Plant Physiol. (1988) 87, 83–88
0032-0889/88/87/0083/06/$01.00/0

A Mutant of *Arabidopsis thaliana* that Exhibits Chlorosis in Air but Not in Atmospheres Enriched in CO_2[1]

Received for publication November 13, 1987 and in revised form January 14, 1988

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ABSTRACT

A mutant of *Arabidopsis thaliana* (L.) Heynh. which requires a high concentration (2% by volume) of atmospheric CO_2 for growth has been isolated. Unlike previous mutants of this type, this line does not have any apparent defect in photosynthetic CO_2-fixation, photorespiration, or photosynthetic electron transport. The mutant is abnormally susceptible to pigment bleaching in air but not in 2% CO_2. The presence of normal or above-normal levels of antioxidants, carotenoids, and enzymes involved in reactive oxygen detoxification suggests that the mutant is equipped to detoxify activated oxygen species. Although it was not possible to establish a biochemical basis for the lesion, the properties of the mutant suggest the existence of a previously unidentified role for CO_2.

A relatively large number of mutants of higher plants have been described which will only grow in atmospheres enriched with about 1% CO_2. All but one of these are mutants with defects in photorespiratory metabolism, which have been described in *Arabidopsis thaliana* and *Hordeum vulgare* (22). These mutants are nonviable in air, but they are able to grow normally in an atmosphere enriched with CO_2 because ribulose bisphosphate oxygenase activity is suppressed and carbon is not diverted into the photorespiratory pathway. In addition to the mutants with defects in photorespiration, a high CO_2-requiring mutant was isolated that was unaltered in photorespiratory metabolism but that had severely reduced levels of activated Rubisco[2] *in vivo* (25). Characterization of the biochemical lesion in this mutant led to the discovery of Rubisco activase, a protein required for the *in vivo* activation of Rubisco (19).

In this report, we describe another mutant of *A. thaliana* which requires elevated levels of atmospheric CO_2 for growth but has normal photorespiratory metabolism. Although we have not been able to identify the precise biochemical lesion in this mutant, we believe that the properties of the mutant suggest the existence of a previously unknown role for CO_2. A preliminary report on this mutant has appeared (1).

[1] Supported in part by grants from the United States Department of Agriculture (No. 86-CRCR-1-2046), the United States Department of Energy (No. DE-AC02-776ER01338), and the McKnight Foundation.

[2] Abbreviations: Rubisco, ribulosebisphosphate carboxylase/oxygenase; SOD, superoxide dismutase; fwt, fresh weight; WT, wild type; MV, methylviologen; 2,6-DMBQ, 2,6-dimethylbenzoquinone; TMPD, tetramethylphenylenediamine; Q_A, primary electron accepting plastoquinone of PSII; Q_B, second electron accepting plastoquinone of PSII; Q_B protein, 32 kD herbicide binding protein of PSII that binds Q_B.

MATERIALS AND METHODS

Plant Materials and Growth Conditions. The lines of *Arabidopsis thaliana* (L.) Heynh. described here were descended from the Columbia WT. The mutant line CS208 was isolated from an M2 population of plants which had been mutagenized with ethyl methane sulfonate as described previously (10). To reduce the number of background mutations, the mutant line was advanced for several generations by self-fertilization and then backcrossed to the WT and reselected in the F2 generation two times. The glycine decarboxylase deficient line CS116 has been described (24). The mutant line CC126 (7) was generously provided by C. J. Chastain and W. L. Ogren.

Except as noted otherwise, the plants were grown on an artificial medium irrigated with mineral nutrients (10) at 23°C in continuous illumination (80–100 μE m^{-2} s^{-1}) in air enriched with 2% (v/v) CO_2. The CO_2 concentration in the chamber was maintained with a CO_2 regulator (Forma Scientific, Marietta, OH).

For the growth of plants in low O_2, a gas mixture (1500 μl L^{-1} CO_2, 1% O_2, balance N_2) flowed continuously at a rate of 1 L min^{-1} through a 2 L Plexiglas chamber, which contained a 1 L pot with 8 to 10 plants of each genotype. The Plexiglas chamber was contained within a larger plant growth chamber which provided the standard conditions for temperature and light.

Tissue Culture Methods. The media for induction and maintenance of callus have been described (10). Seeds were surface-sterilized and germinated on solid callus induction medium. After 3 weeks, the resulting callus cultures were fragmented and transferred at a density of 1 g per plate to callus maintenance medium overlaid with Whatman No. 1 filters. To measure callus growth rate, the filter papers with callus were weighed under sterile conditions at periodic intervals.

