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Cytoskeletal and Ultrastructural Studies on Regenerating Cells from the Protoplasts of *Valonia* (Siphonocladales, Chlorophyceae)

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Abstract
Ultrastructure and cytoskeletal properties of the coenocytic green alga, *Valonia*, were described using light field, immunofluorescence and electron microscopy to investigate the dynamics among cell wall, cell membrane, and protoplasm during cell regeneration. Protoplasts were artificially induced in three species by cutting thalli and extruding the protoplasm. Protoplasts contracted and formed irregularly shaped masses within 30 minutes concomitant with bundling of actin filaments (AFs), convolution of cortical microtubules (CMTs) and formation of a thin enveloping membrane composed of polysaccharides. Size affected survival rates: protoplasts less than 10 μm in diameter displayed lower viability than larger protoplasts. A new cell wall was produced within 24 hours simultaneous with CMT and AF depolymerization. AFs were reduced to granular structures and aggregates that repolymerized by 48 hours. Concurrently, new CMTs polymerized and attained a parallel arrangement. Actin- and microtubule-destabilizing agents had variable effects on protoplast contraction indicating a minor role of intact cytoskeletons in this process; however, resulting cells exhibited abnormal protoplasm distribution and cell deformation after three days. Rhizoids began to form after 7 days on untreated cells which subsequently produced lateral branch cells that eventually developed into mature thalli.

Key words: actin filament, cortical microtubule, protoplast, *Valonia*

1. Introduction

Coenocytic green algae are composed of multinucleate giant cells that vary in numbers according to species. These cells are constantly exposed to injury caused by various elements in the marine environment which can severely impact cellular integrity and survival. In apparent response to this threat, members of this group have developed mechanisms for wound healing (La Claire 1982, Menzel 1988) where repair of an injured site was shown to involve the following processes: restoration of damaged membrane, contraction of cytoplasm, production of insoluble plugs and formation of a new cell wall. In addition, when a cell is cut and the protoplasm is extruded in seawater, numerous new cells are regenerated such as in *Boergesenia, Ventricaria, Bryopsis*, *Microdictyon* and *Chaetomorpha* (Mizuta *et al*. 1985, Nawata *et al*. 1993, Kim *et al*. 2001, Kim *et al*. 2002, Klotchkova *et al*. 2003). According to recent reports (Kim *et al*. 2002, Klotchkova *et al*. 2003), initial protoplast formation is achieved by agglutination of cell organelles and development of an enveloping semi-permeable membrane followed by construction of a new cell wall before eventually developing into mature thalli. Examining this phenomenon in yet another coenocytic green alga such as *Valonia* can be beneficial in understanding the dynamics of wound healing and protoplasm-cell membrane-cell wall continuum. Members of the genus *Valonia* are marine coenocytic green algae, consisting of large multinucleate cells. Lateral branch cells are produced by primary cells through lenticular cell formation (Okuda *et al*. 1997). Several lenticular cells can arise from a single mother cell and in turn become the origin of younger cells.

Actin filaments have been reported to be directly involved in protoplasmic contraction in *Ernodesmis verticillata* and *Boergesenia forbesii* (La Claire 1989, Goddard & La Claire 1991), *Ventricaria ventricosa* (Sugiyama *et al*. 2000) and *Valonia utricularis* (Satoh *et al*. 2000). On the other hand, La Claire (1987) noted that microtubules do not mediate wound-induced motility in *E. verticillata* and *B. forbseii*. A similar result was also

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reported in *V. utricularis* (Satoh *et al*. 2000). Although drastic changes in cortical microtubule arrangement have been observed during protoplast regeneration (Hayano *et al*. 1988), these were previously found to be unrelated to cellulose microfibril orientation during cell wall construction (Itoh & Brown 1984). Thus, it appears that microtubules are mainly involved in controlling the shape of protoplasts during this process.

In this study, we describe the physiological aspects of cell regeneration from *Valonia* protoplasts with emphasis on the changes in actin and microtubule structures. In addition, we provide a description of a possible mode of initial membrane formation using cytochemical staining, immunofluorescence and electron microscopy.

2. Materials and Methods

1) Algal materials

Specimens used in this study were: *V. macrophysa* Kützing collected from Yokonami Peninsula, Susaki, Kochi, Japan; *V. fastigiata* Harvey ex J. Agardh from Otsuki, Kochi, Japan; and *V. aegagropila* (strain no. 7) from Sta. Ana, Cagayan, Philippines. Zoospores or partheno-gametes released from thalli of these three species were isolated using protocols described by Kawai *et al*. (2005), and cultured in Petri-dishes containing ca. 150 mL of ¼-strength PES (Provasoli's Enhanced Seawater) medium (Provasoli 1968) at 22 °C in long-day (LD) conditions (14:10 light:dark) under cool fluorescent lamps (40-50 μmol photons·m^{-2}·s^{-1}).

2) Size distribution and survival rate estimation

Cells were cut in half in PES using a pair of scissors. The protoplasm was then expelled by squirting medium inside the cells. The resulting protoplasts were counted and measured 10 minutes after cutting using a micrometer and then incubated in the above conditions for two weeks. During this period, the time of formation of cell walls and survival rates were determined. Survival rate was computed by dividing the number of spherical, viable cells after 24 hours with the total number of protoplasts after 10 minutes, multiplied by 100.

3) Cytochemical staining

In order to determine the composition of the initial envelope during regeneration, protoplasts at various stages were fixed with 2.5% paraformaldehyde in PBS (pH 7.2) and stained using periodic acid-Schiff's reaction, Alcian Blue, Acid Fuchsin, and Sudan Black according to McCully *et al*. (1980). After washing and mounting on glass slides, images of samples were taken using a digital camera connected to an Olympus BX-51 microscope (Olympus Optical Co., Ltd, Tokyo).

