Plant Physiol. (1997) 113: 1023–1031

hcf5, a Nuclear Photosynthetic Electron Transport Mutant of *Arabidopsis thaliana* with a Pleiotropic Effect on Chloroplast Gene Expression[1]

Randy D. Dinkins[2,3], Hema Bandaranayake[2,4], Laurence Baeza, Anthony J. F. Griffiths, and Beverley R. Green*

Department of Botany, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z4

A photosynthetic mutant of *Arabidopsis thaliana*, *hcf5*, was isolated by screening M_2 seedlings for high chlorophyll fluorescence. Thylakoid morphology was strikingly abnormal, with large grana stacks and almost no stroma lamellae. Fluorescence induction kinetics, activity assays, and immunoblotting showed that photosystem II was absent. Polypeptides of the photosystem I complex, the Cyt b_6/f complex, coupling factor, and the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase were also severely depleted. However, the nuclear-encoded chlorophyll *a/b* light-harvesting complex polypeptides were unaffected. The *rbcL* transcript was present at very low levels, the pattern of transcripts from the polycistronic *psbB-psbH-petB-petD* operon was abnormal, and the mature *psbH* message was almost completely lacking. This suggests that the *hcf5* locus may encode a product required for the correct expression of several chloroplast genes.

Thylakoid membrane biogenesis requires contributions from both nuclear and chloroplast genomes. Most of the genes for thylakoid proteins have been cloned and sequenced, but we still have little information about the many nuclear genes that are required for chloroplast development (Rochaix, 1992). It has been estimated that at least 200 nuclear genes are essential for thylakoid membrane biogenesis in *Chlamydomonas reinhardtii* (Rochaix and Erickson, 1988). At least 14 nuclear complementation classes are required for the correct splicing of just one chloroplast transcript, that of *psaA* (Choquet et al., 1988).

Most of the well-characterized nuclear mutations affecting chloroplast electron transport have the *hcf* mutant phenotype (Bennoun and Levine, 1967; Miles, 1980). Mutant cells emit high levels of red fluorescence when illuminated with blue light because the absorbed light energy cannot be employed to drive electron transport and to establish an electrochemical potential. Many photosynthetic mutants displaying the *hcf* phenotype have been isolated in the green alga *Chlamydomonas* sp. (Rochaix, 1992) and in several higher plants, particularly maize (Miles, 1994; Barkan et al., 1995) and barley (Simpson and von Wettstein, 1980). In both higher plants and *Chlamydomonas* sp. these mutants have played an important role in establishing the polypeptide composition of the major macromolecular complexes of the thylakoid membrane (Chua and Bennoun, 1975; Chua et al., 1975; Metz and Miles, 1982; Lemaire and Wollman, 1989). Some *Chlamydomonas* sp. *hcf* mutants are defective in the accumulation of a single chloroplast transcript (Rochaix, 1992), whereas several higher plant mutants are defective in one or more polycistronic transcripts (Barkan et al., 1986, 1994, 1995; Rochaix, 1992). Mutants defective in the translation of one (Rochaix, 1992) or many (Barkan, 1993) chloroplast messages have also been reported, suggesting the involvement of nuclear-encoded proteins in translation of certain mRNAs as well as in their processing and stabilization.

The crucifer *Arabidopsis thaliana* has been used extensively as a model system for higher plant genetics and molecular biology because of its small genome size, short generation time, and availability of mutants (Goodman et al., 1995). We have previously reported the isolation and characterization of the Arabidopsis mutant line *hcf2*, which has pleiotropic defects at the level of photosynthetic polypeptides and an over-accumulation of the *petA* transcript (Dinkins et al., 1994). A number of Arabidopsis *hcf* mutants with a variety of phenotypes have been isolated by screening progeny of ethyl methanesulfonate-mutagenized seed (Dinkins, 1992; Meurer et al., 1996b), but in most cases the mutations have no effect on chloroplast transcripts. In this paper we describe one of the exceptions: the mutant *hcf5*, which is deficient in all photosynthetic electron transport complexes as well as in Rubisco. These biochemical defects are correlated with altered steady-state levels of several chloroplast transcripts.

MATERIALS AND METHODS

Plant Growth Conditions

Plants were grown in a controlled environment chamber under fluorescent lights (100–150 μE m^{-2} s^{-1}, 23°C, 16 h of

[1] Supported by Natural Sciences and Engineering Research Council of Canada.

[2] R.D.D. and H.B. contributed equally to this work.

[3] Present address: Plant Cell Biology, Agronomy Department, N109 Science Center North, Lexington, KY 40546–0091.

[4] Present address: Biology Department, Virginia Polytechnic University, Blacksburg, VA 24061.

* Corresponding author; e-mail beverley.green@mtsg.ubc.ca; fax 1–604–822–6089.

Abbreviations: CF, coupling factor; CPII*, CPII, trimer and oligomer forms of LHCII, the major chlorophyll *a/b* protein complex of PSII; CP29, minor chlorophyll *a/b* protein complex of PSII; *hcf*, high chlorophyll fluorescence.

light/8 h of dark) in a vermiculite:peat (3:1, v/v) mixture, watered from the bottom, and given 20–20–20 all-purpose fertilizer (Plant Products, Co., Ontario, Canada) at a rate of 0.8 g L^{-1}, as needed. To obtain mutant tissue for study, seed from heterozygous plants segregating for the *hcf* phenotype was surface-sterilized and sown on plates containing agar (0.8 g L^{-1}), Suc (50 g L^{-1}), and one-half Murashige and Skoog salts (Murashige and Skoog, 1962), supplemented with 100 mg L^{-1} inositol, 1 mg L^{-1} pyridoxine-HCl, 1 mg L^{-1} nicotinic acid, and 10 mg L^{-1} thiamine. Plates were placed in a growth chamber under full-spectrum fluorescent lights (Vita-light, General Electric; 25–40 μE m^{-2} s^{-1}, 9 h of light/15 h of dark) at 23°C. Mutant seedlings were distinguished from their wild-type siblings by their abnormally high red fluorescence when illuminated by near-UV light, as described by Miles (1980). Young plants were then transferred to large-diameter (250-mm) plates to allow for leaf expansion. After approximately 4 weeks (at the onset of bolting), the aerial portions of the plants were harvested. All measurements on *hcf* plants were done in parallel on wild-type siblings growing on the same plates.

Mutant Isolation and Genetic Analysis

Seed of *Arabidopsis thaliana* (L.) Heynh. wild-type Columbia was mutagenized with ethyl methanesulfonate, and a large number of small M_1 bulk populations (5–10 plants) grown from this seed were screened for the *hcf* phenotype by plating a sample of surface-sterilized M_2 seed on Suc-supplemented plates (Dinkins et al., 1994). About 100 M_2 plants from each bulk that showed significant numbers of the *hcf* phenotype were grown, seed was collected individually from each plant, and a small sample was screened on agar. Additional details on Arabidopsis *hcf* mutant screening procedures will be published elsewhere (R.D. Dinkins, unpublished data). Mutant line *hcf5* was derived from M_1 bulk no. 302 and found to be segregating in the M_3 generation at a ratio suggesting a single nuclear recessive. The line was subsequently self-pollinated to eliminate additional background phenotypes prior to the experiments presented here, which were performed on plants from the M_4 to M_6 generations. Because the *hcf* phenotype is seedling-lethal in soil, the line is maintained by screening for heterozygous plants each generation.

