Modification of Protoplast Cell Wall Regeneration by Membrane Perturbation

R. L. Legge* and R. M. Brown, Jr.[1]

Department of Chemical Engineering, University of Waterloo, Ontario

Summary

Protoplasts derived from cells of *Boergesenia forbesii* regenerated aberrant cell walls when treated with cholesteryl hemisuccinate (CHS). Protoplasts treated with CHS, for a short period during the initial stages of cell wall regeneration, developed a patchwork cell wall, possessing regions devoid of cell wall. This effect was reversible, and treated cells ultimately developed a normal, confluent cell wall when removed from the CHS. Freeze fracture studies revealed that for CHS-treated cells, regions without microfibril impressions did possess intramembranous particles (IMP's) but that these regions contained small domains free of IMP's suggestive of lateral phase separation. The data implies that the physical characteristics of the plasma membrane lipid are important to the deposition of cell wall microfibrils during cell wall regeneration. This effect may be attributed to altered lipid-protein interactions, modified membrane fusion characteristics, or altered membrane flow.

Keywords: Cellulose biosynthesis; Terminal complexes; Freeze-fracture; *Boergesenia forbesii*; Membranes.

1. Introduction

Linear terminal complexes (tc's) are linear arrays of intramembranous particles which are revealed in freeze fracture replicas of *Boergesenia forbesii* plasma membranes. These structures, which consist of three rows of up to 50 particles each (Itoh *et al.* 1984), terminate the ends of the microfibril impressions and are contiguous with the cell wall microfibrils in thin section (Kudlicka *et al.* 1987). Based on this evidence, linear terminal complexes are thought to function in the biosynthesis of cellulose microfibrils of the cell wall. Since

the functionality of intrinsic membrane proteins is dependent upon the lipid environment which surrounds them, it is reasonable to predict that the physical properties of the membrane lipid are important in cell wall formation. Here we have studied the effects of membrane perturbation on cell wall regeneration by *B. forbesii* protoplasts using cholesteryl hemisuccinate (CHS) which is known to alter membrane fluidity (Borochov *et al.* 1979).

2. Materials and Methods

The culturing and induction of protoplasts in *Boergesenia forbesii* (Harvey) Feldmann was conducted as previously described (Itoh *et al.* 1986). Cells of approximately 1 to 1.5 cm were selected for the preparation of protoplasts. Protoplast formation was initiated by mechanically wounding the cells by puncturing the cell wall with a needle and then cutting the cells in half. Intact protoplasts were released from the parent wall 1.5 hours following wounding and transferred either to natural seawater with 1/4 strength ES-enrichment (Provasoli *et al.* 1957) or into seawater containing 0.25 mg/ml cholesteryl hemisuccinate (Sigma) and 3.5% w/v polyvinylpyrrolidone (PVP, Mr 40,000, Sigma). Cells were treated with CHS for 30 minutes then washed once with the growth medium and returned to the medium. Cell wall microfibrils were visualized by use of the fluorescent brightening agent Tinopal LPW (Ciba-Geigy) staining at a concentration of 0.0075% (w/v) as previously described (Itoh *et al.* 1986). Microfibril fluorescence was observed with a Zeiss Universal microscope equipped for epifluorescence microscopy.

To obtain freeze-etch replicas, protoplasts were pipetted directly onto gold specimen supports (Balzers, Liechtenstein), allowed to settle, and excess medium wicked away with filter paper. Specimens were frozen in Freon 22 (DuPont Co., Wilmington, Del.) cooled by liquid N_2. Specimens were fractured in a BA 360 M freeze-etch apparatus (Balzers), etched at $-100\,°C$ for 60 seconds, and shadowed unidirectionally at 45° with Pt-C and carbon coated at $1-2 \times 10^{-6}$ Torr. Replicas were cleaned in dichromate-sulfuric acid and mounted on Formvar-coated copper grids for examination with a Philips 420 electron microscope at 80 kV.

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* Correspondence and Reprints: Biochemical Engineering Group, Department of Chemical Engineering, University of Waterloo, Waterloo, Ontario, Canada, N2L 3Gl.

[1] Current Address: Department of Botany, University of Texas at Austin, Austin, TX 78713–7640, U.S.A.

