

# A Mutant of *Arabidopsis thaliana* Which Lacks Activation of RuBP Carboxylase *In Vivo*<sup>1</sup>

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## ABSTRACT

A mutant of *Arabidopsis thaliana* has been isolated in which ribulose-1,5-bisphosphate carboxylase is present in a nonactivatable form *in vivo*. The mutation appears to affect carboxylase activation specifically, and not any other enzyme of the photosynthesis or photorespiratory cycles. The effect of the mutation on carboxylase activation is indirect, inasmuch as the properties of ribulose-1,5-bisphosphate carboxylase purified from the mutant are not distinguishable from those of the wild type enzyme. The mutant requires high levels of atmospheric CO<sub>2</sub> for growth because photosynthesis is severely impaired in atmospheres containing normal levels of CO<sub>2</sub>, irrespective of the atmospheric O<sub>2</sub> concentration. In this respect, the mutant is distinguished from previously described high-CO<sub>2</sub> requiring mutants of *Arabidopsis* which have defects in photorespiratory carbon or nitrogen metabolism.

The regulation of RuBP<sup>2</sup> carboxylase activity by light was originally suggested as a possible explanation for the response of metabolite pools during light/dark transitions in *Chlorella* (15). There have been subsequent reports of light regulation of the enzyme in isolated, intact chloroplasts (2, 5, 6, 11), intact leaves (13, 16), and in a reconstituted chloroplast system (9). However, the physiological significance of light activation remains equivocal. Robinson *et al.* (19) have argued that the magnitude of the increase in RuBP carboxylase activity observed following illumination of leaf protoplasts is too small to be of significance since the enzyme activity in nonilluminated protoplasts is sufficient to support maximal rates of CO<sub>2</sub>-fixation. Also, unlike those enzymes subject to light regulation by thioredoxin or LEM-mediated reduction (3), full activation of purified RuBP carboxylase may be achieved *in vitro* (in the presence of Mg<sup>2+</sup> and CO<sub>2</sub>) without the addition of any other factors (10). The stimulatory effect of light may simply reflect increased carbamate-activation of the enzyme (12) brought about by the light induced increase in stromal Mg<sup>2+</sup> concentration and pH (17, 18, 24).

A major problem with attributing the regulation of RuBP carboxylase activity solely to Mg<sup>2+</sup> and pH effects is that the

purified enzyme rapidly loses activation under assay conditions which mimic those thought to exist in the chloroplast (1, 11, 14). Thus, there has been considerable interest in the possible regulatory significance of various sugar phosphates which affect RuBP carboxylase activation *in vitro* (reviewed in [11]). It has recently been suggested that these compounds affect activation by binding at the substrate (RuBP) site and promoting the formation of the activator carbamate (1). If the enzyme cannot be simultaneously catalytically competent and stabilized in the activated state by these metabolic effectors, as proposed (1), then the significance of sugar phosphate activation observed *in vitro* is not evident.

We have described a method for the isolation of mutants of the C<sub>3</sub> species *Arabidopsis thaliana* (L.) Heyn. which require high levels of atmospheric CO<sub>2</sub> for growth (20–23). Although all previous mutants recovered by this screening method have defects in photorespiratory carbon or nitrogen metabolism, the method also permits the recovery of mutants which require elevated CO<sub>2</sub> because of a reduced affinity of the carboxylation reaction for CO<sub>2</sub>. In this report we describe the properties of such a mutant, in which the *in vivo* activity of RuBP carboxylase is much lower than in the parental wild-type.

## MATERIALS AND METHODS

**Plant Materials and Growth Conditions.** All lines of *Arabidopsis thaliana* (L.) Heyn. described here are descended from the Columbia wild-type. The procedures for isolation and maintenance of the mutant lines have been described (20, 23). The original line CS207 lacked vigor under all growth conditions and exhibited abnormalities in morphology. In order to separate the mutations responsible for these effects from the mutation of interest CS207 was backcrossed to the wild type in the M4 generation and a line with improved vigor, designated CS2071, was selected from the resulting F2 population on the basis of a growth requirement for high levels of atmospheric CO<sub>2</sub>. The line CS2071 is green and generally healthy in appearance when grown under high CO<sub>2</sub> but becomes chlorotic after several days of illumination in standard atmospheric conditions.

**Gas Exchange Measurements.** Intact plants were placed in a transparent chamber maintained at 24°C by immersion in a circulating waterbath, and gassed continuously with 344 μl/l CO<sub>2</sub>, 2% O<sub>2</sub>, balance N<sub>2</sub> humidified to 56% RH. The gas exited sequentially through a copper coil maintained at 30°C, the cuvette of a RH meter, a drying tube, and an IRGA. Flow rate, monitored with a mass flowmeter, was adjusted so as to permit accurate determination of transpiration rate rather than to optimize photosynthesis rate. Illumination (300 μE m<sup>-2</sup> s<sup>-1</sup>, 400–700 nm) was provided by a tungsten flood lamp equipped with an optical filter to prevent transmittance of infrared radiation.

**Labeling of Intact Plants.** Mature plants were placed in the

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<sup>2</sup> Abbreviations: RuBP, ribulose-1,5-bisphosphate; LEM, light effect mediator; Ru5P, ribulose-5-phosphate; SBP, sedoheptulose-1,7-bisphosphate; FBP, fructose-1,6-bisphosphate; IRGA, infrared gas analyzer.

system described above and flushed for 10 min in darkness. At this time, the system was converted to a closed circuit of measured volume (375 ml) which included a gas-tight diaphragm pump and an in-line reservoir containing 8% phosphoric acid.  $^{14}\text{CO}_2$  (2  $\mu\text{Ci}/\mu\text{mol}$ ) was generated at various concentrations by injecting  $\text{NaH}^{14}\text{CO}_3$  through a rubber septum into the phosphoric acid. The pressure resulting from the production of  $\text{CO}_2$  was released by transiently opening the circuit distal to the  $\text{CO}_2$ -generator. The system was then closed and the  $\text{CO}_2$  distributed through the system by the action of the pump. Following 10 min of illumination the plants were quickly (3–5 s) transferred to liquid  $\text{N}_2$ , the roots removed, and the leaf material ground in 80% ethanol. Insolubles were removed by centrifugation and Chl determined. The extract was then acidified with an equal volume of 4 N formic acid and dried under vacuum. The products of  $^{14}\text{CO}_2$  fixation were fractionated by combined ion exchange and TLC as described previously (20, 23), or by HPLC (4). RuBP was distinguished from other bisphosphates on thin layer chromatograms by treating the sugar bisphosphate fraction with alkaline phosphatase and then chromatographing in a solvent which resolved the free sugars (formic acid:methyl-ethyl-ketone:*t*-butanol:water in the ratio 12:24:32:12).

