CPD Photolyase Gene from Spinacia oleracea: Repair of UV-Damaged DNA and Expression in Plant Organs

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DNA repair/Cyclobutyl pyrimidine dimers/Photolyase/Spinacia oleracea.

The UV-B radiation contained in solar radiation has deleterious effects on plant growth, development and physiology. Specific damage to DNA caused by UV radiation involves the cyclobutyl pyrimidine dimers (CPD) and the pyrimidine (6-4) pyrimidone photoproducts. CPDs are repaired by CPD photolyase via a UV-A/blue light-dependent mechanism. The gene for the class II CPD photolyase has been cloned from higher plants such as Arabidopsis, cucumbers and rice. We isolated and characterized the cDNA and a genomic clone encoding the spinach class II CPD photolyase. The gene consisted of 3777 bases and 9 exons. The sequence of amino acids predicted from the nucleotide sequence of the cDNA of the gene was highly homologous to that of the higher plants listed above. When a photolyase-deficient Escherichia coli strain was transformed with the cDNA, photoreactivation activity was partially restored, by the illumination with photoreactivating light, resulting in an increased survival and decreased content of CPDs in the Escherichia coli genome. In both the male and female plants, the gene was highly expressed in leaves and flowers under the condition of 14-h light and 10-h dark cycle. The expression in the roots was quite low compared with the other organs.

INTRODUCTION

Ultraviolet (UV) radiation is cytotoxic and mutagenic for living organisms. Ozone absorbs UV-C (wavelengths shorter than 290 nm) radiation in solar radiation and controls the amount of UV-B (between 290 and 320 nm) radiation reaching the earth. Chlorofluorocarbons and other gases emitted in the process of human industrial activity deplete the stratospheric ozone layer, resulting in increased UV-B radiation reaching the surface of the earth.1,2

Increases in UV-B radiation affect ecosystems and cause a reduction in phytoplankton photosynthesis3 and an increase in UV-induced DNA damage in pelagic icefish eggs.4 UV-B radiation affects plant growth and productivity.5,6 Some species of plants and cultivars of a given species exhibit relatively high sensitivity to UV-B radiation, while others are apparently not affected by enhanced UV-B radiation.7,8 Supplemental UV-B levels corresponding to 16 to 25 percent ozone depletion were reported to affect soybean seed yields.9 Several UV-B-sensitive cultivars of higher plants, including rice9,10,11 and maize,12 have been reported, and removal of the UV-B wavelengths from the light increased plant growth.13,14

UV-B radiation induces the formation of DNA lesions such as cyclobutyl pyrimidine dimers (CPDs), pyrimidine pyrimidone (6-4) photoproducts ((6-4) photoproducts) and Dewar isomers. Among them, the CPD is a major UV-induced photoproduct.15 These lesions block DNA synthesis during replication and interfere with mRNA synthesis during transcription, resulting in cell death if not removed.16 Mutation may occur when the lesion bypass DNA synthesis proceeds beyond the lesion by a translesion DNA polymerase such as the Escherichia coli DNA polymerase IV.16 Living organisms have developed a variety of DNA repair mechanisms during evolution, such as photoreactivation, nucleotide excision repair and recombinational repair,17 of which photoreactivation is a light-dependent process and the others are light-independent processes. Photoreactivation is mediated by damage-specific photolyases, i.e., CPD photolyase
and (6–4) photolyase. CPD photolyases are categorized into class I or class II according to their amino acid sequences. Photolyases over prokaryotes to eukaryotes carry FAD as a chromophore, which is excited by light energy and then monomerizes the CPDs in an electron transfer reaction.

Plants must overcome the injurious effects of UV radiation (principally UV-B) in solar radiation, because they are completely dependent on solar radiation for photosynthesis and cannot escape from UV radiation. The mutation frequency of maize endosperm of pollen grains treated with visible light after UV irradiation was significantly lower than that in an untreated control, suggesting the presence of light-dependent repair mechanisms in plants. Photoreactivation might be an essential repair of UV-induced DNA damage in plants. The activity of photolyase-removing CPDs was found in Arabidopsis. A mutant of Arabidopsis ecotype Lansberg (uvr2-1) defective in the light-dependent repair of CPDs was more hypersensitive to UV-B radiation than the wild-type plants. Recently, the genes for photolyase were isolated from cucumbers and rice and classified into the class II CPD photolyase from the predicted amino acid sequences.

