Biogenesis of Thylakoid Membranes Is Controlled by Light Intensity in the Conditional Chlorophyll b-deficient CD3 Mutant of Wheat

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Abstract. Biogenesis of thylakoid membranes in the conditional chlorophyll b-deficient CD3 mutant of wheat is dramatically altered by relatively small differences in the light intensity under which seedlings are grown. When the CD3 mutant is grown at 400 $\mu E/m^2$ s (high light, about one-fifth full sunlight) plants are deficient in chlorophyll b (chlorophyll a/b ratio > 6.0) and lack or contain greatly reduced amounts of the chlorophyll a/b-binding complexes CPII*/CPII (mobile or peripheral LHCII), CP29, CP24 and LHCI, as shown by mildly denaturing 'green gel' electrophoresis, by fully denaturing SDS-PAGE, and by Western blot analysis. High light CD3 chloroplasts display an unusual morphology characterized by large, sheet-like stromal thylakoids formed into parallel unstacked arrays and a limited number of small grana stacks displaced toward the edges of the arrays. Changes in the supramolecular organization of CD3 thylakoids, seen with freeze-fracture electron microscopy, include a reduction in the size of EFs particles, which correspond to photosystem II centers with variable amounts of attached LHCII, and a redistribution of EF particles from the stacked to the unstacked regions. When CD3 seedlings are grown at 150 $\mu E/m^2$ s (low light) there is a substantial reversal of all of these effects. Thus, chlorophyll b and the chlorophyll a/b-binding proteins accumulate to near wild-type levels (chlorophyll a/b ratio = 3.5–4.5) and thylakoid morphology is more nearly wild type in appearance. Growth of the CD3 mutant in the presence of chloramphenicol stimulates the accumulation of chlorophyll b and its binding proteins (Duysen, M. E., T. P. Freeman, N. D. Williams, and L. L. Huckle. 1985. *Plant Physiol.* 78:531–536). We show that this partial rescue of the CD3 high light phenotype is accompanied by large changes in thylakoid structure. The CD3 mutant, which defines a new class of chlorophyll b-deficient phenotype, is discussed in the more general context of chlorophyll b deficiency.

In 1966 Boardman and Highkin noted that our understanding of "the role of chlorophyll b (chl b)[1] in photosynthesis of higher plants and the green algae is far from complete." Today, despite more than 20 years of scrutiny by a large number of investigators taking advantage of tremendous improvements in methodology, this statement is still largely true. Early studies (reviewed by Thornber, 1975) established that chl b is not essential for the primary photochemical events of photosynthesis but plays a purely light harvesting role. It was further established that the photosynthetic reaction centers bind primarily chl a while the light-harvesting antenna complexes bind both chl a and chl b. Since that time, chl b and its binding proteins, particularly those associated with the major light-harvesting complex (LHC) of PSII (mobile or peripheral LHCII) have been implicated in the regulation of energy distribution between the two photosystems (Staehelin and Arntzen, 1983; Kyle et al., 1983), in thylakoid morphogenesis, and in the maintenance of lateral heterogeneity of thylakoid components (Arntzen, 1978; Barber, 1986). In addition, changes in content of chl b and its binding proteins have been shown to play a major role in adaptation to varying light intensities (Boardman, 1977; Anderson, 1986). The mechanisms underlying some of these processes are becoming increasingly clear, but many problems have not yet been addressed or are still controversial.

A major unresolved issue is the precise role of the LHCII polypeptides in the formation and maintenance of grana stacks. A substantial body of evidence from greening studies (Armond et al., 1977), and studies of plants adapted to varying light intensities (Anderson, 1986) establishes a correlation between amounts of LHCII and the extent of grana stacking. In addition, in vitro studies with isolated thylakoids (Steinback et al., 1979; Carter and Staehelin, 1980) or with LHCII proteoliposomes (McDonnel and Staehelin, 1980; Mullet and Arntzen, 1980; Ryrie et al., 1980) have shown that LHCII is capable of mediating membrane adhesion. This probably occurs via a positively charged, surface exposed fragment that can screen negative surface charges and

1. *Abbreviations used in this paper*: chl, chlorophyll; EFs, E-face stacked thylakoids; EFu, E-face unstacked thylakoids; LHC, Light-harvesting complex; PFs, P-face stacked thylakoids; Pfu, P-face unstacked thylakoids.

so allow close appression of the membranes (Mullet, et al., 1981; Ryrie and Faud, 1982; Barber, 1986). Chlorophyll b-deficiency, induced either genetically (Henningsen et al., 1974) or experimentally (De Greef et al., 1971; Day et al., 1984) consistently leads to a loss of the LHCII polypeptides. In the *chlorina*-f^2 mutant of barley, this loss has been shown to be caused by instability of the LHCII polypeptides once inserted into the membrane in the absence of chl b (Bellemare, et al., 1982). At the morphological level this loss of LHCII leads to a major reduction in the extent of thylakoid stacking in some plants, while having little effect on thylakoid organization in others (Goodchild et al., 1966; Schwartz and Kloppstech, 1982; Nakatani and Baliga, 1985). Thus, while some role for LHCII in the formation of grana stacks seems firmly established, a consideration of the available evidence suggests that there may be other, as yet undescribed factors involved in thylakoid stacking, or at least that the role of LHCII in this process is more subtle than it would appear at first glance. The resolution of this issue will be an essential component of our understanding of the question of thylakoid morphogenesis in general.

We report here on correlative biochemical and ultrastructural studies of the light-sensitive chl b-deficient mutant of wheat designated CD3 (Freeman et al., 1982; Duysen, et al., 1984). Our results show that modulation of the light intensity under which CD3 plants are greened leads to changes both in chl b content and accumulation of chl b-containing protein complexes as well as dramatic changes in the degree of thylakoid stacking and overall thylakoid morphology. We also extend earlier observations on the growth of this mutant in the presence of chloramphenicol, an inhibitor of organellar protein synthesis, conditions that have been shown to stimulate the accumulation of LHCII in the mutant chloroplasts (Duysen et al., 1985; Table I in Duysen et al., 1987). The findings reported here have enabled us to define a new class of phenotype associated with chl b-deficient mutants.

Materials and Methods

Plant Material and Growth Conditions

The CD3 mutant was obtained in a screen for pigment deficiency after treatment of seeds from the line ND496-25 with ethyl methanesulfonate (Freeman et al., 1982). The term 'wild type' in this paper refers to strain ND496-25. Fungicide-treated seeds of either wild-type or CD3 wheat were imbibed for 1 h in sterile distilled water before planting in sterile vermiculite. All plants were grown for 6 d in darkness at 25°C before transfer to continuous light under cool white fluorescent lamps at either 150 μE/m^2 s (low light) or 400 μE/m^2 s (high light) at 25°C. Light intensities were determined with a Li-Cor LI-185B quantum radiometer/photometer (Li-Cor Instruments, Lincoln, NE). For these studies all material was harvested after 4 d of continuous illumination. For the chloramphenicol-treated samples, 8 h before the beginning of the light period an appropriate volume of 250 μg/ml D-threo-chloramphenicol in water was added to give a soil concentration of 50 μg/ml soil. This treatment was repeated with fresh chloramphenicol solution 30 min before the beginning of the light period. Excess liquid was drained off before the second chloramphenicol addition. Treated plants were watered normally during the light period.

Thylakoid Membrane Isolation

For preparation of thylakoid membrane samples for biochemical analyses, leaves were cut into approximately 3-mm segments into ice-cold buffer containing 50 mM sodium phosphate, pH 7.2, 0.3 M sucrose, 0.1 M KCl, and 5 mM $MgCl_2$. Leaf segments were chopped in a VirTis homogenizer fitted with steel blades (Virtis Co., Gardiner, NY), and the macerate was filtered through four layers of Miracloth™ (Calbiochem/Behring, La Jolla, CA). The filtrate was subjected to a low speed spin (300 *g*, 2 min, 4°C) to remove whole cells and debris, and the supernatant was spun at 1,500 *g* for 10 min at 4°C. The resulting green pellet, which was enriched for intact and semi-intact chloroplasts, was resuspended and pelleted twice (3,000 *g*, 10 min., 4°C) in a buffer containing 50 mM Tris, pH 7.6, 10 mM KCl, and 5 mM $MgCl_2$. The resulting membrane pellet was either used fresh or frozen in liquid N_2 and stored at −70°C before use. Chlorophyll concentrations and chlorophyll a/b ratios were determined on fresh material in 80% acetone extracts using the equations of Arnon (1949).

