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Exogenous GA_3 Increases Rubisco Activation in Soybean Leaves

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We studied the effect of exogenous applications of GA_3 on rubisco activation in soybean leaves. Activity at 0.1 μM GA_3 was significantly greater than in leaves receiving no treatment. Rubisco content showed patterns of change similar to that for activity. These data suggest that activity was associated with the amount of rubisco protein, and that the activation and induction of rubisco was promoted by GA_3. The degree of intensity of the 50- and 14.5-kD polypeptides (identified as the large and small subunit, respectively, of rubisco by SDS-PAGE analysis) at 0.1 μM GA_3 was significantly higher than that for the control, indicating that GA_3 affected both subunits. The stimulation effects of rubisco activation by GA_3 seem to be caused by the expression of rubisco genes at the transcriptional level. Assuming that these GA_3 effects were related to rubisco activase, we also determined the activity and content of that enzyme. Its activity at 0.1 μM GA_3 increased more than did the control. A similar pattern of change was observed for rubisco activase content. The intensity of two 46- and 42-kD polypeptide bands at GA_3 was higher than for the corresponding bands at the control. Therefore, the change in rubisco activase levels may lead to altered levels of rubisco.

Keywords: activation, GA_3, rubisco, rubisco activase, SDS-PAGE

The first steps in the competing reactions of photosynthetic carbon assimilation and photorespiration are catalyzed by rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase: EC 4.1.1.39). This enzyme is located in the chloroplast stroma (Woodrow and Berry, 1988). Ribulose-1,5-bisphosphate (RuBP) is the substrate, and 3-phosphoglycerate is the product of the carboxylase reaction of rubisco. Both substrate and product are important organic substances for plant development and growth (Josette et al., 1993).

The rubisco holoenzyme is assembled in a catalytically inactive form and is activated by the binding of activator CO_2 and Mg^{2+} to the ε-amino group of Lys-201 within the active site on the large subunit (Miziorko and Lorimer, 1983; Andrews and Lorimer, 1987). Phosphate is an essential factor for the activation and carbamylation of rubisco (Sawada et al., 1990). This activation process in vivo is catalyzed in two sequential steps by rubisco activase (Somerville et al., 1982) in the presence of ATP (Streusand and Portis, 1987) and RuBP (Portis, 1990). Activation by rubisco activase also requires both electron transport through Photosystem I and the presence of a transthylakoid pH difference (Campbell and Ogren, 1990).

Rubisco activase promotes the dissociation of RuBP and other inhibitory sugar phosphates from the decarbamylated rubisco active site in a process requiring the hydrolysis of ATP (Wang and Portis, 1991, 1992). It also catalyzes the removal of inhibitors such as CA1P (2-carboxyarabinitol 1-phosphate) and CABP (carboxyarabinitol 1,5-bisphosphate) from the active site of rubisco (Portis, 1992). CA1P binds tightly to carbamylated rubisco (Moore and Seemann, 1994), whereas CABP binds to both activated and inactive sites of rubisco (Zhu and Jensen, 1990).

In higher plants gibberellins play a key role in the regulation of embryo growth and seed germination (Chen and Osborne, 1970), shoot and internode elongation (Ingram et al., 1984), flowering (Stoddart, 1966) and synthesis of anthocyanin in the corollas of flowers (Weiss and Halevy, 1989), fruit formation (Jones, 1973; Grabe, 1987), and retardation of leaf senescence (Hooley, 1994).

Gibberellin-controlled enzyme production has been studied extensively in the aleurone layers of cereal seeds. A number of hydrolytic enzymes increase their activities following gibberellin treatment, including α-amylase (Varner 1964; Jones and Carbonell, 1984), protease (Jacobsen and Varner,

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1967), β-glucanase (Taiz and Jones, 1970) and various isozymes of α-amylase (Deikman and Jones, 1986). The role of gibberellin in gene expression has also been studied in α-amylase of wheat (Baulcombe and Buffard, 1983) and oat aleurones (Zwar and Hooley, 1986), as well as flavonoid in petunia corollas (Weiss et al., 1990). Sim and Roh (1985) demonstrated the effect of GA_3 on the activity of ribosomes and elongation factors 1 and 2.

Despite extensive physiological, biochemical and molecular studies of GA_3, the action of this phytohormone at the enzyme level during photosynthesis is still unknown. In this study we applied GA_3 by spray to soybean leaves, then used a spectrophotometric assay to measure its effect on the activities and content of rubisco and rubisco activase. In addition, we analyzed peptide patterns via SDS-PAGE.

