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# Intrageneric fusion of protoplasts from gametophytic blades and development of fusion products in *Porphyra* spp.(Rhodophyta)

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**Abstract**: The protoplasts were isolated enzymatically from the gametophytic blades of the wild type and red mutan of *Porphyra haitanensis* and from the wild type blades of *Porphyra yezoensis*, respectively. Protoplast fusions wer made by treatment with polyethylene glycol (PEG) solution. Many heterokaryocytes formed after the PEG solutio was diluted out. The rate of intrageneric fusion was 9.7% – 12.4% for *P. haitanensis*, and 10% – 11.5% fo *P. yezoensis*. The fusion rate became higher as the molecular weight of PEG increased from 1 540 to 6 000. Fusio products that involved one red and one wild type protoplasts in *P. haitanensis* were picked out and individuall cultured. After 30 days of culture, one of them developed into a chimeral blade composed of two sectors showing re and wild type color. While, a fusion product involving two red protoplasts developed into a red blade that has tw rhizoids at the top and basal ends of the blade. However, protoplasts isolated from the blade, regenerated into re blades, which only have one rhizoid at the basal end of the blade. After culture of 15 – 20 days, intrageneric fusio products of *P. yezoensis* developed into cell-masses, which released many spores to germinate into normal wild-typ blades.

**Key words**: gametophytic blade; polyethylene glycol(PEG); protoplast fusion; plant regeneration; *Porphyra* spp

# 紫菜种内原生质体的融合和融合体再生

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**摘　要**：利用酶解法分离出野生型叶状体和红色突变体的坛紫菜原生质体以及条斑紫菜野生型叶状体的原生质体，再用聚乙二醇(PEG)法进行种内原生质体融合。当加入原生质体液内的 PEG 被稀释除去时，原生质体发生融合并形成许多杂合体。种内原生质体融合率坛紫菜为 9.7% ~ 12.4%，条斑紫菜为 10% ~ 11.5%。PEG 分子量在 1 540 ~ 6 000 之间，随着分子量的提高，原生质体融合率增高。原生质体融合体被挑出来进行单个培养。经过 30 天培养坛紫菜的一个杂合体(含一个红色原生质体和一个野生型原生质体)再生成一个呈红色和野生色的相嵌叶状体。而一个含两个红色原生质体的融合体再生成一个在基部和头部均长假根的叶状体。但来自这个再生体的原生质体只长成具一个假根的叶状体。从原生质体融合体的再生体以及它们无性繁殖体的结果来看，坛紫菜的种内原生质体融合体可能只发生了细胞质融合，而真正的核融合并没有发生。培养 15 ~ 20 天，条斑紫菜的原生质体融合体先再生成细胞团，然后由细胞团释放出许多胞子并萌发成正常野生型叶状体。

**关键词**：叶状体；聚乙二醇；原生质体融合；植株再生；紫菜
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The isolation and culture of protoplasts have been successful in more than 100 species of higher plants. The protoplasts were used as tools in physiological and cytological studies[1,2]. The somatic hybrids through intrageneric and intergeneric protoplast fusion from 45 species were obtained[2,3,7]. In recent years, many progresses of protoplast culture in seaweeds have been made[5-14]. Some researchers have taken in producing the improved strains by culture and fusion of protoplasts of seaweeds, especially *Porphyra*. There were several reports on protoplast fusion of seaweeds. Fujita and Migita had reported fusion of protoplasts from different color type thalli of *P. yezoensis* Ueda and development of the fusion products[15]. Dai et al. had made protoplast fusion between two species of *Porphyra*, but no hybrid plant was obtained[16]. The purposes of the present study are to make intrageneric fusion of protoplasts in *Porphyra haitanensis* and *Porphyra yezoensis*, which were both extensively cultivated in China, and to understand whether the real nuclear fusion of protoplasts in *Porphyra* can occur or not.

## 1 Materials and Methods

### 1.1 Strains of *P. haitanensis* and *P. yezoensis*

The wild type strain of *Porphyra haitanensis* Chang et Zheng was isolated from a cultivated population in Linjiang Fisheries Farm, Fujian Province, and the red variant was obtained by irradiation of protoplasts from the gametophytic blades of the wild type strain with UV[17]. The wild type strain of *Porphyra yezoensis* Ueda was obtained from a cultivated population in Lisi Aquaculture Farm, Jiangsu Province. Stock culture of their free-living conchocelis have been maintained in the laboratory at 20℃ under a photon flux density of 10 μmol photon $m^{-2}s^{-1}$ provided by cool-white, 40-W fluorescent lamps on a 14∶10 h light∶dark cycle (14L∶10D). The stock culture was maintained as previously described[18].