Mildly Denaturing SDS-PAGE

Thylakoid samples were subjected to mildly denaturing 'green gel' electrophoresis as described by Dunahay and Staehelin (1986). Thylakoids were washed twice in ice cold 2 mM Tris-maleate, pH 7.0, and then solubilized for 10 min on ice in 0.88% (wt/vol) *n*-octyl β-D-glucopyranoside (OG)/0.22% (wt/vol) SDS/10% (vol/vol) glycerol in 2 mM Tris-maleate, pH 7.0. Unsolubilized material was removed by centrifugation at 30,000 *g* for 10 min and the supernatant was loaded onto 1.5-mm 10% polyacrylamide gels (15 μg chl/lane). Gels were run at 4°C for 3–4 h at a constant current of 15 mA and photographed through a blue filter without staining.

Analytical Gel Electrophoresis

Polypeptide composition of isolated thylakoids was determined using the gel system of Laemmli (1970) modified by the addition of 6 M urea to the resolving gel. Membrane samples were diluted 1:1 with 2× solubilization buffer (4% SDS, 4% β-mercaptoethanol, 20% glycerol in 1.3 M Tris-HCl, pH 6.8) and incubated for 20 min at 50°C before loading 60 μg protein/lane onto 1.5-mm 10–17.5% polyacrylamide gradient gels. Gels were stained with Coomassie Brilliant Blue R-250 and destained according to standard procedures.

For Western blot analysis, solubilized samples were run on 1.5 mm, 15% polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Richmond, CA). Membranes were blocked with either 3% gelatin or 3% BSA in Tris-buffered saline (20 mM Tris, 0.5 M NaCl, pH 7.5). Primary polyclonal antibodies were applied in the same buffer with 1% gelatin. Secondary antibodies (goat-anti-rabbit conjugated to horse radish peroxidase) and peroxidase color development reagent were obtained from Bio-Rad Laboratories. Polyclonal antisera to CPII* and CP29 were prepared against the spinach apoproteins excised from fully denaturing SDS-polyacrylamide gels (Dunahay and Staehelin, 1987). Polyclonal antisera directed against LHCI and CP24 were prepared against spinach pigment-protein complexes purified by multiple rounds of mildly denaturing 'green gel' electrophoresis in gels of differing polyacrylamide concentration. Both LHCI and CP24 antisera were affinity purified to eliminate cross reacting antibodies. After this procedure no cross reactivity could be detected between LHCI and CP24 (Dunahay, 1986). All antisera were the generous gift of Dr. Terri Dunahay.

Preparation for Electron Microscopy

All samples for electron microscopy were taken at the same time points as were samples for biochemical analysis. For thin section electron microscopy, leaf segments taken 1 cm back from the leaf tip were cut into 2-mm pieces under a drop of ice cold fixative containing 2% glutaraldehyde, 0.25 M sucrose, 0.1 M KCl, 5 mM $MgCl_2$, and 50 mM sodium phosphate, pH 7.2. Tissue segments were fixed on ice for 2 h in the same fixative, washed in the same buffer without glutaraldehyde, postfixed for 1 h in 1% aqueous osmium tetroxide, dehydrated through an ethanol series, washed twice with propylene oxide, and infiltrated with increasing proportions of Spurr's resin in propylene oxide overnight before embedding in Spurr's resin. Silver thin sections were cut using a diamond knife on a Reichert Ultracut ultramicrotome, stained with lead citrate and uranyl acetate according to standard procedures, and examined with a JEOL 100C electron microscope operated at 60 kV. The ratio of stacked to unstacked membranes was determined on micrographs printed to a standard magnification using a map wheel.

For freeze-fracture electron microscopy, leaf segments were briefly homogenized in an isoosmotic buffer as described above, filtered, and subjected to a low speed spin (300 *g*, 2 min, 4°C) to remove debris, pelleted at 1,500 *g* for 10 min, and glycerinated to 35% glycerol for 10 min while keeping the magnesium concentration constant at 5 mM. Glycerinated thy-

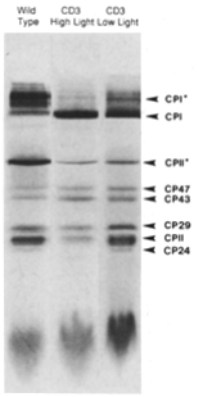


Figure 1. Chlorophyll-protein complexes from wild type and CD3 mutant thylakoid membranes separated by mildly denaturing 'green gel' electrophoresis. Lanes were loaded with solubilized thylakoid membranes containing 15 μg chlorophyll from: the wild type greened at 400 μE/m² s (*Wild Type*); the CD3 mutant greened at 400 μE/m² s (*CD3 High Light*); and from the CD3 mutant greened at 150 μE/m² s (*CD3 Low Light*). CPI* and CPI correspond to the photosystem I reaction center with and without its associated light harvesting antenna, LHCI. CPII and CPII* are monomeric and oligomeric forms of the chl a/b binding LHCII, the main light harvesting antenna of photosystem II. CP47 and CP43 are chl a–containing antennae closely associated with the photosystem II reaction center. CP29 is a chlorophyll–protein complex tightly associated with photosystem II, and CP24 a chl a/b–binding antenna that is found in both photosystem I and II preparations (see text).

lakoids were pelleted at 15,000 g for 15 min and frozen on copper supports by plunging into liquid nitrogen–cooled Freon 12. Samples were fractured and replicated with platinum/carbon in a Balzers BA 360 freeze-etch apparatus according to standard procedures. Replicas were examined in a JEOL 100C electron microscope operated at 80 kV. Particle size measurements were performed as described by Staehelin (1976).

Results

Biochemical Characterization

CD3 plants greened for 4 d at high light intensity (400 μE/m² s, note that the 'high light' conditions used here are only about one-fifth of full sunlight, which is ~2,000 μE/m² s) are pale green with a chl a/b ratio greater than 6.0, the limit of chl b detection with the method of Arnon (1949). Wild-type plants greened at the same light intensity as were dark green, show a greater degree of leaf expansion than the mutant, and display a chl a/b ratio from 3.0 to 3.5. When the CD3 mutant is greened at low light intensity (150 μE/m² s) plants are dark green with markedly increased rates of leaf expansion and chl a/b ratios in the range from 3.5 to 4.5. In general, a decrease in the illumination under which plants are grown results in increased accumulation of chlorophyll b and its binding proteins, along with an increase in the extent of thylakoid appression. However, the relatively small difference between the high and low light intensities used in this study was not sufficient to produce any detectable difference in the composition of wild-type thylakoid membranes. This was determined by comparison of chl a/b ratios, green gel banding patterns, fully denaturing SDS–PAGE profiles, and Western blot analysis using antisera directed against CPII*, CP29, LHCI, and CP24 (data not shown). Thus, for simplicity, all comparisons have been made to the wild type grown at high light intensity.

Analysis of pigment–protein complexes from wild-type wheat by mildly denaturing SDS–PAGE revealed eight major plus several minor green bands plus free pigment (Fig. 1). Data from densitometric scans of this gel are shown in Table I. The bands CPI* and CPI are associated with PSI, corresponding respectively to the P700 apoproteins with and without attached LHCI, the PSI-associated chl a/b–binding antenna complex. The major chl a/b light-harvesting complex of photosystem II (LHCII) is associated with two bands, an oligomeric form, designated CPII*, and the monomer, CPII (Camm and Green, 1980). Results from our laboratory indicate that the bands CPII* and CPII correspond to oligomeric and monomeric forms of peripheral (mobile) LHCII, while CP29, which may bind exclusively chl a, corresponds to bound LHCII (Dunahay et al., 1987). CP24 is a chl a/b–binding complex that has been reported in both PSI and PSII-enriched thylakoid fractions (Dunahay and Staehelin, 1986; Bassi et al., 1985). CP47 and CP43 are chl a–containing complexes tightly associated with PSII (Camm and Green, 1980). A similar analysis of thylakoid extracts from the CD3 mutant grown at high light intensity shows a substantial reduction in levels of mobile LHCII and LHCI, as evidenced by the decreased amounts of pigment associated with CPII* and CPII, and the shift of density from CPI* to CPI (Fig. 1 and Table I). This confirms previous results with this mutant (Duysen et al., 1984) using the gel system of Anderson et al. (1978). With the improved resolution of the gel system used here, a decrease in the amount of pigment associated with CP29 and CP24 is also evident. Thus, all of the chl a/b–binding complexes as well as CP29 are present in significantly reduced amounts in the CD3 mutant grown at high light intensity. It is also interesting to note that the ratio of densities of the chl a–containing bands CP47 and CP43 in the high light CD3 mutant is shifted in favor of CP43 from the usual one-to-one stoichiometry (Thornber, 1986). All of these changes are substantially reversed in the mutant grown at low light intensity. Hence, there is an increased amount of pigment associated with CPI*, CPII*, CPII, CP29, and CP24. The ratio of densities between CP47 and CP43 also returns to normal (Table I). It should be noted that the rescue of the CD3 phenotype is not complete at the low light intensity used here, as evidenced by the slightly elevated chl a/b ratio and the decreased density of the CPI* and CPII* bands relative to the wild type. The CD3 phenotype more closely resembles wild type when CD3 plants are greened at even lower irradiances than used in this study (data not shown).

