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Spatial representation of cellulose synthesis in the red algal cell wall

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Cytological observations on the red alga *Erythrocladia subintegra*, together with improved freeze-fracture studies using rotary and unidirectional shadowing, have led to the proposal of a membrane flow concept for the origin of terminal complexes (TCs). According to this concept, cellulose synthases (as zymogenic particles, or globules) are made in the endoplasmic reticulum, transferred to Golgi cisternae where they are activated, and transported via Golgi vesicles to the plasma membrane. In the plasma membrane, globules (consisting of more subunits) aggregate, swell and unfold, and become closely arranged contributing to TC assembly. 3D modeling is a powerful tool for better understanding and explaining cell structure and function, also useful for educational purposes. An attempt was made to represent the following in a simple 3D model: (1) formation, development and spatial distribution of the linear terminal complexes (TCs) of *Erythrocladia* cells, (2) cellulose microfibril secretion and assembly and (3) TCs cooperation in order to create the crystalline-phase network of the cell wall.

Key words: Cellulose microfibrils, 3D modeling, *Erythrocladia subintegra,* freeze-fracture, linear terminal complexes (TCs), red alga.

INTRODUCTION

The understanding of the function of biological membranes requires a detailed knowledge of their structural organization. Freeze-fracture investigation of many biological membranes has revealed intramembranous particles which are unevenly distributed between the protoplasmic (PF) and the exoplasmic (EF) fracture faces. Some intramembranous particles are aggregated to form specific complexes (for reviews, see Herth, 1985; Brown, 1996; Tsekos, 1996, 1999). Experimental results have confirmed that cellulose polymerization occurs at the plasma membrane-bound cellulose synthase particles (Giddings & Staehelin, 1991; Tsekos, 1996, 1999; Kimura *et al*., 1999).

The red algal cell wall consists of a microfibrillar (crystalline) phase embedded in a rather amorphous phase (the matrix) (Kloareg & Quatrano, 1988; Tsekos *et al*., 1993; Tsekos, 1996, 1999). The microfibrillar phase is composed mostly of cellulose, xylan, or mannan. Cellulose occurs naturally in two polymorphic forms, cellulose I and cellulose II (Richmond, 1991; Kuga *et al*., 1993). Cellulose I, the dominant natural form of cellulose, has parallel chain morphology.

Conventional freeze-fracture studies on the supramolecular organization of the plasma membrane combined with improved methods using rotary shadowing support the view that the rosettes (complexes of six subunits with sixfold rotational symmetry) in higher plants and both the rosettes and the linear terminal complexes (TCs) in lower plants, are the structures that synthesize cellulose and assemble microfibrils (Brown, 1985; Itoh, 1990; Giddings & Staehelin, 1991; Quader, 1991; Okuda & Mizuta, 1993; Tsekos, 1996, 1999; Tsekos *et al*., 1999). The TCs probably represent multienzyme complexes involved in both steps of cellulose synthesis, glucan chain polymerization and crystalliza-

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tion (Brown & Montezinos, 1976; Giddings *et al*., 1980; Hotchkiss & Brown, 1987; Giddings & Staehelin, 1988; Itoh, 1990; Quader, 1991; Tsekos & Reiss, 1992; Okuda *et al*., 1994; Tsekos, 1996, 1999; Kimura *et al*., 1999; Orologas & Tsekos, 2001).

Based on the experimental data given so far an attempt was made to describe in this paper the kinetics of cellulose synthesis in the red algal cell wall aiming at the better understanding of the cellulose biosynthesis phenomenon.

MATERIALS AND METHODS

Culture

A strain of *Erythrocladia subintegra* (= *irregularis*) was provided by Professor G. Tripodi (see Gargiulo *et al.*, 1987). The algae were grown at 20-23 °C on mica sheets or the bottom of petri dishes containing a modified von Stosch culture medium (Guiry & Cunningham, 1984) with an ES enrichment for seawater (Starr & Zeikus, 1987). Light (16 : 8 h LD) was provided by cool white fluorescent lamps with a photon flux density at the culture surface between 10 and 60 $\mu E/m^2.s$.

Freeze fracturing

Thalli of *E. subintegra* grown for 30 days were scraped off the bottom of the petri dishes, immediately mounted on the depression of a 2 mm single replica gold support or on a 2 mm double replica copper support, and then frozen in liquid nitrogen slush without prior chemical fixation or in liquid propane cooled with liquid nitrogen. Frozen material was stored in liquid nitrogen slush until it was fractured with a Balzers BA 360 or a Balzers BAF 400-T (for details, see Okuda & Brown, 1992). The replicas were examined with a Philips EM 420 and a Zeiss EM 9S2 electron microscopes.