4) Electron microscopy

Protoplasts and intact cells were fixed with a solution containing 0.5% glutaraldehyde and 1% osmium tetraoxide in seawater for 1 hour. Fixed materials were rinsed with seawater three times and postfixed with 0.5% osmium tetraoxide in seawater for 24 hours. Samples were then embedded in 2% agar, dehydrated in a graded acetone series and infiltrated with Spurr's resin. Thin sections were obtained using a Leica Ultracut ultramicrotome (Nissei Sangyo, Tokyo, Japan). Sections were stained in uranyl acetate and lead citrate solutions. Ultrastructure was observed using a transmission electron microscope JEM-1010T (JEOL, Tokyo, Japan).

5) Fluorescent staining

To visualize initial membrane polysaccharides, Fluostain I (Dojin East, Tokyo, Japan) was diluted in PES to a concentration of 0.1 mg/ml and used to incubate samples for 1 to 2 hours. Samples were washed three times with PES and mounted in medium, then viewed on the microscope under a fluorescence microscope.

For indirect immunofluorescence staining (see Okuda *et al*. 2000), protoplasts were first fixed with a buffer (MTSB: 587 mM NaCl, 100 mM KCl, 5 mM $MgCl_2$, 5 mM ethylene glycol-bis-(β-aminoethyl ether) - N, N, N',N'-tetraacetic acid and 50 mM piperazine-N, N' – bis- (2-ethanosulfonic acid), pH 7.0) containing 4% paraformaldehyde and 1% glutaraldehyde. Samples were placed in microcentrifuge tubes for washing with 1mg/ml $NaBH_4$ in PBS pH 7.3 (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.46 mM KH_2PO_4, 1.5 mM NaN_3) and 3% Nonidet P40 in PBS. For microtubule visualization, protoplasts were incubated with a primary antibody (a rat monoclonal anti-β-tubulin antibody, YL1/2, Sera Labs, England) followed by treatment with a secondary antibody (a goat polyclonal anti-rat IgG antibody conjugated with FITC, F6258, Sigma Chemical Co.). For actin, a primary antibody (rabbit anti-actin antibody, A2066, Sigma Chemical Co.) and a secondary antibody (goat anti-rabbit IgG antibody conjugated with FITC, F0382, Sigma Chemical Co.) were used. Samples were washed with PBS three times after each incubation step, mounted on a slide with a coverslip and viewed under an Olympus BX-51 epifluorescence microscope (Olympus Optical Co., Ltd, Tokyo, Japan).

6) Cytoskeleton-destabilizing agents

To determine the effect of actin-depolymerizing

chemicals on protoplast regeneration, 10 μM cytochalasin D, 10 μM mycalolide B and 10 μM latrunculin B were used. Against microtubule, 100 μM colchicine, 10 μM oryzalin, and 10 μM aminoprophos methyl (APM) were applied. Stocks were prepared in dimethyl sulfoxide (DMSO) and diluted in the above final concentrations in PES. Controls were set with PES alone and 1% DMSO. Cells were cut and protoplasm was extruded in these supplemented media. Contracted protoplasts were counted after 24 hours and observed for morphological features for 14 days. On the 8th day, media were replaced with PES and observed for another 7 days as a control.

3. Results

1) Cell regeneration from protoplasts

Thalli of *Valonia* are composed of a few to several multinucleate cells, with the mother cell giving rise to lateral cells which in turn produce lateral cells of their own (Fig. 1a). When cells were cut in half and squirted with medium inside (Fig. 1b), the protoplasm was released into the medium resulting in numerous irregularly shaped sheet-like fragments (Fig. 1c). These protoplasts immediately curled and contracted into amorphous masses after 30 minutes (Fig. 1d). Within 1 to 3 hours, most of the protoplasts had attained a compact spherical shape surrounded by a thin transparent envelope (Fig 1e). Approximately 100 protoplasts were formed from the protoplasm of a cell around 3 mm in diameter. The distribution of sizes of protoplasts ranged from less than 10 μm to around 300 μm in diameter. Size impacted protoplast survival rates, with smaller protoplasts (less than 10 μm) having the lowest survival rates (Fig. 2). On the other hand, those larger than 10 μm had mean survival rates higher than 75% with protoplasts ranging from 11 to 150 μm in diameter having almost complete viability. However, when their size surpassed 151 μm, survival rates slowly decreased and became variable. Spherical cells produced cell walls within a week and proceeded to develop primary rhizoids which started as a darkening of a portion of the protoplasm near the glass bottom of incubation vessels (Fig. 1f) and elongated toward the substratum after a few more days (Fig. 1g). After four weeks, the cells produced lateral branch cells which later on gave rise to more lateral cells (Fig. 1h), and eventually grew into new mature thalli.

2) Initial envelope and cell wall formation

Several minutes after extrusion, protoplasts contracted and formed irregularly shaped masses. At that time, amorphous materials were observed to accumulate at various points on the surface of the protoplasts after staining with Fluostain I (Fig. 3a), indicating the presence of polysaccharides. These polymerized further and formed a thin, porous, mat-like membrane surrounding the protoplast after 12 hours (Fig. 3b). After 24 hours, this structure became dense and completely enveloped the protoplast (Fig. 3c). When stained with Alcian Blue, these materials tested positive for the presence of sulfated and carboxylated polysaccharides (Fig. 4a-c, arrows). Staining with periodic acid Schiff's reaction, Acid Fuchsin and Sudan Black did not produce any posi-