Fully denaturing SDS–PAGE analysis of thylakoid extracts

Table I. Green Gel Densitometric Data in Percentage Total Pigment

	HMW‡	CPI*	CPI	CPII*	CP47	CP43	CP29	CPII	CP24	FP§
Wild Type	11.1	13.4	6.0	11.4	3.3	2.9	5.4	7.9	1.5	37.0
CD3 high light	2.2	4.4	20.0	3.5	4.4	6.1	5.8	5.3	1.1	47.7
CD3 low light	8.7	6.8	13.0	4.5	3.6	3.6	5.5	10.1	1.9	42.2

‡ Unidentified high molecular weight bands.
§ FP: Free pigment.

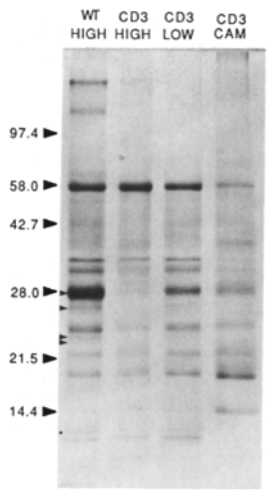


Figure 2. Fully denaturing SDS–PAGE carried out according to Laemmli (1970) in a 10–17.5% polyacrylamide gradient and stained with Coomassie Brillant Blue. Equal amounts of protein are loaded in each lane. Arrowheads indicate polypeptides of LHCII and LHCI that are missing or substantially reduced in the high light–greened CD3 mutant (*CD3 HIGH*) as compared with the wild-type lane (*WT HIGH*). Note that these polypeptides are largely restored in the mutant greened at low light intensity (*CD3 LOW*), or in the CD3 mutant greened at high light in the presence of chloramphenicol (*CD3 CAM*). The polypeptide of ~9 kD indicated with a dot is also greatly reduced in the high light–greened CD3 mutant and restored at low light intensity. Note that this band and a second band in the 9 kD range are both absent from the thylakoids of the CD3 mutant greened at high light in the presence of chloramphenicol. These bands most likely correspond to the chloroplast-encoded cytochrome *b*-559 and the 9–10–kD phosphoprotein of photosystem II (Farchaus and Dilley, 1986).

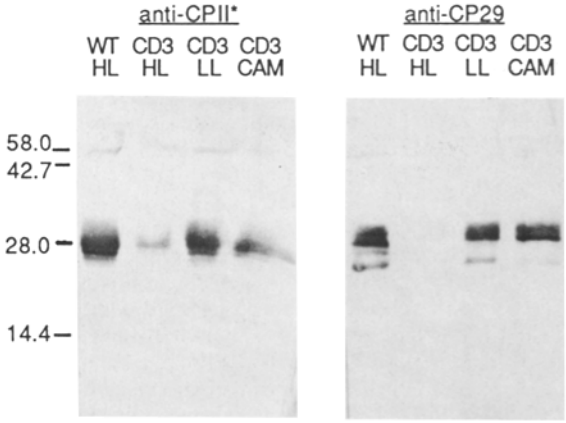


Figure 4. Western blot using polyclonal antisera directed against gel purified spinach LHCI and CP24. Antisera were prepared against green complexes purified by multiple rounds of mildly denaturing 'green gel' electrophoresis in gels of differing polyacrylamide concentrations (see Materials and Methods). Lanes are labeled as in Fig. 3.

confirms that the apoproteins of CPII*, CP29, CP24 and LHCI, all in the range from 31 to 21 kD, are substantially reduced in the high light grown mutant but are present at near wild-type levels in the mutant greened at low light (Fig. 2). This finding was confirmed by immunoblot analysis of thylakoid extracts separated by fully denaturing SDS–PAGE using antisera directed against spinach CPII*, CP29, (Fig. 3) LHCI and CP24 (Fig. 4). Anti–CPII* antisera stain a region from 31 to 24 kD in the wild-type extract. In high light CD3 extracts the overall staining intensity is greatly reduced, with the residual staining centered around 26 to 27 kD. The CD3 mutant grown at low light intensity shows a large increase in CPII* staining, although the wild-type level of staining is not fully restored. Antisera directed against CP29 recognize a diffuse region from 30 to 28 kD and main bands at 25 and 22 kD. These bands are only barely detectable in the high light CD3 thylakoid extracts but are present in nearly wild-type levels in the low light CD3 lane. Anti–LHCI antisera recognize bands at 26 and 23 kD in the wild type. These bands are detectable in slightly reduced amounts in the low light CD3 extracts but are not detectable in high light CD3 extracts. Anti–CP24 antisera recognize a major band at 23 kD and fainter bands at 24.5, 21, and 18 kD. The 18-kD band is a component of the water splitting apparatus that contaminated the original antigen preparation (Dunahay and Staehelin, 1986; Dunahay, T. G., unpublished results). The 23-kD band is greatly reduced in high light CD3 samples and is partially restored in low light samples, but the satellite bands at 24.5 and 21 kD remain relatively constant in the wild type and in the CD3 mutant grown at high and low light intensities.

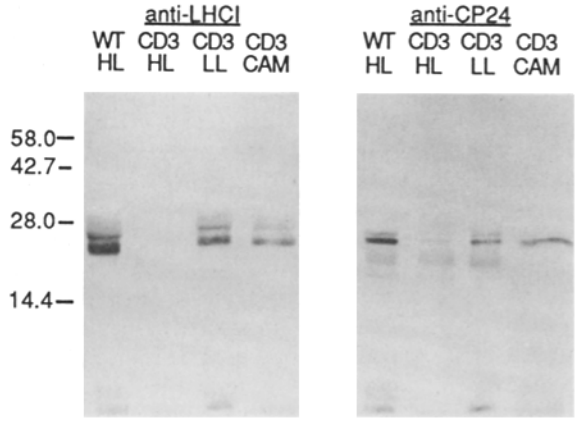


Figure 3. Western blot analysis of isolated thylakoid polypeptides separated by fully denaturing SDS–PAGE and transferred to nitrocellulose membranes. Polyclonal antisera to CPII* and CP29 were prepared against the spinach apoproteins excised from fully denaturing SDS-polyacrylamide gels (see Materials and Methods). Lanes: (*WT HL*) wild-type wheat, high light; (*CD3 HL*) CD3 high light; (*CD3 LL*) CD3 low light; (*CD3 CAM*) CD3 high light plus chloramphenicol.

When the CD3 mutant is greened at high light intensity in the presence of chloramphenicol, total pigment accumulation and leaf expansion, although still somewhat depressed relative to the wild type grown at high light intensity, are much greater than in the untreated mutant plants. Chlorophyll a/b ratios for the chloramphenicol-treated CD3 mutant grown at high light intensity range from 2.5 to 3.5 (compared with >6.0 for the untreated mutant greened at this light intensity). Analysis of fully denaturing SDS–PAGE of thylakoid extracts (Fig. 2) reveals that the light-harvesting polypeptides in the range from 31 to 21 kD that are absent or reduced in the untreated mutant accumulate in the treated mutant, confirming earlier observations (Duysen et al., 1985). These authors found that the chloramphenicol-treated CD3 mutant grown at high light intensity was similar in many respects to the wild type grown at the same light intensity in the presence of chloramphenicol. We further note that a number of bands present in the wild type and in the two untreated mutant lanes are missing or greatly reduced in the chloramphenicol-treated mutant extracts.