Fluorescence Measurements

In vivo fluorescence induction curves were obtained at room temperature on 2- to 3-week-old seedlings plated on 5% Suc-containing medium using the computer-aided fluorescence imaging apparatus described by Fenton and Crofts (1990). Measurements were made on individual cotyledons or first leaves. Similar results were obtained with a modulated fluorescence apparatus (PAM 101, Walz, Effeltrich, Germany) (Schreiber et al., 1986). Petri plates containing *hcf* plants and their wild-type siblings were dark-adapted for 5 min prior to illumination.

EM

EM was carried out on leaves of the mutants and their wild-type siblings grown on 5% Suc agar plates for 2 weeks. The leaf tissue was fixed in 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 1 h and postfixed in 1% (w/v) osmium tetroxide in the same buffer for 1 h. The tissues were then dehydrated in a graded ethanol series, followed by propylene oxide, and then embedded in Spurr's resin. Ultra-thin sections were stained with uranyl acetate and lead citrate and examined in an electron microscope (EM10, Zeiss).

Thylakoid Membranes, Pigment Analysis, and PSII Activity

Thylakoid membranes were isolated by grinding the leaves in 20 mM Tricine (pH 8.0), 10 mM NaCl, and 0.4 M Suc with a mortar and pestle. The homogenate was filtered through 125-μm bolting silk and centrifuged at 4340*g* for 12 min. The pellet was washed once in the same buffer and suspended in 20 mM Tricine (pH 8.0), 150 mM NaCl, and 5 mM $MgCl_2$. Total chlorophyll per plant was determined by assaying the thylakoid membrane fraction and the material retained on the bolting silk by the method of Arnon (1949).

For HPLC analysis of pigments, 3- to 4-week-old *hcf* and wild-type plants were dark-adapted for 1 to 2 h, then leaves were harvested and ground to a fine powder in liquid nitrogen. The powder was dispersed in 80% v/v acetone buffered with 10 mM Hepes, pH 7.5. An equal volume of diethyl ether was added to the acetone extract and the acetone was removed with 3 to 4 volumes of 10% KCl. After extensive washing with water the organic phase was evaporated under nitrogen and the pigments were redissolved in ethanol and used immediately for HPLC analysis. Pigments were separated by reversed phase HPLC using a LiChrospher 100 RP-18 column (5 μm, 4 mm i.d. × 125 mm length) (Merck, Darmstadt, Germany) with a linear gradient starting with 88% organic solvent (75:15:10, acetonitrile:methanol:tetrahydrofuran, v/v/v):12% water (v/v) and ending with 100% organic solvent after 12 min at a flow rate of 2 mL min^{-1}. The pigments were detected at 445 nm using a Waters 994 photodiode array detector. Identification of the pigments was done by a comparison of their absorption spectra and retention behavior to those of purified pigments. Chlorophyll *a* (Sigma) was used as a calibration standard.

PSII activity was assayed spectrophotometrically by the reduction of 2,6-dichlorophenolindophenol monitored at 595 nm in a reaction mixture containing 5 μg mL^{-1} of chlorophyll, 20 mM Mes buffer, pH 6.0, and 0.1 mM 2,6-dichlorophenolindophenol, with either water or 2 mM diphenyl carbazide as electron donors.

Electrophoresis and Immunoblotting

For separation of chlorophyll-protein complexes, thylakoid membrane samples corresponding to 25 μg of chlorophyll were pelleted, washed with 65 mM Tris-maleate (pH 7.0), and solubilized in 88 mM octyl-β-D-glucopyranoside at a detergent:chlorophyll ratio of 30:1 (v/v) (Camm and

Green, 1989). Electrophoresis was at 25 mA for 4 to 5 h, at 4°C in the dark on a 1.5-mm-thick, 10% polyacrylamide gel.

Denaturing gels were run on 10% polyacrylamide gels containing 0.1 M Tricine in the cathode buffer, as described by Schägger and von Jagow (1987), and modified so that the final concentration of Tris (pH 8.25) in the gel was 1.0 M. Thylakoid membrane samples were pelleted and resuspended in 65 mM Tris-HCl (pH 6.8), 20 mM DTT, 10% ethylene glycol, and 2% SDS and heated at 65°C for 15 to 20 min prior to loading onto the gel. For immunoblotting, samples of mutant thylakoids containing 5 μg of chlorophyll were compared with a series of decreasing amounts of wild-type thylakoids. Electrophoresis was at 35 mA for 5 to 6 h at room temperature. Proteins were transferred onto nitrocellulose and visualized, as described by White and Green (1987). For immunodetection of the Rubisco large subunit, total soluble protein from equal fresh weights of normal and mutant tissue were compared.

The antisera used to determine the presence or absence of specific thylakoid polypeptides were prepared against barley CPI (PSI reaction center complex) and CPII, the major chlorophyll *a*/*b* light-harvesting complex associated with PSII (White and Green, 1987); wheat CF_1 (Moase and Green, 1981); spinach PSI subunits II and VI (Bengis and Nelson, 1975); Cyt *f* (gift of R. Malkin); PSII chlorophyll *a*-protein core complexes CP47 and CP43 of *Chlamydomonas* (gift of N.-H. Chua); PSII core complex D1 (gift of A. Eastman), Cyt b_{559} (gift of W. Cramer); and Rubisco large subunit (gift of A. Barkan). Following staining with one antibody, the nitrocellulose filter was stripped overnight in a solution containing 0.1 M Gly (pH 2.2), 20 mM magnesium acetate, and 50 mM KCl (Legocki and Verma, 1981) and reblotted up to three times with other antisera.

RNA Isolation and Northern Blot Analysis

Total RNA from *hcf* mutants and wild-type siblings was isolated from 4- to 5-week-old plants grown on 5% Suc-containing plates. The aerial parts of the plants were harvested, frozen in liquid nitrogen, and ground to a fine powder with a mortar and pestle. The ground tissue was suspended in extraction buffer (10 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 1% SDS) and extracted twice with phenol:$CHCl_3$:isoamyl alcohol (25:24:1, v/v). After ethanol precipitation the pellet was dissolved in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0, and RNA was separated from total nucleic acid by precipitation with LiCl (Sambrook et al., 1989). The RNA was further extracted twice with phenol:$CHCl_3$:isoamyl alcohol (25:24:1, v/v) and once with $CHCl_3$:isoamyl alcohol (24:1, v/v), and precipitated with ethanol. RNA was electrophoresed on formaldehyde-containing agarose (1.2%) gels and blotted onto Hybond-N membrane (Amersham). The filters were hybridized overnight at 65°C in 7% SDS, 0.5 M sodium phosphate (pH 7.0), and 1 mM EDTA, as described by Barkan (1993).

