

UV-B component of sunlight causes measurable damage in field-grown maize (*Zea mays* L.): developmental and cellular heterogeneity of damage and repair

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ABSTRACT

Ultraviolet radiation has diverse morphogenetic and damaging effects on plants. The end point of damage is reduced plant growth, but in the short term UV radiation damages specific cellular components. We measured cyclobutane pyrimidine dimers in maize DNA from plants grown in natural solar radiation. Green maize tissues had detectable DNA damage, roots had less damage, and anthers had much more damage than green leaves. This heterogeneity in damage levels may reflect differences in dose received or in damage repair. The architecture of green tissues had no measurable effects on DNA damage levels, as leaf sheath and leaf blade were equivalent. We observed a slight increase in damage levels in plants sampled at the end of the day, but there was no accumulation of damage over the growing season. We measured photoreactivation, and found substantial levels of this light-dependent repair in both the epidermis and inner cell layers of leaves, and in all organelles that contain DNA – the nucleus, chloroplasts and mitochondria. We conclude that maize has efficient mechanisms for photorepair of daily UV-induced DNA damage that prevent accumulation.

Key-words: *Zea mays*; Gramineae; maize; cyclobutane pyrimidine dimer; DNA repair; UV-B; UV-C.

INTRODUCTION

The measurable diminution of the stratospheric ozone layer and the consequent increase in surface UV radiation (Kerr & McElroy 1993; Lubin & Jensen 1995) have sparked renewed interest in UV-B and its negative effects on the growth, morphology and physiology of plants (Caldwell *et al.* 1995). Reduced photosynthetic efficiency is one of the most commonly measured types of UV-induced damage. Photosynthetic damage is not well correlated with decreased growth after UV exposure (Beyschlag, Barnes & Flint 1988; Ficus & Booker 1995),

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consequently other UV effects must be considered. For example, stimulation of the UV-B photoreceptor may initiate a signal transduction chain that results in slower growth. Another candidate for a primary event that could alter cellular activities is DNA damage. Although much is known about UV-induced DNA damage and subsequent repair in micro-organisms and in cultured mammalian cells (reviewed in Sancar & Sancar 1988; Sancar 1994), we know considerably less about these processes in plants.

The exposure and responses of plants to UV radiation are quite distinct from the treatments of bacteria and mammalian cells in laboratory studies. First, studies in model systems utilize a monolayer of cells receiving equal dosage, usually for a brief period of time. In multicellular higher plants, UV exposure varies by season and position on the plant. Furthermore, the majority of UV radiation is absorbed by the epidermis, resulting in heterogeneity in exposure within organs, a feature that may result in heterogeneity in biological responses. Secondly, unlike bacterial and mammalian cells, intact plants utilize sunlight for photosynthesis with the corollary chronic exposure to the UV present in solar radiation. Because UV-A and UV-B are a natural part of their environment, plants may have novel coping mechanisms. For example, plant pigments such as flavonoids effectively absorb UV-B (Li *et al.* 1993; Stapleton & Walbot 1994). Thirdly, unlike mammals in which the germ cells are set aside early and are completely shielded from radiation, plant cells enter meiosis only after significant vegetative growth. Mutations that accumulate over time in the somatic tissue may be passed to the gametophytes. Thus, the genetic consequences of exposure to chronic UV may be of much greater significance in plants than in animals (Walbot & Cullis 1985).

DNA damage and repair in plants have been examined recently in *Arabidopsis thaliana*, alfalfa and maize exposed to highly mutagenic UV-C as well as the UV-B and UV-A found in solar radiation (reviewed in Britt 1996). Shielding by flavonoids, including anthocyanins, has been demonstrated (Li *et al.* 1993; Stapleton & Walbot 1994) and two types of DNA repair have been confirmed (reviewed in McLennan 1987; Britt 1996). The first type of repair is called 'light' repair or photoreactivation, and it generally occurs within a few hours. Specific photolyase enzymes require UV-A or blue light to energize direct reversal of either cyclobutane pyrimidine dimers (CPD) or

pyrimidine (6,4) pyrimidone dimers (6,4 photoproducts) created in DNA by UV-B exposure (Yasui *et al.* 1994; Sancar 1996; Todo *et al.* 1996). The second type of repair, called 'dark' or excision repair, involves removal of the damaged bases followed by synthesis of a repair patch. The kinetics of this 'dark repair' are considerably slower than those of photoreactivation (Degani, Ben-Hur & Riklis 1980). To advance the study of UV-induced DNA damage in plants, we have begun to examine the heterogeneity of damage and types of repair within and between organs. We report here an analysis of CPD DNA damage levels in different tissue layers of maize leaves, in leaves of varying age over the entire growing season, and in reproductive tissues. In contrast to a recent report on *A. thaliana* seedlings (Chen, Jiang & Britt 1996), we have detected light-activated DNA repair of CPD in the nuclear genome and also in both organellar genomes of maize.

MATERIALS AND METHODS

Plant material

Two maize lines with different backgrounds were used: line A (75% W23 and 25% K55) for all experiments, and line B (100% W23) for some experiments. Neither line could make anthocyanins; line A is homozygous *bronze2* and thus is defective in the last step of anthocyanin biosynthesis. Line B lacks two flavonoid regulatory genes (homozygous for *b* and *pl*). These lines were chosen as they have different plant morphology; line A plants have wider leaves and thicker stems than do line B plants. These lines are available from the authors or from the Maize Genetics Coop, Urbana, IL.

Growth conditions

Plants were grown in a glass greenhouse with supplemental lighting (metal halide and sodium arc lamps with glass covers) or outdoors on the Stanford campus. For the initial set of experiments, kernels were planted weekly and tissue was harvested from all plants on 8 August 1993 after 4, 6 or 9 weeks of growth. For the second round of experiments, kernels were planted weekly as before, and tissue was collected on 15 August 1995 from the top and bottom leaves of plants 3, 4, 6, 9 and 10 weeks after planting. Leaf segments of ≈ 5 g fresh weight were harvested from three individual maize line A and three maize line B plants of each age, and frozen immediately. The three samples from each line were combined for DNA extraction. For the survey of damage levels in different tissues (see Fig. 2), tissue segments of ≈ 5 g fresh weight were harvested from each of three individual maize line A or maize line B plants and frozen immediately. Roots were dug up, rinsed in water and harvested. At collection, anthers had exerted and had shed most of their pollen; tassel samples were the glumes and stem left after the anthers plus filament were harvested. The three samples from individual plants were combined and DNA prepared.

For repair experiments, seedlings were grown in a Percival Model E30b growth chamber (Boone, Iowa) with overhead lighting from six cool-white fluorescent bulbs and two incandescent bulbs. Mylar film was placed below the bulbs to shield the seedlings from UV radiation emitted by the fluorescent tubes; the film was changed after every 60 h of use. The growth chamber contained vertically mounted ballasts for six UV bulbs.

UV irradiation

For data reported in Fig. 4, adult leaf blades were cut into 10 cm by 4 cm sections and irradiated in a solar UV simulator (Sisson & Caldwell 1975), with two sheets of Kodacel cellulose acetate (Kodak, Rochester, NY) covering the bulbs. The spectral output of this simulator is shown in Stapleton & Walbot (1994). Filters were replaced after every 40 h of use.

