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Cytological Features of the Egg Cell Protoplasts Isolated from Gametophytes of Fern, *Struthiopteris niponica* (Kunze) Nakai

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Egg cell protoplasts were isolated from the gametophyte of the fern, *Struthiopteris niponica* (Kunze) Nakai. Archegonia were excised from the prothallia and then digested in an enzyme solution that contained Cellulase "Onozuka" RS and Macerozyme R-10. After 3 hours of incubation, the egg cell protoplasts had separated completely from the somatic cells. The protoplasts had such cytological features as rich cytoplasm containing many granules, the absence of developed chloroplasts, irregular shape of the nucleus, and the presence of one or two spherical nucleoli. These isolated protoplasts gave a positive fluorochromatic reaction in the FDA test and viability was approximately 90%.

Introduction

Reproductive cells of ferns have been studied with particular reference to spermatogenesis and oogenesis. Yamanouchi[1] reported on the spermatogenesis, oogenesis, and fertilization of *Nephrodium molle*. Yuasa[2] studied structural change in the nucleus, border-brim, plastid, and mitochondria during spermatogenesis and fertilization using 13 fern species (*e. g. Adiantum, Pteris, Lygodium*), and reported that the organelle in the egg cell changes during oogenesis. Bell[3] used auto-radiography with tritiated thymidine to study the distribution of DNA in the egg cell of *Pteridium aquilinum* and reported that it is dispersed throughout the mature egg cell. Bell and Mühlethaler[4] reported, with the aid of an electron microscope, that there is degeneration of plastids and mitochondria during fern oogenesis and that the egg cell nucleus evaginates into the cytoplasm. Results of electron microscope studies by Dukett and Bell[5,6] show that the spermatozoid changes its helical form before and after penetration of the egg cell. In the previous studies, the reproductive fern cell has been studied morphologically and cytochemically using fixed materials, but little is known about the morphology of the living egg cell and its relation to physiological activity.

Recently methods to isolate the gametes have been developed using angiosperms, and some cytological features of isolated gametic protoplasts were reported[7]. Faure *et al.*[8] applied the electrofusion technique to gametic protoplasts in maize and demonstrated the process of karyogamy during *in vitro* fertilization. In pteridophytes, methods for the isolation of protoplasts from the prothallium have been developed[9-12]; but, only somatic cells such as thallus, rhizoid, and protonema were used. There has been no report of the isolation of reproductive fern cells. Isolated fern egg cells should give new insights into reproduction in fern. We here report a method

for isolating fern egg cell protoplasts and describe some of their cytological features.

Materials and Methods

1. *Preparation of prothallia*

Spores of *Struthiopteris niponica* (Kunze) Nakai were obtained from fertile fronds. Spores (3~5 mg) were sterilized for 10 minutes in 1% sodium hypochlorite solution, after which they were washed three times then suspended in 10 m*l* of sterile distilled water. One m*l* sample of this spore suspension was inoculated to agar culture medium in a Petri dish. This medium was composed of 2-fold diluted MS medium (Murashige and Skoog)[13] supplemented with 0.5% sucrose and 0.7% agar (pH 5.8). The inoculated dishes were cultured at 25±0.5°C under continuous white light of 1,500~2,000 lux. After 2 months of culture, mature prothallia were obtained. A mature prothallium averaged 15~25 archegonia.

2. *Isolation of the egg cell*

About 30 pieces of unfertilized mature prothallia were used in each experiment. The central cushion parts of the prothallia, which formed many archegonia, were excised with a razor blade under a binocular dissecting microscope. These cushion parts were soaked in 10 m*l* of enzyme solution containing 2.0% Cellulase "Onozuka" RS (Yakult, Tokyo, Japan), 1.0% Macerozyme R-10 (Yakult, Tokyo, Japan), 20 mM MES buffer, 0.6 M mannitol, 10 mM KCl, and 1 mM $CaCl_2$ (pH 5.8) then incubated in a water bath (30°C) with rotary shaking (60 rpm). After 3 hours of incubation the protoplasts were collected by centrifugation at 1,000 rpm (about 300×g) for 3 minutes. Then the protoplasts were washed three times with 2-fold diluted MS medium supplemented with 0.6 M mannitol (pH 5.8). The isolated protoplasts were suspended in 2~3 m*l* of the same medium.

