Ultraviolet-B Sensitivities in Japanese Lowland Rice Cultivars: Cyclobutane Pyrimidine Dimer Photolyase Activity and Gene Mutation

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There is a cultivar difference in the response to ultraviolet-B (UVB: 280–320 nm) in rice (Oryza sativa L.). Among Japanese lowland rice cultivars, Sasanishiki, a leading Japanese rice cultivar, is resistant to the damaging effects of UVB while Norin 1, a close relative, is less resistant. We found previously that Norin 1 was deficient in cyclobutane pyrimidine dimer (CPD) photorepair ability and suggested that the UVB sensitivity in rice depends largely on CPD photorepair ability. In order to verify that suggestion, we examined the correlation between UVB sensitivity and CPD photolyase activity in 17 rice cultivars of progenitors and relatives in breeding of UV-resistant Sasanishiki and UV-sensitive Norin 1. The amino acid at position 126 of the deduced amino acid sequence of CPD photolyase in cultivars including such as Norin 1 was found to be arginine, the CPD photolyase activities of which were lower. The amino acid at that position in cultivars including such as Sasanishiki was glutamine. Furthermore, cultivars more resistant to UVB were found to exhibit higher photolyase activities than less resistant cultivars. These results emphasize that single amino acid alteration from glutamine to arginine leads to a deficit of CPD photolyase activity and that CPD photolyase activity is one of the main factors determining UVB sensitivity in rice.

Keywords: Cyclobutane pyrimidine dimer (CPD) — CPD photolyase — Gene mutation — Rice (Oryza sativa L.) — UVB radiation — UVB sensitivity.

Abbreviations: Arg, arginine; BSA, bovine serum albumin; CPD, cyclobutane pyrimidine dimer; CTAB, cetyl trimethyl ammonium bromide; DTT, dithiothreitol; Gln, glutamine, NIL, near-isogenic line; PAR, photosynthetic active radiation; (6–4) pp, pyrimidine (6–4) pyrimidone photoproducts; QTL, quantitative trait loci; UVB, UV light with wavelengths from 280 to 320 nm.

The nucleotide sequence reported in this paper has been submitted to the DDBJ/EMBL/GenBank database under accession number AB099694.

Introduction

Ultraviolet-B radiation (UVB: 280–320 nm) can damage plants, decreasing growth and productivity. It has been indicated that there is sizeable intraspecific variability as well as wide interspecific variability in crop plants (Teramura 1983, Bornman and Teramura 1993, Mazza et al. 2000). Teramura et al. (1991) have shown that Asian rice cultivars differ in their responses to elevated UVB in terms of growth and physiological processes, and have suggested that geographical location might influence sensitivity to UVB radiation in rice. Conversely, Barnes et al. (1993) and Dai et al. (1995) have shown that rice cultivars originating from regions with higher ambient UVB radiation are not necessarily more tolerant to enhanced UVB. We have also found that various cultivars that belong to the same ecotype and the same group differ in their responses to UVB among rice cultivars belonging to five Asian rice ecotypes (aus, aman and boro from the Bengal region, and tfereh and bulu from Indonesia), and Japanese lowland and upland rice groups (Sato and Kumagai 1993). We have also found that many rice cultivars resistant to UVB belong to the Japanese lowland group and the boro ecotype. Among Japanese lowland rice cultivars, Sasanishiki, a leading variety in northeast Japan, exhibits more resistance to UVB radiation, while Norin 1, a progenitor of many Japanese commercial rice cultivars, is less resistant, although these cultivars are closely related in breeding (Kumagai and Sato 1992, Hidema et al. 1996).

