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# FROM PROTOPLASM TO SWARMER: REGENERATION OF PROTOPLASTS FROM DISINTEGRATED CELLS OF THE MULTICELLULAR MARINE GREEN ALGA *MICRODICTYON UMBILICATUM* (CHLOROPHYTA)[1]

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**Protoplast regeneration from extruded cytoplasm of the multicellular marine green alga *Microdictyon umbilicatum* (Velley) Zanardini (Cladophorales, Anadyomenaceae) was investigated. The early process of protoplast formation is comprised of two steps: agglutination of cell organelles into protoplasmic masses followed by generation of a temporary enclosing envelope around them. Agglutination of cell organelles was mediated by a lectin–carbohydrate complementary system. Three sugars, D-galactosamine, D-glucosamine, and α-D-mannose, inhibited the agglutination process, and three complementary lectins for the above sugars, peanut agglutinin, *Ricinus communis* agglutinin, and concanavalin A, bound to the surfaces of chloroplasts. Agglutination assay using human erythrocytes showed the presence of lectins specific for the above sugars in the algal vacuolar sap. A fluorescent probe 1-(4-trimethylammoniumphenyl)-6-phenyl-a, 3,5-hexatriene revealed that the envelope initially surrounding protoplasts was not a lipid-based cell membrane. However, this developed several hours later. Simultaneous fluorescein diacetate and propidium iodide staining showed that the primary envelope had some characteristics of cell membranes, such as semipermeability and selective transport of materials. Also, fluorescein diacetate staining showed esterase activity in the protoplast and relocation of cell organelles and compartmentalization of cytoplasm during the process of regeneration. Both pH 7–9 and salinity 400–500 mM were found to be essentially important for the development of the protoplast envelope. When the basic regeneration process was accomplished, two alternative pathways of development were seen; about 70% of one-celled protoplasts transformed into reproductive cells within 2 weeks after wounding, whereas others began cell division and grew into typical *Microdictyon* thalli. Quadriflagellate swarmers were liberated from the reproductive cells, and they germinated into mature individuals. It is therefore suggested that this species may use the wound response as a method of propagation and dispersal.**

***Key index words:*** **cell membrane; *Microdictyon*; protoplast regeneration; swarmers; wound-induced reproduction**

***Abbreviations:*** **FDA, fluorescein diacetate; FITC, fluorescein isothiocyanate; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-a, 3,5-hexatriene**

The ability of some coenocytic marine algae to heal wounds has been well studied (e.g. Menzel 1988, La Claire II 1991). When cells are damaged in seawater, the cytoplasm rapidly retracts from the site of injury, and then cells quickly seal the wound with one or more insoluble plugs composed of proteins, polysaccharides, or both to prevent loss of the cell contents (Mariani-Colombo et al. 1980, Pak et al. 1991). In time, the cell recovers its original volume and shape, and a new cell wall is then formed to repair the wounded wall.

Moreover, several coenocytic algae, *Bryopsis* spp., *Cladophoropsis membranacea* (Ag.) Boerg., and *Ernodesmis verticillata* (Kütz.) Boerg., generated protoplasts *in vitro* from the extruded protoplasm (Tatewaki and Nagata 1970, La Claire II 1982a,b, Kobayashi and Kanaizuka 1985, Pak et al. 1991, Kim et al. 2001). When the cells were cut in seawater, the extruded protoplasm spontaneously generated numerous new cells. These protoplasts formed new cell walls around themselves and eventually developed into mature plants. Although some components of the primary envelope of newly generated protoplasts were identified by ultrastructural and biochemical studies in *Bryopsis* cells (Pak et al. 1991, Kim et al. 2001), investigations of the mechanism and functions of this wound response have been extremely fragmentary.

Kim et al. (2001) provided an explanation for the process of protoplast regeneration in *Bryopsis plumosa* and showed how the cell membrane regenerates in seawater. The process of membrane regeneration involved two steps: formation of a temporary polysaccharide envelope that primarily surrounds protoplasts and its subsequent substitution by a lipid-based membrane. The enclosing envelope of the protoplasts was not initially a lipid-based plasma membrane, but it had many characteristics of cell membranes, including semiper-

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meability and selective transport of materials (Kim et al. 2001). However, the mechanism of aggregation of the cell organelles in seawater is not understood.

Considering their large cell size, the importance of wound healing in coenocytic green algae is obvious. To maintain integrity of the cell, these plants need to heal wounds as quickly as possible. Presumably, some species evolved the wound response and protoplast formation as a dispersal mechanism. From quantitative studies of protoplast formation in *Bryopsis*, Kim et al. (2001) suggested that this wound response might be interpreted as a method of propagation, because hundreds of new plants could be regenerated from a single disrupted algal cell. However, before an assessment can be done, it is important to extend the survey of this phenomenon to other algal groups, including those with multicellular systems.

In the present study, the regeneration of protoplasts from the extruded cytoplasm of the multicellular alga *Microdictyon* is described for the first time. Here we try to answer two questions: How do the extruded cell organelles regenerate new cells *in vitro*, and what is the function of protoplast formation in this species?

## MATERIALS AND METHODS

*Plant material and laboratory culture.* *Microdictyon umbilicatum* (Velley) Zanardini was collected in large floating nets in estuarine Burrill Lake, New South Wales, Australia on 14 December 1996. The unialgal isolate was maintained at The University of Melbourne in modified Provasoli's medium (West and McBride 1999) at 23–26° C, 10–15 μmol photons·$m^{-2}$·$s^{-1}$ provided by cool-white fluorescent lamps, and 12:12-h light:dark daily photoperiod up to December 1999. Thereafter, the alga was transferred to Kongju National University, Korea and was grown in IMR medium (Kim and Kim 1999) at 25° C, 30 μmol photons·$m^{-2}$·$s^{-1}$ provided by cool-white fluorescent lamps, and a 16:8-h light:dark photoperiod.

*Protoplast preparation.* Small pieces of thalli were cut with a razor blade and were flattened with a pincette. The extruded protoplasm was collected with a pipette and was transferred to 100 ×

TABLE 1. Percentage of protoplasts with an envelope after treatment with various carbohydrates.

| | |
|---|---|
| Control | 79.8 ± 4.6 |
| D(+)-galactose | 79.2 ± 4.3 |
| D(+)-glucose | 79.5 ± 2.3 |
| *N*-acetyl-D-glucosamine | 78.2 ± 4.6 |
| *N*-acetyl-D-galactosamine | 78.6 ± 3.8 |
| L(−)-fucose | 72.2 ± 5.8 |
| D(+)-galactosamine | 7.2 ± 2.3* |
| D(+)-glucosamine | 6.0 ± 3.3* |
| D(+)-mannose | 3.9 ± 2.3* |

Cytoplasm was extruded into a solution containing each carbohydrate. Number of protoplasts 1 h after wounding was calculated using the equation % = (no. of protoplasmic masses enclosed within envelope / total no. of protoplasmic masses) × 100. Data are the means ± SD of multiple comparisons by Tukey method, $n = 10$, * = $P < 0.001$.

