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Isolation, Culture and Thallus Regeneration of Protoplasts from the Hornwort *Anthoceros punctatus* L. Cultured Cells

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Protoplasts, in which regenerated plants were reported for the first time, were isolated from cultured cells of the hornwort *Anthoceros punctatus*. Like the parent plant the callus and thallus cells from these protoplasts possess a large chloroplast. The chloroplasts in the cultured protoplasts began to divide after 2 days from the start of culture, followed by the first protoplast cell division after 4 days.

Introduction

Protoplasts of bryophytes possess the unique characteristics of a haploid phase and their retention of chlorophyll in culture conditions. The bryophyte protoplasts have usually been isolated mechanically or enzymatically from protonemata or young thalli[1]. Previously, Ono *et al*[2]. presented a technique for isolating protoplasts enzymatically from cultured cells of a liverwort *Marchantia polymorpha* and also reported on the isolation and regeneration of protoplasts using several moss and livewort cultured cells[3,4]. However, there has been no report on the culture and regeneration of protoplasts of hornworts, members of the bryophytes, although Takami *et al*[5]. reported on protoplast isolation from cultured cells of this species. The hornwort *Anthoceros punctatus* is unique among land plants in that the thallus cell usually contains a large chloroplast. Protoplasts of this species, therefore, would be a useful subject for elucidating the correlation between the cell division and chloroplast division of a single cultured cell.

This communication describes the isolation, culture and regeneration of protoplasts from *Anthoceros* cultured cells.

Materials and Methods

The *Anthoceros punctatus* callus tissues in the present study were induced originally in 1986 by culturing aseptic gametophytes on XMSG medium, which consists of major and minor salts and vitamins of Murashige and Skoog's medium (MS)[6], 10^{-6} M 2, 4-D, 4 % glucose and 0. 3 % Gelrite (Kelco, Division of Merck & Co.). The callus tissues were routinely subcultured on the XMSG medium containing 0. 1 % $CaCO_3$ ($XMSGCO_3$ medium)[7] at 30-day intervals at 25°C in continuous light (2, 000 lux). The pH of these media was adjusted to 5. 7 before autoclaving.

The suspension culture was started in June, 1988 from this callus culture with an inoculum of 1 g fresh weight in a 100-m*l* Erlenmeyer flask containing 20 m*l* of liquid $XMSGCO_3$ medium on a gyratory shaker at 110 rpm. The established suspension cells were routinely subcultured at 14-day

intervals by pouring 1 g fresh weight of cells into 100 ml of fresh medium in a 300-ml Erlenmeyer flask. The cells reached a stationary phase after 14 days, showing about a 3-fold increase over the initial value for dry weight.

The protoplast isolation medium consisted of 2 % Cellulase Onozuka RS (Yakult Honsha Co.), 0.3 % Pectolyase Y-23 (Seishin Seiyaku Co.), 0.5 M glucose, 3 mM MES buffer, 6 mM $CaCl_2 \cdot 2H_2O$ and 0.7 mM KH_2PO_4. The pH of the medium was adjusted to 5.6 and the medium was sterilized by filtration through a 0.45-μm Millipore filter.

The protoplast culture medium was the $XKaGCO_3$ medium based on Kao's medium[8], but from which naphthaleneacetic acid, zeatin, casamino acids and coconut water were removed and replaced by 10^{-6} M 2,4-D, 4 % glucose, 5 % mannitol and 0.1 % $CaCO_3$.

Aliquots (0.3 ml) of protoplast suspension with a density of 10^5 (protoplasts/ml) were plated on a Falcon petri dish (60×15 mm) and incubated at 25°C under illumination (1,000 lux).

The viability of protoplasts were surveyed by staining samples with 0.01 % fluorescein diacetate and 0.1 % phenosafranin dissolved in culture media according to Widholm[9], followed by observation with an Olympus fluorescence microscope. The fluorescence of fluorescein diacetate was induced by excitation with light from a 200-W Hg lamp and was observed with a UV excitation filter (360 nm) in combination with a 420-nm suppression filter. The other procedures for protoplast isolation and culture were the same as those described in the previous reports[2,4]. The dividing frequency and the cell wall regeneration of protoplasts were confirmed by adding one drop of 0.1 % Calcofluor White M2 (American Cyanamid Co.) dissolved in 0.5 M mannitol solution to a sample of protoplast suspension on a slide glass and the fluorescence produced with UV light at 360 nm was examined with the same fluorescence microscope. About 200-300 protoplasts per sample were examined and the average frequency of 3 samples was calculated.

