

# Greening under High Light or Cold Temperature Affects the Level of Xanthophyll-Cycle Pigments, Early Light-Inducible Proteins, and Light-Harvesting Polypeptides in Wild-Type Barley and the *Chlorina f2* Mutant<sup>1</sup>

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Etiolated seedlings of wild type and the *chlorina f2* mutant of barley (*Hordeum vulgare*) were exposed to greening at either 5°C or 20°C and continuous illumination varying from 50 to 800  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Exposure to either moderate temperature and high light or low temperature and moderate light inhibited chlorophyll *a* and *b* accumulation in the wild type and in the *f2* mutant. Continuous illumination under these greening conditions resulted in transient accumulations of zeaxanthin, concomitant transient decreases in violaxanthin, and fluctuations in the epoxidation state of the xanthophyll pool. Photoinhibition-induced xanthophyll-cycle activity was detectable after only 3 h of greening at 20°C and 250  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Immunoblot analyses of the accumulation of the 14-kD early light-inducible protein but not the major (Lhcb2) or minor (Lhcb5) light-harvesting polypeptides demonstrated transient kinetics similar to those observed for zeaxanthin accumulation during greening at either 5°C or 20°C for both the wild type and the *f2* mutant. Furthermore, greening of the *f2* mutant at either 5°C or 20°C indicated that Lhcb2 is not essential for the regulation of the xanthophyll cycle in barley. These results are consistent with the thesis that early light-inducible proteins may bind zeaxanthin as well as other xanthophylls and dissipate excess light energy to protect the developing photosynthetic apparatus from excess excitation. We discuss the role of energy balance and photosystem II excitation pressure in the regulation of the xanthophyll cycle during chloroplast biogenesis in wild-type barley and the *f2* mutant.

Exposure of plants to fluctuations in irradiance in excess of that required for photosynthesis generally induces xanthophyll-cycle activity characterized by the reversible, light-dependent de-epoxidation of violaxanthin to antheraxanthin and zeaxanthin. A strong correlation has been established between the nonphotochemical dissipation of excess light energy and zeaxanthin content, which protects PSII reaction centers from overexcitation (Demmig-Adams and Adams, 1992; Gilmore, 1997). Although the mechanism by which zeaxanthin is thought to dissipate excess energy

nonphotochemically is still under debate (Horton et al., 1996; Owens, 1996), there is a general consensus that the antenna systems of PSI and PSII are the primary sites of nonphotochemical energy dissipation. Xanthophyll-cycle pigments are associated with the major and minor Lhcb polypeptides of LHCI and the Lhca polypeptides of the PSI light-harvesting complex (Bassi et al., 1993; Ruban et al., 1994).

Kloppstech and coworkers (Meyer and Kloppstech, 1984; Grimm and Kloppstech, 1987) were the first to report that ELIPs are transiently expressed during greening of etiolated barley (*Hordeum vulgare*) seedlings and mature leaves exposed to high-light stress. Furthermore, ELIPs and the PSII-S protein, which are thylakoid polypeptides induced under high-light stress and related to the Lhcb family of light-harvesting polypeptides, may also bind carotenoids to protect the photochemical apparatus from potential photooxidative damage upon exposure to excess light (Król et al., 1995; Adamska, 1997; Lindahl et al., 1997). In addition to its traditional role as a quencher of absorbed light energy when bound to antenna polypeptides, it has been proposed that unbound zeaxanthin and other carotenoids may also act to stabilize thylakoid membranes against potential peroxidative damage and heat stress (Havaux, 1998).

Angiosperms produce etiolated seedlings when exposed to prolonged darkness (Leech, 1984). Chloroplast biogenesis and assembly of the photosynthetic apparatus has generally been examined in monocots by exposure of etiolated

Abbreviations: 5/50, low-temperature (5°C)/low-light (50  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) treatment; 5/250, low-temperature (5°C)/moderate-light (250  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) treatment; 20/250, moderate-temperature (20°C)/moderate-light (250  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) treatment; 20/800, moderate-temperature (20°C)/high-light (800  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) treatment; Chl, chlorophyll; ELIP, early light-inducible protein;  $F_m$ , maximum PSII fluorescence in the dark-adapted state;  $F_m'$ , maximum PSII fluorescence in the light-adapted state;  $F_v$ , variable PSII fluorescence in the dark-adapted state;  $F_v'$ , variable PSII fluorescence in the light-adapted state;  $F_v/F_m$ , maximum photochemical efficiency of PSII in the dark-adapted state;  $F_v'/F_m'$ , photochemical efficiency of PSII during steady-state illumination; LHCI, PSII light-harvesting complex; qN, nonphotochemical quenching parameter; qP, photochemical quenching parameter.

<sup>1</sup> This research was supported by the Natural Sciences and Engineering Research Council of Canada (grant to N.P.A.H.), by the Swedish Forestry and Agriculture Research Council (grant to S.J.), and by the Deutsche Forschungsgemeinschaft (grant to K.K.).

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seedlings to continuous or intermittent illumination (Akoyunoglou, 1984). Greening of dark-grown seedlings results in the conversion of etioplasts, which are characterized by the presence of prolamellar bodies to mature chloroplasts exhibiting typical granal stacks with intervening stromal thylakoids. The formation of thylakoid membranes during this greening process occurs with the sequential appearance of PSI, followed by PSII, intersystem electron-transport components, and finally the assembly of LHCII and the PSI light-harvesting complex. Maximum rates of CO<sub>2</sub> assimilation occur after the biogenesis of thylakoid membranes is complete (Baker, 1984). Although chloroplast biogenesis at low temperature in winter rye does not alter this sequence of assembly, PSI appearance occurs in parallel with Chl accumulation during thylakoid assembly at low temperature (5°C) (Król et al., 1987), whereas it normally occurs antiparallel to Chl accumulation during greening at moderate temperature (20°C) (Baker, 1984; Król et al., 1987). Furthermore, LCHII appears to be inserted initially in the monomeric form and is subsequently stabilized in its mature, oligomeric form (Król et al., 1988; Dreyfuss and Thornber, 1994).

Recently, we reported that photosynthetic acclimation to either low temperature or high light in wheat and rye can be explained as a response to PSII excitation pressure (Gray et al., 1996; Huner et al., 1998). Plants grown at either 5/250 or 20/800 were photosynthetically adjusted to high PSII excitation pressure (measured as  $1 - qP$ , the relative reduction state of PSII). In contrast, plants grown at either 5/250 or 20/250 were acclimated to growth at low PSII excitation pressure (Huner et al., 1998). Although there were no significant differences in pigment composition or Lhcb content, plants grown under high PSII excitation pressure exhibited greater tolerance for photoinhibition than plants grown under low PSII excitation pressure. This appears to be a consequence of increased photosynthetic capacity and increased  $qN$  induced upon growth at high PSII excitation pressure (Gray et al., 1997). However, the increased  $qN$  under steady-state growth conditions could not be explained on the basis of zeaxanthin/antheraxanthin accumulation in wheat and rye (Hurry et al., 1992; Gray et al., 1996). Recently, Streb et al. (1998) reported similar conclusions for the alpine species *Ranunculus glacialis* acclimated to low temperature.