For repair experiments (data reported in Figs 5 & 6), irradiation of seedlings (four 10-d-old seedlings per treatment) was performed in the Percival growth chamber, with six vertically mounted GL15 UV-C bulbs (total UV-C irradiance 360 W m^{-2}), and recovery in the same chamber, with visible light provided from four cool-white fluorescent bulbs and two 40 W incandescent bulbs. A sheet of 2 mm Plexiglass was placed below the lights to prevent any UV from reaching the plants. Ten-day-old maize line A seedlings were irradiated for 10 or 20 min with six germicidal UV-C bulbs that were mounted vertically in the growth chamber, for a total dose of 108 or 216 kJ m^{-2} . The three emerged leaves from three seedlings were pooled and frozen in liquid nitrogen. The remaining seedlings were returned to the growth chamber for 2 or 24 h (with the lights on continuously) to recover; the dark recovery seedlings were placed in light-tight metal containers inside the growth chamber. DNA was prepared and the amount of repair in specific DNA fragments was measured as described.

Epidermal peels

Adult leaves were cut into sections 0.5 cm by 3 cm, and the sections were taped to the laboratory bench. A new razor blade was used to peel off the top epidermis, working in the direction of the major veins (longitudinally along the leaf). The epidermal peel and the remaining mesophyll and lower epidermis were separated and each sample was immediately frozen in liquid nitrogen. An unpeeled section of the same leaf was also frozen.

DNA extraction

DNA was extracted from frozen tissues as described in Stapleton, Mori & Walbot (1993). In brief, tissue was ground to a powder in liquid nitrogen, the powder was added to an equal volume of urea/SDS lysis buffer, and then the mixture was extracted with phenol-chloroform and the DNA precipitated in ethanol. No special precau-

tions were needed to prevent photoreactivation of samples once they were frozen, as frozen ground samples were dropped directly into lysis buffer and photolyase is not active in lysis buffer.

Antibody assay of DNA damage

UV-induced CPD damage was measured using a monoclonal antibody (TDM-2) that specifically recognizes CPD, as described in Stapleton *et al.* (1993). To measure the concentration of the extracted DNA it was compared to standards of known concentration by electrophoresis on agarose gels, staining with ethidium bromide (Sigma, St. Louis, MO), photography with Type 55 negative film (Polaroid, Rochester NY) and quantification of the genomic DNA band amount using the program NIH Image 1.44. Up to 500 ng DNA was immobilized on Hybond-N (Amersham, Chicago, IL) using a dot-blotting apparatus (Schleicher and Schuell, Keene, NH). The blot was blocked and reacted with the monoclonal TDM-2 antibody that recognizes CPD. TDM-2 antibody binding was detected using a ^{35}S -labelled secondary antibody from sheep that recognizes mouse Ig (Amersham, Chicago, IL); the signal was measured on a Molecular Dynamics PhosphorImager (Sunnyvale, CA). Damage levels were determined using plasmid standards with known damage levels. DNA amounts were normalized (within the sample set on each blot) by hybridization to a genomic DNA probe made by random-priming with the Random Primed DNA Labeling kit according to the manufacturer's instructions (United States Biochemical, Cleveland, OH). Each graph in the figures represents data collected from one blot. Thus comparisons within a graph are 'intra-assay' and comparisons between graphs are 'inter-assay'.

TEV assay of DNA damage

DNA damage was measured in specific DNA fragments by adapting a technique used to measure repair in mammalian cells (Bohr & Okumoto 1988). For our assay it was not necessary to separate replicated DNA from unreplicated DNA, as somatic plant tissues have very little DNA replication. In brief, DNA from irradiated plant material is digested with a restriction enzyme and separated into two equal aliquots. One aliquot is treated with an endonuclease (TEV) that specifically cleaves DNA at CPD damage. Loading controls were as described (Bohr & Okumoto 1988). The DNA samples are separated by size, using gel electrophoresis, blotted to a filter and hybridized to a gene-specific probe that detects the restriction fragment of interest. For each sample, signal intensity in the band corresponding to the full-length restriction fragment in the lane containing DNA treated with TEV (TEV+) is compared to the intensity of the band in the lane containing DNA that was not treated with TEV (TEV-). The frequency of CPDs is reflected in the sensitivity of the DNA to TEV digestion; data are reported as enzyme sensitive sites (ESS) per unit of DNA (Bohr & Okumoto 1988). We used *HpaI* (BRL,

Gaithersburg, MD) for restriction endonuclease digestion of our DNA samples. The TEV (batch 26) was kindly provided by Ann Ganesan (Stanford University). All reagents used were from Sigma (St. Louis, MO) unless otherwise noted. Samples were electrophoresed on a 0.8% alkaline agarose gel with buffer recirculation. The gel was neutralized, stained with $2 \mu\text{g cm}^{-3}$ ethidium bromide, photographed, denatured and neutralized again for 30 min according to standard procedures (Sambrook, Fritsch & Maniatis 1989). The DNA was transferred to HybondN (Amersham, Chicago, IL) and UV-crosslinked to the filter in a Stratalinker (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Prehybridization and hybridization were carried out at 42 °C in a rotisserie oven (Bachofer, Germany) in a hybridization solution of 50% formamide (BRL, Gaithersburg, MD), 3X SSPE, 5X Denhardt's, 0.1 mg cm^{-3} denatured sheared salmon testes DNA and 0.5% SDS (Sambrook *et al.* 1989). Probes were prepared by random priming with the Random Primed DNA Labeling kit according to the manufacturer's instructions (United States Biochemical, Cleveland, OH), using ^{32}P dCTP (Amersham, Chicago, IL). Blots were hybridized overnight, then washed twice for 20 min in $0.1 \times \text{SSC}$ (Sambrook *et al.* 1989) plus 0.1% SDS at 63 °C.

As a nuclear probe we used the insert from pbz1c, a 0.4 kb fragment of the maize *Bronze1* gene (Fedoroff, Furtek & Nelson 1984). The chloroplast probe was the insert from pATP5, a 0.65 kb fragment of the maize *atpF* gene (Barkan 1989). For our mitochondrial probe we used the insert from p. 117B, a 0.7 kb fragment of the maize *cob* gene (Mulligan, Maloney & Walbot 1988). Each of these probes hybridized to a single large band (10–15 kb) in line A maize DNA restricted with *HpaI* under our standard wash conditions. We confirmed our results with a second set of probes: pBz300, a 0.3 kb fragment of the maize *Bronze2* gene (Nash, Luehrson & Walbot 1990), pAB8, a 0.8 kb fragment of the chloroplast *petA* gene (Barkan 1989) and p. 26, a 2.4 kb fragment of the maize mitochondrial *coxII* gene (Fox & Leaver 1981). We also tested each of these probes for hybridization to DNA from the correct cellular compartment by preparing DNA from purified chloroplasts (Mourad & Polacco 1989) and from purified mitochondria (Newton 1993) and observing enhanced signal with only the appropriate probe.

Statistical analysis

Data were analysed for variance between replicate sample sets by single-factor ANOVA using Microsoft Excel version 5.1. TEV repair data was also analysed by Tukey's HSD test using the SAS program.

