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Little or No Repair of Cyclobutyl Pyrimidine Dimers Is Observed in the Organellar Genomes of the Young Arabidopsis Seedling[1]

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A Southern-blot-based, site-specific assay for ultraviolet (UV)-induced cyclobutyl pyrimidine dimers (CPDs), employing the CPD-specific enzyme T4 endonuclease V, was used to follow the repair of this lesion in particular DNA sequences in 5- to 6-d-old *Arabidopsis thaliana* seedlings. CPDs, measured as enzyme-sensitive sites, in nuclear sequences were removed rapidly in the light but were repaired slowly, if at all, in the dark. This result was identical to that obtained in prior analyses of CPDs in total cellular DNA. Assay of representative chloroplast and mitochondrial sequences in the same DNA preparations revealed that, in contrast to nuclear sequences, enzyme-sensitive sites are inefficiently eliminated in both the presence and absence of visible light. These observations suggest that Arabidopsis seedlings possess little or no capacity for the repair of CPDs in the organellar genomes. Given the fact that the UV dose employed only marginally affected the growth of the seedlings, we suggest that Arabidopsis seedlings must possess very efficient mechanism(s) for the tolerance of UV-induced DNA damage.

Plant cells contain three distinct genomes encoded by the nucleus, the plastid, and the mitochondrion. All three genomes inevitably suffer damage due to the actions of UV light, oxidative damage, and spontaneous hydrolysis (Britt, 1996). Because damaged bases can act as blocks to both DNA replication and transcription, each of these three organelles must have one or more pathway(s) for the repair and/or tolerance of DNA damage.

UV-B induces several different kinds of DNA damage products. Among these, CPDs and 6–4 photoproducts are the most abundant. Both of these lesions have been shown to block the progress of RNA polymerase in mammalian systems (Protic-Sabljic and Kraemer, 1986; Mitchell, et al., 1989). By assaying repair of total cellular DNA extracted from *Arabidopsis thaliana* seedlings, it has been established that this plant maintains two distinct photorepair systems that specifically and efficiently eliminate both classes of dimers (Pang and Hays, 1991; Chen, et al., 1994). At least some fraction of this repair must represent photorepair of nDNA, because more than 70% of these lesions are repaired within 2 h of exposure to visible light. Arabidopsis also has been shown to possess light-independent ("dark") repair pathways (Pang and Hays, 1991; Britt, et al., 1993). In the dark, Arabidopsis seedlings eliminate 6–4 photoproducts much more efficiently than CPDs from their total cellular DNA. This is also the case in mammalian, particularly rodent, cell lines (Mitchell, et al., 1985).

Studies of repair in Arabidopsis have so far been limited to the analysis of total cellular DNA. Because no repair proteins are known to be encoded by the organellar genomes, and because any nuclear-encoded organellar proteins must be specifically targeted to the organelle, it is likely that each of the three plant genomes maintains a very different battery of DNA damage repair and tolerance pathways. To study DNA repair in a single genetic compartment, we must be able to distinguish its repair events from those of the other organelles. Although it is possible to purify the organelles away from each other and from the nucleus, the procedure is not only laborious, but also takes a significant amount of time, during which repair may occur. In the unicellular alga *Chlamydomonas*, radiolabeled TMP specifically incorporates into ctDNA, and for this reason DNA repair in chloroplast can be detected without organellar fractionation. Employing this elegant system, Small and co-workers determined that the plastid genome is subject to both dark repair and photoreactivation, and that a gene required for photoreactivation of the nuclear genome is not required for repair of the plastid genome (Small, 1987).

We have employed a Southern-blot-based, site-specific assay (Bohr et al., 1986) that enables us to compare, in a single DNA preparation, the rate of repair of CPDs (measured as T4 endo V-sensitive sites) in mitochondrial, plastid, and nuclear sequences. We found that the rate of repair of nuclear sequences corresponds to that obtained for the repair of total cellular extracts, i.e. CPDs are efficiently photoreactivated, but dark repair of CPDs is so slow as to be experimentally insignificant. In contrast, and quite surprisingly, we found that dimers induced in the organellar genomes persisted over a 24-h period; no significant repair was observed in either organellar genome in the presence or absence of visible light.

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Abbreviations: CPD, cyclobutyl pyrimidine dimer; ctDNA, chloroplast DNA; ESS, enzyme-sensitive sites; rDNA, DNA encoding rRNA; 6–4 photoproduct, pyrimidine [6–4]pyrimidinone dimer; T4 endo V, phage T4 endonuclease V.

