Temperature-Sensitive Photoreactivation of Cyclobutane Thymine Dimer in Soybean

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UV radiation induces the formation of two classes of photoproducts in DNA, the cyclobutane pyrimidine dimer (CPD) and the pyrimidine 6-4 pyrimidone photoproduct. CPDs in plants are repaired by class II CPD photolyase via a UV-A/blue light-dependent mechanism. The genes for the class II CPD photolyase have been cloned from higher plants such as Arabidopsis, Cucumis sativus (cucumber), Oryza sativa (rice) and Spinacia oleracea (spinach). Flavin adenine dinucleotide (FAD) has been identified as a cofactor. Here we report the isolation and characterization of the CPD photolyase cDNA from soybean (Glycin max). The sequence of amino acids predicted from the cDNA sequence was highly homologous to sequences of higher plant class II CPD photolyases. When the cDNA was expressed in a photolyase-deficient Escherichia coli, photoreactivation activity was partially restored by illumination with a fluorescent light. The purified enzyme showed CPD binding and light-dependent photoreactivation activities in vitro. When soybean CPD photolyase was heat-treated in vitro from 25°C to 45°C for 3 min, thymine dimer-binding activity and photoreactivation activity were decreased, and FAD was released from the enzyme. On the other hand, when the enzyme-CPD complex was heat-treated, photoreactivation activity was stable. We argue that FAD in the soybean CPD photolyase is labile for temperature, but once the enzyme-CPD complex has formed, FAD becomes tightly bound to the enzyme or complex.

INTRODUCTION

The predicted reduction in the stratospheric ozone layer will result in increased levels of ultraviolet-B (UV-B) radiation (280–320 nm) reaching the Earth’s surface.1) The rising concentration of greenhouse gases may also result in an increase in surface temperature and global climate change. UV-B radiation has a number of deleterious effects on physiological processes in plants such as the inhibition of photosynthesis,2) growth,3) and crop yields.4,5) Direct absorption of UV-B causes DNA damage such as the cyclobutane pyrimidine dimer (CPD) and pyrimidine pyrimidone 6-4 photoproducts (6-4 adducts) which are known to kill cells by blocking DNA replication and transcription,6) and if damaged DNA is replicated, this may lead to mutagenic effects.7,8)

Higher plants have evolved to counteract the deleterious effects of UV-B on cellular DNA. One of the most widely distributed repair enzymes is photolyase, which utilizes UV-A/blue light to monomerize CPD9,10) or 6-4 adducts:10,11) the former reaction is mediated by CPD photolyase and the latter 6-4 adduct photolyase. CPD photolyases are categorized into class I and class II according to their amino acid sequences.12) Class I photolyases are mostly found in bacteria and archea, whereas class II photolyases are found in some bacteria, archea, plants, green algae, insects, and vertebrates.12–17) Both class I and class II photolyases generally have reduced flavin adenine dinucleotide (FAD) as a chromophore,11,18) which is excited by UV-A/blue light energy and monomerizes the CPDs in an electron transfer reaction.19) It is known that photolyase unbound to FAD cannot bind CPD.20)

Plants are inevitably exposed to solar UV radiation, because they are dependent on solar energy for photosynthesis. Genes for CPD photolyase have been cloned and characterized from Arabidopsis thaliana,14) cucumber,21) rice,22) and spinach.23) The predicted amino acid sequences from plant photolyases show strong sequence similarity to class II photolyases. In higher plants, the photoreactivation is the
major DNA repair pathway in non-proliferating cells. A mutant of Arabidopsis lacking this gene exhibits hypersensitivity to UV radiation. We have previously demonstrated that three rice cultivars, UV-resistant Sasanishiki, UV-sensitive Norin 1, and UV-more sensitive Surjamkhi, differed in sensitivity to UV-B, and the differences were due to differences in the amino acid sequences of their CPD photolyases. For example, Sasanishiki has Gln at 126 and Gln at 296, Norin 1 has Arg at 126 and Gln at 296, and Surjamkhi has Arg at 126 and His at 296. We then argued that the alterations of Gln126Arg may lead to a defect in binding to a second chromophore, and Gln296His may lead to a defect in binding to FAD. It should also be mentioned that CPD photolyase activities have previously been reported to be temperature sensitive in Arabidopsis, cucumber, and rice. A reduced capability for photoreactivation of CPDs could have severe repercussions for crop productivity especially as increases in levels of UV-B reaching the Earth’s surface may coincide with elevated surface temperatures due to rising concentrations of greenhouse gases.

