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# UV-B-Inducible and Temperature-Sensitive Photoreactivation of Cyclobutane Pyrimidine Dimers in *Arabidopsis thaliana*[1]

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## ABSTRACT

**Removal of cyclobutane pyrimidine dimers (CBPDs) *in vivo* from the DNA of UV-irradiated eight-leaf seedlings of *Arabidopsis thaliana* was rapid in the presence of visible light (half-life about 1 hour); removal of CBPDs in the dark, presumably via excision repair, was an order of magnitude slower. Extracts of plants contained significant photolyase *in vitro*, as assayed by restoration of transforming activity to UV-irradiated *Escherichia coli* plasmids; activity was maximal from four-leaf to 12-leaf stages. UV-B treatment of seedlings for 6 hours increased photolyase specific activity in extracts twofold. *Arabidopsis* photolyase was markedly temperature-sensitive, both *in vitro* (half-life at 30°C about 12 minutes) and *in vivo* (half-life at 30°C, 30 to 45 minutes). The wavelength dependency of the photoreactivation cross-section showed a broad peak at 375 to 400 nm, and is thus similar to that for maize pollen; it overlaps bacterial and yeast photolyase action spectra.**

Stratospheric ozone depletion projected for the early 21st century is expected to increase the amount of DNA-damaging UV-B (290–320 nm) radiation at the earth's surface by as much as 20 to 50% (2, 10, 33). This has renewed interest in the UV-resistance mechanisms of plants, which will be exposed continually to increased UV-B fluxes.

Repair of ultraviolet-light-induced DNA damage is a feature of life at all levels of complexity. Studies of bacteria (particularly *Escherichia coli*), yeast, and mammalian cells have elucidated four distinct mechanisms by which cells cope with UV-damaged DNA (for reviews see refs. 9, 29). In photoreactivation, a single enzyme, photolyase, uses light energy in the 300 to 500 nm range to reverse a specific class of UV photoproducts—*cis, syn* CBPDs.[2] In excision repair, an ensemble of proteins act together to remove a wide variety of helix-distorting lesions, including at least two UV photoproducts—CBPDs and pyrimidine-(6–4)-pyrimidone photoproducts (6–4 photoproducts). Photoproducts that block DNA replication are tolerated, rather than repaired, by recombinational filling of daughter-strand gaps or by (necessarily error-prone) DNA synthesis past the lesions. Photoreactivation has been demonstrated in maize pollen (15), pinto bean sprouts (27), water plants (*Wolffia* and *Spirodela*) (5), and wild carrot (14), cultured tobacco (36), and *Ginkgo* (37) cells, but has not been detectable in several plant species (26, 27). Excision repair has been less readily demonstrated, with positive results for water plants (5), grass pea (34), and carrot protoplasts (14). There is some indirect evidence for recombinational repair in *Chlamydomonas* (25). The observation that photoreactivation reduces the frequency of mutation of UV-irradiated pollen grains is consistent with error-prone synthesis past UV photoproducts (16).

The small crucifer *Arabidopsis thaliana* has become recognized as a useful model green plant, because of the many features that make it an attractive subject for genetic studies, and its amenability to the techniques of molecular biology. Thus, it seems a logical starting point for systematic investigations of the genetics and biochemistry of plant resistance to ultraviolet light.

Photoreactivation is the simplest UV repair response, and obviously plants are supplied with abundant light of the appropriate wavelengths. We have tested *Arabidopsis* extracts for photolyase, and whole plants for light-dependent removal of CBPDs. Photoreactivation proves to be the predominant mode of CBPD repair in *Arabidopsis*. The activity is markedly temperature-sensitive, both *in vivo* and *in vitro*, but it is increased in plants treated with UV-B light.

## MATERIALS AND METHODS

### Growth of Plants

All plants were *Arabidopsis thaliana* ecotype Columbia (from Walt Ream, Oregon State University), or C-10 subline of Columbia (from David Mount, University of Arizona). Young plants were usually grown in 10 × 2.5 cm plastic Petri dishes on MSS-agar (0.43% Murashige-Skoog salt mixture [pH 5.7, Gibco Labs], 1% sucrose, 0.001% nicotinic acid, 0.01% pyridoxine-HCl, 0.004% glycine, 0.0001% thiamine-HCl, 0.01% myo-inositol, and 0.8% agar [Difco]) at 22°C in Conviron 8601 growth chambers, at 30 cm from a bank of eight Philips Westinghouse cool-white lamps (F48112/cw/ho) with 16-h light, 8-h dark periods. Before growth-chamber incubation, seeds were sterilized (70% ethanol, 2 min; 5% NaOCl + 0.5% SDS, 15 min; $H_2O$ rinse, 5 times), and vernalized on the agar surface for 7 d at 4°C. Growth rates of

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[2] Abbreviations: CBPD, cyclobutane pyrimidine dimer; ESS, endonuclease-sensitive site.

individual plants on a plate (typically 20–25 plants per plate) and on duplicate plates were quite similar. Mature plants were obtained by growth in a greenhouse on commercial garden soil (after vernalization), in pots with $H_2O$ subirrigation, supplemented every 7 d with MSS solution subirrigation. For UV-B experiments, plants were grown in 9.5 × 9.5 × 9.5 cm plant-tissue-culture containers (Flow Laboratories), containing about 3 cm of MSS-agar. The container material proves to be an excellent cut-off filter at about 295 mm: transmittances at 290 and 300 nm were 0.1 and 47%, respectively; these are, respectively, less and greater than transmittance by cellulose acetate. To provide for aeration and heat escape, covers were separated from containers by about 1.2 cm, and the space sealed with gas-permeable 3M filter tape (Carolina Biological Supply).

