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Biochemical analysis and photosynthetic activity of chloroplasts and Photosystem II particles from a barley mutant lacking chlorophyll *b*

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Ultrastructural, biochemical and photochemical characteristics of chloroplasts from a natural chlorophyll *b*-less mutant of barley (*Hordeum vulgare* L.) have been investigated by comparing them to the wild genotype. Photosystem II (PS II) particles and fractions enriched in light-harvesting chlorophyll *a*/*b*-protein complex (LHC II fractions), isolated from the two genotypes, have been studied. Mutant chloroplasts were characterized by the presence of macrograna and a 2-fold lower membrane density than in wild-type chloroplasts. In mutant plastids, gel electrophoresis showed a marked decrease in the 25 and 27 kDa polypeptides linked to LHC II and in the 24 kDa polypeptide assigned to Photosystem I (PS I) antennae. The use of a non-denaturating method, including solubilization of thylakoids by digitonin followed by electrophoresis in the presence of deoxycholate, as well as the analysis of LHC II fractions, confirmed the high lability of the protein-pigment associations of the PS II antenna in the mutant. Analysis of the kinetics of fluorescence induction of plastid suspensions from the two genotypes indicated an important reduction, both in size and number, of PS II antennae in the mutant. The amount of acyl lipids was 2-fold higher in mutant chloroplasts, but only slight changes were observed in their fatty acid content. Similar photosynthetic activities were noted in chloroplasts from the two genotypes, although the ratio PS I/PS II was significantly higher in the mutant.

Formation of macroganal structures in chlorophyll *b*-less plastids suggests a reorganization of the intraplastidial lamellar system to optimize energy distribution between the two photosystems.

Introduction

Chlorophyll *b* is an accessory pigment playing an important role in the harvesting of light energy in higher plants and green algea. Viable mutants deficient or lacking Chl *b* have been of great interest in the study the location and function of this pigment in plants. Gel electrophoresis of SDS-solubilized thylakoid membranes from these mutants showed the absence of green bands corre-

Abbreviations: BQ, benzoquinone; 16:0, palmitic acid; 16:1*tr*, *trans*-Δ_3-hexadecenoic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid; Chl, chlorophyll; CP I and CP II, chlorophyll protein complexes of Photosystem I and Photosystem II, respectively; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP, 2,6-dichlorophenol indophenol; DGDG, digalactosyldiacylglycerol; FeCy, ferricyanide; LDS, lithium dodecyl sulfate; LHC I and LHC II, light-harvesting chlorophyll *a*/*b*-protein complexes of photosystem I and photosystem II, respectively; Mes, 4-morphinoethanesulfonic acid; MGDG, monogalactosyldiacylglycerol; OGP, octylglucoside, *n*-octyl-β-D-glucopyranoside; PC, phosphatidylcholine; PG, phosphatidylglycerol; PS I, photosystem I; PS II, photosystem II; SQDG, sulfoquinovosyldiacylglycerol; SDS, sodium dodecyl sulfate; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-glycine.

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sponding to the light-harvesting Chl *a/b*-protein complex of photosystem II (LHC II) [1–4]. A gross reduction of polypeptides with an apparent molecular mass of 24–27 kDa, assigned to the major components of the LHC II [5], and of the polypeptides 20–22 kDa, constituents of the PS I-chlorophyll antennae (LHC I) [6], was simultaneously observed [5,7–14]. However Chl *b*-less mutants of *Chlamydomonas* which are lacking the green LHC II bands [15] do not show an altered polypeptide pattern of the LHC II, and some higher plant mutants deficient in Chl *b* and lacking the major polypeptides of LHC II have active protein kinase activities [10,12,16]. Likewise Chl *b*-less mutants show good photosynthetic rates [15,17–19], but appear inefficient at low irradiance [11], with a reduced capacity to regulate quantum energy distribution [5,18]. Alteration in the decay kinetics of chlorophyll fluorescence shows substantial reduction in the Chl light-harvesting antennae of PS I and PS II, and an alteration in the stoichiometry of reaction centers of both photosystems in Chl *b*-less plastids [20–22]. The ultrastructure of chloroplasts from Chl *b*-deficient mutants can be affected or not. Chloroplasts appear either intensively stacked [3,19,23], or grana deficient [4,7,13,23–25], or unchanged [26,27]. As regards the lipid composition of these Chl *b*-less barley mutants, only slight variations have been reported [25]. In this paper, in order to characterize a Chl *b*-less mutant of barley, ultrastructure, proteins, acyl lipid content and photochemical properties of chloroplasts from the mutant and wild genotypes have been compared. Moreover, to characterize the changes occurring in components of the antennae, photosystem II preparations (PS II particles), and fractions enriched in LHC II (LHC II fractions), have been isolated from the two genotypes and analyzed.

