High-Speed Video Analysis of the Flagellar Movement of Isogametes during Fertilization of the Marine Green Macroalga, *Chaetomorpha spiralis* (Ulvophyceae, Chlorophyta)

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Summary Flagellar movement and mating behavior of the biflagellate gametes of the marine green macroalga, *Chaetomorpha spiralis* were studied using high-speed video microscopy and field emission scanning electron microscopy (FE-SEM) to clarify the role of flagella during the rapid fertilization of marine green macroalgae. Discharged isogametes always swung their flagella backward during forward swimming. The beat pattern was flagellar beat with undulatory waves produced at the flagellar base being propagated toward the tip. One beat cycle took 15 ms. When the suspension of the gametes was mixed with that of the opposite mating types, the gametes immediately agglutinated to form gamete clumps or mating pairs within 10–20 s. Initial contacts between the two gametes took place at one of the flagellar tips. In the mating gamete pair, the two gametes maintained mutual contact at the flagellar tips and subsequently at their anterior end of the cell bodies. The gametes stuck together, even though they continued to move their flagella and oscillate their cell bodies. Then the two flagella derived from the opposite mating types became a pair and gradually beat synchronously. Finally, the gamete pair lay side-by-side with their longitudinal axes nearly parallel mutually and became a quadriflagellate planozygote. Such features of gamete behavior and their flagellar movement during fertilization resemble those of *Ulva*.

Key words Cladophorales, Field emission scanning electron microscopy, Marine green macroalgae, Mating pair, Ulvophyceae.

Fertilization of marine green macroalgae (Ulvophyceae) inhabiting the littoral zone involves the union of biflagellate gametes of opposite mating types, leading to the subsequent formation of quadriflagellate motile zygote (planozygote) (Clayton 1992, Fletcher and Callow 1992). This fertilization process, studied mainly in *Ulva* (Ulvales) (Bråten 1971, Lovlie and Bryhni 1976, Melkonian 1980, Miyamura 2004, Mogi et al. 2008), closely resembles that of the fresh water green flagellate *Chlamydomonas* (Brown et al. 1968, Friedmann et al. 1968). In both genera, the gamete has two flagella, which play a critically important role for gamete–gamete recognition and adhesion. The initial contact between mating gametes involves the flagellar tips of both mating types, which engenders sexual agglutination and finally gamete adhesion and fusion. Because the basic fertilization process is common in these phylogenetically distantly related genera, *Chlamydomonas* (Chlorophyceae) and *Ulva* (Ulvophyceae), it is assumed that the gamete flagella also play a similarly important role for gamete–gamete recognition and adhesion in other green macroalgae. Actually, agglutination of gametes at the flagellar tips has been reported in the ulvophycean alga, *Caulerpa* (Goldstein and Morrall 1970).

However, in contrast to *Chlamydomonas*, gamete adhesion and fusion occurs within a very short time in marine green macroalgae (Bråten 1971, Melkonian 1980, Harper and Pienaar 1985, Hori 1988, Miyamura 2004). For instance, cell fusion in *U. mutabilis* is initiated in most cells within 10–20 s after initial mixing of the male and the female gametes. It is completed within 5 min (Bråten 1971). Because of this characteristic nature of fertilization in the marine green macroalgae, it is difficult to elucidate how the living gametes use their flagella to find mating partners and make contact to cell fusion. One solution to this difficulty is the application of high-speed video microscopy, which has supported detailed observations of flagellar movement (Inouye and Hori 1991). Reproductive cells of marine green macroalgae swim forward using "flagellar beat," which is the flagellar movement forming undulatory waves that pass from the base to the tip of the flagellum (Inouye and Hori 1991). This type of flagellar movement differs from the
When the gametes originating from the different mating behaviors were recorded at 23°C, the fertilization involved the union of biflagellate isogametes of opposite mating types leading to the subsequent formation of quadriflagellate motile zygote (Hartmann 1929, Hirose 1954, Chihara 1958). However, the function of gamete flagella for fertilization remains obscure. Therefore, we used high-speed video microscopy to record the flagellar movement and the behavior of gametes of *C. spiralis* to clarify the function of gamete flagella during the fertilization of marine green macroalgae.

Materials and methods

Gametophytes of *Chaetomorpha spiralis* were collected at Oarai (36°19′20″N: 140°35′90″E), Ibaraki Prefecture, Japan, in May, June and July during 1995–2005. Each gametophyte was placed in a separate vessel containing filtered seawater and was maintained under natural daylight conditions or with a 14:10 h light/dark cycle, ca. 50 μmol photons m⁻² s⁻¹ at 20°C until liberation of the gametes. Within a few days to one month of collection, the cells of distal parts of the gametophyte began to start gametogenesis and to release biflagellate isogametes. The gamete accumulated to the illuminated side of the vessel. The gametes were collected and were then used for crossing experiments to find the gametes belonging to the opposite mating types (mt⁺ or mt⁻).

When the gametes originating from the different gametophytes were mutually crossed and produced zygotes, each gamete was treated as a different mating type and was used for observations.

