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UV-B Radiation Affects Chlorophyll and Activation of Rubisco by Rubisco Activase in *Canavalia ensiformis* L. Leaves

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We studied the influence of UV-B radiation on chlorophyll and rubisco activation by rubisco activase in the leaves of jackbean (*Canavalia ensiformis*). Chlorophyll content was decreased, indicating that the synthesis of those molecules may have been degraded or repressed after exposure. Rubisco content was significantly lower in radiated tissue compared with the untreated control; rubisco activity showed a similar pattern of change. Based on these data, we suggest that rubisco activity is associated with the level of rubisco protein, and that UV-B inhibits its activation and induction, as well as that of rubisco activase. Therefore, we propose that the inhibitory effect of rubisco by UV-B may be caused by rubisco activase.

Keywords: *Canavalia ensiformis* L., chlorophyll, rubisco, rubisco activase, UV-B

The impact of UV-B radiation (280 to 320 nm) at the earth's surface is predicted to increase because of the anthropogenic depletion of stratospheric ozone caused by industrial emissions of atmospheric pollutants, particularly chlorofluorocarbons (McKenzie et al., 1999). Environmental factors, such as light, temperature, nutrients, heavy metals, CO_2, and ozone, modify the effect of this radiation (Kulandaivelu et al., 1997). These effects may include decreased penetration of PAR (400 to 700 nm), a reduction in accessory pigments, altered stomatal conductance, and, indirectly, the retardation of photosynthetic carbon assimilation (Teramura and Sullivan, 1994). UV-B radiation can alter several metabolic processes in leaves that ultimately change the levels of many foliar constituents, e.g., carbohydrates, photosynthetic pigments, UV-B-absorbing components, and free radicals (Mazza et al., 2000). Olsson et al. (1999) have suggested that UV-B-induced accumulation of specific UV-absorbing constituents protects the photosynthetic apparatus from the damaging effects of this radiation source.

UV-B radiation has considerable photobiological influences on plant growth and development (Jordan, 1996; Jayakumar et al., 2003), as well as the light reaction of photosynthesis (McNamara and Hill, 2000). Its predominant effect is the inhibition of electron transport. When this radiation is absorbed directly by the thylakoid-membrane components, membrane organization is damaged (Kulandaivelu and Noorudeen, 1983). This may include reductions in chlorophyll, D1 protein, the light-harvesting chlorophyll *a*/*b* binding protein of PSII, rubisco levels (Strid et al., 1990), and the expression of photosynthetic genes (Mackerness et al., 1999).

It is poorly understood how UV-B interacts with chlorophyll, rubisco, and the photosynthetic process. Therefore, the objective of our study was to determine the effect of this radiation source by measuring chlorophyll content and the activities and contents of rubisco and rubisco activase in irradiated and untreated plants.

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MATERIALS AND METHODS

Plant Growth and UV-B Radiation

Plants of the jackbean (*Canavalia ensiformis* L.) were raised in a growth chamber under the following conditions: PFD of 800 to 1200 $\mu M/m^2/s$, 350 ppm CO_2, 60% relative humidity, 16-h photoperiod, and day/night temperatures of 26/18°C (Roh et al., 2001). At 15 weeks after sowing, plants were exposed to 170 mW m^{-2} UV-B radiation (via Philips TL20W10/RS lamps) for 3 h at 25°C. To remove radiation of <280-nm quality, the UV-B tube was wrapped in cellulose acetate (0.13 mm thickness) to prevent increased transmission of the shorter wavelengths. Plexiglas (FBL. 2458, Rohm Gmbh, Chemische Fabrik, Germany) was positioned between the UV lamps and the cellulose acetate to maintain its optical stability. All experiments were independently duplicated.

