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Citation	Phycological research, 64(1), 19-25 https://doi.org/10.1111/pre.12115
Issue Date	2016-01-28
Doc URL	http://hdl.handle.net/2115/64400
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Three-dimensional organization of flagellar basal apparatus in *Ectocarpus* gametes

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Running title: Flagellar basal apparatus in *Ectocarpus*

Abstract

The flagellar basal apparatus of the brown alga *Ectocarpus siliculosus* was re-investigated in details using transmission electron microscopy and electron tomography. As a result, three-dimensional structures with spatial arrangement of bands and microtubular flagellar rootlets were observed. Fibrous structures linking the anterior flagellar basal body to the major anterior rootlet (R3) or the bypassing rootlet was newly discovered in this study. A direct attachment from the minor anterior rootlet (R4) to the anterior and posterior basal bodies was also discovered, as were attachments from the minor posterior rootlet (R1) to the deltoid striated band and from the major posterior rootlet (R2) to the posterior fibrous band. The microtubular flagellar rootlets were connected to the bands and to the anterior or posterior basal body. These bands may have a role in maintaining the spatial arrangement of the anterior and posterior flagellar basal bodies and the microtubular flagellar rootlets. A numbering system of the basal body triplets was established by tracing axonemal doublets in the serial sections. From these observations, the precise position of two flagellar basal bodies, bands, and flagellar rootlets was determined.

Key words: anterior flagellum; flagellar basal apparatus; brown algae; *Ectocarpus* gametes; posterior flagellum

Introduction

Sophisticated flagellum and basal apparatus regulate the locomotion of swarmers, including various unicellular algae, protozoa, gametes and zoospores of algae, and animal sperm. Motile gametes and zoospores of brown algae usually have two different flagella: the anterior flagellum and the posterior one (Kawai 1992). Flagella of brown algal gametes are crucial for phototaxis (Kawai & Kreimer 2000; Matsunaga *et al.* 2010), chemotaxis (Geller & Müller 1981; Maier & Müller 1990; Kreimer 1994; Kinoshita *et al.* 2015), and the first contact between male and female gametes (Müller & Falk 1973).

The ultrastructure of flagella and basal apparatus of the brown algae has been examined extensively (Manton 1956, 1957, 1959, 1964; Manton & Clarke 1950, 1951, 1956; Cheignon 1964; Henry & Cole 1982a, b; O'Kelly & Floyd 1984; Motomura 1989; O'Kelly 1989; Maier 1997a, b). The flagellar basal apparatus of *Laminaria digitata* (Hudson) J. V. Lamouroux zoospores and *Ectocarpus siliculosus* (Dillwyn) Lyngbye male gametes were observed by O'Kelly and Floyd (1984), O'Kelly (1989) and Maier (1997b), respectively, who made schematic representations with the number of microtubules (MTs) in each of the microtubular flagellar rootlets and the morphology of several bands connecting two flagellar basal bodies. Moestrup (2000) possessed a new numbering system of the flagellar basal bodies and the rootles in various algal groups. These authors obtained basic structural information on flagellar basal apparatus of the brown algal swarmers using conventional transmission electron microscopy and chemical fixation methods. However, such methods have a limited resolution, and a higher resolution would be necessary to observe the microstructural features in detail.

It is known that numbering system for doublet MTs of the axoneme can

provide a better understanding of the spatial arrangement of flagellum and basal apparatus, as well as the flagellar movement (Wooly 2010). In this study, we performed electron tomography to re-observe the spatial relation of each flagellar component in *E. siliculosus* gametes under a high-resolution. Moreover, a numbering system comprising nine triplet MTs of basal bodies was adapted to identify the precise position of these flagellar components.

Materials and methods

Plant material and laboratory culture

A gametophyte of *E. siliculosus* (Es400 male) was obtained from Dr. A. F. Peters (Bezhin Rosko, France) and was cultured in half strength PES medium (Provasoli, 1968) under cool-white fluorescent lamps ($30\text{--}40 \mu\text{mol m}^{-2} \text{ s}^{-1}$) at 15°C in long day condition (14 h light:10 h dark). To induce the release of gametes, fertile gametophytes bearing mature plurilocular gametangia were transferred to Petri dishes filled with new culture medium. After 2–3 days, gametes were released from plurilocular gametangia 2 h after light on. Liberated gametes were collected into 1.5 mL microtubes using a micropipette.

