

# EVIDENCE FOR AN INTRAMEMBRANE COMPONENT ASSOCIATED WITH A CELLULOSE MICROFIBRIL-SYNTHESIZING COMPLEX IN HIGHER PLANTS

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## ABSTRACT

Freeze-fracture of rapidly frozen, untreated plant cells reveals terminal complexes on E-fracture faces and intramembrane particle rosettes on P-fracture faces. Terminal complexes and rosettes are associated with the ends of individual microfibril impressions on the plasma membrane. In addition, terminal complexes and rosettes are associated with the impressions of new orientations of microfibrils. These structures are sparse within pit fields where few microfibril impressions are observed, but are abundant over adjacent impressions of microfibrils. It is proposed that intramembrane rosettes function in association with terminal complexes to synthesize microfibrils. The presence of a cellulosic microfibril system in *Zea mays* root segments is confirmed by degradation experiments with *Trichoderma* cellulase.

**KEY WORDS** freeze-fracture · cellulose  
microfibril synthesis · terminal synthesizing  
complex · intramembrane rosette subunits

Cellulose synthesis is an integral process for the regulation of growth and morphogenesis in higher plants. Crystalline cellulosic microfibrils provide the architectural framework of the cell wall, and, together with noncellulosic matrix materials and cytoskeletal components, they are determinants of cell shape (9). In spite of the abundance of data available on cellulose structure and fiber modification in relation to industrial applications, very little is known about the fundamental mechanisms of microfibril assembly at the cellular level. Attempts to solubilize enzyme systems from the plasma membrane and to synthesize cellulose in vitro have met with little success (6).

Using improved freeze-fracture procedures, re-

cent studies have reported the visualization of presumptive cellulose-synthesizing complexes in association with the plasma membrane (2). Unusually ordered complex structures at the ends of growing microfibrils have been observed in algae, and the structural information gained has been useful to the investigation of cellulose synthesis (12, 25). Terminal complexes also have been observed in higher plant cells (13, 24, 26), but to date it has been considerably more difficult to obtain evidence for their structure and function.

This paper presents new structural information on the terminal complexes in higher plant cells. Particle rosettes on P-fracture faces are located at the ends of microfibril impressions, suggesting that they are complementary to the E-fracture face terminal complexes. Thus, the microfibril-synthesizing complex may comprise a transmembrane component. Additional evidence is presented

which supports the interpretation that terminal complexes synthesize cellulose microfibrils in association with the plasma membrane.

## MATERIALS AND METHODS

Corn (*Zea mays*, Burpee's Snowcross) and mung bean (*Phaseolus aureus*) seeds were surface sterilized in a 10% Clorox solution, rinsed three or more times in deionized water, and soaked for ~6 h in deionized water. Seeds were germinated in the dark, 3 or 4 d for corn and 7 d for mung beans. Pine (*Pinus taeda*) seeds were germinated and grown aseptically for ~1 mo before use.

Small segments of tissue were cut and placed immediately on 1-mm gold specimen holders (in water) and, without any prior treatment with fixative or cryoprotectant, were quickly frozen in liquid Freon 22 maintained at liquid nitrogen temperatures, then transferred to liquid nitrogen for storage.

Alternatively, segments of tissue were cut and placed in the center of a pair of single-hole (0.5 or 1.0 mm) gold carriers for double-replica freeze-fracturing. The grids were manufactured by Balzers Corp. (Nashua, N. H.). Liquid nitrogen was supercooled to a solid by placing the liquid into a specially designed styrofoam container and pumping to 80 Torr in a Kinney vacuum evaporator (Kinney Vacuum Co., Boston, Mass.). The container of solid nitrogen was brought to atmosphere, and the sandwiched grids were plunged into the solid nitrogen layer for 10 s. No gaseous nitrogen was produced. Specimens were transferred to the hinged double-replica device (Balzers Corp.) under liquid nitrogen until transferred to the specimen stage.

A Balzers BA 360 M freeze-etch apparatus was used as follows: For conventional freeze-etch, the tissue was fractured, etched for 0.5 min at  $-106^{\circ}\text{C}$ , shadowed with platinum-carbon, and coated with carbon at  $10^{-6}$  Torr. For double-replica freeze-fracture, the fractured halves were replicated immediately as described above. Replicas were cleaned in a 5% sodium dichromate (wt/vol)-50% sulfuric acid mixture and mounted on uncoated or Formvar-coated copper grids.

Fixations were carried out on small pieces of corn root tissue by first fixing in 2% glutaraldehyde in 0.05 M cacodylate buffer, pH 6.8, with 0.25% tannic acid at room temperature for 30–60 min in the dark followed by an additional 1.5–2.0 h in the cold and dark. The tissue was washed in the same buffer and postfixed in 2% osmium tetroxide in the same buffer for 2 h in the cold and dark. Dehydration was done with an ethanol-acetone series. The tissue was infiltrated and polymerized in Spurr's resin (21). Silver-gray sections were poststained in uranyl acetate and lead citrate after mounting on uncoated grids. Thin-section and freeze-fracture material was photographed with a Hitachi HU-11E-1 electron microscope.

Particle dimensions were measured in a minimum of 30 samples, and the mean particle dimensions along with the standard deviation are presented.

The presence of cellulose in *Zea mays* root segments was determined using the complete cellulase system of *Trichoderma reesei*. Root segments were frozen in liquid nitrogen, then ground with a mortar and pestle. The wall fragments were treated with 4% NaOH in a boiling water bath for 10 min, after which they were washed twice with deionized water. These fragments were subsequently treated with the *Trichoderma* cellulase system (1 mg/ml) for 1, 2, 4, and 7 min. Wall fragments and microfibril degradation were followed by electron microscopy using negative staining. Glucose release from 3-d incubations was measured using the Statzyme glucose test.

## RESULTS

Previously, we demonstrated the presence of terminal complexes in higher plant tissue (13). Impressions of complexes associated with the ends of microfibril impressions were observed on E-fracture faces of plasma membranes from cells of corn root stelar tissue. The terminal complex attached to a microfibril occasionally was torn back through the exoplasmic leaflet of the membrane during the fracture process, leaving behind an enlarged hole in the membrane and an associated tear. Thus, microfibril tears with terminal complexes are continuous with impressions, demonstrating that individual microfibrils and their complexes are resolved on membrane fracture faces.

In the present study, supercooled liquid nitrogen combined with double-replica freeze-fracture produced larger expanses of membrane with reduced freezing and mechanical deformation, although some poorly preserved areas were occasionally observed. Increased quality of preservation was also obtained with conventional freeze-fracture when small samples of tissue were frozen in nearly solidified Freon 22 and the tissue was fractured as closely as possible to the gold grid.