### Preparation of Protein Extracts and Assay of Photolyase

Stems and leaves were collected at specified growth stages and frozen in liquid nitrogen. Material (typically 0.5 g from 35–40 plants) was subsequently ground to a fine powder, using a mortar (70-mm o.d.) and pestle under liquid nitrogen, and suspended in 1 mL PX-Buffer (100 mM Tris-HCL [pH 7.4], 2 mM $Na_2EDTA$, 20 mM β-mercaptoethanol, 20% glycerol, 10 mM dithiothreitol); debris was removed by sedimentation in a microfuge at 13,000 rpm for 15 min at 4°C. Protein concentrations were determined by the method of Lowry (20). Extracts are stable for months at −80°C, but gradually lose activity at 4°C, where 15% activity remained after 2 weeks.

Substrates for photolyase assays were pUC19 plasmid (23) DNA (radiolabeled with [$^3$H] thymidine, typically to 50,000 cpm per μg), irradiated with 254-nm light at 1 J per $m^2$/s, at DNA concentrations of 65 to 70 μg/mL, in TE buffer (10 mM Tris-HCl [pH 7.4], 1 mM $Na_2EDTA$ [pH 7.4]). Extracts were diluted with PHR buffer (50 mM Tris-HCl [pH 7.4], 1 mM $Na_2EDTA$, 10 mM NaCl, 10 mM dithiothreitol), mixed with DNA substrate to give a final volume of 100 μL, and incubated at 23°C under two 20-W GE Black Lights (F20T-12-BL) at a distance of 15 cm, with cellulose acetate shielding. Dark-control samples were incubated under red light for 3 min and wrapped in aluminum foil. Aliquots of reaction mixtures were used directly for transformation of *Escherichia coli* strain FD2566, as described (12). This strain is completely UV-repair-deficient (Δ*uvr*B Δ*rec*A Δ*phr*), hence cannot be transformed with plasmids containing even a single lethal UV lesion. The relative transformation efficiently $t$, defined as the ratio of the specific transformation efficiency (CFU [colony-forming units] per ng of [$^3$H]DNA) for UV-irradiated DNA to the specific efficiency for unirradiated DNA, is expected to be given by $\exp(p)$, where $p$ is the average number of randomly (Poisson) distributed lethal UV lesions per plasmid. Thus $p = -ln\ (t)$. Typical transformation efficiencies were 100 CFU per ng for unirradiated DNA, and 0.3 CFU per ng (5.5 lethal lesions per plasmid) and 0.025 CFU per ng (8.8 lethal lesions), for DNA irradiated to 100 J/$m^2$ and 150 J/$m^2$, respectively.

### Heat Inactivation of Photolyase in Crude Extracts

Crude extracts, typically 200 μg protein in 500 μL, were heated in a water bath at specified temperatures; 40-μg aliquots were removed at various times. These, and unheated 40-μg control samples, were assayed with 100 ng of UV-irradiated 100 J/$m^2$ plasmid DNA as described above. In DNA protection experiments, 200 μg of extract protein were incubated for 10 min at room temperature under red light with 200 ng of unirradiated or irradiated (400 J/$m^2$) calf-thymus DNA before mixtures were heated and 40-μg aliquots assayed as above; unheated control samples (40 μg) were assayed in the presence of 40 ng calf thymus DNA. The effect of the protease inhibitor PMSF on heat inactivation of photolyase was tested by adding PMSF (0.1 M in acetone) to a concentration of 0.2 mM to 200 μg of plant extract in 500 μL, incubating at 23°C for 10 min, heating as specified, and assaying 40-μg aliquots for photolyase activity as described above.

### Determination of Wavelength Dependence of Photoreactivation Cross-Section

Aliquots of extracts from eight-leaf *Arabidopsis* (200 μg in 500 μL), in a quartz spectrophotometer cuvette, were assayed for their ability to photoreactivate 500 ng of UV-irradiated (100 J/$m^2$) plasmid DNA during a 20-min incubation in the sample chamber of a Bausch and Lomb model 500 monochromator, while illuminated with light of a specific wavelength. The energy at each wavelength was kept approximately constant at 2 mW/$cm^2$ (measured with a YSI-Kettering model 65 radiometer) by adjusting the entrance slit. In parallel experiments *Arabidopsis* extracts were heated at 50°C for 8 min, cooled, and mixed with 2 ng purified *E. coli* photolyase (gift of Aziz Sancar, University of North Carolina) or 15 ng purified *Anacystis nidulans* photolyase (gift of APM Eker, Delft University), and assayed using light of specific wavelengths.

### Extractions of Plant DNA and Assay for UV-ESS

Stems and leaves (about 0.5 g), typically from 35–40 plants grown to the 8- to 10-leaf stage on MSS-agar plus [$^3$H] thymidine (2 μCi per mL), were ground under liquid nitrogen, suspended in 0.5 mL of 2 × CTAB buffer (CTAB buffer is 1% cetyltrimethylammonium bromide, 50 mM Tris-HCl [pH 8.0], 10 mM $Na_2EDTA$, 0.7 mNaCl, 0.5% polyvinylpyrrolidone [mol wt 40,000]), and heated at 65°C for 3 min in the presence of 50 ng tRNA carrier. Extraction with chloroform: isoamyl alcohol (24:1), precipitation of polysaccharide with 0.1 volume of a solution of 10% CTAB in 0.7 M NaCl, precipitation of DNA with one volume of a solution of 1% CTAB in 50 mM Tris-HCl (pH 8.0), 10 mM $Na_2EDTA$, solubilization of the DNA pellet in TES (10 mm Tris-HCl [pH 8.0], 1 mM $Na_2EDTA$, 1 M NaC1), precipitation in ethanol-NaAcetate, and solubilization in 1/10 × TE were essentially as described by Rogers and Bendich (24). In order to prevent the accumulation of single-strand breaks during storage, plant DNA preparations were treated with 0.3 mg/mL pronase (previously self-digested) for 1 h at 37°C, then extracted with one volume of phenol in the presence of 0.1% SDS, precipitated with ethanol-Na acetate, and redissolved in 1/10 TE. DNA concentrations were determined by the dye-fluorescence method of Labarca and Paigen (18). Typical

yields from 0.5 g of material were 1.5 to 2.5 μg of DNA, average mol wt 25 to 30 × $10^6$, specific activity 2 to 3 × $10^4$ cpm/μg. Mol wts were estimated by sedimentation in neutral sucrose gradients in the presence of $^{14}$C-labeled phage λ-DNA.