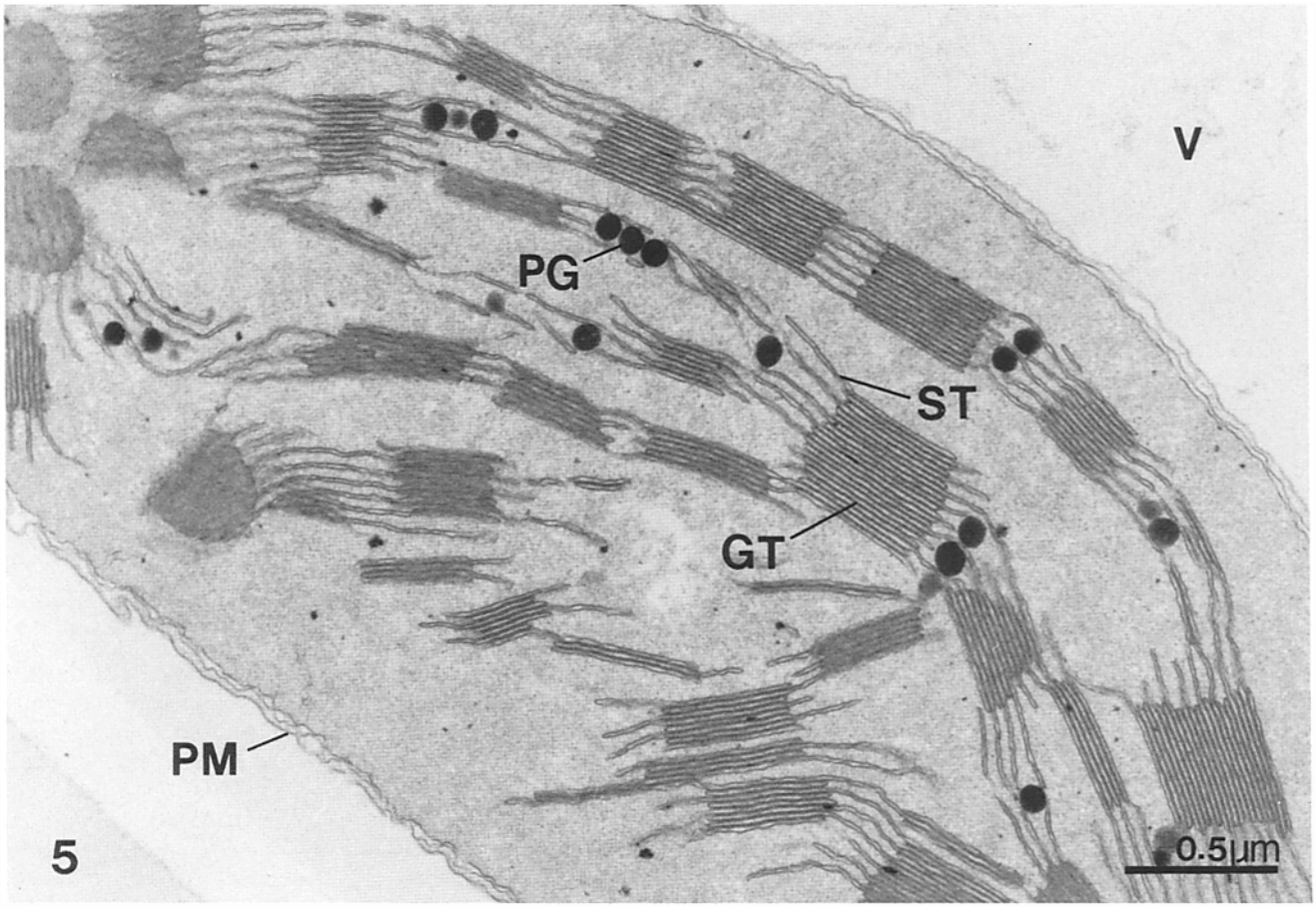


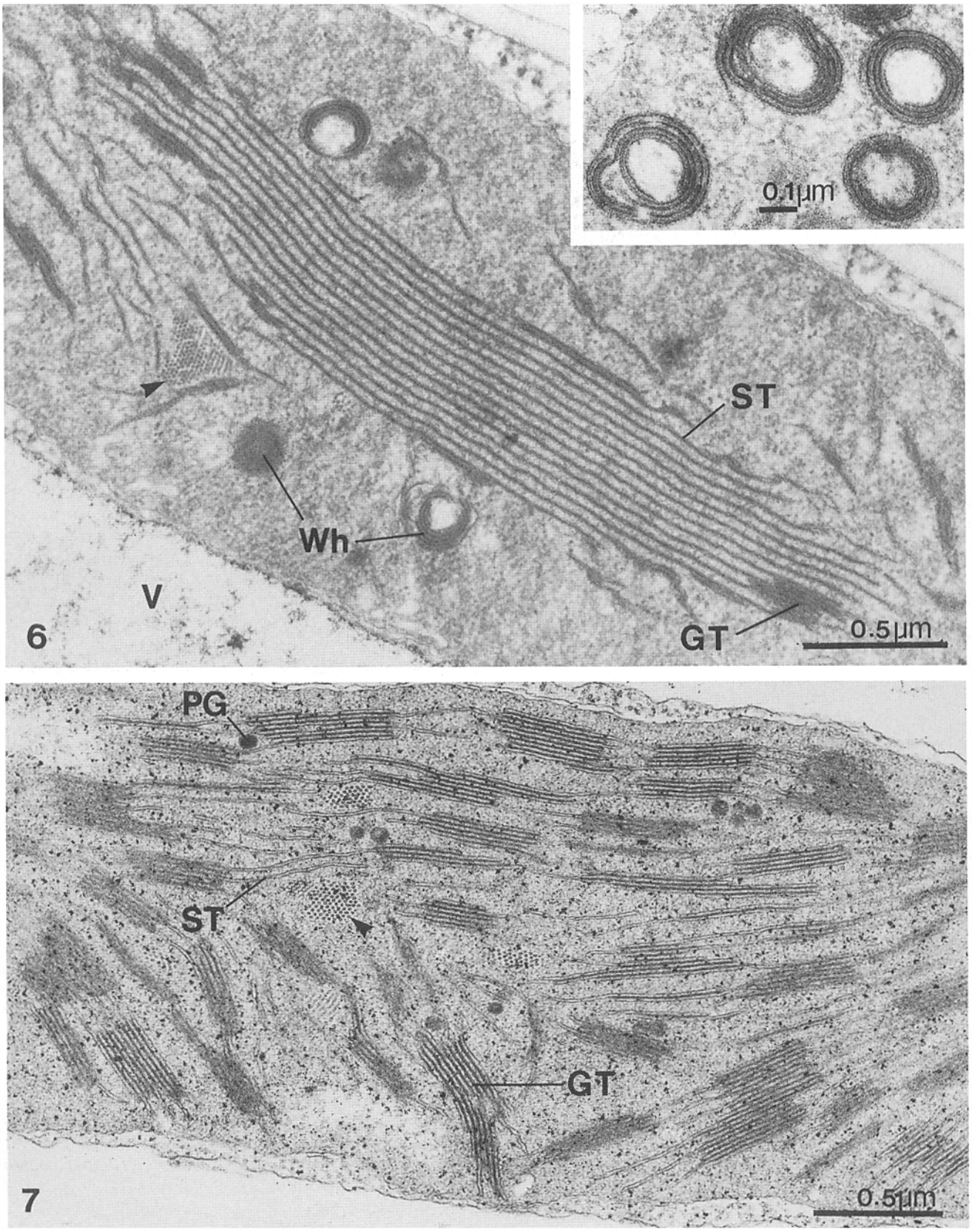
Figure 5. Thin section electron micrograph of a chloroplast from wild-type wheat greened at 400 μE/m² s. The internal chloroplast membranes are differentiated into stacked grana thylakoid (*GT*) regions, and unstacked stromal thylakoid (*ST*) regions. (*PG*) Osmiophilic plastoglobuli; (*V*) vacuole; (*PM*) plasma membrane.

These observations are confirmed and extended by immunoblot analysis of thylakoid extracts separated by fully denaturing SDS–PAGE (Figs. 3 and 4). Immunoreactive material staining with anti-CPII* antisera is reduced in level in the chloramphenicol-treated mutant, but is several-fold higher than in the untreated mutant at this light intensity. The level of staining with anti-CP29 antisera is close to that of both wild type and the mutant greened at low light intensity. Staining with anti-LHCI antisera is reduced relative to the wild type, but the untreated mutant at high light intensity does not contain detectable levels of LHCI immunoreactive material. Interestingly, anti-CP24 antisera stain only the main band at 23 kD in the chloramphenicol-treated mutant. The three satellite bands, which were relatively unaffected in the untreated high light CD3 samples, are not detectable in this tissue.

Thin Section Chloroplast Morphology

Fig. 5 shows a thin section electron micrograph of a mature chloroplast taken from wild-type wheat greened for 4 d under an illumination of 400 μE/m² s (high light). The internal chloroplast membranes show the typical differentiation into appressed grana thylakoid regions and nonappressed stromal lamellae. While there is little starch storage in these chloroplasts at this stage of development, osmiophilic plastoglobuli are evident in most sections. Thin section electron microscopy of CD3 chloroplasts greened for 4 d at high light intensity reveals an unusual morphology characterized by a tremendous reduction in the amount of chloroplast stacking and a virtual absence of starch granules or plastoglobuli (Fig. 6). Three types of membrane structures are observed in these chloroplasts. The predominant structures at this stage of greening are parallel unstacked thylakoid arrays with small stacked regions displaced toward the edges of the array. These stacked regions are typically just doublet or triplet stacks, but in some sections may contain up to 10 short grana discs. The unstacked thylakoids in these arrays show a remarkably regular interthylakoid spacing of ~18 nm and a periodicity (measuring from lumen to lumen) of 32 nm.

