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USE OF A NUCLEAR MUTANT OF MAIZE TO IDENTIFY COMPONENTS OF PHOTOSYSTEM II

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Previous analysis of a high fluorescent, nuclear mutant of maize, designated *hcf-3*, indicated that the primary alterations of the photosynthetic membranes were associated with Photosystem (PS) II (Leto, K. and Miles, C.D. (1980) Plant Physiol. 66, 18–24). Further investigation reveals that the mutant thylakoids contain less than 20% of the wild-type manganese level and are missing most or all of at least six major polypeptides with apparent molecular masses of 49, 45, 34, 32, 16 and 10 kDa. The data provide evidence for the regulated production of a physiological PS II unit made up of these polypeptides and in terms of electron transport, extending from the water-splitting apparatus up to but not including the plastoquinone pool. This mutant may aid in the identification of membrane components associated with PS II as well as provide information concerning nuclear control of production of thylakoid complexes.

Introduction

The photosynthetic electron-transport chain can be conveniently divided into three regions; the PS I and PS II complexes, organized around their respective reaction centers, and the so-called cytochrome f-b_6 complex. Additionally, the thylakoids contain two complexes not directly involved in electron transport; the light-harvesting chlorophyll a/b-protein complexes and the CF_1CF_0-ATP synthetase. Information concerning the modes of action, composition and regulated synthesis of these supramolecular entities has been obtained through the use of a variety of techniques. The use of genetic mutants altered in specific portions of the photosynthetic system can be particularly rewarding, since this approach utilizes the organism's own regulatory systems to define a physiological rather than an operational organization in the membrane.

Classically, mutational studies have employed the microalgae which can be manipulated with standard microbiological techniques [1,2]. Recently, however, a wide range of mutants have been selected in maize [3] and barley [4] allowing for the extension of this method to higher plants. In this study we have utilized a mutant of *Zea mays* to investigate the composition of PS II. This mutant was originally selected due to its increased fluorescence relative to normal plants with unimpaired photosynthetic electron transport [5].

Previous reports concerning this high fluorescent, nuclear mutant, designated *hcf-3*, have shown that the alterations of its thylakoid system are primarily associated with PS II [3,6–8]. The mutant exhibited no PS II electron-transport activity, a high square fluorescence induction curve with no variable component and the loss of cyto-

Abbreviations: Chl, chlorophyll; CP I, chlorophyll-protein complex I; CP a/b, light-harvesting Chl a/b-protein complex; *hcf*, high chlorophyll fluorescence; kDa, kilodalton; LDS, lithium dodecyl sulfate; PS, photosystem; Tricine, *N*-tris(hydroxymethyl)methylglycine.

chrome *b*-559. Electrophoretic analysis of the thylakoids, using a sodium dodecyl sulfate polyacrylamide gel technique, revealed the loss of a 32 kDa polypeptide as well as alterations of several lower molecular mass polypeptides. The mutant possessed wild-type levels of PS I electron-transport activity, somewhat higher than normal levels of plastoquinone as well as cytochromes *f* and b_6. Electrophoresis of membrane samples established the presence of the majority of the thylakoid polypeptides as well as the chlorophyll-protein complexes associated with PS I and the light-harvesting apparatus. All of the observed modifications were restricted to PS II, however, previous work has not shown if only the reducing side of PS II has been affected or if the mutant is missing the entire PS II complex [7].

By using LDS-polyacrylamide gel electrophoretic technique of Delepelaire and Chua [9], we show the alteration in the polypeptide pattern of *hcf-3* is more extensive than previously believed. At least six and possibly seven major thylakoid protein bands are missing or greatly reduced in *hcf-3*. Several of these represent known or inferred components of PS II while others are undescribed. The lower Chl *a*/*b* ratio and the loss of polypeptides with apparent molecular masses of 49 and 45 kDa (which have been shown to bind Chl *a* and β-carotene in the green algae) [9] suggest a loss of a specific set of Chl *a* molecules associated with PS II. Additionally, EDTA-washed thylakoids of the mutant contain only 18% of the manganese normally bound to wild-type membranes, indicating that the oxidizing side of PS II has also been affected. In combination, these data suggest that the entire PS II unit is lacking or greatly reduced in *hcf-3*.