3. *Observation of the isolated egg cells*

Samples of the cell suspension were observed under a Nomarski differential interference contrast (DIC) microscope, then the protoplasts were stained with 1% propionic orcein to show the shape of the nucleus or with 0.1% fluorescein brightener 28[11] to check for the presence of the cell wall. The viability of the isolated egg cell protoplasts was examined after staining them with FDA (*f*luorescein *dia*cetate, 0.02 mg/m*l*)[12]. These fluorescent dyes were separately dissolved in the same medium as cell suspension. The stained, isolated protoplasts were observed under a fluorescence microscope.

Undigested intact egg cells were also observed. After fixation with Carnoy's fluid, the central cushion part of the prothallium was excised by free hand section then stained with 1% acetic orcein.

4. *Osmotic effects of the isolated egg cells and the spermatozoids*

The concentration of mannitol in the medium was reduced gradually by 0.1 M before each observation (0.6~0 M), then the structural change of the isolated egg cell was observed. The prothallia were also immersed in the same media and the discharge of spermatozoids from the antheridia was examined.

Results

1. *Isolation process*

Within 2 hours of enzymatic digestion, the prothallial and jacket cells of the archegonium were degenerated, and the egg cell could be observed from the outside under a DIC microscope (**Fig. 1-A**). After 3 hours of incubation, the cell wall was digested and heterogeneous protoplasts from the egg cell (arrowhead) and somatic cells were obtained (**Fig. 1-B**). The protoplasts ranged in diameter from 15 to 65 μm.

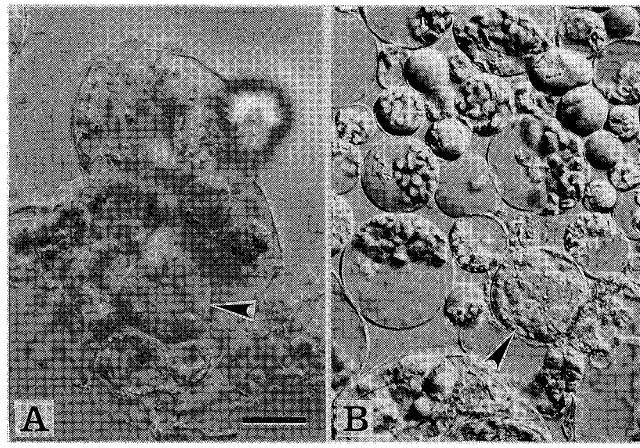

Fig. 1 Egg cells of *Struthiopteris niponica* during enzymatic digestion.
A: The archegonium in the early stage of digestion. The egg cell (arrowhead) is located in the venter. B: An egg cell protoplast (arrowhead) and many somatic protoplasts after 3 hours of digestion. Bar=20 μm.

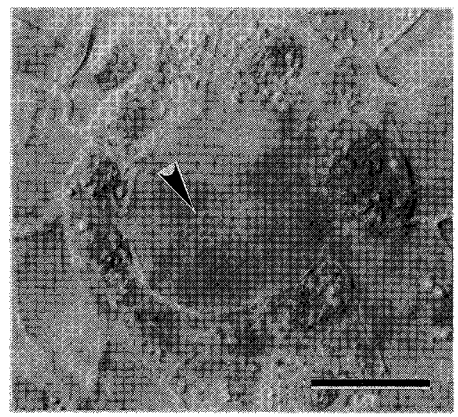

Fig. 2 An undigested, intact egg cell stained with acetic orcein after fixation with Carnoy's fluid.
A mass of chromatin (arrowhead) and a spherical nucleolus can be seen. Bar=20 μm.

2. *Observation of intact egg cells*

An undigested egg cell is shown in **Fig. 2**. Its cytoplasm is stained with acetic orcein more deeply than the cytoplasms of the sometic cells. A mass of chromatin and a nucleolus with a diameter of approximately 4 μm are present. The nuclear membrane does not show clearly. Some chloroplasts are developed in the somatic cells but not in the egg cell.

3. *Observation of isolated egg cells*

Isolated egg cells are shown in **Fig. 3**. In **Fig. 3-A**, undigested cell wall and debris cling to the surface, and the cell is not spherical. **Fig. 3-B~D** show isolated egg cell protoplasts with spherical shapes and diameters ranging from about 35-40 μm. Abundant opaque cytoplasm containing numerous granules and a translucent nucleus are present in **3-B** and **3-C**. In the cell represented by **Fig. 3-B**, there was Brownian movement of the granules, but no movement in the cell in **3-C**. The granules are distributed along the periphery of the cell. There are no developed chloroplasts in the egg cells. The cytoplasm and nucleus are distinguishable because of the presence of the granules and the transparency of the cell.

