Enhanced protection against oxidative stress in an astaxanthin-overproduction <i>Haematococcus</i> mutant (Chlorophyceae)
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Enhanced protection against oxidative stress in an astaxanthin-overproduction *Haematococcus* mutant (Chlorophyceae)

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Many unicellular green algae can become yellow or red in various natural habitats due to mass accumulation of a secondary carotenoid, such as lutein, or astaxanthin. The accumulation of secondary carotenoids is generally thought to be a survival strategy of the algae under photo-oxidative stress or other adverse environmental conditions. The physiological role of the carotenoids in stress response is less well understood at the subcellular or molecular level. In this study, a stable astaxanthin overproduction mutant (MT 2877) was isolated by chemical mutagenesis of a wild type (WT) of the green microalga *Haematococcus pluvialis* Flotow NIES-144. MT 2877 was identical to the WT with respect to morphology, pigment composition, and growth kinetics during the early vegetative stage of the life cycle. However, it had the ability to synthesize and accumulate about twice the astaxanthin content of the WT under high light, or under high light in the presence of excess amounts of ferrous sulphate and sodium acetate. Under stress, the mutant exhibited higher photosynthetic activities than the WT, based on considerably higher chlorophyll fluorescence induction, chlorophyll autofluorescence intensities, and oxygen evolution rates. Cell mortality caused by stress was reduced by half in the mutant culture compared with the WT. Enhanced protection of the mutant against stress is attributed to its accelerated carotenogenesis and accumulation of astaxanthin. Our results suggest that MT 2877, or other astaxanthin overproduction *Haematococcus* mutants, may offer dual benefits, as compared with the wild type, by increasing cellular astaxanthin content while reducing cell mortality during stress-induced carotenogenesis.

**Key words:** astaxanthin, carotenogenesis, flow cytometry, *Haematococcus pluvialis*, oxidative stress, pigment mutant

**Introduction**

Many unicellular green algae can exhibit yellow to red pigmentation in various natural habitats, such as surfaces of snow, soil, tree trunks or rocks, or hyper-saline water (Weiss, 1983; Chapman, 1984; Ginzburg, 1987; Borowitzka, 1997; Graham & Wilcox, 2000). The yellow to red appearance of green algae is due to the formation of a group of carotenoids, such as \(\beta\)-carotene, lutein, zeaxanthin and astaxanthin (Johnson & An, 1991; Schroeder & Johnson, 1995; Margalith, 1999). An individual algal species/strain may synthesize and accumulate a particular carotenoid in quantities ranging from several to tens of milligrams per gram of dry weight along with several minor or trace amounts of other carotenoids. Because carotenogenesis of green algae is frequently associated with adverse environmental conditions, such as nutrient depletion, salinity, low temperatures and/or high solar radiation (Schroeder & Johnson, 1995), it is generally believed that synthesis and accumulation of orange to red carotenoids is a survival strategy of the alga under changing or adverse environmental conditions. However, the possible protective role of carotenogenesis or its products (carotenoids) in the algal cell is not well understood at the subcellular or molecular level.

In the past decade, the green alga, *Haematococcus pluvialis* Flotow has been used as a model system to study carotenogenesis and the possible physiological role of carotenoids, in particular astaxanthin, in stress response. A photo-protective role for astaxanthin has been suggested, along with lower lipid peroxidation (Hagen et al., 1993b), a higher Fv/Fm...
value (Hagen et al., 1994), or a higher oxygen evolution rate (Hagen et al., 2000; Wang et al., 2003) has been associated with astaxanthin-rich cells compared with astaxanthin-free vegetative cells. Contrastingly, in some other investigations, the photosynthetic activity was shown to decrease during the accumulation of astaxanthin in H. pluvialis, as demonstrated by a lower maximum photosynthetic rate, $P_{\text{max}}$ (Zlotnik et al., 1993), a lower Fv/Fm value (Fan et al., 1998), or a lower net oxygen evolution rate (Tan et al., 1995; Fan et al., 1998; Qiu & Li, 2006). Although different strains of H. pluvialis, different experimental settings, or analytical approaches employed among the studies might account partially for the lack of agreement in elucidating the possible role of astaxanthin, the direct comparison between astaxanthin-rich cells with astaxanthin-free vegetative cells commonly used in previous studies was problematic. In addition to the presence of astaxanthin, red cells differ largely from green vegetative cells by cell size, cell wall, subcellular ultrastructure, biochemical composition, and metabolism. Any difference in response of red cysts from green vegetative cells to oxidative stress cannot be solely attributed to the presence or quantity of astaxanthin.

