CHAPTER 10

A PERSPECTIVE ON THE ASSEMBLY OF CELLULOSE-SYNTHESIZING COMPLEXES: POSSIBLE ROLE OF KORRIGAN AND MICROTUBULES IN CELLULOSE SYNTHESIS IN PLANTS

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Abstract

Cellulose is synthesized on the plasma membrane by protein complexes referred to as terminal complexes (TCs). In plants, the TCs are visualized by freeze-fracture electron microscopy as rosettes with a sixfold symmetry. Each rosette synthesizes a cellulose microfibril containing approximately 36 glucan chains. So far, only the cellulose synthase catalytic subunit (CesA) is shown to be localized to the rosette complex, and it is suggested that at least 36 CesA molecules are present in each rosette. Moreover, from analysis of the *CesA* genes, it is predicted that at least three different CesAs are required for assembly of the rosette and the cellulose microfibril. How the different CesA subunits assemble into a rosette structure is not clearly understood. In our view, the assembly of the rosette proceeds in stages, beginning from the rough endoplasmic reticulum (ER) to the plasma membrane, with the final assembly of the rosette structure taking place on the plasma membrane. The membrane-localized endo-1,4-β-D-glucanase, KORRIGAN is probably involved in digesting the noncrystalline cellulose product formed from an assembly of six CesA subunits that compose a rosette particle and is transported to the plasma membrane via vesicles. These rosette particles then assemble into a complete rosette TC in the plasma membrane when the glucan chains synthesized from closely placed particles associate to form crystalline cellulose I microfibrils. The role of microtubules in aligning cellulose microfibrils has been widely debated, and we believe that microtubules probably are involved in aligning the cellulose microfibrils in an indirect manner by "channelizing" the direction of microfibril assembly.

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1 INTRODUCTION

Cellulose may not be a universal biomacromolecule, yet the capability to synthesize cellulose is a much more universal property than previously understood. The realization that the capability to synthesize cellulose may be more widespread has occurred essentially from sequencing of genomes of a large number of organisms, mostly microorganisms. From these studies, genes encoding the cellulose synthase and a few other proteins have been identified in many bacterial species (Römling 2002). Although the genes for cellulose synthesis are present in these organisms, it is not known if all these organisms do in fact synthesize cellulose. Cellulose synthesis has been known for some time in bacterial species such as *Acetobacter xylinum* and *Agrobacterium tumefaciens* (Ross et al. 1991), but recently it has been demonstrated in bacteria such as *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas fluorescens* and others, and in many of these cases the cellulose is found associated with biofilms (Zogaj et al. 2001; Spiers and Rainey 2005). Unlike bacterial cells, cellulose produced by plant cells is a structural component of the cell wall and the direction of cellulose synthesis helps determine cell growth and elongation.

In general, organisms that synthesize cellulose microfibrils do so from organized cellulose-synthesizing sites on the membrane that are often referred to as terminal complexes (TCs). The crystalline nature of cellulose implies an ordered arrangement of glucan chains in cellulose microfibrils and hence the suggestion that the cellulose-synthesizing sites in the cell are organized such that the glucan chains are able to interact with each other and form a crystalline structure while they are being synthesized (coupled polymerization-crystallization). The first organized site of cellulose synthesis was observed as a linear arrangement of particles in three rows in the green alga *Oocystis apiculata* (Brown, Jr. and Montezinos 1976). Two major arrangements of cellulose-synthesizing sites (linear and rosette) have since been identified in most cellulose-synthesizing organisms, and the cellulose synthase has been localized to the rosette complex in plants (Kimura et al. 1999). While it is obvious that an ordered array of cellulose synthases is required for synthesis of cellulose microfibrils, it is not very clear as to how these enzyme molecules are organized in TCs in the different cellulose producing organisms. Considering what is known about the role of different genes and proteins during cellulose synthesis, we wish to present a renewed perspective on the assembly of TCs in cellulose-synthesizing organisms, and more specifically the assembly of the rosette TC and the role that KORRIGAN (KOR) and the microtubules may have in influencing this and cellulose microfibril assembly in plants.