RESULTS AND DISCUSSION

Classical methods for measuring DNA damage using chromatography are not sufficiently sensitive to measure solar UV-induced damage. One very sensitive method to measure damage requires expensive custom-built

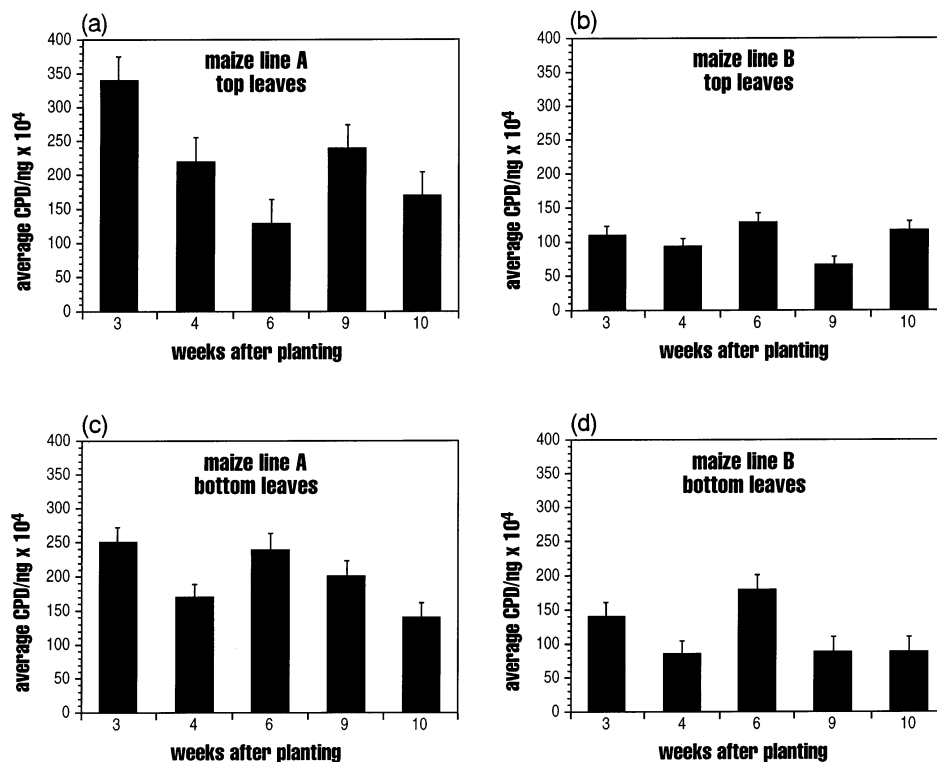


Figure 1. Measurement of DNA damage levels in leaves from plants of various ages. Seeds were planted in the field each week, and the plants were all harvested at the same time. Leaf segments were harvested from three individual maize line A and three maize line B plants and frozen immediately. The three samples from each line were combined for DNA extraction. CPD levels in each DNA sample were measured with the antibody assay as described in 'Materials and methods'. Samples were taken from the top and bottom leaves of plants 3, 4, 6, 9 and 10 weeks after planting. The average of four replicates from each sample is shown. Error bars are standard error. (a) Top leaf samples from maize line A. When compared by ANOVA these samples were significantly different ($P = 3.33 \times 10^{-5}$). (b) Top leaf samples from maize line B. When compared by ANOVA the samples from the five different weeks were significantly different ($P = 2.63 \times 10^{-4}$) from each other. (c) Bottom leaf samples from maize line A. When compared by ANOVA these samples were significantly different ($P = 0.010$) from each other. (d) Bottom leaf samples from maize line B. These samples were significantly different ($P = 0.0024$) from each other. While comparison of samples from the different weeks reveals significant differences there is no trend apparent; for example, average damage levels in week 6 top leaf samples are lowest in line A and highest in line B.

equipment and very high molecular weight DNA (Quaite, Sutherland & Sutherland 1992). In contrast, our assay for damage using antibodies to UV-induced DNA lesions is both sensitive and simple to perform.

DNA damage in leaves of different ages

We measured DNA damage levels in field-grown plants from two different maize lines planted weekly and harvested on one day (Fig. 1). Preliminary experiments with line A showed that there was no significant difference ($P = 0.5$) in DNA damage levels in leaf samples that had been exposed to solar UV for 4, 6 or 9 weeks (Table 1). We extended these experiments in 1995, with two different maize lines and samples from plants 3, 4, 6, 9 and 10 weeks old (Fig. 1). We chose these particular ages for our analysis as they include leaves from three life stages – embryonic, juvenile and adult. We examined both the top (youngest) and the bottom (oldest) leaves. The key finding is that there is no trend towards increased damage with increased age

(Fig. 1). There were some significant differences in damage levels between plants ($P < 0.0001$), but the magnitudes were only about 2-fold and were not reproducible in different growing seasons (compare Table 1 to Fig. 1). This suggests that specific growth conditions or other, unidentified conditions affect the steady-state levels of UV-induced DNA damage in individual plants.

Because there is no progressive increase in UV-induced DNA damage in leaves over the course of a growing

Table 1. Level of CPD DNA damage in field-grown maize leaves. CPD levels were measured as described in 'Materials and methods'. The average of three replicate determinations on each sample is shown. Comparison of samples with each other using ANOVA gave $P = 0.5$

Weeks after planting	Four	Six	Nine
Average antibody signal $\times 10^4$	3.83	3.24	2.80

season, we infer that DNA repair is an active process in field-grown plants. Two additional important points can be inferred from these data. Comparison of Figs 1a & c with b & d shows that at most stages there is less damage in line B samples than in line A samples. Either genotypic and/or phenotypic differences between the lines can affect the level of damage accumulated. The second point is that there are only modest differences in steady-state damage levels in embryonic, juvenile and adult leaves, implying that the balance of damage minus repair is similar despite differences in wax deposition, leaf thickness, and other biochemical and structural features.

Density of damage in field-grown plants

Solar UV produces damage that we can detect in plants grown under normal field conditions. In young leaf tissue we found 1.1×10^6 to 3.4×10^6 CPD ng^{-1} . This corresponds to 1.2–3.7 CPD Mb^{-1} . Using a different measuring technique, Takayanagi *et al.* (1994) found similar results (1.8, 4.2 and 9.0 TEV ESS sites Mb^{-1}) in alfalfa seedlings grown outdoors. The range 1.2–3.7 CPD Mb^{-1} in damage density corresponds to about 3000–10 000 CPD per maize haploid genome or about 300–1000 CPD per maize chromosome. CPD lesions are significant as this damage blocks both transcription and replication; if any CPD are formed in genes that are essential, especially in genes for which transcription of both copies is required, that damage must be repaired for cellular survival. Although the steady-state level of CPD seems high, incident radiation should produce many more CPD. We calculate that solar radiation could cause 800 CPD $\text{Mb}^{-1} \text{d}^{-1}$ in any plant tissue, assuming $8000 \text{ J m}^{-2} \text{d}^{-1}$ (weighted across the UV spectrum), 30 ESS (TEV sites) per $10^{10} \text{ Da J}^{-1} \text{m}^{-2}$ (Mitchell, Jen & Cleaver 1991) and 90% protection of interior DNA by tissue thickness. In fact, the highest tissue damage we observed was in anthers of line A: 1.05×10^7 CPD ng^{-1} , which corresponds to 12.1 CPD Mb^{-1} . This anther level is 60-fold less damage than we predict should occur each day. The striking differences between our theoretical calculation and actual damage levels strongly suggests that maize DNA damage is quickly repaired.