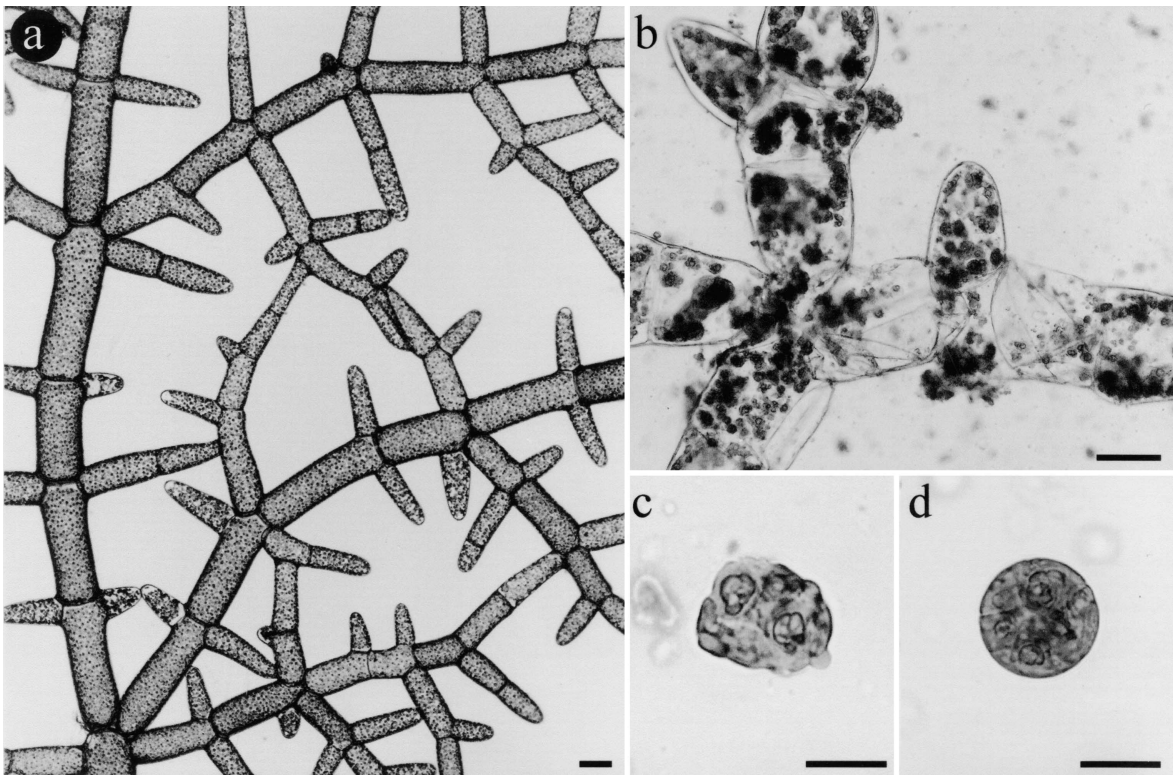



FIG. 1. The sequential process of protoplast regeneration from disrupted cells of *Microdictyon umbilicatum*. (a) Vegetative plant. (b) Protoplasm is expelled from the damaged cells and spreads in seawater. (c) Extruded cell organelles aggregate in seawater. (d) Regenerated protoplast forms an envelope within 10 min after wounding. Scale bars: a = 100 μm, b = 50 μm, c–d = 10 μm.

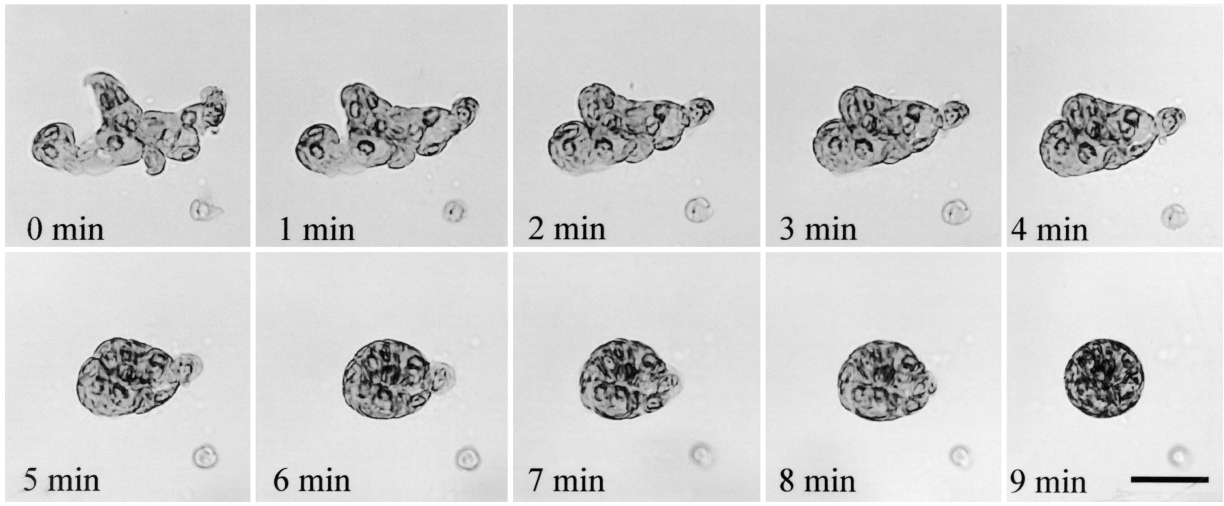



FIG. 2. Time-lapse photography of early protoplast regeneration. Scale bar, 10 μm.

20-mm Petri dishes containing seawater. The protoplasts that developed were checked under the microscope, and all remnants of the disrupted plants were carefully removed from the dish. Culture of the protoplasts was done under the same conditions as specified above. The treatments were repeated a minimum of 20 times.

*Effects of pH, salinity, and ions on protoplasts regeneration.* The effects of pH, salinity, and ions on regeneration were studied using artificial seawater, composed of distilled water 100 mL, NaCl 2.63 g, $MgCl_2$ 0.609 g, $MgSO_4$ 0.193 g, KCl 0.074 g, $CaCl_2$ 0.11 g, Tris 0.1 g. pH was adjusted with HCl or NaOH. Four ions, calcium, magnesium, potassium, and sulfur, were tested throughout the experiments. To study the effects of calcium, potassium, and sulfur ions on regeneration, one salt, $CaCl_2$, KCl, and $MgSO_4$, was deleted from the artificial seawater in each case, respectively. In case of magnesium, both $MgCl_2$ and $MgSO_4$ were deleted. Extruded protoplasm was transferred into a testing medium, and percentage of protoplasts enclosed within an envelope 1 h after wounding was calculated using the equation % = (no. of protoplasmic masses enclosed within envelope / total no. of protoplasmic masses) × 100.

*Carbohydrate experiment.* The carbohydrates used in this study are listed in Table 1. Each carbohydrate was dissolved in seawater to a final concentration of 0.1 M. Sodium hydroxide (NaOH) was used to adjust pH of the solutions containing each sugar to 8. Protoplasm was extruded into a solution containing each sugar, and the number of regenerated protoplasts was counted 1 h after wounding.

*Agglutination assay and monosaccharide inhibition of crude extract.* To prepare a crude extract of the algal vacuolar sap, 20 g of fresh material were cut into small pieces with scissors in 900 mM NaCl artificial seawater, pH 6, and squeezed through several layers of a nylon mesh (90 μm pore size) to remove the cell walls. Thereafter, the extract was centrifuged at 5000*g* for 20 min to sediment out cell organelles and debris, and the supernatant was adjusted to 450 mM NaCl and pH 8, followed by centrifugation at 12,000*g* for 30 min. The supernatant was concentrated to 4 mL by ultrafiltration using an Amicon filter model 8200 (Millipore Corp., Bedford, MA), 75 psi (5.3 $kg \cdot cm^{-2}$) and used for agglutination assay.

