Eyespot-Assembly Mutants in *Chlamydomonas reinhardtii*

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**ABSTRACT**

*Chlamydomonas reinhardtii* is a single-celled green alga that phototaxes toward light by means of a light-sensitive organelle, the eyespot. The eyespot is composed of photoreceptor and Ca$^{2+}$-channel signal transduction components in the plasma membrane of the cell and reflective carotenoid pigment layers in an underlying region of the large chloroplast. To identify components important for the positioning and assembly of a functional eyespot, a large collection of nonphototactic mutants was screened for those with aberrant pigment spots. Four loci were identified. *eye2* and *eye3* mutants have no pigmented eyespots. *min1* mutants have smaller than wild-type eyespots. *mlt1(ptx4)* mutants have multiple eyespots. The *MIN1*, *MLT1(PTX4)*, and *EYE2* loci are closely linked to each other; *EYE3* is unlinked to the other three loci. The *eye2* and *eye3* mutants are epistatic to *min1* and *mlt1* mutations; all double mutants are eyeless. *min1* double mutants have a synthetic phenotype; they are eyeless or have very small, misplaced eyespots. Ultrastructural studies revealed that the *min1* mutants are defective in the physical connection between the plasma membrane and the chloroplast envelope membranes in the region of the pigment granules. Characterization of these four loci will provide a beginning for the understanding of eyespot assembly and localization in the cell.

*Chlamydomonas reinhardtii* is a phototactic, single-celled green alga. The light-sensing eyespot is a complex sandwich composed of plasma membrane, outer and inner chloroplast membranes, and multiple layers of granules in the stroma of the chloroplast intercalated with thylakoid membranes (see Figure 1A). The portion of the eyespot visible as a round orange patch by light microscopy is located in the stroma of the chloroplast. It is formed by a hexagonal array of carotenoid-filled pigment granules (Nakamura et al. 1973). In wild-type *Chlamydomonas* cells, two to four layers of carotenoid lipid granules are observed, each layer subtended by a thylakoid membrane. In the region of the eyespot, both of the chloroplast membranes and the plasma membrane are closely apposed. The photoreceptor, *Chlamydomonas* rhodopsin-like “chlamyopsin,” localizes to the plasma membrane in this region (Deininger et al. 1995). As shown by suction pipette recording, the light-triggered calcium channel is also in the plasma membrane overlying the region of the pigmented eyespot (Harz and Hegemann 1991). Accordingly, an increased density of membrane proteins in the plasma membrane in the region of the pigmented eyespot has been observed by freeze-fracture electron microscopy (Melkonian and Robenek 1980).

In addition to the complex organization of the eyespot components across the membranes of the cell and chloroplast, the eyespot has a precise location in the cell relative to the cytoskeleton. The eyespot is equatorial with respect to the polar location of the basal bodies and flagella (see Figure 1B). A cruciate microtubule “rootlet” structure is oriented at a 45° angle to the plane of the flagellum. One four-membered microtubule rootlet and one two-membered rootlet are associated with each of the basal bodies (mother and daughter) and extend from the basal body region to the equator of the cell. The eyespot is found just clockwise of the daughter four-membered microtubule rootlet (see Figure 1C; Holmes and Dutcher 1989).

The eyespot is oriented to detect light striking the cell surface orthogonal to the eyespot. *Chlamydomonas* cells rotate twice per second as they breaststroke forward, scanning the environment perpendicular to the swimming path much as a radar antenna scans the sky. The carotenoid granule layers were hypothesized to have two functions: they reflect photons that pass unabsorbed through the plasma membrane back onto the photoreceptors in the plasma membrane, and they block light coming through the cell body from other directions. The spacing and periodicity of the carotenoid granule and thylakoid membrane layers were hypothesized to act thus as a “one-way mirror” or what in the physics of light is called a quarter-wave plate (Foster and Smyth 1980). Confocal microscopy studies have confirmed that the pigmented layers act as a reflector of light (Kreimer and Melkonian 1990; Schaller and Uhl 1997). Analysis of a mutant strain with defective eyespots supports the idea that the eyespot also enhances the shielding.
properties of the chloroplast to block light coming through the cell body (Kr eimer et al. 1992; reviewed in Kreimer 1999). Together, the reflective property of the carotenoid pigment layer and the absorptive property of the eyespot and cell body give up to an eightfold enhancement of the light striking the plasma membrane over the eyespot relative to the light coming through the cell from the other side (Har z et al. 1992).

