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Catalysis of Ribulosebisphosphate Carboxylase/Oxygenase Activation by the Product of a Rubisco Activase cDNA Clone Expressed in *Escherichia coli*

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ABSTRACT

Ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) activase activity was obtained from a partially purified extract of *Escherichia coli* transformed with a 1.6-kilobase spinach (*Spinacia oleracea* L.) cDNA clone. This activity was ATP-dependent. Catalysis of rubisco activation by spinach and cloned rubisco activase was accompanied by the same extent of carboxyarabinitol bisphosphate-trapped $^{14}CO_2$ as occurred in spontaneous activation, indicating that rubisco carbamylation is one facet of the rubisco activase reaction. The CO_2 concentration required for one-half maximal rubisco activase activity was about 8 micromolar CO_2. These observations are consistent with the postulated role of rubisco activase in regulating rubisco activity *in vivo*.

The initial reactions in photosynthetic CO_2 reduction and photorespiratory carbon oxidation are catalyzed by rubisco[1] (6). Catalysis of carboxylation or oxygenation by rubisco, and thus of photosynthesis and photorespiration, occurs only when the enzyme is in an activated state. Rubisco activation *in vitro* can be achieved by the spontaneous addition of CO_2 in the presence of Mg^{2+} to form a carbamylated enzyme (4), and a similar mechanism was proposed for activation *in vivo* (4, 8). However, research in this laboratory identified a mutant strain of the small mustard plant *Arabidopsis thaliana* that is incapable of activating rubisco (15) and demonstrated that a chloroplast stromal extract from wild-type *Arabidopsis*, but not the mutant, restored rubisco activation in a reconstituted chloroplast system (12). Further, a protein possessing this activity was purified from spinach chloroplasts (14). Thus, it was concluded that rubisco activation *in vivo* did not occur spontaneously but required the presence of a soluble chloroplast protein which was designated rubisco activase (10, 12, 14). We have now further established the existence of rubisco activase by isolating, from *Escherichia coli* transformed with a specific spinach leaf cDNA clone, a protein fraction which promotes the activation of rubisco.

MATERIALS AND METHODS

Rubisco Activase Purification and Assays. All purification procedures were conducted at 4°C. Spinach (*Spinacia oleracea* L.) rubisco activase was obtained by lysing intact chloroplasts (24 mg Chl) with a 10-fold dilution into a lysis buffer of 20 mM bis-Tris-HCl (pH 7.0), 4 mM BME, 1 mM ATP, 1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, and 0.5 μM antipain. After centrifugation at 30,000*g* for 15 min, the supernatant was loaded onto a QAE-Sepharose[2] column (2.5 × 10 cm) equilibrated with 20 mM bis-Tris-HCl, pH 7.0, and 4 mM BME. The protein was eluted with a 0 to 400 mM linear KCl gradient in 2 L of the column equilibration buffer. Fractions eluting at 170 to 190 mM KCl were pooled, concentrated to about 1 mg/ml by ultrafiltration, and stored in liquid N_2.

Escherichia coli rubisco activase was obtained from *E. coli* strain JM101 transformed with the plasmid pRCA1.6 (17). Cultures were grown overnight at 37°C in Luria-Bertani broth containing 0.2 g/L ampicillin and brought to 1 mM isopropyl-β-D-thiogalactopyranoside 3 h prior to extraction. Cells (14 g fresh weight) were suspended in 140 ml of the lysis buffer described above, exposed to 500 psi N_2 in a pressure bomb, and ruptured upon pressure release. Following centrifugation at 30,000*g* for 15 min, the supernatant was fractioned, concentrated, and stored as described above for the spinach stromal extract.

Spinach rubisco was purified and then deactivated by gel filtration on Sephadex G-25 with N_2-sparged 50 mM Tricine-NaOH, pH 8.0, containing 4 mM BME. RuBP was added to a final concentration of 3 mM. Reactions were initiated with deactivated rubisco-RuBP. After 16 min at 25°C, 50 μl aliquots were removed and rubisco activity was determined (10). Complete assay mixtures contained 50 mM Tricine-NaOH (pH 8.0), 10 mM $MgCl_2$, 3 mM $NaHCO_3$, 3 mM RuBP, 2 mM ATP, an ATP regenerating system (12 mM phosphocreatine and 0.3 unit creatine phosphokinase), 19 μg deactivated rubisco, and the indicated amount of rubisco activase protein in a final volume of 125 μl.