### 1.2 Culture of gametophytic blades and protoplast isolation

An aliquot of stock conchocelis filaments was incubated at 28℃ for *P. haitanensis* and at 23℃ for *P. yezoensis* under 15 μmol photon $m^{-2}s^{-1}$ (10L∶14D) to induce formation of conchosporangia. Once the conchosporangia were formed, the conchocelis filaments were transferred into a 300 mL Erlenmeyer flask containing 150 mL of the MES medium[13] and 5 vinylon monofilaments (ca. 5 cm long), and cultured with aeration at 25℃ for *P. haitanensis* and at 20℃ for *P. yezoensis*, under 25 μmol photon $m^{-2}s^{-1}$ (10L∶14D) to release conchospores. The conchospores attached onto the monofilaments were then cultured to obtain gametophytic blades at 22℃ for *P. haitanensis* and at 15℃ for *P. yezoensis*, under 40 μmol photon $m^{-2}s^{-1}$ (12L∶12D).

**Tab.1 Components of enzyme solutions for isolation of protoplasts in *P. haitanensis* and *P. yezoensis***

| Components | *P. haitanensis* | | *P. yezoensis* |
|---|---|---|---|
| | Wild type | Red type | Wild type |
| Abalone powder(%) | 1.0-1.5 | 2.0 | - |
| Bacterial enzymes | - | - | + * |
| Cellulase Onozuka(R-10) | 2.0 | 2.0-3.0 | 3.0 |
| Glucose(mol·$L^{-1}$) | 2.0 | 2.0 | - |
| Mannitol(mol·$L^{-1}$) | - | - | 0.8 |
| Seawater | - | - | + |
| Distilled water | + | + | - |
| pH | 6.0 | 6.0 | 6.2-6.5 |

*: used

The components of the enzyme solutions for isolation of protoplasts of *P. haitanensis* and *P. yezoensis* were given in Tab.1. Crude bacterial enzymes were prepared as described previously[19]. The procedure to prepare abalone gut enzymes was showed as follows.

When the blades reached 0.5-2.0 cm in length, some of them were used to isolate protoplasts. After being cleaned with a soft brush in fresh sterile seawater more than five times and dried off seawater on the blade suface, the blades were chopped into small pieces(1-2 $mm^2$) and immersed in the enzyme solution for isolating protoplasts. About 0.2 g fresh weight of the blades was incubated in a small Petri dish containing 5 mL of enzyme solution. Incubation temperature was 26℃ for *P.*

*haitanensis* and 24℃ for *P. yezoensis*. After 2.5 – 3 h of incubation, during which time the tissue was gently swirled on a rotary shaker (80 r·min$^{-1}$, the enzyme-protoplast mixtures were passed through a 25 μm nylon mesh to remove the undigested blade pieces. The filtrates were then centrifuged (800 r·min$^{-1}$, 5 min) to sediment the protoplasts. The supernatants were discarded and the protoplasts were resuspended in the MES medium at a higher salinity (45.5), and washed three times in the same ways continuously. The protoplasts thus isolated were ready for fusion.

### 1.3 Protoplast fusion and culture of fusion products

The protoplasts with different color were respectively isolated from the red mutant and the wild type blades of *P. haitanensis*. The protoplasts of *P. yezoensis* were isolated from the wild type blades. A part of protoplasts of *P. yezoensis* were stained with 0.5% neutral red solution containing glucose in 2.0 mol·L$^{-1}$ for 5 min to be red color. The method reported by Kao et al.[20] was modified for protoplast fusion. The protoplasts with red and wild type color in *P. haitanensis* were mixed in the ratio of 1 : 1. Approximately 150 μL of the mixed protoplast suspension was transferred into a slide, which was placed in a Petri dish, to reduce evaporation. After the protoplasts had settled to the slide (ca. 5 min), 450 μl of the PEG solution showed in Tab.2 was slowly added to the protoplast culture. Then, the protoplasts were incubated in the PEG solution for 20 – 30 min at 25 – 28℃ in the dark. About 500 μL of the washing solution showed in Tab.3 was gently added to this mixture. After another 10 min, the protoplasts were washed several times with a total of 10 mL of MES medium. The Petri dish containing the treated protoplasts was added with 20 mL of MES medium and was maintained at 20 ± 1℃, under 20 μmol photon m$^{-2}$s$^{-1}$ (12 L:12 D) for 40 h. And then, the rate of protoplast fusion was examined microscopically. Identification of heteroplasmic fusion was readily possible because the heterokaryocytes contained both red-purple chromatoplast from the red protoplast and light purple-brown chromatoplast from the wild type protoplast in *P. haitanensis*. The fusion products were transferred with a