CO_2 Exchange and $^{14}CO_2$ Labeling. Photosynthetic CO_2 exchange was measured on whole plants at 23°C and 200 μE m^{-2} s^{-1} with an infrared gas analyzer (Analytical Development Co., Hoddesdon, England). Photoassimilation of $^{14}CO_2$ (1 mCi $mmol^{-1}$) was performed in 330 μl L^{-1} CO_2, 50% O_2, balance N_2, at 24°C. For labeling plants during the induction phase of photosynthesis, plants were equilibrated with the atmosphere in darkness, then label was added at the start of illumination. The incorporation period lasted 23 min. To label during steady state photosynthesis, plants were illuminated for 30 min prior to the addition of label. The incorporation period lasted 20 min. Methods for gas exchange measurements, $^{14}CO_2$ feeding in the light, and analysis of the water soluble products of ^{14}C labeling have been described in detail (23). For dark $^{14}CO_2$ assimilation, a 12.5 cm pot with approximately 20 mutant and 20 WT plants was enclosed in a covered Plexiglas chamber that was in line with a pump and a flask containing 8% phosphoric acid. After 20 min of preequilibration, the system was closed and sodium [^{14}C]bicarbonate was injected into the acid to yield a specific activity of 3.5 mCi $mmol^{-1}$

and a CO_2 concentration of 390 μl L^{-1}. Sixty min later, additional sodium [^{14}C]bicarbonate was injected to bring the specific activity up to approximately 5 mCi $mmol^{-1}$. After another 30 min, the plants were harvested and processed as for plants labeled in light (23).

Leaf Fluorescence and Thylakoid Electron Transport. Fluorescence was measured on detached leaves after a 1 h dark incubation using a portable fluorometer (Richard Brancker Research Ltd). The voltage output of the photodiode was monitored on a Nicolet model 206 digital oscilloscope. Electron transport was measured on thylakoids that were prepared as described (16) except that 0.1% (w/v) BSA was included in the grinding buffer. The assays (whole chain, PSI, and PSII) were performed as described (16) except that the standard assay medium contained 0.02 M Hepes-KOH (pH 7.9), 0.3 M sorbitol, 0.01 M NaCl, 2 mM $MgCl_2$, 2.5 mM EDTA, and 0.1% BSA.

Carotenoid Measurements. Total carotenoids were measured quantitatively in 80% acetone extracts by monitoring the absorbance at 480 nm and correcting for the absorbance by Chl (12). Carotenoids in acetone extracts were separated by reverse phase HPLC on a 4.6 × 20 cm C18 column (Varian, MCH-5-n-cap) using a linear gradient of 90% aqueous methanol to 100% ethylacetate over 20 min at 1 ml min^{-1} (6).

Enzyme Assays. Carbonic anhydrase was assayed according to Wilbur and Anderson (27) by monitoring the change in pH during the course of the reaction. Leaf extracts were prepared by homogenizing 50 mg ml^{-1} leaf material in 20 mM sodium phosphate (pH 7.0), 1 mM EDTA, and 1 mM ascorbic acid. A unit of activity is defined as follows: $U = 10[E/N) - 1]$ where E and N are the times required for the enzymic and nonenzymic reactions, respectively, to change the pH of the medium from 8.0 to 7.0. Catalase was assayed by monitoring O_2 evolution from H_2O_2 with an O_2 electrode. Leaf material was homogenized on ice in 0.05 M potassium phosphate (pH 7.2) at a fwt to volume ratio of 50 mg ml^{-1}. The extracts were centrifuged for 10 min at 10,000g. The assay medium contained 0.05 M potassium phosphate (pH 7.2), 0.2% H_2O_2, and extract. Superoxide dismutase was assayed by measuring the inhibition of the reduction of nitro blue tetrazolium by superoxide as described (9). Leaf material was homogenized on ice in 0.1 M potassium phosphate (pH 8.3), 0.5 mM EDTA at a fwt to volume ratio of 50 mg ml^{-1}, and centrifuged 15 min at 13,000g before assay. Dehydroascorbate reductase was assayed spectrophotometrically by measuring the change in absorbance at 265 nm due to the production of ascorbate (17). Extracts were prepared by homogenizing leaf material on ice in 0.05 M potassium phosphate (pH 7.0), 0.1 mM EDTA, centrifuged for 10 min at 18,000g, and dialyzed 4 h against the same buffer. Glutathione reductase was assayed spectrophotometrically by measuring the change in absorbance at 340 nm due to oxidation of NADPH. Leaf material was homogenized on ice in 0.1 M Hepes-KOH (pH 7.5), 3 mM EDTA, and 2 mM GSSG. The GSSG was essential to prevent irreversible inactivation of the enzyme. The extract was filtered through Miracloth and centrifuged 10 min at 11,000g. The assay medium contained 0.05 M Hepes-KOH (pH 8.0), 1 mM GSSG, 1 mM dithiothreitol, 0.24 mM NADPH, and extract.

