Eyespot-dependent determination of the phototactic sign in *Chlamydomonas reinhardtii*

Noriko Ueki[a,b,1], Takahiro Ide[a,1], Shota Mochiji[c], Yuki Kobayashi[a], Ryutaro Tokutsu[d,e,f], Norikazu Ohnishi[d,2], Katsushi Yamaguchi[g], Shuji Shigenobu[e,g], Kan Tanaka[a,f], Jun Minagawa[d,e,f], Toru Hisabori[a,f], Masafumi Hirono[h], and Ken-ichi Wakabayashi[a,3]

[a]Laboratory for Chemistry and Life Science, Institute of Innovative Research, Tokyo Institute of Technology, Yokohama 226-8503, Japan; [b]Department of Biological Sciences, Graduate School of Science and Engineering, Chuo University, Tokyo 112-8551, Japan; [c]Department of Biological Sciences, Graduate School of Science, University of Tokyo, Tokyo 113-0033, Japan; [d]Division of Environmental Photobiology, National Institute for Basic Biology, Okazaki 444-8585, Japan; [e]Department of Basic Biology, Faculty of Life Science, SOKENDAI (The Graduate University for Advanced Studies), Okazaki 444-8585, Japan; [f]Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Chiyoda-Ku, Tokyo 102-0076, Japan; [g]Functional Genomics Facility, National Institute for Basic Biology, Okazaki 444-8585, Japan; and [h]Department of Frontier Bioscience, Hosei University, Tokyo 184-8584, Japan

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The biflagellate green alga *Chlamydomonas reinhardtii* exhibits both positive and negative phototaxis to inhabit areas with proper light conditions. It has been shown that treatment of cells with reactive oxygen species (ROS) reagents biases the phototactic sign to positive, whereas that with ROS scavengers biases it to negative. Taking advantage of this property, we isolated a mutant, *lts1-211*, which displays a reduction-oxidation (redox) dependent phototactic sign opposite to that of the wild type. This mutant has a single amino acid substitution in phytoene synthase, an enzyme that functions in the carotenoid-biosynthesis pathway. The eyespot contains large amounts of carotenoids and is crucial for phototaxis. Most *lts1-211* cells have no detectable eyespot and reduced carotenoid levels. Interestingly, the reversed phototactic-sign phenotype of *lts1-211* is shared by other eyespot-less mutants. In addition, we directly showed that the cell body acts as a convex lens. The lens effect of the cell body condenses the light coming from the rear onto the photoreceptor in the absence of carotenoid layers, which can account for the reversed-phototactic-sign phenotype of the mutants. These results suggest that light-shielding property of the eyespot is essential for determination of phototactic sign.

Chlamydomonas | eyespot | lens | phototaxis | carotenoids

The biflagellate unicellular green alga *Chlamydomonas reinhardtii* exhibits both positive and negative phototaxis (i.e., swimming toward and away from the light source) to inhabit areas with the proper light conditions for photosynthesis. The phototactic response is triggered by photoreception by an elaborate subcellular organelle, the eyespot (Fig. 1). This organelle is observed as an orange spot located near the cell equator. It contains the carotenoid-rich granule layers in the chloroplast and the channelrhodopsin photoreceptor proteins ChR1 and ChR2 in the plasma membrane (1–4). The carotenoid layers of the eyespot function as a light reflector (5).

Recent studies suggested that the *Chlamydomonas* phototactic pathway primarily consists of four steps: (*i*) photoreception by ChRs; (*ii*) excitation of the cellular membrane; (*iii*) increase in intraflagellar [Ca^{2+}]; and (*iv*) a change in the beating balance between the two flagella, i.e., the *cis*-flagellum (the one closest to the eyespot) and the *trans*-flagellum (the one farthest from the eyespot) (Fig. 1) (6–9). During step 1, the eyespot plays a crucial role in directional photoreception. ChRs localize to the plasma membrane over the carotenoid layers, which reflect and amplify the light signal coming from the outside of the cell (the "front side" of ChRs) while blocking the light from the inside of the cell (the "rear side" of ChRs) (Fig. 1). Rotation of the *Chlamydomonas* cell around its long axis during swimming, and light reflection and blocking at the carotenoid layers, produce a periodic modulation of the light intensity received by ChRs. This light signal modulation decreases in amplitude as the cell's swimming path becomes closer to parallel to the light beam. According to the prevailing theory, phototaxis results from the cell's response minimizing the amplitude of light signal modulation (5, 10).