DNA damage in different tissues of field-grown plants

We next examined DNA damage levels in various tissues of maize (Fig. 2). Maize is exceptionally useful for such studies because the organs are large and accessible. Our initial experiments showed that root samples had the least damage (Fig. 2a); root damage levels were about 15% as high as leaf damage levels ($P = 0.015$). Leaf blade and sheath were not significantly different from each other (Fig. 2b). Anther tissue had significantly more damage than any other tissue, with $P = 0.001$ for comparison to leaf samples (Fig. 2c). We repeated these experiments using both line A (Fig. 2d) and line B (Fig. 2e). The relative amount of damage was again greatest in the anther, less in

the leaf, and least in the root. Anther samples have 5–10-fold more damage than adjacent tassel (Figs 2d & e). Tissue architecture could contribute to the high damage levels observed in anthers, as this is the thinnest tissue we measured. It is also possible that anthers might have less repair than leaves; additional experiments will be required to elucidate the mechanism(s) generating the high CPD levels in anthers.

Although roots contain the least damage, and in soil the UV-B fluence should be extremely low, CPD are detectable. Possibly roots are unable to repair any damage received; if this is the case, root damage would be cumulative. In preliminary experiments we have found that roots have much less photoreactivation repair than green tissues (data not shown); excision repair has not been measured. A second possibility is that some CPD were formed during sample processing. No precautions were taken to maintain UV-free conditions, because tissue extraction and DNA handling conditions should have affected all samples equally.

DNA damage at different times of day in field-grown plants

Although DNA damage does not accumulate over a growing season, there might be a daily dynamic of daylight exposure and nighttime recovery. We were interested in whether the cumulative UV dose over the course of a day would be reflected in a parallel increase in damage, or whether repair capacity was sufficient to maintain a constant steady-state level of damage throughout the day. We compared CPD levels in leaves from plants harvested in the morning and in the afternoon (at the time of maximum UV exposure) (Fig. 3a). We saw a trend towards increased average damage levels at 1400 h on two different dates, but these differences were not significant ($P = 0.18$ and $P = 0.29$). A more extensive sampling (Fig. 3b) of three plants from each maize line at 0700, 1200 and 1900 h showed significant differences ($P = 0.012$ for line A and $P = 0.045$ for line B) between the 0700 and 1900 h samples. In absolute terms, this difference was not dramatic (see legend to Fig. 3 for details). We conclude that there is a slight but significant accumulation of DNA damage levels in maize leaves during the day. This damage must be repaired by photoreactivation in early evening or by excision repair during the night, as no accumulation occurs over the growing season.

Effects of tissue architecture on DNA damage after UV-B exposure

To study the effects of epidermal architecture and of shielding compounds on DNA damage in more detail, we utilized controlled UV exposure. The basal portion of a maize leaf is the sheath; it is thicker than the leaf blade, has wider epidermal cells, and lacks hairs (Freeling & Lane 1994). Blade and sheath tissue samples from two line A plants were irradiated side by side in a solar UV simulator.