2 STRUCTURE AND COMPOSITION OF CELLULOSE-SYNTHESIZING COMPLEXES

Cellulose-synthesizing TCs were first observed by freeze-fracture electron microscopy. In general, they are visualized as intramembranous particles that are organized either as a linear row or as a rosette-like structure on the P-fracture face of the plasma membrane (Brown, Jr. 1996). Lately it has also been possible to visualize the cytosolic side of the rosette TCs in membrane sheets prepared from plants (http://www.botany.utexas.edu/facstaff/facpages/mbrown/bowling/default2.html). While bacteria, such as *A. xylinum,* have a single row of particles organized as a linear TC and all land plants have a hexameric rosette TC, great diversity in TC architecture is observed in the algae (Tsekos 1999). Both linear and rosette TCs are found in the algae, with the linear TCs and rosettes in turn being organized in rows in certain algae.

In spite of the fact that TCs are relatively large membrane-embedded structures, biochemical approaches to isolate and identify the protein composition of the TCs has been challenging. However, a protein aggregate suggestive of the TC has been found attached to cellulose microfibrils synthesized *in vitro* using membrane extracts from plants (Lai-Kee-Him J 2002; Laosinchai 2002) and cellulose synthase was localized to this protein aggregate (Laosinchai 2002).

Much of our understanding of the TCs has, in fact, been obtained from genetic and microscopic analyses. Interestingly, genetic analysis of cellulose-deficient mutants in plants led to the proposal that the rosette TC contains three different nonredundant cellulose synthases, and mutation in any one of these results in a defect in the assembly of cellulose microfibrils (Taylor et al. 2003). In addition, the cellulose synthases identified for cellulose synthesis in the primary cell wall are different from the cellulose synthases required for cellulose synthesis in the secondary cell wall (Robert et al. 2004). In spite of the failure to localize any other protein except the cellulose synthase to the rosette TC, other proteins have been predicted by mutant analysis to be associated with the complex and these may have a direct or indirect role in cellulose synthesis.

One of the intriguing proteins that is suggested to have a role in cellulose synthesis is KOR, a membrane-bound endo-1,4-β-D-glucanase. Mutations in the *KOR* gene lead to an altered phenotype and a reduction in the amount of cellulose (Nicol et al. 1998). Based on the features of the mutants, the KOR protein has been suggested to be involved in cellulose biosynthesis (Mølhøj et al. 2002). However, the mechanism of KOR function during cellulose synthesis is not clearly understood. Recent experiments using GFP-labeled KOR demonstrate that this protein is present in intracellular compartments and probably undergoes cycling between these compartments and the plasma membrane (Robert et al. 2005). In a later section, we will suggest a possible role of KOR in cellulose biosynthesis.

The intramembrane particles in a linear TC or in a rosette have been observed quite well by freeze-fracture, but not much is known with respect to the cytoplasmic face of these particles. From sequence analysis of cellulose synthases, a

large globular region of this protein is predicted to be present in the cytosol and as such the TC is predicted to be much larger on the cytosolic side than what is observed on the cell surface following freeze-fracture (Saxena and Brown, Jr. 2005). Since the globular region contains the putative active site of the cellulose synthase and possibly other site(s) for protein–protein interaction, knowledge of the cytosolic region of TCs is crucial for understanding not only the mechanism of cellulose synthesis but also as to how the TC is assembled and regulated. In the absence of isolated TCs, electron microscopic observations provide some very interesting clues to the nature of the cytosolic side of these complexes. Early evidence with respect to the dimensions of the cytosolic region of TCs was obtained from thin sections of the linear TCs in the alga *Boergesenia forbesii* (Kudlicka et al. 1987). Recently, using membrane fragments it was possible to visualize the cytoplasmic face of the rosette complex in plants and the dimensions observed in these studies suggest that a much larger region of the complex extends into the cytoplasm (Bowling 2005). In addition, cortical microtubules and clathrin-coated vesicles are clearly observed on the cytosolic side of the membrane fragments (Bowling 2005). Even though no other proteins have been identified in association with the cytosolic region of the TCs, it is possible that this region may interact with a variety of proteins, including the cytoskeleton, either directly or indirectly through other proteins.

