Carotenoids in the eyespot apparatus are required for triggering phototaxis in *Euglena gracilis*

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Received 6 September 2019; accepted 8 October 2019; published online 20 October 2019.
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SUMMARY

Carotenoids are the most universal and most widespread pigments in nature. They have played pivotal roles in the evolution of photosensing mechanisms in microbes and of vision in animals. Several groups of phytoflagellates developed a photoreceptive organelle called the eyespot apparatus (EA) consisting of two separable components: the eyespot, a cluster of carotenoid-rich globules that acts as a reflector device, and actual photoreceptors for photobehaviors. Unlike other algal eyespots, the eyespot of Euglenophyta lacks reflective properties and is generally considered to act as a shading device for the photoreceptor (parflagellar body, PFB) for major photomovements. However, the function of the eyespot of Euglenophyta has not yet been fully proven. Here, we report that the blocking carotenoid biosynthesis in *Euglena gracilis* by suppressing the phytoene synthase gene (*crtB*) caused a defect in eyespot function resulting in a loss of phototaxis. Raman spectroscopy and transmission electron microscopy suggested that Eg*crtB*-suppressed cells formed eyespot globules but had a defect in the accumulation of carotenoids in those packets. Motion analysis revealed the loss of phototaxis in Eg*crtB*-suppressed cells: a defect in the initiation of turning movements immediately after a change in light direction, rather than a defect in the termination of cell turning at the appropriate position due to a loss of the shading effect on the PFB. This study revealed that carotenoids are essential for light perception by the EA for the initiation of phototactic movement by *E. gracilis*, suggesting one possible photosensory role of carotenoids in the EA for the phototaxis.

Keywords: phototaxis, eyespot, carotenoid, microfluidic device, video tracking, electron microscopy.

INTRODUCTION

Carotenoids, a group of isoprenoid compounds with 40-carbon backbones, are the most universal and most widespread pigments in nature. They are synthesized in various photosynthetic and non-photosynthetic organisms, including bacteria, archaea, fungi, algae, and land plants. In those organisms, carotenoids play crucial roles in light harvesting and photoprotection in photosynthesis, the scavenging of reactive oxygen species, the modulation of chloroplast membranes, the attraction of pollinators and seed dispersers, and growth and development as the precursor of bioactive carotenoid derivatives (abscisic acid, strigolactones, and apocarotenoids) in land plants (Esteban *et al.*, 2015). Carotenoids are also widely distributed in the animal kingdom, but animals cannot synthesize them *de novo*. They are an essential nutrient for vertebrates and humans as the precursor of retinol (vitamin A) and its
metabolites (retinoids) to maintain normal growth, development, reproduction, epidermal integrity, immune system, and vision (Blomhoff and Blomhoff, 2006).

In the eyes of animals, retinol is converted into retinal, which then binds to opsin apoproteins as the chromophore to form cone opsins and rhodopsins (vonLintig, 2012). In addition to animals, various organisms carry rhodopsins and utilize them as photosensing mechanisms (Govorunova et al., 2017). For example, channel rhodopsins of the unicellular chlorophyte alga *Chlamydomonas reinhardtii* (CrCCRs, referred to as *CrChR1* and 2) have been shown to function as photoreceptors for the phototaxis and photoshock response that cause membrane depolarization, resulting in changes in flagellar beating patterns and waveforms (Sineshchekov et al., 2002; Ueki and Wakabayashi, 2018).

In addition to retinal in rhodopsins, intact carotenoids also play an important role in photosensing byphototactic microalgae. Several groups of phytoflagellates have developed a characteristic photoperceptive organelle called the eyespot apparatus (EA), which contains carotenoids (Jékely, 2009; Kreimer, 2009; Colley and Nilsson, 2016). The EA consists of two separable components: a cluster of carotenoid-rich lipid globules called the eyespot (or stigma) in the strict sense and actual photoreceptors for the photobehaviors of algal cells (Kreimer, 2009). EAs are found in many flagellated algae that exhibit three-dimensional phototaxis, including chlorophytes, chromalveolates, and excavates (Jékely, 2009; Kreimer, 2009; Colley and Nilsson, 2016). Evolutionarily, EAs are considered to have been acquired independently by those phototactic algae and thus vary considerably in shape, size, and position in the cells among algal groups (Kreimer, 1994; Jékely, 2009; Colley and Nilsson, 2016). In chlorophytes, the carotenoid-rich globules of the eyespot are hexagonally packed in interthylakoid spaces of the chloroplast facing the plasma membrane, forming one to several layers that act as a quarter-wave interference reflector (Foster and Smyth, 1980; Hegemann and Harz, 1998). Surprisingly, the Warnowiaceae dinoflagellates developed the most elaborate EA, an eye-like ocelloid. The ocelloid resembles camera-type eyes in animals and is built from subcellular analogs to a cornea made of mitochondria, a pigmented retinal body made of plastids, a focusing lens, and iris rings (Gavelis et al., 2015). So far, the ocelloid is one of the most complex known subcellular structures of organisms.

