

# Chloramphenicol Stimulation of Light Harvesting Chlorophyll Protein Complex Accumulation in a Chlorophyll *b* Deficient Wheat Mutant<sup>1</sup>

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## ABSTRACT

As compared with normal wheat leaves, the chlorina wheat mutant, designated CD3, has a high chlorophyll *a/b* ratio and a deficiency in the light harvesting chlorophyll protein (LHCP) complex. Applications of 200 micrograms per milliliter of D-threo-chloramphenicol to etiolated seedlings decreased the chlorophyll *a/b* ratio and increased the accumulation of the 27 kilodalton LHCP polypeptide and the LHCP complex in thylakoids of the mutant during greening. These data led to the suggestion that a protein encoded in chloroplast genes impaired either transcriptional, translational, or posttranslational events in CD3 wheat limiting the accumulation of the LHCP complex. The LHCP complex which accumulated in chloramphenicol treated wheat appeared functional even though chlorophyll protein complex accumulations were altered greatly in the wheat thylakoids. LHCP polypeptides were phosphorylated by action of a membrane protein kinase but yet photosystem II electron transport was impaired. The chloramphenicol treatment increased the photosystem I/photosystem II ratio of electron transport and the fluorescence emission ratio at 740 to 686 nanometers relative to those of untreated wheat. Chloramphenicol prevented development of normal granal thylakoids in normal wheat chloroplasts but not in those of the CD3 mutant. Elongated stacked thylakoids were observed in normal wheat. Net-like membranes and vesicles were noted in the stroma of chloroplasts from treated mutant seedlings.

Most chloroplast proteins are encoded in nuclear genes and are imported from the cytoplasm (11). The proteins of the LHCP complex are synthesized under nuclear gene and cytoplasmic controls, but those of the reaction centers are encoded in chloroplast genes and are synthesized in chloroplasts (8). CAP inhibits the synthesis of chloroplast proteins for the assembly of PSI and PSII reaction centers but not those of the LHCP complex (13). We applied CAP to the CD3 wheat mutant to probe gene expression for production of the LHCP complex. An impairment of all Chl protein complex accumulations in the CD3 wheat under CAP treatment could suggest that the mutation restricted nucleocytoplasmic or posttranslational events in the production of the LHCP complex. We report here that CAP stimulates the accumulation of the LHCP complex in CD3 wheat chloroplasts. Furthermore, the LHCP complex of these plastids appears to function in thylakoid membrane reactions that have been attributed to the LHCP complex. They became phosphorylated by a membrane protein kinase and functioned in the distribution of a greater proportion of excitation energy to PSI than PSII (state II). In addition, the LHCP complex accumulated in concert with an increased ratio of grana to stroma thylakoids.

## MATERIALS AND METHODS

A nonlethal Chl deficient mutant of hexaploid wheat (*Triticum aestivum* L.) was obtained through treatment of line ND 496-25 with the chemical mutagen, ethyl methanesulfonate (12). Seeds of this chlorina mutant (designated CD3) and those of normal wheat were germinated in vermiculite and watered with half-strength Hoagland complete nutrient solution. The seedlings were grown in the dark for 6 d at 25°C and high RH (≈80%). The etiolated seedlings were wetted twice with 200 μg/ml CAP, 7 h before and immediately prior to the light treatment. The wheat was greened under 1200 μmol s<sup>-1</sup> m<sup>-2</sup> irradiance in a greenhouse. A 16-h photoperiod was maintained using supplemental cool-white fluorescent lamps. Primary leaves were harvested after 4 d unless indicated otherwise.

**Electron Microscopy.** Leaf tissue was prepared for transmission electron microscopy using techniques described previously (12). Palisade mesophyll cells near the midvascular bundle and 1 cm from the leaf tip were examined.