A reduced yield of crop plants because of UV-B radiation affects the global food supply. Demonstration of UV sensitivity was unique to a rice cultivar, Sasanishiki and Norin 1, which showed different UV sensitivity and that the differences were due to varying levels of CPD photolyase activity. Norin 1, carrying defective activity of the CPD photolyase, exhibited a lower growth rate under UVB irradiation than Sasanishiki with wild-type photolyase activity. These results suggest the importance of photoreactivation for the growth of higher plants irradiated with UV-B. Takahashi et al. characterized the cDNA encoding the cucumber CPD photolyase, and demonstrated that the photoreactivation activity in the first leaves of cucumber plants changed during the diurnal cycle.

The photoreactivation activity of CPDs in spinach seedlings and the localization of CPD photolyase activity in spinach cells have previously been investigated. Spinach is a dicotyledonous plant, in which male and female flowers are generated on different roots, unlike Arabidopsis and cucumbers, which are monoeious and monococious plants, respectively. A detailed investigation of photoreactivation, including the isolation and characterization of the gene in plants, has been limited to Arabidopsis, cucumbers and rice. Like cucumbers, spinach is relatively sensitive to UV-B radiation, both plants are essential foods for many people worldwide. It is necessary to determine the nucleotide sequence of the gene and predict the amino acid sequence in order to characterize the spinach CPD photolyase and to compare it with that of other organisms.

In this study, we isolated the cDNA encoding the spinach CPD photolyase and examined its photoreactivation ability following the complementation of Escherichia coli deficient in photoreactivation and by enzyme-linked immunosorbent assay (ELISA). The expression of the gene for CPD photolyase in the leaf, flower, stem and root was examined separately in male and female plants.

MATERIALS AND METHODS

Plant materials and growth conditions

The plant growth room was kept at 23–25°C. Spinach seeds of Spinacia oleracea L., cv Orai were sown on vermiculite in a plastic tray and germinated in the dark. The seedlings were illuminated by 4 fluorescent lamps (40 w) at a 50 cm height under 14-h light and 10-h dark cycle. Hypoxen (N:P:K= 10:5:10) diluted 1000-fold was used as a fertil-izer.

Escherichia coli strain and media

Escherichia coli NK3002 (recA uvrA phr) was used for the complementation assay of the CPD photolyase gene of spinach. Luria broth (L broth) and buffer were used as described previously.

UV irradiation

A germicidal lamp (15 w, Toshiba) was used to irradiate the DNA and Escherichia coli cells with UV-C radiation.

Extraction of genome DNA from spinach leaves

Spinach genome DNA was prepared from approximately 5 g of one-month-old young leaves according to the cetyltrimethylammonium bromide (CTBA) procedures with slight modifications. The leaves were ground in liquid nitrogen and mixed with 5 ml of 2 x CTAB extraction buffer (2% CTAB, 0.1 M Tris-HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl and 1% polyvinyl pyrrolidone) heated to 70°C, and then the mixture was incubated at 55°C for 10 min with gentle mixing. The precipitates were dissolved at 55°C in 5 ml of 1 M NaCl-TE (10 mM Tris-HCl, pH 8.0, 1 M NaCl, 1 mM EDTA) after extraction with chloroform/isoamyl alcohol. The DNA was stored at –20°C after treatment with RNase, phenol extraction and ethanol precipitation.