MATERIALS AND METHODS

Plant Culture

Seeds of soybean (*Glycine max* L.) were germinated and grown in a growth chamber as described previously (Roh et al., 1996, 1997). Plants were illuminated under mixed metal-halide and incandescent lamps at 800 to 1,200 $\mu M/m^2/s$ PFD, with 350 ppm CO_2 provided 24-h per d, relative humidity of 60%, and 26°C for the 16-h day and 18°C at night. Leaves were sprayed manually with a 0.1 μM GA_3 solution daily. Fully expanded leaves from mature plants were used for our experiments.

Purification of Rubisco

Rubisco and rubisco activase were purified from the leaves, following a modification of the method of Wang et al. (1992). Leaf tissue was ground to a fine powder in a pre-cooled mortar and pestle in liquid nitrogen, then extracted in a buffer containing 50 mM BTP (pH 7.0), 10 mM $NaHCO_3$, 10 mM $MgCl_2$, 1 mM EDTA, 0.5 mM ATP, 10 mM DTT, 1 mM PMSF, 1 mM benzamidine, 0.01 mM leupeptin, 1.5% PVPP, and 3 mM MBT. This leaf slurry was filtered through four layers of cheesecloth and one layer of Miracloth. The filtered solution was centrifuged at 30,000*g* for 40 min. $(NH_4)_2SO_4$ powder was slowly added into the supernatant to 35% saturation and stirred for 30 min. Both supernatant and pellet were then collected by centrifugation at 8,000*g* for 10 min. The supernatant contained rubisco and the resuspended pellet contained rubisco activase.

The collected supernatant was brought to 55% saturation of $(NH_4)_2SO_4$ by adding the powder. The pellect was resuspended in 5 mL of 20 mM BTP (pH 7.0) containing 0.2 mM ATP, 10 mM $MgCl_2$, and 2 mM MBT (buffer A). 50% PEG-10K was added to a final concentration of 18%. The resulting precipitate was collected by centrifugation at 8,000*g* for 10 min and resuspended in buffer A.

Resuspended solution was loaded onto a Q-Sepharose column equilibrated with 20 mM Tris (pH 7.5), 10 mM $MgCl_2$, and 10 mM $NaHCO_3$. This column was washed with the same buffer containing 0.1 M NaCl. Elution was then started with a linear gradient from 0.1 to 0.5 M NaCl and at a flow rate of 1 mL/min. Three mL fractions were pooled, and assayed for rubisco activity and contents.

Purification of Rubisco Activase

50% (w/v) PEG-10K was added into buffer A that contained the resuspended pellet. This mixture was made up to a final concentration of 18%, stirred 5 min, and centrifuged at 8,000*g* for 10 min. The pellet was dissolved in 5 mL of buffer A, and the solution was cleared by spinning it at 20,000*g* for 10 min. The pellet was resuspended again in 5 mL of buffer A and the solution was cleared again.

The collected supernatants were loaded onto a 20 mL Q-Sepharose column equilibrated with 20 mM BTP (pH 7.0). This column was 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). Three mL fractions were pooled, and assayed for rubisco activase activity and contents.

All the processes for purification were done at 4°C, except as indicated.

Assay of Rubisco Activity

Rubisco activity was determined at 25°C according to the method of Racker (1962). The purified rubisco solution was added to an assay medium with a final volume of 1 mL that contained 1 M Tris buffer (pH 7.8), 0.006 M NADH, 0.1 M GSH, 0.5% glyceraldehyde-3-phosphate dehydrogenase, 0.025 M 3-phosphoglycerate kinase, 0.05% α-glycerophosphate dehydrogenase-triose phosphate isomerase, 0.025 M RuBP, 0.2 M ATP, 0.5 M $MgCl_2$, and 0.5 M $KHCO_3$. Oxidation of NADH was monitored at 340 nm during the conversion of 3-phosphoglycerate to glycerol

3-phosphate. One unit was defined as the amount that catalyzed the cleavage of 1 μM RuBP per min.

Assay of Rubisco Activase Activity

Rubisco activase activity was defined as the ability to produce ADP in an ATP-dependent reaction, as measured at an absorption of 340 nm and following the procedure of Robinson and Portis (1989). The purified rubisco activase solution was added to a total volume of 0.4 mL of an activation reaction mixture that contained 50 mM Tricine (pH 8.0), 20 mM KCl, 10 mM $MgCl_2$, 1 mM ATP, 1 mM phospho*enol*pyruvate, 0.3 mM NADH, 40 units/mL pyruvate kinase, and 40 units/mL lactate dehydrogenase. One unit was defined as 1 μM ATP hydrolyzed per min.