**Activation State of RuBP Carboxylase.** Protoplasts were prepared as described previously (21) and resuspended in 450 mM sorbitol, 30 mM Mops (pH 7.2), 1 mM  $\text{CaCl}_2$ , 0.1% BSA. Aliquots (50  $\mu\text{l}$ ) were placed in a series of sealed round bottomed polystyrene tubes in a temperature-controlled waterbath and gassed continuously with 350  $\mu\text{l}$   $\text{CO}_2$ , 2%  $\text{O}_2$ , balance  $\text{N}_2$ . Illumination was from below. RuBP carboxylase activity was determined by simultaneously disconnecting the gas supply from a tube and injecting 450  $\mu\text{l}$  of assay mixture containing 50 mM Tricine (pH 8.1), 10 mM  $\text{MgCl}_2$ , 0.5 mM EDTA, 0.4 mM RuBP, 0.2% Triton X-100, and 2 mM  $\text{NaH}^{14}\text{CO}_3$  (4  $\mu\text{Ci}/\text{mol}$ ). The reaction was terminated after 30 s with 200  $\mu\text{l}$  6 N acetic acid, dried at 60°C and dpm determined.

Experiments with intact plants were performed using the procedure described above. Following exposure to various periods of illumination plants were quickly removed from the plant chamber and the leaves ground in 800  $\mu\text{l}$  of 100 mM Tricine (pH 8.1), 10 mM  $\text{MgCl}_2$ . The extracts were clarified by 15 s centrifugation in a microfuge. The carboxylase assay was initiated by injecting 50  $\mu\text{l}$  extract into 450  $\mu\text{l}$  100 mM Tricine (pH 8.1), 10 mM  $\text{MgCl}_2$ , 0.4 mM RuBP, 1 mM  $\text{NaH}^{14}\text{CO}_3$  (4  $\mu\text{Ci}/\mu\text{mol}$ ) in a serum-stoppered minivial. The assay was terminated after 60 s by injection of acid. The elapsed time between removal of the plant from the chamber and the initiation of the assay was 75 s. Plants were held in darkness for 6 h before assay to permit the wild-type and mutant plants to arrive at a common level of metabolic activity.

RuBP carboxylase in the remaining portion of the sample was activated by adding  $\text{NaHCO}_3$  to a final concentration of 10 mM. After 20 min of incubation at 22°C the extracts were assayed as above.

**Purification of RuBP Carboxylase.** Leaf material was ground in 50 mM Tricine (pH 8.1), 2 mM  $\text{MgCl}_2$ , 2 mM  $\beta$ -mercaptoethanol and clarified by centrifugation at 30,000g for 30 min. The extract was loaded on a 10 to 30% linear sucrose gradient containing the same buffer constituents and centrifuged for 24 h at 97,000g<sub>av</sub> in a Sorvall AH627 rotor. The gradient was fractionated by standard methods and RuBP carboxylase identified as a peak of A at 254 nm. Carboxylase prepared in this manner gave only two discernible bands, corresponding to the subunits of the enzyme, following polyacrylamide electrophoresis under conditions leading to dissociation of the subunits. Approximately 33% of the protein in the extracts was recovered as RuBP carboxylase.

**Measurement of Kinetic Constants.** For the determination of  $K_m$  ( $\text{HCO}_3^-$ ) and  $K_a$  ( $\text{HCO}_3^-$ ) the enzyme solution was adjusted to 10 mM  $\text{MgCl}_2$  and then purged with  $\text{CO}_2$ -free argon. For assays

initiated with activated enzyme, a portion of the enzyme solution was adjusted to 10 mM  $\text{NaHCO}_3$  and incubated 30 min at 22°C. Assays, which contained 50 mM Tricine (pH 8.1), 10 mM  $\text{MgCl}_2$ , 0.4 mM RuBP and bicarbonate (4  $\mu\text{Ci}/\mu\text{mol}$ ), were initiated with 15  $\mu\text{l}$  enzyme and stopped after 60 s by acidification. The same conditions were used for assays initiated with RuBP except that the degassed enzyme was incubated in the complete reaction mixture (as above but lacking RuBP) for 30 min at 22°C, then the reaction was initiated with RuBP.  $K_m$  (RuBP) and  $K_m$  ( $\text{Mg}^{2+}$ ) were determined by standard methods using fully activated enzyme and other substrates at saturating concentrations (*i.e.* 0.4 mM RuBP, 10 mM  $\text{Mg}^{2+}$ , 10 mM  $\text{NaHCO}_3$ ). The calculation of  $K_a$  ( $\text{HCO}_3^-$ ) was performed as described by Jordan and Ogren (7).

**$^{32}\text{P}$  Labeling of Chloroplasts.** Washed chloroplasts were prepared from protoplasts as described (21). As some difficulty was encountered in stripping the epidermis from leaves of the mutant CS2071, protoplasts of both wild-type and mutant were obtained from razor chopped material. For this reason,  $\text{CO}_2$ -fixation rates were reduced in comparison with the rates obtained previously (21).

