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SHORT COMMUNICATION

Immunoblot Analysis of the Expression of Genes for Barley Rubisco Activase in *E. coli*

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Rubisco activity during photosynthesis is regulated by the rubisco activase, which facilitates the dissociation of RuBP and other inhibitory sugar phosphates from the active site of rubisco in an ATP-dependent reaction. In this paper, barley *Rca* genes (*Rca*A1, *Rca*A2 and *Rca*B) were expressed in *E. coli* and the activity of rubisco activase expressed was assayed biochemically by chromatography. Then the protein was identified electrophoretically by SDS-PAGE and detected immunologically by Western blot analysis using polyclonal antibodies raised against the kidney bean rubisco activase as probe. The band pattern of purified proteins on the polyacrylamide gel showed two polypeptides of 46 kD and 42 kD. Anti-rubisco activase antibodies reacted specifically with both polypeptides of 46 kD and 42 kD present in the crude extracts of *E. coli* transformants. Therefore, it was found that the genes of barley rubisco activase was successfully expressed in *E. coli* as active forms of 46 kD and 42 kD.

Keywords: *E. coli*, *Rca*A, *Rca*B, rubisco activase, immuno blot

Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) activation involves the formation of a carbamate (Werneke *et al.*, 1988a), presumably on lys 201 of the large subunit (Lorimer and Miziorko, 1980) in regulating the photosynthetic process (Stitt *et al.*, 1991). This process in vivo can not occur spontaneously but is catalyzed by rubisco activase (Rca) (Portis *et al.*, 1986; Salvucci *et al.*, 1985, 1987) in an ATP-dependent reaction (Steusand and Portis, 1987).

Rubisco activase facillitates the dissociation of RuBP and other tight-binding inhibitory sugar phosphates from the active site of decarbamylated rubisco (Lilly and Portis, 1990; Wang and Potris, 1991, 1992; Andrews *et al.*, 1995; Salvucci and Orgen, 1996) in a process requiring the hydrolysis of ATP (Streusand and Portis, 1987; Robinson and Portis, 1989a; Portis, 1992). The release of sugar phosphates promotes carbamylation of an active site lysyl residues (Portis *et al.*, 1986; Werneke *et al.*, 1988a; Robinson and Portis, 1989b), thereby completing the binding site for CO_2 (Wang *et al.*, 1992) and Mg^{2+} more rapid access (Knight *et al.*, 1990).

Rubisco activase also restores activity to carbamylated rubisco that bound with the inhibitor 2-carboxyarabinitol 1-phophate (CA1P) *in vitro* (Robinson and Potris, 1988; Portis, 1992) and 2-carboxyarabinitol 1,5-bisphophate (Roh *et al.*, 1996), but control of CA1P binding *in vivo* may be shared between activase and CA1P phosphatase (Moore and Seemann, 1994).

Rubisco activase was first identified in *Arabidopsis thaliana* as the enzyme missing in a high CO_2 requiring Rca mutant (Somerville *et al.*, 1982; Salvucci *et al.*, 1985). Subsequent analyses showed that rubisco activase protein, a soluble, nuclear-encoded chloroplast stroma enzyme, was present in most higher plants (Salvucci *et al.*, 1987). The protein is abundant, accounting for about 2% of the total soluble leaf protein (Robinson *et al.*, 1989). Rubisco activase comprise two defferent-sized polypeptides of approximately 45 kD and 41 kD in

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spinach and *Arabidopsis* (Salvucci *et al.*, 1987). The two activase polypetides in spinach are identical except for 37 additional amino acids on the C-terminal end of the 45 kD form (Crafts-Brandner *et al.*, 1997). Both the 45 and the 41 kD forms of spinach activase exhibit different kinetics toward ATP concentration (Shen *et al.*, 1991), whereas maize and the green alga *Chlamydomonas reinhardtii* contain only the 42 kD form as a single polypeptide (Roesler and Ogren, 1990; Salvucci *et al.*, 1987).

