J. Plant Res. **113**: 311-317, 2000

Journal of Plant Research

Enhancement of the Tolerance to Oxidative Stress in Cucumber (*Cucumis sativus* L.) Seedlings by UV-B Irradiation: Possible Involvement of Phenolic Compounds and Antioxidative Enzymes

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Cucumber (*Cucumis sativus* L.) seedlings were irradiated or not irradiated with UV-B for several days in environment-controlled growth chambers. The first leaves irradiated with UV-B were retarded in growth but simultaneously acquired a remarkably high tolerance to oxidative stress, as induced by paraquat treatment, compared with the non-irradiated leaves. This enhanced tolerance was observed within 1 d after the start of UV-B irradiation and was maintained during the 12 d period of UV-B treatment. The effects of UV-B on several antioxidative enzymes were examined, and activities of superoxide dismutase, ascorbate peroxidase and guaiacol peroxidase, but not of glutathione reductase, were found to be enhanced. However, activation of these enzymes occurred only from 6 d after the start of irradiation. In contrast, accumulation of phenolic compounds was observed within 1 d after the start of UV-B irradiation. HPLC analysis of phenolic compounds showed the distinct enhancement of a substance, which may have antioxidative properties in cucumber seedlings irradiated with UV-B. On the basis of these results, we conclude that not only antioxidative enzymes but also other factors in cucumber seedlings irradiated with UV-B, such as phenolic compounds, may participate in the enhanced tolerance to oxidative stress.

Key words: Antioxidative enzymes — Cucumber (*Cucumis sativus* L.) — Oxidative stress — Paraquat — Phenolic compounds — UV-B

A decrease in the concentration of stratospheric ozone has led to an increase in the amount of UV-B irradiation (290-315 nm) that reaches the earth's surface. Numerous studies have demonstrated several detrimental effects of UV-B on plants, including the inhibition of photosynthetic activity (Teramura 1980, Smillie 1982, He *et al.* 1993) and growth (Sisson 1981, Takeuchi *et al.* 1989). Therefore, such an increase in UV-B is expected to have a negative impact on plants. It is well known that UV-B induces DNA damage by the formation of DNA photoproducts (Britt 1996). In addition, it was suggested that harmful free radicals would participate, at least partly, in the growth reduction of cucumber cotyledons by UV-B (Takeuchi *et al.* 1995), although it is only recently that UV-B irradiation has been shown to also induce the production of free radicals in plants (Hideg and Vass 1996, Hideg *et al.* 1997).

In order to prevent these harmful effects of UV-B irradiation, plants have developed several defense mechanisms. Plants accumulate UV-absorbing phenolic compounds, mainly in the vacuoles of epidermal cells, in order to prevent the penetration of UV-B into the internal tissue (Reuber *et al.* 1996a, b). The accumulation of these phenolic compounds in various plant species has been shown to be stimulated by UV-B (Bornman and Teramura 1993). Plants also have ability to repair DNA damages, caused by UV-B irradiation, using DNA repair enzymes, such as photolyase (Taylor *et al.* 1997).

The UV-B induction of antioxidative enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase (GR), has also been reported in some plants (Strid 1993, Rao *et al.* 1996, Takeuchi *et al.* 1996, Kubo *et al.* 1999), and is assumed to constitute a defense response of plants to free radicals generated by UV-B. In addition, it has recently been suggested that phenolic compounds contribute to the UV-B adaptation of plants not only by UV-absorption but also through their other functions, such as radical scavenging (Markham *et al.* 1998, Olsson *et al.* 1998). Indeed some phenolic compounds are known to have antioxidative ability (Larson 1988). However, it is not yet been investigated whether the plants whose activity of oxidative enzymes or accumulation of phenolic compounds was increased by UV-B could actually acquire enhanced tolerance to oxidative stress.

In the present study, we examined the effect of UV-B irradiation on plant tolerance to oxidative stress, using the free radical generator, paraquat. We also investigated the

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Abbreviations: APX, ascorbate peroxidase; GR, glutathione reductase; PER, guaiacol peroxidase; SOD, superoxide dismutase; UV-B, ultraviolet-B.

effects of UV-B on antioxidative enzymes and phenolic compounds, and discuss their possible role in plant tolerance to oxidative stress.

Most data presented in the present paper have been reported elsewhere (Kawashima *et al.* 2000).

