A Mutant of the Green Alga *Dunaliella salina* Constitutively Accumulates Zeaxanthin Under All Growth Conditions

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Abstract: A novel mutant (*zea1*) of the halotolerant unicellular green alga *Dunaliella salina* is impaired in the zeaxanthin epoxidation reaction, thereby lacking a number of the β-branch xanthophylls. HPLC analysis revealed that the *zea1* mutant lacks neoxanthin (N), violaxanthin (V) and antheraxanthin (A) but constitutively accumulates zeaxanthin (Z). Under low-light physiological growth conditions, the *zea1* (6 mg Z per g dry weight or 8×10^{-16} mol Z/cell) had a substantially higher Z content than the wild type (0.2 mg Z per g dry weight or 0.5×10^{-16} mol Z/cell). Lack of N, V, and A did not affect photosynthesis or growth of the *zea1* strain. Biochemical analyses suggested that Z constitutively and quantitatively substitutes for N, V, and A in the *zea1* strain. This mutant is discussed in terms of its commercial value and potential utilization by the algal biotechnology industry for the production of zeaxanthin, a high-value bioproduct. © 2002 Wiley Periodicals, Inc. *Biotechnol Bioeng* **81**: 115–124, 2003.

Keywords: carotenoid biosynthesis; *Dunaliella salina;* green alga; photosynthesis; mass culture; zeaxanthin

INTRODUCTION

Xanthophylls are oxygenated carotenoids, containing one or more oxygen atoms per molecule. They serve in a variety of functions in photosynthetic organisms and are essential for light-dependent growth. The most important function of these molecules is protection against photo-oxidative damage, chiefly by quenching undesirable chlorophyll triplets, thus preventing the formation of highly reactive singlet oxygen species (Goodwin, 1980; Hirschberg and Chamovitz, 1994). Xanthophylls are also thought to dissipate excess radiant energy, thus mitigating against photo-oxidative damage in the photosynthetic apparatus (Niyogi et al., 1997a). Further, some of the xanthophylls are known to function as light-harvesting antenna molecules and to transfer absorbed light energy to chlorophyll *a* molecules.

When photosynthetic irradiance is greater than that required for the saturation of photosynthesis, vascular plant and green alga chloroplasts undergo a reversible violaxanthin (V) de-epoxidation reaction to form antheraxanthin (A) and subsequently zeaxanthin (Z), resulting in the accumulation of zeaxanthin in the chloroplast thylakoids. The enzyme that catalyzes this reaction, "violaxanthin de-epoxidase," is localized in the lumen of the chloroplast thylakoids (Hager and Holocher, 1994). Formation of both A and Z is closely associated with excitation energy dissipation in the pigment bed of photosynthesis (Demmig et al., 1987; Gilmore and Yamamoto, 1993; Goss et al., 1998). When absorbed irradiance is lower than that required for the saturation of photosynthesis, Z is converted back to V by the enzyme zeaxanthin epoxidase (Hager, 1980), with the monoepoxide A being an intermediate in this reversible oxidation-reduction process. Thus, the xanthophyll cycle is a dynamically regulated reversible interconversion of V to A to Z, it occurs in the thylakoid membrane of photosynthesis and plays a role in photoprotection (Demmig-Adams et al., 1996; Demmig et al., 1987; Gilmore and Yamamoto, 1993; Goss et al., 1998; Havaux and Niyogi, 1999). Genetic studies in *A. thaliana* (Rock and Zeevaart, 1991), *N. plubaginifolia* (Marin et al., 1996) and the green alga *Chlamydomonas reinhardtii* (Niyogi et al., 1997b) revealed the presence of a single gene coding for the zeaxanthin epoxidase enzyme. Thus, a single gene product is apparently responsible for both the biosynthesis of V during growth and development of the organism, and the epoxidation reaction leading to the return of Z via A to V following irradiance stress.

Naturally occurring carotenoids are of significant commercial interest as coloring agents in food and cosmetics, and as pharmaceuticals, neutraceuticals, and additives in animal feed (Armstrong, 1997). Because of their antioxidant properties (Miller et al., 1996), certain xanthophylls have been implicated in the prevention of chronic disease (Rao and Agarwal, 1999). Some of these pigments are currently produced for commercial purposes by chemical synthesis (Bernhard, 1989; Nelis and De Leenheer, 1991), although the stereochemistry of the synthetic pigment may differ from the naturally occurring one (Ben-Amotz and Avron,

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1990). Zeaxanthin, as a potent antioxidant and a high-value bioproduct, is not commercially available owing to its very low level of occurrence in green tissues and unavailability by chemical synthesis. The recent elucidation of the Car biosynthetic pathway in several prokaryotic organisms, including cyanobacteria, may offer new opportunities for the metabolic engineering of xanthophyll and zeaxanthin production in vivo (for a review, see Armstrong, 1997). Generation of Z has been attempted in noncarotenogenic bacteria and in the cyanobacterium *Synechocystis* (Albrecht et al., 1999; Lagarde et al., 2000; Ruther et al., 1997). Currently, the maximum yield of zeaxanthin production in *E. coli* is about 1.6 mg Z per gram dry weight (Albrecht et al., 1999).

Eukaryotic microalgae are unexplored and unexploited in the commercial production of zeaxanthin. Microalgae are attractive for commercial exploitation because they grow fast, typically doubling their biomass once a day, and they require minimal mineral nutrients for growth. They can produce a vast array of natural products such as pigments, enzymes, unique fatty acids, vitamins, etc. Some microalgae are being exploited for the production of high-value pigments, such as astaxanthin from *Haematococcus pluvialis* and β-carotene from *Dunaliella bardawill*. Lutein from *Muriellipsis* sp. is being considered for commercial exploitation (Del Campo et al., 2001).