3 STAGES IN THE ASSEMBLY OF THE ROSETTE TERMINAL COMPLEX IN PLANTS

It is assumed that each rosette TC in plants contains 36 cellulose synthase molecules, each of which is presumably involved in the synthesis of a single glucan chain. Each rosette is composed of six particles and each particle is therefore considered to be an assemblage of six cellulose synthase molecules. The current view holds that the rosette TCs in plants are assembled in the Golgi apparatus, where they exist in an inactive state (Haigler and Brown, Jr. 1986). The rosette TCs are subsequently transported via cytoplasmic vesicles from the Golgi apparatus to the plasma membrane where they are activated for cellulose synthesis. While rosette TCs also have been observed in vesicles in the alga *Micrasterias denticulata* (Giddings et al. 1980), a linear row of particles representing TC precursors have been observed in large, dense cytoplasmic vesicles, quite different from the Golgi vesicles, in the alga *Botrydiopsis intercedens* (Okuda et al. 2004).

The observation of TCs in vesicles suggests that the TCs are assembled prior to their insertion in the plasma membrane and are transported from the Golgi apparatus via an exocytic pathway. However, at this point it is not completely certain if the vesicles containing the TCs are part of an exocytic or an endocytic pathway. Clathrin-coated endocytic vesicles are known to form at the plasma membrane, and in certain cases recycling of components have been reported to occur from the plasma membrane to the Golgi apparatus (Neumann et al. 2003). Moreover, it is not very clear as to how the cellulose synthases stay inactive until they are present in

the plasma membrane, although suggestions have been made that the activation of cellulose synthases is regulated by their phosphorylation state (Somerville 2006).

In our view, being membrane proteins, the cellulose synthase polypeptide chains are synthesized on the rough endoplasmic reticulum (ER), where they undergo folding and probably assemble into a higher order structure. Although assembly of the cellulose synthases into a rosette can proceed completely in the ER or in the Golgi apparatus or the plasma membrane or can take place in stages, it is more likely that at least some assembly occurs in the ER. The ER ensures proper folding and assembly of proteins through a rather strict quality control mechanism in which unfolded or misassembled proteins are transported to the cytosol where they are targeted for destruction in the proteasome (Lord et al. 2000). ER-associated protein degradation (ERAD) is well documented in yeast and mammalian cells, and is now shown in plants as well (Di Cola et al. 2005; Müller et al. 2005). That the cellulose synthases or some component(s) of the cellulose-synthesizing machinery undergoes modification in the ER is evident from analysis of cellulose-deficient mutants such as *cyt1*, *knf* and *rsw3* that have defects in genes encoding mannose-1-phosphate guanylyltransferase (Lukowitz et al. 2001), α-glucosidase I (Gillmor et al. 2002) and glucosidase II (Burn et al. 2002) respectively. All these enzymes are required for the processing of *N*-linked glycans on ER-synthesized proteins, and this processing is essential for the proper folding and assembly of these proteins.

If a significant role is assigned to the quality control mechanism for proper folding and assembly of cellulose synthases in the ER, and some role is assigned to the influence of glucan chain crystallization on the rosette structure, then the assembly of the fully functional rosette TC can be visualized to take place in two stages. In the first stage, two copies each of three different cellulose synthases assemble to form a complex (rosette particle) containing six cellulose synthase molecules in the ER. The cellulose synthases in a single rosette particle assemble by protein–protein interaction using either the RING finger motif or another motif present in the cellulose synthases (Doblin et al. 2002). Requirement for three different cellulose synthases is based upon genetic and biochemical analyses that suggest interaction of specific cellulose synthases in the ER before they are transported (Taylor et al. 2003). The complex, assembled in the ER is then transported to the Golgi apparatus either by COPII vesicles or by direct ER-Golgi connections, and from the Golgi to the plasma membrane by Golgi-derived vesicles (Neumann et al. 2003). In any case, the rosette particle composed of six cellulose synthase molecules does not assemble into a higher-order structure (the rosette) in the intracellular compartments in the absence of crystalline cellulose formation. The single rosette particles cannot be differentiated from other intramembranous particles, and with freeze fracture, only the fully assembled rosette TC with six particles can be definitively shown to be associated with cellulose microfibrils (Mueller and Brown, Jr. 1980). The topology of the assembled complex of cellulose synthases in the ER, Golgi and the vesicles is such that the globular region containing their active site faces the cytosol. In the presence of UDP-glucose in the cytosol, cellulose synthase

