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Inhibition of Violaxanthin Deepoxidation by Ultraviolet-B Radiation in Isolated Chloroplasts and Intact Leaves[1]

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ABSTRACT

The effect of pretreatment with ultraviolet-B (UV-B) light (280–320 nanometers) on the enzymatic conversion of the diepoxyxanthophyll violaxanthin to the epoxy-free zeaxanthin occurring in thylakoid membranes was investigated. When isolated chloroplasts of pea (*Pisum sativum*) were exposed to UV-B, a biologically effective fluence of 7000 joules per square meter caused about 50% inhibition of the activity of the violaxanthin deepoxidase, measured as the first order rate constant of the absorbance change at 505 nanometers. The dose requirement for the inhibition of the deepoxidase in intact leaves, however, was about 2 orders of magnitude higher. The inhibition of the rate constant was observed for both the dark deepoxidation at pH 5, and for the light-driven deepoxidation induced by the lumen acidification due to electron transport from H_2O to methylviologen or due to a photosystem I partial reaction with duroquinol as the electron donor. The availability of violaxanthin was not directly affected by UV-B radiation, as shown for UV-B-treated chloroplasts by the final extent of the 505 nanometer change measured in the dark at pH 5 or by the partial photosystem I reaction. A significant decrease in the violaxanthin availability was observed when lumen acidification was caused by electron transport from H_2O to methylviologen. That effect was probably caused by the well-known UV-B inhibition of photosystem II with a subsequent decreased ability to reduce the plastoquinone pool, the redox state of which is believed to regulate the final amount of converted violaxanthin.

The release of halogenated carbon compounds into the atmosphere appears to be the main cause of the reduction of the stratospheric ozone layer (25). Ozone depletion will result in an increase of the radiation intensity primarily in the UV-B range (280–320 nm), because even if 90% of the present ozone layer is destroyed, virtually all radiation shorter than 280 nm will be absorbed by the remaining ozone (4). Because UV radiation is known to induce deleterious photochemical reactions in various biomolecules (2, 4), the understanding of UV-B action on living organisms has become a matter of general interest. In the case of higher plants, it is known that UV-B light inhibits photosynthetic electron transport, with PSII as the major site of UV-B damage (2).

Considering the natural situation in which plants are always irradiated simultaneously by UV and visible light, Mirecki and Teramura (16) found that soybean leaves that are concomitantly irradiated with UV-B and high intensities of visible light were resistant to UV-B damage, whereas leaves irradiated concomitantly with UV-B and low intensities of visible light were sensitive. In contrast, Warner and Caldwell (27), also working with soybean leaves, showed an increased inhibitory effect of UV-B light on photosynthesis when high but non-photoinhibitory visible light was present during the UV-B treatment. These results indicate the complexity of photoinhibition in the presence of visible and UV light and suggest the involvement of protective or repair mechanisms that can be modulated by either of the two light qualities.

Taking into account these considerations, we investigated the action of UV-B light on the violaxanthin cycle, which has been proposed to act as a protective mechanism against photoinhibition by visible light (9). It is thought that the xanthophyll zeaxanthin, which arises from the light-dependent interconversion of violaxanthin (5,6,5′,6′-diepoxyzeaxanthin) via antheraxanthin (5,6-monoepoxyzeaxanthin), dissipates some of the absorbed light energy and thus prevents damage due to excess excitation.

The whole violaxanthin cycle consists of the deepoxidation sequence and its light-independent reversal, each catalyzed by a different enzyme (13, 23, 29). In isolated chloroplasts, the deepoxidation reaction requires ascorbate as a co-substrate (31). According to the transmembrane model of the violaxanthin cycle, the violaxanthin deepoxidase, which has its activity optimum at pH 5 (12), is located at the lumen side of the thylakoid membrane and is activated by light-dependent proton pumping, whereas the epoxidation reaction, which has a pH optimum of 7.5 (22), takes place at the stroma side.

In this paper, we demonstrate the inhibition of the violaxanthin deepoxidase by UV-B radiation and further show that the amount of violaxanthin available for deepoxidation is not directly affected by UV-B irradiation, but may be reduced by indirect effects.

MATERIALS AND METHODS

Plant Material

Pea plants (*Pisum sativum* cv Little Marvel) were planted in sterilized garden soil and grown under two different conditions. For studies of UV light effects on isolated chloroplasts, 15-d-old plants cultivated in a growth chamber were used. Irradiation was provided by commercial incandescent bulbs and Sylvania Cool-white fluorescent tubes with a 10-h photoperiod. Photon flux density measured with a Li-Cor quantum radiometer model LI-189 was 450 $\mu E/(m^2 \cdot s)$. The tem-

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perature was maintained at 15 and 20°C for dark and light conditions, respectively. For experiments in which whole plants were exposed to UV-B light, 16- to 26-d-old plants grown in a greenhouse were used. During daytime, the greenhouse plants received additional light of 100 $\mu E/(m^2 \cdot s)$ provided by a 400-W high pressure sodium lamp (Energy Technics, York, PA). Plants were kept in the dark for 8 h before all experiments.

Chloroplast Isolation

Chloroplasts used for UV treatments were isolated according to Robinson and Yocum (19), with a modified grinding medium containing 300 mM NaCl, 30 mM Tricine-KOH (pH 7.8), 3 mM $MgCl_2$, 0.5 mM EDTA, and 0.5 g/L BSA. Chloroplasts of UV-treated plants were isolated as described by Ort and Izawa (17). In both cases, the chloroplast pellet was resuspended in a medium (resuspension buffer) containing 200 mM sucrose, 5 mM Hepes-KOH (pH 7.5), 3 mM $MgCl_2$, and 0.5 g/L BSA.

Chl Determination

Pigments were extracted from isolated chloroplasts with 80% acetone. Chl concentrations and Chl *a*/*b* ratios were determined as described by Arnon (1).