Materials and Methods

Plant material and growth conditions

Seeds of cucumber (*Cucumis sativus* L. cv. Hokushin) were sown on artificial soil in plastic pots (7×11 cm). The seedlings were grown in a growth chamber (KG-50HLA; KOITO INDUSTRIES, Tokyo, Japan) with a temperature of 25°C, 70± 7% RH, 160 μmol m^{-2} s^{-1} PAR, and with a 12 h light period. The seedlings were watered every 2 or 3 days with fertilizers using 0.1% volumes of hyponex (Hyponex Japan, Osaka, Japan). When about 8-days old, seedlings were transferred to another chamber to be grown under a 20/15°C (light/dark) temperature regime.

UV-B irradiation

Approximately 10-day-old seedlings were used for the UV-B treatments. Control plants were grown under visible light only, while treated plants were irradiated with supplementary UV-B during the 12 h light periods. UV-B was provided by two fluorescent UV-lamps (FL20S • E; Toshiba, Tokyo, Japan), suspended 53 cm above the plant seedlings. The radiation was filtered through 2 mm-thick quartz glass (UV-29; Hoya, Tokyo, Japan) to remove wavelengths below 290 nm. The UV-B fluence rate, at the height of seedlings, was measured to be 0.2 W m^{-2} s^{-1} using a UV monitor (MCPD-1000; Otsuka Electric, Tokyo, Japan).

Chlorophyll fluorescence

The quantum yield of PSII electron transport was assessed by measuring the chlorophyll fluorescence (F_v/F_m) with a pulse amplitude modulation fluorometer (PAM 2000; Heinz Walz GmbH, Germany) (Takeuchi *et al.* 1995). Leaves were dark adapted for 5 min prior to measurement.

Examination of paraquat sensitivity

The sensitivity of leaf tissues to paraquat (1, 1′-dimethyl-4, 4′-bipyridinium dichloride; Sigma Chemical, USA) was evaluated as described by Aono *et al.* (1993). Leaf discs (1.2 cm in diameter), excised from cucumber first leaves, were incubated with their adaxial side surface up in 2.5 ml of 0.1% Tween 20 (Sigma Chemical, USA) with or without various concentrations of paraquat in 24-well cluster dishes. The leaf discs were incubated for 1 h in the dark and then for 30 h in light (250 μmol m^{-2} s^{-1}) at 25°C, after which visible damage (bleaching) to the discs was examined.

Enzyme assays

To determine the activities of SOD, GR, PER and APX, the cucumber first leaves were harvested at noon and used. Tissues were homogenized at 4°C in a buffer (100 mM potassium phosphate, pH 7.8 or supplemented with 1 mM ascorbate and 1.1 M sorbitol for APX activity determination), and the supernatant obtained after centrifugation of the homogenate at 22,000×g for 30 min at 4°C was used as a crude extract for enzyme assays. Protein concentration was determined using a Protein Assay kit (BioRad Laboratories, USA) with BSA as a standard. For the measurement of SOD activity, crude extracts were dialyzed overnight against 20 mM potassium phosphate buffer (pH 7.8) at 4°C and the SOD activity was then measured spectrophotometrically using the SOD-525 method (Bioxitech S.A., Bonneuil/Marne, France). The assay for GR activity was performed according to Tanaka *et al.* (1982), using a reaction mixture containing 100 mM potassium phosphate (pH 7.8), 0.2 mM NADPH, 0.5 mM GSSG, and an appropriate amount of crude extract. PER activity was determined according to Chance and Maehly (1955), using a reaction mixture at 25°C containing 100 mM potassium phosphate (pH 6.5), 5 mM guaiacol, 2 mM hydrogen peroxide, and an appropriate amount of crude extract. APX activity was assayed according to the method described by Nakano and Asada (1987), in a reaction mixture containing 50 mM potassium phosphate (pH 7.0), 0.5 mM ascorbate, 0.5 mM hydrogen peroxide and crude extract.

Extraction and quantification of UV-absorbing pigments

Cucumber first leaves, excised from control and UV-B treated seedlings, were cut into pieces, and then incubated in 80% (v/v) methanol for 24 h at 4°C. The extraction was performed twice and, after two extracts were combined, the extract was filtered through a membrane filter (Samprep-LCR25-LH; MILLIPORE, USA).

Quantification of UV-absorbing pigments was performed by measuring the relative absorbance of extract at 300 nm with a spectrophotometer (DU Series 60; Beckman Instruments, USA).