Similar conclusions regarding the role of PSII excitation pressure have been reported for thermal and light acclimation in *Chlorella vulgaris*, *Dunaliella salina* (Maxwell et al., 1995a, 1995b), and *Laminaria saccharina* (Machalek et al., 1996), as well as for the regulation of ELIP expression in barley (Montane et al., 1997). However, unlike in cereals, the increased tolerance of photoinhibition observed in *C. vulgaris* and *D. salina* grown at high PSII excitation pressure appeared to be caused by an increase in the capacity for zeaxanthin-induced  $qN$  combined with a decrease in light-harvesting capacity (Maxwell et al., 1995a, 1995b). Although the Cyt  $b_6/f$  complex has been implicated as the possible thylakoid redox sensor (Escoubas et al., 1995), the precise sensing/signaling mechanism remains to be elucidated.

The photosynthetic apparatus should be most susceptible to excessive irradiance during the biosynthesis and assembly of the photochemical apparatus. We hypothesized that greening under conditions of continuous, excessive irradiance created by exposure to either low temperature or high light may induce specific mechanisms to protect the photochemical apparatus during the early stages of chloroplast biogenesis. Therefore, as an initial approach to this problem, we examined the kinetics of xanthophyll-cycle pigment accumulation in relation to the accumulation of Lhcb and ELIPs during greening of wild-type barley and the *chlorina f2* mutant under conditions of either potentially excessive continuous excitation (5/250 and 20/800) or low to moderate continuous excitation (5/50 and 20/250).

## MATERIALS AND METHODS

### Plant Material

Wild-type barley (*Hordeum vulgare* L.) and the *chlorina f2* mutant were germinated and grown in the dark for 6 d at 20°C and for 21 d at 5°C. After this time, both populations of etiolated seedlings were approximately 8 cm in height. Etiolated seedlings were transferred to controlled-growth chambers and allowed to green for various times in continuous light at 20/250, 20/800, 5/50, or 5/250.

### Thylakoid Preparation and SDS-PAGE

Thylakoid membranes from the mid-portion of primary leaves of wild-type barley and the *f2* mutant were isolated according to the method of Harrison and Melis (1992) at different stages of development. Benzamidine and aminocaproic acid were present in the homogenization buffer at concentrations of 2 mM. SDS-PAGE was prepared according to the method of Laemmli (1970) using 12% (w/v) polyacrylamide and 6 M urea in the separating gel. Etioplast membranes and chloroplast thylakoids were solubilized with SDS (SDS:protein, 5:1). Protein concentration was determined using the Bio-Rad protein-assay kit, and 7 µg of protein was loaded per lane.

### Immunoblotting

Immunoblotting was performed by transferring the proteins from SDS-PAGE to the nitrocellulose membrane (Bio-Rad) according to the method of Towbin (1979) using a Mini Trans Blot cell (Bio-Rad). The proteins were then probed with monoclonal antibodies raised against Lhcb2 and Lhcb5 (1:500 dilution) (Jansson, 1994) and ELIPs (1:1000 dilution) (Potter and Kloppstech, 1993). Polypeptides were visualized using the ECL detection kit (Amersham) as prescribed using a peroxidase-linked anti-rabbit secondary antibody (Sigma). The relative contents of the 14-kD ELIP were estimated using an imaging program (Photoshop 5.0, Adobe Systems, Mountain View, CA). Because we were unable to estimate the absolute levels of the 14-kD ELIP from our immunoblots, the intensity of the ELIP signal was normalized to the averaged intensity of the background

signal for each immunoblot. This allowed comparisons within individual blots only.

### Pigment Analysis

Pigments from barley leaves were extracted with 100% acetone at 4°C under a green safelight. After centrifugation at 10,000g for 10 min at 4°C, the supernatant was filtered through a 0.22- $\mu\text{m}$  syringe filter and samples were stored at -80°C until analysis. Pigments were separated and quantified by HPLC according to the method of Gilmore and Yamamoto (1991) with minor modifications (Gray et al., 1996). The system consisted of a programmable solvent module (System Gold 126, Beckman), a diode detector (module 168, Beckman), and a reverse-phase column (5- $\mu\text{m}$  particle size; 25  $\times$  0.46 cm i.d.; CSC-Spherisorb ODS-1, Beckman) with a guard column (Upchurch Perisorb A, Chromatographic Specialties, Concord, Ontario, Canada). Samples were injected using a sample-injection valve (model 210A, Beckman) with a 20- $\mu\text{L}$  sample loop.

Pigments were eluted isocratically for 6 min with a solvent system consisting of acetonitrile:methanol:Tris (0.1 M) (72:8:3.5, v/v) at pH 8.0, followed by a 2-min linear gradient to 100% methanol:hexane (75:25, v/v), which continued isocratically for 4 min. Total run time was 12 min, and the flow rate was 2  $\text{cm}^3 \text{min}^{-1}$ .  $A_{440}$  was detected and peak areas were integrated by Beckman System Gold software. The retention times and response factors of Chl *a*, Chl *b*, lutein, and  $\beta$ -carotene were determined by injection of known amounts of pure standards purchased from Sigma. The retention times of zeaxanthin, antheraxanthin, violaxanthin, and neoxanthin were determined using pigments purified by TLC (Gray et al., 1996). Epoxidation states were calculated as  $V + 0.5A/V + A + Z$ , where V indicates violaxanthin, A indicates antheraxanthin, and Z indicates zeaxanthin.

### Modulated Chl Fluorescence

Chl *a* fluorescence of dark-adapted (30 min) wild-type and *f2* barley leaves was measured under ambient  $\text{CO}_2$  conditions using a modulated Chl-fluorescence measuring system (PAM 101, Heinz Walz, Effeltrich, Germany) (Schreiber et al., 1986). Minimum PSII fluorescence in the dark-adapted state was excited by a nonactinic, modulated measuring beam ( $0.12 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) at 1.6 kHz.  $F_m$  was induced by saturating white-light pulses (800 ms,  $2800 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) provided by a lamp (KL 1500, Schott Glaswerke, Mainz, Germany) and controlled from a trigger control unit (PAM 103, Heinz Walz). The actinic light corresponded to the growth irradiance of 50, 250, or 800  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . All measurements were performed at the corresponding growth temperature of either 5°C or 20°C. The qP and qN parameters were corrected for quenching of minimum PSII fluorescence in the dark-adapted state, as described previously (Gray et al., 1996). PSII excitation pressure, i.e. the relative reduction state of PSII, was estimated as  $1 - \text{qP}$  and was measured at the growth temperature and irradiance. All fluorescence parameters obtained from

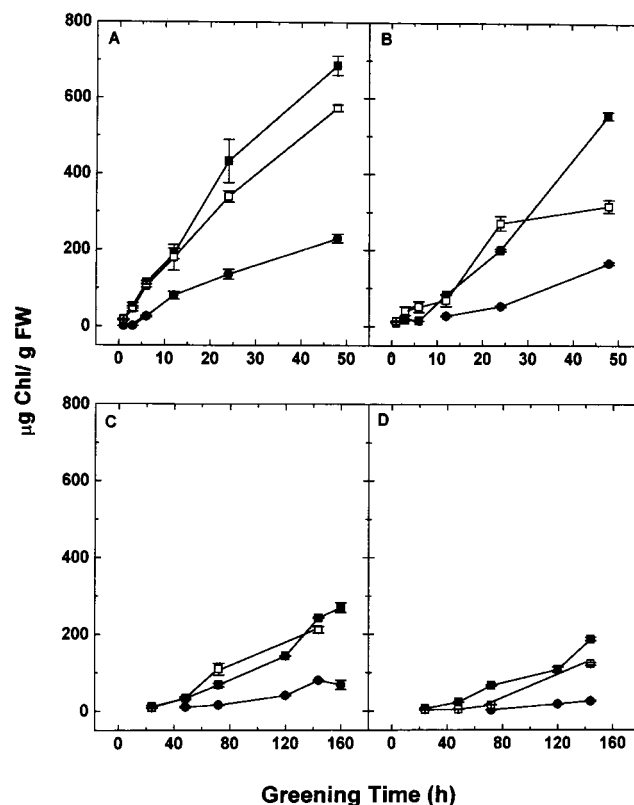
leaves exposed to actinic light were calculated after steady-state photosynthesis had been attained.

