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CO_2 Assimilation and Activities of Photosynthetic Enzymes in High Chlorophyll Fluorescence Mutants of Maize Having Low Levels of Ribulose 1,5-Bisphosphate Carboxylase[1]

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ABSTRACT

Photosynthetic properties were examined in several hcf (high chlorophyll fluorescence 11, 21, 42 and 45) nuclear recessive mutants of maize which were previously found to have normal photochemistry and low CO_2 fixation. Mutants usually either died after depletion of seed reserves (about 18 days after planting), or survived with slow growth up to 7 or 8 weeks. Both the activity and quantity of ribulose 1,5-bisphosphate carboxylase (Rubisco) were low in the mutants (5–25% of the normal siblings on a leaf area basis) and the loss of Rubisco tended to parallel the reduction in photosynthetic capacity. The Rubisco content in the mutants was often marginal for photosynthetic carbon gain, with some leaves and positions along a leaf having no net photosynthesis, while other leaves had a low carbon gain. Conversely, the activities of C_4 cycle enzymes, phosphoenolpyruvate carboxylase, pyruvate,Pi dikinase, NADP-malate dehydrogenase, and NADP-malic enzyme, were the same or only slightly reduced compared to the normal siblings. The mutants had about half as much chlorophyll content per leaf area as the normal green plants. However, the Rubisco activity in the mutants was low on both a leaf area and chlorophyll basis. Low Rubisco activity and lower chlorophyll content may both contribute to the low rates of photosynthesis in the mutants on a leaf area basis.

Mutant plants are useful tools for studies on photosynthesis. In maize, nuclear recessive lethal mutants of photosynthesis have been isolated which have high Chl fluorescence (hcf).[3] These can be studied while growing on seed reserves, and stocks maintained by selfing heterozygotes (10, 11). Miles (11) divided the hcf mutants of maize into five general groups based on various types of mutations in the photosynthetic machinery. One of these, which is not well characterized, is composed of mutants which appear to have normal light reactions, including electron transport and photophosphorylation, but low CO_2 assimilation. This group was classified as carbon dioxide fixation mutants with normal photochemistry (11).

Although the primary lesions in the mutants have not been identified, preliminary analyses indicated hcf 11, 21, and 42 had low Rubisco protein content (25–36% of normal siblings based on percentage of total protein, activity not determined) (13). The present study was aimed at further characterizing several mutants of this group (hcf 11, 21, 42, and 45). We have examined the photosynthetic capacity, Rubisco activity and protein content, and the activities of several C_4 pathway enzymes in these mutants.

MATERIALS AND METHODS

Plant Material. *Zea mays* L. seeds were kindly provided by D. Miles, University of Missouri, St. Louis. These seeds, obtained from selfed heterozygotes, provided normal siblings (including both wild-type and heterozygotes) and the homozygous mutants. The homozygous mutant seedlings were detectable by a yellow green phenotype and/or low photosynthesis. The nuclear recessive mutants studied were hcf 11 (family 1305-33), hcf 21 (family 1307-3, 1307-6), hcf 42 (families 1207-8, 1309-33, 1309-42), and hcf 45 (family 3:45-3). Hcf 21 (chromosome arm 5L) and 42 (chromosome arm 9L) are located on different chromosome arms (12), and are presumably nonallelic.

Growth Conditions. Plants were grown either in a greenhouse or growth chamber as specified in the tables and figures. The greenhouse had a maximum temperature of 26°C and a minimum temperature of 22°C. Direct sunlight measured inside the greenhouse on sunny days in September and October was approximately 1000 to 1700 μmol quanta m^{-2} s^{-1}. The growth rooms, which contained fluorescent lamps, were programmed at 25°C and a photoperiod of 20 h light and 4 h dark. A long photoperiod was used in an effort to extend the survival of mutants which had limited capacity for photosynthesis. For the experiment of Table I, plants were grown in plastic pots in vermiculite. Otherwise, peat pots (5 × 5 cm) were used with one seedling per pot. A "compost" was used which contained 10X compost, 1X peat, 1X perlite/vermiculite/river sand and approximately 1.5 g fertilizer (Aboska)/kg.

Photosynthesis Measurements. The rate of photosynthesis was measured in a growth room at approximately 25°C with an ADC gas analyzer (LCA2) in an open system with a clamp on leaf cuvette (Anri Instruments and Controls, Melbourne, Australia). The leaf chamber contained a small fan and was 25 × 25 mm (6.25 cm^2). CO_2 was provided either from a tank containing 338 μl/L or by mixing CO_2-free air and 0.1% CO_2 for the desired concentration. The flow rate was either 200 or 400 ml/min. Plants had been preilluminated for several hours under growth chamber lights prior to making measurements on photosynthesis. With a

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[3] Abbreviations: hcf, high chlorophyll fluorescence; PEP, phosphoenolpyruvate; Rubisco, ribulose 1,5-bisphosphate carboxylase oxygenase.

single leaf in the cuvette, measurements were made after steady-state photosynthesis was reached, usually less than 10 min after insertion of the leaf. Measurements were made on the CO_2 differential, temperature and percent humidity in the chamber, and on the light intensity, using a built-in quantum sensor which was adjacent to the leaf chamber. The light intensities (see tables and figures) were provided by fluorescent lights in the chamber plus a 150 W incandescent lamp. Different light intensities were obtained by altering the height of the incandescent lamp and using layers of nylon screens. A crystalyzing dish containing 3 to 4 ml of water was mounted above the leaf cuvette to minimize the heat from infrared light. The rate of photosynthesis was calculated according to von Caemmerer and Farquhar (17), including correction for dilution of CO_2 by water vapor from transpiration, except for the data in Table I. The intercellular CO_2 (see Fig. 3) was calculated according to methods outlined in the ADC infrared gas analyzer manual for calculating leaf temperature and according to equations of von Caemmerer and Farquhar (17).