Chlorophyll Measurement

Chlorophyll contents were measured spectrophotometrically, using specific absorption coefficients of 664.5 and 647.0 nm (Inskeep and Bloom, 1985). Fully expanded leaves from mature plants were frozen, then transferred to DMF and stored at 5°C in darkness. Extracts were centrifuged for 5 min at 8000*g*. The following equations were used to estimate the concentrations of chlorophyll *a*, chlorophyll *b*, and total chlorophyll from the supernatants.

Chlorophyll *a* (mg/g fw) = $12.70\ A_{664.5} - 2.79\ A_{647}$
Chlorophyll *b* (mg/g fw) = $20.70\ A_{647} - 4.62\ A_{664.5}$
Total chlorophyll (mg/g fw) = $17.90\ A_{647} + 8.08\ A_{664.5}$

Purification of Rubisco and Rubisco Activase

Rubisco and rubisco activase were purified at 4°C according to the method described by Wang et al. (1992). Briefly, frozen leaf tissue was pulverized in a mortar under liquid nitrogen, then extracted in 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. The leaf slurry was filtered through four layers of cheesecloth and one layer of Miracloth. This filtered solution was then centrifuged at 30,000*g* for 40 min. $(NH_4)_2SO_4$ powder was slowly added to the supernatant, to 35% saturation, and stirred for 30 min. Supernatant and pellet were both collected by centrifugation at 8000*g* for 10 min. The supernatant (containing rubisco) was then brought to 55% saturation by adding $(NH_4)_2SO_4$ powder. Following centrifugation, the pellet was re-suspended 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). Afterward, 50% PEG-10K was added to a final concentration of 18%. The resulting precipitate was collected by centrifugation at 8000*g* for 10 min and re-suspended in Buffer A. This solution was loaded onto a Q-Sepharose column equilibrated with 20 mM Tris (pH 7.5), 10 mM $MgCl_2$, and 10 mM $NaHCO_3$. The column was then washed with the same buffer containing 0.1 M NaCl. Elution was started with a linear gradient of 0.1 to 0.5 M NaCl, and a flow rate of 1 mL/min. The 3-mL fractions were pooled and assayed for rubisco activity and content.

To quantify rubisco activase in the re-suspended pellet obtained above, 50% (w/v) PEG-10K was added to Buffer A to a final concentration of 18%. It was then stirred 5 min, and centrifuged at 8000*g* for 10 min. The pellet was dissolved in 5 mL of Buffer A. This solution was cleared by spinning it at 20,000*g* for 10 min. Afterward, the pellet was re-suspended 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). Elution proceeded 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 (0 to 0.5 M NaCl) in 20 mM BTP (pH 7.0). The 3-mL fractions were pooled and assayed for rubisco activase activity and content.

Activity Assays

Rubisco activity was determined spectrophotometrically at 25°C by monitoring NADH oxidation at 340 nm (Racker, 1962). The assay medium 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 $MgCl_2$, 0.5 M $KHCO_3$, and purified rubisco solution in a final volume of 1 mL. One unit of enzyme was defined as the amount of enzyme producing 1 μM of RuBP per min. Rubisco activase activity was defined as the ability to produce ADP in an ATP-dependent reaction at an absorption of 340 nm (Robinson and Portis, 1989). The purified rubisco activase solution was added to 0.4 mL of an activation-reaction mixture containing 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 the amount that catalyzed the cleavage of 1 μM ATP per min.

ELISA

The contents of rubisco and rubisco activase were detected by ELISA. For coating of the antigen, 100 μL of different dilutions of two enzymes in 0.1 M sodium carbonate-bicarbonate coating buffer (pH 9.5) was added to each microplate well. After incubating overnight at room temperature (RT), 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. The plate was then washed, and 50 μL of various dilutions of two enzymes in 0.01 M PBS (pH 7.4) was added. Approximately 50 μL of different dilutions of a rabbit anti-rubisco and anti-rubisco activase antiserum (as the primary antibody; Roh et al., 1997) was then added to each well, and incubated for 30 min at 37°C. After the plate was

again washed as described above, 100 µL of peroxidase-conjugated goat anti-rabbit IgG [as 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 again washed, 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% of H_2O_2] was added. After incubation at RT in the dark for 20 min, the reaction was terminated by adding 0.1 mL of 1 N HCl. Absorbance at 490 nm was determined by an ELISA microplate reader (Bio-Rad Model 3550-UV).

