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Antioxidants Stimulated by UV-B Radiation in Rice Seedling

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ABSTRACT: In order to investigate low molecular antioxidants synthesized by enhanced UV-B radiation, we used the seedlings of two rice varieties. Woonjangbyeo, UV-tolerant, and Hwajoongbyeo, UV-susceptible, were subjected under supplemental UV-B irradiation. When rice seedlings were irradiated with UV light for short period, biosynthesis of total phenolic compound, ascorbate and glutathione were momently reduced. With an increase of UV-B radiation, however, those were slightly synthesized. The content of lipid peroxides in UV-challenged rice leaves was considerably increased after 12 hrs of UV-B treatment. Lipoxygenase activity under supplemental UV-B radiation was differently responded on rice varieties.

Keywords: rice seedling, UV-B, phenolic compound, ascorbate, glutathione, lipid peroxidation

In plant, exposure to UV light is expected to be a comparatively mild stimulus for the synthesis of phenolic compounds. Induction of the general phenylpropanoid and flavonoid glycoside pathways and the consequential accumulation of UV-protective flavonoids and other forms of phenolic compounds have been regarded as the only major metabolic response (Logemann *et al.*, 1999). UV irradiation results in the generation of intracellular reactive oxygen species (ROS) leading to oxidative stress. Plants metabolize ROS by invoking the antioxidative defence system (Rao et al., 1996). It results in the accumulation of certain phenolic compounds, flavonoids, sinapic acid esters and anthocyanin pigments that selectively attenuates UV radiation (Mackerness, 2000).

Ascorbate is a major primary antioxidant, reacting directly with hydroxyl radicals, superoxide and singlet oxygen, and it is also a powerful secondary antioxidant, reducing the oxidized form of -tocopherol. Ascorbate and glutathione are closely related, since they are both constituents of the antioxidative ascorbate glutathione cycle, which detoxifies hydrogen peroxide through a series of enzyme reactions known as Halliwell-Asada pathway. Glutathione (GSH) turnover seems to be enhanced by UV-B radiation with no net change in GSH concentration; the hypothesis of UV-B radiation enhancing glutathione degradation was confirmed in the experiments where BSO (a transition state inhibitor of -ECs) was used (Masi *et al.*, 2002).

Lipid peroxidation is commonly regarded as a deleterious process (Sevanian & Ursini, 2000; Benzie, 1996) leading to structural modification of complex lipid-protein assemblies like biological membranes and lipoproteins, and is usually associated with cellular malfunction. During lipid peroxidation a polar oxygen moiety (hydroperoxy group) is introduced into the hydrophobic tails of unsaturated fatty acids. When the lipid bilayer of biological membranes is oxidized, it may lose its barrier function and thus put the integrity of subcellular organelles or of the entire cell in danger. Enzymatic oxidation of lipophilic cellular constituents is an universal principle in the metabolism of living organisms, and a variety of enzyme systems have been created for this purpose. Among these systems, lipoxygenases (LOXs) are somewhat unique because they catalyze the specific deoxygenation of polyenoic fatty acids using atmospheric deoxygenation as second substrate.

The aims of this present study were to determine whether contents of phenolic compounds, ascorbate and glutathione are affected by enhanced UV-B radiation, and to measure the lipid oxidation in UV-stressed leaf tissues.

MATERIALS AND METHODS

Plant materials and growth condition

On the basis of the previous study that estimated UV-B-stressed damage (data not shown) of rice cultivars at seedling stage, Woonjangbyeo and Hwajoongbyeo were determined as UV-B-tolerant and UV-B-susceptible cultivars, respectively. Seeds of those cultivars were surface-sterilized with 2% (w/v) solution of sodium hypochlorite, and then placed on an incubator kept at 30°C for 3 days and watered with a distilled water in everyday. The germinated seeds

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were planted on bed soil (Boonong CO., LTD., Korea) for the cultivation of rice seedling packed with trays. The trays were transferred to a controlled environment chamber and grown at day/night temperatures of 25/20°C, 70% relative humidity and 300 ~ 400 μmol photons m^{-2} s^{-1} under 12 photoperiod for 15 days.