SOD Isozymes. To resolve isozymes of SOD in mutant and WT *Arabidopsis*, leaf extracts were prepared by homogenizing 500 mg leaf material in 3 ml 0.1 M potassium phosphate (pH 7.8), 0.1 mM EDTA, and clarifying by centrifugation for 15 min at 13,000g. Samples containing 300 μg protein were electrophoresed on an (7.2%) acrylamide gel, and the regions of SOD activity were localized (3).

Antioxidant Assays. Assays for glutathione and ascorbic acid were performed as described (14) on acid leaf extracts prepared by homogenizing 100 mg leaf material in 1.5 ml of either 5% (w/v) sulfosalicylic acid and 6 mM EDTA for glutathione or 3.5% (w/v) trichloroacetic acid for ascorbic acid. For the isolation of α-tocopherol, leaves were extracted in methanol:chloroform (2:1, v/v), filtered, and partioned against water. The chloroform layer was dried onto alumina powder. The latter steps of the isolation, beginning with the transfer of the dried powder to a neutral Al_2O_3 chromatography column, was performed as described by Wise and Naylor (29). α-Tocopherol was then quantified by HPLC as for the carotenoids except the detection was at 280 nm.

Other Measurements. Chl was measured in 95% ethanol or in 80% acetone (28). Protein was measured with a die binding assay (BioRad) according to the manufacturer's instructions. Leaf area was measured with a Licor portable area meter (Lambda Instruments Corporation).

RESULTS

Phenotype of the Mutant. The mutant line CS208 was isolated by screening a mutagenized population for individuals which grow in 1% CO_2 but become chlorotic in air. When grown in air enriched with 2% (v/v) CO_2, the mutant is slightly lighter in color than the WT but otherwise appears healthy. However, the leaves become chlorotic and rapidly develop necrotic regions when the mutant is transferred to ordinary air. Chl and carotenoid contents of mutant plants grown in 2% CO_2 were 80% of the WT level. After 5 d of continuous illumination in air, the Chl and carotenoid content of the mutant was reduced by 60 and 40%, but was unchanged in the WT (Fig. 1). Similar results were obtained with

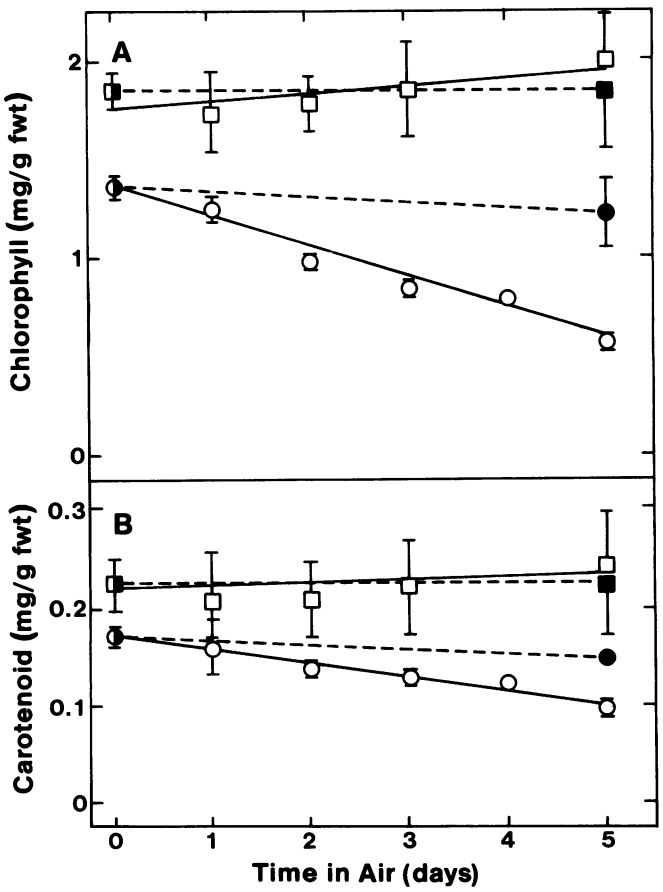

FIG. 1. Chl (A) and carotenoid (B) content in WT (□, ■) and mutant (○, ●) *Arabidopsis* following transfer of plants from 2% CO_2 to air (solid lines). Controls (closed symbols, dashed lines) remained in 2% CO_2. Values are means ± SD (n = 2).

a 16 h photoperiod (results not shown).