Materials and Methods

Materials

Seeds of wild barley (*Hordeum vulgare* L. cv. Menuet, purchased from Secrobra, France) and of a 'natural' barley mutant lacking Chl *b* (a gift of Dr. A. Faludi-Daniel, Institute of Plant Physiology, Biological Research Centre, Szeged, Hungary), were germinated at 22° C in vermiculite moistened with tap water. The light was from fluorescent lamps that provided an energy of 20 $W \cdot m^{-2}$ at the top of the seedlings. Chloroplasts were isolated from the first leaf of 10-day-old seedlings as in Ref. 28, using sorbitol instead of sucrose, and Tricine buffer instead of phosphate. Oxygen-evolving photosystem II preparations (PS II particles) were isolated from chloroplasts of both genotypes using the non-ionic detergent Triton X-100 according to Ref. 29, but the concentration of the detergent was lowered to 7 mg · per mg Chl.

A fraction enriched in PS II light-harvesting chlorophyll protein complex (LHC II fraction) was also isolated from chloroplasts after solubilization of thylakoid membranes with the non-ionic detergent *n*-octyl-β-D-glucopyranoside as described by Camm and Green [30].

Electron microscopy

For ultrastructure study, leaf slices (1 mm thick) from the different regions of the seedling primary leaves were fixed as described in Ref. 31. The biometric analysis of plastid ultrastructure was carried out from electron micrographs as in Ref. 28.

Pigment and lipid analysis

Chlorophyll concentration was determined according to Mackinney [32]. Absorption spectra of plastid fractions were measured with an Aminco DW-2A spectrophotometer, interfaced to a Midan microprocessor analyzer to obtain the fourth derivative of the absorption spectra. The band pass was 2.0 nm. Plastid fractions (10 μg Chl/ml) were suspended in 100 mM sorbitol/10 mM NaCl/10 mM Tricine-NaOH (pH 6.5).

For lipid analysis, individual lipid classes were separated by two-dimensional thin-layer chromatography on silica gel G plates (Merck). Fatty acid methyl esters, obtained after transmethylation of the lipid samples, were separated by gas chromatography in a silica capillary column as in Ref. 31.

Protein analysis

Protein content was determined according to Koch and Macmeekin [33]. Polypeptide analysis was performed by polyacrylamide gel electro-

phoresis [34], using a 10–11% LDS-polyacrylamide gel. A milder method involving electrophoresis on deoxycholate-polyacrylamide gel was also used to improve the resolution of Chl-protein complexes of thylakoids containing fragile photosystems [35]. In this case, preparation of washed thylakoids, solubilization by digitonin (detergent/Chl = 40) and separation of protein pigment complexes were performed as in Ref. 36. Gels were stained with Coomassie brilliant blue. Densitometric traces were obtained with a Vernon densitometer using standard markers (14–92 kDa).

Photosynthetic activity

Steady-state O_2 evolution ($H_2O \rightarrow FeCy + BQ$), O_2 uptake or Mehler ($DCPIPH_2 \rightarrow$ methyl viologen) reactions were measured at 25 °C with an oxygen electrode (Hansatech) as described [31]. Saturating light was provided by a cold light source (Schott).

Fluorescence induction

Fluorescence induction was assayed at 20 °C [37]. Excitation energy was provided by a 250 W quartz-iodine lamp; low intensity illuminations were obtained by adding neutral density filters. Fluorescence emission was detected by a photomultiplier (RTC XP 1017) through a red filter (Oriel 690 nm).

