Differences in the Sensitivity to UVB Radiation of Two Cultivars of Rice (*Oryza Sativa* L.) based on Observation of Long-Lived Radicals

JUN KUMAGAI¹*, HIROMI KATOH¹, TETSUO MIYAZAKI¹, JUN HIDEKA² and TADASHI KUMAGAI²

¹Department of Applied Chemistry, Graduate School of Engineering, Nagoya University, Furo-Cho Chikusa-ku, Nagoya 464–8603, Japan
²Institute of Genetic Ecology, Tohoku University, Katahira, Aoba-ku, Sendai 980–8577, Japan

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Free radicals with a long lifetime were observed in the leaves of two rice cultivars (*Oryza sativa* L.), Sasanishiki (UVB resistant) and Norin-1 (UVB sensitive), by electron spin resonance spectroscopy. The leaves of both cultivars grown with visible light show very similar ESR spectra composed of radical 1 (R1) and radical 2 (R2), which may be attributable to P₇₀₀ cation radicals in the reaction center of photosystem I, and tyrosine cation radicals in the reaction center of photosystem II, respectively. The ESR spectrum composed of R1 and R2 radicals in the leaves of Sasanishiki grown under visible light with supplemental UVB was similar to that in the plant grown without supplemental UVB. On the other hand, the amount of R2 radicals in the leaves of Norin-1 grown under visible light with supplemental UVB was significantly smaller than that in the plant grown without supplemental UVB. It is suggested that the loss of R2 radicals in Norin-1 upon UVB irradiation is related to the instability of the plant.

INTRODUCTION

The amount of UVB (280–320 nm) reaching the earth’s surface has been increasing due to the loss of stratospheric ozone caused by human activities. This is a cause of concern because of the potential for not only an increase in the incidence of carcinogenesis in humans and mammalians¹,² but also crop failure. Thus, it is important to study the effects of UVB irradiation on mammalians and plants.
Kumagai et al. have studied the effect of UVB on the growth of rice, and found a significant difference between two Japanese lowland rice cultivars (Oryza sativa L.). They have investigated the effect of supplemental UVB radiation on the amount of total leaf nitrogen (N) and on the partitioning of nitrogen into soluble protein N, and Rubisco N in the eighth leaves of the Sasanishiki and Norin-1 cultivars. Although the amounts of each kind of nitrogen decreased in both cultivars when plants were grown under a UVB dose of 0.24 W m\(^{-2}\) with a UV29 filter, the degree of the reduction for both kinds of nitrogen was significantly greater in Norin-1 than in Sasanishiki. Thus, Sasanishiki, one of the most popular rice cultivars in Japan, exhibited strong resistance to UVB irradiation, while Norin-1 was much less resistant, although these two cultivars were closely related.

It has been generally accepted that free radicals play an important role when biological systems are subjected to some kind of stress such as ionizing radiation. The most well known radicals, which may cause biological effects, are active oxygen species, such as OH and O\(_2^-\) radicals. Since the lifetime of the active oxygen is usually less than one millisecond, it is difficult to observe it directly in biological systems. Recently Miyazaki et al. observed long-lived radicals directly by Electron Spin Resonance Spectroscopy (ESR) in biological systems and pointed out the importance of their biological effects. For example, a reaction of the radicals with metallothionein in murine liver may be related to the marked radiation tolerance of mice. Matsumoto et al. and Koyama et al. reported that long-lived radicals produced in \(\gamma\)-irradiated mammalian cells caused DNA mutation and transformation. The decay behavior of the long-lived radicals produced in \(\gamma\)-ray irradiated Arabidopsis thaliana seeds is related to the strong radiation resistance of the seeds. Thus, it is expected that the study of long-lived radicals in rice will provide for the elucidation of the difference in UVB sensitivity between the two rice cultivars, Sasanishiki and Norin-1.

The purpose of this study is to observe directly the long-lived radicals in the leaves of the two Japanese lowland rice cultivars, Sasanishiki and Norin-1, to determine their absolute concentrations by use of ESR, and then to find a relationship between the ESR parameters and different UVB sensitivities of the two rice species.

