スサビノリ(紅藻)プロトプラストの生残率及び生長に及ぼす紫外線の影響

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Effect of Ultraviolet Irradiation on the Survival Rate and Growth of Protoplasts of *Porphyra yezoensis* (Rhodophyta)

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**Abstract:** The effects of ultraviolet (UV) light on the survival rate and growth of protoplasts of *Porphyra yezoensis* (laver) were examined. Although UV (UV-C, 254 nm) irradiation showed strong cytotoxic effects on the protoplasts, we obtained eight surviving protoplasts from approximately 5 × 10⁶ ones under the irradiation conditions of 130 W/cm² for 7 min. Five of them developed to thalli with higher growth rates than those of the UV untreated protoplasts. These thalli consisted of extremely smaller cells (< 5 μm) than those in normal thalli (> 10 μm), and showed different characteristics in shape and color from those of normal thalli. The RAPD analysis of total DNA showed that the electrophoretic patterns of the amplified DNA were slightly different between DNA of thalli developing respectively from UV treated and UV untreated protoplasts. These results suggest that the UV irradiation to protoplasts could bring about genetic variation with characteristics different from those of normal thalli.

**Key words:** *Porphyra yezoensis*; UV irradiation; Mutation; RAPD analysis

*Prophyra yezoensis*, 'laver', is an economically important marine red alga and is extensively cultured along the coastal regions of Eastern Asia, especially in the nearshore bay areas in Japan. One of the important subjects on the culture of this alga is how to produce economically useful cultivars and improve these cultivars for stable production. For this purpose, many efforts have been made during about last three decades to obtain prominent cultivars in growth, color, shape and disease resistance by the selection of spontaneously growing variants in the culture fields\(^1\). In addition, recent techniques on protoplast regeneration and cell fusion\(^2,3\) have been applied to the breeding of this alga.

A vast amount of studies have reported on the effects of ultraviolet (UV) irradiation on plants. It is well known to date that UV plays a significant role in mutagenesis, growth, productivity and morphogenic characters of plants, and the main effect of these UV irradiation is the dimerization of DNA bases, leading to the formation of such perimidine dimmers as TT, CT, TC and CC\(^4,5\)). Mutation is one of the important methods for breeding of many plants and germicidal UV-C (200-280 nm) has been utilized as a tool of UV irradiation and potential mutagen of these plants\(^6,7\). For agricultural plants, many cultivars and variants have been produced by the application of UV irradiation\(^8\). However, except for several reports\(^9,10\), little information on the use of UV irradiation have been available for marine alga breeding. In this paper, we describe the production of high growth rate thalli of *P. yezoensis* by UV irradiation and discuss the usefulness of UV irradiation as a tool for seaweed breeding.
Materials and Methods

Alga
The culture strain of Porphyra yezoensis, Saga-5 used in this study was procured from the Saga Prefectural Ariake Fisheries Research and Development Center (Saga, Japan). The alga was cultured in sea farms in Ariake Bay, Japan. When it grew about 3 cm in length, it was harvested, sun-dried for about 3 h and then stored at -20°C until used.

Isolation and culture of protoplasts
The frozen thalli were incubated overnight in sea water at 15°C in the dark. Thalli about 3 cm in length were selected and used for the protoplast isolation. The detailed procedures of the protoplast isolation have been described previously[10]. In brief, thalli were treated first with papain and then with wall-lytic enzymes until protoplasts were produced. The produced protoplasts were purified by passing them through a 10µm mesh nylon gauze, and the resultant suspension was adjusted to 1-2 x 10^6/ml with 0.8 M mannitol solution. 50µl of the protoplast suspension was mixed with 1 ml of prewarmed (35°C) agarose medium which consisted of 0.8% low melting point agarose in SWM medium (top layer). The protoplast agarose mixture was poured and spread quickly over the surface of a flat, preset surface of 1.1 cm agarose in SWM medium (bottom layer) in a 9 cm diameter petri dish. Both the top and bottom layers were sterilized and contained streptomycin and penicillin G (each 50 µg/ml).

UV irradiation and culture of thalli
The frozen thalli were incubated overnight in sea water at 15°C. These thalli were then transferred to sterilized plastic dish (5 thalli in a dish), placed on an automatic turn table 52 cm below a 15 W germicidal light (Toshiba GL15, 254 nm, Toshiba Inc., Japan) in a clean-bench which provided a UV intensity of 130 µW/cm² and irradiated with the UV light for 4, 8, 12 and 16 min. During the irradiation, the dish was turned at 4 rpm/min. After the irradiation, the thalli were transferred to liquid SWM medium in culture bottle and incubated for 10 days at 15°C under a day-night control (light: dark =11h:13h) with 25 to 40 µmol/m²/s with continuous aeration in a photochamber.