Materials and Methods

Kernels of maize (*Z. mays* L.) were germinated in soil and grown to the three-leaf stage as described previously [6]. The nuclear gene mutant, designated *hcf-3*, resembles the wild-type sibling plants at this stage, and the mutant plants are identified by their high fluorescent nature upon irradiation with long-wave ultraviolet light [5]. The mutation is lethal once endosperm nutrient reserves are exhausted, and it is therefore maintained in the heterozygous condition. *hcf-3* was originally assigned the isolation number E-846 by Neuffer [10], who induced the mutation by treatment of pollen with ethyl diethanesulfonate, and supplied the original material for screening.

Thylakoid membranes for manganese determinations were isolated by grinding 1 cm pieces of leaf material in a cold solution of 0.4 M sucrose, 20 mM Tricine-NaOH (pH 7.8), 10 mM NaCl, and 5 mM dithiothreitol using a Sorvall Omni-Mixer. After filtering through two layers of Miracloth, the suspension was centrifuged briefly at $500 \times g$ to remove remaining cell debris, and then at $5000 \times g$ for 10 min to pellet the membranes. The thylakoids were washed three times by suspending, with a glass homogenizer, in 10 mM Tricine-NaOH (pH 7.8), 10 mM NaCl, 1 mM EDTA, and centrifuging at $5000 \times g$ for 10 min followed by one similar wash in 1 mM EDTA. The pellet was suspended in deionized water and the chlorophyll concentration determined [11]. The samples were dry ashed, dissolved in redistilled HCl and the manganese concentration determined by atomic emission using a Jarrell-Ash No. 82-000 spectrophotometer with a nitrous oxide-acetylene flame [12]. The manganese emission line at 403.1 nm was used. Matrix interference was checked by intensity measurements 0.2 nm either side of the 403.1 nm line and were found to be negligible.

A similar grinding and centrifugation routine was used to prepare samples for electrophoresis. However, the breaking solution consisted of 0.33 M sucrose, 30 mM Tricine-NaOH (pH 7.8), 1 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 5 mM β-mercaptoethanol, and the membranes were washed three times in 10 mM Tricine-NaOH (pH 7.8), 10 mM NaCl. The washed membranes were suspended in 10 mM Tricine-NaOH (pH 7.8), 10% sucrose to a final chlorophyll concentration of near 0.5 mg/ml, and stored at −70°C in 0.1 ml aliquots until needed. Just prior to electrophoresis, the samples were thawed, and LDS and dithiothreitol added from stock solutions to yield final concentrations of 2% and 30 mM, respectively. Samples were either maintained at 4°C or heated to 70°C for 4 min prior to loading on the gel. Electrophoresis was as described by Delepelaire and Chua [9] except that a 10–15% acrylamide gradient was employed and the concentration of

EDTA in the upper reservoid buffer was 0.4 mM. A Bio-Rad Protean slab gel electrophoresis cell, with 1.5-mm spacers was used and electrophoresis carried out at 4 W of constant power for approx. 18 h at 4°C. The gels were stained for peroxidase activity using a modification [13] of the technique described by Thomas et al. [14]. The same gels were then stained with Coomassie brillant blue R-250 without removing the peroxidase activity stain. Apparent molecular masses of protein bands were determined by comparing the direct migration distances of the bands with those of a set of molecular mass protein standards run on the same gel (standards from Bio-Rad Laboratories, included phosphorylase *b*, 92500; bovine serum albumin, 66200; ovalbumin, 45000; carbonic anhydrase, 31000; soybean trypsin inhibitor, 21500; and lysozyme, 14400). The band corresponding to cytochrome *b*-559 was identified by electrophoresis of the purified protein isolated from maize using the method of Singh and Wasserman [15].

Results

The manganese contents of EDTA-washed thylakoid membranes isolated from *hcf-3* and the wild-type sibling controls were determined using atomic emission spectroscopy. A value of 3.9 manganese atoms per 400 chlorophylls (i.e., per PS II or PS I reaction center) was obtained for the wild-type membranes. This value is in good agreement with those published by other workers for higher plants [16,17] as well as for the green algae [18]. In contrast, the thylakoids of *hcf-3* contain only 0.7 manganese atoms per 400 chlorophylls, representing a decrease of approx. 82% when compared to the wild-type levels. The values are normalized, based on chlorophyll content of the membranes and assume a similar organization of these pigments in the wild type and mutant. However, the Chl a/b ratio is consistently much lower in the mutant (Chl $a/b = 2.4$) than in the wild type (Chl $a/b = 3.4$). It is likely that this change is due to the loss of a specific set of Chl *a* molecules including those closely associated with PS II. Therefore, comparisons between *hcf-3* and the wild type based on chlorophyll concentration alone will lead to an overestimate of the mutant values. For example, if *hcf-3* is missing 80 Chl *a* molecules associated with PS II, then the manganese content should be based on a 'unit' size of 320 rather than 400, yielding a value of 0.55 manganese atoms per 320 chlorophylls (see also the section on gel electrophoresis).