The amount of shadowing metal and the angle at which it is deposited affect the appearance of membrane structures. When the longitudinal axis of a corn root (and therefore of individual cells) is placed parallel to the knife arc in a conventional freeze-fracture run, the angle of shadow is perpendicular to the cell axis. Thus, a concave E-fracture face shows a gradient in the angle of metal deposition (Fig. 1*a*). In the left and right regions of Fig. 1*a*, membrane morphology is revealed by shallow- and steep-shadowing angles, respectively (see Fig. 1*b* and *c*). Because more details are revealed with a shallow-shadowing angle, electron micrographs were made of these membrane faces.

An awareness of artifact-inducing forces is very important when analysis is made of membrane structures postulated to be associated with microfibril synthesis. We have judged membranes to be well preserved by the following criteria: (*a*) The presence of microfibril impressions on the plasma membrane, suggesting that the cell was functioning normally and was turgid just before freezing; (*b*) the absence of ice crystals between the wall and the plasma membrane; (*c*) the absence of any obvious plastic deformation of intramembrane particles (see references 7 and 23); (*d*) the absence of striations on smooth portions of the membrane;

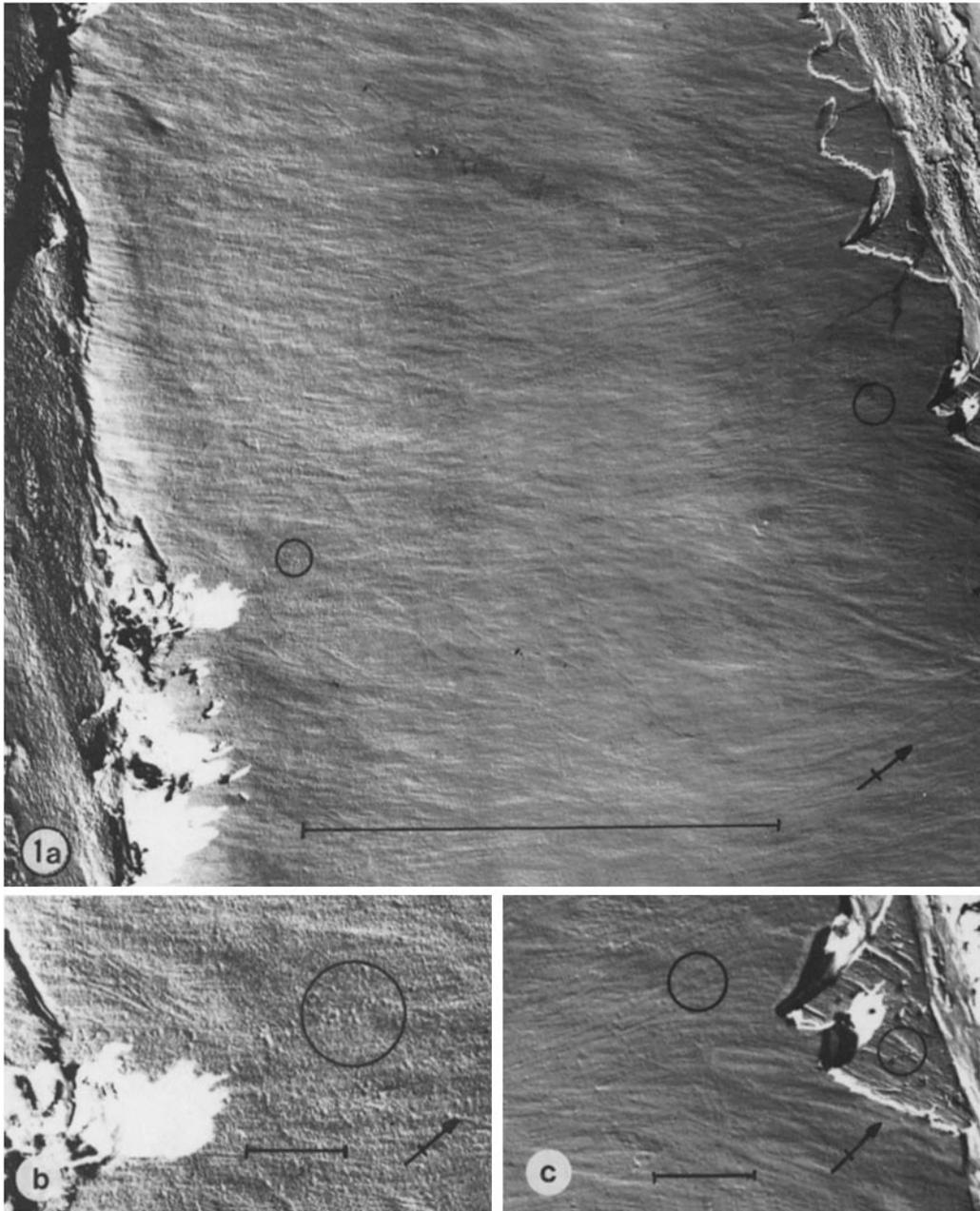


FIGURE 1 (a) Freeze-etch of a corn root cortex. An E-fracture face reveals microfibril impressions with orientations approximately transverse to the cell axis. The cell wall is observed in cross fracture (left) and in tangential fracture (right). Bar,  $1 \mu\text{m}$ .  $\times 67,500$ . (b and c) Note apparent differences in membrane morphology which result from the deposition of shadowing metal on a concave surface. On the left (1 b) the shadowing angle was shallow and on the right (c) it was steep. More details of the membrane are revealed with shallow angles of shadowing depicted in Fig. 1 b. Circle (left) denotes a group of terminal complexes. (b and c)  $\times 140,400$ . For all figures, magnification bars are  $0.1 \mu\text{m}$  unless otherwise noted. Arrows indicate direction of shadow unless otherwise noted.

(e) the absence of knife marks on the membrane accompanied by micromelting.

E-fracture faces of plasma membranes from corn mesocotyl parenchyma reveal terminal complexes in association with impressions of the most recently synthesized microfibrils (Fig. 2). In contrast, impressions of older microfibrils lacking terminal complex impressions are viewed adjacent to the newer microfibril bands. Fig. 2 (inset) shows three terminal complexes at high magnification. These particular terminal complexes measure 24 nm and appear very well preserved. Terminal complexes on less well-preserved membranes generally are smoother in appearance (13). Size measurements are difficult to make because the terminal complex is less clearly defined when viewed as an impression upon the membrane. Although well-preserved terminal complexes measure ~22–24 nm in diameter, the average diameter of terminal complexes including those from poorly preserved membranes is  $28 \pm 3.0$  nm.

P-fracture faces (Fig. 3) of corn mesocotyl plasma membrane reveal intramembrane particle rosettes over the impressions of newly deposited microfibrils. Fig. 3 (inset) represents well-preserved rosettes consisting of six intramembrane particles surrounding a central, non-shadowed region. The mean diameter of the rosette (measured from the outer edge of particles on either side of the depression) is  $24 \pm 2.5$  nm. The same criteria for identification of a well-preserved membrane are applied for P-fracture faces as for E-fracture faces. Unlike the size variation of EF terminal complexes, the rosette dimensions are constant in membranes which reveal different states of preservation. The intramembrane particles of the rosette, however, are indistinct on less well-preserved plasma membranes (13; Fig. 5) and are hard to identify.