To determine if the bleaching of the mutant in air was dependent on light intensity, the Chl content of mutant and WT was compared after 4 d of exposure to various light intensities in normal atmospheric conditions (Fig. 2). Chl was reduced by approximately 40% in both mutant and WT in darkness. The lowest light intensity that had no effect on the Chl content of the WT (30 $\mu E\ m^{-2}\ s^{-1}$) resulted in a 40% reduction of Chl in the mutant. Lower intensities (0.1–20 $\mu E\ m^{-2}\ s^{-1}$) caused Chl loss in WT as well as the mutant (results not shown). The mutant exhibited a 60% loss of Chl in the highest light intensity (270 $\mu E\ m^{-2}\ s^{-1}$) compared to a 15% reduction in the WT. Since varying the light intensity from 0 to 270 $\mu E^{-2}\ s^{-1}$ had little if any effect on the Chl content of CS208, we cannot conclude that light is required for the injurious effects of normal atmospheric conditions. However, the bleaching caused by the mutation is not additive with the normal reduction of Chl that occurs following transfer of plants to darkness. In addition, necrotic lesions are not produced in darkness as they are in light. Light intensity in the range from 75 to 270 $\mu E\ m^{-2}\ s^{-1}$ did not affect Chl content of mutant or WT plants kept in high CO_2 (results not shown).

To determine whether the phenotype is expressed in nonphotosynthetic tissue, we examined the effect of atmospheric CO_2 on calli growth and primary root elongation. In air, calli of mutant and WT had growth rates of 98 ± 21 and 85 ± 22 mg 100 $mg^{-1}\ d^{-1}$, respectively (mean ± SD, n = 4 plates). Similarly, after 4 d in air, the mean (n = 10) primary root lengths for mutant and WT seedlings were 5.7 ± 0.9 and 5.3 ± 0.6 (SD) mm, respectively. These results suggest that the phenotype is expressed only in photosynthetic tissue.

In other high CO_2-requiring mutants of *Arabidopsis*, loss of Chl was readily reversed by returning plants that had become chlorotic in air to an atmosphere enriched with CO_2. By contrast, illumination of this mutant in air for as little as 1 d leads to irreversible injury to the vegetative tissue. Once the leaves become chlorotic, they do not recover when placed back in high CO_2. However, the plants survive by growing new leaves. In this respect, the mutant line CS208 differs from the previously described mutants which require high levels of CO_2 for growth, with the exceptions of two lines identified by Chastain (CC112 and CC126) (7).

Genetic Analysis. The genetic basis for the mutant phenotype was examined by crossing the line CS208 with WT. The resulting F_1 plants were able to grow normally in air. The F_2 progeny resulting from self-fertilization of the F_1 heterozygotes were scored for the mutant phenotype by growing the F_2 plants for 16 d in 2% CO_2 then transferring them to air. Of 260 F_2 individuals examined in this way, 198 grew normally in air and 62 turned chlorotic and died. This excellent fit to the 3:1 hypothesis (χ^2 = 0.13; $P > 0.70$) indicates that the phenotype is due to a single, recessive nuclear mutation at a locus we have designated *hcr*l ('*h*igh CO_2 *r*equiring').

Chastain (7) has isolated a mutant line (CC126) that has a similar phenotype to CS208 except that CC126 becomes chlorotic in air at a significantly slower rate than CS208. The biochemical basis of the lesion in CC126 is not known. F_1 hybrids obtained by crossing CS208 with CC126 had the mutant phenotype indicating that the two mutant lines carry defective alleles at the same locus. We have designated the alleles *hcr1-1* and *hcr1-2* for CS208 and CC126, respectively.

Effects of Oxygen Concentration. The flux of carbon into the photorespiratory pathway can be suppressed by high CO_2 or low O_2. Thus, if the high CO_2 requirement of the mutant was related to a defect in the C_2 pathway, it should be possible to grow the mutant in an atmosphere in which the O_2 concentration is reduced to the point that the ratio of CO_2 to O_2 is equal to or greater than that in air containing 2% CO_2. Therefore, we examined the phenotype of plants that were grown in air containing 2% CO_2 then transferred for 2.5 d to an atmosphere of 0.15% CO_2, 1% O_2, balance N_2. This yields a CO_2/O_2 ratio of 0.15 compared to a ratio of 0.10 in 2% CO_2. The combination of 0.15% CO_2 and 1% O_2 did not prevent the development of chlorosis in the mutant (Table I). As a control, to ensure that the atmospheric conditions in the chamber were maintained under conditions that would effectively prevent the flux of carbon into the photorespiratory pathway, a line of *Arabidopsis* with a defect in glycine decarboxylase activity (24) was included. The observation that this mutant remained green and healthy in the low O_2 and high CO_2 chambers throughout the course of the experiment indicated that the atmospheric conditions were suitable (results not shown).