Photosynthetic Euglenoids (Euglenophyta) also possess an eyespot that consists of a cluster of reddish-orange granular structures, and this alga was therefore named for ‘eu (beautiful) + glena (eyes)’ in Latin. *Euglena* sp. was first described by Antonie van Leeuwenhoek in 1670s (Lane, 2015). Since then, Euglenophyta has long been one of the best studied model organisms for photobehavior research. Whereas Euglenophyta acquired a chlorophyte-derived chloroplast through a secondary endosymbiosis event (Turmel et al., 2009), the eyespot of Euglenophyta is localized in the cytosol separately from, but near, the photoreceptor organelle called the paraflagellar body (PFB) near the base of the major flagellum (Walne and Arnott, 1967; Kivic and Vesk, 1972). Unlike most flagellated algae, the photoreceptor for photomovements of Euglenophyta has been predicted to be a flavoprotein but not a rhodopsin (Hegemann, 1997). Finally, photoactivated adenylyl cyclase (PAC), which contains a flavin chromophore, was identified from the PFB of *Euglena gracilis* and was shown to be the photoreceptor for major photobehaviors of this alga, including negative and positive phototaxis and the step-up photophobic response (abrupt changes in swimming direction in response to a sudden increase in light intensity, resulting in photoavoidance) (Iseki et al., 2002; Ntefidou et al., 2003). Judging from its arrangement in Euglenophyta cells, the eyespot is generally considered to act as a shading device to enable the photoreceptor PAC in the PFB to discriminate the light direction during helical swimming (Kreimer, 2009). However, the function of the eyespot of Euglenophyta and its significance in the phototaxis of this alga has not yet been fully proven and remains controversial (Häder, 1993; Ntefidou et al., 2003; Häder and Iseki, 2017). Elucidating photosensing mechanisms that are independently developed in different phylogenetic groups of phototactic algae and their significance in the photobehaviors of those algae would contribute to understanding the diverse history of vision evolution. As mentioned above, carotenoids play a central role in photosensing mechanisms in a wide variety of organisms, from bacteria to animals, and must be one of the key players in the evolution of vision and eyes. We previously reported that the suppression of the phytoene synthase gene (*crtB*), whose product catalyzes the first committed and rate-limiting step in carotenoid synthesis by the suppression of carotenoid-rich globules but had a marked decrease in cellular carotenoid content and yielded a colorless cell phenotype (Kato et al., 2017). Following the previous study, we speculated that the blocking of carotenoid synthesis by the suppression of *crtB* in *E. gracilis* may cause some defects in the formation or function of the eyespot and in the photobehaviors of this alga. To test this hypothesis, we investigated the ultrastructure of the eyespot in *crtB*-suppressed cells of *E. gracilis* using transmission electron microscopy and examined the photobehaviors of those cells by a video tracking technique in a microfluidic chip (Ozasa et al., 2014; Ozasa et al., 2017).

