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SHORT COMMUNICATION

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Lycopene accumulation and cyclic carotenoid deficiency in heterotrophic *Chlorella* treated with nicotine

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Abstract We studied the effects of nicotine on *Chlorella regularis* Y-21 grown under heterotrophic conditions. Nicotine repressed growth, doubled cell size, and changed the culture coloration from dark-green to orange. These effects are likely due to the change of the chloroplast into a red irregular vesicle. This morphological change was associated with alteration of the carotenoid composition. Lycopene accounted for more than 80% of total carotenoids in nicotine-treated cells. The red irregular vesicles had a high electron density; we supposed them to be immature chloroplasts accumulating lycopene.

Keywords Chloroplast · Red irregular vesicle · Cyclic carotenoid deficiency · Aberrant cell morphology

Introduction

Carotenoids are chloroplast constituents and are disposed differentially within chloroplasts. Among functions assigned to carotenoid molecules, an especially important role is to protect the chloroplast and plant from chlorophyll-sensitized photo-oxidation. Any aberration in carotenoid synthesis can therefore be detrimental to the plant. Nicotine, which blocks the conversion of lycopene to α- and β-carotene, kills plants through the accumulation of lycopene [3–5]. Although the chromophores of lycopene are theoretically long enough to protect plants against photo-oxidation and photodynamic damage, death cannot be prevented, presumably because lycopene cannot occupy the correct structural site in the thylakoid pigment-protein complex [6].

There are several reports of the effects of nicotine on algae. Shaish et al. [18] described the effects of nicotine on the carotenoid composition of *Dunaliella bardawil* under photoautotrophic conditions, and identified intermediates in carotenoid synthesis pathways. Rise et al. [16] isolated a nicotine-resistant mutant of *Chlorella emersonii* under photoautotrophic conditions, and examined photosynthesis and carotenoid synthesis in this mutant. Incidentally, some *Chlorella* species produce photosynthetic pigments and generate a chloroplast under heterotrophic conditions [9, 7, 17]. Because carotenoids are nonessential for heterotrophic *Chlorella* free from photo-oxidation, inhibitors of carotenoid biosynthesis should not be lethal to them, i.e., heterotrophic *Chlorella* would be useful for lycopene production, because heterotrophic *Chlorella* can possibly grow in the presence of nicotine. However, little effort has been made to apply heterotrophic *Chlorella* to commercial lycopene production, despite the rapidly expanding market for lycopene supplements due to the recent finding that lycopene is a biologically active phytochemical in the prevention of prostate cancer [11].

The present study was aimed at lycopene production by heterotrophic *Chlorella* treated with nicotine. In addition, the effect of lycopene accumulation on cellular morphology of heterotrophic *Chlorella* was investigated.

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Materials and methods

Algal strains and culture conditions

The algal strain *Chlorella regularis* Y-21, which was isolated as a mutant producing high amounts of chlorophyll and carotenoids [13], was grown from a stock maintained at the Yakult Central Institute for Microbiological Research (Tokyo, Japan). The medium used

for cultivation was described previously [1]. Nicotine (in the range of 1–10 mM) was added to the medium after autoclaving. A 1-ml portion of a day-3 heterotrophic stock culture was inoculated into 100 ml fresh medium in a 300-ml Erlenmeyer flask, stoppered with a cotton plug, and incubated at 30°C on a rotary shaker (180 rpm) without illumination.

Analysis

Biomass was estimated from dry cell weight or packed cell volume (ml l^{-1}) measured by centrifugation (1,400 *g*, 30 min) in a hematocrit. Centrifuged algal cells were ground in 90% (v/v) acetone in water with a mortar and pestle and thereafter allowed to rest for 1 h before extraction of chlorophyll and carotenoids [14]. Concentrations of these pigments were determined by their absorption at wavelengths of 750, 663, 645, 630, and 480 nm [19]. Carotenoid composition was determined by reverse-phase high performance liquid chromatography (HPLC) [2]. Duplicate analyses were carried out for each culture, and the results were averaged.

Electron microscopy

Cells were fixed with glutaraldehyde and osmium tetroxide and then embedded in epoxide resin (TAAB 812), using *n*-butylglycidylether and ethanol [8, 20]. Ultrathin sections of embedded cells were examined through a transmission electron microscope (JEOL-1200EX; JEOL, Tokyo, Japan).

Chemicals

Carotenoid standards (α-carotene, β-carotene, lutein, and lycopene) were purchased from Sigma (St. Louis, Mo.). All other reagents came from Wako Pure Chemical Industries (Osaka, Japan).

