Growth, Ultrastructure and Carotenoid Spectra of the Chlorophyll-less y-y Mutant of *Chlamydomonas reinhardtii*

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The isolation of a stable, chlorophyll-less Mendelian mutant (y-y) of *Chlamydomonas reinhardtii* is described and its growth, carotenoid spectra and ultrastructure are compared to chlorophyll-containing strains. Unlike most chlorophyll-less mutants, y-y did not die in the light but grew as bright yellow colonies. It was unable to grow in a mineral medium with CO₂ and it required acetate for growth. When compared to green *C. reinhardtii* strains, y-y grew slower in the light but at about the same rate in the dark. Light-grown and dark-grown y-y cells had no detectable chlorophyll and similar amounts of total carotenoids, which were 20–35% of the values for the green strains. The only noticeable difference in ultrastructure between y-y and the green cells was in the inner chloroplast membranes. In the light and dark y-y lacked lamellae, although the outer chloroplast membrane was retained.

**INTRODUCTION**

The unicellular alga, *Chlamydomonas reinhardtii*, produces chlorophyll in the dark as well as in the light. A mutant, y-1, synthesizes normal levels of chlorophyll in the light but has no chlorophyll in the dark due to a single, Mendelian mutation (Sager, 1955). Dark-grown y-1 cells grow as yellow colonies on acetate-supplemented medium.

The y-1 mutant was first described by Sager & Palade (1954). Subsequently, a number of spontaneous and induced y-1 type mutants have been reported (Sager & Tsubo, 1962; Stolbova, 1971; Wang *et al.*, 1977; Gyurján *et al.*, 1979; Ford & Wang, 1980a), demonstrating that *C. reinhardtii* mutants lacking chlorophyll in the dark are not rare.

However, few stable *C. reinhardtii* mutants without chlorophyll in the light are known. They are typically photosensitive or unable to produce viable offspring (Sager, 1961; Gross & Dugger, 1969; Wang *et al.*, 1974; Ford & Wang, 1980b; Wilson *et al.*, 1980; Spreitzer & Mets, 1981).

A *C. reinhardtii* mutant (y-y) was isolated from y-1 cells and forms yellow colonies in the light. It may be induced to mate and lacks chlorophyll in the light because of a single, Mendelian mutation (Nicholson-Guthrie & Hudock, 1980).

This paper reports on how y-y was isolated and on its ultrastructure, carotenoid spectra, and growth in various media in the light and dark. Data on wild-type and y-1 cells are given for comparison.

**METHODS**

Organisms and cultural conditions. Mating type plus strains of *Chlamydomonas reinhardtii* 137c wild-type and y-1 were used. In the electron microscopic studies, *Polytoma uvela* (Indiana University Algae Culture No. L19), a colourless relative of *Chlamydomonas*, was used as a comparative alga having no chlorophyll or lamellae. The *C. reinhardtii* strains were grown and maintained on enriched medium (0-2%, w/v, each of anhydrous sodium

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acetate, Difco yeast extract, and Difco bacto-tryptone) which was solidified with 1-5% (w/v) Difco bacto-agar at room temperature (25-29 °C) under continuous, cool white fluorescent lights (1.08-2.15 klx). Details are given in the text where liquid enriched medium was used and where conditions of illumination were varied. *Polytona* *auteella* was grown as described by Starr (1964).

Before the start of an experiment, the organisms had been recently cloned from a single cell isolate. Samples of the cultures were removed at the start (for electron microscopy and spectral analysis) or at the end (for growth studies) of each experiment and plated to check for spontaneous revertants or pigment mutations (green cells in *y-y* cultures, wild-type in *y-I*, or *y-I* in wild-type) or contamination by bacteria or moulds. Revertants (1% or less) only occurred in *y-I* samples. Data do not include any cultures with contamination.