Chromatography was performed with an HPLC system (GL Sciences, Tokyo, Japan). Separation of the compounds was performed at 35°C through a 4.6×150 mm Inertsil ODS-3 column (GL Sciences, Tokyo, Japan) at a flow rate of 0.8 ml min^{-1} and with a mobile phase of distilled water containing 10% (v/v) acetonitrile and 1% (v/v) phosphoric acid. Phenolic compounds were detected at 300 nm. Absorption spectra of the chromatographical peaks obtained were recorded on-line using a photodiode array detector (Shimadzu, Tokyo, Japan).

Results

Effect of UV-B on cucumber seedlings

In response to UV-B irradiation, a growth inhibition of cucumber first leaves was observed from about 3 d after start of UV-B treatment (34% decrease compared with controls), as reported by Murase *et al.* (1997). However, visible injury, such as chlorosis, which is likely to have been mediated by the generation of free radicals, was not observed throughout the 12 d of UV-B treatment.

Photosynthetic damage of cucumber first leaves

To investigate the effect of UV-B on the electron transport

of photosystem, we measured the Fv/Fm ratio of cucumber first leaves during the 12 d period of UV-B treatment. UV-B irradiation caused no change in the F_v/F_m ratio for the first 6 d (Table 1). However, the F_v/F_m ratio decreased by 13% compared to the control after 9 d, and was kept to be lower than the control level until 12 d.

Tolerance of cucumber first leaves to oxidative stress

In order to examine the effect of UV-B irradiation on acquired plant tolerance to oxidative stress, we treated leaf discs excised from UV-B irradiated or non-irradiated (control) cucumber first leaves with various concentrations of paraquat in the light.

Nearly all leaf discs exhibited chlorosis (bleaching) at high concentrations of paraquat (Table 2), although the extent of chlorosis was always much lower in leaf discs from UV-B treated plants than in those from control plants. Enhanced tolerance to paraquat in UV-B treated plants was observed from 1 d after the start of the UV-B irradiation. While leaf discs from control leaves exhibited visible damage in response to 50 μM paraquat, discs from UV-B treated leaves were still relatively tolerant to 200 μM paraquat for the first 6 d. This enhanced tolerance was maintained even up to 12 d of UV-B treatment.

Table 1 Effect of UV-B irradiation on the Fv/Fm ratio of cucumber first leaves

Days of UV-B	Quantum yield of PSII electron transport (Fv/Fm)		
	Control (A)	+UV-B (B)	B/A ratio
1	0.75±0.01	0.76±0.00	1.01
3	0.75±0.02	0.78±0.01	1.03
6	0.77±0.01	0.75±0.03	0.98
9	0.79±0.01	0.69±0.02	0.87
12	0.81±0.01	0.71±0.02	0.88

Each value represents the average of 3 separate samples. Means±SD are shown.

Table 2 Effects of paraquat concentrations on leaf discs from cucumber first leaves

UV-B treatment		Extent of bleaching					
		Paraquat concentration (μM)					
		0	5	10	50	100	200
1 day	+UV-B	n	n	n	n	n	n
	Control	n	n	n	‡	‡	‡
3 day	+UV-B	n	n	n	n	+	‡
	Control	n	n	‡	‡	‡	‡
6 day	+UV-B	n	n	n	n	n	n
	Control	n	n	‡	‡	‡	‡
9 day	+UV-B	n	n	n	+	‡	‡
	Control	n	+	‡	‡	‡	‡
12 day	+UV-B	n	n	n	+	‡	‡
	Control	n	+	‡	‡	‡	‡

Leaf discs from cucumber leaves irradiated or non-irradiated with UV-B for 1, 3, 6, 9 and 12 d were treated with solutions that contained the indicated concentrations of paraquat. The extent of bleaching of discs represents the extent of oxidative damage induced by paraquat treatment. n : non-bleached, + : partially bleached, ‡ : markedly bleached, ‡ : completely bleached

Effect of UV-B irradiation on antioxidative enzymes

The effect of UV-B on the activities of several antioxidative enzymes in cucumber first leaves was examined during the 12 d period of UV-B treatment. UV-B irradiation began to enhance the activities of SOD and APX drastically from 3 d after start of UV-B treatment, attaining about 4.5-fold increases, compared with controls, after 12 d of irradiation (Fig. 1A, B). In contrast, UV-B irradiation had no significant effect on GR activity during the 12 d of irradiation period (Fig. 1C). PER activity was also enhanced from 3 d after the start of UV-B treatment, attaining 33.5-fold the levels of controls after 12 d (Fig. 1D).

