Intrageneric fusions of isolated protoplasts from *Ulva* and *Porphyra* by electrofusion method

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Isolated algal protoplasts of 1. *Ulva pertusa* (sterile mutant) and *U. conglobata*, 2. *Porphyra yezoensis* normal and *P. yezoensis* green type, were electrically fused. The protoplasts from each alga were mixed together with its algal partner in a 1 : 1 ratio in low conductivity electrofusion solution at a density of $1\times10^{5-6}$ cells/*ml*. Protoplasts were aligned into short chains in high frequency (1 MHz) alternate current (AC) field and sudsequently fused by the application of a single short duration direct current (DC) pulse. Protoplasts aligned at 200 V for 10 s and 40 V for 20 s yielded maximum pairs about 25 and 40% in *Ulva* and *Porphyra* respectively. The application of 20-25 μ s duration DC pulse of 200 V resulted optimum binary fusion percentages about 12% in *Ulva* and whereas 250 V of 40 μ s duration yielded maximum fusions about 16% in *Porphyra*. The application of a high intensity DC pulse (> 300 V of 30 μ s duration for *Ulva* and > 350 V of 40 μ s duration for *Porphyra*) to the aligned protoplasts induced protoplast lysis.

Key words: electrofusion, protoplasts, *Ulva, Porphyra*

Introduction

Somatic hybridization in higher plants has been accomplished through protoplast fusion to develop new plants with greater genetic diversity[1]. A number of fusion methods have recently been described for inducing protoplast fusions in higher plants[2-4]. Among all fusion methods, polyethylene glycol (PEG) mediated fusion[5] has been widely applied for accomplishing protoplast fusion in higher plants[6]. There are several reports have recently been published on protoplast fusion of algae by PEG method[7-9]. However all fusion methods are non specific and have either variable fusion frequencies or caused cytotoxic effects to the treated protoplasts. Therefore an electrofusion technique which has been successfully used to fuse protoplasts of higher plants[10-12] was employed to fuse algal protoplasts. This study essentially investigates the suitable electrical conditions required for inducing binucleate heterokaryons between the protoplasts of *Ulva pertusa* Kjellm. with *U. conglobata* Kjellm. and between *Porphyra yezoensis* Ueda normal with *P. yezoensis* green type.

Meterials and Methods

Vegetative thalli: Young clean vegetative thalli of *U. pertusa* (sterile mutant)[13], *U. conglobata, P. yezoensis* normal and green type were used for the isolation of protoplasts. All the above mentioned plants are being grown as unialgal cultures in our laboratory.

Isolation of protoplasts: Protoplasts from *U. pertusa* and *U. conglobata* were separately produced by incubating the thallus (about 25 mg

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fresh wt.) in 5% cellulase R-10 and 2% abalone crude enzyme powder[14]. The isolated protoplasts from both the species were further incubated in 1% protease P6 (Amano pharmacy Co., Japan) enzyme prepared after Fujita and Saito[15] for about 30 min in dark prior to start of the electrofusion. Similarly protoplasts from *P. yezoensis* normal and green type were prepared following the methods of Fujita and Saito[15].

