Role of the Reversible Xanthophyll Cycle in the Photosystem II Damage and Repair Cycle in *Dunaliella salina*[1]

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The *Dunaliella salina* photosynthetic apparatus organization and function was investigated in wild type (WT) and a mutant (*zea1*) lacking all β,β-epoxycarotenoids derived from zeaxanthin (Z). The *zea1* mutant lacked antheraxanthin, violaxanthin, and neoxanthin from its thylakoid membranes but constitutively accumulated Z instead. It also lacked the so-called xanthophyll cycle, which, upon irradiance stress, reversibly converts violaxanthin to Z via a de-epoxidation reaction. Despite the pronounced difference observed in the composition of β,β-epoxycarotenoids between WT and *zea1*, no discernible difference could be observed between the two strains in terms of growth, photosynthesis, organization of the photosynthetic apparatus, photo-acclimation, sensitivity to photodamage, or recovery from photo-inhibition. WT and *zea1* were probed for the above parameters over a broad range of growth irradiance and upon light shift experiments (low light to high light shift and vice versa). A constitutive accumulation of Z in the *zea1* strain did not affect the acclimation of the photosynthetic apparatus to irradiance, as evidenced by indistinguishable irradiance-dependent adjustments in the chlorophyll antenna size and photosystem content of WT and *zea1* strain. In addition, a constitutive accumulation of Z in the *zea1* strain did not affect rates of photodamage or the recovery of the photosynthetic apparatus from photo-inhibition. However, Z in the WT accumulated in parallel with the accumulation of photodamaged PSII centers in the chloroplast thylakoids and decayed in tandem with a chloroplast recovery from photo-inhibition. These results suggest a role for Z in the protection of photodamaged and disassembled PSII reaction centers, apparently needed while PSII is in the process of degradation and replacement of the D1/32-kD reaction center protein.

Organisms of oxygenic photosynthesis convert the energy of sunlight into chemical energy, which supports most life on earth. In photosynthetic membranes of green algae and plants, incident irradiance is absorbed by chlorophyll (Chl)-binding light-harvesting antenna complexes (LHCs) associated with the reaction centers of PSII and PSI. However, when the photosynthetic apparatus absorbs irradiance in excess of that required for the saturation of photosynthesis, singlet oxygen is generated, and PSII is subject to an irreversible photooxidative damage (Vass et al., 1992; Telfer et al., 1994; Melis, 1999). This photodamage selectively impairs the function of the D1/32-kD reaction center protein of PSII and has the potential to lower rates of photosynthesis and diminish plant growth and productivity (Powles and Critchley, 1980; Powles, 1984).

The probability of photooxidative damage in chloroplasts depends on the oxidation reduction state of the primary electron-accepting plastoquinone of PSII (Q_A), which is the parameter that controls photodamage under a variety of physiological and environmental conditions. When Q_A is oxidized under continuous illumination, photochemical electron transport from the reaction center Chl (P680) converts excitation energy into chemical form. Under these conditions, there is a low probability of excitation transfer to molecular oxygen. When Q_A is reduced under continuous illumination, there is a relatively higher probability that exited Chl molecules in the triplet state would relax through energy transfer to oxygen, thus generating reactive singlet oxygen. Singlet oxygen adversely affects PSII by covalent modification of the photochemical reaction center Chl in the D1 protein (Aro et al., 1993). Under steady-state photosynthesis conditions, the reduction state of Q_A increases linearly with irradiance, thereby causing a correspondingly linear increase in the probability of photodamage (Huner et al., 1998; Melis, 1999). Organisms of oxygenic photosynthesis overcome this irreversible modification upon a molecular repair of the adversely affected PSII centers. The repair process entails disassembly of the PSII holocomplex and the selective removal and replacement of the photodamaged D1 protein (Mattoo and Edelman, 1987), constituting the so-called PSII damage-and-repair cycle (Guenther and Melis, 1990).

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When photooxidative damage to PSII occurs faster than its enzymatic repair, the photosynthetic capacity and quantum yield of photosynthesis are lowered, causing a condition known as photo-inhibition (Powles, 1984; Aro et al., 1993). To avoid or minimize photo-inhibition, photosynthetic organisms have evolved several strategies (Demmig-Adams and Adams, 1992; Horton et al., 1996; Niyogi et al., 1997b, 2001). These are distinguished between short- and long-term responses, aimed at diminishing overexcitation of the reaction centers. Short-term responses include a mechanism known as energy-dependent "non-photochemical quenching," which can help dissipate excess absorbed light energy. In addition, within minutes upon exposure of plants to excessive irradiance, an irradiance-dependent xanthophyll cycle is activated, which involves reversible de-epoxidation of violaxanthin (V) and formation of zeaxanthin (Z) via antheraxanthin (A). Z is believed to play a photoprotective role via dissipation of excessive light energy as heat (Yamamoto, 1979; Demmig-Adams, 1990; Gilmore et al., 1995; Niyogi, 1999). When levels of absorbed irradiance become lower than those required for the saturation of photosynthesis, Z is converted back to V by the enzyme Z epoxidase (Hager, 1980). This xanthophyll cycle is a dynamically regulated and reversible interconversion of V to A to Z, occurring in the thylakoid membrane of photosynthesis (Demmig et al., 1987; Gilmore and Yamamoto, 1993; Demmig-Adams et al., 1996; Goss et al., 1998; Havaux and Niyogi, 1999). Another acclimation mechanism entails reversible changes in the Chl antenna size of the PSs. In this process, long-term high irradiance elicits modulation of gene expression, leading to a reduction in the amount of Chl and in the number of the LHC proteins in the photosynthetic apparatus (Melis, 1991). This causes the assembly of a smaller functional light-harvesting Chl antenna size in the chloroplast thylakoids, effectively diminishing overexcitation of the PSs (Smith et al., 1990; Neidhardt et al., 1998).

As a consequence, mutants with lesions in the Z epoxidase gene are deficient not only in A and V but also fail to synthesize neoxanthin (N; Rock and Zeevaart, 1991; Marin et al., 1996; Niyogi et al., 1997a; Jin et al., 2003). In addition, such mutants accumulate Z, even under low-light (LL) growth conditions. Higher plants with an impaired Z epoxidase are not affected in terms of photosynthesis (Rock et al., 1992; Tardy and Havaux, 1996; Hurry et al., 1997). Analogous mutations resulting in Z accumulation have been described in the green alga *Scenedesmus obliquus* (Bishop et al., 1998), *Chlamydomonas reinhardtii* (Niyogi et al., 1997a), and *Dunaliella salina* (Jin et al., 2003). Under LL growth conditions, no significant differences in the properties of photosynthesis in these organisms could be observed. Such earlier investigations suggested that Z could structurally and functionally replace the missing epoxy-carotenoids A, V, and N in mutants of both higher plant and green algae.