Electrophoresis. After fractionation of proteins from spinach stroma and transformed *E. coli*, 10-μl aliquots of selected fractions were electrophoresed in 12.5% acrylamide resolving gels using the buffers described by Laemmli (3). In one set of gels (Fig. 1, top) the protein was visualized by a silver stain procedure. In replicate gels (Fig. 1, bottom), the rubisco activase proteins were detected after electroblotting onto nitrocellulose, probing the blot with mouse anti-rubisco activase polyclonal antibodies, and visualizing with goat anti-mouse IgG alkaline phosphatase conjugate (14).

Activator $^{14}CO_2$ Trapping with CABP. Reaction mixtures con-

[1] Abbreviations: rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; CABP, carboxyarabinitol-1,5-bisphosphate; RuBP, ribulose-1,5-bisphosphate; BME, β-mercaptoethanol

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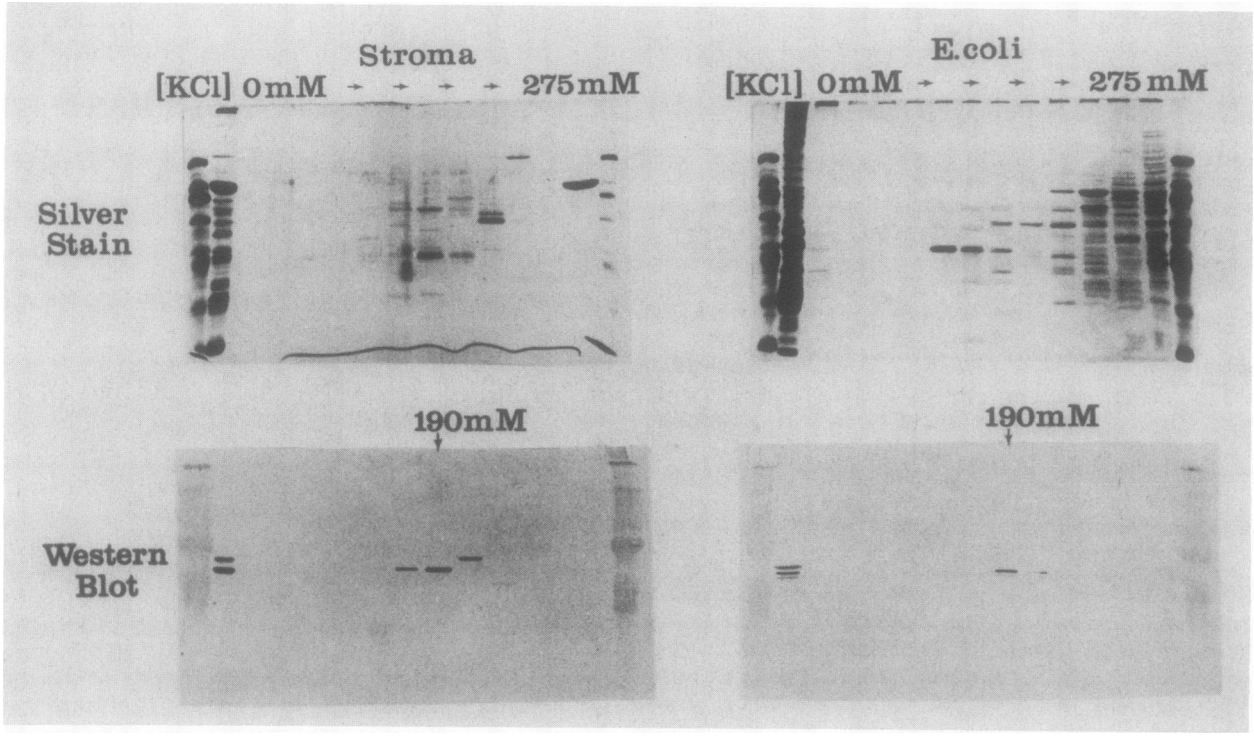

FIG. 1. Gel electrophoresis and immunoblots of rubisco activase fractions isolated from spinach chloroplasts and transformed *E. coli*. Lanes 1 and 15 contained M_r markers ranging from 86 to 26 kD, lane 2 contained the crude extracts, and lanes 3 to 14 contained selected fractions eluted with increasing KCl concentrations between 0 and 275 mM.