Light absorption by the rhodopsin-like photoreceptor leads to the transient opening of calcium channels in the plasma membrane over the eyespot. The influx of calcium triggers the transient opening of voltage-gated calcium channels in the flagella (Har z and Hegemann 1991). The subsequent change in intracellular calcium concentration is thought to affect the activity of the two flagella differentially, as has been demonstrated in vitro (Kamiya and Wit man 1984). The cis (near the eyespot) flagellum beats weakly and the trans (away from the eyespot) beats strongly to cause the cell to turn toward the light as it swims forward. If the light intensity is too strong, the response of the two flagella is reversed and the cell turns away from the light (Rüffer and Nultsch 1997). In-depth coverage of eyespot structure, localization, and function in green algae can be found in two excellent recent reviews (Hegemann and Har z 1998; Kreimer 1999).

Our interest in the Chlamydomonas eyespot is in how the signal transduction components in the plasma membrane are assembled with the chloroplast envelope, reflective carotenoid pigment granule layers, and thylakoid membranes to form a functional light-sensing organelle. To identify genes important in the assembly pathway, we have identified nonphototactic mutant strains of Chlamydomonas reinhardtii and screened for those with aberrant carotenoid pigment patterns. Here we report the identification of four genes governing the assembly of the pigment layer. Mutations in two genes result in the absence of the reflective pigment layer, mutations in the third gene result in small pigment patches, and mutations in a fourth gene result in multiple, pigmented eyespots.

MATERIALS AND METHODS

Media, culture conditions, and strains: M medium is Sager and Granick medium I with Hutner's trace elements (Har r i s 1989). R medium is M medium with 0.1% sodium acetate added. In RNA and DNA medium the NH₄Cl concentration is reduced 10-fold and 200 μg/ml of arginine is added. Solid media contain 1.5% agar (R, RNA), 2% agar (D, DNA), or 4% agar (Z).

Both liquid cultures and plates were incubated at 25°C in continuous white fluorescent light. All of the mutant strains described in this article were isolated in strain 137c mt¹ except strain 6-9, which was isolated in mt². Strains bearing intragenic
complementing arg7 alleles were used to recover diploids for complementation analyses of eyespot-assembly mutations (Matagne 1978; Harris 1989).

**Mutagenesis, enrichment, and screening for phototaxis-negative mutants:** Mutagenesis: Strain 137c was grown on R plates for two days. The cells were scraped from the surface and resuspended in liquid M medium. Between 2 and 7 × 10^7 cells in 1 ml were pipetted into 3-cm-diameter petri dishes and exposed to UV radiation at a distance of ~10 cm for 45 sec. A kill curve showed ~80% survival for this exposure time. The cultures were kept in the dark for 8 hr and then transferred to 30 ml of liquid M medium in large test tubes (25 × 150 mm) and grown at 25°C in light.

Enrichment: At 2-day intervals the tubes were masked with black paper except for the bottom 1 cm of the tube and placed 40 cm from a fluorescent bar lamp for 20 min. Most of the cells phototaxed to the lighted bottom portion of the tube. One milliliter of culture was removed from the top surface and transferred to 30 ml of fresh M medium (see Stavis and Hirschberg 1973). In all cases, cultures were tested at an approximate density of 1 × 10^6 cells/ml.

