Chloroplast Reactions of Photosynthetic Mutants in Zea mays

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ABSTRACT

Three seedling lethal mutants of Zea mays with impaired photosynthesis are described. These recessive mutants were selected on the basis of high chlorophyll fluorescence. They have normal chlorophyll pigmentation but are unable to fix CO2 fully. Evidence is presented from fluorescence characteristics of isolated chloroplasts that both photosystem I and II mutants were isolated. Using conventional measures of photosynthetic electron transport, we suggest that the photosystem I mutant has limited ability to reduce NADP. The other two mutants are clearly blocked in photosystem II, one possibly lacking the primary electron acceptor.

The use of genetic mutants is an indispensable tool in the study of complex biochemical reactions. This approach for gaining insight into the mechanism of the photosynthetic process has been successfully employed, most notably by Levine (13) with Chlamydomonas and by Bishop (6) with Scenedesmus. However, very little work has been done using genetic mutants of higher plants (14). Most of the work with higher plants has involved mutants selected on the basis of pigment abnormality. A mutant of Vicia faba which appears to be inhibited in the ferredoxin-NADP oxidoreductase has been described (11). Homann and Schmid (12) have reported a mutant of Nicotiana which lacks Hill activity and a series of mutants Oenothera were reported (8) which resulted in blocks of either photosystem I or II. These were the major photosynthetic mutants reported in higher plants and all showed some degree of pigment and structure abnormalities.

Following the development of an acceptable screening technique for selection of mutants in higher plants (20), we have isolated a number of full green photosynthetic mutants of maize and here report on the characterization of three such mutants.

MATERIALS AND METHODS

Plant Material. Maize seedlings were grown to the three leaf stage (about 10 days) in a controlled environment chamber. They were subject to 14 hr of light at 30 C and 10 hr of darkness at 25 C. Light was provided by fluorescent (cool-white type) and incandescent lamps at an intensity of 5 x 10^4 ergs·cm⁻²·sec⁻¹. Kernels were germinated and grown in vermiculite contained in 7- x 38-cm plastic trays. Plants were provided with a nutrient solution (Ortho 12-6-6) weekly.

The maize stocks used were from lines treated with the mutagen EMS, and most were previously selected as necrotic or seedling lethal. The method of mutant induction and the genetic handling of the material have been described by Neuffer (22). Briefly, it consists of treating pollen with mutagen, pollinating, selfing all the progeny of treated plants to make any mutation homozygous, and examining the resulting plants.

Mutant Selection. Suspected mutant plants from the above maize stocks were screened for inactivation of photosynthesis by a technique described elsewhere (20). The procedure is a modification of that used for green algae (4, 9), and selects for plants with abnormally high Chl fluorescence.

Chloroplast Isolation. Twice-washed leaves of maize seedlings were macerated in a chilled TenBroeck glass homogenizer. The grinding medium contained 0.8 M sucrose, 0.01 M NaCl, 0.02 M Tricine buffer (pH 7.8), and 1 mg of defatted bovine serum albumin. The resulting homogenate was strained through fiberglass cloth, and chloroplasts were isolated from the filtrate by a standard centrifugation procedure (18) at 4 C. After isolation, chloroplasts were suspended in the grinding mixture, minus albumin. The final concentration of Chl in the chloroplast suspension was determined using the absorption coefficients of MacKinney (16).

Carbon Dioxide Fixation. Photosynthetic reduction of CO2 by whole plants was determined by infrared gas analysis (Beckman 215A) using a two-pump open system (24). Plants were illuminated at 1 x 10⁶ ergs·cm⁻²·sec⁻¹ in 1-liter glass chambers placed in a controlled environment room for temperature regulation. The amount of CO₂ fixed was always calculated on a leaf area basis to conserve plant material for other studies.

Fluorescence Measurements. The kinetics of fluorescence induction in isolated maize chloroplasts were recorded at room temperature with a filter fluorometer. A 1-cm square, quartz fluorescence cuvette was exposed to exciting light from a tungsten lamp. A Rohm and Haas 2045 blue acrylic filter was used which provides light at a peak wavelength of 465 nm. The intensity as measured with a Yellow Springs-Kettering radiometer was 5 x 10⁵ erg·cm⁻²·sec⁻¹. Fluorescent emission was measured at a right angle to the exciting beam with an EMI 9558C photomultiplier tube (S-20 cathode). The tube was blocked with a Baird-Atomic 680 nm narrow band width (10 nm) interference filter. The anode signal of the tube was amplified by Philbrick operational amplifiers and either viewed on an oscilloscope or recorded with a Houston 2000, X-Y plotter.

