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DNA Damage Levels Determine Cyclobutyl Pyrimidine Dimer Repair Mechanisms in Alfalfa Seedlings

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Ultraviolet radiation in sunlight damages DNA in plants, but little is understood about the types, lesion capacity, and coordination of repair pathways. We challenged intact alfalfa seedlings with UV doses that induced different initial levels of cyclobutyl pyrimidine dimers and measured repair by excision and photoreactivation. By using alkaline gel electrophoresis of nonradioactive DNAs treated with a cyclobutyl pyrimidine dimer–specific UV endonuclease, we quantitated ethidium-stained DNA by electronic imaging and calculated lesion frequencies from the number average molecular lengths. At low initial dimer frequencies (less than ~30 dimers per million bases), the seedlings used only photoreactivation to repair dimers; excision repair was not significant. At higher damage levels, both excision and photorepair contributed significantly. This strategy would allow plants with low damage levels to use error-free repair requiring only an external light energy source, whereas seedlings subjected to higher damage frequencies could call on additional repair processes requiring cellular energy. Characterization of repair in plants thus requires an investigation of a range of conditions, including the level of initial damage.

INTRODUCTION

During sunlight exposure, plants receive both photosynthetically active radiation and ultraviolet radiation. Decreasing levels of stratospheric ozone will increase the intensity of ultraviolet B (UV-B, 290 to 320 nm) reaching the earth's surface without changing photosynthetically active radiation significantly. UV-B damages cellular constituents, including DNA, producing cyclobutyl pyrimidine dimers (CPDs) as well as other DNA damages. To deal with such damages, most organisms have developed a variety of repair mechanisms. Unrepaired damage resulting from increased UV-B levels may decrease plant metabolism, growth, and yields.

Predicting the consequences of increased UV-B on plants requires knowledge of the changing UV spectrum, the efficiency of each UV wavelength in inducing each type of damage, and the capacity of plants to deal with such damages. If current UV-B levels inflict damage levels that challenge the repair capacity of a plant, then increased levels of UV-B could lead to serious short- and long-term consequences. If, however, the repair capacity of a plant substantially exceeds the current damage level, some additional damage might be readily repaired and thus not pose a serious problem.

It is essential to understand the repair pathways available to a plant species and the quantitative capacities of each pathway for DNA damages, such as CPDs. In several plants, photoreactivation has been reported to be the predominant, if not the only, enzymatic path employed for repair of UV-induced damages, especially CPDs. Excision of CPDs was not detected in ginkgo (Trosko et al., 1969), *Nicotiana* (Trosko et al., 1968), or *Chlamydomonas* (Swinton and Hanawalt, 1973). Low levels of CPD excision in carrot protoplasts were observed by Howland (1975) and by Eastwood and McLennan (1985); Small and Greimann (1977) found photorepair and excision in chloroplasts. Degani et al. (1980) reported substantial levels of excision but even more rapid photorepair in the water plants *Wolffia microscopica* and *Spirodela polyrhiza*. In wild-type Arabidopsis, Pang and Hays (1991) found that excision was detectable, but photorepair predominated; in the strain *transparent testa* (which is defective in production of UV-absorbing flavonoids), Britt et al. (1993) reported efficient photoreactivation, but excision was not significant in CPD removal.

To understand the capacity of alfalfa to deal with various levels of UV-B in the environment, we exposed seedlings to increasing doses of UV-B radiation and then followed the repair of CPDs either in the dark or in the presence of photoreactivating light (filtered to remove wavelengths that could induce DNA damage). We found that alfalfa seedlings repair CPDs using both excision and photorepair, with the relative contribution of these repair pathways dependent upon the initial damage level. At lower levels of UV damage, photoreactiva-

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tion rapidly repaired CPDs, whereas excision was undetectable. At higher levels of damage, CPDs were repaired by both excision and photoreactivation.