It is well known that UVB radiation is capable of inducing photodamage in DNA, including damage to the cyclobutane pyrimidine dimer (CPD) and the pyrimidine (6–4) pyrimidone photoproducts [6–4] pp. CPDs that are formed between adjacent pyrimidines on the same strand are a major UV-induced DNA lesion in simple or complex organisms, producing lethality or mutations (Brash et al. 1987). Two major mechanisms that repair such CPDs are excision repair and photoreactivation. CPD photoreactivation is the major pathway in plants for repairing UV-radiation-induced DNA damage (Britt 1996, Britt 1999). An enzyme, photolyase, which binds to a dimer to form a complex that is stable in the absence of light, mediates this repair path. When a photon in the wavelength range 300–600 nm is absorbed, the dimer is reversed to monomer pyrimidines and the enzyme is released (Sancar 1994). We found previously that the UV-sensitive Norin 1 is deficient in CPD photorepair ability compared with the UV-resistant Sasanishiki in vivo and in vitro (Hidema et al. 1997, Hidema and Kumagai 1998, Hidema et al. 2000). Similar results were obtained from experiments in which CPD photorepair abilities in the UV-
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sensitive cultivar Surjamkhi and the UV-resistant cultivar Marich-bati, both of which are Indica rice cultivars that belong to the aus ecotype (Hidema et al. 2001). Consequently, using the method of photoflash analysis (Harm et al. 1971), Hidema et al. (2000) studied the molecular origin of deficiency in CPD photorepair in Norin 1 and demonstrated that it results from a structure/function alteration of photolyase rather than from a regulatory mutation (corresponding to fewer photolyase molecules per cell). These results were confirmed by results that showed that the Norin 1 photolyase–dimer complex was highly thermolabile relative to the Sasanishiki photolyase. We then isolated and cloned a homolog of the CPD photolyase gene from the Sasanishiki cDNA library by the PCR-based dilution–amplification method which we recently devised (Hirouchi et al. 2003; DDBJ/EMBL/GenBank database accession no. AB096003).

We have attempted (i) to test whether CPD photolyase ability is one of the main factors for determining UVB sensitivity in rice cultivars, and (ii) to clarify the origin and inheritance of characteristics exhibiting stronger resistance to UVB radiation in terms of growth and higher CPD photorepair ability in the cultivar Sasanishiki. In the present experiment, first, we decided a mutation site of the CPD photolyase gene between UV-resistant Sasanishiki and UV-sensitive Norin 1. Furthermore, we examined the correlations between UVB sensitivity, CPD photolyase activity and the sequence of the CPD photolyase gene in progenitors and relatives in the breeding of UV-resistant Sasanishiki and UV-sensitive Norin 1.

**Results**

UVB sensitivity among Japanese rice cultivars

The effects of supplemental UVB in visible radiation on the growth of some progenitors and relatives in the breeding of UV-resistant Sasanishiki and UV-sensitive Norin 1 were examined. Experimental plants were grown for 30 d under irradiation with visible radiation supplemented or not supplemented with UVB in a phytotron. Fig. 1 shows the effects of supplemental UVB radiation on the growth of rice cultivars, Sasanishiki, Sasashigure, Kamenoo 4, Norin 22, Norin 1, Aikoku, Hatsunishiki and Ginbouzu. UVB sensitivity varied widely among Japanese rice cultivars examined here, although all these cultivars are closely related (see Fig. 6). There were significant differences in UVB sensitivity between rice cultivars. For rice plants, tiller number and dry weight of above-ground parts of plants are highly correlated with yield (Hidema et al. 1996, Kumagai et al. 2001). Therefore, the UVB sensitivity index was determined by summing the percentage changes in tiller number and plant dry weight of above-ground parts of

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**Fig. 1** Effects of UVB radiation on rice cultivars (*Oryza sativa* L. cv), Sasanishiki (A), Sasashigure (B), Kamenoo 4 (C), Norin 22 (D), Norin 1 (E), Aikoku (F), Hatsunishiki (G) and Ginbouzu (H). Rice cultivars were grown for 30 d in a phytotron under visible radiation (350 µmol photon m⁻² s⁻¹) without (–UVB) and with supplemented UVB radiation (+UVB).

**Fig. 2** Organization of *O. sativa* CPD photolyase gene and alignment of the nucleotide sequences from 370 to 384 of exon 2 of the CPD photolyase genes of rice cultivars Sasanishiki and Norin 1. The nucleotide adenine at position 377 in Sasanishiki changed to guanine in Norin 1, thereby, the codon CAG, which encodes glutamine (Q), changed to the codon CGG, which encodes arginine (R).
plants [percentage change = (control–treatment)/control × 100; Barnes et al. 1993]. Control values were derived from plants that had been grown in visible radiation without supplemental UVB. Table 1 shows the ranking of rice cultivars with respect to UVB sensitivity; the percentage changes in tiller number, plant dry weight and UVB sensitivity index. The calculated UVB sensitivity index ranged from 69 (the percentage changes in tiller number and dry weight were 25 and 44, respectively) for the least affected cultivar (Sasanishiki) to 154 (the percentage changes in tiller number and dry weight were 70 and 84, respectively) for the most affected cultivar (Ginbouzu).