In this study, we report the cloning and characterization of a CPD photolyase gene from soybean, which is major agricultural crop, in the context of these anticipated changes in environmental conditions. The isolated cDNA expresses an active CPD photolyase in E. coli. The soybean CPD photolyase purified from E. coli exhibited thermostable binding and repair activities toward a DNA substrate containing the thymine dimer.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media.

The E. coli strains NKJ3002 (phr uvrA recA) and KY20 (phr) were used as hosts for the cloning of the soybean photolyase gene, overexpression and purification of a GST-photolyase fusion protein, and complementation of the soybean photolyase gene in E. coli. The plasmid pGEX-4T-2 (Amersham Biosciences) was used for the glutathione-S-transferase (GST)-fused constructs. The plasmid pKY1 carries E. coli phr gene.

Luria (L) broth, L agar, and 0.067 M phosphate buffer were prepared as described previously. Ampicillin (50 μg/ml) was included if necessary in the L broth and L agar.

Soybean CPD photolyase gene

A cDNA library of soybean (purchased from Life Technology) was screened using oligonucleotide primers derived from the highly conserved amino acid sequence of Arabidopsis thaliana, and Chlamydomonas class II CPD photolyases; AC2, 5'-GTIGAYGCICAYAYGTIGTICC-3' and AC4R, 5'-CATRAAICCRTGCATYTTICC-3', which gave a 641-bp fragment after PCR amplification. Using a dilution amplification method and the 641-bp fragment as a marker of amplification, we obtained a clone of the soybean CPD photolyase gene with a 1698-bp insert containing a 1503-bp ORF coding for 500 amino acid residues (DDBJ/EMBL/GenBank databases under accession No. AB359233). Details on the screening of the library have been described previously.

Purification of the GST-soybean CPD photolyase fusion protein

cDNA of the cloned CPD photolyase gene in pBlueScript SK(--) was amplified using two primers annealing near the putative start and stop codons. These primers introduced new BamHI and EcoRI restriction sites. The resulting 1.5-kb DNA fragment was digested with BamHI and EcoRI, and cloned into the BamHI/EcoRI sites of pGEX-4T-2 to obtain the plasmid pGSTSbCPD. Purification of soybean CPD photolyase was performed as described previously. All operations were carried out at 4°C or on ice. A portion of the protein sample was subjected to SDS-PAGE, and the purity and concentration were estimated with an Image-analyzer and the Bradford method, respectively.

Photoreactivation effect of soybean CPD photolyases in E. coli

NKJ3002 (phr uvrA recA) cells were transformed with pGSTSbCPD, and the transformants were grown to log phase in L broth containing ampicillin. NKJ3002 samples of 5.0 ml were irradiated in 90-mm diameter petri dishes with UV light (254 nm) provided by a germicidal lamp, illuminated with a fluorescent lamp for 30 min as described previously, plated on L agar and incubated at 37°C overnight. The UV fluence rate was 0.025 J/m²/s.

DNA substrate

Oligonucleotides (30 mer) containing a centrally located thymine dimer were used as a substrate. The sequence of the substrate is 5’-CACGTACGCATCCTTTCTGACGACATCAGTCC-3’ (the two T residues that make up the cyclobutane thymine dimer are underlined). Thirty pmol of oligonucleotide containing the thymine dimer was 5’-end-labeled with [γ-32P]ATP and T4 polynucleotide kinase (TaKaRa), then annealed with 50 pmol of complementary oligomer in annealing buffer (10 mM Tris-HCl, pH 8.3, and 10 mM MgCl₂) by heating at 95°C for 3 min and cooling to 30°C over a 30-min period. The duplex DNA was ethanol precipitated and resuspended in deionized distilled water.

