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Fusion of protoplasts from thalli of two different color types in *Porphyra yezoensis* UEDA and development of fusion products*

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Protoplasts isolated enzymatically from thalli of wild type and green type mutant in *Porphyra yezoensis* were fused by polyethylene glycol (PEG) treatment. Many heterokaryocytes (up to 12.0%) were formed soon after the addition of washing medium to the protoplast-PEG preparation. Fusion products involving one wild type and one green type protoplast grew to form a mass of cells after regenerating cell walls. When cultured in aerated medium, the masses of cells grew as a callus-like cell aggregate. Cell aggregates differentiated into a number of full-grown thalli. Those thalli were microscopically chimeral thalli irregularly variegated with greenish cell and reddish purple cell groups. Carpospores released from mature chimeral thalli grew into greenish conchocelis, and the greater part of F_1 thalli produced from the conchocelis mostly consisted of greenish cells.

Key Index Words: Genetic engineering; *polyethylene glycol*; Porphyra yezoensis; *protoplast fusion*; *Rhodophyceae.*

Protoplasts produced from different tissues in higher plants and the resulting induced callus have been used as tools in physiological and cytological studies as well as in the research of protoplast fusion to produce somatic hybrids in the field of plant genetic engineering (GALUN 1981, AHUIA 1982, POTRYKUS *et al.* 1983). Somatic hybridization by protoplast fusion offers great promise for achieving wide crosses between species that are difficult or impossible to hybridize conventionally, with the hope to produce new crop varieties. There have been a large number of successful intraspecific and interspecific somatic hybrid experiments in higher plants reported to date (EVANS 1983, GLEBA and SYTNIC 1984).

Protoplast researches in algae have been limited so far to those of fresh water and marine unicellular algae (ADAMICH and HEMMINGSEN 1980, BERLINER 1981, BRADLY 1983), and only a very few dealing with marine multicellular algae (so-called "seaweeds") (CHENEY 1986). In the last few years, however, the isolation of protoplasts from seaweeds has been increasingly reported, and CHENEY (1986) listed a total of 15 species (nine genera) from which protoplasts have been isolated. The number of algae in which successful protoplast fusion has been reported is very limited; *Polyphysa* (=*Acetabularia*) *cliftonii* (PRIMKE *et al.* 1978), *Zygnema extenue* and *Spirogyra gracilis* (OHIWA 1978), *Chlamydomonas reinhardi* (MATAGNE *et al.* 1979), *Ulva linza* and *Monostroma angicava* (ZHANG 1983), and

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Porphyra yezoensis and *Enteromorpha intestinalis* (SAGA *et al.* 1986).

The red seaweed *Porphyra* "Nori" is being extensively cultured in Japan as one of the important edible seaweeds. Recently, however, *Porphyra* cultivation has frequently suffered from damage due to pathogenic microorganisms and unusual weather. It is hoped that strains of cultivated *Porphyra* can be improved by means of genetic engineering. We have already reported on the isolation and culture of the protoplasts in *Porphyra yezoensis* (FUJITA and MIGITA 1985). In this paper, we will further report on the fusion of protoplasts isolated from wild type and green type thalli in *P. yezoensis*, and on the development of fusion products cultured *in vitro*.

Materials and Methods

Vegetative thallus of Porphyra yezoensis UEDA

Two kinds of specimens were used in the present study, one of wild type (normal color) and the other green type (color mutant). They were both collected from "Nori-nets" experimentally set at the culture farm in Kashima City, Saga Prefecture, Western Kyushu, Japan. Two kinds of shell-living conchocelis filaments for making nursery nets were prepared by using free-living conchocelis filaments kept in our laboratory. The thalli, about 10 cm long, were collected from the "Nori-nets", washed with sterile sea water, drained to semi-dry condition (30–40% moisture content) and stored at −20°C until used. After thawing, the thalli were used for isolation of protoplasts.

