Isolation of a novel UVB-tolerant rice mutant obtained by exposure to carbon-ion beams

Nao TAKANO¹, Yuko TAKAHASHI¹, Mitsuru YAMAMOTO¹, Mika TERANISHI¹, Hiroko YAMAGUCHI¹, Ayako N. SAKAMOTO², Yoshihiro HASE², Hiroko FUJISAWA³, Jianzhong WU³, Takashi MATSUMOTO³, Seiichi TOKI³ and Jun HIDEIMA¹,*

¹Graduate School of Life Sciences, Tohoku University, Sendai, 980-8577, Japan
²Quantum Beam Science Directorate, Japan Atomic Energy Agency, Takasaki, 370-1292, Japan
³Agrogenomics Research Center, National Institute of Agrobiological Sciences, Tsukuba, 305-8602, Japan
*Corresponding author. Graduate School of Life Sciences, Tohoku University, Sendai 980-8577, Japan.
Tel: +81-22-217-5690; Fax: +81-22-217-5691; Email: j-hidema@ige.tohoku.ac.jp

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UVB radiation suppresses photosynthesis and protein biosynthesis in plants, which in turn decreases growth and productivity. Here, an ultraviolet-B (UVB) -tolerant rice mutant, utr319 (UV Tolerant Rice 319), was isolated from a mutagenized population derived from 2500 M₁ seeds (of the UVB-resistant cultivar 'Sasanishiki') that were exposed to carbon ions. The utr319 mutant was more tolerant to UVB than the wild type. Neither the levels of UVB-induced cyclobutane pyrimidine dimers (CPDs) or (6-4) pyrimidine-pyrimidone photodimers [(6-4) photoproducts], nor the repair of CPDs or (6-4) photoproducts, was altered in the utr319 mutant. Thus, the utr319 mutant may be impaired in the production of a previously unidentified factor that confers UVB tolerance. To identify the mutated region in the utr319 mutant, microarray-based comparative genomic hybridization analysis was performed. Two adjacent genes on chromosome 7, Os07g0264900 and Os07g0265100, were predicted to represent the mutant allele. Sequence analysis of the chromosome region in utr319 revealed a deletion of 45 419 bp. RNAi analysis indicated that Os07g0265100 is most likely the mutated gene. Database analysis indicated that the Os07g0265100 gene, UTR319, encodes a putative protein with unknown characteristics or function. In addition, the homologs of UTR319 are conserved only among land plants. Therefore, utr319 is a novel UVB-tolerant rice mutant and UTR319 may be crucial for the determination of UVB sensitivity in rice, although the function of UTR319 has not yet been determined.

Keywords: carbon-ion beam; rice mutant; root bending assay; UVB-tolerance

INTRODUCTION

A reduction of the stratospheric ozone layer increases the amount of ultraviolet-B (UVB: 280–320 nm) that reaches the earth’s surface [1]. Unprecedented ozone loss occurred over the Arctic in the spring of 2011, with a greater than 80% loss over 18–20 kilometers altitude [2]. UVB radiation suppresses photosynthesis and protein biosynthesis in plants, which in turn decreases growth and productivity [3]. There is considerable intra- and inter-specific variability in the sensitivity of crop plants, including rice, to UVB [4, 5]. Consequently, plants with decreased resistance to UVB damage may be less productive under current environmental conditions and may be severely damaged when UVB radiation is high, such as when stratospheric ozone is further depleted.

UVB radiation can affect various biological processes in plants because UVB is readily absorbed by a number of important biological macromolecules, such as DNA and protein. Among these macromolecules, DNA is a major target of UVB damage. UVB radiation induces photodamage in DNA, including the formation of cyclobutane pyrimidine dimers (CPDs) and (6-4) pyrimidine-pyrimidone photodimers [(6-4) photoproducts] [6]. Most DNA damage (approximately 75%) consists of CPD formation, whereas the formation of (6-4) photoproducts accounts for most of...
the remaining damage [7]. Such DNA damage can be lethal or mutagenic to organisms [8] and can impede DNA replication and transcription [9, 10]. Plants have various mechanisms to cope with UVB-induced DNA damage, including photoreactivation (photorepair) and nucleotide excision repair (also referred to as dark repair) [11]. Photorepair is the major DNA repair mechanism that occurs in plants. Photorepair is mediated by photolyase enzymes, which use blue/UVA (320–400 nm) light as an energy source to convert photodimers back to monomers. Previously, we demonstrated that UVB-induced CPDs are one of the principal causes of UVB-induced growth inhibition in rice plants grown under supplementary UVB radiation, and increasing CPD photolyase activity can significantly alleviate UVB-induced growth inhibition in rice [12]. However, the beneficial effects of CPD photolyase are limited, and further protection from UVB-induced damage may require the genetic enhancement of other systems as well.