Results

Effects of nicotine on growth under heterotrophic conditions

Nicotine repressed the growth of *C. regularis* Y-21 under heterotrophic conditions at 5 and 10 mM, whereas mild repression was observed at 2 mM (Fig. 1), and changed the culture color from dark-green to orange at 5 mM (Fig. 2a). Under photoautotrophic conditions, however, *C. regularis* Y-21 was unable to grow in the presence of nicotine at 5 mM (results not shown). Nicotine also changed cellular morphology under heterotrophic conditions. The diameter of nicotine-treated cells (17.14 ± 4.11 μm) was twice that of normal cells

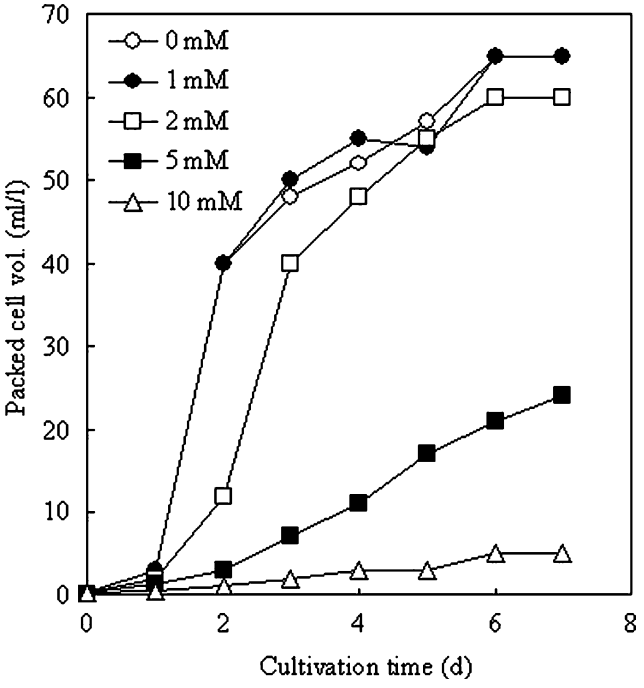

Fig. 1 Effects of nicotine on growth of *Chlorella regularis* Y-21 under heterotrophic conditions. At concentrations greater than 5 mM, nicotine severely repressed algal growth

(10.14 ± 1.82 μm). A red vesicle appeared within nicotine-treated cells (Fig. 2c) in place of the green chloroplast usually visible in normal cells (Fig. 2b). Thus, nicotine affected chloroplast generation as well as growth and coloration of *C. regularis* Y-21.

Effects of nicotine on biosynthesis of photosynthetic pigments

Since chloroplast biogenesis was abnormal in nicotine-treated *C. regularis* Y-21, we examined the photosynthetic pigment content in nicotine-treated cells. Chlorophyll content decreased as the supplied nicotine concentration increased; *C. regularis* Y-21 contained little chlorophyll at 5 mM nicotine (Table 1). On the other hand, total carotenoid content decreased at 1 and 2 mM nicotine but increased at 5 mM (Table 1); this increase was based on the accumulation of lycopene as described below. α-Carotene, β-carotene, lutein, and two unknown carotenoids thought to be xanthophylls such as violaxanthin and neoxanthin, were the major carotenoids in normal cells and in cells treated with nicotine at 1 mM. At 2 mM nicotine, a trace of lycopene was detected in addition to these major carotenoids. At 5 and 10 mM nicotine, however, lycopene accounted for more than 80% of total carotenoids, but no other carotenoids except for lutein were detected (Table 2). The apparent increase in carotenoid content at 5 mM nicotine observed photometrically (Table 1) is likely a consequence of accumulation of lycopene, whose absorption coefficient is 1.5 times more than other carotenoids [10], i.e., the carotenoid content