Chloroplasts containing 40  $\mu\text{g}$  Chl were added to 1 ml of 0.3 M sorbitol, 30 mM HEPES (pH 7.6), 2.5 mM EDTA, 0.1% BSA, 300 units/ml catalase, 0.1 mM  $^{32}\text{PO}_4$  (100  $\mu\text{Ci}/\mu\text{mol}$ ), and 0.1 or 5 mM  $\text{NaHCO}_3$  in an illuminated  $\text{O}_2$  electrode. After 7 min of illumination, 150  $\mu\text{l}$  samples were withdrawn, layered on 150  $\mu\text{l}$  of silicone oil (20:80 mixture of Wacker AR20:AR200) contained in a 400  $\mu\text{l}$  microfuge tube, and centrifuged into 20  $\mu\text{l}$  of 10%  $\text{HClO}_4$ . This procedure eliminated the excess  $^{32}\text{PO}_4$  which would otherwise hamper analysis of labeled metabolites. The pellets were resuspended in water, frozen, centrifuged, neutralized with  $\text{NaHCO}_3$  and products separated by HPLC (4).

## RESULTS

**Photosynthesis Measurements.** All of the previously characterized mutants of *Arabidopsis* which were isolated on the basis of a

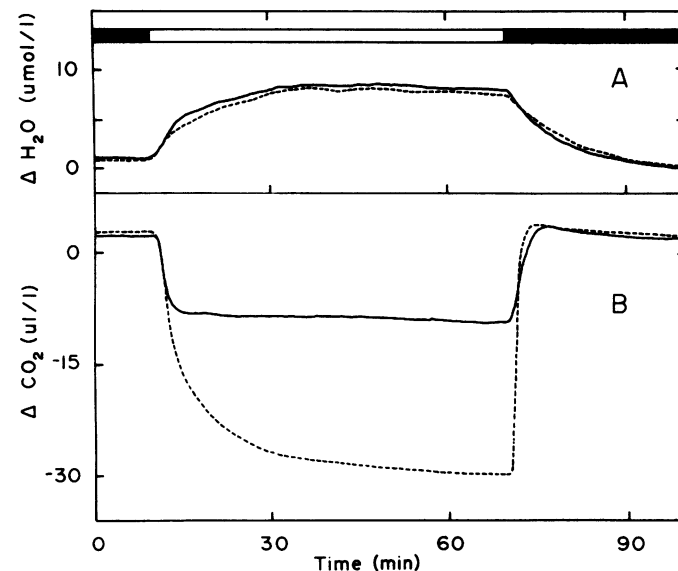


FIG. 1. Measurement of transpiration (A) and photosynthesis (B) rates in wild-type and mutant *Arabidopsis*. The plants were flushed continuously with 344  $\mu\text{l}/\text{l}$   $\text{CO}_2$ , 2%  $\text{O}_2$ , balance  $\text{N}_2$ . The response of the wild-type is represented by the broken line (---) and that of the mutant CS2071 by the solid line (—). The bar inserted in (A) represents conditions of illumination. The maximum photosynthesis and transpiration rates were 100.6  $\mu\text{mol CO}_2 \text{ mg}^{-1} \text{ Chl h}^{-1}$  and 600  $\mu\text{mol H}_2\text{O mg}^{-1} \text{ Chl h}^{-1}$  for wild-type, and 25.0  $\mu\text{mol CO}_2 \text{ mg}^{-1} \text{ Chl h}^{-1}$  and 617  $\mu\text{mol H}_2\text{O mg}^{-1} \text{ Chl h}^{-1}$  for CS2071.

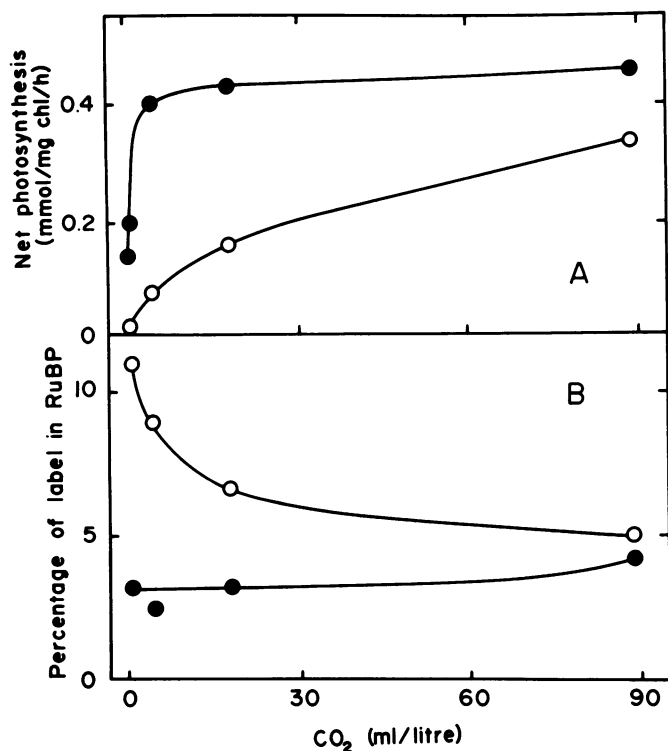


FIG. 2. The effect of CO<sub>2</sub> concentration on photosynthesis rate (A) and RuBP levels (B) in wild-type and mutant *Arabidopsis*. Intact plants were illuminated for 10 min in various concentrations of <sup>14</sup>CO<sub>2</sub>, then killed, extracted, and the resulting labeled products quantitated and identified. (●), wild-type, (○), mutant CS2071.

Table I. Distribution of Label in Products Resulting from <sup>14</sup>CO<sub>2</sub>-fixation by Mutant and Wild-type *Arabidopsis* Plants

Photosynthesis was carried out in an atmosphere containing 400 μl/l CO<sub>2</sub>, 2% O<sub>2</sub>, balance N<sub>2</sub> for 13 min.

