Regulated trafficking of cellulose synthases
EF Crowell², M Gonneau¹, Y-D Stierhof³, H Höfte¹ and S Vernhettes¹

New findings reveal that proteins involved in cellulose biosynthesis undergo regulated trafficking between intracellular compartments and the plasma membrane. The coordinated secretion and internalization of these proteins involve both the actin and cortical microtubule cytoskeletons. This regulated trafficking allows the dynamic remodeling of cellulose synthase complex (CSC) secretion during cell expansion and differentiation. Several new actors of the cellulose synthase machinery have been recently identified.

Introduction
Cellulose consists of a paracrystalline assembly of parallel β-1,4-linked glucan chains, which coalesce to form microfibrils. Microfibril orientation is highly regulated and determines the growth direction of cells and, after growth cessation, the mechanical properties of cell walls and organs. Recent findings provide new insights into the organization and regulation of the cellulose synthase complex (CSC), which underlie the fascinating properties of cellulose microfibrils. Cellulose synthesis has already been extensively covered in several excellent reviews [1,2,3,4]. This review will focus on the most recent findings in this field.

The cellulose synthase complex is more complex than previously thought
Cellulose in land plants is synthesized from a hexameric membrane-bound protein complex CSC or rosette observable in the inner leaflet of freeze-fractured plasma membranes (Table 1) [5]. The complex contains multiple cellulose synthase catalytic subunits (CESAs), which form a large gene family in land plants [6]. Three distinct CESA isoforms interact to form the primary cell wall CSC: CESA1, CESA3, and a member of the CESA6-like clade [7,8]. Preliminary data obtained by quantitative LC–MS/MS suggest that these three isoforms are present in a 1:1:1 ratio in the primary cell wall CSC (unpublished). Three other isoforms, CESA4, CESA7, and CESA8, form a CSC with a specialized role in the deposition of secondary walls. However, the distinction between primary and secondary cell wall CSCs is not so clear-cut: for instance ‘primary wall CESAs’ are the only CESAs present during secondary wall deposition in trichomes [9] and CESA9, a member of the CESA6 clade is required for normal secondary cell wall deposition in the epidermal seed coat [10]. In addition, primary and secondary cell wall CSCs can co-exist in certain cell types [11].

Why are three isoforms required for a functional CSC? It has been suggested that different CESA isoforms provide distinct binding sites for specific intra-particle and inter-particle interactions required for hexameric rosette assembly [12,13,14,15], catalyze distinct chain initiation and elongation reactions [16] or add adjacent glucose residues in opposite orientations [17]. Cellulose-producing bacteria, animals and most algae have linear CSCs. The appearance of hexameric rosettes precedes the land plants since they are found in the Charophyta, the green algae most closely related to land plants. The moss Physcomitrella like vascular plants has multiple CESA genes. The isoform classes, however, do not cluster with vascular plant isoforms, suggesting that they have evolved independently in the moss and vascular plant lineages [18]. Functional rosettes therefore existed before the divergence of the CESA isoforms suggesting that the functional specialization of CESA isoforms is not related to geometrical or biochemical constraints within the rosette but rather reflects specific regulatory functions such as interactions with the cytoskeleton or components of the secretory pathway.

Thus far, co-immunoprecipitation (co-IP) experiments have revealed interactions between CESAs, but have not identified any other proteins in the complex [7,19]. Yeast two-hybrid experiments using the CESA6 catalytic domain as bait identified a large protein named cellulose synthase interactive protein 1 (CSI1) [20]. This protein also interacts with CESA1 and more weakly with CESA3. Loss-of-function csi1 mutants show growth defects, a reduced cellulose content and a greatly reduced velocity of YFP:CESA6 in the plasma membrane. A RFP:CSI1