RESULTS

CPD formation in DNA in intact seedlings exposed to UV radiation was quantitated by alkaline agarose gel electrophoresis (Freeman et al., 1986; Quaite et al., 1992, 1994). By using this method, DNA containing pyrimidine dimers is treated with a CPD-specific UV endonuclease, or it is incubated under identical conditions without endonuclease, denatured by alkali, and electrophoresed on an alkaline agarose gel along with molecular length standards. After renaturation of the DNA and staining with ethidium bromide, the profiles of DNA in each lane are obtained using an electronic imaging system that, unlike photography, gives a direct measure of the quantity of DNA at each distance along the direction of electrophoresis (Sutherland et al., 1987a).

Figure 1 shows representative data and their analysis. Figure 1A shows the profile of DNA length standards. Their known molecular lengths and measured distances of electrophoretic migration were used to construct the smooth curve relating molecular length to distance of migration, that is, the electrophoretic dispersion function for this gel.

Figure 1B shows lane profiles for DNA from 302-nm–irradiated seedlings, with and without UV endonuclease treatment. Because both the quantity of DNA and the length of the DNA molecules at each distance in sample lanes were known, we could compute the number average molecular length of the population of DNA molecules in each lane using Equation 1, and from the differences in number average molecular lengths, we could determine the frequency of CPDs in the DNA sample (Equation 2). Number–average–length analysis of these DNA profiles indicates that the CPD frequency in this seedling DNA sample was 18 CPD per megabase (Mb). We used this method to quantitate CPD induction and repair in intact alfalfa seedlings.

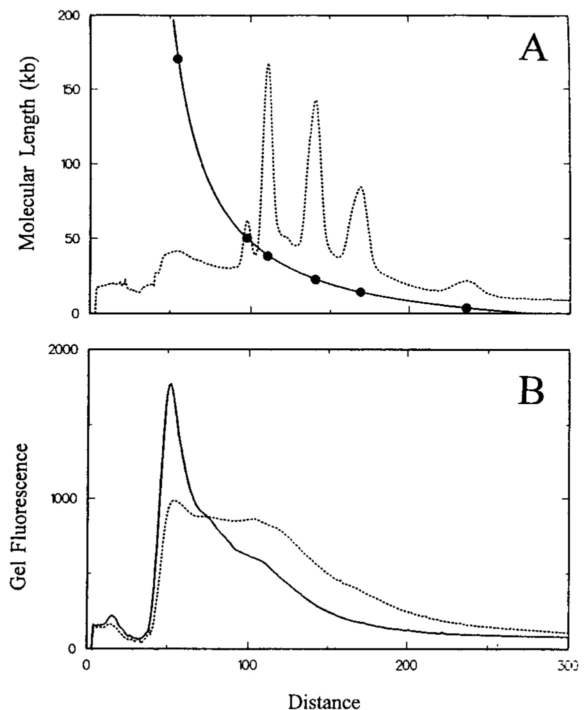

Figure 1. Quantitation of CPDs in DNA from UV-Irradiated Alfalfa Seedlings by Alkaline Agarose Gel Electrophoresis.

(A) Electrophoretic lane profile of DNA molecular length standards (T4, 170 kb; λ, 48.5 kb; T7, 40 kb; and a BglI digest of T7, 22.5, 13.5, and 4 kb) (dotted line). Their migration distance and molecular lengths (●) were used to determine the electrophoretic dispersion function (solid line) for this gel.

(B) DNA from alfalfa seedlings exposed to 302 nm UV radiation, extracted, and either treated (dotted line) or not treated (solid line) with CPD-specific UV endonuclease. The electrophoretic dispersion function in **(A)** and the profiles of the endonuclease-treated and -untreated DNAs were used to determine the number average molecular length (L_n) of each DNA population using Equation 1; the frequency of dimers was then calculated from these number average lengths using Equation 2. The DNA in the sample lanes was determined by these procedures to contain ~18 CPD per Mb.