**MATERIALS AND METHODS**

*Plant culture*

Two cultivars of Japanese lowland rice (Oryza sativa L.), Sasanishiki and Norin-1, were used as experimental plants. The plants were initially grown for 16 days in pots in a mixture of vermiculite and fertilized soil (2 : 1, v/v) in a growth cabinet (Tabai Espec Ltd. Co., Osaka, Japan) (12-h photoperiod, day/night temperature 27/17°C). No UVB was irradiated during the initial period of 16 days. After the initial 16 days, plants were grown under visible light without or with supplemental UVB provided by three UVB bulbs (Toshiba FL 20 SE; Toshiba Ltd. Co., Tokyo) placed 15 cm apart and above the plants. Plants receiving UVB were grown with the same photoperiod under visible radiation. Under the UVB bulbs, a UV29 glass filter (Toshiba Grass Co./Shizuoka, Japan) reduced 290 nm radiation by 50%. The UVB intensity
at the level of the plants was 51.8 or 324 kJ m$^{-2}$ day$^{-1}$. The UVB intensity between 280 and 315 nm was measured with a UVB sensor (MS-210D, Eiko Seiki Co., Tokyo, Japan). The ratio of the irradiance in the region of UVB and UVC in the UVB filtered through a UV29 glass filter was 1 : 0.025$^{17}$. The photosynthetic photon flux density (PPFD) was measured with a data logger (LI-1000; Li-Cor, Lincoln, NE, USA) and an LI-190SA sensor (Li-Cor). The PPFD was usually adjusted to about 350 µmol m$^{-2}$ s$^{-1}$. When the fourth leaves were fully expanded, they were harvested and used for measurement of ESR. Although periods for UVB irradiation differed a little depending on the growth of individual plants, they totaled 48 h during 4 days on average for both Sasanishiki and Norin-1.

**ESR measurement**

The rice leaves, frozen by liquid nitrogen immediately after the pruning, were put into o.d. 5 mm x i.d. 3.1 mm high purity Suprasil quartz tubes for ESR measurement, and then the tubes sealed without exhaustion. The sample weight in a tube is around 0.1 g. Each tube was frozen at 77 K to reduce dielectric loss of the microwaves by water during X-band ESR measurement. The ESR spectra were measured by using a JEOL JES-RE1X ESR spectrometer at 77 K. The ESR parameters for the measurements avoiding saturation of the signals are magnetic modulation of 0.04 mT at 100 kHz and microwave power of 0.1 mW. The yields of radicals were obtained by double integration of the signals using an ES-PRIT425 computer system (JEOL). Absolute concentrations of radicals in the leaves were determined by comparing to the $G$-value of hexyl radicals produced by the $\gamma$-irradiation of $n$-hexane, which is reported as 4.8 at 77 K$^{15-17}$. The difference of ESR sensitivity between the leaf samples and the $n$-hexane sample due to the difference in degree of dielectric loss in the ESR cavity is corrected by the intensity of a Mn$^{2+}$/MgO marker sample which is located in the constant position in the cavity for all ESR measurements. ESR spectra of rice leaves generally consist of two radical species, R1 and R2. R1 shows a large singlet signal with a $g$-value of 2.0029. R2 shows a broad signal composed of five lines with a $g$-value of 2.0056 at the center, and it appears as humps at both sides of the R1 singlet signal. The absolute concentration of R2 radicals was determined by subtracting a typical spectrum of R1 radicals from each observed spectrum of leaf sample. The rational behind the assignment and concentration measurement of these radicals is described in the Results and Discussion section.

**RESULTS AND DISCUSSION**

In the present study, we analyzed the yields of long-lived radicals in leaves of Sasanishiki and Norin-1 grown under UVB irradiation to elucidate the correlation between radical parameters and their growth sensitivity to UVB.

Figure 1 shows the ESR spectra at 77 K of the leaves of two rice cultivars, Sasanishiki and Norin-1. Figure 1(A) shows the spectrum of Sasanishiki grown under visible light without UVB, and Fig. 1(B) the spectrum of Sasanishiki grown under visible light with supplemental UVB at 51.8 kJ m$^{-2}$ day$^{-1}$. The two spectra are almost the same. The spectrum in Fig. 1(A)
shows one large singlet signal, and a broad signal which appears as humps at both sides of the large signal. It is composed of a singlet signal with a $g$-value of 2.0029 and another signal composed of five lines with a $g$-value of 2.0056 in the center. The radical species corresponding to the former and the latter signals are denoted here as R1 and R2 radicals, respectively.

Figure 1(C) shows the spectrum of Norin-1 grown without UVB, and Fig. 1(D) the spectrum of Norin-1 grown under visible light with supplemental UVB at 51.8 kJ m$^{-2}$ day$^{-1}$. The spectrum (C) of Norin-1 grown under visible light without UVB is very similar to the spectrum (A) of Sasanishiki grown without UVB. The spectrum (C) is also composed of two signals due to R1 and R2 radicals. The spectrum (D) with supplemental UVB is different from the spectrum (C) without UVB. The amount of R2 radicals in spectrum (D) was much smaller than that in spectrum (C).