Three dimensional (3D) modeling

On the basis of electron microscope observations done after freeze-fracture and the data selected from relevant papers (Kiermayer & Dobberstein, 1973; Kiermayer & Sleytr, 1979; Giddings *et al*., 1980; Tsekos & Reiss, 1992; Tsekos *et al*., 1996, 1999; Tsekos, 1996, 1999; Graham & Wilcox, 2000), a three-dimensional model of the formation of terminal complexes and cellulose biosynthesis was built in the computer by means of a 3D modeling-rendering program (Orologas-Stavrou, 2002).

RESULTS AND DISCUSSION

Randomly distributed linear protein particle aggregates (TCs) have been observed in the plasma membrane of *Erythrocladia subintegra* (Fig. 1), *Erythrotrichia carnea* and *Porphyra* thallus cells (Tsekos & Reiss, 1992, 1994; Tsekos *et al*., 1993). These linear TCs were often associated with the ends of microfibril impressions. The length of TCs varied widely, while their width was almost constant (Fig. 1). It has been clearly shown that some microfibrils start at the ends of the TCs (Tsekos & Reiss, 1992, 1994; Okuda *et al.,* 1994; Tsekos *et al*., 1996). TCs appear to be formed by repetition of transverse rows of four (*Erythrocladia*, Fig. 1; *Erythrotrichia*) (cf. also Tsekos & Reiss, 1992; Okuda *et al*., 1994), three or two (*Por-*

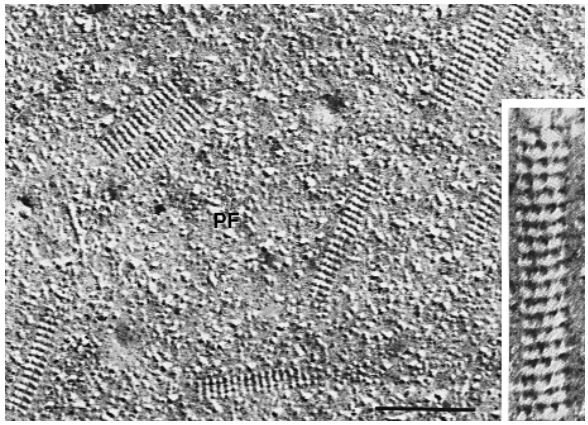


FIG. 1. *Erythrocladia subintegra*. Fracture through a freeze-etched cell at the stage of cell wall synthesis showing the PF face of the plasma membrane. Several linear TCs randomly oriented are visible. Scale bar = 150 nm. **Inset**: A TC at higher magnification. X 198.000.

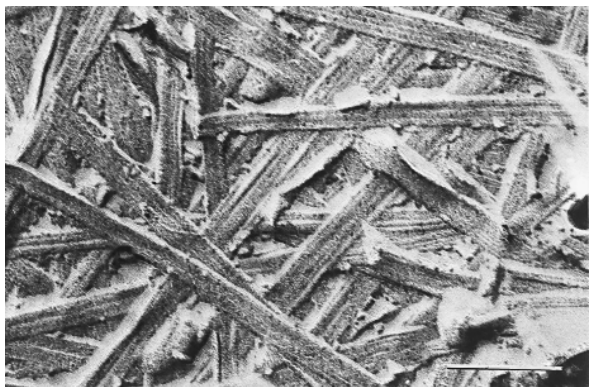


FIG. 2. *Erythrocladia subintegra*. Fracture through the cell wall showing randomly distributed, ribbon-shaped microfibrils. Some fibrils clearly have several linear subcomponents. Note that the width of microfibrils is variable. Scale bar = 150 nm.

phyra yezoensis and *Porphyra leucosticta*) (cf. also Tsekos & Reiss, 1994; Tsekos *et al*., 1999) TC subunits. The number of transverse rows varies between 8-35 (*Erythrocladia*), 7-28 *(Erythrotrichia*), and 3-11 (*P. yezoensis* and *P. leucosticta*), corresponding to variations in TC length (Fig. 1) and microfibril width (Fig. 2).