One amino acid residue alteration of CPD photolyase leads to a deficit of CPD photolyase activity in rice
We demonstrated previously that the photorepair deficiency in UVB-sensitive Norin 1 results from an alteration in the structure of the photolyase enzyme using photoflash analysis (Hidema et al. 2000). At that time, we could not compare the sequences of CPD photolyase genes from the UV-resistant and UV-sensitive cultivars, because the rice photolyase gene had not been cloned. Recently, we isolated and cloned the CPD photolyase gene from Sasanishiki cDNA library (Hirouchi et al. 2003). Here, to compare the nucleotide sequence of the CPD photolyase gene of UV-sensitive Norin 1 with that of UV-resistant Sasanishiki, the complete sequence of the CPD photolyase gene of Norin 1, a 1518-bp ORF coding for 506 amino acid residues, was determined (DDBJ/EMBL/GenBank database accession no. AB099694) using oligonucleotide primers based on the sequence of the CPD photolyase gene in Sasanishiki.

Fig. 2 shows the organization of CPD photolyase gene and parts of the alignments of nucleotide sequences and deduced amino acid sequences on exon 2 of the cultivars Sasanishiki and Norin 1. The rice CPD photolyase gene is composed of 10 exons and nine introns, and CPD photolyase is encoded by a single-copy gene in rice (Teranishi, Hidema and Kungai unpublished result). One nucleotide adenine at nucleotide position 377 on exon 2 in Sasanishiki was changed to guanine in Norin 1. This alteration of nucleotide led to an amino acid change at position 126; the codon CAG in Sasanishiki encodes glutamine (Gln), and the codon CGG in Norin 1 encodes arginine (Arg).

Next, to test whether an amino acid change at position 126 from Gln to Arg in the photolyase gene leads to lower CPD photolyase ability, we analyzed the correlation between the sequences and the activity of CPD photolyase among cultivars tested in Table 1. The CPD photolyase activity of each cultivar was determined as the initial rate of CPD photoreactivation in blue light using enzyme extract prepared from the fully expanded fourth leaves of each cultivar. Crude extract from each cultivar was adjusted to the same protein concentration (1.3 µg µl⁻¹, see Materials and Methods).

Fig. 3 A shows the

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**Fig. 3**  CPD photorepair activities in extracts in vitro and genotypes of CPD photolyase gene in rice cultivars. (A) Extracts of rice cultivars were mixed with UV-irradiated λDNA, incubated in the dark for 15 min at 28°C for photolyase–CPD complex formation and then exposed to continuous blue light; CPD repair was measured as a function of time. Values represent the mean ± SD of three samples for Sasanishiki (closed triangle), Norin 8 (inverted closed triangle), Norin 1 (open circle) and Aikoku (open square). (B) The relationships between the initial rates of CPD photorepair in blue light exposure and the genotypes of the CPD photolyase gene (‘Sasa’-type or ‘Nori’-type) in rice cultivars examined in Table 1. Each value is the average, and the error bars indicate population SD (n = 3–6). White columns show the cultivars for which there is no significant difference in the initial rate of CPD photorepair compared with that of Sasanishiki at the 0.01 level according to the LSD test. Black columns show the cultivars for which there is no significant difference in the initial rate of CPD photorepair compared with that of Norin 1 at the 0.01 level according to the LSD test. * or # indicates significant difference when compared with the initial rate of CPD photorepair of Norin 1 or Sasanishiki, respectively (P < 0.01). All the cultivars with the ‘Nori’-type CPD photolyase gene exhibited lower CPD photorepair ability, while all the cultivars with the ‘Sasa’-type CPD photolyase gene exhibited higher CPD photorepair ability.
time course of CPD repair by extracts of UV-resistant Sasanishiki and Norin 8, and UV-sensitive Norin 1 and Aikoku. The CPD levels repaired in each sample increased linearly with exposure time to blue light until 90 min after the beginning of the irradiation. The rates of CPD photorepair in Sasanishiki and Norin 8 were similar and faster than those in Norin 1 and Aikoku.