UV Light Source

UV radiation was provided by four UVB-313 fluorescent lamps (Q-Panel Company, Cleveland, OH). UV-C emission of the light source was always blocked by a 0.13 mm cellulose diacetate film (Cadillac Plastics, Troy, MI), which does not allow transmission of wavelengths shorter than 290 nm (Fig. 1). Because prolonged UV exposure decreased the UV-B transmission of the cellulose diacetate film, these filters were changed every 5 h in experiments with isolated chloroplasts and every 12 h in long-term treatments. After a 12-h time interval, the light transmission of the cellulose diacetate film at 300 nm was decreased by about 35%. For control samples, UV-B radiation was filtered out by a 0.13 mm polyester film (Cadillac Plastics), which transmits only wavelengths longer than 315 nm (Fig. 1). The spectral irradiance of the UV source was measured at a distance of 0.1 m with a dome window sensor of an OL 752 UV/VIS spectroradiometer (Optronics Laboratories, Inc., Orlando, FL). The spectroradiometer was configured with 0.25 mm slits that produced a band width of 3 nm. Calibration was against a tungsten filament quartz halogen lamp that itself was calibrated against a standard light source (National Institute of Standards and Technology, Washington, DC). Within 280 and 400 nm, the total irradiance of the light source was 11.2 and 3.1 W/m^2 for cellulose diacetate and cellulose diacetate plus polyester, respectively. The biologically effective UV-B irradiance was obtained by weighing the absolute irradiance spectra with the generalized plant-damage action spectrum (5) normalized to unity at 300 nm. From this, total effective irradiances of 1.9 and 0.0 W/m^2 were obtained for UV-B treatment and control, respectively. Consequently, 1 h of UV-B treatment results in a fluence of about 6800 J/m^2, which corresponds to the biologically effective UV-B dose for a cloudless summer day at a latitude of 40° and an elevation of 1500 m (6). In the visible region, the UV source showed distinct emissions at 404, 436, 546, and 578 nm with a quantum flux density of 10, 39, 28, and 9 $\mu E/(m^2 \cdot s)$, respectively.

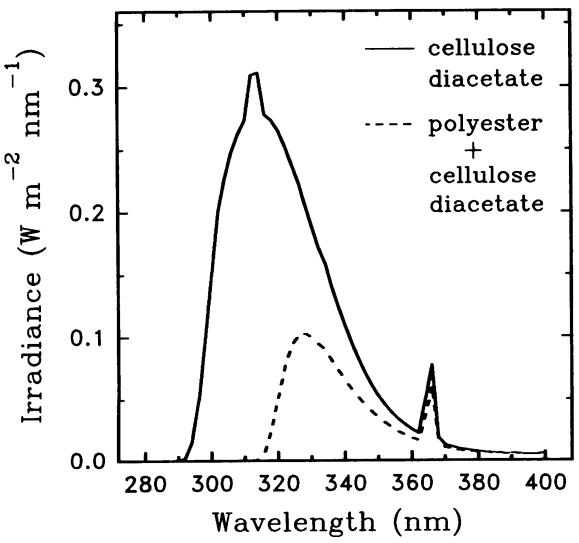

Figure 1. Unweighted spectral irradiance of UV-B-313 fluorescent lamps measured at a distance of 0.1 m filtered through either 0.13 mm cellulose diacetate (solid line) or 0.13 mm diacetate plus 0.13 mm polyester during UV-B treatments (dashed line).

UV-B Treatment

For UV-B exposure of isolated chloroplasts, the preparations were diluted with resuspension buffer to a Chl concentration of 40 $\mu g/mL$. Forty-milliliter aliquots were transferred into 9-cm glass Petri dishes. During the treatment, the samples were kept on ice on a platform of an orbital shaker operated at low frequency. Although all samples remained on the platform for the same amount of time, different intervals of UV-B exposure were achieved by successively covering corresponding Petri dishes with polyester filters. After UV exposure, the chloroplasts were sedimented and the pellet was diluted to a Chl concentration of 1 mg/mL with resuspension buffer containing 20% glycerol. Aliquots of 100 μL were kept in liquid nitrogen until used for measurements.

For whole leaf exposure, flats of plants were positioned at a distance of about 10 cm between the upper unfolded leaves and the light source. For each UV-B treatment, half of the plants were covered with polyester film and used as a control. After the UV treatment, the upper completely unfolded leaves were harvested. Chloroplasts of the leaves were isolated and stored in liquid nitrogen as described above.

Violaxanthin Deepoxidation

Violaxanthin deepoxidation was measured in osmotically shocked chloroplasts according to Siefermann and Yamamoto (20). The absorbance change at 505 nm, with 540 nm as the reference wavelength, was recorded with an Aminco DW-2 UV/VIS spectrophotometer in the dual beam mode

using a band width of 3 nm. During the measurements, chloroplast suspensions were maintained at 18°C and continuously stirred.

Actinic light from a 150-W projection lamp was passed through 2.5 cm of 3% $CuSO_4$ and a Schott RG 695 red glass filter and guided by fiber optics with a divergent endpiece to the cuvette. The resulting quantum flux density, measured with a Li-Cor quantum radiometer model LI-189, was 60 μE/($m^2 \cdot s$) and was saturating for the light-driven violaxanthin deepoxidation. The photomultiplier tube was shielded against actinic light by a Corning CS 4–96 blue glass filter.

The dark violaxanthin deepoxidation was carried out in a buffer containing 50 mM citrate-KOH (pH 5.0), 100 mM sorbitol, 3 mM $MgCl_2$, and the xanthophyll conversion was started by adding ascorbate to a final concentration of 40 mM. Light-driven deepoxidation was carried out in a reaction medium containing 50 mM Hepes-KOH (pH 7.5), 100 mM sorbitol, 3 mM $MgCl_2$, 40 mM ascorbate, and 0.1 mM MV.[2] In experiments in which deepoxidation was driven by PSI alone, 2 μM DCMU and 0.5 mM DQH_2, an electron donor to the Cyt b_6-f complex (15, 28), were present. DQH_2 was prepared from the quinone as described by White *et al.* (28). Ascorbate stock solutions were prepared from free acid and titrated with NaOH to the pH of the respective reaction medium.

Siefermann and Yamamoto (22) introduced the absorbance change at 505 nm as a quantitative measure for the zeaxanthin formation by establishing a proportional relationship between the 505 nm change and the chromatographically determined zeaxanthin concentration. Our chloroplast preparations, however, occasionally showed light-induced absorbance changes at 505 nm either in the absence of ascorbate, the co-substrate of violaxanthin deepoxidation, or in the presence of 5 mM DTT, an inhibitor of the deepoxidase (30). If the DTT-insensitive absorbance change amounted to more than 10% of the absorbance change in the absence of DTT, the former was subtracted from the latter before evaluating the kinetics.