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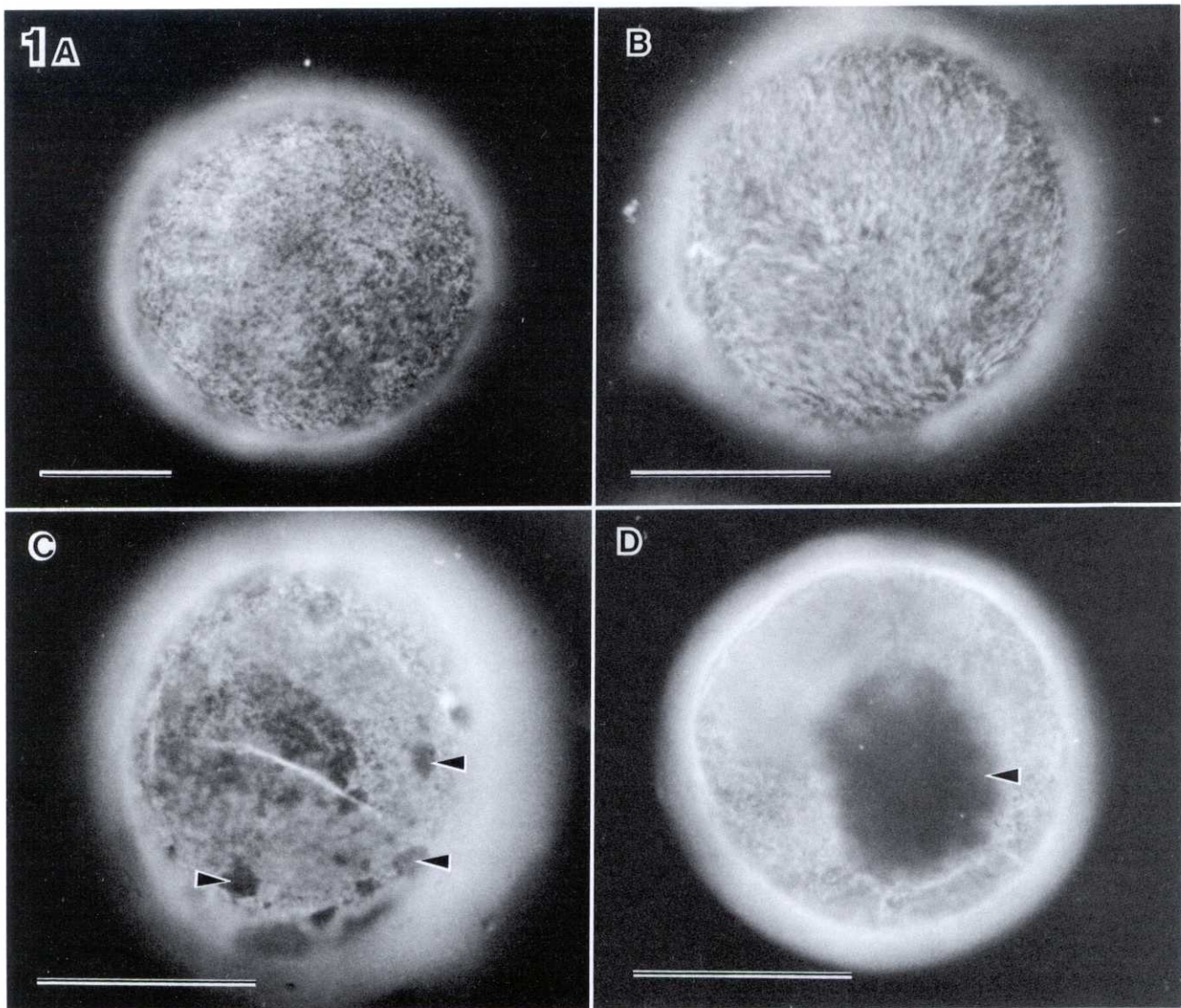
Fig. 1. Effect of cholesteryl hemisuccinate on cell wall regeneration in *B. forbesii* protoplasts based on fluorescence visualization using Tinopal LPW. (*A*) Fluorescence micrograph of a typical protoplast 3 hours following wounding showing a confluent cell wall consisting of randomly oriented microfibrils; (*B*) fluorescence micrograph of a typical cell 4 hours following wounding, showing a confluent cell wall in which the ordered swirling pattern to the microfibrils can be distinguished; (*C*) CHS-treated protoplasts, 3 hours following wounding showing a patchwork-like regenerated cell wall with arrows designating zones on the surface of the cell devoid of cell wall; and (*D*) a CHS-treated protoplast 4 hours postwounding showing a large patch devoid of cell wall. Bar equals 100 μm