Here, we showed that Eg*crtB*-suppressed cells were able to form packet structures of eyespot globules but had a defect in the accumulation of carotenoids in those globules. We also revealed that Eg*crtB*-suppressed cells exhibited no negative phototaxis, whereas the photoavoidance
Euglena requires carotenoids to trigger phototaxis

To elucidate the impact of the unusual form of eyespot globules on the phototactic response, we carried out a motion analysis of the photobehaviors of Egcrtb-suppressed cells using a microfluidic device and a real-time video tracking system (Ozasa et al., 2017). Figure 3(a) shows a schematic representation of the experimental setup of the microfluidic device for motion analysis. In brief, a cell suspension of E. gracilis (c. 0.5 µl containing 20–50 cells) was loaded in a cylindrical polydimethylsiloxane (PDMS) microchamber (2.5 mm diameter, 100 µm depth) and irradiated with actinic light (465–475 nm) from two opposite sides of the microchamber (LED A and B shown in Figure 3a). The light source direction was alternated every 30 sec. The swimming cells were tracked from the top of the microchamber under visible light in the yellow-red region (570–700 nm). Diehn (1969) reported that the action spectra of positive/negative phototaxis of this alga exhibited a major broad peak at c. 375/365 nm and several peaks in the visible region at 480 nm/412, 450, and 480 nm and that no phototactic responses of E. gracilis were observed at wavelengths greater than 550 nm. Therefore, in our motion analysis, we used 570–700 nm for the observation light and 465–475 nm for the actinic light. The number of traced pixels of cell swimming per unit time was quantified as the trace momentum (TM). Cells swimming straight produce line-shape traces with relatively small overlaps and large TM values (Figure 3b). The photoavoidance response, an abrupt change in swimming direction in response to a sudden increase in light intensity (Iseki et al., 2002), is observed as an onsite rotation movement in this system and produces dot-like traces with relatively large overlaps, resulting in relatively small TM values (Figure 3c). In this study, the phototactic response was evaluated by the spatial oscillation amplitude of the center of the cell distribution (the center of gravity), synchronized to switching of the light direction (Figure 3d).

Our motion analyses clearly showed that the control cells of E. gracilis responded to the switching of light direction and swam along the light vector, moving away from the light sources (i.e. negative phototaxis) (Figure 4 middle row and Movie S1). When the control cells were illuminated with blue light at 360 µmol photon m⁻² sec⁻¹, the
Figure 1. Detection of the Raman bands of carotenoids and autofluorescence of flavin at the anterior end of *Euglena gracilis* cells. (a) Bright-field microscopy of the cell appearance of control and *crtB*-suppressed cells (*Eg*crtB-KD) of *E. gracilis* (Top). Bottom: magnified images of the anterior ends of control and *crtB*-suppressed cells (dotted squares shown in the top panel images). The arrowhead indicates the eyespot. Scale bars, 20 µm. (b) Autofluorescence at 520 nm (arrowheads) in control and Eg*crtB*-suppressed cells. Scale bar, 20 µm. (c, d) Raman spectroscopy of the eyespots of control and Eg*crtB*-suppressed cells. (c) Region of interest (ROI, blue open circle) in Raman spectroscopy of the anterior end of the control (Top) and Eg*crtB*-suppressed cells (Bottom). Middle: magnified image of an ROI at the anterior end of the control cells. Arrowheads indicate the eyespots. (d) Raman shift spectra of authentic β-carotene and ROIs at the anterior ends of control and Eg*crtB*-suppressed cells. The Raman spectra were acquired from ROIs 2 µm in diameter (blue open circle shown in panel c). Data are the average value of each ROI in technical duplicate and representative of at least five individual cells.
centrifugal for the position of the swimming traces moved exclusively on the y-axis, which was parallel to the direction of the light vector (Figure 5a). However, these phototactic responses were not observed in EgcrB-suppressed cells under blue light at 360 μmol photon m⁻² sec⁻¹ (Figures 4 and 5a; Movie S2). We did not observe any periodic movement of the centrifugal position on either the x-axis or y-axis in EgcrB-suppressed cells, but EgcrB-suppressed cells swam actively in the presence or absence of blue light (Figures 5a and 6a; Movie S2). We further characterized the photobehaviors of E. gracilis cells under various actinic light intensities in a range from 160 to 2780 μmol photon m⁻² sec⁻¹. When the control cells were irradiated with blue light at 160, 360, and 720 μmol photon m⁻² sec⁻¹, the centrifugal position periodically oscillated with an amplitude of 32–35 pixels on the y-axis (Figure 6b, control). In other words, the control cells exhibited an obvious phototactic response to those irradiation conditions. However, we did not observe oscillation of the centrifugal position of EgcrB-suppressed cells under blue light irradiation (Figure 6b, EgcrB-KD). When irradiated with actinic light above 1470 μmol photon m⁻² sec⁻¹, the control cells showed onsite rotation movement, that is, a photoavoidance response (Figure 6c; Movie S1). Under actinic light in the same intensity range, EgcrB-suppressed cells also rotated on the spot in the same way as the control cells (Figure 6c and Movie S2). In both the control and EgcrB-suppressed cells, the percentage of onsite-rotating cells with respect to total swimming cells increased gradually in a light intensity-dependent manner and reached c. 60–80% under actinic light above 1470 μmol photon m⁻² sec⁻¹ (Figure 6c). Consistent with this result, the average swimming speed, which was calculated from the length of swimming traces per unit time, was gradually decreased in a light intensity-dependent manner (Figure 6a). These results indicated that EgcrB-suppressed cells retain a photoavoidance response similar to that of the control cells.