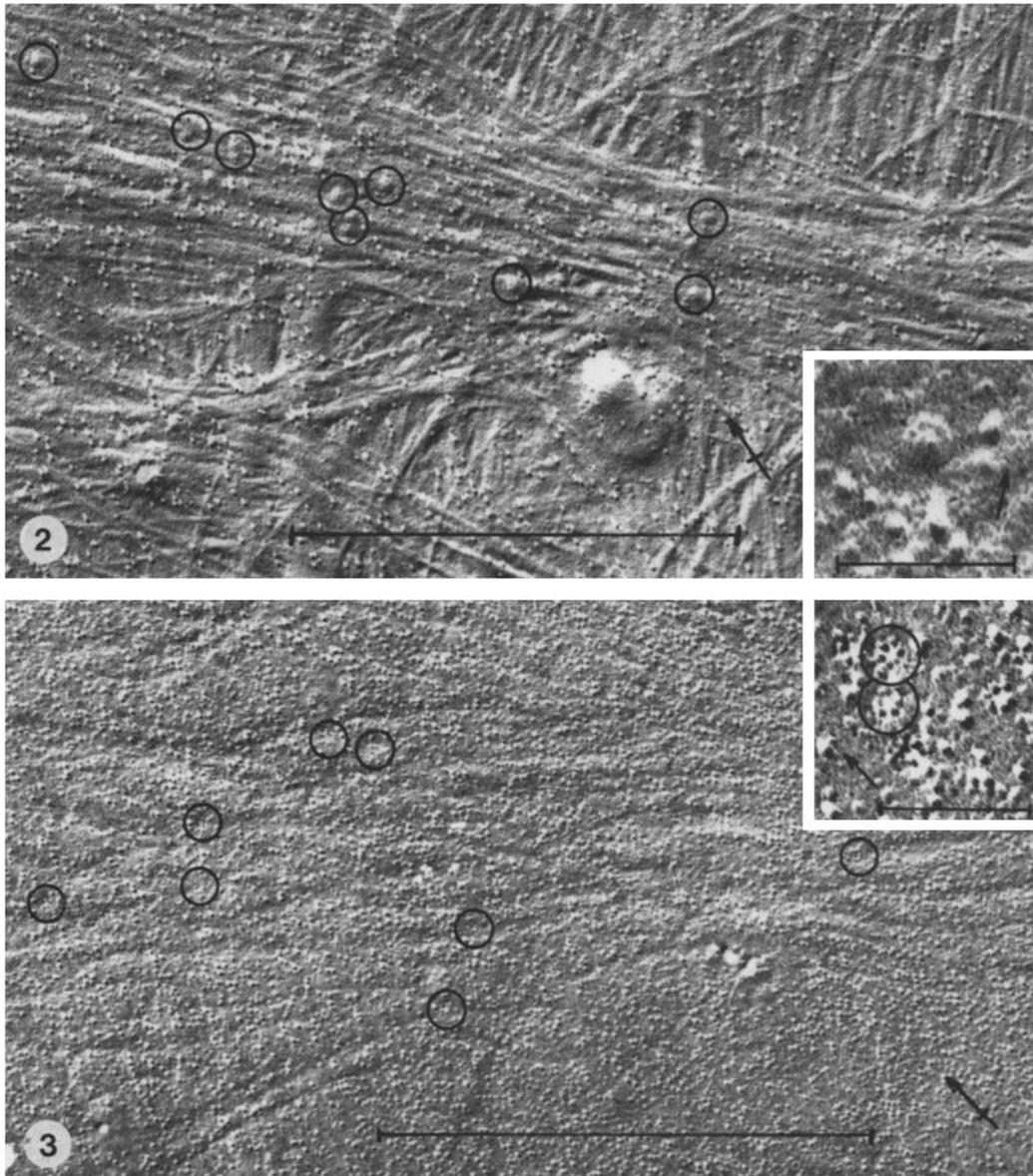
Although the preservation of both terminal complexes and rosettes may depend on the rate of freezing, the visualization of terminal complexes may be dependent upon the turgidity of the cell before freezing (26). When small segments of corn root tissue are exposed to 20% glycerol for 1 min before freezing, the cells lose turgidity, and microfibril impressions are no longer observed (22; Figs. 4 and 5). Whereas terminal complexes were not observed on an E-fracture face, under these conditions rosettes were present on a P-fracture face from an adjacent cell. In contrast, Fig. 2 reveals a cell that was apparently quite turgid before freezing, and terminal complexes are easily seen.

Many terminal complexes from well-preserved membranes appear to contain a central particle surrounded by a peripheral ring of material (Fig. 2, inset). The central particle remains attached to the microfibril when they are both torn through the outer leaflet of the membrane (Fig. 6). The shadowed central particle measures  $16 \pm 2.3$  nm when it is observed attached to an individual microfibril (Fig. 6). The membrane tear reveals a hole in the center of the terminal complex (Fig. 6). It appears that the peripheral ring of material remains behind on the surface of the membrane after the central particle and terminal complex are torn away (Fig. 6; see Figs. 3 and 4 in reference 13).

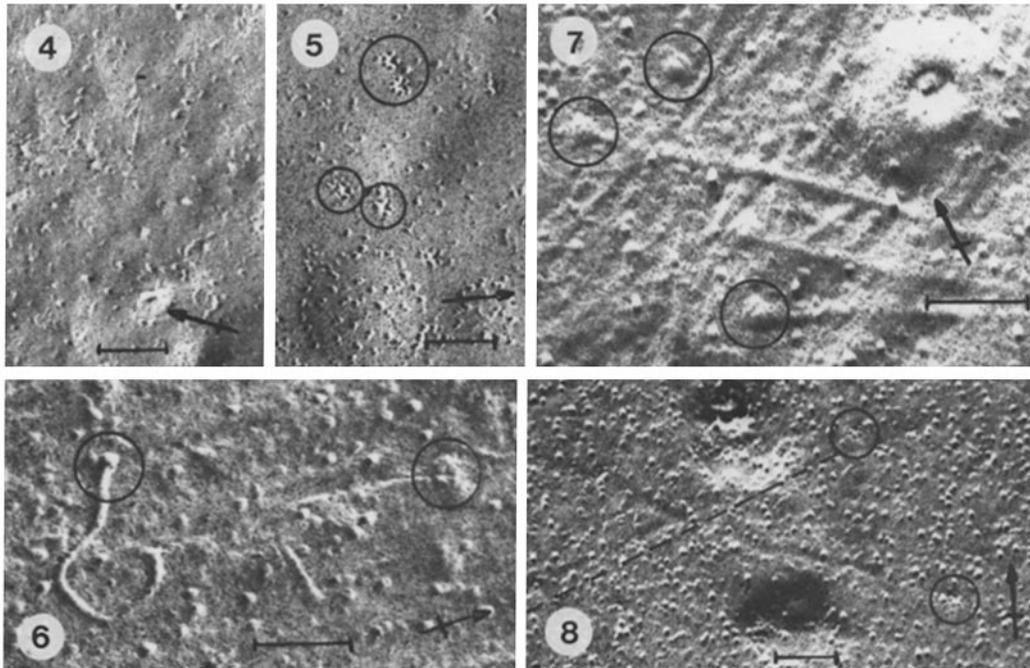
It is difficult to discern an association of terminal complexes or rosettes with specific microfibril termini in parallel orientations of microfibrils; however, individual microfibrils occasionally have orientations which differ from the predominant axis of microfibril orientation. Terminal complexes (Fig. 7) or rosettes (Fig. 8) associated with the termini of these microfibrils are easily observed. Confirmation of the complementarity of rosettes and terminal complexes by the double-replica technique was not obtained.

