

An *Arabidopsis* photolyase mutant is hypersensitive to ultraviolet-B radiation

[blue light/cyclobutylpyrimidine dimer/DNA repair/pyrimidine pyrimidinone (6–4) dimer/*uvr2-1*]

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ABSTRACT Photolyases are DNA repair enzymes that use energy from blue light to repair pyrimidine dimers. We report the isolation of an *Arabidopsis thaliana* mutant (*uvr2-1*) that is defective in photorepair of cyclobutylpyrimidine dimers (CPDs). Whereas *uvr2-1* is indistinguishable from wild type in the absence of UV light, low UV-B levels inhibit growth and cause leaf necrosis. *uvr2-1* is more sensitive to UV-B than wild type when placed under white light after UV-B treatment. In contrast, recovery in darkness or in light lacking photo-reactivating blue light results in equal injury in *uvr2-1* and wild type. The *uvr2-1* mutant is unable to remove CPDs *in vivo*, and plant extracts lack detectable photolyase activity. This recessive mutation segregates as a single gene located near the top of chromosome 1, and is a structural gene mutation in the type II CPD photolyase *PHR1*. This mutant provides evidence that CPD photolyase is required for plant survival in the presence of UV-B light.

Ultraviolet-B (UV-B; 280–320 nm) radiation, which is increasing in the biosphere as a result of stratospheric ozone depletion by chlorofluorocarbons (1), damages DNA and is toxic and mutagenic to cells. It is not surprising that plants, which require sunlight for photosynthesis, have developed unique strategies such as the inducible synthesis of UV-absorptive secondary metabolite sunscreens (2) to avoid the detrimental effects of UV-B. In spite of these protective filters, some UV-B penetrates into plant cells and causes DNA damage. While significant progress has been made in elucidating mechanisms of DNA repair in bacteria and mammals, our understanding of DNA repair processes in plants remains rudimentary (3).

The major type of DNA damage caused by exposure to UV-B is the formation of dimers between adjacent pyrimidines, i.e., the cyclobutylpyrimidine dimer (CPD) and the pyrimidine pyrimidinone (6–4') dimer [(6–4) photoproduct]. Unrepaired dimers are lethal to cells because they deform the DNA helix, interfering with both replication and transcription (3). Photolyases use energy from blue light (400–500 nm) to directly reverse pyrimidine dimer bonds, accurately repairing the damage (4). Whereas most of the known photolyases are specific for the removal of CPDs, a photolyase gene that encodes a (6–4) photoproduct-specific enzyme was recently sequenced from *Drosophila* (5), and there is evidence for a similar enzyme activity in *Arabidopsis* (6), *Xenopus laevis* (clawed toad), and *Crotalis atrox* (rattlesnake) (7).

Although light-dependent DNA repair is documented in bacteria, yeast, animals, and plants, it is apparently not universal (4). While the presence of photolyase in humans remains a controversial topic (8, 9), a human homolog of the *Drosophila* (6–4) photolyase was recently cloned (5). The photolyase genes characterized to date fall into two classes based on sequence homology: class I “microbial” enzymes (including *Escherichia coli*, yeast, and *Neurospora crassa*) and the class II “higher eukaryotic” enzymes (10–12). Interestingly, the blue light photoreceptor of plants has strong sequence similarity to class I photolyases, presumably reflecting their common use of blue light for catalysis (13).

Mutants can help to elucidate the biochemical mechanisms that plants employ for protection from UV-B. This approach recently allowed assessment of the importance of UV-absorptive sunscreens in *Arabidopsis* and maize (14, 15), and identification of a UV-sensitive *Arabidopsis* mutant defective in dark repair of (6–4) photoproducts (16). In this study we report the isolation of a mutant of *Arabidopsis* that is hypersensitive to UV-B and defective in photorepair of CPDs. These results demonstrate that repair of CPDs is critical for survival of *Arabidopsis*, and presumably other plants as well, under UV-B radiation.