Numerous studies have been performed with Arabidopsis mutants impaired in factors affecting UVB resistance. These studies have identified candidate genes or factors involved in UVB resistance. For example, the Arabidopsis mutant uvu1 has constitutively increased levels of flavonoids and other phenolics, which may act as UVB filters, and tolerates UVB radiation [13]. However, purple rice, which has higher levels of flavonoids (such as anthocyanins) than wild-type rice, is sensitive to UVB radiation [14]. The excess accumulation of flavonoids in purple rice reduces the amount of blue/UVA radiation that reaches cells, thereby decreasing the occurrence of CPD photorepair in its leaves [14]. UVB radiation can also produce reactive oxygen species (ROS) that induce oxidative damage in DNA, protein and other cellular components [15–17]. Thus, plants grown in the presence of UVB radiation require the activity of various ROS-scavenging systems [18]. Indeed, a study by Fujibe et al. [19] showed that a methyl viologen-resistant Arabidopsis mutant (rcd1-2) with increased plastidic Cu/Zn superoxide dismutase and stromal ascorbate peroxidase activity is more tolerant to short-term UVB treatment than the wild type. Furthermore, the Arabidopsis mutant uvi1, which exhibits greater levels of CPD photorepair and dark repair of (6–4) photoproducts than the wild type, is UVB-tolerant [20]. However, it is unclear whether the systems used to repair DNA damage and scavenge ROS are associated with UVB resistance in rice, and whether the genetic enhancement of these systems would result in a superior UVB-resistant rice cultivar. In addition, UVB-resistant rice has not been developed (with the exception of rice with increased CPD photolyase activity) [12].

Recently, heavy ions beams with high-linear energy transfer have been used to generate new mutants and varieties of higher plants [21]. Novel Arabidopsis and rice mutants have been obtained by exposing seeds to heavy ion beams [20, 22–26]. In this study, we produced a carbon-ion beam-induced UVB hyper-resistant (UVB-tolerant) rice mutant using the UVB-resistant Japanese rice cultivar ‘Sasanishiki’ as the wild type. We describe the isolation and characterization of a new rice mutant that is more resistant to UVB than the wild type.

**MATERIALS AND METHODS**

**Determining the optimal radiation dosage for the production of rice mutants**

Dry rice seeds of the rice cultivar Sasanishiki (Oryza sativa L., japonica) were employed in this study. Approximately 150 seeds were placed in a Petri dish with their embryos facing upward. Irradiation apparatus for dry seeds, which was connected to a vertical beam line of an azimuthally varying field (AVF) cyclotron (Japan Atomic Energy Agency, Takasaki, Japan) [27], was used for carbon-ion irradiation with the total energy of 320 MeV (26.7 MeV/μ). The linear energy transfer was calculated as 76 keV/μm at the sample surface. Carbon-ion irradiation was performed under atmospheric pressure at the following doses: 20, 40, 60, 80, 100, 120, 140, 160, 180 and 200 gray (Gy).

After irradiation, the seeds in each lot were placed onto wet filter-paper in a Petri dish and maintained at 30°C for 2 days. The seeds were then planted in fertilized soil in a plastic tray and placed in a phytotron (Taba Espec Ltd, Osaka, Japan) under visible radiation (12/12 h photoperiod, at 27°C day/17°C night) as described by Hidema et al. [4]. Photosynthetic active radiation (PAR) was measured using a data logger (Li-1000; Li-Cor, Lincoln, NE, USA) and an L1-190SA sensor (Li-Cor). The PAR was adjusted to approximately 350 μmol m⁻² sec⁻¹ at the tops of the plants.