There is a sizable literature on the role of the reversible xanthophyll cycle in photoprotection (for review, see Demmig-Adams and Adams, 1992; Horton et al., 1996; Gilmore, 1997; Niyogi, 1999). The proposed photoprotective function of Z entails a direct quenching of excitation energy in the pigment bed of photosynthesis (Demmig-Adams, 1990; Horton et al., 1996), thereby protecting the photosynthetic apparatus from the consequences of overexcitation. However, the precise mechanism of photoprotection by Z is not well understood. It has been assumed that de-epoxidation of V to Z (Bugos and Yamamoto, 1996) helps to guard against a photooxidative damage to the PSII reaction center complex. Mechanistic aspects of this hypothesis were not rigorously investigated, though. Recent preliminary work from this laboratory suggested a role for Z after photodamage and while PSII occurred in the disassembled state before repair (Jin et al., 2001). In the present work, the role of Z in the protection of PSII was further investigated with the *zea1* mutant of the green alga *D. salina*. This mutant is apparently aberrant in the Z epoxidase reaction and, irrespective of the growth or irradiance stress conditions, accumulates Z. Biochemical analyses showed that Z constitutively and quantitatively substituted for N, V, and A in the *zea1* strain (Jin et al., 2003). These previous measurements also showed similar rates of growth and light saturation curves of photosynthesis for wild type (WT) and *zea1* mutant in the light intensity range between 0 and 3,000 μmol photons m^{-2} s^{-1}. Thus, this mutant offered an opportunity to rigorously study the role of Z in photo-acclimation and the PSII damage and repair properties of the cells. Results showed that a constitutive accumulation of Z in the thylakoid membrane does not alter the green alga photoacclimation properties, sensitivity to irradiance stress, kinetics of photodamage, or recovery from photoinhibition in *D. salina*. The results are discussed in terms of the accumulation of Z, which, in the WT, occurred in parallel with the accumulation of photodamaged PSII and the return of Z to V, which occurred in tandem with the recovery from photo-inhibition.

RESULTS

Pigment Composition and PS Stoichiometry

The *zea1* mutant of *D. salina* was unable to synthesize detectable amounts of the epoxy-xanthophylls A, V, and N but constitutively accumulated Z (Jin et al., 2003). When cells were grown under 100 μmol photons m^{-2} s^{-1} (LL growth conditions), the Chl content (approximately 3×10^{-15} mol $cell^{-1}$) and the Chl *a*/*b* ratio (approximately 4:1) were about the same in WT and *zea1* mutant (Table I). Total Car content (approximately 1.5×10^{-15} mol $cell^{-1}$) was also similar in

Table I. *Pigment content, Chl a/b ratio, de-epoxidation state, and photosystem stoichiometry in D. salina wild type and zea1 mutant*

Cells were grown under 100 μmol photons m^{-2} s^{-1} (LL).

Parameter	WT	*zea1*
mol Chl $cell^{-1} \times 10^{-15}$	2.56 ± 0.2	2.82 ± 0.12
Chl *a*/*b*	4.2 ± 0.1	4.1 ± 0.2
mol carotenoid (Car)/cell $\times 10^{-15}$	1.5 ± 0.21	1.49 ± 0.1
mol Z $cell^{-1} \times 10^{-16}$	0.3 ± 0.1	6.9 ± 0.1
Z/(V + A + Z)	0.18 ± 0.01	1 ± 0.0
mmol Q_A mol Chl^{-1}	2.6 ± 0.07	2.8 ± 0.15
mmol P700 mol Chl^{-1}	2.1 ± 0.04	2.07 ± 0.15
PSII/PSI (Q_A/P700)	1.23	1.35

WT and *zea1* mutant. However, the Z content of the mutant (Z, approximately 6.9×10^{-16} mol $cell^{-1}$) was about 23-fold greater than that in the WT (Z, approximately 0.3×10^{-16} mol $cell^{-1}$, Table I). The de-epoxidation state ([Z]/[V + A + Z] ratio) was 0.18:1 in the WT and 1:1 in the mutant. These results suggest a quantitative substitution of A, V, and N by Z in the thylakoid membrane of the *zea1* strain.

Photochemical apparatus organization in the two strains was compared upon analysis of PS concentration. Light-induced absorbance difference measurements were used to determine the concentrations of Q_A and P700 as a measure of functional PSII and PSI reaction centers, respectively. Table I shows that, under LL growth conditions, WT and the *zea1* mutant had similar PSII and PSI concentrations either on a per Chl or on a per cell basis. Similar Chl *a*/*b* and PSII/PSI ratios in the thylakoid membranes of WT and *zea1* mutant suggest that the level of LHCII and LHCI were about the same in the two strains (Neidhardt et al., 1998; Jin et al., 2001). Taken together, these results show that absence of the epoxyxanthophylls A, V, and N and constitutive expression of Z did not bring about changes in the PSII/PSI ratio, level of the LHC proteins, or functional Chl antenna size of the PSs in the chloroplast thylakoids.

Photosynthesis Characteristics

The quantum yield and productivity of photosynthesis in WT and *zea1* were assessed upon comparative analysis of the light saturation curve of photosynthesis in the two strains. In such presentation, the rate of O_2 evolution was 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 (not shown), information was obtained about the relative quantum yield of photosynthesis (Φ) in WT and *zea1* (Table II). The light-saturated rate of oxygen evolution (Pmax) defined the capacity of photosynthesis in the two strains. As shown in Table II, WT (Φ = 0.36) and *zea1* (Φ = 0.36) had similar Φ. However, the photosynthetic capacity of the mutant (Pmax = 95 ± 7.2 mmol O_2 mol Chl^{-1} s^{-1}) was about 15% greater than that of the WT (Pmax = 80 ± 3.2 mmol O_2 mol Chl^{-1} s^{-1}). Chl fluorescence activity of WT and *zea1* was measured in vivo. The fluorescence parameter (F_v/F_m) offers a nonintrusive method for the measurement of photochemical charge separation efficiency at PSII. Table II shows that PSII photochemical charge separation efficiency of the mutant (F_v/F_m = 0.68) was slightly higher than that of the WT (F_v/F_m = 0.62). These results provide evidence that constitutive expression and assembly of Z in the thylakoid membrane of *D. salina*, occurring instead of A, V, and N, does not bring about a permanent quenching of excitation in the pigment bed or otherwise affect the function of photosynthesis.

Photo-Acclimation of *D. salina* WT and *zea1* Mutant

Under the LL growth conditions (100 μmol photons m^{-2} s^{-1}) employed in this work, WT and *zea1* were indistinguishable on the basis of their photosynthesis characteristics. Measurements were extended to include the photo-acclimation of the cells to different levels of growth irradiance, in the range of 100 to 2,000 μmol photons m^{-2} s^{-1}. Figure 1, A and B, show the pigment (Chl and total Car) content in the two strains as a function of growth irradiance. Levels of Chl in both WT and mutant declined as a function of growth irradiance from 3×10^{-15} to 0.3×10^{-15} mol $cell^{-1}$ (Fig. 1A). Total Car in the cells also declined as a function of growth irradiance, from 1.5×10^{-15} to 0.8×10^{-15} mol $cell^{-1}$ (Fig. 1B). It is noteworthy that cellular Chl content decreased considerably more than that of Car as a function of growth irradiance. No significant difference could be detected in the irradiance-dependent adjustment of Chl and total Car content between WT and *zea1* mutant, suggesting that the mutation did not affect the ability of the cells to acclimate to the level of irradiance.

