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Cloning and characterization of a gene (*UVR3*) required for photorepair of 6–4 photoproducts in *Arabidopsis thaliana*

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ABSTRACT

UV radiation induces two major classes of pyrimidine dimers: the pyrimidine [6–4] pyrimidone photoproduct (6–4 product) and the cyclobutane pyrimidine dimer (CPD). Many organisms produce enzymes, termed photolyases, that specifically bind to these damage products and split them via a UV-A/blue light-dependent mechanism, thereby reversing the damage. These photolyases are specific for either CPDs or 6–4 products. A gene that expresses a protein with 6–4 photolyase activity *in vitro* was recently cloned from *Drosophila melanogaster* and *Xenopus laevis*. We report here the isolation of a homolog of this gene, cloned on the basis of sequence similarity, from the higher plant *Arabidopsis thaliana*. This cloned gene produces a protein with 6–4 photolyase activity when expressed in *Escherichia coli*. We also find that a previously described mutant of *Arabidopsis* (*uvr3*) that is defective in photoreactivation of 6–4 products carries a nonsense mutation in this 6–4 photolyase homolog. We have therefore termed this gene *UVR3*. Although homologs of this gene have previously been shown to produce a functional 6–4 photolyase when expressed in heterologous systems, this is the first demonstration of a requirement for this gene for photoreactivation of 6–4 products *in vivo*.

INTRODUCTION

The ultraviolet (UV) component of sunlight produces cytotoxic, mutagenic and carcinogenic lesions in DNA. Various repair mechanisms have evolved to counteract the deleterious effects of UV radiation on cellular DNA. One of the most widely distributed repair enzymes is DNA photolyase, which utilizes near UV/blue light to monomerize UV-induced cyclobutane pyrimidine dimers (CPDs) (1) or UV-induced pyrimidine [6–4] pyrimidone photoproducts (6–4 products) (2), which constitute 70–80 and 20–30% of total UV photoproducts respectively. The genes encoding the CPD photolyase are widely distributed amongst species, including *Escherichia coli*, *Saccharomyces cerevisiae*, *Drosophila melanogaster*, *Arabidopsis thaliana* and *Carassius auratus*, and have been characterized in detail (2–6). 6–4 Photolyase activity has been found in extracts from *Drosophila*, *Xenopus*, rattlesnake (*Crotalus atrox*) and fish, and light-dependent repair of 6–4 products has been observed *in vivo* in the higher plant *A.thaliana* (2,7–9), but only the *Drosophila* and *Xenopus* genes have been cloned and sequenced (10,11).

The CPD photolyase genes characterized so far could be grouped, based on sequence homology, into two classes, microbial (class I) and metazoan (class II) genes (5), although these terms are somewhat misleading, as the various eubacterial CPD photolyases may fall into one or the other class. The 6–4 photolyase from *Drosophila* has strong sequence similarity to class I CPD photolyases (10). The blue light photoreceptor (cryptochrome, HY4) of *Arabidopsis* also has a remarkable sequence similarity to the *Drosophila* 6–4 photolyase and class I CPD photolyases (12). Thus microbial CPD photolyases, 6–4 photolyases and the blue light photoreceptor constitute a class I photolyase/photoreceptor family (6,10).

Previous reports have demonstrated that photoreactivation of UV-induced dimers occurs in higher plants (13–15), including photoreactivation of 6–4 products as well as CPDs (9). Plant genes with homology to class I CPD photolyases have been cloned from *Arabidopsis* and *Sinapsis alba*. None of these cloned genes produced significant photolyase activity on expression in *E.coli* and one is known to encode a blue light photoreceptor (12,16–18). Recently an *Arabidopsis* CPD photolyase gene with strong sequence similarity to metazoan class II photolyases was

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cloned (6). The two *uvr2* mutants of *Arabidopsis*, which are defective in photoreactivation of CPDs, carry nonsense mutations in this gene (19,20).

In this study we report cloning of a 6–4 photolyase homolog from *Arabidopsis*, as well as characterization of this plant 6–4 photolyase obtained by expression in *E.coli*. Furthermore, we demonstrate that an *Arabidopsis* mutant (*uvr3*) defective in 6–4 photoproduct repair carries a nonsense mutation in this 6–4 photolyase homolog.