Six different samples, for which absorbance increases between 0.31 and 0.83/(mg Chl·mL) were measured as described, were analyzed chromatographically for their zeaxanthin content according to Gilmore and Yamamoto (10). A linear relation between absorbance increase at 505 nm and zeaxanthin formation was obtained (regression coefficient $r = 0.903$). The calculated *in vivo* extinction coefficient was 21.3 $mM^{-1} \cdot cm^{-1}$ and is in good agreement with the value of 28.8 $mM^{-1} \cdot cm^{-1}$ as published by Siefermann and Yamamoto (22).

The $\Delta A_{505}(t)$ were analyzed in terms of the $\Delta A_{505}\infty$ and the ^1k, as proposed by Siefermann and Yamamoto (20, 21). The parameter $\Delta A_{505}\infty$ was estimated by fitting a single exponential curve to the actual data points, and ^1k was read from the slope of a first order regression to the plot of ln ($\Delta A_{505}\infty - \Delta A_{505}(t)$) *versus* reaction time.

[2] Abbreviations: MV, methyl-viologen; DQH_2, duroquinol (tetramethyl-*p*-hydroquinone); PQ, plastoquinone; TMPD, *N,N,N′,N′*, tetramethyl-*p*-phenylenediamine; ^1k, first order rate constant; $\Delta A_{505}(t)$, absorbance difference at 505 nm as a function of time; $\Delta A_{505}\infty$, asymptote value of $\Delta A_{505}(t)$.

Electron Transport Measurement

Uncoupled electron transport was measured at 18°C in a Clark-type oxygen electrode (Hansatech, Norfolk, UK). The pH 7.5 reaction mixtures described for deepoxidation were used, except ascorbate was omitted, and 5 mM NH_4Cl and 5 mM NaN_3 were present. Additionally, PSI electron transport was carried out using 0.5 mM TMPD as an electron donor to plastocyanine (14). TMPD was kept reduced by 5 mM ascorbate. When an artificial electron donor was used, 15 units/μg Chl superoxide dismutase was present. Actinic light from a 500-W projection lamp was filtered through a Corning CS 2–64 red glass filter and a round bottom flask containing 3% $CuSO_4$. The resulting quantum flux density, which was light-saturating, was 240 μE/($m^2 \cdot s$).

RESULTS

Violaxanthin Deepoxidation in UV-Treated Chloroplasts

The absorbance increase at 505 nm caused by the enzymatic zeaxanthin formation followed first order kinetics in isolated chloroplasts and confirmed the results of Siefermann and Yamamoto (20, 21). This observation is valid for control and UV-B treatments when the deepoxidation reaction was driven in the absence of light at pH 5 (Fig. 2) or with light at pH 7.5 (Figs. 3 and 4). Consequently, each of the deepoxidation kinetics is defined by its ^1k and $\Delta A_{505}\infty$.

For the violaxanthin deepoxidation in the dark at pH 5, the ^1k was found to be progressively decreased with increasing preexposure of chloroplasts to UV-B light and, after 60 min of UV-B exposure, was 52% of the control (Fig. 5B). Under the same reaction conditions, the final extent of the 505 nm change remained constant (Fig. 5A).

The ^1k for light-driven deepoxidation under conditions of electron flow through PSII and PSI (Fig. 3), or through PSI alone with DQH_2 as the electron donor (Fig. 4), were also sensitive to UV-B exposure. However, the control values for light-driven deepoxidation were about 15% lower than the corresponding rate constant measured under dark conditions (Fig. 5B). After 60 min of UV-B treatment, the rate constant dropped to 64 and 73% of the control for whole chain and PSI electron flow, respectively (Fig. 5B).

Whereas in the dark the final extent of the 505 nm change was insensitive to UV-B light, under conditions of whole chain electron transport, this parameter decreased markedly to 56% of the control after 60 min of UV-B irradiation (Fig. 5A). When electron transport was mediated by PSI with DQH_2 as electron donor, however, the total absorbance change decreased only 13% after 60 min of UV-B preillumination.

Electron Transport Rates in UV-B-Treated Chloroplasts

Electron transport rates were measured in the same chloroplasts that were used for the deepoxidation experiments (Fig. 5C). In accordance with the well-known sensitivity of PSII to UV-B (2), whole chain electron transport rates were strongly inhibited by UV-B irradiation and dropped to 21% of the control after 60 min preexposure. PSI electron transport, however, was hardly impaired.