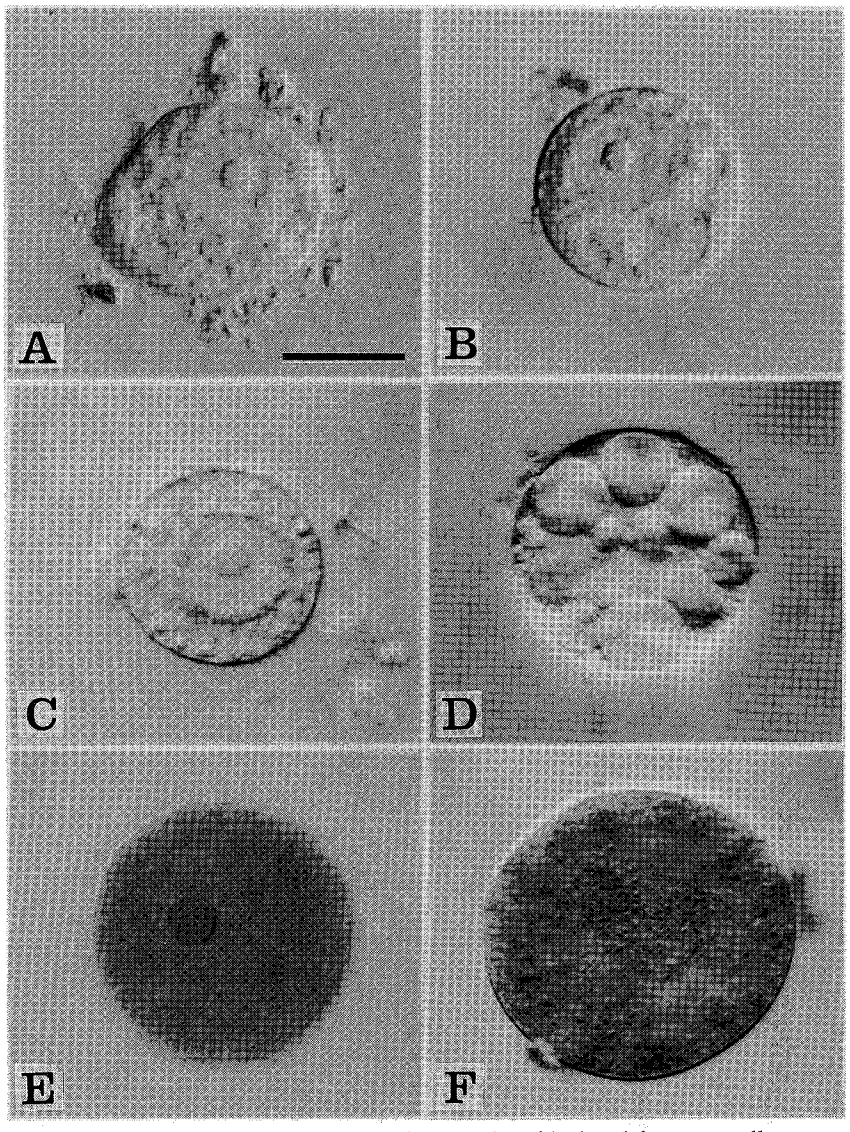

Fig. 3 Nomarski differential interference micrographs of isolated fern egg cells.
A–D: Without staining. E and F: Stained with 1% propionic orcein. A: After 2 hours of digestion the cell is not yet spherical. Undigested cell wall clings to the surface. B–F:Spherical egg cell protoplasts obtained after 3 hours of digestion. B: The boundary between the cytoplasm and nucleus is not clear, and there was Brownian movement of the granules. C: The boundary between the cytoplasm and nucleus is clear, and there was no Brownian movement. D: Some developed vacuoles can be seen. E: The spherical nucleolus is more deeply stained than the cytoplasm and nucleus. F: Thready chromatin is present in the nucleus. Bar=20 μm.

