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CHLOROPHYLL-PROTEINS OF THYLAKOIDS FROM WILD-TYPE AND MUTANTS OF BARLEY (HORDEUM VULGARE L.)

by

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A sodium dodecyl sulphate-polyacrylamide gel electrophoresis system is described that resolves wild-type barley thylakoid components into ten chlorophyll-containing bands, plus free chlorophyll. Many of these bands have been characterised with respect to their absorption spectra and polypeptide composition. The identity and probable function of eight bands were established by comparison of the wild-type pattern with those of isolated light-harvesting chlorophyll *a*/*b*-protein complex and a number of nuclear gene mutants of barley. It was found that the mutant *chlorina-f2*, which lacks chlorophyll *b*, contains only three chlorophyll *a*-proteins designated Chl_a-P1, Chl_a-P2 and Chl_a-P3 according to a new system of nomenclature proposed in this paper. Chl_a-P1 contains the reaction centre of photosystem I (P700) and with the aid of the mutant *viridis-m*^{29}, Chl_a-P3 was deduced to be the most likely site of the reaction centre (P680) of photosystem II.
Two chlorophyll *a*/*b*-proteins were found, which differed in their *a*/*b* ratios and which are thought to play a role in light-harvesting or light-focusing. These were designated $Chl_{a/b}$-P1 and $Chl_{a/b}$-P2, the latter being found in multimeric complexes designated $Chl_{a/b}$-P2*, $Chl_{a/b}$-P2** and $Chl_{a/b}$-P2***, in order of increasing apparent molecular weight. In addition to these eight bands, there were two chlorophyll-containing bands in the wild-type pattern that were not $Chl_{a/b}$-P2 complexes and which were called Chl-P bands until further characterised.

Abbreviations: Chl = chlorophyll, Chl-P = chlorophyll-protein, CPI = chlorophyll-protein complex I, CPII = chlorophyll-protein complex II, DTT = dithiothreitol, HEPES = N-(2-hydroxyethyl)-piperazine-N'-2-ethane sulphonic acid, kD = kilodalton, LHC = light-harvesting complex, SDS = sodium dodecyl sulphate, TEMED = N, N, N', N'-tetramethylethylenediamine, Tricine = N-(tris-(hydroxymethyl)-methyl)glycine, Tris = tris-(hydroxymethyl)aminomethane.

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1. INTRODUCTION

Chloroplast thylakoids can be solubilised by the ionic detergent sodium dodecyl sulphate (SDS) and the polypeptide components separated by polyacrylamide gel electrophoresis. The procedures used until recently were capable of resolving only three chlorophyll-containing bands (8, 27, 34) designated, in order of increasing electrophoretic mobility, chlorophyll-protein complex I (CPI), chlorophyll-protein complex II (CPII) and free chlorophyll (34). These chlorophyll proteins have been isolated from many species, and are sensitive to denaturation by heat (11), unfavourable pH (22) and strongly reducing conditions (MACHOLD, unpublished results).

The chlorophyll-proteins observed on polyacrylamide gels are believed to reflect the existence of chlorophyll-proteins in the native membrane, but much or all of the free chlorophyll on the gel may derive from denaturation of CPI and CPII and other unknown chlorophyll-proteins (see ref. 25). With this possible denaturation in mind, electrophoretic procedures have been designed to increase the stability of the native chlorophyll-proteins. This has been achieved mainly by decreasing the ratio of SDS to protein when solubilising the thylakoids (13, 15, 24, 29), running the gels at low temperature (2, 12, 13, 15, 16, 18), and/or by substituting some or all of the SDS with non-ionic detergents (2, 13, 18, 25), usually in combination with thicker gels. Under such conditions, between one and three new chlorophyll-containing bands have been reported with electrophoretic mobilities between CPI and CPII (2, 12, 13, 15, 16, 17, 18, 27, 29, 30, 36) and up to two bands with mobilities lower than CPI (2, 28). It is difficult, however, to compare the results from different laboratories because of differences in plant material, solubilisation procedures, type of buffer system, and conditions during electrophoresis.

We have examined the chlorophyll-protein composition of wild-type barley thylakoids under conditions of gentle solubilisation and electrophoresis which yield ten chlorophyll-containing bands, plus free chlorophyll. Many of these bands were identified by comparison with the chlorophyll-containing band patterns of isolated light-harvesting complex (LHC) and certain barley nuclear gene mutants. A new nomenclature system was devised since published nomenclature is inadequate to name the large number of new chlorophyll-containing bands described in this paper.

2. MATERIALS AND METHODS

2.1. Plant material

Seeds of wild-type barley (Hordeum vulgare cv. Svalöfs Bonus) were germinated in plastic trays containing vermiculite moistened with tap water. Seedlings were harvested after growing for 7 days at 20 °C in continuous white light (1,700 lux provided by Sylvania F48T12-GRO-VHO Gro-lux fluorescent lights). Seedlings from the barley mutant *chlorina-f2*, which lacks chlorophyll *b* (5), were grown under the same conditions. The nuclear gene mutants *viridis-m^{29}, -n^{34} and -zd^{69}* do not survive in the field and the stocks are maintained in the heterozygous condition. The mutant progeny arising from heterozygous plants were identified by their paler green colour and separated from the wild-type phenotype after growing for 7 days at 20 °C in continuous white light.

2.2. Isolation of thylakoids

The method for isolation and purification of thylakoids was a modification of that previously described (20). The grinding medium consisted of 0.4M-sucrose, 200mM-NaCl and 50mM-Tricine/NaOH, pH7.9. Precooled leaves were homogenised in 3 volumes of grinding medium at 4 °C, using a kitchen blender with replaceable razor blades (21). The resulting slurry was filtered through 2 layers of 31 µm nylon gauze and centrifuged 5 min at 1,400g (wild-type) or 8,000g (mutants). The chloroplast pellet was suspended and osmotically shocked in 25mM-HEPES/NaOH, pH7.5, 10mM-EDTA and centrifuged 5 min at 10,000g. After two additional washes, the final pellet was suspended in a small volume (3–10 ml) of 1.90M-sucrose, 25mM-HEPES/NaOH, pH7.5, 5mM-EDTA using a motor driven teflon pestle. To each centrifuge tube was added approximately 5 ml of thylakoid suspension, which was overlayered and topped up with 1.14M-sucrose, 25mM-HEPES/NaOH, pH7.5, 5mM-EDTA (wild-type) or 0.93M-

sucrose, 25mM-HEPES/NaOH, pH7.5, 5mM-EDTA (mutants), and centrifuged at 4 °C for 30 min at 140,000g using an SW-40 rotor in a Beckman ultracentrifuge. The thylakoids floated to the sucrose-sucrose interface and were collected with a syringe. The sample was diluted with 25mM-HEPES/NaOH, pH7.5, 10mM-EDTA and centrifuged for 10 min at 48,000g. In some experiments, this final wash was replaced by two washes with distilled water to remove as much of the bound ribulose-1,5-bisphosphate carboxylase as possible.

2.3. Solubilisation procedures

The solubilisation procedure depended on the type of gel system being used. When it was desired to separate chlorophyll-proteins, the freshly prepared thylakoid pellets from the final centrifugation were resuspended until fluid in a small volume of distilled water using a glass rod. A sufficient volume of this suspension was added to distilled water containing an amount of stock solution of 10% SDS in 1.5M-Tris/ H_2SO_4, pH9.00 buffer, so that the final ratio of SDS: chlorophyll (w/w) was 3.6:1, and the final concentration of SDS was 1%. Typically this involved adding 60 µl of 10% SDS stock solution to 340 µl of distilled water, followed by 200 µl of wild-type thylakoid suspension. When using mutant thylakoids, an SDS: protein ratio of about 0.7: 1 was used, and for isolated light-harvesting complex, an SDS: protein ratio of 1.1: 1, with a final concentration of SDS of 1%. The resulting mixtures were gently stirred with a glass rod and centrifuged at 18 °C for 15 min at 40,000 rpm in a 50Ti rotor in a Beckman ultracentrifuge. This resulted in a small pellet of non-solubilised material and a dark green clear supernatant which was used immediately for polyacrylamide gel electrophoresis. It was found that chlorophyll-proteins could be obtained from stored material if a small amount of solid DTT was added to the concentrated purified thylakoid suspension and aliquots frozen in liquid nitrogen prior to storage at −20 °C. For gel electrophoresis under more strongly denaturing conditions, the amount of SDS was doubled so that the final concentration was 2%. In addition, solid DTT was added to the samples, to a final concentration of 2–4%, prior to electrophoresis. Because the material was so concentrated, sample volume per track varied between 2 and 5 µl.

2.4. Gel electrophoresis

Three different electrophoretic systems were used in the course of these experiments, and are listed in Table I. A slab-gel apparatus, modified from the design of STUDIER (32) was used for all three gel types. The components of the Tris-glycine/Tris-sulphate gel (system IV) are given in Table II, and those for systems II and III are as previously described (23).