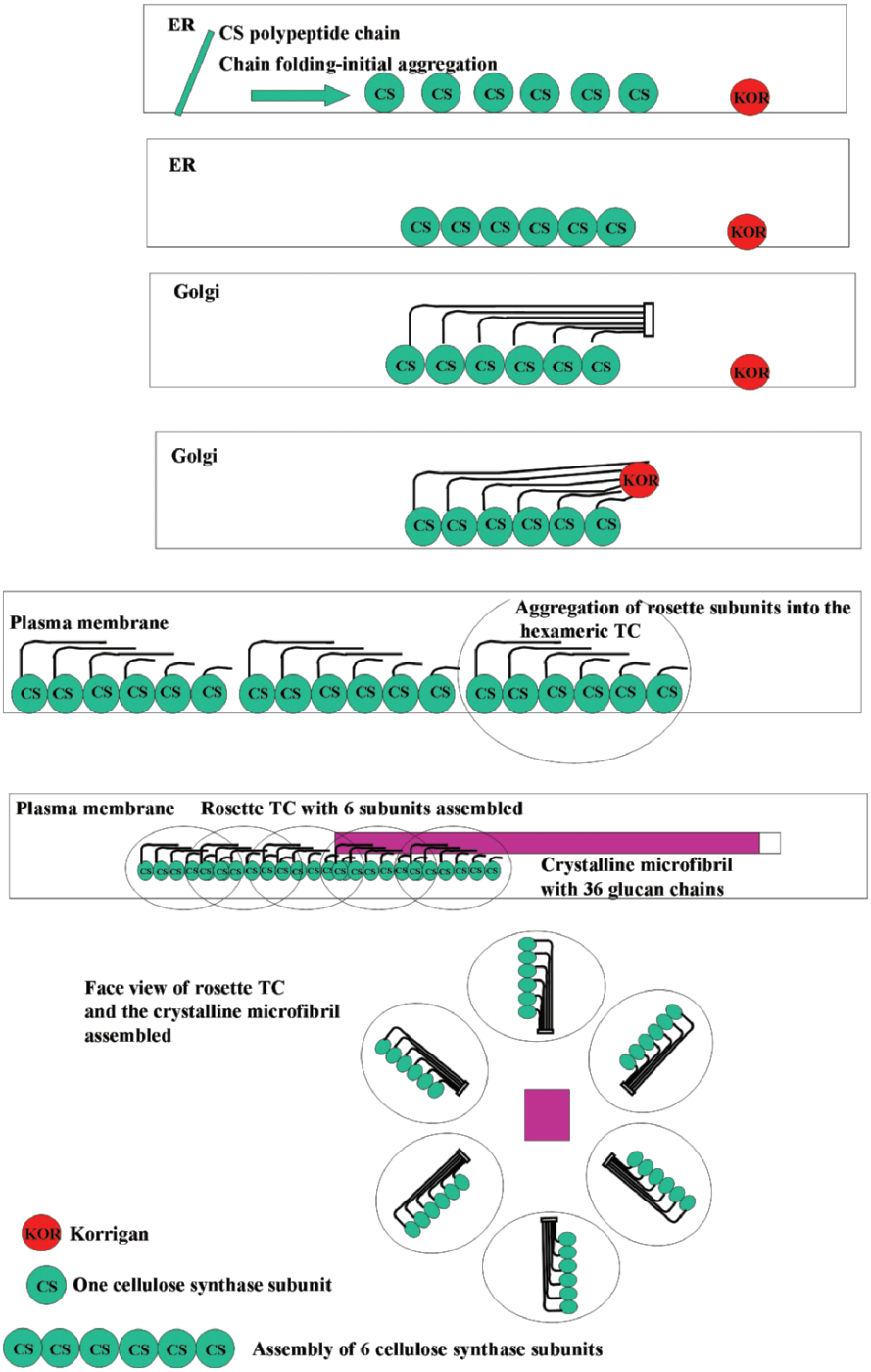
molecules may be able to synthesize and secrete glucan chains into the lumen of these intracellular compartments, or they may stay in an inactive state. If glucan chain synthesis is initiated in the ER, the individual cellulose synthases would still be able to assemble into a single rosette particle, but not into the complete rosette TC. In this case, the glucan chains attached to cellulose synthases would only form glucan chain aggregates with six glucan chains and this would not be a crystalline product. Alternatively, if no glucan chains are synthesized in the intracellular compartments, no cellulose will be formed and as a result the rosette particles would not assemble into a rosette TC. Interestingly, intracellular synthesis of cellulose has been observed during scale formation in the Golgi apparatus in *Pleurochrysis* where crystalline cellulose forms a complex with other components in a spatial and temporal manner (Brown, Jr. and Romanovicz 1976; Romanovicz and Brown, Jr. 1976). Although scales are not found in plants, limited synthesis of noncrystalline cellulose may certainly take place in intracellular compartments in plants. In any case, the Golgi-derived vesicles containing the rosette particles fuse with the plasma membrane and the rosette particles can now assemble into the rosette TC in the plasma membrane. In the plasma membrane, the rosette particles may still be attached to the glucan chains, if synthesis occurred in the intracellular compartments. Alternatively, synthesis may initiate in the plasma membrane if the cellulose synthases are activated in the plasma membrane.