UV-B stimulation of phenolic compound accumulation

In first leaves of cucumber seedlings grown with supplementary UV-B irradiation, the contents of total phenolic compounds began to rise from about 6 h after the start of UV-B irradiation (Fig. 2A), and reached a maximum level (1.4-fold) after 1 d of irradiation (Fig. 2B). Subsequently, however, the levels of phenolic compounds began to decline reaching the control levels by 9 d of UV-B irradiation.

The HPLC profiles of UV-absorbing phenolic compounds from cucumber first leaves are shown in Fig. 3 with (Fig. 3B) or without (Fig. 3A) UV-B irradiation for 1 d. Although UV-B irradiation did not affect the number of peaks, the amplitude of each peak increased to 1.2 to 1.8-fold that of control plants. One peak (indicated by arrow) showed a particularly distinct increase (3.5-fold) compared with the other peaks. To determine the possible type of compound represented by this peak, a spectrum of the peak was obtained by photodiode array detection (insert of Fig. 3B). The peak was found to have a very similar spectrum to that of chlorogenic acid, and so it was assumed to correspond to chlorogenic acid itself or to one of its related compounds.

Discussion

In order to evaluate the effect of UV-B on acquired plant tolerance to oxidative stress, we performed experiments using paraquat, which is known to be reduced by the photosystem and so, generating oxygen free radicals in chloroplasts of plant cells (Dodge 1975), induce oxidative damage to plants. Treatment of leaf discs with various concentrations of paraquat (Table 1) indicated that cucumber first leaves irradiated with supplementary UV-B acquired a higher tolerance to paraquat compared with control leaves. This enhanced tolerance was observed even after 1 d of UV-B irradiation, and was maintained up to 12 d of irradiation. Quantum yield of PSII electron transport (F_v/F_m) was not affected by UV-B at the early stages of irradiation, and only

