Behavior of Flagella and Eyespot during Fertilization of Anisogametes in *Caulerpa racemosa* (Forsskål) J. Agardh var. *laetevirens* (Montagne) (Ulvophyceae, Chlorophyta) Revealed by Field Emission Scanning Electron Microscopy

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Summary Respective fates of male and female flagella and the eyespot during fertilization of ulvophycean alga *Caulerpa racemosa* (Forsskål) J. Agardh var. *laetevirens* (Montagne) were studied using field emission scanning electron microscopy (FE-SEM). FE-SEM allowed visualization of the eyespot of the biflagellate gamete. The female gamete has one eyespot on a posterior position of the cell body; the male gamete does not. The female gamete eyespot was oriented to the direction of the plane of the flagellar beat. The female gamete is larger than the male one and has the only eyespot. For those reasons, the author followed the fate of the flagella and eyespot during fertilization. When both gametes were mixed, the initial cytoplasmic contact and cell fusion between them usually took place at the anterior end slightly below the flagellar base. In most mating pairs, the female gamete fuses at the left side of the eyespot and male gamete at a cell surface that is perpendicular to the plane of the flagellar beat. As fusion proceeds, the gamete pair is transformed into a quadriflagellate planozygote in which two flagella each from the male and female gametes point in nearly the same direction. In particular, the no. 2 flagellum of the female gamete and one male flagellum were directed to the eyespot. These observations indicated that cell fusion at the particular area of the gamete is important for proper arrangement of the flagella and eyespot in the planozygote.

Key words *Caulerpa*, Eyespot, Fertilization, Flagella, Gamete, Planozygote.

In isogamous and anisogamous green algae, fusing gametes usually become entangled in their flagella and frequently meet end to end. Nevertheless, in most cases, the two cells subsequently become laterally apposed to one another: fusion occurs in this position (Fritsch 1935). The cell fusion sites of gametes in some green algae have been observed with electron microscopy to be differentiated to specialized cell membrane (*Chlamydomonas reinhardtii* Dangeard; Friedmann *et al.* 1968, Cavalier-Smith 1975, Goodenough and Weiss 1975, *Ch. moewusii* Gerloff; Triemer and Brown 1975, *Hydrodictyon reticulatum* (L.) Lagerh.; Marchant and Pickett-Heaps 1971). In *Ch. reinhardtii*, this portion of the plasma membrane is designated as the “mating structure” (Goodenough and Weiss 1975). This structure has been reported in subsequent studies of other green algae (e.g., Melkonian 1980, 1981, O’Kelly *et al.* 1984, Hori 1988), but it has not been found in some green algae (Gaffal and Schneider 1978, Roberts *et al.* 1981).

The mating structure occupies a specific locale in the gamete (Goodenough and Weiss 1978, Melkonian 1984). Its position is presumably defined by microtubular roots that radiate out from the basal bodies (*Ch. reinhardtii*; Goodenough and Weiss 1978, *Ulva lactuca* L.; Melkonian 1980, *Bryopsis lynchbyei* Hornemann (female gamete); Melkonian 1981, *Ulvaria obscura* (Kütz.) Gayral var. *blyttii* (Aresch.) Blid., *Monostroma bullosum* (Ruth) Thur., *M. greville* (Thur.) Wittr., *M. oxyspermum* (Kütz.) Doty; O’Kelly *et al.* 1984, *Spongomorpha duriuscula* (Ruprecht) Collins; Miyaji and

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Hori 1984, *Collinsiella cava* (Yendo) Printz; Nakayama and Inouye 2000 and *Bolbocoleon piliferum* N. Pringsheim; O'Kelly *et al.* 2004). All mating structures that have been investigated ultra-structurally have been linked to one type of microtubular root: the d root.

In *Ch. reinhardtii*, the mating type plus structure is associated with the 2d root, whereas the minus structure is in contact with the 1d root, creating an asymmetric localization of the mating structure or the cell fusion site (Holmes and Dutcher 1989). Similar asymmetry has been observed in other green algae including *Co. cava* (Nakayama and Inouye 2000), *Ulva arasakii* Chihara (Miyamura *et al.* 2003), *Enteromorpha compressa* (L.) Nees (Miyamura 2003), *Bo. piliferum* (O'Kelly *et al.* 2004), and *Nephroselmis olivacea* Stein (Suda *et al.* 2004).