**Pigment Protein Complexes.** Anderson's (2) method was used to extract and separate the pigment protein complexes from wheat thylakoids by using slab SDS-PAGE. The SDS/Chl ratio for solubilization of the samples was 20/1 (w/w). Each lane was loaded with 12.5 μg Chl and the absorbance of the pigmented bands on the gels, and the area of each relative to the total was determined using procedures previously described (10). Proteins were stained in a solution of 2% (w/v) Coomassie brilliant blue

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Chloroplasts of the chlorina wheat mutant, designated CD3, are deficient in the light harvesting Chl protein complexes, LHCP1<sup>2</sup> and LHCP2 (10). The LHCP is known to function in the capture of radiant energy, in thylakoid stacking, and in the distribution of excitation energy between the two photosystems. CD3 wheat chloroplasts exhibit near normal thylakoid stacking but are inefficient in the collection of radiant energy at low irradiances (12). Excitation energy absorbed at a high irradiance appears to be distributed to the two photosystem reaction centers in mutant chloroplasts in a manner similar to that in normal wheat chloroplasts (10).

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<sup>2</sup> Abbreviations: LHCP, light harvesting Chl protein(s) complex that includes LHCP1, LHCP2, and LHCP3; CP1, P700 containing Chl protein complex; CP1a, suspected oligomeric form of CP1; CPa, a Chl protein complex of PSII and an associated light harvesting Chl complex; CAP, D-threo chloramphenicol.

R 250 and 50% (v/v) methanol. The relative molecular mobility of the protein bands was determined by comparing the migration distance to that of protein standards.

**Pigment Analysis.** Plastid pigments were extracted under dim light in 80% acetone (v/v) that was saturated with NaHCO<sub>3</sub>. Chl concentrations were calculated per g fresh weight and the Chl *a/b* ratios were determined using previously developed techniques (9).

**Thylakoid Protein Phosphorylation.** Phosphorylation of the thylakoid polypeptides was accomplished by adding 10  $\mu$ Ci ( $\gamma$ -<sup>32</sup>P)ATP (20–40 Ci/mmol) to washed thylakoids, 200  $\mu$ g Chl/ml, in 15 mM Tricine (pH 8.0), 10 mM NaCl, 5 mM MgCl<sub>2</sub>, 10 mM NaF, 0.1 M sorbitol, and 0.2 mM ATP according to the technique of Steinback *et al.* (21). In some experiments, 2 mM K<sub>3</sub>Fe(CN)<sub>6</sub> was added before the sample was exposed to 500  $\mu$ mol s<sup>-1</sup> m<sup>-2</sup> irradiance. After 10 min, thylakoids were pelleted, 10,000g centrifugation, and washed twice with cold 15 mM Tricine buffer (pH 8.0). Polypeptides were extracted by heating the thylakoids to 35°C for 5 min in 0.5 M Tris (pH 6.8), 10% (v/v) glycerol, 1%  $\beta$ -mercaptoethanol, and 2% (w/v) lithium dodecyl sulfate. The polypeptides (20  $\mu$ g/lane) were separated on 10 to 20% gradient polyacrylamide slab gels and stained with Coomassie brilliant blue R 250 (2%, w/v) in 50% methanol (v/v). The radioactivity in the polypeptide bands of dried gels was detected by Kodak X-Omat AR film<sup>3</sup>.

**77 K Fluorescence.** The fluorescence emission of the wheat thylakoids was determined in capillary tubes at 77 K following phosphorylation of the thylakoid membrane proteins as described previously (10). The excitation radiation was 440 nm; the relative fluorescence emissions at 740 and 686 nm were observed. Thylakoids were prepared having 20  $\mu$ g Chl/ml in a solution of 30% glycerol, 10 mM MgCl<sub>2</sub>, 50 mM Tricine (pH 8.0), 0.2 mM ATP, 10 mM NaF and, in some experiments, 2 mM K<sub>3</sub>Fe(CN)<sub>6</sub>. An Aminco-Bowman spectrofluorometer equipped with a red sensitive (S-20 response) photomultiplier tube was used to monitor fluorescence.