Fig. 3B shows the relationship between the initial rate of CPD photorepair in blue light exposure and the genotypes of CPD photolyase genes. The initial rate of CPD photorepair ranged from 0.98 CPD Mb⁻¹ min⁻¹, the highest activity in the cultivar Sasashigure, to 0.48 CPD Mb⁻¹ min⁻¹, the lowest activity in the cultivar Hatsunishiki. On the basis of these results, the cultivars examined here were clearly divided into two groups (significant difference: P < 0.01). One group consists of the cultivars Sasanishiki, Asahi I, Kameno, 4, Koshihikari, Norin 1, Aikoku, Moritawase, Norin 6 and Rikuu 22, which have CPD photolyase activity higher than 0.87 CPD Mb⁻¹ min⁻¹. The other consists of cultivars, Norin 1, Aikoku, Asahi II, Ginbouzu, Hatsunishiki, Kameno, Moritawase, Norin 6 and Rikuu 132, which have CPD photolyase activity lower than 0.64 CPD Mb⁻¹ min⁻¹. Next, the sequences of CPD photolyase genes of other cultivars tested in Table 1 were determined, according to the same method as above (see Materials and Methods). It was evident that there was no cultivar with a CPD photolyase gene different from that of either Sasanishiki (‘Sasa’ type) or Norin 1 (‘Nori’ type) (Fig. 3B). Furthermore, it is interesting to note that all the cultivars with the ‘Nori’-type CPD photolyase gene showed lower CPD photorepair ability, while all cultivars with the ‘Sasa’-type CPD photolyase gene showed higher CPD photorepair ability. These results strongly show that one amino acid residue at position 126 plays an important role in determining CPD photolyase activity in rice.

**CPD photorepair activity correlates with UVB sensitivity**

Our previous reports (Hidema et al. 1997, Hidema et al. 2001) suggested the possibility that UVB-resistant rice cultivars might exhibit higher CPD photorepair ability compared with sensitive ones. Thus, we compared CPD photolyase activities in rice cultivars shown in Fig. 3B with the different UVB sensitivity indices shown in Table 1. Analysis of the data presented in Fig. 4 shows a significant correlation between CPD photorepair ability and UVB sensitivity (P < 0.01 for linear model; R² = 0.658). We can observe the tendency that the cultivars with higher CPD photorepair ability exhibited the lower UVB sensitivity index values (UVB resistant), while the cultivars with lower CPD photorepair ability exhibited higher UVB sensitivity index values (UVB sensitive).

We next examined the correlation between UVB sensitivity and the content of UV absorbing compounds, which are

### Table 1 Effects of supplemental UVB radiation on the changes in tiller number and plant dry weight of above-ground parts of plants, and the ranking of rice cultivars with respect to UVB-sensitivity index

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Tiller number</th>
<th>Dry weight (g)</th>
<th>UVB-sensitivity index (^a) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-UV (\pm SD)</td>
<td>+UV (\pm SD)</td>
<td>% (^b)</td>
</tr>
<tr>
<td>Sasanishiki</td>
<td>6.0 ± 2.4</td>
<td>4.5 ± 0.5</td>
<td>25 0.62 ± 0.07 (^c)</td>
</tr>
<tr>
<td>Norin 8</td>
<td>6.1 ± 0.3</td>
<td>4.3 ± 0.3</td>
<td>30 0.71 ± 0.11</td>
</tr>
<tr>
<td>Sasashigure</td>
<td>7.0 ± 0.1</td>
<td>5.0 ± 0.8</td>
<td>29 0.71 ± 0.08</td>
</tr>
<tr>
<td>Asahi I</td>
<td>5.2 ± 0.6</td>
<td>3.7 ± 0.3</td>
<td>29 0.57 ± 0.09</td>
</tr>
<tr>
<td>Kamenoo 4</td>
<td>5.3 ± 0.5</td>
<td>3.5 ± 0.5</td>
<td>34 0.71 ± 0.12</td>
</tr>
<tr>
<td>Norin 6</td>
<td>5.5 ± 0.1</td>
<td>3.3 ± 0.3</td>
<td>40 0.63 ± 0.01</td>
</tr>
<tr>
<td>Norin 22</td>
<td>5.0 ± 0.5</td>
<td>2.9 ± 0.6</td>
<td>42 0.62 ± 0.06</td>
</tr>
<tr>
<td>Koshihikari</td>
<td>5.3 ± 0.6</td>
<td>2.9 ± 0.6</td>
<td>45 0.63 ± 0.12</td>
</tr>
<tr>
<td>Moritawase</td>
<td>7.7 ± 0.5</td>
<td>3.7 ± 1.1</td>
<td>52 1.11 ± 0.08</td>
</tr>
<tr>
<td>Norin 1</td>
<td>6.0 ± 0.1</td>
<td>2.6 ± 0.2</td>
<td>57 0.65 ± 0.08</td>
</tr>
<tr>
<td>Rikuu 132</td>
<td>5.1 ± 0.9</td>
<td>2.4 ± 0.6</td>
<td>53 0.63 ± 0.21</td>
</tr>
<tr>
<td>Kamenoo</td>
<td>6.8 ± 0.9</td>
<td>2.9 ± 0.1</td>
<td>57 0.78 ± 0.09</td>
</tr>
<tr>
<td>Asahi II</td>
<td>6.1 ± 0.1</td>
<td>2.6 ± 0.5</td>
<td>57 0.83 ± 0.06</td>
</tr>
<tr>
<td>Rikuu 22</td>
<td>5.2 ± 0.5</td>
<td>2.3 ± 0.5</td>
<td>56 0.64 ± 0.13</td>
</tr>
<tr>
<td>Aikoku</td>
<td>4.9 ± 0.5</td>
<td>1.9 ± 0.1</td>
<td>61 0.69 ± 0.03</td>
</tr>
<tr>
<td>Hatsunishiki</td>
<td>6.3 ± 0.6</td>
<td>1.8 ± 1.0</td>
<td>71 0.81 ± 0.15</td>
</tr>
<tr>
<td>Ginbouzu</td>
<td>7.0 ± 0.9</td>
<td>2.1 ± 0.7</td>
<td>70 0.83 ± 0.08</td>
</tr>
</tbody>
</table>