Specific hybidization probes were prepared by labeling DNA with ^{32}P using the random primer method following the manufacturer's recommendation (Gibco-BRL). The filters were washed several times at 65°C in 1 × SSC (0.15 M NaCl, 0.015 M sodium citrate), 0.1% SDS, and exposed to film (XAR-5, Kodak) at −90°C with an intensifying screen.

DNA probes for nuclear genes used in this study were *cab*11, a 0.7-kb *Pst*/*Xba* fragment of the tomato *Lhca4* gene encoding a PSI light-harvesting protein (Schwartz et al., 1991); *cab3c*, one of the *Lhcb1* genes encoding the type I LHCII protein (Pichersky et al., 1985); and *psaD*, a 0.3-kb *Eco*RI fragment of the tomato PSI subunit II gene (Hoffman et al., 1988). DNA probes for chloroplast genes used were *petB*, a 309-bp *Xho*I fragment containing the spinach Cyt b_6 gene (Heinemeyer et al., 1984); *petD*, a 268-bp *Bam*HI/*Xba*I fragment derived from a 1109-bp *Xho*I fragment containing subunit IV of the Cyt complex of spinach (Westhoff and Herrmann, 1988); *psaB*, a 1.7-kb *Bam*HI fragment of the spinach 82-kD PSI reaction center polypeptide (Kirsch et al., 1986); *psbA*, a 0.7-kb *Hin*dIII fragment of the D1 protein of PSII (Nixon et al., 1990); *psbB*, a 1.1-kb *Bam*HI/*Xba*I fragment containing the PSII 47-kD polypeptide gene from spinach (Morris and Herrmann, 1984); *psbH*, a 320-bp *Sal*I/*Eco*RI fragment derived from a 764-bp *Xba*I/*Eco*RI fragment containing the 9-kD phosphoprotein of spinach PSII (Westhoff et al., 1986); *psbD*, a 989-bp *Eco*R1/*Pvu*II fragment containing the PSII D2 core protein gene from spinach (Alt et al., 1984); and *rbcL*, a 1520-bp *Pst*I/*Bam*HI fragment of Rubisco large subunit (gift of P. Westhoff).

RESULTS

Physical Characteristics and EM

The mutant line containing *hcf5* was isolated by screening the M_2 progeny of ethyl methanesulfonate-mutagenized seeds for high chlorophyll (red) fluorescence under UV-A light (Dinkins et al., 1994). Because homozygous mutant plants (*hcf*/*hcf*) are seedling-lethal in soil (due to inability to support autotrophic growth), the line was maintained by selfing individual plants and screening the seed for each generation to identify the heterozygotes. A red filter was used to verify the difference between chlorophyll fluorescence (with a maximum at approximately 685 nm) and the blue fluorescence emitted by other fluorescing phenotypes such as the Trp synthesis mutants (e.g. Bender and Fink, 1995). Under visible light mutant plants are lighter green than their wild-type siblings. All measurements reported here were from mutant plants and their wild-type siblings grown under sterile conditions on the same plates (Dinkins et al., 1994).

When the seedlings were still small, *hcf* and wild-type plants were transferred to fresh medium in large-diameter (250-mm) Petri plates to allow for leaf expansion. Table I shows that after 4 weeks the mutant plants were significantly smaller than their wild-type siblings in spite of being grown on 5% Suc. They had only 21% of the normal amount of chlorophyll per milligram fresh weight and the chlorophyll *a*/*b* ratio was significantly lower. Mutant plants occasionally formed flowers when grown in culture, but did not set seed.

Figure 1 shows that there are striking differences in chloroplast ultrastructure between *hcf5* and wild-type siblings. The mutant thylakoid membrane system (Fig. 1B)

Table I. *Physical characteristics of hcf5 mutant and wild-type siblings*

In each experiment, 40 to 80 *hcf* and wild-type plants were harvested at approximately 4 weeks after germination. Values are means ± SD (n = 3). Fresh weight refers to aerial portion of plant. Chlorophyll was determined by the method of Arnon (1949). PSII activity was measured as light-driven reduction of 2,6-dichlorophenol-indophenol (mmol mg^{-1} chlorophyll h^{-1}) with 2 mM diphenyl carbazide as electron donor at 595 nm, pH 6.0.

Plants	Plant Fresh Wt	Chlorophyll per Fresh Wt	Chlorophyll *a/b* Ratio	PSII Activity
	g	*μg mg⁻¹*		
hcf5	17.6 ± 7.7	0.22 ± 0.02	1.60 ± 0.08	0
Wild type	54.3 ± 4.1	1.04 ± 0.07	2.67 ± 0.17	147 ± 0.17
% (*hcf5*/wild type)	32	21		

consists almost exclusively of large stacks (grana) of tightly appressed membranes. The stacks do not appear to be connected to each other via single, unappressed thylakoids as they are in the normal chloroplast (Fig. 1A). There are some apparently disconnected, unappressed, single membrane vesicles in the stroma surrounding the abnormal grana. This abnormal morphology is more extreme than that reported in most PSII and PSI mutants of barley (Simpson and von Wettstein, 1980).

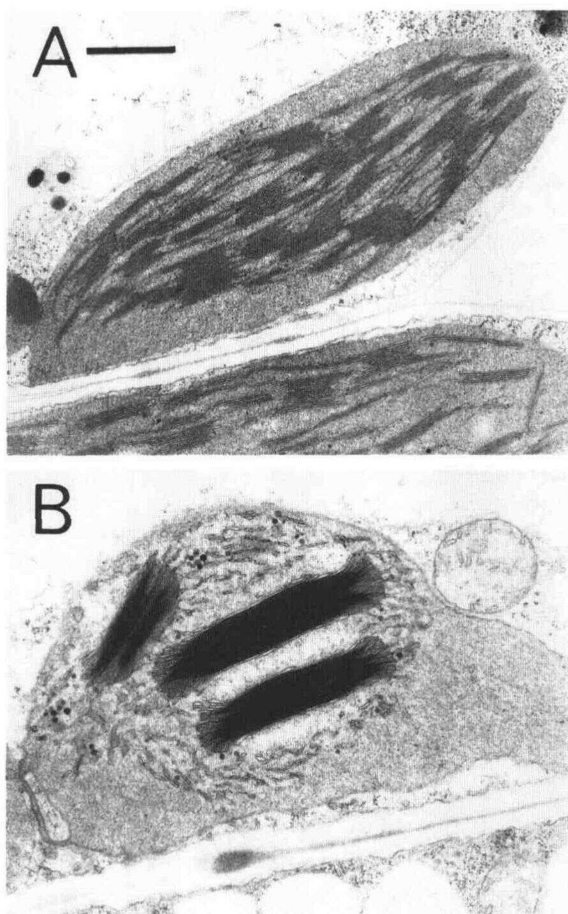

Figure 1. Ultrastructure of typical wild-type (A) and *hcf5* (B) chloroplasts. Bar = 1 μm.

