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Ultraviolet B-Sensitive Rice Cultivar Deficient in Cyclobutyl Pyrimidine Dimer Repair[1]

Jun Hidema, Tadashi Kumagai, John C. Sutherland, and Betsy M. Sutherland*

Institute of Genetic Ecology, Tohoku University, Katahira, Aoba, Sendai 980, Japan (J.H., T.K.); and Biology Department, Brookhaven National Laboratory, Upton, New York 11973–5000 (J.C.S., B.M.S.)

Repair of cyclobutyl pyrimidine dimers (CPDs) in DNA is essential in most organisms to prevent biological damage by ultraviolet (UV) light. In higher plants tested thus far, UV-sensitive strains had higher initial damage levels or deficient repair of nondimer DNA lesions but normal CPD repair. This suggested that CPDs might not be important for biological lesions. The photosynthetic apparatus has also been proposed as a critical target. We have analyzed CPD induction and repair in the UV-sensitive rice (*Oryza sativa* L.) cultivar Norin 1 and its close relative UV-resistant Sasanishiki using alkaline agarose gel electrophoresis. Norin 1 is deficient in cyclobutyl pyrimidine dimer photoreactivation and excision; thus, UV sensitivity correlates with deficient dimer repair.

UV radiation can damage plants, decreasing biomass and productivity (Teramura, 1983; Teramura et al., 1991; Kumagai and Sato, 1992; Barnes et al., 1993; for a recent review, see Britt, 1996). Plants with a decreased resistance to UV damage may show less productivity under current environmental conditions and could show severe damage under increased UV, e.g. in the case of stratospheric ozone depletion. CPDs that are formed between adjacent pyrimidines on the same DNA strand are a major UV-induced DNA lesion in simple and complex organisms, producing lethality and mutations (Brash et al., 1987) and inducing cancers in humans (Brash et al., 1991; Ziegler et al., 1993, 1994). However, their importance in UV-induced biological damage in higher plants has been questioned. Britt et al. (1993) showed that a UV-sensitive mutant of *Arabidopsis thaliana* had normal CPD repair but defective repair of pyrimidine [6–4] pyrimidones, suggesting that pyrimidine [6–4] pyrimidones rather than CPDs were the biologically important lesion. Li et al. (1993) showed that flavonoid-deficient mutants of Arabidopsis were hypersensitive to UVB, and D'Surney et al. (1993) reported that a UV-sensitive soybean cultivar was deficient in the production of UV-absorbing pigments. Recently, it was suggested that UV radiation damage to the light-harvesting complex of the cyanobacterium *Anabaena* might be approximately 20-fold higher than the calculated dimer induction in DNA (Lao and Glazer, 1996).

Rice (*Oryza sativa* L.) cultivars differ in their responses to UVB radiation, with sensitive strains showing leaf browning, decreased chlorophyll production, and reduced accumulation of biomass (Teramura et al., 1991). Norin 1, which is UV-sensitive, is a grandparent of Sasanishiki, which is UV-tolerant (Kumagai and Sato, 1992). We used these closely related rice cultivars to test the relationship of UV light sensitivity to CPD induction and repair.

MATERIALS AND METHODS

For assessment of UV light sensitivity, plants of two rice cultivars (*Oryza sativa* L. cv Sasanishiki and cv Norin 1) were grown for 35 d in pots in vermiculite:fertilized soil (2:1, v/v) in a phytotron (Tabai Expec Ltd., Osaka, Japan) (12-h photoperiod, day/night temperatures 27/17°C). Plants were grown under metal halide lamps (400 W, MT400DL/BUD, Isawaki Electric, Kyoto, Japan) providing 350 μmol PAR m^{-2} s^{-1}, positioned in the top of the chamber, with heat-absorbing filters (Tabai Expec) below them. Plants were grown without or with supplemental UVB provided by six FL20SE bulbs (Toshiba, Tokyo, Japan) 25 cm apart, located above the plants. Plants receiving UVB were provided with the same photoperiod as under metal halide lamps. Under the UVB bulbs, a UV-29 glass filter (Toshiba Glass, Tokyo, Japan), 30 cm above the plants, reduced 290 nm radiation by 50%. The UVB intensity at the level of the plants was 1.12 W m^{-2} below the UVB bulbs and UV-29 filter and 0.02 W m^{-2} not under the UVB bulbs. The UVB irradiance was measured with a spectroradiometer (SS-25, Japan Spectroscopic, Tokyo, Japan), and the biologically effective UVB (39.5 kJ m^{-2} d^{-1}) was calculated using the plant action spectrum of Caldwell (1971) normalized to unity at 300 nm. UVC was 0.025 times the UVB irradiance.