Pigment mutants in cyanobacteria and green algae have been used extensively to study the biogenesis and function of photosynthetic complexes (Chitnis et al., 1997; Hippler et al., 1998; Pakrasi, 1995; Sun et al., 1998). However, so far only a few mutants of the genus *Dunaliella* have been identified and employed (Brown et al., 1987; Hard and Gilmour, 1991; Shaish et al., 1991; Jin et al., 2001). In this work, we report the isolation and characterization of *zea1,* a zeaxanthin-accumulating mutant of the halotolerant green alga *Dunaliella salina*. Biochemical analyses suggested that Z constitutively and quantitatively substitutes for N, V, and A in the *zea1* strain. This mutant is discussed in terms of its commercial value and potential utilization by the alga biotechnology industry for the production of zeaxanthin, a high-value bioproduct.

MATERIALS AND METHODS

Cell Growth Conditions

The unicellular green alga *Dunaliella salina* Teod. (Starr, 1978) was grown photoautotrophically in hypersaline medium (Pick et al., 1986) in the presence of 25 m*M* $NaHCO_3$ as the inorganic carbon source. Cells were grown in flat 1-L bottles (3-cm optical-path length) at 28°C under continuous low light (LL: 100 μmol photons $m^{-2}s^{-1}$) or high-light (HL: 2000 μmol photons $m^{-2}s^{-1}$) conditions. Irradiance was measured with a LI–COR Model LI-185B radiometer. Cells were harvested when they were at a density of $3 - 3.5 \times 10^6$ cells mL^{-1}. Cell counting was done with a Neubauer ultraplane hemacytometer.

Cell dry weight was measured as follows: *D. salina* cell culture (200 mL) was harvested by centrifugation at 3000*g* (Beckman, JS13) for 5 min at 20°C. Supernatant media were carefully decanted, and pellets were resuspended in 14 mL of 0.3*M* NaCl. Resuspended cells were transferred to a 15 mL preweighed Falcon-tube (A). Cells were harvested by centrifugation at maximum speed (about 3000*g* for 5 min, tabletop centrifuge; International Clinical Centrifuge, International Equipment Co.). Supernatant was decanted completely without disturbing the pellet. Falcon-tubes, which contained the *D. salina* pellet, were weighed (B) after drying for 24 h at 80 C. The net dry weight was calculated by B−A**.** The above procedure was repeated 3 times with 200-mL cell culture to yield an average value for the dry weight of the cells. Dry weight/cell was calculated (C). Zeaxanthin/cell (D) was determined after HPLC analysis. Finally, Z/dry weight was calculated as C/D.

Mutagenesis and Mutant Selection

Wild-type *D. salina* cells in the log phase of growth were harvested by centrifugation at 1000*g* for 3 min. The pelleted cells were resuspended in growth medium at a cell density of 2×10^8 cells per mL. Cells were incubated with ethyl methyl sulfonate (EMS) at a final concentration of 0.2*M* for 2 h in the dark. This EMS-mutagenesis treatment resulted in a 5% survival rate. Following the mutagenesis treatment, cells were washed with growth medium 3 times and then incubated overnight on a shaker under 50 μmol photons m^{-2} s^{-1}. Subsequently, cells were plated on an agar-containing (1.5% w/w) growth medium in Petri plates and allowed to developed colonies under 100 μmol photons m^{-2} s^{-1} over a period of 2 weeks. Colonies were transferred into a microtiter well plate (96-well culture plate; Falcon Franklin Lake, NJ) containing *D. salina* growth media. Following incubation and growth for 10 d, cells were spotted onto an agar plate. A grid of independent colonies (colony size of 0.4 cm diameter) was assembled (48 colonies per Petri-plate). Two weeks after plating on a grid, the colonies were screened visually to assess color appearance. Pale green or yellow-green colonies were selected for further analysis of pigment composition.

Pigment Analysis

For pigment determination, cells or thylakoid membranes were extracted in 80% acetone and debris were removed by centrifugation at 10,000g for 3 min. The absorbance of the supernatant was measured with a Shimadzu UV-160U spectrophotometer. The chlorophyll (*a* and *b*) concentration of the samples was determined according to Arnon (1949) with equations corrected as in Melis et al. (1987).

For HPLC analysis, a Hewlett-Packard HPLC Series model 1100 equipped with a Waters Spherisorb S5 ODS1 4.6 × 250 mm Cartridge Column was used. Two milliliters of algal suspension were centrifuged in an Eppendorf centrifuge at 14,000 RPM for 2 min. Pigments were extracted

from the algal cells upon addition to the pellet of 200 μL 90% filtered (0.2 μm nylon filter) acetone and by vortexing at maximum speed for 1 min. The extract was centrifuged in an Eppendorf centrifuge at 14,000 RPM and 15 μL of the filtered supernatant was subjected to HPLC analysis. The pigments were separated using a solvent mixture of 0.1*M* Tris-HCl pH 8.0 ddH_2O, acetonitrile, methanol, and ethylacetate. During the run, the solvent concentrations were 14% 0.1*M* Tris HCl, 84% acetonitrile, and 2% methanol from 0 to 15 min. From 15 min to 19 min the solvent mixture consisted of 68% methanol and 32% acetonitrile. A post-run followed for 6 min with the initial solvent mixture. The flow rate was constant at 1.2 mL per min. Pigments were detected at $\lambda = 445$ nm with a reference at $\lambda = 550$ nm. Concentration of individual pigments was determined from the HPLC profiles calibrated with standard samples of chlorophyll and carotenoids.

Thylakoid Membrane Isolation

Cells were harvested by centrifugation at 1000*g* for 3 min at 4°C. Samples were diluted with sonication buffer containing 100 m*M* Tris-HCl (pH 6.8), 100 m*M* NaCl, 5 m*M* $MgCl_2$, 0.2% polyvinylpyrrolidone-40, 0.2% sodium ascorbate, 1 m*M* aminocaproic acid, 1 m*M* aminobenzamidine, and 100 μ*M* phenylmethylsulfonylfluoride (PMSF). Cells were broken by sonication in a Branson 200 Cell Disrupter operated at 4°C for 30 s (pulse mode, 50% duty cycle, output power 5). Unbroken cells and starch grains were removed by centrifugation at 3000*g* for 4 min at 4°C. The thylakoid membranes were collected by centrifugation of the supernatant at 75,000*g* for 30 min at 4°C. The thylakoid membrane pellet was resuspended in a buffer containing 250 m*M* Tris-HCl (pH 6.8), 20% glycerol, 7% SDS and 2 *M* urea. Solubilization of thylakoid proteins was carried out for 30 min at room temperature. Samples were centrifuged in a microfuge for 5 min to remove unsolubilized material, β-mercaptoethanol was added to yield a final concentration of 10% and the samples were stored at −80°C.

