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Protoplast isolation and fusion in *Porphyra* (Bangiales, Rhodophyta)

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Abstract

The isolation and fusion of protoplasts in several species of *Porphyra* is described. Protoplasts from all species in the present study were obtained by treating thalli with commercial protease and bacterial crude enzyme prepared in the laboratory. Protoplast fusion was accomplished by following polyethylene glycol (PEG) and electrofusion methods. In the electrofusion method, protoplasts aligned in an AC field (1 MHz, 40 V for 20 s) and subsequently fused with a DC pulse of 250 V for 40 μs yielded optimum (about 20%) binary or trinary fusion products compared to the PEG method (about 8–10%). Chromosome counts and absorption spectra of crude phycobilins of most regenerated plants were identical to one or the other of the parental types, but several chimeral thalli showed a mixture of chromosome numbers and pigmentation.

Introduction

Protoplasts have been isolated from different species of *Porphyra* by a variety of enzymatic techniques, and they have regenerated normal plants (Zhao & Zhan, 1981; Saga & Sakai, 1984; Polne-Fuller & Gibor, 1984; Fujita & Migita, 1985; Chen, 1987; Chen *et al.*, 1988). Fujita & Migita (1987) reported protoplast fusion and regeneration of fusion products from *Porphyra yezoensis* Ueda. However, there are no reports of development of somatic hybrids of macrophytic algae to date. Therefore, the present study was undertaken to fuse protoplasts from several species of *Porphyra* to generate somatic hybrids. Thus, this paper describes detailed investigation of (1) improved techniques for protoplast isolation, (2) different fusion methods (*i.e.*, electro- and chemical-induced fusion), and (3) some morphological characteristics of regenerated plants from heteroplasmic fusion products obtained from fusion of several species of *Porphyra*.

Materials and methods

Vegetative thalli

Seven species of *Porphyra* (including one color mutant) were used in the present study (Table 1). Conchospores released from mature shell-dwelling conchocelis grown in the laboratory were collected on synthetic fibers (Cremona monofilament 4 cm long), and germlings were later transferred to side-arm, flat-bottom, aerated flasks containing modified PES medium (Provasoli, 1968). All cultures were usually maintained under daylight white fluorescent lamps at 100 μmol m^{-2} s^{-1}, 12 : 12 L : D, 18–20 °C unless otherwise men-

Table 1. Protoplast yield and regeneration rate in species of *Porphyra*.

Species	Protoplast yield 0.1 g fresh wt^{-1}	Percentage of cell-wall regenerated protoplasts
P. yezoensis Ueda (normal type)	1.2×10^{7}[a]	70.5[b]
P. yezoensis Ueda (green type)	1.8×10^{6}	69.5
P. suborbiculata Kjellman *f. latifolia* Tanaka	1.5×10^{7}	71.5
P. seriata Kjellman	5.0×10^{5}	35.5
P. okamurae Ueda	4.2×10^{6}	63.2
P. pseudolinearis Ueda	4.2×10^{5}	65.1
P. tenuipedalis Miura	2.6×10^{6}	45.0

[a] Protoplast yield after 4 h incubation in bacterial crude enzyme following 1 h protease treatment;
[b] Data represent regenerated protoplasts after 10 days in culture at 20 °C, 120 μmol $m^{-2} S^{-1}$ (12 : 12 L : D).

tioned. Thalli grown to about 10 cm long were used for protoplast isolation.

Isolation of protoplasts

Protoplasts from all species were prepared according to the methods of Fujita & Migita (1987) with the following modifications. Vegetative thalli of about 50 mg each were cut into pieces and were pretreated with 4 mL of 5% protease P (Amano Pharmacy Co., Japan) dissolved in 50 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid buffer (Wako Pure Chem. Indust. Ltd., Japan) pH 8.0 in seawater containing 0.6 M mannitol for one hour prior to incubation in 4 mL of bacterial crude enzyme for protoplast isolation. Potassium dextran sulfate (Nacalai Tesque, Inc., Japan) 0.5% was also included in the bacterial crude enzyme. The rest of the conditions and purification methods followed Fujita & Migita (1987).

Fusion of protoplasts

(A) Polyethylene glycol (PEG) method. Protoplasts from each species were prepared separately and later suspended at a 1 : 1 ratio in seawater containing 0.6 M mannitol at a density of $1 \times 10^{5-6}$ cells mL^{-1} each for the two species being fused. Approximately 100 μL of protoplast mixtures were placed at the center of 60 mm untreated plastic Petri dishes. About 30–40 mg of PEG 4000 powder (Wako) or 100 μL of PEG 4000 fusion solution (Kao *et al.*, 1974) were added to the protoplast mixture and incubated for about 15–20 min. After incubation, 2–5 mL of PEG eluting solution (Kameya *et al.*, 1981) were added to the protoplast-PEG preparation. This mixture was then diluted with modified f/2 enriched seawater medium (Waaland & Watson, 1980).

