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Experimental Hybridization between Some Marine Coenocytic Green Algae Using Protoplasms Extruded *in vitro*

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Some marine coenocytic green algae could form protoplasts from the extruded protoplasm in seawater. The dissociated cell components of the coenocytic protoplasm could be reunited into live cells and, hence, the formation of new species by mixing protoplasms from different coenocytic cells has been predicted. Our results showed that an incompatibility barrier was present during protoplast formation in coenocytic algae to exclude foreign inorganic particles or alien cell components. No inorganic particles or alien cell components were incorporated into protoplast formed spontaneously in seawater. Even when the inorganic particles or alien cell and/or cell component were incorporated into protoplast in some experimental condition, they were expelled from the protoplast or degenerated within several days. A species-specific cytotoxicity was observed during protoplast hybridization between the protoplasms of *Bryopsis* spp. and *Microdictyon umbilicatum*. The cell sap of *M. umbilicatum* could destroy the cell components of *Bryopsis* spp., but had no effect on *Chaetomorpha moniligera*. Species *C. moniligera* and *Bryopsis* did not affect protoplast generation of either species. The wound-induced protoplast formation *in vitro* might have evolved in some coenocytic algae as a dispersal method, and the incompatibility barrier to alien particles or cell and/or cell component could serve as a protective mechanism for successful propagation.

Key Words: coenocytic green algae, cytotoxicity, hybridization, protoplasm, protoplast formation

INTRODUCTION

A unique ability to form protoplasts *in vitro* from the extruded protoplasm has been found in a number of the marine coenocytic green algae (Tatewaki and Nagata 1970; Rietema 1973; Ishizawa and Wada 1979; Kim and Klotchkova 2004). After cell injury, extruded cell organelles in seawater aggregate to form compact protoplasmic masses; a process seems to be mediated by a lectin-carbohydrate complementary system (Kim *et al.* 2002; Klotchkova *et al.* 2003).

The agglutinated protoplasmic masses soon change into spheres, i.e., protoplasts, surrounded with an envelope (Kobayashi and Kanaizuka 1985; Pak *et al.* 1991). Cytochemical and ultrastructural studies revealed that this envelope is not a lipid-based plasma membrane but is a membrane composed of some polysaccharides (Kim *et al.* 2001; Klotchkova *et al.* 2003). The envelope transforms into a plasma membrane within several hours, presumably by incorporation of disintegrated plasma membrane of the precursor cell from the interior of protoplast onto its surface (Kim *et al.* 2001; Klotchkova *et al.* 2003). The protoplasts develop a new cell wall in a day or two.

In some species these cells grow into plants, whereas in other species they lose the ability to grow but remain as an enlarged single cell, producing numerous aplanospores or swarmers inside. It was therefore suggested that wound-induced protoplast formation *in vitro* may be a natural dispersal mechanism (Kim and Klotchkova 2004). Because coenocytic greens often grow in close proximity and their protoplasms may come into physical contact in nature, it is important to investigate if they can intermix while assembling the protoplast.

The dissociated cell components of the coenocytic protoplasm, separated by forcing it through sucrose gradient solution by ultracentrifugation, could be reunited into live cells and thus the formation of new species by mixing cell components from different

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Table 1. List of algae used in this study, their collection site or source and conditions for laboratory culture

Species	Conditions for laboratory culture			Collection site or source
	Temperature, 0°C	Irradiance, μmol photons $m^{-2} \cdot s^{-1}$	Light: Dark photoperiod, h	
Bryopsis hypnoides (Lamouroux)	23, 25	30	12 : 12	Peter the Great Bay, East Sea, Russia, September 2003, col. T.A. Klochkova
Bryopsis plumosa (Hudson) Agardh	23, 25	30	12 : 12	South Sea, Kachon, Korea, May 2000, col. T.A. Klochkova
Chaetomorpha moniligera Kjellman	15, 20	< 15	12 : 12	East Sea, Kangneung, Korea, June 2002, col. T.A. Klochkova
Microdictyon boergesenii Setchell	23	30	12 : 12	Bahamas, June 1975, col. S. Brawley (No. 1557)
Microdictyon umbilicatum (Velley) Zanardini	23	30	12 : 12	Burill Lake, NSW, Australia, August 1996, col. J.A. West (No. 3674)
Chlamydomonas sp.[1]	12, 23	< 2-4, < 15-30	12 : 12	Daecheon (West coast), Korea, 2003
Porphyridium purpureum (Bory) Drew et Ross[1]	23	30	12 : 12	Daecheon (West coast), Korea, 1998
Chlorococcum sp.	20, 25	15	12 : 12	Miruksazi stupa, Iksan, Korea, April 2002, col. T.A. Klochkova

[1]Epiphytic microalgae found during culture of other isolates.

coenocytic cells was predicted by Kobayashi and Kanaizuka (1985). The production of hybrid organisms by means of the induced fusion of somatic animal cells, or plant protoplasts prepared by enzyme digestion of the cell wall has been widely exploited in connection with cellular and genetic manipulation techniques (*e.g.* Ephrussy 1972; Ringerts and Savage 1976; Markert and Petters 1978; Gleba and Sytnik 1984; Kalthoff 2001). It is a completely artificial method and bypasses the natural restrictions inherent in sexual crosses. The fusion techniques are known to be quite complex, requiring specific conditions and stabilizing factors in some cases (*e.g.* Gleba and Sytnik 1984; Kalthoff 2001).