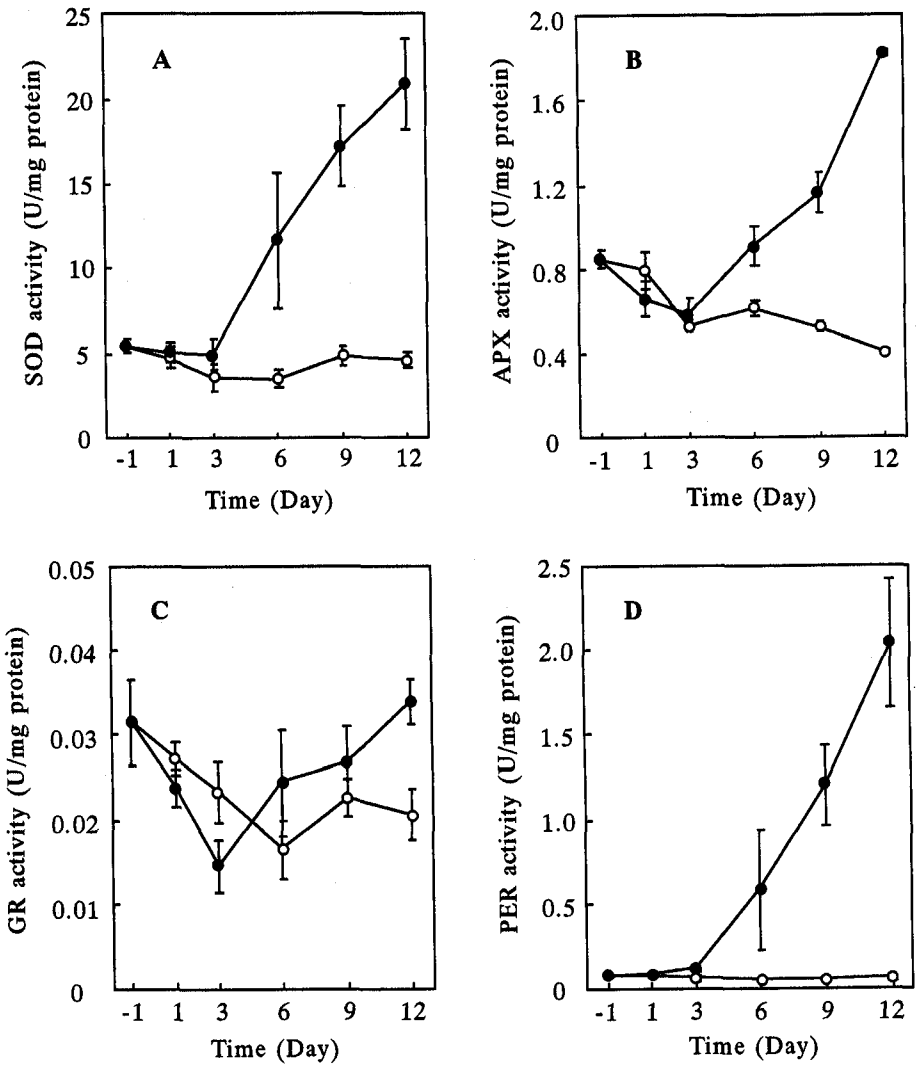

Fig. 1 Changes in SOD (A), APX (B), GR (C) and 'ER (D) activities in cucumber first leaves during UV-B treatment. UV-B irradiation was initiated at 0 d. The unit (U) of enzyme activity was expressed as μmol substrate catalyzed per minute. ●, UV-B irradiated; ○, UV-B non-irradiated (control). Each value is the average of results from 3 separate samples. Vertical bars represent ±SD.

showed slight decreases after 9 d (Table 2), as observed in wheat seedlings (Sharma *et al.* 1998). It has been shown that UV-B mainly inhibits PSII activity without affecting PSI (Noorudeen and Kulandaivelu 1982). Thus, in the present experiment, photosystem is considered not to have been particularly affected by UV-B irradiation, and it is unlikely therefore that the suppressed paraquat effect in UV-B irradiated leaves was due to a decrease in electron transport from the PSI to paraquat. These results therefore indicate that UV-B irradiation enhanced the tolerance of cucumber to oxidative stress, and that this enhancement was rapidly induced and continued for at least 12 d during the irradiation period. It has been shown that UV-B irradiation induces the production of free radicals in *Vicia faba* (Hideg and Vass 1996, Hideg *et al.* 1997). Although we did not determine whether free radical generation was actually enhanced by UV-B or not, the rapid enhancement of tolerance to oxidative stress suggests that free radicals were generated in cucumber early on after the start of UV-B irradiation as already suggested by Takeuchi *et al.* (1996).

Therefore, the next problem is whether the increased tolerance to oxidative stress was associated with increased activities of some antioxidative enzymes. In chloroplasts, SOD, APX and GR are regarded as key enzymes of the scavenging pathway, and UV-B irradiation is known to induce their activities in various plant species; for example, GR in *Pisum sativum* (Strid 1993), *Nicotiana plumbagirifolia* L. (Willekens *et al.* 1994) and *Triticum aestivum* L. (Barabás *et al.* 1998), and SOD and APX in *Arabidopsis thaliana* (Rao *et al.* 1996, Kubo *et al.* 1999). The antioxidative enzymes induced by UV-B are likely to be specific to the plant species. In cucumber cotyledons, the activities of APX and SOD were found to be enhanced by UV-B irradiation (Takeuchi *et al.* 1996). Also, in the present experiments using cucumber first