For the investigation of agglutinating activity, we followed protocols described by Hori et al. (1986). U-shaped microtiter plate wells were used in this test. These well plates are designed in such a way that if there is no agglutination, the cells sink to the bottom; therefore a dot is created. When there is agglutination, the agglutinated cells disperse in the solution and distribute evenly on the bottom of the well without forming a dot. A serial 2-fold dilution of the crude extract was made in a final volume of 25 μL saline in microtiter plate wells, and 25 μL erythrocyte suspension was added sequentially to each well. For the monosaccharide inhibition test, a serial 2-fold dilution of inhibitory sugar solution (0.2 M) was made in a final volume of

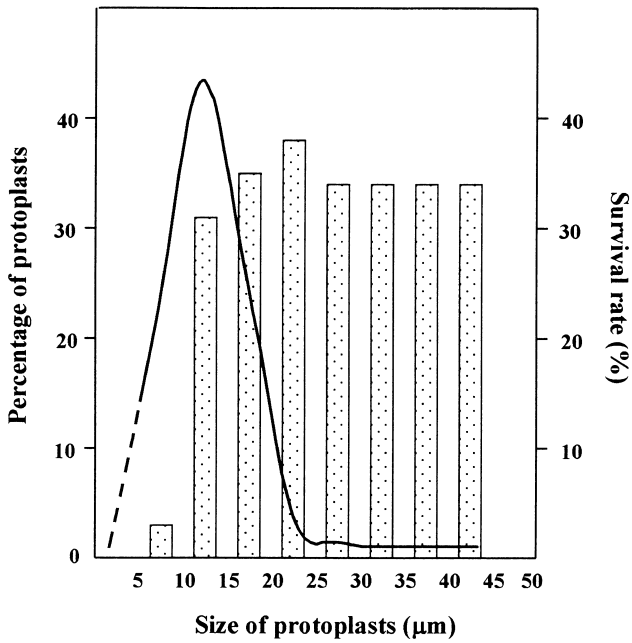



Fig. 3. The size distribution and survival rate of the regenerated protoplasts. Size distribution (line) was studied 1 h after wounding as follows: the total number of protoplasts that were generated from the extruded cytoplasm was considered as 100%, and the percentage of protoplasts of each size group was calculated from the total number of protoplasts. For survival rate (bars), the number of protoplasts of each size group was considered as 100%, and the percentage of protoplasts, which remained intact 24 h after formation, was calculated in each size group using the equation % = (no. of cells 24 h/no. of cells 1 h) × 100.

25 μL saline in microtiter plate wells, and 20 μL erythrocyte suspension and 5 μL crude extract were added sequentially to each well. Saline solution (25 μL) was added instead of the inhibitory sugar solution as the control.

*Application of fluorescent probes.* For cellulosic cell wall staining, Calcofluor White M2R (Sigma Chemical Co., Vienna, Austria) was diluted in artificial seawater at pH 8 to a concentration of 0.1 mg·mL$^{-1}$ and was used for 5–10 min. After a seawater wash, protoplasts were mounted in enriched seawater and examined under a UV filter (U-MWG, Olympus [Tokyo, Japan], 330–380 nm excitation filter, 420 nm barrier filter). As an indication of the viability of protoplasts, fluorescein diacetate (FDA) and propidium iodide staining was performed over a time course (Oparka and Read 1994). FDA is a vital stain that detects specific enzyme reactions within the cytosol. FDA is a nonfluorescent molecule taken up passively into the cell. If the cell is alive, the cell membrane transports this substrate actively into the cytosol, where it is divided by esterases and subsequently fluoresces green when excited under a UV light. For FDA staining, one drop of the stock solution of FDA (Sigma; 1–5 mg·mL$^{-1}$ in acetone) was diluted in artificial seawater at pH 8 and was applied to the regenerated protoplasts over time. For propidium iodide staining, 1–5 mg·mL$^{-1}$ stock solution of propidium iodide (Sigma) dissolved in distilled water was diluted to a concentration of 100 μg·mL$^{-1}$ with enriched seawater. The fluorochrome was applied to the protoplasts for 5 min and then examined under a blue filter (U-MWIG, Olympus, 450 nm excitation filter, 520 nm barrier filter) while still immersed in the stain. Algal nuclei were stained with the DNA-specific fluorochrome DAPI (4′,6-diamidino-2-phenylindole, Sigma) using a heat fixation method. Regenerated protoplasts that attached to coverslips were dipped in 5 μg·mL$^{-1}$ DAPI solution in seawater for 5 min, and then the coverslips were slightly heated over a boiler for a few seconds. After staining, protoplasts were mounted on slides in the DAPI solution and were examined under a UV filter. For membrane staining, 1-(4-trimethylammoniumphenyl)-6-phenyl-a, 3,5-hexatriene (TMA-DPH, Sigma) was dissolved in DMSO (Sigma) to a final concentration of 3 mM. The fluorochrome was applied to the regenerated protoplasts at various times after wounding and cells were examined under a UV filter.

To stain the carbohydrates on the surface of chloroplasts, the vacuolar-sap-free cell organelles were obtained as follows. Protoplasm was extruded in 900 mM NaCl artificial seawater of pH 6 and centrifuged at 8000*g* for 2 min. The supernatant was removed, and the chloroplasts were resuspended in 450 mM NaCl artificial seawater of pH 6. Fluorescein isothiocyanate (FITC)-labeled lectins, concanavalin A, peanut agglutinin, and *Ricinus communis* agglutinin (Vector Laboratories, Burlingame, CA), were diluted in PBS to a final concentration of approximately 10 μg·mL$^{-1}$. The lectins were added to the vacuolar-sap-free chloroplasts and were incubated for 1 h at room temperature. The unbound lectin was removed by a seawater wash, and materials were examined under a blue filter.

*Microscopy.* An Olympus BX50 microscope was used for fluorescence microscopy. FDA staining of protoplasts and FITC-labeled lectin staining of chloroplasts were examined with a Fluoview II (Olympus) confocal microscope. Photographs were taken with Polaroid digital camera (Polaroid, Cambridge, MA) and Olympus PM-C35 photocamera with Kodak Gold III 100 color film (Kodak, Rochester, NY).

## RESULTS

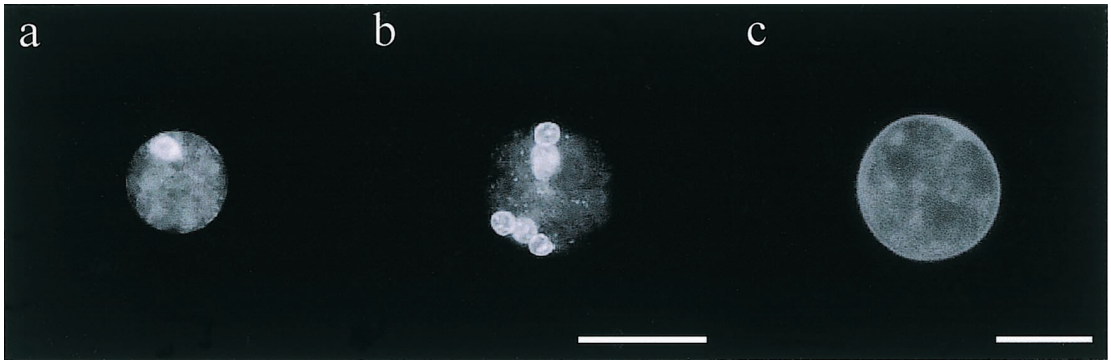
*Microdictyon umbilicatum* has flat, reticulate, monostromatic blades formed by multibranched filaments of multinucleate cylindrical cells. The growing tips of cells fuse to adjacent cells by adhesion of cell wall polysaccharides. Branches do not fit closely to each other, so that a rather regular open network is formed (Fig. 1a). Reproduction has never been recorded for this species, although a life history involving a gametophyte phase with biflagellate isogametes and a sporophyte phase with quadriflagellate spores is recorded for other species, *M. okamurae* Setchell and *M. japonicum* Setchell (see Hori 1994).