Although attempted, the recovery of intact, complementary replicas was rendered extremely difficult because of the disintegration of replicas during tissue thawing. Although it is important to test the relationship between rosettes and terminal complexes by the double-replica technique, this may better be accomplished with single-celled plants using matched grid pairs. In thawing corn tissue the replica frequently broke along the cell walls. Thus, replicas of single files of cells resulted. When these replicas were transferred to single-holed grids, matched pairs could not be obtained.

The possibility that the rosette and terminal complex structures are complementary is strongly suggested by their distribution over the newest impressions of microfibrils (Figs. 2 and 3) and by their association with the termini of individual microfibril impressions (Figs. 7 and 8). In addition, few terminal complexes (Figs. 9 and 10) or rosettes (Figs. 11 and 12) are observed on plasma membranes amidst the plasmodesmata of primary pit fields (regions generally lacking microfibrils), whereas many are observed over adjacent impressions of microfibrils. Terminal complexes and rosettes, therefore, appear to be associated with areas of the plasma membrane overlying the newest microfibrils.



FIGURES 2 and 3 Terminal complexes and rosettes are localized over impressions of new microfibrils. (Double-replica technique). Fig. 2: Freeze-fracture of corn mesocotyl. In a parenchyma cell the E-fracture face of the plasma membrane shows impressions of microfibrils from the longitudinal wall. Terminal complexes (circles) are localized over the new microfibril impressions. Bar,  $1\ \mu\text{m}$ .  $\times 60,000$ . *Inset*: Detail of a terminal complex on E-fracture face (not from Fig. 1). Note central subunit.  $\times 244,000$ . Fig. 3: Freeze-fracture of corn mesocotyl. In a parenchyma cell the P-fracture face shows impressions of microfibrils from the longitudinal wall. Intramembrane particle rosettes (circles) are localized over impressions of new microfibrils. Bar,  $1\ \mu\text{m}$ .  $\times 66,000$ . *Inset*: Two rosettes are visualized on P-fracture face. Note that the center of the rosette is not shadowed.  $\times 205,900$ .



FIGURES 4 and 5 Plasma membranes after exposure to 20% glycerol. Fig. 4: Freeze-etch of corn root stelar tissue. Terminal complexes were not visualized on E-fracture faces when fresh tissue segments were incubated for one minute in 20% glycerol.  $\times 92,400$ . Fig. 5: Freeze-etch of corn root stelar tissue. Rosettes (encircled) were visualized on a P-fracture face in a cell adjacent to that shown in Fig. 4. Large circlets of intramembrane particles were observed only after glycerol treatment.  $\times 92,000$ .

FIGURE 6 (Double-replica technique). Freeze-fracture of a corn root cortex. The central subunit of the terminal complex attached to a microfibril (left circle) is torn through the outer (E) leaflet of the plasma membrane. The circle to the right indicates the remaining portion of the terminal complex surrounding the tear left by the central subunit.  $\times 129,600$ .

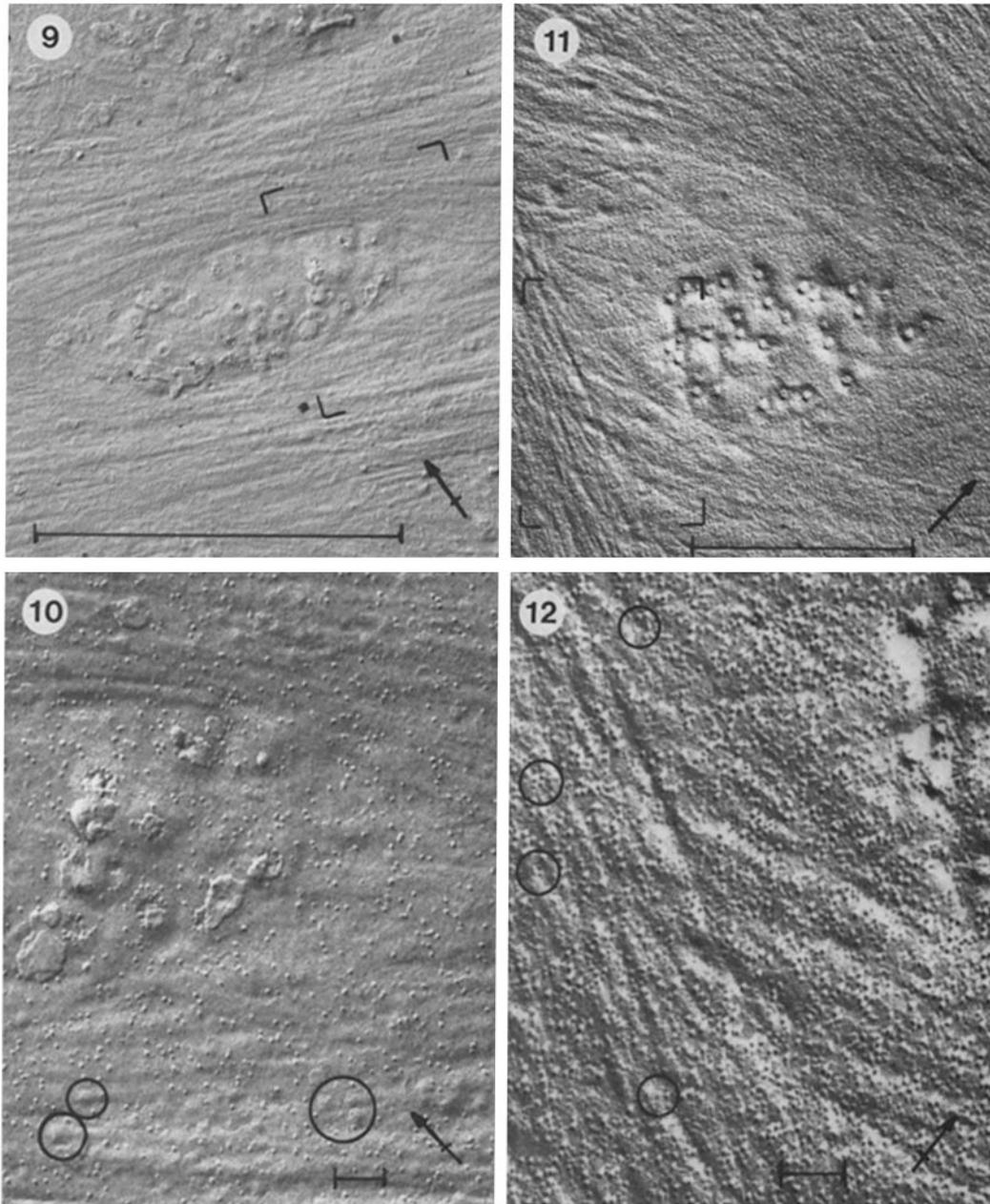
FIGURES 7 and 8 Terminal complexes as well as rosettes are associated with the ends of individual microfibril impressions. (Double-replica technique). Fig. 7: Freeze-fracture of a corn root cortex. Terminal complexes are at the ends of microfibril impressions on E-fracture faces. A plasmodesma is in the field of view at upper right.  $\times 134,400$ . Fig. 8: Freeze-fracture of a corn root cortex. Rosettes (circles) are at the ends of microfibril impressions on the P-fracture face. Two plasmodesmata are in the field of view. Note crossing of microfibril impressions. One is indicated by dashed line.  $\times 81,000$ .