**Electron Transport.** PSI and PSII electron transport rates were determined using techniques previously described (10) and are reported on a per mg Chl basis. A Clark-type electrode was used to monitor the O<sub>2</sub> flux. The PSII uncoupled electron transport rates were obtained by determining the O<sub>2</sub> evolved at pH 8.4 with 5 mM methylamine and 3 mM K<sub>3</sub>Fe(CN)<sub>6</sub>. PSI electron flow rates were calculated from the O<sub>2</sub> consumed using 2.66 mM ascorbic acid, 133  $\mu$ M 2,6-dichlorophenolindophenol, 13  $\mu$ M DCMU, 6.65 mM NaN<sub>3</sub>, 5 mM methylamine, and 133  $\mu$ M methylviologen.

## RESULTS

After 4 d greening, chloroplasts of the normal wheat (Fig. 1A) and those of the CD3 mutant wheat (Fig. 1B) were structurally similar. Numerous grana with an average of six thylakoids developed. The application of 200  $\mu$ g/ml of CAP impaired normal development of thylakoids in normal wheat. Grana with extended thylakoids were produced in these plants (Fig. 1C) and the grana/stroma thylakoid ratio was increased by CAP treatment of both wheat genotypes (Fig. 1, C and D). Numerous vesicles and netlike membranes were apparent in the stroma of mutant plastids (Fig. 1D).

The CAP treatment of normal wheat seedlings reduced Chl accumulation (Fig. 2), but had little effect on the Chl *a/b* ratio during greening of normal wheat (Fig. 3). Chl accumulation (Fig. 2), particularly Chl *b* (Fig. 3), was increased by CAP treatment

of CD3 wheat relative to that of the untreated mutant seedlings. CAP impaired pigment accumulation in CP1a, CP1, and CPa (Table I). We reported previously that the mutant was deficient in the pigment protein complexes CP1a, LHCP1, and LHCP2 (10). CAP stimulated the accumulation of LHCP in both normal and mutant chloroplasts. However, a greater amount of free pigment was noted in lanes loaded with extracts prepared from CAP-treated plants (Table I).

The CAP treatment of both normal and CD3 mutant wheat impaired accumulations of several thylakoid membrane polypeptides, including those of the reaction centers (110 kD, CP1; 46 and 50 kD, PSII), the coupling factor CF<sub>1</sub> (13, 20, 36, 54, and 58 kD), and those associated with PSII (34 kD) (Fig. 4, A–D). It was reported previously that the CD3 mutant is deficient in the LHCP polypeptides (10), but CAP treatment of the mutant stimulated an accumulation of these polypeptides (Fig. 4, C and D).

Numerous membrane polypeptides were phosphorylated by exposing wheat thylakoids to ( $\gamma$ -<sup>32</sup>P)ATP in the light (Fig. 5, lanes 1, 2, 5, and 6). Polypeptides that contained label were CP1 apoprotein, PSII (46 and 34 kD), CF<sub>1</sub> (13, 20, 36, 54, and 58 kD), and LHCP (27–29 kD). Polypeptides contained more label following the light exposure of thylakoids without ferricyanide ion, an electron transfer chain acceptor (lanes 2 and 6), than with an acceptor (lanes 1 and 5). The LHCP polypeptide (27–29 kD) was most heavily labeled. The CAP treatment impaired phosphorylation of membrane polypeptides except for the 27 to 29 kD LHCP polypeptides that were phosphorylated without an electron transfer chain acceptor (lanes 4 and 8).

The ratio of the peak fluorescence emitted at 740 to that emitted at 686 nm from chloroplasts of the normal and CD3 wheat following the light-induced phosphorylation with or without ferricyanide ion is shown in Table II. The F740/F686 ratio was higher in the normal wheat without ferricyanide ion than with ferricyanide (10) (Table II). Furthermore, the F740/F686 ratio of the mutant was low and relatively insensitive to the redox state of electron transport carriers. The F740/F686 ratio of CAP treated plants was extremely high with or without an electron transport chain acceptor. The fluorescence emitted at 686 nm was a shoulder on the major long (F740) wavelength fluorescence peak. The long wavelength fluorescence emission maximum of CAP-treated plants was blue-shifted by approximately 5 nm.

