UV Radiation–Sensitive Norin 1 Rice Contains Defective Cyclobutane Pyrimidine Dimer Photolyase

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Norin 1, a progenitor of many economically important Japanese rice strains, is highly sensitive to the damaging effects of UVB radiation (wavelengths 290 to 320 nm). Norin 1 seedlings are deficient in photorepair of cyclobutane pyrimidine dimers. However, the molecular origin of this deficiency was not known and, because rice photolyase genes have not been cloned and sequenced, could not be determined by examining photolyase structural genes or upstream regulatory elements for mutations. We therefore used a photoflash approach, which showed that the deficiency in photorepair in vivo resulted from a functionally altered photolyase. These results were confirmed by studies with extracts, which showed that the Norin 1 photolyase–dimer complex was highly thermolabile relative to the wild-type Sasanishiki photolyase. This deficiency results from a structure/function alteration of photolyase rather than of nonspecific repair, photolytic, or regulatory elements. Thus, the molecular origin of this plant DNA repair deficiency, resulting from a spontaneously occurring mutation to UV radiation sensitivity, is defective photolyase.

INTRODUCTION

UV radiation can damage plants, decreasing growth and productivity (Teramura, 1983). UV radiation–augmentation studies have identified many UV radiation–sensitive cultivars of higher plants that are of economic importance, including rice (Kumagai and Sato, 1992; Bornman and Teramura, 1993; Hidema et al., 1996; Correia et al., 1998), and UV radiation–exclusion studies indicate that prevention of such damage can increase plant growth (Krizek et al., 1997, 1998). UV radiation also induces photodamage in DNA, including damage to the cyclobutane pyrimidine dimer (CPD) and the (6-4)-pyrimidine-pyrimidone photodimer. Such damage can be lethal or mutagenic to simple and complex organisms; it can also impede replication and transcription, a possible mechanism for the adverse effects observed in higher plants.

Photoreactivation is the major pathway in plants for repairing UV radiation–induced DNA damage (reviewed in Britt, 1996, 1999). This one-enzyme repair path is mediated by an enzyme, photolyase, which binds to a dimer to form a complex that is stable in the absence of light. When a photon in the wavelength range of 300 to 600 nm is absorbed (Saito and Werbin, 1969; Pang and Hays, 1991; Takeuchi et al., 1998), the dimer is reversed to two monomer pyrimidines and the enzyme is released. Photorepair of CPDs has been reported in several plant species, including gingko (Trosko and Mansour, 1969), Arabidopsis (Pang and Hays, 1991; Britt et al., 1993), alfalfa (Quaite et al., 1994b), soybean (Sutherland et al., 1996), cucumber (Takeuchi et al., 1996), rice (Hidema et al., 1997), maize (Stapleton and Walbot, 1994; Stapleton, 1997), and wheat (Taylor, 1996).

Increased sensitivity to UV radiation may result from failure to repair photodamage in DNA. The UV radiation–sensitive *uvr1* mutant of Arabidopsis cannot photorepair (6-4)-pyrimidine-pyrimidone photodimers (Britt et al., 1993). Landry et al. (1997) showed that another Arabidopsis UV radiation–sensitive mutant, *uvr2-1*, was deficient in photorepair of CPDs, and cloning and sequencing of the photolyase genes from UV radiation–sensitive and wild-type strains indicated alterations of DNA sequence that led to defective photolyase protein (Ahmad et al., 1997).

These data suggest that such deficiencies might be ameliorated by restoration of photolyase function in photorepair-deficient strains. However, for such restoration, exact knowledge of the molecular origin of their photorepair deficiency is essential. Is the deficiency in the photolyase protein itself, or does it result from some other cause—such as a regulatory mutation (resulting in the production of fewer normal photolyase molecules per cell)? Previous data show that the UV radiation–sensitive rice cultivar Norin 1, a progenitor of many Japanese commercial rice strains (Kumagai and Sato, 1992; Sato and Kumagai, 1993; Hidema et al., 1996), is deficient in photorepair of CPDs (Hidema et al., 1997; Hidema and Kumagai, 1998). These studies did not

address the biochemical nature of the photorepair deficiency. Because the rice photolyase gene has not been cloned, we could not compare the sequences of photolyase genes from the UV radiation–resistant and UV radiation–sensitive cultivars.

However, a powerful approach using photoflash analysis (Harm et al., 1971) allows dissection of photolyase function and structure. We have used this approach in rice plants in vivo and in extracts of the cultivars. The results suggest that the photorepair deficiency in Norin 1 results from an alteration in the structure of the photolyase enzyme rather than from a regulatory mutation. This finding indicates that a strategy of increasing the activity of photolyase, a one-enzyme repair pathway, through selective breeding or engineering could increase resistance to UV radiation and perhaps the productivity of such cultivars.

RESULTS

Norin 1 is highly sensitive to UV radiation–induced damage (Kumagai and Sato, 1992; Hidema et al., 1996). Figure 1 shows the effect of supplementary UVB radiation on the growth of Norin 1 (Figure 1A) and its close relative Sasanishiki (Figure 1C). Furthermore, a comparison of Figures 1B and 1D shows that the UV radiation–sensitive Norin 1 seedlings also are deficient in CPD photorepair, the principal path for repair of UV damage in plants. Over this time scale, nucleotide excision repair is not significant in either strain.

