CHAPTER 14

IMMUNOGOLD LABELING OF CELLULOSE-SYNTHESIZING TERMINAL COMPLEXES

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Abstract

Rosette TCs that are visualized exclusively by freeze-fracture electron microscopy have long been thought of as "putative" cellulose-synthesizing terminal complexes (TCs). We succeeded in directly demonstrating that the TCs contain cellulose synthases by the application of novel techniques combined with both freeze-fracture and immunogold labeling. Since the purification of cellulose synthases has not succeeded in higher plant cells, we cloned *GhCesA* using cotton cDNA libraries and prepared polyclonal antibodies against the GhCesA protein. We were successful in applying SDS-FRL techniques to plant cells for the first time by using mixtures of cellulases and pectolyases to digest cell walls after freeze replication and immunogold labeling. Using SDS-FRL, the antibodies of cellulose synthases specifically labeled the rosette TCs in the plasma membrane of higher plant cells. This provided direct evidence that the rosettes contain the catalytic subunit of the cellulose synthase. In this chapter, we have analyzed the mechanism of labeling compared with actual dimensions of rosettes, gold particles, and antibodies.

Keywords

cellulose biosynthesis, fracture labeling, Gh*CesA*, rosette, terminal complexes, *Vigna angularis*.

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1   INTRODUCTION

It is well known that the rosette and linear terminal complexes (TCs) can be observed by the freeze-fracture replication technique. The structures revealed by this technique are known as "putative" cellulose-synthesizing TCs. Kimura et al. (1999) demonstrated that TCs in vascular plants contain cellulose synthases using a novel technique of sodium dodecyl sulfate (SDS)-solubilized freeze frac- ture replica labeling (SDS-FRL). The localization of the cellulose synthase to the TC was accomplished almost 40 years after the hypothesis of Roelofsen (1958) in which he stated that enzyme complexes could be involved in cellulose bio- synthesis. It has been more than 30 years since the discovery of the first TC by Brown, Jr. and Montezinos (1976) and in particular, 26 years after the discovery of rosette TCs in plants by Mueller and Brown, Jr. (1980).

Until recently the only way to visualize linear and rosette TCs depended on the application of the freeze-fracture replication technique. We have now succeeded in visualizing the components of TCs by applying freeze replica labeling tech- niques coupled with the solubilization of plant materials with SDS. In contrast, it has not been possible yet to prepare and purify cellulose synthases from plant cells. Success with the SDS-FRL technique in localizing cellulose synthases to the rosette TCs in plants was based on the ability to clone and express a region of the cotton cellulose synthase (GhCesA) and to obtain polyclonal antibodies against this region of the cellulose synthase. These antibodies allowed specific labeling of the cellulose synthases in the rosette TCs on the plasma membrane of Azuki beans (*Vigna angularis*) (Kimura et al. 1999). Similarly, polyclonal antibodies raised against a 93-kd protein, obtained during purification of cel- lulose synthase activity from the bacterium *Acetobacter xylinum,* was found to be useful in labeling the linear TCs in this bacterium by the SDS-FRL technique (Kimura et al. 2001). Based on these experiments we now have direct proof that the rosette TCs contain cellulose synthase subunits that catalyze cellulose bio- synthesis, and linear TCs in *A. xylinum* contain subunits of cyclic-di-guanylic acid (c-di-GMP) binding proteins that activate cellulose biosynthesis.

In the present article we will discuss the difficulties associated with under- standing the composition of the TCs and the application of the SDS-FRL technique in unraveling the contents of the TCs.

2   THE CELLULOSE-SYNTHESIZING MACHINERY (TERMINAL COMPLEXES)

Roelofsen (1958) first suggested that native cellulose might be assembled, polym- erized and crystallized by the action of a large enzyme complex located at the growing tip of the microfibril. Interestingly, Dobberstein and Kiermayer (1972) visualized ordered particle complexes within "f-vesicles" of the Golgi apparatus in the green alga *Micrasterias denticulata,* and these particles were implicated in the biosynthesis of cellulose. This work is significant historically, because what

was later to be beautifully imaged by freeze fracture was first observed in sectioned material. Preston (1964) presented the ordered granule hypothesis based on the his observations of freeze-dried replicas of the innermost cell wall layer after plasmolysis in the marine algae *Valonia* and *Chaetomorpha*. Three-layered and rectangular enzyme particle-complexes were thought to be involved in cellulose biosynthesis, and according to this model, cellulose microfibrils were thought to be synthesized in three different directions. It is well known now that microfibrils are synthesized unidirectionally with parallel or antiparallel directions (Brown, Jr. 1978; Itoh and Brown, Jr. 1984).