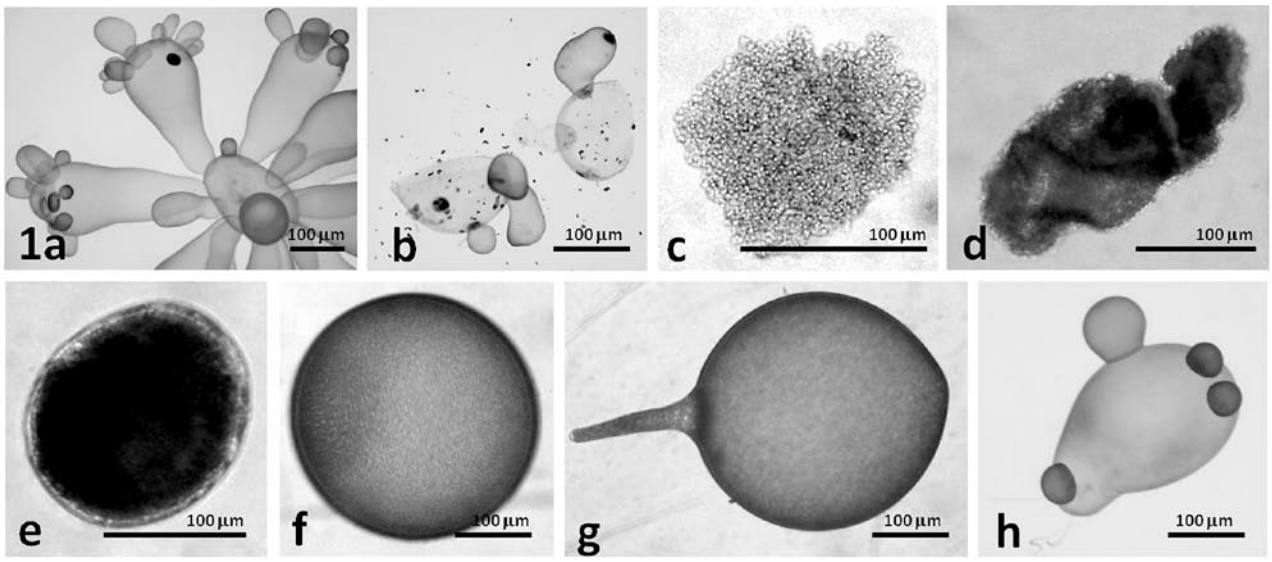

Fig. 1. Stages of regeneration of cells from protoplasts.
A vegetative thallus (a) was cut, releasing the protoplasm into the medium, producing numerous protoplasts (b,c). A protoplast contracts after 15-30 min (d), was enveloped by a membranous structure after 24 hours (e) and a cell wall after 24 h (f). After 7-10 days rhizoids were formed (g), followed by lateral cells within 3-4 weeks (h).

tive result for polysaccharides with vicinal hydroxyl groups, proteins or lipid/lipoproteins, respectively (Fig. 4d-f).

Electron microscopic observations indicated that immediately after extrusion protoplasts lacked any continuous enveloping membrane (Fig. 5a), although thin fragmented membranous structures were observed along the external edges of the protoplast (arrow). Fusion of free amorphous materials with the initial membrane appeared to occur as these materials were abundant around the edges of protoplasts (Fig. 5b, arrow). This membrane proceeded to envelop the protoplast after 24 hours (Fig. 5c) as the cell wall started reconstruction (Fig. 5d). After 72 hours several layers of the cell wall formed while the membrane was still visible (Fig. 5e,f).

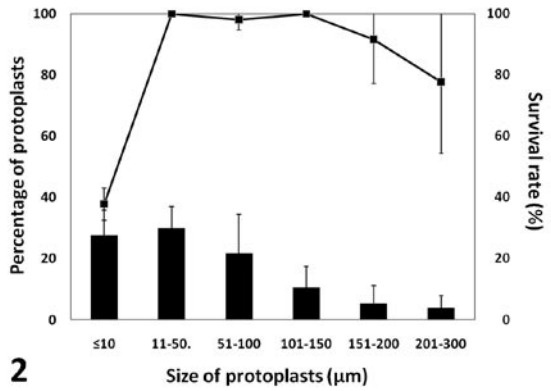

Fig. 2. Size distribution (bars) and survival rate (line) of protoplasts in each size range 24 hours after extrusion.
Data are means from *V. macrophysa, V. fastigiata and V. aegagropila*. Results are expressed as means ± S.D.

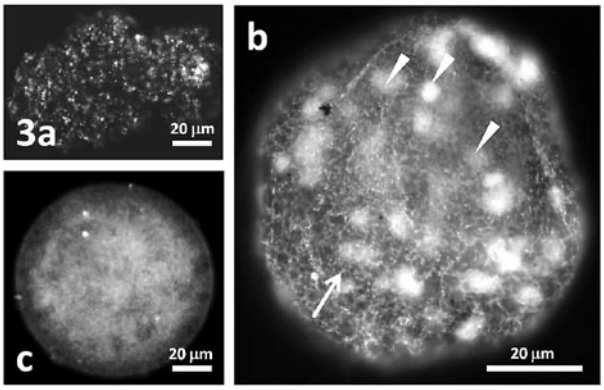

Fig. 3. Staining of surface materials on protoplasts.
Fluostain I - treated cells displayed labeling of surface materials 3 hours after extrusion (a) which formed a porous cover after 12 hours (b, arrow) and a spherical envelope at 24 hours (c). Traces of DAPI-stained nuclei were also visualized (b, arrowhead).