MATERIALS AND METHODS

Growth Conditions, Mutant Isolation Strategy, and Genetic Characterization. *Arabidopsis thaliana* Landsberg *erecta* (*Ler*) was the wild-type (WT) plant used in this study. *uvr1*, a mutant defective in dark repair of (6–4) photoproducts (16), was used as a control in root growth assays. Plants were grown under continuous light from CW1500 cool-white fluorescent lamps (General Electric; approximately 100 $\mu\text{mol m}^{-2}\text{sec}^{-1}$ photosynthetically active radiation) filtered through 0.13-mm thick mylar (AIN Plastics, Mt. Vernon, NY; removes wavelengths <310 nm) in Cornell growing mix in pots or on nutrient agar medium in plates as described (14). Supplemental UV-B light from F40UVB fluorescent lamps (Phillips, Somerset, NJ) or T12F40 UVB lamps (Ultraviolet Resources International, Cleveland), filtered through either glass or cellulose acetate (to remove wavelengths <280 nm), was monitored using a spectroradiometer (model OL 752; Optronics Laboratory,

Abbreviations: (6–4') photoproduct, pyrimidine pyrimidinone (6–4) dimer; CPD, cyclobutylpyrimidine dimer; UV-B, ultraviolet-B; UV-BBE, biologically effective UV-B; WT, wild type.

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Orlando, FL) calibrated with an OL 752–150 calibration module and OL 752–10 spectral irradiance standard as described (14). For comparison with previous work, UV-B irradiance was converted to biologically effective UV-B [kilojoules (kJ) biologically effective UV-B (UV-B_{BE})⁻²·h⁻¹] using the Caldwell action spectra normalization (17). Unweighted UV-B doses are also provided, expressed in kJ·m⁻². Spectra from the F40UVB lamps with the filters described above are as described (14), and the T12F40 UVB lamps have a fluence spectrum similar to the F40UVB lamps.

To screen for UV-B-sensitive mutants, M₂ plants derived from ethyl methanesulfonate mutagenesis of *Ler* (18) were grown in soil for 5 days under constant illumination from mylar-filtered cool-white fluorescent lamps. The plants were then subjected to supplemental UV-B treatment by turning on F40UVB lamps (1.5 kJ·m⁻²·h⁻¹ unweighted UV-B and 0.4 kJ UV-B_{BE}·m⁻²·h⁻¹) for 1 or more days. Seedlings exhibiting symptoms of UV-B sensitivity, such as growth inhibition and browning or bleaching of cotyledons and leaves, were allowed to recover and self-pollinate in the absence of UV-B. The resulting M₃ progeny were grown with or without UV-B as described above to test the phenotypic uniformity and specificity of the UV-sensitivity trait. More than 200 individuals were chosen for progeny testing from approximately 150,000 M₂ seeds screened, and 14 met the criteria for UV-B sensitivity after the M₃ screen. Because it is one of the most highly UV sensitive of the mutants identified, *uvr2-1* was chosen for further characterization. The mutant line used in all experiments was derived by backcrossing once to *Ler*. Genetic mapping was conducted using the codominant microsatellite markers *nga59* and *nga63* (19) and cleaved amplified polymorphic sequence markers NCC1 and ATEAT1 (20) for an F₂ population derived from a cross between *uvr2-1* and the WT Columbia ecotype.

Root Sensitivity to UV-B. Root sensitivity to UV-B was determined as described by Britt *et al.* (16) with the following modifications: seedlings were germinated under Sylvania F40GO gold lights (6 μmol·m⁻²·sec⁻¹ photosynthetically active radiation); UV-B (1.32 kJ·m⁻²·min⁻¹) was supplied by a model UVT750-M Ultraviolet Transilluminator (International Biotechnologies) with a peak output of 314 nm, filtered through 0.13-mm-thick cellulose acetate (<0.2 J·m⁻²·min⁻¹ from λ < 280 nm). Following UV-B treatment, plants were either wrapped in foil or placed under CW1500 cool-white lamps with mylar filters to allow photorepair to occur in the absence of UV-B. Approximately 35 roots per experiment were measured for each treatment (except for *uvr1*, for which approximately 15 roots were measured per treatment).

