to be associated with cellulose microfibrils (Mueller and Brown 1980; Brown 1996), the rosettes observed in the Golgi did not seem to synthesize any cellulose (Haigler and Brown 1986). Thus, rosettes are thought to be functional in the plasma membrane only. This does not mean however that cellulose biosynthesis does not begin in intracellular compartments. This is rarely considered as a possibility, essentially because strictly linear chains of β-(1→4)-glucan tend to form microfibrillar structures, and such structures have never been observed in the intracellular compartments of higher plants. It is only in the case of the marine alga *Pleurochrysis scherffelii* that a complex network of cellulose microfibrils forming circular or ellipsoidal scales has been shown to be produced in the Golgi apparatus (Brown et al. 1969). But despite these considerations, the possibility that cellulose biosynthesis begins in the Golgi apparatus in higher plants cannot be ruled out. Indeed, non-crystalline chains of β-(1→4)-glucan may be synthesized in the Golgi by catalytic subunits of the cellulose synthase that have not yet been assembled in the form of rosettes. In this case, several possibilities can be considered to explain how the assembly of β-(1→4)-glucan chains as microfibrillar structures may be prevented in the Golgi. But before considering these different possibilities, it is important to keep in mind that the process of assembly of individual β-(1→4)-glucan chains as microfibrils is poorly understood. It may be occurring spontaneously through the formation of interchain hydrogen bonds when a number of chains sufficient to form an elementary microfibril such as those observed in primary walls (ca 3 nm diameter) are in close proximity. Such autoassembly may not be possible if the synthesized chains are produced by spatially separated individual catalytic subunits distributed randomly along the Golgi membrane. Alternatively, the formation of microfibrils may require the assembly of complete rosettes with the reported six-fold symmetry, which may form essentially at the plasma membrane (supported by the fact that very few rosettes only have been visualized in the Golgi (Haigler and Brown 1986)), or the involvement of accessory proteins that are not functional or present in the Golgi apparatus. Data obtained in *Arabidopsis* support the hypothesis that the
rosette structures are required for the formation of microfibrils (Arioli et al. 1998). Indeed, mutants carrying a point mutation in one of the cellulose synthase catalytic subunits have been shown to be able to form β-(1→4)-glucan chains that however do not crystallize as microfibrils (Arioli et al. 1998). In yet another hypothesis, cellulose biosynthesis could be initiated in the Golgi apparatus in the form of oligosaccharides that are too short to form microfibrillar strings. Another possible explanation for the absence of formation of cellulosic microfibrils in the Golgi is that the β-(1→4)-glucan chains may interact in the organelle with other compounds, for instance other carbohydrates or an aglycone that would prevent microfibril formation. In conclusion, cellulose biosynthesis is considered as occurring exclusively at the plasma membrane because of the lack of experimental evidence for the formation of the polymer in intracellular compartments.

What is the Very First Step of Cellulose Polymerization?

Cellulose synthase catalytic subunits polymerize β-(1→4)-glucan chains from the activated sugar donor UDP-glucose. The substrate is synthesized by the cytosoluble enzyme UDP-glucose pyrophosphorylase from UTP and glucose-1-phosphate (Figure 1). Sucrose synthase, which can synthesize UDP-glucose from sucrose and UDP has been proposed to be directly involved in cellulose biosynthesis by channeling UDP-glucose to the cellulose synthase catalytic subunit(s) (Figure 1) (Amor et al. 1995). The existence of a membrane-bound form of sucrose synthase potentially involved in this process has been reported, and this may make cellulose polymerization more efficient (Amor et al. 1995). In addition, several reports describe the localization of sucrose synthase in sites where cellulose synthesis is high, either close to the plasma membrane or in cell walls (Salnikov et al. 2001, 2003; Albrecht and Mustroph 2003; Persia et al. 2008). Other data based on gene expression analyses reveal a high expression of several isoforms of sucrose synthase in tissues actively producing high amounts of cellulose, such as for instance tension wood (Geisler-Lee et al. 2006). The overexpression of sucrose synthase genes in poplar (Coleman et al. 2009b) and tobacco (Coleman et al. 2006, 2009a) has revealed an effect on biomass production, which suggests a link to cell wall formation and cellulose biosynthesis. Altogether, these data and observations support a role of sucrose synthase in carbon partitioning and the involvement of several isoforms of the enzyme in cell wall formation, including cellulose biosynthesis. But this involvement is most likely indirect and a consequence of the ability of sucrose synthase
to catalyze the formation of UDP-glucose. Indeed, UDP-glucose is not only the direct substrate of glucosyltransferases such as cellulose synthase or callose synthase, but also a key precursor for the different nucleotide-sugars and the corresponding noncellulosic cell wall carbohydrates (Seifert 2004). Thus, it is expected that the levels of expression of at least some sucrose synthase isoforms would have an impact on cell wall formation by altering the availability of the substrates (UDP-glucose and nucleotide-sugar derivatives) of the cell wall synthesizing enzymes. There is no doubt that part of the UDP-glucose generated by sucrose synthase is used by carbohydrate synthases in addition to the UDP-glucose produced by UDP-glucose pyrophosphorylase (reviewed in Haigler et al. 2001). It remains however, that the direct physical association of sucrose synthase with the cellulose synthase machinery has never been experimentally demonstrated.