For determination of cyclobutane pyrimidine dimers, DNA samples (100–200 ng; about 4000 CPM) in 100 μL UVE buffer (10 mM Tris-HCl [pH 7.6], 20 mM $Na_2EDTA$ [pH 7.6], 50 mM NaCl) were treated with dilute highly purified phage T4 endonuclease V (gift of Dr. Stephen Lloyd, Vanderbilt University) for 90 min at 37°C, then mixed with 10 μL of 1 M NaOH and 3000 CPM of phage λ-[$^{14}$C] DNA (mol wt 3 × $10^7$), layered on top of 5 mL of a 5 to 20% alkaline sucrose gradient, and sedimented for an appropriate time (typically 3 h) at 45,000 rpm in a Beckman SW 50.1 rotor. Radioactivity profiles for 30 fractions were used to determine apparent weight-average mol wt, assuming mol wt proportional to the 0.35 power of the sedimentation coefficient (13). The average frequencies of ESS per nucleotide were calculated from the formula ESS/nt = $2[1/X_w - 1/X_o]$, where $X_o$ and $X_w$ are the weight-average numbers of nucleotides in single-stranded DNA before and after UV endonuclease treatment, respectively (35). The assumption of this equation, that mol wt are approximately randomly distributed, in treated and untreated samples, was supported by the sedimentation profiles. Doubling the amount of UV endonuclease did not significantly increase apparent ESS frequencies. Growth-chamber and greenhouse light intensity in the photoreactivation-active region was measured with a YSI-Kettering radiometer, with light below 300 nm and above 500 nm cut off by a K-1 filter.

### UV Irradiation of Plants

Plants grown to about eight-leaf stage on [$^3$H]MSS-agar at 22°C were irradiated at a rate of 10 J/m$^2$/s, typically to 1000 J/m$^2$, using a single 15-W germicidal lamp (254 nm), and incubated in a growth chamber until plants were harvested for UV endonuclease assays. For assay of postirradiation dark repair, plates were wrapped in aluminum foil. For measurement of temperature effects, plants were shifted to 30 or 37°C growth chambers or to a greenhouse for 1 h (sufficient to bring agar to ambient temperatures [34°C in the greenhouse]), irradiated to 1000 J/m$^2$ and incubated, with or without wrapping in foil, at elevated temperature for various times. The irradiation manipulations required plants to be out of the heated growth chambers for less than 2 min.

For testing the effect of UV-B treatment on photolyase levels, plants in MSS-agar-containing plant-tissue-culture containers were incubated in a growth chamber at 22°C, with or without complete filtering of UV-B frequencies by 0.13-mm polyester film (optically equivalent to Mylar D), then transferred to a greenhouse at the Environmental Protection Agency Corvallis Laboratory. In the greenhouse, containers were placed equidistant from two broad-band fluorescent lamps (Q-panel UVB-313) at a vertical distance of 30 cm, shielded either with 0.13-mm cellulose acetate (diacetate type) to block UV-C radiation or with 0.13-mm Mylar equivalent. Additional illumination was by 400-W high-pressure sodium vapor lamps and full June sunlight. Intensity, measured by a double-monochromater spectroradiometer (Optronics model 742), and weighted according to the biologically effective UV-B weighting function of Setlow (33), was 11 mW/m$^2$, equivalent to solar noon at high altitudes in the tropics. Plants were irradiated for 7 h with container tops removed. This corresponds to an effective fluence of 280 J/m$^2$, approximately equivalent to the daily UV-B fluence expected for 40% ozone depletion at temperate latitudes (10). The agar temperature increased to 29°C during the treatment, with either cellulose acetate or Mylar shielding. Plants were returned to a 22°C growth chamber for 2 h before leaves and stems were frozen for subsequent extract preparation.

## RESULTS

### Photoreactivation and Dark Repair in Growing Plants

The amount of 254-nm irradiation reaching the earth's surface is insignificant, but laboratory sources of this radiation induce, with high efficiency, CBPDs and other DNA photoproducts of biological interest. Since repair of the former is of interest, we used 254-nm irradiation as a convenient source of CBPDs. To determine whether the UV fluences used had gross biological effects as well, we irradiated to various extents young (six-leaf) plants growing on Petri plates, by covering portions of the plates with foil during the irradiation. The plates were subsequently incubated under photoperiodic light, or in the dark. After 1 week growth in light, plants previously irradiated to 500 J/m$^2$ showed some leaf yellowing but were about the same size as unirradiated plants, those irradiated to 1000 J/m$^2$ showed some diminution in size, and those previously irradiated to 2000 J/m$^2$ were markedly reduced in size—leaves appeared to be about half the width of those on control plants. Unirradiated plants grew poorly in the dark, and the damage resulting from prior UV irradiation was more obvious: even 500 J/m$^2$ caused severe yellowing and some relative growth reduction, and 2000 J/m$^2$ virtually eliminated growth. The extent to which these effects were the result of induction of CBPDs in DNA is not known, and the apparent exacerbation of UV effects by the absence of light could have several explanations besides the absence of photoreactivation.