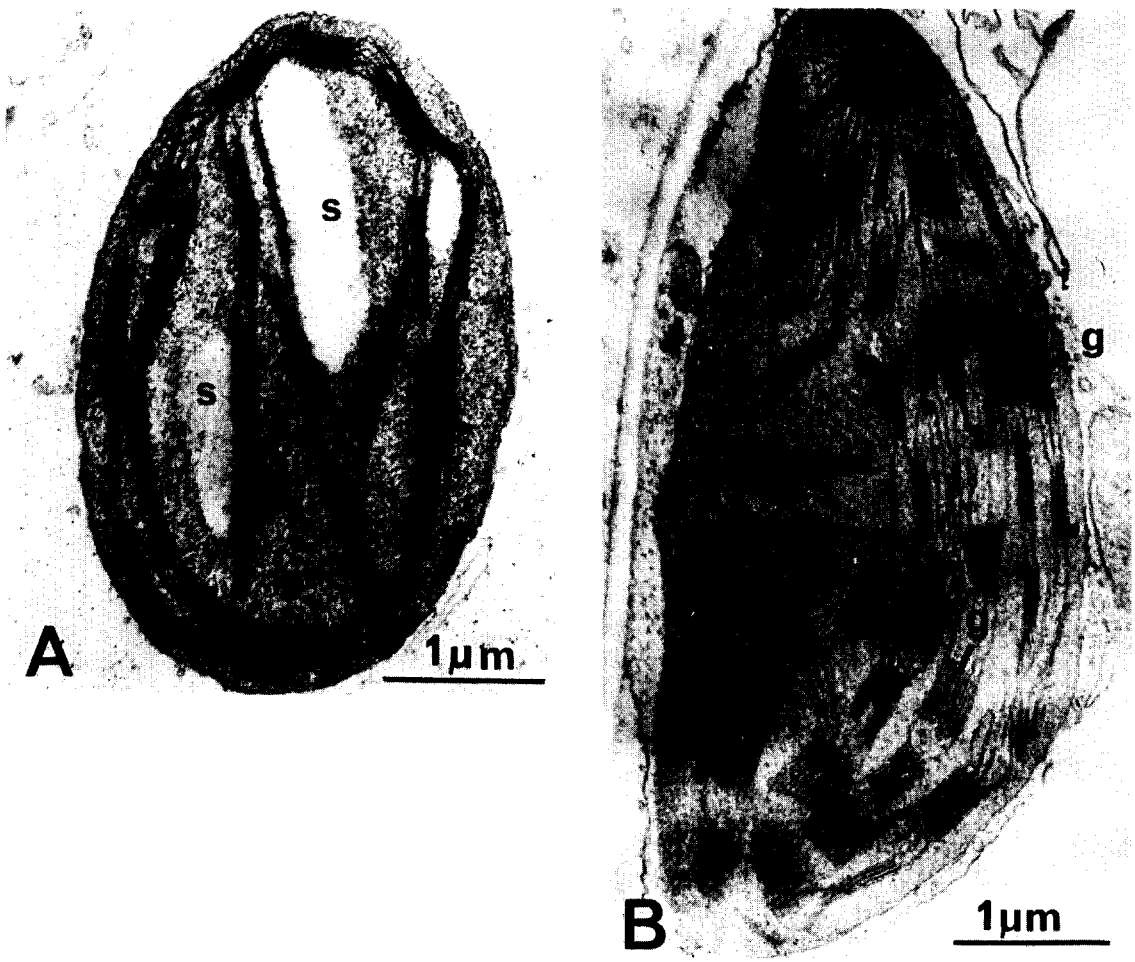

Fig. 1. Electron micrographs of chloroplasts from the basal (A) and the apical (B) regions of the primary leaf of 10-day-old wild barley seedings. Note the small size and the reduced lamellar system of developing plastids from the basal region (A) as compared to mature plastids from the apical region (B); i, intergranal thylakoids; g, granum; s, starch granule.

Results

Leaf ultrastructure

As described previously [31], near the leaf base, young chloroplasts (Fig. 1A) appeared much smaller and with less-developed granal stacks than mature chloroplasts localized near the tip of the same leaf (Fig. 1B). Many differences appeared in the mutant during plastid development as compared to the wild-type genotype. In the mutant plastids from the basal cells, only rudimentary grana, made of two thylakoids stacked on the whole length of the plastid, were present. A prolamellar body was often visible in these plastids (Fig. 2A), as in those from the middle part of the leaf, which contained numerous macrograna resulting from the stacking of five to seven thylakoids (Fig. 2B). Older plastids from the apical region contained impaired grana with up to 15 stacked thylakoids, external thylakoids being swollen and scattered in the stroma (Fig. 2C). In these degenerated organelles, swollen stroma thylakoids were often arranged in an extensive 'peripheric reticulum' [38,39].

The biometric study of plastid ultrastructure showed, per surface unit, a membrane density 2-fold lower in the mutant than in the wild type, but the ratio of grana to stroma thylakoids ap-