Screening: After four or five enrichments, individual cultures were diluted and spread on R plates. Ten single colonies from each original mutagenized culture were transferred into 2.5 ml of M medium in 13 × 100-mm tubes and grown for 2 days at 25°C in 80 μE/m²/sec light. On the second day, the tubes were placed in a box with a 0.5-cm-wide slit that illuminated the tubes at the bottom. After 20 min of illumination at 40 cm from a fluorescent bar lamp, the tubes were removed from the box and examined. Cultures of cells that did not form a band at the bottom were scored as phototaxis negative. Only one mutant strain was kept from each original mutagenized culture.

**Screening for mutants with an aberrant eyespot morphology:** Each of the original mutant strains was grown on liquid medium, fixed with 0.1% glutaraldehyde in phosphate-buffered saline (PBS), and observed at ×1000 magnification by oil immersion light microscopy. The strains with no eyespot, multiple eyespots, or smaller than normal eyespot were characterized genetically.

**Linkage and complementation analyses:** Each of the mutants with aberrant eyespot morphology was crossed to each of two strains bearing complementing arg7 alleles, arg7-8 and arg7-2 (Matagne 1978; Harris 1989). Haploid phototaxis-negative Arg- progeny of opposite mating types were mated and diploids were recovered on medium lacking arginine. Ten diploid colonies from each cross were tested for phototaxis as described in the screening section above. If the diploid was able to phototax, the two mutants were placed in different complementation groups; if the diploid was unable to phototax, the mutants were placed in the same group. To test linkage, haploid, phototaxis-negative strains of opposite mating type were crossed and allowed to go through meiosis. C. reinhardtii produces haploid progeny in tetrads, which can be dissected and analyzed. Approximately 20 tetrads were analyzed for recombination between two different phototaxis-negative mutations. Methods for mating, recovery of diploids, and tetrad dissection were as described (Harris 1989).

**Electron microscopy:** Cultures of each strain were grown for 8 days in M medium or for 3 days in R medium. Cells were harvested at 5000 rpm and mixed 1:1 with 2% formaldehyde (100% paraformaldehyde stock diluted in heat-treated water and neutralized with sodium hydroxide), 0.25% glutaraldehyde (50% stock) in 50 mm cacodylate, and microwaved three times for 30 sec to accelerate fixation (Gibson et al. 1997). Paraformaldehyde and glutaraldehyde were purchased from Electron Microscopy Sciences (Fort Washington, PA). Cells were rinsed three times with H MEEK buffer (30 mm Hepes, pH 7.4, 5 mm MgSO_4_, 1 mm EGTA, 0.1 mm EDTA, 25 mm KCl, 1 mm DTT). Cells were incubated with microwaving in 0.5% OsO_4_, 0.8% K_2Fe(CN)_6_ in H MEEK (25 sec, 15°C, three times), rinsed with H MEEK three times, and incubated in 0.5% tannic acid in H MEEK for 1 min at room temperature (RT). Cells were rinsed with H MEEK three times and deionized water four times, and then incubated in 2% uranyl acetate (2 hr, RT, in the dark). Dehydration was carried out in aceton, and cells were embedded in Epon-Araldite resin using microwave treatment to accelerate the process. Sections were cut by D. Bentley (Imaging Facility, University of Arizona), mounted on copper grids, and stained with a saturated solution of uranyl acetate (10 min, RT) and Reynolds’s lead citrate (1.5 min, RT). In Figure 4A and Figure 5A the tannic acid incubation was omitted and cells were imbedded in LR White instead of Epon-Araldite. In Figure 4D (provided by K. E. Huey), microwaving was omitted. Imaging was performed on a Phillips 420 transmission electron microscope at 80 kV.

**Light microscopy:** Cultures of each strain were grown for 6 days in M medium or for 2 days in R medium. Glutaraldehyde (1 μl) was added to 1 ml of each culture, and cells were harvested at 5000 rpm. A yellow-green filter (Zeiss 467806) was used at the light source to enhance the contrast of the eyespots. Photographs were taken with Kodak Technical Pan film.