There are several conflicting studies debating the importance of the reflective and absorptive properties of the eyespot in determining the direction (or "sign") of phototaxis by the cell (11). These properties are important because positive phototaxis requires that the *trans*-flagellum beat more strongly than the *cis*-flagellum when the eyespot is facing the light source, and vice versa. Based on the hypothesis put forward originally by Foster and Smyth (5), the asymmetric layered structure of the eyespot was thought to act as a quarter wave-plate, reflecting light from the front back onto the photoreceptors in the plasma membrane and blocking light from the back coming through the cell. Data collected by Schaller and Uhl (12), with cells held on micropipettes, was used to argue that reflection does little to enhance photoreception from the front and that the pigment granule layers do not shield the photoreceptors from the backside. These authors suggested that the chlorophyll pigments in the cell were responsible for absorbing "backside" light. In another study with cells lacking both chlorophyll and pigment granule layers, active photoreceptors were reconstituted in the plasma membrane with exogenously added retinal. Interestingly, the sign of phototaxis of these rescued,

Significance

The phototactic behavior of the unicellular green alga *Chlamydomonas reinhardtii* is thought to rely on photoreception by the eyespot apparatus. Here, we isolated an eyespot-less mutant that clearly exhibits phototaxis. Intriguingly, the phototactic sign (the direction of cell migration) in this mutant is opposite to that of the wild type after treatment with reagents that enhance the sign, a property that we also detected in previously reported eyespot-less mutants. The reversed phototactic-sign phenotype was attributed to the fact that the photoreceptors were exposed to condensed light from their rear side. This report demonstrates the importance of the eyespot, in which carotenoid layers shield the photoreceptors from light condensed by the cell body, which functions as a convex lens.

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[1]N.U. and T.I. contributed equally to this work.

[2]Present address: Institute of Plant Science and Resources, Okayama University, Kurashiki 710-0046, Japan.

[3]To whom correspondence should be addressed. Email: wakaba@res.titech.ac.jp.

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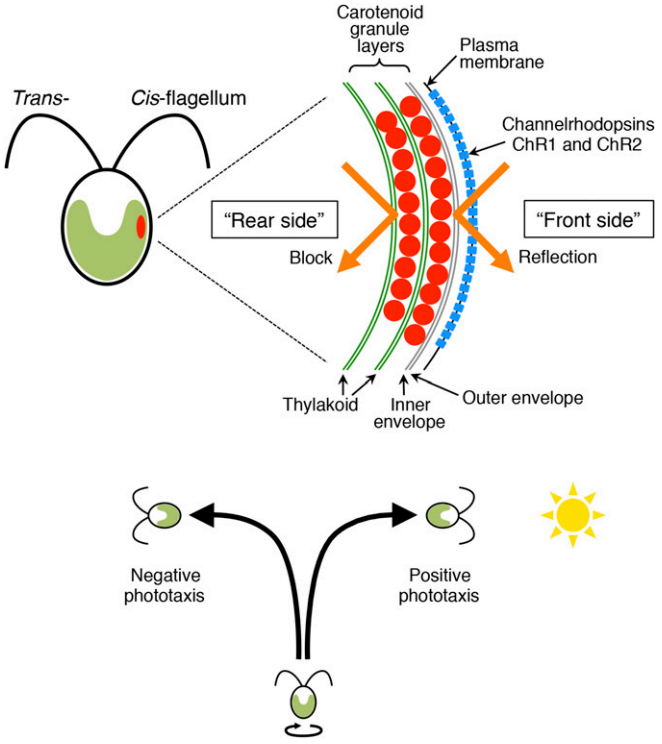

Fig. 1. Schematic diagrams of a *Chlamydomonas* cell and its phototactic behavior. (*Top*) The eyespot is located near the cell equator and contains the carotenoid granule layers (red) and photoreceptor proteins, channelrhodopsins (ChR1 and ChR2; blue). The carotenoid layers reflect a light beam (orange arrows) and amplify the light signal from the outside of the cell on ChR (the "front side") and block the light from the inside of the cell (the "rear side"). The flagellum closest to the eyespot is called the *cis*-flagellum, whereas the other one is called the *trans*-flagellum. Modified from refs. 24 and 41. (*Bottom*) As the cell swims with self-rotation, the eyespot apparatus scans the incident light around the cell's swimming path. After photoreception by the channelrhodopsins, the cell changes the beating balance of the two flagella and exhibits either positive or negative phototaxis (swimming toward or away from the light source, respectively).

clear cells was reversed relative to that of wild-type green cells (13). It was hypothesized that a "lens effect" or "focusing effect" of the transparent cell body was condensing light on the backside of the photoreceptors on the other side of the cell. However, it had been questioned earlier whether the refractive index of the cell was much different from the surrounding water, which would be required for the cell to act as a convex lens (5). Here, we sought to show whether green *Chlamydomonas* cells can act as lenses, because we found that several strains with missing eyespot granule layers, including newly isolated *lts1-211*, demonstrated a reversal in the sign of phototaxis.