For damage and repair studies seedlings were grown on a plastic net floating on tap water, pH 5.0 to 5.5, in an environmental chamber (16-h photoperiod, day/night temperatures 28/22°C). Illumination was provided by cool-white fluorescent lamps (Sylvania/GTE, Danvers, MA) filtered by UF4 Plexiglas (Rohm and Haas, Philadelphia, PA), which excludes wavelengths shorter than approximately

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* Corresponding author; e-mail sutherl3@bnl.gov; fax 1–516–344–3407.

Abbreviations: CPD(s), cyclobutyl pyrimidine dimer(s); UPFE, unidirectional pulsed field gel electrophoresis; UVB, 290 to 320 nm; UVC, 220 to 290 nm.

400 nm. The third fully expanded leaves of 12-d-old seedlings were used for irradiation experiments. For each damage or repair study, two or more independent experiments, containing different batches of seedlings, were carried out; for each independent experiment, CPD frequencies were determined at least in duplicate (two- to five-replicate UV endonuclease digestions, gel electrophoreses, electronic imagings, and calculations of the number average molecular lengths, see below).

UV Irradiation and Photoreactivation

Each seedling was centered in a test tube with its roots submerged in water and the shoot protruding vertically. Each leaf was irradiated singly on a revolving turntable with narrow-band UV radiation (302 ± 1.5 nm) from a high-intensity monochromator (Johns and Rauth, 1965a, 1965b) at a rate of approximately 25 W m^{-2}. UV fluxes from the monochromator were measured using a pyroelectric radiometer (PR200, Molectron, Sunnyvale, CA). After UV irradiation seedlings were harvested immediately or after being kept in a dark box or exposed to photoreactivating light from two blue fluorescent lamps (15T8/B, North American Philips lighting, Somerset, NJ), filtered through a UF4 Plexiglas filter. The tips of the plants were approximately 15 cm below the filter, and the intensity of the photoreactivating light, measured with the Molectron radiometer, was approximately 1.5 W m^{-2}. All subsequent manipulations were carried out in dim yellow or red light (General Electric) to minimize uncontrolled photoreactivation of pyrimidine dimers.

DNA Isolation

Rice DNA was isolated by a modification of the methods of Quaite et al. (1994a) and Takayanagi et al. (1994). The top 4 cm of each leaf was sliced in 80 μL of 10 mM Tris-HCl, pH 8.0, containing 0.5 M EDTA, 1% sarcosyl, and 1 mg mL^{-1} proteinase K (Boehringer Mannheim) on a sterile Petri dish with a scalpel, then vacuum-infused for 1 min, and incubated at 45°C for 10 min. The slurry was mixed with an equal volume of 2% low-melting-point agarose (SeaPlaque; FMC, Rockland, ME) and agarose plugs were prepared. The plugs were digested, rinsed with TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA), followed by TE containing 2.5 mM PMSF, then TE buffer again, and finally UV endonuclease buffer (30 mM Tris-HCl, pH 8.0, 40 mM NaCl, and 1 mM EDTA).

Treatment of Rice DNA with UV Endonuclease

UV endonuclease was partially purified from *Micrococcus luteus* by streptomycin and ammonium sulfate precipitations. Its specificity and activity toward pyrimidine dimers and lack of nonspecific cleavage were determined using supercoiled DNA containing and lacking dimers (Sutherland et al., 1992) and by analysis of cleavage using DNA-sequencing gels (Sutherland and Bennett, 1995). Plugs containing rice DNA were digested with UV endonuclease by modifications of the methods of Bennett and Sutherland (1993) and Takayanagi et al. (1994). Plugs were preincubated in UV endonuclease buffer containing 1 mM DTT and 0.1 mg mL^{-1} BSA for 1 h at 4°C, then incubated in 20 μL of UV endonuclease buffer with DTT and BSA containing sufficient endonuclease to yield complete cleavage at all dimer sites (activity, 4×10^{15} CPD cleaved μL^{-1} h^{-1}) for 15 min on ice, and then incubated for 30 min at 37°C. Additional (1 μL) UV endonuclease was added and the plug was incubated for 30 min at 37°C. Duplicate plugs were incubated in the buffer without endonuclease under identical conditions. Reactions were stopped and the DNA was denatured by the addition of alkaline stop mixture (0.5 M NaOH, 50% [v/v] glycerol, and 0.25% [w/v] bromocresol green) and incubation for 30 min at 37°C.