Preparation of bacterial crude enzyme solution

Protoplasts from the thalli of the two different types were produced by using a crude enzyme prepared from the cultured supernatant of *Pseudomonas* sp. strain P-1 which causes "Green spot rotting" on *Porphyra* thalli. The crude enzyme solution was prepared by a slightly modified procedures reported by FUJITA and MIGITA (1985). The strain was inoculated in a 200 m*l* Erlenmeyer flask containing 50 m*l* of ZOBELL 2216E broth and precultured for 3 days at 20°C. All of the preculture was inoculated in 1 *l* of medium which was composed of 0.1% potassium nitrate, 0.01% yeast extract, 0.01% potassium phosphate (dibasic), 0.001% ferric chloride, 0.1% tris(hydroxymethyl)aminomethane and 0.2% "Nori" powder in 75% sea water (pH 7.5) in 2 *l* Erlenmeyer flask and incubated with stirring for a week at 20°C. The culture was centrifuged at 12,000×g for 30 min. Ammonium sulfate (80% saturation) was added to the supernatant. The precipitate was collected and dissolved in 50 m*l* sea water containing 0.01% tris(hydroxymethyl)aminomethane (pH 7.2). The enzyme solution was dialyzed with the same sea water for 12 hr.

Protoplast isolation

Protoplasts from the vegetative thalli of the two kinds were produced separately. Basal parts of the thalli were removed in advance and the remaining parts washed several times by shaking in a semi-solid agar layer, then in sterile sea water. After washing three times in sea water, thalli were cut into small pieces (1–2 mm^2) with a razor blade. About 0.2 g (fresh weight) of the small pieces was incubated in a test tube (10×150 mm) containing 10 m*l* of the bacterial crude enzyme solution containing 0.8 M mannitol, 6,000 IU penicillin G potassium and 10 mg streptomycin sulfate. The incubation was carried out at 18–20°C on a reciprocating shaker, shaking it at about 120 cycles per min. After 3–4 hr incubation, the enzyme-protoplast mixture was passed through a

nylon mesh (25 μm opening) to remove undigested cell wall clumps and cell wall debris. The pellet was washed three times with sterile sea water containing 0.8 M mannitol. The washed cells were re-suspended at the concentration of 10^{5-6} cells per m*l* of the mannitol/sea water.

Protoplast fusion and culture of fusion products

To induce protoplast fusion the modified method of KAO and MICHAYLUK (1974) was used. Approximately 100 μl of each protoplast suspension was placed on a cover slip (24×24 mm) in a Petri dish (9×2 cm) and allowed to settle for 10 min then 200 μl of polyethylene glycol (PEG) solution [30% PEG 4000 or PEG 6000 (Wako Pure Chem. Indust. Ltd., Osaka, Japan) 0.2 M mannitol in sea water] was slowly added. After the protoplasts were incubated in PEG solution for 20 min, 10 m*l* of washing medium (KAMEYA *et al.* 1981) was added to the protoplast-PEG preparation. The mixture was then diluted with 10 m*l* of modified PROVASOLI's enriched sea water (PES) medium (PROVASOLI 1968) containing 0.8 M mannitol, 1,200 IU penicillin G potassium and 2 mg streptomycin sulfate. The Petri dishes containing protoplasts were maintained under continuous light (1,000 lux) at 15°C for a period of 24–48 hr. The frequency of protoplast fusion was examined microscopically. Identification of heteroplasmic fusion was easily possible, since the heterokaryocytes contained both reddish purple chloroplast(s) from the wild type protoplast(s) and green chloroplast(s) from the green type protoplast(s). Fusion products were transferred by using a fine pipet to Petri dish (6×2 cm) containing 12 m*l* of PES medium, and the cultures were maintained at 4,000 lux, 12:12 hr LD, 18°C. After 1–2 months plants developed from fusion products were transferred to flat bottom flasks containing 200 m*l* or 1 *l* of PES medium, and the cultures were maintained at 6,000 lux, 12:12 hr LD, 18°C and aerated. All cultures were changed to fresh medium weekly and placed under the "daylight" fluorescent lamps.

Results and Discussion

Immediately after the addition of PEG 4000 or PEG 6000 solution to the suspension of protoplasts of two types, adjacent protoplasts began to aggregate and the number of aggregates increased with time (Fig. 1A, B). Aggregates tightly adhered to the surface of the cover slip. As long as protoplasts were being incubated in PEG solution, however, very few fusion occurred. The greatest number of fusion occurred as the aggregates began to leave the surface of the cover slip soon after the addition of washing medium to the protoplast-PEG preparation. The cells that fused had initially oval or irregular shape (Fig. 1C, E), but gradually became spherical (Fig. 1D). The constituents of the chloroplast mixture intermixed very slowly even 48 hr after the addition of washing medium, so there was little difficulty in identifying the fusion products which held two different chloroplasts. The frequency of heterokaryocyte formation due to PEG 4000 and PEG 6000 were 10.4% and 12.0% respectively. The fusion products involved two to four or more protoplasts. Seven fusion products which involved only one wild type and one green type protoplast were selected and transferred to PES medium in separate Petri dishes for cultivation. The two different chloroplasts in the fusion products became yellowish brown through the gradual mixing. Four of seven fusion products perished after a few days. The reason for the death of four