In Vivo Dimer Repair. Plants were grown on nutrient agar plates for 12 days under CW1500 cool-white lamps with mylar filters, and then treated with 1.32 kJ·m⁻² unweighted UV-B using the transilluminator. Plates were harvested immediately (0 hr control), or allowed to recover either wrapped in aluminum foil or replaced under cool-white lamps. All plants were harvested under F40GO gold lights by pouring liquid nitrogen directly onto the plates, scraping seedlings (50–100 per sample) into chilled tubes, and freezing immediately at –80°C. DNA was extracted from frozen tissue and dimers quantified using CPD- or (6–4) photoproduct-specific monoclonal antibodies (21). Samples were measured in quadruplicate and compared by ANOVA within each experiment using the computer program STATISTICA. The Newman–Keuls procedure was used for pairwise comparison (22). In three experiments, initial average CPD damage levels were 1.9, 1.3, and 1.7 dimers per 10⁵ bases in *Ler* and 2.3, 1.2, and 1.8 dimers per 10⁵ bases in *uvr2-1*.

Photolyase Activity. Three-week-old plants grown on nutrient agar plates were either maintained under CW1500 cool-white lamps filtered through mylar, immediately wrapped in foil, or treated with supplemental UV-B from F40UVB lamps

(1.5 kJ·m⁻²·h⁻¹ unweighted UV-B; 0.4 kJ·UV-B_{BE}·m⁻²·h⁻¹) for 24 hr and then harvested using liquid nitrogen. Extracts were prepared as described (23), and a chromatographic assay for removal of CPDs from heavily irradiated bacterial DNA substrate was performed as described (24). Assay incubation times (up to 19 hr) were chosen such that activity was proportional to the amount of extract (typically 100–600 μg) for at least three time points. Dark-incubated control extracts showed no CPD removal.

RESULTS

Isolation and Genetic Characterization of *uvr2-1*. The *uvr2-1* mutant was identified in a screen for plants whose growth is strongly inhibited when treated with supplemental UV-B. *uvr2-1* is very sensitive to UV-B: chronic exposure to daily UV-B doses lower than that experienced by plants under clear-sky sunlight is sufficient to dramatically inhibit growth of *uvr2-1* seedlings (Fig. 1A). Symptoms of UV-B sensitivity are also observed following short-term UV-B treatment: as little as 4 hr of irradiation produces withering and necrosis of expanded leaves within 6 days after UV-B (Fig. 1B Middle). UV-B sensitivity is observed throughout development, from early vegetative growth through flowering. The mutant is indistinguishable from the WT when grown in the absence of UV-B (Fig. 1B Top), demonstrating that the injury is caused by UV-B.

The *uvr2-1* recessive mutation defines a single genetic locus. When *uvr2-1* and WT were crossed, F₁ progeny were UV-resistant; self-pollinated F₂ progeny segregated in a 3:1 ratio of resistant:sensitive plants (*n* = 715, χ² = 0.029). The mutation mapped to the top of chromosome 1, with only one recombination event occurring between *uvr2-1* and NCC1 in 266 F₂ plants. This places the two loci within 0.2 centimorgans of each other (Fig. 1C). One hundred of the *uvr2-1/uvr2-1* F₂ plants were also analyzed for segregation of *nga63* and ATEAT1 (Fig. 1C). Although a previously reported UV-B sensitivity mutation (*uvh6*) is also located at the top of chromosome 1, it is not tightly linked to *uvr2*, mapping 12 centimorgans from NCC1 (M. Jenkins and D. Mount, personal communication) and 1.6 centimorgans from *nga59* (25).