Terminal complexes and rosettes have been observed in parenchyma from corn roots (Figs. 6–10 and 17–19), coleoptiles (S. C. Mueller and R. M. Brown, unpublished observations), mesocotyls (Figs. 2, 3, 11, and 12), and in mung bean hypocotyls (Figs. 13 and 14). Terminal complexes have been observed in pine root parenchyma on E-fracture faces (Fig. 15). In addition, 30-nm depressions, resembling the rosette depressions, have been observed on P-fracture faces of pine roots. Several of these depressions appear to contain rosettes (Fig. 16). Particle rosettes may be more deeply embedded in P-fracture faces of pine

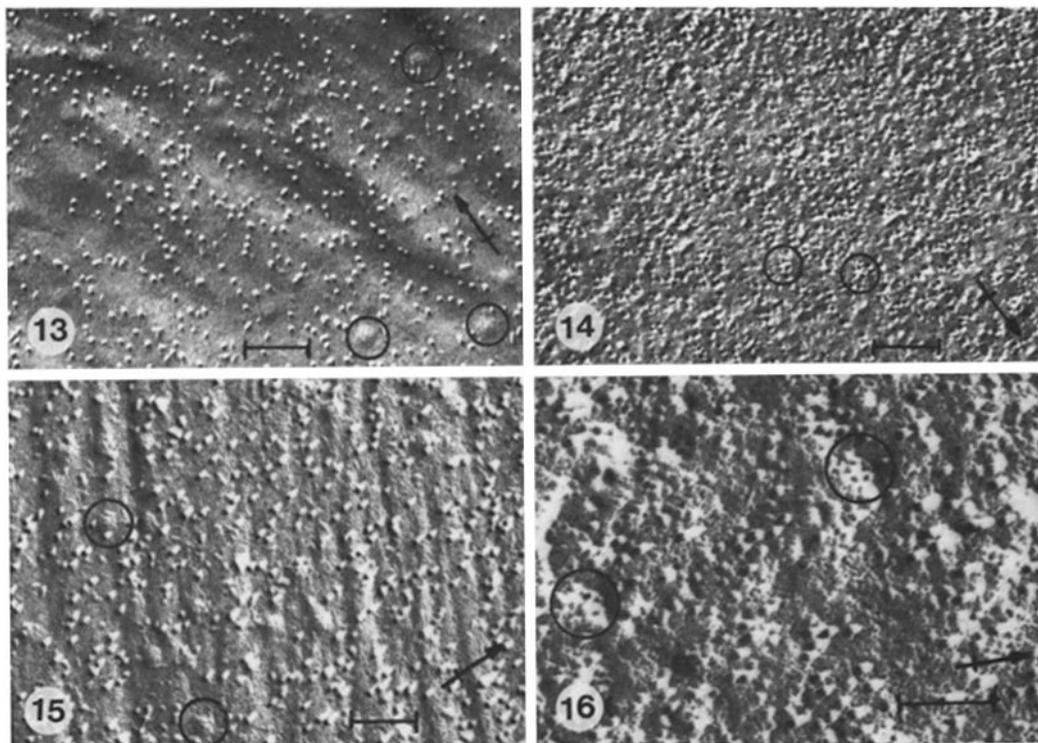
plasma membranes compared to corn, and thus are observed less frequently. In both pine (Fig. 16) and mung bean (Fig. 14), small pits have been observed on P-fracture faces of some cells.

Freeze-fracture reveals groups of 50- to 100-nm depressions on P-fracture faces of corn roots (Fig. 17). We believe that some of the smaller depressions (Fig. 18) contain 24-nm rosettes identical in morphology to those distributed over the membrane face (Fig. 19). Details of the fracture face are not observed in other, larger depressions because of the shadowing angle (Fig. 17).

Ultrathin sections of fixed specimens reveal



FIGURES 9-12 Terminal complexes and rosettes are less numerous within pit fields. (Double-replica technique). Fig. 9: Freeze-fracture of a corn root cortex. An E-fracture face reveals a pit field. Bar,  $1 \mu\text{m}$ .  $\times 51,600$ . Fig. 10: Higher magnification of region bracketed in Fig. 9. Terminal complexes (encircled) are associated with parallel orientations of microfibrils adjacent to the pit field).  $\times 69,600$ . Fig. 11: Freeze-fracture of a corn mesocotyl cell. A P-fracture face reveals a pit field. Bar,  $1 \mu\text{m}$ .  $\times 31,500$ . Fig. 12: Higher magnification view of field bracketed in Fig. 11. Rosettes (encircled) are associated with microfibril impressions adjacent to the pit field.  $\times 82,900$ .



FIGURES 13 and 14 Freeze-fracture of a mung bean hypocotyl. (Double-replica technique). Fig. 13: Terminal complexes are present on an E-fracture face (some encircled).  $\times 84,000$ . Fig. 14: Rosettes are present on a P-fracture face (some encircled).  $\times 84,000$ .