Photoflash Analysis in Rice Seedlings

The photorepair deficiency of Norin 1 could result from either a regulatory mutation (corresponding to fewer photolyase molecules per cell) or an alteration in the photolyase protein itself. Photoflash analysis offers a powerful tool for analysis of photorepair both in vivo and in vitro. Figure 2 shows a scheme for photoflash analysis: UV radiation induces CPDs in DNA. Photolyase molecules bind to CPDs, forming enzyme–substrate complexes that are stable in the absence of light. An intense flash of visible light lasting less than a millisecond photolyses all the productive photolyase–CPD complexes present at the instant of the flash, resulting in repair of each CPD in those complexes and release of the photolyase molecules. Because the flash is very short, each active photolyase acts only once and cannot bind to a second dimer during the flash (Harm et al., 1971). Thus, the number of complexes present at the time of the flash can be determined directly from the number of CPDs repaired. For this analysis, two requirements must be met: first, the level of CPDs present must be sufficiently high to allow all active photolyase molecules to form enzyme–substrate complexes; second, the flash must be of sufficient intensity for
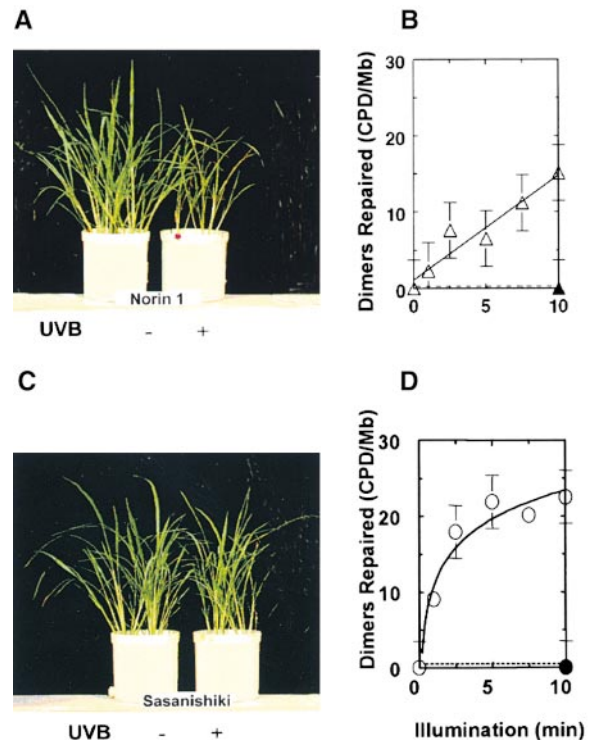
Figure 1. UV Radiation Damage and Photorepair in UV Radiation–Sensitive and –Resistant Rice Cultivars.

(A) UVB radiation induces growth stunting and browning of UV radiation–sensitive Norin 1.
(B) Slow dimer photorepair (open triangles) in Norin 1 seedlings; dimer removal in the dark (filled triangle) was not observed.
(C) UVB radiation has little effect on UV radiation–resistant Sasanishiki seedlings.
**(D)** Rapid dimer photorepair (open circles) in Sasanishiki seedlings; dimer removal in the dark (filled circle) was not observed.
(A) and **(C)** Plants were grown for 35 days in a phytotron under visible light without (−) or with (+) supplemental UVB radiation. **(B)** and **(D)** Seedlings exposed to UV radiation at 302 nm were harvested immediately and kept in a dark box (filled triangle and filled circle) or exposed to blue fluorescent lamps filtered through UF4 plexiglass (open triangles and open circles). Seedlings were harvested at increasing times, their DNA isolated, and CPD frequencies were measured in at least duplicate samples. Lines for photorepair are least-squares fits. Lines fit to the excision data are superimposed on the x axis; the lines shown were displaced slightly upward for clarity. Symbols show averages with error bars; individual determinations are shown without error bars.
Error bars indicate population standard deviations.

photolysis of all complexes present at the instant of the flash.

Using UV radiation–resistant Sasanishiki seedlings, we determined conditions for the initial frequency of dimers and for photoflash intensities necessary to meet these requirements. Figure 3 shows that the magnitude of repair by one
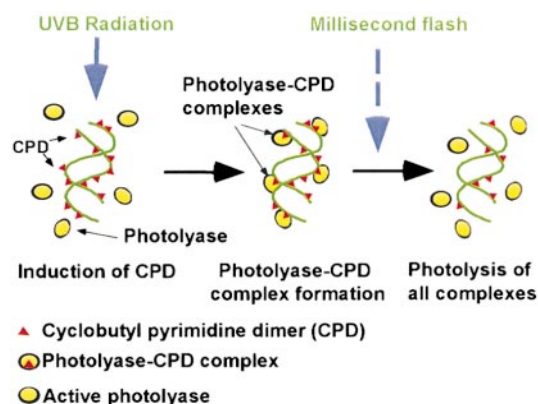
**Figure 2.** Schematic Representation of Photoflash Analysis.

DNA in plants (or in vitro) is irradiated with UV to produce high CPD frequencies. Samples are incubated in the dark to allow formation of photolyase–CPD (enzyme–substrate) complexes. A high-intensity (sufficient for photolysis of all existing enzyme–substrate complexes), sub-millisecond (time short enough that no photolyase can bind to a second CPD) photoflash is administered to the complexes. Because each photolyase–CPD complex repairs one dimer, measurement of the level of CPD photolyzed directly reflects the concentration of active photolyase–CPD complexes at the instant of the flash.

full photoflash depends on initial CPD contents in the seedlings. Dimer frequencies greater than or the same as ∼40 CPD Mb^{-1} in the seedlings support photorepair of a constant level of dimers; that is, these initial dimer frequencies correspond to saturating concentrations of substrate. At such initial dimer concentrations, the number of photorepaired CPDs reflects the concentration of active photolyases in the cell. Using a CPD frequency of 80 CPD Mb^{-1}, ascertained in the previous experiments to be a saturating substrate concentration, we determined the flash intensity sufficient to photolyse all the complexes. We used the Varipower VP-1 module of a photographic flash unit to obtain fractional relative flash intensities and two flash units with a ganged firing system to provide relative flash intensities of 2. The insert in Figure 3 shows that although fractional flash intensities were not adequate to photolyse all photolyase–CPD complexes, either one or two full flashes (intensities of 1 or 2) yielded the same amount of repair (cf. the data in the main portion of Figure 3). The repair of 15 (or 30) CPD Mb^{-1} in the rice genome (haploid, 400 Mb; diploid, 800 Mb) indicates that Sasanishiki seedlings contain ∼24,000 active photolyase molecules per cell.

