Plant Physiol. (1986) 82, 19–27
0032-0889/86/82/0019/09/$01.00/0

Developmental Loss of Photosystem II Activity and Structure in a Chloroplast-Encoded Tobacco Mutant, *Lutescens-1*[1]

Received for publication December 19, 1985 and in revised form April 21, 1986

CATHERINE P. CHIA*[2], JOHN H. DUESING[3], AND CHARLES J. ARNTZEN
MSU/DOE Plant Research Laboratory (C.P.C., J.H.D.), Michigan State University, E. Lansing, Michigan 48824-1312; and E. I. du Pont de Nemours (C.J.A.), Central Research and Development, Wilmington, Delaware 19898

ABSTRACT

***Lutescens-1*, a tobacco mutant with a maternally inherited dysfunction, displayed an unusual developmental phenotype. *In vivo* measurement of chlorophyll fluorescence revealed deterioration in photosystem II (PSII) function as leaves expanded. Analysis of thylakoid membrane proteins by polyacrylamide gel electrophoresis indicated the physical loss of nuclear- and chloroplast-encoded polypeptides comprising the PSII core complex concomitant with loss of activity. Freeze fracture electron micrographs of mutant thylakoids showed a reduced density, compared to wild type, of the EF_s particles which have been shown previously to be the structural entity containing PSII core complexes and associated pigment-proteins. The selective loss of PSII cores from thylakoids resulted in a higher ratio of antenna chlorophyll to reaction centers and an altered 77 K chlorophyll fluorescence emission spectra; these data are interpreted to indicate functional isolation of light-harvesting chlorophyll *a/b* complexes in the absence of PSII centers. Examination of PSII reaction centers (which were present at lower levels in mutant membranes) by monitoring the light-dependent phosphorylation of PSII polypeptides and flash-induced O_2 evolution patterns demonstrated that the PSII cores which were assembled in mutant thylakoids were functionally identical to those of wild type. We conclude that the *lutescens-1* mutation affected the correct stoichiometry of PSII centers, in relation to other membrane constituents, by disrupting the proper assembly and maintenance of PSII complexes in *lutescens-1* thylakoid membranes.**

The formation of the photosynthetic apparatus in higher plants requires coordinated gene expression of both the nuclear and chloroplast genomes. To evaluate the contribution of the plastome (the plastid genome) to the biogenesis and maintenance of photosynthetic membranes, we have analyzed a series of chloroplast-encoded photosynthetic mutants (6). One of these was initially characterized by a developmentally expressed phenotype; this mutant, *lutescens-1*, is described in more detail herein.

Photosynthetic mutants have been used as tools in numerous studies to understand plastid development and elucidate functional properties of the chloroplast (13, 15). In almost all cases, however, the materials studied were nuclear-encoded mutations. For higher plants there have been only a few thorough studies of plastome mutants affecting photosynthetic function. These include pigment-deficient mutants of *Oenothera* (12), and *Nicotiana* (26), and herbicide-resistant mutants of *Brassica* (8), *Amaranthus* (14), and *Solanum* (10). In all these cases, uniparental inheritance of the mutation was presumptive evidence of a plastome mutation.

The mutant *lut-1*[4] belongs to a collection of tobacco chloroplast mutants, described in Chia *et al.* (6), generated through ethyl methane sulfonate mutagenesis of *Nicotiana tabacum* seed. The mutants were initially identified by the appearance of leaf sectors showing Chl deficiencies. Reciprocal genetic crosses showed that these traits were maternally inherited, thereby indicating mutations in the plastid genome. Preliminary results have shown that most of the mutants from this collection have reduced electron transport activity (primarily in PSII), altered Chl *a/b* ratios, and the loss or depletion of certain thylakoid membrane proteins as compared to WT tobacco grown under the same conditions.

What made *lut-1* subject to more intensive examination was the obvious and consistent loss of Chl exhibited by mutant leaves as a function of leaf age. This suggested that a developmentally controlled biochemical change, occurring over the lifetime of the mutant chloroplasts, was resposible for the loss of Chl.

MATERIALS AND METHODS

***In Vivo* Analysis.** Mature plants of WT and mutant *lut-1* (previously designated TP-13, Ref. 6) tobacco (*Nicotiana tabacum*, var L.C. from the Connecticut Agricultural Experiment Station) were grown in a greenhouse under natural light conditions (temperature 25 ± 5°C) in soil. Plants were watered daily. Mutant plants were maintained as variegated sectorial and/or periclinal chimeras, and pruned to force growth of mutant axillary buds which gave rise to completely mutant shoots suitable for chloroplast analysis.

In vivo Chl fluorescence induction transients were monitored with a model SF-10 Plant Productivity Fluorometer (R. Brancker, Ltd., Ottawa, Canada) as described earlier (6). Transients were recorded with a Nicolet Explorer III digital oscillo-

[1] Supported, in part, by Department of Energy Contract No. DE-AC02-76ERO-1338 to Michigan State University and a grant from E. I. du Pont de Nemours, Inc. C. P. C. received partial support from a United States Office of Education Graduate and Professionals Opportunities Program Fellowship.

[2] Current address: Biology Department, Princeton University, Princeton, NJ 08544.

[3] Current address: Biotechnology Research, CIBA-GEIGY Corp., P. O. Box 12257, Research Triangle Park, NC 27709-2257.

[4] Abbreviations: *lut-1, lutescens-1*; WT, wild type; DPIP, dichlorophenolindolphenol; MV, methyl viologen; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; LDS, lithium dodecyl sulfate; RC, reaction center; Q_B, secondary quinone acceptor; F_o, initial fluorescence; F_p, peak fluorescence (*in vivo*); Q_A, primary quinone acceptor; F_v, variable fluorescence; F_m, maximum fluorescence (*in vitro*); LHC-II, light harvesting complex serving PSII; PQ, plastoquinone.

scope and plotted with an x-y recorder. A minimum of four different sections of a given leaf were assayed; if dissimilar fluorescence transients were found within the same leaf, that leaf was not used for further analysis. Tissue from leaves having similar fluorescence transients were pooled for chloroplast isolation.

Chloroplast Membrane (Thylakoid) Isolation. Thylakoid membranes were isolated as previously described (6) in an isolation medium containing 400 mM sorbitol, 100 mM Tricine-NaOH (pH 7.8), and 10 mM NaCl. The chloroplast pellet obtained was washed with 10 mM Tricine-NaOH (pH 7.8), 10 mM NaCl, and 5 mM $MgCl_2$. All buffers contained 2 mM phenylmethylsulfonyl fluoride and 1 mM *p*-aminobenzamidine as protease inhibitors. Chl was assayed by the method of MacKinney (23). Protein determinations were performed using a modified Lowry assay (24).

Photosynthetic Reactions. PSII-dependent DPIP reduction was measured at 580 nm as previously described using a Hitachi model 100-60 spectrophotometer modified for direct sample illumination with an actinic beam (28). Chloroplasts (5–10 μg Chl) were added to 2 ml of a solution containing 100 mM sorbitol, 50 mM K-phosphate (pH 6.8), 0.4 mM DPIP, 10 mM NaCl, 5 mM $MgCl_2$, 0.5 mM NH_4Cl, and 0.1 μM gramicidin-D.

Whole chain electron transport (H_2O to MV) and PSI-mediated electron transport (0.5 mM TMPD, reduced with 2.5 mM sodium ascorbate [pH 7.8], as electron donor and MV as electron acceptor in the presence of 10 μM DCMU) were measured by continuous recording of O_2 uptake using a water-jacketed O_2 electrode. Illumination was provided by a high intensity microscope lamp. Chloroplasts (25–40 μg Chl) were added to 1 ml of a reaction mixture containing 100 mM sorbitol, 50 mM Tricine-NaOH (pH 7.8), 10 mM NaCl, 5 mM $MgCl_2$, 0.5 mM MV, 0.5 mM NH_4Cl, 0.1 μM gramicidin-D, 0.5 mM NaN_3, and 5 to 10 μg of superoxide dismutase (approximately 3000 units/mg).