\(^a\) The sensitivity index was determined by summing the percentage changes in tiller number and plant dry weight.  
\(^b\) Percentage change = [(control (–UV) – UV treatment (+UV))/control (–UV)] × 100.  
\(^c\) Mean ± SD, n = 3–7.

*The methods (Hidema et al. 1997, Hidema et al. 2001) suggested the possibility that UVB-resistant rice cultivar might exhibit higher CPD photorepair ability compared with sensitive ones. Thus, we compared CPD photolyase activities in rice cultivars shown in Fig. 3B with the different UVB sensitivity indices shown in Table 1. Analysis of the data presented in Fig. 4 shows a significant correlation between CPD photorepair ability and UVB sensitivity (P < 0.01 for linear model; R² = 0.658). We can observe the tendency that the cultivars with higher CPD photorepair ability exhibited the lower UVB sensitivity index values (UVB resistant), while the cultivars with lower CPD photorepair ability exhibited higher UVB sensitivity index values (UVB sensitive).*
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thought to play a role in coping with UVB-induced damage (Bornman and Teramura 1993, D’Surney et al. 1993, Li et al. 1993, Lois and Buchanan 1994, Mazza et al. 2000, Bieza and Lois 2001). The contents of UV-absorbing compounds in crude acid–methanol extracts (OD 330) were similar in the rice cultivars tested (Fig. 5). There was no significant correlation between UVB sensitivity and the content of UV-absorbing compounds. As a result, the UVB sensitivities of all cultivars examined here were shown to be dependent on the degree of CPD photorepair ability, and to be independent of the content of UV-absorbing compounds, suggesting that UVB sensitivity in rice correlates with CPD photorepair ability.