It is admitted that UDP-glucose is used directly by the cellulose synthase catalytic subunits to elongate cellulose in a repetitive manner, leading to chains that consist of 800 to up to 10,000 glucosyl units depending on the origin of cellulose (Klemm et al. 2005). But before such polymerization process takes place, cellulose biosynthesis must be initiated and this may require a primer (Figure 1). Biochemical approaches have historically been used to assess the possibility that cellobiose, which increases the rate of β-glucan polymerization in vitro, might be used by glucan synthases, including cellulose synthase, as a primer (McLachlan 1982). However, all approaches used have shown that cellobiose most likely acts as an effector of cellulose and callose synthase activities but that it is not required for the formation of β-glucan chains, which also implies that it is not used by the synthases as a primer (Morrow and Lucas 1986; Hayashi et al. 1987; Li and Brown 1993; Ng et al. 1996; Lai Kee Him et al., 2001). In a later work, sitosterol-β-glucoside was proposed as a primer for cellulose biosynthesis (Peng et al. 2002). In these experiments, radioactive sterolcellodextrins could be synthesized in vitro when crude membranes from cotton fibers were incubated in the presence of sitosterol-β-[14C]glucoside and nonradioactive UDP-glucose. Furthermore, membranes from yeast cells that express a cotton cellulose synthase catalytic subunit were shown to catalyze the same reaction in vitro, while yeast membranes carrying a mutated form of the cellulose synthase catalytic subunit did not exhibit such catalytic activity (Peng et al. 2002). These data provide evidence that sitosterol-β-glucoside can be used by cellulose synthase as an acceptor in vitro, which does not necessarily mean
that it actually acts as a primer for cellulose polymerization in vivo. Indeed, compounds other
than sitosterol-β-glucoside may also be used by the enzyme as glucosyl acceptors in similar
in vitro reactions. Enzymes are well known to have at least a certain degree of promiscuity
with respect to their substrates, and this may also be the case for cellulose synthase in in vitro
reactions with respect to its acceptor substrate. Thus, the role of sitosterol-β-glucoside as a
primer for cellulose biosynthesis remains to be firmly demonstrated in vivo.

How are Cellulose Chains Polymerized?

The glycosyltransferase responsible for cellulose polymerization uses UDP-glucose as the
monosaccharide donor. In this substrate, the glucosyl residue linked to the pyrophosphate
moiety of the molecule is in the α configuration while cellulose contains exclusively β
linkages. For this reason, the enzyme and its corresponding mechanism is described as being
“inverting”, with the presumed involvement of a single nucleophilic substitution at the
anomic carbon of the glucosyl unit of the donor, by analogy with better characterized
inverting glycosyltransferases and comparable to the mechanism of inverting glycoside
hydrolases (Lairson et al. 2008).

The stereochemistry of the β-(1→4) glycosidic bonds that link the glucosyl units in
cellulose induces the formation of a regular two-fold screw axis along the glucan chain.
Thus, monomers are rotated by 180° with respect to their neighbors. For this reason,
cellobiose rather than glucose is sometimes considered as the repeating unit of cellulose. This
structural feature raises the question of the mode of polymerization of cellulose chains. A
model describing the simultaneous addition of two glucosyl units on the growing glucan
chain was proposed to explain the formation of the 2-fold screw axis (Saxena et al. 1995).
This supposes the existence of two UDP-glucose binding sites in the catalytic subunit of the
cellulose synthase that would be spatially organized to allow the formation of the 2-fold
screw axis, concomitantly with the dual monosaccharide addition and with a minimum
energy requirement. However, this attractive model remains purely hypothetical in the
absence of structural data on cellulose synthases. The authors suggested that cellulose
polymerization might occur by the addition of glucosyl units at the reducing end of the
chains (Saxena et al. 1995). This possibility was however ruled out by experimental data
obtained in the bacterium G. xylinus (Koyama et al. 1997) and using enzymatic preparations
from blackberry that were able to produce cellulose chains in vitro (Lai Kee Him et al. 2002).

Indeed, in both cases the results based on the specific labeling of the reducing ends of growing cellulose chains strongly suggested an elongation of the polysaccharide from its nonreducing end (see also Figure 1), which is consistent with the mode of elongation of other carbohydrate polymers such as for instance chitin (Imai et al. 2003).

The polymerization is often described to proceed by the addition of the monomers directly to the carbohydrate chains, although a hypothetical model in which the sugars are first transferred to Ser or Thr residues of the enzyme has also been proposed (Albersheim et al. 1997). In fact, there is no experimental evidence for either of these possibilities. Solving such detailed molecular mechanisms would require the possibility of performing enzymology on pure and active cellulose synthase catalytic subunits, but this has been a holy grail in the field of plant sciences due to the difficulty to express and manipulate such unstable proteins with multiple transmembrane domains.

A rarely envisaged alternative to the models discussed above, and by analogy with some bacterial systems (Matthysse et al. 1995), the polymerization of cellulose chains in higher plants could be initiated on lipid acceptors. Cello-oligosaccharides would then be transferred to the nonreducing end of the elongating glucan chains by cellulose synthase, which would then rather act as a transglycosylase than as a strict glycosyltransferase. Such hypothesis would be consistent with the potential role of sitosterol-β-glucoside in cellulose biosynthesis (Peng et al. 2002), or with the involvement of other lipids similar to those involved in bacterial systems (Matthysse et al. 1995) such as for instance polyisoprenes and their phosphorylated forms. It remains nonetheless that, apart from the report describing sitosterol-β-glucoside as a primer for cellulose biosynthesis (Peng et al. 2002), there is no data in the literature that support the involvement of lipid acceptors in the process of cellulose polymerization in higher plants.

On Which Side of the Plasma Membrane Does Catalysis Occur?