In this paper, we describe the results of protoplasm hybridization between five different coenocytic species to determine whether or not intergeneric and interspecific protoplasm crosses can occur, as well as data on fusion of the protoplasts with unicellular algae or fluorescent beads. This investigation may lead to a better understanding of dispersal by the wound-induced protoplast formation and isolating mechanisms in this group of algae.

MATERIALS AND METHODS

Plant materials

The algae used in this research, their source and conditions for laboratory culture are given in Table 1. All isolates were maintained in IMR medium (Klochkova *et al.* in press). The isolate of *Chlorococcum* sp. used in protoplast hybridization experiment was grown in a liquid ATCC Medium 625 (www-cyanosite.bio.purdue.edu).

Protoplast preparation

For preparation of protoplasts of the coenocytes, the algal thalli were cut with a razor blade, squeezed out and the protoplasmic clumps were transferred to 90 x 15-mm plastic Petri dishes containing IMR medium. The protoplasts that formed were checked under an inverted microscope and all remnants of the disrupted cell wall were removed from the dish. The newly generated protoplasts were moved to the culture chamber and kept at the same conditions as specified in Table 1 for the original plants. Thereafter, the cells were checked daily. All treatments described were repeated a minimum of 10 times. Numerical data present the means of multiple comparisons ($n \geq 10$).

Protoplast hybridization experiment

Species combinations used in the hybridization experiment are given in Fig. 1.

Ability of protoplasm to take up inorganic particles

To determine the ability of the protoplasm to take up inert particles spontaneously during formation, the algal thalli were cut in IMR medium containing 1-2 μL of FluoSpheres of 0.02-2 μm in diameter (yellow-green

Combination for hybridization	*B. hyp*	*M. boer*	*C. mon*	*M. umb*	*Chlamy*	*Chloroc*	*Porphy*	Fluosph
Bryopsis plumosa	-	-	-	-	+	+	-	++
Bryopsis hypnoides		-	-	-	+	+	+	++
Chaetomorpha moniligera				-	-	-	-	+
Microdictyon umbilicatum					-	-	-	+

- Species-specific cytotoxicity

Fig. 1. Species combinations used for the hybridization experiments and the results obtained. Symbols '-' and '+' mean negative and positive hybridization, respectively; symbol '++' implies that hybridization was very successful. Abbreviations, *B. hyp - Bryopsis hypnoides*; *M. boer - Microdictyon boergesenii*; *C. mon - Chaetomorpha moniligera*; *M. umb - Microdictyon umbilicatum*; *Chlamy - Chlamydomonas* sp.; *Chloroc - Chlorococcum* sp.; *Porphy - Porphyridium purpureum*; *Fluosph - FluoSpheres.*

fluorescing carboxylate-modified microspheres, Molecular Probes). These FluoSpheres are inert particles used for observations of intracellular transportation (www.probes.com). Protoplasts that formed were checked under the blue filter (filter set BA510IF and BA510-540) to detect if the beads were taken up.

In another experimental set, protoplasm was extruded in artificial seawater of pH 6 to loosen the binding between cell organelles (Kim *et al.* 2002), and centrifuged at 2,000g for 2 min in 1-mL plastic tubes. The cell organelles in the pellet were re-suspended in new artificial seawater of pH 6, and 1-2 μL of ddH_2O containing FluoSpheres was added. The tube was shaken vigorously for several minutes to incorporate the fluorescent beads into protoplasmic masses and then centrifuged at 2,000g for 2 min. The pellet containing protoplasmic masses and fluorescent beads was re-suspended in IMR medium. Then, the protoplasts that formed were checked under the blue filter to detect if the beads were taken up.

Hybridization of protoplasts from coenocytic algae with unicellular algae

Unicellular microalgae *Chlamydomonas* sp., *Chlorococcum* sp., and *Porphyridium purpureum* were used to determine the ability of the protoplasts to include other living organisms during formation. *Chlorococcum* sp. from soil was used in hybridization experiment because of its ability to tolerate salinity stress (Klochkova *et al.* in press).

As the cells of *P. purpureum* were surrounded with a mucilaginous sheath, they were first centrifuged at 1,500g for 1 min and washed for 3-5 min with 0.5% Triton X-100 diluted in IMR medium. The procedures for protoplasm extrusion and fusion with the unicellular algae were performed as described above for FluoSpheres, using IMR medium and artificial seawater of pH 6 for protoplasm extrusion. Protoplasts formed were checked under the light microscope according to time.

Hybridization of protoplasts of different coenocytic algae

To hybridize protoplasm between different coenocytic green algae, the thalli of each species were placed together on a slide glass and cut with a razor blade in several drops of IMR medium. Protoplasm extruded from the thalli was transferred to 90 x 15-mm Petri dishes containing IMR medium and examined under the inverted microscope over time intervals (5, 10, 20, 30, 40, 50, and 60 min).

To observe the interactions between vacuolar-sap-free cell organelles of different algae, protoplasm of each species was extruded separately in artificial seawater of pH 6, centrifuged at 2,000g for 2 min and washed with the same solution twice. The cell organelles were aggregated very loosely or detached from each other after the treatment. Thereafter, artificial seawater was removed and the cell organelles of different species were re-suspended and mixed together in a small volume of IMR medium and observed under the light microscope.

Hybridization of protoplasm with foreign cell sap

The effect of the cell sap of one coenocytic cell on the cell organelles of another species was studied as follows. The cell sap of *Microdictyon umbilicatum* was obtained by centrifuging the protoplasm extruded in IMR medium at 2,000 g for 2 min and removing the pellet. The cell sap was mixed with the chloroplasts of *Bryopsis* spp. and *Chaetomorpha moniligera*. The mixture was directly transferred into 90 x 15-mm Petri dishes containing IMR medium and examined under the inverted microscope over time intervals (5, 10, 20, 30, 40, 50, and 60 min).