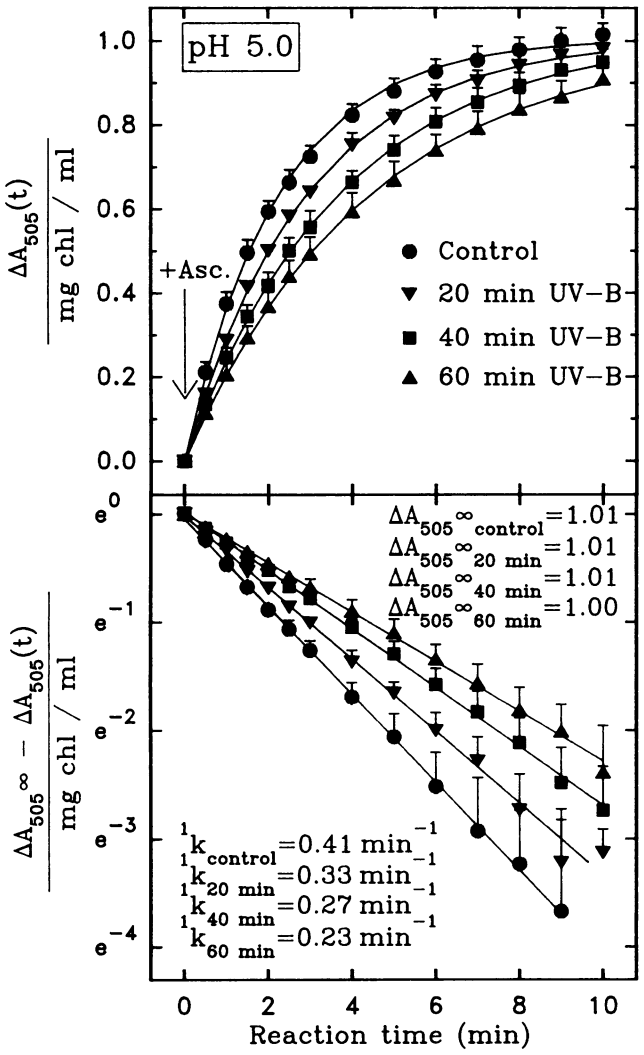

Figure 2. Effect of UV-B pretreatment of isolated chloroplasts on violaxanthin deepoxidation at pH 5.0 in the dark. Isolated chloroplasts were pretreated with UV-B light for the indicated times as described in "Materials and Methods." Violaxanthin deepoxidation was followed by recording the $\Delta A_{505}(t)$ with 540 nm as the reference wavelength (upper panel). The data points of the semilogarithmic plots (lower panel) were obtained by subtracting the $\Delta A_{505}(t)$ values from the $\Delta A_{505}\infty$. ^1k values were obtained from first order regressions. Regression coefficients were 0.9996, 0.9968, 0.9994, and 0.9989 for control, 20 min UV-B-treated, 40 min UV-B-treated, and 60 min UV-B-treated samples, respectively. Each data point corresponds to the mean of at least three values normalized to the Chl concentration of 1 mg/mL (±SD). The deepoxidation reactions were carried out at pH 5 in a medium containing 50 mM citrate-KOH, 100 mM sorbitol, and 3 mM $MgCl_2$ and were started by the addition of ascorbate (final concentration, 40 mM). Chl concentration of the individual samples was 22 to 25 μg/mL.

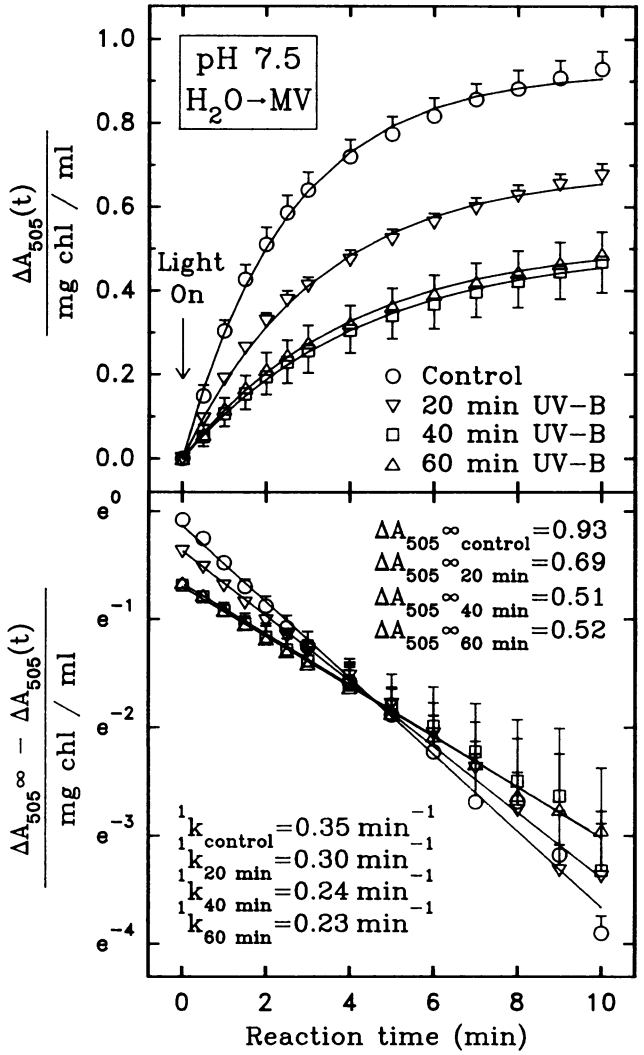

Figure 3. Effect of UV-B pretreatment of isolated chloroplasts on violaxanthin deepoxidation under conditions of whole chain electron transport. Isolated chloroplasts were pretreated with UV-B light for the indicated times. Violaxanthin deepoxidation was measured as the $\Delta A_{505}(t)$ (upper panel). The data points for the semilogarithmic plots (lower panel) were calculated as described in Figure 2. ^1k values were obtained from first order regressions to these plots. Regression coefficients were 0.9951, 0.9963, 0.9914, and 0.9989 for control, 20 min UV-B-treated, 40 min UV-B-treated, and 60 min UV-B-treated samples, respectively. Each data point is the mean (±SD) of at least three values. The deepoxidation reactions were carried out at pH 7.5 in a medium containing 50 mM Hepes-KOH, 100 mM sorbitol, 3 mM $MgCl_2$, 40 mM ascorbate, and 0.1 mM MV and were driven by red light of 60 $\mu E/(m^2 \cdot s)$. Chl concentration of the individual samples was 22 to 25 μg/mL.