MATERIALS AND METHODS

Plant Materials

For DNA repair assays, we used *Arabidopsis thaliana* ecotype Landsberg *erecta transparent testa-5*, which was isolated by Koornneef (1990). Seeds were sterilized in 1.25% (v/v) sodium hypochlorite and 0.02% (v/v) Triton X-100 for 5 min and, after five washes with sterilized water, germinated on vertically placed 0.7% nutritive agarose plates (Kranz and Kirchheim, 1987) (Appligene, Pleasanton, CA) under cool-white fluorescent light (model F72T12/CW; Philips, Somerset, NJ) filtered with Mylar (320 nm long pass) at 22°C.

Light Sources and Filters

Broad-spectrum UV-B, the cellulose acetate filter, and UV probes were as described by Britt et al. (1993).

Measurement of Root Growth

Seedlings were grown on 1.2% agar vertical plates under cool-white fluorescent light filtered with Mylar. Sixty-five hours after imbibition, the seedlings were irradiated with 1.4 kJ m^{-2} broad-spectrum UV-B and immediately placed in the dark. Root growth was measured on photographs taken at the indicated times. The seedlings were exposed to white light from four 120-W incandescent photography lamps (General Electric EBV no. 2) for no more than 5 s for each data point.

DNA Extraction

Each set of seedlings was frozen in liquid nitrogen under dim red light (Photolamp 6W, C.P.M., Dallas, TX). DNA extraction was performed via the urea extraction procedure (Shure et al., 1983).

T4 Endonuclease V Preparation

An *Escherichia coli* strain that overproduces T4 endo V (a protein that specifically nicks DNA at CPDs) (Valerie et al., 1985) was a gift from Dr. Anne Ganesan of Dr. Philip Hanawalt's laboratory (Stanford University, Stanford, CA). T4 endo V was induced by adding isopropyl-1-thio-β-D-galactopyranoside into cell culture at mid-logarithmic phase (optical density at 650 nm = 0.4) for another 3 h of incubation at 37°C. The cells were collected by centrifugation, resuspended in 100 mM NaCl, 10 mM EDTA, 10 mM Tris-HCl (pH 8.0), and 150 mM Suc, and then lysed by 0.23% Brij 58 (Sigma) and 23 μg mL^{-1} lysozyme. Cell debris was removed by centrifugation at 40,000 rpm for 45 min at 10°C in a Beckman 70.1 Ti rotor. The supernatant was dialyzed against 100 mM NaCl, 10 mM EDTA, 10 mM Tris (pH 8.0), and 10% (v/v) ethylene glycol (Ganesan et al., 1981). We thank Charles Martin (Section of Plant Biology, University of California, Davis) for providing a T4 endonuclease preparation.

Southern Blot Assay of CPDs

Each DNA sample, after restriction digestion, was divided into two equal volumes and the buffer was adjusted to 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 100 mM NaCl. This high level of EDTA was apparently sufficient to inhibit any contaminating nonspecific DNases, as shown by the lack of digestion of non-UV-irradiated DNA samples (see "Results"). At least a 4-fold excess activity of T4 endo V was added to one of these two aliquots and the dialysis solution for the enzyme preparation was added to the other. The reaction was performed at 37°C and stopped by adding alkaline loading buffer to a final concentration of 50 mM NaOH, 1 mM EDTA, 2.5% Ficoll, and 0.25% bromcresol purple. Samples were electrophoresed on 0.5% alkaline agarose gel in 30 mM NaOH and 1 mM EDTA at 22 V. After Southern blotting to Hybond-N+ nylon membranes (Amersham), hybridization was performed in 0.5 M phosphate buffer (pH 7.0), 7% SDS, and 1 mM EDTA and washed in 0.1× SSPE at 58°C. Membranes were exposed to Kodak XAR-5 x-ray film at −70°C without intensifying screens.