Scanning electron microscopic observations

Dense suspensions of protoplasts were placed on the cover slips coated with 3% 3-aminopropyltriethoxy-sylane (Sigma) diluted in acetone and were then fixed with 3.7% formaldehyde (Junsei Chemical Co., Japan) in

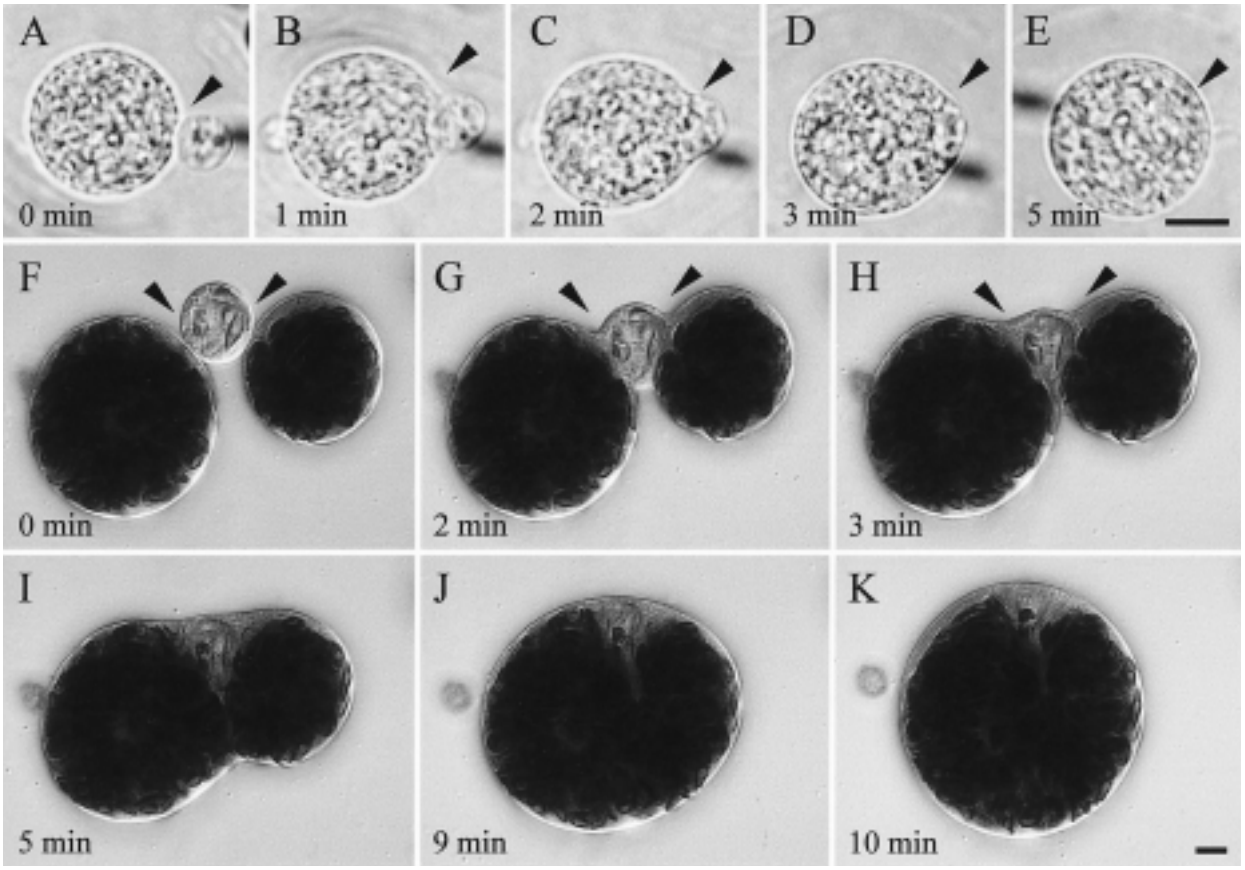

Fig. 2. Fusion of the leucoplasts (A-E) and double fusion of the protoplasts (F-K) of *Bryopsis plumosa*. The time after initiation of fusion is indicated. The leucoplasts and protoplasts were 10 and 20-min old, respectively, before they started to fuse. Scale bars, 10 μm.

IMR medium in room temperature for 2 h. The fixative was washed off and the materials were dehydrated in a graded ethyl alcohol series, followed by 3-Methylbutyl acetate (Junsei Chemical Co.) series. Thereafter, the cover slips were freeze-dried in the automated freeze dryer (Viritis model No. 010-10, Viritis Co., NY) at -60 to -70°C overnight. Samples were sputter-coated with gold and viewed on a JEOL-35 Scanning Electron Microscope (JEOL, Tokyo, Japan).

Microscopy

Microscopes Olympus BX50 and Olympus BH-2 were used. Fluorescence of FluoSpheres was examined with confocal laser scanning microscope (Fluoview, v. 2.0.28, Olympus). Micrographs were taken with Olympus DP50 digital camera equipped to microscope Olympus BX50 using Viewfinder Lite and Studio Lite computer programs. Inverted microscope Olympus IX70 was also used for the observation of materials.

For time-lapse video-microscopy, the protoplasm and protoplasts were placed on a glass slide and a coverslip was lowered and sealed with VALAP (1:1:1; Vaseline: Lanolin:Paraffin) melted on a hot plate at 70°C. The slide preparations were examined on an Olympus BH-2 microscope under the oil immersion x40 objective lens and recorded on a Digital Imaging Time-Lapse Recorder (DITRS, TCS Korea, Daejeon, Korea) via a JVC color video camera (KY-F550). For reproduction, video clips were exported to a computer using RECO Desktop software and single video frames were then captured.