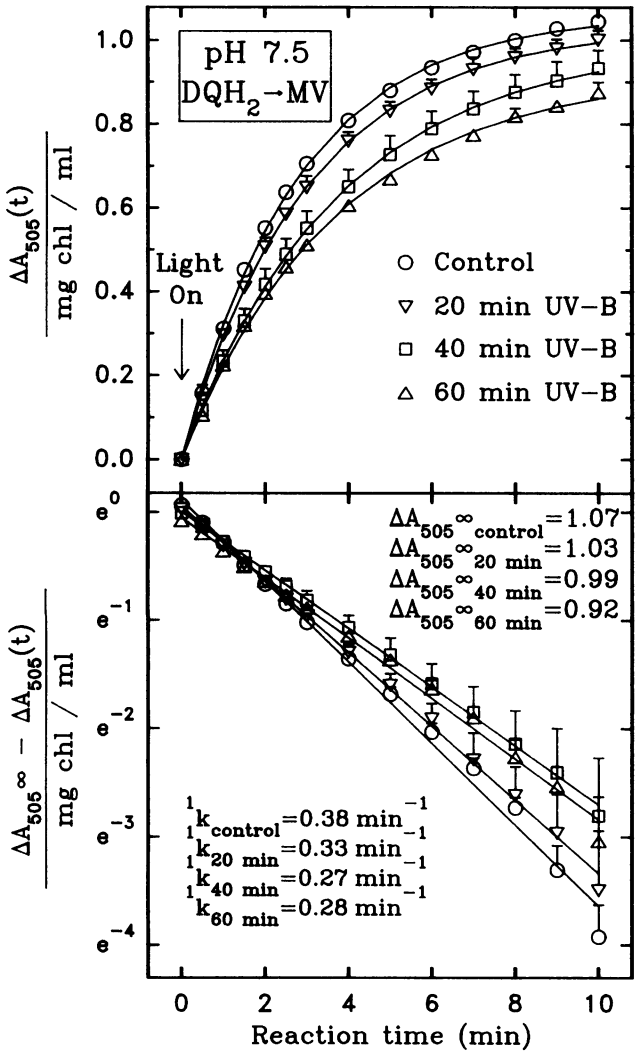

Figure 4. Effect of UV-B pretreatment of isolated chloroplasts on violaxanthin deepoxidation under conditions of PSI electron transport. Isolated chloroplasts were pretreated with UV-B light for the indicated times and were suspended in the pH 7.5 medium described in Figure 3 containing additionally 2 μM DCMU and 0.5 mM DQH_2. For further assay conditions and Chl concentrations, see Figure 3. Regression coefficients for the semi-logarithmic plots were 0.9961, 0.9989, 0.9992, and 0.9969 for control, 20 min UV-B-treated, 40 min UV-B-treated, and 60 min UV-B-treated samples, respectively.

Violaxanthin Deepoxidation in Chloroplasts Isolated from UV-B-Treated Intact Plants

The ^1k for violaxanthin deepoxidation of chloroplasts isolated from UV-B-treated plants showed significant differences between the dark and the light-driven reaction within the same preparation (Table I). Furthermore, differences in the rate constants among the various control samples tested under the same reaction conditions were observed (Table I). No pattern underlying this variability was found. For the three reaction conditions, however, the ^1k measured in chloroplasts isolated from UV-B-treated plants showed a decrease in ^1k for 24 and 64 h preexposure when compared with the respective controls (Table I). After 64 h of UV-B light, the rate decreased to values between 70 and 79% of the control, depending on the reaction condition applied (Table I). For all three reaction conditions, no inhibition was found after 12 h of UV-B treatment.

The control values of the $\Delta A_{505}\infty$ varied with different sample preparations and with reaction conditions (Table I). The $\Delta A_{505}\infty$ value of the UV-B-exposed samples relative to the respective controls was higher for the 12- and 24-h UV-B treatment. After 64 h of pretreatment, no such increase was observed, and the final $\Delta A_{505}\infty$ measured under PSI reaction conditions even dropped to 78% of the control value.

Rate of Electron Transport in Chloroplasts Isolated from UV-B-Treated Plants

As observed for the deepoxidation parameters, the control rate of electron transport was different among the various

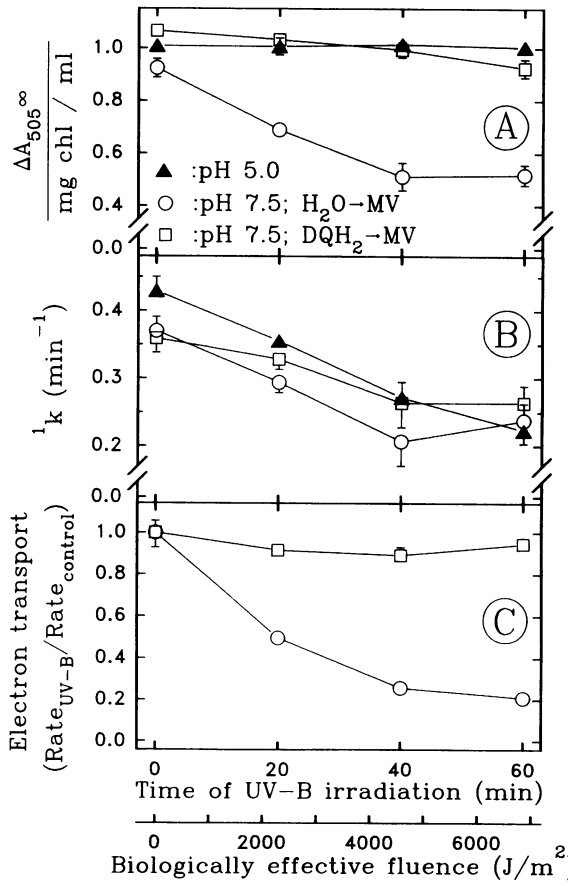

Figure 5. Effect of UV-B pretreatment of isolated chloroplasts on violaxanthin deepoxidation and electron transport measured under various experimental conditions. The $\Delta A_{505}\infty$ (A), ^1k (B), and uncoupled electron transport rates (C) were plotted *versus* the time of UV-B pretreatment. Reactions were carried out at pH 5 in the dark (denoted pH 5.0) or at pH 7.5 under conditions of whole chain electron transport (denoted pH 7.5; $H_2O \rightarrow MV$) or under conditions of PSI-mediated electron transport (denoted pH 7.5; $DQH_2 \rightarrow MV$). Each data point represents the mean (±SD) of three to six individual measurements of deepoxidation kinetics or electron transport rates. Actual electron transport rates were 182 and 451 μmol e^-/(mg Chl·h) for whole chain and PSI electron transport, respectively.

Table I. *Effect of UV-B Irradiation of Intact Plants on Violaxanthin Deepoxidation and Uncoupled Electron Transport Rates*

The ^1k, the final extent of the 505 nm changes normalized to 1 mg Chl/mL (violaxanthin availability), and the electron transport rates of chloroplasts isolated from UV-B-exposed plants are shown. The reaction conditions were as described for Figure 5. Each value represents the mean ± SD of three measurements.