Gels containing chlorophyll-protein bands were photographed without removing the glass plates using Agfacolor 50S professional film with transmitted diffuse illumination from fluorescent tubes. The fluorescence from the chlorophyll-proteins was recorded using the same film with a combination of UV (B + W 010) and dark red (B + W 091) filters and gels were illuminated from below with long wavelength UV light from a Chromato-Vue transilluminator, Model C-62 (Ultra-Violet Products, Inc., San Gabriel, California, USA).

Absorption spectra of the chlorophyll-protein bands in the gels were recorded with an Aminco-Chance DW-2a dual wavelength spectrophotometer. Chlorophyll-proteins for re-electrophoresis were obtained by cutting out the appropriate green bands from 2.4 mm thick unfixed gels with a scalpel and homogenising in a small volume of Na borate/HC1 substance buffer (pH 8.9) in a conical Potter-Elvehjem homogeniser with a motor driven teflon pestle. The resulting suspension was centrifuged in a benchtop centrifuge. After re-extraction of the resulting pellet, followed by centrifugation, the supernatants were pooled. The supernatant was then centrifuged in a SS-34 rotor (Sorvall) for 10 min at 48,000g to pellet the residual polyacrylamide, and filtered through a Millipore filter (GSWT01300, 0.2 µm pore size) attached to a syringe. The green filtrate was loaded into an Amicon microultrafiltration system 8 MC operated at 5 $kg \cdot cm^{-2}$ with a PM10 filter, and concentrated to about 200 µl.

Gels were fixed in methanol: acetic acid: water (5:5:1) and stained in either Coomassie brilliant blue G-250 or R-250(0.05% solution in fixative) and destained with 7% acetic acid, followed by

Table I

Description of electrophoretic systems

	System II	System III	System IV
Upper reservoir buffer	Tris-borate, pH 8.64 0.1% SDS	borate-HCl, pH 8.00 0.1% SDS	Tris-glycine, pH 9.00 0.1% SDS
Stacking gel buffer	Tris-sulphate, pH 6.10	Tris-HCl, pH 8.90	Tris-sulphate, pH 9.00
Stacking gel	6% acrylamide acryl/bis = 30: 0.8 0.1% SDS 12 mm	6% acrylamide acryl/bis = 30: 0.8 0.1% SDS 4 mm	5% acrylamide acryl/bis = 30: 0.75 0.1% SDS 20 mm
Separation gel buffer	Tris-HCl, pH 9.00	Tris-HCl, pH 8.90	Tris-sulphate, pH 9.00
Separation gel	gradient 12–18% acryl/bis = 30: 0.8 0.1% SDS	gradient 12–18% acryl/bis = 30: 0.8 0.1% SDS + 6M-urea	gradient 10–20% acryl/bis = 30: 0.75 0.1% SDS
Lower reservoir buffer	Tris-HCl, pH 9.18	borate-HCl, pH 8.00	Tris-glycine, pH 9.00
Gel length	22 cm	22 cm	15 cm
Gel thickness	0.95 mm	0.95 mm	2.4 mm
Temperature	18 °C	18 °C	5 °C
Current (initial)	1 mA/100 mm^2	2.5 mA/100 mm^2	1 mA/100 mm^2
(after penetration)	3.5 mA/100 mm^2	12 mA/100 mm^2	7 mA/100 mm^2

30 min in 10% trichloroacetic acid prior to photographing. SDS (Ferak) was recrystallised from ethanol before use. Acrylamide, N,N'-methylene-bis-acrylamide and Coomassie brilliant blue were obtained from Sigma and used without further purification.

The apparent molecular weight of the various chlorophyll-proteins and their polypeptide components were calculated using the following values for known polypeptides of wild-type barley (37): chlorophyll *a*-protein 1 (110kD), chlorophyll *a*-protein 1 apoprotein (68kD), coupling factor 1 β subunit (57kD), large subunit of ribulose-1,5-bisphosphate carboxylase (55kD), cytochrome *f* (33kD) and the small subunit of ribulose-1,5-bisphosphate carboxylase (14kD). These in turn had been calibrated using the following standards: bovine serum albumin (68kD), ovalbumin (45kD), aldolase (40kD) and chymotrypsinogen A (25kD).

Table II

Components of electrophoresis system IV

	stacking gel (5%)	separation gel (10%)	separation gel (20%)
gel buffer (ml)	2.55	7.5	7.5
sucrose (g)	0	1.5	5.25
acrylamide (ml)	1.25	7.5	15.0
ammonium persulphate (μl)	50	150	150
water	to 10 ml	to 30 ml	to 30 ml

Stock solutions:
gel buffer: Tris (36.3 g); 1N-H_2SO_4 (62 ml); TEMED (0.48 ml); SDS (0.80 g) and water to 200 ml
acrylamide: acrylamide (40 g); bis-acrylamide (1 g) and water to 100 ml
ammonium persulphate: 100 mg·ml^{-1}
reservoir buffer: Tris (121.0 g); glycine (115.6 g) and water to 1000 ml
upper reservoir buffer: Tris-glycine stock (21.0 ml); SDS (0.5 g) and water to 500 ml
lower reservoir buffer: Tris-glycine stock (168.0 ml) and water to 4000 ml
solubilisation buffer: gel buffer stock plus SDS (10%)

2.5. Isolation of the light-harvesting complex (LHC)

The light-harvesting chlorophyll *a*/*b*-protein complex (LHC) from barley was isolated and purified according to the method of BURKE, DITTO & ARNTZEN (6) and stored in 30% glycerol at −15 °C until needed.

3. RESULTS

3.1. Wild-type thylakoids

Ten chlorophyll-protein bands, plus free chlorophyll, could be resolved when purified wild-type barley thylakoids solubilised with a limiting amount of SDS, as described in section 2.3, were subjected to 6h of electrophoresis using the Tris-glycine/Tris-sulphate system (Figure 1a). It can be seen that there are at least seven chlorophyll-containing bands with electrophoretic mobilities between Chl_a-P1 (CPI) and $Chl_{a/b}$-P2 (CPII).

Since metalloporphyrins, such as chlorophyll, usually fluoresce red when illuminated with ultra-violet light, the gel was also photographed under conditions suitable for visualising fluorescence. This resulted in the type of image seen in Figure 1b, where each chlorophyll-containing band in Figure 1a has given rise to a red fluorescing band, except for Chl_a-P1 (CPI). Fixation of the gel in 7% acetic acid converted metalloporphyrins to the free base, which also fluoresces red (14), and the resulting image is seen in Figure 1c. It can now be seen that Chl_a-P1 (CPI) is fluorescent and there are several extra fluorescent bands not seen in Figure 1b, and which do not correspond to chlorophyll-containing bands in Figure 1a. Figure 1d shows the polypeptide bands in the same gel, as revealed by Coomassie brilliant blue staining after fixation.

3.2. Isolated light-harvesting complex (LHC)

The multiplicity of chlorophyll-protein bands from wild-type barley thylakoids led us to examine systems with less complexity in order to attempt an identification of some of these bands. The purified light-harvesting complex from barley contains mainly $Chl_{a/b}$-P2 (CPII) and a minor amount of non-chlorophyll-protein material. When subjected to the same electrophoretic conditions as in Figure 1, up to four chlorophyll-protein bands were seen (Figure 2), although there were quantitative variations in the relative amounts of each of these bands from run to run. The patterns from two separate experiments are given in Figures 2b and 2e. By comparing the position of these bands with the Coomassie blue staining polypeptides in the wild-type pattern, it was possible to assign apparent molecular weights of 29kD, 50kD, 71kD and 107kD to the four chlorophyll-containing bands from light-harvesting complex. The absorption spectra of these bands are presented in Figure 3. They all showed high levels of chlorophyll *b*, although there were differences in the chlorophyll *a* to *b* ratios and in the wavelengths of the maxima.

It was possible to correlate the chlorophyll-protein bands from the isolated light-harvesting complex with some of the bands present in wild-type thylakoids (Figure 2a and 2b). The band with an apparent molecular weight of 71 kD ($Chl_{a/b}$-P2**) was clearly visible in the wild-type pattern and the absorption spectra are similar (Figures 3 and 6). The two minor bands ($Chl_{a/b}$-P2*** and $Chl_{a/b}$-P2*) of the light-harvesting complex are obscured in the wild-type pattern by Chl_a-P1 and Chl_a-P2.