For fluorescence measurements, isolated chloroplasts containing 35 to 50 μg of Chl were suspended in 3 ml of 0.2 M sucrose, 5 mM NaCl, and 10 mM Tricine buffer (pH 7.8).

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2 Abbreviations: EMS: ethyl methanesulfonate; DCIP: 2,6-dichlorophenolindophenol.
Where indicated DCMU was added to a final concentration of 1 μM.

An assessment of the influence of photosystem I on the fluorescence transient was accomplished by measuring the ability of a far red light (710 nm) to lower the initial fluorescence level. This was performed by orienting a second light beam (in addition to the exciting blue light), on the cuvette from the side opposite the photodetector. The second beam was provided by an Instrumentation Laboratories 330-cm grating monochromator at 710 nm with a light intensity of 2 × 10⁵ erg cm⁻² sec⁻¹.

**Oxygen Evolution.** Photosynthetic oxygen evolution was measured at 10 C with a Clark electrode (Yellow Springs-Kettering 4044) using isolated chloroplasts in a previously described apparatus (19). The 5-ml reaction medium contained 250 μmole of Tricine (pH 7.8), 150 μmole of NaCl, 12 μmole of MgCl₂, and maize chloroplasts with 100 μg of Chl. Either 10 μmole of potassium ferricyanide, or 0.5 μmole of DCIP, was added as the electron acceptor.

**Photo reductions.** Light-dependent reduction of methyl viologen by maize chloroplasts was measured by monitoring oxygen uptake with the described Clark electrode technique (19). The reaction mixture (5 ml) was identical to that used for oxygen evolution except that 1 μmole of methyl viologen and 2.5 μmole of NaN₂ were included. The electron donor was either water or ascorbate-reduced DCIP (0.4 μmole) as indicated.

Photo reduction of NADP as ascorbate-reduced DCIP as the electron donor was performed exactly as reported for maize by Bishop et al. (5). Spinach ferredoxin was obtained from Sigma Chemical Co. NADP absorbance change at 340 nm was measured with a Beckman DK2-A spectrophotometer.

Ferricyanide photoreduction was followed by the sensitive assay for ferrocyanide production used by Avron and Shavit (2). The resulting absorbance at 535 nm was read on the Beckman DK2-A.

For chloroplast photoreduction of DCIP, the standard procedure involving decreased absorbance at 580 nm was used. The time course of this absorbance change was recorded on a Beckman DK2-A equipped for side illumination of the sample cuvette. The reaction mixture and procedure used were described elsewhere (21).

**NADPH Diaphorase.** Enzyme activity was assayed with DCIP as the electron acceptor by the absorbance decrease at 620 nm. Time course for the change was measured with a dual-beam spectrophotometer. The procedures for assay and enzyme extraction were those of Avron and Jagendorf (1). Purification was carried through the acetone precipitation step. The 3-ml reaction mixture contained 16 mm tris-HCl (pH 8.5), 100 μmole NADPH, 30 mm MgCl₂, DCIP for an absorbance change at 620 nm of about 1.5, and 1 ml of enzyme. Protein was determined by the method of Lowry et al. (15).

**Delayed fluorescence.** A syringe technique was used to measure the slow phase of the delay light emission with a modification of a described apparatus (17). A red-sensitive photomultiplier, EM1-9781R, was the detector. The signal was amplified by a Keithley 414A picoammeter and was followed by either an oscilloscope or a fast-response recorder. Chloroplasts were illuminated for 30 sec at an intensity of 1 × 10⁵ erg cm⁻² sec⁻¹ in a syringe before being injected into a cuvette in front of the photomultiplier.

**Electron Microscopy.** Leaf material from 10-day-old plants was fixed in 5% glutaraldehyde, 1.5% acrolein, 1.5% paraformaldehyde, and was stained with OsO₄. It was embedded in Epon and thin sections were examined on a RCA EMU-3G microscope.