For high-speed video-microscopy for flagellar movement analysis, 40–60 μL gamete suspensions were placed in 26-mm-long, 20-mm-wide, and 0.1-mm-deep observation chambers to insure free-swimming mobility. Swimming behavior, flagellar movement, and mating behavior were recorded at 23°C using high-speed video microscopy (MHS-200; nac Image Technology, Inc., Tokyo, Japan) with strobooscopic light to achieve 200 frames per second, attached to microscope (Optiphot; Nikon Corp., Tokyo, Japan). Videotapes were played back at slow speed for flagellar movement analysis using a videocassette recorder (SVO-5800; Sony Corp., Tokyo, Japan). All analog images were converted to digital images using an analog-digital converter (ADVC-100; Canopus Co., Ltd., Kobe, Japan). Figures of flagellar movements were produced by selecting representative sequences and still frames using iMovie (Apple Inc., Cupertino, CA, USA).

For field emission scanning electron microscopy (FE-SEM), one volume of suspension of cells was mixed with an equal volume of 6% glutaraldehyde comprising 3% NaCl in 0.1 M cacodylate buffer, pH 7.1, on a Nuclepore polycarbonate membrane (Whatman Japan KK, Tokyo, Japan) that was coated with 0.1% poly-l-lysine (Sigma Chemical Co., St. Louis, MO, USA). For fertilization experiments, one volume of suspension of gametes was mixed with one volume of opposite mating type gametes on a Nuclepore polycarbonate membrane and was fixed with two volumes of fixative at 1 min after mixing. The cells were fixed at 23°C. After removing the supernatant, the cells were fixed further with 2.5% glutaraldehyde, 3% NaCl, 0.05 M cacodylate buffer, pH 7.1, for 6 h at 23°C. They were 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% OsO₄ 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 an E-1030 or E-1045 sputter-coating unit (Hitachi High-Technologies Corp., Tokyo, Japan). Observations were made respectively using a field-emission scanning electron microscope (S5000; Hitachi High-Technologies Corp., Tokyo, Japan; or JSM6330F; JEOL, Tokyo, Japan) at 2 or 5 kV.

All digital images were minimally adjusted for brightness and contrast, trimmed (Photoshop CS6; Adobe Systems Inc., San Jose, USA) and reduced from their original size (Adobe Illustrator CS6; Adobe Systems Inc.).

Results

Morphology of the gametes belonging to the opposite mating types was identical under light microscopy to that reported by Chihara (1958) (Fig. 1a, b). The gamete was pear or ovoid-shaped, lacking a cell wall. Its anterior end displayed a papilla with two isokont flagella arising from opposite sides. The gamete had a chloroplast and an eyespot that was present at the lateral side of the gamete. A hair-point occurred at the flagellar tip (Fig. 1c). Figure 1d shows sequential movie frames of the beat cycles of the gamete exhibiting forward swimming. During forward swimming the gametes always swung their flagella backward. The beat pattern was the flagellar...
Fig. 1. Light microscopic (a, b), FE-SEM (c) and high-speed video (d, e) images of the gametes of *C. spiralis*. (a) The gamete. (b) The gamete of the opposite mating type to (a). (c) Hair-point of flagellar tip. (d) Sequential movie frames of the beat cycles of the gamete moving by forward swimming. (e) Sequential movie frames of a single beat cycle of the gamete moving by backward swimming. Arrows indicate the direction of the swimming. Arrowheads indicate the wave propagation from the flagellar base toward the tip. E, eyespots; F, flagella; H, hair-point. Scale bars=10 µm (a, b, d, e) and 1 µm (c).

Fig. 2. Light microscopic (a) and high-speed video (b, c) images of the gamete clump and mating pair formation. (a) The gamete clumps. Arrowheads indicate the clumps formed within 10–20 s after mixing the gametes. (b) Enlarged images of the mating pairs and clump. Double arrowheads indicate the separation of a gamete from the clump. (c) Sequential movie frames showing the contact of two gametes by flagella. The gamete indicated by asterisk swam toward another gamete and contacted by their flagella. White arrows indicate the contact site of two flagella. C, clump; G, gametes; M, mating pairs. Scale bars=100 µm (a) and 10 µm (b, c).
beat, as described previously in other marine green macroalgae (Inouye and Hori 1991, Miyamura 2004). One beat cycle took 15 ms in both mating types. When the gamete moved by forward swimming, there was usually a phase difference between two opposite flagella. The longitudinal axis of the cell bodies oscillated periodically. When the gametes swam backward, the gametes always swung their flagella forward (Fig. 1e). The beat pattern was a flagellar beat. One beat cycle took 15 ms. The beat pattern of the gametes was identical in both mating types (mt+ and mt−).