***uvr2-1* UV-B Hypersensitivity Depends on Light Conditions.** Because it is known that repair of pyrimidine dimers by photolyase requires blue light, we asked whether light influences the UV-B sensitivity of *uvr2-1*. WT and *uvr2-1* were grown without UV-B until the seedlings had two to four fully-expanded true leaves and then were treated with 1.7 kJ·UV-B_{BE}·m⁻²·h⁻¹ (5.0 kJ·m⁻²·h⁻¹ unweighted UV-B) from two T12F40 UVB lamps for 0.5, 1, 4, 8, or 16 hr. When plants recovered in the light, cotyledons and leaves of *uvr2-1* that had previously been exposed to UV-B for ≥ 4 hr turned brown and died, whereas WT was unaffected by this treatment (Fig. 1B Middle). The shorter treatments had no effect on either genotype. Eight- and 16-hr UV-B treatments caused injury to WT leaves, yet *uvr2-1* was more severely damaged than WT. In contrast, both WT and *uvr2-1* were injured by 4 hr UV-B when allowed to recover under gold lights, which lack photoreactivating wavelengths (Fig. 1B Bottom). Under these nonphotoreactivating conditions, *uvr2-1* was indistinguishable from WT for all UV-B doses tested.

Although roots receive only a low fluence of UV-B and blue light in soil, *uvr2-1* roots also exhibit light-dependent UV-B sensitivity (Fig. 2). Consistent with a defect in a light-dependent repair process, *uvr2-1* did not exhibit the light-stimulated recovery seen in the WT. Following treatment with 2.64 kJ·m⁻² unweighted UV-B, new growth of *uvr2-1* roots was approximately 50% that of WT. As expected, the light-independent (6–4) photoproduct repair mutant *uvr1* (16) was more sensitive to UV-B than WT in the dark, but not in the light (Fig. 2).

