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A CLOVER MUTANT LACKING THE CHLOROPHYLL a- and b-CONTAINING PROTEIN ANTENNA COMPLEXES

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SUMMARY. A chlorophyll deficient mutant of clover has been examined by SDS-PAGE, spectrophotometric and electron microscopic methods. By a comparison of the absorption and first derivative spectra of acetone extracts from the mutant and normal biotypes, we observed a deficiency in chlorophyll b for the mutant biotype. The calculated chlorophyll a/b ratios, using the method of Arnon (Plant Physiol. 24, 1-15, 1949), approached infinity for the mutant whereas it ranged from 3.0-3.3 in the wild-type. The low temperature (77°K) fluorescence emission bands in the 685-695 nm region could not be differentiated for the biotypes; however, the long wavelength emission band (near 740 nm in the wild-type) was shifted to shorter wavelengths (ca 720-725 nm) in the mutant indicating loss of photosystem I antenna. The SDS-PAGE profile of the mutant biotype showed a dramatic decline in the Coomassie stained polypeptides of apparent molecular weights similar to those of LHC II. Transmission electron micrographs of the mutant and normal tissue exhibited similar extents of grana-stacking, indicating that a component(s) other than the LHC II may be responsible for membrane adhesion in this mutant.

LHC II has been assigned a number of roles in photosynthesis. In addition to its role in light energy absorption and transfer, it has been implicated in the regulation of quantal energy distribution between PS II and PS I. This latter role has also been implicated with the structural membrane characteristics of granal and stromal lamellae and the physical separation of the two photosystems. (1) Phosphorylation and dephosphorylation of the LHC II protein(s) have recently been argued to be involved in the regulation of quantal distribution and associated physical membrane changes. This latter phenomenon has received wide

ABBREVIATIONS:
LHC II and I, chlorophyll a and b containing light harvesting antenna complexes associated with photosystem II and I, respectively; PS II, photosystem II; PS I, photosystem I; Chl, chlorophyll; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; pI, isoelectric point; TEM, transmission electron microscopy; ImL, intermittant light.

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attention ever since Bennett (2) initially observed the in-vitro phosphorylation of a number of chloroplast proteins.

Moreover, others have reported the involvement of the LHC II in structural reorganization of the chloroplast membrane from cation studies with liposomes which had been incorporated with isolated LHC II (7,8). Much of our knowledge of the LHC II has come from mutant studies, in particular, in mutants deficient in chlorophyll b. The most intensive studies have been conducted with the chlorina f mutant of barley (3) and greening studies including the use of ImL conditions during growth (4). Characterization of a chlorophyll deficient mutant of wheat (5,6) has recently been reported. In addition, these latter studies and those pertaining to greening, provided us with some clues as to the nature of the PS I chlorophyll a/b containing antenna complex (LHC I) and the origin of the long-wavelength fluorescence emission from PS I at low temperature (77°K). The data suggested that a shift in the long-wavelength emission from 735-740 nm to 720-725 nm is indicative of a loss of the antenna complex serving PS I (LHC I), see ref. 9. The blue-shifted maximum emission band likely originates from the PS I reaction center as previously reported (7,10).

The studies conducted in this report pertain to a mutant of clover which is deficient in chlorophyll b (See also 11).

MATERIALS AND METHODS

Chloroplasts were isolated from greenhouse grown normal (U389) and a chlorophyll b-deficient mutant (U374) of clover, Melilotus alba. The seeds were kindly supplied by Drs. John Markwell, H. J. Gorz and F. A. Haskins, University of Nebraska-Lincoln.

Thylakoid membranes were obtained as previously described for spinach (12). Chl concentration and Chl a/b ratios were determined using the method of Arnon (13). Protein concentrations were obtained using the Bio-Rad protein assay method (14).

Absorption spectroscopy was conducted on a Shimadzu 260 spectrophotometer and/or UV 3000 dual wavelength spectrophotometer. Fluorescence (77°K) emission spectra were obtained courtesy of Drs. L. Valerino and L. Decola, Virginia Commonwealth University, Richmond, Va. using an SLM-Aminco 8000 fluorimeter. Acrylamide gel electrophoresis was conducted as previously described (15,16) using premade gels (10-20% acrylamide), Integrated Separation Systems, Newton, Ma.