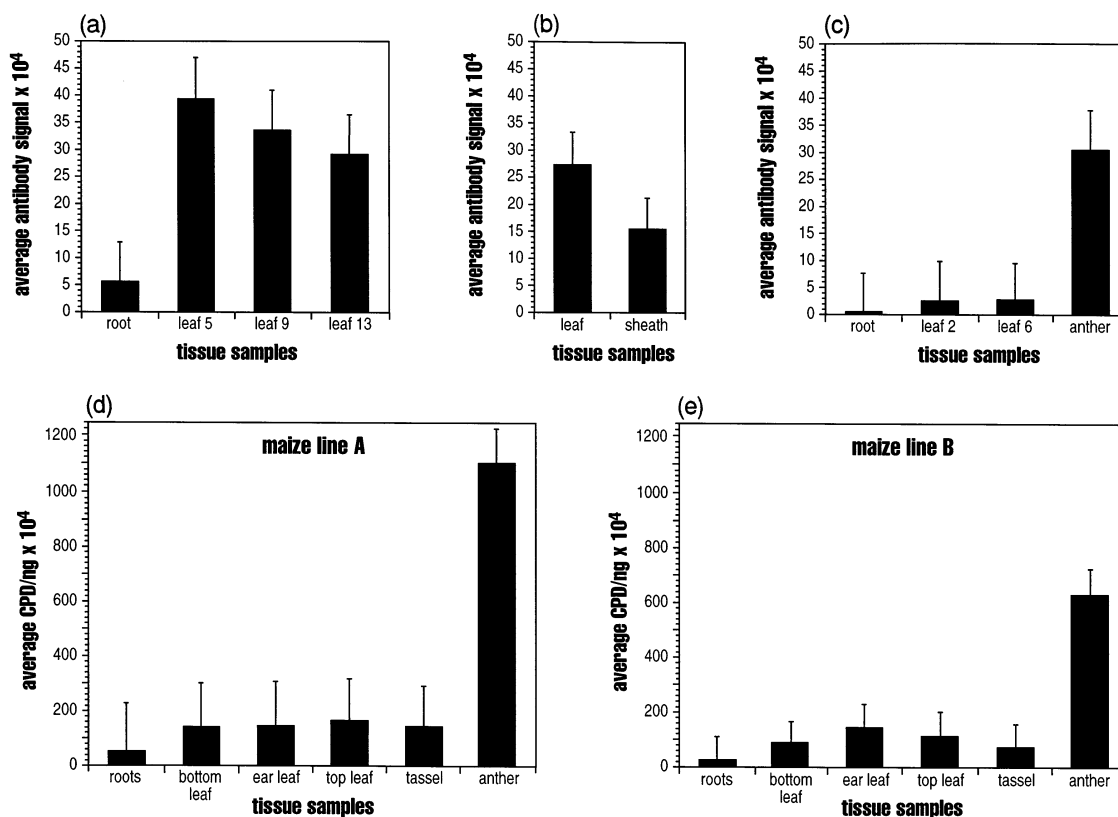


Figure 2. Measurement of DNA damage levels in various maize tissues. Seeds were planted in the field in successive weeks, and all the plants were harvested at one time. Tissue segments were harvested from each of three individual maize line A or maize line B plants and frozen immediately. Roots were dug up, rinsed in water and harvested. At collection, anthers had exerted and had shed most of their pollen; tassel samples were the glumes and stem left after the anthers plus filament were harvested. The three samples from individual plants were combined, DNA prepared, and CPD levels measured as described in 'Materials and methods'. For the CPD determination shown in (a-c) (the 1993 samples), standards with known amounts of CPD damage were not available, so no conversion of counts to CPD ng^{-1} was possible. Each set of CPD determinations shown in each graph was from the same blot so the counts were comparable. (a) Leaf and root samples taken in mid-afternoon at 6 weeks of age in 1993. The average of three replicates from each sample is shown. Comparison of leaf blade samples to each other gave $P = 0.5$. Comparison of root samples to leaf blade samples gave $P = 0.015$. (b) Leaf blade and sheath samples from leaf 6 (a juvenile leaf) were taken in mid-afternoon 6 weeks after planting in 1993. The average of three replicate determinations from each sample is shown. Comparison of leaf blade to sheath samples gave $P = 0.42$. (c) Root, leaf and anther tissue samples taken at week 9 in 1993. The average of three replicate determinations from each DNA sample is shown. Error bars are standard error. Comparison of root with leaf gave $P = 0.2$, whereas comparison of anther to either leaf or root gave $P < 0.001$. (d) Anther, tassel, leaf and root samples from maize line A harvested at 10 weeks in 1995. Averages of four replicate determinations from each DNA sample are shown. Tassel, top leaf, ear leaf and bottom leaf compared gave $P = 0.56$, and root samples compared to bottom leaf samples gave $P = 6.62 \times 10^{-6}$. (e) Anther, tassel, leaf and root samples from maize line B harvested at 10 weeks in 1995. The average of four replicate determinations from each DNA sample is shown. For tassel, top leaf, ear leaf and bottom leaf $P = 1.13 \times 10^{-6}$, for top ear and bottom leaf $P = 1.37 \times 10^{-4}$, for top leaf compared to bottom leaf $P = 0.009$, for tassel compared to bottom leaf $P = 0.06$, and for root compared to bottom leaf $P = 2.5 \times 10^{-4}$.

There was a trend towards more UV damage in the sheath (Table 2), but the differences were not significant. This result confirms our previous observation that there was no significant difference in damage levels between blade and sheath in field samples, as shown in Fig. 2. Thus differences in epidermal architecture in leaf blade versus leaf sheath do not affect the levels of damage observed.

Although these maize lines do not synthesize anthocyanins, other flavonoid compounds might act as sunscreens. UV-absorbing compounds were assessed by measuring the absorbance at 300 nm of methanol-extracts of

leaf blade and sheath sections (data not shown). We found more UV-absorbing material in leaf blades, more than enough to account for the trend for greater DNA damage in the sheath.

Where does most damage occur in the leaf after controlled irradiation? We irradiated leaf blades of line A in the solar UV simulator and separated the upper (abaxial) epidermal layer from the remainder of the leaf (Fig. 4a). We then compared the DNA damage in the epidermal peel, in the remainder of the peeled leaf, and in an intact leaf sample. As we increased the simulator UV dose, we found

more damage in the epidermal peel. No significant difference was found in the DNA damage levels in the remainder of the leaf at these irradiation levels (which are comparable to the cumulative UV-B in sunlight for 15 or 30 min). The epidermal peel experiment was repeated with both maize lines, and no significant difference was found in line A versus line B (data not shown). These data indicate that the epidermal layer effectively shields the interior, at least for short time periods at current fluence levels of UV-B.

There is always some detectable damage in the interior samples even without UV treatment; it is similar to that found in root samples from field studies. We do not see this

background in DNA samples from immature maize ears that were collected and extracted under 'UV-free' conditions (in a darkroom with a red safelight) (data not shown). We conclude that these low levels are probably damage incurred during harvesting, which is not carried out under UV-free conditions.

Repair of DNA damage in organelles

How quickly does maize repair DNA damage? Available data implicate photoreactivation as the major repair pathway in plants in the light. We performed preliminary tests of repair by irradiating leaves on an intact plant with high doses (108 kJ m^{-2}) of UV-C, followed by a recovery period in a glasshouse in the light. This radiation results in measurable CPD throughout the leaf. We measured initial damage and recovery in the peeled epidermal layers and in the rest of the leaf (data not shown). About 80% of the damage in the epidermis was removed after 7 h in the light. As with the epidermis, we observed significant but not complete repair in the internal leaf tissue within 7 h, indicating that both epidermal and internal tissues of lines W23 and K55/W23 can repair CPD in the light.

For a further analysis of photoreactivation in total maize DNA, we used seedlings for ease of manipulation. Based on the whole-plant experiments, we used a 108 or 216 kJ m^{-2} UV-C dose in order to induce rapidly sufficient damage to assess repair. As shown in Fig. 5, we indeed observed photoreactivation in intact seedlings: 30–50% of the total DNA damage is removed after a 24 h incubation