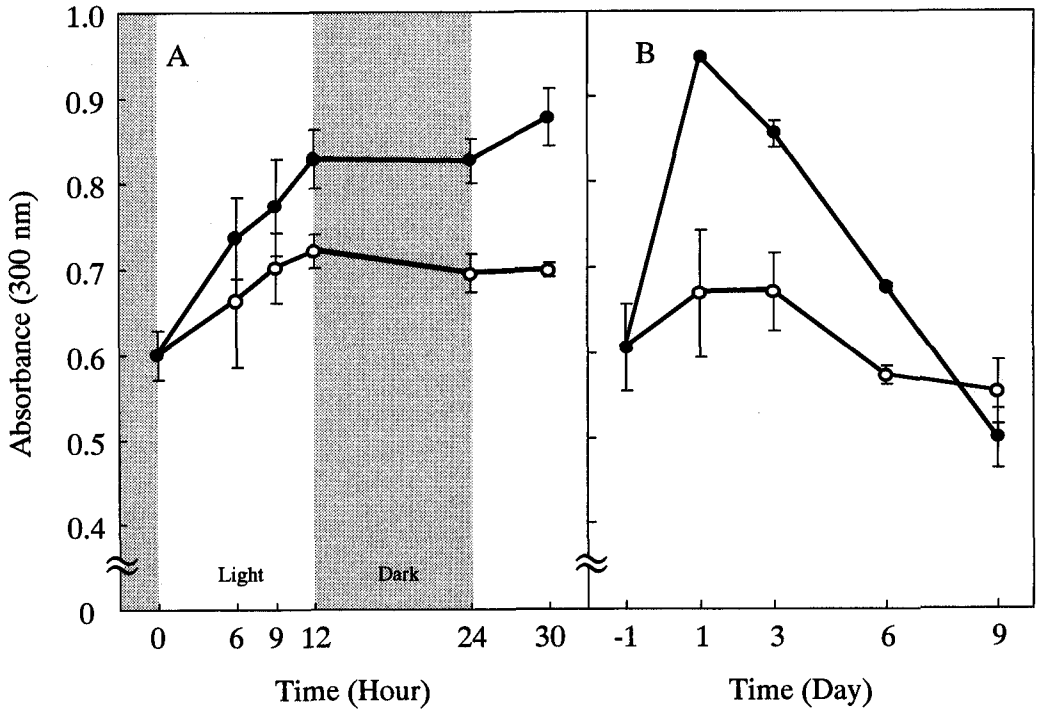

Fig. 2 Changes in the accumulation of total UV-absorbing phenolic compounds in cucumber first leaves during UV-B treatment. Extraction was performed during 0 h~30 h (A) and −1 d~9 d (B) after start of UV-B irradiation. Absorption at 300 nm of MeOH extracts was considered to represent the relative quantity of total UV-absorbing phenolic compounds. ●, UV-B irradiated; ○, UV-B non-irradiated (control). Each value represents the average of 3 separate samples. Vertical bars represent ±SD.

leaves, activities of SOD and APX were significantly enhanced by UV-B irradiation (Fig. 1A, B), while the activity of GR was generally unaffected during the 12 d treatment with UV-B (Fig. 1C). The activity of PER, a general peroxidase, which was measured using guaiacol as substrate, was also drastically enhanced by UV-B irradiation (Fig. 1D). However the activities of these antioxidative enzymes were enhanced only at a relatively late stage (6 d), whereas the cucumber first leaves acquired high tolerance to oxidative stress from a very early stage of UV-B irradiation (Table 1). Therefore, at least in the early stages of UV-B irradiation, enhanced tolerance to oxidative stress in cucumber cannot be explained by these antioxidative enzymes but appears to be caused by factors other than these enzymes examined.

Phenolic compounds began to accumulate from about 6 h after the start of UV-B irradiation, reaching a maximum after 1 d, and thence declining to the control level (Fig. 2A, B). Hence, this increase in phenolic compounds may have contributed to the enhanced UV-filtering effect in cucumber only over a short-term period, but not over a long term. There are only a limited number of studies that have examined the UV-B effect on the accumulation of phenolic compounds over several days. In our experiments, the accumulation of phenolic compounds occurred rapidly compared with the induction of antioxidative enzymes and, just as the levels of phenolic compounds began to decrease, the antioxidative enzyme activities began to rise. We therefore assume that the early accumulation of phenolic compounds may, by UV-filtering or by other functions, such as radical scavenging, have delayed the need for induction of the antioxidative enzymes. Rao *et al.* (1996) reported that antioxidative enzyme activities in the flavonoid-deficient *Arabidopsis* mutant tt5 were enhanced earlier than in wild-type LER in response to UV-B irradiation.

HPLC analysis of phenolic compounds (Fig. 3) indicated that UV-B irradiation increased nearly all the phenolic compounds examined by the same extent, except for one substance which was particularly increased compared to the others by UV-B treatment. The absorption spectrum of this peak closely resembled that of chlorogenic acid, which, together with its related compounds, is known to have high antioxidative ability (Ohnishi *et al.* 1994, Kono *et al.* 1997). While it is possible that this specifically accumulating compound also has antioxidative ability, further analysis is required to evaluate its direct role in the scavenging pathway. The selective enhancement of other phenolic compounds with antioxidative ability in response to UV-B has also been observed in other plants (Liu *et al.* 1995, Reuber *et al.* 1996b, Olsson *et al.* 1998), but there are limited reports on the selective induction of chlorogenic acid by UV-B (Lavola *et al.* 1997). The physiological functions of chlorogenic acid in plants is not yet clear, but Grace *et al.* (1998) recently proposed that chlorogenic acid is a powerful antioxidant, that may play an important role in mitigating the effects of oxidative stress in *Mahonia repens*.