The two rubisco activase polypetides in barley is encoded by two tandemly oriented nuclear genes, *Rca*A and *Rca*B (Warncke *et al.*, 1988, 1989; Rundle and Zielinski, 1991a). The *Rca*A gene transcript is alternatively spilced to yield *Rca*A1 and *Rca*A2 mRNA that encode mature proteins of 46 and 42 kD, respectively. The *Rca*B gene transcript is spliced to yield a single mRNA encoding a 42 kD (Rundle and Zielinski, 1991b).

In this paper, the expression of *Rca* genes of barley rubisco activase in *E. coli* were studied by analysis of the polypeptide profiles of SDS-PAGE and Western blotting assay using the probe with rabbit polyclonal antibodies to rubisco activase.

MATERIALS AND METHODS

Transformation of *E. coli*

Introduction of plasmid pGEM-4Z containing *Rca*A1 gene and pBluescript-KS$^+$ containing *Rca*A2 and *Rca*B gene into the competent *E. coli* strain DH 5α were performed by method as described by Sambrook *et al.* (1989). The growing cells were suspended in 50% glycerol, and either used immediately or stored at -80°C freezer until the next experiments. The transformation was confirmed by appearance of ampicillin resistant colonies.

Culture of *E. coli* Transformant

E. coli transformed was precultured in 150 mL of LB medium. The precultures were inoculated into 4 L cultures containing 100 μg/mL ampicillin and 1 mM IPTG (isoprophyl-β-D-thiogalactopyranoside), and incubated at 37°C with shaking overnight. Growth rates were determined by measuring the OD_{600} of *E. coli* cultures at different times in its growth cycle. All experiments were repeated at least two times.

Isolation of Plasmid DNA

Plasmid DNA was isolated from *E. coli* cells transformed by method as described by Birnboim and Doly (1979). The transformant *E. coli* cells were cultured in 3 mL of LB medium containing 50 μg/mL ampicillin at 37°C for 12 h.

DNA was digested with *Eco*RI. The digested sample was load into a 1.7% agarose gel and electrophoresed at 8 V/cm for 2.5 h. A 1 kb DNA ladder was used as molecular size markers.

Isolation of Crude Extracts for Rubisco Activase from *E. coli*

The crude extracts containing rubisco activase were isolated from *E. coli* transformed as described by procedure of Wang *et al.* (1992). The transformant *E. coli* cells harvested by centrifugation at 7,000 g for 5 min was resuspended into 25 mL of extraction buffer [40 mM BTP (pH 7.0), 5 mM $MgCl_2$, 0.5 M ATP, 5 mM DTT, 2 mM benzamidine, 0.01 mM leupeptin, 2 mM PMSF and 10 μg/mL of antipain]. Then, 50,000 units/mL of lysozyme, 20 units/mL of RNase and 4.5 mL of glycerol were added, and the solution was stirred for 20 min at 4°C. The cells were disrupted by sonic treatment for 3 min bursts. A crude lysates were clarified by centrifugation for 15 min at 30,000 g. A $(NH_4)_2SO_4$ powder was slowly added into the supernatant to 40% saturation and stirred for 30 min. The precipitate was collected by centrifugation for 10 min at 10,000 g. The pellet was dissolved in 6 mL of extraction buffer and precipitated by addition of PEG-10,000 to 17%. The precipitate was collected by centrifugation for 8 min at 10,000 g, and resuspended in 10 mL of extraction buffer. The resuspended pellet contains rubisco activase.

Purification of Rubisco Activase

50% (w/v) PEG-10,000 was added into the resuspended pellet with continuous stirring to 18%, and centrifuged for 10 min at 8,000 g. The pellet was dissolved in 5 mL of buffer A [20 mM BTP (pH 7.0) containing 0.2 mM ATP, 10 mM $MgCl_2$ and 2 mM MBT]. The supernatants of centrifugation for 10 min at 20,000 g were loaded onto a 20 mL Q-Sepharose column equilibrated with 20 mM BTP (pH 7.0) and eluted with 40 mL of 20 mM BTP (pH 7.0) at a flow rate of 1 mL/min before continuing with 140 mL of a linear gradient from 0 to 0.5 M NaCl in 20 mM BTP (pH 7.0). Only a single fraction (3 mL) having the highest rubisco

activase activity was separated by SDS-PAGE.