Fraction	<sup>14</sup> CO <sub>2</sub> Incorporated	
	Wild-type	Mutant CS2071
	% of total	
Neutral	34.3	20.8
Basic	14.0	18.9
Weak acids	23.9	24.8
Strong acids	9.8	29.5
RuBP	2.5	19.8
Insoluble	17.0	4.6
Recovery	99.0	98.6

growth requirement for high levels of atmospheric CO<sub>2</sub> exhibit reduced photosynthesis in standard atmospheric conditions but have normal rates of photosynthesis in atmospheres containing low concentrations of O<sub>2</sub> (*i.e.* 350 μl/l CO<sub>2</sub>, 2% O<sub>2</sub>, balance N<sub>2</sub>). These mutants have defects in photorespiratory carbon or nitrogen metabolism which lead to the accumulation of toxic compounds or depletion of required compounds under photorespiratory conditions (20, 22, 23) Since photorespiration is suppressed in atmospheres containing high levels of CO<sub>2</sub> or low levels of O<sub>2</sub>, the effects of the mutation are not expressed under these atmospheric conditions.

In the exceptional mutant line CS2071, photosynthesis is greatly inhibited irrespective of the atmospheric O<sub>2</sub> concentration. This is illustrated for low O<sub>2</sub> concentration in Figure 1, in which the gas exchange characteristics of the mutant and the wild-type are compared under nonphotorespiratory conditions (344 μl/l CO<sub>2</sub>,

Table II. Kinetic Parameters of RuBP Carboxylase from Wild-type and Mutant *Arabidopsis*

$K_m$  (NaHCO<sub>3</sub>) and  $K_a$  (NaHCO<sub>3</sub>) were determined from the data in Figure 3 as previously described (7).

	Wild-type	Mutant CS2071
$V_{max}$ μmol min <sup>-1</sup> mg <sup>-1</sup> protein	0.87	0.86
$K_m$ (NaHCO <sub>3</sub> ), mM	0.78	0.83
$K_a$ (NaHCO <sub>3</sub> ), mM	1.07	1.17
$K_m$ (Mg <sup>2+</sup> ), mM	0.50	0.62
$K_m$ (RuBP), μM	83.0	88.0

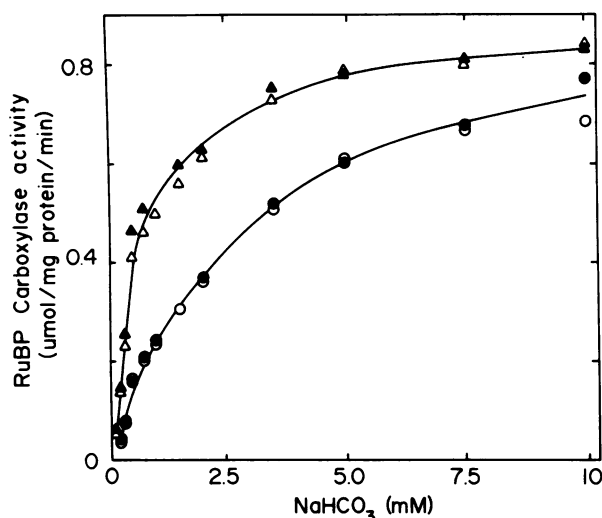


FIG. 3. CO<sub>2</sub>-saturation curves for RuBP carboxylase assays initiated with RuBP (●, ○) or with bicarbonate activated enzyme (▲, △) from wild-type (●, ▲) and mutant (○, △) *Arabidopsis*.

2% O<sub>2</sub>, balance N<sub>2</sub>). The strong inhibition of the photosynthesis rate in the mutant (Fig. 1B) is accompanied by a corresponding reduction in the rate of photorespiration as measured by the rate of release of CO<sub>2</sub> into a CO<sub>2</sub>-free gas stream (results not presented). However, the transpiration rates of the mutant and the wild-type were similar (Fig. 1A), indicating normal stomatal function in the mutant. Analysis of leaf extracts indicated that a normal level of carbonic anhydrase was present in the mutant (results not presented). Thus, something other than the availability of intracellular CO<sub>2</sub> was limiting the rates of carboxylation and oxygenation of RuBP in the mutant. In contrast to the other CO<sub>2</sub>-requiring mutants (20, 22), the mutant CS2071 does not exhibit the progressive inhibition of photosynthesis rate associated with accumulation or depletion of a metabolite. Rather, the photosynthesis rate increases almost linearly for the first several min of illumination, then abruptly stabilizes. In this respect, the response of the mutant is distinguishable from that of the wild type not only in maximal rate, but also in the time required to attain maximal rate.

Because preliminary gas exchange measurements of photosynthesis indicated CO<sub>2</sub> saturation at concentrations greater than could be measured by an IRGA, the CO<sub>2</sub>-response curve of photosynthesis was measured as net <sup>14</sup>CO<sub>2</sub> fixation (Fig. 2A). In contrast to the wild type, in which the photosynthesis rate saturated at a relatively low CO<sub>2</sub> concentration, the rate of CO<sub>2</sub>-fixation by the mutant was apparently not saturated at CO<sub>2</sub> concentrations several hundred-fold higher than normal air levels. At the highest CO<sub>2</sub> concentration, the photosynthesis rate of the mutant was similar to that of the wild-type. This observation is taken as evidence that the mutant is not defective in the light harvesting reactions of photosynthesis but has a defect which reduces the affinity of the carboxylation reaction for CO<sub>2</sub>.

**Labeling Studies.** Analysis of the products of <sup>14</sup>CO<sub>2</sub> fixation in

the experiment above, and other similar experiments (Table I), revealed an abnormal distribution of label among the products of photosynthesis. In short term (10 min) labeling experiments, conducted in nonphotorespiratory conditions, the mutant showed a dramatic increase in the proportion of label which accumulated in RuBP, and reduced accumulation of label in the carbohydrate fractions (neutral and insoluble). The accumulation of label in RuBP by the mutant was largely reversed when the plants were labeled in atmospheres containing high levels of CO<sub>2</sub> (Fig. 2B), whereas the labeling of RuBP in the wild-type was unaffected by the external CO<sub>2</sub> concentration. Similarly, the proportion of label in the carbohydrate fractions in the mutant increased to wild-type levels when plants were labeled in high CO<sub>2</sub> (results not presented).

**RuBP Carboxylase Activity *In Vitro*.** To examine the possibility that the mutant line has an alteration in RuBP carboxylase, the enzyme was purified by sedimentation and the kinetic constants of the enzymes from wild-type and mutant were determined. It was found that the enzymes from the two sources were indistinguishable with respect to  $V_{max}$  and  $K_m$  values for CO<sub>2</sub>, RuBP, and Mg<sup>2+</sup> (Table II).