**UV treatment.** Cells of the *y-I* strain growing exponentially in continuous light in liquid enriched medium, were harvested by centrifugation, washed and resuspended in 0-13 M-potassium phosphate pH 6-8 (phosphate buffer) to a concentration of 3-4 × 10⁶ ml⁻¹. Cell suspension (4 ml) was placed in a sterile 15 × 60 mm glass Petri dish and irradiated at room temperature with one 15 W General Electric germicidal bulb (rated output is approximately 0-37 μJ s⁻¹ mm⁻² at 1 m; 90% output at 253-7 nm) placed 91 cm from the bottom centre of the Petri dish. During irradiation, the cells were stirred constantly with a small magnetic stirrer. UV treatment and subsequent sampling took place in the dark. The UV method was modified from Lyman et al. (1961).

At various times after irradiation, duplicate 0-1 ml samples were removed, diluted in 9-9 ml liquid enriched medium, and placed for 3 or 6 d in the dark or red light (a single layer of red gel type filter over four cool, white fluorescent lights). At the end of the dark or red light period, the cells were plated on enriched medium and placed in continuous white light (2-15-4-3 klx). After 2-3 weeks, the plates were scored by eye for mutant colonies with decreased chlorophyll content. Mutant colonies were picked off into 2 ml enriched medium in continuous white light for 1-2 weeks and then replated. After this second plating, some putative mutant isolates gave greener colonies or a mixture of mutant and greener colonies.

**Growth studies.** Cells from approximately 1 week old plates were suspended in 2-5 ml phosphate buffer in the light. After 4-6 h, 0-5 ml cell suspension was inoculated into 30 ml medium in 300 ml Bellco Nephelco flasks and placed on gyratory shakers (medium speed) in the light (3-2-5-4 klx) or dark at room temperature. Growth was measured on a Bausch and Lomb 20 colorimeter at 750 nm (negligible pigment absorption at this wavelength) until stationary phase (maximum cell density) had been maintained for several days. Initial absorbance in the growth flasks was 0-01-0-05.

**Carotenoid spectra.** Cells grown to mid-exponential phase in liquid, enriched medium were washed and resuspended in phosphate buffer, and the cell count was determined with a haemocytometer. A minimum of 3 × 10⁸ total cells was centrifuged and extracted at least three times with about 15 ml total ice-cold 80% (v/v) reagent grade acetone. Dark-grown cultures were extracted in the dark. Absorbance was determined on a Cary model 14 recording spectrophotometer with a 10 mm path length.

**Electron microscopy.** Cells were grown in liquid, enriched medium in the light (1-08-4-3 klx) or dark and treated as described previously (Nicholson-Guthrie et al., 1975) except that following exposure to 2% (w/v) OsO₄ vapour and agar layering, the cells were fixed for 1-2 h in 2% (w/v) KMnO₄ at 4 °C. The samples were then washed, dehydrated with graded ethanol, stained in 70% (v/v) ethyl alcohol saturated with lead subacetate for 6 h (F. R. Turner, personal communication), followed by dehydration in 95% and 100% ethanol and 100% acetone. Thin sections, embedded in Spurr low viscosity medium, were examined without further staining.

**RESULTS**

**Isolation of the *y-y* mutant**

Over 27000 colonies arising from irradiated *y-I* cells were examined and only one colony was found containing viable cells unable to produce chlorophyll (Table 1). This mutant, referred to as *y-y*, was found after 16 min UV treatment followed by 6 d red light, conditions under which no reduction in viability due to irradiation was observed.

Although cells were irradiated for up to 21 min, only a small number of mutants (less than 1%) with lowered chlorophyll content were found (Table 1). In preliminary experiments using shorter UV exposures than reported here (0-5 min) and dark incubation for 1, 2, 3 d, only one mutant colony out of several thousand colonies examined had less chlorophyll than the control colonies. It appears that *C. reinhardtii* mutants with reduced or no chlorophyll content are rare and difficult to induce with a low UV dose.