Quantitation of Band Intensity via Phosphoimagery

The autoradiographs shown are for the purposes of illustration only; all data were obtained from multiple lanes (minimally three, each representing an independently derived DNA sample) quantified via phosophoimagery. As illustrated in "Results," band intensity was measured on a BAS1000 bioimagery system (Fuji Medical Systems, USA, Stamford, CT) by drawing a box around the P_o band (indicating those fragments that were not digested by T4 endo V and therefore do not contain a dimer) of the first lane (a no-UV, no-enzyme sample) and calculating the counts within that area. The same box was then shifted from one lane to the next to calculate the intensity of all P_o bands on the gel. The region of the gel directly above each band was also quantified and its value was subtracted as "background." The frequency of enzyme-sensitive sites was calculated from the signal ratio of DNA fragments of T4 endo V-treated (I_+) to untreated (I_-) aliquots. CPD content was calculated as $-\ln (I_+/I_-)$ (Mellon et al., 1987).

Probes

Probes used for Southern-blot hybridizations were as follows: nuclear 18S and 25S rDNA was probed with the purified phage λbAt002 DNA (phage plus insert) containing 1.5 repeat units of 9.9-kb rDNA (Pruitt and Meyerowitz, 1986). A 1-kb Arabidopsis cDNA for the *AMAG* gene (Santerre and Britt, 1994) was also employed as a probe for the repair of nuclear sequences. A cloned *Brassica campestris* 15-kb *Sac*I ctDNA fragment 3′ of the *rbcL* gene was employed as a probe for a 9.2-kb Arabidopsis chloroplast *Bam*HI DNA fragment. A cloned *B. campestris* 3.0-kb mitochondrial *Pst*I/*Eco*RI DNA fragment was employed as a probe for an 18.7-kb Arabidopsis mtDNA fragment. The detected chloroplast and mtDNA regions in Arabidopsis are described in further detail in "Results." Both *B. campestris* probes were a generous gift of Dr. Jeffrey Palmer

(Department of Biology, Indiana University, Bloomington). λbAt002 was a generous gift of Dr. E.M. Meyerowitz (California Institute of Technology, Pasadena). All probes were ^{32}P labeled by the random-primer method as described by the manufacturer (GIBCO-BRL).

RESULTS

UV-B Induction of Dimers in the Arabidopsis Seedling Follows a Poisson Distribution

We chose to use the Southern-blot-type assay developed by the Hanawalt laboratory (Hanawalt, 1989) for the assay for DNA repair in specific sequences. This assay, generally used for the study of repair in cells grown in tissue culture, is based on the quantitative comparison of the intensity of T4 endo V-digested versus undigested bands of specific DNA fragments run on an alkaline (denaturing) gel. Unlike the alkaline Suc gradient assay (Ganesan et al., 1981), which determines the average density of dimers from the average molecular weight of T4 endo V-digested DNA, the Southern-blot assay does not take the size of the digested fragments into account but instead relies solely on the measurement of the "zero class" (P_o), that is, those fragments that were not digested by T4 endo V and therefore do not contain a dimer. The average frequency of dimers can then be predicted using the Poisson distribution only if the induction of dimers was truly random. If, for example, most of the dimers were induced in the outer layers of the seedling, and very few were induced in the interior tissues, the zero class would be artifactually large and the density of dimers would be severely underestimated. To determine whether we could apply this site-specific, quantitative assay to intact Arabidopsis seedlings, we compared the induction of dimers as calculated via the Poisson distribution (using the site-specific assay with rDNA sequences as a probe) and as calculated via the actual change in molecular weight of total DNA (using the alkaline Suc gradient technique). The results, presented in Figures 1 and 2, show that the two techniques give approximately the same value for the induction of dimers, suggesting that UV-B irradiation of 5-d-old Arabidopsis seedlings produces a Poisson distribution of dimers. In addition, the linear increase in dimer frequency with UV-B dose observed with the Southern-blot assay also indicates that the distribution of dimers within the young seedlings is random; if there were a substantial population of relatively UV-protected cells, the apparent (but not the actual) yield of dimers per unit of UV would diminish with increasing dose. The same DNA preparations were also probed with mitochondrial and plastid DNA sequences, and we found that similar densities of dimers were induced by a given UV-B dose in each of the three genetic compartments (Table I).

Seedlings Can Recover from the Challenge Dose

When measuring repair rates in vivo, it is important to use as low a UV dose as possible, because very high doses of UV-B may generate substantial damage in cellular components other than DNA and may directly interfere with the repair apparatus. The amount of damage might also saturate the repair capacity of the cell. The sensitivity of the Southern-blot assay is directly proportional to the size of the restriction fragment under investigation. We chose to look at approximately 10-kb fragments. To determine whether the induction of dimers at a significant density in these 10-kb fragments was lethal to the seedlings, we measured the rate of growth after irradiation. Five- to 6-d-old Arabidopsis seedlings, grown on vertically placed agarose plates under cool-white fluorescent light filtered with Mylar, were irradiated for 40 s with broad-spectrum UV-B at the fluence of 35 W m^{-2} to produce a final density of dimers of approximately 0.6/10 kb (single stranded). As shown in Figure 3, growth of the Arabidopsis seedlings was slightly inhibited by this UV dose, but growth continued and no lethality was observed (data not shown).