More than 10 years after the presentation of Preston's hypothesis, Brown, Jr. and Montezinos (1976) first discovered a plasma membrane particle complex associated with the ends of cellulose microfibrils in the alga, *Oocystis apiculata*. This complex was a linear multimeric structure, termed as a linear terminal complex (TC), and it consisted of three rows of subunit particles. The complex was found to be intimately associated with microfibrils, as clearly evidenced by impressions of microfibrils leading from and associated with the complex. In the same year, Brown, Jr. et al. (1976) also observed a single row of particles in the outer membrane of *A. xylinum* and showed that this type of particle row is the cellulose-synthesizing machinery that is involved in cellulose biosynthesis. In 1980, Mueller and Brown, Jr. found a different arrangement of particles, a cluster or rosette of six particles, associated with the terminus of cellulose microfibril impressions on the P-fracture face of the plasma membrane in root tip cells of maize that were actively involved in cellulose synthesis. In the same year, Giddings et al. (1980) found octagonal arrays of rosettes in the plasma membrane of *M. denticulata*, and these arrays are involved in the synthesis of banded cellulose microfibrils in this alga. Since then, numerous studies have implicated rosette TCs (frequently called rosettes) in cellulose microfibril assembly (Emons 1991, 1994; Herth 1984, 1985a, b; 1987, 1989; Herth and Weber 1984; Hotchkiss and Brown, Jr. 1987, 1988; Hotchkiss et al. 1989; Itoh 1990; Itoh and Brown, Jr. 1984; Mizuta et al. 1989; Mueller and Brown, Jr. 1982; Rudolph et al. 1989; Tsekos 1999; Tsekos and Reiss 1992; Okuda et al. 1994; Brown, Jr. 1996). As shown in Figure 14-1, different types of TCs have been identified in cellulose-producing organisms; however, TCs are categorized into two types, linear and rosette. Almost all species of land plants that include vascular plants, Pterophyta and Bryophyta have solitary rosette TCs. On the contrary, a variety of linear and rosette types of TCs are found in Protista. Different arrangements of linear rows of subunit particles form linear TCs and different groupings of rosette TCs from single to more than ten rosettes organized in regular arrays are observed in this group of organisms (Tsekos 1999; Grimson et al. 1996).

A. xylinum is the only organism in Monera that shows TCs with a single row of particles. To date, genes for cellulose synthesis have been identified in many bacterial species including *A. xylinum*, *Agrobacterium tumefaciens*, *Rhizobium* spp. (Ross et al. 1991); *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhimurium* (Zogaj et al. 2001); and cyanobacteria (Nobles et al. 2001). In particular, *Acetobacter*
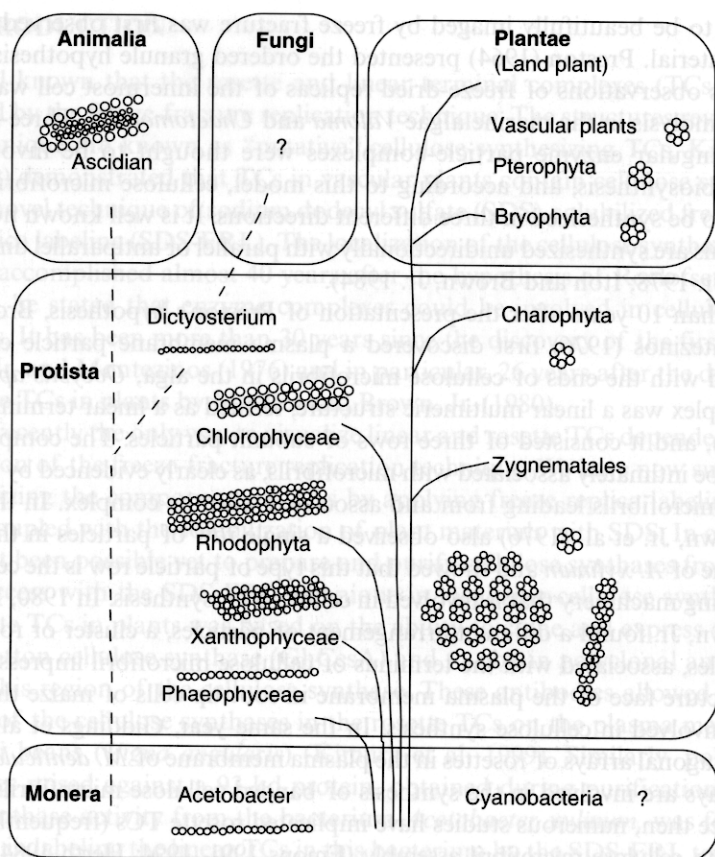
Figure 14-1. Different morphological forms of cellulose-synthesizing terminal complexes in five different biological kingdoms. (Figure 1 from: Itoh, T. 2002. Immunogold labeling of terminal cellulose-synthesizing complexes-demonstration coupled by freeze fracture and immunogold labeling techniques. Regulation of Plant Growth and Development (in Japanese) 37:44–50. Reproduced with kind permission of The Japanese Society for Chemical Regulation of Plants.)

and *Agrobacterium* have been used as model organisms for studying cellulose biosynthesis from a molecular biological and biochemical perspective. However, in spite of great progress in understanding cellulose biosynthesis in *Agrobacterium*, the TCs in this bacterium have not been well characterized. The inability to visualize TCs in *Agrobacterium* may be due to the difference between the actual fractured membrane and the location of TCs particles. The occurrence of cellulosic microfibrils has also been reported in cyanobacteria, but the presence of TCs is not confirmed in this group of organisms. Cyanobacteria would be a key group to understand the structure and function of TCs in relation to evolution because the origin of green plants is traced back to these organisms.

Furthermore, we have found specific types of linear TCs in ascidians that are members of the animal kingdom and that are phylogenetically apart from plants (Kimura and Itoh 1996). In short, almost all cellulosic organisms have shown the presence of TCs. However, TCs have not been observed in any kind of cellulosic fungi. It has long been thought that Oomycetes are a cellulose-synthesizing fungus; however, recent advances in molecular phylogeny show that Oomycetes cluster with the stramenophiles group in protista together with Heterokonta, based on the morphology of zoospore and analysis of the 18S rRNA sequence (Peer and Wacher 1997; Saunders 1997). This means that fungi are the only group of organisms that do not show the occurrence of TCs as well as cellulose biosynthesis.