RESULTS

Ability of protoplasm to take up inorganic particles

The early process of protoplast formation occurred similarly in all algae used in this study. The difference was in the motility of protoplasm and time required for the protoplasmic masses to adopt a spherical shape and become surrounded with an envelope. Using time-lapse video-microscopy an active swirling movement of cell organelles was observed in the protoplasmic masses during protoplast formation in *Bryopsis* spp. Such protoplasmic motility was not readily observed in the protoplasmic masses of *Microdictyon* spp. and

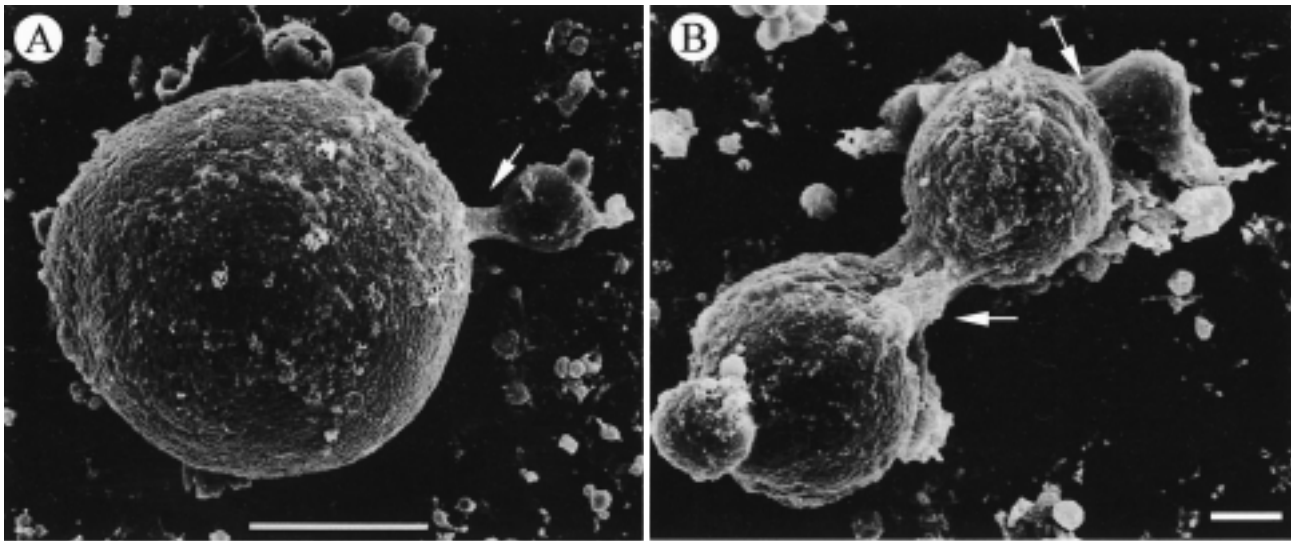

Fig. 3. Scanning electron micrographs of the fusing protoplasts of *Bryopsis plumosa.* A. Protoplasts of different sizes could fuse. B. Double fusion. Arrows point to the fusion areas. Scale bars, A = 50 μm; B = 10 μm.

Chaetomorpha moniligera used in this study. An interesting attribute in *Bryopsis* spp. was the presence of numerous protoplasts without chloroplasts, so-called leucoplasts, which contained all other cell organelles (Figs 2A-E). The protoplasts and leucoplasts of *Bryopsis* spp. fused easily with each other during the first 20-30 min after formation (Figs 2 and 3). Thereafter, the fusion among the primary envelopes stopped, although the protoplast surfaces were in direct contact for many hours.

No fusion among the primary envelopes of the protoplasts of *C. moniligera* or *Microdictyon* spp. was observed when these were in direct contact for many hours. Therefore, *Bryopsis* protoplasm was the most suitable recipient in the hybridization experiment.

When the algal cells were cut and the protoplasm was extruded in IMR medium containing FluoSpheres of various sizes, the beads were never taken up by the protoplasts. However, when the protoplasm was extruded in artificial seawater of pH 6, centrifuged and mixed with the beads and then re-suspended in IMR medium, some protoplasts containing FluoSpheres were formed (Figs 4A-F). This occurred because low pH prevented tight binding of extruded cell organelles in the protoplasmic masses, allowing the beads to be incorporated into the protoplasm. Thereafter, the protoplasmic masses were transferred in enriched seawater of pH 8 (IMR medium) that induced tight binding between cell organelles and formation of the primary envelope on their surface.

About 2% of the protoplasts of *Bryopsis hypnoides* and *B. plumosa* contained FluoSpheres of different sizes. There was no significant correlation between the size of FluoSpheres and the success of incorporation. In case of *Bryopsis* spp., FluoSpheres from 0.5 to 2 μm were used because they were easier to be counted under the microscope. The maximum number of FluoSpheres of 2 μm in diameter found in a protoplast of *Bryopsis hypnoides* was 38. The sizes of protoplasts containing FluoSpheres were usually over 40 μm in diameter.