Table 1 shows the concentration of R1 and R2 radicals in the rice leaves. The separation of R1 and R2 radicals from the observed spectra was achieved as follows. The spectrum
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which shows only R1 radicals was subtracted from each observed spectrum to reproduce the spectrum shape of the tyrosine cation radical (see Fig. 2(D)) by changing the amplitude of the spectrum of R1 radicals. The spectrum which shows only R1 radicals was observed in one of the UVB (51.8 kJ m$^{-2}$ day$^{-1}$) irradiated Norin-1 leaves. Total concentrations of radicals which correspond to the sum of R1 and R2 radicals in Sasanishiki and Norin-1 were not affected by the additional UVB (51.8 kJ m$^{-2}$ day$^{-1}$). Although the absolute concentration of R2 radicals in Sasanishiki grown with the additional UVB of 51.8 kJ m$^{-2}$ day$^{-1}$ (8 ± 3 nmol g$^{-1}$) was similar to that (6 ± 2 nmol g$^{-1}$) in the plant without UVB, the concentration (2 ± 1 nmol g$^{-1}$) in Norin-1 grown with the additional UVB of 51.8 kJ m$^{-2}$ day$^{-1}$ was much smaller than that (8 ± 2 nmol g$^{-1}$) in the plant without UVB. When Sasanishiki was irradiated with much stronger UVB light at 324 kJ m$^{-2}$ day$^{-1}$, however, the concentration of R2 radicals decreased to 2 ± 1 nmol g$^{-1}$. It is notable that the absolute concentration (20–27 nmol g$^{-1}$) in the leaves was ten times that (2 nmol g$^{-1}$) of long-lived radicals in murine liver$^{11}$.

Assignment of R1 and R2 radicals

The typical ESR spectra of rice leaves grown under visible light (Figs. 1(A) and 1(C)) are similar to those observed in *Chlorella pyrenoidosa*, in which the spectra also consisted of two radical components, related to the photosynthetic system$^{18,19}$.

Figure 2(A) shows the ESR spectra of Sasanishiki leaves at 77 K grown without UVB, and Fig. 2(B) the ESR spectra of Sasanishiki leaves at 77 K grown without UVB and then kept in the dark for 24 h at room temperature. Figure 2(C) shows the spectrum when (B) was subtracted from (A). Figure 2(D) shows the ESR spectrum of the tyrosine cation radical, the so called Tyr-D, in thylakoid membranes at 20 K reported by Isogai et al in 1990$^{20}$.

Table 1. Absolute concentration of radicals in the rice leaves

<table>
<thead>
<tr>
<th>cultivars</th>
<th>kind of radicals</th>
<th>Radical Concentration/nmol g$^{-1}$</th>
<th>UVB (^{a}) (51.8 kJ m$^{-2}$ day$^{-1}$)</th>
<th>UVB (^{a}) (324 kJ m$^{-2}$ day$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R1</td>
<td>17 ± 6</td>
<td>19 ± 4</td>
<td>18 ± 6</td>
</tr>
<tr>
<td>Sasanishiki</td>
<td>R2</td>
<td>6 ± 2</td>
<td>8 ± 3</td>
<td>2 ± 1</td>
</tr>
<tr>
<td></td>
<td>total</td>
<td>23 ± 8</td>
<td>27 ± 7</td>
<td>20 ± 7</td>
</tr>
<tr>
<td>Norin-1</td>
<td>R1</td>
<td>14 ± 3</td>
<td>24 ± 7</td>
<td>24 ± 18</td>
</tr>
<tr>
<td></td>
<td>R2</td>
<td>8 ± 2</td>
<td>2 ± 1</td>
<td>1 ± 1</td>
</tr>
<tr>
<td></td>
<td>total</td>
<td>23 ± 5</td>
<td>26 ± 8</td>
<td>25 ± 19</td>
</tr>
<tr>
<td></td>
<td>(9 samples)</td>
<td>(4 samples)</td>
<td>(4 samples)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(10 samples)</td>
<td>(6 samples)</td>
<td>(3 samples)</td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\) Determination of the absolute concentrations of the sum of R1 and R2 radicals was described in the methods section. Rational of the assignment and concentration measurement of R1 and R2 radicals was described in the discussion at *Assignment of R1 and R2 radicals*.

\(^{b}\) Grown under visible light without UVB.

