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Increase in CPD photolyase activity functions effectively to prevent growth inhibition caused by UVB radiation

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Summary

Rice cultivars vary widely in their sensitivity to ultraviolet B (UVB) and this has been correlated with cyclobutane pyrimidine dimer (CPD) photolyase mutations that alter the structure/function of this photorepair enzyme. Here, we tested whether CPD photolyase function determines the UVB sensitivity of rice (*Oryza sativa*) by generating transgenic rice plants bearing the CPD photolyase gene of the UV-resistant rice cultivar Sasanishiki in the sense orientation (S-B and S-C lines) or the antisense orientation (AS-D line). The S-B and S-C plants had 5.1- and 45.7-fold higher CPD photolyase activities than the wild-type, respectively, were significantly more resistant to UVB-induced growth damage, and maintained significantly lower CPD levels in their leaves during growth under elevated UVB radiation. Conversely, the AS-D plant had little photolyase activity, was severely damaged by elevated UVB radiation, and maintained higher CPD levels in its leaves during growth under UVB radiation. Notably, the S-C plant was not more resistant to UVB-induced growth inhibition than the S-B plant, even though it had much higher CPD photolyase activity. These results strongly indicate that UVB-induced CPDs are one of principal causes of UVB-induced growth inhibition in rice plants grown under supplementary UVB radiation, and that increasing CPD photolyase activity can significantly alleviate UVB-caused growth inhibition in rice. However, further protection from UVB-induced damage may require the genetic enhancement of other systems as well.

Keywords: CPD photolyase, cyclobutane pyrimidine dimer, *Oryza sativa*, transgenic rice plant, ultraviolet B, UVB resistance.

Introduction

Ultraviolet B radiation (UVB, 280–320 nm) can damage plants, resulting in decreased growth and productivity (Teramura, 1983). Consequently, plants with decreased resistance to UVB damage may be less productive under current environmental conditions and may become severely damaged when UVB radiation is high, such when stratospheric ozone is depleted.

DNA is one of the major targets of UV damage, and UVB radiation is capable of directly altering its structure. Two of the main UVB-induced photoproducts form between adjacent pyrimidines on the same strand, namely cyclobutane pyrimidine dimers (CPDs) and pyrimidine-pyrimidone (6–4) photoproducts, also termed (6–4) photoproducts (Britt, 1996). The majority of DNA damage consists of CPDs (approximately 75%), while the (6–4) photoproducts account for most of the remainder (Mitchell and Nairn, 1989). Such DNA damage may be lethal or mutagenic to organisms and may impede replication and transcription (Hoeijmakers, 2001; Sancar *et al*., 2004). Plants have various mechanisms for coping with such UVB-induced DNA damage, including photoreactivation (photorepair) and nucleotide excision repair (dark repair). Photorepair is the major mechanism (Britt, 1996) and is mediated by an one-enzyme 'photolyase' pathway that monomerizes dimers by using blue/UVA light as the energy source. In dark repair, the dimers are replaced by *de novo* synthesis in which the undamaged complementary strand is employed as the template.

The sensitivity of rice to UVB radiation varies widely among cultivars (Dai *et al*., 1992; Sato and Kumagai, 1993). Genetic analysis of progeny from a cross between the UV-resistant 'Sasanishiki' (*Oryza sativa* L. *japonica*) and UV-sensitive 'Norin 1' (*O. sativa* L. *japonica*) cultivars has

revealed that UVB sensitivity is controlled by more than two major recessive genes (Sato *et al.*, 1994). We previously reported that UV-resistant rice cultivars are better able to repair CPDs through photorepair than are UV-sensitive cultivars (Hidema *et al.*, 1997, 2000), and that this is due to enhanced photolyase function that results from spontaneously occurring mutations in the CPD photolyase gene (Hidema *et al.*, 2005; Teranishi *et al.*, 2004; Ueda *et al.*, 2005). Thus, increasing the photolyase function in rice might improve the UVB resistance of rice. To test this possibility, we generated three transgenic rice plant strains: two had higher CPD photolyase activity than the wild-type (WT) UV-resistant rice cultivar Sasanishiki and one had lower activity. We report here that the two former strains were much more resistant to UVB-induced growth inhibition than the WT cultivar, while the third was much more sensitive.

Results

Generation of CPD photolyase sense or antisense transgenic rice plants

Sense or antisense cDNA of the CPD photolyase of the Sasanishiki cultivar (Hirouchi *et al.*, 2003) was subcloned into the binary vector pPZP2Ha3, with a hygromycin phosphotransferase gene (HPT) as a selection marker (Fuse *et al.*, 2001) (Figure 1a). The subclones were transferred into *Agrobacterium* and then infected into the callus of the UVB-resistant Sasanishiki strain, which has higher CPD photolyase activity than many other rice cultivars (Hidema *et al.*, 2005; Teranishi *et al.*, 2004). Twenty lines generated from hygromycin-resistant callus (T_0 plants) were selected, and the copy numbers of the transferred CPD photolyase gene were determined by Southern blot analysis, which involved digestion of genomic DNAs isolated from the T_0 plants with *Eco*RI (Figures 1b and 1c) or *Apa*I (data not shown). Figure 1 shows the results of Southern blot analysis with *Eco*RI digestion of the sense T_0 plants (A–E, Figure 1b) or antisense T_0 plants (A–E, Figure 1c). All sense or antisense T_0 plants had 1–3 copies of the photolyase sense or antisense gene. We selected for further study two sense lines that carried three copies or one copy of the photolyase sense gene, respectively; these lines were named S-B and S-C, respectively (Figure 1b). We also selected an antisense line carrying a single copy of the antisense gene; this line was named AS-D (Figure 1c). Self-fertilized plants (T_1) from each T_0 plant were grown, and homozygous plants were selected. T_2 or T_3 lines generated by self-fertilization of each T_1 plant were used as experimental materials.

