Jpn. J. Phycol. (Sôrui) 41: 231–236, September 20, 1993

Use of fluorescent staining to monitor the temporal pattern of cell wall resynthesis in *Ulva fasciata* (Ulvales, Chlorophyta) protoplasts

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Chen, Y. C. and Chen, C. S. 1993. Use of fluorescent staining to monitor the temporal pattern of cell wall resynthesis in *Ulva fasciata* (Chlorophyta: Ulvales, Ulvaceae) protoplasts. Jpn. J. Phycol. 41: 231–236.

Fluorescent brightener agent (FBA) was used to continuously check the development of new cell walls of an *Ulva fasciata* protoplast population. Cell wall resynthesis began within 6 hrs after the isolation of protoplasts. Maximum cell wall formation was reached at the 8th day of incubation, when about 81% of the protoplasts had formed new cell walls. Resynthesis of cell walls was delayed when they were stained earlier than 4 hrs after isolation. However, after 24 hrs, the influence was small.

Key Index Words: Chlorophyta—fluorescent brightener agent—new cell wall—protoplasts—stain—Ulva fasciata.

Algal protoplasts have considerable potential for use in physiological investigations. Practical applications are varied. Protoplasts can be subjected to genetic modification, used for mass production of protoplast-fusion hybrids (Saga *et al.* 1986) or for establishment of cell suspensions (Chen, L. C-M. 1989). However, since they lack a cell wall, protoplasts are fragile, and in culture they are particularly susceptible to changes in the osmotic concentration of the medium. Thus, protoplasts regenerate only when incubated in a medium with suitable and well-regulated osmotic oncentration (Ahuia 1982; Evans and Bravo 1983; Chen, L. C-M. 1989). Also, when protoplasts form new cell walls, they must be transferred from the hyperosmotic medium and incubated in enriched seawater (i.e. Provasoli 1968). This counteracts the increase in turgor pressure resulting from the regeneration of the cell walls (Kirst and Bission 1979, Berliner 1981). In a previous study of the green alga *Ulva fasciata* (Chen and Chen 1991). It was found that protoplasts grow best at hyperosmotic concentrations, but that the osmotic concentration shoud be gradually decreased as cell-wall resynthesis occurs to obtain a high number of regenerated protoplasts (Chen, Y. C. 1989). However, neither the optimal time to begin decreasing the osmolarity of the medium nor the rate at which the reduction should occur are known and no doubt differ with taxon and culture conditions. If there was a way to identify those protoplasts that had already formed cell walls, and these protoplasts were directly transferred to enriched seawater, then it might be easier to obtain a high yield of regenerated protoplasts. This paper investigates one possible method.

FBA was used to follow the course of resynthesis of cell wall in protoplast cultures. This agent specifically binds with cell wall materials (Maeda and Ishida 1967). Fluorescent staining, to distinguish whether or not the protoplasts have regenerated new cell walls, can be used to address these problems in batch culture to which FBA has been added. Fluorescent staining has often been used to observe the biosynthesis of cell walls with an electron microscope (Berliner *et al.* 1978, Haigler and

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Brown 1980, Herth 1980, Galbraith 1981, Itoh *et al.* 1984), and to distinguish whether or not protoplasts have retained remnants of the mother-cell walls. However, they have rarely been used to follow, *in vivo*, the development of the new walls of protoplasts during incubation.

The purpose of this paper is to describe a method of fluorescent staining which can be used with protoplasts of *Ulva fasciata* and similar Chlorophyta to determine the temporal pattern of cell wall resynthesis in a process of culture procedure, with less negative side effects.

Materials and Methods

Fronds of the marine macroalgae *Ulva fasciata* Delile were collected at Keelung, Taiwan on May 5, 1989. Immediately after the collection, plants were washed with autoclaved seawater several times, and transported to the laboratory.