Fluorescence and Electron Transport

The room temperature fluorescence-induction curves of mutant *hcf5* plants and their normal siblings are shown in Figure 2. To record the kinetics of the fluorescence rise, a computer-aided video fluorescence imaging system (Fenton and Crofts, 1990) was employed. When normal, dark-adapted plants are exposed to light, their fluorescence rapidly rises from a low initial fluorescence (F_0) to maximum fluorescence (F_M), then drops to a steady-state level due to reoxidation of PSII electron acceptors and the establishment of the transmembrane potential (Krause and Weis, 1991). The wild-type siblings on 5% Suc displayed typical normal fluorescence kinetics compared with plants in soil (compare with Artus and Somerville, 1988), indicating that the conditions on these plates do not adversely affect the development of the electron transport system. Plants with the *hcf5* mutant phenotype, on the other hand, had a high initial fluorescence (F_0), and essentially no variable fluorescence (F_M–F_0). This characteristic pattern is typical of a blockage of electron flow

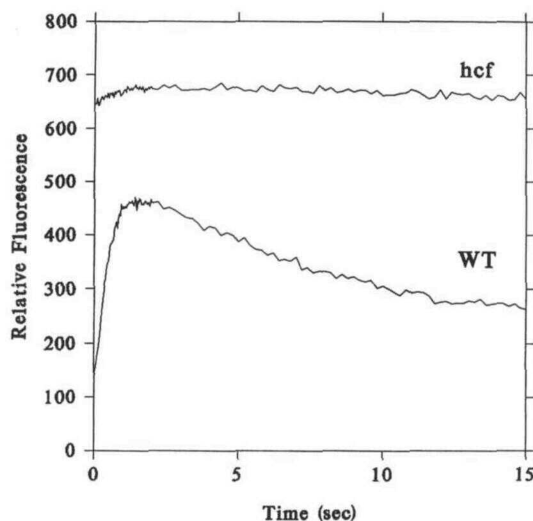

Figure 2. Fluorescence induction kinetics recorded from wild-type (WT) and *hcf5* (hcf) mutant leaves using the computer-aided fluorescence imaging apparatus described by Fenton and Crofts (1990). Traces shown are averages of emission curves from five (WT) and three (*hcf5*) plants.

through PSII (Chua and Bennoun, 1975; Miles, 1980); it was confirmed by in vitro assays of PSII activity in thylakoids (Table I). PSII electron transport activity was undetectable above the background level even using the artificial donor diphenylcarbazide.

Pigments and Chlorophyll-Protein Complexes

Table II shows that all the pigments normally present in wild-type plants were also present in the mutants, although in different proportions. The HPLC analyses confirm that the chlorophyll *a/b* ratio is significantly lower. When expressed on a fresh weight basis, β-carotene was the pigment most severely depleted in the mutant, with only 6% of the wild-type level (calculated from Tables I and II). The xanthophylls neoxanthin, violaxanthin, and lutein were at 34, 41, and 45% of wild-type levels, respectively, although they were enriched relative to chlorophyll *a* (Table II).

Electrophoresis on nondenaturing ("green") gels was used to analyze PSI and PSII pigment-protein complexes in mutant and wild-type plants (Camm and Green, 1989). Figure 3 shows that bands corresponding to CP47 and CP43, the core chlorophyll *a*-binding proteins of PSII, were greatly reduced. The CPI band (PSI reaction center and internal antenna) was also depleted, but the chlorophyll *a/b* antenna complexes CPII*, CPII, and CP29 appeared to be normal. These results are consistent with the depletion of β-carotene, which is found mainly in PSI and PSII core complexes, and suggest that most of the chlorophyll is associated with the light-harvesting complexes, particularly LHCII, which has a low chlorophyll *a*/*b* ratio (Camm and Green, 1989).

Photosynthetic Proteins

Thylakoid polypeptides were completely denatured, separated by gel electrophoresis, and immunoblotted successively with several antibodies to determine the presence or absence of specific polypeptides. Figure 4 shows that two PSII proteins, the chlorophyll *a*-binding apoprotein of CP47 and the Cyt b_{559} polypeptide, were depleted, with the former being almost undetectable in the mutant. The apoproteins of CP43 and the reaction center polypeptide D1

Table II. *Pigment analysis by HPLC*

Average of four to nine preparations of wild-type chloroplasts or two extracts of *hcf5* chloroplasts, each injected twice.

Pigment	Pigment Content	
	Wild-type	Mutant
	mol (100 mol chlorophyll a)$^{-1}$	
Chlorophyll *a*	100	100
Chlorophyll *b*	33.9	56.0
β-Carotene	10.1	3.4
Lutein	18.2	36.7
Neoxanthin	4.8	9.4
Violaxanthin	4.1	9.5
cis-Lutein	1.5	4.8
Antheraxanthin	0.09	1.7
Vitamin K_1	0.72	3.0

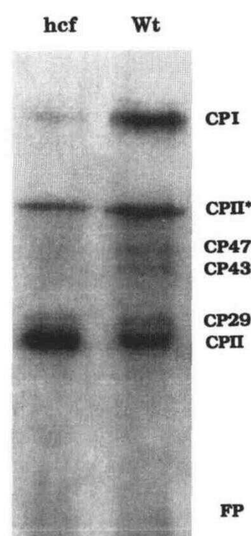

Figure 3. Chlorophyll-protein complexes of *hcf5* mutant (hcf) and wild-type (Wt) thylakoids resolved on nondenaturing "green gel" (unstained). Each lane is loaded with thylakoid membranes containing 25 μg of total chlorophyll, solubilized with 88 mM octyl glucoside.

were also present at very low levels (data not shown). Thus, it appears that all polypeptides associated with the PSII core are missing or severely reduced. This was not unexpected, as it has been previously reported that mutations affecting any component of PSII have a pleiotropic effect, leading to the loss of all PSII-associated polypeptides (Metz and Miles, 1982; Jensen et al., 1986; Rochaix and Erickson, 1988).