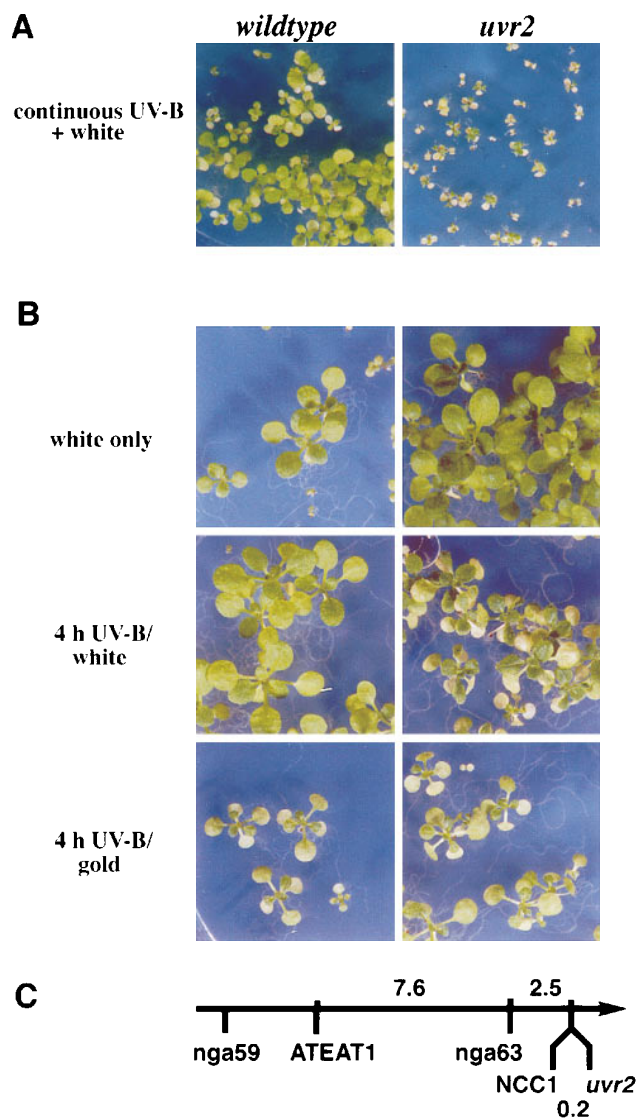


FIG. 1. Phenotypes and map location of *uvr2-1*. (A) Seed were germinated on nutrient agar plates; beginning on day 3 seedlings were exposed to continuous UV-B from F40UVB lamps ($0.13 \text{ kJ-UV-B}_{\text{BE}} \text{ m}^{-2}\text{-h}^{-1}$; $0.64 \text{ kJ-m}^{-2}\text{-h}^{-1}$ unweighted UV-B) and photographed 10 days later. This daily UV-B_{BE} dose is approximately one-half that of a clear mid-summer day in Ithaca, New York. (B) Thirteen-day-old, light-grown plants with two to four fully expanded true leaves were treated with UV-B from two T12F40 ($1.7 \text{ kJ-UV-B}_{\text{BE}}\text{m}^{-2}\text{-h}^{-1}$; $5.0 \text{ kJ-m}^{-2}\text{-h}^{-1}$ unweighted UV-B) for 0.5, 1, 4, 8, or 16 hr (4-hr treatment shown), and then either returned to grow under light from CW1500 cool-white lamps filtered through mylar (Middle) or placed under F40GO gold fluorescent lights (Bottom) for 7 days. Plants of the same age grown continuously under CW1500 cool-white lamps with mylar filters are shown for comparison (Top). (C) Map locations of *uvr2-1* and nearby markers on the top of chromosome 1. Distances are in centimorgans. The arrow points toward the centromere; the relative map position of *nga59* is shown for orientation. Map distances to ATEAT1 and *nga63* were determined using 100 *uvr2-1/uvr2-1* F₂ plants.

***uvr2-1* Is Defective in CPD Repair.** The light dependence of differences in UV-B sensitivity between WT and *uvr2-1* was consistent with the hypothesis that this mutant is defective in photorepair of UV-induced DNA damage. To test this, CPDs and (6-4) photoproducts were measured in plants treated with an acute dose of UV-B, followed by recovery in the light or dark. Fig. 3 illustrates that, in contrast to WT, *uvr2-1* showed no statistically significant removal of CPDs in the light ($P > 0.05$ in each experiment after 6 hr of recovery). The observa-