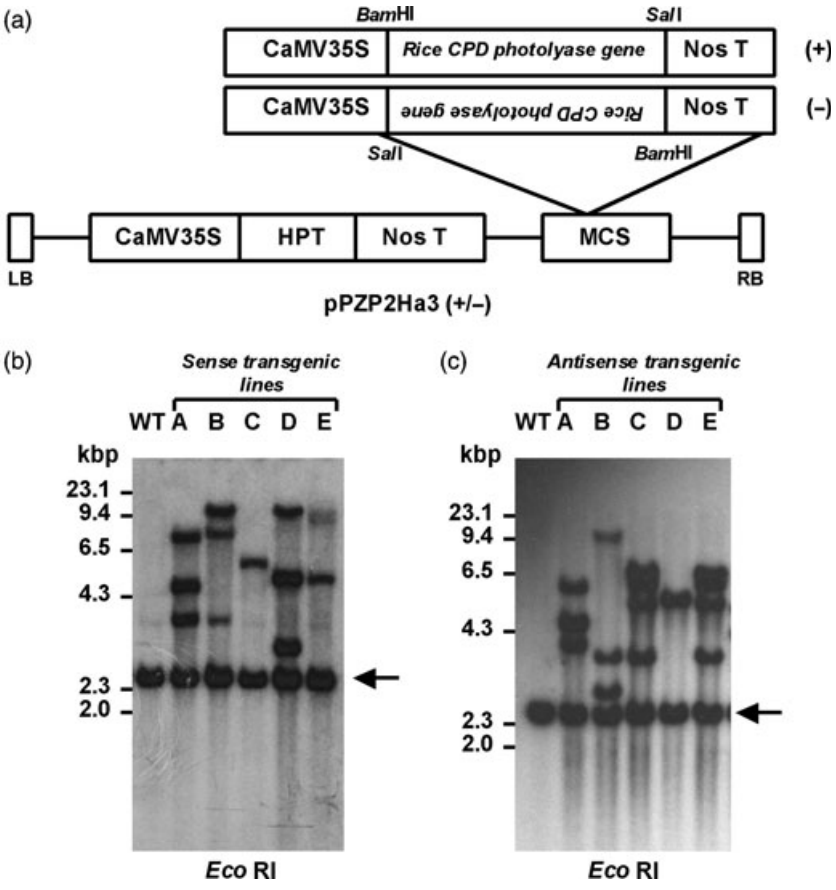

Figure 1. Analysis of the rice cyclobutane pyrimidine dimer (CPD) photolyase gene copy number in sense and antisense transgenic rice plants.
(a) Schematic representation of the expression vector pPZP2Ha3 that was used to transform rice with cDNA encoding the CPD photolyase of the Sasanishiki cultivar. The cDNA was subcloned into a multi-cloning site (MCS) of the binary vector pPZP2Ha3 in the sense (+) or antisense (–) orientation.
(b,c) Southern blot hybridization of the rice CPD photolyase gene in WT and in sense (b) or antisense (c) transgenic plants. Genomic DNA (20 μg) isolated from each hygromycin-resistant callus (T_0 plant) was digested with *Eco*RI and then separated on a 0.8% agarose gel. After transfer to a nylon membrane, the DNA blot was hybridized with a ^{32}P-labeled CPD photolyase gene probe. The arrows show the CPD photolyase gene bands.

CPD photolyase transcript levels and CPD photorepair activities in transgenic rice plants

The homozygous transgenic plants, S-B, S-C and AS-D, and a wild-type plant, WT, were grown under visible radiation alone, and the 4th leaves of 16-day-old seedlings of these plants were subjected to various analyses. First, CPD photolyase transcripts in transgenic plants were measured by quantitative real-time RT-PCR. The CPD photolyase transcript levels (calculated as the ratios of CPD photolyase transcripts of transgenic plants to that of the WT plant) in S-B and S-C plants were 20- and 149-fold higher than in the WT plant, respectively (Figure 2a). The antisense transgenic plant AS-D had 14-fold higher levels than the WT plant (Figure 2a), which indicates that this plant had increased antisense CPD photolyase transcript levels. The *in vitro* CPD photolyase activities in the cell-free extracts of the transgenic and WT 4th leaves were then measured using UV-irradiated λDNA as a substrate followed by alkaline agarose gel electrophoresis (Freeman *et al.*, 1986). The inset in Figure 2(b) shows the relationships between the amounts of CPD (CPD Mb^{-1} min^{-1}) repaired by blue irradiation and the soluble protein concentration in cell-free extracts (μg protein per μl cell-free extract) of transgenic and WT plants in the reaction mixture. The amounts of CPD photorepaired by the S-C, S-B and WT leaf cell extracts increased linearly with the protein concentration of the cell-free extracts but at markedly different rates. This is shown by the equations of the lines, namely $y = 2.2 \times 10^2\ x$, $y = 1.9 \times 10^3\ x$ and $y = 4.2 \times 10^1\ x$, respectively, where x is the soluble protein concentration of the cell-free extract in the reaction mixture and y is the amount of CPD. Relative to the WT plant, the *in vitro* photolyase activities (CPD Mb^{-1} min^{-1} per μg protein per μl cell-free extract) of S-B and S-C were 5.1- and 45.7-fold higher, respectively (Figure 2b). Thus, over-expressing CPD photolyase enhances the *in vitro* photorepair ability of rice. In contrast, the AS-D extracts could not photorepair the CPDs within the range of protein concentrations examined.