Assay of ATP Hydrolysis by Rubisco Activase

ATP hydrolysis by rubisco activase was assayed spetrophotometrically by coupling ADP formation to NADH oxidation as indicated by decrease in absorption at 340 nm (Robinson and Portis, 1989a). Reactions contained 50 mM Tricine (pH 8.0), 20 mM KCl, 10 mM $MgCl_2$, 1 mM ATP, 1 mM phosphoenolpyruvate, 0.3 mM NADH, 40 units/mL pyruvate kinase, and 40 units/mL lactate dehydrogenase in a total volume of 0.4 mL. The reaction was initiated with addition of rubisco activase. One unit of activity was defined as 1 μM ATP hydrolyzed per min.

SDS-polyacrylamide Gel Electrophoresis

SDS-PAGE was done with 13% gel by the method of Laemmli (1970). The gel was stained with Coomassie brilliant blue R-250 to visualize the proteins.

Western Blot Analysis

Western blot analysis was carried out by the method of Harlow and Lane (1988). Proteins on gel was electrophoretically transferred to 0.2 μm nitrocellulose membrane in 48 mM Tris containing 39 mM glycine, 0.037% SDS and 20% methanol at 0.25 A of constant current for 1 h using a semi-dry transblot apparatus (Bio-Rad Lab. Hercules, CA, U. S.A.). Nitrocellulose membrane was blocked with PBS [137 mM NaCl, 2.68 mM KCl, 1.76 mM KH_2PO_4, 4 mM Na_2HPO_4 (pH 7.2)] containing 5% (w/v) nonfat dry milk, 0.2% Tween 20 and 0.02% sodium azide. Then, the protein blot was incubated with a 1:1,000 dilution of a rabbit anti-rubisco activase antiserum against kidney bean as a primary antibody (Roh *et al.*, 1997) at room temperature for 1 h, followed by four washes for 5 min each with PBS.

The membrane was incubated with a 1:5000 dilution of goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma, U.S.A.) as a secondary antibody at room temperature for 1 h, and then washed for 10 min in TBS [150 mM NaCl, 50 mM Tris (pH 7.5)]. The bound antibody was visualized by addition of 10 mL of alkaline phosphatase buffer [100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris (pH 9.5)] supplemented with 66 μL of 50 mg NBT/mL of 70% DMF and 33 μL of 50 mg BCIP/mL of 100% DMF. To stop the reaction, PBS containing 20 mM Mg-EDTA added.

All procedures were conducted at 4°C except as indicated.

RESULTS AND DISCUSSION

The gene for rubisco activase have been cloned and sequenced from *Arabidopsis* (Werneke and Ogren, 1989; Werneke *et al.*, 1989), spinch (Werneke *et al.*, 1988b, 1989), *Chlamydomonas reinhardtii* (Roesler and Ogren, 1990), barley