Abalones
↓
Digestive organs
↓
Freezing twice at -20℃
↓
Smash digestive organs and add six times volume of distilled water(4℃)
↓ Centrifugation at 12000 r·min$^{-1}$ for 30 min, at 4℃
Supernatants Ⅰ
↓ 30% saturation ammonium sulphate at 4℃, centrifugation at 12000 r·min$^{-1}$ for 30 min
Supernatants Ⅱ
↓ 80% saturation ammonium sulphate at 4℃, centrifugation at 12000 r·min$^{-1}$ for 30 min
Precipitate
↓ Dissolution in distilled water
Crude enzyme solution
↓
Desalting by Sephadex column $G_{25}$
↓ Lyophilisation
Abalone enzyme power

procedure for preparing abalone gut enzymes

**Tab.2 PEG solutions for protoplast fusion**

| Components | PEG solutions | | | |
|---|---|---|---|---|
| | $P_1$ | $P_2$ | $P_3$ | $P_4$ |
| $PEG_{6000}$(%) | 30 | – | – | – |
| $PEG_{4000}$(%) | – | 30 | – | – |
| $PEG_{2000}$(%) | – | – | 30 | 30 |
| Mannitol(Mol/L) | – | – | – | 0.6 |
| $CaCl_2 \cdot H_2O$(mMol/L) | – | – | – | 5.0 |
| $KH_2PO_4$(mMol/L) | – | – | – | 0.7 |
| Sea water | + | + | + | – |
| Distilled water | – | – | – | + |
| pH | 6.5 | 6.5 | 6.5 | 6.5 |

* : used

**Tab.3 Solution for washing PEG**

| Components | Washing solutions | | | |
|---|---|---|---|---|
| | $W_1$ | $W_2$ | $W_3$ | $W_4$ |
| Seawater | + * | + | + | + |
| Salinity | 45.5 | 45.5 | 32.0 | 32.0 |
| Mannitol(mol/L) | – | – | 0.6 | 0.6 |
| pH | 7.0 | 9.0 | 9.8 | 10.5 |

* : used

fine pipette to a Petri dish containing 5 mL of MES medium, and cultured at 20 ± 1℃ under 30 μmol photon $m^{-2}s^{-1}$ (12L:12D).

The neutral-red-stained protoplasts were mixed with the wild type protoplasts in the proportion of 1:1 in *P. yezoensis*. The fusion methods and culture conditions for fusion products were the same as described above. The fusion products in *P. yezoensis* were identified according to whether the fusion products contained red-stained chromatoplast from the neutral-red-stained protoplast or not.

## 2 Results and Discussion

### 2.1 Protoplast fusion and culture of fusion products in *P. haitanensis*

The adhesion of protoplasts occurred immediately after introduction of PEG solution in the mixture of red and the wild type protoplasts. Not only two protoplasts aggregated together, but also more than two protoplasts adhered tightly. The protoplasts with different color adhered over a large area of their surface as well as did the protoplasts with the same color (Plate Ⅰ a – b). As the incubation of protoplasts in PEG solution prolonged, a few of protoplast fusion occurred. However, a great number of protoplast fusion occurred after the PEG solution was diluted by the washing solution (Plate Ⅰ c). The fusing cell was 8-shaped initially, however, as the fusion did further, the shape became to be oval, and gradually to be spherical (Plate Ⅰ d – g). In the fusion products, the chromatoplasts in different colors intermixed very slowly, and they could be distinguished even 46 h after addition of the washing solution to the protoplast-PEG preparation. Therefore, it was easy to identify the fusion products containing two chromatoplasts with different color. The molecular weight (MW) of PEG played an important role in protoplast fusion of *Porphyra*. The frequency of heterkaryocyte formation was 4.6%, 11.5% and 11.8%, respectively, when the protoplasts were incubated in the solution containing 30% PEG in MW of 1 540, 4 000 and 6 000. The higher the MW of PEG, the higher the fusion rate. Besides, the pH value and $Ca^{2+}$ concentration in the PEG solutions also influenced the rate of protoplast fusion. As showed in Tab.4, the higher the pH value and $Ca^{2+}$ concentration, the higher the fusion rate. These results were the same as those of high plant[21]. Incubation temperature also affected the protoplast fusion. The optimal incubation temperature was 28℃ for *P. haitanensis*. A relative higher fusion frequency was obtained when incubated the protoplasts in PEG solution at 28℃ for 25 min. When the incubation temperature went up to 30 ℃, the fusion rate relatively increased, however, the survival rate of fusion products was very low and sometimes the treated protoplasts died off within 12 h. Three fusion products containing only a red and a wild type protoplasts, and two fusion products involving two red protoplasts were picked out and transferred into Petri dishes for culture individually. One of the former regenerated cell wall in 5 days, and subsequently began to cell division. In 30 days of culture, it developed into a chimeral blade composed uf a red and a wild type sectors, which was compartmentalized by a clear line(Plate Ⅱ b). One of the latter regenerated cell wall in 6 days and developed into a red blade that have two rhizoids at both ends of the blade (Plate Ⅱ a). After 1 month of culture, it was transferred and cultured in a flat bottom flask to get large blade. When it grew up to 5 cm long, it was used to produce protoplasts again. All of the protoplasts developed into red blades. Most of them have only one rhizoid, and a few of the blades had no rhizoid. However, no blades had two rhizoids (Plate Ⅱ c).

