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Analysis of xanthophyll cycle carotenoids and chlorophyll fluorescence in light intensity-dependent chlorophyll-deficient mutants of wheat and barley

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Abstract

Three light intensity-dependent Chl *b*-deficient mutants, two in wheat and one in barley, were analyzed for their xanthophyll cycle carotenoids and Chl fluorescence characteristics under two different growth PFDs (30 versus 600 μmol photons m^{-2} s^{-1} incident light). Mutants grown under low light possessed lower levels of total Chls and carotenoids per unit leaf area compared to wild type plants, but the relative proportions of the two did not vary markedly between strains. In contrast, mutants grown under high light had much lower levels of Chl, leading to markedly greater carotenoid to Chl ratios in the mutants when compared to wild type. Under low light conditions the carotenoids of the xanthophyll cycle comprised approximately 15% of the total carotenoids in all strains; under high light the xanthophyll cycle pool increased to over 30% of the total carotenoids in wild type plants and to over 50% of the total carotenoids in the three mutant strains. Whereas the xanthophyll cycle remained fairly epoxidized in all plants grown under low light, plants grown under high light exhibited a considerable degree of conversion of the xanthophyll cycle into antheraxanthin and zeaxanthin during the diurnal cycle, with almost complete conversion (over 90%) occurring only in the mutants. 50 to 95% of the xanthophyll cycle was retained as antheraxanthin and zeaxanthin overnight in these mutants which also exhibited sustained depressions in PS II photochemical efficiency (F_v/F_m), which may have resulted from a sustained high level of photoprotective energy dissipation activity. The relatively larger xanthophyll cycle pool in the Chl *b*-deficient mutant could result in part from the reported concentration of the xanthophyll cycle in the inner antenna complexes, given that the Chl *b*-deficient mutants are deficient in the peripheral LHC-II complexes.

Abbreviations: A – antheraxanthin; Chl – chlorophyll; F_o and F_m – minimal yield (at open PS II reaction centers) and maximal yield (at closed centers) of chlorophyll fluorescence in darkness; F – level of fluorescence during illumination with photosynthetically active radiation; F_m' – maximal yield (at closed centers) of chlorophyll fluorescence during illumination with photosynthetically active radiation; $(F_m'-F)/F_m'$ – actual efficiency of PS II during illumination with photosynthetically active radiation; $F_v/F_m = (F_m - F_o)/F_m$ – intrinsic efficiency of PS II in darkness; LHC-II – light-harvesting chlorophyll-protein complex of Photosystem II; PFD – photon flux density (between 400 and 700 nm); PS I – Photosystem I; PS II – Photosystem II; V – violaxanthin; Z – zeaxanthin

Introduction

In the dark, or when light is not excessive, the xanthophyll cycle pool typically remains in the epoxidized form as violaxanthin in the thylakoid membranes of photosynthetic tissues (Yamamoto 1979). When the light absorbed by chlorophyll reaches excessive levels, the carotenoid violaxanthin is de-epoxidized to antheraxanthin and then to zeaxanthin as a part of the xanthophyll cycle. The degree to which the

pool of violaxanthin becomes converted to antheraxanthin and zeaxanthin is dependent upon the degree to which the absorbed light is excessive. The latter two carotenoids have been implicated in a photoprotective process whereby excess excitation energy absorbed by Chl is harmlessly dissipated as heat (Demmig-Adams and Adams 1992a; Gilmore and Yamamoto 1993). Upon return to non-excessive light levels, zeaxanthin becomes converted back to antheraxanthin and in turn to violaxanthin. This cycle of enzymatic epoxidation and de-epoxidation of the xanthophyll cycle carotenoids occurs continuously in response to the light environment (Adams and Demmig-Adams 1992; Adams et al. 1992). These changes in the epoxidation state of the xanthophyll cycle pool are typically accompanied by changes in the yield of Chl fluorescence emission, indicative of decreases in photochemical efficiency associated with increases in the level of thermal energy dissipation (Demmig-Adams and Adams 1994).

Chl *b*-deficient mutants offer an interesting system to examine the response of the xanthophyll cycle to PFD. Such mutants have a partial block in the synthesis of Chl, resulting in a decrease in Chl *b* and light harvesting complexes (Falbel and Staehelin 1994). What is the state of the xanthophyll cycle in mutants that lack or have diminished light harvesting antennae? We have examined the carotenoid composition and Chl fluorescence characteristics of two wheat mutants and a barley mutant which are all deficient in Chl *b* and light-harvesting complexes, with an emphasis on the status of the xanthophyll cycle under two different growth PFDs.

Materials and methods

Growth conditions

For carotenoid determinations and ambient-temperature fluorescence measurements, wild type wheat (*Triticum aestivum* L.) and the CD3 and Driscoll mutant strains (provided by M. Duysen at North Dakota State University) as well as wild type barley (*Hordeum vulgare* L.) and the chlorina-104 mutant strain (provided by D. Simpson, Carlsberg Laboratories, Copenhagen) were grown from seed for ten days on a 14-h light, 10-h dark cycle under two different light conditions in Conviron E-15 growth chambers. High light (~600 μmol photons m^{-2} s^{-1} incident upon the leaves) was provided by metal halide lamps, and low light (~30 μmol photons m^{-2} s^{-1} incident upon the leaves) was provided by a mixture of cool white fluorescent and incandescent lamps. 'Dark' ('predawn') samples were taken for measurement at the end of the dark cycle on the tenth day, and 'light' samples were taken for measurement approximately three hours into the light cycle after the 'dark' samples were taken.