The electron transport rates of chloroplasts isolated from wheat treated with CAP are presented in Table III. The PSII electron transport rates were more sensitive to the CAP treatment than the PSI rates. This resulted in high ratios of PSI/PSII electron transport rates. PSI electron transport in normal wheat was affected little by the CAP treatment, however, the CD3 mutant was inhibited by 50%. It would appear that the mutation increased the sensitivity of PSI to CAP inhibition.

## DISCUSSION

The tight coupling of physiological and morphological events in the chloroplast biogenesis suggests a coordination of the genetically controlled programs of biosynthetic activities. The biosynthesis of Chl and carotenoids proceeds exclusively in chloroplasts, but proteins of cytoplasmic and chloroplastic origin are produced and inserted with plastid pigments into thylakoid membranes during development (19). The current concept of chloroplast biogenesis assigns a dominant role to the nucleus since most chloroplast polypeptides are encoded in nuclear genes and expressed through cytoplasmic protein synthesis (11). A protein signal of chloroplast origin has been reported to influence nuclear gene activities required for organelle biogenesis in a feedback loop (6). Our studies suggest that a protein signal originating in chloroplasts regulates the accumulation of the

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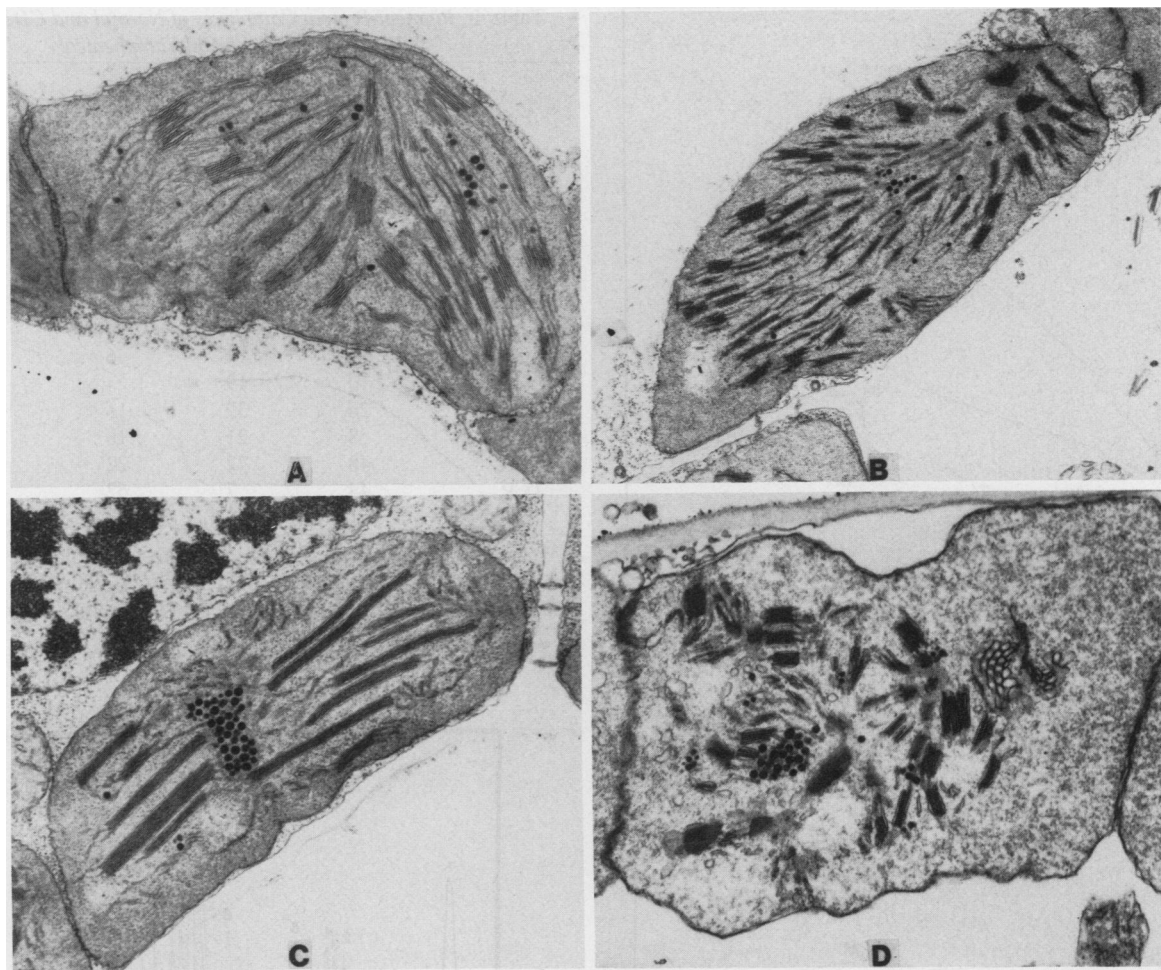