**RESULTS**

**Efficient method yields 168 independent nonphototactic strains:** C. reinhardtii wild-type strain 137c was mutagenized with ultraviolet light and enriched for nonphototactic cells as described in materials and methods (Stavis and Hirschberg 1973). From 200 separate mutagenized cultures, 168 independent mutant strains were collected. Since the strains were grown on minimal medium without an added carbon source throughout the mutagenesis and enrichment procedure, all of the mutants retain photosynthetic capability. Because carotenoid biosynthetic pathway mutants are defective in photosynthesis (Sager and Zalokar 1958; Spritz and Metz 1981), the collection should not include strains defective in steps leading up to carotene biosynthesis. Since the enrichment and screening procedures required that the cells be able to swim, mutant strains with gross defects in swimming should not be included in the collection. We expect that this collection should include mutations that affect the signal transduction pathway, those that affect the response of the flagella to the light-activated calcium influx, and those governing the assembly and localization of the eyespot.

**Mutants with aberrant eyespot morphology:** The 168 mutant strains were screened visually with an oil immersion objective (×1000 magnification) for eyespot pigment patches that were other than wild type in size and/or location. In this screen, three aberrant phenotypes were identified: (1) the “eyeless” phenotype, or cells with no pigment patches observable by light microscopy; (2) the “miniature” phenotype, or cells with a smaller than normal eyespot; (3) the “multiple” phenotype, or cells with multiple eyespots. In all, 27 mutant strains had one of these three different phenotypes. Seventeen
TABLE 1

Complementation/recombination groups of mutants with aberrant eyespots

<table>
<thead>
<tr>
<th></th>
<th>eye1-</th>
<th>eye2-</th>
<th>eye3-</th>
<th>min1-</th>
<th>mlt1-</th>
<th>ND</th>
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<td>1</td>
<td>ey-1-</td>
<td>1, 10-18, C</td>
<td>1, 12-6, RC</td>
<td>1, 10-13, RC</td>
<td>8-11</td>
<td></td>
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<td>ey-550, C</td>
<td>2, 6-9, C</td>
<td>2, 12-12, RC</td>
<td>2, 11-15, RC</td>
<td>8-15</td>
<td></td>
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<tr>
<td>3</td>
<td>ey-627, C</td>
<td>3, 10-6, RC</td>
<td>3, 15-2, RC</td>
<td>3, 12-10, RC</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>4, 10-11, C</td>
<td>4, 15-12, RC</td>
<td>4, 13-14, RC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5, 10-15, C</td>
<td></td>
<td>5, 14-14, RC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6, 11-5, C</td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>7, 11-8, C</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>8, 12-5, RC</td>
<td></td>
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<td></td>
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<td>9, 12-7, C</td>
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<td>10, 12-18, RC</td>
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<td></td>
<td></td>
<td>15, 14-18, R</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

ND, not determined; 8-11 has a multi-eyed phenotype; 8-15 has an eyeless phenotype. R, determined by recombination. C, determined by complementation.

Eyespot morphology mutants define four loci: Both linkage and complementation analyses were used to characterize the 27 morphologically defective mutant strains. All of the mutations were shown to be recessive in diploids obtained by crossing the mutant strains to wild-type 137c. As shown in Table 1, the 27 strains fall into four complementation/ linkage groups. Fifteen eyeless mutants comprise one group. One additional eyeless mutant strain is in a complementation group by itself. Five multiple eyespot mutants are all in the same group and the fourth group is composed of the four miniature eyespot mutants. It should be noted that mutant 10-13 was not identified originally as a multiple eye mutant. The addition of this mutant to the group occurred during analysis of a linkage matrix of ten mutants in the large nonphototactic collection chosen at random. In logarithmically growing cultures, 10-13 cells had mostly single eyespots. Stationary phase cultures contained cells with multiple eyespots. Therefore, it may be possible that additional members of the multiple eye group and/or the other three groups have not been identified in the collection as a whole.