For 77K fluorescence measurements wild-type and CD3 wheat were grown for 16 days under high light provided in a greenhouse supplemented with metal halide lamps (~1600 μmol photons m^{-2} s^{-1}) or low light provided by cool white fluorescent lamps (~100 μmol photons m^{-2} s^{-1}).

Fluorescence measurements

In situ measurements of Chl fluorescence were performed with a PAM-2000 portable Chl fluorometer (Walz, Effeltrich, Germany) prior to taking samples for carotenoid determinations. Fluorescence was measured in triplicate for both dark-adapted ('predawn') and light-exposed plants from the three strains of wheat (two mutant and one wild type) and two strains of barley (one mutant and one wild type) grown under both light environments.

For 77K fluorescence measurements, frozen aliquots of wild type wheat and CD3 mutant thylakoids were used. Thylakoids were prepared from plants homogenized in 0.4 M sorbitol, 100 mM tricine-KOH pH 7.5, 10 mM NaCl, 5mM $MgCl_2$. After filtering through nylon mesh and centrifugation, chloroplasts were washed in 5 mM Hepes-KOH pH 7.5, 10 mM NaCl, 5 mM $MgCl_2$ and recentrifuged. Finally, thylakoids were washed in 5 mM Hepes-KOH pH 7.5, 10 mM EDTA, centrifuged and resuspended in the same buffer with 10% glycerol added. Samples were stored at −80 °C in small aliquots.

Fluorescence spectra from samples frozen at 77K were obtained by diluting (in duplicate) thylakoids thawed from freezer stocks to about 50 μg Chl/ml into a buffer containing 100 mM tricine-KOH pH 7.5, 10 mM NaCl, 5 mM $MgCl_2$, 0.4 M sorbitol and protease inhibitors (1 mM benzamidine HCL and 5 mM ϵ-aminocaproic acid). All samples were dark-adapted for 15 minutes, and half of the samples remained in the dark at 25 °C for an additional 20 minutes. To the other half of the samples 10 mM ATP and 10 mM NaF (to inhibit protein phosphatase) were added. These samples were incubated under a mixture of incandescent (high-intensity tungsten) and fluorescent light at 25 °C for 20 minutes. Pasteur pipets with sealed tips were

dipped into each suspension and frozen in liquid N_2. From the thin film of dilute thylakoid suspension frozen on the outside of the pipet, emission spectra (excitation at 438 nm) were recorded on a Perkin-Elmer MPF43 fluorimeter, or on a Spex Fluorolog 1 fluorimeter corrected for instrument response.

Carotenoid determinations

Triplicate leaf discs were sampled from leaves and rapidly wrapped in aluminum foil and frozen in liquid N_2. Upon thawing, samples were ground immediately in 85% ice cold aqueous acetone with a hand-held glass tissue homogenizer and re-extracted with 100% acetone, following the procedure of Thayer and Björkman (1990). After the volumes were measured and the samples filtered and bubbled with nitrogen gas, 20 μl samples were injected onto a non-endcapped C18 HPLC column using the method described by Gilmore and Yamamoto (1991). Carotenoids and Chls separated by this method were quantified by comparing peak areas to calibrated values. Data were finally expressed as μmoles carotenoid per m^2 leaf area. Daytime and nighttime values from the same plants were first adjusted so that the total carotenoid content of those samples remained the same in order to account for diurnal changes in leaf volume due to shrinkage, which was significant in the high-light grown leaves.

Results

Pigment determinations

The wild-type wheat and barley leaves exhibited lower Chl *a/b* ratios when grown under low PFD than when grown under high PFD (approximately 2.8 for low light vs. 3.4 for high light for both species; Table 1), which is typical of the differences in this ratio observed between sun and shade acclimated leaves of most species (Anderson 1986; Anderson and Osmond 1987; Leong and Anderson 1986). Furthermore, as had been shown previously (Falbel and Staehelin 1994), the three Chl *b*-deficient mutant strains possessed higher Chl *a/b* ratios than the wild type strains, particularly when grown under high PFD (Table 1). This was most extreme in the Driscoll's chlorina mutant of wheat, which accumulated no detectable Chl *b* and almost no Chl *a* under high PFD.

The total carotenoid pool (the sum of neoxanthin, lutein, β-carotene, and the xanthophyll cycle carotenoids (V+A+Z)) and total Chl content were lower in the mutants compared to wild type, for both growth conditions, when expressed on a leaf area basis (Table 1 and Fig. 1). Interestingly, there was little variation in the total carotenoid levels (per unit leaf area) between the two growth PFDs for a given strain (Table 1 and Fig. 1), whereas total Chl per unit leaf area was lower in the mutant than in wild type in a light-intensity dependent manner (Table 1), consistent with earlier reports (Falbel and Staehelin 1994). Under low light the carotenoid and Chl contents were reduced by similar amounts yielding similar molar ratios of carotenoids to Chl in all plants. However under high light much less Chl accumulated in the mutants, yielding higher levels of carotenoids per Chl for mutants compared to wild type.

The individual carotenoid components (except for the xanthophyll cycle carotenoids which were pooled) for the plants grown under each light regime are depicted in Fig. 1. Absolute amounts per unit leaf area are shown as well as the fraction of each carotenoid relative to the total carotenoid pool. The amounts of neoxanthin and lutein were reduced in the mutants compared to wild type when expressed on a leaf area basis (Fig. 1). When expressed as a fraction of the total carotenoid pool (Fig. 1, pies) the amount of neoxanthin was lower in mutants than wild type for both growth conditions. Lutein was also present as a smaller fraction of the total carotenoids in the mutant plants compared to wild type plants grown in low light, yet it was present in the same or higher proportions in mutant plants grown in high light. Thus, the amount of lutein was not as tightly correlated to the amount of light-harvesting chlorophyll protein complexes as was the amount of neoxanthin. β-carotene was present as a greater proportion of the total carotenoid pool in the mutant plants compared to wild-type plants grown under low light conditions (Fig. 1, pies). In contrast, growth under high PFD resulted in a much larger fraction of the total carotenoids being present as components of the xanthophyll cycle in the mutants relative to wild-type (Fig. 1).