For transmission electron microscopy, leaflets were obtained from the mid-stalk position of two month old plants and placed into 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). The leaflets were diced into 1 mm X 5 mm strips while immersed in the above fixative solution and fixed overnight at 4°C. The samples were then washed 3X in 0.1 M cacodylate buffer and post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2) for two hours. The samples

were washed again 3X in 0.1 M cacodylate buffer. The samples were then dehydrated through a sequential series of ethanol concentrations in water: 50% (2X), 70%, 85%, 95% (3X) and 100% (3X). This was followed by two changes in 100% acetone. Samples were infiltrated with Spurr epoxy resin (17) through a sequential series of Spurr concentrations in acetone: 33% (overnight), 66% (6 h.) and 100% (overnight). The samples were then embedded in Spurr and allowed to polymerize at 65°C for 48 hours. 100 nm thick sections were sliced using a LKB-3 ultramicrotome and placed on copper grids. The sections were subsequently stained with 4% uranyl acetate in 50% methanol (H_2O) followed by 0.2% lead citrate in H_2O. The leaf-cross sections were examined with the use of a JEOL 100CX TEM. Cross-sectional thickness measurements on the appressed membrane pairs in the two biotypes were measured from TEM negatives of the membranes taken at 33,000X and projected to 5X the original. Means and standard deviations were calculated from 34 different grana stacks in 11 different cells for the wild type clover and 24 different grana stacks in 6 different cells for the mutant material.

LHC I (tobacco) was obtained by subjecting a PS I enriched preparation to column isoelectric focusing (1% Pharmalyte) on a stepwise sucrose gradient (decreasing in 10% steps) layered on a 60% sucrose cushion. 150V were applied for 1 hour followed by 250V overnight. 20 mN NaOH was used as the cathode electrode solution and ~1% H_3PO_4 was used as the cathode electrode solution. Green bands were removed manually by use of a capillary pipette.

RESULTS AND DISCUSSION

SPECTROSCOPY

The deficiency of chlorophyll b in the sweet clover mutant is observed by comparison of the absorption and first derivative spectra for the normal and mutant biotypes of clover. This is shown in Fig. 1 by the shoulder in the first derivative spectra near 640 nm for the wild-type clover which is absent in the mutant. The Chl a/b ratio for the wild-type material was approximately (3.0 - 3.3) whereas the mutant exhibited a ratio approaching infinity.

Fluorescence spectroscopy has been widely used as a diagnostic tool of the chlorophyll complexes present in photosynthetic tissue. We examined the two biotypes to see whether or not there were any apparent differences especially in the short wavelength chlorophyll fluorescence emission region (680-695 nm). However, from the results shown in Fig. 2, very little difference could be discerned in this region. The fluorescence emission band maximum for the ImL treated materials (4), generally has shown a blue shift (peak near 680 nm) in this region which has been attributed to the lack of integration of LHC II complexes with PS II. A more obvious difference in the fluorescence spectra, however, is the shift of the long-wavelength emission maximum, i.e., from 730-740 nm to around 725 nm. As reported in a previous paper (9), a shift in the fluorescence emission band from the PS I reaction center was found to be indicative of