Application of inhibitors to certain enzymes involved in carotenogenesis represents another common approach to depict the possible role of astaxanthin in stress response and also assist in identifying genuine intermediates in the pathway of astaxanthin biosynthesis (Fan et al., 1995, 1998; Harker & Young, 1995; Hagen & Grunewald, 2000; Zhekisheva et al., 2005). The increase in susceptibility or cell mortality of inhibitor-treated algal cells to high light and/or other environmental stimuli was used as evidence to support the photoprotective role of astaxanthin in untreated ones. However, the proven toxic side-effect and/or limited inhibition-specificity of these tested inhibitors made the above observation less conclusive (Sandmann & Boger, 1989; Fan et al., 1995; Harker & Young, 1995; Zhekisheva et al., 2005).

A possible role of astaxanthin as an antioxidant against stress-induced excessive reactive oxygen species (ROS) was also proposed (Kobayashi et al., 1997; Kobayashi, 2000). The concern was that the different responses of mature (astaxanthin-rich) and immature (astaxanthin-poor) Haematococcus cells or cell-free extracts to exogenously introduced ROS or ROS-inducing chemicals may not necessarily reflect real physiological responses of individual forms of the cells to endogenous ROS under natural environmental stress conditions.

In this study, we have isolated a stable astaxanthin overproduction mutant (MT 2877) by chemical mutagenesis of the wild type (WT) of H. pluvialis Flotow NIES-144. MT 2877 was identical to the WT with respect to cell morphology, pigment composition, and growth kinetics during the early vegetative stage of the life cycle. However, the mutant had the ability to synthesize and accumulate about 100% more astaxanthin than the WT under high light or other stress conditions (e.g. heavy metal and high salinity). A comparative study of MT 2877 and the WT provided new insight into the physiological role of carotenogenesis and astaxanthin in the stress response. Based on our observations, MT 2877 or a similar astaxanthin overproduction mutant may provide a distinct advantage over the wild type for commercial production of astaxanthin.

Materials and methods

Organism, growth medium, and culture conditions

Haematococcus pluvialis Flotow NIES-144 was obtained from the National Institute for Environmental Studies in Tsukuba, Japan. A basal growth medium described by Kobayashi et al. (1991) was used: 14.6 mM sodium acetate; 2.7 mM L-asperagine; 2 g L$^{-1}$ yeast extract; 0.985 mM MgCl$_2$; 0.135 mM CaCl$_2$; 0.036 mM FeSO$_4$; pH 6.8. The cells were grown in 250 ml Erlenmeyer flasks containing 100 ml of growth medium. Cultures were incubated in a Percival growth chamber (model: 1–35LLVL; Boone, IA, USA) at 22°C and 20 µmol photons m$^{-2}$ s$^{-1}$ of light (20 W white fluorescent lamps) under a 12:12-h light-dark cycle. Cultures were shaken manually twice a day. For induction of astaxanthin biosynthesis and red cyst formation, exponentially growing cultures (cell density of approximately 5 x 10$^5$ cells ml$^{-1}$) were exposed to continuous illumination of 250 µmol photons m$^{-2}$ s$^{-1}$ in the presence or absence of sodium acetate and ferrous sulphate at a final concentration of 45 mM and 450 µM, respectively. As a control, some cultures were maintained throughout the experiment period under continuous, low light intensity of 20 µmol photons m$^{-2}$ s$^{-1}$ (LL).

Mutagenesis and isolation of putative pigment mutants

Chemical mutagenesis of H. pluvialis was performed using the chemical mutagen, N-methyl-N-nitro-N-nitrosoguanidine (MNNG). MNNG was able to induce mutations through base-pair substitutions with high frequencies and little lethality. It has been suggested as one of the best mutagens to generate mutant strains of bacteria, yeasts, plants, and microalgae (Loppes & Deltour, 1981; Ito et al., 1994). Cells in the exponential growth phase were incubated with 50 mg ml$^{-1}$ MNNG at 25°C for 30 min. Mutagenesis was terminated by adding an equal volume of freshly made 10% w/v filter-sterilized sodium thiosulphate into the reaction solution. Treated cells were collected by centrifugation (2000 g, 10 min). For expression of mutations, the mutagenized cells were incubated on agar
plates containing the acetate basal medium and 20 mg ml$^{-1}$ of ampicillin (sodium salt). When mutagenized colonies developed on the agar plate, they were transferred individually into test tubes containing 5 ml of liquid acetate basal medium and incubated in a Percival growth chamber at 22°C and 20 μmol photons m$^{-2}$ s$^{-1}$ of light under a 12:12-h light-dark cycle. Screening for mutants was performed by examining cells from individual colonies by light microscopy and pigment analysis using reverse-phase HPLC.