The cytological observations and the improvements of the freeze-fracture technique (rotary and unidirectional shadowing) in *Micrasterias* on the one hand (Kiermayer & Dobberstein, 1973; Kiermayer & Sleytr, 1979; Giddings *et al*., 1980; see also Haigler & Brown, 1985 for higher plants) and in *Erythrocladia* on the other hand (Tsekos, 1996, 1999; Tsekos *et al*., 1996) have proposed the membrane flow concept (Morré, 1975; Morré *et al*., 1979). The TCs in *Erythrocladia* seem to be assembled from zymogenic precursors, the globules (Figs 3 and 4) (Tsekos *et al*., 1996). The globules may represent a unique packaging device for the transport of TC subunits from the ER via the Golgi apparatus and transport vesicles to the plasma membrane (Fig. 5) (Giddings *et al*., 1980; Haigler & Brown, 1985; Tsekos *et al*., 1996; Tsekos, 1999). The condensed packing of the subunits into a small unit (Figs 3 and 4) seems appropriate because the TCs themselves are rather large for convenient handling by the involved compartments. The dense packing may, in addition, prevent the activating processes, which initiate operating subunits from occurring at the wrong location and time. Therefore, activating processes are thought to be initiated by or after the unfolding of the globules.

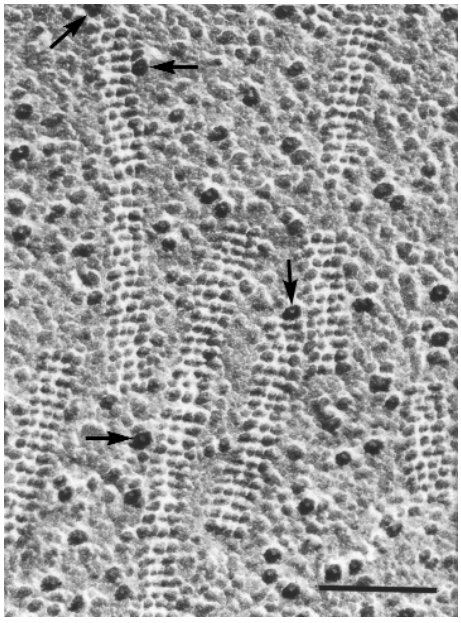


FIG. 3. *Erythrocladia subintegra*. Rotary shadowed PF face of the plasma membrane displaying eight microfibril-active TCs with many globules attached to TCs (arrows). Scale bar = 100 nm.

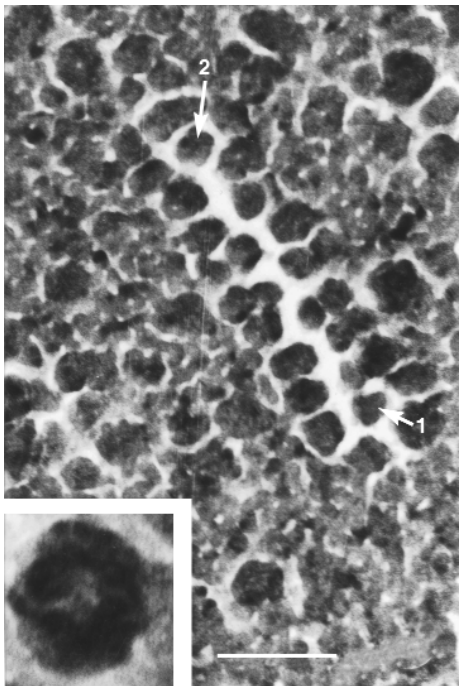


FIG. 4. *Erythrocladia subintegra.* Rotary shadowed PF face of the plasma membrane showing the assembly of a TC. Note the presence of a double row-formation center (arrows 1 and 2) and attachment and unfolding of globules. Scale bar = 30 nm. **Inset**: Details of a large globule consisting of multiple subunits. X 1.200.000.

The initiating factor of unfolding the globules is still obscure. However, based on the present data we tentatively suppose that contact between two or three globules may cause their swelling and unfolding to start (Tsekos *et al*., 1996). In *Erythrocladia,* the globule ridge is probably edged by 8 particles which form 4 doublets (Figs 4 and 8), and after unfolding constitute a single transverse row of TC subunits. However, the possibility that the 8 detectable particles already constitute doublets cannot be excluded. The unfolding of a globule would then give rise to two transverse subunit rows in a TC (Figs 9-11). After the first 2-4 transverse rows of subunits are established (Figs 12-14), globules can attach to both ends of the nascent, still immature TC, to create a double row-formation center (Figs 15, 16, 17) (cf. also Mizuta & Brown, 1992). In *Erythrocladia*, the latter seems to disappear after 15-17 transverse rows of the TC are completed. This contrasts with the situation in *Vaucheria* where the double row-formation