*Process of protoplast regeneration.* When the plant cells were cut and squeezed out, the protoplasm flowed away from them and spread in seawater (Fig. 1b). The extruded cell organelles aggregated rapidly, and numerous tiny irregularly shaped protoplasmic masses were formed (Fig. 1c). The binding between cell organelles became tighter within several minutes, and protoplasmic masses changed from irregular to spherical in shape and were covered with an envelope in 5–10 min (Figs. 1d and 2). When protoplasts were mechanically disrupted again in seawater, new protoplasts were never regenerated. About 10 to 20 protoplasts were formed from the protoplasm extruded from a single disintegrated cell of approximately 130 × 65 μm (length/width).

Sizes of the resultant protoplasts were rather constant, averaging about 10–20 μm in diameter, throughout the experiments regardless of the wounding conditions (Fig. 3). Survival of the protoplasts was partially dependent on their size. No protoplast less than 9 μm in size survived more than 24 h after wounding. The survival rate of protoplasts did not increase with size greater than 20 μm diameter (Fig. 3). The number of nuclei in the protoplasts was irregular and varied even among the cells of the same size. Thus, protoplasts of approximately 10 μm diameter usually contained only one nucleus and larger cells contained 1 to 3 or more nuclei (Fig. 4, a and b). These results suggest that the cell organelles come out independently, not in regular clusters, when the cells are damaged, and protoplast assembly occurs spontaneously.

FDA staining was used to examine the viability of newly generated protoplasts and the permeability of the primary envelope. A dramatic decrease in the number of viable protoplasts was observed within the first 3 h after wounding, as shown by FDA staining (Table 2). About 35% of the protoplasts lost viability by 3 h after formation. Although the cells were still enclosed within an envelope and outwardly they looked intact, esterase activity was lacking. The nonviable protoplasts degenerated by 12 h after wounding, but those that were able to survive until that time began to develop a cell wall.

Calcofluor White staining revealed that regeneration of the cell wall began around 20 h after the protoplast formation. About 11% of cells were labeled at that time, and over 90% developed a cell wall 48 h after wounding (Fig. 4c). The development of a cell wall was not dependent on initial size of the protoplasts. Once the protoplasts developed a cell wall, most (over 80%) survived and began to grow in enriched seawater. During the next week after wounding, the newly regenerated cells increased in size several times from their original volume (up to 140 μm). Thereafter, two alternative pathways of development were seen. About 70% of one-celled protoplasts became reproductive and formed quadriflagellate zoo-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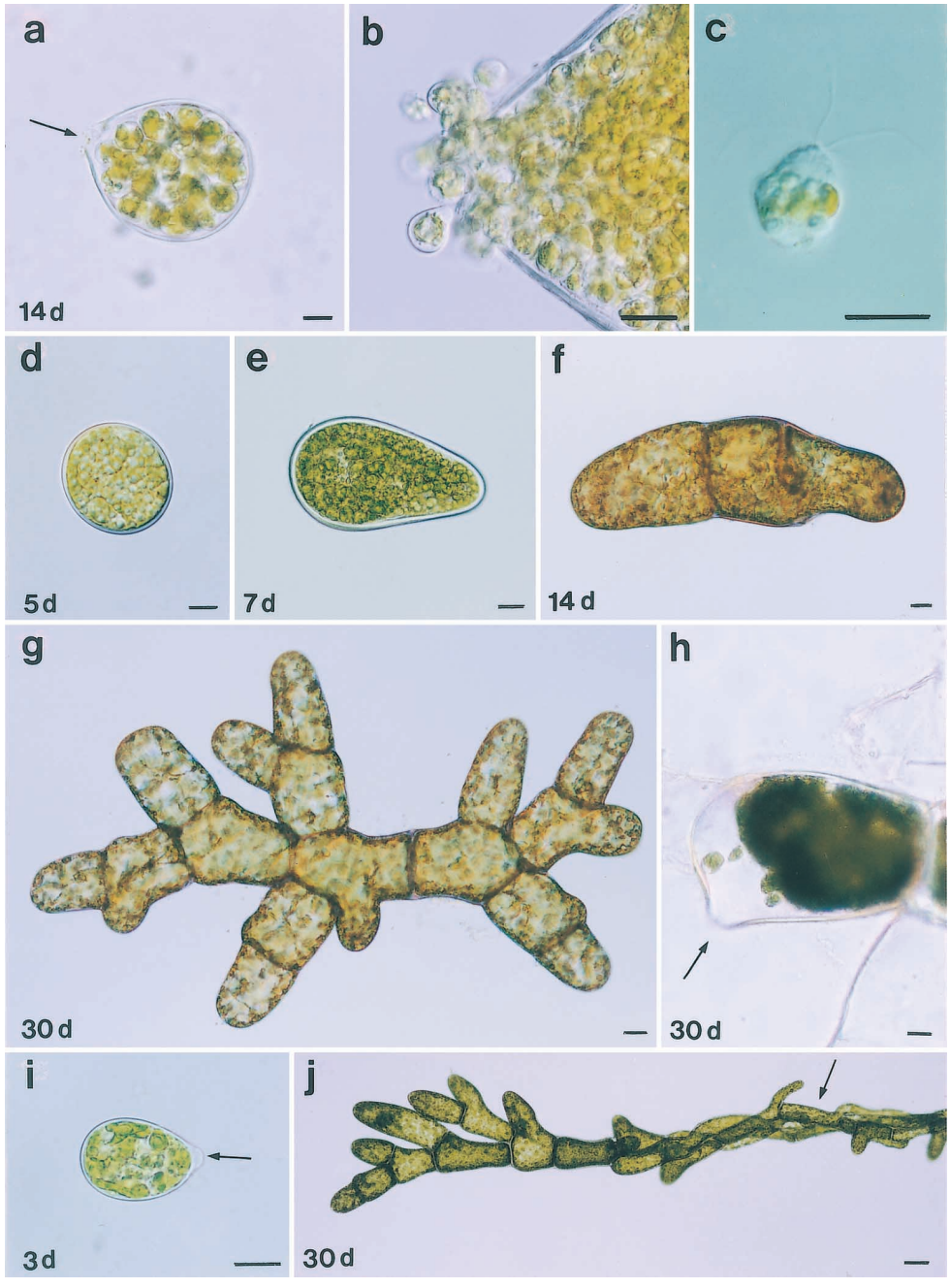
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FIG. 4. DAPI staining and Calcofluor White staining of the regenerated protoplasts. (a and b) DAPI staining shows irregular number of nuclei in protoplasts. Note that protoplasts of almost similar size have different number of nuclei. (c) Calcofluor White staining indicates that regeneration of the protoplast cell wall begins 20 h after wounding. A bright blue labeling can be seen 48 h after the protoplast formation. Scale bars: a–c = 10 μm.