Acclimation of photosynthetic organisms to irradiance also entails changes in Z content. The cellular content of Z in the WT increased as a function of growth irradiance from 0.3 to 3.2×10^{-16} mol $cell^{-1}$ (Fig. 1C), reflecting a shift in the de-epoxidation state of the Car present, more toward Z. In the *zea1* mutant, Z per cell was constant as a function of growth irradiance in the 100 to 400 μmol photons m^{-2} s^{-1} (about 7×10^{-16} mol $cell^{-1}$). It gradually declined at higher light intensities to about 4×10^{-16} mol $cell^{-1}$

Table II. *Photosynthetic capacity, relative photon use efficiency, and PSII efficiency of D. salina wild type and zea1 mutant*

Parameter Measured	LL	
	WT	*zea1*
Photon use efficiency (Φ), arbitrary units	0.36 ± 0.02	0.36 ± 0.02
Photosynthetic capacity (Pmax), mmol O_2 mol Chl^{-1} s^{-1}	80 ± 3.2	95 ± 7.2
F_v/F_m	0.62 ± 0.04	0.68 ± 0.02

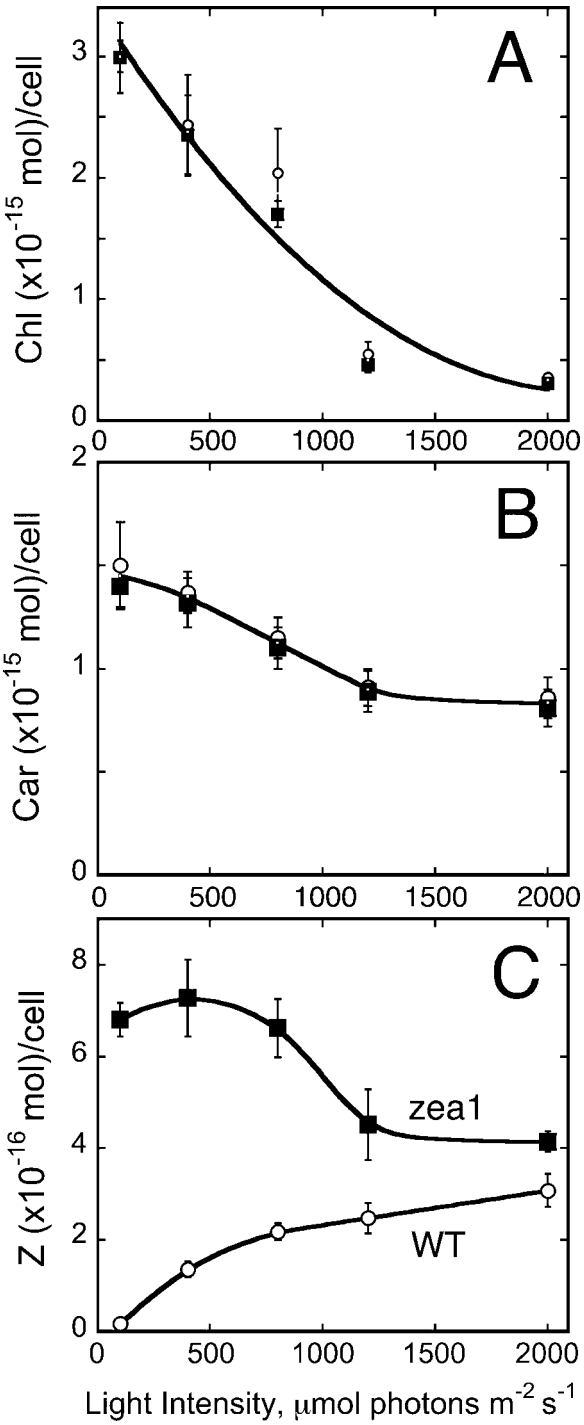

Figure 1. Chl and Car content of *D. salina* cultures. The effect of growth irradiance on the cellular Chl content (A), total Car content (B), and Z content (C) of WT (white circle) and *zea1* mutant (squares) is shown.

at 2,000 μmol photons m^{-2} s^{-1} (Fig. 1C), consistent with the overall decline of total Car per cell as a function of growth irradiance.

The de-epoxidation state of the xanthophyll cycle Car was also determined in cells grown under different levels of irradiance. In the WT, the de-epoxidation state increased as a function of growth irradiance from 0.18 to 0.95 (Fig. 2A). In the *zea1* mutant, the de-epoxidation state remained at 1.0 irrespective of the growth irradiance regime. The latter is consistent with a *zea1* lesion in the Z epoxidase gene, which prevents the epoxidation of Z to V and also renders the xanthophyll cycle inoperative. Figure 2B shows the F_v/F_m ratio of dark-adapted WT and *zea1* cells grown under different irradiance regimes. The ratio declined as a function of growth irradiance from F_v/F_m = 0.65 at 100 μmol photons m^{-2} s^{-1} to F_v/F_m = 0.25 at 2,000 μmol photons m^{-2} s^{-1}. This response, attributed to the slowly reversible excitation quenching, was identical in WT and *zea1* mutant, showing that constitutive accumulation of Z in the mutant did not affect in any way the development of this excitation quenching as a function of growth irradiance.

To further probe the effect of a constitutive accumulation of Z on the photo-acclimation properties of *D. salina*, the light-saturated rate and the Φ were measured as a function of growth irradiance. Figure 2C shows that, in the range from 100 to 2,000 μmol photons m^{-2} s^{-1}, Pmax increased as a function of growth irradiance from about 90 to about 180 mmol O_2 mol Chl^{-1} s^{-1}, whereas the Φ declined from 0.36 to 0.22 (Fig. 2D, arbitrary units). The increase in Pmax is attributed to a lowering of the Chl content (Pmax measurement is on a per Chl basis). The lowering of the Φ is attributed to steady-state photo-inhibition of photosynthesis in these green algae, which is accentuated with growth irradiance (Vasilikiotis and Melis, 1994; Baroli and Melis, 1996). It is concluded that a constitutive accumulation of Z in *D. salina* does not alter the photo-acclimation response of the cells.

The above analyses showed a substantially different Z content between WT and *zea1* mutant (Figs. 1C

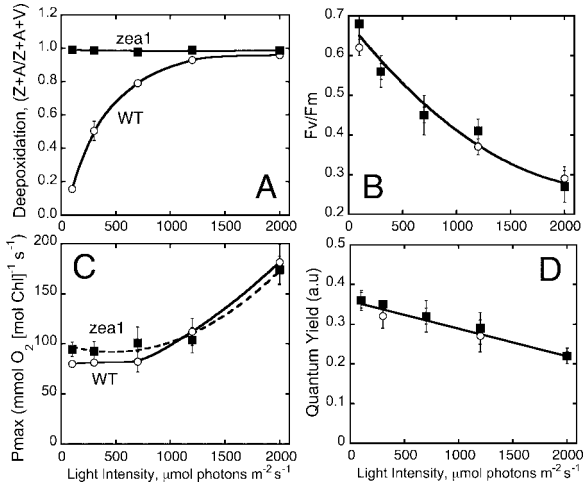

Figure 2. Effects of growth irradiance on de-epoxidation state of the xanthophyll cycle (A); PSII photochemical charge separation efficiency, as measured by the Chl fluorescence F_v/F_m ratio (B); Pmax of cellular photosynthesis (C); and Φ of photosynthesis (D) in WT (white circles) and *zea1* mutant (squares) of *D. salina*.

and 2A). However, there were no discernible differences between the two strains in PSII photochemical charge separation efficiency of the acclimated cells (Fig. 2B) or photo-inhibition of photosynthesis as a function of growth irradiance (Fig. 2D). Considering the proposed role of Z in photoprotection (Demmig et al., 1987; Gilmore and Yamamoto, 1993; Demmig-Adams et al., 1996; Goss et al., 1998; Havaux and Niyogi, 1999), these results indicate that a constitutive presence of Z does not confer enhanced resistance to photooxidative damage in the *zea1* mutant. To more rigorously assess the role of Z in the protection against photooxidative damage of PSII in chloroplasts, further detailed analysis was undertaken at the thylakoid membrane and molecular levels.