As an explanation for the asymmetric arrangement of the cell fusion site, Holmes and Dutcher (1989) speculated that this asymmetry ensures that the gamete fuse with a fixed polarity such that their two eyespots - one each from a male and a female gamete - lie on the same side of the zygote, and that phototactic steering mechanisms (Kamiya and Witman 1984) are not compromised.

Furthermore, in *Bryopsis maxima* Okamura, in which the male gamete has no eyespot, cell fusion usually occurs between the anterior left side of the eyespot in the female gamete and a broader face of the male gamete (Miyamura *et al.* 2005). This observation suggests the possibility that the cell fusion site of the gamete occupies a specialized position even if one gamete does not have an eyespot. However, this possibility has not been substantiated in other green algae in which only the female gamete has the eyespot.

Caulerpa racemosa (Forsskål) J. Agardh var. *laetevirens* (Montagne) is a monoecious species of ulvophycean siphonous alga that lives in the littoral zone of the tropical and temperate coast. The matured thallus of this alga releases anisogamous gametes, in which only the female gamete has an eyespot (Enomoto and Ohba 1987). Therefore, in this study, the author observed fertilization of *C. racemosa* var. *laetevirens* using field emission scanning electron microscopy.

Materials and methods

Plant material

Thalli of *Caulerpa racemosa* (Forsskål) J. Agardh var. *laetevirens* (Montagne) were collected at Amami-Oshima, Kagoshima Prefecture, Japan, between June 2003—June 2004. Samples were brought back to the laboratory and kept in a tank of seawater provided with air through diffusers under natural daylight conditions for several days. Subsequently, each thallus was placed in a separate vessel containing seawater and was maintained under a 12:12 light/darkness photoregime, ca. 30 $\mu\text{mol}/\text{m}^2/\text{s}$, at 22°C until liberation of the gametes. Within one month of collection, cytoplasm of all parts of a thallus, except in most of the rhizoidal portion, began to accumulate to form a network. Viscous material containing biflagellate male and female gametes was released through discharge tubes and precipitated on the bottom of the vessel. Male and female gametes mated immediately after discharge.

Scanning electron microscopy

One volume of sediment containing male and female gametes were mixed on a Nuclepore polycarbonate membrane (Whatman Japan KK, Tokyo, Japan) which was coated with 0.1% poly (L)-lysine (Sigma Chemical Co., St. Louis, MO, USA) and fixed with one volume of fixatives (6% glutaraldehyde, 3% NaCl, 0.1 M cacodylate buffer, adjusted to pH 7.1) at 0, 1, 5, 10 min after mixing. Cells were fixed for 1 h at room temperature. After removing the supernatant, the cells on the membrane were fixed with fixatives (3% glutaraldehyde, 3% NaCl, 0.05 M cacodylate buffer, adjusted to pH 7.1) for 1.5 h and then washed in a series of 0.05 M cacodylate buffer solutions containing 3, 2, 1, and 0% NaCl; each step took 30 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 with a freeze-dryer (VFD-21S; Vacuum Device Corp., Ibaraki, Japan) and coated with platinum-palladium in a Hitachi ion sputter-coating unit E102 (Hitachi High-Technologies Corp., Tokyo, Japan). Observations were made at 5 kv using a JSM6330F field emission scanning electron microscope (JEOL, Tokyo, Japan). The SEM images were exported to Adobe Photoshop and mounted using Adobe Illustrator. After preparation for FE-SEM, the cells were ca. 72% the size of living cells.

Results and discussion

Gametes of *Caulerpa racemosa* (Forsskål) J. Agardh var. *laetevirens* (Montagne) were pear-shaped (Figs. 1 and 2). The anterior end of the gametes displayed a papilla with two isokont flagella protruding from its opposite sides (Figs. 1 and 2). In accordance with other recorded cases of anisogamy, the smaller cells will be regarded as “male” while the larger will be “female”. Measurement by FE-SEM showed that male gametes were $4.5 \pm 0.4 \mu\text{m}$ (mean $\pm$ SD) ($n=50$) long and $1.8 \pm 0.2 \mu\text{m}$ ($n=50$) wide; female gametes were $5.5 \pm 0.4 \mu\text{m}$ ($n=50$) long and $2.2 \pm 0.2 \mu\text{m}$ ($n=50$) wide. In both gametes, the anterior end of the papilla was depressed, forming a groove (arrows in Figs. 1 and 2). The groove was oriented roughly perpendicular to the plane of the beat of the flagella.