(B) Electrofusion method. Protoplast fusion was induced by electric fields using a Shimadzu somatic hybridizer SSH-2. Protoplasts suspended in seawater-mannitol were washed twice with electrofusion solution [0.7 M mannitol, 0.2 mM tris (hydroxymethyl)aminomethane, 0.1 mM $CaCl_2 \cdot 2H_2O$ and 0.1 mM $MgCl_2 \cdot 6H_2O$ in distilled water, adjusted to pH 7.5] and finally resuspended in the same solution in a 1 : 1 proportion at the concentration of $1 \times 10^{5-6}$ cells mL^{-1}. Aliquots of 200 μL protoplast suspension prepared as above were placed between two electrodes (1 mm spacing) in a fusion chamber and allowed to settle for 5 min. Protoplasts were subsequently aligned in an AC field at different voltages at 1 MHz (Table 2) to obtain chains consisting of 2–3 protoplasts rather than multiple protoplasts.

Heterokaryon culture

Heterokaryons were sorted out on the basis of natural pigmentation and occasionally stained with neutral red (0.001% for 5 min) to ascertain viability. Fusion products were transferred to Petri dishes using fine-tip pipettes and cultured in 10 mL of modified f/2 enriched seawater medium

Table 2. Parameters calibrated to obtain optimum fusion frequencies of binary fusion products from normal and green types of *Porphyra yezoensis* by electrofusion method.

Electrical parameters	Tested range	Optimum range
Frequency	1 MHz	1 MHz
Voltage AC	10–40 V	40 V
Initial time	10–25 s	20 s
Voltage DC	150–350 V	250 V
Electric field strength	1.5–3.5 Kv cm^{-1}	2.5 Kv cm^{-1}
Pulse width	10–70 μs	40 μs

at 100 μmol m^{-2} s^{-1}, 12:12 L:D, 18–20 °C. Plantlets grown to about 1 mm long from callus-like structures were transferred to aerated cultures. *Chromosome staining and measurement of crude phycobilin spectra of thalli regenerated from fused cells:* Chromosomes were stained with haematoxylin (Wittmann, 1965). Crude phycobilin pigments were extracted in 50 mM phosphate buffer (pH 6.5) by grinding the thalli with pestle and mortar. The slurry was centrifuged for 30 min at 9500 × g, and the absorption spectrum of the supernatant was measured from 350 to 750 nm by a Hitachi 220 A Spectrophotometer.

Results

Protoplast yields from all species of *Porphyra* are presented in Table 1. The isolated protoplasts from all plants were spherical in shape as shown in Fig. 1C, D. Protoplast yield varied from species to species and ranged from 4.2×10^5 to 1.5×10^7 cells 0.1 g fresh wt^{-1} of plant. Similarly, regeneration rates of protoplasts (Table 1) also varied from species to species; the highest, 71.5% and 70.5%, occurred in *P. suborbiculata* Kjellman f. *latifolia* Tanaka and *P. yezoensis*, respectively.

Protoplast fusion was induced by the addition of PEG 4000 solution to the protoplast suspension. The highest number of fusions (about 8%) occurred soon after the addition of PEG eluting solution to the protoplast-PEG preparation. In another instance, addition of PEG powder directly to the protoplast suspension induced more fusions (about 10%) than the PEG solution.

In the electrofusion method, initial experiments focused on determination of optimal electrical parameters (Table 2) to obtain binary fusion products using normal and green type *Porphyra yezoensis*. Protoplasts aligned in an AC field at 1 MHz, 40 V for 20 s and subsequently fused with a DC pulse of 250 V for 40 μs yielded optimum fusions (about 20%). The higher pulse voltages (> 300 V) induced either protoplast lysis or produced nonviable fusion products that gradually perished within 24–48 h. Hence, thereafter the same electrical parameters were chosen for all other species to induce fusions.