Band shift assay and in vitro photoreactivation assay

DNA 30 mer substrates which have a single thymine dimer as mentioned above were used in the band shift assay and the photoreactivation assay in vitro. Total volume in reaction mixture was 10 μl. For the band shift assay, 0 to 12 pmol of partially-purified GST-fused protein was added to 150 fmol of the 32P-labeled DNA substrates in a reaction buffer containing 50 mM Tris-HCl pH 7.4, 50 mM NaCl, 1
repair assay, we took advantage of the fact that the thymine dimer can be specifically cleaved by T4 UV endonuclease V (T4 endV), and removal of the thymine dimer by soybean photolyase can be used to measure photolyase enzymatic activity. In this assay, 150 fmol of the 30-mer substrates mentioned above was mixed with 2 pmol of partially purified GST-photolyase in a reaction buffer containing 50 mM Tris-HCl pH 7.4, 50 mM NaCl, 1 mM EDTA, and 1 mM DTT. The reaction mixture was either kept in the dark or exposed to a fluorescent lamp at room temperature for 10 and 30 min. After treatment with 2 μg of proteinase K, the DNA was extracted with phenol/chloroform, precipitated with ethanol and resuspended in T4 endV buffer (2.5 mM NaPO₄, pH 6.8, 0.1 mM EDTA, 10 mM NaCl, 0.1 mM DTT, and 0.01 mg/ml BSA), and digested with 2 units of T4 endV (purchased from TREVIGEN, INC.) for 90 min at 37°C. The reaction products were denatured by heating at 95°C for 3 min then electrophoresed on an acrylamide gel containing 7 M urea. The levels of band shifting and of repair were determined from the intensity of the radiolabeled oligonucleotides using a BAS 2000 imaging analyzer (Fuji Film).

Spectroscopic analysis
The absorption spectra were recorded with a ND-1000 spectrophotometer (NanoDrop Technologies). For the spectroscopic observation of free chromophore, the chromophore was released by boiling the enzyme for 3 min with SDS, and the precipitate was removed by centrifugation and the supernatant was used for measuring the absorption spectrum. For measuring the thermosensitivity of the protein, the soybean photolyase was treated at 45°C for 3 min in a buffer (50 mM Tris-HCl pH 7.4, 50 mM NaCl, 1 mM EDTA, and 1 mM DTT), and the precipitate was collected by centrifugation using ULTRAFREE 0.5 centrifugal filter device (MILLIPORE, USA), which is a filtration/concentration membrane that allows free passage of FAD. The precipitate was resuspended into the buffer and was used for measuring the absorption spectrum.

RESULTS

Cloning, sequencing, and primary structure of the CPD photolyase gene from soybean
The cloning of a CPD photolyase gene from the soybean cDNA library by dilution-amplification method yielded a 1698-bp insert containing a single 1503-bp ORF coding for 500 amino acids (data not shown, but see accession No. AB359233). The soybean CPD photolyase shows a high degree of sequence homology to the photolyases from Arabidopsis (71%), cucumber (74%), rice (62%), spinach (71%), and Chlamidomonas (57%). Comparing the amino acids sequence at positions 126 and 296 with rice photolyases, soybean photolyase has Arg at 126 and Gln at 296 which indicates the same compositions as UV-sensitive Norin 1.

Expression of soybean photolyase gene in UV-sensitive E. coli cells
To determine whether the 1698-bp cDNA clone of the 1503-bp ORF encodes for a CPD photolyase, the cDNA was inserted into the glutathione S-transferase (GST) fusion vector pGEX4T-2, yielding pGSTSbCPD, and expressed in E. coli NKJ3002 cells, which are deficient in phr, uvrA, and recA. The survival of NKJ3002/pGSTSbCPD cells was increased on illumination with visible light for 30 minutes after UV irradiation (Fig. 1), although the conferred resistance was weak as compared to that by E. coli CPD photolyase used as the positive control, suggesting that soybean CPD photolyase in the experimental conditions cannot completely repair the CPDs formed in the E. coli chromosome.

Fig. 1. Photoreactivation of UV-irradiated E. coli strain NKJ3002 (phr uvrA recA) expressing the soybean GST CPD photolyase. After UV irradiation at 254 nm, the NKJ3002 cells carrying the plasmids, pGSTSbCPD (square) and pKY1 (circle), were kept in the dark (closed symbol) or illuminated for 30 min with a day-light fluorescent bulb (open symbol). Experiments were carried out 3–5 times and were reproducible. The results represent one experiment done in parallel.

Spectroscopic properties of soybean photolyase
The soybean CPD photolyase was purified from extracts of E. coli KY20 (phr) cells by affinity chromatography on a glutathione-sepharose column, yielding a band of 85 kDa (Fig. 2 insert). The purified GST fusion protein was used for in vitro analysis.