Optical sectioning of the protoplast with the confocal laser scanning microscope showed that all FluoSpheres were located just beneath the primary envelope as soon as it was formed (Figs 4A-F). The number of protoplasts containing FluoSpheres decreased by 4 to 6 hours after protoplast formation, and most beads were attached to the protoplasts' outer surface and only several beads were inside (Figs 4G-L). None of them were found inside by 24 hours after protoplast formation, but numerous beads were attached to the growing cells. As the number of FluoSpheres inside the protoplasts was decreasing over time and, at the same time, the beads were appearing on the protoplast outer surface and as the protoplast mortality was not higher than usual, it was suggested that the beads were expelled from the protoplasts.

In *C. moniligera* and *M. umbilicatum*, several protoplasts containing small beads of 0.2 μm in diameter were found only twice, although the experiments were repeated more than 10 times for each species. When those protoplasts with beads were incubated overnight, they all died in 12 hours. No protoplast containing

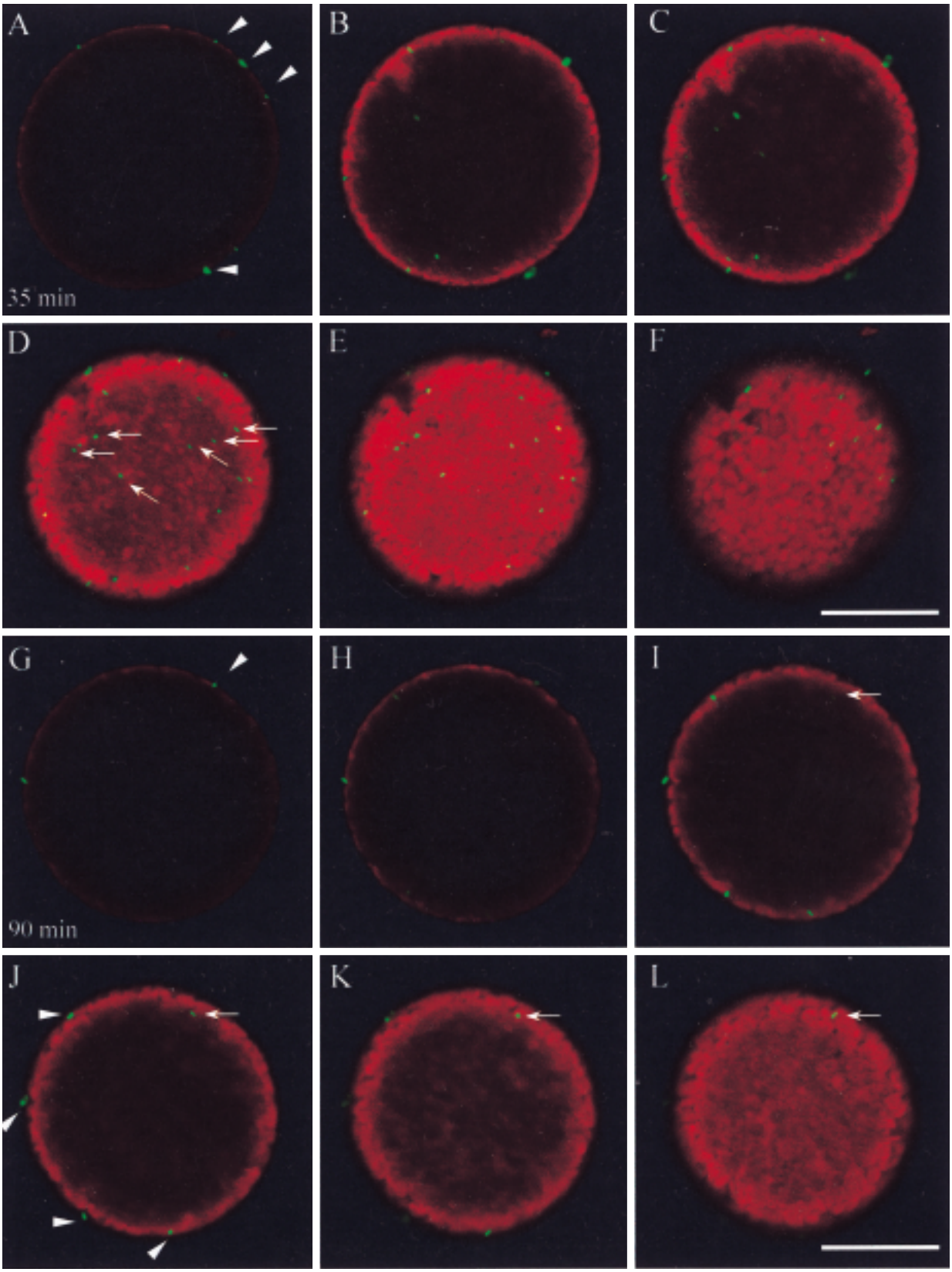

Fig. 4. Optical sectioning with confocal laser scanning microscope of the protoplasts of *Bryopsis hypnoides* containing FluoSpheres at different times after formation. A-F. In 35-min old protoplast, the beads were localized beneath the primary envelope between the chloroplasts (arrows). Several beads were attached to the protoplast's outer surface (arrowheads). G-L. In 90-min old protoplast, except one bead (arrow) all beads were expelled from the protoplast and were located on its outer surface (arrowheads). Scale bars, 20 μm.