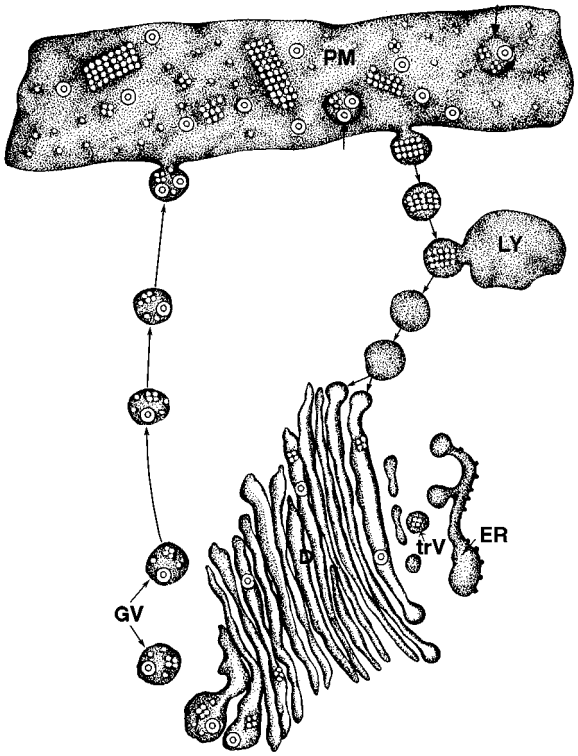


FIG. 5. Scheme showing various possible routes for membrane flow in developing cells of *Erythrocladia* which could lead to TC formation. 1. Membrane flow via transitional vesicles (trV) from endoplasmic reticulum (ER) to dictyosome (D). The membranes of the transitional vesicles probably contain "particle tetrads". 2. Membrane flow via Golgi vesicles from dictyosome to plasma membrane (PM) (exocytosis). The membranes of the Golgi cisternae and Golgi vesicles contain large globules and "particle tetrads". The unfolding multi-subunit globules aggregate in the plasma membrane to form TCs. The depressions (arrows) in the plasma membrane may have arisen from Golgi vesicle fusion. 3. Patches of plasmalemma (membrane subunits with TCs) are recovered by endocytosis and fuse first with the membrane of lysosomes (LY) and then with that of Golgi cisternae.

center is reduced to a single row-formation center after about 10 rows of subunits are established in the plasma membrane. Although globules seem to be attached to both sides of the assembling or mature TCs it cannot be ascertained whether these globules also unfold as in the case of *Vaucheria* (Mizuta & Brown, 1992).

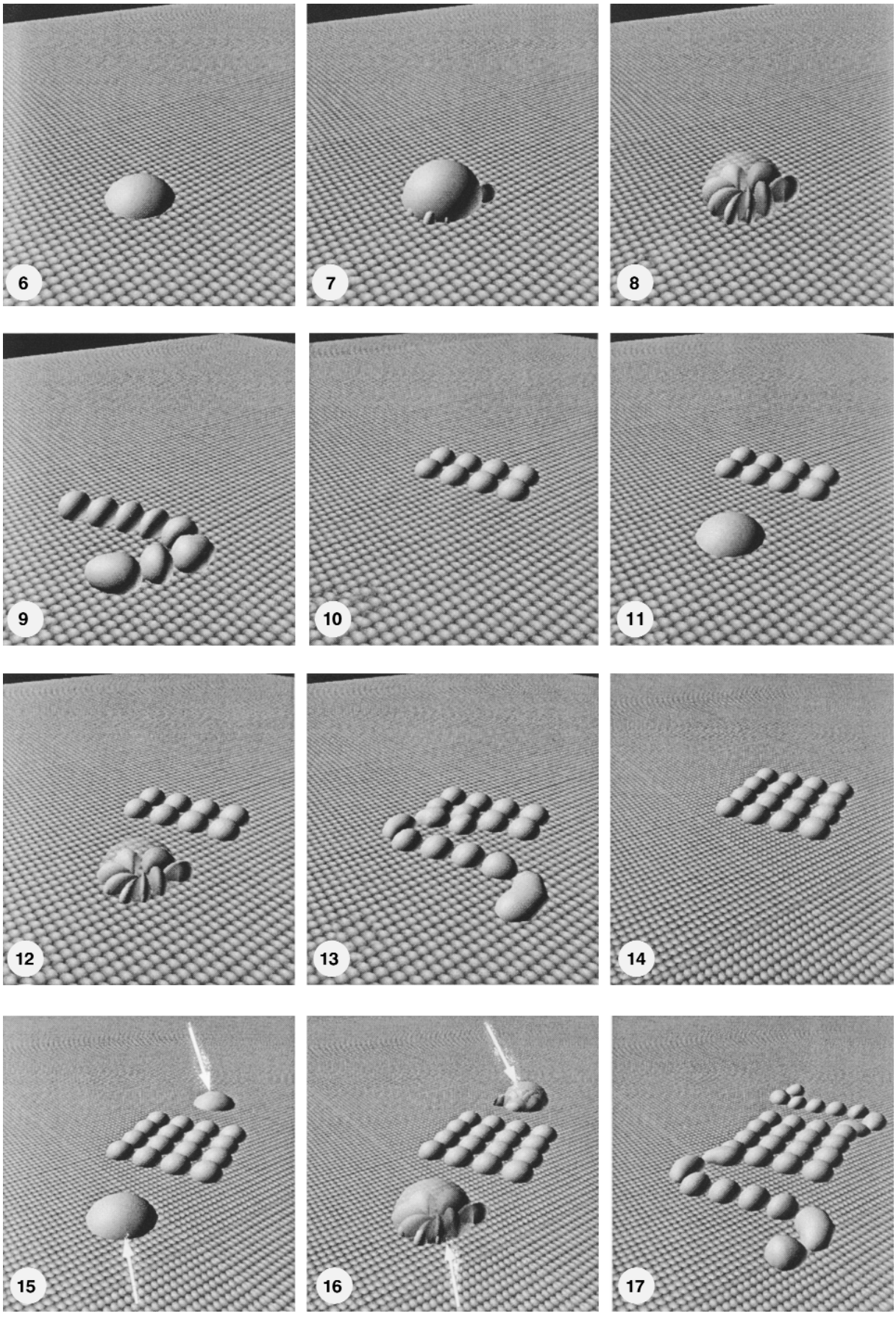
In *Erythrocladia,* the TCs seem to start cellulose synthesis at a time when the double row-formation centers are still visible at the TC ends, whereas in *Vaucheria,* microfibril synthesis seems to begin after the double center is reduced to a "single" row-formation center (Mizuta & Brown, 1992).