TABLE 2. Percentage of viable protoplasts determined by FDA staining over time.

| 1 h | 2 h | 3 h | 6 h | 9 h | 12 h |
|---|---|---|---|---|---|
| 68.45 ± 3.3 | 46.5 ± 3.5 | 33.3 ± 2.9 | 33.6 ± 0.5 | 30.9 ± 2.3 | 92.0 ± 3.9 |

FDA was applied to the regenerated protoplasts at various times after wounding. Although all protoplasts were enclosed within envelopes at 1–9 h after wounding, some cells did not show esterase activity. At 12 h, all nonviable cells lost the enclosing membrane, and most cells that had a membrane possessed esterase activity. The % viability was calculated using the equation % = (no. of fluorescing cells/ total no. of cells) × 100. Values are the means of 10 comparisons ± SD.

spores within 2 weeks after wounding (Fig. 5, a–c). The others began cell division and grew into typical *Microdictyon* thalli that also formed similar reproductive cells eventually (Fig. 5, d–h). In both cases, quadriflagellate swarmers were liberated through an aperture that developed at the end of slightly protruded portion of the cell wall (Fig. 5, a–c, h). These zoospores germinated into mature plants that were morphologically identical to those developed from the protoplasts except that the lower segments of their thalli produced numerous rhizoids (Fig. 5, i and j). Usually, one reproductive cell produced up to 100 swarmers. The swarmers were of oval shape, 10–15 μm in length, which is almost the same as that of most protoplasts regenerated from the extruded cytoplasm.

*Characteristics of protoplast enclosing envelope.* FDA and propidium iodide were applied to the protoplasts to test for the integrity of the enclosing envelope. When protoplasts were suspended in seawater containing FDA at the first 1 h after formation, strong labeling appeared within the cells in 5 min (Fig. 6, a and b). The labeling increased significantly during the next hour and was evenly distributed within the cell (Fig. 6c). The amount and distribution of fluorescence did not change until 10 h after the protoplast formation. Thereafter, it was observed only in the peripheral region of the cells, indicating that their cytoplasm became compartmentalized and a vacuole was formed (Fig. 6d). Interestingly, esterase activity was not observed inside the vacuole. The protoplasts were not stained with propidium iodide, indicating their envelopes were not leaky.

To determine the nature of the protoplast enclosing envelope, TMA-DPH was applied to the regenerated protoplasts at various times after wounding. This fluorescent probe specifically stains cell membranes (Mulders et al. 1986). When TMA-DPH is applied to a cell, its molecules embed between the lipid bilayer and fluoresce blue under UV excitation, thereby visualizing the cell membrane. When TMA-DPH was applied to the protoplasts during the first 2 h after wounding, no staining of the envelopes occurred (Fig. 7a). Thereafter, a very thin dim labeling in patches was visible on the protoplast surfaces, but this was unstable and faded easily (Fig. 7b). The labeling became stronger around 12 h after wounding, and a thin homogeneous line on the surfaces of protoplasts was observed, but it was still dim and quite unstable under a UV light. Strong labeling appeared 24 h after the protoplast formation, the time when development of the cell wall began (Fig. 7c).

*Aggregation of cell organelles.* The aggregation of cell organelles and the following formation of the primary envelope were pH dependent (Fig. 8). When the algal cells were chopped and extruded into artificial seawater, the cell organelles aggregated best at pH 7–9, which is the normal pH of seawater, but vacuolar-sap-free cell organelles could not aggregate in this condition. The tight binding between cell organelles was not observed at a pH lower than 6 or higher than 10. Therefore, we hypothesized that some materials in the vacuolar sap mediate aggregation of the cell organelles.

To determine if aggregation of the cell organelles is mediated by a lectin–carbohydrate complementary system, a carbohydrate inhibition experiment was performed with various sugars (Table 1). Protoplasm was extruded into a solution containing each sugar, and the number of regenerated protoplasts was counted 1 h after wounding. The agglutination of cell organelles and formation of protoplast envelopes were inhibited by D-galactosamine, D-glucosamine, and α-D-mannose.

FIG. 5. Development of the protoplast over time. (a) Protoplast with swarmers 2 weeks after wounding. Arrow shows liberation aperture for the swarmers. (b) Liberation of swarmers. (c) Quadriflagellate swarmer released from the protoplast. (d–g) Micrographs of the protoplasts taken at different times after wounding. (h) Some cells become reproductive over time and swarmers are formed simultaneously in large numbers from the ordinary segments of the thallus. (i) The swarmers lost flagella and began to grow (arrow). (j) Swarmers eventually grew into mature plants identical to those developed from the protoplasts except that the lower segments of their thalli developed numerous rhizoids (arrow). Scale bars: a–f, h–i = 10 μm; g, j = 50 μm.

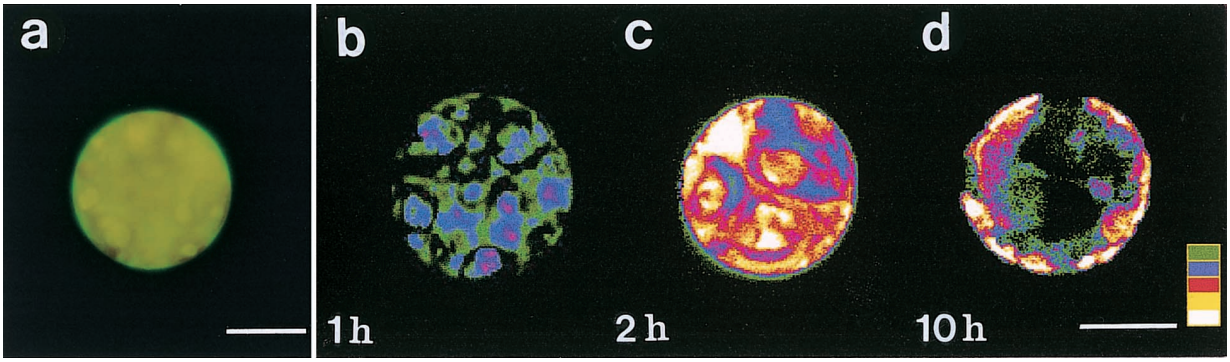


FIG. 6. Fluorescence micrograph of FDA staining of the protoplast. (a) FDA is transported into the cell and subsequently fluoresces green when divided by cellular esterases. (b–d) Confocal laser scanning micrographs of FDA staining of the protoplasts taken at various times after wounding. Artificial colors indicate fluorescence intensity inside the protoplast that increases from green to white. (b) Strong labeling appears within the protoplast at 1 h after wounding. (c) Esterase activity inside the protoplast increases over time and therefore labeling becomes stronger. (d) Cytoplasm becomes compartmentalized 10 h after the protoplast formation and labeling occurs only on the periphery of the cell. Scale bars, 10 μm.

Percentage of protoplasts decreased dramatically from 79.8% in control to 3%–7% in treatment with those sugars. The presence of the above sugar moieties on the surface of chloroplasts was verified with their complementary FITC–lectins (Fig. 9, a–d).

When crude extract of the vacuolar sap was added to human erythrocytes, strong agglutinating activity was observed, but this was inhibited when the above sugars were added to the crude extract (Fig. 10). The inhibitory effect of D-galactosamine and D-glucosamine was very strong. Even 0.01 M solution of those sugars could inhibit the agglutinating activity of the crude extract. The treatment of α-D-mannose showed the inhibitory effect only at 0.2 M solution (Fig. 10). The inhibitory effect did not become stronger even when all three sugars were treated simultaneously. When crude extract was treated to vacuolar-sap-free chloroplasts, it could aggregate chloroplasts effectively at pH 7–9. These results suggest that there are lectins in the vacuolar sap that mediate the aggregation of cell organelles when exposed to seawater.