UV-B radiation was supplied with UV-B emitting fluorescent tubes (310 ± 10 nm) with filtering through 0.1 mm-thick cellulose diacetate film (Cadillac Plastic Co., Baltimore, MD, USA), which absorbs UV radiation below 290 nm. The irradiance in the UV-B region of the supplemental UV-B radiation at the plant canopy level was 1.2 W m^{-2}. Rice seedlings were subjected to the UV-B radiation for 0, 3, 6 12 and 24 hrs in photoperiod and biologically effective energy of UV-B was 0, 12.9, 25.9, 51.8 and 103.5 $kJm^{-2}d^{-1}$, respectively. After UV-B exposure, shoots of rice seedlings were immediately harvested, frozen liquid nitrogen and stored at -70°C for further analysis.

Assay of total phenolic compounds

Total phenolic compounds were determined using a modified method originally developed by Chandler & Dodds (1983). Fifty mg of plant material was placed in 2.5 ml of 95% ethanol and kept in a freezer for 72 hrs. Each sample was homogenized and centrifuged at 13,000 rpm for 10 min. After centrifugation, 1 ml of the supernatant was transferred to a test tube, and mixed with 1 ml of 95% ethanol and 5 ml of distilled water. And 0.5 ml of 50% Folin-Ciocalteu reagent (Sigma, USA) was added, vortexed and incubated for 5 min at room temperature. Finally, 1 ml of 5% (w/v) Na_2CO_3 was added to each sample, vortexed and incubated covered with an aluminum foil in dark for 1 hr. After 60 min, samples were vortexed again and absorbance was measured at 725 nm using an UV spectrophotometer (UV-2510, HP). Standard curves were established for each assay using concentrations of 25, 50, 75, 100, 150 and 200 ml^{-1} of gallic acid in 95% ethanol. Absorbance values were converted to mg of phenolics per gram of fresh weight of tissue.

Ascorbate assay

For ascorbate estimation (Mukherjee & Choudhari, 1983), fresh leaf sample (0.5 g) preserved in liquid nitrogen were extracted with 10 ml of 6% trichloroacetic acid. Four ml of the extract was mixed with 2 ml of 2% dinitrophenylhydrazine (in acidic medium) followed by the addition of 1 drop of 10% thiourea (in 70% ethanol). The mixture was boiled for 15 min and cooled to room temperature. Then, five ml of 80% (v/v) H_2SO_4 was added to the mixture at 0 (in an ice bath). The absorbance was recorded at 530 nm. The concentration of ascorbate was calculated from a standard curve plotted with known concentration of ascorbate.

Glutathione (Nonprotein-thiol) assay

The assay of acid-soluble sulfhydryl content (nonprotein thiols, of which GSH occupies ca. 90%) was estimated by the method of Sparnins et al. (1982). The plant material (200 mg) was homogenized in 50 mM sodium phosphate buffer (pH 7.0), 1 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 2% (w/v) PVP, 1% (v/v) glycerol and 0.1% (v/v) tween-20. The extract was centrifuged at 10.000 g for 20 min and the supernatant was immediately used for the assay of nonprotein thiol content. To 500 ml of the crude extract, as prepared for the GST assay, 100 ml of 25% (w/v) TCA was added. The precipitate was centrifuged at 12,000 g for 20 min at 4°C, and 300 ml of the supernatant was added to 2.7 ml of 0.6 mM 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) prepared in 0.1M Na-phosphate buffer, pH 8.0. The sulfhydryl content was quantified spectrophotometrically at 412 nm.

Lipoxygenase activity

The plant material (200 mg) was homogenized in 50 mM sodium phosphate buffer (pH 7.0), 1 mM EDTA, 0.1 mM PMSF, 2% (w/v) PVP, 1% (v/v) glycerol and 0.1% (v/v) tween-20. The extract was centrifuged at 10.000 g for 20 min and the supernatant was immediately used for the assay of lipoxygenase activity according to the method of Ederli *et al.*(1997). LOX (E.C. 1.13.11.12) activity was measured spectroscopically at room temperature by the addition of 1mM linoleic acid in 0.1M sodium acetate buffer (pH 5.6) to the extract and by measuring the increase of absorbance at 234 nm. The extinction coefficient of (25 mM^{-1} cm^{-1}) was used to convert absorbance values to micromols of conjugated diene. One unit of activity was defined as the amount of enzyme catalyzing the synthesis of 1 μmol of hydroperoxide (HPOD) min^{-1}.