**Tab.4 Effect of washing solution on the fusion rate of protoplasts**

| Washing solution | pH | Salinity | Fusion rate(%) |
|---|---|---|---|
| $W_1$ | 7.0 | 45.5 | 9.7 |
| $W_2$ | 9.0 | 45.5 | 11.4 |
| $W_3$ | 9.8 | 32.0 | 12.4 |
| $W_4$ | 10.5 | 32.0 | 11.8 |

## 2.2 Protoplast fusion and plant regeneration in *P. yezoensis*

A great deal of protoplasts aggregated as soon as the PEG solution was added into the mixed protoplast suspension containing the neutral-red-stained and wild type protoplasts (Plate Ⅲ a – b). When the protoplasts were incubated in 30% of $PEG_{6000}$ solution at 26℃ for 25 min, and diluted with $W_2$ or $W_3$ solution (Tab.3), the fusion rate was 10% – 11.4%. Fusion process between two protoplasts with different colors was observed. The fusion process of two protoplasts, from 8-shaped to oval shape and finally became spherical, were completed within 55 min after addition of the washing solution to the protoplast-PEG preparation (Plate Ⅲ c – f). Three fusion products containing two protoplasts of one neutral-red-stained and one wild type, were picked out and individually cultured. Two of them began to cell division in 7 days of culture, and developed into cell-masses in three weeks (Plate Ⅲ g). Many spores released from the cell-masses lately, and germinated into normal wild type blades (Plate Ⅲ h). This result was similar to that obtained by Fujita and Migita[15].

## 2.3 Influence factors on protoplast fusion and analysis of the regenerated plants from fusion products

Not only the MW of PEG, pH value and $Ca^{2+}$ concentration affected protoplast fusion, but also did the following factors. The percentage of true protoplasts was the most important to make fusion. Protoplasts isolated from the young blades cultured in the lab (0.5 cm long, aged 25 – 30 days) were the best materials for fusion. The fusion rate of such protoplasts was much higher than the protoplasts from the blades, which were cultivated in nori-net in the sea. The blades growing on the nori-net were thicker than those growing in the lab even in the same length and at the same age, because the former are often exposed to the air for several hours every day. Therefore, the percent of true protoplasts from the latter was higher than from the former.

The tool enzymes were one of the most important factors to obtain good true protoplasts. According to the results of the repeated experiments, more than 90% of true protoplasts of *P. haitanensis* could be obtained by using the enzyme mixture containing abalone enzyme and cellulase (R-10). However, using the same enzyme mixture to produce *P. yezoensis* protoplasts, only 80.3% of true protoplasts were obtained. Similarly, 98% of true protoplasts of *P. yezoensis* could be isolated by enzyme mixture of bacterial enzymes and cellulose (R-10), but using the same enzyme mixture only 50% – 64% of true protoplasts of *P. haitanensis* were obtained. These results indicate that the cell wall composition of *P. haitanensis* is different from that of *P. yezoensis*. The better enzyme to produce protoplasts, is abalone enzymes for *P. haitanensis*, but is the bacterial enzymes for *P. yezoensis*. For *P. haitanensis*, using the protoplasts isolated by enzyme mixture of abalone enzyme and cellulase (R-10), resulted much more heterokaryocyte and higher fusion rate than using protoplasts isolated by enzyme mixture of bacterial enzymes and cellulose (R-10). However, the contrary results were obtained in *P. yezoensis*.