We observed the motion of the cells in more detail at higher magnification at the time points when the light direction was switched. When the source direction of the actinic light (160 μmol photon m⁻² sec⁻¹) was switched to the opposite side, the control cells made both clockwise and counterclockwise turns several times and then swam away from the light source (Movie S3). Conversely, when irradiated with actinic light at the same intensity, EgcrB-suppressed cells were unresponsive to the switching of the light direction and did not turn (Movie S4). To compare the turning response upon switching of the actinic light direction, we quantified the angle of the swimming tracks of cells as shown in Figure 7(a). In this study, we define the initiation of turning movements as the transition of swimming mode from straightforward swimming [also called helix swimming in some literature (e.g., Tsang et al., 2018)] to onsite rotation [also called localized spinning (e.g., Tsang et al., 2018)]. Straightforward swimming is characterized by a mostly constant swimming speed with small fluctuations in swimming direction, whereas onsite rotation is characterized by a swimming speed near zero with continuous/frequent changes in the direction of the cell body.
and Movie S4. These observations clearly indicated that the direction of the light was changed, as shown in Figure 5. Pressed cells continued straightforward swimming when the direction (Figure 7b, Eg

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noids were reported to be the major carotenoids in the eyespot globules of E. gracilis (Heelis et al., 1979). Common carotenoids are shown to have similar Raman bands at 1500–1510 cm

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We previously revealed that β-carotene, neoxanthin, diadinoxanthin, and diatoxanthin were the major carotenoids in E. gracilis cells and that the suppression of EgcrtB by RNA interference caused marked decreases in the cellular content of those four carotenoids (Kato et al., 2017). Among them, β-carotene, diatoxanthin, and diadinoxanthin were reported to be the major carotenoids in the eyespot globules of E. gracilis. The Plant Journal published by Society for Experimental Biology and John Wiley & Sons Ltd, The Plant Journal, (2020), 101, 1091–1102

Figure 3. Motion analysis of phototactic and photoavoidance responses of Euglena gracilis in the microfluidic device. (a) Schematic illustration of the experimental setup of a microchamber containing E. gracilis cells. Blue light illumination was provided from both sides of the microchamber (LED A, B). (b, c) Schematic illustration of swimming traces of E. gracilis cells swimming straight (b) and exhibiting a photoavoidance response (c). (d) Schematic illustration of the transition of the centroid position (red filled circle) of swimming traces in motion analysis

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DISCUSSION

Structure of eyespot in crtB-suppressed cells of Euglena gracilis

The eyespot of Euglenophyta consists of packets of carotenoid-rich globules and is positioned in the cytosol separately from the actual photoreceptive PFB that is located near the base of the major flagellum (Walne and Arnott, 1967; Kivic and Ves, 1972; Kreimer, 1994). Kivic and Vesk (1972) reported that the individual eyespot globules of E. gracilis measure c. 0.4 μm in diameter. The results of these previous studies are compatible with our observations of eyespot structures in control cells (Figure 2). Our TEM observations showed that EgcrtB-suppressed cells possess monolayered packet structures in the cytosol near the flagellum. These packets contained several globules surrounded by a monolayer envelope with a diameter similar to that of the eyespot granules in the control cells. Considering the structural similarities of those packets in EgcrtB-suppressed cells to the eyespot globules, those structures appeared to be the eyespots of the colorless cells. The origin of the membrane of the packet structure and eyespot globules has not yet been identified. Kivic and Vesk (1972) reported that the eyespot membrane of E. gracilis had no structural similarity to chloroplasts and that the formation of eyespot structure was independent of chloroplast development in the alga. Walne and Arnott (1967) observed that the endoplasmic reticulum is closely associated with the developing eyespot packets. In EgcrtB-suppressed cells, we observed enlarged vesicles and monolayer membrane structures in the cytosol close to the reservoir (Figure 2g,h). This observation indicated that those structures might originate from the endoplasmic reticulum and that the formation of abnormal eyespot globules in EgcrtB-suppressed cells does not seem to result from a reduction in chloroplast development.