The susceptibility of the transgenic and WT plants to UV-induced CPDs was then determined by exposing the detached 4th leaves to UV radiation for various time periods to induce CPDs, after which the DNA was isolated and the CPD levels were determined. The CPD levels increased with lengthening of the duration of UV illumination, and the transgenic and WT plants showed similar dose–response curves (Figure 3a). This is because the UV-absorbing compounds and chlorophylls in their leaves did not differ significantly (data not shown). Thus, the transgenic plants show equivalent susceptibility to UV-induced CPD formation. We then examined the *in vivo* ability of the plants to repair CPDs by blue radiation. To do this, the 4th leaves of the plants were exposed to UV radiation for 15 min to induce approximately 50 CPD Mb^{-1}, then illuminated with blue

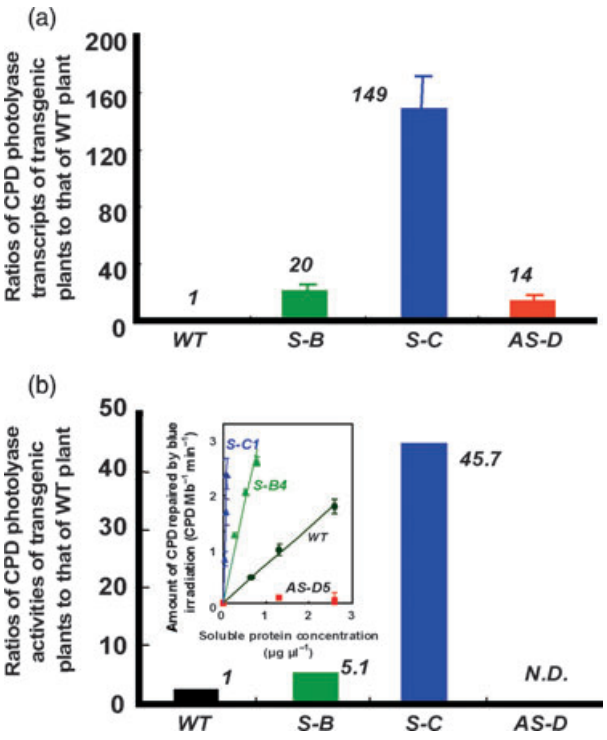

Figure 2. Cyclobutane pyrimidine dimers (CPD) photolyase transcript levels and *in vitro* CPD photolyase activities of transgenic (S-B, S-C and AS-D) and wild-type (WT) rice plants.
(a) Ratios of CPD photolyase transcript levels of transgenic plants relative to that of the WT plant. Transcripts were measured by quantitative real-time RT-PCR analysis. 18S rRNA was used as a control. The level of WT was taken as 1. Values represent the mean ± SD of at least four samples.
(b) Ratios of CPD photolyase activities (CPD Mb^{-1} min^{-1} per 20 μl cell-free extract) of transgenic plants relative to that of the WT plant. The photolyase activity of the WT was taken as 1. The cell-free extracts of the fully expanded 4th leaves of each experimental plant were mixed with UV-irradiated λ DNA, incubated in the dark for 15 min at 30°C to facilitate photolyase–CPD complex formation, and then exposed to continuous blue light for 30 min. Immediately thereafter, the DNA was isolated and the CPDs were measured. The inset shows the relationship between the amount of CPD repaired by blue irradiation (CPD Mb^{-1} min^{-1}) and the soluble protein concentrations in the cell-free extracts (μg μl^{-1}) in the reaction mixture. The enzyme activities (CPD Mb^{-1} min^{-1} μg^{-1} soluble protein in the cell-free extract) of the sense transgenic and WT plants were determined from the results shown in the inset. The values represent the mean ± SD of at least three samples for S-B (green triangle), S-C (blue triangle), AS-D (red square) and WT (black circle). N.D., not detected.

light for various lengths of time. The degree of CPD repair was then determined. The S-B and S-C seedlings showed significantly increased photorepair compared with the WT seedlings (Figure 3b), as the WT seedlings repaired about 8 CPD Mb^{-1} in less than 15 min while the S-B and S-C seedlings repaired approximately 20 and 40 CPD Mb^{-1}, respectively. In contrast, the AS-D seedlings showed little photorepair, even after 4 h of blue light illumination (Figure 3b). None of the seedlings showed any CPD repair in the dark at the 4 h time point (Figure 3b). Thus, the sense transgenic plants had much higher *in vivo* CPD photorepair

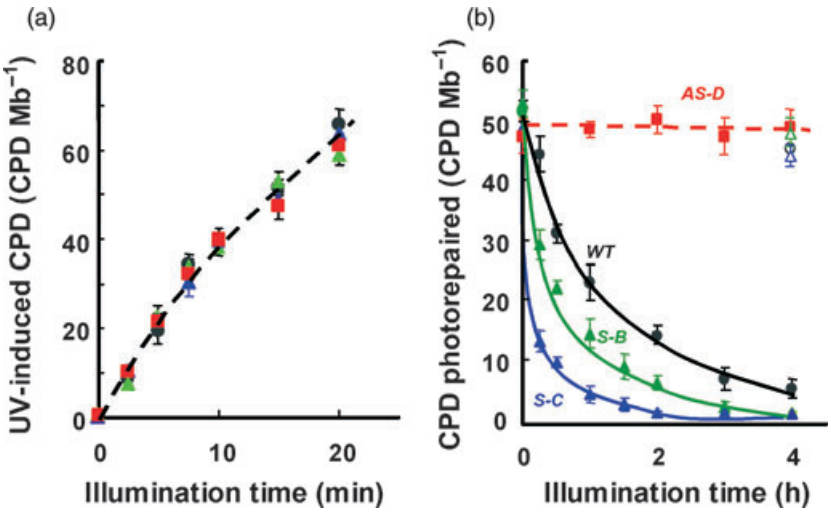

Figure 3. Susceptibility to UV-induced cyclobutane pyrimidine dimers (CPD) and *in vivo* CPD photorepair activities of transgenic and wild-type (WT) seedlings.
(a) Susceptibility to UV-induced CPD. The detached 4th leaves were exposed to UV radiation (germicidal lamp) at a distance of 10 cm for 0–20 min, DNA was isolated, and then the CPD levels were determined. The values shown are means ± SD of at least three samples.
(b) *In vivo* CPD photorepair activities. The detached 4th leaves were exposed to UV radiation for 15 min to induce approximately 50 CPD Mb^{-1} and then exposed to blue light (60 μmol m^{-2} sec^{-1}) for various lengths of time (closed symbols) or kept in the dark for 4 h (open symbols). Samples were harvested at the indicated times, and the CPD frequencies in the DNAs were measured. The values shown are means ± SD of at least three samples.
S-B, green triangle; S-C, blue triangle; AS-D, red square; WT, black circle.

activities than WT, while the antisense transgenic plant had little CPD photolyase activity.