The ratio of total carotenoid to Chl in the Driscoll's chlorina mutant grown under high light (Table 1, (*)) was very large as the Chl level in the high-light grown Driscoll's chlorina mutant plants was quite low (i.e. we were dividing by a number close to zero). The Driscoll's chlorina plants died a week later under these conditions. Lutein and zeaxanthin were the two predominant carotenoids remaining in these plants (Figs. 1 and 2).

Table 1. Chlorophyll and carotenoid contents of wild type and mutant wheat and barley plants grown under low light (30 μmol photons m^{-2} s^{-1}) or high light (600 μmol photons m^{-2} s^{-1}). Values reported are means of triplicate determinations ± standard deviations. Driscoll's chlorina mutant (*) grown under high light conditions exhibited considerable variability in its small amount of total chlorophyll, resulting in highly variable carotenoid to chlorophyll ratios

Material	Chl *a/b*	Total carotenoids (μmol m^{-2})	Total Chl (μmol m^{-2})	Carotenoid/Chl (mmol mol^{-1})
Wheat				
Low light-grown				
Wild type	2.9±0.1	103±8	262±29	392±21
CD3	4.1±0.1	60±9	142±18	424±15
Driscoll	4.4±0.8	29±3	58±9	502±50
High light-grown				
Wild type	3.5±0.2	129±17	211±25	611±39
CD3	12.9±3.5	91±10	27±10	3528±884
Driscoll	∞	27±3	1±1	18887±9954*
Barley				
Low light-grown				
Wild type	2.7±0.2	111±10	264±27	421±12
Chlorina-104	3.3±0.2	93±10	229±30	407±14
High light-grown				
Wild type	3.3±0.1	126±7	194±19	655±41
Chlorina-104	12.7±8.7	93±5	54±4	1654±170

Xanthophyll cycle carotenoid levels in wheat and barley mutants

No differences were noted during the dark or light periods for the amounts of neoxanthin, lutein, β-carotene, or the total xanthophyll cycle pool (V+A+Z) and hence these 'dark' and 'light' data were combined for Fig. 1. Furthermore, plants grown under the low light conditions showed no difference between samples taken at the end of the dark cycle or taken during the light cycle in the composition (status) of the xanthophyll cycle (Fig. 2). Mutant plants exhibited the same relative carotenoid composition as wild type plants, but with fewer carotenoids per unit leaf area. The ratio of xanthophyll cycle carotenoids (violaxanthin + antheraxanthin + zeaxanthin) to the total carotenoid pool was slightly higher in mutant plants than in wild type for these low light-grown plants (Fig. 1 pies, V+A+Z).

Under high-light growth conditions, however, the status of the xanthophyll cycle pool was strikingly different between dark-adapted and light-exposed plants in most cases (see, however, Driscoll's chlorina mutant). The high-light conditions were above saturating light levels and induced the de-epoxidation of violaxanthin in both wild type and mutant plants. In both dark- and light-adapted plants, the relative epoxidation state of the xanthophyll cycle pigments was much lower (more zeaxanthin, less violaxanthin) for the mutants than the wild type. The most extreme wheat mutant, Driscoll's chlorina, had a xanthophyll cycle pool that was almost entirely composed of zeaxanthin in both dark- and light-adapted samples (Fig. 2).

The differences in the degree to which the xanthophyll cycle was de-epoxidized between strains and growth PFDs was also apparent when zeaxanthin (Z) and antheraxanthin + zeaxanthin (A+Z) were expressed on a total Chl basis or compared to the total xanthophyll cycle pool (Table 2). The variability in these values was fairly great in the mutant strains growing in high light, due primarily to the variation in leaf Chl content (Table 1).

Photosystem II fluorescence

The Chl fluorescence parameters shown in Table 2 were determined from leaves examined in darkness and during exposure to their growth light just before removal of leaf discs for the carotenoid analyses described above. Both wild-type and mutant strains had higher values of intrinsic PS II efficiency, measured as the ratio of F_v/F_m, for plants grown under low light than under high light. During exposure to

Table 2. Zeaxanthin and antheraxanthin contents in wild type and mutant wheat and barley plants grown under low light (30 μmol photons m^{-2} s^{-1}) or high light (600 μmol photons m^{-2} s^{-1}) conditions. Measurements were taken either at the end of the dark cycle or after 2–3 hours of exposure to the light growth conditions. Also shown are the intrinsic efficiency of PS II after 10 h of darkness (F_v/F_m), and the actual efficiency of PS II in the light ($[F_m'-F]/F_m'$). Values reported are means of triplicate determinations ± standard deviations. Because chlorophyll contents varied greatly between leaves for mutants grown under high light conditions, the carotenoid to chlorophyll ratios exhibited a great deal of variability