All photolyases characterized to date contain FAD as a catalytic chromophore. We have previously shown that rice photolyases contain FAD¹²,³⁰ if expressed in E. coli. The soybean CPD photolyase absorption spectrum exhibited...
peaks in the near-UV/blue light region, at 360 nm, 450 nm and 475 nm, and a shoulder at 420 nm (Fig. 2a). The GST-fusion protein was denatured by boiling for 5 min in the presence of SDS, the precipitate was removed by centrifugation, and the absorption spectrum was measured. The absorption spectrum of the supernatant (Fig. 2b) was typical of that of flavin with maxima at 375 nm and 450 nm which is identical to that of fully oxidized FAD. Thus, the GST-fusion soybean CPD photolyase expressed in *E. coli* had FAD as a chromophore.

### Binding of photolyase to thymine dimer

We investigated the thymine dimer-binding activity of the soybean CPD photolyase, using a gel mobility shift assay. As shown in Fig. 3A, the purified soybean CPD photolyase bound to the thymine dimer substrate in an enzyme concentration-dependent manner. The equilibrium binding constant, $K_b$, for the formation of a photolyase-thymine dimer complex (E-S) was determined by incubating increased amounts of enzyme with a constant amount of substrate (150 fmol) and quantifying the fraction of E-S at each concentration (Fig. 3B). From the Scatchard plots shown in Fig. 3C, we obtained a $K_b$ value of $7.5 \times 10^6$ M$^{-1}$ for binding to a thymine dimer. We have previously determined a $K_b$ value of $1.4 \times 10^7$ M$^{-1}$ for a photolyase derived from Sasanishiki, a UV-resistant *japonica* rice cultivar, *japonica* rice cultivar, 1.1 $\times 10^7$ M$^{-1}$ for a photolyase derived from UV-sensitive Norin 1, a progenitor of Sasanishiki, and 1.8 $\times 10^6$ M$^{-1}$ for a photolyase derived from Surjamkhi, a UV-sensitive *indica* rice cultivar.$^{30}$ Thus, the soybean photolyase may bind to UV-damaged DNA as almost same extent as Norin 1.

### The effect of temperature on photoreactivation in vitro

The light-dependent repair of CPDs in *Arabidopsis*, cucumber cotyledons, and rice has been reported to be markedly temperature sensitive.$^{27,31,33}$ Photolyase-mediated repair of CPD was reported to decline between 22°C and 30°C, and to be negligible when *Arabidopsis* plants were transferred from 22°C to 37°C for 1–3 h.$^{31}$ Thus, these results suggest that photolyase activity might be temperature sensitive.

A GST-fused soybean CPD photolyase was placed at 25°C, 37°C, 40°C and 45°C for 0–30 min, and its activity to bind and repair the thymine dimer was investigated. The binding of the soybean CPD photolyase to the dimer appeared to be quite thermosensitive. The ability to bind the thymine dimer was decreased to 50% within 3.5 min and 40 sec when placed at 40°C and 45°C, respectively (Fig. 4). We next observed the thermostability of thymine dimer repair by the soybean photolyase. In this case, a 30-mer DNA containing the thymine dimer was sensitive to digestion with T4 endV, but the site of damage became resistant to T4 endV after the photoreactivation with photolyase (Fig. 5 insert). As shown in Fig. 5, thymine dimer repair was efficient when E-S was first made at room temperature for 15 min then treated at 25°C or 45°C for 3 min. On the other hand, when the enzyme alone was treated at 45°C for 3 min, the repair capability was greatly reduced. These results are consistent with earlier studies mentioned above.$^{31–33}$ When the crude extract derived from 12-day-old rice seedlings and a UV-
irradiated lambda phage DNA mixture were incubated at 28°C for 15 min and then placed at 0, 28, 45 or 60°C for 0–30 min, Hidema et al.\textsuperscript{27} further observed an efficient photoreactivation even at 45°C for 30 min. Thus, the enzyme alone is unstable, but once it binds to a substrate, enzyme becomes thermostable.

Next we measured the binding of FAD to the enzyme during heat treatment. In this case, the soybean CPD photolyase was treated at 45°C for 3 min, the protein was collected by centrifugation using an ULTRAFREE 0.5 centrifugal filter device, and the protein fraction was resolved in buffer (50 mM Tris-HCl pH 7.4, 50 mM NaCl, 1 mM EDTA, and 1 mM DTT). As shown in the absorption spectrum (Fig. 2c), no FAD peaks were observed. We argue that heat treatment

Fig. 3. Gel shift analysis showing thymine dimer-binding activity of GST-soybean photolyase. A 30-mer substrate (150 fmol) with one thymine dimer was mixed with 0 to 12 pmol of GST-photolyase. The photolyase-30-mer complexes were separated from the free 30-mer substrate by the gel shift method (A). The averages of three gel shift experiments were plotted as a saturation curve (B). Gel shift data were then analyzed by using Scatchard plots (C).