Chlorophyll Fluorescence

The initial (F_o), variable (F_v) and maximal (F_m) chlorophyll fluorescence yield of intact cells was measured upon excitation of the cultures with green light (CS 4-96 and CS 3-69 Corning Filters, actinic light intensity of 75 μmol photons m^{-2} s^{-1}). An aliquot from the culture was incubated in the dark for 10 min prior to the measurement, and the chlorophyll fluorescence was recorded in the absence or presence of DCMU (2.5 μ*M* final concentration).

Oxygen Evolution Measurements

Oxygen evolution of the cultures was measured at 26°C with a Clark-type oxygen electrode illuminated with a slide projector lamp. Yellow actinic excitation was provided by a CS 3-69 Corning cut-off filter in combination with an Ealing 35-5453 VIQ5-8 filter. An aliquot of 5-mL cell suspension (2 μ*M* Chl) was transferred to the oxygen electrode chamber. To ensure that oxygen evolution was not limited by the carbon source available to the cells, 100 μL of 0.5*M* sodium bicarbonate solution (pH 7.4) was added to the suspension prior to the oxygen evolution measurements. The measurement of the light-saturation curve of photosynthesis was implemented with the oxygen electrode, beginning with the registration of dark respiration in the cell suspension, and followed by measurements of the rate of oxygen evolution at 30, 60, 125, 225, 330, 500, 750, 1100, 1300, 1800, 2220, and 3000 μmol photons m^{-2} s^{-1}. Registration of the rate (slope) of oxygen evolution at each light intensity step was recorded for about 2 min. The photon use efficiency of the cells was calculated from the initial slope of the light-saturation curves of photosynthesis.

RESULTS

Isolation and Pigment Characterization of the *D. salina zea1* Strain

Following chemical mutagenesis with EMS, about 500 putative colonies of *D. salina* were identified by their pale-green coloration during the primary visual screening process. These colonies were subjected to pigment analysis. Three stable mutants (*zea1, zea2,* and *zea3*) were selected on the basis of their zeaxanthin (Z) accumulation and growth characteristics (Fig. 1). These mutants lacked neoxanthin (N), violaxanthin (V), and antheraxanthin (A) but accumulated significant amounts of Z compared to the wild type. When grown as colonies on agar under low light (LL: 100 μmol photons m^{-2} s^{-1}), they appeared pale green while the wild type was dark green (Fig. 1). Figure 2 shows the HPLC profile of pigments from LL-grown wild type and

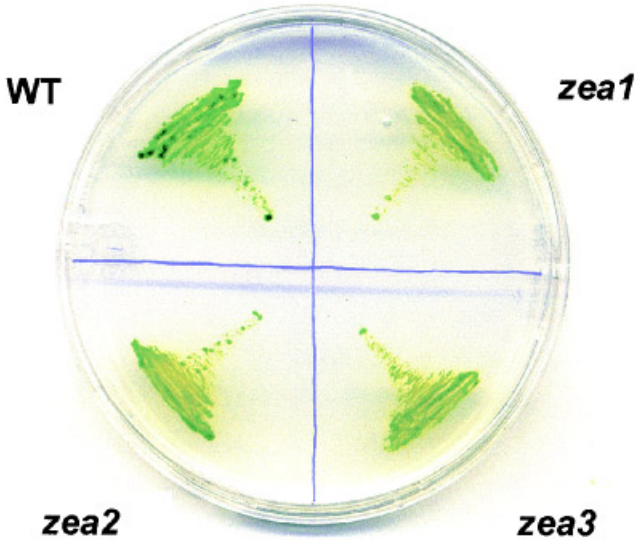

Figure 1. Color appearance of *D. salina* wild type and *zea* colonies on agar plates, grown under 100 μmol photons m^{-2} s^{-1}.

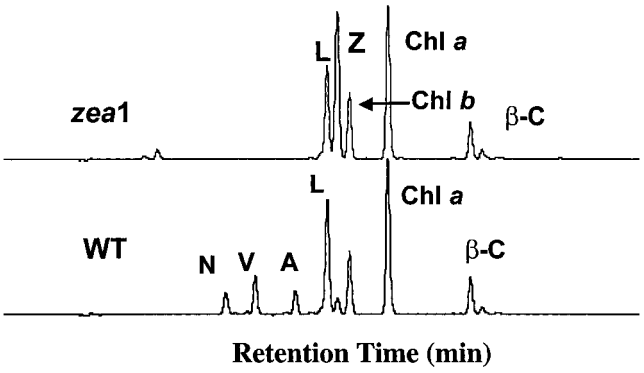

Figure 2. HPLC elution profile of total pigment extract from *D. salina* wild-type and *zea1* mutant. Traces were normalized to the peak of Chl *a*. Identification of lettered peaks is as follows: N, neoxanthin; V, violaxanthin; A, antheraxanthin; L, lutein; Z, zeaxanthin; Chl *b,* chlorophyll *b;* Chl *a,* chlorophyll *a;* β-C, β-carotene.

zea1. It is evident that *zea1* lacks the ββ-epoxycarotenoids, N, V, and A. However, a larger amount of Z is present in the *zea1* strain when compared to the wild type. Qualitatively, it appears that Z has replaced N, V, and A in the mutant. The peak amplitude of the other pigments (L, Chl *a,* Chl *b,* and β-carotene) remained unchanged relative to that in the wild type. The HPLC profile of pigments in *zea2* and *zea3* was nearly identical to that of *zea1*. Thus, all further biochemical and physiological characterizations were performed on *zea1* only.