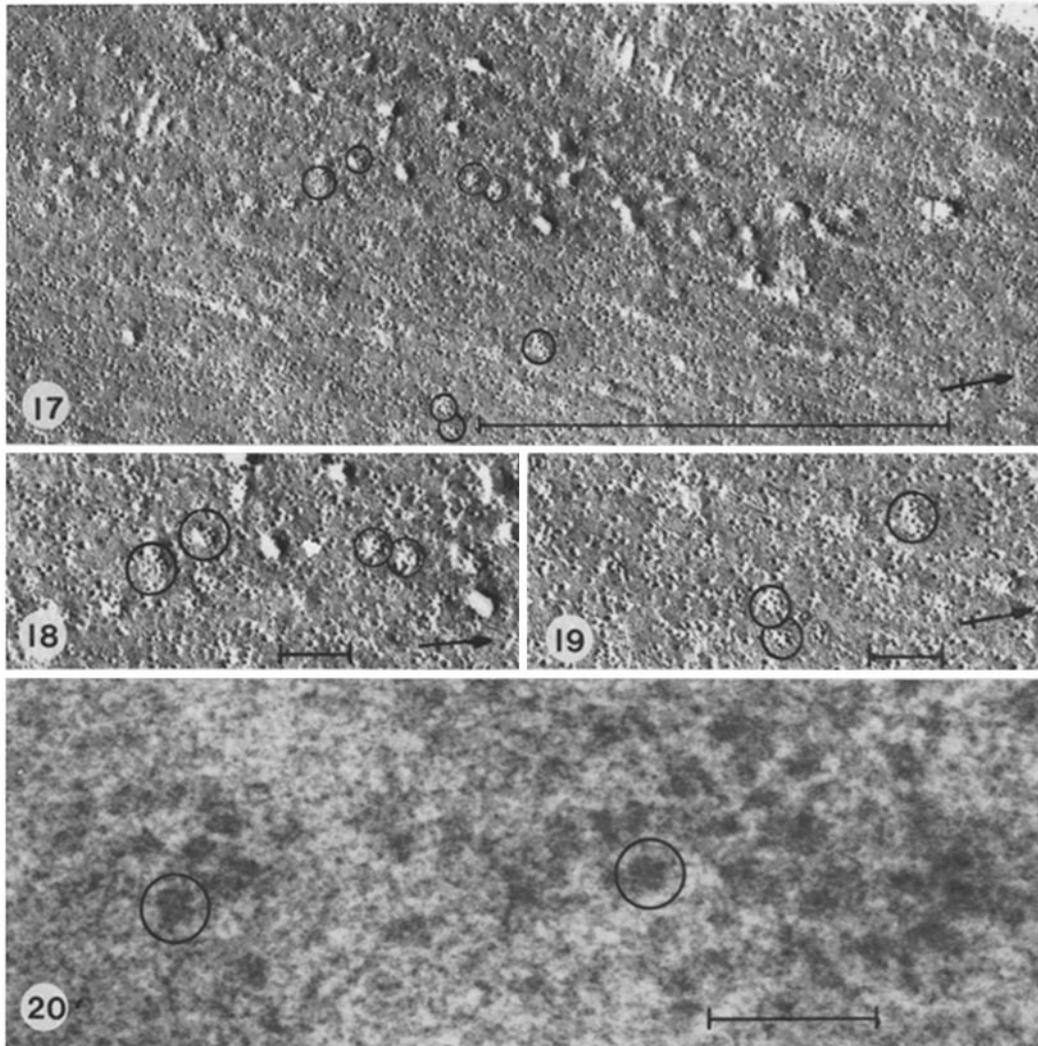
FIGURES 15 and 16 Freeze-fracture of pine root tips. Fig. 15: Terminal complexes are present on an E-fracture face (Some are encircled. Note central subunit.)  $\times 87,000$ . Fig. 16: Depressions in the P-fracture face appear to contain rosettes (encircled).  $\times 128,700$ .

electron-dense profiles on the membrane. In grazing sections through the plasma membrane and cell wall,  $18 \pm 6.3$  nm electron-dense structures are observed (Fig. 20). The observed dimensions of these structures are variable because of the plane of sectioning and degree of staining. In general, the size, frequency, and distribution of these structures coincide with those of the complexes visualized by freeze-fracture.

Although it is generally known that the cell walls of most higher plants are comprised, in part, of cellulose microfibrils, there are apparently no data, at least for the Burpee's Snowcross variety of *Zea mays*, to confirm the presence of cellulose microfibrils. Root segments were treated with NaOH to remove most of the noncellulosic polysaccharides. These segments still remained intact, although the flexibility of the roots was greatly increased. Light microscope examination of the

NaOH-treated root segments revealed normal cell wall structure similar to that of untreated roots. Treatment of these intact root segments for 3-d with the *Trichoderma* complete cellulase system (containing endoglucanase, cellobiohydrolase, and B-glucosidase) completely dissolved the cell walls (and released glucose as measured by the Statzyme test). Examination of this preparation with the electron microscope revealed occasional microfibril fragments but no intact walls.

Cell wall fragments prepared before treatment with NaOH were used to study the progressive degradation of microfibrils by the cellulase system. The control consisted of NaOH-treated wall fragments not incubated with cellulase. Examination of the control by negative staining revealed tightly bound microfibrillar bundles, similar to those within intact cell walls as observed with freeze-fracturing. After 1-min incubation with the cellu-



FIGURES 17-19 Depressions on the plasma membrane of a corn root parenchyma cell which may correspond to vesicles in the process of fusing with the plasma membrane. Fig. 17: Freeze-etch of a corn root cortex. Pronounced band of depressions on P-fracture face. Microfibril orientation is predominantly transverse in this cell. Bar, 1  $\mu\text{m}$ .  $\times 62,700$ . Fig. 18: Higher magnification view of region encircled in Fig. 17 above. Circles indicate two of several depressions in which rosettes are visible.  $\times 92,400$ . Fig. 19: Higher magnification view of region encircled in Fig. 17 above. Circles indicate three rosettes present on the membrane in a region not occupied by the depressions.  $\times 92,400$ .

FIGURE 20 Ultrathin section of glutaraldehyde-tannic acid-OsO<sub>4</sub>-fixed corn root cortex. A grazing section of the plasma membrane and cell wall contains electron-dense profiles (two encircled are 21-24 nm in diameter).  $\times 225,700$ .

lase, microfibrils were completely coated with the enzymes, and the microfibril structure was very difficult to discern. After 2-min incubation, the microfibrils became separated into small bundles, and individual microfibrils were coated with small

particles (presumably the cellulases). After 4-min incubation, there were numerous short microfibril fragments with many breakage loci, and after 7-min incubation, microfibrils could not be detected. Thus, the microfibril component of the Burpee

Snowcross variety of *Zea mays* is cellulose. Although it can be argued that the microfibrillar impressions with terminal complexes are not confirmed directly to represent cellulosic microfibrils, the indirect evidence for their cellulosic nature is quite strong.

## DISCUSSION

Freeze-fractured plasma membranes from corn seedling tissue reveal rosettes and terminal complexes associated with the ends of microfibril impressions. Intramembrane particle rosettes are observed on the protoplasmic leaflet of the fractured plasma membrane, while terminal complexes are observed on the outer surface of the exoplasmic leaflet of the plasma membrane. The complementarity of rosettes and terminal complexes, although not demonstrated unequivocally by double replicas, is strongly suggested by the fact that both are associated with the ends of microfibril impressions (Figs. 7 and 8). The suggestion that rosettes and terminal complexes are complementary is strengthened further by the observed association of both these structures with newly deposited microfibrils (Figs. 2, 3, and 9–12). New orientations of microfibrils frequently appear on plasma membranes as bands. The bands overlie older layers of microfibrils. Terminal complexes on E-fracture faces and rosettes on P-fracture faces are associated with these new bands, but are absent on adjacent fracture faces overlying older microfibril orientations (Figs. 2 and 3). Additionally, rosettes and terminal complexes are scarce within pit fields where fewer microfibril impressions are observed, but are abundant over microfibril impressions adjacent to the pit field (Figs. 9–12).

