RESEARCH ARTICLE

Immunogold Labeling of Rosette Terminal Cellulose-Synthesizing Complexes in the Vascular Plant *Vigna angularis*

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The catalytic subunit of cellulose synthase is shown to be associated with the putative cellulose-synthesizing complex (rosette terminal complex [TC]) in vascular plants. The catalytic subunit domain of cotton cellulose synthase was cloned using a primer based on a rice expressed sequence tag (D41261) from which a specific primer was constructed to run a polymerase chain reaction that used a cDNA library from 24 days postanthesis cotton fibers as a template. The catalytic region of cotton cellulose synthase was expressed in *Escherichia coli*, and polyclonal antisera were produced. Colloidal gold coupled to goat anti–rabbit secondary antibodies provided a tag for visualization of the catalytic region of cellulose synthase during transmission electron microscopy. With a freeze-fracture replica labeling technique, the antibodies specifically localized to rosette TCs in the plasma membrane on the P-fracture face. Antibodies did not specifically label any structures on the E-fracture face. Significantly, a greater number of immune probes labeled the rosette TCs (i.e., gold particles were 20 nm or closer to the edge of the rosette TC) than did preimmune probes. These experiments confirm the long-held hypothesis that cellulose synthase is a component of the rosette TC in vascular plants, proving that the enzyme complex resides within the structure first described by freeze fracture in 1980. In addition, this study provides independent proof that the *CelA* gene is in fact one of the genes for cellulose synthase in vascular plants.

INTRODUCTION

Cellulose is the most abundant biopolymer on earth and is the major constituent of the plant cell wall (Franz and Blaschek, 1990; Brown, 1996). Cellulose is a biopolymer consisting only of β-1,4-glucans. Approximately 40 β-glucan chains are synthesized from a multimeric enzyme complex, and these chains associate immediately upon synthesis to form the crystalline entity known as a microfibril (Brown, 1996). Crystalline cellulose is defined by its various allomorphs, with cellulose I denoting the most abundant native crystalline form (Brown et al., 1996).

One of the great enigmas in plant biology is the biosynthesis of cellulose. During the past 50 years, the site of cellulose synthesis has been investigated intensively, but until now,

there has been no direct proof for the existence of a multimeric enzyme complex located in the plasma membrane of vascular plant cells. Roelofsen (1958) first suggested that cellulose might be assembled by a large enzyme complex at the growing tip. As early as 1972, Dobberstein and Kiermayer (1972) had visualized ordered particle complexes within "f-vesicles" of the Golgi apparatus in the green alga *Micrasterias denticulata*. These particles were implicated in the biosynthesis of cellulose. This work is significant historically, because the first observation of what was later to be beautifully imaged by freeze fracture was from sectioned material. Thus, the ordered granule complex, first postulated by Preston (1964), was found only eight years later, but it was not until the freeze-fracture technique was used that the involvement of an organized particle complex in cellulose biosynthesis was confirmed. Brown 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 a linear terminal complex (TC), and consisted of three rows of subunit particles. The complex was intimately associated with microfibrils, as clearly evidenced by impressions of microfibrils leading from and associated with the complex.

Subsequently, Mueller and Brown (1980) found a different arrangement of particles, a cluster or rosette of six particles, associated with cellulose microfibril impressions in the plasma membrane of a vascular plant. They named these complexes rosette TCs. Since this time, numerous studies have implicated rosette TCs (frequently called simply rosettes) in cellulose microfibril assembly (Giddings et al., 1980; Mueller and Brown, 1982; Herth, 1984, 1985a, 1985b, 1987, 1989; Herth and Weber, 1984; Hotchkiss and Brown, 1987, 1988; Hotchkiss et al., 1989; Rudolph et al., 1989; Emons, 1991, 1994; see also Dobberstein and Kiermayer, 1972, and Kiermayer and Sleyter, 1979, for interesting historical perspectives); however, all evidence to date has been indirect, and independent confirmation that the rosette TC contains the catalytic subunit for cellulose assembly has awaited the development of immunocytochemical approaches that could be coupled with the freeze-fracture technique.

Unfortunately, the production of antibodies to purified cellulose synthase has had to take an indirect route because over the years researchers have had great difficulty with the biochemical identification and purification of this enzyme in vascular plants. The only successful approach has been the design of a primer based on a rice expressed sequence tag (EST) that shows similarity to the *Acetobacter xylinum* catalytic region of cellulose synthase (Saxena et al., 1990, 1991, 1994). This primer was used to clone a cotton gene that is related to the *A. xylinum* gene and that has a strong similarity to the *CelA* gene recently isolated and characterized from cotton (Pear et al., 1996). This investigation confirms by immunocytochemical labeling of proteins attached to freeze-fractured replicas that the catalytic unit for cellulose synthase is localized in the rosette TC of vascular plant cells.

RESULTS

Protein Gel Blot Analysis

Figure 1 shows the results of protein gel blot analyses with the control preimmune serum and the immune serum. Figure 1A illustrates protein gel blot analysis against an *Escherichia coli* lysate. Note that two prominent 45- and 53-kD bands are labeled with the antibody. According to the sequencing data, the 45-kD band is the truncated band. The preimmune serum does not react with any proteins in the gel blot, demonstrating the specificity of the immune sera to the antigen.