## RESULTS

### Effects of Light and Temperature on Chl *a* and *b* Accumulation

Chl accumulation is an indicator of chloroplast development in angiosperms. Both wild-type and *f2* etiolated seedlings exhibited similar kinetics for Chl *a* accumulation during greening at 20/250 (Fig. 1A), with a lag time of 1 h or less. In contrast to Chl *a*, Chl *b* accumulated with a lag time of about 6 h, with no Chl *b* accumulation detected in the *f2* mutant. However, an increase in the irradiance during greening at 20/800 resulted in an increased lag time for both Chl *a* and Chl *b* accumulation (Fig. 1B). During greening of the wild type at 20/250 and 20/800, the Chl *a/b* ratio decreased from about 20 to a final value of 3.8 and 4.3, respectively, when greening was complete (Table I).

As expected, when the irradiance was maintained at 250  $\mu\text{mol m}^{-2} \text{s}^{-1}$  but the temperature was decreased from 20°C to 5°C (5/250) during greening (Fig. 1D), Chl *a* and Chl *b* accumulation exhibited an extended lag time of about 40 to 60 h and a reduced rate compared with greening at



**Figure 1.** Effects of growth regime on the kinetics of Chl accumulation during greening of etiolated wild-type barley and the *f2* mutant. A, 20/250; B, 20/800; C, 5/50; D, 5/250. All data are means  $\pm$  SD of three to five replicate plants in one experiment. The experiment was repeated at least once. ■, Wild-type Chl *a*; ●, wild-type Chl *b*; □, *f2* Chl *a*. FW, Fresh weight.

**Table I.** Pigment content of wild-type barley after development under various growth regimes

Pigments were separated and quantified by HPLC, as described in "Materials and Methods." The results are presented as means  $\pm$  SD of three replicate measurements from three different plants. Numbers in parentheses represent the percentages of the total xanthophyll-pool size (V+A+Z).

Pigment	20/250	20/800	5/50	5/250
	$\mu\text{g g}^{-1}$ fresh wt			
Chl <i>a/b</i>	3.84 $\pm$ 0.22	4.30 $\pm$ 0.64	3.15 $\pm$ 0.25	3.27 $\pm$ 0.12
Neoxanthin	19.5 $\pm$ 2.3	12.2 $\pm$ 1.4	21.3 $\pm$ 1.8	24.7 $\pm$ 1.3
Lutein	128 $\pm$ 3	109 $\pm$ 9	119 $\pm$ 8	191 $\pm$ 9
$\beta$ -Carotene	122 $\pm$ 2	76 $\pm$ 4	92 $\pm$ 3	86 $\pm$ 2
Violaxanthin	61 $\pm$ 2 (97 $\pm$ 1)	37 $\pm$ 3 (46 $\pm$ 3)	56 $\pm$ 3 (100 $\pm$ 3)	112 $\pm$ 2 (91 $\pm$ 1)
Antheraxanthin	nd <sup>a</sup> (nd)	nd (nd)	nd (nd)	5.6 $\pm$ 1 (4.5 $\pm$ 0.2)
Zeaxanthin	3.1 $\pm$ 0.1 (5 $\pm$ 0.1)	44 $\pm$ 5 (54 $\pm$ 5)	nd (nd)	6 $\pm$ 2 (5 $\pm$ 2)
V+A+Z	64 $\pm$ 2	81 $\pm$ 2	56 $\pm$ 3	123 $\pm$ 3

<sup>a</sup> nd, Not determined.

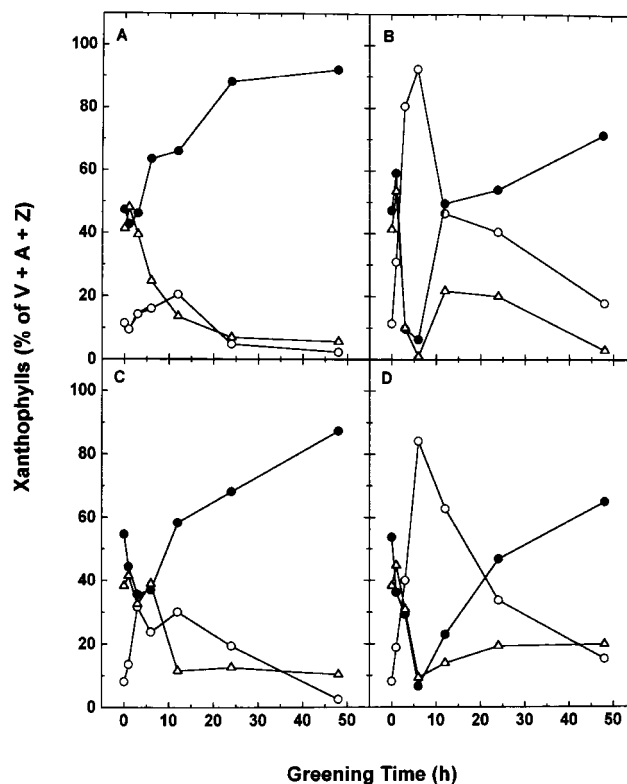
20/250 (Fig. 1A). However, a reduction in the irradiance during greening at low temperature (5/50) (Fig. 1C) caused an increase in the rates of Chl *a* and *b* accumulation in both the wild type and the *f2* mutant compared with greening at 5/250. Greening of the wild type at either 5/250 or 5/50 resulted in a final Chl *a/b* ratio of about 3.2 after greening was complete (Table I).

#### Effects of Light and Temperature on the Accumulation of Xanthophyll-Cycle Pigments in the Wild Type and the *f2* Mutant

The pattern of accumulation of each xanthophyll-cycle pigment during chloroplast biogenesis at 20°C was examined by calculating the content of violaxanthin, antheraxanthin, and zeaxanthin as a percentage of the total xanthophyll-cycle pool size (V+A+Z) and plotted as a function of greening time (Fig. 2). The relative content of violaxanthin increased in the wild type as a function of greening time at 20/250, from about 45% in etiolated leaves to about 95% after 48 h of greening. This was associated with a concomitant decrease in the proportion of antheraxanthin, from about 40% in etiolated wild-type barley leaves to less than 10% after 48 h of greening (Fig. 2A). Although the proportion of zeaxanthin was less than 20% throughout the greening period (Fig. 2A), a transient accumulation was observed after 12 h of greening. When greening was complete at 20/250, zeaxanthin represented only about 5% of the total xanthophyll pool (V+A+Z). Analyses of washed thylakoid membranes isolated at various stages during greening at 20/250 indicated that about 85% of the xanthophyll pool of total leaf extracts was accounted for in the thylakoid membrane fraction (data not shown).