UV irradiation of protoplasts
The protoplasts in the top layer of agarose plate were placed on an automatic turn table and irradiated with the UV light for 2, 4 and 7 min in the same manner as those described above for thalli. After the irradiation, they were incubated at 15°C in a photochamber. After 4-6 weeks of incubation, the multi-cellular bodies which developed from the UV-treated and untreated protoplasts in the top layer were picked up from agarose plates as described previously[10], transferred to liquid SWM medium in 100 ml flasks and incubated at 15°C with continuous aeration.

DNA extraction and RAPD analysis
DNA was extracted from the thalli according to the CTAB method described previously[20]. Thalli were frozen, powdered in liquid nitrogen and mixed with 5 ml of CTAB solution (3% cetyl trimethyl ammonium bromide, 1.4 M NaCl, 20 mM EDTA, 10 mM Tris-HCl and 0.2% -mercaptoethanol, pH 7.5). This mixture was incubated at 65°C for 10 min and then at 40°C for 30 min with occasional swirling. An equal volume of phenol/chloroform (1:1) was added to the mixture and gently shaken for 10 min. After centrifugation at 3000 rpm for 10 min, the DNA in the aqueous phase was precipitated with isopropanol, pelleted by centrifugation, washed in 80% ethanol and dissolved in 10 mM Tris-HCl - 1 mM EDTA (pH 7.5). The DNA was then purified by CsCl gradient centrifugation. Ten-base oligonucleotide primers purchased from Operon Technologies (Alameda, California, USA) were used for PCR reactions as described previously[31]. The sequences of these primers are as follows: A-12, TCGGGTATAG; B-1, GTTTCCGCTCC. The reaction components were 0.2 mM of dNTP, 0.2 mM primer, 1 unit of Taq polymerase and 2.5 ng of genomic DNA in Taq polymerase buffer.
Amplification was performed in a Perkin Elmer Cetus DNA thermal cycler and the products were electrophoresed in 1.2% agarose gel.

**Results**

*Effect of UV irradiation on the growth of thalli and on the survival of protoplasts*

In the present study, we used a germicidal lamp as a source of UV. Figure 1 shows that when thalli (approximately 3 cm in length) were irradiated by a germicidal lamp (UV) for 4, 8, 12 and 16 min, the growth rate of them were reduced by approximately 20, 36, 40 and 71%, respectively, compared to normal thalli with S.D. of 1.1-3.7 after 10 days of culture. Figure 2 shows the effects of UV irradiation on the survival rate of the protoplasts under the same conditions with those described above. By the 4 min-irradiation, the survival rate was less than 50% after one week of incubation. In the 7 min-irradiation treatment, a survival rate of only

![Fig. 1. Effects of UV irradiation on the growth of thalli. Thalli were irradiated by UV-lamp for 4, 8, 12 and 16 min. After ten days of culture, the lengths of thalli were measured. Values are indicated mean lengths of 5 thalli, respectively. The vertical bars indicate the standard deviations.](image1)

![Fig. 2. Effects of UV irradiation on the survival rates of protoplasts. The protoplasts were irradiated by UV-lamp for 0 ( ), 2 ( ), 4 ( ) and 7 ( ) min after one hour of plating of them in the agarose medium, and incubated at 15°C. The survival rate was estimated at the appropriate times of the incubation. The points indicate the mean values of three plates. The vertical bars indicate the standard deviations.](image2)

![Fig. 3. Growth of protoplast after UV irradiation. The protoplast was cultured for 2 (A and B) and 4 (C) weeks at 15°C after UV irradiation for 7 min. (B) is the same protoplast with (A), but at a higher magnification. The scale bars indicate 0.5 mm.](image3)
several percent after one week was observed. These results indicate that the growths of thalli and protoplasts are strongly effected by short time (several minutes) UV irradiation ($130 \mu W/cm^2$) and the latter are more sensitive and weak to the UV irradiation than the former.

**Growth of UV treated protoplast**

Eight protoplasts from approximately $5 \times 10^6$ protoplasts were detected as surviving cells after two weeks of irradiation. Figure 3A and 3B show a small callus-like tissue which developed from a UV irradiated protoplast. These callus-like tissues grew gradually to large multicellular bodies which could be detected by the naked eyes as shown in Figure 3C. The shapes of these callus-like tissues and multicellular bodies were very similar with those developed from UV untreated protoplasts. These multicellular bodies were then picked up from agarose plates, transferred to liquid medium and cultured with continuous aeration. Five multicellular bodies grew comparatively larger than the others. Figure 4 shows two typical thalli of them growing in liquid medium from the multicellular bodies as shown in Figure 3C. One of them looked more dark black, slender and twisted, and the other seemed to be more broad, thick and hard than normal thalli. The growth of these regenerated thalli were compared with those of thalli regenerated from UV untreated protoplasts. Figure 5 shows that the growth of the former were considerably promoted in comparison with those of the latter. The growth rate of the former was somewhat higher than that.
of the latter after 27 days of culture. Thus, the growth rate and the morphological characteristics were remarkably different between thalli developed from UV irradiated and untreated protoplasts.