Estimation of lipid peroxidation

Lipid peroxidation was measured as the amount of (MDA) determined by the thiobarbituric acid (TBA) reaction as described by Heath & Packer (1968). Control and treated leaf tissues (0.5 g) were homogenized in 5 ml of 20% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 3,500 g for 20 min. To 1 ml of the aliquot of the supernatant, 2 ml of 20% TCA containing 0.5% (w/v) TBA and 100 μl 1% (w/v) buthylated hydroxytoluene (BHT) in ethanol were added. The mixture was heated at 95°C for 30

min, and then quickly cooled on ice. The reactants were centrifuged at 5,000 g for 15 min, and the absorbance was measured at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The concentration of thiobarbituric acid reactive substances (TBARS) was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

RESULTS

Total phenolic compounds

The content of Total phenolic compounds was higher in UV-B susceptible Hwajoongbyeo than UV-B tolerant Woonjangbyeo, i.e. 32 mg g^{-1} FW and 25 mg g^{-1}FW, respectively. Within 3 hrs of UV-B treatment the phenolic content sharply decreased to nearly 20 to 24 mg g^{-1}FW (20 to 25% reduction). From 3 hrs after UV-B radiation total phenolics in Hwajoongbyeo seedlings were slightly increased with continuous UV-B treatment. Thereafter, those were recovered to 96% level compared to the control. Whereas total phenolics in Woonjangbyo seedlings were declined until 6 hrs after UV-B radiation, and then kept in a constant level.

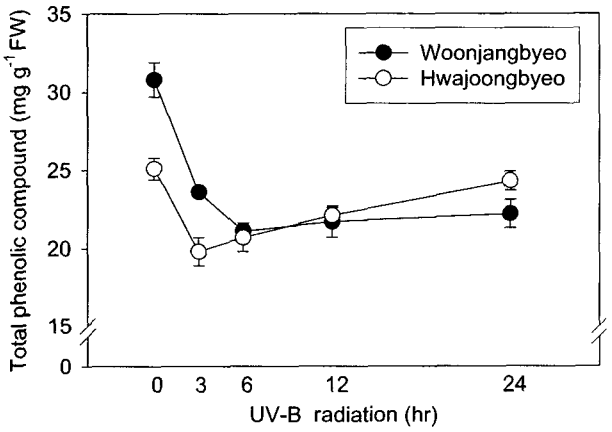

Fig. 1. Changes in the content of total phenolic compounds in leaves of rice seedlings under continuous UV-B radiation. Data are mean S.D. (n=6).

Ascorbate and glutathione contents

The levels of low-molecular antioxidants like ascorbate and glutathione in both rice seedlings were significantly different (Fig. 2-a). For tolerant Woonjangbyeo, ascorbate concentration was slightly reduced until 6 hrs of UV-B radiation. After 6 hrs of UV-B treatment, ascorbate contents, however, was reversed. In other word, Woonjangbyeo induced swiftly ascorbate accumulation, but in Hwajoongbyeo it was continuously decreased until 12 hrs of UV-B radiation, and then increased.

Glutathione contents in UV-B-stressed rice seedlings were assayed (Fig. 2-b). Woonjangbyeo synthesized 1.5 times more actively compared to Hwajoongbyeo without supplemental UV-B radiation suggested that such high level of glutathione was responsible for the UV-B tolerance of the cultivar. Glutathione contents for Woonjangbyeo were momently reduced within 3 hrs. It was, however, rapidly elevated to almost 1.4-fold after 6 hrs of UV-B treatment, and then slightly declined with an increase of UV-B radiation. The contents for Hwajoongbyeo were considerably influenced by UV-B treatment. Within 3 hrs, its content was decreased to 50% compared to the control seedlings. Its biosynthesis was continuously inhibited until 12 hrs of UV-B radiation, and then slightly increased.