FIG. 1. Transmission electron micrographs of chloroplasts from leaves of: (A), normal wheat; (B), CD3 wheat mutant; (C), normal wheat treated with 200  $\mu\text{g/ml}$  CAP; and (D), CD3 wheat mutant treated with 200  $\mu\text{g/ml}$  CAP. Magnifications are: (A),  $\times 11,700$ ; (B),  $\times 10,000$ ; (C),  $\times 11,900$ ; and (D),  $\times 13,500$ .

LHCP complex in wheat thylakoid membranes. CAP, an inhibitor of protein synthesis on 70S chloroplast ribosomes, impaired the accumulation of CP1a, CP1, and CPa but stimulated the accumulation of the LHCP complex in a wheat mutant (CD3) deficient in the LHCP complex (Table I). The accumulations of pigment protein complexes in normal wheat and the CD3 mutant were similar following the CAP treatment. It was reported previously that both CAP (18) and lincomycin (15) impaired the production of CP1 protein. We observed increased accumulations of the 24, and 27 to 29 kD light harvesting polypeptides (Fig. 4), and the LHCP complex (Table I) in response to CAP treatment of both wheat genotypes. The Chl content of the CD3 wheat mutant relative to that of the normal wheat was significantly reduced, and the Chl *a/b* ratio was increased (12) (Figs. 2 and 3). CAP impaired Chl accumulation (Fig. 2) but had little effect on the Chl *a/b* ratio (Fig. 3) in normal wheat leaves. CAP stimulated the accumulation of Chl *b* which decreased the Chl *a/b* ratio in CD3 wheat leaves to nearly that of normal wheat (Fig. 3). DNA of the nucleus encodes protein for the LHCP complex (14, 17). It appears that CAP impaired the translation of a protein encoded by chloroplast DNA and either stimulated the translation and/or insertion of nuclear encoded LHCP protein into thylakoid membranes or impaired the rate of LHCP complex turnover in wheat chloroplasts. The mutant phenotype is conditioned by a single recessive nuclear gene located on chromosome 7D (22). It is possible that homeoallelic genes on

group 7 chromosomes of either genome A and/or B were expressed under CAP treatment of CD3 mutant wheat to yield higher accumulations of the LHCP complex.

Polypeptides of the LHCP complex (27–29 kD) become phosphorylated by a membrane bound protein kinase in the light under the control of the redox state of plastoquinone (4, 5, 16). The LHCP polypeptides (27–29 kD) of CAP-treated wheat were phosphorylated by action of a membrane protein kinase in light without ferricyanide ion (Fig. 5, lanes 4 and 8), but were not with ferricyanide ion (Fig. 5, lanes 3 and 7). CAP impaired the phosphorylation of polypeptides other than those of the LHCP complex (Fig. 5, lanes 1, 2, 5, and 6). The accumulations of these polypeptides were also impaired by the CAP treatment (Fig. 4).