A second prominent feature of these chloroplasts is the presence in the stroma of membrane whorls consisting of concentrically arranged appressed thylakoids, often enclosing an electron lucent space (Fig. 6 and inset). These whorls, which typically contain from three to six thylakoids, are most often seen in the stroma around the margins of the plastid, though in some sections they appear to be budding off the end of thylakoid arrays. The third membrane structure observed in these plastids consists of loosely organized, usu-



Figures 6 and 7 (Fig. 6). Thin section electron micrograph of a chloroplast from the CD3 mutant greened at high light intensity (400 μE/m² s). Stromal thylakoids (ST) are arranged into parallel unstacked arrays with small grana thylakoid stacks (*GT*) excluded to the edges of the array. Note that in this preparation the thylakoid lumen is highly electron dense (cf. Figs. 5 or 7). The semi-crystalline array (*arrowhead*) resembles the stromacenters described by Gunning (1965). In longitudinal section these arrays are seen to be composed of tubular elements. The tightly appressed membrane whorls (*Wh*) seen here are present in the stroma of virtually every chloroplast section

ally short, singlet and doublet thylakoids dispersed in the stroma. In most sections these thylakoids (visible in the left hand margin of the chloroplast shown in Fig. 6) are seen around the margins of the chloroplast, but in some sections these are the predominant membrane structures observed. When all of these three membrane structures are quantitated, including the membrane whorls as stacked membrane, it is found that the stacked to unstacked ratio is 24% stacked versus 76% unstacked. Quantitation of thylakoid membranes from wild-type wheat grown at the same light intensity (400 $\mu E/m^2$ s) yielded a ratio of 61% stacked vs. 39% unstacked membranes, characteristic of most higher plant chloroplasts.

After 4 d of greening at low light intensity, CD3 chloroplast ultrastructure appears similar to wild type (Fig. 7). In particular, the percentage of stacked membranes approaches that of wild-type thylakoids, and plastoglobuli but no starch granules are observed in the stroma. An intriguing feature of these plastids is the generally poor overall organization of the thylakoid membranes as compared to wild type. Individual grana stacks are oriented more or less at random angles relative to each other and the interconnecting stromal membranes do not appear as well developed. The parallel unstacked arrays and membrane whorls characteristic of the CD3 mutant grown at high light intensity are never observed in the mutant grown at low light intensity.

Freeze-Fracture Electron Microscopy of Thylakoid Membranes

Changes in the composition and overall morphology of membrane systems are frequently accompanied by changes in supramolecular organization. With this in mind, thylakoid membranes were isolated from the wild type and the CD3 mutant greened at high light intensity, and from the chloramphenicol-treated CD3 mutant grown at high light intensity, and were examined by freeze-fracture electron microscopy. Replicas from wild-type thylakoids (Fig. 8) were essentially identical in appearance and particle distribution to those reported for other plant species (reviewed in Staehelin, 1986). In contrast, substantial differences in particle sizes and densities were observed in thylakoid membranes from the CD3 mutant greened at high light intensity (Fig. 9). Quantitation of wild-type and mutant freeze-fracture particles is shown in Table II. Particle size distribution histograms are shown in Fig. 10. To quantitate changes in particle populations between the mutant and the wild-type membranes, it was necessary to take into account the large differences in particle densities and in the ratios of stacked vs. unstacked membrane regions (61% stacked for the wild type vs. only 24% stacked in the high light–greened mutant). Appropriately weighted difference histograms are shown in Fig. 11. It should be emphasized that these weighted difference histograms reflect changes in populations of particles rather than simple changes in size distribution for a particular fracture face.

Table II. Particle Density Measurements In Average Number of Particles Per Square Micron of Membrane

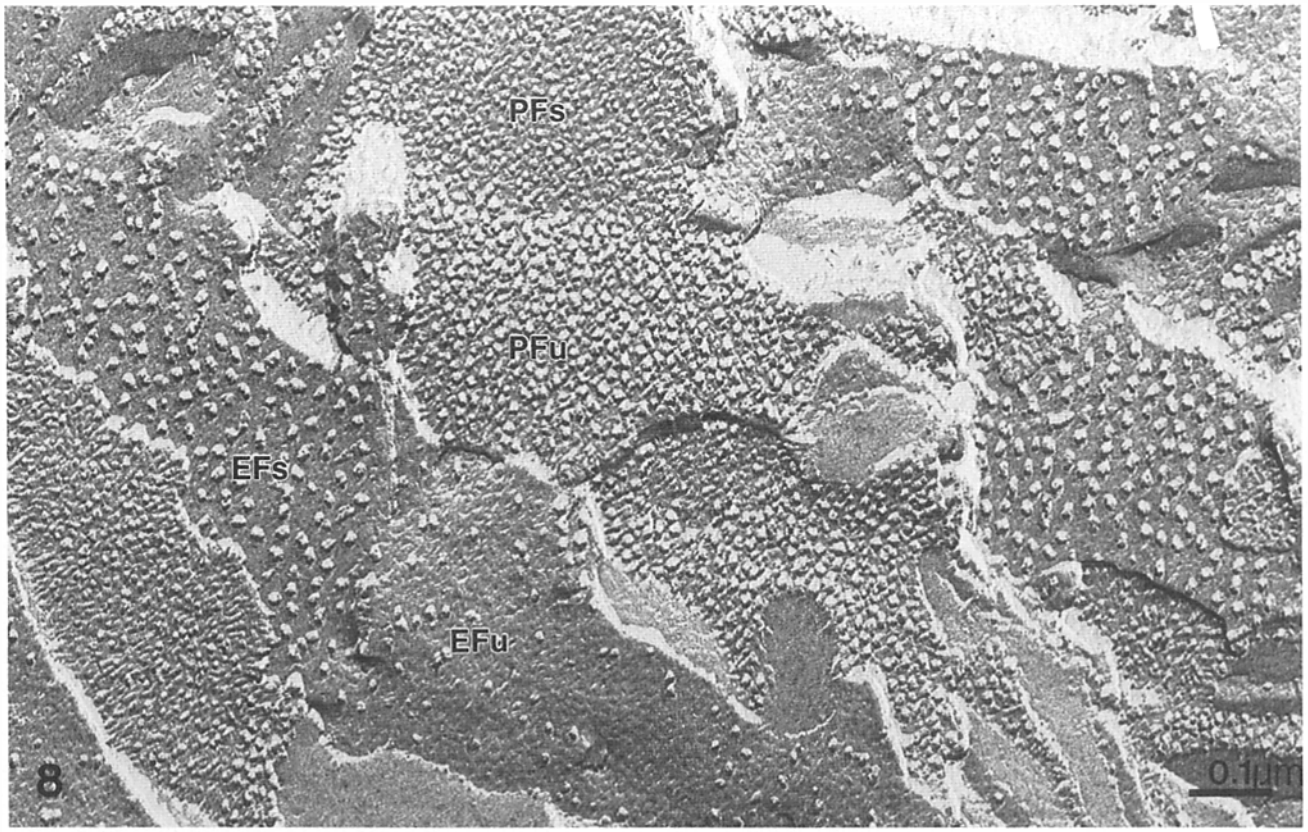
	Fracture Face			
	EFs	EFu	PFs	PFu
Wild type high light*	1,125	415	1,752	2,445
CD3 high light	1,325	773	1,200	1,615
CD3 high light + CAM‡	136	n.d.§	1,467	n.d.