Protoplasts were obtained by digestion of the cell walls with enzymes as decribed by Chen and Chen (1991). Selected pieces of healthy fronds (approx. 2 cm^2) were thoroughly cleaned in filtered seawater. Then they were incubated for 24 hrs in 100 ml of autoclaved Provasoli's enriched seawater (PES) medium (Provasoli 1968) containing 10 ml of antiboitic mixture (Polne-Fuller and Gibor 1987). The culture room was 24°C and had a 12 : $\overline{12}$ L : D regime with irradiation of 20 $\mu Em^{-2}s^{-1}$. Fronds of *U. fasciata* were cut to 0.5–1 mm square pieces on a clean bench with a sterile knife blade. To obtain protoplasts 0.1 g of pieces was incubated on a rotary shaker (50 rpm) for 12 hrs in darkness at 24 °C, in 10 ml of sorbitol-enzyme solution (1.2 M sorbitol, 4% cellulase, Onozuka R-10, 2% marcerozyme, Onozuka R-10). Then the protoplast-enzyme suspension was layered onto the top of a 35% (w/v) density buffer (Ficoll-400, Sigma) solution, and centrifuged at 200 × g (or 1200 rpm, HERMLEZ 320) for 30 min, to remove detritus. Protoplasts were separated (purified) from the interface between the density buffer and the enzyme solution with a sterile Pasteur pipette. Purified protoplasts of *U. fasciata* were cultured in mannitol-PES medium. Osmotic concentration was adjusted to 0.84 M mannitol. Fluorescent brightening agent (FBA) (Calcofluor White ST, Sigma) was used to stain the new cell wall of protoplasts.

One-tenth of a ml of stock 1% FBA per 10 ml of culture medium was added to six cultures of purified protoplasts at 0, 2, 4, 8, 12 and 24 hrs after isolation. After staining, protoplasts with resynthesized cell walls appear yellow or green when viewed with a fluorescent microscope. The protoplasts without cell walls appear red. Changes in the number of fluorescent protoplasts in each culture were monitored continuously until the 8th day of incubation. At each monitoring period, five fields (ca. 100–500 cells in a field) were randomly sampled from each of the six cultures. In addition, another seven cultures of purified protoplasts were incubated in FBA-free media until stained with 0.01% FBA at days 2, 3, 4, 5, 6, 7, and 8 after isolation, and the average percentages with new cell walls were determined immediately. These groups provide comparative information to determine whether or not FBA influences the process of cell wall formation. They are considered to be control groups. An inverted fluorescent microscope (Nikon, Diaphot-TMD with TMD-EF) was used to examine protoplasts for cell wall synthesis. Except for protoplast isolation all laboratory procedures were carried out under 12 : $\overline{12}$ L : D regime and irradiation of 166 $\mu Em^{-2}s^{-1}$ at 24°C in a culture room.

Results and Discussion

Newly synthesized cell walls of algal protoplast are difficult to observe under a normal optical microscope. Fluorescent complexes, resulting from the binding of FBA with amorphous cellulose, facilitate the observation of the cell wall resynthesis. With FBA as the staining agent, protoplasts with new cell walls exhibit either green or yellow fluorescence, depending on the stage of formation of the cell

Table 1. Percentage of appearance of new cell wall in FBA-stained *U. fasciata* protoplasts at hrs 0, 2, 4, 8, 12 and 24 after isolation.

Age of protoplasts (hrs)	Percentage* of protoplasts with new cell walls					
	Hours after isolation for protoplasts staining					
	0 hr	2 hrs	4 hrs	8 hrs	12 hrs	24 hrs
6		0.46	0.48			
8	0.29	0.62	0.66	0.50		
10	0.38	0.94	1.16	1.56		
12	0.48	1	1.48	1.65	0.62	
14	0.57	1.06	1.53	1.85	0.82	
16	0.67	0.12	1.57	5.08	1.97	
18	0.76	1.18	1.62	8.13	9.06	
20	0.86	1.23	1.66	11.18	16.46	
24	1.05	1.35	1.75	17.28	20.19	
48	16.89	16	19.34	46.63	49.26	37.91
72	21.97	33.78	39.36	52.92	53.96	47.21
96	28.16	38.21	45.63	59.01	58.83	64.83
120	32.6	42.17	59.09	63.62	63.72	68.34
144	43.01	49.05	62.84	66.82	68.72	73.28
168	49.67	51.27	64.05	68.65	73.49	78.52
192	49.67	51.42	64.24	72.46	74.83	80.85

Culture conditions: 166 $\mu Em^{-2}s^{-1}$, 12 : $\overline{12}$ L : D at 24°C.