This approach can be used to distinguish among possible origins for the decreased photorepair in Norin 1. Figure 4 shows two sets of possible photoflash results, corresponding to different origins of photorepair deficiencies in Norin 1. A regulatory mutation could decrease the number of photolyase molecules per cell; in that case, no matter how long

photolyase–CPD complex formation was allowed, there would always be fewer complexes in Norin 1 than in Sasanishiki. Figure 4A shows the results expected for photoflash analysis in such plants with a regulatory mutation in photolyase compared with wild-type plants. However, a major decrease in photolytic efficiency of the enzyme, or a substantial decrease in the stability of the enzyme–substrate complexes at room temperature, could give similar results. A decrease in both CPD binding and complex stability would both change the initial binding rates and decrease the final concentrations of complexes.

If, however, a structural mutation in Norin 1 photolyase decreased enzyme functionality—for example, through decreasing the rate of binding to dimers (but without altering the number of photolyase molecules)—then given sufficient time, the mutant enzyme might form the same number of productive complexes as the wild type. Figure 4B shows the results expected for a structural mutation in photolyase.
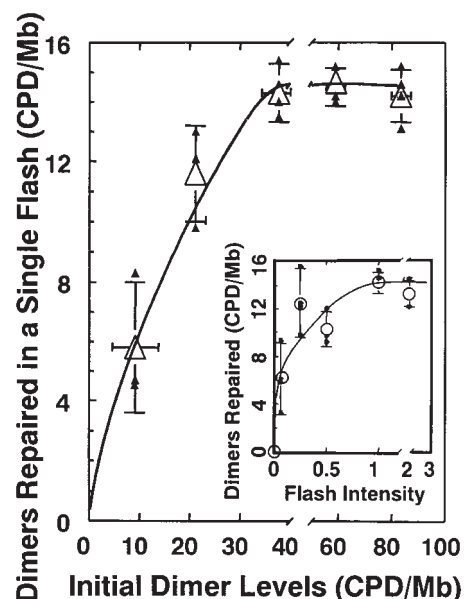
Figure 3. Determination of CPD Levels and Flash Intensities Required for Photoflash Analysis in Rice Seedlings.

Sasanishiki seedlings were irradiated with increasing UVB doses, kept in the dark for 15 min to allow formation of photolyase–CPD complexes, and then exposed to a single, full-intensity photoflash. The level of photorepaired CPDs was measured in at least replicate determinations, and the no-UV radiation (no dimer) values were subtracted from each initial dimer value. The vertical error bars show standard deviations of photorepair obtained in the flash; the horizontal error bars show the measured range of initial net CPD concentrations. The inset shows the amount of photorepaired CPD (initial value, 80 CPD Mb^{-1}) versus the intensity of the photoflash. Lines were fit by least squares (insert) or provided to guide the eye (main graph). Small filled symbols indicate individual data points; large open symbols indicate averages.
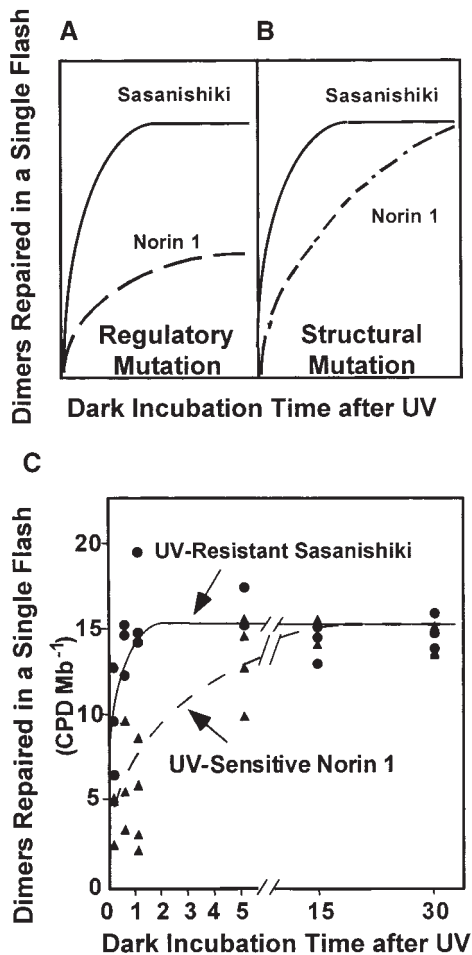
Figure 4. Anticipated and Experimental Time Courses of Association of Photolyases with CPD in Rice Seedlings.

(A) Anticipated kinetics of photolyase–substrate complex formation if Norin 1 contains a regulatory mutation that affects the cellular photolyase content. The final amount of complexes differs between Norin 1 and Sasanishiki.

(B) Anticipated association kinetics if Norin 1 contains a structural mutation in photolyase protein. The rate of association is slower in Norin 1, but the final amount of complexes is similar to that in Sasanishiki.