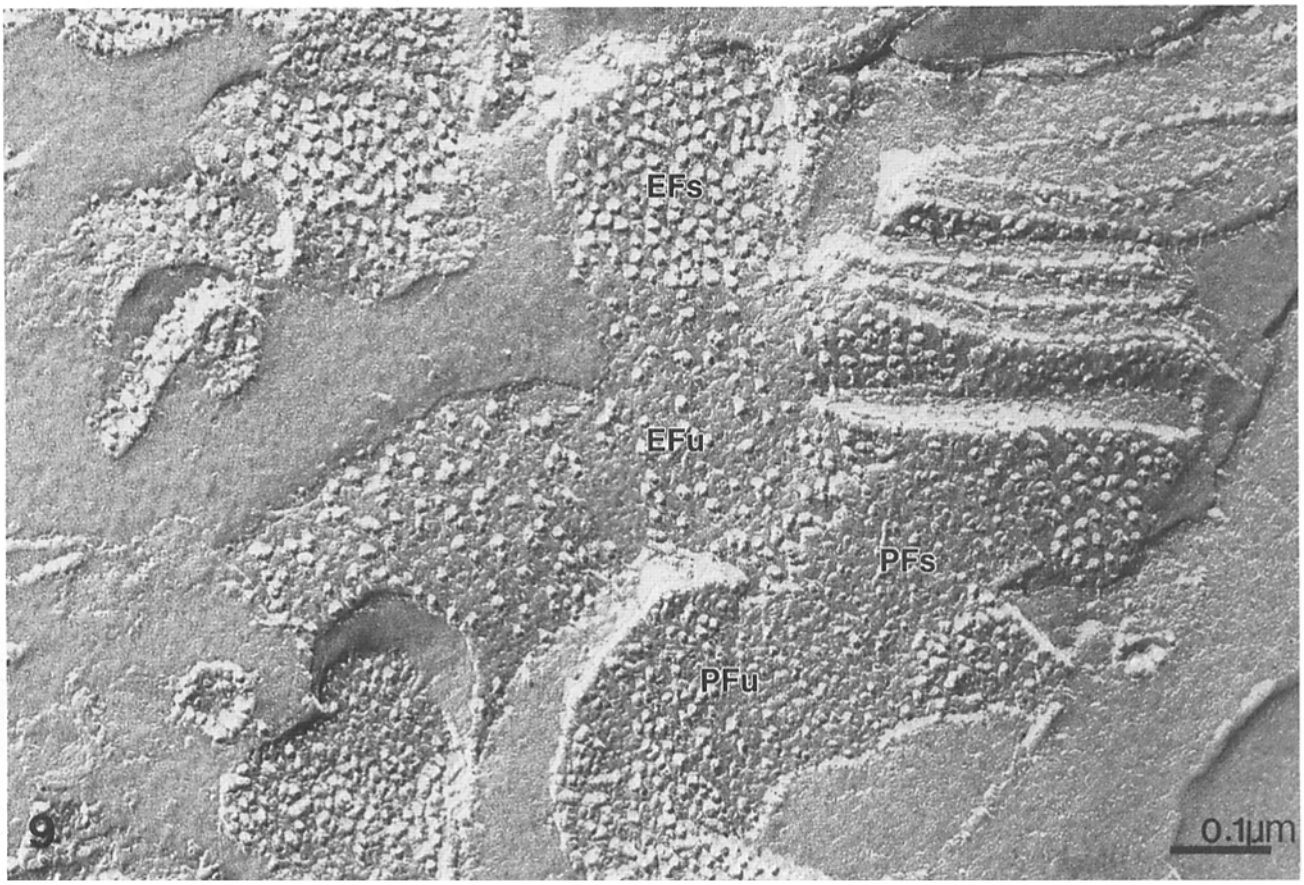
* High light = 400 $\mu E/m^2$ s.
‡ CAM: chloramphenicol.
§ n.d.: no data.

One of the most striking differences between the wild type and the untreated CD3 mutant thylakoids was in the size and distribution of E-face thylakoid (EF) particles. As shown in Fig. 10, the average size of EF stacked particles decreases from 13.7 nm in the wild type to 12.4 nm in the mutant. Accompanying this is an increase in the average size of EF unstacked (EFu) particles from 11.0 to 11.6 nm. The change in EFs mean particle sizes was determined to be significant at the <0.0001 level by the Kolmogoroff/Smirnoff test of two independent samples. The difference in EFu mean particle sizes was significant at the <0.001 level. Even more striking than these size changes, and readily apparent in the freeze-fracture micrographs shown in Figs. 8 and 9, are the large changes in the density of EFs and EFu particles. Specifically, density of EFs particles increases by 18%, from 1,125 particles/μm^2 in the wild type to 1,325 particles/μm^2 in the mutant. Accompanying this is an 86% increase in EFu particle density from 415 particles/μm^2 to 773 particles/μm^2 (Table II). As shown in Fig. 11, this large increase in EFu particle density coupled with the increase in proportion of unstacked membrane area from 39 to 76% in the mutant gives rise to a substantial increase in the total number of EFu particles in the mutant. On the other hand, the modest increase in EFs particle density does not fully compensate for the observed decrease in stacked membrane area. Thus, in the high light grown CD3 mutant a net loss of EFs particles coincides with an increase in the numbers of large, 'new' EFu particles.

Changes were also noted in the distribution of P-face thylakoid (PF) particles. Although the high light CD3 mutant thylakoids displayed no statistically significant change in the average size of PF stacked (PFs) particles, there was a subtle shift toward larger particles apparent in the size frequency histograms shown in Fig. 10. More importantly, there was a 32% decrease in the density of PFs particles from 1,752 particles/μm^2 to 1,200 particles/μm^2. Taken together with the large decrease in the proportion of stacked membrane area, this translates into a large net loss of PFs particles, evident in the weighted difference histogram shown in Fig. 11. The shape of the distribution of missing particles is very similar to the shape of the normal PFs particle distribution (compare

observed in this tissue. (*Inset*) Higher magnification view of membrane whorls. (Fig. 7) Thin section electron micrograph of a chloroplast from the CD3 mutant greened at low light intensity (150 $\mu E/m^2$ s). The degree of grana formation is close to that of the wild type (cf. Fig. 5). Plastoglobuli (*PG*) are frequently observed in the stroma. The membrane whorls seen in the high light CD3 chloroplasts (Fig. 6) are never observed in low light CD3 chloroplasts. (*GT*) grana (stacked) thylakoids. (*ST*) Stroma (unstacked) thylakoids. The arrowhead indicates a tubular semi-crystalline array, possibly a stromacenter.





Figures 8 and 9. (Fig. 8). Freeze-fracture electron micrograph of thylakoid membranes from wild-type wheat greened at high light intensity. The four fracture faces, identifiable by the characteristic size and density of particles on each face, and by their spatial orientation with respect to each other, are indicated (see Staehelin, 1986 for review). (*EFs*) E-face, stacked thylakoids; (*PFs*) P-face; stacked thylakoids;

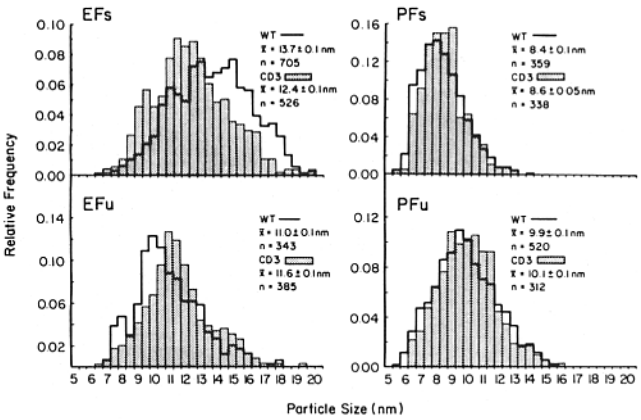


Figure 10. Histograms showing the distribution of freeze-fracture particle sizes on the four fracture faces of the wild-type and the CD3 mutant grown at high light intensity. Fracture faces are as labeled in Fig. 8.

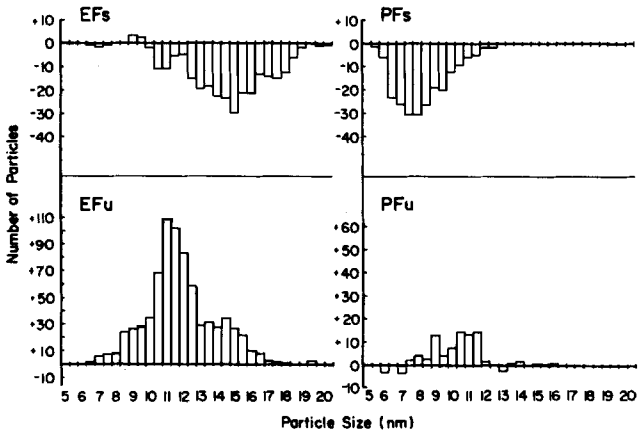


Figure 11. Weighted difference histograms (CD3-wild type) showing the changes in populations of freeze-fracture particles observed in the CD3 mutant grown at high light intensity. Fracture faces are as labeled in Fig. 8.

Figs. 10 and 11), indicating a loss of the entire range of PFs particle size classes. the small increase in average PF unstacked (PFu) particle size from 9.9 to 10.1 nm was not statistically significant. There was, however, a 43% drop in PFu particle density from 2,856 particles/μm^2 to 1,615 particles/μm^2. This decreased particle density was offset by the large increase in the proportion of unstacked membrane area to yield a modest net appearance of PFu particles in the 9 to 11.5 nm size range.

Ultrastructure of CD3 Chloroplasts Greened at High Light in the Presence of Chloramphenicol

Treatment of CD3 plants with chloramphenicol just before a 4-d high light greening period, while stimulating the accumulation of chl b and its binding proteins (see Figs. 2–4), does not result in the formation of normal thylakoid morphology. Chloramphenicol treatment results in 'hyperstacking' of thylakoid membranes and what would appear to be a general depression of membrane expansion (Fig. 12). The majority of the thylakoids are arranged into large stacked regions which may extend half the length of the chloroplast. The remainder of the membranes are arranged in loosely organized tubular networks that appear in some sections to be remnants of the prolamellar body. Numerous plastoglobuli, often packed into hexagonal arrays, are observed in these plastids.