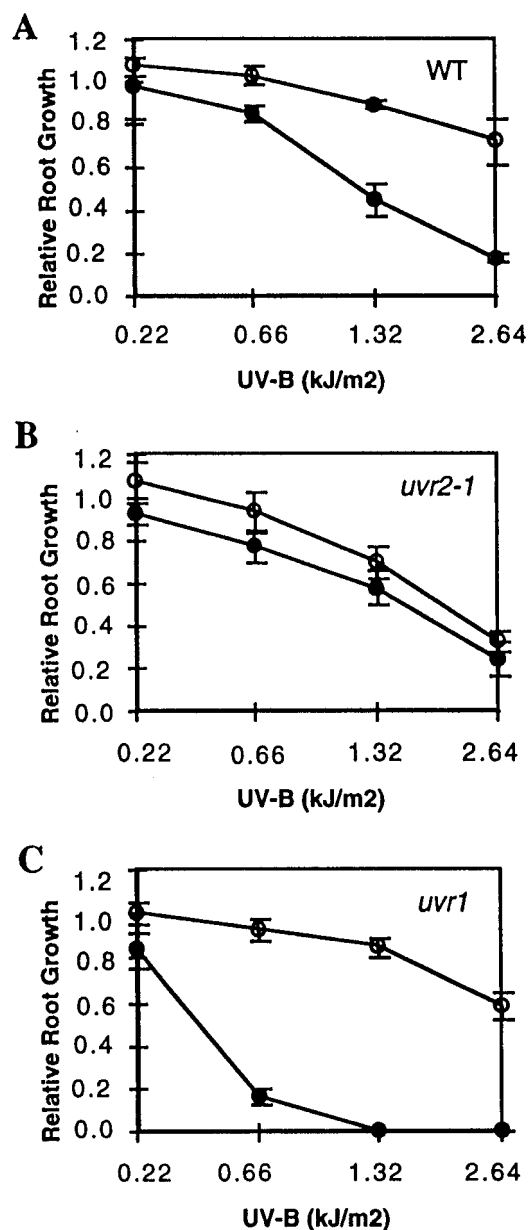


FIG. 2. Root growth of *uvr2-1* is affected by UV-B during recovery in the light, but not in the dark. Three-day-old seedlings were treated with 0.22 – 2.62 kJ-m^{-2} unweighted UV-B ($1.32 \text{ kJ-m}^{-2}\text{-min}^{-1}$) from a transilluminator followed by 24 hr incubation under CW1500 cool-white lamps with mylar filtration or in the dark, to allow new root growth. (A) WT. (B) *uvr2-1*. (C) *uvr1*. Open symbols, light; solid symbols, dark. Standard error is indicated with vertical lines. Data are the means of three experiments with root growth normalized to that of untreated plants.

tion that both WT and *uvr2-1* removed (6-4) photoproducts in the light indicates that *uvr2-1* specifically affects light repair of CPDs. Repair, rather than shielding, is implicated because UV-B generated a similar number of CPDs in WT and *uvr2-1* tissues. Furthermore, HPLC analysis of flavonoids and hydroxycinnamic acids also indicated that UV-B sunscreens are normal in *uvr2-1* (L.G.L. and R.L.L., unpublished results). Neither WT nor *uvr2-1* showed statistically significant repair of CPDs or (6-4) photoproducts in the dark.

***uvr2-1* Lacks Photolyase Activity.** Persistence of CPDs in irradiated *uvr2-1* tissue suggested a defect in photolyase. This activity was assayed in extracts from plants adapted to darkness, maintained in the light, or treated with UV-B (Table 1).