(C) Experimental photoflash data for photolyase–CPD complex formation in Norin 1 (filled triangles) and Sasanishiki (filled circles). The third leaves of seedlings were exposed to 6 kJ m^{-2} of unfiltered UVB radiation, inducing ~80 CPD Mb^{-1}; then they were kept in darkness until, at various times (shown in minutes) after UVB irradiation, they were exposed to a single full flash. DNAs were isolated, and CPD frequencies were measured in at least replicate determinations. The points indicate individual measurements; lines are provided to guide the eye. The experimental data fulfill the prediction of **(B)** for a structural mutation.

We thus tested the origin of the mutation in photolyase in Norin 1 by monitoring the kinetics of the association of photolyase molecules with CPD to form productive enzyme–substrate complexes. Seedlings were UV irradiated to produce initial concentrations of ~80 CPD Mb^{-1}; then, as a function of time after UV radiation, they were subjected to a single, full-intensity photoflash, harvested immediately, and used to determine dimer levels. Figure 4C shows the results of such an experiment. In Sasanishiki, the complete amount of enzyme–substrate complexes, formed by incubation for ~1 min after UV exposure, corresponds to ~15 Mb^{-1}. In Norin 1, formation of the enzyme–substrate complex is substantially slower, but the eventual content is essentially equal to that in Sasanishiki. The fact that the amount of CPD photolysis in Norin 1 under these conditions equals that in Sasanishiki argues against significant decreases in Norin 1 in photolysis or in stability of the complexes at room temperature. These results thus suggest that the mutation in Norin 1 affects the function of photolyase, manifested as a decreased rate of binding to CPD and thus a slower formation of enzyme–substrate complexes.

Photoflash Analysis of Photolyase–CPD Complex Stability

The results shown in Figure 4C could also result from some cellular factor interfering with enzyme access or binding to dimers in DNA. Such a factor might impede complex formation, but once complexes were formed, it should not affect their stability. Furthermore, if the Norin 1 mutant photolyase had less affinity for CPD (resulting in slower formation of the enzyme–substrate complex, as shown in Figure 4C), the Norin 1 photolyase–CPD complexes might be less stable to higher temperatures than are the Sasanishiki photolyase complexes.

The scheme in Figure 5A shows the use of photoflash to determine the stability of preformed photolyase–CPD complexes. Photolyase is mixed with DNA containing CPDs, and after sufficient time is allowed for all active enzyme molecules to form productive photolyase–CPD complexes, aliquots of the mixture are placed at different temperatures. At various times thereafter, samples containing the enzyme–substrate complex are removed and subjected to one full photoflash to determine the number of active complexes present at that moment. This approach allows determination of the stability of the photolyase–CPD complexes formed by the enzymes from the two cultivars.

These experiments require testing of the photolyase–CPD complexes by determining their stability at different temperatures. However, heating the intact UV-irradiated seedlings could affect DNA isolation or perhaps lead to induction of complicating heat responses. We thus determined whether we could assay photolyase in extracts of the seedlings and whether the deficient photorepair observed in the intact Norin 1 plants was manifested as decreased photolyase ac-

A

▲ Cyclobutyl pyrimidine dimer (CPD)
◐ Photolyase-CPD complex
● Active photolyase
✿ Inactive photolyase

B
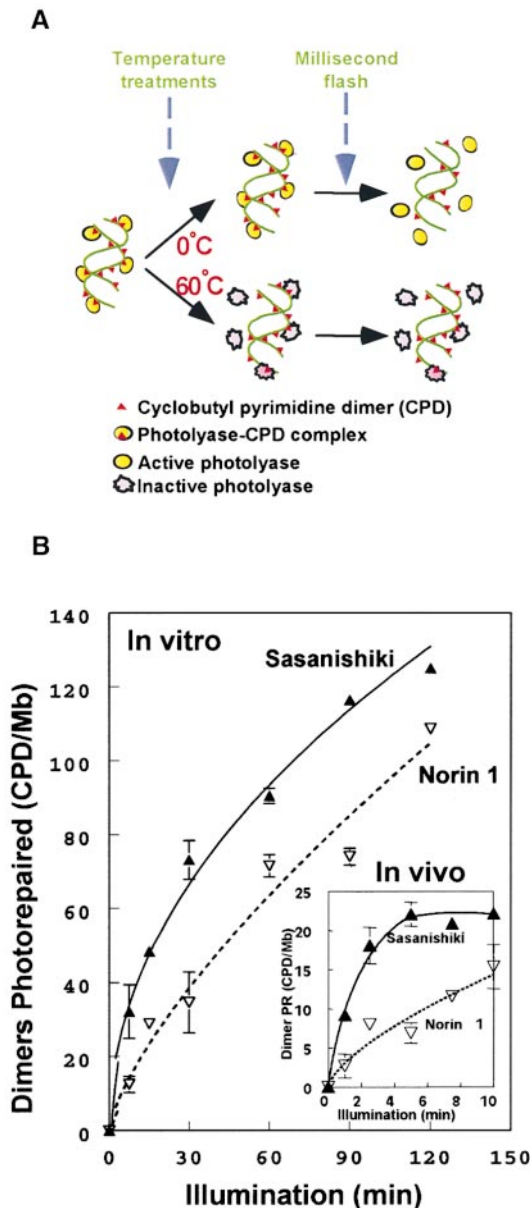
Figure 5. Photoflash Analysis of Thermal Stability of Photolyase–CPD Complexes in Vitro.