Results

Isolation of a *Chlamydomonas* Mutant with a Reversed Sign of Phototaxis. Several years ago, we showed that cellular reduction-oxidation (redox) poise acts as a strong signal that determines the phototactic sign: Cells show positive phototaxis after treatment with reactive oxygen species (ROS), whereas they show negative phototaxis after treatment with ROS-scavenging reagents (14). Although the molecular basis of this redox-based sign switching of phototaxis remains to be clarified, the effects of ROS/ROS scavengers (hereafter referred to as "redox reagents") on the phototactic sign are intense. With the goal of isolating mutants defective in the signal transduction pathway affected by ROS, we screened for mutants defective in sign switching and isolated a mutant (*lts1-211*) exhibiting an opposite phototactic sign change (compared with the wild type) after treatment with redox reagents, i.e., positive phototaxis after treatment with ROS scavengers and negative phototaxis after treatment with ROS.

We generated a panel of mutants by UV irradiation and screened for cells showing an opposite sign of phototaxis to that of the wild

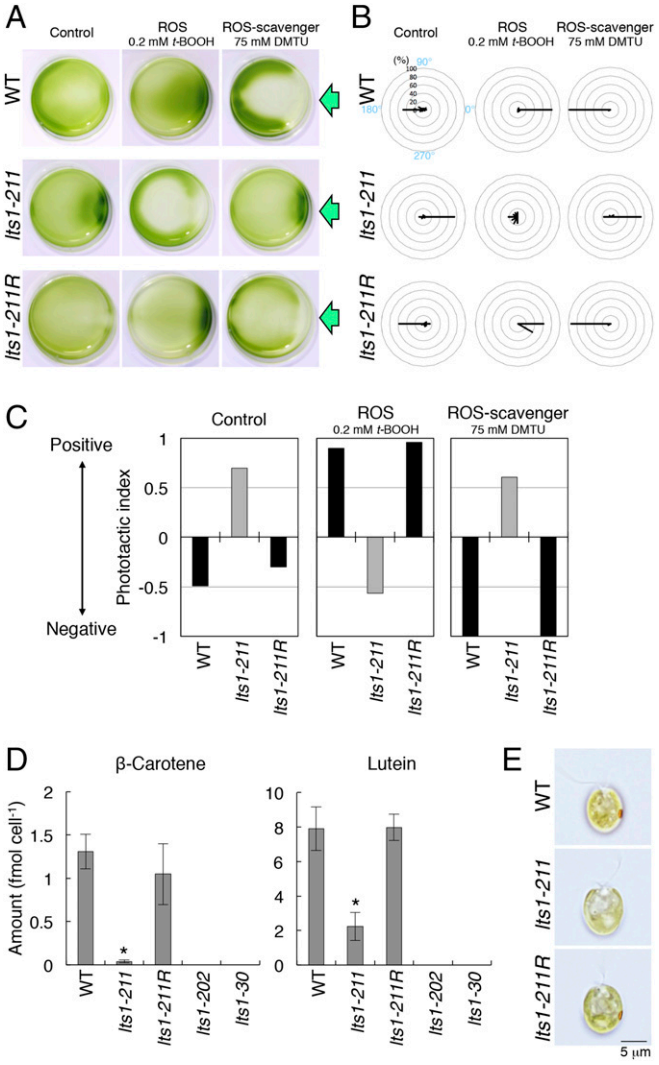

Fig. 2. The *lts1-211* mutant lacks eyespots and exhibits the opposite sign of phototaxis relative to the wild type. (*A*) Dish phototaxis assays of the wild type, *lts1-211*, and *lts1-211R* (rescued strain) with or without treatment with redox reagents. Cell suspensions in Petri dishes were photographed after illumination with a green light-emitting diode (LED) from the side for 5 min (green arrows). The areas without cells on the horizontal axis (e.g., ROS scavenger-treated *lts1-211R*) are likely caused by the photophobic responses of some cells. (*B*) Polar histograms representing the percentage of cells moving in a particular direction relative to light illumination from the right (12 bins of 30°; $n = 20$–30 cells per condition) for 1.5 s following 15-s illumination. (*C*) The sign of phototactic index in *lts1-211* (gray) is opposite to that of WT or *lts-211R* (black) with or without treatment with redox reagents. The phototactic index was calculated as an average value of $\cos\theta$ in *B*. When cells are not illuminated and swim in random directions, the phototactic index should be ~0. When 100% of cells show clear positive or negative phototaxis, the phototactic index is 1 or −1, respectively. (*D*) *lts1-211* produces less carotenoids than the wild type. β-Carotene and lutein levels in each strain (PSY null mutants *lts1-202* and *lts1-30* cells were grown in the dark) are shown [average values ± SEM for six (WT, *lts1-211* and *lts1-211R*) or three (PSY null mutants) independently prepared samples]. Asterisks represent significant differences ($P < 0.05$, paired *t* test). (*E*) Bright-field images of the wild-type, *lts1-211*, and *lts1-211R* cells. Note that *lts1-211* is eyespot-less.