3.3. *Chlorina-f2* mutant

The nature of some of the other chlorophyll-protein bands in the wild-type pattern was investigated by a comparison with the pattern from the nuclear gene mutant *chlorina-f2*. This mutant contains no chlorophyll *b* (5), so any chlorophyll-protein bands present would contain only chlorophyll *a*. When the pattern from these thylakoids was examined, the major difference was the expected absence of the $Chl_{a/b}$-P2 (CPII) band (Figure 4). In addition, Figure 4 illustrates that several other bands were missing, including those of low electrophoretic mobility associated with LHC. The main bands present are those designated Chl_a-P1 (CPI), Chl_a-P2 and Chl_a-P3 (see Discussion for explanation of nomenclature) and two minor bands with mobilities slightly slower than Chl_a-P2. Absorption spectra of the three major bands (Figure 5) confirmed that they contained only chlorophyll *a* and the wavelength at the absorption maximum was characteristic for each band. The absorption spectra of the wild-type chlorophyll-protein bands not found in

Figure 1. Separation of wild-type barley thylakoid components by SDS-polyacrylamide gel electrophoresis using the Tris-glycine/Tris-sulphate system. All photographs are of the same gel and are enlarged 1.38 times. a). Chlorophyll-protein bands appear as green regions on the gel and are named according to the nomenclature summarised in Table III. b). Appearance of the gel illuminated with long wavelength UV light and photographed through a red filter. All chlorophyll-containing bands fluoresce red, except for Chl_a-P1 in which fluorescence is quenched by P700. The dark region in the $Chl_{a/b}$-P2 band results from self-absorption due to the high chlorophyll concentration. c). The same gel after fixation in 7% acetic acid. The Chl_a-P1 band is now fluorescent, due to the destruction of P700 and an additional band has appeared (arrowhead) which may be due to cytochrome *f*. Fixation has removed much of the free chlorophyll below $Chl_{a/b}$-P2, revealing discrete fluorescent bands corresponding to the major polypeptide bands in Figure 1d. d). The polypeptide pattern revealed by staining with Coomassie brilliant blue R-250.

Figure 2. Identification of $Chl_{a/b}$-P2 and its complexes by electrophoresis of isolated light-harvesting complex from wild-type barley. a). Chlorophyll-protein bands of wild-type barley thylakoids. b). Chlorophyll-protein bands of isolated light-harvesting complex run on the same gel. c). The fluorescence pattern from Figure 2b. d). The polypeptide pattern of the same gel after staining with Coomassie blue G-250. The band below $Chl_{a/b}$-P2 is thought to be a contaminant and was not seen in every gel run. e, f, g). Gels from another electrophoretic separation of light-harvesting complex, corresponding to Figures 2b, c and d respectively. On this occasion there was a higher amount of $Chl_{a/b}$-P2**.

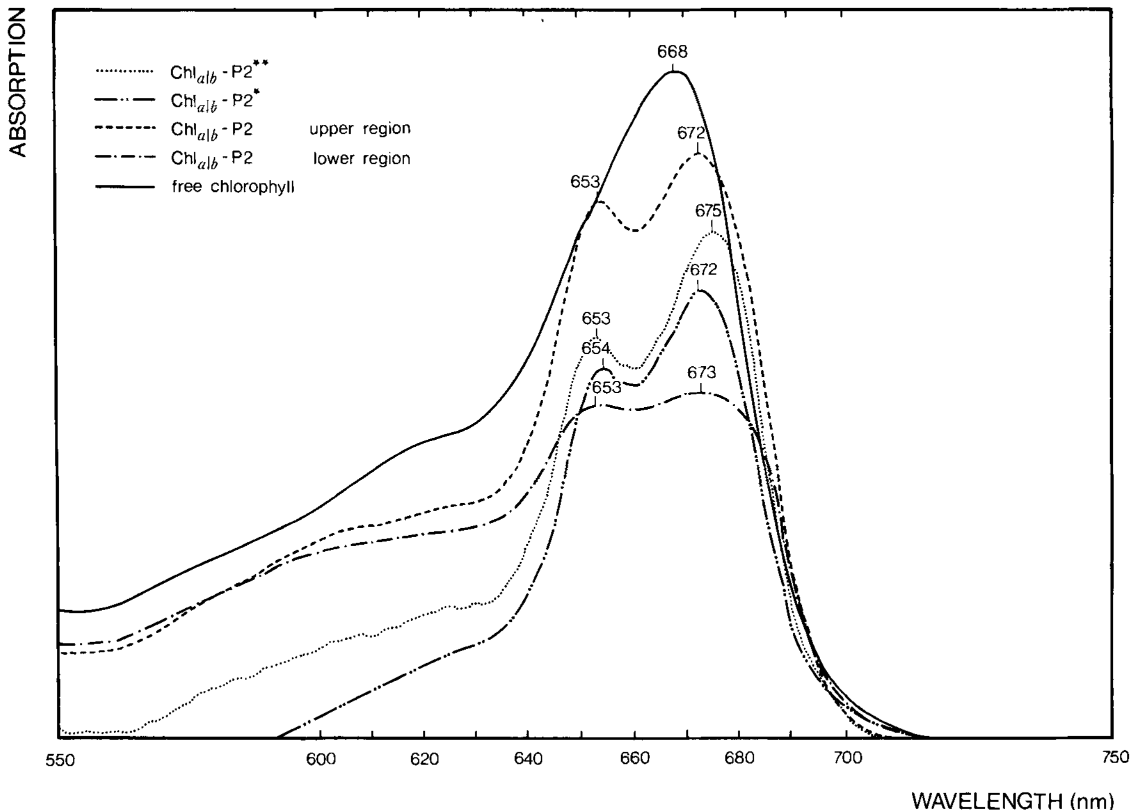


Figure 3. Absorption spectra of the chlorophyll-containing bands seen in Figure 2b measured in the gel. The peak wavelength values for $Chl_{a/b}$-P2** differ from those of $Chl_{a/b}$-P2. There is a decrease in the chlorophyll *a*/*b* ratio as one goes from the top to the bottom of the $Chl_{a/b}$-P2 band, and a high chlorophyll *a*/*b* ratio in the free chlorophyll band, indicating a selective loss of chlorophyll *a* from $Chl_{a/b}$-P2 during electrophoresis.

Figure 4. Comparison of the chlorophyll-protein band patterns of wild-type barley and the *chlorina-f2* mutant which lacks chlorophyll *b*. As in Figure 2, the same two tracks from the same gel are shown to compare the chlorophyll-protein bands, the fluorescent bands and the Coomassie G-250 staining polypeptide patterns. The *chlorina-f2* mutant contains Chl_a-P1, Chl_a-P2 and Chl_a-P3 and a minor Chl-P band above Chl_a-P2 (arrowhead). This band immediately above Chl_a-P2, which does not seem to correspond to a Chl-P band in Figure 1, may be an electrophoretic artefact causing free chlorophyll to focus at this point. The absence of $Chl_{a/b}$-P2* improves the resolution of Chl_a-P2 and Chl_a-P3 in the *chlorina-f2* pattern. Since this mutant is photosynthetically competent, and Chl_a-P1 is known to contain the reaction centre of photosystem I, it follows that the reaction centre of photosystem II is probably associated with Chl_a-P2 and/or Chl_a-P3.

the *chlorina-f2* mutant pattern reveal a peak or shoulder at 654 nm, indicating the presence of chlorophyll *b* (Figure 6). The chlorophyll *b* in Chl_a-P2 from wild-type was probably due to contamination by the 50kD chlorophyll-protein band associated with LHC (Figure 2), since the absorption spectrum from the corresponding band from *chlorina-f2* shows no chlorophyll *b* shoulder (Figure 5). There were three chlorophyll-containing bands in the wild-type pattern that were not found in *chlorina-f2* or isolated LHC. These bands have been designated $Chl_{a/b}$-P1 and Chl-P bands (see Discussion). Thus electrophoresis of wild-type barley thylakoids yields three chlorophyll *a*-proteins, two chlorophyll *a*/*b*-containing bands, three extra bands associated with one of these (in LHC), and two uncharacterised chlorophyll-containing bands.

Since oligomeric forms of CPII were found in the Tris-glycine/Tris-sulphate gel system (Figure 2), it was necessary to establish that Chl_a-P2 and Chl_a-P3 are unique chlorophyll-proteins and not