In contrast to greening of the wild type at 20/250, greening of etiolated wild-type barley at 20/800 (Fig. 2B) resulted in a rapid but transient decrease in the relative content of violaxanthin, from about 46% to about 5%, with a minimum occurring after about 3 h of greening. This was followed by a subsequent recovery in the proportion of violaxanthin after 48 h of greening, accompanied by a rapid decrease in antheraxanthin, which subsequently remained at less than 5% after 48 h at 20/800. The transient decrease in violaxanthin was mirrored by a rapid and transient

increase in the relative content of zeaxanthin, from about 10% to about 95%, with a maximum accumulation after 3 to 6 h of greening at 20/800 followed by a decrease to 17% after 48 h of greening (Fig. 2B). When greening was complete at 20/800, the xanthophyll pool size was about 27% greater than that observed after greening at 20/250 (Table I). Furthermore, zeaxanthin represented 54% of the total



**Figure 2.** Dynamics of the accumulation of xanthophyll-cycle intermediates during greening of etiolated wild-type barley and the *f2* mutant. A, Wild type at 20/250; B, wild type at 20/800; C, *f2* at 20/250; D, *f2* at 20/800. ●, Violaxanthin; △, antheraxanthin; ○, zeaxanthin. All data are expressed as a percentages of the total xanthophyll pool (V+A+Z) and are the averages of three to five replicate plants. For clarity of presentation, the error bars were omitted. The errors averaged less than 10%.

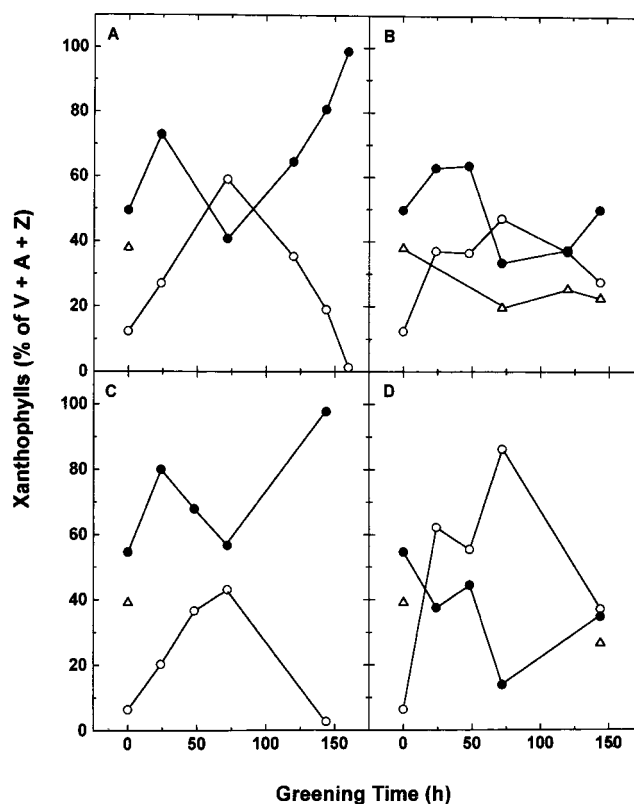


xanthophyll pool, which was greater than that observed after completion of greening of the wild type at 20/250 (Table I). Similar transients were observed for the accumulation of violaxanthin and zeaxanthin during the greening of the *f2* mutant under either 20/250 or 20/800 (Fig. 2, C and D; Table II).

When etiolated wild-type barley was exposed to greening at 5/250 (Fig. 3B), the relative violaxanthin content exhibited a transient minimum of about 40% after 72 h of greening. This was accompanied by a concomitant transient maximum of about 60% in the relative zeaxanthin content after 72 h of greening, with minimal changes in the relative content of antheraxanthin. Even though the final relative zeaxanthin contents (5%) were similar in wild-type plants exposed to greening at either 5/250 or 20/250, the final absolute level of zeaxanthin in wild-type plants exposed to 5/250 was almost double that observed in wild-type plants exposed to 20/250 (Table I). Similar trends with respect to transient fluctuations in the relative contents and absolute levels of violaxanthin and zeaxanthin estimated on a per gram fresh weight basis were observed during greening of wild-type barley at 5/50 (Fig. 3A). After greening was complete, the xanthophyll-pool size in wild-type plants exposed to 5/50 was about one-half that observed for wild-type plants exposed to 5/250 (Table I). Greening of the *f2* mutant under conditions of either 5/250 or 5/50 exhibited similar transients in violaxanthin and zeaxanthin accumulation (Fig. 3, C and D; Table II).

### Effects of Greening on the Epoxidation State of the Xanthophyll Pool

The epoxidation state of the xanthophyll pool is thought to be sensitive primarily to fluctuations in irradiance (Demmig-Adams and Adams, 1992). Figure 4 illustrates that, even during greening under continuous illumination at either 20/250 or 20/800, both the wild type and the *f2* mutant exhibited transient fluctuations in the epoxidation state. However, the extent of these fluctuations was dependent on the irradiance experienced during greening at 20°C, with the minimum epoxidation state occurring after 6 h of greening at 800  $\mu\text{mol m}^{-2} \text{s}^{-1}$  in both the wild type and the *f2* mutant. Similar trends in transient fluctuations



**Figure 3.** Dynamics of the accumulation of xanthophyll-cycle intermediates during greening of etiolated wild-type barley and the *f2* mutant. A, Wild type at 5/50; B, wild type at 5/250; C, *f2* at 5/50; D, *f2* at 5/250. ●, Violaxanthin; △, antheraxanthin; ○, zeaxanthin. All data are expressed as a percentages of the total xanthophyll pool (V+A+Z) and are the averages of three to five replicate plants. For clarity of presentation, the error bars were omitted. The errors averaged less than 10%.

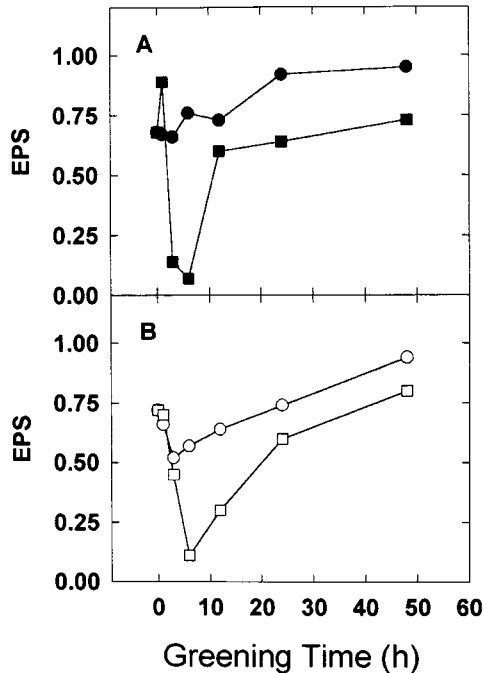
of the epoxidation state were observed during greening of both the wild type and the *f2* mutant at 5°C, except that the minimum epoxidation state during greening at either 5/50 (0.57) or 5/250 (0.14) occurred after 72 h (data not shown). Furthermore, the extent of the transient fluctuation in the epoxidation state was greatest during greening at 5/250

**Table II.** Pigment content of the *f2* mutant after development under various growth regimes

Pigments were separated and quantified by HPLC, as described in "Materials and Methods." The results are presented as means  $\pm$  SD of three replicate measurements from three different plants. Numbers in parentheses represent the percentages of the total xanthophyll-pool size (V+A+Z).