Partial cloning of a gene for the class II CPD photolyase from spinach by PCR

A partial region of a gene for the CPD photolyase in spinach was amplified by PCR using degenerate PCR primers designed from highly conserved amino acid sequences in Arabidopsis and Chlamydomonas CPD photolyase. The primer sequences were AC2 (5’-GTIGAYGCICAYAAYGT-IGTICC-3’) and AC4R (5’-CATRAAICCRTGCATYTTTICC-3’). About 1.4 kbp of PCR products was subcloned into a pGEM-T Easy vector (Promega) and sequenced.
Isolation of cDNA encoding spinach CPD photolyase and determination of its nucleotide sequence

Total RNA was prepared from 2g of spinach leaves using TRIZOL reagent (Invitrogen). Poly(A)⁺ RNA was purified from total RNA using a QuickPrep Micro mRNA purification kit (Pharmacia Biotech). cDNA was synthesized from poly(A)⁺ RNA by 5’-RACE and 3’-RACE. Double-stranded cDNA was synthesized from poly(A)⁺ RNA using the cDNA Synthesis Kit (Stratagene) and inserted into pGEM-T Easy Vector (Promega). After the cDNA was sequenced, it was excised at an EcoRI site and cloned into the EcoRI site of pBluescript II SK+, named pSpCPDPRI-7.

Determination of the genome sequence of the CPD photolyase gene of spinach

Five DNA fragments, including a part of the spinach CPD photolyase gene, were amplified using five pairs of PCR primers and cloned on pGEM-T Easy Vectors. These primers were designed on the basis of the sequence of cDNA for the spinach CPD photolyase. Each PCR product consisted of 1077 bp, 867bp, 713 bp, 708bp and 542 bp.

Complementation of Escherichia coli mutant deficient in photoreactivation with the gene for spinach CPD photolyase

We introduced pSpCPDPRI-7 into Escherichia coli strain NKJ3002. The transformants were grown to the medium log phase in LB medium containing ampicillin and 5 mM IPTG at 37°C. The cultures were diluted and spread on LB agar medium. The Escherichia coli on the agar plates were exposed to UV-C radiation from a germicidal lamp at a dose rate of 0.003 Jm⁻² s⁻¹ and thereafter illuminated with white light from a fluorescent daylight lamp for 60 min to allow photoreactivation.

Repair of CPD from genomic DNA of Escherichia coli carrying the spinach photolyase gene

Escherichia coli NKJ3002 transformed with pSpCPDPRI-7 was grown in L broth with ampicillin and 1 mM IPTG to the late log phase. The cells re-suspended in phosphate-buffered saline (PBS) were irradiated with 0.1 Jm⁻² of UV-C in a plastic dish with gentle agitation. The irradiated cells were illuminated with a fluorescent daylight lamp for 18 hrs. The UV region included in the fluorescent light was cut off with an acrylic plastic plate. After the extraction of Escherichia coli DNA, the relative amount of CPDs was determined by ELISA. The CPD-specific antibody used was MX-thymine dimer-HRP (Kyowa Medex Co., Japan).

Determination of the expression of the gene for CPD photolyase in organs of spinach by real-time PCR

All leaves, flowers, stems and roots were harvested during flowering from one plant body of spinach grown under the conditions mentioned above. The stems were part of the plant that did not have any leaves, flowers or roots. The total RNA was isolated separately from these organs using TRIZOL Reagent (Invitrogen). Template cDNA for real-time PCR was synthesized using the Thermoscript™ RT-PCR System (Invitrogen). The primers for CPD photolyase were SpCPD-34R (5’-CTTCATCAACGGCGTGGATTAGA-3’). The reference standard was spinach cytosolic ribosomal protein L23 mRNA. The relative quantity of spinach CPD photolyase mRNA was determined by real-time PCR (GeneAmp 5700 Sequence Detection System, PE Biosystems) following RT-PCR. The primers were SpCPD-34R and 5’-ATGACCTCACAACCGGTACC-3’. Double-strand DNA-specific dye SYBR Green was used to detect and quantitate the PCR products as fluorescent molecular probes (SYBR Green PCR Core Reagents, PE Biosystems). The relative extent of the gene expression for the spinach CPD photolyase was expressed as the ratio of the initial amount of mRNA for the CPD photolyase to that for the cytosolic ribosomal protein L23.