Photo-Inhibition Status as a Function of Growth Irradiance

To further analyze the effect of growth irradiance on the photosynthetic apparatus of WT and *zea1* mutant, thylakoid membranes were isolated from WT and *zea1* cells grown under different levels of irradiance. SDS-PAGE analysis of the thylakoid membrane proteins was performed with samples loaded on an equal Chl basis, followed by western-blot analyses with specific polyclonal antibodies. Figure 3 shows western blots with specific polyclonal antibodies against the D1 reaction center protein of PSII (Kim et al., 1993) or against a 160-kD PSII repair intermediate (Kim et al., 1993; Melis and Nemson, 1995; Yokthongwattana et al., 2001). The 160-kD protein complex is known to contain a photodamaged but as yet undegraded D1 protein (Kim et al., 1993) and the D2 protein of the PSII reaction center (Melis and Nemson, 1995). This unusual property has permitted, for the first time to our knowledge, an SDS-PAGE-based quantitation of photodamaged versus active D1 in chloroplast thylakoids (Baroli and Melis, 1996). It was postulated that formation of such a 160-kD protein complex might reflect PSII conformational changes that occur as a direct consequence of photodamage and the ensuing partial disassembly of PSII (Yokthongwattana et al., 2001). The quantitative measurement of the 160-kD protein complex provides a convenient way by which to assess the extent of the in vivo photo-inhibition in *D. salina*. Results from such quantitative western-blot analysis in Figure 3A show increasing steady-state levels of the 160-kD complex as a function of growth irradiance. However, there were no obvious differences in the amount of the 160-kD protein complex accumulating in the *zea1* thylakoid membrane versus that of the WT. Moreover, levels of the active D1 protein in the *zea1* thylakoids showed no significant difference compared with those of the WT (Fig. 3B). It is concluded that a constitutive accumulation of Z in the *zea1* mutant does not confer enhanced advantage over the WT in terms of protection from photo-inhibition.

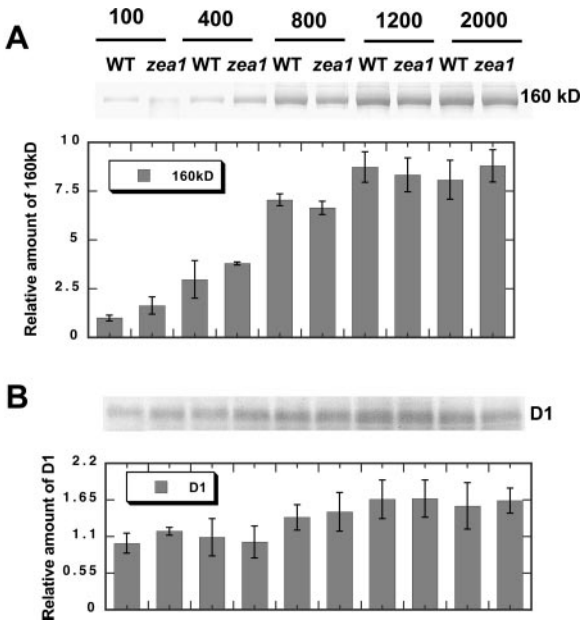

Figure 3. Quantitative western-blot analysis of thylakoid membrane proteins from *D. salina* WT and *zea1* mutant grown under different light intensities. Proteins were probed with specific polyclonal antibodies against the 160-kD PSII repair intermediate (A) or against the PSII D1/32-kD reaction center protein (B). The corresponding densitometric quantitations of the bands are given as a bar graph on the bottom of each panel. Lanes were loaded on an equal Chl basis (4 nmol Chl lane^{-1}).

The amount and composition of the LHCII and Cbr proteins in WT and the *zea1* mutant were estimated from western-blot analyses with specific polyclonal antibodies raised against the apoproteins of the LHCII and against the Cbr protein (Fig. 4). LHCII proteins in both WT and the *zea1* mutant declined as a function of growth irradiance (Fig. 5A). Among four distinct bands of the LHCII (termed according to electrophoretic mobility as LHCII-1–4), the amount of LHCII-1 declined faster than LHCII-2 to 4 as a function of growth irradiance. This is consistent with the photo-acclimation response of the photosynthetic apparatus (Anderson, 1986; Tanaka and Melis, 1997). This response appeared to be similar in WT and *zea1* mutant.

The Cbr protein is homologous to higher plant ELIP proteins and belongs to the LHC super family (Green and Kühlbrandt, 1995; Banet et al., 2000). Cbr proteins accumulate when *D. salina* cells are stressed by HL. Therefore, the Cbr protein is thought to be an indicator of irradiance stress (Lers et al., 1991; Levy et al., 1992, 1993). Thylakoid membrane proteins of WT and *zea1* cells were probed with a Cbr antibody, and levels of the Cbr protein in WT and mutant were quantified from western-blot analyses. Figure 4B shows that Cbr proteins were practically absent in both WT and *zea1* mutant when grown at 100 μmol photons m^{-2} s^{-1}, whereas the levels of Cbr increased substantially with growth irradiance. This increase in

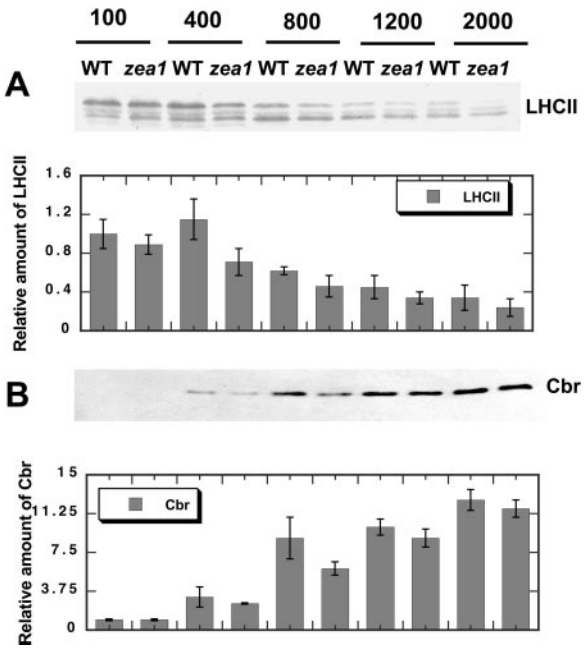

Figure 4. Quantitative western-blot analysis of thylakoid membrane proteins from *D. salina* WT and *zea1* mutant grown under different light intensities. Proteins were probed with specific polyclonal antibodies raised against the LHC-II (A) and Cbr protein (B). The corresponding densitometric quantification of the bands is given as a bar graph on the bottom of each panel. Lanes were loaded on an equal Chl basis (4 nmol Chl lane^{-1}).

the level of the Cbr protein was nearly identical in the WT and *zea1* mutant (Fig. 4B).