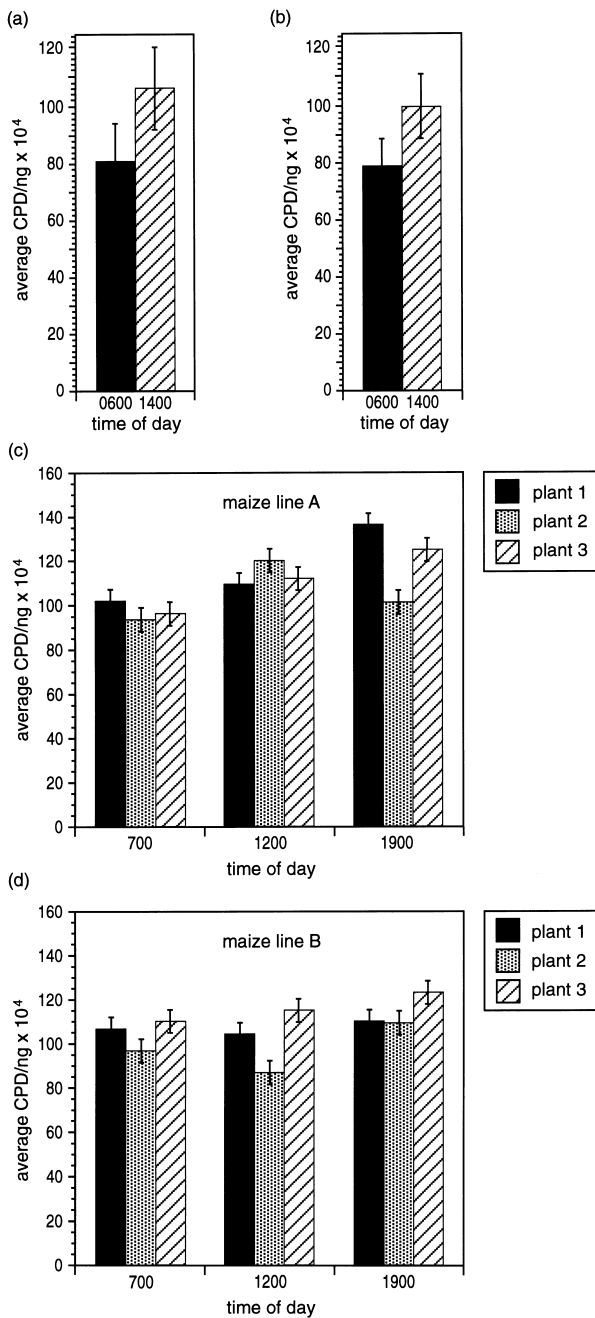


Figure 3. Measurement of DNA damage levels in plants during the day. Samples were harvested from the top leaf of 8-week-old plants at the times indicated and frozen immediately after harvest, DNA was prepared and CPD levels in each DNA sample were measured with the antibody assay as described in 'Materials and methods'. Error bars define standard error. (a) Leaf samples taken from three maize line A plants on 1 July 1992, 4 weeks after planting, at 0600 and 1400 h. The average of three replicate determinations from each DNA sample is shown. Comparison of these samples by ANOVA gave $P = 0.18$. (b) Leaf samples taken from three maize line A plants on 27 August 1992, 12 weeks after planting, 0600 and 1400 h. The average of three replicate determinations from each DNA sample is shown. Comparison of am to pm samples gave $P = 0.29$. (c) Samples taken individually from the top leaf of three line A plants at 0700, 1200 and 1900 h on 22 July 1995, 7 weeks after planting. DNA was prepared from each leaf segment separately, and the average damage levels (from four replicate determinations) for each of the three leaves is shown. The samples from each plant are not significantly different from each other (0700 h, $P = 0.75$; 1200 h, $P = 0.38$; 1900 h, $P = 0.66$). When 0700 h samples are compared to 1900 h samples, $P = 0.012$. (d) Samples taken individually from three line B plants at 0700, 1200 and 1900 h on 22 July 1995, 7 weeks after planting. DNA was prepared from each leaf segment separately, and the average damage levels (from four replicate determinations) for each of the three leaves is shown. The samples from each plant are not significantly different from each other (0700 h, $P = 0.073$; 1900 h, $P = 0.24$) except for the noon samples, where $P = 0.0017$. When 0700 h samples were compared to 1900 h samples, $P = 0.045$.

(a) Plant 1			
Tissue	Average CPD ng ⁻¹ × 10 ⁴		
	Ambient UV	600 Jm ⁻² UV-B	1200 Jm ⁻² UV-B
Leaf blade	4.8	15.4	30.6
Leaf sheath	7.6	23.3	49.5
(b) Plant 2			
Tissue	Average CPD ng ⁻¹ × 10 ⁴		
	Ambient UV	600 Jm ⁻² UV-B	1200 Jm ⁻² UV-B
Leaf blade	11.4	12.8	19.0
Leaf sheath	17.2	21.3	28.2

Table 2. Comparison of CPD DNA damage levels in the leaf blade and the leaf sheath for (a) plant 1 and (b) plant 2. Leaf and sheath samples were removed from the greenhouse-grown plants and UV-irradiated in a solar simulator. CPD levels were measured as described in 'Materials and methods'. The average of four replicate determinations on each sample is shown. Comparison of plant 1 samples using ANOVA gave $P=0.244$ for leaf versus sheath with no UV, $P=0.074$ for 600 J m⁻² UV-B and $P=0.203$ for 1200 J m⁻² UV-B treatment. Comparison of plant 2 samples using ANOVA gave $P=0.647$ for leaf versus sheath with no UV, $P=0.130$ for 600 J m⁻² UV-B and $P=0.095$ for 1200 J m⁻² UV-B treatment

in the light, but there is no significant repair in the dark controls.

Photoreactivation repair has been observed in both the nucleus and chloroplast of the microorganisms *Chlamydomonas reinhardtii* (Small & Greimann 1977) and *Euglena gracilis* (Nicholas *et al.* 1980). In fungi, photoreactivation has been demonstrated in both the nucleus and

mitochondria of *Saccharomyces cerevisiae* (Waters & Moustacchi 1974; Prakash 1975). In contrast, it has recently been reported that there is no organellar DNA repair in *A. thaliana* (Chen, Jiang & Britt 1996).

To assess the contribution of nuclear and organellar DNA damage and repair we adapted a technique designed to measure repair in specific DNA fragments (Bohr &

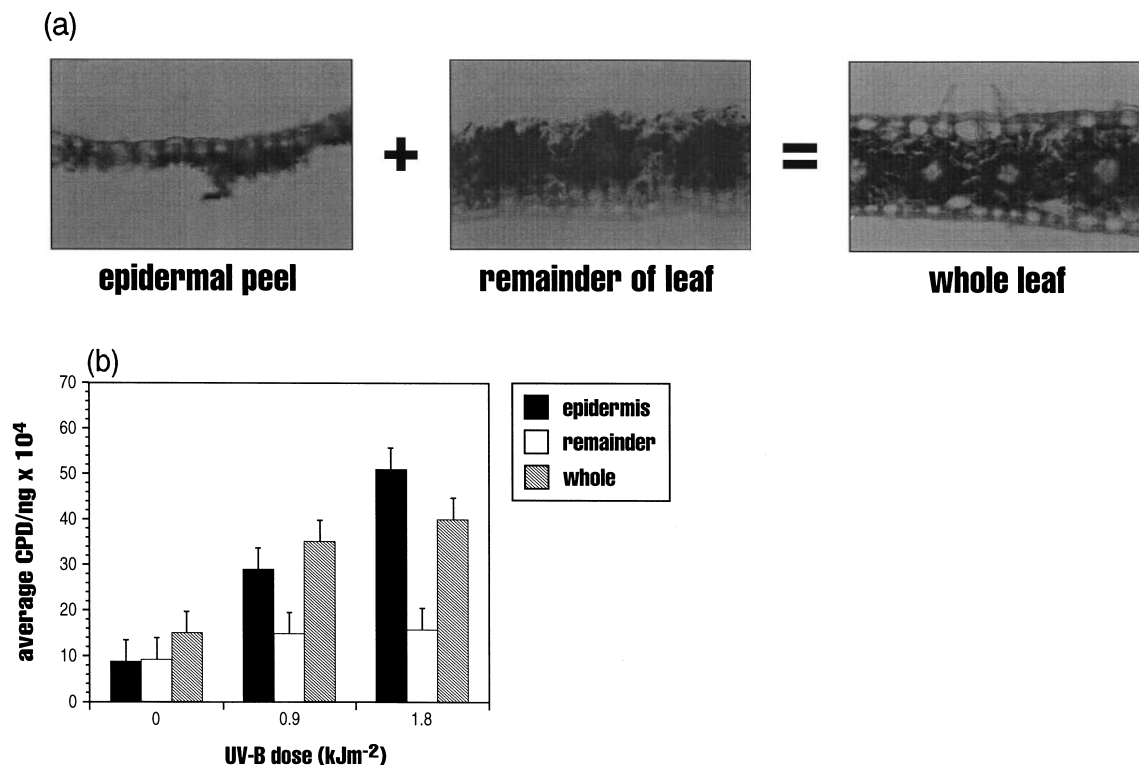


Figure 4. DNA damage in the outer and inner leaf cell layers. (a) Transverse sections of an epidermal peel, remainder of leaf and whole leaf sample. Leaf epidermal peels were prepared as described in 'Materials and methods', hand-sectioned and photographed at 20X magnification. (b) Leaves were cut from plants that had been grown in the greenhouse for 8 weeks; 7 cm × 20 cm sections of the leaves were irradiated in a solar simulator for 15 min (0.9 kJ m⁻²) or 30 min (1.8 kJ m⁻²). A subset of the leaf sections were separated into epidermal peels and the remainder of the leaf. DNA was prepared and damage was assayed with the antibody method as described in 'Materials and methods'. The average of six replicates of each sample is shown. The error bars are standard error. Comparison of all three no-UV samples gave $P=0.21$. Comparison of the epidermal sample to the remainder of leaf sample gave $P=0.064$ for 0.9 kJ m⁻² and $P=0.022$ for 1.8 kJ m⁻².