Fig. 1 shows a typical electrophoretic separation of wild-type and mutant membranes using samples that have either been heated prior to separation or maintained at 4°C. The gel has not been stained for protein but has been stained for peroxidase activity (see Materials and Methods). The chlorophyll-protein complexes associated with PS I (CP I) and the light-harvesting Chl a/b-protein (CP

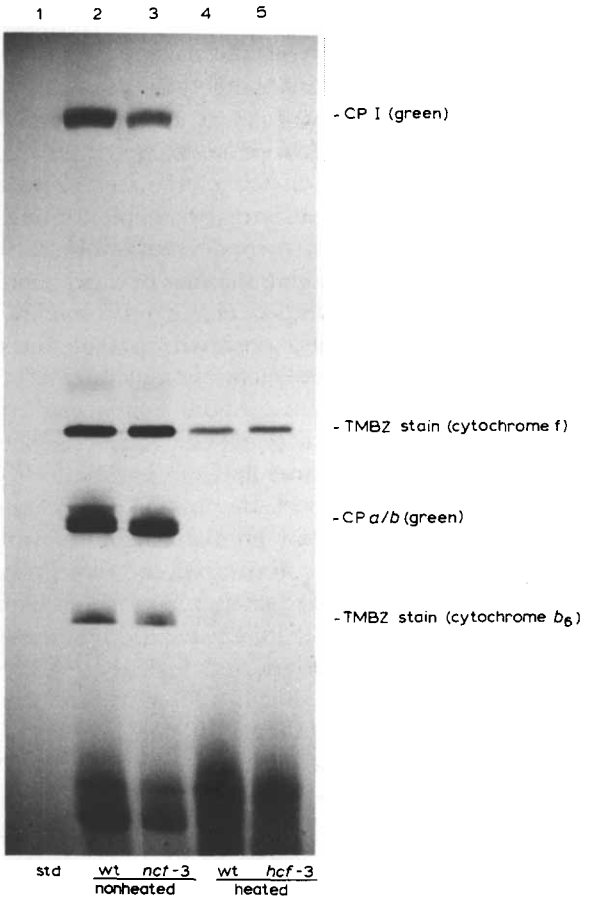

Fig. 1. Chlorophyll-protein complexes and peroxidase activity bands (3,3',5,5'-tetramethylbenzene (TMBZ) stain) of thylakoid membrane samples from mutant *hcf-3* and normal siblings. Separation was by LDS-polyacrylamide gel electrophoresis at 4°C. Samples were either maintained at 4°C (nonheated) or heated to 70°C for 4 min (heated) prior to the separation. std, standard; wt, wild type.

a/b) are present in both the wild type and the mutant although the CP I complex is slightly reduced in the mutant.

There are two blue bands which appear in these gels upon staining for peroxidase activity. The band at 33 kDa has been suggested to be cytochrome *f* [19] and we have verified this in maize by comparison with spectroscopically pure maize cytochrome *f* co-electrophoresed with whole membrane samples (data not shown). The band with higher mobility (apparent molecular mass 23 kDa) has been correlated with the presence of cytochrome b_6 [19]. The presence of both peroxidase activity bands in the wild-type and mutant samples confirms the spectroscopic data of Leto and Miles [7] and allows the unambiguous identification of a 33 kDa polypeptide as cytochrome *f*. With the specific staining method employed most of the peroxidase activity is lost upon heating of the samples prior to electrophoresis. This is seen in lanes 4 and 5 in Fig. 1. The chlorophyll-protein complexes are also dissociated by sample heating.

Fig. 2. shows the polypeptide profile obtained by Coomassie brillant blue staining of a gel similar to that shown in Fig. 1. The 25–30 predominant bands consistently observed in these gels represent the major polypeptide constituents of the thylakoid membranes. Many bands can be correlated to known functional components of the photosynthetic membranes and this correlation is indicated in Fig. 2. There are several differences between the mutant and normal sibling control profiles. The mutant is missing or shows a great reduction in polypeptides with apparent molecular masses of 49, 45, 34, 32, 16, 12 and 10 kDa (these bands are marked with asterisks in Fig. 2). Additionally, at least two bands (17 and 13 kDa) appear to stain more intensely in the samples of the mutant. With one exception, the alterations discussed here have been observed consistently in four independent experiments. The exception being the alteration of the band at 12 kDa, which was evident in only three of the four gels. Also, the use of gels containing high concentrations of urea, which allows improved resolution of the lower molecular mass polypeptides, did not reveal any further differences between the normal and mutant membranes (data not shown).