(A) Scheme for photoflash analysis of photolyase–CPD complexes. Complexes are incubated at different temperatures and after various times are subjected to a single flash. The level of photorepaired CPDs indicates the level of complexes persisting after incubation at that temperature for the times tested.
(B) Photorepair in rice seedlings in extracts in vitro and in plants in vivo. Extracts of Sasanishiki or Norin 1 were mixed with UV-irradiated λ DNA, incubated for 15 min at 28°C for photolyase–CPD complex formation, and then exposed to continuous illumination; CPD repair was measured as a function of time. Values represent the mean ±SD of three samples for Norin 1 (open triangles) and Sasanishiki (filled triangles). The inset shows CPD photorepair in vivo of both cultivars (data from Figure 1B).

tivity in vitro. Extracts prepared from the two cultivars were adjusted to the same protein concentration, and photolyase activity was determined by using UV-irradiated λ DNA as substrate. Figure 5B compares the photorepair in the extracts with that in the seedlings. These data indicate that not only can photolyase activity readily be determined in the extracts but that the decreased photorepair in Norin 1 seedlings is reflected in the lower amount of photolyase activity in extracts of those seedlings.

We then determined the stability of the photolyase–CPD complexes from the two strains at different temperatures. Extracts were mixed with UV-irradiated λ DNA and incubated in the dark for 15 min at room temperature, which had been determined to be sufficient time for formation of all potential photolyase–CPD complexes (Hidema and Sutherland, unpublished results). Aliquots of these mixtures were then placed at 0, 28, 45, or 60°C; at various times, samples were exposed to a single photoflash and then kept in the dark during UV endonuclease digestion until the addition of alkaline stop mixture. Figures 6A to 6D show the results of such experiments. At 0°C (and in the absence of light), complexes from Norin 1 and Sasanishiki were stable. Thus, photoflashes applied after 30 min of incubation at 0°C showed the presence of enzyme–substrate complexes in numbers approximately equal to those present at the beginning of this incubation (15 min after the mixing of DNA and extract). Similarly, at 28°C, complexes from both strains were stable. However, at 45°C, the Norin 1 photolyase–CPD complex was less stable than that of Sasanishiki photolyase–CPD; incubation of the Norin 1 complexes for 7.5 min under these conditions led to loss of function, perhaps by dissociation, of ∼50% of the productive photolyase–CPD complexes. At 60°C, the complexes of both strains lost activity, but even at this temperature, the Sasanishiki photolyase–CPD complexes were inactivated less rapidly than the Norin 1 complexes.

DISCUSSION

UV radiation can kill and produce mutations in simple and complex organisms, principally by inducing photodamage in DNA. Because reproductive cells and meristematic tissue in many higher plants are well shielded from UV radiation exposure, and DNA in higher plant leaves is not replicated after early developmental stages, the impact of UV radiation on higher plants might be anticipated to be unimportant. However, UV radiation–induced DNA photolesions can also block transcription (Sauerbier and Hercules, 1978; Jordan, 1996), so unrepaired DNA damage in exposed leaves could decrease growth and productivity.

Indeed, studies of UV radiation shielding indicate that many plants show greater productivity in the absence of UV radiation (Krizek et al., 1997, 1998). Because plants in the field are exposed to UV radiation during daily exposure to sunlight, adequate defense against the detrimental effects of UV
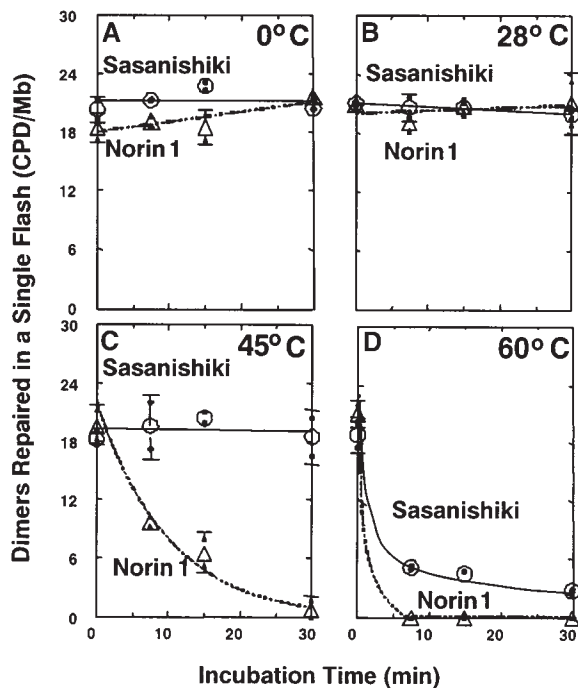
Figure 6. Photoflash Analysis of Thermostability of Complexes Containing CPD and Photolyases from Norin 1 or Sasanishiki.

Extracts of the two cultivars were mixed with λ DNA containing CPD, incubated for 15 min at 28°C for formation of photolyase–CPD complexes, and then underwent different treatments.

(A) Incubation of extracts was shifted to 0°C.
(B) Incubated extracts continued to be incubated at 28°C.
(C) Incubation of extracts was shifted to 45°C.
(D) Incubation of extracts was shifted to 60°C.

At various times, samples were exposed to a single photoflash, and CPD frequencies were measured. The photorepair activity of UV radiation–resistant Sasanishiki (circles) and Norin 1 (triangles) was measured as a function of time after the shift in temperature. Lines in **(A)** to **(C)** are least-squares fits; lines in **(D)** guide the eye. Error bars indicate SD.

radiation is essential. Photoreactivation of UV radiation–induced DNA damage is the major repair path in higher plants, dealing with CPD at all damage levels (Quaite et al., 1994a, 1994b; Britt, 1996; Sutherland et al., 1996; Hidema et al., 1997), whereas nucleotide excision repair apparently is important principally at greater damage levels (Quaite et al., 1994b; Sutherland et al., 1996; Hidema et al., 1999).