Resistance of transgenic rice plants to UVB radiation

To test the resistance of the transgenic plants to UVB radiation, they were grown for 40 days in a growth chamber under visible radiation with or without 20.3 or 42.5 kJ m^{-2} UVB per day, after which their growth was examined. All transgenic plants grew as well as the WT plant when grown only under visible radiation (-UVB; Figure 4a). This was confirmed by determining the tiller numbers and FW of aerial parts, which did not differ significantly between the WT and transgenic plants (Figure 4b,c). In contrast, the sense transgenic plants grew significantly better than the WT plant when exposed to UVB radiation, while the growth of the antisense transgenic plant was severely damaged (Figure 4a). Supplementation with 20.3 kJ m^{-2} UVB per day reduced the tiller numbers of WT, S-B, S-C and AS-D plants by 48, 26, 20 and 93% (n = 7–8), respectively, while supplementation with 42.5 kJ m^{-2} UVB per day reduced the tiller numbers by 60, 32, 36 and 93% (n = 6–8), respectively (Figure 4b). Similarly, supplementation with 20.3 kJ m^{-2} UVB per day reduced the FW of the aerial parts of WT, S-B, S-C, and AS-D plants by 59, 44, 40 and 92% (n = 7–8), respectively, while supplementation with 42.5 kJ m^{-2} UVB per day resulted in 75, 61, 61 and 92% reductions (n = 6–8), respectively (Figure 4c). Thus, CPD photolyase-over-expressing plants are significantly more resistant to the growth-impairing effects of UVB radiation than the WT plant. However, the S-C plants, which had much higher CPD photolyase transcription and activity than the S-B plants, were not more resistant to UVB radiation than the S-B plants. Thus, there is a limit to the UVB resistance that can be generated by altering CPD photolyase levels. In contrast, the AS-D plant was much more susceptible to UVB-induced growth inhibition than the WT plant. These results strongly suggest that UVB-induced CPDs are one of the principal causes of UVB-induced growth inhibition in rice plants grown under supplementary UVB radiation, and elevating CPD photolyase function can increase resistance to UVB radiation, probably because it lowers the CPD levels.

Steady-state CPD levels in the leaves of transgenic and WT plants

To test the possibility that elevating CPD photolyase function increases resistance to UVB radiation because it lowers the CPD levels, we measured the steady-state CPD levels in the leaves of transgenic and WT plants grown with or without supplementary UVB radiation. The plants were grown under visible radiation until the 4th leaves had expanded fully, after which some of the potted plants were grown under visible radiation supplemented with UVB radiation. The remaining plants received only visible radiation. Over the following 2 days, the 4th leaves were detached at various time points and the CPD levels were measured (Figure 5). When grown under visible radiation only, all plants showed equivalent steady-state CPD levels that remained constant at less than 1 CPD Mb^{-1} during the