Before 1999 only indirect evidence was available for the involvement of TCs in cellulose biosynthesis. Firstly, TCs could be observed at the terminus of cellulose microfibril impressions. Secondly, the linear TCs in *Oocystis apiculata* could be observed as a pair in different developmental stages of TC growth, suggesting that individual TCs in a pair may move in opposite directions by producing cellulose microfibrils (Brown, Jr. 1978). Thirdly, the length of linear TCs was shown to increase during the transition from primary wall to secondary wall formation in *Valonia* and *Boergesenia* (Itoh and Brown, Jr. 1988). There was no direct evidence to prove that cellulose synthases are localized in TCs on the plasma membrane until 1999 when Kimura et al. demonstrated that rosette TCs are labeled by CesA antibodies using freeze replica labeling techniques.

3 ADVANCES IN THE UNDERSTANDING OF CELLULOSE SYNTHASES

Research on cellulose biosynthesis has greatly advanced with the use of the bacterium *A. xylinum* as an experimental material. This bacterium produces a ribbon-like cellulose product at the surface of the cell. In 1987, c-di-GMP was shown to accelerate cellulose biosynthesis (Ross et al. 1987) and it is now possible to demonstrate *in vitro* cellulose-synthesizing activity in membrane and purified fractions obtained from this bacterium. Cellulose synthases are supposed to be attached to the terminus of cellulose microfibrils and based on the heavy weight of the cellulose, product entrapment was used to purify cellulose synthases from the other proteins in a manner similar to that applied for purification of chitin synthases (Kang et al. 1984). Two proteins that are required for cellulose synthesis were isolated and purified from *A. xylinum* by two groups in 1990 and 1991. One of these proteins was identified as the catalytic subunit of cellulose synthase (Lin et al. 1990), and the other protein was shown to bind c-di-GMP and activate the catalytic subunit (Mayer 1991). The gene encoding the cellulose synthase catalytic subunit of *A. xylinum* was identified by determining the partial amino acid sequence of the cellulose synthase catalytic subunit and by analysis of mutants defective in cellulose production. At present, a number of proteins are suggested to be involved in cellulose biosynthesis in *A. xylinum*. Genes encoding these proteins are organized in an operon. The cellulose-synthesizing operon contains four

structural genes *bcsA*, *bcsB*, *bcsC*, and *bcsD*. In addition, other genes such as those that encode for an endoglucanase and a protein of an unknown function are also required for cellulose biosynthesis. The *bcsA* gene encodes for the cellulose synthase catalytic subunit and the *bcs B* gene encodes for the c-di-GMP-binding protein that activates the cellulose synthase. (Wong et al. 1990). The *bcsC* and *bcsD* genes are thought to be involved in the secretion of cellulose microfibrils by controlling either crystallization or polymerization.

The identification of genes that encode cellulose synthases in *A. xylinum* made possible the molecular biological approach for identification of cellulose-synthase genes in plants. The screening for cellulose-synthase genes in plants was actively pursued using antibodies and nucleic acid probes derived from *A. xylinum*. However, it was not found possible to isolate the cellulose synthase genes from plants using the *A. xylinum* gene as a probe (Delmer and Amor 1995). In parallel with these experiments, amino acid sequence analysis was performed to identify conserved regions in enzymes that catalyze formation of β-1,4 linkages including cellulose synthase, hyaluronan synthase and chitin synthase (Delmer and Amor 1995; Saxena et al., 1995). In 1996, Pear et al. performed random sequencing of cDNA libraries made from cotton fibers during secondary wall formation and isolated cDNA clones that encoded amino acid sequences that contained the conserved regions identified in the *A. xylinum* cellulose synthase.

The cotton cellulose-synthase (Gh*CesA*) genes isolated by Pear et al. (1996) were found to be homologous to the *CesA* genes identified in *Arabidopsis* by analysis of mutants affected in cellulose biosynthesis. (Arioli et al. 1998; Taylor et al. 1999). Recently, it was confirmed that the GhCesA proteins show cellulose-synthesizing activity and it is suggested that although cellulose is directly synthesized from the precursor UDP-glucose, a lipid intermediate sitosterol-β-glucoside works as a primer for cellulose synthesis (Peng et al., 2002; Read and Basic, 2002).

4   HOW TO PROVE IF THE ROSETTE OR LINEAR TC IS THE CELLULOSE-SYNTHESIZING MACHINERY?

So far the evidences to suggest that TCs contain cellulose synthases were all indirect. In many cases, rosette TCs can not be visualized easily by freeze fracture replication technique even during stages of active cellulose biosynthesis in some plant cells. Therefore, we had to await for advances in immunocyto-chemical techniques coupled with freeze-fracture electron microscopy to demon-strate directly that rosette TCs contain the catalytic subunit of cellulose synthase and the linear TCs in *A. xylinum* contain the c-di-GMP-binding protein that activates cellulose synthesis.