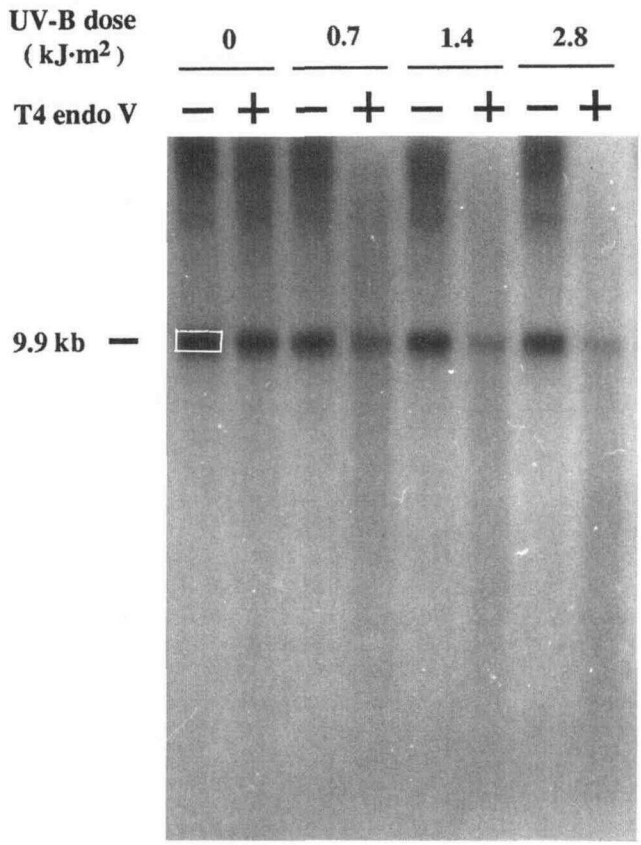

Figure 1. UV-B induction of CPDs in the nuclear rDNA. Five- to 6-d-old Arabidopsis seedlings grown on 0.7% agarose plates were exposed to broad-spectrum UV-B at an intensity of 35 W m^{-2}. The seedlings were harvested in dim red light immediately after UV-B irradiation and frozen in liquid nitrogen. DNA samples were digested with *Hin*dIII, and half of each sample was subsequently digested with T4 endo V. The digests were run on alkaline gels, and Southern blots were probed with ^{32}P-labeled λbAt002 DNA (rDNA). The "0 UV-B" lane in this and other figures serves as a control for nonspecific nuclease activities in the T4 endo V preparation.

Repair of a Nuclear-Encoded Sequence Is Similar to Repair of Total Cellular DNA

In Arabidopsis, 18S and 25S nuclear rRNA genes are present in high copy number and are arranged in two

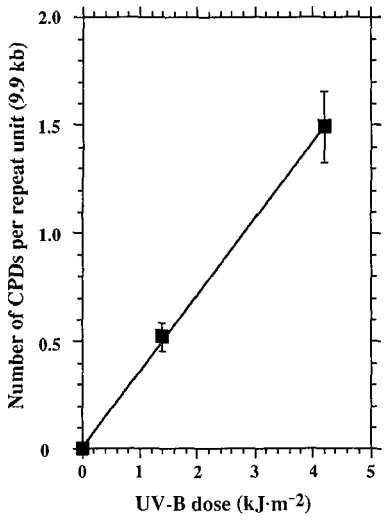

Figure 2. The induction of CPDs in the nuclear rDNA linearly increased with UV-B dose. Treatments were the same as those described in Figure 1. CPD content was determined by quantitation of band signals via phosphoimagery (Fujix BAS1000). Each data point is the average value of four pairs of lanes produced from one DNA sample. Error bars represent 1 SD.

tandem arrays of 9.9-kb repeat units distributed on chromosomes 2 and 4. In total, they make up about 7% of the nuclear genome (Pruitt and Meyerowitz, 1986). Arabidopsis seedlings were irradiated with the challenge dose (1.4 kJ m^{-2} UV-B), total DNA was extracted at various times, and the frequency of ESS in rDNA sequences was assayed over a 24-h period. As shown in Table I and Figure 4, no significant loss of ESS was observed in the dark. This result was similar to that derived via radioimmunoassay of total cellular DNA (Britt et al., 1993). In contrast, seedlings incubated in the presence of visible light rapidly eliminated ESS from rDNA sequences (Fig. 4). This result also matched that observed via radioimmunoassay of total cellular DNA from light-grown Arabidopsis seedlings (Britt et al., 1993).