RESULTS

Cloning of the CPD photolyase gene of spinach

Amino acid sequences of the CPD photolyase in Arabidopsis and Chlamydomonas were applied to design PCR primers for isolation of the CPD photolyase gene from spinach. A 1.4-kbp DNA fragment was successfully amplified from spinach genome DNA by using two degenerate primers, AC2 and AC4R. The amplified DNA fragment contained a sequence similar to the class II CPD photolyase of Arabidopsis. Sequences of the fragment were employed to design primers for cloning cDNA by RT-PCR. Unknown regions of the 5’ side and 3’ side of cDNA were determined by 5’-RACE and 3’-RACE using the primers listed above. The entire cDNA was obtained by joining three parts of cDNA, i.e., the 5’ region, the 3’ region and the region cloned by the AC2 and AC4R primers. The full-length cDNA consisted of 1476 bases containing a single ORF of 491 amino acids. The cDNA sequence has been registered in the GenBank database, under accession number AF267198.

Fourteen primers including forward and reverse directions were designed from the cDNA sequences. Genome DNA was subjected to PCR using these primers, resulting in the amplification of seven DNA fragments. The nucleotide sequence of each fragment was determined and aligned according to its cDNA sequence. The nucleotide sequence from the initiation codon, ATG, to the termination codon, TGA, consisted of 3776 bases. The spinach photolyase gene was defined to include nine exons. Furthermore, a ca. 400-bp region upstream from the initiation codon was determined by Thermal Asymmetrical Interlaced PCR, although the regulatory region of the gene has not been analyzed. The genome sequence has also been registered in the GenBank database.
Table 1 shows the sequence of exon-intron boundaries. All eight introns contain a 5'-GT and 3'-AG sequence. There was a pyrimidine-rich sequence upstream of the AG in many organisms.42

![Image]

**Table 1.** Sequence of exon-intron boundaries of spinach CPD photolyase gene.

| No. | exon(bp) | 5’ side spliced out | intron(bp) | 3’ side spliced out |
|-----|----------|---------------------|------------|--------------------|
| 1   | 319      | TTTCCAGgtaacc       | 586        | tatacagGGAAG       |
| 2   | 341      | GTCTCAAgtaagat      | 434        | tgattAGAAAAGA      |
| 3   | 240      | TCCTGAgttgtc        | 132        | ttgaagGCAGTTG      |
| 4   | 158      | TTGTACAgtaagta      | 79         | actgcagGAGGAG       |
| 5   | 37       | TGATCCGgttaacc      | 147        | tttcacCTATGGA      |
| 6   | 59       | TTATGCGgttaagta     | 75         | tttcgatTATGTC      |
| 7   | 82       | TGACAAAGgttaactc    | 86         | tttcgatTATGAGA     |
| 8   | 72       | TGACCAGGtttctc      | 121        | tttaagGGGGTGGC     |
| 9   | 177      |                     |            |                    |

1) base number of exons and introns, 2) sequences of 5’ side and 3’ side of intron, Uppercase and lowercase indicate exon and intron sequences, respectively. Bold face indicates conserved sequence in splice junction.

Fig. 1. Alignment of predicted amino acid sequences of CPD photolyases from higher plants: spinach, this work; Arabidopsis, GenBank accession number: AF053365; cucumbers27; rice.28 Screened letters indicate identical amino acid residues to the spinach CPD photolyase. The putative nuclear localizing signal is marked with a + symbol. The alignment of the spinach CPD photolyase was constructed by the Clustal W Program (http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html).

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Spinach photolase shows a high degree of homology to other class II CPD photolases from Arabidopsis (71%), cucumbers (71%), rice (64%) (Fig. 1), Drosophila (48%), Chlamydomonas (58%) and Monodelphis (53%) (data not shown). The spinach photolase in this study was therefore shown to be a class II CPD photolase (Fig. 2). At the C-terminus of the photolase, we predicted a nuclear localization signal by using the Prediction of Protein Sorting Signals and Localization Sites in Amino Acid Sequences Program (Fig. 1), which suggests targeting the spinach photolase in the nucleus.