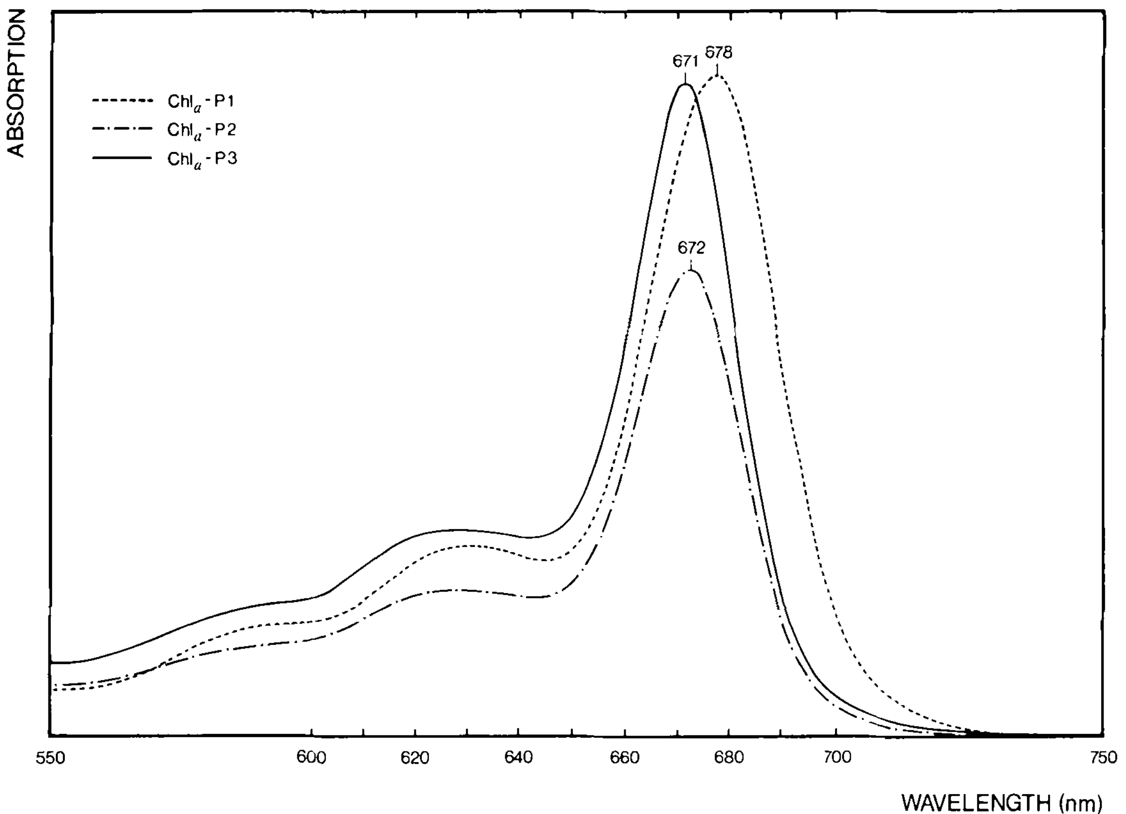


Figure 5. Absorption spectra of the chlorophyll *a*-proteins of *chlorina-f2* recorded in situ in the gel. Note that each band has a characteristic peak wavelength value.