Figure 1B depicts the results of protein gel blot analysis against plasma membrane fractions isolated from cotton
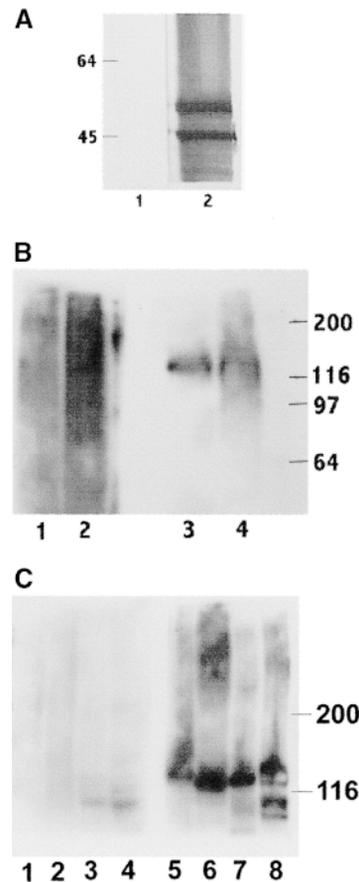
Figure 1. Protein Gel Blot Analysis of the Preimmune Serum and Antibody-Containing Serum.

(A) Protein gel blot analysis of *E. coli* lysate–transformed BL21 (DE3). Lane 1, control, preimmune serum; lane 2, immune serum. Molecular weight markers in kilodaltons at left.

(B) Protein gel blot analysis of cotton primary wall (14 DPA) and secondary wall (24 DPA) plasma membrane fractions. Lane 1, control, preimmune serum, membrane fraction from cotton 24 DPA stage; lane 2, control, preimmune serum, membrane fraction from cotton 14 DPA stage; lane 3, immune serum, membrane fraction from cotton 24 DPA stage; and lane 4, immune serum, membrane fraction from cotton 14 DPA stage. Molecular weight markers in kilodaltons at right.

(C) Protein gel blot analysis of cotton primary and secondary wall fractions and Arabidopsis and *V. radiata* membrane fractions. Lane 1, control, preimmune serum, membrane fraction from cotton 14 DPA stage; lane 2, control, preimmune serum, membrane fraction from cotton 24 DPA stage; lane 3, control, preimmune serum, membrane fraction from Arabidopsis; lane 4, control, preimmune serum, membrane fraction from *V. radiata*; lane 5, immune serum, membrane fraction from cotton 14 DPA stage; lane 6, immune serum, membrane fraction from cotton 24 DPA; lane 7, immune serum, membrane fraction from Arabidopsis; and lane 8, immune serum, membrane fraction from *V. radiata*. Molecular weight markers in kilodaltons at right.

primary wall (at 14 days postanthesis [DPA]) and secondary wall (at 24 DPA). Note in lane 3 that there is a prominent band of ~130 kD. The migration of this band is consistent with the CelA molecular mass predicted from the nucleotide sequence (110 kD) (Pear et al., 1996). Variations in molecular weight may be due to glycosylation or some other modifications to the protein. The CelA protein also is highly expressed in the cotton secondary cell wall (i.e., 24 DPA) stage. In lane 4, there is also a band of ~130 kD, indicating that the predicted CelA protein is present during primary cell wall development. Protein gel blotting of extracts from *Vigna radiata* and Arabidopsis also showed a prominent band at ~130 kD, indicating that the catalytic domain of cellulose synthase may be highly conserved (Figure 1C).

Freeze-Fracture Labeling Analysis

In this study, we have pioneered the fracture labeling technique termed SDS-FRL to plant cells for the first time. 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 the technique to plant cells has been difficult because the cell wall remains after SDS treatment and obscures evaluation of the replicas of the fractured face of the membrane. This difficulty has been overcome by treating the tissue attached to the replicas with a cellulase mixture. Subsequent SDS treatment was successful in removing the cell wall materials and bulk cytoplasm attached to the replicas, allowing the replicas to be clearly viewed. Replicas obtained in this manner appear similar to those obtained by using conventional freeze-fracture techniques, in which harsh acid treatments customarily have been used to remove cell and tissue components. Antibody interactions with the gold label and the rosette TC are shown diagrammatically and to scale in Figure 2. The SDS-FRL technique as applied to plants is shown diagrammatically in Figures 3A to 3I.

The CelA antibodies specifically recognized rosette TCs on the P-fracture face (Figures 4A and 4B). Gold particles were observed directly over or close to rosette TCs, usually <20 nm from the edge of these structures. Individual rosette TCs were labeled by one to four gold particles. A preimmune serum, which was obtained from the same rabbit before injection with the CelA protein, was tested in a control experiment. This preimmune serum showed labeling of rosette TCs for only 2% of 98 gold particles on the P-fracture face (Figures 5A and 5B).