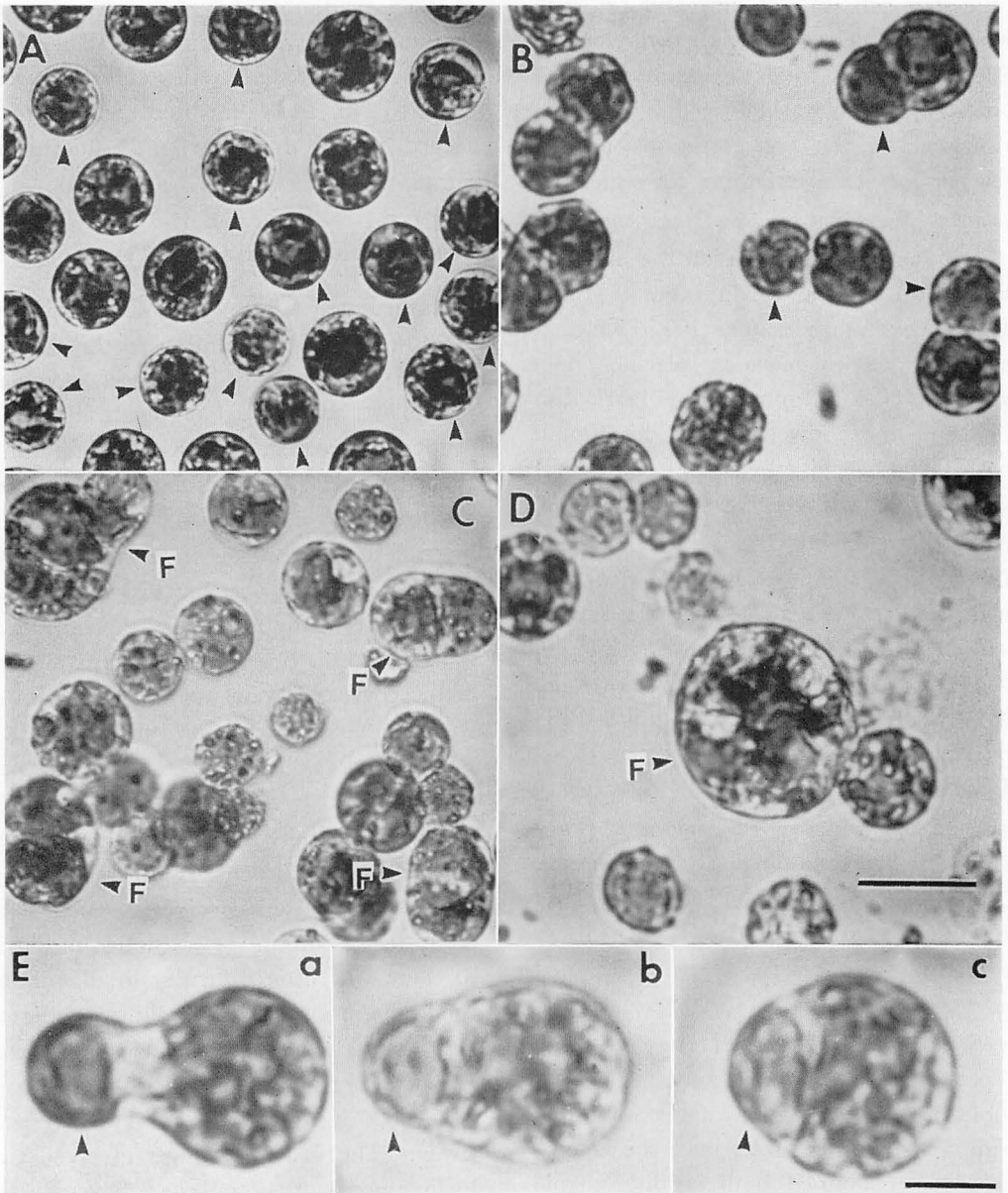

Fig. 1. Freshly isolated protoplasts and fusion events.