Heteroplasmic fusion products were obtained through the electrofusion and/or PEG method for *Porphyra yezoensis* (normal type) with *P. yezoensis* (green type), *P. pseudolinearis* Ueda, *P. suborbiculata* f. *latifolia* and *P. tenuipedalis* Miura. The heteroplasmic fusion products of *P. yezoensis* (normal) with *P. suborbiculata* f. *latifolia* and *P. tenuipedalis* have not yet regenerated adult plants, whereas *P. yezoensis* (normal) with green type and *P. pseudolinearis* have regenerated adult plants. The development and morphogenetic patterns of heterokaryons of *P. yezoensis* (normal) and green type were similar to those reported by Fujita & Migita (1987). Hence, this paper essentially describes developmental morphology of heteroplasmic fusion products of *P. yezoensis* (normal, Fig. 1A, purplish red thallus) and *P. pseudolinearis* (Fig. 1B, red thallus). Protoplasts of the two species (Fig. 1E) were electrically fused (Fig. 1F, G, H) to obtain fusion products. The heterokaryons initially had two separate chloroplasts, but they gradually mixed with each other and became reddish purple overnight. The heteroplasmic fusion product slowly regenerated cell walls (Fig. 1I) within seven days and subsequently underwent cell divisions (Fig. 1J) to form cell masses (Fig. 1K). The masses were later grown as callus-like structures (Fig. 1L) and gave rise to a number of young thalli (Fig. 1M) within two months. Many of the regenerated plants (four out of five) were similar