Okumoto 1988). The UV-C dose we used gave $\approx 1\text{--}2$ ESS per 10 kb. This high dose is optimal for measuring repair in 10–20 kb fragments, using DNA hybridization with individual gene probes. After UV treatment, seedlings were incubated in the light or in the dark for 2 and 24 h, and the DNA was extracted for an endonuclease T4 digestion experiment (see 'Materials and methods'). We confirmed that bulk DNA was repaired, based on the intensity of ethidium bromide fluorescence in high-molecular-weight DNA visualized after electrophoresis in preparation for blotting (data not shown). To assess repair in each com-

partment, we performed DNA blot hybridization using probes derived from nuclear, chloroplast and mitochondrial genes. As shown in Fig. 6, by 24 h we saw nearly complete repair of all three DNA segments in the light. In contrast, repair after 24 h in the dark was only 21% in the nuclear gene *Bronze1*, 3% in the plastid gene *AtpF*, and 12% in the mitochondrial *Cob* gene. With a second set of probes (nuclear *Bronze2*, plastid *PetA* and mitochondrial *Cox2*) we confirmed light repair in another region of each genome (data not shown). Based on the TEV assay, it is clear that CPD in all genes assessed are nearly fully repaired by 24 h in the light.

The antibody and TEV assays provide different damage assessments. For example, the TEV assays of individual genes indicated nearly 100% photoreactivation repair in 24 h, but we saw only 30 to 40% repair of bulk DNA using the antibody assay on the same DNA samples (Fig. 5a). This difference suggests that there is heterogeneity of damage or repair in maize DNA. Preferential repair of genes compared to non-coding DNA is a well-established phenomenon in mammalian cells, bacteria and yeast (Hanawalt, Donahue & Sweder 1994). As established in other organisms (Hanawalt *et al.* 1994), it is quite possible that DNA in plant genes is more accessible or is targeted by repair enzymes. As a consequence, the genomic regions within 10 kb of known genes, which were assessed by the TEV assay, could be repaired more efficiently than the bulk genome assessed by the antibody repair assay.

We also know (Fig. 4) that damage is not distributed evenly between the epidermis and the interior of maize leaves, a feature that compromises calculation of the rate of repair using the TEV assay. All calculations of the rate of repair in specific fragments using the TEV assay depend on a Poisson distribution of damage in the DNA. Dimer

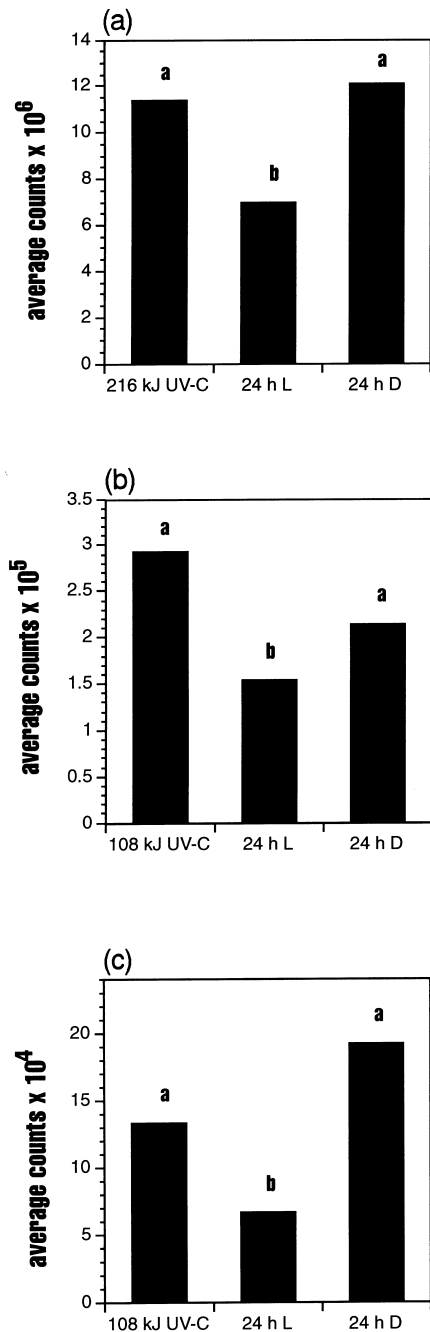


Figure 5. DNA damage in line A seedlings after exposure to UV-C radiation. Damage levels were measured as described in 'Materials and methods'. L = recovery in the light, D = recovery in the dark. (a) CPD levels, in normalized counts, in DNA samples that were also used for determination of damage in nuclear, chloroplast and mitochondrial genes (Fig. 5). The average of six replicate determinations from seedling set 1 (growth and harvest conditions described in 'Materials and methods') is shown. Columns marked with different letters are significantly different by ANOVA; for 216 kJ m⁻² compared to 24 h L $P = 0.00014$, and for 216 kJ m⁻² compared to 24 h D $P = 0.73$. (b) CPD damage levels, in normalized counts, in seedlings exposed to 108 kJ m⁻² of UV-C, and then allowed to recover in the light or dark for 24 h. The average of three replicate determinations is shown. Columns marked with different letters are significantly different by ANOVA; for 108 kJ m⁻² compared to 24 h L $P = 0.013$, and for 108 kJ m⁻² compared to 24 h D $P = 0.06$. (c) CPD damage levels, in normalized counts, in a second set of seedlings exposed to 108 kJ m⁻² of UV-C, and then allowed to recover in the light or dark for 24 h. The average of three replicate determinations is shown. Columns marked with different letters are significantly different by ANOVA; for 108 kJ m⁻² compared to 24 h L $P = 0.019$, and for 108 kJ m⁻² compared to 24 h D $P = 0.091$.