Fluorescence Measurements. Room temperature fluorescence induction transients with stroma-free thylakoids were measured in the absence or presence or 5 μM DCMU as previously described (28). Chloroplast membranes (10 μg Chl) were dark-adapted for 10 min in 2 ml of 100 mM sorbitol, 50 mM Tricine-NaOH (pH 7.8), 10 mM NaCl, and 5 mM $MgCl_2$. Transients were reported with a Nicolet oscilloscope. A minimum of three replicates of each sample were recorded.

Low temperature (77 K) fluorescence emission spectra were acquired using a System 4800 scanning spectrofluorometer (SLM Instruments, Urbana, IL). Samples (10 μg Chl/ml, 60% v/v glycerol, 50 mM Tricine-NaOH [pH 7.8], 10 mM NaCl, and 5 mM $MgCl_2$) were frozen in 0.5 mm i.d. capillary tubes and excited at 440 nm. Sodium fluorescein (1 μM) was used as an internal standard (17).

Flash-Induced Oxygen Production. O_2 evolution patterns induced by a series of single-turnover flashes were measured with a Joliot-type O_2 electrode (31). Freshly prepared thylakoid membranes (250 μg Chl/ml), transferred to the Pt-electrode surface in dim light, were given a train of 40 flashes at 1 Hz and then dark-adapted for 15 min before recording the O_2-evolution patterns with an x-y recorder. Illumination was provided by a Xenon flash lamp (General Radio Stroboslave model 1539A). Calculations for double hits, misses, and S-state fractions from the O_2-evolution patterns induced by the first 10 to 12 flashes after dark adaptation were made using a computer program, provided by W. F. J. Vermaas, for a Hewlett-Packard 85 computer. Experimental data were fitted to O_2-evolution values calculated from the classical model developed by Kok *et al.* (16).

Polypeptide Analysis. SDS-PAGE of denatured membrane polypeptides was performed using the discontinuous buffer system of Laemmli (20) as described previously (6). Mol wt markers were: BSA, 68 kD; ovalbumin, 45 kD; carbonic anhydrase, 29 kD; and Cyt *c*, 12 kD. Extraction of PSII Chl-protein complexes was accomplished using octyl-β-D-glucopyranoside following the procedure of Camm and Green (5). For our analyses, isolated membrane fragments were washed twice with 300 mM sucrose, 5 mM MOPS (3-[N-Morpholino]propanesulfonic acid) (pH 7.0) before solubilization. Chl-protein complexes were separated on polyacrylamide slab gels run at 4°C in the presence of LDS rather than SDS.

Protein Phosphorylation. Phosphorylation of WT and *lut-1* thylakoid membrane proteins was carried out using [γ-^{32}P]ATP. The protocol was as previously described (19) except that the final ATP concentration was 100 μM rather than 200 μM and 5 mM NaF was added to prevent dephosphorylation of phosphorylated samples (except as indicated). Samples were illuminated with white light (500 $\mu E/m^2 \cdot s$) for 20 min in the presence or absence of 10 μM DCMU. Samples were analyzed by SDS-PAGE (10 μg Chl/lane) and phosphoproteins were detected by autoradiography.

Protein Detection by Immunoblotting. Proteins from denaturing SDS polyacrylamide gels were transferred to nitrocellulose sheets (0.45 μm pore size, Schleicher & Schuell, Keene, NH) for 6 to 10 h at 60 V with a Transphor Electrophoresis Cell (Hoefer Scientific Instruments, San Francisco, CA). The transfer buffer was 25 mM Tris, 192 mM glycine, 20% v/v methanol (pH 8.3). Nitrocellulose filters were quenched at room temperature for a minimum of 4 h in 20 mM Tris, 0.9% (w/v) NaCl (pH 7.4) (Tris-saline), and 3% (w/v) BSA. Blots were incubated with primary antibody (see below; typically dilutions of 1/500 or 1/1000 were used) in Tris-saline, 1% (w/v) BSA at 37°C for 1 to 3 h. Blots were washed three times, 15 min each, with Tris-saline, 0.1% (w/v) BSA, 0.1% (w/v) Triton X-100 (30 ml per 100 cm^2) before addition of alkaline phosphatase conjugated to protein A (Sigma; 10 units/100 ml) for 3 h at room temperature. Filters were washed three times, 15 min each, with 100 mM Tris (pH 7.5), 100 mM NaCl, 2 mM $MgCl_2$, and then washed twice, 10 min each, in alkaline phosphatase buffer (designated AP 9.5) containing 100 mM Tris (pH 9.5), 100 mM NaCl, 5 mM $MgCl_2$ at room temperature. The color detection of the immune complexes (21) was performed using 0.33 mg nitro blue tetrazolium/ml of AP 9.5 and 16.7 mg 5-bromo-4-chloro-3-indoxyl phosphate per 333 μl dimethylformamide per 100 ml of AP 9.5. After 15 min of color development in the dark, the reaction was stopped by washing filters in 10 mM Tris (pH 7.5), 1 mM EDTA for 15 min Blots were stored in 10 mM Tris (pH 9.5), 5 mM EDTA before air drying.

Antisera Production. Antisera to the PSII RC polypeptides and a 33 kD polypeptide were prepared as described by Chua and Blomberg (7). PSII enriched preparations were derived from detergent fractionation of corn (*Zea mays*) thylakoid membranes (2). These PSII preparations were subjected to preparative LDS-PAGE (10–17.5% slab gels). Coomassie blue staining bands at 51, 45, and 33 kD were excised and pooled; protein was electroeluted, concentrated and checked for purity by rerunning using analytical LDS-PAGE. Antibodies were raised in female New Zealand rabbits. One week prior to immunization, rabbits were bled to obtain preimmune sera. The initial injection contained 50 μg of the purified polypeptide in 0.5 ml 10 mM sodium phosphate (pH 7.0), and 1% (w/v) SDS mixed with an equal volume of complete Freund's adjuvant. The emulsion was injected into the subcapsular space of each rabbit (3 sites). The injection was repeated using incomplete adjuvant on d 20, 34, and 60. Rabbits were bled 4 d after each booster injection. Antisera to the 32 kD Q_B-binding protein was provided by J. Hirschberg and L. McIntosh, Michigan State University, E. Lansing, MI.

Electron Microscopy. Membrane samples were diluted with glycerol to a final concentration of 35% (v/v) glycerol before

freezing in liquid N_2 cooled Freon 12. Freeze-fracture replicas were prepared with a Balzers 360 M device at −115°C. Replicas were cleaned with commercial bleach, distilled H_2O and 1:1 chloroform/methanol (v/v). Magnification of micrographs is indicated in the figure legend.

RESULTS

Stage-Specific Changes in Chl Fluoresence in *lut-1*. The youngest leaves of tobacco plastome mutant *lut-1* closely resembled the dark green color of WT tobacco leaves of equivalent age, whereas successively older leaves along a mutant stem had less and less Chl. The oldest leaves were nearly colorless. We have investigated whether or not an underlying functional alteration of the mutant chloroplasts preceded this pigment loss (*i.e.* that pigment loss is a secondary effect of the mutation). To accomplish this, it became necessary to establish adequate criteria to be used in pooling sufficient quantities of leaf material which could be used for biochemical characterization studies.

Chl fluorescence induction transients were used as a nondestructive technique (6) to monitor the photosynthetic status of homoplastidic intact leaves from completely mutant shoots of *lut-1*. Fluorescence transients of young and mature WT leaves and four leaves of *lut-1* of increasing age (designated I–IV) are shown in Figure 1. These traces have been normalized to the same F_o (initial level of Chl fluorescence after dark adaptation; indicated by the horizontal arrow). The transients of young and old leaves of WT are considered to be insignificantly different. The transients showed a biphasic increase to F_p (the maximal or peak fluorescence level, achieved at the time when the primary acceptor, a plastoquinone called Q_A, of PSII is largely reduced). Under our experimental conditions, F_p was reached after 2 to 3 s of illumination of WT leaves, or after 4 to 5 s of mutant leaves. Variable fluorescence (F_v) is defined as $F_p - F_o$ (11).