We directly assayed *in vivo* UV repair processes, by incubating plants in a growth chamber on [$^3$H]thymidine-containing agar (which efficiently radiolabeled plant DNA), irradiating to a fluence of 1000 J/m$^2$, continuing incubation in the growth chambers, treating extracted plant DNA with (CBPD-specific) UV endonuclease, and analyzing the frequency of UV-ESS by alkaline-sucrose sedimentation (Fig. 1). Light-dependent removal of CBPDs was relatively rapid—50% removal in 1 h, and essentially complete repair (90% removal) in 6 h. Removal of CBPDs in the dark, presumably via excision repair, was much slower—50% removal in 8 to 17 h, and only 85% removal after 96 h. Thus, photoreactivation appears to be the predominant means by which CBPDs are repaired in *Arabidopsis*.

### Photolyase Activity in *Arabidopsis* Extracts

To measure repair activity in plant tissue extracts, we used an assay previously employed in studies of UV repair in oocytes of the frog *Xenopus laevis* (12). Mutants of the bacterium *Escherichia coli* that lack photoreactivation, excision

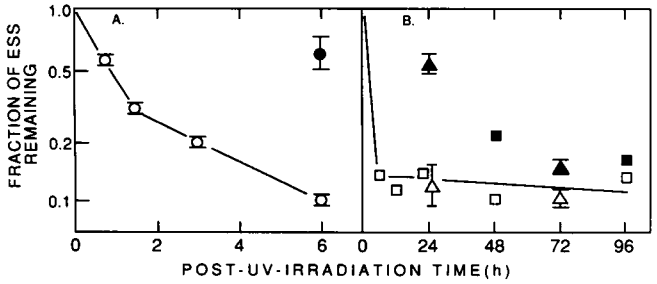


**Figure 1.** Repair of cyclobutane pyrimidine dimers *in vivo*. Plants were grown at 22°C on MSS-agar plates containing [$^3$H]thymidine (2 $\mu$Ci/mL) to 8-leaf stage, irradiated at 254 nm to 1000 J/m$^2$, and incubated at 22°C under growth-chamber illumination (open symbols) or wrapped in foil (filled symbols). Extraction of DNA from 30 to 50 plants harvested at indicated times, and measurement of ESS, by treatment with phage T4 UV endonuclease and sedimentation in alkaline sucrose, were as described under "Materials and Methods." A, Short-term experiment, with continuous illumination. Data (○, ●) correspond to averages from two or three ESS determinations, with standard deviations indicated. B, Long-term experiments, with photoperiodic illumination. Data are a composite from two experiments and correspond to one ESS determination (□, ■) or average of two determinations (△, ▲) with ranges indicated. Relative ESS of 1.0 corresponds to 51 (△), 70 (□), or 57 (○) ESS per 10$^6$ nucleotides. DNA from unirradiated plants contained less than 2 ESS per 10$^6$ nucleotides except for 24 h (dark) and 24 h (light), 5.5 and 2.2 per 10$^6$ nucleotides, respectively. Single-strand weight-average mol wts (before UV-endonuclease treatment) were 13 to 15 × 10$^6$, and did not vary significantly with UV-irradiation, incubation time, or exposure to light.

repair, and recombinational "repair" (Δ*phr*Δ*uvrB*Δ*recA*) cannot be transformed by plasmids that contain even a single UV lesion. The transformation frequency for irradiated plasmids should, therefore, equal the fraction of plasmids containing no lesions, $exp(-p)$, if an average of $p$ UV lesions per plasmid are randomly (Poisson) distributed. The lethal lesions thus defined by the natural logarithm of the relative transformation frequency include both CBPDs and pyrimidine (6–4) pyrimidone photoproducts (6–4 photoproducts), since the mutant bacteria can repair neither. The number of lethal lesions so defined is a linear function of UV fluence, as predicted, and exceeds the number of CBPDs (measured by UV-endonuclease assay) by 15 to 25% (12), consistent with expected ratios of CBPDs to 6–4 photoproducts (1, 22).

By this assay, extracts of *Arabidopsis* shoots showed good repair activity in the presence of 365-nm light (Fig. 2). The rate of repair was constant for at least 40 min at room temperature; restoration of transformability reached a plateau corresponding to removal of 65% of lethal lesions. The rate of reaction was proportional to the amount of protein present if less than 20 $\mu$g was used, and remained constant when protein was increased from 80 to as much as 200 $\mu$g (data not shown). There was no repair activity in boiled extracts, or when reactions were performed in the dark (data not shown), indicating that all of the repair observed correspond to photoreactivation rather than excision repair. We do not know why the plateau for the *E. coli* photolyase reaction corresponded to a slightly higher restoration of transformability. Perhaps some plasmids were inactivated by aborted excision repair in *Arabidopsis* extracts.

*Arabidopsis* photolyase activity was absent from seeds, was submaximal in 2-leaf plants, and was approximately constant from the 4-leaf (7 d growth) to the 12-leaf stage (15 d) (Fig. 3). Even 20-d-old and 30-d-old plants showed one-half and one-third maximal activity, respectively. In a second experiment, a different set of plant extracts, assayed with a plasmid irradiated to 150 J/m$^2$, showed about 50% higher activities, but again showed half-maximal activity at the 2-leaf stage and a plateau at the 4-leaf to 12-leaf stages. There was no detectable photolyase in seeds.

### Thermosensitivity of *Arabidopsis* Photolyase

The photolyase activity appeared quite temperature-sensitive in extracts—half-lives were about 12 min at 30°C and 1.3 min at 50°C (Fig. 4), and about 1.8 min at 40°C (data not shown). *Arabidopsis photolyase* activity was partially protected from heat inactivation by its substrate, UV-irradiated DNA, but not by unirradiated DNA (Fig. 4). Although we cannot rule out thermal activation of proteolysis, addition of the proteolytic inhibitor PMSF did not significantly affect inactivation kinetics; half-lives at 30°C were 11.2 min in the presence of PMSF and 13 min in its absence, and were 1.4 min in both cases at 50°C.