MATERIALS AND METHODS

Bacterial strains and plasmids

The *E.coli* strains KY29 (JM107 + *phr19*::Cm *recA56*) (21), NKJ3002 (JM107 + *phr20*::Kan *uvrA*::Kan Δ*recA*) and XL1-Blue MRF′ (Clonetech) were used as host strains for cloning of the 6–4 photolyase gene, overexpression and purification of recombinant proteins. NKJ3002 was constructed as follows. NKJ2999 (JM107 *phr20*::Kan), was infected with P1 phage carrying *uvrA*::Kan *malE*::Tn*10* alleles, selecting for tetracycline (Tc)-resistant and UV-sensitive phenotypes. The Tn*10* transposon was then eliminated according to Bochner *et al.* (22). The resulting strain, NKJ3000 (NKJ2999 + *uvrA*::Kan) was infected with P1 phage carrying Δ(*srlR-recA*)$_{306}$::Tn*10*, selecting for Tc resistance. The Tn*10* transposon was once again eliminated. The resultant *phr*$^-$ *uvrA*$^-$ *recA*$^-$ phenotype was confirmed by UV survival and photoreactivation and was named NKJ3002. Plasmid pKY137 was constructed by inserting a 2.4 kb *Pvu*II–*Hin*cII fragment containing the *E.coli* CPD photolyase gene (23) into the *Hin*dIII and *Hin*cII sites of pACYC184. The pGEX-4T-2 plasmid used for glutathione S-transferase gene fusion was purchased from Pharmacia Biotech (Sweden).

Cloning of the *Arabidopsis* 6–4 photolyase gene

The following oligonucleotides derived from the highly conserved sequence of CPD photolyases and *Drosophila* 6–4 photolyase were used as PCR primers: 64F1, 5′-CCYTCYTCCCANSWDATC-CA-3′; 64M2, 5′-TGNCKGGCNAGRTGRTGNATCCA-3′; 64C3, 5′-TGGMGNGARTTYTWYTAYAC-3′. Total RNA was isolated from hypocotyl segments, hypocotyl-derived callus, hypocotyl explants induced to form shoots, hypocotyl explants forming shoots and hypocotyl explants forming roots of *Arabidopsis* (ecotype Landberg *erecta*) by the procedure described by Ozeki *et al.* (24). After these samples of total RNA were combined, poly(A)$^+$ RNA was separated from total RNA using oligo(dT)-tailed latex beads (Oligotex-dT30 super; Japan Synthetic Rubber) according to the manufacturer's instructions. Double-stranded cDNA was synthesized from 0.5 μg poly(A)$^+$ RNA using the instructions provided with a Clonetech Marathon™ cDNA Amplification Kit (Clontech). The cDNA was firstly used for amplification with primers 64F1 and 64C3 in 30 PCR cycles of 1 min 94°C, 1 min 53°C and 1 min 72°C. An aliquot of the PCR product was then amplified with primers 64M2 and 64C3 in 20 PCR cycles of 1 min 94°C, 1 min 53°C and 1 min 72°C. A 204 bp amplified DNA fragment was gel purified and cloned into pMOSBlue T vector (Amersham) for sequencing. To isolate the entire transcribed region 5′- and 3′-RACE was performed on the double-stranded cDNA.

Purification of the GST–6–4 photolyase fusion protein

cDNA of the cloned 6–4 photolyase gene in vector pMOSBlue T was amplified using two primers annealing near the putative start and stop codons. These primers introduced new *Eco*RI and *Sal*I restriction sites. The resulting 1.6 kb DNA fragment was digested with *Eco*RI and *Sal*I and cloned into the *Eco*RI and *Sal*I sites of vector pGEX-4T-2 to obtain plasmid pGEXAt64.

Escherichia coli NKJ3002 transformed with pGEXAt64 was grown at 25°C in 2 l LB medium with 50 mg ampicillin to an OD_{600} of 0.6. IPTG (0.1 mM) was then added to induce expression of the chimeric gene and the culture incubated overnight. Cells were harvested, resuspended in phosphate-buffered saline and sonicated with a Bronson sonicator. The cell debris was removed by centrifugation. The cell-free extract was loaded onto glutathione–Sepharose 4B (Pharmacia, Sweden) and heparin–Sepharose CL-6B (Pharmacia, Sweden) columns. The fusion proteins were located by SDS–PAGE and Coomassie staining. Fractions containing 6–4 photolyase were combined and concentrated using VIVAPORE 10 (Vivascience), which is a filtration/concentration membrane that allows free passage of molecules <5 kDa. The GST fusion protein was not sensitive to cleavage with thrombin (data not shown).