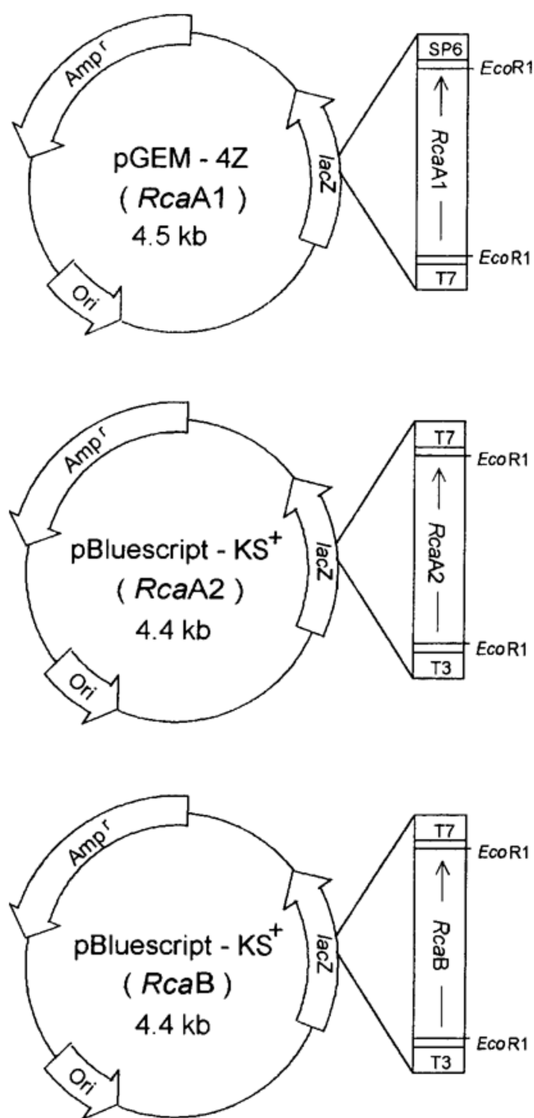

Fig. 1. Maps of the recombinant expression vector. Each expression vector was constructed by inserting *Rca*A1 or *Rca*A2 and *Rca*B gene into *Eco*RI site of either pGEM-4Z or pBluescript-KS$^+$. An arrow in each plasmid indicates transcriptional orientation.

(Rundle and Zielinski, 1991) and tobacco (Qian and Rodermel, 1993). The spinach cDNA clone, and distinct full length (*Rca*A2, *Rca*B) and near full length (*Rca*A1) cDNAs encoding barley rubisco activase were isolated from a λgt11 (Werneke *et al.*, 1988b) and a λgt10 cDNA library (Rundle and Zielinski, 1991), respectively. The three cDNAs code for tobacco rubisco activase that are from 85 to 94% similar to other activase proteins of higher plant (Qian and Rodermel, 1993).

The vectors used in this study were constructed by ligating of barley rubisco activase gene *Rca*A1, *Rca*A2, and *Rca*B into pGEM-4Z and pBluescript-KS$^+$ plasmid, utilizing the lacZ promoter for trascription (a gift from Dr. Raymond E. Zielinski at Dept. of Plant Biology, University of Illinois at Urbana-Champaign, U.S.A.). As shown in Fig 1, *Rca*A1 gene was inserted into *Eco*RI site of pGEM-4Z plasmid. *Rca*A2 and *Rca*B genes were inserted into *Eco*RI site of pBluescript-KS$^+$ plasmid, respectively.

In order to determine the optimum culture condition of *E. coli*, the growth rate was measured the OD at 660 nm during its growth. *E. coli* for the isolation of rubisco activase was cultured until the absorbance of the cultures reached 1.0. *E. coli* cells were collected at this stage and then broken with lysozyme.

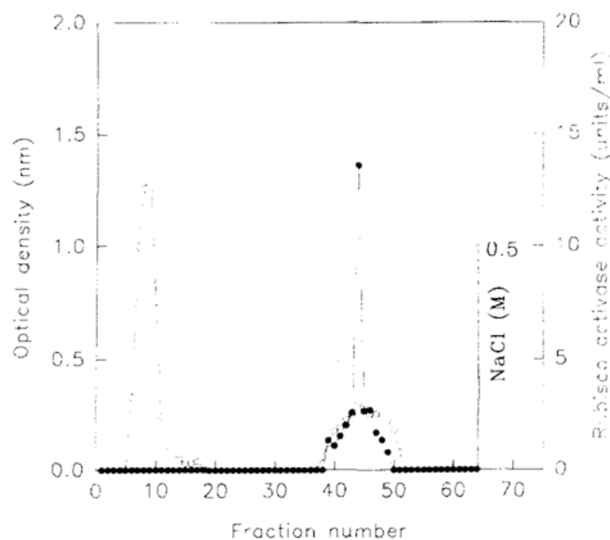

Fig. 2. Elution profile of rubisco activase from anion exchange chromatography on Q-Sepharose column. The sample was eluted from the column by increasing NaCl concentration from 0 to 0.5 M in 20 mM BTP (pH 7.0). Protein concentration (○) was monitored by absorbance at 280 nm. The activity of rubisco activase (●) was detected by assay of ATP hydrolysis at 340 nm.