3. Results and Discussion

Protoplast formation can be naturally induced in the coenocytic marine alga *B. forbesii* by physical wounding. These protoplasts begin to regenerate a new cellulosic wall characterized by randomly oriented microfibrils (which measure approximately 30 nm in width) 2–3 hours following the induction of protoplast formation (Fig. 1 *A*). Within 4 hours the cell wall begins to exhibit secondary cell wall-like characteristics with microfibrils being deposited in an ordered manner distinguished by swirling patterns on the surface of the protoplast (Fig. 1 *B*). Protoplasts treated with CHS just prior to the initiation of cell wall deposition regenerate a cell wall which is frequently aberrant (50% of the cells) containing regions devoid of cell wall (Fig. 1 *C*). These regions are commonly distributed uniformly over the surface of the protoplast, but may also be manifest as a single large domain on the surface (Fig. 1 *D*). Cells are capable of recovering from the effects of this treatment, with an ordered, confluent secondary cell wall being evident within 5–6 hours analogous to that shown
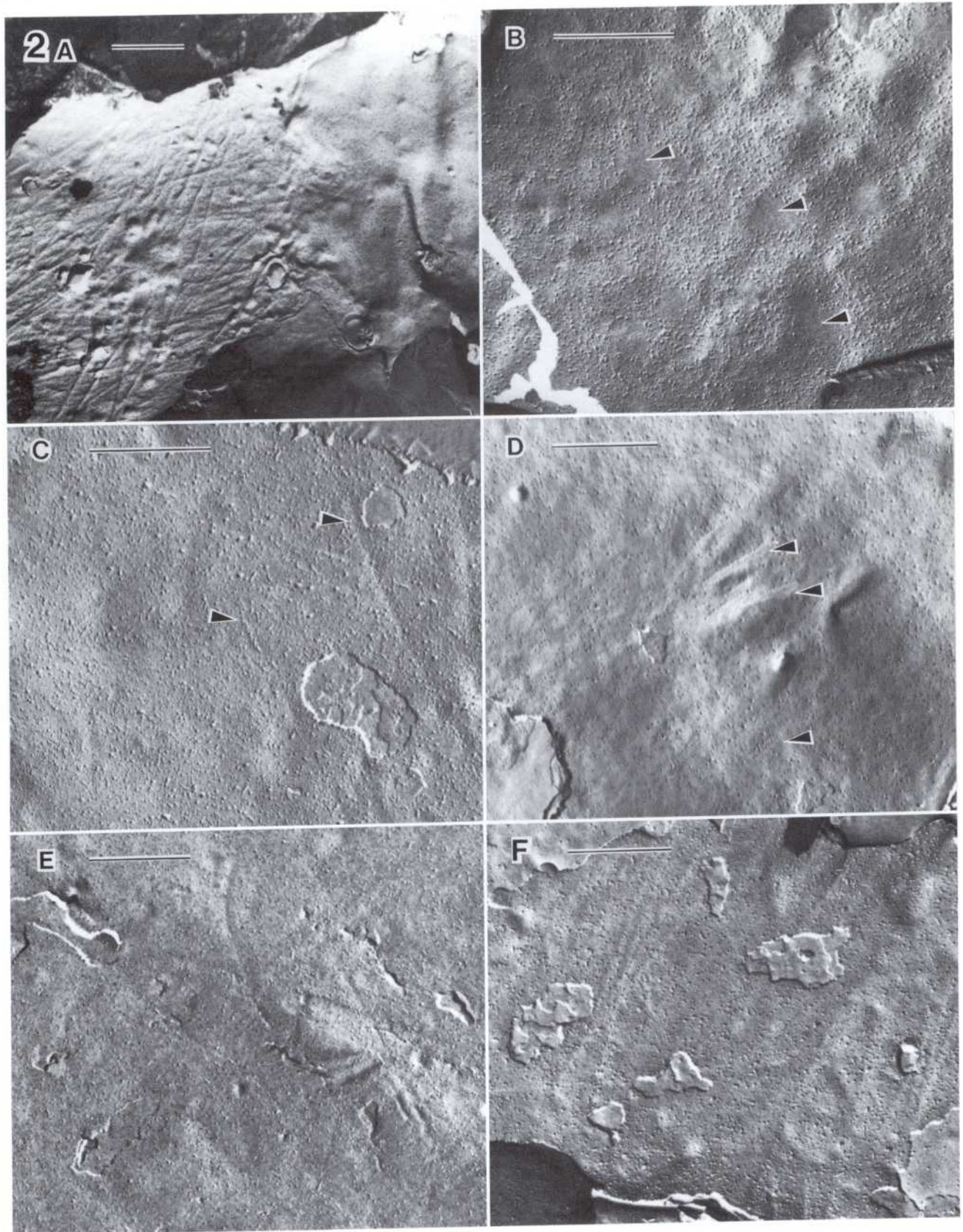
Fig. 2. (A) PF-fracture face of a CHS-treated B. forbesii protoplast 3 hours after wounding. Note randomly-oriented microfibril impressions on the left, and the absence of microfibril impressions in the adjoining region. (B) PF-fracture face of a CHS-treated B. forbesii protoplast 4 hours after wounding. Arrows designate IMP-free microdomains. (C) PF-fracture face of a CHS-treated B. forbesii protoplast 3 hours after wounding. Arrows designate linear terminal complexes, in some instances terminating the ends of microfibril impressions. (D) PF-fracture face of a CHS-treated B. forbesii protoplast 3 hours after wounding. Numerous linear terminal complexes (arrows) are evident. (E) PF-fracture face of an untreated B. forbesii protoplast 3 hours after wounding showing numerous terminal complexes. (F) PF-fracture face of an untreated B. forbesii protoplast 4 hours after wounding. Fig. 2 A bar equals 1.0 μm; Figs. 2 B–2F bar equals 0.5 μm