RuBP carboxylase also possesses a CO<sub>2</sub>-binding site which has been distinguished from the binding site for catalysis and is involved in altering the kinetic constants of the catalytic reaction (10, 12). The kinetic parameters associated with this regulatory site are not readily amenable to measurement. However, a parameter designated  $K_a$  (7) may be obtained by comparing the CO<sub>2</sub>-response curves for catalysis obtained by initiating assays with bicarbonate-activated enzyme or with RuBP. Measurements of this parameter (Table II), obtained from the data in Figure 3, were similar for the enzymes from the mutant and the wild-type.

The enzymes from the mutant and the wild-type were also compared by isoelectrophoresis in narrow (5–7) pH range denaturing polyacrylamide gels. No differences were found in the isoelectric points of the large or the small subunit (results not presented). From this we concluded that the mutation in CS2071 probably does not directly affect the structure of the RuBP carboxylase enzyme.

**RuBP Carboxylase Activity *In Vivo*.** Although *in vitro* analyses did not show that the mutant was deficient in RuBP carboxylase activity, the evidence obtained from measurements of CO<sub>2</sub> ex-

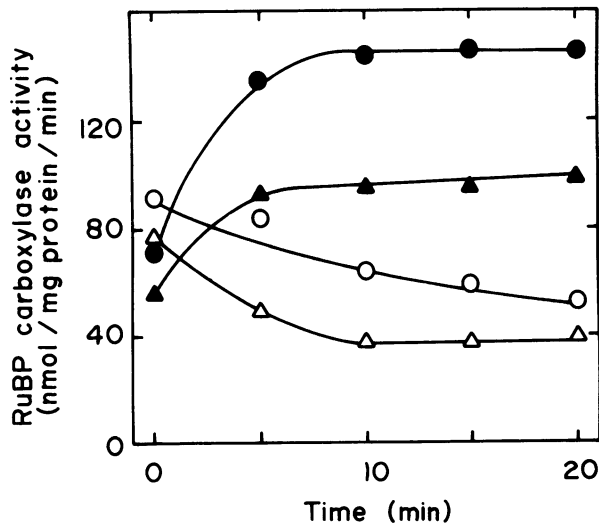


FIG. 4. The effect of light and CO<sub>2</sub>-concentration on activation state of RuBP carboxylase in intact plants of wild-type and mutant *Arabidopsis*. Plants were illuminated in air (△, ▲) or in 1% CO<sub>2</sub>, balance N<sub>2</sub> (○, ●) and at the indicated intervals were quickly homogenized and the extract was assayed for RuBP carboxylase activity. (●, ▲), wild-type; (○, △), mutant CS2071.

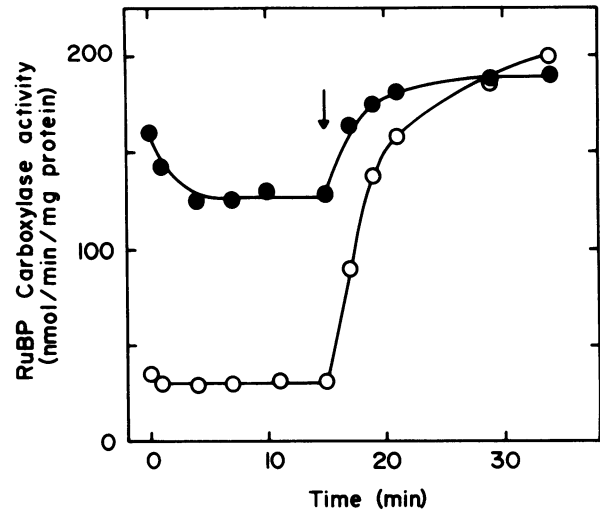


FIG. 5. Bicarbonate activation of RuBP carboxylase in crude extracts of leaves from wild-type and mutant *Arabidopsis*. Plants were illuminated for 30 min in standard atmospheric conditions. The leaves were then homogenized in cold 50 mM Tricine (pH 8.1) and 5 mM MgCl<sub>2</sub>, clarified by centrifugation for 15 s, chilled to 0°C, and assayed for activity at the indicated intervals. After 15 min, bicarbonate was added to a final concentration of 10 mM and the extract was brought to 23°C. (●), wild-type; (○), mutant CS2071.

change and metabolite levels suggested that the enzyme was not normally active *in vivo*. This hypothesis was tested by employing methods which permitted the rapid release and assay of RuBP carboxylase from leaves, protoplasts, and isolated chloroplasts. The approach is predicated on the general observation that, for several metabolite regulated enzymes, the transition from active to inactive form is a sufficiently slow process that may be observed by employing rapid extraction and assay techniques.

The effect of light and CO<sub>2</sub> concentration on rapidly extractable RuBP carboxylase activity in intact plants is presented in Figure 4. Prior to illumination, both mutant and wild-type have similar levels of activity. Following the onset of illumination, wild-type activity increases, whereas activity in the mutant decreases. High levels of atmospheric CO<sub>2</sub> (1%) stimulate the activity observed in wild-type extracts but do not prevent the decline of activity observed in mutant extracts. From these results it is apparent that the *in vivo* activation state of RuBP carboxylase can be modulated by light and CO<sub>2</sub>. It is also apparent that the mutant either lacks this mechanism for activating carboxylase or light brings about production of an inhibitor of RuBP carboxylase activity. Whatever the precise mechanism, nothing prevents the bicarbonate stimulated activation of the enzyme from the mutant to normal levels of activity once it is released from the cell. This is indicated in Figure 5. In this experiment, leaf homogenates were prepared from illuminated wild-type and mutant plants as described above. After 15 min of incubation, during which time no change took place in the activation state of the enzyme from the mutant, bicarbonate was added to the extracts, whereupon the carboxylase activity of the mutant extract rapidly increased to an approximately wild-type level. Thus, it seems that something in the chloroplast milieu is specifically preventing bicarbonate activation of the enzyme *in vivo*, even at external CO<sub>2</sub> concentrations 30-fold greater than that normally found in air.