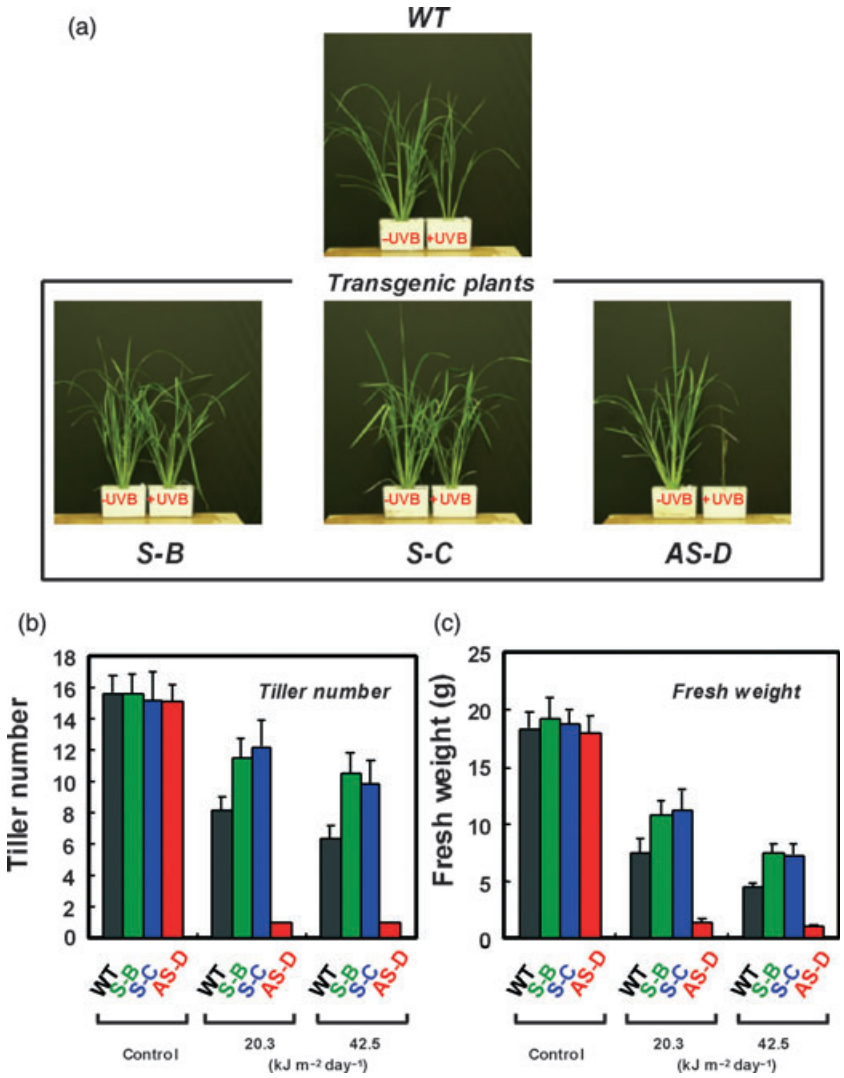

Figure 4. Effects of supplementary UVB radiation on the growth of transgenic (S-B, S-C and AS-D) and wild-type (WT) plants.
(a) Photographs of plants grown for 40 days under visible radiation supplemented with (+UVB) or without 42.5 kJ m^{-2} UVB per day (-UVB).
(b,c) Effects of supplementary UVB radiation on tiller numbers (b) and FW of aerial parts (c) of the transgenic plants (S-B, green column; S-C, blue column; AS-D, red column) and WT plants (black column). Plants were grown for 40 days under visible radiation with or without 20.3 or 42.5 kJ m^{-2} UVB per day. The values shown are means ± SD of at least six samples.

day and night (Figure 5a for S-B, S-C and WT; Figure 5c for AS-D). When the WT plant was grown under supplementary UVB radiation (42.5 kJ m^{-2} UVB per day), its CPD levels increased to between 4 and 6 CPD Mb^{-1} during the first day, and these levels were maintained during the night and the following day (Figure 5b). In contrast, the CPD levels of the S-B and S-C plants remained at less than 1 CPD Mb^{-1} during both day and night. Thus, the CPD photolyase activity of the S-B and S-C plants was sufficient to remove the CPDs induced by UVB radiation under these culture conditions. In contrast, in the AS-D plant, the CPDs accumulated much faster than in the WT plant, and reached 25 CPD Mb^{-1} at 8 PM on the first day (Figure 5c). These levels remained constant during the night but started to climb further at 8 AM on the second day (onset of illumination); by 8 PM on the second day, the CPD levels had reached 45 CPD Mb^{-1}. Thus, upon UVB radiation, the AS-D plant accumulates significantly higher CPD levels than the WT plant, while the S-B and S-C plants eliminate the CPDs more efficiently than WT.

Discussion

Here, using the UVB-resistant *O. sativa* L. *japonica* rice cultivar 'Sasanishiki' as our WT plant, we produced three CPD photolyase transgenic rice plants that ubiquitously express the CPD photolyase gene of Sasanishiki. This gene was expressed in all tissues because we used the CaMV 35S promoter. Two of the transgenic rice plants had the transgene in the sense orientation; the S-B plant carried three copies while the S-C plant carried a single copy (Figure 1). The third transgenic plant (AS-D) had a single copy of the transgene in the antisense orientation (Figure 1). Compared to the WT plant, the sense transgenic plants had much higher CPD photolyase transcript levels and CPD photolyase activities in 2-day-old seedlings, mature 6th and 8th leaves, and the root (data not shown), as well as in the fully expanded 4th leaves of 16-day-old seedlings. In contrast, all of tissues of the antisense transgenic plant exhibited much lower CPD photolyase activity (data not shown). Notably,

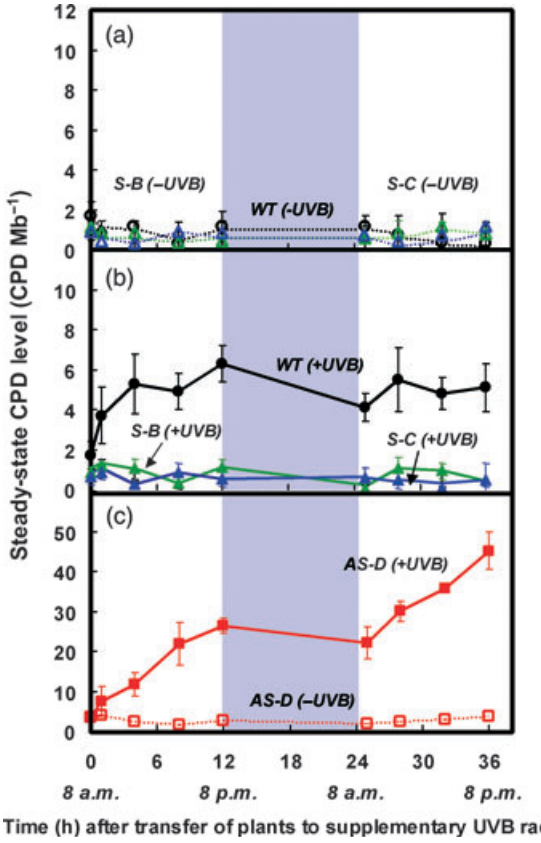

Figure 5. Changes in steady-state CPD levels in the 4th leaves of transgenic plants (S-B, green triangle; S-C, blue triangle; AS-D, red square) and WT plants (black circle) growing with (+UVB; closed symbols) or without (-UVB; open symbols) supplementary UVB radiation.
Experimental plants were first grown under visible radiation without supplementary UVB radiation in a large growth cabinet until the 4th leaves had expanded fully. Thereafter, some of the potted plants were cultured under visible radiation with supplementary UVB radiation (42.5 kJ m^{-2} UVB day^{-1}). The remaining plants were maintained in unsupplemented visible radiation. Over the next 2 days, the 4th leaves of groups of plants were detached every 3 h during the day (8 AM to 8 PM). The CPD levels in the leaf DNAs were then determined. The values shown are means ± SD of at least three samples.
(a,b) Changes in steady-state CPD levels in sense transgenic and WT plants growing without (a) and with (b) supplementary UVB radiation.
(c) Changes in steady-state CPD levels in antisense transgenic plants growing with or without supplementary UVB radiation.