Sampling of Leaves and Extraction for Enzyme Assays and Assay of Chl. A 25 mm longitudinal section of the leaf (as specified in the results) was marked lightly with a red felt pen and the average width of the leaf measured. The leaf was inserted for gas exchange measurements so that the marked section was clamped in the chamber. Afterward, the leaf was removed from the cuvette, and the marked section was cut out with scissors, placed in a small glass vial (20 ml), and immediately immersed in liquid N_2. A cap was loosely screwed on the vial, which was then stored in a −80°C freezer until sampling for enzyme assays and Chl content. The time from removal of the leaf from the cuvette to killing the section in liquid nitrogen was about 10 s.

The frozen leaf tissue was added to a prechilled mortar with 0.1 ml of grinding medium. The grinding medium contained 25 mM Hepes pH 7.4, 5 mM DTT, 5 mM $MgCl_2$, 0.5% BSA, and 1 mM pyruvate. After grinding for 30 to 40 s, an additional 0.5 ml of grinding medium was added. The extract was added to a microfuge tube and a 50 μl aliquot was taken for Chl determination. The tubes were centrifuged in an Eppendorf microfuge at full speed for 30 s. The supernatant fraction was used for enzyme assays.

Enzyme Assays, Rubisco Protein, and Chl Determination. For the assay of NADP-malate dehydrogenase and pyruvate,Pi dikinase, the activity was determined following extraction and after incubation at 30°C for approximately 120 min under activating conditions (for activation of NADP-malate dehydrogenase, an additional 20 mM DTT was added; for activation of pyruvate,Pi dikinase 2 mM Pi was added to an aliquot of the extract). In most treatments, there were substantial activities in the initial extract; the maximum activities are reported. Experiments were not performed to determine the capacity for *in vivo* light activation (light *versus* dark treatments followed by immediate extraction and assay without storage of leaf samples).

The enzymes were assayed in a temperature controlled room at approximately 25°C according to the following procedure: NADP-malate dehydrogenase (15) except 5 mM DTT was included in the assay mix, NAD malate dehydrogenase (9), pyruvate,Pi dikinase (8), PEP carboxylase (6), NADP-malic enzyme (2), and aspartate aminotransferase (5).

For phosphoribulokinase the assay mixture contained 25 mM Hepes-KOH (pH 7.8), 40 mM KCl, 8 mM $MgCl_2$, 4 mM DTT, 1 mM ATP, 1 mM PEP, 2 mM ribose 5-P, 0.25 mM NADH, ribose 5-P isomerase (4 units ml^{-1}), pyruvate kinase (2 units ml^{-1}) and lactate dehydrogenase (1 unit ml^{-1}). The observed rates for phosphoribulokinase were corrected for a background rate (due to PEP carboxylase) in the absence of ribose 5-P. The assay for NADP-glyceraldehyde 3-P dehydrogenase contained 25 mM Hepes-KOH (pH 8.1), 8 mM $MgCl_2$, 6 mM DTT, 2.5 mM glycerate 3-P,, 1.25 mM ATP, 5 mM creatine-P, creatine phosphokinase (3 units ml^{-1}), 0.25 mM NADPH, glycerate 3-P kinase (10 units ml^{-1}), and triose-P isomerase (10 units ml^{-1}). For assay of glycerate 3-P kinase the assay mixture contained 25 mM Hepes-KOH (pH 7.8), 5 mM DTT, 5 mM $MgCl_2$, 1 mM NaF, 1 mM KH_2PO_4, 1.25 mM glycerate 3-P, 1 mM ATP, 5 mM creatine-P, 0.25 mM NADH, creatine phosphokinase (3 units ml^{-1}), glyceraldehyde-P dehydrogenase (3 units ml^{-1}), and triose-P isomerase (6 units ml^{-1}).

Rubisco was assayed spectrophotometrically in a medium containing 25 mM Tricine (pH 8.3, 10 mM KCl, 1 mM EDTA, 15 mM $MgCl_2$, 5 mM DTT, 0.2 mM NADH, 5 mM ATP, 20 mM $NaHCO_3$, 5 mM P-creatine, 15 units ml^{-1} glycerate 3-P kinase, 6 units ml^{-1} NAD-glyceraldehyde 3-P dehydrogenase, 15 units ml^{-1} triose-P isomerase, 5 units ml^{-1} glycerol-P dehydrogenase, 3 units ml^{-1} creatine-P kinase, 25 μg carbonic anhydrase, leaf extract (stored on ice) and 0.5 mM RuBP. The reaction was initiated by addition of RuBP (stoichiometry of the coupled assay system was 4 NADH oxidized per CO_2 fixed). In tests with normal and yellow green seedlings there was no increase in activity by preincubating extracts with 20 mM $NaHCO_3$, 10 mM DTT, and 20 mM $MgCl_2$ for 10 min at room temperature prior to assay. Initially, the coupling enzymes, which were stored in $(NH_4)_2SO_4$, were centrifuged for 5 min at full speed in an Eppendorf microfuge, following which the pellet was resuspended in 50 mM phosphate (pH 7.5), 1 mM EDTA, and desalted on Sephadex G-25 preequilibrated with the same medium. The mixture of coupling enzymes was brought to 20% glycerol and stored at −20°C.