The predicted topology of cellulose synthase catalytic subunits suggests the existence of 8 transmembrane helices that anchor the proteins in the plasma membrane (Delmer 1999). This even number of helices means that the N-terminal and C-terminal ends of the catalytic subunits face the same side of the plasma membrane. The proteins also contain a large
domain predicted to be soluble and that carries the conserved D,D,D,QXXRW motif
involved in the catalytic event and common to all known putative cellulose synthase catalytic
subunits (Campbell et al. 1997). This domain is typically expected to face the cytoplasmic
side of the plasma membrane due to the localization of UDP-glucose pyrophosphorylase,
which is primarily involved in the biosynthesis of the UDP-glucose substrate of cellulose
synthase (Figure 1). In addition, there is no known transporter of UDP-glucose in the plasma
membrane that would allow the transfer of the substrate across the membrane. However,
despite these observations, the possibility that the catalysis of cellulose polymerization
occurs on the extracellular side of the plasma membrane cannot be completely ruled out. In
this case, both the N-terminal and C-terminal ends as well as the catalytic domain of the
enzyme would be exposed in the cell wall, which is theoretically possible. There may exist
yet unidentified transporters of UDP-glucose in the plasma membrane that would deliver the
substrate synthesized by the cytosoluble UDP-glucose pyrophosphorylase to the extracellular
catalytic domain. An alternative to this hypothesis, or a concomitant process, would be the
involvement of a cell-wall-located sucrose synthase in the biosynthesis of UDP-glucose
outside the cell. Interestingly, the occurrence of sucrose synthases in plant cell walls has been
reported recently (Persia et al. 2008). However, this would suppose the existence of
additional transporter systems coupled to the action of sucrose synthase that would deliver
the substrates of this enzyme, i.e. UDP and sucrose, to the cell wall. This process remains
purely speculative at this stage, and the current knowledge rather supports a localization of
the catalytic event on the cytoplasmic side of the plasma membrane, although this cannot be
firmly admitted until the topology of the enzyme has been experimentally demonstrated.

Catalysis at the cytoplasmic side of the membrane implies that the elongating cellulose
chains are translocated across the membrane to reach the cell wall (Figure 1). Several
hypothetical models that involve the action of porin-like proteins or flippases have been
proposed to explain this process (Brown and Saxena, 2000). In another speculative model,
the predicted 8 transmembrane helices of each individual cellulose synthase catalytic subunit
assemble in the membrane to delimit a pore through which the elongating glucan chains are
transported (Figure 1) (Delmer 1999). In the models involving a pore type of structure, the
polymerization reaction itself would provide the energy required for the movement and
extrusion of the cellulose chains. There is virtually no strong experimental evidence
supporting any of the proposed models, and the mechanism of translocation of the cellulose chains across the plasma membrane has yet to be elucidated.

How Do Cellulose Microfibrils Form and Crystallize?

The term microfibril is widely used to describe cellulose structure, but the precise definition is sometimes unclear. In the context of this review, we define a microfibril as the morphological entity that corresponds to the minimum number of $\beta$-(1→4) glucan chains required to form a crystalline structure. By extension, a microbibril may also be considered to be the elementary structure produced by an individual cellulose synthase complex defined as a rosette in higher plants. Other terms such as ribbons, fibrils or macrofibrils, refer to aggregated microfibrils and these structures may have a large range of lateral sizes depending on their origin. The width of cellulose microfibrils from cell walls of various higher plants have been determined using different techniques such as transmission electron microscopy, X-ray scattering and solid-state NMR, and most reports describe lateral dimensions in the range 2-4 nm (see for instance Roland et al. 1975; Chanzy et al. 1978, 1979; Revol et al. 1987; Emons 1988; Boylston and Hebert 1995; Ha et al. 1998; Thimm et al. 2002; Kennedy et al. 2007). Figure 2A shows an example of a network of cellulose microfibrils from primary walls of suspension cultures of blackberry cells, which exhibit a width that was also estimated to be in the range 2-4 nm (Lai Kee Him et al. 2002). Interestingly, detergent extracts of membranes from the same cells were able to synthesize in vitro microfibrils of a similar size, but of about 10% higher crystallinity, compared to the microfibrils produced in vivo (Figure 2B; Lai Kee Him et al. 2002). These data demonstrated the possibility of isolating cellulose synthase complexes that are functional in vitro and able to produce similar microfibrils as enzymes embedded in the plasma membrane. However, the reason for the observed higher crystallinity of the microfibrils synthesized in vitro remains to be determined.

Cellulose chains are extruded from the catalytic subunits that compose the rosettes and their crystallization occurs at some distance from the enzyme complex itself. According to freeze-fracture experiments, a rosette exhibits a typical diameter in the range 25-30 nm and consists of 6 globular structures arranged with a six-fold symmetry (Mueller and Brown 1980; Brown 1996). However, this represents only the average dimensions of the part of the
complex that is embedded in the plasma membrane. Indeed, each rosette also comprises a hexagonal cytoplasmic domain of 45-50 nm in diameter (Bowling and Brown 2008). Each of the 6 globules of a rosette is possibly composed of 6 cellulose synthase catalytic subunits (Figure 1), which implies that a rosette would be responsible for the simultaneous elongation of 36 β-(1→4) glucan chains that would co-crystallize to form a microfibril (Delmer 1999). This number of 36 chains is compatible with the size of microfibrils isolated from most primary walls (~ 3.5-4 nm) (Delmer 1999). However, other data are more consistent with the packing of 18 chains or even less per microfibril (Chanzy et al. 1978, 1979; Ha et al. 1998; Thimm et al. 2002; Kennedy et al. 2007). It is in fact the number of active catalytic subunits per rosette that determines the number of chains that are extruded from each enzyme complex. But the actual stoichiometry of the cellulose synthase catalytic subunits in the rosettes, and the number of active enzyme molecules per rosette, have never been experimentally demonstrated. Thus, the generally admitted concept that individual cellulose microfibrils consist of 36 chains is essentially based on the observed six-fold symmetry of the rosette structures and the estimated lateral size of microfibrils from primary walls. It is well possible that different rosettes contain a lower number of catalytically active subunits than assumed in this model, leading to the formation of thinner microfibrils. In fact, if we assume a square cross section for cellulose microfibrils, the packing of 4 x 4 chains, corresponding to a total of 16 chains per microfibril, is the minimum requirement to form a crystalline structure. This number of chains is compatible with a lateral size of 2 to 2.5 nm for an individual microfibril and consistent with the width typically observed for microfibrils from primary walls (Chanzy et al. 1978, 1979; Ha et al. 1998; Thimm et al. 2002; Kennedy et al. 2007).