Photoreactivation of UV-induced damage in *E.coli* expressing the *Arabidopsis* 6–4 photolyase homolog: survival curves

NKJ3002 cells with pKY137, which carries the wild-type *E.coli* CPD photolyase gene, were transformed with pGEXAt64 and the transformants grown in LB medium to an OD_{600} of 0.6. Expression of the transgene was then induced by adding IPTG to 1 mM and incubating the cells for an additional 3.5 h with shaking. Cells were washed and resuspended to 3×10^5 cells/ml in phosphate buffer. Samples of 2.5 ml cells were irradiated in a Petri dish with 0.05 J/m^2/s UV-C, provided by a germicidal lamp. The cells were then illuminated with daylight fluorescent lamps for 30 min as previously described (25). Samples were plated on LB agar and incubated overnight to score surviving colonies. All experiments, except for photoreactivating treatments, were done under yellow light. Fractional survival was calculated from the average of more than five trials.

Preparation of DNA containing 6–4 product

Oligonucleotides (49mers) containing centrally located 6–4 products were prepared as previously described (26). The sequence of the substrate is AGCTACCATGCCTGCCTGCACGAATTAAGCA-ATTCGTAATCATGGTCATAGCT (the two T residues that make up the 6–4 photoproduct are underlined). One nanomole of oligonucleotide containing 6–4 photoproduct was annealed with 2 nmol complementary oligomer in annealing buffer (10 mM Tris, pH 7.5, 100 mM NaCl) by heating at 75°C for 10 min and cooling to 30°C over a 2–3 h period. Duplex DNA was separated from non-hybridized single-strand oligomers by electrophoresis on a 10% polyacrylamide gel and purified using DE51 paper (Whatman) (27). The purified DNA was ethanol precipitated and resuspended in annealing buffer. Aliquots of the purified DNA (~5 pmol) were 5′-end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Labeled DNA was ethanol precipitated and resuspended in annealing buffer and used as substrate.