Effects on Photosynthetic CO_2 Assimilation. Since a major requirement for CO_2 is in photosynthesis, we examined the possibility that high CO_2 is needed to enhance Rubisco activity in the mutant. It is conceivable that if there were a high resistance to CO_2 diffusion in the mutant, or if there were a biochemical impairment in carbon fixation, high CO_2 might be required to maintain an adequate level of CO_2 fixation by Rubisco. These possibilities were examined by measuring the rate of photosynthetic gas exchange by WT and mutant plants grown in high CO_2. The rate of photosynthetic CO_2-fixation in air was similar in WT and mutant when expressed on a protein, leaf area, or fwt basis (Table II). When expressed on a Chl basis, the photosynthetic CO_2-fixation rate was higher in the mutant since the mutant was slightly chlorotic (Fig. 1). The rate of gas exchange remained stable during a continuous 20 h measurement in both WT and mutant (results not shown). Thus, it appears that whatever leads to the injurious effects of air in the mutant does not directly

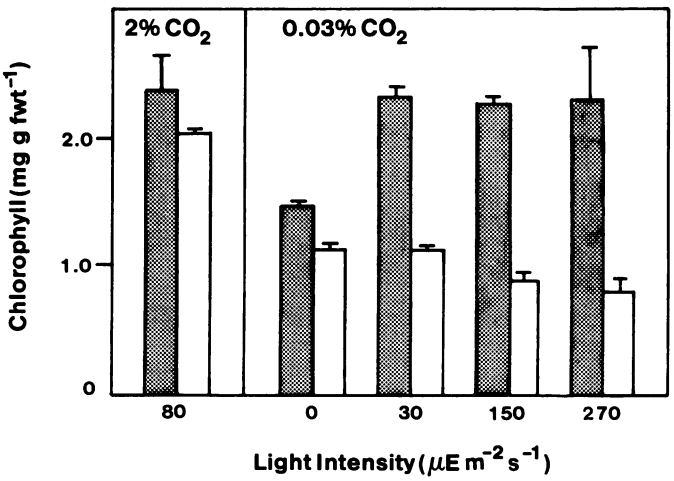

FIG. 2. Effect of light intensity on Chl content of WT (closed bars) and mutant (open bars) *Arabidopsis* 4 d after transfer from 2% CO_2 to air (0.03% CO_2). Values are means ± SD (n = 2).

Table I. *Chl Concentration in WT and Mutant Arabidopsis Grown in Air Containing 2% CO_2 then Transferred for 2.5 d to Air or 1% O_2*

Values are means ± SD (n = 2).

Atmospheric Conditions		Chl Content	
		WT	CS208
CO_2 ($\mu l\ L^{-1}$)	O_2 (%)	mg g fwt^{-1}	
20,000	21	2.0 ± 0.1	1.9 ± 0.1
350	21	2.2 ± 0.1	1.1 ± 0.1
1,500	1	1.8 ± 0.1	1.2 ± 0.1

Table II. *Photosynthetic Gas Exchange in Air of WT and Mutant Arabidopsis*

Values are means ± SD (n = 3)

Strain	$\mu g\ CO_2\ g\ fwt^{-1}\ h^{-1}$	$\mu g\ CO_2\ cm^{-2}\ h^{-1}$	$\mu g\ CO_2\ mg\ protein^{-1}\ h^{-1}$	$\mu g\ CO_2\ mg\ Chl^{-1}\ h^{-1}$
WT	4,900 ± 800	76 ± 8	212 ± 16	2600 ± 300
CS208	4,600 ± 500	75 ± 10	200 ± 26	3100 ± 400

affect the activity of the C_3 cycle or the diffusion of CO_2 to the chloroplast stroma.

The interconversion of CO_2 and H_2CO_3 is catalyzed by carbonic anhydrase. A defective carbonic anhydrase in a higher plant may, in principle, result in a requirement for elevated CO_2. However, carbonic anhydrase activities in leaf extracts were 210 U mg protein^{-1} for the mutant and 220 U mg protein^{-1} for WT. (See "Enzyme Assays" in "Materials and Methods" section for definition of U.)

Labeling Studies. Since CO_2 exerts an effect on the phenotype, it seemed possible that CO_2 metabolism was altered in the mutant. Therefore, we examined the products of $^{14}CO_2$ fixation in both light and dark conditions. WT and mutant plants were labeled with $^{14}CO_2$ under photorespiratory conditions (330 μl L^{-1} CO_2, 50% O_2, balance N_2) either during induction of photosynthesis or during steady state photosynthesis. The distribution of label revealed no difference between mutant and WT (Table III). Resolution of the labeled constituents of the various fractions by two-dimensional TLC also failed to reveal a significant difference in the amount of any of the major constituents (results not presented).

Mutant and WT plants were labeled with $^{14}CO_2$ in the dark for 1.5 h. The average incorporation of $^{14}CO_2$ in mutant plants was 278 dpm $\mu g\ Chl^{-1}$ compared to 232 dpm $\mu g\ Chl^{-1}$ in WT plants. The 20% higher incorporation in the mutant is due to the 15% reduction in Chl. The distribution of label between fractions was the same for mutant and WT, with 78% of the label in the basic fraction. Thus, there is no obvious quantitative defect in any of the major leaf carboxylases that operate in the absence of light.