**Cell counting, cell dry weight measurement, and pigment analysis**

The cell number was determined using a haemocytometer under a light microscope (Olympus BH-2, Olympus, Tokyo, Japan). For cell dry weight measurement, a 10 ml aliquot of culture was filtered through pre-weighed Whatman GF/C filter paper. The filter paper was dried overnight in an oven at 80°C till a constant weight was reached. The difference between the final weight and the weight before filtration was the dry weight of the sample. Pigment composition of the cells was analysed by HPLC according to the method of Yuan et al. (2002). Algal cells were harvested and extracted in the solvent mixture of dichloromethane and methanol (25:75, v/v). The pigment extracts (20 μl aliquots) were separated and analysed by using a Beckman Ultrasphere C18 column (250 mm long, 4.6 mm i.d.; 5 μm; Beckman Instruments, Fullerton, CA, USA) at 25°C. The mobile phase consisted of solvent A (dichloromethane/methanol/acetonitrile/water, 5.0: 85.0: 5.5: 4.5, v/v) and solvent B (dichloromethane/methanol/acetonitrile/water, 25.0: 28.0: 42.5: 4.5, v/v). The flow rate was 1.0 ml min$^{-1}$. The three dimensional chromatogram was monitored from 250 to 750 nm. Peaks were measured at a wavelength of 480 nm to facilitate the detection of astaxanthin species, lutein and β-carotene. Chlorophyll a and b were measured at 450 nm according to Yuan et al. (1997). Chromatographic peaks were identified by comparing retention times and spectra against known standards or by comparing their spectra with published data.

**RNA isolation and real-time PCR analysis**

*Haematococcus* cells were collected by centrifugation and the resulting cell pellet was frozen and subsequently ground under liquid nitrogen using a mortar and pestle. RNA was then isolated according to the miniprep RNA extraction procedure (Sokolowsky et al., 1990) with minor changes: RNA extract was treated with DNase-I (Invitrogen, Carlsbad, CA, USA) at 37°C for 30 min, and then mixed thoroughly with an equal volume of phenol:chloroform (1:1). After centrifuging (10,000 g, 10 min at 4°C), 0.75 ml volumes of 8 M LiCl were added for precipitation. Nuclear acids were quantified by NanoDrop 3.0.0 (NanoDrop, Wilmington, DE, USA).

For real-time (RT) PCR analysis, first strand cDNA synthesis was carried out using a TaqMan® Reverse Transcription system (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instruction. 250 ng of total RNA was used in a 10 μl reaction system. Controls received water instead of reverse transcriptase to assess any contamination of genomic DNA. Primers were designed by using the Primer Express® Software Version 2.0 (Applied Biosystems, Foster City, CA, USA) except primers for *pds* were from Grunewald et al. (2000). The primer concentrations were determined when specific amplification relative to primer dimers was maximal in a positive versus negative control experiment. Primer sequences, concentrations, and gene accession numbers are summarized in Table 1.

Plasmids (from TA Cloning Kit, Invitrogen, Carlsbad, CA, USA) containing cDNAs from each gene were used as the templates for standards. Equimolar quantities of the standards were 10-fold serially diluted and used to generate standard curves. RT-PCR was performed in triplicate on cDNA samples and controls on an ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA, USA). In each experiment, a standard dilution series of plasmids containing specific PCR fragments and 25 ng cDNA (total RNA equivalent) of unknown samples were amplified in a 20 μl reaction containing 1 × SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The experiments were repeated independently at least twice. Data were captured as amplification plots. Transcription levels of the target genes were calculated from threshold cycle by interpolation from the standard curve. To standardize the results, the relative abundance of 18S rRNA gene was also determined and used as the normalization standard. All calculations and statistical analysis were performed as described in the ABI 7900 sequence detection system User Bulletin 2 (Applied Biosystems, Foster City, CA, USA).

**Chlorophyll fluorescence**

Cells were harvested by centrifugation and resuspended in fresh BG-11 medium to obtain an O.D. of 2.8 at 730 nm. Chlorophyll fluorescence induction curves