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presence of carotenoids in the eyespots of the control cells. However, no characteristic Raman signals arising from carotenoids were detected in the EgcrtB-suppressed cells (Figure 1c,d). Judging from Raman spectroscopy and TEM observations, EgcrtB-suppressed cells seemed to be defective in the accumulation of carotenoids in eyespot globules due to the blocking of carotenoid biosynthesis, although those cells were able to form the packet structures of eyespot globules in the cytosol.

**Motion analyses of photobehaviors of EgcrtB-suppressed cells**

The structure of EAs and their functions in photobehavior have been well studied in chlorophytes, especially in *C. reinhardtii*. The typical eyespot of chlorophytes consists of one to several layers of hexagonally arranged carotenoid-rich globules in the interthylakoid spaces of the chloroplast and is, because of those arrangements, considered to function as a quarter-wave interference reflector (Foster and Smyth, 1980; Hegemann and Harz, 1998). In fact, Ueki et al. (2016) experimentally demonstrated that layers of carotenoid droplets in the eyespot of *C. reinhardtii* shield the photoreceptor channel rhodopsins (CrChR1 and 2) for phototaxis on the plasma membrane from light coming from inside the algal cell, which acts as a convex lens. Furthermore, Ueki et al. (2016) revealed that the eyespot is essential for the determination of the phototactic sign of *C. reinhardtii*, using a mutant *lts1-211* with a defect in the phytoene synthase gene.

In contrast with the eyespots of chlorophytes, the eyespot globules of Euglenophyta are irregularly and loosely arranged in the cytosol (Walne and Arnott, 1967; Kivic and Vesk, 1972) and seem to lack reflective properties, whereas eyespot reflection has been detected in all other algal groups examined to date (Kreimer, 1994). Therefore, for decades, the eyespot of Euglenophyta has been speculated to be device that shades the photoreceptor for phototaxis. In *E. gracilis*, several studies have noted that the absorption spectra of eyespot globules and eyespot pigments closely match the action spectrum for phototaxis of this alga, indicating the similarity to carotenoid properties (Batra and Tollin, 1964; Diehn, 1969; Benedetti et al., 1976). Benedetti et al. (1976) assessed the pigment concentration in the eyespot globules of *E. gracilis* at c. 0.02–0.04 μmol and estimated the visible light transmittance of the eyespot to be c. 50–70%. Spectroscopic analysis revealed that the screening efficiency of the eyespot of *E. gracilis in vivo* is limited in visible light in a range of 400–600 nm and that those absorption spectra clearly matched the properties of carotenoids (Benedetti et al., 1976). Furthermore, Heelis et al.
(1979) revealed that major carotenoids (β-carotene, diatoxanthin, and diadinoxanthin) comprised c. 60% of the eyespot pigments. It can easily be suggested that carotenoid screening pigments might be essential for the regular function of the eyespot in the phototaxis of *E. gracilis*. Accordingly, we hypothesized that *Eg*crtB-suppressed cells possessing abnormal eyespots might show a different phototactic sign to that of the control cells or might turn in a random direction regardless of the light vector.

Unexpectedly, *Eg*crtB-suppressed cells exhibited no phototactic response, although the cells showed a phototactic response (Figures 4 and 5; Movie S2). Ntefidou *et al.* (2003) demonstrated that silencing of the PAC gene of *E. gracilis* completely suppressed both negative and positive phototaxis as well as the step-up photophobic response (photoavoidance response) of this alga. Our TEM analyses indicated that *Eg*crtB-suppressed cells possessed PFBs, as observed in the control cells (Figure 2). Under excitation at 488 nm, we clearly detected a speckle of autofluorescence (emission wavelength c. 520 nm) at the anterior end of *Eg*crtB-suppressed cells, indicating the presence of flavin and PAC in the base of the major flagellum (Figure 1b). Furthermore, our motion analyses showed that *Eg*crtB-suppressed cells exhibited a photoavoidance response similar to that of the control cells without any marked shift in the dose–response relationship for the photoavoidance response to a lower light intensity range (Figure 6a,c). Hence, these data indicated that PFB functions properly in *Eg*crtB-suppressed cells and that the eyespot and carotenoids seemed to be irrelevant to the photoavoidance response of *E. gracilis* cells.