Eyeless mutations define two new loci, EYE2 and EYE3: As described by Hartshorne (1953), one of the first mutant strains of Chlamydomonas to be characterized was an eyeless strain. Named ey-1, this mutant strain is still available from the stock center, as are strains named ey-550 and ey-627 (Morel-Laurens and Bird 1984; Kreimer et al. 1992). All three of these strains are eyeless and nonphototactic in logarithmic culture, but acquire phototactic ability and eyespots in stationary phase cultures. By complementation testing, we determined that the ey-1 strain, ey-550, and ey-627 harbor noncomplementing mutations and thus contain ey-1 alleles on linkage group X (Smyth et al. 1975). We propose that this locus be renamed EYE1 to reflect the recommendation for three-letter Chlamydomonas gene names. The alleles thus should be renamed ey-1 (ey-1), ey-2 (ey-550), and ey-3 (ey-627). Our two eyeless loci are unlinked to, and the mutations complement, ey-1 alleles (see Table 1). Therefore, we have named the new loci EYE2 and EYE3. EYE2 is defined by the mutation in the 10-18 strain and an insertion mutation, H9-8, obtained in a later transformation/ mutagenesis scheme (D. G. W. Roberts and C. L. Dieckmann, unpublished data). ey-2 mutants never acquire eyespots or the ability to phototax during extended culturing. ey-3 mutant strains do acquire phototactic ability after extended time in stationary phase; however, we have never observed them to acquire pigmented eyespots.

Multi-eyed, mlt1, and mini-eyed, min1, mutants: Actively growing cultures of the multi-eyed mutant cells, named mlt1, display a characteristic phenotype only within the population. Scoring over 600 cells, 72% of a logarithmic culture of the mlt1-3 multi-eyed mutant had two eyespots. Of these two-eyespot cells, the majority (63% of the total cell number) had one eyespot in an equatorial position and an additional eyespot in a region of the chloroplast near the base of the flagella. The additional eyespot was located either on the same side and in line with the equatorial eyespot (36%) or 180° from the equatorial eyespot (27%). In addition to the two-eyespot cells, 14% had one eyespot, and 13% had more than two eyespots. Cells in stationary phase cultures acquire additional eyespots with time in culture. The multi-eyed eye mutant, named min1, have small eyespots located most often in the equatorial position.
**TABLE 2**

**Linkage data for the min1-mlt1-eye2 cluster**

<table>
<thead>
<tr>
<th>Cross</th>
<th>PD:NPD:TT</th>
<th>Map units</th>
</tr>
</thead>
<tbody>
<tr>
<td>min-1 (12-6, 12-12) × mlt-1 (12-10)</td>
<td>115:0:9</td>
<td>3.6</td>
</tr>
<tr>
<td>min-1 (12-6, 12-12) × ey-2 (10-18)</td>
<td>115:0:11</td>
<td>4.7</td>
</tr>
<tr>
<td>mlt-1 (12-10) × ey-2 (10-18)</td>
<td>132:0:2</td>
<td>0.75</td>
</tr>
</tbody>
</table>

**Eyespot assembly gene cluster:** Surprisingly, the mlt1 and min1 mutations are linked to the eye2 mutations. To determine the map distances between these loci, two-point crosses of strains mutant at the three loci were analyzed (see Table 2). The three loci are tightly linked and most likely in the order min1-mlt1-eye2. We are uncertain of the order with respect to the centromere. Recently, a probe prepared from sequence adjacent to the EYE2 locus hybridized to two BAC clones that were also identified with a PTX4 probe (D. G. W. Roberts, G. J. Pazour, G. B. Witman and C. L. Dieckmann, unpublished results). ptx4 mutations result in multiple eyespots and are allelic with mlt1 mutations (Pazour et al. 1995; C. L. Dieckmann, unpublished results). This physically places the MIN1-MLT1(PTX4)-EYE2 gene cluster on the left arm of linkage group XII/XIII (Pushpa Kathir, map position of PTX4 with a probe provided by G. Pazour, reported on gene maps provided on the Chlamydomonas Stock Center webpage http://genome.cornell.edu/cgi-bin/WebAce/webace?db=chlamydb).