The germination rate was measured at 2 days after irradiation in a Petri dish. To assess plant growth, plants were grown in plastic trays (62 cm wide × 41 cm long × 7 cm high) or pots (15 cm wide × 6 cm long × 10 cm high) containing fertilized soil in a phytotron, with a 12/12 h photoperiod, at 27°C day/17°C night. The survival rate of the plants was measured by counting the number of viable plants at 3 weeks after germination. Growth analysis was performed by determining the number of panicles, the percentage of ripening, the culm length and the dry mass of aerial parts of the plants. Aerial parts of the plants were dried at 80°C for 3 days, and the dry mass was then measured. At least three independent experiments were performed for each dose of irradiation.

**Mutant isolation**

Dry seeds of the rice cultivar Sasanishiki (wild type; WT-Sa) were irradiated with carbon-ions at a dose of 80 Gy under atmospheric pressure. Approximately 2500 M₁ seeds were planted, and the plants were grown to maturity and harvested. Twenty M₂ seeds derived from each M₁
plant were subjected to the first screening using a root-bending assay according to the method described by Britt et al. [28]. Two-day-old seedlings that were grown vertically on 1.5% agar plates under visible radiation (350 μmol m⁻² s⁻¹) were exposed to UVB radiation supplied by UVB bulbs (FL20SE; Toshiba, Tokyo, Japan) at a total dose of 9.6 kJ m⁻² (2.0 W m⁻² for 80 min). The UVB intensity, which ranged from 280–320 nm, was measured with a UVB sensor (MS-210D, Eiko Seiki Co., Tokyo, Japan). After UVB exposure, the plates were rotated 90° and incubated for 4 days under visible radiation at 25°C. The amount of new root growth after UVB exposure was then measured. Three plants that showed greater increases in root length than WT-Sa were selected for further analysis. These lines were grown individually under normal conditions, self-pollinated and harvested to yield M₃ seeds. Second and third screenings were carried out using 10 to 20 seeds of the M₂ through M₃ progeny using the root-bending assay and the long-term exposure assay described below.

For the root-bending assay of the M₃ generation, 1-day-old seedlings that were grown vertically on 1.5% agar plates under visible radiation were exposed to UVB radiation supplied by UVB bulbs (FL20SE) at a total dose of 2.2, 3.2, 4.3 or 5.4 kJ m⁻² (1.8 W m⁻² for 20, 30, 40 or 50 min). After UVB exposure, the plates were rotated 90° and incubated for 3 days under visible radiation at 25°C. For the long-term exposure assay, 10 seeds of each putative mutant line were soaked in water at 30°C for 2 days and sown in pots as described above. Three seedlings were grown in each pot under visible light, with or without supplementary UVB radiation supplied by UVB bulbs (FL20SE) filtered through a UV29 glass filter (Toshiba Glass Co., Shizuoka, Japan), for 30 days [biologically effective UVB radiation (UVBBE) = 42.5 kJ m⁻² day⁻¹] [29]. UVBBE was calculated using the plant action spectrum of Caldwell [30] normalized to unity at 300 nm. Plants receiving UVB were subjected to the same photoperiod as those receiving visible radiation. The PAR was adjusted to approximately 350 μmol m⁻² s⁻¹ at the tops of the plants. Growth analysis was performed, including the determination of the number of tillers and fresh weights of the aerial parts of the plants. The relative growth rates [(growth following UVB treatment (UVB treatment))/growth following visible radiation treatment (UVB control)] × 100 (%) were measured.

Analysis of induction and repair activities of CPDs and (6-4) photoproducts
To induce photodamage in the plants, detached, fully expanded third leaves were placed on wet filter-paper and irradiated using unfiltered UVB bulbs (FL20SE) at a total dose of 0–9 kJ m⁻² (0–10 W m⁻² for 15 min). For repair experiments, the detached leaves were exposed to 4.5 kJ m⁻² of UVB. Immediately after UVB exposure, the leaves were exposed to blue irradiation (60 μmol m⁻² s⁻¹) from blue fluorescent tubes (20B-F, Toshiba) or maintained in a light-tight box. After exposure to UVB or blue irradiation, the leaves were harvested immediately and stored in liquid nitrogen until analysis. All subsequent manipulations were carried out in red light to minimize uncontrolled photoreactivation.

The induction and repair of CPDs and (6-4) photoproducts were analyzed by enzyme-linked immunosorbent assay (ELISA). Rice DNA was extracted from each sample as previously described [5] and 50 μl of the extracted DNA was placed into each well of the ELISA plate. CPDs and (6-4) photoproducts were detected using the specific antibodies TDM-2 and 64M-2, respectively [31]. The DNA concentration was adjusted to 0.5 μg ml⁻¹ for incubation with TDM-2 and 3 μg ml⁻¹ for incubation with 64M-2. ELISA was performed as previously described [32].