Table 1. *Chlamydomonas reinhardtii* strains used in this study

Strain	Description	Source
WT	A progeny from the mating of two wild-type strains, CC124 (mt-) and CC125 (mt+), devoid of the *agg1* mutation	This study
CC124	A wild-type strain that carries *agg1-, nit1-, nit2-*, shows strong negative phototaxis and cannot grow on nitrate as sole nitrogen source (mt-)	33–36
CC125	Basic wild-type strain that carries *nit1-, nit2-* and cannot grow on nitrate as sole nitrogen source (mt+)	33–36
lts1-211	Point mutation in phytoene synthase	This study
lts1-30	Null mutant of phytoene synthase	16, 37
lts1-202 (a.k.a. *FN68)*	Null mutant of phytoene synthase	16, 38
eye1-1	Lacks eyespots during logarithmic growth; phototactic orientation impaired	39
eye2-1	Eyespots not formed; defect in thioredoxin-like protein	22, 23
eye3	Eyespots not formed; defect in putative ABC1 kinase	23, 24

type. After treatment with oxidizing reagents, wild-type cells exhibited strong positive phototaxis (Fig. 2 *A*–*C*) (14). We chose isolates showing negative phototaxis after treatment with 0.2 mM tertiary-butylhydroperoxide (*t*-BOOH), a ROS reagent. One clone always exhibited a reversed-phototactic sign after treatment with redox reagents: positive after treatment with ROS scavengers and negative after treatment with ROS (Fig. 2 *A*–*C*).

The mutation in this strain was mapped (by a PCR-based method) to a region (~131 kb) on chromosome 11 (Fig. S1*A*, see *SI Materials and Methods* for details) (15). We also performed whole genome sequencing of this mutant as well as wild-type strains (CC124 and WT, a progeny from the cross CC124 × CC125; Table 1). Comparisons with the *Chlamydomonas* genome sequence database (based on the CC503/*cw92* strain) (https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Creinhardtii), as well as pairwise comparisons with wild-type strains to remove CC125-specific SNPs from candidate mutations, revealed a two-base substitution in the phytoene synthase (PSY) gene that produces a single amino acid substitution (P159I) in the catalytic domain of PSY (Fig. 3 *A* and *B* and Fig. S1*B*).

Phytoene is an intermediate in the carotenoid-biosynthesis pathway (Fig. 3*C*). A series of mutants lacking PSY (named *lts1* mutants) was reported (16). However, the growth phenotype of *lts1-211* is different from that of previously reported PSY null mutants. These null mutants, *lts1-30* and *lts1-201* through *lts1-210*, cannot grow in the light and are white or pale green when grown in the dark (referred to as "white mutants") (16). By contrast, *lts1-211* cells grow in the light, and their green color is indistinguishable from that of the wild type (Fig. 3*D*). In the dark, *lts1-211* cells appear pale green (Fig. 3*D*). As a previously unidentified *lts1* allele, we thus named this mutant *lts1-211*.

Without application of the redox reagents, *lts1-211* did not show significant phototaxis in low light (~0.3 μmol photons·m^{-2}·s^{-1}), whereas WT cells showed positive phototaxis (Fig. S2). In stronger light (>5 μmol photons·m^{-2}·s^{-1}), WT cells showed negative phototaxis, whereas *lts1-211* showed positive phototaxis (Fig. S2). Thus, as far as cells show phototaxis, *lts1-211* almost always showed phototaxis with a sign opposite to that of WT. For ease of phototactic sign analyses, strong light (~10 μmol photons·m^{-2}·s^{-1} for polar histograms and ~100 μmol photons·m^{-2}·s^{-1} for dish assays) was used in the following analyses (except for Fig. S2).

***lts1-211* Has Low Levels of Carotenoid and Defective Eyespot Formation.** To confirm that *lts1-211* impaired PSY activity, we quantified the carotenoid contents in the cells by reversed phase chromatography. β-Carotene and lutein are the two major carotenoids in wild-type cells (17, 18). Both carotenoids were absent in the two strains of the PSY null mutants, and their levels were significantly reduced in *lts1-211* cells compared with wild-type cells (β-carotene, 3%; lutein, 28%; Fig. 2*D*).