When a suspension of the gametes was mixed with that of the opposite mating types, the gametes immediately agglutinated to form gamete clumps or mating pairs within 10–20 s (Fig. 2a). The clumps comprised more than three gametes, in which mating pairs were formed. Afterwards, the clump disintegrated completely. For instance in Fig. 2b, the two gametes became a pair in the clump composed of three gametes at 0 ms. After 80 ms, the other gamete started to leave the clump (double arrowheads in Fig. 2b). Because the clump or mating pair formation occurred very rapidly, we were unable to record the moment of the encounter of the gametes with the other mating type gamete and subsequent clump or mating pair formation in one shot. Figure 2c shows one of the chance observations. When the gamete encountered the other gamete (55 ms in Fig. 2c), the gamete denoted by the asterisk turned to the lower left (175 ms). Then contact between the two gametes occurred at the flagellar tips (190 ms in Fig. 2c). The gametes continued to contact with the other gamete at their flagella during forward swimming (190–220 ms in Fig. 2c), but the
attachment at the flagellar tips finally detached in this gamete pair.

In some mating pairs, two gametes became attached to each other at the distal part of their flagella and at the cell anterior (Fig. 3a). The gametes continued to beat their flagella and rub the cell body against the other gamete. The adhesion of the flagella was so strong that the flagella were not separate from each other during flagellar movement. No backward swimming was observed during this process. In most cases, the contact site of the two cell bodies was fixed to the anterior end of the cell bodies (Fig. 3b, c). The gametes remained stuck together, even though they continued to move their flagella and oscillated their cell bodies. Their beat pattern was flagellar beat with undulatory waves produced at the flagellar base being propagated toward the tip, which takes ca. 15 ms. However, the waveform of the mating gametes was slightly different from that of the free-swimming gametes (Figs. 1d and 3b); the flagella of the mating gametes were mainly extended but still partly bended at their tips at the later stage of the wave propagation (15 ms in Fig. 3b). Finally, two attached flagella aligned parallel and beat synchronously in one of the two flagellar pairs or both pairs (Fig. 3c). Contact of two gametes was also observed using FE-SEM. Figures 3d and 3e show an FE-SEM image of mating gametes 1 min after mixing the gametes. The two gametes were attached to each other at the anterior end of the cell bodies (Fig. 3d) and the distal part of the flagella (Fig. 3d, e), confirming the results of high-speed video observations. Finally, the gamete pair lay side-by-side with their longitudinal axes nearly parallel to one another and became a planozygote (Fig. 4a). Occasionally, three cells attached at the anterior end of the cell one another. Figures 4b and 4c respectively portray FE-SEM and high-speed video images of such cells. These cells continued to beat their flagella in the clump, in which two gametes maintained mutual contact at the flagellar tips (0–15 ms in Fig. 4c). Although we were unable to follow the fate of these cells in the clump, it is probable that gamete fusion might sometimes occur among three gametes because planozygotes with six flagella were sometimes observed after mixing the gametes (Fig. 4d).

Discussion

High-speed video microscopy of the C. spiralis fertilization process revealed that the fertilization proceeds in order from the initial contact between the flagella of the opposite mating type gametes to cell adhesion and fusion between the anterior portion of the gamete cell bodies. The initial contact between mating gametes involves the flagellar tips of both mating types. Subsequent cytoplasmic adhesion between the compatible gametes takes place nearly simultaneously with the adhesion of the flagella. We were unable to observe the stage from flagellar adhesion to cell fusion in one shot, probably because this step proceeded very rapidly. The fertilization process of C. spiralis closely resembles those of U. arasakii (Miyanura 2004), C. moewusii (Brown et al. 1968), and C. reinhardtii (Friedmann et al. 1968). In common with these species, the flagella of the gamete play an important role for gamete–gamete recognition and adhesion. The only difference from Chlamydomonas is that the gamete lacks a cell wall and the duration of copulation is very short. This feature is common to U. arasakii. In C. moewusii and C. reinhardtii, gamete pairs might form instantaneously when sexually activated compat-
ible populations are mixed, whereas the establishment of the cytoplasmic contact by the elongation of fertilization tubules from the mating structure (cell fusion apparatus) takes a few minutes (Brown et al. 1968, Friedmann et al. 1968, Goodenough and Weiss 1975). This feature stands in sharp contrast with C. spiralis and U. arasaikii, for which the fertilization tubules are not present in the gametes and for which the cytoplasmic adhesion between the compatible gametes takes place nearly simultaneously with agglutination of the flagella. This fact might explain why the duration of copulation in the gametes of these marine green macroalgae is very short.

In marine green macroalgae, the adhesion and fusion site of the gamete is generally restricted to the cell anterior under the basal portion of the papilla, where the mating structure is reportedly present (Melkonian 1980, Mogi et al. 2008, Miyamura et al. 2015). Although the mating structure has not been reported in C. spiralis, it is expected to be present in this alga because the putative mating structure is present in the gamete of closely related species, Cladophora dalmatica (Floyd et al. 1985) and because cell fusion always started from the cell anterior under the basal portion of the papilla in C. spiralis. If the cell fusion in C. spiralis occurs in the region of mating structures, then the results of the present observations suggest that the signal for the induction of gamete–gamete adhesion and fusion will be transmitted rapidly from the flagellar tip to the plasma membrane of the mating structure because adhesion of flagellar tips and cell bodies occurred in this order in most of the mating pairs almost simultaneously after mixing the gametes.

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