Fig. 4. Temperature-sensitive thymine dimer-binding activity of GST-soybean photolyase. Two pmol of photolyase was kept at 25°C (open circle), 37°C (closed diamond), 40°C (closed triangle) and 45°C (closed circle) for 0 to 30 min, then mixed with 150 fmol of 30-mer substrate with one thymine dimer at room temperature (~25°C) for 15 min. A gel shift assay was performed, and the amount of the E-S complex was determined as Fig. 3. Heating time at 0 min was set as a value of 100% shift band. Experiments were carried out 3–5 times and were reproducible. The results represent one experiment done in parallel.
can release FAD from the photolyase. It has been reported previously that a photolyase without FAD had no affinity for CPD.20)

**DISCUSSION**

We isolated and characterized the gene for a class II CPD photolyase from soybean, which consists of 1698 bases containing a single 1503-bp ORF coding for 500 amino acids. The deduced amino acid sequence was quite similar to that of the class II CPD photolyases of higher plants including Arabidopsis, cucumber, rice, and spinach. The cDNA was expressed as a GST fusion protein in *E. coli* NKJ3002 (*phr uvrA recA*), and the resistance of NKJ3002 to UV was enhanced in a fluorescent light-dependent manner (Fig. 1). The purified GST fusion protein bound a 30-mer substrate containing one thymine dimer in a concentration-dependent manner (Fig. 3), and to repair thymine dimer with a fluorescent light-dependent manner (Fig. 5). Finally, the absorption spectrum of the GST fusion protein is consistent with the presence of a flavin as a chromophore (Fig. 2). Thus, the cDNA we isolated from the soybean library encodes a functional CPD photolyase.

From the Scatchard plots shown in Fig. 3C, the $K_a$ value for binding to the thymine dimer was $7.5 \times 10^6 \text{ M}^{-1}$. Previously, we calculated the $K_a$ of the photolyase of UV-resistant rice (Sasanishiki) as $1.4 \times 10^7 \text{ M}^{-1}$, UV-sensitive rice (Norin 1) as $1.1 \times 10^7 \text{ M}^{-1}$, UV-more sensitive rice (Surjamkhi) as $1.8 \times 10^6 \text{ M}^{-1}$, and UV-supersensitive rice (Gulfmont) as $3.7 \times 10^5 \text{ M}^{-1}$. Thus, the soybean CPD photolyase seems to be as efficient as UV-sensitive Norin 1. Actually, it has been mentioned that cultivated vegetables including soybean are sensitive to UV-B radiation.38) Atmospheric pollution is expected to result in both increased UV-B levels reaching the earth’s surface through depletion of the stratospheric ozone layer and higher temperature due to greenhouse warming. If photolyase-mediated repair of UV-B-induced DNA damage is sensitive to temperature increases, as previously reported for Arabidopsis,31,32) cucumber,33) and rice,27) then this could have implications for crop yields and biomass production through adverse effects on growth. Our *in vitro* experiments indicated that soybean CPD photolyase-binding activity and photoreactivation activity were temperature sensitive (Figs. 4 and 5). The deficiency of the activity at high temperature was due to the temperature-dependent release of FAD from the holoenzyme (Fig. 2c). It has been reported that *E. coli* photolyase without FAD had no affinity for CPD.20)

Since plants cannot flee from UV-B radiation, photoreactivation facilitated by UV-A/blue light solar radiation is assumed to be the most efficient repair system for UV-induced DNA lesions. The rice cultivar Norin 1 is more...
sensitive to UV-B than is Sasanishiki, resulting in a lower growth rate under UV-B.\textsuperscript{39,40} The hypersensitivity of Norin I was ascribed to a mutation in the rice CPD gene.\textsuperscript{29,30} Thus, these results suggest the importance of photoreactivation for the growth of higher plants irradiated with solar UV-B radiation, at low as well as high temperature. Since photoreactivation is the major pathway that protects plants from the deleterious effects of UV-B radiation,\textsuperscript{10} the introduction of a soybean CPD photolyase which has been manipulated to be thermostable, into cultivars by genetic engineering may ameliorate UV-B sensitivity, thus leading to substantial increases in the productivity of crops with increases in UV-B levels and global warming.

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