Alkaline UPFE

Rice DNAs were dispersed according to their single-strand molecular lengths by alkaline agarose gel (0.4%, w/v) electrophoresis using UPFE (Sutherland et al., 1987b). Molecular length markers were DNAs from bacteriophages G (750 kb), T4 (170 kb), λ (48.5 kb), *Hin*dIII digest of λ (23.1, 9.4, 6.6, 4.3, and 2.3 kb), T7 (39.9 kb), and a *Bgl*I digest of T7 (22.5, 13.5, and 4 kb). DNAs were first subjected to static-field electrophoresis (typically 5 V cm^{-1}, 40 min) to allow the DNA to enter the gel; the plugs were then removed from the wells, and the DNAs were separated according to single-strand molecular length by electrophoresis for 16 h by alkaline UPFE (typically 15 V cm^{-1}; 0.3-s pulse, 10-s interpulse period; 10°C with buffer recirculation). Gels were neutralized (two 30-min changes of 0.1 M Tris-HCl, pH 8.0), stained with ethidium bromide (1 μg mL^{-1}) for 30 min, and destained (two 30-min changes of H_2O).

Electronic Imaging of Gels and Pyrimidine Dimer Analysis

An image of the distribution of the fluorescence of ethidium bromide to DNA in the destained gels was recorded using an improved version of the electronic imaging system described previously (Sutherland et al., 1987a). CPD frequencies were calculated as previously described (Quaite et al., 1992, 1994a) using the method of moments (Freeman et al., 1986). DNA profiles of sample and length-standard lanes were obtained from the quantitative image data; a dispersion function (molecular length versus migration position) was constructed from the molecular length standards. Using this curve and the quantity of DNA at each migration position from the quantitative image data, we calculated the number average molecular length, L_n, of each DNA distribution from the equation

$$L_n^{-1} = \frac{\int \rho(x) \cdot dx}{\int \frac{\rho(x)}{L(x)} \cdot dx} \quad (1)$$

where $L(x)$ is the length of the DNA molecules that migrated to position x and $\rho(x)$ is the intensity of ethidium fluorescence at position x. From the number average lengths of the DNA populations, the frequency of the lesions is obtained from the equation

$$\phi = L_n^{-1}(+e) - L_n^{-1}(-e), \quad (2)$$

where ϕ is the frequency of cyclobutyl pyrimidine dimers, $L_n(+e)$ is the number average length of the population of molecules treated with the UV endonuclease, and $L_n(-e)$ is the number average length of the population of molecules not treated with the enzyme. DNA lengths, $L(x)$ and L_n, are expressed in units of kilobases or megabases. Thus, ϕ is expressed in units of CPD kb^{-1} or CPD Mb^{-1}.

RESULTS

We determined the effect of UV radiation on plants of the two cultivars grown in a phytotron under visible light alone or with supplemental UVB radiation. Figure 1 shows 35-d-old Norin 1 and Sasanishiki plants grown under these conditions. Supplemental UV light induced little damage in Sasanishiki seedlings in comparison with plants grown under visible light alone. In visible light alone, Norin 1 plants grew as well as Sasanishiki, and under supplemental UV light Norin 1 plants suffered leaf browning and growth inhibition.

If this UV radiation sensitivity results from a decrease in UV-absorbing pigments, UVB light should produce more DNA damage in Norin 1 than in Sasanishiki. We grew seedlings in a UV-free chamber, exposed them to 0 to 10 kJ m^{-2} of 302-nm UVB light, and quantitated CPD levels in the leaves using alkaline agarose gel techniques adapted for plants. UVB light produced similar dimer levels in the two cultivars (Fig. 2), indicating that seedlings grown under these conditions do not show major differences in baseline levels of UV-absorbing pigments. Thus, higher initial DNA damage levels are unlikely to be the major cause of the UV sensitivity of Norin 1.