	Sampled at the end of the dark cycle (10 hours of darkness)				
	mmol mol^{-1} Chl		Z	A+Z	
Material	Z	A+Z	V+A+Z	V+A+Z	F_v/F_m
Low light-grown					
Wheat					
Wild type	2±4	5±4	0.03±0.06	0.07±0.06	0.790±0.007
CD3	0±0	9±2	0.00±0.00	0.13±0.02	0.850±0.010
Driscoll	0±0	10±3	0.00±0.00	0.11±0.03	0.857±0.008
Barley					
Wild type	0±0	2±2	0.00±0.00	0.03±0.03	0.784±0.005
Chlorina-104	0±0	5±0	0.00±0.00	0.08±0.00	0.822±0.003
High light-grown					
Wheat					
Wild type	23±7	36±6	0.10±0.03	0.16±0.02	0.719±0.054
CD3	716±442	1549±668	0.36±0.13	0.80±0.12	0.536±0.050
Driscoll	4120±2721	5220±3169	0.73±0.05	0.95±0.03	not detectable
Barley					
Wild type	19±6	38±6	0.08±0.02	0.17±0.01	0.765±0.002
Chlorina-104	116±86	336±212	0.16±0.10	0.48±0.24	0.741±0.091

	Sampled 2–3 hours into the light period				
	mmol mol^{-1} Chl		Z	A+Z	
Material	Z	A+Z	V+A+Z	V+A+Z	$(F_m'-F)/F_m'$
Low light-grown					
Wheat					
Wild type	0±0	4±1	0.00±0.00	0.06±0.01	0.724±0.005
CD3	0±0	8±1	0.00±0.00	0.13±0.02	0.777±0.004
Driscoll	0±0	9±1	0.00±0.00	0.10±0.03	0.767±0.029
Barley					
Wild type	0±0	3±0	0.00±0.00	0.05±0.00	0.730±0.003
Chlorina-104	0±0	5±1	0.00±0.00	0.08±0.01	0.768±0.010
High light-grown					
Wheat					
Wild type	36±4	82±7	0.20±0.02	0.43±0.03	0.463±0.084
CD3	1740±651	1804±680	0.94±0.02	0.98±0.01	0.305±0.027
Driscoll	10667±3497	13348±4082	0.79±0.02	0.98±0.01	not detectable
Barley					
Wild type	71±28	128±34	0.38±0.11	0.69±0.12	0.454±0.024
Chlorina-104	612±193	680±129	0.81±0.22	0.90±0.13	0.405±0.098

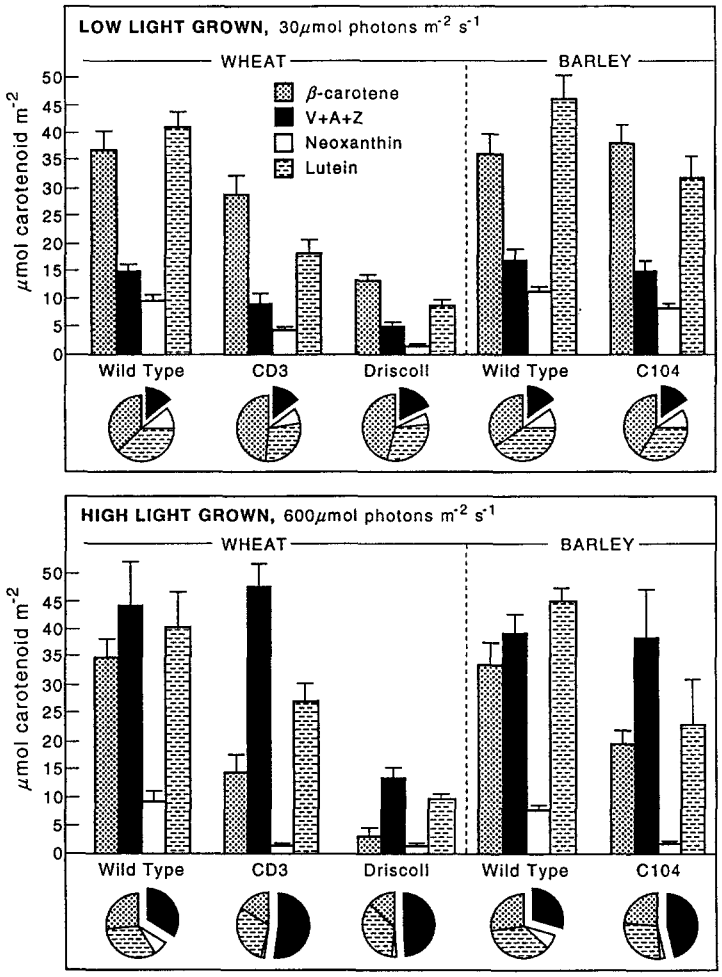

Fig. 1. Leaf carotenoid composition (neoxanthin, lutein, β-carotene, and sum of the xanthophyll cycle carotenoids (V+A+Z)) of wild type wheat and barley and three chlorophyll *b*-deficient mutants growing under low (30 μmol m^{-2} s^{-1}) or high (600 μmol m^{-2} s^{-1}) PFD expressed per unit leaf area. Measurements were performed in triplicate from dark- and light-adapted leaves. Error bars represent one standard deviation (n = 6). The pie diagrams depict the same carotenoids expressed as relative proportions of the total carotenoid pool. V+A+Z = Violaxanthin + Antheraxanthin + Zeaxanthin

their growth PFD, wild-type and mutant strains under both growth conditions exhibited a lower value of actual efficiency $(F_m'-F)/F_m'$, although plants grown under low light conditions responded with a smaller depression in efficiency than plants grown under high light.

When grown under low light, the dark-adapted CD3 and Driscoll's chlorina mutants, as well as the chlorina-104 mutant, showed significantly higher values of F_v/F_m than the corresponding wild type controls. Using this technique, PS I fluorescence also contributes to F_o, and in the section below we demonstrate that for these mutants fluorescence from PS I was greatly reduced. Thus, with a lower value for F_o, $F_v/F_m$was greater for the mutants than for wild type.