Pigment	20/250	20/800	5/50	5/250
	$\mu\text{g g}^{-1}$ fresh wt			
Chl <i>a/b</i>	>20	>20	>20	>20
Neoxanthin	3.9 $\pm$ 0.5	4.4 $\pm$ 0.4	nd <sup>a</sup>	3.5 $\pm$ 0.5
Lutein	59 $\pm$ 3	64 $\pm$ 5	38 $\pm$ 3	89 $\pm$ 3
$\beta$ -Carotene	77 $\pm$ 4	70 $\pm$ 3	44 $\pm$ 2	44 $\pm$ 2
Violaxanthin	40 $\pm$ 2 (100 $\pm$ 2)	28 $\pm$ 2 (53 $\pm$ 2)	25 $\pm$ 2 (100 $\pm$ 2)	68 $\pm$ 4 (90 $\pm$ 1)
Antheraxanthin	nd (nd)	7.9 $\pm$ 1.0 (15 $\pm$ 1)	nd (nd)	6.1 $\pm$ 0.4 (5 $\pm$ 1)
Zeaxanthin	nd (nd)	16.8 $\pm$ 1.5 (32 $\pm$ 2)	nd (nd)	4.5 $\pm$ 0.3 (5 $\pm$ 1)
V+A+Z	40 $\pm$ 2	53 $\pm$ 2	25 $\pm$ 2	75 $\pm$ 4

<sup>a</sup> nd, Not determined.



**Figure 4.** Fluctuations in epoxidation state (EPS) during greening of wild-type barley and the *f2* mutant. A, Wild-type barley at 20/250 (●) and 20/800 (■). B, *f2* mutant at 20/250 (○) and 20/800 (□). Data were calculated from data in Figure 2.

compared with greening at 5/50 (data not shown) in both the wild type and the *f2* mutant.

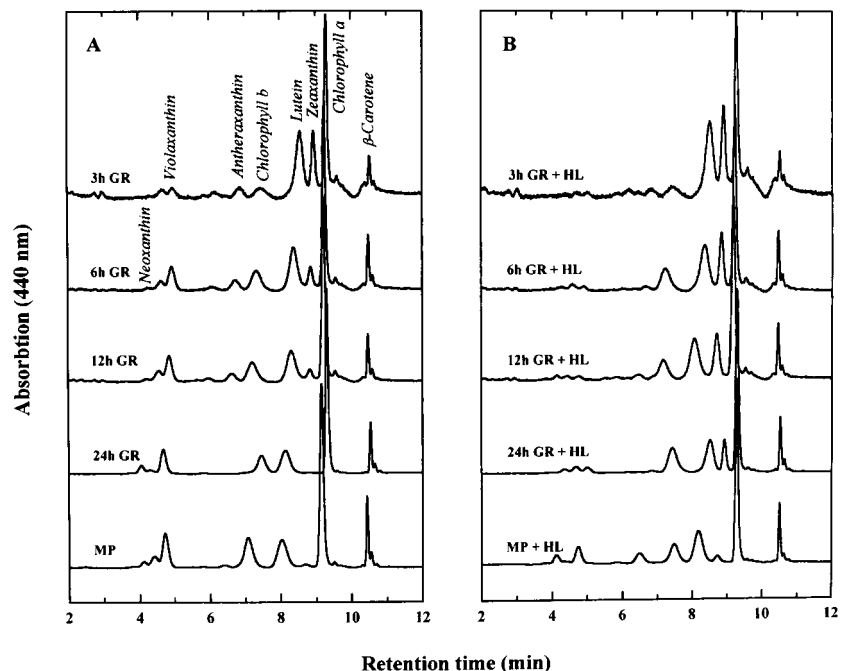
Because we examined these fluctuations in the epoxidation state as a function of greening in the wild type and the *f2* mutant, changes in the epoxidation state could be attributable to either de novo synthesis of zeaxanthin or the

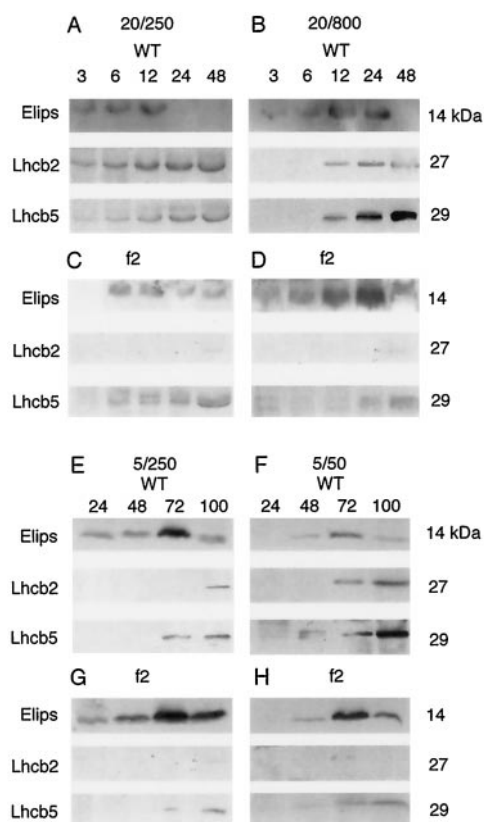
concomitant conversion of violaxanthin to zeaxanthin through the xanthophyll cycle. To determine if the xanthophyll cycle was operative during greening, wild-type barley was shifted to photoinhibitory conditions at various times during greening (Fig. 5). Figure 5A shows typical HPLC results of pigments extracted and separated from wild-type barley leaves at various stages during greening at 20/250. Comparison of the chromatograms in Figure 5A with the respective chromatograms obtained from wild-type leaves after photoinhibition (Fig. 5B) indicates that even after only 3 h of greening, exposure to photoinhibition resulted in an increase in the zeaxanthin peak and a concomitant decrease in the violaxanthin peak. Thus, the capacity to convert violaxanthin to zeaxanthin was expressed early during the greening process. This is consistent with the recent results of Farber and Jahns (1998).

#### Effects of Light and Temperature on the Accumulation of ELIPs

Chloroplast development at 20/250 induced a transient accumulation of a 14-kD ELIP polypeptide in the wild type and the *f2* mutant (Fig. 6, A and C). Maximum levels of this ELIP polypeptide were observed after 6 to 12 h of greening, but after 24 h of illumination at 20/250, the 14-kD ELIP was not detectable in the wild type (Fig. 6A). In contrast, the 14-kD ELIP still was detectable after 24 and 48 h of greening in the *f2* mutant, albeit at lower levels than after 6 and 12 h of greening (Fig. 6C). Similar trends for the transient accumulation of the 14-kD ELIP were observed during greening at 20/800, except that the levels of this ELIP appeared to be generally higher than during greening at 20/250 and the maximum time for accumulation was shifted to between 12 and 24 h of greening (Fig. 6, B and D).