Many organisms can perform both excision and photorepair, but in plants photorepair has been reported as either the predominant or the only repair pathway. To determine the relative roles of excision and photorepair in alfalfa, we tested the ability of the seedlings to remove CPDs in the absence of visible light as well as in its presence. Seedlings were exposed to low doses of UV-B, producing about 10 CPD per Mb. The seedlings were then harvested immediately, kept in the dark, or exposed to visible light from filtered blue bulbs. Samples were harvested at 0, 30, 60, or 120 min in the light or in the dark, and the CPD frequencies were determined. Figure 2A shows that UV-B-irradiated seedlings exposed to visible light effectively perform photoreactivation of CPDs at a rate of 0.12 ± 0.05 CPD per Mb per min. Figure 2E shows, however, that in the absence of visible light, there is little or no CPD removal by excision (slope = −0.0045 ± 0.018 CPD per Mb per min). Similar results were obtained in experiments in which seedlings were irradiated with UV-B to produce initial CPD frequencies up to ~20 dimers per Mb; photoreactivation was effective in dimer repair, but excision was not detected (cf. Figures 2B [light] and 2F [dark]).

We also examined the roles of excision and photorepair in alfalfa seedlings containing higher levels of CPDs. To stay within the physiological range of responses, we used UV exposures that did not significantly affect growth of the alfalfa seedlings (even without photoreactivating light treatment). Seedlings were exposed to 302 nm radiation to induce 30 to 60 CPD per Mb and then harvested immediately, kept in the dark, or exposed to visible light as usual. Seedlings were harvested at increasing times after UV treatment, and the CPD frequencies were

