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Eyespot Behavior during the Fertilization of Gametes in Ulvalean Alga *Enteromorpha compressa* (Linnaeus) Nees Revealed by Field Emission Scanning Electron Microscopy

Shinichi Miyamura

Institute of Biological Sciences, University of Tsukuba, Tsukuba, Ibaraki, 305–8572 Japan

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Summary Fate of the eyespots during the fertilization of ulvalean alga *Enteromorpha compressa* was studied using field emission scanning electron microscopy (FE-SEM). FE-SEM enabled the visualization of the eyespot of biflagellate male and female gamete. The smaller male gamete has 1 protruded smaller eyespot and the larger female gamete has larger one on a posterior position of the cell. The cell membrane over the eyespot region is relatively smooth compared to other part of the cell body and exhibits hexagonal arranged lipid globules. Since the size of cell body and the eyespot is slightly different between male and female gamete, the author could follow the fate of the eyespots during the fertilization. In most of the mating pairs, larger female gamete has always fused along the same side as the eyespot, while smaller male gamete along the side away from its eyespot. As fusion proceeds, the gamete pair is transformed into the quadriflagellate planozygote, in which the eyespots are positioned side by side on the region of cell fusion. These observations indicated that the opposite positioning of the eyespot relative to the cell fusion site in male and female gametes is important for the proper arrangement of the eyespots in the planozygote.

Key words Eyespot, Fertilization, Field emission scanning electron microscopy, Gamete, *Enteromorpha compressa*, Planozygote.

Eyespot of green algae is an orange or red pigmented spot visible in the light microscope and locates at the cell surface of flagellated swarmer (Foster and Smyth 1980, Melkonian and Robenek 1984, Kreimer 2001). It is part of photoreceptive apparatus and its location is fundamental importance for the reception of a light signal for phototaxis (Hegemann 1997).

The gametes of most ulvalean algae have usually 1 eyespot and they are positively phototactic (Melkonian and Robenek 1984, Clayton 1992). Upon sexual fusion of gametes, 2 eyespots from male and female gamete lie side by side on one side of the planozygote in *Enteromorpha* (Fig. 40 in Miyake and Kunieda 1931), *Chaetomorpha okamurai* (Figs. 22, 23 in Hirose 1954), *Ulva* (Kornmann and Sahling 1977), *U. arasakii* (Miyamura *et al.* 2003) and *Collinsiella cava* (Nakayama and Inouye 2000). After gamete fusion, phototaxis of the resultant planozygote is reversed and partially fused gamete pairs swim away from the light source (Miyake and Kunieda 1931, Smith 1947, Hirose 1954, Chihara 1969). Robenek and Melkonian (1981) suggested that the 2 eyespots in the planozygote might be cooperating in shading photoreceptor.

In the previous study, we analyzed the behavior of the male and female eyespots during the fertilization of bifflagellate gametes of *U. arasakii* using field emission scanning microscopy (FE-SEM) (Miyamura *et al.* 2003). Each gamete contains a single eyespot, which always locates on one side of the cell body bisecting the flagellar beat. The female gamete fuses along the same side as the eyespot, whereas the male gamete along the side away from its eyespot, resulting in the location of 2 eyespots on the same side of the planozygote (Miyamura *et al.* 2003). The opposite positioning

* Corresponding author,e-mail: miyamura@sakura.cc.tsukuba.ac.jp

of the eyespot relative to the cell fusion site in each sex gamete has been also shown in *C. cava* using transmission electron microscopy (Nakayama and Inouye 2000).

However, it has been reported that the eyespot is on the same side as a mating structure (cell fusion apparatus) in the gametes of *U. lactuca* irrespective of the sex types (Robenek and Melkonian 1981), whereas the eyespot is always located on the other side in *Ulvaria obscura* (Kutz.) Gayral var. *blyttii*, *Monostroma bullosum*, *M. greville* and *M. oxyspermum* (O'Kelly *et al.* 1984). Therefore, it remains an unsettled question in ulvalean algae how 2 eyespots from male and female gamete locate on the same side of the planozygote.