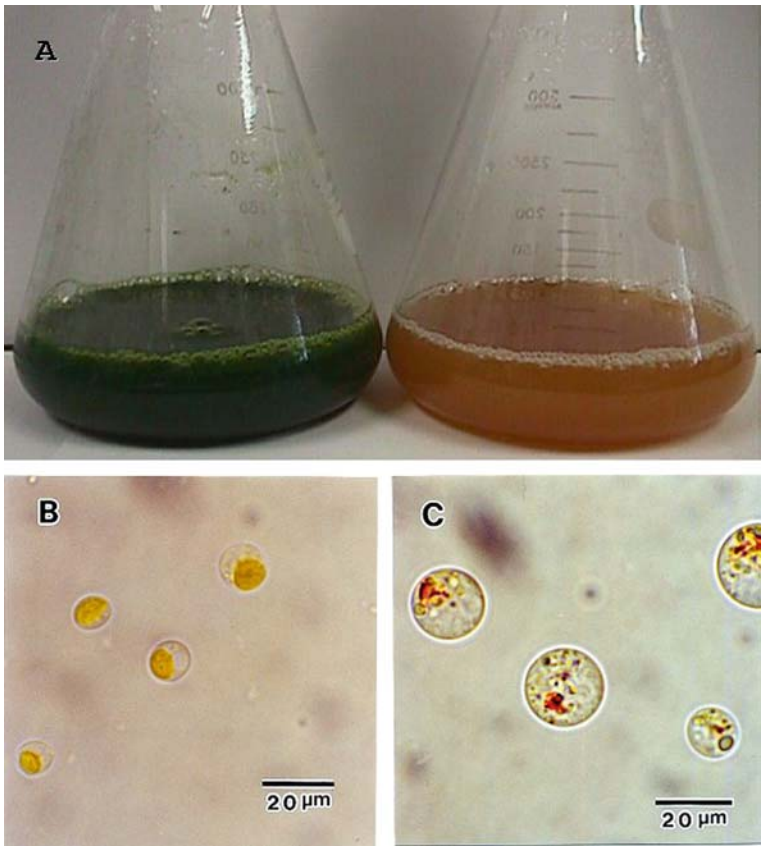

Fig. 2a–c Changes of culture coloration and cell morphology of *C. regularis* Y-21 in the presence of 5 mM nicotine. **a** *Left* Culture containing no nicotine, *right* culture containing 5 mM nicotine. **b** Cells in the absence of nicotine. **c** Cells in presence of 5 mM nicotine

Table 1 Effects of nicotine on chlorophyll and total carotenoid contents under heterotrophic cultivation conditions. Chlorophyll and carotenoid content were estimated photometrically

Nicotine (mM)	0	1	2	5	10
Chlorophyll (mg/g)[a, b]	51.95	48.12	28.99	5.28	1.65
Carotenoids[b]	7.12	6.21	4.26	6.45	5.45

[a]Per dry cell weight
[b]Calculated according to Stickland and Parsons [19]

Table 2 Effects of nicotine on carotenoid composition under heterotrophic cultivation. Each carotenoid content was determined by reverse-phase high performance liquid chromatography (HPLC)

Nicotine (mM)	0	1	2	5	10
Lycopene (mg/g)[a]	0.00	0.00	0.03	1.67	1.86
α-Carotene	0.53	0.44	0.28	0.07	0.01
β-Carotene	0.70	0.49	0.31	0.05	0.02
Lutein	3.16	1.72	1.20	0.40	0.22
Unknown 1[b]	1.81	1.31	0.91	0.26	0.11
Unknown 2[b]	0.45	0.25	0.21	0.11	0.07
Total carotenoids[c]	6.65	4.21	2.94	2.56	2.29

[a]Per dry cell weight
[b]Calculated as equivalent to lutein
[c]The total sum of each carotenoid

at 5 and 10 mM given in Table 1 will be over-estimated owing to lycopene, since the carotenoid content in Table 1 is somewhat greater than that in Table 2. These observations suggest that photometric estimation of carotenoid content is inadequate for lycopene-accumulating *Chlorella*. In any case, nicotine had a clear influence on carotenoid composition as well as on chloroplast generation.

Electron microscopy of nicotine-treated cells

Nicotine treatment caused changes in cellular morphology. We observed further details of these morphological changes by electron microscopy. The cell wall of nicotine-treated cells was thicker than that of normal cells (70 vs 28 nm). The nucleus did not differ, but organelles such as mitochondria and Golgi apparatus were relatively obscure in nicotine-treated cells (Fig. 3a–c). Nicotine-treated cells had a long, slender organelle with an extremely high electron density but no normal chloroplast (Fig. 3d). This organelle corresponded to the red vesicle observed under the light microscope in Fig. 2.