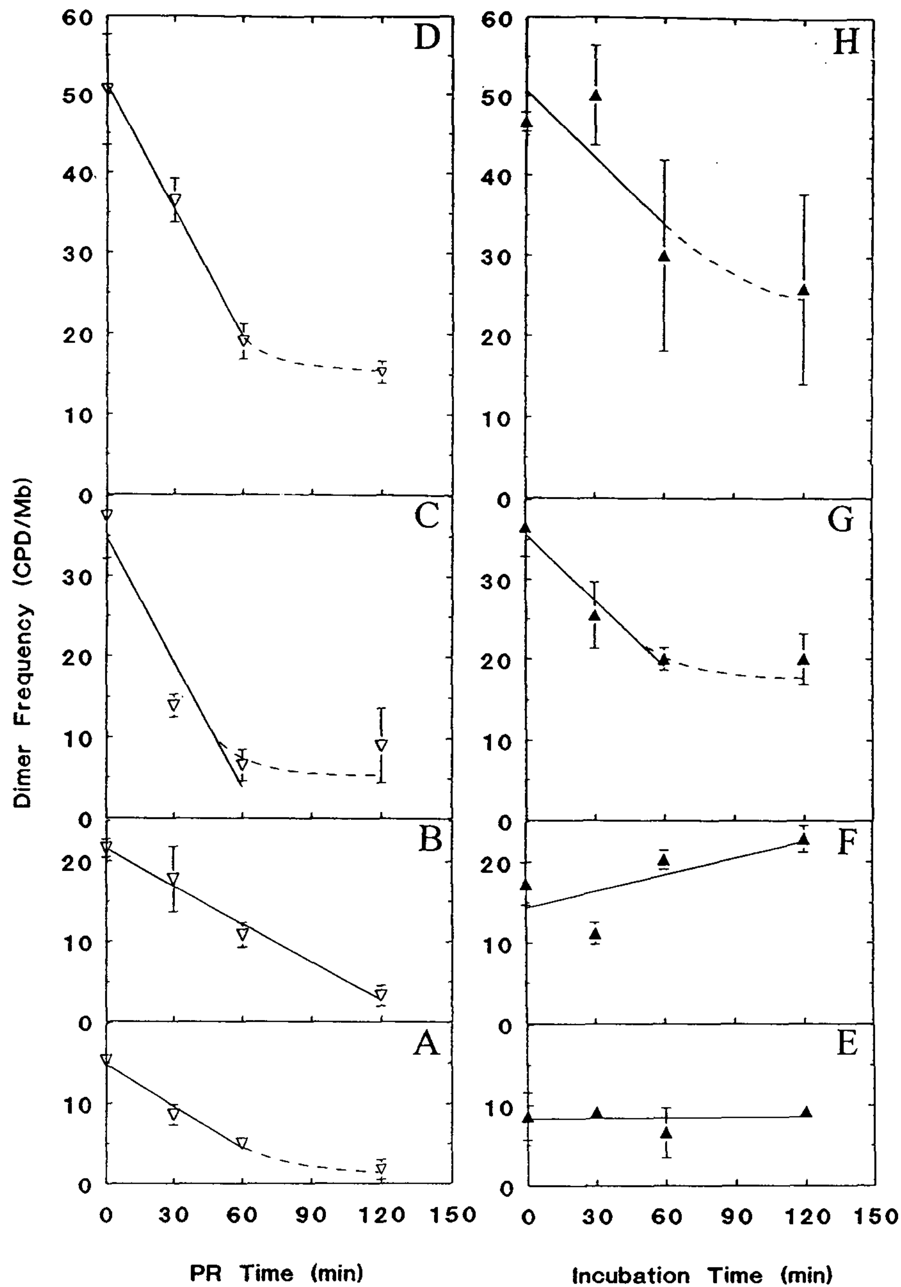

Figure 2. Photorepair and Excision of Cyclobutyl Pyrimidine Dimers in Alfalfa Seedlings Exposed to Low or High Initial Levels of UV-B Radiation.

CPD frequencies as a function of time after UV irradiation were determined for seedlings exposed to photoreactivating light (open symbols) or kept in the dark (closed symbols). The data points are the means of duplicate or triplicate determinations; the standard deviations are shown. The linear least-squares line is drawn through the points used in the calculation of the line (solid line), and a dashed curve was fit by eye to the remaining data points. PR, photorepair.

(A) and **(E)** Repair of CPDs in seedlings exposed to UV-B to give an initial level of ~10 dimers per Mb and then to blue photoreactivating light **(A)** or held in the dark **(E)**.

(B) and **(F)** Repair of CPDs in seedlings with an initial level of ~20 dimers per Mb.

(C) and **(G)** Repair in seedlings with an initial level of ~30 dimers per Mb.

(D) and **(H)** Repair of CPDs in seedlings with an initial level of ~50 dimers per Mb.

determined. Figures 2C and 2G show that at an initial dimer level of ~35 CPD per Mb, photorepair is still predominant, with an overall repair rate of ~0.5 ± 0.12 CPD per Mb per min. However, even in the absence of light, removal by excision is readily detectable at a rate of 0.28 ± .05 CPD per Mb per min. Similarly, at an initial CPD level of ~50 lesions per Mb per min, both photorepair and excision are readily detected (Figures 2D and 2H).

Because the relative roles of excision and photorepair seemed to depend on the damage level, that is, the intracellular concentration of substrate for the two repair systems, we determined the initial repair rates at additional induced dimer frequencies. Groups of seedlings were exposed to increasing doses of 302 nm radiation and harvested immediately or allowed to repair as described above; the DNAs were purified and the CPD content was determined by gel electrophoresis using triplicate or duplicate independent gels. The frequencies of dimers remaining were plotted as a function of repair time, and the initial slopes of the repair function were determined by linear least-squares analysis.

Figure 3 shows the resulting data in which the initial repair rates (the slopes of the linear least-square lines, in CPDs [removed] per Mb per min) and the standard deviation of the slopes are plotted as a function of initial CPD frequency in the seedlings. The rates of CPD repair in the presence of visible light increase with the initial damage frequency, with a slope of ~0.0123 min^{-1} as determined by regression analysis, which also indicated that this slope is different from 0 with a P value of 0.0148.