**The eye2 and eye3 phenotype is epistatic to that of min1 and mlt1:** To investigate whether the four gene products function in the same or different eyespot assembly or positioning pathways, double-mutant strains were constructed and the eyespot phenotypes were examined. Both eye2 and eye3 were found to be epistatic to min1 and mlt1; i.e., any combination of an eyeless mutation and a mini- or multi-eye mutation resulted in the eyesless phenotype. min1 mlt1 double mutants have a synthetic phenotype different from either parent. In a logarithmically growing culture, ~43% of the population of min1 mlt1 cells had no eyespot visible under oil immersion at ×1500 magnification (100 cells analyzed). An equivalent fraction had very tiny pigment spots that were displaced in the cell toward the region of the chloroplast closest to the base of the flagella. In a min1 mlt1 culture that remained at stationary phase for 2 days, the percentage of cells with eyespots decreased to 26% (100 cells analyzed). As might be expected, eye2 eye3 double mutants are eyeless and do not acquire the ability to phototax in stationary phase, which is similar to eye2 single mutants. Light micrographs of the single-mutant strains and the min1 mlt1 double-mutant strain are shown in Figure 2.

**Eyeless mutants have pigment granules:** To examine the ultrastructure of the eyespots in the eyespot-assembly mutant strains, late-log or early stationary phase cells were fixed and prepared for electron microscopy. As shown in Figure 4A, wild-type eyespots show a close apposition of the plasma membrane with the chloroplast envelope membranes. In cross-section, the carotenoid-filled lipid granules (arrowheads) are in a closely packed, linear array directly beneath the inner envelope membrane. As shown here, multiple pigment granule layers are separated by thylakoid membranes. Surpris-
R as shown by light microscopy (Figure 3). The number of pigment granules is significantly less than that of wild type as shown by electron microscopy (Figures 4B and 5, A and B). Eyespots in cells grown on acetate medium have nearly wild-type morphology with apposed membranes (Figure 5B). However, eyespots of cells grown in acetate-free medium have an aberrant morphology in which cytoplasm is often apparent between the plasma membrane and the underlying chloroplast envelope (Figure 4B), and thylakoid membranes and stroma are seen between the chloroplast envelope and the pigment granules, which are often jumbled rather than in neat layers (Figures 4B and 5A). The loss of contact between the plasma membrane and the chloroplast envelope membrane is suggestive of a defect in the organization of the eyespot components across the three usually apposed membranes.

The min1 phenotype is exacerbated when the mutation is in combination with a mlt1 allele. As shown in Figure 4D, the pigment granule arrays in the double mutant 12-12,12-10 are small, disordered, and not associated with the chloroplast envelope, similar to the min1 single mutant (Figure 4B). In general, the double-mutant eyespots are smaller and more disordered than those of min1 single mutants. The pigment granule patches often appear to be randomly located within the stroma of the chloroplast.

DISCUSSION

One of the obstacles on the road from endosymbiont to becoming an organelle is to ensure segregation into new cells at mitosis and meiosis. The mechanism for the segregation of mitochondria and chloroplasts and other organelles during division is still a mystery. Organelles such as flagella and light-sensing eyespots must also be correctly positioned in the new cells for proper function. In C. reinhardtii, the eyespot is located at the equator of the cell if the flagella are centered at one pole (see Figure 1B). The positioning of this structure is complex, in both a spatial and a temporal sense. The eyespot is positioned just clockwise of the daughter four-membered microtubule rootlet as viewed from the flagellar pole (see Figure 1C). During mitosis, the cleavage furrow forms along the four-membered microtubule plane and the eyespot is resorbed. In the two daughter cells, the new eyespots appear again just clockwise of the new daughter four-membered rootlet. While the daughter cells are still attached to one another, these new eyespots are 180° from each other and 90° from the cleavage plane and the location of the old eyespot (Holmes and Dutcher 1989). The eyespot is organized across the plasma membrane and both chloroplast envelope membranes to include pigment granules in the stroma. These pigment granules are organized in hexagonal arrays and are subtended by thylakoid membranes (Nakamura et al. 1973).
Our interest is in understanding both the structural basis of the organization of the eyespot across the cell/organelle boundary and the timing and localization of the organelle in the cell during growth and cell division. To this end, we have isolated and characterized several mutant strains with aberrant eyespot assembly. From 168 nonphototactic mutants, 27 eyespot mutants were found. Three different phenotypes were observed: no eyespots, multiple eyespots, or smaller than wild-type eyespots. These mutations could eliminate a component of the eyespot structure or disrupt a regulatory pathway necessary for proper assembly of the structure.