Transmission electron microscopy

Freeze fixation and freeze substitution were conducted as previously described by Nagasato and Motomura (2002) and Ueki *et al.* (2008) with some modifications. Released male gametes were spun down ($6000 g$, 1 min, 4°C) (Himac CF 15R, Hitachi, Tokyo, Japan) and the pellet of gametes was placed on formvar-coated gold loops (5–10 mm diameter). Samples were rapidly frozen by placing the loop into liquid propane

pre-cooled to -180°C. The loop was transferred to liquid nitrogen, and then submerged in cooled acetone (-85°C) containing 4% OsO₄. After 2 days, the samples were transferred to -20°C for 2 h, followed by 4°C for 2 h, and finally to the room temperature. They were detached from the formvar-coated gold loops, washed several times with acetone at room temperature, and infiltrated with Spurr's epoxy resin at room temperature using the following conditions: 20% resin in acetone for 12 h; 30% for 5 h; 40% for 5 h; 50% for 12 h; 70% for 5 h; 80% for 5 h; and 100% for 2 days. The final two steps of infiltration were carried out in desiccators. Samples were finally embedded in Spurr's epoxy resin on dishes made of aluminum foil. Successfully frozen samples were selected using a light microscope, and serial sections were cut using a diamond knife (Micro Star, Micro Star Tech, TX, USA) on an ULTRACUT ultramicrotome (Reichert-Jung, Depew, NY, USA). The serial sections were mounted on formvar-coated slot grids, stained with TI blue (Nissin EM Corporation, Tokyo, Japan) for 40 min at room temperature, followed by lead citrate (Reynolds, 1963) for 10 min at room temperature. Finally, they were observed using JEM-1011 electron microscope (JEOL, Tokyo, Japan).

Electron tomography

Serial sections (90–190 nm) were cut for JEM-2100 electron microscope (JEOL, Tokyo, Japan). The sections were mounted on formvar-coated one-slot grid and stained with 5% hafnium tetrachloride in 100% methanol for 40 min, followed by lead citrate staining for 10 min at room temperature, or stained with 2% uranyl acetate for 1 hr at 60°C and lead citrate for 3 min at room temperature. After staining, the side of the grid carrying sections was coated with formvar to sandwich them. Then, 15 nm

colloidal gold particles were attached to both sides of the grid, and the grids were coated with carbon on both sides. These samples were used to obtain tilted images for tomographic analysis.

Image acquisition and analysis

Samples were placed on a tilt-rotate specimen holder and observed using the JEM-2100 electron microscope with 200 kV acceleration voltages. Specimens were oriented from -60° to 60° at 1° interval and images were captured using a Veleta digital camera with a resolution of 2,048 × 2,048 pixels (Olympus Soft Imaging Solutions, Münster, Germany) at ×50,000, ×80,000 and ×100,000 magnification, and the pixel sizes were 1.254 nm, 0.784 nm and 0.627 nm, respectively. Dual-axis electron tomography was performed around two orthogonal axes in order to obtain high resolution images and to reduce the “missing wedge” artifact (Mastronarde 1997).

Using the colloidal gold particles as fiducial markers, the tilt series (2D images) were aligned and combined to reconstruct a double-axis tomogram using the computer software IMOD (Kremer & Mastronarde 1996).

Results and discussion

In this study, we observed the ultrastructure of the flagellar basal body in *E. siliculosus* from 23 tomograms. Our schematic representation of the flagellar apparatus is shown in Figure 1. The terminology by O’Kelly and Floyd (1984), Maier (1997b) and Moestrup (2000) were adopted for the components of the flagellar basal apparatus. The flagellar basal body was located at the center of the cell’s ventral surface, in close association with the nucleus, chloroplast, and Golgi body. The longitudinal axes of two

basal bodies, as well as the anterior flagellar basal body (AB) and the posterior flagellar basal body (PB) were arranged more or less parallel to the ventral surface of the cell, but not on the co-planar position (Fig. 2). When viewed from the dorsal face of the cell, the PB always appeared to the left of the AB (Fig. 2a–h). The PB was dorsally shifted by approximately one-third of the basal body diameter (Fig. 2i–k).