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**RESULTS**

We report here characterization of three new mutants of maize which exhibit abnormally high Chl fluorescence. These are being designated hcf₁, hcf₂, and hcf₃, to denote high Chl fluorescence. Mutant hcf₁ and hcf₂, at this point appear to be the same type of general photosystem II block as reported in *Euglena* (26, 27), *Scenedesmus* (23) and *Chlamydomonas* (14). The mutant hcf₃, is unlike previously described algal mutants.

Along with several other high fluorescent mutants, these were selected from abnormal plants which arose from EMS treatment. The mutant genes show segregation and are carried as recessives by selfing normal sibs of the high fluorescent plants. Mutant seedlings grow normally until starch reserves are exhausted. At that time hcf₁, and hcf₂ are lethal. The hcf₃ mutant is not lethal but shows a marked decrease in growth rate following starch depletion. Under optimal growing conditions, hcf₁ may grow to 40 cm. Up to this time, growth and general morphology of all three mutants appears normal.

Therefore, before the first chlorosis appears (a withering and drying beginning from the leaf tip) homozygous mutants can only be differentiated from normal plants by their high level of leaf fluorescence as shown in Figure 1.

Chlorophyll was extracted from both high and normal fluorescent leaves by grinding with 10% acetone and was determined spectrophotometrically (16), or pigments were separated on sucrose columns (29) and absorption spectra were determined for individual pigments. Total Chl content varied from 2 to 2.5 mg/g fresh weight and Chl a/b ratios were from 2.8 to 3.1. No consistent pigment differences could be detected for the high fluorescent mutants.

When CO₂ fixation was measured by infrared gas analysis and comparisons made between high fluorescent plants and normal plants of the same family, the high fluorescent plants fixed little or no CO₂. The rate of photosynthesis in mg CO₂ fixed per sq. cm of leaf per hr was 18 for the wild type, 0.92 for hcf₁, 0.33 for hcf₂, and 0.0 for hcf₃. Thus, hcf₁, and hcf₂ did not significantly fix CO₂ over respiration rates, but hcf₃ usually compensated respiration even though it was much reduced from the normal rate. Each gas-analysis chamber contained three to five plants and the data represents a combined average of three experiments.

Because all mutants showed high levels of fluorescence and

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![Fig. 1. Comparison of leaf fluorescence from mutant and normal maize. hcf₁: center; hcf₂: left; hcf₃: right. For each a high fluorescence (light colored) and normal plant (very dark) are shown. Fluorescence was activated by a long wave ultraviolet lamp and photographed through a red cut-off filter (610 nm).](attachment:figure1.jpg)
did not normally fix CO₂, it seemed plausible that they were limited in the light reaction of photosynthesis. Next we examined the fluorescent transients to see generally where in the light reaction electron transport might be blocked.

Chloroplasts were isolated from mutant or normal leaves and suspended at the same concentration of Chl. The induction of fluorescence was measured after dark adaption of the chloroplasts as indicated in "Materials and Methods." The maximum level of fluorescence ($F_{\text{max}}$) for the mutants was two to three times higher than normal chloroplasts (Fig. 2). However, when fluorescence kinetics of the mutant chloroplasts were examined, there were two different types. The fluorescence of hcf₂ (Fig. 2B) resembled wild type, with a fast rise to the initial level ($F_0$) followed by a slower increase to $F_{\text{max}}$. Also in hcf₂ chloroplasts, as with normal chloroplasts, there was a large increase in the level of $F_0$ following DCMU treatment (broken lines, Fig. 2). These two results indicate that hcf₂, like wild type, has a large variable fluorescence but the mutant has a higher over-all yield of fluorescence on a Chl basis. In contrast to these results, chloroplasts prepared from hcf₁ and hcf₃ mutants showed little or no variable fluorescence (Fig. 2, C and D) as is typical for photosystem II mutants of algae. $F_0$ was nearly the same as $F_{\text{max}}$ and DCMU treatment had little or no effect on $F_0$. The fluorescence tracing for hcf₃ was unchanged from that indicated in Figure 2D following addition of DCMU. However, hcf₃ always had a 5 to 10% change in $F_0$ after DCMU treatment. Therefore, variable fluorescence is missing in hcf₁ and severely reduced in hcf₃ chloroplasts.