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession number</th>
<th>Forward primer (5′–3′)</th>
<th>Reverse primer (5′–3′)</th>
<th>Primer concentration (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S</td>
<td>AF159369</td>
<td>TGCCCTAGTAAACCGCGAGTCA</td>
<td>CCCACCGCTAAAGTCATCC</td>
<td>100</td>
</tr>
<tr>
<td>cre O</td>
<td>D58881</td>
<td>ACGCTCACAACCTCCAGCA</td>
<td>AAAAACCTCGGTCCAGGTG</td>
<td>100</td>
</tr>
<tr>
<td>cre R-b</td>
<td>AF162276</td>
<td>GGGCTGAACCTGGACGTTTG</td>
<td>GCCAGGTCATTGCTTACAA</td>
<td>50</td>
</tr>
<tr>
<td>ipi</td>
<td>AB019034</td>
<td>GTGATGTCAGCCGGAGGAG</td>
<td>TCCCCAATCTCGTGTGGT</td>
<td>250</td>
</tr>
<tr>
<td>psy</td>
<td>AF305430</td>
<td>ACCAGAGCTTCCGAGGCTG</td>
<td>TGCCACATGACAGGCATAGTC</td>
<td>150</td>
</tr>
<tr>
<td>pds</td>
<td>X86783</td>
<td>TCCGCTAGGGCTGCTGC</td>
<td>GGCGAGGTCTTGAGGCT</td>
<td>250</td>
</tr>
</tbody>
</table>
were measured using a PAM Chlorophyll Fluorometer (Heinz Walz GmbH, Effeltrich, Germany) in the presence or absence of 50 μM 3-(3,4-dichlorophenyl)-1,1-dimethyurea (DCMU) at room temperature. Chlorophyll concentration was about 10 μg ml⁻¹.

**Oxygen evolution measurements**

Green and red cells were harvested by centrifugation (3000g, 2 min), and the pellets were resuspended in fresh acetate basal medium to obtain the same cell numbers or the same chlorophyll concentrations. Oxygen evolution rate of each sample was determined with an O₂ Oxygraph (Hansatech Instruments Ltd, Norfolk, UK) at 30°C, 2000 μmol photons m⁻²s⁻¹ according to Vonshak et al. (1988). Red or white light was used as separate light sources in different experiments. Chlorophyll concentration was about 10 μg ml⁻¹.

**Flow cytometric analysis**

*In vivo* chlorophyll autofluorescence was analysed using a flow cytometer (FACScalibur, Becton-Dickinson, San Jose, CA, US) equipped with an ion argon laser (excitation 488 nm, 30 mW power). Autofluorescence was displayed in a histogram using FL3 as the chlorophyll fluorescence emitted at 675 ± 20 nm. A small volume (5 ml, ∼10⁶ cells ml⁻¹) of *Haematococcus* cell suspension was withdrawn from the culture at time intervals during the cell cycle. Cells were washed twice by centrifugation with deionized water, then resuspended and transferred to polystyrene tubes. For the flow cytometric measurements 20,000–50,000 events were acquired.

**Results**

**Generation of astaxanthin over-production mutant**

*N*-methyl-N-nitro- N-nitrosoguanidine (MNNG) has been previously used as an effective mutagen to generate numerous algal mutants such as cell wall-deficient mutants of the unicellular green algae *Chlamydomonas reinhardtii* (Hyams & Davies, 1972) and *H. pluvialis* (Wang et al., 2005), as well as pigment mutants from several species of the red algal genus *Porphyra* (Mitman & Vandermeer, 1994; Yan et al., 2000). In this study, MNNG was applied to *H. pluvialis* to obtain astaxanthin over-production mutants. Treatment with 50 mg ml⁻¹ MNNG at 25°C for 30 min resulted in a mortality rate of ca. 95% in *H. pluvialis*. About 3000 mutant colonies were obtained and subjected to screening using spectrophotometric and HPLC analyses.

Individual mutant colonies were picked up from agar plates and transferred separately into 10-ml test tubes containing 5 ml of the basal growth medium. The test tubes were first maintained under the optimal growth conditions for 7 days. Exponentially growing cells were then subjected to high light of 250 μmol photons m⁻²s⁻¹ (HL), or high light in the presence of sodium acetate and ferrous sulphate at final concentrations of 45 mM and 450 μM (HL + SA + FE), respectively, for astaxanthin induction. These stress treatments have previously been used for maximum induction of astaxanthin formation while minimizing mortality in the culture (Kobayashi et al., 1991; Kobayashi, 2000; Steinbrenner & Linden, 2001; Wang et al., 2004). As a result, several putative pigment mutants were obtained, one of which (MT 2877) exhibiting the highest astaxanthin content (3.9 ± 0.5% of dry weight) at a high light intensity of 350 μmol photons m⁻²s⁻¹ and comparable morphology to the WT, was subjected to further investigation.

**Pigment profile and quantification**

Compared with WT, the MT 2877 culture appeared somewhat yellowish in colour during late exponential growth phase (ca. a 4-day old culture) under optimal growth conditions (Fig. 1a). Spectrophotometric analysis of whole cell extracts normalized on a chlorophyll a basis revealed that MT 2877 possessed noticeably more carotenoids in green vegetative cells (Fig. 1c), and accumulated larger amounts of carotenoids in cysts, compared with the WT (Figs. 1b, d).