Lipoxygenase activity and lipid peroxidation

In two rice seedlings, the activity of LOX seemed to response differently (Fig. 3). Enzyme activity in Woonjangbyeo was initially increased within 3 hrs of UV-B radiation, and then was constantly maintained to the end of UV-B radiation. Its activity in Hwajoongbyeo, however, was consider-

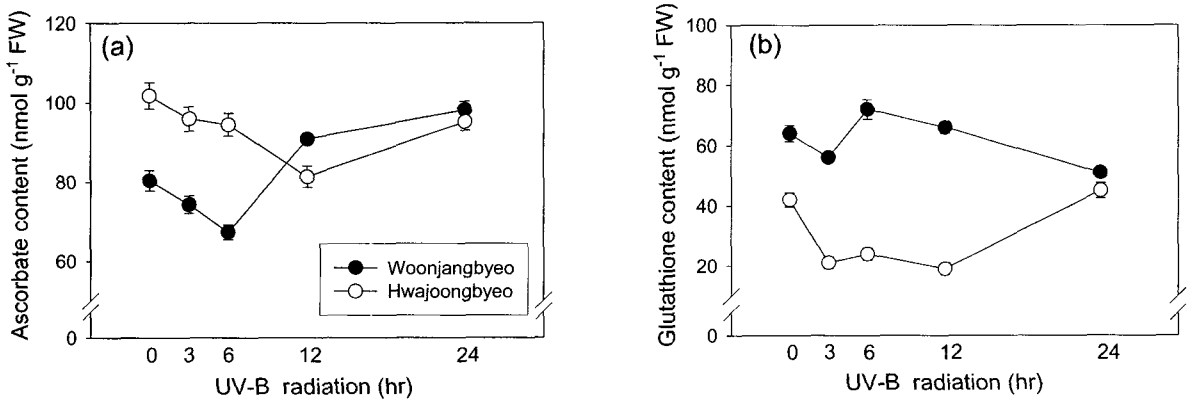

Fig. 2. Changes in ascorbate and glutathione contents synthesized in leaves of two rice seedlings with an increase of UV-B radiation. Data are mean S.D. (n=6).

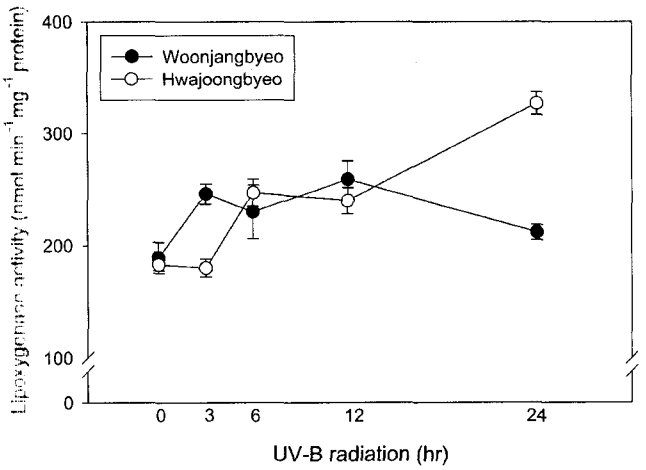

Fig. 3. Lipoxygenase activities in rice seedlings exposed to UV-B radiation. Data are mean S.D. (n=6).

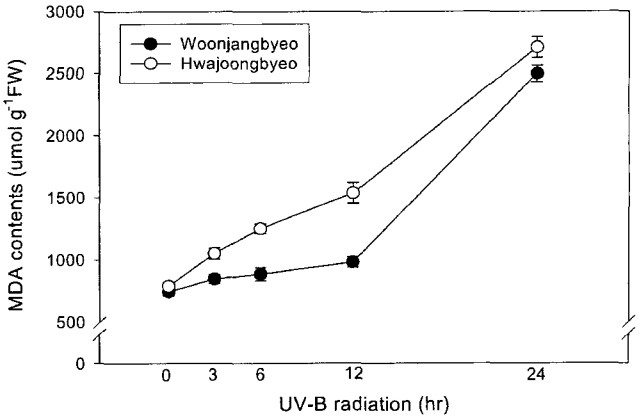

Fig. 4. Lipid peroxidation based on the MDA content in rice seedlings subjected to UV-B radiation. Data are mean S.D. (n=6).

ably affected, a two fold higher activity of the end of UV-B radiation as compared to the control seedlings (0 hr UV-B radiation).