**Growth of *y-y***

The mutant grew in a mineral salts medium with acetate as carbon source, but not when supplied only with CO₂ (Table 2). In the light, wild-type and *y-I* grew better than *y-y*, but in the
Chlorophyll-less mutant of Chlamydomonas

Table 1. *UV-induced chlorophyll mutants of y-1 C. reinhardtii*

<table>
<thead>
<tr>
<th>Treatment after UV</th>
<th>Total colonies examined*</th>
<th>Pale green</th>
<th>White†</th>
<th>Yellowish-green</th>
<th>Yellow</th>
<th>Total</th>
<th>Percentage mutants found</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dark (3 d)</td>
<td>14400</td>
<td>7</td>
<td>34</td>
<td>6</td>
<td>0</td>
<td>47</td>
<td>0.33</td>
</tr>
<tr>
<td>Dark (6 d)</td>
<td>800</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>0.25</td>
</tr>
<tr>
<td>Red light (3 d)</td>
<td>6300</td>
<td>23</td>
<td>8</td>
<td>13</td>
<td>0</td>
<td>44</td>
<td>0.70</td>
</tr>
<tr>
<td>Red light (6 d)</td>
<td>5800</td>
<td>22</td>
<td>4</td>
<td>12</td>
<td>1‡</td>
<td>39</td>
<td>0.67</td>
</tr>
<tr>
<td>Total</td>
<td>27300</td>
<td>53</td>
<td>46</td>
<td>32</td>
<td>1</td>
<td>132</td>
<td></td>
</tr>
</tbody>
</table>

* From cells exposed to UV from 5 to 21 min.
† Viable colonies which turned green on transfer to fresh medium.
‡ Origin of y-y mutant. This mutant colony was isolated and grown in 2 ml enriched medium in continuous dim, white light for 1–2 weeks and then replated on enriched medium plus agar. Besides the all yellow colonies of y-y, some green colonies were found. One of the all yellow colonies was selected and used in all subsequent experiments.

Table 2. *Growth analysis of C. reinhardtii wild-type and mutants*

<table>
<thead>
<tr>
<th>Medium*</th>
<th>Strain</th>
<th>Average doubling time (h)</th>
<th>Maximum cell density ($A_{750}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Light Dark</td>
<td>Light Dark</td>
<td></td>
</tr>
<tr>
<td>Salt</td>
<td>Wild-type</td>
<td>36</td>
<td>0-063‡</td>
</tr>
<tr>
<td></td>
<td>y-1</td>
<td>34</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>y-y‡</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Salt +</td>
<td>Wild-type</td>
<td>10</td>
<td>16</td>
</tr>
<tr>
<td>acetate</td>
<td>y-1</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>y-y</td>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td>Salt +</td>
<td>Wild-type</td>
<td>11‡</td>
<td>22</td>
</tr>
<tr>
<td>acetate</td>
<td>y-1</td>
<td>8</td>
<td>18‡</td>
</tr>
<tr>
<td></td>
<td>y-y</td>
<td>28†</td>
<td>30†</td>
</tr>
<tr>
<td>Acetate +</td>
<td>Wild-type</td>
<td>10</td>
<td>17</td>
</tr>
<tr>
<td>yeast extract</td>
<td>y-1</td>
<td>9</td>
<td>15†</td>
</tr>
<tr>
<td>bacto-tryptone</td>
<td>y-y</td>
<td>25</td>
<td>20</td>
</tr>
</tbody>
</table>

* Salt medium: 0-925 g K2HPO4, 3H2O; 0-363 g KH2PO4; 0-40 g NH4Cl; 0-10 g MgSO4·7H2O; 0-065 g CaCl2·2H2O; 1 ml Hutner's trace elements (Hutner et al., 1950); distilled water to 1 l. Anhydrous sodium acetate, Difco yeast extract and Difco bacto-tryptone (0-2%, w/v each) were used.
† The mean deviations were within ±10% (±20% where indicated by a dagger).
‡ Values after up to 17 d incubation. Also no growth occurred when y-y cells were illuminated in salt medium with 5% (v/v) CO2 for 8 d.
§ Values after about 30 d growth.

dark the growth patterns of the three cell types were similar. Light decreased the final cell density of y-y cells but in general had little effect on doubling time. For example, when y-y was grown in liquid enriched medium at different light intensities (0-22, 2-4, 7-1 klx), no significant differences in doubling times were found (data not shown).