However, we had to overcome two major difficulties before applying the freeze-fracture technique coupled with immunogold labeling. First, it has been believed that conventional freeze-fracture technique does not allow for immunogold labeling. This is because the replica membrane made of platinum and carbon does not show any reaction with the antibody. This difficulty was overcome by utilizing

the SDS-solubilized freeze-fracture replica labeling technique first developed by Fujimoto (1995). The most important point about the immunochemical reaction as applicable to replica membrane is that the inner half of the cell membrane that is physically fixed by platinum-shadowing during freeze fracture will not dissolve with SDS. According to Fujimoto et al. (1996), artificial membranes produced by a mixture of α-phosphatidylcholine and L-α-phosphatidyl-L-serine and 100% L-α-phosphatidyl-L-serine did not dissolve after they were strengthened by a platinum-carbon replica membrane.

Second, it has not been possible to isolate and purify cellulose synthases from higher plants and so it has been difficult to prepare antibodies against this protein. This problem was overcome by expressing a fragment of the cotton CesA cDNA that encoded the catalytic region of a cotton cellulose synthase in *E. coli* and using the recombinant protein for obtaining polyclonal antibodies.

5   LABELING OF FREEZE FRACTURE REPLICAS

The authors applied the fracture labeling technique termed SDS-FRL to plant cells for the first time (Kimura et al. 1999). This technique was initially developed for animal cells by Fujimoto (1995) to bridge the gap between biochemistry and the unique morphology that is revealed by splitting the bimolecular leaflet of membranes. However, the application of this technique to plant cells has been difficult because the cell wall remains after the SDS treatment and obscures evaluation of the replicas of the fractured face of the membrane. The harsh acid treatments customarily used for conventional freeze-fracture techniques will dissolve completely not only the cell wall materials but also the bulk of the antigens present in the cytosol and the plasma membrane that may be recognized by specific antibodies.

The authors overcame this difficulty by treating the tissue attached to the replicas with a cellulase mixture commonly used for obtaining plant protoplasts. Figure 14-2 shows the comparison between conventional freeze-fracture and SDS-FRL techniques. The procedures of freeze-fracturing (a) followed by shadowing (b) are the same in both techniques. In the conventional freeze-fracture technique, plant tissues attached to the replica membrane are dissolved completely after chromic acid (Figure shows sulfuric acid and does not show the labeling of steps) treatment (c), including cell wall and cytoplasmic materials. In contrast, replica membrane attached to plant tissue are treated by a mixture of cellulase, pectolyase and proteinase inhibitor (c') in SDS-FRL technique. The replica membrane is further treated with SDS after dissolving the cell wall. After washing, the replica membrane is labeled with the CesA antibody or preimmune serum (d). Following labeling, the replica membrane is washed and treated with a secondary antibody conjugated to colloidal gold (10nm). The replica membrane is washed with PBS after this step, fixed with 0.5% glutaraldehyde, washed with distilled water twice, and placed on Formvar-coated grids for observing under transmission electron microscope. Using this protocol, the rosette TC was
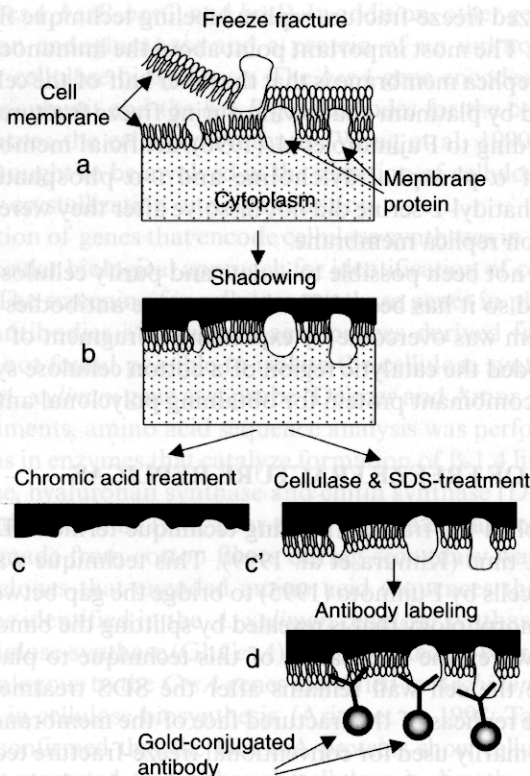
Freeze fracture

Figure 14-2. Comparison of SDS-FRL and conventional freeze-fracture techniques **(a)** freeze fracturing, **(b)** shadowing, **(c)** chromic acid treatment, **(c′)** Cellulase and SDS treatment, **(d)** antibody labeling. (Figure 2 from: Itoh, T. and Kimura S. 2001. Cellulose synthases are localized in terminal complexes. Journal of Plant Research: 114:483–489. Reproduced with kind permission of Springer Science and Business Media and the Botanical Society of Japan).

labeled with CesA antibodies on the P-fracture face of cells in the elongating hypocotyls of Azuki bean (Figure 14-3).

It was observed that 74% of gold particles were attached to rosettes or within 20 nm from the edges of rosette particles. Individual rosette was labeled with 1–4 gold particles, but usually with 1–2 gold particles (inset of Figure 14-3).