Microarray-based comparative genomic hybridization (array CGH) analysis
Genomic DNA was isolated from utr319 mutant or WT-Sa seedlings as previously described [5]. The quantity and quality of the prepared genomic DNAs were checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Genomic DNA (1.5 μg) was digested with RsaI and AluI, and the digested DNA was labeled using a Genomic DNA Labeling Kit (Agilent Technologies) according to the manufacturer’s instructions. The digested genomic DNA of utr319 was labeled with cyanin-3 (Cy3)-dUTP, and the digested genomic DNA of WT-Sa was labeled with Cy5-dUTP for use as an experimental reference. Cy3- or Cy5-labeled DNA was purified using Microcon YM-30 Filter Units (Millipore, Billerica, MA, USA), and the quality of the labeled DNA was examined using a NanoDrop ND-1000 UV-VIS Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). A mixture of Cy3-labeled DNA and Cy5-labeled DNA (approximately 3.3 μg each) was hybridized to a slide containing a rice 44K oligo microarray (G2519F#15241; Agilent Technologies), which contained approximately 42,000 oligonucleotides, based on the nucleotide sequence and full-length cDNA data from the Rice Annotation Project Database (RAP-DB; http://rapdb.dna.affrc.go.jp/); the hybridization was performed at 65°C for 24 h. Hybridization and washing of the hybridized slide were performed according to the manufacturer’s instructions. The slide was scanned on an Agilent G2505B DNA microarray scanner (Agilent Technologies). The signals were quantified and normalized with Feature Extraction Software version 10.5.1.1 (Agilent Technologies).

PCR analysis of genomic DNA
PCR analysis was performed using ExTaq DNA polymerase (Takara Bio Inc. Shiga, Japan) according to the
manufacturer’s instructions. The primer sequences are shown in Supplemental Table 1. The PCR products were subjected to electrophoresis on 1.5% agarose gels.

**Construction of bacterial artificial chromosome libraries**

Two bacterial artificial chromosome (BAC) libraries were constructed using young leaves respectively from WT-Sa and utr319 mutant with the method as described before by Zhang et al. [33] and Peterson et al. (http://wheat.pw.usda.gov/jag/papers00/paper300/indexpage300.html) [34].

**Generation of RNA interference lines**

To construct a plasmid for the production of Os07g0265100 RNA interference (RNAi) transgenic rice plants, a full-length cDNA clone (GenBank/EMBL/DDBJ accession number AK11251) was obtained from the Rice Genome Resource Center (National institute of Agrobiological Science; http://www.rgrc.dna.affrc.go.jp/jp/index.html). Part of the sequence of Os07g0265100 (nucleotides 1 to 350) was amplified by PCR using the primers shown in Supplemental Table 1. The PCR product was inserted into the pANDA vector [35]. Nipponbare rice was transformed with the construct using an Agrobacterium tumefaciens (strain: EHA101)-mediated procedure, as described by Toki et al. [36].

**Gene expression analysis**

Total RNA was extracted from fully expanded third leaves of 2-week-old plants using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. DNA in the RNA samples was digested with RNase-free DNase (Qiagen). Total RNA (500 ng) was used for cDNA synthesis using a PrimeScript RT Reagent Kit (Takara Bio Inc.) and subjected to RT-PCR. Quantitative real-time PCR analysis was performed using a KAPA SYBR FAST qPCR Kit (Kapa Biosystems, Boston, MA, USA) in a C1000TM Thermal Cycler (Bio-Rad, Hercules, CA, USA). Primers for real-time PCR analysis are shown in Supplemental Table 1. β-tubulin mRNA was used as a control [37].