*The percentage is expressed as the average value of five randomly selected fields under an inverted microscope with fluorescent equipment.

wall. In protoplast populations of *U. fasciata*, cell wall resynthesis in FBA-enhanced medium does not proceed synchronously, due to the variant physiological conditions of cells.

The resynthesis of cell walls of *U. fasciata* protoplasts that were stained at 2 hrs after isolation, was initiated at hour 6 after isolation (Talbe 1). At this time about 0.5% of the protoplasts had cell walls. This could be due to the remaining old cell walls. However, the percentage of regenerated cell walls increased at hr 8, verifying that the protoplasts had formed new walls. The highest percentage (81%) was reached after 8 days of incubation in the group of protoplasts that was stained after 24 hrs of isolation.

Through eight days of monitoring, the number of green fluorescent protoplasts (those with a cell wall) increased in every experimental group irrespective of the time of staining (Fig. 1). Protoplasts stained at 0, 2 and 4 hrs after isolation took the longest time after staining (ca. 48-44 hrs) for evident appearance (11-16%) of green fluorescent protoplasts. Protoplasts stained at 8, 12 and 24 hrs after isolation took 8, 6 and 3 hrs, respectively. This indicates that there is considerable inhibition of cell wall resynthesis in protoplasts stained earlier than 4 hrs after isolation.

Initiation of cell wall resynthesis in *Boergesenia forbesii* protoplasts occurs within 2 to 3 hrs of incubation (Itoh *et al.* 1986). The temporal differences in resynthesis of cell walls between the studies of *B. forbesii* and this study of *U. fasciata* could reflect the considerable taxonomic differences between the species, or differences in the techniques used to obtain the protoplasts, the osmotic concentration, pH, the physiological status and growth stage of the plant material. Itoh *et al.* (1984, 1986) physically cut the coenocytic plant of *B. forbesii* to obtain protoplasts. However, the protoplasts from *Ulva* which is not coenocytic, were obtained through the digestion of the original cell wall and subsequent centrifugation to separate the protoplasts from the debris. The protoplasts of *Ulva* were, there-

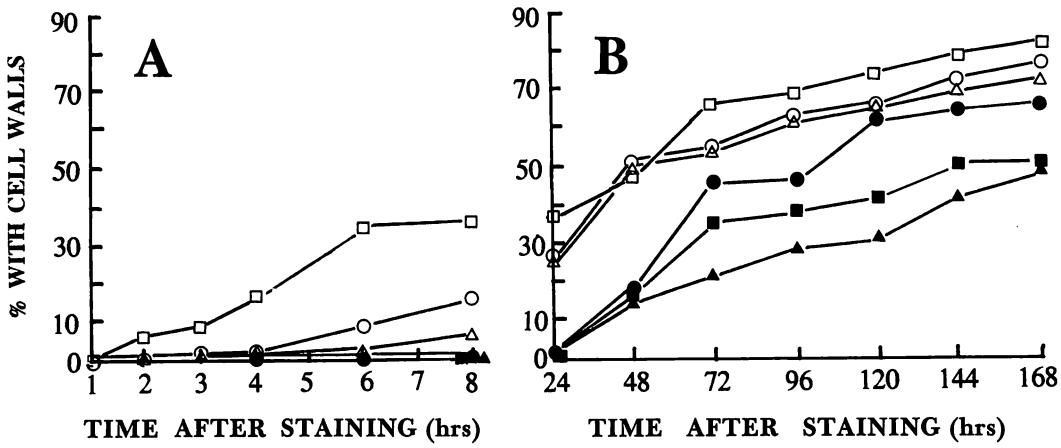

Fig. 1. Appearance of new cell walls of *Ulva fasciata* protoplasts stained with FBA at hrs 0, 2, 4, 8, 12 and 24 after isolation under 166 $\mu Em^{-2}s^{-1}$, 12 : $\overline{12}$ L : D at 24°C conditions (A, early stage; B, late stage). The percentage is expressed as the average value of five randomly sampled fields under a microscope. ▲, protoplasts stained at 0 hr after isolation; ■, stained at 2 hrs; ●, stained at 4 hrs; △, stained at 8 hrs; ○, stained at 12 hrs; □, stained at 24 hrs.

fore, subjected to considerably more stress than were those of *B. forbesii*. Such stresses are known to affect the physiology of the protoplasts (Galun 1981).