For confirmation of the presence of rubisco activase in the transformant *E. coli*, the rubisco activase was purified from the *E. coli* lysate by passage through a Q-Sepharose column. The elution profiles for protein and an ATP-dependent activity of rubisco activase are seen in Fig. 2. The highest peak of activity in the fractions was detected at 0.28 M NaCl concentration.

The various fractions in the purification steps of rubisco activase were analyzed by SDS-PAGE as shown in Fig. 3. The fraction with the highest rubisco activase activity from the ion exchange Q-Sepharose (Fig. 2) showed two predominant bands at 46 and 42 kD, which were both subunits of rubisco activase in all steps.

To determine whether these two polypeptides are related, the band pattern of purified proteins was compared by Western blot as shown in Fig. 4. The band pattern of purified proteins analyzed by SDS-PAGE showed two polypeptides at 46 kD and 42 kD polypeptide in the protein gel (Fig. 4. lane 1). These results were similar to the report obtained in stromal protein extracts isolated from intact barley chloroplast by Rundle and Zielinski (1991a).

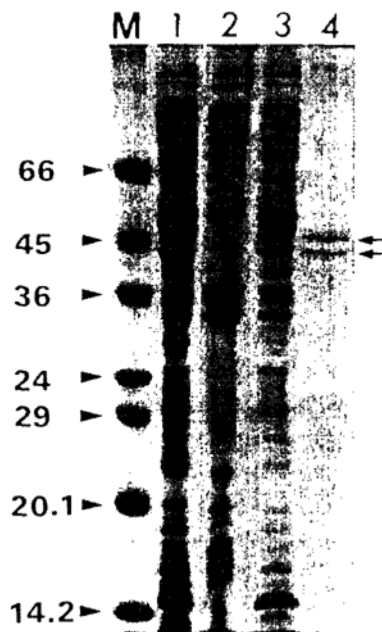

Fig. 3. SDS-PAGE analysis of fractions in the purification steps of rubisco activase from E. coli transformed. M, molecular weight marker; Lane 1, crude lysate; Lane 2, ammonium sulfate precipitate; Lane 3, PEG-10,000 precipitate; Lane 4, peak fraction on Q-Sepharose column. Electrophoresis was carried out at 100 V in 13% SDS-polyacrylamide gel. The arrow indicates the position of the rubisco activase.

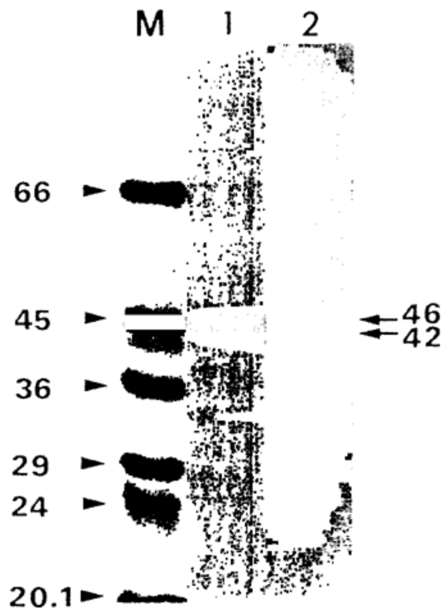

Fig. 4. Comparison of rubisco activase analyzed by SDS-PAGE (lane 1) and immunological detection by Western blot (lane 2) in *E. coli* transformed with *Rca* genes. SDS-PAGE was performed with high rubisco activase and ATP hydrolysis fractions. For Western blotting assay, proteins from crude extracts were separated by electrophoresis and then transferred onto nitrocellulose membrane. Nitrocellulose membrane was stained with BCIP and NBT as substrate. M, molecular weight marker (in kD). The arrow indicates the position of the rubisco activase.