As mentioned above, a three-dimensional model of the formation of terminal complexes (TCs) and cellulose biosynthesis was built in the computer by means of a 3D modeling-rendering program (Figs 6-27, cf also Fig. 16-15 of Graham & Wilcox, 2000; Orologas-Stavrou, 2002).

More specifically, our experimental data on *Erythrocladia subintegra* have shown that the ribbon-shaped microfibrils consist of cellulose (Tsekos, 1999). In the same species, the geometry of linear terminal complexes (rectangular) determines the architecture of ribbon-shaped microfibrils with constant thickness (1-1,5 nm) and variable width (13-81 nm). It appears that every transverse row of four TC subunits contributes to the formation of a glucan chain minicrystal (Tsekos *et al*., 1999). The number of minicrystals (elementary fibrils) comprised of a single TC and coupled laterally, equals the number of transverse particle rows. As each row of transverse subunits adds minicrystalls laterally to the microfibril, its width becomes increased. It is suggested that elementary fibrils fasciate during biosynthesis to form microfibrils in such a way that each microfibril behaves as a single crystal.

TC length gradually increases in the plasma membrane (Figs 14-20) and when it reaches 8 to 10 rows of subunits (particles) (Figs 18-20) biosynthesis of β-1,4-glucan chains commences in the form of ribbon-shaped cellulose microfibrils (Figs 18-27). It is