Young leaves of *lut-1*, with transients showing F_v values of 50% or more of WT values, were designated as stage I. Mutant leaves with little or no variable fluorescence were grouped into stage IV. Stages designated as II and III were leaves having a reduced amplitude of variable fluorescence (F_v values of 25–50% and 10–25% of WT, respectively). Changes in F_v were related to leaf age in the mutant (monitored by determining the number of days between emergence of a morphologically distinct leaf from the apex to the time at which samples of that leaf were analyzed); results are presented in Table I. Loss of variable fluorescence was clearly a function of leaf age and was directly correlated with loss of Chl on a fresh weight basis. The mutant chloroplasts generally had Chl *a*/*b* ratios ranging from 2.2 to 2.8 (Table I). Membranes from younger tissue had a relatively high ratio, whereas those from older leaves had a lower ratio. WT membranes had Chl *a*/*b* ratios ranging from 3.1 to 3.3. It should be noted that both mutant and WT leaves expanded to maximal surface area by d 20; *i.e.* the mutation did not affect patterns of leaf cell division or expansion.

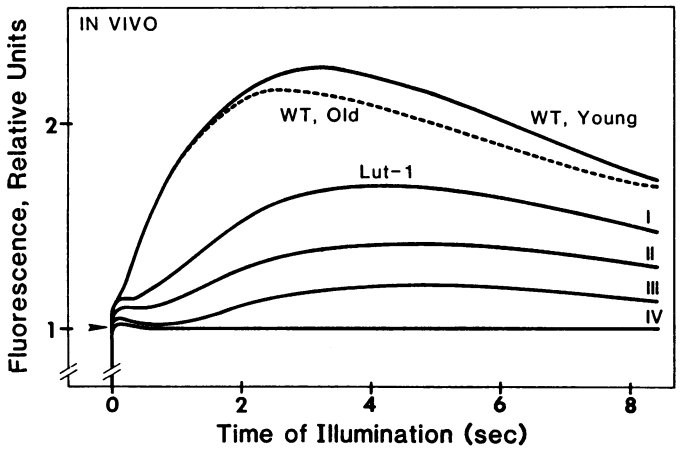

FIG. 1. Leaf Chl fluorescence induction transients used to establish the classification scheme for homoplastidic mutant *lut-1* leaves. Traces have been normalized to the same F_o (arrow) in order to show more clearly the loss of variable fluorescence ($F_v = F_p - F_o$) occurring during leaf maturation. F_p is defined as the peak or maximal intensity of fluorescence observed for each sample. Age did not significantly affect the F_v of WT leaves.

The four mutant 'stages' of fluorescence transients for whole leaves were easily monitored while harvesting mutant tissue. The technique was therefore used as the basis for pooling leaf tissue to ensure sufficient homogeneity of chloroplasts for isolation of comparable samples for biochemical analyses. It is important to emphasize that discrete leaf categories did not exist *in vivo*; rather these stages were arbitrary designations made to provide an initial quantitative classification of mutant leaves. The reduction in variable fluorescence did not appear as a series of single events in *lut-1* but as a progressive alteration in photosynthesis during leaf growth.

Because equivalent amounts of Chl were compared, *in vitro* Chl fluorescence measurements (Table I) allowed a more rigorous assessment of photochemical efficiency than *in vivo* measurements. These results reinforced the classification scheme which emerged from the leaf fluorescence data. Mutant chloroplasts isolated from successively older mutant leaves exhibited the same progressive reduction in F_v as seen at the intact tissue level. Mutant chloroplasts had high F_o values compared to WT, even in the youngest stage I leaves. This indicated that an increased fraction of the excitation energy received by the light-harvesting pigment-proteins (primarily serving PSII) was not used to drive electron transport, but was reemitted as fluorescence. The observed 3- to 4-fold difference in F_o levels between mutant and WT chloroplasts is not strictly proportional to the amount of unused excitation energy. That is, a small population of the light-harvesting pigment-proteins may be inactive and thus be responsible for increased F_o values. Similarly, the majority of the pigment-proteins may be effectively inactive (as in stage IV mutant chloroplasts; Fig. 2) yet F_o values of stage IV mutant chloroplasts are only 50% larger than stage I chloroplasts (Table I). Thus, it is not possible to make a quantitative correlation between F_o values and the fraction of antennae which are inactive. The high F_m values observed with mutant chloroplasts were related to the high F_o values; *i.e.* to an increased fluorescence yield from a functionally inactive pigment bed.

To determine if there was an excess of light-harvesting antennae in the mutant, half-rise times of Chl fluorescence inductions (rise time to one-half F_v, where $F_v = F_m - F_o$) of stage I *lut-1* and WT thylakoids, (measured in the presence of DCMU) were determined. The values obtained were 2.7 and 4.6 ms, for mutant and WT, respectively. Since the $t_{1/2}$ for inductions is a relative (inverse) measure of the size of the antennae bed serving each PSII trap (25), we can conclude that there were more light-harvesting pigments serving each PSII center in the mutant, with efficient energy transfer to the trap.

At low temperature (77 K), there are three Chl fluorescence emission maxima from chloroplast membranes: F685 and F695 which are jointly assigned to the light-harvesting and PSII core complexes, and a broad band centered near 730 nm which is assigned to PSI (4). Fluorescence emission spectra at 77 K of WT and mutant thylakoids are shown in Figure 2a. These spectra have offset baselines, but have been normalized such that the amplitude of the peak emission at 680 to 685 nm was equal in all samples. In parallel experiments using fluorescein as an internal standard (17), the absolute fluorescence yield was found to increase during mutant progression from stage II to IV (Fig.

Table I. *Chl Content and Room Temperature Chl Fluorescence (in Vivo and in Vitro) of WT and Plastome Mutant lut-1*

	Leaf Age[a]	Chl content	Chl *a/b*	Chl Fluorescence[b]			
				In vivo	*In vitro*		
				F_v/F_o	F_o	F_m	F_v/F_o
	d	*mg/g fresh wt*					
WT	5–7	2.2	3.3	1.8 ± 0.10	920 ± 30	3942 ± 45	3.28
	15–20	2.4	3.1	1.7 ± 0.09	1055 ± 4	4628 ± 16	3.29
lut-1							
Stage I[c]	5–7	1.8	2.8	0.67 ± 0.17	3709 ± 23	6581 ± 133	0.77
Stage II	8–14	1.2	2.6	0.41 ± 0.08	3871 ± 240	6260 ± 75	0.62
Stage III	15–20	0.9	2.3	0.25 ± 0.09	3809 ± 98	5789 ± 104	0.52
Stage IV	21–28	0.3	2.2	0.06 ± 0.04	5461 ± 134	6973 ± 162	0.28

[a] Growth during late spring and summer seasons. [b] Measurements are the average of 3 replicates and are given ± SD; $F_v = F_m - F_o$. [c] Stages of mutant leaf tissue, which were established using Chl fluorescence data, broadly paralleled leaf age and Chl content.