The depletion of salts, magnesium, potassium, and sulfur from artificial seawater showed no effect on aggregation of the cell organelles, suggesting that the lectins may not need these salts for agglutinating activity. However, in artificial seawater lacking calcium and magnesium, the number of protoplasts surrounded by an envelope was lower than that in the solutions lacking potassium and sulfur (Fig. 11). The protoplasts degenerated very rapidly in the solutions that lacked calcium and magnesium. The optimum salinity for the aggregation of the cell organelles was 400–500 mM NaCl, which is the normal salinity of seawater. These data indicate that these lectins become effective in the salinity condition close to seawater. Also, protoplasts swelled or shrank according to the salinity of the solution, indicating that the primary envelope is semipermeable.

## DISCUSSION

The process of protoplast regeneration from the disrupted cells in *M. umbilicatum* was similar to that in *B. plumosa* (Huds.) Ag. except for the initial size of protoplasts (Kim et al. 2001). The regeneration process was basically composed of five stages: aggregation of the cell organelles in seawater, changes in protoplasmic masses from an irregular to a spherical shape, formation of a primary envelope, development of a lipid-based cell membrane, and development of a cell wall.

Considering that the cell organelles are expelled independently but not in regular clusters when the plant cells are damaged, it is essential to assemble all important cell organelles in a very short time and to

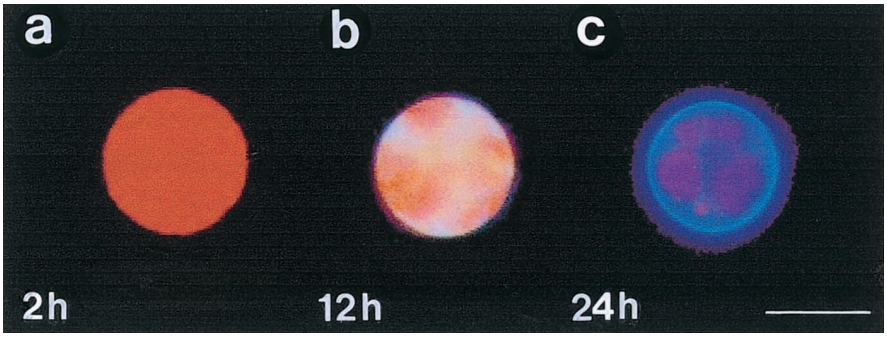


FIG. 7. TMA-DPH staining of the regenerated protoplasts. (a) The absence of blue color on the surface of the protoplast indicates that the enclosing envelope is initially not a lipid-based cell membrane. (b) Some fragments of lipid membrane begin to incorporate into the envelope forming patches. (c) Regeneration of the protoplast membrane is complete and a bright blue labeling appears. Scale bar, 10 μm.

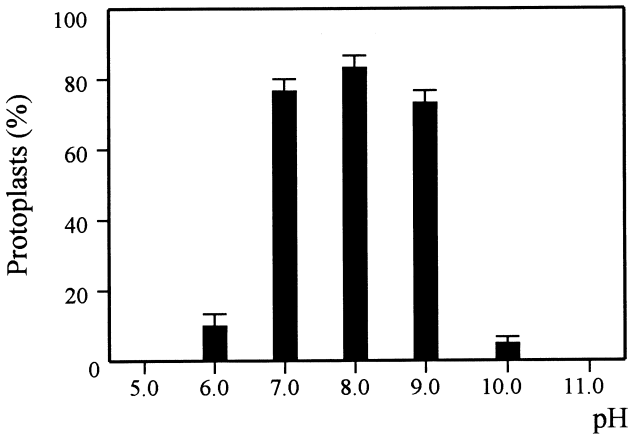


FIG. 8. Effects of pH on aggregation of extruded protoplasm and formation of protoplasts. Bars on the top show standard deviation. $n = 10$.

exclude other contaminating particles in seawater. Therefore, the presence of a specific recognition/binding system between cell organelles is expected. Our data indicate that agglutination of cell organelles might be mediated by a lectin–carbohydrate complementary system, because the agglutination process was inhibited when D-galactosamine, D-glucosamine, and α-D-mannose were added to the solution. Complementary FITC–lectin labeling showed the presence of these sugar moieties on the surface of chloroplasts. An agglutination assay using human erythrocytes showed that lectins specific for the above sugars were present in the vacuolar sap. Partial purification of lectins by using agarose bound sugar affinity chromatography showed more than two types of lectins involved in the aggregation of cell organelles in this species (unpublished data).

Cell surface carbohydrates have been reported as primary markers for cell–cell recognition events in many organisms (Wassarman 1987, Sharon and Lis 1989, Karlsson 1991). Such recognition systems depend on complementary binding between carbohydrate moieties of glycoconjugates on one cell with specific sugar-binding proteins (lectins) on another cell (Sharon and Lis 1989, Kim 1997). Gamete recognition in some algae is mediated by such a carbohydrate-receptor system (Wiese and Shoemaker 1970, Bolwell et al. 1979, 1980, Kim and Fritz 1993a,b, Schmid 1993, Kim et al. 1996, Kim and Kim 1999). It is interesting because our data suggest that such a recognition system might work on even the chloroplast membranous system. Further work is required to isolate and characterize the individual molecules of this recognition mechanism.

Our data from experiments using TMA-DPH indicate that the primary envelope is not a lipid membrane. It initially is composed of other molecules and then changes to a lipid-based membrane. In our previous study using Nile Red and various enzymes, we showed that the primary envelope of protoplasts in *B. plumosa* is composed of polysaccharides, then changes to a polysaccharide–lipid complex, and finally is substituted by a lipid-based membrane (Kim et al. 2001). From an ultrastructural study on protoplast regenera-

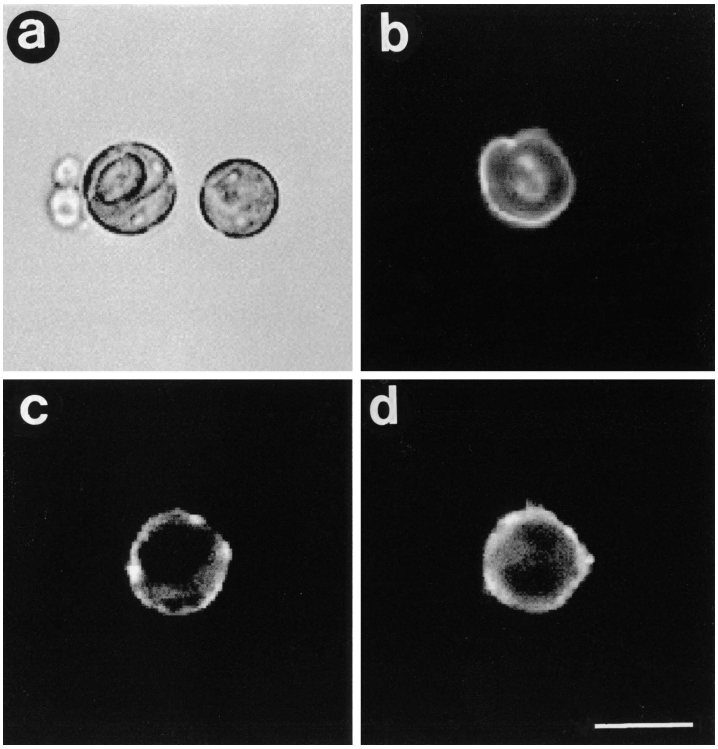


FIG. 9. The binding of FITC-conjugated lectins to the chloroplasts. (a) Differential interference micrograph of the chloroplasts. (b–d) Confocal laser scanning micrographs of the chloroplasts labeled with FITC-concanavalin A (b), FITC-peanut agglutinin (c), FITC-*Ricinus communis* agglutinin (d). Scale bar, 5 μm.