Rocket Immunoelectrophoresis. Rubisco protein in the extracts was measured by rocket immunoelectrophoresis using a Bio-Rad model 1415 electrophoresis apparatus according to the instructions supplied by the manufacturer. The gels (100 × 100 × 1.5 mm) were prepared in a buffer, pH 8.6, containing 24 mM tricine, 81 mM Tris, 0.5 mM $CaCl_2$, 0.02% NaN_3, 1% agarose, and 0.5% rabbit antiserum raised against purified spinach Rubisco. Extracts and calibration standards of spinach Rubisco were diluted appropriately and 5 μl samples were applied to the wells. Electrophoresis was conducted for 4 h at 200 V. The gels were then blotted, rinsed, dried, stained with Coomassie brilliant blue, and the peak heights measured. The calibration plot was linear to 160 ng of spinach Rubisco.

Hill Reaction Activity. When the Hill reaction was to be measured, the leaf section was divided longitudinally into two equal parts along the midrib following gas exchange. One half was stored in a vial with a small amount of water and subsequently used for the Hill reaction assay, while the other half was killed in liquid N_2 and stored for enzyme assays. To assay the Hill reaction activity, the tissue was gently ground in a mortar with 0.2 ml of grinding medium containing 0.33 M sorbitol, 50 mM Hepes-KOH (pH 7.6), 10 mM KCl, 2 mM EDTA, 5 mM ascorbate (added fresh), 4 mM cysteine (added fresh), and 0.2% BSA. After addition of 0.4 ml of grinding medium the homogenate was filtered through an 88 μ net, and then centrifuged in an Eppendorf microfuge for 30 s at full speed. The supernatant pellet was discarded and the chloroplast pellet was resuspended in 100 μl of 0.33 M sorbitol, 50 mM Hepes-KOH (pH 7.6), 10 mM KCl, 2 mM EDTA, 1 mM $MgCl_2$, and 1 mM $MnCl_2$. A 25 μl aliquot was taken for Chl determination. The light dependent O_2 evolution was measured at 30°C with a Rank Brothers Clark-type O_2 electrode in a reaction volume of 1 ml containing the chloroplast resuspension medium plus 3 mM ferricyanide. After initiating the reaction by addition of 75 μl of chloroplasts, 5 mM NH_4Cl was added after 1 to 2 min of illumination and the maximum rate of O_2 evolution determined. Preliminary experiments showed that rates with sorbitol in the assay medium were as high or higher than in the absence of sorbitol which suggests chlo-

roplasts isolated by this means did not have an intact envelope which would exclude ferricyanide.

Chl was determined by the method of Arnon (1).

RESULTS

Hcf 21 is a virescent mutant (D Miles, personal communication) and in the present study the plants were grown with low light intensities to decrease photooxidative damage, and under a 20 h photoperiod in order to maximize any potential for carbon gain and to minimize losses of carbon from dark respiration (rates of dark respiration measured during our study were about 0.5 to 2 μmol m^{-2} s^{-1} with the higher rates occuring in normal siblings). There was increased greening as the mutants developed and they either died two to three weeks after planting or lived and grew slowly for 5 to 6 weeks. One mutant having no net photosynthesis as a 12 d old seedling, had a rate 25% of normal after 19 d, and showed substantial growth over a three month period (tassling with ear formation). This indicates the mutation causes a slowing of development and that mutants can survive provided that they can develop sufficient photosynthetic capacity before exhausting their seed reserves.

The rate of photosynthesis and activities of several photosynthetic enzymes were measured on the primary leaf of 12 d old seedlings of hcf 21 (Table I). The mutants had about half the Chl content of normal siblings. There was no net photosynthesis in the mutant seedlings. The activity of Rubisco in mutant plants on a leaf area basis was only 17% of normal, while on a Chl basis the activity was 33% of the normal seedlings. Three other enzymes of the reductive pentose-P pathway were examined. There was a moderate reduction in the activity of phosphoribulokinase on a leaf area basis in the mutant seedlings, while the activities of glycerate 3-P kinase and NADP-triose-P dehydrogenase were equivalent to, or higher than, that of the normal seedlings. Despite the mutant having only half as much Chl and no net photosynthesis, the activities of five C_4 cycle enzymes (NADP-malic enzyme, PEP carboxylase, pyruvate,Pi dikinase, NADP-malate dehydrogenase and aspartate aminotransferase) on a leaf area basis were equivalent to, if not somewhat higher than, the normal activities. With the exception of Rubisco, the activities of all enzymes assayed were higher in the mutants on a Chl basis. Therefore, among the parameters measured, the lack of photosynthesis was only associated with low Rubisco activity.