HPLC analysis of pigment extracts of the two strains showed that the total chlorophyll content (including chlorophyll a and b) on a per dry weight basis was 11.3% lower in MT 2877 than in WT, although the chlorophyll a/b ratios were similar between the two in the green vegetative stage (Table 2). Major carotenoids in *H. pluvialis* at the green stage were lutein and β-carotene. Quantitatively, the lutein and β-carotene contents were comparable in the MT 2877 and WT (Table 2).

After stress induction, the chlorophyll content in MT and WT cells decreased considerably, yet the rate of decrease was higher in the WT than in the MT 2877. After 3 days of HL stress, the total chlorophyll content in the WT was approximately 57.2% of that in green vegetative cells, whereas the MT 2877 had approximately 72.9% of the chlorophyll measured in green vegetative cells (Table 2). Under HL stress, the oxygenated carotenoid astaxanthin occurred and rapidly became the dominant carotenoid. The lutein and β-carotene content remained more or less constant in the two strains (Table 2).

Astaxanthin appeared earlier with a higher production rate in MT 2877 than in the WT under stress conditions. After exposure to HL (i.e. 250 μmol photons m⁻²s⁻¹) for 4 days, the cellular
content of astaxanthin in MT 2877 and WT was about 1.6% and 0.8% of dry weight, respectively (Fig. 2). As light intensity increased to 350 μmol photons m⁻² s⁻¹, the maximum content of astaxanthin in MT 2877 and WT reached 3.9 ± 0.5% and 2.0 ± 0.3% of dry weight, respectively, after 7 days of cultivation. On the other hand, the pigment composition of MT 2877 was identical to that of the WT, as indicated by identical absorption spectra and retention time of each peak in HPLC chromatograms of the two strains (Figs. 3a, b).

Expression of the major genes involved in carotenoid biosynthesis

Relative expression of five genes involved in carotenoid biosynthesis was investigated in both strains under stress conditions. They were *ipi*, *psy*, *pds*, *crtO*, and *crtR-b* encoding isopentenyl-diphosphate δ-isomerase (IPI), phytoene synthase (Psy), phytoene desaturase (Pds), beta-carotene oxygenase (CrtO), and β-carotene hydroxylase (CrtR-b), respectively. The transient increase in mRNA transcripts of these genes occurred in both strains in response to oxidative stress, and the major results are presented in Table 3. The increase in maximum mRNA transcripts of *psy* and *crtR-b* was more than 28-fold, whereas the increase in that of *ipi*, *pds* and *crtO* was within ca. 10-fold, relative to that measured just before HL treatment. A comparison of the two strains at the late vegetative stage revealed that the mutant cells already showed higher expression levels of *ipi*, *pds* and *crtR-b* than the WT. Under HL + SA + FE, the maximum relative transcript levels of *ipi* and *pds* were nearly double and that of *crtR-b* was quadrupled in the mutant compared with the WT. On the other hand, the relative transcript levels of *psy* and *crtO* were similar in the two strains. The transient up-regulation of these carotenoid biosynthetic genes was correlated with the occurrence and accumulation of astaxanthin in the *Haematococcus* cell.

Table 2. Pigment content of WT and MT 2877 of *Haematococcus pluvialis* at different culture stages.

<table>
<thead>
<tr>
<th></th>
<th>Chl a content (mg g⁻¹ DW)</th>
<th>Chl b content (mg g⁻¹ DW)</th>
<th>Chl a/b ratio</th>
<th>Lutein (mg g⁻¹ DW)</th>
<th>β-carotene (mg g⁻¹ DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT Green¹</td>
<td>18.89 ± 0.6</td>
<td>8.8 ± 0.33</td>
<td>2.15</td>
<td>4.18 ± 0.35</td>
<td>0.88 ± 0.09</td>
</tr>
<tr>
<td>MT Green¹</td>
<td>16.85 ± 0.65</td>
<td>8.03 ± 0.41</td>
<td>2.10</td>
<td>4.35 ± 0.35</td>
<td>0.90 ± 0.09</td>
</tr>
<tr>
<td>WT Red²</td>
<td>11.39 ± 0.12</td>
<td>4.46 ± 0.23</td>
<td>2.55</td>
<td>4.20 ± 0.23</td>
<td>1.04 ± 0.12</td>
</tr>
<tr>
<td>MT Red²</td>
<td>12.78 ± 0.9</td>
<td>5.35 ± 0.41</td>
<td>2.39</td>
<td>4.50 ± 0.19</td>
<td>0.85 ± 0.12</td>
</tr>
</tbody>
</table>

¹Green stage cells were collected after 4 days of optimal growth.
²Red stage cells were collected 3 days after high-light induction.
Chlorophyll fluorescence induction