In most plants CPDs are repaired principally by photoreactivation. In photorepair the enzyme photolyase uses light as energy to monomerize dimers. Plants also repair DNA by excision, with replacement of damaged bases by de novo synthesis using the undamaged complementary strand as a template. Excision of CPD was not detected in *Ginkgo* (Trosko and Mansour, 1969), *Nicotiana* (Trosko and Mansour, 1968), or *Chlamydomonas* (Swinton and Hanawalt, 1973), but low levels of CPD excision were found in carrot protoplasts (Howland, 1975; Eastwood and McLennan, 1985). Degani et al. (1980) found substantial levels of excision but even more rapid photorepair in the water plants *Wolffia microscopica* and *Spirodela polyrhiza*. In wild-type Arabidopsis, Pang and Hays (1991) found that excision was detectable, but photorepair predominated; in the strain transparent testa (which is defective in production of UV-absorbing flavonoids) Britt et al. (1993) found efficient photoreactivation, but excision was not significant in CPD removal.

Since a deficiency in either of these pathways could result in UV sensitivity, we determined the capacity of Norin 1 and Sasanishiki plants to photorepair and excise cyclobutyl pyrimidine dimers. Seedlings were exposed to 302-nm UVB and then to visible light for increasing times. Companion UV-irradiated seedlings were also kept in the dark for measurement of excision repair. CPD levels were determined immediately after UV irradiation and after repair in the light and in the dark. Figure 3A shows that under our conditions Sasanishiki carried out rapid photoreactivation of dimers, removing approximately 75% of the dimers in less than 5 min. However, Norin 1 photoreactivated CPD at an initial rate of only about one-fifth that of

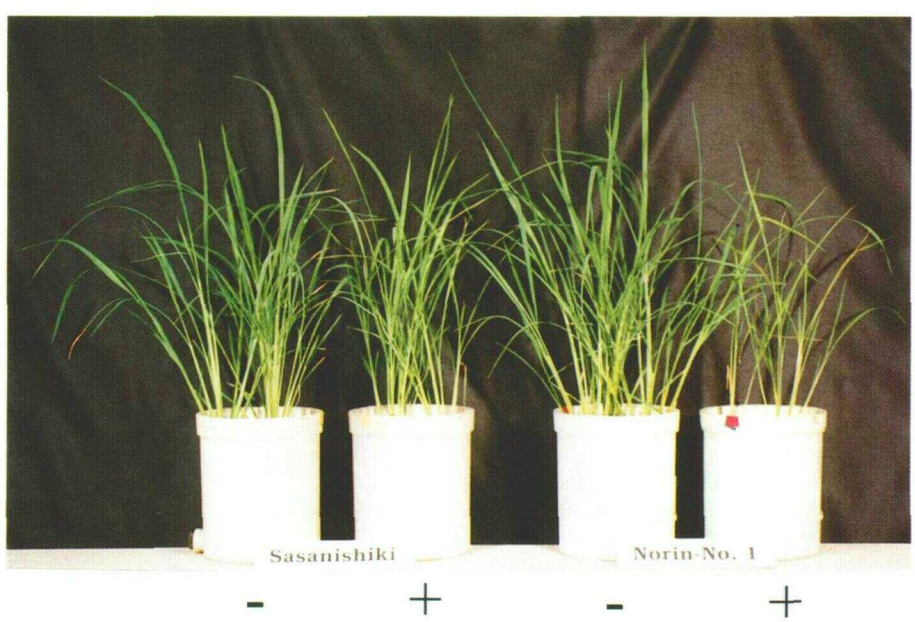

Figure 1. UVB radiation effects on UV-resistant Sasanishiki and UV-sensitive Norin 1 rice (*O. sativa* L.) seedlings. Plants were grown for 35 d in a phytotron (12-h photoperiod, day/night temperatures 27/17°C) under metal halide lamp radiation (400 W, MT400DL/BUD) at 350 μmol PAR m^{-2} s^{-1} without (−) or with (+) supplemental UVB radiation. In the absence of added UV light, the growth of the two cultivars was similar; supplemental UV light decreased growth and induced browning of Norin 1 and had little effect on Sasanishiki.

Sasanishiki. Since photoreactivation is the major CPD repair mechanism in plants, this deficiency could seriously decrease UV light resistance.