The motion analysis revealed that, immediately after the switching of the projected light direction, the control cells turned in both directions (clockwise and counterclockwise) several times and then swam away from the light source, resulting in negative phototaxis. Conversely, *Eg*crtB-suppressed cells showed no turning response when the light direction was switched to the opposite side. These observations indicated that the eyespot is involved in the phototaxis of *E. gracilis*. In the past, as discussed by Walne and Arnott (1967), two possible functions of the eyespot of Euglenophyta had been proposed: (i) eyespot globules function as the primary photoreceptor in the phototaxis of Euglenophyta (photoreceptor hypothesis), and (ii) the PFB is the photoreceptor for phototaxis and is shaded by the eyespot as the swimming cell changes its orientation (shading hypothesis). Later, the photoreceptor hypothesis for the eyespot was rejected upon the discovery of PAC, which is responsible for the negative and positive phototaxis of *E. gracilis* as well as the step-up photophobic response (Iseki *et al.*, 2002; Ntefidou *et al.*, 2003).

In both the step-up photophobic response and phototactic responses, the primary role of PAC is the formation of cyclic AMP, which activates a downstream signaling pathway, resulting in cell turning through modulation of flagellar beating (Häder and Iseki, 2017). Based on the shading hypothesis, without shading carotenoid pigments in the eyespot, all PACs in the PFB might be continuously...
Figure 6. Dose–response curve of (a) swimming speed, (b) amplitude of centroid movement, and (c) percentage of cells showing onsite rotation among total swimming cells. The control and crtB-suppressed cells of Euglena gracilis were irradiated with blue light at 0, 160, 360, 720, 1470, or 2780 µmol photon m$^{-2}$ s$^{-1}$. Blue light illumination was alternately provided from both sides of the microchamber every 30 sec for 30 min. Each open circle and open square indicate the average value of data for the middle 20 min of the measurement time. Blue (control) and red (EgcrtB-KD) line symbols indicate the median of data from 36 (0 µmol photon m$^{-2}$ s$^{-1}$) and 6 (160–2780 µmol photon m$^{-2}$ s$^{-1}$) individual measurements.

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photosensitized in helical swimming. If the eyespot functions as a shading device for the photoreceptive PFB in phototaxis, EgcrtB-suppressed cells would keep turning because the PACs would not become desensitized during irradiation with actinic light. However, we did not observe such responses in the motion analyses of EgcrtB-suppressed cells (Movie S4). In contrast, EgcrtB-suppressed cells were insensitive to changes in the light source direction and did not show any apparent changes in the swimming direction (Figure 7b; Movie S4). The classical shading hypothesis does not fully explain our observations in EgcrtB-suppressed cells. All things considered, it is reasonable to assume that carotenoids in the eyespot of E. gracilis have an additional role in initiating the phototactic response in addition to a shading function for the PFB. Carotenoids in the eyespot could protect the eyespot or EA against reactive oxygen species generated by the flavin chromophore of PAC proteins. We cannot rule out the possibility that carotenoids also accumulate in the PFB. Carotenoids might modulate the local stabilization and inactivation of PACs by quenching the excitation energy or scavenging of reactive oxygen species in the PFB. Otherwise, as proposed previously, we may assume that the eyespot is a photoreceptive organelle for the phototactic response of E. gracilis and that carotenoids in the eyespot (or the EA) have a photosensory role.

In conclusion, the present study clearly indicated by single-cell tracking and motion analysis techniques that the eyespot is necessary for the phototaxis of E. gracilis. Furthermore, we revealed that carotenoids are essential for intact EA function, allowing directional light perception to initiate the phototactic movement of E. gracilis. These findings suggest the hypothesis that carotenoids in the EA have a photosensory role in phototaxis.