Furthermore, the similar dimensions of rosettes and terminal complexes are consistent with the interpretation that these structures may be complementary. Fig. 21 *a* illustrates how rosettes and terminal complexes might be juxtaposed within the plasma membrane. The membrane, microfibrils, and the microfibril-associated structures are presented approximately to scale. The dimensions of the rosette, the terminal complex, and the microfibril are derived from freeze-fracture images. Fig. 21 *b* suggests a probable fracture plane which would be consistent with the observed structure in fractured membranes. Two of the six subunits of the rosette (*C*) are diagrammed in cross section. The E half of the membrane contains the central subunit (*A*) associated with a microfibril terminus (Fig. 6). The central subunit could be complemen-

tary to and project into the rosette center. This would account for the nonshadowed central portion of the rosette observed on P-fracture faces. The central subunit also is depicted to be surrounded by a peripheral ring of material (*B*) which is associated with the exoplasmic leaflet of the membrane. This peripheral ring could comprise discrete particles as suggested by Fig. 2 (inset), or it may result from the deformation of the plasma membrane around the central particle. Since microfibril-terminal complex tears are observed not only with conventional fracturing with knife blades but also in double-replica freeze-fracturing, the tendency of the terminal complex and its microfibril to tear through the E membrane half may result from an affinity of the central subunit for the rosette.

The association of terminal complexes and rosettes with the most recently deposited microfibrils and their absence in pit fields where the cell wall is thinner suggest evidence for their function as

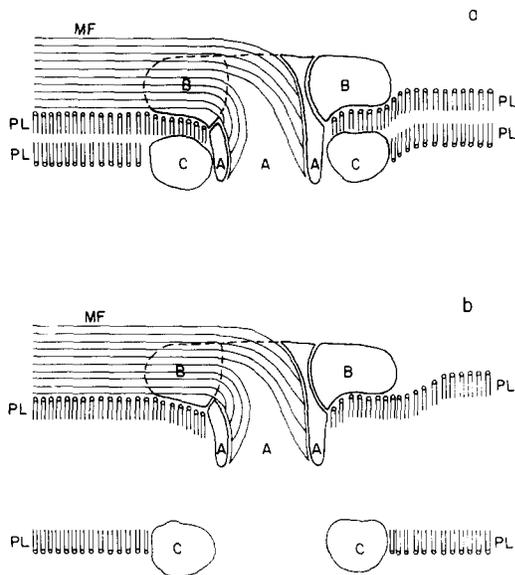


FIGURE 21 A model demonstrating the proposed relationship of terminal complexes and rosettes in association with the plasma membrane. The membrane, microfibrils, and microfibril-associated structures are drawn approximately to equivalent scale. The dimensions of the rosettes (*C*), the terminal complex (*A* and *B*) and the microfibril (*MF*) are derived from freeze-fracture images. The upper diagram illustrates the terminal complex-rosette as it might exist in the intact plasma membrane. The lower diagram reveals our interpretation of the fracture plane which provided the observed freeze-fracture images.

well as their complementarity. It has been proposed that terminal complexes are responsible for the synthesis of wall microfibrils in corn stelar tissue (13). The results from the present study extend that hypothesis to include rosettes functioning in association with terminal complexes to synthesize microfibrils. Furthermore, these membrane-associated complexes are observed in mung bean hypocotyls, pine roots, and in various tissues of corn seedlings. These structures are therefore features of several different higher plants known to be engaged in cellulose synthesis (11). Although microfibrillar wall components in fungi and several algae may comprise chitin or different polysaccharides (17), it is generally accepted that higher plant microfibrils comprise crystalline cellulose (14, 17). Thus, the observation of microfibril-synthesizing complexes implies circumstantially that these complexes could be synthesizing cellulose. This notion is considerably strengthened by the fact that cellulases degrade microfibrillar wall components of the precise region of the root segments of the same variety of *Zea mays* examined by freeze-fracture. Direct evidence that the observed microfibrillar impressions are created by underlying cellulose microfibrils awaits the development of a specific cellulose "stain." The possibility of an immunological approach using ferritin-conjugated antibodies to the *Trichoderma* cellulase system offers an attractive method to identify microfibrils, particularly those that have torn back through the plasma membrane.

The presence of an intramembrane microfibril-synthesizing complex in higher plants is consistent with evidence for complexes in the algae *Oocystis* (1) and *Glaucocystis* (25); however, the intramembrane complexes of these algae are linear and are associated with cellulose microfibrils of larger dimensions. *Acetobacter xylinum*, a cellulose-producing bacterium, also has an integral membrane linear array of particles in association with the newly synthesized cellulose ribbon (3, 27); however, the synthesizing complex is fixed with respect to the cell surface and traverses not only the plasma membrane but also the cell envelope (2). Complexes in higher plants (13) and algae (1, 25) are proposed to be mobile in the fluid plane of the membrane.

Structures postulated to be involved in microfibril synthesis have been reported previously. Willison and Grout (26) have confirmed the association of terminal complexes with microfibril termini in radish roots, and Willison and Brown (24) have

observed globular complexes in cotton fibers. Thin-sectioned particles have been seen in higher plants associated with the outer surface of the plasma membrane (19). These particles may be equivalent to those observed in the present study. Thin-sectioned linear complexes associated with *Oocystis* plasma membranes have been observed (12). These linear complexes have overall structures equivalent to freeze-fractured terminal synthesizing complexes (12). In higher plants, however, different techniques are required to determine the relationship between thin-sectioned and freeze-fractured structures. In the alga, *Micrasterias*, thin-sectioned particles have been observed as ordered arrays on Golgi membranes, and they have been hypothesized to be responsible for the ordered synthesis of microfibrils on the cell surface (10). Additionally, Giddings and Staehelin (see presentation in this issue) have found rosettes on P-fracture faces of *Micrasterias* associated with the ends of microfibril bundles. Because intramembrane particles usually are not identified as such in ultrathin cross sections of the plasma membrane, particles visualized in the above examples may only correspond to the portion of the terminal complex on the membrane surface.

The structure and location of the intramembrane-synthesizing complex could reflect its function in the incorporation of cytoplasmic precursors to form crystalline microfibrils on the surface of the plasma membrane. Deep impressions of the terminal complex and rosette in the fluid lipid bilayer are observed in freeze-fracture (for example, Figs. 2 and 3). The position of the terminal complex and rosette in the membrane may facilitate interactions of the complex with cytoplasmic components. The terminal complex may actually be a transmembrane complex. UDP-glucose precursors are not incorporated into  $\beta$ -1,4-glucans by intact cells but only by cut or damaged cells (4, 18). Raymond et al. hypothesize that pea epicotyl slices incorporate UDP-glucose only through cut cells because the precursor must first be utilized from the protoplasmic side of the plasma membrane (18). Furthermore, freeze-fracture results suggest that the loss of microfibril-synthesizing capacity might be expected if intramembrane rosettes were separated from the microfibril-associated terminal complex subunits during cell fractionation procedures.