Experimental Condition	Exposure Time		^1k		Violaxanthin Availability		Electron Transport	
			^1k (±SD)	$\frac{^1k_{UV\text{-}B}}{^1k_{control}}$	$\Delta A_{505}\infty$ (±SD)	$\frac{\Delta A_{505}\infty_{UV\text{-}B}}{\Delta A_{505}\infty_{control}}$	Rate (±SD)	$\frac{Rate_{UV\text{-}B}}{Rate_{control}}$
			min^{-1}		mL/mg Chl		μmol e^-/(mg Chl·h)	
pH 5.0 (dark)	12 h	Control	0.28 (±0.02)		1.14 (±0.04)			
		UV-B	0.31 (±0.01)	1.11	1.32 (±0.12)	1.16		
	24 h	Control	0.28 (±0.03)		0.46 (±0.09)			
		UV-B	0.20 (±0.03)	0.71	0.52 (±0.08)	1.13		
	64 h	Control	0.27 (±0.03)		0.64 (±0.05)			
		UV-B	0.19 (±0.02)	0.70	0.64 (±0.05)	1.00		
pH 7.5 $H_2O \rightarrow MV$	12 h	Control	0.36 (±0.05)		0.96 (±0.04)		405 (±20)	
		UV-B	0.35 (±0.05)	0.97	1.12 (±0.01)	1.17	463 (±10)	1.14
	24 h	Control	0.31 (±0.01)		0.43 (±0.05)		221 (± 4)	
		UV-B	0.27 (±0.02)	0.87	0.62 (±0.02)	1.44	187 (±15)	0.85
	64 h	Control	0.43 (±0.01)		0.50 (±0.03)		463 (±12)	
		UV-B	0.34 (±0.03)	0.79	0.49 (±0.02)	0.98	202 (±22)	0.44
pH 7.5 $DQH_2 \rightarrow MV$	12 h	Control	0.28 (±0.01)		0.88 (±0.01)		578 (±15)	
		UV-B	0.31 (±0.03)	1.11	1.12 (±0.04)	1.27	695 (±35)	1.20
	24 h	Control	0.23 (±0.05)		0.40 (±0.03)		291 (± 5)	
		UV-B	0.20 (±0.03)	0.87	0.53 (±0.01)	1.33	258 (±19)	0.89
	64 h	Control	0.39 (±0.01)		0.49 (±0.02)		732 (±25)	
		UV-B	0.29 (±0.06)	0.74	0.38 (±0.02)	0.78	406 (±25)	0.55

chloroplast isolations (Table I). Compared with the respective controls, whole chain electron flow, as well as PSI-mediated electron transport with DQH_2 as the electron donor, was significantly inhibited by UV-B treatment. After 64 h of exposure, the rates were decreased to 44 and 55% of the initial rates for whole chain and PSI reaction, respectively (Table I).

Comparison of UV-B Effects on Isolated Thylakoids and Whole Leaves

A significantly higher inhibition of PSI-mediated electron transport was observed when whole leaves were UV-B treated compared with the UV-B treatment of isolated chloroplasts (Fig. 5C, Table I). These observations have been verified in an experiment in which plants were exposed to the usual UV treatment for 60 h. Subsequently, chloroplasts isolated from the control plants were subjected for 30 min to UV-B or control treatment. Both pretreatments decreased the rate of whole chain electron transport ($H_2O \rightarrow MV$) to about 35% (Fig. 6). However, UV-B exposure of the whole leaves decreased PSI electron transport ($DQH_2 \rightarrow MV$) to 46% of the control activity, whereas the treatment of isolated chloroplasts gave a value of 75%. Likewise, electron flow from TMPD to MV was inhibited more in the whole leaf experiment (65% activity remained) compared with the exposure of isolated thylakoids, which did not show significant inhibition.

The different pattern of data obtained for the UV-B treatment of isolated chloroplasts (Fig. 5) compared with the leaf exposure (Table I) is more obvious when the various inhibition time courses, normalized to the control value, are plotted *versus* the corresponding inhibition time course of whole chain electron transport (Fig. 7). Figure 7C shows the already-mentioned difference in UV-B susceptibility of PSI electron flow between the whole leaf treatment and the chloroplast experiments. A similar difference was found for the Chl *a*/*b* ratio, which was significantly reduced in the whole leaf inhibition (−9%), whereas the Chl *a*/*b* ratio remained virtually constant in the isolated chloroplast experiment (Fig. 7D). No significant difference in the inhibition pattern between the two experimental setups was found for the ^1k of light-driven deepoxidation (Fig. 7B). This is also valid for the rate constants measured under dark conditions (data not plotted in Fig. 7). When isolated chloroplasts were subjected to UV-B irradiation, the $\Delta A_{505}\infty$ was reduced only under conditions of whole chain electron transport (Fig. 5A). A different pattern was found for the leaf treatment, where, with increasing UV-B exposure, the $\Delta A_{505}\infty$ measured under conditions of PSI electron transport dropped below the corresponding whole chain value.

DISCUSSION

In this paper, we have investigated the kinetics of violaxanthin deepoxidation in chloroplasts pretreated with UV-B light and in chloroplasts isolated from UV-B-treated plants. The deepoxidation of violaxanthin to zeaxanthin measured as $\Delta A_{505}(t)$ followed first order kinetics (Figs. 2–4), as was already shown by Siefermann and Yamamoto (20). Conse-

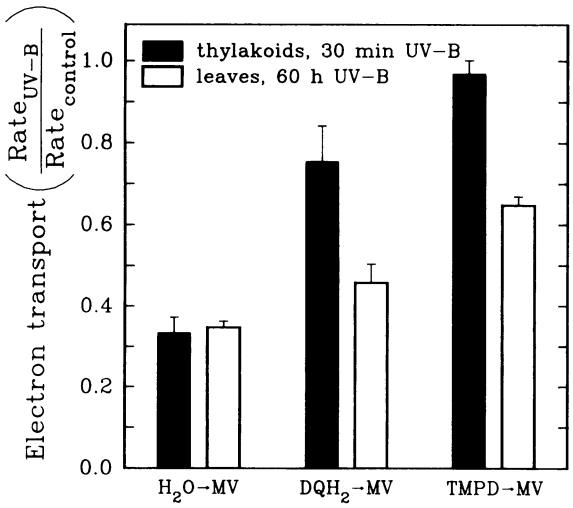

Figure 6. Inhibition of electron transport rates in isolated chloroplasts pretreated with UV-B light and in chloroplasts isolated from plants treated with UV-B light. Chloroplasts isolated from the controls of UV-B exposure of whole plants (open bars) were used for UV-B irradiation of chloroplasts (closed bars). Uncoupled whole chain electron transport and uncoupled PSI electron transport with DQH_2 or TMPD as the electron donor were measured. UV-B treatment was 30 min and 60 h for isolated chloroplasts and whole plants, respectively. Control rates for the treatment of isolated chloroplasts were 151, 302, and 323 μmol e^-/(mg Chl·h), and for chloroplasts isolated from treated plants 297, 431, and 436 μmol e^-/(mg Chl·h), for electron transport with water, DQH_2, and TMPD as the electron donor, respectively.