A. Mixture of wild type and green type protoplasts.

B. Aggregated protoplasts 15 min after the addition of the PEG solution to protoplast mixture.

C. Fusion cells (F) 20 min after the addition of the washing medium to protoplast-PEG preparation.

D. Nearly spherical fusion product (F) involving four or more protoplasts 12 hr after stage C. Scale bar= 20 μm.

E. Progressive stages of fusion between one wild type and one green type protoplast: a–c; 10, 25, 45 min after the addition of the washing medium to protoplast-PEG preparation. Scale bar=10 μm.

Arrows in A, B and E indicate wild type protoplasts. Scale bar in D applies also to A, B and C.

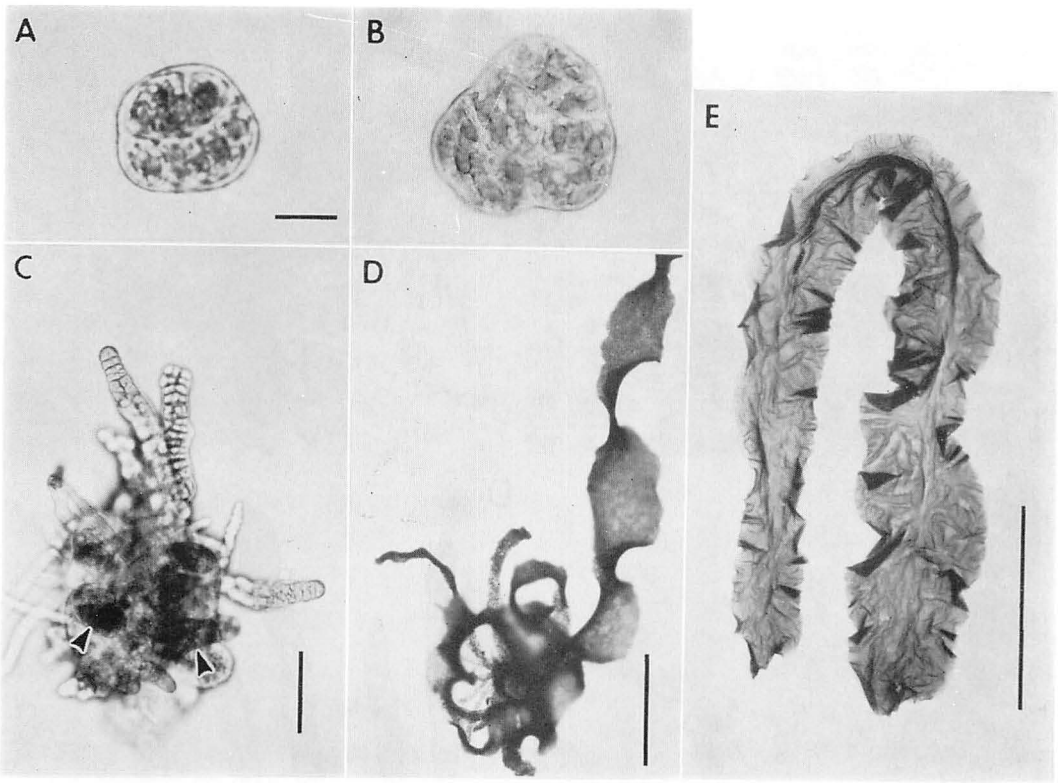

Fig. 2. Various stages in development of fusion product involving one wild type and one green type protoplast.
A and B. 20-day old and 30-day old germlings "masses of cells" from fusion product cultured in Petri dish.
C–E. Thalli differentiated from "callus-like cell aggregate" (arrows) induced after transfer "mass of cells" stage in B to aerated culture. C; 25 days, D; 65 days, E; 125 days after transfer to aerated culture.
Scale bar: 20 μm for A; 150 μm for C; 1 mm for D; 10 cm for E. Scale bar in A applies also to B.

fusion products may be the increase of bacteria in the culture medium. The remaining three fusion products divided very slowly after regenerating a cell wall and grew to form a mass of 10–30 cells after 20–30 days (Fig. 2A, B). The masses were composed of greenish and reddish purple cells of various diameters. The masses grew as callus-like cell aggregates in medium with continuous aeration. The callus-like cell aggregates are similar to the callus-like clumps of cells induced from the isolated cells of *Porphyra perforata* by POLNE-FULLER *et al.* (1984).