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Table 1

<table>
<thead>
<tr>
<th>Cellulose synthase complex parameters</th>
<th>At least 3 CESA isoforms and, upon arrival in plasma membrane, an ARM-repeat protein (CSI1) and a cellulose (KOR1)</th>
<th>(7,8,19,20), unpublished</th>
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<tbody>
<tr>
<td>Composition</td>
<td>Dimensions – membrane component: 25 nm in diameter; Dimensions – cytoplasmic component: 45-50 nm in diameter, 30-35 nm in height</td>
<td>[5]</td>
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<tr>
<td>Localization (GFP:CESA3)</td>
<td>Plasma membrane (PM), Golgi apparatus, trans-Golgi network, microtubule-associated cellulose synthase compartments (MASCs)</td>
<td>[28*,31]</td>
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<tr>
<td>Velocity in the PM (GFP:CESA3)</td>
<td>270–330 nm/min at 20 °C</td>
<td>[7]</td>
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<tr>
<td>Delivery rate to PM (GFP:CESA3)</td>
<td>4.8 ± 0.7 per μm² per h</td>
<td>[29**]</td>
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<td>Effect of microtubule depolymerization (oryzalin)</td>
<td>Disorganization of CSC trajectories in PM at low oryzalin concentrations, self-organization in parallel oblique trajectories at high concentrations.</td>
<td>[31]</td>
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<tr>
<td>Effect of microtubule stabilization (taxol)</td>
<td>Randomization of CSC insertion into PM</td>
<td>[28**]</td>
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<tr>
<td>Effect of actin depolymerization (LatB, CytD)</td>
<td>Stabilization of CSC trajectories in PM</td>
<td>[28**,29**]</td>
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<tr>
<td>Effect of isoxaben, thaxtomin or CGA cellulose synthesis inhibitors</td>
<td>Rapid internalization of CSCs from PM to MASCs</td>
<td>[28**,31,44]</td>
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<td>Effect of DCB cellulose synthesis inhibitor</td>
<td>Inhibition of CSC movement in PM</td>
<td>[27]</td>
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...fusion protein labeled motile punctae that colocalized with GFP:CESA3 in the plasma membrane. In contrast to GFP:CESA3 however, no labeling of intracellular compartments was observed. This suggests that the CSC interacts with CSI1 only upon arrival in the plasma membrane. CSI1 has 10 predicted armadillo (ARM) repeats and a C2 binding domain at its C-terminus. ARM repeats often participate in protein–protein interactions and C2 domains in Ca²⁺-dependent phospholipid binding. The authors speculate that CSI1 may play a role as an assembly platform for the CSC, in the targeting of the CSC to the plasma membrane or in its interaction with microtubules.

Sucrose synthase provides the major source of UDP-Glc (the substrate for cellulose synthesis) in sink cells and is a potential interactor of the CSC [12,21]. Fujii et al. (2010) isolated a detergent-soluble fraction with in vitro cellulose synthase activity from Azuki bean epicotyl plasma membranes. This fraction contained 10 nm particles enriched for sucrose synthase. The detergent-insoluble fraction was shown to contain 30–40 nm hexameric structures reminiscent of CSFs. This fraction did not show in vitro cellulose synthase activity. Upon mixing the two fractions, sucrose synthase (SUSY) became associated with the hexameric structures as shown by immunolocalization. The authors propose that the membrane-associated hexameric structure is the rosette onto which the 10 nm particles containing the cellulose synthase catalytic activity and sucrose synthase are docked. These results are intriguing, in particular in view of the presence of cellulose synthase activity in a cytosolic fraction, which suggests that the catalytic domain has been cleaved off from the membrane. Interestingly, Bessouille et al. (2009), observed a correlation between in vitro cellulose synthase activity and the presence of a 57 kDa processed version of CESA in detergent-resistant membranes from hybrid aspen cells [22*]. Thus, the 10 nm particles could contain the processed CESA catalytic domain. Before accepting this rather unexpected scenario, it is essential to demonstrate the presence of CESA fragments and not only SUSY in this active fraction.

The membrane-bound cellulase KORRIGAN1 (KOR1), which is essential for cellulose synthesis in both primary and secondary cell walls, is also a component of the CSC (unpublished). We have demonstrated the interactions between KOR1 and CESAs both by yeast two-hybrid and by bimolecular fluorescence complementation (BiFC). Interestingly, the transmembrane domain of KOR1 is required for these interactions, thus explaining why detergent-based purification techniques previously failed to identify KOR1 as a member of the CSC. Additionally, we observed GFP:KOR1 colocalizing and migrating with CESAs proteins in the plasma membrane. Overexpressed GFP:KOR1 does not localize to the plasma membrane [23], but GFP-KOR1 expressed with its endogenous promoter in a kor1 mutant background is present at the plasma membrane. This suggests that the localization of GFP:KOR1 is tightly controlled according to its expression level. It has been previously proposed that KOR1 plays a role in cellulose synthesis initiation through the recycling of a primer [24], by removing non-crystalline glucan chains, or by detaching the CSC from the ends of fully synthesized microfibrils. The finding that KOR1 is present in the CSC during cellulose synthesis will help distinguish between these different possible roles.