RESULTS AND DISCUSSION

Chlorophyll

Depending on the particular crop species, chlorophyll content can either increase or decrease in response to enhanced UV-B radiation (Larsson et al., 1998; Sun and Payn, 1999; Barsig and Malz, 2000). In our study, the chlorophyll *a*:*b* ratio decreased when plants were irradiated (Table 1). This may have been a result of faster breakdown or decreased synthesis of Chl *a* compared with Chl *b*, even though the level of the latter also rose here. Decreased amounts of total chlorophyll in UV-treated leaves have been associated with the inhibition of aminolevulinic acid synthesis (Stobart et al., 1985) or a reduction in protochlorophyllides (Boddi et al., 1995). Our data suggest that chlorophyll synthesis may have been degraded or repressed after UV-B exposure. These results agree with those of Strid et al. (1990), who showed that UV radiation causes the total-chlorophyll content to decline significantly in pea leaves. Strid and Porra (1992) have also proposed that UV-B radiation influences the genetic regulation of the chlorophyll-binding protein, thereby leading to chlorophyll destruction. Likewise, Dube and Bornman (1991) have reported that the addition of Cd in combination with UV-B radiation reduces chlorophyll concentrations.

Table 1. Effect of UV-B on chlorophyll content in jackbean leaves. Plant was grown for 15 weeks.

Treatment	Chl *a*	Chl *b*	Chl *a*/*b*	Total chl
	(mg/g fw)			
Control	15.03	19.02	0.79	34.05
UV	13.20	19.31	0.68	32.51

Rubisco and Rubisco Activase

Rubisco content was greatly reduced by treatment with UV-B (Fig. 1), which suggests that this exposure caused a reduction in the synthesis and/or expression of rubisco. This result agrees with that of Takeuchi et al. (2002), who showed that rubisco synthesis is significantly suppressed by supplementary UV-B radiation in rice leaves. Jordan et al. (1992) have also demonstrated that UV-B radiation lowers the mRNA transcripts for rubisco subunits in pea. Rubisco activity also declined in response to irradiation (Fig. 2), which seems to indicate that decreased activity plays an important role in UV-induced inhibition of photosynthesis. This result is consistent with that of Nogues and Baker (1995), who have reported that UV-B radiation affects carboxylating efficiency not only by changing rubisco activity, but also by degrading rubisco in pea leaves. UV-B exposure has also been linked to rubisco damage either by direct mechanisms or, indirectly, through the formation of reactive oxygen species within the cell (Lesser, 1996; Allen et al., 1998).

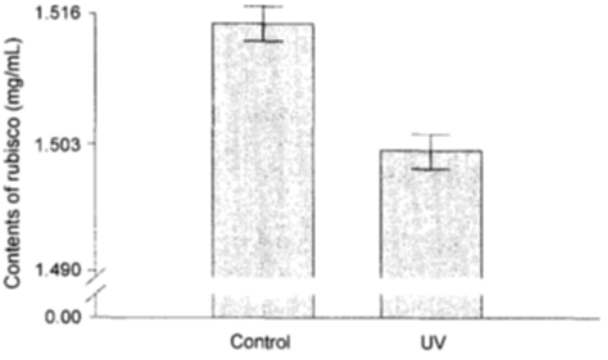

Figure 1. Effect of UV-B on rubisco content in jackbean. Plant was grown for 15 weeks. Experimental leaves were irradiated with UV-B (280 - 320 nm) at 25°C for 3 h.