The formation of plant chloroplasts is mainly controlled by nuclear genes[12]. The fusion product of *P. haitanensis*, which involved a red and a wild type protoplasts, developed into a chimeral blade with two sectors showing red and wild type color, which probably resulted from cytoplasmic fusion and no real nuclear fusion occurred. The red hybrid plant of *P. haitanensis*, which had two rhizoids at both ends of the blades, is also considered as result of a cytoplamic fusion, because none of the same blade appeared in the blades regenerated from the protoplasts of it. Isoenzyme analysis and chromosome behavior of the hybrid plants in *P. haitanensis* need to do.

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## References

[1] Li S H, Xu Z H. Tissue culture of economic plant[M]. Beijing: Science Press House, 1988. 1 – 50 (in Chinese).

[2] Xia Z H. Advancement of protoplast fusion in high plants [J]. Plant Physiology Communicati ns, 1989, 2: 1 – 6 (in Chinese).

[3] Evans D, Branvn J, Gleba Y. Int Rev Cytol[C]. 1983, 16(Suppl.): 143 – 159.

[4] Harms C. Protoplasts[J]. Birkhauser Verlag, Basel, 1983, 69 – 84.

[5] Chen L C M. Protoplast morphogenesis of *Porphyra leucostica*[J]. Bot Mar, 1987, 30(5): 399 – 403.

[6] Chency D P, Mar E, Saga N, van der Meer J. Protoplast isolation and cell division in the agar-producing seaweed *Gracilaria* (Rhodophyta)[J]. J Phycol, 1986, 22: 238 – 243.

[7] Fujita Y, Migita S. Isolation and culture of protoplasts from some seaweeds[J]. Bull Fac Fish Nagasaki Univ, 1985, 57: 39 – 45 (in Japanese with English abstract).

[8] Polne-Fuller M, Giber A. Development studies in *Porphyra* 1. Blade differentiation in *Porphyra perforta* as expressed by morphology, enzymic digestion, and protoplast regeneration[J]. J Phycol, 1984, 20: 609 – 616.

[9] Polne-Fuller, M, Giber A. Calluses, cells, and protoplasts in studies towards genetic improvement of seaweeds[J]. Aquac, 1986, 57: 117 – 123

[10] Saga N. Isolatinn of protoplasts from edible seaweeds[J]. Bot Mag Tokyo, 1984, 67: 423 – 427.

[11] Saga N, Sakai Y. Isolation of protoplasts from Laminaria and *Porphyra*[J]. Bull Jap Soc Fish, 1984, 50(6): 1085.

[12] Tang Y L. Isolatinn and cultivation of the vegetative cells and protoplasts of *Porphyra suborbiculate* Kjellm[J]. J Shanglong Coll Oceanol, 1982, 12 (4): 37 – 50(in Chinese with English abstract).

[13] Wang S J, Zhang X P, Xu Z D, et al. Studies on the cultivation of the vegetative cells and protoplasts of *Porphyra haitanensis* Chang et Zheng[J]. Chin J Oceanol Limnol, 1986, 17(3): 217 – 221 (in Chinese with English abstract).

[14] Yan X H, Wang S J. Studies on the development and differentiation of the somatic cells in *Porphyra* spp. (Rhodophyta)[J]. Marine Sciences, 1990, 2(3): 195 – 208.

[15] Fujita Y, Migita S. Fusion of protoplasts from thalli of two different color types in *Porphyra yezoensis* Ueda and development of fusion products[J]. Jap J Phycol, 1987, 35: 201 – 208.

[16] Dai J X, Zhang Q Q, Bao Z M, et al. Studies on the pure line culture, mutagenization and interspecific fusion of *Porphyra* protoplasts[J]. China J Oceanol Limnol, 1990, 21(3): 293 – 296(in Chinese).

[17] Yan X H. Studies on color type variants from mutagenized protoplasts of *Porphyra haitanensis* Chang et Zheng & *P. yezoensis* Ueda(Rhodophyceae)[J]. Chin J Oceanol Limnol, 1993, 11(3): 235 – 244.

[18] Kato M, Aruga Y. Comparative studies on the growth and photosynthesis of the pigmentation mutants of *Porphyra yezoensis* in laboratory culture[J]. Jap J Phycol, 1984, 32: 333 – 47.

[19] Yan X H. Preparation of bacterial enzymes, isolation and culture of protoplasts in *Porphyra* spp.[A]. 1991, Abstract of the 1st Symposium of Chinese Marine Biotechnology[C]. 17 (in Chinese).