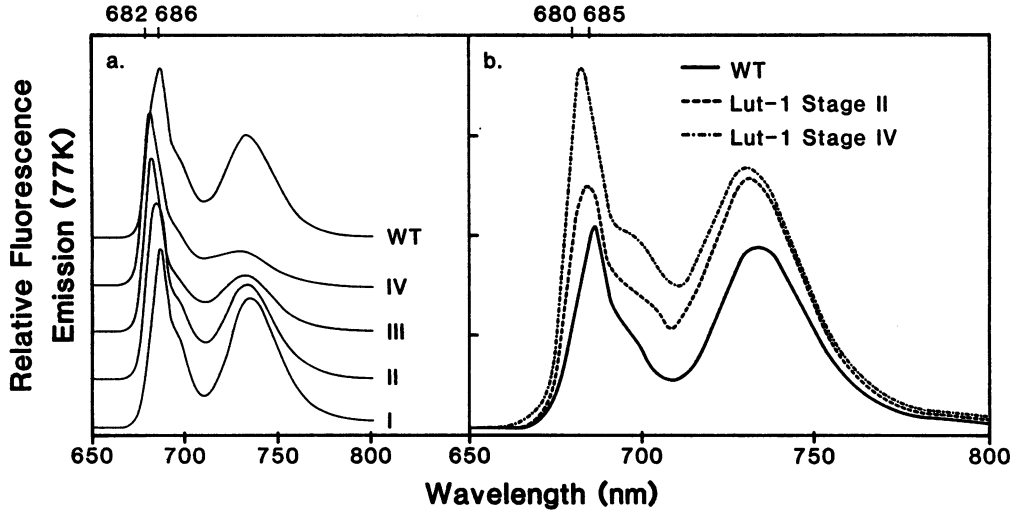

FIG. 2. Liquid N_2 (77 K) Chl fluorescence emission spectra of isolated WT and mutant thylakoids. a, Normalized at the low wavelength emission peak in order to show more clearly the gradual shift in the peak from 686 nm (WT and stage I) to 682 nm (stage IV) with increasing mutant stage. This blue-shift of the 686 nm Chl emission peak, which arises from the light-harvesting complexes serving PSII, is indicative of a functional disconnection between the antennae and RC (3, 22). The fluorescence emission yield at the long-wavelength peak appeared to diminish as a result of this normalization process. b, Normalized to the emission peak of an internal standard, fluorescein (17) in order to show the increase in fluorescence emission yield at both peak wavelengths. The dramatic increase in yield from the low-wavelength peak is typical of inhibited excitation energy transfer from the antenna Chl to the PSII centers. The increase in long wavelength (PSI) emission was probably due to increased PSI sensitization by free LHC-II.

2b). At the most extreme state of mutant expression, the ratio of emission of *lut-1*/WT in the 680 to 685 nm emission band was found to be 1.8 (in stage IV thylakoids). In stage I mutant membranes, the short wavelength Chl emission was centered at 686 nm—identical to WT samples. This peak shifted progressively to shorter wavelengths in stages II, III, and IV (684, 683, and 682 nm, respectively). This indicated an increased amount of 'free LHC-II' in the mutant membranes (the purified form of which shows a peak emission at 680 nm (3); this type of spectral shift has also been reported in a PSII-deficient mutant of maize (22) and was correlated to an increase in free LHC-II).

Electron Transport Properties of *lut-1* Membranes. Assays were performed to determine electron transport partial reactions (on a Chl basis) for chloroplasts prepared from stage I through IV leaves of *lut-1* in comparison to those from WT. From Table II, it can be seen that whole chain electron transport and PSII activity declined from 93 to 92% of WT activity in stage I to 6% in stage IV. In contrast, PSI activity was present at greater than WT levels through all four stages. The ratio of protein to Chl (in purified thylakoid preparations) is shown in Table II. The higher PSI rates in later stages of mutant chloroplasts were correlated to the increased protein to Chl ratio. Measurements of Chl/PSI RC (Chl/P700) made by monitoring chemical oxidation/reduction absorbance changes at 700 nm (data not presented) indicated that WT and mutant chloroplasts had similar ratios of functional P700 on a protein basis at all mutant stages. Since PSI activity on a Chl basis (Table II) increased more than the change in protein/Chl, however, we conclude that the electron donor and/or acceptor used in assaying PSI activity had greater access to electron transport components associated with PSI in late stage mutant thylakoids. As the loss of whole chain activity closely paralleled the loss of PSII activity, the decline of the latter was likely responsible for the overall decrease.

Efficiency of PSII-Dependent Reactions. Since PSII centers

Table II. *Protein Content and Photosynthetic Activity of Tobacco Plastome Mutant lut-1 Thylakoids Compared to WT Tobacco Thylakoids*

	Protein Content	Activity[a]		
		Whole chain	PSII-dependent	PSI-dependent
	$\mu g/\mu g$ Chl[b]		%WT	
lut-1				
Stage I	5.1	93	92	130
Stage II	6.2	72	65	230
Stage III	7.4	21	23	280
Stage IV	9.3	6	6	250

[a] Activity measurements are expressed as percent WT activity. WT activities: 269 and 415 μmol O_2 mg^{-1} Chl h^{-1}, whole chain and PSI-dependent, respectively; 288 μmol DPIP reduced mg^{-1} Chl h^{-1}, PSII-dependent. [b] WT value: 5.5 μg protein/μg Chl.

appeared to be lost during chloroplast development in *lut-1*, we were interested in learning if the centers at early stages of leaf development had some functional impairment not detectable by fluorescence or partial reactions measured at saturating light. Under flash illumination, chloroplasts are known to evolve O_2 in a pattern expressing a periodicity of four (16); this process requires the concerted reactions of several PSII constituents. Except for the diminished O_2 yield/flash of mutant chloroplasts (data not shown), the flash yield patterns of mutant (stage I) and WT chloroplasts were similar (Fig. 3). Maximum O_2 yield occurred upon the third flash after dark adaptation, and peaked thereafter every four flashes.

In experiments measuring O_2 flash yield, the periodicity of O_2-evolution damps out after many flashes due to a combination of factors. First, centers can occasionally undergo two turnovers (double hits) during the duration of the flash; this results in an extra advance of the flash yield sequence in a subfraction of the total number of centers. Second, some centers may not undergo a charge separation during the flash (misses). Using the classical model of Kok (16) defining the 'S-states' of O_2-evolution, the values for misses and double hit probabilities were calculated. Miss probabilities, 0.12 and 0.14, respectively, were similar for *lut-1* and WT. The double hit parameter for *lut-1* (0.05) was less than one-half of WT (0.11); this finding accounted for the higher probability for a single step transition for *lut-1* centers than WT centers (0.83 and 0.75, respectively). In summary, the measurement of O_2 flash yields in *lut-1* chloroplasts indicated that the active PSII centers were nearly indistinguishable from WT, and that there were no major changes in the rate constants for the processes involved in electron transport from water to the plastoquinone pool.

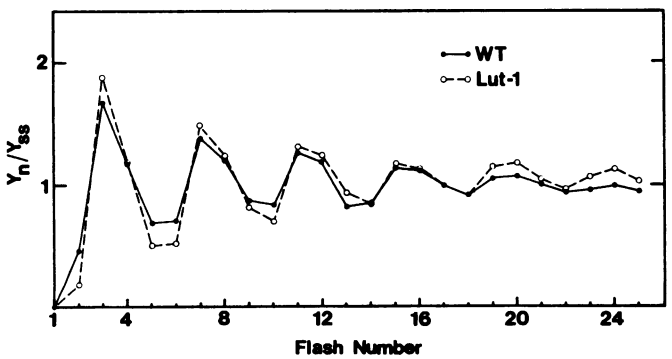

FIG. 3. O_2 yield as a function of saturating 1 Hz light flashes for dark-adapted WT and stage I *lut-1* thylakoids. O_2 yield after each flash (Y_n) was normalized to a steady state flash yield (Y_{ss}), acquired after the oscillations damped out (generally after 40 flashes). The WT and mutant patterns both displayed the typical oscillatory pattern of O_2 evolution, with peaks at the third flash and every fourth flash thereafter.

The ability of isolated WT and mutant chloroplast membranes to phosphorylate several thylakoid polypeptides, due to light activation of a thylakoid-bound kinase (reviewed in Ref. 29), was a second PSII-associated property assayed to assess the PSII characteristics of the mutant. Using [γ-^{32}P]ATP, WT and *lut-1* (stage I) thylakoids were phosphorylated in the light, and the membrane proteins separated by SDS-PAGE. The autoradiogram of the gel is shown in Figure 4. Lanes 1 and 5 show that in the absence of DCMU, the same proteins of WT and *lut-1* chloroplasts, respectively, were radiolabeled.