See Figure 10-1 for an illustration of two-step assembly of the rosette TC.

4 POSSIBLE ROLE OF KORRIGAN IN THE DIGESTION OF GLUCAN CHAINS AND IN THE SECOND STAGE OF THE ASSEMBLY OF THE TERMINAL COMPLEX

Most workers in the field agree that KOR probably has an indirect role in cellulose biosynthesis *in vivo*, but so far it has not been possible to assign a specific role to this protein in this process. In simple terms, it is believed that KOR hydrolyzes

Figure 10-1. Two-step assembly of the rosette terminal complex (TC) in plants. In the first stage, a cellulose synthase particle complex containing six cellulose synthase molecules is assembled in the ER by protein–protein interactions. Single rosette particles synthesize glucan chains that will stay attached as noncrystalline cellulose in the ER and other intracellular compartments. KOR is also synthesized in the ER and it may be transported from the Golgi apparatus in vesicles with or without the rosette particles. The vesicles carrying the rosette particles and/or KOR may be directed to sites of cellulose synthesis by the microtubules. Once the vesicles carrying the cellulose synthases and KOR fuse with the plasma membrane and the rosette particles are present in the plasma membrane, KOR is able to digest the noncrystalline cellulose. In the second stage, the assembly of the individual rosette particles into a hexameric rosette TC structure is favored by the assembly of glucan chains into a crystalline cellulose product. KOR can also digest the noncrystalline cellulose while in the Golgi apparatus, and under these circumstances assembly of the rosette can take place in the Golgi apparatus itself. KOR does not digest crystalline cellulose. The assembled rosette TC continues to synthesize cellulose microfibril and the movement of the cellulose synthase in the plasma membrane is governed by the rate of cellulose synthesis. The rosette particles, KOR, and in some cases even the rosette TC may be recycled from the plasma membrane (*See Color Plate of this figure beginning on page 355*)


ER
CS polypeptide chain
Chain folding-initial aggregation
CS
KOR
ER
Golgi
Golgi
Plasma membrane
Aggregation of rosette subunits into the hexameric TC
Plasma membrane
Rosette TC with 6 subunits assembled
Crystalline microfibril with 36 glucan chains
Face view of rosette TC and the crystalline microfibril assembled
Korrigan
One cellulose synthase subunit
Assembly of 6 cellulose synthase subunits

glucan chains in noncrystalline cellulose, and may possibly relieve stress generated during assembly of glucan chains in cellulose microfibrils (Mølhøj et al. 2002). Enzymatic analysis of a recombinantly-obtained soluble form of a KOR homolog from *Brassica napus* showed that this protein has substrate specificity for low substituted carboxymethyl cellulose and amorphous cellulose, but does not hydrolyze crystalline cellulose or the oligosaccharides cellotriose, cellotetraose and cellopentaose (Mølhøj et al. 2001). Similar properties also have been observed in a soluble form of the KOR homolog obtained from *Populus* (Master et al. 2004).