The female gamete has an eyespot; the male does not (Enomoto and Ohba 1987, Figs. 1 and 2). Its position in the female gamete is easily recognizable using FE-SEM (Fig. 2) because of its oval shape and smooth surface (Hori 1981). Using FE-SEM, one can faintly ascertain the lipid globules that comprise the eyespot (Fig. 3). They are located directly beneath the cell membrane and two chloroplast envelopes.

The eyespot is on one side of the cell; it is posterior of the female gamete correspondent to the plane of the beat of the flagella. The long axis of the eyespots is always parallel to the longitudinal axis in female gametes (Fig. 2). A streak extends down to the cell posterior from the flagellar base and attaches to the left side of the eyespot (arrowheads in Figs. 2 and 3). This streak may correspond to the 2s root because the gamete of *Caulerpa* has four cruciately arranged microtubular root system. In addition, its eyespot is associated with the 2s and 2d root (Hori 1981), which extends down to the cell posterior from the basal body and is located directly beneath the cell membrane, as in other siphonous green algae (Melkonian and Robenek 1984, Kreimer 2001). Therefore, the left and right flagella in Fig. 2 correspond respectively to no. 1 and no. 2 flagella in the numbering convention of Moestrup and Hori (1989). This numbering of flagella is based on the eyespot position, which is probably consistent in advanced chlorophytes (Melkonian and Robenek 1984, Holmes and Dutcher 1989, Melkonian 1989, Kreimer 2001). However, this numbering system is inapplicable to the male gametes because they have no eyespot.

The fate of each gamete and the eyespot can be followed during gamete fusion because FE-SEM images of the size of cell body and the presence or absence of the eyespot are characteristic of female and male gametes. Furthermore, the no. 1 and no. 2 flagella in the female gamete are identifiable, and it is possible to determine the precise orientation of the gametes during fertilization. When both gametes were mixed, the initial cytoplasmic contact and cell fusion between the two gametes usually occurred at the anterior end slightly below the flagellar base (Figs. 4 and 5). In most mating pairs, the gametes fused by folding together in a jackknife-like manner. The female gamete always fused at the anterior left side of the eyespot and the male gamete at one of the cell surfaces perpendicular to the plane of the flagellar beat. This fusion site was positioned between the 2s and 1d root in the female gamete (Figs. 4 and 5). Thereby, the gamete pairs were situated side-by-side with their longitudinal axes nearly parallel (Fig. 6), as observed previously in this (Enomoto and Ohba 1987) and other species of *Caulerpa* using light microscopy (Miyake and Kunieda 1937, Goldstein and Morrall 1970). This fusion pattern was observed in 128 mating pairs ($n=131$) fixed

Figs. 1–8. Field emission scanning electron microscope images showing fertilization of gametes of *C. racemosa* var. *laetevirens*. 1) Male gamete. 2) Female gamete. 3) Enlarged image of the female gamete's eyespot region. 4) Mating pair viewed from the eyespot side of the female gamete. Attachment of the male gamete (♂) to the anterior left side of the eyespot in the female gamete (♀). 5) Mating pair viewed from the opposite side of the eyespot of the female gamete. 6) Planozygote continuing cell fusion. 7) Planozygote after completing cell fusion. No. 2 flagellum of the female gamete and one male flagellum are directed to the eyespot. 8) Papilla of the planozygote. As the cytoplasmic fusion proceeded, two papillae fused to one papilla and the grooves in the papillae also fused to one long groove. Cells were fixed at 0 min (1–3, 5, 6 and 8), 1 min (7) and 5 min (4) after mixing sediments containing male and female gametes. E: eyespot, ♂: Male gamete, ♀: Female gamete, #1: no. 1 flagellum. #2: no. 2 flagellum. Arrows: Groove in the center of the papilla. Arrowheads: 2s root (Figs. 2–4) or 1s root (Fig. 5). Double arrowheads: 1d root. Scale bars, 1 μ m (Figs. 1, 2, 4–7), 500 nm (Figs. 3 and 8).

0–10 min after mixing the gamete sediment. Fusion at the right side of the eyespot in the female gamete was observed in three mating pairs. Gametes fusion was rapid: the stages shown in Figs. 4–6 were apparent in the specimen fixed immediately after mixing gamete sediment.