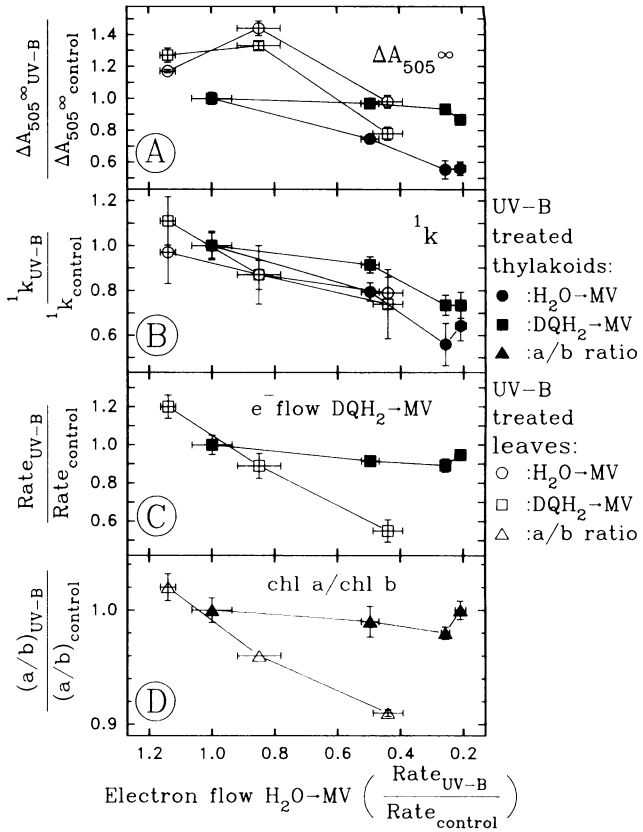

Figure 7. Comparison of the effect of UV-B treatment of isolated chloroplasts and intact plants. The various inhibition time courses obtained for UV-B treatment of isolated chloroplasts and of intact plants are plotted *versus* the respective inhibition values of whole chain electron transport. Electron transport data and parameters of the deepoxidation kinetics are taken from Figure 5 and Table I, for UV-B-treated chloroplasts and UV-B-treated leaves, respectively. Control values for the Chl *a/b* ratio were 3.7 and 3.4, for the chloroplast treatment and the leaf treatment, respectively.

quently, deepoxidation kinetics were analyzed in terms of their ^1k and their $\Delta A_{505}\infty$, which correspond to the activity of the violaxanthin deepoxidase and the maximum amount of violaxanthin available for deepoxidation, respectively (20, 21).

In isolated thylakoids at pH 5, the pH optimum of the deepoxidase (12), violaxanthin deepoxidation can be induced in the dark by adding the co-substrate ascorbate, thus excluding influences of UV-B photoinhibition on the photosynthetic apparatus. When isolated thylakoids were pretreated with UV-B light and tested under dark conditions, the ^1k decreased with increasing UV-B preexposure of the chloroplasts, whereas the availability of violaxanthin remained constant (Figs. 2 and 5). From this, it follows that the violaxanthin deepoxidase is inhibited by UV-B radiation and that no immediate action of UV-B light on the violaxanthin pool *per se* takes place. The unchanged violaxanthin availability together with the monophasic single exponential deepoxidation kinetics observed throughout the time course of UV-B inhibition can be most simply explained by assuming that many deepoxidase enzymes are sharing a common violaxanthin pool. In this case, the apparent rate constant would be the mean of the rate constant of the fraction of noninhibited, and the rate constant of the fraction of inhibited, enzymes.

When deepoxidation was activated at physiological pH by proton pumping caused by electron flow through both photosystems, however, UV-B irradiation of isolated chloroplasts resulted in a marked drop in the apparent availability of violaxanthin (Figs. 3 and 5A). Under conditions in which PSII was blocked by DCMU and electrons were donated by DQH_2 as an analog to plastoquinol (15, 28), the violaxanthin availability was restored to the control level (Figs. 4 and 5A), although the enzymatic rate constant was still inhibited.

It is known that UV-B irradiation affects the electron transport chain predominantly at the level of PSII (2). In agreement with those reports, the UV-B treatment of isolated chloroplasts inhibited primarily whole chain electron flow, whereas PSI electron transport with DQH_2 as electron donor was hardly impaired (Fig. 5C). From this, we presume that the decrease of the violaxanthin availability results from PSII damage. Siefermann and Yamamoto (21) concluded from their results that the availability of violaxanthin is controlled by the redox state of PQ or an electron carrier near PQ, *i.e.* a highly oxidized state corresponded to a lesser violaxanthin availability. In the present study, an impaired PQ reduction by PSII due to UV-B inactivation, together with an unimpaired PQH_2-oxidizing function of the remaining electron transport chain, could lead to a relatively more oxidized PQ pool and thus account for the decrease in the availability. The

observation that no decrease in the violaxanthin availability occurred after 40 min UV-B exposure, although the rate of whole chain electron transport continued to drop (Fig. 5), may be due to electron donation to PQ by ascorbate, which at this greater extent of PSII inhibition overcomes the PSII-mediated redox control.

When isolated chloroplasts were treated with UV-B light, the inhibition time courses of the first order rate constant measured for light-driven deepoxidation were in good agreement with the time course obtained for the dark experiments (Fig. 5B). The reasons for the decreased initial rate constants of the light-driven reactions may be suboptimal coupling due to the pretreatment of the chloroplasts and the storage in liquid nitrogen.