The phosphoproteins observed in WT and mutant tobacco thylakoids corresponded to those phosphoproteins observed in other higher plant chloroplasts (30). These included species at 45, 32, 28, 27, and 10 kD, all of which have been identified either as PSII constituents or apoproteins of the LHC-II. The protein kinase is believed to be activated by a reduced PQ pool (29). If the reduction of the PQ pool was prevented, either by inhibition with DCMU or darkness, the kinase was inactive and no phosphorylation was observed in either WT or mutant membranes (Fig. 4; plus DCMU: lanes 2, 6 [WT and *lut-1*, respectively]; unilluminated: lane 4 [WT]). A phosphorylated WT sample allowed to dephosphorylate in the dark was run to show the reversibility of the reaction (lane 3). The interpretation of these data is that the mutant membranes have a normal pattern of protein phosphorylation control; *i.e.* the mutant, in early stages of development, has an apparently normal functioning PSII complex.

Structural Analysis of Thylakoid Membranes. When membrane preparations were examined by freeze-fracture electron microscopy, marked differences were evident in membrane substructure. Figure 5, a and b, show, respectively, WT and stage II *lut-1* membranes. Although grana stacks were evident, the density of EF_s particles, considered to be PSII core complexes plus bound light-harvesting Chl-protein complexes (29), decreased in mutant lamellae during chloroplast maturation. These results were consistent with our interpretation of biochemical and biophysical data suggesting a reduction in PSII complexes.

Polypeptide Content of Thylakoids. PSII-associated Chl-protein complexes were gently extracted by solubilization of isolated mutant and WT thylakoids with octyl-glucopyranoside (5). These complexes were then subjected to LDS-PAGE. The two PSII RC Chl-protein complexes, CP47 and CP43 (whose apoproteins migrated at 51 and 45 kD, respectively, in our gel system), were clearly present in stage I *lut-1* chloroplasts (Fig. 6a). Their migration was identical to the PSII complexes of WT membranes (data not shown). This was additional structural evidence indicating the physical integrity of PSII centers in young mutant chloroplasts. CP47 and CP43 were not observed when late stage mutant membranes were similarly solubilized with octyl-glucopyranoside (data not shown).

The polypeptide profiles of WT and mutant membranes were

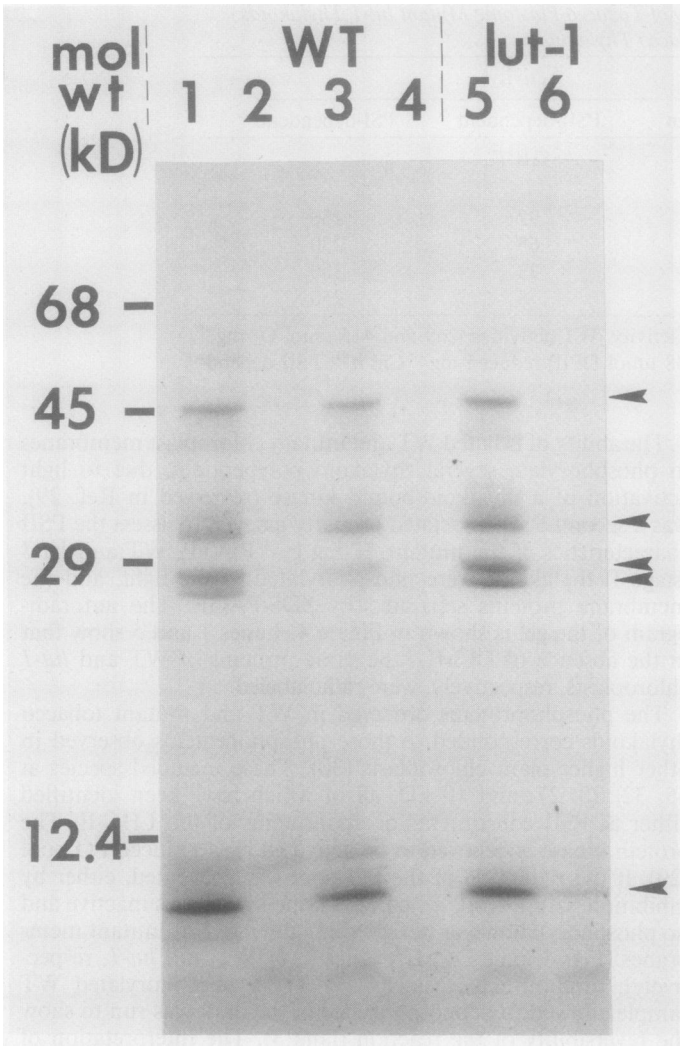

FIG. 4. Autoradiogram of a denaturing SDS polyacrylamide gel, containing 4 M urea, showing the radiolabeled phosphoproteins of WT and *lut-1* (stage I) thylakoids. Lanes were loaded on an equal thylakoid protein basis. Lanes 1 and 5, WT and *lut-1*, respectively, illuminated samples minus DCMU; lanes 2 and 6, WT and *lut-1*, respectively, illuminated in the presence of DCMU (phosphorylation was inhibited because DCMU blocks electron transport between the Q_B-binding protein and the plastoquinone pool, keeping the plastoquinone pool largely oxidized). Lane 3 was a sample which was phosphorylated in the light and then allowed to dephosphorylate for 20 min in the dark (NaF was not included in this sample). Lane 4 was a nonilluminated WT sample run to demonstrate the requirement for light-activation of the protein kinase. Arrows indicate the major phosphoprotein species at 45, 32, 28, 27, and 10 kD, which were present in both WT and mutant membranes.

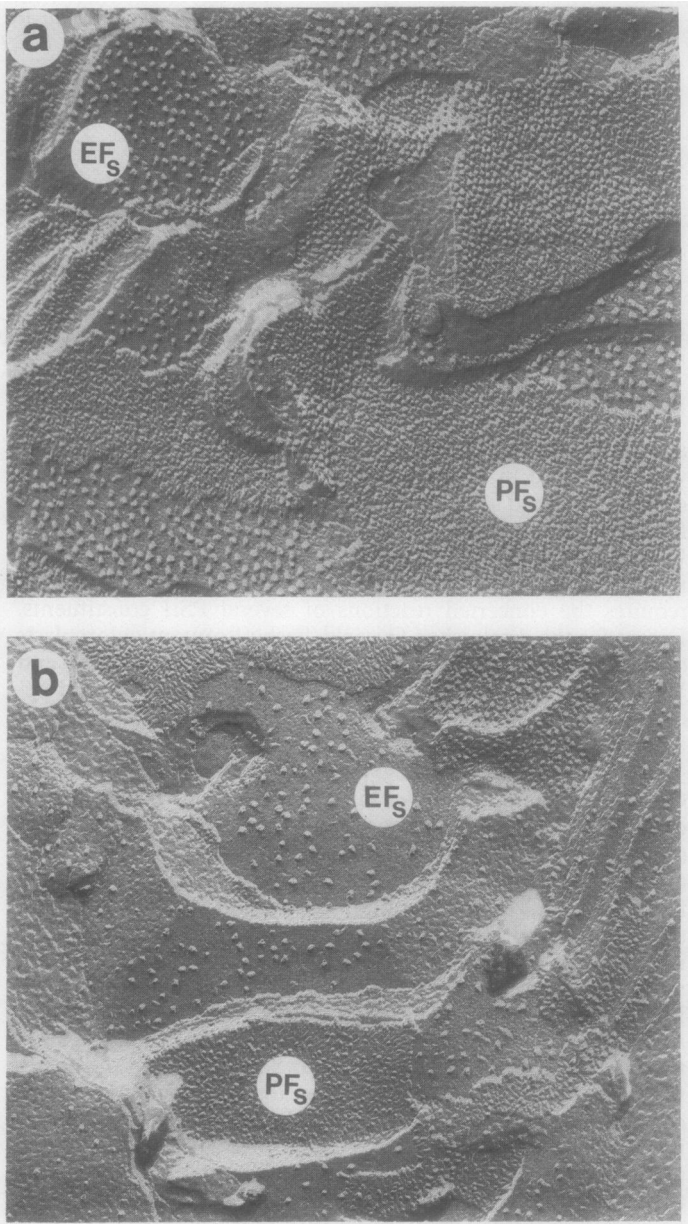

FIG. 5. Freeze-fracture micrographs of (a) WT and (b) stage II *lut-1* thylakoids. The density of the particles on the EF_s fracture face was reduced in mutant membranes in comparison to WT (×100,000).