In order to determine whether *Arabidopsis* photolyase was thermosensitive *in vivo,* we grew plants on [$^3$H]thymidine-containing plates at 22°C to the eight-leaf stage, shifted them to 30°C or 37°C for 1 h, irradiated them to a fluence of 1000 J/m$^2$, and continued growth-chamber incubation at the respective temperatures. Samples of control plants (postirradiation incubation at 22°C), withdrawn at 45 min and 90 min for UV-endonuclease assays of CBPDs, showed the expected photoreactivation (Fig. 5). However, 30°C plants exhibited reduced photolyase activity during the first 45 min, and none

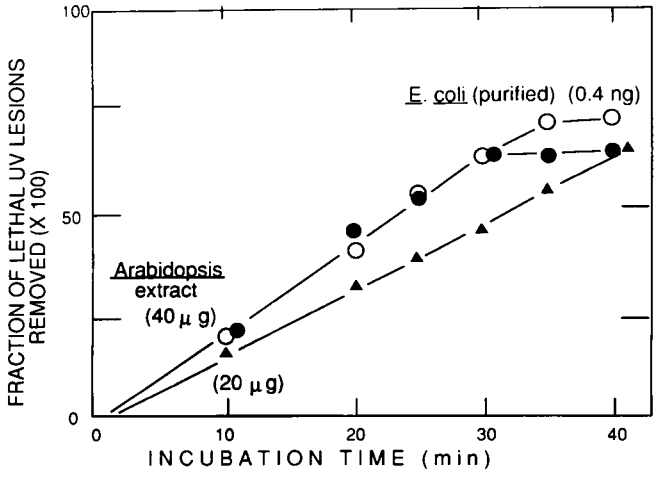


**Figure 2.** Time course of photoreactivation *in vitro*. Preparation of extracts from stems and leaves of 8-leaf-stage plants, and assay at 23°C of 40 $\mu$g (●) or 20 $\mu$g (▲) of extract using 100 ng of UV-irradiated plasmid DNA was as described under "Materials and Methods." Purified *E. coli* photolyase (0.4 ng) (gift of A. Sancar) was assayed in parallel (○).

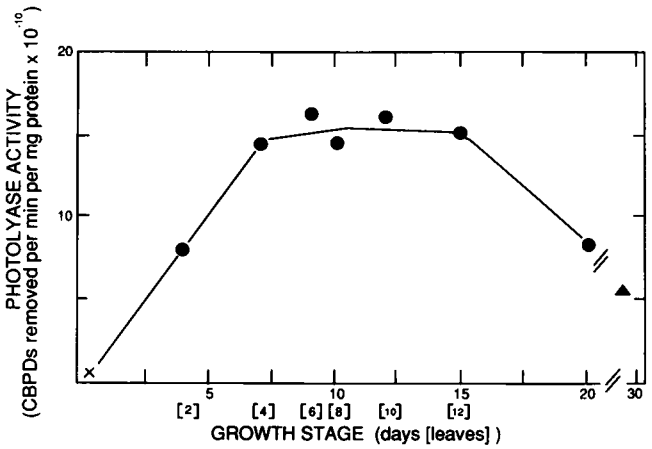


**Figure 3.** *Arabidopsis* photolyase activity at various growth stages. Extracts were prepared from plants harvested at indicated times, and photoreactivation of UV-irradiated plasmid DNA was assayed under conditions where activity was directly proportional to extract protein (20 μg, 30 min), as described under "Materials and Methods." (×), extract of seeds; (●), extracts from plants grown on MSS-agar in growth chamber; (▲), extract of plants transferred to soil after 20 d and grown under room lighting. Data correspond to averages of duplicate assays, with range ±3 to 11%, except for 20-d (±20%) and 30-d (±25%) samples.

thereafter; 37°C plants were almost completely inactive from the start (Fig. 5). Plants incubated in a greenhouse, under solar illumination only, attained a temperature of 34°C; their photolyase activity was intermediate between the activities of the 30 and 37°C growth-chamber plants. Light intensities between 300 and 500 nm were about $1.3 \times 10^5$ ergs/cm$^2$/s in the growth chamber and $1.2 \times 10^6$ ergs/cm$^2$/s in the greenhouse. The intensity of the black light used for photoreactivation *in vitro* was about $1.3 \times 10^4$ ergs/cm$^2$/s.

Presumptive excision repair, measured at a single dark time point, appeared to be slightly increased at 30 and 34°C, but to be inactivated at 37°C (Fig. 5).

### Wave-Length Dependence of Photoreactivation Cross-Section of *Arabidopsis* Photolyase *in Vitro*

Photolyases characterized thus far appear to fall into two classes with respect to their essential chromophores (6). All photolyases appear to contain flavin adenine dinucleotide. Those of one class, exemplified by the enzymes from *E. coli* and the yeast *Saccharomyces cerevisiae,* show maximum activity in the presence of light at about 370 nm in *E. coli,* reflecting activity of a pterin second chromophore (17). The yeast photolyase polypeptide is active in *E. coli* (29), and the *E. coli* polypeptide is active in yeast (19), consistent with the notion that the two enzymes contain similar second chromophores. Photolyases of the second class, of which enzymes from the mold *Streptomyces griseus* and the cyanobacterium *Anacystis nidulans* are examples, show maximum activity at about 445 nm, corresponding to a deazaflavin second chromophore (7).