FIGS 6-27. Fragmentary schemes from a 3-dimensional model showing the kinetics and spatial representation of cellulose synthesis in the red algal cell wall.
FIGS 6-17: 3D-model of linear TC assembly in the plasma membrane of *Erythrocladia*. FIGS 6-8: Exocytosis and incorporation of a large globular particle (globule) in the plasma membrane (Fig. 6); globule swelling (Fig. 7) and distinction of 8 subunits (Fig. 8). FIGS 9, 10: Unfolding of the globule gives rise to two transverse subunit rows in a TC. FIGS 11-17: After the first 2 transverse rows of subunits are established, globules can attach to both ends of the nascent, still immature TC (Figs 15 and 16, arrows 1, 2) to create a double row-formation center. ⟶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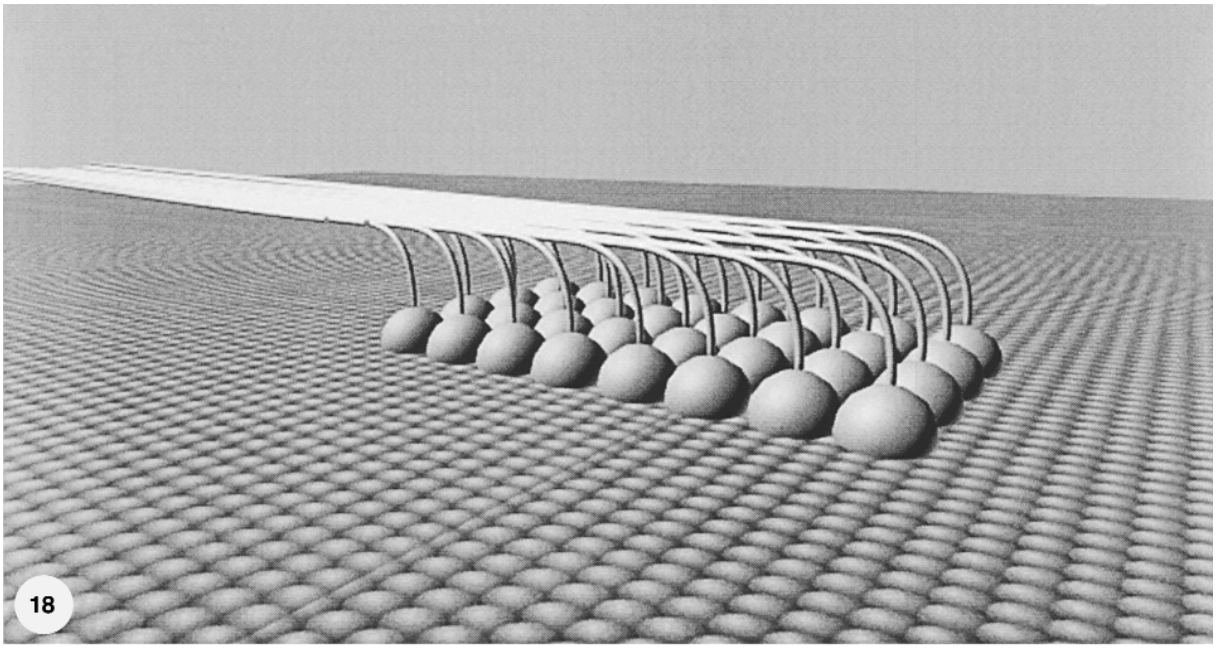
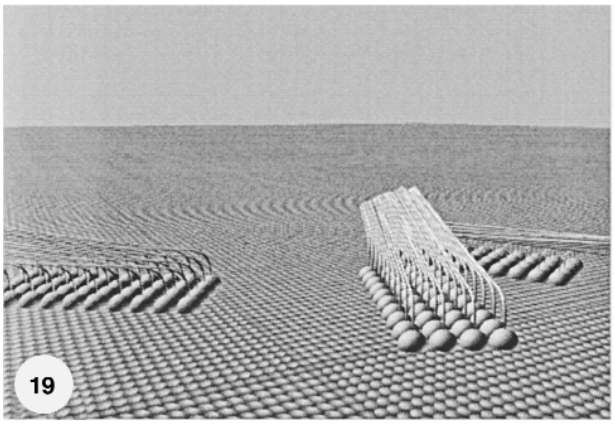
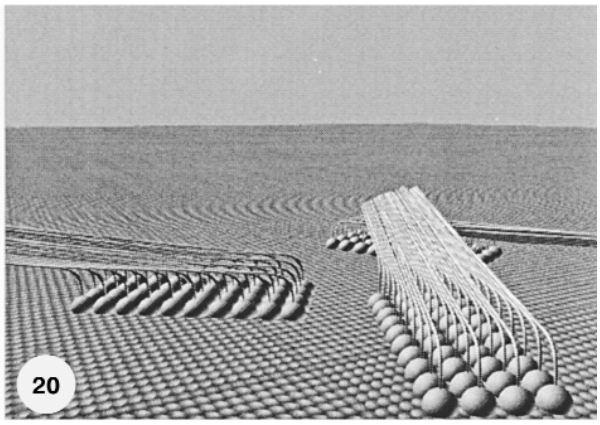
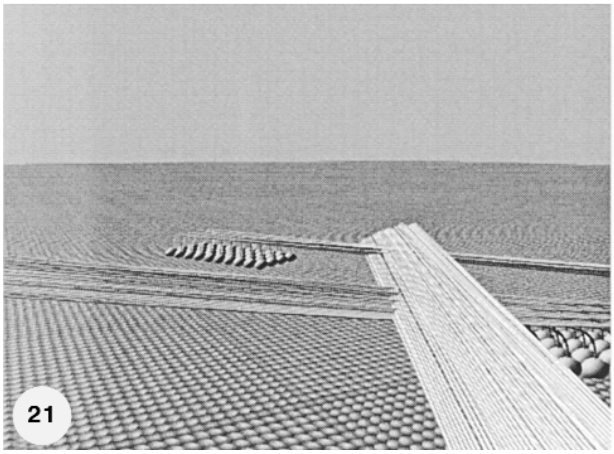
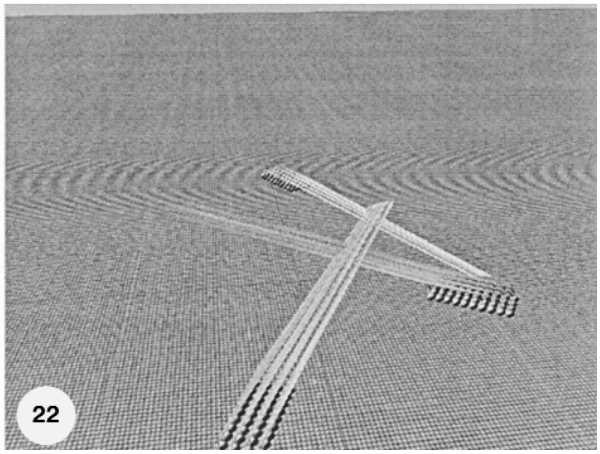