determined by SDS-PAGE in the presence of 4 M urea (Fig. 6b). Compared to WT (lane 1), mutant thylakoids (lanes 2 through 5) showed a progressive depletion of polypeptides at 45, 32 to 34, and approximately 10 kD. Judging by their Coomassie blue staining intensity, the 45 kD and 32 kD (Q_B-binding polypeptide) appeared to be significantly depleted in stage I thylakoids whereas the 33 and 10 kD species were present in chloroplasts from stage II leaves. A polypeptide of 51 kD decreased only slightly in staining intensity in the late stage mutant membranes. A protein migrating slightly faster than the 32 kD (Q_B-binding polypeptide) was evident in the WT polypeptide profile (lane 1) and in the PSII-enriched preparation (lane 6), but not in any of the mutant thylakoid samples. We do not know the identity of this polypeptide.

Two lines of investigation were used to show that the polypeptides which were lost during mutant thylakoid maturation were associated with PSII. First, an isolated grana fraction from WT thylakoids which almost exclusively contained PSII (prepared by the method of Berthold *et al.* [2]; lane 6 of Fig. 6b) contained proteins co-migrating with the polypeptides which were depleted over time in *lut-1*. Second, protein blots probed with antibodies raised against authentic PSII polypeptides demonstrated the identity of the depleted proteins (Fig. 7).

Antisera raised against the 51 kD protein (thought to be the P680-binding RC protein (9, 27) reacted with two closely migrating species on protein blots of mutant membranes isolated from young, intermediate, and mature *lut-1* leaves (corresponding to stages I, II, and IV, respectively; lanes 2, 3, and 4 in Fig. 7a). The intensity of the antibody reaction decreased slightly with increasing stage (lanes were loaded on an equal protein basis) which indicated a slight reduction in the levels of these

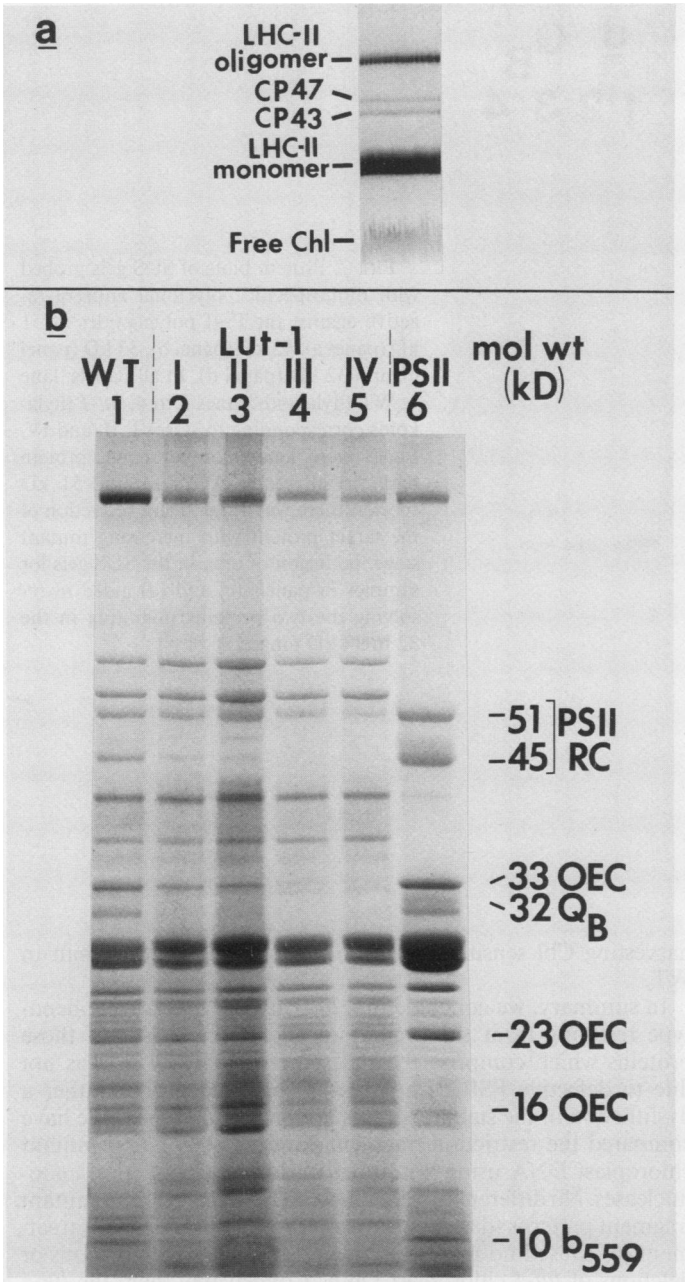

FIG. 6. PAGE of: a, PSII Chl-protein complexes extracted with octylglucopyranoside (5) from stage I *lut-1* thylakoids and run on an LDS gel under conditions in which Chl remained stably bound to Chl-binding polypeptides. Both RC Chl-protein complexes, CP47, and CP43, were present in young mutant chloroplast membranes, indicating proper cofactor and complex associations. b, Separation of thylakoids on an SDS gel containing 4 M urea. WT, the four mutant stages, and a PSII-enriched grana fraction from WT are shown in lanes 1, 2 to 5, and 6, respectively. The PSII-enriched fraction was isolated as described in Ref. 2. As judged by Coomassie blue staining intensity, PSII-associated proteins at 45, 33, 32, and 10 kD were lost from mutant membranes with increasing age. Additional immunoblotting analyses (data not shown) indicated the loss of polypeptide species at 23 and 16 kD which are part of the O_2 evolving complex (1).

species. The doublet signal was not observed on blots in which samples were heated and/or boiled before loading on gels. These harsher denaturing conditions generally yielded a smeared and diffuse staining region. We consider the two reacting species to be forms of the same protein. Possibly because of residual Chl (not removed by the gentle solubilization procedure required for sharper banding patterns), the 51 kD polypeptide migrated as two distinct populations which could be detected by the more sensitive immunoblotting methods. There was a broad, diffuse immunologically tagged polypeptide of approximately 42 kD in the mutant samples (Fig. 7a, lanes 2, 3, and 4). We interpret these to be degradation products of the 51 kD protein, but cannot determine whether they formed during sample preparation or *in vivo*.

In contrast to the 51 kD polypeptide, results using the antisera raised against the 45 kD protein indicated a more significant depletion of this Chl *a*-binding RC protein with increasing mutant stage (Fig. 7b, lanes 2, 3, and 4). Patterns for the 33 kD polypeptide, a nuclear-encoded component of the water oxidizing complex (1), and the 32 kD Q_B-binding protein paralleled that of the 45 kD protein, confirming the loss of other PSII constituents in mutant chloroplasts during *lut-1* leaf expansion (Fig. 7, c and d). In the late stages of *lut-1* membrane ontogeny, the anti-33 sera reacted with a larger size polypeptide (approximately 34 kD) Fig. 7c, lane 4). We believe this is a partially processed form of the precursor to the mature 33 kD protein; this concept will be discussed in greater detail in a subsequent manuscript.

DISCUSSION

This study has shown that *lut-1* is a developmentally expressed plastome mutant. The biochemical phenotype is a loss of PSII function due to a selective reduction in the concentration of PSII core complexes in thylakoids. This phenomenon only occurred as leaves (plastids) matured. We did not observe this alteration in PSII stoichiometry in chloroplasts isolated from WT leaves during maturation and do not consider this mutant behavior to be mimicking a senescence process.