Depressions measuring 50- to 100-nm on P-fracture faces of corn roots may correspond to vesicles in the process of fusing with the plasma mem-

brane. These depressions are the same size as microvesicles found in the cortical cytoplasm of higher plants (15, 8). Furthermore, the P-fracture face depressions have a morphology similar to that of presumed vesicle fusion sites in several other organisms (16, 20).

The possibility exists that microfibril-synthesizing precursors are transported by cytoplasmic vesicles since rosettes are observed in some of the smaller depressions. Structural details within the deeper depressions, unfortunately, are not revealed because of the absence of shadowing metal. However, approaches combining freeze-fracture techniques with biochemical and cytological techniques will undoubtedly elucidate the role of cytoplasmic elements in cell wall biosynthesis.

In conclusion, it is proposed that coordinated intramembrane complexes are located at microfibril termini and are responsible for the synthesis of microfibrils. The visualization of these structures by freeze-fracture is dependent upon the quality of preservation of the plasma membrane. Terminal complex rosettes are common features of higher plant cells engaged in cellulose synthesis. We have observed them in corn, pine, and mung bean seedlings. Thus, these complexes may represent cellulose-synthesizing systems. Data on the orientation mechanism for microfibrils will be presented elsewhere.

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## REFERENCES

- BROWN, R. M., JR., and D. MONTEZINOS. 1976. Cellulose microfibrils: visualization of biosynthetic and orienting complexes in association with the plasma membrane. *Proc. Natl. Acad. Sci. U. S. A.* **73**:143-147.
- BROWN, R. M., JR., and J. H. M. WILLISON. 1977. Golgi apparatus and plasma membrane involvement in secretion and cell surface deposition, with special emphasis on cellulose. In *International Cell Biology, 1976-77*. B. R. Brinkley and K. R. Porter, editors. The Rockefeller University Press, New York.
- BROWN, R. M., JR., J. H. M. WILLISON, and C. L. RICHARDSON. 1976. Cellulose biosynthesis in *Acetobacter xylinum*: visualization of the site of synthesis and direct measurement of the *in vivo* process. *Proc. Natl. Acad. Sci. U. S. A.* **73**:4565-4569.
- COOPER, K. M., and R. M. BROWN, JR. 1978. UDP-G utilization by damaged radish root hairs. *J. Cell Biol.* **79**(2, Pt. 2):241 a. (Abstr.).
- COSTELLO, M. J., and J. M. CORLESS. 1978. The direct measurement of temperature changes within freeze-fracture specimens during rapid quenching in liquid coolants. *J. Microsc. (Oxf.)* **112**:17-37.
- DELMER, D. P. 1977. The biosynthesis of cellulose and other plant cell wall polysaccharides. In *Recent Advances in Phytochemistry*. F. A. Loewus and V. C. Runeckles, editors. Plenum Publishing Corp., New York.
- GROSS, H., E. B. BAS, and H. MOOR. 1978. Freeze-fracturing in ultrahigh vacuum at  $-196^{\circ}\text{C}$ . *J. Cell Biol.* **76**:712-728.
- GUNNING, B. E. S., HARDHAM, A. R., and J. E. HUGHES. 1978. Evidence for initiation of microtubules in discrete regions of the cell cortex in *Azolla* root-tip cells, and an hypothesis on the development of cortical arrays of microtubules. *Planta (Berl.)* **143**:161-180.
- HEPLER, P. K., and B. A. PALEVITZ. 1974. Microtubules and microfilaments. *Annu. Rev. Plant Physiol.* **25**:309-362.
- KIERMAYER, O., and B. DOBBERSTEIN. 1973. Membrankomplexe dictyosomaler Herkunft als "Matrizen" für die extraplasmatische Synthese und Orientierung von Mikrofilamenten. *Protoplasma* **77**:437-451.
- LAMPORT, D. T. A. 1970. Cell wall metabolism. *Annu. Rev. Plant Physiol.* **21**:235-270.
- MONTEZINOS, D., and R. M. BROWN, JR. 1976. Surface architecture of the plant cell: biogenesis of the cell wall with special emphasis on the role of the plasma membrane in cellulose biosynthesis. *J. Supramol. Struct.* **5**:277(229)-290(242).
- MUELLER, S. C., R. M. BROWN, JR., and T. K. SCOTT. 1976. Cellulose microfibrils: nascent stages of synthesis in a higher plant cell. *Science (Wash. D. C.)* **194**:949-951.
- NORTHCOPE, D. H. 1972. Chemistry of the plant cell wall. *Annu. Rev. Plant Physiol.* **23**:113-132.
- PALEVITZ, B. A. 1978. Cortical microtubules in plant cells: a high voltage electron microscope (HVEM) study. *J. Cell Biol.* **79**(2, Pt. 2): 278 a. (Abstr.).
- PINTO DA SILVA, P., and M. L. NOGUEIRA. 1977. Membrane fusion during secretion. A hypothesis based on electron microscopic observation of *Phytophthora palmivora* zoospores during encystment. *J. Cell Biol.* **73**:161-181.
- PRESTON, R. D. 1974. *The Physical Biology of Plant Cell Walls*. Chapman and Hall Ltd., London.
- RAYMOND, Y., G. B. FINCHER, and G. A. MACLACHLAN. 1978. Tissue slice and particulate  $\beta$ -1,4-glucan synthetase activities from *Pisum* epicotyls. *Plant Physiol.* **61**:938-942.
- ROBARDS, A. W. 1969. Particles associated with developing plant cell walls. *Planta (Berl.)* **88**:376-379.
- SATIR, B. 1974. Membrane events during the secretory process. *Symp. Soc. Exp. Biol.* **28**:399-418.
- SPURR, A. R. 1969. A low viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.* **26**:31-43.
- WILLISON, J. H. M. 1975. Plant cell-wall microfibril deposition revealed by freeze-fractured plasmalemma not treated with glycerol. *Planta (Berl.)* **126**:93-96.
- WILLISON, J. H. M. 1976. An examination of the relationship between freeze-fractured plasmalemma and cell-wall microfibrils. *Protoplasma* **88**:187-200.
- WILLISON, J. H. M., and R. M. BROWN, JR. 1977. An examination of the developing cotton fiber: wall and plasmalemma. *Protoplasma* **92**: 21-41.
- WILLISON, J. H. M., and R. M. BROWN, JR. 1978. Cell wall structure and deposition in *Glaucozystis*. *J. Cell Biol.* **78**:103-119.
- WILLISON, J. H. M., and B. W. W. GROUT. 1978. Further observations on cell-wall formation around isolated protoplasts of tobacco and tomato. *Planta (Berl.)* **140**:57-58.
- ZAR, K. 1979. Visualization of pores (export sites) correlated with cellulose production in the envelope of the gram-negative bacterium *Acetobacter xylinum*. *J. Cell Biol.* **80**:773-777.