Electrophoresis

SDS-PAGE was performed on a 13% polyacrylamide gel at room temperature, using the method of Laemmli (1970). The protein samples were boiled for 10 min before being loaded onto the gel. Proteins were stained by Coomassie Brilliant Blue R-250, then destained with 7.5% acetic acid.

ELISA

The contents of rubisco and rubisco activase were detected by ELISA. To coat the antigen, 100 μL of different dilutions of two enzymes in a 0.1 M sodium carbonate-bicarbonate coating buffer (pH 9.5) was added to each well of the microplate. After incubation overnight at room temperature, the plate was washed with 0.01 M PBS (pH 7.4) containing 0.05% Tween 20. To eliminate nonspecific binding, 250 μL of 0.1% BSA in 0.01 M PBS (pH 7.4) was added to each well and incubated for 1 h at 37°C. After washing, 50 μL of various dilutions of two enzymes were added in 0.01 M PBS (pH 7.4), 50 μL of different dilutions of a rabbit anti-rubisco and anti-rubisco activase antiserum, serving as a primary antibody (Roh et al., 1997) was added to each well, and incubated for 30 min at 37°C. The plate was again washed as described above, Afterward, 100 μL of peroxidase-conjugated goat anti-rabbit IgG, diluted to 1:20,000 in 0.01 M PBS (pH 7.4) that contained 0.1% BSA, was added and incubated for 30 min at 37°C. The plate was washed as previously described and 100 μL of peroxidase substrate [OPD tablets in 10 mL of 0.05 M citrate/0.1 M sodium phosphate buffer (pH 5.0) containing 30% H_2O_2] was added. After incubation at room temperature in the dark for 10 to 20 min, the reaction was terminated by adding 0.1 mL 1 N HCl. Absorbance at 490 nm was determined by an ELISA microplate reader (Bio-Rad Model 3550-UV).

RESULTS AND DISCUSSION

Gibberellin produces a large number of physiological responses in higher plants. In this study, we used exogenous applications of GA_3 to identify its possible role in the processes of biosynthesis, transport and metabolism, as well as its action mechanism in the growing plant. Measurements of activity, carbamylation and levels of rubisco would also benefit photosynthesis studies.

To determine an optimum concentration to use in our subsequent experiments, we first tested the effect of various GA_3 concentrations on rubisco activity. Our preliminary data (not shown) identified 0.1 μM GA_3 as the optimum. Elution profiles for protein and the activity of rubisco are found in Figure 1. The fraction with the highest peak of activity, detected at 0.134 M NaCl, was used for measuring rubisco activity and content.

Rubisco activity at 0.1 μM GA_3 was significantly greater than that in the control (Fig. 2). Rubisco content (Fig. 3) followed patterns of change similar to that for activity. These data suggest that activity was associated with the amount of rubisco protein (Downton et al., 1980), and that activity and content were directly correlated with GA_3 concentration. Therefore, the activation and induction of rubisco is probably caused

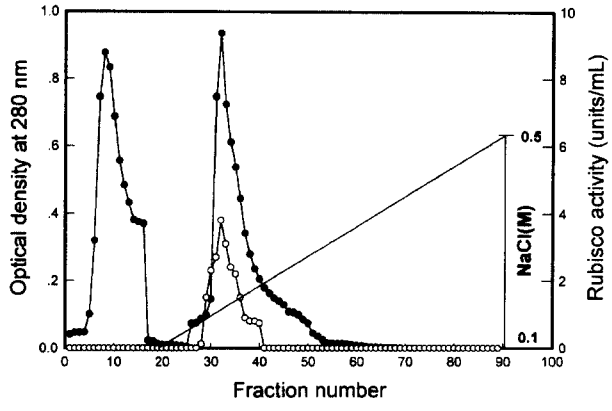

Figure 1. Elution profile for protein (●) and rubisco activity (○) from anion exchange chromatography on Q-Sepharose column. Rubisco was purified from soybean leaves treated with 0.1 μM GA_3. Its activity was detected by oxidation of NADH at 340 nm. The straight line indicates the 0.1-0.5 M NaCl gradient in 20 mM Tris (pH 7.5).