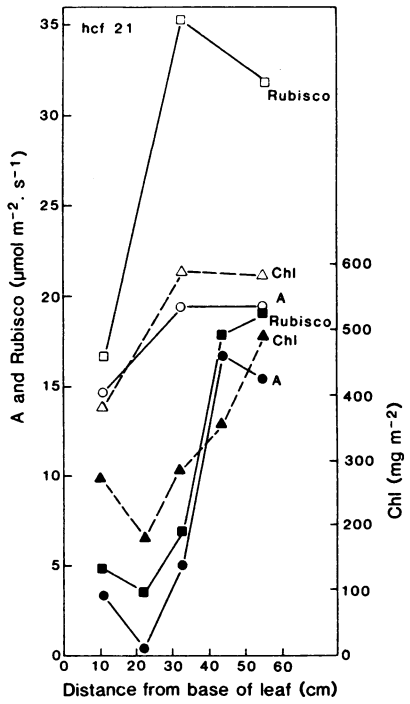

FIG. 1. The influence of age along the leaf on photosynthesis (A), Chl content, and Rubisco activity in an hcf 21 mutant (closed symbols) compared to a normal sibling (open symbols). Plants were 2 months old, measurements were made on the 13th leaf from the base, and each leaf was 65 cm long. The light intensity was 1000 μmol quanta m^{-2} s^{-1}. The CO_2 partial pressure in the leaf curvette for the individual measurements was 285 to 314 μbar and the temperature in the cuvette was 30.1 to 33.0°C.

Table I. *Photosynthesis and Enzyme Activities of hcf 21 Mutants and Normal Siblings of Maize*

The rate of photosynthesis was measured under 975 μmol quanta m^{-2} s^{-1}, over a range of 277 to 307 μbar of CO_2 and at a cuvette temperature of 22.9 to 25.9°C in individual experiments. The data are the average from two separate seedlings. Photosynthesis rates (in this case not corrected for dilution of CO_2 by water vapor from transpiration, which will cause a slight overestimate of the rate) and enzyme activities were measured on the same leaf segment. Measurements were made on the primary leaf of 12 d old plants grown in a growth room in vermiculite. The average Chl content was 299 mg m^{-2} for normal siblings and 156 mg m^{-2} for the mutants.

Measurement	Normal	Mutant	Normal	Mutant
	(μmol $m^{-2}s^{-1}$)		(μmol mg Chl^{-1} min^{-1})	
Photosynthesis rate	9.1	0	1.8	0
Rubisco activity	11.4	2.0	2.3	0.77
Phosphoribulokinase	114.3	73.7	22.9	28.3
Glycerate 3-P kinase	286.6	342.6	57.5	131.7
NADP-triose-P dehydrogenase	57.3	67.1	11.5	25.8
NADP-malic enzyme	63.7	70.4	12.8	27.1
PEP carboxylase	57.4	122.4	11.5	47.1
Pyruvate,Pi dikinase	18.2	24.6	3.7	9.5
NADP-malate dehydrogenase	111.8	124.1	22.4	47.7
Aspartate aminotransferase	20.4	32.7	4.1	12.6
NAD-malate dehydrogenase	257.0	234.8	51.6	90.3

This was further examined by studying development along a single leaf. Using a viable hcf 21 mutant, the Chl content, rate of photosynthesis, and activity of Rubisco from the base towards the tip of the thirteenth leaf were measured, and compared with those of a normal sibling two months after planting (Fig. 1). Towards the tip of the leaf, the rate of photosynthesis and Chl content were only slightly lower in the mutant compared to the normal sibling. However, in the middle of the leaf and towards the base the mutant had a very low rates of photosynthesis and very low Rubisco activity. The Chl decreased from the tip towards the base, but less than the carboxylase and photosynthesis activities. The activities of the C_4 cycle enzymes NADP-malic enzyme, PEP carboxylase, pyruvate,Pi dikinase, and NADP-malate dehydrogenase were also determined. For each of these the activity along the leaf was quite similar, as were the activities between samples of the mutant and normal sibling (data not shown). The results show how the mutation delays the development of photosynthesis, Rubisco, and Chl in this mutant leading to a virescent phenotype.

The Chl content, photosynthesis rate, and activities of several enzymes were determined on growth chamber grown hcf 11, 21, 42, and 45 mutants of maize in comparison to their normal sib-

Table II. *Photosynthesis and Enzyme Activities of hcf 11, 21, 42, and 45 Mutants (M) versus Normal (N) Siblings of Maize*

The rate of photosynthesis was measured at near atmospheric levels of CO_2 (outlet from chamber was generally 265–319 μbar) and 1000 μmol quanta $m^{-2}s^{-1}$. The temperature inside the cuvette was 30.3 to 33.4°C among the different measurements. Photosynthesis rates and enzyme activities were measured on the same leaf segment. The number of replications are indicated in parentheses. Each replication represents a sample from a separate seedling except as otherwise mentioned. The age of plants and leaves sampled were as follows: hcf 11 (16 d after planting, measurements on the third leaf); hcf 21 (16 d after planting, measurements on the third leaf, two of the replications were 6 cm and 24 cm from the base of a leaf 24 cm long); hcf 42 (measurements on fourth and seventh leaves 34 d after planting or the second, third and fourth leaves 15 d after planting); hcf 45 (measurements on the fifth leaf of 34 d old plants). Plants were grown in a growth room, except part of the hcf 42 seedlings were grown for 12 d in a Greenhouse and transferred to the growth room 3 d prior to measurement.