To further substantiate the efficiency of photoreactivation of nuclear sequences, a 1-kb Arabidopsis cDNA encoding a methyladenine glycosylase (Santerre and Britt, 1994) was employed as a second probe for the assay of repair. This double-stranded probe hybridizes to a 12-kb *Kpn*I fragment. This fragment includes both transcribed and nontranscribed sequences. As observed for the nuclear rRNA probe, Southern-blot assay of three independent DNA preparations indicated that the photoreactivaton of ESS was rapid, whereas the dark repair of ESS in a 24-h period was too low to be measured via this technique (Table I).

No Significant Repair of CPDs Was Observed in Organellar DNAs

The same DNA samples analyzed for repair of nuclear rDNA sequences were subjected to Southern-blot assay for the repair of mitochondrial and plastid DNA. A 3-kb *B. campestris* mtDNA probe, including the *rrn18* gene, was employed to detect an 18.7-kb Arabidopsis mitochondrial *Bam*HI fragment that also encodes the *nad7*, *trnH*, and *rrn5* genes (Unseld et al., 1993; Palmer et al., 1994). Like the nuclear sequence, this mitochondrial sequence was not substantially repaired during a 24-h incubation in the dark (Fig. 5). However, in contrast to the nuclear sequence assayed in the same DNA preparation, no significant light-induced repair of mitochondrial sequences was observed in an 8-h period (Fig. 5). Similar results were obtained using a plastid sequence probe. An Arabidopsis *Bam*HI digest was probed with a *B. campestris* fragment hybridizing to the plastid genes *psaI,J*, *petA,G*, *psbE,F,J,L*, *trnW,P*, and *rpl33,20* (Palmer et al., 1994). No significant repair of this 9.2-kb fragment was observed after 24 h of incubation in the dark or after 8 h of incubation in the light (Fig. 6). This lack of significant photoreactivation of ESS in the organellar genomes contrasts sharply with what is observed in total DNA extracts from the Arabidopsis seedling, where less than 50% of the original lesions persist after 20 min of incubation in the light (Chen et al., 1994).

DISCUSSION

We employed a site-specific assay for repair of cyclobutyl dimers (measured as T4 endo V-sensitive sites) to study repair in each of the three genetic compartments of the Arabidopsis seedling. ESS were rapidly photoreactivated in both the repetitive nuclear rDNA sequences and a randomly selected unique nuclear sequence, whereas light-independent loss of ESS was quite limited (Table I; Fig. 4). This is consistent with our previous

Table I. *The induction and repair of ESS*

The data for "Total cellular DNA" were obtained using the alkaline Suc gradient procedure. All other data were obtained via the Southern-blot assay. This table summarizes the data presented in Figures 4 through 6, as well as the data obtained using the nuclear AMAG probe. All numbers are normalized to ESS per 10 kb to facilitate direct comparison.

Sequence Probed	ESS per 10 kb at Time after UV Irradiation (Treatment)			
	0 h	2 h (light)	8 h (light)	24 h (dark)
Total cellular DNA	0.61[a]			
Nuclear rDNA	0.56 ± 0.20	0.15 ± 0.14	0.04 ± 0.02	0.41 ± 0.06
Nuclear *AMAG* and surrounding DNA	0.56 ± 0.16	0.047 ± 0.04	0.025 ± 0.14	0.41 ± 0.05
A chloroplast DNA fragment	0.58 ± 0.06	0.59 ± 0.07	0.60 ± 0.04	0.52 ± 0.12
A mitochondrial DNA fragment	0.55 ± 0.17	0.50 ± 0.06	0.51 ± 0.07	0.52 ± 0.05

[a] From Britt et al. (1993).