although the S-B plant carried three copies of the sense transgene, its CPD photolyase transcript levels and activity were lower than those of the S-C plant, which carried only a single copy of the transgene. This may be due to homology-dependent gene silencing (Matzke and Matzke, 1995), which has been widely observed in multicellular eukaryotes, including higher plants, fungi and mammalian cells (Garrick *et al.*, 1998). Significantly, the S-B and S-C plants removed UVB-induced CPDs by photoreactivation more rapidly than the UV-resistant Sasanishiki rice plants did, while the antisense transgenic rice plants had hardly any photorepair ability (Figures 2 and 3).

We found that the antisense transgenic rice plants were severely damaged by UVB radiation (Figure 4). Similarly, Landry *et al.* (1997) have reported that a CPD photolyase-deficient mutant of Arabidopsis is hypersensitive to UVB radiation. Thus, CPD photolyase is indispensable for plant growth under UVB radiation. Moreover, increasing the CPD photolyase activity in rice conferred added protection from the deleterious effects of UVB radiation. However, we found that the S-C plant, which has much more CPD photolyase activity than the S-B plant, was not more resistant to UVB radiation than the S-B plant (Figure 4), and the CPD photolyase activities of both the S-B and S-C plants were sufficient to remove the CPDs induced by UVB radiation under these culture conditions (Figure 5). Thus, while increasing the CPD photolyase activity in rice does elevate UVB resistance, there is a limit to this beneficial effect.

These observations suggest that to develop plants with greater UVB resistance, it may be necessary to examine factors other than CPD photolyase that promote UVB resistance in plants. For example, it may be beneficial to enhance the levels of UV-absorbing compounds (certain phenylpropanoid compounds such as flavonoids and anthocyanins) in the epidermal and sub-epidermal cell layers. These compounds are believed to function as UV filters and to help mitigate UVB-induced damage (Bornman and Teramura, 1993). Indeed, Li *et al.* (1993) have shown that flavonoid-deficient mutants of Arabidopsis are hypersensitive to UVB radiation. Moreover, an Arabidopsis mutant that has constitutively elevated levels of flavonoids and other phenolics has been shown to tolerate UVB radiation (Bieza and Lois, 2001). However, it is not clear whether increasing the levels of UV-absorbing compounds would also enhance the UVB resistance of rice as excess accumulation of flavonoids (such as anthocyanins) reduces the amount of blue/UVA radiation reaching cells and effectively lowered CPD photorepair in purple rice leaves (Hada *et al.*, 2003). Another way to generate UVB-resistant rice plants may be to neutralize factors other than CPD that contribute to UVB-induced growth inhibition. UVB radiation can induce DNA lesions other than CPD, including (6–4) photoproducts. Britt *et al.* (1993) have shown that, while the UV-sensitive mutant of Arabidopsis *uvr1* has normal CPD photorepair, its repair of (6–4) photoproducts is defective. Moreover, mutations in AtXPF (Gallego *et al.*, 2000; Liu *et al.*, 2000), AtXPG (Liu *et al.*, 2001) or AtXPD (Liu *et al.*, 2003), which are related to nucleotide excision repair enzymes, result in UV sensitivity. Similarly, the UV-sensitive mutant *rev3-1* is deficient in a DNA polymerase ζ (*AtREV3*) for translesion synthesis (Sakamoto *et al.*, 2003). Conversely, the Arabidopsis mutant *uvi-1*, which shows enhanced dark repair of (6–4) photoproducts and CPD photorepair, is UV-tolerant (Tanaka *et al.*, 2002). Thus, all of the systems used by plants to repair DNA damage are indispensable for their growth under UVB radiation, and the simultaneous enhancement of these

systems may help generate a highly resistant rice plant. Moreover, UVB radiation can produce reactive oxygen species (ROS) that induce oxidative damage to DNA, proteins and so on (Caldwell, 1993; Foyer *et al.*, 1994). Thus, plant growth under UVB radiation also requires various ROS-scavenging systems, which include superoxide dismutase, catalase, ascorbate peroxidase, glutathione-*S*-transferase and low-molecular-weight antioxidants such as ascorbate, glutathione and carotenoids (Asada, 1999). Indeed, a recent study by Fujibe *et al.* (2004) showed that a methyl viologen-resistant Arabidopsis mutant (*rcd1-2*) with enhanced plastidic Cu/Zn superoxide dismutase and stromal ascorbate peroxidase activity had elevated tolerance to short-term UVB treatment. However, it remains unclear whether the above-described systems used to repair DNA damage and scavenge ROS are associated with UVB resistance in rice and whether their genetic enhancement would result in a superior UVB-resistant rice cultivar.

In the present study, we showed the sense transgenic rice plants had much higher UVB resistance, while the antisense transgenic plant was markedly more susceptible. These results show unequivocally that CPDs are a major cause of UVB-induced growth inhibition in rice plants. How CPDs inhibit plant growth remains to be clarified. Recent studies of mammalian cells have revealed that UVB-induced CPDs interfere with processes such as transcription and replication, thereby reducing RNA synthesis, arresting cell-cycle progression, inducing apoptosis and promoting skin cancer (Jans *et al.*, 2005; Sancar *et al.*, 2004). Such CPD-mediated interference with transcription and replication may also affect the biological processes of higher plants. Indeed, it has been shown that, in plants, UVB radiation reduces gene expression (Strid *et al.*, 1996) and inhibits protein biosynthesis (Takeuchi *et al.*, 2002). Alternatively, CPD may act indirectly as the initial signal that activates a number of UVB signal transduction pathways (Conconi *et al.*, 1996) that ultimately up- or downregulate gene expression (http://www.Arabidopsis.org/info/expression/ATGenExpress.jsp). UV-induced DNA damage has also been proposed to be the initial step in the activation of a particular class of genes in animal systems (Garinis *et al.*, 2005). How these possibilities relate to higher plants should be examined in the near future. The CPD photolyase transgenic plants produced in this work may be highly useful tools for such studies.