PSII Function. In young leaves of *lut-1*, PSII activity was nearly normal, based upon *in vivo* fluorescence transients (Fig. 1) and the fluorescence and partial reactions measured with isolated thylakoids (Tables I, II). Chl was properly associated with RC proteins as indicated by the presence of CP43 and CP47 in early stage *lut-1* membranes (Fig. 6a). When O_2 flash yield measurements were used to study early-stage chloroplasts, the oscillatory pattern of O_2 evolution was identical to that of WT, and only small changes in the kinetic parameters of this process were detected (Fig. 3). The most significant difference was the reduced steady state O_2 flash yield in the mutant thylakoids which simply indicates loss of PSII centers.

Since the evolution of O_2 results from the integration of many reaction steps, the O_2 flash yield experiments strongly indicate that the *lut-1* chloroplasts have the capacity to produce a normal PSII center (*i.e.* the genes for PSII proteins are transcribed and translated in early stage tissue and functionally normal proteins are synthesized and assembled in mutant thylakoids).

The early stage membranes also carried out light-dependent phosphorylation of PSII-associated polypeptides (Fig. 4). This process has previously been shown to be a regulatory step controlling light harvesting (29). An active protein phosphorylation in *lut-1* suggests that overall membrane processes coupling electron flow to energy distribution in the chloroplast function properly in early stage *lut-1* chloroplasts.

We are left with the conclusion that PSII centers in *lut-1* initially assemble and function properly, but then are selectively lost during chloroplast maturation. Since the loss of functional activity was specific for PSII (PSI and light harvesting Chl *a/b* pigment-proteins were retained; Tables I, II; and Fig. 6b), we sought evidence to explain why PSII function degenerated over time during leaf maturation in *lut-1*.

Evidence for Physical Loss of PSII Centers. The loss of PSII

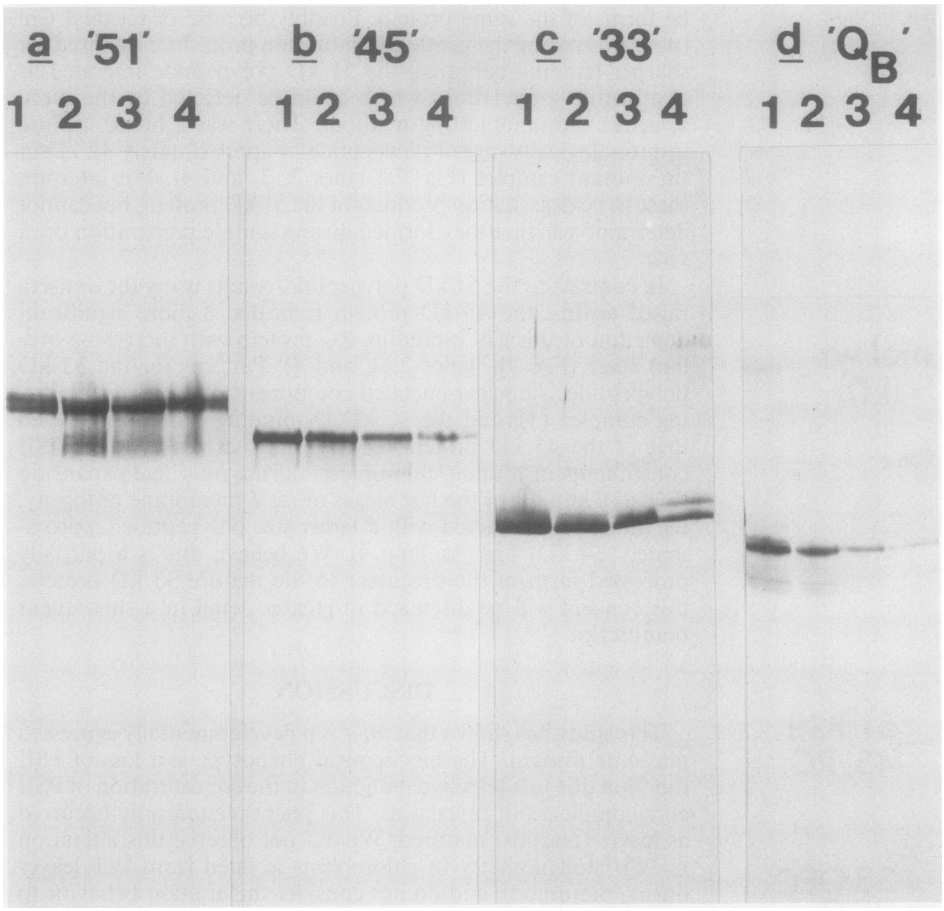

FIG. 7. Protein blots of SDS gels probed with monospecific polyclonal antisera reactive against the PSII polypeptides at 51 kD (panel a), 45 kD (panel b), 33 kD (panel c), and 32 kD (panel d). In all panels: lane 1, WT thylakoids; lanes 2 to 4, *lut-1* thylakoids corresponding to stages I, II, and IV. Lanes were loaded on an equal protein basis. In all cases, except for the 51 kD species, there was a significant reduction of the target protein with increasing mutant stage. Inclusion of urea in the SDS gels for samples in panels (c) and (d) aided in resolving the two proteins migrating in the 32 to 34 kD range.

activity in *lut-1* membranes corresponded directly with the physical depletion of the PSII core complexes from the thylakoids. This conclusion is based upon three lines of evidence. (a) A comparison of *lut-1* and WT thylakoid ultrastructure by freeze-fraction analysis showed a reduced number of particles on the EF_s fracture face (Fig. 5); these particles have been shown to be PSII core complexes and associated light-harvesting pigment proteins (reviewed in Ref. 29); (b) the polypeptide profile of *lut-1* thylakoids isolated from increasingly older leaf tissue showed a progressive depletion of Coomassie blue staining polypeptides typically ascribed to the PSII RC core complex (Fig. 6b) (except for the putative RC apoprotein at 51 kD); (c) immunoblots of polypeptides separated by PAGE confirmed the depletion of proteins at 45 and 33 kD as well as the diffusely staining 32 kD Q_B-binding protein considered the apoprotein of the secondary electron acceptor in the PSII RC (18).

The physical absence of PSII structural units is consistent with the alterations in the Chl fluorescence properties and diminished photosynthetic electron transport activities, over time, of mutant chloroplasts. The changes in the 77 K fluorescence spectra of chloroplasts from the different mutant leaf stages included a blue-shift of the short-wavelength emission peak from 685 nm (stage I) to 682 nm (stage IV) (Fig. 2, a and b). This is characteristic of membranes in which the light-harvesting complexes are functionally disconnected from PSII RC complexes (22). The resulting pool of 'free' light-harvesting complex has an increased Chl fluorescence emission yield (Fig. 2b) at 77 K and at room temperature (see high F_o and F_m values for *in vitro* Chl fluorescence in Table II).

A reduced number of PSII centers per unit membrane was also indicated by the faster rise times to one-half F_v in the younger *lut-1* membranes, compared to WT; *i.e.* there was more light-harvesting Chl sensitizing the PSII RC in the mutant than in WT.

In summary, we conclude that *lut-1* displayed a novel phenotype resulting from the premature and selective loss of those proteins which comprise the PSII core complex. This was not due to defective PSII complexes or constituents, but rather a dysfunction in the stability of this lipoprotein complex. We have compared the restriction fragment patterns of WT and mutant chloroplast DNA using a number of different restriction endonucleases. No differences were observed between WT and mutant fragment patterns (data not shown). Thus, the mutagenesis treatment (6) caused no major rearrangements or gross insertions or deletions of *lut-1* chloroplast DNA. Conceivably then, the *lut-1* mutation is a point mutation (or several point mutations). If such a mutation alters the primary sequence of a PSII polypeptide, the altered protein may disrupt the stability of the PSII complex. However, because chloroplast-encoded PSII polypeptides are present and functional in chloroplasts of young mutant leaves, we believe that these proteins are structurally normal.