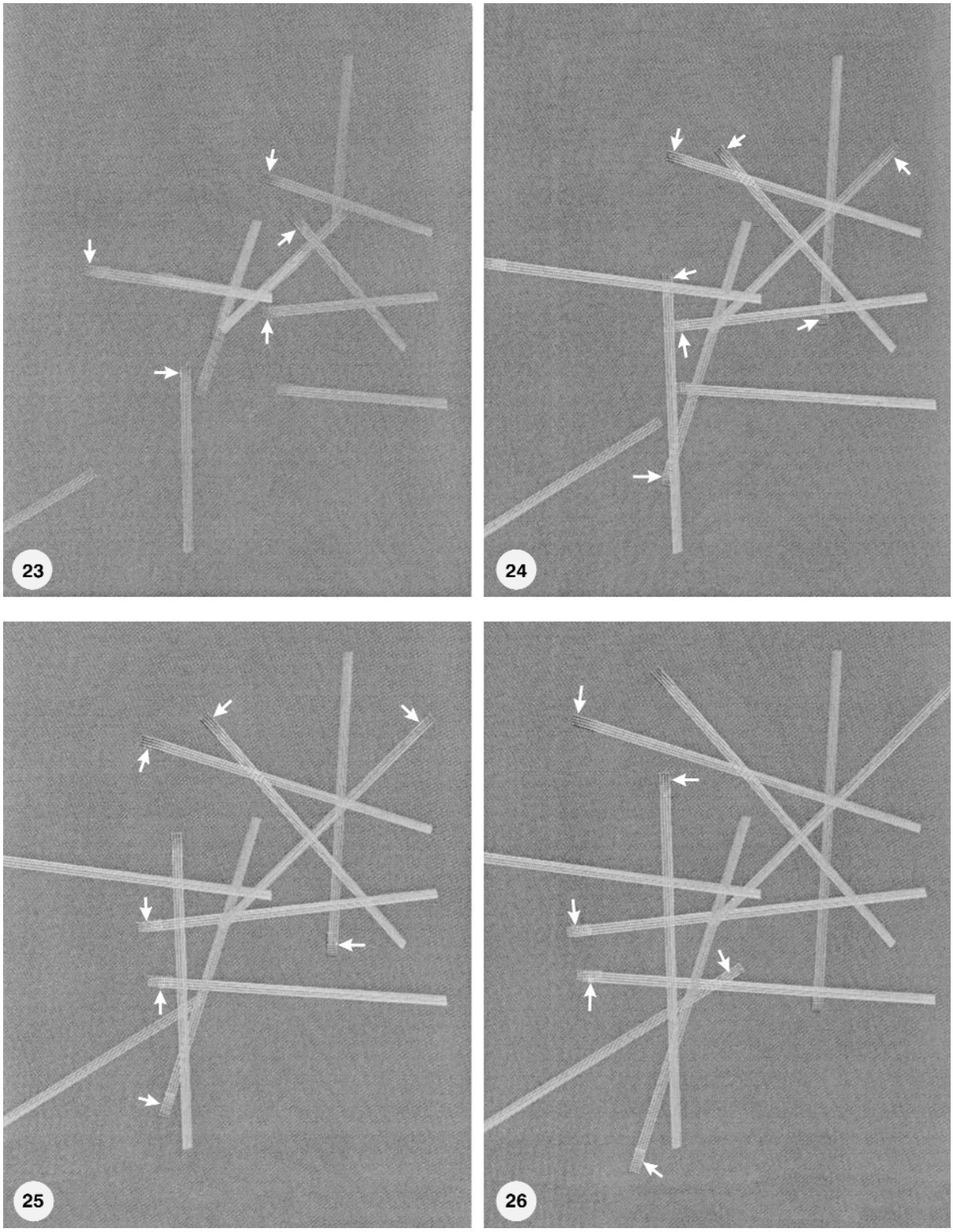




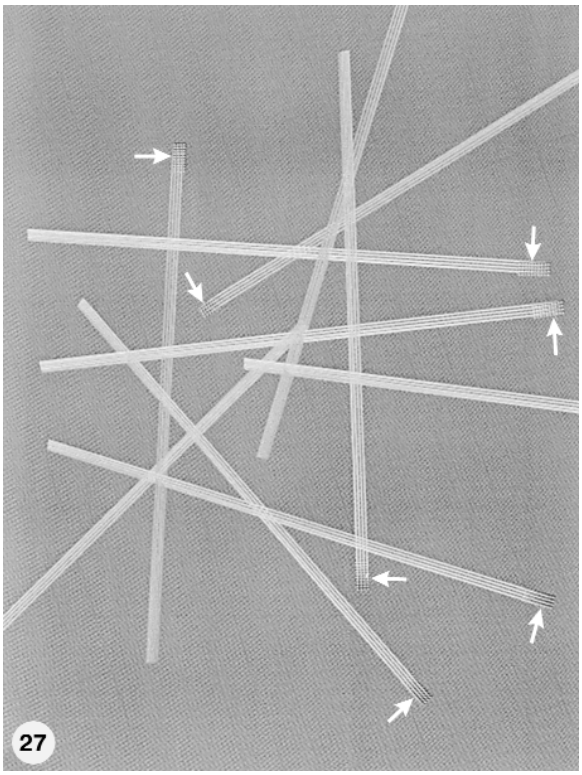




FIGS 18-22: 3D-model of cellulose microfibril secretion and assembly in *Erythrocladia*. After the first 8-10 transverse rows of subunits in a linear TC are established, the TC seems to start cellulose synthesis. The rectangular terminal complexes, characteristic of red algae, spin out ribbon-shaped cellulose microfibrils (β-1,4-glucan chains). The four subunits of the transverse row generate a glucan chain minicrystal that contains 12 glucan chains (Tsekos *et al*., 1999). Note that both the linear TCs and the cellulose microfibrils are randomly distributed.



FIGS 23-27: Progressive formation and development of cellulose synthase aggregates (linear terminal complexes) resulting in the gradual growth of cellulose microfibrils (Figs 23-26). Linear TCs (arrows) occur at the ends of newly synthesized microfibrils. The TCs are laterally displaced because of the polymerization and crystallization of the β-1,4-glucan chains, supporting the "fluid mosaic" membrane model. The random distribution of the TCs in the plasma membrane (FIG. 27) generates a network of randomly distributed cellulose microfibrils (cell wall crystalline phase).



observed that cellulose microfibril length increases with time.