**RESULTS**

**Optimal radiation dosage of carbon-ion beams for the production of rice mutants**

To produce a variety of rice mutants using carbon-ion beams as a mutagen, it was important to first investigate the effects of carbon ions on biological processes such as germination, survival, ripening and subsequent mutations in the Sasanishiki cultivar of rice (WT-Sa). Fig. 1a shows the effects of 0–180 Gy carbon-ion beams on the growth of rice plants. Rice plants generated from seeds exposed to more than 160 Gy were severely damaged, although the germination rates of all the exposed seeds were over 90% (Fig. 1b). Fig. 1c shows the survival rates of rice plants generated from seeds exposed to carbon-ion beams. The survival rate dropped sharply when the seeds were exposed to more than 120 Gy. Table 1 shows various yield parameters of rice plants germinated from irradiated seeds. Dry mass, panicle number and percentage of ripening was significantly lower in plants at doses over 100, 80 and 80 Gy, respectively, while culm length was not affected in these plants. Yamaguchi et al. [38] reported that the most effective doses for obtaining mutants in rice cultivar Hitomebore were around shoulder of survival curves of seeds irradiated with ion beams and gamma-rays. Our results suggest that the optimal radiation dosage for inducing mutations without unnecessary damage
Isolation of the UVB-tolerant mutant, utr319

A total of 2500 dry rice seeds were irradiated with an 80-Gy carbon-ion beam and germinated. After flowering, the plants were self-pollinated, and the M2 seeds were harvested. For the first screening, we performed a root-bending assay [28] using seedlings germinated from 20 M2 seeds per M1 plant. The three candidate UVB-tolerant mutants with longer (>20%) roots than those of WT-Sa were selected for further analysis. To confirm the genetic stability of the UVB-tolerance trait, candidates from the M2 through M4 generations were tested for UVB tolerance using both a root-bending assay and a long-term exposure assay. In the M5 generation, two UVB-tolerant mutant lines, named UV Tolerant Rice (utr), were selected. The UVB-tolerant mutant utr319 was chosen for further analysis.

Fig. 2 shows the results of a root-bending assay and a long-term exposure assay of utr319 (M5 generation). One-day-old seedlings of Sasanishiki and utr319, which contained a primary root and a shoot, were irradiated with 0, 2.2, 3.2, 4.3 or 5.4 kJ m⁻² of UVB radiation. When grown under visible radiation, the roots of the utr319 mutant grew as well as those of the WT-Sa plant (Fig. 2a). By contrast, roots of utr319 plants that were irradiated with 3.2, 4.3 or 5.4 kJ m⁻² of UVB radiation grew significantly better than those of UVB-irradiated WT-Sa plants (Fig. 2b). Furthermore, to confirm the UVB tolerance of utr319, we performed a long-term UVB exposure assay (Fig. 2c and d). WT-Sa and utr319 were grown for 30 days in a phytotron under visible irradiation with (+UVB) or without (–UVB) supplementary 42.5 kJ m⁻² UVB radiation per day. The utr319 mutant grew as well as the WT-Sa plant under visible radiation alone. This was confirmed by determining the tiller numbers and fresh weights of aerial parts. The utr319 mutant grew significantly better than WT-Sa when exposed to visible radiation supplemented with 42.5 kJ m⁻² UVB radiation. Under these conditions, the relative growth rates [(UVB treatment)/(UVB control) × 100 (%)] in the tiller numbers of WT-Sa and utr319 were 58% and 84%, respectively. Additionally, when exposed to supplementary UVB radiation, the increases in fresh weights of the aerial parts of WT-Sa and utr319 were 40% and 62%, respectively. These data indicate that the utr319 mutant is more tolerant to UVB than WT-Sa.

We previously demonstrated that UVB-induced CPDs are a principal cause of UVB-induced growth inhibition in rice grown under supplemental UVB radiation, and that increasing the activity of CPD photolyase in rice elevates UVB resistance [12]. We therefore examined whether the UVB resistance of this mutant could be attributed to a mutation of the gene encoding CPD photolyase. Furthermore, we investigated the photoreactivation of (6-4) photoproducts, which are other UVB-induced photoproducts. To
determine the susceptibility of *utr319* to CPD or (6-4) photoproduct induction, the detached leaves of *utr319* and WT-Sa were exposed to 0–9 kJ m\(^{-2}\) of UVB radiation. Similar levels of CPDs and (6-4) photoproducts were produced in WT-Sa and *utr319* following UVB radiation (Fig. 3a), indicating that there were no significant differences in the levels of UV-absorbing compounds between these lines. Furthermore, there was no difference in the ability of *utr319* or WT-Sa to perform photorepair or dark repair of CPDs or (6-4) photoproducts (Fig. 3b). Therefore, the mutation in *utr319* did not affect the amount of UV-absorbing compounds, nor the repair of CPDs or (6-4) photoproducts.