To determine, without obtaining a rigorous action spectrum, whether the *Arabidopsis* photolyase fell into either of these classes, we took advantage of the thermosensitivity of the *Arabidopsis* activity. Extracts were heated for 8 min at 50°C, to inactivate over 98% of *Arabidopsis* photolyase, then mixed with photolyase purified from either *E. coli* or *A. nidulans* (kind gifts of A Sancar and APM Eker, respectively). We compared the wavelength dependence of photoreactivation catalyzed by these mixtures with the wavelength of photoreactivation by unheated *Arabidopsis* extracts, on the assumption that effects of turbidity, absorbance, wave-length dispersion at high slit widths, and other nonidealities would be approximately the same (Fig. 6). The *Arabidopsis* enzyme (solid triangles) showed a broad peak at 375 to 400 nm. Thus, although the wavelength dependence of *Arabidopsis* photolyase was not identical to that of the *E. coli* enzyme (solid circles), it was more similar to the latter than to the *A. nidulans* enzyme (solid squares). Despite the nonrigorous nature of the determination, the data is in good agreement with the respective published action spectra for the enzymes from *E. coli* (dotted lines) (28), and *A. nidulans* (dashed lines) (7), especially in the region of interest from 375 to 450 nm. The crude spectra show some broadening, particularly at the highest wavelengths.

### UV-B Induction of Photolyase

UV-B light induces increased synthesis of enzymes involved in flavonoid biosynthesis, resulting in increases in levels of these UV-absorbing compounds (11, 21). Flavonoids appear

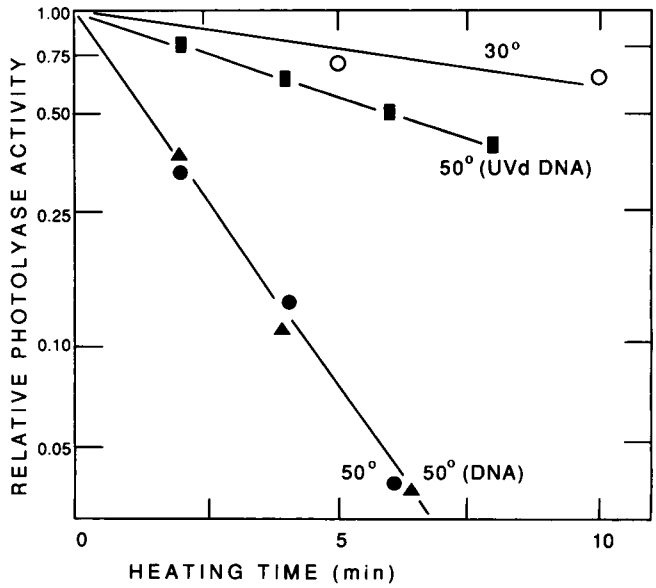


**Figure 4.** Temperature sensitivity of *Arabidopsis* photolyase *in vitro*. Extracts of 8-leaf-stage plants in PHR buffer (200 μg in 500 μL) were heated in the absence of added DNA at 30°C (○) or 50°C (●) or at 50°C in the presence of 200 ng of unirradiated calf thymus DNA (▲) or of calf thymus DNA irradiated to 400 J/m$^2$ (■), for the times indicated, and cooled immediately to 0°C. Aliquots (40 μg) were assayed for photolyase activity by incubation with 100 ng UV-irradiated plasmid DNA for 30 min at 23°C, as described under "Materials and Methods." (UV-irradiated calf thymus DNA was also present during assay of the appropriate unheated control sample.)

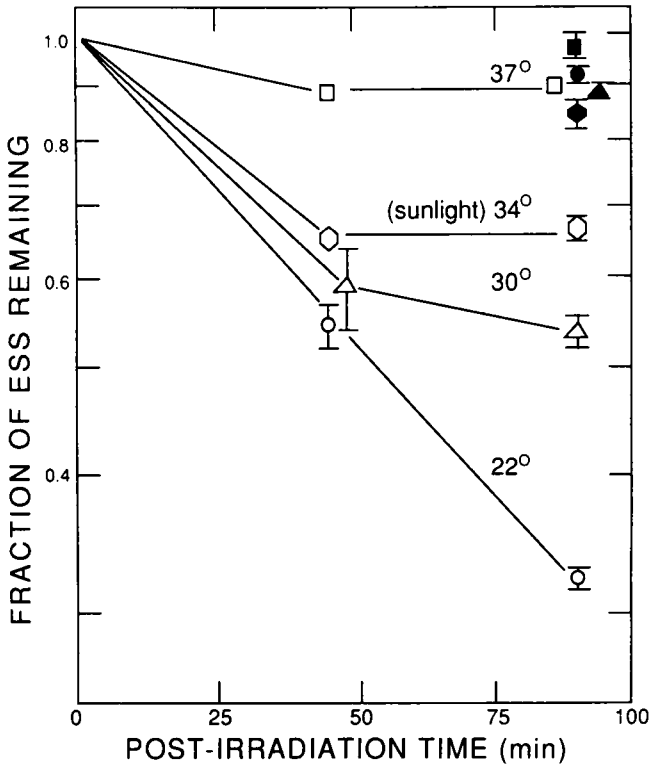


**Figure 5.** Temperature sensitivity of *in vivo* photoreactivation by *Arabidopsis*. Plants were grown on MSS-agar plates containing [$^3$H] thymidine (2 $\mu$Ci/mL) to 8-leaf stage and shifted to a growth-chamber warmed to 30°C (△, ▲) or 37°C (□, ■) or to a greenhouse under direct sunlight (○, ●) for 1 h, by which time the agar medium had come to the chamber temperature, or to 34°C, in the sunlight sample. Control plants (○, ●) remained at 22°C. Plants were then irradiated at 254 nm to 1000 J/m$^2$, and incubation at the same temperature continued for the indicated times, in the light (open symbols) or dark (filled symbols). Extraction of DNA and analysis for UV-ESS was as described under "Materials and Methods" and the legend to Figure 1. Relative ESS frequency of 1.0 corresponds to 53 (○), 52 (△), 58 (□), or 54 (○) ESS per $10^6$ nucleotides. Single-strand weight-average mol wts (before UV endonuclease treatment) were all 13 to 14 × $10^6$. Data correspond to averages for two ESS determinations, error bars indicate ranges. Where indicated, the ranges between determinations were less than the size of the symbol.