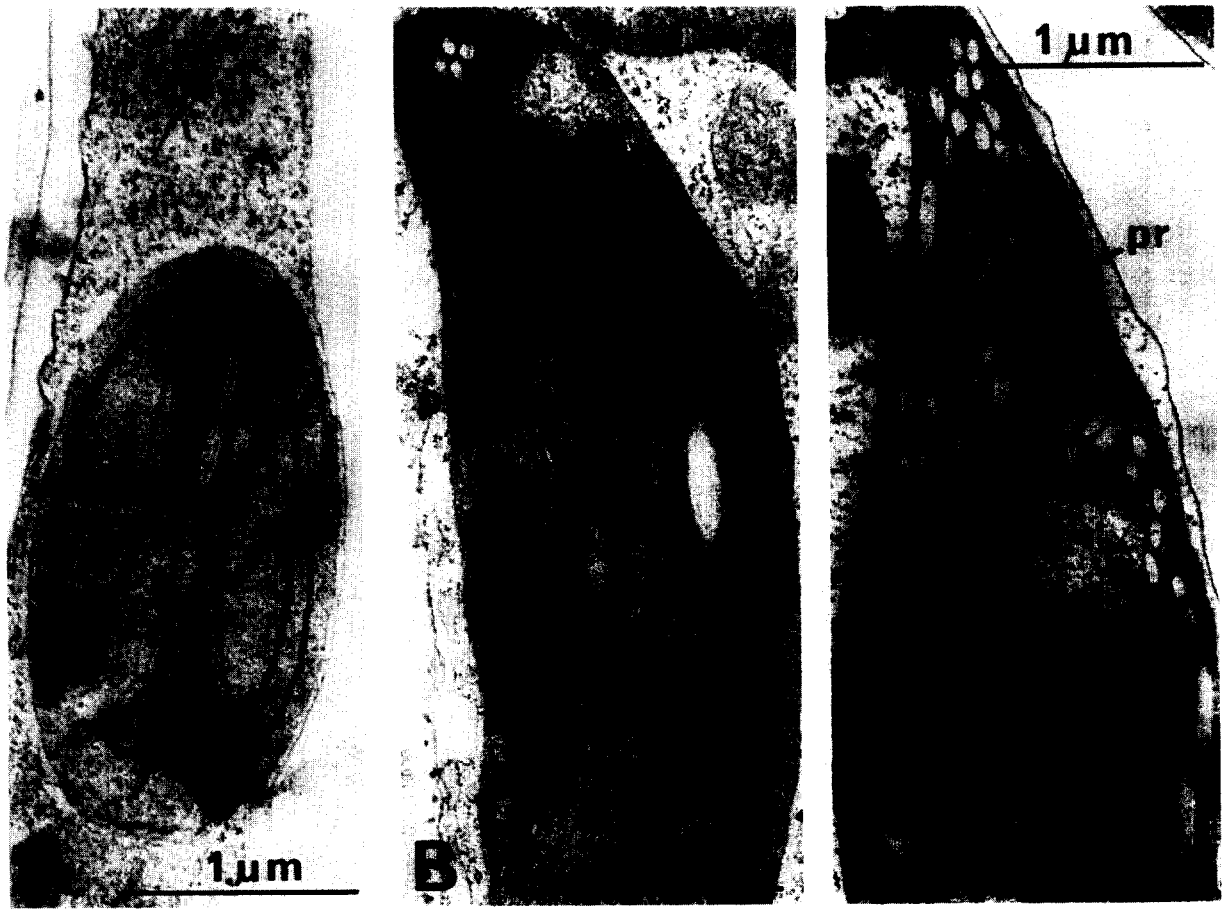

Fig. 2. Electron micrographs of chloroplasts from the different regions of the primary leaf of 10-day-old Chl *b*-less barley seedings. In chloroplasts from the basal region (A), only rudimentary grana are observed. Note the persistence of the prolamellar body (pb) in these plastids as in the chloroplasts from the middle region (B) that display numerous macrogana (mg). In older chloroplasts from the apical region (C), grana, with external swollen thylakoids (arrows), are scattered in the stroma. At the plastid extremities, swollen stroma thylakoids are arranged like a 'peripheric reticulum' (pr).

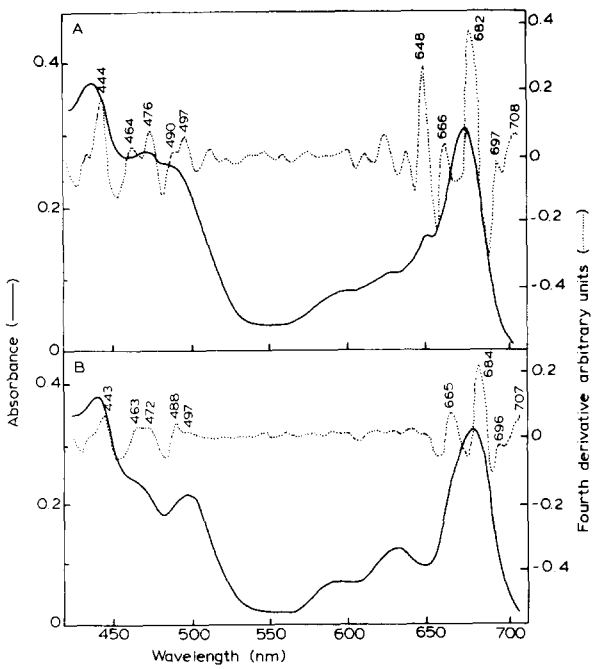

Fig. 3. Absorption and fourth derivative spectra at 77 K of chloroplasts isolated from wild (A) and mutant (B) barley. In mutant chloroplasts the spectrum lacks a band at 650 nm and no maximum at 648 nm is observed on its fourth derivative.

peared similar for the two genotypes, whatever the region of the leaf [31].

Absorption spectra

Low temperature absorption spectra with their fourth derivatives of chloroplasts isolated from the two types of barley are shown in Fig. 3. The absorption spectrum of the Chl *b*-less mutant lacked a distinct peak at 650 nm and, instead, showed a clear through at 645 nm as described in a Chl *b*-less rice mutant [40]. Moreover, no maximum corresponding to this peak was found in the fourth-derivative of the spectrum, indicating no detectable amount of Chl *b* in the mutant.