We believe that the KOR protein present in the plasma membrane is indirectly involved in the assembly of the rosette TC in plants by digesting the noncrystalline cellulose product attached to the rosette particles. It is known that polymerization and crystallization are coupled steps, and that crystallization influences the rate of polymerization during cellulose synthesis (Benziman et al. 1980). In the absence of crystallization, the polymerization reaction probably lasts only for a short period of time even though the enzyme may be fully active. Removal of the noncrystalline product attached to the cellulose synthase allows polymerization to continue, but in a more directed and controlled sense. Here we have a type of "editing mechanism" whereby the KOR protein removes disordered glucan chains that are not yet crystallized but that were critical in keeping the rosette particles in close proximity for final assembly which can take place once a fully crystalline ordered cellulose I microfibril begins to emerge from the TC. Once the noncrystalline cellulose is removed from the rosette particles, and where sufficient numbers of rosette particles are present to simultaneously produce the glucan chain aggregates, the glucan chain aggregates (we suggest a glucan chain aggregate consisting of six glucan chains bound by hydrophobic interactions) (Cousins and Brown, Jr. 1995) from each particle are able to associate with glucan chain aggregates from the other particles. This now becomes the second stage of crystallization, namely a hydrogen bonding interaction between the glucan chain aggregates to result in the crystallization of a cellulose I microfibril (Cousins and Brown, Jr. 1997a, b) and consequently, the polymerization-crystallization-induced assembly of a complete rosette TC. Therefore the cellulose–cellulose glucan chain association could ultimately lead to the final assembly of the hexameric rosette TC.

Obviously, crystallization of glucan chains is not the only mechanism required for assembly of the rosette TC. Most likely, certain features of the cellulose synthase protein influences either the assembly or the stability of the rosette TC, as is very clearly demonstrated in the disassembly of the rosette TCs in the *rsw1* mutant of *Arabidopsis* at restrictive temperature (Arioli et al. 1998). An even more interesting observation is the increase in the amount of noncrystalline cellulose, probably produced by the rosette particles in this mutant at the restrictive temperature (Arioli et al. 1998). In wild-type cells, a significant amount of the noncrystalline cellulose is digested by KOR on the plasma membrane. Recycling of the KOR from the plasma membrane occurs much more frequently and although most of the KOR is shown by microscopy to be localized in intracellular vesicles (Robert et al. 2005), its presence has been shown in isolated membrane fractions

as well (Nicol et al. 1998). It is expected that in the absence of KOR activity, many more particles with attached glucan chains will be present in the plasma membrane, and this will result in increase in the production of noncrystalline cellulose. Although a defect in KOR would affect the assembly of the particles into the rosette structure, reduced assembly of rosette TCs and cellulose microfibrils would proceed as long as some rosette particles are brought sufficiently close together before the synthesis of the glucan chains takes place. To a lesser extent, other proteins with an endoglucanase activity may be able to remove the noncrystalline cellulose and allow assembly of the rosette TC. Interestingly, *Arabidopsis* root-swelling mutants defective in KOR (*rsw2*) are cellulose-deficient but produce increased amounts of noncrystalline cellulose in comparison to wild-type cells (Lane et al. 2001), suggesting a role for KOR in the assembly of cellulose microfibrils possibly via assembly of the rosette TC.