At low damage levels (up to ~20 CPD per Mb), little or no excision was detected, although photorepair was measured readily. At higher damage levels, the rate of excision repair increased, and at the highest UV doses tested, excision appeared to contribute substantially to the repair of CPDs in alfalfa seedlings. Indeed, regression analysis indicated that the slope (0.0078 min^{-1}) of the line through the data points was significantly different from 0 (P = 0.0035). Although from the present data set we cannot determine the exact shape of the repair function for dimer levels above the threshold value, it is clear that excision repair of CPDs is significant at higher initial damage levels.

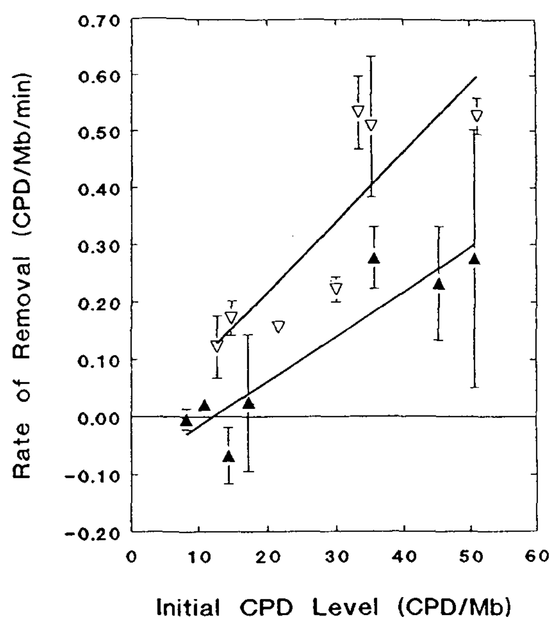

Figure 3. Dependence of Repair Rates on Initial CPD Level.

Seedlings were irradiated with UV-B and either exposed to visible light (open symbols) or kept in the dark (closed symbols). The initial slopes of the repair time courses were determined from individual repair experiments, for example, those in Figure 2, using linear least-squares analysis procedures. The standard deviations of the slopes are also shown. The slope of the photoreactivation kinetics line is 0.0123 min^{-1}; linear regression analysis indicated that this slope is significantly different from 0 (P = 0.0148); the slope of the excision kinetics line is 0.0078 min^{-1}, and regression analysis also indicated that this value is significantly different from 0 (P = 0.0035). At lower initial dimer levels, no significant excision was detected, but photorepair was observed; at higher initial dimer levels, both excision and photorepair contributed to dimer removal.

DISCUSSION

Photoreactivation in alfalfa seedlings efficiently repairs CPDs over a wide range of initial dimer frequencies. The initial rate of photorepair in the seedlings increases as a roughly linear function of dimer level (Figure 3). Photoreactivation of a CPD in DNA is performed by a single protein molecule, with a slow (dark) step, in which the photoreactivating enzyme locates a CPD and forms a productive enzyme–substrate complex. In the presence of saturating levels of light, the second step, dimer photolysis, is rapid. Because the intensity of visible light was constant in our experiments, the increase in the photorepair rate with an increasing initial damage level is presumably a result of the rate of enzyme–dimer complex formation, most likely to the increased concentration of the substrate and thus decreased time required for enzyme association.

Our preliminary experiments on repair in alfalfa seedlings exposed to low UV doses (see Figures 2E and 2F) suggested that excision of CPDs might be entirely absent in this plant, as has been reported in other plant species (Trosko et al., 1968, 1969; Swinton and Hanawalt, 1973). However, additional experiments at higher UV exposures, corresponding to initial CPD levels greater than ~30 CPD per Mb, indicated that excision can be measured readily and its contribution is almost as high as that of photorepair at high damage levels. Similarly, Eastwood and McLennan (1985) found that the rate of excision in carrot protoplasts also increased as a function of UV dose, reaching a maximum at an initial 254-nm dose of 100 J/m^2, which produced 0.26% thymines converted to dimers

(~400 thymine-containing dimers per Mb). Paterson et al. (1973) and Wani et al. (1987) showed that the rate of excision in human cells also depended on the dimer frequency. Thus, the rate of excision in carrot and in human cells depends on initial dose as it does in alfalfa seedlings. However, the relative roles of excision and photorepair cannot be evaluated from their data, because photorepair rates were not determined in the same experiments. Preliminary data (S. Takayanagi and B.M. Sutherland, unpublished data) show that the rate of repair in soybean seedlings also depends on the initial CPD frequency, but the relative importance of photorepair and excision is different in two cultivars.