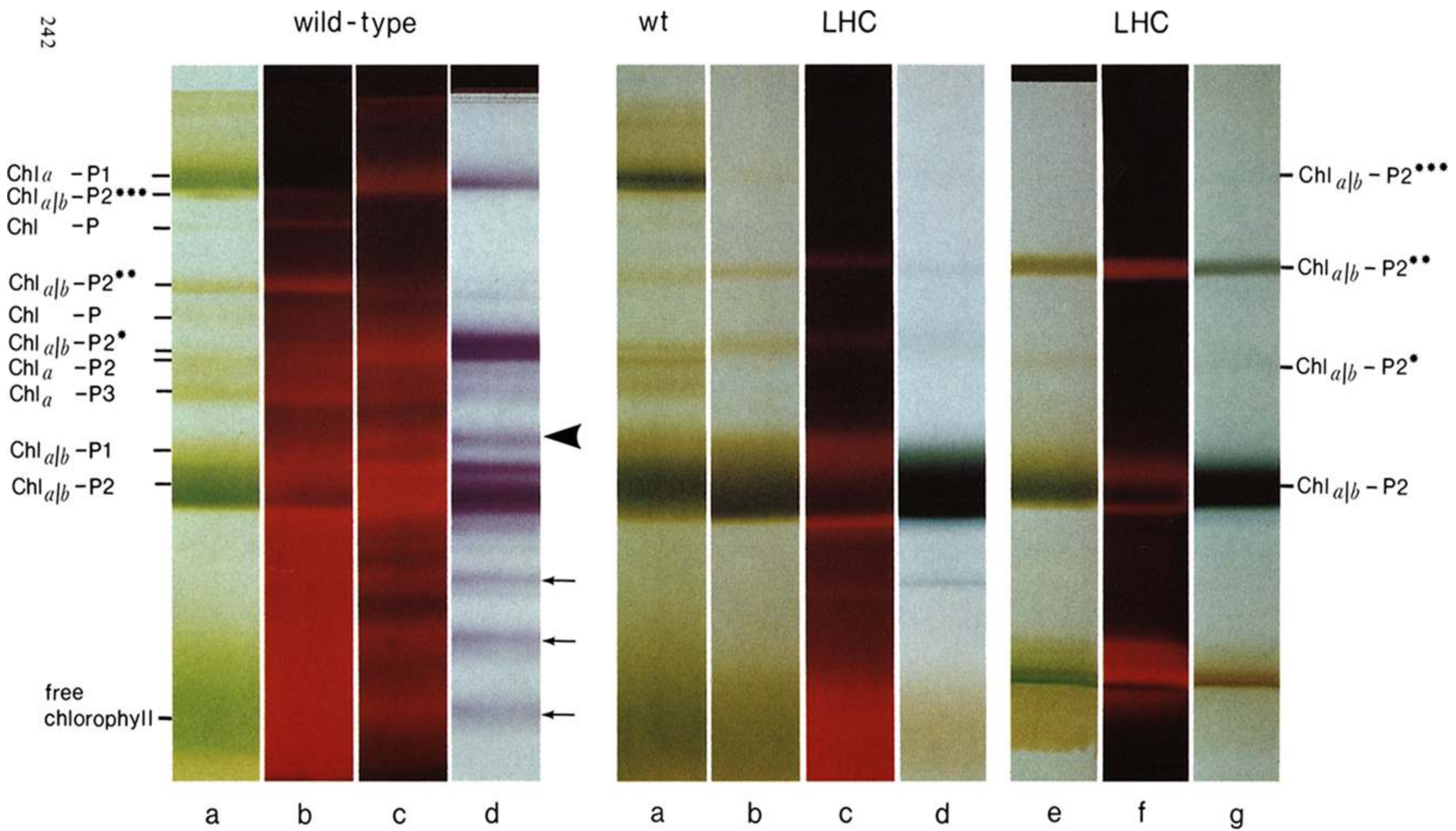


fig. 1

fig. 2

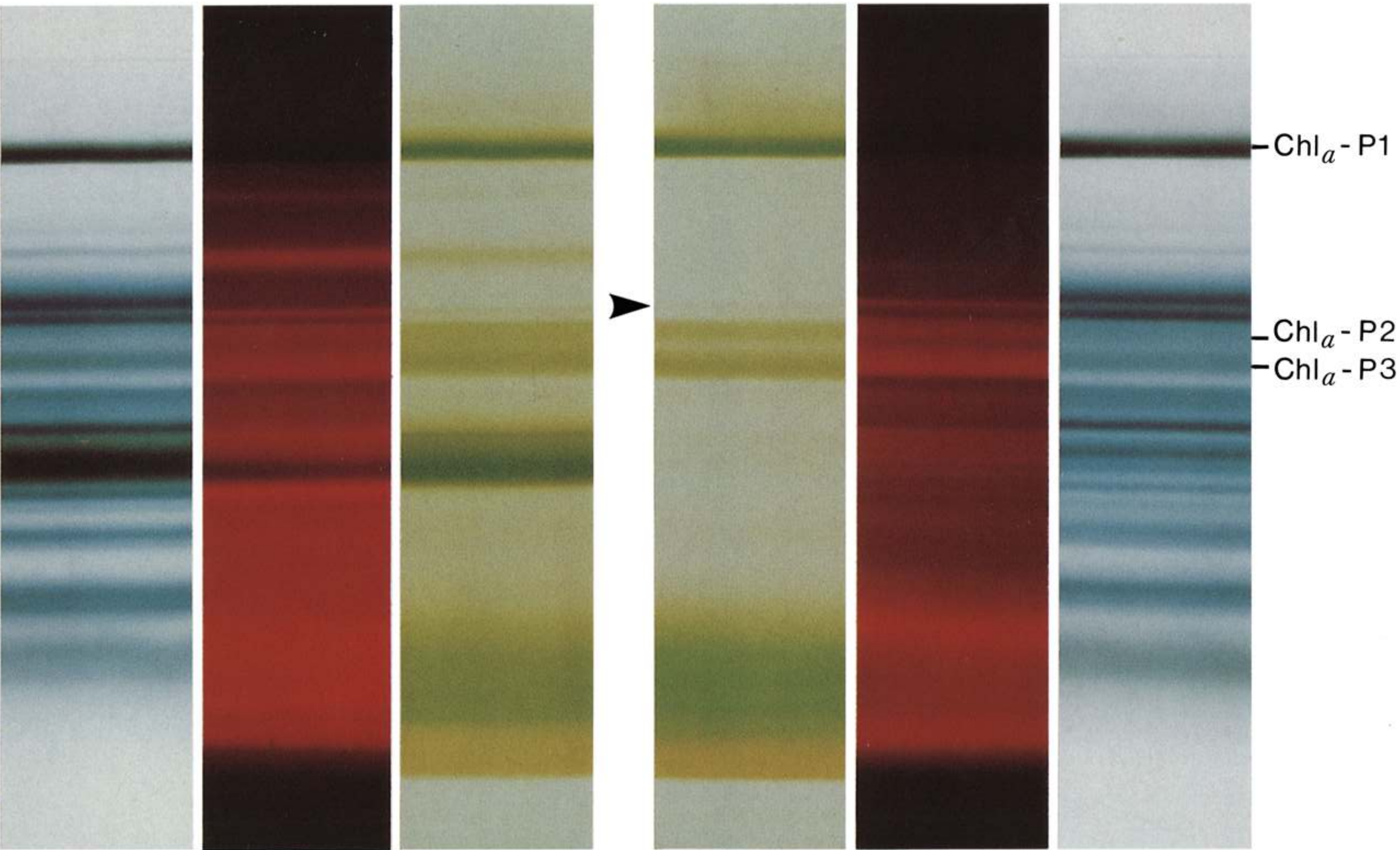


fig. 4

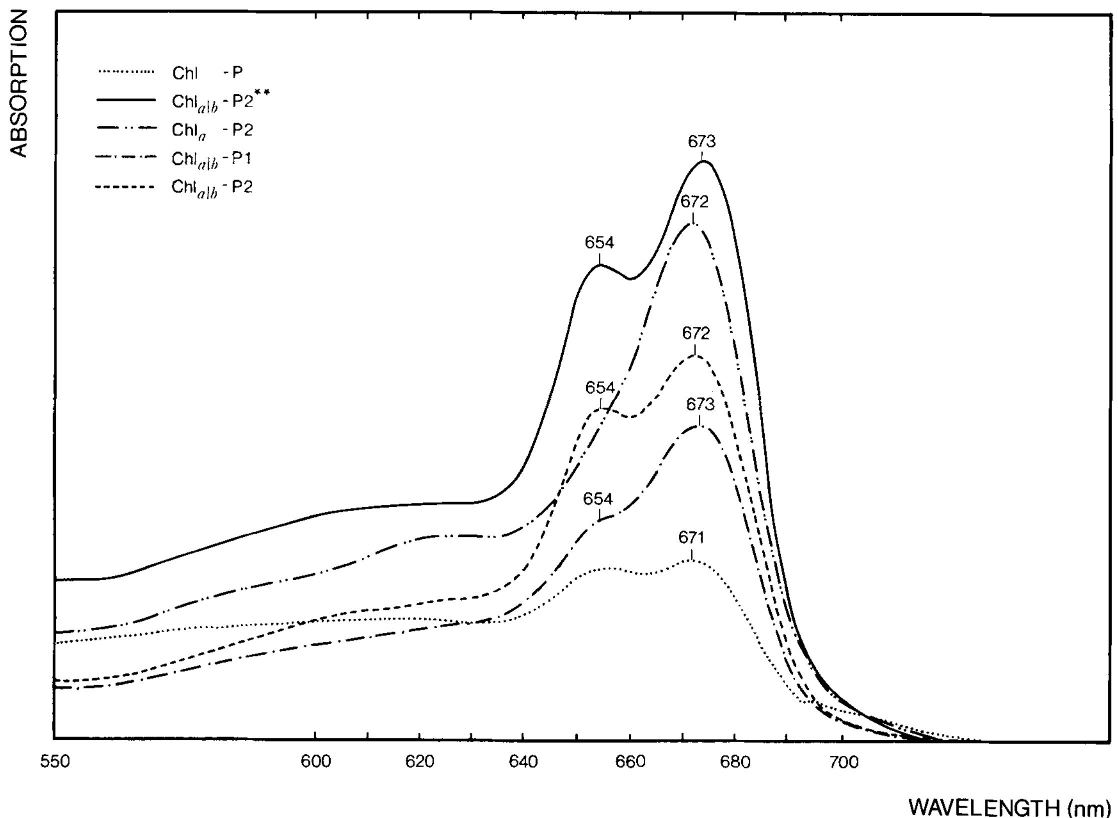
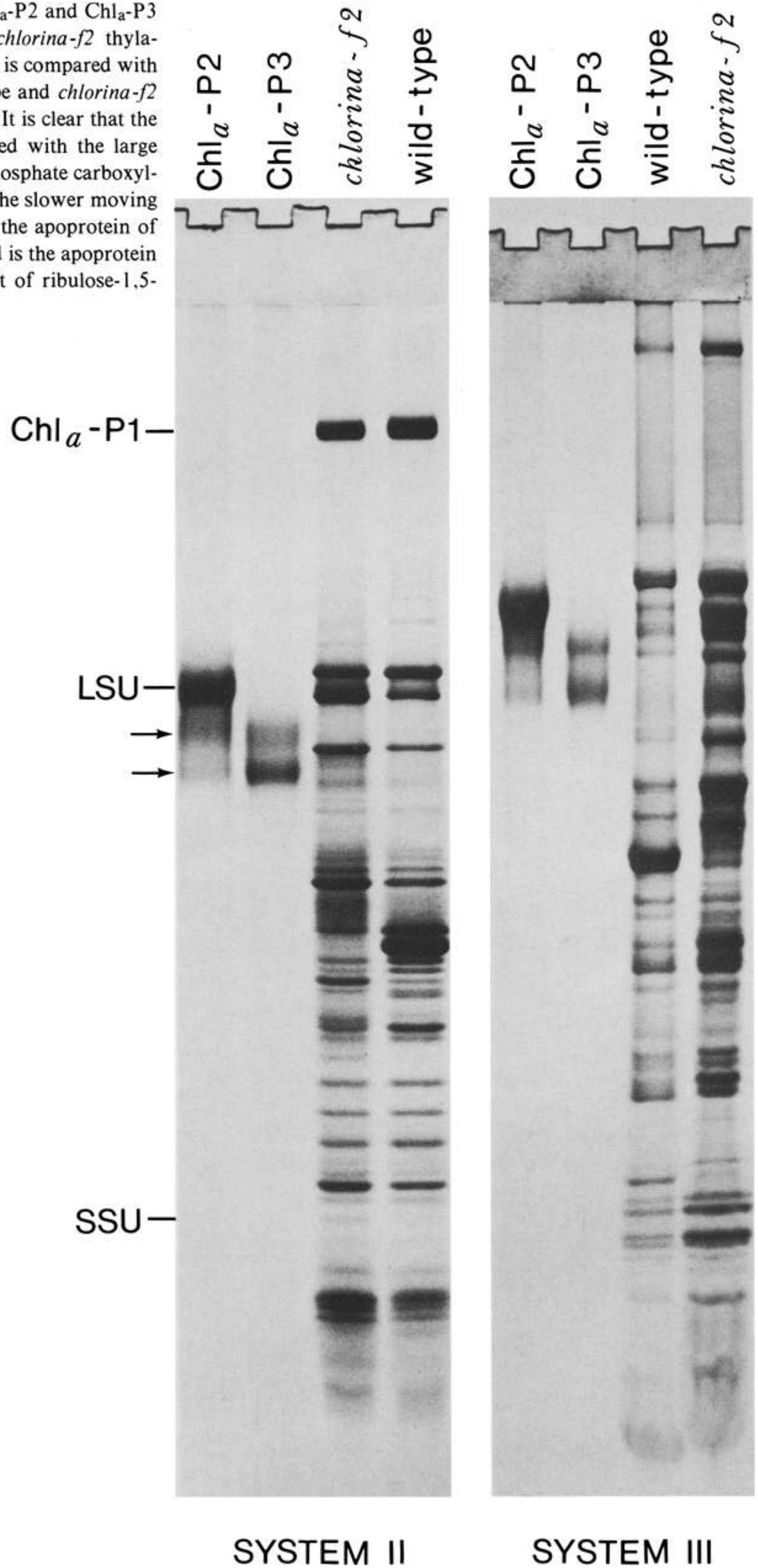


Figure 6. Absorption spectra of the chlorophyll-containing bands of wild-type barley not present in *chlorina-f2*. All these bands contain chlorophyll *b* and probably have a light-harvesting or light-focusing role for the photosynthetic reaction centres. Also shown is the spectrum of Chl_a-P2 showing a chlorophyll *b* shoulder due to the presence of $Chl_{a/b}$-P2* in this region of the gel. Chl-P is the band in Figure 1 just below $Chl_{a/b}$-P2***.

derived from other chlorophyll-proteins. The green bands corresponding to Chl_a-P2 and Chl_a-P3 were cut out from 3 preparative gels of SDS-solubilised *chlorina-f2* thylakoids and the protein extracted and concentrated as described in section 2.4. This mutant was chosen instead of the wild-type because in the mutant, Chl_a-P2 and Chl_a-P3 are well resolved and not contaminated by the 50kD band derived from LHC. The polypeptide composition of the isolated chlorophyll-proteins is shown in Figure 7 and can be compared with the total thylakoid pattern of *chlorina-f2* electrophoresed under 2 different gel conditions (see Table I). Chl_a-P2 contained the 55kD large subunit of ribulose-1,5-bisphosphate carboxylase (probably due to insufficient washing of the thylakoids) and two bands of higher electrophoretic mobility. These two bands were also present in isolated Chl_a-P3, but with an enrichment of the faster moving polypeptide as judged by relative staining intensity. Calculation of the molecular weights of these polypeptides yielded values of 49kD and 45kD in system II, and 47kD and 41kD in system III for the slower and faster moving polypeptides respectively. It was therefore concluded that the slower of the 2 bands corresponded to the protein moiety of Chl_a-P2 and the faster moving band to the protein moiety of Chl_a-P3.