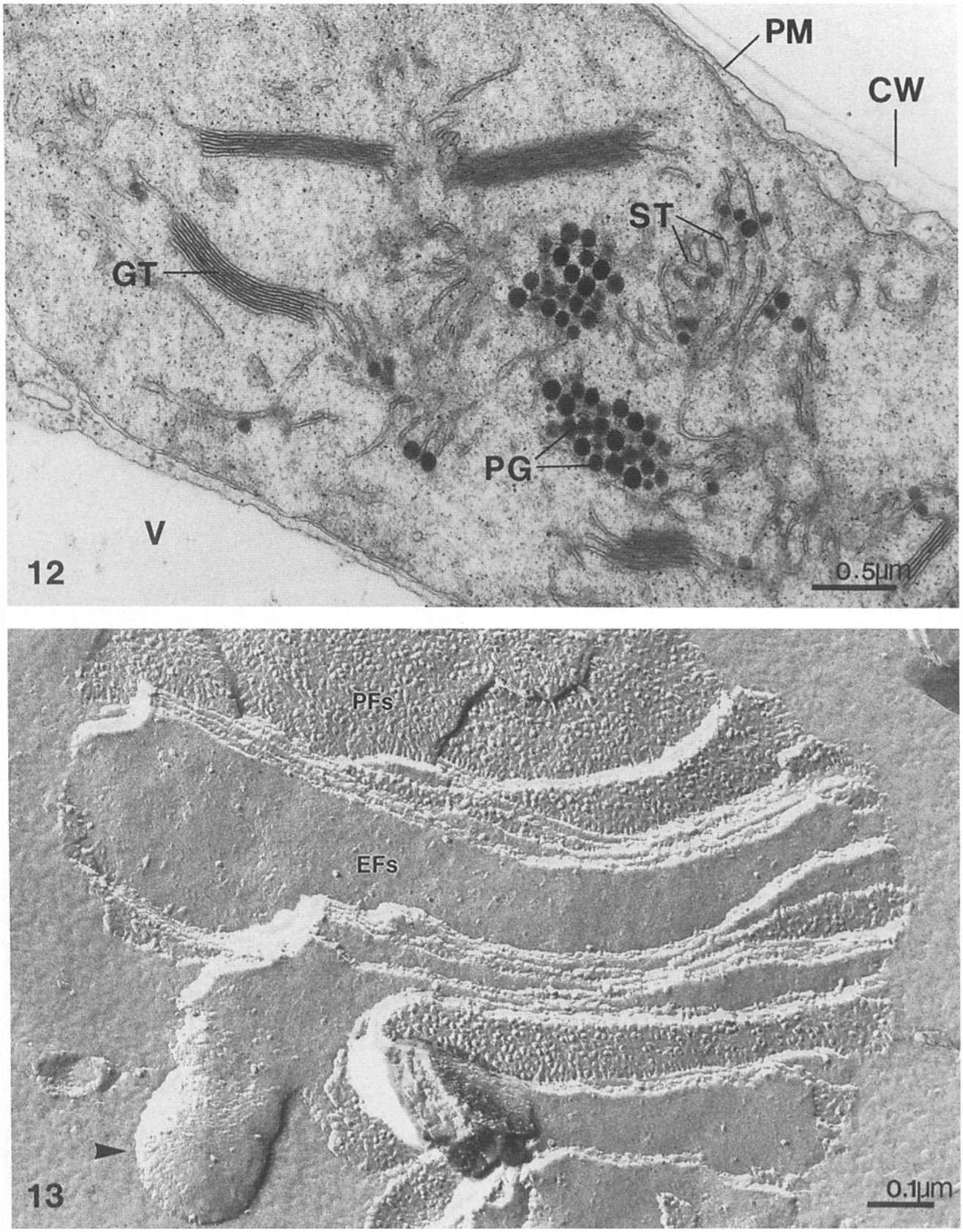
A complete analysis of the freeze-fractured thylakoids from the chloramphenicol-treated CD3 mutant greened at high light intensity was rendered impossible by the bizarre morphology of this tissue. While PFs and EFs faces were readily apparent in fractures through giant grana stacks (Fig. 13), it was very difficult to find more than small blebs of unambiguously identifiable EFu and PFu regions. It is likely that these unstacked membranes, visible as indistinct tubules in thin sections, survived poorly in the processing steps before freezing, although remnants are occasionally observed around the margins of a stack (arrowhead in Fig. 13). Unstacked membranes also exist at the top and the bottom of these giant stacks, but the number of such fractures was sufficiently small to render analysis of these faces impractical. The most striking feature of these replicas is an 88% reduction in the density of EFs particles and a 16% reduction in the density of PFs particles. Due to the small number of EFs particles present in these replicas we were not able to obtain a statistically significant average particle size for this fracture face. Measurements of PFs particles showed an increase from 8.4 nm in the wild type to 9.6 nm in the chloramphenicol-treated mutant (Fig. 14).

Discussion

Nature of the CD3 Mutation

The pleiotropic nature of the CD3 high light phenotype, affecting the entire range of chl a/b–binding proteins, makes it very unlikely that the CD3 mutation is one that completely blocks the expression or function of a single light-harvesting polypeptide. Studies with the *chlorina*-f^2 mutant of barley have established that chl b plays a role in stabilizing chl b–binding proteins, and hence in regulating the accumulation of these proteins (Bellemare, et al., 1982). Thus, the most likely explanation for the decreased accumulation of the chl a/b–binding antenna polypeptides in the high light CD3 mutant is that the mutation leads to a decrease in chl b levels at high light intensity that secondarily affects the stability of chl b–containing complexes in the membranes. It does not appear, however, that the primary lesion in the CD3 mutant completely disrupts the expression or function of a gene product required for chl b biosynthesis, given that chl b accumulates to near normal levels when CD3 plants are greened at low light intensity (150 $\mu E/m^2$ s), or at high light (400 $\mu E/m^2$ s) in the presence of chloramphenicol, an in-

(*EFu*) E-face, unstacked thylakoids; (*PFu*) P-face, unstacked thylakoids. (Fig. 9) Freeze-fracture electron micrograph of thylakoid membranes from the CD3 mutant greened at high light intensity. The four fracture faces are labeled as in Fig. 8. Note the increased density of particles on the EFs and EFu faces, and the decreased density of particles on the PFs face.



Figures 12 and 13. (Fig. 12) Thin section electron micrograph of a chloroplast from the CD3 mutant greened at high light intensity in the presence of chloramphenicol, an inhibitor of organellar ribosomes. The formation of stacked grana thylakoids (*GT*) is greatly stimulated (cf. Fig. 6), but unstacked stroma thylakoids (*ST*) are present only as loosely organized tubular networks. Overall, there appears to be a general depression of membrane formation. Plastoglobuli (*PG*) are very abundant in these chloroplasts. (*V*) Vacuole; (*PM*) plasma mem-

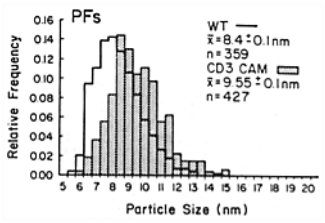


Figure 14. Histogram showing the size distribution of PFs particles in thylakoids of the CD3 mutant greened at high light intensity in the presence of chloramphenicol.

hibitor of organellar ribosomes. It should also be noted in this regard that greening of the CD3 mutant at high light intensity for several weeks results in accumulation of chl b and its binding proteins to near wild-type levels (data not shown). Thus, it appears that the mutation makes the light intensity-dependent pathway of chl b accumulation overresponsive to increases in light intensity, and that this amplification of the photoadaptation response is gradually overcome with sufficiently long growth periods at high light intensity.

One possible explanation for the light-sensitive phenotype of the CD3 mutant is that the lesion affects the accumulation of photoprotective carotenoid pigments. Hence, the decreased chlorophyll accumulation would be caused by increased rates of photooxidation of antenna chlorophylls. Several considerations argue against this hypothesis. Firstly, none of the carotenoid deficient mutants described to date would survive, much less accumulate chlorophyll pigments, at the 'low light' intensity used in our experiments (150 μE/m² s). Mayfield et al. (1986) found that in maize made carotenoid deficient by treatment with herbicides, maximal chlorophyll accumulation occurred at 0.011 μE/m² s, while exposure of these plants to light levels of 96 μE/m² s for 24 h resulted in 90% photooxidation of chlorophyll pigments. Second, the carotenoid deficiencies reported to date affect accumulation of both chls a and b. To our knowledge there are no carotenoid deficiencies resulting in a preferential loss of chl b. Lastly, the ratio of total carotenoid to chlorophyll actually increases relative to the wild type in the CD3 mutant grown at high light intensity (Freeman et al., 1982). Thus, it seems unlikely to us that the primary lesion in the CD3 mutant disrupts carotenoid biosynthesis.