**EXPERIMENTAL PROCEDURES**

**Biological materials**

crtB-suppressed cells of E. gracilis Klebs (strain Z) were prepared as reported in our previous study (Kato et al., 2017) with some modifications. In total, 5 × 10^6 cells were electroporated twice at 0.4 kV with 15 µg of double-stranded RNA (dsRNA) directed toward a part of the EgcrtB cDNA (DDBJ accession no. LC062707) in 100 µl of Cramer-Myers medium (Cramer and Myers, 1952) in a 0.2-cm gap cuvette (Bio-Rad, Hercules, CA, USA) with an electroporator (Micropulser, Bio-Rad). For the electron microscopy, cells treated with and without EgcrtB-dsRNA were inoculated in 100 ml of CM medium containing 0.1% ethanol at an initial concentration of 3 × 10^6 cells ml$^{-1}$ and cultured for 7 days at 25°C under continuous illumination at 55 µmol photon m$^{-2}$ s$^{-1}$ with agitation (90 rpm), as we reported previously (Kato et al., 2017). For the motion analysis, algal cells were cultured in 15–20 ml of CM medium containing 0.1% ethanol at room temperature without agitation and illuminated at c. 10 µmol photon m$^{-2}$ s$^{-1}$ for 10 h daily.
Confocal laser microscopy

The control and EgcrB-suppressed cells were treated with 20 µm cytochalasin B for 30 min at room temperature to halt euglenoid movements. Subsequently, the algal cells were treated with 100 µm nickel chloride to stop flagellar movements immediately before observation. The cells were observed under excitation at 488 nm under a TCS SP8X (Leica, Wetzlar, Germany) equipped with a highly flexible pulsed white-light laser (commercial, WLL). To emphasize the cell shape of the control cells (colored) and EgcrB-suppressed cells (colorless) in the bright-field images, two different powers of WLL were used: 21% and 14% for the control and EgcrB-suppressed cells, respectively. The emission spectra of flavin and chlorophylls were detected at 520-550 nm using a conventional photomultiplier tube (PMT), respectively. For the detection of flavin autofluorescence, chlorophyll autofluorescence was eliminated by time-gated fluorescence imaging (gate-on time: 0.3–12.0 nsec) (Kodama, 2016). The fluorescence images were acquired at 400 Hz (400 lines sec⁻¹) with 3x line accumulation.

Raman spectroscopy

*Euglena gracilis* cells immobilized in 0.6% agarose were used for Raman spectroscopy analyses. Data were collected using a JASCO NRS-4100 laser Raman spectrometer (JASCO, Tokyo, Japan) in the spectral range of 750–1750 cm⁻¹ at a resolution of 1 cm⁻¹. A 532 nm laser at 3.4 mW was used for excitation. For each spectrum, the acquisition time was 3 sec, and the number of averaged spectra was two. The β-carotene standard was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

Electron microscopy

*Euglena gracilis* cells treated with and without EgcrB-dsRNA were fixed with 1.7% glutaraldehyde in sodium cacodylate buffer (50 mm, pH 7.0) for 2 h at 25°C. After washing with the same buffer, cells were embedded in low-melting agarose and cut into 1 mm cubes. The samples were post-fixed in 1% osmium tetroxide in the same buffer for 2 h at 25°C and subsequently dehydrated in an ethanol series (25, 50, 75, 90, 100%). The samples were embedded in Epon812 resin (TAAB, Berkshire, UK). For the TEM study, 60–80 nm thick ultrathin sections were cut with a diamond knife on an ultramicrotome EM UC7 (Leica) and mounted on formvar-coated one-slot copper grids. Sections were stained with 4% uranyl acetate for 12 min and Reynold’s lead citrate for three min at 25°C. Sections were examined on a JEM-1400 (JEOL, Tokyo, Japan) at 80 kV.