Photodamage and Photo-Inhibition after an LL → HL Shift

To gain a better insight into the potential role of a constitutive accumulation of Z in the thylakoid membrane, light shift experiments were performed and the effect on rate of photodamage or recovery from photo-inhibition were recorded. The rate of PSII photodamage was compared in WT and *zea1* mutant after an LL → HL shift of the cultures. In such measurements, the amount of D1 protein was quantified as a function of time in the presence of lincomycin, a chloroplast protein biosynthesis inhibitor. In the presence of lincomycin, de novo biosynthesis of the D1 protein is prevented and, therefore, the repair process is inhibited. After photodamage, there is a loss to the D1 protein from the 32-kD position because the latter is converted into a 160-kD protein complex (Kim et al., 1993; Baroli and Melis, 1996). This process can be monitored by western-blot analysis from the kinetics of the D1 loss from the 32-kD position (Kim et al., 1993). Figure 5 shows results from such measurements. WT and *zea1* mutant cells were transferred from LL → HL at 0 min. Samples were harvested at the indicated times, and thylakoid membranes were isolated and subjected to western-blot analysis (Fig. 5, upper). Quantitation of the D1 decay kinetics (Fig. 5, lower) showed half times of about 32 ± 12 min for WT and 45 ± 15 min for the *zea1* mutant. These results may suggest that a constitutive accumulation of Z in the thylakoid membrane would cause somewhat slower kinetics in the processing of the inert D1 protein after photodamage.

In addition to the analysis on D1 processing after photodamage in the presence of lincomycin (Fig. 5), changes in photochemical activity of WT and *zea1* were measured in situ after an LL → HL shift of the cultures in the absence of this inhibitor. Figure 6A shows that functional PSII centers (Q_A/Chl) are lowered by about 60% within 6 h after the LL → HL shift (absence of lincomycin). At longer incubation times under HL, the Q_A/Chl ratio was stabilized or gradually increased because of a lowering in the Chl content of the cells. This is consistent with earlier results from this laboratory (Kim et al., 1993). The effect of an LL → HL shift on the PSI photochemical activity is also shown (Fig. 6B). Loss in P700 activity also occurs as a result of the sudden change in the level of irradiance. The amplitude of this adverse effect, however, is less than that for PSII. It is important to note that no significant difference was observed between WT and *zea1* mutant in this experimentation.

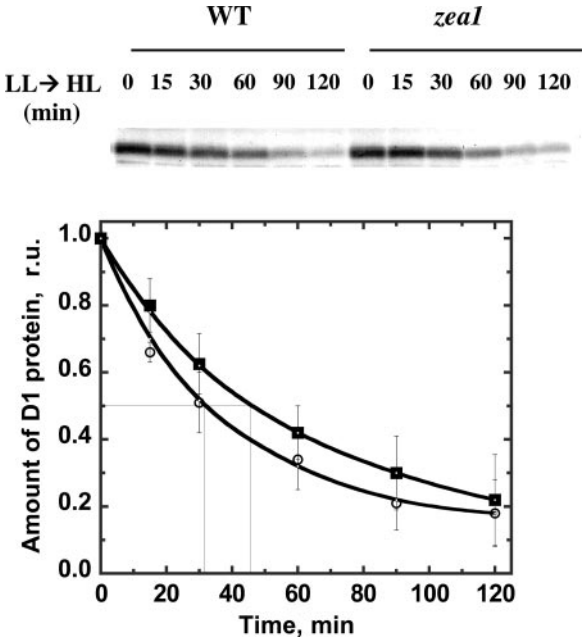

Figure 5. Time course for the loss of the D1 protein from its 32-kD position after an LL → high light (HL) shift of the cultures. *D. salina* WT (white circles) and *zea1* mutant (squares) were grown under LL to the late log phase. Cells were suspended in the presence of lincomycin immediately before an LL → HL shift. Western blots probed with polyclonal antibodies against the D1 protein are show in the upper panel. Densitometric quantification of the corresponding western blots for WT (white circles) and *zea1* mutant (squares) are shown in the lower panel. The half time of the 32-kD protein loss was 32 ± 12 min for the WT and 45 ± 15 min for the *zea1* mutant.

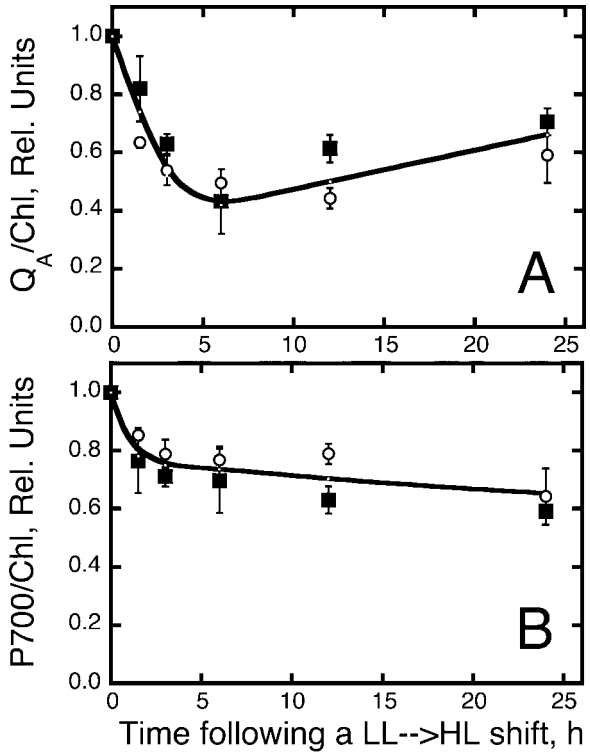

Figure 6. Time course of PS concentration (Q_A and P700) after an LL → HL shift of WT (white circles) and *zea1* mutant (squares) of *D. salina*. A, Photochemically competent PSII measured from the amplitude of Q_A photoreduction. B, Photochemically competent PSI measured from the amplitude of P700 photoreduction.

Photodamage and photo-inhibition in WT and *zea1* mutant were investigated further after an LL → HL shift from the Pmax and Φ of photosynthesis. Figure 7A shows a transient loss in Pmax, reaching a low after about 6 h in HL. Subsequently, Pmax gradually increased, partly because of the establishment of a steady state between photodamage and repair, and in part due to the lowering in the Chl content (Fig. 1A) as cells acclimate to HL. Figure 7B shows a substantial loss in Φ, reaching a low of only 15% of the control after about 6 h incubation under HL. This precipitous loss in Φ most likely reflects the ensuing dissociation of the LHC-II antenna from the photodamaged PSII core complex (Melis, 1991). A dissociated LHC-II antenna would absorb light but would not contribute to the Φ. Once again, this characteristic photodamage and photo-inhibition phenomenology was invariant between WT and *zea1* mutant.

PSII Repair and Recovery from Photo-Inhibition

The preceding results suggested that constitutive accumulation of Z in the *zea1* strain did not enhance protection of PSII from photodamage. We investigated whether constitutive accumulation of Z may affect the repair of PSII from photodamage and/or the recovery of photosynthesis from photo-inhibition. In such experiments, both WT and *zea1* mutant were grown under continuous irradiance stress conditions (2,500 μmol photons m^{-2} s^{-1}). Under these conditions, growth occurs while the yellowish cells exist in steady-state photo-inhibition, with up to 80% of all PSII centers being photochemically inert at any given point in time (Vasilikiotis and Melis, 1994). Such HL-grown cells were shifted to LL growth conditions (HL → LL). Samples were collected at different time intervals after the HL → LL shift for analysis. Two main photosynthesis parameters, Pmax and Φ, were measured as a way by which to monitor the recovery of photosynthesis from photo-inhibition. Figure 8A shows the adjustment of the Pmax in cells after an HL → LL transition. It is evident that Pmax increased promptly as a function of time upon the LL → HL transition, reaching a 50% greater value within approximately 2 h under the LL. This change reflects the repair of photodamaged PSII centers, which results in a greater capacity for photosynthesis (Neidhardt et al., 1998). Incubation of the cultures for more than approximately 2 h under LL conditions caused a gradual decline in the value of Pmax (Fig. 8A), reflecting the accumulation of Chl in the chloroplasts (Fig. 1A), and increase in the light-harvesting Chl antenna size, which resulted in a lower per Chl Pmax value.