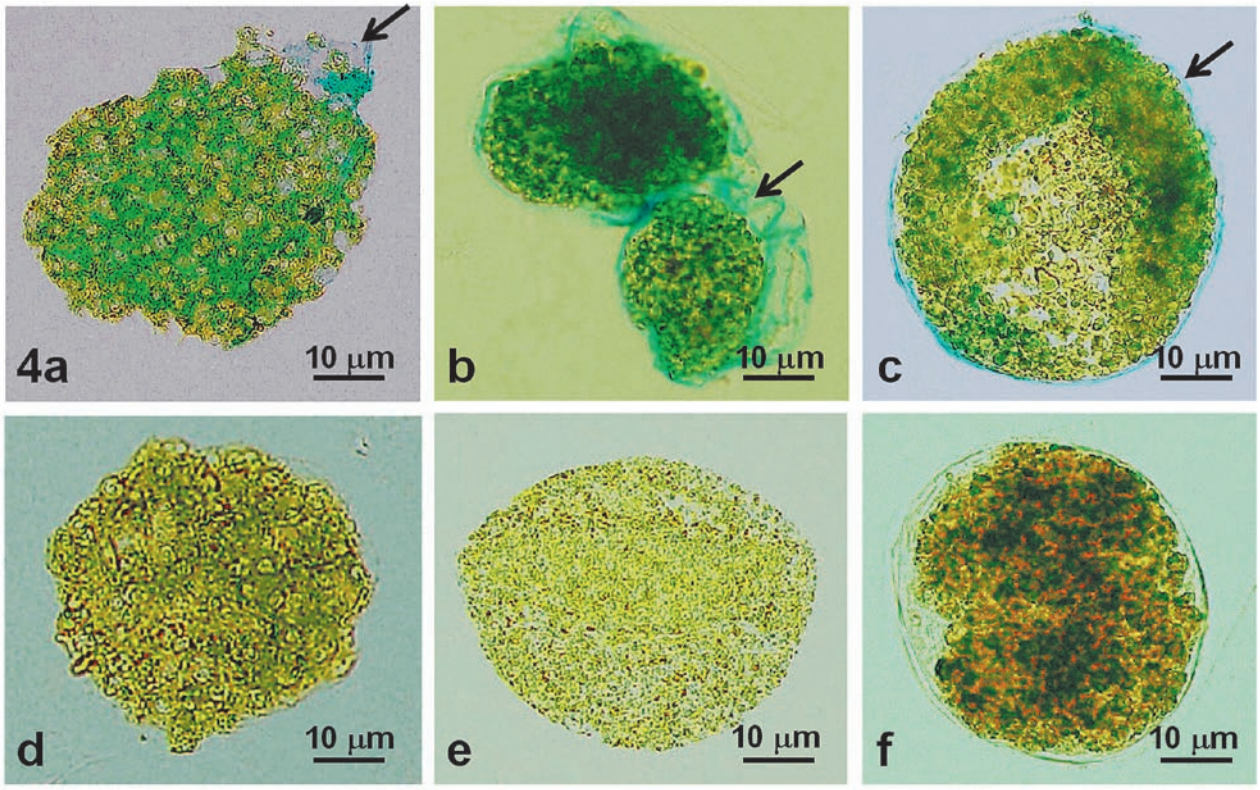

Fig. 4. Initial wall membrane detection in regenerating cells from protoplasts.
Membranous structures (arrows) stained with Alcian Blue in protoplasts 15 minutes (a), 6 hours (b) and 24 hours (c) after extrusion. Negative reactions resulted from Acid Fuchsin (d), Sudan Black (e) and periodic acid Schiff's (f) treatments of 24-hour-old protoplasts.

3) Cytoskeletal modifications

Actin filaments formed distinct networks like a scaffold supporting the protoplasm of intact cells (Fig. 6a). Filaments were relatively thin and uniform along orthogonal strands, forming semicircular gaps. Immediately after extruding the protoplasm, actin filaments retracted and coiled forming rings and loops, obscuring the inner network (Fig. 6b). After 15 minutes, long actin cables were constructed as the protoplast contracted (Fig. 6c). Numerous highly conspicuous ring-like actin structures were distributed randomly as the previously thin actin networks thickened after 12 hours (Fig. 6d). After 24 h, the actin networks depolymerized, leaving specks, short fragments of the actin filaments and actin ring structures (Fig. 6e). Actin filaments appeared to repolymerize after 48 h as protoplasts attained an evenly distributed globular shape (Fig. 6f). At this time, actin networks became prominent as the semicircular matrices and distinct spaces re-emerged.

Intact *Valonia* protoplasm displayed parallel cortical microtubules (CMTs) (Fig. 7a). After cutting the cells, CMTs convoluted into a wavy configuration, spanning the whole surface area of protoplasts (Fig. 7b). This occurred during the early stages when the protoplasts were starting to contract, appearing as thick, flat or curved sheets. After 12 hours of contraction, CMTs