When there was Brownian movement, the boundary between the cytoplasm and nucleus was not clear (**3-B**), but when there was no movement the boundary showed clearly (**3-C**). Many of the isolated egg cells have a spherical nucleolus (*ca.* 2~4 μm in diameter), and some have more than two nucleoli. In **Fig. 3-D**, there are many conspicuous vacuoles. The isolated irregularly shaped egg cell (**3-A**) stained with fluorescein brightener 28 shows faint fluorescence on its surface. The spherical cell (**3-B**) shows no fluorescence, evidenced that it is a protoplast.

The isolated egg cell treated with propionic orcein (**3-E, F**) is stained pale red throughout. The

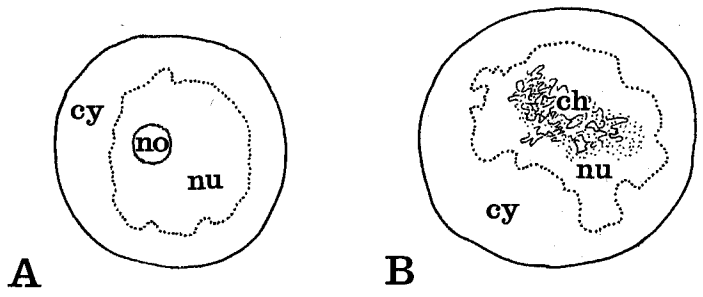

Fig. 4 Diagram of **Fig. 3-E** and **F**.
The nuclei are irregular in shape. nu: nucleus, no: nucleolus, ch: chromatin, cy: cytoplasm.

nucleolus shows a deeper stain than the cytoplasm and nucleus. The nucleus is stained most faintly in the cell (**Fig. 3-E**). The shape of the nucleus is irregular (**Figs. 3-E, F** and **4-A, B**).

The isolated egg cell stained with FDA fluoresces yellow-green, evidence that it is viable. Among 73 egg cell protoplasts examined, 65 showed yellow-green fluorescence in the FDA test (viability: approximately 90%) about 24 houre after isolation. Isolated egg cells that gave a negative reaction differed from those reacting positively in morphology. The negative cells shrank and had irregular shape. Brownian movement was not observed in cytoplasms of negative cells.

4. *Osmotic effects of isolated egg cells and spermatozoids*

The isolated egg cell kept its spherical shape in the media containing 0.3～0.6 M mannitol, but most cells burst below 0.2 M.

Spermatozoids were discharged from antheridia and swam freely in the media containing below 0.1 M mannitol. As concentration of mannitol increased more than 0.2 M, spermatozoids decreased to discharge from antheridia and few were discharged over 0.4 M even in 60 minutes after immersion. In high osmotic condition more than 0.4 M mannitol, flagella should remarkably slow movement and the spermatozoid could not swim.

Discussion

Unfertilized egg cells of the fern *Struthiopteris niponica* (Kunze) Nakai could be isolated from the archegonia by enzymatic digestion. Enzyme combination is similar to the case of prothallial somatic cells of *Pteridium aquilinum*[9], *Lygodium japonicum*[10]. The egg cells were separated easily from surrounding cells as well as somatic cells. However, some isolated egg cells (*e. g.* **Fig. 3-A**) showed irregular shape and reacted faintly to fluorescein brightener 28 even when most somatic cells became protoplasts. This result suggests that components of the cell wall around egg cell are different from the somatic cell. Cave and Bell[14] showed the mature egg of *Pteridium* is surrounded by an acetolysis-resistant membrane which consists of lipid.

Isolated egg cell protoplasts reveal some morphological features: a rich cytoplasm containing many granules, the absence of developed chloroplasts, irregular shape of the nucleus, and the presence of one or more nucleoli in the nucleus.

The fixed intact egg cell (**Fig. 2**) is concaved on the side of ventral canal cell, although isolated egg cells are spherical. The appearance of the organelle and nucleus is almost the same between the intact and isolated egg cell. Yuasa[2] reported the presence of leucoplasts in fern intact egg cytoplasm. Bell and Mühlethaler[4] showed by electron microscopy that small vesicular plastids and mitochondria are present in the intact egg cytoplasm of *Pteridium aquilinum*. A comparison of our results with those of these studies shows that the granules in the cytoplasms of isolated egg cell protoplasts have a similar distribution. In the isolated egg cell protoplast cytoplasm is opaque and

white, and there is no developed chloroplast.