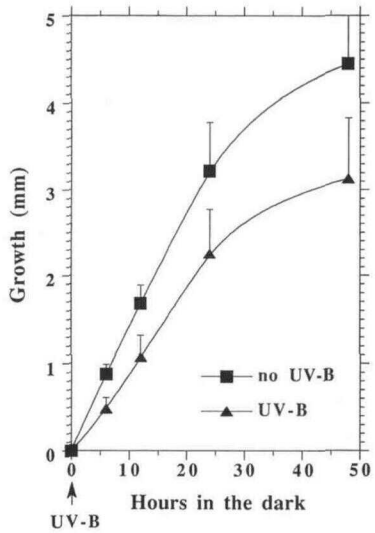

Figure 3. Root gowth in the dark after irradiation of 1.4 kJ m^{-2} of broad-spectrum UV-B. Detailed procedures are described in "Materials and Methods." Error bars represent 1 SD.

radioimmunoassay work on the repair of total cellular DNA (Britt et al., 1993; Chen et al., 1994). Site-specific assay of ESS in organellar sequences, however, indicated that repair of these sites in both the mitochondrial and plastid genomes was slow or nonexistent (Table I; Figs. 5 and 6). No dark repair was observed over a 24-h period, and no light repair was observed over an 8-h period. This result suggests, quite surprisingly, that the organelles of the Arabidopsis seedling lack any effective ability to repair this very significant and, under natural

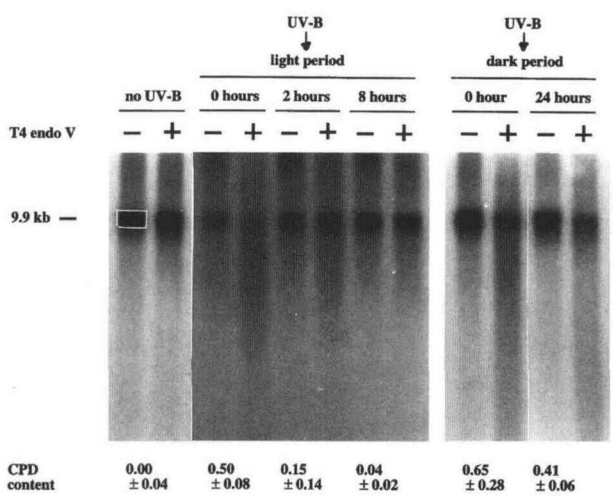

Figure 4. The repair of CPDs in nuclear rDNA. Five- to 6-d-old Arabidopsis seedlings grown under cool-white fluorescent light filtered with Mylar were irradiated with 1.4 kJ m^{-2} UV-B and then immediately placed either in the dark or under cool-white fluorescent light filtered with Mylar (PAR = 90 μE m^{-2} s^{-1}). Seedlings were harvested at the indicated times. The indicated values represent the average (±SD) CPD number per 9.9 kb in the rDNA of three to four samples. Each "sample" is an independent DNA preparation from separate batches of seedlings.

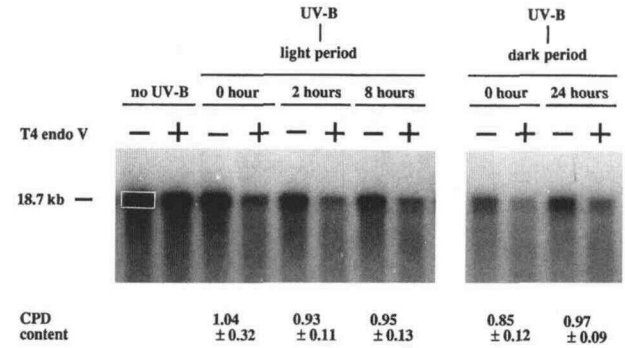

Figure 5. No significant repair of CPDs was observed in an 18.7-kb *Bam*HI mtDNA fragment. Treatments and DNA preparations were the same as in Figure 3. The detected mtDNA region was described in the text. CPD content was the average (±SD) CPD number per 18.7 kb, as assayed in four DNA samples.

conditions, unavoidable lesion. This result may be specific to our test system: to generate a uniform distribution of dimers, we are restricted to the use of the very small and UV-B-transparent Arabidopsis seedling. It is possible that other species (particularly those that grow under bright light) or more mature growth stages of Arabidopsis do repair organellar cyclobutyl dimers in an efficient and effective manner. Evidence for the photoreactivation of organellar ESS in mature leaves of maize (a plant that, unlike Arabidopsis, requires high light conditions for growth) has been obtained (A. Stapleton and V. Walbot, unpublished results). Photoreactivation of plastid DNA has also been detected in cultured soybean cells, although the repair of these sequences is considerably less efficient than the photoreactivation of nuclear sequences (Hedrick et al., 1996).