It should be noted that the amount of sample

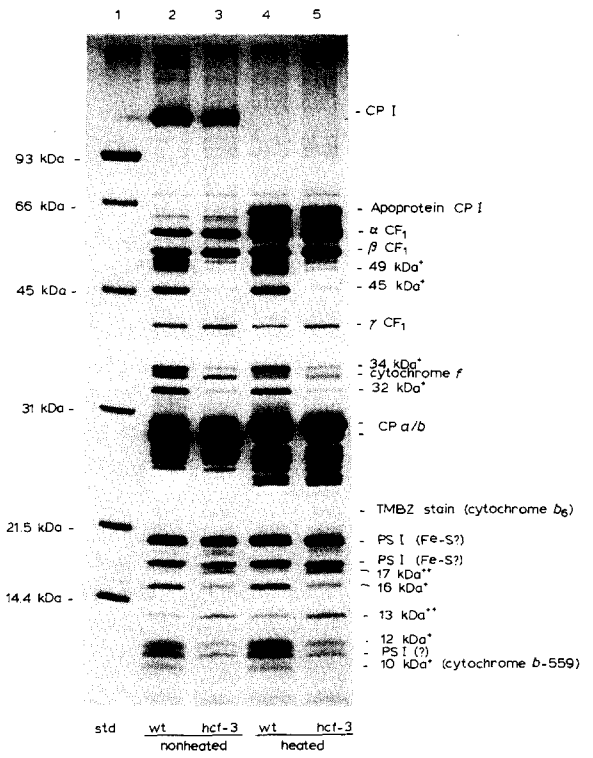

Fig. 2. Thylakoid membrane polypeptides of mutant *hcf-3* and the normal sibling plants separated by LDS-polyacrylamide gel electrophoresis at 4°C and revealed by staining with Coomassie brillant blue R-250. Known or tentatively identified polypeptides are indicated to the right of the gel. Bands missing or greatly reduced in the mutant thylakoids are marked with (*) while those staining more intensely in the mutant are indicated with (**). Lane 1 contains protein molecular mass standards.

loaded per slot on the gels was based on a chlorophyll determination, and that less chlorophyll was loaded in the case of the mutant (12 μg vs. 15 μg for the wild type). In previous experiments when equal amounts of chlorophyll (or protein) were placed in the wild-type and mutant samples, many of the polypeptide bands of the mutant stained more intensely than the corresponding bands of the wild type. This was obvious for the α-, β- and γ-subunits of the coupling factor, for bands stained with peroxidase activity method as well as several others. When 20% less chlorophyll is loaded in the *hcf-3* samples, as in the gel shown in Fig. 2, the protein bands mentioned above appear to stain with about the same intensity as corresponding bands of the wild-type sample. The visual match-

ing of the intensity of staining of these bands was thus treated as a standardization method. As was noted, the CP I complex appears to be somewhat decreased in the gels when this method of standardization is used, however, the reduction is minor when compared to the losses of the bands which can be associated with PS II. Additionally, the availability of mutants of maize which lack the PS I reaction center and closely associated polypeptides (mutant *hcf-50* described in Ref. 3), as well as the work of others [20–22], has aided the identification of the polypeptides of PS I. These bands are indicated in Fig. 1 and all show the same relative degree of reduction in *hcf-3* as was observed for the CP I complex.

Discussion

The identification of the biochemical composition of PS II has proven to be more difficult than for any of the other complexes of the thylakoids. For example, components of PS I have been identified using both particle-isolation techniques [21–23] and mutant analysis [20,24]. Also, the cytochrome b_6-f complex has recently been isolated and analyzed [25]. The general lability of the water-splitting system, the close physical association of PS II with the light-harvesting Chl a/b complex and the tendency of the CF_1 complex to follow PS II through many biochemical purification procedures have all contributed to the problem of PS II isolation. However, several procedures have been reported for obtaining fractions enriched in PS II, though none are as clean as the PS I preparations [26–30].