[20] Kao K N, Michayluk M R. A method for high-frequency intergeneric fusion of plant protoplasts[J]. Plants, 1974, 115(4): 335 – 367.

[21] Zhen C C. Cytobiology[M]. Beijing: People Education Press House, 1980. 165 – 185 (in Chinese).

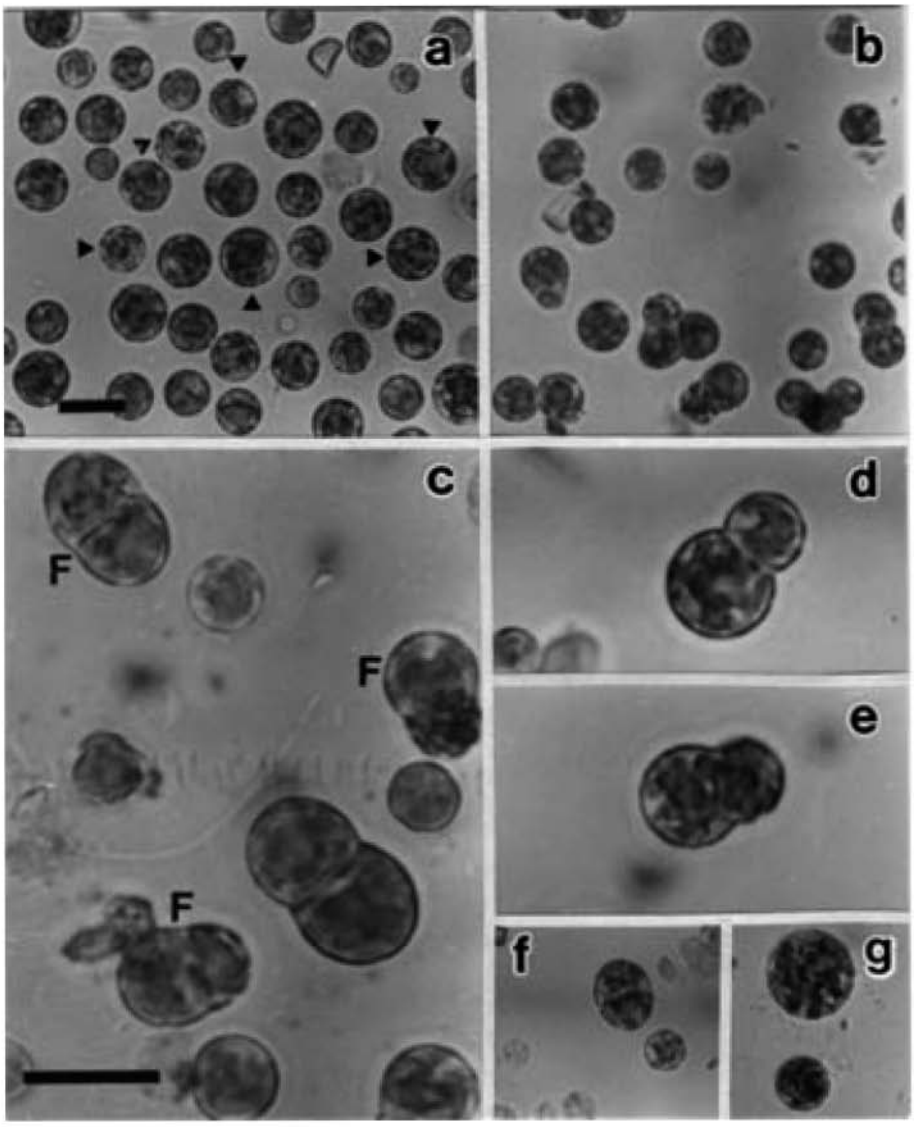


**Plate Ⅰ　Freshly isolated protoplasts and protoplast fusion in *P. haitanensis***

a. Mixture of the red and wild type protoplasts. Arrows indicate the red protoplasts.

b. Aggregated protoplasts 20 min after addition of PEG solution to the mixture of protoplasts.

c. Fusion cells (F) 10 min after addition of the washing solution to the protoplast-PEG preparation.

d–g. Progressive stages of fusion between one red and one wild protoplasts, 10, 20, 25, 35 and 50 min, respectively, after addition of the washing solution to the protoplast–PEG preparation.

a, b and f, at the same magnification. c, d, e and g, at the magnification. Scale bars, 25μm in a, and 20μm in c.