Regardless of the precise number of chains that form individual microfibrils, the packing of a sufficient number of chains into a crystalline structure is most likely spontaneous and not assisted by proteins, although this remains to be demonstrated (Figure 1). It is expected that adjacent chains protruding from the same enzymatic complex self assemble through hydrogen and van der Waals bonds to obtain the most stable structure. Crystallization can be prevented using dyes that interact with cellulose chains, such as for instance Calcofluor White (Roberts et al. 1982). However, the use of such compounds does not prevent polymerization, and leads to the formation of noncrystalline structures. Thus, based on this
observation, polymerization and crystallization can be considered as two different events, which are however tightly coordinated. Cellulose synthase complexes move in the plasma membrane, as observed experimentally (Paredez et al. 2006, 2008; DeBolt et al. 2007a; Crowell et al. 2009; Gutierrez et al. 2009), and a biophysical model has been proposed in which the movement of the enzyme complex is driven by the polymerization and crystallization events (Diotallevi and Mulder, 2007). In this model, the predicted rate of movement of individual cellulose synthase complexes matches with experimental values deduced from optical imaging techniques (in the range 150-500 nm/min). However, due to the rather low resolution of confocal microscopy (~200 nm), it is unlikely that the structures that have been observed as moving in the membrane correspond to individual rosettes whose dimensions are in the range 25-30 nm. This suggests that aggregates of microfibrils form simultaneously as they are spun from groups of rosettes spatially close in a confined environment. Interestingly, it has been shown recently that cellulose synthase is located in lateral patches of the plasma membrane that are resistant to detergents and exhibit similar properties as lipid rafts in animal cells (Bessueille et al. 2009). Thus, it is tempting to speculate that groups of rosettes co-localized in raft-like structures are responsible for the coordinated synthesis of multiple microfibrils that coalesce shortly after having been extruded from individual enzyme complexes to form fibrils or ribbons of cellulose. Due to the rate of synthesis of microfibrils, the intrinsic affinity of cellulose microfibrils with each other and their tendency to form aggregates, the process of fibril formation is most likely spontaneous, especially if multiple rosettes occur in close proximity as suggested above. Similarly, individual microfibrils most likely crystallize spontaneously, although a process in which proteins would assist the crystallization event cannot be ruled out. But this would require additional energy to be provided to the system. In addition, no protein involved in the packing of the chains per se has been identified so far.

What Are the Protein Components of the Cellulose Synthase Machinery?

The catalytic subunits of plant cellulose synthases are encoded by CesA genes (reviewed in Delmer 1999 and Doblin et al. 2002). These genes were first identified in the cotton fiber (Pear et al. 1996) by exploiting their similarity with the bacterial cellulose synthase genes isolated and characterized earlier (Saxena et al. 1990; Wong et al. 1990). The occurrence of
some of the CesA gene products in rosettes was demonstrated by freeze-fracture combined with antibody labeling (Kimura et al. 1999). Since these seminal reports, multiple homologues of the CesA genes have been identified in numerous higher plants, essentially by sequence similarity (see for instance Arioli et al. 1998; Taylor et al. 1999, 2000; Fagard et al. 2000; Holland et al. 2000; Doblin et al. 2001; Scheible et al. 2001; Burton et al. 2004; Djerbi et al. 2004, 2005). Functional studies of CesA genes have typically involved the production and characterization of mutants in which cellulose biosynthesis is perturbed or that are resistant to the action of drugs that affect cellulose biosynthesis. Genome analyses and EST sequencing have revealed that higher plants systematically contain families of CesA genes whose function has been firmly elucidated in a limited number of cases. For instance, the genome of Arabidopsis contains 10 different CesA genes (Richmond 2000) while as many as 18 CesA genes have been identified in the genome of Populus trichocarpa (black cottonwood) (Djerbi et al. 2005). Unfortunately, the nomenclature used for these genes, especially their numbering, is not uniform across species and this is sometimes at the origin of confusion when comparisons are made between orthologues. To clarify the situation, a nomenclature has been proposed for the CesA genes from different poplar species, for which the numbering was so far extremely confusing (Kumar et al. 2009). This facilitates also the comparison with the Arabidopsis orthologues, which have been the most studied to date. The analysis of mutants of Arabidopsis in which cellulose biosynthesis is perturbed in primary walls, and immunoprecipitation experiments combined with the use of mutants, have demonstrated that a group of 3 different gene products, namely AtCesA1, 3 and 6, is required for cellulose biosynthesis in primary walls (Desprez et al. 2007). Based on these immunoprecipitation experiments and genetic evidence, it has been proposed that these 3 proteins form a complex in which AtCesA2 and AtCesA5, which have been reported to be partially redundant with AtCesA6, may compete for the same position in the enzyme complex (Desprez et al. 2007; Persson et al. 2007). The occurrence of putative zinc-finger domains rich in Cys at the N-terminus of the CesA proteins and the analysis of their redox states, have suggested a role of these domains in the dimerization of the CesA subunits (Kurek et al. 2002). Such dimerization would represent an early step of the formation of the rosette complex. A different combination of 3 CesAs, each required for secondary wall formation in the same Arabidopsis cells (AtCesA4, 7 and 8), are associated in a complex, as
shown by immunoprecipitation experiments (Taylor et al. 2003). Expression profiling revealed that the 3 proteins are co-regulated during secondary cell wall formation, which is consistent with their association in a complex (Brown et al. 2005; Persson et al. 2005). Interestingly, recent work on AtCEsA4, 7 and 8 based on yeast two-hybrid experiments have confirmed that the 3 proteins can indeed interact with each other, but only AtCesA4 is able to form homodimers (Timmers et al. 2009). In addition, somehow contradictory with the proposed role of the zinc-finger domains in the dimerization of CesA subunits (Kurek et al. 2002), mutations in several Cys positions in the domain of AtCesA7 decreased but did not abolish the interactions between the secondary cell wall associated CesA subunits (Timmers et al. 2009). This suggests that the zinc-finger domains or at least their Cys residues are not essential for the interaction between CesA subunits. However, recent work performed on the purification of the cellulose synthase complex involved in secondary cell wall biosynthesis using a dual epitope tagging strategy combined with a two-step purification procedure, and the analysis of the purified complex, suggests that disulfide bonds contribute to the stability of the complex and further supports their involvement in the oligomerization of the CesA subunits (Atanassov et al. 2009). But it was not possible to determine whether the disulphide bonds were actually intermolecular.