The spatial arrangements of all rootles were analyzed using more than three tomograms. All microtubular rootlets, a major anterior rootlet (MAR, R3), a minor anterior rootlet (mar, R4), a major posterior rootlet (MPR, R2), a minor posterior rootlet (mpr, R1), and a bypassing rootlet (BR), were closely associated with AB and PB (Fig. 2a–h). The mar, which was composed of a single microtubule (MT), originated from the left side of AB and extended anterior along the left side of AB (Fig. 2d, e) from the dorsal view. The angle between the longitudinal axis of AB and the mar measured about 20°, and the basal part of the mar directly attached to the proximal part of the AB and PB (Fig. 2d, e). Cap structure could not be detected at the basal end of MT of the mar. The mpr, which was composed of a single MT, originated from a deltoid striated band (DB) (O'Kelly & Floyd 1984; Maier 1997b), and extended toward the left side of the cell at an angle of about 10° to the longitudinal axes of PB (Fig. 2d–g). The original MAR component was composed of approximately 8 MTs and attached to AB with darkly stained material (Maier 1997b). This structure could be clearly observed by electron tomography (Fig. 2d, e, double arrowheads). Connecting structures of AB-BR and AB-mar were detected (Fig. 2i–k). The MPR originated at the posterior fibrous band (pfb) near the PB and was composed of three MTs (O'Kelly and Floyd 1984; Maier 1997b) (Figs 2h, 3b, c).

When viewed from the dorsal side, striated strap-shaped band (SB) was

observed. It spread from the proximal parts of AB to PB (Figs 2b, c, 4a, b), and the darkly stained V-shaped segment was conspicuous (Fig. 2b, c). The deltoid striated band (DB) was observed between AB and PB (Figs 2f, 3d, 4f–h). It was mainly attached to the proximal part of PB and mpr. It showed a regular striation pattern, which had a periodicity in the range of 29–44 nm from analyses of two tomographs. All flagellar rootlets were attached to these bands and connected to AB or PB. Therefore, these bands might have a role to maintain spatial arrangement of AB, PB, and the rootlet MTs. A connecting structure between PB and the chloroplast was observed in two tomograms (Fig. 4i–k). Katsaros *et al.* (1993) suggested that in *E. siliculosus* gametes nucleus-basal body connector, DB and SB contained centrin, which is Ca^{2+} -mediated contractile protein (Salisbury *et al.* 1984, 1988). Nagasato and Motomura (2004) reported that centrin was localized between the proximal sites of AB and PB in gametes of *Scytoniphon lomentaria* (Lyngbye) Link.

In our study, basal plates of AB and PB showed the same appearance and existed at their distal parts. They were plate-like structures with small holes attached to the plasma membrane. Moreover, electron dense material accumulated at the center of the plate, and attached to the central pair of flagellar axoneme (Figs 2f, g, 3c).

There are two numbering systems for designating doublet MTs in the flagellar axonemes (Lin *et al.* 2012). In sea urchin sperm flagella, axoneme of doublet No. 1 was decided as a doublet MT that located at an equal distance from the central pair, and numbering proceeded in the direction of the dynein arms (Afzelius 1959; Sale 1986). The remaining eight doublets were consecutively numbered in a clockwise direction when observing the flagellum from the proximal to the distal sides. A bridge exists between doublet MTs No. 5 and No. 6. Another numbering system is based on the