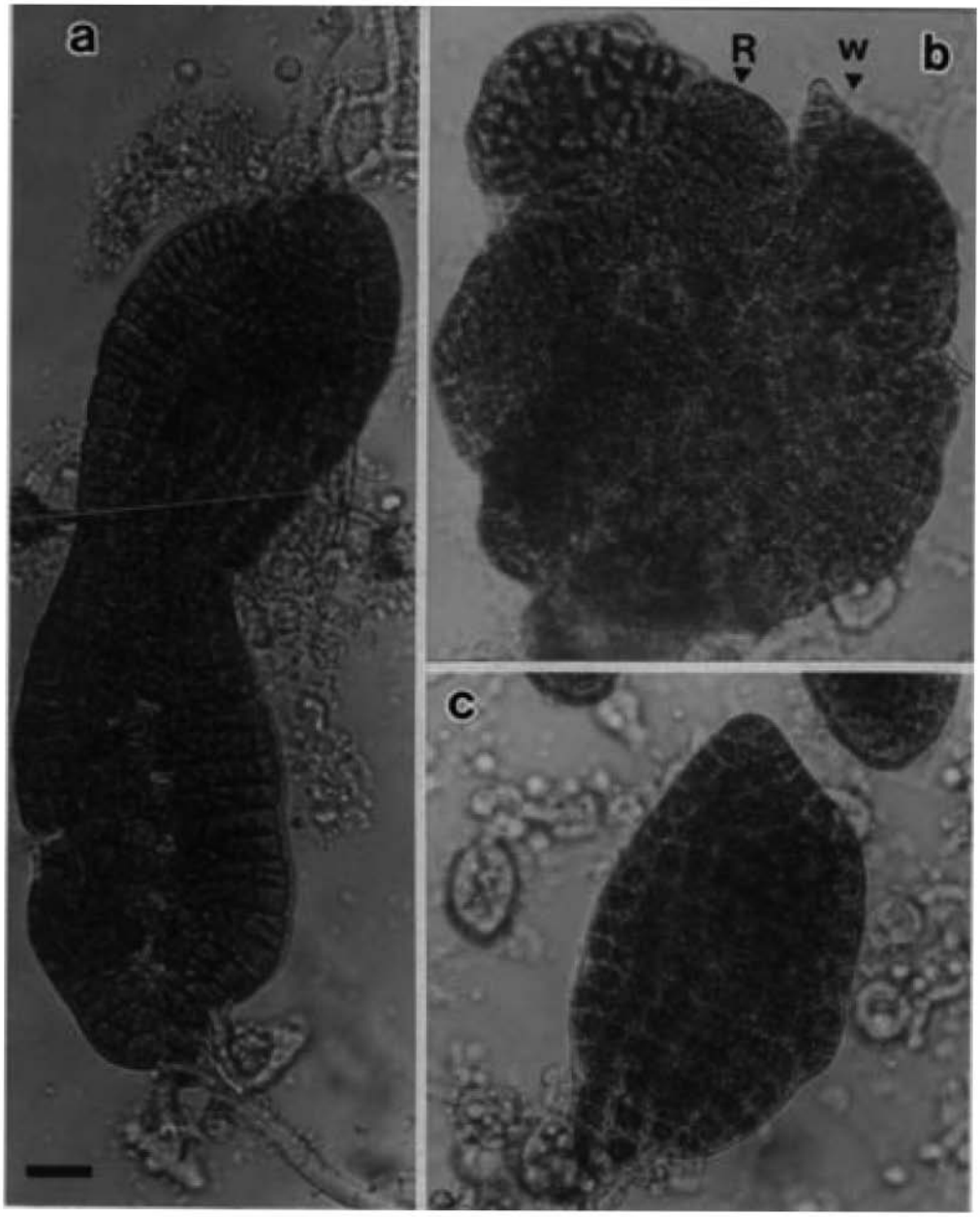


**Plate Ⅱ Regenerating blade from fusion product in *P. haitanensis***

a. The regenerated blade (age, 30 days) with two rhizoids at both ends of the blade, which developed from a fusion product involving two red protoplasts

b. The regenerated blade (age, 36 days) composed of two sectors in red(R) and wild type(W) color, which developed from a fusion product involving one red and one wild type protoplasts

c. A red blade (age, 25 days) regenerated from a protoplast of the blade showed in Plate Ⅱ a.

a—c, at the same magnification. Scale bar, 25 μm in a.