Measurement	hcf 11		hcf 21		hcf 42		hcf 45	
	N (2)	M (2)	N (1)	M (3)	N (3)	M (5)	N (1)	M (1)
					mg m^{-2}			
Chl	357	180	241	172	351	230	281	153
					μmol $m^{-2}s^{-1}$			
Photosynthesis rate	26.0	4.5	25.4	10.5	26.9	5.2	27.1	0
Rubisco activity	26.8	5.1	19.8	9.7	26.3	6.5	33.6	1.7
NADP-malic enzyme	64.3	42.5	54.0	54.5	53.2	52.5	46.3	22.7
PEP carboxylase	62.4	49.6	49.0	38.4	57.3	68.6	33.7	19.6
Pyruvate,Pi dikinase	15.1	9.9	13.2	10.9	8.8	9.2	9.8	6.1
NADP-malate dehydrogenase	91.3	83.1	98.0	92.6	31.0	57.5	45.4	19.6
Hill reaction	16.7	3.9	11.2	7.7	10.1[a]	2.6[a]		
					μmol mg Chl^{-1} min^{-1}			
Photosynthesis rate	4.5	1.5	6.3	3.7	4.6	1.4	5.8	0
Rubisco activity	4.5	1.7	4.9	3.4	4.5	1.7	7.2	0.67
NADP-malic enzyme	10.8	14.3	13.4	19.0	9.1	13.7	9.9	8.9
PEP carboxylase	10.5	16.6	12.2	13.4	9.8	17.9	7.2	7.7
Pyruvate,Pi dikinase	2.5	3.3	3.3	3.9	1.5	2.4	2.1	2.4
NADP-malate dehydrogenase	15.3	27.7	24.4	31.8	5.2	15.0	9.7	7.7
Hill reaction	2.8	1.3	2.8	2.7	2.2[a]	0.68[a]		

[a] The Hill reaction was determined in a different experiment (two replications for normal siblings and three replications for mutants).

42, and 45 mutants of maize in comparison to their normal siblings (Table II). In this case, mutants of hcf 21 were evaluated which had developed some capacity for photosynthesis. There was a very similar pattern among all the mutants in that the loss of photosynthetic capacity on a leaf area or Chl basis was paralleled by a decrease in Rubisco activity. With four enzymes of the C_4 cycle, the activities in the mutants on a leaf area basis were generally equivalent to those of the normal siblings, or only partially reduced. In normal siblings the activities of pyruvate, Pi dikinase were substantially below recorded photosynthesis rates. This may in part be reconciled by the fact that photosynthesis was measured at substantially higher temperature than enzyme activity (30–33°C *versus* 25°C). The normal siblings of hcf 42 had relatively low activity of NADP-malate dehydrogenase compared to normal siblings of other groups. This was also observed in greenhouse grown plants. Also, the normal siblings of hcf 21 in Table II had higher rates of photosynthesis and Rubisco activity than those in Table I which may be due in part to differences between families (family 1307-6 in Table I and family 1307-3 in Table II) or the difference in leaf temperature during photosynthesis measurements.

Results of greenhouse grown plants of hcf 42 were similar to that of growth chamber plants, except the losses in Rubisco, Chl and photosynthesis were more pronounced in greenhouse grown mutants. On sunny days the light intensity in the greenhouse was 5 to 8 times higher than in the growth rooms. Compared to

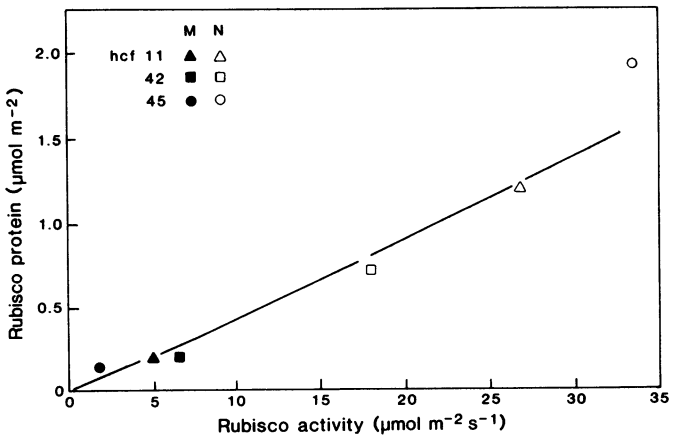

FIG. 2. The activity of Rubisco *versus* Rubisco protein in mutant and normal siblings of hcf 11, 42, and 45. Samples from hcf 11 and hcf 45 were as described in Table II. Samples of hcf 42 were from 40 d old growth chamber grown seedlings; measurements on the mutant were made on the sixth leaf, measurements on the normal sibling were made on the ninth leaf. N = normal, M = mutant.

normal seedlings (n = 5), the mutants (n = 9) had on average only 10% as much Chl, 2% of the rate of photosynthesis, 6% Rubisco activity, while the activities of C_4 pathway enzymes were 50% up to equivalent levels on a leaf area basis. These results indicate that, at least in hcf 42, growth conditions can influence the extent of effects caused by the mutation.

The activities of the four C_4 cycle enzymes in the hcf 45 mutant were lower than the normal sibling (Table II). However, in another mutant of this group which had higher photosynthetic capacity at the time of sampling (50% compared to wild type), the level of Rubisco was reduced by 40% while the activities of the C_4 cycle enzymes in the mutant, on a leaf area basis, were equivalent to those of the normal sibling (results not shown).

The Hill reaction measured on leaf extracts (Table II) was lower on a leaf area basis in the mutant seedlings of hcf 11, 21, and 42 than in the respective normal siblings (not measured in hcf 45). In hcf 21 the Hill reaction activity was similar on a Chl basis in the mutant and normal seedlings. However, the Hill reaction was also lower on a Chl basis in hcf 11 and 42 mutants compared to the normal siblings and this, along with Rubisco activity, may contribute to the lower rate of photosynthesis on both a leaf area and Chl basis.