Effect of Chloramphenicol

Greening of CD3 plants at high light intensity in the presence of chloramphenicol brings about a substantial accumulation of chl b and its binding proteins, as previously reported (Duysen et al., 1985; Duysen et al., 1987). But it is important to note that this treatment does not result in a complete rescue of the mutant phenotype. The polypeptide profile of isolated thylakoids from this tissue (Fig. 2) shows a number of differences from the wild-type control, as would be expected if the chloramphenicol inhibition of organellar ribosomes were effective. Further, thylakoid morphology is substantially altered after this treatment (Figs. 12 and 13). The 'hyperstacking' evident in thin section micrographs has also been reported for wild-type barley treated with chloramphenicol (Henningsen et al., 1974). The profusion of plastoglobuli and the presence of poorly organized tubular membranes resembling remnants of prolammelar bodies suggests that this treatment interferes with normal membrane formation in these plastids. The near absence of EFs particles, which correspond to PSII core complexes with varying amounts of bound LHCII, in fractures through the large stacked regions is not particularly surprising in light of the fact that much of the PSII reaction center is encoded in the chloroplast genome (Palmer, 1985) and that the stable assembly of the PSII complex is dependent on the chloroplast-encoded subunits (Jensen et al., 1986). A reduction in activities of PSI and PSII has been observed in *Chlamydomonas* cells greened in the presence of chloramphenicol (Miller and Ohad, 1978). This loss of activity was correlated with a reduction in the number of EFs particles. Nuclear PSII-deficient mutants of barley (Simpson et al., 1978), pea (Popov et al., 1981), and maize (Leto et al., 1982) produce hyperstacked thylakoid membranes, with dramatically reduced numbers of EFs particles. EFs particles are also missing in a PSII-deficient mutant of tobacco that does not display hyperstacking (Miller and Cushman, 1979).

The simplest model to account for the thylakoid morphology of chloramphenicol-treated CD3 plants greened at high light intensity is that the accumulation of LHCII leads to a large increase in stacking, but that the normal controls on the extent of thylakoid appression are eliminated as a result of chloramphenicol treatment. The mechanism(s) underlying the accumulation of chl b and its binding proteins under a light regime that would normally produce chl b deficiency in this mutant is still obscure. One possibility is that the reduction in the amounts of the chl a–binding proteins associated with PSI and PSII eases the demand for chl precursors which can then be shunted to chl b biosynthesis. Whatever the cause, the stimulation of accumulation of chl b and polypeptides not present in the untreated mutant is a factor which must be taken into consideration in thinking about the nature of the lesion giving rise to the CD3 phenotype.

Freeze-Fracture Electron Microscopy

Based on previous correlative structure-composition studies of thylakoid membranes (reviewed by Staehelin, 1986) it is possible to relate the changes in freeze-fracture particle sizes and distribution observed in the CD3 mutant grown at high light intensity to the observed decreases in chl b–containing pigment-protein complexes as evidenced by changes in the green bands seen on mildly denaturing SDS-PAGE (Fig. 1) and by Western blot analysis of fully denaturing SDS gels (Figs. 3 and 4). The reduction in mean diameter of the EFs particles (Fig. 10), which correspond to PSII units with varying numbers of bound LHCII antennae, has also been reported for the *chlorina*-f² mutant (Simpson, 1979), and can be attributed to the loss of bound LHCII. The decrease in numbers of PFs particles (Fig. 11), also observed in the *chlorina*-f² mutant as well as in the OY-YG mutant of maize

brane; (*CW*) cell wall (Fig. 13) Freeze-fracture electron micrograph showing the internal structure of a giant grana stack from the CD3 mutant greened at high light intensity in the presence of chloramphenicol. EFs and PFs faces are labeled as in Fig. 8. Note the tremendous reduction in the number of particles observed on the EFs face as compared to either the wild type (Fig. 8) or the untreated CD3 mutant (Fig. 9). The arrowhead indicates a small region of unstacked membrane.

(Greene et al., 1988), can be attributed to a loss of peripheral LHCII particles, which are thought to partition with the P-face during freeze-fracture, giving rise to 8-nm PFs particles. The net redistribution of EF particles from the stacked to the unstacked regions appears to be a unique feature of the high light CD3 mutant, as might be expected in light of the fact that the other two chl b-deficient mutants for which freeze-fracture data are available (Simpson 1979; Greene et al., 1988) do not show large changes in the degree of thylakoid appression. Since peripheral LHCII appears to play a major role in the formation of appressed thylakoid membranes (Steinback et al., 1979; McDonnel and Staehelin, 1980), and hence in helping to maintain lateral heterogeneity of the thylakoid membrane, this redistribution of EF particles may be a consequence of the loss of peripheral and possibly bound LHCII. The observed increase in EFs particle density might represent the maximum possible packing of EF particles into the available stacked regions. Thus the remaining EFs particles, which would normally partition into the grana, would end up in the unstacked regions by simple physical exclusion. The net result of this postulated lateral transfer of EFs particles would be the appearance of 'new,' larger particles on the EFu fracture face (Fig. 11).

Comparison of the CD3 Mutant with Other chl b-deficient Systems

The arrangement of high light CD3 thylakoids into parallel unstacked arrays with small stacked regions displaced toward the edges of the array has also been observed as a characteristic of a few other chl b-deficient systems. For example, when etiolated bean leaves are greened in far red light, there is very slow accumulation of photosynthetic pigments, with chl b accumulation lagging significantly behind chl a (de Greef et al., 1971). Parallel unstacked arrays begin to form in these plastids at about the same time as chl b becomes detectable by the method of Arnon (1949), and the first small stacks are formed. This suggests that the formation of these small marginal stacks is required to bring the primary thylakoids together into arrays. Similar structures have also been observed in the *viridis-k*[23] and *xantha-l*[35] mutants of barley (Henningsen et al., 1974; Simpson and von Wettstein, 1980). The common feature these systems share with the CD3 high light plants is a general reduction in photosynthetic pigment accumulation and a specific deficiency in chl b (chl a/b $\geqslant$ 6.0). So the tremendous reduction in thylakoid stacking and the formation of parallel unstacked arrays characteristic of these systems would appear to be a common class of phenotype associated with chl b deficiency. On the other hand, a number of chl b-deficient mutants have been reported that show large reductions in the extent of grana formation, but do not form the parallel unstacked arrays seen in the CD3 high light plants (see Hopkins et al., 1980, and references therein).

There are also several chl b-deficient mutants that do not display significant reductions in the degree of membrane adhesion even though the accumulation of the LHCII polypeptides is significantly impaired. The most extensively studied example of this is the *chlorina*-f[2] mutant of barley (Goodchild et al., 1966; Miller et al., 1976; Simpson, 1979; Bassi et al., 1985), but also included in this group is a chl b-deficient mutant of pea (Schwartz and Kloppstech, 1982) and of clover (Nakatani and Baliga, 1985), as well as the OY-YG mutant of maize harvested after two or three weeks of growth at very high light intensity (Greene et al., 1987). These mutants define another class of phenotype associated with chl b-deficiency, with the caveat that the OY-YG mutant may be differentiated from the other members of this group in that, like the CD3 mutant, its phenotype is modulated by light intensity.

One conclusion to be drawn from this sort of comparison among chl b-deficient mutants is that these systems are not a monolithic group, and therefore that knowledge of photosynthetic pigment content does not constitute a complete description of a mutant or experimentally induced phenotype. Further, the fact that apparently similar biochemical deficiencies are associated with very different effects on thylakoid architecture suggests that there are discrete biochemical differences among these different systems that have so far escaped the resolution of SDS-PAGE analysis as applied to date. We feel that a more detailed correlative analysis of the changes in polypeptide composition and membrane architecture in these systems is warranted.

A role for the mobile LHCII polypeptides, and hence of chl b, in thylakoid morphogenesis seems well established. But the fact that major changes in LHCII content do not lead to predictable structural changes indicates that we do not yet fully understand the precise nature of this role. Barber (1986) has argued that surface charge distribution controls both the lateral separation of thylakoid membrane components and the formation of grana. This suggests that any complex with a surface exposed charged segment may play a role in thylakoid structure. Therefore PSII and LHCII, located primarily in the grana regions and carrying a relatively low net negative surface charge (see Barber, 1986 and references therein), may both participate in grana formation. Further, PSI, primarily located in the stromal lamellae and carrying a higher net negative surface charge may act to limit the extent of grana formation. In this view there may be many protein complexes involved in the formation of thylakoid architecture, and so the final structure results from a balance between forces favoring stacked membranes and those favoring unstacked membranes. The multiplicity of players in the determination of the final structural organization of thylakoid membranes makes it reasonable to assume that compositional changes more subtle than those described to date are responsible for the complex structural alterations observed in photosynthetic mutants. Further examination of mutants such as the CD3 mutant, which lead to dramatic perturbations of the thylakoid architecture, should help in the elucidation of the factors governing the formation of this three-dimensional membrane network.

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