Electrofusion: Protoplasts of *U. pertusa* with *U. conglobata* and *P. yezoensis* normal with green type were subjected to electrofusion at 20°C using a Shimadzu somatic hybridizer SSH-2 (Shimadzu Co., Japan). Protoplasts from each fusion partner were mixed together with its algal partner in 1 : 1 ratio in the electrofusion solution (0.2 mM tris (hydroxymethyl) aminomethane, 1.0 mM $CaCl_2 \cdot 2H_2O$ and 1.0 mM $MgCl_2 \cdot 6H_2O$ and 0.9 M mannitol (0.7 M for *Porphyra*) in distilled water, adjusted to pH 7.5) at a density of $1.0 \times 10^{5-6}$ cells/m*l*. Aliquots of 200 μl protoplast suspension of the two fusion partners were placed between the two electrodes (1 mm spacing) in a fusion chamber (FTC-02 of Shimadzu Co.) and allowed to settle for few minutes prior to the start of the electrofusion. Protoplasts were initially aligned into short chains preferably pairs in alternate current (AC) field and subsequently fused by the application of a single short duration direct current (DC) pulse. To investigate the necessary AC and DC fields required for induction of protoplast alignment and fusions, protoplast suspension of each combination were initially aligned in an AC field (1 MHz) at different voltages ranging from 10 to 40 V for different durations ranging from 10 to 25 s to find out the appropriate voltage and length of electric fields necessary for establishing protoplast pairs. Similarly pulse voltage (100 -350V) and pulse width (10-60μs) have also been calibrated to obtain optimum fusion frequencies of binucleate fusion products, in order to facilitate easy regeneration and subsequent genetic analysis of regenerated plants. Generally, five random microscopic fields (each with about 100-150 cells) were counted for every fusion event to caliculate the rate of protoplast alignment and fusions.

The rate of protoplast alignment and fusion were caliculated as follows and expressed as a percentage. Total alignment rate = (total number of protoplasts involved in alignment into chains) / (total number of protoplasts) × 100. Total fusion frequency = (total number of protoplasts involved in binary and multi (>3 cells) fusion products) / (total number of protoplasts involved in fusion event + number of unfused cells) × 100. Binary fusion products = (total number of protoplasts involved in binary fusions) / (total number of protoplasts involved in binary fusions + number of unfused cells) × 100.

Results

Electrofusion of *Ulva* and *Porphyra* protoplasts was performed in two steps. In the first step protoplast adhesion with adjacent protoplast (Figs. 1A, C) was generated by dielectrophoresis in an AC field at 1 MHz. The results of alignment rate of protoplasts as a function of the interaction of alignment voltage and time are shown in Table 1 for *Ulva* and *Porphyra* respectively. The number of protoplasts in chain increased with alignment voltage and time. The application of low AC fields (20V) for shorter duration (10s) yielded high percentage of (about 25%) of paired protoplasts (Fig. 1B) in *Ulva*. Maximum percentage (about 40%) of paired protoplasts (Fig. 1D) in *Porphyra* were obtained at higher AC field (40V) and longer duration (20s). The prolonged exposure (>25s) to above mentioned respective AC fields induced long protoplast chains (Figs. 1A, C) in both *Ulva* and *Porphyra*. Though the percentage of total aligned protoplasts and multi-protoplast chains increased with AC field strength and time, but the latter one however reduced the pairing protoplasts number (Table 1) than the former one.

The second step was induction of protoplast fusion by the application of a single high intensity DC rectangular pulse of microsecond duration.