As for *Arabidopsis*, gene expression analyses in other plant species reveal the co-regulation of multiple CesA genes, which would also support the association of several different CesA proteins in a given complex. The observation that 4 different CesA genes are more specifically expressed during xylogenesis in hybrid aspen may reflect some level of functional redundancy (Djerbi et al. 2004), as observed for primary wall biosynthesis in *Arabidopsis* (Desprez et al. 2007). Also, it is possible that rosettes with different compositions coexist in the same membrane, particularly if some of the genes are redundant and co-expressed.

If the involvement of at least some CesA genes in cellulose biosynthesis is well supported, it remains however that the catalytic activity of individual proteins has never been directly demonstrated. This would require the expression of individual catalytic subunits in an active form and their characterization *in vitro*, an objective that may be achieved in the future with the development of more efficient expression systems for polytopic membrane-bound proteins. In addition, if some CesA proteins have been shown to be directly associated
with the rosette structures and associated in the form of complexes, the function of the other CesA gene products remains to be elucidated. Some of the CesA proteins may be involved at different steps of the biosynthetic process such as in the biosynthesis of primers to initiate cellulose polymerization (see Taylor et al. 1999, 2000, 2003; Peng et al. 2002; Read and Bacic 2002).

The cellulose synthase machinery is almost systematically described as a heteromultimeric complex. Molecular genetics has provided an alternative to the challenging biochemical purification of intact cellulose synthase complexes in an active form. The characterization of numerous mutants affected in cellulose biosynthesis has led to the conclusion that proteins other than CesAs are part of the complex, but in all cases the evidence is indirect and the physical association of all possible candidates to the complex remains to be demonstrated (see also Figure 1). Among the multiple proteins that have been proposed to be associated to the cellulose synthase machinery are a membrane-bound sucrose synthase (Amor et al. 1995), a membrane-bound endo-β-(1→4)-glucanase (Korrigan in Arabidopsis and its orthologues in other species) (Nicol et al. 1998), annexins (Hofmann et al. 2003), actin, tubulin, a putative lipid transfer protein, etc (for details related to the possible roles of these proteins see Haigler et al. 2001; Doblin et al. 2002; Mølhøj et al. 2002). In addition, Arabidopsis gene products such as KOBITO1 and COBRA or COBRA-LIKE have been shown to be potentially involved in cellulose biosynthesis in relation with cell expansion (Pagant et al. 2002; Roudier et al. 2002, 2005; Brown et al. 2005; Persson et al. 2005). But the precise roles of these proteins have not been determined and the effect of the corresponding gene mutations on cellulose biosynthesis may be indirect. Interestingly, the recent purification to homogeneity of the cellulose synthase complex involved in secondary cell wall biosynthesis in Arabidopsis has revealed that the isolated complex contains no other proteins than the 3 previously identified CesA subunits, namely AtCesA4, 7 and 8 (Atanassov et al. 2009). As mentioned by the authors, this does not preclude the transient association of other proteins to the complex, such as the candidates mentioned above. In this work, complexes whose size was consistent with the expected size of dimers, tetramers and possibly hexamers of the CesA proteins were isolated, but the actual stoichiometry of the CesA proteins in the isolated complexes could not be determined. In addition, the efficiency of the purification procedure was not sufficient to search for enzymatic activity, and therefore
it was not possible to determine whether proteins other than the 3 CesAs forming the
complex are required for activity (Atanassov et al. 2009). This important contribution
suggests that the 3 CesA subunits are tightly interacting and probably represent the minimum
required structure for catalytic activity. The other proteins that are possibly transiently
associated to the complex most likely play a role in vivo, but are certainly not required for
cellulose polymerization per se.

Of particular interest is the endoglucanase Korrigan whose function remains puzzling. This
protein has been proposed to exert different possible roles, such as for instance the release of
the newly synthesized cellulose microfibrils in the cell wall, the removal of a primer, the
post-synthetic trimming of imperfections along the newly synthesized microfibrils, the
control of the degree of polymerization of the β-(1→4)-glucan chains, etc (Mølhøj et al.
2002). Its association to the cellulose synthase complex could indeed be transitory, justified
by its requirement only at a specific stage of the cellulose biosynthesis process.