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. However, as many of the UVB-sensitive rice cultivars belong to the *aus* and *aman* ecotypes originating from Bengal regions, it is not necessarily true that rice cultivars originating from regions with higher ambient UVB radiation are more UVB-tolerant (Sato and Kumagai, 1993). Furthermore, the disparate UVB sensitivities of rice cultivars is tightly correlated to their CPD photorepair ability, which varies due to amino acid changes (Hidema *et al.*, 2005; Teranishi *et al.*, 2004; Ueda *et al.*, 2005). In this study, we demonstrated that increasing CPD photolyase activity in rice was able to produce alleviation of UVB-induced growth inhibition in rice. However, this study only examined the effect of elevated UVB radiation in a growth chamber. Thus, a study of the effect of increasing CPD photolyase activity on the growth and yield of rice grown under natural sunlight should be conducted in the future.

Experimental procedures

Construction of vectors and transformation of rice plants

Binary vector pPZP2Ha3 (Fuse *et al.*, 2001) was used for rice transformation. This vector contains the hygromycin phosphotransferase (*hpt*) gene as a selection marker under the control of the CaMV 35S promoter. The cDNA of the Sasanishiki CPD photolyase (Hirouchi *et al.*, 2003) was subcloned into pPZP2Ha3 in the sense (+) or antisense (-) orientation, and subclones were transferred into the *Agrobacterium* strain EHA101 (Figure 1) (Toki, 1997). The UVB-resistant rice cultivar Sasanishiki was then transformed essentially as described by Kojima *et al.* (2000), and 20 lines generated from hygromycin-resistant callus (T_0 plants) were selected. Two sense transgenic lines (S-B and S-C) and one antisense transgenic line (AS-D) were then chosen, and self-fertilized plants (T_1) of each T_0 plant were generated. Homozygous T_1 lines were selected and self-fertilized, and the resulting T_2 or T_3 lines were used as the experimental materials.

Growth conditions

To assess sensitivity to UVB radiation, transgenic and WT rice plants were grown for 40 days in pots (15 × 6 cm, 10 cm deep) containing fertilized soil in a growth cabinet (Koito Ind. Ltd Co.; http://www.koito-ind.co.jp) (12 h photoperiod, day/night temperatures 27 /17°C). Visible light in the growth cabinet was supplied by a combination of metal halide lamps (MT 400DL/BUD, Iwasaki Electric Ltd Co.; http:///www.iwasaki.co.jp) and higher-pressure sodium lamps (NH360DL, Iwasaki Electric Ltd Co.) positioned at the top of the chamber. There was also a heat-absorbing filter that eliminated radiation below 350 nm (Kang *et al.*, 1998). Photosynthetic active radiation (PAR) was measured using a data logger (LI-1000; Li-Cor; http://www.licor.com) and an L1-190SA sensor (Li-Cor). The PAR was adjusted to about 350 μmol PAR m^{-2} sec^{-1} at the top of the plants. Plants were grown with or without supplementary UVB radiation from three UVB bulbs (FL20SE; Toshiba Electric Ltd Co; http://www.toshiba.co.jp.) filtered through a 0.1 mm cellulose diacetate film (Cadillac Plastic Co., Baltimore, MD, USA) [biologically effective UVB radiation (UVB_{BE}) = 20.3 kJ m^{-2} per day] or a UV29 glass filter (Toshiba Glass Co.; http://www.jgc.co.jp) (UVB_{BE} = 42.5 kJ m^{-2} day^{-1}). UVB_{BE} was calculated using the plant action spectrum of Caldwell (1971) normalized to unity at 300 nm. The UVB intensity between 280 and 320 nm was measured with a UVB sensor (MS-210D, Eiko Seiki Co.; http://www.eko.co.jp).

To measure the transcription of the CPD photolyase gene and UV-induced DNA damage and repair, transgenic and WT seedlings were grown for 16 days under visible radiation in a

large growth cabinet until the 4th leaves had expanded fully. These fully expanded 4th leaves were then subjected to various assays.

To analyze the steady-state CPD levels in the 4th leaves of transgenic and WT plants, the plants were grown under visible radiation without supplementary UVB radiation in the large growth cabinet until the 4th leaves had fully expanded. After full expansion of the 4th leaves, some of the potted plants were grown under visible radiation supplemented with UVB radiation (42.5 kJ m^{-2} day^{-1}), while the remaining plants were maintained in unsupplemented visible radiation. Over the next 2 days, the 4th leaves of groups of plants were detached every 3 h during the day (8 AM to 8 PM). The CPD levels in the leaf DNAs were then determined as described below.

Southern blot analysis

For Southern blot analysis, genomic DNA was isolated from each hygromycin-resistant callus (T_0 plant) by the CTAB methods described in detail elsewhere (Teranishi *et al.*, 2004). Aliquots (20 μg) of the isolated genomic DNA were digested with *Eco*RI or *Apa*I, which was then separated on a 0.8% agarose gel. After being transferred to nylon membranes, the DNA blots were hybridized with a ^{32}P-labeled CPD photolyase gene probe, which was prepared by the random priming method (Feinberg and Vogelstein, 1983) using [α-^{32}P]dCTP (110 Tbq/mmol; Amersham Biosciences; http://www5.amershambiosciences.com/). The primers used to amplify the CPD photolyase gene were 5′-CCGTCGATGCTTTCTTGGAG-3′ (forward) and 5′-CCGAGCTCGTGGTATACCACACAAAGAAATG-3′ (reverse), and these produce a 638 bp fragment.