Labeling of the linear TCs of *A. xylinum* was performed using an antibody raised against the 93-kd protein that is suggested to be the c-di-GMP-binding protein. Procedures for labeling of the linear TCs of *A. xylinum* are similar to that utilized for labeling of the rosette TCs of plants except for the added steps for digestion of the peptidoglycan. The digestion of the peptidoglycan by lysozyme prior to SDS solubilization was found to be a prerequisite for freeze-fracture labeling of membrane proteins in *A. xylinum*. Lysozyme digests the
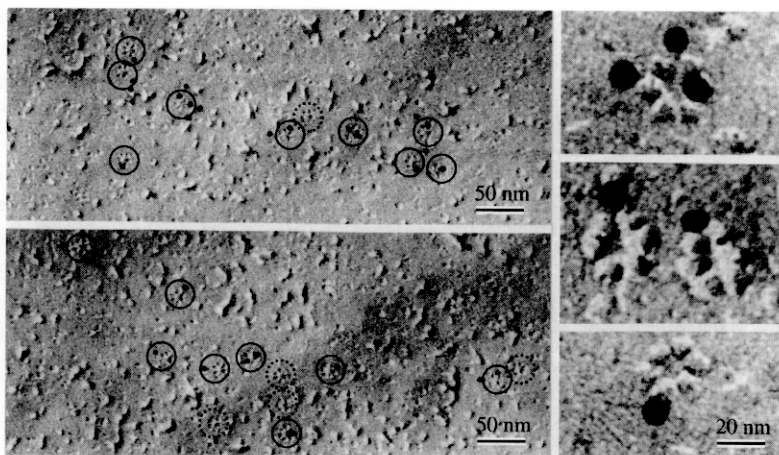
Figure 14-3. Numerous rosette TCs were labeled. Only one (*dotted circle*) of ten clearly visible rosette TCs is not labeled (*upper left*). Four (*dotted circle*) of twelve clearly visible rosette TCs are not labeled (*lower left*). A higher magnification of a rosette TC labeled with three gold particles is shown in upper right of the figure. A higher magnification of two rosette TCs each labeled with single gold particle is shown in middle right of the figure. A higher magnification of a rosette TC labeled with single gold particle is shown in *lower right* of the figure. (Itoh, T., Kimura, S., and Brown, Jr. R.M. 2004. Theoretical considerations of immunogold labeling of cellulose-synthesizing terminal complexes. Cellulose 11:385–394. Reproduced with kind permission of Springer Science and Business Media).

peptidoglycan and therefore allows the cell debris to be removed, which is done by harsh acid treatments in conventional freeze-fracture. The replicas obtained by freeze replica labeling appear similar to those obtained by conventional freeze-fracture techniques. The linear TCs of *A. xylinum* exhibit ordered particle arrays within single or double rows. The bacterial cell in Figure 14-4a shows the PF-face of its outer membrane (OM) and a single row of TC subunits with a cellulose ribbon attached at its terminus (arrow). Upon closer examination, the gold particles are observed to be attached along a single row of TCs. In the case of *A. xylinum*, almost all of the fractured planes occur through the outer membrane. In other words, the fractured cytoplasmic membrane is rarely observed in *A. xylinum* although it is more commonly observed in other Gram negative bacteria (Beveridge, 1999). The frequency of cytoplasmic membrane fractures is less than 5% based on the observation of more than one hundred cells. Even in the case where we successfully visualized the fractured plane of the cytoplasmic membrane, only part of this membrane was exposed. Furthermore, TC structures were never observed on the cytoplasmic membrane (CM) of *A. xylinum*. Figure 14-4b and 14-4c show a fractured plane occurring in the OM. Antibody labeling of TCs was not observed in a typical row of particles (Figure 14-4b). However, TCs showing a line of depressions or pits with an indistinct particle arrays were positively labeled (arrows, Figure 14-4c).
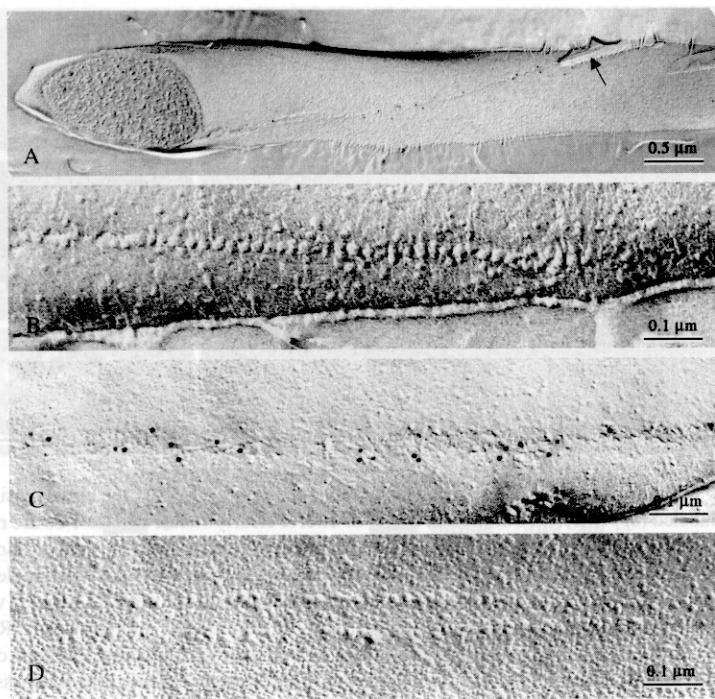
Figure 14-4. Fracture-labeled images (PF face, panels A to C; EF-face, panel D) showing reaction with the c-di-GMP-binding protein (93-kDa protein) antibody. Panel A and C show a typical fracture-labeled image of *A. xylinum*. The TC appears to be a single row along the longitudinal axis of the bacterial cell. A ribbon (arrow) of cellulose microfibrils is attached to the end of the TC row (Panel A). The 93-kDa protein antibody is distributed along the TC row on the PF face of the OM. The labeled TCs are visible as a row of slight depressions (or pits) with indistinct particles and small holes that may be due to particle displacement (Panel C). The TCs showing a distinct particle row on the PF face of the OM are not labeled with antibodies (Panel B). Panel D shows TCs with double rows on the EF face of the OM. The TCs on the EF face of the OM are never labeled by the antibodies (Figure 2a from: Kimura, S., Chen, H P., Saxena, I.M., Brown, Jr. R.M., and Itoh.T. 2001. Localization of c-di-GMP-binding protein with the linear terminal complexes of Acetobacter xylinum. J Bacteriol 183:5668–5674. Reproduced with kind permission of the American Society for Microbiology).