Effects on Electron Transport. Bicarbonate is required for electron transport on the reducing side of PSII (4). If thylakoids are depleted of bicarbonate, electron transport between Q_A and Q_B is reversibly inhibited. It has been proposed (4) that bicarbonate binds to the Q_B protein or a nearby protein causing a conformational change in Q_B. In bicarbonate-depleted thylakoids, the induction transient for room temperature fluorescence rises rapidly, resembling a fluorescent transient in the presence of the Q_A to Q_B electron transport inhibitor, DCMU (26). In order to examine the possibility that the mutant was altered in bicarbonate-stimulated electron transport, fluorescence induction of detached leaves was monitored at ambient CO_2 in the absence of DCMU. For 13 out of 15 mutant leaves, the rise in fluorescence was slower than that for 12 WT leaves examined (Fig. 3). This result suggests that the bicarbonate binding site is not altered in the mutant. This conclusion is supported by the observation that rates for PSII electron transport measured from H_2O to 2,6-DMBQ were similar in the WT and mutant (Table IV). Similarly, there was no apparent difference in the rates of whole chain (H_2O to MV) or PSI (TMPD to MV) uncoupled electron transport between WT and mutant (Table IV).

Table III. *Distribution of ^{14}C Label in Water Soluble Products of $^{14}CO_2$ Photoassimilation by WT and Mutant Arabidopsis*

Labeling was performed either during the induction of photosynthesis (0–23 min after illumination) or during 20 min of steady state photosynthesis. Values are averages of two samples.

Fraction	Induction		Steady State	
	WT	CS208	WT	CS208
	% of total incorporation			
Basic	48	45	44	48
Neutral (sucrose)	24	25	21	23
Acid 1 (organic acids)	6	4	9	6
Acid 2 (acid phosphates)	14	15	15	11
Acid 3 (sugar bisphosphates)	6	7	6	7

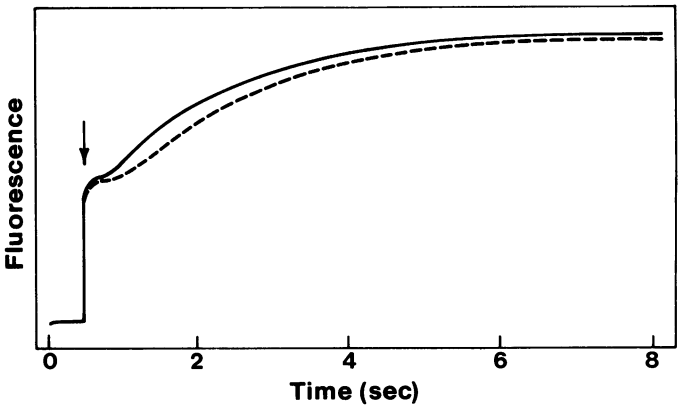

FIG. 3. Typical fluorescence induction kinetics of leaves from WT (——) and mutant (– – –) *Arabidopsis*.

Table IV. *Electron Transport Rates of Thylakoids from WT and Mutant Arabidopsis*

Values are means ± SD (n = 3–6).

	WT	CS208
	$\mu eq\ mg\ Chl^{-1}\ h^{-1}$	
Whole chain		
$H_2O \rightarrow MV$	570 ± 40	640 ± 50
PSII		
$H_2O \rightarrow$ 2,6-DMBQ	520 ± 40	510 ± 50
PSI		
TMPD → MV	1250 ± 150	1290 ± 230

Ability to Detoxify Reactive Oxygen. The tissue damage displayed by CS208 in air is suggestive of the production of reactive O_2 species. A mutant of barley that lacks 90% of the leaf catalase activity exhibits a similar phenotype (*i.e.* brown lesions, bleaching, and ultimate death of the leaves) (11). Reactive O_2 is produced either by reduction of molecular O_2 or by photodynamic excitation of O_2 to the singlet state (*e.g.* by intersystem energy transfer from triplet Chl) (8). If the injurious effects of low CO_2 on the mutant were caused by reactive O_2, then either the mutant is not capable of detoxifying a particular species of reactive O_2 or it overproduces reactive O_2. We examined the possibility that the mutant is deficient in some aspect of the detoxification. Plants grown in 2% CO_2 were assayed for the scavenger enzymes catalase and superoxide dismutase and the antioxidant regenerating enzymes dehydroascorbate reductase and glutathione reductase. The soluble antioxidants ascorbate and glutathione, and α-tocopherol and the carotenoids, singlet O_2 scavengers located on membranes, were also examined. The results are presented in Tables V to VII. The activities of the four enzymes were similar

Table V. *Activities of Enzymes Involved in Detoxification of Activated Oxygen Species*

Values are means ± SD (n = 2–5).