Chlorophyll and protein content

The amounts of chlorophyll, expressed on a protein basis, appeared to be much higher for wild genotype fractions than for mutant fractions (Table I). However, when data were expressed as the chlorophyll content per plastic for each genotype results were similar. Whatever the mode of reference, the protein content was much higher in mutant than in wild-type fractions, showing an accumulation of proteins in mutant plastids.

Polypeptide analysis

After staining of the gels from LDS-solubilized thylakoids with Coomassie blue, mutant chloroplasts showed a large reduction in the amount of the 25–27 kDa polypeptide characteristic of LHC II, as well as of the 125 kDa and 24 kDa polypeptides, respectively constituents of PS I center and antennae [41,42] (Fig. 4A, B). The electrophoretic analysis of PS II particles isolated from chloroplasts of the two genotypes confirms the above results with whole thylakoids. Electrophoresis on deoxycholate-polyacrylamide gels did not shown, in the mutant, the presence of the fraction 3 described by Acker et al. [43] and corresponding to the PS II antenna (data not shown). LHC II

TABLE I

CHLOROPHYLL AND PROTEIN CONTENT OF CHLOROPLASTS AND PS II PARTICLES FROM WILD AND MUTANT BARLEY LEAVES

Chloroplasts and PS II particles were isolated and the plastid number was estimated as in Materials and Methods. Mean values of three experiments ± S.D.

Barley	Fraction	Chlorophyll		Chl *a*/Chl *b* ratio	Protein		
		mg per mg protein	mol·10^{-6} per plastid		mg per mg Chl *a* + *b*	mg per mg Chl *a*	pg per plastid
Wild	chloroplasts	0.18 ± 0.02	395 ± 120	3.5 ± 0.8	5.9 ± 0.6	7.6 ± 0.4	7.4 ± 0.7
	PS II particles	0.24 ± 0.04	–	2.8 ± 0.4	4.7 ± 0.2	6.4 ± 0.6	–
Mutant	chloroplasts	0.09 ± 0.01	675 ± 110	–	–	11.8 ± 1.2	10.7 ± 1.2
	PS II particles	0.05 ± 0.01	–	–	–	18.9 ± 2.1	–

TABLE II

POLAR LIPID COMPOSITIONS OF CHLOROPLASTS AND PS II PARTICLES FROM WILD AND MUTANT BARLEY LEAVES

Mean values of three experiments ± S.D.

Barley	Fraction	Polar lipids mmol per mol Chl	(% of total polar lipids)					MGDG/DGDG
			MGDG	DGDG	SQDG	PC	PG	
Wild	chloroplasts	2906 ± 738	53.3 ± 9.9	28.4 ± 6.5	6.9 ± 0.4	5.5 ± 2.4	6.4 ± 2.7	2.0 ± 0.9
	PS II particles	2016 ± 1500	46.1 ± 13.3	21.2 ± 8.0	10.1 ± 2.1	9.5 ± 10.1	13.1 ± 4.3	2.1 ± 0.9
Mutant	chloroplasts	4478 ± 265	56.8 ± 0.3	22.2 ± 4.8	10.4 ± 2.5	3.5 ± 1.0	7.2 ± 1.5	2.7 ± 0.6
	PSII particles	4498 ± 453	55.1 ± 4.2	22.0 ± 7.2	9.4 ± 2.2	4.2 ± 1.3	9.3 ± 0.5	2.7 ± 1.0

fractions (Fig. 5A, B) isolated from the mutant with octylglucoside showed no green bands with apparent molecular masses of 27 and 64 kDa, corresponding respectively to the LHC II monomer and oligomer [30]. After staining with Coomassie blue, the polypeptides corresponding to the 27 kDa band appeared particularly deficient in LHC II fractions from the mutant (Fig. 5C, D).

TABLE III

FATTY ACID COMPOSITIONS OF POLAR LIPIDS OF CHLOROPLASTS AND PS II PARTICLES ISOLATED FROM WILD AND MUTANT BARLEY LEAVES

Data are average of two determinations with maximum deviation of 5%

Lipids	Barley	Fraction	Relative content (mol%)					
			16:0	16: *ltr*	18:0	18:1	18:2	18:3
MGDG	wild	chloroplasts	1	0	t	0	5	94
		PS II particles	2	0	0	0	6	92
	mutant	chloroplasts	2	0	1	0	2	95
		PS II particles	2	0	1	0	2	95
DGDG	wild	chloroplasts	15	0	2	0	4	79
		PS II particles	19	0	2	0	3	76
	mutant	chloroplasts	23	0	3	1	2	71
		PS II particles	23	0	3	t	2	72
SQDG	wild	chloroplasts	40	0	3	0	4	53
		PS II particles	46	0	3	0	5	46
	mutant	chloroplasts	46	0	2	0	2	50
		PS II particles	53	0	3	0	1	43
PC	wild	chloroplasts	38	0	t	0	19	43
		PS II particles	48	0	4	5	23	20
	mutant	chloroplasts	38	0	4	2	18	38
		PS II particles	36	0	4	2	20	38
PG	wild	chloroplasts	16	38	2	0	3	41
		PS II particles	22	40	1	0	4	33
	mutant	chloroplasts	10	46	2	1	2	38
		PS II particles	11	50	2	0	2	35