Figure 7. Re-electrophoresis of Chl_a-P2 and Chl_a-P3 isolated from preparative gels of *chlorina-f2* thylakoids. The polypeptide composition is compared with that of the total pattern of wild-type and *chlorina-f2* barley in two different gel systems. It is clear that the Chl_a-P2 preparation is contaminated with the large subunit (LSU) of ribulose-1,5-bisphosphate carboxylase. It is tentatively concluded that the slower moving of the other two bands (arrows) is the apoprotein of Chl_a-P2 and the faster moving band is the apoprotein of Chl_a-P3. (SSU = small subunit of ribulose-1,5-bisphosphate carboxylase).



3.4. *Viridis* mutants

Additional evidence for the identity of the protein moieties of Chl_a-P2 and Chl_a-P3 was sought by studying the chlorophyll-protein composition and polypeptide patterns of certain nuclear gene mutants of barley. The mutants *viridis-m*^{29}, -*n*^{34} and -*zd*^{69} were chosen on the basis of their high chlorophyll content and known photosynthetic deficiencies (31, 37). It was reasoned that these mutants, which are single gene mutations from wild-type barley, might be lacking Chl_a-P2 and/or Chl_a-P3 and this might be correlated with the polypeptide pattern, which was not expected to be greatly different from that of wild-type barley thylakoids. The chlorophyll-protein band pattern, the fluorescent band pattern and the Coomassie blue staining polypeptide bands, all from the same gel, are shown in Figure 8. All 3 mutants contained Chl_a-P3, but only *viridis-m*^{29} lacked a chlorophyll-protein band in the region corresponding to Chl_a-P2.

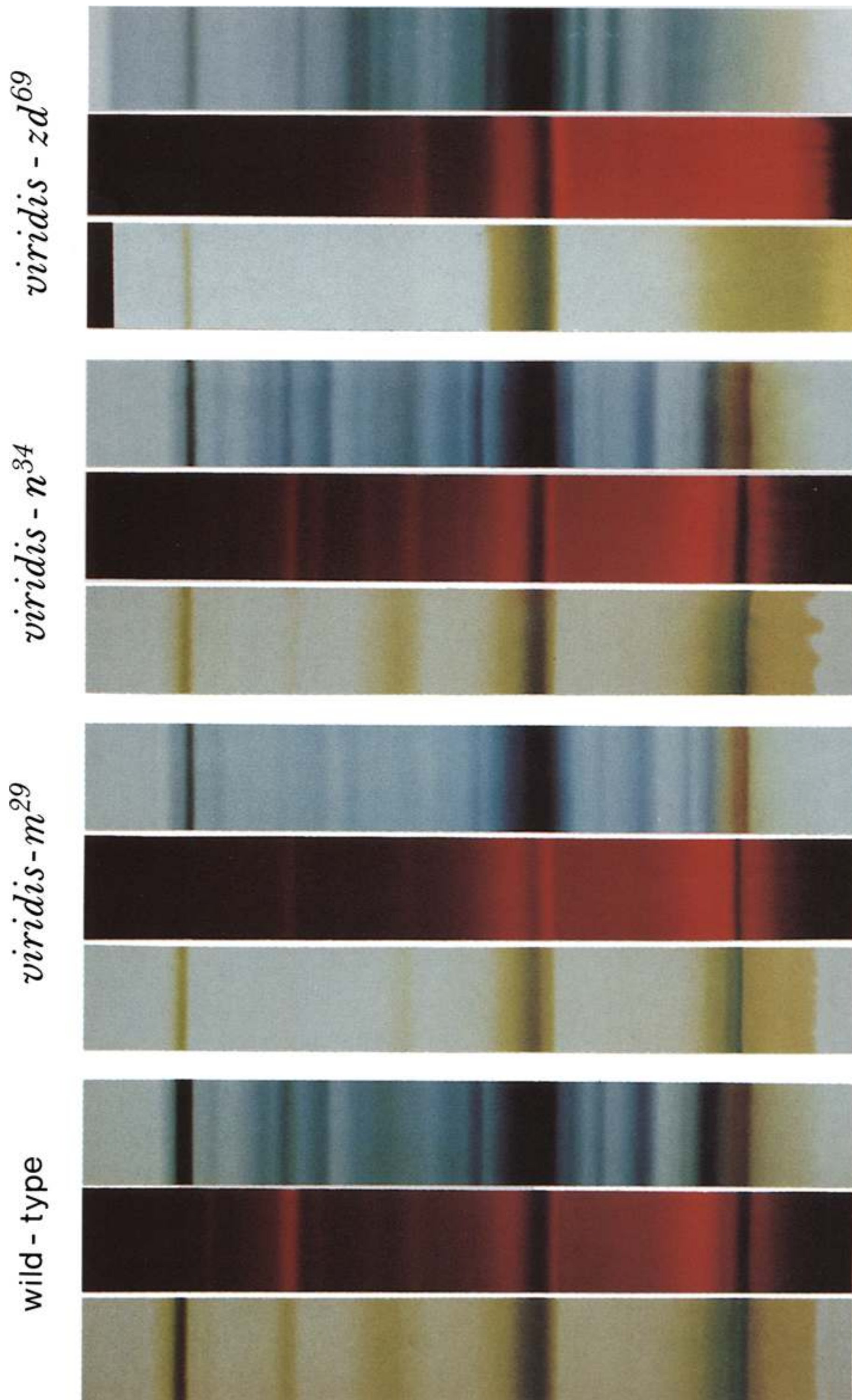
The polypeptide compositions of the purified thylakoids isolated from wild-type and the 4 mutants of barley were examined using high resolution polyacrylamide gel electrophoresis, both with and without 6M-urea. The presence of urea improves the separation of polypeptides in the low molecular weight region. The positions of polypeptides corresponding to the protein moieties of Chl_a-P2 and Chl_a-P3, as predicted from re-electrophoresis of the isolated material (Figure 7), are indicated in Figure 9. These bands are characteristically diffuse (compare with the apoprotein of Chl_a-P1 (CPI) in Figure 9), but the polypeptide from Chl_a-P3 can be seen in all 4 mutants. The polypeptide of Chl_a-P2 was particularly intense in *chlorina-f2*, and could readily be seen in the wild-type and *viridis-n*^{34} patterns (figure 9). It was strongly reduced in intensity or missing from the *viridis-m*^{29} and *viridis-zd*^{69} patterns.

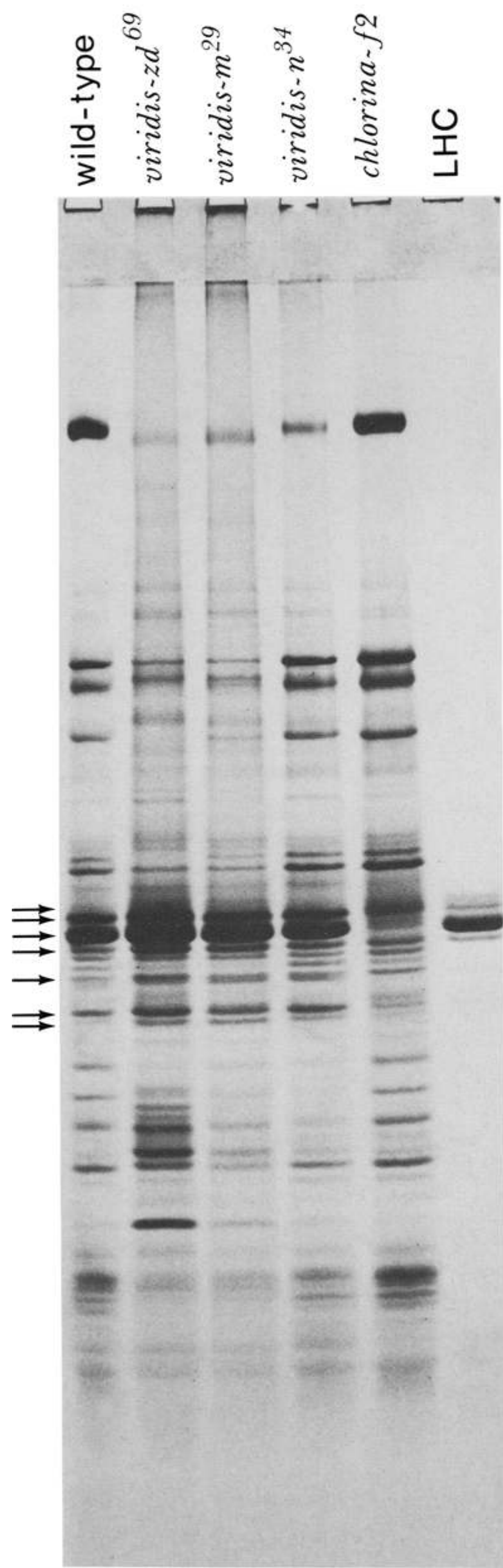
4. DISCUSSION

Ten chlorophyll-containing bands can be reproducibly obtained from SDS-solubilised thylakoids of wild-type barley using the polyacrylamide gel electrophoresis system described in this paper. Five of these bands are unique in their polypeptide composition and absorption spectra. It is useful when describing these different bands to refer to each one by a specific name. Since many workers have more or less simultaneously discovered, and named, new chlorophyll-containing bands in the past 2–3 years, there is no uniform system of nomenclature. Not only has the same chlorophyll-protein been called different names by various authors, but sometimes the same name has been given to two different bands (see Table III). It is evident from the data presented in this paper that no single nomenclature system published to date is adequate for classifying the different chlorophyll-containing bands of barley thylakoids. Therefore, the following nomenclature system for the chlorophyll-containing bands separated by SDS-polyacrylamide gel electrophoresis has been adopted:

Chlorophyll-protein: This term is used for those chlorophyll-containing bands which are sufficiently well characterised to establish that they have a unique polypeptide composition. It is helpful if, in addition, a chlorophyll-protein can be shown to be antigenically unrelated to all other chlorophyll-containing bands (9, 12). In the case where several chlorophyll-containing bands can be shown to consist of identical polypeptides, the band with the highest electrophoretic mobility (i.e., the lowest apparent molecular weight) is called a chlorophyll-protein. Such a band can be expected to represent the monomeric state. The chlorophyll-proteins so far discovered can be divided into two classes: those containing only chlorophyll *a*, and those containing both chlorophyll *a* and *b*. The different

Figure 8. Comparison of the chlorophyll-protein patterns of wild-type barley and the three nuclear gene mutants *viridis-m*^{29}, *viridis-n*^{34} and *viridis-zd*^{69}. Also shown are the fluorescent band patterns and polypeptide patterns from the same gels. The main feature of this figure is the absence of Chl_a-P2 from the mutant *viridis-m*^{29}, which although deficient in photosystem II, has sufficient activity to enable the plant to live. This is evidence that the reaction centre of photosystem II lies in Chl_a-P3.


viridis - zd69
viridis - n34
viridis - m29
wild - type



wild-type
viridis-zd69
viridis-m29
viridis-n34
chlorina-f2
LHC
LHC
wild-type
viridis-zd69
viridis-m29
viridis-n34
chlorina-f2
LHC
wild-type
Chl$_a$-P1
1
α,β-CF$_1$
2
3
SYSTEM II
SYSTEM III

Figure 9. The polypeptide composition of wild-type barley and the four nuclear gene mutants examined in this paper, as well as isolated LHC, is compared in two different gel systems. The position of Chl_a-P1 and the α and β subunits of chloroplast coupling factor 1 (CF_1) are indicated, as is the position of the apoproteins of Chl_a-P1, Chl_a-P2 and Chl_a-P3 (arrowheads 1, 2 and 3 respectively). The arrows indicate those bands in the total wild-type pattern that are found in isolated light-harvesting complex (LHC). Some of the minor bands are considered to be contaminants. The thylakoids were well-washed, to remove as much of the ribulose-1,5-bisphosphate carboxylase as possible since the large subunit of this protein has an electrophoretic mobility close to that of the apoprotein of Chl_a-P2. Of particular interest is the absence of the band corresponding to the apoprotein of Chl_a-P2 from the *viridis-m*[29] pattern. This mutant also lacks the green band corresponding to Chl_a-P2.

chlorophyll *a*-containing chlorophyll-proteins are designated Chl_a-P1, Chl_a-P2 and Chl_a-P3 in order of increasing electrophoretic mobility. Chlorophyll-proteins containing both chlorophyll *a* and *b* are, in the same way, designated $Chl_{a/b}$-P1 and $Chl_{a/b}$-P2 (Figure 1 and Table III). Where spectral or other evidence has indicated a function or property of a chlorophyll-protein, this can be incorporated into the name e.g., P-700 chlorophyll *a*-protein 1 and light-harvesting chlorophyll *a/b*-protein 2.

Apoprotein: There are no covalent bonds between chlorophyll and the protein moiety of higher plant chlorophyll-proteins since chlorophyll can be dissociated from the protein under relatively mild conditions, such as brief boiling. The polypeptide to which the chlorophyll is attached is called the apoprotein of the chlorophyll-protein. When there is only a single polypeptide component of a chlorophyll-protein, there is no ambiguity in identifying it as the apoprotein of the chlorophyll-protein. If a chlorophyll-protein dissociates into two (or more) polypeptides upon denaturation, one or both might be apoproteins. Usually an apoprotein will have a different electrophoretic mobility from the native chlorophyll-protein. The size of the difference is the result of the relative contributions of two opposing phenomena: substitution of SDS for chlorophyll produces a higher charge /mass ratio and a higher electrophoretic mobility, while denaturation and unfolding of the polypeptide chain increases its Stokes radius and lowers the electrophoretic mobility. Thus if a purified chlorophyll-protein yields two polypeptides which have a mobility slightly different from that of the chlorophyll-protein, both polypeptides may be considered to be apoproteins. The implication then, is that the original chlorophyll-containing band consists not of one, but two different chlorophyll-proteins with identical electrophoretic mobility. If one polypeptide has the same mobility as the original chlorophyll-protein, the possibility that it is a contaminant co-migrating with the chlorophyll-protein must be considered. The presence of several polypeptides, one or more of which has a mobility much lower than the original chlorophyll-protein, is more difficult to interpret and may mean that in the native state, an apoprotein plus chlorophyll is complexed with non-chlorophyll-containing polypeptides by bonds that are stable in the presence of SDS (see below).

The apoprotein of a chlorophyll-protein may be abbreviated by inserting a capital 'A' in the abbreviation for the corresponding chlorophyll-protein. Thus the apoprotein of chlorophyll *a*-protein 1 (Chl_a-P1) is abbreviated to Chl_a-AP1, and the apoprotein of chlorophyll *a/b*-protein 2 ($Chl_{a/b}$-P2) is represented by $Chl_{a/b}$-AP2, and so on.

Only five of the chlorophyll-containing bands from barley thylakoids can be classified as chlorophyll-proteins (Figure 1 and Table III). The P700-chlorophyll *a*-protein 1 (Chl_a-P1) has an apparent molecular weight of 110kD and an apoprotein of 68kD (Figure 7). The antenna chlorophylls in this band do not fluoresce, indicating the presence of an efficient quencher. This is likely to be P700, since it has been shown to be present in the Chl_a-P1 band from tobacco thylakoids (19, 26). Conversion of the P700 chlorophylls to pheophytin by acetic acid destroys the quenching ability and the antenna chlorophylls (pheophytins) can then emit red fluorescent light (Figure 1c). The highly efficient quenching of fluorescence from antenna chloro-

Table III
Chlorophyll-protein nomenclature

Anderson (2)	Chua (12)	Hayden (15)	Henriques (16)	Machold (23)	Remy (28)	Thornber (34)	Chlorophyll-proteins§	Complexes	Not Characterised	Apparent mol. wt.+
					Ia			Chl_a-P1**		
CP1a					Ib			Chl_a-P1*		
CP1	CPI	I	CPI	CPI	Ic	CPI	Chl_a-P1			110kD
								$Chl_{a/b}$-P2***		107kD
									Chl-P band	92kD
LHCP[1]		II[1]	IIb		IIa			$Chl_{a/b}$-P2**		71kD
									Chl-P band	62kD
LHCP[2]			IIa					$Chl_{a/b}$-P2*		50kD
	CPIII				IIb_1		Chl_a-P2			49kD
CPa		IV	A							
	CPIV				IIb_2		Chl_a-P3			41kD
				CPIII			$Chl_{a/b}$-P1			32kD
LHCP[3]	CPII	II	II	CPII'	CPII	CPII	$Chl_{a/b}$-P2			29kD

§ The apoprotein is indicated by the abbreviation Chl_a-AP1, Chl_a-AP2 etc.
+ These values are approximate only, and apply to barley thylakoids.

phyll indicates that Chl_a-P1 has retained its native conformation, as previously indicated by its low temperature fluorescence emission spectrum (2) and P700 difference spectrum (28).

The apparent molecular weight of Chl_a-P2 is approximately 49kD and Chl_a-P3 has an apparent molecular weight of about 41kD. The electrophoretic mobility of each of these chlorophyll-proteins is at least as high as their corresponding apoproteins, in contrast to Chl_a-P1 and its apoprotein. In Chlamydomonas (12) both of these chlorophyll-proteins (Chl_a-P2 and Chl_a-P3) have a faster electrophoretic mobility than their corresponding apoproteins. These two chlorophyll-proteins are good candidates for the site of the reaction centre of photosystem II (P680), since along with Chl_a-P1 (the site of the reaction centre of photosystem I), these are the only major chlorophyll-proteins in the *chlorina-f2* mutant (Figure 4). Chl_a-P2 is absent from the mutant *viridis-m[29]* (Figure 8), which although low in photosystem II activity (B. Carlsen, cand. scient. thesis), is nevertheless autotrophic and will produce seeds under suitable growth conditions (7). It would therefore appear that Chl_a-P3 is the most likely site of the reaction centre of photosystem II. This band, also called CPIV (12), or complex IIb_2 (28), or a component of CPa (2), or A (16), or complex IV (15) has previously been implicated as being involved in photosystem II. In all instances, Chl_a-P3 lacks chlorophyll *b* and has a chlorophyll absorption peak at 671 nm (Figure 5). Conclusive evidence for the site of the photosystem II reaction centre will come from the demonstration of P680 in this band, and/or a low temperature fluorescence emission peak at 695 nm.