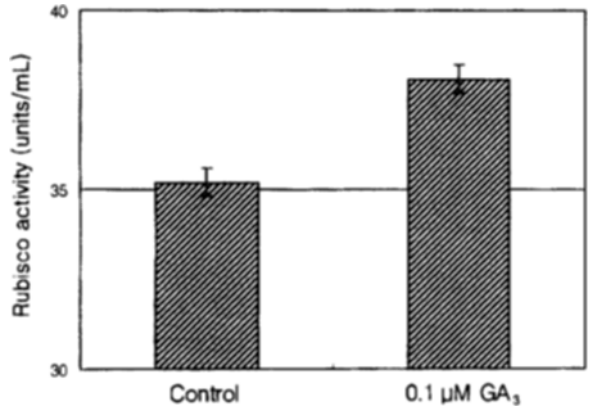

Figure 2. Effect of GA_3 on rubisco activity in soybean leaves. The purified rubisco solution was added to assay medium of a final volume of 1 mL. Oxidation of NADH was monitored at 340 nm.

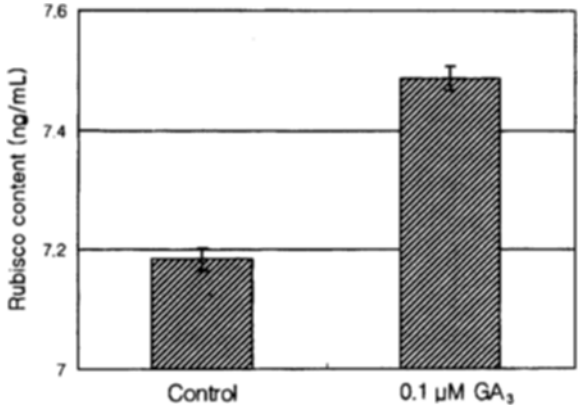

Figure 3. Effect of GA_3 on rubisco content in soybean leaves. Content was detected by ELISA at an absorption of 490 nm.

by GA_3. Similar results were reported in a study of rubisco using benzyladenine in etiolated cucumber cotyledons (Ohya and Suzuki, 1991). Unlike our result, however, ABA depressed the accumulation of rubisco in embryonic cotyledons of the kidney bean (Medford and Sussex, 1989).

Hormonal control of enzyme levels is mediated at least in part by the rate of transcription of its genes. GA_3 may control enzyme activity and transcriptional gene induction for enzymes. For example, during germination of cereal seeds, GA_3 is released from the embryo and diffused to the aleurone cells, where hydrolytic enzymes are synthesized or activated (Baulcombe and Buffard, 1983). These enzymes then diffuse into the endosperm, where they catalyze the digestion of stored macromolecules.

Deikman and Jones (1986) have reported that GA_3 regulates α-amylase mRNA accumulation in barley aleurones. Increased mRNA levels in oat aleurone protoplasts result from transcriptional induction of the α-amylase gene (Zwar and Hooley, 1986). Likewise, the increase in synthesis of anthocyanin pigments by GA_3 during the development of petunia corollas is correlated with phenylalanine ammonia-lyase, chalcone synthase, chalcone isomerase, flavanone 3-hydroxylase, and UDP-glucose: 3-*O*-flavonoid glucosyltransferase (van Weely et al., 1983; Weiss and Halevy, 1989). GA_3 also regulates gene expression of both flavonoids (Weiss et al., 1990), and triosephosphate isomerase (Ben-Nissan and Weiss, 1995) in the corollas of *Petunia hybrida*. This has also been shown by Zwar and Hooley (1986).

Berry et al. (1985) have suggested that transcriptional regulation plays an important role in the expression of genes that encode the large and small subunits of rubisco. Although BA promotes rapid accumulation of mRNAs that encode the small subunit, it hardly affects mRNA levels for the large subunit (Ohya and Suzuki, 1991). In contrast, ABA mediates a negative regulation of *rbc*S in tomato leaves (Bartholomew et al., 1991). In our study, the stimulation effects of rubisco activation by GA_3 (Fig. 2) also seem to be caused by the expression of rubisco genes at the transcriptional level. An increase in enzyme activity is preceded by an increase in specific mRNAs (Beld et al., 1989). Further research will focus on the regulation of rubisco gene expression by GA_3 at the transcriptional level.