The mutation affected other photosynthetic polypeptides as well. Cyt *f* was less than 10% of the control, and the α

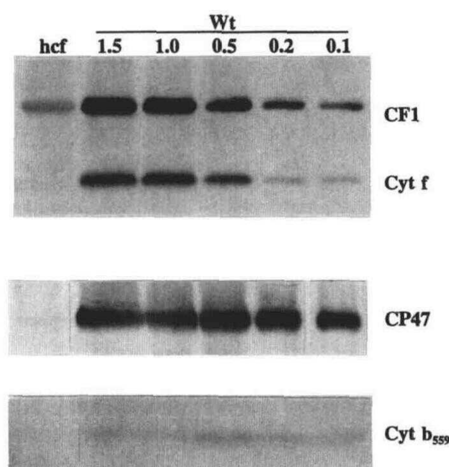

Figure 4. Immunoblots of photosynthetic membrane proteins. Washed thylakoids (5 μg of chlorophyll) of mutant (*hcf5/hcf5*) plants were compared with wild-type (Wt) thylakoids corresponding to a fraction of the mutant chlorophyll, i.e. 7.5, 5.0, 2.5, 1.0, and 0.5 μg of chlorophyll, respectively. Samples were solubilized in 2% SDS, separated on SDS-PAGE, transferred to nitrocellulose, and immunostained with antisera to CF_1, Cyt f (*petA* protein), CP47 (*psbB* protein), and Cyt b_{559} (*psbE* protein).

and β subunits of CF_1, which are not resolved from each other on this gel system, were 10 to 20% of the control (Fig. 4). Because equal amounts of chlorophyll were loaded in mutant and control lanes and the mutant is depleted in core chlorophyll *a* complexes, the other thylakoid polypeptides should have been overrepresented in the mutant lanes if they were unaffected by the mutation. PSI polypeptides were also at significantly lower levels in the mutant (Fig. 5). The polypeptides of CPI, the PSI reaction center, were almost undetectable, and the PSI subunit II polypeptide PsaD was about 10% of normal levels. The PSI "Subunit VI" antiserum (Bengis and Nelson, 1975) reacts with three polypeptides in wild-type thylakoids, but these were below the limit of detection in the mutant. Thylakoid membrane proteins are not the only photosynthetic proteins affected in this mutant; immunoblotting of the chloroplast soluble fraction showed that the large subunit of Rubisco was present at less than 6% of normal levels on a fresh weight basis (Fig. 5).

Steady-State RNA Levels

To determine if any of the observed differences in the *hcf5* mutant could be due to an effect of the mutation on chloroplast transcripts, total leaf RNA was isolated and probed with specific nuclear and ctDNA sequences. After hybridization to one or more probes, filters were stripped and hybridized with a chloroplast rDNA probe to verify that differences were not due to a general decrease in chloroplast RNA in the mutant. Figure 6 shows that *rbcL*

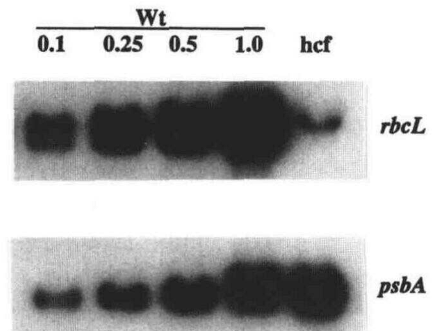

Figure 6. RNA blots of wild-type (Wt) and *hcf5* mutant. Total RNA (10 μg) from mutant and wild-type plants grown on the same plates was fractionated on formaldehyde-containing agarose gels, transferred to nylon membrane, and hybridized with ^{32}P-labeled probes for *rbcL* and *psbA*.

message was only 1 to 3% of wild-type levels, expressed as a fraction of total RNA. In contrast, the *psb*A message appeared to be present at normal levels in *hcf5* plants.

Like other plants, Arabidopsis has a polycistronic *psb*B-*psb*H-*pet*B-*pet*D transcript that undergoes a complex series of processing events (Tanaka et al., 1987; Barkan, 1988; Westhoff and Herrmann, 1988). Figure 7 shows RNA blots

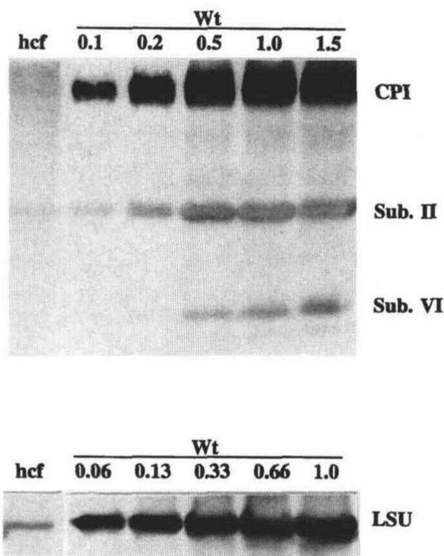

Figure 5. Immunoblots of photosynthetic proteins. Top, Washed thylakoids (5 μg of chlorophyll) of mutant (*hcf5/hcf5*) plants were compared with wild-type (Wt) thylakoids corresponding to a fraction of the mutant thylakoids, i.e. 0.5, 1.0, 2.5, 5.0, and 7.5 μg of chlorophyll. Samples were solubilized in 2% SDS, separated on SDS-PAGE, transferred to nitrocellulose, and immunostained successively with antisera to CPI (*psaA/psaB* proteins), PSI subunit II (*psaD* protein), and PSI subunit VI. Bottom, Soluble protein fraction loaded on an equivalent fresh weight basis, immunostained with antiserum raised to Rubisco large subunit (LSU).

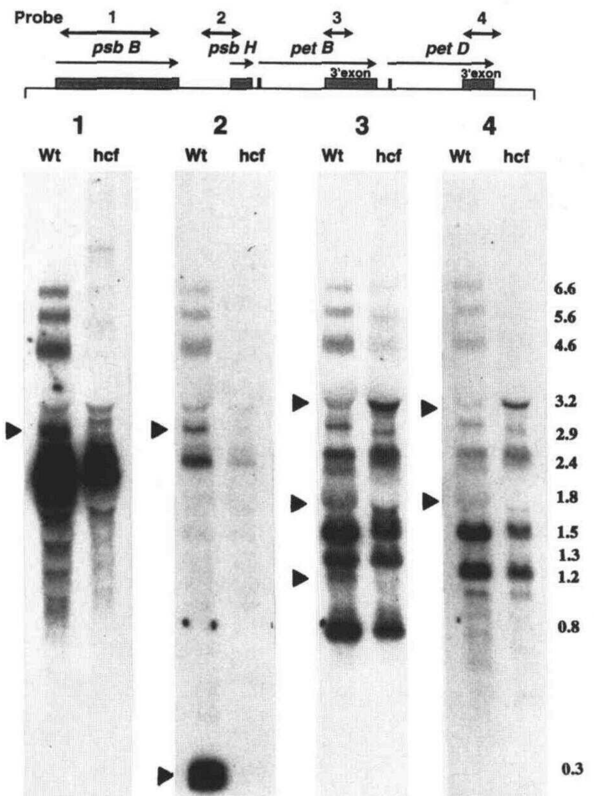

Figure 7. RNA blots of wild-type (Wt) and hcf (*hcf5/hcf5*) mutant. Total RNA (10 μg) from mutant and wild-type plants grown on the same plates was fractionated on formaldehyde-containing agarose gels, transferred to nylon membrane, and hybridized with ^{32}P-labeled probes as indicated in the upper panel. Arrowheads indicate major differences between mutant and wild-type bands.

from mutant and wild-type siblings hybridized with the probes shown as double-headed arrows in the map. The pattern of bands seen with wild-type Arabidopsis RNA was very similar to that of tobacco mRNA run on the same gel (Tanaka et al., 1987; data not shown).