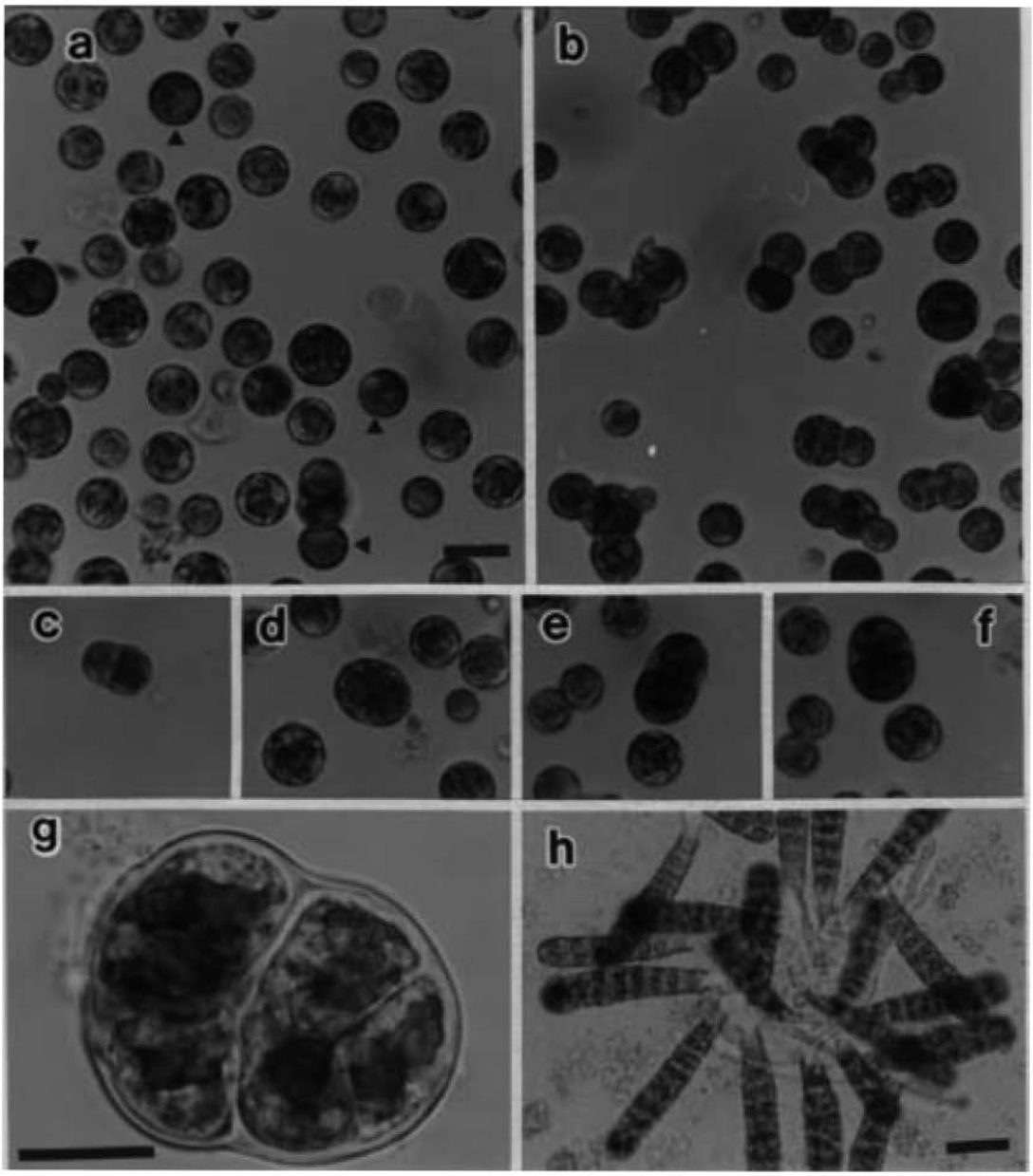


**Plate Ⅲ　Freshly isolated protoplasts and protoplast fusion in *P. yezoensis***

a. Mixture of the neutral-red-stained and the wild type protoplasts. Arrows indicate the neutral-red-stained protoplasts.

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

c – d. Progressive stages of fusion between one neutral-red-stained and one wild type protoplasts, 15 and 45 min respectively, after addition of the washing solution to the protoplast-PEG preparation.

e – f. Progressive stages of fusion between two neutral-red-stained protoplasts, 30 and 50 min respectively, after fusion of the washing solution to the protoplast-PEG preparation.

g. A cell-mass (age, 18 days) developed from a fusion product involving two neutral-red-stained protoplasts.

h. Young blades (age, 15 days) developed from the spores released by the blade showed in Plate Ⅲ g.

a – f, at the same magnification. Scale bars, 25 μm in a, 20 μm in g, 50 μm in h.