A relationship between cytoskeleton-related proteins and cellulose biosynthesis has been
implied for many years and more and more experimental evidence is being accumulated,
supporting this concept. As discussed in more detail in the next section, multiple recent
reports suggest an involvement of cortical microtubules in the movement of the cellulose
synthase complex in the plasma membrane (Paredez et al. 2006, 2008; DeBolt et al. 2007a;
Crowell et al. 2009; Gutierrez et al. 2009). In addition, actin has recently been shown to
participate in the delivery of organelles that carry cellulose synthase subunits to bands of
secondary cell wall thickenings in developing xylem cells (Wightman and Turner 2008).
Thus, the abovementioned possible association of tubulin and actin to the cellulose synthase
complexes, most likely in a transient manner, is somehow expected in light of the recent cell
biology developments related to cellulose formation.

In summary, the protein composition of the cellulose synthase machinery may vary
between different steps of the biosynthetic process. The certitude is that several CesA
proteins are part of a given complex, but their association in the form of a rosette is not a
requirement for polymerization since non-crystalline β-(1→4)-glucans can be formed in the
absence of a complete rosette (Arioli et al. 1998). The oligomerization of the CesA proteins
as rosettes most likely represents a minimum structural state required for the concomitant
polymerization of a sufficient number of cellulose chains to allow for the formation of crystalline microfibrils.

**How is the Cellulose Synthase Machinery Coupled to the Cytoskeleton?**

Plant development and growth depend on the coordination of cytoskeleton dynamics in the cell cortex and cellulose microfibril deposition in the cell wall. Numerous reports describe the involvement of the cytoskeleton in the organization of plant cell wall and of microtubule-associated proteins (MAPs) in microtubule orientation, with direct consequences on plant growth and architecture (see for instance Sedbrook et al. 2004; Korolev et al. 2007; Perrin et al. 2007; Wang et al. 2007; Rajangam et al. 2008). Interestingly, a MAP from poplar, denoted PttMAP20, particularly abundant in cells forming secondary walls was shown to bind the cellulose synthesis inhibitor 2,6-dichlorobenzonitrile (DCB) *in vitro* (Rajangam et al. 2008). It was suggested that the protein represents a link between the cytoskeleton and cellulose biosynthesis, thereby providing an explanation of the possible mechanism of action of DCB. But the precise mode of interaction of PttMAP20 with the cellulose synthase machinery, either direct or indirect, remains to be elucidated.

Live cell imaging and advanced analyses of mutants and overexpressors have provided new insights in the parallelism between cellulose biosynthesis and cytoskeleton (Emons et al. 2007; Lindeboom et al. 2008). AtCesA6 fused to YFP was shown to move along tracks defined by microtubules, and the pattern of the movement was affected, but not prevented, upon microtubule disruption with oryzalin (Paredez et al. 2006). In addition, upon complete depolymerization of microtubules, cellulose synthase complexes move according to an apparently self-organized pattern (Paredez et al. 2006; Gutierrez et al., 2009). It was proposed that microtubules provide a “scaffold” for the movement of the cellulose synthase complexes, but are not required for the cellulose synthase motility *per se* (Paredez et al. 2006). Other studies based on the use of DCB have shown that the drug treatment affects the movement of CesA6:YFP, without influencing the microtubule dynamics and alignment (DeBolt et al. 2007b). A screening of small molecules causing the swelling of organs led to the identification of the drug morlin, which triggers a series of cytoskeletal defects, namely the reorientation of the cortical array and formation of shorter and more bundled microtubules, together with a decrease in the mean velocity of CesA6:YFP (De Bolt et al.
Morlin-treated plants that had been pre-incubated with oryzalin also exhibited a decrease in the velocity of cellulose synthase, demonstrating that morlin compromises the movement of the cellulose synthase complex independently of its effects on microtubules (De Bolt et al. 2007a). A further support to the existence of a crosstalk between the cortical cytoskeleton and cellulose biosynthesis comes from the discovery of microtubule-associated cellulose synthase compartments designated MASCs whose movement was shown to depend on the cortical microtubule array (Crowell et al. 2009). A transient interaction of the cellulose synthase complexes with the microtubules, rather than a passive movement of the enzyme between the microtubules, was proposed (Crowell et al. 2009). In addition, SmaCCs, i.e. “small compartments carrying cellulose synthase complexes” (as designated by Gutierrez et al. 2009), probably identical to MASCs, were shown to be involved in the delivery of the cellulose synthase complexes to the plasma membrane and are associated with cortical microtubules (Gutierrez et al. 2009).

As mentioned above, actin has been proposed to be associated to the cellulose synthase machinery, at least transiently (Doblin et al. 2002). However, little information is available about its role in cellulose biosynthesis. In *Zinnia* tracheary elements, actin filaments have been shown to align transversely beneath the sites of future cell wall deposition (Kobayashi et al. 1988). More recently, actin cables have been proposed to mediate the intracellular trafficking of cellulose synthase-containing organelles, which pause at sites marked by transverse actin (Wightman and Turner 2008). However, the disassembly of the actin cytoskeleton does not prevent the delivery of the cellulose synthase complex to the plasma membrane but alters the global distribution of the enzyme complexes (Gutierrez et al. 2009). Independently from the latter report, Crowell et al. (2009) have also shown that the actomyosin network is involved in the distribution of the Golgi-born cellulose synthase complexes throughout the cell. Altogether, these recent data firmly confirm the importance of the actin cytoskeleton in the regulation of cellulose synthesis. Interestingly, a rate of 7 µm/s was determined for particles bearing cellulose synthase complexes beneath the nascent secondary wall in developing xylem vessels (Wightman and Turner 2009). This rate is several orders of magnitude higher than those reported for the primary wall cellulose
synthase complexes and is probably due to the involvement of a highly motile cytoplasmic compartment (Wightman and Turner 2009).