tained 50 mM Tricine-NaOH (pH 8.0), 1 mM $NaHCO_3$, 20 mM $MgCl_2$, and 0.25 mg/ml of deactivated rubisco. In assays with rubisco activase, the deactivated rubisco was mixed with 3 mM RuBP and 0.25 mg/ml rubisco activase. The complete reaction mixtures (1.0 ml) also contained the ATP regenerating system described above. Reaction mixtures without ATP did not contain the ATP regenerating system. Reactions were initiated by the addition of deactivated rubisco. Rubisco activity was determined by removing aliquots at the indicated times and measuring activity. Determination of activator CO_2 binding was carried out in the same activation mixtures supplemented with $NaH^{14}CO_3$ (15 Ci/mol). Aliquots (100 μl) were removed at the indicated times and mixed with 100 μl of a trapping solution containing 50 mM Tricine-NaOH (pH 8.0), 20 mM $MgCl_2$, 120 mM $NaHCO_3$, and 4 mM CABP. The samples were incubated at 25°C for 16 h. The rubisco-CO_2-Mg^{2+}-CABP quaternary complex was purified by Sephadex G-50 gel filtration in 50 mM Tricine-NaOH, pH 8, containing 100 mM $NaHCO_3$ and 1 mM DTT. Radioactivity in this complex was determined by liquid scintillation counting.

CO_2 Response Curve. Reactions were carried out in 2-ml serum-capped tubes flushed continuously with 30 ml/min of N_2 or the appropriate CO_2-air mixture as quantitated with an inline infrared gas analyzer. All solutions were prepared from boiled, N_2-sparged water, and maintained under soda lime traps to exclude CO_2. Rubisco activase reaction mixtures contained 50 mM Tricine-NaOH (pH 8.0), 10 mM $MgCl_2$, 3 mM RuBP, the ATP regenerating system described above, and 7.5 μg of deactivated rubisco. The free Mg^{2+} concentration was calculated to be about 3 mM. The reaction mixtures (40 μl) were flushed with the appropriate gas for 10 min at 25°C, and 7.5 μg of spinach rubisco activase was added to start the reactions. Rubisco activity was determined after 16 min of incubation with gas flushing. Activity at 0 μM added CO_2 indicated that 1 to 2 μM CO_2 still remained in the reaction mixtures after the N_2-sparging treatment.

RESULTS AND DISCUSSION

A 1.6-kb spinach cDNA clone coding for rubisco activase was isolated immunologically from a λgt11 cDNA library (17). *E. coli* was transformed with a plasmid harboring this cDNA, pRCA1.6, and was grown and used as a source of rubisco activase. The rubisco activase protein was partially purified from the *E. coli* lysate, and chloroplast stromal extracts, by passage through a QAE-Sepharose column (Fig. 1). Fractions eluting at 170 to 190 mM KCl were pooled, concentrated, and assayed. These fractions contained polypeptides with apparent M_r of 41 and 45 kD for spinach and 45 kD for *E. coli*. The pooled fractions from both chloroplasts and *E. coli* catalyzed an ATP-dependent activation of rubisco (Table I). The extent of activation was dependent upon the amount of *E. coli* protein added. Because the amount of rubisco activase protein was approximately 20-fold less in *E. coli* lysates than in stromal extracts, the specific

Table I. *Rubisco Activation*

Rubisco activation catalyzed by the pooled 170-190 mM KCl fractions of extracts of spinach stromas and of *E. coli* transformed with a specific spinach leaf cDNA.

Rubisco Activase Source	Protein	Reaction Mixture	Rubisco Activity
	μg		$\mu mol\ CO_2/min \cdot mg$ protein
Spinach	7.5	Complete	0.385
	7.5	Minus ATP	0.030
E. coli	75	Complete	0.163
	38	Complete	0.077
	8	Complete	0.004
	75	Minus ATP	0.001
(boiled)	75	Complete	0

activity of *E. coli* protein was much less than stromal protein. Boiled *E. coli* protein did not catalyze rubisco activation (Table I). Further, untransformed *E. coli* cells did not produce any anti-rubisco activase immunoreactive polypeptides (17). Thus, the cDNA in the pRCA1.6 construct encodes a protein possessing rubisco activase activity. As reported previously for the spinach enzyme (11, 16), ATP is required for activity.