Complementation of mutant Escherichia coli deficient in photoreactivation by the cloned cDNA

Escherichia coli NKJ3002 (recA, uvrA, phr) was transformed with pSpCPDPR1-7 so we could investigate whether the cloned cDNA contained photoreactivation activity. The survival of NKJ3002 and NKJ3002/pBluescript II SK+ cells irradiated with 0.15 J m⁻² of UV-C light was about 10⁻¹ with or without photolysis. The survival of NKJ3002/pSpCPDPR1-7 irradiated with 0.15 J m⁻² of UV-C light was 5 × 10⁻³ when they were illuminated with white light (Fig. 2). These results demonstrate that the cDNA isolated from spinach encodes protein with photoreactivation activity.

Light-dependent removal of CPDs from genome DNA in UV-irradiated NKJ3002/pSpCPDPR1-7 cells

We investigated the repair of CPD on the genome DNA of Escherichia coli NKJ3002 carrying pSpCPDPR1-7. The remaining CPDs in NKJ3002/pSpCPDPR1-7 cells illuminated with white light for 18 h after 0.1 J m⁻² of UV-C irradiation was approximately 50% of the unilluminated control (Fig. 3). The repair of CPDs was not enhanced in NKJ3002/pBluescript II SK+ by white light illumination (Fig. 3). The number of living cells in phosphate-buffered saline did not decrease during the 18-h white light illumination. Therefore, it was demonstrated that NKJ3002/pSpCPDPR1-7 showed an enhanced repair activity of CPDs by white light illumination. We concluded that the cDNA isolated from spinach was the gene for the class II CPD photolase.

Expression of the gene for CPD photolase in the leaves, flowers, stems and roots of spinach

The amount of expression of the CPD photolase gene

| leaf | flower | root | stem |
|------|--------|------|------|
| male | 2.2 ± 1.2 | 1.8 ± 0.7 | 0.5 ± 0.2 | 1 |
| female | 2.5 ± 1.3 | 1.2 ± 0.7 | 0.1 ± 0.1 | 1 |

Spinach was grown under a 14-h light and 10-h dark cycle at 23–25°C. RNA was extracted from the leaves, flowers, stems and roots which were harvested at flowering. The amount of mRNA for CPD photolase in each organ was represented as a relative amount to that of the mRNA in the stem. Figures are a mean value of the quarterly independent experiments and the standard error.

Fig. 2. Photoreactivation of UV-irradiated Escherichia coli NKJ3002 (phr, uvrA, recA) transformed with pSpCPDPR1-7. UV-irradiated Escherichia coli cells were illuminated with fluorescent white light for 60 min (closed symbols) or kept in the dark (open symbols). Symbols: circles, NKJ3002/pSpCPDPR1-7; triangles, NKJ3002/pBluescript II SK+; squares, NKJ3002.

Table 2. Expression of CPD photolase gene in male and female spinach.
was determined in the leaves, flowers, stems and roots of spinach grown under 14-h light and 10-h dark conditions. As shown in Table 2, in both males and females, the level of expression of the CPD photolyase gene in spinach was higher in the leaves and flowers than in the roots in each quarterly independent experiment, although the expression level varied widely in the leaves and flowers among the experiments (Table 2).

**DISCUSSION**

We isolated and characterized the gene for class II CPD photolyase from spinach, which consisted of 3776 bases. The cDNA consisting of 1476 bases encoded the protein of 491 amino acid residues, which was quite similar to the class II CPD photolyase of higher plants, including *Arabidopsis*, cucumbers and rice. The genome sequence of the gene consisted of 9 exons, which was the same as the class II CPD photolyase gene of *Arabidopsis* and cucumbers (GenBank AB084265).

The cDNA was introduced into *Escherichia coli* strain NKJ3002, which was defective in photoreactivation. When NKJ3002 carrying the gene was illuminated with white light after irradiation with UV-C light, the survival significantly increased compared with that of NKJ3002 transformed with an empty vector and an unilluminated NKJ3002 (Fig. 2). Therefore, we demonstrated that the cDNA conferred the photoreactivation activity to *Escherichia coli*.