**Mutated DNA region in *utr319***

Carbon-ions deposit high energy locally compared to gamma-rays or X-rays, and induce mutations such as deletions, translocations and point mutations in plants [39]. CGH was developed for genome-wide analysis of DNA sequence copy number and has been utilized to screen various mutants for multiple chromosomal aberrations [40–41]. To deduce the mutated region in *utr319*, we first performed array CGH analysis using the rice 44K oligo microarray, which was designed for expression array analysis. The resulting DNA array profile revealed that there were almost no differences in the signal intensities of genes between WT-Sa and *utr319*, except for two adjacent genes, Os07g0264900 and Os07g0465100, on chromosome 7. The signal intensities of Os07g0264900 and Os07g0465100 in *utr319* were extremely low (6 and 44, respectively), while those in WT-Sa were 1464 and 681, respectively (Table 2). These results suggest that the region on chromosome 7 that includes these two genes may be deleted in *utr319*.

To test this possibility, we investigated the existence of mutated genes in the putative mutated region of chromosome 7 in *utr319*. PCR-based markers (t3PS1, t3PS2, t3PS3, t3PS4 and t3PS5) positioned between two genes, Os07g0264800 and Os07g0265600, were designed based on data from the RAP-DB, and PCR analysis was performed using genomic DNA prepared from WT-Sa and *utr319* plants (Fig. 4a). The PCR products of the PCR-based markers t3PS2, t3PS3 and t3PS4, which were positioned at Os07g0264900 and Os07g0465100 in *utr319* were extremely low (6 and 44, respectively), while those in WT-Sa were 1464 and 681, respectively (Table 2). These results suggest that the region on chromosome 7 that includes these two genes may be deleted in *utr319*.

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To examine the mutated DNA region in *utr319* in detail, we constructed BAC libraries of *utr319* and WT-Sa. A BAC clone from each BAC library containing a region of approximately 200 kb, including Os07g0264800 and Os07g0265600, was then screened using the PCR-based markers t3PS1 and t3PS5. Sequence analysis of the BAC clones revealed a deletion of 45,419 bp (from 9,430,500–9,475,918 or 9,430,501–9,475,920) including Os07g0264900 and Os07g0465100 on chromosome 7 in *utr319*; the breakpoints appeared to be rejoined based on the shared-end terminal homology of ‘AC’ sequences (Fig. 4c).

**UVB tolerance of Os07g0265100 RNAi transgenic plants**

Next, we sought to identify the characteristics and functions of the predicted proteins encoded by Os07g0264900 and Os07g0465100 (GenBank/EMBL/DDBJ accession numbers AK071462 and AK111251, respectively) using the MSU Rice Genome Annotation Project Database (http://rice.plantbiology.msu.edu/). The putative amino acid sequences of Os07g0264900 and Os07g0265100 (NCBI reference sequence number NM_001065867 and NP_001059333, respectively) were annotated as hypothetical proteins of 155...
Table 2. Part of the array CGH profile of WT-Sa and utr319

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<th>Description</th>
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<tr>
<td>Os07g0262600</td>
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<tr>
<td>Os07g0264100</td>
<td>D-isomer specific 2-hydroxyacid dehydrogenase NAD-binding domain containing protein</td>
<td>646 599</td>
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# = Signal intensities are normalized signal by Feature Extraction Software version 10.5.1.1 (Agilent Technologies). * = The signal intensities in utr319 were extremely low.

Fig. 4. Analysis of the mutation site of utr319. (a) Schematic diagram of a region of chromosome 7 in WT-Sa and utr319. The predicted genes from the RAP-DB are indicated by black and blue bars. Arrows indicate the direction of transcription. The positions of primer sets are indicated by red arrows. (b) Agarose gel electrophoresis analysis of PCR products amplified using the primer sets indicated in (a). (c) A partial sequence near the mutation site. The breakpoints appeared to be rejoined, based on the shared-end terminal homology of the ‘AC’ sequences. The size of the deleted region was 45 419 bp (9 430 499–9 475 918 or 9 430 501–9 475 920).
and 136 amino acids, respectively. In addition, the Os07g0265100 gene was grouped into the category ‘response to stress’, whereas the gene Os07g0264900 was not grouped into a specific category, in the Gene Ontology (GO) project database (http://www.geneontology.org), which provides standardized vocabulary to describe the attributes of genes and their products in any organism. Furthermore, a BLAST search against the genomic database (http://genome.jgi.doe.gov/) revealed that the homologs of the Os07g0265100 sequence were commonly conserved only in upland plants (Fig. 5), and the homologs of the Os07g0264900 sequence were conserved only in Gramineae (Supplemental Fig. 1). Thus, we subsequently focused on Os07g0265100.