**Figure 7.** Quantification of the cell turning response in the phototaxis of *Euglena gracilis*. (a) Time-sequential images of the control cell before and after the change in light direction. Images are excised from Movie S3 for a typical single cell. The actinic light direction was switched from the bottom side of the image to the top side at 12.3 sec. Longer (shorter) white lines indicate the body direction of the head (tail) of *E. gracilis* cells. (b) Histogram of swimming direction distribution 10 sec before, 2 sec after, and 10 sec after the change in light direction evaluated for the experiment shown in Figure 5(a) with 360 µmol photon m⁻² sec⁻¹. The swimming direction was binned into 10 sections of π/5 starting from the x-axis. Thirty events of light switching from LED B to A were evaluated by the algorithm described in Figure S1.
to negative phototaxis. Therefore, the value Yc oscillated temporarily with an oscillation period of 60 sec, as shown in Figure 5. The strength of the phototactic response was evaluated from this temporal oscillation of the cell distribution along the y-axis, that is Yc, by applying Fourier transformation. The only component for the 60-sec oscillation period was calculated by Fourier transformation from the temporal data series of Yc, and the amplitude was used as a measure of phototactic strength. Note that the amplitude depends on swimming speed as well as phototactic directionality. When the swimming speed is increased/decreased by photokinesis effects or the photovoidance response, it also affects the phototactic strength used in this study.

We automatically deduced the swimming speed and percentage of rotating cells from the trace images by an algorithm we developed previously (Ozasa et al., 2015). First, the swimming traces in a trace image were sectioned into single traces by rectangles, as shown in Figure S1(a). The number of rectangles obtained was regarded as the number of cells in the image. Second, the positions of the head and body of each trace were determined from the pixel positions of the trace, as shown in Figure S1(b). The swimming speed of the cell was calculated from the two positions. Third, the aspect ratio (L/D) and filling factor (FF) of the rectangle were calculated as shown in Figure S1(c). The aspect ratio was the ratio of the horizontal length L to the vertical length D of the rectangle. The FF was defined as the ratio of the pixel number of the trace in the rectangle to that of the whole rectangle area. We empirically determined a criterion to categorize the swimming motion of the cell into straightforward swimming and rotating. The criterion was that the rotating cell satisfied the two conditions below simultaneously:

\[
1/\alpha < L/D < \alpha \]  
(1)

\[
FF > \beta \]  
(2)

The values of alpha and beta were 1.7 and 0.38, respectively, which were determined empirically to optimize the categorization. The accuracy of the categorization was typically 80-90% (Ozasa et al., 2015). By using the algorithm above, the swimming speed and swimming motion (straightforward or rotating) were derived for each cell in the trace image. We averaged the values for all the cells in the image and time averaged the values for each experimental condition.

ACKNOWLEDGEMENTS

The authors thank Professor S. Song at Hanyang University for microchip preparation. The authors are grateful to M. Wakahazi (RIKEN) for her kind support in performing transmission electron microscopy. A part of this work was supported by a grant from the Japan Society for the Promotion of Science [grant number 17K07945] to T. Shinomura, a grant from the Ministry of Education, Culture, Sports, Science and Technology [grant number 15K131014] to T. Shinomura, a grant for pioneering projects ‘Fundamental Principles Underlying the Hierarchy of Matter: A Comprehensive Experimental Study’ provided by RIKEN to K. Ozasa, and JST ERATO [grant number NPMJER1602] for M. Higuchi-Takeuchi, Y. Kodama, and K. Numata.

AUTHOR CONTRIBUTIONS

SK, KO, and TS designed the research. SK and KO performed most part of the experiments. YT, ST, and YK performed bright-field and confocal laser microscopic observations. MHT and KN performed Raman spectroscopy. MS and KT performed electron microscopy. MM and TS supervised the research. SK and KO wrote the manuscript.

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The Plant Journal published by Society for Experimental Biology and John Wiley & Sons Ltd, The Plant Journal (2020), 101, 1091–1102

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

DATA AVAILABILITY

The data that support the findings of this study are included within this article and in the supporting information files.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Algorithm for swimming speed evaluation and straightforward/rotating swimming categorization.

Movie S1. Traces of cell swimming of Euglena gracilis illuminated with blue light at 0, 360, or 2780 μmol photon m⁻² sec⁻¹ (LED current at 0, 2, or 16 mA, respectively).

Movie S2. Traces of cell swimming of EgrcrtB-suppressed cells illuminated with blue light at 0, 360, or 2780 μmol photon m⁻² sec⁻¹ (LED current at 0, 2, or 16 mA, respectively).

Movie S3. Clockwise and counterclockwise turns of E. gracilis cells immediately after switching of the light source direction.

Movie S4. Swimming movements of EgrcrtB-suppressed cells before and after switching of the light source direction.

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