Evidence for understanding the topology of the bacterial membranes is furthermore obtained from a very rare case of freeze fracture which shows a distinct row of TC particles and pits in a single line at the same time on the PF-face of OM (Figure 14-5). The TCs in this region of the pits were exclusively labeled with antibodies. Two different features associated with the linear rows on the PF- face of OM are noted: (i) a pit or depressed region with indistinct particles (Figure 14-5b); and, (ii) a distinct, single row of particles (Figure 14-5c). The gold particles are localized only in the former, but not in the latter. In addition, these same TC particles can be found associated with the outer leaflet of the OM in all cases; however, TCs on the E-fracture face are never labeled in any case
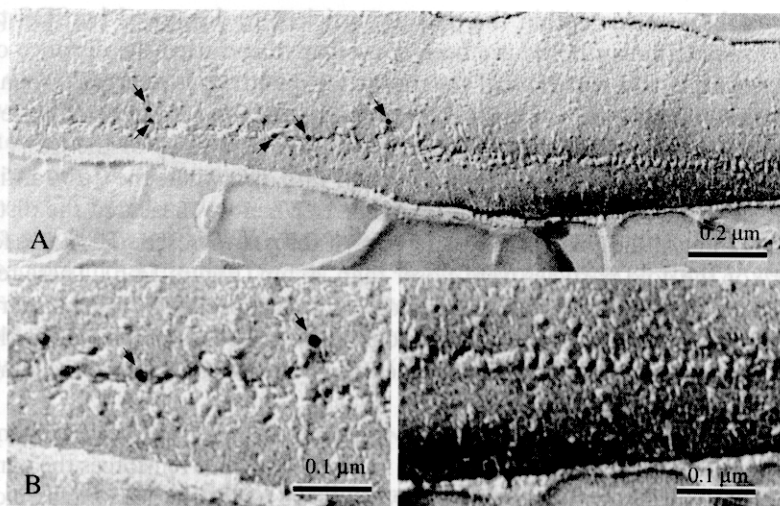
Figure 14-5. Fracture labeled image showing two types of TCs structure revealed on the PF-face of the OM. The distinct TC particles (*right half* of Figure 14-5a, Figure 14–5c) and depressions (or pits) with particles (left half of Figure 14-5a, Figure 14-5b) are visualized in a single row on the same PF-face of the OM. Moreover, the labeling of gold particles can be seen only in the region with depressions (*arrows* in Figure 14-5a and 14-5b). (Figure 5a is Figure 3 from: Kimura, S., Chen, H P., Saxena, I.M., Brown, Jr. R.M., and Itoh,T. 2001. Localization of c-di-GMP-binding protein with the linear terminal complexes of Acetobacter xylinum. J Bacteriol 183:5668–5674. Reproduced with kind permission of the American Society for Microbiology).

(Figure 14-4d). These membrane particles often appear complementary with the pit-like structures or depressions in the PF-face of OM.

6 SPECIFIC LABELING OF ROSETTE TCS

According to preliminary western blot analysis, the antibody to the recombinant cellulose synthase recognized an antigen of 130kd from three vascular plants - cotton, *Arabidopsis* and *Vigna radiata*. These results suggest that the catalytic subunit is conserved among a number of vascular plants. The antibody also recognized proteins obtained from cotton fibers during primary and secondary wall development. The specific labeling of rosette TCs on the P-fracture face of plasma membrane by the CesA antibody therefore provides direct proof that the rosette TC contains the catalytic subunit of cellulose synthases.

Labeling of the rosettes with gold-conjugated secondary antibody showed that the gold particles were present not only on the rosette TCs but also within 20 nm from the edge of the rosettes. The distance of 20 nm from the edge of the rosettes is within the range of the sum of the lengths of the primary plus secondary antibodies that has been determined to be 27 nm (Sarma et al. 1971). In fact, the actual distance from the antigens, cellulose synthases, may extend to more than

27 nm as the proteins within the rosette particles are denatured by SDS treatment. However, the authors have been conservative and used the distance of 20 nm between the gold particle and the rosette for quantitative analysis. When preimmune serum was used as the primary antibody, less than 2% of the rosette TCs were labeled with gold particles, which supports the specificity of the CesA antibodies. When the preimmune data is compared with data where the CesA antibodies were used the results are even more distinct. When we measured the distance from the center of the gold particle to the edge of the nearest rosette TC, it was found that 84% of the gold particles were closer than 20 nm (Figure 14-6). These results demonstrate that the antibodies to cellulose synthase specifically label a morphological structure that has been independently identified as associated with the end of cellulose microfibrils and suggested to be the site of the enzyme complex (Mueller and Brown, Jr., 1980).