Since all these mutants had markedly lower activities of Rubisco, the relationship between the activity and quantity of Rubisco protein was determined for mutant and normal siblings (Fig. 2). The low activity of Rubisco in the mutant seedlings was paralleled by a low level of Rubisco protein. Therefore, there was no evidence that the mutants were making inactive enzyme. Since the protein content was determined by rocket immunoelectrophoresis using antibody derived against spinach Rubisco, an absolute kcat value for the maize enzyme was not calculated.

To further define the photosynthetic characteristics of some of these mutants, the effects of varying light intensity and pCO_2 were examined. The light response curves for photosynthesis in mutant hcf 21 relative to normal plants are shown in Figure 3. In the mutant, measurements were made from the base toward the tip of the sixth leaf. At 12 cm from the base, no carbon was assimilated in the light in the mutant since the net rate of CO_2 loss was the same in the light and dark. At 14.5 cm from the base, the rate of photosynthesis was just sufficient to compensate for dark respiration, there was no net gain in carbon, and the rate saturated at about 100 μmol quanta m^{-2} s^{-1}. Further from the leaf base the rate of photosynthesis was higher and saturated at 500 μmol quanta $M^{-2}s^{-1}$. There was good agreement between the maximum increase in photosynthesis with increasing light intensity in the mutant leaves and the extractable activity of Rubisco (see figure legend). Even though the mutant, at 19.5 cm from the base of the leaf, and the normal sibling had similar Chl content, the rate of photosynthesis was much lower in the mutant, as was the Rubisco activity (see figure legend). In this case, the initial slopes at low light were similar, indicating there was little difference in the maximum efficiences of utilizing light. This suggests the mutant has similar photochemical efficiency, and that the limitation on photosynthesis under high light is due to the lower Rubisco activity. However, toward the base of the leaf the initial slope of the light response curve in the mutant was much lower, which indicates the capacity to utilize light photochemically was reduced along with the reduction in Chl content. The low light saturation of the normal seedling may have been due to loss of vigor from selfing of heterozygotes, since some other normal siblings showed higher light saturation responses. Other measurements on maximum rates of photosynthesis in normal plants showed the rates along the leaf were within 75% of the maximum rate obtained on a leaf area basis (Fig. 1 and data not shown), in sharp contrast to results with the mutant.

Light responses for photosynthesis were also measured on an

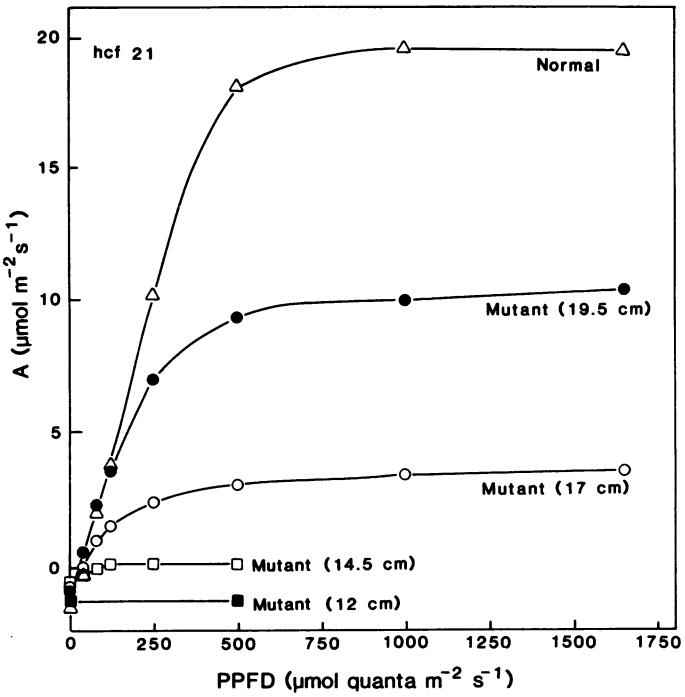

FIG. 3. The influence of light intensity on photosynthesis (A) in an hcf 21 mutant *versus* a normal sibling. Measurements were made on the sixth leaf of 6 week old plants. The values in parentheses indicate the distance (cm) from the base of the leaf on which measurements were made on the mutant plant (leaf approximately 44 cm long). The light response of the normal sibling was in the middle of the leaf (22 cm from the base). The outlet pCO_2 from the cuvette among the individual measurements was 250 to 319 μbar, and the temperature in the cuvette was 29.6 to 32.6°C. The Chl in the samples was 311 in normal, and 121, 133, 222, and 297 mg m^{-2} in the mutant leaf, progressing from the base toward the tip. The Rubisco activities in the leaf samples were 52 in the normal and 0.8, 1.6, 4, and 8 μmol m^{-2} s^{-1} in the mutant, progressing from the base toward the tip of the leaf.

hcf 42 mutant, using several leaves at various stages of development, and compared with leaves of a normal plant. In the mutant the rate of photosynthesis was very low at three different leaf positions and it tended to reach a maximum at 125 to 250 μmol quanta m^{-2} s^{-1} (Fig. 4a). In the more mature second leaf of the normal sibling, photosynthesis reached a high rate and responded up to near full sunlight. However, in the fourth leaf, which was just emerging and had about half the Chl content, the rate of photosynthesis was much lower and reached near saturation at much lower light intensity. The fourth leaf of the normal sibling had about half the Chl content and half as much Rubisco activity compared to the second leaf (see figure legend). The Chl content of the fourth leaf of the normal plant was similar to that of the second, third and fourth leaves of the mutant. However, the activities of Rubisco in the mutant leaves were only 30 to 40% of that in the emerging fourth leaf of the normal sibling. The second, third and fourth leaves of the mutant had lower initial slopes of photosynthesis *versus* light intensity than the fourth leaf of the normal siblings, which suggests a lower photochemical efficiency in the mutants. Both low Rubisco and lower capacity for photochemical generation of energy likely limit photosynthesis in these mutants.