	WT	CS208
Catalase mmol O_2 min^{-1} g fwt^{-1}	1.7 ± 0.2	1.8 ± 0.1
Superoxide dismutase units min^{-1} g fwt^{-1}[a]	1.4 ± 0.4	1.2 ± 0.1
Dehydroascorbate reductase μmol min^{-1} g fwt^{-1}	4.0 ± 0.5	3.7 ± 0.5
Glutathione reductase μmol min^{-1} g fwt^{-1}	2.1 ± 0.1	2.1 ± 0.2

[a] One unit is the amount of activity that causes 50% inhibition of NBT photoreduction.

Table VI. *Levels of Antioxidants in Leaves of WT and Mutant*

Values are means ± SD (n = 3) or means (n = 2).

	WT	CS208
	μmol g fwt^{-1}	
Ascorbic acid		
Reduced	7.2 ± 1.0	7.3 ± 0.2
Oxidized	0.2	0.2
Glutathione		
Reduced	0.20 ± .01	0.30 ± 0.01
Oxidized	0.004	0.017
α-Tocopherol	0.019 ± .004	0.033 ± 0.001

Table VII. *Relative Concentrations of Carotenoids in WT and Mutant Arabidopsis*

Values are averages (n = 3) of percentages of total carotenoids in the sample. The mutant line CS208 contained 0.17 mg and the WT 0.22 mg of carotenoids per g fwt.

	WT	CS208
	% of total carotenoids	
Lutein	49	46
β-Carotene	24	24
Violoxanthin	14	15
Neoxanthin	13	15

in the mutant and WT when grown in 2% CO_2 (Table V). Since there are several isozymes of SOD in the cell (9) and since the activities of the various isozymes may increase as a result of various environmental stresses or increased O_2 radical production (8), the SOD activity in a crude extract may not reveal a deficiency in one particular isozyme. For this reason, leaf extracts were analyzed on a native acrylamide gel and stained for SOD activity. Four bands were discernible from both mutant and WT *Arabidopsis*. The bands from the mutant were indistinguishable from those obtained from WT with respect to both mobility and intensity (results not shown).

The level of ascorbate in the mutant was similar to that in the WT (Table VI). However, GSH and α-tocopherol were elevated 1.5- and 2-fold, respectively, in the mutant compared to the WT (Table VI). The four major carotenoids were present in similar proportions in mutant and WT (Table VII). The total concentration of carotenoids in the mutant was 80 to 90% of the WT when grown in 2% CO_2 (Fig. 1). Because the amount of carotenoids declined when the mutant was in air, we attribute the reduction in the carotenoid concentration observed in 2% CO_2 to be a secondary effect of the mutation rather than the primary defect. The elevated level of α-tocopherol is probably a response to a powerful oxidant in the thylakoid membranes, where α-tocopherol is located (30). Together, the data in Tables V, VI, and VII indicate that CS208 has normal or greater than normal levels of the enzymes and metabolites thought to be involved in the detoxification of reactive O_2 species.

DISCUSSION

In attempting to determine the basis for the requirement for high CO_2 and the biochemical defect caused by the *hcr1* mutation, we examined the possibility of a lesion in photorespiration or photosynthetic CO_2 fixation. While CS208 grows well in air containing 2% CO_2, an atmosphere low in O_2 offered no protection against the deleterious effects of low CO_2 (Table I). Hence, the high CO_2 requirement of the mutant does not appear to be due to a lesion in photorespiratory metabolism.

The lack of protection by low O_2 also suggests that the mutant does not require a rate of CO_2 assimilation higher than the normal rate in 21% O_2 and 350 μl L^{-1} CO_2, since suppression of photorespiration enhances the net CO_2 fixation rate.

A disruption in either the C_3 cycle or photorespiration would be revealed by a modified distribution of label relative to the WT after $^{14}CO_2$ feeding in the light, as well as by a reduction in the rate of photosynthesis. However, the ^{14}C labeling pattern revealed no anomalies in the mutant (Table III), and the rate of CO_2 exchange was very similar in the mutant to that in the WT (Table II). The normal photosynthetic rate also reveals that there is no barrier in the diffusion of CO_2 to the stroma. The presence of normal levels of carbonic anhydrase indicated that the CO_2-HCO_3^- interconversion is not limiting. Considered together, these observations indicate that the biochemical lesion imposed by the *hcr1* mutation is not in photosynthetic or photorespiratory carbon metabolism.

We investigated other processes that involve CO_2. PSII electron transport requires the binding of bicarbonate on or near the Q_B protein (4). However, we have ruled out the possibility that the bicarbonate binding site is altered in the mutant since photosynthetic electron transport was not impaired (Table IV), and the fluorescence induction transient was consistently slower in the mutant than the WT (Fig. 3). This conclusion is also supported by the observation that the mutation is of nuclear origin, whereas all of the peptides known to occur on the reducing side of PSII are chloroplast encoded (18).