The reversible phosphorylation of the LHCP complex was suggested to control the distribution of excitation energy to ensure an energy balance between the two photosystems (4, 5, 16). The phosphorylated LHCP complex was believed to direct a greater proportion of excitation energy to PSI than to PSII, and the unphosphorylated form was suggested to partition a greater fraction to PSII than to PSI. The reduction of plastoquinone in the electron transfer chain correlated with an active membrane protein kinase (1). The PSII electron transport rate of CAP-treated wheat was decreased (Table III) but yet must have been sufficient to activate the membrane protein kinase for phosphorylation of the 27 to 29 kD LHCP polypeptides without the terminal electron transfer chain acceptor (Fig. 5, lanes 4 and

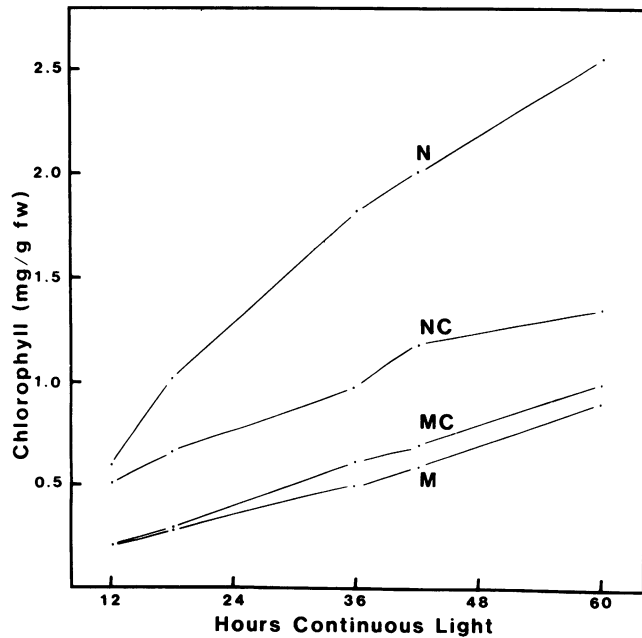


FIG. 2. Chl accumulation over a 60-h exposure period of etiolated seedlings to continuous light. Greening in normal wheat (N), normal wheat treated with 200  $\mu\text{g/ml}$  CAP (NC), CD3 wheat mutant (M), and CD3 wheat mutant treated with 200  $\mu\text{g/ml}$  CAP (MC).

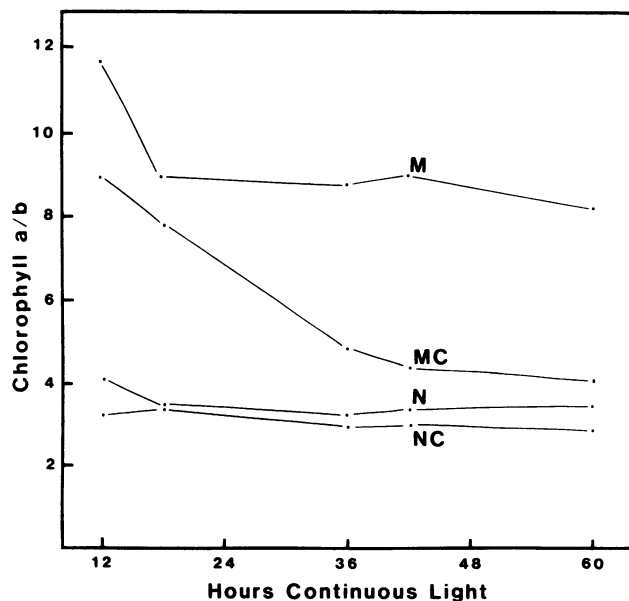


FIG. 3. Chl *a/b* ratio of etiolated seedlings exposed to continuous light over a 60-h greening period. Ratios of normal wheat (N), normal wheat treated with 200  $\mu\text{g/ml}$  CAP (NC), CD3 wheat mutant (M), and CD3 wheat mutant treated with 200  $\mu\text{g/ml}$  CAP (MC).

8).