The response of photosynthesis to intercellular pCO_2 was examined in mutant seedlings of hcf 42 (Fig. 4b). Although difficult to determine accurately, the carboxylation efficiency at very low levels of CO_2 appeared similar in the normal and mutant seed-

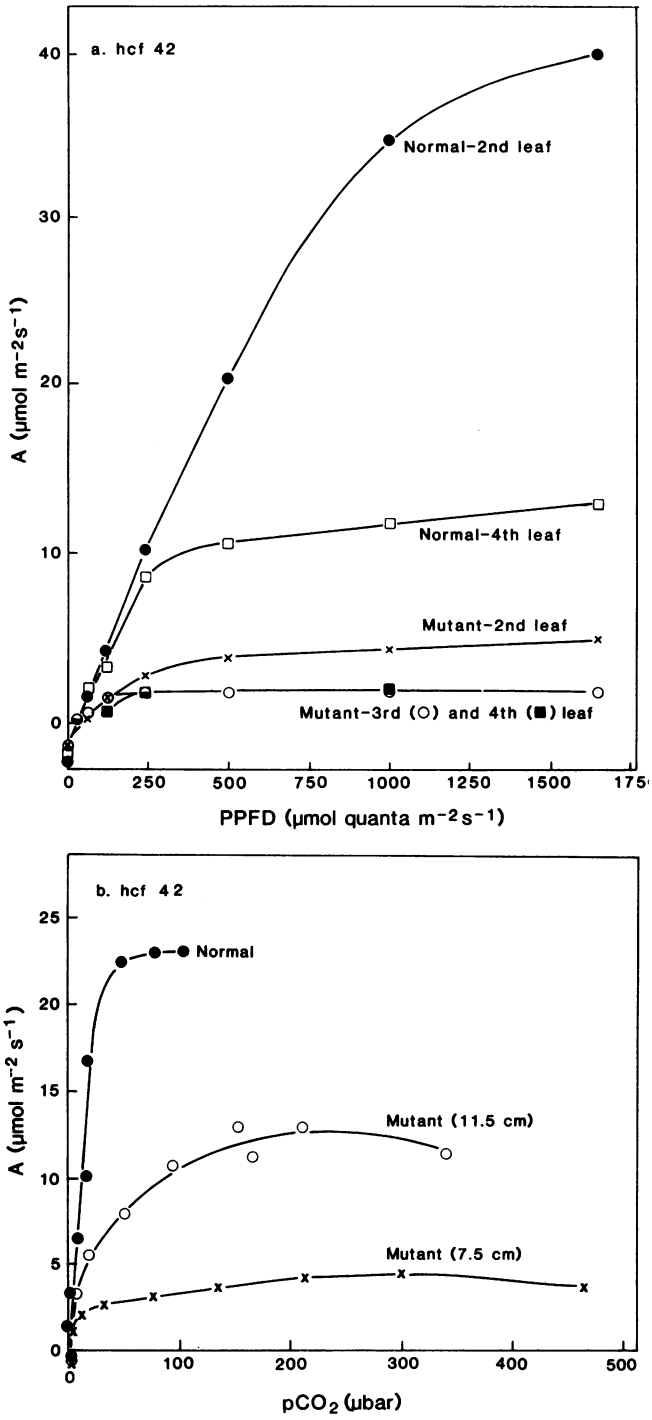

FIG. 4. Photosynthesis (A) in an hcf 42 mutant versus normal sibling. a, The influence of light intensity. The temperature range in the cuvette between individual measurements was 27.5 to 31.4°C. The outlet pCO_2 from the cuvette was 303 to 319 μbar with the mutant and 205 to 319 μbar with the normal sibling. The activities of Rubisco were 24 and 11 μmol m^{-2} s^{-1} in the second and fourth leaf of the normal sibling, and 4.5, 3.3, and 4.0 μmol m^{-2} s^{-1} in the second, third and fourth leaf of the mutant seedling. The Chl content was 426 and 216 mg m^{-2} on the second and fourth leaf of the normal sibling and 180, 252, and 239 mg m^{-2} on the second, third and fourth leaf of the mutant. b, The influence of intercellular levels of CO_2. Measurements were made on the fourth leaf of the mutant and the seventh leaf of the normal sibling 34 d after planting. The temperature range in the cuvette between individual measurements was 31.5 to 33.4°C and the light intensity was 1000 μmol quanta m^{-2} s^{-1}. Measurements were made sequentially from low to high pCO_2. The activity of Rubisco was 34.9 in the normal sibling, and in the mutant seedling, 7.3 (7.5 cm from the base) and 12.3 (11.5 cm from the base) μmol m^{-2} s^{-1}. The Chl content was 365 mg m^{-2} in the normal sibling, and, in the mutant seedling, 172 (7.5 cm from the base) and 305 (11.5 cm from the base) mg m^{-2}. At the highest intercellular CO_2 concentration shown for each leaf the respective level of atmospheric CO_2 was 617 μbar for the normal leaf, 617 μbar for the mutant (11.5 cm) and 630 μbar for the mutant (7.5 cm).

lings. The rates at saturating CO_2 were lower in the leaf of the mutant seedling. As the experiments were carried out over a similar range of atmospheric pCO_2 for both mutant and normal plants (see legend to Fig. 4b) it is apparent that, at a given level of atmospheric CO_2 the normal seedling with its higher photosynthetic capacity has a lower intercellular pCO_2 than leaves of the mutant.