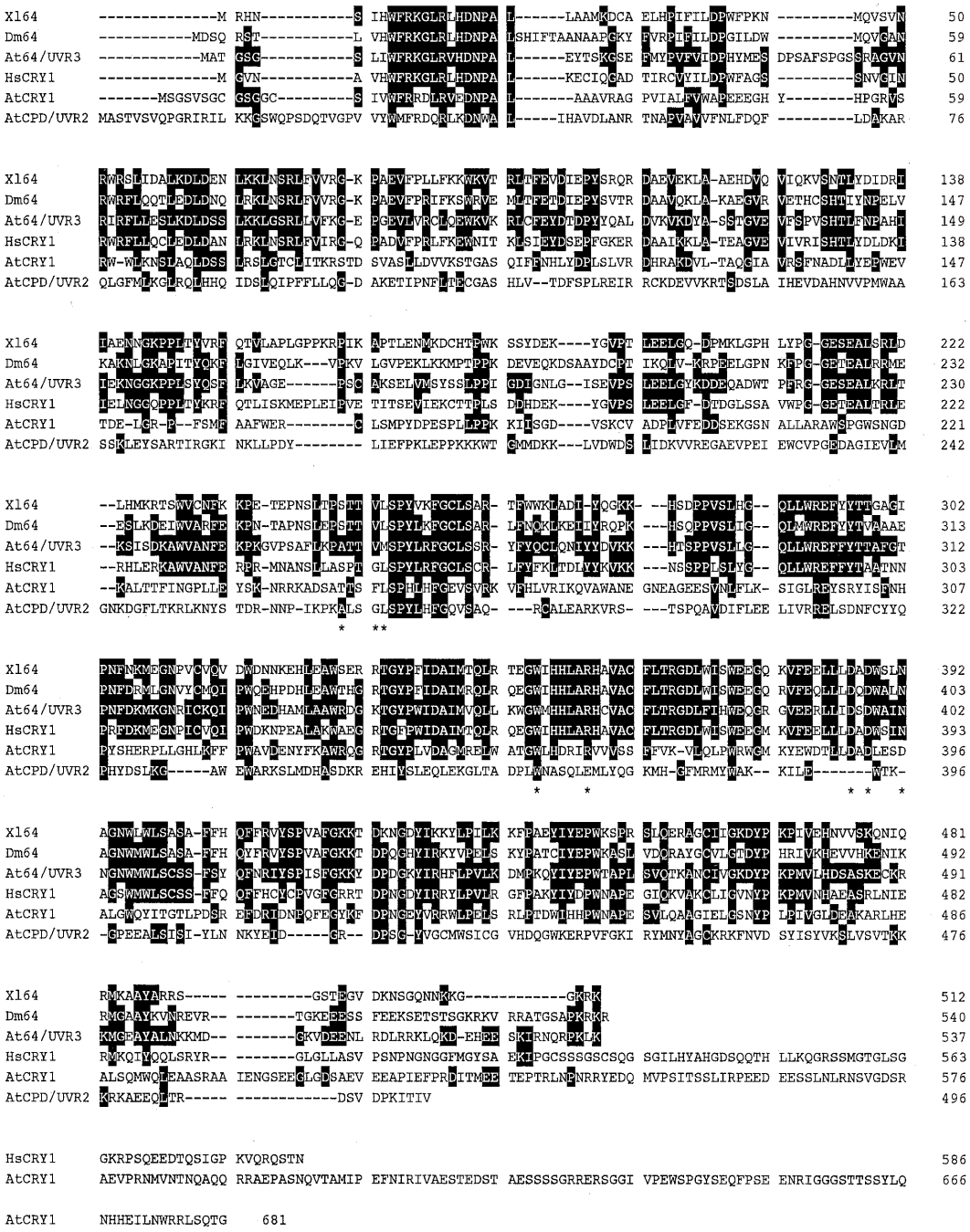

Figure 1. Alignment of the amino acid sequence of the *Arabidopsis* 6–4 photolyase (At64/UVR3) with sequences of the photolyase/photoreceptor family, showing *Drosophila* 6–4 photolyase (Dm 64) (10), *Xenopus* 6–4 photolyase (Xl 64) (11), *Arabidopsis* CRY1 (At CRY1) (12), *Homo sapiens* CRY1 (Hs CRY1) (10) and *Arabidopsis* CPD photolyase (At CPD/UVR2) (6). Amino acids identical to those in *Arabidopsis* 6–4 photolyase are shown as white letters on a black background. Amino acids predicted to interact with FAD are marked with an asterisk below the alignment. Alignment was achieved with the ClustalW program, available on http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html, and by eye. The DDBJ/EMBL/GenBank database accession no. of the *Arabidopsis* 6–4 photolyase nucleotide sequence is AB003687.

Characterization of GST–*Arabidopsis* 6–4 photolyase

Binding activity was measured with the UV-irradiated 73 bp double-stranded TC-3 probe gel shift assay (10). TC-3 was end-labeled by filling recessed 3′-ends using [α-^{32}P]dCTP and Klenow fragment. Reaction mixtures contained UV-irradiated TC-3 (15 kJ/m^2 UV-C), 6–4 photolyase and buffer (10 mM Tris–HCl, pH 7.4, 50 mM NaCl, salmon sperm DNA) and were analyzed by electrophoresis on a 5% polyacrylamide gel.

6–4 Product repair activity was measured using a 49mer oligonucleotide containing one [T6–4T] product at an *Mse*I site. Reaction mixtures containing the 49mer 6–4 oligonucleotide, 6–4

photolyase and buffer (50 mM Tris–HCl, pH 7.4, 1 mM DTT) were photoreactivated at room temperature for 60 min. The reactions were precipitated with ethanol, digested with *Mse*I and analyzed by electrophoresis on a 10% polyacrylamide gel containing 7 M urea.

Amplification and sequencing of the *Arabidopsis* 6–4 photolyase gene in the photoreactivation-deficient *uvr3* mutant

Primers bordering the coding region of the *Arabidopsis* 6–4 photolyase homolog were used for PCR amplification of the corresponding region from total genomic DNA. The forward and reverse primers have the sequences 5′-TAACAATACACAC-TTCATCAAT-3′ and 5′-CGATTCTGCGTCTGTTCACCTT-3′ respectively. These primers produce a 3.1 kb genomic DNA fragment. Two independently amplified genomic DNA products were generated and directly sequenced from the progenitor line Landsberg *erecta tt5 uvr1* and the 6–4 photoreactivation-deficient Landsberg *erecta tt5 uvr1 uvr3* mutant. Both transcribed and non-transcribed strands were sequenced.

RESULTS

Cloning of the *Arabidopsis* 6–4 photolyase gene and sequence comparisons

The similarity between *Drosophila* 6–4 photolyase and class I CPD photolyase genes was utilized to isolate a 6–4 photolyase homolog from *Arabidopsis* by PCR. A 204 bp DNA fragment was amplified directly from *Arabidopsis* cDNA. Sequences derived from this fragment were then employed to design primers for 5′- and 3′-RACE extension of the clone and a 1914 bp fragment containing a single 1611 bp ORF of 537 amino acids was obtained. Southern analysis of *Arabidopsis* DNA confirmed the origin of the cloned cDNA (data not shown). Sequence comparison of this gene with the *Drosophila* and *Xenopus* 6–4 photolyase genes, the blue light receptor (HY4/CRY1) gene of *Arabidopsis*, a human 6–4 photolyase homolog of unknown function (hsCRY1) and a class II CPD photolyase of *Arabidopsis* (UVR2) is shown in Figure 1. The *Arabidopsis* 6–4 photolyase shows a high degree of sequence homology to the *Drosophila* 6–4 photolyase (45% sequence identity), the *Xenopus* 6–4 photolyase (47%) and the human homolog (50%). Lesser degrees of identity were observed for the *Arabidopsis* blue light receptor HY4 (26%) and the *Arabidopsis* class II CPD photolyase UVR2 (16%).