In 15-day-old seedlings of two rice cultivars, Woonjangbyeo and Hwajoongbyeo, the levels of lipid peroxides measured in terms of MDA concentration were enhanced with an increase in the exposure of supplemental UV-B radiation (Fig. 4). Before UV-B exposure, the amounts of lipid peroxides of two rice seedlings were similar. With the persisting UV-B radiation, lipid peroxides in both seedlings, however, became obviously higher than that of the control plants. Actually, for Woonjangbyeo, UV-radiation for 12 hrs had no effects on MDA contents. Thereafter, they were dramatically increased to about 4.0 times higher level in 24 hrs UV-B treated seedlings than the control seedlings. For Hwajoongbyeo, MDA contents were immediately increased with supplemental UV-B treatment and retained as same as Woonjangbyeo under continuous UV-B radiation for 24 hrs.

DISCUSSION

In plants, the protective role of flavonoid polyphenolics in the expression of tolerance to UV-B radiation has been shown repeatedly (Duell-Pfaff & Wellmann, 1982; Li *et al.*, 1993). It has been reported in many studies that flavonoids and related phenolic compounds specifically increase when plants are exposed to enhanced UV-B radiation (Beggs & Wellmann, 1994; Reuber *et al.*, 1996; Markham *et al.*, 1998). Both rice seedlings, Woonjangbyeo and Hwajoongbyeo, accumulated lower levels of phenolic compounds when exposed to supplemental UV-B radiation compared to the control (Fig. 1) showing different tendency from the general hypothesis. However, the contradictory results regarding the effects of UV-B on plants may be due to methodological differences, including disparate levels of UV-B, photosynthetically active radiation (PAR) and interactions with other environmental factors (Greenberg *et al.*, 1997). Many protective mechanisms are dependent on PAR, degree of photorepair, accumulation of UV-absorbing pigments and leaf anatomy (Warner & Caldwell, 1983). Cen & Bornman (1990) showed that in beans, the levels of PAR less than 500 mol m^{-2} s^{-1}, combined with UV-B radiation, were too low to support significant flavonoid synthesis or, alternatively, flavonoid synthesis itself could have been damaged by the UV-B radiation under the low light condition. It should be noted that rice seedlings in this experiment were grown in 350 mol m^{-2} s^{-1}.

In both rice seedlings, ascorbate and glutathione concentration was unstable and affected strongly by supplemental UV-B radiation (Fig. 2). However, Carletti *et al.* (2003) reported that in maize seedling ascorbate contents were not correlated with UV-B treatment. In addition, it can be assumed that glutathione concentration also responded differently depending on rice varieties and UV-B radiation time. Therefore, the excess oxidative events induced by the UV-B irradiation in our experimental conditions may elicit changes in the total ascorbate and glutathione pool. The increase in MDA content is more precisely a direct indicator of a general UV-B-induced oxidative damage due to the damage of cell defence system. Changes in MDA contents was the apparent evidence that, in our experimental conditions, UV-B radiation induced oxidative stress (Fig. 4). After 12 hrs of UV-B radiation, the MDA content showed to be increased distinctly in both rice seedlings. However, lipoxygenase activities responded differently to lipid peroxides generation although it in Hwajoongbyeo seemed to be the similar pattern (Fig. 3). Also, lipid peroxides can also be detoxified through conjugation with glutathione. In fact, some GSTs have a substrate preference for hydroperoxides, the toxic byproducts of lipid peroxidation in plants

(Marrs, 1996). In conclusion, our results suggest that in rice seedlings, UV-B radiation inhibits initial accumulation of phenolic compounds, ascorbate and glutathione. These compounds are likely to afford protection to the cells by absorbing in the UV-B region of the light spectrum and scavenging reactive oxygen species generated by UV-B radiation.

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