The frequency distribution of gold particles associated with the immune serum is shown in Figure 6A. Seventy-four percent of 217 gold particles were found within 20 nm of the edge of the rosette TC. These results summarize distance measurements from 20 photographs representing the fractured plasma membranes of >20 different cells. The distribution of labeling distances is rather narrow, centering at
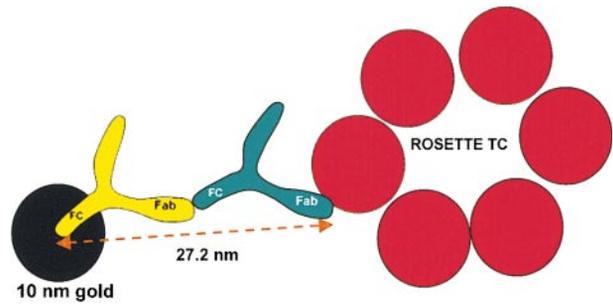
Figure 2. Scale Model Depicting Typical Primary and Secondary Antibody Dimensions Relative to the 10-nm Gold and Rosette TC Dimensions.

See the antibody dimensions given by Sarma et al. (1971).

~15 to 20 nm, with a median distance of 6.5 nm from the edge of the rosette TC.

The distribution of gold particles associated with the preimmune serum is presented in Figure 6B. Although some gold particles were observed on the replica membrane, those particles were distributed randomly and nonspecifically on the P-fracture face (Figure 5). Fewer than 2% of the 98 gold particles measured from 10 different cells were within 20 nm of a rosette. The distribution of gold particles to the nearest rosette was broad (median = 145 nm), as would be expected by nonspecific labeling.

No significant differences were detected within the distributions of either the preimmune or the immune cells; thus, their individual distributions were lumped to test whether the overall distributions of preimmune and immune labels differed. The Pearson chi squared test of the polyclonal immune and preimmune sera was highly significant (degrees of freedom = 1; ψ^2 = 152.2; P < 0.0001), indicating the immune serum was much more effective at labeling rosettes.

DISCUSSION

Specificity of Labeling and Confirmation of the Site of Cellulose Synthase in Association with the Rosette TC

In this study, we have successfully labeled rosette TCs with the anti-CelA antibody on freeze-fractured replicas. Specific labeling with low background was demonstrated between the anti-CelA antibody and rosettes on the P-fracture face of the plasma membrane. This provides direct evidence that the rosette TCs contain the cellulose synthase catalytic subunit.

Gold particles were observed not only directly over the rosette TCs but also <20 nm from the edge of rosettes. This distance is considered permissible for specific labeling of CelA antibody against rosette TCs because the size of both primary and secondary antibodies is ~27 nm (Figure 2)

(Sarma et al., 1971). The actual distance of the gold particle from the antigen, cellulose synthase, could be >27 nm because the proteins in the rosette TC are denatured and extended by SDS treatment (see Figure 3). Nevertheless, we selected 20 nm or less as a conservative distance for a "labeled" rosette TC in our qualitative analyses.

The chi squared test on the distributions of the preimmune and immune sera samples rules out the possibility that the preimmune sera and the immune sera are the same in their behavior. It also shows that the immune serum labeled significantly more rosette TCs, lending strong support

to the conclusion that cellulose synthase is associated with the rosette TC. It is clear from the preimmune sera data that fewer than 2% of the gold particles were sufficiently close to a rosette TC to be counted as labeled. Furthermore, these data came from >10 different photographs that represented P-fracture surfaces from at least eight different cells. When the preimmune data are compared with data for the antibodies against cellulose synthase, the results are dramatic. If the distance from the gold particle to the center of the rosette TC is considered, >80% of the gold particles are closer than 20 nm; however, we wanted to be more conser-
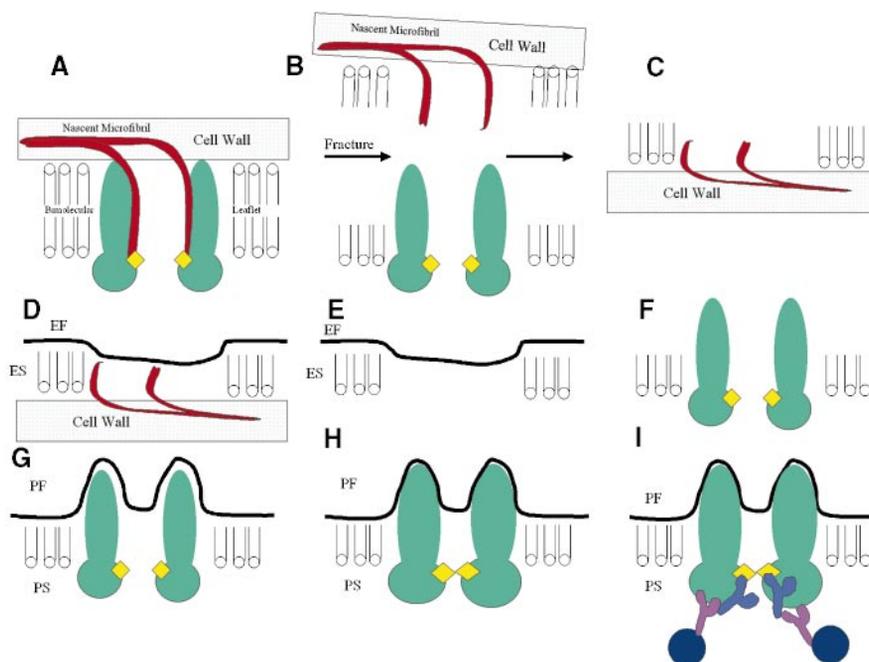
Figure 3. Diagram of the Methodology of SDS-FRL Technique Used in the Immunocytochemical Localization of Cellulose Synthase.