A comparison was made between dark reoxidation of Q and reoxidation in the presence of photosystem I light. Figure 3 shows that 710 nm of light increased the rate of Q reoxidation over the dark rate of normal chloroplasts. This reoxidation was inhibited by DCMU (data similar to hcf₈ in Fig. 3). There was, of course, dark reoxidation with hcf₁ chloroplasts but photosystem I light did not increase the rate further. Even though hcf₈ chloroplasts reoxidized Q only to a small extent, there was a definite and reproducible increase in the rate of reoxidation in 710 nm light. There was no reoxidation of Q in light or dark with hcf₈ chloroplasts. This lack of change for hcf₈ was similar to DCMU-inhibited chloroplasts.

The above procedures measure light-induced change of the oxidation-reduction state of Q, and relative function of photosystem I or II, but in addition we measured the functional amount of Q and the secondary pool of electron acceptors by chemical oxidation or reduction. A comparison was made between the usual mutant fluorescent induction curve and those in the presence of potassium ferricyanide or sodium hydrosulfite (Fig. 4). Ferricyanide oxidizes Q and lowers the level of fluorescence while hydrosulfite reduces Q and increases the fluorescence yield. This response is seen with wild type, with hcf₁, and to a very limited extent with hcf₃. However, hcf₈ showed essentially no functional electron acceptor which could be oxidized or reduced.

A characteristic of photosystem II mutants is the lack of delayed light emission (10). When delayed light was measured, as outlined in "Materials and Methods," using chloroplasts from mutant plants, those which appear to be photosystem II mutants, hcf₃ and hcf₆, showed very low levels of delayed...

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**Fig. 2.** Induction of fluorescence in isolated maize chloroplasts from normal and high fluorescence mutants. A: wild type chloroplast; B: hcf₁; C: hcf₂; D: hcf₃. In each, the upper solid line represents mutant chloroplasts and the lower one represents low fluorescence chloroplasts from the same family. The broken line shows the effect of adding DCMU. ↓: light on; ↑: light off.
light (Fig. 5). The mutant hcf₁, on the other hand, had a normal or even enhanced level of delayed light.

In viewing all data of fluorescence induction, delayed light, the effects of photosystem I light, or oxidizing and reducing agents on fluorescence induction, we can separate the high fluorescence mutants into those abnormal in photosystem II or those abnormal in photosystem I. The mutant hcf₁ appears to have normal photosystem II but limited photosystem I inasmuch as far red light does not increase the rate of Q reoxidation over dark controls. The mutant hcf₃ has an abnormal but not completely blocked photosystem II and a functional photosystem I. The mutant hcf₂ appears to be completely blocked in photosystem II function.

With these data from fluorescence induction, we proceeded to conventional assay methods for photosynthetic electron transport.

Chloroplasts of hcf₁ plants evolved O₂ at a normal rate with either potassium ferricyanide or DCIP as the electron acceptor; however, only very small amounts of O₂ could be measured with hcf₂ or hcf₃ chloroplasts (Table I). Further, when photoreduction of these electron acceptors as well as methyl viologen was measured, hcf₁ reduced all three dyes but hcf₂ and hcf₃ chloroplasts showed zero to low rates. Since hcf₁ could carry on these photoreductions and oxygen evolution, it has a functional photosystem II. The mutants hcf₂ and hcf₃ did not perform these reactions, and could be considered devoid of photosystem II function.

When methyl viologen reduction was measured with ascorbate-reduced DCIP as the electron donor to bypass photosystem II, all high fluorescent mutants carried on good rates of electron transport (Table I). Since all mutants reduced methyl viologen from the electron donor system, they must have functional photosystem I up to the point at which methyl
viologen accepts electrons from the chain. To test the remaining portion of the photosystem I electron transport chain from the site where methyl viologen accepts electron, we measured NADP photoreduction. Photosystem II involvement was again eliminated by using ascorbate-reduced DCIP as an electron donor and DCMU. In this reaction, hcf reduced NADP at a near normal rate, while hcf always showed a rate of about 50% of wild type (Table I).

These data agree with the fluorescence induction results that hcf is limited in photosystem I electron transport while hcf is blocked in photosystem II. Further they suggest that hcf, has an inactive NADP reductase enzyme system.

The NADP reductase has other enzyme activities when isolated; therefore, if its activity is reduced, a reduction in NADPH diaphorase should be detectable (I). Figure 6 shows the decrease in absorbance associated with DCIP reduction which is the standard electron acceptor for NADPH diaphorase. The mutant hcf, showed a 50% rate of normal maize. In this figure, enzyme activity is compared on a leaf fresh weight basis. When compared on a protein basis, hcf had 37% of control activity (data not shown).