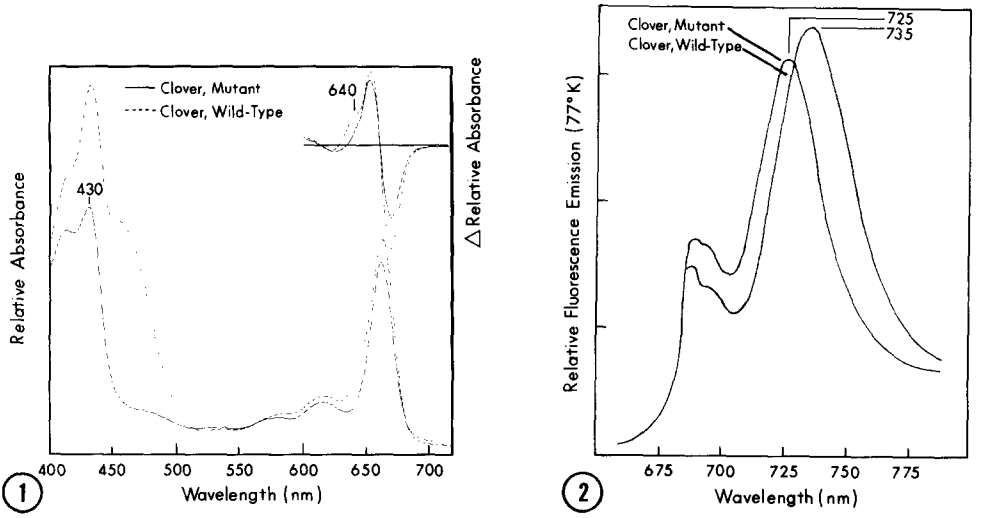

Figure 1. Absorption spectra of 80% acetone extracts of thylakoids obtained from wild-type and mutant clover. First derivative spectra are shown in upper right for the red-region of the absorption spectra for both biotypes (note shoulder at 640 nm for the mutant).

Figure 2. Low temperature (77°C) chlorophyll fluorescence emission spectra of thylakoids from the wild-type and mutant clover (note shift in the long-wavelength emission band maximum.

a loss of LHC I, a specific chlorophyll a/b protein complex serving PS I (10, 18,19). Upon examination of previous spectra for chlorophyll b deficient mutants and ImL treated material, a shift from long wavelengths (730-740 nm) to shorter wavelengths (720-730 nm) is apparent in all cases. This phenomenon, therefore, is apparently diagnostic of a loss of the antenna complex serving PS I. Freeman et. al (5) have recently reported on a chlorophyll b-deficient mutant of wheat, CD3, in which a shift in the long wavelength emission band was observed. Monomeric forms of the LHC II complex were still present, indicating the presence of chlorophyll b in the mutant (6, and personal communication, Markwell and coworkers).

SDS-PAGE PROFILES

As a consequence of the deficiency in Chl b in the mutant clover, the apoproteins associated with LHC II and LHC I would also be expected to be deficient or lacking. Extremely faint Coomassie stainable - proteins (lane 4, mutant) appear to have similar electrophoretic behavior to those associated with the LHC

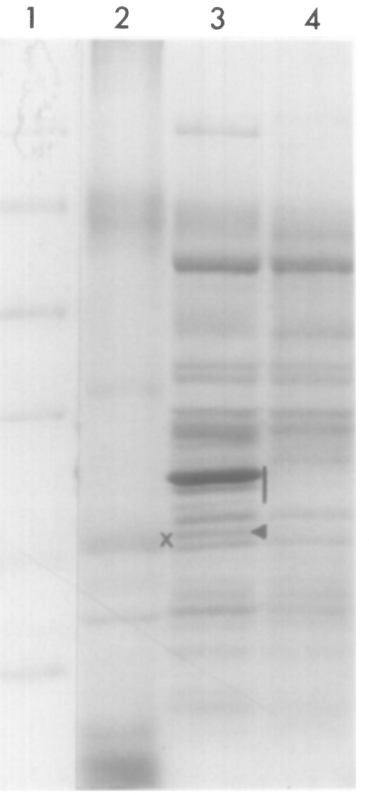

Figure 3. SDS-PAGE profile in lane 1 (molecular weight markers 92.5, 66.2, 45, 31, 21.5 and 14.4 kDa, BioRad), Lane 2 (LHC I obtained from tobacco by isoelectric focusing), lane 3 (wild type clover chloroplast thylakoids), lane 4 (mutant clover chloroplast thylakoids). Bar denotes LHC II size class, ▲ denotes polypeptide band (having a similar molecular weight to that for an LHC I apoprotein (from tobacco) denoted by X), missing in the mutant. Non-dissociated LHC II has an apparent molecular weight in the wild type clover of ~29 kDa.

II (lane 3, wild-type) apoproteins denoted by bar (~23-25 kDa) in Fig. 3; whether these are, in fact, due to a residual amounts of LHC II is difficult to assess.