By maintaining the aerated cultures, a large number of young thalli differentiated from the callus-like cell aggregates (Fig. 2C). Those young thalli grew well in aerated culture to the length of 2–5 mm after two months (Fig. 2D) and the largest of those attained to about 60 cm in length after four months (Fig. 2E). They were microscopically chimeral thalli irregularly variegated with greenish and reddish purple cell groups.

Four months after the differentiation of young thalli, some chimeral thalli matured, forming carpogonia and spermatangia (Fig. 3A). Carpospores released from mature thalli grew into greenish conchocelis-phase filaments which formed conchosporangial branches (Fig. 3B). The conchospores

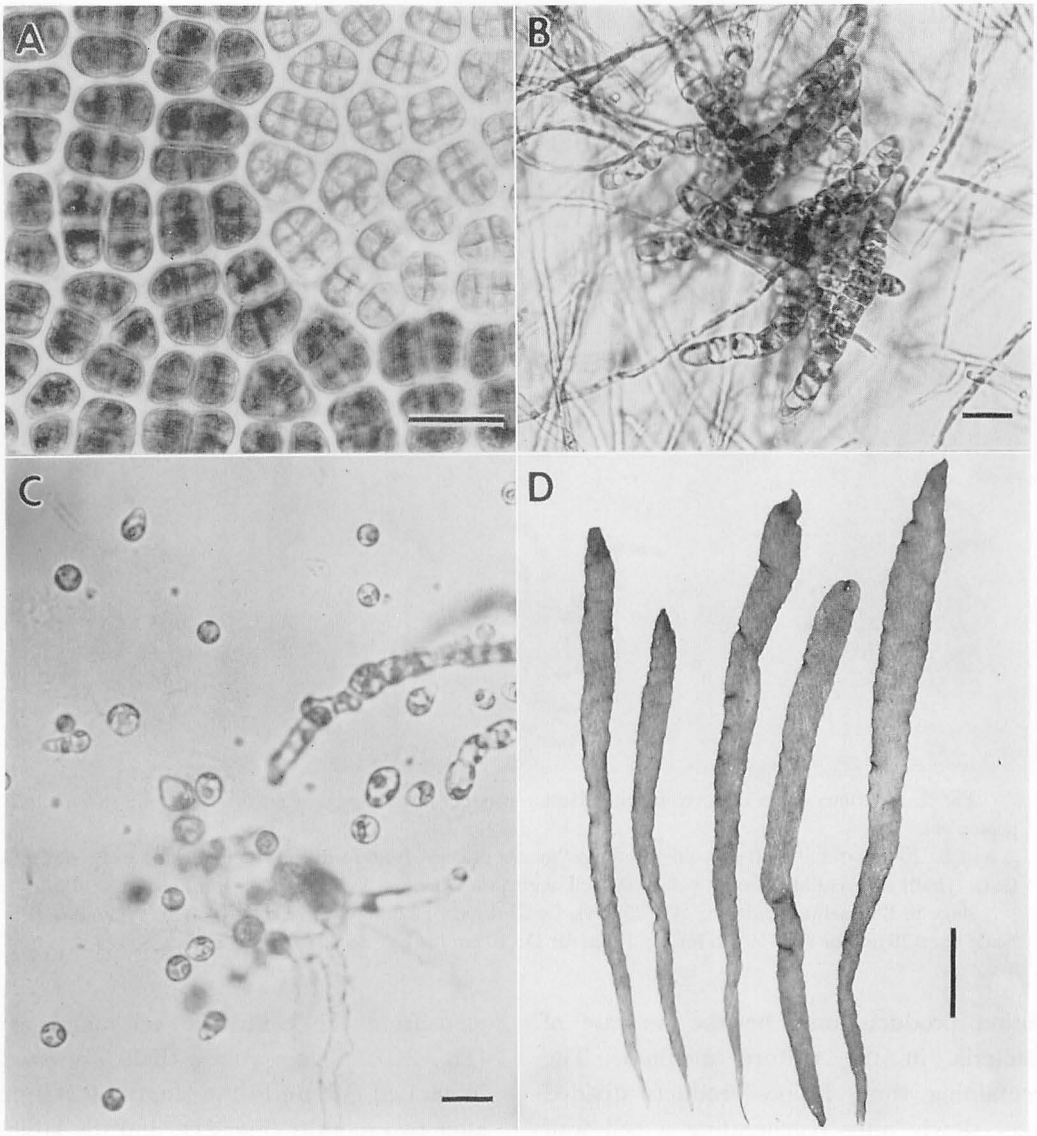

Fig. 3. Further development of thallus differentiated from "callus-like cell aggregates".
A. A portion of mature thallus in Fig. 2E showing carpogonia and spermatangia.
B. Conchosporangial branches formed on the conchocelis filaments derived from the carpospores.
C. Conchospores released from the conchosporangia.
D. F_1 thalli arisen from the germinated conchospores.
Scale bar: 20 μm for A–C; 1 cm for D.

(Fig. 3C) released from the conchosporangia of wild-like type and that of the greenish type could be distinguished by the difference in their color. Conchospores attached to synthetic fibers ("Cremona" monofilaments) of about 4 cm length grew into thalli (F_1) of oblanceolate or linear oblanceolate shape in aerated culture (Fig. 3D), similar to germlings from conchospores of original wild and green types. The greater part of those thalli was mostly composed of greenish

cells, while in thalli derived from the conchospores of wild-like type only a few cells at the basic part appeared reddish purple. There was no appreciable difference in color and morphology between the plants arisen from the three fusion products.

The phenomenon of chimeral thalli regenerated from the protoplast fusion products is regarded as a result of the occurrence of two type of cells with parental colors. This may be traced back to the gradual process of segregation of two different colored chloroplasts that had once undergone fusion. It is hypothesized that during the cell-divisions in the thalli and the growth of the conchocelis-phase the green type chloroplasts gained predominance over the wild type ones. As a result, in the F_1 thalli the greater part was replaced by cells of green type. The segregation to one or the other parental type of chloroplast has been often observed in regenerated hybrid plants and in the progeny of somatic hybrids of higher plants (cf., GLEBA and EVANS 1983, COCKING 1983).