Chlorophyll a fluorescence of dark-adapted MT 2877 and WT cells was measured in the presence, or absence, of 50 μM 3-(3,4-dichlorophenyl)-1,1-dimethyurea (DCMU), which is commonly adopted to measure the potential quantum efficiency of photo-system II (PSII). At the green vegetative stage, MT 2877 cells emitted weaker fluorescence with a lower $F_v/F_m$ value than the WT in the presence or absence of DCMU, indicative of its weaker photosynthetic activity (Fig. 4a). A reverse situation occurred when MT 2877 cells underwent the transformation from the green vegetative stage to the resting red cyst stage. Figure 4B shows that the chlorophyll fluorescence of MT 2877 red cysts under HL + SA + FE for 4 days was much stronger than that of the WT, with higher $F_v/F_m$ value in the presence...
or absence of DCMU, suggesting that the overproduction of astaxanthin in the mutant enhanced its photosynthetic efficiency under stress (Fig. 4b).

Table 3. Maximum mRNA transcript level of carotenoid biosynthetic genes of *Haematococcus pluvialis* under HL + SA + FE.

<table>
<thead>
<tr>
<th>Gene</th>
<th>MT 2877 0h</th>
<th>WT 0h</th>
<th>Maximum expression point (h)</th>
<th>MT 2877 0h</th>
<th>WT 0h</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ipi</em></td>
<td>3.18 ± 0.35</td>
<td>1.61 ± 0.33</td>
<td>12</td>
<td>0.95 ± 0.08</td>
<td>0.52 ± 0.1</td>
</tr>
<tr>
<td><em>psy</em></td>
<td>6.01 ± 0.7</td>
<td>6.08 ± 0.75</td>
<td>48</td>
<td>0.19 ± 0.06</td>
<td>0.21 ± 0.03</td>
</tr>
<tr>
<td><em>pds</em></td>
<td>1.05 ± 0.36</td>
<td>0.6 ± 0.07</td>
<td>12</td>
<td>0.18 ± 0.03</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td><em>crtO</em></td>
<td>1.28 ± 0.12</td>
<td>1.17 ± 0.16</td>
<td>48</td>
<td>0.13 ± 0.02</td>
<td>0.1 ± 0.02</td>
</tr>
<tr>
<td><em>crtR-b</em></td>
<td>639.3 ± 58.27</td>
<td>143.6 ± 9.5</td>
<td>48</td>
<td>5.93 ± 0.42</td>
<td>2.01 ± 0.16</td>
</tr>
</tbody>
</table>

aGene transcript level was measured at 0 h, 12 h, 24 h and 48 h. (Relative amounts were calculated and normalized with respect to one thousandth of 18S gene transcript levels. Data shown represent mean values obtained from three independent amplification reactions, and the error bars indicate the SE of the mean.)

bMaximum expression point was expressed as hours after stress induction.

cFor comparison, the basal mRNA levels of the genes before stress induction (4-day old green vegetative culture) were measured as MT 2877 0 h or WT 0 h.

**Oxygen evolution**

Consistent with the chlorophyll fluorescence measurement, the maximum O₂ evolution rate of the mutant was lower, on a per chlorophyll basis, than that of the WT at the green vegetative stage (Fig. 5a). However, the reverse situation occurred in red cysts, where the maximum O₂ evolution rate of the mutant was almost double that of the WT (Fig. 5b). O₂ evolution measurements using white or red light source, or normalized on a per cell basis showed a similar trend (data not shown).

**Flow cytometric analysis**

When excited with 488 nm light, both WT and MT2877 cells maintained under optimal growth conditions emitted autofluorescence (M2) at the maximum wavelength of ca. 670 nm, as shown in Fig. 6a,b. It is believed that the autofluorescence was derived from photosynthetically functional chlorophyll or living cells. When the cells were subjected to HL + SA + FE treatment, weak chlorophyll autofluorescence (M1) was detected in addition to the strong autofluorescence (M2), as detected in non-stressed cells. The weak auto-fluorescence is speculated to be derived from partially degraded chlorophyll molecules, impaired chlorophyll-protein complexes, or damaged cells. Thus, after 6 days stress induction, two subpopulations of the culture (i.e. healthy and damaged) were present in the WT (Fig. 6c) and MT2877 cultures (Fig. 6d), one emitting strong chlorophyll auto-fluorescence (M2) the other emitting weak chlorophyll fluorescence (M1). From a quantitative perspective, the higher cell counts with strong chlorophyll autofluorescence were measured in the MT2877 culture than in the WT, suggesting that higher photosynthetic activity or more living cells remained in the former than the latter. As a result, little chlorophyll autofluorescence emission was detected in the WT culture after 15 days of stress induction (Fig. 6e), whereas more chlorophyll...
autofluorescence occurred in the mutant culture (Fig. 6f).