Taking into consideration that the violaxanthin deepoxidase is susceptible to inhibition by the sulfhydryl reagent DTT (30), one may infer that the enzyme contains at least one cistine disulfide bridge, which plays a key role for protein conformation or enzyme activity. Cistine is a relatively poor absorber in the UV-B region but undergoes UV-induced photolysis at a high quantum efficiency (7, 11, 18, 26). We suggest, as a hypothesis, that the rupture of a disulfide bridge of the deepoxidase may be the inactivation mechanism of UV-B action.

When intact leaves were irradiated with UV-B light, the inhibition of the rate constant of violaxanthin deepoxidation required much higher doses compared with the UV-B exposure of isolated chloroplasts. After the leaves were treated for 64 h with UV-B light, the rate constant was decreased to about 75% of the control value (Table I). UV-B exposure of isolated chloroplasts for less than 2% of this time interval caused a comparable inhibition of the rate constant (Fig. 5). Similarly, the dose requirement of the inhibition of whole chain electron transport was much smaller when isolated chloroplasts were UV-B treated (Table I, Fig 5). The relatively low UV-B susceptibility of intact leaves is most probably due to selective screening of short wavelength radiation by the epidermis and the upper cell layers of the leaf (8).

After 12 h of UV-B treatment of intact leaves, the rate constant of violaxanthin deepoxidation measured under the various reaction conditions was unchanged or slightly increased compared with the control (Table I). It is obvious from the corresponding SDs listed in Table I that the observed increases are not significant and are probably due to experimental error.

Whole chain and PSI electron transport rates measured under light-saturating conditions, however, were significantly increased after 12 h UV-B irradiation (Table I). Because these electron transport rates were normalized to equal Chl concentration, a loss in bulk Chl that proceeds faster than the inactivation of the electron transport chain could mimic an increased electron transport rate. In fact, Strid *et al.* (24) found in pea leaves a delay of 1 d for the onset of UV-B inhibition of the maximum photosynthetic rate, whereas the total Chl content was reduced within this time interval.

Moreover, the increase in violaxanthin availability found for the 12 and the 24 h treatment may be due to a more rapid decay of Chl molecules compared with carotenoids during UV-B exposure. Actually, a decrease of the Chl/carotenoid ratio during UV-B irradiation of pea plants was also reported by Strid *et al.* (24). No stimulation of the apparent violaxanthin availability was observed after 64 h of UV-B treatment. At this stage of inhibition, the actual availability may be decreased by general damage to the thylakoid membranes (3).

In addition to the difference in the dose requirement of the inhibition of whole chain electron transport and deepoxidase activity, PSI electron transport was decreased more when the UV-B treatment was carried out with intact leaves compared with the UV-B exposure of isolated thylakoids (Fig. 5, Table I).

We verified the electron transport data in an experiment in which identical plant material was used for the UV exposure of whole leaves and chloroplasts. Fluences that yield about the same extent of inhibition of whole chain electron transport for both experimental setups were chosen (approximately 65% activity loss, Fig. 6). Similar to what had been found in the previous experiments, PSI electron transport with DQH_2 was inhibited twice as much in the leaf exposure compared with the UV-B treatment of isolated chloroplasts (−55% and −25%, respectively, Fig. 6). PSI-mediated electron transport, with TMPD as electron donor to plastocyanin, was inhibited only in the whole leaf treatment. The fact that the inhibition of electron transport with DQH_2 as the electron donor was greater compared with the rates measured with TMPD (Fig. 6) indicates that the Cyt b_6-f complex, as well as PSI, are inhibited by UV-B irradiation of intact leaves.

It is obvious from our results that doses that cause the same whole chain inhibition yield different inhibition of PSI electron transport, depending on whether intact leaves or isolated chloroplasts are exposed to UV-B light. One may assume that equal inhibition of whole chain electron flow obtained by UV-B irradiation of isolated chloroplasts or by UV-B irradiation of intact leaves corresponds approximately to the same cumulative dose of UV-B radiation that reached the thylakoid membrane. Consequently, it can be concluded that the different pattern of UV-B inhibition as demonstrated by plotting the various inhibition time courses *versus* the corresponding values of whole chain inhibition (Fig. 7) is not caused by direct action of UV-B light. One possibility we can propose is that the deleterious effect of UV-B irradiation on the ultrastructure of plant cells (3) may result in the release of potentially inhibitory substances that are usually confined to specific cell compartments. Because UV-B light generally can interact with proteins and nucleic acids, the inhibition of reactions that are required for the regular biochemical turnover of chloroplast constituents may additionally play a role in the long-term exposure of whole plants.

In UV-B-treated isolated chloroplasts, a marked decrease of the violaxanthin availability was observed under conditions of whole chain electron transport, but electron donation by DQH_2 restored the availability to the control level (Fig. 5A). When whole leaves were UV-B treated for 24 or 64 h, the violaxanthin availability for PSI electron transport was smaller than the corresponding whole chain value (Table I, Fig. 7). These observations may be explained if one assumes that the UV-B inhibited electron transport through the Cyt b_6-f complex and PSI results in not oxidizing the PQ pool to the extent that is sufficient to decrease the violaxanthin availability under conditions of whole chain electron transport.

CONCLUDING REMARKS

We conclude from our results that the enzyme violaxanthin deepoxidase in intact leaves and in isolated chloroplasts is inhibited by UV-B light. The dose required for the inhibition in intact leaves, however, is about 2 orders of magnitude higher compared with isolated chloroplasts. The size of the violaxanthin pool available for deepoxidation is not affected by UV-B irradiation. A decrease of the violaxanthin availability by indirect UV-B action mediated by a decrease of PSII activity or by general membrane damage, however, may occur.

It has been suggested (9) that xanthophyll cycles play an important role in protection against photoinhibition by visible light. Our findings clearly show that this putative protection mechanism itself is a target of photoinhibitory radiation, namely UV-B light. Consequently, the exposure of plants to sunlight with a high UV-B portion may result in an increased susceptibility to photoinhibition by visible light and, thus, amplify the separate, potentially deleterious actions of UV-B and visible light.

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