The most striking changes in the *hcf5* RNAs were the almost complete absence of the monocistronic *psbH* transcript at 0.3 kb and the marked decrease of all other transcripts containing *psbH*. This included bands at 3.2, 2.8, and 2.4 kb that hybridized with both *psbB* and *psbH* probes, as well as the three large transcripts of 6.6, 5.6, and 4.6 kb that hybridized with all four probes (Fig. 7). By analogy with other plants, the latter probably represent the complete polycistronic transcript (6.6 kb) and polycistronic transcripts from which one or two introns have been spliced out (Tanaka et al., 1987; Barkan, 1988; Westhoff and Herrmann, 1988). The steady-state levels of all three species were decreased in the *hcf5* plants. Although there were some changes in intensity of the smaller bands detected only by the *psbB* probe, they were not as marked. The *psbB* probe terminates before the 3′ end of the gene, so it would not detect any monocistronic transcripts originating from *ycf8* (also known as *orf31*, *orf38*, or *psbT*), the gene for a small PSII protein that lies downstream of and is cotranscribed with *psbB* (Hird et al., 1991; Monod et al., 1992).

The *petB* and *petD* probes hybridized with bands of 3.2, 2.9, 2.4, 1.8, and 1.5 kb. The 3.2-kb band was increased in the mutant, in contrast to the 3.2-kb band detected with the *psbB* and *psbH* probes. It also hybridized to a probe specific for the *petD* intron (data not shown). The 2.9-kb band just below it did not hybridize with the intron-specific probe. This band was missing in the mutant but appeared to be replaced with a band of similar intensity migrating slightly faster. The rather diffuse band at 1.8 kb was also missing in the mutant, as was a 1.1-kb band that hybridized only with the *petB* probe. However, the 0.8-kb band, which presumably represents the final *petB* transcript, was unchanged. The mutation had little effect on bands hybridizing only with the *petD* probe.

Not all chloroplast transcripts were affected. In addition to *psbA*, only minor differences were found with probes for *psaB*, *petA*, *psbD*, and *atpA* (data not shown). Nuclear genes *lhcb1* and *lhca4* (encoding polypeptides of the peripheral light-harvesting complexes of PSII and PSI, respectively), *psaD* (encoding an essential polypeptide of PSI), and *rbcS* appeared to be expressed at wild-type levels (data not shown).

DISCUSSION

In this paper we describe the isolation and characterization of the *A. thaliana* high chlorophyll fluorescence mutant *hcf5*. The kinetics of fluorescence induction as well as the absence of PSII activity showed that PSII is severely affected, and immunoblotting confirmed that all PSII polypeptides are either lacking or at very low levels compared to wild type. However, the mutation appears to have a pleiotropic effect on other components of the electron transport system. Polypeptides of PSI, the Cyt f-b_6 complex, and CF_1 were also depleted or absent (Fig. 4). In contrast, the chlorophyll *a*/*b* antenna complexes LHCII and CP29 appeared normal on nondenaturing gels (Fig. 3), and transcripts of the nuclear genes encoding an LHCII polypeptide (*Lhcb1*) and a PSI LHCI polypeptide (*Lhca4*) were present in normal amounts in the mutant. This shows that the pleiotropic nature of the mutant is not due to an underlying defect in chlorophyll synthesis.

Besides high fluorescence, the most striking phenotypic trait was the drastic alteration of thylakoid membrane ultrastructure (Fig. 1). Almost all the thylakoids were appressed, although a few single thylakoids were observed around the periphery of the thylakoid stack. The chloroplast morphology of *hcf5* most closely resembles the barley PSII mutants at the *viridis-e* locus, but there are also some barley PSI mutants with diminished amounts of stroma thylakoids, e.g. *viridis-h*[15] (Simpson and von Wettstein, 1980). Although the ultrastructural defects of the barley mutants cannot be related to specific photosynthetic defects (Simpson and von Wettstein, 1980), the high degree of thylakoid appression in *hcf5* is probably due to the severe depletion of both photosystems and the consequent predominance of LHCII in the thylakoid membrane, because inter-thylakoid interaction of LHCII is known to be one of the major factors in thylakoid adhesion (Staehelin, 1987).

The *hcf5* mutant is unique in having decreased levels of all *psbB* operon transcripts containing *psb*H, no detectable *psb*H monocistronic transcript, and extremely low levels of *rbcL* transcript. In addition, there is an overaccumulation of the 3.2-kb *petB-petD* transcript and a decrease in two of the smaller transcripts containing *petB*, although the *petB* monocistronic transcript appears to be present in normal amounts. These defects suggest that the normal Hcf5 gene function is involved in both the processing and the stability of selected chloroplast transcripts. Because these processes have been shown to be mediated in vitro by proteins interacting with the 3′ end of the mRNA (Schuster and Gruissem, 1991; Hayes et al., 1996), RNA blots from *hcf5* and normal siblings were probed with Arabidopsis genes for two of these proteins, 28RNP (Schuster and Gruissem, 1991; S. Abrahamson, personal communication) and 33RBP (Cheng et al., 1994). No differences in transcript abundance were detected (S. Abrahamson, personal communication; A. DeLisle, personal communication). Other proteins binding to the 5′ end of chloroplast transcripts have been implicated in translational initiation and stability in *Chlamydomonas* (Nickelsen et al., 1994; Zerges and Rochaix, 1994; Mayfield et al., 1995; Yohn et al., 1996). If *hcf5* were defective in a protein required for the translatability of a number of chloroplast messages while affecting the stability of only certain ones, it could account for the pleiotropic effects of this mutation on PSI, PSII, and the Cyt complex.

The lowered levels of the *rbcL* transcript could be an indirect effect of a decrease in translation, because it has been shown that in spinach this mRNA is highly sensitive to changes in chloroplast translation and processing (Klaff and Gruissem, 1991; Schuster and Gruissem, 1991). Several maize mutants with general defects in chloroplast protein

synthesis due to decreased association of mRNAs with ribosomes have lowered amounts of *rbcL* message (Barkan, 1993). It has been suggested that this message, but not other chloroplast messages, was destabilized when not associated with ribosomes (Barkan, 1993). If *hcf5* were such a mutation, it would explain the low levels of *rbcL* message but not the depletion of *psbH*-containing transcripts or what appears to be a splicing or endonucleolytic process affecting *petB-petD* transcripts.