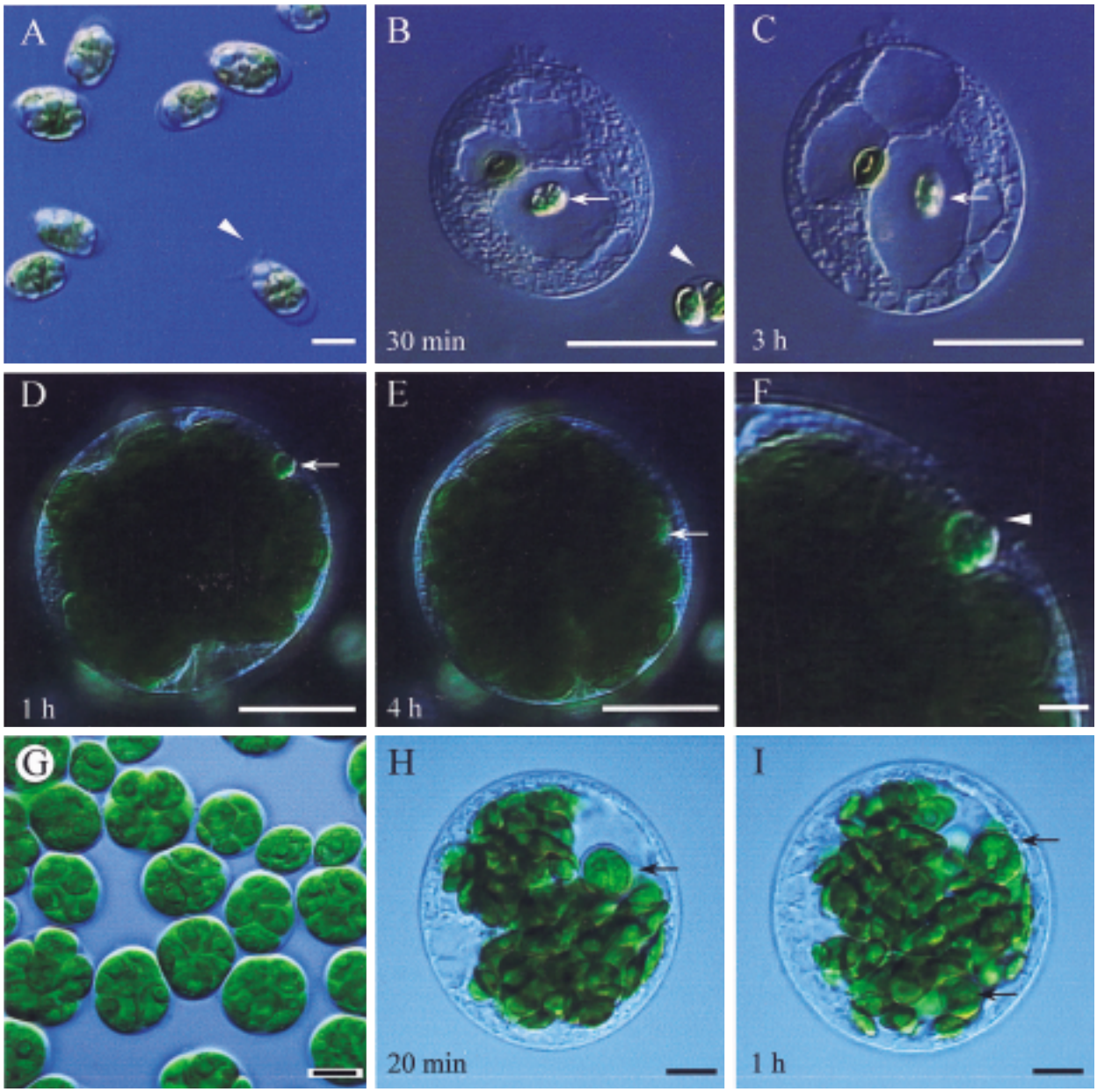

Fig. 5. Protoplasts of *Bryopsis plumosa* containing *Chlamydomonas* (A-F) and *Chlorococcum* cells (G-I). A. Motile flagellated *Chlamydomonas* cells (arrowhead) were never incorporated into the protoplasts. B-F. Arrows point to *Chlamydomonas* inside the protoplasts. B-C. Single chloroplast lies in separate compartment from the alien cell. The time indicates age of the protoplasts. Arrowhead on Fig. B points to the aplanospore of *Chlamydomonas* that did not enter the protoplast. F. Arrow points to *Chlamydomonas* cell (enlarged image D). G. Microphotograph of the *Chlorococcum* cells used in the experiment. H-I. Arrows point to *Chlorococcum* cells inside the protoplast. Scale bars, A, F = 2 μm; B-E, G-I = 10 μm.

FluoSpheres inside survived, but the other protoplasts survived and started to grow.

Hybridization of protoplasts from coenocytic algae with unicellular algae

The protoplasts could take up some unicellular algae only in some experimental condition. *Chlamydomonas* sp., *Chlorococcum* sp., and *Porphyridium purpureum* were chosen for their small cell sizes. The protoplasts of all coenocytic algae did not take up the unicells when the protoplasm was extruded in IMR medium. However, *Bryopsis* protoplasts engulfed *Chlamydomonas* and *Chlorococcum* cells in artificial seawater of pH 6 (Fig. 5). Large protoplasts over 100 μm in diameter could contain 3 to 4 foreign cells.