The pRCA1.6 vector (Fig. 2) was constructed by ligating a 1.6-kb cDNA, representing most of the coding region of rubisco activase mRNA, into the *Eco*RI site of the multiple cloning region of plasmid pUC8, utilizing the lacZ promoter for transcription (17). The fusion protein synthesized by this construction has several more amino acids at the N-terminal end than the mature wild-type polypeptide. At the N-terminal end of the cloned rubisco activase there are 7 amino acids derived from the pUC8 plasmid, 4 from a cloning artifact, and 19 from the 58-amino acid transit peptide. The last 36 residues of the 414-amino acid mature polypeptide (17) are missing because of early termination. However, the cloned rubisco activase polypeptide contained two consensus nucleotide binding sites, the only known features of the protein other than the transit peptide, and exhibited enzymatic activity (Table I, Fig. 3). The complete derived amino acid sequence of the protein synthesized by pRCA1.6 is given in Werneke *et al.* (17).

The spontaneous activation of rubisco is accompanied by carbamylation of lysine-201 near the active site (5). This activating CO_2, which is distinct from substrate CO_2 (5), can be trapped on rubisco by the addition of CABP, an analog of the carboxylation reaction intermediate (9). Rubisco activation catalyzed by the enzyme isolated from spinach chloroplasts and by the enzyme from cloned spinach cDNA was also accompanied by CABP-trapped activator CO_2, with an identical quantitative relationship between CO_2 trapped and the extent of activation in all cases (Fig. 3). In the absence of ATP, there was neither activation of rubisco nor CABP-trapped CO_2 (Fig. 3). Because these experiments were conducted at limiting CO_2 concentration, the extent of carbamylation at maximum activation state was about 1.1 mol CO_2/mol rubisco for the spontaneous and spinach rubisco activase catalyzed reactions, and about 0.3 mol CO_2/mol enzyme for the reaction with cloned rubisco activase.

The discovery of an enzyme catalyzing rubisco activation, and the partial characterization of this process (10, 12, 16), suggests resolutions to two puzzles encountered in attempting to explain activation *in vivo* by the spontaneous mechanism. First, the substrate RuBP is a potent inhibitor of spontaneous activation

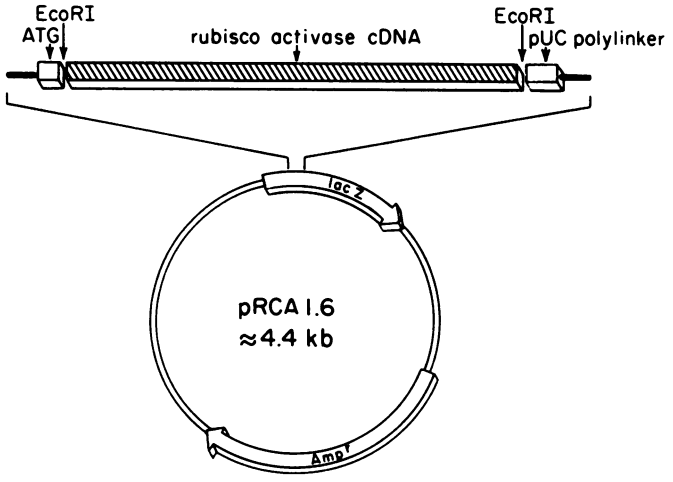

FIG. 2. Construction of the pRCA1.6 vector. Experimental details of the construction and the derived amino acid sequence of the cloned rubisco activase gene product are given in Werneke (17).