In UVB radiation–augmentation tests, Norin 1, a progenitor of many commercially important rice strains, was found to be sensitive to UV radiation–induced damage (Kumagai and Sato, 1992; Hidema et al., 1996, 1997; Hidema and Kumagai, 1998). Furthermore, supplementation of Norin 1 plants with visible light was less effective in ameliorating UVB radiation–induced damage than it was in other cultivars (Kumagai and Sato, 1992). Given that (among its many ef-

fects on plants) visible light is highly effective as a light source for photoreactivation, these data suggested that the UV radiation sensitivity of Norin 1 might stem from a deficiency in photorepair. Previous results indicated that Norin 1 is indeed deficient in photorepair of CPDs (Hidema et al., 1997) and that leaves at different developmental stages all show this deficiency (Hidema and Kumagai, 1998). However, these data did not distinguish between regulatory mutations affecting the number of photolyase molecules per cell and structural mutations altering the functional integrity of the photolyase. Because the rice photolyase gene has not been cloned, we could not compare the sequence of the corresponding genes of the two cultivars to look for mutations. However, photoflash analysis (Harm et al., 1971) provides a powerful tool for analysis of the identity and origin of repair deficiencies, even in the absence of cloning and sequencing of the photolyase genes.

Norin 1 Photorepair Deficiency Results from a Structural/Functional Mutation in Photolyase

Study of the kinetics of association of photolyase and CPDs in seedlings shows that the rate of formation of productive photolyase–CPD complexes is slower in the UV radiation–sensitive Norin 1 than in the UV radiation–resistant Sasanishiki (see Figure 4C). However, the final concentration of complexes is similar in both strains. Furthermore, because the number of complexes is determined from the number of CPDs repaired in a single flash, this implies that the photolytic efficiency of the complexes is similar in both strains. It is unlikely that an altered cofactor of photolyase is responsible for the decreased photorepair. Because cofactors of photolyases are frequently important in general cellular metabolism, a mutation in such a molecule would be expected to affect plant growth adversely. However, in the absence of supplemental UV radiation, growth of Norin 1 appears similar to that of Sasanishiki. Further, as mentioned above, photolyase–DNA complexes in seedlings of Norin 1 and Sasanishiki have equal photolytic efficiency (Figure 4C). A postulated altered cofactor would have to alter binding without changing the photolytic efficiency, which seems unlikely. Thus, the data suggest that the photorepair deficiency in Norin 1 seedlings results from a structural/functional alteration of the enzyme, specifically in the binding of the photolyase to CPD in DNA; the data argue against other possibilities, such as an altered cofactor, an alteration in photolytic efficiency, or a mutation in a regulatory gene that would affect the quantity of cellular photolyase present.

Norin 1 Photolyase–CPD Complexes Show Decreased Thermostability

Because the decreased rate of binding could reflect the presence of some interfering or competing factor in Norin 1,

we allowed enzymes in extracts of the two cultivars sufficient time to form complexes then determined their thermostability. Photoflash analysis of complexes formed between photolyases in extracts of Norin 1 or Sasanishiki with CPDs in λ DNA in vitro allows determination of the stability of the complexes at increasing temperatures. Figures 6C and 6D clearly show the decreased stability of the complexes formed by the Norin 1 photolyase and CPD in DNA, indicating inactivation of the enzyme or decreased affinity of the enzyme for its substrate.

UV radiation–sensitive mutants derived by ethyl methanesulfonate mutagenesis of wild-type Arabidopsis have been shown to be defective in photorepair of (6-4)-pyrimidine-pyrimidone adducts (uvr1) (Britt et al., 1993) or CPDs (uvr2-1) (Landry et al., 1997). In the latter case, the alleles from the wild-type and mutant *PHR1* genes have been sequenced, and marked differences between the wild-type and mutant sequences indicate alterations of the photolyase apoprotein (Ahmad et al., 1997). UV radiation hypersensitivity in higher plants is not necessarily associated with deficiencies in photolyases, however; *uvh1* mutants of Arabidopsis have normal photorepair and are sensitive to other damaging agents as well (Harlow et al., 1994). Furthermore, some UV radiation–sensitive soybean cultivars may lack UV radiation protective pigments (D'Surney et al., 1993).

Many cultivars of a wide variety of plant species, including economically important crop plants, have been shown to have higher UV radiation sensitivity than do related cultivars (Teramura, 1983; Bornman and Teramura, 1993). Furthermore, UV radiation exclusion studies indicate that the UV component of sunlight may decrease growth and productivity in some crop plants (Krizek et al., 1997, 1998). Given the exposure of plants to UV radiation along with the other wavelengths in sunlight, this result suggests that plant productivity could be limited by the ability to shield against or to repair UV radiation damage successfully.

Determining the origin of UV radiation sensitivity in specific plant cultivars should provide a rational basis for increasing their ability to resist UV radiation damage. In higher plants, photorepair is the major mechanism for dealing with UV radiation–induced damage to DNA. Thus, if UV radiation sensitivity in a plant results from photorepair deficiency, restoration of the ability to perform dimer photoreactivation should increase UV radiation resistance. Because photorepair is mediated by a one-enzyme repair path, it makes an excellent candidate for restoration or augmentation by way of selective breeding or engineering of the responsible enzyme, photolyase. Photoflash analysis provides a rapid and powerful method for identifying deficiencies in photorepair as well as determining the functional origin of such deficiencies. Along with advances in genomics of higher plants, for example, sequencing of genomes, including that of rice, these approaches to improving the ability to repair UV radiation–induced damage could allow substantial increases in productivity of economically important crop plants.