To examine the physiological consequence of a lack in the epoxy-carotenoids N, V, and A, we compared rates of growth for the *zea1* and wild type under different light-intensity conditions. Figure 3 shows growth curves of wild type and *zea1* strain in liquid media under a light intensity of 100 μmol m^{-2} s^{-1} (LL, solid symbols), or 2000 μmol m^{-2} s^{-1} (HL, open symbols). Such comparative growth measurements showed that wild type and the *zea1* strain grow with approximately equal rates under LL or HL-conditions. Thus, HL-growth did not appear to either favor or disadvantage *zea1* cells relative to the wild type.

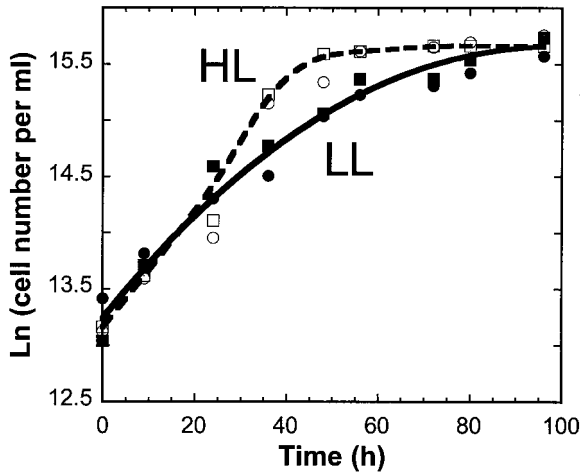

Figure 3. Growth curves of *D. salina* wild type and *zea1* mutant. The natural logarithm of cell number is plotted as a function of time during growth. Cells were cultivated either under 100 (LL) or 2,000 μmol photons m^{-2} s^{-1} (HL). LL-grown wild type (solid circles), LL-grown mutant (solid squares), HL-grown wild-type (open circles) and HL-grown mutant (open squares) are shown.

Table I compares the chlorophyll (Chl) and total carotenoid (Car) content in wild-type and *zea1* strain, grown under different irradiance conditions. In LL-growth, Chl *a*/Chl *b* ratio ($= \approx 3.4/1$) and Chl/cell ($\approx 3.9 \times 10^{-15}$ mol/cell) were similar in wild-type and the *zea1* mutant. The total Car/cell ($\approx 2 \times 10^{-15}$ mol/cell) and the Car/Chl ratio ($=0.5/1$) were also similar between the two strains. To compare the response of the two strains to irradiance stress, we performed a LL→HL shift, followed by incubation of the cultures under HL for 24 h. Pigment analysis and other photosynthesis characteristics were assayed at the end of the 24-h incubation period. It is known that HL exposure, under our growth conditions, causes a lowering of the cellular Chl content and a concomitant increase in the de-epoxidation state of the xanthophyll-cycle carotenoids (Krol et al., 1997; Jin et al., 2001). The latter predominantly reflects conversion of violaxanthin to zeaxanthin under irradiance stress. Table I (LL→HL) shows that a 24-h incubation under HL resulted in a higher Chl *a*/Chl *b* ratio, lower Chl/cell content and a higher Car/Chl ratio in the cells. These changes were more pronounced in the *zea1* mutant than in the wild type. Taken together, these results (Fig. 2 and Table I) show that constitutive accumulation of Z instead of N, V, and A in the *zea1* mutant did not prevent or inhibit the photo-acclimation response in *D. salina.*

Efficiency and Productivity of Photosynthesis in *D. salina* Wild-Type and *zea1* Mutant

The efficiency and productivity of photosynthesis in *D. salina* wild-type and *zea1* mutant was measured from the light saturation curves of photosynthesis in the two strains. In such a presentation, the rate of O_2 evolution is measured and plotted as a function of incident light intensity, thus obtaining the photosynthesis versus irradiance curve. From the slope of the initial linear part of the light-saturation curve of photosynthesis, information was obtained about the relative photon use efficiency of photosynthesis (Φ) in wild-type

Table I. Photosynthetic apparatus parameters in *D. salina* wild-type and *zea1* mutant. Cells were grown under either 100 μmol photons m^{-2} s^{-1} (LL) or exposed to 2000 μmol m^{-2} s^{-1} (HL) for 24 h following a LL→HL shift. Values represent means ± SD (n = 3–5).

	LL		LL→HL	
Parameter	WT	*zea1*	WT	*zea1*
Chl *a*/Chl *b*	3.4 ± 0.11	3.37 ± 0.1	5.85 ± 0.62	6.95 ± 0.61
mol Chl × 10^{-15}/cell	4.07 ± 0.43	3.79 ± 0.46	1.24 ± 0.34	1.05 ± 0.1
mol Car × 10^{-15}/cell	2.05 ± 0.2	1.86 ± 0.2	1.23 ± 0.07	1.31 ± 0.07
Car/Chl	0.50	0.49	0.99	1.24

and mutant cells (Bjorkman and Demmig, 1987; Neale et al., 1993). The light-saturated rate of oxygen evolution defined Pmax, i.e., the capacity of photosynthesis, in the two strains (Powles and Critchley, 1980). Figure 4 shows the light-saturation curves of photosynthesis for wild type and mutant grown under different light regimes. Table II summarizes critical parameters from the respective light-saturation curves. LL-grown wild type ($\Phi = 0.31$) and mutant ($\Phi = 0.31$) had identical quantum yields of photosynthesis. Pmax was slightly higher in the *zea1* (113 mmol O_2 [mol Chl]$^{-1}$ s^{-1}) than in the wild type (81 mmol O_2 [mol Chl]$^{-1}$ s^{-1}. However, other parameters such as the yield of primary photochemistry at PSII (Fv/Fm) and rates of cellular respiration were about the same in the two strains. Following a LL→HL shift and incubation for 24 h under HL, photon use efficiencies (Φ) and the yield of primary PSII photochemistry (Fv/Fm) decreased by about 50% in both strains. Lowering of Φ and of Fv/Fm is known to result from a HL-induced photoinhibition of photosynthesis in *D. salina* (Neidhardt et al., 1998). A similar "index of photoinhibition" in wild type and *zea1* following a LL→HL shift would suggest that the *zea1* strain shows the same susceptibility to photoinhibition as the wild type, despite a constitutive accumulation of Z. This unexpected result and apparent inability of accumulated zeaxanthin to protect against photo-damage needs to be more rigorously investigated. Following a LL→HL shift, Pmax and the rate of dark respiration increased in both wild type and *zea1,* primarily a consequence of the lower Chl content in the cells following incubation for 24 h under HL.