DISCUSSION

We have examined several hcf mutants which were reported to have normal photochemistry and low CO_2 fixation capacity. It was previously shown that, on a Chl basis, the mutants have normal, noncyclic electron transport capacity and normal levels of cyclic photophosphorylation (10, 11). These mutants, while having slightly higher fluorescence than normal plants, have much lower fluorescence than various other photosynthetic mutants of maize which have lesions directly impairing the photochemistry (10) (D. Miles personal communication). In the present study, the hcf 11, 21, 42, and 45 mutants of this group had very low Rubisco activity relative to the normal siblings, and this may be a major basis for the low photosynthetic rates. There was generally a very good correlation between the photosynthesis rate, at 1000 μmol m^{-2} s^{-1} and atmospheric levels of CO_2, and the extractable activity of Rubisco. When Rubisco activity is very low there may be little or no net photosynthesis, since dark respiration is about 5% of the maximum rate of photosynthesis (Figs. 3 and 4). The mutants also had a lower Chl content per leaf area. Thus, even if the photochemical capacity on a Chl basis is normal, the lower Chl content could contribute to the lower rates of photosynthesis on a leaf area basis. Although this study was not focused on analysis of photochemistry, mutants of hcf 11 and 42 had lower Hill reaction activity on a Chl basis with ferricyanide as an oxidant, and this may also have limited photosynthesis. It is clear that, of the enzymes assayed, the mutations cause a selective reduction in Rubisco activity and Rubisco protein content. Whether these nuclear encoded lesions specifically impair the synthesis of Rubisco, with Chl deficiency as a secondary effect, or whether they directly impair both Rubisco and Chl synthesis is unknown. A *Chlamydomonas reinhardii* mutant lacking functional Rubisco has been shown to be photosensitive and acetate requiring (16). With low Rubisco which limits utilization of photochemically produced energy, other components of the photosynthetic apparatus may be unstable. It is of interest that plastome mutants of *Oenothera*, which are Rubisco deficient, have some reduction in Chl content (15–32%) and an altered photochemistry which may be due to secondary effects (4).

With respect to hcf 21, there are several virescent mutants of maize which have been shown to be deficient in 70S ribosomes at lower temperatures (7). If the hcf 21 mutant has some deficiency in 70S ribosomes, it could explain why it has a virescent phenotype where there is a decrease in Chl along with a selective loss of Rubisco activity (since synthesis of the large subunit is dependent on 70S ribosomes) among the enzymes examined. The enzymes of the C_4 cycle, as well as the other C_3 pathway enzymes

examined in the present study, are found in high activity in another virescent mutant (v16/v16) of maize when grown at a temperature causing a deficiency in 70S ribosomes (3). In contrast, there is some evidence that hcf 42 mutants have low levels of nuclear encoded mRNA for the small subunit, but normal levels of chloroplast encoded mRNA for the large subunit (personal communication in Refs. 11 and 14). If confirmed, this would suggest the mutation in hcf 42 may be affecting expression of small subunit genes of Rubisco rather than at the chloroplast level which would presumably occur in 70S ribosome-deficient, virescent mutants.

The results indicate when several parameters are being measured in these photosynthetic mutants it is important to make analyses on the same leaf segment. Often there is sufficient variation between seedlings, leaf number, and position along a leaf such that discrepancies would occur if each factor were measured on separate leaves. Also, growth conditions can influence the appearance and the viability of these mutants.

The light response curves of hcf 21 and 42 indicate that when there is a severe loss of photosynthetic capacity in the mutants light saturation occurs at a much lower intensity, and their maximum photochemical efficiency is lower (Figs. 3 and 4a). Under these conditions it is uncertain to what extent photosynthesis is limited by Rubisco activity *versus* photochemistry. Where there is a severe reduction in photosynthesis, Rubisco and photochemistry are likely colimiting. With a more moderate decrease in photosynthesis by mutation (e.g., the higher rates of photosynthesis by the mutant segments in Figs. 3 and 4, where mutant and normal tissue had similar levels of Chl), photosynthesis may be limited mainly by the loss of Rubisco activity. When photosynthesis is plotted against intercellular pCO_2 in order to consider carboxylation efficiency on a biochemical basis, the maximum carboxylation efficiency at very low pCO_2 in the hcf 42 mutant is similar to the normal siblings (Fig. 4b) while the maximum capacity is decreased. This is consistent with normal operation of the C_4 pathway but lower Rubisco levels in the mutant.

In the future, it would be of interest to determine the levels of mRNA for the large and small subunit of Rubisco in these mutants in order to assess the basis for the limited synthesis of this protein. It would also be of value to determine whether any of the mutants are allelic by crossing different heterozygotes.

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