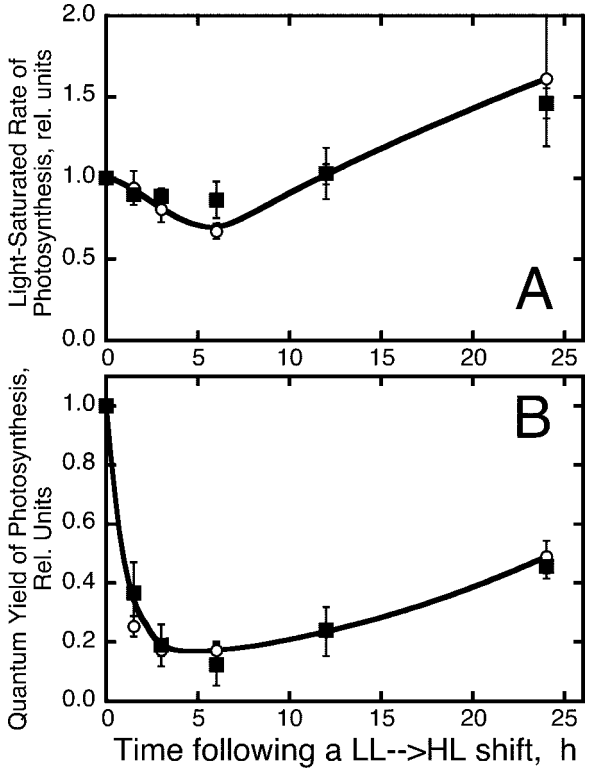

Figure 7. Changes in the Pmax and Φ of photosynthesis after an LL → HL shift of the cultures. A, Measurement of the light-saturated rate of photosynthesis in WT (white circles) and *zea1* mutant (squares). B, Measurement of the Φ in WT (white circles) and *zea1* mutant (squares).

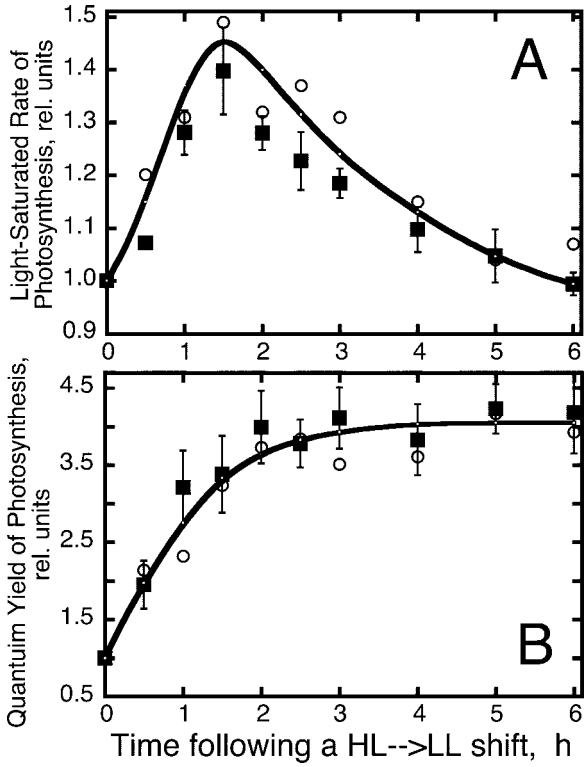

Figure 8. Changes in Pmax and Φ of photosynthesis after an HL → LL shift of the cultures. A, Measurement of the light-saturated rate of photosynthesis in WT (white circles) and *zea1* mutant (squares). B, Measurement of the Φ in WT (white circles) and *zea1* mutant (squares).

Figure 8B shows the adjustment of the Φ in *D. salina* WT and *zea1* mutant after an HL → LL transition. Φ increased exponentially from a low relative value (Φ = 1) in HL to a high relative value (Φ = 4) after about 2 to 3 h in LL, i.e. a change by a factor of about 4, which underscores the difference between control and photo-inhibited samples. This HL → LL-dependent transition in the value of Φ is also consistent with the repair of photodamaged PSII centers, which now contribute to useful photochemistry, thereby resulting into a greater Φ. (Note that the Φ is independent of the Chl antenna size and remains at the 4 "relative units" level, even though the antenna size of the PSs continues to expand in the 2–6-h interval after the HL → LL shift.) The kinetics of this adjustment in Φ showed a half time of approximately 1 h, consistent with earlier findings on the half time of the PSII repair from photodamage (Sundby et al., 1993; Vasilikiotis and Melis, 1994; Baroli and Melis, 1996; Neidhardt et al., 1998).

The above characteristics and related phenomenology pertaining to the recovery of photosynthesis from photo-inhibition were invariant between WT and the *zea1* mutant, suggesting that a constitutive accumulation of Z instead of A, V, and N does not in any substantial way affect repair and recovery of PSII from photo-inhibition.

Kinetics of PSII Photodamage/Recovery and Z Accumulation/Decay

The above results suggested that constitutive accumulation of Z in the thylakoid membrane of the *zea1* strain, occurring in the place of A, V, and N, did not bring about an effect on the properties of photoacclimation, photodamage, or repair of the photosynthetic apparatus from photo-inhibition. The first part of this work, however, suggested parity between relative amount of Z, as measured from the steady-state epoxidation level (Figs. 1C, 2A, and 4B) and the fraction of photodamaged PSII centers in the WT thylakoids (Figs. 2, B and D, and 3A). These results suggested that Z accumulates in direct proportion to the photodamaged PSII reaction centers in the chloroplast thylakoids. To explore this notion with a different experimental approach, light shift experiments were conducted, and the kinetics of V loss and Z accumulation were noted in relation to the kinetics of PSII photodamage measured upon an LL → HL shift of WT *D. salina* cultures. Figure 9A shows identical kinetics of PSII photodamage ($1 - Q_A$), Z accumulation, and V loss occurring in vivo with a half time of about 100 min upon an LL → HL shift of *D. salina* cultures. These results suggest that photodamage and the prompt disassembly of the PSII holocomplex were accompanied by a V de-epoxidation to Z.

A quantitative analysis of Car pool levels after such an LL → HL shift was undertaken. After about 8 h following the LL → HL shift, when nearly 90% of the PSII reaction centers had accumulated in a photodamaged state (Fig. 9A), the pool of Z had increased by about 5.2×10^{-16} mol cell^{-1}. Concomitantly, the pool of V decreased by about 4.0×10^{-16} mol cell^{-1}. Thus, there was a 1.2×10^{-16} mol cell^{-1} Z formed that could not be accounted for by the corresponding loss in V. This persistent lack of quantitative parity between Z accumulation and V loss after an LL → HL shift could be explained by a conversion of β-carotene to Z, occurring upon the disassembly of the photodamaged PSII reaction centers, as proposed by Depka et al. (1998). Consistent with this interpretation, we found a corresponding lowering of the β-carotene pool size, by about 1.4×10^{-16} mol cell^{-1}, after an LL → HL shift (not shown).