To test whether the xanthophyll cycle was operational and whether a LL→HL shift altered the amount of Z in the

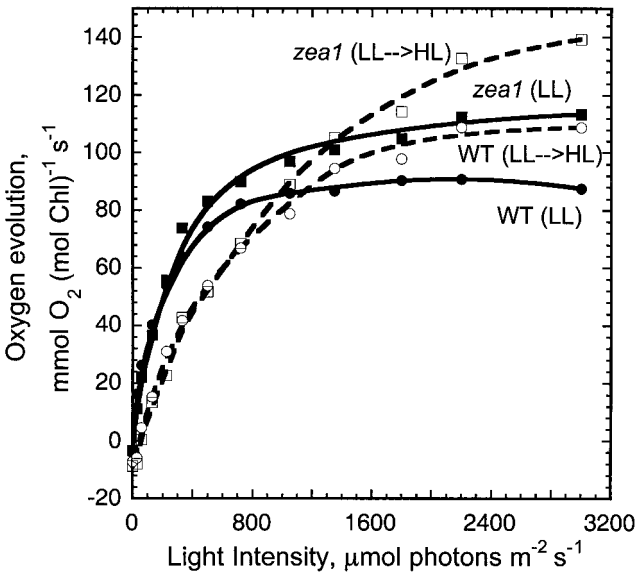

Figure 4. The light-saturation curves of photosynthesis in LL-grown wild-type (solid circles), LL-grown mutant (solid squares), LL→HL-shifted wild-type (open circles) and LL→HL-shifted mutant (open squares) *D. salina*. Rates of oxygen evolution on a per chlorophyll basis were measured as a function of actinic (incident) light intensity.

zea1 mutant, a carotenoid compositional analysis was undertaken before and after 24 h exposure to HL. As shown in Table III, WT (LL) has appreciable amounts of N, V, and A (totaling 0.62×10^{-15} mol/cell) but very low levels of Z (0.05×10^{-15} mol/cell). The *zea1* strain has no measurable amounts of N, V and A but constitutively accumulated Z ($= 0.81 \times 10^{-15}$ mol/cell). Thus, Z accumulation in the mutant is more than enough for the quantitative replacement of N, V, and A. Lutein (L) and β-carotene content were about the same in WT and *zea1*. Following a LL→HL shift and a 24 h incubation under HL, WT content in V was specifically lowered while Z content increased by an equivalent amount (Table III, WT [LL→HL]), suggesting an irradiance-dependent de-epoxidation of V to Z. In the *zea1* strain, no changes in carotenoid epoxidation were noted and the ratio of L/Z remained unchanged in LL-grown and following the LL→HL shift (Table III).

Figure 5 shows kinetics of pigment change in *D. salina* wild-type and *zea1* mutant following a LL→HL shift. In the wild type, Z/cell increased from a low value (0.5×10^{-16} mol/cell) to a maximum ($\approx 5 \times 10^{-16}$mol/cell), attained after about 6 h under HL. At longer HL-incubation times, Z content declined slightly and reached a steady-state value of about 4×10^{-16} mol/cell (Fig. 5A). The *zea1* mutant (Z/cell $= 8 \times 10^{-16}$ mol/cell) showed a small transient increase upon the LL→HL shift (0–3 h). At longer HL-incubation times, Z content in the *zea1* also declined slightly and reached a steady-state value of about 6×10^{-16} mol/cell (Fig. 5A). Figures 5B and 5C show changes in the level of Chl and total carotenoid, respectively, following a LL→HL shift. Levels of Chl in both wild type and mutant declined with a half time of about 10 h from 4×10^{-15} mol/cell to a steady-state of 1×10^{-15} mol/cell (Fig. 5B). No significant difference could be detected in the kinetics of Chl adjustment between wild type and *zea1* mutant, suggesting that the mutation did not affect the ability of the cells to acclimate to the level of irradiance. Total Car in the cells also declined, albeit only slightly (Fig. 5C), as a function of time under HL. Here again, no statistical difference could be detected in the kinetics of total Car adjustment between wild-type and *zea1* mutant. The substantial loss of Chl and the partial loss of carotenoid from the cells following a LL→HL shift may underline down-regulation of thylakoid membrane in the chloroplast as an acclimation response to HL.

The de-epoxidation state of the xanthophyll cycle carotenoids was also measured as a function of time following a LL→HL shift of the cultures. In the wild type, de-epoxidation state increased from 30% to 90% within about 6 h from the light shift (Fig. 6). In the *zea1* mutant, de-epoxidation state remained at 100% irrespective of the light regime to which the cells were exposed. The latter is consistent with a lesion in the zeaxanthin epoxidase gene, which prevents the epoxidation of zeaxanthin to violaxanthin and renders the xanthophyll cycle inoperable.

In photosynthetic organisms, carotenoids bind noncovalently but specifically with thylakoid membrane proteins

Table II. Photosynthetic capacity (P_{max}), relative photon-use efficiency (Φ) and PSII photochemical efficiency (Fv/Fm) in *D. salina* wild-type and *zea1* mutant. Cells were grown under either 100 μmol photons m^{-2} s^{-1} (LL) or exposed to 2000 μmol m^{-2} s^{-1} (HL) for 24 h following a LL → HL shift. The values shown represent the mean ± SD.