(A) Before fracturing. The intact rosette TC is presented with its major subunits (green) embedded preferentially in the inner leaflet of the plasma membrane and the catalytic subunit (yellow) exposed to the cytoplasm (based on sequence analysis). Nascent glucan chains originating from the catalytic subunit site (depicted in red) originate from the globular domain, which is predicted to lie in the cytoplasmic volume. More specifically, the glucan chains are diagrammed to be in close association with the individual rosette TC subunits (whether or not they pass through closed channels or surface depressions is unknown at present). Lipid bilayer molecules are also indicated.
(B) The bimolecular leaflet halves separating at the moment of fracture, leaving the nascent cellulose separated from the rosette TC.
(C) An inverted orientation of the fractured cellulose and outer plasma membrane leaflet before platinum–carbon evaporation.
(D) The platinum–carbon replica film (dark black line) and attached replicate after evaporation. Note that the typical fracture view is labeled, indicating that the exoplasmic face (EF) has been replicated. Frequently, a depression is coated with platinum–carbon in which the rosette TC subunit once existed. ES indicates a view of the exoplasmic surface that would have been replicated had the membrane not been fractured.
(E) The replica after cell wall digestion and SDS treatment. No organic materials remain attached to the replica.
(F) The orientation of the fractured inner plasma membrane leaflet before replication. Note that the rosette TC hydrophobic transmembrane domains remain and have sufficient projection to create the replica of a rosette TC structure.
(G) The fractured inner plasma membrane leaflet and the embedded rosette TC structure after replication (dark black line). Note also that the typical view revealing a rosette TC is the PF or fracture surface facing the protoplasm. PS, membrane surface adjacent to the cell interior.
(H) The encased subunits remain attached to the replica after swelling from cell wall digestion and SDS treatment. This leaves the antigenic sites available for antibody labeling.
(I) Antibody labeling. Note that the primary antibody (light blue) binds to the partially denatured catalytic domain (yellow diamond). Secondary antibodies (pink) with their attached colloidal gold (blue) complete the labeling of the rosette TC.
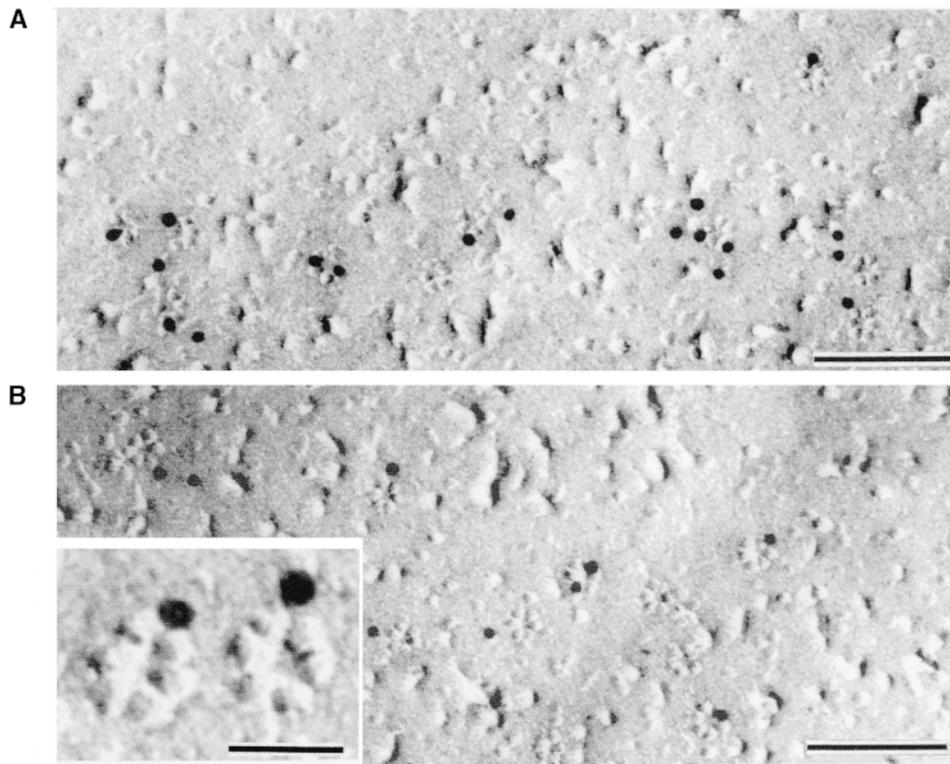
Figure 4. Fracture-Labeled Replicas (PF Views) Showing Reactions with the Cellulose Synthase Catalytic Subunit Antibodies.

(A) Numerous rosette TCs labeled. Only one rosette TC in the center of the photograph is not labeled (of eight clearly labeled rosette TCs).
(B) Another field of labeled rosettes with seven clearly labeled rosettes and one unlabeled rosette. The inset shows an enlarged view of two selected rosette TCs with antibody labels.
Bars in **(A)** and **(B)** = 0.1 μm; bar in inset = 30 nm.

vative, and so we measured the distance from the center of the gold particle to the edge of the nearest rosette TC. Even these measurements revealed that >74% of the gold particles were closer than 20 nm, thus demonstrating 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, 1980).