The analysis of mutants altered in PS II function avoids the problems associated with particle isolation and such mutants have been widely used in attempts to elucidate the protein composition of PS II [31–34]. The pleiotropic effect which presumably single-site mutations exert on thylakoid membranes has been noted many times, and it is known that mutations at nonallelic sites of the nuclear genome can result in nearly identical phenotypes [7,24]. It seems apparent now that in many cases the mutation has affected some factor required for the production, assembly or biochemical stability of a multicomponent unit of the membranes. Whether the mutated factor represents a component of the unit, an enzyme involved in posttranslational modification or is more directly involved in regulation at a translational or transcriptional level is not known. However, it is clear that several distinct factors can yield the same result, i.e., the loss of a complex unit in the membrane. Such seems to be the case for *hcf-3*, in which a single-site nuclear mutation has resulted in the loss of all the components of the membrane which can be associated with PS II, while the other major membrane complexes are qualitatively unaffected.

Manganese is required for photosynthetic electron-transport activity and has been associated with PS II [35]. Between four and six manganese atoms per PS II reaction center (based on the assumption of 400 Chl/PS II reaction center) remain bound to photosynthetic membranes even after several washes with 1 mM EDTA [16,17]. Experimental correlation of the loss of thylakoid manganese with loss of water-splitting activity upon treatment with various specific inhibitors suggest that 60–70% of the manganese is directly involved with that reaction [36]. Also, Metz and Bishop [37] found that static mutants of *Scenedesmus* which were unable to carry out the water-splitting reactions, but possessed otherwise intact photosynthetic membranes, were also missing between 60 and 70% of the normal manganese levels. The thylakoids of *hcf-3* show a loss of 82% of the manganese normally associated with the membranes of the wild-type plant. This indicates that not only has the water-splitting apparatus been affected by the mutation but also that an additional manganese binding site has been lost. The function of the manganese not associated with the water-splitting reactions is not clear but it has been suggested to function in PS II, possibly on the oxidizing side between the water-splitting site and the reaction center [16,38,39].

In a previous publication [7], the lamellar polypeptide composition of wild type and mutant *hcf-3* was analyzed using the electrophoretic system described by Laemmli [40]. However, with thylakoids isolated from maize, this method can result in the loss of a specific set of major polypeptides, presumably by causing aggregation of the proteins and thereby preventing them from entering the gel. The use of LDS-polyacrylamide gel electrophoresis

as described by Delepelaire and Chua [9] consistently reveals several major bands not seen with the method of Laemmli [40]. In maize, these include the apoprotein of CP I and three other polypeptides with apparent molecular masses of 34, 45 and 49 kDa (compare Fig. 8 of Ref. 7 with Fig. 2 of this report). Therefore, the comparison of the polypeptide composition of the thylakoids of *hcf-3* with those of the normal sibling control plants now reveals a loss or reduction of several major components of the membranes (Fig. 2).

Delepelaire and Chua [9], using the LDS-polyacrylamide gel electrophoretic procedure at 4°C, observed two new Chl *a*-protein complexes in *Chlamydomonas*. The apoproteins of these complexes had apparent molecular masses of 50 and 47 kDa. Previous work using high fluorescent mutants had indicated that these two proteins were required for PS II reaction center activity [31,32] and that the proteins were synthesized on chloroplast ribosomes [41]. Using immunological techniques it was shown that homologous polypeptides were present in higher plants [9]. Thylakoids of *hcf-3* are missing major polypeptides with apparent molecular masses of 49 and 45 kDa and these are the only bands missing within the 40–50 kDa range. It is clear that they represent polypeptides homologous with the reaction center proteins of *Chlamydomonas*. In maize, we do not observe the two Chl *a*-protein complexes comparable to those of *Chlamydomonas*, however, *hcf-3* has a lower Chl *a*/*b* ratio than that of the wild-type control plants suggesting a loss of Chl *a* molecules, presumably inducing those which would be associated with these polypeptides.