To verify that GA_3 concentration indeed caused an increase in rubisco content, we tested the SDS-PAGE rubisco profile for the control and for 0.1 μM GA_3. Two major polypeptide bands of 50- and 14.5-kD were identified as the large and small subunit of rubisco, respectively (Fig. 4). This result is consistent with that of Roh et al. (1996) in the kidney bean. The large and small subunits of this enzyme are coded by the chloroplast gene *rbc*L and the nuclear *rbc*S gene, respectively (Dean et al., 1989). The degree of intensity for both bands at 0.1 μM GA_3 was significantly higher than for the control, indicating that exogenous GA_3 increased the amounts of both subunits. GA_3-controlled rubisco production in soybean leaves was direct evidence for GA_3 action at the biochemical level.

The activation of rubisco requires ATP-dependent rubisco activase (Lilly and Portis, 1990; Portis, 1992). Assuming that the effects of GA_3 on rubisco may be related to rubisco activase, we purified this enzyme, and determined its activity and content. Elution pro-

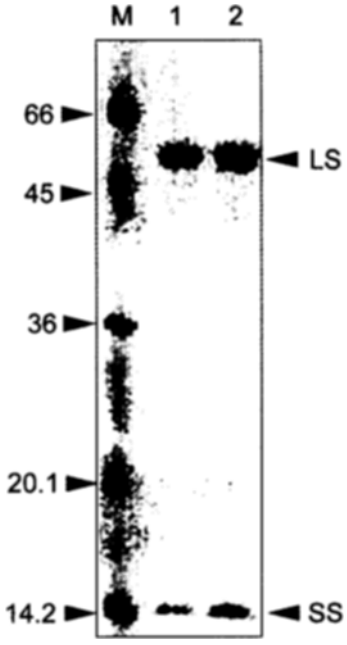

Figure 4. SDS-PAGE detection of rubisco purified from soybean leaves. Proteins (20 μg) were separated on 13% SDS-PAGE gels. M, molecular weight standards; Lane 1, no treatment; Lane 2, GA_3 treatment. Large and small subunits of rubisco are indicated by an arrow.

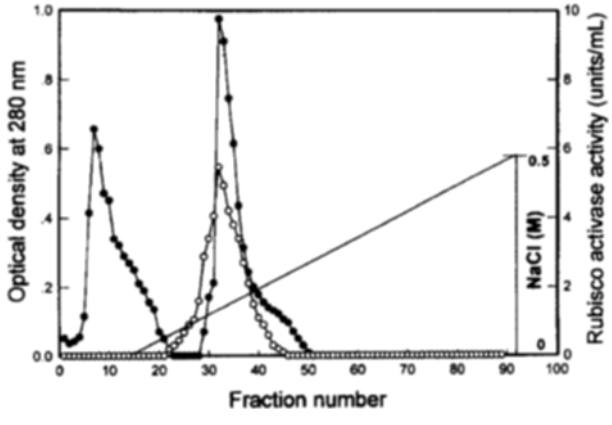

Figure 5. Elution profile for protein (●) and rubisco activase activity (○) from anion exchange chromatography on Q-Sepharose column. Rubisco activase was purified from soybean leaves treated with 0.1 μM GA_3. Its activity was detected by ATP hydrolysis at 340 nm. The straight line indicates the 0-0.5 M NaCl gradient in 20 mM BTP (pH 7.0).

files and ATP-dependent activity are presented in Figure 5. Enzyme activity was detected in a single peak at 0-0.5 M NaCl. Maximal elution was at 0.137 M, which is lower than 0.38 M for the kidney bean (Roh et al., 1997).

The assay of rubisco activase is based on its ability

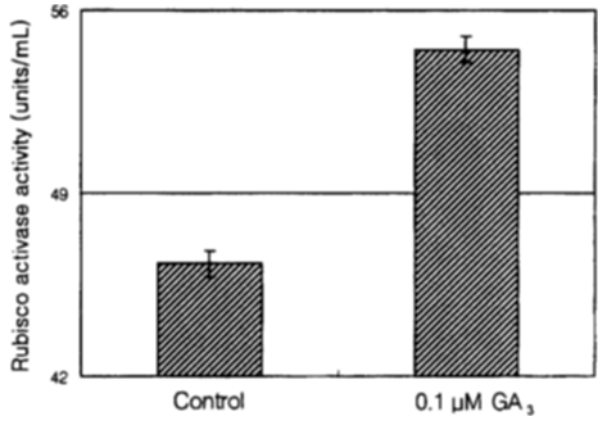

Figure 6. Effect of GA_3 on the activity of rubisco activase in soybean leaves. The purified rubisco activase solution was added to a total volume of 0.4 mL of the activation reaction mixture. Activity was assayed as the ability to produce ADP in an ATP-dependent reaction in absorption at 340 nm.