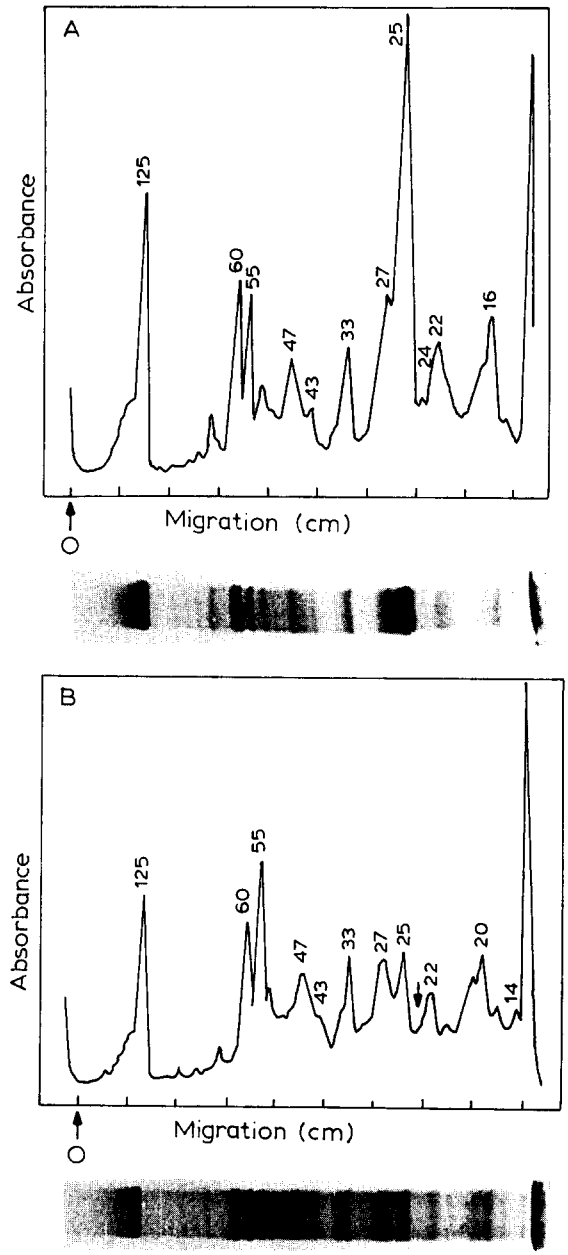

Fig. 4. LDS-polyacrylamide slab gels and their densitometric tracings from chloroplast after staining with Coomassie Brilliant Blue R250 (cf. Materials and Methods). Numbers are molecular mass (kDa). A and B, respectively, are chloroplasts from wild and mutant barley. Note the deficiency in 24, and 25–27 kDa polypeptides of chloroplasts from mutant.

Lipid analysis

The most striking difference is the polar lipid content that appeared to be about twice higher in chloroplasts and PS II particles from the mutant than in those from the wild barley (Table II). When data were expressed on a plastid number, this enrichment was more than 2-fold, and was even more marked, since the amount of polar lipids was $3.0 \cdot 10^9$ molecules per plastid for mutant chloroplasts, and $1.2 \cdot 10^9$ molecules per plastid for wild-type chloroplasts. A slight increase in the proportion of PG and SQDG and a concomitant enhancement of the MGDG/DGDG ratio was also noticed in mutant fractions.

No important differences were noticed in the fatty acid contents of galactolipids from the fractions of the two genotypes (Table III). As observed by Bolton et al. [25], a decrease in the proportions of linolenic acid in SQDG, PC and PG was associated with an increase in palmitic and linoleic acid contents. These results were particularly marked in PS II particles from the mutant. Moreover, in PG molecules of PS II fractions from the two genotypes the proportion of 16 : 1*tr* reached 40 and 50% of total fatty acids, showing that almost all PG molecules linked to these fractions contained 16 : 1*tr*.

Photosynthetic activity

High activities of the Hill and the Mehler reactions were observed with chloroplasts from the two genotypes (Table IV). Although results varied widely from one experiment to another, as shown by high standard deviations, the PS I/PS II ratio appeared significantly higher in the mutant than in the wild-type. PS II particles from the mutant exhibited the highest activity as compared to other fractions.