Under high light growth conditions, Driscoll's chlorina mutant (which contained barely measurable amounts of Chl) showed no Chl fluorescence for measurements taken during the dark cycle or the light cycle. The high light-grown, dark-adapted CD3 wheat mutant and the chlorina-104 barley mutant both showed lower values of F_v/F_m than the wild type controls, and the chlorina-104 mutant was affected to a lesser extent than the CD3 mutant. There were also significant amounts of antheraxanthin and zeaxanthin retained during the dark cycle in the mutant leaves (Fig. 2, Table 2). During high light exposure, both mutant and

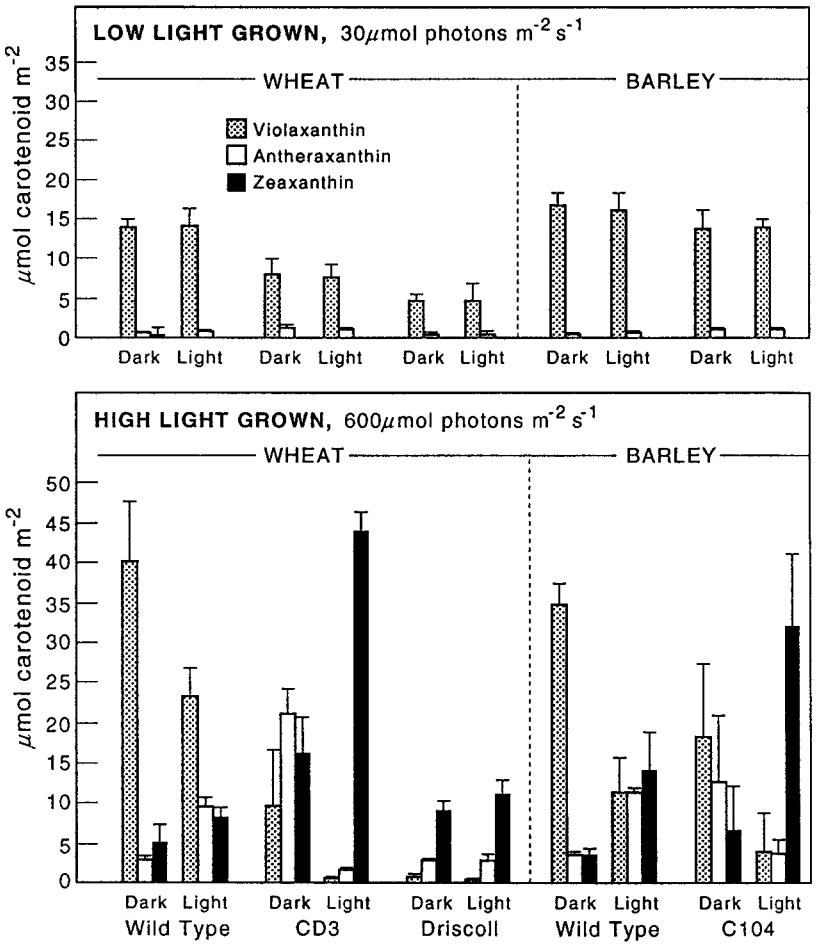

Fig. 2. Xanthophyll cycle carotenoids expressed per unit leaf area for wheat and barley plants grown under low versus high light conditions. Samples labelled Dark were taken at the end of the dark cycle, and samples labelled Light were taken 2–3 hours into the light cycle, on the same day that the dark samples were taken. Measurements were performed in triplicate, and error bars represent one standard deviation.

wild type plants exhibited reductions in actual PS II efficiency ($(F_m' - F)/F_m'$), which accompanied further de-epoxidation of the violaxanthin pool.

77K fluorescence measurements

Three distinct fluorescence peaks can be noted from the frozen thylakoid samples, at F_{685}, F_{695}, and F_{735} shown in Fig. 3A, and a shoulder at F_{680} is visible in some spectra. The F_{735} band is a broad band and can sometimes be resolved into two components, F_{735} and F_{720}. The origin of these bands is not well understood, but they appear to be related to specific Chl protein complexes (Krause and Weis 1991). The F_{680} band is assigned to LHC-II, the F_{685} and F_{695} bands are assigned to the PS II reaction center core, the F_{720} band is associated with the PS I reaction center core, and the F_{735} band is probably associated with PS I and its antenna.

When spectra taken from dark-adapted wild type and CD3 thylakoids isolated from plants grown under low light are overlayed, and normalized to the 685 nm peak (Fig. 3A), it is clear that the large PS I-associated 735 nm component found in wild type plants (solid line) was considerably reduced in the mutant (dotted line). All that remained in the mutant was a 720 nm component. The same phenomenon was observed, but to a greater extent, when comparing wild type and mutant spectra from dark adapted thylakoids isolated from plants grown under high light (Fig. 3B). Both wild type and mutant plants grown under low light exhibited a slight shoulder at 680 nm (Fig. 3A), which