The lack of effective repair of cyclobutyl dimers is not unprecedented; the organelles of mammalian cells completely fail to repair bulky lesions such as CPDs and 6–4 photoproducts (LeDoux et al., 1992), whereas they effectively repair smaller DNA-damage products such as *N*-methylpurines. This suggests that the organelles of mammalian cells may lack a general nucleotide excision-repair

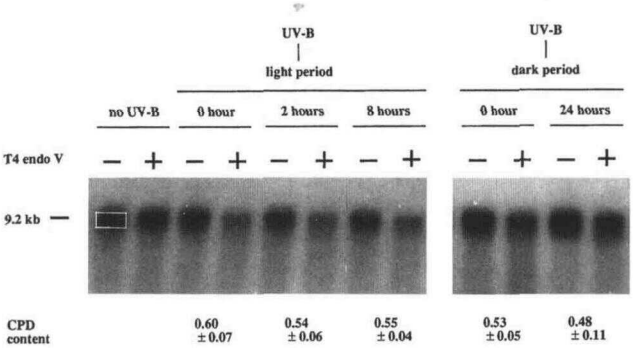

Figure 6. No significant repair of CPDs was observed in a 9.2-kb *Bam*HI ctDNA fragment. Treatments and DNA preparations were the same as in Figure 3. The detected ctDNA region was described in the text. CPD content was the average (±SD) CPD number per 9.2 kb, as assayed in four DNA samples.

pathway but that they possess certain specialized base-excision-repair pathways. Mammalian mitochondria also appear to lack any type of photolyase activity (Clayton et al., 1974). In fact, there is considerable debate as to whether photoreactivation of UV-induced damage occurs in even the nuclear genes of placental mammals (Li and Sancar, 1993). In contrast, both the nucleus and the mitochondria of *Saccharomyces cerevisiae* appear to efficiently photoreactivate CPDs (Prakash, 1975), and both the plastid and nuclear genomes of *Chlamydomonas* undergo photoreactivation (Small, 1987).

Our UV-B "challenge" dose (1.4 kJ m^{-2}) generated about 0.6 CPDs per 10 kb in all three of the sequences investigated, as well as in the total cell extracts. Given a size of 153 kb for the plastid genome and 372 kD for the mitochondrial genome (Palmer et al., 1994), this means that only a vanishingly small fraction of the organellar genomes would escape damage completely (10^{-4} for the plastid genome, 10^{-10} for the mitochondrial genome). For these genomes to replicate in spite of the persistence of damage, some sort of damage-tolerance mechanism must be invoked. It is possible that the organellar DNA polymerases are insensitive to dimers and capable of (mutagenic) translesion synthesis. Similarly, daughter-strand gaps might be filled by donation of an undamaged stretch of DNA from a second copy of the genome via a process misleadingly termed recombinational "repair." Both of these mechanisms can permit a genome to replicate in spite of the persistence of DNA damage. Neither mechanism has been directly assayed in an organellar genome, but a plastid-targeted homolog of the *E. coli* RecA protein has been cloned from Arabidopsis (Cerutti et al., 1992), and a protein with immunological cross-reactivity to RecA has been shown to be UV inducible in pea, suggesting a role for this protein in DNA damage tolerance or repair (Cerutti et al., 1993).

What are the toxic effects of dimers on transcription? The number of plastid genomes per organelle for Arabidopsis seedlings grown under our conditions is not known but is generally estimated to be between 5 and 100 copies per organelle (Herrmann, 1992; Staub and Maliga, 1992). Virtually all of the mitochondrial genes are expressed as monocistronic transcriptional units and do not present very large targets for UV-induced damage. Because each individual organelle maintains, minimally, several copies of its genome, the chance that every copy of any particular transcriptional unit in an organelle has incurred damage is quite small. The plastid does encode some longer polycistronic transcripts (Herrmann, 1992), but these transcriptional units contain internal promoters and so may be able to compensate for DNA damage by simply reinitiating transcription downstream of a persisting lesion.

It has never been entirely clear why the organellar genomes are always present in 5- to 100-fold molar excess of the nuclear genome, even up to 10,000-fold excess if one directly compares the copy number per cell rather than the copy number per organelle. It is possible that what appears to be a gratuitously large number of genomes actually represents a compensatory mechanism for an inadequate DNA repair capacity.

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