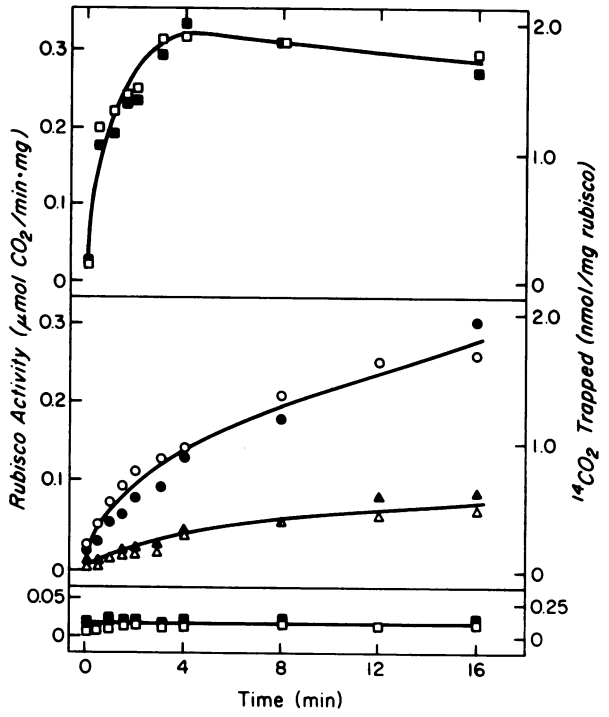

FIG. 3. The kinetics of rubisco activation and CABP-trapped CO_2 in the presence or absence of rubisco activase. Upper panel, spontaneous rubisco activation (□) and CABP-trapped $^{14}CO_2$ (■) of spinach rubisco; middle panel, activation of rubisco-RuBP catalyzed by ATP and rubisco activase from spinach (○) and from *E. coli* (△), and CABP-trapped $^{14}CO_2$ (●, ▲) from the same reaction mixtures; lower panel, activation of rubisco-RuBP (□) and CABP-trapped $^{14}CO_2$ (■) in the absence of ATP.

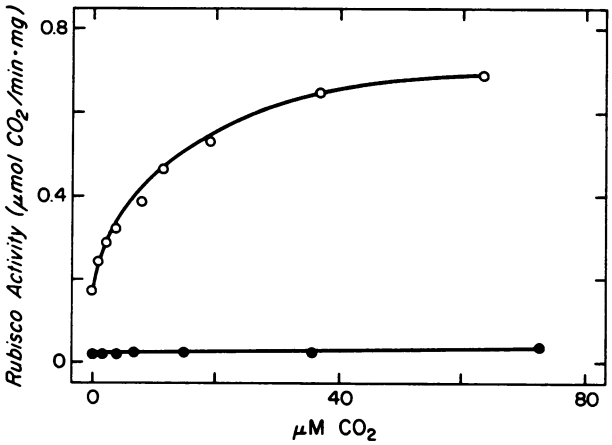

FIG. 4. The effect of CO_2 concentration on rubisco activation at 3 mM RuBP in the presence (○) or absence (●) of spinach rubisco activase.

(1), forming a stable and inactive rubisco-RuBP complex. Little or no spontaneous activation of this complex occurred at the typical chloroplast RuBP concentration of 3 to 6 mM (Figs. 3 and 4) (16). It is evident that this inhibition of activation was overcome by the presence of rubisco activase and ATP (Fig. 3) (16). Second, the CO_2 concentration required for the spontaneous activation of rubisco to one-half of full activity is 25 to 30 μM CO_2 (2, 4), but the enzyme in a leaf is nearly fully activated at the atmospheric CO_2 concentration of 8 to 10 μM (8, 13). In a reconstituted chloroplast system the CO_2 concentration for one-half rubisco activation catalyzed by rubisco activase was about 5 μM CO_2 (10). In the soluble system used here this value was

about 8 μM CO_2 (Fig. 4). Thus, the affinity for CO_2 in the rubisco activase catalyzed activation is substantially greater than in spontaneous activation and in the range of physiological CO_2 concentration. Rubisco activation did not occur spontaneously over a wide range of CO_2 concentrations in the presence of RuBP at concentrations found in illuminated chloroplasts (Fig. 4).

The ATP-dependent catalysis of rubisco activation by the product of a spinach gene expressed in *E. coli* confirms the existence of rubisco activase activity. As was observed during spontaneous activation (5), the reaction intermediate analog CABP trapped CO_2 on the activated rubisco, and the extent of activation per bound CO_2 was similar in both the spontaneous and rubisco activase-catalyzed activation processes. Further, rubisco was activated by rubisco activase and ATP at RuBP concentrations observed in chloroplasts of illuminated leaves, but not by the spontaneous activation process. These observations suggest that ATP provides the energy required to activate the enzyme-RuBP complex, either by directed carbamylation or by creating a conformational change in the rubisco-RuBP complex which is stabilized by subsequent carbamylation.