Intracellular trafficking of the cellulose synthase complex

The identification of the gene encoding cellulose synthases and their fusion to fluorescent proteins has paved the way for fascinating studies of the intracellular dynamics of CSFs [7,8,25–27,28*,29**,30]. YFP:CESA6-labeled and GFP:CESA3-labeled particles can be seen to glide through the plasma membrane in strikingly well-organized arrays [7,31]. Since GFP:CESA particles travel...
along cortical microtubules, and rapidly change direction as the cortical microtubule array reorients [31], cortical microtubules are thought to guide the movement of CSCs and thus determine the orientation of cellulose microfibrils deposited in the cell wall [32].

Fluorescently labeled CSCs are found not only in the plasma membrane, but also in intracellular organelles, giving us insight into the regulation of the delivery of CSCs to the site of cellulose synthesis (Figure 1). GFP:CESA3 is found at the periphery of Golgi cisternae, yielding a ring-shaped fluorescent labeling pattern [28**]. GFP:CESA3 also colocalized with a subpopulation of compartments labeled by VHA-a1, a marker for the trans-Golgi network (TGN). In plants the TGN is both a secretory and endosomal organelle, which is highly motile and can be physically independent from the Golgi bodies [28**,33,34*]. It is not clear whether the VHA-a1 compartment is involved in the secretion of CSCs, or in their internalization from the plasma membrane. Interestingly, these GFP:CESA3/VHA-a1 double-labeled compartments rapidly associate with and dissociate from Golgi bodies, suggesting that exchange of cargo may take place during these interactions [28**].

Finally, two independent groups describe a population of cortical microtubule-associated compartments labeled by GFP:CESA3 [28**,29**]. We termed this compartment the microtubule-associated cellulose synthase compartment (MASC), while Gutierrez et al. described a more diverse population of small CESA compartments (SmaCCs) that were initially free in the cytosol, and became microtubule-associated only after osmotic stress or treatment with the cellulose synthesis inhibitor iso-xaben [29**]. While MASCs are defined as having strictly linear trajectories [28**], SmaCCs can have either linear or curved trajectories [29**]. Gutierrez et al. report that SmaCCs partially colocalize with markers for the TGN, thus suggesting that SmaCCs represent a heterogeneous population comprising both MASCs and the VHA-a1/GFP-CESA3-containing compartments.

In our study, MASCs were never observed to colocalize with the VHA-a1 marker, and are thus distinct from the TGN/early endosome compartment [28**]. MASCs indeed appear to represent a unique endosomal compartment, whose movement is dependent on cortical microtubules. The role of MASCs in CESA trafficking is further discussed below.

**Plasma membrane insertion of the cellulose synthase complex**

The delivery of cellulose synthase complexes to the PM is regulated by both major cytoskeletal components in higher plants: actin filaments and cortical microtubules. It has been demonstrated that the actin cytoskeleton is required for the uniform distribution of CSC secretory organelles throughout the cortex during primary cell wall cellulose synthesis [28**,29**], and is similarly required for the transport of CSC-containing organelles to sites of secondary cell wall thickenings [30]. Cortical microtubules undeniably play the leading role in regulating the dynamics of cellulose synthase complexes, by targeting their secretion through interactions with the Golgi apparatus [28**], guiding their movement through the PM [31], and mediating the internalization of CSCs into MASCs [28**,29**]. There have been observations of de novo insertion of CSCs in the PM from both the Golgi apparatus and MASCs [28**,29**]. A vesicular intermediate with the same cortical microtubule-binding properties as a MASC may exist during the transfer of a cellulose synthase complex from the Golgi apparatus to the PM [28**]. Another possibility is that MASCs are recycling endosomes, capable of internalizing and re-inserting cellulose synthase complexes in the PM independent of the Golgi apparatus [29**].