The ratio of the peak fluorescence emitted at 740 nm (PSI) to that emitted at 686 nm (LHCP of PSII) was reported to correlate with the organization of Chl protein complexes in thylakoid membranes (3). The high F740/F686 ratio is associated with a predominance of the oligomeric forms of pigment protein complexes. The CAP treatment decreased wheat thylakoid Chl fluorescence at 686 nm and increased that at 740 nm (Table I). We did find a higher amount of the oligomeric forms of LHCP (LHCP1 and LHCP2) (Table I) in thylakoids of the CAP treatment than in untreated wheat which correlated with the high

Table I. Pigment-Protein Complexes of Normal and CD3 Mutant Wheat Treated with Chloramphenicol

Pigment Protein Complex	Treatment			
	Normal wheat	Normal wheat + CAP (200 $\mu\text{g/ml}$ )	CD3 mutant	CD3 mutant + CAP (200 $\mu\text{g/ml}$ )
	% of Chl <sup>a</sup>			
CP1a	13	3	8	5
CP1	19	4	24	4
CPa	12	<sup>b</sup>	30	-
LHCP1	12	14	6	6
LHCP2	10	19	-	16
LHCP3	26	39	16	41
FP <sup>c</sup>	8	21	16	28
TLH	48	72	22	63
TCP	44	7	62	9
TLH/TCP	1.1	10.3	0.4	7.0

<sup>a</sup> Chl is a percentage of the total absorbance at 675 nm. <sup>b</sup> -, data are missing for pigment-protein complexes with less than 1% Chl. <sup>c</sup> FP, free pigment; TLH, total light harvesting Chl *a/b* complex; TCP, total Chl of CP1a, CP1, and CPa.

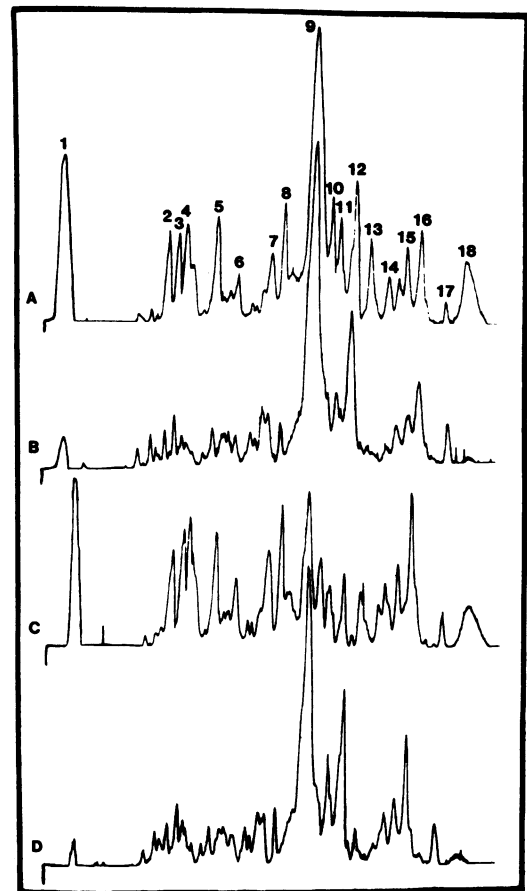


FIG. 4. Density tracings of thylakoid membrane polypeptides extracted and separated from chloroplasts of normal wheat (A), CAP-treated normal wheat (B), CD3 mutant wheat (C), and CD3 mutant wheat treated with CAP. Peaks are: (1), CP1; (2), CF1 $\alpha$ ; (3), CF1 $\beta$ ; (4), 50 kD; (5), 46 kD; (6), 43-40 kD; (7), 36 kD; (8), 34 kD; (9), 29-27 kD light harvesting Chl; (10-11), 26-25 kD; (12), 24 kD; (13), 22 kD; (14), 20 kD; (15), 18 kD; (16), 16 kD; and (17-18), 14-12 kD.