For observation of chloroplast behavior, protoplasts suspended in a liquid Kao's medium containing 2 % glucose, 7 % mannitol and 10 mM succinic acid disodium salt (KaG2M7SA) were placed on a Cuprak dish (60×15 mm, Coster Co.) and examined by an Olympus inverted microscope every 24 hours.

Inorganic medium containing a half concentration of Knop-II salts (1/2 KN-II)[10] was used for obtaining regenerated thalli from callus cells derived from protoplasts.

Results and Discussion

Protoplast isolation was carried out using 10-day-old suspension cells. In the isolation medium, 1 g fresh weight of cells yielded only about 14 mg fresh weight of protoplasts, i.e. the yield was 1.4 %, although 40-45 % protoplast yields were obtained from *Marchantia* suspension cells and 40 % from *Atrichum* protonema cells[3,4]. However, this yield was sufficient to examine the regeneration of protoplasts. When young thallus tissues were used for materials, we could not obtain any protoplasts, although Bopp and Vicktor[11] recently were successful in isolating a small amount of protoplasts from young thallus tissues of *Marchantia polymorpha*. From the results of our experiment, we think that cultured cells and protonemata are the suitable materials for isolating viable bryophyte protoplasts.

After 28 days from the start of culture, the viability and dividing frequency of protoplasts were examined. The value of viability was 31.1 % and that of dividing frequency was 15.5 %. In the present study, the protoplast and cell cultures of *Anthoceros punctatus* were found to require the addition of 0.1 % $CaCO_3$ to a culture medium to prevent the pH of the medium dropping rapidly to a very acidic value as the culture aged. This agrees with the result for *Jungermannia sublata*