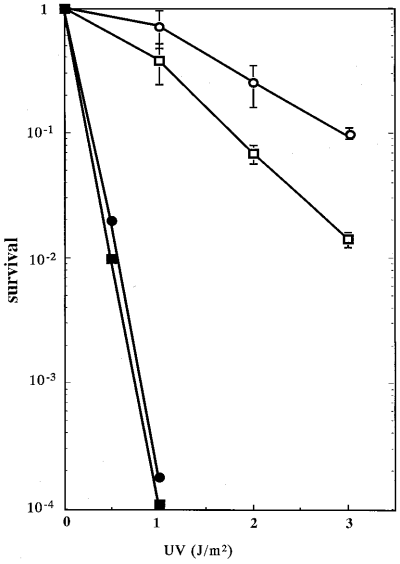

Figure 2. Photoreactivation of UV-induced damage in repair-defective *E.coli* strain NKJ3002, with and without *E.coli* CPD photolyase (pKY137) and *Arabidopsis* 6–4 photolyase–GST fusion (pGEXAt64). After UV irradiation the *E.coli* cells were kept in the dark (closed symbols) or illuminated with white light (open symbols). NKJ3002(pKY137) (■, □); NKJ3002(pKY137 and pGEXAt64) (●, ○).

Purification of the recombinant *Arabidopsis* 6–4 photolyase

To determine whether the isolated cDNA clone encodes a 6–4 photolyase the cDNA was inserted into the glutathione S-transferase (GST) fusion vector pGEX-4T-2 and expressed in *E.coli*. The resultant plasmid was named pGEXAt64. The simultaneous presence of pGEXAt64 and an *E.coli* CPD photolyase plasmid (pKY137) in the *uvrA*⁻ *recA*⁻ *phr*⁻ host strain *E.coli* NKJ3002 resulted in an increase in UV resistance after photoreactivation relative to NKJ3002, carrying only the CPD photolyase plasmid (Fig. 2).

The recombinant protein was purified from *E.coli* KY29 extract by affinity chromatography on glutathione–Sepharose and heparin–Sepharose columns, yielding a single band of 90 kDa (Fig. 3). Incubation of the GST fusion protein with thrombin did not lead to cleavage of the protein (data not shown). Thus we used the intact fusion protein for *in vitro* analysis of protein activity.

The purified GST fusion protein bound to the UV-irradiated 73 bp DNA probe TC-3 (10) but not to probes from which 6–4 photoproducts were removed with *Xenopus* 6–4 photolyase (Fig. 4). The *Xenopus* 6–4 photolyase has no significant CPD photolyase activity (11). Thus GST fusion protein binds specifically to DNA containing 6–4 products. The purified GST fusion protein was also able to repair 6–4 products. A 49 bp DNA containing a 6–4 product at a TT sequence in the *Mse*I site (5′-TTAA-3′) was resistant to digestion with *Mse*I (lane 1), whereas the site became *Mse*I-sensitive after photoreactivation with the protein (Fig. 5, lanes 2–4). As a control, *Xenopus* 6–4 photolyase responded in essentially the

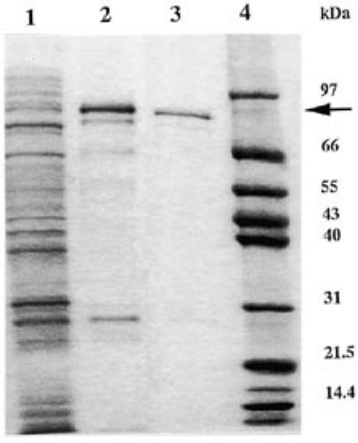

Figure 3. Coomassie blue staining of GST–*Arabidopsis* 6–4 photolyase separated on a 4–20% gradient SDS–PAGE of *E.coli* pGEXAt64 extract (lane 1) and eluates of glutathione–Sepharose (lane 2) and heparin–Sepharose (lane 3) columns. Lane 4 contains marker proteins. Arrow indicates the 90 kDa protein band.