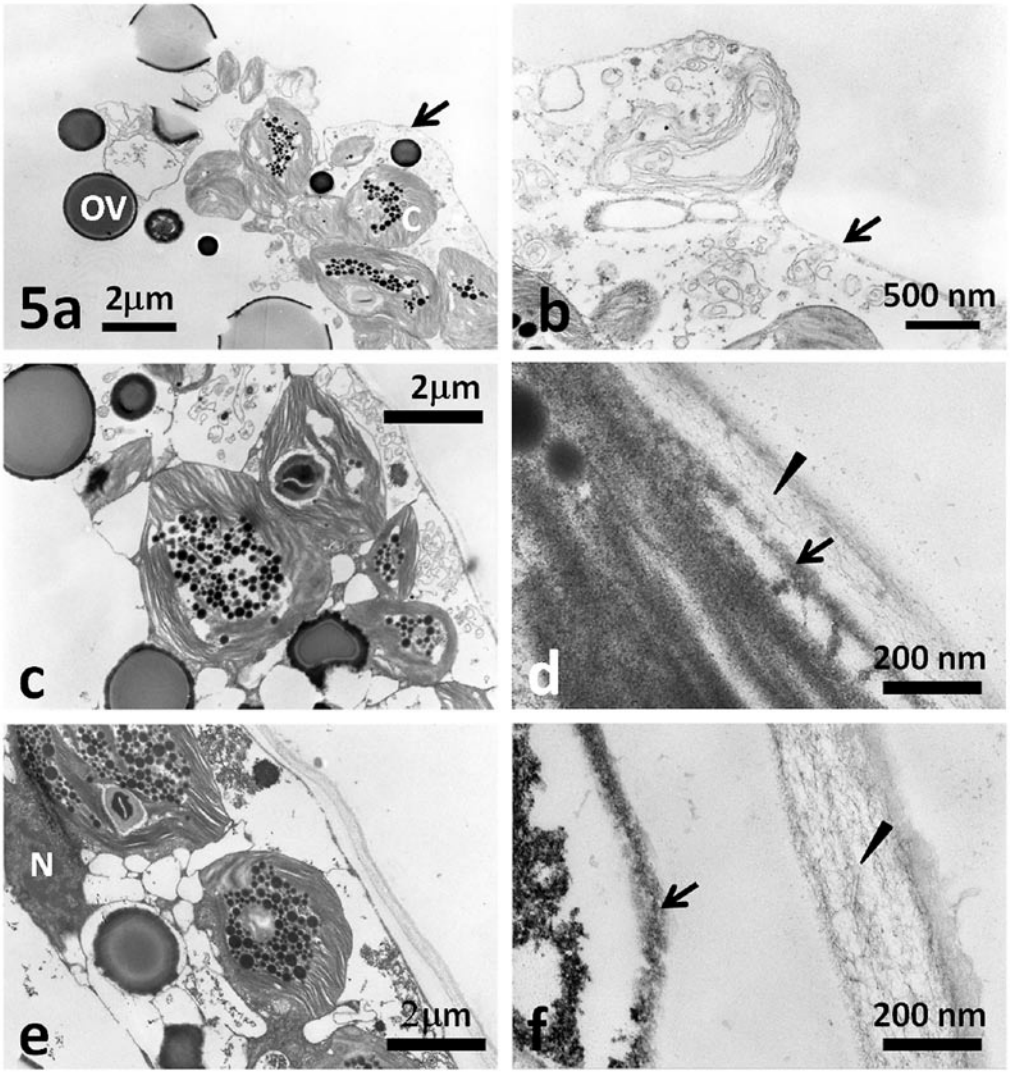

Fig. 5. Initial envelope and cell wall formation on protoplasts.
Electron micrographs revealed the absence of a continuous enveloping membrane around the protoplast 15 minutes after extrusion although some fragments partially covering organelles were observed (a, arrow) which appeared to polymerize and fuse with free amorphous structures (b, arrow). After 24 hours, a continuous membrane surrounded the organelles (c). Adjacent to this membranous structure (d, arrow), a thin layer of cell wall formed (d, arrowhead). The initial envelope and amorphous structures remained after 72 hours (e). At this stage, remnants of the initial envelope were visible (f, arrow) while the cell wall attained multiple layers (f, arrowhead). C: chloroplast, OV: osmiophilic vesicle, N: nucleus.

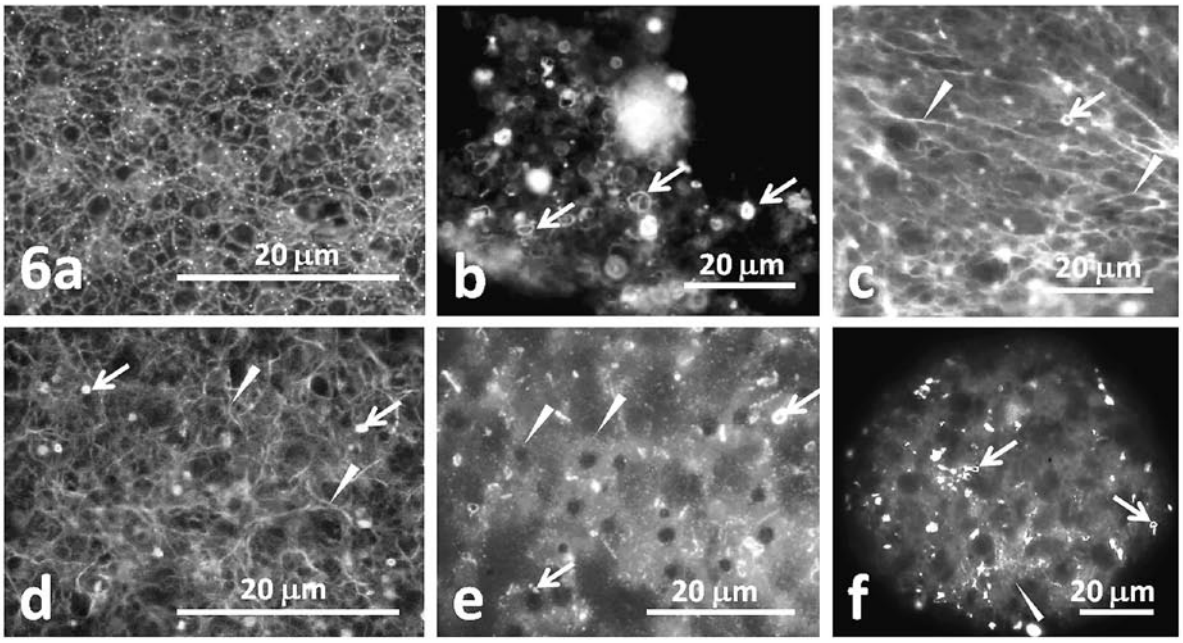

Fig. 6. Modifications in actin filaments during cell regeneration from protoplasts.
Actin filaments forming networks in intact cells (a). Actin rings occurring in a protoplast immediately after extrusion of protoplasm (b, arrows) with actin networks disappearing. Actin cables (c, arrowheads) in a protoplast 15 minutes after extrusion. Actin cables shortened and thickened to become bundles (d, arrowheads) after 3 hours while actin rings still remained (arrows). After 24 hours, the bundles disappeared and were replaced by granular structures (e, arrowheads) and actin aggregates (arrows). These aggregates remained on the surface of the protoplasm after 48 hours (f, arrows) when actin filaments started repolymerizing (arrowhead).