**Activation State of Carboxylase in Protoplasts.** To examine the lack of activation of RuBP carboxylase in an experimental system more amenable to kinetic studies, the effect was reexamined in protoplasts isolated from mutant and the wild-type leaves. Commonly used assay methods, in which samples are withdrawn from an illuminated cuvette (19), were found to be unsatisfactory,

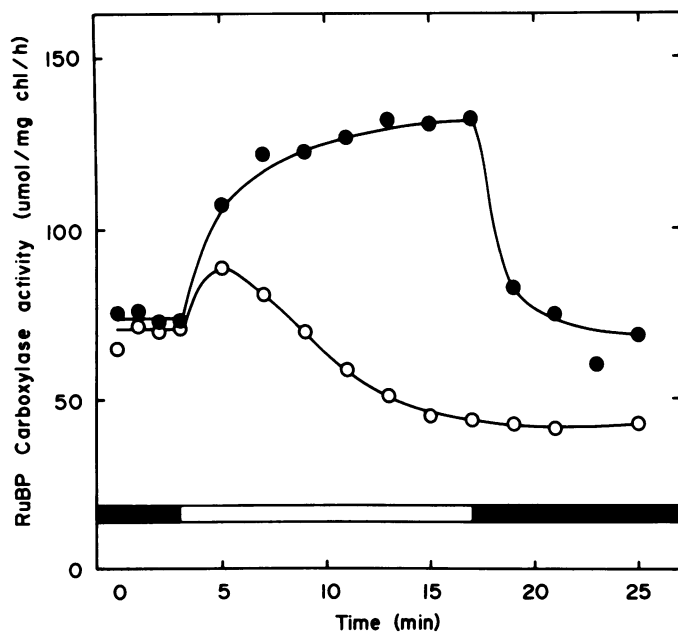


FIG. 6. Light-induced changes in the activation state of RuBP carboxylase in protoplasts of wild-type and mutant *Arabidopsis*. The bar inserted in the figure represents the conditions of illumination. Each point represents the average of two independent experiments. (●), wild-type; (○), mutant CS2071.

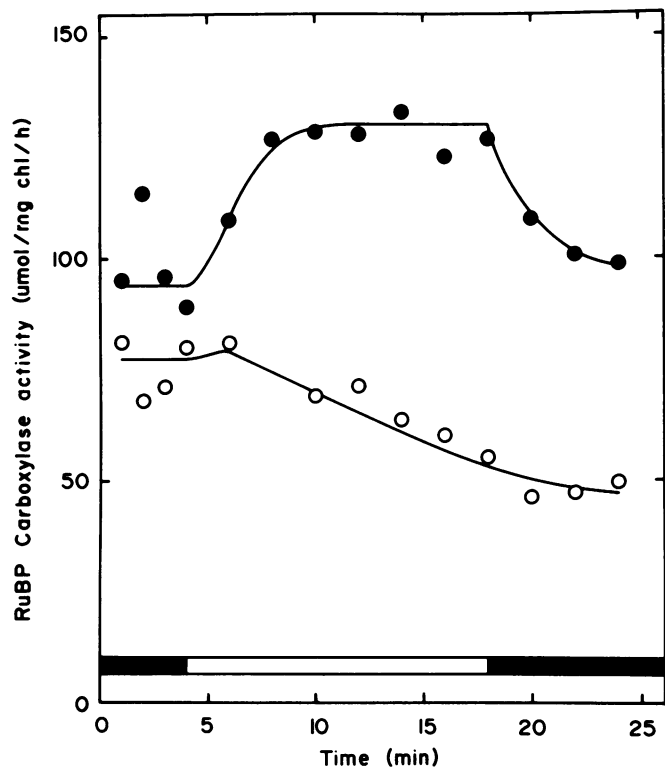


FIG. 7. The effect of high levels of bicarbonate on the activation state of RuBP carboxylase in protoplasts of wild-type and mutant *Arabidopsis*. (●), wild-type; (○), mutant CS2071.

inasmuch as the protoplasts were subject to mechanical injury and the CO<sub>2</sub>-bicarbonate level could not be maintained at low concentrations because of photosynthetic depletion. These problems were circumvented by distributing protoplast suspensions of low Chl concentration as small droplets which were continually flushed with a gas stream of the desired composition (350 μl/l CO<sub>2</sub>, 2%

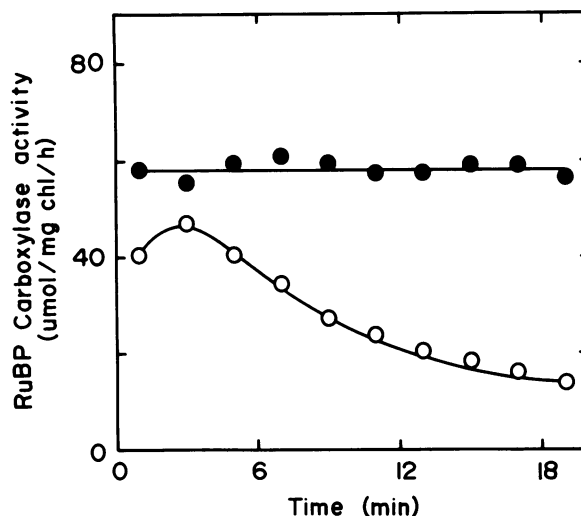


FIG. 8. RuBP carboxylase activity in isolated chloroplasts from wild-type and mutant *Arabidopsis*. Small (50 μl) aliquots of chloroplast suspensions were illuminated at 25°C, and gassed continuously with 350 μl/l CO<sub>2</sub>, 2% O<sub>2</sub>, balance N<sub>2</sub>. At the indicated intervals RuBP carboxylase activity was determined. (●), wild-type; (○), mutant CS2071.