It is also important to note that there is no specificity of antibody labeling on the E-fracture face of plasma membrane in *V. radiata*. This reinforces the concept that the catalytic region of the cellulose synthase lies truly on the cytoplasmic side of the plasma membrane, an observation that is congruent with the site of the catalytic domain predicted from sequencing data (Pear et al. 1996).
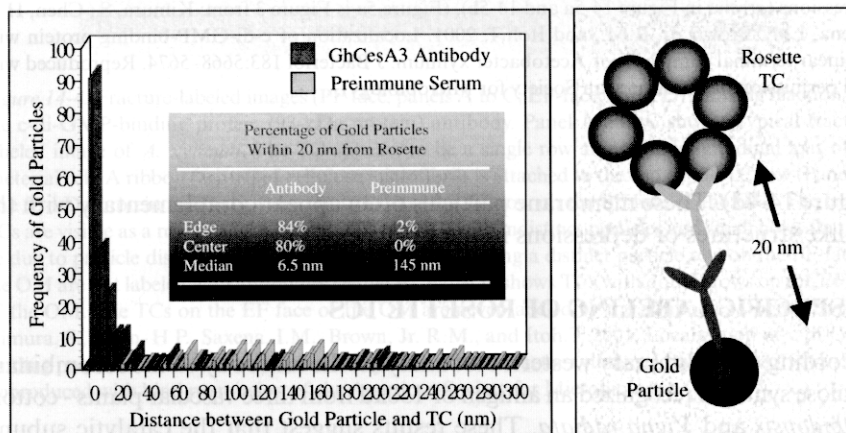

Figure 14-6. Frequency distributions of the number of gold particles associated with the immune serum containing antibodies to cellulose synthase and the preimmune control serum as a function of the measured distance to the center and the edge of the nearest rosette TC (*left*). Schematic diagram for the measurement of the distance between gold particles and rosette TC (*right*) is also shown (green:rosette, pink:primary antibody to cellulose synthase, blue:secondary antibody, red: gold particle the 93 kDa antibody-labeled particles. (**a**) Schematic diagram for the measurement of the distance between gold particles and linear TCs. The distance (double arrowheads) between the edge of gold particles and the linear TCs is indicated by the dotted line. (**b**) Frequency distribution of the number of gold particles associated with the 93 kDa protein antibody is shown as a function of the measured distance (nanometers) to the linear TC. Total number of gold particles measured was 277, taken from 30 different cells (*See Color Plate of this figure beginning on page 355*)

7 SPECIFIC LABELING OF LINEAR TCs

The distribution of gold particles associated with linear TCs in *A. xylinum* is shown in Figure 14-7. The distance between the TCs and gold particles was calculated by measuring the vertical distance between the edge of gold particles and a linear row of TC particles (Figure 14-7a, double arrowheads). For frequency analysis, 277 gold particles were randomly sampled from 30 different cells that had a single row of TCs. We neglected the measurement of this distance where the bacterium had double rows of TCs. Seventy five percent of the 277 gold particles were found within 20 nm of the linear row (shown as a dotted line in Figure 14-7a) of TCs. Most gold particles were found within 10–14 nm, with a median distance of 9.3 nm from the linear row (Figure 14-7b).

8 THE MECHANISM OF LABELING OF CELLULOSE SYNTHASES

The key point of antibody labeling in SDS-FRL is to label the cytoplasmic region of cellulose synthases using the modified freeze-fracture technique combined with cell wall digestion and SDS treatment. Transmembrane proteins
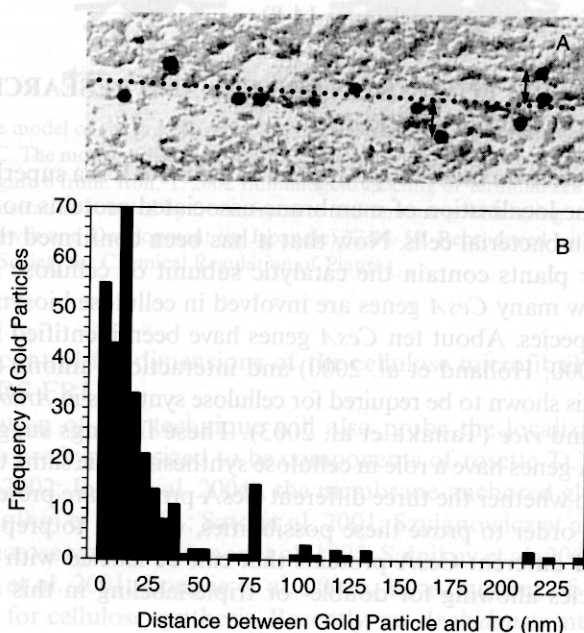

Figure 14-7. The distribution of gold particles associated with linear TCs in *A. xylinum*. (Figure 4 from: Kimura, S., Chen, H.P., Saxena, I.M., Brown, Jr. R.M., and Itoh, T. 2001. Localization of c-di-GMP-binding protein with the linear terminal complexes of *Acetobacter xylinum*. J Bacteriol 183:5668–5674. Reproduced with kind permission of the American Society for Microbiology).

present in the rosette TC and that have large regions exposed to the cytoplasmic side were labeled by this method. SDS treatment is a prerequisite for this method to work and it is also important that the rosette TC proteins stay attached to the replica membrane. It is extremely important to note that Fujimoto et al (1996) demonstrated by analysis of membrane proteins and lipids that these components are not removed by SDS treatment from the inner half of the fractured plasma membrane. This suggests that the semimembrane lipoprotein complex may be enzymatically active. The suitability of this idea can be demonstrated if the inner half of fractured plasma membrane synthesizes *in vitro* cellulose upon addition of UDP-glucose and we are in the process of testing this idea.