Most of the *hcf* mutants isolated in higher plants are missing or depleted in one of the macromolecular complexes of the photosynthetic membrane but do not show any effect at the level of chloroplast mRNA (Barkan et al., 1995). Only three mutants of maize, *hcf38*, *crp1*, and *crp2* (Barkan et al., 1986, 1994), appear to be deficient in the accumulation of chloroplast transcripts. The *hcf38* mutant lacks most of the *psbB* containing products of the *psbB* polycistronic operon and has lower levels of *petA*, *atpB/E*, and *psaA* transcripts, but has normal levels of the *rbcL* message. The *crp1* mutation appears to affect the accumulation of the monocistronic *petB* and *petD* products and the translatability of *petA* and *petD*, but not *petB* messages (Barkan et al., 1994). While our paper was in preparation, another Arabidopsis mutant, *hcf109*, which is defective in the *psbB* operon transcripts, was reported (Meurer et al., 1996a). In *hcf109* the transcripts containing *psbB* rather than *psbH* were depleted, and *petB* and *petD* were unaffected. In addition, three other polycistronic operons (*psbD/C*, *ndhC*, and *ndhH*) showed selective depletion of certain transcripts. All of the higher plant mutants are therefore different from each other and are similar only in that the mutations all affect polycistronic transcripts and all have pleiotropic effects. In no case do the alterations in chloroplast transcripts explain all the downstream effects on the steady-state levels of the major thylakoid membrane complexes. This indicates that the mutated genes encode proteins involved in complex processes, possibly requiring gene-specific interactions of a number of protein factors. Further exploration of these complex mutant phenotypes should be greatly aided by the isolation of T-DNA or transposon tagged alleles (Goodman et al., 1995).

ACKNOWLEDGMENTS

We thank our many colleagues who generously provided antisera or probes: N. Nelson, R. Malkin, N.-H. Chua, A. Eastman, W. Cramer, R. Herrmann, E. Pichersky, A. Barkan, and J. Tonkyn. We particularly thank P. Westhoff for his generosity in supplying specific probes for chloroplast genes, and S. Abrahamson and A. DeLisle for probing our blots with genes for RNA-binding proteins. We also thank M. Weis and S. Weilesko for help with electron microscopy, J. Fenton and A. Crofts for use of the fluorescence video imaging apparatus, and I. Damm for performing the pigment analyses.

Received August 26, 1996; accepted November 12, 1996.
Copyright Clearance Center: 0032-0889/97/113/1023/09.

LITERATURE CITED

Alt J, Morris J, Westhoff P, Herrmann RG (1984) Nucleotide sequence of the clustered genes for the 44 kd chlorophyll a apoprotein and the "32 kd"-like protein of the photosystem II reaction center in the spinach plastid chromosome. Curr Genet **8:** 597–606

Arnon DI (1949) Copper enzymes in isolated chloroplasts. Polyphenol-oxidase in Beta vulgaris. Plant Physiol **24:** 1–15

Artus NN, Somerville CR (1988) A mutant of Arabidopsis that exhibits chlorosis in air but not in atmospheres enriched in CO_2. Plant Physiol **87:** 83–88

Barkan A (1988) Proteins encoded by a complex chloroplast transcription unit are each transcribed from both monocistronic and polycistronic mRNAs. EMBO J **7:** 2637–2644

Barkan A (1993) Nuclear mutants of maize with defects in chloroplast polysome assembly have altered chloroplast RNA metabolism. Plant Cell **5:** 389–402

Barkan A, Miles D, Taylor WC (1986) Chloroplast gene expression in nuclear, photosynthetic mutants in maize. EMBO J **5:** 1421–1427

Barkan A, Voelker R, Mendel-Hartvig J, Johnson D, Walker M (1995) Genetic analysis of chloroplast biogenesis in higher plants. Physiol Plant **93:** 163–170

Barkan A, Walker M, Nolasco M, Johnson D (1994) A nuclear mutation in maize blocks the processing and translation of several chloroplast mRNAs and provides evidence for the differential translation of alternative mRNA forms. EMBO J **13:** 3170–3181

Bender J, Fink GR (1995) Epigenetic control of an endogenous gene family is revealed by a novel blue fluorescent mutant of *Arabidopsis*. Cell **83:** 725–734

Bengis C, Nelson N (1975) Purification and properties of the photosystem I reaction center from chloroplasts. J Biol Chem **250:** 2783–2788

Bennoun P, Levine RP (1967) Detecting mutants that have impaired photosynthesis by their increased level of fluorescence. Plant Physiol **42:** 1284–1287

Camm EL, Green BR (1989) The chlorophyll a/b complex, CP29, is associated with the photosystem II reaction centre core. Biochem Biophys Acta **974:** 180–184

Cheng S-H, Cline K, DeLisle AJ (1994) An *Arabidopsis* chloroplast RNA-binding protein gene encodes multiple mRNAs with different 5′ ends. Plant Physiol **106:** 303–311

Choquet Y, Goldschmidt-Clermont M, Girard-Bascou J, Kuck U, Bennoun P, Rochaix J-D (1988) Mutant phenotypes support a *trans*-splicing mechanism for the expression of the tripartite *psa*A gene in the *C. reinhardtii* chloroplast. Cell **52:** 903–913

Chua N-H, Bennoun P (1975) Thylakoid membrane polypeptides of *Chlamydomonas reinhardtii*: wild-type and mutant strains deficient in photosystem II reaction center. Proc Natl Acad Sci USA **72:** 2175–2179

Chua N-H, Matlin K, Bennoun P (1975) A chlorophyll-protein complex lacking in photosystem I mutants of *Chlamydomonas reinhardtii*. J Cell Biol **67:** 361–377

Dinkins RD (1992) Isolation and characterization of high chlorophyll fluorescence mutants of *Arabidopsis thaliana*. PhD thesis. University of British Columbia, Vancouver

Dinkins RD, Bandaranayake H, Green BR, Griffiths AJF (1994) A nuclear photosynthetic electron transport mutants of *Arabidopsis thaliana* with altered expression of the chloroplast *petA* gene. Curr Genet **25:** 282–288

Fenton JM, Crofts AR (1990) Computer-aided fluorescence imaging of photosynthetic systems: applications of video imaging to the study of fluorescence induction in green plants and photosynthetic bacteria. Photosynth Res **26:** 59–66

Goodman HM, Ecker JR, Dean C (1995) The genome of *Arabidopsis thaliana*. Proc Natl Acad Sci USA **92:** 10831–10835

Hayes R, Kudla J, Schuster G, Gabay L, Maliga P, Gruissem W (1996) Chloroplast mRNA 3′-end processing by a high molecular weight protein complex is regulated by nuclear encoded RNA binding proteins. EMBO J **15:** 1132–1141

Heinemeyer W, Alt J, Herrmann R (1984) Nucleotide sequence of the clustered genes for apocytochrome b6 and subunit 4 of the cytochrome b6/f complex in the spinach plastid chromosome. Curr Genet **8:** 543–549