Fluorescence induction

After excitation with low or high energy, the variable fluorescence in presence of DCMU was more decreased (5–6-fold) in mutant than in wild-type chloroplasts (Table V). Moreover, the half-rise time appeared much slower in the mutant than in the wild genotype. This difference was increased under high light conditions. The ratio of variable fluorescence to maximum fluorescence (F_v/F_m ratio) was lower in mutant chloroplasts,

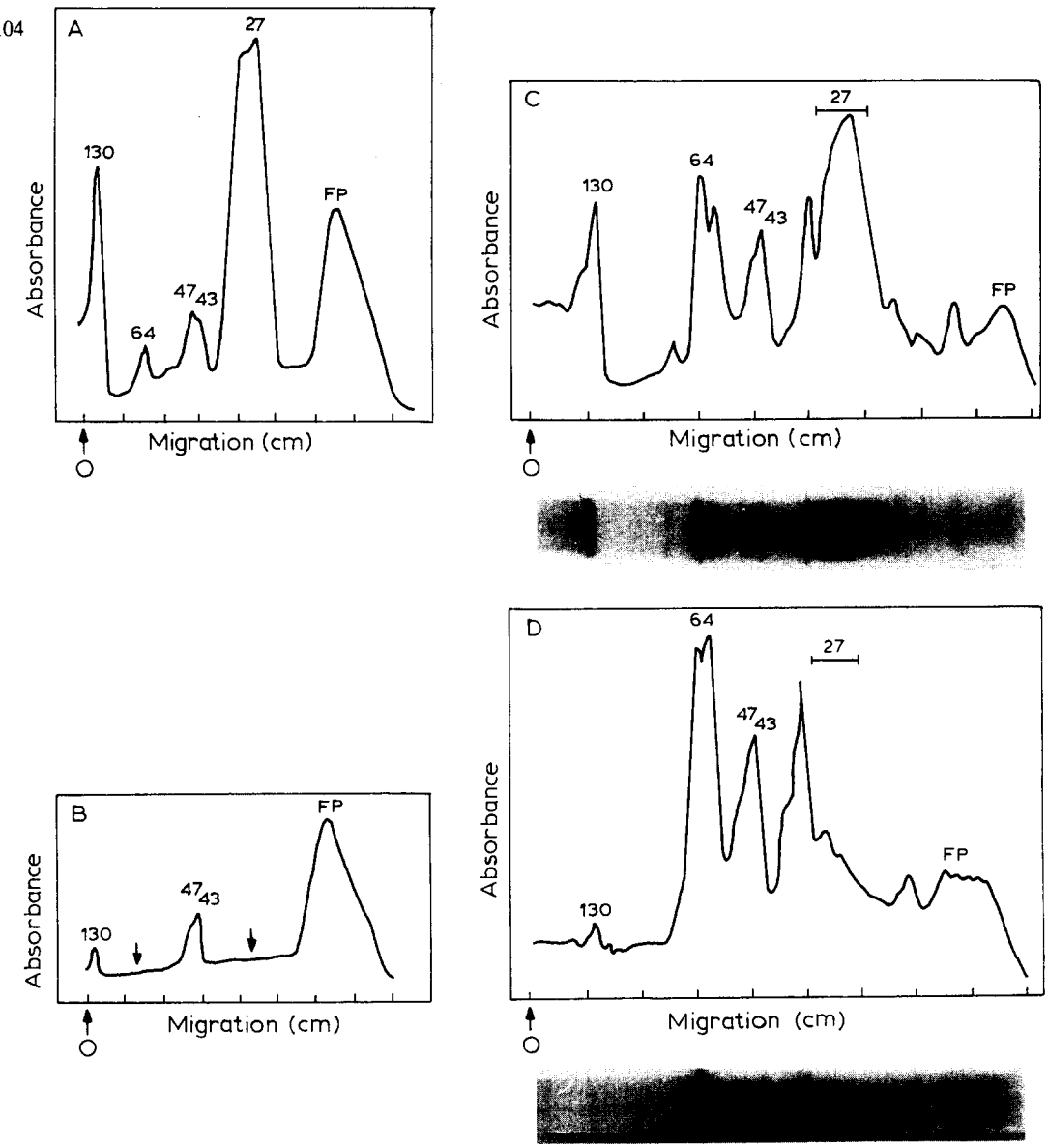

Fig. 5. LDS-polyacrylamide slab gels and their densitometric scans of unstained gels of LHC II fractions from wild (A) and mutant (B) barley (cf. Materials and Methods). No green bands at 27 and 64 kDa, corresponding to the LHC II monomer and oligomer respectively, as in Ref. 30, are visible in the mutant. After staining by Coomassie brilliant blue R250 of the gels from the LHC II fractions of the wild type (C) and mutant (D) barley, a deficiency of polypeptides corresponding to the 27 kDa band appears in the mutant.