Another point in understanding the mechanism of CesA antibody labeling is to determine the number of gold particles that can be associated with a single TC. The diameter of both the gold particles and antibodies is 10 nm. Therefore, the diameter of the complex of gold particle and antibodies is approximately 30 nm (lower left, Figure 14-8). The diameter of a rosette TC is 25 nm. The actual image of a rosette labeled with these gold particles is shown in upper right of Figure 14-8. When we drew a Figure using the above mentioned dimensions, the illustration as shown in the middle right of Figure 14-8 was obtained. The lower right of Figure 14-8 shows the side view. It is surprising to see such a close match between the actual image (upper right of Figure 14-8) and its illustration (or modified) (middle right of Figure 14-8).

9   FUTURE PERSPECTIVES ON SDS-FRL AND RESEARCH IN CELLULOSE BIOSYNTHESIS

Immuno-gold electron microscopy applied to SDS-FRL is a superb technique to demonstrate the localization of membrane-associated proteins not only in plant cells but also in bacterial cells. Now that it has been confirmed that the rosette TCs of higher plants contain the catalytic subunit of cellulose synthases, the question is how many *CesA* genes are involved in cellulose biosynthesis in individual plant species. About ten *CesA* genes have been identified in *Arabidopsis* (Richmond 2000; Holland et al. 2000) and interactions among three different CesA proteins is shown to be required for cellulose synthesis in *Arabidopsis* (Taylor et al., 2003) and *rice* (Tanaka et al. 2003). These findings suggest that three different CesA genes have a role in cellulose synthesis at the same time. However, it is not known whether the three different CesA proteins are present in the same TC or not. In order to prove these possibilities, we need to prepare antibodies against several different CesA proteins that can be labeled with different sizes of gold particles allowing for double- or triple-labeling in this application of SDS-FRL.

The specific labeling of c-di-GMP-binding protein to a single row of cellulose-synthesizing TCs in the outer fractured membrane of *A. xylinum* should allow localization of other proteins in these complexes (Kimura et al., 2001). In the near future, it will be possible to localize crystallization proteins and pore
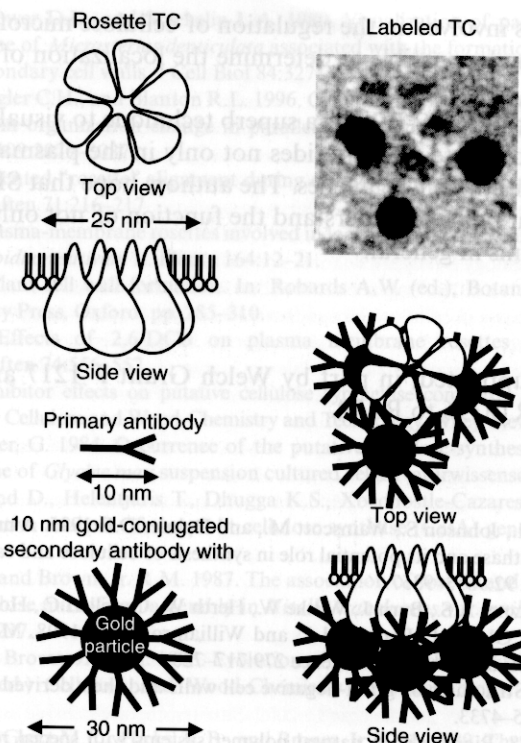
Figure 14-8. Scale model of the gold particle, antibodies, rosette TC, gold-conjugated antibody, and labeled rosette TC. The model of labeled rosette TC (*middle right*) closely resembles the actual image (*upper right*). (Figure 6 from: Itoh, T. 2002 Immunogold labeling of terminal cellulose-synthesizing complexes-demonstration coupled by freeze fracture and immunogold labeling techniques. Regulation of Plant Growth and Development (in Japanese) 37:44–50. Reproduced with kind permission of The Japanese Society for Chemical Regulation of Plants.)

proteins that control the dimensions of the cellulose microfibril (Saxena et al. 1994) using SDS-FRL.

The application of this technique will also probe the localization of other proteins which are hypothesized to be components of rosette TCs in plant cells (Doblin et al. 2002; Joshi et al. 2004), the membrane-anchored glucanase (Lane et al. 2001; Mølhøj et al. 2001; Sato et al. 2001; Szyjanowicz et al. 2004; Rober et al. 2005), sucrose synthase (Amor et al. 1995; Salnikov et al. 2001), and callose synthase (Cui et al. 2001; Zonglie et al. 2001a, b). A number of these proteins are important for cellulose synthesis. Recently, an *Arabidopsis* mutant defective in the COBRA protein was isolated (Roudier et al. 2005). This mutant exhibits disorganization of the orientation of cellulose microfibrils and subsequent reduction of crystalline cellulose. It is suggested that the COBRA protein is a GPI-anchored protein localized on the plasma membrane. This means that

COBRA protein is involved in the regulation of cellulose microfibril arrays and in the future it may be possible to determine the localization of this protein by SDS-FRL.

Generally speaking, SDS-FRL is a superb technique to visualize the localization of any kind of protein that resides not only in the plasma membrane but also the membranes of cell organelles. The authors hope that SDS-FRL will be applied in different fields to understand the function of not only plant cells but also other organisms in general.

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