**Figure 5.** Chromatograms of HPLC pigment separations illustrating the effects of exposure to low-temperature photoinhibition on the conversion of violaxanthin to zeaxanthin at various times during greening (GR) of etiolated wild-type barley. Photoinhibition conditions consisted of exposure to  $1600 \mu\text{mol m}^{-2} \text{s}^{-1}$  at  $5^\circ\text{C}$  (high light [HL]) for 2 h. A, Wild-type barley controls before exposure to photoinhibition exposed to greening at 20/250. B, Wild-type barley after exposure to photoinhibition. MP, Mature wild-type barley plants after greening had been completed at 20/250. The data are the results of a single experiment.





**Figure 6.** The effects of greening on the accumulation of ELIPs, Lhcb2, and Lhcb5. Thylakoids were isolated at various times (3, 6, 12, 24, and 48 h) from wild-type (WT) barley and the *f2* mutant exposed to greening at either 20/250 (A and C) or 20/800 (B and D). Similarly, thylakoids were isolated at various times (24, 48, 72, and 100 h) from wild-type barley and the *f2* mutant exposed to greening at either 5/250 (E and G) or 5/50 (F and H). Immunoblots from SDS-PAGE were probed with polyclonal antibodies raised against ELIP (14 kD), Lhcb2 (27 kD), and Lhcb5 (29 kD).

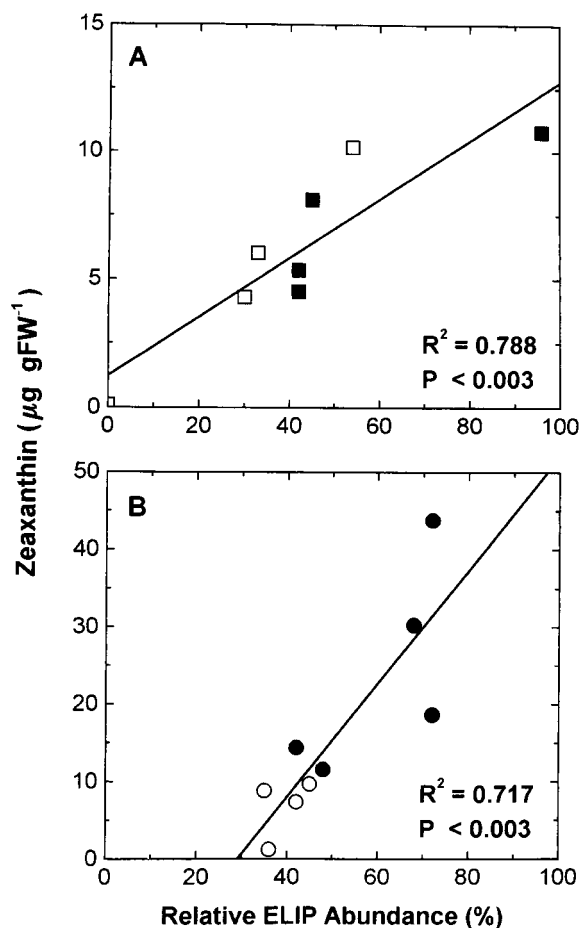
Exposure of etiolated leaves of wild-type barley and the *f2* mutant to 5/250 also induced a transient accumulation of the 14-kD ELIP, with maximum accumulation occurring after about 72 h (Fig. 6, E and G). In addition, the maximum levels of this ELIP were higher during greening at 5/250 than at 20/250 in both the wild type and the *f2* mutant (Fig. 6). Furthermore, exposure to greening under 5/50 again resulted in a transient accumulation of the 14-kD ELIP, with maximum accumulation occurring after 72 h of greening (Fig. 6, F and H). However, the maximum level of this polypeptide was lower during greening at 5/50 than during greening at 5/250 in both the wild type and the *f2* mutant (Fig. 6).

Although the 14-kD ELIP and zeaxanthin exhibited similar transient kinetics for accumulation during greening of wild-type barley and the *f2* mutant, we examined the correlation between levels of the 14-kD ELIP and zeaxanthin. As illustrated in Figure 7, there was a good correlation between zeaxanthin and the relative abundance of the 14-kD ELIP for wild-type barley grown at 5°C (Fig. 7A) and the *f2* mutant grown at 20°C (Fig. 7B). However, these apparent correlations between zeaxanthin and ELIP accu-

mulation were less robust for the wild-type plant grown at 20°C and the *f2* mutant grown at 5°C (data not shown).

### Effects of Light and Temperature on the Accumulation of Lhcb2 and Lhcb5

As illustrated in Figure 6A, the major light-harvesting polypeptide of LHCII, Lhcb2, and the minor light-harvesting polypeptide, Lhcb5, were detected after about 3 h of greening of the wild type at 20/250. However, unlike the 14-kD ELIP, the accumulation of Lhcb2 and Lhcb5 did not exhibit transient kinetics but, rather, increased gradually as a function of time (Fig. 6A). This was accompanied by a decrease in the Chl *a/b* ratio from an initial value of about 20 to a value of about 4 after 48 h (data not shown). As expected, the Lhcb2 polypeptide was not detected during greening in the *f2* mutant, but the level of Lhcb5 increased gradually in the *f2* mutant as a function of greening time at 20/250 (Fig. 6C). Similar trends were observed for the wild type (Fig. 6B), in which greening occurred at 20/800. However, the level of Lhcb2 was lower and that of Lhcb5 was higher during greening of the wild type at



**Figure 7.** Correlation between zeaxanthin and relative ELIP abundance. A, Wild-type barley at 5/50 (□) and 5/250 (■). B, *f2* mutant at 20/250 (○) and 20/800 (●). ELIP abundance was estimated from immunoblots using a specific probe for the 14-kD ELIP, as described in "Materials and Methods." FW, Fresh weight.

20/800 compared with 20/250. Furthermore, greening at high light caused a delay in the appearance of the Lhcb2 and Lhcb5 polypeptides (Fig. 6B). Greening under high light also caused a delay in the maximum accumulation of Lhcb5 in the *f2* mutant (Fig. 6D).

When etiolated primary leaves of wild-type barley were illuminated at 5/250, the appearance of Lhcb2 was delayed until 100 h of greening, whereas the appearance of Lhcb5 was delayed to about 72 h (Fig. 6E). In contrast, during greening of the wild type at 5/50, Lhcb2 was detected after 72 h, whereas Lhcb5 was detectable after 24 to 48 h (Fig. 6F). Although Lhcb2 was not detected in the *f2* mutant, trends similar to those observed for greening of the wild type at 5/250 and 5/50 were also observed for the detection of Lhcb5 during greening of the *f2* mutant at either 5/250 or 5/50 (Fig. 6, G and H).