Characteristics of the regenerated thalli from UV irradiated protoplasts.

The surfaces of thalli shown in Figure 4 were observed with a photomicroscope at 27 days of culture. Figure 6 shows that thalli developed from UV untreated protoplasts consisted of comparatively large cells (> 10 μm) which could be clearly distinguished from each other and arranged regularly throughout the thallus, while regenerated thalli from UV treated protoplasts consisted of densely packed and extremely small cells (< 5 μm) which were arranged very irregularly. These differences could be observed throughout the surfaces of thallus. Thus, the size and pattern of cell arrangement were different between these thalli. Differences of nucleotide sequences of the total DNA was examined between the normal and the regenerated thalli developed from UV treated protoplasts by PCR-RAPD analyses. Figure 7 shows that the RAPD patterns of the regenerated thalli developed from the UV treated protoplasts were slightly different from those of normal (cultivar name: Porphyra
Saga-5) thalli from which protoplasts were prepared, while RAPD patterns were very similar among the three individual normal (cultivar Saga-5) thalli developed from individual normal protoplast. These differences of RAPD patterns were detected with 9 primers out of the 30 primers examined.

**Discussion**

In the present study, we used a germicidical lamp as a source of UV. The germicidal lamp emits UV-C (≈ 280 nm) and is known to cause injury, necrosis, mutation and death to plant cells. However, most of these studies have been conducted with land plants and microorganisms, and there is little information on the effect of UV-C on growth and mutation of marine algal protoplasts.

Although almost all of protoplasts died by the 7 min-UV irradiation, we found that the growth of irradiated intact whole thalli (about 3 cm in length) was inhibited only less than 50% of unirradiated control thalli by the 8 min-UV irradiation. Therefore, protoplasts were more sensitive to UV irradiation than intact whole thalli. It has been reported with red algae that some components of the cell wall protect cells against UV-damage\(^{14-16}\). The evidence described above could be interpreted as due to the lack of a cell wall for the protoplasts. From about 5 \(10^6\) UV irradiated protoplasts, we obtained eight thalli in total. Five of these thalli showed higher growth rates than those of thalli developed from UV untreated protoplasts and consisted of extremely small cells which were irregularly arranged throughout the thalli. RAPD analyses showed that the RAPD patterns of the thalli DNA were different between normal and the regenerated thalli from UV treated protoplasts. This evidence suggests that the UV irradiation to the protoplasts brought about a mutation.

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*Fig. 7. Comparison of RAPD patterns of total DNA between thalli developed from normal and UV irradiated protoplasts.*

1 to 3: DNA from 3 individual thalli developed from normal protoplasts. 4: DNA from thallus developed from a UV-irradiated protoplast. A: A-12 primer, B: B-1 primer. Size markers on the left are given in Kb.
in the protoplast genome and the regenerated thalli developed from these protoplasts were genetic variants affected by the changes in DNA base sequences, and showing different morphological and growth characteristics.

We could not obtain conchocelis from the thalli which developed from UV irradiated protoplasts. These thalli died before the formation of conchocelis at their margins. The genes or gene expressions which are concerned with the carpospore formation and maturation might have been damaged by the UV irradiation. Because of this, the inheritance of the nucleotide sequences and morphogenetic characteristics of these thalli could not be examined using the products of fertilization. It is interesting to determine which and how many genes were affected mutagenesis by such UV irradiation and which genes were related to the growth rate, shape and color of the thalli.

Infertile mutant alga is not available for the direct use in commercial farm-culture. Therefore, the selection and breeding of fertile variants is hereafter necessary prior to any commercial use and precise analysis of the mutation.

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References

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紫外線ランプ（UV-C）を用いてプロトプラストの生残率及び生長に及ぼす紫外線（UV）の影響を調べた。130 W/cm² 照射下では、照射時間が増すに従ってプロトプラストの生残率が低下した。7 分間照射では、大部分のプロトプラストが2 週間以内に死滅したが、約5 ×10⁶個のうち8 個のプロトプラストの生残と葉体への生長が観察された。生長した8 枚の葉体のうち5 枚は、正常の葉体に比べ、高い生長率及び異なる色や形態を示した。これらの葉体は、葉体全体が不規則に並んだ小さな（＜5 µm）細胞からなり、また、全 DNA の RAPD 解析においても正常葉体のそれらと異なる RAPD パターンを示した。これらの結果から、プロトプラストへの UV 照射は、プロトプラストへ遺伝的変異をもたらし、再分化した葉体の生長や形態へ影響を与ええたものと推察された。