At a very early stage of gamete adhesion and fusion, papillae from both male and female gametes were easily distinguishable in the gamete pair (Figs. 4 and 5). As the cytoplasmic fusion proceeded from the cell anterior to posterior (Fig. 6), two papillae fused to one papilla and the grooves in the papillae also fused to one long groove (arrow in Fig. 8). Finally, the gametes fused completely (Fig. 7) and one flagellum each from the male and the female gametes became a pair (Fig. 8). In particular, the no. 2 flagellum of the female gamete and one of the male flagella are pointed in the direction of the eyespot (Fig. 7). In contrast to this, the no. 1 flagellum of the female gamete and the other flagellum of the male oriented to the nearly opposite direction to the eyespot: consequently, their mutual angle became ca. 90°.

The present study using FE-SEM revealed the behavior of male and female flagella and the eyespot during fertilization of *C. racemosa* var. *laetevirens*. These results are consistent with previous observations of *B. maxima* that the female gamete is always fused at the anterior left side of the eyespot with a broader surface of the male gamete (Miyamura *et al.* 2005). In both species, the cell fusion site of the female gamete probably corresponds to the cell anterior between 1d and 2s roots. This position is almost identical to the cell fusion site of the female gamete of *U. arasaki* (Miyamura *et al.* 2003), *E. compressa* (Miyamura 2003), a sex type of *Co. cava* (Nakayama and Inouye 2000) and *Bo. piliferum* (O'Kelly *et al.* 2004), the mating type minus of *Ch. reinhardtii* (Holmes and Dutcher 1989) and the mating type plus gamete of *N. olivacea* (Suda *et al.* 2004). In contrast to those gametes, the cell fusion site of the opposite sex gamete occupies the opposite positioning in these algae. Therefore, it is likely that two types of gametes, in terms of the position of cell fusion site, exist in these green algae. Nakayama and Inouye (2000) proposed that the presence of opposite positioning of the mating structure in each sex gamete is a universal feature of advanced chlorophytes. Results of this study support their proposal. Furthermore, they indicate that the cell fusion site of the four ulvophyceean female gametes (Ulvaes: *U. arasaki* and *E. compressa*, Caulerpales: *B. maxima* and *C. racemosa* var. *laetevirens*) always occupies the common spatial position. Therefore, it is likely that such features are a characteristic that specifies females in Ulvaes and Caulerpales.

In contrast to the algae described above, Goodenough (1991) suggested that both mating types of *Ch. eugametos* Moewus produce apical papillae that fuse in a far more symmetrical fashion than that observed with *Ch. reinhardtii*. Therefore, the cell fusion site of *Ch. eugametos* might not occupy an asymmetrical position between the opposite sex gametes because the cytoplasmic bridge forms medially rather than to one side of the basal apparatus. It remains unknown whether *Ch. eugametos* is the exceptional case to the asymmetric localization of the cell fusion site in the opposite sex gamete of isogamous and anisogamous algae.

In the planozygote of *C. racemosa* var. *laetevirens*, two flagella—one each from the male and the female gamete—become a pair; particularly, the no. 2 flagellum of the female gamete and a male flagellum points to the direction of the eyespot (Fig. 7). Such arrangement of flagella in the planozygote has also been observed in *B. maxima* (Miyamura *et al.* 2005) and may be important for phototactic steering. The planozygotes of both species show negative phototaxis and migrate away from the light source (Enomoto and Ohba 1987, Miyamura *et al.* 2005). Phototactic tracking of the algal flagellate is based on the cell's ability to sense the direction of a light beam (Foster and Smyth 1980). As the cells swim, they rotate along their long axis and the eyespot apparatus, as a photoreceptor, scans the light intensity around the cell. In *Ch. reinhardtii*, two flagella have different Ca²⁺ sensitivity and the turning mechanism of the cell appears to be inherent to *trans* and *cis* flagella (Kamiya and Witman 1984), which correspond respectively to no. 1 and no. 2 flagella. The physiological difference between no. 1 and no. 2 flagella has not been established in ulvophyceean algae,

such inherence of the flagella may be present for a correct turning mechanism of the swarmer. Therefore, co-operating functions of the male and female flagella may be necessary for the correct turning response at the proper point during phototactic steering. This hypothesis seems to explain the flagellar arrangement of the planozygote observed in *C. racemosa* var. *laetevirens* and *B. maxima*. Finally, it is likely that the cell fusion site of the gamete occupies a specialized position for proper arrangement of flagella and the eyespot in the planozygote, even if one mating partner has no eyespot.

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