Pang and Hays (1991) and Britt et al. (1993) measured both excision and photorepair in Arabidopsis (using only a single UV dose). Pang and Hays (1991) found that excision was only ~20% of the rate of photorepair at 254-nm doses producing 50 to 70 CPD per Mb, corresponding to rates of ~0.1 and 0.5 CPD per Mb per min, respectively. On the other hand, Britt et al. (1993) found no significant excision of CPDs in the 20-hr course of their experiment, although assays using antibodies specific for pyrimidine (6-4) pyrimidones indicated that these adducts were removed efficiently. Whether the difference in CPD removal reflects a difference between strains or whether the apparent excision observed by Pang and Hays (1991) could also include new DNA synthesis is not clear. Degani et al. (1980) found that after exposure to doses of 254 nm radiation producing ~600 dimers per Mb, the water plant *W. microscopica* removed dimers by excision at a rate approximately two thirds that of photorepair, which is similar to the relative rates we measured in alfalfa for the highest doses we used.

Early investigations indicated that excision could not be detected in several plant species. These investigations were limited to high dimer levels (2×10^2 to 11×10^3 dimers per Mb) by the relative insensitivity of the chromatographic procedures used for measuring photoproducts. Even the most rapid excision rates (~0.3 CPD per Mb per min) we measured in alfalfa seedlings subjected to higher initial doses would not have yielded substantial decreases in these damage levels during the times of the experiments. Thus, it is conceivable that excision might be present in these plants, although it was not detectable in the early experiments.

The apparent absence of excision in alfalfa at low UV doses is not a result of experimental difficulties in quantitating lesions at low levels, because photorepair can be easily measured at the same initial lesion levels. Excision of CPDs at rates comparable to those observed after higher doses (e.g., 0.3 CPD per Mb per min) would certainly be detected after 120 min but were not observed in our experiments (Figures 2E and 2F). The apparent absence of excision at these damage levels may result from the lower affinity of excision enzymes for CPDs relative to other lesions, to lower accessibility of the lesions to excision enzymes, or to participation of these enzymes in nonrepair-related DNA metabolism.

This differential repair response could reflect an efficient and beneficial UV strategy: at low damage levels, the seedlings employ primarily photorepair, which requires only external energy, visible (photoreactivating) light, which is abundant in the solar spectrum. The use of photorepair as a primary repair path should also minimize repair-generated mutations because photoreversal of cyclobutyl dimers to parental pyrimidine monomers is inherently error free and thus nonmutagenic. It might also promote rapid repair of non-CPD lesions by the excision system simultaneously with CPD reversal by photoreactivation; rapid repair would be facilitated by making more excision complexes available to deal with non-CPD damages.

Furthermore, nicks induced during the excision process can trigger UV stress responses that can result in cessation of macromolecular synthesis and can halt growth (Kastan et al., 1991; Nelson and Kastan, 1994). Plants respond to ionizing radiation exposure by delaying cell division: this radiation-induced response has multiple components, with some but not all being dependent on de novo protein synthesis (Van't Hof and Kovacs, 1970). Because plants are exposed to low levels of UV continuously during the day, repair by excision might initiate responses that could depress plant growth, whereas photorepair would permit CPD reversal and thus bypass interruption of growth. At higher damage levels at which the capacity of photorepair is saturated (either because of limiting photoreactivating light levels or limited numbers of photolyase molecules), these plants also use excision, which requires cellular energy in the form of ATP. This overall strategy would represent an efficient use of cellular resources in response to low and high levels of UV-induced damage.