On enriched agar plates, y-y colonies are bright yellow during growth in the light and yellowish-orange in the dark. After prolonged growth, light-grown colonies turn whitish and eventually die. Cells of y-y revert at a low frequency (<10⁻⁷) to cells containing chlorophyll. When they occur, the revertant green colonies are easily distinguished from y-y colonies (Nicholson-Guthrie et al., 1975).

The best conditions for maintaining the mutant are growth on agar plates containing about 30 ml enriched medium, at room temperature, in dim light (about 0-08 klx), on a 12 h light–12 h dark cycle, and transferred to fresh medium every 3–4 weeks.
The number of cells per ml of acetone extract were: wild-type, $6.5 \times 10^6$; y-I, $5.6 \times 10^6$; y-y, $10 \times 10^6$.

**Fig. 1.** Typical absorption spectra of light-grown (about 1.5 klx) *Chlamydomonas reinhardtii* strains. The number of cells per ml of acetone extract were: wild-type, $6.5 \times 10^6$; y-I, $5.6 \times 10^6$; y-y, $10 \times 10^6$.

**Fig. 2.** Typical absorption spectra of dark-grown strains. The number of cells per ml of acetone extract were: wild-type, $10 \times 10^6$; y-I, $10 \times 10^6$; y-y, $15 \times 10^6$.

**Spectral analysis of y-y**

In the region (400–500 nm) where carotenoids absorb, the spectra of acetone extracts of light-grown and dark-grown y-y cultures were essentially identical, with the major absorption peak occurring at 447 nm (Figs 1 and 2). In contrast wild-type and y-I cultures produced more pigment during light growth, and their maximal absorption occurred at 435 nm.

Although the carotenoid spectra of light-grown y-y and y-I differed, they were similar for dark-grown cultures. The absorbance (average absorbance for $10^6$ cells ml$^{-1}$ acetone extract) for dark-grown cells at 435 nm was 0.048 for y-y and 0.045 for y-I and at 447 nm was 0.052 for y-y and 0.055 for y-I.

Chlorophyll was not found in acetone extracts of light-grown and dark-grown y-y cells. Occasionally very small absorbance readings at 652 nm were noted. For example, an absorbance of 0.024 (about 3% of the absorbance value of wild-type and y-I) was recorded for the 80% acetone extract containing $2 \times 10^7$ light-grown y-y cells ml$^{-1}$. This reading was not considered significant since similar absorbance measurements for dark-grown y-y and y-I cells were found.

**Ultrastructure of y-y**

The lamellar membranes in the single chloroplast of a green, light-grown y-I cell were found as stacks of long, flat discs (Fig. 3a), as reported by Sager & Palade (1954). During growth in the dark, y-I cells lost lamellae and chlorophyll. Dark staining material, thought to be lamellar remnants, was scattered throughout the chloroplast (Fig. 3b).

The y-y mutant cells contained no lamellae, whether grown in the light (Fig. 3c) or dark (Fig. 3d); however, the envelope of the single cup-shaped plastid was retained. Chloroplasts of light-grown y-y cells (Fig. 3c) were remarkably similar to dark-grown y-I cells (Fig. 3b) and contained material appearing as lamellar remnants. Occasionally other membrane structures were found inside the y-y chloroplast: a lamellar-like membrane next to the chloroplast envelope and structures appearing as circles in cross sections (Fig. 3f). Except for the chloroplast, the other