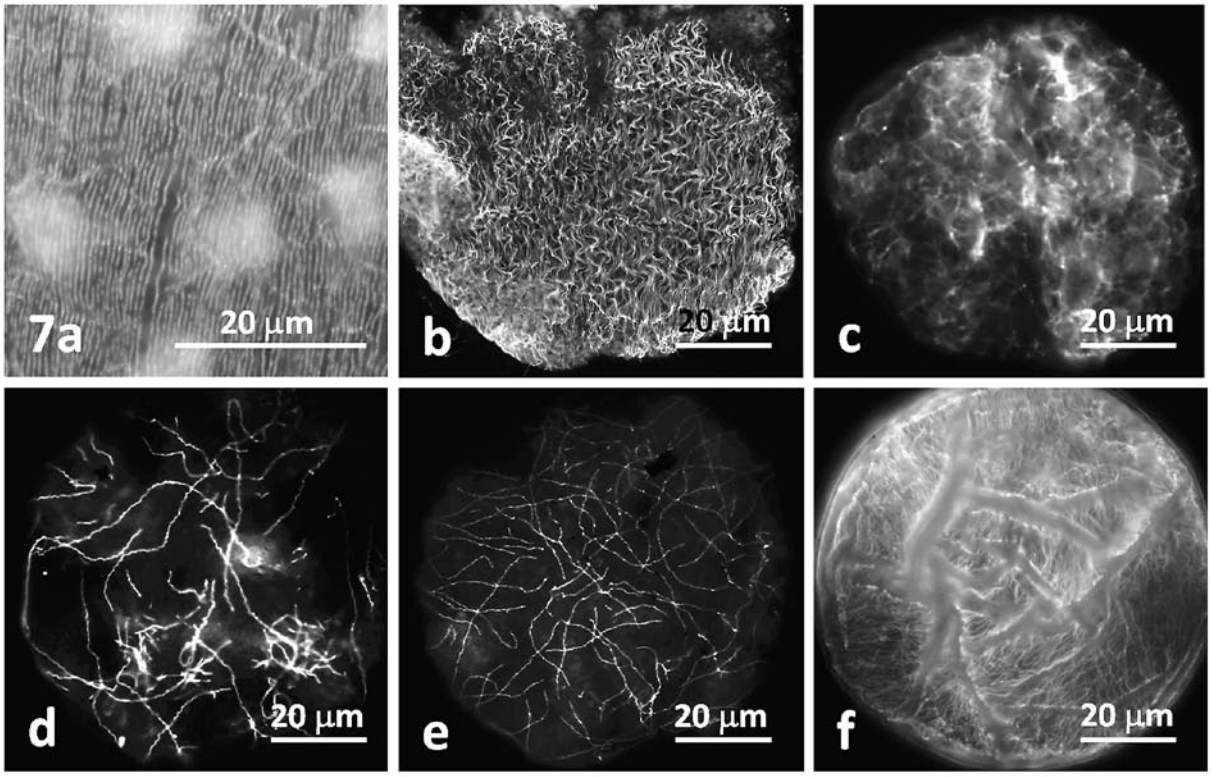

Fig. 7. Rearrangement of cortical microtubules during cell regeneration from protoplast.
Cortical microtubules (a) oriented parallel in an intact cell. CMTs undulating in a protoplast 15 min after extrusion (b), became random after 12 h (c), depolymerized after 24 h (d), repolymerized at 36 h (e) and reverted to parallel arrangement in places after 48 h (f).

Table 1. Percentage of contracted protoplasts when protoplasmic masses extruded from cells were incubated in medium with or without actin- and microtubule-depolymerizing agents for 24 hours.

	V. macrophysa	*V. fastigiata*	*V. aegagropila*
Cytochalasin D 10 μM	87.7 ± 7.8	86.7 ± 5.6	87.0 ± 0.4
Latrunculin B 10 μM	81.0 ± 6.5	78.4 ± 5.1	79.9 ± 7.6
Mycalolide B 10 μM	66.1 ± 2.2	70.8 ± 3.0	65.1 ± 1.3
APM 10 μM	79.2 ± 4.8	83.1 ± 2.3	85.0 ± 3.1
Colchicine 100 μM	87.0 ± 3.4	90.6 ± 3.4	86.3 ± 1.1
Oryzalin 10 μM	89.2 ± 3.2	88.3 ± 6.7	84.5 ± 1.2
DMSO 1%	88.8 ± 2.8	89.4 ± 3.6	96.8 ± 0.5
PES	94.6 ± 0.5	96.4 ± 0.5	94.4 ± 0.9

Data are the means ± SD (n=3).
APM, aminoprophos methyl; DMSO, dimethyl sulfoxide; PES, Provasoli's Enhanced Seawater.

Table 2. Cell shape and protoplasm distribution in three *Valonia* species three to 14 days after extrusion in actin- and microtubule-depolymerizing agents.

	3 days		7 days		14 days*	
	Cell Shape	Protoplasm	Cell Shape	Protoplasm	Cell Shape	Protoplasm
Cytochalasin D 10 μM	spherical	fragmented	spherical	fragmented	spherical	intact
Mycalolide B 10 μM	non-viable		non-viable		non-viable	
Latrunculin B 10 μM	spherical	collapsed	spherical	collapsed	spherical	intact
Colchicine 100 μM	spherical	fragmented	spherical	fragmented	spherical	intact
Oryzalin 10 μM	irregular	intact	irregular	intact	irregular	intact
APM 10 μM	spherical	fragmented	spherical	fragmented	spherical	intact
DMSO 1%	spherical	intact	spherical	intact	spherical	intact
PES	spherical	intact	spherical	intact	spherical	intact

*Medium for every treatment was replaced with PES at the 8th day. Cells in unchanged media showed no recovery after 14 days.
APM, aminoprophos methyl; DMSO, dimethyl sulfoxide; PES, Provasoli's Enhanced Seawater

attained a random orientation (Fig. 7c), long strands of which were laid curled in a tangled cytoskeletal mass. At this point, protoplasts were still irregularly shaped although initial enveloping membranes were already spherical. MTs depolymerized after 24 hours (Fig. 7d), showing randomly arranged short and long fragments. After 36 hours, MTs started to polymerize again (Fig. 7e). When the protoplasts had formed an even, globular structure 48 hours after extrusion, CMTs began forming a parallel arrangement (Fig. 7f).