We examined the extent of the repair of CPDs in genome DNA in NKJ3002 carrying the cDNA. The amount of CPDs decreased in white light-illuminated cells by less than 50% of the amount in the unilluminated NKJ3002 (Fig. 3). These results indicate that the cDNA complemented the deficiency of photoreactivation in NKJ3002. Therefore, we concluded that the cDNA cloned in the present study encodes the class II CPD photolyase.

The level of photoreactivation of *Escherichia coli* NKJ3002 transformed with the spinach photolyase gene was lower than that observed in *Escherichia coli* with the gene for cucumbers and rice (Fig. 2). The vector pBluescript II SK+ used for cloning contains bacteriophage T3 and T7 promoters in opposite orientations. The direction of the gene inserted in the vector may affect the expression of the photolyase gene on pSpCPDPR1-7. In the examination of the light-dependent removal of CPDs from the *Escherichia coli* genome DNA, white light was illuminated for a long time, 18 h, to allow the full, light-dependent repair of CPDs (Table 2).

The CPD photolyase protein has been reported to be the most abundant in floral tissues and at a very low level in the roots of *Arabidopsis*. The amount of photolyase in *Arabidopsis* was observed when *Arabidopsis* cells were exposed to UV-A and white light after UV-B-irradiation, although these photoproteins were slightly reduced in the dark. CPD photolyases repair CPDs much more rapidly than the light-independent repair process in plant tissues. Seedlings of the UV-B-resistant mutant *uvi* of *Arabidopsis* grown under white light exhibited a more rapid removal of CPDs than that in wild-type seedlings, indicating an increased photoreactivation activity in *uvi* *Arabidopsis*. The CPD photolyase gene expression in the *uvi* mutant was higher than in the wild-type plants. UV-B radiation is known to induce the expression of the gene for the CPD photolyase, which suggests that a defense system against UV is induced by UV itself. Plant leaves efficiently receive solar energy for photosynthesis and are simultaneously exposed to a massive dose of solar UV radiation. Flowers, the sexual organs which produce seeds, should be protected from UV damage.

Since plants cannot flee from the UV radiation found in solar radiation, photoreactivation facilitated by the white light in solar radiation is assumed to be the most efficient repair system of UV-induced DNA lesions. Rice cultivar Norin 1 is quite a bit more sensitive to UV-B radiation than Sasanishiki, resulting in a lower growth rate for Norin 1 under UV-B irradiation than for Sasanishiki. The hypersensitivity of Norin 1 was ascribed to a defect in the light-dependent repair of CPDs caused by functionally altered CPD photolyase. These results strongly suggest the importance of photoreactivation for the growth of higher plants irradiated with solar UV-B radiation under natural conditions. Cultivated vegetables such as soybeans, tomatoes, spinach and cucumbers are sensitive to UV-B radiation. Alleviation of the reduced yield of crop plants caused by solar UV-B radiation is required to ensure the future global food supply. If the resistance of agricultural plants to UV radiation increases, the yield will likely increase. Introducing the gene for photolyase into plants to enhance their

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repair activity could prove a powerful strategy for producing plants resistant to UV radiation.

ACKNOWLEDGEMENTS

We would like to thank Dr. Toshio Mori, Nara Medical University, for his valuable advice on determining CPDs by the ELISA method. We are grateful to Prof. Takeshi Todo, Kyoto University, and Dr. Kenji Matsui, Yamaguchi University, for their kind advice and input. We would also like to thank Prof. Yasuho Kiso and Dr. Eiichi Hondo for kindly supplying the real-time PCR apparatus. This work was supported by Grants-in-Aid for Scientific Research nos. 13680628 and 15201010.

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Received on October 26, 2004
1st Revision received on January 31, 2005
Accepted on February 2, 2005

J. Radiat. Res., Vol. 46, No. 2 (2005); http://jrr.jstage.jst.go.jp