RNA isolation and quantitative real-time RT-PCR analysis

Total RNA was extracted from the 4th leaves by the RNeasy Plant Mini Kit (Qiagen Inc.; www.qiagen.com/), and cDNAs were synthesized using SuperScript II RNase H reverse transcriptase (Invitrogen; http://www.invitrogen.com/) according to the manufacturer's instructions. The reverse transcription PCR primers were 5′-CCGTCGATGCTTTCTTGGAGG-3′ and 5′-CATCTCCAACTGCGAT GCATTCCA-3′, which amplify the coding region of CPD photolyase (nucleotides 935–1159). Constitutively expressed 18S rRNA served as a normalization control (Kim *et al.*, 2003). Quantitative real-time RT-PCR was performed by using the DNA Engine Opticon™ System (MJ Research Inc.; http://www.gmi-inc.com).

Analysis of photolyase activity in leaf extracts

Preparation of protein extracts from rice leaves and all procedures for the treatment of DNA with UV endonuclease and alkaline agarose gel electrophoresis have been described in detail elsewhere (Hidema *et al.*, 2000). The fully expanded 4th leaves (typically 0.08 g FW) were homogenized using a chilled mortar and pestle in 400 μl of 80 mM potassium phosphate buffer, pH 7.2, containing 5 mM EDTA, 2 mM DTT, 0.2 mg ml^{-1} BSA and 10% glycerol. The homogenate was centrifuged for 15 min at 4°C and 20 000 ***g***, and the supernatant was desalted by passage through a Bio-Gel P6DG spin-column (Bio-Rad; http://www.bio-rad.com/). The filtrate was used for *in vitro* assays of photolyase activity. The soluble protein content was determined using the method described by Bradford (1976) with BSA as the standard. λ DNA (50 μg ml^{-1} in 0.1 × TE buffer) irradiated with 10 J m^{-2} of 254 nm radiation, which produces 150 CPD Mb^{-1}, was used as the substrate. The DNA was diluted with an equal volume of 2 × reaction buffer (1 × reaction buffer consists of 40 mM potassium phosphate buffer, pH 7.2, 5 mM EDTA, 2 mM DTT, 0.2 mg ml^{-1} BSA and 80 mM NaCl), and then mixed with the extract at a ratio of substrate solution to extract of 9:1 (v/v). The mixture was incubated in the dark for 15 min at 30°C to facilitate photolyase–CPD complex formation, and then exposed to continuous blue light (four blue fluorescent tubes, 20B-F, at a distance of 20 cm; Toshiba Electric Ltd Co.) for 30 min. CPD photorepair was measured as a function of soluble protein concentration. All manipulations were carried out in dim red lighting to minimize uncontrolled photorepair.

UV endonuclease was partially purified from *Micrococcus luteus* by streptomycin and ammonium sulfate precipitations. The activity was 7×10^{-14} CPD cleaved μl^{-1} h^{-1}.

λ DNA molecules were separated according to their single-strand molecular lengths by alkaline agarose gel (0.7%) electrophoresis using static-field electrophoresis. The molecular length markers were λ DNA (48.5 kb) and a *Hin*dIII digest of λ DNA (23.1, 9.4, 6.6, 4.3 and 2.3 kb).

Analysis of the in vivo *accumulation of UV-induced CPDs by rice seedlings and their photorepair ability*

To analyze the effect of UV radiation on the *in vivo* CPD levels in the lines, the detached fully expanded 4th leaves were placed on wet filter paper and irradiated with UV radiation emitted by a germicidal lamp (Toshiba GL20; Toshiba Electric Ltd Co.) at a distance of 10 cm for 0–20 min. To analyze the CPD photorepair ability of the lines, the detached fully expanded 4th leaves were irradiated with UV radiation for 15 min to induce approximately 50 CPD Mb^{-1} and were harvested immediately. Alternatively, they were exposed to blue irradiation from blue fluorescent tubes (20B-F; Toshiba Electric Ltd Co.). The intensity of blue irradiation was adjusted to about 60 μmol m^{-2} sec^{-1}, which provides saturating light levels for CPD photorepair. After exposure to UV or blue radiation, 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. At least three independent experiments were performed and the data were averaged.

DNA extraction, preparation of agarose plugs, treatment of rice DNA with UV endonuclease, and alkaline gel electrophoresis

All procedures for rice DNA extraction, the preparation of agarose plugs, and the treatment of DNA with UV endonuclease have been previously described in detail (Hidema *et al.*, 2000). Rice DNA molecules were dispersed according to their single-strand molecular lengths by alkaline agarose gel (0.5%) electrophoresis using static-field electrophoresis and biased sinusoidal field gel electrophoresis (Genofield; ATTO Co.; http://www.atto.co.jp) (Hidema and Kumagai, 1998). The molecular length markers were DNA from *Hansenula wingei* chromosomes (smallest 1.05 Mb) (Bio-Rad), T4 (170 kb), λ DNA (48.5 kb), and the *Hin*dIII digest of λ DNA (23.1, 9.4, 6.6, 4.3 and 2.3 kb).

CPD analysis

The CPD frequencies were determined using a DNA damage analysis system constructed by Tohoku Electric Co.; http://www.tei-c.com as previously described in detail (Hidema and Kumagai, 1998). CPD frequencies were calculated using the molecular length

standard curve and the quantity of DNA at each migration position as shown by the quantitative image data (Freeman *et al.*, 1986). The CPD frequencies are expressed in units of CPD Mb^{-1}.

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