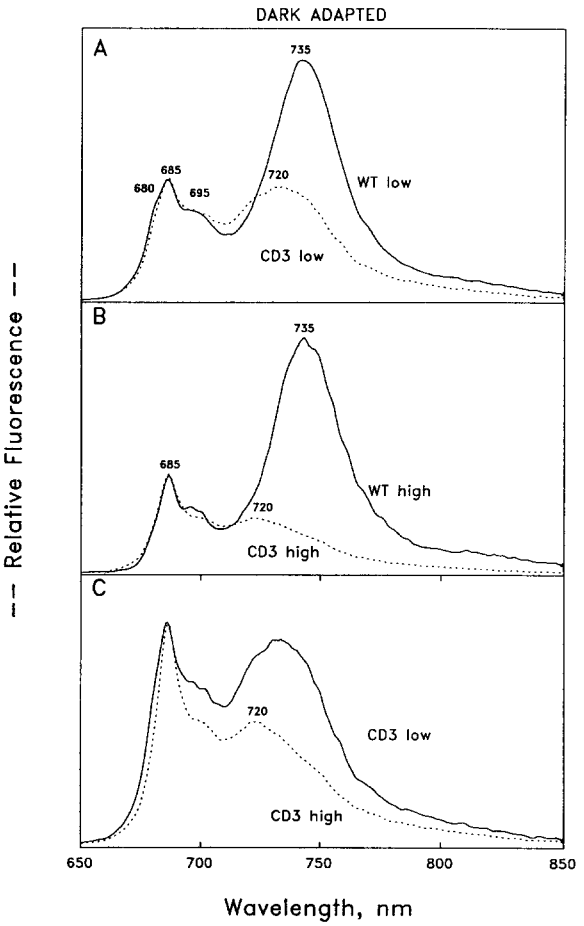

Fig. 3. 77K fluorescence spectra from suspensions of thylakoids isolated from wild type wheat and CD3 mutant wheat grown under low (100 μmol m^{-2} s^{-1}) and high (1600 μmol m^{-2} s^{-1}) PFD. Samples were dark-adapted for 20 minutes before spectra were taken. The excitation wavelength was 438 nm. Spectra were normalized at 685 nm. This experiment was performed twice from fresh aliquots of these samples, with similar results in each trial. (A) Wild type (solid line) and mutant (dotted line) thylakoids from plants grown under low light. (B) Wild type (solid line) and mutant (dotted line) thylakoids from plants grown under high light. (C) CD3 mutant thylakoids grown under low light (solid line) or high light (dotted line). These spectra are the same spectra shown for the CD3 mutant in part A (high light) and part B (low light), but plotted on an expanded scale to facilitate comparison. For the samples in (B) and (C) grown under low light conditions, a small shoulder is visible at 680 nm, which is thought to correspond to PS II antenna.

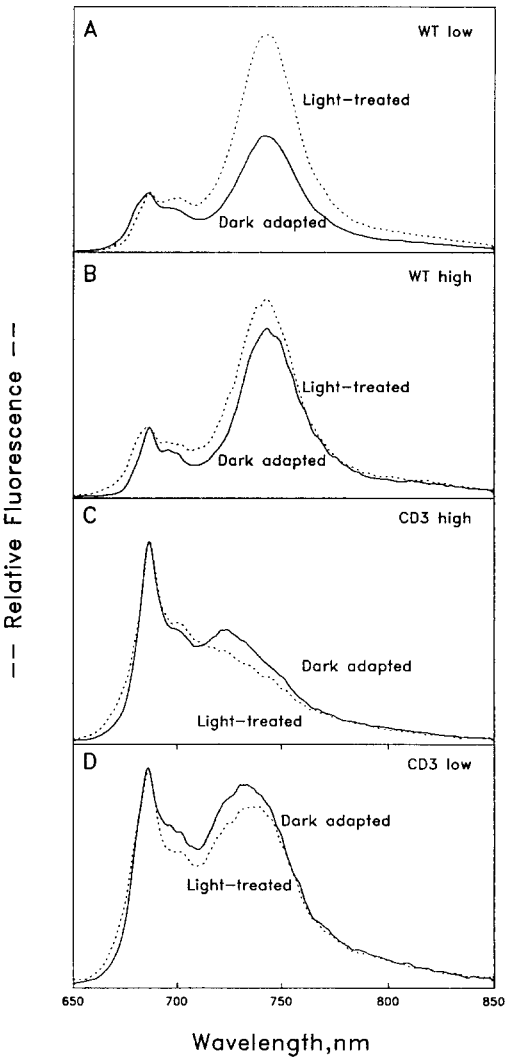

Fig. 4. 77K fluorescence spectra from suspensions of thylakoids isolated from wild type wheat and CD3 mutant wheat grown under low and high PFD (see legend of Fig. 3). Solid lines indicate samples that were dark-adapted for 20 minutes before spectra were taken (thylakoids in State 1). Dotted lines indicate samples incubated with ATP and NaF in the light for 20 minutes (thylakoids in State 2). The excitation wavelength was 438 nm. Spectra were normalized at 685 nm. This experiment was performed twice from fresh aliquots of these samples. All solid lines are the same spectra as were shown for dark-adapted samples in Fig. 3. (A) Wild type thylakoids from plants grown under low light conditions. (B) Wild type thylakoids from plants grown under high light conditions. (C) Mutant thylakoids from plants grown under high light conditions. (D) Mutant thylakoids from plants grown under low light conditions.

could be attributable to a greater amount of LHC-II in those plants than in plants grown under high light. Figure 3C compares thylakoids from CD3 plants grown under both light conditions and shows a reduction of the 735 nm component and 'blue shift' of the peak to 720 nm.

All of the 77K spectra shown thus far were from dark-adapted samples. Additional spectra were obtained to investigate 'state transitions' in the CD3 mutants which, according to Markwell et al. (1985), do

not occur in these plants. The samples were incubated in the light in the presence of 10 mM ATP and 10 mM NaF because state transitions require ATP for LHC-II phosphorylation and NaF was included to inhibit the thylakoid phosphatase that acts on LHC-II. Wild type wheat grown in low light seemed to undergo a classical state transition when spectra from dark adapted plants were compared to the spectra of plants exposed to light (Fig. 4A). Relative to the PS II-associated fluorescence (F_{685}, F_{695}) the PS I-associated fluorescence (F_{735}) increased or possibly the PS I associated fluorescence decreased relatively less than the fluorescence associated with PS II. The high light-grown wild type wheat exhibited little or no such state transition, but there was a curious, reproducible, slight blue-shift of the 735 nm component (Fig. 4B).