In this study, the author reports the visualization of eyespots in the gametes of *E. compressa* using FE-SEM and also describe the behavior of eyespots during the fertilization.

Materials and methods

Materials

Fertile thalli of *Enteromorpha compressa* was collected at Obitsugawa mudflat, Chiba Prefecture, Japan in 2001 and 2002, and immediately brought back to the laboratory. After arrival at the laboratory, thalli were cultured at 20°C in sea water under fluorescent lamps (55 μmol m^{-2} s^{-1}) with a 12 : 12 h light/dark cycle. Next afternoon, matured thalli released gametes, which immediately swam to the light source.

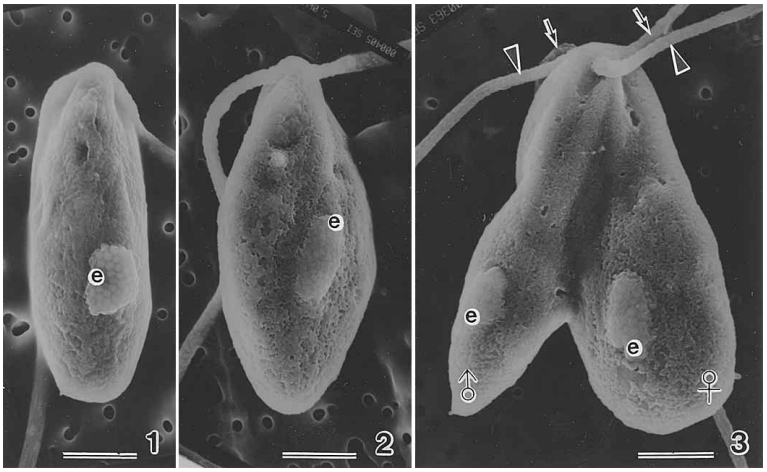
Scanning electron microscopy

One volume of suspension of male or female gametes were mixed with an equal volume of 6% glutaraldehyde made up in 3% NaCl in 0.1 M cacodylate buffer, pH 7.1, on the Nuclepore polycarbonate membrane (Costar Scientific Corporation) which were coated with 0.1% poly-L-lysine (Sigma). For the fertilization experiments, 1 volume of suspension of male gametes and 1 volume of female gametes were mixed on the Nuclepore polycarbonate membrane and fixed with 2 volumes of the fixatives at 0, 10, 30, 60, 120 and 420 s after mixing. The cells were fixed at room temperature. After removing the supernatant, the cells on membrane were further fixed with 3% glutaraldehyde, 3% NaCl, 0.05 M cacodylate buffer, adjusted to pH 7.1 overnight at 4°C and then washed in a series of 0.05 M cacodylate buffer solutions containing 3, 2.25, 1.5, 0.75 and 0% NaCl, each step taking 15–20 min. Post-fixation was made in 1% OsO_4 dissolved in 0.05 M cacodylate buffer, pH 7.1, overnight at 4°C. After dehydration through a graded series of ethanol, the cells were infiltrated with *t*-butyl alcohol, freeze-dried at 4°C and coated with platinum-palladium in a Hitachi E-102 sputter-coating unit. Observations were made on a JEOL JSM-6330F field emission scanning electron microscope at 5 kV.

Results and discussion

Gametes of *Enteromorpha compressa* are pear-shaped (Figs. 1, 2). The anterior end of gamete displays a papilla, from whose opposite site 2 isokont flagella arise (Figs. 1, 2). Although the gametes of *E. compressa* have been reported to be isogamous (Tatewaki 1994), the author could observe the 2 slightly different sizes of gamete under FE-SEM. In accordance with other recorded cases of anisogamy, the smaller cells will be regarded as "male" while the larger will be "female". Male gametes are 5.2 ± 0.5 (mean $\pm$ S.D.) μm ($n=400$) in length and 2.2 ± 0.3 μm ($n=400$) in width (Fig. 1), and female gametes 5.3 ± 0.6 μm ($n=400$) in length and 2.4 ± 0.3 μm ($n=400$) in width (Fig. 2).