These data prompted us to examine whether *lts1-211* has a normal eyespot. As shown in Fig. 1, the *Chlamydomonas* eyespot contains multiple layers of carotenoid-rich granules, the main component of which is β-carotene (19). As expected, most *lts1-211* cells did not have a detectable eyespot (Fig. 2*E*). Approximately <1% of *lts1-211* cells had a faint orange spot on the cell surface, suggesting that these cells have eyespots containing only small amounts of carotenoids.

The phenotype of *lts1-211* was rescued by transformation with a wild-type genome fragment containing the PSY gene (Fig. 3*A*). A rescued strain, *lts1-211R*, contained a normal level of carotenoids, normal eyespots, and showed the same sign of phototaxis as the wild type, with or without redox-reagent treatment (Fig. 2 and Fig. S2). These data suggest that PSY carrying the P159I mutation has significantly reduced activity.

All Eyeless Mutants Exhibit Redox-Dependent Reversal of Phototactic Sign. Why do *lts1-211* cells show an opposite sign of phototaxis compared with that of the wild type? The swimming velocity and flagellar beat frequency of this mutant did not significantly differ from those of the wild type (Fig. S3 *A* and *B*). Similarly, Ca^{2+}-dependent dominance switching between the two flagella, which is thought to be the basis for phototactic turning of the cell, apparently occurs normally in *lts1-211*, as assessed by using demembranated and reactivated "cell models" (Fig. S3*C*) (20). These cells also exhibited a normal photophobic response, which is characterized by transient backward swimming upon sudden light stimulation of ChRs (mainly ChR1) (6). Therefore, overall, the motility of *lts1-211* cells appears to be normal.

We thus surmised that the lack of eyespot pigments alone caused reversal of the phototactic sign. When the mutant displays phototaxis in a direction opposite to that of wild type, the photoreceptors may sense the light from the rear side, i.e., the light coming through the cell body, more strongly than from the front side. Such rear-side stimulation could take place if the cell body acts as a convex lens and condenses light on the photoreceptor. In fact, previous studies suggested that the cell bodies of *lts1* null mutants are almost transparent and act as convex lenses, which condense light on the farthest side of the cell (13). It is possible, however, that such a lens effect is not limited to the transparent cell body of the mutant; the cell bodies of other *Chlamydomonas* strains with normal (green) pigmentation may also function as convex lenses, which help stimulate the photoreceptor from the rear side. If this hypothesis is the case, other eyeless mutants with green cell bodies might also exhibit a reversed sign of phototaxis. We thus examined the phototactic signs in eyeless mutants *eye1*, *eye2*, and *eye3*. Intriguingly, all three *eye* mutants exhibited an opposite sign of phototaxis compared with the wild type after treatment with redox reagents (Fig. 4).

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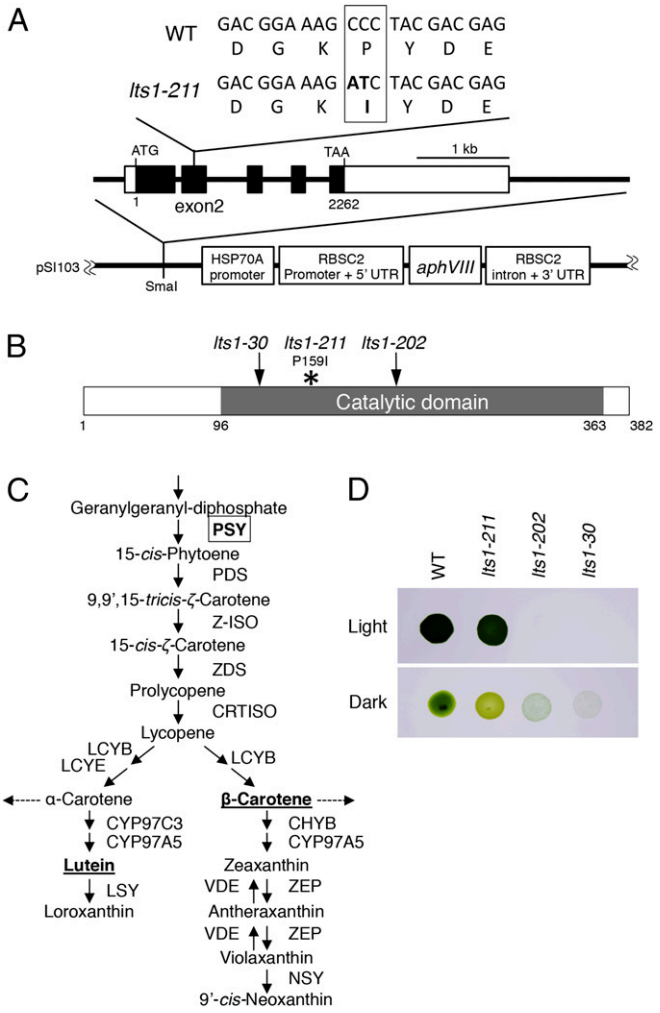