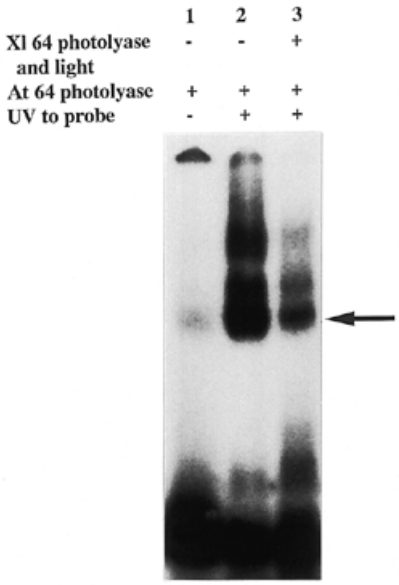

Figure 4. Gel shift analysis showing UV-irradiated DNA binding activity of GST–*Arabidopsis* 6–4 photolyase. Non-irradiated (lane 1) or UV-irradiated (lane 2) TC-3 DNA probe, 1.5 µg/reaction tube, was assayed for GST fusion protein binding. UV-irradiated TC-3 DNA probe was mixed with 2 µg/reaction tube *Xenopus* 6–4 photolyase (11) and exposed to fluorescent light for 30 min to remove 6–4 products. The TC-3 DNA was extracted with phenol, phenol/chloroform and chloroform, precipitated with ethanol and assayed for GST fusion protein binding by gel shift analysis (lane 3). The faint shifted band seen in lane 3 is due to remaining 6–4 products in TC-3 after incomplete photoreactivation with *Xenopus* 6–4 photolyase. An arrow indicates the shifted band.

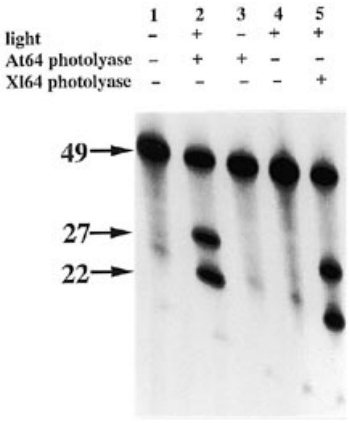

Figure 5. Photoenzymatic repair of T6–4T in 49mer DNA by GST–*Arabidopsis* 6–4 photolyase. The 49mer DNAs were mixed with protein and exposed to fluorescent light for 60 min. The DNA was extracted with phenol, phenol/chloroform and chloroform, precipitated with ethanol and digested with *Mse*I, which cuts only repaired DNA. Lane 1, no enzyme, no white light; lane 2, GST–*Arabidopsis* 6–4 photolyase and white light; lane 3, GST–*Arabidopsis* 6–4 photolyase, no white light; lane 4, no enzyme and white light; lane 5, *Xenopus* 6–4 photolyase and white light. Arrows indicate undigested 49mer and digested 27mer and 22mer bands.

same way as the GST fusion protein (Fig. 5, lane 5). Together these results indicate that the fusion protein is an active 6–4 photolyase.

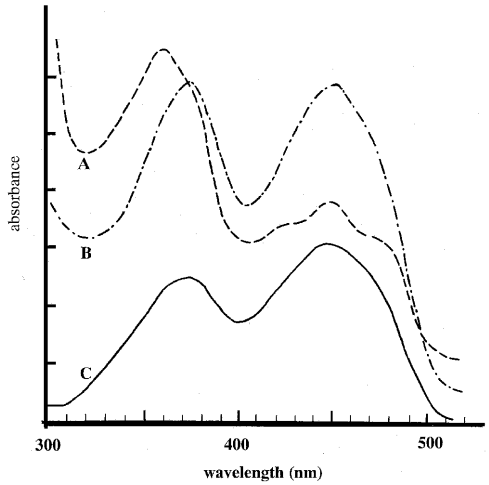

Figure 6. Spectroscopic analyses of (**A**) native GST–*Arabidopsis* 6–4 photolyase and (**B**) GST–*Arabidopsis* 6–4 photolyase boiled for 5 min, compared with that of FAD (**C**).