In conclusion, the relationship between cortical microtubules, the actin network and cellulose biosynthesis remains to be elucidated in greater details (Figure 1). But a new era that opens great opportunities to tackle this problem has just begun owing to the development of advanced methodologies for live cell imaging, combined with the analysis of the continuously expanding collection of plant mutants, and the use of novel bioactive drugs that affect specific steps in cytoskeleton dynamics and cellulose formation.

In Vitro Approaches to Study Cellulose Biosynthesis: Frustrations, New Opportunities and Prospects for the Future

Attempts to characterize plant cellulose synthases the conventional way, i.e. through protein purification, biochemical analyses and protein sequencing prior to gene isolation have been particularly frustrating for several decades. In the 90’s, approaches have evolved towards molecular genetics in the broad sense and this has allowed considerable progress in the area. However, the development of such approaches to study plant cellulose synthases has become possible only after the isolation of the bacterial cellulose synthase genes (Saxena et al. 1990; Wong et al. 1990), which undoubtedly represents a major breakthrough in the field.

Plant cellulose synthases have traditionally been difficult to study in vitro using biochemical approaches for several reasons. First, the enzymes are membrane-bound complexes which are in addition particularly unstable upon extraction from the plasma membrane (Delmer 1999; Bessueille and Bulone 2008). The other major difficulty is the occurrence of callose synthase in the plasma membrane, which uses the same substrate (UDP-glucose) as cellulose synthase and which exhibits high activity in vitro (Okuda et al. 1993; Lai Kee Him et al. 2001, 2002; Colombani et al. 2004) although callose is only a minor component of plant cell walls. Callose plays important roles in vivo. For instance, it is synthesized transiently during cell division, contributing to the formation of the cell plate prior to the establishment of the primary cell wall between the daughter cells, as well as in specific cell types or in response to various stresses such as infection or wound (Stone and
Clarke 1992). The reason why a low abundant cell wall polymer like callose is synthesized abundantly by cell free extracts while the major cell wall component cellulose cannot be easily produced in vitro has been an intriguing, yet unsolved, question. For many years, it was believed that the same enzyme might be responsible for callose and cellulose biosynthesis, and that the switch between both activities in vitro might be the result of a loss of accessory proteins required for cellulose biosynthesis or some change of conformation during protein extraction (Delmer 1999). However, it is now clear that the genes that code for cellulose synthase catalytic subunits and callose synthase are different, which does not preclude though a co-regulation mechanism or/and the transient association of similar or identical proteins in both complexes (reviewed in Bessueille and Bulone 2008). In addition, the characterization of the glucans synthesized in vitro by cell free extracts has not always been most rigorous, and the incorporation of radioactive glucose into ethanol-insoluble polysaccharides has sometimes been associated to cellulose biosynthesis while the polymer that was synthesized was in fact callose (reviewed in Colombani et al. 2004 and Bulone 2007). Thus, the distinction between $\beta-(1\rightarrow3)$ and $\beta-(1\rightarrow4)$ linkages is essential when studying callose and cellulose synthases in vitro.

The development of an assay specific for cellulose synthase has been one of the great challenges in the field. Progress on this particular aspect has been slow, but methods that allow in vitro synthesis of cellulose have been developed (Kudlicka et al. 1995, 1996; Kudlicka and Brown 1997; Lai Kee Him et al. 2002; Colombani et al. 2004). However, cellulose synthase still cannot be assayed routinely in a high-throughput manner because the assays always need to be accompanied by careful product characterization for distinguishing callose from cellulose synthesis (Bulone 2007). Indeed, there is currently no in vitro method available that allows synthesis of cellulose without the co-formation of callose. After the first successful synthesis of cellulose in vitro (Kudlicka et al. 1995, 1996; Kudlicka and Brown 1997), the method was significantly improved by the careful selection of adapted detergents that allowed the recovery of mg amounts of in vitro synthesized cellulose and the thorough characterization of the polymer (Lai Kee Him et al. 2002). Interestingly, the cellulose produced in vitro from detergent-extracts of blackberry cell membranes was of a significantly higher crystallinity than its counterpart isolated from the primary walls of the same cells (Lai Kee Him et al. 2002). The morphology of these 2 types of microfibrils is shown in Figure 2.
Additional work using hybrid aspen cells harvested at their stationary growth phase confirmed the possibility of increasing the proportion of cellulose synthesized in vitro to up to 70% of the total glucans produced (Colombani et al. 2004). The method has more recently been optimized for in vitro synthesis of cellulose from detergent extracts of tobacco BY2 cells (Cifuentes et al. 2010). Despite these significant advances, it seems that the conditions for cellulose biosynthesis need to be optimized for different plant models (Lai Kee Him et al. 2002; Colombani et al. 2004). However, accumulated evidence has now demonstrated the possibility of following cellulose synthase activity in vitro from several plant species. This opens a great opportunity to analyze the biochemical properties of the enzyme, particularly in light of the recent progress made in the purification of intact complexes in Arabidopsis using dual epitope tagging and the specific corresponding purification steps (Atanassov et al. 2009). Efforts in our group are also being made on the expression of individual catalytic subunits for biochemical characterization, and the availability of a specific assay will definitely be an asset for characterizing in great detail the recombinant proteins.