The structure of chloroplasts in mutant leaves was examined and compared to low fluorescent sibs. Figure 7 shows representative electron micrographs of granal chloroplasts from mesophyll and agranal chloroplasts from bundle sheath cells. The nonlethal hcf mutant has essentially normal chloroplasts in both cell types, but there may be some separation of membranes in the agranal bundle sheath chloroplasts (arrow, Fig. 7B). The mutant hcf has the maximum alteration of structure. There were gross separations of lamellae in bundle sheath chloroplasts and abnormal stacking of membranes in mesophyll chloroplasts even though whole leaf pigmentation appeared normal. The mutant hcf has quite normal-appearing bundle sheath chloroplasts but lacked proper grana stacking in mesophyll chloroplasts both in size and compactness (arrow, Fig. 7D). This situation is considered usual for photosystem II mutants (12).

**DISCUSSION**

Three high fluorescent mutants of maize have been isolated and their photosynthetic electron transport reactions have been characterized. All possess normal pigmentation but do not fix CO$_2$ at full rates.

hcf. This mutant has high fluorescence yield and normal two-phase fluorescence induction. Large changes in fluorescence can be seen in response to oxidizing or reducing agents, and chloroplasts have normal levels of delayed light. Good rates of oxygen evolution and Hill acceptor reduction can be measured. From these data, hcf must have a functional photosystem II. The mutant hcf, also reduces methyl viologen, which indicates that electron transport on the reducing side of photosystem I is operational through the ferredoxin-reducing substance (30) step. However, this mutant reduces NADP at low rates even in the presence of spinach ferredoxin, and it has a much reduced rate of NADPH diaphorase activity. Therefore we suggest that the hcf, mutation is due to limited ferredoxin-NADP oxidoreductase activity. We did not detect a complete block in electron transport which would be expected because the mutation is not a seedling lethal. It has grown to 40 cm but has not reproduced. It is interesting to note that a limitation of electron transport this far from Q

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**Fig. 5.** Chloroplast delayed fluorescence emission from wild type or high fluorescence mutants of maize.

**Fig. 6.** Comparative rate of NADPH diaphorase activity in enzyme extracts from hcf mutant and normal maize leaves. Absorbance change at 620 nm due to DCIP reduction is plotted versus time.

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**Table 1. Photosynthetic Electron Transport Reactions in Wild Type and High Fluorescent Mutants of Maize**

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Electron Acceptor</th>
<th>Electron Donor</th>
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<th>hcf,</th>
<th>hcf,</th>
<th>Wild Type</th>
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<td>157</td>
<td>4</td>
<td>2</td>
<td>186</td>
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<td>40</td>
<td>4</td>
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<td>113</td>
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<tr>
<td></td>
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<td>11</td>
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<td>122</td>
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can cause a high fluorescence yield. It is also possible that there are other blocks such as in photophosphorylation with this mutant.

A previously described mutation in *Vicia faba* also is blocked only in NADP reduction (11). It has normal photosystem II electron transport and cyclic photophosphorylation in photosystem I. However the *Vicia* mutant is different, having completely lost the ability to reduce NADP and having abnormal structure and pigmentation. Of the many mutants reported from the green algae (7, 14), none is blocked in NADP reduction.

**hcf**, **hcf**. These mutants are generally the same as the well known photosystem II mutants of algae such as the P, mutant of *Euglena* (26, 27), *Scenedesmus* No. 11 or 40 (23), and the acetate mutants of *Chlamydomonas*, ac-115 or ac-141 (14). The mutant hcf<sub>3</sub> is more completely blocked in electron transport than hcf<sub>2</sub>, and may have its effect at or very near the primary electron acceptor.

The mutant hcf<sub>3</sub> causes more chloroplast structural changes. Mesophyll plastids appear very similar to those reported from the *iojap* mutant of maize (25). Shumway and Weier (28) showed chloroplasts from green segments of leaves with *iojap*
have the same long parallel runs of lamellae (arrow, Fig. 7C), and generally a smaller, spherical appearance. This general shape and structure of the plastid is also seen in the pastel mutant of maize (3).

Chloroplasts of hcf, are more normal than those of hcf, but have the usual photosystem II mutant characteristic of small, less compact grana (12).

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LITERATURE CITED