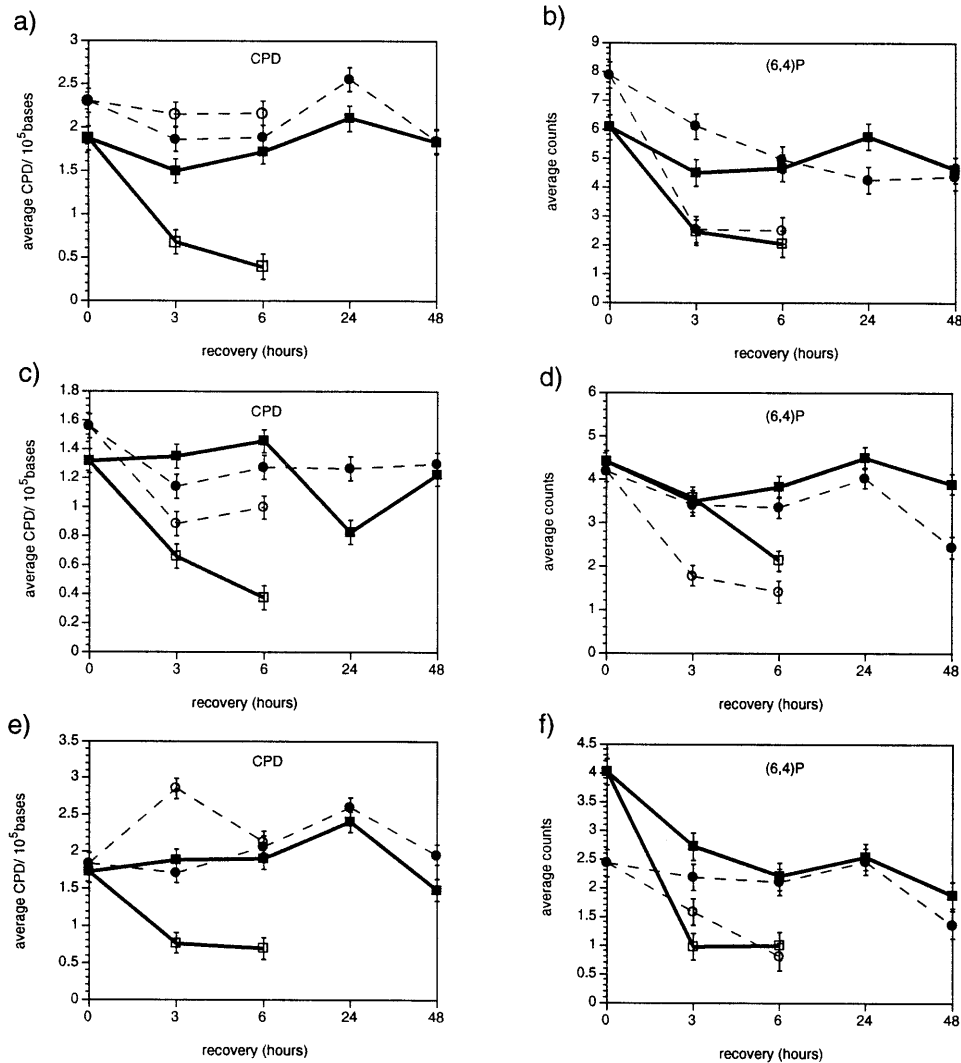


FIG. 3. CPD and (6-4) photoproduct levels in *Ler* and *uvr2-1* during recovery. WT (squares/solid lines) and *uvr2-1* (circles/dashed lines) plants were irradiated with $1.32 \text{ kJ}\cdot\text{m}^{-2}$ UV-B from a transilluminator and allowed to recover under CW1500 cool-white lamps with mylar filtration (open symbols) or in the dark (solid symbols). The data show three experiments with monoclonal antibody detection for CPDs (a, c, and e) and (6-4) photoproducts (b, d, and f). Values are the means of four measurements for each sample; vertical bars are standard error.

As predicted, extracts from *uvr2-1* plants had no detectable photolyase activity when assayed under saturating levels of both dimer substrate and photoreactivating photons. Light-grown wild-type plants had approximately 4-fold higher photolyase activity than those adapted to the dark for 24 hr. UV-B

Table 1. Photolyase activities in plant extracts

Extract	Dimers (10^6) removed per hr per μg of protein*		
	Dark [†]	Light [†]	Light + UV-B [†]
WT	215 (± 24)	783 (± 54)	750 (± 66)
<i>uvr2-1</i>	<14	<10	<12

Three-week-old seedlings were either wrapped in foil for 24 hr (dark), maintained under CW1500 cool-white lamps with mylar filters (light), or supplemented with UV-B from F40UVB lamps ($2 \text{ kJ}\cdot\text{m}^{-2}\cdot\text{h}^{-1}$ unweighted UV-B; $0.6 \text{ kJ}\cdot\text{UV-B}_{\text{BE}}\cdot\text{m}^{-2}\cdot\text{h}^{-1}$; light + UV-B) for 24 hr.

*Means for four trials each. Standard deviations for WT given in parentheses.

[†]Using a second set of extracts, mixing WT and *uvr2-1* (1:1), yielded 93% (dark), 100% (light), and 102% (+UV-B) of the expected WT values with *uvr2-1* samples contributing no activity or inhibition.

treatment apparently did not increase photolyase activity, although it is possible that we underestimated activities of UV-B treated extracts, i.e., from competition between dimers in endogenous plant DNA and the irradiated bacterial DNA substrate. In an independent experiment, mixing equal volumes of WT and *uvr2-1* extracts yielded 93% (dark adapted), 100% (light grown), and 102% (UV-B treated) of the expected WT values with *uvr2-1* samples contributing no activity or inhibition. These data indicate that *uvr2-1* does not contain a photolyase inhibitor and that WT extracts do not supply a cofactor missing in *uvr2-1*, consistent with a defect in the photolyase apoprotein.

DISCUSSION

uvr2-1 is a UV-B-sensitive mutant of *Arabidopsis* that completely lacks detectable photolyase activity and light-dependent repair of CPDs. *uvr2-1* is defective in the light-mediated repair of CPDs, but not (6-4) photoproducts, and is more sensitive to UV-B than WT in the light, but not in the dark. These characteristics distinguish *uvr2-1* from *uvr1*, which defines a gene involved in dark repair of the (6-4) photoproduct (16). *uvr2-1* appears to be a novel mutation because it is genetically unlinked to both *uvr1* and the previously reported

UV-sensitivity mutations *uvh1* (25) and *uvh6* (M. Jenkins and D. Mount, personal communication.).