Many plants remove UV radiation damage by light-independent excision repair, usually more slowly than by photorepair (Quaite et al., 1994b). Figure 3A shows that no excision was detected in either strain during the time required for substantial photorepair. In both alfalfa (*Medicago sativa* L.) and soybean (*Glycine max* L.), excision is low or undetectable at low initial damage levels. However, at high initial CPD levels, the rate of excision can increase dramatically (Quaite et al., 1994b; Sutherland et al., 1996). We therefore measured excision at initial damage frequencies of approximately 55 CPD Mb^{-1} (compare Fig. 3A, initial frequency approximately 30 CPD Mb^{-1}). Seedlings of UV-resistant Sasanishiki removed dimers by excision at an initial rate of approximately 0.4 CPD Mb^{-1} min^{-1}. However, UV-irradiated Norin 1 seedlings excised dimers at an initial rate of approximately 0.08 CPD Mb^{-1} min^{-1}, about 20% of that of the UV-resistant strain.

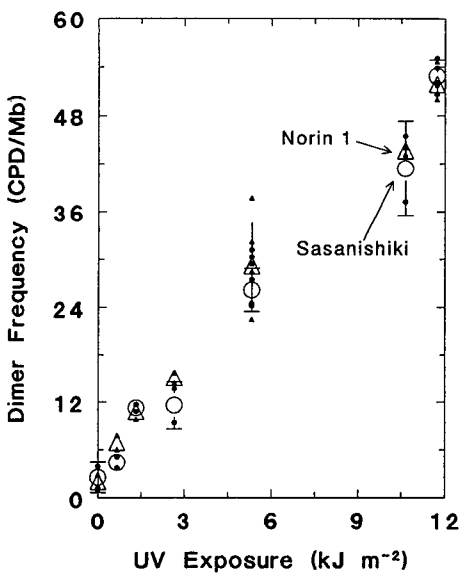

Figure 2. Pyrimidine dimer induction in DNA in rice seedlings. Twelve-day-old seedlings grown in a phytotron (16-h photoperiod, day/night temperatures 28/22°C; cool-white lamp, filtered by UF4 Plexiglas to exclude $\lambda <$ approximately 400 nm), were irradiated singly on a turntable with 302 nm UVB radiation. Manipulations were carried out in dim red light. The third fully expanded leaves were harvested immediately after exposure to UV light, DNA was isolated, and the CPD frequency was determined by alkaline agarose electrophoresis (Sutherland et al., 1987b), quantitative electronic imaging of the gel (Sutherland et al., 1987a), and calculation of the number average molecular lengths (Freeman et al., 1986) (△, Norin 1; ○, Sasanishiki). Data were taken from two independent experiments, using at least replicate determinations of the dimer frequency in each. Small symbols, Individual data; large symbols, averages; error bars, SDs; curves were fitted by eye. The frequencies of dimers per UV light exposure produced in the two strains were indistinguishable.