OHME *et al.* (1986) reported that the heterozygous conchocelis from the sexual cross of the green and wild types in *P. yezoensis* were all the wild type, and frequently produced sectorially variegated chimeral thalli composed of parental color sectors. The difference between their results and ours suggests that the mechanisms of the formation of chimeral thalli and the presentation of colors in the progeny are different in the protoplast fusion and in the sexual cross between the green type and the wild type.

In the present study, only distinct green plants were obtained. If many more fusion products had been successfully regenerated, it might have been possible to find some that gave the intensive wild type color. We plan to engage in continuous research on further details of new fusion products and their regenerated plants with respect to the behavior of chloroplasts, nuclei and chromosomes and to the biochemical characters (e.g., constituents of pigments).

The PEG method has been used principally for the fusion of protoplasts to produce somatic hybrids, especially in higher plants. In recent years, attempts to accomplish algal protoplast fusion based on PEG treatment have been successful in *Zygnema extenue* and *Spirogyra gracilis* (OHIWA 1978), *Chlamydomonas reinhardi* (MATAGNE *et al.* 1979), *Ulva linza* and *Monostroma angicava* (ZHANG 1983), and *Porphyra yezoensis* and *Enteromorpha intestinalis* (SAGA *et al.* 1986). We have shown that the PEG method is also suitable for producing intraspecific parasexual hybrids of *Porphyra* by means of protoplast fusion.

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藤田雄二・右田清治：スサビノリの二つの色彩型葉体から分離されたプロトプラストの融合ならびに融合細胞の発生

スサビノリ *Porphyra yezoensis* UEDA の野生型と緑色型葉体から酵素法によって分離されたプロトプラストは，ポリエチレングリコール処理によって融合した。野生型と緑色型の各1個のプロトプラストからなる融合細胞は，細胞壁を再生した後，分裂し，1か月後に約30細胞の細胞小塊となった。その細胞小塊から誘導されたカルス状の細胞塊からは，よく生長する多数の葉体が発生した。それらの葉体は，顕微鏡的に赤紫色の細胞群と緑色がかった細胞群からなるキメラ葉体であった。成熟キメラ葉体からの果胞子は緑色を帯びた糸状体に生長した。その糸状体からの殻胞子は赤紫色あるいは緑色とみなされたが，いずれも発芽するとほとんど緑色を帯びた細胞からなる葉体となった。（852 長崎市文教町1-14　長崎大学水産学部藻類増殖学研究室）