4) Cytoskeleton-disrupting chemicals

When actin- and microtubule-destabilizing reagents were introduced during extrusion of the protoplasm, the rate of protoplast contraction after 24 hours was variably affected (Table 1). For actin, addition of 10 μM cytochalasin D, 10 μM mycalolide B and 10 μM latrunculin B resulted in contraction of protoplasts in the range of 65-88% with mycalolide B having the highest effect. When 10 μM APM, 100 μM colchicine and 10 μM oryzalin were introduced, 79-90% of protoplasts contracted.

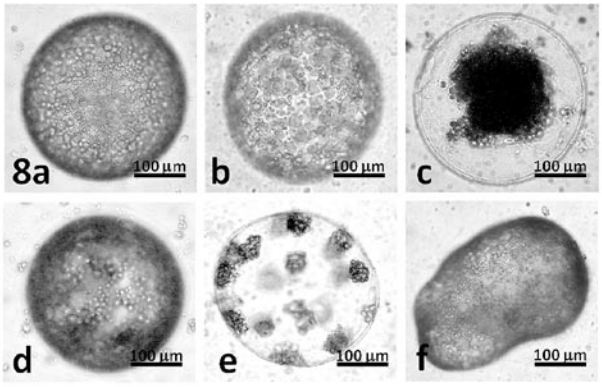

Fig. 8. Cytomorphological effects of cytoskeleton-disrupting chemicals.
After three days, cells incubated in PES had a spherical shape and an even distribution of protoplasm (a). When treated with actin-depolymerizing agents, the protoplasm was disrupted (cytochalasin D (b)) or detached from the cell wall (latrunculin B (c)). Clusters of protoplasmic mass attached to the cell wall were produced in microtubule-disrupting chemicals, APM (d) and colchicine (e), while a distorted shape was induced in oryzalin (f).

Controls, made up of 1% DMSO in PES or PES only, had 89-97% contraction.

After several days, other cytomorphological effects due to these chemicals were observed (Table 2). On the third day, spherical cells were produced in cells treated with most of the chemicals, except in cells treated with mycalolide B, where none of the cells survived, and in cells treated with oryzalin, where cells had irregular shapes. Protoplasm at this stage was fragmented in cells treated with cytochalasin D (Fig. 8b) while collapsed in the center in cells treated with latrunculin B (Fig. 8c). With colchicine and APM, protoplasm was fragmented (Fig. 8 d,e), but it was evenly distributed in cells with oryzalin (Fig. 8f) and in the control (Fig. 8a). After 7 days, the cells had the same shape and protoplasm distribution as on the 3rd day. The incubation medium was replaced with PES to remove actin- and microtubule-destabilizing reagents 8 days after the beginning of the experiments. After 14 days (6 days after cells were transferred into drug-free PES), the protoplasm in all cells treated with these chemicals reverted to an even distribution as with the controls. However, the irregular shape of oryzalin-treated cells did not change.

4. Discussion

Valonia exhibited regeneration from protoplasts through artificial extrusion of the protoplasm, as previously described in coenocytic green algae such as *Boergesenia, Bryopsis, Chaetomorpha* and *Microdictyon* (Enomoto & Hirose 1972, Tatewaki & Nagata 1970, Kim *et al*. 2001, Kim *et al*. 2002, Klotchkova *et al*. 2003). When *Valonia* cells were cut and the contents were released into the medium, numerous protoplasts contracted into irregularly shaped masses, which were enveloped by a membranous structure. These protoplasts produced new cell walls as the protoplasms reverted to an even parietal distribution. Such a mechanism is seen as advantageous to these siphonous algae which are constantly exposed to the harsh marine environment. Furthermore, this provides a good opportunity for propagation in the wounded cells as previously suggested by Kim *et al*. (2002).

Even before completely contracting to a compact mass, protoplasts began the accumulation of amorphous β-glucan residues which polymerized into a membranous structure containing sulfated and carboxylated polysaccharides, confirming earlier observations (Shepherd *et al*. 2004). By the time the protoplast attained a nearly round configuration these acidic polysaccharides had collected around the protoplast, polymerized and served as its initial cover for the next 24 hours as reconstruction of the cell wall started. According to Kim *et al*. (2002) and Klotchkova *et al*. (2003), these polysaccharides are part of a possible recognition/binding system between organelles that is mediated by a lectin-carbohydrate complementary system. However, some of these sugar moieties appeared to be inherently located on the outer surface of the protoplast even before contraction, manifesting as sheet-like structures in some protoplasts (Fig. 4a). The present study has provided a demonstration of how such a mechanism can occur through the polymerization of β-glucan residues into an enveloping membrane also containing acidic polysaccharides. These polysaccharides may either be originally present on organelle surfaces or released from the punctured vacuole. Actin filaments may anchor this polysaccharide membrane close to the protoplast as proposed below.

Immunofluorescence studies indicated that when cells were wounded, actin filaments immediately contracted and formed actin cables which pulled the actin network into a clump. This has been previously demonstrated in puncture wounds of *V. utricularis* (Satoh *et al*. 2000). According to La Claire (1989), such a response in *E. verticillata* and *B. forbesii* is a result of a hierarchical assembly of actin filaments into bundles that is Ca^{2+}-dependent. In *Ventricaria ventricosa*, a Ca^{2+} receptor involved in wound healing, calcium-dependent protein kinase, has been reported (Sugiyama *et al*. 2000). Treatment with actin depolymerizing agents did not completely inhibit the contraction of protoplasts but caused distortion or death of protoplasts after 3 days in the present study. Whereas disruption of actin filaments was expected, the death of protoplasts due to a strong chemical such as mycalolide B implies a complete disintegration of actin impacting cellular functions dependent on them.