Western blots of leaf extracts from several higher plants separated by SDS-PAGE were probed for the presence of two rubisco activase polypeptides using the rubisco activase antibodies (Salvucci *et al.*, 1987). The cross-immunoreactivity was found in extracts of all higher plants examined (Salvucci *et al.*, 1987). The expression of barley *Rca* genes in *E. coli* was detected immunologically by Western blot analyses using antibodies produced against purified kidney bean rubisco activase as probe (Roh *et al.*, 1997). Anti-rubisco activase antibodies reacted specifically with both polypeptides of 46 and 42 kD present in *E. coli* transformed on immunoblots (Fig. 4. lane 2). As a result, bands correspondig to the 46 kD and 42 kD polypeptide on immunoblots of crude extracts from *E. coli* were visible in the protein gel. These 46 kD and 42 kD polypeptide were encoded from 1.8 kb *Rca*A1, and 1.5 kb *Rca*A2 and *Rca*B gene, respectively (Rundle and Zielinski, 1991b). The result presented here are consistent with reports showing that the spinach rubisco activase polypeptide synthesized from a cloned cDNA in *E. coil* was capable of activating rubisco in an ATP-dependent reaction *in vitro* (Werneke *et al.*, 1988a). Previously, we reported that the analysis of Western immunoblotting in kidney bean leaves showed two major polypeptides at 46 and 42 kD (Roh *et al.*, 1997) which were independently capable of catalyzing rubisco activation *in vitro* (Zielinski *et al.*, 1989).

Rubisco activase is encoded by a single mRNA of 1.9 kb in spinach, pea and Arabidopsis (Werneke *et al.*, 1988b). The mRNAs of *Rca* mutant of Arabidopsis are about 200 nucleotides larger and smaller than the wild type rubisco activase mRNA (Werneke *et al.*, 1988b). Many chloroplast polypeptides are encoded by the plant nuclear genome, initially synthesized as a precussor in the cytoplasm, and then imported into the chloroplast posttranslationally (Ellis, 1981). The 51 kD precursor polypeptide, generated from a spinach cDNA clone, was produced both a major polypeptide of 45 kD and a minor polypeptide of 41 kD by alternative splicing of the same primary transcript within the stroma (Werneke *et al.*, 1988b).

Pre-mRNA produced by one of the genes in barley is alternatively spliced, thus yielding a total of three distinct *Rca* mRNAs (Crafts-Brandner *et al.*, 1997). The expression of three Rca mRNAs differ during development of the first leaf as well as on a diurnal basis. These changes involve differential accumulation of transcripts from two distinct *Rca* genes (Rundle and Zielinski, 1991b). *Rca*A mRNAs were present at levels 20-100 fold more abundant than *Rca*B mRNA in the mature regions of barley leaves (Zielinski *et al.*, 1989). The two mRNAs, *Rca*A1 and *Rca*A2 mRNA, encoded by *Rca*A also accumulate progressively from the base to the tip in barley leaves. This result suggests that *Rca*A1 mRNA is much less stable than *Rca*A2 mRNA (Rundle and Zielinski, 1991a). *Rca*A1 mRNA could be preferentially translated, resulting in the production of proportionally more of the 46 kD polypeptide per unit of mRNA. On the other hand, the 42 kD *Rca* polypeptides may be turned over more rapidly than the 46 kD polypeptide (Rundle and Zielinski, 1991a). Zielinski *et al.* (1989) initially observed reduced amounts of the 46 kD rubisco activase polypeptide compared with the 42 kD polypeptide in barley leaf cell.

Because it is unclear that the physiological significance of the two 42 kD rubisco activase polypeptides in barley, further experiments will be focussed to determine the biochemical and

physiological differences between the 42 kD polypeptides encoded by *Rca*A2 and *Rca*B.

In summary, activation of rubisco is catalyzed by rubisco activase in the presence of ATP. Rubisco activase was isolated from extracts of the competent *E. coli* transformed with pGEM-4Z containing *Rca*A 1 (1.8 kb), and pBluescript-KS$^+$ plasmids containing *Rca*A2 (1.5 kb) and *Rca*B (1.5 kb). Rubisco activase, polypeptide of 46 kD and 42 kD, were detected by Western immunoblot. *Rca*A1, and *Rca*A2 and *Rca*B encoded polypeptide of 46 kD and 42 kD, respectively. It has been confirmed that the genes of rubisco activase expressed in transformed *E. coli.*

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