As the eyespot of *Enteromorpha* is composed of many hexagonal shaped lipid globules (McArthur and Moss 1979) and protrudes slightly from the cell surface, I could easily recognize its oval shape and smooth surface by FE-SEM (Figs. 1, 2). The eyespot in both gametes of *E. compressa* lies on one side of a plane bisecting the flagellar beat (Figs. 1, 2). The long axis of the eyespots



Figs. 1–3. Field emission scanning electron microscope images showing fertilization of gametes of *Enteromorpha compressa*. 1) Male gamete. 2) Female gamete. 3) Planozygote fixed 7 min after mixing gametes. Gamete pair lies side by side with their longitudinal axes nearly parallel. Such planozygotes can be found 30 s after mixing. e: eyespot, ♂: male gametes, ♀: female gametes, arrowheads: male flagella, arrows: female flagella. Bars indicate 1 μm.

always lies parallel or slightly oblique to the longitudinal axis in both gametes. Using FE-SEM one can visualize hexagonal arranged lipid globules that comprise the eyespot and which are located directly beneath the cell membrane and 2 chloroplast envelopes. The eyespots of male gametes (Fig. 1) are slightly smaller than those of females (Fig. 2), male and female eyespots being $1.3\pm0.2\,\mu$m ($n=22$) and $1.4\pm0.2\,\mu$m ($n=22$) in length, and $0.7\pm0.1\,\mu$m ($n=22$) and $0.8\pm0.1\,\mu$m ($n=22$) in width, respectively.

As FE-SEM images of the size of cell body and eyespot are slightly different between the male and the female gametes, it is possible to follow the fate of the each gamete and its eyespot during gamete fusion. When both gametes are mixed together, the initial cytoplasmic contact and cell fusion between the 2 gametes takes place at the anterior end slightly below the flagellar base (Fig. 3). Fusion of the 2 gametes is rapid and stages like the one shown in Fig. 3 can be found 30 s after mixing and still observed 7 min later. The arrangement of gamete during fusion followed 2 distinct patterns as observed previously in *Ulva mutabilis* (Figs. 4, 5 in Bråten 1971), *U. lactuca* (Melkonian 1980) and *U. arasakii* (Figs. 3, 4 in Miyamura *et al.* 2003). Most of the gamete pairs lie side by side with their longitudinal axes nearly parallel (Fig. 3), while the rests are oriented antiparallel. In both cases, larger female gamete has fused along the same side as the eyespot, while smaller male gamete has fused along the side away from its eyespot. This fusion pattern is observed in 45 mating pairs ($n=49$) fixed 30–420 s after mixing together the 2 sexes. In 4 mating pairs, the author could not recognize the significant difference in size between 2 gametes. When the cytoplasmic fusion proceeds from the cell anterior to posterior of both gametes, the eyespots lie side by side adjacent to the region of cell fusion.

The present study using FE-SEM has revealed that the sex specific and site specific positioning of the eyespot in the mating gametes of *E. compressa*. This observation is in contrast to the previous observations of *U. lactuca* (Robenek and Melkonian 1981), *Ulvaria obscura* (Kutz.) Gayral var. *blyttii*, *Monostroma bullosum*, *M. greville* and *M. oxyspermum* (O'Kelly *et al.* 1984), in which eyespot is always located on the same side as the mating structure or the side away from it irrespective of the sex types. The reason why the sex specific and site specific positioning of the eyespot to the cell fusion site/mating structure has not been observed in these algae is not clear. One possibility is

that the opposite positioning of the eyespot to the mating structure in each sex gamete may have been missed, because mating pairs or planozygotes were not observed in these previous studies (Robenek and Melkonian 1981, O'Kelly *et al.* 1984).