Taken together, our results suggest that not only antiox-

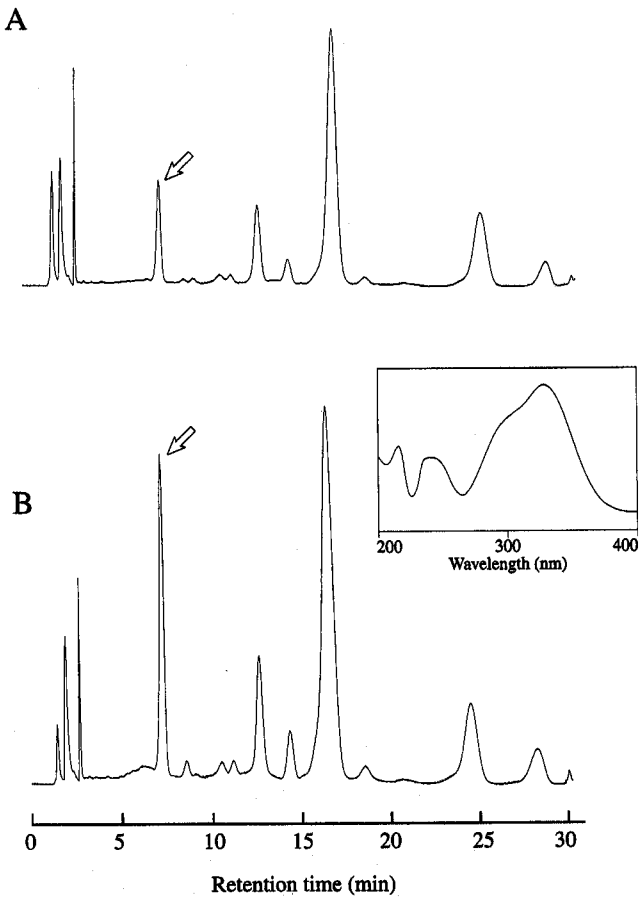

Fig. 3 HPLC analysis of phenolic compounds from cucumber first leaves irradiated with (B) or without (A) UV-B for 1 d. Arrows indicate the compound which showed a particularly distinct increase in response to UV-B irradiation. Insert: Absorption spectrum of indicated compound obtained by a photodiode array detector.

idative enzymes but also other antioxidative systems, for example phenolic compounds, may participate in the observed enhancement of tolerance to oxidative stress. However, the site at which phenolic compounds accumulate must be examined, although such accumulation is generally thought to occur mainly in epidermal cells, since the generation of oxygen free radicals by paraquat occurs mainly in chloroplasts of mesophyll cells. Aono *et al.* (1991, 1993) reported that increased activity of GR localized not only in the chloroplasts but also in cytoplasmic matrix of transgenic tobacco plant cell caused enhanced plant tolerance to oxidative stress by paraquat. In addition to the above examined factors, other substances or enzymes with known antioxidative abilities, such as α-tocopherol, β-carotene, ascorbic acid and catalase, may also be involved in the acquisition of oxidative stress tolerance after UV-B irradiation, and we will also therefore examine their role in this phenomenon.

In the field experiments, the influence of enhanced UV-B irradiation on the yield of soybean was examined by Teramura (1983). The results obtained were different depending on the cultivars and on the experimental years, so that enhanced UV-B caused not only the decrease in crop yield but also the increase in the yield. So far, few researches have been done on the mechanism of the positive effect of UV-B. High intensity of light and the dryness in the fields often increase the formation of reactive oxygen species in plant cells and thereby have a harmful effects on the growth of plants. There is a possibility that the increase of tolerance to the reactive oxygen species by UV-B, which was shown by the present research, might mitigate the damages to plants by high light intensity and/or dryness. It is also necessary to examine this point in the future.

We thank Dr. Mitsuko Aono, Dr. Akihiro Kubo and Dr. Hideyuki Shimizu, National Institute for Environmental Studies, for instruction in the enzyme assay methods and for Prof. Kosaku Takeda and Dr. Fumi Nakanishi, Tokyo Gakugei University, for guidance in photodiode array detection.

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(Received June 10, 2000; accepted July 1, 2000)