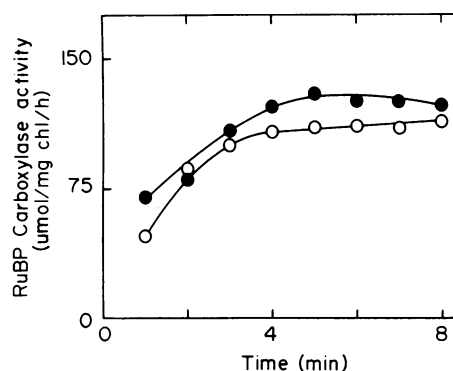


FIG. 9. RuBP carboxylase activity in isolated chloroplasts from mutant and wild-type *Arabidopsis* in presence of high levels of bicarbonate. Chloroplasts were gassed with CO<sub>2</sub>-free argon for 15 min at 23°C, then placed in an illuminated cuvette containing 0.1 mM Pi and 5 mM NaHCO<sub>3</sub>. At the indicated times, aliquots were withdrawn and assayed for RuBP carboxylase activity. (●), wild-type; (○), mutant CS2071.

O<sub>2</sub>, balance N<sub>2</sub>). The activation state of RuBP carboxylase was then determined by lysing the protoplasts with an assay mixture containing detergent.

Upon illumination of wild-type protoplasts, RuBP carboxylase activity increased by about 70% (Fig. 6). Cessation of illumination caused a rapid decline in activity to preillumination levels. Thus, the activation state of wild-type carboxylase is clearly modulated by light.

Illumination of protoplasts from the mutant invokes a transient increase of carboxylase activation state which subsequently declines to about 30% of the maximal level of wild-type activity. Cessation of illumination did not cause any immediately apparent change of activity levels. These results are therefore consistent with similar experiments performed on intact plants but show, in addition, that light induces a transient increase in the activation state of carboxylase. The kinetics of this response suggest the depletion or accumulation of a regulatory metabolite rather than a defect in a regulatory protein or other structural component of the chloroplast. As with intact plants, the provision of high (5 mM) levels of bicarbonate to the protoplast suspensions did not overcome the decline in activation state of RuBP carboxylase in the mutant (Fig. 7).

**Chloroplast Studies.** In addition to  $\text{CO}_2$ ,  $\text{Mg}^{2+}$  and Pi have been reported to be required for activation of RuBP carboxylase (5, 11). Inasmuch as protoplasts are impermeable to these compounds, we sought to establish conditions for reproducing the effect in chloroplasts so that possible regulatory compounds, such as Pi, could be provided exogenously. By employing conditions similar to those used with protoplasts and maintaining the  $\text{CO}_2$  concentration at nearly normal levels, the chloroplasts from the mutant were clearly distinguishable from those of the wild-type in that RuBP carboxylase activity declined during illumination in the mutant but remained stable in the wild-type (Fig. 8). Thus, the effect of the mutation in CS2071 is expressed in isolated chloroplasts provided with optimal levels of exogenous Pi. We conclude that the loss of RuBP carboxylase activity in illuminated protoplasts is due to a defect in some aspect of chloroplast structure or metabolism.

Experiments with chloroplasts maintained in normal levels of  $\text{CO}_2$  were subject to more variability than with protoplasts. For instance, carboxylase activity in wild-type chloroplasts occasionally exhibited a bicarbonate-reversible decline during illumination which seemed to be due to photosynthetic  $\text{CO}_2$  depletion of the medium. Thus, it was important to use low Chl concentrations and rates of gassing high enough to permit  $\text{CO}_2$  replenishment but not so high as to result in substantial evaporation from the chloroplast suspensions. The stimulation of RuBP carboxylase activity by light was also very variable with chloroplasts and appeared to depend primarily on the preincubation conditions. For instance, in the experiment presented in Figure 8, the carboxylase activity in wild-type chloroplasts showed no increase following illumination. In contrast, chloroplasts which were preincubated in  $\text{CO}_2$ -free conditions before use showed a relatively low level of RuBP carboxylase activity which increased during subsequent illumination in high (5 mM) levels of bicarbonate (Fig. 9). It appears that chloroplasts differ in some significant way from protoplasts with respect to the effect of illumination on RuBP carboxylase activity. The cause of this difference is not known.

The results presented in Figure 9 are of interest in that the provision of high levels of bicarbonate apparently prevented the decline of RuBP carboxylase activity in chloroplasts from the mutant. This observation suggests that the inactivation phenomenon is not obligatory and is probably due to a metabolic defect rather than to a structural defect in the chloroplast. The effect of high  $\text{CO}_2$  could be due to the prevention of the accumulation of an inhibitor of carboxylase activation, or,  $\text{CO}_2$  could overcome a deficiency of a required activator in the mutant.

**Chloroplast Labeling Studies.** The data suggest that loss of activation potential of carboxylase in the mutant is due to a metabolic effect. However, aside from the accumulation of RuBP, labeling studies with intact plants did not reveal the presence of

any unusual compounds or the absence of any commonly observed metabolites. Such studies are of restricted utility in that they measure only those compounds which are rapidly labeled with  $^{14}\text{CO}_2$ . In order to extend the range of measurable compounds, and to confine the analysis to chloroplast metabolites, we measured the levels of chloroplast metabolites by illuminating isolated chloroplasts from mutant and wild-type in medium containing  $^{32}\text{Pi}$  and different levels of bicarbonate. The results of this experiment are present in Table III. The results are consistent with labeling studies performed on whole plants in that RuBP levels are significantly higher in the chloroplasts from the mutant than in wild-type chloroplasts. The distribution of label is otherwise very similar between the two chloroplast types except that the mutant has a significantly higher ATP:ADP ratio. This appears to be due to an increase in the absolute amount of ATP rather than a decrease in ADP. Thus, although the ATP:ADP ratio is anomalous, this experimental approach failed to resolve the presence of a potentially regulatory compound.