5 ROLE OF MICROTUBULES IN CELLULOSE BIOSYNTHESIS

Understanding the relationship between cellulose microfibrils and cortical microtubules in plant cells has been an area of great interest and debate (Baskin 2001; Wasteneys 2004). While almost everyone agrees that the anisotropic growth of plant cells is dependent on the synthesis of cellulose microfibrils in a direction perpendicular to the elongating axis, what is not clear is the mechanism that determines the direction of cellulose biosynthesis or in other words, the ordering of the cellulose-synthesizing complexes on the plasma membrane. Evidence supporting the view that microtubules influence the direction of cellulose synthesis has come mostly from studies using inhibitors of microtubule formation. Although no direct interaction has been observed between microtubules and the cellulose-synthesizing complexes, two different versions of how microtubules may guide the cellulose-synthesizing complexes in the plasma membrane are prevalent in the literature (Baskin 2001). In one case, it is believed that during cellulose synthesis, the cellulose-synthesizing complexes move in the plasma membrane within tracks created by cortical microtubules associating with the plasma membrane. The result is that the cellulose microfibrils co-align with the underlying microtubules. In this case, the interaction between the microtubules and the cellulose-synthesizing complexes is indirect. Alternatively, the cellulose-synthesizing complexes are thought to be attached to the microtubules directly or indirectly through other protein(s) and the movement of the cellulose-synthesizing complexes during cellulose synthesis is guided by these microtubules (Paredez et al. 2006). However, in this case, the rate of movement of the rosette TCs in plants would be governed by the rate of synthesis of the cellulose microfibrils, and this movement would be affected if the TCs are attached to the microtubules. In fact, we know that the rate-limiting step in cellulose polymerization is directly controlled by the crystallization process (Benziman et al. 1980). In this work, it was demonstrated that the rate of polymerization increases up to four times the control rate when crystallization is inhibited by Calcofluor. From such observations, it is obvious

that the rate-limiting step in cellulose biosynthesis is the crystallization step. If the rosette TCs were directly associated with microtubules, the rate of cellulose synthesis would be greatly reduced or even terminated; however, this does not appear to be the case. Thus, it appears highly unlikely that any direct association of rosette TCs takes place with microtubules but rather, these act indirectly by "channelizing" the direction of microfibril assembly. One interesting indirect consequence of such "channelization" would be that aggregates of rosette TCs, each with their parallel cellulose microfibrils, could indirectly control the overall rate of cellulose synthesis. If clusters of cellulose microfibrils held together by hydrogen bonds occurs, then the overall synthesis of cellulose in this case would be rate-limiting.

While these two models suggest that it is the microtubules that guide the cellulose-synthesizing complexes and determine the orientation of the cellulose microfibrils in the plant cell wall, other models suggest that the cellulose microfibrils that are attached to the cellulose synthases determine the direction of movement of the cellulose-synthesizing complexes in the plasma membrane and as such the microtubules have no direct role in determining the direction of cellulose microfibril synthesis (Emons and Mulder 1998). This model is based on geometrical constraints and it states that the microfibrils are "deposited along paths determined by the geometry of the cell alone" and a function for microtubules is not clearly understood. In our view, favoring the indirect model that cellulose microfibrils do align with the microtubules, it is more likely that the microtubules have additional indirect functions in cellulose biosynthesis, especially in exocytosis by directing transport of vesicles containing the cellulose synthases and the KOR proteins to sites of cellulose synthesis in the plasma membrane (Robert et al. 2005).

6 SUMMARY

Since the discovery of TCs as cellulose-synthesizing sites almost three decades ago, we are now beginning to get a better understanding of their composition and how they may be assembled. The localization of the cellulose synthase (Kimura et al. 1999) and the analyses suggesting the presence of three nonredundant cellulose synthases (Taylor et al. 2003) in the rosette TC in plants were major milestones, but it is only now that we have begun to get a look at the cytosolic side of these large multimeric complexes (Bowling 2005). From the dimensions of the cytosolic region of the rosette TCs, it is apparent that they may not be attached to any cytoskeletal structure. In fact, the rosettes extend so deeply into the cytoplasm that they would actually displace the parallel array of cortical microtubules. That plasma membrane recycling occurs much more frequently is validated by the occurrence of a large number of clathrin-coated pits and vesicles observed in membrane sheets (Bowling 2005). Although more information is beginning to be obtained about the cellular localization and dynamic properties of cellulose synthase complexes (Paredez et al. 2006), it is not entirely clear as to how these complexes are assembled. We believe that the glucan chains attached to the cellulose synthases play a

major role in the final assembly of these complexes and propose that the initiation of cellulose synthesis via glucan chain polymerization and limited aggregation could lead to the hierarchical assembly of the sixfold complete rosette TC in plants. KOR may be involved in an editing function by removal of noncrystalline cellulose attached to single rosette particles and allowing the particles to assemble into a rosette when crystalline cellulose is formed.

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