**Internalization of the cellulose synthase complex from the plasma membrane**

The internalization of cellulose synthase complexes in response to environmental stress seems to provide a mechanism for rapidly shutting down cellulose synthesis in conditions that are unfavorable for growth. For example, CSC internalization is induced by osmotic stress or by treatment with cellulose synthesis inhibitors [28**,29**]. Exactly by what mechanism CSCs are internalized remains an open question. It is unlikely to be a clathrin-dependent mechanism, since the dimensions of the CSC would be predicted to prevent clathrin coat formation [28**]. However, some evidence suggests that dynamin, a small GTPase that catalyzes membrane fission, is required for internalization of components of the cellulose synthesis machinery. The rsw9 null allele of the gene encoding dynamin DRP1A is cellulose deficient and has incomplete cell walls [35]. This mutant is also deficient in the uptake of FM4-64, an endocytic tracer dye, consistent with the idea that the cellulose deficiency results from a perturbation of endocytosis. Likewise, a mutation in the dynamin OsDRP2B gene provokes a cellulose deficiency in culms, leaves and roots of rice seedlings [36]. Interestingly, the dynamins DRP2B and DRP1A participate in clathrin-dependent endocytosis in plants [37], suggesting that the internalization of at least some components of the cellulose synthesis machinery may involve clathrin.

**How do cortical microtubules guide the movement of the cellulose synthase complex?**

We now have convincing evidence that cortical microtubules define the trajectories of CSCs, thereby indirectly orienting cellulose deposition [31]. However, the mechanism by which cortical microtubules guide CSC movement remains as mysterious as ever. The tight regulation of CSC trajectories by cortical microtubules, and the presence of CSCs in microtubule-associated compartments, suggest that a component of the CSC may interact with a component of the cortical microtubule array. Interestingly, results of Zhong et al. [38] showed that a mutation in a kinesin-like protein affected the deposition of cellulose microfibrils in secondary cell walls in Arabidopsis. More recently, it has been shown that the bc12 mutation in Kinesin-4 results in an alteration of cellulose microfibril deposition in rice and a brittle phenotype [39]. Discovering how CSC-containing intracellular compartments interact with cortical microtubules during the secretion and internalization of CSCs may finally reveal the mechanism by which cortical microtubules guide the movement of CSCs in the PM.

**Conclusion**

In the past four years, we have made major steps forward in our understanding of cellulose synthesis. We now have in hand a more complete picture of the trafficking of CESA proteins, and their assembly into an active CSC. In addition, new actors in cellulose synthesis and new members of the CSC have been identified. It is now of the utmost importance to fully characterize the interactions between CESA proteins and partners such as KOR1 and CSI1, which are also essential for cellulose synthesis.
These pieces of the puzzle must be fitted into a developmental context, in order to successfully model the dynamics of cellulose synthesis and deposition during plant growth. Research in this area will enable the engineering of plants with modified cell wall properties for use in multiple applications.

Acknowledgements

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest


Using membrane-based yeast two-hybrid (MyYTH) and bimolecular fluorescence complementation (BiFC), the authors show that all secondary CESAs can interact with each other, and that only CES4A is able to form homodimers. A new model for the secondary cell wall rosette structure is proposed.


22. Bessueille L, Sindt N, Guichardant M, Djerbi S, Teeri TT, Bulone V: Plasma membrane microdomains from hybrid aspen cells are involved in cell wall polysaccharide biosynthesis. Biochim J 2009, 420:93-103. The authors report the first isolation of detergent-resistant plasma membrane microdomains (DRM) from a tree species. The DRMs exhibit similar properties as DRMs from other species and contain key carbohydrate synthases namely callose [(1→3)-(1→4)-beta-D-glucan] and cellulose synthase. More than 70% of the total glucan synthase activities present in the original plasma membranes are associated with the DRM fraction.


This study and reference [29] use live-cell imaging of fluorescently labelled CESA and tubulin proteins to demonstrate a role for microtubules, the Golgi apparatus, endosomal and novel microtubule-associated compartments in the regulation of plasma membrane delivery and internalization of CSCs.


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An elegant study using electron microscopy and confocal laser scanning microscopy imaging of a brassinosteroid receptor and a boron exporter showing that both endocytic and secretory cargo transit through the trans-Golgi network.