Spectroscopic properties of the GST–*Arabidopsis* 6–4 photolyase

All known photolyases contain a flavin cofactor. The GST–*Arabidopsis* 6–4 photolyase has an absorption spectrum with near-UV/blue light peaks at 360 and 450 nm and a shoulder at 475 nm, which is similar to the spectra of many flavoproteins (Fig. 6A). The protein was denatured by boiling for 5 min, the precipitate (denatured protein) was removed by centrifugation and the absorption spectrum of the supernatant was measured. The absorption spectrum was similar to that of fully oxidized FAD, with 375 and 450 nm peaks (Fig. 6B and C). *Arabidopsis* 6–4 photolyase was denatured at pH 3.0 by heating at 65°C and the released chromophore was recovered. When *E.coli* photolyase apoenzyme was mixed with the chromophore the resulting reconstituted photolyase restored photoreactivating activity (data not shown). *Escherichia coli* photolyase required bound FAD as a catalytic cofactor. Thus, like other photolyases, the *Arabidopsis* protein contains a non-covalently bound FAD.

Identification of a mutation in the *Arabidopsis* 6–4 photorepair-deficient *uvr3* mutant

A photoreactivation-deficient mutant (*uvr3*) was isolated in a screen for UV-sensitive mutants of *Arabidopsis* (19). The *uvr3* mutant displays a visible light-dependent UV-sensitive phenotype observed as browning, puckering and rolling of the leaf edges in response to relatively low UV-C doses (200 J/m^2). The *uvr3* mutant is deficient in photoreactivation of 6–4 products but proficient in repair of CPDs (19). In order to determine whether this 6–4 photoreactivation-deficient mutant carried an alteration in the sequence of the 6–4 photolyase gene we compared the sequence of this gene in the mutant with that of its Landsberg *erecta tt5 uvr1* progenitor. The mutant carries a single base pair alteration (a G→A transition), as shown in Figure 7. This single base change was confirmed from two independently amplified PCR products sequenced from both transcribed and non-transcribed strands. This mutation results in a nonsense (W→stop) mutation in codon 359 of the 6–4 photolyase

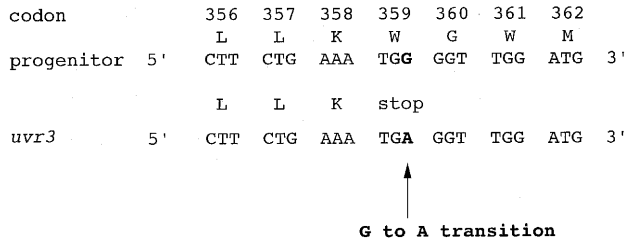

Figure 7. Sequence change in the *uvr3* 6–4 photoreactivation-deficient mutant. DNA from the 6–4 photolyase gene was amplified by PCR from the *uvr3* mutant and genomic DNA of its progenitor. The position of the mutation is indicated in bold.

gene. This result strongly suggests that the 6–4 photolyase homolog is indeed required for photoreactivation *in vivo*.

DISCUSSION

Although photoreactivation of CPDs is a thoroughly characterized and well-understood phenomenon, the existence of a 6–4 photolyase activity was only recently discovered, in *Drosophila* (2). A later report describing photoreactivation of 6–4 products in *Arabidopsis* (9) suggested that this phenomenon was not limited to insects and indeed this activity has since been described in extracts from fish (8), amphibians and reptiles. Genes expressing 6–4 photolyase activity have been cloned from both *Drosophila* and *Xenopus* and a gene with a high degree of sequence similarity (termed hsCRY1) has been cloned from humans, although its function remains unknown. We have cloned a 6–4 photolyase homolog from the higher plant *Arabidopsis*. Two lines of evidence indicate that this clone produces a functional 6–4 photolyase upon expression in *E.coli*. First, expression of the clone in *uvrA*$^{-}$, *recA*$^{-}$, *phr*$^{-}$ *E.coli* strain NKJ3002 enhanced UV resistance of this strain in a visible light-dependent manner (Fig. 2). Secondly, purified GST fusion protein was shown to bind specifically to DNA containing 6–4 products (Fig. 4) and to repair these lesions in a visible light-dependent manner (Fig. 5).

We have also shown that a mutant line of *Arabidopsis* (*uvr3*) that is defective in photoreactivation of 6–4 products carries a nonsense mutation in this gene. From this data we conclude that the 6–4 photolyase homolog sequence corresponds to the *UVR3* gene. Although previously cloned 6–4 photolyase genes have been shown to produce a functional photolyase in extracts from heterologous expression systems, this constitutes the first demonstration of a requirement for this sequence for photoreactivation of 6–4 products *in vivo*.