	LL		LL→HL	
Parameter measured	WT	*zea1*	WT	*zea1*
Photon use efficiency, Φ, rel. units	0.31	0.31	0.17	0.18
Photosynthetic capacity (P_{max}) mmol O_2 (mol Chl)$^{-1}$ s^{-1}	81 ± 11	113 ± 9.5	108 ± 11	139 ± 15
F_v/F_m	0.71 ± 0.02	0.71 ± 0.03	0.44 ± 0.07	0.43 ± 0.01
Respiration (mmol O_2 (mol Chl)$^{-1}$ s^{-1})	3.9 ± 0.6	3.3 ± 0.12	7.2 ± 1.7	8.7 ± 2.1

(Armstrong, 1997). Carotenoid distribution and localization in the photosynthetic membranes vary from one organism to another. Predominantly, carotenes are associated with the photosynthetic reaction center and photosystem-core complexes (Hirschberg and Chamovitz, 1994). In vascular plants and algae, xanthophylls are associated mainly with the auxiliary light-harvesting antenna complexes (Armstrong, 1997; Havaux, 1998). Since the major carotenoid in the *zea1* mutant is Z, which appears to constitutively accumulate in lieu of N, V and A, we addressed the question of whether Z is bound to the thylakoid membrane in this mutant strain. The carotenoid composition of isolated thylakoid membranes from wild type and mutant were analyzed by HPLC, both in LL-grown samples and following a LL→HL shift. Table IV shows that the relative content and composition of carotenoids in the thylakoid membrane of WT and *zea1* mutant is essentially the same as that of the whole cell extracts (Table III). This analysis suggests that the bulk of Z in the *zea1* mutant is bound to the thylakoid membrane, probably within specialized chlorophyll-xanthophyll-protein complexes.

Table V further compares the total Car and Z content per cell dry weight in wild-type and *zea1* mutant grown under LL and following a LL→HL shift. Under LL-growth, mutant and wild type each contained about 13 mg of total carotenoid per g dry weight. The wild type contained 0.23 mg Z per g dry weight, while the *zea1* strain contained about 6 mg Z per g dry weight (Table V). This value (0.6% of dry weight) is close to the 1% value (i.e., 10 mg Z per g dry weight), thought to represent the minimum cellular content of zeaxanthin for a viable commercial exploitation of this product (Ben-Amotz and Avron, 1990).

DISCUSSION

Green plants and algae reversibly convert violaxanthin via antheraxanthin to zeaxanthin in a so-called xanthophyll de-epoxidation cycle (Demmig et al., 1987; Niyogi, 1999; Yamamoto 1979; 1985). De-epoxidation of violaxanthin to zeaxanthin is triggered by irradiance stress while LL-conditions elicit a return of Z to V in the chloroplast of eukaryotic photosynthetic organisms. The green alga *Dunaliella salina* also operates the reversible xanthophyll cycle (Jin et al., 2001; Krol et al., 1997). A xanthophyll-aberrant mutant of the green alga *Dunaliella salina* (*zea1*) was isolated and studied in this work. This strain lacks a number of ββ-epoxycarotenoids (N, V, and A) and constitutively accumulates zeaxanthin (Z) under all irradiance growth conditions. In the *zea1* strain, N, V, and A were quantitatively replaced by Z, so that total carotenoid and chlorophyll content in the mutant were about the same as those in the wild type. The biochemical analyses conducted in this work strongly suggest that *zea1* is a specific zeaxanthin epoxidase mutant. The lesion in *zea1* and the respective xanthophyll biosynthetic pathway that has become affected are shown in Figure 7. In this respect, the yellow–green coloration of the *zea1, zea2,* and *zea3* colonies (Fig. 1) could not be attributed to lower chlorophyll content or a higher overall carotenoid content in the mutants vs. the wild type (Table I). Rather, the more yellow coloration of the mutants is probably a consequence of the accumulation of zeaxanthin at the expense of neoxanthin, violaxanthin, and antheraxanthin. The former (Z) more strongly absorbs light in the blue region of the spectrum than N, V, or A due to its

Table III. Carotenoid composition in *D. salina* wild type and *zea1* mutant. Whole-cell extracts were analyzed for pigment content. Cells were grown under either 100 μmol photons m^{-2} s^{-1} (LL) or exposed to 2000 μmol m^{-2} s^{-1} (HL) for 24 h following a LL→HL shift. Carotenoid pigment data are presented on a per cell basis (mol Car per cell). Abbreviations used: N, neoxanthin; V, violaxanthin; A, antheraxanthin; L, lutein; Z, zeaxanthin; β-Car, β-carotene. Values represent means ± SD (n = 3–5).

	N	V	A	L	Z	β-Car
Growth conditions	10^{-15} mol/cell					
WT (LL)	0.21 ± 0.027	0.34 ± 0.014	0.07 ± 0.011	0.77 ± 0.008	0.05 ± 0.001	0.33 ± 0.028
zea1 (LL)	0	0	0	0.66 ± 0.07	0.81 ± 0.09	0.33 ± 0.01
WT (LL→HL)	0.17 ± 0.015	0.027 ± 0.006	0.11 ± 0.001	0.47 ± 0.05	0.38 ± 0.02	0.17 ± 0.01
zea1 (LL→HL)	0	0	0	0.48 ± 0.03	0.6 ± 0.03	0.19 ± 0.003