Previous reports demonstrated photolyase activity in higher plants (for examples see refs. 3, 23, and 26), but this is the first direct evidence that CPD removal is necessary for survival. Although plant genes with homology to class I photolyase have been described from *Arabidopsis* and mustard, all available data indicate that these encode blue-light photoreceptors (13, 27–29). Recently, an *Arabidopsis* gene with strong similarity to higher eukaryotic class II photolyases was cloned (*PHR1*) (30), and recent results indicated that *uvr2-1* is allelic to this photolyase gene. For example, *PHR1* is genetically linked to *uvr2-1*, the *uvr2-1* mutant has a frameshift mutation in *PHR1* (30) with a concomitant reduction of *PHR1* mRNA (L.G.L., J.L., and R.L.L., unpublished results).

The absence of photolyase activity in plant extracts (Table 1) and the lack of *in vivo* CPD repair (Fig. 3) in *uvr2-1* suggest that there is only one CPD-specific photolyase in *Arabidopsis*. While the existence of independent photolyases for nuclear and chloroplastic DNA was suggested by a *Chlamydomonas* mutant deficient in photolyase activity (31), recent experiments indicate that young *Arabidopsis* seedlings do not repair CPDs in mitochondrial or chloroplastic DNA (32), and may require only a nuclear CPD photolyase. *uvr2-1* is fully capable of light repair of the second major form of pyrimidine dimer, the (6–4) photoproduct (Fig. 3), suggesting that like class I photolyases, the class II *Arabidopsis* enzyme only repairs CPDs.

Although light-independent repair of (6–4) photoproducts was reported in *Arabidopsis* and wheat (16, 33), our results suggest that this did not occur under our experimental conditions. While a great deal of variability was seen for light-independent (6–4) photoproduct repair, there was no statistically significant repair in WT, nor significant differences observed between *uvr2-1* and WT. While this discrepancy with published results may reflect technical differences [for example, our transilluminator emitted a significant amount of 314 nm light, which could affect the proportion of (6–4) dimers in the Dewar isomer form], the disparity in plant developmental stage may also be relevant. Published studies demonstrating dark repair of (6–4) photoproducts used germinating seedlings, whereas we used older plants. It is possible that the relative amount of DNA repair completed by dark- vs. light-dependent mechanisms is developmentally regulated in *Arabidopsis*.

Differences in the quality or quantity of UV-B radiation may also be responsible for the differences reported with respect to dark repair of CPDs. For example, in agreement with our results, previously published work with *Arabidopsis* (16) showed no repair of CPDs in the absence of light when plants were treated with $1.3 \text{ kJ}\cdot\text{m}^{-2}$ unweighted UV-B from a transilluminator (peak output at 305 nm). In contrast, dark repair of CPDs was detected in *Arabidopsis* plants treated with $1 \text{ kJ}\cdot\text{m}^{-2}$ UV from a germicidal lamp (peak output 254 nm; ref. 23), and wheat seedlings treated with approximately $4 \text{ kJ}\cdot\text{m}^{-2}$ UV-B_{BE} from a fluorescent lamp (33). It was previously demonstrated that UV-B dose can affect the amount of CPDs repaired in the dark: at low UV-B doses dark repair was undetectable in alfalfa, but at higher doses dark repair of CPDs was readily observed (26). It thus is likely that the spectral quality of UV as well as the total dose, may affect induction of dark repair mechanisms. These complexities illustrate that much remains to be learned about the repair of DNA in plants. The *uvr2-1* mutant, in addition to showing for the first time that light repair is essential for the survival of a higher plant, will

enhance these studies, because this mutant must rely solely on dark repair to remove CPDs.

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