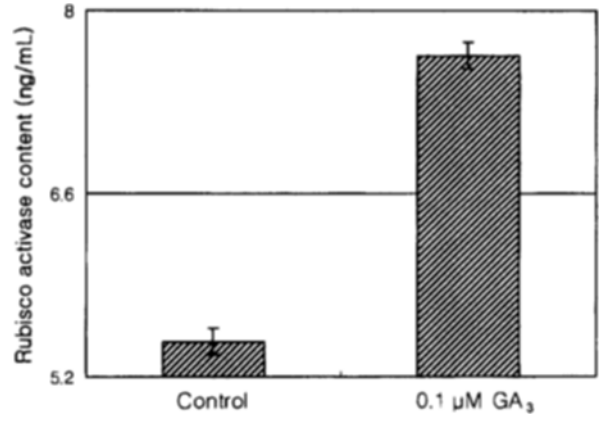

Figure 7. Effect of GA_3 on the content of rubisco activase in soybean leaves. Content was detected by ELISA at an absorption of 490 nm.

to produce ADP in the presence of ATP (Robinson and Portis, 1989). In our study, rubisco activase activity at 0.1 μM GA_3 was much higher than with the control (Fig. 6). A similar change pattern was also observed in its content (Fig. 7) and specific activity (data not shown). These data indicate that the application of GA_3 had a stimulation effect similar to that found with rubisco. Therefore, we would propose that rubisco activase is required for GA_3-induced rubisco activity. Furthermore, these results provide direct support for the suggestion that increased activity and content of rubisco, caused by GA_3, may be due to an increase in rubisco activase.

As confirmed by SDS-PAGE, the fraction with the highest rubisco activase activity from the ion

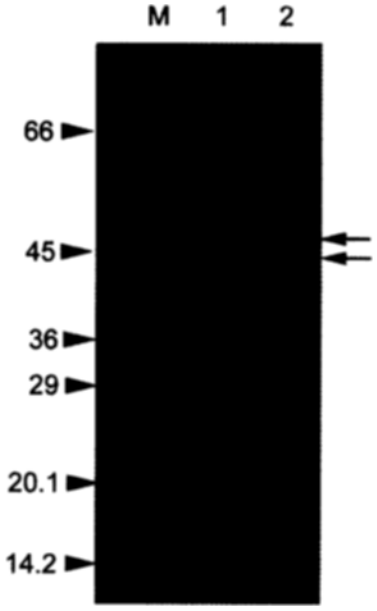

Figure 8. SDS-PAGE detection of rubisco activase purified from soybean leaves. Proteins (20 μg) were separated on 13% SDS-PAGE gels. M, molecular weight standards; Lane 1, no treatment; Lane 2, GA_3 treatment. Rubisco activase indicated by an arrow.

exchange Q-Sepharose (Fig. 5) had only 46- and 42-kD bands (Fig. 8). Immunological detection using purified activase antibodies from several higher plant species have been probed for the presence of activase subunits (Salvucci et al., 1987; Roh et al., 1997, 1998). Two polypeptides, also at 46- and 42-kD, have been detected in leaf extracts of *Arabidopsis* (wild type), spinach, tobacco, soybean, pea, celery, oat, barley, and kidney bean, as well as in crude extracts of *Escherichia coli* transformants. Those bands are similar to those shown in Figure 8. The two cross-reacting polypeptides have also been found in extracts of pigweed, purslane, dandelion, sorghum, and crabgrass.

These two rubisco activase polypeptides are encoded by tandemly oriented nuclear *Rca*A and *Rca*B (Werneke et al., 1989; Rundle and Zielinski, 1991a). The *Rca*A gene transcript is alternatively spliced to yield 1.8 kb *Rca*A1 and 1.5 kb *Rca*A2 mRNA which encode mature proteins of 46- and 42-kD, respectively. The 1.5 kb *Rca*B gene transcript is spliced to yield a single mRNA encoding a 42-kD band (Rundle and Zielinski, 1991b). Because the intensity of our 46- and 42-kD polypeptide bands for GA_3 was higher than with the bands for the control (Fig. 8), we suggest that the change in the levels of rubisco activase leads to a subsequent alteration of rubisco levels.

In conclusion, exogenous applications of GA_3 induced increases in rubisco activity and content in soybean leaves. This effect was accompanied by a coordinated increase in the activity and content of rubisco activase. Therefore, we suggest that the stimulation of the rubisco activation level by exogenous GA_3 is caused not only by the expression of rubisco genes at the transcriptional level, but also by the level of rubisco activase.

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