\(^{c}\) Grown under visible light with supplemental UVB.

which shows only R1 radicals was subtracted from each observed spectrum to reproduce the spectrum shape of the tyrosine cation radical (see Fig. 2(D)) by changing the amplitude of the spectrum of R1 radicals. The spectrum which shows only R1 radicals was observed in one of the UVB (51.8 kJ m$^{-2}$ day$^{-1}$) irradiated Norin-1 leaves. Total concentrations of radicals which correspond to the sum of R1 and R2 radicals in Sasanishiki and Norin-1 were not affected by the additional UVB (51.8 kJ m$^{-2}$ day$^{-1}$). Although the absolute concentration of R2 radicals in Sasanishiki grown with the additional UVB of 51.8 kJ m$^{-2}$ day$^{-1}$ (8 ± 3 nmol g$^{-1}$) was similar to that (6 ± 2 nmol g$^{-1}$) in the plant without UVB, the concentration (2 ± 1 nmol g$^{-1}$) in Norin-1 grown with the additional UVB of 51.8 kJ m$^{-2}$ day$^{-1}$ was much smaller than that (8 ± 2 nmol g$^{-1}$) in the plant without UVB. When Sasanishiki was irradiated with much stronger UVB light at 324 kJ m$^{-2}$ day$^{-1}$, however, the concentration of R2 radicals decreased to 2 ± 1 nmol g$^{-1}$. It is notable that the absolute concentration (20–27 nmol g$^{-1}$) in the leaves was ten times that (2 nmol g$^{-1}$) of long-lived radicals in murine liver$^{11}$. Assignment of R1 and R2 radicals

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Since the ESR parameters of R1 radicals; a $g$-value of 2.0029 and maximum slope line width ($\Delta H_{\text{msl}}$) of 0.80 mT, shown in Fig. 2(B), are very similar to those of P 700 cation radicals, a $g$-value of 2.0026 and $\Delta H_{\text{msl}}$ of 0.80 mT, in the photoreaction center of photosystem I ($^{21-24}$), R1 radicals may be P 700$^{+}$ cation radicals. However the ESR spectral information on R1 radicals is limited to the $g$-value and the line width. And the senescencing leaves of Sasanishiki or Norin-1 show a singlet signal, a $g$-value of 2.0050 and $\Delta H_{\text{msl}}$ of 0.87, which is different from that of R1 radicals at a $g$-value of 2.0029. Thus, R1 radicals cannot be the long-lived radicals in senescencing leaves.

Regarding the assignment of R2 radicals, the $g$-value of 2.0056 at the center of spectrum (C) which corresponds to R2 radicals is close to that (2.0046) of spectrum (D) of tyrosine cation radical (Tyr-D) in thylakoid membranes at 20 K observed by Isogai et al.$^{20}$. The hyperfine splittings in spectrum (C) are similar to those in spectrum (D). Therefore, R2 radicals are the Tyr$^{+}$ cation radicals (Tyr-D) in the polypeptide near the manganese cluster for oxygen generation in photosystem II ($^{20,25}$).

Fig. 2. ESR spectra at 77 K (A) Sasanishiki leaves grown under visible light without UVB, (B) Sasanishiki leaves grown under visible light without UVB and then kept in the dark for 24 h at room temperature, (C) spectrum when (B) is subtracted from (A), and (D) the spectrum due to tyrosine cation radicals (Tyr$^{+}$) in thylakoid membranes observed at 20 K by Isogai et al.$^{20}$. 
UVB effect on radical production in Sasanishiki and Norin-1

The observed changes in the yields of R1 and R2 radicals following UVB irradiation (Table 1) clearly show that the decrease of R2 radicals in the rice leaves is related to the effect of UVB irradiation. The yields of R2 radicals in Norin-1 are suppressed markedly upon the exposure to additional UVB at 51.8 kJ m$^{-2}$ day$^{-1}$. The R2 radicals that are considered to be Tyr-D are located at the photoreaction center polypeptide of photosystem II, and participate in electron transfer between the reaction center chlorophyll (P680) and the manganese center of the oxygen-evolving complex$^{20,25}$. Thus, it is suggested that the decrease in the amount of R2 radicals in Norin-1 caused by UVB during growth corresponds to the demolition of the reaction center polypeptide of photosystem II, resulting in a decrease in photosynthetic activity. Vass and co-workers$^{26}$ reported that UVB radiation inhibited electron transport in isolated spinach photosystem II. Their report is consistent with our results for Norin-1. Therefore, the marked decrease in the yields of R2 radicals in Norin-1 upon additional UVB irradiation that corresponds to the demolition of photosystem II may be related to the weakness of Norin-1 against UVB. Since this study of the effects of UVB on rice by the measurement of long-lived radicals is the first of its type, there is not enough information available on these radicals to identify the mechanism of the effects. Further studies are needed.

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