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**Fig. 3.** (a) A light-grown y-I cell. (b) A dark-grown y-I cell. (c) A light-grown y-y cell. (d) A dividing dark-grown y-y cell. (e) A light-grown *Polytoma uvella* cell. (f) Sections of a chloroplast of a light-grown y-y cell showing atypical membranes (arrows). C, chloroplast; L, lamellae; M, mitochondrion; D, dictyosome; S, starch granule; LP, leucoplast; LR, lamellar remnants. The bar markers represent 1.0 μm except in (f) where it represents 0.5 μm.
Chlorophyll-less mutant of Chlamydomonas

(a) 

(b) 

(c) 

(d) 

(e) 

(f)
cellular features of \( y-y \) were not unusual. The nuclei, mitochondria, eyespots, dictyosomes, flagella, cellular envelopes, pyrenoids and starch plates were comparable in \( y-y \) and \( y-I \) cells.

*Polytoma uvella* is a naturally occurring chlorophyll-less, unicellular alga and a relative of *Chlamydomonas*. Under light microscopy, \( y-y \) and *P. uvella* cells looked similar; their ultrastructures, however, differed (Fig. 3c, e). The leucoplast of *P. uvella*, as described by Lang (1963), had no lamellar remnants and contained starch granules in light-grown cultures (Fig. 3e). Also the mitochondria of *P. uvella* and \( y-y \) differed.

**DISCUSSION**

*Chlamydomonas reinhardtii* mutants lacking chlorophyll usually cannot survive in the light. For example, the brs-1 mutant grows as yellowish-brown colonies in very dim light (<10 lx) but is killed by 48 h in higher light exposures (Wang *et al.*, 1974). Recently isolated temperature-sensitive mutants which have chlorophyll at 25 °C but not at 33 °C die after several generations at the higher temperature in the light (Ford *et al.*, 1981).

In contrast, \( y-y \) grew in liquid medium in light intensities as high as 7.1 klx. It did not have chlorophyll even when grown in an enriched medium or in dim light to avoid possible destruction of chlorophyll by photooxidation. Strain \( y-y \) appears to be an exception to the premise of Wang *et al.* (1974) that *C. reinhardtii* cannot survive in the light in the absence of chlorophyll.

Gross & Dugger (1969) isolated a *C. reinhardtii* mutant (U3N) which apparently lacks chlorophyll and survives light growth. Although electron micrographs of light-grown U3N cells have not been published, the chloroplasts of dark-grown cells have small lamellar regions (Wilson *et al.*, 1980) which were not seen in \( y-y \) cells. Genetic analysis on U3N has not been possible because its zygotes fail to germinate.

Although a few uniparental mutants with reduced chlorophyll levels have been reported (Gyurján *et al.*, 1979; Spreitzer & Mets, 1981), most chlorophyll deficiencies in *C. reinhardtii* are due to single, Mendelian genes (Sager, 1955; Wang *et al.*, 1974; Wang, 1978; Ford & Wang, 1980a, b; Ford *et al.*, 1981). The \( y-y \) mutation causing a lack of chlorophyll in the light in \( y-y \) cells segregates as a single, Mendelian gene (Nicholson-Guthrie & Hudock, 1980). The \( y-y \) mutant, isolated from \( y-I \) and characterized in this report, has been shown to be a double mutant, carrying \( y-1 \) and \( y-y \) mutations (unpublished observation).

Light induces carotenoid synthesis in some uni- and multicellular plants, including permanently-bleached *Euglena* mutants (Gibor & Granick, 1962; Goodwin, 1965). In this report, light-grown wild-type and \( y-I \) contained more carotenoids than their dark-grown cells, but \( y-y \) did not show light-induced carotenogenesis. Since the role of the carotenoids is not completely understood, the \( y-y \) mutant could be used to study carotenoids, without the interference of chlorophyll pigments.

Furthermore, as a unicellular mutant plant which lacks chlorophyll and lamellae but is able to grow and survive in the light, \( y-y \) might provide information on the chlorophyll biosynthetic pathway and membrane synthesis.

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