As seen from Figs 5B-C, the *Chlamydomonas* cell is contained in a separate compartment within the protoplast, and single chloroplast of *Bryopsis* is in the

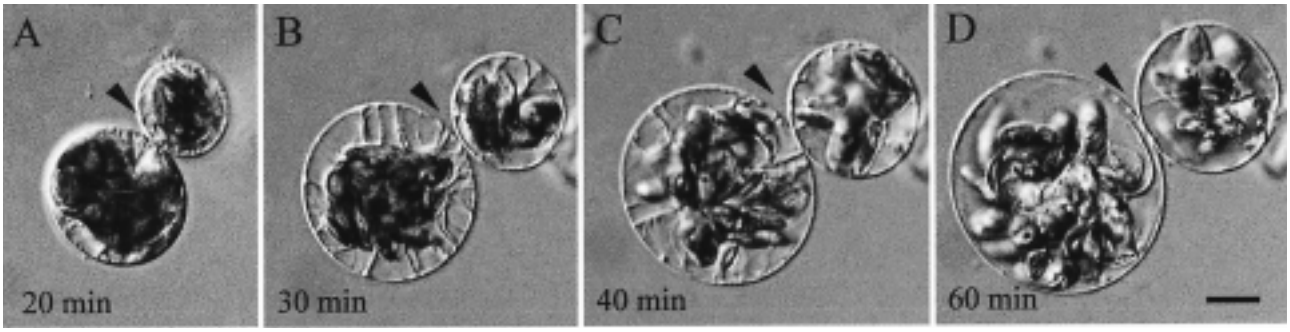

Fig. 6. These protoplasts of *Bryopsis plumosa* were exposed to the *Microdictyon umbilicatum* protoplasm and thus swelled 1.5 times within 40 min. Also, they did not fuse with each other. Scale bar, 10 μm.

other compartment. It is noteworthy that these *Chlamydomonas* cells were non-motile and lacked flagella. When motile flagellated *Chlamydomonas* cells were used (Fig. 5A) and same experiments were performed, they were never incorporated into the protoplasts. The protoplasts of *Bryopsis* spp. containing foreign cells were transferred to the culture chamber and grown for several days. The filamentous plants developed from the protoplasts, but all foreign cells disappeared at this stage. The foreign cells may have been expelled from the protoplasts because several cells attached to the surface of the growing filaments were observed at this stage.

Porphyridium purpureum cells were taken up by the protoplasts of *B. hypnoides* only once (not shown), although the experiments were repeated more than 20 times. The mucilage on the cell surface of *P. purpureum* was largely washed off with Triton X-100, and protoplasm hybridization was performed in the artificial seawater of pH 6.

Hybridization of protoplasts between different coenocytic algae

When protoplasms of *Bryopsis* spp. and *C. moniligera* were mixed together, chimerical protoplasts were not obtained regardless of the experimental conditions (Fig. 1). Same results were obtained by mixing protoplasms of *C. moniligera* and *M. umbilicatum* (Fig. 1). In most cases, the unbound free chloroplasts belonging to different species dispersed in the Petri dish. Although *Bryopsis* protoplasts fused easily with each other at the early developing stage, they never fused with any foreign protoplasts. The spontaneous co-aggregation of the vacuolar-sap-free chloroplasts of different species did not occur as well.

A small number of protoplasts formed when the protoplasms of two *Bryopsis* species were mixed together. These protoplasts did not attach to substrate and kept floating in the water even when the control protoplasts (unialgal culture) attached to the bottom and began to grow. The protoplasts could survive for several weeks but they never grew into individual plants.

Some species-specific cytotoxicity was observed during protoplast hybridization. When protoplasms of *Bryopsis* spp. and *M. umbilicatum* were mixed together, all chloroplasts of *Bryopsis* degenerated within 10-30 min, depending on the volume of *Microdictyon* plants used. Protoplasts of *M. umbilicatum* were formed and their development proceeded normally, but no protoplast of *Bryopsis* was formed in this condition. Only when the amount of *M. umbilicatum* protoplasm was several times smaller than that of *Bryopsis* could the protoplasts of the latter be generated, but they were swollen (Fig. 6) or contained few chloroplasts. These protoplasts of *Bryopsis* did not develop into individual plants.

The chloroplasts of *Bryopsis* spp. remained intact for up to 2 hours when vacuolar-sap-free chloroplasts of both species were mixed together. Intact chloroplasts of *Bryopsis* and *M. umbilicatum* laid in direct contact with each other for hours. On the contrary, when vacuolar sap of *M. umbilicatum* was mixed with *Bryopsis* chloroplasts, the latter degenerated. Therefore, a substance that killed the chloroplasts of *Bryopsis* appears to be localized in the vacuolar sap of *M. umbilicatum*.

When the protoplasms of *C. moniligera* and *M. umbilicatum* were mixed together, both species produced live protoplasts. The hybridization of protoplasms of *Bryopsis* and *Chaetomorpha* did not affect protoplast generation of either species. It is noteworthy that when protoplasms of different species were mixed together the number of surviving protoplasts was always smaller than in the unialgal experiment, and their sizes were smaller.

To detect if other *Microdictyon* species possesses the same cytotoxicity to *Bryopsis* protoplasm, *M. boergesenii*

was used. When protoplasm of *M. boergesenii* was extruded, several protoplasts were formed in each trial, although the experiments were repeated more than 10 times. When protoplasms of *Bryopsis* spp. and *M. boergesenii* were mixed together, *Bryopsis* protoplasts formed normally. However, within the next 3 hours all of them swelled up to 10 times their original size. For example, 633 swollen protoplasts containing few chloroplasts were counted in one experimental set, and only 7 protoplasts did not swell. The latter were smaller than 20 μm in diameter and they soon degenerated. About 38% of the swollen protoplasts survived for 10 days after formation. At this time, the cells 60-270 μm in diameter containing 4-100 chloroplasts were floating in the water and many of them lacked turgor pressure. All cells degenerated by 19th day after formation. Therefore, being exposed to foreign protoplasm altered the development of *Bryopsis* protoplasts even when the second plant, *M. boergesenii*, did not produce viable protoplasts at all. However, the cytotoxicity such as that of *M. umbilicatum* was not observed.