Fig. 3. Phytene synthtase gene in *lts1-211* and genetic/phenotypic differences from the other *lts1* alleles. (*A*) Structure of the *Chlamydomonas* PSY gene and the mutation in *lts1-211* (mid). DNA and amino acid sequences in the vicinity of the mutation in exon 2 in the wild-type and *lts1-211* genomes (*Top*) are shown. For the rescue experiment, *lts1-211* was transformed with a 6,000-kb DNA fragment containing the PSY gene, which was cloned into pSI103 plasmid (*Bottom*) (42). (*B*) Domain structure of PSY. The P159I mutation in *lts1-211* occurs in the catalytic domain of PSY. Mutations in the previously reported PSY null mutants are also shown as follows: In *lts1-30*, W123 is substituted for a stop codon, whereas in *lts1-202* (previously called *FN68*), a frameshift occurs (16). (*C*) Part of the carotenoid-biosynthesis pathway in *Chlamydomonas* modified from ref. 19. PSY (boxed) synthesizes phytoene from geranylgeranyl-diphosphate. β-Carotene and lutein, the two major carotenoids in *Chlamydomonas* analyzed in Fig. 2*D*, are underlined. (*D*) Growth phenotypes of the wild type, *lts1-211*, and two PSY null mutants. Cell suspensions from each mutant containing ~10^5 cells were spotted onto TAP-agar plates and incubated in the light (~50 μmol photons·m^{-2}·sec^{-1}; *Top*) or dark (*Bottom*) for 3 d.

To confirm the presence of a lens effect in "green" cells, we observed the wild-type and the *lts-211* cells under a microscope by using sideways illumination (Fig. 5*A*). Regardless of the location of the eyespot, a small, bright area appeared on the side of each cell edge opposite the light source. Furthermore, we observed images of an object in the light path of the microscope, which were formed by the cellular lens effect (Fig. 5*B*, Fig. S4, and Movie S1). These observations indicate that even a normally pigmented cell body acts as a convex lens. The redox-dependent reversal of phototactic sign in *lts1-211* and the three *eye* mutants suggests that the carotenoid layers of the eyespot play a crucial role in determining the phototactic sign in *Chlamydomonas* (Fig. 6).

Discussion

Screening for *Chlamydomonas* mutants defective in phototactic sign switching resulted in the isolation of *lts1-211*, a weak-allele mutant of the PSY gene. The mutant cells contained low amounts of carotenoids, and most lacked detectable eyespots. These cells displayed phototaxis against light stronger than ~5 μmol photons·m^{-2}·s^{-1}, but its sign was opposite to that of wild-type cells with or without the application of redox reagents, which strongly biases the sign of phototaxis. Interestingly, all previously known *eye* mutants also exhibited the same phenotype after redox-reagent treatment.

Previously isolated *eye* mutants were reported to exhibit weak or no phototaxis. The *eye1* mutant exhibits weak phototaxis because of