Do other plants show similar excision and photorepair relationships? Preliminary results with soybean seedlings grown in our laboratory under the same conditions as the alfalfa seedlings indicate that the rates of both excision and photorepair correlate with the initial CPD level; however, the absolute rates of both repair processes are much more rapid in soybean. Further, the relative roles of excision and photorepair vary in two cultivars with different UV-B sensitivities. Thus, other plant species may differ from alfalfa in both absolute repair rates and in the relative roles allocated to photorepair and excision. Plants whose preferred habitat is shade might repair more slowly and use photorepair less extensively, whereas plants whose natural environment contains high UV (and thus high total levels of sunlight including visible and long-wavelength UV effective in photoreactivation) levels might repair more rapidly and use photorepair preferentially.

The changing roles of excision and photorepair at different doses mean that their relative importance in a particular plant cannot be determined from data obtained at only one UV dose. Consideration of only the low-dose data in alfalfa would lead us to conclude that excision is absent in this plant, whereas data from only high doses would suggest that excision is comparable in importance to photorepair. Our data show that understanding repair in plants—and perhaps in other organisms as well—requires measurement of excision and photorepair under different conditions, including a substantial range of initial UV doses.

METHODS

Plant Material

Alfalfa (*Medicago sativa*) seed were germinated on Whatman No. 3 (Whatman, Clifton, NJ) filter paper discs in sterile Petri dishes in a growth chamber. Illumination was provided by Sylvania (GTE Products, Danvers, MA) cool-white fluorescent lamps filtered by UF4 Plexiglas (Rohm and Haas, Philadelphia, PA), which excludes most radiation shorter than ~400 nm. The seedlings were grown (20°C; 16-hr photoperiod) for 6 to 7 days; the fully expanded cotyledons were then used for irradiation experiments. Plants were irradiated in four experimental series, each containing one higher and one lower UV exposure. After UV treatment, the plants within each treatment group were divided; one part was exposed to photoreactivating light and the other was kept in the dark, and samples were obtained at increasing times after UV treatment.

UV Irradiation

Seedlings in the open Petri dishes were irradiated with narrow-band UV radiation from a high-intensity monochromator (Johns and Rauth, 1965a, 1965b). UV fluxes from the monochromator were measured using a Molectron PR200 pyroelectric radiometer (Molectron Corp., Sunnyvale, CA). A typical exposure rate was 2 mW/cm^2, and irradiations required from 50 sec to 10 min. In some experiments, plants were irradiated using a Westinghouse (Westinghouse Electric, Lamp Division, Bloomfield, NJ) FS20 lamp emitting broad spectrum UV-B radiation, which was monitored with a Jagger meter (Jagger, 1961) calibrated versus the Molectron radiometer. All subsequent manipulations were performed in dim yellow (General Electric, Cleveland, OH; $\lambda > 500$ nm) or red (General Electric 25W) light to minimize uncontrolled photoreactivation.

Photoreactivation

Samples to be photoreactivated were exposed to UV as described above and then to light from two Philips 15T8/B blue-fluorescent lamps (North American Philips Lighting, Somerset, NJ) filtered through a UF4 Plexiglas filter, resulting in a spectrum with a maximum at ~450 nm and little radiation below ~400 nm. The combination of blue lamps and the UF4 filter provided visible light for photorepair while excluding almost all long wavelength UV radiation.

DNA Extraction

This DNA extraction method has been described in detail elsewhere (Quaite et al., 1992, 1994). In brief, alfalfa cotyledons were sliced in buffer A (0.5 M EDTA [Mallinckrodt, Paris, KY], 10 mM Tris-HCl [United States Biochemical Corp., enzyme grade], pH 8.0, 1% sarcosyl [Sigma], 1 mg/mL proteinase K [Boehringer Mannheim]), vacuum infused for 30 to 40 sec, and incubated at 45°C for 30 min. The slurry was mixed with an equal volume of 1.4% low-melting-point agarose (SeaKem LE or SeaPlaque; FMC, Rockland, ME), and agarose plugs were prepared. The plugs were digested, rinsed with TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA), and then rinsed with TE containing 2.5 mM phenylmethylsulfonyl fluoride, then TE, and finally UV endonuclease buffer (30 mM Tris-HCl, pH 8.0, 40 mM NaCl, 1 mM EDTA).