Itoh *et al.* (1984) also reported that cell wall synthesis can be negatively affected by FBA in studies of the green alga *B. forbesii*. They found that 95 μM FBA (ca. 0.01%) was the highest concentration which was not toxic to terminal complexes. However, even at these concentrations, FBA disfigured the microfibrils. This presumably also influenced cell wall resynthesis.

Cell wall formation of the protoplasts that were stained at 24 hrs after isolation showed no apparent negative influence. In fact cell wall resynthesis was almost the same between this group and the control groups (Fig. 2). The rates of increase in presence of cell walls are similar, indicating that the delay in cell wall synthesis occurs only in the early phases of resynthesis. Although early staining with FBA delays the resynthesis of cell wall, the method described here has proved faster and less harmful to the protoplasts than other methods, such as those which distinguish the

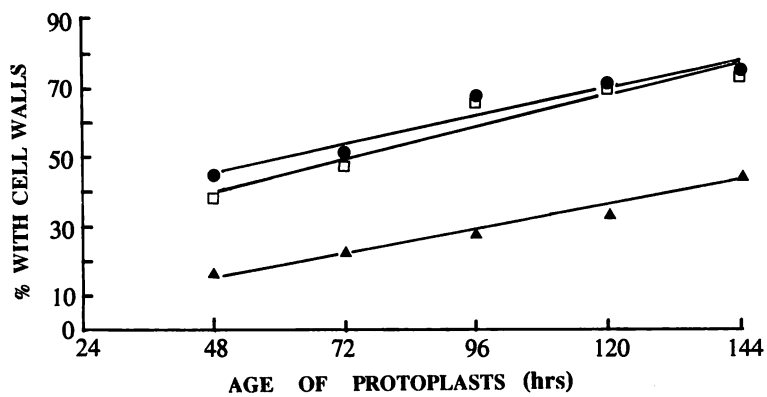

Fig. 2. The relationship between time (hr) and increase rate in protoplast with new cell walls from group 0, 24 and control groups. Group 0 and group 24 were stained at the start of incubation (0 hr) and after 24 hrs of isolation respectively. Control groups were incubated in FBA-free madia until they were stained at days 2, 3, 4, 5 and 6 after isolation. For group 0, $Y=2.29+0.276X$ with $r^2=0.98$, for group 24, $Y=25.07+0.339X$ with $r^2=0.929$, and for control groups, $Y=32.86+0.285X$ with $r^2=0.925$. (▲, Group 0; □, Group 24; ●, Control groups.

new cell walls through the use of electron microscopes (Burgess *et al.* 1978, Itoh *et al.* 1984) and protein-synthesis inhibitors (Itoh *et al.* 1986). The information provided here should encourage use of the FBA staining technique in continuously recording the number (percentage) of protoplasts with new cell walls of *Ulva fasciata* and of other marine macroalgae.

Information on the time of cell wall resynthesis will allow us to transfer protoplasts from a hyperosmotic concentration to normal enriched seawater with optimal timing. This should lead to more efficient propagation of marine algae.

Acknowledgments

Sincere gratitude is extended to Dr. Jane, E. Lewis, Institute of Marine Biology, National Taiwan Ocean University, and Dr. Stephen G. Nelson, Marine Laboratory, University of Guam, for helpful suggestions on the manuscript.

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陳　衍昌・陳　忠信：リボンアオサ（緑色植物門，アオサ目，アオサ科）のプロトプラストの細胞壁再成を経時的に調べるための蛍光染色

リボンアオサのプロトプラストを蛍光染色 (FBA) を含むメディウム中で培養し，細胞壁の再生を経時的に追跡した。細胞壁再生はプロトプラスト単離後 6 時間以内に始まった。細胞壁の形成率は 8 日後に最大となり，約 81％のプロトプラストが細胞壁を再生した。細胞壁の再生は，プロトプラスト単離後 4 時間以内に染色した場合には遅れたが，24時間後に染色したものでは，染色による阻害は小さかった。この方法は，細胞壁再生状態を知るための簡便法として利用できる。(Institute of Aquaculture, National Taiwan Ocean University, Keelung Taiwan, Republic of Chaina)

(*Received November 19, 1992, Accepted June 14, 1993*)