It seems unlikely that the mutation affects a carboxylase which operates in the absence of light, since equivalent amounts of $^{14}CO_2$ were incorporated into mutant and WT plants in an experiment performed in the dark, and the distribution of label in the various fractions was identical. In addition, high CO_2 was not required for normal growth of callus tissue or for seedling root elongation.

We considered the possibility that the chlorosis which occurred in air may be attributed to senescence. Senescence in plants is characterized, in part, by enhanced sensitivity to ethylene and enhanced production of ethylene. Ethylene action is inhibited by CO_2 with a K_i of 0.49 mM or 1.55% in the gas phase (5). It is conceivable that the mutant is hypersensitive to ethylene so that it senesces unless high CO_2 is present. We consider this unlikely for the following reasons: First, WT *Arabidopsis* leaves and plants were insensitive to 5 μl L^{-1} ethylene when illuminated at 100 μE m^{-2} s^1 for 8 d, whereas leaves kept at 8 μE m^{-2} s^{-1} lost all of their Chl within 3 d. Control leaves at 8 μE m^{-2} s^{-1} were only slightly affected (results not shown). Second, an inhibitor of ethylene action, 2,5-norbornadiene (20), did not prevent the bleaching of mutant leaves in ambient CO_2 at a concentration of 10 μl L^{-1} (results not shown). Third, ethylene action in pea, measured as growth inhibition, was markedly reduced by lowering the partial pressure of O_2 to 5% (5). However, an O_2 concentration of 1% offered no protection to the *hcr1* mutant in this study (Table I).

It also seems unlikely that the pigment loss in the mutant is a

senescenic phenomenon caused by a factor other than sensitivity to ethylene. It is well known that both light and cytokinins delay senescence of leaves. However, neither 0.5 mM benzyladenine applied to the foliage of mutant plants (results not shown) nor light (Fig. 1) offered protection against bleaching in air.

Since the necrotic lesions displayed by the mutant in air are indicative of oxidative damage in the cells, we examined the ability of the mutant to detoxify reactive O_2 species. In the chloroplast, superoxide anions, which are produced on the reducing side of PSI, are detoxified by a series of reactions involving superoxide dismutase, ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase, and glutathione reductase (2). Based on the activities of some of these enzymes (Table V) and the levels of glutathione and ascorbate (Table VI), this system appears to be functional in the *hcr1* mutant. Singlet O_2, produced when O_2 quenches triplet Chl (13), is itself quenched by the carotenoids and α-tocopherol (8). The carotenoids and α-tocopherol were also present at adequate levels in the mutant (Fig. 1; Tables VI and VII). It was interesting to find that the mutant contained more glutathione and α-tocopherol than WT (Table VI). The glutathione level (1) and the glutathione reductase activity (results not shown) continued to increase after mutant plants were transferred to air. These protective responses by the mutant indicate that the mutant may overproduce strong oxidants. A barley mutant deficient in catalase activity and WT barley fed an inhibitor of catalase produce an elevated level of glutathione in response to an excess of H_2O_2 (21). Since we have examined the known mechanisms for detoxification of reactive O_2 species and found no deficiency, it is possible that the *hcr1* mutation somehow causes an overproduction of reactive O_2 or a defect in an aspect of detoxification that we have not examined.

If excess reactive O_2 species are produced in the mutant in air, it is difficult to understand how CO_2 prevents it. Perhaps CO_2 or bicarbonate has an as yet undiscovered function as an effector for some process in leaves. Bicarbonate may provide a needed countercharge to the positive charge of a basic amino acid residue. This has been proposed (4) for the Q_B apoprotein of PSII, which has an arginyl residue buried within a hydrophobic portion so that its positive charge is uncompensated by a nearby countercharge. The binding of bicarbonate to the arginyl residue is believed to cause a configurational change in the Q_B protein. Alternatively, CO_2 may be required to form a carbamate on a protein as it does with Rubisco and hemoglobin (15). A carbamate is formed as the result of a nucleophilic attack of an uncharged amine upon CO_2. In the case of Rubisco, carbamate formation occurs with the ε-amino group of a lysyl residue on the large subunit and serves to activate the enzyme. Carbamates occur on the α-amino groups on the N-termini of the hemoglobin subunits and cause a decrease in the affinity for O_2. The regulation of Rubisco and hemoglobin by CO_2 was no doubt discovered because these are extensively studied proteins. Lorimer (15) has speculated that carbamate formation "may be considerably more widespread and of greater biological significance than was previously realized." Thus, it is possible that the *hcr1* mutation results in an altered affinity for CO_2 or HCO_3^- by a protein that requires a carbamate or HCO_3^- for activity so that the mutant must be grown in a high concentration of CO_2 in order to activate the protein.

Acknowledgments—We thank Drs. A. Bleecker, A. Hanson, H. Kende, N. E. Tolbert, and J. Zeevaart for their suggestions.

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