METHODS

Plant Materials and Growth Conditions

For assessment of UV radiation sensitivity, plants of two rice cultivars (*Oryza sativa* cv Sasanishiki and cv Norin 1) were grown for 35 days in pots of vermiculite/fertilized soil (2:1 [v/v]) in a phytotron (Tabai Expec Ltd., Osaka, Japan), with a 12-hr-day/12-hr-night photoperiod, with temperatures at 27 and 17°C, respectively. Plants were grown under 400-W metal halide lamps (model MT400DL/BUD; Iwasaki Electric, Kyoto, Japan), which provided 350 μmol PAR m^{-2} sec^{-1}. Plants were grown without or with supplemental radiation from six UVB bulbs (model FL20SE; Toshiba, Tokyo, Japan) filtered by UV-29 glass (Toshiba Glass) 30 cm above the plants. This filter reduced 290-nm radiation by 50% (Kang et al., 1998). The UVB radiation intensity at plant level was 1.12 W m^{-2}, as measured with a spectroradiometer (model SS-25; Japan Spectroscopic, Tokyo, Japan), and the biologically effective UVB radiation (39.5 kJ m^{-2} day^{-1}) was calculated using the plant action spectrum of Caldwell (1971) normalized to unity at 300 nm.

For repair studies, seedlings were grown on a plastic net floating on tap water, pH 5.0 to 5.5, in an environmental chamber (16-hr-day/8-hr-night photoperiod, with temperatures 28 and 22°C, respectively). Illumination was provided by cool white fluorescent lamps (Sylvania/GTE, Danvers, MA) filtered by UF4 Plexiglas (Rohm and Haas, Philadelphia, PA) that excludes wavelengths <400 nm (Takayanagi et al., 1994). The third fully expanded leaves of 12-day-old seedlings were used for all experiments.

Preparation of Protein Extracts

Six leaves of 12-day-old seedlings (typically 0.08 g fresh weight) were homogenized in 400 μL of 40 mM potassium phosphate buffer, pH 7.2, containing 5 mM EDTA, 2 mM DTT, 0.2 mg mL^{-1} BSA, and 25% glycerol by using a chilled mortar and pestle. The homogenate was centrifuged for 15 min at 4°C and 27,000g, and the supernatant was used as cell extracts for assays of photolyase in vitro. Extracts were stable for 1 week at 4°C and for at least 2 weeks at −20°C. Protein concentrations were determined by the method of Lowry et al. (1951).

Assay of Photolyase in Vitro

DNA (50 μg mL^{-1} in 0.1 × TE buffer [1 × TE is 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA]) was irradiated with 10 J m^{-2} of 254-nm radiation, producing 150 cyclobutane pyrimidine dimers (CPDs) per megabase. DNA was diluted with an equal volume of 2 × reaction buffer (1 × reaction buffer is 40 mM potassium phosphate buffer, pH 7.2, 5 mM EDTA, 2 mM DTT, 0.2 mg mL^{-1} BSA, and 80 mM NaCl) and then mixed with extract at the ratio of substrate solution/extract of 9:1 (v/v). All manipulations were performed in dim red light to minimize uncontrolled photorepair. The reaction mixture was incubated in the dark for 15 min at room temperature (28°C), which had been ascertained to allow complete formation of photolyase–CPD complexes in extracts from both strains (19.7 ± 1.7 complexes Mb^{-1} for Sasanishiki and 19.7 ± 1.6 for Norin 1; Hidema and Sutherland, unpublished results). In experiments determining photolyase–CPD association, a flash was applied at various times after mixing. Aliquots

(15 μL) were placed in 0.2-mL polypropylene tubes and either incubated at 28°C in the dark, with or without exposure to flash illumination, or exposed to continuous illumination.

For continuous illumination, samples were incubated in a 28°C water bath in the presence of photoreactivating light (four 150-W Sylvania spot lamps at a distance of 20 cm). For photoflash experiments, enzyme–substrate complexes were incubated at 28°C, then exposed to fractional, single full flashes or to multiple flashes of 33-μsec duration from a photographic flash (Vivitar, Santa Monica, CA) at a 3-cm distance, filtered though UF4 Plexiglas. Fractional flash intensities were obtained using a VP-1 Varipower (Vivitar) module attached to the flash unit.

The levels of pyrimidine dimers were determined by adding to each reaction mixture 15 μL of 2 × UV endonuclease buffer (1 × UV endonuclease buffer is 30 mM Tris-HCl, pH 7.5, 40 mM NaCl, and 1 mM EDTA) and dividing the sample into two parts. To one part, 1 μL of *Micrococcus luteus* UV endonuclease (Carrier and Setlow, 1970) was added at sufficient concentrations (activity, 4×10^{15} CPDs cleaved μL^{-1} hr^{-1}) to cleave the dimers quantitatively at CPD sites; this mixture was incubated at 37°C for 1 hr. The remaining part was incubated without UV endonuclease. DNAs were then denatured by adding alkaline stop mixture (0.5 M NaOH, 25% glycerol, and 0.25% [w/v] bromocresol green) and incubating for 30 min at 37°C.

An agarose gel (0.7%) was prepared in 1 mM EDTA plus 50 mM NaCl and equilibrated with alkaline electrophoresis solution (2 mM EDTA and 30 mM NaOH) for 30 min. Sample DNAs and molecular length markers (λ DNA, 48.5 kb; HindIII digest of λ DNA, 23.1, 9.4, 6.6, 4.3, and 2.3 kb) were subjected to static-field electrophoresis (typically 2.1 V cm^{-1}) for 3 hr. Gels were neutralized with two 30-min changes of 0.1 M Tris-HCl, pH 7.5, stained with ethidium bromide (1 μg mL^{-1}) for 30 min, and destained with two 30-min changes of water.