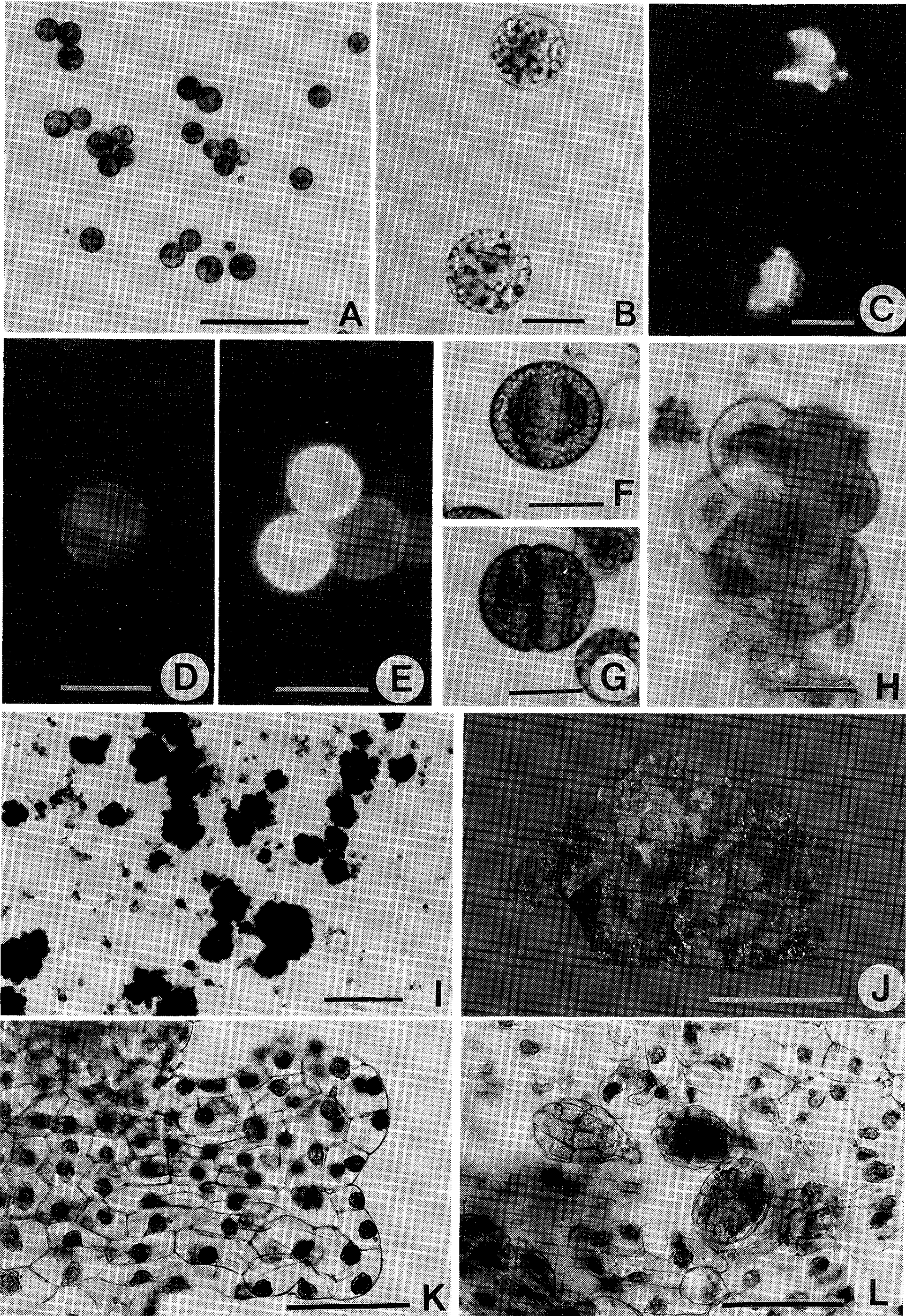

Fig. 1 Isolation and development of protoplasts from cell suspension cultures of *Anthoceros punctatus*. A and B) Freshly isolated protoplasts. C-E) Protoplasts treated with Calcoflour White M2: C shows the same protoplast as B. A large chloroplast glowing red is visible in each protoplast: D shows the initiation of cell wall regeneration in a protoplast after 12 hours of culture: E shows cell wall regeneration in protoplasts after 96 hours of culture. F) A protoplast with 2 chloroplasts. G) First cell division in a protoplast after 10 days of culture. H) Cell cluster from a protoplast after 1 month of culture. I) Calli from protoplasts after 2 months. J and K) Young thalli differentiated from callus cells 1 month after from transfer to an inorganic 1/2 KN-II medium. L) Antheridia developed in a thallus. Bars indicate 100 μm in A, K and L, 20 μm in B-H, 200 μm in I and 5 mm in J, respectively.

cultured cells[3,7].

Anthoceros protoplasts from cultured cells, like thallus cells, usually possess a large chloroplast, although at the early stage of culture its outline was not very clear with light microscopy because of interference by many granules in the cytoplasm (**Fig. 1A and B**). When observed with a fluorescence microscope, however, the presence of chloroplasts was obvious from the autofluorescence of chlorophyll (**Fig. 1C, D and E**). With the passage of culture, chloroplasts could be clearly observed by light microscopy owing to the disappearance of the granules (**Fig. 1F**).

In a freshly isolated protoplast, the cell was not observed in preparations stained with Calcoflour white M2 because it was digested by enzymes (**Fig. 1C**).

After 12 hours in the above preparation the beginning of cell wall regeneration was observed as spots glowing white at the surface of the protoplast (**Fig. 1D**), and the newly regenerated cell wall entirely surrounded the surface of the protoplast after 96 hours (**Fig. 1E**).

Fig. 2 shows the frequencies of 3 types of cells with the passage of protoplast culture, that is, the cell with a chloroplast (Type A), the cell with 2 chloroplasts (Type B) and the cell resulting from cell division (Type C). In freshly isolated and 1-day-old protoplasts, the Type A and Type B cells were observed in frequencies of 93 % and 7 %, respectively. After 2 days of culture, the frequency of Type B cells gradually began to increase (10 %) with decrease of that of Type A cells. The frequency of Type B cells rose rapidly after 3 to 4 days (15 to 25 %) and reached an equilibrium state (about 30 %) in the subsequent culture. Type C cells began to occur in frequency of 2 % after 3 days. Their frequency increased rapidly after 4 days of culture (5 %). Peak values of 37 % were observed after 10 days and this value was appreciably unchanged in the subsequent culture.