Treatment of Alfalfa DNA with UV Endonuclease from *Micrococcus luteus*

M. luteus UV endonuclease was partially purified by streptomycin and ammonium sulfate precipitations; its activity toward CPDs and lack of nonspecific activity were determined using cleavage of supercoiled DNA containing and lacking CPDs (Sutherland et al., 1992). Plugs containing alfalfa DNA samples were digested for 30 min at 37°C in 10 µL of UV endonuclease buffer containing sufficient endonuclease to yield complete cleavage at all CPD sites, and a duplicate sample was incubated in 10 µL of UV endonuclease buffer (without endonuclease) under identical conditions. Reactions were stopped and the DNA was denatured by adding 10 µL of alkaline stop mix (0.5 M NaOH, 50% [v/v] glycerol, and 0.25% [w/v] bromocresol green) and incubating for 30 min at 37°C.

Alkaline Unidirectional Pulsed Field Electrophoresis

The plant DNA was dispersed according to its single-strand molecular length by alkaline agarose unidirectional pulsed field electrophoresis (Sutherland et al., 1987b). Agarose (SeaKem LE) gels (0.4% [w/v]) were prepared in 1 mM EDTA, 50 mM NaCl and equilibrated with the alkaline electrophoresis solution (2 mM EDTA, 30 mM NaOH) for 30 min. Molecular length markers (DNAs from bacteriophages T4 [170 kb], λ [48.5 kb], T7 [40 kb], and a BglI digest of T7 [22.5, 13.5, and 4 kb]) were included on all gels. Electrophoresis (15 V/cm; 0.3-sec pulse, 10-sec interpulse period; 10°C with buffer recirculation) was performed for 16 hr; the sample slices were then removed from the wells and electrophoresis resumed using the same conditions for 8 hr (Quaite et al., 1992, 1994). Gels were neutralized (twice for 30 min in 0.1 M Tris-HCl, pH 8), stained with ethidium bromide (1 µg/mL) for 30 min, and destained in H_2O overnight at 4°C. All data are the averages of either duplicate or triplicate gels from the same plant samples.

Electronic Imaging of Gels and Cyclobutyl Pyrimidine Dimer Analysis

An image of the distribution of the fluorescence of ethidium bound to DNA in the destained gels was recorded using an improved version of the electronic imaging system described previously (Sutherland et al., 1987a). Cyclobutyl pyrimidine dimer (CPD) frequencies were calculated as previously described (Quaite et al., 1992, 1994) using the method of moments (Freeman et al., 1986): DNA profiles of sample and length standard lanes were obtained from the quantitative image data (see Figure 1); a dispersion function (molecular length versus migration position) was constructed from the molecular length standards. Using this curve and the quantity of DNA at each molecular length position from the quantitative image data, the number average molecular length, L_n, of each DNA distribution was calculated from the equation

$$L_n^{-1} = \frac{\int \frac{\rho(x) \cdot dx}{L(x)}}{\int \rho(x) \cdot dx}, \quad (1)$$

where $L(x)$ is the length of the DNA molecules that migrated to position x, and $\rho(x)$ is the intensity of ethidium fluorescence from DNA molecules that have migrated to position x. From the number average

lengths of the DNA populations, the frequency of lesions was obtained from the equation

$$\Phi = L_n^{-1}(+e) - L_n^{-1}(-e), \qquad (2)$$

where Φ is the frequency of CPDs, $L_n(+e)$ is the number average length of the population of molecules treated with the UV endonuclease, and $L_n(-e)$ is the number average length of the population of molecules not treated with the enzyme. DNA lengths, $L(x)$ and L_n, are expressed in units of kilobases or megabases. Thus, Φ is expressed in units of CPD per kilobase or CPD per megabase.

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