Discussion

As described above, sensitivity in response to UVB in progenitors and relatives in the breeding of cultivars Sasanishiki and Norin 1 is highly dependent on CPD photorepair ability. However, the UVB sensitivity index among cultivars that have the ‘Sasa’-type CPD photolyase gene with the higher CPD photorepair ability, varied from 69 for the least affected cultivar Sasanishiki to 129 for the most affected cultivar Rikuu 132, while the UVB sensitivity index among cultivars that have the ‘Nori’-type CPD photolyase gene with lower CPD photorepair ability, varied from 107 (Norin 6) to 154 (Ginbouzu) (Fig. 4). These results mean that there are some unknown factors that exceed the function of CPD photorepair ability in determining UVB sensitivity in rice. In genetic studies of the UVB sensitivities of the progeny of crosses between the cultivars Sasanishiki and Norin 1, we found previously that more than two major genes were involved in the control of UVB sensitivity in rice (Sato et al. 1994). We recently reported three putative quantitative trait loci (QTL) regions associated with UVB resistance in rice (Sato et al. 2003), and among them qUVR-10 (the QTL for UVB resistance on chromosome 10) showed the largest allelic difference. Furthermore, Ueda et al. (2003) found that the CPD photolyase gene and four putative chalcone synthase genes were located in the candidate genomic region of the qUVR-10 by linkage mapping, although qUVR-10 has not been cloned yet. An accumulation of certain phenylpropanoid compounds (such as flavonoids and anthocyanins) in the vacuoles of the epidermal and subepidermal cell layers, which are thought to act as UVB filters, play a role in coping with UVB-induced damage (Bornman and Teramura 1993, D’Surney et al. 1993, Li et al. 1993, Lois and Buchanan 1994). An Arabidopsis mutant tolerant to lethal UVB radiation showed constitutively elevated accumulation of flavonoids and other phenolics (Bieza and Lois 2001). In contrast, Dai et al. (1992) reported that the differences in sensitivity to UVB radiation among four rice cultivars could not explain the quantitative differences in flavonoid shielding compounds. Markham et al. (1998) reported that flavonoids might play a more subtle role in plant UVB protection than simple UVB screening in a UV-tolerant rice cultivar M202 (Caasi-Lit et al. 1997). Furthermore, we showed previously that the growth of a near-isogenic line (NIL) for purple leaf gene pl of rice with a genetic background of Taichung 65 (T-65) rice was significantly retarded by supplementary UVB, despite the fact that the amounts of UV-absorbing
compounds and anthocyanins in the NIL were significantly higher than those in T-65. The retardation of growth in the NIL resulted from its lower ability to photorepair CPD by higher amounts of anthocyanins, and accumulation of anthocyanins and UV-absorbing compounds did not effectively function as screening against damage caused by elevated UVB radiation in the NIL (Hada et al. 2003). As shown in Fig. 5, it was clear that there was no significant correlation between UVB sensitivity and UV-absorbing compound content among rice cultivars examined here. Thus, their importance in protection mechanisms against UV-induced biological damage in rice plants has been questioned. To date, numerous studies on factors effectively acting in UVB resistance in plants have been reported: (6–4) photolyase (Britt et al. 1993, Jiang et al. 1997), dark repair for (6–4) pp (Tanaka et al. 2002), nucleotide excision repair (NER) enzymes (Galligo et al. 2000, Liu et al. 2000), DNA polymerase ζ (AREV3) in translesion synthesis (Sakamoto et al. 2003), active oxygen species (AOS)-scavenging enzymes (Fujibe et al. 2004) and gibberellin level (Suge et al. 1991). We are interested in what factors other than CPD photolyase would effectively act to increase UVB sensitivity in rice, a question that should be answered in the near future. At any rate, the results shown above emphasize the possibility that CPD photorepair ability is one of the main factors determining UVB sensitivity in rice.

It is also evident that there is a single alteration of amino acid residue at position 126 in the deduced amino acid sequences of CPD photolyase in UV-resistant Sasanishiki and UV-sensitive Norin 1 (Fig. 2). This result suggests that such a single amino acid alteration could be closely related to the difference in CPD photorepair ability between these two cultivars. This suggestion was supported by the experiment in which a significant correlation was shown between CPD photorepair activity and the deduced amino acid sequence of various cultivars (Fig. 3B). It was thus shown that all cultivars that exhibited higher CPD photolyase activities, had the same deduced amino acid sequence as that of Sasanishiki (‘Sasa’-type). Furthermore, all cultivars that exhibited lower CPD photolyase activities had the same deduced amino acid sequence as that of Norin 1 (‘Nori’-type).

To infer the function of an altered amino acid residue, it is sometimes useful to compare the CPD photolyase sequence of rice with that of other organisms, in which the functions of sequence motif are known. One report for yeast has shown that the carboxy-terminal region (amino acid residues 326–505) binds to FAD as an essential catalytic cofactor. The amino-terminal region (amino acid residues 15–326) binds to DNA and 5,10-methenyltetrahydrofolate (MTHF), which has the function of absorbing light and transferring energy to the FAD cofactor (Malhotra et al. 1992). The CPD photolyase genes are classified into two classes, microbial (Class I) and metazoan (Class II) genes, based on the sequence similarity (Yasui et al. 1994). The CPD photolyase of yeast is classified as a Class I gene, while the CPD photolyase of rice is classified as a Class II gene (Hirouchi et al. 2003). It is therefore uncertain whether the function of the sequence motif of Sasanishiki CPD photolyase can be compared with that of yeast CPD photolyase. However, we demonstrated previously that the mutation in Norin 1 photolyase affects the function of photolyase, manifested as a decreased rate of binding to CPD and thus a slower formation of enzyme–substrate complexes, as shown by photoflash analysis (Hidema et al. 2000). It is therefore speculated that the amino-terminal region of CPD photolyase, which contains the amino acid residue 126, could bind to DNA, similarly to the case of yeast CPD photolyase.