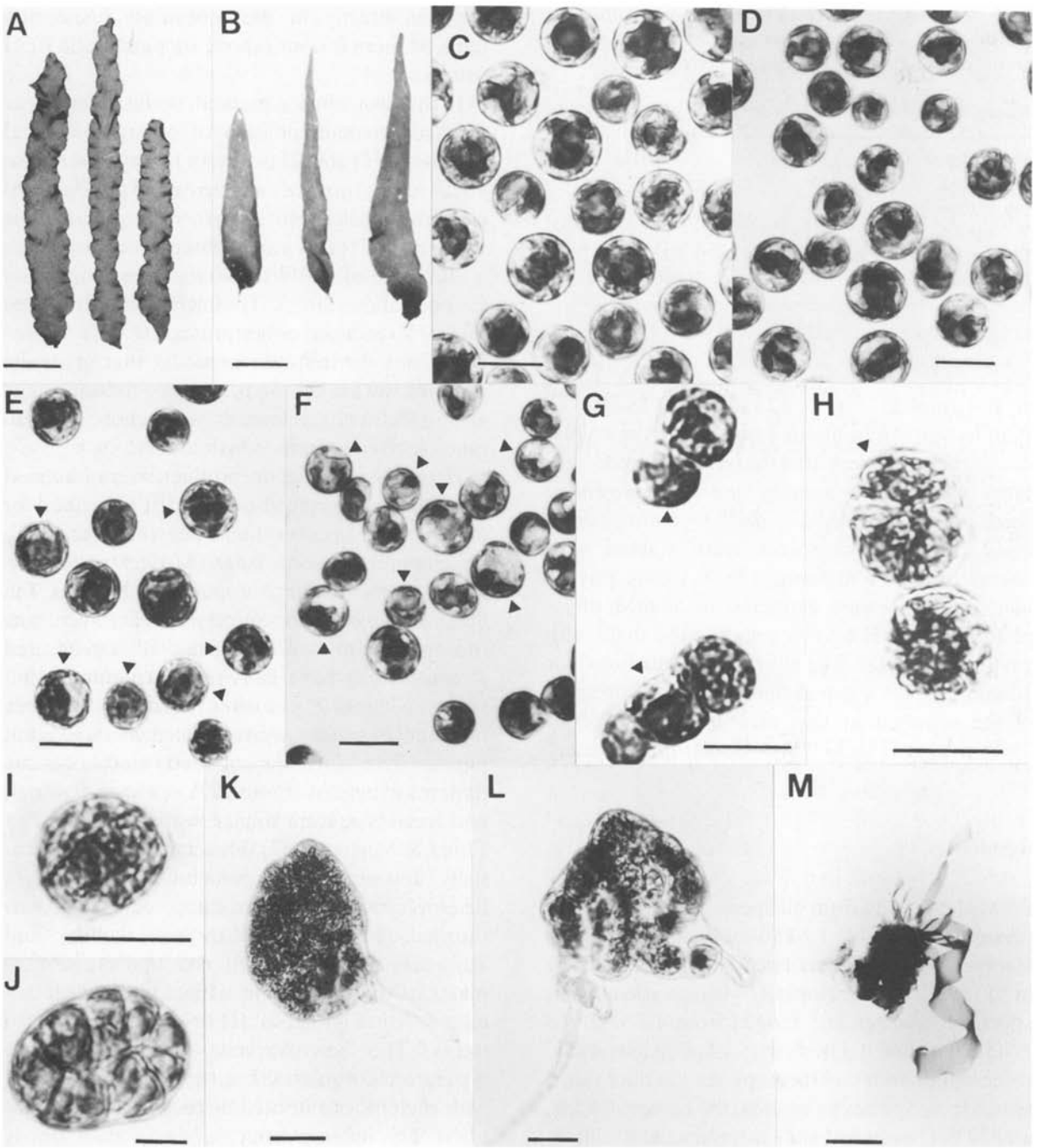

Fig. 1. Electrofusion and regeneration of heteroplasmic fusion products of *Porphyra yezoensis* and *P. pseudolinearis*. A. *P. yezoensis* (normal type) protoplast source plants. Bar = 1 cm. B. *P. pseudolinearis* protoplast source plants. Bar = 1 cm. C. Freshly isolated protoplasts of *P. yezoensis*. Bar = 15 μm. D. Freshly isolated protoplasts of *P. pseudolinearis*. Bar = 15 μm. E. Protoplast suspension of both *P. yezoensis* (with arrows) and *P. pseudolinearis*. Bar = 15 μm. F. Protoplast alignment in an AC field at 1 MHz, 40 V for 20 s. Bar = 15 μm. G. Immediately after high intensity DC pulse of 250 V for 40 μs. Bar = 15 μm. H. Round fusion products 5 min. after stage G. Bar = 25 μm. I. Heteroplasmic fusion cell after 6 days in culture. Bar = 25 μm. J. Heteroplasmic cell divisions after 20 days in culture. Bar = 10 μm. K. 40-day old germling from heteroplasmic cell. Bar = 60 μm. L. Callus-like cell aggregates after 50 days in culture. Bar = 60 μm. M. Differentiation of young thalli induced from callus-like cell aggregates after transferring to aerated cultures (60-day old). Bar = 5 mm.

to *P. pseudolinearis* in color and shape of thallus. However, the fifth one, unlike the other four, gave rise to a different type of thallus, most of which were similar to *P. yezoensis* but a few were chimeral (a mosaic of greenish purple cells all over the reddish thallus).

Chromosome counts of all five regenerated plants from heterokaryons of *Porphyra yezoensis* and *P. pseudolinearis* were either 3 or 4 like one of the parents. The chimeral type thalli showed variable chromosome counts of 3 and 4. The major absorption maximum of phycobilins, *i.e.* phycoerythrin and phycocyanin, was 497, 564 and 614 nm in *P. yezoensis* and 497, 564 and 618 nm in *P. pseudolinearis*. The phycobilin spectrum of four regenerated plants was the same as *P. pseudolinearis* and one was identical to *P. yezoensis*. The chimeral type was not measured due to the dual pigment system in the same thalli.

Discussion

The protoplast yield varied from species to species. *Porphyra* thalli generally consist of a single layer of thick-walled cells embedded in a mucilaginous matrix with a tough outer cuticle. The cell walls have microfibrils with amorphous areas (Mukai *et al.*, 1981). The major cell wall components have been identified as β-1,3-xylan, β-1,4-mannan, sulfated D-L galactan and protein (Peat *et al.*, 1961; Frei & Preston, 1964; Hanic & Craigie, 1969). The bacterial crude enzyme has shown relatively high activity for protease, β-1,4-mannanase, β-1,3-xylanase and porphyranase (Fujita, unpubl. data). Therefore, variation in the protoplast yield in the present study might be due to quantitative variation in cell wall composition of the species. Similarly, variation in regeneration rate in different species might be due to variation in morphogenetic capacity and growth rate of individual plants.

Although PEG has been used widely for induction of protoplast fusion in land plants, it did not yield satisfactory fusion frequencies in *Porphyra*. Therefore an alternate, suitable method that offers high fusion frequencies and viable fusion products has been investigated. The electrofusion method has been employed successfully for the first time to produce viable fusion products in large numbers in *Porphyra*. The fusion and the regeneration rate of fusion products obtained through this system was always higher than the PEG method. Hence, fusions were accomplished by the electrofusion method in addition to the PEG method in all investigated species.

The results of crude phycobilin spectra, thallus shape and chromosome counts of all regenerated plants (except the chimeral type) that developed from heterokaryons were similar to one or the other of the parental types. The development of such plants from heterokaryons might be due to the absence of genetic variation resulting from the gradual elimination of one nucleus and chloroplast of one of the parents during the course of development to adult plant. The occurrence of a few chimeral thalli from heterokaryons has already been reported (Fujita & Migita, 1987) in *Porphyra yezoensis* normal and green types. The development of such chimeral thalli suggests the possibility of some degree of variation in combination of pigment constituents, unlike the other regenerated plants. Furthermore, the dual chromosome count in adult chimeral thalli suggests the coexistence of both nuclei at initial stages, which later might have segregated to produce two different chromosome counts in the same thallus. It is also yet unknown which color cells in the thalli have 3 and 4 chromosomes. Thus, it is difficult to explain the mechanism that controls nuclear elimination and chimeral-type thallus formation in *Porphyra*. It is evident from this study that further investigations using isoenzyme analysis and other cytological and biochemical techniques on regenerated plants (including the chimeral type) are needed to categorize interspecific hybrid plants in *Porphyra*.

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