We have shown that *Arabidopsis* 6–4 photolyase, like other photolyases and the cryptochrome blue light receptor, is a flavoprotein (Fig. 6; 1,11,16,28). These proteins utilize reduced FAD as a chromophore. In the *E.coli* CPD photolyase FADH^{-} is the active form, which donates an electron to the CPD resulting in splitting of the cyclobutane ring (1). In the *Arabidopsis* blue light receptor oscillation of FAD between its different redox states determines the response wavelength for each plant cell (28). In the *Drosophila* 6–4 photolyase electron transfer from excited FAD to a 6–4 product which has been thermally converted to an oxetane intermediate restores the original form (29). The *Arabidopsis* 6–4 photolyase probably functions via the same mechanism.

The *UVR3* gene encodes a polypeptide of 62 kDa with high sequence homology to 6–4 photolyases from *Drosophila* and *Xenopus* (45 and 47% amino acid identity respectively). It also

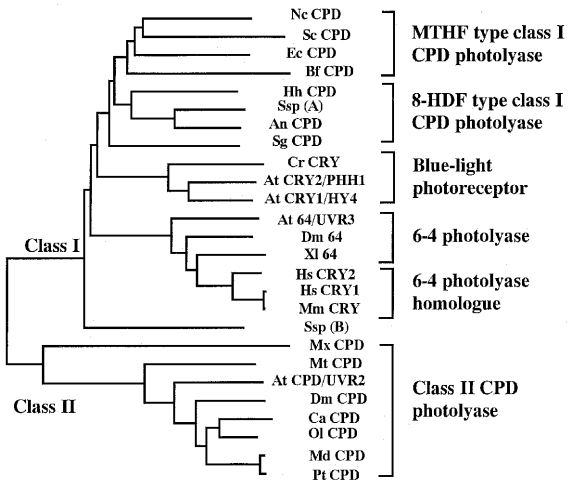

Figure 8. An unrooted phylogenetic tree based on alignment of photolyase/blue light photoreceptors. The tree was generated by software package ODEN (National Institute of Genetics, Japan). Species abbreviations are: Hs, *Homo sapiens*; Dm, *Drosophila melanogaster*; Xl, *Xenopus laevis*; At, *Arabidopsis thaliana*; Cr, *Chlamydomonas reinhardtii*; Hh, *Halobacterium halobium*; An, *Anacystis nidurans*; Ec, *Escherichia coli*; Nc, *Neurospora crassa*; Pt, *Potorous tridactylus*; Ol, *Oryzias latipes*; Mx, *Myxococcus xanthus*; Sc, *Saccharomyces cerevisiae*; Bf, *Bacillus firmus*; Ssp, *Synechocystis* spp.; Sg, *Streptomyces griseus*; Mt, *Methanobacterium thermoautotrophicum*; Ca, *Carassius auratus*; Md, *Monodelphis domestica*; Mm, *Mus musculus*. The GenBank accession no. of Ssp (A) is U51943 and Ssp (B) D90909.

shares a lesser degree of amino acid identity (30%) with the *Arabidopsis* CRY1 (HY4) photoreceptor and CRY2 (PHH1) (a gene of unknown function) genes, as well as 17% with the *Arabidopsis* class II CPD photolyase (Fig. 1). The CRY1 and CRY2 proteins have no significant photoreactivation activity (16,17). The *Arabidopsis* class II photolyase protein, encoded by gene *UVR2*, is required for photoreactivation of CPDs but not 6–4 products (6,19,20). In contrast, the *UVR3* gene is required for repair of 6–4 products but not for repair of CPDs. Thus these two photolyase genes, although they undoubtedly originated from a single gene, have, during the course of evolution, developed specialized and non-overlapping substrate specificities.

In fact, *Arabidopsis* has four photolyase-related sequences: two of these are the functional photolyases described above (*UVR2* and *UVR3*); a third (*CRY1*/*HY4*) is a blue light receptor and the function of the fourth homolog (*CRY2*/*PHH1*) is unknown. Interestingly, the *Arabidopsis* 6–4 photolyase is more closely related in sequence to the blue light receptor than to the CPD photolyase, while the blue light receptors are themselves very similar in sequence to the microbial class I CPD photolyases. A phylogenetic tree obtained from amino acid sequence comparison shows the clear separation of class I and class II photolyases, as well as subdivision of class I sequences into CPD photolyases, blue light photoreceptor(s) and 6–4 photolyases (Fig. 8). The class I CPD photolyases can be further divided into two subclasses, carrying MTHF or 8-HDF second chromophores. A similar result was reported recently by Kanai *et al.* (30), who determined that photolyase/blue light photoreceptor family genes originated from what was presumably an ancestral CPD photolyase gene via at least eight gene duplication events early in evolution, yielding the photolyases and photoreceptors found in extant organisms.

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