in Fig. 1 B. This represents a 2 hour delay in cell wall regeneration relative to controls. The plasma membrane of B. forbesii is particularly dynamic during protoplast formation with approximately a 40% reduction in plasma membrane surface area during the initial wounding response (O'NEIL and LA CLAIRE 1984). This may account for the ability to recover from the effects of the CHS treatment.

It has been demonstrated that one of the effects of CHS is to increase the viscosity of the membrane, resulting in a vertical displacement of the membrane proteins (SHINITZKY and RIVNAY 1977). It has not been suggested that CHS results in the formation of gel phase lipid although some of the results for CHS-treated systems would be in accord with this. Freeze fracture evidence in this study would imply that CHS-treatment of B. forbesii does result in the formation of regions devoid of IMP's suggestive of lateral phase separation due to the presence of gel phase lipid. The fluorescence micrographs of cell wall deposition suggest that the primary consequency of CHS-treatment is the induction of large-scale membrane heterogeneity resulting in regions unfavourable for the deposition of cell wall. Figure 2 A reveals a PF-face of the plasma membrane spanning adjoining domains with and without microfibril impressions. The regions devoid of microfibril impressions do, however, contain IMP's. A common feature of zones lacking microfibil impressions was the presence of particle-depleted regions, averaging 0.18 μm in diameter (Fig. 2 B). Similar observations have been made in freeze fracture studies of chilling injured Avocado (PLATT-ALOIA and THOMPSON 1987). It has been suggested that these regions form due to an aggregation of gel phase lipids into domains resulting in the lateral displacement of proteins.

Normal terminal complexes could be distinguished during the early stages of cell wall regeneration in both controls (Fig. 2 E and F) and in CHS-treated protoplasts (Fig. 2 C). Those which could be distinguished terminated microfibril impressions implying that they were active in microfibril synthesis. Fully assembled terminal complexes could also be distinguished in fracture planes lacking microfibril impressions in CHS-treated cells (Fig. 2 D). This implies that membrane perturbation with CHS does not inhibit terminal complex assembly but does affect some membrane-related aspect of cellulose biosynthesis. This observation also discounts the possibility that terminal complexes are an aggregation of IMP's resulting from a passive association with the terminal end of the cellulose microfibril.

Table 1. Effet of cholesteryl hemisuccinate on IMP density in fracture zones possessing microfibril impressions*

Treatment	IMP Density (IMP's/μm²)
Control (t = 3)	900 ± 108
Control (t = 4)	1,790 ± 70
CHS (t = 3)	1,910 ± 65
CHS (t = 4)	1,335 ± 132

* Particle counts were determined using an IBAS system (distributed by Carl Zeiss, Inc., Thornwood, NY). Images were input from freeze fracture micrographs using a digital image averaging procedure, the resulting stored image was then subjected to a low pass filter and then background shade corrected. A binary image was generated interactively to discriminate the IMP's. Particle distribution analysis was performed on areas equivalent to 0.85 μm² for a minimum of 6 individual fracture fields.

Examination of the IMP density in freeze fracture zones possessing microfibril impressions revealed a significant difference between treated and untreated cells (Table 1). IMP densities were found to be 2 times greater in CHS-treated cells, relative to normal protoplasts 3 hours following wounding. This is the period during which the patchwork-like cell wall was most evident. This modified particle distribution reflects a CHS-mediated lateral redistribution of proteins in the plasma membrane. IMP densities are dynamic within this system doubling during the period when a transition from primary to secondary cell wall is occurring. During this same period in CHS-treated cells the IMP density decreases, correlating with the recovery phase.

It is apparent that the plasma membrane lipid plays an important role in the synthesis and/or deposition of the cellulose microfibrils during cell wall regeneration. Perturbation of the membrane lipid with CHS results in a redistribution of IMP's both with respect to overall density and the formation of particle-depleted regions indicative of lateral phase separation due to gel phase lipid. It is not clear from this study if perturbed wall synthesis is a consequence of altered protein-lipid interactions, modification of membrane fusion phenomena or effects on membrane flow. Membrane fluidity and membrane flow have been invoked to play a role in microfibril deposition. MIZUTA (1985) observed intramembranous protein distributions in Boergesenia when orientation of the microfibrils was changing. Similar conclusions were made for higher plants based on asymmetric microfibril patterns found around pit fields which resembled flow patterns (MUELLER and BROWN 1982).

Acknowledgements

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