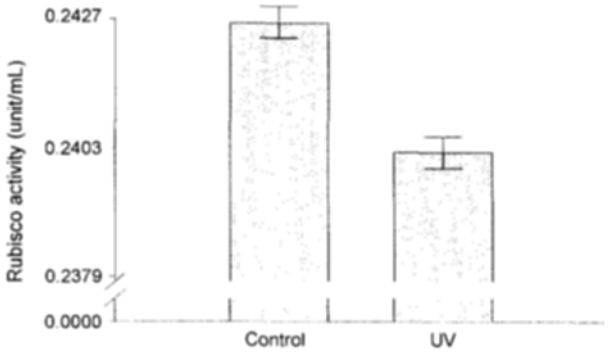

Figure 2. Effect of UV-B on rubisco activity in jackbean. Plant was grown for 15 weeks. Experimental leaves were irradiated with UV-B (280 - 320 nm) at 25°C for 3 h.

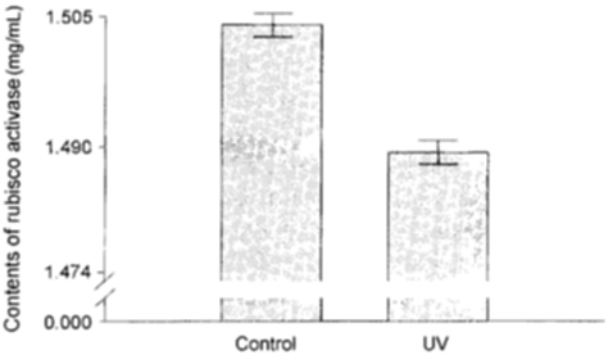

Figure 3. Effect of UV-B on rubisco activase content in jackbean. Plant was grown for 15 weeks. Experimental leaves were irradiated with UV-B (280 - 320 nm) at 25°C for 3 h.

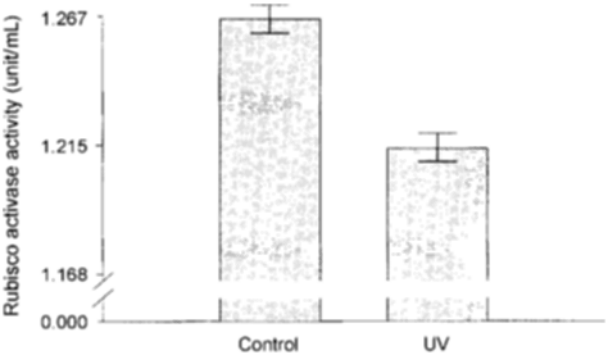

Figure 4. Effect of UV-B on rubisco activase activity in jackbean. Plant was grown for 15 weeks. Experimental leaves were irradiated with UV-B (280 - 320 nm) at 25°C for 3 h.

Jordan (1996) found that UV-B exposure causes rubisco activity to decrease more rapidly than the rate at which the enzyme disappears. Likewise, Strid et al. (1990) have shown that radiation decreases photosynthetic capacity by damaging PS II and total rubisco activity in pea leaves, although relative activation of the remaining rubisco may increase. Allen et al. (1997) have reported that a UV-B-induced decrease in the light-saturated rate of CO_2 assimilation is accompanied by declines in both rubisco content and activity. The rubisco holoenzyme 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 (Andrews and Lorimer, 1987). Rubisco inactivation likely lowers photosynthetic activity (Aro et al., 1993).

We have previously hypothesized that the UV-induced decrease in rubisco content and activity is associated with rubisco activase (1997, 2001). In the current study, rubisco activase content was lower in UV-treated tissue than in the control (Fig. 3). A similar change was observed in the activity of rubisco activase (Fig. 4). Portis (2003) has determined that rubisco activase is one of a new type of chaperone required for rubisco activation. This enzyme binds preferentially to inactive rubisco and dissociates after ATP hydrolysis (Sanchez et al., 1995). Therefore, our observation that the reduction in rubisco activase led to a large decline in rubisco activity further supports the theory that these processes inhibit photosynthesis and growth (Eckardt et al., 1997; He et al., 1997).

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