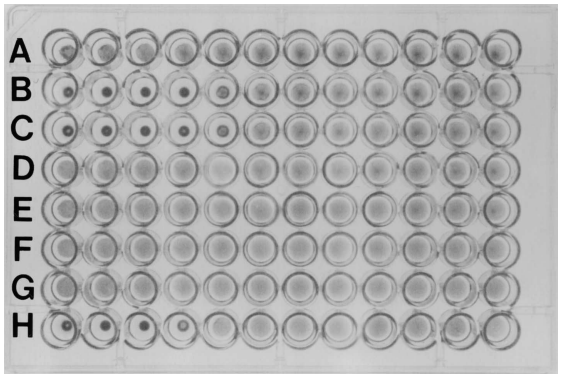


FIG. 10. Inhibition experiment of hemagglutination activity of the crude extract by various sugars. A serial 2-fold dilution of inhibitory sugar solution starting from 0.2 M was made from left to right. Dotted wells represent the inhibition of hemagglutination activity. Three sugars, α-D-mannose, D-galactosamine, and D-glucosamine, showed inhibition. Lane A, α-D-mannose; B, D-galactosamine; C, D-glucosamine; D, D-galactose; E, D-glucose; F, *N*-acetyl-d-glucosamine; G, L-fucose; H, One third titer of α-D-mannose, D-galactosamine, and D-glucosamine treated simultaneously.

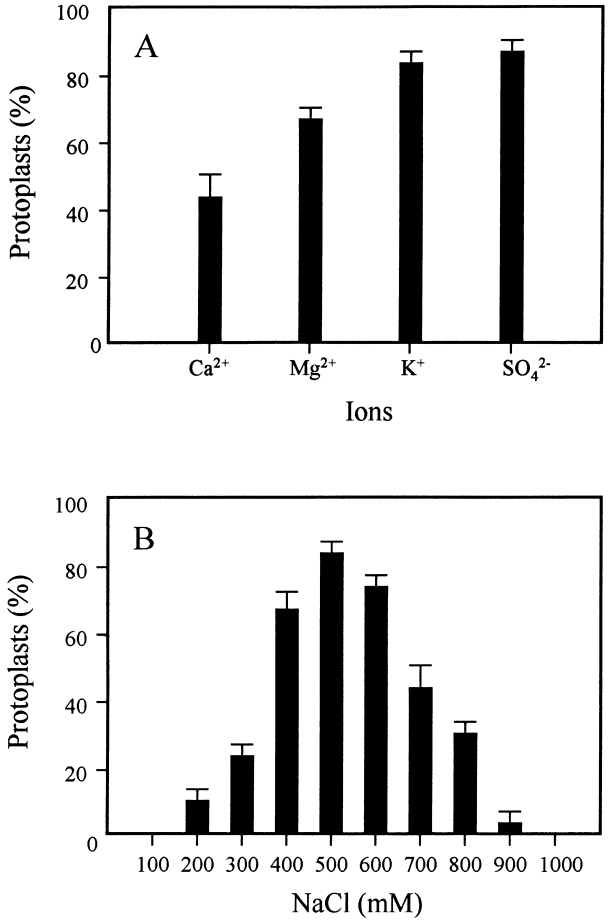


FIG. 11. Effects of ions (A) and salinity (B) on aggregation of extruded protoplasm and formation of protoplasts. (A) The abscissa represents ions missing from the artificial seawater. (B) The abscissa represents molar concentration of sodium chloride in the artificial seawater. The number of protoplasts in control treatment was considered as 100% and the percentage of protoplasts, which remained intact 24 h after formation was calculated in each treatment group using the equation % = (no. of cells 24 h / no. of cells 1 h) × 100. Bars on the top show standard deviation. $n = 10$.

tion in *Bryopsis maxima* Okamura, Pak et al. (1991) also suggested that the primary envelope might not be a lipid membrane. These results indicate that development of the protoplast membrane requires at least several hours and during this time the enclosing envelope performs the functions of cell membranes, that is, it defines the protoplast boundaries and maintains the essential differences between the cytosol and the extracellular environment.

The primary envelope is initially not a lipid membrane; nevertheless, it has many characteristics of the cell membrane. The protoplasts swelled or shrank according to the salinity of the solution, indicating that the primary envelope is semipermeable. Moreover, FDA staining showed that the primary envelope allowed entry of this substrate into the protoplast. The esterase activity, as shown by FDA staining, appeared at the very beginning of the regeneration process. The polarization of cytoplasm required a longer time than the protoplasts of *B. plumosa*, which seem to develop a vacuole as soon as the protoplast becomes surrounded by primary envelope. This may be due to the small size of the protoplasts in *Microdictyon*. However, propidium iodide could not penetrate the envelope when the protoplasts were alive. It is possible that the fragment of cell membrane embedded in the polysaccharide envelope is responsible for this transmembrane movement of selected substances. The fragmentary insertion of cell membrane into the primary envelope during the first 12 h as shown by TMA-DPH staining may support this idea.

Wound response has been studied for many different groups of algae, but it was considered only to be a healing process to maintain the integrity of the thallus (O'Neil and La Claire II 1984, Shihira-Ishikawa 1987, Menzel 1988, Kim and Fritz 1993c, Nawata et al. 1993, Kim et al. 1995, Waaland 1990). However, in coenocytic green algae, the wound-induced protoplast formation seems to play a significant role in asexual reproduction. There are reports on intracellular protoplasm reticulation and subsequent contraction into numerous aplanospores/protoplasts in two marine green algae, *Boergesenia forbesii* (Harvey) Feldmann and *Ventricaria ventricosa* (Olsen *et* West), caused by incubation of the algae in the double concentrated seawater or by perforation of their cell wall with a pincette or a needle (Enomoto and Hirose 1972, La Claire II 1982a,b, 1987, O'Neil and La Claire II 1984, Nawata et al. 1993). However, the nature of the resulting spheres was not identified, and they were termed either as aplanospores (Enomoto and Hirose 1972, Nawata et al. 1993) or protoplasts (La Claire II 1982a,b, 1987, Nawata et al. 1993).

Although there are brief mentions of protoplast formation *in vitro* in *Cladophoropsis membranacea* and *Ernodesmis verticillata* (La Claire II 1982a,b), the ability to form protoplasts from the protoplasm extruded directly into the seawater (*in vitro*) was described in details only in *Bryopsis* spp. (Tatewaki and Nagata 1970, Kobayashi and Kanaizuka 1985, Pak et al. 1991, Kim et al. 2001). The process of protoplast regeneration from disrupted cells may be an even more effective propagation method if the protoplast can generate numerous swarmers in a short time. In *Microdictyon*, about 70% of one-celled protoplasts transformed into reproductive cells within 2 weeks after wounding, whereas others began cell division and grew into mature plants that eventually started reproduction. On average, each reproductive cell produced about 100 quadriflagellate swarmers that germinated into mature individuals. Other evidence supporting this hypothesis is that the protoplast regeneration process occurs best in ordinary seawater at normal pH and salinity. It is noteworthy that this *Microdictyon* strain never showed any reproduction during years of laboratory culture before this experiment.