It is also interesting to consider that if dispersed rosette TC subunits are present, the immune sera may also be labeling these; however, we have no morphological grounds to determine whether a dispersed particle revealed in the plasma membrane fracture truly is a part of cellulose synthase. Perhaps in the future, careful comparisons of the dimensions and shapes of single particles may reveal criteria that can be used to make the identification by antibodies meaningful at the subunit level of resolution.

It is also important to note that on the E-fracture face, there was no specificity of antibody labeling (data not shown). This reinforces the concept that the catalytic subunit for 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).

Antibody Labeling Specifies a Highly Conserved Catalytic Subunit among Four Genera of Vascular Plants

Protein gel blot analysis shows that our recombinant cellulose synthase antibody clearly recognizes antigens of ~130 kD from at least two other genera of vascular plants (*Gossypium* and Arabidopsis). Thus, the catalytic subunit may be highly conserved within all vascular plants. Moreover, the antibodies recognize proteins from cotton fibers in primary and secondary stages of cell wall development. Although the proteins were recognized in both stages of development and were of the same size, the quantity of proteins was found to be higher during secondary wall development.
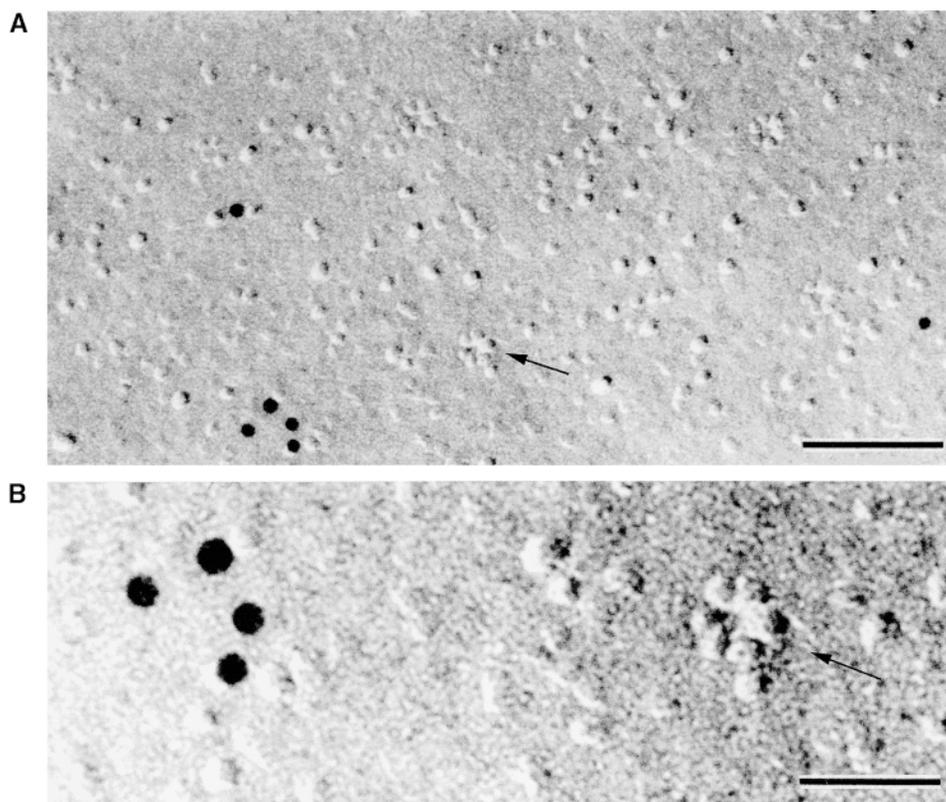
Figure 5. Fracture-Labeled Replicas (PF Views) Showing Reactions with the Control Preimmune Serum.

(A) Five clearly unlabeled rosette TCs and random labeling with gold. One unlabeled rosette TC (arrow) is enlarged in **(B)**.
(B) An enlargement of **(A)** (rotated slightly counterclockwise), clearly showing that the antibody label does not associate with the rosette TC to the right (arrow).
Bar in **(A)** = 0.1 μm; bar in **(B)** = 50 nm.

The successful immunolabeling of a rosette TC required two fortuitous circumstances: (1) the development of a novel fracture labeling method that enabled viewing of the replica while keeping the proteins in place for labeling; and (2) the production of polyclonal antisera against a large recombinant polypeptide constructed from the catalytic domain. Having a larger polypeptide increased the probability of exposing antigenic sites of interest for antibody production. Obviously, a large number of polyclonal antisera are made to various regions of the antigen, in this case to 45- and 53-kD recombinant polypeptides. Because both contain the catalytic domain, there is a higher probability of producing antibodies to cellulose synthase.

Fracture Labeling of a Cytoplasmic Component of Cellulose Synthase

The modified fracture labeling method used in this work has enabled the immunolabeling of a predominantly cytoplasmic component of cellulose synthase. Cellulase treatment is necessary to effect sufficient removal of cellular debris from the replica, while maintaining the adhesion of the rosette TC proteins to the replica. It is interesting that Fujimoto (1995) demonstrated through analyses of membrane proteins and lipids that SDS does not remove lipids or proteins from the split membrane halves. Thus, it is possible that the phospholipid–protein "semi-membrane" complex might still be enzymatically active. Experiments are in progress to determine whether the rosette proteins encapsulated in the platinum–carbon replica cages can still enzymatically use UDP-glucose to synthesize cellulose microfibrils. It might be possible to use the FRL technique as a nano-platform for enzymatic reactions.