### Effects of Light and Temperature on the Chl *a* Fluorescence Characteristics of the Wild Type and the *f2* Mutant

Table III shows that growth regime had a minimal effect on the  $F_v/F_m$  of the wild type. However, the wild type developed at 5/50 did exhibit an  $F_v/F_m$  ratio that was about 7% lower than that of the wild type grown at 20/250. In contrast, increasing growth irradiance at 20°C resulted in a small (5%) increase in  $F_v/F_m$  (Table III). Furthermore, the *f2* mutant developed at 5°C exhibited an approximately 13% lower  $F_v/F_m$  than the *f2* mutant developed at 20/250 (Table III). As expected, the  $F_v'/F_m'$  decreased as a function of increasing growth irradiance at both 20°C and 5°C (Table III). Although the  $F_v'/F_m'$  of the *f2* mutant grown at 5°C exhibited comparable sensitivity to irradiance and temperature as the wild type, the  $F_v'/F_m'$  of the *f2* mutant was less sensitive to irradiance at 20°C than the  $F_v'/F_m'$  of the wild type (Table III).

As expected, the proportion of closed PSII reaction centers, estimated as  $1 - qP$ , increased as a function of irradiance at 20°C, which was correlated with a concomitant increase in  $qN$  (Table III). However, the apparent effects of high light on both  $1 - qP$  and  $qN$  were mimicked by exposing the wild type to 5/250. Similar trends for  $1 - qP$

and  $qN$  were observed for the *f2* mutant developed at 20°C and various growth irradiances or after development at 5/250 (Table III). As a consequence, the extent of  $qN$  induced under the various developmental conditions in the wild type and the *f2* mutant exhibited a positive, linear correlation with the proportion of PSII reaction closure ( $1 - qP$ ) experienced in these leaves (Fig. 8A). Although the data from Tables I, II, and III indicate no apparent correlation between the levels of zeaxanthin, antheraxanthin, or zeaxanthin plus antheraxanthin and the extent of  $qN$ , the results in Figure 8B indicate a positive correlation between the total xanthophyll-pool size and the extent of  $qN$  developed in both the wild type and the *f2* mutant.

### DISCUSSION

Zeaxanthin and antheraxanthin, which are formed from the light-dependent conversion of violaxanthin, have been implicated in the down-regulation of PSII photochemistry and the subsequent dissipation of excess light as heat (Demmig-Adams and Adams, 1992; Frank et al., 1994; Owens, 1996; Gilmore, 1997). It has been assumed that stimulation of the xanthophyll cycle is a consequence of sudden exposures to high-light stress in mature, fully developed plants (Demmig-Adams and Adams, 1992). Furthermore, major and minor components of LHCII have been implicated in the regulation of the xanthophyll cycle (Gruszecki and Krupa, 1993; Król et al., 1995; Heyde and Jahns, 1998).

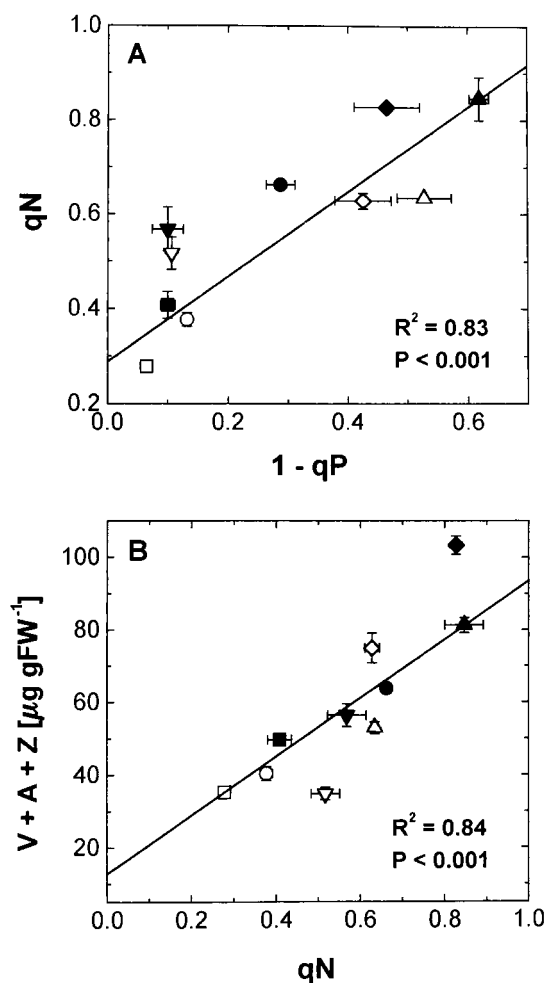
We have shown that greening of the wild type and the *f2* mutant of barley under continuous illumination at either 20°C or 5°C resulted in transient accumulation of zeaxanthin, accompanied by concomitant transient decreases in the levels of violaxanthin during continuous illumination (Figs. 2 and 3), which is consistent with the results of Farber and Jahns (1998). However, these decreases were both light and temperature dependent. Furthermore, the transients in xanthophyll-cycle pigments were associated with the transient accumulation of the 14-kD ELIP (Fig. 6). These patterns of accumulation for zeaxanthin and the 14-kD ELIP are consistent with the notion that ELIPs may be zeaxanthin-binding proteins (Król et al., 1995; Adamska,

**Table III.** Steady-state Chl fluorescence characteristics of wild-type barley and the *f2* mutant after development under various growth regimes

$qP$  and  $qN$  were calculated as described in "Materials and Methods." All measurements were performed at the growth temperature and irradiance. Data are the means  $\pm$  SD of three replicate measurements from three different plants.

Fluorescence Parameter	20/50	20/250	20/800	5/50	5/250
Wild Type					
$F_v/F_m$	0.804 $\pm$ 0.003	0.780 $\pm$ 0.002	0.782 $\pm$ 0.009	0.729 $\pm$ 0.005	0.778 $\pm$ 0.003
$F_v'/F_m'$	0.705 $\pm$ 0.011	0.603 $\pm$ 0.015	0.427 $\pm$ 0.012	0.544 $\pm$ 0.025	0.436 $\pm$ 0.007
$1 - qP$	0.100 $\pm$ 0.012	0.287 $\pm$ 0.024	0.619 $\pm$ 0.016	0.100 $\pm$ 0.026	0.465 $\pm$ 0.055
$qN$	0.408 $\pm$ 0.028	0.662 $\pm$ 0.004	0.846 $\pm$ 0.004	0.568 $\pm$ 0.046	0.827 $\pm$ 0.004
<i>f2</i>					
$F_v/F_m$	0.759 $\pm$ 0.002	0.775 $\pm$ 0.006	0.795 $\pm$ 0.009	0.692 $\pm$ 0.009	0.681 $\pm$ 0.004
$F_v'/F_m'$	0.694 $\pm$ 0.001	0.697 $\pm$ 0.009	0.611 $\pm$ 0.002	0.560 $\pm$ 0.025	0.456 $\pm$ 0.010
$1 - qP$	0.065 $\pm$ 0.003	0.132 $\pm$ 0.003	0.527 $\pm$ 0.045	0.106 $\pm$ 0.002	0.425 $\pm$ 0.047
$qN$	0.278 $\pm$ 0.008	0.377 $\pm$ 0.014	0.634 $\pm$ 0.009	0.517 $\pm$ 0.034	0.628 $\pm$ 0.017





**Figure 8.** Correlation between  $qN$  and  $1 - qP$  (A) and  $qN$  and the total xanthophyll-pool size ( $V+A+Z$ ) (B) in wild-type barley (closed symbols) and the  $f2$  mutant (open symbols) developed under various growth regimes. ■, □, 20/50; ●, ○, 20/250; ▲, △, 20/800; ▼, ▽, 5/50; ◆, ◇, 5/250. All values of  $qN$  and  $1 - qP$  were obtained under growth conditions and represent means  $\pm$  SE from three independent experiments. FW, Fresh weight.