The CD3 mutant showed quite reproducible changes in its 77K fluorescence spectra when incubated in the light. The amplitude of the 720 nm PS I component lessened upon light treatment in the presence of ATP and NaF. CD3 high light membranes showed a reduction in the 720 nm component after light treatment (Fig. 4C). CD3 low light membranes exhibited an apparent red shift in the broad peak at 735 nm which is likely to be due to reduced fluorescence of the 720 nm component (Fig. 4D). Thus, when CD3 mutant thylakoids from both high light-grown and low light-grown plants were exposed to light, something appeared to be quenching the source of the 720 nm PS I-associated fluorescence. There was no change in fluorescence observable at 720 nm in wild type thylakoids.

Discussion

Carotenoid composition and chlorophyll fluorescence

The differences in the carotenoid composition of the wild type strains of wheat and barley grown under low versus high PFD are similar to those observed between shade and sun plants (Demmig-Adams and Adams 1992b; Thayer and Björkman 1990). The components of the xanthophyll cycle responded most strongly to the difference in growth light regime, with an almost threefold larger pool of these carotenoids present in the high light-grown plants (Fig. 1). The difference in the size of this pool was even greater between the mutant strains grown at the two different PFDs, with the pool representing as much as half of the total carotenoids in the mutants grown at high light. This apparently occurred at the expense of β-carotene, the biochemical precursor in the synthesis of zeaxanthin, which was present at levels that were two- to three-fold lower under high light than low light. The total carotenoid pools on a leaf area basis were, however, roughly the same for plants grown under the two different PFDs (Table 1; see, however, Schindler et al. 1994).

Whereas the xanthophyll cycle remained almost totally epoxidized as violaxanthin in all plants grown under low light (Fig. 2, Table 2), plants grown under high light experienced de-epoxidation of violaxanthin to antheraxanthin and zeaxanthin, and in the mutants large amounts of the latter two were retained even after 10 hours of darkness each night. Furthermore, the mutants also exhibited a sustained depression in PS II efficiency, F_v/F_m. It is tempting to speculate that sustained high levels of xanthophyll cycle-associated energy dissipation contribute to this lowering of F_v/F_m. Although xanthophyll cycle-associated energy dissipation serves a photoprotective function, it also results in a lowering of the efficiency of photosynthetic energy conversion, as energy is diverted away from the reaction centers (Demmig-Adams and Adams 1992a). Thus it is conceivable that the reductions in F_v/F_m can be accounted for by the engagement of xanthophyll cycle-associated energy dissipation. A reduction in F_v/F_m associated with a decrease in the epoxidation state of the xanthophyll pool was also observed in the chorophyll *b*-less chlorina f2 barley mutant (Leverenz et al. 1992).

It was suggested previously that F_v/F_m values determined by the ambient temperature technique used in this study were lower than those determined from PS II using a 77K technique (Björkman and Demmig 1987) due to the fact that fluorescence from PS I contributes to the F_o signal at ambient temperature, thereby lowering the ratio of F_v/F_m (Adams et al. 1990). The 77K fluorescence spectra of the CD3 mutant relative to wild type wheat, that exhibited a much lower PS I than PS II emission (Figs. 3A and 3B), are consistent with this interpretation given that these mutants also exhibited a much higher F_v/F_m value than the wild type plants under low light conditions (Table 2), similar to the maximal reported values of 0.86 or 0.87 determined from 77K fluorescence.

Carotenoids and chlorophyll-protein complexes

The relatively smaller fraction of the total carotenoids present as neoxanthin and lutein, and greater fraction

present as β-carotene in mutant plants compared to wild type plants grown under low light are consistent with a reduction of light-harvesting complexes and an increase in the relative level of reaction center polypeptides (Allen et al. 1988; Bassi et al. 1993; Knoetzel and Simpson 1991; Koyama 1991). Under the high light conditions used in this study very little Chl was present in the wheat mutants. Therefore the lowered absolute amount of β-carotene as well as a lower proportion of β-carotene as a fraction of the total carotenoid pool in the high light-grown mutants is probably a reflection of fewer reaction centers per leaf area for the mutant plants grown under high light conditions.

To investigate the mechanism whereby xanthophyll cycle pigments bring about thermal energy dissipation several laboratories have attempted to determine with which complexes these carotenoids are associated. Bassi et al. (1993) have shown that violaxanthin is associated mostly with the inner PS II antenna CP29, CP26, and CP24 in dark-adapted maize leaves, and Ruban et al. (1994) have reported that the inner antenna complexes contain a greater relative proportion of such pigments than the peripheral LHC-II complexes. Yet other groups have demonstrated that the xanthophyll cycle operates in both PS II and PS I (Siefermann and Yamamoto 1976; Thayer and Björkman 1992). Studies of Chl *b*-deficient and Chl *b*-less mutants might be able to shed further light on where the xanthophyll cycle pigments operate, since their Chl-protein composition can be manipulated by light (Allen et al. 1988; Greene et al. 1988a; Knoetzel and Simpson 1991). CP29, CP26, and CP24 were reportedly absent in the barley chorina f2 mutant grown under intermittent light (Harrison and Melis 1992), yet the xanthophyll cycle carotenoids were still present and experienced epoxidation and de-epoxidation (Krol et al. 1993). This suggests that, while these polypeptides might be associated with the xanthophyll cycle carotenoids for some part of their function, the cycle is also functional in other locations as well.