Hird SM, Webber AN, Wilson RJ, Dyer TA, Gray JC (1991) Differential expression of the *psbB* and *psb*H genes encoding the 47 kDa chlorophyll a-protein and the 10 kDa phosphoprotein of photosystem II during chloroplast development in wheat. Curr Genet **19:** 199–206

Hoffman NE, Pichersky E, Malik VS, Ko K, Cashmore AR (1988) Isolation and sequence of a tomato cDNA clone encoding the subunit II of the photosystem I reaction center. Plant Mol Biol **10:** 435–445

Jensen KH, Herrin DL, Plumley FG, Schmidt GW (1986) Biogenesis of photosystem II complexes: transcriptional, translational, and posttranslational regulation. J Cell Biol **103:** 1315–1325

Kirsch W, Seyer P, Herrmann RG (1986) Nucleotide sequence of the clustered genes for two P700 chlorophyll a apoproteins of the photosystem I reaction center and the ribosomal protein s14 of the spinach plastid chromosome. Curr Genet **10:** 843–855

Klaff P, Gruissem W (1991) Changes in chloroplast mRNA stability during leaf development. Plant Cell **3:** 517–529

Krause GH, Weis E (1991) Chlorophyll fluorescence and photosynthesis: the basics. Annu Rev Plant Physiol Plant Mol Biol **42:** 313–349

Legocki RP, Verma DPS (1981) Multiple immunoreplica technique: screening for specific proteins with a series of different antibodies using one polyacrylamide gel. Anal Biochem **111:** 385–392

Lemaire C, Wollman F-A (1989) The chloroplast ATP synthase in *Chlamydomonas reinhardtii*. II. Biochemical studies on its biogenesis using mutants defective in photophosphorylation. J Biol Chem **264:** 10235–10242

Mayfield SP, Yohn CB, Cohen A, Danon A (1995) Regulation of chloroplast gene expression. Annu Rev Plant Physiol Plant Mol Biol **46:** 147–166

Metz JG, Miles D (1982) Use of a nuclear mutant in maize to identify components of photosystem II. Biochem Biophys Acta **681:** 95–102

Meurer J, Berger A, Westhoff P (1996a) A nuclear mutant of *Arabidopsis* with impaired stability on distinct transcripts of the plastid *psbB, psbD/C, ndhH,* and *ndhC* operons. Plant Cell **8:** 1193–1207

Meurer J, Meierhoff K, Westhoff P (1996b) Isolation of high-chlorophyll-fluorescence mutants of *Arabidopsis thaliana* and their characterisation by spectroscopy, immunoblotting and Northern hybridisation. Planta **198:** 385–396

Miles D (1980) Mutants of higher plants: maize. Methods Enzymol **69:** 3–23

Miles D (1994) The role of high chlorophyll fluorescence photosynthesis mutants in the analysis of chloroplast thylakoid membrane assembly and function. Maydica **39:** 35–45

Moase EH, Green BR (1981) Isolation and properties of the chloroplast coupling factor from wheat. Eur J Biochem **119:** 145–150

Monod C, Goldschmidt-Clermont M, Rochaix J-D (1992) Accumulation of chloroplast *psbB* RNA requires a nuclear factor in *Chlamydomonas reinhardtii*. Mol Gen Genet **231:** 449–459

Morris J, Herrmann RG (1984) Nucleotide sequence of the gene for the P680 chlorophyll a apoprotein of the photosystem II reaction center of spinach. Nucleic Acids Res **12:** 2837–2850

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant **15:** 493–497

Nickelsen J, van Dillewijn J, Rahire M, Rochaix J-D (1994) Determinants for stability of the chloroplast *psbD* RNA are located within its short leader region in *Chlamydomonas reinhardtii*. EMBO J **13:** 3182–3191

Nixon PJ, Metz JG, Roegner M, Diner BA (1990) A *Synechocystis* PCC 6803 deletion mutant and its transformation with a *psbA* gene from a higher plant. Curr Res Photosynth **1:** 471–474

Pichersky E, Bernatzky R, Tanksley SD, Breidenbach RB, Kausch AP, Cashmore AR (1985) Molecular characterization and genetic mapping of two clusters of genes encoding chlorophyll a/b-binding proteins in Lycopersicon esculentum (tomato). Gene **40:** 247–258

Rochaix J-D (1992) Post-transcriptional steps in the expression of chloroplast genes. Annu Rev Cell Biol **8:** 1–28

Rochaix J-D, Erickson J (1988) Function and assembly of photosystem II: genetic and molecular analysis. Trends Biochem Sci **13:** 56–59

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Schägger H, von Jagow G (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem **166:** 368–379

Schreiber U, Schliwa U, Bilger W (1986) Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer. Photosynth Res **10:** 51–62

Schuster G, Gruissem W (1991) Chloroplast mRNA 3′-end processing requires a nuclear-encoded RNA-binding protein. EMBO J **10:** 1493–1502

Schwarz E, Shen D, Aebersold R, McGrath JM, Pichersky E, Green, BR (1991) Nucleotide sequence and chromosomal location of Cab11 and Cab12: the genes for the fourth polypeptide of the photosystem I light-harvesting antenna (LHCI). FEBS Lett **280:** 229–234

Simpson DJ, von Wettstein D (1980) Macromolecular physiology of plastids XIV. viridis mutants in barley: genetic, fluoroscopic and ultrastructural characterization. Carlsberg Res Commun **45:** 238–314

Staehelin LA (1987) Chloroplast structure and supramolecular organization of photosynthetic membranes. *In* LA Staehelin, CJ Arntzen, eds, Encyclopedia of Plant Physiology, New Series, Vol 19. Springer, Berlin, pp 1–84

Tanaka M, Obokata J, Chunwongse J, Shinozaki K, Sugiura M (1987) Rapid splicing and stepwise processing of a transcript from the *psbB* operon in tobacco chloroplasts: determination of the intron sites in *petB* and *petD*. Mol Gen Genet **209:** 427–431

Westhoff P, Farchaus JW, Herrmann RG (1986) The gene for the Mr 10,000 phosphoprotein associated with photosystem II is part of the psbB operon of the spinach plastid chromosome. Curr Genet **11:** 165–169

Westhoff P, Herrmann RG (1988) Complex RNA maturation in chloroplasts. Eur J Biochem **171:** 551–564

White MJ, Green BR (1987) Antibodies to the photosystem I chlorophyll a+b cross-react with polypeptides of CP29 and LHCII. Eur J Biochem **163:** 545–551

Yohn CB, Cohen A, Danon A, Mayfield SP (1996) Altered mRNA binding activity and decreased translation initiation in a nuclear mutant lacking translation of the chloroplast *psbA* mRNA. Mol Cell Biol **16:** 3560–3566

Zerges W, Rochaix J-D (1994) The 5′ leader of a chloroplast mRA mediates the translational requirements for two nucleus-encoded functions in *Chlamydomonas reinhardtii*. Mol Cell Biol **14:** 5268–5277