Considering the large size of the cell in the coenocytic green algae, the importance and benefit of propagation by protoplast formation is obvious; however, more studies at a field ecology level are necessary for a better understanding. Regeneration of protoplasts from the subcellular fractions may be an important model system for the study of such processes as the interaction of various cell organelles, formation of various hybrid cell types, and especially development of the cell membrane.

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Bolwell, G. P., Callow, J. A., Callow, M. E. & Evans, L. V. 1979. Fertilization in brown algae. II. Evidence for lectin sensitive complementary receptors involved in gamete receptors involved in gamete recognition in *Fucus serratus*. *J. Cell Sci.* 36:19–30.

Bolwell, G. P., Callow, J. A. & Evans, L. V. 1980. Fertilization in brown algae. III. Preliminary characterization of putative gamete receptor from eggs and sperm of *Fucus serratus*. *J. Cell Sci.* 43:209–24.

Enomoto, S. & Hirose, H. 1972. Culture studies on artificially induced aplanospores and their development in the marine alga *Boergesenia forbesii* (Harvey) Feldmann (Chlorophyceae, Siphonocladales). *Phycologia* 11:119–22.

Hori, T. 1994. *An Illustrated Atlas of the Life History of Algae.* Vol. 1. *Green Algae.* Uchida Rokakuho, Tokyo, xvii + 367 pp.

Hori, K., Miyazawa, K. & Ito, K. 1986. Preliminary characterization of agglutinins from seven marine algal species. *Bull. Jpn. Soc. Sci. Fish.* 52:232–42.

Karlsson, K. A. 1991. Glycobiology: a growing field for drug design. *Trends Biochem. Sci.* 12:265–72.

Kim, G. H. 1997. Gamete recognition and signal transduction during fertilization in red algae. *Algae* 12:263–8.

Kim, G. H. & Fritz, L. 1993a. Gamete recognition during fertilization in a red alga *Antithamnion nipponicum*. *Protoplasma* 174:69–73.

Kim, G. H. & Fritz, L. 1993b. Ultrastructure and cytochemistry of early spermatangial development in *Antithamnion nipponicum* (Ceramiaceae, Rhodophyta). *J. Phycol.* 29:799–805.

Kim, G. H. & Fritz, L. 1993c. A signal glycoprotein with D-mannosyl residues is involved in the wound-healing response of *Antithamnion sparsum* (Ceramiales, Rhodophyta). *J. Phycol.* 29:85–90.

Kim, G. H., Hwang, M. S., Fritz, L. & Lee, I. K. 1995. The wound healing response of *Antithamnion nipponicum* and *Griffithsia pacipica* (Ceramiales, Rhodophyta) monitored by lectins. *Phycol. Res.* 43:161–6.

Kim, G. H., Klotchkova, T. A. & Kang, Y.-M. 2001. Life without a cell membrane: regeneration of protoplasts from disintegrated cells of the marine green alga *Bryopsis plumosa*. *J. Cell Sci.* 114:2009–14.

Kim, S.-H. & Kim, G. H. 1999. Cell-cell recognition during the fertilization in a red alga *Aglaothamnion oosumiense* (Ceramiaceae, Rhodophyta). *Hydrobiologia* 398/399:81–9.

Kim, G. H., Lee, I. K. & Fritz, L. 1996. Cell-cell recognition during fertilization in a red alga *Antithamnion sparsum* (Ceramiaceae, Rhodophyta). *Plant Cell Physiol.* 37:621–8.

Kobayashi, K. & Kanaizuka, Y. 1985. Reunification of sub-cellular fractions of *Bryopsis* into viable cells. *Plant Sci.* 40:129–35.

La Claire II, J. W. 1991. Immunolocalization of myosin in intact and wounded cells of the green alga *Ernodesmis verticillata* (Kutzing) Borgesen. *Planta* 184:209–17.

La Claire II, J. W. 1987. Microtubule cytoskeleton in intact and wounded coenocytic green algae. *Planta* 171:30–42.

La Claire II, J. W. 1982a. Cytomorphological aspects of wound healing in selected siphonocladales (Chlorophyceae). *J. Phycol.* 18:379–84.

La Claire II, J. W. 1982b. Wound-healing motility in the green alga *Ernodesmis*: calcium ions and metabolic energy are required. *Planta* 156:466–74.

Mariani-Colombo, P., Vannini, G. L. & Mares, D. 1980. A cytochemical approach to the wound repair mechanism in *Udotea petiolata* (Siphonales). *Protoplasma* 104:105–17.

Menzel, D. 1988. How do giant plant cells cope with injury? The wound response in siphonous green algae. *Protoplasma* 144:73–91.

Mulders, F., van Langen, H., van Ginkel, G. & Levine, Y. K. 1986. The static and dynamic behaviour of fluorescent probe molecules in lipid bilayers. *Biochimica et Biophysica Acta* 859:209–18.

Nawata, T., Kikuyama, M. & Shihira-Ishikawa, I. 1993. Behavior of protoplasm for survival in injured cells of *Valonia ventricosa*: involvement of turgor pressure. *Protoplasma* 176:116–24.

O'Neil, R. M. & La Claire II, J. W. 1984. Mechanical wounding induces the formation of extensive coated membranes in giant cell. *Science* 255:331–3.

Oparka, K. J. & Read, N. D. 1994. The use of fluorescent probes for studies of living plant cells. *In* Harris, N. & Oparka, K. J. [Eds.] *Plant Cell Biology; A Practical Approach.* Oxford University Press, New York, pp. 27–50.

Pak, J. Y., Solorzano, C., Arai, M. & Nitta, T. 1991. Two distinct steps for spontaneous generation of subprotoplasts from a disintegrated *Bryopsis* cell. *Plant Physiol.* 96:819–25.

Schmid, C. 1993. Cell-cell recognition during fertilization in *Ectocarpus siliculosus* (Phaeophyceae). *Hydrobiologia* 260/261:437–43.

Sharon, N. & Lis, H. 1989. Lectins as cell recognition molecules. *Science* 177:949–59.

Shihira-Ishikawa, I. 1987. Cytoskeleton in cell morphogenesis of the coenocytic green alga *Valonia ventricosa*. Two microtubule systems and their roles in positioning of chloroplasts and nuclei. *Jpn. J. Phycol.* 35:251–8.

Tatewaki, M. & Nagata, K. 1970. Surviving protoplasts in vitro and their development in *Bryopsis*. *J. Phycol.* 6:401–3.

Waaland, S. D. 1990. Development. *In* Cole, K. M. & Sheath, R. G. [Eds.] *Biology of the Red Algae.* Cambridge University Press, New York, pp. 259–73.

Wassarman, P. M. 1987. The biology and chemistry of fertilization. *Nature* 235:553–60.

West, J. A. & McBride, D. 1999. Long-term and diurnal carpospore discharge patterns in the Ceramiaceae, Rhodomelaceae and Delesseriaceae (Rhodophyta). *Hydrobiologia* 298/299:101–13.

Wiese, L. & Shoemaker, D. W. 1970. On sexual agglutination and mating-type substance (gamones) in isogamous heterothalic *Chlamydomonas*. II. The effect of concanavalin A upon the mating-type reaction. *Biol. Bull.* 138:88–95.