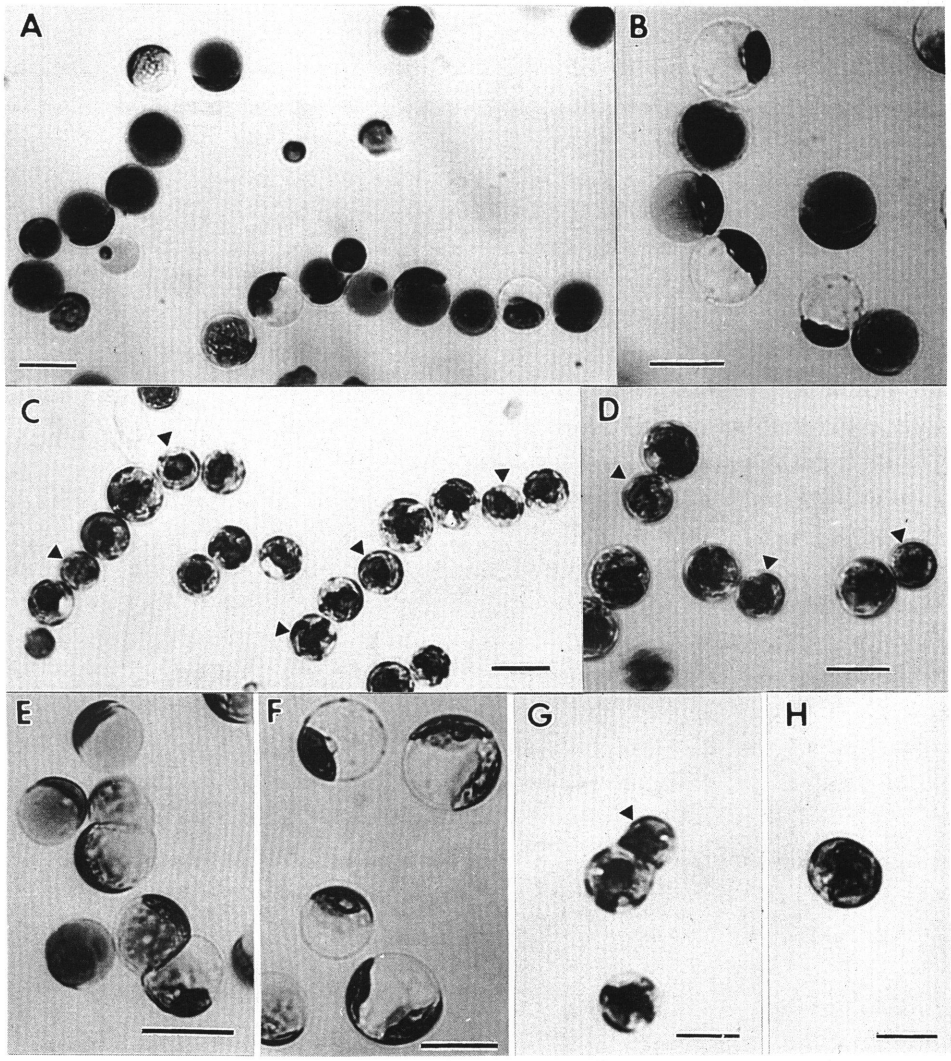

Fig. 1. Intrageneric electrofusion of *Ulva* and *Porphyra* protoplasts. A: Protoplast alignment into long chains of *U. pertusa* (stained with neutral red) and *U. conglobata*, exposed to long durations to high frequency AC fields at 1 MHz, 20 V for 25 s. B: Induction of protoplast pairs of *U. pertusa* (stained with neutral red) and *U. conglobata*, exposed to short durations to AC field at 1 MHz, 20 V for 10 s. C: Protoplast alignment into long chains of *P. yezoensis* normal and green (with arrows) type, exposed to long durations to high frequency AC fields at 1 MHz, 40 V for 25 s. D: Induction of protoplasts pairs of *P. yezoensis* normal and green (with arrows) type, exposed to short durations to AC fields at 1 MHz, 40 V for 20 s. E: Protoplast fusion of *U. pertusa* with *U. conglobata* soon after application of a single DC pulse of 200 V for 20 μ s duration. F: Round heterokaryons of *Ulva*, 3 minutes after application of DC pulse. G: Protoplast fusion of *P. yezoensis* normal with green soon after application of a single DC pulse of 250 V for 40 μ s duration. H: Round heterokaryons of *Porphyra*, 5 minutes after application of DC pulse.
Bar in all figures is 20 μm.

The yield of fusion products as a result of the interaction of pulse voltage and pulse width are shown in Fig. 2 A & B for *Ulva* and *Porphyra* respectively. The fusion process in *Ulva* protoplasts was initiated by the application of a DC pulse of >150 V of 15 μ s duration (Fig. 1E). The delivery of a short duration (20-25μ s) DC pulse of 200 V to aligned protoplasts in *Ulva* resulted optimum binary fusions about 12% (Fig. 1F), and whereas 250 V of 40 μ s duration yielded optimum binary fusions about 16% in *Porphyra* (Figs. 1G, H). Although percentage of heterokaryons were not determined, but 40-50% of total fusion products were found to be heterokaryons. However the application of a high intensity DC pulse (> 300V of 30μ s duration for *Ulva* >350 V of 40μs and duration for *Porphyra*) to aligned protoplasts induced protoplast lysis.