1997) that protect the developing photosynthetic apparatus from overexcitation (Adamska, 1997; Lindahl et al., 1997). Although the kinetics of zeaxanthin and ELIP accumulation are qualitatively similar, zeaxanthin and ELIP do not appear to be quantitatively correlated under all of the conditions examined in this study. This appears to be attributable in part to the large variations in the absolute levels of zeaxanthin that we observed during greening of both the wild type and the  $f2$  mutant under the various conditions examined. Because Lhcb2 is not present in the  $f2$  mutant of barley, we conclude that this LHCII polypeptide is not essential for the regulation of the xanthophyll cycle in barley.

A comparison of the kinetics for the accumulation of Chl  $a$  and  $b$  in the wild type and Chl  $a$  in the  $f2$  mutant indicated that the initial lag in Chl accumulation was sensitive to both temperature and light (Fig. 1). Greening under either high light (20/800) or low temperature (5/250, 5/50)

caused an increase in the lag time before maximum Chl  $a$  and Chl  $b$  accumulation relative to greening in controls (20/250). Furthermore, a decrease in irradiance during greening at low temperature (5/50) resulted in a doubling in the rate of Chl accumulation in both the wild type and the  $f2$  mutant. These results indicate that, although light is required for Chl synthesis in angiosperms, it can also inhibit Chl accumulation and the development of the photochemical apparatus. More importantly, the optimal irradiance for greening appears to be temperature dependent, indicating an important interaction between light and temperature during chloroplast biogenesis.

Assuming that the transient fluctuations in the epoxidation state during greening (Fig. 4) were primarily caused by the modulation of the xanthophyll cycle, the signal that induced the observed fluctuation in the epoxidation state could not be attributable to either light or temperature per se. We suggest that the fluctuations in the epoxidation state and ELIP levels reflect a response to transient imbalances between energy absorbed by the photochemical apparatus and energy used by metabolism because of limitations in photosynthetic capacity during various stages of chloroplast biogenesis (Huner et al., 1998). This is supported by the fact that development at either 20/800 or 5/250 resulted in a higher reduction state of PSII than development at either 20/250 or 5/50. This conclusion is also consistent with the recent report of Montane et al. (1997) regarding the regulation of ELIP accumulation by PSII excitation pressure rather than by light or temperature. Although our *in vivo* data preclude the identification of the precise nature of the molecular signal that controls the epoxidation state and ELIP accumulation during chloroplast development under various PSII excitation pressures, the transthylakoid pH gradient is probably the major signal that regulates the epoxidation state (Gilmore, 1997). Whether the change in pH or a thylakoid redox signal controls ELIP expression remains to be determined.

The capacity to stimulate the xanthophyll cycle can occur very early during chloroplast development, as indicated here by the conversion of violaxanthin to zeaxanthin upon exposure to photoinhibition (Fig. 5). We suggest that fluctuations in the epoxidation state during this time (Fig. 4) most likely reflect an active xanthophyll cycle. However, we cannot exclude the contribution of *de novo* synthesis of xanthophylls to the transient fluctuations in the apparent epoxidation state observed during greening at either 20°C or 5°C. The consistently greater xanthophyll-pool sizes induced during greening at either 20/800 or 5/250 in the wild type and the  $f2$  mutant probably reflect enhanced *de novo* synthesis of xanthophyll-cycle intermediates.

The results presented in this report are consistent with the premise that both low temperature and excessive light can mediate photooxidative stress (Koroleva et al., 1995). Furthermore, the transient stimulation of zeaxanthin and ELIP accumulation by either high light or low temperature occurs during the very early stages of assembly of the photochemical apparatus (Król et al., 1987, 1988), presumably at a time when the potential for photooxidative damage is high. We suggest that the initial lag phase observed for Chl  $a$  and  $b$  accumulation (Fig. 1) represents the time of

maximal photooxidative stress during greening. Thus, maximal rates of greening occur only after sufficient photoprotection is in place in the form of zeaxanthin associated with ELIPs. This occurs within the first 6 to 12 h of greening at 20°C and after about 72 h of greening at 5°C, when the potential for the absorption of light energy exceeds that for the use of light energy by photosynthetic electron transport and carbon metabolism. During this early stage of chloroplast development, photoprotective mechanisms such as energy dissipation by zeaxanthin are induced, presumably to reduce potential damage to the developing photosynthetic apparatus. The subsequent decrease in both zeaxanthin and ELIP content and the increase in violaxanthin probably reflect the increased capacity of the developing chloroplast to use the absorbed light energy metabolically.

Dissipation of excess light through qN has been proposed to be important in the down-regulation of PSII photochemical efficiency to protect this reaction center from potential photodamage (Demmig-Adams and Adams, 1992; Eskling et al., 1997; Gilmore, 1997). The general consensus is that qN is a response to high-light stress. As expected, we show that in mature, fully expanded primary leaves of either the wild type or the *f2* mutant, the capacity for maximum qN is indeed dependent on the irradiance experienced during greening irrespective of the growth temperature (Table III). However, the qN exhibited after development at 20/800 was comparable to that observed after development at 5/250. Thus, the development of qN cannot be explained as a response to high light per se. Although the wild type and the *f2* mutant exposed to either 20/800 or 5/250 were grown at substantially different growth irradiances and temperatures, they experienced comparably high levels of PSII closure, measured as 1 – qP. In other words, the wild type and the *f2* mutant experienced the highest PSII excitation pressures when developed at 20/800 or 5/250 (Huner et al., 1998). This is supported by the fact that there was a strong, positive correlation between qN and 1 – qP irrespective of whether the wild type or the *f2* mutant was used (Fig. 8A).

There was little correlation between the accumulation of zeaxanthin, antheraxanthin, or zeaxanthin plus antheraxanthin, the epoxidation state, and the development of qN in either the wild type or the *f2* mutant (Tables I–III). However, we did observe a good correlation between qN and the total xanthophyll-pool size when measured on a micrograms per gram fresh weight basis (Fig. 8B). The lack of a correlation between zeaxanthin and the development of qN is probably the result of the fact that the available binding sites for zeaxanthin involved in qN become saturated at low concentrations of zeaxanthin (Gilmore, 1997). Consequently, zeaxanthin and antheraxanthin can be present in excess, and therefore, a linear correlation between zeaxanthin, antheraxanthin, and qN would not be expected. Furthermore, in addition to zeaxanthin and antheraxanthin, other xanthophylls such as lutein may also contribute significantly to qN (Pogson et al., 1998). Further studies will be needed to distinguish the possible contributions of the various xanthophylls to the observed qN.

## ACKNOWLEDGMENTS

We are grateful to Prof. Gunnar Öquist (Department of Plant Physiology, University of Umeå) for his support during the initial stages of this research; to Dr. David Simpson (Carlsberg Laboratories, Copenhagen, Denmark) for seeds of the *chlorina f2* mutant of barley; and to Ian Craig (Science Pics, University of Western Ontario) for his help in preparing Figure 6 and the estimation of relative ELIP content.

Received July 31, 1998; accepted February 16, 1999.

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