Electronic Imaging of Gels and Pyrimidine Dimer Analysis

An image of the fluorescence of ethidium bromide bound to DNA was recorded by using an improved version of an electronic imaging system described previously (Sutherland et al., 1987a). CPD frequencies were calculated as previously described (Quaite et al., 1992), using the method of moments (Freeman et al., 1986): DNA profiles and the length-standard lanes were obtained from the quantitative image data, and coefficients of a dispersion function (molecular length versus migration position) were constructed from the observed migrations of the molecular length standards. Using this curve and the quantity of DNA at each migration position from the quantitative image data, the number average molecular length, L_n, of each DNA distribution was calculated from Equation 1

$$L_n = \frac{\int f(x)\, dx}{\int \frac{f(x)}{L(x)}\, dx} \qquad (1)$$

where $L(x)$ is the length of the DNA molecules that migrated to position x and $f(x)$ is the intensity of ethidium fluorescence at position x. From the number average lengths of the DNA populations, the frequency of lesions was obtained from Equation 2

$$\Phi = L_n^{-1}(+T) - L_n^{-1}(-T) \qquad (2)$$

where Φ is the frequency of CPDs, and $L_n(+T)$ and $L_n(-T)$ are the number average lengths of the populations of molecules treated with or without the UV endonuclease, respectively. Because the DNA

lengths, $L(x)$ and L_n, are expressed in units of kb or Mb, Φ is in units of CPD kb^{-1} or CPD Mb^{-1}, respectively.

Assay of Photoreactivation in Rice Seedlings in Vivo

For assaying photoreactivation in vivo under continuous light, each third leaf was irradiated singly with narrow-band UV radiation (302 ± 1.5 nm) from a high-intensity monochromator (Johns and Rauth, 1965a, 1965b) at ~25 W m^{-2}, as measured by a pyroelectric radiometer (model PR200; Molectron, Sunnyvale, CA). After UV irradiation, seedlings were harvested either immediately, or after having been kept in a dark box, or after exposure to light from two blue fluorescent lamps (model 15T8/B; North American Philips Lighting, Somerset, NJ) filtered through UF4 Plexiglas. The tips of the plants were ~15 cm below the filter; the intensity of the light, measured with the Molectron radiometer, was ~1.5 W m^{-2}.

For assaying photoreactivation by a single flash in seedlings, the third leaves of seedlings were exposed to 6 kJ m^{-2} of unfiltered UVB radiation (Westinghouse FS20) to induce ~80 CPD Mb^{-1}. Radiation was monitored with a Jagger meter (Jagger, 1961) calibrated against a YSI radiometer (Yellow Springs Instruments, Yellow Springs, OH). Immediately after UVB irradiation, seedlings were placed in a light-tight box, and at various times after UV irradiation, they were exposed to a single flash of visible light as described above. The leaves were immediately placed in aluminum foil envelopes and stored in liquid nitrogen. All subsequent manipulations were performed in dim red light to minimize uncontrolled photoreactivation.

DNA Extraction and Preparation of Agarose Plugs

Rice DNA was isolated by a method described in detail elsewhere (Bennett et al., 2000). In brief, three liquid nitrogen–frozen leaves were ground to a fine powder with a mortar and pestle chilled in liquid nitrogen. A drop of lysis buffer A (10 mM Tris-HCl, pH 8.0, 0.83 M EDTA, 2% sarcosyl, 13% mannitol, and 1 mg mL^{-1} proteinase K [Boehringer Mannheim, Indianapolis, IN]) was placed in a plastic dish, the rice leaf powder was transferred to the buffer, and the mixture was vacuum-infused for 1 min. The vacuum-infused plant slurry was mixed with an equal volume of 95°C stock agarose solution (2% low-melting-point agarose [SeaPlaque; FMC, Rockland, ME] in TE) and formed into plugs. The agarose plugs were incubated in buffer B (10 mM Tris-HCl, pH 7.5, 0.5 M EDTA, 1% sarcosyl, and 1 mg mL^{-1} proteinase K) at 45°C for at least 72 hr, with daily changes of buffer B. After rinsing the plugs with TE, they were incubated with TE containing 2.5 mM phenylmethylsulfonyl fluoride at 45°C for 30 min, then TE again, and finally UV endonuclease buffer.

Pyrimidine Dimer Quantification in Rice DNA

Plugs containing rice DNA were incubated for 15 min on ice in 20 μL of UV endonuclease buffer with 1 mM DTT and 0.1 mg mL^{-1} BSA containing sufficient endonuclease to yield complete cleavage at all dimer sites (activity, 4×10^{15} CPDs cleaved μL^{-1} hr^{-1}) and then incubated for another 30 min at 37°C. Additional UV endonuclease (1 μL) was added, and the plugs were incubated for 30 min at 37°C. Companion plugs were incubated in the buffer without endonuclease under identical conditions; two or more sets of plus and minus endonuclease plugs were used for each determination. Reactions

were stopped and the DNA was denatured by adding the alkaline stop mixture and incubating for 30 min at 37°C.

Sample DNAs and molecular length markers—*Hansenula wingei* (Bio-Rad) chromosomes (smallest at 1.05 Mb), T4 (170 kb), λ (48.5 kb), BglI digest of T7 (22.5, 13.5, and 4 kb), and HindIII digest of λ DNA (23.1, 9.4, 6.6, 4.3, and 2.3 kb)—were first subjected to static-field electrophoresis (typically 3.8 V cm^{-1} for 40 min) to allow the DNA to enter the gel. The plugs containing sample DNAs were then removed from the wells, and the DNAs were separated according to single-strand molecular length by using alkaline unidirectional pulsed-field gel electrophoresis (typically 13 V cm^{-1}; 0.3-sec pulse, with a 10-sec interpulse period; 10°C with buffer recirculation) for 13.5 hr (Sutherland et al., 1987b; Quaite et al., 1994a). Gels were neutralized, stained with ethidium bromide for 30 min, destained, and imaged, and the CPD frequencies were determined as described above.

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