Discussion

Electrofusion has been developed to an efficient and routine technique to fuse both animal cells and plant protoplasts[16,17]. Though in one instance electrofusion of *Enteromorpha* protoplasts has been reported[18], however there are no detailed studies on optimizing the electrical conditions for obtaining high fusion frequencies of viable fusion products in algal protoplasts. Protoplasts from normal *P. yezoensis* were previously fused with green type following the PEG

Table 1. Effect of AC voltage and AC voltage applied time on protoplast induction into pairs in *Ulva* and *Porphyra*

Parameters	% protoplasts involved in pairs	
	Protoplast suspension of	
	Ulva	*Porphyra*
Voltage (V)[1]		
10	19 (33)*	0 (0)*
20	25 (44)	32 (68)
30	15 (50)	36 (80)
40	10 (47)	40 (98)
Time (s)[2]		
10	25 (44)	18 (44)
15	16 (48)	35 (90)
20	10 (52)	40 (95)
25	9 (60)	28 (97)

Protplasts of *Ulva* and *Porphyra* were constantly exposed 1) to AC fields for 10 and 20s and 2) to AC voltages 20 and 40 V respectively.
* : % total protoplasts involed in long and short (pairs) chains.

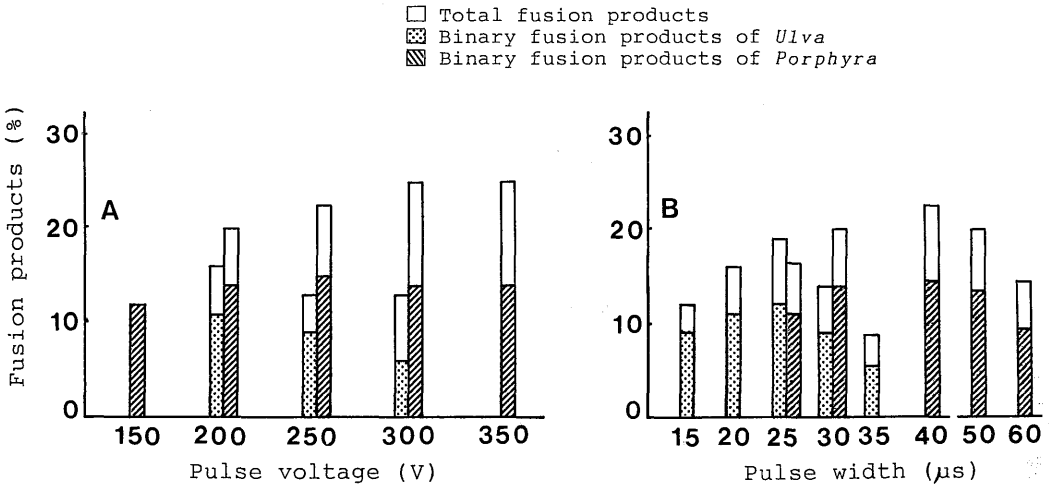

Fig. 2. Effect of pulse voltage (A) and pulse width (B) on fusion frequencies of prealigned protoplasts of *Ulva* and *Porphyra*. Prior to fusion pulse, protoplasts of both *Ulva* and *Porphyra* were aligned to pairs by applying 20 and 40 V AC fields for 10 and 20 s respectively. Pulse width in (A) for *Ulva* is 20 μ s and for *Porphyra* is 40μ s, similarly pulse votage in (B) is 200 V for *Ulva* and 250 V for *Porphyra*.