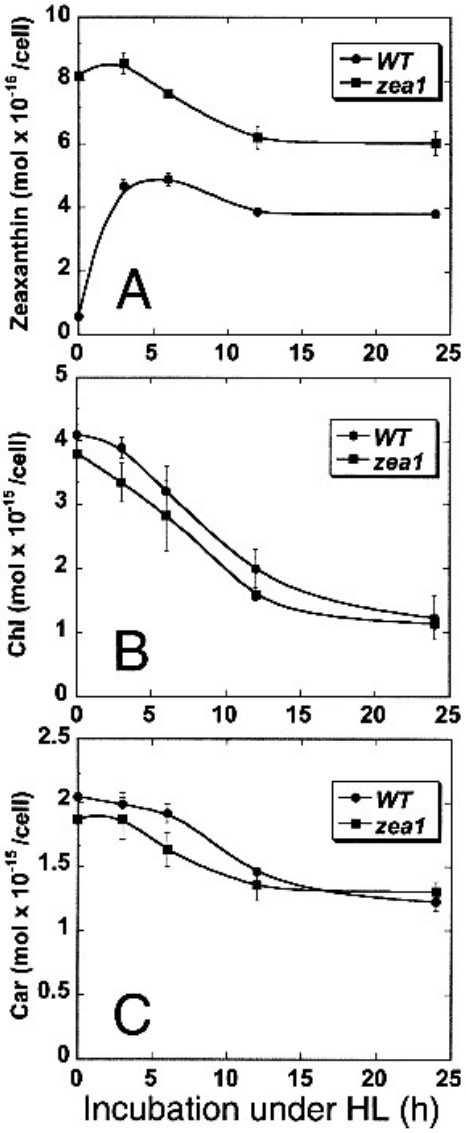

Figure 5. (A) Kinetics of Z/cell, (B) Chl/cell, and (C) Car/cell in wild-type and *zea1* mutant of *D. salina.* The two strains were grown under LL and then shifted to HL. Aliquots were sampled at the indicated time points following the LL→HL shift.

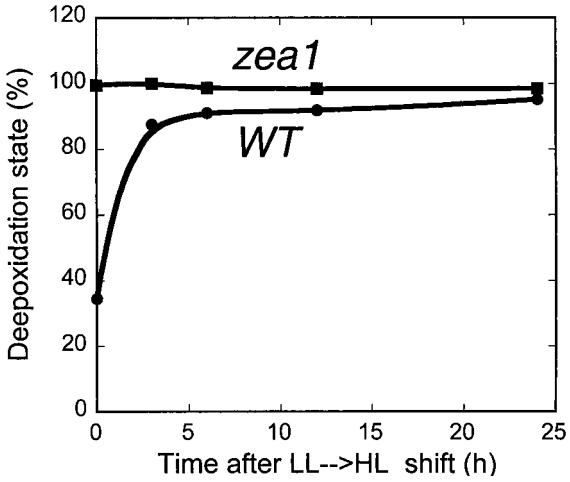

Figure 6. De-epoxidation kinetics of the xanthophyll cycle carotenoids in wild type and *zea1* mutant of *D. salina.* The two strains were grown under LL and then shifted to HL conditions. Aliquots were sampled at the indicated time points following the LL→HL shift.

longer conjugated double-bond system and the better developed π-orbital configuration (Fig. 7).

Under physiological irradiance conditions, Z occurs only in trace amounts within the auxiliary light-harvesting complex (LHC) of green plants and algae (Lee and Thornber 1995; Ruban et al., 1994; Verhoeven et al., 1999). However, upon exposure to irradiance stress, zeaxanthin is formed upon de-epoxidation of violaxanthin (Demmig et al., 1987; Yamamoto 1979, 1985). Although Z is thought to be hydrophobically bound to the LHC, such association is only transient and it is readily reversible to V during incubation in low-light or darkness (Yamamoto, 1985). Because Z is present only under irradiance stress conditions, and even then in relatively small amounts, its specific association with LHC proteins is difficult to ascertain. Nevertheless, it was concluded from recent in vitro studies (Croce et al., 1999b; Hobe et al., 2000), upon analysis of Z accumulating *Arabidopsis thaliana* mutants (Tardy and Havaux, 1996), and from research with the green algae *Scenedesmus obliquus* (Bishop et al., 1998) and *Chlamydomonas reinhardtii* (Polle et al., 2001) that Z could replace V in the LHC of the photosynthetic apparatus. Comparative HPLC analysis of whole cell extracts and of isolated thylakoid membranes from the *zea1* mutant suggested that Z is localized in the chloroplast thylakoids (Tables III and IV). The apparently quantitative replacement of N, V, and A by Z in the *zea1* strain (Tables III–V) suggests that further enhancement in the amount of Z in *D. salina* could be achieved only upon a secondary mutation that prevents the biosynthesis of lutein (Fig. 7). In a lutein-less and zeaxanthin epoxidase double mutant, L would also be replaced by Z, such that the Z content of the cells would reach up to 11 mg per g dry weight, significantly enhancing the prospects of *D. salina* utilization in the commercial production of zeaxanthin. This upper ceiling in Z content was arrived at upon the assumption of a strict association of Z with the LHC proteins of photosystem-II and photosystem-I in the photosynthetic apparatus.

However, it is not entirely clear whether Z is strictly associated with the xanthophyll-binding sites of the LHC-II proteins (Connelly et al., 1997; Croce et al., 1999a; 1999b), or whether some of these sites could be vacant in the *zea1* strain, with the surplus amount of Z occurring in the thylakoid lipid bilayer. Previous studies have shown that xanthophyll-cycle carotenoids are localized within the minor LHC (Bassi et al., 1993; Verhoeven et al., 1999) and/or may be found as free pigment in the lipid matrix of the thylakoid membrane (Bassi and Caffarri, 2000; Havaux and Tardy, 1995; Tardy and Havaux, 1996). Comparative analysis of different *aba* mutants from *A. thaliana* pointed out a potential function of Z in the protection against lipid peroxidation by a mechanism other than quenching of excitation energy

Table IV. Carotenoid composition of thylakoid membranes in *D. salina* wild-type and *zea1* mutant. Thylakoid membranes were isolated and analyzed for pigment content. Cells were grown under either 100 μmol photons m^{-2} s^{-1} (LL) or exposed to 2000 μmol m^{-2} s^{-1} (HL) for 24 h following a LL→HL shift. Carotenoid pigment data are presented on a total chlorophyll basis (mmol Car per mol Chl). Abbreviations used: N, neoxanthin; V, violaxanthin; A, antheraxanthin; L, lutein; Z, zeaxanthin; β-Car, β-carotene. Values represent means ± SD (n = 3–5).