The dynamic process of cellulose microfibril orientation (random) is attributed to the distribution of linear terminal complexes (Figs 1, 18-22). TCs move laterally within the plasma membrane [(see Singer and Nicolson's (1972) fluid mosaic model)] at the ends of the assembled microfibrils (Figs 19-22), probably pushed by the pressure, resulting from glucan chain polymerization and crystallization. Finally, the function of linear TCs in the plasma membrane generates a network of randomly distributed cellulose microfibrils (cell wall crystalline phase) (Figs 2, 23-27).

It should be noted that the globules are inserted into the plasma membrane along with "tetrads" (Tsekos & Reiss, 1993; Tsekos *et al*., 1996) and intramembrane particles by the fusion of Golgi vesicles (Fig. 5). The "membrane tetrads" seem to be involved in the synthesis of the amorphous matrix polysaccharides of *Erythrocladia* cell wall (Tsekos & Reiss, 1993). The simulataneous presence of globules and "tetrads" in Golgi cisternae and Golgi vesicles supports earlier suggestions that synthesis of the non-crystalline and crystalline components of the cell wall is a co-ordinated cell activity (Tsekos *et al*., 1996).

After the synthetic function of the linear TC is accomplished, the structure may be recycled back into the cytoplasm via an endocytotic process (Fig. 5) (Tsekos *et al*., 1996) (breakdown of TCs allows protein conservation by the cell) (Northcote, 1991).

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