The depletion of PSII-associated proteins from *lut-1* thylakoids may be due to one of two processes. One possible explanation would be a reduction in the rate of synthesis, occurring either at the level of transcription or translation, of one or more plastome-encoded proteins which are part of PSII. Accordingly, the absence of PSII components(s) would alter the normal assembly of the membrane protein complex. This explanation would require that the mutation be in a regulatory process controlling protein synthesis, and that the regulation be specific for PSII proteins. A second equally plausible explanation which would account for the reduction in PSII complexes would be the presence of a faulty enzyme which is involved in protein processing needed for assembly and stabilization of the PSII complex or for its

normal turnover. If some required enzyme-mediated protein-protein associations were not correctly formed, we would expect these PSII units to be unstable, resulting in their deterioration over time. If degradation of membrane proteins was involved, it was a relatively efficient and rapid process since breakdown products of most PSII polypeptides were not observed on immunoblots.

Studies are underway to distinguish between these two different mechanisms by which premature PSII degradation could occur. Preliminary data indicate that the mutation does not affect the synthesis of PSII proteins, and that processing of nuclear-encoded polypeptides associated with the water oxidation process is arrested in *lut-1* plastids. These data will be presented in a subsequent manuscript.

Acknowledgments—We thank S. Sprague for freeze-fracture microscopy, T. Wydryzinski for advice on Joliot oxygen electrode measurements, and L. McIntosh and J. Hirschberg for providing antisera to the Q_B-binding protein.

LITERATURE CITED

1. ANDERSSON B, C JANSSON, U LJUNGBERG, H-E AKERLUND 1985 Polypeptides in water oxidation. *In* KE Steinback, S Bonitz, CJ Arntzen, L Bogorad, eds, Molecular Biology of the Photosynthetic Apparatus. Cold Spring Harbor Laboratory, pp 21–31
2. BERTHOLD DA, GT BABCOCK, CF YOCUM 1981 A highly resolved, oxygen-evolving photosystem II preparation from spinach thylakoid membranes. EPR and electron-transport properties. FEBS Lett 134: 231–234
3. BURKE JJ, KE STEINBACK, I OHAD, CJ ARNTZEN 1978 Control of photosynthetic competence in the Y-1 mutant of *Chlamydomonas reinhardi*. *In* G Akoyunoglou, JH Argyoudi-Akoyunoglou, eds, Chloroplast Development. Elsevier, Amsterdam, pp 413–418
4. BUTLER, WL 1978 Energy distribution in the photochemical apparatus of photosynthesis. Annu Rev Plant Physiol 29: 345–378
5. CAMM EL, BR GREEN 1980 Fractionation of thylakoid membranes with the nonionic detergent octyl-β-D-glucopyranoside. Plant Physiol 66: 428–432
6. CHIA CP, JH DUESING, JL WATSON, R GUY, CJ ARNTZEN 1986 Characterization of cytoplasmic mutants of *Nicotiana tabacum* with altered photosynthetic function. Curr Genet 10:469–479
7. CHUA N-H, F BLOMBERG 1979 Immunochemical studies of thylakoid membrane polypeptides from spinach and *Chlamydomonas reinhardtii*. J Biol Chem 254: 215–223
8. DARR SC, VS MACHADO, CJ ARNTZEN 1981 Uniparental inheritance of a chloroplast photosystem II polypeptide controlling herbicide binding. Biochim Biophys Acta 634: 219–228
9. DE VITRY C, F-A WOLLMAN, P DELEPELAIRE 1984 Function of the polypeptides of the photosystem II reaction center in *Chlamydomonas reinhardtii*. Localization of the primary reactants. Biochim Biophys Acta 767: 415–422
10. GOLOUBINOFF P, M EDELMAN, RB HALLICK 1984 Chloroplast-coded atrazine resistance in *Solanum nigrum*: *psb*A loci from susceptible and resistant biotypes are isogenic except for a single codon change. Nucleic Acids Res 12: 9489–9496
11. GOVINDJEE, G PAPAGEORGIOU 1971 Chlorophyll fluorescence and photosynthesis: fluorescence transients. *In* AC Giese, ed, Photophysiology, Vol 6. Academic Press, New York, pp 1–46
12. HALLIER MW, UW HEBER 1977 Cytochrome *f* deficient plastome mutants of *Oenothera*. Plant Cell Physiol 3: 257–273
13. HENNINGSEN KW, BM STUMMANN 1982 Use of mutants in the study of chloroplast biogenesis. *In* B Parthier, D Boulter, eds, Encyclopedia of Plant Physiology, New Series, Vol 14B. Springer-Verlag, New York, pp 597–644
14. HIRSCHBERG J, L MCINTOSH 1983 Molecular basis of herbicide resistance in *Amaranthus hybridus*. Science 222: 1346–1349
15. KIRK JTO 1978 Genetic control and plastid biochemistry. *In* JTO Kirk, RAE Tilney-Bassett, eds, The Plastids. Elsevier/North-Holland Biomedical Press, Amsterdam, pp 560–614
16. KOK B, B FORBUSH, M MCGLOIN 1970 Cooperation of charges in photosynthetic O_2 evolution. A linear four step mechanism. Photochem Photobiol 11: 457–475
17. KRAUSE GH, J-M BRIANTAIS, C VERNOTTE 1983 Characterization of chlorophyll fluorescence quenching in chloroplasts by fluorescence spectroscopy at 77 K. I. pH-dependent quenching. Biochim Biophys Acta 723: 169–175
18. KYLE DJ 1984 The 32000 dalton Q_B protein of photosystem II. Photochem Photobiol 41: 107–116
19. KYLE DJ, P HAWORTH, CJ ARNTZEN 1982 Thylakoid membrane protein phosphorylation leads to a decrease in connectivity between photosystem II reaction centers. Biochim Biophys Acta 680: 336–342
20. LAEMMLI UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227: 680–685
21. LEARY JJ, DJ BRIGATI, DC WARD 1983 Rapid and sensitive colorimetric method for visualizing biotin labeled DNA probes hybridized to DNA or RNA immobilized on nitrocellulose: bio-blots. Proc Natl Acad Sci USA 80:4045–4049
22. LETO K, CJ ARNTZEN 1981 Cation-mediated regulation of excitation energy distribution in chloroplasts lacking organized photosystem II complexes. Biochim Biophys Acta 637: 107–117
23. MACKINNEY G 1941 Absorption of light by chlorophyll solutions. J Biol Chem 140: 315–322
24. MARKWELL MAK, SM HAAS, LL BIEBER, NE TOLBERT 1978 A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal Biochem 87: 206–210
25. MELIS A, APGM THIELEN 1980 The relative cross-section of photosystem I and photosystem II in chloroplasts from three types of *Nicotiana tabacum*. Biochim Biophys Acta 589: 275–286
26. MILLER KR, RA CUSHMAN 1979 A chloroplast membrane lacking photosystem II. Thylakoid stacking in the absence of the photosystem II particle. Biochim Biophys Acta 546: 481–497
27. NAKATANI HY, B KE, E DOLAN, CJ ARNTZEN 1984 Identity of the photosystem II reaction center complex polypeptide. Biochim Biophys Acta 765: 347–352
28. PATERSON DR, CJ ARNTZEN 1982 Detection of altered inhibition of photosystem II reactions in herbicide-resistant plants. *In* M Edelman, RB Hallick, N-H Chua, eds, Methods in Chloroplast Molecular Biology. Elsevier Biomedical Press, Amsterdam, pp 109–118
29. STAEHELIN LA, CJ ARNTZEN 1983 Regulation of chloroplast membrane function: protein phosphorylation changes the spatial organization of membrane components. J Cell Biol 97: 1327–1337
30. STEINBACK KE, S BOSE, DJ KYLE 1982 Phosphorylation of the light-harvesting Chlorophyll-protein regulates excitation energy distribution between photosystem II and photosystem I. Arch Biochem Biophys 216; 356–361
31. VERMAAS WFJ, G RENGER, G DOHNT 1984 The reduction of the oxygen-evolving system in chloroplast by thylakoid components. Biochim Biophys Acta 76: 194–202