The irregular shape of the nucleus (**Figs. 3-E, F** and **4-A, B**) is consistent with the previous reports[1,4]. These reports revealed that the intact mature egg cell has an expanded and irregularly shaped nucleus. An irregularly shaped nucleus sometimes can be detected in viable egg cell protoplasts even without staining. Therefore the egg cell protoplasts in **Figs. 3-B, E, F** are considered to be mature.

Yamanouchi[1] also reported that the fern egg nucleus shows transfromation into a system of anastomosing and branching fine strands; however there has been no recent report on the morphology of the fern egg nucleus. **Figs. 3-F** and **4-B** show a mass of intertwined thready chromatin. A similar structure can be seen in the intact egg cell shown in **Fig. 2**.

We found the entire egg cell protoplast to be stained with propionic orcein; the nucleolus stained fairly deeply, the expanded nucleus very faintly. Bell[3] reported a similar cytochemical feature that the intact fern egg cell nucleus is not stained by the Feulgen reagent.

It is suggested from the morphological observation of isolated egg cell that the boundary between cytoplasm and nucleus relates to its viability: the physiologically active cells show an irregular boundary, however inactive cells show a spherical boundary.

The fern egg cell protoplasts that we obtained by enzymatic isolation, have very high viability, about 90%. Somatic cell protoplasts isolated from prothallia can be cultured successfully, and gametophytes regenerate within 50 days in *Lygodium japonicum*[10]. If viable egg cell protoplasts can be cultured, it may be possible to examine the maturation, structural change of egg cells, and *in vitro* fertilization of the fern. By using angiosperm maize, *in vitro* fusion of isolated male gametes with isolated egg cell protoplast is induced successfully and the fusion product develops into a plant[15]. In this study the method for isolation of egg cell and discharge of motile spermatozoids has been carried out, but for the purpose of *in vitro* r l *fertilization, establishment of an osmotic condition needs to be solved.*

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References

1) *Yamanouchi, S., 1908. Bot. Gaz.,* **45**: 145-175.
2) Yuasa, A., 1937. Jap. J. Bot., **5**: 17-35.
3) Bell, P. R., 1961. Proc. Roy. Soc. London, Series B **153**: 421-432.
4) Bell, P. R., K. Mühlethaler, 1962. J. Ultrastruct. Res., **7**: 452-466.
5) Duckett, J. G., P. R. Bell, 1971. Cytobiologie, **4**: 421-436.
6) Duckett, J. G., P. R. Bell, 1972. Cytobiologie, **6**: 35-50.
7) Theunis, C. H., E. S. Pierson, M. Cresti, 1991. Sex. Plant Reprod., **4**: 145-154.
8) Faure, J. -E., H. L. Mogensen, C. Dumas, H. Lorz, E. Kranz, 1993. The Plant Cell, **5**: 747-755.
9) Partanen, C. R., J. B. Power, E. C. Cocking, 1980. Plant Sci. Lett., **17**: 333-338.
10) Maeda, M., M. Ito, 1981. Bot. Mag. Tokyo, **94**: 35-40.
11) Huckaby, C.S., A. R. Bassel, J. H. Miller, 1982. Plant Sci. Lett., **25**: 203-208.
12) Kadota, A., M. Wada, 1989. Plant Cell Physiol., **30**: 1107-1113.
13) Murashige, T., F. Skoog, 1962. Physiol. Plant., **15**: 473-497.
14) Cave, C. F., P. R. Bell, 1974. Ann. Bot., **38**: 17-21.

15) Kranz, E., H. Lorz, 1993. The Plant Cell, 5: 739-746.

《和文要約》

シダ(シシガシラ *Struthiopteris niponica* (Kunze) Nakai)配偶体から単離した卵細胞プラトプラストの細胞学的特徴

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シダの配偶体から酵素処理によって卵細胞プロトプラストを単離することを試みた．シシガシラ *Struthiopteris niponica* (Kunze) Nakai の前葉体を材料にして，セルラーゼ“オノズカ”RS とマセロザイム R-10 を含んだ酵素液で約 3 時間の振盪培養を行った結果，他の前葉体の細胞から完全に単離された卵細胞プロトプラストが得られた．卵細胞プロトプラストでは，多数の顆粒を含む細胞質に富むこと，細胞質中に発達した葉緑体が認められないこと，核が不規則な外形を示すこと，核内に 1 または 2 個以上の核小体が存在すること，などの細胞学的な特徴が確認された．また，卵細胞プロトプラストは生理活性を維持しており，単離 24 時間後で約 90% の生存率を示した．