It is said that systematic breeding of commercial rice cultivars in Japan was commenced under the supervision of the Japanese government around 120 years ago. Before that time, in their own way from several ancestor strains, devoted farmers had bred cultivars tolerant to low temperatures and pathogenic fungi and insects, and with good productivity, taste and kernel texture. When the government commenced the systematic breeding of rice, about six ancient strains, including Asahi, Ginbouzu, Aikoku and Kamenoo, which had been developed previously by farmers, were ordinarily used. It is therefore questionable how pure the genetic background of each ancient strain was. As a matter of fact, it was confirmed in this experiment that there are two different strains of Asahi and Kamenoo having either ‘Sasa’-type (Asahi I and Kamenoo 4) or ‘Nori’-type (Asahi II and Kamenoo) CPD photolyase genes (see Fig. 3B, 6). A phylogeny of progenitors and relatives in the breeding of Sasanishiki and Norin 1 is shown in Fig. 6. It is speculated that the ‘Sasa’-type CPD photolyase gene originated from the cultivar Asahi, while the ‘Nori’-type CPD photolyase gene was inherited from the cultivar Moritawase. It has also been shown that the CPD photolyase genes of Ginbouzu and Aikoku are ‘Nori’-type. Finally, it is surprising that Japanese cultivars tested in this experiment showed a considerably positive correlation between UVB sensitivity, CPD photorepair ability and genotypes of CPD photolyases. It is also significant that the rice cultivars with the ‘Sasa’-type CPD photolyase gene and higher photorepair ability potentially possess resistance to elevated UVB. These results should be quite helpful for bioengineering UV-tolerant cultivars.
Materials and Methods

Plant materials and growth conditions

Seventeen lowland Japanese cultivars of rice (Table 1) were used as experimental materials. Seeds of each cultivar, soaked in water at 30°C for 2 d, were sown for 30 d in pots (15 cm wide x 6 cm deep x 10 cm high) containing fertilized soil in a large growth cabinet (Tabai Espec Ltd., Osaka, Japan), with a 12/12 h photoperiod, with temperatures at 27/17°C. Five seedlings were grown in each pot under visible light supplied by a combination of metal halide lamps (MT 400 DL/BUD; Iwasaki Electric Ltd Co., Saiitama, Japan) and higher-pressure sodium lamps (NH360DL; Iwasaki Electric Ltd Co.) positioned at the top of the chamber, with a heat-absorbing filter (Tabai Espec Ltd. Co., Osaka, Japan). The heat-absorbing filter eliminated radiation below 350 nm (Kang et al. 1998). Photosynthetic active radiation (PAR) was recorded with a data logger (LI-1000; Li-Cor Inc., Lincoln, NE, U.S.A.) and an LI-1900A sensor (Li-Cor Inc.). The PAR was adjusted to about 350 μmol photon m⁻² s⁻¹ at the top of the plants. When necessary, plants were grown under visible light supplemented with UVB radiation using three UVB bulbs (FL20SE; Toshiba, Tokyo, Japan) 20 cm apart, located above the plants. Plants receiving UVB were subjected to the same photoperiod with visible radiation. Under UVB bulbs, a UV29 glass filter (Toshiba Glass Co., Shizuoka, Japan) was used for each cultivar, soaked in water at 27°C for 2 h, and mixed gently with 400 μl of 2× SS25; Japan Spectroscopic, Tokyo, Japan). Biologically effective UVB intensity was measured with a data logger (LI-1000) and a SD-104B sensor (Li-Cor Inc.). The PAR was adjusted to give a biologically effective UVB intensity of 0.2 g liter⁻¹ bovine serum albumin (BSA) (Seikagaku Co., Tokyo, Japan) and 10% (v/v) glycerol] using a chilled mortar and pestle. The homogenate was centrifuged for 20 min at 20,000×g at 4°C, and the supernatant was desalted by passage through a Bio-Gel P6DG spin-column (Bio-Rad). The filtrate was used as an extract for assay of photolyase activity. The soluble protein content was determined by the method of Bradford (1976) using BSA as the standard.

Preparation of protein extracts

The fully expanded fourth leaves (typically 0.08 g fresh weight) were homogenized in 400 μl of homogenization buffer (40 mM potassium phosphate, pH 7.2, 5 mM EDTA, 2 mM dithiothreitol (DTT), 0.2 g liter⁻¹ bovine serum albumin (BSA) (Seikagaku Co., Tokyo, Japan) and 10% (v/v) glycerol] using a chilled mortar and pestle. The homogenate was centrifuged for 20 min at 20,000×g at 4°C, and the supernatant was desalted by passage through a Bio-Gel P6DG spin-column (Bio-Rad). The filtrate was used as an extract for assay of photolyase activity. The soluble protein content was determined by the method of Bradford (1976) using BSA as the standard.