If Z is a component of the PSII repair process, as the above results would strongly suggest, then decay of Z (a reversible epoxidation to V) should follow a chloroplast recovery from photo-inhibition. Figure 9B shows the decay kinetics of Z (conversion to V) upon an HL → LL shift of *D. salina*. The half time for the decay of Z was measured to be about 2 h under these conditions. The repair of the photodamaged PSII was measured to occur with a half time of about 1 h (Fig. 8B), whereas growth of the light-harvesting Chl antenna size after the HL → LL shift took more than 6 h (Fig. 8A; Neidhardt et al., 1998). Therefore, it is likely that a temporal sequence of events, after an HL → LL shift, is first repair of PSII reaction centers

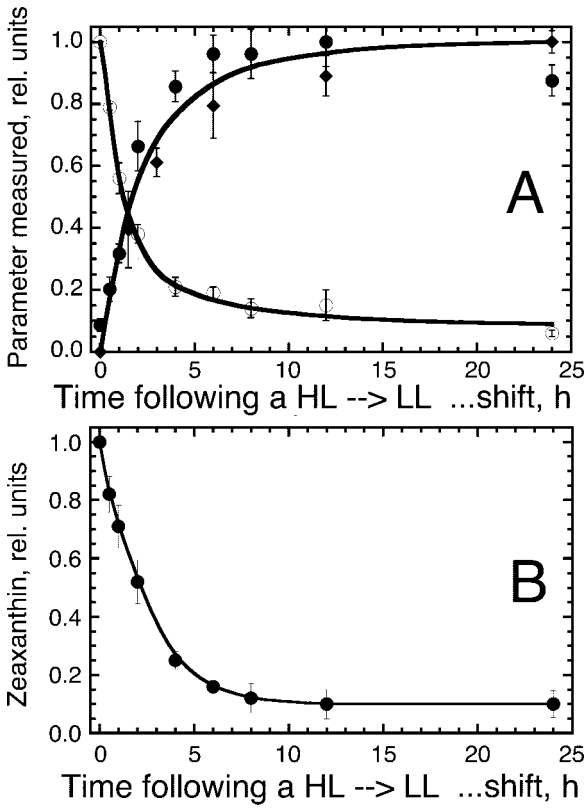

Figure 9. A, Comparative kinetic analysis of the accumulation of Z (solid circles), accumulation of photodamaged PSII reaction centers (solid diamonds), and loss of V (white circles) after an LL → HL shift of *D. salina* WT cultures. B, Decay kinetics of Z to V conversion after an HL → LL shift of the *D. salina* WT cultures.

followed by Z epoxidation to V. The latter apparently occurs soon after the repair and functional recovery of the photodamaged PSII reaction centers and precedes the build-up of the light-harvesting Chl antenna size.

DISCUSSION

A xanthophyll aberrant mutant (*zea1*) of *D. salina* was unable to synthesize any of the epoxyxanthophylls A, V, and N but constitutively accumulated Z instead. The *zea1* mutant did not show any discernible difference from the WT in terms of growth either under LL or HL conditions (Jin et al., 2003). This work provided evidence that WT and *zea1* could not be distinguished on the basis of rate or Φ of photosynthesis, efficiency of PSII charge separation, or photo-acclimation characteristics. Constitutive accumulation of Z in *zea1* occurred without any changes in total cellular Chl or Car content and without affecting the Chl *a*/*b* or PSII/PSI ratio in the thylakoid membrane. WT and *zea1* could not be distinguished on the basis of susceptibility to photodamage or recovery from photo-inhibition. However, it was noted that Z in the WT accumulated in parallel with the accumulation of photodamaged PSII centers in the chloroplast thylakoids and decayed in tandem with the chloroplast recovery from photo-inhibition. There was a clear correlation between the reversible xanthophyll cycle and the PSII repair cycle and a lack of correlation between xanthophyll cycle and Chl antenna size in the thylakoid membrane. These results would suggest, therefore, that Z is a component of the PSII repair process (Jahns et al., 2000; Jin et al., 2001). It is proposed that the "photoprotective" mechanism of Z in the chloroplast thylakoids operates after PSII photodamage and disassembly has occurred and before functional recovery and reconstitution of the PSII holocomplex.

Our working hypothesis is that at least a fraction of the Z pool accumulating in the WT confers photoprotection to the disassembled PSII from further and possibly irreversible photobleaching (Jin et al., 2001). This hypothesis is consistent with the observation that V de-epoxidation occurs immediately upon photodamage, and Z accumulation occurs in parallel with the accumulation of photodamaged PSII reaction centers in the chloroplast thylakoids. A prompt disassembly of the PSII holocomplex (Melis, 1991; Aro et al., 1993) and formation of a PSII repair intermediate (Melis, 1999; Yokthongwattana et al., 2001) are known to follow photodamage. Highly photoactive tetrapyrrole pigments are released by the PSII D1/D2 reaction center in the course of reaction center disassembly and D1 degradation and replacement. It could be argued that quenching of tetrapyrrole excitation by Z (Wentworth et al., 2000) is needed at this stage because this repair intermediate stage renders PSII most vulnerable to massive and irreparable photooxidative bleaching. According to this hypothesis, Z might play a role in the photoprotection of the photodamaged and disassembled PSII core, including the D2, CP47, and CP43 Chl proteins. This hypothesis is also consistent with the effect of high photon flux densities on mutants that are deficient in the V de-epoxidase enzyme. Upon photooxidative stress, the *npq1* mutant of Arabidopsis was subject to a loss of bulk Chl, bleaching, and/or pronounced lipid peroxidation (Havaux and Niyogi, 1999; Havaux et al., 2000). Similarly (Verhoeven et al., 2001), transgenic tobacco (*Nicotiana tabacum*) with suppressed Z formation was found to be susceptible to stress-induced photo-inhibition, consistent with the notion of Z being a photoprotective pigment functioning in the PSII repair process. Our proposed hypothesis is also consistent with the role of the Cbr protein, which appears upon photooxidative damage (Fig. 4B) and was reported to involve stabilization of assemblies highly enriched in Z and possibly containing other unbound pigments (Banet et al., 2000).

Thus, Cbr and Z may participate in the PSII repair process and may be critical for the protection of PSII because the latter is in the process of degrading and replacing nonfunctional D1 reaction center proteins

(Jin et al., 2001). A return of Z to V in the WT, and a removal of the Cbr protein from the thylakoid membrane, would logically follow the repair of PSII from photodamage and the recovery of the chloroplast from photo-inhibition. Such a mechanism must also operate in the *zea1* strain, evidenced by the onset of photodamage upon an LL → HL shift (Fig. 5), accumulation of photo-inhibited PSII centers (Fig. 3A), and parallel accumulation of the Cbr protein (Fig. 4B) in HL. The converse is observed during the repair and recovery of the photosynthetic apparatus upon a HL → LL shift. The only difference (without a functional consequence) in this respect was the constitutive presence of Z and the absence of an irradiance-induced V de-epoxidation in the *zea1* strain.