Further Applications of the SDS-FRL Technique

The SDS-FRL method allows the positive identification of membrane proteins in plant cells. Extending this technique

may reveal the presence of cellulose synthases that are not necessarily organized into the rosette TC. For example, individual subunits may also function in cellulose biosynthesis and still could be active in the temperature-sensitive mutant of Arabidopsis that synthesizes less crystalline cellulose but possibly produces an abundant quantity of noncrystalline amorphous cellulose (Arioli et al., 1998). Using map-based cloning, these workers identified a gene that is mutated in a temperature-sensitive radial-swelling mutant of Arabidopsis and that encodes the catalytic subunit of cellulose synthase. The temperature-sensitive mutants had reduced amounts of cellulose but increased amounts noncrystalline β-1,4-glucans (Arioli et al., 1998). Significantly, the mutants also lacked organized rosette TCs. Thus, an intact TC appears to be required to produce the metastable crystalline cellulose microfibril.

Many questions remain regarding the structure and function of the rosette TC. For instance, the regulators of cellulose synthase also may reside within the subunit structure and have important functions, and antibodies to these can help to answer further questions: (1) Are annexin-like components involved (Shin and Brown, 1999)? (2) Where is sucrose synthase located (Amor et al., 1995)? (3) Is the presumed 2,6-dichlorobenzonitrile binding 18-kD peptide colocalized with the TC rosette (Delmer et al., 1987)? (4) What is the fate of antibody labeling under the conditions of TC assembly and breakdown pathways? (5) Can other regulatory proteins be identified in association with the rosette TC?

Application of the SDS-FRL technique could help in the identification of other forms of cellulose synthase not presently recognized. It should be noted that earlier freeze-fracture investigations of the cotton fiber (Willison and Brown, 1977) failed to demonstrate conclusively rosette TCs in the plasma membrane of fibers undergoing secondary wall deposition, which is the most active cellulose biosynthesis phase. In this case, the cellulose synthase particles might simply aggregate into nonrosette, supramolecular complexes to synthesize bands of microfibril that have been seen by freeze fracture (K. Okuda and R.M. Brown, unpublished observation).

With antibodies to a highly conserved region of cellulose synthase, it also may be possible to localize the cellulose-synthesizing structures in a number of other interesting organisms such as tunicates (Kimura and Itoh, 1996); humans (Hall and Saxl, 1960); various algae (Brown, 1990); and *Acetobacter xylinum* and other bacteria, including some of the more primitive Archaebacteria (Gupta et al., 1994). Of particular interest is the antibody localization of rosette TCs discovered in advanced algae such as *M. denticulata*, *Nitella* spp, and *Coleochaete scutata*, which are thought to be progenitors of vascular plants (Hotchkiss and Brown, 1987, 1988; Hotchkiss et al., 1989). The antibody approach could help to dissect the evolution of cellulose biosynthesis and rosette TC architecture (see also Herth, 1985b; Brown, 1990).
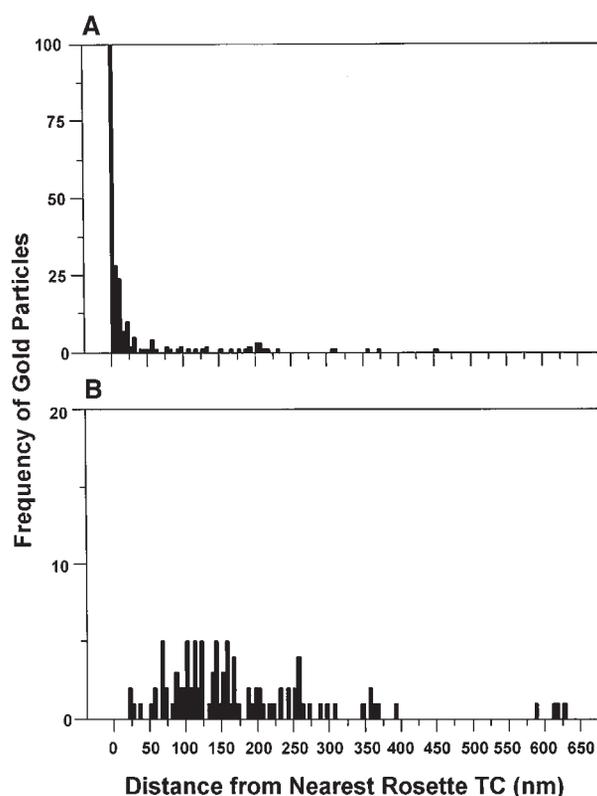
Figure 6. Frequency Distributions for Immune and Preimmune Sera.

(A) Frequency distribution of the number of gold particles associated with the immune serum containing antibodies to cellulose synthase as a function of the measured distance (nanometers) to the edge of the nearest rosette TC. Two hundred and seventeen gold particles were counted.
(B) Frequency distribution of the number of gold particles associated with the preimmune control serum as a function of the measured distance (nanometers) to the edge of the nearest rosette TC. Ninety-eight gold particles were counted.
Note the distribution of the preimmune labels, which show almost no labeling within 20 nm of a rosette TC, and the different vertical scales for each graph.