In vitro assay of photolyase

Photolyase activity in vitro was measured by a method described in detail elsewhere (Hada et al. 2003). A DNA (50 mg liter⁻¹ in 0.1× TE buffer) was irradiated with 10 J m⁻² of 254-nm radiation, producing 150 CPD Mb⁻¹. The DNA was diluted with an equal volume of 2× reaction buffer (1× reaction buffer is 40 mM potassium phosphate buffer, pH 7.2, 5 mM EDTA, 2 mM DTT, 0.2 g liter⁻¹ BSA, 80 mM NaCl and then mixed with extract at a ratio of substrate solution to extract of 9:1 (v/v). All manipulations were carried out using dim red lighting to minimize uncontrolled photorepair. The reaction mixture was incubated in the dark for 15 min at 30°C, ascertained to allow complete formation of photolyase–CPD complexes in extracts. Aliquots (20 μl) were placed in 0.2 ml polypropylene tubes, incubated at 30°C in the dark, or exposed to continuous illumination. For continuous illumination, the samples were incubated in a 30°C waterbath in the presence of photoreactivating light [four blue fluorescent tubes (20B-F; Toshiba, Tokyo, Japan)] at a distance of 20 cm. The CPD level repaired by blue radiation for 90 min increased with increasing protein concentration in the enzyme extract prepared from the cultivar Sasanishiki, which exhibited the highest CPD photolyase activity, until reaching 26 μg in 20 μl of reaction mixture (data not shown). Thus, each enzyme assay contained 1.3 μg liter⁻¹ protein concentration.

CPD levels were determined by adding 15 μl of 2× UV endonuclease buffer (1× UV endonuclease buffer is 30 mM Tris–HCl, pH 7.5, 40 mM NaCl, 1 mM EDTA) to each reaction mixture, and the mixture was divided into two parts. One microliter of Micrococcus luteus UV endonuclease (Carrier and Setlow 1970) was added to one part, which was then incubated at 37°C for 1 h, while the remaining mixture was incubated without UV endonuclease. DNAs were then denatured by the addition of alkaline stop mixture [0.5 M NaOH, 25% (v/v) glycerol and 0.25% (v/v) bromocresol green] and incubated for 30 min at 37°C.

Rice DNA molecules were dispersed according to their single-strand molecular lengths by alkaline agarose gel (0.7% [v/v]) electrophoresis, using static-field electrophoresis. Molecular length markers were λDNA (48.5 kb) and a HindIII digest of λDNA (23.1, 9.4, 6.6, 4.3 and 2.3 kb). Gels were neutralized (two 30 min changes of 0.1 M Tris–HCl, pH 7.5), stained with ethidium bromide (1 mg liter⁻¹) for 30 min, and destained (two 30 min changes of H₂O).
**CPD analysis**

The CPD frequencies were determined using a DNA damage analysis system made by Tohoku Electric Co. (Miyagi, Japan) as described previously (Hidema and Kumagai 1998). CPD frequencies were calculated using the molecular length standards curve and the quantity of DNA at each migration position as shown by the quantitative image data (Quaite et al. 1992, Quaite et al. 1994). The CPD frequencies are expressed in units of CPD Mb⁻¹.

**Quantification of UV-absorbing compounds**

Quantification of UV-absorbing compounds have been described in detail elsewhere (Kang et al. 1998). In brief, the fully expanded fourth leaf was homogenized in homogenization buffer (50 mM sodium phosphate, pH 7.2 and 10% (v/v) glycerol) at a ratio of leaf to buffer of 1 : 15 (g : ml) using a chilled mortar and pestle. The UV-absorbing compounds were extracted with 1% (v/v) HCl in 70% (v/v) sodium phosphate, pH 7.2 and 10% (v/v) glycerol) at a ratio of leaf to buffer of 1 : 15 (g : ml) using a chilled mortar and pestle. The UV-absorbing compounds were extracted with 1% (v/v) HCl in 70% (v/v) methanol (final concentration) at 4°C for 24 h. The extract was centrifuged at 1,800 × g for 15 min, and the amounts of UV-absorbing compounds in the supernatant were spectrophotometrically measured at 330 nm. Absorbance was measured with a JASCO V-550 UV/VIS spectrophotometer (JASCO Ltd. Co., Tokyo, Japan).

**Statistical analyses**

Statistical analyses were performed using the Excel statistical analysis 97 package (SSRI Ltd. Co., Tokyo, Japan).

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**References**


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