flagella of the green alga *Chlamydomonas reinhardtii* Dangeard (Hoops & Witman 1983; Bui *et al.* 2009). In *Chlamydomonas* flagella, beak-like structure can be detected in three out of nine doublet MTs. One of these three doublet MTs, marked as No.1, characteristically lack outer dynein arm, and eight doublets were consecutively labeled in clockwise direction, similar to the case of sea urchin's sperm. There is a bridge between No.1 and No. 2 doublet MTs. Melkonian (1984) widely adopted this flagellar numbering system for the green algae. However, flagellar axoneme of the brown algal swarmers does not possess morphologically distinct doublet MTs. Fu *et al.* (2013) defined doublet No. 1 as a line extending through the central pair of MTs of the axoneme, at the level of the paraflagellar body of PF, and consecutively labeled the rest of the doublet MTs in a clockwise direction, when observing the flagellum from the distal to the proximal sides. In the present study, we consecutively labeled doublet MTs in a clockwise direction when observing the flagellum from the proximal side to the distal one, after doublet No. 1 was decided as a doublet MT that located at an equal distance from the central pair (Afzelius 1959).

A numbering system of the basal body triplets could be adopted by tracing flagellar axonemal doublets in serial sections (Fig. 5). PB existed against the side of No. 7–9 triplet MTs of AB (Fig. 5e, f). The MAR and BR were passing near the side of No. 2–4 of AB and No. 5–7 of AB, respectively (Fig. 5c, e, f). The mar existed between No. 8 and No. 9 of AB (Fig. 5f). The MPR and BR were passing near the side of No. 5–7 of PF (Fig. 5g). The DB and SB were attached to No. 7–8 and No. 9–1 of AB, respectively (Fig. 5e, f). Fibrous material was elongated from a part of the chloroplast to No. 1 of PB (Fig. 5h). Eyespot and crystallized material region in the paraflagellar body (Fu *et al.* 2013) were facing to the side of No. 6–9 of PF (Fig. 5i). These data will facilitate the

precise mapping of previously identified structures, as well as novel basal apparatus proteins.

Acknowledgements

We would like to thank Dr. Tatyana Klochkova, Kamchatka State Technical University, Russia, who kindly gave us many valuable comments and corrected our English.

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Figure legends

Fig. 1. A schematic representation of the flagellar apparatus from the dorsal side of male gamete of *Ectocarpus siliculosus*. Transverse images of flagellar axonemes are from the distal to the proximal parts of the anterior flagellar (AF) and posterior flagellar (PF), respectively, with a new numbering system for the nine doublet MTs of the flagellar axoneme. AB, anterior flagellar basal body; BR, bypassing rootlet; DB, deltoid band; MAR, major anterior rootlet, mar, minor anterior rootlet; mpr, minor posterior rootlet; PB, posterior flagellar basal body; pfb, posterior fibrous band; SB striated strap-shaped band.

Fig. 2. Longitudinal and transverse sections of AB and PB. (a–h, i–k). Two serial tomograms, viewed from the dorsal side of male gametes (0.627 nm-thick tomogram slices). Arrowheads and double arrowheads in d, e indicate the connection of mar to AB and PB, and MAR to AB, respectively. Arrowhead in g shows electron dense material at the center of the basal plate. (i–k) Three tomogram slices (0.784 nm-thick) viewed from the distal side of AB. Arrows show the connection between AB and each of microtubular rootlets (mar, MAR and BR). Arrowheads indicate BR and MAR. Ch, chloroplast; G, Golgi body.

Fig. 3. Four tomogram slices of PB (0.627 nm-thick) viewed from the dorsal side of the male gamete. Arrowhead in c indicates electron dense material at the center of the basal plate.

Fig.4. Longitudinal sections of AB and PB. (a–d) Four tomogram slices showing SB

(0.627 nm-thick). (e–h) Four tomogram slices showing DB and pfb (0.784 nm-thick). (i–k) Three tomogram slices showing the connection between PB and chloroplast (1.25 nm-thick). Ch, chloroplast; G, Golgi body; N, nucleus.

Fig. 5. Transverse sections of AB and PB with a conventional transmission electron microscope observation. The new numbering system for outer doublets of MTs is adopted. (a–d) Serial cross-sections of AB. (e, f) Two serial sections of AB and PB. (e) Section showing the position of BR, MAR and DB. Inset is the enlarged image. (f) Section showing the position of BR, MAR, mar and SB and mar. Inset is the enlarged image. (g, h) Two serial sections of PB. An arrow in inset shows connection between chloroplast and PB. (i) Section showing position of electron-dense material and crystallized material in paraflagellar body of PF.