The eye2 and eye3 mutants do not assemble a pigment granule apparatus observable by light microscopy. Quite different from the appearance of eyespots in stationary cultures of eye1 mutants, we have never observed pigment spots in the eye2 or eye3 strains in our collection. However, eye3 cells starved for nitrogen or kept in sta-
tionary phase for 2 days recover the ability to phototax. It is clear that the signal transduction machinery, at least in starved cells, is sufficient for phototaxis. In addition, we have found both eye2 and eye3 strains will photoshock at high levels of light (D. G. W. Roberts and C. L. Dieckmann, unpublished data), also suggestive that signal transduction is intact in these mutants. Thus, the eye2 and eye3 mutants are primarily defective in pigment granule organization in the chloroplast. These EYE genes could code for essential structural components of the eyespot or positively regulate the pathway of assembly. We expected that we might identify several more loci of this type. Identifying only three loci may suggest the following: (1) that the structure is quite simple, (2) that other structural or regulatory elements of the system are essential gene products and would not have been identified in this analysis, or (3) that the genome has not been saturated for mutations that would result in the eyeless phenotype.

The min1 mutants have smaller than normal eyespots, which are highly disordered when the cells are grown in medium lacking acetate. The product of the MIN1 gene could be a structural component that is involved in the apposition of the plasma membrane with the chloroplast envelope. This hypothesis would explain why we observe cytoplasm between the plasma membrane and the chloroplast in min1 mutant strains. However, min1 cells grown on acetate assemble small but more normal-looking eyespots with the membranes opposed. Future investigations will focus on discovering the basis of the phenotypic variation.

The mit1 mutants are defective in the regulation of eyespot number. Occasionally in a wild-type population of cells a cell with two eyespots is observed following phototaxis (Holmes and Dutcher 1989). The additional eyespot is the result of retention of the parental eyespot in the daughter cell along with the newlyformed eyespot. In logarithmically growing cultures of the mit1 strains, most cells have two eyespots, 36% with both on line on the same side of the cell, one in the normal equatorial location and the other closer to the flagella. We have observed that, like wild-type cells, mit1 cells absorb the eyespots during division. Appearance of new eyespots seems delayed relative to wild-type cells. Therefore, supernumerary eyespots are not due to being retained from the parental cell. Future investigation of the mit1 mutants during mitosis should clarify the timing of the appearance and placement of eyespot relative to the cleavage furrow and four-membered microtubule rootlets. That 62% of the cells examined have eyespots that are aligned or are 180° from each other and are all in the flagellar hemisphere is suggestive that some positional cues have been retained. Perhaps the signal that designates the position of the four-membered rootlets or the four-membered rootlets themselves are cues for eyespot localization. Recently we discovered that mit1 mutations and ptx4 mutations are allelic (Pazour et al. 1995; C. L. Dieckmann, unpublished data), which places the MLT1/PTX4 locus on the left arm of linkage group XII/XIII (P. Kathir, Chlamydomonas Stock Center webpage).

We find it intriguing that the MIN1, MLT1(PTX4), and EYE2 loci are closely linked to each other; this clustering may allow cell-cycle or other coordinated con-
trol of expression. Alternatively, this group could have a common genetic origin, perhaps from the genome of the endosymbiont/organellae.

This study is a beginning for the understanding of eyespot structure and function in *C. reinhardtii* and other related algae. The continued analysis of genes described here will increase our general knowledge of the cell-organellae interactions required for light reception, signal transduction, and eyespot assembly and positioning in the cell.

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**LITERATURE CITED**


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