**Growth and cell mortality under stress**

Growth kinetics of the WT and MT 2877 are shown in Fig. 7. Little difference was observed in the two strains and the maximum specific growth rate was about 0.031 h\(^{-1}\) under optimal growth conditions. Reduction in growth (cell number) by about 10% occurred in the MT 2877 culture after day 4, compared with WT. Upon stress induction, both WT and mutant cells exhibited moderate growth for 2–3 days. As stress continued, significant cell mortality occurred in both strains, with the cell mortality being higher in the WT than in the mutant culture. After 10 days in HL + SA + FE, the WT culture lost about 40% of its population due to oxidative damage of the cells. In contrast, the MT 2877 culture retained about 80% of its population (Fig. 7a). Light microscopy revealed a remarkably higher number of photo-bleached cells in the WT culture than in the mutant culture (Fig. 7b, c), although the thickness and morphology of the cell wall were similar in the two strains. As a result, the biomass production of the mutant was about twice that of the WT under oxidative stress conditions. The WT culture was completely bleached after being under HL + SA + FE for 1 month, whereas the mutant culture remained pigmented and viable (Fig. 7d).

**Discussion**

In this study, an astaxanthin overproduction mutant, MT 2877, has been obtained using chemical mutagenesis and exhibited stable phenotypic characteristics for over five years in our laboratory. Under HL or HL + SA + FE, MT 2877 cells exhibited higher photosynthetic activities and lower cell mortality than the WT. As the mutant cells had similar morphologies and growth kinetics to the WT during the early vegetative stage, the increase in tolerance of the mutant to oxidative stress may be attributed largely to enhanced carotenoid biosynthesis. The advantage of using an astaxanthin overproduction mutant to study the physiological role of carotenogenesis or astaxanthin is that it allows the comparison with the WT to be based upon characteristics of the cells at the same stage of the life cycle, in contrast to previous investigations which compared red cysts with green vegetative cells that are morphologically, physiologically, and biochemically different (Hagen et al., 1993a; Zlotnik et al., 1993; Tan et al., 1995; Kobayashi et al., 1997; Hagen & Grunewald, 2000; Kobayashi, 2000; Wang et al., 2003; Qiu & Li, 2006). Also, the negative effect caused by the application of inhibitors to certain enzymes involved in carotenogenesis can be avoided.

The MT 2877 mutant can be used as a model system to study the expression and regulation of the genes involved in pigment metabolism, in particular carotenogenesis and astaxanthin formation. Compared with the WT, MT 2877 had a reduced amount of Chl and slightly higher (relative to Chl content) content of carotenoids (mainly lutein and beta-carotene) at the late vegetative stage. This may reflect the fact that MT 2877 was more sensitive than the WT to environmental stress signals, such as depletion of certain nutrient(s) necessary for cell division and growth, or accumulation of certain inhibitory metabolites in the culture medium. The environmental stress signals may in turn trigger carotenogenesis while at the same time reducing chlorophyll biosynthesis. Indeed, maintaining the WT culture in low light (20\(\mu\)mol photons m\(^{-2}\) s\(^{-1}\)) for an extended period of time (1–2 weeks) also resulted in decreased chlorophyll content with concomitant
formation of astaxanthin, albeit at a minimum level (data not shown). HL or HL + SA + FE were the stresses introduced in this study to induce or accelerate carotenogenesis and formation of astaxanthin, but apparently they were not the only factors that triggered the changes in pigment metabolism in our experimental system. A similar H. pluvialis mutant, Car-3, was previously isolated by Sun et al. (1998). In the vegetative stage, Car-3 strain had a similar carotenoid: chlorophyll ratio to WT. Upon transfer to high-light of 150 μmol photons m⁻² s⁻¹, Car-3 cells were precocious in carotenoid accumulation, resulting in a ca. 2-fold increase in carotenoid: chlorophyll ratio relative to the WT after 24 h of induction (Sun et al., 1998). However, the genetic background of Car-3 was unknown and no further physiological characteristics of Car-3 were available.

Although ipi, psy, pds, crtO and crtR-b all underwent transient up-regulation at the transcription level under HL or HL + SA + FE, the extent to which individual genes responded was different. The increase in the maximum transcript levels of ipi, pds, and crtO was within an order of magnitude, whereas that of psy and crtR-b were over an order of magnitude higher, relative to that measured just before stress induction. Furthermore, the maximum transcript levels of ipi and pds were doubled and that of crtR-b was quadrupled in the mutant over that in the WT, resulting in twice the amount of astaxanthin in the former. Also, the maximum transcript levels of ipi and pds occurred at 12 h, whereas those of psy, crtO, and crtR-b occurred at 48 h, reflecting the possible co-existence of multiple regulatory mechanisms among these carotenoid biosynthetic genes at the transcription level. Little difference in the transcripts of psy and crtO between MT 2877 and the WT suggests that Psy and CrtO may not be under tight transcriptional control. Previously, the
transient up-regulation of an *ipi* gene encoding a 32.5-kDa isoform of IPI (Sun et al., 1998) and *pds* gene in *H. pluvialis* (Grunewald et al., 2000) was reported to result in enhanced cellular astaxanthin accumulation.