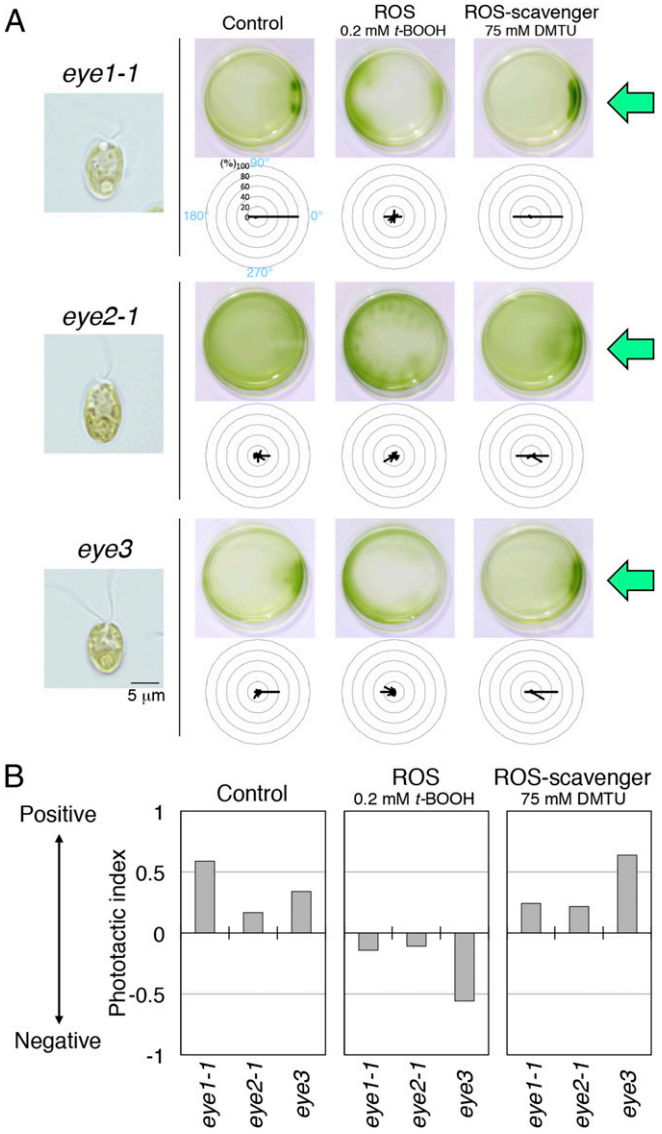

Fig. 4. All eyespot-deficient mutants show a redox-dependent sign of phototaxis opposite to that of the wild type. (*A*) Cell images, dish phototaxis assays, and polar histograms of *eye1-1*, *eye2-1*, and *eye3*, with or without treatment with redox reagents (12 bins of 30°; $n = 24$–56 cells per condition). (*B*) Phototactic index calculated as an average value of cosθ measured in *A*. After treatment with redox reagents, all eyeless mutants showed signs of phototaxis opposite to those of strains with eyespots (wild type and *lts1-211R*) and same as *lts1-211* (Fig. 2*C*).

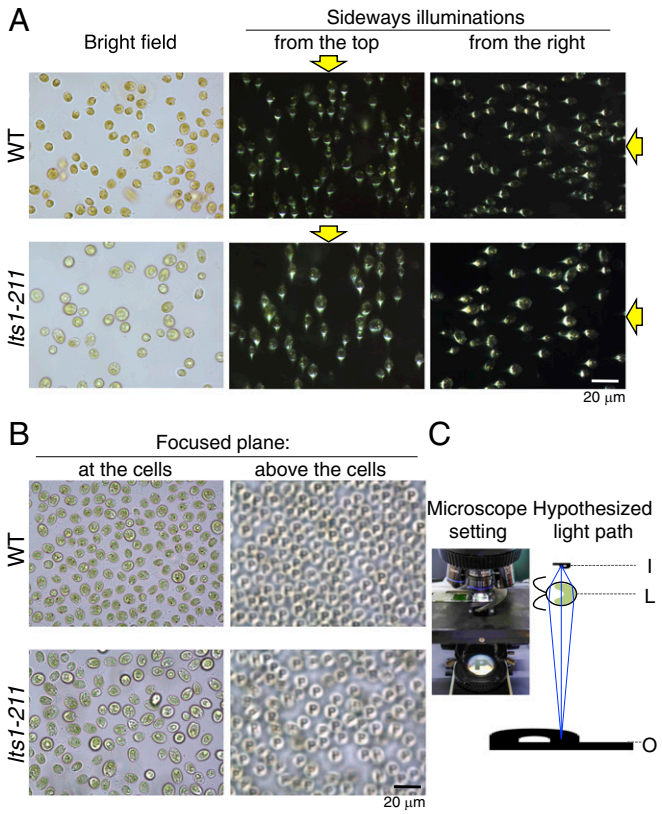

Fig. 5. The *Chlamydomonas* cell body has a lens effect. (*A*) Wild-type and *lts1-211* cells were observed with bright-field illumination (*Left*) or with sideways illumination (*Middle* and *Right*; yellow arrows indicate the direction of illumination). A small bright area is observed in each cell on the side opposite the light source. (*B*) The letter "P" (for "photo") set on a field stop ring of the microscope was imaged through the cells of both strains by the lens effect. The letter "P" appeared on each cell as the plane of focus was moved from the cells (*Left*) to above the cells (*Right*). (*C*) The setting of the microscope and a hypothetical optical path are shown. I, image; L, cell as a lens; O, object.

the less precise orientation of the cells' swimming direction (21). The *eye2* mutant also exhibits weak phototaxis because it is ∼100-fold less sensitive to light than the wild type (22). The *eye3* mutant does not exhibit phototaxis unless it is under special conditions (e.g., nitrogen starvation or a prolonged incubation at the stationary phase) (23). In the *eye2* and *eye3* mutants, ChR1 localizes to several patches around the "correct" position where the eyespot would normally occur, suggesting that the focused localization of channelrhodopsins, but not their approximate localization, requires the presence of the carotenoid layers (24, 25). Individual ChR1 molecules present in the membrane of a cell without a detectable eyespot appear to function normally, because *eye1* exhibits a normal photophobic response (6, 21). In contrast to previous studies, in the present study, the use of redox reagents produced rather strong (but oppositely directed) phototaxis in all eyeless mutants examined, including the newly isolated mutant *lts1-211*. Strong phototaxis in eyeless mutants was detected in this study, most likely because redox reagents fixed the phototactic sign and, thereby, stabilized this behavior (14). In the dish phototaxis assay without redox reagent, only *eye2* did not exhibit obvious positive phototaxis among eyespot-less mutants (Fig. 2*A* and Fig. 4*A*). A previous study showed that *eye2* shows weak negative phototaxis at approximately 120∼150 μmol photons·m^{-2}·s^{-1} (22). Because Eye2p is a thioredoxin family protein, its absence may change the intracellular redox poise (22).