The quantitative replacement of A, V, and N by Z in the *zea1* strain is consistent with findings by Bishop et al. (1998) pertaining to the properties of *S. obliquus* mutants with deletions in Car biosynthesis. They are also consistent with results from Z-accumulating mutants of *C. reinhardtii* (Polle et al., 2001) and Arabidopsis (Lokstein et al., 2002), demonstrating replacement of at least V and A in the respective LHC-binding sites by Z. The quantitative replacement of A, V, and N by Z would suggest that Z occupies positions that are normally occupied by A, V, and N in the *Lhcb* and *Lhca* gene products. However, this remains a hypothesis, and the results in the present work cannot exclude the possibility of vacancy in the former A, V, or N positions and a placement of at least part of the Z pool in a different domain, e.g. the lipid bilayer of the *zea1* chloroplast thylakoids. Previous studies have shown that xanthophylls cycle Car 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 (Tardy and Havaux, 1996; Bassi and Caffarri, 2000). Relevant in this respect are the findings of Havaux and Niyogi (1999) and Havaux et al. (2000), who showed that the *npq1* mutant of Arabidopsis, which cannot perform a de-epoxidation of V to Z, is subject to enhanced lipid peroxidation upon photooxidative stress. It is also possible that Z, forming upon de-epoxidation of V during irradiance stress, occupies a thylakoid membrane domain that is different from that occupied by V (Jahns et al., 2001) under physiological conditions. The question of translocation of pigments and of different domain localization for V and Z, dynamically occurring as part of the reversible xanthophyll cycle, has not been thoroughly considered. In this respect, Z forming upon de-epoxidation of V at HL may have substantially different properties from Z that constitutively accumulates in the *zea1* strain under LL conditions. Clearly, more research is needed to delineate between these alternatives and to fully elucidate the role of the reversible xanthophyll cycle and the photoprotective role of Z in the thylakoid membrane of photosynthesis.

Why possess a xanthophyll cycle and not simply constitutively retain Z in the pigment bed of photosynthesis if it makes no apparent difference in the performance of the organism? Obviously, the reasons are not totally clear. It is possible that subtle quenching effects do occur under physiological conditions when Z is the only xanthophyll present, e.g. the *npq2 lor1* strain of *C. reinhardtii* (Polle et al., 2001). It could be argued that even subtle quenching effects would tend to give a slight but significant competitive advantage to organisms that possess the xanthophyll cycle because these would benefit from the photoprotection afforded by Z while minimizing the quenching upon Z epoxidation to V under physiological conditions. It is also possible that a constitutive expression of Z slows down specific steps of the PSII repair process, e.g. Figure 5, which might become limiting under certain conditions. Alternatively, one cannot exclude the possibility that, under conditions of irradiance stress combined with additional and fluctuating environmental stresses, an active xanthophyll cycle would confer advantage over a constitutive expression of Z (Niyogi et al., 1998).

In summary, Z accumulation and absence of β,β-epoxy xanthophylls in the *zea1* mutant of *D. salina* did not affect rates of photodamage or cell recovery from photo-inhibition. The acclimation of the photosynthetic apparatus to the level of irradiance was not affected by the constitutive accumulation of Z in the *zea1* strain either, as evidenced upon the irradiance-depended adjustment in the amount of the LHCII in WT and *zea1* thylakoids. However, results strongly support the notion that Z is a component of the PSII repair process. Z forms in situ upon photodamage and stays in association with the disassembled and photochemically inert PSII-core, until such time when the repair of the affected PSII center permits the return of individual units into the pool of functional PSII. In the WT strain, Z returns to V at the end of the PSII damage and repair cycle. This and other related possibilities on the functional role of Z in chloroplast thylakoids are currently under investigation.

MATERIALS AND METHODS

Algal Strains and Growth Conditions

The unicellular green alga *Dunaliella salina* Teod. strain 1644 was obtained from the UTEX culture collection (Starr, 1978). The *zea1* mutant strain of *D. salina* was isolated in this laboratory after chemical mutagenesis and screening (Jin et al., 2003). Strains were grown photoautotrophically in hypersaline medium (Pick et al., 1986) in the presence of 25 mM $NaHCO_3$ as a supplemental inorganic carbon source in 1-L Roux Bottles at a light intensity range of 100 to 2,000 μmol photons m^{-2} s^{-1}. Irradiance was measured with a model LI-185B radiometer (LI-COR, Lincoln, NE). The cultures were shaken to ensure uniform illumination of the cells. Cells were harvested at a density of 2 to 2.5 × 10^6 cells mL^{-1}. Cell density was monitored via a Neubauer ultraplane hemacytometer (Reichert, Buffalo, NY). To block translation of the chloroplast-encoded D1 protein, lincomycin, an inhibitor of plastidic protein biosynthesis, was added to the *D. salina* cultures as recently described (Baroli and Melis, 1996).

Pigment Analyses

For pigment determination, cells or thylakoid membranes were extracted in 80% (v/v) acetone, and debris was removed by centrifugation at 10,000*g* for 3 min. The absorbance of the supernatant was measured with a UV-160U spectrophotometer (Shimadzu, Columbia, MD). The Chl (*a* and *b*) concentration of the samples was determined according to Arnon (1949), with equations corrected as in Melis et al. (1987). HPLC analysis was done as recently described (Jin et al., 2001).

Thylakoid Membrane Isolation

Cells were harvested by centrifugation at 1,000*g* for 3 min at 4°C. Samples were diluted with sonication buffer containing 100 mM Tris-HCl (pH 6.8), 10 mM NaCl, 5 mM $MgCl_2$, 0.2% (w/v) polyvinylpyrrolidone 40, 0.2% (w/v) sodium ascorbate, 1 mM aminocaproic acid, 1 mM aminobenzamidine, and 100 μM phenylmethylsulfonylfluoride. Cells were broken by sonication in a Branson 200 Cell Disruptor (Branson Ultrasonics Corporation, Danbury, CT) 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 3,000*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 mM Tris-HCl (pH 6.8), 20% (w/v) glycerol, 7% (w/v) 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% (v/v), and the samples were stored at −80°C.

SDS-PAGE and Western-Blot Analysis

Samples were brought to room temperature before loading for electrophoresis and diluted accordingly to yield equal Chl concentrations. Gel lanes were loaded with an equal amount of Chl per lane. SDS-PAGE analysis was carried out according to Laemmli (1970). Gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R for protein visualization. Identification of thylakoid membrane proteins was accomplished with specific polyclonal antibodies raised in rabbit in this laboratory against the isolated reaction center D1 protein and the LHC-II apoproteins (Kim et al., 1993). Anti-Cbr antibody was kindly provided by Dr. Ada Zamir (Weizmann Institute of Science, Rehovot, Israel). Immunoreactive bands were detected either by enhanced chemiluminesence employing horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech, Piscataway, NJ) or by cross-reaction with the antibodies was detected by a chromogenic reaction with anti-IgG secondary antibodies conjugated with alkaline phosphatase (Bio-Rad, Hercules, CA). Immunoblots were scanned with an HP Scan Jet 5300C optical scanner (Hewlett-Packard, Palo Alto, CA) connected to a MacIntosh/G3 computer (Apple Computer, Cupertino, CA). The NIH Image version 1.6 program (National Institutes of Health, Bethesda, MD) was used for the deconvolution and quantitation of the bands.

Spectrophotometric Analyses

For spectrophotometric measurements, the thylakoid membrane pellet was resuspended in a buffer containing 50 mM Tricine (pH 7.8), 10 mM NaCl, and 5 mM $MgCl_2$. The amount of functional PSI and PSII reaction centers was estimated from the light-minus-dark absorbance difference measurements of P700 photooxidation and Q_A photoreduction, respectively (Melis, 1989).

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 cut-off filter (Corning, Corning, NY) in combination with an Ealing 35–5453 VIQ5–8 filter (Ealing, Inc., Rocklin, CA). An aliquot of 5 mL of cell suspension (2 μM 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 before the oxygen evolution measurements. The light saturation curve of photosynthesis was obtained 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 sequentially increasing irradiance levels. Registration and the rate (slope) of oxygen evolution at each light intensity step were 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.

Statistical Analyses

Results shown are the average of three to five independent experiments ± SE.

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