Conclusion

This study represents the apex of a lengthy search for the genes involved in cellulose biosynthesis. The first purification of a cellulose synthase, by Lin and Brown (1989), led to the acquisition of the N-terminal sequence and cloning of the gene from *A. xylinum* by Saxena et al. (1990). The catalytic subunit of cellulose synthase first sequenced in *A. xylinum* was used to screen ESTs leading to the close match with the rice D41261 EST. Saxena et al. (1995) subjected the *A. xylinum* sequence to hydrophobic cluster analysis to reveal clearly a predictable pattern in processive β-glycosylation

reactions. The conserved information relating to the D-D-D-QXXRW motif from Saxena et al. (1995) confirmed the identification of a presumptive cellulose synthase from a vascular plant (Pear et al., 1996). Independently, Arioli et al. (1998) found the same gene using an Arabidopsis mutant. Kudlicka and Brown (1997) reported the separation of β-1,3- and β-1,4-glucan synthase activities using gel electrophoresis in nondenaturing conditions. These gels could be incubated in UDP-glucose and the resulting in vitro products visualized with the electron microscope. They identified multiparticulate complexes in association with the in vitro–synthesized cellulose and only single particles in association with callose synthesis. Some of the particles associated with cellulose assembly resembled rosette TCs. With the in situ localization of polyclonal antisera directed against a specific recombinant cellulose synthase, the story returns to the original concept of a multimeric enzyme complex postulated by Roelofsen in 1958. That complex now has been identified conclusively as the rosette TC in vascular plant cells.

METHODS

Plant Material

Cotton plants (*Gossypium hirsutum* cv Texas Marker-1) were grown in the greenhouse under the following light and temperature conditions: 14 hr of light (incandescent and fluorescent lamps) at 32°C and 10 hr of darkness at 20°C. Flowers were tagged on the day of anthesis. At a specified fiber developmental stage, bolls were removed, and locules were stored in liquid nitrogen until the time of fiber harvesting. *Arabidopsis thaliana* suspension cultures were grown at 25°C, and *Vigna radiata* seedlings were germinated in darkness for 3 to 5 days at 25°C.

Isolation of Cotton Fiber RNA

Total RNA from cotton fibers at 24 days postanthesis (DPA) was extracted from frozen tissues according to Hughes and Galau (1988). Polysaccharides were eliminated with the following procedures. After LiCl precipitation, the RNA pellet was dissolved in 30 mM sodium acetate, pH 5.5, and 0.5 volume of ethanol was added. The mixture was incubated on ice for 20 min and then centrifuged at 12,000g for 20 min at 4°C. The supernatant was collected for ethanol precipitation of RNA. mRNA was isolated from the total RNA with the Poly-A-Tract mRNA isolation system (Promega, Madison, WI).

cDNA Library Construction

A cotton fiber cDNA library corresponding to the secondary (24 DPA) wall stage was constructed in the Uni-ZAP XR λ vector (Stratagene, La Jolla, CA) as specified by the manufacturer.

Cloning of Cotton Cellulose Synthase

The catalytic subunit domain of *Acetobacter xylinum* was used to screen expressed sequence tags (ESTs) from the database. A rice EST (D41261) gave a close match. Then, polymerase chain reaction (PCR) was performed by using a specific primer from the rice EST (5′-GATTACCCAGTTGADAAGGTT-3′; note that D represents A, G, or T), the T7 primer, and a cotton cDNA library as the template. The cDNA library was obtained from 24 DPA cotton fibers. The PCR product was cloned into pCR-Script SK+ (Stratagene) and sequenced.

Expression of the Catalytic Subunit Region of Cotton Cellulose Synthase

PCR was performed to obtain the catalytic subunit domain using the specific primers primer 1 (5′-CAGTCATATGGATTACCCAGTTGA-GAAGGTT-3′) and primer 2 (5′-GCCAAAGCTTCCTACCACTATAAC-CATACCA-3′). The recombinant plasmid mentioned above was used as a template. The PCR product was cloned into pET-21a(+) (Novagen, Madison, WI) at the NdeI and HindIII sites. This product also had a six-histidine tag. The recombinant plasmid was transformed into *Escherichia coli* BL21(DE3). The transformed cells were cultured in Luria-Bertani medium until the OD_{600} reached 0.4 to 0.6. Then, cells were induced with 1 mM isopropyl-β-thiogalactopyranoside and incubated for another 4 hr. These induced cells were collected and used to purify recombinant proteins.

Antibody Production

The recombinant protein was purified using Ni+ affinity chromatography column according to the manufacturer's protocol (Novagen). The purified recombinant protein was injected into rabbits to produce antibody according to standard protocols (HTI Bio-Products, Inc., Ramona, CA). The preimmune antiserum was collected before the first injection, and the antisera were collected after each of three injections.

Plasma Membrane Protein Isolation

The plasma membrane proteins from 14 and 24 DPA cotton fibers as well as Arabidopsis and *V. radiata* were prepared according to Okuda et al. (1993).