The development of these assays and the availability of specific antibodies have allowed the demonstration that callose and cellulose synthases are located in plasma membrane microdomains resistant to detergents like Triton X-100, and that exhibit similar biochemical features as lipid rafts from animal cells (Bessueille et al. 2009). This novel concept suggests a function of plant membrane microdomains in cell growth and morphogenesis. The isolated microdomains are functional in vitro and their lipid composition reveals an enrichment in sterols and sphingolipids, which possibly interact directly with callose and cellulose synthases in either the same or different subpopulations of detergent-resistant microdomains (Bessueille et al. 2009). This suggests that such lipids participate in the stability of the cellulose synthase complex in the plasma membrane. Interestingly and consistent with this hypothesis, sterols have been shown to be crucial for cellulose synthesis in Arabidopsis (Schrick et al. 2004). Varying lipid compositions between different plant species and their membrane microdomains might be the reason for the need of a careful choice of the detergents to achieve a successful extraction of cellulose synthases active in vitro. The isolation of these membrane microdomains represents a great opportunity to perform quantitative proteomics analyses on intact or fractionated detergent-resistant structures. Such experiments are ongoing in our laboratory and expected to unveil the specific enrichment of...
proteins in membrane microdomains compared to the rest of the plasma membrane, and thus to allow the firm identification of proteins that are at least transiently associated to the cellulose synthase complex.

Very little information is available on the biochemical regulation of the cellulose synthase machinery. Recently, phosphorylation events have been shown to be involved in the regulation of the callose and cellulose synthase activities in tobacco cells using *in vitro* approaches, but the mode of action of the corresponding wall-bound phosphatase, either direct or indirect, remains to be determined (Kaida et al. 2009). Interestingly, in *A. thaliana* the phosphorylation of two Ser has been proposed to play an important role in the regulation by proteolysis of the secondary cell wall cellulose synthase complex (Taylor 2007). In addition, biochemical data based on the characterization of the detergent-resistant membrane microdomains discussed above suggest that the intact cellulose synthase catalytic subunits of 120 kDa are catalytically inactive, and that they undergo a proteolytic process to yield a catalytically active form of ~57-kDa that is associated to the membrane microdomains (Bessueille et al. 2009). In *Arabidopsis* (Taylor 2007) and the cotton fiber (Jacob-Wilk et al. 2006), proteolytic processes have been proposed to be involved in the turnover and/or degradation of the enzyme complexes rather than in a proteolytic maturation leading to enzyme activation. In the case of the cotton fiber, the proteolytic event may be controlled by the state of dimerization of the cellulose synthase catalytic subunits that may occur through their N-terminal zinc-binding domains (Jacob-Wilk et al. 2006).

In conclusion, the availability of the biochemical tools mentioned above, combined with the use of specific antibodies such as those that have already been exploited in immunoprecipitation experiments, opens the opportunity to (finally) gain detailed biochemical information on the cellulose synthase machinery. For instance, it is expected that fundamental questions such as the stoichiometry of the cellulose synthase catalytic subunits within the complexes and the composition of the cellulose synthase machinery will be solved in the near future owing to the availability of immunoprecipitated or otherwise purified complexes. Longer term but promising areas are the isolation of recombinant individual catalytic subunits in an active form, which will not only make possible the complete determination of the biochemical properties and molecular mechanisms of the CesA proteins,
but also, hopefully, lead to the determination of the three-dimensional structure of the enzyme.
References


Figure legends

Figure 1. Hypothetical model for the biosynthesis of cellulose in higher plants. A schematic top-view of a rosette, which is naturally embedded in the plasma membrane, is represented in the top left corner of the Figure, with its hypothetical organization consisting of 36 catalytic subunits. The protein with 8 putative transmembrane helices represents one of the 6 catalytic CesA proteins whose association presumably forms one of the 6 rosettes subunits of ~8 nm each. The catalytic site of this CesA protein is represented facing the cytoplasmic side of the plasma membrane, which is most consistent with the assumed site of synthesis of the substrate UDP-glucose (see main text for discussion). (?) refers to aspects that remain to be elucidated including (a) the requirement of a soluble or membrane-bound primer to initiate polymerization (broken arrows); (b) the orientation of the cellulose chains being extruded; (c) the mechanism of transport of the cellulose chains from the cytoplasm to the cell wall (a pore involving the 8 putative transmembrane helices of the catalytic subunits is represented as proposed earlier by Delmer (1999)); (d) the association of yet unidentified membrane-bound proteins to the CesA subunits, including (e) a membrane-bound form of sucrose synthase (SuSy) that may interact with the membrane through another membrane-bound protein, as represented in the model, or directly; (f) the mode of interaction of the cellulose synthase machinery with microtubules and actin; (g) the mode of assembly and crystallization of cellulose chains into microfibrils; (h) the involvement of structural lipids stabilizing the complex; (i) the possible roles of Korrigan (Kor). Other potential protein components of the complex, such as regulation subunits, are not represented. The stoichiometry of the different proteins in the complex is not known (adapted and modified from Bessueille and Bulone 2008).

Figure 2. Electron micrographs of cellulose microfibrils (negative staining with 2% uranyl acetate). (A) Microfibrils extracted from primary walls of blackberry (Rubus fruticosus) cells grown as suspension cultures. (B) Microfibrils synthesized in vitro using detergent extracts of plasma membranes from the same blackberry cells as a source of enzyme. The lateral size of individual microfibrils was estimated to be of no more than 2-4 nm (Lai Kee Him et al.
Rosette (~25 nm) composed of the products of 3 different CesA genes

Assembly and crystallization into microfibrils (?)

Possible roles of Kor:
- release of primer (?)
- release of microfibril (?)
- control of chain length (?)
- trimming of microfibrils

Plasma membrane

Cell wall

Figure 1
Figure 2B