In addition, a comparison of the polypeptides associated with LHC I obtained from tobacco chloroplasts is shown in lane 2 of Fig. 3. The LHC I (pI$\underline{\sim}$6.2) was obtained by subjecting a PS I enriched preparation obtained by Triton-X 100 fractionation of tobacco chloroplasts to isoelectric focusing on a sucrose support medium. There appear to be polypeptides in the wild type clover with similar molecular weights to those of LHC I isolated from tobacco. The band denoted by X (~20 kDa) in lane 2 (Fig. 3) from the tobacco LHC I complex may be analogous to the band, denoted by Δ, observed in lane 3 of Fig. 3 which is apparently absent in the mutant, see lane 4 (9,19). The calculated value of X is at

variance with the actual experimental position of the marker, soybean trypsin inhibitor-BioRad, which migrated anamolously in our gel system.

TRANSMISSION ELECTRON MICROSCOPY

Ultrastructural studies of the wild type and mutant clovers, using TEM, show that both biotypes possess stacked grana (Fig. 4). The number of grana per chloroplast and the number of paired thylakoid membranes per granum were found to be variable in both biotypes; however, no statistical analysis was undertaken. An analysis of the spacing between membrane pairs, measured as the cross-sectional thickness of the appressed membranes and half the distance between the next appressed membrane pair, appeared to be different for the two biotypes. The mean and standard deviation were 143Å ± 12.6Å and 171Å ± 12.3Å for the wild-type and mutant clover, respectively. This is in contrast to the results reported by Goodchild et al. (20) from their studies on barley. This slightly greater separation between membrane pairs may indicate weaker electrostatic and hydrophobic interactions at the membrane surface of the mutant clover (see Ref. 22). Although no statistical analysis was conducted, the mutant appeared to contain fewer starch granules and more electron dense (oil) droplets present in each

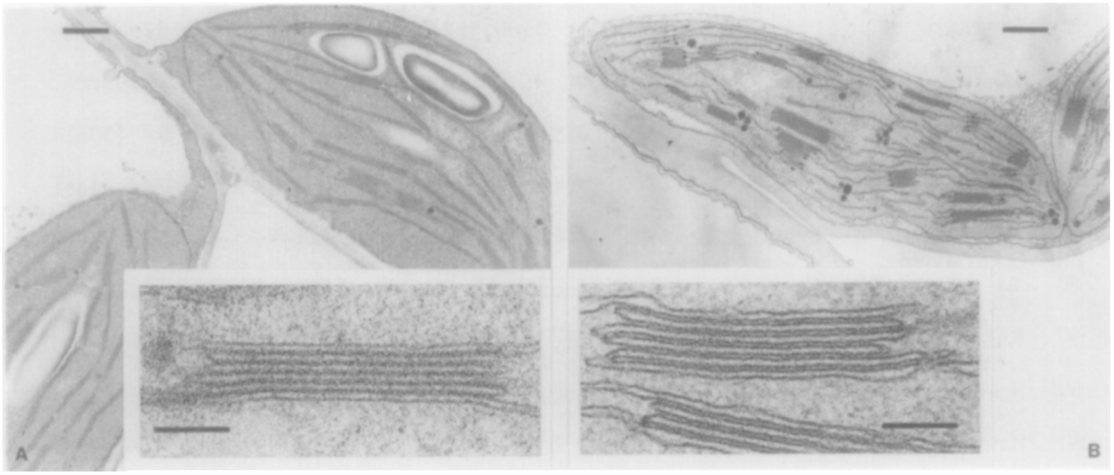

Figure 4. Chloroplasts from wild type and mutant clover. A. Wild type chloroplasts from spongy cell. Bar denotes 0.5 μ. Insert illustrates the close positioning of appressed membranes, bar denotes 0.1 μ. B. Mutant chloroplast from spongy cell. Bar denotes 0.5 μ. Insert demonstrates wider spacing (16% more) between the membrane pairs than the wild type. Bar denotes 0.1 μ.

individual chlorophast than the wild type. It has been postulated that grana stacking is dependent on the presence of LHC II (23). It has also been suggested that phosphorylation and dephosphorylation of LHC II is the key factor in the regulation of quantal energy distribution between PSI and PS II (23). The results in this study indicate that the clover mutant contains no chloropyll b, and is deficient in the apoproteins of the two chlorophyll a/b containing protein complexes, LHC I and LHC II. Since both biotypes have appressed grana we suggest that some component other than LHC II is involved in grana stacking.

This clover mutant appears to be useful for continued studies on the mechanism of membrane appression and the characterization of the photosynthetic apparatus.

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