Protein Gel Blot Assay

The *E. coli* BL21(DE3) cell lysate as well as plasma membrane fractions from cotton, Arabidopsis, and *V. radiata* were subjected to 6.0 to 7.5% and 10% SDS-PAGE and electrotransferred to a nitrocellulose membrane (Novex, San Diego, CA). After blotting, proteins and molecular weight markers were stained in 0.2% Ponceau S to visualize proteins and molecular mass markers. The protein blot was blocked in 5% blocking reagent in Tris-buffered saline (TBS; ECL Western Blotting Protocols; Amersham) and 0.1% Tween-20 overnight. Then, the blots were incubated with either antibody (1:1000) or

preimmune serum (1:1000) and incubated on a shaker for 1 hr. The blots were washed three times for 10 min each in TBS and 0.1% Tween-20. For chemiluminescent detection, the blots were incubated with a goat anti–rabbit IgG (H+L) horseradish peroxidase–conjugated secondary antibody (Bio-Rad) for another hour. The blots were washed three times for 10 min each in TBS and 0.1% Tween-20. The blots were incubated in development reagent according to the manufacturer's instructions (Amersham, Piscataway, NJ). For color development, the blots were incubated with a goat anti–rabbit IgG (H+L) alkaline phosphatase–conjugated secondary antibody (Bio-Rad) for another hour. The blots were washed three times for 10 min each in TBS and 0.1% Tween-20. The blots were washed once with TBS for 5 min and rinsed with distilled water. The blots were incubated with 100 mL of 100 mM Tris-HCl, pH 9.5, 0.5 mM $MgCl_2$ buffer, 1 mL of 15 mg/mL 5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt, and 1 mL of 30 mg/mL p-nitro blue tetrazolium chloride. The reaction was stopped by rinsing the blots with distilled water. Results of protein gel blot analysis are shown in Figure 1.

Freeze Fracture

The SDS-digested freeze-fracture replica labeling technique was conducted according to the methods of Fujimoto (1995), except that the replicas were pretreated with a cell wall digestive solution containing 2% Cellulase Onozuka RS (Yakult, Tokyo, Japan), 0.5% Hemicellulase (Nakarai Chemical, Kyoto, Japan), 0.5% Pectolyase Y-23 (Seishin Corporation, Tokyo, Japan), protease inhibitor cocktail tablet Complete (Boehringer Mannheim, Mannheim, Germany), and 50 mM acetate buffer, pH 5.5. Segments 2 to 3 mm long were cut from elongating epicotyls of Azuki bean (*Vigna angularis* Takarawase) grown in a growth chamber under continuous light at 27°C for 5 days. The segments were placed on the specimen carrier and then quick-frozen by liquid propane in a Reichert KF80 quick-freezing unit (Leica, Germany). The frozen samples were fractured in a Balzers BAF 400D freeze-etch unit (Balzers Union, Liechtenstein) at −110°C, replicated by evaporation of platinum–carbon from an electron beam gun positioned at a 45° angle, followed by carbon coating. To release the replicas from the specimen carrier, the carrier was immersed gently in PBS. The replicas attached with samples were transferred to the cell wall digestive solution. Cell wall digestion was performed for 2 hr at room temperature with continuous shaking on a rotary shaker at 100 to 200 rpm.

After cell wall digestion, the pieces of replica were transferred to 2.5% SDS containing 10 mM Tris-HCl, pH 8.3. SDS digestion was conducted for 2 to 12 hr at room temperature with continuous shaking on a rotary shaker at 100 to 200 rpm. After treatment with SDS, replicas were washed four or more times with PBS and placed on drops of 1% BSA in PBS (BSA-PBS) for 30 min at room temperature. The replicas were then labeled with CelA antibody or preimmune serum diluted 1:100 in BSA-PBS overnight at 4°C. After labeling, the replicas were washed three times with PBS and incubated for 2 hr at room temperature with the secondary antibody–conjugated 10-nm colloidal gold (Zymed Laboratories, San Francisco, CA) diluted 1:50 in BSA-PBS. After immunogold labeling, the replicas were washed three times with PBS, fixed with 0.5% glutaraldehyde in 50 mM phosphate buffer, pH 7.4, for 10 min at room temperature, washed twice with distilled water, and picked up onto Formvar-coated grids. For details of the novel procedure, see Figure 1. Figure 2 provides spatial information on the labeling components, the 10-nm gold, the primary and secondary antibodies, and the rosette TC. The replicas

were observed with a transmission electron microscope (model 2000EXII; JEOL, Akishima, Japan).

Quantative Analysis of Gold Labeling

For quantitative analysis of the gold labeling, the distance was measured from the gold particles to the edge of the nearest rosette. This process was continued until all gold particles within a given photograph were counted. For the preimmune sera, 98 gold particles from 10 different photographs representing eight different cells were analyzed. For the immune sera, 217 gold particles from 20 different photographs representing 20 different cells were counted. The data are summarized in Figure 6.

To test whether the distributions of the immune and preimmune particles within 20 nm of rosette TCs were significantly different, we first tested whether the individual cell distributions for each treatment were significantly different. Individual gold particles were classified as either close enough to a rosette to have labeled it (<20 nm) or too distant (>20 nm). A Kruskal-Wallis test was then performed on the numbers of particles that were close and far for the cells of each treatment. Nonsignificant results (P > 0.29 for both immune and preimmune cells) indicated that the data for all the cells of a treatment could be lumped. A chi square test on the distribution of near and far gold labels for the immune and preimmune sera was performed.

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