**Genetic Analysis.** The genetic basis for the defect in mutant CS2071 was examined by measuring the frequency of the growth requirement for  $\text{CO}_2$  in a segregating population which resulted from a cross between the wild-type and CS2071. The F1 plants resulting from this cross were of wild-type phenotype, suggesting a recessive mutation. The phenotype segregated in the resulting F2 population 205:72 (wild-type:mutant). The excellent agreement ( $\chi^2 = 0.15$ ;  $p > 0.9$ ) with the expected 3:1 ratio indicates that a single, recessive nuclear mutation is responsible for the mutant phenotype. We have tentatively designated the altered gene by the symbol *rca* (regulation of carboxylase activation).

## DISCUSSION

The primary difference between the *rca* mutant and the wild-type parent is the apparent inability of the mutant to activate RuBP carboxylase *in vivo*. RuBP carboxylase appeared to be the only chloroplast enzyme affected, inasmuch as the results of labeling experiments did not reveal any perturbations of Calvin cycle activity which could not be explained on this basis. Also, the provision of high levels of  $\text{CO}_2$  overcame the deleterious effects of the mutation on photosynthesis. The simplest interpretation of this is that high  $\text{CO}_2$  restored photosynthesis by overcoming the high  $K_m(\text{CO}_2)$  of the nonactivated form of RuBP carboxylase. This indicates that the other enzymes of the Calvin cycle are normally active in the mutant. Thus, it is extremely unlikely that the *rca* mutation affects chloroplast  $\text{Mg}^{2+}$  levels or stomal pH, factors which have previously been suggested to be involved in the regulation of RuBP carboxylase and several other chloroplast enzymes (3). Our results do not exclude the possible involvement of  $\text{Mg}^{2+}$  or pH but indicate the participation of another, enzyme specific, regulatory agent.

It is not at present clear whether the altered regulation of RuBP carboxylase in the mutant reflects the presence of an inhibitor of activation or the absence of an activator. No inhibition of bicarbonate activation was observed in crude extracts of leaves. However, an inhibitor may have been diluted or inactivated during enzyme extraction. The decline in the activation state of RuBP carboxylase following illumination of protoplasts (Fig. 6) is probably most simply explained by the accumulation of an inhibitor of activation. In this respect it is worth noting that the concentration of the proposed inhibitor would have to be present in approximately stoichiometric amounts with the number of RuBP carboxylase active sites, and would have to be a compound which undergoes a quantitative or qualitative change during illumination of chloroplasts. In this context, the *rca* mutation could be envisioned as affecting an enzyme which normally prevents the accumulation of the inhibitory compound by promoting conversion to something innocuous, or by preventing the synthesis of the inhibitory substance. Whatever the case, it is particularly intriguing

Table III. Incorporation of  $^{32}\text{Pi}$  into Chloroplast Metabolites in Chloroplasts from Mutant and Wild-type *Arabidopsis*

Metabolite	0.1 mM $\text{NaHCO}_3$		5 mM $\text{NaHCO}_3$	
	Wild-type	CS2071	Wild-type	CS2071
	$\%$ total $^{32}\text{Pi}$ in chloroplast			
Sugar monophosphates	6.6	5.7	6.9	6.1
Pi	47.2	44.2	41.9	44.6
P-glycevate	26.9	17.6	34.7	22.3
SBP + FBP	5.1	5.7	4.9	6.3
RuBP	6.9	15.9	2.2	9.9
ADP	5.7	4.7	6.2	5.2
ATP	1.8	6.1	2.1	5.6
	$\mu\text{mol mg}^{-1} \text{Chl h}^{-1}$			
Photosynthesis rate	25.9	24.9	48.7	44.3

that it is activation, rather than catalysis, which appears to be altered in the mutant.

Several attempts to identify a regulatory compound were not successful. The only compounds which were found to be present in abnormal amounts were RuBP and perhaps ATP. The simplest explanation for the accumulation of these compounds is reduced utilization because of reduced carboxylase activity. It is, however, also possible that the accumulation of RuBP is the cause of reduced carboxylase activity rather than the effect. Laing and Christeller (8) reported that RuBP may bind the nonactivated form of the enzyme and prevent subsequent carbamate activation. A similar argument has been invoked to explain dithioerythritol-induced loss of RuBP carboxylase activation in isolated chloroplasts (6). The dithioerythritol presumably exerted this effect by activating phosphoribulokinase under conditions which permitted RuBP synthesis to exceed utilization by the carboxylase. A mutation which altered the regulation of Ru5P kinase might be expected to produce the same effect.

Comparison of the results obtained with intact leaves, protoplasts, and isolated chloroplasts suggests a discrepancy with respect to the effect of high CO<sub>2</sub> on the activation state of RuBP carboxylase in the mutant. Illumination of leaves in 1% CO<sub>2</sub>, or of protoplasts in a medium containing high bicarbonate concentrations did not overcome the defect. However, when chloroplasts from the mutant were illuminated in media containing high levels of bicarbonate, the activation state of the carboxylase was not distinguishable from that observed in the wild-type. The effect was apparently due only to bicarbonate levels since illumination of chloroplasts in the same medium, but lacking added bicarbonate, caused loss of activation of the carboxylase. This apparent discrepancy suggests the possible involvement of an extrachloroplastic factor such as Pi, which has previously been implicated in the regulation of RuBP carboxylase in isolated chloroplasts (5). Although we were unable to find a difference between the mutant and the wild-type in leaf Pi levels or in the kinetics of Pi transport by isolated chloroplasts (results not presented), it remains possible that the intracellular distribution of Pi or some other metabolite is abnormal in the mutant. Alternatively, the highly artificial conditions employed in experiments with isolated chloroplasts may have resulted in an artifactual suppression of the defect with little or no physiological significance.

The characteristics of the *rca* mutant are not explicable on the basis of our current understanding of the properties of RuBP carboxylase and the chloroplast milieu. Although we have been unable to establish the nature of the primary genetic lesion, the characteristics of the mutant must be accounted for in any future models concerning the regulation of Calvin cycle activity. The mutant may also be useful as parental material in future searches for mutations which increase the rate of CO<sub>2</sub>-fixation. That is, since the factor limiting CO<sub>2</sub>-fixation by the mutant is RuBP carboxylase, it may be possible to select directly for an alteration in the carboxylase which renders it independent of the regulatory agent.

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