In contrast, the result of the present study is consistent with that of *Collinsiella cava* (Nakayama and Inouye 2000) and *U. arasakii* (Miyamura *et al.* 2003). In these algae, the position of the eyespot relative to the cell fusion site/mating structure is converse between 2 mating types. This phenomenon is already known in chlorophycean alga *Chlamydomonas reinhardtii* (Holmes and Dutcher 1989). In *Ch. reinhardtii*, each gamete has a single eyespot at the periphery of the cell closer to one basal body (*cis* basal body) than the other (*trans* basal body). The minus gamete fuses along the eyespot side, whereas the plus gamete do along the opposite side, a combination that results in the 2 eyespots coming to lie on the same side of the planozygote (Holmes and Dutcher 1989). Therefore, it is likely that ulvalean algae use the same mechanism as that of *Ch. reinhardtii* in order to locate male and female eyespot on the same side of the planozygote after fusion. This mechanism may be important for the phototaxis of the planozygote.

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References

Bråten, T. 1971. The ultrastructure of fertilization and zygote formation in the green alga *Ulva mutabilis* Føyn. J. Cell Sci. **9**: 621–635.

Chihara, M. 1969. *Ulva arasakii*, a new species of green algae: its life history and taxonomy. Bull. Nat. Sci. Mus. Tokyo **12**: 849–863.

Clayton, M. N. 1992. Propagules of marine macroalgae: structure and development. Br. Phycol. J. **27**: 219–232.

Foster, K. W. and Smyth, R. D. 1980. Light antennas in phototactic algae. Microbiol. Rev. **44**: 572–630.

Hegemann, P. 1997. Vision in microalgae. Planta **203**: 265–274.

Hirose, H. 1954. Studies on the morphology and behavior of the reproductive cells of *Chaetomorpha okamurai* Ueda. Cytologia **19**: 358–369.

Holmes, J. A. and Dutcher, S. K. 1989. Cellular asymmetry in *Chlamydomonas*. J. Cell Sci. **94**: 273–285.

Kornmann, P. and Sahling, P. H. 1977. Meeresalgen von Helgoland. Helgolander wiss. Meeresunters. **29**: 1–289.

Kreimer, G. 2001. Light Perception and Signal Modulation during Photoorientation of Flagellate. In: Häder, D. P. and Lebert, M. (eds.). Photomovement, Elsevier Science B.V., Amsterdam, New York. pp. 193–227.

McArthur, D. M. and Moss, B. L. 1979. Gametogenesis and gamete structure of *Enteromorpha untestinalis* (L.) Link. Br. Phycol. J. **14**: 43–57.

Melkonian, M. 1980. Flagellar roots, mating structure and gametic fusion in the green alga *Ulva lactuca* (Ulvales). J. Cell Sci. **46**: 149–169.

— and Robenek, H. 1984. The eyespot apparatus of flagellated green algae: a critical review. Prog. Phycol. Res. **3**: 193–268.

Miyake, K. and Kunieda, H. 1931. On the conjugation of the gametes and the development of the zoospores in Ulvaceae. J. Coll. Agric. **11**: 341–357.

Miyamura, S., Hori, T. and Nagumo, T. 2003. Eyespot behavior during the fertilization of gametes in *Ulva arasakii* Chihara (Ulvophyceae, Chlorophyta). Phycol. Res. (in press).

Nakayama, T. and Inouye, I. 2000. Ultrastructure of the biflagellate gametes of *Collinsiella cava* (Ulvophyceae, Chlorophyta). Phycol. Res. **48**: 63–73.

O'Kelly, C. J., Floyd, G. L. and Dube, M. A. 1984. The fine structure of motile cells in the genera *Ulvaria* and *Monostroma*, with special reference to the taxonomic position of *Monostroma oxyspermum* (Ulvophyceae, Chlorophyta). Pl. Syst. Evol. **144**: 179–199.

Robenek, H. and Melkonian, M. 1981. Comparative ultrastructure of eyespot membranes in gametes and zoospores of the green alga *Ulva lactuca* (Ulvales). J. Cell Sci. **50**: 149–164.

Smith, G. M. 1947. On the reproduction of some Pacific coast species of *Ulva*. Amer. J. Bot. **34**: 80–87.

Tatewaki, M. 1994. *Enteromorpha compressa* (Linnaeus) Greville. In: Hori, T. (ed.). An Illustrated Atlas of the Life History of Algae, Vol. 1, Uchida Rokakuho Publish. Co. Ltd., Tokyo. pp. 192–193.