method[8]. Later protoplasts from several species of *Porphyra* were electrically fused and high fusion frequencies were reported. The regeneration rate of post fusion products was also higher than with the PEG method[15]. Although PEG induced fusions to occur in *U. pertusa* with *U. conglobata*, it did not yield satisfactory fusion frequencies and viable fusion products[19]. Consequently electrofusion has been performed as an alternative to the PEG method. Protoplasts of *U. pertusa* with *U. conglobata* and *P. yezoensis* normal with green type were electrically fused by a combined approach of 1. cell adhesion by AC fields and 2. subsequent cell fusion by DC pulses. The fusion medium prepared in seawater with mannitol did not induce protoplast fusion due to high conductivity. The subsequent preparation of protoplast suspension in low conductivity medium prepared in distilled water however induced electrofusions in both cases. The protoplast alignment and fusions occurred at lower field strength in *Ulva* than in *Porphyra*. However the alignment rate and fusion percentages in *Porphyra* were greater than *Ulva*. The rate of cell alignment is usually attributed to both the magnitude of the electric field and ionic strength of the fusion medium. The former is however dependent on the radius of the cell (the smaller the cell, the larger the electric field that must be applied to achieve alignment). The induction of protoplast alignment and fusion at lower voltages in *Ulva* despite the same cell size and fusion medium (except mannitol concentration) might be due to the differences between protoplast membranes (composition and structure) of *Ulva* and *Porphyra*. Secondly the protease treatment of *Ulva* protoplasts prior to the fusion might have resulted in the induction of alignment and fusion at lower voltages. It is assumed that the protease treatment prior to fusion, enhances the fusion ability of protoplasts by removing the surface glycoproteins[20,21] or generation of fusogenic polypeptides[22] on membrane component. However the electrofusion frequencies in *P. yezoensis* normal with *P. pseudolinearis* varied with $CaCl_2$ and $MgCl_2$ concentration in the fusion solution[23]. Electrofusion in *Ulva* was completely blocked by increasing the $CaCl_2$ and $MgCl_2$ concentration to 3 mM in the fusion medium. Similarly fusion medium without $CaCl_2$ also limited the electrofusion (<1%) in *Ulva*. Therefore it is essential to investigate the right concentrations of $CaCl_2$ needed for inducing high fusion rates. The frequency of protoplast fusion by PEG method in higher plants has been reported to vary with the nature (i. e. ultra structure) of the protoplasts[24] and fusion conditions. Unlike the *Porphyra* protoplasts *Ulva* protoplasts with their big vacuoles might have less tendency to involve in fusion events. Thus the fusion percentage of *Ulva* protoplasts is comparatively less than the *Porphyra*. The regeneration and development of heterokaryons, following the electrofusion method were earlier reported for *Porphyra*[15] and *Ulva*[19]. Thus this study demonstrates the suitability of electrofusion methods for fusing algal protoplasts as in higher plants.

Acknowledgements

This work was partially supported by a grant in Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan. We are indebted to Prof A. Gibor, University of California for critically reading and revising the manuscript. We are also thankful to Mrs. Jhansi Lakshmi for skilfully typing this manuscript on word processor.

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電気刺激法によるアオサとアマノリのプロトプラストの属内融合

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1．アナアオサ *Ulva pertusa*（不稔型）とボタンアオサ *U. conglobata*，2．スサビノリ *Porphyra yezoensis* の野生型と緑色変異型との間で，葉体から単離したプロトプラストを電気刺激法によって融合させた。それぞれの葉体から単離したプロトプラストは、低導電性の融合緩衝液で洗浄後 $1\times10^{5-6}$ cells/ml に調製し，1：1の割合で混合した。融合チャンバーに滴下したプロトプラスト混合懸濁液に高周波電圧（AC）を印加することによってプロトプラストチェーンが形成され，パルス電圧（DC）の印加により融合が開始された。アオサではAC200 V，10 s の印加，スサビノリでは40 V，20 s の印加により，それぞれ最大25%および40%のプロトプラスト対が形成された。そしてアオサではDC200V，20～25μs の印加で最大約12%，スサビノリでは250 V，40μs の印加で約16%の融合率が示された。またアオサでは300 V，30 μs，スサビノリでは350 V，40μs 以上の印加によって細胞の破壊が生じた。

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