to protect plants from UV damage (2), so photolyase, an enzyme that repairs UV damage, might also be UV-B-inducible. In an initial experiment, plants growing in plant-tissue-culture containers (which absorbs strongly at 290 nm and below, but passes most light above 300 nm, were treated with UV-B in a greenhouse at the Environmental Protection Agency (EPA) Corvallis Laboratory. Plants subjected for 6 h to a lamp-(cellulose acetate) filter arrangement that models UV-B-enhanced sunlight, showed a modest increase (25%) in photolyase specific activity in extracts (data not shown). UV-B irradiation for only 3 h, or Mylar-filtered UV-B, had no effect, and irradiation for 9 h (with or without subsequent 3-h or 16-h growth-chamber incubation) did not further increase photolyase activity. Although the apparent UV-B effect was small, it corresponded to a 60 to 70% increase in the transformation efficiency of UV-irradiated plasmids treated with the respective extracts, and thus seemed significant.

The apparent low induction factor could reflect a high basal level of photolyase, perhaps induced by a small UV-B component in the growth chamber illumination, or suboptimal inducing radiation. We addressed both of these possibilities by incubating plants in a growth chamber with four different Mylar shielding protocols for 9 d (8-leaf stage) then treating them for 7 h with UV-B in topless containers, in the EPA greenhouse. The UV-B intensity corresponded to the highest currently expected on the earth (solar noon, equator, high altitudes). There was little difference between the photolyase specific activities of growth-chamber plants incubated entirely or partially without Mylar shielding (Fig. 7A, open symbols) and the activities of plants incubated with shielding (filled symbols). However, subsequent UV-B treatment of the four corresponding sets of plants caused in every case a doubling of the rate of photoreactivation of lethal UV lesions by extracts, corresponding to a fourfold increase in the transformation efficiency of photoreactivated plasmids (Fig. 7B, open symbols), whereas Mylar-filtered UV-B had no effect (filled symbols). Extracts from UV-B-treated plants were able to repair as many as 80% of total lesions, in contrast to the 65% plateau observed previously (Fig. 1).

## DISCUSSION

We have subjected a model green plant, *Arabidopsis thaliana,* to UV light treatments that induced significant frequen-

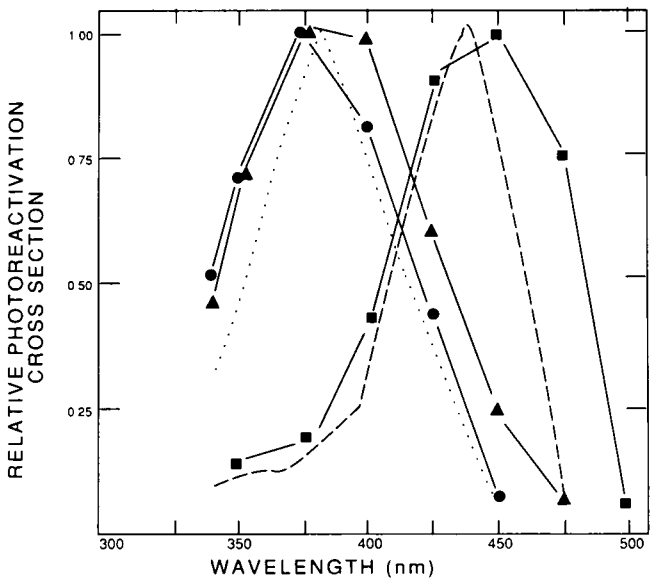


**Figure 6.** Wavelength dependence of photoreactivation: Photoreactivation of UV-irradiated plasmids by *Arabidopsis* extracts (from 8-leaf plants grown on agar at 22°C) (▲) or extracts heated at 50°C for 8 min and supplemented with purified *E. coli* photolyase (●) or purified *A. nidulans* photolyase (■) were assayed in the presence of equal-energy light at the indicated wavelengths, under conditions where activity was directly proportional to amount of protein, and values corrected for energy per photon, as described under "Materials and Methods." Published action spectra for the *E. coli* (28) and *A. nidulans* (7) enzymes are reproduced (· · · and – – –, respectively).