Discussion

Although trace amounts of lycopene were accumulated in photoautotrophic *Chlorella emersonii* [16] and *Dunaliella bardawil* [18], the present study is the first report of hyper-accumulation of lycopene (more than 80% of

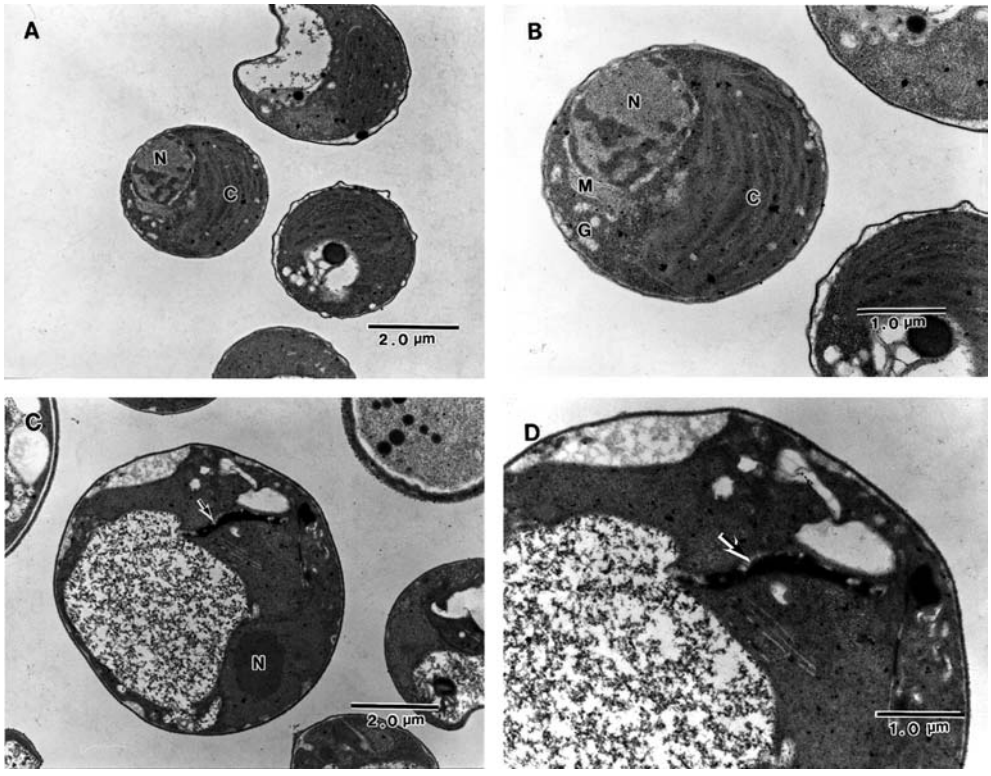

Fig. 3a–d Electron micrographs of cell morphology. **a**, **b** Normal *Chlorella*, **c**, **d** nicotine-treated *Chlorella*. The *arrow* indicates a red vesicle with an extremely high electron density. *C* Chloroplast, *G* Golgi apparatus, *M* mitochondria, *N* nucleus

total carotenoids) in algae. This finding is not specific to strain Y-21, as nicotine causes lycopene accumulation and cyclic carotenoid deficiency among other *Chlorella* spp. grown heterotrophically (results not shown). These observations suggest that heterotrophic *Chlorella* treated with nicotine is useful for lycopene production, although nicotine severely repressed growth.

Nicotine inhibits carotenoid cyclization, and cyclic carotenoids are necessary for assembly of active photosystems [6]. Therefore, the deficiency of cyclic carotenoids caused by nicotine is lethal to *Chlorella* under photoautotrophic conditions. However, heterotrophic *Chlorella* metabolizes glucose as an energy source, so cells can grow even if they possess inactive photosystems. Consequently, lycopene that is not incorporated into photosystems will aggregate inside the immature chloroplast to form a red vesicle in the presence of nicotine under heterotrophic conditions. As described above, heterotrophic *Chlorella* exhibits a unique cell morphology in response to inhibition of carotenoid synthesis. Many inhibitors of carotenoid synthesis are used as herbicides in agriculture [3–5]. Therefore, heterotrophic *Chlorella* might be suitable for screening large numbers of potential herbicides.

Role of cyclic carotenoids in assembly of photosystems

In green algae, lutein is bound to the light-harvesting chlorophyll- *a*/*b*-carotenoid-protein complex, whereas *β*-carotene is primarily associated with the chlorophyll-*a*/*β*-carotene-protein complex located in the reaction centers of photosystem (PS) I and PS II [15]. Strong evidence has been presented that lutein is an essential component in the assembly of the active PS II unit [12]. Therefore, failure of chloroplast generation in the presence of nicotine may result from the lack of lutein to assemble an active PS II unit. Although we do not yet know why nicotine enlarged the cell size and inhibited chlorophyll synthesis, heterotrophic culture of *Chlorella* will be useful in expanding our knowledge of the roles of carotenoids in the construction of the photosynthetic apparatus.

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