DISCUSSION

In the present study, an attempt was made to hybridize protoplasms of five different coenocytic green algae with each other and fuse their protoplasts with unicellular algae or fluorescent beads. Previously, Kobayashi and Kanaizuka (1985) suggested possibility of new species formation by mixing dissociated cell components of different coenocytic species. In higher plants, more than two protoplasts, prepared by enzyme digestion of the cell wall, may fuse during the parasexual hybridization process and various types of hybrid plants may be generated from these fusion products (Gleba and Sytnik 1984).

In our experiment, protoplasms of different coenocytic greens were not likely to hybridize even in the experimental conditions. Moreover, there was species-specific cytotoxicity between coenocytic cells, as shown in hybridizations between *Microdictyon umbilicatum* and *Bryopsis* spp.

When the cells were cut and the protoplasm was squeezed into IMR medium containing dense suspensions of FluoSpheres or unicellular algae, the protoplasts did not engulf them. Some beads and non-motile unicells were attached to the surface of the protoplasmic masses, but they were expelled during the process of protoplast formation. The same results were obtained by mixing protoplasms of *Bryopsis* spp. with *Chaetomorpha moniligera* or *C. moniligera* with *M. umbilicatum*. Protoplasts of each species formed separately. Hybrid protoplasts were not formed even when vacuolar-sap-free chloroplasts of different species were mixed together, regardless of the experimental conditions.

From the previous studies, it is known that identical carbohydrates inhibited agglutination of cell organelles and identical FITC-labeled lectins bound to the membranes of chloroplasts in *M. umbilicatum* and *C. aerea* (Kim *et al.* 2002; Klotchkova *et al.* 2003). However, spontaneous hybridization of their protoplasms did not occur regardless of the experimental conditions. Thus, an incompatibility barrier might be present during protoplast formation to exclude foreign inorganic particles, or alien cell components. One should consider that the number of foreign materials used was much bigger than could be expected in the natural seawater environment.

When protoplasms of *Bryopsis hypnoides* and *B. plumosa* were mixed together, the protoplasts that formed swelled and did not grow into plants. Unfortunately, it was hard to distinguish between the chloroplasts of *Bryopsis* spp. because of similar morphology and color of the plastids. An attempt to label chloroplast surface before the hybridization experiment did not succeed. Thus, it was unclear whether or not hybrid protoplasts were formed. Whatever the case, the fact that normal *Bryopsis* plants failed to develop suggests existence of a selective recognition system to eliminate the possibility of hybridization even with closely related species.

Similar abnormal protoplasts were formed when protoplasms of *Bryopsis* spp. and *M. boergesenii* were mixed together, although the latter produced 2-3 protoplasts, or often did not produce them at all and the risk of contamination would be minimized.

Several studies have dealt with the fate of cytoplasmic organelles in the process of fusion and culture of the hybrid cells in higher plants (Fowke *et al.* 1975, 1976, 1977; Sidorov *et al.* 1978; Kajita *et al.* 1980). There were no pronounced incompatibility processes during the first several days of cultivation of the hybrids and they contained plastids and nuclei from both parents. Thereafter, selective destruction or propagation of plastids from one or the other parent occurred (see Gleba and Sytnik 1984).

We have been able to develop a method of insertion of the silica beads and non-motile *Chlamydomonas*,

Chlorococcum, and *Porphyridium purpureum* cells into the protoplasts. This could be induced using artificial seawater of pH 6 and centrifugation at 2,000 g. As was previously described, agglutination of extruded cell organelles was inhibited by low pH condition (Kim *et al.* 2001, 2002; Klotchkova *et al.* 2003) and, therefore, this acidic solution was used to loosen the chloroplast binding and allow the beads and unicells to enter the protoplasmic masses. Then, the acidic solution was replaced by normal seawater and protoplast formation began immediately. Foreign materials that got deep inside the protoplasmic masses were incorporated into the protoplasts.

The results showed that the protoplasts containing FluoSpheres degenerated by 12 hours after formation, or the beads were expelled, although these carboxylate-modified silica microspheres are biologically inert and physically durable (www.probes.com). They are widely used for biological and medical purposes as long-term tracers. The protoplasts of *Bryopsis* spp. that contained *Chlamydomonas* and *Chlorococcum* cells grew into normal plants after all the foreign cells disappeared from them. Several foreign cells were observed attached to the surface of the growing *Bryopsis* filaments, thus the foreign cells may have been ejected from the protoplasts. Therefore, some compartmentalization process might be involved to eliminate alien cells and particles during protoplast formation process.

The existence of symbiotic organisms that live within the cells of algae is known (see Dodge 1973). As to the coenocytic greens, there are reports on symbiotic bacteria living within the cytoplasm of the vegetative cells or in the gametes (*e.g.* Burr and West 1970; Turner and Friedmann 1974; Menzel 1987). Each bacterial cell was intact inside the host body and often surrounded by a mucilaginous sheath. They did not become part of the alga or fused with its intracellular components (see Dodge 1973). As present experiments showed, it was possible to insert inorganic particles and/or unicellular algae into the coenocytic protoplast in experimental conditions. Moreover, when the alien materials were incorporated, they were ejected from the protoplast or degenerated. These results show that only some chosen cells or particles could incorporate into coenocytic cells.

Wound-induced protoplast formation *in vitro* was presumed to be not just an artifact observed in certain experimental conditions, but a result of an effective adaptation, using the hazardous effect of wounding as a chance for dispersal (Kim and Klotchkova 2004). Hence, the incompatibility barrier to alien particles or cell and/or cell components found in this study might secure successful dispersal.

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