The larger ratio of the xanthophyll cycle pool to total chlorophyll content in the Chl *b*-deficient mutants relative to the wild type plants grown under high light may simply be due, in part, to the absence of the peripheral LHC-II complexes in the mutants. If, as suggested by the above reports (Bassi et al. 1993; Ruban et al. 1994), the xanthophyll cycle is associated most strongly with the inner antenna complexes, the presence of the peripheral LHC-II complexes would tend to dilute the total xanthophyll cycle concentration when expressed on a chlorophyll basis. Thus, if one were to exclude the peripheral LHC-II complexes from the analysis of the xanthophyll cycle content of wild type plants one would expect the total xanthophyll cycle pool to comprise a larger proportion of the total pigment pool. The Chl *b*-deficient mutants are analogous to such a scenario given that they accumulate lesser amounts of the outer LHC-II complexes. Likewise, the greater conversion state of the cycle to antheraxanthin and zeaxanthin in the mutants under high light relative to the wild type plants might result from a pool of violaxanthin in the wild type plants, located in the peripheral LHC-II complexes, that does not become de-epoxidized.

Carotenoids and state transitions

Several changes in the membrane occur during a state transition, the best characterized of which is the phosphorylation of a subset of LHC-II complexes that results in their functional and physical disconnection from the PS II reaction center (Staehelin and Arntzen 1983). The light energy that is absorbed by phosphorylated LHC-II is presumably either dissipated or it is donated to PS I (reviewed by Allen 1992). One regulatory factor controlling LHC-II phosphorylation is thought to be the reduction state of the plastoquinone pool (Bennett et al. 1988). In low light-acclimated plants a subfraction of LHC-II becomes phosphorylated and disconnects from PS II when the plastoquinone pool becomes reduced beyond a certain point. Such disconnection presumably results in a decrease in the donation of excitation energy to PS II, which in effect slows the reduction of plastoquinone. On the other hand, for soybean acclimated to high light, dephosphorylation of LHC-II occurred upon exposure of leaves to excessive light that resulted in increased energy dissipation (Cleland et al. 1990). Leaves acclimated to high light have a large capacity for xanthophyll cycle-associated energy dissipation, and such a capacity may be sufficient for dissipating most of the excess excitation energy under the growth light regime. For plants acclimated to low light and suddenly exposed to a light environment that exceeds that experienced during growth, energy dissipation through the xanthophyll cycle may be quite insufficient, and the peripheral LHC-II complexes become phosphorylated and apparently remain in such a state. And indeed, the wild type wheat grown under low light exhibited a shift in the relative fluorescence emanating from PS II versus PS I, those grown under high light exhibited very little shift, and the CD3 Chl *b*-deficient mutant, which pre-

sumably had no capacity for state shifts given that it possessed little or no peripheral LHC-II complexes in the first place, exhibited none of the characteristics of a classical state shift (Fig. 4).

In the wild type thylakoid membrane, LHC-II is the most abundant light-harvesting chlorophyll-protein complex, hence the antenna system associated with PS II is much larger than that associated with PS I. As a result of the regulatory mechanisms discussed in the preceding section, this large PS II antenna system provides the photosynthetic apparatus with a large amount of flexibility to cope with short-term changes in the light environment. This is particularly important for adjusting the turnover rates of PS I and PS II, which are linked through the electron transport chain. Thus, the lack of antenna systems would be expected to impart a lack of flexibility to the photosynthetic apparatus that may only be compensated for by changing the reaction center stoichiometry. The chlorina f2 mutant and the OY-YG mutant have both been found to significantly increase their PS II/PS I ratios to compensate for missing light harvesting complexes (Ghirardi and Melis 1988; Greene et al. 1988b). The shift toward more PS II can be explained based on the fact that LHC-II constitutes a larger antenna system than does the light-harvesting complex of PS I and therefore requires greater compensatory changes in PS II. Such changes in photosystem stoichiometry are also consistent with the relative decrease in the contribution of fluorescence from PS I in the mutants observed in this study (Figs. 3 and 4).

Light sensitivity of the mutants

Possibly the most fascinating aspect of Chl *b*-deficient, but not Chl *b*-less, mutants is their tremendous diversity of phenotypes. Thylakoids of such mutants have been shown to be not only Chl *b*-deficient, but also LHC-deficient and grana-deficient (Allen et al. 1988; Greene et al. 1988a; Knoetzel and Simpson 1991). Usually these phenotypes are expressed in a light-intensity dependent and temperature dependent manner (Allen et al. 1988; Greene et al. 1988a; Knoetzel and Simpson 1991).

In a separate report (Falbel and Staehelin 1994), we have demonstrated that a set of allelic wheat mutants chlorina-1, chlorina-214, and CD3, and the barley mutant chlorina-104, all have a bottleneck in Chl synthesis at the level of the Mg-insertion step, which causes a buildup of protoporphyrin IX. When protoporphyrin IX absorbs light energy, its excited triplet state can react to result in the production of singlet oxygen which may cause damage to the surrounding proteins and lipids (Emiliani and Delmelle 1983; Hopf and Whitten 1978). We suggested, therefore, that the light intensity-dependent nature of these mutants was most likely due to the plants being photosensitized by the higher than normal pool of Chl precursors. A second factor that may contribute to the photosensitivity of these mutants is that when the mutant plants are subjected to high light growth conditions, the xanthophyll cycle is pushed to its maximum capacity in the sense that the carotenoids are converted entirely to zeaxanthin (Fig. 2, Table 2) at much lower light intensities than in wildtype plants. Although mutant plants have larger carotenoid to Chl ratios, and larger xanthophyll cycle pools, than the wild type plants (Table 1), even this potential capacity for photoprotection may be insufficient to dissipate all of the excess excitation energy absorbed by the Chls when growing under high light.

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