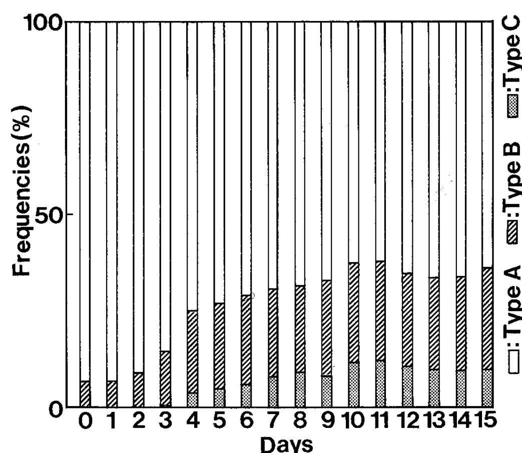

Fig. 2 Time course of frequencies (%) of 3 different cell types (Type A, B and C) in the *Anthoceros punctatus* cultured protoplasts. Type A: The cell with a chloroplast. Type B: The cell with 2 chloroplasts. Type C: The cell resulting from cell division. About 500 viable protoplasts were observed in a experiment. The figure is described on basis of mean values of 3 replicate experiments.

In view of the above results, it is considered that chloroplasts in *Anthoceros punctatus* protoplasts begin to divide after 2 days from the start of culture and the first cell division of protoplasts were observed after 3 or 4 days (**Fig. 1G**). The beginning of both cell wall regeneration and cell division of protoplasts in this species was fast compared with *Marchantia* and *Jungermannia* protoplasts previously reported[2,3]. In protoplasts of the latter two, cell walls began to regenerate after 2 days from the start of culture and to divide after 7 days. The cells of *Anthoceros* derived from protoplasts divided repeatedly and formed cell clusters like calli after 2 months of culture (**Fig. 1H and I**).

For obtaining regeneraed thalli decrived from protoplasts, calli were transferred to an inorganic 1/2 KN-II medium. After a month, many young thalli differentiated from callus cells (**Fig. 1J**) and developed to complete thalli. In regenerated thalli each cell contained a large chloroplast as in normal thallus cells (**Fig. 1K**). In addition, the formation of antheridia was observed in thalli (**Fig. 1L**) but not archegonia.

In the present study, we demonstrated an effective method for isolation, culture and regeneration of protoplasts from suspension cultured cells of *Anthoceros punctatus*. Using this cell culture, we are now examining the correlation between cell and chloroplast division in a single cultured cell and are also planning to clarify the mode of replication and division of chloroplast DNA by taking advantage of the unique cell characteristics.

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References

1) Chopra, R. N., P. K. Kumra, 1988. Protoplast culture. In Biology of Bryphytes. Written by Chopra, R. N., P. K. Kumra, pp. 268-284. Wiley Eastern Limited, New Delhi.
2) Ono, K., K. Ohyama, O. L. Gamborg, 1979. Plant Science Letters, **14**: 225-229.
3) Ono, K., K. Okamoto, 1984. J. Hattori Bot. Lab., **56**: 201-207.
4) Ono, K., Y. Murasaki, K. Kawauchi, 1987. Bot. Mag. Tokyo, **100**: 217-221.
5) Takami, S., M. Yasunaga, S. Takio, J. Kimura, S. Hino, 1988. J. Hattori Bot. Lab., **64**: 429-435.
6) Murashige, T., F. Skoog, 1962. Physiol. Plant., **15**: 473-497.
7) Ono, K., Y. Murasaki, M. Takamiya, 1988. J. Hattori Bot. Lab., **65**: 391-401.
8) Kao, K. N. 1982. Plant protoplast fusion and isolation of heterokaryocytes. In Plant Tissue Culture Methods. Ed. by Wetter, L. R., F. Constabel, pp. 49-56. National Research Council of Canada, Ottawa.
9) Widholm, J. M. 1972. Strain Technol., **47**: 189-194.
10) Hurey-Py, G. 1948. C. R. Acad. Sc., **227**: 1256-1258.
11) Bopp, M., R. Vicktor, 1988. Plant Cell Physiol., **29**: 497-501.

《和文要約》

ツノゴケ類ナガサキツノゴケ培養細胞からのプロトプラストの単離，培養
および葉状体の再生

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ツノゴケ類ナガサキツノゴケ (*Anthoceros punctatus*) の葉状体は，陸上植物としては珍しく，1細胞中に1個の大型葉緑体を有している．本研究では，本種の懸濁培養細胞からプロトプラストを単離・培養し，プロトプラストの細胞分裂にともなう核分裂と葉緑体分裂の様子を観察した．プロトプラストは培養12時間後に細胞壁の再生を開始し，96時間後には再生を完了した．葉緑体は培養2日後から分裂を始め，続いて4日後に最初の細胞分裂が観察された．培養プロトプラストは，細胞分裂を繰り返し，カルス塊を経て再生葉状体を分化させた．