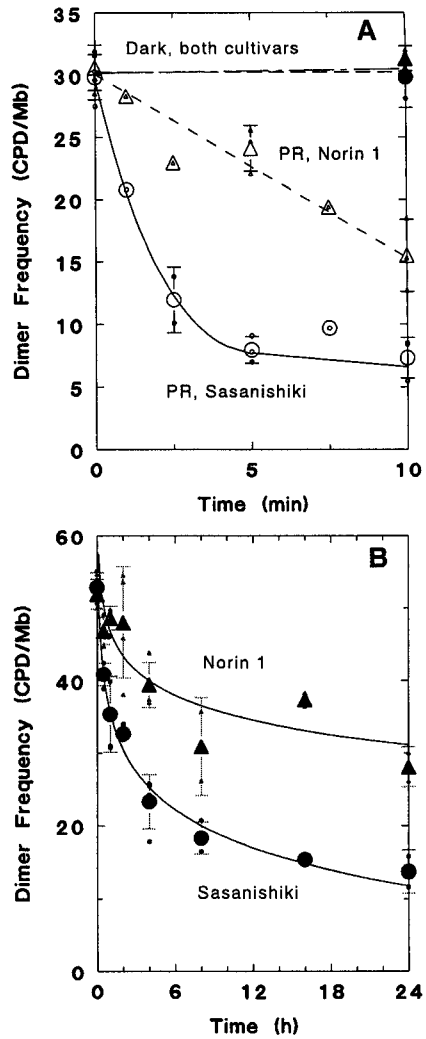

Figure 3. Pyrimidine dimer repair in UV-sensitive Norin 1 and UV-resistant Sasanishiki rice strains. Seedlings exposed to 302 nm UV light were harvested immediately, kept in a dark box (solid symbols), or exposed to two Philips 15T8/B blue fluorescent lamps filtered through UF4 plexiglass (open symbols). Seedlings were harvested at increasing times, their DNA was isolated, and CPD frequencies were measured using at least replicate determinations. A, Dimer frequencies in plants incubated in light: Sasanishiki (○) and Norin 1 (△) seedlings; dimer levels in plants kept in the dark (●, ▲). B, Dimer frequencies in seedlings of Sasanishiki (●) and Norin 1 (▲) incubated in the dark for longer periods than in A (initial CPD level, approximately 55 CPD Mb^{-1}). Small symbols, Individual data; large symbols, averages; error bars, SDs; curves were fitted using a logarithmic function. Compared with Sasanishiki, Norin 1 is deficient in photorepair and in excision repair of pyrimidine dimers.

DISCUSSION

Our data show that Norin 1 is deficient in both photorepair and excision repair compared with Sasanishiki. In repair-proficient plants these paths are complementary: during the day visible light allows plants to remove CPD from their DNA by photorepair. At night residual damage can be removed by excision, which, in higher plants, is generally much less efficient, especially at low dimer fre-

quencies (Quaite et al., 1994b; Sutherland et al., 1996). The low photorepair capacity of Norin 1 would decrease the portion of dimers repaired during daylight hours, whereas its lessened excision efficiency would further decrease the number of lesions removed over the course of the night. The UV sensitivity of Norin 1, thus, likely reflects the dual deficiencies of photorepair and excision repair of pyrimidine dimers.

Indeed, genetic studies indicate that UV light sensitivity of Norin 1 is polygenic (Sato et al., 1994); however, it is not clear whether its repair defects correspond to independent loci. Since the rates of excision and of photorepair in Norin 1 are both about 20% of that of Sasanishiki, it is possible that both of these defects have a common molecular origin, which also might affect the net repair of pyrimidine [6–4] pyrimidones. Other factors, e.g. GA levels, may also affect UV light responses in rice (Suge et al., 1991). Lao and Glazer (1996) detected UV-induced damage to the light-harvesting complex of the cyanobacterium *Anabaena* and suggested that such damage might be a major effect of UV on plants. Currently, however, the biological impact of damage to the photosynthetic apparatus is not known, nor have differences in the sensitivity of plant genotypes to such damage been demonstrated. Thus, the contribution of such damages to UV light sensitivity and resistance in plants remains speculative.

However, in the case of Norin 1, UV sensitivity correlates with the decreased repair of cyclobutyl pyrimidine dimers, a DNA photoproduct known to kill and produce mutations in many organisms. This suggests that DNA damage accounts for a substantial portion of UVB-induced damage, thus implicating damage to DNA rather than to the photosynthetic apparatus. Since UV sensitivity in this cultivar is polygenic, it is important that genetic studies testing the co-segregation of UV sensitivity and DNA repair deficiencies be carried out.

Rice is the world's most important crop for human nutrition (Hseih et al., 1982). Several major Japanese rice cultivars are descendants of Norin 1. Some of these, e.g. Koshihikari, show intermediate UV sensitivity (Sato and Kumagai, 1993). Norin 1 was chosen for breeding because of its low-temperature resistance, ease of hybridization, and the shape, taste, and texture of the kernel. It also conveyed upon some of its progeny a reduced ability to withstand UVB radiation. Most studies of UV light sensitivity in these cultivars have been carried out in phytotrons. However, a 5-year investigation of their field sensitivity is underway, and the results will be critical in assessing potential sensitivity or resistance of these cultivars to current levels of UVB light and increased levels resulting from various alterations of the biosphere. Since photorepair is the major repair path for CPDs in plants, it would be an excellent candidate for directed bioengineering in rice. Cyclobutyl pyrimidine dimer photorepair is a pathway made up of a single enzymatic step, and many photolyase genes have been cloned and expressed in heterologous species.

UV-sensitive cultivars of many other important plant species have been found in field and laboratory tests. In most cases the origin of the sensitivity is unknown, making the design of bioengineering or breeding programs difficult for improving UV light resistance. Determination of the susceptibility of such plants to UV-induced DNA damages and their capacities to repair these lesions should allow complementation of deficiencies to increase UV light resistance to increase crop yields while retaining other desirable traits. Such developments could provide cost-effective increases in the world's food supply for existing environmental conditions and for possible increases in solar UV light.

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