It is not known at this point if MT 2877 was the result of a single- or multi-gene mutation. Complementation tests through inter-crossing MNNG-induced *C. reinhardtii* and *H. pluvialis* mutants with their wild-type counterparts suggested that those mutants resulted from a single-gene mutation (Mittman & Vandermeer, 1994; Yan et al., 2000). If this is the case for MT 2877, it would be unlikely that the mutated gene was a structural gene involved in an individual reaction in either chlorophyll biosynthesis or carotenogenesis. Instead, it must be a key regulatory gene that controls these two pathways, thereby redirecting carbon partitioning from chlorophyll synthesis, and possibly the carbohydrate pathway, into carotenogenesis. We cannot, however, exclude the possibility of mutations in multiple genes regulating these pathways.

MT 2877 protected itself better from oxidative stress than the WT. Although cell mortality occurred in both the WT and MT 2877 cultures after being transferred from the optimal growth conditions to HL or HL + SA + FE for 10 days, the mutant culture retained twice as many viable cells as the WT culture. Significant cell mortality was also observed by other investigators working with the same algal strain (*H. pluvialis* NIES 144). Kobayashi et al. (1993) reported a loss of ca. 20% of the cells after the culture was maintained in HL + SA + FE for 5 days. Hata et al. (2001) observed that the population density of this strain was reduced by ca. 35% or ca. 70% after cysts or green vegetative cells, respectively, were maintained in a photobioreactor exposed to a high light intensity of 950 µmol photons m⁻² s⁻¹ for 5 days.

We propose that the greater tolerance of MT 2877 to oxidative stress was due in part to its accumulation of ca. 100% more astaxanthin than the WT. Astaxanthin stored in lipid bodies located in the peripheral region of the cell may serve as a ‘sunscreen’ preventing an excess of photons from impinging on the chloroplast under stress conditions, as suggested by a number of investigators (Yong & Lee, 1991; Hagen et al., 1994; Wang et al., 2003). Astaxanthin could also serve in carbon and energy storage under stress conditions (Hagen et al., 1993b). Perhaps an equally, or even more, important protective role under stress may be played by the carotenogenesis process rather than the end-product astaxanthin. Our preliminary experimental results from a comparative study of the WT and MT 2877 (Li et al., 2008) suggest that the multiple steps of desaturation and oxygenation involved in astaxanthin biosynthesis and the coupling of the astaxanthin synthesis pathway with the photosynthetic and/or chlororespiratory electron transfer chain through the plastoquinone pool may serve as a primary protective mechanism against the formation of reactive oxygen species in the chloroplast. These reaction steps could consume as much as 10% of photosynthetically evolved molecular oxygen near thylakoid membranes which may otherwise be used to form excess reactive oxygen species under stress. Our observation is in line with the hypothesis made by Fan
et al. (1998) that the biosynthetic pathway of astaxanthin, rather than astaxanthin itself, could be the predominant antioxidative mechanism in *H. pluvialis*.

The generation and application of the MT 2877 mutant or other carotenoid-overproduction mutants has far-reaching biotechnological significance. While various astaxanthin products derived from *H. pluvialis* have been commercially available (Lorenz & Cysewski, 2000; Guerin et al., 2003), the current astaxanthin production system using the wild type strain of *H. pluvialis* Flotow NIES144 suffers from a low cellular astaxanthin content (ca. 1.5–2.0% dry weight) (Johnson & An, 1991; Kobayashi et al., 1991; Steinbrenner & Linden, 2003; Wang et al., 2004) and low biomass productivity (Margarith, 1999; Liu & Lee, 2000; Hata et al., 2001), which increases production costs and the astaxanthin market price. The MT 2877 strain has the ability to accumulate about 100% more cellular astaxanthin and sustain about 100% more viable cells than the WT. As a result, a 3-fold increase in volumetric productivity of astaxanthin by the MT 2877 strain may be anticipated under culture conditions identical to those for the WT strain. The enhanced tolerance of MT 2877 to oxidative stress may also make it particularly suitable for mass culture outdoors, where the intensity of solar radiation may be as high as 2200 μmol photons m⁻² s⁻¹. The WT would be more susceptible to over-saturation from solar radiation than MT 2877, resulting in photo-inhibition of photosynthesis and ultimately photo-oxidative death of the cells.

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**References**