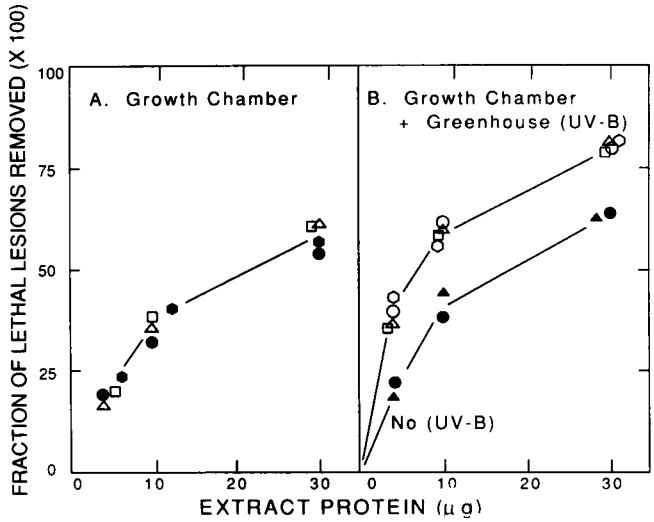


**Figure 7.** UV-B induction of photolyase. A, Effect of shielding against UV-B during growth chamber incubation. Incubation of plants in MSS-agar-containing tissue-culture containers in a growth chamber for 10 d at 22°C under cool-white lamps, with indicated shielding conditions, preparation of extracts, and (plasmid) assay for photolyase were as described under "Materials and Methods." (●), Container shielded with 0.13-mm-thick Mylar D equivalent during d 1 to 10; (△), no additional shielding d 1 to 10; (□), Mylar during d 1 to 7, no shielding d 8 to 10; (●), no shielding d 1 to 7, Mylar during d 8 to 10. B, Effect of postgrowth UV-B irradiation. Plants were incubated 9 d in a growth chamber as described under (A) above [corresponding symbol shapes indicate same growth conditions as in (A), except d 10 omitted], irradiated in topless containers with a UV-B source at the Environmental Protection Agency, Corvallis Laboratory, with or without Mylar shielding, and extracts prepared and assayed for photolyase, as described under "Materials and Methods." Open symbols, 7 h unfiltered UV-B (approximately 280 J/m$^2$); filled symbols, 7 h Mylar-filtered light. The 25% repair corresponds to removal of about $2.5 \times 10^9$ lethal lesions per minute.

cies of CBPDs in its DNA, and determined some characteristics of the CBPD repair processes: (a) UV (254 nm) fluences that result in easily measurable CBPD levels (about one per $2 \times 10^4$ nucleotides) have significant physiological effects; (b) CBPDs are rapidly removed from plant DNA in the light (1 h half-life), but are removed an order of magnitude more slowly in the dark; (c) photolyase activity, as judged by assays of crude extracts of plant tissue, appears early, but not immediately, during development, and persists for at least 30 d; UV-B treatment significantly increases photolyase levels; (d) *Arabidopsis* photolyase is markedly temperature sensitive, both *in vitro* and *in vivo*; (e) light in the 375- to 400-nm range is maximally effective for photoreactivation.

Cyclobutane pyrimidine dimers are generated in DNA at UV-B wavelengths that correspond to terrestrial-surface sunlight (8), as well as by 254-nm irradiation. Whether the observed effects on plant growth and appearance are consequences of DNA damage, of destruction of the photosynthetic apparatus, or of other processes, remains to be determined. Presumably the CBPD levels measured represent averages between high frequencies in cells near leaf surfaces and low frequencies in interior cells, and an average for genomic, chloroplast, and mitochondrial DNA. It would be of interest to determine the extent to which physiological effects of UV irradiation correlate with cell damage and frequencies of induced CBPDs in specific tissues and organelles.

The predominance of photoreactivation of CBPDs over excision repair in *Arabidopsis* is similar to the situation in *E. coli*. In the latter bacterium, dark repair removes 5 to 20 CBPDs per minute (13), whereas photoreactivation can remove as many as 125 to 250 per minute, if light is saturating (29). The approximate equality of sunlight and growth-chamber *in vivo* rates of photoreactivation by *Arabidopsis* suggests that light is not the rate-limiting factor. Thus there appears to be scope for increasing the intrinsic resistance of plants to UV-induced DNA damage by increasing levels of plant photolyase.

In pinto beans, photolyase activity is relatively high in extracts from sprouts, but low in those from 2-week-old leaves, and negligible in those from 6-week-old leaves (27). The persistence of *Arabidopsis* photolyase in mature plants suggests that it will be practical to obtain enough material for biochemical studies.

The surprising temperature sensitivity of *Arabidopsis* photolyase may have important implications with respect to predicted changes in the terrestrial atmosphere. Stratospheric ozone depletion seems likely to increase biologically effective terrestrial UV-B irradiation by 20 to 50% in the early 21st century (2), and increases in $CO_2$ and other greenhouse gases may cause surface air temperatures to be 1.5 to 5.5°C higher (31). The effects of these changes on the biosphere have usually been considered independently of one another. However, if CBPD induction is a major factor in UV-B damage to plants, and if temperature-sensitive photoreactivation proves to be an important defense against DNA damage in food plants, then the combination of increased UV-B and increased temperatures may be highly deleterious to such plants and the animals that depend on them. In the experiments reported here, photoreactivation ceased completely *in vivo* about an hour after the temperature was increased from 22 to 30°C.

The inducibility of *Arabidopsis* photolyase by treatment with a terrestrially attainable UV-B intensity suggests that plants might adapt in part to stratospheric ozone depletion by increasing levels of DNA repair activities, as well as UV-shielding compounds (3). E Wellman (personal communication) has observed UV-B induction of photoreactivation in parsley, but in *E. coli* photolyase appears not to be part of the UV-induced SOS response (29). Recently G Sancar and coworkers (32) have demonstrated substantial increases in expression of the yeast photolyase gene after treatment with UV radiation or UV-mimetic chemicals. The relatively modest induction factor observed for *Arabidopsis* photolyase may reflect high constitutive expression, a suboptimal induction protocol, the high temperatures (about 30°C) during the UV-B treatment in the greenhouse, or a combination of these factors.

The broad wavelength-dependence curve for *Arabidopsis* is virtually identical to the photoreactivation action spectrum reported for maize pollen extracts (15). These two plant spectra are nearly coincident with the low-wavelength half of the much sharper *E. coli* spectrum (Fig. 6). Interestingly, the

broad *Arabidopsis* and maize pollen spectra are coincident with the high-wavelength half of the relatively sharp pinto-bean-sprout spectrum (27). Thus, the maize and *Arabidopsis* spectra may correspond to two photolyases, one similar to the *E. coli* (and yeast) enzymes, one similar to pinto-bean photolyase. There is evidence for two distinct photolyases—one nuclear, one chloroplast-specific—in Chlamydomonas (4), and one aim of future work will be to determine whether such is the case in *Arabidopsis*.

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