Os07g0465100 RNAi transgenic rice plants were produced to determine whether the utr319 mutant was tolerant to UVB due to a deletion of the Os07g0465100 gene. To produce these RNAi transgenic plants, the japonica rice cultivar ‘Nipponbare’ was used as the wild type (WT-Ni), because experimental techniques for producing RNAi transgenic rice plants were established in Nipponbare rice.

A construct containing two inverted repeats of 350 nucleotides in the 5’ coding region of Os07g0265100 was transferred into Agrobacterium, which was then used to transform WT-Ni callus. Two T3 RNAi lines [lines 3 (RNAi-3) and 34 (RNAi-34)] that were generated by self-fertilization of each independent T0 plant were selected for further study. First, the levels of Os07g0265100 expression in the RNAi lines were measured by quantitative RT-PCR. The relative gene expression levels, which were calculated as the ratio of the Os07g0265100 expression levels of each RNAi transgenic line to that of the WT-Ni plant, were approximately 29 and 32% in RNAi-3 and RNAi-34, respectively (Fig. 6a). On the other hand, the expression levels of Os07g0264900, as a control gene, were similar to those in the wild type (Fig. 6a). Next, we performed a root-bending assay using RNAi-3, RNAi-34 and WT-Ni. One-day-old seedlings of WT-Ni, RNAi-3 and RNAi-34 were irradiated with 3.2 kJ m−2 UVB radiation and grown for 3 days at 25°C under continuous white light. The relative root growth of the RNAi lines was significantly higher than that of WT-Ni; the relative root growth of WT-Ni, RNAi-3 and RNAi-34 was 39, 50 and 52%, respectively (Fig. 6b). Under the same conditions, utr319 was more tolerant to UVB than WT-Sa; the relative root growth of WT-Sa and utr319 was 49 and 57%, respectively (Fig. 2b). These results suggest that the UVB tolerance phenotype in utr319 was caused by deletion of Os07g0265100. Based on these results, we designated the mutant gene in utr319 as UTR319.

**DISCUSSION**

Using carbon-ion beam mutagenesis of 2500 M1 seeds of the UVB-resistant japonica rice cultivar ‘Sasanishiki’, we produced a novel, nontransgenic rice mutant that was more tolerant to UVB than the wild type. To our knowledge, no UV-tolerant mutant has previously been reported in rice, while four UV-tolerant mutants (uvil, rcdl-2, uviol and uv4) have been reported in Arabidopsis. The uvil and uv4
mutants, which were isolated from plants generated from 1280 M$_1$ Arabidopsis seeds irradiated with a 150-Gy carbon-ion beam, are unique, UVB-tolerant mutants [20]. The uvi1 mutant has an increased capacity for dark repair of (6-4) photoproducts and photoreactivation of CPDs than the wild type [20]. Therefore, UVI1 may be a negative regulator of two different repair systems, i.e. photoreactivation and dark repair, in Arabidopsis. The uvi4 mutant lacks UVI4, which is necessary for the maintenance of the mitotic state, and is tolerant to UVB due to increased levels of endoreduplication [23].

We previously demonstrated that the CPD photolase (PHR) overexpressing transgenic rice lines (S-B and S-C) are more tolerant to UVB than the Sasanishiki rice cultivar [12]. When the transgenic rice lines and WT-Sa are grown under the same supplementary UVB light conditions employed in the present study, the inhibitory effects of supplementary UVB light on growth observed in WT-Sa are alleviated in the transgenic lines; the supplementation of 42.5 kJ m$^{-2}$ UVB per day reduces the fresh weight of the aerial parts of S-B, S-C and WT-Sa by 61, 61 and 75%, respectively [12]. Therefore, the degree of growth alleviation is 14% higher in the transgenic lines than in WT-Sa, indicating that loss of UTR319 may be crucial for UVB sensitivity in rice. Indeed, this factor may be more important for UVB sensitivity than CPD photolase.