In Chlamydomonas Chl_a-P2 and Chl_a-P3 (called CPIII and CPIV respectively) each contain a single unique polypeptide (polypeptide 5 and 6 respectively) (12). The apoprotein of Chl_a-P3 (CPIV) in Chlamydomonas cross-reacts immunochemically with the apoprotein of barley Chl_a-P3 (31) and it is probable that the apoprotein of Chlamydomonas Chl_a-P2 (CPIII) corresponds to the apoprotein of barley Chl_a-P2. Genetic evidence has indicated that polypeptide 6, and possibly polypeptide 5 of Chlamydomonas, are involved in the reaction centre of photosystem II (8). Genetic (8) and inhibitor studies (10) have shown that these polypeptides

are coded for by chloroplast DNA and are translated on chloroplast ribosomes. Since *viridis-m*[29], which lacks Chl_a-P2, is a nuclear gene mutant, nuclear DNA is involved in the incorporation of Chl_a-P2 into the thylakoid membrane. The involvement of both chloroplastic and nuclear genes in the formation of another chlorophyll-protein (Chl_a-P1) has already been reported (4, 11).

There are two chlorophyll *a*/*b*-proteins (Table III). Chlorophyll *a*/*b*-protein 1 ($Chl_{a/b}$-P1) has an electrophoretic mobility slightly lower than $Chl_{a/b}$-P2 (Figure 1) and a higher chlorophyll *a*/*b* ratio, but is not completely resolved in gel system IV. It has been shown with Vicia faba (23) that isolated $Chl_{a/b}$-P1 is composed of a single polypeptide which has the same mobility as one of the two polypeptides of $Chl_{a/b}$-P2. It has been demonstrated, however, that the $Chl_{a/b}$-P1 apoprotein is different from the $Chl_{a/b}$-P2 polypeptides by using the improved resolution of the urea gel system (23). The function of this chlorophyll-protein is not known, but its absence from the *chlorina-f2* pattern (Figure 4) indicates that it is not essential for photosynthesis, but might play a role in light-harvesting or light-focusing for photosystem I or II.

The light-harvesting chlorophyll *a*/*b*-protein 2 ($Chl_{a/b}$-P2) is the major chlorophyll-protein of wild-type thylakoids, containing about 50% of the total chlorophyll (2, 18) and most, but not all, of the chlorophyll *b*. It was originally believed to be the reaction centre of photosystem II, but since it is not found in the photosynthetically competent *chlorina-f2* mutant, it has been assigned a light-harvesting function (35). Its properties will be discussed below in connection with chlorophyll-protein complexes.

Chlorophyll-protein complexes: Originally the chlorophyll-containing bands separated by gel electrophoresis were called chlorophyll-protein complexes (33, 34), indicating that chlorophyll and a protein formed a complex. For some time it was thought that such bands on polyacrylamide gels were identical to chlorophyll-protein complexes isolated by detergent treatment of thylakoids followed by purification on sucrose gradients or by column chromatography. The use of the term 'complex' for both chlorophyll-proteins and the preparations containing chlorophyll-proteins associated with other polypeptides has led to confusion between, for example, light-harvesting chlorophyll *a*/*b* protein complex (CPII or LHCP) and the light-harvesting complex (LHC), and between Chl_a-P1 (CPI) and photosystem I particles (3) containing Chl_a-P1.

The term chlorophyll-protein complex is limited here to a chlorophyll-protein associated with itself to form an oligomer, or with another chlorophyll-protein, or with non-chlorophyll-containing polypeptides. A chlorophyll-containing band is designated a complex by placing one or more asterisks after the name of the chlorophyll-protein from which it is derived (see Table III). Many chlorophyll-protein complexes are isolated by gentle detergent treatment of thylakoids, yielding upon purification, a uniform population of particles consisting of stoichiometric quantities of a chlorophyll-protein and non-chlorophyll-containing proteins. Such complexes seem to exist within the thylakoid membrane as structural (and functional) units (3, 6). In addition, the tendency for some chlorophyll-proteins to self-aggregate (6), may lead to the formation of artefactual higher order complexes that are stable under the conditions used for electrophoresis.

The chlorophyll-protein complexes related to $Chl_{a/b}$-P2 were investigated by electrophoresis of the light-harvesting complex isolated by Triton-X100 solubilisation and precipitation with Mg^{++} (6). When electrophoresed in system II (Figure 9) this preparation was resolved into one major polypeptide component of molecular weight 25kD and two minor bands at 26kD and 24kD. The four bands at 27kD, 22kD, 20kD and 19kD are probably contaminants that co-precipitate during the isolation. The same preparation run in a urea gel (system III) yields two major bands at 27kD and 26kD with minor bands at 25kD and 24kD and probable contaminants at 31kD, 21kD and 20kD (Figure 9). It is difficult to make a direct correlation between the bands in the two different gels, but it seems probable that the major band in system II has been resolved into two bands in the urea system. There is indirect evidence from Vicia faba that $Chl_{a/b}$-P2 consists of two distinct apoproteins and hence two chlorophyll-proteins (23). The chlorophyll *a*/*b* ratio decreases as one moves down the $Chl_{a/b}$-P2 band, but the relatively high

chlorophyll a/b ratio of the free chlorophyll (Figure 3) could mean that there is a selective loss of chlorophyll a from $Chl_{a/b}$-P2 during electrophoresis. While APEL (1) has found two components of the $Chl_{a/b}$-P2 complex in Acetabularia thylakoids, only one of them was considered to be an apoprotein. There is evidence, however, that the $Chl_{a/b}$-P2 region of Chlamydomonas consists of several chlorophyll-containing bands when electrophoresed at low temperature in the presence of lithium dodecyl sulphate (12.

It is interesting that there is a small amount of red fluorescing material at the position of $Chl_{a/b}$-P2 in the *chlorina-f2* mutant (Figure 4) and that it has a chlorophyll a spectrum. The site of the primary lesion in *chlorina-f2* is not known, but in view of the absence of so many chlorophyll b-containing chlorophyll-proteins, it would appear to be the inability to convert chlorophyll a to chlorophyll b. It is possible that without chlorophyll b, chlorophyll b-containing proteins are not synthesised, or fail to become incorporated into the thylakoid membrane.

The slower moving chlorophyll-protein complexes of $Chl_{a/b}$-P2 have been designated $Chl_{a/b}$-P2*, $Chl_{a/b}$-P2**, and $Chl_{a/b}$-P2***, in order of increasing molecular weight (Table III). These are considered to be multimers of $Chl_{a/b}$-P2 on the basis of absorption spectra and polypeptide composition of both the complexes and the isolated light-harvesting complex. Although one might speculate that the complexes represent dimers, trimers and tetramers of $Chl_{a/b}$-P2 on the basis of their apparent molecular weights (29kD, 50kD, 71kD and 107kD) it should be remembered that chlorophyll-proteins show anomolous electrophoretic behaviour in polyacrylamide gels (11). Since the actual method for purification of this material is based on Mg^{++}-induced self-aggregation of LHC, it is difficult to decide whether these complexes of $Chl_{a/b}$-P2 are artefacts, or represent forms which are closer to the in vivo state, as suggested (2, 28) from spectral evidence.

On occasion, a chlorophyll-containing band was seen above Chl_a-P1, and on the basis of its fluorescence quenching is most likely a higher order complex of Chl_a-P1, and should be designated Chl_a-P1*. Such complexes have been seen previously in tobacco (28) and spinach (2) and Vicia faba.

Chlorophyll-protein bands: It was agreed by participants of the EMBO Workshop on Chloroplast Membrane Polypeptides (Carlsberg Laboratory, Copenhagen, June 11, 1979) that a chlorophyll-containing region in polyacrylamide gels should be given the general name of chlorophyll-protein bands until sufficient evidence had accumulated to establish its identity as either a chlorophyll-protein or a chlorophyll-protein complex. In the case of barley thylakoids, there are two chlorophyll-containing protein bands, designated Chl-P (Figures 1 and 6, Table III), that are not complexes of $Chl_{a/b}$-P2 and have not yet been characterised sufficiently well to determine whether they are unique chlorophyll-proteins. They could be artefactual chlorophyll-containing bands formed by the focusing of free chlorophyll released from chlorophyll-proteins, by ion boundaries or high local concentrations of polypeptides. The occurrence of the same red fluorescent bands between $Chl_{a/b}$-P2 and free chlorophyll, in both wild-type and *chlorina-f2*, may be the result of these phenomena, particularly since these bands correspond with the major Coomassie-staining polypeptide bands in this region of the gel (Figure 1). The appearance of a fluorescent band with a position between Chl_a-P3 and $Chl_{a/b}$-P1 after fixation in acetic acid, is consistent with the known electrophoretic mobility of barley cytochrome f (37) which becomes fluorescent when converted to the free base.

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