Growth conditions	N	V	A	L	Z	β-Car
	Car/Chl (mmol/mol)					
WT (LL)	45 ± 7.1	75 ± 3.2	29 ± 1.1	195 ± 9.9	11 ± 0.8	112 ± 3.0
zea1 (LL)	0	0	0	246 ± 3.8	338 ± 3.5	145 ± 2.7
WT (LL→HL)	64 ± 6.2	23 ± 3.2	89 ± 11	448 ± 51	362 ± 41	170 ± 19
zea1 (LL→HL)	0	0	0	509 ± 45	749 ± 73	212 ± 21

(Havaux and Niyogi, 1999; Jahns et al., 2001). Such protective function of Z could transcend its association with the LHC Chl antenna proteins and could involve direct localization in the thylakoid membrane lipid bilayer (Bassi and Caffarri, 2000; Bassi et al., 1993; Havaux and Tardy, 1995; Tardy and Havaux, 1996). Localization of Z in the lipid bilayer would readily explain the relatively high content of zeaxanthin in LHC Chl antenna-depleted vascular plants and algae, where only few xanthophyll-binding Chl proteins are present (Jahns, 1995; Jin et al. 2001; Smith et al., 1990). In the lipid bilayer, Z could interact structurally with the diglyceride fatty acids. Thus, it may play an important antioxidant role by scavenging reactive oxygen species and/or by terminating lipid peroxidation chain reactions. A natural localization of Z in the lipid bilayer would lift the upper limit of Z content in green algae and would raise the prospect of a much greater than 11 mg per g dry weight accumulation of zeaxanthin under the proper conditions.

Mutations similar to that in *zea1* affecting Z epoxidation have been identified in *Arabidopsis* (*aba1-3*). The *aba1-3* mutants were originally identified as abscisic acid-deficient mutants (Koornneef et al., 1982). They appeared to be significantly different in terms of the effect of N and V xanthophyll deficiency from corresponding mutants in unicellular green algae. The *aba1* mutant accumulated Z but was virescent and exhibited a delayed greening during photomorphogenesis (Pogson et al., 1998). In contrast to these higher plant mutants, greening and photoautotrophic growth of the *zea1* mutant was not slowed-down relative to the wild type, both under LL- and HL-growth conditions. This work thus demonstrated that the xanthophylls N, V, and A are not essential for the survival of *Dunaliella salina.* Moreover, wild type and *zea1* mutant were practically indistinguishable in terms of growth under LL or HL conditions. They were also indistinguishable in terms of their photoacclimation to irradiance response. These findings are consistent with the results of Bishop et al. (1998), in which deletions of secondary carotenoid biosynthesis enzymes did not affect photosynthetic efficiency or photosynthetic capacity in the green alga *Scenedesmus obliquus.* It may be concluded that Z accumulation does not, in itself, alleviate the perception of and response to irradiance in *D. salina*. These biochemical considerations on the localization, concentration, and function of zeaxanthin relate to the commercial production of this high-value pigment. Zeaxanthin, as a bio-product, could find important applications in the pharmaceutical, neutraceutical and animal feed industries as well as a food coloring agent and in the medical treatment of specific diseases (Armstrong, 1997; Ben-Amotz and Avron, 1990).

Table V. Total carotenoid (Car) and zeaxanthin (Z) content per cell dry weight in *D. salina* wild-type and *zea1* strain. Cells were grown under either 100 μmol photons m^{-2} s^{-1} (LL) or exposed to 2000 μmol m^{-2} s^{-1} (HL) for 24 h following a LL→HL shift. Values represent means ± SD (n = 3–5).

Pigment content	LL		LL→HL	
	WT	*zea1*	WT	*zea1*
Car/cell, mg/g dry weight	13.6 ± 2.8	12.8 ± 2.4	10.7 ± 0.1	11.0 ± 0.4
Z/cell, mg/g dry weight	0.23 ± 0.07	5.9 ± 0.5	2.62 ± 0.18	4.18 ± 0.33

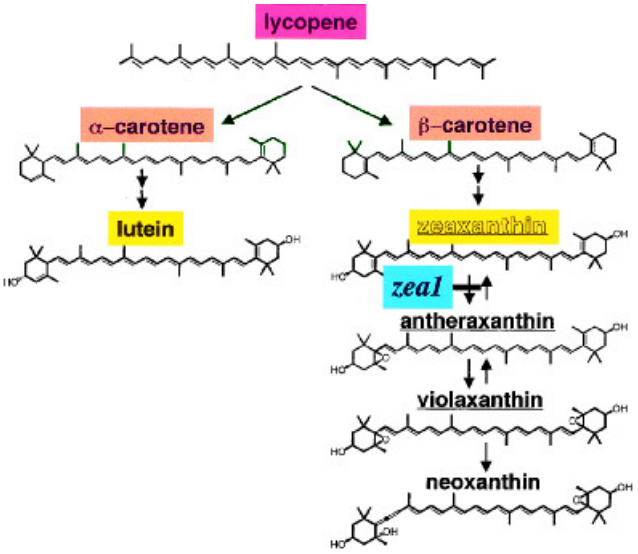

Figure 7. The carotenoid biosynthesis pathway in *D. salina* with the α and β branches of carotene and xanthophyll formation. The reversible xanthophyll-cycle reactions in the β branch (Z, A, and V) are underlined. Note that the *zea1* mutant has an apparent lesion in the zeaxanthin epoxidation to antheraxanthin step.

We wish to thank Dr. Kris Niyogi for making available HPLC equipment.

NOMENCLATURE

Car	carotenoids
Chl	chlorophyll
HL	high-light
LL	low-light
LHC	light-harvesting complex
LHC-II	light-harvesting complex of photosystem II
PMSF	phenylmethylsulfonylfluoride
V	violaxanthin
L	lutein
A	antheraxanthin
Z	zeaxanthin
N	neoxanthin

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