The reversed-phototactic sign in the eyeless mutants after treatment with redox reagents can be explained by the lens effect of the cell body (Figs. 5 and 6), which was previously found in several organisms including cyanobacteria, fungi, dinoflagellates, colonial Volvocine algae, and colorless *Chlamydomonas* mutants (12, 13, 26–28). The present study directly demonstrates that a normally pigmented *Chlamydomonas* cell can also function as a convex lens such that light illuminated sideways on the cell is condensed on the farther side, forming a small, bright patch, and that the images of an object are formed through the cells. In an apparent contradiction to our observations, a previous study (12) concluded that chlorophylls or other pigments in the cell body, rather than the eyespot, act as shields against light from the rear. However, our results indicate that shielding by chlorophylls or other pigments dispersed throughout chloroplast is insufficient to cancel the cellular lens effect on the ChRs, and that the carotenoid layers underneath the ChRs, where the incident light most strongly concentrates in the cell, are necessary.

Because we directly observed the lens effect of the cell bodies (Fig. 5*B*), we then were able to estimate that the refractive index of *Chlamydomonas* cells is 1.47, which is closed to the refractive index of most of the cells of green algae *Dunaliella salina* (1.46) and *Chlorella* sp. (1.40–1.45), as well as plants (1.48) (Fig. S4) (29–31). This value is higher than the previously reported value for *Chlamydomonas* estimated by a laser scanning flow cytometer (1.39∼1.43) (32). Our method can be applied to evaluation of refractive indices of other spheroidal organisms without special equipment.

In conclusion, a new screening method using redox reagents allowed us to isolate a previously unidentified *Chlamydomonas* mutant and to detect a previously unknown aspect of eyespot function affecting phototaxis. The isolation of the mutant, *lts1-211*, revealed that the cellular lens effect affects cellular behavior in the absence of carotenoid layers. The carotenoid pigment granules therefore have a crucial role in determining the sign of phototaxis, by shielding the ChRs in the plasma membrane from light condensed by the cellular lens onto the back of the eyespot.

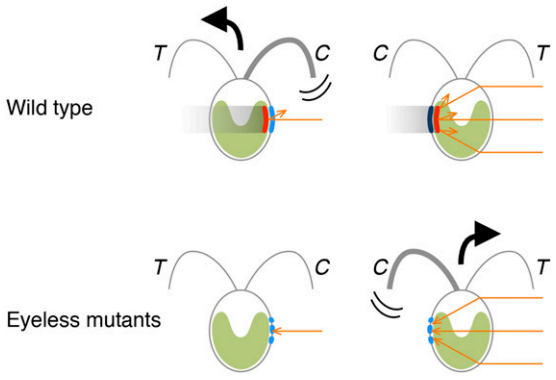

Fig. 6. Model illustrating the effect of light illumination on the photoreceptors and the phototactic sign of the wild type (*Top*) and eyeless mutants (*Bottom*). Carotenoid layers (red) reflect and amplify the light signal (orange arrows) onto the photoreceptors (blue) when the eyespot faces the light source. These layers shield the photoreceptors from the light condensed by the lens effect of the cell when the eyespot faces the side opposite the light source. The photoreceptors in an eyeless mutant cell localize to several patches around the "correct" position but function normally (24). The photoreceptors receive stronger light stimulation when facing away from the light source, i.e., in an opposite manner to that of wild-type photoreception. When the wild type cells are illuminated by strong light, they show negative phototaxis by beating the *cis*-flagellum (*C*) stronger than the *trans*-flagellum (*T*) when the eyespot faces the light source (*Top Left*). In contrast, the eyeless mutant cells show positive phototaxis by beating the *cis*-flagellum stronger than the *trans*-flagellum when the eyespot faces the side opposite the light source (*Bottom Right*).

Materials and Methods

The strains used in this study are listed in Table 1. All cells were grown in Tris-acetate-phosphate (TAP) medium (40) with aeration at 22 °C under a 12 h/12 h light/dark cycle, except for *lts1-202* and *lts1-30*, which were grown in the dark for pigment and growth-phenotype analyses.

See *SI Materials and Methods* for more information.

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