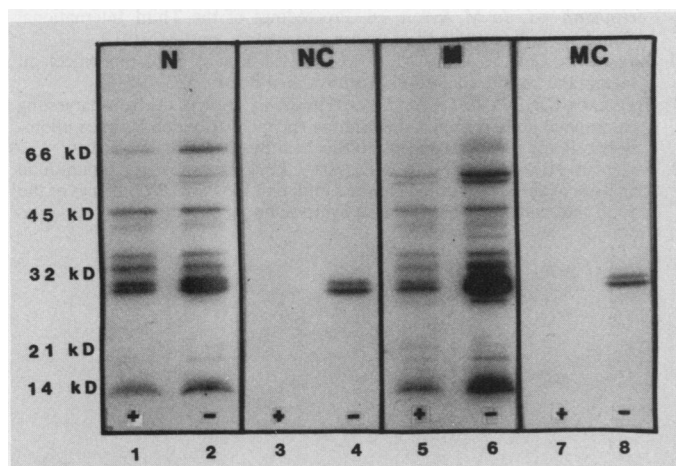


FIG. 5. Autoradiogram from LiDS polyacrylamide gels of normal wheat (N), CAP-treated normal wheat (NC), CD3 mutant wheat (M), and CAP-treated CD3 mutant wheat (MC) chloroplast membrane polypeptides following phosphorylation with ( $\gamma$ - $^{32}$ P)ATP. Membrane polypeptides were phosphorylated with (+) or without (-) 2 mM  $K_3Fe(CN)_6$  as a terminal electron transport chain acceptor in the light.

Table II. Effect of CAP on the Chloroplast Fluorescence Emission Ratio at 740 to 686 nm at 77K

Treatment	Fluorescence		Peak Wavelengths	
	-FeCN	+FeCN	740/686 nm	nm
Normal wheat	1.29	0.92	686	740
Normal wheat + CAP (200 $\mu$ g/ml)	2.40	2.36	686	736
CD3 wheat	0.97	0.83	686	735
CD3 wheat + CAP (200 $\mu$ g/ml)	2.84	2.56	686	729

Table III. Electron Transport Rates of CAP Treated Normal and CD3 Wheat Mutant Chloroplasts at 600 w/m<sup>2</sup>

Each value is the mean of three replicates.

Treatment	Electron Transport		PSI/PSII
	PSII	PSI	
	$\mu$ eq/mg Chl·h		ratio
Normal wheat	412	651	1.6
Normal wheat + CAP (200 $\mu$ g/ml)	50	608	12.2
CD3 mutant	1193	1443	1.2
CD3 mutant + CAP (200 $\mu$ g/ml)	44	763	17.3

F740/F686 ratio (Table II) for thylakoids of CAP-treated wheat. The maximum peak fluorescence emission was shifted from 686 nm in CD3 wheat mutant thylakoids (10) to 729 nm (Table II) by the CAP application. It is possible that the CAP treatment altered the association of Chl protein complexes of PSI in both the normal and mutant wheat genotypes as compared to the untreated wheat. This could direct a greater proportion of exci-

tation energy to PSI than PSII (Table II). The high ratio of PSI/PSII electron transport rates (Table III) could suggest that CAP decreased the concentration of active PSII centers. In fact, the CPa concentration (Table I) correlated with the high PSII activity of CD3 wheat chloroplasts relative to that of the normal wheat and also with the severe inhibition of PSII activity by the CAP treatment (Table III). The large difference between the F740/F686 ratio of normal wheat thylakoids following phosphorylation of thylakoid proteins with and without ferricyanide ion (electron transport acceptor) was not observed from chloroplasts of CAP-treated plants (Table II). It could be that the CAP-treated wheat had an altered membrane complex that prevented the lateral movement and/or interconnection of LHCP for either distribution of excitation energy to PSII or fluorescence emission from PSII.

The application of CAP to normal wheat seedlings either stimulated the development of grana with extended but stacked thylakoids (Fig. 1C), or impaired normal thylakoid development from the primary thylakoids. This CAP effect has been reported in other plants (7, 20). Numerous vesicles and net-like membranes were apparent in the stroma of CAP-treated CD3 wheat chloroplasts, but granal thylakoids were not elongated (Fig. 1D). The ratio of appressed to nonappressed thylakoids was increased by the CAP treatment of both wheat genotypes. The CAP treatment impaired the synthesis and normal assembly of the same pigment protein complexes (Table I), in chloroplasts of both wheat genotypes even though granal thylakoids were different between the CAP-treated normal and CAP-treated CD3 mutant wheat (Fig. 1).

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