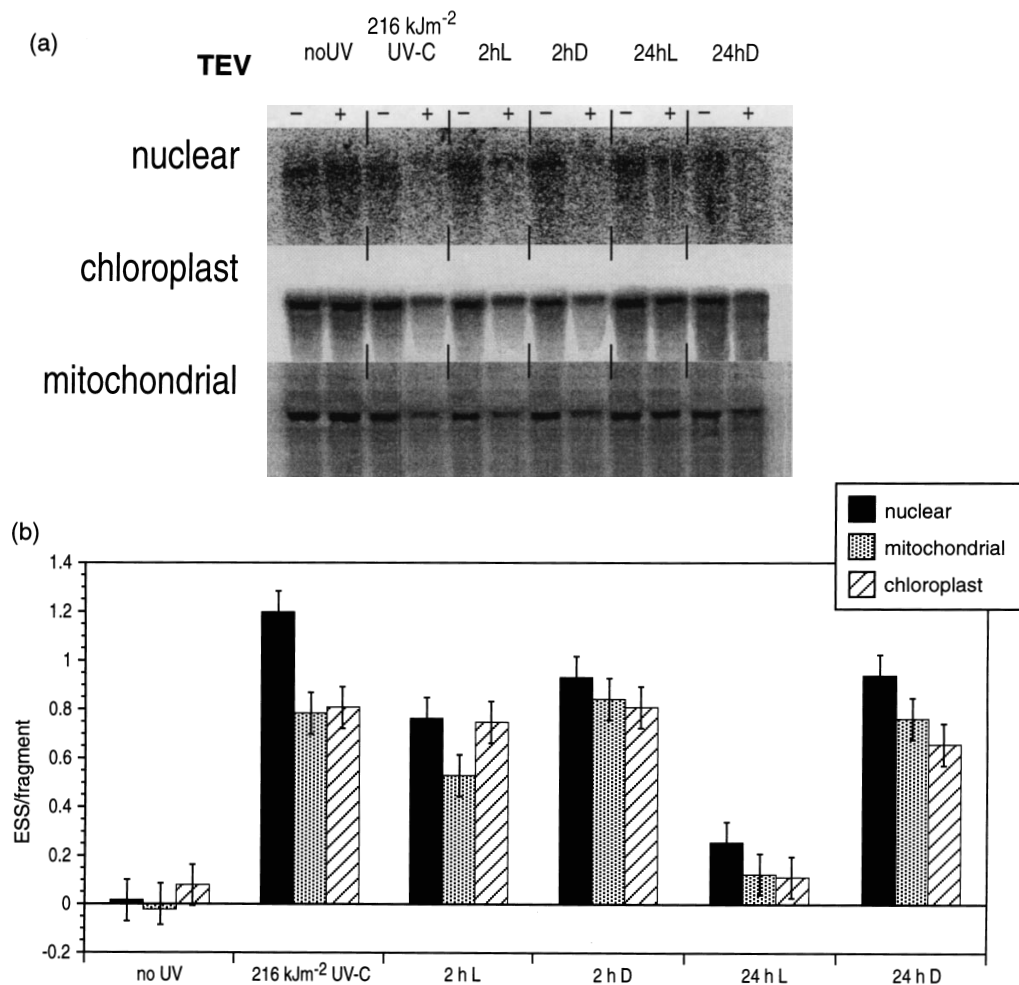


Figure 6. Repair in nuclear, chloroplast and mitochondrial DNA. Ten-day-old maize line A seedlings were irradiated for 20 min with six germicidal UV-C bulbs that were mounted vertically in the growth chamber, for a total dose of 216 kJ m⁻². The three emerged leaves from three seedlings were pooled and frozen in liquid nitrogen. The remaining seedlings were returned to the growth chamber for 2 or 24 h (with the lights on continuously) to recover; the dark recovery seedlings were placed into light-tight metal containers inside the growth chamber. DNA was prepared and the amount of repair in specific DNA fragments was measured as described in 'Materials and methods'. L = recovery in the light, D = recovery in the dark. Error bars are standard error. (a) Southern blots hybridized to probes from each cellular genome. The DNA sample from each treatment was restricted with *HpaI* and half was treated with TEV. The treated and untreated aliquots were then electrophoresed side by side, blotted and hybridized. The same blot was hybridized with the nuclear probe first, then stripped and reprobed with the chloroplast probe, and then finally stripped and reprobed with the mitochondrial probe. (b) The average of three blots of two sets of seedlings is shown. There were no significant differences between the DNA damage levels measured in the two sets of seedlings ($P = 0.59$), so the data were combined. There is no significant difference between the DNA damage levels when nuclear, chloroplast and mitochondrial probes were used on any given sample ($P = 0.058$). Using Tukey's HSD test for each probe individually the no-UV samples are significantly different from the 216 kJ m⁻² samples, the 2 h L samples, the 2 h D samples and the 24 h D samples. The 24 h L samples are significantly different ($P < 0.05$) from the 216 kJ m⁻² samples, the 2 h L samples, the 2 h D samples and the 24 h D samples. The no-UV samples and the 24 h L samples are not significantly different ($P > 0.05$), indicating that photoreactivation repair is virtually complete.

density is underestimated by the TEV assay if the induction of dimers does not follow a Poisson distribution. In our samples, damage levels immediately after irradiation are $3.4 \pm 1.8 \times 10^8$ CPD ng⁻¹ with the antibody assay, and as expected this is somewhat higher than when the same samples are measured with the TEV assay ($1.09 \pm 0.32 \times 10^8$ CPD ng⁻¹ for the *Bronze1* nuclear probe). Presumably the skew in the distribution would be the same for all the treatments of maize seedlings. Thus we cannot use our TEV data to compare the rate of repair of

individual genes in maize to the rates in samples such as monolayer bacteria, mammalian cultured cells or other tissues with low shielding. We can compare relative damage levels in maize seedlings and demonstrate that repair does occur. Although the heterogeneity of damage in maize and the possibility of differences in repair rate makes the calculation of absolute repair rate uncertain, it is clear from the TEV assays that in maize seedlings DNA from the nucleus, mitochondria and chloroplasts is repaired in the light more quickly than in the dark.

Conclusion

Using a sensitive antibody assay for CPD we established that solar UV radiation damages DNA in field-grown maize plants and that photoreactivation is the main repair pathway. Plants receive sufficient UV-B radiation every day to cause about 60-fold more damage than is actually observed, and damage does not accumulate over time. The epidermal cells contain the most damage, about 95% of the total, and appear to shield effectively interior cells from most UV in the solar spectrum. Consequently the combination of shielding and DNA repair is very effective in ameliorating the impact of this deleterious radiation.

Photoreactivation is the main pathway for removal of CPD in total DNA of mature *A. thaliana* leaves (Pang & Hays 1991), in alfalfa cotyledons irradiated with low levels of UV (Quaite *et al.* 1994), in soybean tissue culture cells (Cannon, Hedrick & Heinhorst 1995) and in wheat leaves (Taylor *et al.* 1996). Our experiments establish that photoreactivation is also the primary repair mechanism in individual maize genes: by the TEV assay an average of 83% of UV-induced damage is reversed in the light within 24 h, as compared to $\approx 10\%$ removal in the dark. We report the first demonstration of photoreactivation repair of UV-induced damage in the mitochondrial and chloroplast DNA of maize. In seedlings, 85% of induced CPD are removed in 24 h from individual genes in the nuclear, mitochondrial and plastid genomes.

Because photoreactivation activity is found in all three maize organelles, one question of interest is the nature of the photolyase(s). Are there organellar genes for photolyase or is the protein imported from the nucleus? If the protein is imported, are there separate nuclear genes for proteins targeted to each organelle or is there differential processing? Future research will be directed towards answering these questions.

Recently, Chen *et al.* (1996) reported that no photoreactivation repair occurs in organellar DNA of young *A. thaliana* seedlings. Interestingly, a survey of plant resistance to increased UV found that resistance in monocots exceeds that in dicots, and this difference could not be explained by differences in flavonoid shielding compounds (Musil 1995). If a more diverse distribution of photorepair enzymes is a general feature of monocots even at the seedling stage, we could explain their general increased resistance to UV. It will be interesting to learn if more mature *A. thaliana* plants eventually develop organellar repair capacities equivalent to those we observed in maize, or if the differences present at the seedling stage persist in adult monocots and dicots.

To understand how increased UV-B may affect ecosystem structure, a much wider survey of repair of all types of UV-induced DNA damage in more species will be required. The extent of the correlation between DNA repair activity and differences in UV-induced growth inhibition remains to be tested. If some species have more active repair systems than others, this difference could contribute to changes in species distribution in areas

affected by the increased solar UV caused by depletion of the stratospheric ozone layer.

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