Apart from the actin cables, numerous ring structures were also observed as the protoplasts contracted. These were located on the surface alongside actin bundles and remained highly visible even after the protoplast had formed a spherical structure when actin networks began to depolymerize. Actin rings have been thought to originate from the successive bundling of actin filament fragments due to various modes of cell disturbance such as mechanical isolation of mesophyll cells in *Zinnia* (Frost & Roberts 1996) and aluminum toxicity in *Vaucheria longicaulis* var. *macounii* (Alessa & Oliveira 2001). However, the significance of these actin rings is unknown especially with regard to actin network - cell membrane - cell wall interaction. Therefore, the issue is whether or not actin is directly fastened to the cell mem-

brane. Information on the cytoskeleton-plasma membrane-cell wall continuum in plant cells is still patchy at present although several integration protein candidates have been forwarded (Baluska *et al*. 2003). Nonetheless, Kobayashi (1996) reported that actin filaments are indeed bound to the plasma membrane in Zinnia cells while in *Saprolegnia ferax* integrin and spectrin homologues were found to facilitate such connections (Kaminskyj & Heath 1995). A plausible scenario in *Valonia* would entail that during the contraction of actin, some filaments may break free or remain attached to the plasma membrane. Thus, it is probable that actin rings may come from fragmented actin aggregates produced from the breakage of potential connections with the cell membrane. Fixed filaments, on the other hand, could have contracted and pulled a portion of the cell membrane together with the protoplast. Cells treated with latrunculin B for 3 days resulted in protoplasm collapsing to the center of the cell, appearing as though connections between the wall and the protoplasm had been degraded. In addition, these chemicals could have inhibited the repolymerization of G-actin to F-actin after 24-48 hours.

In terms of microtubule reorganization, CMTs did not appear to play a pivotal role during the initial part of protoplast formation. Immediately after extrusion, parallel MTs convoluted apparently as a result of, or to give way to, actin contraction as La Claire (1987) previously posited. Treatment with MT-depolymerizing chemicals resulted in a minimal reduction in the number of contracted protoplasts. However, observations on wound healing using APM in *V. utricularis* (Satoh *et al*. 2000) and protoplast formation in *E. verticillata* and *B. forbeseii* (La Claire 1987) indicated no effect on contraction. Ruling out any effect of MT degradation on protoplast contraction would mean that no possible actin-MT interaction exists which recent studies have proven otherwise (Petrášek & Schwarzerová 2009). For instance, cross-linking of MTs and AFs in plants was found to be facilitated by proteins called formins (Deeks *et al*. 2010). If a similar or homologous protein is present in *Valonia*, the destruction of MTs would therefore cause a decrease in efficiency in hauling protoplasts into an inward-curling semicircular sheet which may lead to straight- or reverse-curling protoplasts. Aside from potentially providing a cause behind the convoluted form of CMTs, it may also present another explanation on the presence of actin rings. As the CMTs bend due to the pull of actin cables, some of these cables may lose attachment due to breakage of possible cross-links, producing loose-end actin fragments which then aggregate and form rings. More of such rings should be generated when CMTs subsequently randomize and depolymerize before new parallel CMTs are organized.

Microtubules are known to control cell shape in higher plants (Goddard *et al*. 1994, Paradez *et al*. 2006) as in algal protoplasts (La Claire 1987, Hayano *et al*. 1988, Mizuta *et al*. 1985). The distribution of parallel CMTs provides the structural framework for the protoplasm and cell membrane around which the cell wall is constructed. In *Valonia*, this parallel arrangement of CMTs coincides with the attainment of a spherical cell shape (Fig. 5f). As expected, treatment with oryzalin resulted in a misshapen cell (Fig. 8f). However, APM and colchicine induced a different effect in which spherical cells contained fragmented protoplasmic clusters (Fig. 8d,e). Lower and higher concentrations of these two chemicals did not produce irregularly shaped cells (data not shown). This suggests that CMTs may be involved in supporting the even distribution of protoplasm – an interesting complement to the AF-MT cross-linkage argument above. In addition, APM and colchicine could have had a delayed effect compared to oryzalin, which impacted MTs before the cell shape was determined. Another possibility is that it was readily absorbed by the protoplast just as MT repolymerization commenced.

The direction of MTs is not believed to directly orient the synthesis of microfibrils in *Valonia macrophysa* (Itoh & Brown 1984), *Boodlea coacta* (Mizuta & Okuda 1987), *Chaetomorpha moniligera* (Okuda & Mizuta 1987) and *Chamaedoris orientalis* (Okuda *et al*. 1990). Results indicated that cellulosic materials began forming while CMTs were still in a random state. Intense cell wall synthesis occurred as CMTs began to assume a parallel arrangement and continued to thicken thereafter. These observations concurred with the previous studies in that CMTs were not yet in place to facilitate a defined orientation of microfibril synthesis to be of considerable impact. However, initial membrane formation always preceded the construction of the cell wall, as expected. This suggests that the cell membrane plays a significant role in the rapid synthesis of microfibrils as indicated by the multiple layers of the cell wall after 72 hours. Traces of the initial membrane remained during the formation of the cell wall and could have, in addition, provided the attachment between the protoplasm and the cell wall.

In conclusion, several concurrent modifications in the architecture of protoplasts can transpire in such a short span of time. Illustrating the mechanisms involved contributes towards understanding the dynamics of plant cytoskeleton-membrane-cell wall interactions. In addition, biochemical studies detailing the regulation of the cascade of enumerated events can be of great advantage

in explaining how a cell can cross the threshold between a simple wound response and a complete rearrangement towards a new cell.

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