In addition to CPD photolase activity, other important factors that help determine UVB sensitivity, such as 6-4 photolase activity and the level of UV-absorbing compounds, were similar in the utr319 mutant and WT-Sa. RNAi analysis suggests that Os07g0265100 on chromosome 7 is most likely UTR319, which was lost in the mutant. Database analysis indicates that UTR319 encodes a putative protein with unknown significant domain sequences, characteristics or function. In addition, since the homologs of Os07g0265100 sequences are commonly conserved, especially in the middle region of the sequence, only among upland plants, UTR319 is likely to be a plant-specific protein. Luhua et al. [42] produced transgenic plants that overexpress At2g21195, which is homologous to UTR319, with which they performed several stress-response assays. Plants that overexpress At2g21195 are resistant to paraquat and t-butyl hydroperoxide and sensitive to high- and low-temperature stress [42]. Therefore, the function of UTR319 may be related to stress resistance.

The relative root growth of Sasanishiki was higher than that of Nipponbare during the root-bending assay (Fig. 6b); the relative root growth of WT-Ni and WT-Sa was 39 and 49%, respectively. This may be attributed to Nipponbare having a lower level of CPD photolase activity than Sasanishiki. The deficiency in CPD photolase activity in Nipponbare is due to a single nucleotide change (at

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**Fig. 6.** Analysis of Os07g0265100 RNAi transgenic plants of rice. (a) Relative expression of Os07g0265100 and Os07g0264900 genes in WT-Ni, RNAi-3, RNAi-34, WT-Sa and utr319. Total RNA was extracted from the fully expanded third leaves of 2-week-old plants. Transcript levels were measured by quantitative RT-PCR analysis. β-tubulin mRNA was used as a control. The level of the transcript in the WT plant (WT-Ni for RNAi-3 and RNAi-34; WT-Sa for utr319) was set at 100 (%). Each value represents the mean ± SD of at least three samples. ND = not detected. (b) Relative root growth represents the percentage of root growth of UVB-irradiated in comparison with that of unirradiated WT plants (WT-Ni or WT-Sa). One-day-old seedlings were exposed to 0 (control) or 3.2 kJ m$^{-2}$ UVB radiation and incubated under continuous white light for 3 days. Each value represents the mean ± SD of at least six samples. * and ** indicate significantly different from the WT at $P<0.05$ and $P<0.01$, respectively.
position 377) in the photolyase gene (GenBank/EMBL/DDBJ accession number AB198744), which changes the amino acid at position 126 in the putative protein from glutamine to arginine [43]. Furthermore, the recovery of UVB-induced root-growth inhibition of each RNAi line seems slightly higher than that of utr319 compared with respective wild type, while the relative UTR319 gene expression level of each RNAi line is higher than that of the utr319 mutant. At this moment, the relationship between the deficient levels of UTR319 and UVB tolerance can’t be explained; to understand the functions of UTR319, we need to clarify the relationship. Since the relative root growth of RNAi lines exposed to UVB radiation were alleviated, RNAi analysis suggests that the UVB tolerance phenotype in utr319 was caused by deletion of Os07g0265100.

UTR319 may be a negative regulator of an unknown factor related to the growth of rice under UVB stress. To help elucidate the function of UTR319, the levels of gene expression should be compared between WT-Sa and utr319 plants grown under visible radiation, with or without supplemental UVB radiation, using microarray analysis. In addition, the responses of utr319 to various stresses should be investigated. Indeed, UTR319 may be a crucial factor that helps determine the UVB sensitivity of rice. The results of the present study suggest that it may be possible to develop UVB-hypertolerant rice plants by overexpressing crucial UVB-tolerance genes, such as the gene encoding CPD photolyase, in the utr319 mutant.

Finally, rice is one of the world’s most important staple food grains, and is widely cultivated in various regions throughout Asia. It is grown not only in regions of moderate climate at middle latitudes but also in tropical climates, such as Indonesia or the Bengal region, where there is more UVB radiation in the sunlight. Thus, we should conduct a study on the effect of natural sunlight or supplemental UVB radiation on the growth and yield of UVB-tolerant utr319 rice mutant in the field in the future.

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**SUPPLEMENTARY DATA**

Supplementary data is available at the Journal of Radiation Research online.

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