There are many reports in the literature of thylakoid polypeptides with molecular masses in the 30–34 kDa regions [15,18, 42–44] and it is often difficult to compare the results obtained from different laboratories. In our gel system, three distinct polypeptides with apparent molecular masses of 34, 33 and 32 kDa are observed in this region. The availability of a stain for peroxidase permits the unambiguous identification of the 33 kDa band as cytochrome *f*. As expected from the spectroscopic data of Leto and Miles [7] this band is present in equivalent amounts in the wild type and mutant. In contrast, the polypeptides at 34 and 32 kDa are greatly reduced in *hcf-3*. Metz and Bishop [37] found that static, low fluorescent mutants of *Scenedesmus* which were unable to oxidize water, but which possessed otherwise intact photosynthetic membranes, were lacking a 34 kDa thylakoid protein. Since these mutants were also missing from 60 to 70% of the manganese normally bound to the wild-type membranes, they proposed that the 34 kDa polypeptide was involved in the water-splitting reaction. Åkerlund and Jansson [44] also suggested a role in water photolysis for a 34 kDa polypeptide, which is released from Tris-treated, inside-out thylakoid preparations of spinach. *hcf-3* shows no water-splitting activity and is also deficient in thylakoid-bound manganese, suggesting a similar role for the 34 kDa protein of maize.

Although there have been reports of the involvement in the water-splitting reactions of proteins with molecular masses of 65 kDa [45] and 58 kDa [46], we have not observed any differences between the wild-type and *hcf-3* membranes in these regions.

The intensely stained protein band with an apparent molecular mass of 32 kDa in Fig. 2 has been identified as a rapidly turning over, photoinducible polypeptide which contains the binding site for the triazine herbicides [43,47,48]. It may be directly involved in electron transport on the reducing side of PS II between the primary acceptors and the plastoquinone pool, and also may act as a cap over the PS II reaction center [49]. The gene coding for this protein is located in the chloroplast genome [50]. As was noted previously [7], thylakoids of *hcf-3* are missing most of this protein.

hcf-3 also has reduced amounts of polypeptides with apparent molecular masses of 16 and 12 kDa. The alteration of the 16 kDa band was consistently observed while the change at 12 kDa was observed in three out of four experiments. Functions associated with these proteins have not been proposed in the literature although alterations of polypeptides in this region have been observed in PS II-deficient algal mutants [31] and are present in PS II-enriched particle preparations from the cyanobacteria [26,27] and spinach [30].

The function of cytochrome *b*-559 in the photosynthetic membranes is unresolved at this time, although several roles have been proposed [51–53].

It is physically close to the PS II reaction center and at low temperatures can donate electrons to the reaction center [54]. The polypeptides of this cytochrome are synthesized in the chloroplast, possibly in a precursor form [55]. Pure samples of cytochrome *b*-559 yield a single protein-staining band upon LDS-polyacrylamide gel electrophoresis which migrates at the position indicated in Fig. 2, yielding an apparent molecular mass, in this gel system, of 10 kDa. The mutant is missing most of the Coomassie blue-stainable material at this position in the gel, verifying the loss of the polypeptides of the cytochrome.

hcf-3 shows increases in polypeptides with molecular masses of 17 and 13 kDa. The relationship of these bands to others in the profile, or their functions in the membranes is not known at this time.

In summary, the mutation in *hcf-3* has resulted in the loss of all components of the photosynthetic membranes which can be associated with PS II. These include the 49 and 45 kDa polypeptides of the reaction center, the 32 kDa herbicide-binding protein, over 80% of the thylakoid-bound manganese and the polypeptides of cytochrome *b*-559. Additionally, they lack a 34 kDa polypeptide which may be involved in the manganese dependent water-splitting reactions. The presence of known constituents of the other major complexes of the thylakoids (coupling factor, PS I, CP *a*/*b* and cytochromes *f* and b_6) suggest that all of the major alterations of these membranes will be associated with PS II.

That a single-site, nuclear mutation can result in the loss of a group of polypeptides, several of which have been shown to be synthesized on chloroplast ribosomes, suggests that PS II exists as a physiological unit, whose presence in the membrane is regulated independently of the other major complexes of the thylakoids. Trace amounts of all of the PS II polypeptides are present in the photosynthetic membranes of *hcf-3*, indicating that the PS II particle may be synthesized in the mutant, but at a very low level. Two regulatory systems controlling the production of the cytochrome aa_3 complex of the mitochondria have been proposed [56]. One system controls the basal or 'constitutive' level while the other coordinates the synthesis with respect to other components of the membrane. A similar situation could be operating in the case of the chloroplast membranes. The mutation in *hcf-3* while not preventing the production of minor amounts of the PS II unit may have altered the regulatory system involved in coordinating the level of PS II production with that of the other complexes of the thylakoid membranes. Further analysis of mutants such as *hcf-3* may provide useful information as to the mechanisms which govern biochemical regulation at this level.

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