The activation state of rubisco *in vivo* is an important regulator of photosynthesis. It has been shown, for example, that rubisco activation state and leaf photosynthesis in *Arabidopsis* exhibit near identical responses to incident light intensity (13). Such modulation of rubisco activity is necessary because if the reactions catalyzed by rubisco proceeded at maximal velocity under conditions where the rate of RuBP synthesis is low, such as at low light intensity, then RuBP would become less than saturating and lead to oscillations in the photosynthesis rate (7). Previous research in this laboratory (10, 12) led to the suggestion that the regulation of rubisco activation and deactivation was provided by rubisco activase and RuBP, respectively. In this conceptual framework, RuBP inhibits rubisco activity by forming the inactive rubisco-RuBP complex, and the complex is activated to an appropriate extent by rubisco activase and ATP. In this manner RuBP and rubisco activase maintain the essential balance between the rates of RuBP synthesis by the photosynthetic carbon cycle and of RuBP utilization by carboxylation and oxygenation. The mechanisms by which environmental stimuli such as light intensity are sensed and measured by the rubisco activase system remains to be determined.

LITERATURE CITED

1. JORDAN DB, R CHOLLET 1983 Inhibition of ribulose bisphosphate carboxylase by substrate ribulose 1,5-bisphosphate. J Biol Chem 258: 13752–13758
2. JORDAN DB, WL OGREN 1981 A sensitive assay procedure for simultaneous determination of ribulose-1,5-bisphosphate carboxylase and oxygenase activities. Plant Physiol 67: 237–245
3. LAEMMLI UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685
4. LORIMER GH, MR BADGER, TJ ANDREWS 1976 The activation of ribulose 1,5-bisphosphate carboxylase by carbon dioxide and magnesium ions. Equilibria kinetics, a suggested mechanism and physiological implications. Biochemistry 15: 529–536
5. LORIMER GH, HM MIZIORKO 1980 Carbamate formation on the ϵ-amino group of a lysyl residue as the basis for the activation of ribulosebisphosphate carboxylase by CO_2 and Mg^{2+}. Biochemistry 19: 5321–5328
6. OGREN WL 1984 Photorespiration: pathways, regulation and modification. Annu Rev Plant Physiol 35: 415–442
7. OGREN WL, ME SALVUCCI, AR PORTIS JR 1986 The regulation of rubisco activity. Phil Trans R Soc Lond B313: 337–346
8. PERCHOROWICZ JT, DA RAYNES, RG JENSEN 1981 Light limitation of photosynthesis and activation of ribulose bisphosphate carboxylase in wheat seedlings. Proc Natl Acad Sci USA 78: 2985–2989
9. PIERCE J, NE TOLBERT, R BARKER 1980 Interaction of ribulose bisphosphate carboxylase/oxygenase with transition state analogues. Biochemistry 19: 934–942
10. PORTIS AR JR, ME SALVUCCI, WL OGREN 1986 Activation of ribulosebisphosphate carboxylase/oxygenase at physiological CO_2 and ribulosebisphosphate concentrations by rubisco activase. Plant Physiol 82: 967–971
11. ROBINSON SP, AR PORTIS JR 1988 Involvement of stromal ATP in the light activation of ribulose-1,5-bisphosphate carboxylase/oxygenase in intact isolated chloroplasts. Plant Physiol 86: 293–298
12. SALVUCCI ME, AR PORTIS JR, WL OGREN 1985 A soluble chloroplast protein catalyzes activation of ribulosebisphosphate carboxylase/oxygenase *in vivo*. Photosynth Res 7: 193–201
13. SALVUCCI ME, AR PORTIS JR, WL OGREN 1986 Light and CO_2 response of ribulose 1,5-bisphosphate carboxylase/oxygenase activation in *Arabidopsis* leaves. Plant Physiol 80: 655–659
14. SALVUCCI ME, JM WERNEKE, WL OGREN, AR PORTIS JR 1987 Purification and species distribution of rubisco activase. Plant Physiol 84: 930–936
15. SOMERVILLE CR, AR PORTIS JR, WL OGREN 1982 A mutant of *Arabidopsis thaliana* which lacks activation of RuBP carboxylase *in vivo*. Plant Physiol 70: 381–387
16. STREUSAND VJ, AR PORTIS JR 1987 Rubisco activase mediates ATP-dependent activation of ribulosebisphosphate carboxylase. Plant Physiol 85: 152–154
17. WERNEKE JM, RE ZIELINSKI, WL OGREN 1988 Structure and expression of spinach leaf cDNA encoding rubisco activase. Proc Natl Acad Sci USA 85: 787–791