TABLE IV

PHOTOSYNTHETIC ACTIVITIES OF CHLOROPLASTS AND OF PS II PARTICLES FROM WILD AND MUTANT BARLEY LEAVES

Mean data of three experiments with S.D. Reaction medium and measurements as in Materials and Methods. MV, methyl viologen.

Barley	Fraction	Activity, e^- μeq/mg Chl per h		PS I/PS II
		PS II ($H_2O \rightarrow FeCy + BQ$)	PS I($DCPIPH_2 \rightarrow MV$)	
Wild	chloroplasts	437 ± 51	460 ± 166	1.0 ± 0.2
	PS II particles	396 ± 51	0	–
Mutant	chloroplasts	359 ± 81	307 ± 167	0.8 ± 0.4
	PS II particles	635 ± 251	0	–

TABLE V

VARIABLE FLUORESCENCE AND HALF-RISE TIMES IN WILD AND MUTANT CHLOROPLASTS

The data are from a representative experiment. Measurements were performed as in Refs. 37, 47. I, excitation energy: 80 $W \cdot m^{-2}$; II, excitation energy: 30 $W \cdot m^{-2}$. Variable fluorescence (F_v), maximum fluorescence (F_m) and half-rise times were deducted from the curve of kinetics of fluorescence. The percentage of active chlorophylls given by $F_v/F_m \times 100$ decreased in mutant.

Barley	Conditions	F_v (Arbitrary units)	$F_v/F_m \times 100$	Half-rise time (ms)
Wild	I	3.07	74	14
	II	0.91	68	6
Mutant	I	0.53	58	34
	II	0.16	45	89

which required higher light intensities for saturation.

Discussion

Large granal thylakoids, named 'macrograna', were formed in mature chloroplasts from the barley mutant. The development of these structures, already observed in other barley mutants deficient in Chl *b* [19,23], in tobacco mutants developing under high or suboptimal light [39], and in shade plants [28,44] suggests a reorganization of the thylakoid components in order to improve light energy collection by the chloroplasts. The fast granal degeneration and the swelling of stroma thylakoids that occurs in aged chloroplasts in the mutant could be a response to the photodestruction of plastids [38,39].

A total deficiency of Chl *b* has been demonstrated by the analysis of the low temperature absorption spectra. Simultaneously, the polypeptides of the light-harvesting Chl *a*/*b*-protein complex, as those linked to the antenna of PS I, are highly reduced. Analysis of fractions enriched in LHC II, as well as electrophoresis in the presence of deoxycholate, also confirms the loss of the polypeptides linked to LHC II in the mutant. Recently, Duranton and Brown [45], by adding protease inhibitors to the cloroplast isolated media and during electrophoresis and by decreasing the amount of digitonin during solubilization of thylakoids, could detect two green bands attributed to LHC II. All these results demonstrate the extreme lability of the protein-pigment associations of the PS II antenna in the absence of Chl *b*. Therefore, Chl *b* appears as a prime factor to LHC II formation [46], and especially to stabilize the antennae proteins [14,35]. From the comparison of the kinetics of fluorescence induction of plastid suspensions from wild-type and mutant genotypes, one may also conclude that there is a large reduction in size and number of antennae in the mutant, resulting in poor light harvesting by mutant plastids [47], as demonstrated on the CD3 of mutant of wheat [11] or on the *chlorina* f2 mutant of barley [22].

As regards the whole chloroplasts, an increase in chlorophyll, total protein and acyl lipid content was observed in the mutant in parallel to a decrease of the membrane density of plastids. From these data, in the absence of Chl *b* great amounts of these components could be accumulated, without being integrated into the intraplastidial lamellar system. No striking changes could be detected in the acyl lipids from the mutant plastids, except for a higher amount of 16 : 1*tr* in PG molecules. This was particularly obvious at the level of PS II particles where almost all PG molecules contained this fatty acid. Our results confirm the hypothesis of Tuquet et al. [48], demonstrating the presence of this lipid in granal structures. Implicated in the efficiency of light capture [49], PG with 16 : 1*tr* could interfere with the energy transfer between the antenna and the reaction center of PS II rather than with the O_2-evolving system [50].

Mutant chloroplasts showed good photosynthetic activities (PS I and PS II) as measured by the electron transport rates in the light. Therefore, Chl *b* appears not to be essential for the manifestation of photosynthetic activity as suggested by Highkin and Frenkel [17]. In the absence of Chl *b*, macrograna rich in mutant chloroplasts could ensure contact between the two reaction centers of PS I and PS II complexes, and optimize the energy distribution between the two photosystems as suggested by Waldron and Anderson [35].

In order to define the regulation processes of the assembling of the LHC II polypeptides in

thylakoids, we are now studying the effect of chloramphenicol during the greening of etioplasts from the wild and mutant barley genotypes. It has indeed been shown that this inhibitor induces an accumulation of the 27 kDa LHC II polypeptides during greening of the wheat plastids [27].

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